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In vitro screening of the open source Pathogen Box identifies novel compounds with profound activities against *Neospora caninum*

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ABSTRACT

Neospora caninum is a major cause of abortion in cattle and represents an important veterinary health problem of great economic significance. The Medicines for Malaria Venture (MMV) Pathogen Box, an open-source collection of 400 compounds with proven anti-infective properties against a wide range of pathogens, was screened against a *N. caninum* beta-galactosidase reporter strain grown in human foreskin fibroblasts. A primary screening carried out at 1 μM yielded 40 compounds that were effective against *N. caninum* tachyzoites. However, 30 of these compounds also affected the viability of the host cells. The 10 remaining compounds exhibited IC_{50} values between 4 and 43 nM. Three compounds with IC_{50} values below 10 nM, namely MMV676602, MMV688762 and MMV671636, were further characterized in vitro in more detail with respect to inhibition of invasion versus intracellular proliferation, and only MMV671636 had an impact on intracellular proliferation of tachyzoites. This was confirmed by transmission electron microscopy, showing that the primary target of MMV671636 was the mitochondrion. MMV671636 treatment of experimentally infected mice significantly reduced the number of animals with lung and brain infection, and these mice also exhibited a significantly reduced titer of antibodies directed against *N. caninum* antigens. Thus, MMV671636 is a promising starting point for the development of a future neosporosis therapy.

1. Introduction

Apicomplexan parasites such as *Neospora*, *Toxoplasma*, *Cryptosporidium*, *Eimeria*, *Plasmodium*, *Theileria* and others are of outstanding veterinary and/or human medical importance. *Neospora caninum* is phylogenetically closely related to *Toxoplasma gondii*, but several biological features distinguish these two species, including elements of their life cycle, host range, pathogenicity and ultrastructure (Hemphill et al., 2016, 2013). Canids, namely dogs, wolves, dingoes and coyotes, represent definitive hosts of *N. caninum*, while asexual proliferation occurs in a wide range of intermediates hosts including cattle, sheep, water buffaloes and many more (Buxton et al., 2002; Dubey, 2003). Extensive proliferation of tachyzoites can lead to cellular destruction, tissue damage and immunopathology, and thus disease. *Neospora* infections have never been demonstrated in humans. However, *N. caninum* has considerable economic significance as one of the most important causes of abortion, stillbirth and/or birth of weak offspring in cattle, with estimated losses of approximately 1.28 billion US Dollars in 10 countries (Reichel et al., 2013).

Strategies that are currently considered for the prevention and treatment of bovine neosporosis are (i) testing and culling of seropositive animals, (ii) discontinued breeding with offspring from seropositive cows, (iii) vaccination of susceptible and infected animals, and (iv) chemotherapeutical treatment of calves from seropositive cows (Hemphill et al., 2016). The latter two would be economically viable provided suitable targets and effective formulations for vaccination and/or drug treatments were identified (Häsler et al., 2006). Since ab initio drug development against neosporosis would certainly be too costly, repurposing of compounds effective against other pathogens may constitute a suitable approach. A wide range of compound classes were shown to exhibit interesting effects against tachyzoites in vitro (for review see Hemphill et al., 2016), but only a few have been demonstrated to be effective in vivo. One promising candidate for such a drug repurposing approach is buparvaquone (BPQ), a drug marketed in Africa against bovine theileriosis. BPQ is effective against *N. caninum* in vitro and in vivo (Müller et al., 2015a) and inhibits vertical transmission of *N. caninum* in a pregnant mouse model (Müller et al.,

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2016). Moreover, inhibitors of calcium-dependent protein kinases (CDPKs), which are effective against *Plasmodium* (Ojo et al., 2012), *Toxoplasma* (Ojo et al., 2010; Vidadala et al., 2016), *Cryptosporidium parvum* (Castellanos-Gonzalez et al., 2016; Schaefer et al., 2016), *Babesia bovis* (Pedroni et al., 2016), *Theileria equi* (Hines et al., 2015) and *Sarcocystis neurona* (Ojo et al., 2016), are also effective against *N. caninum* in vitro as well as in pregnant and non-pregnant neosporosis mouse models (Ojo et al., 2014; Winzer et al., 2015; Müller et al., 2017).

To stimulate drug discovery beyond malaria, Medicines for Malaria Venture (MMV) has, in 2011, created the Malaria box. This is a collection of 200 drug-like and 200 probe-like compounds with proven activities against *Plasmodium falciparum* blood stages, which was made freely accessible around the globe, and since then has been tested against a wide range of pathogens including apicomplexan parasites of medical and economic importance (Van Voorhis et al., 2017). Data on activities, structures, chemical properties, cytotoxicity, biodistribution and pharmacokinetic properties were made openly available, and this has resulted in a considerable pipeline of preclinical and clinical candidates for a wide range of diseases. In December 2015, MMV launched the Pathogen Box, a collection of 400 diverse drug-like molecules, which is also provided to researchers at no cost (www.pathogenbox.org). Each of the 400 compounds in the Pathogen Box has confirmed activity against one or more pathogens causing devastating diseases such as tuberculosis, malaria, sleeping sickness, leishmaniasis, schistosomiasis, hookworm disease, toxoplasmosis and/or cryptosporidiosis.

