



Multitarget approach to babesiosis therapy: An *in vitro* study of therapeutic compounds

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Babesiosis is a vector-borne parasitic disease caused by intraerythrocytic protozoa of the genus *Babesia* and represents a significant threat to veterinary health and zoonotic safety. Among the clinically most relevant species in Europe, *Babesia divergens* is of particular concern due to increasing reports of high prevalence, highlighting the urgent need for novel treatment strategies. In this study, we evaluated the *in vitro* antiparasitic activity of two compounds with distinct mechanisms of action: atovaquone, a well-established inhibitor of the mitochondrial bc₁ complex, and deferoxamine, a trivalent iron chelator with the potential to disrupt essential intracellular metabolic processes of the parasite. *B. divergens* was cultivated in bovine erythrocytes, and parasite growth was quantitatively assessed using DNA-specific fluorescent staining combined with flow cytometry. Atovaquone demonstrated potent antiparasitic activity at low concentrations, whereas deferoxamine exhibited moderate but statistically significant inhibitory effects at higher concentrations, likely through interference with iron-dependent metabolic pathways. Notably, combined exposure to atovaquone and deferoxamine at subtherapeutic concentrations resulted in an additive inhibitory effect, achieving over 82% suppression of parasite growth, suggesting complementary modes of action. In addition, this study highlights methodological challenges associated with the standardization of *in vitro* *Babesia* culture systems in Ukraine, including optimization of cultivation conditions, incubation parameters, staining protocols and analytical strategies. Collectively, these findings support the potential of multitarget approaches that simultaneously disrupt parasite energy metabolism and iron homeostasis and provide a framework for the development of novel antibabesial agents and harmonized *in vitro* screening methodologies.

Keywords: *Babesia divergens*; babesiosis; atovaquone; deferoxamine; flow cytometry; *in vitro* study; antiparasitic therapy.

Introduction

Babesiosis is a zoonotic parasitic disease caused by intraerythrocytic protozoa of the genus *Babesia* and represents a major challenge for both human and veterinary medicine. Traditionally, the disease has been most prevalent in temperate climatic zones; however, over recent decades, a marked increase in the number of reported cases and a gradual expansion of its geographic distribution have been documented (Gray et al., 2019; Hildebrandt et al., 2021). These trends are largely attributed to changes in vector ecology, climate conditions, and increased awareness and diagnostic capacity.

Babesiosis of cattle and small ruminants remains one of the leading parasitic causes of economic losses in livestock production, resulting in reduced productivity, increased mortality, and substantial financial burdens associated with treatment, prevention, and control of tick vectors. The cumulative costs incurred by farmers and veterinary services significantly amplify the economic impact of the disease at both regional and national levels (Bock et al., 2008).

Despite the evolving epizootiological situation and the growing relevance of babesiosis, the repertoire of available antibabesial drugs has remained largely unchanged for several decades. In human medicine, the combination of atovaquone and azithromycin is currently considered the first-line therapy, whereas clindamycin in combination with quinine is applied as an alternative regimen. Nevertheless, the effectiveness of these approaches is supported mainly by limited clinical observations, and their use is frequently associated with a high incidence of adverse reactions (Krause et al., 2019; Bloch et al., 2021).

In veterinary practice, the treatment of babesiosis across different animal species faces similar limitations, with the restricted availability of effective antiprotozoal agents being a persistent concern. In dogs, imidocarb dipropionate is most commonly used; however, its efficacy varies depending on the infecting *Babesia* species, and treatment is often accompanied by pronounced side effects. For infections caused by *Babesia gibsoni*, combined regimens based on atovaquone and azithromycin have gained increasing acceptance in European practice,

demonstrating improved parasite clearance and reduced relapse rates. Prolonged courses of clindamycin in combination with doxycycline and metronidazole have also been employed, although these approaches still require standardization and validation in larger cohorts (Jefferies et al., 2007; Carli et al., 2025).

