Delivery of antimicrobials into parasites

B. U. Samuel*†, B. Hearn†‡, D. Mack*†, P. Wender‡§¶, J. Rothbard‡§, M. J. Kirisits*, E. Mui*, S. Wernimont*, C. W. Roberts‖, S. P. Muench**, D. W. Rice**, S. T. Prigge††, A. B. Law††, and R. McLeod*¶

*Department of Visual Sciences, University of Chicago, 5841 South Maryland, AMB S-208, Chicago, IL 60637; ‡Department of Chemistry, Stanford University, Stanford, CA 94305; §Cell Gate Inc., 552 Del Rey Avenue, Sunnyvale, CA 94086; ‖Department of Immunology, University of Strathclyde, G4 ONR Glasgow, Scotland; **Department of Molecular Biology, Sheffield University, Firth Court, Western Bank, Sheffield S10 2TN, United Kingdom; and ††Malaria Research Institute, The Johns Hopkins University School of Public Health, Baltimore, MD 21205

Contributed by P. Wender, September 25, 2003

To eliminate apicomplexan parasites, inhibitory compounds must cross host cell, parasitophorous vacuole, and parasite membranes and cyst walls, making delivery challenging. Here, we show that short oligomers of arginine enter *Toxoplasma gondii* tachyzoites and encysted bradyzoites. Triclosan, which inhibits enoyl-ACP reductase (ENR), conjugated to arginine oligomers enters extracellular tachyzoites, host cells, tachyzoites inside parasitophorous vacuoles within host cells, extracellular bradyzoites, and bradyzoites within cysts. We identify, clone, and sequence *T. gondii enr* and produce and characterize enzymatically active, recombinant ENR. This enzyme has the requisite amino acids to bind triclosan. Triclosan released after conjugation to octaarginine via a readily hydrolyzable ester linkage inhibits ENR activity, tachyzoites *in vitro*, and tachyzoites in mice. Delivery of an inhibitor to a microorganism via conjugation to octaarginine provides an approach to transporting antimicrobials and other small molecules to sequestered parasites, a model system to characterize transport across multiple membrane barriers and structures, a widely applicable paradigm for treatment of active and encysted apicomplexan and other infections, and a generic proof of principle for a mechanism of medicine delivery.

The vast majority of interesting small molecules will never be used for therapeutics because they cannot traverse biologic barriers to reach their targets. A special challenge for the development of antimicrobials effective against apicomplexan parasites is delivery. Effective agents have to gain entry to host cells, cross the parasitophorous vacuole, and enter parasites and their specialized organelles. This is potentially more difficult with *Toxoplasma gondii* bradyzoites, which reside in cysts composed partly of host and partly of parasite constituents. The studies described herein were performed to determine whether short oligomers of arginine (Fig. 1*A*) could provide a solution to these challenges. Arginine oligomers linked to immunomodulatory and antitumor compounds can actively transport such compounds across a variety of epithelial barriers, including skin, lung, and eye (1–4). Oligoarginine-medicine conjugates have been administered safely to experimental animals and humans (ref. 1 and unpublished data).

Better approaches for treating apicomplexan parasite infections (e.g., toxoplasmosis and malaria) are needed. For example, *T. gondii* bradyzoites infect 30–50% of people throughout the world, but no currently used medicines eradicate this slowly growing, encysted, latent parasite that causes chronic, lifelong infections in brain and eye with recrudescence causing severe illness (5). Available antimicrobial agents to eliminate rapidly growing *T. gondii* tachyzoites, which cause tissue destruction during active infection, are limited by toxicity and allergy (5).

Enoyl-ACP reductase (ENR) is a key enzyme active in fatty acid synthesis (6). Bacterial and apicomplexan fatty acid synthesis occurs by the type II pathway (6–10) (Fig. 5, which is published as supporting information on the PNAS web site) and depends on monofunctional polypeptides, including ENR. In contrast, mammalian fatty acid synthesis occurs by the type I pathway in which the key enzymes are present on a polyfunctional single polypeptide (6–10). In apicomplexans, type II fatty acid synthesis enzymes are in a plastid organelle (6, 8).

Some monofunctional type II enzymes had been identified in *Plasmodia* and *T. gondii* (6–10), but *T. gondii* ENR (TgENR) had not been identified. The antimicrobial agent 5-chloro-2-[2,4-dichlorophenoxy]phenol (triclosan) had been found to inhibit bacterial ENRs (11). We (6) and Surolia and Surolia (9) had found that μM concentrations of triclosan dissolved in DMSO inhibit replication of apicomplexan parasites, presumably by binding to, and inhibiting, apicomplexan ENR. Triclosan was active only when solubilized in DMSO (6, 9). Structures of triclosan, oligomers, and conjugates of oligoarginines are shown in Fig. 1 (see also Fig. 5).

Materials and Methods

Short Arginine Oligomers, Conjugates, and Controls. Peptides were synthesized by using solid-phase techniques and commercially available fluorenylmethoxycarbonyl amino acids, resins, and reagents with an Applied Biosystems 433 peptide synthesizer. Fastmoc cycles were used with *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU). Peptides and conjugates were cleaved from resin by using 95% trifluoroacetic acid and 5% triisopropylsilane for 24 h. Peptides were subsequently filtered from resin, precipitated with diethyl ether, purified by using HPLC reverse-phase columns, and characterized by using ^{1}H NMR and electrospray MS.

