Molecular target validation, antimicrobial delivery, and potential treatment of *Toxoplasma gondii* infections

Bo-Shiun Lai, William H. Witola[1], Kamal El Bissati, Ying Zhou, Ernest Mui, Alina Fomovska, and Rima McLeod[2]

Department of Surgery, University of Chicago, Chicago, IL 60637

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Toxoplasma gondii **persistently infects over two billion people worldwide. It can cause substantial morbidity and mortality. Existing treatments have associated toxicities and hypersensitivity and do not eliminate encysted bradyzoites that recrudesce. New, improved medicines are needed. Transductive peptides carry small molecule cargos across multiple membranes to enter intracellular tachyzoites and encysted bradyzoites. They also carry cargos into retina when applied topically to eyes, and cross blood brain barrier when administered intravenously. Phosphorodiamidate morpholino oligomers (PMO) inhibit gene expression in a sequence-specific manner. Herein, effect of transductive peptide conjugated PMO (PPMO) on tachyzoite protein expression and replication in vitro and in vivo was studied. Initially, sequence-specific PPMO successfully reduced transfected *T. gondii*'s fluorescence and luminescence. PPMO directed against *T. gondii*'s dihydrofolate reductase (DHFR), an enzyme necessary for folate synthesis, limited tachyzoite replication. Rescue with exogenous folate demonstrated DHFR PPMO's specificity. PPMO directed against enoyl-ACP reductase (ENR), an enzyme of type II fatty acid synthesis that is structurally distinct in *T. gondii* from ENR in mammalian cells was investigated. PPMO directed against plant-like Apetela 2 (AP2) domain transcription factor XI-3 (AP2XI-3), not present in human cells, was characterized. ENR and AP2XI-3 PPMO each restricted intracellular parasite replication validating these molecular targets in tachyzoites. DHFR-specific PPMO administered to infected mice diminished parasite burden. Thus, these antisense oligomers are a versatile approach to validate *T. gondii* molecular targets, reduce essential *T. gondii* proteins in vitro and in vivo, and have potential for development as curative medicines.**

protozoan | Apicomplexan | toxoplasmosis | protein translation

The Apicomplexan parasite *Toxoplasma gondii* infects approximately one-third of the world's human population. These parasites persist throughout a person's life. Disease caused by *T. gondii*, called toxoplasmosis, occurs in some infected persons. When a pregnant woman acquires this infection for the first time during gestation, *T. gondii* can be transmitted to her fetus. Then, fetal ocular or brain damage, or even death, may occur. Recrudescence of persistent, encysted bradyzoites also can cause disease. Recrudescent or new infection in immune-compromised persons may be life-threatening.

Pyrimethamine and sulfadiazine are the gold-standard medicines used to treat toxoplasmosis. Although highly effective against tachyzoites, these medicines may cause hematological side effects, other toxicities, and hypersensitivity. Furthermore, no medicines are effective in eliminating *T. gondii* encysted bradyzoites. To cure *T. gondii* infections, anti-*Toxoplasma* medicines must cross the placenta, enter the retina, and traverse the blood-brain barrier as well as cyst walls and bradyzoite membranes. The medicines also must cross the host cell membrane, the parasitophorous vacuole, and tachyzoite membranes. Medicine development can be complex, costly, and time-consuming, with pitfalls arising along the way with target validation, off-target effects and suboptimal or adverse ADMET (absorption, metabolism, distribution, excretion, toxicity) properties for lead candidate compounds. Improved medicines with significantly less toxicity, greater efficacy against tachyzoites and encysted bradyzoites, and access to infected tissues are urgently needed. A rapid and direct antisense system for target validation would also be useful.

Phosphorodiamidate morpholino oligomers (PMO) (Fig. 1) are an antisense knockdown approach that disrupts mRNA translation. PMO differ from RNA molecules structurally: PMO have deoxyribonucleic instead of ribonucleic bases. Because they inhibit their targets through Watson/Crick base-pairing mechanisms, PMO knockdown is a form of reverse genetic inhibition. Most importantly, the phosphorodiamidate groups on morpholino oligomers are neutral and hydrophilic, making PMO highly stable and water-soluble inhibitors (1). Because they contain stable phosphate backbones, PMO can be stored at room temperature without degradation. Furthermore, PMO have six-member instead of five-member rings; they also have nitrogen atoms, which are absent in RNA molecules (1). PMO can be administered intravenously, intramuscularly, and intranasally to effectively restrict their targets. They also are nontoxic and nonimmunogenic.

Transductive peptides attached to the 3′ ends of PMO allow the oligomers to readily enter mammalian cells and microorganisms by crossing multiple membrane barriers. A transductive peptide has been shown to facilitate entry of molecular cargos into encysted, latent bradyzoites and deliver cargos across the blood-brain barrier (2, 3). These peptides also have been shown to deliver antimicrobial compounds to retina when applied topically. Transductive peptides' versatility, in combination with PMO's stability, has promise for clinical application (4).

T. gondii can be transfected stably with YFP or luciferase, which can be easily measured using standard reagents. As a result, knocking down YFP and luciferase gene products could be a first proof of the principle that transductive peptide-conjugated PMO (PPMO) could reach intracellular parasites and successfully abrogate transfected fluorescent or luminescent gene expressions. PPMO is used herein to refer to transductive peptide PMO, including Vivo-PMO (Gene Tools), which uses a transductive dendrimer. Because YFP is a protein that, when excited at a certain wavelength, exhibits yellow fluorescence, it is easily quantifiable within intracellular parasites by fluorometry or immunofluorescence analysis. Similarly, because luciferase is an enzyme that catalyzes luciferin to produce bioluminescence, inhibition of luciferase is quantifiable.

