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Antimalarial Action of Hydrophilic Drugs: Involvement of Aqueous Access Routes to Intracellular Parasites

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SUMMARY

The antimalarial action and intracellular distribution of the hydrophilic agents phloridzin (PHL) (a bioflavonoid glycoside) and desferrioxamine (DFO) (an iron chelator) were studied in cultures of *Plasmodium falciparum*-infected human erythrocytes. When added to cultures, these agents arrested parasite growth with IC_{50} values of 12 μM (PHL) and 22 μM (DFO). At 37°, PHL (40 μM) was virtually impermeant to uninfected cells but permeated with a mean $t_{1/2}$ of 1.5 hr in trophozoites (30% accessible cell volume) and 8 hr in rings (10% of accessible cell volume). PHL, in analogy with DFO, was demonstrably permeant to infected cells harboring mature forms of the parasites. Permeation was restricted to only a fraction of the infected cell volume. PHL elicited inhibition of nucleic acid synthesis within 1 hr of exposure of trophozoites to PHL (40 μM) and in >8 hr of exposure of rings. Red cell containers into which millimolar concentrations of PHL or DFO were encapsulated demonstrably supported parasite invasion and subsequent parasite growth and maturation (48-hr incubation). Under culture conditions, uninfected or parasite-infected red cell containers that were loaded with either agent retained the drugs for at least 42 hr at hundred-micromolar concentrations. The agent present in the cells was fully active after release from cells and administration to test cultures of parasites. PHL added to parasite cultures was active at micromolar concentrations, but when present intracellularly it was virtually inactive even at millimolar concentrations. The data presented are consistent with direct access of hydrophilic agents from medium to parasite, a process referred to as fenestration. Permeation into parasites might constitute the rate-limiting step in drug uptake and drug-mediated arrest of parasite growth by PHL and DFO. The putative role of the parasitophorous duct in providing aqueous access routes from medium to parasites is discussed.

The development of novel chemotherapies for life-threatening malaria depends largely on the identification of potential drug targets and elucidation of the mechanisms by which the drugs gain access to intracellular parasites. The strategy of targeting potential cytotoxic materials to infected cells first requires basic knowledge about the permeability properties of the HPM and in particular how those properties differ in infected, compared with normal, cells (1–4). The malaria-infected red blood cell is classically viewed as a highly compartmentalized system in which the intracellular parasite is apparently surrounded by the PVM and the HPM, which faces the external medium (2). Thus, to reach the parasite cytosol a drug would have to sequentially cross the concentric compartments enclosed within the various membranes. This process encompasses both membrane permeation steps and diffusion across aqueous compartments. However, as was recently suggested, membranous extensions of the PVM could be far reaching (5, 6), so as to interact with the HPM and thus provide an aqueous duct (5) for direct communication between the cytosol of the parasite and the external medium, via the PPM. Although the maintenance of such membranous structures remains controversial (7–9), the direct access of hydrophilic agents from medium to parasite was recently demonstrated (10, 11). The hydrophilic iron chelator DFO (Fig. 1) was shown to preferentially access "intracellular parasites" from the medium and exert a major and specific cytotoxic effect on parasites (11). That effect was demonstrably dependent on parasite growth stage (12, 13).

Based on the information described above, it can be assumed that a drug affecting the parasite could reach the PPM by the direct route and/or by the indirect route (via the host), depending on the biophysical properties of the relevant membranes. In this work we explored the nature of the antimalarial effect caused by the hydrophilic bioflavonoid glycoside PHL, in relation to its mode of entry into parasitized cells. PHL and its various structural congeners were previously shown to markedly and irreversibly affect intracellular parasite development (1, 14, 15). In parallel with inhibition of growth, the drug was

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ABBREVIATIONS: HPM, host plasma membrane; PVM, parasitophorous vacuole membrane; PPM, parasite plasma membrane; DFO, desferrioxamine; PHL, phloridzin; RCC, red cell container; TCA, trichloroacetic acid; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

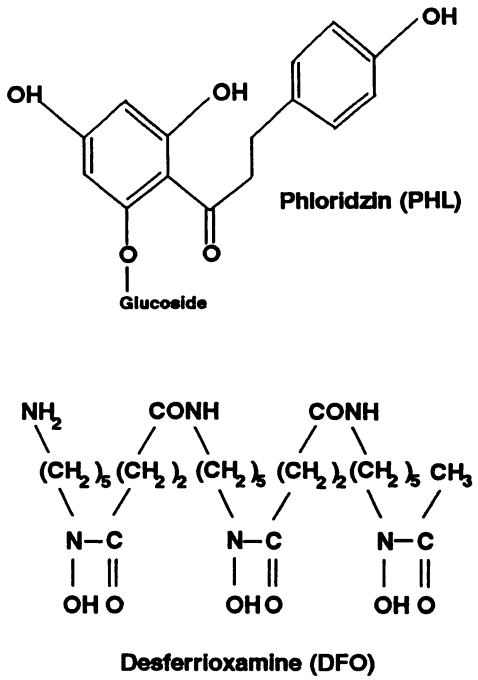

Fig. 1. Schematic structures of PHL and DFO.

shown to block some of the novel permeation pathways that are induced by parasites in the HPM (1, 14). Although the biological effect on parasite growth was initially attributed to blockage of essential routes of nutrient traffic (16), the fact that PHL affected parasite metabolism *per se* in HPM-permeabilized cells (14) indicated that the HPM was probably not the sole pharmacological target of the drug. Moreover, the demonstrably restricted entry of the hydrophilic drug to virus-permeabilized infected cells (14), and the newly recognized direct route to the parasite surface provided by the aqueous ducts, prompted us to consider the latter as the most plausible access route of PHL into infected cells. The rate-limiting step in the entry into the parasites would therefore be permeation across the PPM. Thus, assessment of how the PPM permeability properties differ from those of the HPM could serve as a potential means for specifically targeting cytotoxic drugs to parasites.

