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Differential In Vitro Activities of Ionophore Compounds against *Plasmodium falciparum* and Mammalian Cells

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Twenty-two ionophore compounds were screened for their antimalarial activities. They consisted of true ionophores (mobile carriers) and channel-forming quasi-ionophores with different ionic specificities. Eleven of the compounds were found to be extremely efficient inhibitors of *Plasmodium falciparum* growth in vitro, with 50% inhibitory concentrations of less than 10 ng/ml. Gramicidin D was the most active compound tested, with 50% inhibitory concentration of 0.035 ng/ml. Compounds with identical ionic specificities generally had similar levels of antimalarial activity, and ionophores specific to monovalent cations were the most active. Compounds were further tested to determine their in vitro toxicities against mammalian lymphoblast and macrophage cell lines. Nine of the 22 compounds, i.e., alborixin, lonomycin, nigericin, narasin, monensin and its methylated derivative, lasalocid and its bromo derivative, and gramicidin D, most specific to monovalent cations, were at least 35-fold more active in vitro against *P. falciparum* than against the two other mammalian cell lines. The enhanced ability to penetrate the erythrocyte membrane after infection could be a factor that determines ionophore selectivity for infected erythrocytes.

The intraerythrocytic stage of the malaria-causing parasite *Plasmodium falciparum* is the longest period in the vertebrate host and corresponds to the pathological phase of the disease. Within the erythrocyte, the parasite constantly ingests nutrients while evading the defense mechanisms developed by the host immune system. However, its intracellular location creates some obstacles that must be overcome. The parasite must have access to various extracellular nutrients and ions, while it inevitably must reject catabolites from its jail. It thus benefits from, or sets up, mechanisms within the host erythrocyte plasma membrane to promote its intracellular growth. Consequently, erythrocyte membrane changes during malarial infection allow the parasite to survive intracellularly.

As an example, erythrocytes continuously expend energy to maintain an internal ionic environment distinct from that of the external medium. After plasmodial infection, the physical properties of the erythrocyte membrane are modified; e.g., increased fluidity, decreased surface pressure (28), and new ways of being permeable appear (4, 14). The permeability of the erythrocyte membrane to alkaline cations changes, and the ionic imbalance becomes less clear. As the parasite matures, especially during the schizont stage, the cation composition of the infected erythrocyte cytosol is drastically altered. The potassium level in the host cytosol decreases, whereas that of Na^+ increases. In addition, the parasite cytosol seems to contain relatively high K^+ levels and low Na^+ levels (13, 23, 37). The Ca^{2+} content of the whole infected erythrocyte increases as the parasite matures (by 20- to 40-fold at schizogony), and this increase is almost exclusively localized in the parasite compartment (1, 21, 37). These changes may be limiting factors for intraerythrocytic parasite development since the ionic composition of the erythrocyte-infected cytosol must reach a critical level that differs from that of the uninfected erythrocytes to allow parasite proliferation. Modification of the cytosol concentration by the addition of Ca^{2+} ionophores, e.g., A23187 or ionomycin (38, 41), irreversibly arrests parasite development. In the same way, depletion of extracellular Ca^{2+} from the culture medium with a Ca^{2+} chelator (42) or the use of Ca^{2+} antagonists (22, 42) also prevents *Plasmodium* development. The calcium level therefore must be finely controlled so as not to be cytotoxic to the intracellular parasite.

Ionophores are well-known biochemical tools that can be used to modify cation concentrations in cells. Therapeutic applications have been reported in the veterinary field for carboxylic true ionophores, which are widely used for the treatment of poultry coccidiosis (36). These ionophores are also important in livestock farming for improving cattle feed (15).

The question then arises as to whether ionophores that allow the transfer of ions from one side of the membrane to the other can systematically and specifically affect intracellular malarial parasite viability. Owing to their lipophilicities, these compounds are incorporated into membranes and facilitate the diffusion of ions such as H^+, Na^+, K^+, and Ca^{2+}; this then leads to ionic gradient and content modifications. Most ionophores are natural products isolated from microorganisms; some of them are modified by hemisynthesis. The most useful classification for biologists is that of Pressman (30), which distinguishes true ionophores (acting as mobile carriers) from the quasi-ionophores (channel-forming compounds) according to their mechanisms of action. Different types of ionophores lead to marked ionic selectivity and transport rate differences. Neutral ionophores, such as valinomycin, catalyze diffusion through membranes of alkaline cations, generally K^+, according to the membrane potential. Carboxylic ionophores are subdivided into different classes according to their cation selectivities and structures (43) and act through an antiport mechanism

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with H^+. Quasi-ionophores form channels that cross the membrane, allowing much faster ion diffusion but lower ionic selectivity than those for true ionophores (30).

