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Characterization of the Potent In Vitro and In Vivo Antimalarial Activities of Ionophore Compounds

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Large-scale in vitro screening of different types of ionophores previously pinpointed nine compounds that were very active and selective in vitro against *Plasmodium falciparum*; their in vitro and in vivo antimalarial effects were further studied. Addition of the ionophores to synchronized *P. falciparum* suspensions revealed that all *P. falciparum* stages were sensitive to the drugs. However, the schizont stages were three- to ninefold more sensitive, and 12 h was required for complete parasite clearance. Pretreatment of healthy erythrocytes with toxic doses of ionophores for 24 to 48 h showed that the activity was not due to an irreversible effect on the host erythrocyte. No preferential ionophore adsorption in infected or uninfected erythrocytes occurred. On the other hand, ionophore molecules strongly bound to serum proteins since increasing the serum concentration from 2 to 50% led to almost a 25-fold parallel increase in the ionophore 50% inhibitory concentration. Mice infected with the malaria parasites *Plasmodium vinckei petteri* or *Plasmodium chabaudi* were successfully treated with eight ionophores in a 4-day suppressive test. The 50% effective dose after intraperitoneal administration ranged from 0.4 to 4.1 mg/kg of body weight, and the therapeutic indices were about 5 for all ionophores except monensin A methyl ether, 5-bromo lasalocid A, and gramicidin D, whose therapeutic indices were 12, 18, and 344, respectively. These three compounds were found to be curative, with no recrudescence. Gramicidin D, which presented impressive antimalarial activity, requires parenteral administration, while 5-bromo lasalocid A has the major advantage of being active after oral administration. Overall, the acceptable levels of toxicity and the good in vivo therapeutic indices in the rodent model highlight the interesting potential of these ionophores for the treatment of malaria in higher animals.

Malaria is the most important parasitic disease of subtropical countries, and it is constantly progressing, especially through the development of parasite cross-resistance to standard antimalarial agents (27). New strategies for producing molecules with original mechanisms of action are essential for the eradication of this endemic disease. During its development, the parasite modifies the erythrocyte plasma membrane, mainly to allow for the entry of nutrients and the egress of waste products, and it sets up new permeability pathways which are necessary for its own survival (9). The capacity of normal erythrocytes to transport cations is probably not suitable for parasite survival since this capacity is considerably altered in the presence of the parasite. An increase in K^+ leaks and in Na^+-K^+-Cl^- transport systems, along with inhibition of the Na^+-K^+ ATPase pump at the erythrocytic plasma membrane, have been reported (29). The Ca^{2+} content of whole infected erythrocytes increases through high-level Ca^{2+} entry and a reduction in the level of active extrusion by Ca^{2+} ATPase (1, 9, 15, 29). Furthermore, the intraerythrocytic parasite constitutes an intracellular organism, and new pumps or carriers are set up to establish new ionic gradients at the expense of the infected erythrocyte cytosol (29). There is generally a marked decrease in the K^+ level and a decrease in the Na^+ level in the host cell compartment, whereas the parasite compartment has high K^+ and Ca^{2+} levels and low Na^+ levels (10, 17, 29).

In this context, perturbation of the cation gradient and/or content in *Plasmodium falciparum*-infected human erythrocytes provides a basis for developing an original rational pharmacological approach. We recently showed that ionophores that are membrane effectors, allowing transmembrane ion transfer, have a strong effect on intracellular malarial parasite viability. The in vitro antimalarial activities of ionophores are especially high for those showing marked specificity for monovalent cations (H^+, K^+, and Na^+). Among the 22 ionophore compounds of different classes that were tested, 9 compounds were at least 35-fold more toxic in vitro against *P. falciparum* than against mammalian cells, indicating selectivity for *Plasmodium* (12). Modification of erythrocytic membrane properties after infection, e.g., a decrease in surface pressure favoring ionophore integration in infected erythrocyte membranes, could be the basis of the selectivity of the compound with regard to mammalian cells (12a).

The in vivo effectiveness of several carboxylic ionophores (natural and hemisynthetic derivatives) as anticoccidial agents and ruminant growth enhancers has long been established, and they have been shown to have commercial applications (11, 28). A large-scale antiparasitic study of hemisynthetic derivatives of monensin revealed in vivo antiplasmodial activity, and some of these compounds have been patented (19). Among the ionophores, gramicidin D is quite safe for local clinical use because of its extraordinary bacteriostatic activity (13).

In the present study, nine leader compounds were selected for further investigation on the basis of their good in vitro 50%

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inhibitory concentrations (IC_{50}s; in the nanomolar range) and good differential activity in vitro (>35). The aim of the present study was to further determine the characteristics of the in vitro antimalarial activity of compounds presenting different structures and ion selectivities. In vivo studies were also carried out to assess both their tolerance and activity in a malaria-infected murine model.

MATERIALS AND METHODS

Chemicals. The sodium salt of nigericin and gramicidin D were obtained from Sigma Chemical Co. (St. Louis, Mo.). The other ionophores were stock samples from our Laboratoire de Synthèse et Etude des Systèmes à Intérêt Biologique, URA CNRS 485. Most of them were in the acid form, namely, alborixin, lonomycin A, nigericin, narasin A, monensin A, and lasalocid A. Monensin A methyl ether (or 25-*O*-methyl monensin A) and 5-bromo lasalocid A were modified by hemisynthesis as described previously (7, 32). [G-^{3}H]hypoxanthine was purchased from Amersham Corp. (Les Ulis, France). RPMI 1640 was obtained from GIBCO Laboratories (Eragny, France) and was supplemented with 25 mM HEPES buffer (pH 7.4) and 10% type AB-positive serum (complete medium). All other reagents were of analytical grade.

