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Simple, Fast, and Accurate Fluorometric Method To Determine Drug Susceptibility of *Plasmodium falciparum* in 24-Well Suspension Cultures

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An in vitro test which quantifies drug inhibition of *Plasmodium falciparum* replication by measuring the fluorescence intensity of Hoechst 33258 dye bound to DNA is described. The procedure does not require expensive reagents or equipment and can be completed in less than 10 min. The assay was highly accurate and sensitive: cultures with as few as 0.4% schizont-infected erythrocytes could reliably be analyzed. The method was not biased by the actual parasite stage used; i.e., the amount of fluorescence detected in a sample of a culture of mature schizonts equaled the amount detected with the ring form culture derived from these schizonts. Even the presence of large proportions of free merozoites, which are easily neglected in microscopic estimates, did not bias the results. Furthermore, measurement of the chloroquine susceptibility of the multidrug-resistant K1 strain and the chloroquine-susceptible NF54 strain showed that the method is most suitable for quantifying the drug resistance of *P. falciparum*.

Infection with the parasite *Plasmodium falciparum* still causes one of the most serious life-threatening and widespread diseases. Rapid development of resistance against former active antimalarial drugs and slow progress in the development of an antimalarial vaccine indicate that malaria will remain a serious health problem in the near future. Increased efforts are therefore needed to develop new drugs, to study the efficacies of drug combinations, and to explore the possibility of reversing drug resistance. An accurate in vitro drug-screening assay is a prerequisite in this field of research. Two methods based on (i) microscopic examination of Giemsa-stained blood smears and (ii) incorporation of tritium-labeled hypoxanthine (5) are most frequently used to assess the drug susceptibility of the parasite in vitro. Examination of Giemsa-stained thin smears is tedious and time-consuming and requires the additional step of the subjective differentiation of parasite stages to correct the bias from equal evaluations of multinucleated schizonts and newly invaded ring forms. Analysis of drug-induced inhibition of incorporation of the nucleotide precursor [^{3}H]hypoxanthine into both RNA and DNA excludes this bias. This method (5), performed under less optimal static culture conditions (8), however, requires the use of a hazardous radioactive compound. In addition, this method is complicated by a two-step culture procedure, one without the growth-stimulating (7, 21) purine nucleotide precursor hypoxanthine followed by a phase with suboptimal concentrations of ^{3}H-labeled hypoxanthine. Furthermore, because ^{3}H incorporation in both DNA and RNA is measured, the assay primarily determines inhibition of metabolic activity rather than inhibition of parasite replication. Recently, a rapid drug susceptibility assay which does not require radioactive compounds and which is based on the measurement of plasmodial lactate dehydrogenase activity has been developed (1). This method, however, also determines the arrest of metabolic activity (1).

In contrast, analysis of DNA content versus drug concentration correlates directly with drug-induced inhibition of replication, even when a unicellular organism with multinucleated stages is measured. As [^{3}H]thymidine, which is generally applied to determine the extent of DNA synthesis, is not incorporated by *Plasmodium* parasites (17), alternative methods based on measuring the fluorescence of DNA-bound fluorochromes like ethidium bromide (20) and Hoechst 33258 (10) have been developed. Fluorometric analysis of DNA content with Hoechst 33258 is simple, is sensitive down to a nanogram-per-milligram level (2, 12), and is highly DNA specific because the fluorescence contribution of DNA over that of RNA is more than 100-fold (2, 3, 11). A Hoechst 33258-based method with a fluorescence-activated cell sorter (FACS), which measures the DNA content of individual infected erythrocytes, has been developed to determine the 50% inhibitory concentrations (IC_{50}s) of antimalarial drugs (16). This method, although very elegant, requires the availability of a FACS and highly specialized technical personnel to keep it operating. This report describes an alternative, equally fast, and highly accurate fluorescence-based assay that can be used to determine the drug-induced inhibition of parasite replication in semimicro 24-well suspension cultures. The method is very simple and does not require expensive equipment.

MATERIALS AND METHODS

***P. falciparum* cultures.** The multidrug-resistant K1 strain, a UV-induced (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)-adenine [(*S*)-HPMPA]-resistant clone derived from it, and the chloroquine-susceptible NF54 strain were grown in modified (8) RPMI 1640 medium–35 μM hypoxanthine–10% human type AB Rhesus-positive serum–2% hematocrit (human type O^+ erythrocytes) at 37°C under 2% O_2–5% CO_2–93% N_2. Parasites were synchronized with sorbitol as described previously (14).

