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

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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. Transdusive 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 transdusive 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 enol-ACP reductase (ENR), an enzyme for fatty acid biosynthesis (FAS) pathway in Apicomplexan parasites, such as Toxoplasma, was characterized. ENR and AP2XI-3 PPMO each restricted main transcription factor XI-3 (AP2XI-3), not present in human cells, was characterized. ENR and AP2XI-3 PPMO each restricted main transcription factor XI-3 (AP2XI-3), not present in human cells.

DHFR, an enzyme necessary for folate synthesis, limited tachyzoite replication. Rescue with exogenous folate demonstrated DHFR PPMO’s specificity. PPMO directed against enol-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 mammalian 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.

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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. Recrudescence 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. Fortunately, 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 acid 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.

Transdusive peptides attached to the 3′ ends of PMO allow the oligomers to readily enter mammalian cells and microorganisms by crossing multiple membrane barriers. A transdusive 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. Transdusive 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 transdusive peptide-conjugated PMO (PPMO) could reach intracellular parasites and successfully abrogate transfected fluorescent or luminescent gene expressions. PPMO is used herein to refer to transdusive 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. Enol-ACP reductase (ENR) is involved in the type II fatty acid biosynthesis (FAS) pathway in Apicomplexan parasites, such as Toxoplasma. As a result, blocking ENR′s activity in these parasites could inhibit their replication.


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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 antimalarial 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. 2A). Human foreskin fibroblasts (HFF) infected with type 1 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 altered 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. S1A). 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. S1B). 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 allowed us to distinguish between 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. 2B). HFF infected with 2,000 RH parasites stably transfected with YFP but not treated with any PPMO had 13,065 relative fluorescence units (RFU) 96 h postinfection (Fig. 2B). 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, $P = 0.0494$). 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: 400x.) (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. S1C. Lack of effect of YFP PPMO on host cell viability is shown in Fig. S1D. Luciferase PPMO inhibits luminescence. Standardization of this assay ($R^2 = 0.88$) is shown in Fig. S1E. Lack of effect of luciferase PPMO on HFF viability is shown in Fig. S1F. 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 $4,414 \pm 463$ RFU, and similarly, untreated control cultures had $4,777 \pm 1,358$ 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. 2Bi). Average uracil uptake of parasites treated with 5 μM of YFP-specific PPMO was $19,466 \pm 1,477$ 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 ($20,638 \pm 1,240$ 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. S1C) ($P > 0.05$). 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. S1D) ($P > 0.05$ for all comparisons). Pairwise statistical tests demonstrated no significant difference between the corresponding on-target and off-target PPMO 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. S1E). 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. S1E). 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. 2C). 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. 2Ci). Parasite and host cell viability assays were conducted using luciferase-specific PPMO (Fig. 2Ci and Fig. S1F). 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. 3A). To test whether active PPMO 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 $19,714 \pm 1,331$ 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. 3A). Type II parasites treated with luciferase-specific or off-target antisense PPMO had RFU of $18,207 \pm 1,168$ and $18,729 \pm 829$, 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 25,590 ± 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 affect 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...
stably transfected YFP parasites to determine efficacy of antisense oligomers on a known, essential molecular target (Fig. 4Ai). 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. 4Aii).

To study specificity of PPMO targeting DHFR, knockdown of DHFR was product rescued with exogenous folic acid (Fig. 4B). 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 ± 4,248 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. 4C) 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 AP2XI3 were studied to determine whether they contribute to parasite replication (Fig. S4i, Left). PPMO specific to ENR or AP2XI3 reduced parasite replication at 3.75 μM and 5 μM 96 h postinfection (Fig. S4i, Right).
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. Intrapleural fluid was collected and parasites were quantified by fluorometry (Fig. 6A) and hemocytometer (Fig. 6B). Fluorometry indicated that with DHFR-specific PPMO, there were 83% fewer parasites 96 h postinfection (Fig. 6A) \( (P = 5.2 \times 10^{-6}, n = 10) \), data from replicate experiments are 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. 6B) \( (P = 1.3 \times 10^{-10}, n = 10) \), 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 transactive 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. 5. PPMO targeting 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 < 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.
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 retinas 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 delivery 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 cargo 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)_{4}BX 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 (agctagatTCTAAAAATGTGGACAGAAGGGCGACG; 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 (gattgcgtCATGAGAGAAGCGCCAAACACATAAAA); DHFR (cttgGAAAGTGTCAGAGCGGTTCgtgct; ENR (aatctgaAAATGTGGTTTCTAAACCTCCGctcc); APX2I-3 (gctgtgcTCTGTTCCGTGCCTGCGGATGAGTgga); and control-off target (TATAAAGTTGACTGAGGTAAAGAGG) was provided by Gene Tools. Conjugating transductive peptide to PPMO 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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**Supporting Information**