The objective of the study described here was to investigate the effects of 22 ionophores with different chemical structures, ionic selectivities, and mechanisms of action on the intracellular parasite by systematic screening. Compounds with good antimalarial activities were then tested against two mammalian cell lines to determine the possible selectivities of these compounds toward infected erythrocytes.

The results of the present study indicate that ionophores carrying monovalent cations are the most active against the in vitro growth of *P. falciparum* and present selective toxicity in comparison with their toxicities against mammalian cells. Their selective actions could partly be explained by the higher amounts of ionophore integrated into the membranes of *Plasmodium*-infected erythrocytes.

MATERIALS AND METHODS

Chemicals. Nonactin, valinomycin, nigericin (sodium salt), salinomycin, ionomycin, calcimycin, alamethicin, gramicidin S, and gramicidin D were obtained from Sigma Chemical Co. (St. Louis, Mo.). The other ionophore products were stock samples from the Laboratoire de Chimie Organique Biologique, Centre National de la Recherche Scientifique URA 485. Most of them were in the acid form, namely, cationomycin, monensin A, nigericin, grisorixin, narasin A, alborixin, lonomycin A, lasalocid A, and X14547 A, whereas narasin, lysocellin, and X14885 A were sodium salts. Monensin A methyl ether (25-*O*-methylmonensin), monensin A lactone (25-dehydroxymethyl-25-oxomonensin) (11), and 5-bromolasalocid (44) were obtained by hemisynthesis as described previously. [G-^{3}H]hypoxanthine and [^{3}H]thymidine were purchased from Amersham Corp. (Les Ulis, France). RPMI 1640 medium was obtained from Gibco Laboratories (Eragny, France). Complete medium consisted of RPMI 1640 medium supplemented with HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (25 mM; pH 7.4), and 10% type AB^+ serum. All other reagents were of analytical grade.

Biological materials. Human blood and type AB^+ human serum came from the local blood bank. The Nigerian strain of *P. falciparum* (32) 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 (17). The levels of parasitemia were routinely monitored on blood smears by using a 10% Giemsa azure type B stain in phosphate buffer (pH 7.2).

Human Jurkat lymphoblasts and U937 macrophage cell lines were provided by J. Dornan (Institut National de la Santé et de la Recherche Médicale U431, Montpellier, France), and cells were routinely cultured at 37°C in RPMI 1640 medium complemented with 50 μM β-mercaptoethanol, 1 mM fresh glutamine, and 10% fetal calf serum (Gibco).

In vitro antimalarial activity against *P. falciparum*. The effects of the drugs tested on the growth of *P. falciparum* in vitro were measured in microtiter plates as described by Desjardins et al. (6); the incorporation of radioactive hypoxanthine (precursor of purine base of *P. falciparum* through the purine salvage pathway [12]) into nucleic acids was used as a viability measurement. 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 and 0.3 to 0.8% parasitemia). 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 period. The cells were then subsequently lysed, and the parasite macromolecules, including radioactive nucleic acids, were retained on glass fiber filters (Whatman GF/C) by using an automatic cell harvester (Skatron "Macro 96"). The filters were then counted for radioactivity after adding 2 ml of scintillation cocktail (Packard) in a Beckman 5000 liquid scintillation spectrometer. The radioactive background was obtained from incubations of normal erythrocytes under the same hematocrit and medium conditions. Growth inhibition in the presence of the different compounds was expressed as a percentage of the level of parasitemia observed in the controls without drug. The IC_{50}s, which represent the drug concentrations required to inhibit parasite growth by 50%, were evaluated from the plot of log dose versus parasite growth expressed as a percentage of the value for the control and are the means of at least two independent experiments performed in triplicate with different stock drug solutions.