For synthesis of triclosan-Orn(FITC)-Arg$_n$-CONH$_2$, **4** and **5**, triclosan was reacted with α-bromoacetic acid to provide the desired α-substituted acetic acid product. Attachment of the resin-bound carrier was accomplished by reaction of this acetic acid product with the appropriate oligomer of L-arginine(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, Pbf) containing an N-terminal L-ornithine(methyltrityl). After cleavage of the side-chain methyltrityl protecting group with 1% trifluoroacetic acid in dichloromethane, the conjugates were labeled with FITC in the presence of diisopropylethylamine (DIPEA). The conjugates were then deprotected and cleaved from the resin by using the previously described protocol. For the synthesis of triclosan-Orn(tetramethylrhodamine isothiocyanate)-Arg$_8$-CONH$_2$, **ent-6**, tetramethylrhodamine isothiocyanate was reacted in the solution phase with triclosan-D-Orn-D-Arg$_8$-CONH$_2$ in the presence of DIPEA. Triclosan was reacted with glutaric anhydride (GA) in the presence of DIPEA to provide the desired glutaric acid product, i.e., triclosan-GA-Arg$_n$-CONH$_2$, **7** and **8**. Attachment of the carrier was accomplished by the solution-phase reaction with the appropriate oligomer of D- or L-arginine. For the synthesis of triclosan-acetic

Abbreviations: ENR, enoyl-ACP reductase; TgENR, *Toxoplasma gondii* ENR; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY372520).

†B.U.S., B.H., and D.M. contributed equally to this work.

¶To whom correspondence should be addressed. E-mail: wenderp@leland.stanford.edu or rmcleod@midway.uchicago.edu.

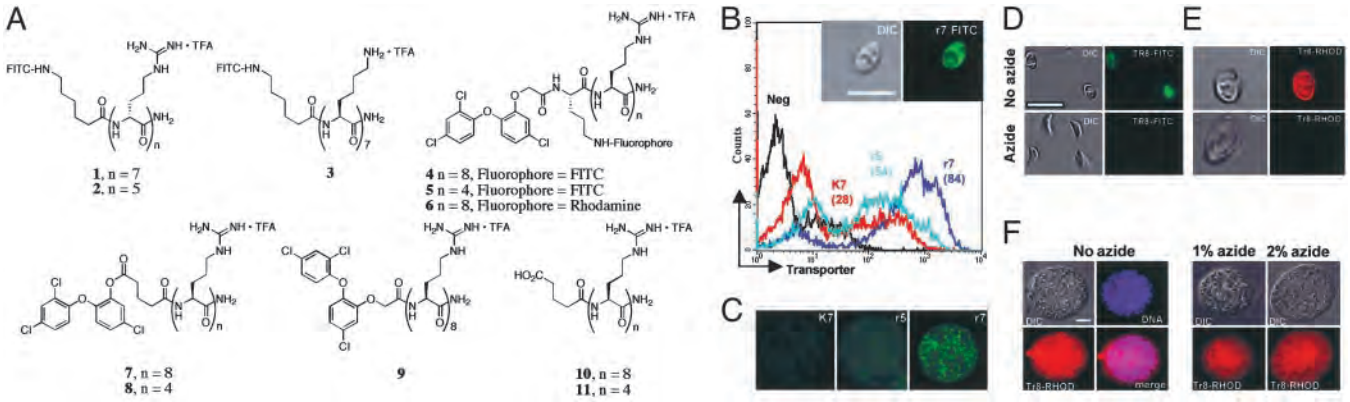

Fig. 1. Schematic representations of oligomers and conjugates, uptake of short oligomers of arginine and lysine by *T. gondii* tachyzoites, encysted bradyzoites, and effect of azide on uptake of arginine triclosan conjugates by parasites. (*A*) Structures of short oligomers of arginine and conjugates used in these studies. Releasable conjugates use readily hydrolyzable ester linkages for the attachment of triclosan to the oligoarginine carrier (structures **7** and **8**). Nonreleasable triclosan conjugates use nonhydrolyzable ether bonds for the attachment of triclosan (structures **4**, **5**, **6**, and **9**). Control compounds **10** and **11** are the hydrolysis products of releasable conjugates **7** and **8**, respectively. Ent designates the dextro isomer. Synthetic details are in *Materials and Methods*. (*B*) Comparative flow cytometry to measure labeling of *T. gondii* tachyzoites incubated with FITC-conjugated polyamino acid compounds. Numbers in parentheses indicate percentage of positive cells. (*Inset*) Fluorescence and differential interference contrast (DIC) microscopy assays of uptake of r7 FITC, compound **1**, by *T. gondii* tachyzoites. (*C*) Fluorescence images showing uptake of FITC-conjugated polyamino acid compounds by *T. gondii* tissue cysts. (Scale bar = 10 μm.) (*D–F*) Fluorescein and DIC microscopy images showing inhibition of uptake of arginine triclosan conjugates by azide in *T. gondii*. (*D*) Tachyzoites. (*E*) Excysted bradyzoites. (*F*) Uptake of r8 triclosan (compound **7**) and less inhibition in isolated brain cysts. The arginine carrier was conjugated to fluorophore and triclosan in a nonreleasable manner (**4** and **ent-6**). (Scale bar = 10 μm.)

acid-Arg_8-$CONH_2$, **9**, the previously described α-substituted acetic acid product was attached to the carrier by reaction with the resin-bound octa-L-arginine(Pbf). The conjugate was subsequently cleaved from the resin, and the Pbf sulfonamides were removed. For the synthesis of GA-Arg_n-$CONH_2$, **10** and **11**, the resin-bound oligomer of L-arginine(Pbf) was reacted with GA in the presence of DIPEA, followed by cleavage of the resin and Pbf deprotection.