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**SI Methods**

**Principles and Design of Peptide Conjugated Phosphorodiamidate Morpholino Oligomers (PPMO) and PPMO Sequences Used in This Study.** We summarize herein the guidelines used for designing PPMO. These guidelines were published elsewhere in more detail (1) (GeneTools Web site): Morpholinos of 25-mers are the usual choice, although in bacterial systems 11-mers or 12-mers were found to be optimal. These morpholinos can be decreased by a few bases as needed. Shorter oligomers are better for high CG content. Lower CG means that the oligomers’ affinities might be too low to block processes, whereas higher CG content favors nonspecific binding of subsequences. There is a shift toward lower CG for organisms living below 37 °C. Up to 36% G is acceptable. Higher G causes loss of water solubility. The upper end of the acceptable range is avoided if possible. Three sub- sequential Gs are maximum because runs of four or more G can form tetramers and reduce oligo efficacies. When possible, a G or A on the 3′ end of a modified oligo is avoided as these may decrease coupling efficiency of the modification.

Upstream (5′ UTR) sequences can be targeted and are usually successful. A morpholino can target anywhere between the 5′ cap and the start codon. The morpholino also can extend downstream into the coding sequence as long as the start codon is covered. The reason this process works is related to the steps at the beginning of translation. A group of proteins and the small ribosomal subunit bind at the 5′ cap and then other initiation factors bind, forming the initiation complex. The initiation complex scans through the UTR to the start codon. At the start codon, the large subunit binds, the initiation factors dissociate, and translation proceeds through the coding region. Interfering with the initiation complex by binding a morpholino to the UTR prevents the initiation from reaching the start codon, but once the large subunit binds and forms an entire ribosome then a morpholino oligomer cannot stop its progression. In the latter case, the ribosome just displaces the downstream oligo from the mRNA and reads through. This is why the targetable region for translation blocking extends from the 5′ cap to the start +25 bases.

There are two reasons why targeting at the start is preferable. First, the quality of sequencing deposited in public databases is often poor in the UTR. Sometimes, cloning vector sequences are reported as being in the UTR. Second, although rare in vertebrate genomes, internal ribosome entry sites do exist and can allow translation of mRNAs that are not included in PPMO designs, are indented in lowercase letters); luciferase (gatggctgtgagcaagggcgcag); PPMO sequence is bolded; starting codon is underlined; sequences before and after the target genes, which are not included in PPMO designs, are indicated in lowercase letters); luciferase (gatggctgtgagcaagggcgcag); PPMO sequence is bolded; starting codon is underlined; sequences before and after the target genes, which are not included in PPMO designs, are indicated in lowercase letters). For example, when targeting DHFR in type II Toxoplasma gondii parasites that are a clonally derived stable line expressing cystolic YFP (3). When inhibiting YFP fluorescence in type II Prugneaud parasites stably transfected with YFP, HFF were infected with 3,500 instead of 2,000 tachyzoites, because type II parasites grow slower than type I tachyzoites. After 2 h of infection, wells were separately treated with 1.5 μM, 2.5 μM, 3.75 μM, and 5 μM of PPMO targeting the correlating gene product. All wells were rocked gently to ensure even distribution of oligomers without disturbing HFF monolayers. When knocking down luciferase luminescence, fibroblasts were seeded in white, opaque 96-well microplates. Each well was infected with 3,500 Type 2 T. gondii parasites that were stably transfected with luciferase. After 2 h of infection, wells were separately treated with 1.5 μM, 2.5 μM, 3.75 μM, and 5 μM of luciferase-specific and off-target PPMO. Off-target PPMO was used in all knockdown experiments as a control.

