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Characterization of anti-*Toxoplasma* Activity of SDZ 215-918, a Cyclosporin Derivative Lacking Immunosuppressive and Peptidyl-Prolyl-Isomerase-Inhibiting Activity: Possible Role of a P Glycoprotein in *Toxoplasma* Physiology

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The immunosuppressive agent cyclosporin A (CsA) also possesses broad-spectrum antimicrobial activity. Previous investigators have reported that the obligate intracellular protozoan *Toxoplasma gondii* is sensitive to CsA. We have measured the sensitivity of *Toxoplasma* to 26 CsA derivatives that maintain only a subset of the parent compound's activity. We identified one compound, SDZ 215-918, that is a particularly potent inhibitor of parasite invasion and replication, with a 50% inhibitory concentration of 0.45 μg/ml, which is 10-fold lower than that of CsA. Kinetic studies demonstrate that activity has a rapid onset (half-life, ≤20 min) and is initially reversible, although long-term exposure (>24 h) to 5 μg/ml is lethal; in contrast, this concentration had no effect on host cell protein synthesis or cell division. SDZ 215-918 acts directly on the parasite, as demonstrated by inhibition of macromolecular synthesis in host-free extracellular parasites. Inhibition of invasion is due to a reduction in parasite motility. SDZ 215-918 does not bind to cyclophilins, the ubiquitous cyclosporin-binding proteins, but is a potent inhibitor of the mammalian P glycoprotein, a member of the ATP binding cassette transporter superfamily and the pump responsible for multidrug resistance in cancer and parasite cell lines. SDZ 215-918 blocks the efflux of rhodamine 123 from extracellular parasites, consistent with inhibition of a P glycoprotein-like pump. We suggest that a P glycoprotein or a related transporter plays a crucial role in the biology of *Toxoplasma* and may be a novel target for antiparasitic compounds. Preliminary studies with animals indicate that SDZ 215-918 inhibits parasite growth in vivo; its relationship to CsA may make it suitable for clinical development.

Toxoplasma gondii is a ubiquitous human pathogen and a significant hazard to immunocompromised individuals, particularly transplant patients and persons with AIDS (2). Currently existing chemotherapeutic regimens, while effective at controlling the parasite, are poorly tolerated, particularly by immunocompromised individuals. Further understanding of host-pathogen interactions and identification of novel parasite biochemical pathways may lead to more specific treatments.

Toxoplasma is an obligate intracellular protozoan parasite. Entry of *Toxoplasma* into target cells occurs by a poorly understood process of active invasion, followed by multiplication within the parasitophorous vacuole, a specialized membrane-bound compartment (15, 46). The mechanisms by which *Toxoplasma* invades cells and modifies the host environment to establish productive infection are largely unknown.

Previous research has indicated that *T. gondii* is sensitive to high levels of cyclosporin A (CsA) (30, 32). CsA is a potent immunosuppressive drug that has revolutionized solid-organ transplantation. CsA affects a wide range of cellular processes, from T-cell activation to regulated secretion (29). Three distinct biochemical activities have been assigned to CsA to date: (i) Complexes formed between CsA and cyclosporin-binding proteins (cyclophilins) interfere with the activity of the calcium-calmodulin-dependent protein phosphatase calcineurin and inhibit signal transduction (44). (ii) The cyclophilins are peptidyl-prolyl *cis-trans* isomerases (PPIases) and are believed to be involved in protein folding, oligomer formation, and secretion. CsA can inhibit the PPIase activities of the cyclophilins and alter their chaperonin function (39). (iii) CsA has been shown to inhibit the action of the P glycoprotein, the pump that confers multidrug resistance (MDR) on cancer cells and parasitic protozoa (5, 20, 48). The P glycoprotein is a transporter of hydrophobic molecules and is associated with chloride channel activity. Its normal function in protozoa is not understood, although the mammalian forms have been shown to act as lipid translocators (40, 50).

Although best known as an immunosuppressive agent, CsA was originally isolated as an antifungal agent and has antimicrobial activity against a range of fungi, bacteria, and protozoa, including *Toxoplasma*, *Plasmodium*, and *Leishmania* (7, 23, 24, 35). The basis of this activity is unknown, but it could be due to any of the mechanisms described above. We are engaged in an effort to characterize the activity of CsA against *Toxoplasma*. Here, we describe a CsA analog that is a potent anti-*Toxoplasma* agent, capable of blocking parasite invasion and replication. SDZ 215-918 lacks the ability to interact with cyclophilins, but it is a potent inhibitor of the P glycoprotein, suggesting that a P-glycoprotein homolog plays an important role in the biology of *Toxoplasma*, including the invasion process.

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MATERIALS AND METHODS

Parasites and tissue culture. The RH strain of *T. gondii* was maintained by serial passage in Vero cells (African green monkey kidney cells; ATCC 1587) maintained in α-minimal essential medium (MEM) supplemented with nonessential amino acids and 7.5% fetal bovine serum at 37°C under 5% CO_2 or by peritoneal passage in Swiss Webster mice (27). For experiments with mycophenolic acid, the HXGPRT$^-$ strain (an RH derivative lacking hypoxanthine, xanthine, and guanine phosphoribosyltransferase; courtesy of David Roos, University of Pennsylvania [14]) was used.

Uracil incorporation assays. Parasite viability was measured by the incorporation of tritiated uracil by the method of Pfefferkorn and Pfefferkorn (38). Briefly, Vero cell monolayers grown in 24-well plates were infected at a multiplicity of infection (MOI) of 1 in the presence of test compounds for 24 h prior to labeling with [^{3}H]uracil (0.75 μCi per well) for an additional 6 h. Monolayers were washed with phosphate-buffered saline (PBS) prior to treatment with 10% trichloroacetic acid (TCA). Following TCA precipitation, monolayers were solubilized with 0.2 N NaOH, and the level of incorporation of [^{3}H]uracil into TCA-precipitable material was measured by liquid scintillation counting. Control wells were incubated with medium plus sufficient ethanol (the solvent for CsA and SDZ 215-918) to match the maximum concentration of ethanol in the drug samples. Wells lacking parasites incorporated <0.1% of the [^{3}H]uracil incorporated by the control.

For short-time-course assays, Vero cell monolayers were infected at an MOI of 3 for 24 h with the HXGPRT$^-$ strain in the absence of inhibitors. Monolayers were washed prior to the addition of fresh medium plus inhibitors (see legend to Fig. 2 for details). During the final 15 min of drug exposure, parasites were labeled via the addition of 1 μCi of [^{3}H]uracil per well (for the sample taken at 15 min, label and drug were added simultaneously). TCA-precipitable material was assayed as described above. Ethanol-treated control wells incorporated approximately 2,000 cpm under these labeling conditions.

PPIase assays. *Toxoplasma* extracts enriched for cyclophilins were prepared by passage over an 8-ornithino–CsA affinity matrix as previously described (25), followed by elution at pH 3.3 (elution was directly into alkali buffer to neutralize the pH). PPIase activity was determined by measuring isomerization of *N*-succinyl-Ala-Ala-Pro-Phe (*p*-nitroanilide) in a coupled chymotrypsin cleavage assay at 4°C as described previously (28). For inhibitor studies, CsA or SDZ 215-918 was added to extracts at a final concentration of 500 nM, and the mixture was incubated for 5 min before the addition of substrate.