Materials and Methods

Chemicals. All biochemicals were from Sigma Chemical Co. (St. Louis, MO) or were of the best available grade. PHL was recrystallized three times from ethanolic solutions. DFO was procured from Ciba-Geigy (Basel, Switzerland). [^{3}H]PHL (5 Ci/mmol; The Radiochemical Center, Amersham) was kindly provided by Dr. A. Moran, Ben-Gurion University (Beer-Sheva, Israel). Antibodies against PHL were prepared in rabbits by injection of PHL-thioureido-thyroglobulin. The latter was prepared by modification of thyroglobulin with PHL-isothiocyanate (14) in phosphate-borate buffer, pH 8.5, at a 100:1 molar ratio. The solution was stirred for 5 hr at room temperature, followed by overnight incubation at 5° and extensive dialysis against PBS. The conjugated material was analyzed by spectrophotometry at 290 nm (absorption coefficient of PHL, 17,000 M^{-1} cm^{-1}). The antigen (10 hapten molecules/protein, 2 mg of protein) was prepared in 0.5 ml of PBS, emulsified with 0.5 ml of complete Freund's adjuvant, and injected subcutaneously into rabbits (2.5–3 kg). All subsequent injections at weeks 2 and 4 and booster injections were done with incomplete adjuvant. The first bleeding was in week 6. After overnight blood coagulation, the serum was centrifuged (10 min at 2000 × *g*), treated with a 2 M solution of $(NH_4)_2SO_4$, dialyzed against PBS, and analyzed by fluorescent enzyme-linked immunosorbent assay, using egg phoridzin-ovalbumin as the test antigen (17).

Cultures. Cultures of *Plasmodium falciparum* (ITG-2F6 cloned strain from Brazil, originally obtained from Dr. L. H. Miller National, Institute of Health, Bethesda, MD) were grown in culture flasks containing growth medium (RPMI 1640 medium; Sigma) supplemented with 25 mM HEPES, 23 mM sodium bicarbonate, 10 mM glucose, and 10% heat-inactivated human plasma (O positive or A positive), with washed human erythrocytes (A positive) at 2–2.5% hematocrit levels. The growth medium was replenished daily and gassed with a mixture of 90% N_2/5% CO_2/5% O_2. Cells were usually harvested or subcultured when the parasitemia reached 15–20%, as determined microscopically by thin blood smears stained with Giemsa. Trophozoite and early schizont stages were routinely isolated by the gelatin flotation method (18). Percoll isopycnic separation of all parasite developmental stages was carried out as reported elsewhere (19), with few modifications. Briefly, the Percoll layers contained (from bottom to top) 90%, 80%, 70%, and 60% Percoll solutions in RPMI 1640 medium containing 6% alanine. Before separation, cell suspensions were preincubated for 5 min at 37° with PBS containing 6% alanine (to allow optimal entry of alanine into parasitized red cells). Red blood cells were layered on top of the 60% Percoll layer in 8-ml plastic tubes and centrifuged at 5000 rpm for 15 min in a Sorvall centrifuge (DuPont Instruments).

Encapsulation of drugs into RCCs. The procedure was performed aseptically, as described previously (11). Briefly, packed red blood cells were washed three times with buffer containing 150 mM NaCl, 5 mM glucose, and 5 mM sodium phosphate, pH 7.0 (PBSG), adjusted to 75% hematocrit with the same buffer, and dialyzed against 5 mM sodium phosphate, pH 7.0, for 45 min. Spectra/Por 2 dialysis tubing (Spectrum, Los Angeles, CA) was routinely used for that purpose. Dialysis was initiated at room temperature and completed at 10–12°, to reduce spontaneous resealing of cells. The cells were aseptically removed from the dialysis bag, placed on ice, and mixed with stock solutions of DFO or PHL in dimethylsulfoxide to reach the final concentrations required. After supplementation with ATP (2 mM final) and 10-min incubation, the cells were resealed by addition of KCl (final concentration, 150 mM), washed with PBSG, and resuspended in RPMI 1640 medium with or without indicator. After 2 hr of incubation at 37°, the cells were washed twice with prewarmed growth medium to remove adsorbed, loosely bound, or membrane-entrapped drug. Average entrapment efficiencies for DFO and PHL varied between 25 and 45%, as assessed by radioactivity (with [^{3}H]PHL) or by spectrophotometry after perchloric acid treatment followed by neutralization of extracts with saturated K_2CO_3 and complexation of the solution with iron (11).

Measurement of [^{3}H]PHL ingress into infected cells. [^{3}H]PHL (40 μM, 5 × 10^6 dpm/ml) was added to cultures of uninfected or infected cells (rings-parasitemia, 50%; trophozoite-schizonts, >97%) (10% hematocrit) in complete growth medium at 37°. At the indicated times, 300-μl aliquots were withdrawn, and the cells were washed several times with ice-cold RPMI 1640 medium, lysed in water, and processed for hemoglobin measurement (at 410 nm) and for radioactivity counting after TCA (final concentration, 5%) precipitation.

