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# Anion Transport Inhibitors as Suppressors of *Plasmodium falciparum* Growth in *in Vitro* Cultures

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SUMMARY

A series of inhibitors of red blood cell anion-transport were tested as possible inhibitors of intraerythrocytic growth of *Plasmodium falciparum* parasites in *in vitro* human cultures. Two classes of compounds were used in this study: Class I, hydrophilic compounds which are impermeant to uninfected cells (e.g., disulfonic stilbene derivatives and phlorizin), and Class II, relatively lipophilic compounds to which membranes are permeable (e.g., phloretin, furosemide, niflumic acid,and indacrinone. (MK-196). Good correlation between the inhibitory power on anion transport and on parasite growth was obtained for the reversibly acting 4,4′-dinitro-2,2′-stilbenedisulfonic acid and the irreversibly acting analogue, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid. Phlorizin; a potent inhibitor of sugar transport but a rather poor inhibitor of anion transport, was the most efficient antimalarial agent. Alternatively, niflumic acid and MK-196, both good inhibitors of anion transport, and phloretin, a potent inhibitor of anion and sugar transport, were relatively less efficient in arresting parasite growth. Studies of growth rates and of metabolic activities conducted with synchronously grown cultures indicate that the Class I inhibitors arrest parasite development and propagation by the two following modes of inhibition: at the host cell membrane level, by blocking the anion transport (Mode A), and at the cellular level, by direct interference with schizont development and propagation (Mode B). The latter is made possible by an observed permselectivity change in the host cell membrane, which appears after trophozoite formation and persists throughout schizogony. Stilbene disulfonates act by both modes, whereas phlorizin and niflumic acid act primarily by Mode B. Our work indicates that anion transport inhibitors which act in a reversible fashion and which are impermeant to uninfected cells are useful antimalarial agents in *in vitro* systems. The work provides a rational basis for the design of drugs of potential therapeutic use.

## INTRODUCTION

The development of malaria parasites inside red blood cells depends on a metabolically balanced intraerythrocytic environment. The host cell, in general, and its membrane, in particular, play a major role in maintaining this environment by providing routes of supply of nutrients and electrolytes and the means for disposing of the catabolites produced by the developing parasites. In this work we explored new means for selectively impairing intraerythrocytic parasite growth, primarily by the use of agents which are demonstrably impermeant to uninfected human red blood cells and whose known action on these cells is the blockade of red cell anion permeability (1, 2).

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Anion transport is the predominant red blood cell membrane function involved in the $Cl^-$-$HCO_3^-$ exchange which subserves the removal of $CO_2$ from tissues to lungs (see refs. 1 and 2 for review). In addition, this membrane transport system provides red cells the only access route for other important inorganic anions such as phosphate and sulfate, as well as a minor route for some amino acids (e.g., glycine and cysteine) (3) and for catabolites such as lactate (4, 5). Since the development of parasites demands a relatively high input of nutrients and output of catabolites (6), we hypothesized that partial blockade of the above routes by agents impermeant to uninfected cells should have deleterious effects on parasite growth, while marginally affecting other cells (7).

In this work we present evidence for the antimalarial activity of red blood cell anion transport blockers such as the highly membrane-specific impermeant disulfonic stilbenes (8) and the less specific niflumic acid, a nonsteroidal anti-inflammatory drug (9), phlorizin, and phlore-

tin (1), as well as the diuretics furosemide (10) and MK-196[1] (11). We also attempted to determine the *modus operandi* of apparently impermeant agents by studying their effect on anion transport, glucose consumption, lactic acid production, and amino acid incorporation into parasitized cells at defined stages of parasite growth. The results of this work indicate that the inhibition of intraerythrocytic parasite growth by disulfonic stilbenes and phlorizin can only partially be explained by direct blocking of host membrane anion transport, which indirectly affects the metabolic activity of parasitized cells. The additional mode of action of these agents is by their direct interference with intracellular events occurring at the advanced stages of parasite growth (trophozoite and schizont stages). In these stages a discrete change in the permselectivity properties of the host cell membrane makes the intracellular compartment demonstrably accessible to the otherwise impermeant substances (12).

## MATERIALS AND METHODS

### *Chemicals*

The disulfonic stilbenes DADS, DIDS, $H_2$DIDS, DNDS, and [$^3$H]$H_2$DIDS were synthesized according to previously published methods (8, 13). NBD-Taurine was prepared as described before (14). Other chemicals were from the following sources: phloretin, K & K Chemicals; RPMI-1640, GIBCO: phlorizin, Fluka; furosemide, Farbwerke Hoechst AG; MK-196 was a generous gift from Merck, Sharp & Dohm (Rahway, N. J.). Other chemicals were of the best available grade.

### *Cultures*

*Plasmodium falciparum* was grown in 75 cm$^2$ screw-capped flasks (Nunc) with human erythrocytes (2–2.5% hematocrit) and the growth medium of Trager and Jensen (15) supplemented with inosine (1 mM) and heat-inactivated human plasma (AB$^+$). Medium was changed daily and flushed with the following gas mixture: $N_2$, 90%; $CO_2$, 5%; and $O_2$, 5%. Enrichment of parasitized cells (trophozoites and schizonts) was performed on Ficoll-Hypaque cushions (16). Synchronization of growth was accomplished by a recently published method (12). The percentage of infected cells (i.e., parasitemia) was determined on thin smears stained with Giemsa stain by counting at least 1000 cells.

### *Chemical Modification of Cells*

Red blood cells (infected or uninfected) were washed with PBS containing 20 mM phosphate/135 mM NaCl (pH 7.4) and were allowed to react with DIDS, $H_2$DIDS, or [$^3$H]$H_2$DIDS at the indicated concentrations for 30 min at 37° and at a final hematocrit of 10%. The cells were subsequently washed with PBS and with growth medium. All of these steps were conducted under sterile conditions.

