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Original Article

Atovaquone, chloroquine, primaquine, quinine and tetracycline: antiproliferative effects of relevant antimalarials on *Neospora caninum*

Atovaquona, cloroquina, primaquina, quinino e tetraciclina: efeitos antiproliferativos de antimaláricos relevantes em *Neospora caninum*

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Abstract

Neospora caninum is an apicomplexan parasite that causes abortion in cattle, resulting in significant economic losses. There is no commercial treatment for neosporosis, and drug repositioning is a fast strategy to test possible candidates against *N. caninum*. In this article, we describe the effects of atovaquone, chloroquine, quinine, primaquine and tetracycline on *N. caninum* proliferation. The IC_{50} concentrations in *N. caninum* were compared to the current information based on previous studies for *Plasmodium* and *Toxoplasma gondii*, correlating to the described mechanisms of action of each tested drug. The inhibitory patterns indicate similarities and differences among *N. caninum*, *Plasmodium* and *T. gondii*. For example, atovaquone demonstrates high antiparasitic activity in all the analyzed models, while chloroquine does not inhibit *N. caninum*. On the other hand, tetracycline is effective against *Plasmodium* and *N. caninum*, despite its low activity in *T. gondii* models. The repurposing of antimalarial drugs in *N. caninum* is a fast and inexpensive way to develop novel formulations using well-established compounds.

Keywords: *Neospora caninum*, atovaquone, chloroquine, quinine, primaquine, tetracycline.

Resumo

Neospora caninum é um parasita Apicomplexa relacionado a abortos no gado bovino, que resultam em impactos econômicos. Não há tratamento comercial para neosporosis e o reposicionamento de drogas indica uma estratégia rápida para testar candidatos anti-*N. caninum*. Neste artigo, são descritos os efeitos da atovaquona, cloroquina, quinino, primaquine e tetraciclina na proliferação de *N. caninum*. As concentrações IC_{50} em *N. caninum* foram comparadas com a informação disponível, baseada em estudos publicados previamente para *Plasmodium* e *Toxoplasma gondii*, incluindo a correlação com os mecanismos de ação descritos para cada droga testada. Os padrões de inibição indicam pontos de similaridades e diferenças entre *N. caninum*, *Plasmodium* e *T. gondii*. Por exemplo, a atovaquona demonstra uma alta atividade antiparasitária em todos os modelos testados, enquanto a cloroquina não inibe *N. caninum*. Por outro lado, a tetraciclina é efetiva contra *Plasmodium* e *N. caninum*, em contraste com a baixa atividade em modelos de *T. gondii*. O reposicionamento de drogas antimaláricas em *N. caninum* é uma forma rápida e de baixo custo para o desenvolvimento de novas formulações que usam compostos bem estabelecidos.

Palavras-chave: *Neospora caninum*, atovaquona, cloroquina, quinino, primaquine, tetraciclina.

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Introduction

Neospora caninum is an obligate intracellular protozoan and a member of the phylum Apicomplexa. Canids are the definitive host of *N. caninum*, while ruminants are infected by the non-sexual forms of the parasite (Dubey & Schares, 2011; Marugan-Hernandez, 2017). In intermediate hosts, neosporosis causes abortion and impairs fertility, thus strongly affecting livestock productivity (Reichel et al., 2013).

