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Original Article

FGI-104: a broad-spectrum small molecule inhibitor of viral infection

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Abstract: The treatment of viral diseases remains an intractable problem facing the medical community. Conventional antivirals focus upon selective targeting of virus-encoded targets. However, the plasticity of viral nucleic acid mutation, coupled with the large number of progeny that can emerge from a single infected cells, often conspire to render conventional antivirals ineffective as resistant variants emerge. Compounding this, new viral pathogens are increasingly recognized and it is highly improbable that conventional approaches could address emerging pathogens in a timely manner. Our laboratories have adopted an orthogonal approach to combat viral disease: Target the host to deny the pathogen the ability to cause disease. The advantages of this novel approach are many-fold, including the potential to identify host pathways that are applicable to a broad-spectrum of pathogens. The acquisition of drug resistance might also be minimized since selective pressure is not directly placed upon the viral pathogen. Herein, we utilized this strategy of host-oriented therapeutics to screen small molecules for their abilities to block infection by multiple, unrelated virus types and identified FGI-104. FGI-104 demonstrates broad-spectrum inhibition of multiple blood-borne pathogens (HCV, HBV, HIV) as well as emerging biothreats (Ebola, VEE, Cowpox, PRRSV infection). We also demonstrate that FGI-104 displays an ability to prevent lethality from Ebola *in vivo*. Altogether, these findings reinforce the concept of host-oriented therapeutics and present a much-needed opportunity to identify antiviral drugs that are broad-spectrum and durable in their application.

Key Words: Antiviral, HCV, Ebola virus, HBV, hepatitis, HIV

Introduction

Effective treatment of viral diseases remains a primary obstacle confronting the medical community. Following infection, viruses hijack the host cell machinery to facilitate their replication and dissemination. As such, viruses have generally adapted to encode for a minimal number of genes. In an effort to minimize cross-reactivity with host molecules, conventional antivirals have been designed to target the relatively small number of virally-encoded targets and mechanisms. Successful examples include therapeutic targeting of retroviral reverse transcriptase (in the case of retroviruses) or thymidine kinase (in the case of herpesviruses) [1, 2]. Unfortunately, conventional antivirals are often themselves toxic and more importantly, targeting of viral targets places direct selective pressure upon the virus to minimize the impact of the drug via random mutation of the target molecule [3, 4]. Viral polymerases generally display poor fidelity and compounding this, emerging evidence indicates that antiviral therapy itself further selectively promotes the growth of mutant variants [5]. Consequently, antiviral therapeutics are often rendered ineffective

over time as resistant viruses become more prevalent within an infected community. To counter this, combination therapies using multiple drugs have been successfully applied but as demonstrated by the situation with HAART therapy for HIV/AIDS, issues of fundamental biology combined with decreased compliance in asymptomatic individuals, has facilitated the emergence of variants that are resistant even to combination therapy [6]. These outcomes are not limited to HIV. Similarly, the recent emergence of H5N1 influenza has reinforced the idea of rapid resistance since drug resistant strains of H5N1 rapidly became prominent in the months since the virus first emerged [7, 8].

The difficulty in treating infections is compounded further when the viral pathogen is emerging or unknown. The past few decades have witnessed many emerging pathogens, including Ebola, VEE, SARS and PRRS viruses [9-11]. Compounding these natural infections, viral pathogens may be manipulated in the laboratory to increase their pathogenicity or transmissibility and virus-based bioweapons are increasingly recognized as a potential threat. These facts emphasize the need for new antivirals that can be used to treat both known and unknowable viral pathogens. Such needs are seemingly inconsistent with conventional approaches to develop therapies based on virus-encoded targets since the targets themselves may not be identifiable.

Host-directed therapeutics may provide a much-needed means of combating both established and emerging viral diseases. The fundamental concepts underlying host-oriented therapeutics are well-established and grounded in a basic understanding of viral biology. While minimizing the number of virally-encoded mechanisms, most viruses have developed means to exploit routine host-encoded "housekeeping" molecules [12-14]. In doing so, however, the virus often alters the expression or function of these housekeeping molecules. An example of this "hijacking" function is portrayed by events associated with TSG101 [15, 16].

TSG101 is a housekeeping protein that normally functions to escort internalized proteins that have been fated for degradation within proteosomes [17-20]. As such, TSG101 is restricted to the cell interior, where it resides in structures known as multivesicular bodies. Enveloped viruses encounter the opposite situation than that of internalized membrane proteins since they are assembled inside the cell but need to transit to and through the cell membrane [15, 16]. Consequently, many viruses have converged upon a mechanism by which the function of TSG101 is "hijacked" in a manner that facilitates a retrograde movement of viruses from the cell interior to the outer membrane [16]. Indeed, a well-developed literature has demonstrated that TSG101 plays an essential role in the viral life cycle and that this role is independent of its normal role in proteosomal transport [21]. Moreover, this mechanism is shared by a surprisingly broad spectrum of different viruses encompassing families virtually from all subsets defined by the standard Baltimore classification system. This remarkable utilization of a single mechanism presumably reflects a convergent evolution by different virus types towards a fundamental mechanism necessary for the viral life cycle. Based on these findings, we sought to identify potential therapeutics that might target TSG101 and be broadly-relevant to unrelated virus types. We demonstrated herein the identification of FGI-104, a small molecule antiviral with broad-spectrum applicability and unprecedented ability to prevent viral pathogenesis *in vivo*.