**Measuring YFP and Luminescence.** Ninety-six hours after parasite infection, fluorescence and luminescence were respectively measured for YFP and luciferase inhibition using Synergy H4 Hybrid Multi-Mode Microplate Reader. This time-frame was chosen because a reasonable estimate of the time that PPMO remain effective in cells and animals had been found to be 4–5 d in an earlier work (4). YFP fluorescence was read at 540 nm. Luciferin stock solution was created by diluting 33 mg of luciferin potassium salt in 1 mL of nuclease-free, deionized, distilled water. Stock solution was diluted 1:20, and 20 μL was added to every well. Thirty minutes after adding luciferin, luminescence was read 10 s per well at 420 nm, and at 37 °C for optimal signal.

**Immunofluorescence.** HFF were grown on cover-slips in 24-well multiwell plates. When confluent, the plates were infected with 10^3 YFP-transfected RH tachyzoites with or without 3.75 μM of YFP-, DHFR-, or ENR-specific PPMO. Medium was aspirated under sterile conditions after 48 h of treatment. HFF were then treated with 3% (wt/vol) paraformaldehyde for 30 min at room temperature. The HFF were then permeabilized with 0.2% Triton X-100 in PBS at room temperature for 10 min, and blocked with 0.2% Triton X-100 and 3% (wt/vol) BSA in PBS overnight at 4 °C. Antibodies specific to DHFR or ENR, diluted 1:500, were in buffer. The antibodies were further blocked with Texas red secondary antibody diluted 1:1,000 in blocking solution. DAPI stain was diluted 1:1,000 in blocking solution and applied for 1 h at room temperature. Cells were rinsed three times in PBS, and cover-slips were mounted using nail polish. Slides were examined using Nikon Eclipse Ti.

**Assays to Assess Effects of PPMO on T. gondii Tachyzoite Replication in Vitro.** Tritiated uracil uptake assays were conducted as previously described (5, 6).

**Assays to Assess Effects of PPMO on Host Cell Viability in Vitro.** HFF viability was measured using Premixed WST-1 Cell Proliferation

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**Cell Culture.** Human foreskin fibroblasts (HFF) were cultured in Isocove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% (vol/vol) heat-inactivated FBS (Invitrogen), 1% (vol/vol) glutamax, and 1% (vol/vol) PSF. When conducting knockdown assays, medium was aspirated and changed to IMDM free of Phenol red supplemented with 10% heat-inactivated FBS, 1% Glutamax and 1% PSF. Parasites were resuspended in Phenol-free medium as well.

PPMO Knockdown. When knocking down YFP fluorescence, DHFR, ENR, and AP2XI-3 gene products in type I RH tachyzoites, HFF were seeded in black, flat-bottom 96-well microplates. Each well was infected with 2,000 type 1 *Toxoplasma gondii* parasites that are a clonally derived stable line expressing cystotic YFP (3). When inhibiting YFP fluorescence in type II Prugneaud parasites stably transfected with YFP, HFF were infected with 3,500 instead of 2,000 tachyzoites, because type II parasites grow slower than type I tachyzoites. After 2 h of infection, wells were separately treated with 1.5 μM, 2.5 μM, 3.75 μM, and 5 μM of PPMO targeting the correlating gene product. All wells were rocked gently to ensure even distribution of oligomers without disturbing HFF monolayers. When knocking down luciferase luminescence, fibroblasts were seeded in white, opaque 96-well microplates. Each well was infected with 3,500 Type 2 *T. gondii* parasites that were stably transfected with luciferase. After 2 h of infection, wells were separately treated with 1.5 μM, 2.5 μM, 3.75 μM, and 5 μM of luciferase-specific and off-target PPMO. Off-target PPMO was used in all knockdown experiments as a control.

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Reagent. Tetrazolium salt WST-1 is converted to formazan in mitochondria only in viable cells. Viability assays involving WST-1 reagent work by measuring the absorption of formazan dye in cells. High absorption signifies cell viability. The numbers of viable cells positively correlate with the quantities of formazan dye absorbed.