Invasion assays. Vero cell monolayers grown on 12-mm glass coverslips were infected for 2 h in the presence of test compounds and were then washed well with PBS and fixed with 100% methanol. Parasites were visualized by staining with rabbit polyclonal antisera to dense granule antigen GRA3 (36) and fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody; GRA3 is secreted into the vacuolar space following entry, but it is seen only as a particulate distribution in extracellular parasites. Host and parasite nuclei were visualized by staining with 4′,6-diamidino-2-phenylindole (DAPI). Monolayers were assayed by immunofluorescence microscopy; fields were chosen at random under DAPI illumination and counted for host cells before intracellular parasites were counted under fluorescein isothiocyanate illumination. Data are expressed as the percentage of cells infected. For pretreatment assays, 3×10^6 HXGPRT$^-$ parasites were incubated at 37°C under 5% CO_2 in 1 ml of MEM plus inhibitor in a loosely capped tube for 60 min with agitation at 15-min intervals. Following incubation, parasites were washed once in 1 ml of MEM without inhibitor and were then resuspended in 1 ml of MEM without inhibitor. Vero cell monolayers were challenged at an MOI of 3 and were assayed as described above.

Replication assays. Vero cell monolayers were infected as described above for the invasion assays, but in the absence of inhibitors. Following removal of extracellular parasites, fresh medium plus inhibitor at the indicated concentration was added, and cells were incubated at 37°C for 18 to 20 h before being fixed, stained, and examined as described above for the invasion assays; data are expressed as the average number of parasite doublings per vacuole. For long-term reversibility assays, intracellular parasites were exposed to inhibitors for 24, 36, or 48 h; inhibitors were removed and parasite replication was measured microscopically 24 h later, as described above. Viable vacuoles were defined as containing four or more parasites 24 h after removal of the inhibitor.

Attachment assays. Attachment assays were performed by the method of Mineo and Kasper (33) following preincubation with 5 μg of SDZ 215-918 per ml or a 1:40 dilution of rabbit antisera to whole *Toxoplasma* (antibody coated) for 30 min at 37°C under 5% CO_2. Parasites were visualized by staining with monoclonal antibody to the surface antigen SAG1 (12); host cell nuclei were stained with DAPI. Fields were chosen at random under DAPI illumination; 300 cells were counted per replicate. Data are expressed as an attachment index, which is the number of parasites/100 cells.

Motility assay. The effects on parasite motility were determined by microscopic observation of live organisms. Parasites were harvested from tissue culture and were maintained on ice. *T. gondii* cells were added to MEM plus appropriate drugs, and the mixture was preincubated for 10 min at 37°C under 5% CO_2 prior to transfer to petri dishes (with or without Vero cell monolayers) for observation; alternatively, no pretreatment occurred. Petri dishes were placed on a heated microscope stage and were observed for up to 60 min; motility was defined by the characteristic corkscrew motion of *T. gondii*. At least two dishes per treatment were observed. The experiment was repeated on separate days with independent parasite preparations.

Extracellular uracil incorporation and protein synthesis assays. Freshly harvested parasites were resuspended at 10^7/ml in artificial intracellular salt solution (10) plus amino acids but lacking cysteine and methionine. Parasites were pretreated as required at 37°C prior to labeling for 10 min with 1 μCi of [^{3}H]uracil or [^{35}S]methionine. For uracil labeling, incorporation was stopped by the addition of TCA to 10%. Samples were harvested on glass microfiber filters and washed with TCA and ethanol, and the level of incorporation was measured by liquid scintillation counting. For protein synthesis, samples were processed as described previously (3).

Rhodamine 123 efflux. Parasites were preincubated in MEM at 37°C under 5% CO_2 (control, SDZ 215-918-treated, and verapamil-treated parasites) or 65°C for 30 min (heat-killed parasites) prior to labeling with rhodamine 123 at 10 ng/ml for 30 min at 37°C under 5% CO_2. Parasites were washed twice to remove excess dye and were then resuspended in MEM or MEM plus inhibitors and incubated for the indicated times. Efflux was stopped by the addition of ice-cold MEM; parasites were pelleted and resuspended in 1 ml of ice-cold MEM and were held on ice until the end of the assay. Fluorescence was determined at an excitation of 388 nm and an emission of 430 nm in a Hitachi F-2000 spectrofluorimeter. Fluorescence values were normalized to the absolute reading at time zero. Readings for all time points were taken in quadruplicate.

In vivo assays. For studies on its in vivo activity, SDZ 215-918 was dissolved in 25% ethanol–10% polyethylene glycol 300–10% polyethoxylated castor oil cremaphore)–PBS. Female Swiss Webster mice (weight, 22 to 25 g; Charles River Laboratories) were treated with 0, 0.072, or 0.72 mg (approximately 0, 3, or 30 mg per kg of body weight) 30 min prior to inoculation with 500 RH strain parasites. Mice were injected with additional drug at 24-h intervals; injections were given in two doses 2 h apart to reduce the toxicity from ethanol. The assay was continued until all mice (seven per test group) were dead.

Materials. Radioisotopes were purchased from Amersham (Arlington Heights, Ill.). CsA and CsA derivatives were provided by Sandoz Pharma, Ltd., Basel, Switzerland, and Robert Handschumacher, Yale University School of Medicine. Mitotracker was from Molecular Probes (Eugene, Oreg.); all other reagents were purchased from Sigma (St. Louis, Mo.).

RESULTS

The anti-*Toxoplasma* activities of CsA derivatives correlate with P-glycoprotein inhibition. Three distinct biochemical activities have been described for CsA: inhibition of the PPIase activity of cyclophilins, inhibition of the calcium-calmodulin-dependent protein phosphatase calcineurin (via complex formation with cyclophilins), and inhibition of the transport activity of the P glycoprotein. Any of these activities could conceivably confer antiparasitic activity upon the compound. In order to determine the likely pathway of anti-*Toxoplasma* activity, the sensitivity of *T. gondii* to CsA derivatives that maintain only a subset of the activities of the parental compound was measured. Susceptibility was determined by measuring uracil incorporation by intracellular parasites following 24-h exposure to the test compounds. The results are presented in Fig. 1 for three compounds: CsA, SDZ 215-918 (the most effective analog tested), and cyclosporin H (one of the least effective analogs tested). A 50% inhibitory concentration (IC_{50}) of approximately 4.5 μg/ml was determined for CsA, whereas SDZ 215-918 had an IC_{50} of 0.45 μg/ml. Comparison of IC_{50}s, however, does not fully indicate the differences among these compounds: we also note that high concentrations (5 μg/ml) of SDZ 215-918 more completely inhibit parasite metabolic activity (<5% of that of the control) than large doses (10 μg/ml) of CsA (≈20% of that of control).

A total of 26 CsA derivatives were tested; the IC_{50}s of 10 compounds are listed in Table 1. Other chemical and biological properties are as reported to us by the manufacturer, Sandoz Pharma Ltd. (53), and are based on tests with mammalian cell lines. Strikingly, the ability to inhibit *Toxoplasma* does not correlate with PPIase inhibition or immunosuppression, suggesting that drug sensitivity is not via a cyclophilin-mediated pathway. Instead, the four most effective compounds are devoid of immunosuppressive or PPIase inhibitory activities but are excellent inhibitors of the P glycoprotein, suggesting that a P-glycoprotein homolog plays a significant role in *Toxoplasma*