Materials and Methods

In vitro anti-HBV evaluation

HepG2 2.2.15 cells were plated in 96-well microtiter plates. Only the interior wells were utilized to reduce "edge effects" observed during cell culture; the exterior wells are filled with complete medium to help minimize sample evaporation. After 16-24 hours the confluent monolayer of HepG2-2.2.15 cells was washed and the medium was replaced with complete medium containing six dilutions of the test compound in triplicate. Lamivudine (2', 3'-dideoxy-3'-thiacytidine) was used as the positive control, while media alone was added to cells as a negative control. Three days later the culture medium was replaced with fresh medium containing the appropriately diluted drug. Six days following the initial administration of the test compound, the cell culture supernatant and the cells were collected to measure copy number of intracellular and extracellular HBV DNA, respectively, in a real-time qPCR (TaqMan) assay. Antiviral activity was calculated from

the reduction in HBV DNA levels (IC50). Compound cytotoxicity (TC50) was assessed in parallel by measuring cell viability with Cell Titer 96 (Promega, Madison, WI). The therapeutic index (TI) is calculated as TC50/IC50.

Evaluation of HCV

The ET cell line harboring HCV subgenomic replicon was obtained from Dr. Ralf Bartenschlager at University of Heidelberger [22]. The HCV replicon assay evaluated compounds at six half-log concentrations each. Human interferon alpha-2b is included in each run as a positive control compound. Briefly, sub-confluent cultures of the ET line were plated out into 96-well plates that were dedicated for the analysis of cell numbers (cytotoxicity) or antiviral activity. On the following day, compounds were added to the appropriate wells. Cells were processed 72 hr later when the cells were still sub-confluent. HCV replicon-derived luciferase activity was measured to derive applicable IC50 and IC90 (concentrations resulting in 50% and 90% inhibition, respectively). The cytotoxic effects were assessed using the CytoTox-1 cell proliferation assay (Promega) according to manufacture's instructions.

To evaluate release of infectious HCV particles, human hepatoma cells (Huh7) were infected with a recombinant hepatitis C virus (HCV) that expresses a renilla luciferase. At 2 hours post-infection, HCV-infected cells were incubated with compounds at concentrations from 30 to 0.04 μM in a 3-fold serial dilution (30, 10, 3.33, 1.11, 0.37, 0.12, and 0.04 μM). At 3 days post-infection, cells were lysed in a buffer included in a Promega luciferase assay kit. The level of luciferase expression was determined by luciferase assay using a kit derived from Promega. To evaluate reduction of HCV protein, Huh7 cells were infected with HCV. At 2 hours post-infection, HCV was removed and cells were washed once with PBS. HCV-infected cells were then incubated with inhibitors at indicated concentrations at 37oC for three days. Cell lysate was prepared by extracting cells in 50 μl RIPA buffer. The level of HCV NS5A protein was determined by Western blot analysis using an NS5A-specific monoclonal antibody.

In vitro testing of HIV

MT4 cells were infected with HIV-1 NL4–3 at MOI of 0.001 by low speed centrifugation (1,200g) for 1hr. Cells were seeded in a 96-well plate (1.5x10^5 in 100ul culture medium per well)[23]. Serial dilution of FGI-104 was immediately added in triplicate in 50ul culture medium per well. †Half of the supernatants were refreshed every day starting from day 3 pi in the presence of same concentration of FGI-104. The collected supernatants were then transferred to the TZM-bl indicator cell line (reference below) for examination of viral production in FGI-104 treated samples. Relative Luminescence Unit (RLU) was obtained on TZM-bl cells after they were treated with Bright-Glo Luciferase Assay System (Promega) 3 days later. The percentages of inhibition of viral production by FGI-104 were calculated as: (RLU from mock-treated samples - RLU from FGI-104 treated samples)/RLU from mock-treated samples *100. Normal MT4 cells were treated with serial dilution of FGI-104 as same as above and its cytotoxicity was measured by CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's instruction.

In vitro testing of PRRS virus

The fluorescent focus inhibition assays were performed as previously described with minor modifications [24]. Anti-viral compounds were diluted in Eagle's Minimum Essential Medium (MEM) containing 2% horse serum. 100ul of selected dilutions were added to duplicate wells of 96-well tissue culture plates and an equal volume of the selected PRRSV isolate (North American Type 2 strain SD-23983 or European-like Type 1 strain SD 01-08) at 2000 TCID50/mL was then added to each well. The contents of each plate were then transferred to corresponding wells of another 96-well plate containing confluent monolayers of MARC-145 cells or primary porcine macrophages set 48 hrs prior to infection. Plates were incubated for 24 hrs at 37C, then fixed with 80% acetone in water and stained with fluorescein-conjugated anti-PRRSV monoclonal antibody SDOW-17 [25]. Endpoints were interpreted as the greatest dilution of anti-viral compound resulting in a 90% or 100% reduction in fluorescent foci of virus infected cells relative to control wells. All assays were conducted with a 2 hr pre-incubation of cells with the compound and with the compounds being added at the same time as the virus and no

differences in endpoints were noted.

