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Hexamminecobalt (III) Chloride as a Broad-Spectrum Antiviral Complex

Eddie L. Chang[1]*, Gene G. Olinger[2], Lisa E. Hensley[2], Calli M. Lear[2], Corinne E. Scully[2], Marie K. Mankowski[3], Roger G. Ptak[3], Dzung C. Thach[4] and D. Andrew Knight[5]

[1]Center for Bio/Molecular Science and Engineering, 4555 Overlook Avenue SW, Washington, DC20375, USA
[2]United States Army Medical Research Institute of Infectious Diseases, Division of Virology, 1425 Porter Street, Frederick, Maryland, USA
[3]Infectious Disease Research Department, Southern Research Institute, 431 Aviation Way, Frederick, MD, 21701, USA
[4]Laboratory of Infectious Diseases, National Institute of Allergies and Infectious Diseases, NIH, Department of Health and Human Services, Bethesda, MD 20892, USA
[5]Chemistry Department, Florida Institute of Technology, 150 West University Boulevard, Melbourne, FL 32922, USA

Abstract

Metal ion complexes have the potential to form novel types of antiviral compounds, due to their ability to form octahedral and square-planar molecular geometries and their intrinsic charge density. Previously it has been shown that hexamminecobalt(III) chloride (Cohex) has antiviral properties against Sindbis virus (SINV) and adenovirus. Here, we report that Cohex also exhibits activities against two isolates of human immunodeficiency virus (HIV) and the Zaire Ebola (ZEBOV) strain expressing green fluorescent protein (GFP). The therapeutic indices for antiviral activity against the HIV isolates were similar to that found for SINV. Cohex was also effective in decreasing the host translation of viral GFP in four different cell-lines infected with ZEBOV. Toxicity studies in mice found no deleterious effects at up to 8 mg/kg. The 8 mg/kg concentration also prolonged survival of mice infected with ZEBOV. These results point to the potential of Cohex as a new type of broad-spectrum antibiotic compounds.

Keywords: Broad-spectrum antiviral drugs; Ebolavirus; HIV; hexamminecobalt (III) chloride; Therapeutic index

Introduction

The rapidity with which microorganisms develop resistance to existing drugs necessitates the continuing search for new compounds that have antibacterial or antiviral properties. This problem is particularly acute for viruses [4], for which there are few available antimicrobial choices. Thus, there is a need to look for new classes of antiviral drugs, especially those that show broad-spectrum properties. One area that has potential for development of new drugs is the formation of metal coordination complexes. These compounds differ in properties from carbon-based compounds because of the intrinsic charged state of the central ion, the potential to form octahedral and square planar molecular geometries, and the ability to make these compounds both small enough and with unique molecular geometries to contact regions that have unusual (non-tetrahedral or simpler based) geometric requirements for inhibition.

We have been investigating hexamminecobalt(III) chloride (Cohex) (1, Chart 1), a fully coordinated complex of Co(III) ion surrounded by six ammonia ligands, as a potential broad-spectrum antiviral compound and have reported on its antiviral activity against both Sindbis virus (SINV) and adenovirus [2,5]. Due to its full octahedral coordination, the Co (III) ion interacts with its environment via outer-sphere coordination and through its triply positive charge. One consequence is that, unlike previously studied systems [13], Cohex does not hydrolyze nucleotides, but does show potent inhibition of protein synthesis and dose-dependent antiviral properties [2].
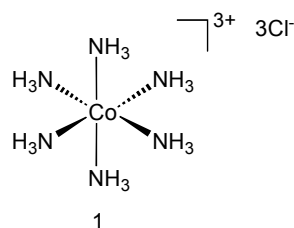
The status of the biological activities of Co(III) complexes formed with mono and polydentate ligands has recently been reviewed. The simple Co^{3+} ion is unstable in water, but can be stabilized against reduction to Co^{2+} by coordination to monodentate ligands or chelators. By far the most common ligand type used to stabilize the cobalt (III) ion in aqueous solution is the chelating N,O donor ligand. A previously reported series of Co(III) complexes, based on a chelating Schiff base, was reported to be effective in the treatment of epithelial herpetic keratitis, one of the major causes of blindness in industrial nations. In 2006, Epstein also reported the activity of another compound in the series, CTC-96, that was effective against adenovirus in a cell culture model and also against adenovirus keratoconjunctivitis in a rabbit model [1]. In 1965, Hawthorne reported a new class of cluster known as the metal bis(1,2-carbollides); while Cigler and Rezacova have described the use of cobalt bis (1,2-carbollides) as antiviral therapeutics, specifically against HIV protease [43].

Cohex is a different type of Co(III) compound from those previously reported to have antiviral activity. For example, in contrast to the Co(III) Schiff base complexes, whose axial ligands are readily exchanged for histidine ligands, Cohex forms a very stable and non-labile complex with its six ammonia ligands. In addition, two of the Co(III) charges are neutralized by inner-shell coordination, whereas the Cohex complex retains a net charge of +3. We previously explored the basis of Cohex's antiviral properties by examining several key structure-activity relationships, such as whether the full coordination of ammonia ligands with the Co(III) ion is necessary for its activity

Chart 1: Hexamminecobalt (III) chloride (Cohex).

or whether Co(III) is unique in its ability to elicit antiviral effects in a hexammine configuration. We found that macrocyclic or mixed macrocyclic–monodentate analogs showed no antiviral activity, although substituting a water molecule for an NH_3 molecule in Cohex only somewhat diminished the activity. Analog ammine complexes with either Ni(II) or Ru(III) ion centers exhibited some antiviral activity, but were generally more toxic than Cohex.