The half-life of conjugate **7** decomposition in PBS was determined by using HPLC to monitor the change in the ratio of conjugate-to-internal standards (benzoic acid) over time.

***T. gondii* Parasites.** Tachyzoites of the RH strain were maintained and used in experiments as described (12, 13). GFP-, plastid-labeled RH strain parasites were kindly provided by B. Streipen and D. Roos (University of Pennsylvania, Philadelphia). Production and isolation of *T. gondii* cysts were as described (14).

Studies on the Uptake of Short Arginine and Lysine Oligomers into *T. gondii*. Live cells incubated with conjugates were examined by using a Zeiss Axiovert inverted fluorescence microscope or Zeiss Axioplan equipped with cooled charge-coupled device camera. For flow cytometry, parasites and cells were incubated in PBS/2% FCS containing 12.5 μM of each oligomer at 18°C for 10 min, washed in cold PBS three times, resuspended in PBS, and then analyzed by flow cytometry with a FACScan and CELL QUEST software. Before analysis, intracellular parasites were exposed to 12.5 μM conjugate for 10 or 30 min.

Treatment with Azide. Parasites were preincubated for 30 min with 0.5% azide (tachyzoites, bradyzoites) or 0.5–2% azide (cysts), all in 2% FCS/PBS buffer, before the addition of 12.5 μM conjugate.

Cloning and Sequencing of TgENR. A cDNA library was screened to identify and characterize the TgENR gene as described (15, 16) (see *Supporting Text*, which is published as supporting information on the PNAS web site).

Expression and Purification of Recombinant TgENR. A construct of TgENR containing residues 103–417 (and lacking the putative signal and transit peptides) was designed for *in vivo* cleavage by tobacco etch virus (TEV) protease. Amplified DNA encoding residues 103–417 of TgENR was ligated into a modified version of the pMALc2x vector (pMALcHT) in which the linker region was altered to contain nucleotides encoding a TEV protease cleavage site followed by a six-histidine tag (17). The resulting ligation product, pSTP8, was transformed into BL21 Star (DE3) cells (Invitrogen). These cells were cotransformed with the pRIL plasmid from BL21-CodonPlus (DE3) cells (Stratagene) and a plasmid (pKM586) encoding the TEV protease (18). Cells were grown, harvested, and lysed as described (17). TgENR was purified by using metal chelate and anion exchange chromatography (see Fig. 3*B*).

Effect of r8-Triclosan on Recombinant TgENR. The activity of TgENR was assayed by using crotonyl-CoA as a substrate and monitoring the consumption of the NADH cofactor. Inhibition by r8-triclosan over a 24-h period was measured by incubating TgENR at 37°C with different concentrations of r8-triclosan. Aliquots were removed at 0, 12, and 24 h and assayed for TgENR activity. Briefly, 10 μl of 1 mM NADH and 10 μl of 1 mM crotonyl-CoA were added to 80 μl of incubated TgENR/r8-triclosan, and the reaction was followed for 120 s in a Beckman DU-640 spectrophotometer. Final concentration of TgENR was 11.6 nM, and the final concentrations of r8-triclosan were 2 μM, 0.4 μM, 80 nM, 16 nM, 3.2 nM, 640 pM, and 128 pM. TgENR was inhibited by r8-triclosan with an IC_{50} value >1 μM at 0 h, an IC_{50} value = 40 nM ± 10 nM at 12 h, and an IC_{50} value <16 nM at 24 h (see Fig. 3*C*). The IC_{50} value at 24 h is an upper limit because inhibition cannot be properly measured near the concentration of TgENR used in these assays (11.6 nM).

Assays to Assess Inhibition of *T. gondii* Tachyzoite Growth and Effect of Antimicrobial Agents on Host Cells *in Vitro*. These studies were performed as described (12, 13).

Effect of r8-Triclosan on Tachyzoites *in Vivo*. One thousand RH strain parasites were inoculated i.p. into five female SW mice

(each ≈25 g and between 2 and 4 months of age). Lypholized releasable r8-triclosan or triclosan were resuspended in Dulbecco's PBS. Forty milligrams per kilogram of r8-triclosan or control PBS was inoculated i.p. each day. On the fourth day, 1.5 ml of PBS (pH 7.4) was inoculated. All available peritoneal fluid plus PBS was withdrawn on the fourth day. Total numbers of parasites and concentrations of parasites were quantitated microscopically.

Analysis of Data and Statistics. Statistical analysis was with Student's *t* test, χ^2 analyses, one-way ANOVA, or Tukey's test.

Detailed Methods. See *Supporting Text* for more details on materials and methods used.