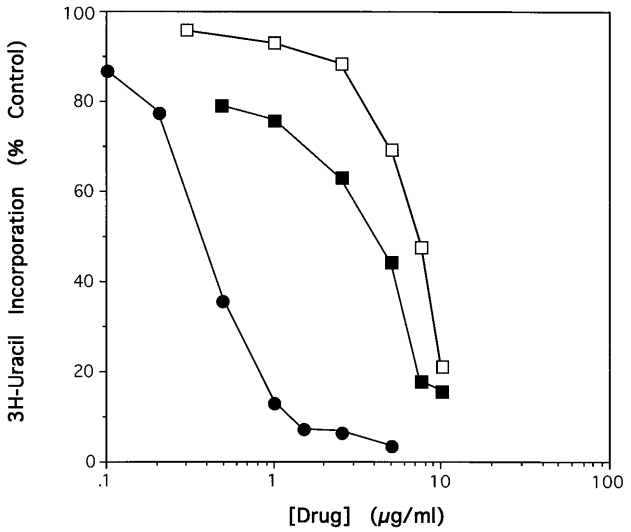

FIG. 1. SDZ 215-918 is a potent inhibitor of *T. gondii* and is more effective than its parent compound, CsA. Incorporation of tritiated uracil by intracellular *Toxoplasma* was measured following a 24-h exposure to SDZ 215-918 (●), CsA (■), or cyclosporin H (□). Control wells were incubated with medium plus sufficient ethanol (the solvent for CsA and SDZ 215-918) to match the maximum concentration of ethanol in the samples containing drug. Wells lacking parasites incorporated <0.1% of the ^{3}H-uracil incorporated by the control. Assays were performed at least four times (n = 4), in triplicate; data from representative assays are presented.

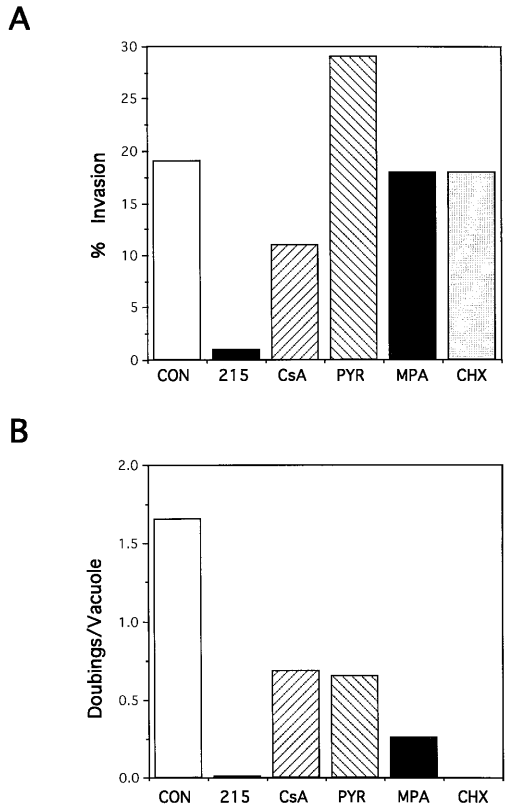

FIG. 2. SDZ 215-918 inhibits invasion of host cells by extracellular parasites and replication of intracellular parasites. Invasion (A) and replication (B) assays were performed as described in Materials and Methods. CON, control; 215, SDZ 215-918, 5 μg/ml; CsA, 10 μg/ml; PYR, pyrimethamine, 1 μM; MPA, mycophenolic acid, 25 μg/ml; CHX, cycloheximide, 10 μM. Data from representative assays (n = 3) are presented.

physiology. Consistent with this, cyclosporin H, which is a poor P-glycoprotein inhibitor, is less effective than CsA at inhibiting parasite growth.

To verify that the information reported to us was relevant to studies on protozoans, we directly tested the ability of SDZ 215-918 to inhibit the PPIase activity of detergent extracts enriched with parasite cyclophilins. As described previously (25), the PPIase activity of *Toxoplasma* cyclophilins is completely inhibited by 500 nM CsA; however, 500 nM SDZ 215-918 had no effect, confirming its inability to interact with parasite cyclophilins.

We describe below the further characterization of the activity of SDZ 215-918.

TABLE 1. Sensitivity to CsA analogs does not correlate with immunosuppression or inhibition of PPIase activity[a]

Compound[b]	IC_{50} (μg/ml)	Immunosuppression	PPIase inhibition	Cyclophilin binding	P-glycoprotein inhibition
SDZ 215-918	0.45	None	None	None	++++
SDZ 216-070	0.65	None	None	None	++++
SDZ 217-383	0.85	None	None	None	++++
DihydroCsA	1.2	0.5	0.5	0.5	ND[c]
PSC-833	2.7	None	None	None	++++
D-MeSer-3-Abu-2-CsA	3.2	1	5–10	5–10	ND
CsA	4.5	1	1	1	++
$MeAla_6$-CsA	5.8	None	0.2–0.5	0.2–0.5	++
Cyclosporin H	8.3	None	None	None	+
FurfurylCsA	>>10	0.02	0.04	0.04	ND

[a] *T. gondii* RH was tested for sensitivity to the indicated compounds as described in Materials and Methods and in the legend to Fig. 1. IC_{50} indicates the dose required to reduce uracil incorporation by 50%; other properties were provided by the manufacturer (53). All values (other than IC_{50}s) are normalized to those for CsA. The structures of dihydroCsA, PSC-833, and cyclosporin H have been reported previously (17, 31, 51).

[b] In addition to the compounds listed here, the following compounds were found to have IC_{50}s not significantly different from that of CsA (between 2 and 5 μg/ml): cyclosporin G (Nva-2-CsA), cyclosporin D (Val-2-CsA), SDZ 215-764, SDZ 217-666, SDZ 090-004, D-Ser-8-CsA, MeVal-4-CsA, Val-2-D-MeAla-3-CsA, Bm2t-CsA, MeBmt(6,7-dihydro-8-azido)-1-CsA, MeBmt(6,7-dihydro)-1-Val-2-CsA, MeBmt(6,7-dihydro)-1-Thr-2-CsA, MeBmt (6,7-dihydro-8-)-Ome)-1-CsA, MeBmt(7-desmethyl-7-phenyl)-1-CsA, SDZ 280-446, and Thr-2-Leu-5-D-Hiv-8-Leu-10-CsA (where MeBmt indicates (4R)-4[(E)-butenyl]-4, *N*-dimethyl-L-threonine, MeBm2t indicates (2S, 3R, 6E)-4,4-dimethyl-3-hydroxy-2-(N-methylamino)-6-octenoic acid, Nva indicates norvaline, and Hiv indicates 2-hydroxyvaleric acid). Structures for some derivatives have been reported previously (1, 52).

[c] ND, not determined.

SDZ 215-918 inhibits parasite invasion and replication. In our initial susceptibility assays, parasites were exposed to CsA derivatives throughout the infection cycle. Under these conditions, reduced uracil incorporation could be due to inhibition of invasion of host cells or to a reduction in parasite replication. As indicated in Fig. 2, SDZ 215-918 and CsA can inhibit both invasion and replication. Treatment with cycloheximide or mycophenolic acid, capable of inhibiting protein synthesis and nucleoside salvage, respectively, have no effect on invasion, suggesting that these pathways are not the direct targets of action of SDZ 215-918. This is the first report of an anti-*Toxoplasma* agent capable of disrupting both invasion and replication and suggests that the target may be involved in both aspects of the life cycle.

