ApicoWplexa virtual Meeting, October 15, 2020
Drugs and drug targets

Abstracts

Keynote 1

Identification and genetic validation of novel drug targets in *Cryptosporidium parvum*

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Cryptosporidium parvum, a protozoan parasite, is a leading cause of diarrheal disease in neonatal calves and young children. There are currently no effective drugs available to treat *Cryptosporidium* infection (cryptosporidiosis) in animals and humans. This calls for the development of new therapeutics to reduce the morbidity and mortality associated with cryptosporidiosis. In my laboratory, we utilize a combination of genetics and cellular biology approaches to understand the parasite biology, and identify and validate new drug targets. We use the powerful CRISPR/Cas9 genome editing and an immunocompromised mouse model system to genetically manipulate *Cryptosporidium,* and propagate transgenic parasites. Leveraging on transgenic *C. parvum* reporter strains, we have established a multi-well plate assay and a robust animal model to identify several compounds that effectively kill the parasite. These include pyrazolopyridines that inhibit the parasite PI4(K) lipid kinase, bumped kinase inhibitors that target calcium-dependent protein kinase-1, and compounds that target aminoacyl tRNA synthetases. Recently, we have developed a conditional system that now allows study of essential genes in C*ryptosporidum* and validation of drug targets.

Keynote 2:

Endochin-like quinolones: mechanism, selectivity and efficacy against apicomplexan pathogens

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Endochin-like quinolones (ELQ) are highly effective compounds that are broadly active against apicomplexan pathogens. ELQs inhibit parasite proliferation by competing with the cytochrome bc_1 complex substrates, ubiquinone and ubiquinol. Key structural features of ELQs determine whether ELQs inhibit the quinone reduction (Qi) site of cytochrome *b* or the quinol oxidation (Qo) site of cytochrome *b*, the target of atovaquone, a clinically used drug for malaria and toxoplasmosis. ELQs that target the Qi site are specific for the apicomplexan cytochrome bc_1 compared to mammalian cytochrome bc_1. This specificity is likely due to Qi site amino acids that are unique to Apicomplexa. ELQ-316 binds to the Qi site and has been identified as the most promising ELQ for the treatment of many apicomplexan pathogens. To advance ELQ-316 toward clinical use, the oral bioavailability of ELQ-316 was improved by creating carbonate ester prodrugs that increased the plasma concentrations of ELQs six-fold. Clinical potential has also been identified through testing the combination of ELQ-316 and the clinically used drugs, atovaquone and pyrimethamine. ELQ-316 prodrugs are promising preclinical candidates for apicomplexan pathogens that cause devastating diseases in animals.

Short presentation 1:

Bumped kinase inhibitors and endochin-like quinolones: individual and combined treatments investigated *in vitro* and in the pregnant mouse model for neosporosis

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Bumped kinase inhibitors (BKIs) and endochin-like quinolones (ELQs) are promising drug classes that act against a number of apicomplexan species, including *N. caninum*, *in vitro* and *in vivo*. BKIs target calcium-dependent protein kinase 1, which plays a key role in host cell invasion and egress, and BKIs induce the formation of intracellular multinucleated complexes (MNCs). In contrast, ELQs affect the cytochrome *bc1* complex and thus oxidative phosphorylation in the mitochondrion. *In vitro* studies were carried out to investigate whether BKIs and ELQs could act in synergy against proliferating *N. caninum* tachyzoites constitutively expressing β–Galactosidase. BKI-1748 applied alone had an IC_{50} of 165 nM. ELQ-316 was shown to exhibit an IC_{50} of 1.55 nM, while ELQ-334 and ELQ-422, both acting as prodrugs of ELQ-316, display IC_{50}s of 54.3 and 15.6 nM, respectively. Combined application of BKI-1748 with ELQ-316 and ELQ-422 showed synergistic effects and inhibited proliferation almost completely at concentrations of 0.2-0.002 nM, while no synergy was seen with the BKI-1748/ELQ-422 combination. However, immunofluorescence and TEM demonstrated that the BKI-1748/ELQ-422 resulted in the formation of MNCs that were much smaller than in cultures treated with BKI-1748 alone. The application of the drugs in the pregnant mouse model, either individually or in combination, showed that the treatments did not notably affect fertility, but neonatal pup mortality was slightly increased in the combination treatment groups (approx. 18%) compared to BKI-1748 (5.6%, identical to the control), and treatments with individual ELQs resulted in 12% neonatal mortality. Postnatal survival in the BKI-1748 treated group was seen for 70% of pups, while postnatal survival of pups was higher (>90%) in the groups treated with individual ELQs or BKI-ELQ combinations. Notably, 100% postnatal survival and a complete (100%) inhibition of vertical transmission was noted in the group treated with the BKI-1748/ELQ-334 combination. Analyses of drug levels in plasma samples collected shortly after the final drug application showed that in the BKI-1748/ELQ-334 group drug levels of BKI-1748 and ELQ-334 were roughly twice as high

compared to the groups that had received individual BKI-1748 or ELQ-334 treatments. This indicates that combined application of these compounds impact on plasma levels and this could influence neonatal mortality as well as efficacy in the pregnant neosporosis mouse model.

