Evaluation of methylene blue, pyrimethamine and its combination on an *in vitro Neospora caninum* model

LUIZ MIGUEL PEREIRA[1,2], ISABEL CRISTINA VIGATO-FERREIRA[1,2], GABRIELA DE LUCA[1], CÁSSIA MARIANA BRONZON DA COSTA[1] *and* ANA PATRÍCIA YATSUDA[1,2]*

[1] *Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Av do Café, s/n, 14040-903, Ribeirão Preto, SP, Brazil*
[2] *Núcleo de Apoio à Pesquisa em Produtos Naturais e Sintéticos, Universidade de São Paulo, Ribeirão Preto, SP, Brazil*

(*Received 23 July 2016; revised 10 November 2016; accepted 3 December 2016; first published online 11 January 2017*)

SUMMARY

Neospora caninum is an apicomplexan parasite strongly related to reproductive problems in cattle. The neosporosis control is not well established and several fronts are under development, predominantly based on immune protection, immunomodulation and chemotherapy. The use of anti-malarial drugs as therapeutic sources has, in theory, considerable potential for any apicomplexan. Drugs such as methylene blue (MB) and pyrimethamine (Pyr) represent therapeutic options for malaria; thus, their use for neosporosis should be assessed. In this work, we tested the effects of MB and Pyr on *N. caninum* proliferation and clearance, using LacZ-tagged tachyzoites. The drugs inhibited at nanomolar dosages and its combination demonstrated an antagonistic interaction in proliferation assays, according to the Chou and Talalay method for drug combination index. However, the drug combination significantly improved the parasite *in vitro* clearance. The repositioning of well-established drugs opens a short-term strategy to obtain low-cost therapeutics approaches against neosporosis.

Key words: *Neospora caninum*, methylene blue, pyrimethamine, repositioning, drug combination.

INTRODUCTION

Neospora caninum is an intracellular obligate Apicomplexa, observed in 1984 (Bjerkås *et al*. 1984) and identified in 1988 (Dubey *et al*. 1988), strongly related to an abortive syndrome in cattle, which leads to significant economic losses (Reichel *et al*. 2013). Compared with the phylum counterparts, the neosporosis combat lacks effective treatments, despite the dedication of several groups aimed at developing methods to inhibit the parasite cycle. Some approaches, such as immune protection (Jimenez-Ruiz *et al*. 2012; Reichel *et al*. 2015), immunomodulation (Cardoso *et al*. 2012), inhibition of invasion (Pereira *et al*. 2011) or chemotherapy (Strohbusch *et al*. 2009; Schorer *et al*. 2012; Muller *et al*. 2015*a*, *b*), have been developed; however, none of them are commercially available.

Methylene blue (MB) and pyrimethamine (Pyr) are drugs that are commonly applied for, respectively, malaria (Ginimuge and Jyothi, 2010) and toxoplasmosis research (Rajapakse *et al*. 2013), but sparingly applied for neosporosis research (Lindsay and Dubey, 1989; Lindsay *et al*. 1994; Lindsay *et al*. 1996). The development of tagged LacZ *N. caninum* employing plasmids based on promoter regions from the parasite (Pereira & Yatsuda, 2014; Pereira *et al*. 2014) are suitable for drug screening. In this work, we applied the CPRG (chlorophenolred-β-D-galactopyranoside) to establish the IC_{50} of MB and/or Pyr on *N. caninum* proliferation and the effects on the invasion process. Additionally, we compared the combination of drugs on tachyzoites resistant and susceptible to Pyr.

The potential use of anti-malarial drugs on neosporosis control might shorten the time required for clinical tests, as these compounds have an extensive background related to side-effects; most of them are not patent protected anymore. MB and Pyr demonstrated activity at a nanomolar scale and are suitable for combinations, indicating the viability of the repositioning of low cost alternatives for neosporosis control or for complementation of protective or immunomodulatory therapies.

MATERIAL AND METHODS

Neospora caninum

For the invasion, proliferation and clearance assays, two strains of Nc-1 *N. caninum* expressing β-galactosidase, either resistant to chloramphenicol (NcLacZCAT) (Pereira and Yatsuda, 2014) or Pyr (NcLacZM2M3) (Pereira *et al*. 2014), respectively, were applied. The tachyzoites were cultivated in Vero cell cultures, purified by filtration (5 μm) and

* Corresponding author: Departamento de Análises Clínicas, Bromatológicas e Toxicológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 14040-903, Brazil. E-mail: ayatsuda@fcfrp.usp.br

Parasitology (2017), **144**, 827–833.
doi:10.1017/S0031182016002584

counted in a haemocytometer. The strain resistant to Pyr (NcLacZM2M3) was employed as a positive control. The strain resistant to chloramphenicol (NcLacZCAT) was the reference for the evaluation of MB and Pyr.

Drugs

MB and Pyr were purchased from Sigma-Aldrich. The stock solutions of 10 mg mL^{-1} (for proliferation and invasion assays) or 20 mg mL^{-1} (for (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) assay) were constituted of MB and Pyr diluted in absolute ethanol and Dimethyl Sulfoxide (DMSO), respectively.

