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Antiviral properties of cobalt(III)-complexes

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Abstract—We have investigated the potential antiviral activity of three cobalt(III) compounds. Two compounds, Co(III)–cyclen–methylbenzoic acid and its methyl ester derivative, are based on the macrocyclic chelator, cyclen, and were synthesized in our laboratory. Both compounds have been shown to bind tightly to nucleic acids and to hydrolyze phosphodiester bonds. However, neither compound exhibited any significant antiviral activity in an in vitro model of Sindbis virus replication. In contrast, a third compound, Co(III)hexammine, significantly inhibited Sindbis virus replication in baby hamster kidney (BHK) cells in a dose- and time-dependent manner. In plaque assays, the incubation of Co(III)hexammine with Sindbis virus resulted in a dose-dependent decrease in virus replication when measured at both 24 and 48-h post-infection. Over the concentration range of 0–5 mM Co(III)hexammine, the IC_{50} for the inhibition of viral replication was determined to be 0.10 ± 0.04 mM at 48 h. Additionally, when BHK cell monolayers were pretreated with Co(III)hexammine for 6 h prior to Sindbis infection, optimal cellular morphology and plasma membrane integrity were observed at 0.6–1.2 mM Co(III)hexammine. Analysis by flow cytometry confirmed that Co(III)hexammine mediated a concomitant dose-dependent increase in BHK cell viability and a decrease in the percentage of Sindbis virus-infected cells ($IC_{50} = 0.13 \pm 0.04$ mM). Our findings demonstrate for the first time that Co(III)hexammine possesses potent antiviral activity. We discuss our findings within the context of the ability to further functionalize Co(III)hexammine to render it a highly specific antiviral therapeutic reagent.
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1. Introduction

The identification of new antiviral drugs is a challenging endeavor that aims to strike a balance between potent antiviral activity and minimal toxicity against host tissues. We have been developing novel anti-transcription and anti-translation compounds based on small cobalt (III)-chelator complexes that possess a high positive charge-density. We have previously shown that cobalt (III), when complexed with functionalized derivatives of the tetraazamacrocyle chelator, cyclen, efficiently hydrolyzes the phosphodiester bonds in DNA[1] and RNA[2] and also inhibits RNA translation in a cell-free system via its high affinity binding interaction with the RNA template.[2] We have also demonstrated that these cobalt(III)-cyclen systems can be covalently coupled to solid supports (e.g., agarose beads) via carbodiimide chemistry.[3] Hence, these cobalt(III) systems can potentially be attached to oligonucleotide probes to produce sequence-specific gene silencing reagents for therapeutic and functional screening applications.

Previous reports have described the use of cobalt(III)-based systems for antiviral applications. The most effective among these systems is the series of cobalt compounds known as the CTC series of cobalt complexes, which has been shown to possess anti-inflammatory[4] and antiviral activity.[5] Several CTC complexes have moderate in vitro and in vivo activity against herpes simplex virus types 1 and 2, varicella-zoster virus, cytomegalovirus, and Epstein–Barr virus.[4–6] Interestingly, the mode of action of these novel compounds appears to differ from that of currently available antiviral nucleoside analogs and protease inhibitors. For example, it has been shown that several CTC compounds bind irreversibly to and inhibit Sp1, a zinc finger-containing transcription factor,[7] suggesting a role for the cobalt(III) complexes in disrupting the function of zinc finger-containing proteins. More recently, an alternative mechanism of inhibition has been postulated wherein the

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CTC compounds inhibit the initial fusion event between the virus and the target cell.[8]

Here, we present a study on the antiviral effects of three candidate cobalt(III) compounds on Sindbis virus replication in baby hamster kidney (BHK) cells. Sindbis virus is an alphavirus containing a positive, single-stranded RNA genome and it was specifically chosen for these studies as it replicates entirely in the cytoplasm and is useful as a model for Venezuelan equine encephalitis virus, a potential biothreat agent. Upon binding to target cells via high affinity interactions with the glycosaminoglycan heparan sulfate, the virion is endocytosed.[9] After endosomal acidification, the viral RNA is released into the cytoplasm where it serves as a template for the synthesis of viral proteins. Sindbis virus induces apoptosis-mediated cell death characterized by the rounding and detachment of infected cells from the tissue culture substrate.

Two of the compounds investigated in this study, Co(III)–cyclen–methylbenzoic acid (Co(III)cycmba) and its methyl ester derivative (Co(III)cycmmb), are hydrolytic cyclen complexes in which the chelator ligands occupy four of the six coordination sites on the Co(III) ion, leaving two equatorial *cis* positions available for nucleotide binding and hydrolysis. The third compound, Co(III)hexammine (Co(III)hex), is a commercially available cobalt(III) complex that does not possess free cobalt(III) coordination sites and does not hydrolyze oligonucleotides. These compounds were specifically chosen to assess the relative importance of nucleic acid hydrolysis, nucleic acid coordination, and net metal-complex charge in mediating antiviral activity. While the two Co(III)–cyclen compounds displayed minimal antiviral activity, Co(III)hex exhibited a potent dose-dependent inhibition of Sindbis virus replication.

