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Reaction of Antimalarial Endoperoxides with Specific Parasite Proteins

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The endoperoxides are a new class of antimalarial agents, of which artemisinin (qinghaosu) is the prototype. We have previously shown that artemisinin is capable of alkylating proteins in model reactions. In the present study, we showed that when *Plasmodium falciparum*-infected erythrocytes are treated with a radiolabeled antimalarial endoperoxide, either arteether, dihydroartemisinin, or Ro 42-1611 (arteflene), the radioactivity is largely coverted into a form which can be extracted with sodium dodecyl sulfate (SDS). Autoradiograms of SDS-polyacrylamide gels showed that six malarial proteins are radioactively labeled by the three endoperoxides. This labeling occurs at physiological concentrations of drug and is not stage nor strain specific. The labeled proteins were not the most abundant proteins seen on Coomassie-stained gels. No proteins were labeled when uninfected erythrocytes were treated with these drugs, nor when infected erythrocytes were treated with the inactive analog deoxyarteether. Thus, the antimalarial endoperoxides appear to react with specific malarial proteins.

The endoperoxides are a new class of antimalarial agents, of which the prototype, artemisinin (qinghaosu; Fig. 1), was first isolated from an ancient Chinese herbal remedy (13, 18). Several semisynthetic derivatives, including artemether, arteether, and artesunate (Fig. 1), are already in development or use (13). Artemether is the most widely used of these compounds; it is particularly effective against cerebral malaria and no clinically relevant drug resistance has yet been observed (13). Artemether has been administered to more than 3 million patients in the People's Republic of China and is now undergoing phase II and III development by Rhone-Poulenc (13). In addition, a number of synthetic endoperoxides have also been prepared and tested, of which one, Ro 42-1611 (arteflene; Fig. 1), is undergoing phase II and III clinical evaluation (3, 13).

The endoperoxide moiety is necessary for antimalarial activity, since analogs which lack this group are inactive (2, 5). The endoperoxide may explain why the drug is selectively toxic to the parasites that cause malaria, since the parasites are rich in heme and heme catalyzes the reductive decomposition of the endoperoxide bridge (31). The drug decomposes into a free radical (25, 27) and other electrophilic intermediates (26). In model systems, we have obtained evidence that the artemisinin-derived intermediate can act as an alkylating agent, forming adducts in vitro with human serum albumin (30), proteins in isolated erythrocyte membranes (1), and heme (15). Here we provide the first biological evidence that the drug may react with specific proteins in malaria-infected erythrocytes.

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MATERIALS AND METHODS

Materials. [12-^{3}H]dihydroartemisinin (15 Ci/mmol) and [^{14}C]Ro 42-1611 (56 mCi/mmol) were gifts from A. Benakis, University of Geneva, and R. Ridley, Hoffmann-La Roche, Basel, Switzerland, respectively. The position at which Ro 42-1611 is labeled is depicted in Fig. 1. RPMI 1640 was obtained from Gibco/BRL (Grand Island, N.Y.). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise noted.

[14-^{3}H]arteether (650 mCi/mmol) was prepared by a modification of a previously published procedure (16). To a solution of the tosylate of 14-hydroxyarteether (20 mg, 0.04 mmol) in dry dimethyl sulfoxide (6.0 ml) was added NaB^3H_4 (350 mCi; specific activity, 712 mCi/mmol). The suspension was stirred and was incubated at 80°C for 5 h. The reaction was quenched by the addition of methanol (1.0 ml) and was diluted with $CHCl_3$ (10 ml). The organic layer was separated, washed with water, dried over Na_2SO_4, and concentrated. The crude product was purified by preparative silica thin-layer chromatography by using hexane-ethyl acetate (7:3) as the solvent to afford [14-^{3}H]arteether (5.0 mg; specific activity, 700 mCi/mmol.)

A portion of the labeled arteether was converted to [14-^{3}H]deoxyarteether. A mixture of [14-^{3}H]arteether (3.0 mg) and 1 mg of 5% palladium-charcoal in ethyl acetate was saturated with H_2, and the mixture was stirred for 2 h. The mixture was filtered and the filtrate was concentrated. The crude reaction mixture was purified by preparative silica thin-layer chromatography by using hexane-ethyl ether (2:1) as the solvent to yield 1 mg of [14-^{3}H]deoxyarteether. The specific activity of the product was presumed to be identical to that of the labeled arteether, since no radioactivity was lost during the reduction.