In cattle from Western and Central Europe, imidocarb- or diminazene-based treatments continue to be widely applied, with therapeutic outcomes strongly dependent on early diagnosis and disease severity. In contrast to Ukraine, where the use of certain chemotherapeutic agents is restricted or tightly regulated, several European countries have implemented well-defined protocols for monitoring drug residues in animal products, allowing a broader application of effective antibabesial compounds under intensive farming conditions (Bock et al., 2008). Similar therapeutic strategies are employed in small ruminants, including sheep and goats, where imidocarb and diminazene aceturate remain the mainstay of treatment. Recent European efforts have focused on dose optimization and toxicity reduction, whereas in Ukraine treatment protocols largely remain conventional and do not always account for species-specific differences in disease progression.

The frequent occurrence of relapses, reinfections, and chronic carrier states in dogs, cattle, and small ruminants underscores the urgent need for the development of novel antibabesial agents and the implementation of modern therapeutic strategies. Such approaches are actively explored in European research but remain largely underutilized in Ukrainian veterinary practice (Carli et al., 2025; Rimal et al., 2025).

Prolonged reliance on uniform chemotherapeutic regimens has increasingly revealed signs of emerging drug resistance in *Babesia* spp. Studies conducted on *B. microti* isolates have identified mutations in genes associated with the mechanisms of action of atovaquone and macrolides, potentially reducing parasite susceptibility and complicating treatment, particularly in relapsing infections and immunocompromised patients (Simon et al., 2017). These molecular adaptations raise concerns regarding the gradual loss of efficacy of current therapeutic strategies and highlight the necessity of integrating anti-re-

sistance principles into experimental *in vitro* platforms. Contemporary European research increasingly emphasizes the investigation of alternative inhibitors, targeted molecules, and combination pharmacological strategies, enabling the modeling of resistance risks and the identification of regimens with a higher likelihood of sustained therapeutic success (Renard & Ben Mamoun, 2021; Cardillo et al., 2025).

The advancement of *in vitro* cultivation systems for *Babesia* spp. has substantially expanded opportunities for screening antiparasitic compounds, elucidating their mechanisms of action, and evaluating pharmacodynamic properties. Historically, drug efficacy was primarily assessed using isotopic methods, such as measuring hypoxanthine incorporation in *Babesia*-infected erythrocyte cultures (Munkhjargal et al., 2013). In recent years, fluorescence-based assays employing DNA-binding dyes, including SYBR Green I, have become predominant due to their high sensitivity, reproducibility, and suitability for high-throughput screening (Rizk et al., 2015; Alvarez et al., 2020).

The application of flow cytometry combined with nucleic acid stains further extends the dynamic range of parasitemia detection and ensures robust reproducibility even at low infection levels (Rizk et al., 2020). Beyond quantitative assessment, cytometric approaches enable discrimination between viable and degenerative parasite forms, thereby substantially enhancing the informational value of experiments evaluating novel antiparasitic compounds. Together with light and fluorescence microscopy, which retain importance for morphological analysis, these techniques constitute the current standard for laboratory screening and have been successfully applied in studies involving *B. bovis*, *B. divergens*, *B. microti*, and *B. duncani* (Nguyen et al., 2019).

Despite considerable progress in *Babesia* cultivation methodologies, the lack of standardized *in vitro* protocols remains a critical issue for Ukraine. Experimental outcomes are highly sensitive to multiple variables, including hematocrit levels, erythrocyte source, culture medium composition, serum concentration, and incubation gas conditions, which complicates cross-study comparisons (Alvarez et al., 2020). Additional factors such as parasite strain selection, life-cycle stage, incubation duration, and data normalization or modeling methods can result in substantial variability in IC_{50} values, even for reference compounds. Consequently, the establishment of unified evaluation criteria, including standard control drugs, predefined sampling time points, statistical analysis parameters, and dose–response curve models, is essential to ensure reproducibility and comparability of future studies (Rizk et al., 2016; Rizk et al., 2020).

In this context, the present study provides an experimental evaluation of the antiparasitic activity of atovaquone and deferoxamine against *B. divergens* and examines the potential additive effects of their combined application. In addition, morphological alterations in infected erythrocytes were assessed, and modern quantitative approaches to parasitemia analysis were applied to substantiate the feasibility of a multitarget therapeutic strategy for babesiosis.