Biological materials. Human blood and type AB-positive human serum came from the local blood bank. The Nigerian strain of *P. falciparum* (25) (IC_{50} of chloroquine, 40 nM; unpublished data) was maintained by serial passages in human erythrocytes cultured at 7% hematocrit in complete medium at 37°C by the petri dish-candle jar method (14). The levels of parasitemia were routinely monitored on blood smears with a 10% Giemsa azure type B stain in phosphate buffer (pH 7.2).

For in vivo studies, male Swiss albino mice (weight, 30 to 40 g) were obtained from C. E. R. Janvier (Le Gerest-St. Isle, France). *Plasmodium chabaudi* 864VD and *Plasmodium vinckei petteri* 279BY (4) strains were provided by I. Landau (Paris, France).

In vitro antimalarial activity against *P. falciparum*. The effects of the drugs on in vitro *P. falciparum* growth were measured in microtiter plates as described by Desjardins et al. (6). The final volume in each well was 200 μl, consisting of 50 μl of complete medium with or without the drug (control) and 150 μl of *P. falciparum*-infected erythrocyte suspension (1 to 2% final hematocrit, which is the ratio of the packed cell volume to the total volume, and 0.3 to 0.8% parasitemia, which is the percentage of infected cells). The drugs were dissolved in dimethyl sulfoxide and were then further diluted in culture medium so that the final dimethyl sulfoxide concentration never exceeded 0.25%. After 48 h of incubation at 37°C, 30 μl of complete medium containing 0.8 μCi of [^{3}H]hypoxanthine was added to each well, and candle jar incubations were continued for an additional 12 to 18 h. The cells were subsequently lysed, and radioactive nucleic acids were retained on glass fiber filters (Whatman GF/C) with an automatic cell harvester (Skatron Macro 96). The radioactivity on the filters was then counted after adding 2 ml of scintillation cocktail in a Beckman 5000 liquid scintillation spectrometer. Radioactive backgrounds were obtained from incubations of normal erythrocytes under similar conditions. The level of [^{3}H]hypoxanthine incorporation varied in the different experiments as a function of the parasite stage to which radioactive precursor was added and as a function of the level of parasitemia, percentage of serum, and time of incubation with [^{3}H]hypoxanthine. Under standard conditions, the level of [^{3}H]hypoxanthine incorporation ranges from 10,000 to 50,000 dpm, which is much higher than the corresponding level of incorporation in the control preparation consisting of noninfected erythrocytes, which always had levels of incorporation lower than 400 dpm. In some cases, especially at low levels of parasitemia (0.15%) or in the presence of a high percentage of serum in the medium, the level of [^{3}H]hypoxanthine incorporation was lower, ranging from 4,000 to 10,000 dpm, and the levels were compared to those for the appropriate controls. IC_{50}s, i.e., the concentrations of drug inhibiting parasite growth by 50%, were evaluated from plots of parasite growth (expressed as a percentage of control growth) versus the log dose and are the means of at least two independent experiments conducted in triplicate with different stock drug solutions. Student's *t* test was used for all statistical analyses. The significance threshold was set at $P < 0.005$.

Time course of in vitro parasite growth inhibition by ionophores. A 1-ml aliquot of suspensions of *P. falciparum*-infected erythrocytes at 1% hematocrit and 0.3 to 0.5% parasitemia were injected at time zero into 24-well plates. A total of 500 μl of complete medium without (control) or with the ionophore compound were added. After various incubation times at 37°C, the culture medium was twice removed and replaced with fresh medium without drug. Cell suspensions were then transferred to 96-well plates (200 μl/well), and [^{3}H]hypoxanthine (1.2 μCi/well) was added either immediately after drug removal or at 52 h. Reactions were stopped by cell filtration at 76 h, as described above.

In some experiments, *P. falciparum*-infected erythrocytes were synchronized by three successive treatments with 5% D-sorbitol (16) at times of −82, −43, and −1 h and were then incubated with the drug as described above for various times starting at time zero for the ring, stage, 24 h for the trophozoite stage, and 36 h for the schizont stage. At 52 h, cell suspensions were transferred to 96-well plates (200 μl/well) and [^{3}H]hypoxanthine (1.5 μCi/well) was added.

Inhibition of synchronous *P. falciparum* growth as a function of the ionophore concentration at three parasite development stages. *P. falciparum*-infected erythrocytes were synchronized. At time zero, 1-ml aliquots of suspensions of synchronized infected erythrocytes at 1.5% hematocrit and 0.3% initial parasitemia (100% rings) were transferred to 24-well plates; 500 μl of complete medium containing drug at various concentrations was added when the parasites were in the ring (time 0 h), trophozoite (24 h), or schizont (36 h) stage. After 9 h of incubation in the presence of the drug, the medium was replaced twice with fresh complete medium, and the parasites were cultured for an additional 15 h at 37°C. At times of 24, 48, and 60 h for the ring, trophozoite, and schizont stages, respectively, cell suspensions were transferred to 96-well plates (200 μl/well) and [^{3}H]hypoxanthine was added. Incubations were finally stopped at 76 h as described above. The IC_{50}s for each stage were evaluated.