To check the effects of the various experimental conditions, we first investigated the antimalarial activity of a carboxylic ionophore, nigericin, when it was added in acid form or as a Na^+ salt in various solvents, either dimethyl sulfoxide (DMSO), ethanol, or DMSO-ethanol (50:50; vol/vol). In each case, stock drug solutions were then diluted in complete medium so that the concentration of the solvent never exceeded 0.25%. The IC_{50} of each form of the compound and the stock solvent solution was determined. The effect of the drug form (acid or Na^+ salt) was also studied for narasin, another class 1a carboxylic ionophore. In all cases, regardless of the initial forms (Na^+ salt or acid) and stock solution solvents (i.e., DMSO, DMSO-ethanol, or ethanol), no significant differences in the IC_{50}s were observed (e.g., the nigericin IC_{50} ranged between 0.7 and 1.1 ng/ml, irrespective of the experimental conditions). Viability tests were thus performed with stock ionophore solutions, usually in the acid form, solubilized in DMSO; valinomycin, however, was solubilized in ethanol.

In vitro toxicities against U937 macrophages and lymphoblast cell lines (Jurkat strain). For both cell types, the effects of the drugs on cell viability were measured in microtiter plates following [^{3}H]thymidine incorporation into the DNAs of the cell suspension (6,000 cells per well). Cell suspensions (150 μl) were exposed to various drug concentrations (50 μl in medium culture) for 24 h at 37°C, and then 30 μl of [^{3}H]thymidine (0.75 μCi per well) was added for an additional 5- to 6-h period. The cells were then lysed, and the eukaryotic cell macromolecules including radioactive nucleic acids were recovered on glass fiber filters by using the cell harvester as described earlier. Cell viability was expressed as MV_{50} or LV_{50}, which is the drug concentration leading to 50% inhibition of [^{3}H]thymidine incorporation into the DNA of macrophages or lymphoblasts, respectively.

RESULTS

Effects of the different classes of ionophores on *P. falciparum* growth in vitro. The effects of 22 different ionophores belonging to both classes (true ionophores and quasi-ionophores) were evaluated by incubating nonsynchronized *P. falciparum*-infected erythrocyte suspensions in their presence for 48 h, corresponding to a full asexual parasite multiplication cycle. Their IC_{50}s are listed in Table 1. All 22 compounds possessed antimalarial activities, with IC_{50}s ranging between 2,000 ng/ml (micromolar range) and 0.035 ng/ml. For most of them, the antimalarial effect occurred over a very narrow concentration range (Fig. 1), which probably indicates a very precise target with a specific effect rather than an overall disturbance of parasite metabolism. Eleven ionophores were shown to be highly active in inhibiting *P. falciparum* growth in vitro, with their IC_{50}s being less than 10 ng/ml (generally in the nanomolar range).

In the neutral true ionophore class, the cyclic dodecadepsipeptide valinomycin was very active, with an IC_{50} of 5.3 ng/ml, whereas nonactin, a cyclic polyester, exhibited significantly lower activity, with an IC_{50} of 440 ng/ml.

Carboxylic ionophores specific to monovalent cations (class 1a) were among the most active of the ionophores tested against *P. falciparum*, with antimalarial activity generally being found at a concentration of about 1 ng/ml. In this class, alborixin presented the highest antimalarial activity (IC_{50} = 0.6 ng/ml). Lonomycin was also very active, with an IC_{50} of 1.4 ng/ml. Nigericin, regardless of the form (acid or sodium salt), and a compound very closely related to nigericin, grisorixin, which lacks a hydroxyl group (a hydroxyl group is present in nigericin at position 29), were also very active in the same concentration range (IC_{50}s = 1 ng/ml). Narasin, regardless of the form (acid and sodium salt), and salinomycin (a close derivative of narasin that is methylated at position 4) were active to similar extents, with IC_{50}s of 1 and 2.8 ng/ml, respectively. By contrast, cationomycin, a polyether antibiotic consisting of an unusual aromatic ester, was moderately active, with an IC_{50} of 35 ng/ml.

