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## Original Article

# Gene-Specific Antisense Oligonucleotide Therapy Targeting AMA1: A Potential Malaria Treatment

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***Abstract***

***Background:*** We evaluated the effects of 2'-O-methyl (2'-OMe) antisense oligonucleotides targeting the apical membrane antigen 1 (AMA1) gene of *Plasmodium berghei* using *In vivo* and *In vivo* models.

***Methods:*** This study was conducted from 2019 to 2022 in Tehran, Iran. Antisense gapmer oligonucleotides targeting conserved regions of the AMA1 gene were designed using Vector NTI, mfold, and sfold and synthesized with 2'-OMe modifications. *P. berghei* ANKA cultures were treated with various ASO concentrations, and parasitemia was assessed by Giemsa-stained smears and real-time PCR. *In vivo* efficacy was evaluated in BALB/c mice infected intraperitoneally with *P. berghei* and treated subcutaneously with ASOs or control oligonucleotides; parasitemia was monitored microscopically.

***Results:*** Both antisense oligonucleotides showed low cytotoxicity, with cell viability exceeding 89% at the tested concentrations. In vitro, both antisense oligonucleotides significantly reduced parasitemia compared with the sense control ($P$=0.01), with no significant difference between the two antisense sequences ($P$>0.05). Both oligonucleotides also reduced AMA1 expression, with the 183–202 oligonucleotide showing the greatest inhibitory effect. In vivo, the AMA1 183–202-treated group showed significantly lower parasitemia on day 2 post-infection ($P$=0.048), while lower parasitemia was observed in the antisense-treated groups on subsequent assessment days.

***Conclusion:*** 2'-O-methyl antisense oligonucleotides targeting AMA1 reduced parasite growth and AMA1 expression *In vivo* and were associated with reduced parasitemia in *P. berghei*-infected mice. These preliminary findings support further investigation of AMA1-directed antisense therapy as a potential gene-specific antimalarial strategy. Larger, adequately powered studies are required to confirm efficacy and evaluate ASO delivery, stability, safety, and durability of gene silencing.

## Introduction

Malaria remains one of the most prevalent parasitic diseases in tropical and subtropical regions. In 2023, approximately 263 million cases were reported, corresponding to 60.4 cases per 1,000 population at risk (1). The emergence of antimalarial drug resistance, particularly in *P. falciparum*, remains a major challenge. Resistance to chloroquine and sulfadoxine–pyrimethamine (SP) in *P. falciparum*, as well as reduced susceptibility of *P. vivax* to chloroquine, has also been documented in southern Iran (2-5).

Mutations in the *dihydrofolate reductase* (DHFR) and *dihydropteroate synthase* (DHPS) genes of *P. falciparum* are associated with antifolate treatment failure and SP resistance, with resistant genotypes identified in Iranian isolates. No routine malaria vaccination program is currently available, largely due to antigenic and genetic diversity among *Plasmodium* species. Consequently, malaria control remains dependent on pharmacological interventions, increasingly challenged by drug resistance and highlighting the need for novel therapies (6-11).

Genetic manipulation has gained attention as a promising approach to combat malaria by inhibiting or suppressing genes essential for parasite survival (12-15). Antisense oligonucleotides (As-ODNs) are short synthetic sequences designed to complement target mRNA, leading to mRNA cleavage or inactivation and inhibition of gene expression. Their specificity and rapid design make them promising candidates for treating parasitic diseases, including malaria (16). Thus, antisense technology represents a potential strategy for diseases complicated by drug resistance and the absence of effective vaccines (12,13,16,17).

To enhance the stability, efficacy, and bioavailability of As-ODNs, various chemical modifications have been introduced, categorized into first, second, and third generations. Second-generation As-ODNs incorporate alkyl modifications at the 2' position of the ribose sugar, which activates RNase H, an enzyme that degrades RNA in RNA-DNA hybrids. These As-ODNs feature a "gapmer" design, with a central DNA gap flanked by chemically modified arms resistant to nuclease degradation. Common modifications include 2'-O-Methyl (2'-OME) and 2'-O-Methoxyethyl (2'-MOE), which improve enzymatic stability, half-life in vivo, mRNA binding affinity, tissue uptake, and reduce toxicity relative to first-generation As-ODNs (16,17).