WhileSINV isa single-strand (+) RNA Alphavirus, HIV is a lentivirus of theretroviridae family. HIV also has a single-strand (+) RNA genome, but at two copies per virion, and is reverse-transcribed into cDNA by virusRNA-dependent DNA polymerasein the host cell. Thevirus cDNA serves as a template for DNA-dependent DNA polymerase to make virusdouble-stranded DNA (dsDNA), which is transported to the nucleus where it integrates into the cell's genome. Viralreplication is initiated when the integrated DNA provirus is transcribed into mRNA. Ebola viruses belong to the –RNA filoviridae family with five known strains (subtypes): Bundibugyo, Côte d'Ivoire, Sudan, Zaïre, and Reston. The Bundibugyo, Sudan, and Zaïre strains have caused outbreaks of Ebola hemorrhagic fever among humans in Africa, killing up to 90% of those infected, with the Zaire strain being the most virulent. In this paper, we report that Cohex, in addition to possessing antiviral effects against SINV and adenovirus, has the ability to decrease both HIV and Ebola virus replication *in vitro*, and is also able to enhance survival rates in a mouse model for pathogenic Ebola virus infection.

Materials and Methods

Cohex HIV preparation

Cohex was tested in a standard PBMC (peripheral blood mononuclear cell) cell-based microtiter anti-HIV assay against HIV-1_{NL4-3} [subtype B, CXCR4-tropic, molecular clone] and HIV-1_{Ba-L} (Subtype B, CCR5-tropic, isolated from primary culture of adherent cells grown from explanted human infant lung tissue)as described previously [4]. The following reagents were obtained through the AIDS Research and Reference Reagnt program, Division of AIDS, NIAID, NIH: [1] pNL4-3 [molecular clone of HIV-1_{NL4-3}] from Dr. Malcolm Martin; and [2] HIV-1_{Ba-L} from Dr. Suzanne Gartner, Dr. Mikulas Popovic and Dr. Robert Gallo [4760]. For this study PBMCs were pre-treated with the compound for 2 h before infection. HIV-1_{NL4-3} stocks were prepared by transfection of pNL4-3 into HeLa-CD4-LTR-βgal cells and subsequent amplification in fresh human PBMCs. HIV-1_{Ba-L} stocks were also prepared in fresh human PBMCs.

Cohex was stored at 4°C as a powder and solubilized in sterile distilled water for tests. The solubilized stock was stored at -20°C until the day of the assay. Stocks were thawed at room temperature on each day of assay setup and were used to generate working drug dilutions used in the assays. Working dilutions were made fresh for each experiment and were not stored for re-use in subsequent experiments performed on different days. Cohex was evaluated using a 3 mM [3,000 µM] high-test concentration with 8 additional serial half-log dilutions in the PBMC assays.

PBMC assay

Freshly prepared PBMCs were centrifuged and suspended in RPMI 1640 with 15% fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, non-essential amino acids (MEM/NEAA; Hyclone; catalog # SH30238.01), and 20 U/mL recombinant human interleukin (IL)-2. PBMCs were maintained in this medium at a concentration of 1-2 x 10^6 cells/mL, with twice-weekly medium changes until they

were used in the assay protocol. Monocyte-derived-macrophages were depleted from the culture as the result of adherence to the cell culture flask.

The cells were plated in the interior wells of a 96-well round bottom microplate at 50 µL/well (5 x 10^4 cells/well) in a standard format developed by the Infectious Disease Research department of Southern Research Institute. Each plate contained virus control wells (cells plus virus) and experimental wells (drug plus cells plus virus). Test drug dilutions were prepared at a 2X concentration in microtiter tubes and 100 µL of each concentration was placed in appropriate wells using the standard format. 50 µL of a predetermined dilution of virus stock was placed in each test well (final multiplicity of infection ~ 0.1). Separate plates were prepared identically without virus for drug cytotoxicity studies using an MTS assay system (described below; cytotoxicity plates also included compound control wells containing drug plus medium without cells to control for colored compounds that could affect the MTS assay). The PBMC cultures were maintained for 7 days after infection at 37°C, 5% CO_2. After this period, cell-free supernatant samples were collected for analysis of reverse transcriptase activity and compound cytotoxicity was measured by addition of MTS to the separate cytotoxicity plates for determination of cell viability. Wells were also examined microscopically and any abnormalities were noted.

Reverse transcriptase activity assay

A microtiter plate-based reverse transcriptase (RT) reaction was utilized [3]. Tritiated thymidine triphosphate (3H-TTP, 80 Ci/mmol, NEN) was received in 1:1 dH_2O: ethanol at 1 mCi/ml. Poly rA:oligo dT template:primer (Pharmacia) was prepared as a stock solution by combining 150 µl poly rA [20 mg/ml] with 0.5 ml oligo dT [20 units/ml] and 5.35 ml sterile dH_2O followed by aliquoting [1.0 m] and storage at -20°C. The RT reaction buffer was prepared fresh daily and consisted of 125 µl 1.0 M EGTA, 125 µl of $dH2O$, 125 µl of 20% Triton X100, 50 µl of 1.0 M Tris (pH 7.4), 50 µl of 1.0 M DTT, and 40 µl of 1.0 M $MgCl_2$. The final reaction mixture was prepared by combining one part 3H-TTP, four parts dH_2O, 2.5 parts poly rA:oligo dT stock and 2.5 parts reaction buffer. Ten microliters of this reaction mixture was placed in a round bottom microtiter plate and 15 µl of virus-containing supernatant was added and mixed. The plate was incubated at 37°C for 60 min. After incubation, the reaction volume was spotted onto DE81 filter- mats (Wallac), washed five times for 5 min each in a 5% sodium phosphate buffer or 2X SSC (Life Technologies), two times for 1 min each in distilled water, two times for 1 min each in 70% ethanol, and then dried. Incorporated radioactivity (counts per min, CPM) was measuredby standard liquid scintillation techniques.

