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# A simple assay for determining antiviral activity against Crimean-Congo hemorrhagic fever virus☆

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**Abstract**

Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne virus that is emerging as a significant human pathogen in many regions of the world, including Africa, Asia, and Europe. In this report, we describe a simple screening method for discovering new antiviral compounds directed against CCHFV. Antiviral activity was determined by assaying infected SW-13 cells (human adrenal gland carcinoma) for protection from cytopathic effect (CPE). By using an in vitro neutral red uptake assay, we were able to quantitatively measure CPE induced by CCHFV. As a proof of concept, we used this method to evaluate the antiviral activity of ribavirin and a series of structural analogs (ribamidine, 6-azauridine, selenazofurin, and tiazofurin) against four geographically diverse strains of CCHFV. Ribavirin inhibited the replication of CCHFV as reported previously using plaque reduction assays. One drug, ribamidine, showed antiviral activity that was 4.5- to 8-fold less than that of ribavirin, and the other three drugs (6-azauridine, selenazofurin, and tiazofurin) did not show significant antiviral activity. There were no significant differences in drug sensitivities among the CCHFV strains. Development of this simple and reliable assay will potentially allow high-throughput screening for discovering additional antiviral drugs to combat this important public health threat. Published by Elsevier B.V.

*Keywords:* Antiviral; Crimean-Congo hemorrhagic fever virus; Ribavirin; High-throughput screening; Bunyavirus; Tick-borne disease

## 1. Introduction

Crimean-Congo hemorrhagic fever (CCHF) is a zoonotic disease that can develop into a severe hemorrhagic fever in humans. CCHF virus (CCHFV) is a member of the genus *Nairovirus*, family *Bunyaviridae*. This tick-borne pathogen is transmitted to humans primarily by the bite of an infected Ixodid tick (mostly of the *Hyalomma* genus). Human infection can also result from direct contact with blood or tissues from infected humans or livestock (Nichol, 2001). The virus is widely distributed and occurs in scattered enzootic foci over sub-Saharan Africa, the Middle East, and southern Eurasia (Hoogstraal, 1979; Watts et al., 1988). The capacity for human-to-human transmission has made nosocomial infections a significant public health concern, and infection transmitted by contact with infected humans has been reported to result in a more severe disease with increased mortality (Burney et al., 1980; Shepherd et al., 1985).

CCHF is a severe human disease resulting in a death rate of 13–50%. After a brief incubation period, the patient has sudden onset of fever, myalgia, nausea, and severe headache. Within 3–6 days of the onset of illness, development of a petechial rash and hemorrhagic symptoms such as epistaxis, hematemesis, and melena may be apparent. The most severely ill patients enter multiorgan failure characterized by shock, hemorrhage, and coma (Swanepoel et al., 1989).

The highly pathogenic nature of the CCHFV has led to the fear that it might be used as an agent of bioterrorism and/or biowarfare and has resulted in its inclusion as a CDC/NIAID Category C Priority Pathogen. CCHFV can be transmitted from person to person, has a high case-fatality rate, and may be transmissible by small-particle aerosol; but, its inability to replicate to high concentrations in cell culture is cited as a major impediment to its development as a mass casualty weapon (Borio et al., 2002), and thus precludes its classification as a Category A or B pathogen. The highly lethal nature of the virus has restricted research to biosafety level-4

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(BSL-4) laboratories and has consequently had limited research investigations.

Treatment options for CCHF are limited. Immunotherapy has been attempted via passive transfer of CCHF survivor convalescent plasma (Vassilenko et al., 1990). Although seven patients with severe CCHF who received immune plasma recovered, this was an uncontrolled experiment, and firm evidence of its value is lacking. There is currently no specific antiviral therapy for CCHFV approved for use in humans by the U.S. Food and Drug Administration (FDA). However, ribavirin has been shown to inhibit in vitro viral replication in Vero cells (Watts et al., 1989) and reduce the mean time to death in a suckling mouse model of CCHF (Tignor and Hanham, 1993). Additionally, several case reports have been published that suggest oral or intravenous ribavirin is effective for treating CCHFV infections (Fisher-Hoch et al., 1995; Papa et al., 2002a; Mardani et al., 2003; Tang et al., 2003). For example, in Pakistan, three nosocomial cases of CCHF were treated with oral ribavirin for 10 days, and they made a complete recovery (Fisher-Hoch et al., 1995). More recently, in a large cohort study in Iran, the efficacy of oral ribavirin was 80% among patients with confirmed CCHF (Mardani et al., 2003). But, to date, no randomized, controlled studies have been performed to confirm the efficacy of ribavirin for treating CCHF.