Results and Discussion

Initially, uptake of fluoresceinated hepta-D-arginine (r7, compound **1**, Fig. 1*A*) and control compounds penta-D-arginine (r5, compound **2**, Fig. 1*A*) and hepta-L-lysine (K7, compound **3**, Fig. 1*A*) into extracellular and intracellular tachyzoites, and extracellular and encysted bradyzoites, was studied by using deconvolution fluorescence microscopy and flow cytometry (Fig. 1 *B–F*). R designates levo isomer, r designates dextro isomer, and the number after r indicates the number of arginines in the compound. Short arginine oligomers were found to cross all membranes and to enter each life cycle stage and compartment (Fig. 1 *B–F*). Deconvolution fluorescence microscopy revealed that r7 facilitated uptake of fluoresceinated compounds into tachyzoites (Fig. 1*B Inset*). Flow cytometric analysis (Fig. 1*B*) demonstrated that uptake of r7 was greater than that of r5 or K7 (seven arginines ≫ five arginines or seven lysines). This finding was consistent with earlier studies demonstrating that longer oligomers of arginines (seven or eight) entered certain eukaryotic cells better than shorter oligomers (one to four). Deconvolution microscopy demonstrated that short arginine oligomers could cross cyst walls and enter encysted bradyzoites (Fig. 1*C* and Movie 1, which is published as supporting information on the PNAS web site). Uptake of short arginine oligomers into tachyzoites and bradyzoites was very rapid (seconds), and in bradyzoites, the fluoresceinated compound moved from the cytoplasm to the nucleus over the first day (data not shown).

Floresceinated, nonreleasable triclosan R8, conjugate **4**, then was studied by microscopic analyses. The conjugated triclosan was taken up by extracellular tachyzoites and bradyzoites within cysts ≈10 s after addition (Fig. 1 *D–F*). Treatment with azide blocked uptake of short arginine oligomers linked to triclosan by tachyzoites (Fig. 1*D*) and bradyzoites (Fig. 1*E*) but did not completely inhibit uptake into encysted bradyzoites under the conditions studied (Fig. 1*F*). Thus, uptake into tachyzoites and bradyzoites is a facilitated process. Uptake of fluoresceinated triclosan R8 (conjugate **4**) into tachyzoites was substantially greater (one and a half logs) than triclosan R4 (conjugate **5**), after incubation for 10 s, 1 min, and 10 min. Uptake of triclosan R8 increased over time (data not shown). As uptake into isolated bradyzoites was blocked by azide, the inability of azide to completely abrogate uptake by encysted bradyzoites is surprising and suggested that more than a single mechanism of uptake might be involved in uptake across the cyst wall.

Fluorescein-tagged conjugate **4** and its enantiomer both entered host fibroblasts. *T. gondii* is an obligate intracellular parasite and resides inside a specialized vacuolar compartment known as the parasitophorous vacuole delimited by the parasitophorous vacuole membrane. To determine whether the carrier conjugated to triclosan can enter this specialized compartment, tachyzoites within a vacuole in human fibroblasts were studied. Intracellular tachyzoites took up fluoresceinated r8-triclosan (conjugate **ent-4**) within minutes (Fig. 2*A*). This finding was also confirmed by flow cytometric analysis (Fig. 2*B*).

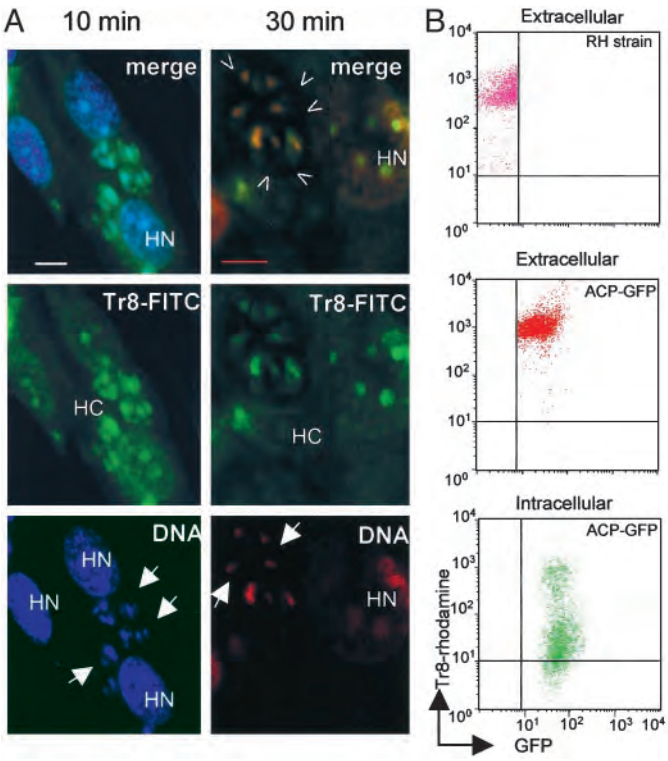

Fig. 2. Intracellular and extracellular tachyzoites take up triclosan conjugated to r8. (*A*) Fluorescence images of live intracellular tachyzoites after 10 or 30 min of exposure to r8-triclosan conjugate (nonreleasable conjugate **4**). Parasite nuclei (arrows) and host cell nuclei (HN) were stained with Hoechst. FITC signal is seen in host cell cytoplasm (HC) and nuclei (HN), and in parasites residing in parasitophorous vacuole (v). (Scale bar = 10 μm.) (*B*) Uptake of triclosan r8 conjugate (nonreleasable conjugate **4**) by extracellular and intracellular tachyzoites. Flow cytometric analysis showing representative dot blots of RH strain (pink) and transgenic parasites expressing the GFP (ACP-GFP, extracellular, red; intracellular, green) exposed to r8-triclosan conjugated to rhodamine (**ent-6**) for 10 min. Intracellular parasites were exposed to the carrier and then released from the host cells before analyses.

