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Potent inhibition of respiratory syncytial virus by combination treatment with 2–5A antisense and ribavirin

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Abstract

Respiratory syncytial virus (RSV) is a major cause of lower respiratory diseases in infants, young children, and the elderly. Ribavirin, the only currently approved drug for the treatment of RSV infections in the U.S., requires high doses to be effective. Therefore, it has only a limited clinical efficacy in the treatment of RSV infections. It has been shown that a cellular ribonuclease, RNase L, can be recruited by 2′–5′ linked tetra-adenylates (2–5A) attached to an antisense sequence complementary to the RSV genome to specifically cleave RSV genomic RNA. Here we confirm the antiviral activity of the lead 2–5A antisense compound, RBI034, by using several different viral assays. We demonstrate that RBI034 is more efficient than antisense lacking 2–5A or small interfering dsRNA (siRNA) in inhibiting RSV replication. Although the best antiviral activity of RBI034 was observed with co-treatment of RSV infection, it remained effective even when administrated 24 h after the initiation of infection. Interestingly, the activity of RBI034 can be further enhanced by a combination treatment with ribavirin. At suboptimal concentrations, neither ribavirin nor RBI034 was effective in suppressing RSV replication. However, a combination of these two drugs at the same suboptimal concentrations showed a potent inhibitory activity. The potent reduction of RSV replication by combination treatment was also confirmed in primary human airway epithelial cells. Therefore, a combination therapy of the 2–5A antisense compound RBI034 and ribavirin might be a more effective therapeutic approach for treating RSV infections than ribavirin alone.

Keywords: RNase L; 2–5A antisense; RSV; Ribavirin; Combination therapy

1. Introduction

Respiratory syncytial virus (RSV) is a non-segmented negative strand RNA virus belonging to the family Paramyxoviridae, subfamily Pneumoviridae (McIntosh and Chanock, 1990). The host cells for RSV infection are primarily ciliary epithelial cells lining the respiratory tract (Zhang et al., 2002). The cytopathic effect (CPE) of RSV is due to the fusion of the membranes of neighboring cells, resulting in giant cells (syncytia) with multiple nuclei. The life cycle of RSV is entirely cytoplasmic. The RSV genome consists of a 15.2 kb single-stranded RNA of negative polarity encoding 10 viral structural and non-structural proteins named from 3′ to 5′: NS1, NS2, N, P, M, SH, G, F, M2, and L protein (Collins et al., 1996). Transcription of the RSV genomic RNA is mediated by virion-associated protein RNA-dependent RNA polymerase (L, P) and this enzyme has two modes of transcription (Banerjee et al., 1991). Immediately after entering the cell, the RNA-dependent RNA polymerase transcribes 10 distinctive mRNA species by recognizing GS (gene starting) and GE (gene ending) elements of each gene (Collins et al., 1986). The first nine RSV genes have a conservative intergenic GS element (AUUUGCCCC) in the 5′ region of each individual gene. In a mechanism not fully understood, later during infection, the viral RNA-dependent RNA polymerase is triggered to synthesize a full-length RNA instead of individual mRNAs. The full-length RNA is an uncapped positive-strand RNA or anti-genome strand that subsequently serves as template for the replication of numerous copies of RSV negative-strand RNA genomes.

Human RSV is a highly contagious virus, which infects all age groups. Outbreaks in the United States frequently reach epidemic proportions during the winter months. RSV causes severe respiratory disease in infants, children and the elderly with weakened immune function (Wyde, 1998; Falsey and Walsh, 2000). RSV infections do not induce long-term completely protective immunity. By age two, nearly all children have been exposed to and infected with RSV (Glezen et al., 1986). RSV is a serious cause of lower respiratory tract diseases and infections, accounting for 40–50% of hospital-

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ization for bronchiolitis and 25% of pediatric hospitalization for viral pneumonia. Infections by RSV was previously estimated to result in about 90,000 hospitalizations and 4500 deaths annually (Collins et al., 1996; Shay et al., 1999). A recent study suggests that the actual mortality rate associated with RSV infection on average is over 17,000 per year, much higher than previously estimated due to a significant number of cases in elderly patients (>65 years old) mistakenly associated with influenza infection (Thompson et al., 2003). Therefore, RSV imposes a significant threat to public health.

Unfortunately, no effective treatment for RSV infections exists. The benefits of the only approved drug, ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), in the U.S., have been questioned (Dominguez and Mercier, 1999). Ribavirin is a nucleoside analog, which inhibits IMP dehydrogenase. It has been proposed that ribavirin interferes with viral mRNA capping or causes mutations to the viral RNA (Crotty et al., 2000). Recently, it has been shown that ribavirin causes error catastrophe in the replication of viral RNAs in HCV (Zhou et al., 2003), human Hantaan virus (Severson et al., 2003), and polivirus (Crotty et al., 2001). The humanized anti-RSV monoclonal antibody, Palivizumab (Synagis™), was recently approved for preventing RSV infections in infants, but its benefit in patients already infected with RSV remains unknown. Moreover, the cost of prophylaxis exceeds the savings from reduced hospital admissions, unless Palivizumab is given only to extremely high-risk infants (Greenough, 2002; Roeckl-Wiedmann et al., 2003).

Therefore, the need for new anti-RSV drugs or therapeutic measures remains high. We and others have previously described a promising antiviral strategy for the treatment of RSV infections using a targeted therapeutic, 2–5A antisense, that recruits the cellular antiviral nuclease RNase L and directs it to specifically cleave RSV RNA (Cirino et al., 1997; Player et al., 1998; Barnard et al., 1999; Leaman et al., 2002). RNase L is a ubiquitous cellular ribonuclease which is activated by 2′,5′-linked oligoadenylates (2–5A) (Silverman, 1997). However, RNase L has little sequence specificity, cleaving single-stranded RNA preferably at UU or UA dinucleotides. In order to recruit RNase L for the sequence specific RNA cleavage, we linked the 2–5A moiety to a guide sequence complementary to the targeted RNA. Thereby, the 2–5A moiety binds and activates RNase L, while the antisense portion directs the RNase L to the targeted RNA molecule, resulting in degradation of the RNA (Torrence et al., 1993, 1997; Maran et al., 1994). The utility of 2–5A antisense targeting RSV was initially evaluated with antisense targeting RSV mRNA (Cirino et al., 1997). Later it was found that a compound (NIH351) targeting the consensus intergenic element, which is present at multiple sites in the RSV genome, was more efficient (Player et al., 1998). The current lead 2–5A anti-RSV compound, RBI034, is an RNA version of NIH351. It has an identical antisense sequence (AAAAUGGGGCAAAUAA) as NIH351 but advanced chemistry. The antisense part of RBI034 is entirely composed of 2′-*O*-methyl nucleosides including several phosphothioate internucleotide linkages. This results in an increase of in vivo stability of RBI034, a greatly improved affinity for the RSV genomic RNA and enhanced cleavage activity. When administrated intranasally, RBI034 greatly reduced RSV viral titer in African green monkeys (Leaman et al., 2002).