Pretreatment of normal erythrocytes with monensin A. Normal erythrocytes were incubated at 1.5% final hematocrit in complete medium in the absence (control) or the presence of 7 ng of monensin A per ml for the following times: −48 to −24 h, −24 to 0 h, or −48 to 0 h. At time zero, erythrocytes were washed twice with complete fresh medium and were then infected at 0.5% initial parasitemia with a highly parasitized suspension. The added infected cells accounted for less than 2% of the total erythrocytes. Infected suspensions (200 μl) were then distributed into a 96-well culture plate and were incubated for 48 h by the candle jar technique. Parasite growth was monitored by the incorporation of [^{3}H]hypoxanthine over the 48- to 72-h period.

Effects of hematocrit, parasitemia, and serum levels on ionophore in vitro antimalarial activity. The effect of the hematocrit level was determined by carrying out experiments in a final volume of 200 μl, consisting of 50 μl of complete medium with or without the drug (control) and 150 μl of a *P. falciparum*-erythrocyte suspension at different levels of hematocrit. The erythrocyte suspension was at either 0.5% initial hematocrit (2% parasitized), 2% hematocrit (0.5% parasitized), or 10% hematocrit (0.1% parasitized). Under these conditions, the final hematocrit varied by 0.32, 1.3, and 6.5%, respectively, whereas the total amount of infected erythrocytes present in the suspension remained constant, irrespective of the hematocrit (1.5×10^5 parasites/well).

The effect of the level of parasitemia was evaluated by carrying out experiments under the same conditions and at constant hematocrit (1%), except that the level of parasitemia of the erythrocyte suspension varied (0.15, 0.3, 0.6, or 1.2%), so the total amount of erythrocytes present in the suspension remained constant (1.5×10^7 erythrocytes/well).

To evaluate the effect of the serum concentration, 50 μl of HEPES-buffered RPMI 1640 medium containing 2% serum with or without (control) the drug was added to 150 μl of an infected cell suspension (1.5% final hematocrit and 0.6% parasitemia) suspended in RPMI 1640 medium containing 2, 12.7, or 66% serum, so the final serum concentration was 2, 10, or 50%, respectively.

In every case, parasite viability was assessed by adding [^{3}H]hypoxanthine at 48 h and further incubation for 12 to 18 h at 37°C.

Assessment of acute and subacute toxicity in mice. Acute toxicity was determined after a single injection of drug to four noninfected, randomly bred male Swiss albino mice weighing 30 to 40 g and was expressed as the 50% lethal dose (LD_{50}), which corresponds to the dose leading to 50% deaths 10 days after drug injection. Subacute toxicity was evaluated after twice-daily drug injection for 4 consecutive days (eight doses) to four mice. Routinely, the drugs were dissolved in 10% (wt/vol) gum arabic in water or in 0.5% Tween 80. Stock solutions were ultrasonicated (Vibracell; Bioblock Scientific) for 2 to 5 min. The drugs were administered intraperitoneally (250 μl) or orally (500 μl).

In vivo antimalarial activity against mouse-derived *Plasmodium*. In vivo antimalarial activity was determined against two different rodent-derived strains, *P. chabaudi* and *P. vinckei petteri*, according to a slightly modified version of the 4-day suppressive test of Peters et al. (22). Swiss mice were inoculated intravenously with 10^7 parasitized erythrocytes (resuspended in 0.2 ml of HEPES-buffered RPMI 1640 medium). The animals were then administered the drug twice daily for 4 consecutive days, beginning on the day of infection. The first drug injection started 1.5 h after parasite inoculation, and the second injection was given 8 h later. Parasitemia levels were determined on the day following the last treatment. The ED_{50}, which is the dose leading to 50% parasite growth inhibition compared to growth in the controls (treated with an equal volume of vehicle), was evaluated from a plot of activity (expressed as a percentage of the activity in the controls) versus the log dose. Three animals were treated with each dose, while six control animals were tested (21 mice in one test with five drug doses). Treatment was considered curative when no parasites were detected after 60 days. In some cases, mice were again challenged at 60 days by intravenous inoculation of 10^7 infected erythrocytes to check whether they were fully susceptible to a new infection.

RESULTS

We previously determined the in vitro effects of 22 ionophores from different classes against the intraerythrocytic stage of *P. falciparum*. Most of them were found to be very active, with IC_{50}s in the nanomolar range. Nine compounds presented high antimalarial activity (IC_{50}s, ≤70 ng/ml) and much higher

TABLE 1. Antimalarial activities and toxicities of various ionophore compounds[a]

Compound	In vitro antimalarial activity (*P. falciparum* IC_{50} [ng/ml])	In vitro differential activity (mean)	LD_{50} (mg/kg) in mice		ED_{50} (mg/kg)		TI
			Acute	Subacute	*P. chabaudi*	*P. vinckei petteri*	
Carboxylic ionophores							
Class 1a							
Alborixin	0.6	57	<1 (i.p.)	ND[b]	ND	ND	
Lonomycin A	1.4	202	10 (i.p.)	2.5 (i.p.)	0.44 (i.p.)		5.7 (i.p.)
Nigericin	1	141	14.2 (i.p.)	ND	2.3 (i.p.)		6 (i.p.)[c]
						1.1 (i.p.)	4.5 (i.p.)
Narasin	1	402	4 (i.p.)	1.75 (i.p.)	0.42 (i.p.)	ND	4.2 (i.p.)
Monensin A	1.5	80	8.5 (i.p.)	>6 (i.p.)	4.1 (i.p.)	ND	2.1 (i.p.)[c]
			51 (p.o.)[d]	>30 (p.o.)	10.1 (p.o.)	ND	5 (p.o.)[c]
Monensin A methyl ether	6.5	73	30 (i.p.)	>17.5 (i.p.)	ND	2.5 (i.p.)	12 (i.p.)[c]
Class 2							
Lasalocid A	28	43	30 (i.p.)	13.3 (i.p.)	4 (i.p.)	ND	3.3 (i.p.)
5-Bromo lasalocid A	69	55	80 (i.p.)	30 (i.p.)	1.4 (i.p.)		22 (i.p.)
						2 (i.p.)	14 (i.p.)
			613 (p.o.)	230 (p.o.)	ND	11 (p.o.)	21 (p.o.)
Quasi-ionophore, gramicidin D	0.035	1,328	482 (i.p.)	>140 (i.p.)	ND	1.4 (i.p.)	344 (i.p.)[c]
			>1,000 (p.o.)		ND	>100 (p.o.)	

