NOVEL DRUG COMPOUNDS AGAINST *NEOSPORA CANINUM* AND *TOXOPLASMA GONDII IN VITRO*

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Abstract. *Neospora caninum* has recently been identified as an important cause of abortion in cattle worldwide. This parasite is closely related to *Toxoplasma gondii*. To identify the drug compounds for potential use against both parasites *in vitro*, nine novel drug compounds were incubated with either parasite on microtiter plate. The number of extracellular tachyzoites and the quantities of Vero cells left in the wells after incubating with those nine drugs were compared to the conventional drug control, a combination of sulfadiazine 25µg/ml and pyrimethamine 0.1µg/ml. The most effective drugs against both *N. caninum* and *T. gondii* in this study were trifluralin analogues.

INTRODUCTION

Phylogenetic studies have shown that *Neospora caninum* is very closely related to *Toxoplasma gondii* although they cause quite different biological diseases (Dubey, 2003). Neosporosis is now considered as a major cause of abortion in cattle worldwide and it can also cause neurological symptoms in dogs whereas toxoplasmosis is more often associated with diseases in humans and sheep (Esteban-Redondo and Innes, 1997; Innes, 1997; Anderson *et al*, 2000; Dubey, 2003).

Little information is available on the efficacy of drugs for the chemotherapy of *N. caninum* infected animals even though sulfadiazine and pyrimethamine were found to be effective in some dogs at the early stage of neosporosis but there is no drug that can treat neosporosis in cattle at present (Lindsay *et al*, 1994; Thate and Laanen, 1998; Kim *et al*, 2002; Darius *et al*, 2004; Mui *et al*, 2005). The current usage of sulfadiazine and pyrimethamine for the treatment of toxoplasmosis also has many disadvantages (Mui *et al*, 2005).

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The cell culture-based assays were performed in this present study to evaluate the novel drugs to determine which are effective to inhibit the growth of *N. caninum* and *T. gondii in vitro*.

MATERIALS AND METHODS

Microtiter plate assay

N. caninum (NC1 strain) and *T. gondii* (RH strain) were grown and maintained in Vero cells. For microtiter assays, the host cells were plated (104 cells/well) into flat-bottomed 96-well tissue culture plates with Dulbecco's modified eagle medium; with 10% fetal bovine serum, 1% L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin; at 37°C; and with 5%CO2 until a complete monolayer was visible under an inverted microscope. Monolayers were inoculated with 104 tachyzoites of *N. caninum* or *T. gondii* per well. Two hours post-inoculation, 50 µl of the medium was aspirated and replaced with the same volume of medium that contained either drug A, B, C, D, E, F, G, H, or I. All stock dilutions of the drugs were made in DMSO at a 10 mM or 20 mM concentration. The compounds were tested at a final concentration of 10 µM, 1 µM, and 0.1 µM in each well. Working dilutions were freshly prepared for each experiment in culture media. The drugs were incubated with the parasites for 72 hours. In each culture plate, three controls were included: (i) uninfected monolayer, (ii)

infected monolayers treated with a combination of pyrimethamine (0.1 µg/ml) and sulfadiazine (25 µg/ml), and (iii) untreated of infected monolayer (Derouin and Chastang, 1988; Mui *et al*, 2005). After a 72-hour incubation period, the number of extracellular tachyzoites in each well was assessed with a hematocytometer. The percentage of growth inhibition was calculated using the formula (Sarciron *et al*, 2002):

% growth inhibition = 100 - (100 x number of extracellular tachyzoites in treated well/number of extracellular tachyzoites in control well)

Crystal violet assay

Plates were visually monitored every day, and the assay was stopped when 90-100% of the untreated infected cells had lysed (three days post-inoculation). The crystal violet assay was performed as previously described with some modification (Linsay and Dubey, 1999; Zarubin *et al*, 2005). Briefly, the culture medium and extracellular tachyzoites were removed from wells. Adhering Vero cells were washed with 1xPBS and then fixed in 100% methanol for 5 minutes. The crystal violet solution was added and incubated at room temperature for 5 minutes. Cells were washed twice with 1xPBS. The 50% glacial acetic acid solution was added and incubated at room temperature for 1 hour. An ELISA plate reader operating at 595 nm was used to quantitate the amount of crystal violet present. The percentage of cell viability was calculated using the formula (Akca *et al*, 2003):

% cell viability = 100 x A1/A0
(When A1 is the OD of treated infected well and A0 is the OD of untreated uninfected well).

Cytotoxicity assay for Vero cells

To determine the cytotoxicity of these drug compounds against Vero cells, the MTT assay was used. Approximately 1 x 10^4 Verocell/well were applied to 96-well, microtiter plates that were treated with either drug at the final concentration of 10 µM and incubated at 37°C for 24 hours. The MTT assay was performed according to manufacturer's instructions. The absorbance was measured on an ELISA plate reader with a test wavelength of 595 nm and a reference wavelength of 620 nm. Cells treated with the medium only were used as the control.