Plaque reduction assays were performed as previously described [26] with the following modifications. Selected anti-viral dilutions were mixed with input viruses at an M.O.I. of 0.1. Results were interpreted as a percentage reduction in visible plaques relative to control wells receiving no anti-viral compound.

In Vitro testing against Ebola virus

Ebola Virus expressing the eGFP was derived by reverse genetics to generate a full-length cDNA clone inserted with a foreign reporter gene, eGFP (EboZ-eGFP). Generation of EboZ-eGFP, showed similar viral characteristics to the wild-type virus yet causes infected cell to fluoresce green when stimulated. EboZ-eGFP has provided a more sensitive quantitative assay to rapidly screen multiple drugs in less time [27].

Vero E6 cells were seeded at 40,000 cells/well in 96-black well plates and incubated at 37°C with 5% CO_2 with EMEM provided by (Invitrogen) complete with 10% FBS + 5% L-Glutamine and Hepes buffer (EMEM-C). Primary drug plates were received from Functional Genetics Inc. packaged on dry ice containing 25µl of 1mM compounds of interest. Dilution plates were generated by transferring 20µl of each compound from the primary drug plate to 80µl of EMEM and mixing for a final dilution of 50.00µm. Further dilution of each compound by transferring 33µl of the previous concentration into 66µl of EMEM and repeating three more times, created a range of concentrations to assess at which dose viral inhibition or cell toxicity is greatest. The final concentration range was 50.00µm, 16.5µm, 5.56µm, 1.85µm, and 0.62µm. After 24hour incubation of seeded cells and proper dilution of the compounds, 25µl from the dilution plate was transferred to two cell plates; one cell plate would be used for the EboZ-eGFP infection and the second for the Cell Cytotoxicity assay described below. The plates used for the EboZ-eGFP infection were transferred into Bio-Safety Level-4 containment (BSL-4), whereas, those used for the Cell Cytotoxicity assay remained at Bio-Safety Level-2 containment until the 72 hour time point. At BSL-4, 25µl of EboZ-eGFP virus diluted in EMEM at 1:16 was added to each infection plate, with an additional 50µl EMEM-C afterwards. Infected EboZ-eGFP assay plates incubate for 72 hours at 37°C with 5% CO_2; after incubation, plates are read for fluorescence on a Gemini EM provided by Molecular Devices. At the same time, plates remaining in BSL-2 are used for the below described Cell Cytotoxicity assay.

CellTiter-Glo Luminescent Cell Viability Assay provided by Promega is a method of determining the number of viable cells based on the quantitation of the ATP present, which signals the presence of metabolically active cells (Promega). CellTiter-Glo Buffer and lyophilized CellTiter-Glo substrate are removed from -20°C storage into -4°C for thawing 24 hours before use. After proper thawing and equilibration to room temperature the CellTiter-Glo buffer is added to the substrate and mixed by inverting the closed bottle, making the CellTiter-Glo Reagent mixture. All media and compound concentrations added to the assay plate, as described below, are discarded by flicking into a waste container of MicroChem disinfectant after 72 hour incubation with compounds. CellTiter-Glo reagent mixture and DMEM (Invitrogen) are added in equal amounts 1:1 for a final volume of 200µl/well. After addition of the reagent and media, the samples were mixed using an orbital shaker for two minutes; after mixing, plates incubate at room temperature for 10 minutes to stabilize the luminescence signal. Plates are then read for luminescence on the Safire2 provided by Tecan.

In Vivo testing in lethal mouse model of Ebola challenge

Mouse-adapted Ebola virus was derived by serial passage of Ebola Zaire '76 in suckling mice; virus was obtained from Dr. Mike Bray [28]. Female C57BL/6 mice (4-8 weeks of age) were obtained from the Animal Production Area at the National Cancer Institute (Frederick, MD) and housed in sterile cages with sterile food and water. All research was conducted in accordance with the Animal Welfare Act and the principles stated in the Guide for the Care and Use of Laboratory Animals in an AAALAC accredited facility. During infection, mice were transferred to a Bio-Safety Level-4 containment area. Mice were treated with FGI-104 (10mg/kg) by 200.0µl I.P. injection then exposed to 1000 PFU of mouse-adapted Ebola virus by 200.0 µL I.P. injection two hours after the initial

