Ligands of the Peripheral Benzodiazepine Receptor Are Potent Inhibitors of *Plasmodium falciparum* and *Toxoplasma gondii* In Vitro

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The increase in resistance of the malaria parasite *Plasmodium falciparum* to currently available drugs demands the development of new antimalarial agents. In this quest, we have found that ligands to the peripheral benzodiazepine receptor such as flurazepam, an agonist of the benzodiazepine family, and PK11195, an antagonist derived from isoquinoline, were active against *Plasmodium falciparum*. These two compounds effectively and rapidly inhibited parasite growth in vitro, irrespective of parasite resistance to chloroquine and mefloquine. Treatment with both drugs induced a sharp and consistent decline in parasitemia, a complete inhibition of parasite replication, and the destruction of parasites within the host red blood cells. Using electron microscopy, we showed that dramatic morphological changes, involving swollen endoplasmic reticulum and the reduction of hemozoin, were consistent with parasite death. The potent activities of flurazepam and PK11195 were also evaluated for antagonist or synergistic effects with currently used antimalarial drugs such as chloroquine and mefloquine. Moreover, flurazepam was found to be active against *Toxoplasma gondii*, another member of the phylum Apicomplexa. Taken together, our results indicated that benzodiazepines could be considered promising candidates in the treatment of both malaria and toxoplasmosis.

Among apicomplexan parasites are numerous pathogens such as *Plasmodium* species (causative agents of malaria), *Toxoplasma gondii* (an important opportunistic pathogen associated with AIDS and congenital birth defects), *Eimeria* (agent of coccidiosis), and *Cryptosporidium* (an opportunistic intestinal pathogen). Malaria is an ancient and intractable vector-borne infectious disease that has thwarted most efforts to combat the causative agents, *Plasmodium* spp. Each year, malaria causes 2 million to 3 million deaths, mostly in children, and hundreds of millions of people are debilitated by infection (27). Infection by *T. gondii* can lead to severe syndromes including congenital malformations such as blindness, mental retardation, and hydrocephaly in children exposed to the parasite in utero. Recently, more attention has been given to *T. gondii* because toxoplasmosis is the most common cause of focal encephalitis or focal central nervous system abnormalities in immunocompromised patients with AIDS or in transplant recipients (12, 13). The complex biology and life cycle of apicomplexan parasites has hindered attempts to control infections and prevent transmission. In addition, there is increasing resistance of the malaria parasite *Plasmodium falciparum*

to currently available drugs. As a result, there is an urgent need for the development of chemotherapeutic regimens that can control the parasite. Therefore, the identification of antiparasitic drugs already in use for other therapeutic purposes represents an attractive approach with potentially rapid clinical or prospective application.

Benzodiazepines (BDZ) are best known for their action as anxiolytics, anticonvulsants, antispasmodics, and hypnotics, leading to their widespread clinical use. Their physiological effects are mediated by their binding to two types of receptors named the central BDZ receptor (CBR), which is associated with the GABA_A receptor of the central nervous system, and the peripheral BDZ receptor (PBR), localized in the outer membrane of the mitochondrion in peripheral cells such as hemopoietic cells (5, 11). The pharmacology of the CBR associated with the GABA_A receptor complex and the PBR was extensively probed using diazepam, clonazepam, flurazepam, or PK11195, an antagonist derived from isoquinoline. Although extensively characterized pharmacologically and biochemically and implicated in numerous biological processes, the precise function of the PBR remains an enigma (6). For example, possible roles of the PBR in cell proliferation, calcium channel activity, immune responses, porphyrin transport and heme biosynthesis, anion transport, regulation of steroid biosynthesis, and regulation of mitochondrial oxidative phosphorylation have been described (6, 30). There have been many reports that BDZ affect cell growth, proliferation, and differentiation in a number of cell types. A strongly positive correlation between inhibition of cell proliferation and the

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affinities of BDZ for the PBR has been described (10). However, BDZ exhibited nanomolar affinities for PBR on these cells, whereas micromolar concentrations of BDZ were necessary to elicit these antiproliferative effects (10, 26). In addition, it has been described that protoporphyrin IX, an endogenous ligand of the peripheral BDZ receptor, potentiates induction of the mitochondrial permeability transition and the killing of cultured hepatocytes (19).