Clearly there is a critical need to identify new effective antiviral drugs for the treatment of this disease. Many successful antiviral discovery programs depend on cell culture-based assays of viral replication. Plaque counting and virus yield reduction assays are labor-intensive and thereby limit the number of compounds that can be screened. In this report we describe a simple and reliable method, based on neutral red uptake, for measuring inhibition of CCHFV replication by potential antiviral compounds. This method was originally developed to measure antiviral activity of interferons (Rubinstein et al., 1981), and we now describe its application to screen for compounds with potential antiviral activity to the *Bunyavirus*, CCHFV.

## 2. Materials and methods

### 2.1. Cells and viruses

The CCHFV strains used in this study were IbAr 10200 (isolated from a *Hyalomma impeltatum* tick from Nigeria), Hy-13 (isolated from a *Hyalomma asiaticum* tick from China), UG3010 (isolated from a human from Uganda), and Spu128/81 (isolated from a *Hyalomma marginatum* tick from South Africa) and were obtained from the USAMRIID collection. The E6 line of Vero African green monkey kidney cells, Vero C1008 (ATCC CRL-1586), SW-13 (human adrenal gland carcinoma; ATCC CLL-105), BHK-21 (hamster kidney fibroblast; ATCC CCL-10), and 293T/17 (human kidney; ATCC CRL-11268) were propagated Eagle's minimum essential medium with Earle's salts (EMEM) containing 10% fetal calf serum (FCS), nonessential amino acids, L-glutamine, penicillin, and streptomycin at 37 °C in a 5% $CO_2$ environment. The same medium only with 2% FCS was used as replacement medium after viral infection. Virus preparations were quantitated by standard plaque assay on Vero E6 cells. After viral infection, cells were incubated at 35 °C with 5% $CO_2$ until complete cytopathic effect (CPE) was observed (usually 3–4 days). All work with CCHFV was performed in the biosafety level 4 (BSL-4) laboratories at the USAMRIID, Fort Detrick, MD. Personnel wore positive-pressure protective suits (ILC, Dover, Frederica, DE) supplied with umbilical-fed air (Fig. 1).

### 2.2. Antiviral compounds

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole) was provided by Dr. Humberto Fernandez, ICN Pharmaceuticals, Costa Mesa, CA. Ribamidine (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine), 6-azauridine (2-β-D-ribofuranosyl-1,2,4-triazine-3,5(2*H*,4*H*)-dione, selenazofurin (2-β-D-ribofuranosylselenazole-4-carboxamide), and tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) were described in previous studies (Baker et al., 2003). All compounds were dissolved in dimethyl sulfoxide (DMSO) to 300 mM.

### 2.3. Viral growth curves

Various cell lines were infected with CCHFV strain IbAr 10200 at multiplicity of infection (MOI) of 0.1 and 0.001. At 72 h postinfection (p.i.), 250 μl of infected cell culture supernatants were harvested and viral titers were determined by plaque assay on Vero E6 cells.

### 2.4. Neutral red uptake assay

The neutral red uptake assay was performed as previously described (Baker et al., 2003) with minor modifications. Briefly, antiviral compounds at 300 mM were serially diluted three-fold in EMEM, and 50 μl was added to 96-well microtiter plates of confluent SW-13 cells already containing 100 μl of medium. At each drug concentration, three wells were infected with $1 \times 10^3$ plaque-forming units (pfu) (MOI = 0.001) of virus in 50 μl of medium, while three wells were left uninfected for toxicity determination. Plates were incubated at 35 °C and examined daily until the virus-infected, untreated cells showed 4+ CPE (approximately 3–4 days). Neutral red (Invitrogen, Carlsbad, CA) was added to the wells to give a final concentration of 1.11 mg/ml, and the plates were returned to the incubator for 1 h. After the incubation period, the medium was removed, wells were rinsed twice with phosphate-buffered saline, and the retained stain was solubilized by adding 100 μl of a 50% ethanol, 50% 0.001 M ammonium phosphate ($NH_4H_2PO_4$) (pH 3.5) solution. Plates were rocked for 15–30 min at 150 rpm, and the optical density (OD)