Previous in vitro studies (Cirino et al., 1997; Player et al., 1998; Leaman et al., 2002) on 2–5A antisense targeting RSV were mostly based on neutral red uptake assay, which measures host cell metabolism and therefore viral infection indirectly. Thus, it is compellingly important to cross validate the activity of the 2–5A antisense with other viral assays. Therefore, in this study, we evaluated anti-RSV activity of RBI034 using a number of other methods including TaqMan® real time polymerase chain reaction (PCR) to quantitatively measure RSV RNA, enzyme-linked immunosorbent assay (ELISA) against the fusion protein to examine RSV viral protein expression and progeny titer to determine virus yield. We confirmed that RBI034 is a non-toxic potent anti-RSV compound that works at low concentrations. Its anti-RSV activity is significantly superior to corresponding non-2–5A antisense compound or small interfering dsRNA. To maximally exploit the antiviral properties of the 2–5A antisense compound, we also evaluated the efficacy of RBI034 administrated at different time points after the initiation of RSV infection. Additionally, we found that combination therapy of RBI034 and ribavirin resulted in significantly enhanced antiviral activity. These observations suggest that combination therapy with RBI034 and ribavirin is a promising strategy for the treatment of RSV infections.

2. Materials and methods

2.1. Compounds and synthesis

The compounds used in this study are listed in Table 1. 2–5A antisense compounds were synthesized in house as described previously (Leaman et al., 2002). All siRNA compounds were synthesized by Xeragon, Inc. (Germantown, MD, USA).

2.2. Cell culture and virus propagation

HEp-2 cells were maintained in MEM medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2. Normal human bronchial epithelium cells (NHBE) were purchased from Clonetics (San Diego, CA, USA) and maintained in a medium according to the manufacturer's instructions. RSV strain A_2 was purchased from ATCC (Manassas, VA, USA) and propagated in HEp-2 cells. Virus stock was prepared from the supernatant of infected HEp-2 cell cultures and stored at −80 °C.

Table 1
2–5A antisense and siRNA compounds

Compounds	Code	Target	Sequence and structure
2–5A antisense	RBI034	Genome	spA_4-Bu_2-$(A_sA_sA_sAAUGGGGCAAA_sU_sA_sA)_m$
Scrambled control of 2–5A antisense	RBI066	None	spA_4-Bu_2-$(G_sA_sU_sAGAAAUAGAA_sG_sC_sA)_m$
Non-2–5A antisense	RBI065	Genome	$(A_sA_sA_sAAUGGGGCAAA_sU_sA_sA)_m$
Small interference dsRNA	si245	Genome	5′-AUUUAUUUGCCCCAUUUUUTT-3′ 3′-TTUAAAUAAACGGGGUAAAAA-5′
Small interference dsRNA	siP	mRNA	5′-CGAUAAUAUAACUGCAAGATT-3′ 3′-TTGCUAUUAUAUUGACGUUCU-5′
Small interference dsRNA	siM2	mRNA	5′-AAACAACCCAAAUAACCAUTT-3′ 3′-TTUUUGUUGGGUUUAUUGGUA-5′

s: phosphothioate linkage; m: 2′-*O*-methyl modification at the ribose ring; spA_4: 2′,5′-linked tetraadenylate containing a phosphorothiate group at the 5′ terminus; Bu_2: two butanediol molecules linking the 2–5A moiety with the oligonucleotide moiety and having the formula $–O(CH_2)_4OPO(CH_2)_4O–$.

2.3. Treatment of cells with anti-RSV compounds

Cells were seeded in 96-well plates at 2×10^4 cells per well and grown overnight. Culture medium was removed from the cells and replaced with 100 μl of fresh medium with a reduced serum concentration of 2% and containing the appropriate drug concentration. Cells were then infected with 10 μl of RSV (multiplicity of infection; m.o.i. = 0.01 unless specified) diluted in MEM with 2% fetal bovine serum (FBS). For post-infection administration of the drug, cells were first exposed to RSV (m.o.i. = 0.01 unless specified) in 100 μl of 2% FBS MEM. After 2 h or other specified time periods, the medium was removed and replaced with fresh medium containing drug.

2.4. Treatment of cells with siRNA compounds

siRNA compounds (siP, siM, and si245) were synthesized by Xeragon, Inc. (Germantown) with sense and antisense strands separately and annealed to obtain duplexes according to manufacture's instruction. For the direct treatment of HEp-2 cells with siRNA compounds in the absence of transfection reagent, the procedure was followed as described above for the 2–5A antisense compound except that a higher RSV m.o.i. (0.5) was used. For treatment with transfection, HEp-2 cells were seeded in 96-well plates at 2×10^4 cells per well and grown overnight. Transfection of siRNA compounds was performed using TransMessenger™ (Xeragon) according to the instructions provided. Cells in each well were transfected with 0.5, 0.1, or 0.02 μM of individual siRNA compound in 100 μl of transfection medium. The ratio of siRNA compound to TransMessenger™ was roughly 1:4 (μg:μl). Therefore, the final amount of TransMessenger™ added to the cell culture was 0.56 μl for 0.5 μM, 0.11 μl for 0.1 μM, or 0.02 μl for 0.02 μM of siRNA transfection. A master mixture for 0.5 μM of siRNA transfection medium was prepared and then diluted with serum-free medium to 0.1 or 0.02 μM, and aliquoted to each well. TransMessenger™ controls without siRNA compound were also included by using the same amount of transfection reagent. After 4 h of transfection, 20 μl of RSV infection medium (RSV suspended in 10% FBS-MEM) was added to the cells without removing transfection medium. After incubation for 4–5 days, culture supernatants were removed for measuring the progeny viral titers.