The PBR is an 18-kDa protein that displays a strong homology to the CrtK/TspO protein of the outer membrane of *Rhodobacter sphaeroides* and *R. capsulatus* (2). When expressed in *R. sphaeroides*, the 18-kDa protein was shown to bind to BDZ, leading to an alteration in the photosynthetic apparatus (diminution of the B800-B850 complex) and to an orientation of the rhodobacteria toward aerobic metabolism (28, 29). Given these considerations, especially the potential cytotoxic properties of PBR ligands, we aimed to investigate whether some of these compounds may exert an antiparasitic activity on *P. falciparum* and *T. gondii*. Our rationale was that as members of the apicomplexan phylum, these parasites, which possess both an apicoplast and a mitochondrion, might be sensitive to PBR ligands. Our results indicate that flurazepam, an agonist of BDZ receptors, and the specific PBR antagonist PK11195 have profound deleterious effects on *P. falciparum* and *T. gondii* growth, suggesting that these molecules might be of potential value alone or in combination with other antimalarial or anti-*Toxoplasma* drugs.

MATERIALS AND METHODS

Antimicrobial agents. The following antimicrobial agents were used: chloroquine, flurazepam (Fz), 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isouquinoline-carboxamide (PK11195) (Sigma Chemical Co., St Louis, Mo.) and mefloquine (a kind gift of D. Dive, Pasteur Institute of Lille, Lille, France). PK11195 was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 3.3 mg/ml. Fz was dissolved in Dulbecco modified Eagle medium (DMEM) (Biowhittaker) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Dutscher), 2 mM glutamine (Sigma), and 0.05 mg of gentamicin (Sigma) per ml, at a concentration of 5 mg/ml. The same culture medium (DMEM-FCS) described above was used to culture intracellular tachyzoites of *T. gondii*. Chloroquine and mefloquine were dissolved in 70% methanol at 5 mg/ml. DMEM-FCS or RPMI 1640-FCS (Gibco BRL) containing DMSO and methanol necessary to dissolve the drugs tested were shown to have no effect on *T. gondii* and *P. falciparum* growth and morphology.

In vitro culture of *P. falciparum* and drug assays. The experiments were performed with two tissue culture-adapted strains of *P. falciparum*: the chloroquine-sensitive strain HB3 and the mefloquine-chloroquine resistant strain Dd2. The parasites were maintained on human type O⁺ erythrocytes in RPMI 1640 culture medium supplemented with 27.5 mM NaHCO₃, 20 mM HEPES (pH 7.4), 11 mM glucose, and 7.5% (vol/vol) heat-inactivated human AB⁺ serum under 5% CO₂-5% O₂-90% N₂ at 37°C (25). The assays were conducted in 96-well plates. The different drug concentrations prepared as described above were added to asynchronous or synchronous parasite cultures (0.5% parasitemia and 1.8% hematocrit) in the presence of 0.5 μ Ci of [³H]hypoxanthine (Amersham; 1 mCi/ml) per well. After incubation for 48 h at 37°C, the cells were harvested from each well with an automatic cell harvester (1450 Microbeta; Wallac) onto fiberglass filters. The radioactivity was measured by scintillation counting of dried filters. All drug concentrations were tested three times in triplicate for each experiment. At least three independent experiments were performed for all drugs tested. Inhibition of parasite growth was determined for each concentration by comparing the radioactivity incorporated in the treated cultures with those in the control cultures (without drug) maintained on the same plate. The concentrations corresponding to 50% inhibition (IC₅₀) were determined graphically.

In vitro culture of *T. gondii* and drug assays. Tachyzoites of 76K strain of *T. gondii* were grown in human foreskin fibroblasts (HFF) in DMEM-FCS. The assays were conducted in 24-well plates. The monolayer of HFF cells was in-

fectured with 2×10^5 parasites per well, and 4 to 6 h later, drugs were added at the range of concentrations described above, alone or in combination. After a further 48 h, the intracellular tachyzoites were pulse-labeled with 2 μ Ci of [³H]uracil (Amersham) per well for 6 h. The lack of uracil phosphoribosyltransferase in the host cells allows the specific labeling of *T. gondii* (20). After labeling, lysis was performed in the wells using 1% sodium dodecyl sulfate-100 μ g of uracil per ml. Radiolabeled nucleic acids were precipitated with trichloroacetic acid overnight at 4°C and then recovered on fiberglass filters. Radioactivity was measured by scintillation counting (14). All drugs were tested in triplicate for each experiment, which was repeated three times.