Proliferation assay

Purified *N. caninum* tachyzoites (NcLacZ M2M3 or NcLacZCAT) were distributed (1×10^3 $well^{-1}$) on Vero cells in a 96-well plate and incubated for 2 h to allow the invasion. For *N. caninum* resistant to Pyr (NcLacZM2M3), the combination of MB and Pyr was composed of three distinct dilutions of MB (0·6, 0·3, 0·15 μM) added to serial dilutions of Pyr (starting from 8 μM) and incubated on Vero cells (previously invaded by *N. caninum* tachyzoites) for 72 h at 37 °C. The positive control was Pyr in the absence of MB. For *N. caninum* susceptible to Pyr (NcLacZCAT), seven fractional inhibitory concentrations (0·7, 0·56, 0·45, 0·35, 0·24, 0·17 and 0·07 μM) of each drug alone and combined were applied to proliferating tachyzoites for 72 h.

After the drug-step, the wells were washed twice with phosphate-buffered saline (PBS) and CPRG lysis buffer [125 μL of 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, pH 8·0; 1 mM $CaCl_2$; 1% Triton X-100, 0·5% SDS; 5 mM DTT (dithiothreitol)] was added for 1 h, at 50 °C. The lysates were incubated with 125 μL of CPRG buffer (5 mM CPRG in lysis buffer) for 1 h and the plates read at 570 nm in an enzyme-linked immunosorbent assay (ELISA) reader (Sunrise, Tecan). Two independent assays were performed for each drug evaluation and combination.

Cytotoxicity

MTT assay (Mosmann, 1983) was employed to evaluate the toxicity of MB, Pyr, DMSO and ethanol. Briefly, 5×10^3 Vero cells/well were cultivated in 96-well plates in complete RPMI (RPMI supplemented with 5% fetal bovine serum). After reaching cell confluence, the media was discarded and the cells incubated with dilutions of MB, Pyr (starting from 1 mM), DMSO or ethanol (starting from 20%) for 72 h. The media was carefully discarded and the wells incubated with 100 μL of MTT solution (500 μg mL^{-1}) for 4 h, 37 °C and the formazan crystals diluted in acidified isopropanol (HCl 50 mM). The reaction was read at 570 nm in an ELISA reader (Sunrise, Tecan) and the percentage of cytotoxicity calculated. Two independent assays were performed for each drug and solvent.

Invasion assay

The assay followed our previous work (Pereira and Yatsuda, 2014). Purified tachyzoites (NcLacZCAT, 1×10^6/tube) were incubated with serial dilutions of MB or Pyr (1 mM $well^{-1}$) for 30 min at 37 °C in phenol red-free RPMI. The parasites, in triplicate, were placed in contact with Vero cell monolayers in a 24-well plate for 2 h at 37 °C for invasion. The plates were washed twice with PBS and the cells incubated in 350 μL lysis buffer for 1 h at 50 °C. In a 96-well plate, a duplicate aliquot of 20 μL was mixed with 230 μL of CPRG buffer for 4 h, at 37 °C, and the plate was read at 570 nm. The percentage of invasion was calculated as indicated in statistical analyses and plotted against the respective drug concentration. Two independent assays were performed for each drug.

Tachyzoite clearance assay

Tachyzoites (NcLacZCAT, 1×10^6 $well^{-1}$) were incubated with three dilutions of MB, Pyr or the combination (1·6, 0·8 and 0·4 μM) in Vero cell monolayers cultured in 24-well plates for 72 h, at 37 °C, with 5% CO_2. After the lytic cycle, the wells were scrapped, washed twice with PBS and the extracellular tachyzoites were counted in a haemocytometer. For all the drug concentrations, the absolute number of tachyzoites was obtained and the percentage of inhibition was calculated compared with the wells with no drug.

The number of extracellular tachyzoites counted for the wells incubated with 1·6 μM (the higher drug concentration and consequently, higher inhibition) at the end of the first round was used as the reference to initiate the second round for all the concentrations. New plates with fresh Vero cells received the same number of tachyzoites for the three drug concentrations (1·6, 0·8 and 0·4 μM), under the same conditions (24-well plates for 72 h, at 37 °C, with 5% CO_2). In total, four consecutive cycle rounds were performed and the percentages of inhibition for all the concentrations were calculated. Additionally, at the end of the fourth round, the final parasite load was measured by incubation with CPRG for 30 min and 24 h (Supplementary Fig. 1).

Drug combination quantification and statistical analyses

The percentages of proliferation, toxicity and invasion were calculated by the formula [$(ABS_{control} - ABS_{sample}/ABS_{control}) \times 100$], where $ABS_{control}$

https://doi.org/10.1017/S0031182016002584 Published online by Cambridge University Press

Table 1. *In vitro* efficacy and toxicity of pyrimethamine (Pyr) or methylene blue (MB) on *Neospora caninum* and Vero cells

	IC_{50}			Selectivity index	
	N. caninum (NcLacZM2M3)	*N. caninum* (NcLacZCAT)	CC_{50} Vero cells	*N. caninum* (NcLacZM2M3)	*N. caninum* (NcLacZCAT)
Pyr (μM)	32·49 ± 8·57	0·312 ± 0·017	194·8 ± 64·1	5·99	624·3
MB (μM)	0·371 ± 0·115	0·349 ± 0·084	>62·5	>168·4	>179·1
DMSO (%)	s/n	s/n	1·88 ± 0·44	s/n	s/n
Ethanol (%)	s/n	s/n	7·94 ± 1·84	s/n	s/n