Dihydrofolate reductase (DHFR) is an enzyme that is essential for the synthesis of *T. gondii* tetrahydrofolate, which is critical for production of purines, thymidylic acid, and certain amino acids. Because DHFR is a known, validated antimicrobial target downstream from the *T. gondii* shikimate pathway, it would be suitable as a next target in a proof-of-principle investigation.

Enoyl-ACP reductase (ENR) is involved in the type II fatty-acid biosynthesis (FAS) pathway in Apicomplexan parasites, such

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[1]Present address: Department of Agricultural Sciences, Tuskegee University, Tuskegee, AL 36088.

[2]To whom correspondence should be addressed. E-mail: rmcleod@uchicago.edu.

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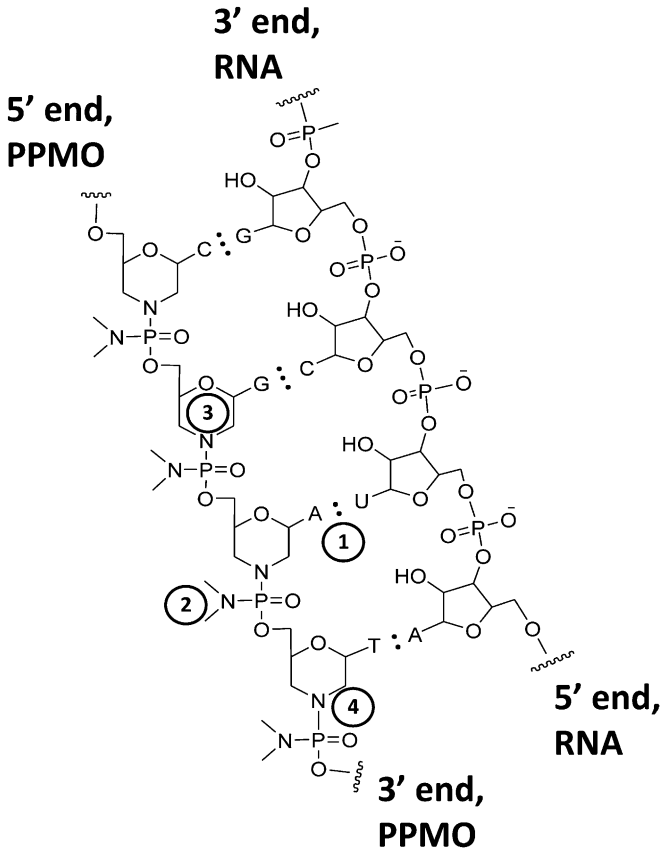

Fig. 1. Schematic representation of inhibitory mechanisms of PPMO. PPMO bind to target frames via Watson/Crick base-pairing, binding their deoxyribonucleic bases with mRNA's ribonucleic bases (labeled "1"). The base-pairing process is stabilized by PPMO's neutral phosphate backbones (labeled "2"), which provide stability not evident in other inhibitory strategies. Transductive peptides are conjugated at the 3′ ends of PPMO, allowing antisense oligomers to enter tachyzoites. PPMO have six-member carbon rings, whereas RNA targets have five member rings (labeled "3"). Nitrogen is absent on RNA but present on PPMO (labeled "4").

as *T. gondii* and *Plasmodium falciparum*. *T. gondii* ENR is a single polypeptide, whereas the ENR activity in mammalian cells is subserved by a multidomain enzyme. These structural differences have been exploited in the development of antimicrobial agents effective against type II FAS. ENR is predominantly localized inside the *T. gondii* apicoplast, an organelle evolutionarily derived from endosymbiotic algae and that has four surrounding membranes in *T.gondii*. Inhibiting ENR using PPMO would be a simple and direct way to determine whether ENR is essential and a valid molecular target for future medicine development.

Apetela 2 (AP2) domain transcription factor XI-3 (AP2XI-3) is a member of the plant-like Apicomplexan Apetela 2 (ApiAP2) transcription factor family (5, 6). ApiAP2 do not have homologs in mammalian cells. This family of ApiAP2 transcription factors regulates cell cycle, replication, and maintenance of the tachyzoite life-cycle stage. These transcription factors also regulate differentiation, and switch to and maintenance of the bradyzoite life-cycle stage in Apicomplexan parasites (5). AP2XI-3 is believed to play a key role in tachyzoite replication (5).

Transcription factors have been considered difficult to target because they are intracellular proteins that each binds to a short DNA motif that could occur in multiple species including humans. Successful specific inhibition of AP2XI-3 through PPMO would be of considerable value because the antisense approach could then be used to abrogate transcription factors, which are difficult to target through other means. Transcription factors of interest are essential for sustaining tachyzoites and encysted bradyzoites such as AP2 transcription factors (5). Three transcription factors are important for maintaining the tachyzoite stage, and three transcription factors appear to be important for maintaining the bradyzoite stage.

Applying PPMO would make it possible to determine whether either or both ENR and AP2XI-3 are essential for tachyzoites. This approach could establish the enzyme and transcription factor as promising targets for future medicine development and useful tools to study their biological functions. If effective, it might be possible to further optimize this technique for widespread use for target validation or expand this to larger scale screening efforts. The technique could provide the unique advantage of being able to target any gene product alone or several together, including gene products enclosed by multiple membranes, as well as transcription factors. This approach then also might be amenable to development of compounds capable of inhibiting or killing multiple life-cycle stages of this parasite or for therapeutic applications. We describe effects of PPMO targeting stably transfected YFP, luciferase and parasite DHFR, ENR, and an AP2 transcription factor.