The work presented herein identifies Co(III)hex as a new cobalt(III)-based compound possessing potent antiviral activity. This compound is structurally quite different from the CTC series of cobalt(III)-complexes. For example, CTC-96, the most effective compound of the CTC series, has two labile axial ligands in the form of 2-methylimidazoles. CTC-96 has been shown to inhibit the infectivity of herpes simplex virus types 1 and 2 by blocking virus entry at the point of membrane fusion, independent of the cellular receptors present.[8] While substitution of the labile imidazole residues by target proteins is thought to play an important role in CTC-96's antiviral activity, the exact molecular mechanism by which CTC-96 prevents membrane fusion remains unclear. In the Co(III)hex complex, by contrast, the Co(III) ion forms a rather stable complex with its six ammonia ligands.[10] This complex mediates a simultaneous dose-dependent increase in BHK cell viability and a decrease in Sindbis virus protein synthesis during viral replication. We discuss our findings within the context of our ability to further functionalize Co(III)hex with targeting biomolecules in order to render it a highly specific antiviral agent.

2. Results

2.1. Cobalt(III) compounds

In this study, we examined the potential antiviral effects of three candidate cobalt(III) compounds in an in vitro model of Sindbis virus replication. Two of the compounds (Co(III)–cyclenmba (Fig. 1a) and Co(III)–cyclenmmb (Fig. 1b), referred to herein as Co(III)cycmba and Co(III)cycmmb, respectively) are based on the macrocyclic chelator, cyclen. The synthesis of these compounds has been described in detail elsewhere. The reader is referred to Knight et al.[1] for a description of the synthesis of Co(III)cycmba and to Deschamps et al.[11] for details on the Co(III)cycmmb complex. The third compound, Co(III)hex (Fig. 1c), was purchased commercially and used as supplied. For both

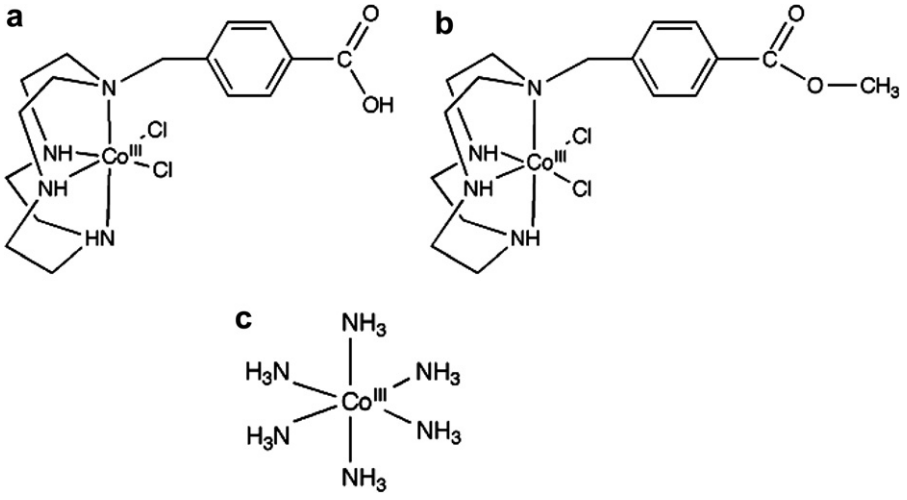

Figure 1. Structures of cobalt(III) compounds used in this study. Two cobalt(III) compounds based on the macrocyclic chelator, cyclen, are shown: (a) Co(III)cycmba and (b) Co(III)cycmmb. The coordination geometry about the Co(III) ion is a distorted octahedral and the chloride ligands are oriented in a *cis* conformation. (c) Co(III)hex bears six ammonia ligands about the Co(III) ion in an octahedral geometry.

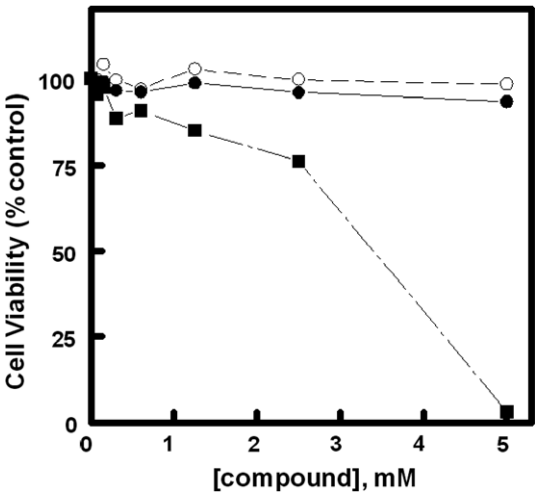

Figure 2. Cytotoxicity of cobalt(III) compounds. Cytotoxicity was assessed using a colorimetric cell proliferation assay. BHK cells were incubated with each compound for 72 h at the concentrations indicated prior to assaying for viability. Symbols correspond to Co(III)cycmba (open circle), Co(III)cycmmb (closed circle), and Co(III)hex (square). Each data point represents means ± S.D. of four replicate measurements. In all cases, the error bars are smaller than the width of the data points.