MTS staining for PBMC viability to measure cytotoxicity

At assay termination, the uninfected assay plates were stained with the soluble tetrazolium-based dye MTS [CellTiter 96 Reagent, Promega] to determine cell viability and assay compound toxicity. MTS is metabolized by the mitochondria enzymes of metabolically active cells to yield a soluble formazan product, allowing the rapid quantitative analysis of cell viability and compound cytotoxicity. This reagent is a stable, single solution that does not require preparation before use. At termination of the assay, 20-25 µL of MTS reagent was added per well and the microtiter plates were then incubated for 4-6 h at 37°C, 5% CO_2 to assess cell viability. Adhesive plate sealers were used in place of the lids, the sealed plate was inverted several times to mix the soluble formazan product, and the plate was read spectrophotometrically at 490/650 nm (absorbance/reference) with a Molecular Devices SPECTRAmax plate reader.

In vitro high throughput zebov assay

Ebola virus expressing green fluorescent protein (GFP) was derived by reverse genetics. A full-length cDNA clone of Zaire Ebola virus (Mayinga strain) was generated and rescued containing the foreign reporter gene, eGFP (EboZ-eGFP). Generation of EboZ-eGFP, showed similar viral characteristics to that of the wild-type virus yet causes infected cells to fluoresce green when stimulated. EboZ-eGFP has provided a more sensitive quantitative assay to rapidly screen multiple compounds in shorter period of time.

In the evaluation of Co (III), Vero E6 and 293T cells were plated in black 96-well plates at 40,000 cells/well with a volume of 100μl EMEM (Invitrogen) containing 10% fetal bovine serum (FBS) + 5% L-Glutamine and Hepes buffer. A549 and HepG2 cells were plated in black 96-well plates at 40,000 cells/well with a volume of 100μl DMEM (Invitrogen) containing 10% fetal bovine serum (FBS) + 5% L-Glutamine and Hepes buffer. Plates were incubated for 24 hours at 37°C with 5%CO_2 before infection. After 24 hours, cells were treated in triplicate, with concentrations of Cohex ranging from 2.5 μM to 5 mM for one hour and then infected with a 1:16 dilution of EBOZ-EGFP (MOI ~0.1). Plates were placed back into the incubator at 37°C with 5% CO_2 and read for fluore scence (Excitation: 485nm; Emission: 515; Cut-off: 495) at 48 hours post infection on a Gemini EM plate reader (Molecular Devices).
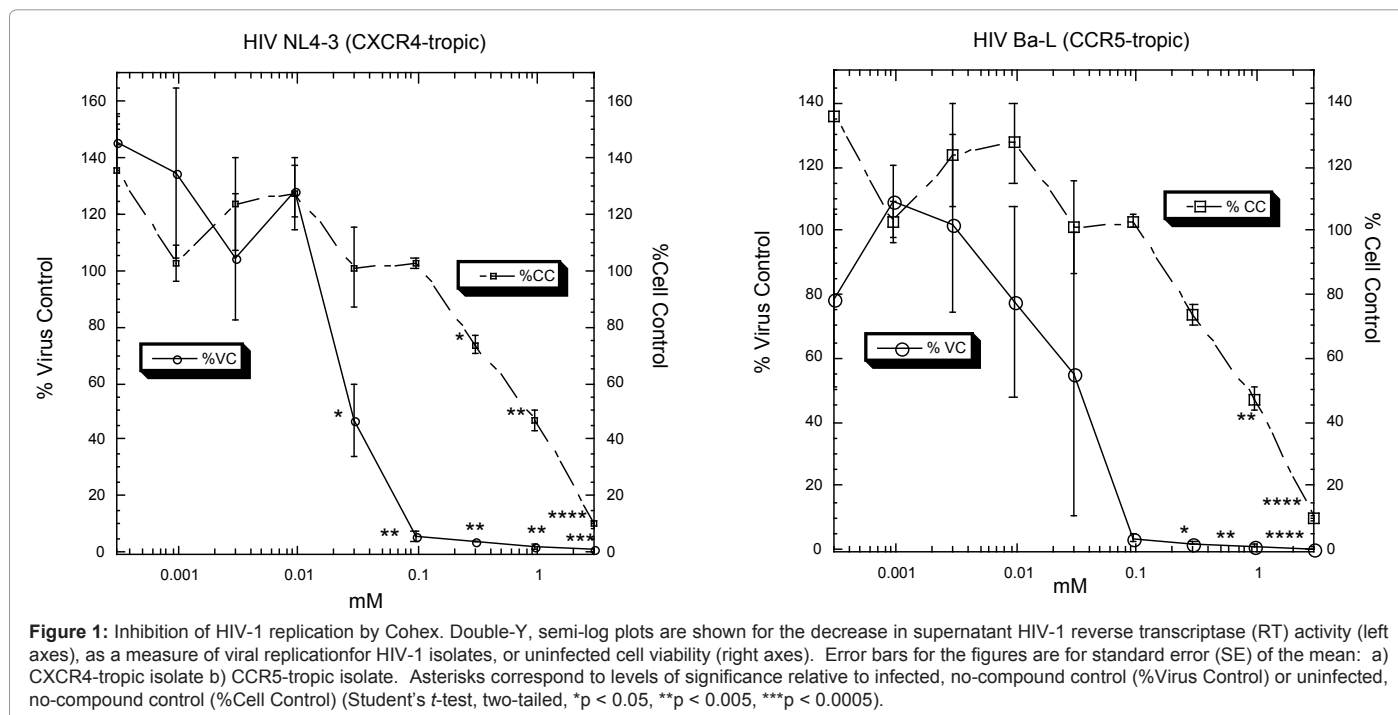
Cell viability assay