[a] Ionophores are classified by Pressman (23) as true ionophores (mobile carriers) and channel-forming quasi-ionophores. Carboxylic ionophores are subdivided into class 1a for ionophores specific to monovalent cations and class 2 for ionophores able to complex mono- and divalent cations (31). The in vitro IC_{50} against the intraerythrocytic stage of *P. falciparum* and differential activity between mammalian cells (U937 macrophages and Jurkat lymphoblasts) and *P. falciparum* were determined previously (12). Acute and subacute LD_{50}s were determined for mice (i.p., intraperitoneal administration; p.o., oral administration). ED_{50}s were determined by a 4-day suppressive test with *P. chabaudi*- or *P. vinckei-petteri* infected mice. The TI is the LD_{50}/ED_{50} ratio and is based on subacute toxicity.

[b] ND, not determined.

[c] TI is based on the acute LD_{50} and represents an upper limit of the TI. Drugs were dissolved in 10% (wt/vol) arabic gum in water unless indicated otherwise.

[d] Vehicle corresponds to 0.5% Tween 80.

toxicity against *P. falciparum* cells than against mammalian cells, such as Jurkat lymphoblasts or U937 macrophages (12). Eight of them were carboxylic true ionophores of class 1a and 2, and one, gramicidin D, was a channel-forming quasi-ionophore specific to monovalent cations. These compounds and their IC_{50}s for the intraerythrocytic stage of *P. falciparum* are listed in Table 1.

Time course of in vitro *P. falciparum* growth inhibition as a function of parasite development. Monensin A, a Na^+-specific ionophore, was added at 11.6 ng/ml (about sevenfold its IC_{50}) for pulse-inhibitory times to an infected erythrocyte suspension (Fig. 1). After 4 h of treatment, parasite viability had already decreased by as much as 60%. Longer treatment progressively killed higher percentages of parasites, but 30 h was necessary for complete inhibition. The observed inhibition was quite similar when hypoxanthine, used to monitor parasite viability, was added either immediately after removal of the drug or only late (52 to 76 h) in the period after parasite culture in the absence of drug. Parasite viability and hypoxanthine incorporation were thus simultaneously and equally affected, suggesting a cytocidal rather than a cytostatic effect. A very close time-dependent inhibition pattern was obtained with cationomycin, a K^+-specific ionophore, at fourfold its IC_{50} (data not shown).

Monensin A was then added at sevenfold its IC_{50} for various times to synchronized erythrocyte suspensions infected with *P. falciparum* at the ring (time zero), trophozoite (24 h), or schizont (36 h) stage to determine whether any specific parasite stage was more sensitive to the drug (Fig. 2). The three successive treatments with sorbitol at 35- to 40-h intervals allowed a tight synchronization window of about 5 h.

The ring stage was the least sensitive stage since, even after 20 h of contact with monensin A, parasitemia still amounted to 20% of that for the control. On the other hand, 15 h of contact of the trophozoite stage with the drug led to complete disappearance of the parasite. The schizont stage appeared to be the most sensitive stage, since inhibition was higher than 70% after 3 h of contact and complete inhibition occurred after 12 h of drug exposure.

Cationomycin was also tested under similar conditions at fourfold its IC_{50}. As noted for monensin A, the lethal effect

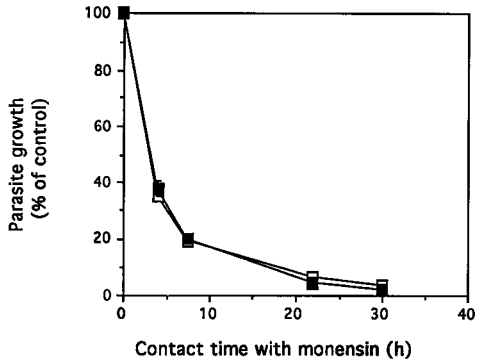

FIG. 1. Time course for in vitro *P. falciparum* growth inhibition by monensin A. Monensin A at 11.6 ng/ml was added at time zero to *P. falciparum*-infected erythrocyte suspensions. At the indicated times, the culture medium was changed (twice) and was replaced with fresh medium without drug. [^{3}H]hypoxanthine (1.2 μCi/well) was added either immediately after drug removal (□) or at 52 h (■). Reactions were stopped by filtration at 76 h, when the level of parasitemia was 1.5% in the controls. Results are means ± standard errors of the means for one typical experiment carried out in triplicate and expressed as a percentage of the hypoxanthine incorporation in the absence of the ionophore. When they are not visible, error bars fell within the symbol.