2.5. Neutral red uptake assay

Neutral red uptake assay was used to measure the reduction in RSV-induced cell killing (CPE) due to drug treatment essentially as described previously (Player et al., 1998). This method was also used for measuring the toxicity of compounds to cells when virus was not added. Briefly, cells were maintained in 96-well plates, infected with RSV and then treated with 2–5A antisense compounds as mentioned above. Plates were incubated for 4–5 days or until control cells infected with the virus alone were all dead. Neutral red (100 μl 0.034% solution in culture medium) was added to the cells and the plates were incubated for 2 h. After removing the fluid, the cells were carefully washed twice with PBS. Neutral red was extracted from the cells by adding 0.2 ml of extraction buffer in the dark at room temperature for at least 30 min. Photo absorbance was read using a microplate reader at a wavelength of 540 nm. The percentage of relative cell viability was calculated by subtracting the mean of infected cell control (0%) from the measured absorbance. The resulting number was divided by the uninfected cell control (100%). The mean values and the standard error of mean (S.E.M.) are shown in the figures.

2.6. Cytotoxicity assay

Cytotoxicity of the compounds to the cells was measured using neutral red uptake assay as described above except that no virus was added to the cells.

2.7. Enzyme-linked immunosorbent assay (ELISA)

An ELISA against RSV fusion protein F was used for measuring viral replication and viral protein expression in RSV-infected cells. Cells were seeded in 96-well plates and treated with RSV and drug compound as described above. Cells were incubated for approximately 3 days or until CPE began to emerge. Culture medium was re-

moved and the cells were fixed with 100 μl of ice-cold methanol for 30 min. Plates were blocked with 5% milk in phosphate buffered saline (PBS) for 1 h. Immunostaining was carried out by adding primary goat anti-human RSV antibody (Biogenesis, Poole, England) in PBS with 5% milk. The cells were then incubated at room temperature for 1 h, washed with 5% milk in PBS for 3 times and then incubated with anti-goat horse raddish peroxidase (HRP) conjugated secondary antibody for 1 h. The plates were washed three times with PBS. Chromogenic reaction was then initiated by adding 100 μl of TMB solution (DAKO USA, Carpinteria, CA, USA) to each well and incubating the plate at room temperature for 15 min. The plates were read in a microplate reader at a wavelength of 650 nm.

2.8. Quantitative measurement of RSV genomic and mRNA levels by TaqMan® PCR

HEp-2 cells were maintained in 96-well plates. Cells were infected with RSV and treated with drugs as described above. After infection for 24 h, total RNA was isolated from the plates by using RNeasy 96 RNA isolation kit following manufacturer's instructions (Qiagen, Valencia, CA, USA). For cells grown and treated in 24-well plates, total RNA was isolated by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was determined using Ribogreen following manufacture's instructions (Molecular Probes, Eugene, OR, USA). Reverse transcription reactions were performed by using a kit from Applied Biosystems (Foster City, CA, USA) in 20 μl of reaction volume with 50–100 ng of total RNA, 2.5 μM reverse transcription primer (either oligo dT primer for measuring RSV mRNA or RSV gene specific primer P-RT for measuring RSV genomic RNA), one unit of reverse transcriptase at 48 °C for 30 min. Gene specific reverse transcription primer P-RT (5′-GTTACAAAGGCTTACTACCCAAGGA-3′) was derived from the complementary sequence of RSV N genomic RNA region. About 10 ng of input cDNA was used for TaqMan® quantitative PCR (Applied Biosystems). Amplification was targeted to RSV N genomic RNA or mRNA using primer pairs P1 (5′-AGACTCCCCACCGTAACATCAC-3′) and P2 (5′-GTGGCAGTAGAGTTGAAGGGATTTT-3′). RSV TaqMan® probe (5′-FAM-CTGCACCATAGGCATTCATAAACAATCCTGC-TAMRA-3′) has dual labels of reporter dye 6-FAM at 5′ end and quencher dye TAMRA at 3′ end. Primers and probes were ordered from Integrated DNA Technology, Inc. (Coralville, IA, USA). For measuring RSV mRNA, multiplex TaqMan® PCR of GAPDH (Applied Biosystems) was included at the same tube as endogenous control. Two-step TaqMan® PCR was performed at a 25 μl reaction volume using the ABI Prism 7700 sequence detection system. Reaction conditions were: 50 °C hold for 2 min, 95 °C hold for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The critical threshold value was determined for each sample and data were analyzed according to manufacture's protocol.

2.9. Progeny virus titer determination

Culture medium from cells infected with RSV was recovered and assayed for viral titer. Ten-fold serial dilutions were prepared and used to infect HEp-2 cell monolayer as described above. After 90 min, the infection medium was removed and MEM containing 2% FBS and 1% methyl cellulose was added to the cells. The cells were further incubated for 3–4 days or until syncytia began to appear. The plaques were visualized by immunostaining using ELISA procedures described above except that in the last step a chromogen (DAKO USA) was added. RSV plaques were counted for determining the titer of original viral solution.

3. Results

3.1. Inhibition of RSV by 2–5A anti-RSV antisense compound RBI034

In previous in vitro studies neutral red uptake assays or CPE examinations were predominately used for evaluating 2–5A antisense oligonucleotides on anti-RSV activity (Cirino et al., 1997; Player et al., 1998; Leaman et al., 2002). Progeny RSV titer reduction in cell culture by 2–5A antisense is not well documented in literature. However, viral titer determinations are considered to be the "golden assay" in antiviral research. Therefore, we carried out experiments to measure the release of progeny RSV in HEp-2 cells after treatment with the lead 2–5A antisense compound, RBI034. To confirm that 2–5A antisense mediated cleavage of the RSV RNA is sequence specific as well as 2–5A-dependent, we also synthesized two control compounds: sequence scrambled 2–5A control, RBI066, and antisense control lacking 2–5A, RBI065, and evaluated their ability to inhibit RSV replication in HEp-2 cells by direct measurement of progeny RSV titers. To be effective, RBI034 is needed at a concentration above 0.1 μM when administered as single-dose using the neutral red assay (EC_{50} is 0.15–0.3 μM), with 1 μM of RBI034 achieving about 80% inhibition of RSV replication (data not shown), which is comparable to previous reports (Player et al., 1998; Leaman et al., 2002). RBI034 at concentrations less than 0.1 μM suffers a dramatic loss of potency. To compare 2–5A antisense with a non-2–5A and a scrambled control, RBI034, RBI065, and RBI066 were used at concentrations of 0.6 μM. As shown in Fig. 1A, RBI034 was by far the most active compound. It reduced virus release nearly 100-fold in comparison to untreated cells, whereas RBI065 (non-2–5A control) decreased the virus yield about 10-fold. Since RBI065 is a partially phosphorothioated 2′-*O*-methyl oligonucleotide that does not support RNase H-mediated RNA degradation, its activity is likely due to steric blocking of transcription (Cramer et al., 1999). The scrambled control RBI066 also