In the evaluation of Co (III) cell toxicity, Vero E6 and 293T cells were plated in black 96-well plates at 40,000 cells/well with a volume of 100μl EMEM (Invitrogen) containing 10% fetal bovine serum (FBS) + 5% L-Glutamine and Hepes buffer. A549 and HepG2 cells were plated in black 96-well plates at 40,000 cells/well with a volume of 100μl DMEM (Invitrogen) containing 10% fetal bovine serum (FBS) + 5% L-Glutamine and Hepes buffer. Plates were incubated for 24 hours at 37°C with 5% CO_2 before being treated in triplicate with concentrations

of Cohex ranging from 2.5 μM to 5 mM for 48 hours. After 48 hours, cells were evaluated for viability using the Promega CellTiter-Glo Luminescent Cell Viability 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, revised 1/07). 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, are discarded by flicking into a waste container of MicroChem disinfectant after 48 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, plates are mixed by orbital shaker for two minutes; after mixing, plates incubate at room temperature for 10 minutes to stabilize the luminescence signal. Plates are read for luminescence by Safire[2] provided by Tecan.

Rodent tolerability and zebov efficacy

Initial studies focused on the tolerability of Cohex treatment in mice. Uninfected mice were treated with Cohex in biosafety level (BSL)-2 conditions and the weights and clinical signs were observed daily before and after treatment. 10 animals were treated via intraperitoneal (i.p.) injection with 0.5, 1, 2, 4, or 8 mg/kg of Cohex via once a day for 10 continous days. Once the tolerability was found to be acceptable, the efficacy of Cohex treatment against Ebola virus infection in the murine mouse model was performed. We utilized the mouse-adapted Ebola Zaire virus generated by Bray, et al. Animals were treated i.p. with 2, 4, and 8 mg/kg of Cohex approximately 1 h before Ebola virus exposure (1000 PFU); treatments continued once a day for 10 continuousdays.

Figure 1: Inhibition of HIV-1 replication by Cohex. Double-Y, semi-log plots are shown for the decrease in supernatant HIV-1 reverse transcriptase (RT) activity (left axes), as a measure of viral replicationfor HIV-1 isolates, or uninfected cell viability (right axes). Error bars for the figures are for standard error (SE) of the mean: a) CXCR4-tropic isolate b) CCR5-tropic isolate. Asterisks correspond to levels of significance relative to infected, no-compound control (%Virus Control) or uninfected, no-compound control (%Cell Control) (Student's t-test, two-tailed, *p < 0.05, **p < 0.005, ***p < 0.0005).

Mice were observed once daily for changes in weight and physical appearance for the onset of signs of disease. Mice were observed twice daily once the onset of symptoms occurred.

Mouse-adapted Ebola virus was obtained from Dr. Mike Bray. Female C57BL/6 mice (5-8-weeks old) were obtained from the National Cancer Institute (Frederick, MD.) and housed under specific pathogen-free conditions. For infection, mice were inoculated i.p. with 1000 PFU (30,000 LD_{50}) of mouse-adapted Ebola virus in a BSL-4 laboratory. All

infections were performed at BSL-4 facilities; personnel wore positive-pressure protective suits (ILC Dover, Frederica, DE) fitted with HEPA filters and umbilical-fed air.

Research was conducted in compliance with the Animal Welfare Act and other federal statues and regulations relating to animals and experiments involving animals, and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully
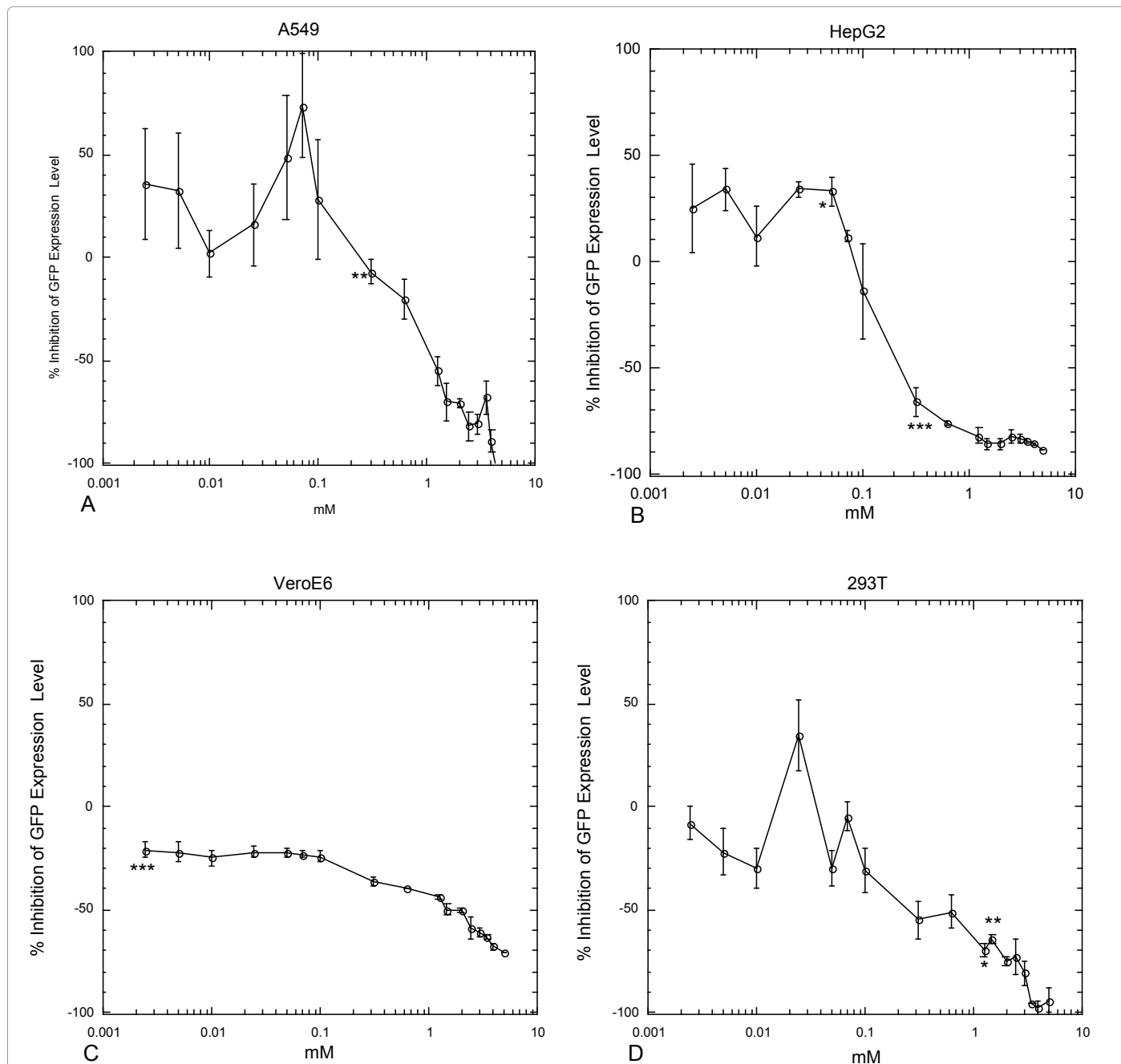