The IC_{50}, CC_{50} and selectivity index were calculated for MB, Pyr, ethanol and DMSO on *N. caninum* and Vero cells. *Neospora caninum*-resistant strains to Pyr (NcLacZM2M3) or chloramphenicol (NcLacZCAT) were used. Tachyzoites or Vero cells were incubated with the molecules for 72 h, at 37 °C, with 5% CO_2, and the proliferation (tachyzoites) or toxicity (Vero cells) was measured after CRPG or MTT assays respectively. The percentage of inhibition was calculated in comparison to the non-treated controls in two independent assays. The ethanol and DMSO toxicity were evaluated only in Vero cells; thus, the columns with tachyzoites were represented by s/n.

represents the mean absorbance of the drug-free control and ABS_{sample} the absorbance from each drug treatment. The percentages of proliferation or toxicity values were plotted against the respective drug concentration in Graphpad 5·0 software. The IC_{50}, CC_{50} and the selective index (CC_{50}/IC_{50}) were calculated using Compusyn software (http://www.combosyn.com/). The interaction of the drugs was evaluated through the combination index (CI), represented by the formula (IC_{50} of drug A associated with B/IC_{50} of drug A alone) + (IC_{50} of drug B associated with A/IC_{50} of drug B alone) (Berenbaum, 1978; Chou *et al*. 1983). Three types of interactions might be obtained from the CI values: Synergism (CI < 0·5); additivity (CI = 1) and antagonism (CI > 1) (Chou, 2010).

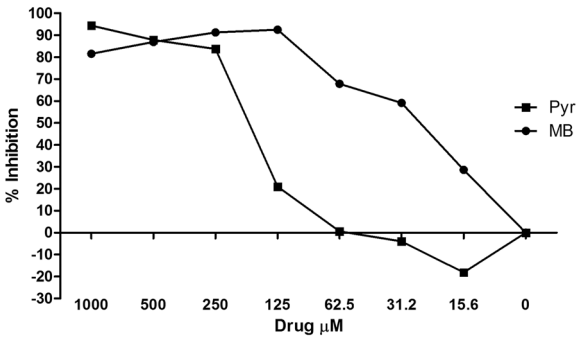

Fig. 1. Inhibition of *Neospora caninum* invasion by pyrimethamine (Pyr) or methylene blue (MB). *Neospora caninum* LacZ (NcLacZCAT) were incubated with serial dilutions of Pyr (square) or MB (circle) for 30 min and allowed to invade Vero cells for 2 h in 24-well plates. The invasion was measured by CPRG reaction and the percentage of inhibition calculated in comparison with the drug free control.

RESULTS

Proliferation and cytotoxicity

MB and Pyr demonstrated a robust inhibitory effect on tachyzoite proliferation. MB displayed an IC_{50} for chloramphenicol (NcLacZCAT) and Pyr (NcLacZM2M3) resistant strains, respectively, of 0·349 and 0·371 μM (Table 1). The *N. caninum* resistant to Pyr (NcLacZM2M3) demonstrated an IC_{50} 104-fold higher for Pyr when compared with the concentration on the susceptible strain (NcLacZCAT) (respectively, 32·49 and 0·312 μM; Table 1). The values indicated the suitability of NcLacZCAT strain for both drugs and confirmed the resistance of NcLacZM2M3 to Pyr.

No toxic effects of MB on Vero cells were verified below 62·5 μM, whereas Pyr indicated a CC_{50} of 194·8 μM. The selectivity index for MB and Pyr, were, respectively, higher than 168·4 and 5·99 for *N. caninum* resistant to Pyr (NcLacZM2M3) and higher than 179·1 and 624·3 for *N. caninum* resistant to chloramphenicol (NcLacZCAT). The percentage of solvents in the CC_{50} of Pyr (DMSO, 0·15%) or MB (ethanol, 0·62%) did not influence the activities of these drugs, since the CC_{50} of DMSO and ethanol were 1·88 and 7·94%, respectively (Table 1).

Inhibition of invasion

MB inhibited the invasion of *N. caninum* with an IC_{50} of 25·9 (±4·2) μM and the effects of Pyr on the tachyzoites were not observed below 125 μM (Fig. 1).

Effects of the combination of MB and Pyr on the proliferation of the parasites

The drug combination of MB + Pyr exhibited more discernible effects on the proliferation of *N. caninum* (NcLacZCAT) when compared with Pyr alone for all the concentrations (Fig. 2A). The IC_{50} of the combination was 0·264 (±0·055) μM, whereas for MB or Pyr were 0·304 (±0·033) μM or 0·368 (±0·074) μM, respectively, with a CI of 1·58. When

https://doi.org/10.1017/S0031182016002584 Published online by Cambridge University Press