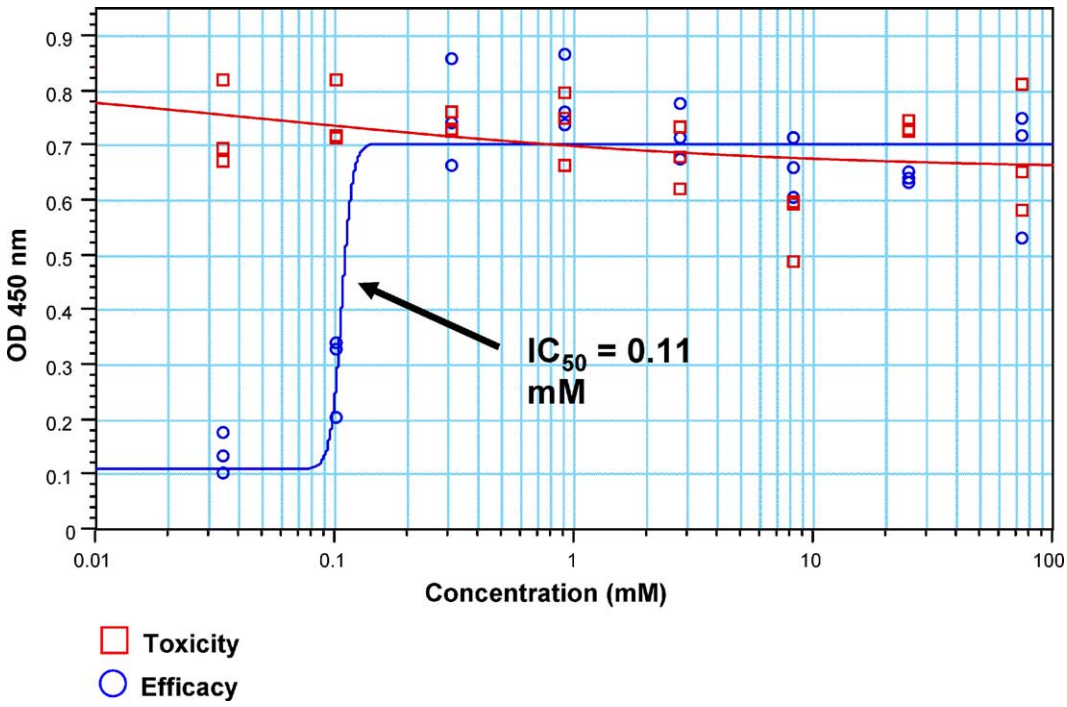


Fig. 1. Representative data from a single experiment for ribavirin against the IbAr 10200 strain of CCHFV on SW-13 cells. The $IC_{50}$ for this experiment is shown, as is the $R^2$ fit of the points to the curve.

at a wavelength of 450 nm was measured using a plate reader.

### 2.5. Data analysis

The data were analyzed using the four parameter-logit option of a curve-fitting program (SOFTmax PRO, version 4.0; Molecular Devices, Menlo Park, CA) to determine the 50% inhibitory ($IC_{50}$) and toxic ($TC_{50}$) drug concentrations. $TC_{50}$ and $IC_{50}$ were determined from the average of three replicates on each plate. The therapeutic index (TI) was calculated as a ratio of $TC_{50}$ to $IC_{50}$. Compounds with a TI $>$ 10 were considered active, and compounds with a TI $<$ 2 were considered inactive. A TI value between 2 and 10 was considered moderately active.

