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Original Article

Potential of *RH5* Antisense on *Plasmodium falciparum* Proliferation Abatement

Sepand Razavi Vakhshourpour [1], *Mehdi Nateghpour [1,2], *Nader Shahrokhi [3], Afsaneh Motevalli Haghi [1], Mehdi Mohebali [1], Haleh Hanifian [1]

1. *Department of Medical Parasitology & Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
2. *Research Center of Quran, Hadith, and Medicine, Tehran University of Medical Sciences, Tehran, Iran*
3. *Department of Molecular Biology, Pasteur Institute of Iran, Tehran, Iran*

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***Correspondence Email:**
nateghpourm@sina.tums.ac.ir;
shahrokhi@pasteur.ac.ir

Abstract

Background: Infections by *Plasmodium falciparum*, are becoming increasingly difficult to treat. Therefore, there is an urgent need for novel antimalarial agents' discovery against infection. In present study, we described a 2'-O-Methyl gapmer phosphorothioate oligonucleotide antisense targeting translation initiation region of 3D7 strain *RH5* gene.

Methods: The study was conducted in Pasteur Institute of Iran in 2020. ODNs effects were measured by microscopic examination and real time RT-PCR. For microscopy, microplates were charged with 2'-OMe ODNs at different dilutions. Unsynchronized parasites were added to a total of 0.4 ml (0.4% parasitemia, 5% red blood cells), and slides were prepared. Proportion of infected cells was measured by counting at least 500 red blood cells.

Results: *RH5* genes start codon regions selected as conserved region besed on alignment results. Gap-*RH5*-As which was complementary to sequence surrounding AUG *RH5* start codon significantly reduced parasite growth (>90% at 50 nM) compared to sense sequence control (Gap-*RH5*-Se) (17%), (P<0.001). *RH5* transcripts were dramatically reduced after exposed to ODNs at a concentration of 5-500 nM for 48 h.

Conclusion: Gemnosis delivery of a chimeric gapmer PS-ODN with 2'-OMe modifications at both sides had high antisense activity at low concentrations (10-100 nM) and shown a good efficiency to reach to target mRNA in human RBCs. Anti-parasite effect was correlated to reduction of target gene mRNA level. In addition, 2'-OMe ODNs free delivery is an effective way and does not need any carrier molecules or particles.

Introduction

Antisense (AS) oligodeoxynucleotides (ODNs) are a promising alternative chemotherapeutic strategy for inhibiting specific mRNA translation to protein (1). They are single-stranded DNA or RNA molecules paired with a target gene, consisting of about 15-25 nucleotides. Via blockade of transcription or translation of target genes, they may inhibit gene expression at RNA level. Since their high specificity and effectiveness on one hand, and low toxicity, on or hand, antisense oligonucleotides are very good candidates to use in gene and chemotherapy (2). Furthermore, it is easily possible to create new AS ODNs, based on every new point mutation to remove mutation results. There are some unstable, non/poor-specific, and potentially toxic natural antisense oligonucleotides (ASOs). Therefore, in vivo, they are quickly degraded with some side effects (3). Drug ASOs, try to overcome problems of natural ones by different modifications in different generations. Most popular form of first-generation antisense ODNs was phosphorothioate oligodeoxynucleotides (S-ODNs) (4) which their main disadvantage was relatively low stability of duplexes with complementary RNA and some toxic effects. This disadvantage could be linked to non-specific protein-S-ODN interactions (5).

2'-O-sugar modifications which are common in second-generation antisense ODNs, responsible to improve stability of duplexes with complementary RNA (6). Since these duplexes lack structure required to trigger RNase H, they are often interrupted with a gap of phosphorothioate nucleotides lack sugar modification. Chimeric gapmer S-ODNs, 2'-O modifications had antisense efficacy (7). In 2'-O-methyl chimeras, a gap size of eight was needed for optimal target mRNA reduction. As it is cost-effective, 2'-O-methyl modification remains one of most valuable 2'-O-sugar modifications (8).

P. falciparum's genome contains approximately 80% AT bp, making it one of most AT-rich genomes known makes it possible to use sequence-specific antisense oligonucleotides to target parasite's genome(9). ODNs may be highly specific to several parasite-specific mRNA targets, resulting in drug candidates are less toxic, highly specific, and easy to combine to target multiple genes for increased efficacy (10).