Co(III)cycmmb and Co(III)hex, the net charge for the complexes at neutral pH is 3^+, while for Co(III)cycmba, the net charge is 2^+.

2.2. Cytotoxicity of cobalt(III) compounds

We first assessed the potential cytotoxicity of the candidate cobalt(III) compounds by monitoring their ability to inhibit the proliferation of BHK cells. Using a colorimetric tetrazolium-based cell proliferation assay, the toxicity of each compound was determined after 72 h of continuous incubation with the cells. As shown in Figure 2, Co(III)cycmba and Co(III)cycmmb elicited minimal toxicity; greater than 95% cell viability was observed at the highest concentration tested (5 mM). Co(III)hex exhibited no significant toxicity at concentrations below 0.3 mM. At 2.5 mM, Co(III)hex mediated approximately 80% cell viability while the maximum toxicity was observed at 5 mM.

2.3. Antiviral activity of cobalt(III) compounds

2.3.1. Inhibition of Sindbis virus replication in plaque assays. Plaque assays were performed to determine the minimal inhibitory concentrations of each cobalt(III) compound in an in vitro model of Sindbis virus replication. BHK cells were simultaneously incubated with Sindbis virus in the absence or presence of a dose range of each cobalt(III) compound for either 24 or 48 h prior to assaying for the number of Sindbis virus pfu. Figures 3a and b show the number of virus pfu (expressed as $\log_{10}$ pfu) at 24 and 48-h post-infection (p.i.), respectively. At 24-h p.i., a slight increase in viral pfu relative to control was noted for all three compounds at concentrations up to 0.3 mM. At concentrations greater than 0.3 mM, however, Co(III)hex was the only compound

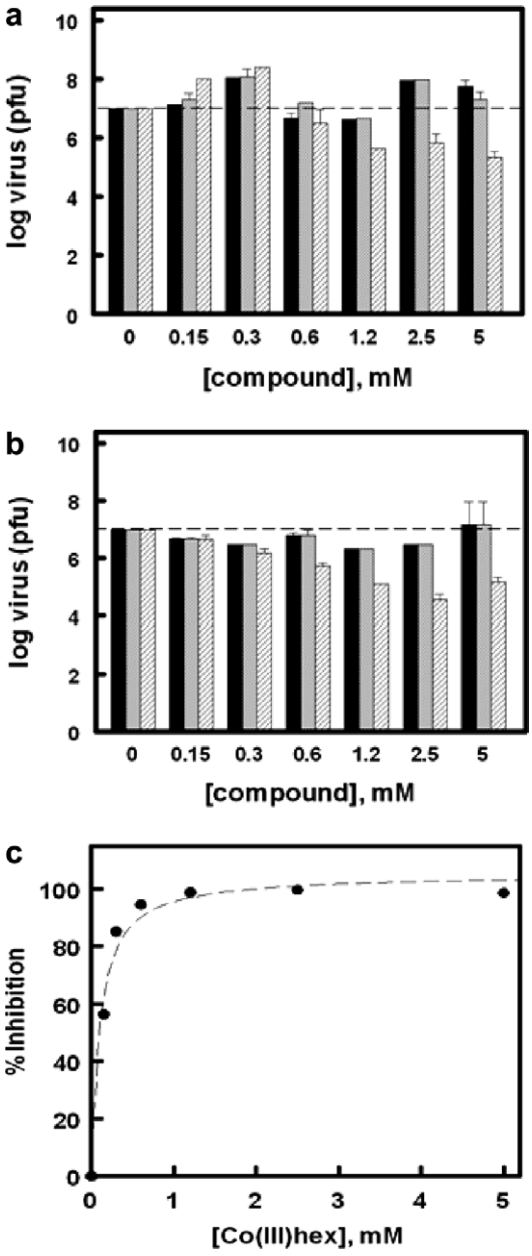

Figure 3. Effect of cobalt(III) compounds on Sindbis virus (SV) replication in BHK cells. Plaque assays were performed in which BHK cells were infected with SV in the presence of the cobalt(III) compounds. At (a) 24 h and (b) 48-h p.i., the number of SV pfu was determined. Pfu are expressed in $\log_{10}$ units. Symbols correspond to Co(III)cycmba (black bar), Co(III)cycmmb (gray bar), and Co(III)hex (hatched bar). (c) The degree of inhibition of SV plaque formation is plotted as a function of Co(III)Hex concentration for the 48-h data set. The IC_{50} for inhibition was determined to be 0.10 ± 0.04 mM Co(III)Hex using a one-site dose response logistic curve fit function.

which produced a corresponding decrease in viral pfu with the most pronounced effect observed at concentrations of 1.25 mM and greater. In these instances, the decrease in viral pfu relative to the untreated control was greater than 10-fold (one $\log_{10}$ unit). Over this same concentration range, the greatest decrease in pfu observed for either Co(III)cycmba or Co(III)cycmmb was 0.38 $\log_{10}$ units. At 48-h p.i., these same general trends were observed. Neither of the cyclen compounds elicited

any significant reduction in pfu while the presence of increasing concentrations of Co(III)hex resulted in significantly larger reductions in viral pfu. The maximal response was noted at 2.5 mM (a decrease of ~2.5 $\log_{10}$ units in viral pfu) and decreased slightly to a reduction of 1.8 $\log_{10}$ units in viral pfu at 5 mM. At 48-h p.i., the 50% inhibitory concentration (IC_{50}) for Co(III)hex inhibition of Sindbis virus plaque formation was determined to be 0.10 ± 0.04 mM (Fig. 3c). Although in vitro findings do not always correlate with in vivo results, Co(III)hex-mediated reductions in viral pfu of these magnitudes in vitro suggest in vivo significance. Previous studies have shown that a 10-fold decrease (one $\log_{10}$ unit) in viral load in murine brains correlates with survival of infected animals.[12–14]