## 3. Results

### 3.1. Comparative replication of CCHFV in BHK-21, 293T/17, Vero E6, and SW-13 cells

Various cell lines were examined for their ability to support the productive replication of CCHFV strain IbAr10200. The results are summarized in Table 1. At an MOI of 0.1, CCHFV replicated to an approximately 10-fold higher level in both 293T/17 and SW-13 cell lines than in BHK-21 cells. However, at an MOI of 0.001, the virus replicated to approximately 100-fold higher in SW-13 cells than in 293T/17 cells, and BHK-21 cells failed to support CCHFV replication. Thus, SW-13 cells were apparently the most able to support high levels of replication of CCHFV. Additionally, the virus produced clearly discernible plaques in SW-13 cells and caused extensive CPE; consequently, these cells were used in the assays throughout the study.

### 3.2. Inhibition of CCHFV replication by ribavirin and comparison of drug sensitivities of CCHFV strains to ribavirin and structural analogs

Ribavirin is a known inhibitor of CCHFV replication in vitro. As a proof of principle, we determined the $IC_{50}$ and $TC_{50}$ of ribavirin against CCHFV using the neutral red uptake assay. Ribavirin was active against all four CCHFV strains (IbAr 10200, Spu 128/81, Hy-13, and UG3010) tested with $IC_{50}$ values ranging from 0.04 to 0.11 mM (Table 2). To evaluate potential differential drug sensitivities of CCHFV strains, $IC_{50}$ values were determined for all five drugs tested against the CCHFV strains IbAr 10200, Spu 128/81,

Table 1
Comparative replication of CCHFV in various cell lines[a]

| Cell lines | MOI | |
|---|---|---|
| | 0.1 | 0.001 |
| BHK-21 | $2.5 \times 10^4$ | 0 |
| 293T/17 | $2.0 \times 10^5$ | $1.0 \times 10^2$ |
| SW-13 | $1.9 \times 10^5$ | $1.4 \times 10^4$ |

[a] Cell lines were infected with CCHFV strain IbAr 10200 at differing MOIs, and at 75 h p.i., supernatants were plaque titered on Vero E6 cells. Values represent pfu/ml.

Table 2
Comparison of the sensitivities of CCHFV strains for ribavirin and structural analogs[a]

| Drug | CCHFV Strains | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | IbAr 10200 | | | Spu 128/81 | | | Hy-13 | | | UG3010 | | |
| | $IC_{50}$ | $TC_{50}$ | TI | $IC_{50}$ | $TC_{50}$ | TI | $IC_{50}$ | $TC_{50}$ | TI | $IC_{50}$ | $TC_{50}$ | TI |
| Ribavirin | 0.11 (±0.03) | >75 | >682 | 0.07 (±0.02) | >75 | >1071 | 0.04 (±0.01) | >75 | >1875 | 0.06 (±0.02) | >75 | >1250 |
| Ribamidine | 0.60 (±0.25) | >75 | >125 | 0.57 (±0.18) | >75 | >132 | 0.18 (±0.14) | >75 | >417 | 0.40 (±0.03) | >75 | >188 |
| 6-Azauridine | >75 | >75 | >1 | >75 | >75 | >1 | >75 | >75 | >1 | >75 | >75 | >1 |
| Selenazofurin | 17.34 (±7.29) | >75 | >4 | 37.41 (±12.04) | >75 | >2 | 5.08 (±0.88) | >75 | >15 | 8.37 (±6.37) | >75 | >9 |
| Tiazofurin | 18.85 (±3.56) | >75 | >4 | 19.98 (±3.24) | >75 | >4 | 3.21 (±1.90) | >75 | >23 | 8.85 (±0.35) | >75 | >9 |

$IC_{50}$: inhibitory concentration (mM) at 50% cell viability; $TC_{50}$: toxic concentration (mM) at 50% cell viability; TI: $TC_{50}/IC_{50}$.
[a] The data represent the mean of three experiments (±S.D.).

Hy-13, and UG3010 (Table 2). Ribamidine showed antiviral activity against all strains (0.18 to 0.60 mM), but was 4.5- to 8-fold less active than ribavirin. Selenazofurin and tiazofurin were moderately active with $IC_{50}$ values ranging from 5.08 to 37.41 mM and 3.21 to 19.98 mM, respectively. 6-Azauridine was uniformly inactive against all four strains tested and only slightly toxic at the highest concentrations. Additionally, there was no cytotoxicity seen with ribavirin, ribamidine, selenazofurin, or tiazofurin in concentrations up to 75 mM.