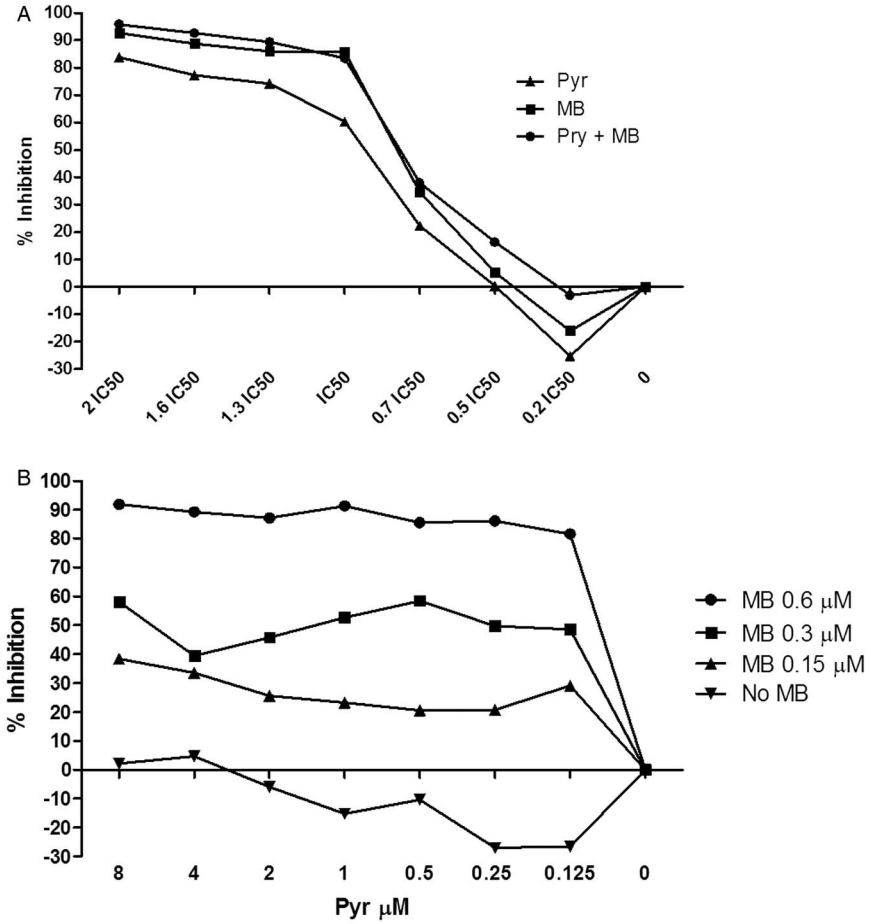

Fig. 2. Combination of pyrimethamine (Pyr) and methylene blue (MB) on *N. caninum* proliferation. To evaluate the general effects of the MB alone or associated with Pyr, Pyr-susceptible (NcLacZCAT, A) and -resistant (NcLacZM2M3, B) *N. caninum* LacZ tachyzoites were incubated with dilutions of MB and Pyr. (A) For susceptible tachyzoites, 0·7, 0·56, 0·45, 0·35, 0·24, 0·15 and 0·07 μM (based on $IC_{50} \sim 0{\cdot}35\ \mu$M) of MB and/or Pyr were incubated for 72 h, 37 °C, 5% CO_2. (B) For resistant parasites, Pyr (8, 4, 2, 1, 0·5, 0·25, 0·125 μM) was associated with MB 0·6, 0·3 and 0·15 μM under the same conditions. The proliferation was measured by CPRG reaction and the percentage of inhibition calculated from the absorbance of treated samples compared with the free drug control.

Pyr was applied to Pyr-resistant tachyzoites (NcLacZM2M3), no inhibition effects were detected. The activity of MB on the Pyr-resistant tachyzoites (NcLacZM2M3) followed a similar pattern when compared with the susceptible ones (NcLacZCAT) (Fig. 2B). MB maintained inhibition rates about 87·5 (±6·5), 50·3 (±12·7) and 27·2 (±16·4)% for 0·6, 0·3 and 0·15 μM, respectively (Fig. 2B).

Tachyzoite clearance assay

MB and Pyr demonstrated different patterns for the elimination of the parasites. Pyr removed over 90% of *N. caninum* after the fourth lytic cycle at 1·6 and 0·8 μM (Fig. 3B) and no visible tachyzoites were detected (Fig. 3D) with a high percentage (>95%) of elimination at the first cycle (Fig. 3B). In contrast, for MB the clearance was moderate when compared with Pyr, indicating ~80% of inhibition for the highest dose of 1·6 μM (Fig. 3A) and extracellular tachyzoites were still detected after the fourth cycle (Fig. 3D). The elimination of tachyzoites with 0·4 μM of drug followed a similar pattern for MB and Pyr (Fig. 3A and B, respectively); however, for 0·8 μM, the treatment with Pyr elevated the inhibition rate such as observed for 1·6 μM. Pyr at 1·6 μM cleared the tachyzoites, whereas the β-galactosidase activity was observed for all the concentrations of MB (Fig. 3E and F).

The drug combination eliminated the tachyzoites likewise as for Pyr at 1·6 μM (Fig. 3C and D), but the clearance effect was also positively observed for 0·8 μM (Fig. 3E and F).