Results

Knockdown of Transfected YFP Resulted in Diminished Fluorescence. Immunofluorescence staining was conducted to visualize YFP knockdown (Fig. 2*A*). Human foreskin fibroblasts (HFF) were infected with type I RH parasites that were stably transfected with YFP. These parasite cultures were treated with YFP-specific or off-target PPMO. Off-target antisense oligomers showed no observable effects on intracellular tachyzoites' fluorescent intensities in comparison with those of untreated parasites. However, YFP stably transfected parasites treated with YFP-specific PPMO showed reduced amounts of fluorescence. Neither off-target nor YFP-specific PPMO alterred the parasites' ability to invade HFF.

In addition to being qualitatively assessed, YFP fluorescence also was standardized using increasing concentrations of YFP-transfected parasites and measuring amounts of fluorescence using a fluorometer (Fig. S1*A*). Fluorometry detected increasing amounts of RH-YFP parasites, with a strong, positive correlation between the number of stably transfected RH-YFP parasites and their fluorescence 96 h after infection ($R^2 = 0.97$).

Death of HFF where parasites reside could spuriously appear to demonstrate effects of PPMO. Therefore, a parallel assay that measures host cell viability was used (Fig. S1*B*). HFF viability was quantifiable using a WST-1 cell proliferation assay. This assay was standardized with increasing dimethyl sulfoxide (DMSO) concentrations. DMSO concentrations less than 1% were not toxic, but concentrations over this amount were toxic to HFF, with greater toxicity at higher concentrations. The WST-1 cell proliferation assay could detect decreasing HFF viability by staining for formazan production in host cell mitochondria ($R^2 = 0.78$). The WST-1 cell proliferation assay was able to distinguish HFF grown under toxic conditions from those that were not, as the HFF grown in media containing 1% DMSO and 0.5% showed no toxicity. In contrast, those grown in media containing more than 1% DMSO were less viable. This assay was used to assess each PPMO's effect on HFF viability.

Fluorescence intensities were quantified to corroborate effectiveness of YFP-specific PPMO against fluorescence (Fig. 2*Bi*). HFF infected with 2,000 RH parasites stably transfected with YFP but not treated with any PPMO had 15,065 relative fluorescence units (RFU) 96 h postinfection (Fig. 2*Bi*). Fluorescence diminished with increasing concentrations of YFP-specific PPMO. When parasites stably transfected with YFP were treated with 3.75 μM and 5 μM of YFP-specific PPMO, fluorescence was reduced by 40% and 63%, respectively, 96 h postinfection. These reductions were statistically significant compared with untreated RH-YFP parasites (at 3.75 μM, $P = 0.021$; at 5 μM, $P = 0.0049$). Off-target PPMO did not have an inhibitory effect on intracellular tachyzoites' fluorescence in comparison with infected fibroblasts without PPMO ($P = 0.47$).

PPMO with mutations were not effective in reducing fluorescence. For example, for cultures treated with PPMO with a mismatched sequence that contained 13 point mutations at a concentration of