[1] The abbreviations used are: MK-196, indacrinone; DADS, 4,4′-diamino-2,2′-stilbenedisulfonic acid; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; DNDS, 4,4′-dinitro-2,2′-stilbenedisulfonic acid; $H_2$DIDS, 4,4′-diisothiocyano-2,2′-dihydrostilbenedisulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline (20 mM $NaH_2PO_4$/140 mM NaCl, pH 7.4); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

### *Effects of Inhibitors on Parasite Growth*

*Asynchronous cultures.* Reversibly binding inhibitors were added to the growth medium at the concentrations indicated and were present throughout all periods of growth unless otherwise stated. Irreversibly binding inhibitors were used to chemically modify uninfected cells, as shown above. Infection was initiated by adding an inoculum of infected cells. Parasite growth was assessed as shown above and is given as percentage of parasitemia (namely, the percentage of cells in culture containing parasites).

*Synchronous cultures.* Inhibitors were added to cultures at defined stages of parasitic growth. Reversibly binding inhibitors were present during all subsequent growth periods, whereas irreversibly binding inhibitors were used as described under Chemical Modification of Cells.

### *Effect of Inhibitors of Anion Transport*

Anion transport and its susceptibility to irreversibly binding inhibitors were assessed with the aid of the anion fluorescent probe NBD-taurine and the method of continuous monitoring of transport by fluorescence (14, 17). Briefly stated, cells were loaded with NBD-taurine (1 mM final concentration) in PBS medium (1 hr, 37°, 10% hematocrit). Prior to efflux measurements, the extracellular probe was removed by repeated washes in Hepes medium (150 mM Hepes, pH 7.4, 0°). Aliquots of cells (~$10^7$ in 10 μl of Hepes medium) were injected into cuvettes containing 2 ml of PBS (± inhibitor) and placed in the 37° thermostated cuvette compartment of an MPF-4 Perkin-Elmer spectrofluorimeter. Efflux was monitored spectrofluorimetrically on four parallel systems. Rate constants were computed from nonlinear regression analysis of the exponential fluorescence traces with the aid of a Wang PCSII system. In a few instances the efflux medium PBS contained either inactivated human plasma or human serum albumin (Sigma Chemical Company).

### *Effect of Inhibitors on Amino Acid Incorporation into Proteins*

Short-term and long-term effects of inhibitors on parasite development were assessed, after removal of the extracellular probe (18), by [$^3$H]isoleucine incorporation into trichloroacetic acid-precipitable material.

### *Effect of Inhibitors on Glucose Consumption and Lactate production*

Normal or infected cells were incubated at a 10% hematocrit concentration in wash medium (growth medium without plasma) in the presence of absence of inhibitors. After various intervals of incubation at 37°, aliquots of 2 ml were centrifuged, and the supernatant was brought to 5% trichloracetic acid and recentrifuged. Glucose was determined by use of the glucose/oxidase (EC 1.1.3.4)/peroxidase (EC 1.11.1.7) method (19). Lactate was determined on 0.25 ml of whole cell suspensions after the addition of 15 μl of 1.0 M perchloric acid. After a brief centrifugation, 0.2 ml of the supernatant was neutralized with 15 μl of 0.7 M KOH. Lactate was deter-

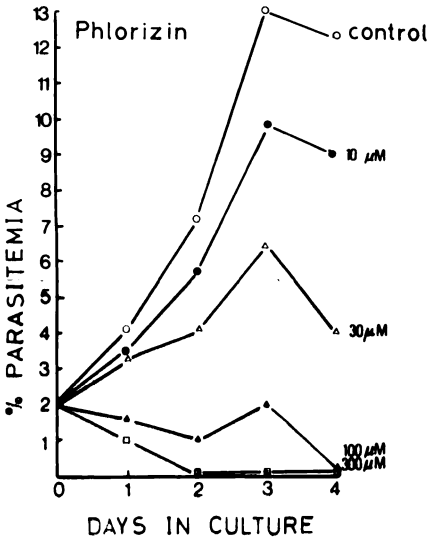


FIG. 1. *In vitro growth profile of Plasmodium falciparum in the presence of phlorizin*

A suspension of erythrocytes (2.5% hematocrit) was inoculated with *P. falciparum*-infected cells to a final parasitemia of 2% and incubated in growth medium in the presence of the indicated concentrations of phlorizin. The parasitemia was followed on a daily basis.

mined enzymatically with lactate dehydrogenase (EC 1.1.1.27) (20). Glucose consumption and lactate production were calculated and were related to the concentration of cells in the assay systems, as determined by counting the cells in a hemocytometer. All enzymes and reagents were obtained from Sigma Chemical Company.

## RESULTS

*Effects of red blood cell impermeant anion transport inhibitors.* Typical growth curves of *P. falciparum* in the presence of phlorizin, a reversibly acting agent which is impermeant to uninfected red blood cells (21), are depicted in Fig. 1. In the presence of phlorizin, parasite growth is reduced in a concentration-dependent manner and is fully arrested at about 100 μM probe. To compare the inhibitory power of phlorizin with that of other agents, we used the parasitemia values (the percentage of parasitized cells in a given cell culture) at the 3rd day of cultivation with the drug, and we displayed the data as parasitemia relative to control versus drug concentration (Fig. 2). The dose-response curves of three reversibly acting drugs to uninfected cells, namely phlorizin, the dinitrostilbene disulfonate DNDS, and the diamino analogue DADS, showed marked differences in their inhibitory potencies. To assess whether these effects could be correlated to the inhibitory activity of these drugs on anion transport, we compared both inhibitory effects on Dixon plots (Fig. 3). As shown, phlorizin was approximately 3-fold more potent in arresting growth than in blocking anion transport. Conversely, DNDS was apparently 3-fold less potent in arresting growth than in blocking anion transport. However, this was found to result from binding of DNDS to plasma components present in the growth medium but absent in the standard transport assay medium. As depicted in Fig. 3, the addition of human serum albumin to the standard transport assay medium, to approximately the same final concentration as in the growth medium, brought about the DNDS inhibitory effect on anion transport to virtually the same level as that observed on parasite growth. The specificity of both inhibitory effects of DNDS are supported also by the fact that its diamino analogue, DADS, led to no measurable change in either property in the indicated concentration range (Fig. 3) and even in the millimolar range (data not shown).