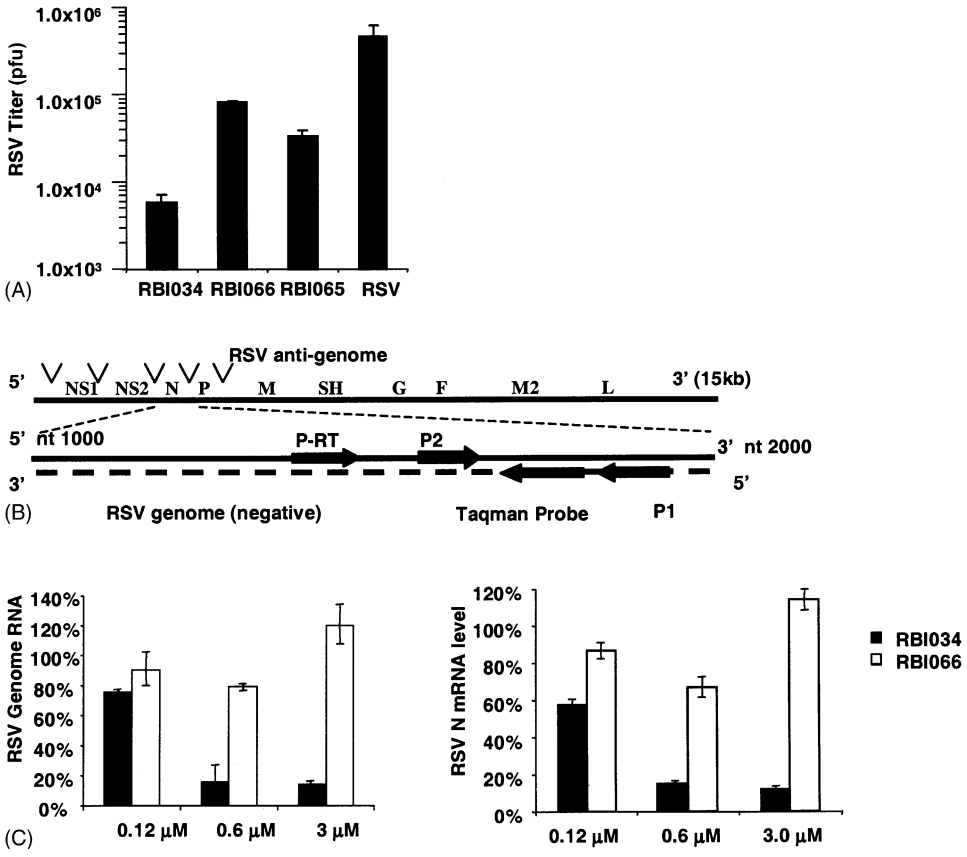


Fig. 1. Inhibition of RSV by 2–5A antisense compound RBI034. (A) HEp-2 cells were infected with RSV (m.o.i. = 0.02) and treated with RBI034, scrambled 2–5A control RBI066 and antisense control RBI065 at 0.6 μM. After 4 days of incubation, culture media were collected and progeny RSV viral titers were measured by immunostaining of RSV syncytia. Treatments were performed in quadruples, samples were pooled and viral titers measured in triplicates. The mean values and S.E.M. are shown. (B) Schematic illustration of RSV RNA RT TaqMan® PCR design. The 15 kb antigenome sequence of RSV is shown on top indicating all 10 coding viral proteins. The arrows indicate the intergenic target sites of RBI034. The RT PCR region is located in the N gene and enlarged at the bottom. RSV genome (negative strand) is represented as dotted line and shown in 3′–5′ direction. Total RNA prepared from RSV-infected cells was used for reverse transcription and real time TaqMan® quantitative PCR. To assess RSV genomic RNA degradation, reverse transcription was performed using an RSV gene specific primer P-RT annealed specifically to RSV negative genomic RNA. To assess RSV mRNA levels, reverse transcription was performed using an oligo-dT primer. Both TaqMan® assays were carried out with PCR primers P1, P2, and a TaqMan® probe. (C) Inhibition of RSV RNA by 2–5A antisense compounds. HEp-2 cells were infected with RSV-A2 (m.o.i. = 0.01). RBI034 (black bars) or its sequence scrambled control compound RBI066 (white bars) was added at 3, 0.6, or 0.12 μM 2 h after infection. Total RNA was prepared 20 h after infection and assayed for RSV N mRNA level or genomic RNA level by TaqMan® quantitative PCR. The abundance of RNA is shown in percentage level relative to that of untreated RSV infection cell control (100%). The levels of RSV N mRNA are shown after normalization to GAPDH mRNA. Mean and S.E.M. are derived from three independent TaqMan® PCR measurements.

showed some activity. However, its activity is more than 10-fold lower than RBI034. The slight activity of RBI066 might be due to non-specific activation of RNase L by the 2–5A moiety. Taking together, these results demonstrate that the antiviral effect of RBI034 is sequence specific and 2–5A-dependent.

To confirm that the anti-viral activity of RBI034 is directly related to the compound's ability to induce the cleavage of RSV genomic RNA and consequently reduce RSV mRNA transcription, we quantitatively measured RSV RNA levels in RBI034-treated cells using TaqMan® real time RT-PCR (Fig. 1B). Both genomic RNA and mRNA levels were investigated. For measuring RSV genomic RNA levels, we used a reverse transcription primer (P-RT) that specifically annealed to the genomic RNA. For measuring RSV mRNA levels, we used oligo-dT as reverse transcription primer. Specific degradation of RSV RNA was examined using RNA samples prepared from RSV-infected HEp-2 cells, which were treated with RBI034 or scrambled control compound RBI066 2 h after RSV infection. Cells treated with 0.6 or 3 μM of RBI034 showed a significant reduction in both RSV genomic RNA and mRNA compared to untreated controls. Scrambled control compound RBI066 did not show a significant reduction in either RSV RNA (Fig. 1C).