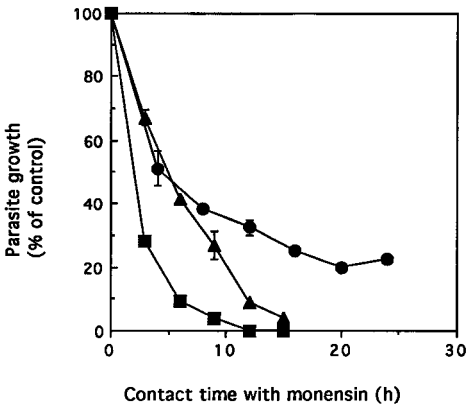

FIG. 2. Time dependence of monensin A-induced growth arrest at the ring, trophozoite, and schizont stages. Cells were synchronized at −82, −43, and −1 h (initial parasitemia, 0.5%). The ring, trophozoite, and schizont stages, which were obtained at time zero, 24 h, and 36 h, were exposed to 10 ng of monensin A per ml for the indicated times, and then the drug was removed, as described in Materials and Methods. The parasites were labeled with [^{3}H]hypoxanthine from 51 h (when the level of parasitemia was 2.2%) until 72 h. Hypoxanthine incorporation is expressed as a percentage of that in the control (without drug) for the ring (●), trophozoite (▲), or schizont (■) stage.

was much more rapid for mature stages of the parasite than for the ring stages (data not shown).

This partial inhibition of ring-stage growth by monensin A led us to investigate the sensitivity of parasites at each of the three development stages. Increasing drug concentrations were thus added to synchronized *P. falciparum*-infected erythrocyte suspensions for 9 h, allowing an IC_{50} determination for each parasite stage. Leader ionophores representative of each class (with different ion specificities) were tested as a function of the parasite stage (Table 2).

Nigericin exerted significant activity against all *P. falciparum* development stages in vitro. The *P. falciparum* schizont stage was the stage most susceptible to the drug, with an IC_{50} of 1.6 ng/ml, while the ring and trophozoite stages were about fivefold less sensitive, with respective IC_{50}s of 7 and 9 ng/ml. With 5-bromo lasalocid A and gramicidin D, the schizont stage was also significantly more sensitive (three- to ninefold) to the drug than the ring stage.

Effect of ionophore pretreatment of erythrocytes on their ability to support parasite growth. Considering that ionophores are membrane effectors that alter the intraerythrocytic ion content, we investigated whether they could modify the properties of the host cell, possibly hindering its ability to sustain parasite invasion and/or growth. Normal erythrocytes were thus preincubated for 24 or 48 h in the presence of 7 ng of monensin A per ml and were then washed and infected with highly parasitized erythrocyte suspensions (data not shown). In the first set of experiments, normal erythrocytes were placed in contact with monensin A from −48 to −24 h and were then cultured for an additional 24 h without drug before infection at time zero. Compared to untreated controls, no change in the level of parasitemia was observed at time 72 h, indicating that 24 h of pretreatment of erythrocytes with a lethal dose of monensin A did not affect the ability of erythrocytes to support parasite growth. In the second and third sets of experiments, normal erythrocytes were exposed to monensin A for 24 or 48 h immediately before *P. falciparum* infection. The presence of monensin A for the whole 48 h before plasmodial infection weakly but significantly inhibited parasite growth (−28%). Surprisingly, addition of drug during the period from −24 to 0 h, i.e., just before infection, led to significant enhancement (by 30%) of the level of parasitemia on day 3. In these two cases, the levels of parasitemia obtained at time 72 h were significantly ($P < 0.005$) different from those in the controls (absence of drug), according to the standard Student's *t* test.

Effects of levels of hematocrit, parasitemia, and serum on ionophore antimalarial activity in vitro. Before determining the antimalarial activity in vivo, we assessed the effects of the levels of hematocrit, parasitemia, and serum since usual in vitro tests were carried out at hematocrits lower than 2% and a serum concentration of 10%. In the presence of nigericin, the same IC_{50} was obtained (1.2 ng/ml) for 0.32, 1.3, and 6.5% hematocrits, i.e., after a 20-fold increase in the hematocrit (Fig. 3A). This was also the case for monensin A, whose IC_{50} remained unchanged, irrespective of the hematocrit level (data not shown). The effect of increasing parasitemia level was investigated at a fixed hematocrit level (1%) and four parasitemia levels (0.15, 0.3, 0.6, and 1.2%). This increase in the level of parasitemia of as much as eightfold did not significantly modify the IC_{50} of nigericin (Fig. 3B).

The effect of increasing the serum concentration from 2 to 50% was investigated at fixed hematocrit (1.5%) and parasitemia (0.6%) levels. The results presented in Fig. 3C indicate that increasing the serum concentration from 2 to 10% led to a 6-fold increase in the IC_{50} of nigericin (from 0.22 to 1.3 ng/ml) and a 4.5-fold increase (IC_{50}, 6 ng/ml) when the serum concentration was increased from 10 to 50%. The effect of serum was also determined for lonomycin A, narasin A, monensin A, lasalocid A, 5-bromo lasalocid A, and gramicidin D. Irrespective of the class, an increased serum concentration always shifted the IC_{50} toward higher concentrations, indicating an apparent decrease of in vitro antimalarial activity (data not shown). Mean IC_{50}s were increased by 5.2 ± 0.6-fold when the serum concentration was increased from 2 to 10% and from 10 to 50%.

In vivo activities of the different ionophores in mice. Preliminary tests were performed to determine the acute toxicities of these compounds (expressed as LD_{50}s) after a single drug administration by the intraperitoneal or oral route. In preliminary experiments, the same LD_{50} was obtained after intraperitoneal injection when monensin was dissolved in Tween 80 and gum arabic (data not shown).