In vitro culture of *P. falciparum* for morphological assessment and hemozoin production. Two 150-cm² flasks containing 6% hematocrit and 0.5% parasitemia were mock treated or treated with 50 μ g of Fz per ml and cultured at 37°C as described above. The culture medium was changed and the drug was replenished daily. After 72 h, both mock- and drug-treated cultures were diluted to a hematocrit of 3% in two new flasks and cultured as described for the standard growth conditions with or without the drug. The medium was changed twice daily for both the mock- and drug-treated flasks for 72 h until the parasitemia reached 16% in the mock-treated flask while that of the Fz-treated culture was reduced to 0.5%. Samples were then processed for morphological assessment and hemozoin measurements.

Assessment of *P. falciparum* and *T. gondii* morphology, development, and replication. Morphology, development, and replication of asynchronous or synchronous cultures of *P. falciparum* were evaluated in cultures by light microscopy of Giemsa-stained thin blood smears. Smears from drug-free cultures were used as controls. Parasitemia was measured by counting 1,000 erythrocytes and reported as the percentage of total parasitized erythrocytes. Morphologically normal and abnormal parasites were included in the measurements. Parasite proliferation was based on difference in the level of parasitemia, and parasite development within the erythrocyte was evaluated by examining the number of the various developmental stages: rings, trophozoites, and schizonts.

For transmission electron microscopy, tachyzoites of *T. gondii* (76K strain) grown in HFF, or in erythrocytes infected by *P. falciparum* (HB3 strain) were treated with Fz or PK11195 for 2 days. The treated intracellular parasites were fixed in 2.5% glutaraldehyde prepared in 0.1M cacodylate buffer (pH 7.3) and postfixed in 1% OsO₄ in the same buffer. After ethanol dehydration, the pellet was embedded in Epon. Thin sections were cut using a Reichert Ultracut E ultramicrotome and collected on 100-mesh grids. After being stained with 2% uranyl acetate prepared in 50% ethanol and lead citrate, sections were observed in a Hitachi H-600 electron microscope.

Isolation and measurement of hemozoin in mock-treated and drug-treated *P. falciparum*. The parasites, treated as described above, were collected, washed with phosphate-buffered saline and harvested by saponin lysis. After PBS washing, a crude extract of hemozoin was prepared by the method described by Slater et al. (22). Briefly, the saponin-treated parasites were resuspended in buffer A (Tris.HCl [pH 7.4]), sonicated for 15 min, and centrifuged at $25,000 \times g$ at 4°C for 30 min. To solubilize any contaminating membranes, crude hemozoin was extracted twice for 2 h at room temperature in buffer A containing 2% sodium dodecyl sulfate. The hemozoin pellet was washed three times in buffer A, and residual proteins were removed by an overnight digestion in buffer A containing proteinase K at 1 mg/ml. Insoluble material was recovered and washed as described above before being extracted in 6 M urea for 3 h at 4°C. The purified hemozoin was pelleted by centrifugation, and washed with distilled water. One-fourth of the hemozoin was solubilized by DMSO and analyzed by matrix-assisted laser desorption mass spectroscopy (MALDI-MS) on a Vision 2000 time-of-flight (TOF) instrument (Finnigan Mat, Bremen, Germany) equipped with a 337-nm UV laser. The remaining hemozoin was solubilized with 0.1 M NaOH, and the UV-visible absorbance spectrum was determined.

Statistics. The significance of differences was evaluated by statistical significance using Student's *t* test. *P* < 0.05 was considered significant.

RESULTS

Fz and PK11195 inhibit *P. falciparum* growth in vitro. In the course of the investigation of the effects of PBR ligands such as BDZ on the growth of *P. falciparum* and *T. gondii*, we used Fz (Fig. 1A) and the isoquinoline carboxamide (PK11195) (Fig. 1B). Parasite growth was assessed in HB3 strain of *P. falciparum* by measuring the incorporation of [³H]hypoxanthine in growing asynchronous culture. Both Fz and PK11195 inhibited the radiolabel incorporation by the parasite in a dose-depen-

FIG. 1. Chemical structures of the two archetypic PBR ligands. (A) Fz. (B) PK11195.