3.2. RBI034 is more efficient than small interference RNA in inhibiting RSV

Double-stranded RNA interference (RNAi) is a recently discovered mechanism of eukaryotic cells to degrade tar-

geted cellular mRNA with complementary sequence to one strand of the introduced double-stranded RNA (Hammond et al., 2000). In mammalian cells, small interfering RNA (siRNA), which is composed of double-stranded RNA with 19–21 nucleotides in length, can sufficiently induce an RNA interference response (Elbashir et al., 2001). siRNA targeting viral RNA has also been reported to inhibit virus replication including HIV (Jacque et al., 2002; Novina et al., 2002), polivirus (Gitlin et al., 2002), and RSV (Bitko and Barik, 2001). In order to compare the activity of RSV inhibition by 2–5A antisense with siRNA, we obtained three siRNA compounds targeting the RSV genome (si245), P (siP), and M2 (siM2) mRNA. Sequences of si245 and siM2 were selected following the standard guideline for choosing siRNA originally described by Elbashir et al. (2001). si245 has the identical target site as RBI034. siP is equivalent to the small interference RNA described by Bitko and Barik, who demonstrated potent inhibition of RSV when transfecting cells with this siRNA compound (Bitko and Barik, 2001). The integrity of these three RNA duplexes and RBI034 was confirmed by both HPLC and PAGE (data not shown). First, we evaluated each compound's anti-viral activity by adding it to RSV-infected HEp-2 cells immediately after infection and measuring progeny RSV titers. Without transfection reagent, none of the siRNA compounds showed any RSV inhibitory activity even at a concentration in the micromolar range. On the contrary, RBI034 demonstrated a potent reduction of RSV titers under the same conditions (Fig. 2A). siRNAs' lack of activity may be explained by siRNAs' inability to enter cells without the help of transfection reagents. Therefore, in the next experiment, we reevaluated the antiviral effect of siRNA compounds formulated with transfection reagent TransMessenger™. TransMessenger™ was selected after comparing a panel of frequently used transfection reagents in facilitating a fluorescently-labeled 2–5A antisense compound's entry into HEp-2 cells (data not shown). For reasons that are unclear, HEp-2 cells transfected with TransMessenger™ formulated RBI034 showed severe cytotoxicity (data not shown). Therefore, no transfection agent was used for RBI034-treated HEp-2 cell control. We did not observe significant inhibition of RSV replication by any of the siRNA compounds even in the presence of transfection reagent. siM2, siP as well as si245 siRNA only showed a reduction of RSV titer by 1 log unit when used at a concentration of 0.5 μM. However, TransMessenger™ alone also showed similar reduction at the same concentration (Fig. 2B). Therefore, we concluded that the 2–5A antisense compound RBI034 is significantly more efficient in inhibiting RSV replication than any of the siRNA compounds tested in this study.

3.3. Anti-viral activity of RBI034 and the time of its administration

To assess the influence of the time of drug administration on the activity of RBI034, the compound was added to

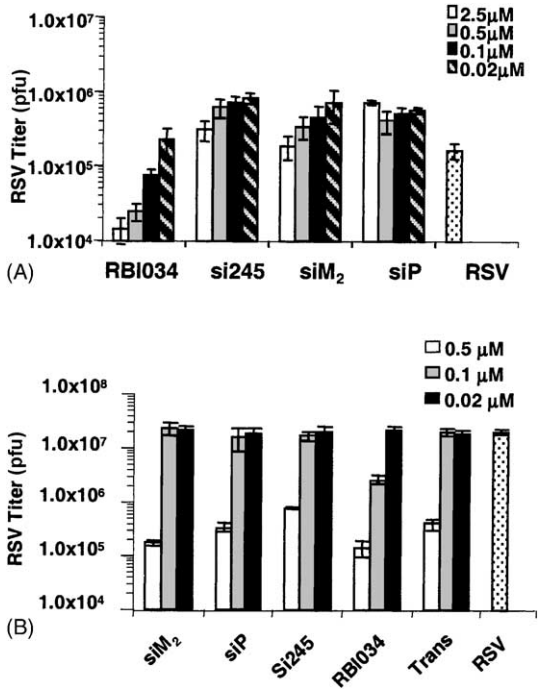


Fig. 2. Effect of siRNA compounds on RSV replication. (A) Progeny viral titer determination from RSV-infected HEp-2 cells treated with siRNA compounds or RBI034. HEp-2 cells were grown in 96-well plates and infected with RSV-A2 (m.o.i. = 0.5) followed by immediate administration of RBI034 and siRNA compounds (si245, siM2, and siP) at a final concentration of 2.5, 0.5, 0.1, or 0.02 μM. Cells continued to grow for about 4 days. At that time RSV infection control cells showed 100% CPE. Culture supernatants were collected for determining progeny virus titers by immunostaining of RSV syncytium. (B) RSV progeny viral titer determination from infected HEp-2 cells transfected with siRNA compounds. HEp-2 cells were grown in 96-well plates and transfected with different siRNA compounds (si245, siM2, or siP) at final concentrations of 0.5, 0.1, or 0.02 μM using TransMessenger™. Transfection medium was prepared according to manufacture's instructions (see Section 2). TransMessenger™ no-oligonucleotide control (Trans) was also included at concentrations of 0.56 (white), 0.11 (grey), or 0.02 μl (black) in 100 μl of medium for each well. Those amounts equal the siRNA transfection concentrations at 0.5, 0.1, or 0.02 μM. Transfection of RBI034 using TransMessenger™ showed severe cytotoxicity. Therefore, a treatment of RBI034 without TransMessenger™ was used to compare its activity with transfected siRNA compounds. Cells were infected with RSV at an m.o.i. of 0.5 at 4 h after transfection. Progeny viral release was determined by immunostaining of RSV syncytia when RSV infection control showed 100% CPE. Treatments in (A) and (B) were performed in quadruples. Samples were pooled and the titer determined in triplicates.

RSV-infected HEp-2 cells at 0, 2, 7, or 24 h after infection. After 4 days of incubation, cells were examined visually for cytopathic effects. RBI034 retained anti-RSV activity even when administered 24 h following RSV infection. In a more quantitative measurement, the progeny virus yields were determined. Viral yield was 10-fold lower in cells treated with RBI034 24 h after the initiation of RSV infection compared to the untreated control. Adding RBI034 immediately after RSV infection was most effective in inhibiting RSV replication and the RSV titer decreased 300-fold (Fig. 3A).

