96

High-throughput *In Vitro* HIV Rev-multimerization Assay

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The HIV Rev protein is essential for efficient viral replication. Rev-monomers multimerize on viral RNAs to export these from the nucleus to the cytoplasm of the infected cell through interaction with the cellular CRM1-mediated pathway for nuclear export. Efforts to develop inhibitors of the Rev-function were focussed mainly on the Rev-RNA and the Rev-CRM1 interaction. However an important aspect to the function of Rev is its necessity to multimerize. So far, no inhibitors targeting the Rev-oligomerization process have been discovered. Therefore, we have developed a solid high-throughput *in vitro* Rev-multimerization assay based on FRET (Fluorescence Resonance Energy Transfer). This technique requires two fluorophores to be in close proximity of each other. Upon excitation of the donor fluorophore, energy is transferred to the acceptor fluorophore from which emission is measured. In our multimerization assay fusion proteins of Rev to ECFP (FRET-donor) and Rev to EYFP (FRET-acceptor) are mixed allowing interaction of Rev-monomers, which results in high FRET-efficiencies. Samples containing combinations of ECFP and EYFP-Rev, ECFP-Rev and EYFP or ECFP and EYFP are used as negative controls. To validate this FRET-assay for the screening of Rev-multimerization inhibitors, increasing amounts of unlabeled Rev were added to the ECFP-Rev/EYFP-Rev sample leading to a dose-dependent inhibition of the FRET-signal. This fast and solid Rev-multimerization assay is adaptable to 96-well plate, 386-well plate and 1536-well plate formats, making the assay suitable for high-throughput screening of Rev-multimerization inhibitors. The discovery of a Rev-oligomerization inhibitor will allow the validation of HIV Rev-multimerization as target for antiviral chemotherapy. The concept of this assay is also widely applicable to the discovery of inhibitors of other protein–protein interactions.

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97

Pre-clinical Development of IQP-0410, a Highly Potent Dual-acting Agent for the Therapy of HIV-1 Infection

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The primary problems associated with anti-HIV therapy continue to be drug toxicity, drug–drug interactions, patient compliance with prescribed treatment regimens, and the appearance of drug resistant viruses. ImQuest Pharmaceuticals is developing IQP-0410, a highly potent non-nucleoside pyrimidinedione inhibitor of both HIV-1 and HIV-2. Oral dose of IQP-0410 administered via gavage, up to a maximum feasible dose level of 1000 mg/kg, was well tolerated in Beagle dogs. There were no test article-related findings noted during the evaluation of in-life data, clinical pathology or necropsy data. Histopathology examination of selected tissues revealed no drug-related microscopic findings. Preliminary pharmacokinetics studies of IQP-0410 in dogs showed that a significant amount of the compound remained in plasma at 24 h on Day 6, with an average of 37 ng/mL remaining. C_{max} values for Day 1 ranged from 52 to 113 ng/mL and from 71 to 165 ng/mL on Day 7 and the calculated EC95 value for IQP-0410 was 1 ng/mL. Metabolic stability and reaction phenotyping in human liver microsomes indicated that CYP3A4 is a major enzyme responsible for the metabolism of IQP-0410. Safety pharmacology studies showed no signs of pharmacological and toxicological activity. All genotoxicology tests of IQP-0410 were negative except for the mouse lymphoma test that was positive but below the range normally seen with a known positive control compound. This favorable pre-clinical profile suggests that IQP-0410 will be an important addition to the currently available therapeutic regimens used to treat HIV.

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Poster Session 2: Herpesviruses, Poxviruses, Other Antiviral Agents, Medicinal Chemistry and Topical Microbicides

98

Antiviral Activity of Monoterpene Components of Essential Oils Against Herpes Simplex Virus

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Herpes simplex virus (HSV) is an important pathogen for humans causing labial herpetic infections and is a serious disease in immunosuppressed patients. The development of resistant strains of HSV to the available drugs, especially acyclovir, has further attempted to identify and develop new alternative agents for management of HSV infections. Essential oils and their components are potential antiviral agents. Eleven monoterpenenes including alpha-terpinene, gamma-terpinene, alpha-pinene, beta-pinene, alpha-terpineol, terpinene-4-ol, limonene, thymol, p-cymene, citral and 1,8-cineol, with hydrocarbon, alcohol, aldehyde and ether structure, which are major components of essential oils, were evaluated for anti-HSV activity. All monoterpenes were examined *in vitro* on RC-37 cells, cytotoxicity of components were evaluated by a standard neutral red assay. The 50% inhibitory concentrations (IC_{50}) of the monoterpenes for HSV plaque formation were determined in dose–response studies. Ten monoterpenes revealed high antiviral activity against free HSV. At maximum noncytotoxic concentration, all monoterpenenes reduced plaque formation by 80–100 %, except for monoterpene ether. The experimental data exhibited a significant higher susceptibility of HSV against the monoterpene hydrocarbons in comparison to oxidized components. In order to identify the mode of antiviral action, the monoterpenes were added to host cells or viruses at different stages of infection. In time addition experiments, the viral infection was reduced when monoterpenes interacted with free virus. Our studies suggest that monoterpenes might be suitable for topical treatment of herpetic infections.