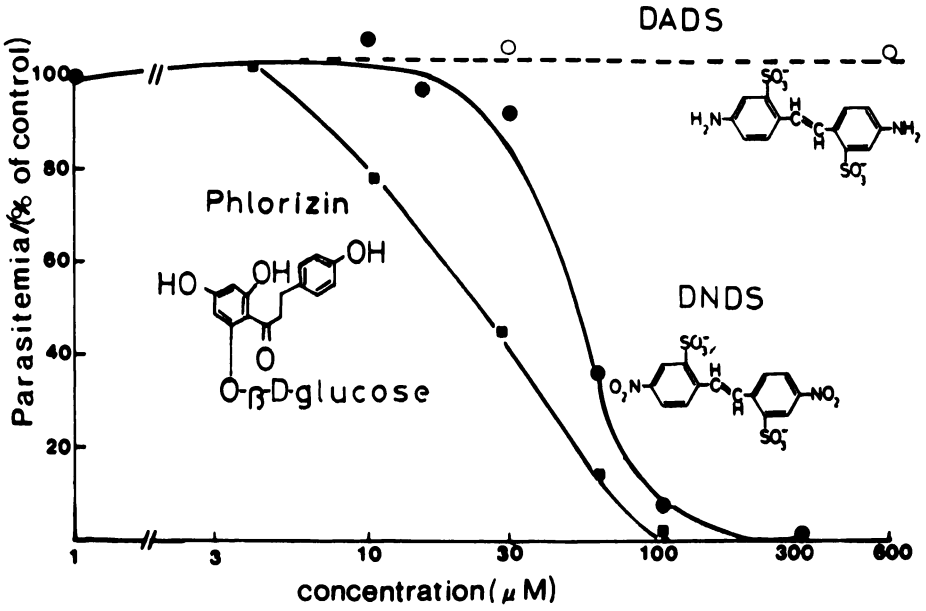


FIG. 2. *Dose-response profile of Plasmodium falciparum growth in the presence of various anion transport inhibitors impermeant to uninfected red cells*

*In vitro* growth profiles of *P. falciparum* were carried out in the presence of various agents, essentially as described for phlorizin in Fig. 1. The dose-response curves for the various drugs were constructed from the parasitemia values obtained at either the 3rd or 4th day of the cultures (Fig. 1).

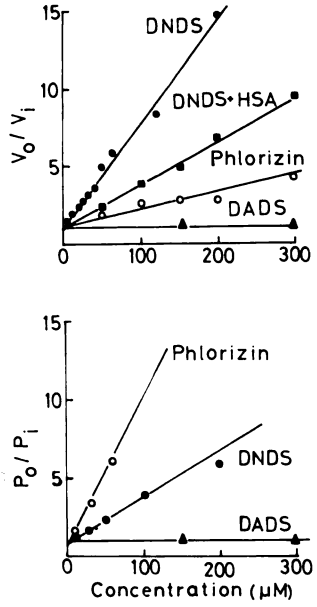


FIG. 3. *Correlation between inhibition of anion transport and inhibition of parasite growth by impermeant agents*

Upper half. The effect of agents on anion transport of uninfected cells was determined at 37° by the continuous monitoring of transport fluorescence method (17). Inhibitors were present during the flux measurements at the indicated concentrations. In one instance, human serum albumin (5 g/liter) was present in the flux medium. Data are given as Dixon plots of $V_0/V_i$ versus the DNDS concentrations, where $V$ is the initial rate of NBD-taurine efflux obtained either in the absence ($V_0$) or in the presence ($V_i$) of inhibitor. The experimental data were analyzed by a nonlinear regression program with a Wang PCSII computer. The $x$-intercepts give the concentration of probe required to inhibit transport by 50% ($IC_{50}$): phlorizin, 120 ± 40 μM; DADS > 1 mM; DNDS, 15 ± 4 μM; and DNDS + human serum albumin (*HSA*), 39 ± 13 μM.

Lower half. Dixon plot of data shown in Fig. 2, given here as $P_0/P_i$ versus DNDS concentration, where $P_0$ and $P_i$ are the percentages of parasitemia in the absence and presence of inhibitor, respectively. Data analyzed as in the upper half gave the following $IC_{50}$ values: phlorizin, 16 ± 7 μM; DNDS, 35 ± 9 μM; and DADS > 10 mM.