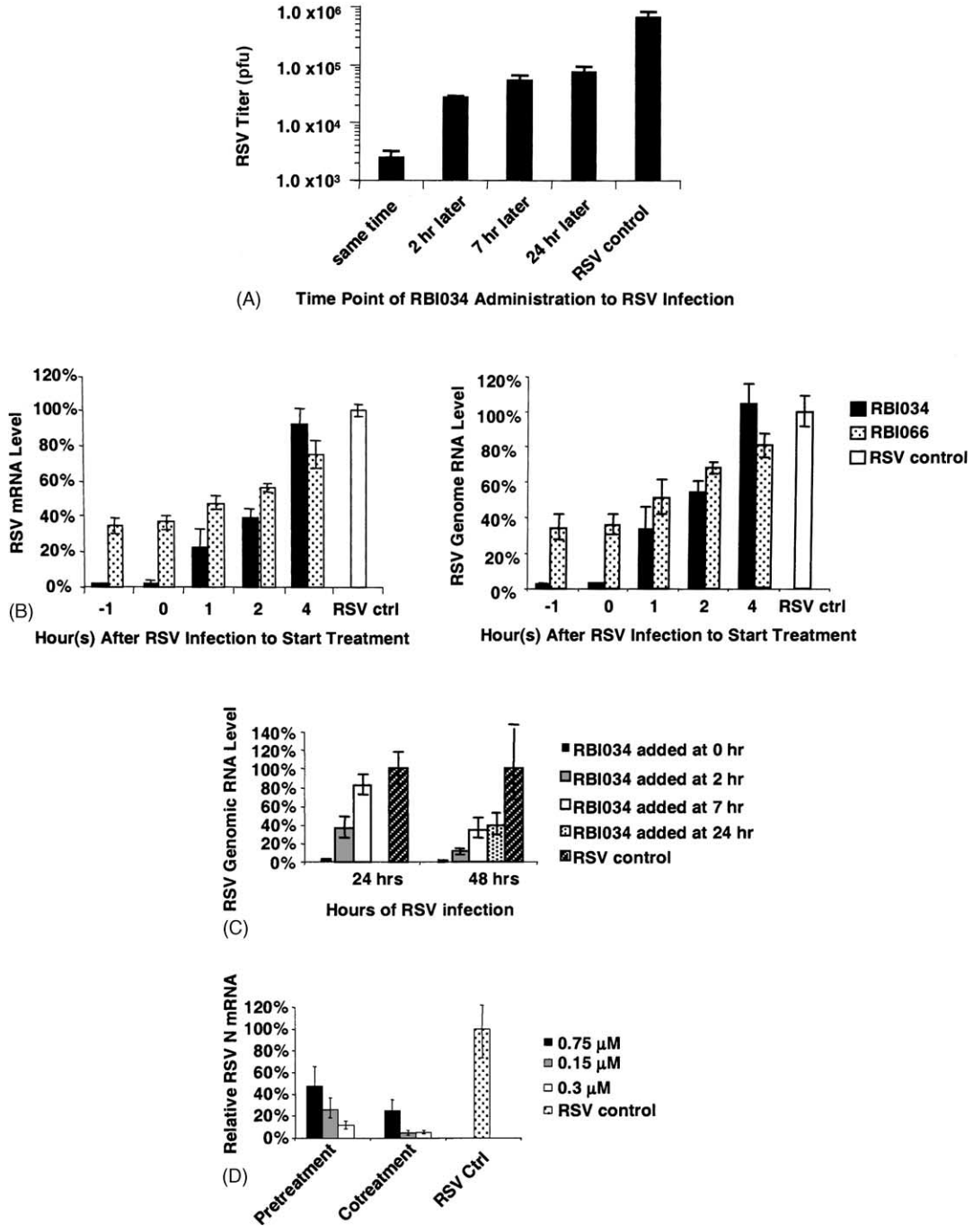


Fig. 3. Antiviral activity of RBI034 administered at different time points after RSV infection. (A) HEp-2 cells were infected with RSV-A2 at an m.o.i. of 0.01. RBI034 was added to cells at a final concentration of 0.6 μM at different time points (0, 2, 7, or 24 h) after initiation of RSV infection. After 4 days of incubation, culture supernatants were recovered and assayed for progeny RSV viral titer by immune staining of RSV syncytia. Mean and S.E.M. were derived from three independent samples. (B) Degradation of RSV RNA by RBI034 administrated at different time points during viral infection using TaqMan® assay. HEp-2 cells were infected with RSV at an m.o.i. of 0.01. RBI034 or control compound RBI066 was added to cells at a final concentration of 0.6 μM at the following time points: 1 h before infection, immediately following infection, 1, 2, or 4 h after infection. RNA was isolated 24 h after RSV infection. RSV N mRNA and genomic RNA levels were evaluated by TaqMan® quantitative PCR. Mean values and S.E.M. were derived from three observations. (C) Activity dependency on time of administration. HEp-2 cells were infected with RSV at an m.o.i. of 0.01 and treated with RBI034 at 0.6 μM at different time points: 0 h (black), 2 h (shade), 7 h (blank), or 24 h (dot) after RSV infection. Total cellular RNA was isolated at 24 or 48 h after infection. RSV genomic RNA levels were evaluated using TaqMan® real time PCR. The figure shows the relative abundance of RSV genomic RNA in comparison to RSV-infected cell control (100%). RNA level differences between RSV control at 24 and 48 h after infection are not shown (the viral RNA level at 48 h after infection is about 20 times higher than that at 24 h after infection). Mean values and S.E.M. were derived from three independent observations. (D) RBI034 as prophylactic agent. HEp-2 cells were pre-treated with RBI034 at 0.3, 0.15, or 0.075 μM. After 24 h, RSV (m.o.i. = 0.01) was added directly to the culture. Cellular RNA was isolated 24 h after RSV infection and viral RNA evaluated using TaqMan® PCR. Mean values and S.E.M. were derived from three independent observations.

We confirmed the viral yield results by measuring viral RNA levels using TaqMan® real time PCR. We compared RSV genomic RNA and mRNA levels derived from infected cells treated with RBI034 at different time points (1 h pre-infection and 0, 1, 2, and 4 h post-infection). Pre- and coadministration of RBI034 led to the most dramatic reduction in RSV RNA levels (greater than 95% in comparison to untreated RSV control). However, the reduction in viral RNA was not as pronounced if RBI034 was added more than 1 h after initiating RSV infection. Adding RBI034 4 h post-infection did not result in a significant reduction of RSV RNA levels (Fig. 3B). Although the data seem inconsistent with the observation that RBI034 is effective in reducing viral yield even when administrated 24 h post-infection, it is important to note that total RNA for the TaqMan® assay was collected at 24 h post-infection, which might not be sufficient time for RBI034 to act on the viral RNA due to the unique RSV life cycle that involves synthesis of anti-genomic RNA, which is used as template for replicating the RSV genomic RNA. Indeed, when levels of RSV genomic RNA were analyzed 48 h after infection, we noticed that viral genomic RNA amounts were significantly reduced, even in cells treated with RBI034 at up to 24 h post-infection (Fig. 3C).