RESULTS

Anti-protozoa activities of drug compounds determined by the percentage of growth inhibition

At 10 µM, drug B was the most effective drug against *N. caninum* in cell culture, whereas drug F was the most effective drug against *T. gondii* (Table 1).

Anti-protozoa activities of drug compounds determined by the crystal violet assay

Results of the crystal violet assay demonstrated that drug B was the most effective drug against *N. caninum*. Drugs F and G were also shown to inhibit *N. caninum* development. For *T. gondii* growth inhibition, drug F was the most effective drug, while drug B and H also demonstrated some effectiveness (Table 2).

Cytotoxicity test

None of the nine drug compounds showed any toxicity when used up to 10 µM. The morphology

Table 1
Percentage growth inhibition of the parasites after 72-hour incubation with each drug.

Drug (10 µM)	*N. caninum*	*T. gondii*
Drug A	60.8	70.8
Drug B	88.6	81.5
Drug C	78.5	79.2
Drug D	62.0	0.8
Drug E	78.5	77.7
Drug F	75.9	92.3
Drug G	79.7	59.2
Drug H	43.0	63.1
Drug I	45.6	53.1
Sulfadiazine and pyrimethamine (control)	84.8	88.5

Table 2
Vero cell viability of the drug test to *N. caninum* and *T. gondii*.

Drug	*N. caninum*			*T. gondii*		
	10 µM	1 µM	0.1 µM	10 µM	1 µM	0.1 µM
A	78.2%	71.7%	71.7%	53.8%	34.5%	40.6%
B	90.7%	86.6%	86.6%	89.0%	34.3%	31.7%
C	39.4%	68.3%	68.3%	17.5%	28.9%	31.9%
D	46.9%	79.1%	79.1%	27.8%	27.3%	49.6%
E	77.2%	83.7%	83.7%	31.1%	20.9%	33.1%
F	80.8%	84.0%	84.0%	91.7%	31.9%	37.1%
G	81.2%	90.5%	90.5%	66.0%	44.6%	38.6%
H	76.5%	84.3%	84.3%	83.0%	80.7%	82.8%
I	78.8%	77.7%	77.7%	74.4%	44.7%	36.8%
Sulfadiazine and pyrimethamine (control)		97.4%			99.0%	
Untreated uninfected Vero cells		100%			100%	

and growth rate of both treated and untreated Vero cells were not different (Fig 1).

DISCUSSION

In the present study, the cultured host cells, which were infected with *N. caninum* or *T. gondii*, were treated with various concentrations of nine drug compounds to examine the efficacy of drugs against *N. caninum* and *T. gondii* tachyzoites intracellular multiplication. A combination of sulfonamides and pyrimethamine has been well known as being effective in inhibiting the growth of *T. gondii* and *N. caninum* (Lindsay and Dubey, 1989; Lindsay *et al*, 1994). It also showed an excellent effect to inhibit the development of both *N. caninum* and *T. gondii* in our study. However, there are many disadvantages of this drug combination as it can block folic acid metabolism of host cells in long-term usage or at high doses, and it is associated with bone marrow suppression (Montoya and Liesenfeld, 2004; Mui *et al*, 2005). Moreover, pyrimethamine is teratogenic when used in pregnant woman, and it is not specifically approved for veterinary use (Toribio *et al*, 1998). Alternative drugs that are

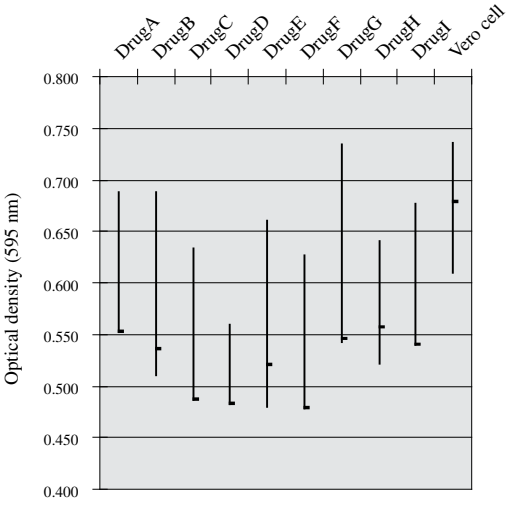

Fig 1- Effect of nine drugs on Vero cell *in vitro*. Cytotoxicity of drug compounds was measured by the MTT assay.

safer and more potent are needed. We examined nine new drug compounds that are effective in inhibiting the growth of other parasites *in vitro* in our laboratory (data not shown). Drugs B, F, G and H showed satisfactory effects to inhibit the development of *N. caninum* and/or *T. gondii*

in Vero cell culture. Drug B, F, G and H are trifluralin analogues. Trifluralin is used primarily as an herbicide on grass. It ranks as one of the five best-selling herbicides in the US (Extoxnet, 2001). It prevents weed growth by inhibiting root development through the interruption of mitosis.

These novel drug compounds might be of value in the prevention and treatment of neosporosis and toxoplasmosis in the future. For that purpose, antiprotozoal activity of these drugs in in vivo and the mechanism of action should be initiated.

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