Drug susceptibility test. A synchronized ring form culture (parasitemia, 1 to 1.5%) was used to prepare serial drug dilutions in 25-cm^2 culture flasks or 24-well culture plates (6- and 1-ml culture volumes, respectively). The culture plates were placed in a humidified, airtight container. The normal gas atmosphere was replaced by three rounds of brief vacuum extraction and flushing with the appropriate gas mixture. Culture flasks were flushed for 1 min. Both types of cultures were grown in suspension. After a 48-h incubation period, parasites from control and drug-treated cultures (samples of 0.8 ml in 2-ml Eppendorf tubes) were liberated from the erythrocytes by the addition of 1 ml of 0.08% saponin–phosphate-buffered saline. Free parasites were collected by centrifugation (4 min at 15,800 × *g*), and the supernatant was removed by aspiration.

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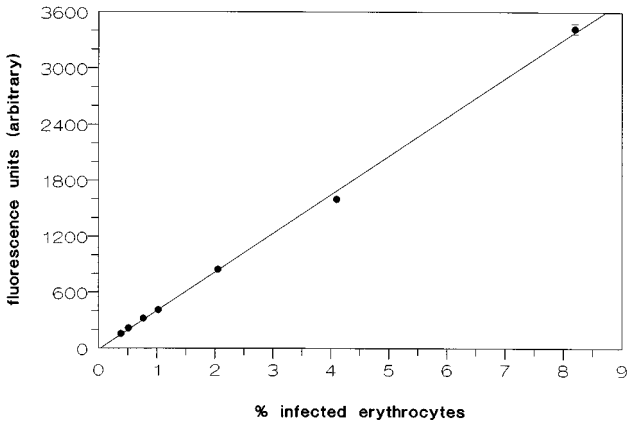

FIG. 1. Assessment of the linear relationship between number of infected erythrocytes and fluorescence intensity. A serial twofold dilution of an infected culture (8.2% midschizonts) with noninfected erythrocytes was made. Note that intermediate dilutions of 0.77 and 0.385% infected erythrocytes were included. Bars (omitted when they were <20 units) indicate the deviation of the mean for two independently processed samples.

Parasite pellets were either stored at −20°C or immediately processed. The parasite pellet was solubilized in 25 μl of 6 M guanidinium-HCl–sodium acetate (pH 5.5; 1 to 2 s on a mechanical cell homogenizer). Next, 2 ml of 2 M NaCl–50 mM Tris-HCl (pH 7.8)–0.33 μg of Hoechst 33258 (Polysciences, Inc., Warrington, Pa.) ml^{-1}, and 50 μl of chloroform-isoamyl alcohol (24:1) was added. The sample was mixed, and interfering hemozoin was removed by brief centrifugation (1 min at 15,800 × *g*). The amount of DNA present in the supernatant was quantified by measuring Hoechst 33258 fluorescence with a minifluorometer (model TKO 100; Hoefer Scientific Instruments, San Francisco, Calif.; emission spectrum mercury lamp, 365 ± 50 nm; detection of Hoechst 33258 fluorescence at 460 ± 5 nm). Samples from cultures exposed to a high concentration (2 μM) of chloroquine (or from control cultures at time zero) were used to correct for the background fluorescence derived from parasites (and erythrocytes) already present at the start of the experiment.

IC_{50} determination. Dose-response curves were constructed manually or by the statistical computer program Number Cruncher Statistical System (Kaysville, Utah) by plotting drug concentrations versus net fluorescence values. The IC_{50} was defined as the drug concentration which corresponds to 50% of the net amount of fluorescence of nontreated control cultures.

RESULTS

Several procedures were tested during development of the assay. Essential prerequisites are that all DNA be extracted from the parasites and that there be an accurate, ideally linear relationship between the amount of DNA and the measured fluorescence. Extraction of DNA from nuclei collected after hypotonic detergent treatment, advocated as a simple, rapid, and efficient method (13), did not result in the complete recovery of DNA when it was applied to *Plasmodium*-infected erythrocytes. Moreover, residues of the detergent Triton X-100 caused some disturbance of linearity between the amount of DNA and the fluorescence value. A similar disturbance was observed when sodium dodecyl sulfate was used to solubilize free parasites collected after saponin treatment (data not shown).

The final method guaranteed the essential linear relationship between the amount of DNA in serially diluted samples of strictly synchronized cultures ranging from 24 to 1% ring form-infected erythrocytes and fluorescence intensity (data not shown). Samples with as little as 0.4% infected erythrocytes (Fig. 1; in this case midterm schizonts were used) could be analyzed. The method also proved to be highly reproducible: six independently processed samples from the same culture gave a standard deviation of <1% (data not shown). Pretreatment of samples with DNase I resulted in a decline in the fluorescence intensity to the background (noninfected erythrocyte) level (data not shown).