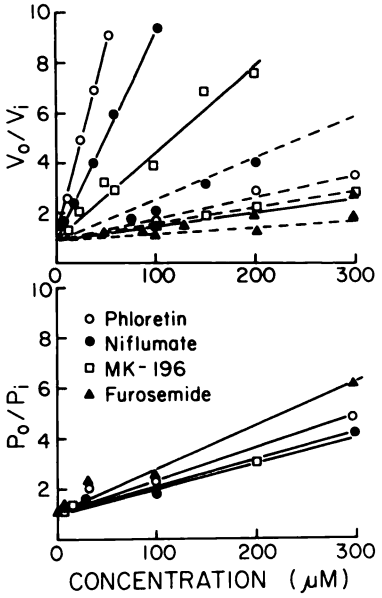


FIG. 4. *Correlation between inhibition of anion transport and inhibition of parasite growth by various agents*

The effects of various agents on parasite growth (lower half) and on anion transport in protein-free medium (upper half, ——) were determined essentially as described in Fig. 3. The corresponding effects on anion transport in human serum albumin-containing medium (5 g/liter) were performed in an analogous fashion (upper half, - - -). Data are displayed in terms of Dixon plots of either $V_0/V_i$ (anion transport) or $P_0/P_i$ (parasitemia) versus drug concentration, the lines indicating the best fit through the experimental points obtained by linear least-squares analysis. The $x$-intercepts denote the $IC_{50}$ (micromolar). For anion transport in protein-free medium the $IC_{50}$ values were as follows: phloretin, 7.1 ± 0.9; niflumate, 12 ± 0.2; MK-196, 28 ± 2; and furosemide, 300 ± 80. For anion transport in human serum albumin-containing medium the $IC_{50}$ values were as follows: phloretin, 112 ± 24; niflumate, 65 ± 15; MK-196, 170 ± 40; and furosemide, 420 ± 95. For parasitemia the $IC_{50}$ values were as follows: phloretin, 56 ± 16; niflumate, 60 ± 23; MK-196, 72 ± 25; and furosemide, 50 ± 13.

*Effect of other anion transport inhibitors.* A comparison between the effects of a variety of anion transport inhibitors on both transport and parasite growth is shown in Fig. 4 in terms of Dixon plots. Phloretin, niflumic acid, and MK-196, all of which are potent reversible inhibitors of anion transport, in serum-free media (Fig. 4, *upper half*, ——) were markedly less inhibitory in medium containing human serum albumin (Fig. 4, *upper half*, - - -). However, MK-196 correlated reasonably well with inhibition of parasite growth (Fig. 4, *lower half*). Conversely, the diuretic sulfonamide, furosemide (22), showed demonstrably more potent effects on parasite growth than on anion transport—this despite the fact that in growth media this sulfonamide is sequestered by serum-albumin and is therefore less available for binding to red blood cell membrane components.

*Mechanism of growth inhibition by disulfonic stilbenes.* The only known common denominator for all of the agents used in this work is their inhibitory effect on red blood cell anion transport. We selected to study the effect of disulfonic stilbenes because of their selective effects on membranes, their high specificity for the anion transporter, and the body of information available on their molecular mode of action (2). Although the inhibitory effect of DNDS on red cell anion transport was shown to be of a reversible nature (23), it was important to verify that DNDS inhibition of parasite growth was not caused by irreversible damage to uninfected cells. As shown in Fig. 5, preincubation of uninfected cells with DNDS under culture conditions affected only temporarily the subsequent growth of parasites after removal of the drug. Cells precultivated with DNDS for 48 hr and subsequently cultivated in DNDS-free growth medium supported parasite growth either after a 24-hr or a 48-hr recovery period, depending upon the concentration of DNDS present during precultivation. In order to assess whether growth inhibition by DNDS was as fast as on transport (23) or whether it manifested itself only after prolonged incubations of the cells with the drug and increased with time of incubation, we followed the *in vitro* growth of parasites as a function of time after the addition of DNDS (Fig. 6). The drug was added at the 3rd day of an asynchronously grown culture, when the parasitemia reached a 9% value. While control cells continued to grow, parasitized cells exhibited an arrest of parasite growth within 24 hr after addition of the drug.

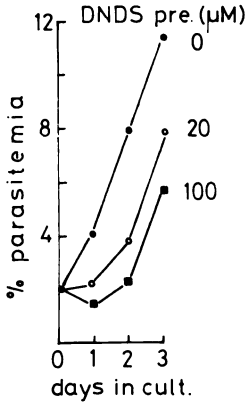


FIG. 5. *Reversal of DNDS effects on uninfected cells as reflected by their subsequent infection with malaria parasites*

Uninfected cells were cultivated for 2 days in culture medium containing the indicated concentrations of DNDS. At the end of this period, the cells were washed free of external DNDS, resuspended in growth medium, and infected with an aliquot of a 20% parasitemia cell suspension (2% final parasitemia). Parasite propagation was determined daily and is given as percentage of parasitemia.

Since the life cycle of *P. falciparum* is 48 hr, it is evident that the propagation of the parasites was impaired by DNDS with no apparent delay. This was corroborated by the fact that very few ring-forms were present in the culture 14 hr after the addition of DNDS (data not shown).

A more direct test to evaluate the particular developmental stage at which DNDS affected parasite growth was carried out on synchronously grown cultures, using amino acid incorporation into proteins as a measure for parasite development. As shown in Fig. 7, DNDS had virtually no effect on ring development but markedly arrested the development of the metabolically more active and advanced stages of trophozoites and schizonts. Further proof for the above interference of DNDS with the metabolic activity of infected cells (trophozoites and schizonts) was obtained by following lactic acid production, which provides a measure for the glycolytic activity

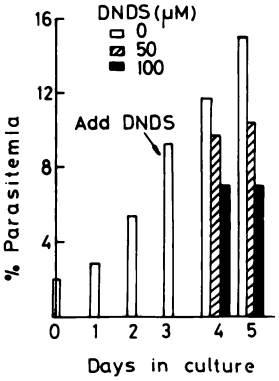


FIG. 6. *Effect of DNDS on cells preincubated under culture conditions*

Cultures (2.5% hematocrit) were cultivated for 3 days with daily change of growth medium (parasitemia reached 9%). Thereafter, cultivation was continued in the presence of DNDS at the indicated concentration. Parasitemia was determined on Giemsa stains taken from samples withdrawn prior to the daily change of growth medium.