dent manner, allowing the calculation of their IC_{50} , estimated at 40 and 20 $\mu\text{g/ml}$, respectively (Fig. 2). When the two drugs were tested at a range of concentrations below 10 $\mu\text{g/ml}$, no significant difference was noticed between treated and untreated parasites. A sharp decline in [^3H]hypoxanthine incor-

poration was observed when a combination of Fz and PK11195 was tested (Fig. 2). These data indicated no significant synergy between Fz and PK11195. However, a cumulative inhibition was observed, as evidenced by a complete inhibition (>98%) of incorporation of [^3H]hypoxanthine at 50 μg of each drug per

FIG. 2. Inhibitory effect of Fz and PK11195 on asynchronous cultures of *P. falciparum* (HB3 strain). Fz was tested alone at doses of 0, 10, 20, 25, 30, 35, 40, 45, 50, and 100 $\mu\text{g/ml}$. PK11195 was tested alone at the same doses as indicated above. The combination of Fz and PK11195 added to the culture at the concentrations from 10 to 100 $\mu\text{g/ml}$ actually represent 20 to 200 μg of total drug per ml. [^3H]hypoxanthine and drugs were added, and the labeled parasites were harvested after a 48-h exposure. Incorporation by parasite cultures in the absence of drug was used as the 100% values. Error bars represent the means and standard deviations of triplicate determinations of a representative of three reproducible experiments ($P < 0.05$). Where error bars are not seen, they are smaller than the symbol.

ml (a total concentration of 100 $\mu\text{g/ml}$), whereas, at the same concentration, 10 to 30% of radiolabel was still incorporated by the parasites when PK11195 and Fz were used alone.

Fz and PK11195 alter *P. falciparum* morphology and development. To determine the effect of the drugs on parasite proliferation, the level of parasitemia in treated and untreated cultures was evaluated in Giemsa-stained smears. After 72 h, the parasitemia of mock-treated culture reached 6% while that of the drug-treated culture was below 1%. After dilution and culturing for a further 72 h, the parasitemia reached 16% in the mocked-treated samples while remaining at or below 0.5% in the drug-treated sample. Parasite viability was not discernible from morphological criteria alone, and so morphologically normal and abnormal parasites were included in the measurements. Therefore, the percentage of viable parasites in the drug-treated cultures may be lower than that mentioned above.

To study the effects on parasite development within the red cell, asynchronized samples were examined after 48 hours of treatment. The profound effects of Fz on the parasite growth are demonstrated by the light micrographs shown in Fig. 3 (compare Fig. 3A and C). While all parasitic stages, rings, trophozoites, and also schizonts (not seen on the fields shown here) were observed in the untreated culture (Fig. 3A and B), only a few parasitic forms were detected in the treated culture (Fig. 3C and D). The uninfected erythrocytes showed normal morphology in both treated and untreated cultures (Fig. 3, white arrows). The deleterious effects of Fz on *P. falciparum* were confirmed by electron microscopy, which revealed that the few remaining parasites in the Fz-treated culture lacked large food vacuoles containing hemozoin crystals, in contrast to the parasites observed in the untreated sample (Fig. 4B to D). This conclusion is supported by the observation that in a random sample of 20 infected cells from the treated sample, none of the parasites contained hemozoin, in contrast to the control sample, where the majority of parasites contained hemozoin (Fig. 4A). Furthermore, swollen endoplasmic reticulum was also evident (Fig. 4C). These profound morphological changes can lead to degeneration and complete destruction of parasites within a single asexual cycle (Fig. 4D). The morphology of *P. falciparum* was also profoundly altered by PK11195. A similar pattern of degeneration and the presence of dead parasites like that shown in Fig. 4D were observed (data not shown).

Fz treatment of *P. falciparum* also leads to a decrease of hemozoin. To provide more evidence for the decrease or absence of hemozoin in treated *P. falciparum*, hemozoin was purified from both mock-treated and treated parasites (see Materials and Methods). The UV-visible absorbance spectrum of a base-solubilized (0.1 M NaOH) suspension of purified material from treated parasites gave no absorbance, while the untreated or control parasites revealed absorbance values between 325 and 410 nm, with the characteristic *P. falciparum* hemozoin peak at 390 nm (Fig. 5A). When one-fourth of the purified crude extract from the untreated parasites was solubilized with DMSO and analyzed by mass spectrometry, the major molecular ion was at m/z 616.1 (Fig. 5B), identical to the characteristic molecular ion of hemozoin (22). Again, no hemozoin could be detected in the Fz-treated parasites. Taken together, these data demonstrate that the amount of hemozoin is considerably reduced when cultures of human erythrocytes

infected with *P. falciparum* are treated with Fz. It is most likely that the reduction in the amount of reflects the decrease in parasite growth and proliferation in the drug-treated cultures. In other words, this reduction is probably one of multiple morphological changes observed in the drug-treated parasites, consistent with the ultrastructural changes described above.