A key strategy for malaria management is targeting the asexual erythrocytic stages responsible for clinical disease. Proteins involved in merozoite invasion of red blood cells (RBCs) are promising therapeutic targets, as rhoptry and microneme proteins mediate host-cell recognition and invasion (18,19). Among these, apical membrane antigen-1 (AMA-1) is a critical invasion protein and major vaccine candidate; antibodies against its ectodomain can inhibit *P. falciparum* merozoite invasion (20). Notably, high nucleotide and haplotype diversity of PfAMA-1 and allelic diversity of PvAMA-1 have been reported in Iranian isolates (21,22).

This study represents the first use of both *In vivo* and *In vivo* approaches to investigate malaria antisense therapy targeting the AMA-1 gene and addresses a significant gap in current research by exploring the efficacy of antisense technology in malaria treatment.

## Materials and Methods

### *Mice and Ethical Considerations*

This study was conducted from 2019 to 2022 in Tehran, Iran. Male BALB/c mice aged 6–8 weeks and weighing 25 ± 5 g were obtained from the Razi Vaccine and Serum Research Institute (Karaj, Iran). Ten mice were used for parasite propagation and preparation of infected blood for *In vivo* culture, while 15 mice were randomly assigned to five groups (n=3/group) for the *In vivo* experiment. Animals were housed under standard laboratory conditions with a 12-h light/dark cycle at 22 ± 2 °C and

*ad libitum* access to standard rodent diet and water. All procedures were conducted in accordance with the Standard Guidelines for the Care and Use of Laboratory Animals and the ARRIVE guidelines. Before euthanasia, mice were anesthetized with ketamine (75 mg/kg, subcutaneously) and euthanized by cervical dislocation. All efforts were made to minimize animal suffering and distress. The study was approved by the Ethics Committee of Tehran University of Medical Sciences (Ethics No. IR.TUMS.VCR.REC.1397.1071).

### *Maintenance of Parasites in In vivo Culture*

The *P. berghei* ANKA strain was propagated in BALB/c mice by intraperitoneal inoculation of $1 \times 10^6$ infected red blood cells (iRBCs). At 7 days post-infection, when parasitemia exceeded 10%, blood was collected by cardiac puncture under ketamine anesthesia (75 mg/kg). After removal of the buffy coat, parasites were cultured in RPMI 1640-based complete culture medium containing hypoxanthine, gentamicin, and 10% fetal bovine serum, as previously described (23). Cultures were maintained at 34 °C in a candle jar with shaking at 100 rpm for 20 h. The medium was replaced daily, hematocrit was maintained at 10% using erythrocytes from healthy mice, and Albumax II (50 µL) was added every 3 days to support parasite growth (23).

### *Computational Design and Selection of AMA1 Target Sites*

Conserved regions of the *P. berghei* AMA1 gene were identified from NCBI sequences and analyzed using Vector NTI version 11 (Invitrogen) and BLAST. Potential target sites were selected based on sequence conservation, GC content (40%–60%), predicted secondary structure, and mRNA accessibility. AMA1 mRNA secondary structure was predicted using mFold, while sFold was used to identify favorable target sites based on accessibility and hybridization probability. The Target Finder server, using the mRNA Accessible Site Tagging (MAST) algorithm, was subsequently used to identify accessible target regions within conserved AMA1 motifs. Binding free energies (ΔG) of As-ODN/AMA1 mRNA duplexes and potential As-ODN self-interactions were calculated using OligoWalk within the RNAstructure package to select sequences with favorable thermodynamic properties for hybridization at 37 °C. A sense-strand oligonucleotide was designed as a negative control.

### *Synthesis of Antisense Oligonucleotides*

Classic gapmer antisense oligonucleotides consisted of a phosphorothioate-modified DNA core flanked by 2'-O-methyl (2'-OMe)-modified RNA wings. Oligonucleotides were synthesized by Microsynth (Switzerland), purified by reversed-phase high-performance liquid chromatography (HPLC), and characterized by HPLC and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. All oligonucleotides had >95% purity and molecular masses consistent with their theoretical values (Table 1).