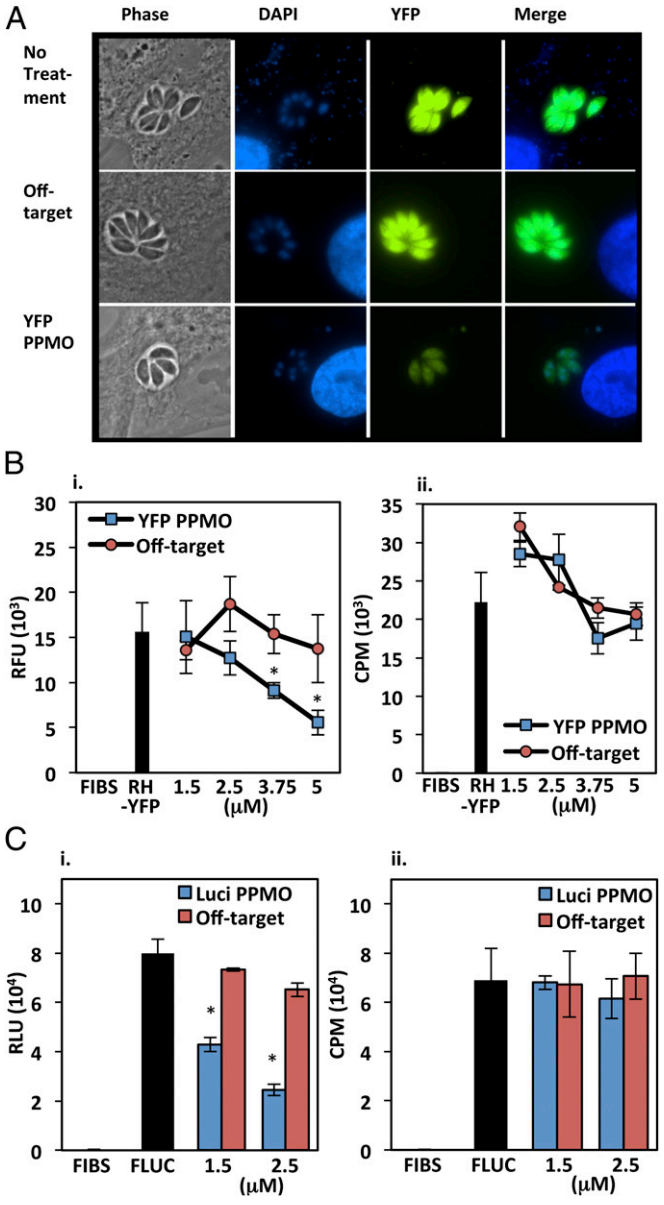

Fig. 2. PPMO inhibit gene expression of YFP and luciferase. (*A*) Immunofluorescence staining. YFP-specific PPMO (labeled YFP PPMO) reduce fluorescence in intracellular parasites. Standardization of methods quantifying fluorescence and HFF viability using WST-cell proliferation reagent are in Fig. S1 *A* and *B*, respectively. (Magnification: 400×.) (*B*) Effects of YFP-specific PPMO on parasite fluorescence and lack of effect on parasite viability in uracil uptake assay measuring tachyzoite replication. Microscopic preparations are confirmed in Fig. S1*C*. Lack of effect of YFP PPMO on host cell viability is shown in Fig. S1*D*. (*C*) Luciferase PPMO inhibits luminescence. Standardization of this assay ($R^2 = 0.86$) is shown in Fig. S1*E*. Lack of effect of luciferase PPMO on HFF viability is shown in Fig. S1*F*. FIBS, uninfected HFF; RH-YFP, untreated RH-YFP cultures; and FLUC, untreated luciferase-transfected tachyzoites-infected cultures. Comparisons to the PBS control were made using Student *t* test. No adjustments for multiple comparisons were made. Asterisks represent values that are statistically significant ($P < 0.05$).

3.75 μM, there were 4,414 ± 463 RFU, and similarly, untreated control cultures had 4,777 ± 1,358 RFU ($P > 0.05$).

Uracil is used by *T. gondii* but not by mammalian cells, so it reflects parasite replication and survival. Uracil uptake was assayed as described to examine effects of PPMO on tachyzoites' replication (Fig. 2*Bii*). Average uracil uptake of parasites treated with 5 μM of YFP-specific PPMO was 19,466 ± 1,477 cpm, similar to uracil uptake of untreated YFP parasites ($P = 0.16$). Parasites treated with off-target PPMO also had comparable amounts of uracil uptake (20,638 ± 2,140 cpm) to untreated *T. gondii* stably transfected with YFP ($P = 0.27$). Pairwise statistical test results demonstrated no difference between on-target and off-target PPMO ($P > 0.05$). Further measurement of mean number of parasites per vacuole and percent of infected cells evaluated microscopically did not differ (Fig. S1*C*) ($P > 0.05$). The WST-1 cell proliferation assay was conducted to measure PPMO's effect on HFF host cell viability (Fig. S1 *B* and *D*). Absorption of formazan dye with 1.5 μM, 2.5 μM, and 3.75 μM of YFP-specific PPMO and all off-target PPMO's concentrations tested were not lower than the absorption of untreated HFF 96 h postinfection (Fig. S1*D*) ($P > 0.05$ for all comparisons). Pairwise statistical tests demonstrated no significant difference between the corresponding on-target and off-target PMO results ($P > 0.05$).

Knockdown of Transfected Luciferase Reduces Parasite Luminescence. The luciferase–luciferin interaction was standardized to distinguish various concentrations of Prugneaud type II parasites stably transfected with luciferase (Fig. S1*E*). The assay was successful in distinguishing different numbers of type II Prugneaud parasites stably transfected with firefly luciferase (FLUC); the level of luminescence had a positive correlation with increasing numbers of luciferase-transfected tachyzoites ($R^2 = 0.88$) (Fig. S1*E*). This standardized assay was then used to measure effects of luciferase-specific PPMO in knocking down luciferase gene expression in tachyzoites. HFF were infected with 3,500 FLUC and were treated with 2.5 μM of luciferase-specific PPMO. Luminescence was significantly reduced from 65,162 relative luminescence units (RLU) in untreated parasites to 24,517 RLU 96 h postinfection among those treated with luciferase-specific PPMO ($P = 0.0082$) (Fig. 2*Ci*). Off-target PPMO had no effects on parasite luminescence at 2.5 μM and 1.5 μM compared with untreated parasites ($P = 1.00$ and $P = 0.38$, respectively) (Fig. 2*Ci*). Parasite and host cell viability assays were conducted using luciferase-specific PPMO (Fig. 2*Cii* and Fig. S1*F*). It was found that luciferase-specific and off-target PPMO had no adverse effects on parasites' uracil incorporation and HFF's formazan production in mitochondria compared with HFF not grown in DMSO-containing media ($P > 0.05$ for all comparisons).

Knockdown of Transfected YFP in Different Parasite Strains Demonstrates Efficacy in Genetically Diverse Parasites Known to Have Different Virulence. To investigate effects of PPMO targeting YFP in a strain other than RH type I *T. gondii*, YFP-specific PPMO were used to abrogate YFP expression in Prugneaud type II parasites stably transfected with YFP instead of RH Type I *T. gondii* (Fig. 3*A*). To test whether active PPMO not targeting YFP would have off-target effects, luciferase-specific oligomers, in addition to off-target PPMO, were used as controls. HFF infected with 3,500 Prugneaud type II parasites stably transfected with YFP had 19,714 ± 1,331 RFU 96 h postinfection. Parasites treated with 3.75 μM YFP-specific PPMO showed an 80% reduction in fluorescence compared with untreated infected cultures ($P < 0.05$) (Fig. 3*A*). Type II parasites treated with luciferase-specific or off-target antisense PPMO had RFU of 18,207 ± 1,168 and 18,729 ± 829, respectively. Their luminescence was not significantly different from untreated type II Prugneaud parasites ($P = 0.13$ and $P = 0.32$, respectively).