Combined effects of Fz and PK11195 with other antimalarial compounds. The combination of Fz or PK11195 with chloroquine against the *P. falciparum* chloroquine-sensitive HB3 strain demonstrated significantly increased inhibition of [^3H]hypoxanthine incorporation in the treated parasites compared to those incubated with chloroquine alone (Fig. 6A). Specifically, treatment with 19 ng of chloroquine per ml had no obvious effect on parasite growth while the addition of 20 μg of Fz or PK11195 per ml significantly reduced the [^3H]hypoxanthine incorporation to 80 to 90% compared to the untreated parasites (Fig. 6A). The same pattern of parasite growth inhibition was observed when the data for mefloquine alone were compared with those obtained with mefloquine combined with either Fz or PK11195 (Fig. 6B). Collectively, these experiments indicate that neither Fz nor PK11195 displays antagonistic effects with chloroquine and mefloquine, two drugs already used in the field against malaria. Rather, the data obtained on the interaction of Fz or PK11195 with mefloquine or chloroquine reveal substantial additive effects depending on drug concentrations. The inhibitory effect of Fz and PK11195 can be readily seen when applying concentrations of chloroquine as low as 19 to 37 ng/ml. At these concentrations, chloroquine alone appears to be ineffective under our experimental conditions.

Fz and PK11195 inhibit mefloquine- and chloroquine-resistant *P. falciparum* as well. The effects of Fz and PK11195 on the mefloquine- and chloroquine-resistant Dd2 strain were evaluated by measuring the incorporation of [^3H]hypoxanthine in growing asynchronous parasite culture. When parasites of the Dd2 strain were incubated with increasing chloroquine concentrations ranging from 9 to 150 ng/ml, the strain incorporated [^3H]hypoxanthine at the same level as the untreated parasites, demonstrating that the Dd2 strain is fully resistant to chloroquine, as expected (Fig. 7). In contrast, linear and sharp decrease of [^3H]hypoxanthine incorporation was observed when the Dd2 strain was incubated with Fz or PK11195 (Fig. 7). Interestingly, additional inhibition was obtained when Fz and PK11195 were mixed during the assay, suggesting cumulative damage of this mefloquine-chloroquine resistant strain by the two drugs (Fig. 7).

Effect of Fz and PK11195 on another apicomplexan parasite, *T. gondii*. The effects of Fz and PK11195 on in vitro growth of *T. gondii* were investigated. Inhibition of the growth of intracellular tachyzoites of *T. gondii* 76K by Fz was indicated by a reduction in the incorporation of [^3H]juracil (Fig. 8A). The IC_{50} of Fz for *T. gondii* is estimated at approximately 40 $\mu\text{g/ml}$. Since Fz is known for its activity on the host cell mitochondria, we evaluated its toxicity on the uninfected HFF used as host cells for *T. gondii* growth, using [^3H]hypoxanthine incorporation in the presence and absence of the two drugs. No difference was detected in [^3H]hypoxanthine incorporated by treated and untreated fibroblast cells in the presence of Fz (data not shown), suggesting that the drug had no detectable toxicity on the host cells. When PK11195 was tested, a 20 to

FIG. 3. Morphological appearance of *P. falciparum* cultures (HB3 strain) after a 48-h incubation with Fz. Parasites were tested as asynchronized cultures containing ring, trophozoite, and schizont stages. Shown is the morphology of Giemsa-stained thin blood smears from drug-free control cultures (A and B) and cultures incubated with 50 µg of Fz per ml (C and D). Note the absence of detectable alterations of the host blood cells in the drug-treated cultures and the reduced parasitemia when the two fields are compared. The views are shown at magnifications of $\times 100$ and $\times 500$. White arrows indicate uninfected erythrocytes, and black arrows show infected erythrocytes.

FIG. 4. Morphological alterations of *P. falciparum* (HB3) cultures using transmission electron microscopy after a 48-h incubation with Fz at 10 $\mu\text{g/ml}$ (B and C) or 50 $\mu\text{g/ml}$ (D) or without drug (control) (A). Note the presence of hemozoin (stars) in the untreated parasites; two distinct trophozoites are present in this infected erythrocyte (A). The views also show the disorganization of the endoplasmic reticulum (C; arrow), the absence of hemozoin (B to D), and total degeneration of the treated parasites (D).