Since 1990s, ODNs are used to target a variety of genes in *P. falciparum*. Two major families of *P. falciparum* adhesions, released most likely from microneme organelles in response to intracellular signaling, are responsible for initial close contact with erythrocyte (11).

Reticulocyte-binding protein homolog 5 family as one of these groups belongs to *P. falciparum* reticulocyte-binding-like homolog (Rh) family of ligands, which are released by parasite during invasion and are necessary for host cell recognition by interacting directly with receptor proteins on erythrocyte surface (12-14). Merozoite invasion of human red blood cell (RBC) is a critical step in complex life cycle of merozoite, which necessitates several interactions between merozoite invasion-related ligands and host receptors (15). *RH5* and erythrocyte-binding-likes (EBLs) are well-known among large number of invasion proteins play a role in this process (16). To classify appropriate RBCs, *RH5* performs an initial sensing role (17). Furthermore, by controlling merozoite Ca2+ signaling, *RH5* have been related to merozoite invasion. Cell division, protein trafficking, transcription, and cell motility have all were regulated by Ca2+ signaling in eukaryotes (18). *RH5* is only member of RH family has yet to be knocked out of all parasite strains studied. *RH5* is expressed in rhoptry and secreted to merozoites' surface during invasion, according to some researches (19).

In this study, we used S-ODN sequences with some modifications to decrease its side effects and increasing its effectiveness (2'-O-methyl modifications). Fortunately were successful in decrease *RH5* protein levels in *P. falciparum* cultures in human red blood cells and shows effectiveness of S-ODNs in blocking attachment of parasites to RBCs and consequently lowering its invasion and multiplication within (20). This is the first report of 2'-*O*-methyl modification, which results in a reduction of important non-specific effects on *P. falciparum* growth.

Materials and Methods

Cultivation of P. falciparum 3D7 strain

3D7 strain of *P. falciparum* was provided by National Malaria Reference Laboratory (21). Giemsa staining was used to stain malaria parasites in red blood cells in National Malaria Laboratory of Tehran University of Medical Sciences, Tehran, Iran in 2019. To determine adaptation time to reach maximum parasitemia, highest percentage of parasitemia in ring stage considered more than 85%.

For cultivation of *P. falciparum* parasites, small plates with a capacity of 3-4 ml of culture medium were used. 3D7 strain of *P. falciparum* parasite was added to culture plate containing complete culture medium, washed red blood cells and Albumax. Some amounts of washed RBCs were added to suspensions until contents to being reached 10% hematocrit. Plates were incubated in jar at 37 °C. Ten percent parasitemia was cut off to transfer half of parasites to new ones to avoid acidic products of parasite metabolism. Refreshment of culture medium and transfer of parasites continues until reaches an adequate number of parasites for experiments.

P. falciparum culture medium refreshing

Upper layer of biphasic mix including metabolism products of parasites and lower layer including pellet contains red blood cells and parasites. After removing supernatant, thin smears were prepared and evaluated under microscope to check growth condition of parasites.

Parasitemia estimation

One drop of red blood cells containing parasite was taken and placed on a clean slide. Uniform thin smears were prepared and stained by Giemsa staining method. Parasitemia of 50 microscopic fields and 10,000 red blood cells counted. Each parasite inside or outside of red blood cell and each schizont was counted as one parasite. Ratio was calculated in percent.

Evaluation of Rh5 gene structure

Different available sequences of *Rh5* gene were downloaded from NCBI databases to find conserved regions of *RH5* gene as targets of antisense ODNs. They were analyzed by Vector NTI 11 software (Invitrogen).

Computational screening of accessible regions in mRNA of target genes

Target genes sequence alignment (*P. falciparum RH5* gene, GenBank AC: KU526814.1) done by BLAST and Clustal W. *P. falciparum RH5* mRNA Secondary structure predicted by mfold web server (http://www.unafold.org) and reconfirmed by RNA structure 5. An oligonucleotide from sense strand sequence was used as a negative control.