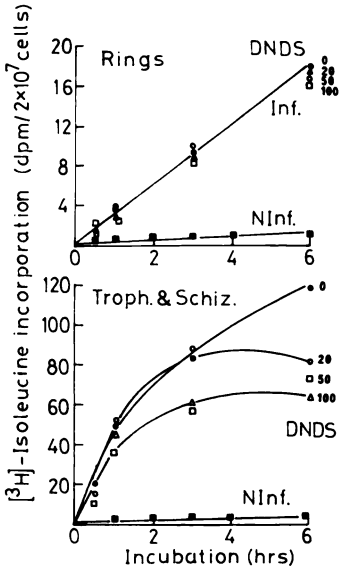


FIG. 7. *Stage dependence of DNDS effect on amino acid incorporation into parasitized cells*

Parasite growth was synchronized as described under Materials and Methods. Cells which reached the indicated stage of growth were analyzed for [$^3$H]isoleucine incorporation for various periods of time in growth media containing the indicated concentrations of DNDS. Uninfected cells cultured in parallel and analyzed for amino acid incorporation, in either the presence or absence of DNDS, gave essentially the same results.

of the parasites (6) and host cells (24). As shown in Fig. 8, neither lactic acid production by ring-forms nor that by uninfected cells was significantly affected by DNDS, whereas trophozoite and schizont lactate production was markedly suppressed by the drug. Comparable results were obtained with glucose consumption (data not shown), which also provided a measure for the metabolic status of the cells.

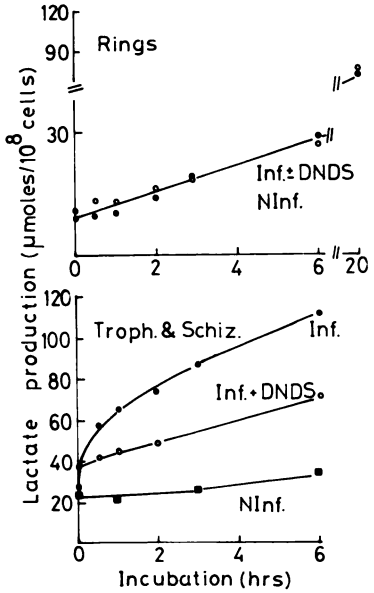


FIG. 8. *Stage dependence of DNDS effect on lactate production by parasitized cells*

The experiments were conducted essentially as described in Fig. 7, except that lactate production (see Materials and Methods) were studied at the different developmental stages of parasite growth.

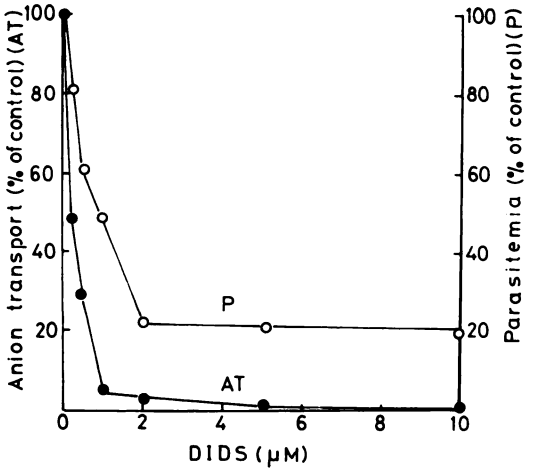


FIG. 9. *Correlation between DIDS inhibition of anion transport and of parasite growth*

Red cells (10% hematocrit in PBS, pH 7.4) were treated with the indicated concentrations of DIDS (30 min at 15°) and were subsequently washed with growth medium. An inoculum of infected cells (20% parasitemia) was added to each of the treated cell preparations to reach a 2.5% final hematocrit and 2% parasitemia, and the suspensions were kept under culture conditions. The parasitemia values given were determined at the 3rd day of cultivation. For measuring anion transport, cells were first loaded with NBD-taurine at 37°, subsequently washed with PBS at 5°, and treated with DIDS as indicated above.

A means to assess whether growth inhibition by the reversibly acting DNDS resulted primarily or exclusively from effects induced at the host-cell level relied on the use of DIDS or $H_2$DIDS—both covalently binding analogs—which were shown to interact with DNDS membrane binding sites (13, 25). These analogues provided, therefore, the tools with which to probe the sites of action of DNDS. Uninfected cells were first labeled with DIDS and then either used for measuring anion transport or were infected with parasitized cells and their growth was followed with time. Data shown in Fig. 9 indicate that anion transport was about 2-fold more susceptible to inhibition than parasite growth. The latter showed about 20% of the cell population as refractory to increasing concentrations of DIDS at levels at which anion transport was already fully blocked by the drug. These apparent differences in susceptibilities were found to result, in part, from the fact that approximately 10% of uninfected cells in the culture were DIDS-untreated cells which were carried along with the inoculum of parasitized cells at the time of infection of the DIDS-treated cells. When this factor was taken into account, the two profiles of inhibition became comparable. These results indicate that modification of the cell surface of uninfected cells by DIDS reduced the ability of these cells to support parasite growth and suggest that one mode of DNDS inhibition of growth is by directly affecting red cell membrane components. Nevertheless, since in the previous profiles of DNDS inhibition both uninfected and infected cells were exposed to the drug, we attempted to evaluate whether DIDS had additional effects on infected cells. Synchronous cultures were grown up to the schizont stage, at which point part of the culture was challenged with DIDS. Aliquots of these cultures were mixed with an equal volume of uninfected cells which were either treated or not treated with DIDS and placed under culture conditions. The percentages of newly formed rings and amino acid incorporation were determined after 14 hr. As shown in Table 1, DIDS modification of uninfected cells did not interfere with ring formation of these cells, whereas DIDS modification of schizonts was reflected as a major reduction in the number of newly formed rings.