30% reduction of [^3H]hypoxanthine incorporation was observed in uninfected HFF. Therefore, we were unable to distinguish between the specific activity of PK11195 on the host cells and *T. gondii*. Although the Fz activity appears more conclusive, only a moderate effect was observed (Fig. 8A). Transmission electron microscopy was used to examine the effect of Fz on parasite morphology. Treatment by Fz did not appear to affect the ultrastructure of the parasites. However there did appear to be an effect on parasite proliferation, with the average number of parasites per parasitophorous vacuole being one-fifth lower in the treated sample (Fig. 8C) than in the untreated sample (Fig. 8B). This would be consistent with a cytostatic effect resulting in a reduction in tachyzoite replication and development. Again, at least 20 vacuoles were observed, and no significant morphological changes could be

identified, except for a considerably increased in the number of phagolysosomes in the treated host cells and a reduction in the number of intracellular parasites in the treated culture.

DISCUSSION

This study shows a potent activity of ligands of PBR, such as Fz and PK11195, against two obligate intracellular apicomplexan parasites, *P. falciparum* and *T. gondii*, when used as single agents or in combination with other antiparasitic drugs. High drug concentrations are required compared to the doses of BDZ measured in the plasma of treated patients. The therapeutic concentrations vary from 0.001 to 3 $\mu\text{g/ml}$, depending on the BDZ used, with toxic levels ranging from 0.05 to 20 $\mu\text{g/ml}$ (15, 23). These concentrations were 0.005 to 0.1 $\mu\text{g/ml}$

FIG. 5. (A) Comparison of the UV-visible absorbance spectrum of hemozoin purified from a *P. falciparum* (HB3 strain) culture treated with 50 μ g of Fz per ml and from the drug-free control culture. (B) Analysis of the purified hemozoin by mass spectrometry showing its characteristic molecular ion at m/z 616.1. The data confirm the complete absence of hemozoin in the drug-treated parasites.

for the therapeutic range of Fz and 0.2 to 0.5 μ g/ml for its toxic level. However, the strong lipophilic properties of these drugs and their capacities to cross the blood-brain barrier and to distribute in tissues indicate that these compounds could be very interesting in the treatment of apicomplexan parasites, such as *P. falciparum* and *T. gondii*, with their complex life

cycles having parasitic stages also localized in the central nervous system or in the liver. It is worth noting that similar high drug concentrations were needed for the inhibition of *Rhodobacter sphaeroides* growth (1, 29). It will be of great relevance to determine whether these drugs are readily effective against *P. falciparum* and *T. gondii* in vivo. Unfortunately,

FIG. 6. Comparative inhibitory effects of chloroquine (CQ) (A) or mefloquine (MQ) (B) alone or combined with Fz or PK11195I. Chloroquine and mefloquine were tested alone at 0, 9, 19, 37, 75, and 150 ng/ml. The series of concentrations of chloroquine or mefloquine were combined with Fz (0 to 100 μg/ml) or with PK11195 (0 to 100 μg/ml) as described in the legend to Fig. 2. The combinations of chloroquine or mefloquine with Fz and PK11195 actually represent, for example, 9 ng of chloroquine per ml plus 10 μg of Fz per ml. [^3H]hypoxanthine and drugs tested were added, and labeled parasites were harvested after a 48-h exposure. Error bars represent the means and standard deviations of triplicate determinations of a representative of three reproducible experiments ($P < 0.05$).

FIG. 7. Incorporation of [³H]hypoxanthine by the chloroquine (CQ)- and mefloquine-resistant *P. falciparum* Dd2 asynchronous cultures in the presence of chloroquine, Fz, PK11195, or Fz mixed with PK11195. Chloroquine, Fz and PK11195 were tested alone at the doses indicated on the x axis. The combination of Fz with PK11195 was also tested as described in the legend to Fig. 2. Error bars represent the means and standard deviations of triplicate determinations of a representative of three reproducible experiments ($P < 0.05$).