2.3.2. Cellular morphology and plasma membrane integrity during Sindbis virus replication. Given the toxicological profile of Co(III)hex, it was likely that the decrease in viral pfu observed at concentrations above 2.5 mM in the plaque assays was attributable to a combination of antiviral activity and Co(III)hex-induced cytotoxicity. To investigate this, we examined both the cellular morphology and the plasma membrane integrity of BHK cells as a measure of cell viability during Sindbis virus infection in the presence of Co(III)hex. Figure 4A shows the resulting cellular morphology of BHK cells upon 6-h pretreatment with Co(III)hex followed by a 48-h period of infection with Sindbis virus. In the absence of virus or Co(III)hex, BHK cells achieved a completely confluent, dense monolayer after 48 h of culture (panel **a**). The cells remained attached to the tissue culture substrate and they adopted an elongated, epithelial cell-like morphology. In contrast, panel **b** shows BHK cells 48-h p.i. in the absence of Co(III)hex. The majority of the cells was detached from the plate and was rounded; characteristic of cells undergoing death by apoptosis induced by Sindbis infection. Panels **c** through **g**, however, depict Sindbis virus-infected cells that, in the presence of an increasing amount of Co(III)hex, adopted morphologies that were intermediate in nature. In the presence of Co(III)hex, cells were elongated and remained attached

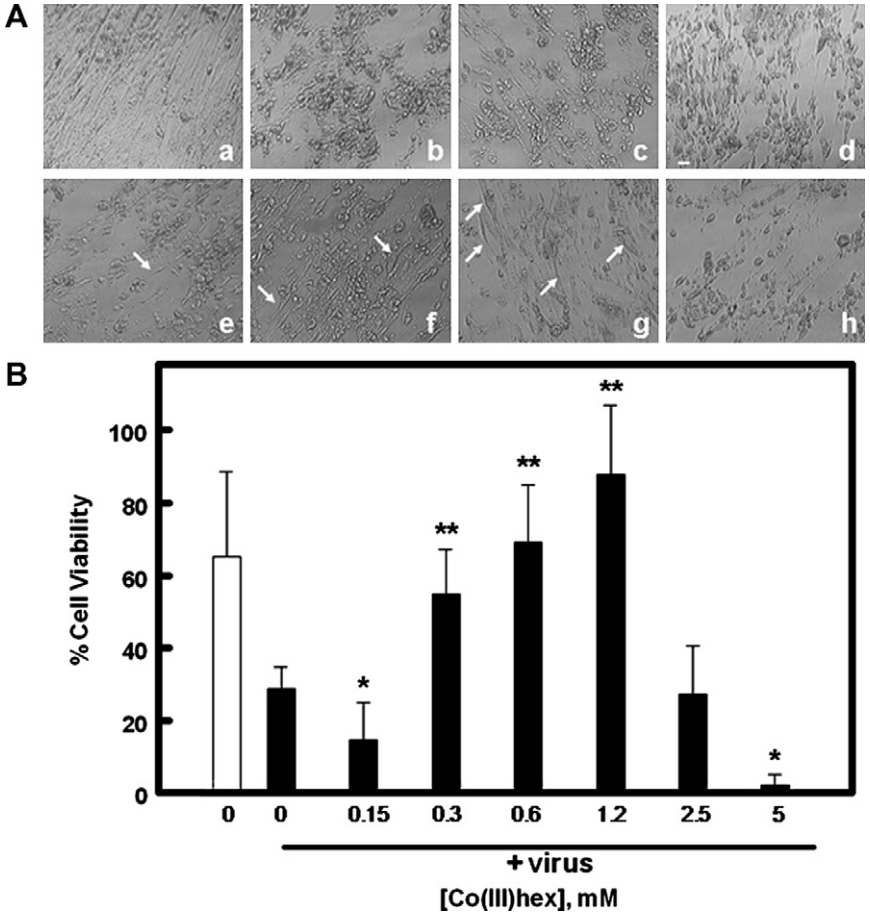

Figure 4. Co(III)hex promotes viable cell morphology and plasma membrane integrity in BHK cells during SV infection. (A) Cellular morphology of BHK cells was examined by Trypan blue staining and light microscopy at 48-h p.i. following 6 h of pretreatment with Co(III)Hex. Panel **a** shows cells in the absence of both SV and Co(III)hex. In panels **b** through **h**, cells were infected with SV following 6-h pretreatment with Co(III)hex at the following concentrations: (b) 0 mM, (c) 0.15 mM, (d) 0.3 mM, (e) 0.6 mM, (f) 1.2 mM, (g) 2.5 mM, and (h) 5 mM. The arrows indicate representative cells that have adopted an elongated and adherent morphology in the presence of SV and Co(III)hex. The scale bar in panel **d** is 5 μm. (B) Plasma membrane integrity of BHK cells was determined by Trypan blue exclusion assay at 48-h p.i. following 6 h pretreatment with Co(III)hex at the concentrations indicated. The asterisks indicate levels of significance relative to the infected, untreated control (as determined by a two-tailed Student's *t*-test): $^{*}(p < 0.05)$; $^{**}(p < 0.005)$. Open bar corresponds to BHK cells in the absence of SV; solid bar denotes BHK cells in the presence of SV.