Microscopic inspections led us to conclude that DIDS affected the schizonts and interfered with their maturation and rupture, inasmuch as many degenerated schizonts were present in the smears stained with Giemsa stain.

To localize the effect of DIDS on schizonts, we used the $^3$H-labeled analogue [$^3$H]$H_2$DIDS, which had effects similar to those of DIDS both on anion transport and on growth suppression. As shown in Fig. 10, the [$^3$H]$H_2$DIDS labeling was performed on an asynchronously grown culture, and subsequently uninfected cells and rings were separated from trophozoites and schizonts by a Ficoll gradient and analyzed by SDS-PAGE. [$^3$H]$H_2$DIDS labeling of rings and uninfected cells was fully localized in the Band 3 protein, the purported anion transporter (2). However, the schizont-rich fraction showed not only labeling of Band 3 but also high labeling of lower molecular weight components. One major component was identified as intracellular hemoglobin, both by molecular weight and by the fact that hemoglobin-free membranes from schizonts showed no labeled band in the hemoglobin area. A major additional labeled component which migrated at the front of the SDS gel reflected membrane material, probably of a lipid nature. These results indicate that the permselectivity properties of the host cell membrane at the schizont stage were markedly altered, so that molecules such as $H_2$DIDS (and probably also DNDS, phlorizin, and niflumic acid) could gain access to the cytoplasm, thus affecting parasite development in a more direct fashion. The latter hypothesis was assessed by testing the effect of these inhibitors on the ability of schizonts to propagate and lead to new ring formation. The results given in Table 2 show that phlorizin, DNDS, and niflumic acid indeed inhibited parasite propagation at the schizont stage. However, because the reversibly

TABLE 1

*Effect of DIDS treatment of uninfected cells and of schizonts on the propagation of parasites*

Cells from infected cultures which reached the schizont stage were either treated or not treated with DIDS (50 μM, 30 min, 37°) and washed with growth medium. A volume of the above cells was diluted with a volume of noninfected cells (N) (either treated or not treated with DIDS as shown above), and cultivated for 16 hr. Invasion was determined by counting both parasitemia (relative number of rings) and [$^3$H]isoleucine incorporation as described under Materials and Methods.

| Treatment | Parasitemia (% ring-forms)[a] | Isoleucine incorporation (dpm/2 × $10^7$ cells)[a] |
|---|---|---|
| N + I | 9.0 (100) | 15,000 (100) |
| $N_{DIDS}$ + I | 8.5 (94) | 13,800 (92) |
| N + $I_{DIDS}$ | 4.2 (47) | 7,300 (49) |
| $N_{DIDS}$ + $I_{DIDS}$ | 4.5 (50) | 8,000 (53) |

[a] Numbers in brackets denote percentage of control (N + I). Values given are averages of quadruplicate determinations (SEM 5–10%).

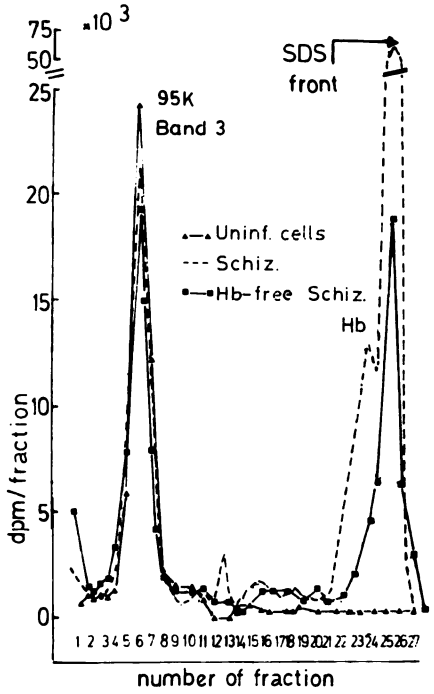


FIG. 10. *SDS-PAGE radioelectrophoretogram of [$^3$H]$H_2$DIDS-labeled cells from infected cultures*

A suspension of infected cells in PBS, pH 7.4 (18% parasitemia: 8% rings, 2% trophozoites, 8% schizonts), was treated with [$^3$H]$H_2$DIDS (20 μM, 5 × $10^9$ dpm/μmole) for 30 min at 37°. Uninfected cells and rings were separated from trophozoites and schizonts by Ficoll-Hypaque cushions. The final parasitemia of the upper layer of the cushion (i.e., trophozoites and schizonts) was 75%. SDS-PAGE was performed either on the same number of whole cells or on membranes isolated from cells by hypotonic lysis (13). Gels were processed for $^3$H counting as previously described (13).

acting inhibitors had to be present during schizogony and reinvasion, it was difficult to ascertain at which of the two stages of the cycle the inhibitory effect was most prominent. The above work with DIDS supports the idea that the schizont stage is apparently the most susceptible one to the aforementioned drugs.

## DISCUSSION

The primary goal of this work was to introduce a new series of antimalarial agents which might be of potential use against a disease that is spreading throughout the world and for which new therapeutic agents are in demand. The rationale on which the present approach is based is the use of agents that are impermeant to animal cells and the antimalarial action of which would therefore be confined primarily to interference with membrane-related processes. Because intraerythrocytic growth of parasites demands a much higher import of nutrients and export of catabolites than in noninfected red cells (6), we reasoned that blockers of the red cell transport processes might differentially affect malaria-infected cells (7).