Table 1. Broad spectrum antiviral activity of FGI-104

Virus	Type	Genome Type	EC50 (μM)	CC50 (μM)	SI
HCV	Flavivirus	(+) sense RNA	0.28	20 uM	71
HBV	Hepadnavirus	dsDNA-RT	0.02	14	700
PRRS	Arterivirus	(+) ssRNA	0.3	>50	>150
Cowpox	Poxvirus	dsDNA	0.25	>25	>100
Ebola	Filovirus	(-) ssRNA	10	>50	>5
VEE	Togavirus	(+) ssRNA	2.5	>25	>10
HIV	Retrovirus	ssRNA-RT	8	43	5

Shown is a summary of FGI-104 antiviral activity using cell-based assays. Please note that SI represents a relative "safety index," defined as the CC50/EC50.

treatment. Every 24 hours, for ten continuous days, mice received a single dose of FGI-104 and were observed daily for morbidity, weights and mortality for a minimum of 3 weeks. Under Bio-Safety Level-4 conditions, all laboratory personnel wore positive-pressure protective suits (ILC Dover, Frederica, DE) fitted with HEPA filters and umbilical-fed air.

Results

Cell-based assays to identify inhibitors of blood-borne pathogens

A focus library of small molecule compounds was tested for their abilities to block viral replication. These compounds were selected in part based on presumed capacity to block the interaction of TSG101 with its viral cognate ligands as determined using computational algorithms. The emphasis upon TSG101 was based on investigation from our laboratory and others, which demonstrate the host-derived TSG101 provides an opportunity to target multiple and different families of viruses that converge upon a common host pathway for viral budding and release [16].

To identify compounds with broad-spectrum activity against multiple virus types, a series of cell-based assays were utilized. The viruses selected for the initial screen were broadly divided into two groups. One group consisted of HIV, Hepatitis B virus (HBV) and Hepatitis C virus (HCV). These particular viruses were selected since they are often co-morbid within the same affected individuals [29]. A second group consisted of Ebola, Cowpox, Porcine Respiratory and Reproductive Syndrome virus (PRRS) and Venezuelan Equine Encephalitis (VEE) viruses. The latter group was emphasized since each represents an emerging virus and/or biothreat agent. Altogether, the pathogens under investigation comprised a broad array of unrelated virus families, which vary with regards when comparing the type of genomic nucleic acid, its strandedness and methods of replication (**Table 1**).

Cell-based assays identified one particular compound with potent activity against multiple virus types. One series of studies sought to determine the ability of the compounds to inhibit HCV infection of host cells. A luciferase-based infection of Huh7 cells with HCVcc (clone JFH-1) was employed. This particular model was selected since most other assay systems for HCV assess the number of replicons [22], which is problematic since TSG101 functions in viral budding and release and thus is downstream of nucleic acid replication. Using this system, one particular compound, known hereafter as FGI-104, demonstrated potent inhibition of HCV release. FGI-104 decreased HCV-based luciferase activity in a dose-dependent manner and achieved 99.5% relative to untreated controls at a concentration of 5 μM (**Figure 1A**). The potency of FGI-104 was also indicated by its relatively low EC50 of 280 nM. Cytotoxicity of the compound was assessed in parallel and no significant cytotoxic effects of FGI-104 were observed at the test concentration range (data not shown). Moreover, the levels of HCV-derived NS5A, a non-structural protein encoded by HCV, were assessed by Western blot analysis (**Figure 1B**). Parallel assessments of β-actin levels provided a matched control for sample loading. Western blot analysis of NS5A confirmed the dose-dependent inhibition of HCV by FGI-104.

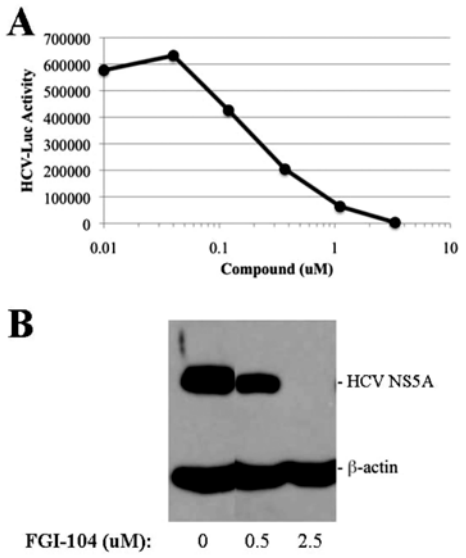


Figure 1. FGI-104 inhibition of HCV in cell-based assays. Huh7 cells were infected with luciferase expressing HCV JFH-1 virus for 72 hours in the presence of absence of FGI-104, with vehicle treated samples providing a negative control. (A) Luciferase-based readouts of viral release into the culture supernatant demonstrated dose-dependent inhibition of HCV replication by FGI-104. (B) The expression levels of virally-encoded NS5A as measured by western blot analysis provided an independent confirmation of dose-dependent inhibition of HCV by FGI-104.