Previously, the only apicomplexan ENR identified was that of *Plasmodium falciparum*. We (17) and others (19) have cloned, sequenced, and solved the structure of *P. falciparum* ENR. It was of interest to compare *P. falciparum* ENR and TgENR for the present study, particularly with reference to their triclosan binding sites. Portions of TgENR were identified in a database, full-length cDNA clones were isolated and sequenced, deduced amino acid sequences were determined, and enzymatically active recombinant protein was produced (Fig. 3). Analysis of the deduced amino acid sequence revealed that key amino acid residues are conserved between *T. gondii* (Fig. 3*A*, GenBank accession no. AY372520), *P. falciparum*, *Brassica napus*, and bacterial ENRs and that 11 amino acid residues involved in triclosan binding are also conserved (Fig. 3*A*). This result suggests that triclosan is likely to have a similar mode of inhibition in *T. gondii* as in other species. In keeping with a plastid localization of this enzyme in other apicomplexans, a bi-partite transit sequence consisting of a cleavable von Heijne secretory signal (the first 27 aa) followed by a chloroplast-like targeting sequence (amino acid numbers 28–66) were identified. Alignment with the genomic DNA sequence in the database indicated that there are four introns. In analyzing the amino acid sequence of *P. falciparum* ENR, several amino acid insertions were found. *B. napus* ENR had inserts corresponding to two of those from *P. falciparum* ENR. The sequence of TgENR has multiple amino acid sequences that coincide with those inserts