Kinetics of YFP-Specific PPMO. YFP-RH tachyzoites were treated with 3.75 μM YFP-specific and off-target PPMO at 2, 8, or 18 h postinfection to investigate kinetics of PPMO against YFP expression. Effects of PPMO targeting YFP added at 2 h were detected from 48 to 72 h, decreasing from 25,596 ± 5,413 to 16,682 ± 1,960 RFU over this period, as were effects of PPMO added at 8 and 18 h postinfection. Off-target PPMO did not effect YFP expression throughout the 96-h time span.

Knockdown of Native DHFR Inhibits Parasite Replication. PPMO targeting endogenous DHFR were cultured with HFF infected with

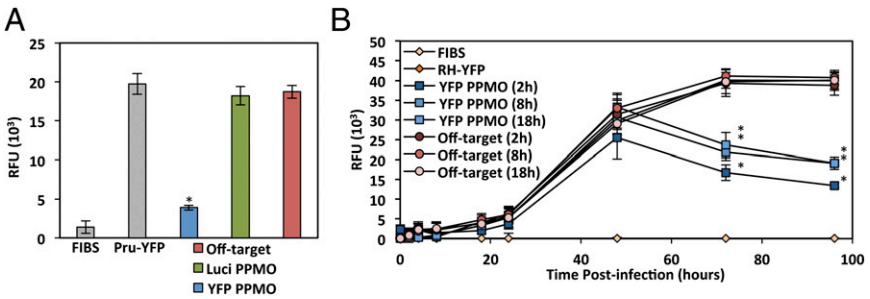

Fig. 3. PPMO targeting YFP effective in type II parasites stably transfected with YFP and an assay demonstrating kinetics of effect of PPMO. (*A*) YFP expression in type II Prugneaud parasites stably transfected with YFP (labeled Pru-YFP) was reduced using YFP-specific PPMO (blue). PPMO targeting firefly luciferase (labeled Luci PPMO, green) and off-target PPMO (red) had no effects on YFP expression in Pru-YFP. Uninfected HFF, labeled FIBS. Asterisks represent values that are statistically significant ($P < 0.05$). (*B*) Kinetics of YFP-specific PPMO. RH-YFP treated with YFP-specific PPMO 2 h postinfection began showing reduction in YFP expression after 48 h. PPMO administered 8 or 18 h postinfection had similar kinetics of effect, which reflects both knockdown and persistence of protein present before PPMO were added.

stably transfected YFP parasites to determine efficacy of antisense oligomers on a known, essential molecular target (Fig. 4*Ai*). Mean SD counts per minute of uracil uptake at 96 h postinfection when 3.75 μM and 5 μM of DHFR-specific PPMO were administered were 20,875 ± 2,417 and 48,283 ± 7,799, respectively. These counts were significantly lower than the counts per minute of untreated parasite cultures (at 3.5 μM, $P = 0.009$; at 5 μM, $P = 0.009$). The counts were also 52.4% and 66.2%, respectively, lower than the counts per minute of off-target PPMO at the same concentrations. Further measurement of mean number of parasites per vacuole and percent-infected cells evaluated microscopically were lower (Fig. S2) ($P < 0.05$). Absorption of formazan dye at all concentrations of both DHFR and off-target PPMO were not statistically different from absorption of untreated HFF ($P > 0.05$ for all values) (Fig. 4*Aii*).

To study specificity of PPMO targeting DHFR, knockdown of DHFR was product rescued with exogenous folic acid (Fig. 4*B*). Untreated RH type I parasites stably transfected with YFP were used as a control. These parasites had 33,956 ± 4,290 RFU 96 h postinfection. With 1.13 mM of exogenous folic acid, untreated YFP parasites had 43,544 ± 3,148 RFU 96 h after infection. Pyrimethamine also targets DHFR, so folic acid-rescue of parasites treated with pyrimethamine and sulfadiazine also was a control. Parasites treated with pyrimethamine and sulfadiazine were fully rescued with 1.13 mM of folic acid, having 40,274 ± 1,305 RFU 96 h postinfection. Similarly, 1.13 mM of folic acid fully rescued parasites treated with 3.75 μM and 5 μM of DHFR-specific PPMO. Without folic acid, parasites treated with 3.75 μM and 5 μM of PPMO targeting DHFR had 16,429 ± 1,589 RFU and 9,058 ± 709 RFU, respectively, 96 h postinfection. With folic acid, parasites treated with 3.75 μM and 5 μM of DHFR-specific PPMO had 47,478 ± 1,406 RFU and 44,957 ± 2,448 RFU, respectively. In comparison with effective rescue of parasites treated with DHFR inhibitors by exogenous folic acid, folic acid had minimal effects on viability of parasites treated with either 3.75 μM or 5 μM off-target PPMO.

Immunofluorescence assay (Fig. 4*C*) was conducted to both visualize effect of knockdown and substantiate specificity of PPMO targeting endogenous DHFR. After 48 h of treatment with DHFR-specific PPMO, DHFR expression of intracellular parasites was markedly reduced. Off-target PPMO had no effects on DHFR expression. Both DHFR and off-target PPMO had no effect on YFP expression.