Promising findings with HCV led us to evaluate the potential of FGI-104 to inhibit other virus types. We first evaluated those virus types, which are often co-morbid with HCV. Cell-based assays of HBV and HIV demonstrated consistent inhibition of each virus type in cell-based assays. For example, FGI-104 inhibited HBV infection of HepG2 cells and in a dose-dependent manner, with an estimated EC50 of 20 nM (**Figure 2A**). Similar to the results with Huh7 cells, the levels of FGI-104 associated with antiviral activity were considerably lower than levels associated with toxicity (with the CC50 estimated at 14 μM, 700-fold higher than the EC50; **Figure 2B**). Similar studies found FGI-104 also decreased HIV infection of MT-4 cells, albeit at higher levels of drug than were required for HBV or HCV (with an estimated EC50 of 8.5 μM).

In the course of evaluating FGI-104 antiviral activity, we investigated further the mechanistic basis of antiviral activity, with emphasis upon its impact on the viral life cycle. Based on the potential link with TSG101 we reasoned that FGI-104 would likely impact a point in the viral life cycle after nucleic acid replication but prior to viral budding and release. To test this hypothesis, the compound was further evaluated with the HCV subgenomic replicon. No appreciable activity was observed against the replicon system, suggesting that FGI-104 has no inhibitory effects on HCV replication (**Figure 3A, B**). The compound was further evaluated for anti-HBV activity by measuring either intracellular or extracellular HBV DNA copy number. Whereas FGI-104 demonstrated potent inhibition of extracellular HBV and virus release, it had no effects on the intracellular accumulation of HBV DNA, or virus replication (**Figure 3D**). These findings are therefore consistent with the idea that FGI-104 blocks a stage of the viral life cycle prior to budding and release but after nucleic acid replication.

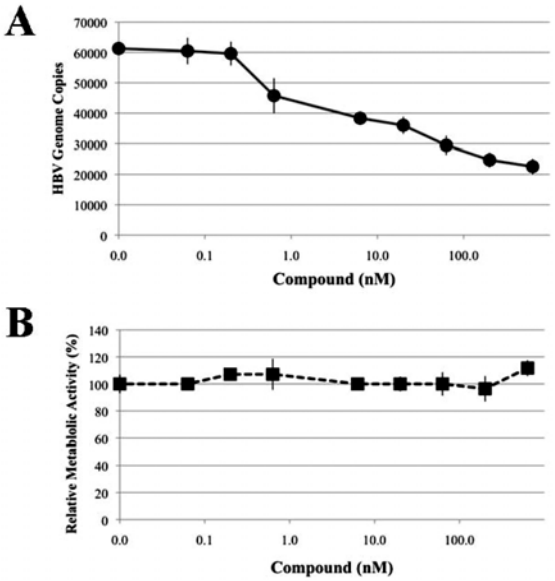
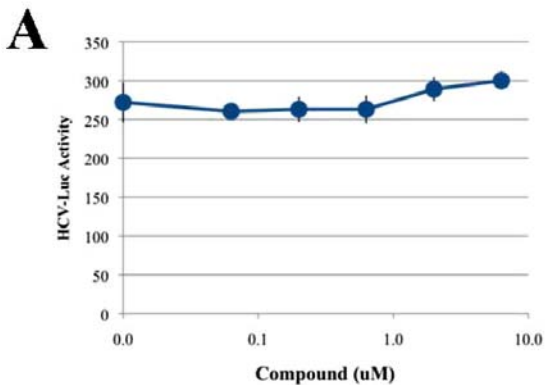


Figure 2. FGI-104 inhibition of HBV. HepG2 cells expressing HBV replicons was treated for 72 hours in the presence or absence of FGI-104, with vehicle treated samples providing a negative control. (A) The number of HBV genomic copies in the culture supernatant was assessed by using quantitative PCR ,, demonstrating a dose-dependent inhibition of viral release. (B) The impact of FGI-104 treatment on overall cell survival and metabolism was assessed to preclude the potential that the observed antiviral activity simply reflected an overall decrease in host cell metabolic activity.



B

HCV

	Viral Release	Replicon
EC_{50}	0.12 μM	>20 μM
CC_{50}	>20 μM	>20 μM

C

HBV

	Viral Release	Replicon
EC_{50}	0.05 μM	>20 μM
CC_{50}	>20 μM	>20 μM

Figure 3. The mechanistic basis of FGI-104 antiviral activity occurs after nucleic acid replication. The antiviral activity of FGI-104 was assessed using standard replicon assays of nucleic acid replication for HCV (A,B) and HBV (C). Note that the data compiled in (B) and (C) represents summaries of multiple experiments. Unlike the potent inhibition observed previously with assay systems that measured release of infectious virions, assessments of intracellular nucleic acid replication did not reveal FGI-104 antiviral activity.