There is no commercial strategy to control neosporosis, despite the recent advances (Anghel et al., 2018; Harmse et al., 2017; Pereira et al., 2020; Sánchez-Sánchez et al., 2018a, b) and the development of management control measures designed to reduce parasite transmission (Reichel et al., 2014). Likewise, there are few options for the treatment of human toxoplasmosis, which is usually treated with compounds (antifolates, clindamycin, and atovaquone) that are toxic, especially to pregnant women (Neville et al., 2015). On the other hand, there is an arsenal of drugs against malaria, targeted to various stages of the parasite, which were developed in response to the side effects (i.e., blue urine and sclera in methylene blue treated patients) or cases of resistance (artemisinin, pyrimethamine, chloroquine) (Bosson-Vanga et al., 2018; Luzzi & Peto, 1993; Takala-Harrison & Laufer, 2015; Wadi et al., 2018). Thus, the application of anti-malarial drugs indicates an interesting source for drug repurposing against *N. caninum*. For example, methylene blue and analogues, pyrimethamine and artemisinin formulations have been successfully tested on *in vitro* (Kim et al., 2002; Lindsay & Dubey, 1989; Pereira et al., 2017, 2018) and *in vivo* (Pereira et al., 2020) models of *N. caninum* infection. Likewise, several novel candidates with anti-*N. caninum* activity were identified from the Malaria Venture (MMV) Pathogen Box, with promising results (Müller et al., 2017, 2020). Moreover, antimalarial drugs also demonstrate activity against *T. gondii* (Holfels et al., 1994; Kadri et al., 2014; Kim et al., 2002; Lindsay et al., 1994; McFadden et al., 1997; Secrieru et al., 2020). As members of the same phylum (Apicomplexa), there are several similarities among *N. caninum, Plasmodium* and *T. gondii* (Morrissette & Sibley, 2002; Reid et al., 2012), which was also observed when drugs were evaluated.

In this study, the widely used antimalarials quinine, chloroquine, primaquine and atovaquone were tested against *N. caninum* using LacZ-tagged tachyzoites and were compared with the current information (based on previous studies) about *Plasmodium* and *T. gondii*, reinforcing the similarities and differences among them. This will underpin the development of common or exclusive therapeutic strategies based on drug repurposing.

Material and Methods

N. caninum culture

N. caninum tachyzoites (Nc1-LacZ) (Pereira & Yatsuda, 2014) were maintained in Vero cells monolayers with 100 µg/mL kanamycin. The Vero cells and parasites were cultured in RPMI-1640 and RPMI-1640 supplemented with 5% fetal calf serum (Sigma), respectively. The tachyzoites were purified from the culture supernatants using a 5 µM syringe filter and counted in a hemocytometer.

Drugs

All the drugs used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). Atovaquone (10 mg/mL, catalogue number: A7986), primaquine (100 mg/mL, catalogue number: 160393) and tetracycline (20 mg/mL, catalogue number: T7660) were diluted in DMSO (Sigma-Aldrich), while chloroquine (10 mg/mL, catalogue number: C6628) and quinine (10 mg/mL, catalogue number: 145904) were diluted in PBS.

Antiproliferative assay

The antiproliferative assay was performed as described by Pereira et al. (2017), using a chlorophenol red-β-D-galactopyranoside (CPRG, Sigma-Aldrich) based assay. Briefly, the purified tachyzoites (5 x 10^3/well) were distributed in 96-well plates with Vero cell monolayers and incubated for 2 h, 37 °C, 5% CO_2 to allow the invasion. Seven serial dilutions of drugs (1:2) were incubated, in triplicate, for 72 h, 37 °C, 5% CO_2. The initial concentrations of atovaquone, chloroquine, quinine, primaquine, and tetracycline were 100 nM, 1 mM, 1 mM, 100 µM and 100 µM, respectively (Figure 1). After treatment, the cells were washed with PBS and lysed with 125 µL CPRG lysis buffer (100 mM 4- (2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 8.0; 1 mM CaCl2; 1% Triton X-100, 0.5% SDS; 5 mM DTT) for 1 h at 50 °C. The lysates were incubated with 125 µL of CPRG buffer (5 mM CPRG and 50 mM $MgSO_4$ in lysis buffer) for 4 h at 37 °C and the plates analyzed at 570 nm in a spectrophotometer (Sunrise, Tecan). Three independent assays were performed for each drug.