In class 1a, monensin is a unique polyether antibiotic that is more specific to sodium, differing from other polyether antibiotics that are more specific to potassium (30). Nevertheless, this Na^+-specific ionophore also showed potent antimalarial activity, with an IC_{50} of 1.5 ng/ml. We also tested hemisynthetic derivatives of monensin, monensin methyl ether, and monensin lactone, which were reported to be equally specific to Na^+ and K^+. Only the methyl ether derivative was very active against *P. falciparum*, with an IC_{50} of 6.5 ng/ml, whereas the

TABLE 1. Differential effects of the various ionophore classes against in vitro growth of *P. falciparum* and two mammalian cell lines[a]

Compound	Selectivity[b]	IC_{50} (ng/ml) for *P. falciparum*	MV_{50} (ng/ml) for macrophage U937	LV_{50} (ng/ml) for Jurkat lymphoblast	In vitro differential activity for U937 and Jurkat cells
Neutral true ionophores					
Nonactin	$K^+ > Na^+$	440	—[c]	—	—
Valinomycin	$K^+ >>> Na^+$	5.3	2.4	0.88	0.4/0.2
Carboxylic true ionophores					
Class 1a					
Alborixin	$K^+ > Na^+$[d]	0.6	23.5	45	39/75
Lonomycin A	$K^+ > Na^+$	1.4	235	330	168/236
Nigericin	$K^+ >> Na^+$	1	193	90	193/90
Grisorixin	$K^+ > Na^+$	0.9	25	140	28/155
Narasin A	$K^+ > Na^+$	1	305	500	305/500
Salinomycin	$K^+ > Na^+$	2.8	—	—	—
Cationomycin	$K^+ > Na^+$[e]	35	40	—	1.1
Monensin A	$Na^+ >> K^+$	1.5	64	175	43/117
Monensin A methyl ether	$K^+ \sim Na^+ \sim 0$[f]	6.5	350	600	54/92
Monensin A lactone	$K^+ \sim Na^+ \sim 0$[f]	340	>10,000	—	>29
Class 2					
Lysocellin	$K^+ > Na^+; > Mg^{2+}\ Ca^{2+}$	7.5	70	330	9.3/44
Lasalocid A (X537A)	$K^+ > Na^+; Ca^{2+} > Mg^{2+}$	28	1,000	1,400	35.7/50
5-bromolasalocid A	$Ca^{2+} > Mg^{2+}$[g]	69	2,550	5,000	37/73
Ionomycin	$Ca^{2+} > Mg^{2+}$	260	—	—	—
Class 3					
A 23187 (calcimycin)	$Ca^{2+} > Mg^{2+}$	150	—	—	—
X14885 A	$Ca^{2+} > Mg^{2+}$[h]	205	260	300	1.3/1.5
X14547 A	$Ca^{2+} > Mg^{2+}$[i]	275	2,000	1,400	7.3/5
Channel-forming quasi-ionophores (peptides)					
Alamethicin	C > A[j]	2,000	—	—	—
Gramicidin S	$H^+ >> K^+ > Na^+$	667	5,400	4,800	8/7.2
Gramicidin D	$H^+ >> K^+ > Na^+$	0.035	28	65	800/1,857

[a] All drugs except valinomycin were dissolved in DMSO. Valinomycin was dissolved in ethanol. Cell viabilities were measured by using labelled hypoxanthine or thymidine as described in Materials and Methods. The MV_{50}/IC_{50} or LV_{50}/IC_{50} ratios correspond to the differential activities of the compounds between mammalian cells (macrophages or lymphoblasts, respectively) and *P. falciparum.*

[b] Selectivity was determined by Pressman (30) and Westley (43) unless indicated otherwise. Only the biologically important cation selectivities are reported. C and A stand for mono- or divalent cation and anion, respectively.

[c] —, not determined.

[d] Selectivity was determined by Chapel et al. (5).

[e] Selectivity was determined by Ubukata et al. (39).

[f] Selectivity was determined by Gaboyard et al. (11).

[g] Selectivity was determined by Juillard et al. (18).

[h] Selectivity was determined by Albrecht-Gary et al. (2).

[i] Selectivity was determined by Bolte et al. (3).

[j] Selectivity was indicated by Woolley and Wallace (45).

lactone derivative was much less active, with an IC_{50} of 340 ng/ml.

