

104

Antiviral Drug Efficacy Evaluation Against Pandemic Influenza A (H1N1) Viruses in MDCK Cells

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New and emerging influenza virus strains, such as the pandemic influenza A (H1N1) virus, require constant vigilance for antiviral drug sensitivity and resistance. These studies evaluated eight isolates of pandemic influenza A virus against seven antiviral drugs: oseltamivir, peramivir, zanamivir, T-705 (favipiravir), ribavirin, amantadine and rimantadine. The pandemic viruses tested include isolates from California, New York, Utah, and an oseltamivir-resistant isolate from Hong Kong. In addition, a mouse-adapted A/CA/04/2009 virus was evaluated. EC₉₀ values for each drug were determined by virus yield reduction assay in three replicate experiments. A broad range of drug sensitivities was observed among the H1N1 viruses tested. However, significantly higher ($P < 0.05$) EC₉₀ values for the neuraminidase inhibitors were only observed for the drug-resistant A/Hong Kong/2369/2009 virus. As expected, all isolates showed resistance to the adamantanes. Dose–response curves also showed that the mouse-adapted strain had consistently higher EC₉₀ values compared to its non-adapted counterpart. We also evaluated the effects of antiviral drug combinations on drug-resistant influenza A/Hong Kong/2369/2009 virus. Drug combinations included T-705 plus one of the neuraminidase inhibitors: oseltamivir, peramivir, or zanamivir. Synergism was observed involving three doses of T-705 (1.0, 3.2, and 10.0 μ M) for all three neuraminidase inhibitors. Oseltamivir and zanamivir were synergistic with T-705 at three concentrations (3.2, 10, and 32 μ M for oseltamivir, or 0.1, 0.32, and 1.0 μ M for zanamivir) and peramivir showed synergism with T-705 at two concentrations (0.32 and 1.0 μ M). These studies demonstrate that pandemic influenza A virus isolates from different regions remain sensitive *in vitro* to commonly used antiviral drugs, and that combination chemotherapy holds promise as a treatment strategy for drug-resistant influenza virus.

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105

In Vitro Combination of ANA598 with Other Anti-HCV Agents can Eliminate the Emergence of Resistant Colonies

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ANA598 is a novel HCV non-nucleoside polymerase inhibitor that is currently in a Phase II combination trial with IFN/RBV in HCV infected patients. Clinical trials in HCV are beginning to explore combinations of two or more direct antivirals, which may provide additional benefit in certain populations and may reduce or eliminate the need for interferon or ribavirin. To better understand the potential of combinations utilizing ANA598, co-selection studies of ANA598 with IFN- α and clinically advanced direct antiviral agents were evaluated *in vitro*. The results provide support for future clinical exploration of combination regimens that include ANA598.

Co-selection studies were conducted using Huh7-Luc/neo wt 1b dicistronic replicon cells. Combinations of ANA598 with IFN- α , the

HCV NS3/4 protease inhibitor telaprevir, and the NS5B nucleoside polymerase inhibitor PSI-6130 were evaluated. These direct antiviral agents have distinct mechanisms of action and non-overlapping resistance profiles. The cells were selected with G418 in the absence or presence of either one or two agents at combinations between their EC₅₀ and EC₉₉. After 3 weeks in culture, cells were either fixed and stained or total cellular RNA was extracted. The NS5B and NS3 coding sequences were determined through direct sequencing of PCR products.

In vitro combination of ANA598 with IFN- α , telaprevir, and PSI-6130 after 3 days in the replicon system showed that the antiviral interaction between the compounds was additive to synergistic (see Patel et al. ICAR 2010 abstract). Treatment of the replicon cells for 21 days with ANA598 or telaprevir alone selected for resistant colonies. The co-selection studies demonstrate that at the EC₉₅ of ANA598, which is readily achievable in the clinic, combination with IFN- α , telaprevir or PSI-6130 led to clearance of the replicon from the cells rather than emergence of resistant colonies.

The *in vitro* co-selection studies demonstrate an elimination of resistant variants and additive to synergistic antiviral effects when ANA598 is combined with other anti-HCV agents. Clinical use of such combinations may produce a greater viral load reduction and potentially allow durable clearance of virus prior to the emergence of drug resistance in most patients.

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106

Development and In Vitro Evaluation of Gel-formulated Saquinavir as Vaginal Microbicide: Anti-HIV-1 Activity and Pharmaceutical Availability in Biorelevant Media

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The HIV protease inhibitor saquinavir is an interesting microbicide candidate for its potent anti-HIV-1 activity and favorable resistance profile. Here, we wanted to evaluate the formulate-ability of this poorly water-soluble compound in vaginal microbicide gels in terms of anti-HIV-1 activity and pharmaceutical availability in biorelevant media. Saquinavir mesylate was dissolved in an aqueous hydroxyethylcellulose gel (pH 4.6) at a concentration up to 2 mg/g (10⁵-fold *in vitro* anti-HIV-1 IC₅₀ values). The *in vitro* anti-HIV-1 activity of saquinavir was preserved in the formulation: comparable mean IC₅₀ values were obtained for formulated and native saquinavir mesylate (10 and 13 ng/ml, respectively). In the vaginal environment, potential precipitation of saquinavir in vaginal fluid or semen may reduce its availability. Therefore, saquinavir concentrations were monitored upon adding the formulation to vaginal fluid simulant (VFS) or semen simulant (SS) (1:1 dilution). Dilution of the saquinavir mesylate solution with VFS resulted in preservation of its antiviral activity, both at low (0.1 mg/g) or high (1 mg/g) dose, without noticeable precipitation. Comparable data were obtained for low dose saquinavir in combination with VFS and SS. However, dilution of high dose saquinavir (1 mg/g) with VFS and SS caused immediate precipitation of saquinavir with subsequent reduction in anti-HIV-1 activity (5-fold increase in IC₅₀ value). Inclusion of the solubilizing excipients polyethylene glycol 1000 (12%) and hydroxypropyl- β -cyclodextrin (2.5%) to the formulation reduced the extent of precipitation of saquinavir and restored its antiviral potency. In conclusion, while the mesylate salt of saquinavir allows the for-