Figure 2: Inhibition of Ebola virus replication by Cohex. Plots of the levels of GFP expression in infected cells, normalized against infected cells with no Cohex (+/- control). Error bars are for standard error (SE) of the mean. Asterisks correspond to levels of significance relative to infected, no-compound control: a) relative GFP levels for A549 **$p < 0.005$ for 0.3 mM and higher Cohex; b) relative GFP levels for HepG2 cells as a function of Cohex concentration; *$p < 0.05$ for 0.025 and 0.05 mM and***$p< 0.0005$ for 0.312 mM and higher concentrations; c) relative GFP levels for VeroE6 cells, all data have ***$p< 0.0005$; d) relative GFP levels for 293T cells, *$p < 0.05$, **$p < 0.005$ for all concentrations 1.5 mM and higher.

accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Results

HIV

Cohex was tested for antiviral effects against one CXCR4-tropic HIV-1 isolate (HIV-1$_{NL4-3}$) and one CCR5-tropic HIV-1 isolate (HIV-1$_{Ba-L}$) in peripheral blood mononuclear cells (PBMCs). For this study, the PBMCs were pre-treated with the compound for 2 h before infection and the raw data were normalized by dividing by either the average untreated infected value for the infection measurements (% viral control) or by the uninfected, untreated value for the cytotoxicity measurements (% cell control), shown in (Figure 1). The normalized values were then analyzed to determine IC$_{50}$ (50% inhibition of viralreplication), CC$_{50}$ (50% cytotoxicity), and therapeutic index values (TI = CC$_{50}$/IC$_{50}$).
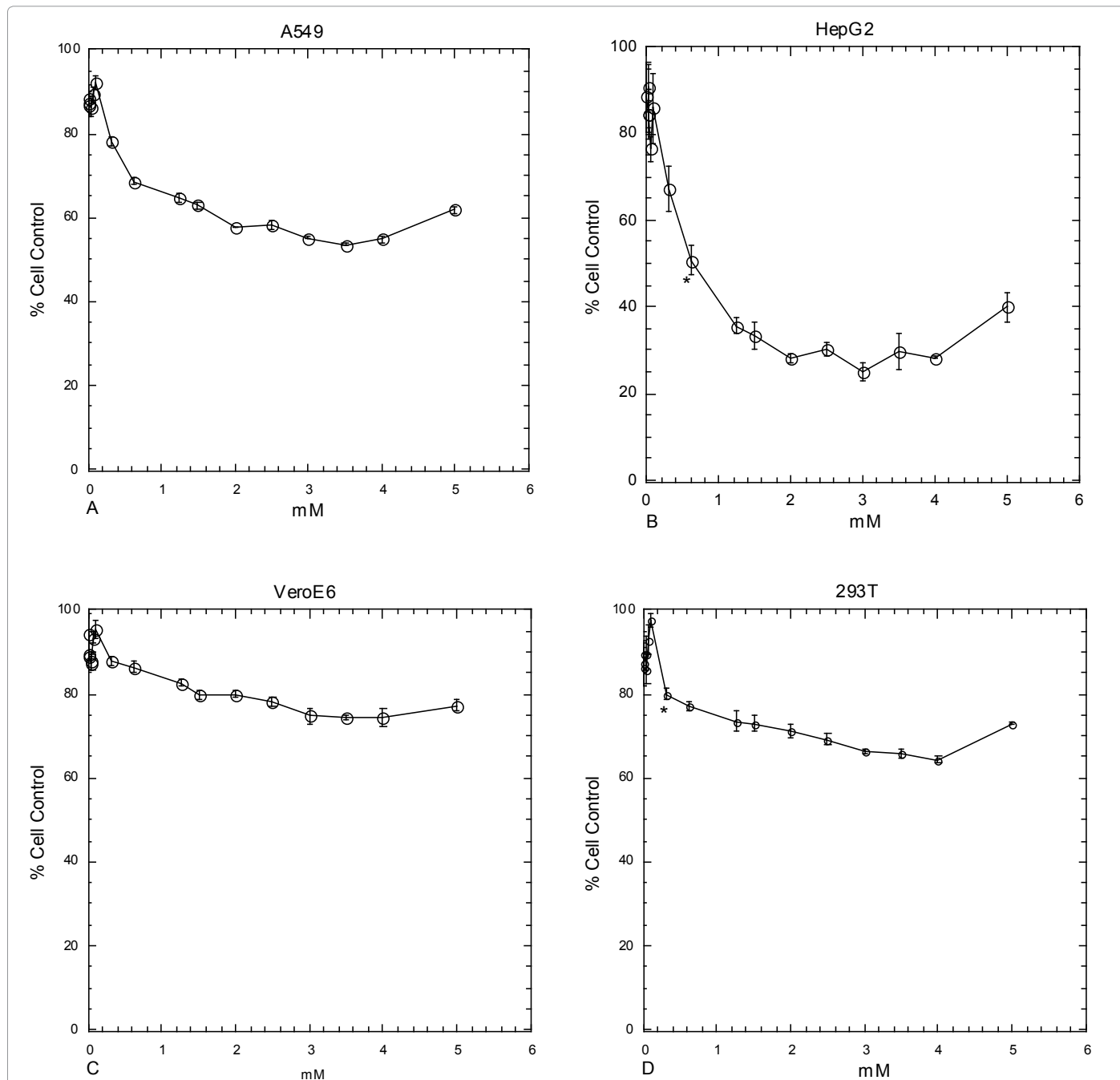