The effects of SDZ 215-918 on *Toxoplasma* are extremely rapid and rapidly reversible. Invasion of host cell monolayers is generally detectable within minutes of parasite inoculation; the nearly complete inhibition of parasite invasion by SDZ 215-918 suggested that its effects might be extremely rapid. As

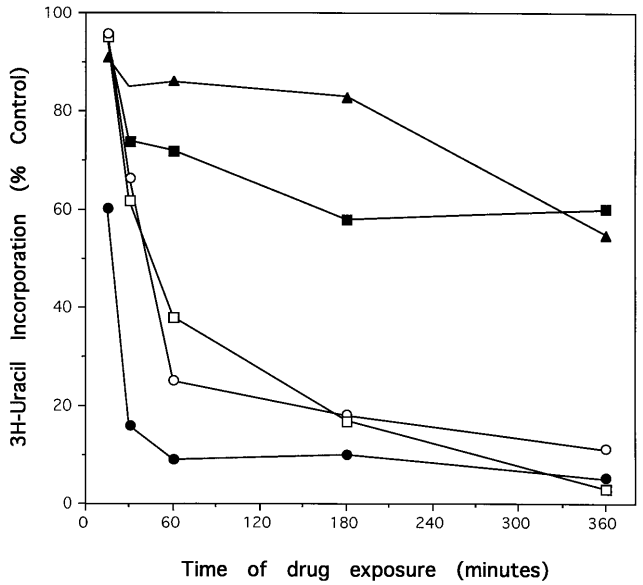

FIG. 3. SDZ 215-918 rapidly inhibits the metabolic activity of intracellular parasites. Incorporation of tritiated uracil by intracellular *Toxoplasma* was measured following short exposure to various inhibitors. ●, SDZ 215-918, 5 μg/ml; □, CsA, 10 μg/ml; ▲, pyrimethamine, 1 μM; ■, mycophenolic acid, 25 μg/ml; ○, cycloheximide, 10 μM. TCA-precipitable material was assayed as described in the legend to Fig. 1. Ethanol-treated control wells incorporated approximately 2,000 cpm under these labeling conditions. Assays were performed in triplicate; data from a representative assay (n = 3) are presented.

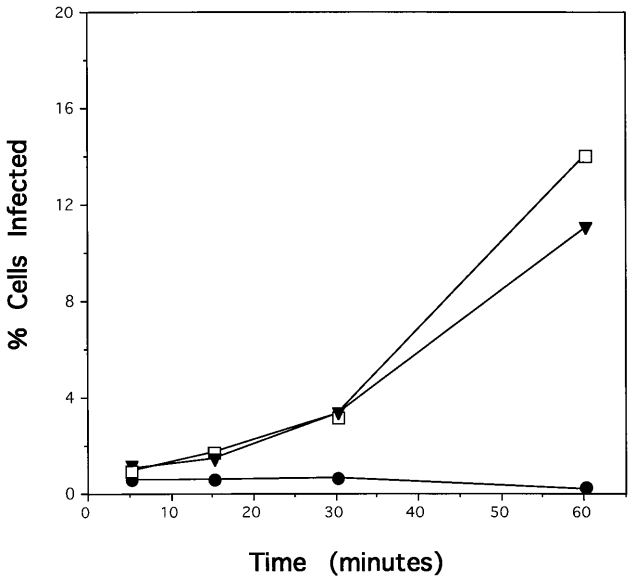

FIG. 4. SDZ 215-918 rapidly inhibits invasion of host cell monolayers. Invasion assays were performed as described in Materials and Methods, with the time of monolayer challenge varied as indicated by the values on the x axis. ●, SDZ 215-918, 5 μg/ml; □, cycloheximide, 10 μM; ▼, control. Assays were performed in duplicate; data from a representative assay (n = 5) are presented.

indicated in Fig. 3, the metabolic activity of intracellular parasites, as measured by the level of incorporation of [^{3}H]uracil, is rapidly reduced, with 40% inhibition seen at the first assayable time point (15 min); this is significantly faster than the effects of mycophenolic acid and pyrimethamine, which affect nucleotide salvage and synthesis pathways, suggesting that these are not the direct target of action of SDZ 215-918. Similarly, differences in the efficiency of invasion by SDZ 215-918-treated parasites can be observed after as little as 5 min of drug treatment (Fig. 4).

Previous investigators have shown that the sensitivity of *Toxoplasma* to certain drugs (e.g., clindamycin [6, 37]) is greatly increased by increasing the time of exposure. In contrast, increasing the time of exposure to SDZ 215-918 from 1 to 5 days does not significantly alter the IC_{50} (data not shown), suggesting that the inhibition seen in short-time-course assays represents the complete effect of the drug.

Reversibility assays were performed to determine whether SDZ 215-918 is parasiticidal or parasitistatic. Pretreatment of extracellular parasites failed to affect invasion or subsequent replication in the absence of SDZ 215-918 (Table 2), suggesting that SDZ 215-918 must be present during monolayer challenge to reduce invasion and indicating the rapid reversibility of the effects of a short exposure. The reversibility of the effects of a long-term exposure was tested with intracellular parasites. After 24 h of exposure to 5 μg of SDZ 215-918 per ml, approximately 25% of the intracellular parasites were capable of division following removal of the inhibitor, whereas after 48 h no viable parasites remained (data not shown). In the same assay, 1 μM pyrimethamine reduced viability to <5% after a 24-h exposure, again indicating a different mechanism of action. These experiments suggest that while SDZ 215-918 is initially parasitistatic, it is eventually parasiticidal; we have not determined the exact point at which treatment becomes irreversible.

SDZ 215-918 inhibits parasite motility but not attachment to host cells. Invasion of host cells by *T. gondii* is a dynamic and poorly understood process involving attachment to target cells by an as yet unknown ligand and requiring parasite actin-based motility (13, 33, 34, 41, 47). We have examined the effect of SDZ 215-918 on both of these processes. SDZ 215-918 had no effect on attachment, which was effectively inhibited by antisera raised to whole parasites. Attachment indices for control (0.1% ethanol), SDZ 215-918-treated (5 μg/ml), and antibody (anti-strain RH)-coated samples were 9.7 ± 2.1, 9.0 ± 2.5, and 1.9 ± 0.5 parasites/100 cells, respectively. These data are the averages of two assays, and samples were taken in duplicate. Effects on motility were monitored by direct microscopic observation. *Toxoplasma* moves along cell monolayers and serum-coated plastic surfaces via a characteristic corkscrew motion, with the posterior end being relatively stationary and the apical end twisting and writhing. In untreated control populations, corkscrewing parasites could be observed for up to 60 min, while the addition of cytochalasin D (1 μg/ml) rendered parasites nonmotile within 1 to 2 min. Strikingly, treatment with SDZ 215-918 (5 μg/ml) resulted in a gradual shutdown of parasite motility: by 10 min posttreatment, no corkscrewing parasites were visible (data not shown). The kinetics of this

TABLE 2. Pretreatment of extracellular parasites with SDZ 215-918 does not affect invasion or replication in the absence of the drug[a]

Treatment	% Cells infected in invasion assay	No. of parasites/vacuole in replication assay
Control	36	3.21
SDZ 215-918 (5 μg/ml)	38	3.14
CsA (10 μg/ml)	35	3.14
Host cell pretreatment (SDZ 215-918, 5 μg/ml)	37	3.06

[a] Pretreatment assays were performed as described in the text. Data from a representative assay are presented (n = 3).