Broad spectrum inhibition of emerging and biothreat agents

We then compared the work using established viruses (HCV, HBV and HIV) with emerging virus types to test the concept of application of TSG101 both to known and unknowable viral pathogens. As an example, a series of studies were conducted using PRRS virus. This particular pathogen was selected since it was first reported in 1987 and based on the far-reaching economic impact of PRRS as evidenced by the catastrophic ongoing outbreak in China [30, 31]. Cell-based assays of PRRS demonstrated potent antiviral activity. For example, FGI-104 treatment of Marc-145 cells could completely inhibit PRRS propagation as indicated using plaque formation assays (**Figure 4A**), with an estimated EC90 of 1.6 μM (**Figure 4B**). Importantly, the potent activity against PRRS was comparably observed using both major strains of the virus (European and North American isolates; data not shown). We further demonstrated that the antiviral activity was not unique to any particular model system since comparable inhibition of PRRS virus was documented using either primary porcine-derived macrophages or the Marc-145 cell model as the host target for infection. The potential breadth of FGI-104 application was demonstrated by inhibition of other emerging or biothreat agents (**Table 1**). For example, FGI-104 demonstrated a dose-dependent inhibition of the propagation of additional viruses, including Cowpox, VEE and Ebola viruses (**Figure 5** and data not shown). However, FGI-104 was not able to inhibit a number of other virus types including multiple and different seasonal variants of influenza (data not shown). Notably, multiple and different assay systems were utilized (e.g., viral titer or assessment of viral killing of host cells) to evaluate these viruses and FGI-104 demonstrated consistent abilities to inhibit viral infection regardless of the experimental

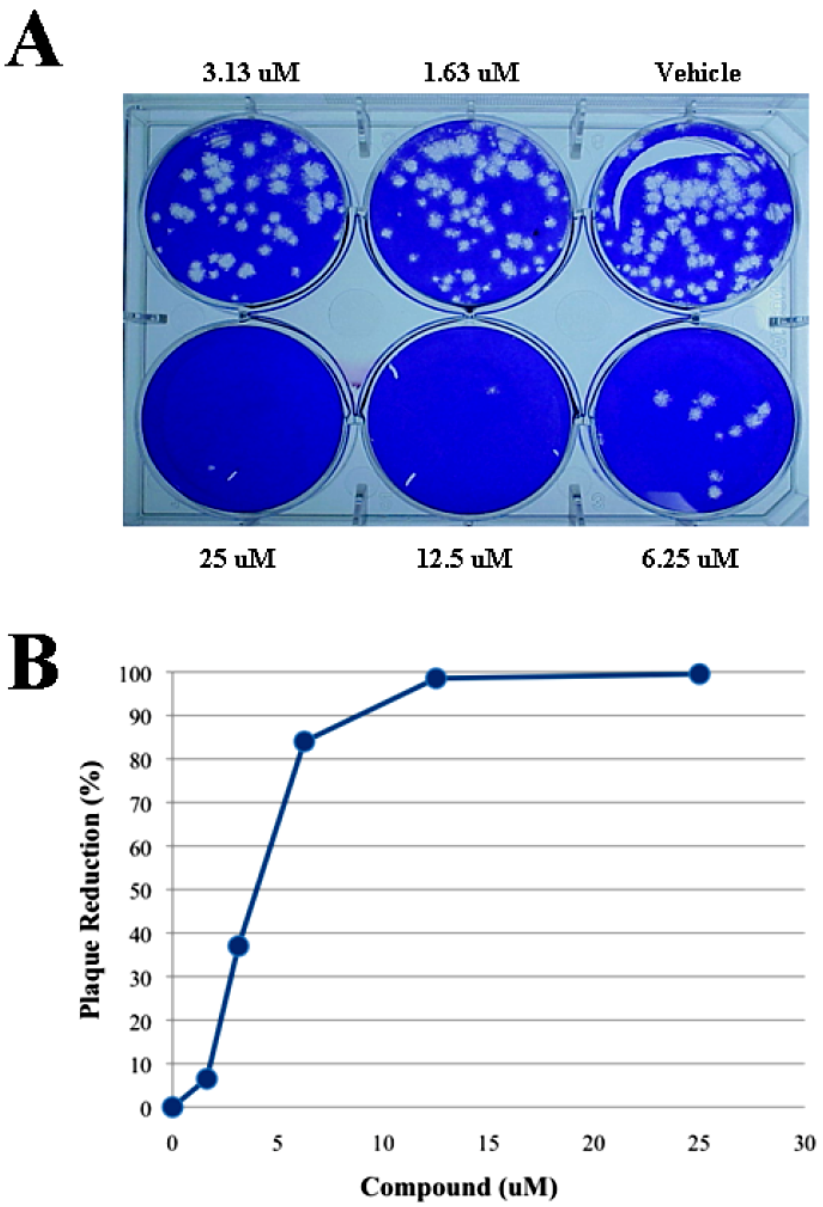


Figure 4. FGI-104 inhibition of PRRS virus. Marc-145 cells were infected with PRRSV virus (23983) for 72 hours in the presence or absence of FGI-104, with vehicle treated samples providing a negative control. Plaque formation was assessed visually (A) and the number of plaques quantified to determine the % reduction by FGI-104 (B).

outcome.