DISCUSSION

Pyr has a current relevant role for the preventive therapy of malaria, the so-called Intermittent preventive therapy (Venkatesan *et al*. 2013) and it is successfully adopted for congenital toxoplasmosis in humans (Kaye, 2011; Kieffer & Wallon, 2013). Pyr inhibits the bifunctional enzyme dihydropholate

https://doi.org/10.1017/S0031182016002584 Published online by Cambridge University Press

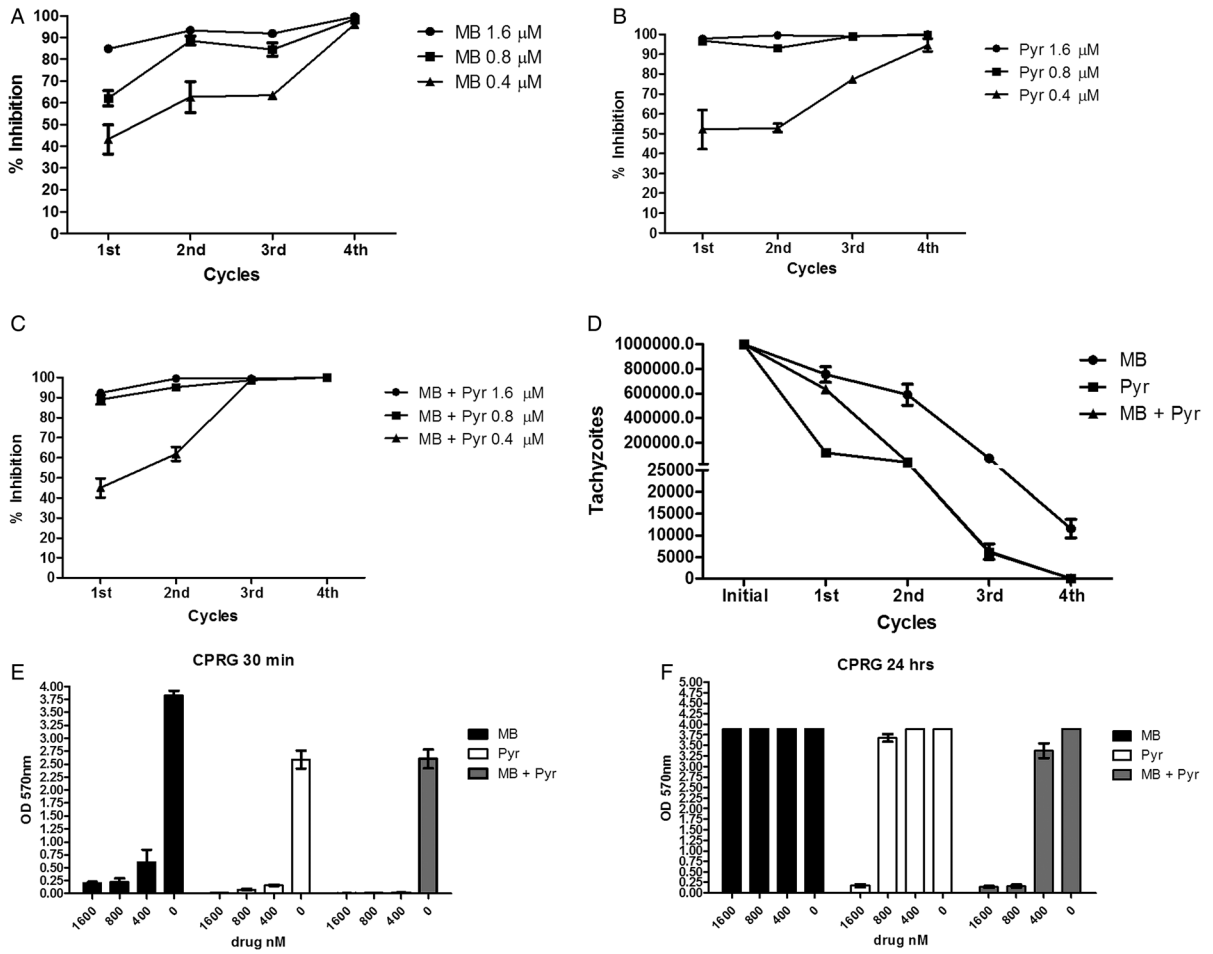

Fig. 3. *In vitro* clearance of tachyzoites. *Neospora caninum* NcLacZCAT (1×10^6 well^{-1}) were incubated with three serial dilutions (1·6, 0·8 and 0·4 μM) of methylene blue (MB) and/or pyrmethamine (Pyr) for 72 h. The parasites were counted and re-incubated under the same conditions for four rounds. At each cycle, the tachyzoites were counted and the percentage of inhibition was calculated. After the fourth cycle, the total tachyzoites were detected by CPRG reaction, incubated at 30 min or 24 h. (A) Percentage of elimination for MB. (B) Percentage of elimination for Pyr. (C) Percentage of elimination for the combination MB + Pyr. (D) Absolute number of tachyzoites after incubation at 1·6 μM. (E, F) Total tachyzoites detected by CPRG reaction after 30 min and 24 h, respectively.

reductase–thymidylate synthase (DHFR–TS) of susceptible protozoans, blocking the conversion of dihydrofolate to tetrahydrofolate, a critical step for thymidine nucleotide biosynthesis (Ivanetich & Santi, 1990). The malaria treatment with Pyr demonstrated limitations due to the increase of resistant *Plasmodium* strains by the 1990s (Peterson *et al*. 1988). For *N. caninum*, Pyr resistance was artificially obtained through point mutations of DHFR–TS (NcLacZM2M3) (Pereira *et al*. 2014). This was the first *N. caninum*-resistant strain, since, to our knowledge, there is no isolation from natural infected host sources. As natural selection occurred after years of Pyr massive treatment for human malaria; this phenomenon might extend to *N. caninum* in bovine neosporosis if massive treatment occurs in a certain microenvironment such as a dairy farm. *In vitro*, the Pyr resistance (NcLacZM2M3) was 104-fold higher when compared with the susceptible strain (NcLacZCAT). Due to the malaria experience, the combination of other anti-*Neospora* drugs with Pyr appears to be a prudent preventive approach for neosporosis control.