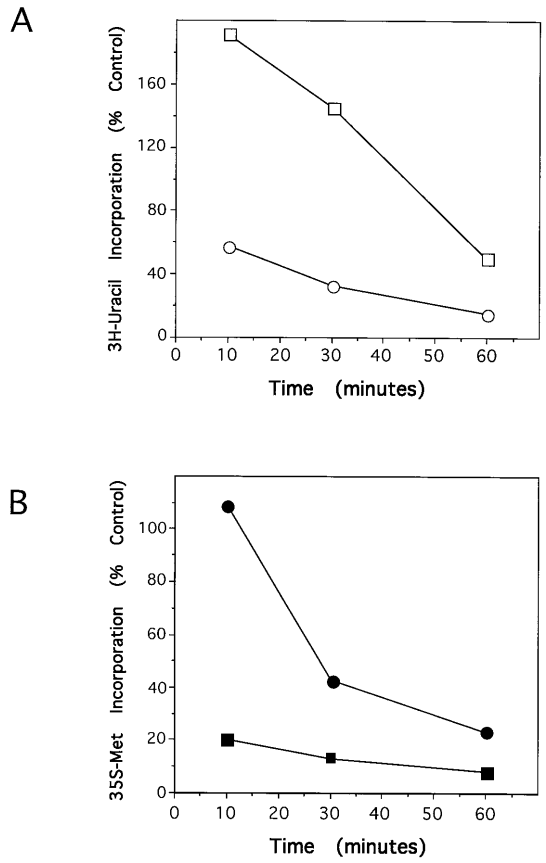

FIG. 5. SDZ 215-918 inhibits macromolecular synthesis in extracellular host-free parasites. Incorporation of [^{3}H]uracil (A) or [^{35}S]methionine (B) into TCA-precipitable material was determined with extracellular parasites liberated from host cells by passage through a 27-gauge needle; mock-infected host cells prepared by syringe passage incorporated <1% of that incorporated by the control. ○, ●, SDZ 215-918, 5 μg/ml; □, ■ cycloheximide, 5 μg/ml. Assays were performed in triplicate; data from a representative assay (n = 4) are presented.

process are consistent with the previously described effects on invasion, and we suggest that the inhibition of invasion is related to the inhibition of parasite motility.

The effect of SDZ 215-918 is not mediated via a host cell target. Because CsA can interact with targets in all eukaryotes, it is possible that the effects observed are due to action via a host cell target. Three lines of data suggest that this is not the case. First, as reported above, direct microscopic examination reveals an alteration in the behavior of extracellular parasites. Second, SDZ 215-918 rapidly and completely inhibits uracil incorporation and protein synthesis by host-free extracellular parasites with kinetics similar to those seen for intracellular parasites (Fig. 5). We noted that the reduction in protein synthesis lagged behind the reduction in uracil incorporation, suggesting that it is a secondary effect of that phenomenon. Finally, incubation of Vero cell monolayers for up to 7 days with SDZ 215-918 (5 μg/ml) had no effect on protein synthesis levels or on doubling rates (data not shown). All of these data strongly indicate that the effects of SDZ 215-918 are directly on *T. gondii* and suggest that the target of the drug either is not present or is not important in *Toxoplasma*'s mammalian host.

***Toxoplasma* is sensitive to verapamil.** The data presented in Table 1 suggest that the sensitivity of *Toxoplasma* to CsA is mediated via a P-glycoprotein homolog. In light of this, we have reexamined the sensitivity of *Toxoplasma* to verapamil, the classic P-glycoprotein inhibitor (11). It has previously been reported that *Toxoplasma* growing in macrophages is insensitive to verapamil at 10 μg/ml, with higher concentrations causing monolayer degradation (26). In contrast, we have found that *T. gondii* growing in Vero cells is sensitive to verapamil (Fig. 6) and that the drug, like CsA, can block both replication and invasion, although not as effectively as CsA derivatives (data not shown). This is consistent with the conclusion that a P glycoprotein or related molecule plays a role in these processes. We do not understand the discrepancy between our results and those published previously.

SDZ 215-918 inhibits rhodamine 123 efflux by *Toxoplasma*. The standard biochemical assay for P-glycoprotein activity is the efflux of the hydrophobic potential-sensitive fluorescent dye rhodamine 123 (16). As indicated in Fig. 7, untreated parasites accumulate rhodamine 123 and rapidly efflux the dye. This efflux is inhibited by SDZ 215-918 (as well as other CsA derivatives [data not shown]) and verapamil. Inhibition is detectable at the earliest time point, indicating rapid activity of the drug. Similar experiments have been performed with the fixable hydrophobic potential-sensitive fluorescent dye Mitotracker, with accumulation detected by fluorescence microscopy. Mitotracker accumulates in the mitochondria of both treated and untreated extracellular parasites, as expected, but in the presence of SDZ 215-918 it accumulates in the cytoplasm as well (data not shown). Again, this is consistent with a functioning efflux system in untreated parasites. We note also that the accumulation of potential-sensitive dyes suggests that SDZ 215-918 treatment does not significantly alter the mitochondrial membrane potential of extracellular parasites.

Inhibition of rhodamine 123 efflux could reflect specific inhibition of a P-glycoprotein homolog, or it could indicate general disruption of membrane transport phenomena in treated parasites. We have found that the uptake of adenosine via facilitated diffusion, the only membrane transport activity characterized in *Toxoplasma* (45), is unaffected by 5 μg of SDZ 215-918 per ml (data not shown), suggesting a specific inhibition.

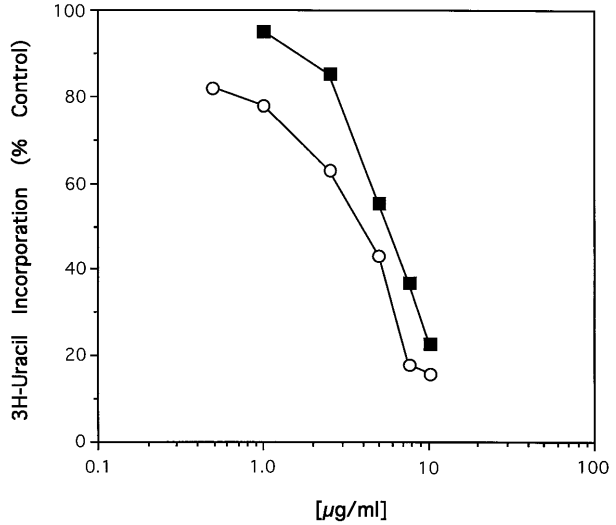

FIG. 6. *Toxoplasma* is sensitive to the P-glycoprotein inhibitor verapamil. Incorporation of tritiated uracil by intracellular parasites was measured as described in the legend to Fig. 1. ■, verapamil; ○, CsA. Assays were performed in triplicate; data from a representative assay are presented (n = 4). Data for CsA are reproduced from Fig. 1 to simplify comparison.

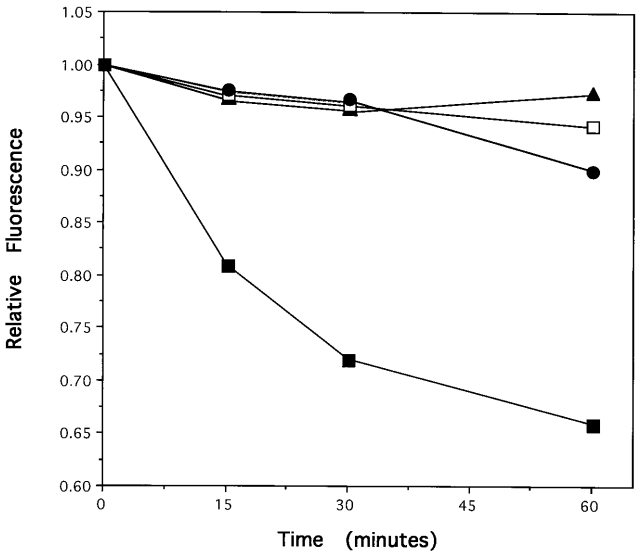

FIG. 7. SDZ 215-918 and verapamil inhibit rhodamine 123 efflux from extracellular *Toxoplasma*. Efflux of the fluorescent dye rhodamine 123 was measured from extracellular parasites. Fluorescence was determined at an excitation of 388 nm and an emission of 430 nm. Fluorescence values were normalized to the absolute reading at time zero (for control, SDZ 215-918-treated, and verapamil-treated parasites, approximately 1.8; for heat-killed parasites, approximately 1.05). Assays were performed in quadruplicate; data from a representative assay are presented ($n = 3$). ●, SDZ 215-918, 5 μg/ml; □, verapamil, 10 μg/ml; ■, control; ▲, heat-killed parasites.