To explore the utility of RBI034 as a prophylactic agent, cells were treated with RBI034 24 h before RSV infection. Using TaqMan® PCR, we found that RBI034 remains very active even when administered a day prior to RSV infection (Fig. 3D). The data suggest that RBI034 may be useful for both treatment and prevention of RSV infection.

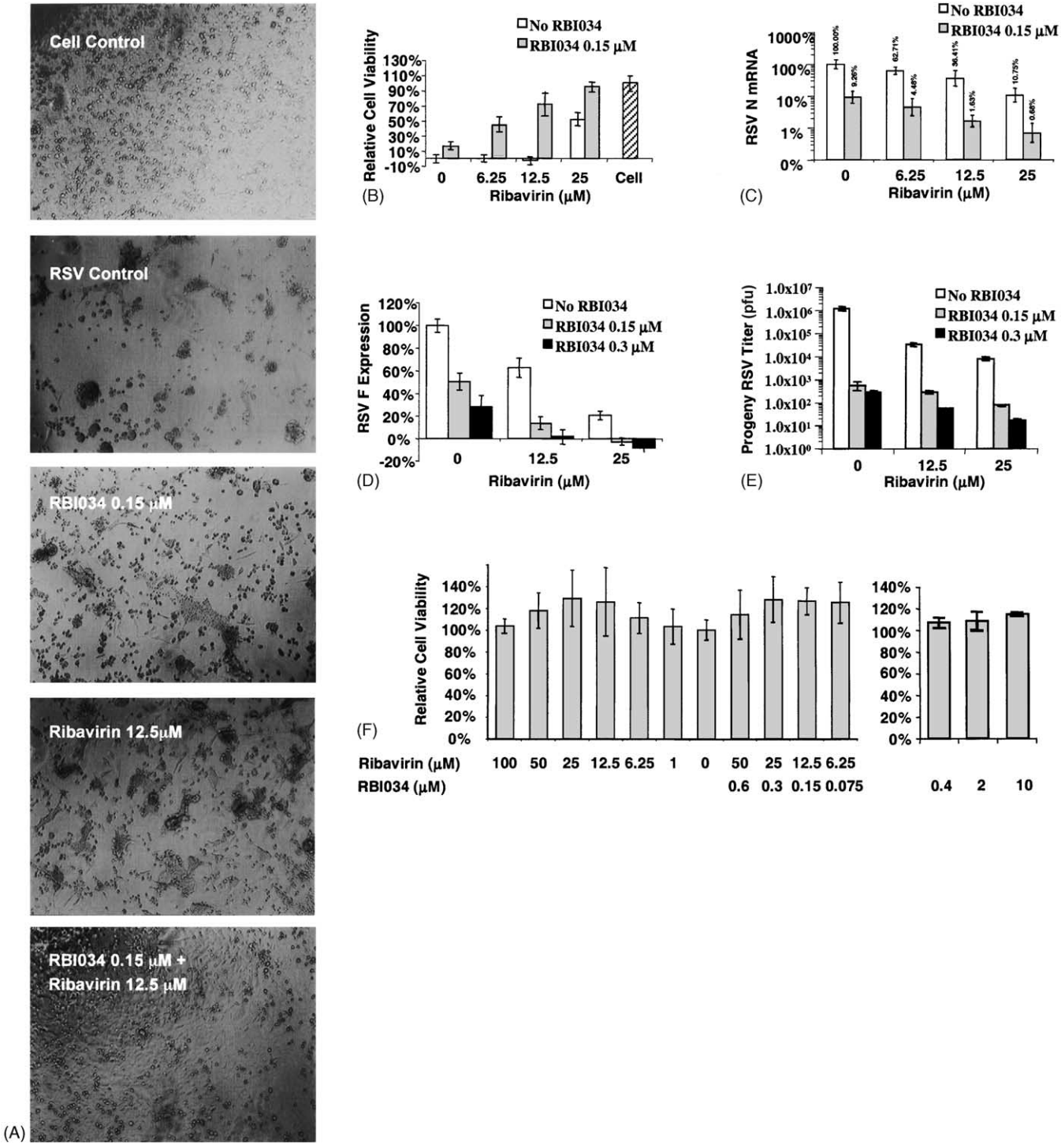
3.4. Combination treatment of RSV infection with RBI034 and ribavirin

Since RBI034 and ribavirin inhibit RSV replication through different and independent mechanisms, we hypothesized that both drugs may act additively or synergistically in blocking viral growth, when administrated together. To test this hypothesis, we first treated RSV-infected HEp-2 cells with RBI034 and/or ribavirin at suboptimal concentrations, 0.15 and 12.5 μM, respectively. At this concentration neither RBI034 nor ribavirin alone was potent enough to completely suppress RSV replication, which made evaluation easier. Indeed, treatment of HEp-2 cells with both ribavirin and RBI034 protected cells from RSV infection, whereas treatment with either drug alone at the same concentration was far less effective (Fig. 4A). To quantitatively measure the antiviral activity of the combination treatment and compare it with either single treatment, we treated cells with varying concentrations of RBI034 plus ribavirin and evaluated their anti-viral activity by examining the cytopathic effect using the neutral red uptake assay (Fig. 4B) and by measuring RSV RNA and viral protein levels using TaqMan® PCR (Fig. 4C), and RSV fusion protein ELISA (Fig. 4D), respectively. The data consistently indicate that combination treatment of RBI034 and ribavirin is more effective in inhibiting RSV replication than either drug by itself. We further determined the amount of virus released from infected cells, and found that RSV replication was almost completely blocked when cells were treated with a combination of both compounds. The resulting viral titers were extremely low, and well below the amount used for the initial infection (Fig. 4E). The cytotoxicity of each compound in HEp-2 cells was also measured by the neutral red assay using different concentrations of ribavirin and/or RBI034. No apparent cytotoxicity was observed for ribavirin nor for RBI034 at the concentrations tested (Fig. 4F). This rules out the possibility that the antiviral activity of the combination treatment resulted from the toxicity of ribavirin or RBI034.