For arteether, deoxyarteether, and Ro 42-1611, the labels are in positions that are stable to exchange, hydrolysis, and oxidation.

Parasite culture. Chloroquine-resistant *Plasmodium falciparum* FCR3 was a gift from Margaret Perkins, Rockefeller University. Chloroquine-susceptible strain D6 was a gift from

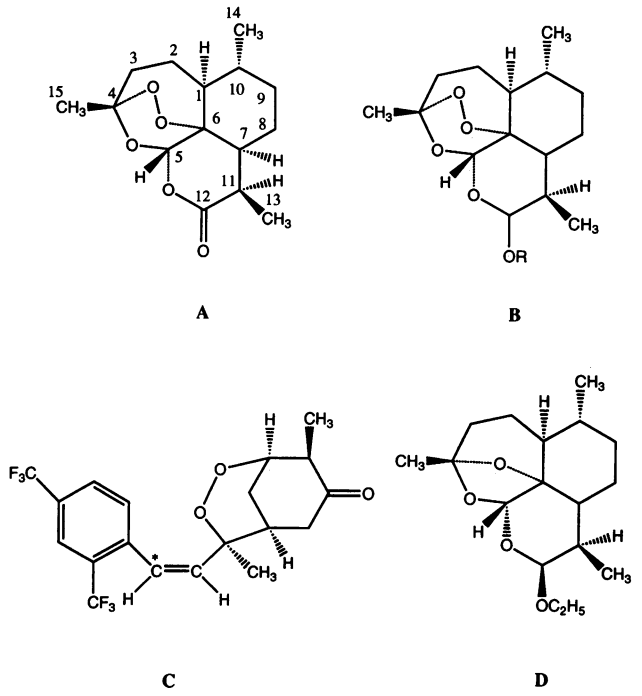

FIG. 1. Structure of artemisinin (A), its semisynthetic derivatives (dihydroartemisinin, R = H; arteether, R = CH_2CH_3; artesunate, R = $COCH_2CH_2COOH$) (B), Ro 42-1611 (the askerisk denotes the position of the ^{14}C label) (C), and deoxyarteether (D).

the Division of Experimental Therapeutics, Walter Reed Army Institute of Research. For all experiments, parasite-infected erythrocytes were cultivated in candle jars (29) and synchronized by sorbitol lysis (21) so that >90% of the parasites were at the same stage.

Incorporation of radiolabeled drug and extraction of radioactivity from parasites. Parasitized erythrocytes were used when they contained mature trophozoites (26 to 32 h of development) or rings (12 to 16 h of development). Infected erythrocytes were incubated at 37°C in culture medium (10% hematocrits with 20 to 30% parasitemias) in the presence of 0.5 μCi of [^{3}H]arteether, [^{3}H]dihydroartemisinin, [^{14}C]Ro 42-1611, or [^{3}H]deoxyarteether per ml. After 6 h, the infected erythrocytes were pelleted by centrifugation at 1,000 × *g* for 5 min and were washed three times with RPMI 1640. Host cell-free parasites were then obtained from the infected erythrocytes by saponin lysis (8) and were washed four times with Dulbecco's phosphate-buffered saline (PBS) by centrifuging at 5,000 × *g* for 10 min at 4°C. The parasites were then lysed by suspension in 20 volumes of hypotonic buffer (5 mM potassium phosphate [pH 7.4]) for 10 min at room temperature. More than 70% of the parasites were disrupted, as determined by phase-contrast microscopy. The lysed parasite suspension was centrifuged (14,000 × *g* for 30 min), and the supernatant was saved (aqueous extract). The pellet was then incubated in 10 volumes of 1% sodium dodecyl sulfate (SDS) for 10 min and was then recentrifuged at 14,000 × *g* for 30 min, and both the supernatant (SDS extract) and the pellet (SDS-insoluble fraction) were saved.