Measurement of [^{3}H]PHL retention in and egress from RCCs. RCCs with PHL encapsulated therein (1.4 mM internal concentration, unless otherwise specified) were suspended in RPMI 1640 medium without indicator (2.5% hematocrit) and were incubated at 37°. At indicated times, duplicate aliquots of 500 μl were transferred to Eppendorf tubes and centrifuged for 10 sec at 10,000 × *g*. The radioactivity in the supernates (egress of material) was measured after TCA treatment (final concentration, 5%). The pellets were lysed in water, samples were taken for hemoglobin measurement, and the radioactivity was measured after TCA precipitation (final concentration, 5%) (drug retention). The egress of drug was expressed in nmol of PHL/10^{10} cells, taking into account the fact that 1 absorbance unit at 410 nm corresponds to 5.5 × 10^6/ml noninfected cells (or rings) (for trophozoites-schizonts, we used the value of 2.5–3 × 10^6/ml). Radioactivity was counted in a liquid β-scintillation counter (Beckman instruments, Inc., Fullerton, CA).

Effect of agents on parasite growth. Upon reaching the trophozoite stage (10–20% parasitemia), the cells were washed with growth medium and resuspended to 2–2.5% hematocrit (2–2.5% parasitemia) in 24-well microtiter plates (0.6 ml/well). Inhibitors were added at the indicated concentrations in duplicate wells, and the plates were transferred to a candle jar and incubated at 37° for 24 hr. [^{3}H]Hypoxanthine was added to a final activity of 5 μCi/ml, and after an additional 24-hr incubation period cells were transferred in triplicate to 96-well plates and harvested with a cell harvester (Dynatech, Inc.). The filters were washed with distilled water, dried for 2 hr at 60°, and transferred into toluene-based scintillation fluid for counting of radioactivity.

The time dependence of PHL effects on parasitized cells was assessed by monitoring parasitemia and growth stages of parasites (by microscopic inspection of Giemsa-stained smears) and nucleic acid synthesis of samples of cultures exposed to PHL for different periods of time, essentially as described above.

The effect of drugs encapsulated in RCCs on parasite growth was tested after hemolysis (freezing and thawing), both with uninfected RCCs that were incubated for different times under culture conditions and with RCCs that were infected with parasites and then separated into the various stages of parasite growth using Percoll-alanine.

To monitor the growth of parasites in RCCs in which DFO or PHL was encapsulated, gelatin-isolated trophozoite-schizonts were mixed with drug-encapsulated cells to final values of 2.5% parasitemia and 2.5% hematocrit (unless otherwise indicated) and were analyzed as described above. Parasite growth was estimated as a ratio of [^{3}H] hypoxanthine incorporation into drug-encapsulated cells to incorporation into sham-encapsulated controls (relative activity). The inhibitory effect of the drugs was assessed by Dixon plot analysis (20). The x-axis intercept gave the IC_{50} value.

Results

The permeation properties of the hydrophilic agent PHL were assessed in uninfected cells and infected cells (Fig. 2). At 40 μM, PHL was virtually impermeant to uninfected cells, in agreement with previous studies (14, 15). Ring forms were found to be somewhat permeable to PHL, however; the $t_{1/2}$ of ingress was >8 hr, and only about 10% of the total cell volume was found to be accessible to the drug over a 10-hr period of incubation (15). At the end of the incubation, formation of some trophozoites became apparent (data not shown). However, trophozoites were demonstrably permeant to PHL ($t_{1/2}$, 1.5 hr) and about 30% of their cell volume was apparently accessible to the drug.

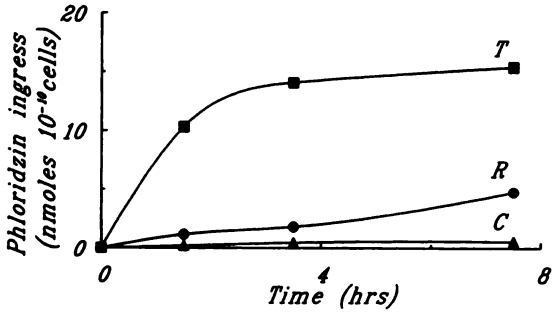

Fig. 2. Ingress of PHL into uninfected and infected red blood cells. The ingress of [^{3}H]PHL into synchronized cultures of *P. falciparum* (*T*, trophozoites; *R*, rings; *C*, uninfected cells) was carried out under culture conditions, as indicated in Materials and Methods. The extracellular concentration of drug was 40 μM. Data are given as nmol of drug/10^{10} cells, using the hemoglobin readings at 410 nm and appropriate correction factors, as described in Materials and Methods. The level of drug attained at 7 hr was 15 μM. Results are means of a representative experiment performed in triplicate (standard error values are within the size of the symbols).