to the substrate, adopting a non-infected cell phenotype. This effect was most pronounced at concentrations of 0.6 and 1.2 mM Co(III)hex and was observed to decrease at 2.5 mM. At 5 mM Co(III)hex, the majority of cells once again adopted a rounded and detached morphology, presumably due to the combination of virus-induced cell death and Co(III)hex cytotoxicity (panel **h**).

The assessment of BHK plasma membrane integrity as a function of Co(III)hex concentration during Sindbis virus infection mirrored the cellular morphology results. In the absence of both Co(III)hex and virus, BHK viability was approximately 65% after a 48-h culture period while the viability decreased to 30% as a result of Sindbis virus infection in the absence of the compound (Fig. 4B). However, an increase in Co(III)hex concentration was coupled with a corresponding increase in BHK membrane integrity, reaching a maximum of 84% at 1.2 mM Co(III)hex. The data demonstrated an overall protective effect of Co(III)hex against Sindbis-induced cell death that reflected our observations in the morphology studies.

2.3.3. Quantification of Sindbis virus replication by flow cytometry. To further validate our plaque assay and cell viability results, we quantified the protective effect of Co(III)hex against Sindbis virus replication using flow cytometry, a more sensitive fluorometric assay. In these assays, Sindbis virus replication was assessed by monitoring viral structural protein synthesis using a recombinant Sindbis virus construct. In this construct, the gene encoding enhanced green fluorescent protein (EGFP) was placed under the control of the same promoter sequence that drives transcription of viral structural proteins. Thus, upon Sindbis virus replication, virally infected cells produce intracellular EGFP at levels that are proportional to the level of viral structural proteins.[15] BHK cells were pretreated with Co(III)hex for 6 h prior to infection with Sindbis virus. After 48-h p.i., both the percentage of Sindbis-infected cells (as evidenced by EGFP fluorescence) and the percentage of viable cells (as determined by the exclusion of propidium iodide (PI)) were determined as a function of Co(III)hex concentration. Representative raw data plots are shown in Figure 5A. Panels **a** and **b** show the distribution of EGFP-positive cells versus forward scatter (FSC) for Sindbis-infected cells (panel **a**, region R1) or Sindbis-infected cells treated with 0.15 mM Co(III)Hex (panel **b**, region R1). In the presence of the compound, a distinct decrease in the percentage of EGFP-positive (i.e., Sindbis-infected) cells was apparent. Quantification of cell viability revealed that during Sindbis infection, relative to infected cells that were not treated with the compound (panel **c**, region R2), cells treated with 0.15 mM Co(III)hex exhibited a significantly lower percentage of PI-positive (non-viable) cells (panel **d**, region R2). Data demonstrating the dose-dependent nature of the Co(III)hex-mediated increase in cell viability and inhibition of EGFP expression (i.e. inhibition of SV replication) as a function of Co(III)hex concentration are presented in Figure 5B and C, respectively. When the data in Figure 5C were fitted to a standard dose–response curve, an IC_{50} of 0.13 ± 0.04 mM was determined. This value agrees well with the IC_{50} value determined by the plaque assay (0.10 ± 0.04 mM).

3. Discussion

Over the last decade, cobalt-containing compounds have begun to show considerable promise as antiviral agents, exhibiting both anti-inflammatory and antiviral activities. Specifically, the CTC series of cobalt chelates has demonstrated significant activity in vitro and in vivo against a number of viruses.[4–6] CTC-96 has been shown to be the most effective and least cytotoxic of these cobalt compounds, with particular efficacy against herpes simplex viruses 1 and 2[5,6] and adenovirus type 5.[16] Schwartz et al.[8] recently reported that CTC-96 targets the initial fusion event between the virus and the cell and also inhibits cell-to-cell spread and syncytium formation. While the exact molecular mechanism of this activity remains unclear, the functionality of CTC-96 is believed to be dependent on the labile 2-methylimidazole ligands which can be exchanged for target proteins. This could allow for the induction of conformational changes in virus glycoproteins and/or cellular receptors required for viral entry as CTC compounds have been shown to selectively unfold proteins in vitro.[17]