Subcellular fractionation. Subcellular fractionation was performed by a modification of the method of Goldberg et al. (10). Host cell-free parasites were prepared as described above, washed with PBS containing 1.5 mM magnesium chloride and 0.5% streptomycin sulfate, and suspended in 0.5 ml of 10 mM sodium phosphate (pH 7.1) containing 0.25 M sucrose and 0.5% streptomycin sulfate. The suspension was homogenized for 10 min at 4°C by using a motorized Teflon pestle at maximum speed. More than 80% of the parasites were disrupted, as determined by phase-contrast microscopy. The homogenate was then mixed with 9.5 ml of 42% Percol (in 0.25 M sucrose–1.5 mM magnesium chloride [pH 7.0]), and the mixture was centrifuged as described previously (10). Ten 1-ml fractions were withdrawn, transferred to new tubes, diluted with 0.5 ml of PBS, and centrifuged at 14,000 × *g* for 30 min. Each pellet was washed once with 1 ml of PBS. A portion of each fraction was assayed for dihydroorotate dehydrogenase activity (17) and heme (7), which are markers for mitochondria (9) and food vacuoles (7), respectively. Pellets from each fraction were then extracted with hypotonic buffer; this was followed by extraction with 1% SDS as described above. The radioactivity in the two extracts and the final pellet was then counted.

Electrophoresis and autoradiography. Synchronized *P. falciparum*-infected erythrocytes were incubated with radiolabeled drug as described above. Host cell-free parasites were isolated, suspended in loading buffer (50 mM Tris-HCl [pH 6.8] with 10% SDS, 10% glycerol, and 0.05% bromophenol blue), boiled for 5 min, and loaded onto SDS-polyacrylamide gels (10 to 15 μg per lane). Some samples were treated with β-mercaptoethanol or β-mercaptoethanol–urea as described previously (1) prior to loading. For some gels, parasites were lysed by freezing and thawing five times and were centrifuged at 14,000 × *g* for 30 min. The pellets were then either loaded onto gels as described above or were sonicated and centrifuged five times to obtain a particulate suspension of hemozoin (11). Discontinuous slab gel SDS-polyacrylamide gel electrophoresis (PAGE) (20) was carried out by using 1.5 M Tris-HCl buffers at pH 8.6 for the 12% separating gel and 0.5 M at pH 6.8 for the 4% stacking gel. The resultant gels were then fixed and stained with Coomassie blue, impregnated with autoradiography enhancer (En^{3}Hance; New England Nuclear-Du Pont, Boston, Mass.), and exposed to X-ray film (Kodak X-Omat AR) at −70°C. Exposure times were between 3 weeks and 1 month.

Miscellaneous assays. Radioactivity was measured in a Beckman LS 7000 scintillation counter (Beckman Instruments, Fullerton, Calif.), with Scinti-Verse BD (Fisher Scientific, Fair Lawn, N.J.) used as the scintillant. Protein was determined by using the DC Protein Assay (Bio-Rad, Rockville Center, N.Y.), with bovine serum albumin used as a standard.

RESULTS

When *P. falciparum*-infected erythrocytes were incubated in vitro for 6 h with the radiolabeled endoperoxides (dihydroartemisinin, arteether, and Ro 42-1611), drug-derived radioactivity was found at high levels in the isolated parasites (Fig. 2). Arteether was taken up most effectively. In contrast, a negligible amount of the inactive deoxyarteether was taken up (Fig. 2), suggesting that drug incorporation by the parasite is dependent on the presence of the endoperoxide bridge. The drugs were not taken up in any measurable amount by uninfected erythrocytes (data not shown).

Most of the drug contained in isolated parasites can be extracted from the parasites with 1% SDS. For all three active drugs, less than 10% of the parasite-associated radioactivity was found in the initial aqueous extract, while 70 to 85% was found in the SDS extract (Fig. 2). All of the parasite-associated protein was found in these two fractions, with approximately 60