mulation of a relatively high concentration (1 mg/g) in vaginal gels, the present study suggests precipitation of the active ingredient in the vaginal environment. Thus, solubilizing excipients are required to avoid precipitation and to preserve the antiviral activity of saquinavir. This study illustrates the importance of evaluating the pharmaceutical availability of poorly water-soluble microbicide candidates in biorelevant media.

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107

Identification of Antiviral Activity of Koe Ken Tang Against Influenza Virus

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Influenza still remains the major plagues as well as the highly pandemic outbreak in the world. Due to the emergence of neuraminidase (NA) and M2 inhibitor resistant viruses in current clinical reports, we have sought to find out the traditional prescriptions used in thousands of years in China to manage the pandemic of influenza viruses. In this study, we have demonstrated that the Chinese herbal mixture Koe Ken Tang (KKT) could not only inhibit cytopathy of MDCK cells but also block the synthesis of cleavage PARP induced by influenza A virus. In addition, according to time of addition assay, we notice that KKT exerts anti-viral effects after viral infection, but does not lead to a general block of transcription/translation or inhibition of viral polymerase activity based on the vRNP activity inhibition assay and primer extension assay. We also figure out that KKT could exert antiviral activity in multiple flu A strains, including Tamiflu resistant virus, but not flu B. Finally we have demonstrated that KKT could suppress the Akt phosphorylation post viral infection and decrease the titers of progeny virus. By IF, we observe that KKT could block the NP export from the nucleus at 9 h post-viral infection. These data suggest that the antiviral effects of KKT was due to its suppression of Akt phosphorylation that leads to viral NP protein accumulation in the nucleus and thus offer the potential for development of a new anti-influenza virus agent.

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108

Anti-influenza Virus Activity by Tricin, Isolated from *Sasa Albo-marginata* in Japan

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Effective new anti-influenza virus agents need to be developed. We examined the in vitro anti-influenza virus properties of triclin, 4',5,7-trihydroxy-3',5'-dimethoxyflavone, isolated from *Sasa albo-marginata*, a bamboo known in Japan. The effects were studied on influenza virus A/Solomon islands/3 (H1N1) and B/Malaysia/2506 replication in the Madin-Darby canine kidney (MDCK) cells. In a plaque-reduction assay, triclin showed dose-dependent inhibitory effects at concentrations ranging from 0.37 μ M to 10 μ M. The predominant inhibitory effect of triclin treatment was observed during 1–3 h after viral infection using time of drug addition assay. Moreover, triclin inhibited the synthesis of viral RNA but had no virucidal effect on cell-free virus (H1N1). Western blot analysis demonstrated

that triclin decreased the amount of HA and M proteins expression in the infected cells. These findings indicate that triclin acts on a late stage of the viral replication cycle resulting in inhibition of progeny virus production from the infected cells. It suggests that triclin is a novel compound with potential anti-influenza virus activity. Future studies should evaluate these findings in vivo.

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109

Synthesis and Biological Evaluation of Triazolo-pyrimidine Derivatives as Novel Inhibitors of Hepatitis B Virus Surface Antigen (HBsAg) Secretion

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Due to the side effects of interferon and the viral resistance to nucleos(t)ide inhibitors of the viral polymerase, current therapies for chronic hepatitis B virus (HBV) infection are still far from satisfactory. Further development of more effective therapeutics needs antiviral drugs that could interfere the viral life cycle through blocking other functions than inhibiting the viral polymerase, whether for monotherapy or combination therapy. The high levels of hepatitis B virus surface antigen (HBsAg)-bearing subviral particles in the serum of chronically infected individuals are thought to play an important role in suppressing the HBV-specific immune response. Inhibitors of HBsAg secretion could enable the therapeutic use of HBV vaccines or be used as combination therapy with nucleoside drugs for the treatment of HBV infection. However, current therapeutics are not directed at reducing this viral antigenemia. In order to achieve this therapeutic goal, our recent high-throughput screening of an in-house small-molecule library identified HBF-0259, 7-(2-chloro-6-fluorophenyl)-5-(4-chlorophenyl)-4,5,6,7-tetrahydro-tetrazolo[1,5-a]pyrimidine, as an effective inhibitor of HBsAg secretion, using the HBV-expressing cell line HepG2.2.15. During our follow-up lead optimization, a series of novel triazolo-pyrimidine analogues of HBF-0259 were synthesized and evaluated for their in vitro inhibitory activity of HBsAg secretion. No cytotoxicity was observed for these compounds at the concentration up to 50 μ M. PBHBV-2-15 was identified as the most potent inhibitor ($EC_{50} = 1.40 \pm 0.41 \mu$ M). PBHBV-001, PBHBV-2-1 and PBHBV-2-15 also exhibited good activities against nucleoside resistant mutants. Now, two analogues, PBHBV-001 and PBHBV-2-15 are undergoing in vivo toxicity and efficacy study.

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