Targeting protein translation, RNA splicing, and degradation by morpholino-based conjugates in *Plasmodium falciparum*

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Identification and genetic validation of new targets from available genome sequences are critical steps toward the development of new potent and selective antimalarials. However, no methods are currently available for large-scale functional analysis of the *Plasmodium falciparum* genome. Here we present evidence for successful use of morpholino oligomers (MO) to mediate degradation of target mRNAs or to inhibit RNA splicing or translation of several genes of *P. falciparum* involved in chloroquine transport, apicoplast biogenesis, and phospholipid biosynthesis. Consistent with their role in the parasite life cycle, down-regulation of these essential genes resulted in inhibition of parasite development. We show that a MO conjugate that targets the chloroquine-resistant transporter *PfCRT* is effective against chloroquine-sensitive and -resistant parasites, causes enlarged digestive vacuoles, and renders chloroquine-resistant strains more sensitive to chloroquine. Similarly, we show that a MO conjugate that targets the *PfDXR* involved in apicoplast biogenesis inhibits parasite growth and that this defect can be rescued by addition of isopentenyl pyrophosphate. MO-based gene regulation is a viable alternative approach to functional analysis of the *P. falciparum* genome.

malaria | intraerythrocytic development | peptide conjugated morpholino oligomer | vivo morpholino oligomer | gene expression

Of the ~5,300 genes encoded by the *Plasmodium falciparum* genome, only a small number of genes have been successfully targeted for genetic modification using available genetic tools. With the lack of RNAi technology in this parasite, forward genetic approaches suitable for large-scale functional analysis are needed to validate possible drug targets and to gain a better understanding of *P. falciparum* pathophysiology (1). Recent efforts aimed to develop such tools include the use of Piggy-Bac, peptide-conjugated morpholino oligomers (PPMO), zinc-finger nucleases, glmS ribozyme, CRISPR-Cas9-mediated genome editing, peptide nucleic acids, and the Tet-R aptamer system (2–8). Each of these methods requires further development and optimization to be used in a large-scale format to assess the function of *P. falciparum* genes. Morpholino oligomers (MO) have identical Watson–Crick base-pair characteristics as DNA or RNA. They are resistant to degradation by nucleases due to the presence of a morpholine ring and have no charge because of the phosphorodiamidate bond in the backbone. The MO-based RNA-targeting approach has been shown to be an excellent alternative to RNAi as morpholinos can bind to RNA with high specificity (9). The sequence of the MO thereby decides the fate of the endogenous transcript. For example, MOs with External Guide Sequence (EGS) conjugated to peptides (PPMOs) have been designed to target essential genes of several pathogenic bacteria and have displayed strong antibacterial activity (10–13). Similarly, PPMOs targeting the *P. falciparum* *PfGyrA* and *PfPAT* RNAs for RNase P-mediated cleavage inhibit parasite growth in the low micromolar range (4, 14–16). Because binding of MOs to their target RNAs can prevent binding of other molecules to the

same targets, MO conjugates have also been used to inhibit RNA splicing and initiation of protein translation (9, 17, 18).

To enhance cellular uptake of morpholino oligomers and other drug-like molecules, arginine-rich peptides and polyguanidino dendrimers have been used (19–23). For morpholino-based antimicrobial activity, two types of conjugates have been developed, PPMOs and vivo morpholino oligomers (VMOs, octa-guanidinium dendrimer-conjugated MOs; *Materials and Methods*). PPMOs are produced following conjugation of a specific MO to a cell-penetrating, arginine-rich peptide, whereas VMOs are synthesized as conjugates between a MO molecule and an octa-guanidinium head group (13, 17, 24). To date, VMOs have been used to down-regulate gene expression in human fibroblasts, mice, zebrafish, *Xenopus* oocytes, *Toxoplasma gondii*, and other model organisms (20, 25–29). However, these conjugates have not yet been assessed for their use in functional analysis of *P. falciparum* genes.