Knockdown of ENR and AP2XI3 Establishes That They Contribute to Parasite Replication. ENR and AP2XI-3 were studied to determine whether they contribute to parasite replication (Fig. 5*Ai*, *Left*). PPMO specific to ENR or AP2XI-3 reduced parasite replication at 3.75 μM and 5 μM 96 h postinfection (Fig. 5*Ai*,

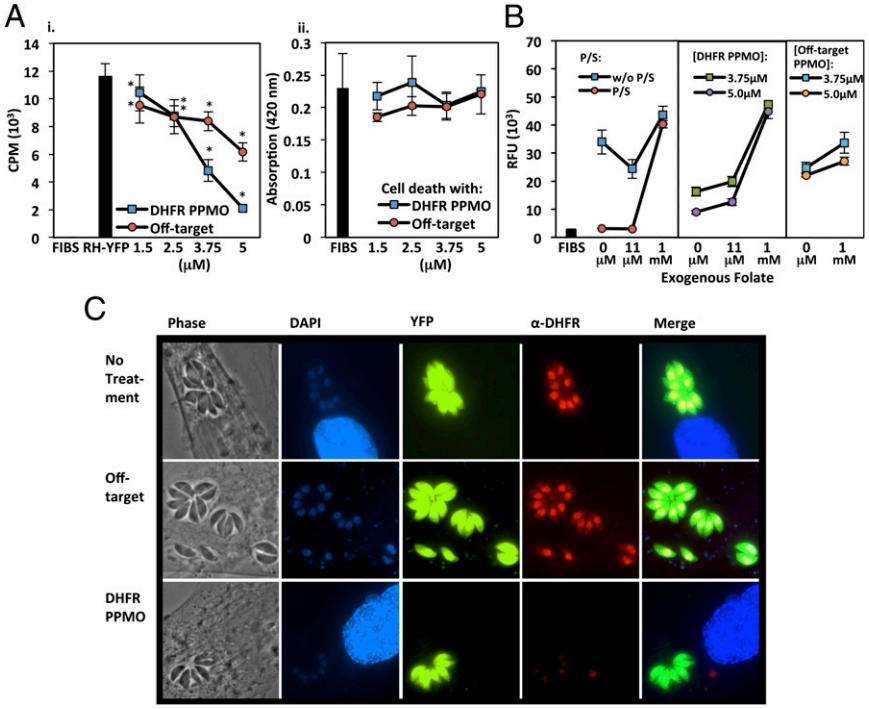

Fig. 4. PPMO designed to knockdown DHFR gene products inhibited parasite replication with oligomers' specificity demonstrated by folic acid rescue. (*A*) Effects of PPMO targeting DHFR (labeled DHFR PPMO, blue) on (*i*) parasite replication and (*ii*) HFF viability are shown. Off-target PPMO data points are colored red. Effect on mean parasite number per vacuole and percent-infected cells are shown in Fig. S2. (*B*) RH-YFP parasites treated with pyrimethamine and sulfadiazine (labeled P/S, red in column to left) were rescued with 1.13 mM of folic acid. Parasites treated with DHFR-specific PPMO (3.75 μM, green; 5.0 μM, purple in the center column) were rescued using folic acid. The 1.13 mM of folic acid fully rescued parasites treated with PPMO. Off-target PPMO 3.75 μM (light blue in the column to the right) and 5.0 μM (orange). (*C*) PPMO against DHFR also reduced immunofluorescence with α-DHFR. (Magnification: 400×.)

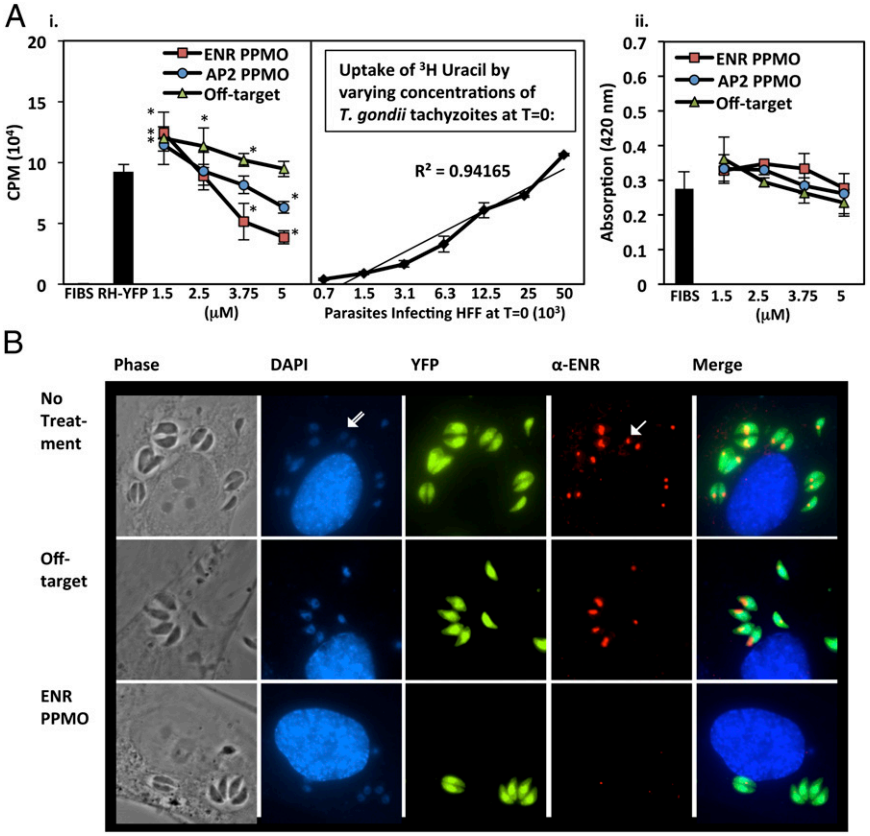

Fig. 5. PPMO targetting ENR and AP2XI-3 reduce tachyzoite replication. (*A*) Effects of PPMO targeting ENR or AP2XI-3 on (*i*) parasite replication and (*ii*) HFF viability. DMSO $\leq$ 1% was nontoxic to HFF and > 1% caused toxicity to HFF. (*B*) Immunofluorescence staining with α-ENR confirmed efficacy of ENR-specific PPMO. (Magnification: 400×.) Parasite nucleus stained with DAPI (double arrow), ENR in plastid with α-ENR (single arrow). *$P < 0.05$relative to RH-YFP (*A*) and FIBS (*B*) ($P < 0.05$). No adjustments for multiple comparisons were made.

