Supplementary information

Supplementary material and methods

WHO-based formulations

WHO I formulation consists of 85% ethanol (v/v), 0.725% glycerol (v/v) and 0.125% hydrogen peroxide (v/v). The isopropyl-based formulation, WHO II, contains 75% isopropanol (w/w), 0.725% glycerol (v/v) and 0.125% hydrogen peroxide (v/v). Both WHO-recommended formulations were prepared by Dr. Brill + Partner GmbH, Institute of Hygiene and Microbiology, Hamburg, Germany strictly following the instructions in the WHO Guidelines [1]. Dilutions of the WHO formulations I and II from 10% to 80% were prepared in sterile water.

Quantitative suspension test and virus titrations

Infectivity of the different viruses was determined by means of end point dilution titration in microtitre plates. At the end of the chosen exposure time (30 seconds), activity of the biocides was immediately stopped by serial dilutions with cell culture medium. Each dilution was placed in a sterile polystyrene flat-bottomed 96-well microtitre plate containing permissive cell suspensions as outlined above for each virus. All tests were conducted in two independent test runs on different days. Virus controls were incorporated after the exposure time. All tests were performed with an organic load of 0.3% bovine serum albumin (BSA). The 50% tissue culture infectious dose ($TCID_{50}$) was determined for each virus and calculated according to the method of Spearman and Kärber [2, 3]. To determine the cytotoxicity of the WHO formulations, two parts of phosphate buffered saline (PBS) were mixed with eight parts of the disinfectant and inoculated into permissive cells. Cytotoxicity was determined by examining permissive cells by microscopy for any significant changes of the cell monolayer. The

cytotoxicity was calculated in analogy to the determination of virus titer [$TCID_{50}$/ml] and is depicted as dashed line in the figures.

Detailed experimental procedures for viral titrations were described previously for HCV Jc1 [4, 5], MVA [6], EBOV trVLP [7], EBOV, MERS-CoV and SARS-CoV [8]. Vero-B4 target cells were inoculated with ZIKV for 72 h before immunostaining was performed. Briefly, cells were washed once with PBS, fixed for 10-20 min with 4% PFA, and washed twice times with PBS containing 0.1% saponin. Cells were incubated with primary mouse anti-flavivirus hybridoma supernatant (Hybridoma mouse anti-flavivirus group antigen, clone 4G2, ATCC HB-112) in 0.3% saponin for 30 min at 37°C. Cells were washed twice with PBS containing 0.1% saponin before HRP-conjugated rabbit anti-mouse antibody (Dako) was added and incubated for 30 min at 37°C. Cells were washed as described above and 3-amino-9-ethylcarbazol (AEC) substrate was added for 15 minutes at RT and the viral titer was determined.

For preparation of BCoV, U373 cells were cultivated with EMEM supplemented with L-glutamine, non-essential amino acids and sodium pyruvate and 10% FCS. Before virus infection, cells were washed two times with PBS, incubated for three hours with EMEM without FCS and were washed once with EMEM supplemented with trypsin. For virus production, BCV strain L9 was added to the prepared monolayer. After an incubation period of 24 to 48 hours cells were lysed by a rapid freeze/thaw cycle. Cellular debris was removed by low speed centrifugation and the supernatant was directly used as the test virus suspension.

To prepare the test virus suspension of H1N1, MDCK cells had been cultured with EMEM with L-glutamine and 10% FCS were inoculated in 175 cm^2 cell culture flasks. Once a cytopathic effect had been induced (approx. 24 hours), freezing and thawing was carried out once. The cell debris was removed by low speed centrifugation at 4 °C and the supernatant was recovered as test virus suspension and stored in aliquots at -80 °C.

Statistical Analyses

Concentrations at which the formulations reached the half maximal virus inactivation effective concentration 50 (EC_{50}) were determined using nonlinear regression employing the robust fitting method on the normalized $TCID_{50}$ data implemented in GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). The mean $TCID_{50}$ of two individual experiments and standard deviation of means were also calculated using GraphPad Prism. Significance of differences in mean EC_{50} obtained for the viruses between WHO formulations I and II was tested using two-tailed Wilcoxon matched-pairs signed rank test (**$p < 0.01$).

Supplementary Figure legends

Supplementary Figure 1

Virucidal activity of WHO formulations I and II against BCoV, H1N1, and MVA. A) WHO formulations I and II were tested for their efficacy in inactivating BCoV. The biocide concentrations ranged from 0% to 80% with an exposure time of 30 seconds. For this inactivation assay, one part virus and one part of organic load was mixed with eight parts of biocide. Residual infectivity was determined by a limiting dilution assay. Viral titers are displayed as 50% tissue culture infectious dose 50 ($TCID_{50}$) values (n.d.: not detected). The cytotoxicity was calculated in analogy to the determination of virus titer [$TCID_{50}$/ml] and is depicted as dashed line. The mean of two independent experiments with standard deviations are shown. Efficacy of WHO formulations I and II against H1N1 (B) and MVA (C) was addressed by a quantitative suspension assay as described for panel A.

Supplementary Figure 2

Effective concentration 50 (EC_{50}) of WHO formulations I and II against various enveloped viruses.

The concentrations at which the products reached the half maximal virus inactivation effective concentration 50 (EC_{50}) was determined. Significance of differences in mean EC_{50} obtained for the viruses between WHO formulation I and WHO formulation II was tested using two-tailed Wilcoxon matched-pairs signed rank test (**$p < 0.01$).

Supplementary Table 1

Overview of viruses and target cell line characteristics used in the study.

Supplementary references

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