Except for alborixin, for all carboxylic ionophores tested, the LD_{50}s after intraperitoneal administration ranged from 4 to 80 mg/kg of body weight; for alborixin the LD_{50} was less than 1 mg/kg (Table 1). Class 1a carboxylic ionophores, specific to monovalent ions, had LD_{50}s of between 4 and 30 mg/kg, while class 2 compounds specific to divalent cations had higher LD_{50}s (between 30 and 80 mg/kg). Gramicidin D was the

TABLE 2. Differential sensitivities of the ring, trophozoite, and schizont stages of *P. falciparum* to various ionophores[a]

Parasite stage	IC_{50} (ng/ml)[b]		
	Ring stage	Trophozoite stage	Schizont stage
Nigericin	7 ± 0.56	9 ± 1.18	1.6 ± 0.16**
5-Bromo lasalocid A	340 ± 46	340 ± 54	80 ± 4.6**
Gramicidin D	0.37 ± 0.044	0.12 ± 0.002	0.042 ± 0.008*

[a] Various drug concentrations were added for 9 h to synchronized *P. falciparum*-infected erythrocyte suspensions when the parasites were in the ring, trophozoite, or schizont stage, and IC_{50}s were determined by measuring the level of hypoxanthine incorporation as described in Materials and Methods.

[b] IC_{50}s are means ± standard errors of the means for at least one experiment carried out in triplicate. Data were analyzed according to the standard Student's *t* test: $P < 0.005$ (*) or $P < 0.001$ (**) compared to the IC_{50} for ring stages.

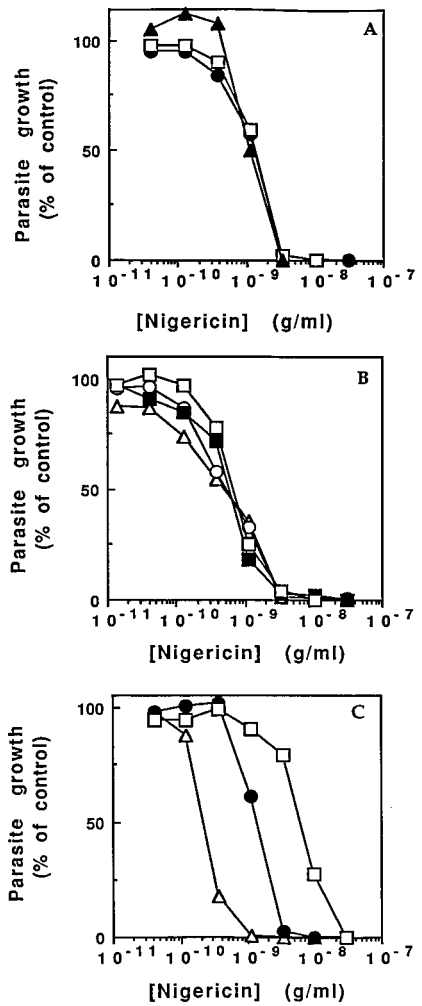

FIG. 3. Effect of hematocrit level (A), parasitemia level (B), and serum concentration (C) on in vitro antimalarial activity of nigericin. (A) The nigericin effect on *P. falciparum* growth in vitro was measured as described in Materials and Methods, except that the final hematocrit was 0.5% (●), 2% (□), or 10% (▲), whereas the amount of infected erythrocytes in the suspension remained constant (1.5×10^5 infected erythrocytes/well). (B) The erythrocyte suspension was at constant hematocrit (1%), but with variable initial levels of parasitemia of 0.15% (□), 0.3% (■), 0.6% (○), or 1.2% (△). (C) The effect was determined at a 2% final hematocrit and a 0.6% parasitemia, but the final serum concentration was 2% (△), 10% (●), or 50% (□).

best-tolerated compound, with an LD_{50} of 482 mg/kg. Subacute toxicity was also measured after twice-daily drug administration for 4 consecutive days. The subacute toxicity was similar to or slightly higher than the acute toxicity (by 1.4- to 4-fold) for the seven compounds tested.

Monensin A, 5-bromo lasalocid A, and gramicidin D were also tested for their toxicities after oral administration. Their LD_{50}s were 51, 613, and greater than 1,000 mg/kg, respectively. The fact that there was very little difference between the toxicities of monensin A and 5-bromo lasalocid A given by the intraperitoneal and oral routes (6- and 7.6-fold, respectively) indicated that there was substantial intestinal absorption of these two compounds after oral administration.

All compounds except alborixin (which was found to be highly toxic, with an LD_{50} of less than 1 mg/kg) were tested for their in vivo antimalarial activities in mice by a modified version of the 4-day suppressive test of Peters et al. (22), in which various drug doses were administered twice daily for 4 days. Drugs were administered at 8 and 18 h, which roughly corresponded to the mature parasite stages, i.e., trophozoite and schizont stages, which are the stages most sensitive to the drug (see above). The level of parasitemia in control mice reached as high as 45% at day 5, with death occurring within the following 4 days.

The seven carboxylic ionophores tested were able to decrease the levels of parasitemia in *P. chabaudi* and/or *P. vinckei petteri*-infected mice on day 5 by more than 70%, allowing us to determine the ED_{50}. The highest dose of nigericin, lasalocid A, monensin A methyl ether, 5-bromo lasalocid A, or gramicidin D (usually the maximal tolerable dose) led to the complete clearance of parasitemia (data not shown). The in vivo activity occurred over less than 2 log drug concentrations, revealing specific targets (data not shown). The ED_{50}s ranged from 0.4 to 4.1 mg/kg after intraperitoneal administration (Table 1). For comparison, nigericin and 5-bromo lasalocid A were tested against both *P. chabaudi* and *P. vinckei petteri*. The data in Table 1 indicate that the ED_{50} obtained for the two compounds did not differ significantly for either mouse-derived *Plasmodium* species.