To examine whether the fluorometric analysis under the chosen assay conditions is biased by parasite stage, especially during the transition from the midterm schizont stage into free merozoites and newly invaded ring forms, measurements were performed on highly synchronized cultures during a 28-h period (Fig. 2). As expected, an increase in fluorescence was observed (from time −8 until 0 h) during the schizont stage, in which the parasite replicates its DNA content. Then, fluorescence levels remain stable while increasing amounts of mature schizonts transform into free merozoites and newly invaded ring forms (peak of merozoite release at time zero) and subsequently develop into young trophozoites, indicating that merozoites, ring forms, and trophozoites, which contain equal amounts of DNA, are indeed analyzed unbiased. This stage independency was achieved after removal of interfering factors like partly solubilized hemozoin, which causes a minor quenching of fluorescence. The elimination of quenching by brief chloroform treatment, which did not cause any detectable loss of DNA, was confirmed by a spectrophotometric scan (200 to 600 nm) overlapping both the emission and excitation optima of the fluorochrome Hoechst 33258 (data not shown).

To evaluate the validity of the drug susceptibility assay, the IC_{50}s of chloroquine for the multidrug-resistant *P. falciparum* K1 strain and the chloroquine-susceptible NF54 strain were determined and were compared with previously published values. From dose-response curves (a typical graph is shown in Fig. 3), an IC_{50} of 459 ± 74 nM was determined for the K1 strain. A closely related value of 367 ± 136 nM (15) determined by [^{3}H]hypoxanthine incorporation has been reported, although values as low as 221 ± 57 nM (6) by the same method and up to greater than 640 nM (9) by microscopic evaluation have been reported for this strain. An IC_{50} of 15.1 ± 3.1 nM was determined for the chloroquine-susceptible NF54 strain (Fig. 3). An equal IC_{50} has been determined for this strain (4), and similar values ranging from 5 up to 31 nM have been reported for the chloroquine-susceptible T9-96 strain (6, 9, 15).

The fluorometric assay was further examined for its capacity to detect the unique delayed inhibition of DNA replication observed during incubation with high concentrations of the

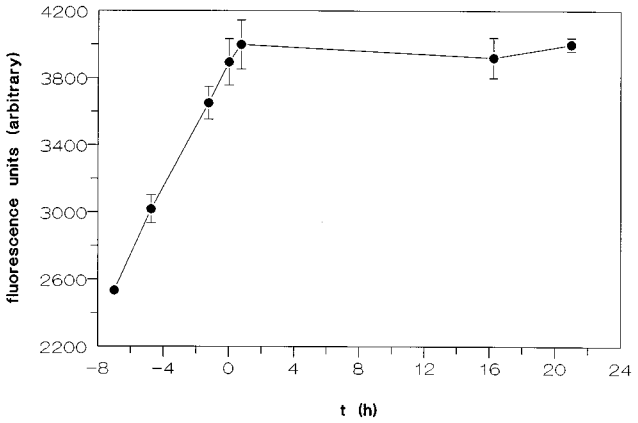

FIG. 2. Evaluation of fluorescence intensity at different parasite stages. Parallel cultures of strictly synchronized parasites were analyzed at the indicated times (*t*). Parasites were characterized by microscopy as near mature schizonts (up to time zero), mature schizonts and free merozoites at time zero, young ring forms (time 0.75 h), and young trophozoites (time 22.25 h). Bars (omitted when they were <30 units) indicate the deviation of the mean for two independently processed samples.

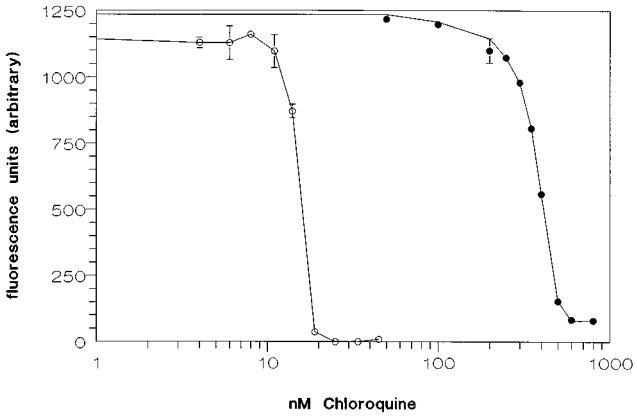

FIG. 3. Inhibitory effect of chloroquine on the multidrug-resistant *P. falciparum* K1 strain and the chloroquine-susceptible NF54 strain. Synchronized cultures of multidrug-resistant K1 parasites (●) and NF54 parasites (○) were exposed for 48 h to the indicated concentrations of chloroquine. Bars (omitted when they were <20 units) indicate the deviation of the mean for two independently processed samples.

nucleotide analog (*S*)-HPMPA (18). The dose-response graph (Fig. 4) of the level of inhibition caused by (*S*)-HPMPA indeed shows that, in contrast to the total reduction of fluorescence observed with chloroquine, even high concentrations of (*S*)-HPMPA do not cause a complete disappearance of fluorescence. Finally, the routinely observed increase in fluorescence of >10-fold during one cycle (data not shown) indicates that culture conditions in these 24-well plates and 25-cm^2 flasks are optimal (8).