The rates of entry of the drug into infected cells were subsequently compared with the rate of action of PHL on parasite growth by monitoring nucleic acid synthesis in the presence of drug (40 μM). Previous studies indicated that PHL arrested parasite growth and inhibited protein synthesis with similar IC_{50} values (15, 21). In this work we followed the course of growth of rings and trophozoites in the presence of PHL, both by determination of parasitemia and growth stages (Fig. 3, *top*) and by measurement of nucleic acid synthesis (Fig. 3, *middle* and *bottom*). As shown in Fig. 3, *top*, rings exposed to PHL progressed normally up to the trophozoite stage. These trophozoites did not mature into schizonts and by 30 hr showed major deformations, which are indicative of cell death. In the control system, trophozoites appeared at 10–24 hr of incubation and within the next 6 hr there was invasion, as manifested by the appearence of new rings. PHL action on trophozoites was already apparent within 2 hr of exposure to drug (Fig. 3, *middle*). Between 4 and 8 hr or 8 and 20 hr of exposure to PHL, the rate of nucleic acid synthesis dropped by >90% (Fig. 3, *middle*, *inset*). These results clearly indicate that PHL arrested trophozoite development. On the other hand, the rate of nucleic acid synthesis in rings, which was markedly smaller than that in trophozoites, was poorly, if at all, affected by PHL (Fig. 3,

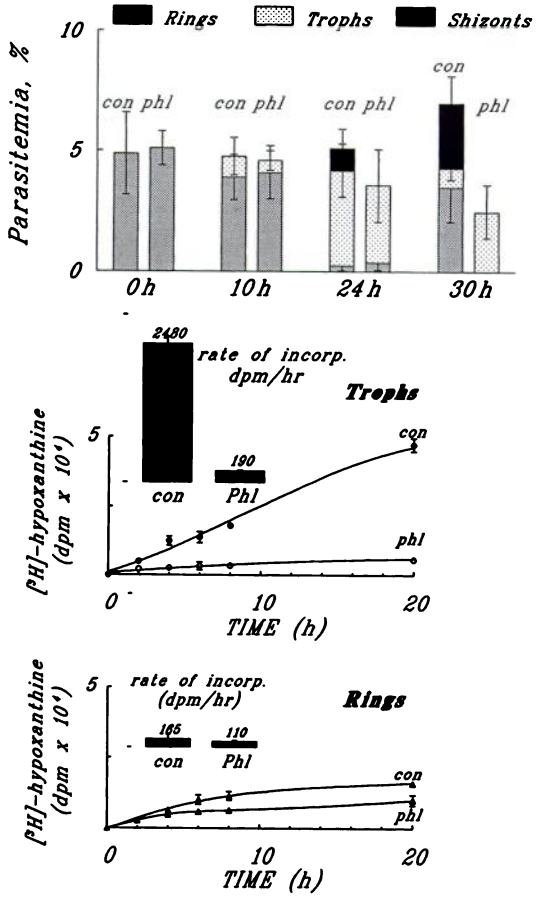

Fig. 3. Profiles of parasitemia and [^{3}H]hypoxanthine incorporation into nucleic acids in the presence of PHL. Ring cultures were exposed to PHL (40 μM) for different periods of time (up to 30 hr) and were analyzed in terms of parasitemia and parasite developmental stages by microscopic examination of Giemsa-stained smears (*top*). Nucleic acid synthesis was monitored in the presence or absence of PHL (40 μM) in cultures of trophozoites (*T*) (*middle*) and rings (*R*) (*bottom*), as described in Materials and Methods. Data are given as dpm of [^{3}H]hypoxanthine incorporated into nucleic acids. *Insets*, rates of incorporation of [^{3}H] hypoxanthine into nucleic acids beween 8 and 20 hr. *con*, control.

bottom). As shown in Fig. 3, *bottom*, *inset*, the rate of nucleic acid synthesis between 8 and 20 hr of exposure was not statistically different between PHL-treated and control parasites. The small PHL-susceptible component of nucleic acid synthesis, which was apparent only at early times of exposure, could be attributed to the presence of a minor (i.e., undetectable) fraction of contaminating trophozoites. This is because trophozoites take up considerably higher levels of nucleic acid precursors than do rings (>10-fold, as seen in Fig. 3). Although at this point a minor inhibitory effect of PHL on rings cannot be fully excluded, its contribution to parasite growth arrest elicited by PHL is virtually insignificant, compared with that obtained with trophozoites.

To determine whether the effect of PHL on parasite growth demanded permeation of PHL into the host cell, as the data described above would indicate, we considered the possibility of using RCCs carrying relatively large amounts of drug as targets for parasite invasion and growth. The feasibility of the approach was previously established using DFOs as test drugs (11). To assess whether PHL-loaded RCCs could support intracellular parasite growth, it was necessary to establish, first, that the encapsulated material could be retained in RCCs under culture conditions and, second, that those RCCs could sustain invasion by parasites and retain substantial amounts of material in the host cell compartment. The egress-retention experiments carried out with DFO-loaded RCCs (11) showed that RCCs were virtually impermeant to DFO and only moderately leaky to more hydrophobic derivatives of DFO. The analogous assay applied to PHL (Fig. 4) revealed an exponential egress or retention profile from RCCs originally encapsulated with 1.4 mM drug. After 24–48 hr under culture conditions, egress of drug reached a plateau level with about 40% of the drug still retained within the RCCs. Under those experimental conditions, the level of drug attained outside was 22 μM, which is in the range of the IC_{50} values of PHL tested on parasite cultures (14). Thus, the feasibility of using RCCs for the aforementioned experiments with parasites would be limited to experimental conditions in which the drug concentration attained extracellularly is negligible, compared with the IC_{50} of the drug. This could be accomplished either by using lower concentrations of encapsulated drug, by sequestrating extracellular drug using anti-PHL antibodies, or by using RCCs after the material ceased to leak out. The initial experiments using PHL-encapsulated RCCs as parasite growth targets indeed revealed a marked inhibition of parasite growth. In fact, the level of growth inhibition attained was commensurate with the amount of drug that had egressed from the RCCs and was present in the medium and definitely not with that estimated to have been retained in the uninfected RCCs (Fig. 4). If, however, RCCs were used after being incubated for 24 hr under culture conditions and being washed free of extracellular probe, full parasite growth support was obtained (data not shown). Moreover, when parasitized cultures of PHL-encapsulated RCCs were supplemented exogenously with anti-PHL antibodies (1/800 dilution of concentrated IgG fraction), the ability of parasites to propagate in RCCs was fully or partially restored, depending on the level of drug originally encapsulated (Fig. 5). Partial reversal of growth inhibition was obtained with a given dilution of antibodies added to a RCC preparation loaded with 4 mM PHL, and full reversal was obtained with a preparation loaded with 0.4 mM PHL. The antibody titer used was that which by itself caused no effect on *P. falciparum* culture growth (data not shown).