**Table 1:** Characteristics of Antisense Oligonucleotides Targeting the AMA1 Gene

| Oligomer | Target Position (nt)[a] | Sequence (5' → 3')[b] | Tm (°C) | Length(nt) |
|---|---|---|---|---|
| AMA1 antisense 1 | 117-136 | CAGTA T*T*T*T*C*C*T*T*T*T*T* GAATC | 42.0 | 20 |
| AMA1 antisense 2 | 183-202 | GCATC T*T*T*T*T*C*T*A*T*A*T* CATAT | 40.0 | 20 |
| **Sense (control)** | **Sense Strand** | **GTCAT A*A*A*G*G*A*A*A*A*A* CTTAG** | **40.0** | **20** |

[a] Position of the first nucleotide according to GenBank accession no. XM_034564734.

[b] Oligomers contained phosphorothioate linkages; starred letters indicate 2'-O-methyl modifications; underlined letters indicate the specified modification

### MTT Assay

Cytotoxicity of the synthesized antisense and sense oligonucleotides was evaluated in human dermal fibroblasts (HDFs) using the MTT assay. Cells (6,000/well) were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin in 96-well plates and incubated at 37 °C with 5% $CO_2$ for 24 h. Oligonucleotides (0.1, 0.5, 1, and 10 μM) were then added and incubated for 48 h. MTT (5 mg/mL) was added for 3 h, followed by DMSO to dissolve the formazan crystals. Absorbance was measured at 570 nm, and cell viability was expressed relative to untreated controls. Each concentration was tested in triplicate.

### In vivo Evaluation of Antisense Oligonucleotides against P. berghei

Antisense oligonucleotides were tested at 0.5, 0.1, 0.05, 0.01, and 0.005 μM in cultures containing 1% parasitemia and 10% hematocrit. Oligonucleotides were reconstituted in RNase- and DNase-free distilled water to prepare a 100 μM stock solution and serially diluted to the desired concentrations. A sense oligonucleotide was used as the negative control. Cultures were incubated at 34 °C in an anaerobic jar for 24 h, and experiments were performed in triplicate. Cell pellets and supernatants were collected for real-time PCR and parasitemia assessment.

### Parasitemia Assessment by Microscopy

Thin blood smears were prepared from cultured parasites, air-dried, methanol-fixed, and stained with 10% Giemsa solution (pH 7.2) for 15 min. Smears were examined at 1000× magnification under oil immersion, and parasitemia was calculated as the percentage of parasitized erythrocytes among 10,000 erythrocytes counted.

### Evaluation of AMA1 Gene Expression by Real-Time PCR

Parasite RNA was extracted using a Qiagen RNA extraction kit according to the manufacturer's instructions and treated with DNase I to remove genomic DNA contamination. Relative AMA1 expression was quantified by real-time PCR using AMA1-specific primers (Table 2) and SYBR Green Master Mix. Quantitative PCR was performed using an Applied Biosystems 7500 system. Relative gene expression was analyzed using the ΔCt and 2^−ΔΔCt methods and REST 2009 software (QIAGEN), with mouse β-actin used as the reference gene.

**Table 2:** Primer sequences for AMA1 and reference genes

| Primer Name | Gene | Sequence (5'-3') |
|---|---|---|
| Pb AMA-F347 | AMA1 | 5'- CCG GTG ATC AGT CAG TGA GA -3` |
| Pb AMA-R551 | AMA1 | 5'- ACA GCG GGG TGT CTA TAT GC -3` |
| Mouse B-actin F | β-actin | 5'-GGC TGT ATT CCC CTC CAT CG-3` |
| **Mouse B-actin R** | **β-actin** | **5'-CCA GTT GGT AAC AAT GCC ATG T-3`** |

### In vivo Evaluation of Antisense Oligonucleotides against P. berghei

Male BALB/c mice (25 ± 5 g) were randomly assigned to five groups (n=3/group) and intraperitoneally infected with 1 × $10^6$ infected red blood cells (iRBCs) from frozen parasite stocks. Three days post-infection, 100 μL of the respective treatment was administered subcutaneously in two doses within 24 h. The treatment groups are described in Table 3.