Our initial studies were carried out with inhibitors of red cell anion transport for the following reasons. (a) Anion transport is a unique transport system for which the red cell is highly specialized and for which a large reportoire of reversibly and irreversibly binding inhibitors, of known mode of action, has been developed. (b)

TABLE 2

*Effect of anion transport inhibitors on the propagation of parasites*

Cells from infected cultures which reached the schizont stage were diluted with an equal volume of noninfected cells and cultured in the presence of the indicated concentrations of inhibitors. Further details as in Table 1 and under Materials and Methods.

| Treatment | Parasitemia (% ring-forms)[a] | Isoleucine incorporation (dpm/2 × $10^7$ cells)[a] |
|---|---|---|
| None | 12.0 (100) | 34,273 (100) |
| DNDS (100 μM) | 7.2 (60) | 18,832 (55) |
| Niflumate (300 μM) | 4.3 (36) | 17,147 (50) |
| Phlorizin (100 μM) | 1.2 (10) | 15,093 (44) |
| MK-196 (300 μM) | 11.8 (98) | 23,678 (70) |

[a] Numbers in brackets denote percentage of control (None). Values given are averages of quadruplicate determinations.

The red cell anion transport system is the main route for phosphate entry into red cells (1–3) and a minor route for lactate exit from (5, 6), and amino acid (i.e., the glutathione precursors glycine and cysteine) entry into, these cells (4). (c) Partial interference with this system should have a more deleterious effect on the metabolically more active parasitized cell than on the uninfected cell.

As shown in this work, the results of testing *in vitro* parasite growth in the presence of classical inhibitors of anion transport support in part the aforementioned hypothesis. Interestingly, the two types of agents which were nonpenetrating to uninfected cells, the glycoside phlorizin and the disulfonic stilbene DNDS, showed the most potent inhibitory action on malaria growth *in vitro* (Fig. 2). In the case of DNDS, the excellent correlation between the inhibitory activity on anion transport and the arrest of intraerythrocytic parasite growth suggests the involvement of anion blockade in growth inhibition (Fig. 3). This was also supported by the fact that normal cells modified with DIDS under conditions known to affect only the anion transporter Band 3 (Fig. 4), or preincubation of noninfected cells with DNDS (Fig. 4), failed to support parasite growth. An interesting feature of the latter treatments is that they reduce considerably the ability of noninfected cells to support subsequent parasite propagation in *in vitro* culture conditions. To some extent, the hypothesis is also supported by the results obtained with a variety of other anion transport inhibitors whose *modus operandi* is not precisely known, particularly with regard to their site of action on the anion transporter and to their permeation properties. However, these agents were chosen preferentially because they are currently in therapeutic use: niflumic acid as a nonsteroidal anti-inflammatory agent (9) and furosemide (26) and MK-196 (11) as diuretics.

In the case of phlorizin,[2] the most potent of the agents used, the inhibition of growth could not be correlated with that of anion transport (Fig. 3), indicating that this drug operates either by a different mechanism or by an

[2] Phlorizin was referred to in *The Merck Index*, 1968 edition (27), as an antimalarial agent whose medical use was discontinued. Our attempts to obtain further information on the pharmacological and pharmaceutical uses of phlorizin as an antimalarial agent were not successful.

additional inhibitory mechanism. This finding with phlorizin does not necessarily contradict the aforementioned hypothesis; it only suggests that the major antimalarial mode of action of the drug is probably not via blockade of host cell anion transport. The possibility that the drug works mainly by blocking host cell monosaccharide transport (28, 29) can also be rejected on the grounds that phloretin, a relatively better blocker of this function (30), is a poor inhibitor of malaria growth, as well as on the basis that the $IC_{50}$ of parasitemia (10 $\mu$M) was markedly smaller than the $IC_{50}$ of sugar transport (140 $\mu$M, ref. 30). A likely target for phlorizin could be an intracellular component, the parasitophorous vacuole membrane, or perhaps the parasite itself, inasmuch as otherwise impermeant molecules of the same molecular size as phlorizin (e.g., $H_2$DIDS) could gain access to the host cytoplasm of either trophozoites or schizonts (Fig. 10).

The change in the permselectivity properties of the host-cell membranes which appears at the trophozoite stage and persists throughout schizogony (12) provides a pattern of inhibition of parasite development that is additional to the aforementioned effect on anion transport. Support for this pattern was obtained when drugs added to schizonts were shown to interfere with their metabolic activity as a short-range effect (Fig. 8; Tables 1 and 2) and with the subsequent development and propagation of parasites as a long-range effect (Fig. 7; Tables 1 and 2). The two suggested patterns of growth inhibition by DNDS, phlorizin, and possibly niflumic acid, one on the anion transporter at the host membrane level and the other on intracellular targets, have the unique feature of relying on drugs which are impermeant to most normal cells and which are likely to produce only reversible effects on noninfected cells. The relative importance of the two patterns, which at present can be given only in qualitative terms, would depend on the drug of choice. Phlorizin's primary mode of action is probably by direct interference with intracellular events in trophozoites and schizonts, which is facilitated by partial permeabilization of the host-cell membrane. Conversely, the effect of DNDS is of a bimodal nature, affecting directly the red cell membrane functions of uninfected cells and of infected cells at all parasite stages and/or affecting intracellular events, as proposed for phlorizin. Although elucidation of the molecular mechanism of growth inhibition by blockers of anion transport would require further investigation, the initial studies with these agents provide a new basis for the design of modern drugs for malaria chemotherapy.