Antisense Synthesis

Antisense oligonucleotide was synthesized by Mycrosynth, Switzerland. Conjugates were purified by HPLC. Characterization in terms of purity and identity was done by HPLC and MALDI-TOF mass spectrometric analyses. All conjugates exhibited masses within experimental error corresponding to calculated mass and were >95% pure by HPLC analysis. We designed classic gapmer ODNs: RNase H activating phosphorothioate DNA domain (DNA gap) is flanked by short RNA sequenc-

es in 2'-OMe backbone at both ends (wings) to protect ODNs from degradation by nucleases (Table 1).

Table 1: Sequence, and melting temperatures of oligomers

Oligomer	*RH5 target position*[a]	*Sequence (5'-3')*[b]	*Tm (°C)*	*Total Length*	*DNA Gap Length (nt)*
Gap-*RH5*-As	-7+11	UUAUUC*T*T*T*A*T*C*A*T*T*C*UAUUA	44.1	20	10
Gap-*RH5*-Se	sen	UAAUAG*A*A*T*G*A*T*A*A*G*AAUAA	44.1	20	10

[a] Position of first nucleotide about GenBank accession no KU526814.1
[b] oligomers contained phosphorothioate linkages; underlined letters, 2'-*O*-methyl; bold letters

In vitro evaluation of challenge of oligonucleotide antisense against P. falciparum parasite strain 3D7

Concentration of oligonucleotide antisense was selected according to similar studies (9). Rate of parasitemia based on 0.6% and hematocrit on 10%. Due to structure of *RH5* gene, two different regions of gene were considered for antisense design. One type of antisense was used as a control for effect of antisense on parasite growth in vitro, which had no attach site with structure of target gene. Antisense arrived in form of lyophilized powder, which was first prepared in a 100 µM stock solution. Stock solution turned to 1 µM, 0.5 µM, 0.1 µM, 0.05 µM, and 0.01 µM solutions. A sense oligonucleotide with an equal concentration was used as a negative control. Challenge of 0.6% parasitemia and 10% hematocrit based on different concentrations of antisenses carried out in a 6 repeats manner. Half of wells were saved for Giemsa staining (corresponding to type of antisense and ir final concentration) and other half for real-time PCR.

Microscopic examination of effect of antisense on P. falciparum parasite 3D7 strain

Thin smears of three wells prepared and counted as follows:

$$\frac{\text{Parasite infected RBCsss}}{\text{Total RBCs}} \times 100$$

Nucleic acid extraction of P. falciparum strain 3D7

For DNA/RNA extraction of *P. falciparum* strain 3D7, QIAquick PCR Purification Kit (QIAGEN, Germany) was used according to manufacturer's user manual on pooled soups and pellets of 3 stored wells of each concentration.

Evaluation of effect of oligonucleotide antisense on P. falciparum strain 3D7 by PCR to assess RH5 gene amplification

Effect of oligonucleotide antisense on growth of *P. falciparum* and subsequently on rate of proliferation and expression of *RH5* gene was investigated. We tried to compare growth rate of subject parasites with non-affected parasites (control group) via using PCR method for amplifying *RH5*. DNA extraction of parasites was performed, DNaseI (KIAGEN IRAN) was added to extracted DNA samples to remove DNA of samples. Remained RNA used in process of expression of gene by relative Real-Time PCR (RELATIVE). *Plasmodium 18s-rRNA* gene was used as a reference gene. Real-time PCR experiments were done with *RH5* specific primers and SYBR green master mix (MERCK) (Table 2).

Real time PCR for assaying expression of RH5 gene

Real-time polymerase chain reaction was carried out with 5 µl of cDNA in a 20 µl reac-

tion consisting of 10 µl QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany), 0.5 µl of each primer (final concentration = 0.25 µM), and 4.5 µl of water using Rotor-Gene Q real-time PCR detection system (QIAGEN, Germany). Program was set as follows: pre-incubation at 95°C, 15 mins, 40 cycles, denaturing at 95 °C, 15 seconds, annealing at 60° C, 15 seconds, elongation at 72 °C, 15 seconds, 8 min final extension.

Statistical analysis was carried out on gene expression levels were normalized to chosen reference gene (18S rRNA) through -ΔCt analysis. Relative expression of each gene was determined by $2^{-\Delta\Delta CT}$ method. We used REST 2009 (QIAGEN Company) software for analyzing data.

