Chloroquine Resistance in Malaria: Accessibility of Drug Receptors to Mefloquine†

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The process of mefloquine accumulation was studied in mouse erythrocytes infected with either Plasmodium berghei CS (chloroquine susceptible) or P. berghei CR (chloroquine resistant). In both cases, mefloquine was accumulated by a saturable process with an apparent dissociation constant of $2.5 \times 10^{-6}$ M and an apparent maximal capacity of 700 μmol per kg of erythrocyte pellet; uninfected mouse erythrocytes accumulated more than half as much mefloquine as infected erythrocytes. The process of accumulation was not stimulated by providing glucose as a substrate, and it was not inhibited in infected erythrocytes by azide, iodoacetate, or incubation at 2°C. Although mefloquine was accumulated more effectively than chloroquine by uninfected erythrocytes and by erythrocytes infected with P. berghei CR, competition between chloroquine and mefloquine was observed, raising the possibility that the same process of accumulation serves both drugs. Chloroquine competitively inhibits mefloquine accumulation, with an apparent inhibitor constant of $1.7 \times 10^{-3}$ M, and mefloquine competitively inhibits chloroquine accumulation, with an apparent inhibitor constant of $2 \times 10^{-6}$ M. The same process of accumulation and the same group of receptors could serve both drugs if mefloquine has greater access than chloroquine to the receptors. Regardless of whether the same process serves both drugs, undiminished accumulation by erythrocytes infected with P. berghei CR provides an explanation for the superiority of mefloquine in treating chloroquine-resistant malaria.

Recent studies of Plasmodium berghei reveal that chloroquine-resistant and susceptible lines of parasites are equally susceptible to chloroquine when they are equally exposed in vitro (6). Under conditions that prevail in vivo, however, resistant parasites receive less exposure because the infected erythrocytes fail to accumulate chloroquine avidly (3, 5, 9, 12, 14). Consequently, chloroquine resistance in this model may be attributed to reduced exposure of the parasites rather than to ineffectiveness of the drug after it reaches the parasites. This mechanism of resistance provides a basis for explaining how drugs in the same chemotherapeutic class with chloroquine could be effective in the treatment of chloroquine-resistant malaria. For example, amodiaquine, quinine, and mefloquine might have exactly the same, albeit unknown, mechanism of action as chloroquine (2) and yet be more effective in the treatment of chloroquine-resistant malaria (1, 13, 16-21) because they penetrate to the parasites. These four drugs all are quinoline derivatives and have many characteristics in common, but they also possess differences in structure that could affect their interactions with parasitized erythrocytes (Fig. 1). We have previously reported that amodiaquine is accumulated more effectively than chloroquine by erythrocytes infected with P. berghei CR (chloroquine resistant) (10) or Plasmodium falciparum CR (5). We now report that mefloquine accumulation is undiminished in erythrocytes infected with P. berghei CR. Mefloquine is a new antimalarial drug (15) that is more effective than chloroquine against P. berghei CR (16) and P. falciparum CR (1, 13, 17-19, 21).

METHODS

To provide erythrocytes for study, 20-g male Swiss-Webster mice (Hilltop Laboratories, Scottsdale, Pa.) were infected with either P. berghei CS (chloroquine susceptible) or P. berghei CR strain NYU-2. The parasites, the characteristics of erythrocytes infected with them, and the methods used to obtain and prepare infected erythrocytes for study have been described previously (3, 12). The P. berghei CR line is resistant to 40 mg of chloroquine per kg of body weight of the mouse daily (12). The P. berghei CS line is susceptible to 3 mg of chloroquine per kg daily (9).

Washed erythrocytes were suspended to a hematocrit of 5% in a medium buffered to pH 7.4 with 50 mM phosphate (8) to study mefloquine accumulation.

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[\textsuperscript{14}C]mefloquine hydrochloride with the label in the methanol carbon (Monsanto Research Corp.; 12.45 mCi/mmol; radiochemical purity greater than 99% by thin-layer chromatography) was used as a tracer to permit measurement of accumulation. To conserve the [\textsuperscript{14}C]mefloquine, it was diluted with nonradioactive mefloquine to obtain a series of [\textsuperscript{14}C]mefloquine preparations with lower specific activities for use in incubations requiring high concentrations of the drug. Other additions and the conditions of incubation are given in the legends for the figures. The incubations were terminated by centrifugation, after which the radioactivity remaining in the medium was measured directly, with a counting error of less than 3%, as described previously for amodiaquine (8, 10). The amount of mefloquine accumulated by the erythrocytes was calculated from the disappearance of radioactivity from the medium. An evaluation of the time course of mefloquine accumulation revealed that steady-state conditions were reached within 60 min with incubation at 25°C.