**Folic Acid Rescue of DHFR Knockdown.** Forty-percent of sodium hydroxide was diluted 1:10 in IMDM 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.

**Effects of PPMO on Tachyzoites in Vivo.** Twenty-week-old C57Bl6J HLA A2 female mice were each infected with $10^3$ YFP-transfected RH parasites. Mice were injected intraperitoneally with 12.5 mg of DHFR-specific PPMO. Further treatment was given 24 h after infection, for a total of two PPMO doses. Intrapertitoneal fluid was collected 96 h postinfection and parasite fluorescence and numbers were measured using a fluorometer and hemocytometer, respectively.

**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 ($R^2$).

Fig. S1. Effect of YFP PPMO and luciferase PPMO on stably transfected tachyzoite YFP and Luciferase. (A) Standardization of measurement of YFP. YFP fluorescence was standardized using increasing concentrations of parasites stably transfected with YFP (RH-YFP). HFF were infected with various concentrations of RH-YFP at t = 0. Relative fluorescent units (RFU) were measured at t = 96 and the fluorescence results were presented in log scale. The fluorometer was able to detect increasing numbers of RH-YFP, because there was a positive correlation between parasite burden infected at t = 0 and log RFU measured at t = 96 ($R^2 = 0.97$). The results established the use of a fluorometer to detect YFP expression as an appropriate and accurate assay to quantify PPMO knockdown of YFP expression. (B) Standardization of measurement of host cell viability. Because *T. gondii* is an obligate intracellular parasite, death of HFF host cells could spuriously appear to demonstrate effects of PPMO. Therefore, a host cell viability assay was standardized as well. HFF viability was quantifiable using a unique WST-1 cell proliferation assay. This assay was standardized with increasing dimethyl sulfoxide (DMSO) concentrations. Concentrations less than 1% were not toxic, but concentrations over this amount were toxic to HFF, with greater toxicity at higher concentrations. HFF not treated with DMSO (labeled FIBS) was used as a control. The WST-1 cell proliferation reagent detects decreasing HFF viability by staining for formazan production in host cells’ mitochondria. Staining of formazan production was measured by measuring absorption at 420 nm 96 h postinfection ($R^2 = 0.78$). 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 herein to assess each PPMO’s effects on HFF viability. (C) Lack of effect of YFP PPMO on a mean number of parasites per vacuole and infected cells per high power field. HFF were grown on cover-slips until confluence and then infected with 5,000 parasites stably transfected with YFP. Parasites were cultured with YFP-specific PPMO (labeled YFP PPMO) or off-target PPMO (labeled Off-target) 2 h postinfection. Untreated YFP-transfected parasites were used as a control (labeled RH-YFP). After 48 h of infection, parasites were fixed with 3% (wt/vol) paraformaldehyde for 30 min at room temperature. Parasites were then permeabilized with blocking buffer containing 0.2% TritonX-100 in PBS at room temperature for 10 min and blocked with 0.2% TritonX-100 and 3% (wt/vol) BSA in PBS overnight at 4 °C. The cover-slips were mounted on slides, and parasites in five high power fields were counted and averaged (Left). The tachyzoites per vacuole in five high power fields were counted and averaged (Right). ANOVA was conducted, and the means of three groups were found to be statistically equivalent ($P_{Left} = 0.73$, $P_{Right} = 0.98$). This finding demonstrated that both YFP-specific PPMO and off-target PPMO did not result in parasite toxicity in the immunofluorescence assays presented. (D) The WST-1 cell proliferation assay was conducted to measure effect of PPMO on HFF host cell viability. Absorption was measured at 420 nm 96 h postinfection. FIBS, uninfected HFF. 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. Furthermore, the absorption values of YFP-specific PPMO were statistically indistinguishable with respect to their corresponding off-target PPMO concentrations ($P > 0.05$ for all comparisons). (E) Standardization of measurement of luciferase in transfected parasites. The luciferase–luciferin interaction was first standardized to distinguish various concentrations of Prugneda type II parasites stably