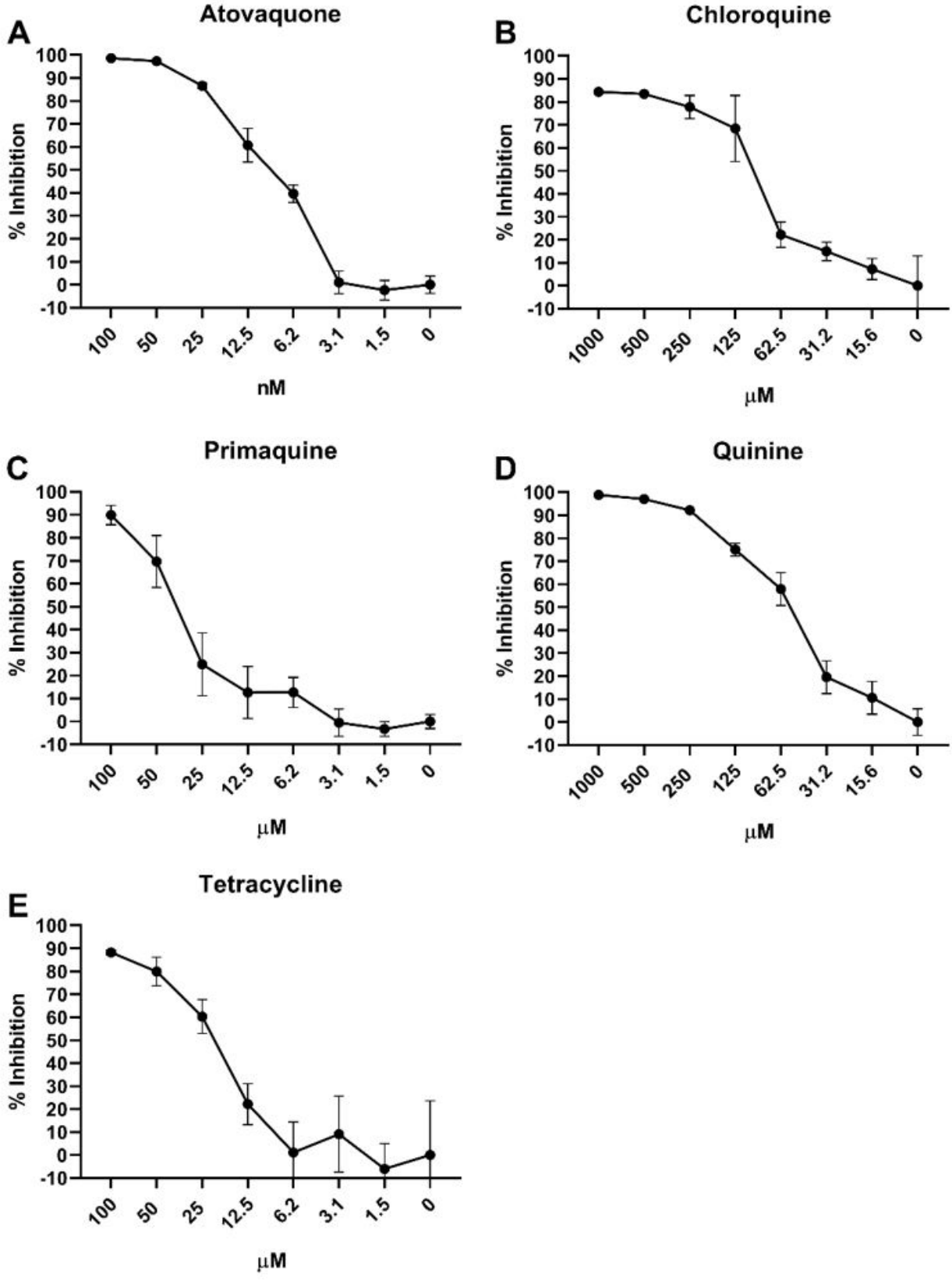

Figure 1. Dose-inhibitory curves of antimalarials against *N. caninum*. *N. caninum* tachyzoites in Vero cells were incubated for 72 h with seven serial dilutions of atovaquone (100-1.5 nM), chloroquine (1000-15.6 µM), primaquine (100-1.5 µM), quinine (1000-15.6 µM) and tetracycline (100-1.5 µM). After incubation, the parasite proliferation was measured by CPRG assay and the percentage of inhibition was calculated in relation to the non-treated controls. The percentage of inhibition values were plotted in relation to the drug concentrations using the GraphPad Prism 5 software.