As observed for Pyr, MB demonstrated an IC_{50} at the nanomolar scale for *N. caninum*, the first synthetic molecule applied against malaria (Buchholz *et al*. 2008). Due to the low cost, MB was extensively used in the Pacific front by USA militaries in the Second World War (Schirmer *et al*. 2011). Although non-systemically deleterious, MB causes visible side-effects, such as turning urine and the sclera green or blue. Therefore, MB was abandoned as a method for the malaria treatment, mainly after the adoption of chloroquine (Ginimuge and Jyothi, 2010). There is no reference of MB resistance in *Plasmodium* (Buchholz *et al*. 2008) and the low cost, efficiency and limited side-effects revived the drug as a candidate for combination with artemisinin (Dormoi *et al*. 2012), quinine, Pyr and chloroquine (Garavito *et al*. 2012).

https://doi.org/10.1017/S0031182016002584 Published online by Cambridge University Press

Bovine neosporosis is important for many developing and especially under developed countries' economies; hence, MB appears as an economic and effective option for the control of this disease. For *N. caninum*, there are few real candidates for chemotherapy and the anti-malarial drugs have been underutilized, despite their effective potential. Our group aims at determining the real capacity of classical drugs on *N. caninum* proliferation, using objective tests such as CPRG. The two LacZ tagged strains developed by our group represent a safe option for initial drug screening.

MB cannot be properly evaluated by assays involving Giemsa or haematoxylin–eosin, since MB acts as a stain, interfering with the identification of *N. caninum*. Due to the advantages of the CPRG assay, MB demonstrated a capacity to block the invasion of the *N. caninum*, a fact that was not observed for Pyr. Therefore, MB probably acts in the invasion process and proliferation, indicating multi-site targets of the parasite cycle. The invasion inhibition probably avoids the disease propagation, acting on mechanisms related to adhesion and/or invasion, which are the usual targets for preventive approaches (Pastor-Fernandez *et al*. 2015; Yoshimoto *et al*. 2015).

The effects of MB with Pyr demonstrates an antagonist interaction in the proliferative assay and a positive pattern in the clearance assay (% of the combination inhibition is higher than % of MB alone + % of Pyr alone), revealing that these drugs probably have some different targets. For *Plasmodium*, the antagonist effect of MB/Pyr combination *in vitro* (Garavito *et al*. 2007) was replaced by a synergic inhibition in *in vivo* tests (Garavito *et al*. 2012).

The use of low-cost and well-established drugs of human apicomplexan diseases for *N. caninum* expands the current narrow therapeutic options. The price of MB (catalogue number M9140) and Pyr (catalogue number 46706) at Sigma-Aldrich website is USD 0·0027 and USD 0·21 per milligram, respectively. MB and Pyr appears as competitive treatment candidates, when compared, for example, to toltrazuril (USD 0·31 per milligram; catalogue number 34000), currently applied against animal coccidiosis (Philippe *et al*. 2014; Rodrigues Fde *et al*. 2016). The price from Sigma usually represents several aspects such as costs for synthesis or extraction and purification, essential factors when scaling up for clinical purposes. The next steps should focus on an *in vivo* treatment model based on MB/Pyr, especially for bovine species affected by *N. caninum*. The establishment of MB/Pyr use will depend on the time required for the complete drug elimination from the animal to guarantee a safe consumption of milk or meat. To avoid commercial limitations, the use of this composition seems more suitable in neosporosis outbreaks, or as part of the quarantine process of animals coming from very endemic regions with neosporosis. The treatment has also an interesting potential on cows with repeated abortion occurrences with high *N. caninum* antibody titration and its calves.

There are several methods for Pyr detection in milk (Koesukwiwat *et al*. 2007*a*, *b*; Azzouz *et al*. 2011), useful in future treatment strategies, especially in commercial herds. MB lacks residue detection approaches in milk or meat due to the sparse application in cattle, although the drug was successfully applied against nitrite intoxication in bovines (Van Dijk *et al*. 1983). The residue management of MB in herds might demand simple adaptations, once there are several sensitive methods of drug detection (Tang and Santschi, 2000; Xu *et al*. 2012), allowing a safety neosporosis control strategy allied to a rational commercial purpose.

A simple repositioning of traditional drugs for the combat of neosporosis is strategic for many economies around the world involved with the livestock economy. After our findings, MB associated with Pyr is highlighted as a possible drug combination candidate for neosporosis control, contributing to the complementation of therapeutic approaches.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0031182016002584

ACKNOWLEDGMENTS

We would like to thank Maraísa Palhão Verri for the excellent technical assistance.

FINNANCIAL SUPPORT

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the Postdoctoral fellowship to LMP (PNPD/CAPES).

REFERENCES

Azzouz, A., Jurado-Sanchez, B., Souhail, B. and Ballesteros, E. (2011). Simultaneous determination of 20 pharmacologically active substances in cow's milk, goat's milk, and human breast milk by gas chromatography-mass spectrometry. *Journal of Agricultural and Food Chemistry* **59**, 5125–5132.

Berenbaum, M. C. (1978). A method for testing for synergy with any number of agents. *Journal of Infectious Diseases* **137**, 122–130.