SDZ 215-918 is active in vivo. The apparent absence of an effect of SDZ 215-918 on mammalian host cells raises the possibility that this drug can be developed for clinical use against toxoplasmosis. As a first step, we have performed preliminary studies to determine if SDZ 215-918 is effective in an animal model of *Toxoplasma* infection. As indicated in Fig. 8, mortality in mice treated with 0.72 mg of SDZ 215-918 per day (approximately 30 mg/kg of body weight) was delayed by approximately 48 h; SDZ 215-918 at 0.072 mg/day had no significant effect, indicating a dose-response. Additionally, the number of parasites present in the peritoneal cavity at 7 days postinfection (indicated by the arrow in Fig. 8) was determined by microscopic examination of peritoneal fluid. Treatment with 0.72 mg of SDZ 215-918 per day reduced parasite loads from 1×10^7 (control; 0.072 mg/day) to 2×10^4, indicating significant reduction in the rate of parasite replication. These results suggest that SDZ 215-918 maintains activity in vivo, although additional study is required to determine the most effective dose and delivery system.

DISCUSSION

The broad-spectrum antimicrobial activity of CsA has been known for some time (23, 24), but the mechanism of this activity and whether the activity can be separated from the immunosuppressive properties of the drug to allow its clinical use as an antibiotic have remained unclear. We report here the identification of a CsA derivative that has anti-*Toxoplasma* activity both in vitro and in vivo. This derivative, SDZ 215-918, lacks a cyclophilin-binding capacity and is nonimmunosuppressive; it may therefore be a strong candidate for development as a clinically useful anti-*Toxoplasma* agent.

The cyclosporins, both immunosuppressive and nonimmunosuppressive, are capable of inhibiting the action of the P glycoprotein (48), a broad-substrate-range transporter capable of acting on most hydrophobic compounds. SDZ 215-918 is a potent inhibitor of P-glycoprotein activity in mammalian cell lines (53). We have shown here that it inhibits rhodamine 123 efflux in extracellular parasites, the classic assay for P-glycoprotein activity, suggesting that its target is a P-glycoprotein homolog. The potent anti-*Toxoplasma* activity of SDZ 215-918 suggests that this target plays an essential role in the biology of both intracellular and extracellular parasites. This represents a novel finding, because P glycoproteins in other systems have not been found to be essential (43). Intriguingly, this observation is not unique to *Toxoplasma*. Bell and coworkers (4) have previously tested the in vitro sensitivity of *Plasmodium falciparum* to a similar panel of CsA derivatives. They reported that PSC-833, a nonimmunosuppressive CsA derivative currently in clinical trials as an MDR reversal agent (9, 19), had the most potent anti-*Plasmodium* activity, while the parasite was relatively resistant to cyclosporin H, a poor P-glycoprotein inhibitor. *Toxoplasma* and *Plasmodium* are related apicomplexan parasites and share many structural and life-cycle features. These data indicate the potential importance of pursuing P-glycoprotein inhibitors as a novel class of drugs for use against this family of pathogens.

To date, no P glycoprotein has been identified in *T. gondii*, nor have multidrug-resistant isolates been reported. We are attempting to demonstrate the existence of such a protein. We have observed specific reactivity by immunofluorescence microscopy between *Toxoplasma* and two antibodies directed against known P glycoproteins: JSB-1, which recognizes the mammalian MDR1 (42), and a polyclonal antibody against *P. falciparum* MDR2 (unpublished data). These cross-reacting species may represent the target of drug action.

We are unable at this time to explain the mechanism of action of SDZ 215-918, although its ability to inhibit both invasion by extracellular parasites and replication of intracellular parasites while not affecting host cells suggests that its mode of action is novel. Other conditions which prevent invasion, such as exposure to cytochalasin D, depletion of energy stores, or antibody treatment (13, 33, 41, 54), have no such specificity or act by interfering with parasite-host attachment, which is not altered by SDZ 215-918. In many of the experiments reported here, we have used the level of incorporation

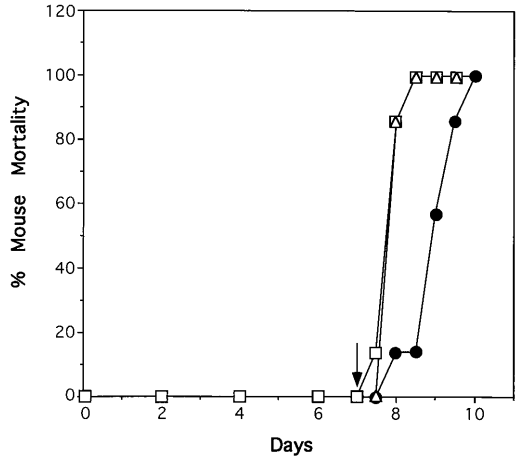

FIG. 8. SDZ 215-918 is active against *Toxoplasma* in vivo. Swiss Webster mice infected with 500 *T. gondii* RH cells were given daily injections of SDZ 215-918 dissolved in 25% ethanol–10% polyethylene glycol 3000–10% cremaphore–PBS. Viability was monitored until all mice in a test group ($n = 7$) were dead. △, 0.072 mg/day; ●, 0.72 mg/day; □, solvent only. Peritoneal fluid of infected mice was sampled on the morning of day 7, indicated by the arrow.

of [^{3}H]uracil as a measure of parasite viability. Pfefferkorn and Pfefferkorn (38) have shown that in short labeling experiments, uracil is mainly incorporated into newly synthesized RNA and can be reasonably used as a measure of gene expression. The effects of SDZ 215-918, however, are clearly different from those of cycloheximide and mycophenolic acid, which also rapidly inhibit uracil incorporation, suggesting that a general effect on gene expression is not the primary mode of action. In addition, while the effects of SDZ 215-918 are rapid and global, measurements of adenosine transport, ATP levels, and cytoplasmic pH (unpublished data) in extracellular parasites indicate that *T. gondii* maintains a number of normal cellular functions following drug treatment, arguing against a disruption of cellular integrity.

Cyclosporin and nearly all CsA derivatives tested possess anti-*Toxoplasma* activity with the same general characteristics: inhibition of invasion and replication, rapid onset of activity, and reversibility of action. They differ, however, in their IC_{50}s. We interpret this to reflect the specificity of interaction between SDZ 215-918 and its molecular target. Previous investigators (8) have reported strikingly different K_is for CsA and PSC-833 interacting with different ATP-dependent transporters, suggestive of specific inhibitor-target interactions. Along with differences in physiology, this may also account for the drug's specificity for the parasite rather than host functions.

Possible clues to the action of SDZ 215-918 may be found in its ability to inhibit invasion. Entry into host cells occurs via an energy-dependent parasite-driven invasion process that uses actin-based motility (13). The ability of SDZ 215-918 to inhibit invasion appears linked to its ability to inhibit parasite motility, which dissipates over approximately 10 min. This is reminiscent of the observation that treatment of parasites with valinomycin or nigericin, agents which abolish H^+ gradients by conducting K^+ ions, also cause a relatively gradual loss of motility, although the effects of these compounds were visible over about 2 min. Endo and Yagita (18) have suggested that *Toxoplasma* requires a plasma membrane potential for motility; it is possible that SDZ 215-918 is acting on some target to alter parasite membrane potential.