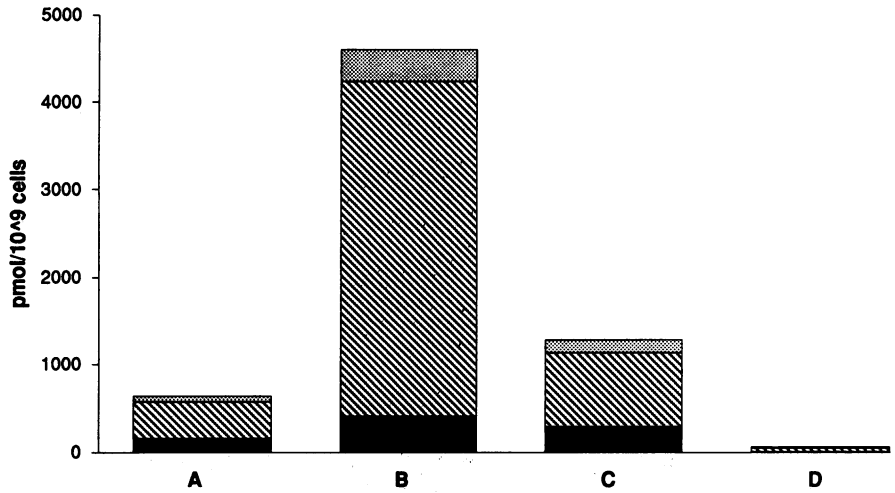

FIG. 2. Incorporation of [^{3}H]dihydroartemisinin (A), [^{3}H]arteether (B), [^{14}C] Ro 42-1611 (C), and [^{3}H]deoxyarteether (D) into aqueous extracts (dotted bars), SDS-soluble extracts (hatched bars), and SDS-insoluble pellets (solid bars) of isolated parasites. Parasites were isolated by saponin lysis from *P. falciparum*-infected cells which were treated with drug and then extracted as described in Materials and Method.

to 65% found in the SDS extract. The SDS-insoluble fraction is almost entirely composed of heme, containing less than 5 μg of protein per μmol of heme, suggesting that this latter component consists predominantly of hemozoin. Only 13 to 15% of the radioactivity was found in this latter fraction. Thus, when treated with radiolabeled drug, most of the parasite-associated radioactivity can be extracted with SDS.

Subcellular fractionation was performed to attempt to better localize the parasite-associated drug (Fig. 3). Dihydroorotate dehydrogenase and heme were used as markers for the mitochondria and food vacuoles, respectively. The SDS-insoluble counts, as expected, were highest in the subcellular fraction which contained parasite food vacuoles and hemozoin. In contrast, neither the aqueous nor the SDS-soluble counts could be localized into any subcellular fraction (data not shown).

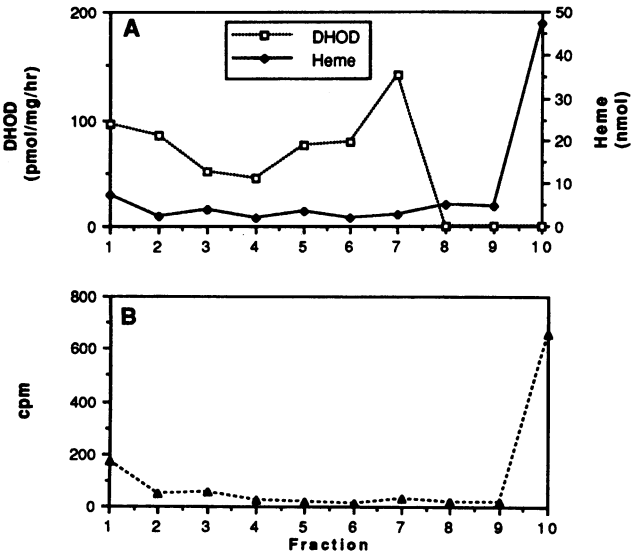

FIG. 3. Subcellular fractionation of [^{3}H]dihydroartemisinin-treated parasites. (A) Distribution of heme and dihydroorotate dehydrogenase (DHOD), which are markers for the food vacuoles and mitochondria, respectively. (B) Distribution of drug-derived SDS-insoluble radioactivity.

In order to determine the ultimate fates of the endoperoxides, drug-treated parasites were analyzed by SDS-PAGE and autoradiography. When trophozoite-infected erythrocytes (FCR3 strain) were incubated with [^{3}H]dihydroartemisinin at a physiological concentration (33 nM) (13), four major protein bands (25, 50, 65, and >200 kDa) and two minor ones (32 and 42 kDa) became labeled (Fig. 4A). The intensities of these bands were not diminished by treatment with mercaptoethanol or 8 M urea (data not shown), suggesting that the drug-protein bond is covalent.

These same labeled bands were found in the pellets obtained after centrifugation of lysed parasites at 14,000 × *g*, which contained both membranes and hemozoin (Fig. 4F). However, no radioactive bands were found in isolated hemozoin (Fig. 4G). Thus, the drug-labeled proteins appear to be associated with parasite membranes and not with hemozoin.

In contrast, no bands were seen when uninfected erythrocytes were incubated with the labeled drugs under identical conditions (data not shown).