Class 2 carboxylic true ionophores are less specific to the cation valence; i.e., they are able to complex both mono and divalent cations. Except for one compound, lysocellin, with an IC_{50} of 7.5 ng/ml, the compounds of this class were less active than most class 1a ionophores against the human parasite. Indeed, lasalocid and its bromo derivative had significantly lower antimalarial activities, with IC_{50}s of 28 and 69 ng/ml, respectively. Ionomycin, which is more specific to Ca^{2+}, was the least active compound of this class, with an IC_{50} of 260 ng/ml. Class 3 ionophores (A23187, X14885, and X14547), which are specific to divalent cations (Ca^{2+} and Mg^{2+}), were much less active against *P. falciparum*, with IC_{50}s ranging between 150 and 275 ng/ml.

We also tested quasi-ionophore compounds, gramicidins, either cyclic (S) or linear (D), and alamethicin, another special linear peptide. These channel-forming peptides reportedly have lower ionic selectivities (30). Alamethicin and gramicidin S were not very active, with IC_{50}s of 2,000 and 667 ng/ml, respectively, whereas gramicidin D with an IC_{50} of 0.035 ng/ml, was shown to be the most potent antimalarial agent of all the ionophores tested in the present study.

Effects of the different ionophores on two mammalian cell lines. We then investigated the toxicities of the different ionophores against two human cell lines, U937 macrophages and Jurkat lymphoblasts, which, like *P. falciparum*, show rapid cell division. The inhibitory effects in vitro were expressed as MV_{50}s and LV_{50}s, as measured by thymidine incorporation. Figure 1 shows the effects of a quasi-ionophore (gramicidin D)

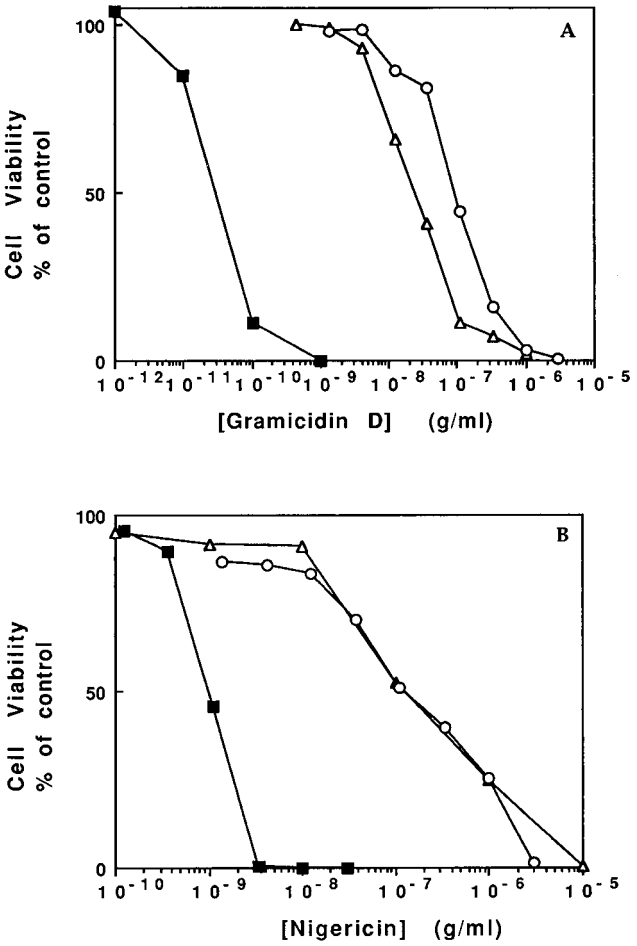

FIG. 1. Inhibition of *P. falciparum*, lymphoblast, and macrophage viability by gramicidin D (A) and nigericin (B). Antimalarial activities (■) were measured by using *P. falciparum*-infected erythrocytes (2% final hematocrit and 0.5% parasitemia) incubated for 48 h at 37°C in the presence of the drug. Inhibitory effects on lymphoblast (○) or macrophage (△) growth were assessed after 24 h of drug contact, with 6,000 cells per well. Cell viabilities were assessed by measuring [^{3}H]hypoxanthine (*P. falciparum*) or [^{3}H]thymidine (macrophage or lymphoblast) incorporation into nucleic acids for 18 to 20 and 6 h, respectively, as described in Materials and Methods. The results are expressed as a percentage of the incorporation of the radioactive precursor observed in controls incubated in the absence of drug and are means of typical experiments carried out in triplicate.