We report here the successful use of VMO or PPMO conjugates designed to target translation, splicing, and degradation of target RNAs in *P. falciparum*. Using these conjugates, we have targeted the *PfDXR*, *PfPMT*, and *PfCRT* genes that play a critical role in apicoplast biogenesis, membrane biosynthesis, and drug/metabolite transport (30–32). We show that VMO (*PfPMT*, *PfCRT*) and PPMO (*PfDXR*) conjugates reduce endogenous levels of their target RNAs and inhibit parasite growth. *PfCRT*-VMO was effective against drug-sensitive and -resistant strains

Significance

Malaria remains a major public health issue worldwide and world health organization estimates ~198 million cases and ~584,000 deaths in the year of 2013 alone due to malaria. Lack of an effective vaccine and rapid emergence of drug resistance are two major causes of this persistent problem. Understanding the biology of the parasite and studies of its gene function are essential to identify potential drug targets. Here we report a morpholino oligomer (MO)-based approach to alter gene expression via inhibition of post-transcriptional processes or by targeting mRNAs for degradation. The ease in design of the MO molecules presents a possibility for their use in large-scale genome functional analyses and possibly in malaria therapy.

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Fig. 1. Inhibition of translation and splicing using VMOs (A–C) Schematic representation of the binding sites of luciferase-VMO. (A), *PfpMT*-VMO (B), and *PfcRT*-VMO (C) on their respective sites on the mRNA or pre-mRNA. Arrows indicate the sites and orientation of the primers used for qPCR. cDNA was made from VMO-treated 3D7 parasites. (D) Luciferase-expressing parasites were treated with 1 or 2 μ M Ctrl-VMO and luciferase-VMO; 72 h (one cycle) and 96 h (two cycles) later parasites lysates were used for luciferase assay. Luciferase activity is plotted after normalization to Ctrl-VMO. (E and G) qPCR studies with primers 2F and 2R to amplify *PfpMT* or *PfcRT* steady-state transcripts. (F and H) qPCR analyses carried out using primers 1F and 1R, which amplify *PfpMT* or *PfcRT* unspliced transcripts. The results represent three independent experiments, and the error bars indicate SE of mean. The level of significance in the graph is indicated with an asterisk (* $P < 0.01$). (I) cDNA from PBS (lane 1), *PfpMT*-VMO (lane 2), and *PfcRT*-VMO (lane 3) treated 3D7 parasites along with genomic DNA (lane 4) were amplified using *PfpMT* 3F and 3R, and the PCR products were separated on a 1% agarose gel.

and rendered a chloroquine-resistant strain more sensitive to the drug.

Results

Inhibition of Luciferase Reporter Activity with a Specific Luciferase-VMO. To assess the specificity of VMO-mediated inhibition, transgenic parasites expressing luciferase (LUC) (33) were used, and a Luc-VMO that binds at the start codon to inhibit translation initiation was designed. (Fig. 1A and Table 1) (18). The morpholino sequence was designed to have less than 16 contiguous hydrogen-bonding bases to limit self-complementarity and no more than nine guanine residues to be water soluble (9). As a control, a VMO with no homology to the parasite genome was used (Ctrl-VMO) (Table 1). Both Luc-VMO and Ctrl-VMO were conjugated to dendritic molecular transporter units with guanidine head groups to facilitate delivery to host cells (24). Parasites expressing luciferase were synchronized and treated at the ring stage with Ctrl-VMO or Luc-VMO at two different concentrations (see legend to Fig. 1), and parasite intraerythrocytic development was monitored after one (Rings $\rightarrow$ Trophozoite $\rightarrow$ Schizont $\rightarrow$ Ring) and two 48-h cycles of parasite

growth. As expected, no differences in growth could be detected after one or two cycles between Luc-VMO- and Ctrl-VMO-treated parasites (see Fig. S1A and B). However, treatment with 2 μ M Luc-VMO resulted in a 17 and 30% reduction in luciferase activity compared with Ctrl-VMO after one and two cycles, respectively (Fig. 1D). Higher concentrations of VMO conjugates altered parasite growth most likely due to the inherent toxicity of the targeting dendrimer as has been previously reported in mice (Fig. S1B) (34).

Gene-Specific Transcript Expression Is Reduced After VMO Treatment.