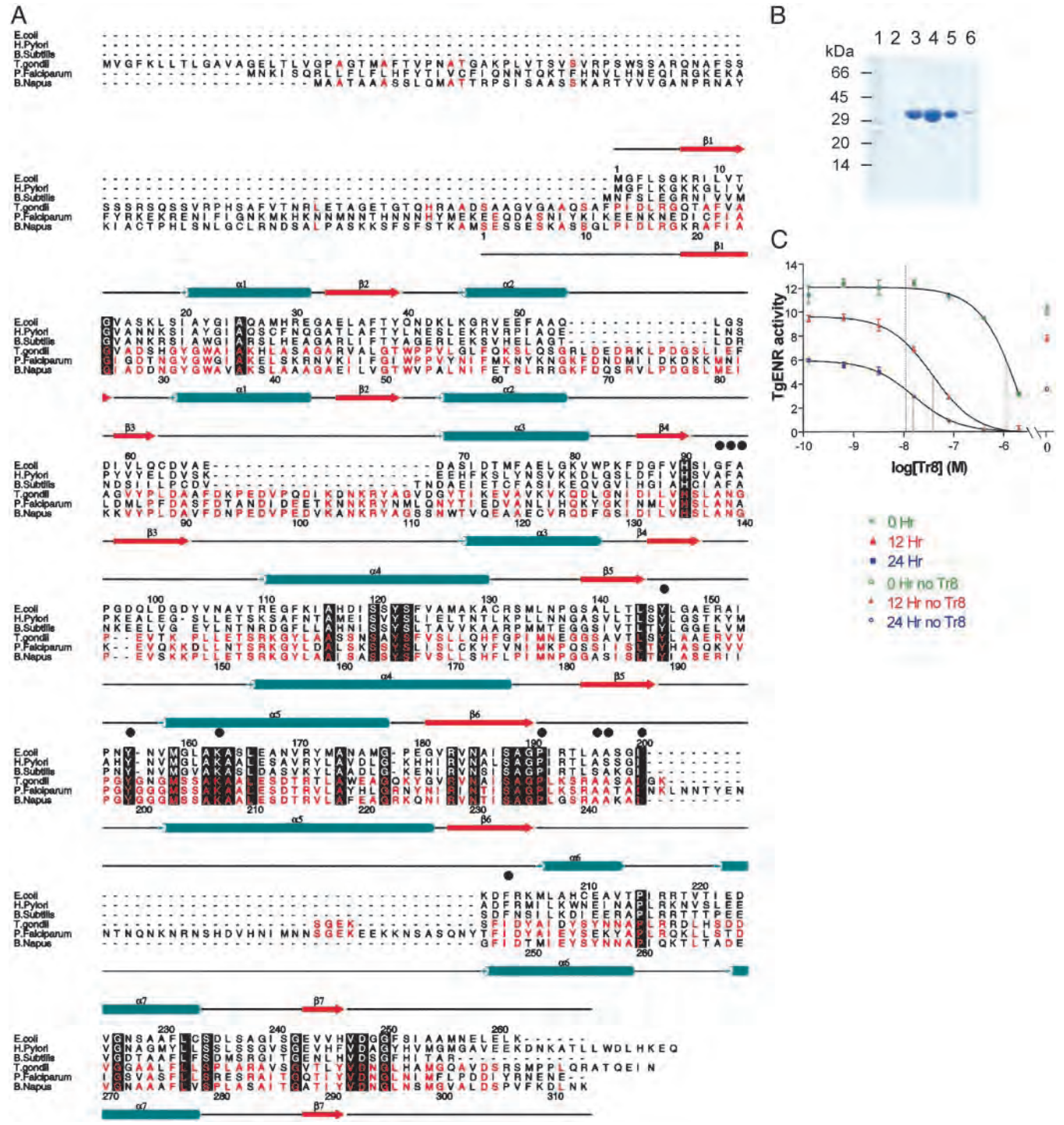

Fig. 3. TgENR: Deduced amino acid sequence and structure, purification of recombinant protein, and kinetics of enzyme activity in the presence of r8 releasable conjugate. (*A*) Multiple structure-based sequence alignment showing deduced amino acid sequence of TgENR and enoyl reductases of *Escherichia coli*, *Helicobacter pylori*, *Bacillus subtilis*, *P. falciparum*, and *B. napus*. This sequence was confirmed by isolation and sequencing of five separate clones from an RH strain *T. gondii* library kindly provided by J. Ajioka (Cambridge University, Cambridge, U.K.). The secondary structures and sequence numbers of the *E. coli* and *B. napus* enzymes are shown above and below the alignment, respectively. The residues that are completely conserved are in reverse type, and those involved in triclosan binding are indicated by ●. The residues in red are conserved between *B. napus* ENR, *P. falciparum* ENR, and TgENR. Red arrows indicate β-sheets, and blue-green cylinders indicate α-helices. (*B*) SDS/PAGE of TgENR purification. SigmaMarker (Sigma) wide-range molecular weight markers are shown in lane 1. Lanes 2–6 show fractions eluted from the HiTrap Q Fast Flow column containing pure TgENR. (*C*) Time course of TgENR inhibition by releasable r8-triclosan conjugate (Tr8), compound **7**. Inhibition curves were measured at 0 h (■), 12 h (▲), and 24 h (●) of incubation at 37°C. The three open symbols to the right show TgENR activity at these time points with no added inhibitor. Enzyme activity is expressed as turnovers per TgENR molecule per s, and inhibitor concentration is expressed as the log of the Tr8 concentration in molar units. IC_{50} values calculated by nonlinear regression analysis are displayed graphically as dashed vertical lines for the three inhibition curves, showing an increase of inhibitory activity with time. A black vertical dashed line marks the concentration of TgENR used in these assays and thus represents a value below which inhibition cannot be properly measured in these assays. Error bars show the variation between triplicate measurements at each point. When not visible, the variation is smaller than the symbol.

