Virucidal Efficacy Assay

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Virus:	Zika (MR766 Uganda); Influenza A/Duck/MN/1525/81 (H5N1)
Samples tested:	Puriton, lot# CP-051917, received 7/6/2017
Contact conditions:	22 ± 2°C; 4 hours, 18 hours

Procedure:
The compound was received as a liquid and tested undiluted and diluted to 50% in water. One mL of each drug dilution was added to tubes in triplicate for each time point and each virus tested. A negative control (water) and positive control (70% Ethanol) were included for each replicate. One set of toxicity control tubes was prepared in the same manner with no virus added to these tubes. Virus was added to the tubes for each time point (4 hour and 18 hour). Ten µl of Zika virus stock and 100 µl influenza A(H5N1) virus stock were added to respective tubes and mixed thoroughly. The H5N1 stock had a lower titer and therefore increased volume was required for testing, so the highest concentration of drug tested was 90% once virus was added, whereas it was 99% for Zika. Tubes were incubated at room temperature for 4 hours or 18 hours. Following incubation, samples were added to cell culture media at a 1/10 dilution and serial log dilutions were performed. Diluted samples were added to 4 wells each of a 96-well plate with 80-90% confluent MDCK cells for influenza and Vero 76 cells for Zika. Toxicity controls were diluted and plated in the same manner described above. Half of the uninfected control wells were spiked with virus (30 $CCID_{50}$/well) to monitor for antiviral activity (neutralization controls) of the compound in the cells. Plates were incubated at 37 ± 2°C with 5% CO_2. Cultures were scored for presence or absence of cytopathic effect (CPE) on day 3 for H5N1 and day 6 for Zika virus. The Reed-Muench method was used to determine end-point titers (50% cell culture infectious dose, $CCID_{50}$) of the samples, and the log reduction value (LRV) of the compound compared to the negative (water) control was calculated.

One-way ANOVA with Dunnett post-test was performed using Prism™ for Mac (GraphPad) to compare all test groups to the negative control.

Results:
Neutralization controls showed that virus was effectively detected in the titer assay. Toxicity controls showed that titer plates were valid and no toxicity was observed on the test plates. Virucidal results are in Tables 1 and 2.

For Zika virus, the 70% ethanol was fully effective, and untreated virus controls were as expected. The undiluted compound and 50% solution were effective virucidals with 4- and 18-hour contact times (Table 1).

For influenza A(H5N1) virus, the 70% ethanol was fully effective, and untreated virus controls were as expected. The undiluted compound was an effective virucidal with 4- and 18-hour contact times and the 50% solution was effective with 18 hours of contact time, but less effective with 4 hours of contact time (Table 2).

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Table 1. Virucidal efficacy of Puriton against Zika virus after 4 or 18 hrs liquid-liquid contact at 22 ± 2°C

	Contact time (hr)	90% Puriton	50% Puriton	70% Ethanol	Water
[a]$CCID_{50}$ per 100 µL	4	0.7 ± 0.0***	0.7 ± 0.0***	0.8 ± 0.2***	5.0 ± 0.00
Log reduction value	4	>4.3	>4.3	4.2	n/a
[a]$CCID_{50}$ per 100 µL	18	[b]<0.7***	0.7 ± 0.0***	[b]<0.7***	5.2 ± 0.3
Log reduction value	18	>4.5	4.5	>4.5	n/a

[a] Log_{10} $CCID_{50}$ of virus per 0.1 mL, average of 3 replicates ± standard deviation
[b] For statistical analysis "<" signs were ignored.
***P < 0.001 by one-way ANOVA and Dunnett post-test compared with untreated virus control (water)

Table 2. Virucidal efficacy of Puriton against Influenza A(H5N1) virus after 4 or 18 hrs liquid-liquid contact at 22 ± 2°C

	Contact time (hr)	90% Puriton	50% Puriton	70% Ethanol	Water
[a]$CCID_{50}$ per 100 µL	4	[b]<0.7***	1.9 ± 0.5***	[b]<0.7***	4.5 ± 0.2
Log reduction value	4	>3.8	2.6	>3.8	n/a
[a]$CCID_{50}$ per 100 µL	18	[b]<0.7***	<0.7***	[b]<0.7***	4.1 ± 0.4
Log reduction value	18	>3.4	>3.4	>3.4	n/a

[a] Log_{10} $CCID_{50}$ of virus per 0.1 mL, average of 3 replicates ± standard deviation
[b] For statistical analysis "<" signs were ignored.
***P < 0.001 by one-way ANOVA and Dunnett post-test compared with untreated virus control (water)