Cytotoxicity

The cytotoxicity of the antimalarial drugs on Vero cells was evaluated by MTT assay (Mosmann, 1983). The drugs were incubated in 96-well plates for 72 h, 37 °C, 5% CO_2 on confluent monolayers of Vero cells in phenol red-free RPMI. After incubation, the supernatant was carefully discarded and the wells were incubated with 100 µL of MTT solution (500 µg/mL) for 4 h, 37 °C, followed by the dilution of formazan crystals with DMSO (Sigma). The plates were read in a spectrophotometer at 570 nm and the percentage of cytotoxicity was calculated (Pereira et al., 2017). The drugs were initially diluted at 20 µM (atovaquone), 1 mM (chloroquine), 2 mM (quinine), 1 mM (primaquine) and 1 mM (tetracycline). Three independent assays were performed for each drug.

Statistical analysis

The percentage of proliferation inhibition and toxicity were calculated using the formula (($ABS_{control}$ – ABS_{sample}/ $ABS_{control}$)*100), where $ABS_{control}$ and ABS_{sample} represent the mean absorbance of the drug-free control and the absorbance from each drug treatment, respectively. The IC_{50} (parasite inhibition) and CC_{50} (Vero cell toxicity) were calculated from the proliferation/toxicity percentages using CompuSyn software (CompuSyn, 2017; Chou, 2010). The selective index (CC_{50}/IC_{50}) was also calculated from the IC_{50} and CC_{50} values. The values are presented as the mean of three independent tests ± SD, calculated using the Graphpad Prism 5 software.

Results and Discussion

Among the tested antimalarials, atovaquone showed the lowest IC_{50} for *N. caninum* (0.008 µM, Table 1 and Figure 1A). This concentration was similar to that reported for *Plasmodium* (0.0007-0.0018 µM) (Basco et al., 1995) and *T. gondii* (0.007-0.021 µM) (McFadden et al., 1997). Atovaquone combined with proguanil (registered as Malarone®) has been used for the treatment of uncomplicated malaria in non-endemic countries and as a preventive strategy for travelers (Thybo et al., 2004). Atovaquone has also exhibited promising results against retinochoroiditis caused by *T. gondii* (Harrell & Carvounis, 2014; Pearson et al., 1999). In the *Plasmodium* model, the molecule causes the mitochondria to collapse, inhibiting electron transport through the cytochrome bc_1 complex (Mather et al., 2005). Indeed, the mutation in the cytochrome bc1 complex is causally associated with atovaquone resistance in malaria patients (Staines et al., 2018). Despite the low CC_{50} of atovaquone in Vero cells (3.3 µM), the drug is usually well-tolerated (Baggish & Hill, 2002). For example, the CC_{50} for HEK293T (human embryonic kidney) is 43 µM (Schuck et al., 2013), indicating a higher susceptibility of Vero cells to the drug, fact also observed in several cell lines of breast cancer (CC_{50}: 11-18 µM) (Gupta & Srivastava, 2019). Indeed, there is a report of atovaquone related nephrotoxicity in allogeneic transplanted patients (Mendorf et al., 2015), indicating the susceptibility of some cells of renal origin. As observed in toxoplasmosis and malaria, atovaquone has an interesting potential against neosporosis. Further studies should elucidate the mechanisms of action.