In the present study, our goal was to perform a comparative analysis of the potential antiviral activity of several other cobalt-containing compounds. Two compounds, Co(III)-cycmba and Co(III)-cycmmb, are based on the macrocyclic chelator, cyclen, and have been synthesized in our laboratory. In these complexes, the tetraazamacrocyle ligand adopts a distorted octahedron about the Co(III) ion while two labile chloride ligands are oriented in a *cis* configuration.[11] This is the ideal orientation to mediate phosphodiester bond cleavage in aqueous solution.[18] Indeed, our previous work has demonstrated that these complexes bind tightly to and efficiently hydrolyze the phosphodiester linkages in both DNA[1] and RNA.[2] A third compound, Co(III)hexammine (Co(III)hex), is a commercially available complex in which six ammonia ligands are arranged in an octahedral geometry about the Co(III) ion. In contrast to the cyclen complexes, in which the labile chloride ligands are readily exchanged for phosphate groups during nucleic acid hydrolysis, the ammonia ligands on Co(III)hex are inert toward ligand exchange.[10] Despite the fact that Co(III)hex lacks the ability to coordinate to and hydrolyze phosphodiester bonds, it does possess the ability to hydrogen bond with the nitrogenous bases of nucleotides and the phosphate backbone of DNA.[10] The compounds examined in this study, therefore, were selected to ascertain the roles of metal ion geometry, nucleic acid coordination, and nucleic acid hydrolysis in mediating antiviral activity.

Of the three compounds investigated herein, Co(III)hex was the only cobalt complex that exhibited significant antiviral activity against Sindbis virus in an in vitro model of viral replication. Given the presence of Sindbis virus RNA in the cytoplasm and the high efficiency with