FGI-104 demonstrates efficacy in vivo

Although the broad-spectrum antiviral activity observed above was compelling, we considered that these results were confined to cell-based assay systems. We sought to overcome this limitation using *in vivo* assays but a lack of relevant animal-based systems precluded investigation of HCV, HIV and most of the other virus types of relevance to FGI-104. Therefore, we focused on animal models of Ebola hemorrhagic fever. Specifically, mouse-adapted Ebola provided a means to interrogate the antiviral properties of FGI-104 *in vivo* [28]. For these studies, mice were infected with Ebola and then treated daily with

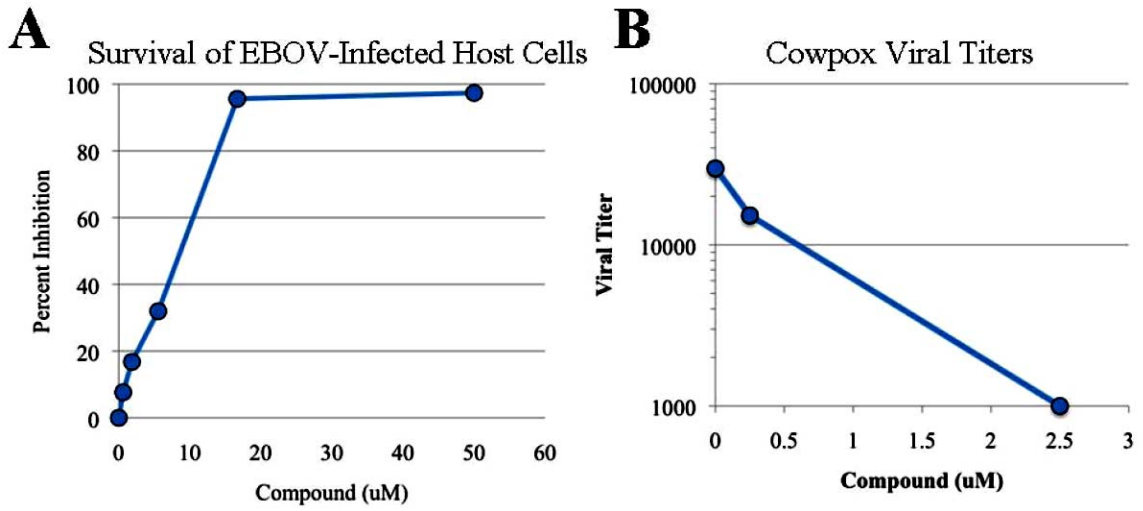


Figure 5. Broad-spectrum inhibition of biothreat viruses by FGI-104. The antiviral activity of FGI-104 was assessed using cell-based assays of Ebola hemorrhagic fever (EBOV) (A) or cowpox (B) viruses. Please note these two particular findings were representative of the breadth of viral inhibition (Ebola is an RNA virus whereas cowpox is a DNA virus) and that FGI-104 demonstrated antiviral activity even when using different types of readouts (Inhibition of virus-mediated killing of host cells or plaque-based readouts of viral titer).

FGI-104. A vehicle-treated group provided a matched negative control. Unfortunately, no positive control was available since no other therapeutic was available that can block Ebola-mediated pathophysiology. Consistent with its efficacy in cell-based assays, FGI-104 treatment prevented Ebola-mediated killing of infected animals. Whereas vehicle-treated animals succumbed within 7 days post-infection, all mice treated with FGI-104 demonstrated lived longer and all animals in the FGI-104 group survived (**Figure 6**). Ebola is relatively immunogenic and those animals that survive beyond 10 days post-infection generally acquire immunity to subsequent challenge. As an additional control, the surviving animals were re-challenged after 14 days with Ebola and each survived, thus verifying that the animals had encountered Ebola but that FGI-104 spared these animals from an otherwise lethal infection.

Discussion

The major finding of our present study is the identification of a small molecule inhibitor of viral disease, with the potential for broad-spectrum application to a wide array of unrelated virus types. We also demonstrate that FGI-104 demonstrates antiviral efficacy at concentrations that are substantially lower than those required to initiate host cell toxicity, thus suggestive of a safe therapeutic window. Consistent with this finding, FGI-104 treatment was sufficient to prevent lethal infections with Ebola virus in animals-based models.

The fundamental principle underlying our investigation centers upon the concept of host-oriented therapeutics for infectious disease. Host-oriented therapeutics seeks to identify host-based mechanisms and therapeutics that deny viral pathogens the ability to cause disease. The advantages of a successful host-oriented therapeutic could be many-fold. For example, it is generally understood that many different viruses are completely dependent upon a relative small subset of host targets and mechanisms. This fact presents an opportunity to identify host targets that are shared among different virus types and inhibition of these pathways could have broad-spectrum application for prevention or therapy. By definition, host-oriented therapeutics impact the host and not a viral target. Unlike their virally-encoded counterparts, host-encoded targets are genetically stable and moreover, these targets are fundamental for the virus and direct selective pressure is not placed upon the viral pathogen. Thus, we anticipate that a successful host-oriented therapeutic will demonstrate reduced