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99

Preclinical Pharmacokinetic, Toxicokinetic and Toxicology Results for Cyclopropavir, a Promising New Agent for the Treatment of Beta- and Gamma-herpesviruses

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Cyclopropavir (CPV, ZSM-I-62, MBX 400) has been shown to be potent against beta- and gamma-herpesviruses (Kern et al.,

2005). We now report that CPV is well tolerated in oral toxicology studies in the rat and dog with overall good oral bioavailability. In single dose pharmacokinetic and bioavailability studies, oral bioavailability in rats and dogs ranged from 22 to 46% and 70 to 91%, respectively. In single dose rat toxicology studies, CPV was well tolerated up to 300 mg/kg, with no changes in clinical signs, body weight, organ weights, food consumption, hematology or clinical pathology (NOAEL ≥ 300 mg/kg). Similarly, in a 14-day multiple dose (10–100 mg/kg, PO) rat study, no CPV-related effects on clinical observations, body weight, food consumption, ophthalmic examinations, hematology, or clinical chemistries were observed at any dose tested (NOAEL ≥ 100 mg/kg). CPV was readily absorbed with T_{max} values ranging from 0.50 to 4.0 h on Day 1 and 0.50 to 2.0 h during Week 2. After reaching C_{max}, $t_{1/2}$ elimination values ranged from 1.51 to 3.74 h on Day 1 and 2.37 to 7.26 h during Week 2. The $t_{1/2}$ generally increased with increasing dose and after repeated dosing. Possible sex differences in $t_{1/2}$ and T_{max} were also observed. Toxicokinetic results from the multiple dose rat study show overall CPV exposure increased with increasing dose. Escalating oral doses of CPV (10, 50, 100, and 300 mg/kg) were evaluated in dogs. At doses of 10–100 mg/kg there were no changes in clinical signs, food consumption, body weight or clinical pathology. CPV administration reaching 300 mg/kg resulted in prominent clinical pathology findings observed on Day 12 which correlated with clinical signs and with the kidney identified as a target organ. The MTD for CPV was determined to be 100 and 300 mg/kg for the female and male dogs, respectively. Overall, toxicology studies have demonstrated a very acceptable margin of safety for the advancement of CPV into human phase I clinical studies.

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100

Phenotyping Human Cytomegalovirus Drug Resistance Mutations Using a Recombinant Virus Incorporating EGFP

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Resistance of human cytomegalovirus (HCMV) to antiviral drugs can be determined either genotypically, by mapping known resistance mutations in genes UL97 and UL54, or phenotypically, by testing viral strains in cell culture in the presence of antiviral drugs. Whereas mutations in UL97 have been well characterised, allowing reliable resistance testing, data about polymerase (UL54) mutations are incomplete. We established a new method for phenotypic characterisation of UL54 mutations. All approved anti-HCMV drugs, Ganciclovir, Cidofovir and Foscarnet, target the viral polymerase. Inhibition of the polymerase leads to reduced synthesis of viral DNA and to reduced expression of late genes. Using a recombinant reporter virus expressing the early late protein pp65 fused to EGFP (vTB65g), we were able to determine drug concentrations required to reduce fluorescence intensity by 50% (IC_{50}). The assay was evaluated regarding MOI dependence, the time of measurement post infection and inter- and intra-test variability and was found to be a highly reproducible, cheap and relatively simple method for the quick analysis of antiviral drug resistance. We were able to determine growth characteristics of vTB65g by generating fluorescence intensity kinetics instead of titrating. In addition, intensity of the pp65-EGFP fluorescence signals strongly correlated with the amount of newly synthesised HCMV genome copies, measured by quantitative realtime PCR, showing that, unlike other assays, our method allows quantitative evaluation of polymerase activity. By combining this assay with powerful markerless BAC mutagenesis, the published phenotype of UL54 mutation E756K was confirmed. To provide the clinically essential link between genotype and resistance phenotype, we generated a database of rules containing all previously published HCMV UL97 and UL54 mutations as well as associated quantitative phenotypic results. Sequence data of UL97 and UL54 from patients' isolates can now be blasted against the database thus allowing a fast screening for drug resistance mutations and corresponding phenotypes. This will provide essential information for an optimum treatment of HCMV-diseased patients.

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101

The Effect of Human Cytomegalovirus Proteins PUL97 and PUL27 on Host Interferon Responses

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Interferon-gamma (IFN-γ), the sole type II interferon, plays an important role in both innate and adaptive immune responses to viral infection. Human cytomegalovirus (HCMV) alters the expression of both type I and II interferons, but the alteration of IFN-γ signaling by the virus is poorly understood. The viral serine/threonine kinase, pUL97, has been shown to impact multiple host functions as has its putative paralog, pUL27. In murine cytomegalovirus, the M27 homolog of pUL27 has been shown to specifically disrupt IFN-γ signaling. Both pUL97 and pUL27 have been shown to confer resistance to maribavir (MBV) which implied that these viral proteins may have interrelated or redundant functions, so we examined their effect on interferon pathways, specifically under IFN-γ stimulation. MBV is an antiviral that is currently in phase III clinical trials for the treatment of HCMV infections and has been shown to inhibit the kinase activity of UL97. We examined the JAK-STAT and IFN-regulated signaling pathways using real-time reverse transcriptase PCR (RT-PCR) with MBV and a recombinant virus that does not express UL27. The absence of UL27 and/or inhibition of UL97 kinase activity with MBV significantly dysregulates key players within IFN-related signaling pathways, such as IRF-1, ISG15, OAS-1, PDGFRα, MMP-3, IRF-1, SOCS-5 and IP-10 (CXCL10). Upon further evaluation of a subset of statistically significant targets, it was evident that UL97 and/or UL27 may both be involved in the disruption of the host antiviral response via the type II IFN pathway. Investigation of these pathways altered by both pUL27 and pUL97 can clarify the roles of both proteins in viral infection and may improve our understanding of why mutations in both these viral proteins confer resistance to MBV.

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