Defining the Concentration of an Antiviral Agent Required to Suppress Virus Infection, Replication, and Transmission

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ABSTRACT

We have previously described a microbicide transmission sterilization assay (MTSA) that has been developed to quantify the concentration of a topical anti-HIV microbicide product that is required to suppress the sexual transmission of HIV-1. Additional development of the MTSA has resulted in the concept that the MTSA-defined sterilizing concentration may approximate the tissue concentration of an active anti-HIV agent that is required at the site of infection to assure complete product efficacy. Data from *in vitro* and *ex vivo* assays support the concept that a microbicide product must attain concentrations that are 100-1000-fold higher that the *in vitro* defined EC_{50} concentration in order to be effective and the tissue concentration achieved is highly dependent on the chemical nature of the product and the formulation employed for delivery to the tissue. Biological evaluations of a variety of potential microbicide products has shown that analogs of highly active products have much different sterilizing concentrations despite possessing similar inhibitory concentrations and similar solubility. In addition, differences in sterilizing concentrations appear within products of the same inhibitory classes, suggesting that the MTSA or definition of the sterilizing concentration may be a critically important component of preclinical drug development algorithms. The assay also has the ability to define the relative ability of compounds to rapidly select for drug resistant virus strains. Biological evaluations of the nature of the sterilizing concentrations support the notion that the results of the MTSA are related to the antiviral potential of the test compound and not merely due to tissue culture artifacts and the sterilizing concentration can be determined within days of the initiation of an acute infection of human target cells. Our *in vitro* and *ex vivo* definition of sterilizing concentrations has continued to evolve to include evaluation of the ability of test compounds or combinations of test compounds to sterilize cultures and have resulted in the expansion of the assays systems to the evaluation of products active against other sexually transmitted infections (HSV-1 and HSV-2), as well as viruses such as influenza. We believe the sterilization assay thus may be an early means of prioritizing compound development based on the relative ability of a compound to prevent the replication of viruses in target cells and tissues as well as to prevent the spread of virus from initially infected cells to surrounding target cells in tissue. The concentration of drug required may also be evaluated in explant or other biological tissue in the biological matrix in which the compound would need to exert antiviral activity.

METHODS

Sterilization Assay

CEM-SS cells or human PBMCs and HIV were incubated for 1 hour at 37˚C, 5% CO2. Following the initial infection, the cells and virus suspension were transferred to a 24-well or 6-well plate containing 5 mL of media and a fixed concentration of experimental or control compound. The concentrations ranged from an initial test concentration set at the EC_{50} concentration (as determined in the CPE inhibition assay) followed by 5 additional concentrations that were each 5-fold higher than the previous concentration. Three days after the initial infection the cultures were observed for virus-induced cytopathic effects (CEM-SS cells) and virus in cell-free supernatant was quantified by RT assay. Twenty percent (20% v/v) of the existing culture was added to 80% (v/v) of fresh CEM-SS cells or PBMCs in medium containing fresh test compound at the same fixed concentration. Each sub-culturing with new cells represented a new virus passage. Passaging was performed every 3 days for a total of 15 passages. Following the 10^{th} passage, test compound was removed and the cells from passages 11 through 15 were observed for virus outgrowth to confirm sterilization of the culture. In addition, PCR was also used to evaluate sterilization of the culture. Sequencing is performed on passage 10 samples to confirm mutations that were selected under drug pressure.

HIV-1 CPE Assay in CEM-SS Cells

CEM-SS cells were added to a 96-well round-bottom microtiter plate at a density of 2.5 x 10^3 cells/well. Serially diluted test compound was added to the cells in triplicate. Virus diluted to a pre-determined titer was then added and the plates were incubated for six days at 37°C, 5% CO2. Following the incubation XTT/PMS solution was added to all wells of the plate. The plates were incubated for an additional 4 hours at 37°C, 5% CO2 prior to being read at 450/650 nm.

Reverse Transcriptase Activity Assay

Reverse transcriptase is measured in cell-free supernatants using a standard radioactive incorporation polymerization assay. The RT reaction buffer was prepared by combining 125 µL of 1 M EGTA, 125 µL of H2O, 125 µL of 20% Triton X-100, 50 µL of 1 M Tris (pH 7.4), 50 µL of 1 M DTT, and 40 µL of 1 M MgCl2. For each reaction, 1 µL of TTP, 4 µL of H2O, 2.5 µL of rAdT and 2.5 µL of reaction buffer were mixed. The reaction mixture and the viral supernatants were incubated at 37°C in a humidified incubator and incubated for 90 minutes. Following the incubation, 10 µL of the reaction volume was spotted onto a DEAE washed and allowed to air dry. The dried filtermat was placed in a plastic sleeve and 4 mL of Opti-Fluor O was added. Incorporated radioactivity was quantified utilizing a Wallac 1450 Microbeta Trilux liquid scintillation counter.