Two additional modes of action have been suggested. CsA has been shown to alter the fluidity of membranes in a variety of systems (22), and it has previously been reported that agents that alter membrane fluidity affect the efficiency of *Toxoplasma* invasion (54). It is possible, therefore, that SDZ 215-918 does not specifically inhibit a target protein but, rather, globally alters parasite membranes. Without detailed knowledge of the membrane-active properties of all of the CsA derivatives tested, we cannot rule out such a possibility, although we note that the previously reported effects on invasion were on the order of two- to threefold rather than the nearly complete inhibition observed here. A second possibility is that SDZ 215-918 is inhibiting the action of a P glycoprotein that is responsible for the removal of an inhibitory metabolite normally generated by *Toxoplasma*. This would be a relatively straightforward explanation of the ability of a P-glycoprotein inhibitor to completely shut down parasite metabolic activity, although we cannot at this time offer a plausible identity for such a compound.

Our efforts to understand the action of SDZ 215-918 would be greatly facilitated by the isolation of a parasite line resistant to its effects. To date, however, our efforts to obtain resistant mutants by both insertional and chemical mutagenesis have been unsuccessful, while under the same circumstances, we have successfully isolated mutants resistant to 5-fluorodeoxyuridine (unpublished data). One possible reason for the failure is that the target of SDZ 215-918 may be essential to parasite viability. A second possibility is that there are two targets: multiple P glycoproteins have been found in most protozoa (49). A mutation that rendered parasites resistant to the invasion defect but that did not overcome the replication block would not be recovered, and vice versa. We are attempting to select resistant mutants by stepwise selection; this approach has been successfully applied to other parasites to identify multidrug-resistant isolates by gene amplification (21).

Toxoplasmosis remains a life-threatening infection for immunocompromised individuals, particularly transplant recipients and persons with AIDS. Although treatable, new pharmacological agents are desirable, because the available therapies are often poorly tolerated. The extensive use of CsA in clinical settings suggests that it may be possible to adapt SDZ 215-918 to this use. Our preliminary studies with animals suggest that the drug maintains anti-*Toxoplasma* activity in vivo, where it is capable of slowing parasite replication and delaying mortality. Additional work is required, however, to determine the proper dosage and the proper route of delivery and to more closely examine possible toxicity and other side effects before its clinical potential can be fully known.

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REFERENCES

1. **Aebi, J. D., D. T. Deyo, C. Q. Sun, D. Guillaume, B. Dunlap, and D. H. Rich.** 1990. Synthesis, conformation, and immunosuppressive activities of three analogues of cyclosporin A modified in the 1-position. J. Med. Chem. **33:** 999–1009.
2. **Beaman, M. H., R. E. McCabe, S.-Y. Wong, and J. S. Remington.** 1995. *Toxoplasma gondii*, p. 2455–2475. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases. Churchill Livingstone, New York, N.Y.
3. **Beckers, C. J. M., D. S. Roos, R. G. K. Donald, B. J. Luft, J. C. Schwab, Y. Cao, and K. A. Joiner.** 1995. Inhibition of cytoplasmic and organellar protein synthesis in *Toxoplasma gondii*: implications for the target of macrolide antibiotics. J. Clin. Invest. **95:**367–376.
4. **Bell, A., B. Weroli, and R. M. Franklin.** 1994. Roles of peptidyl-prolyl *cis-trans* isomerase and calcineurin in the mechanism of antimalarial action of cyclosporin A, FK506, and rapamycin. Biochem. Pharm. **48:**495–503.
5. **Bellamy, W. T.** 1996. P-glycoprotein and multidrug resistance. Annu. Rev. Pharmacol. Toxicol. **36:**161–183.
6. **Blais, J., C. Tardif, and S. Chamberland.** 1993. Effect of clindamycin on intracellular replication, protein synthesis, and infectivity of *Toxoplasma gondii*. Antimicrob. Agents Chemother. **37:**2571–2577.
7. **Bogdan, C., H. Streck, M. Rollingham, and W. Solbach.** 1989. Cyclosporin A enhances elimination of intracellular *L. major* parasites by murine macrophages. Clin. Exp. Immunol. **75:**141–146.
8. **Bohme, M., M. Buchler, M. Muller, and D. Keppler.** 1993. Differential inhibition by cyclosporins of primary-active ATP-dependent transporters in the hepatocyte canalicular membrane. FEBS Lett. **333:**193–196.
9. **Book, D. J., I. F. Dennis, P. R. Twentyman, R. J. Osborne, C. Laborte, S. Hensel, J. F. Smyth, M. H. Brampton, and N. M. Bleeker.** 1996. Phase I study of etoposide with SDZ PSC-833 as modulator of multidrug resistance in patients with cancer. J. Clin. Oncol. **14:**610–618.
10. **Choi, I., and R. B. Mikkelsen.** 1990. *Plasmodium falciparum*: ATP/ADP transport across the parasitophorous vacuole and plasma membranes. Exp. Parasitol. **71:**452–462.
11. **Cornwell, M. M., M. M. Gottesman, and I. H. Pastan.** 1986. Increased vinblastine binding to membrane vesicles from multidrug-resistant KB cells. J. Biol. Chem. **261:**7921–7928.
12. **Couvreur, G., A. Sadak, B. Fortier, and J. F. Dubremetz.** 1988. Surface antigens of *Toxoplasma gondii*. Parasitology **97:**1–10.
13. **Dobrowolski, J. M., and L. D. Sibley.** 1996. *Toxoplasma* invasion of mammalian cells is powered by the actin cytoskeleton of the parasite. Cell **84:** 933–939.