Left) ($P < 0.05$ for all values). There was no effect on host cell HFF viability (Fig. 5*Aii*) ($P > 0.05$ for all comparisons) or reduction by off target PPMO. Control assay with increasing numbers of parasites is in Fig. 5*Ai*, *Right* ($R^2 = 0.9416$). Immunofluorescence assays (Fig. 5*B*) demonstrated that ENR in plastids was robustly knocked-down by ENR-PPMO without adverse effect on YFP expression, but off-target PPMO did not abrogate ENR. Furthermore, $(RXR)_4BX$ peptide as part of ENR PPMO was effective and not toxic at 20 μM (Fig. S3).

PPMO Targeting DHFR Reduce Parasite Burden in Infected Mice. Mice infected with YFP-transfected RH parasites were treated with PPMO targeting DHFR (Fig. 6) to determine whether PPMO were effective in vivo. Intraperitoneal fluid was collected and parasites were quantified by fluorometry (Fig. 6*A*) and hemocytometer (Fig. 6*B*). Fluorometry indicated that with DHFR-specific PPMO, there were 83% fewer parasites 96 h postinfection (Fig. 6*A*) ($P = 5.2 \times 10^{-6}$, $n = 10$, data from replicate experiments were similar and are shown here combined). As a control, off-target PPMO did not inhibit DHFR in vivo ($P = 0.65$). Numbers of parasites measured using a hemocytometer indicated that there was a 97% reduction in number of viable parasites after administering PPMO specific to DHFR (Fig. 6*B*) ($P = 1.3 \times 10^{-10}$, $n = 10$, data from replicate experiments were similar and are shown here combined).

Discussion

Herein, we demonstrate that PPMO targeting YFP, luciferase, DHFR, ENR, and an AP2XI-3 transcription factor reduced these protein products or reduced parasite replication. Study of kinetics of effects of YFP-specific PPMO demonstrated that PPMO effect is not altered by varying times that PPMO are added, from 2 to 18 h after infection. In addition to being effective against type I RH-YFP parasites, PPMO targeting YFP expression were also effective in type II Prugneaud tachyzoites stably transfected with YFP. PPMO inhibited their target frames in a highly sequence-specific manner. We designed PPMO of 25 bases long, as it previously was shown that there has to be a minimum of 14 consecutive bases of sequence conservation for PPMO to be inhibitory. This minimum inhibitory length requirement is more stringent than the nine consecutive bases required for siRNA inhibition to be successful (bases 2–8 for recognition and bases 9–12 for cleavage of RNA target). Because of PPMO greater minimum length requirement, they have fewer off-target effects than other antisense inhibitors.

Uracil incorporation assay demonstrated that DHFR-specific PPMO successfully inhibited tachyzoite replication, a phenotype expected when knocking down DHFR. This assay characterized PPMO as inhibitors against synthesis of specific, essential genes. Successful knockdown of fluorescence, luminescence, and a known essential gene was a first proof-of-concept that established PPMO as a unique, sequence-specific knockdown system that can cross multiple membranes to inhibit gene products within intracellular tachyzoites. Antisense oligomers against another enzyme and a transcription factor also were successful. PPMO specific to ENR or AP2XI-3 successfully reduced parasite replication. Effective inhibition of ENR and AP2XI-3 not only suggested that their gene products contribute to tachyzoite replication, but also demonstrated that this unique inhibitory approach in tachyzoites could be an expeditious tool to screen large numbers of genes for quick target validation. The effect of reducing ENR and AP2XI-3 using transductive peptide-conjugated oligomers underscores that this approach is paradigm-shifting because it opens potential for abrogating any molecular target. Inhibiting transcription factors is difficult through other means. Successful inhibition of ENR demonstrated that PPMO are effective in inhibiting an enzyme of type II FAS. In the case of abrogating ENR, PPMO-targeting ENR were able to cross HFF membranes, the parasitophorous vacuole that surrounds replicating intracellular parasites, and parasite membranes. It will be of interest in future studies to determine where ENR is, when it