Bjerkås, I., Mohn, S. F. and Presthus, J. (1984). Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. *Zeitschrift für Parasitenkunde* **70**, 271–274.

Buchholz, K., Schirmer, R. H., Eubel, J. K., Akoachere, M. B., Dandekar, T., Becker, K. and Gromer, S. (2008). Interactions of methylene blue with human disulfide reductases and their orthologues from *Plasmodium falciparum*. *Antimicrobial Agents and Chemotherapy* **52**, 183–191.

Cardoso, M. R., Mota, C. M., Ribeiro, D. P., Noleto, P. G., Andrade, W. B., Souza, M. A., Silva, N. M., Mineo, T. W., Mineo, J. R. and Silva, D. A. (2012). Adjuvant and immunostimulatory effects of a D-galactose-binding lectin from *Synadenium carinatum* latex (ScLL) in the mouse model of vaccination against neosporosis. *Veterinary Research* **43**, 76.

Chou, T. C. (2010). Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Research* **70**, 440–446.

Chou, J. H., Chou, T. C. and Talalay, P. (1983). Computer simulation of drug effects: quantitation of synergism, summation and antagonism of multiple drugs. *Pharmacologist* **25**, 175.

Dormoi, J., Pascual, A., Briolant, S., Amalvict, R., Charras, S., Baret, E., Huyghues des Etages, E., Feraud, M. and Pradines, B. (2012). Proveblue (methylene blue) as an antimalarial agent: *in vitro* synergy with dihydroartemisinin and atorvastatin. *Antimicrobial Agents and Chemotherapy* **56**, 3467–3469.

Dubey, J. P., Hattel, A. L., Lindsay, D. S. and Topper, M. J. (1988). Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. *Journal of the American Veterinary Medical Association* **193**, 1259–1263.

Garavito, G., Bertani, S., Rincon, J., Maurel, S., Monje, M. C., Landau, I., Valentin, A. and Deharo, E. (2007). Blood schizontocidal activity of methylene blue in combination with antimalarials against *Plasmodium falciparum*. *Parasite* **14**, 135–140. http://dx.doi.org/10.1051/parasite/2007142135

Garavito, G., Bertani, S., Quiliano, M., Valentin, A., Aldana, I. and Deharo, E. (2012). The *in vivo* antimalarial activity of methylene blue combined with pyrimethamine, chloroquine and quinine. *Memórias do Instituto Oswaldo Cruz* **107**, 820–823.

Ginimuge, P. R. and Jyothi, S. D. (2010). Methylene blue: revisited. *Journal of Anaesthesiology, Clinical Pharmacology* **26**, 517–520. PMCID: PMC3087269.

Ivanetich, K. M. and Santi, D. V. (1990). Bifunctional thymidylate synthase-dihydrofolate reductase in protozoa. *FASEB Journal* **4**, 1591–1597.

Jimenez-Ruiz, E., Alvarez-Garcia, G., Aguado-Martinez, A., Salman, H., Irache, J. M., Marugan-Hernandez, V. and Ortega-Mora, L. M. (2012). Low efficacy of NcGRA7, NcSAG4, NcBSR4 and NcSRS9 formulated in poly-epsilon-caprolactone against *Neospora caninum* infection in mice. *Vaccine* **30**, 4983–4992.

Kaye, A. (2011). Toxoplasmosis: diagnosis, treatment, and prevention in congenitally exposed infants. *Journal of Pediatric Health Care* **25**, 355–364.

Kieffer, F. and Wallon, M. (2013). Congenital toxoplasmosis. *Handbook of Clinical Neurology* **112**, 1099–1101.

Koesukwiwat, U., Jayanta, S. and Leepipatpiboon, N. (2007*a*). Solid-phase extraction for multiresidue determination of sulfonamides, tetracyclines, and pyrimethamine in Bovine's milk. *Journal of Chromatography* **1149**, 102–111.

Koesukwiwat, U., Jayanta, S. and Leepipatpiboon, N. (2007*b*). Validation of a liquid chromatography-mass spectrometry multi-residue method for the simultaneous determination of sulfonamides, tetracyclines, and pyrimethamine in milk. *Journal of Chromatography* **1140**, 147–156.

Lindsay, D. S. and Dubey, J. P. (1989). Evaluation of anti-coccidial drugs' inhibition of *Neospora caninum* development in cell cultures. *Journal of Parasitology* **75**, 990–992.

Lindsay, D. S., Rippey, N. S., Cole, R. A., Parsons, L. C., Dubey, J. P., Tidwell, R. R. and Blagburn, B. L. (1994). Examination of the activities of 43 chemotherapeutic agents against *Neospora caninum* tachyzoites in cultured cells. *American Journal of Veterinary Research* **55**, 976–981.

Lindsay, D. S., Butler, J. M., Rippey, N. S. and Blagburn, B. L. (1996). Demonstration of synergistic effects of sulfonamides and dihydrofolate reductase/thymidylate synthase inhibitors against *Neospora caninum* tachyzoites in cultured cells, and characterization of mutants resistant to pyrimethamine. *American Journal of Veterinary Research* **57**, 68–72.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65**, 55–63.

Muller, J., Aguado-Martinez, A., Manser, V., Balmer, V., Winzer, P., Ritler, D., Hostettler, I., Arranz-Solis, D., Ortega-Mora, L. and Hemphill, A. (2015*a*). Buparvaquone is active against *Neospora caninum in vitro* and in experimentally infected mice. *International Journal for Parasitology: Drugs and Drug Resistance* **5**, 16–25.