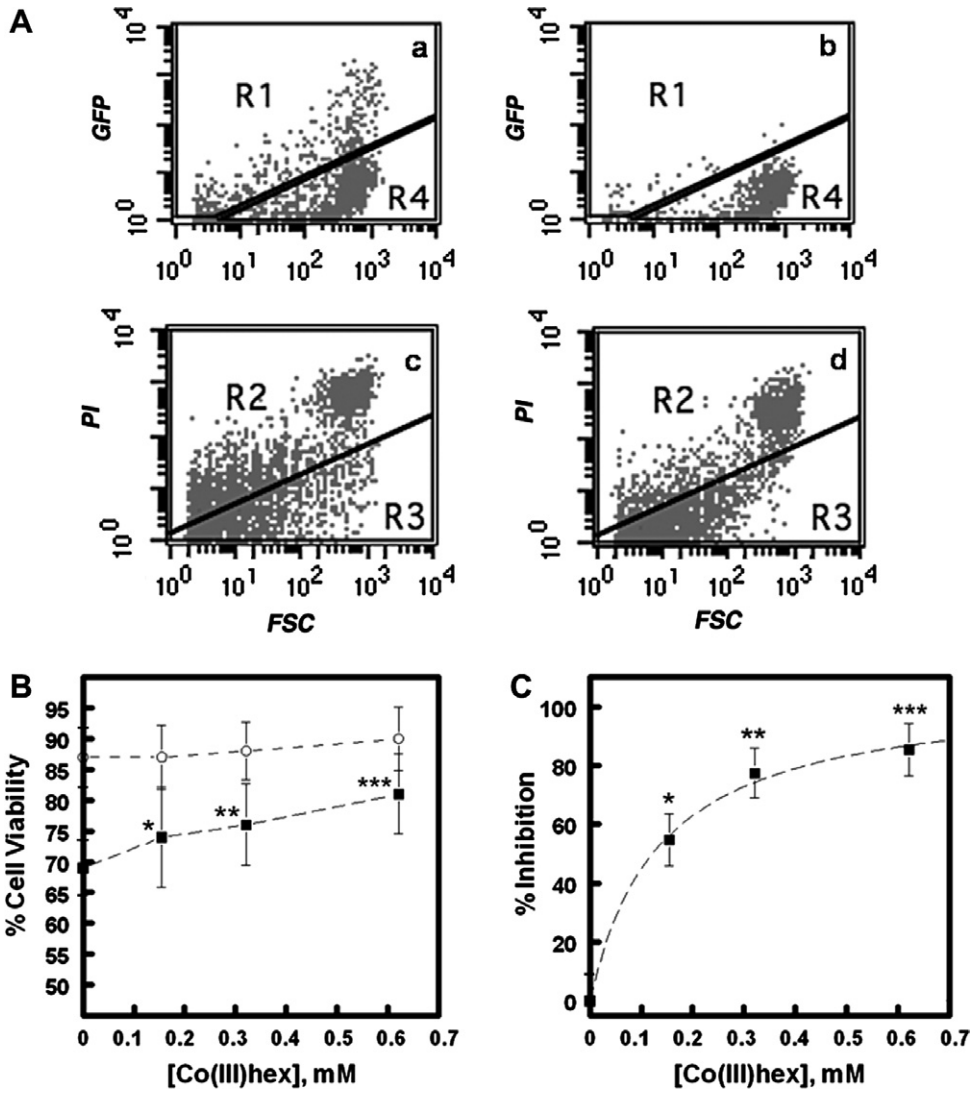

Figure 5. Simultaneous analysis of BHK cell viability and SV protein synthesis during SV infection. (A) BHK cells were pretreated with Co(III)hex for 6 h prior to infection with SV. At 48-h p.i., cells were analyzed by flow cytometry. Representative flow cytometry data of SV-infected BHK cells (**a**) in the absence of Co(III)hex or (**b**) in the presence of 0.15 mM Co(III)hex. The area marked 'R1' corresponds to EGFP-positive (SV-infected) cells. Representative flow cytometry data for the viability of SV-infected BHK cells (**c**) in the absence of Co(III)hex or (**d**) in the presence of 0.15 mM Co(III)hex. The area marked 'R2' corresponds to propidium iodide (PI)-positive (non-viable) cells. In all cases, the *x*-axis corresponds to forward scatter (FSC). (B) Data are shown for the dose-dependent increase in cell viability in SV-infected cells (solid square) as a function of Co(III)hex concentration relative to control, uninfected cells (open circle). (C) Data are shown for the dose-dependent inhibition of EGFP expression in SV-infected cells as a function of Co(III)hex concentration. The IC_{50} for inhibition was determined to be 0.13 ± 0.04 mM Co(III)hex using a one-site dose response logistic curve fit function. In panels (B) and (C), asterisks on the plots for the SV-infected cells correspond to levels of significance relative to the untreated infected control (as determined by a two-tailed Student's *t*-test): $^{*}(p < 0.05)$; $^{**}(p < 0.005)$; $^{***}(p < 0.001)$.

which the Co(III)–cyclen complexes hydrolyze nucleic acids, we expected that they would inhibit viral replication. Yet, neither Co(III)–cyclen complex displayed any appreciable antiviral activity. In our previous studies, Co(III)–cyclenmmb efficiently hydrolyzed an RNA template in a rabbit reticulocyte lysate-based translation system.[2] It is unlikely, therefore, that the lack of antiviral activity by the cyclen complexes is due to their inactivation by cytoplasmic proteins, but is due rather to their inability to gain entry into the cytoplasm. Future investigations will be aimed at assessing the antiviral activity of Co(III)–cyclen complexes that are introduced into cells via transfection.

Despite its inability to hydrolyze phosphodiester bonds, Co(III)hex exhibited significant antiviral activity as evidenced by reduced pfu counts in plaque assays and reduced synthesis of viral structural proteins as evidenced by EGFP reporter assays. These results suggest that Co(III)hex inhibits viral replication via the inhibition of viral structural protein synthesis or some point further upstream in the viral replication cycle. For example, just as CTC-96 acts at the point of viral entry, it is quite possible that the high positive charge density of Co(III)hex allows it to disrupt the interaction of Sindbis virus glycoproteins with highly negatively charged, poly-sulfonated heparan sulfate receptors. Still

another possible mechanism involves the ability of Co(III)hex to compete with hydrated Mg(II) and interfere with virus assembly.[19] Further investigation is required to elucidate the exact impact of Co(III)hex on the various stages of the Sindbis replication cycle.

4. Conclusion

This report describes the comparative analysis of the antiviral activity of several cobalt(III)-containing compounds and it identifies Co(III)hex as a new cobalt(III) complex with potent antiviral activity. While the precise mechanism by which Co(III)hex exerts its anti-Sindbis virus effects remains to be determined, it is clear that the compound inhibits the Sindbis virus replication cycle at or before the point of viral structural protein synthesis. Interestingly, recent work in our laboratory has revealed the ability to synthesize functionalized Co(III)hex derivatives in which one of the ammonia ligands is substituted with a functional group that allows for coupling to biomolecules (e.g., oligonucleotides, peptides). Once the exact point of Co(III)hex's action is identified there is now the exciting possibility of conjugating Co(III)hex to specific targeting moieties, resulting in a highly specific and effective antiviral therapeutic reagent. In addition to identifying a new compound with antiviral activity, this report provides further evidence of the utility of cobalt complexes in the development of novel antiviral therapeutic agents.

5. Experimental

5.1. Materials

Hexammine cobalt(III) chloride, neutral red stain, and antibiotic/antimycotic were purchased from Sigma–Aldrich (St. Louis, MO). Co(III)–cyclenmba and Co(III)–cyclenmmb were synthesized as described in Knight et al.[1] and Deschamps et al.[11], respectively. Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (DPBS), fetal-bovine serum, and baby hamster kidney cells were obtained from ATCC (Manassas, VA). Minimal Essential Medium (MEM), virus production grade serum-free medium (VP-SFM), and Bactoagar were purchased from Invitrogen (Carlsbad, CA). All other reagents were obtained as noted in the text.

5.2. Cell culture

Baby hamster kidney (BHK) cells were cultured as exponentially growing subconfluent monolayers in complete growth medium [DMEM supplemented with 1% (v/v) antibiotic/antimycotic and 10% (v/v) heat-inactivated fetal bovine serum (FBS)]. Cells were grown in either T25 or T75 flasks (Corning) and incubated at 37 °C under 5% CO_2 atmosphere. A subculture was performed every 3–4 days.