Quinine, chloroquine and primaquine are members of the quinolone family, which have traditionally been used to treat malaria. Quinine was originally extracted from the bark of the *Cinchona* (quina-quina) tree, used by native inhabitants of South America for the alleviation of malaria symptoms (Achan et al., 2011). The compound

Table 1. Parasite inhibitory (IC_{50}) and cytotoxic (CC_{50}) doses of antimalarials in *N. caninum*. Purified *N. caninum* (lacZ) tachyzoites were distributed in Vero cell monolayers and incubated with serial dilutions of atovaquone, chloroquine, primaquine, quinine and tetracycline. Proliferation was evaluated by CPRG. In parallel, the cytotoxicity on Vero cells was determined by MTT under the same conditions. The IC_{50} and CC_{50} concentrations were calculated from dose response-curves, using CompuSyn software. SI: Selectivity index.

	***N. caninum* IC_{50} (µM)**	**Vero cell CC_{50} (µM)**	**SI**
Atovaquone	0.008 (± 0.002)	3.3 (± 1.1)	412.5
Chloroquine	112.6 (± 39.4)	483.5 (± 253.7)	4.2
Primaquine	44.4 (± 17.0)	> 1000	> 22.5
Quinine	56.6 (± 11.7)	508.5 (± 155.4)	8.9
Tetracycline	19.6 (± 3.3)	> 500	> 25.5

was isolated by Pierre Joseph Pelletier and Joseph Caventou in 1820 (Krettli, 2001) and completely synthesized in 1945 by Woodward & Doering (1945). Quinine has long been used to treat malaria. However, the drug has been replaced by artesunate or artemether (Esu et al., 2019). Interestingly, quinine has different effects against *Plasmodium* and *T. gondii*. Although quinine shows high activity in *in vitro* models of *Plasmodium* (0.272-5.2 µM, and 0.053-8.1 µM determined by (Touré et al., 2008), (Björkman et al., 1991) and (Menezes et al., 2001), no inhibitory effect on *T. gondii* has been reported (Gomes et al., 2012; Holfels et al., 1994). Against *N. caninum*, the activity of quinine was moderate (56.6 µM, Table 1 and Figure 1D).

The manipulation of the methylene blue structure generated pamaquine and quinaquine, the basic compounds for the synthesis of primaquine and chloroquine, respectively (Al-Bari, 2015). Eventually, methylene blue was replaced with chloroquine, mainly due to the absence of visible side effects (green urine and sclera) of the phenothiazinium dyes (Ginimuge & Jyothi, 2010). Moreover, no activity of methylene blue on hepatic stages of *Plasmodium* sp. was observed, including hypnozoites (Bosson-Vanga et al., 2018), in contrast to its effective *in vitro* activity against *N. caninum* (Pereira et al., 2017). Currently, the use of chloroquine is restricted to non-complicated malaria in regions with no prevalence of drug resistance (Mwanza et al., 2016). Primaquine is used to prevent the relapse of *Plasmodium vivax* and has the unique ability to eliminate the gametocyte form of *Plasmodium falciparum* (Ashley et al., 2014). Chloroquine susceptible strains of *Plasmodium* are usually inhibited in doses below 0.1 µM (Aguiar et al., 2014; Chehuan et al., 2013; Fall et al., 2015). In Brazil, the recommended treatment for *Plasmodium vivax* (83.6% of the reported cases) is based on primaquine and chloroquine combinations to control the hypnozoite and trophozoite forms, respectively (Brasil, 2010; Negreiros et al., 2016). Chloroquine is also active against *T. gondii*, with an IC_{50} of 2.25 µM (Kadri et al., 2014). However, chloroquine showed a robust inhibitory effect against *N. caninum* only at concentrations above 100 µM (Table 1 and Figure 1B). Similarly to quinine, primaquine inhibited *N. caninum* at 44.4 µM (Table 1 and Figure 1C), whereas no effect has been reported against *T. gondii* (Holfels et al., 1994). On the other hand, *Plasmodium falciparum* exhibits higher susceptibility to primaquine (IC_{50} range; 0.46-18.9 µM) than *N. caninum* (Basco et al., 1999; Cabrera & Cui, 2015). Although formulations containing quinine, chloroquine or primaquine have been applied in malaria therapy for more than 50 years, the mechanisms of action are not completely established. The suggested mechanism for quinine and chloroquine is based on the prevention of heme polymerization, converting the toxic molecule to hemozoin (Sullivan et al., 1996). The mechanism of primaquine is not fully understood, but the drug probably interferes with the cellular respiration in *Plasmodium*, which generates oxygen free radicals and deregulates the electron transport (Fernando et al., 2011). The quinolones showed different effects on *N. caninum*, *Plasmodium* and *T. gondii*, indicating species-specific targets. Firstly, the erythrocyte cycle is absent in *N. caninum* and *T. gondii*, requiring future assays to elucidate the mechanism of quinine and chloroquine in coccidian members. The higher activity of quinine and primaquine in *N. caninum* compared to *T. gondii* indicates an interesting group of drugs for the control of neosporosis, with independent strategies compared to toxoplasmosis models. Moreover, there is a wide range of available quinolone derivatives (Chu et al., 2019; Gao et al., 2019; Hu et al., 2017; Wang et al., 2019), amplifying the candidate list for testing against *N. caninum*.