The reaction between the endoperoxides and parasite proteins is specific, because if it were not, the drugs would react with the most abundant parasite proteins. Yet, none of the radiolabeled bands were major bands on Coomassie blue-stained gels (Fig. 4E). Furthermore, bands with similar molecular sizes were labeled when parasites were incubated with either of the two other active drugs, arteether (Fig. 4B) and Ro 42-1611 (Fig. 4C), suggesting that there are specific endoperoxide-targeted malarial proteins.

The endoperoxide targets are neither strain- nor stage-specific proteins. The same bands that were labeled in chloroquine-resistant FCR3 trophozoites were labeled in chloroquine-susceptible D6 strain trophozoites treated with either [^{3}H]arteether (Fig. 4I) or [^{3}H]dihydroartemisinin (data not shown). Protein alkylation also occurred in ring stage FCR3 parasites as well, although only the four major bands (25, 50, 65, and >200 kDa) were seen clearly (Fig. 4H).

Finally, the inactive artemether derivative, [^{3}H]deoxyarteether, does not alkylate any proteins, even when incubations

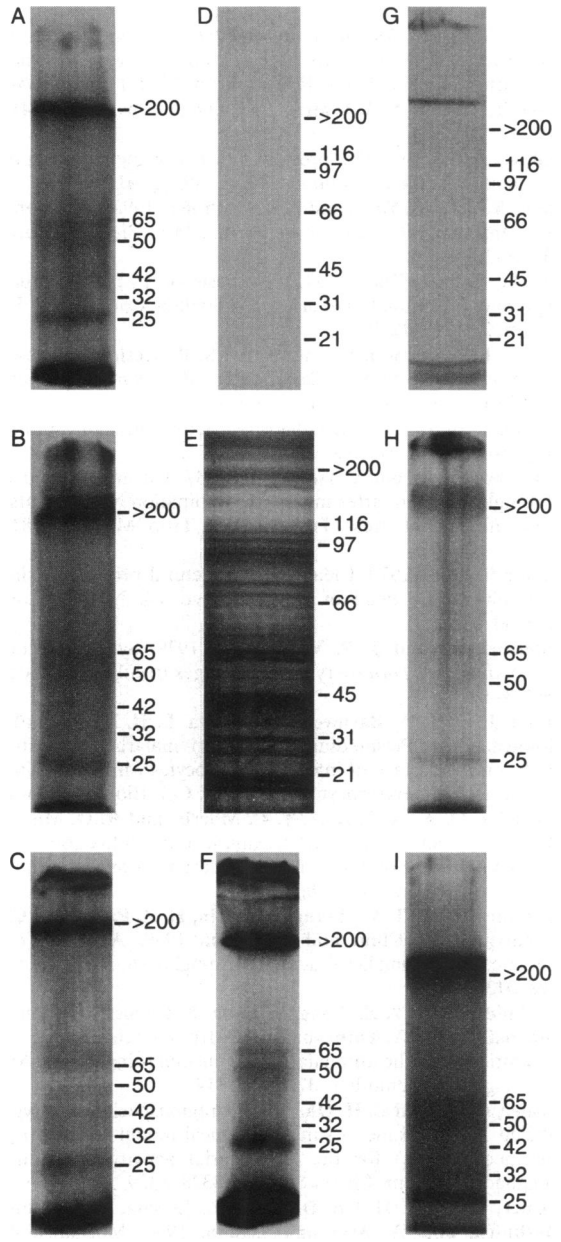

FIG. 4. SDS-PAGE autoradiograms of drug-treated synchronized parasites. The autoradiograms are of FCR3 trophozoites after treatment with [^{3}H]dihydroartemisinin (0.5 μCi/ml) (A), [^{3}H]arteether (0.5 μCi/ml) (B), [^{14}C]Ro 42-1611 (0.5 μCi/ml) (C), and [^{3}H]deoxyarteether (10.0 μCi/ml) (D). Autoradiograms of a pellet of [^{3}H]dihydroartemisinin-treated lysed FCR3 trophozoites obtained after centrifugation at 14,000 × *g* (F), hemozoin isolated from the preparation shown in panel F (G), FCR3 rings after treatment with [^{3}H]arteether (0.5 μCi/ml) (H), and D6 trophozoites after treatment with [^{3}H]arteether (0.5 μCi/ml) (I) are also shown. (E) Coomassie-blue stained gel of [^{3}H]dihydroartemisinin-treated parasites.

contain 20 times as much radioactivity as those used for the endoperoxides (Fig. 4D). Thus, only the active endoperoxides appear to be capable of reacting with malarial proteins.