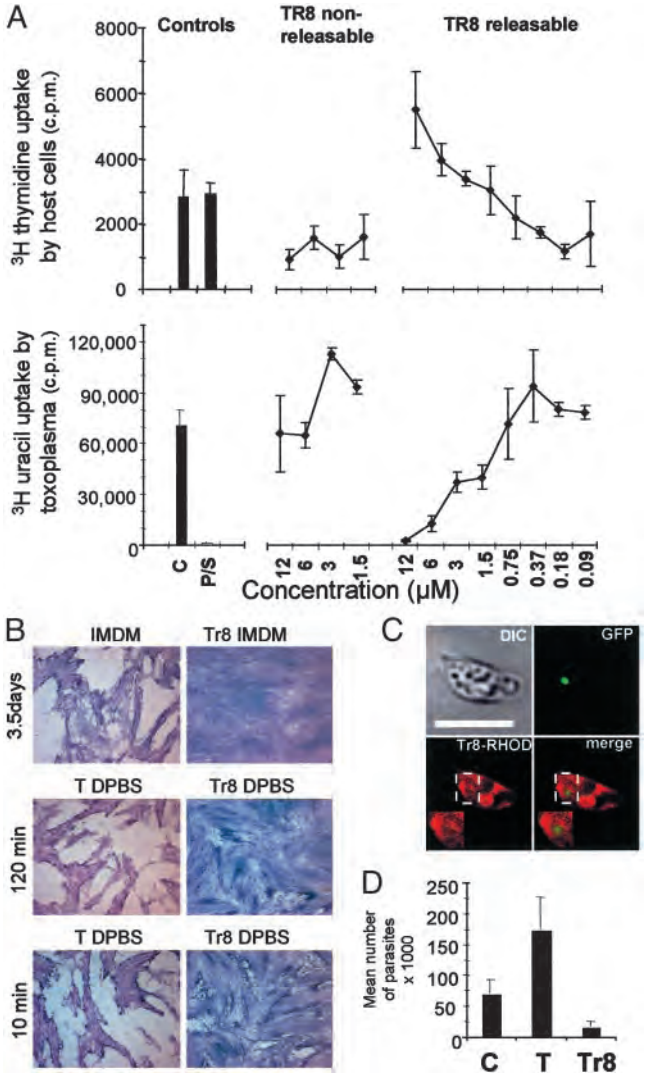

Fig. 4. *In vitro* and *in vivo* effects of octaarginine triclosan conjugates on *T. gondii* growth and subcellular distribution of rhodamine-conjugated octaarginine triclosan. (*A*) R8 conjugated to triclosan, either as a nonreleasable or releasable compound, had little or no toxic effect on replication of host cells as measured by [^{3}H]thymidine uptake. Only releasable R8 triclosan conjugate had an inhibitory effect on parasite growth, as measured by [^{3}H]uracil incorporation. Controls included *T. gondii*-infected host cells in medium (C) or treated with pyrimethamine (0.1 μg/ml) and sulfadiazine (25 μg/ml) (P/S), which inhibit parasite growth. (*B*) Brief exposures of *T. gondii* to releasable r8-triclosan (Tr8) but not triclosan (T) in Dulbecco's PBS had a small inhibitory effect on *T. gondii*. Two hours after infection of host cells with tachyzoites, cultures were incubated with T or Tr8 (12.5 μM) either in PBS or growth medium (Iscove's modified Dulbecco's medium–FCS). For the PBS-exposed cultures (not controls), T or Tr8 PBS then were replaced at varying intervals with Iscove's modified Dulbecco's medium–FCS, which was present for the remainder of the 3.5-day culture period. Parasite growth also was determined by [^{3}H]uracil uptake at the end of 3.5 days of culture in growth medium (data not shown). Brief exposures to Tr8 partially inhibited parasite growth and the 3.5-day exposure (or a 48-h exposure, data not shown) inhibited parasite growth almost completely. Unconjugated triclosan had no effect. The photomicrographs (Giemsa stain) on the right show the ''protective'' effect of Tr8, but not T, in PBS on the infected host cell monolayer. The large empty areas represent host cell destruction by intracellular parasite growth, which is inhibited by r8-conjugated triclosan and not by the triclosan in PBS. (*C*) Subcellular distribution of r8-triclosan conjugate in tachyzoites was determined by deconvolution microscopy using nonreleasable r8-triclosan conjugated to rhodamine, and transgenic parasites expressing the GFP targeted to the parasite plastid organelle. Fluorescence and differential interference contrast (DIC) images showing rhodamine signal surrounds the GFP signal, partly overlapping it. (*Inset*) Higher magnification of the boxed area showing rhodamine signal partly overlapping plastid. (Scale bar = 10 μm.) (*D*) *In vivo* administration of releasable r8-triclosan, **ent-7**, but not triclosan in PBS, reduces numbers of i.p. tachyzoites 5 days after challenge ($P = 0.006$).

present in both *P. falciparum* and *B. napus* ENRs. *P. falciparum* ENR also contained a third polar insert that had no counterpart in other species. Analysis of the TgENR sequence indicates only partial conservation of this larger malarial insert. The function(s) and origins of these inserts are unknown.

The predicted ENR structure suggested a strategy whereby octaarginine could be conjugated to triclosan both in a releasable manner, exposing a key part of the inhibitor essential in the binding of triclosan to ENR only upon its release, and in a nonreleasable manner, preventing access to the critical site where a hydroxyl in triclosan binds apicomplexan ENR (Figs. 1 and 5). Triclosan's insolubility in aqueous media, unless prepared with DMSO or ethanol, limits its clinical use, but provided us with an opportunity to compare efficacy against *T. gondii* of triclosan alone and triclosan delivered by conjugation to octaarginine.

We then studied effects of triclosan conjugated to short oligomers of arginine on activity of recombinant ENR (Fig. 3 *B* and *C*) and on intracellular *T. gondii* (Fig. 4 *A* and *B*). For these studies, a releasable conjugate of triclosan, compound **7**, was synthesized. Attachment of triclosan to octaarginine was achieved by readily hydrolyzable ester bonds so that the triclosan cargo could be released to bind to TgENR and thus to inhibit the parasite.

Inhibition of enzyme activity paralleled kinetics of release of the conjugated triclosan (Fig. 3). Releasable triclosan octaarginine (conjugates **7** and **8**) were effective in inhibiting *T. gondii* tachyzoites (Fig. 4), as was triclosan but only when dissolved in DMSO (data not shown). Neither nonreleasable triclosan R8 (conjugate **9**) nor octaarginine alone was active, indicating the specificity of triclosan (Fig. 4).

As the nonreleasable triclosan R8 (conjugate **9**) was without effect, triclosan must be released from R8 to be active (Fig. 1). It is not sufficient just to make triclosan soluble, and R8 (compound **10**) and R4 (compound **11**) had no effect on *T. gondii* alone.

A kinetic analysis of the pharmacologic effect of the conjugate ($T_{1/2} = 12$ h) revealed that some activity could be detected after short exposure: For example, after a brief (10 min to 2 h) exposure there was a modest effect of r8-triclosan (conjugate **ent-7**) in PBS, but not triclosan in PBS, when uptake of [^{3}H]uracil by *Toxoplasma* tachyzoites was measured in the last 18 h of 3.5 days of culture (data not shown). Two hours was substantially less than the time required for 50% dissociation of r8-triclosan (conjugate **ent-7**) (half-life of dissociation in PBS = 12 h).

Exposure throughout the first 48 h was essential, however, for full efficacy (i.e., IC_{90}) of r8-triclosan (compound **ent-7**) in medium and serum (Fig. 4*B*). This finding is consonant with the ''delayed death phenotype'' seen for antimicrobial agents, such as clindamycin and azithromycin, believed to effect plastid-associated processes.