The results described above suggested that the PHL effect on parasite growth was most probably exerted by extracellular and not intracellular drug. However, for that to be the case, it had to be shown that drug-loaded RCCs were indeed invaded by parasites and that the drug was quantitatively retained (in a pharmacologically active form) in infected cells during parasite growth. This was shown by using RCCs into which [^{3}H]PHL was encapsulated; parasites were added and the distribution of the label was monitored in the infected cells along the developmental stages of parasite growth 24 and 48 hr after initiation of the experiment. To eliminate any possible effects of drug that might have leaked out, the culture medium was washed periodically (at 8–10-hr intervals). Separation of the stages was accomplished by Percoll-alanine isopycnic separation. As shown in Fig. 6, 24 hr after addition of trophozoite-schizonts to PHL-loaded RCCs (initial concentration, 2 mM) about 30% of the drug remained in the noninfected RCCs, with about 22% in the ring-containing fractions (427 μM and 446 μM concentrations for the two subpopulation of rings, i.e., early

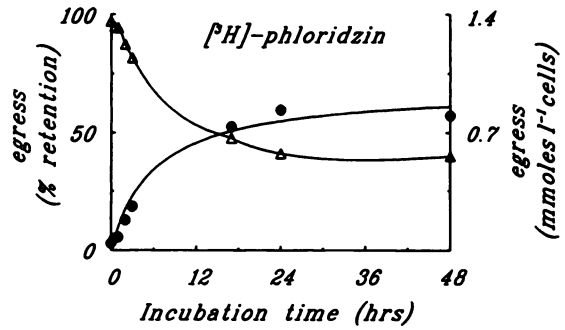

Fig. 4. Permeation of [^{3}H]PHL in RCCs. [^{3}H]PHL and unlabeled drug were encapsulated in RCCs to a final concentration of 1.4 mM, as detailed in Materials and Methods. Cells (2.5% hematocrit) were suspended at 37° in RPMI 1640 medium with no phenol red, and drug permeation was monitored either in the supernates (egress; *filled circles*) or in the cell pellets (retention; *open triangles*) by taking duplicate samples for analysis at the indicated time periods. The points depicted are average values (standard errors are smaller than the symbols) of a representative experiment.

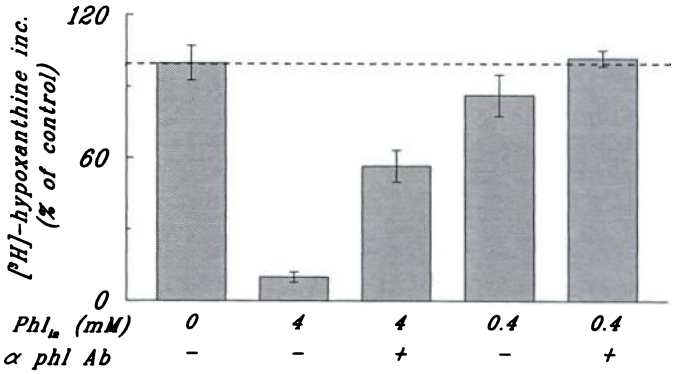

Fig. 5. Effect of anti-PHL antibodies on parasite growth in PHL-loaded RCCs. Parasites isolated as late trophozoites-early schizonts were added to PHL-loaded RCCs (0, 0.4, or 4 mM concentrations, as indicated) (2.5% parasitemia, 2.5% hematocrit), with half of the cultures supplemented with anti-PHL antibodies (α *phl Ab*). Eighteen hours after parasite inoculation [^{3}H]hypoxanthine (5 μCi/ml) was added, and nucleic acid synthesis was measured at the end of a 24-hr incubation period. Data are given as means ± standard errors (six experiments). *Dashed line*, 100% probe accumulation. At 0.4 mM PHL_{in}, the value of [^{3}H]hypoxanthine incorporation in the presence of anti-PHL antibodies (102 ± 3) was significantly different from that in its absence (86 ± 8; $p < 0.05$ by paired *t* test analysis).