Syncytium Forming Unit Assay

CEM-SS cells were cultured with HIV-1_{IIIB} in the presence of 1.0, 0.1, 0.01 and 0.001 µM IQP-0528. Each day for 12 days, serially diluted cells from the infected cultures (10^6 to 10^1 cells) were co-cultured with a fixed number of uninfected CEM-SS cells(10^5 cells) and syncytia were quantified at 48 hours to measure the number of HIV-infected cells in the original infected culture.

CONCLUSIONS

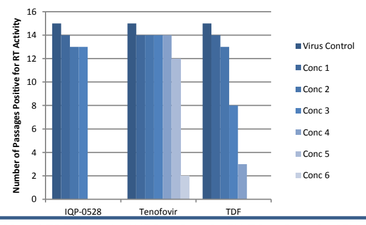
- Sterilizing concentrations range from 5-fold to > 600-fold the EC_{50} concentration depending on the assay system. Lower sterilization concentrations are observed in human PBMCs.
- Three structurally similar pyrimidinediones with identical EC_{50}'s have very different sterilizing concentrations providing direct evidence of the biological relevance to the MTSA.
- Protease Inhibitors appear to be the most active class of molecules in the MTSA, though this may reflect the ability of the test compounds to select for drug-resistant viruses.
- The MTSA may be used to select drug-resistant virus in culture and can be performed with drug combinations.
- The biology of the MTSA has indicated that the sterilizing concentration of a compound can be determined within days (possibly hours) of infection.
- The sterilizing concentration can be correlated to the inhibitory concentration of a compound derived from *in vitro* and *ex vivo* models.
- Studies are in progress to correlate the required dosing concentration with the required amount of compound required to achieve sterilizing concentrations in target tissues.
- Future studies aim to evaluate the sterilizing concentration required to prevent infection of other viruses such as HSV.

STERILIZING CONCENTRATION DETERMINATION

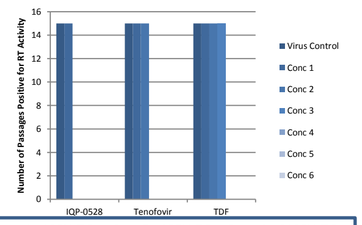
Antiviral Activity of Candidate Microbicides

Antiviral Evaluation	IQP-0528 EC_{50} (µM)	Tenofovir EC_{50} (µM)	TDF EC_{50} (µM)
CEM-SS/HIV-1_{IIIB}	0.002	1.2	0.006
PBMC/HIV-1_{BaL}	0.006	1.9	0.006

CEM-SS/HIV-1_{IIIB}

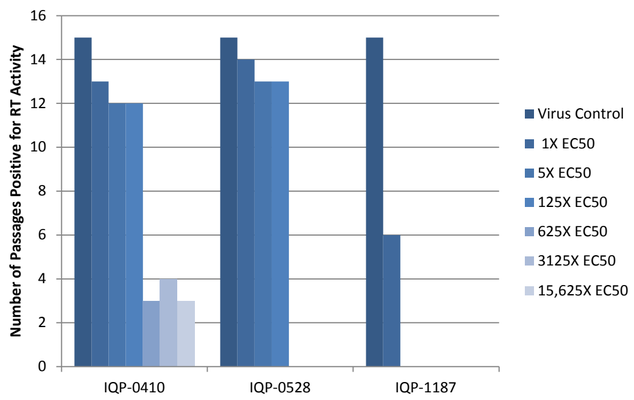


PBMCs/HIV-1_{BaL}



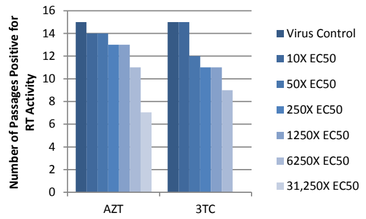
IQP-0528 and TDF Conc 1: 1XEC$_{50}$, Conc2: 5X EC$_{50}$, Conc 3: 25X EC$_{50}$ Conc 4: 125X EC$_{50}$, Conc5: 625X EC$_{50}$ Conc 6: 3125X EC$_{50}$
Tenofovir Conc 1: 1XEC$_{50}$, Conc2: 2.5X EC$_{50}$, Conc 3: 6.25X EC$_{50}$ Conc 4: 15.6X EC$_{50}$, Conc5: 39X EC$_{50}$ Conc 6: 98X EC$_{50}$

Compounds with Similar Structures and Activity Have Varying Sterilization Concentrations