Table 2: Sequences of primers against *RH5* and universal genes

Primer Name	*against*	*Sequence (5'-3')*	*References*
Pf *RH5*-F1007	*RH5 gene*	CAGATGATGAAACCGAAGAGG	This study
Pf *RH5*-R1233		TTG TTCAAAAAGGTT TGTACCATA A	This study
Pl Uni-1956f	*18srRNA*	CTACGTCCCTGCCCTTTGTA	34
Pl Uni-2127r		GATCCTTCCGC GGTTCAC	34
βg1	Human	GGGCTGGGCATAAAAGTCA	34
βg2	beta globin	AATAGACCAATAGGCAGA	34

Statistics

All statistical analyses were carried out in SPSS 17.0 (SPSS, Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD) of biological replicates analyzed using Student's *t*-test. *P* values of ≤ 0.05 were considered significant.

Results

Gapmering ODNs Designed against mRNA of Target Gene

Alignment of start codon regions of *RH5* genes had shown as conserved region and suitable as target for antisense design. Based on predicted secondary structure, start codon region of *RH5* gene was selected with lack of any obvious, stable secondary structure and being potentially *RH5* accessible to complementary antisense. Accordingly, gapmer 2'-OMe-ODNs were designed and used to trigger mRNA degradation through activation or employment of RNase H. RNase H activating phosphorothioate DNA domain (DNA gap) is flanked by short RNA sequences in 2'-OMe backbone at both ends (wings) to protect ODNs from degradation by nucleases. Sense and antisense gapmers ODNs were designed to target start codon of *RH5* gene (Table 1).

Growth inhibition of P. falciparum

P. falciparum 3D7 strain was exposed to different concentrations (5-1000 nM) of 2'-OMe-ODNs against *RH5* mRNA for 48 H to examine growth and proliferation un-synchronized parasites of malaria by microscopic and real time PCR methods. Microscopic measuring of parasitemia had shown significant growth inhibition in parasites incubated with increasing concentration of gap-*RH5* antisense compared with sense control ODN or with medium alone, suggesting sequence specific inhibition (Table 3).

Relative quantitative real time PCR using SYBR green carried out to investigate parasites growth. Specific primers against *Plasmodium* sp. 18sRNA and human β2 globin genes were used as specific and internal control genes respectively.

Table3: Effects of ODN-s on *P. falciparum* growth determined by Microscopy and real time PCR

ODN-S	***Concentration nM***	***Total parasitemia at 48h (%)***	***Inhibition of ring stage formation (%)***	***RH5 expression inhibition (%)***
Gap *RH5* -As	5	3.7	43	40
Gap *RH5* -Se	5	6.2	4.6	5
Gap *RH5* -As	10	1.5	77	55
Gap *RH5* -Se	10	5.9	9.2	10
Gap *RH5* -As	50	0.5	92	78
Gap *RH5* -Se	50	5.4	17	15
RH5 -7+11	100	0.4	94	80
RH5 -7+11 S	100	4.7	35	32
RH5 -7+11	500	0.4	94	83
RH5 -7+11 S	500	3.9	40	38
Control	-	6.5	0.0	0.0

In control assays, parasites proceeded through their full life cycle from ring forms to trophozoite and schizont forms and invasion of red cells to produce next generation of daughter rings.

Gap-*RH5*-As which was complementary to sequence surrounding AUG start codon of *RH5* significantly reduced parasite growth (>90% at 50 nM) compared to sense sequence control (Gap-*RH5*-Se) (17%) or with only medium alone, suggesting a sequence specific inhibition (Fig.1A-C) (P<0.001). Inhibition of parasite growth by this antisense was dose dependent in range of 5-100 nM. IC50 value for Gap-*RH5*-As was between 10 and 50 nM, whereas IC50 values for negative controls were 10-15 fold higher (Fig. 1D). Results from RT-PCR were similar to those from microscopic examination analysis. Inhibition of parasites was due to reduced numbers of ring stage parasites.

A summary of antimalarial activity for test ODNs by growth inhibition assay method is presented in Fig. 1. Designed 2'-OMe ODN which was complementary to sequence surrounding AUG start codon of *RH5* significantly reduced parasite growth (18.6 fold at 0.5 nM) compared to sense sequence control (No decrease) or medium alone (No decrease), suggesting a sequence specific inhibition (Fig.1). Inhibition of parasite growth by this antisense was dose-dependent in range of 0.005–0.5 nM. Results from RT-PCR were similar to those from microscopic examination analysis. Inhibition of parasites was due to reduced numbers of ring-stage parasites.