Therapeutic indices (TIs; i.e., the LD_{50}/ED_{50} ratio) were based on subacute toxicity; for nigericin, monensin A (and its derivative), and gramicidin D, however, subacute toxicities could not be determined due to limited drug supplies. In this case, TIs were calculated on the basis of the acute LD_{50}/ED_{50} ratio, representing upper limits of the TIs. For most of the ionophores, i.e., lonomycin A, nigericin, narasin A, monensin A, and lasalocid A, the TI achieved in the intraperitoneal mode usually ranged from 2 to 6. The TIs of monensin A methyl ether and 5-bromo lasalocid A were more interesting, with values of 12 and 14 to 22, depending on the strain used.

The in vivo activity and toxicity of gramicidin D are presented in Fig. 4. This ionophore was found to be very active against *P. vinckei petteri*, regardless of whether the drug was administered once or twice a day for 4 days, with very close ED_{50}s (1.4 and 2 mg/kg for once- and twice-daily administration, respectively), indicating that repeated daily drug administration was unnecessary. In both cases, antimalarial activity occurred over less than 2 log drug concentrations. For comparison, its toxicity after the administration of a single dose (acute toxicity) was found to be very low (LD_{50}, 482 mg/kg) and the subacute LD_{50} was greater than 140 mg/kg (not tested

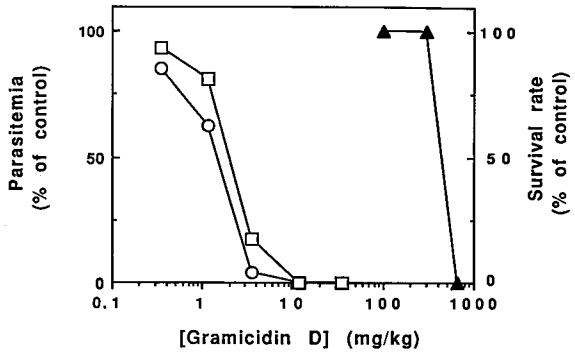

FIG. 4. Acute toxicity and in vivo antimalarial activity of gramicidin D against *P. vinckei petteri* after intraperitoneal administration to mice. Acute toxicity for mice (▲) was measured after a single drug administration. Antimalarial activities were measured as described in Materials and Methods according to the 4-day suppressive test of Peters et al. (22). Drugs were administered once (○) or twice (□) daily. On day 5, the mean level of parasitemia in the six control mice was 45% ± 5% (standard error of the mean).

above) (Table 1). Gramicidin D was thus the compound with the highest level of safety since its TI against *P. vinckei petteri* in mice was 344.

The in vivo antimalarial activities of monensin A and 5-bromo lasalocid A were also investigated after oral administration (very active monensin A methyl ether could not be evaluated due to the limited amount of product available). The ED_{50}s of monensin A and 5-bromo lasalocid A were 10.1 and 11 mg/kg, respectively, corresponding to respective TIs of 5 and 21. In both cases, the TIs after oral administration were in the same range as the TI obtained after intraperitoneal administration. On the other hand, gramicidin D was found to be inactive after oral administration up to at least 100 mg/kg (the highest dose tested).

For drugs that completely cleared the parasitemia and that had good TIs after intraperitoneal administration, parasitemia levels in mice were followed for 60 days to assess possible recrudescence. Monensin A methyl ether, 5-bromo lasalocid A (at the maximal tolerable dose), and gramicidin D (at the acute $LD_{50}/14$) were curative against *P. vinckei petteri*, since no parasites could be detected within 60 days posttreatment. After this time, mice were challenged by intravenous administration of 10^7 *P. vinckei petteri*-infected erythrocytes. Mice treated with 5-bromo lasalocid A and monensin A methyl ether did not develop parasitemia, whereas those that received gramicidin D developed disease, with death occurring within 12 days (death of naive control mice occurred within 8 days).

DISCUSSION

Characterization of the in vitro antimalarial activities of these highly potent and selective ionophores was aimed at determining the minimal drug contact times required to kill the parasite in its different development stages, the possible effects of the ionophores on host erythrocytes, and the influence of parameters such as hematocrit, parasitemia, and serum quantities on their antimalarial activities.

Monensin A required long-duration contact (30 h) to completely kill all parasites in an asynchronous suspension, indicating a cytocidal rather than a cytostatic effect (Fig. 1), as shown by the same patterns of activity obtained when parasite viability was measured immediately after drug removal or at 52 h. These compounds were highly toxic against every intraerythrocytic stage of *Plasmodium*, which indicates that the vital target was present throughout the erythrocytic stage, highlighting the advantages of this approach. Irrespective of the ionophore class, the higher sensitivity (three- to ninefold) of the schizont stage (Table 2) was likely related to the progressive changes in the host membrane properties and ionic contents during parasite development, whose alterations are maximal at the schizont stage (3, 21).

Pretreatment of healthy erythrocytes with lethal concentrations of monensin A had different effects on subsequent parasite growth, depending on the period and pretreatment time. While a 24-h treatment 1 day before malarial infection did not produce irreversible changes in the host cells, pretreatment as long as 48 h appeared to make the host erythrocyte less suitable for parasite growth (data not shown). Ionic changes or energetic depletion could be responsible for these effects. Pretreatment with ionophore for 24 h before infection, leading to slightly higher levels of radioactive hypoxanthine incorporation, could indicate a more efficient invasion process rather than an effect on parasite growth. Indeed, *P. falciparum*-infected erythrocytes possessed the same sensitivity (in terms of IC_{50}) regardless of whether host erythrocytes were pretreated or not pretreated with ionophores (data not shown). Cations, and especially Ca^{2+}, were reported to be involved both in the production of infectious merozoites and in erythrocyte invasion by merozoites (30). In our experiments, pretreatment did not fundamentally change the susceptibilities of the erythrocytes to *P. falciparum* invasion or their ability to support parasite growth, indicating that the toxic effects of the ionophores did not occur at the host cell level. In fact, potential modifications of the host cell properties would be either reversible after drug removal or would have no effect on parasite growth.