DISCUSSION

Although the endoperoxides are an important new class of antimalarial agents, relatively little is known about their modes of action. Dihydroartemisinin is concentrated by the parasite (12). The antimalarial actions of these drugs may be mediated by the generation of free radicals, because free-radical scavengers antagonize the drug both in vitro and in vivo (19, 23, 24). The free-radical formation probably occurs as a result of cleavage of the endoperoxide bridge by intraparasitic heme iron (25–27, 31). Once it is formed, however, it is unclear how the endoperoxide-derived free radicals cause parasite death. In order to gain insight into the modes of action of these drugs, we have been following the fate of radiolabeled drug in malaria-infected erythrocytes.

Artemisinin and dihydroartemisinin have previously been shown to be capable of forming covalent bonds with proteins in aqueous solution (30), with proteins in isolated membranes (1), and with heme (15). In the present study, we showed that reactions between drug and protein may also occur in *P. falciparum*-infected erythrocytes. There are several pieces of evidence that suggest that these reactions are physiologically relevant. First, protein alkylation was seen after infected erythrocytes were treated with physiological concentrations of dihydroartemisinin. Second, the reaction appears to be specific, since the alkylated proteins are not the most abundant proteins in isolated parasites. Third, the three active endoperoxides—dihydroartemisinin, arteether, and Ro 42-1611—all react with the same proteins. Fourth, the same proteins are alkylated in rings and trophozoites and in a different strain of the parasite. Fifth, no proteins are alkylated in drug-treated erythrocytes, confirming our previous observation that dihydroartemisinin does not react with proteins in intact erythrocytes (1). Sixth, no proteins are alkylated in deoxyarteether-treated parasites, even when 20 times as much radioactivity is used. Thus, malaria parasites appear to contain specific proteins that react with endoperoxides.

The bonds between drug and protein observed in the present study are probably covalent, since the presence of SDS during electrophoresis is sufficient to dissociate most noncovalent bonds. Further support for this comes from the observation that the intensities of the bands on autoradiograms were not diminished by pretreatment with either mercaptoethanol or 8 M urea. Thus, the drug-protein bonds resemble the bond between artemisinin and human serum albumin, which was also not dissociable by mercaptoethanol or urea and which was proven to be covalent by electrospray mass spectrometry (30). In contrast, the bonds formed between dihydroartemisinin and proteins in isolated erythrocyte membranes are different in that they can be partially dissociated by these pretreatments (1).

While we do not yet know the identities or functions of the alkylated malaria proteins, a variety of membrane proteins have been identified in *P. falciparum*-infected erythrocytes which have molecular sizes similar to those of the endoperoxide target proteins. These include MSA-1 (180 to 220 kDa) (14), parasite-modified band 3 (65 kDa) (6), MSA-2 (45 to 55 kDa) (28), and CRA.5.1 (23 kDa) (4) and a histidine-rich protein (42 kDa) (22). More work is needed to establish the precise identities of the artemisinin target proteins.

For the three endoperoxides studied here, 13 to 15% of the total parasite-associated radioactivity was found in the SDS-

insoluble hemozoin. As described previously (15), this hemozoin-associated radioactivity consists predominantly of low-molecular-mass artemisinin-heme adducts. This explains why there are no bands seen in gels run on isolated hemozoin. Curiously, significantly more hemozoin-associated radioactivity (48% parasite associated) was found in a previous study (15) than was found in the present study. There are three possible explanations for this. First, the earlier study used a different radiolabeled endoperoxide ([^{14}C]artemisinin), and it is possible that this drug behaves differently from the three endoperoxides that we studied here. Second, the earlier study used an 8-h incubation in comparison with a 6-h incubation used in the present study, and it is possible that uptake into hemozoin occurs later. Third, the earlier study used a complex hemozoin isolation protocol involving several sonication steps, so it is possible that some of the increased association with hemozoin occurred during isolation. Nevertheless, both studies agree that a proportion of drug-derived radioactivity can be found to be associated with hemozoin.

In summary, radiolabeled endoperoxides appear to alkylate several specific membrane-associated material proteins. A better understanding of this process could aid in the development of better antimalarial endoperoxides.

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