and a true ionophore (nigericin) on the viability of U937 macrophages and lymphoblast cells relative to their in vitro antimalarial activities. As already noted, gramicidin D was extremely active against the in vitro growth of *P. falciparum*, with an IC_{50} of 0.03 ng/ml (Fig. 1A). By contrast, inhibition of macrophage and lymphoblast cell growth occurred at much higher concentrations, with an MV_{50} and an LV_{50} of 23 and 90 ng/ml, respectively, i.e., about 1,000-fold higher than the IC_{50}. Similarly, the activity of nigericin against the two mammalian cell lines occurred at a high concentration, with an MV_{50} and an LV_{50} of 110 ng/ml, i.e., 100-fold higher than the antimalarial activity (IC_{50} = 1 ng/ml) (Fig. 1B). These tests were extended to the other ionophores, and the results are summarized in Table 1. The LV_{50} and the MV_{50} were very close for all but two of the compounds tested. The LV_{50} and MV_{50} were significantly different only for grisorixin and lysocellin (by five- to sixfold).

To compare the toxicities of the ionophores against mammalian cell lines (either macrophage or lymphoblast cell lines) and against *P. falciparum*, we introduced the MV_{50}/IC_{50} and LV_{50}/IC_{50} ratios, corresponding to in vitro differential ionophore activity between mammalian cell lines and *P. falciparum*. Only valinomycin (neutral ionophore) was found to be slightly more toxic against mammalian cells than against *P. falciparum*, while cationomycin (class 1a) and X14885 (class 3) had similar toxic effects. By contrast, all of the other compounds clearly inhibited mammalian cell proliferation at concentrations significantly higher than those required to inhibit the intracellular growth of *P. falciparum*, indicating their lower levels of toxicity against mammalian cells. Class 1a carboxylic polyether antibiotics (except cationomycin) exhibited lower levels of toxicity against mammalian cells compared with their antimalarial activities (by 30- to 300-fold for the macrophage cell line and by 75- to 500-fold for the lymphoblast cell line). Among them, three compounds, i.e., lonomycin, nigericin, and narasin, presented very low levels of toxicity against mammalian cells compared with their antimalarial activities, with MV_{50}s and LV_{50}s being at least 168- and 90-fold higher than the IC_{50}s, respectively. Note that similar differential activities between mammalian cell lines and *P. falciparum* were obtained for monensin and its derivatives, even though these compounds exert their toxic effects at quite different concentrations.

For quasi-ionophore compounds, gramicidin S presented weak in vitro differential activity against the two mammalian cells (about eightfold). On the contrary, gramicidin D, the most active compound tested against *P. falciparum*, was much less toxic than the other ionophores tested against mammalian cells (with a differential activity as high as 800-fold between macrophages and *P. falciparum* and 1,857-fold between lymphoblasts and *P. falciparum*).

Nine compounds, namely, alborixin, lonomycin, nigericin, narasin, monensin and its methylated derivative, lasalocid and its bromo derivative, and gramicidin D, showed high levels of antimalarial activity, while they exerted a low level of toxicity on mammalian cells, with in vitro MV_{50}/IC_{50} and LV_{50}/IC_{50} ratios being above 35. The high in vitro ratio indicates a difference in the respective sensitivity thresholds of both kinds of cells and highlights the selectivities of these compounds against *Plasmodium*-infected erythrocytes.

DISCUSSION

Until now, ionophores have only been studied sporadically for their antimalarial properties (7, 24, 27, 41). They have been tested under different assay conditions, according to different objectives and applications, thus complicating comparisons. For instance, the quasi-ionophore gramicidin D (channel-forming compound) was tested by three groups, but the reported IC_{50} varied from 2 ng/ml (24, 27) to 0.02 ng/ml (7), i.e., a 100-fold difference.