Short presentation 2:

DEVELOPMENT OF RUTHENIUM COMPLEXES AS ANTIPARASITIC AGENTS

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After the successful introduction of cisplatin as an anticancer drug, the potential of organometallic compounds for the discovery of new drugs has long been established, not only as anticancer drugs, but also as antibacterial and antiparasitic agents. In particular, ruthenium-based compounds are promising candidates for the development of antiparasitic drugs. Specifically, our cationic trithiolato-bridged dinuclear ruthenium(II)-arene complexes (general formula $[(\eta^6\text{-arene})_2Ru_2(\mu_2\text{-SR})_3]^+$ and, respectively, $[(\eta^6\text{-arene})_2Ru_2(\mu_2\text{-SR}^1)_2(\mu_2\text{-SR}^2)]^+$) present high *in vitro* activity against the apicomplexan parasites such as *Toxoplasma gondii*[1] and *Neospora caninum*[2]. However, their modes of action and cellular targets remain largely unknown and this aspect represents one important axis of our research. One powerful strategy for the "sub-cellular localization" investigation of organometallic compounds is to tag the organometallic moiety with a fluorophore dye, such as coumarins, BODIPYs etc.[3]
In this talk, a series of 13 new conjugates di-ruthenium(II)-arene complexes bearing a coumarin tag, their photophysical and antiparasitic properties are presented.[4] The linking of coumarin to the ruthenium complex considerably quenched the fluorescence of the new conjugates. Nevertheless, fluorescence microscopy investigations of HFF cells treated with some coumarin-ruthenium compounds were performed. A primary screening against a transgenic constitutively β-galactosidase expressing *T. gondii* strain (*T. gondii* β-gal) grown in human foreskin fibroblasts (HFF) at 0.1 and 1 µM, and alamarBlue assay of the cytotoxicity in non-infected HFF and further IC_{50} determination were performed. Compounds displayed IC_{50} values against *T. gondii* β-gal ranged from 105 nM to 735 nM. Interestingly, transmission electron microscopy (TEM) showed important ultrastructural changes in the parasite mitochondrion, resulting in pronounced destruction of tachyzoites, while the mitochondria of HFF remain intact.

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Short presentation 3:

Efficacy of BKI-1294 in a pregnant sheep model of neosporosis

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Control of *N. caninum* infection in ruminants remains without an effective therapeutic or prophylactic alternative. The NcCDPK1 inhibitor BKI-1553 applied by subcutaneous injection in *N. caninum*-infected pregnant sheep was only partially efficacious against abortion and failed to protect transplacental transmission. In contrast to BKI-1553, BKI-1294 demonstrated improved efficacy in a pregnant mouse model of neosporosis. Orally applied BKI-1294 was safe in pregnant sheep and was highly efficacious in a pregnant sheep model of toxoplasmosis. In this work, we present the efficacy of BKI-1294 treatment (dosed 5 times orally every 48 h) starting 48 h after intravenous infection with 10^5 Nc-Spain7 tachyzoites at mid-pregnancy (refined model of infection). In the dams, BKI-1294 plasma concentrations were above the IC50 for *N. caninum* during 12-15 days. We observed a minor increase in rectal temperature, higher IFNγ levels after blood stimulation *in vitro* and a minor increase of the humoral immune response in treated ewes compared to infected but untreated ewes, but only until day 28 post infection. However, the drug

did not protect against abortion (87% fetal death in treated and untreated ewes), did not reduce transplacental transmission, parasite load and lesions in placentomes and fetal brain. These results differ sharply from the high efficacy found in sheep infected orally with *T. gondii* oocysts at mid-pregnancy. We hypothesize that the lack of fetal efficacy could be due to: i) a short systemic exposure in the dams and a probably suboptimal fetal exposure of this parasitostatic drug, which was not able to reduce replication of the likely established *N. caninum* tachyzoites in the fetus at the moment of treatment; and ii) in the pregnant sheep model for neosporosis the challenge is done by the intravenous route and the parasite may reach the placenta shortly after inoculation compared with the toxoplasmosis model, where the challenge was by oral oocysts. We propose that a BKI with lower plasma clearance and good ability to cross the blood-brain and placental barriers should be tested in a model of ovine neosporosis using a more natural route of infection.

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