Tetracycline was active against *N. caninum* (IC_{50} 19.6 µM, Table 1 and Figure 1E and ineffective against *T. gondii* at concentrations above 40 µg/mL (83.1 µM) (Chang et al., 1990). Moreover, tetracycline analogues (doxycycline and minocycline) have demonstrated high activity against *N. caninum*, blocking 100% of the parasite proliferation at doses > 2 µM (Lindsay et al., 1994). For *Plasmodium*, tetracycline inhibits 50% of *in vitro* proliferation at concentrations below 9.8 µM (Ye & Van Dyke, 1994). The drug and derivatives (i.e., doxycycline) are applied as slow-acting blood schizonticidal agents in formulations for the treatment of uncomplicated malaria, usually in combination with quinine (Dahl et al., 2006; Gaillard et al., 2015). Tetracycline has an antagonist affinity at the 30S ribosomal subunit of prokaryotes, preventing the attachment of aminoacyl tRNA to the acceptor (A) site of the organelle (Chopra & Roberts, 2001). In *Plasmodium*, the inhibitory effect of tetracycline is also observed on ribosomes of the parasite apicoplast, inducing the delayed death mechanism (Fichera & Roos, 1997; Ramya et al., 2007; Uddin et al., 2017), and occurs mainly after a cycle of egress and invasion (Botté et al., 2012; Uddin et al., 2017). Apicoplast is an exclusive organelle of the phylum Apicomplexa with a prokaryotic origin and houses singular and essential metabolic pathways to the parasite's survival and virulence, representing a promising target for the control of apicomplexan diseases (Biddau & Sheiner, 2019; Dahl et al., 2006). The differential susceptibility to tetracycline suggests divergences among the apicoplast metabolism of *N. caninum, Plasmodium* and *T. gondii* (Haussig et al., 2011). Although the tetracycline delayed death process is well documented in *Plasmodium*, further experiments are needed to evaluate this phenomenon in *N. caninum* and *T. gondii*.

Excepting atovaquone, all antimalarials tested demonstrated low toxicity in Vero cells (CC_{50} ≥ 483.5 µM) (Table 1). Chloroquine, primaquine, quinine and tetracycline usually lead to toxicity in non-tumoral lineages at concentrations above 51 µM, 395 µM, 200 µM and 225 µM, respectively (Lelièvre et al., 2012; Davanço et al., 2014; Sanders et al., 2014; Ou at al., 2019). However, we must consider that the CC_{50} concentrations vary depending on the cell lineage employed (Florento et al., 2012). Therefore, novel assays focusing on the cytotoxicity on different cells lineages, especially from bovine and canine models, are mandatory to elucidate the safety of antimalarials for the control of neosporosis.