Our preliminary studies (see Material and Methods) showed that nigericin had similar activities regardless of the form of drug presentation (acid or Na^+ salt) and the solvent (DMSO or ethanol) used for the stock solutions. The culture and test conditions (strains, hematocrit, initial level of parasitemia, duration of drug contact) and also the different susceptibilities of the different stages of the parasite could be partially responsible for these discrepancies (31). We thus incubated infected erythrocytes with the drug for a complete parasite cycle before measuring the viability of the parasite. The advantage of the present work was that a main series of ionophores with a wide range of ionic selectivities and different mechanisms of action was tested, thus enabling comparative studies under clearly defined and comparable conditions.

The most active compounds ($IC_{50} \leq 5$ ng/ml, i.e., in the nanomolar range) were ionophores specific to monovalent cations (class 1a), with most of them having a marked preference for K^+. Concerning the two neutral ionophores specific to K^+, only valinomycin, a cyclic dodecadepsipeptide that is a K^+-specific compound (29), was active against *P. falciparum* in the nanomolar range. Among the class 1a ionophores, monensin was the only one which was more selective for Na^+ than for K^+. However, this compound was also very active against *P. falciparum*, with an IC_{50} of 1.5 ng/ml. The monensin derivatives monensin methyl ether (which possesses one intramolecular bond for cyclization) and monensin lactone (in which the cyclization ability of the chain is completely suppressed) (11) had antimalarial activities 4- and 200-fold lower than that of monensin, respectively. The antimalarial activities of monensin and its derivatives thus seem to be correlated with ionophore capacity. Interestingly, the same differential activities of monensin and its two derivatives were obtained for their bacteriostatic activities against *Bacillus cereus* (11).

Class 3 carboxylic ionophores, which can be considered divalent cation ionophores, had moderate antimalarial activities (in the micromolar range). This was also true for ionomycin, a class 2 compound, which also showed a clear preference for Ca^{2+}. Conversely, other class 2 compounds that are able to transport both mono and divalent cations, such as lysocellin and lasalocid, had intermediate antimalarial activities (7.5 and 28 ng/ml, respectively).

Among the three quasi-ionophores tested, two of them, alamethicin and gramicidin S, showed weak antimalarial activity at approximately 1 μg/ml (micromolar range). In contrast, gramicidin D showed extremely potent antimalarial activity, with an IC_{50} of 0.035 ng/ml (~0.01 nM). Gramicidin S is a cyclic compound (10, 16), while gramicidin D and alamethicin have linear structures (34, 45). The main differences between alamethicin and gramicidin D concern their ionic specificities and their voltage-dependent conductivities (34, 45).

Overall, compounds that reportedly modify the contents of divalent cations (Ca^{2+} and Mg^{2+}) kill the parasite at much higher concentrations than compounds that are specific to monovalent cations. True ionophores specific to monovalent cations have higher antimalarial activities (in the nanomolar range). Among the quasi-ionophores, the linear peptide specific to H^+, gramicidin D, was found to be exceptionally active against *P. falciparum*.

The chemotherapy approach is based on compounds that exert greater selective toxicity against the intracellular parasite compared with that against other cells in the mammalian host. Apart from grisorixin and lysocellin, which had LV_{50}s and MV_{50}s that differed by up to sixfold, the in vitro toxic effects against macrophage and lymphoblast cell lines were observed at similar ionophore concentrations. For the 17 ionophores tested, it was striking that there was no correlation between their inhibitory effects on parasite growth and on macrophage and lymphoblast cell viabilities.

Differential ionophore activity between mammalian cells and infected erythrocytes was expressed by the MV_{50}/IC_{50} and LV_{50}/IC_{50} ratios (as defined in the Results), highlighting the general characteristics of each class of carboxylic ionophores. Class 3 compounds (specific to divalent cations) presented weak ratios (<10). Among the class 2 compounds tested, lasalocid and its bromo derivative had good ratios (~50), whereas lysocellin had a lower ratio (~10). By contrast, all class 1a compounds (except cationomycin), and especially lonomycin, nigericin, and narasin, had much higher ratios (between 30 and 500), highlighting their very low levels of toxicity against mammalian cells. The highest in vitro ratios were obtained for gramicidin D, with values of about 1,000. These exceptionally high values seem to be specifically related to an exceptional toxic effect of this drug on *Plasmodium*-infected erythrocytes.