Materials and methods

Babesia divergens was maintained *in vitro* in bovine erythrocytes at 37 °C under a humidified atmosphere containing 5% CO₂. Cultures were propagated in RPMI-1640 medium supplemented with 20% heat-inactivated bovine serum (56 °C for 30 min), gentamicin sulfate (50 µg/mL) and amphotericin B (0.25 µg/mL).

Prior to the initiation of experimental treatments, parasitemia levels were determined using thin blood smears stained with a commercial Diff-Quick kit. Cultures with an initial parasitemia of 2% and a hematocrit of 5% were selected for subsequent experiments.

The *in vitro* antiparasitic activity of test compounds against *B. divergens* cultures was evaluated using a 96-well microplate format. Stock solutions of atovaquone (10 mM) were prepared in dimethyl sulfoxide (DMSO), whereas deferoxamine stock solutions (50 mM) were prepared in sterile distilled water.

Working solutions were freshly prepared in RPMI-1640 medium immediately before use, ensuring that the final concentration of DMSO in culture wells did not exceed 0.5%. Serial dilutions were ge-

nerated to obtain final concentration ranges of 0.001–1 µM for atovaquone and 3.1–100 µM for deferoxamine.

Untreated *B. divergens* culture without inhibitors served as the control. Each well contained 100 µL of infected erythrocyte suspension, consisting of 10 µL of infected erythrocytes and 90 µL of culture medium, followed by 100 µL of the corresponding drug solution to achieve a final volume of 200 µL per well.

Fresh inhibitor solutions were added to the wells at 24-hour intervals throughout the incubation period. Each concentration was tested in triplicate technical replicates, and all experiments were performed independently at least three times.

To enable high-throughput monitoring of parasitemia, flow cytometry was employed. Infected erythrocytes were pelleted by centrifugation, washed with 1× phosphate-buffered saline (PBS), and fixed in a solution containing 4% paraformaldehyde and 0.025% glutaraldehyde in PBS for 30 min at room temperature. Following fixation, cells were washed twice with PBS (600 × g, 3 min) and stored at 4 °C for up to three weeks prior to further analysis. For parasitemia quantification, cells were stained with the DNA-specific dye ethidium homodimer-1 (EthD-1; Biotium) at a final concentration of 0.02 mM diluted in PBS and incubated for 30 min at room temperature. The dye selectively binds to parasitic DNA, allowing clear discrimination between infected and non-infected erythrocytes. After incubation, samples were washed twice with PBS at room temperature (600 × g, 3 min). Flow cytometric analysis was performed using a BD FACSCanto II flow cytometer (Becton Dickinson, USA).

Half-maximal inhibitory concentrations (IC_{50}) and 90% inhibitory concentrations (IC_{90}), defined as the drug concentrations resulting in 50% and 90% inhibition of parasite growth, respectively, were calculated for each compound using nonlinear regression based on a four-parameter logistic model. Data analysis was performed with GraphPad Prism software version 9.0 (GraphPad Software, USA). All results are presented as the mean ± standard deviation (SD) of three independent experiments.

Results

After 48 h of cultivation in the presence of the tested compounds, a clear dose-dependent inhibition of *B. divergens* growth was observed. Microscopic examination of Diff-Quick–stained blood smears revealed varying levels of parasitemia and distinct morphological alterations of parasites in infected erythrocytes following treatment with both compounds (Figs. 1, 2).

In parallel with microscopic examination of Diff-Quick–stained blood smears, parasitemia levels of *B. divergens* were quantitatively assessed by flow cytometry using ethidium-based fluorescent staining. Flow cytometric analysis revealed differences in the proportion of infected erythrocytes among the experimental groups and demonstrated a reduction in parasitemia following treatment with the tested compounds.