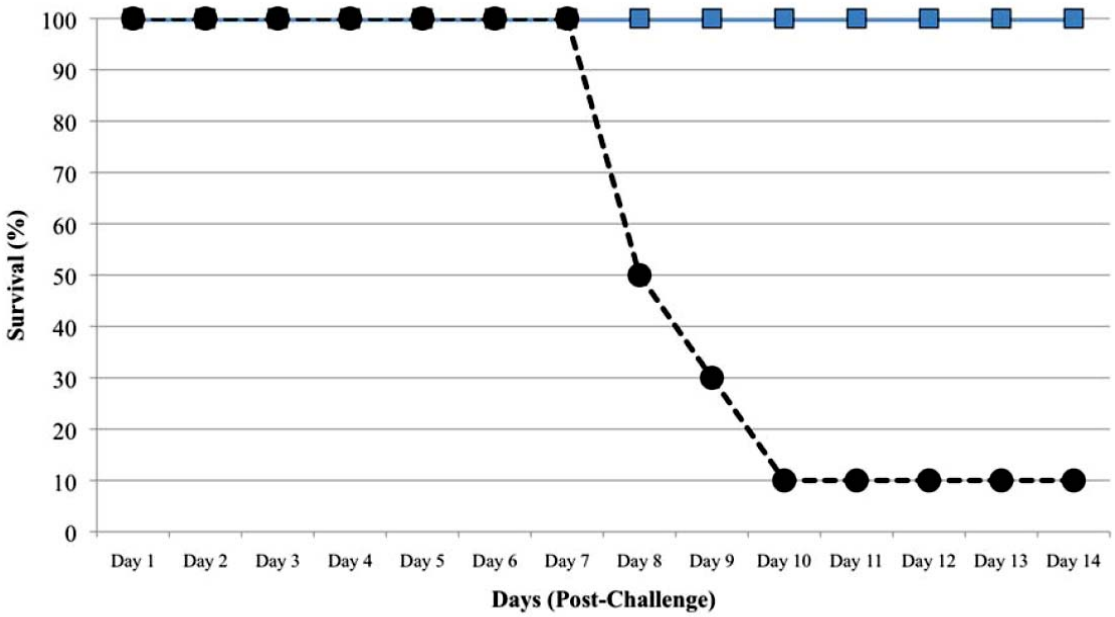


Figure 6. Antiviral efficacy of FGI-104 in animal-based models of Ebola infection. Shown is an assay of FGI-104 efficacy in Mice following a lethal Ebola Challenge. C57Bl/6 mice were challenged with 1000 pfu of mouse adapted Ebola and treated with 10 mg/kg/day of FGI-104. Overall survival was assessed, revealing the characteristic lethality of Ebola 7-8 days post-infection. Whereas vehicle-treated controls succumbed as expected, at least one half of FGI-104-treated animals survived lethal challenge.

susceptibility to viral resistance mechanisms that often thwart the efficacy of conventional virus-oriented therapeutics [32, 33].

One novel aspect of our present study is the demonstration that FGI-104 can inhibit multiple and different viral pathogens. A practical example of the application of such a broad-spectrum application would center upon the treatment of a constellation of blood-borne pathogens. The studies herein represented by the shared efficacy of FGI-104 when target a constellation of blood-borne viral pathogens (HBV, HCV, and HIV) provide a glimpse of the potential application of FGI-104 to treat or prevent viruses, which are often co-morbid within particular populations [29]. Successful development of this strategy could likewise apply to other pathogens such as simultaneous targeting of different pathogens responsible for respiratory infections (e.g., seasonal infection with influenza and RSV), sexually-transmitted diseases (Herpes and HIV) or other constellations of viral-mediated diseases.

Our demonstration of efficacy against Ebola virus *in vivo* is particularly intriguing in light of the well-understood and extreme pathogenicity of Ebola and because Ebola represents an emerging pathogen potential biothreat agent [34, 35]. At the mechanistic level, Ebola has been shown to require TSG101 for its dissemination [36] and this understanding is consistent with the original strategy described herein, which predicted that FGI-104 would inhibit the interaction of TSG101 with its cognate viral ligands, i.e., 'PTAP' late domain sequence in the Ebola VP40. Although future investigation will be required to test the hypothesis that FGI-104 directly inhibits TSG101, such knowledge could have far-reaching implications since genetic-based analysis suggests that TSG101 provides an essential function for many different virus types. However, we cannot preclude that FGI-104 might also impact other pathways and additional investigation will be required to dissect further the mechanistic basis of FGI-104 antiviral activity.

In the course of investigation FGI-104, pharmacokinetics studies have revealed that the molecule is amenable to delivery via oral

or intravenous route (data not shown). FGI-104 is also well-tolerated, with LD50 values greater than 1 g/kg in mice (as compared with the efficacy observed herein at 10 mg/kg). In light of the fact that cell-based assays indicated a much higher EC50 value for Ebola (10 μM) than HBV or HCV, these findings suggest a high relative safety margin for FGI-104. Thus, the work described with a handful of blood-borne or biothreat agents could have a much more far-reaching impact for combating viral diseases.

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