To assess the possible use of VMO conjugates in down-regulation of *P. falciparum* gene expression, two VMO conjugates were designed to target splicing of *PfpMT* and *PfcRT* RNAs, encoding the phosphoethanolamine methyltransferase and digestive vacuole transporter of the parasite, respectively (35, 36). The sequences of *PfcRT* and *PfpMT* VMOs are shown in Table 1. These VMOs were designed to bind to the first exon-intron junction in each gene's pre-mRNA to prevent splicing, resulting in accumulation of unspliced RNAs. Parameters for morpholino design were similar to those described for Luc-VMO. Both VMOs were conjugated to a dendritic molecular transporter with guanidine headgroups to facilitate delivery. The possible outcomes of the VMO treatment on the parasite endogenous transcript are illustrated in Fig. 1B and C. PCR analyses using primers specific to the first intron region were performed to compare levels of unspliced transcripts between control and treated parasites (Fig. 1B and C). Levels of steady-state mRNA were determined by using primer pairs specific to exon regions. A highly synchronized culture of *P. falciparum* was treated with the VMOs for 6 h after which total RNA was isolated for cDNA preparation. Real-time PCR analysis showed 24 and 60% reduction in *PfpMT* and *PfcRT* steady-state mRNA levels, respectively (Fig. 1E and G), whereas the levels of unspliced transcripts (Fig. 1F and H) were 38% higher in both *PfpMT*-VMO or *PfcRT*-VMO-treated parasites compared with parasites treated with Ctrl-VMO. The observed decrease in the steady-state mRNA levels could be due to nonsense-mediated mRNA decay of mis-spliced transcripts (37). PCR amplification using primers 3F and 3R yielded two bands in *PfpMT*-VMO-treated parasites corresponding to unspliced and spliced forms whereas only a single band corresponding to the spliced form was detected in Ctrl-VMO-treated parasites (Fig. 1I). Primers 3F and 3R bind to the first and fourth exon, and the amplification product from genomic DNA includes all three introns, resulting in a 647-base product. Using these primers, a fully spliced mRNA yields a 227-base product, whereas an unspliced RNA containing the first intron following treatment with *PfpMT*-VMO results in a 372-base product. The absence of unspliced *PfpMT* RNA products in untreated or *PfcRT*-VMO-treated parasites indicates that *PfpMT*-VMO mediates gene-specific inhibition of splicing of its target RNA.

Table 1. Sequences of VMOs and PPMOs used in the text

Gene	Conjugate	Sequence of VMO/PPMO (5'-3')
Control	VMO	CCTCTTACCTCAGTTACAATTATTA
Luciferase	VMO	TCATAAACTTTCGAAGTCATGCGGC ⁻⁵
<i>PfpMT</i>	VMO	AAGTTTTTAGCACCTTCATCCGTAT ⁵⁵
<i>PfcRT</i>	VMO	CCATTTTGGATACTTACTTCCTTC ⁸⁴
<i>PfDXR</i>	PPMO	GTCCACGAGGTTGCAATCCTCTATATCC ¹⁴¹
<i>SaGyr</i>	PPMO	ACCTTGGCCAACCA

The boldface letters for *PfDXR* indicate the functional part of the sequence in the EGS. The other letters indicate the sequence needed for RNase P recognition in eukaryotic cells. The number in superscript next to the sequence indicate binding site on the transcript.

VMOs Targeting Splicing of *PfPMT* and *PfCRT* Inhibit Parasite Growth.

Previous genetic studies have shown that the loss of *PfCRT* results in parasite death whereas disruption of *PfPMT* results in a major developmental defect during *P. falciparum* intra-erythrocytic asexual development (30, 32). To examine the effect of VMOs targeting *PfPMT* or *PfCRT* on parasite development, cultures of the *P. falciparum* 3D7 clone were examined in the presence of Ctrl-VMO, *PfCRT*-VMO, or *PfPMT*-VMO after one or two cycles of treatment. A major delay in development could be seen in cultures of parasites treated with *PfPMT*-VMO compared with parasites treated with Ctrl-VMO with most parasites arrested at the trophozoite stage. *PfCRT*-VMO treatment resulted in deformed parasites with large digestive vacuoles (Fig. 2A), a phenotype reminiscent of the effect of cysteine protease inhibitors (38). Using transgenic parasites expressing a luciferase reporter to monitor parasite development, *PfPMT*-VMO and *PfCRT*-VMO were effective in reducing luciferase activity by two- to three-fold compared with Ctrl-VMO after one or two cycles of treatment (Fig. 2B and C). This effect was further confirmed by flow cytometry (Fig. S1C and D). Optimal parasite inhibition was observed with 1.25 μ M *PfPMT*-VMO and 1.75 μ M *PfCRT*-VMO (Fig. 2B–E).