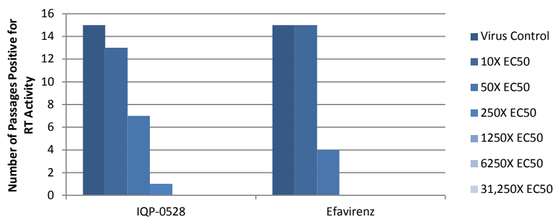


Sterilizing Concentration of Different Classes of HIV Inhibitors

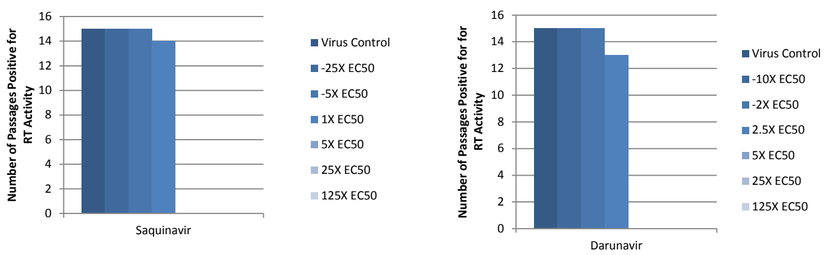
Nucleoside RT Inhibitors



Nonnucleoside RT Inhibitors



Protease Inhibitors



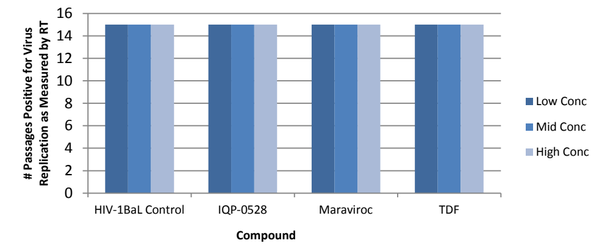
RAPID RESISTANCE SELECTION OF CANDIDATE COMPOUNDS

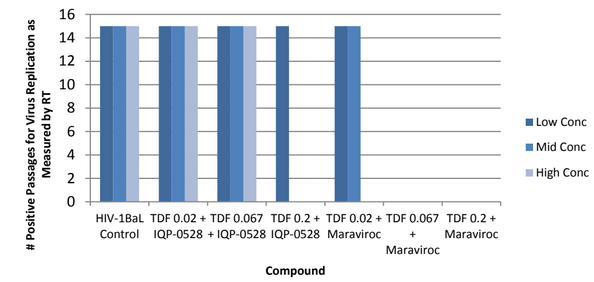
CEM-SS/HIV-1_{IIIB}

Compound	Sterilizing Concentration (Fold Increase Over EC_{50}) (µM)	Concentration Selecting Mutant (µM)	Mutations Identified
IQP-0528	0.5 (250)	0.01	Y181C/G142R
IQP-0410	12.5 (31,250)	0.1	Y181C
IQP-1187	0.02 (50)	0.004	Y181C

EFFECT OF DRUG COMBINATIONS IN THE MTSA

PBMC/HIV-1_{BaL}

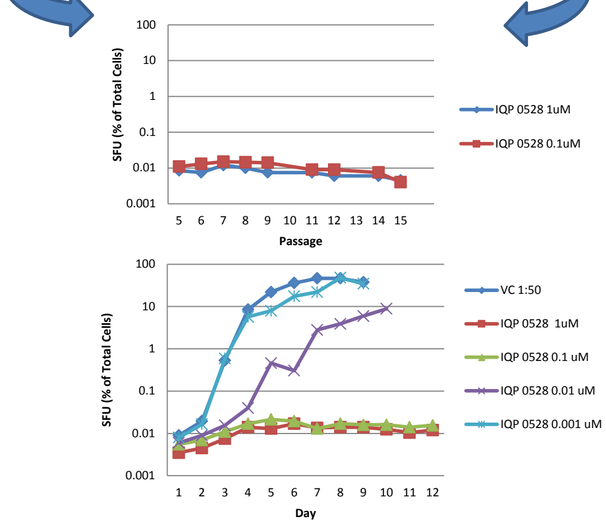




KINETICS OF VIRUS REPLICATION IN THE MTSA

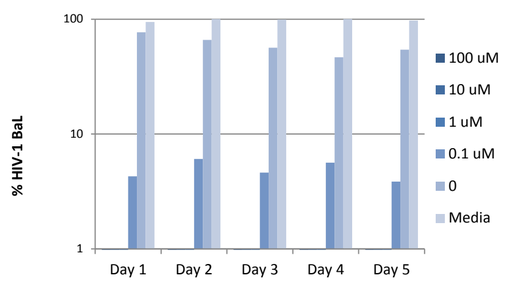
Virus Replication Quantified by Syncytium Forming Units

Removal of IQP-0528 from these cultures does not result in the outgrowth of HIV-1 indicating the cultures are completely sterilized or infected with replication incompetent virus at IQP-0528 concentrations >0.1 µM

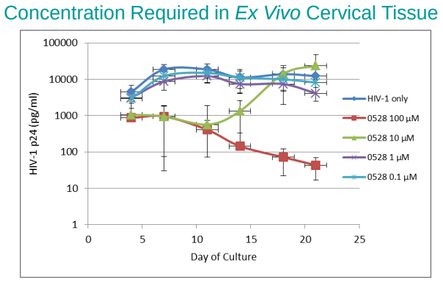


Relationship of Sterilizing Concentration to Pharmacokinetics/Pharmacodynamics Defined in Trans-Well Assays and Explant Cultures

IQP-0528 Inhibition of HIV-1_{BaL} in TZM-bl-FcRI Cells
Trans-Well PK/PD Assay with HEC1A Cells – Wash at 18 Hours



Concentration Required in *Ex Vivo* Cervical Tissue



ACKNOWLEDGMENTS

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