**Table 3:** Experimental groups used to assess the efficacy of antisense and sense oligonucleotides against *Plasmodium berghei* infection

| Group | Treatment Description |
|---|---|
| 1 | Infected mice treated with AMA1 antisense oligonucleotides targeting positions 117–136 at 1.6 µg/g body weight |
| 2 | Infected mice treated with AMA1 antisense oligonucleotides targeting positions 183–202 at 1 µg/g body weight |
| 3 | Infected mice treated with sense oligonucleotides at 1.6 µg/g (negative control) |
| 4 | Infected mice treated with chloroquine (positive control) |
| 5 | **Infected mice without treatment (infection control)** |

### *Parasitemia Assessment by Microscopy*

Blood samples were collected daily from the tail vein. Thin blood smears were prepared, air-dried, methanol-fixed, and stained with 10% Giemsa solution. Smears were examined at 1000× magnification, and parasitemia was calculated as the percentage of parasitized erythrocytes among total erythrocytes counted. Mean parasitemia was determined from three independent slides per sample.

### *Statistical analysis*

Statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD). Comparisons between experimental groups were performed using appropriate parametric tests, with $P \leq 0.05$ considered statistically significant.

## Results

### *In vivo assay*

MTT analysis showed >89% cell viability at all tested concentrations. AMA1 117–136 and AMA1 183–202 antisense oligonucleotides were subsequently evaluated at 0.005, 0.01, 0.05, 0.1, and 0.5 µM, using a sense oligonucleotide as the negative control. Both antisense oligonucleotides significantly reduced parasitemia compared with the sense control ($P=0.01$), with no significant difference between the two antisense groups ($P>0.05$) (Fig. 1).

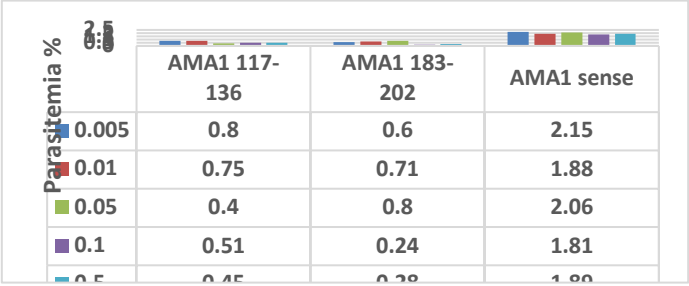


**Fig. 1:** Comparison of parasitemia rates between antisense oligonucleotide-treated groups and the *In vivo* sense oligonucleotide control group

### *Results of AMA1 Gene Expression Analysis In Vitro*

Both antisense oligonucleotides significantly reduced AMA1 gene expression compared with the negative control, with AMA1 183–202 showing the greatest inhibitory effect. The sense oligonucleotide showed no significant difference from the negative control (Fig. 2).

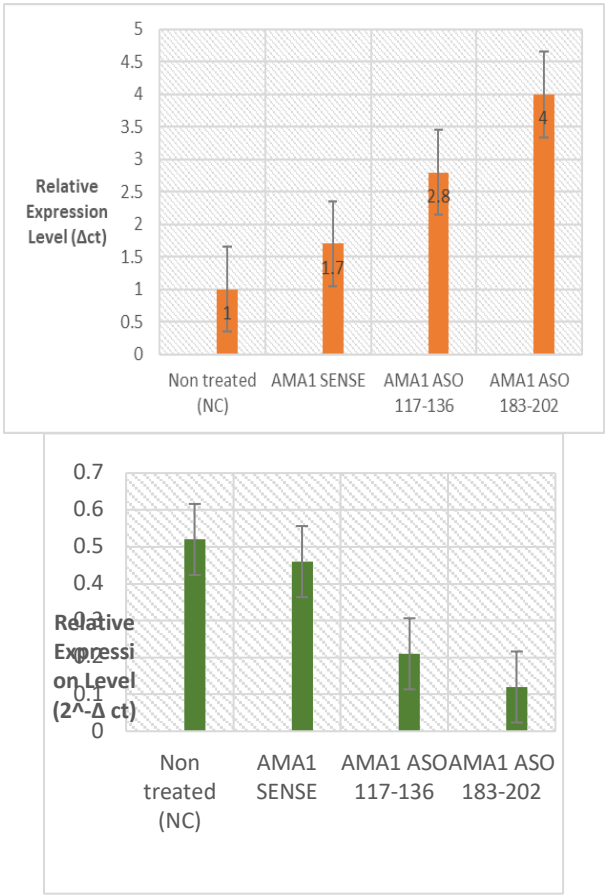


**Fig. 2:** Relative AMA1 expression in *P. berghei* cultures treated with antisense oligonucleotides targeting positions 117–136 and 183–202 compared with sense and negative controls. Data are presented as mean ± SD from three independent experiments. $P \leq 0.05$ was considered statistically significant

### *Relative AMA1 Gene Expression Based on 2−ΔΔCt*

Both antisense oligonucleotides significantly inhibited AMA1 expression compared with the negative control, whereas the sense oligonucleotide did not significantly differ from the control (Fig. 3).