The reasons for the specific susceptibility of the intracellular parasite to most of the ionophores remain to be determined. After infection, the magnitude of the negative membrane potential decreased in the erythrocyte plasma membrane along with the transmembrane monovalent gradient (25, 26). Both of these fluctuations would decrease the efficiency of transport by an ionophore in this membrane. Conversely, there are many possible complex reasons for this higher degree of susceptibility of *Plasmodium*-infected erythrocytes. The localization of true ionophores in the internal organelles of eukaryotic cells has been reported (20), and it is likely that some ionophores will have significant access to the parasite and modify the ionic contents of the various compartments in infected erythrocytes. The existence of direct access of the intracellular parasite to the extracellular medium, either by diffusion or transport through a metabolic window or parasitophorous duct (8), is still controversial, but if such direct access exists, ionophore compounds could be directly internalized, thus affecting the parasite's ionic contents and then its growth. The new ionic gradient established between the parasite cytosol and erythrocyte cytosol is another target for ionophore action. The nature of the cation that is carried appears to be very important, since the best differential activity was observed for ionophores able to carry monovalent cations. Moreover, the sudden change in the erythrocyte's ionic composition after infection probably obliged the parasite to use considerable energy to maintain normal ionic gradients instead of using this energy for growth. This would probably result in the release of massive amounts of lactic acid, since only glycolysis provides ATP molecules for the whole infected erythrocyte (33).

After interaction with the membrane, cation transport by a true ionophore is a multistep process involving complexation of the cation at one water-membrane interface, diffusion of the complex through the membrane, and its dissociation at the other interface. Ionophore incorporation and transport efficiency depend on the structural and physical characteristics of the membrane, e.g., its surface pressure in relation to its lipid and protein composition. The absolute ionic transport rate could thus be increased after membrane fluidization, which occurs in erythrocytes after infection (40), and ionophore molecules could be more able to interact with the erythrocyte membrane after infection or with the parasite membrane. As an example, surface pressures in the outer leaflet of membranes of normal and infected erythrocytes were shown to be 33 and less than 20 mN/m, respectively (28). Using monolayer experiments at a constant surface (35), we have shown that nigericin (specific to K^+), monensin (specific to Na^+), and 5-bromolasalocid exhibited strong interactions with lipid membranes. Their degree of penetration into the phosphatidylcholine monolayer indicated that, at identical ionophore concentrations, there is at least threefold more ionophore present in monolayers with a surface pressure of 20 mN/m than in monolayers with a surface pressure of 33 mN/m (data not shown). The surface pressure of usual mammalian cells is in the same range as the one observed in erythrocytes (between 30 and 40 mN/m) (35). Therefore, a much higher ionophore interaction with and a greater degree of penetration into the erythrocyte membrane are then expected after infection.

The extremely potent antimalarial activity and specificity of gramicidin D warrant consideration of its mechanism of action. Ion rate diffusion was reported to be 10,000-fold higher than that of a neutral true ionophore (30). The ionophore activity of

gramicidin D depends on its ability to form channels and on the stability of the channel structure in the membrane. Channel formation by gramicidin D and its stability are highly dependent on the membrane composition and structure, including its surface pressure (19). For instance, the channel lifetime of gramicidin D increases as the lipid bilayer pressure decreases (9). Moll et al. (27) have shown that the K^+ loss induced by gramicidin was greater in erythrocytes after plasmodial infection, corroborating a stronger effect on infected erythrocytes. The surface pressure of the erythrocyte membrane decreases after infection, and this corresponds to conditions that favor the integration, channel formation, and stability of gramicidin D, leading to enhanced ionic conduction.

In summary, the systematic in vitro screening of the various classes of current ionophores has enabled the selection of nine compounds on the basis of their high levels of antimalarial activity and relatively low levels of toxicity against macrophage and lymphoblast cell lines. The most active compounds were reported to be specific to monovalent cations and include six class 1a and two class 2 carboxylic true ionophores and gramicidin D, a channel-forming quasi-ionophore that was also the most active compound tested, with an IC_{50} of 0.035 ng/ml. Selectivity for infected erythrocytes could involve modified membrane properties, especially the surface pressure of the plasma membrane (which is decreased after infection). The actions of these compounds will be further studied in vitro to specify their mechanisms of action and in vivo to assess their activities and tolerance in the murine model of malaria.

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