Figure 3: Cytotoxicity of Cohex in cell lines used for in vitro Ebola virus studies. Plots of cell viability, normalized against uninfected cells with no Cohex (-/- control; % cell control). Error bars for the figures are for standard error (SE) of the mean: a) the % of viable A549 cells, as a fraction of -/- control, is plotted as a function of increasing Cohex; $p < 0.05$ for all concentrations (down to $p = 1 \times 10^{-6}$ for 5 mM); b) % viable cells for HepG2 cells as a function of Cohex concentration; *$p < .05$ for .63 mM and higher(down to $p = 4 \times 10^{-4}$ for 0.4 mM); c) % viable cells for VerE6 cells as a function of Cohex concentration; all data have $p < 0.05$ except at 0.1 mM Cohex. (lowest $p = 1 \times 10^{-4}$ for 3.5 mM) d) % viable cells for 293T cells as a function of Cohex concentration; *$p < 0.05$ for 0.63 mM and higher(lowest $p = 4 \times 10^{-5}$ for 3.5 mM).

Cohex displayed antiviral activity against both virus isolates evaluated in this study. The IC_{50} values against both virus isolates were similar with an average value of 31.2 μM. Cytotoxicity was observed with the compound at concentrations above 100 μM (CC_{50} = 833 μM), resulting in an average therapeutic index value of 26.7. Summaries of IC_{50}, CC_{50}, and TI are shown in (Table 1a)

Ebola virus

Four different cell-lines were infected with Zaire Ebola GFP virus (ZEBOV-GFP), a viralstrain that encodesthe GFP gene. GFP expression levels were measured 48 h postinfection, with Cohex added right after infection. Cells were treated, in triplicate, withconcentrations of Cohex ranging from 2.5 μM to 5 mM.

The results for A549 cells and HepG2 cells are shown in (Figure 2a,b). There wasa generally flat response from 2.5 μM until around 0.1 mM Cohex, at which point, GFP expression decreaseduntil there wasnearly 100% suppression (-100 %) of viral expression at concentrations above 1mM of Cohex. In 293T cells (Figure 2d), GFP expression decreasedmonotonically with increasing Cohex, starting as low as 2.5 μM Cohex. For VeroE6 cells (Figure 2c), GFP expression also decreasedwith increasing Cohex, but at a lowerratethan the other cells. In A549 and HepG2 cells (Figure 2a,b), at concentrations below 0.1 mM, GFP expression fluctuated between 0 and +50% enhancement with large variations. In contrast, in 293T and VeroE6, at the same concentration range, all butone showed GFP suppression. Thus, at low Cohex concentrations, different cell types exhibited differential viral expression decrease or even increase, but at Cohex concentrations above 0.1 mM, all showed suppression of virus expression.

To determine if the decreasing GFP levels were due to decreasing numbers of viable cells, in vitro cytotoxicity studies using a luminescent cell viability assay were performed for the same cell lines (Figure 3). A decrease in viability was generally observed at concentrations of 0.1 mM, with HepG2 showing the greatest susceptibility, and 293T and VeroE6 the least susceptibility, to increasing concentrations of Cohex. Thus, Cohex affected each cell line differently, but none of the cells was completely killed, even at the highest Cohex concentration (5 mM). Thus, decreasing GFP could not be entirely attributed to lower cell viability. Cohex had much lower IC50 and CC50 values for A549 and HepG2 cell lines than for VeroE6 or 292T cells. HepG2's CC50 values were also lower than the other three cell lines. As a result, the TI value (Table 2) for HepG2 was the lowest of the four cell lines.

In vivo efficacy of cohex against ebola virus

An efficacy study was conducted in mice to determine whether Cohex would havea therapeutic benefit against Ebola virus infection. Tolerability studies were performed intraperitoneally (i.p.) with injections of Cohexonce a day for 10 days at levels of 0.5, 1, 2, 4, and 8 mg/kg. The weight of the mice did not vary over the 14 days of study (Figure 4) and no adverse events were reported.