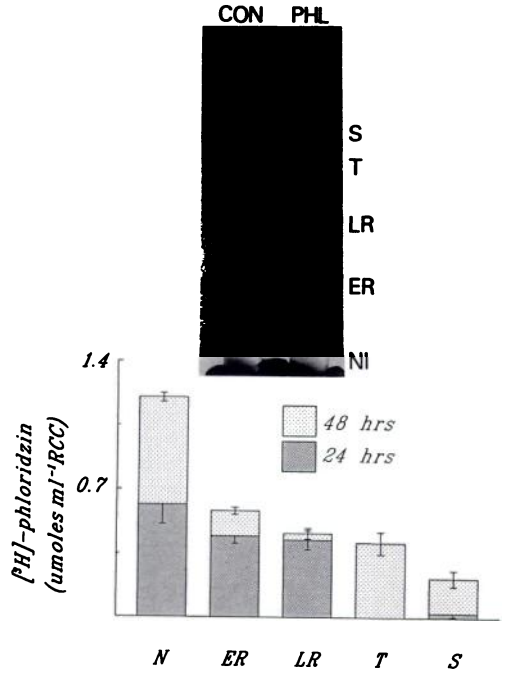

Fig. 6. Stage dependence of the recovery of [^{3}H]PHL in RCCs infected with *P. falciparum*. PHL was encapsulated in RCCs to a final concentration of 2 mM and supplemented with trophozoites, as detailed in Materials and Methods. At 24 and 48 hr after parasite inoculation, samples were withdrawn and cells were subjected to Percoll-alanine separation (*top*). The layers of the gradient obtained after 48 hr corresponded to the following parasite growth stages (from top to bottom): schizonts (*S*), trophozoites (*T*), young trophozoites-late rings (*LR*), early rings (*ER*), and noninfected cells (*NI*). After washing of the Percoll, each layer was analyzed for the number of cells (absorbance of hemolysates at 410 nm) and for radioactivity (*bottom*). Data are given as μmol of PHL/ml of RCCs (two experiments). *CON*, control.

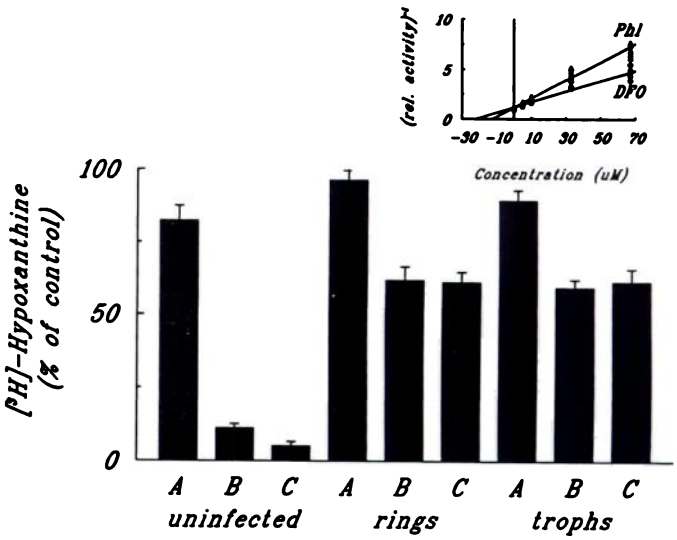

Fig. 7. Biological assay of drug present in hemolysates of RCCs loaded with drugs and infected with parasites. Sham-loaded RCCs or cells loaded with 4 mM DFO or PHL were incubated for 18 or 42 hr in regular RPMI 1640 medium at 37°, either with or without gelatin-isolated trophozoites. Medium replacement was done after 18 hr. At 18 and 42 hr, samples were withdrawn and the cells were subjected to Percoll-alanine separation. Cells were recovered from each Percoll layer, diluted to a 10% hematocrit value, and freeze-thawed. The aliquots of the hemolysates were added to a culture of trophozoites (600 μl, 2.5% parasitemia, 2.5% hematocrit) for determination of the effects on [^{3}H]hypoxanthine incorporation into nucleic acids (24-hr test), i.e., 15 μl of uninfected cells (lysates of 50% hematocrit suspensions) just after encapsulation, 60 μl of rings (24 hr), or 100 μl of trophozoites (48 hr). *A–C*, RCCs encapsulated with no drug (*A*), DFO (*B*), or PHL (*C*). *Inset*, Dixon plots of inhibition of nucleic acid synthesis by extracellularly supplemented DFO or PHL. The relative activity was calculated as the ratio of the [^{3}H]hypoxanthine incorporation at a given concentration of inhibitor to control incorporation. The *x*-intercepts are the experimental IC_{50} concentrations (22 μM for DFO and 12.5 μM for PHL). Data are given as means ± standard errors (six experiments).

and late, respectively). After 48 hr, most of the rings progressed to trophozoites. The level of drug in noninfected cells remained essentially the same as that after 24 hr, and the levels in trophozoites and schizonts were reduced to 411 μM and 191 μM, respectively. The small fractions of label associated with early and late rings most probably represent late invasion events by parasites that matured later than the bulk of trophozoite-schizonts originally added to the RCCs (Fig. 6). These experiments clearly indicate that, despite intracellular drug concentrations in RCCs far beyond those needed to fully inhibit the parasite when added extracellularly, no detectable inhibition of parasite invasion or intracellular growth was observed. Thus, RCCs loaded with PHL demonstrably supported parasite growth.