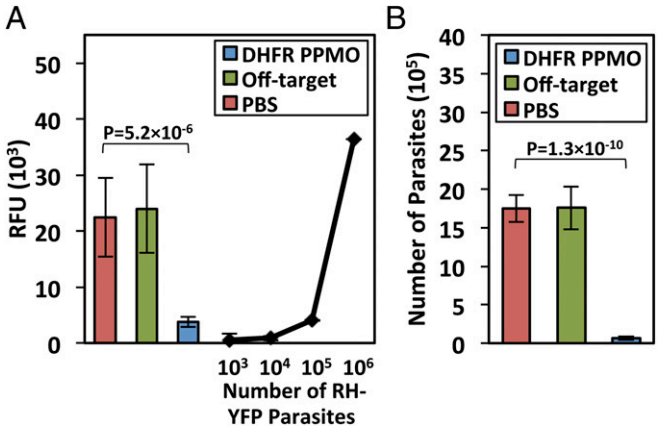

Fig. 6. DHFR PPMO reduces parasite burden in vivo. YFP-parasites quantified using a (*A*) fluorometer and (*B*) hemocytometer. Parasite numbers decreased when mice received DHFR-specific PPMO (blue in both panels). $*P < 0.05$ relative to untreated parasite cultures (labeled PBS, colored red) (*A*: $P = 5.2 \times 10^{-6}$, $n = 10$, data from replicate experiments were similar and are shown here combined). As a control, off-target PPMO did not inhibit DHFR in vivo ($P = 0.65$). Numbers of parasites measured using a hemocytometer indicated that there was a 97% reduction in the number of viable parasites after administering PPMO specific to DHFR (*B*, $P = 1.3 \times 10^{-10}$, $n = 10$, data from replicate experiments were similar and are shown combined herein). Initially, ANOVA was performed, and when $P < 0.000001$, pairwise T comparisons were performed using Student *t* test.

is inhibited, and whether PPMO can cross the four membranes that enclose the apicoplast or other organelles.

Our findings are useful because of the disease burden of *T. gondii* infection, broad applicability of the unique approach for validation of gene function, and the potential of the approach to be developed into a widely used therapeutic modality to treat a variety of diseases, including other Apicomplexan infections. As our manuscript was in revision, another group demonstrated that this approach was effective in *P. falciparum* as well, confirming our findings in another Apicomplexan parasite (7).

In conjunction with our earlier work and other work with transductive peptides (8), our work also opens the possibility of inhibiting parasite molecular targets in tissues, such as retina and brain. Substantial reductions in parasite burden evident in vivo demonstrated that PPMO could be used to target *T. gondii*'s essential genes in animal models. Although PPMO was quite effective in mice, it was with delivery to a location where the parasite was replicating. In terms of therapeutic potential, PPMO have potential to enter retina and could thereby act as a medicine by inhibiting parasite growth with topical application to the eye. Others have shown that transductive peptides with N-terminal rabies virus glycoprotein to target acetylcholine receptors can cross the blood-brain barrier and carry inhibitory molecules to pathogens in the brain (3). Intravenous and intranasal deliveries also have been found to be feasible. This work provides a unique paradigm for solving certain previously unsolvable biologic problems and for development of novel therapeutic approaches for diseases caused by *T. gondii*. In addition, PPMO potentially could inhibit latent bradyzoites. A model system of delivery to bradyzoites was developed earlier by conjugating a small molecule inhibitor to octaarginine (2). This peptide delivered molecular cargos across cyst walls and into dormant parasites and their nucleus (2).

PPMO deliver cargo across multiple membranes to intracellular parasites and inhibit various targets. However, Vivo-PMO (Gene Tools) have a narrow therapeutic-toxic ratio, causing toxicity to host cells at high concentrations. Thus, this antisense system requires further optimization. Toxicity originates from the transductive peptide, known as "Vivo-porter™", used at 3′ ends of antisense oligomers. An effective way to eliminate this toxicity and expand therapeutic range of PPMO is to modify the transductive peptide. Moving toward a stable, versatile, robust, less toxic, inhibitor against *T. gondii*, our preliminary data demonstrate that the $(RXR)_4BX$ peptide as part of the ENR PPMO is effective and not toxic at 20 μM (Fig. S3). Such PPMO or PMO inhibitors have recently entered human clinical trials for Duchenne's muscular dystrophy and to treat Ebola and Marburg virus infections. In these trials, PMOs or PPMOs have been found to be stable, safe, effective, and nonimmunogenic. This finding underscores the promise and unique suitability of this approach for treatment of diseases caused by *T. gondii*.

Methods

Principles of Design, Methods Used Previously for in Vitro and in Vivo Studies. The work with murine model is approved by the University of Chicago Institutional Animal Care and Use Committee. The principles of design and methods used previously were as described previously (1, 9) and are presented in detail in *SI Methods*. Specific PPMO were designed to inhibit gene products of YFP (agctagat**TCTAAAATGGTGAGCAAGGGCGAG**c; PPMO sequence is bolded; starting codon is underlined; sequences before and after target genes, which are not included in PPMO designs, are indicted in lowercase letters); luciferase (gatggct**GTCATGGAAGACGCCAAAAACATA**aagaaa); DHFR (ctgg**GAAGATGCAGAAACCGGTGTCTG**gtcgtc); ENR (aaatcg**AAAATGGTTGGTTTCAAACTCCTC**accctc); AP2XI-3 (cgcg**TCTGTTCCGTGCCGCGATGGAGT**cgga). Control off-target PPMO (**TATAAATTGTAACTGAGGTAAGAGG**) was provided by Gene Tools. Conjugating transductive peptide to PMO was carried out as described in the additional relevant references in *SI Methods*.

Folic Acid Rescue of DHFR Knockdown. Forty-percent of sodium hydroxide was diluted 1:10 in Iscove's Modified Dulbecco's Medium without Phenol Red. Next, 0.5 g of folic acid was incrementally dissolved in every 10 mL of diluted sodium hydroxide, resulting in 113 mM folic acid stock solution. Stock solution was serially diluted and added to wells of 96-well plates. Fluorescence was measured 96 h postinfection.

Analysis of Data and Statistics. For analysis of effect of YFP PPMO for in vitro studies, statistical analysis comparing experimental group data to PBS control was with Student *t* test. No adjustments for multiple comparisons were made. For in vivo experiments, an initial ANOVA was performed when $P < 0.000001$, pairwise T comparisons were performed using Student *t* test. Regression analysis was with coefficient of determination (R^2).

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