our preliminary attempts to test the activity of these drugs in vivo on *T. gondii* failed when mice chronically infected with encysted bradyzoites or with virulent tachyzoites of *T. gondii* were used (our unpublished data). Because the activity of these drugs on *T. gondii* in vitro was moderate in comparison with that on *P. falciparum*, it remains to be determined if Fz and PK11195 are more effective in vivo against *P. falciparum* or other *Plasmodium* spp. We believe that the data reported herein provide at least a foundation for further studies to test the efficacy of these drugs. At the moment, because higher drug concentrations are required to kill apicomplexans than the concentrations achieved in plasma in patients, these compounds can only be envisaged as early compounds in the process of discovering powerful analogs with a better affinity for the PBR-like receptor. Although some BDZ, including Fz, can bind to both the central-type benzodiazepine (CBR) and the PBR receptors, the effect of PK11195 indicates that a related PBR-like molecule may be involved in the activities of these BDZ in these parasites. In addition, it should be noted that when we used flumazenil, a specific CBR antagonist, alone or in combination with flurazepam and PK11195, no activity or no reversal of the inhibitory effects could be seen in the range of concentrations which are not toxic to the host cells (our unpublished results), suggesting that the mechanisms by which these BDZ inhibit parasites are probably different from those occurring in mammalian cells. Whether flurazepam and PK11195 bind to homologues of mammalian PBR in *P. falciparum* and *T. gondii* remains to be determined.

Unfortunately, our first attempts to clone a PBR-like receptor gene in *T. gondii* by PCR amplification using degenerated primers or to identify such a gene or cDNA in *T. gondii* and in *P. falciparum* were unsuccessful. Alternatively, the PBR-like molecules of the parasite might be structurally different from mammalian PBR. We were also unable to identify a PBR-like gene when we examined the almost completed genome sequence of *P. falciparum* and expressed sequence tags from *T. gondii*.

It is intriguing that the treatment of *P. falciparum* with Fz leads to the complete absence of parasitic hemozoin, as demonstrated by UV-visible absorbance spectrum and mass spectrometry, suggesting an impairment of the digestive process. This drug could also modify or interfere with the binding of an endogenous compound, for instance protoporphyrin IX, a molecule which is part of the heme metabolism in mammalian cells and which has been proved to bind PBR (26). Although these molecular mechanisms have not yet been described for *P. falciparum*, it is interesting that heme derivatives are capable of lysing malaria parasites and that (*Plasmodium*) parasite survival relies on its capability to polymerize and store heme within the parasite digestive vacuole (3, 8, 17). It has been shown that other antimalarial compounds may act by inhibition of heme polymerization (7, 9, 21, 24), and it remains possible that such a process might also implicate a PBR-like molecule. The absence of hemozoin in the Fz-treated *P. falciparum* may simply reflect a direct consequence of parasite death, as seen

FIG. 8. (A) Effect of various concentrations of Fz at single doses on the intracellular replication of tachyzoites of *T. gondii* 76K assessed by the incorporation of [³H]uracil. Error bars represent the means and standard deviations of triplicate determinations of three reproducible experiments ($P < 0.05$). (B and C) Ultrastructural morphology of *T. gondii* 76K cultures by transmission electron microscopy after a 48-h incubation with Fz at 50 µg/ml (C) or without drug (control) (B). Note the accumulation of phagolysosomes in the host cell cytoplasm and the reduced number of tachyzoites in treated intracellular parasites (C, arrow) compared to untreated intracellular parasites (B).

by the degeneration of many parasitic organelles including the endoplasmic reticulum. These BDZ also inhibit *T. gondii*, which does not synthesize any hemozoin, suggesting that no direct link can be made between a specific inhibition of heme polymerization and the Fz activities in the parasite.

In conclusion, our preliminary data suggest that Fz and PK11195 are potentially effective in relatively short-term treatments of *P. falciparum* because of the total disappearance of parasites and disintegration of *P. falciparum* after 24 to 48 h of exposure. As discussed above, this effect can only be moderately measured for *T. gondii*. The fact that Fz and PK11195 do not antagonize the other antimalarial drugs indicated that they can be combined with chloroquine and/or mefloquine. Most importantly, it appears that both Fz and PK11195 are effective against the chloroquine- and mefloquine-resistant Dd2 strain of *P. falciparum*. In the quest to identify new antiparasitic molecules, many compounds derived from isoquinoline have already been isolated from plants for their antiplasmodial activity (4, 16). However, the receptors of these inhibitors have not been precisely identified and characterized. Our current work may also shed new light on the molecular and pharmacological mechanisms of conventional quinoline derivatives.

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