5.3. Cytotoxicity of cobalt(III) compounds

The in vitro toxicity of the cobalt(III) compounds was determined using the CellTiter96® Cell Proliferation Assay (Promega), a quantitative colorimetric assay based upon the conversion of a tetrazolium salt substrate into a blue formazan product by viable cells. At the assay endpoint, the absorbance at 570 nm is directly proportional to the number of viable cells. BHK cells were seeded into the wells of a 96-well tissue culture microtiter plate (2×10^4 cells/well) and cultured overnight at 37 °C in a humidified atmosphere containing 5% CO_2. The next day, the compounds were serially diluted into tissue culture media and incubated with the cells for 72 h. For all compounds, triplicate wells were included for each concentration. At the end of the 72-h culture period, 15 μL of tetrazolium substrate was added to each well and the plate was returned to the incubator for 4 h to allow viable cells to convert the substrate into the formazan product. Subsequently, 100 μL of solubilization solution was added to each well and the plate was incubated overnight. The absorbance values for each concentration were divided by the absorbance values from wells with cells cultured in the absence of compound (control wells corresponding to maximum color formation). To control for any absorbance due to the compounds themselves, a second set of control wells containing no cells was included for each compound concentration.

5.4. Antiviral activity of cobalt(III) compounds

5.4.1. Sindbis virus stocks. Recombinant Sindbis virus (SV) (633-EGFP strain) was generously provided by Dr. Diane Griffin (Johns Hopkins University). In this construct, the gene encoding enhanced green fluorescent protein (EGFP) is placed under the control of the same promoter sequence that controls the transcription of the viral structural proteins. Thus, upon replication, virally infected cells produce soluble EGFP at levels that are proportional to the level of viral structural proteins.[15] The virus seed stock was expanded on BHK cells under serum-free conditions. BHK cells were grown in T-150 tissue culture flasks until ~90% confluent. Subsequently, the cells were washed twice with Dulbecco's PBS and infected with SV at a multiplicity of infection (moi) of five plaque forming units (pfu) per cell in 2 ml of serum-free VP-SFM media. After 1 h of incubation at 37 °C, an additional 13 ml of VP-SFM was added. The next day, the cells were observed under light microscopy for signs of cytopathic effects. The presence of EGFP expression in infected cells was confirmed via fluorescence microscopy. Cellular debris was centrifuged and the supernatant was collected, aliquoted, and stored at −80 °C. The majority of cells (>50%) were positive for EGFP fluorescence.

5.4.2. Plaque assay for antiviral activity. BHK cells (1×10^5 cells) were seeded to the wells of a 24-well plate and grown overnight to a confluent monolayer. The next day, the cells were infected with SV at an moi of 5 pfu/cell in DMEM containing 2% FBS in the presence of increasing concentrations of the cobalt(III) compounds. Infection was allowed to proceed for either 24 or 48 h, at which time the supernatants were collected and stored at −80 °C until further use. For virus quantification, the supernatants were thawed and serially diluted in 1% FBS DMEM. The diluted virus was used to infect monolayers of BHK cells grown in 6-well plates. An aliquot

(200 μL) of virus from each selected dilution was incubated with BHK monolayers for 1 h at 37 °C with rocking of plates every 10 min to prevent drying of the monolayers. After 1 h, the cells were overlayed with warm melted 1.2% Bacto agar in water mixed with an equal volume of 2× MEM. The overlay media were allowed to solidify at room temperature. The plates were then incubated at 37 °C for 48 h to allow for plaque formation. Two days later, the cells were stained with neutral red (staining solution per well is 0.5 ml of 2× MEM, 0.5-ml distilled water, and 0.11 ml of 0.33% neutral red (3.3 g/L in D-PBS)). After 1–2 h of staining, the remaining dye solution was aspirated off and plaques were observed as clear foci within the cell monolayer. The number of pfu per well was multiplied by the dilution factor to determine the concentration of pfu in the original supernatant.

5.4.3. Assessment of cell viability during Sindbis virus replication. The viability of BHK cells during SV infection was determined as a function of Co(III)hex concentration by monitoring cellular morphology (via light microscopy) and plasma membrane integrity (using a Trypan blue dye-exclusion assay). BHK cells were seeded to the wells of a 96-well plate (2×10^4 cells/well) and cultured overnight in complete growth medium. The cells were then incubated with increasing concentrations of Co(III)hex for 6 h prior to the addition of SV (1×10^5 pfu/well). After a 48-h infection period, the cells were resuspended and an aliquot of cells (10 μL) was mixed with 90 μL of 0.2% Trypan blue. The number of viable cells was determined by counting with a hemocytometer. A corresponding set of wells was prepared exclusively for observation under light microscopy to assess cellular morphology.

5.4.4. Assessment of Sindbis virus replication by flow cytometry. BHK cells (1×10^5) were plated into the wells of 24-well plates in 1 mL of complete media and incubated with increasing concentrations of Co(III)hex for 6 h prior to infection with SV at an moi of 5 pfu per cell. The infection was allowed to proceed for 48 h. In preparation for analysis by flow cytometry, cells from each well were removed and pelleted, washed with PBS, and resuspended in 500 μL of PBS. One half of each sample was analyzed directly to determine the percentage of EGFP-positive cells (percent infected cells). The other half of each sample was incubated with 5 μM propidium iodide for 1 min prior to the determination of the percentage of non-viable cells. In all cases, analysis was performed on 1×10^4 cells.

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