Muller, J., Balmer, V., Winzer, P., Rahman, M., Manser, V., Haynes, R. K. and Hemphill, A. (2015*b*). *In vitro* effects of new artemisinin derivatives in *Neospora caninum*-infected human fibroblasts. *International Journal of Antimicrobial Agents* **46**, 88–93.

Pastor-Fernandez, I., Regidor-Cerrillo, J., Jimenez-Ruiz, E., Alvarez-Garcia, G., Marugan-Hernandez, V., Hemphill, A. and Ortega-Mora, L. M. (2015). Characterization of the *Neospora caninum* NcROP40 and NcROP2Fam-1 rhoptry proteins during the tachyzoite lytic cycle. *Parasitology* 1–17.

Pereira, L. M. and Yatsuda, A. P. (2014). The chloramphenicol acetyltransferase vector as a tool for stable tagging of *Neospora caninum*. *Molecular and Biochemical Parasitology* **196**, 75–81.

Pereira, L. M., Candido-Silva, J. A., De Vries, E. and Yatsuda, A. P. (2011). A new thrombospondin-related anonymous protein homologue in *Neospora caninum* (NcMIC2-like1). *Parasitology* **138**, 287–297.

Pereira, L. M., Baroni, L. and Yatsuda, A. P. (2014). A transgenic *Neospora caninum* strain based on mutations of the dihydrofolate reductase-thymidylate synthase gene. *Experimental Parasitology* **138**, 40–7.

Peterson, D. S., Walliker, D. and Wellems, T. E. (1988). Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 9114–9118.

Philippe, P., Alzieu, J. P., Taylor, M. A. and Dorchies, P. (2014). Comparative efficacy of diclazuril (Vecoxan (R)) and toltrazuril (Baycox bovis (R)) against natural infections of *Eimeria bovis* and *Eimeria zuernii* in French calves. *Veterinary Parasitology* **206**, 129–137.

Rajapakse, S., Chrishan Shivanthan, M., Samaranayake, N., Rodrigo, C. and Deepika Fernando, S. (2013). Antibiotics for human toxoplasmosis: a systematic review of randomized trials. *Pathogens and Global Health* **107**, 162–169.

Reichel, M. P., Alejandra Ayanegui-Alcerreca, M., Gondim, L. F. and Ellis, J. T. (2013). What is the global economic impact of *Neospora caninum* in cattle – the billion dollar question. *International Journal for Parasitology* **43**, 133–142.

Reichel, M. P., Moore, D. P., Hemphill, A., Ortega-Mora, L. M., Dubey, J. P. and Ellis, J. T. (2015). A live vaccine against *Neospora caninum* abortions in cattle. *Vaccine* **33**, 1299–1301.

Rodrigues Fde, S., Tavares, L. E. and Paiva, F. (2016). Efficacy of treatments with toltrazuril 7·5% and lasalocid sodium in sheep naturally infected with Eimeria spp. *Revista Brasileira Parasitologia Veterinaria* **25**, 293–298.

Schirmer, R. H., Adler, H., Pickhardt, M. and Mandelkow, E. (2011). "Lest we forget you – methylene blue…". *Neurobiology of Aging* **32**, 2327–2316.

Schorer, M., Debache, K., Barna, F., Monney, T., Muller, J., Boykin, D. W., Stephens, C. E. and Hemphill, A. (2012). Di-cationic arylimidamides act against *Neospora caninum* tachyzoites by interference in membrane structure and nucleolar integrity and are active against challenge infection in mice. *International Journal for Parasitology: Drugs and Drug Resistance* **2**, 109–120.

Strohbusch, M., Muller, N., Hemphill, A., Krebber, R., Greif, G. and Gottstein, B. (2009). Toltrazuril treatment of congenitally acquired *Neospora caninum* infection in newborn mice. *Parasitology Research* **104**, 1335–1343.

Tang, D. and Santschi, P. H. (2000). Sensitive determination of dissolved sulfide in estuarine water by solid-phase extraction and high-performance liquid chromatography of methylene blue. *Journal of Chromatography* **883**, 305–309.

Van Dijk, S., Lobsteyn, A. J., Wensing, T. and Breukink, H. J. (1983). Treatment of nitrate intoxication in a cow. *The Veterinary Record* **112**, 272–274.

Venkatesan, M., Alifrangis, M., Roper, C. and Plowe, C. V. (2013). Monitoring antifolate resistance in intermittent preventive therapy for malaria. *Trends in Parasitology* **29**, 497–504.

Xu, Y. J., Tian, X. H., Zhang, X. Z., Gong, X. H., Liu, H. H., Zhang, H. J., Huang, H. and Zhang, L. M. (2012). Simultaneous determination of malachite green, crystal violet, methylene blue and the metabolite residues in aquatic products by ultra-performance liquid chromatography with electrospray ionization tandem mass spectrometry. *Journal of Chromatographic Science* **50**, 591–597.

Yoshimoto, M., Otsuki, T., Itagaki, K., Kato, T., Kohsaka, T., Matsumoto, Y., Ike, K. and Park, E. Y. (2015). Evaluation of recombinant *Neospora caninum* antigens purified from silkworm larvae for the protection of *N. caninum* infection in mice. *Journal of Bioscience and Bioengineering* **120**, 715–719.