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transfected with luciferase. The assay was successful in distinguishing different numbers of type II Prugneaud parasites stably transfected with firefly luciferase (FLUC), as the level of luminescence had a positive correlation with increasing numbers of luciferase-transfected tachyzoites ($R^2 = 0.88$). This standardized assay was then used to measure effects of luciferase-specific PPMO in knocking down luciferase gene translation in tachyzoites. (F) Lack of effect of luciferase PPMO on host cell viability. Cell viability assay through the use of WST-1 Cell Proliferation reagent was conducted. FIBS, HFF not cultured with either luciferase-specific or off-target PPMO. HFF were treated with 1.5 μM or 2.5 μM of luciferase-specific or off-target PPMO at $t = 0$, and absorption at 420 nm was measured at $t = 96$. It was found that luciferase-specific and off-target PPMO had no adverse effects on HFF's formazan production in the mitochondria compared with HFF not grown in DMSO-containing media ($P > 0.05$ for all comparisons).

Fig. S2. Effect of DHFR PPMO on mean number of parasites per vacuole and percent infected cells and lack of effect of off target PPMO. HFF were grown on cover-slips until confluence and then infected with 5,000 parasites stably transfected with YFP. Parasites were cultured with DHFR-specific PPMO (labeled DHFR PPMO) or off-target PPMO (labeled Off-target) 2 h postinfection. Untreated parasites were used as a negative control (labeled RH-YFP). After 48 h of infection, parasites were fixed with 3% paraformaldehyde for 30 min at room temperature. Parasites were then permeabilized with blocking buffer containing 0.2% TritonX-100 in PBS at room temperature for 10 min, and blocked with 0.2% TritonX-100 and 3% BSA in PBS overnight at 4 °C. The cover-slips were mounted on slides, and parasites in five high power fields were counted and averaged (Left). The tachyzoites per vacuole in five high power fields were also quantified and averaged (Right). ANOVA was conducted, and the means between at least a pair of datasets were found to be statistically different ($P_{\text{ANOVA Left}} = 3.0 \times 10^{-5}$, $P_{\text{ANOVA Right}} = 8.6 \times 10^{-3}$). ANOVA was followed by pairwise comparison using Student $t$ test. There were significantly fewer tachyzoites per high power field 48 h postinfection when parasites were treated with DHFR-specific PPMO in comparison with untreated RH-YFP ($P = 2.2 \times 10^{-4}$). On average, there were fewer parasites per vacuole when treated with DHFR-specific PPMO with respect to untreated RH-YFP ($P = 0.014$). This result demonstrated that DHFR-specific PPMO successfully inhibited parasite growth at 48 h postinfection. In contrast, off-target PPMOs did not adversely affect parasite replication and viability with respect to untreated tachyzoite cultures.
Effect of ENR PPMO with transductive peptide (RXR)4XB has effect on replication in vitro without any toxicity to host cells at 30 μM. HFF were grown in 96-well microplates until confluence. Each well was infected with 2,000 parasites stably transfected with YFP (RH-YFP). Uninfected HFF (labeled FIBS) and untreated parasites (labeled RH-YFP) were used as controls. Parasites were treated with ENR-specific PPMO conjugated with (RXR)4XB [labeled (RXR)4XB-ENR, blue] at various concentrations. Fluorescence (RFU), which had been shown to be an accurate measure of RH-YFP parasite viability, was measured 96 h postinfection. Reductions in parasite fluorescence, and consequently parasite replication, were statistically significant above 3 μM of ENR-specific PPMO conjugated with (RXR)4XB with respect to untreated RH-YFP (P < 0.05 for all comparisons). No disturbance of HFF was observed upon PPMO treatment. These findings suggest that the (RXR)4XB transductive peptide may be a promising candidate as we improve the efficacy and minimize toxicity of transductive peptides. To demonstrate that the fluorometer could differentiate various concentrations of RH-YFP, a parasite gradient assay (labeled Parasite Gradient, black) was conducted. HFF were infected with various numbers of RH-YFP at t = 0, and their fluorescence was measured at t = 96. It was found that numbers of parasites positively correlated with fluorescence 96 h postinfection (R² = 0.95).

Suggested Readings