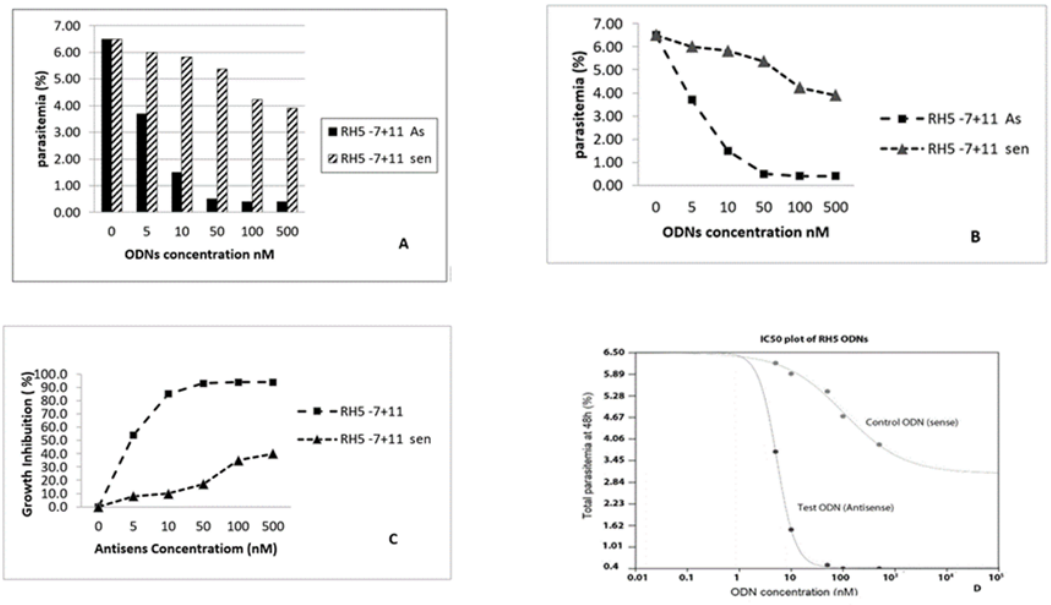

Fig. 1: Effects of 2'-OMe PS ODNs against *P. falciparum RH5*, determined by microscopic examination after 48 hr (A, B) Reduction in parasitemia by ODN specific for start codon site on *P. falciparum RH5* mRNA Starting parasitemia for challenge experiments was 0.6%. Final parasitemia in both control groups reaches 6.5% after 48 hours versus 0.4% to 3.7% in test groups. (C) Growth inhibition of *P.falciparum* in human RBCs using different concentration of 2-OMe ODN targeting *RH5* mRMA. Parasite growth were dramatically reduced after exposed to ODNs at a concentration of 5-500 nM for 48 hr D: IC50 of antisense and sense ODNs against *P.falciparum* culture parasitemia in human RBCs culture. MLA "Quest Graph™ IC50 Calculator." AAT Bioquest, Inc, 12 Apr. 2021, https://www.aatbio.com/tools/ic50- calculator. APA AAT Bioquest, Inc. (2021, April 12). Quest Graph™ IC50 Calculator.". Retrieved from https://www.aatbio.com/tools/ic50-calculator

Down-regulation of P. falciparum RH5 mRNA transcripts by AS-ODN

Molecular antisense mechanism of Gap-RH5 antisese in parasites is believed to rely on degradiation of *RH5* mRNA. In other words, 2' OMe antisense silencing in parasites leads to repression and presumably degrading mRNA.

To test efficiency of 2'-OMe ODNs on suppressing expression of *RH5* gene, we performed relative real-time RT-PCR. RNA samples were isolated from *P. falciparum* cultured in human RBC treated with different concentration of gapmer ODNs. Untreated or sense ODN-treated human RBCs were used as controls.

RH5 transcripts were dramatically reduced after exposed to ODNs at a concentration of 5-500 nM for 48 hr. Therefore, ODNs targeting mRNA achieved more significant suppression on *RH5* transcripts than sense oligose targeting mRNA.