DISCUSSION

Assessment of the drug susceptibility of *P. falciparum* by the fluorometric method described here, in which total DNA is extracted and measured without the use of expensive chemicals or equipment, has both practical and scientific advantages over existing methods. First, it is accurate. Interfering compounds such as hemoglobin and hemozoin are removed by a short and mild saponin treatment, and a rapid chloroform extraction is used to prevent quenching of fluorescence. No measurable loss of DNA is induced by the method; even young trophozoites killed by high levels of chloroquine did not show a loss of DNA until after more than 24 h. Also, only DNA is measured, since RNA-induced fluorescence is negligible; indeed, pretreatment of samples with DNase I results in the complete removal of fluorescence. Second, it is a sensitive method: down to 1% ring form or 0.4% schizont infected erythrocytes can reliably be measured. Third, the amount of fluorescence is directly related to the replication of the parasite; i.e., a mature schizont which within less than 1 h will cause the infection of more than 10 erythrocytes contributes proportionally more to the amount of fluorescence detected than a ring form or a trophozoite does (Fig. 2). This is clearly an advantage over drug susceptibility assays based on the presence of metabolic enzymes such as lactate dehydrogenase, the activity of which remains almost the same during the transition from the early trophozoite to the mature schizont stage (1). Even the presence of a relatively large proportion of free merozoites at the time of reinvasion, or on the first day after (Fig. 2), in synchronized cultures, which are almost certainly neglected in microscopic estimates, does not bias the results.

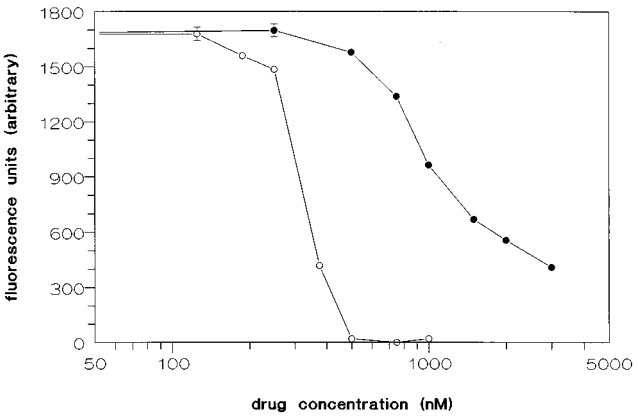

FIG. 4. Inhibitory effects of chloroquine and (*S*)-HPMPA on an (*S*)-HPMPA-resistant C6 clone of the multidrug-resistant *P. falciparum* K1 strain. Synchronized suspension cultures of the (*S*)-HPMPA-resistant C6 clone of the multidrug-resistant *P. falciparum* K1 strain were exposed for 48 h to the indicated concentrations of chloroquine (○) and (S)-HPMPA (●). Bars (omitted when they were <20 units) indicate the deviation of the mean for two independently processed samples.

Determination of the IC_{50} of chloroquine for the multidrug-resistant K1 strain and the chloroquine-susceptible NF54 strain confirmed that the method is most suitable for quantifying the drug resistance of *P. falciparum*. Fluorometric analysis of the antiplasmodial activity of (*S*)-HPMPA, a unique drug which does not inhibit DNA replication immediately (18), showed that the assay indeed detected a delayed inhibition of DNA replication. An accurate IC_{50} of (*S*)-HPMPA therefore could not be established after a 48-h period of incubation, but accurate determination from a sigmoid curve with a steep slope is possible after a 72-h incubation period. Such a prolonged incubation period is also applied in the extended microscopic drug susceptibility test (19) and a semiautomated microdilution technique based on the incorporation of [^{3}H]ethanolamine (7). It is relevant to mention that an extended incubation period is in fact also required in the hypoxanthine incorporation method, because ongoing ^{3}H incorporation occurs during the 24-h labeling period, which coincides with the delayed (*S*)-HPMPA inhibition in the schizont stage.

From a practical point of view, the present fluorometric assessment of the in vitro drug susceptibility of *P. falciparum* parasites is fast, because it takes only 8 min from the time of sampling to actual fluorometry, while large numbers of samples (up to 100, which is equivalent to the determination [in duplicate] of the IC_{50}s of six different drugs) can be handled in 2 h when multidelivery pipets are used. The fluorescence intensity of the Hoechst 33258 dye bound to DNA is stable for at least 24 h, provided that samples are not exposed to strong light (12). The method does not require expensive or radioactively labeled reagents, while it can be performed with a moderately priced minifluorometer. Finally, it avoids bias by the use of a compound like [^{3}H]hypoxanthine for measurements, a compound which in itself influences parasite growth and which can now be used to optimize culture conditions.

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