Ionophore compounds are lipophilic membrane-interacting molecules whose solubility in water is extremely limited. However, increasing the total number of normal erythrocytes by 20-fold had no effect on the IC_{50} of nigericin or monensin A, suggesting the absence of a preferential interaction (or adsorption) of ionophores with uninfected erythrocytes. Alternatively, the absence of an effect of increasing levels of parasitemia on the IC_{50} suggests that ionophores are not adsorbed at the infected erythrocyte surface and that no accumulation in a specific parasite compartment could occur (Fig. 3B). Instead, the in vitro antimalarial effect is directly related to the drug concentration in the medium. On the other hand, irrespective of the compound class and the compounds tested, the increased serum concentration always induced a proportionally related shift in the IC_{50} toward higher concentrations, i.e., an apparent decrease in in vitro antimalarial activity (Fig. 3C), due to strong binding of ionophore molecules to serum proteins. While a higher hematocrit would not be an obstacle for in vivo antimalarial activity, serum protein binding could limit the effects of these kinds of antimalarial drugs under in vivo conditions.

Until now, only three natural ionophore compounds (which are commercially available), i.e., salinomycin, lasalocid A, and gramicidin D, have been tested in vivo against *Plasmodium berghei* or *Plasmodium yoelii*. The first two compounds were shown to be inactive or weakly active (24), and gramicidin D was found to be active against *P. yoelii* when it was administered subcutaneously, with an ED_{50} of 0.75 mg/kg, but the TI was not indicated (20). In addition, three synthetic monensin urethane derivatives that possess a very strong affinity for K^+ were found to be moderately active against *P. berghei* after oral administration (26).

With the aim of obtaining reliable comparative data, selected ionophores were tested in vivo under similar conditions for their antimalarial activities against *P. vinckei petteri* (or *P. chabaudi*)-infected mice. These two mouse-derived strains were chosen because of their marked preference for invading mature erythrocytes and their high degree of synchronization, indicating that they are closer to *P. falciparum* than *P. berghei* or *P. yoelii* (2). After intraperitoneal administration, the acute LD_{50} generally ranged from 4 to 80 mg/kg; however, gramicidin D was by far the compound with the lowest acute toxicity (LD_{50}, 482 mg/kg). Lonomycin A, nigericin, narasin A, monensin A, and lasalocid A had moderate TIs (between 2 and 6) after intraperitoneal administration. Monensin A methyl ether, 5-bromo lasalocid A, and gramicidin D were very active, with good or very good TIs of 12, 14 to 22 (depending on the strain), and 344, respectively. They were also curative, since no parasites were detected after 60 days. Interestingly, the hemisynthetic derivatives, i.e., monensin A methyl ether and 5-bromo lasalocid, were both better tolerated and had higher in vivo TIs than their natural parent products (monensin A and lasalocid A), probably due to chemical modifications.

After oral administration, the TIs of monensin A and 5-bromo lasalocid A were similar to those observed in the intraperitoneal mode. The ED_{50}s were increased by only 2.5- and 5.5-fold, respectively, in comparison to those obtained in

the intraperitoneal mode, indicating high levels of oral absorption for both compounds. On the other hand, at the highest dose tested, i.e., 100 mg/kg, gramicidin D was inactive after oral administration, likely due to its fast degradation because of its polypeptide structure.

The general conclusions concerning these ionophore antibiotics as possible antimalarial agents are therefore positive. The cytocidal effect against all parasite stages appears to be more valuable than an effect at a specific stage, such as drugs that act late in the parasite cycle, e.g., chloroquine or pyrimethamine (8), or at an early stage, such as artemisinin (18, 33).

The levels of toxicity and good TIs highlight the interesting potential for the treatment of malaria with ionophore compounds. It would now be worthwhile to test these compounds against human malarial parasites in vivo in monkeys, since we observed that a series of antimalarial compounds (which affect parasite phospholipid metabolism) had much higher TIs in *P. falciparum*-infected *Aotus* monkeys than in the murine model, probably reflecting a lower sensitivity of murine parasites to such compounds (29a).

Special attention should be given to the quasi-ionophore gramicidin D, since this compound had exceptional in vitro antimalarial activity (IC_{50}, 0.035 ng/ml) and very high differential activity in vitro (1,330) compared to its effect against mammalian cell lines (12). The present in vivo experiments revealed that this compound possesses impressive in vivo antimalarial activity, with no recrudescence, after intraperitoneal injection, with a very low ED_{50} of 1.4 mg/kg, compared to an LD_{50} of 482 mg/kg. Unfortunately, it cannot be administered orally unless a very efficient process for peptide administration is developed through the important peptide research being carried out to improve oral absorption (5).

Pharmacokinetic and metabolism issues were not addressed in the present study. Nevertheless, the fact that the ED_{50}s of gramicidin D and 5-bromo lasalocid obtained after single or double daily intraperitoneal administration were the same suggests advantageous elimination half-lives for these compounds. The broad parasite stage specificity and elimination half-lives of these compounds are properties that could be useful in developing curative antimalarial drugs.

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