After 48 h of incubation, erythrocyte populations were identified based on forward and side scatter parameters (FSC-A/SSC-A), allowing exclusion of cellular debris and aggregates (Fig. 3a). Infected and non-infected erythrocytes were subsequently discriminated according to PerCP-Cy5.5 fluorescence intensity resulting from ethidium binding to the DNA of intracellular parasite forms (Fig. 3d).

A distinct bimodal fluorescence histogram enabled reliable identification of the BDIV ETH⁺ cell population (Fig. 3c), while additional gating based on FSC-A/FSC-H parameters restricted the analysis to single-cell events and minimized the contribution of doublets (Fig. 3d).

Flow cytometric analysis showed that at a concentration of 0.01 µM of atovaquone, the proportion of infected cells decreased by 65–72% compared with the control. At 0.10 µM, parasitemia was reduced by 93–96%, which was consistent with microscopic assessment (94 ± 2%). Maximum inhibition of 98–99% was observed at a concentration of 1 µM.

Dose–response curve analysis of infected cells as a function of atovaquone concentration yielded the following values: $IC_{50} = 0.02 \pm 0.01$ µM and $IC_{90} = 0.10 \pm 0.01$ µM (Fig. 4).

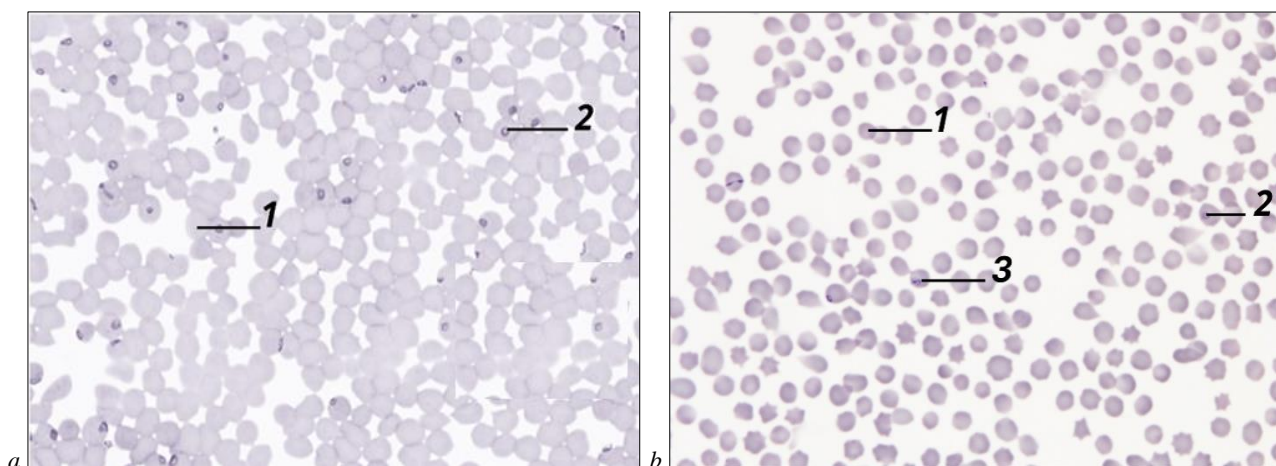


Fig. 1. Effect of atovaquone on *Babesia divergens* parasitemia in erythrocytes: representative Giemsa-stained blood smears showing (a) untreated cultures with numerous intact intraerythrocytic ring and paired forms and (b) cultures treated with atovaquone (0.01 μM , 24 h) with reduced parasitemia and fewer structurally preserved parasites; 1 – non-infected erythrocyte; 2 – erythrocyte infected with *B. divergens*; 3 – erythrocyte containing a structurally altered parasite after drug exposure