To be active against the parasite, triclosan must be targeted to, and released in the vicinity of, or within, the plastid where enzymes of apicomplexan fatty acid synthesis are targeted and active (6–10). Because short oligomers of arginine alone and linked to triclosan rapidly entered the parasite cytoplasm and nucleus, we wanted to determine whether short oligomers of arginine linked to triclosan also were in close proximity to or entered the plastid, as the delayed death phenotype of triclosan suggested that they were likely to act on a plastid associated process. Also, analysis of TgENR suggested that the plastid was a likely site of action of this inhibitor of ENR. To determine

whether short oligomers of arginine delivered triclosan to the vicinity of the plastid, a rhodamine-conjugated r8 linked to triclosan (**ent-6**) was synthesized to facilitate colocalization studies of r8 conjugate, and a parasite in which the plastid was labeled with GFP was examined. These studies showed a dense ring of rhodamine signal at the perimeter of the plastid that colocalized with the outer portion of the plastid with a much less intense signal in the center of the plastid (Fig. 4*C*).

As triclosan conjugate **7** was effective *in vitro*, studies were performed to determine the effect of this triclosan conjugate *in vivo*. For these studies, we developed a model in which tachyzoites of the RH strain were inoculated i.p. into mice. Mice were treated i.p. with r8-triclosan (conjugate **ent-7**) or triclosan (suspended in PBS) or PBS daily for 4 days beginning on the day they were infected. The numbers of parasites present in the peritoneal cavity were determined on the fifth day, after the 4 days of treatment. To develop and test this assay, initially, sulfadiazine was given to mice in their drinking water in the 4 days after infection. Sulfadiazine (data not shown) and r8-triclosan (conjugate **ent-7**), but not triclosan in PBS, reduced subsequent parasite burden ($P < 0.006$) (Fig. 4*D*).

Our studies described herein demonstrate that short oligomers of arginine can transport inhibitory compounds into an apicomplexan parasite, including extracellular *T. gondii* tachyzoites and bradyzoites, intracellular tachyzoites, and encysted bradyzoites. The peptide carrier can deliver to and release lead inhibitory compounds inside tachyzoites that are multiplying within parasitophorous vacuoles, to their cytoplasm and subcellular organelles such as the apicomplexan nucleus and the perimeter of the plastid, where they are efficacious. Furthermore, octaarginine can deliver compounds into bradyzoites in cysts and tachyzoites *in vivo*. Oligoarginine antimicrobial conjugates provide a way to eliminate *T. gondii* tachyzoites and deliver antimicrobial agents to intracellular *T. gondii* tachyzoites and bradyzoites within cysts.

It is noteworthy that after crossing the host cell external membrane, octaarginine can cross each of the obstacles the parasite has specified to create sequestered, protected, intracellular or intracyst niches. Most notably, the carrier with its antimicrobial cargo crosses the walls of the cysts that contain untreatable bradyzoite forms of the parasite. No currently used antimicrobial compound previously has been shown to cross the cyst wall.

We selected, designed, and developed a method of delivery as a generic proof of principle, showing the ability of a carrier to deliver small molecules to microbes that are sequestered by multiple membranes and structures. This proof of principle is based on discoveries reported herein of a promising enzyme target for antimicrobials (TgENR), production and purification of recombinant TgENR, demonstration and characterization of TgENR activity, and kinetics of its inhibition by triclosan that parallels kinetics of the release of triclosan from the octaarginine carrier. Deductions concerning the structure of this promising enzyme target are then used to design an approach for testing efficacy and toxicity of releasable octaarginine inhibitor conjugates (Fig. 4). Identification and characterization of this enzyme in *T. gondii* provides useful information for further development of unique classes of antimicrobial agents effective against apicomplexans. It was not obvious or certain *a priori* that *T. gondii* would have this enzyme as certain organisms, e.g., the *pneumococcus*, that use type II fatty acid synthesis do not have an ENR. As a proof of concept, our observations show the potential for delivery, with efficacy and without toxicity, of an inhibitor of ENR by a releasable carrier *in vitro* and *in vivo* (Figs. 3 and 4).

We demonstrate a mechanism of medicine delivery to an important pathogen target enzyme. Octaarginine delivers a "prodrug" processed by the organism to an enzyme that is essential for pathogen survival. A number of inhibitors of ENR described so far are inherently poorly soluble in part because they reflect the lipophilic nature of the enzyme substrate. One clear advantage of the delivery system is that the specific solubilizing agent attaches a strongly hydrophilic functionality to the drug, which gives rise to freely soluble compounds.

As short oligomers of arginine are being optimized for use in delivery across skin, eye, and blood-brain barrier, results described herein provide potential for significantly improving treatment of apicomplexan, and perhaps other, infections. Future characterization of the mechanisms whereby the parasite cyst wall and multiple host and parasite membranes (of prokaryotic and eukaryotic lineage) are traversed by short arginine oligomers to deliver its antimicrobial agent cargo will be of considerable interest.

We thank B. Streipen and D. Roos for providing ACP-GFP-transfected tachyzoites, the RITA Corporation for donating triclosan, S. Bond in the Digital Light Microscopy Facility at the University of Chicago, and M. Sing, E. Castro, and L. Kallal for their assistance with preparation of this manuscript. This work was supported by the National Institutes of Health National Institute of Allergy and Infectious Diseases TMP program (Grant R01 AI43228), The Research to Prevent Blindness Foundation, National Institutes of Health Grants CA 31841 and 31845, gifts from the Keiweit, Blackmon, Brennan, Koshland, and Langel families, the Biotechnology and Biological Sciences Research Council, and The Wellcome Trust. R.M. is the Jules and Doris Stein RPB Professor at the University of Chicago. B.H. was the recipient of a National Institutes of Health postdoctoral fellowship.

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