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## REFERENCES

1. Deuticke, B. Properties and structural basis of simple diffusion pathways in the erythrocyte membrane. *Rev. Physiol. Biochem. Pharmacol.* **78**:1–97 (1977).
2. Cabantchik, Z. I., P. A. Knauf, and A. Rothstein. The anion transport system of the red blood cell: the role of membrane protein evaluated by the use of "probes." *Biochim. Biophys. Acta* **515**:239–302 (1978).
3. Young, J. D., S. E. M. Jones, and J. C. Ellory. Amino acid transport via the red cell anion transport system. *Biochim. Biophys. Acta* **645**:157–160 (1981).
4. Dubinsky, W. P., and E. Racker. The mechanism of lactate transport in human erythrocytes. *J. Membr. Biol.* **44**:25–36 (1978).
5. Deuticke, B., I. Rickert, and E. Beyer. Stereoselective SH-dependent transfer of lactate in mammalian erythrocytes. *Biochim. Biophys. Acta* **507**:137–155 (1978).
6. Sherman, I. W. Biochemistry of plasmodium (malarial parasites). *Microbiol. Rev.* **43**:453–495 (1979).
7. Ginsburg, H., S. Kutner, M. Krugliak, and Z. I. Cabantchik. Inhibition of *P. falciparum* growth *in vitro* by specific inhibitors of red blood cell anion transport, in *Biochemistry of Parasites* (G. M. Slutzky, ed.). Pergamon Press, Oxford and New York, 85–96 (1981).
8. Barzilay, M., S. Ship, and Z. I. Cabantchik. Anion transport in red blood cells. I. Chemical properties of anion recognition sites as revealed by structure-activity relationships of aromatic sulfonic acids. *Membr. Biochem.* **2**:227–254 (1979).
9. Cousin, J. L., and R. Motais. Inhibition of anion permeability by amphyphilic compounds in human red cells: evidence for an interaction of niflumic acid with the Band 3 protein. *J. Membr. Biol.* **46**:125–153 (1976).
10. Brazy, P. C., and R. B. Gunn. Furosemide inhibition of chloride transport in human red blood cells. *J. Gen. Physiol.* **68**:583–599 (1976).
11. Brooks, B. A., and A. F. Lant. The use of the human erythrocyte as a model for studying the action of diuretics on sodium and chloride transport. *Clin. Sci. Mol. Med.* **54**:679–683 (1978).
12. Kutner, S., D. Baruch, H. Ginsburg, and Z. I. Cabantchik. Alterations in the membrane permeability of malaria-infected human erythrocytes are related to the growth stage of the parasite. *Biochim. Biophys. Acta* **687**:113–117 (1982).
13. Cabantchik, Z. I., and A. Rothstein. The nature of the membrane sites controlling anion permeability of human red blood cells as determined by studies with disulfonic stilbene derivatives. *J. Memb. Biol.* **10**:311–330 (1972).
14. Eidelman, O., M. Zangwill, M. Razin, H. Ginsburg, and Z. I. Cabantchik. The anion transfer system of erythrocyte membranes: NBD-taurine, a fluorescent substrate analog of the system. *Biochem. J.* **195**:503–513 (1981).
15. Trager, W., and J. B. Jensen. Human malaria parasites in continuous culture. *Science (Wash. D. C.)* **193**:673–675 (1976).
16. Howard, R. J., P. M. Smith, and G. F. Mitchell. Removal of leucocytes from red cells in *Plasmodium berghei*-infected mouse blood and purification of schizont-infected cells. *Ann. Trop. Med. Parasitol.* **72**:573–576 (1978).
17. Eidelman, O., and Z. I. Cabantchik. Fluorescence studies of anion transfer in red blood cell membranes. *Anal. Biochem.* **106**:335–341 (1980).
18. Breuer, W. V., H. Ginsburg, and Z. I. Cabantchik. An assay of malaria parasite invasion into human erythrocytes: the effects of chemical and enzymatic modification of erythrocyte membrane components. *Biochim. Biophys. Acta*, in press.
19. Bergmeyer, H. U., and F. Berut. Determination with glucose oxidase and peroxidase, in *Methods of Enzymatic Analysis* (H. U. Bergmeyer, ed.). Academic Press, New York, 123–130 (1963).
20. Henry, R. J. *Clinical Chemistry—Principles and Techniques.* Harper & Row, New York, 664–666 (1968).
21. Lepke, S., and H. Passow. Asymmetric inhibition by phlorizin of sulfate movements across the red cell membrane. *Biochim. Biophys. Acta* **298**:529–533 (1973).
22. Weinstein, L. The sulfonamides, in *The Pharmacological Basis of Therapeutics* (L. S. Goodman and A. Gilman, eds.) Ed. 4. Macmillan, London, 1177–1203 (1970).
23. Barzilay, M., and Z. I. Cabantchik. Anion transport in red blood cells. II. Kinetics of reversible inhibition by nitroaromatic sulfonic acids. *Membr. Biochem.* **2**:255–281 (1979).
24. Minakami, S., C. Suzuki, T. Saito, and H. Yoshikawa. Studies on erythrocyte glycolysis. I. Determination of the glycolytic intermediates in human erythrocytes. *J. Biochem.* **58**:543–550 (1965).
25. Lepke, S., H. Fasold, M. Pring, and H. Passow. A study of the relationship between inhibition of anion exchange and binding to the red blood cell membrane of 4,4′-diisothiocyano stilbene-2,2′-disulfonic acid (DIDS) and its dihydroderivative ($H_2$DIDS). *J. Membr. Biol.* **29**:147–177 (1976).
26. Burg, M., L. Stoner, J. Cardinal, and N. Green. Furosemide effect on isolated perfused tubules. *Am. J. Physiol.* **225**:119–124 (1973).
27. Stecher, P. G. (ed.). *The Merck Index*, Merck & Company, Inc., Rahway, N. J., 821 (1968).
28. Le Fevre, P. G. Active transfer of glycerol and glucose across the human red cell membrane. *J. Gen. Physiol.* **31**:505–527 (1948).
29. Bowyer, F. The kinetics of the penetration of non-electrolytes into the mammalian erythrocyte. *Int. Rev. Cytol.* **6**:469–511 (1957).
30. Basketter, D. A., and W. F. Widdas. Asymmetry of the hexose transfer system in human erythrocytes: comparison of the effect of cytochalasin B, phloretin and maltose as competitive inhibitors. *J. Physiol. (Lond.)* **278**:389–401 (1978).

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