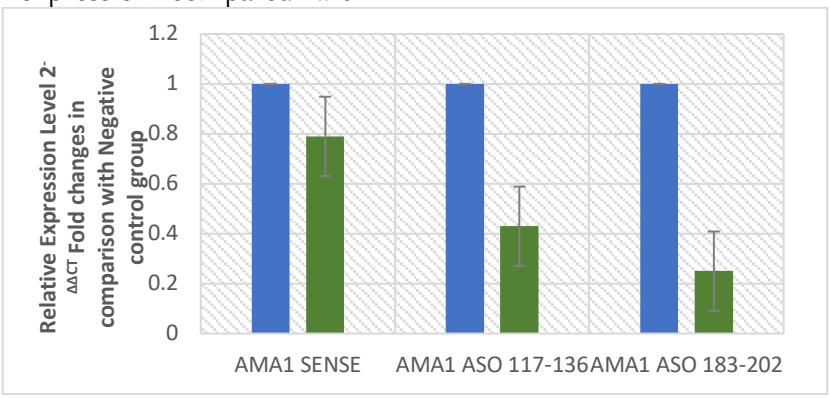


**Fig. 3:** Relative fold change in AMA1 expression in *P. berghei* cultures treated with antisense and sense oligonucleotides compared with the control group

### *In vivo Assay*

Parasitemia was evaluated on days 0, 2, 3, 4, and 7 post-infection. On day 0, no significant differences were observed among groups, with parasitemia ranging from 0.23% to 0.41% ($P$>0.05). On day 2, mice treated with AMA1 183–202 showed significantly lower parasitemia ($P$=0.048). On days 3 and 4, parasitemia was lower in the antisense-treated groups ($P$=0.032), whereas the differences between the antisense and sense groups were not statistically significant ($P$=0.077 and 0.083, respectively) (Fig. 4).

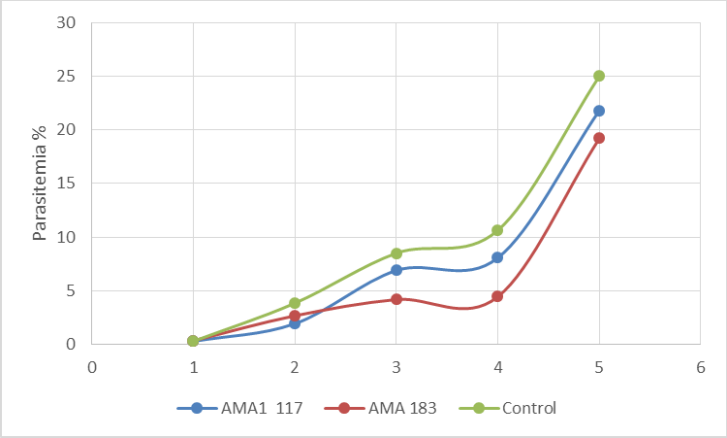


**Fig. 4:** Parasitemia in *P. berghei*-infected BALB/c mice following treatment with AMA1 antisense oligonucleotides and control treatments over the observation period

## Discussion

In this study, antisense oligonucleotides targeting two regions of the *P. berghei* AMA1 gene, AMA1 117–136 and AMA1 183–202, were evaluated for their effects on parasite growth and AMA1 expression using *In vivo* and *In vivo* models. Both antisense oligonucleotides reduced parasitemia and AMA1 expression in vitro, while the *In vivo* experiment showed reduced parasitemia following antisense treatment.

AMA-1 is an invasion-associated micronemal protein that plays an important role in *Plasmodium* host-cell invasion. It interacts with rhoptry neck proteins during merozoite invasion and is considered an important target for antimalarial intervention and vaccine development (24-26). The high immunogenicity and genetic diversity of AMA-1, including substantial diversity reported among Iranian *P. falciparum* and *P. vivax* isolates, further support its biological and immunological importance (27,28). These characteristics provided the rationale for investigating AMA1 as a target for antisense-mediated gene suppression.