The tested antimalarials (Figure 2) exhibited several similarities and differences against *N. caninum*, *Plasmodium* and *T. gondii*, contributing to a specific comprehension of the metabolic mechanisms of each parasite. Our results indicate the potential of atovaquone for use in *in vivo* assays, as well as the demand for investigating effective analogues of chloroquine, primaquine, quinine and tetracycline. The repurposing of antimalarials against *T. gondii* and *N. caninum* is an interesting way to obtain fast and low-cost candidates, since there is a vast body of information about their efficacy and toxicity in *Plasmodium* models (Müller et al., 2017, 2020). The comparison between these related parasites is a valid methodology, which will guide common or exclusive treatment regimens against neosporosis, malaria and toxoplasmosis.

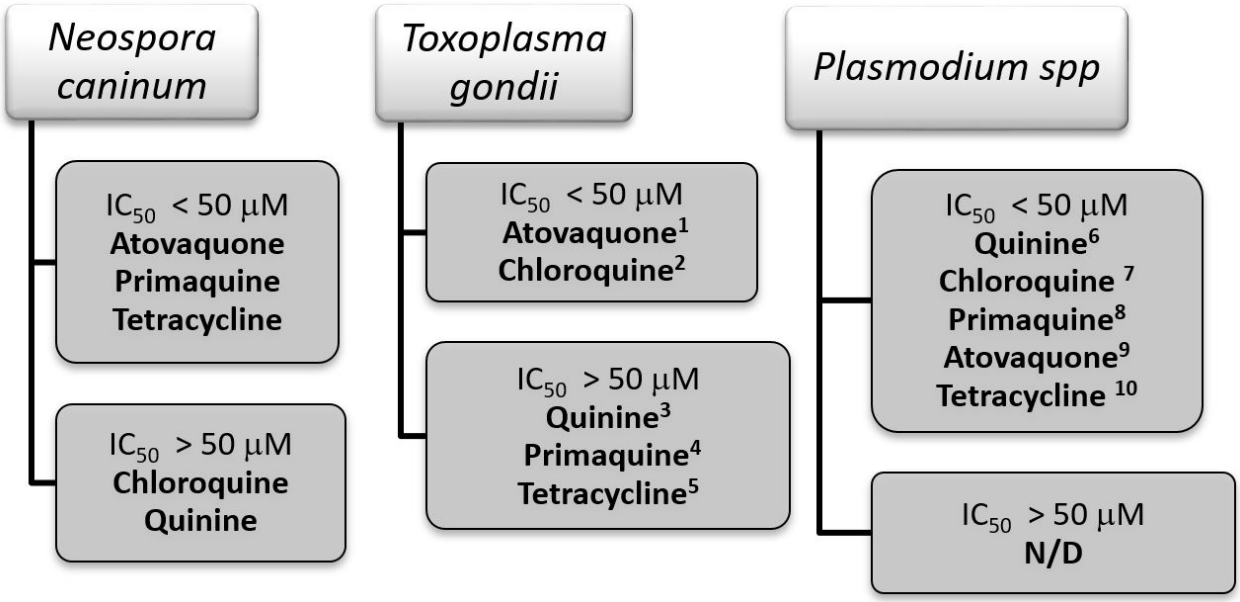

Figure 2. Schematic illustration of the antimalarial inhibition pattern on *N. caninum*, *T. gondii* and *Plasmodium*. Antimalarial drugs tested on *N. caninum* in this study (atovaquone, primaquine, tetracycline, chloroquine and quinine) were grouped according to their inhibitory pattern. Compounds with an IC_{50} > 50 µm were separated from those with higher inhibitory activity (IC_{50} < 50 µm). This classification was also applied to the same compounds with reported assays against *T. gondii* and *Plasmodium*. [1]McFadden et al. (1997); [2]Kadri et al. (2014); [3,4]Holfels et al. (1994); [5]Chang et al. (1990); [6]Björkman et al. (1991); [7]Chehuan et al. (2013); [8]Basco et al. (1999); [9]Basco et al. (1995); [10]Ye & Van Dyke (1994). N/D: no data.

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