14. **Donald, R. G. K., D. Carter, B. Ullman, and D. S. Roos.** 1996. Insertional tagging, cloning and expression of the *Toxoplasma gondii* hypoxanthine-xanthine-guanine phosphoribosyl transferase gene: characterization of a new genetic marker for stable transformation. J. Biol. Chem. **271:**14010–14019.
15. **Dubremetz, J. F.** 1995. *Toxoplasma gondii*: cell biology update, p. 345–368. *In* J. C. Boothroyd and R. Komunieck (ed.), Molecular approaches to parasitology. Wiley-Liss, New York, N.Y.
16. **Efferth, T., H. Lohrke, and M. Volm.** 1989. Recpiprocal correlation between expression of P-glycoprotein and accumulation of rhodamine 123 in human tumors. Anticancer Res. **9:**1633–1637.
17. **Emmer, G., M. A. Grassberger, G. Schulz, D. Boesch, C. Gaveriaux, and F. Loor.** 1994. Derivatives of a novel cyclopeptolide. 2. Synthesis, activity against multidrug resistance in CHO and KB cells *in vitro*, and structure-activity relationships. J. Med. Chem. **37:**1918–1928.
18. **Endo, T., and K. Yagita.** 1990. Effect of extracellular ions on motility and cell entry in *Toxoplasma gondii*. J. Protozool. **37:**133–138.
19. **Ferry, D. R., H. Traunecher, and D. J. Kerr.** 1996. Clinical trials of P-glycoprotein reversal in solid tumours. Eur. J. Cancer **32A:**1070–1081.
20. **Germann, U. A.** 1996. P-glycoprotein: a mediator of multidrug resistance in tumour cells. Eur. J. Cancer **32A:**927–944.
21. **Gueiros-Filho, F. J., J. P. Viola, F. C. Gomes, M. Farina, U. Lins, A. L. Bertho, D. F. Wirth, and U. G. Lopes.** 1995. *Leishmania amazonensis*: multidrug resistance in vinblastine-resistant promastigotes is associated with rhodamine 123 efflux, DNA amplification, and RNA overexpression of a *Leishmania* mdr1 gene. Exp. Parasitol. **81:**480–490.
22. **Haynes, M., L. Fuller, D. H. Haynes, and J. Miller.** 1985. Cyclosporin partitions into phospholipid vesicles and disrupts membrane architecture. Immunol. Lett. **11:**343–349.
23. **High, K. P.** 1994. The immunosuppressive and antimicrobial activities of cyclosporin A, FK506, and rapamycin. Transplantation **57:**1689–1700.
24. **High, K. P., and R. E. Handschumacher.** 1992. Immunity, microbial pathogenesis, and immunophilins: finding the keys, now where are the locks? Infect. Agents Dis. **1:**121–135.
25. **High, K. P., K. A. Joiner, and R. E. Handschumacher.** 1994. Isolation, characterization, and amino acid sequence of the two major cyclosporin binding proteins of *Toxoplasma gondii*. J. Biol. Chem. **269:**9105–9112.
26. **Holfels, E., J. McAuley, D. Mack, W. K. Milhous, and R. McLeod.** 1994. In vitro effects of artemisinin ether, cycloguanil hydrochloride (alone and in combination with sulfadiazine), quinine sulfate, mefloquine, primaquine phosphate, trifluoperazine hydrochloride, and verapamil on *Toxoplasma gondii*. Antimicrob. Agents Chemother. **38:**1392–1396.
27. **Joiner, K. A., S. A. Fuhrman, H. M. Miettinen, L. H. Kasper, and I. Mellman.** 1990. *Toxoplasma gondii*: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. Science **249:**641–646.
28. **Kofron, J. L., P. Kuzmic, V. Kishore, G. Colon-Bonilla, and D. H. Rich.** 1991. Determination of kinetic constants for peptidyl-prolyl *cis-trans* isomerases by an improved spectrophotometric assay. Biochemistry **30:**6127–6134.
29. **Kunz, J., and M. N. Hall.** 1993. Cyclosporin A, FK506, and rapamycin: more than just immunosuppression. Trends Biochem. Sci. **18:**334–338.
30. **Mack, R. G., and R. McLeod.** 1984. New micromethod to study the effect of antimicrobial agents on *Toxoplasma gondii*: comparison of sulfadoxine and sulfadiazine individually and in combination with pyrimethamine and study of clindamycin, metronidazole, and cyclosporin A. Antimicrob. Agents Chemother. **26:**26–30.
31. **Maurer, G.** 1985. Metabolism of cyclosporine. Transplant. Proc. **17**(Suppl. 1)**:**19–26.
32. **McCabe, R. C., B. T. Luft, and J. S. Remington.** 1986. The effects of cyclosporine on *Toxoplasma gondii in vivo* and *in vitro*. Transplantation **41:**611–615.
33. **Mineo, J. R., and L. H. Kasper.** 1994. Attachment of *Toxoplasma gondii* to host cells involves major surface protein, SAG-1 (P30). Exp. Parasitol. **79:** 11–20.
34. **Morisaki, J. H., J. E. Heuser, and L. D. Sibley.** 1995. Invasion of *Toxoplasma gondii* occurs by active penetration of the host cell. J. Cell Sci. **108:**2457–2464.
35. **Nickell, S. P., L. W. Scheibel, and G. A. Cole.** 1982. Inhibition by cyclosporin A of rodent malaria in vivo and human malaria in vitro. Infect. Immun. **37:**1093–1100.
36. **Ossorio, P. N., J.-F. Dubremetz, and K. A. Joiner.** 1994. A soluble secretory protein of the intracellular parasite *Toxoplasma gondii* associates with the parasitophorous vacuole membrane through hydrophobic interactions. J. Biol. Chem. **269:**15350–15357.
37. **Pfefferkorn, E. R., R. F. Nothnagel, and S. E. Borotz.** 1992. Parasiticidal effect of clindamycin on *Toxoplasma gondii* grown in cultured cells and selection of a drug-resistant mutant. Antimicrob. Agents Chemother. **36:** 1091–1096.
38. **Pfefferkorn, E. R., and L. C. Pfefferkorn.** 1977. Specific labeling of intracellular *Toxoplasma gondii* with uracil. J. Protozool. **24:**449–453.
39. **Price, E. R., M. Jin, D. Lim, S. Pati, C. T. Walsh, and F. D. McKeon.** 1994. Cyclophilin B trafficking through the secretory pathway is altered by binding of cyclosporin A. Proc. Natl. Acad. Sci. USA **91:**3931–3935.
40. **Ruetz, S., and P. Gros.** 1994. Phosphatidylcholine translocase: a physiological role for the mdr2 gene. Cell **77:**1071–1081.
41. **Ryning, F. W., and J. S. Remington.** 1978. Effect of cytochalasin D on *Toxoplasma gondii* cell entry. Infect. Immun. **20:**739–743.
42. **Scheper, R. J., J. W. Bulte, J. G. Brakkee, J. J. Quak, E. van der Schoot, A. J. Balm, C. J. Meijer, H. J. Broxterman, C. M. Kuiper, J. Lankelma, and H. M. Pinedo.** 1988. Monoclonal antibody JSB-1 detects a highly conserved epitope on the P-glycoprotein associated with multidrug-resistance. Int. J. Cancer. **42:**389–394.
43. **Schinkel, A. H., C. A. Mol, E. Wagenaar, L. van Deemter, J. J. Smit, and P. Borst.** 1995. Multidrug resistance and the role of P-glycoprotein knockout mice. Eur. J. Cancer. **31A:**1295–1298.
44. **Schrieber, S. L.** 1992. Immunophilin-sensitive protein phosphatase action in cell signalling pathways. Cell **70:**365–368.
45. **Schwab, J. C., M. A. Affifi, G. Pizzorno, R. E. Handshumacher, and K. A. Joiner.** 1995. *Toxoplasma gondii* tachyzoites possess an unusual plasma membrane adenosine transporter. Mol. Biochem. Parasitol. **70:**59–69.
46. **Silverman, J. A., and K. A. Joiner.** 1997. *Toxoplasma*/host-cell interactions, p. 313–338. *In* S. Kaufmann (ed.), Host response to intracellular pathogens. R. G. Landes Company, Austin, Tex.
47. **Tomavo, S., V. Wyls, and C. Torusel.** 1996. Targeted disruption of P43 gene of *Toxoplasma gondii* reduces attachment to host cells and invasion. *In* meeting abstracts of the *Toxoplasma* Workshop.
48. **Twentyman, P. R.** 1992. Cyclosporins as drug resistance modifiers. Biochem. Pharm. **43:**109–117.
49. **Ullman, B.** 1995. Multidrug resistance and P-glycoproteins in parasitic protozoa. J. Bioenerg. Biomembr. **27:**77–84.
50. **van Helvoort, A., A. J. Smith, H. Sprong, I. Fritzsche, A. H. Schinkel, P. Borst, and G. van Meer.** 1996. MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. Cell **87:**507–517.
51. **VonWartburg, A., and R. Traber.** 1986. Chemistry of the natural cyclosporin metabolites. Prog. Allergy **38:**28–35.
52. **Wenger, R.** 1986. Cyclosporine and analogues: structural requirements for immunosuppressive activity. Transplant. Proc. **18**(Suppl. 5)**:**213–218.
53. **Wenger, R.** Personal communication.
54. **Werk, R., and W. Bommer.** 1980. *Toxoplasma gondii*: membrane properties of active energy-dependent invasion of host cells. Tropenmed. Parasitol. **31:**417–420.