PfCRT-VM0 Enhances Susceptibility of the Chloroquine-Resistant Dd2 to Chloroquine. Previous studies have shown that specific muta-

tions in the *PfCRT* gene render *P. falciparum* resistant to chloroquine and other 4-aminoquinolines (35). The ability to reverse the resistance phenotype through direct inhibition of *PfCRT* expression or by blocking drug transport activity is highly desirable. The growth of the chloroquine-resistant Dd2 strain (IC₅₀-131nM) was examined using flow cytometry in the absence or presence of *PfCRT*-VMO. As shown in Fig. 3A, no inhibition of parasite growth was observed after one 48-h cycle. However, after two cycles, *PfCRT*-VMO caused 30% growth inhibition at 2 μ M and 50% inhibition at 3 μ M compared with Ctrl-VMO (Fig. 3B). Because resistant alleles of *PfCRT* impart chloroquine resistance, reduced levels of mutated *PfCRT* protein should result in increased sensitivity to the drug. Dd2 parasites were therefore treated with Ctrl-VMO and *PfCRT*-VMO in the absence or presence of 50 nM chloroquine. As shown in Fig. 3, an approximate twofold increase in sensitivity of Dd2 to chloroquine was detected in the presence of *PfCRT*-VMO. Compared

with Ctrl-VMO, in the absence of chloroquine, *PfCRT*-VMO inhibited parasite growth by 29%, whereas in the presence of chloroquine growth inhibition reached 53% (Fig. 3 C and D).

Antimalarial Activity of *PfDXR*-PPMO Is Reversed by Isopentenyl Pyrophosphate. The *P. falciparum* gene 1-deoxy D-xylulose

Pyrophosphate. The *P. falciparum* gene 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*PfDXR*) is essential for intra-erythrocytic development and encodes an enzyme in the non-mevalonate pathway important for the synthesis of isoprenoids and targeted by the drug fosmidomycin (31). Because the loss of *PfDXR* can be complemented by addition of isopentenyl pyrophosphate (IPP), targeting this gene represents a unique way to validate the specificity of MO-mediated downregulation. An EGS for *PfDXR* was selected from the coding sequence of the gene on the basis of its ability to form a precursor tRNA-like structure when it binds to the target RNA using previously described methods (Table 1) (14). Potential Ribonuclease P cleavage sites within this sequence were determined (15, 39) (Fig. 4 A and B). Once these sites were identified, specific EGSs complementary to the selected sites were prepared and the mRNA-EGS complexes were again assayed to validate the choices. DXR130 was successful as an EGS: the mRNA in the DXR mRNA-DXR130 EGS complex was cleaved specifically as designed. The selected EGS (DXR-130EGS) was subsequently conjugated to a cell-penetrating L-arginine-rich peptide to produce *PfDXR*-PPMO (13) (*Materials and Methods*). Real-time PCR analyses on ring-stage parasites treated with *PfDXR*-PPMO showed a dose-dependent decrease in the *PfDXR* transcript following treatment with *PfDXR*-PPMO with 1 and 5 μ M resulting in 32 and 48% decreases in transcript levels, respectively (Fig. 4C).