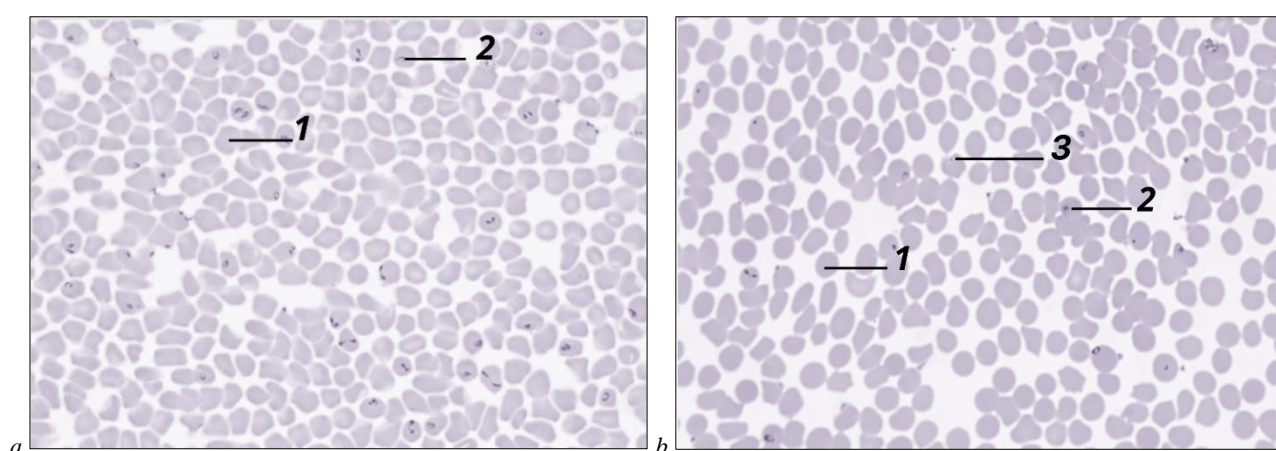


Fig. 2. Effect of deferoxamine on *Babesia divergens* in erythrocytes: representative Giemsa-stained blood smears showing (a) untreated cultures with numerous morphologically preserved intraerythrocytic parasites and (b) cultures treated with deferoxamine (12.5 μM , 24 h) exhibiting reduced merozoite numbers and parasites with drug-induced morphological alterations (see Fig. 1)

Flow cytometric determination of parasitemia following deferoxamine treatment revealed a moderate but statistically significant inhibitory effect. At a concentration of 25 μM , parasitemia decreased by 45–52%, whereas at 50 μM , a reduction of 68–74% was observed, which was consistent with microscopic assessment ($70 \pm 4\%$). Higher concentrations (100 μM) resulted in inhibition levels of 80–85% (Fig. 5). Dose – response analysis yielded the following values: $\text{IC}_{50} = 12.6 \pm 1.4 \mu\text{M}$ and $\text{IC}_{90} = 41.3 \pm 3.7 \mu\text{M}$. These values indicate lower antiparasitic activity of deferoxamine compared with atovaquone, which is consistent with its proposed metabolic mechanism of action.

In the untreated control culture and in the control containing 0.5% DMSO, the proportion of infected erythrocytes increased from an initial value of $2.0 \pm 0.2\%$ to 5.4–7.4% and 5.7–7.7%, respectively, which fully corresponded to the increase in parasitemia recorded by light microscopy.

Flow cytometric analysis demonstrated that combined treatment with atovaquone (0.01 μM) and deferoxamine (10.0 μM) resulted in an 80–85% reduction in parasitemia, indicating an additive or weakly synergistic inhibitory effect. This combined treatment produced a more pronounced decrease in the proportion of infected erythrocytes than either compound applied individually at the corresponding concentrations.

Comparative analysis further confirmed that atovaquone acts as a highly potent inhibitor of *B. divergens* growth *in vitro*, whereas deferoxamine exhibits moderate antiparasitic activity, particularly at higher concentrations. The inhibitory effect of deferoxamine is unlikely to result from direct cytotoxicity but rather from depletion of bioavailable iron required for essential parasite metabolic processes,

including the activity of iron-dependent mitochondrial enzymes. Taken together, these findings suggest that combination strategies targeting both mitochondrial electron transport and iron homeostasis may represent a promising approach for the further optimization of antibabesial therapy.