To examine the effect of Cohex on Ebola virus infection in mice,female C57BL/6 were treated by i.p.injection with either phosphate-buffered saline (PBS) or Cohex resuspended in PBS 1 h before Ebola Zaire virus exposure (1000 PFU given i.p.), and further treated once a day for 9 more days (Figure 4). The untreated control group survived through day 7, but all succumbed to infection by day 8. The treated groups, 2, 4, and 8 mg/kg Cohex-treated mice, had survival rates of 30, 90, and 80% on day eight, respectively. Without further treatment past day 9, two mice in the 8 mg/kg treatment
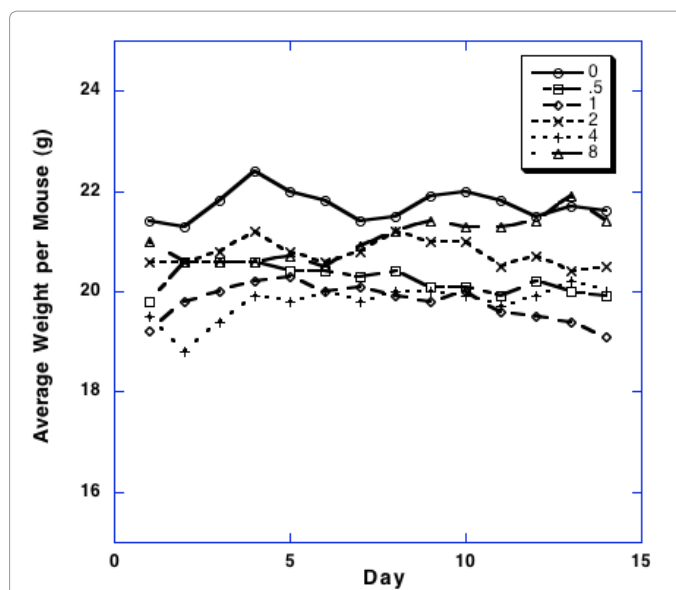

Figure 4: Plot of average weight of mice over 14 days with different concentration of Cohex (0 through 8 mg/kg). Control (0 mg/kg) is PBS.
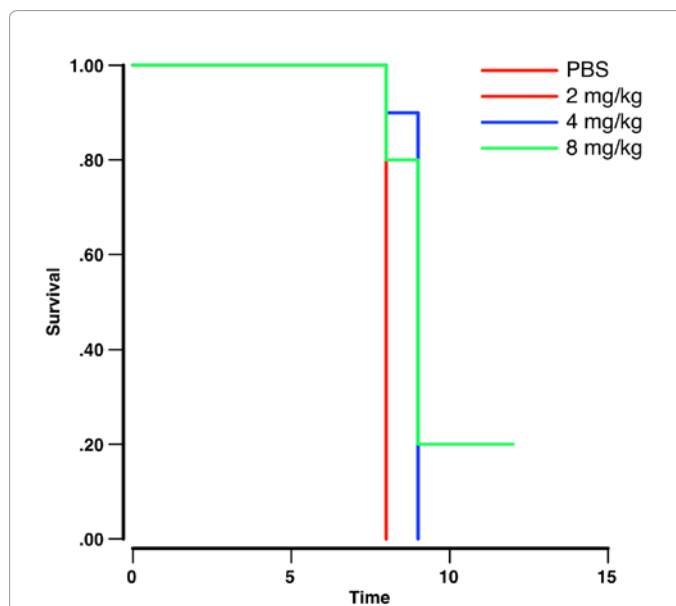

Figure 5: Kaplan-Meier survival plot of mice after Ebola infection (10 in each group), with one control (PBS) and three dose-level groups (2, 4, 8 mg/kg/day, respectively).

group survived through day 12, at which point they were euthanized (Figure 5). In comparing the results of the untreated control mice to those treated with 8 mg/kg of Cohex, it was found to be statistically significant ($p = 0.01$ in a chi-squared test) that the 8 mg/kg treatment improved survival when compared to untreated control mice infected with Ebola virus.

Discussion

Hexamminecobalt (III) chloride is a commercially available compound, with well-known chemical properties, having been extensively studied for its ability to mimic biological magnesium and for its ability to condense nucleotides into unusual toroidal structures.

However, we recently discovered that Cohex also possesses antiviral properties, such as those against SINV and adenovirus [2].

Cohex was assayed for *in vitro* antiviral activity against both a CXCR4-tropic HIV-1 isolate and a CCR5-tropic HIV-1 isolate in PBMCs. CCR5-tropic isolates typically are dominant during the early infection stage of HIV, while CXCR4-tropic isolates are found in the later stages. Cohex showed a concentration-dependent ability to inhibit HIV replication in infected PBMCs. There did not appear to be any difference in the inhibition of HIV replication by the compound based on co-receptor tropism, as Cohex had approximately equal activity against both virus isolates tested. The reported TI for SINV [2] is similar to that for HIV. While both HIV and SINV possess +RNA as their genomic material, they have very different modes of replication. The TI for Cohex in the HIV-1 PBMC assay, while promising, was much less than that of a targeted drug, such as AZT, whose TI was measured simultaneously for this system and was found to be above 1,000 (Table 1b). However, the TI for Cohex indicates the compound has antiviral activity that is separated from its cytotoxicity.

Ebola virus expressing green fluorescent protein (GFP) was derived using reverse genetics. The full-length cDNA clone of Zaire Ebolavirus (Mayinga strain) was generated and rescued containing the foreign reporter gene, eGFP (ZEBOV-GFP). ZEBOV-GFP showed similar viral characteristics to that of the wild-type virus, yet caused infected cells to fluoresce green when infected. ZEBOV-GFP has provided a more sensitive quantitative assay to rapidly screen multiple compounds in shorter period of time than traditional plaque assay methods. In vivo testing for small molecules therapeutics typically starts with tests on small animals, such as a mouse-adapted ZEBOV model. The virus is uniformly lethal to mice and has been used in both vaccine and therapeutic studies as a screening tool.