The antimalarial activity of *PfDXR*-PPMO was also examined by microscopic analysis of Giemsa-stained blood smears as well as by flow cytometry. Dihydroartemisinin (DHA) was used as a positive control, and the *Staphylococcus aureus*-specific *Gyrase*-PPMO (*SaGyr*-PPMO) was used as a negative control. As shown in Fig. 4, *PfDXR*-PPMO inhibited growth of 3D7 parasites whereas the control *SaGyr* had no effect (Fig. 4 *D* and *E*). To assess the effect of this conjugate on drug-resistant parasites, the sensitivity of the artemisinin slow-clearance strain ART-SL₄₀₂₆ to *PfDXR*-PPMO was examined. This strain, which was isolated at the Thailand-Burma border, has a clearance rate of 8.37 h and a wild-type Kelch sequence (40) and grows slowly and asynchronously. DHA treatment

Fig. 2. *PfPMT*-VMO and *PfCRT*-VMO conjugates inhibit parasite growth. (A) 3D7 parasites were treated with 1.75 μ M of control-VMO, *PfPMT*-VMO, or *PfCRT*-VMO. Four representative images of Giemsa-stained smears after two cycles posttreatment are shown. (B–E) Luciferase-expressing parasites were treated with 1.25 or 1.75 μ M of Ctrl-VMO, *PfPMT*-VMO, or *PfCRT*-VMO, and luciferase activity was determined after one or two cycles of intraerythrocytic development. Growth as percentage of luciferase activity of *PfPMT*-VMO– (B and C) or *PfCRT*-VMO– (D and E) treated parasites normalized to Ctrl-VMO is shown. The experiment was carried out three times. The result represents data from a representative experiment; error bars indicate the SD of the average from three biological replicates.

Fig. 3. *PfCRT*-VMO enhances sensitivity of Dd2 parasites to chloroquine. Dd2 parasites were treated with 1, 2, or 3 μ M of Ctrl-VMO or *PfCRT*-VMO. Parasite inhibition was assessed by flow cytometry. Parasite growth in the presence of *PfCRT*-VMO was normalized to Ctrl-VMO as shown after one cycle (A) and two cycles (B). The effect of *PfCRT*-VMO on Dd2 sensitivity to chloroquine (CQ) was examined by treating Dd2 parasites with 2 μ M Ctrl-VMO or *PfCRT*-VMO in the absence or presence of 50 nM CQ (C). Parasite growth was examined by flow cytometry. The percentage inhibition was obtained by calculating the difference between Ctrl-VMO and *PfCRT*-VMO treatments in the absence or presence of CQ as a percentage of Ctrl-VMO treatment. (D) A representative flow plot comparing different treatments is shown. The presence of *PfCRT*-VMO increases parasite sensitivity to CQ, whereas a similar effect is not seen with Ctrl-VMO. The experiment was performed three times. The result represents data from a single experiment with the error bars indicative of the SD of the average from three biological replicates. The level of significance in the graph is indicated with an asterisk (* $P < 0.01$). R: ring-stage parasites; T: trophozoite-stage parasites.

resulted in a further decrease in growth. By 72 h, whereas untreated parasites showed a ring-to-trophozoite ratio of 1:3, DHA-treated parasites had a 1:1 ratio of the two stages. Interestingly, the inhibitory activity of *PfDXR*-PPMO was similar to that of DHA on the artemisinin slow-clearance strain (Fig. 4*F*). Flow cytometry analyses showed that *PfDXR*-PPMO causes 60–70% growth inhibition compared with *SaGyr*-PPMO at 15 μ M (Fig. 5*A* and Fig. S24). In the same assay, 1 μ M fosmidomycin showed 50% growth inhibition (Fig. 5*B*).

The specificity of *PfDXR*-PPMO-mediated inhibition was further demonstrated by the use of IPP (31). Similar to fosmidomycin, *PfDXR*-PPMO-mediated parasite inhibition was abrogated in the presence of IPP (Fig. 5C and D and Fig. S2B). An additive effect in parasite inhibition was observed following treatment with fosmidomycin and *PfDXR*-PPMO but not fosmidomycin and Ctrl-PPMO. As expected, addition of IPP alleviated parasite inhibition mediated by fosmidomycin and *PfDXR*-PPMO (Fig. 5D and Fig. S2B).