In selected instances after incubation with [\textsuperscript{14}C]mefloquine, the erythrocytes were extracted with acetone (10 ml/g of erythrocyte pellet) to verify that radioactivity disappearing from the medium could be quantitatively recovered. More than 97% of the radioactivity calculated to be present in the erythrocytes was recovered by extracting twice with acetone. The radioactive material thus extracted was subjected to thin-layer chromatography on 0.25-mm silica gel plates (precoated, Quanta-Gram thin-layer plates; Arthur H. Thomas Co., Philadelphia, Pa.). All of the radioactivity migrated with the same R\textsubscript{f} as authentic mefloquine in two solvent systems. With methanol-triethylamine (40:1) the R\textsubscript{f} was 0.42; with chloroform-methanol (9:1) the R\textsubscript{f} was 0.14. No degradation of mefloquine was detected in these studies in vitro. Since the specific activities of the [\textsuperscript{14}C]mefloquine preparations in the incubation medium were known, the absence of degradation made it possible to calculate the total concentrations of mefloquine in the medium and the pellet from measurements of radioactivity.

RESULTS

The characteristics of mefloquine accumulation (Fig. 2) differ in several important ways from those reported for chloroquine accumulation (3, 9, 12). Most remarkably, there was no difference in the amounts of mefloquine accumulated by erythrocytes infected with \textit{P. berghei} CR or \textit{P. berghei} CS. Moreover, the curves describing mefloquine accumulation by erythrocytes infected with \textit{P. berghei} CS and \textit{P. berghei} CR were similar in shape. In contrast, erythrocytes infected with \textit{P. berghei} CR accumulate less chloroquine (3, 9, 12), and the curve describing the process is sigmoid (9). Erytho-
cytes infected with *P. berghei* CS accumulate more chloroquine than those infected with *P. berghei* CR, and the curve describing the process is a rectangular hyperbola (3, 9, 11). Lack of stimulation by glucose and lack of inhibition by cold (Fig. 2) also distinguish mefloquine accumulation from chloroquine accumulation (9, 12). In agreement with these findings, mefloquine accumulation was not appreciably inhibited by 1 mM azide or iodoacetate (Fig. 3). Finally, it should be noted that uninfected erythrocytes accumulated more than half as much mefloquine as infected erythrocytes (Fig. 2 and 4). In comparison, uninfected erythrocytes accumulate only trace amounts of chloroquine from the nanomolar concentration range of external chloroquine (3, 9, 12).

In Fig. 2 and 4, the curves describing mefloquine accumulation are curvilinear. In fact, they take the same form as curves describing chloroquine and amodiaquine accumulation by erythrocytes infected with *P. falciparum* (5). A regression equation describing terms for a rectangular hyperbola and for a straight line fits such curves better than the regression equation for a straight line. Thus, the data shown in Fig. 2 and 4 support the existence of at least two components of mefloquine accumulation, one of which is saturable and the other of which is nonsaturable. The binding constants for the saturable component can be estimated by fitting the data directly to the curvilinear regression equation. Alternatively the binding constants can be estimated from a double-reciprocal transformation of the data, which then fit a straight line in the low-concentration range where the nonsaturable component of mefloquine accumulation makes a relatively small contribution to total accumulation. The latter method is suitable both for graphical analysis and for studies of inhibition (4), and it was employed in the present work (Fig. 5).

The results shown in Fig. 5 were obtained with a preparation of erythrocytes infected with *P. berghei* CS, but nearly identical results were obtained when erythrocytes infected with *P. berghei* CR were used. From these experiments nonradioactive mefloquine was found to be a competitive inhibitor of [14C]-mefloquine accumulation, and an apparent dissociation constant of \(2.5 \times 10^{-6} \text{ M}\) with a maximal binding capacity of 700 \(\mu\text{mol/kg}\) of erythrocyte pellet was calculated. A very similar value was calculated for the inhibitor constant of mefloquine \(2 \times 10^{-6} \text{ M}\) in earlier studies in which mefloquine was found to be a competitive inhibitor of chloroquine accumulation (4). In the present work, chloroquine was found to be a competitive inhibitor of mefloquine accumulation, with an apparent inhibitor constant of \(1.7 \times 10^{-3} \text{ M}\). For
The reciprocal of the concentration of mefloquine is shown on the abscissa, and the reciprocal of the amount of mefloquine accumulated is shown on the ordinate (1/μmol per kg).

DISCUSSION

Despite large differences between mefloquine and chloroquine in accessibility to drug receptors and in strengths of binding, we now know that mefloquine competitively inhibits chloroquine accumulation and that chloroquine competitively inhibits mefloquine accumulation. This knowledge raises the question of whether mefloquine and chloroquine are served by the same process of accumulation, possibly using a common group of receptors. The following observations permit an explanation of how a common group of receptors could serve both drugs. In erythrocytes infected with P. berghei, active metabolism ordinarily is required to make high-affinity receptors accessible to chloroquine (9, 11, 12). Without active metabolism, however, receptors of similar affinity and specificity can be made accessible by treating the erythrocyte surface with a nonspecific protease from Strep- tomyces griseus (7, 11). These and related ob-