Discussion

The obtained results confirm that atovaquone is one of the most effective agents for inhibiting the *in vitro* growth of *B. divergens*, whereas deferoxamine, a classical iron chelator, exhibits moderate but statistically significant antiparasitic activity. The determined IC_{50} values ($0.02 \pm 0.01 \mu\text{M}$ for atovaquone and $12.6 \pm 1.4 \mu\text{M}$ for deferoxamine) are consistent with previously reported data on the activity of related compounds against other *Babesia* species (Guswanto et al., 2014; Rizk et al., 2020; Lau et al., 2021).

Atovaquone, a hydroxynaphthoquinone derivative, acts through inhibition of the mitochondrial bc_1 complex, thereby blocking electron transport in the oxidative phosphorylation chain at the level of cytochrome *b* (*cytb*) (Lobo et al., 2017; Korsinczky et al., 2022). This leads to collapse of the mitochondrial membrane potential, disruption of ATP synthesis, and parasite death. *B. divergens* possesses a mitochondrion with a single-copy genome, which makes this complex a particularly vulnerable therapeutic target. Previous studies have shown that mutations in the *cytb* gene of *B. microti* and *B. duncani* may result in reduced sensitivity to atovaquone (Lemieux et al., 2016; Lobo et al., 2017). Therefore, although the present study confirms the high efficacy of atovaquone against *B. divergens*, the potential risk of

resistance development during prolonged or suboptimal use should be taken into account. In this context, combined therapeutic approaches involving atovaquone and other antiparasitic agents or antibiotics,

such as azithromycin or doxycycline, have been proposed to reduce the likelihood of resistant strain emergence (Krause et al., 2021; Simon et al., 2021).