An important point to be determined was whether the drug retained inside the RCCs (normal or infected) was pharmacologically active. As an additional control for the efficiency of drug encapsulation we used DFO, for which the methodologies were originally developed (11). The IC_{50} values for inhibition by externally added PHL or DFO (24-hr test on trophozoites) were 12 μM and 22 μM, respectively (Fig. 7, *inset*). RCCs loaded with 4 mM concentrations of PHL or DFO or without drug were incubated with trophozoites under culture conditions and subjected to Percoll-alanine separation after either 18-hr or 42-hr incubation. As shown in Fig. 6, a regimen of periodic replacement of culture medium was implemented to avoid extracellular effects on parasites exerted by drug that might leak out from intact or damaged RCCs. After freeze-thaw steps, the RCC lysates of the Percoll fractions were tested with synchronized cultures of trophozoites, to attain a final 1/70 to 1/100 dilution of the original lysate (Fig. 7). The lysates of either DFO- or PHL-loaded RCCs, uninfected or infected with parasites, showed a clear inhibitory effect on *P. falciparum* cultures. The levels of inhibition attained with the different lysates were compared with those obtained with extracellularly added drug (Fig. 7, *inset*). The levels in uninfected RCCs after 18 hr were equivalent to about 2 mM PHL or 3.2 mM DFO. In rings and trophozoites the estimated intracellular concentrations were 0.6 mM PHL and 1.2 mM DFO. The differences in potencies of the drugs released from RCCs reflect the fact that leakage from infected RCCs was relatively greater than that from uninfected RCCs. Despite these quantitative differences, the results clearly indicate that most of the PHL retained within the host cell was potentially active throughout most of the growth cycle of the parasite.

The absence of inhibitory effects of drugs encapsulated within cells that were invaded by parasites was also assessed over an entire cycle of parasite growth, in terms of nucleic acid synthesis and microscopic observation of Giemsa-stained smears. RCCs loaded with 2 mM PHL or DFO were supplemented with trophozoites. As with previous experiments, the medium was replaced periodically (Fig. 8). The percentages of reduction in nucleic acid synthesis and parasitemia were comparable for the three systems depicted in Fig. 8. Parasitemia values after 42 hr showed at most 10% reduction in growth, compared with control, sham-loaded RCCs. Nucleic acid syn-

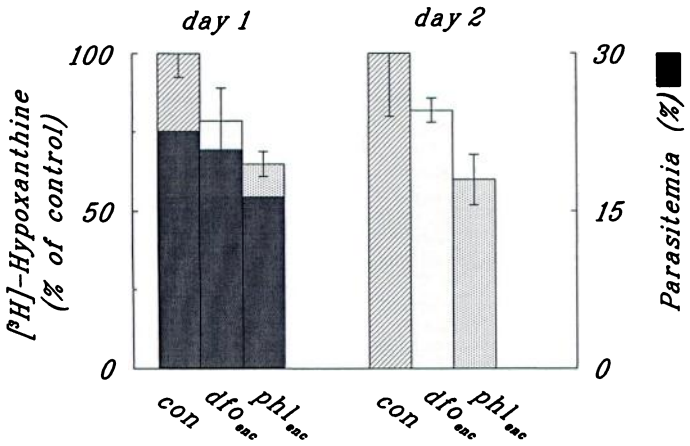

Fig. 8. Effect of encapsulated drugs on *P. falciparum* invasion and growth. DFO and PHL were encapsulated in RCCs (final concentrations, 2 mM) and supplemented with trophozoites. Parasitemia and hematocrit values were each adjusted to 2.5%. All cell suspensions were washed at 5 and 12 hr after inoculation and every 12 hr thereafter. At 18 hr after inoculation, samples were withdrawn and parasites were counted on Giemsa-stained smears (■); nucleic acid synthesis was measured at 24 hr as [^{3}H]hypoxanthine incorporation into macromolecules (*day 1*). The cultures were supplemented with fresh red blood cells at 42 hr after inoculation and were assayed for nucleic acid synthesis after another 24 hr, in the same manner (*day 2*). Data are given as means ± standard errors (six experiments): *con* (control, sham-encapsulated RCC) 100 ± 8 (*day 1*) and 100 ± 20 (*day 2*); dfo_{enc} (DFO encapsulated RCC) 79 ± 10 (*day 1*) and 82 ± 4 (*day 2*); phl_{enc} (PHL encapsulated RCC) 65 ± 4 (*day 1*) and 60 ± 8 (*day 2*).

thesis was, however, inhibited by 15–30% in RCCs encapsulated with either drug. These effects were significant; however, they could be accounted for by the presence of extracellular drug that could have leaked out or been released from damaged cells in the intervals between washes (8–12-hr intervals). However, because even under those conditions the concentrations of drugs retained in the RCCs were in the hundred-micromolar range, clearly most of the intracellular drug would seem to be pharmacologically ineffective *in situ*. The proof that only drug that had leaked out from RCCs was pharmacologically effective was provided by the experiments with anti-PHL antibodies (see Fig. 5).