In the present study, both AMA1 antisense oligonucleotides significantly reduced parasitemia compared with the sense control, with no significant difference between the two target sequences. This finding suggests that both selected regions can support antisense activity under the conditions tested. The reduction in AMA1 expression observed by real-time PCR further supports a gene-specific effect of the antisense oligonucleotides.

Antisense strategies have previously been investigated against several parasitic pathogens. Antisense-mediated inhibition has been reported for *Leishmania major*, while RNA-based approaches have been used to investigate gene function in *Toxoplasma gondii* and antisense peptide nucleic acids have been employed to inhibit gene expression in *Entamoeba histolytica* (29-33). In *P. falciparum*, sense and antisense

noncoding RNAs have also been identified in association with the *var* gene family, suggesting a potential role for antisense transcription in parasite gene regulation (34,35). These findings demonstrate the relevance of antisense and RNA-based mechanisms in parasite biology and support further investigation of sequence-specific nucleic acid approaches for functional gene modulation.

Similar approaches have also been explored in malaria parasites. Antisense oligodeoxynucleotides targeting *P. falciparum* genes have been reported to inhibit parasite growth, while peptide nucleic acids have been used to manipulate gene expression in *P. falciparum* (36,37). More recently, a 2'-O-methyl gapmer targeting the RH5 translation initiation region reduced *P. falciparum* proliferation and RH5 transcript levels, providing further evidence that chemically modified antisense oligonucleotides can be used to interfere with parasite gene expression (38). Together with these previous findings, our results support the feasibility of targeting essential or invasion-associated *Plasmodium* genes using antisense approaches.

In the present study, the chimeric gapmer oligonucleotides containing phosphorothioate and 2'-O-methyl modifications produced biological activity without an additional delivery carrier. This observation is consistent with previous work reporting antiplasmodial activity of antisense oligodeoxynucleotides in the absence of specialized delivery systems (39). However, because cellular uptake and intracellular localization were not directly assessed in the present study, the precise mechanism underlying carrier-free activity remains to be determined.

The *In vivo* findings were generally consistent with the *In vivo* results. Parasitemia was significantly reduced in the AMA1 183–202-treated group on day 2, while lower parasitemia was observed in the antisense-treated groups on subsequent assessment days. No significant difference was observed on day 0, before sufficient exposure to treatment, which is consistent with the experimental design. These findings provide preliminary evidence that AMA1-directed antisense treatment can influence parasite growth in vivo; however, the small sample size warrants cautious interpretation.

Previous studies have also demonstrated the protective potential of AMA1-based interventions. Lee et al. reported that immunization with AMA1-containing malaria virus-like particles induced immune responses and reduced parasitemia in *P. berghei*-infected mice (40). Although immunization and antisense-mediated gene suppression act through different mechanisms, these findings, together with the present results, support AMA1 as a biologically relevant target for antimalarial intervention.

Despite these encouraging findings, several challenges remain before antisense oligonucleotides can be considered for therapeutic development. These include optimization of oligonucleotide stability, cellular uptake, intracellular trafficking, tissue distribution, and target-specific delivery. The present study provides preliminary evidence of biological activity without an additional carrier, but direct assessment of ASO uptake, intracellular localization, pharmacokinetics, and durability of gene suppression will be necessary in future studies.

## Limitations

The main limitation of this study was the small sample size (n=3 per group), reflecting its exploratory pilot design and ethical considerations regarding animal use. The findings should therefore be interpreted cautiously. Larger, adequately powered studies are needed to confirm these results and to further evaluate the efficacy, safety, and reproducibility of AMA1-targeted antisense therapy.

## Conclusion

Antisense oligonucleotides targeting conserved regions of AMA1 reduced gene expression and parasitemia *In vivo* and decreased parasitemia *In vivo* in *P. berghei* infection models. These findings provide preliminary evidence supporting AMA1-directed antisense therapy as a potential gene-specific antimalarial strategy. Larger, adequately powered studies are needed to confirm these findings and evaluate ASO chemistry, delivery, long-term safety, durability of gene silencing, additional molecular targets, and potential combination with established antimalarial drugs.

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## Conflict of interest

The authors declare that there is no conflict of interests.

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