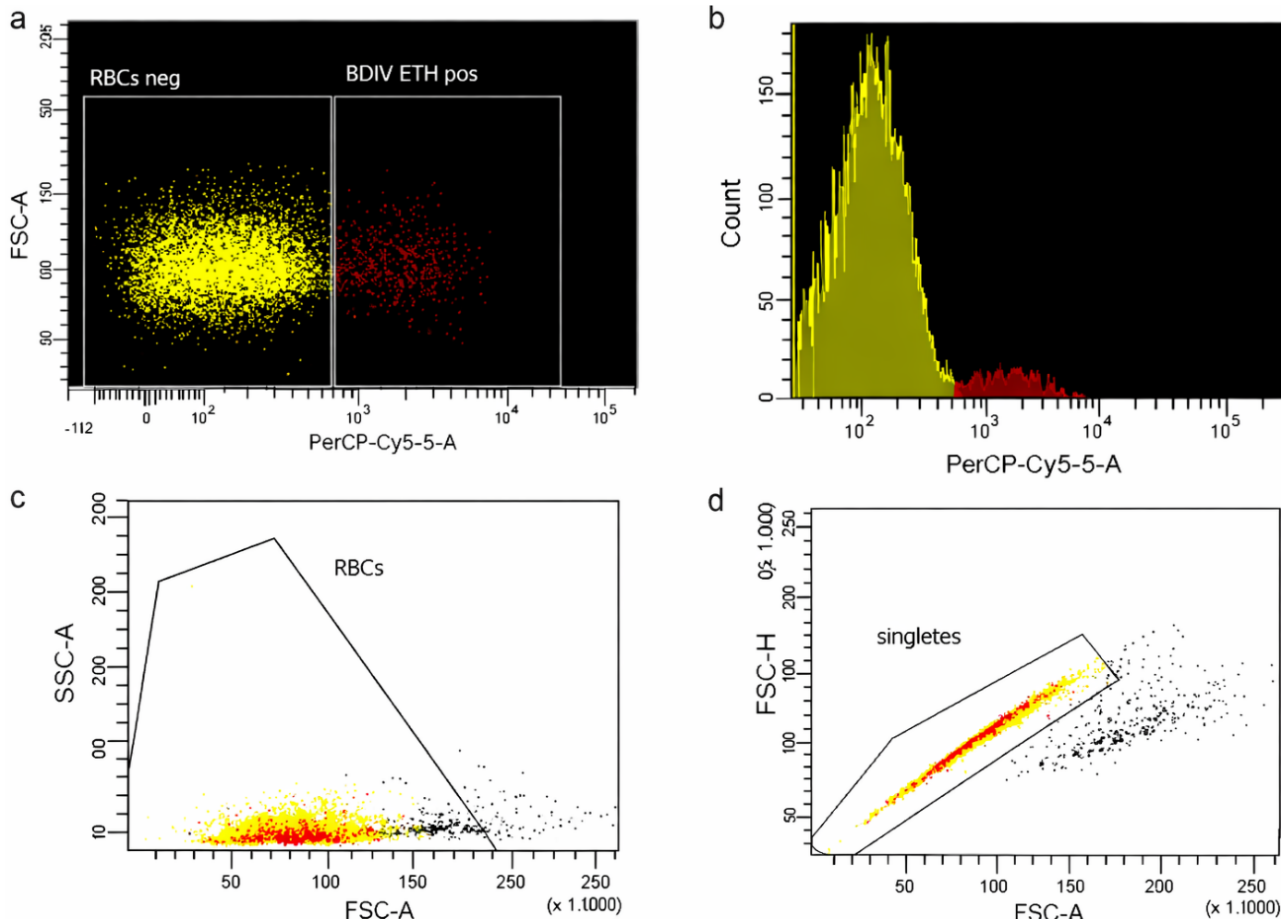


Fig. 3. Flow cytometric analysis of *Babesia divergens*-infected erythrocytes after 48 h of incubation with deferoxamine (DFO) using ethidium bromide staining: *a* – gating of the erythrocyte population based on forward scatter (FSC-A) and side scatter (SSC-A) parameters; *b* – discrimination of non-infected erythrocytes (Eth⁻) and *B. divergens*-infected erythrocytes (Eth⁺) according to PerCP-Cy5.5 fluorescence intensity; *c* – representative PerCP-Cy5.5 fluorescence histograms illustrating the distribution of non-infected and infected cell populations; and *d* – identification of single-cell events (singlets) using FSC-A/FSC-H gating

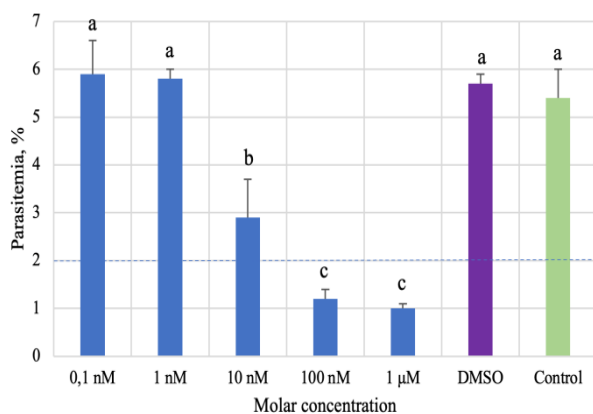


Fig. 4. Dose-dependent effect of atovaquone on *Babesia divergens* parasitemia after 48 h of *in vitro* cultivation: parasitemia (%) was determined in cultures exposed to increasing concentrations of atovaquone (0.1, 1, 10, 100 nM, and 1 μM) for 48 h; a progressive reduction in parasitemia was observed with increasing drug concentration, demonstrating a clear dose-dependent inhibitory effect; DMSO-treated cultures served as the solvent control and untreated cultures were used as the negative control; data are presented as mean ± SD from three independent experiments; data were analyzed using Tukey's test, different letters indicate statistically significant differences between groups ($P < 0.05$)