Discussion

Elucidation of the mode of action of antimalarial agents involves the study of the routes and modes of entry of drugs into infected cells and drug interference with parasite functions. Because modern drug design aims also at specifically targeting drugs with antiparasitic potential, it was of interest to assess whether the permeation properties of normal versus infected cells were sufficiently different to ensure preferential access of drugs to infected cells. Previous biophysical analysis of host cell membrane properties underscored major differences between the normal and infected systems (3, 4, 22). A variety of agents within a wide chemical spectrum were found to be accessible to the host cell cytosol in the infected state (22). Some agents, such as PHL (14) and more recently nitrophenylanthranilates (23), were found to block some of the novel permeation pathways induced by parasites. However, the correlations between blockage of the novel pathways and inhibition of *P. falciparum* growth in culture were only partial, and growth inhibition could not be unequivocally attributed to blockage of those pathways (22). Because agents such as PHL, despite being hydrophilic in nature and poorly permeant to uninfected cells, were nevertheless demonstrably permeant to infected cells, the possibility of PHL directly interfering with parasite functions was specifically invoked (15, 16). Moreover, studies with parasites enclosed in virus-permeabilized host cells showed that PHL indeed caused significant reduction in parasite protein-synthetic capacity (14). Clearly, such effects demanded drug entry into parasite compartments and interference with metabolic processes, primarily at the trophozoite stage, as shown in this work. According to the classical scheme presented above, drug entry into the parasite is achieved by sequential crossing of HPM, the PVM, and the PPM and diffusion across the enclosed compartments (22). However, the recently proposed "direct access route" from medium to the parasite surface (5) seemingly provides a parallel or alternate access route that, for drug action, would require only drug diffusion through the putative duct and crossing of the PPM (11). Permeation and pharmacological data obtained with the hydrophilic DFO (11) in *P. falciparum*-infected cells [and a similar result shown by Scott *et al.* (10) for DFO-dextran] were previously found to be consistent with the existence of such a direct route or window of access, a process referred to as "fenestration." To what extent the fenestration of hydrophilic drugs into parasites occurs via the putative parasitophorous duct remains to be demonstrated. The existence of the parasitophorous duct as a permanent structure present in infected cells has been debated (5, 9, 24–27).

The possibility that, in analogy with DFO, fenestration might also underlie the mode of entry of PHL into infected cells and its action on parasites was experimentally assessed in this work. Studies of the ingress of PHL into red blood cells showed the drug to be impermeant to uninfected cells, poorly permeant to rings, and highly permeant to trophozoites (Fig. 2). The speed of action of PHL on nucleic acid synthesis (Fig. 3) was commensurate with ingress of the drug into cells (Fig. 2). However, permeation into trophozoites was apparently limited to only one third of the cell volume (Fig. 2). This could be interpreted as indicating either that only one third of the infected cell is permeable to PHL or that only one third of the volume of a trophozoite is accessible to drug, as found for DFO (11). Alternatively, the possibility that PHL might have accessed the host cytosol of trophozoites and egressed during cell washing could not be eliminated *a priori*. To distinguish between these alternatives, we studied the properties of PHL fluxes in RCCs into which PHL was encapsulated and in which parasites were shown to enter and grow. Thus, drug encapsulation into RCCs served as a target for parasite invasion and subsequent growth, an approach previously applied to DFO and congeners (11). The result shown in this work indicated that 1) PHL encapsulated into RCCs at millimolar concentrations was largely retained (30–40%) over extended periods of time (24–48 hr), 2) the drug-loaded RCCs (PHL or DFO) supported parasite growth almost as well as did sham-loaded RCCs, 3) PHL was substantially retained also in RCCs infected with parasites, even 48 hr after invasion, 4) PHL retained in infected or uninfected RCCs was demonstrably active after being physically released from the cells, whereas the drug retained within infected cells was innocuous to intracellular parasites, and 5) inhibition of parasite growth by PHL that leaked out from RCCs into medium or was released by damaged cells could be neutralized by anti-PHL antibodies added to the medium. Taken *in toto*, these data indicate that host cell-associated PHL

was ineffective in blocking parasite growth, whereas extracellularly present drug was markedly effective. Moreover, it is inferred that, even if some PHL permeated into the host cytosol of infected cells, it would be rendered biologically ineffective or inaccessible to parasites. These and the aforementioned data are apparently difficult to reconcile with the classical sequential model of drug action, which invokes exclusive entry of drug into host compartments as the first step in drug uptake by infected cells. Moreover, the data obtained with RCCs are supportive of a restricted accessibility of externally added PHL to the host cell interior of trophozoites, as suggested by us previously (15) and demonstrated in the present work. That property does not eliminate the possibility of some PHL entering the host cytosol of infected cells, and the present work does not provide direct evidence for the drug accessing parasites by a particular route. The data are consistent with the notion of a direct route of access of hydrophilic drugs into parasites, the process of fenestration mentioned above. In that process, the proposed parasitophorous duct (5) might provide the actual aqueous corridor for hydrophiles to access the parasite. Surface interaction of PHL with parasite membrane components or parasite cytosolic components might therefore underlie its inhibitory action on parasites. The relative contributions of the effects on parasite growth elicited by PHL by direct action on parasites, compared with those previously observed on the new permeation pathways appearing in infected cells (15), remain to be assessed.

In mammalian cells, PHL affects primarily epithelial sodium-glucose co-transport (28). However, the fact that structural congeners of PHL were more biologically potent against malaria than was the parent compound and at the same time were relatively more ineffective as transport blockers (14) implied no co-transporter involvement in the drug mode of action.

At present, only iron chelators of the DFO type and the dihydrochalcone glycoside PHL have been used as hydrophilic antimalarial agents that might approach parasites by the aforementioned direct route. The modes of action of these agents as antimalarial agents and their chemical structures (Fig. 1) are considerably different. However, their compartmental distributions within infected cells are qualitatively similar, in that both seemingly approach the parasite directly from the medium. On this basis we propose that the chemical design of agents that display both selective admittance by the PPM and potential cytotoxic activity might pave the road for improving both drug efficacy and selectivity in the chemotherapy of malaria.

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