Upon treatment with ODN, as shown in Table 4 a significant ($P< 0.01$) reduction (up to 60%) in level of mRNA was observed compared with sens control.

Table 4: Effect of 2'-OMe ODN of *P. falciparum* parasitemia and relative target

Gap-RH5 As	***Concentration nM***				
	5	10	50	100	500
Parasitemia (%)	3.70	1.50	0.50	0.40	0.44
RQ = $2^{-\Delta\Delta Ct}$	-3.53	-3.00	-1.27	-0.82	-1.05

Quantity (RQ) value of *RH5* mRNA

Discussion

We aimed to examine 2'-OMe ODNs directed against *RH5* gene for ir capacity to inhibit parasite growth using 3D7 strain.

Our data showed growth of asexual blood-stage *P. falciparum* could be inhibited when malarial attachment is inhibited using antisense ODNs. These ODNs targeted gene for *RH5* and inhibit erythrocytes merozoite invasion in *P. falciparum*. Reduction in parasitemia is sequence specific and correlates with reduction in specific mRNA as well as binding activity of parasites (27).

Synthetic oligonucleotides and their chemical modifications have inhibited infectious agents' gene expression by sequence-specific antisense hybridization to target mRNAs (28). Recent work has shown antisense technology has potential to address antibiotic-resistance crisis, since resistance mechanisms for standard antibiotics apparently have no effect on antisense antimicrobials.

Our data shown nuclease-resistant modifications of oligodeoxynucleotide phosphorothioates are effective in nanomolar concentrations (5 to 500 nM) against growth of *P. falciparum*, in vitro. However, non-specific growth inhibition was observed at higher ODNs concentrations.

We reported gemnosis delivery of 2'-OMe oligonucleotide-containing samples in a final concentration of higher than 10 nM significantly reduced parasite growth compared to sense sequence-containing control samples or with medium alone, suggesting sequence specific inhibition (Table 3). This contention is in good accordance with previous studies showing oligonucleotides inhibit cellular gene expression in a sequence specific manner at low concentrations (29-31). At high concentrations (1 μM and more) both sense and antisense ODNs inhibit growth of parasites in a non-specific manner by polyanionic properties of oligonucleotides. Garg et al had shown a 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 biosynsis (31). Kolvzon et al had shown peptide nucleic acids analoges (PNAs) conjugates with a simple octa-D-lysine CPP successfully and specifically down-regulate *P. falciparum* gene expression, which significantly reduced parasites' viability (32). In this study, gmnosis (free) delivery of 2'OMe ODNs clearly demonstrate a high-pronounced sequence specific antisense effect and this could be due to sustained delivery of such ODNs leading to a relatively lower initial concentration. Foger and co-workers formulated topoisomerase II ASO into biocompatible chitosan based nanoparticles (NPs). However, over 50% growth inhibition was observed when using sense oligoes to same gene, indicating a significant non-specific effect (33).

In our study, we selected new target gene *RH5*, in accordance with newly annotated translation initiation site(s) in *P. falciparum RH5 gene*.

Interestingly we found decrease in *RH5* expression was accompanied with detectable changes in levels of its steady state mRNA levels. This implies mechanism by which 2'-OMe ODNs down regulate genes in *P. falciparum* is RNase H dependent.

Present study has identified an antisense oligonucleotide targeting translation initiation site of mRNA of *RH5* gene, which exhibits anti-malarial activity against *P. falciparum*. An antisense effectiveness was confirmed by significantly lower activity of sense controls, reduction of target mRNA levels upon ODNs treatment.

Conclusion

Chimeric gapmer PS-ODN with 2'-OMe modifications at both sides had high antisense

activity at low concentrations and gemnosis delivery of such ODNs had shown a good efficiency to reach to target genes in human RBCs. *RH5* mRNA is an effective antisense target and shows effectiveness of 2'-OMe PS-ODNs in blocking attachment of parasites to RBCs and consequently lowering its invasion and multiplication within RBCs to inhibit significantly growth of parasites in a nucleotide sequence-dependent manner .

Since antisense antimicrobials are a platform technology, they can be rapidly designed and synthesized to target almost any microbe. This reduces drug discovery time, and provides flexibility and a rational approach to drug development.

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Conflict of Interest

The authors declare that there is no conflict of interests.

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