### 3.3. *Effect of pre- and postinfection treatment of ribavirin and ribamidine on CCHFV replication*

To determine whether ribavirin and the most active analog, ribamidine, act at a step early or late in viral replication, time-of-addition experiments were performed. Compounds were added on days −1, 0, 1, 2, and 3 p.i. with CCHFV strain IbAr 10200. Ribavirin was equally active when added either on day –1 or 0 p.i. ($IC_{50}$ of 0.11 mM for both days). Similarly, the activity of ribamidine was comparable on day –1 or 0 p.i. ($IC_{50}$ of 0.42 mM and 0.36 mM, respectively). The antiviral activities of both compounds were significantly diminished when they were added at day 1 p.i. ($IC_{50}$ of 0.36 mM for ribavirin and 1.1 mM for ribamidine) and were completely abolished at day 2 and 3 p.i. ($IC_{50}$ of >100 mM).

## 4. Discussion

The goal of the current work was to develop a simple and reliable method (i.e., neutral red uptake assay) that could be used to screen and identify new compounds with potential antiviral activity against CCHFV. While other antiviral drug discovery programs for CCHFV have depended on viral yield-reduction and plaque assays (Watts et al., 1989) or have employed surrogate viruses (Sidwell et al., 1994), this assay permits testing of potential antiviral compounds directly with CCHFV and is performed in a 96-well plate format, which is amenable to high-throughput screening efforts.

We confirmed the in vitro antiviral activity of ribavirin against CCHFV, and our calculated $IC_{50}$ values (mean = 0.1 mM) were comparable to those in previous reports (Watts et al., 1989). We also showed that although some analogs of ribavirin (i.e., ribamidine) exhibited antiviral activity against CCHFV, others had significantly lower activity or were completely inactive. Although to our knowledge, ribamidine has not been tested against CCHFV previously, it has been reported to have antiviral activity against Punta Toro virus (Sidwell et al., 1988) and Pichinde virus (Smee et al., 1993).

CCHFV isolates, like other bunyaviruses, are known to exhibit extensive genetic diversity (Schwarz et al., 1996; Morikawa et al., 2002; Papa et al., 2002b). For example, some Chinese strains exhibit a 15% difference in nucleotide sequence in the S segment and a 13% difference in the M segment from those of the Nigerian strain IbAr 10200 (Papa et al., 2002b). Given this level of genetic diversity among CCHFV strains, we wanted to examine if it might translate into differential drug sensitivity. In order to address this question, we tested the antiviral activity of ribavirin and four ribavirin analogs against four CCHFV strains that were isolated from diverse geographic locations. Although strains from China are known to be greatly divergent from African strains (Schwarz et al., 1996; Morikawa et al., 2002; Papa et al., 2002b), results did not indicate any difference in drug sensitivity to either of the most active compounds, ribavirin or ribamidine. In addition, there were no significant differences in antiviral activity of either compound among the tick-derived strains (IbAr 10200, Hy-13, and Spu 128/81) and the human-derived strain (UG3010). Based on our limited testing, these data suggest that genetic heterogeneity among these viruses does not necessarily result in differential susceptibility to certain antiviral drugs; however, a larger collection of viruses should be evaluated before these questions can be fully answered.

The availability of a simple and rapid assay to measure antiviral activity against CCHFV will be greatly beneficial to efforts to discover new therapies for this emerging zoonotic virus. The neutral red uptake assay for measuring CCHFV-induced CPE is simple and reliable and should be amenable to adaptation to high-throughput screening techniques. This may be a useful measure to quickly exclude compounds with possible strain-specific effects. Because CCHFV is lytic in SW-13 cells, this assay could also be

modified to use other staining methods (i.e., MTT or crystal violet, etc.). We also believe that this assay is appropriate for screening compounds that act to block viral replication and induce cellular antiviral systems. With the recent outbreaks of CCHFV and reports that nations have evaluated the possibility of weaponzing CCHFV, there is an urgent need for developing specific antiviral compounds against this potential public health threat.

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