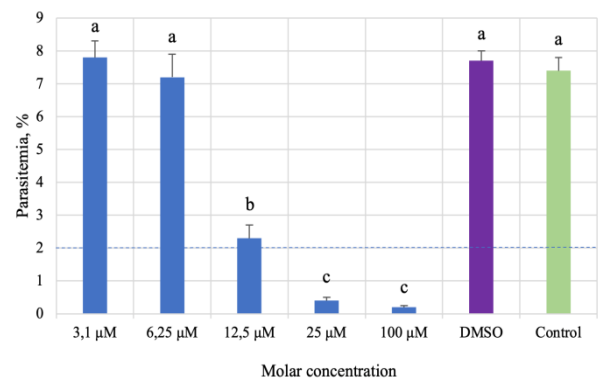


Fig. 5. Concentration-dependent inhibition of *Babesia divergens* growth by deferoxamine after 48 h in culture: parasitemia (%) was quantified following exposure of *B. divergens* cultures to increasing concentrations of deferoxamine (3.10, 6.25, 12.50, 25, and 100 μM) for 48 h; treatment resulted in a marked decline in parasite burden at ≥ 12.5 μM, with near-complete inhibition observed at 25–100 μM (see Fig. 4)

Deferoxamine is a well-characterized chelator of ferric iron (Fe³⁺) widely used in clinical practice for the treatment of iron overload disorders. In apicomplexan parasites such as *Plasmodium* and *Toxoplasma*, restriction of iron availability has been shown to suppress parasite growth, as iron is essential for the activity of respiratory chain enzy-

mes and DNA synthesis (Ganz & Nemeth, 2012; Schaer et al., 2013). In *Babesia*, the role of iron is less extensively studied; however, available evidence suggests that this metal is critical for the survival of intraerythrocytic stages (Arbon et al., 2024). Deferoxamine likely reduces intracellular iron availability, thereby disrupting parasite metabolism, particularly the function of iron-dependent mitochondrial enzymes. Our results demonstrated that high concentrations of deferoxamine ($\geq 50 \mu\text{M}$) significantly reduced parasitemia (by approximately 70%), supporting the hypothesis of iron-dependent growth of *B. divergens*. Similar effects have been reported for *Plasmodium falciparum*, where deferoxamine inhibits parasite growth through impairment of iron-sulfur cluster formation in electron transport enzymes (Tiwari et al., 2020). At the same time, excessive iron chelation may also affect host cells, indicating that the therapeutic window for such approaches may be limited.

Comparison of atovaquone and deferoxamine activity highlights substantial differences in their mechanisms of action, which opens perspectives for their combined use. The combination of an iron chelator with an electron transport inhibitor may induce dual metabolic stress: atovaquone suppresses mitochondrial ATP synthesis, whereas deferoxamine limits the availability of cofactors required for Krebs cycle enzymes and oxidoreductases (Lemieux et al., 2016; Korsinczyk et al., 2022). In the present study, the additive effect of atovaquone (0.01 μM) and deferoxamine (10 μM) was associated with inhibition of *B. divergens* growth at a level of approximately 82%, suggesting a metabolic interaction between respiratory pathways and iron homeostasis. Similar synergistic effects between iron chelators and antimalarial drugs have previously been reported (Guswanto et al., 2014; Tuvshintulga et al., 2019).

Overall, the results emphasize the key role of mitochondrial processes and iron homeostasis in the survival of *B. divergens* within erythrocytes. The high efficacy of atovaquone underscores its importance as a potent inhibitor, while deferoxamine demonstrates potential as a metabolic modulator capable of enhancing parasite susceptibility to other chemotherapeutic agents. Further studies should focus on evaluating combination therapies involving atovaquone with iron chelators or antibiotics, analyzing the expression of genes associated with iron transport and metabolism in *B. divergens*, and elucidating possible compensatory mechanisms of energy metabolism under conditions of respiratory chain inhibition.

Conclusion

This study demonstrates that targeting mitochondrial function and iron homeostasis represents an effective strategy for suppressing *B. divergens* growth under *in vitro* conditions. Atovaquone shows strong antiparasitic activity at low concentrations, whereas deferoxamine exerts a moderate inhibitory effect that becomes pronounced at higher doses. Importantly, their combined application enhances suppression of parasitemia compared with monotherapy, supporting the feasibility of a multitarget therapeutic approach. The integration of complementary quantitative methodologies provides a robust framework for evaluating antiparasitic efficacy and may facilitate the development of optimized treatment strategies for babesiosis.

The authors declare that there is no conflict of interest regarding the publication of this article.

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