We examined the effect of Cohex on cells infected with Zaire Ebola virus and found that the compound exhibited antiviral activity in all four cell-lines tested. The results showed a difference in efficacy of the compound for different cells types. The calculated IC50s (Table 2) for A549 and HepG2 cells (0.48 and 0.24 mM, respectively) were lower than those for VeroE6 and 293T cells (1.66 and 1.28 mM, respectively). Differences in IC_{50} for the cells were mirrored in the susceptibility of the cell-lines to exposure to Cohex alone. Both A549 and HepG2 cells were more susceptible (showed a larger decrease in % of viable cells with increasing Cohex concentration) to Cohex than VeroE6 and 293T. These observed variations in responses of different cell lines to

Cells	Virus	IC_{50}	CC_{50}	Therapeutic Index
PBMC	HIV-1 Ba-L	33.8 µM	833 µM	24.7
	HIV-1 NL4-3	28.6 µM		29.1

Table 1a: Summary of Cohex activity against HIV-1 in PBMCs.

Cells	Virus	IC_{50}	CC_{50}	Therapeutic Index
PBMC	HIV-1 Ba-L	0.26 µM	> 1000 µM	>3,885
	HIV-1 NL4-3	0.73 µM		>1,373

Table 1b: Summary of AZT activity against HIV-1 in PBMCs.

	IC_{50} (mM)	CC_{50} (mM)	TI
A549	0.5	4.0	8.3
HepG2	.24	.62	2.6
VeroE6	1.7	> 5.0	> 3.0
293T	1.3	> 5.0	> 3.9

Table 2: Summary of Cohex activity against Zaire Ebola virus for different cell-lines.

the same compound is not surprising in light of past reports, such as Aljofan, et al that reported on cytotoxic differences (CC50) in Vero and 293T responses to test compounds.

After the successful in vitro results, in vivo studies in mice were pursued. The compound tolerability tests demonstrated that up to 8 mg/kg/day of Cohex had no adverse effects on the mice (data not shown). Based on the tolerability results, we dosed Zaire Ebola (1000 PFU)-infected mice at 2, 4, and 8 mg/kg/day of Cohex for 10 days. Some therapeutic effect was observed at 4 mg/kg/day with 90% of the mice surviving until day 8, one day longer than untreated mice. Interestingly, 20% of the 8 mg/kg/day mice survived until day 12 of the experiment—and 3 days after dosing with Cohex was stopped—after which the mice were euthanized. It has been reported that mice can ingest at least 100 ppm Cohex in water for 14 weeks without fatal effect. In addition, Cohex introduced once daily, up to 20 mg/kg of Cohex, to male albino BALB/c mice via i.p. injections for three consecutive days caused no fatalities. Monitoring of liver and kidney markers showed some signs of liver toxicity. Thus, presumably, a higher dosage of Cohex for the infected mice could have been possible. These results are encouraging, suggesting that Cohex may have the potential to be effective in the treatment of Ebola, a virus for which there is no currently approved therapeutics or vaccines.

The viruses for which Cohex shows activity possess very different replication pathways. ZEBOV_GFP is a(-) stranded RNA virus, adenovirus possesses dsDNA and, while both SINV and HIV are (+) strand RNA viruses, their replication pathways are very different, with the HIV making use of the cell nucleus, while the SINV replication life-cycle takes place entirely in the cytoplasm. Taken together, the results point to Cohex as a potential new broad-spectrum antiviral drug and suggest that this drug may have utility for other high-consequence pathogens beyond ZEBOV. If successful, this would provide the first broad-spectrum intervention for biothreat agents and could greatly facilitate the ability to economically stockpile and respond in the face of a public health or national emergency.

Cohex is a well-known complex that was previously used as a mimic of biological magnesium, e.g., as a substitute for hydrated magnesium ion as a co-factor in RNA biochemistry [2,3]. We have already shown that Cohex can inhibit translation in a cell-free assay [5], most likely by binding to mRNA, while other groups have thoroughly characterized the ability of Cohex to bind tightly to DNA (and condense it at low salt concentrations) [3,4]. These properties, and possibly others, suggest potential mechanisms for Cohex's antiviral activities. However, a single mechanism has not been demonstrated yet. Indeed, due to its broad-spectrum nature, it is possible that Cohex may be affecting viral infection through more than one pathway in the virus-cell lifecycles. This potential for affecting multiple pathways could also make it harder for microorganisms to develop resistance against Cohex. Thus, in determining its potential utility, one must balance the multiple microorganisms Cohex may suppress against a desire for very low IC_{50}. Issues such as this, e.g., selectivity versus multiple-targeting, were previously recognized and discussed.

Conclusion

Whatare the potential uses of a broad-spectrum compound that requires a higher dosage and may not have a TI as high as more specific drugs? The general advantages of such a broad-spectrum drug, like Cohex, are its low-cost, stability, and, more importantly, its ability to attack multiple microorganisms, have efficacy against

novel or emerging diseases, and potentially serve as a breakthrough intervention for agents such as ZEBOV for which previous attempts have been unsuccessful or of limited efficacy. For viruses, such as HIV, where drugs with very high TI already exist, Cohex may be useful in a combination drug therapy regime. There are several advantages to doing this: as a broad-spectrum compound, Cohex can be effective against opportunistic infections by other microorganisms; the possible multiple pathways by which Cohex affect viral replication may make it a natural candidate a part of a combination therapy regime; and, finally, because of the low-cost of Cohex, it become seasier to control costs for combinations using Cohex.

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