Discussion

Strategies aimed to down-regulate expression of genes may provide the ultimate approach to probing the function of all of the genes expressed by *P. falciparum* during its life cycle within human erythrocytes. Here we demonstrate the use of different morpholino-based targeting strategies to down-regulate expression of three *P. falciparum* genes. This is also the first time, to our knowledge, that an octaguanidine group has been used to deliver morpholino oligomer conjugates to *P. falciparum*. The presence of specific unspliced transcripts in the parasite indicates that it can be delivered to the nucleus of the parasite. Efficient parasite inhibition of wild type and the chloroquine-resistant Dd2 strain was obtained with *PfCRT*-VMO designed to alter splicing of *PfCRT*. This conjugate further enhanced sensitivity of this strain to chloroquine. It is noteworthy that modulating *PfCRT* levels in 7G8, another chloroquine-resistant strain, also resulted in a similar increase in chloroquine sensitivity (41).

Similarly, using PPMO-mediated degradation of target mRNA, we have shown successful down-regulation of *PfDXR* and increased sensitivity of the parasites to fosmidomycin.

The ability of *PfDXR*-PPMO to down-regulate gene expression and to inhibit parasite growth was achieved at higher concentrations compared with VMO conjugates. This could be due to differences in the efficiency of the delivery moiety (cell-penetrating peptide in the PPMO conjugates versus the octaguanidine-based dendrimer in the VMO conjugates). However, due to differences in the target sequence, conjugate formulation and approaches used for down-regulation, the potency of VMO and PPMO cannot be accurately compared.

Studies *in vivo* using mixtures of VMO conjugates showed toxicity due to hybridization between morpholinos leading to blood clots. Injections of VMO diluted in saline alleviated toxicity and also eliminated any hybridization potential within or between different VMOs, thus serving as a possible solution to the toxicity problem (34). Because we also observed toxicity at high concentrations, it is best to use these VMOs within the 1–3 μM range.

At times, the presence of unconjugated peptides in the PPMO preparation resulted in nonspecific parasite growth inhibition as was observed with the SaGyr PPMO control (Fig. 5). The nonspecific effect of peptides was not seen in the presence of 15 μ M conjugates or free peptides. However, concentrations higher than that due to carryover of unconjugated peptide resulted in toxicity.

Although PPMO- and VMO-mediated gene regulation has great potential for functional analysis and specific and selective inhibition of parasite growth, the delivery moiety and synthesis methods that result in consistently active conjugates must be optimized.

A great advantage of the methodology described here is the continued function of the MOs after three-point, noncontiguous mutations in the target RNA (42). Four mutations did not work. These facts make a possible therapy more valuable than current strategies where a single mutation in a gene affecting drug resistance converts parasites from drug sensitive to drug resistant or vice versa.

which the amount of target RNA was compared with *Pf-β-actin1*, which served as an internal control as previously described (45). Primers used for quantitative PCR (qPCR) are listed in Table S1.

Luciferase Assay. VMOs were added to a 96-well plate with the final concentration as indicated. A highly synchronized luciferase expressing early ring-stage parasite culture was added to the plate containing conjugate. Plates were incubated for one cycle and two cycles at 37 °C in a gas chamber. The luciferase assay was carried out using Renilla Luciferase assay system (Promega E2820) as described in the assay protocol. Briefly, for data collection, 100 μL of the culture was centrifuged to remove the media. The lysis buffer (30 μL) was added to culture and shaken at room temperature for 15 min. Subsequently, one freeze thaw cycle was used during which lysed culture was stored at –80 °C and thawed at room temperature. After thawing, the culture was kept at room temperature for at least 1 h. Thereafter, 10 μL of the lysate was aliquoted in a luminescence-compatible plate, and 50 μL of the assay buffer was added to the lysate. Plates were read on a Synergy MX Biotek plate reader for luminescence.

Flow Cytometry. For flow cytometry, cultures were treated as described above. After one and two cycles, 25 μL of culture was aliquoted into a U-bottom 96-well plate. The culture was washed with flow buffer (PBS with 1% FBS and 2 μM EDTA) and stained with Hoechst 33342 (Molecular Probes, R37605) or dihydroethidium (Sigma, R37291) for 25 min followed by washing with flow buffer. A 0.05% glutaraldehyde solution was used for fixing the sample before data collection in the STD-13L flow cytometry machine. Flow Jo was used for data analysis.

Statistical Analysis. Statistical analyses was carried out in graphpad using unpaired Student's *t* test.

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