We here report on the in vitro screening of the MMV Pathogen Box against *N. caninum* tachyzoites and on the identification of eight novel compounds that could potentially serve as a starting point for the development of effective drugs against neosporosis. Based on their in vitro properties, three of these compounds were characterized in more detail regarding their effects on the cellular ultrastructure of tachyzoites and the efficacy of two compounds was assessed in the non-pregnant neosporosis mouse model.

2. Materials and methods

2.1. Chemicals

The MMV Pathogen Box contains 400 compounds as 10 mM stock solutions in DMSO and was obtained free of charge from the MMV (Geneva, Switzerland). Supporting information including the plate mapping is available at www.pathogenbox.org. The box was stored at −20 °C. Biochemical reagents were purchased from Sigma (St. Louis, MO, USA). Culture media were from Thermo Fisher Scientific (formerly Gibco; Waltham, MA, USA).

2.2. Cell culture

Human foreskin fibroblasts (HFF) were cultured in DMEM containing 10% FCS. Vero cells were cultured in RPMI medium containing 5% FCS. Both media contained 50 U of penicillin/ml, and 50 μg of streptomycin/ml. The cells were cultured at 37 °C and 5% CO_2 in tissue culture flasks (Sarstedt, Nürnbrecht, Germany). Cultures were passaged at least once each week. Transgenic beta-galactosidase-expressing *N. caninum* (Nc-beta-gal) and *N. caninum* Spain7 strain tachyzoites were maintained by serial passage in Vero cells cultured in RPMI-1640 (Müller et al., 2015a).

2.3. In vitro screening of MMV Pathogen Box compounds

In vitro screening was carried out as previously described (Müller et al., 2015a,b). *Neospora caninum* tachyzoites were harvested by scraping off the infected HFF cell monolayer with a rubber cell scraper, followed by repeated passages through a 25-gauge needle at 4 °C, and separation from intact cells by centrifugation at 200*g* for 1 min at 4 °C. Drug efficacy tests were performed on HFF monolayers grown in 96-well plates. They were infected with 10^3 freshly harvested Nc-beta-gal tachyzoites, and compounds were added at the time point of infection. In parallel, cytotoxicity assays were carried out using HFF monolayers that were kept uninfected. Proliferation of *N. caninum* tachyzoites was measured using the beta-galactosidase assay and host cell viability was assayed using the Alamar Blue assay as described (Müller et al., 2015a,b).

Compounds were regarded as effective against *N. caninum* when the beta-galactosidase values were lower than the mean values of the controls minus three times their S.D. Compounds were regarded as cytotoxic when the Alamar Blue values were lower than the mean values of the controls minus three times their S.D. Subsequently, IC_{50} values (inhibitory concentration of 50% of the solvent control value) of effective and not cytotoxic compounds were determined in the same assay set-up, and were calculated as described (Müller and Hemphill, 2013).

2.4. Assessments on inhibition of invasion versus intracellular proliferation in vitro

Three compounds that were retained in our screen, namely MMV671636, MMV676602 and MMV688762, were tested in a different set-up. HFF cell monolayers grown in 96-well plates were infected with 10^3 tachyzoites and treated with the compounds at a final concentration of 100 nM per well at the time of initiation of infection (0 min), as well as 3 and 24 h after addition of tachyzoites. Control wells with equal volume of DMSO were also included per reaction plate. The plate was then incubated for three more days, and proliferation of *N. caninum* tachyzoites was measured using the beta-galactosidase assay.

2.5. Transmission electron microscopy (TEM)

HFFs were grown to confluence in T25 flasks and infected with Nc-Spain7 tachyzoites as described (Müller et al., 2017). After 24 h, cultures were supplemented with 0.5 μM MMV676602 and MMV671636, and infected cultures treated with the corresponding amounts of DMSO served as controls. After 24, 48 and 72 h of treatment, the monolayers were washed with 100 mM sodium cacodylate buffer (pH 7.3) and fixed with cacodylate buffer containing 2.5% glutaraldehyde for 10 min. The cellular material was then collected using a rubber cell scraper and was centrifuged for 10 min at 1000*g* at room temperature. The supernatant was removed, and infected cells were fixed further in glutaraldehyde-cacodylate at 4 °C overnight. Post fixation in 2% OsO_4, dehydration in a graded series of ethanol and embedding in Epon 820 epoxy resin was done as previously described (Müller et al., 2015a,b). Ultrathin sections were cut on a Reichert and Jung ultramicrotome (Reichert and Jung, Vienna, Austria), placed onto formvar carbon-coated grids (Plano GmbH, Marburg, Germany, and were contrasted with uranyle-acetate and lead citrate. Specimens were viewed on a CM12 transmission electron microscope operating at 80 kV.

2.6. Treatments with MMV676602 and MMV671636 in N. caninum-infected mice

All protocols involving animals were approved by the Animal Welfare Committee of the Canton of Bern, Switzerland (license BE115/14). All animals were handled in strict accordance with practices to minimize suffering.

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