3.5. Anti-RSV activity of RBI034 in normal human bronchial epithelium

Since RSV primarily infects epithelial cells in human respiratory ducts, we tested the activity of RBI034 in pri-

Fig. 4. Anti-viral activity of combination treatment with RBI034 and ribavirin. (A) Protection of HEp-2 cell against RSV infection visualized by a microscope. HEp-2 cells were infected with RSV at an m.o.i. of 0.01 and treated with RBI034 (0.15 μM) and/or ribavirin (12.5 μM) immediately following infection. After 4 days of RSV infection CPE was examined under a microscope. (B) Measuring anti-RSV activity using neutral red uptake assay. HEp-2 cells were infected with RSV at an m.o.i. of 0.01 and treated immediately thereafter with RBI034 (0.15 μM) and/or ribavirin (6.25, 12.5, or 25 μM) following a regime as indicated. After incubation for 5 days, CPE was assessed using a neutral red uptake assay. Shown are cell viabilities at a percentage scale with uninfected cell control set to 100% and RSV infection control set to 0%. Mean values and S.E.M. are derived from at least five independent treatments. (C) Determination of RSV RNA levels using TaqMan® real time PCR. HEp-2 cells were infected with RSV at an m.o.i. of 0.01 and treated with RBI034 and ribavirin as described above. Total cellular RNA was prepared 24 h after infection and used in TaqMan® assay for measuring RSV N mRNA. The chart shows relative RSV N mRNA level in comparison to RSV infection control (100%) at a log scale. Mean values and S.E.M. were derived from six replicates. (D) Viral protein level determination using ELISA of RSV F protein. HEp-2 cells were infected with RSV and treated with RBI034 and ribavirin as described above. Expression of fusion F protein was examined 3 days after infection just when CPE began to show. Absorbance at 650 nm was recorded using a plate reader. The absorbance values were converted into a percentage scale setting RSV infection control to 100% and cell control to 0%. Mean and S.E.M. were derived from six independent treatments. (E) Progeny viral release determination. HEp-2 cells were infected with RSV and treated with RBI034 and ribavirin as described above. Supernatants were removed from cells at day 4 after RSV infection and treatment and assayed for progeny viral titer by syncytia immunostaining. Mean values and S.E.M. were derived from five independent observations. (F) Neutral red uptake assay of the cytotoxicity of ribavirin and RBI034 either alone or in combination at different concentrations in HEp-2 cells. HEp-2 cells were maintained in 96-well plates and treated with ribavirin or RBI034 at concentrations indicated. Cytotoxicity was measured by neutral red uptake assay after incubation for 4 days with ribavirin or ribavirin/RBI034 combination. Treatment with RBI034 alone was performed independently and the incubation lasted for 5 days. Error bars indicate the S.E.M. derived from four independent samples.



mary normal human bronchial epithelial cells (NHBE) by measuring the degradation of RSV RNA using TaqMan® quantitative PCR. NHBE cells were infected with RSV and immediately thereafter treated with RBI034 and/or ribavirin at different concentrations. RBI066, the scrambled antisense oligonucleotide, was used as a control. RSV RNA levels were measured 24 h after infection. Again, the results confirmed RBI034's sequence specific antiviral activity. In addition combination therapy with ribavirin resulted in greatly decreased RSV RNA levels (Fig. 5).

4. Discussion

In our studies we compared, for the first time, the anti-RSV activity of 2–5A antisense (RBI034) with regular antisense (RBI065) and siRNAs (siP, si245, siM2). From

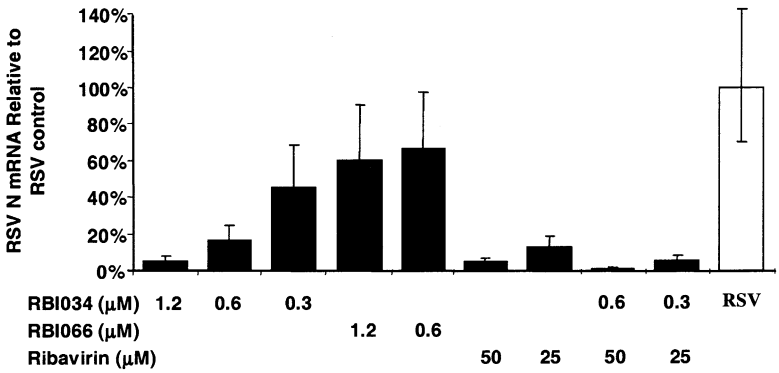


Fig. 5. Inhibition of RSV in primary normal human bronchial epithelial cells (NHBE). Cells were infected with RSV and treated immediately thereafter with RBI034, RBI066, or ribavirin as indicated. After 20 h, total RNA was prepared and Taqman PCR assay for measuring RSV N mRNA was carried out as described earlier (mean ± S.E.M., $n = 6$).

the results presented in this work, it is apparent that RBI034 demonstrated the greatest antiviral activity. Antisense compounds such as RBI065 and its deoxy analog (see Player et al., 1998) showed some antiviral activity. The antiviral activity observed with the all phosphorothioate deoxy analog reported by Jairath et al. (1997) does not seem to be due to a true antisense effect, but instead is related to the phosphorothioate G-quartet (Player et al., 1998). Small interfering RNA has been shown to be effective in inhibiting RSV replication in A549 lung epithelial cells (Bitko and Barik, 2001). Short dsRNA transfected into cells at nanomolar concentration using oligofectamine induced specific ablation of targeted P mRNA and reduction of syncytia formation and viral titers. However, we failed to observe antiviral activity of small interfering dsRNA targeting RSV, using the identical short dsRNA compound (siP) as well as two additional siRNA compounds. Instead, we found that the transfection reagent TransMessenger™ by itself reduced RSV titer by more than 10-fold at a concentration of 0.56 μl in 100 μl of medium. When we examined a number of other commercially available lipid based transfection reagents by TaqMan® quantitative PCR including oligofectamine, we found that all tested transfection reagents reduced RSV replication to similar levels (data not shown). Therefore, it is important to test transfection reagents on activity not only in combination with siRNA but also by themselves.

How time of administration affects the activity of RBI034 or its predecessor 2–5A anti-RSV antisense compound NIH351 (Player et al., 1998) has not been thoroughly investigated before. Here we found that RBI034 remains effective when added to cells before or after initiation of infection, having its highest efficacy when it is added within 2 h after infection. Interestingly, when cells were treated with RBI034 4 h post-infection we were unable to observe any reduction in viral RNA levels in 24 h. Most likely, this is due to the unique RSV life cycle, which involves making anti-genomic RNA shortly after the virus has finished synthesizing individual mRNAs and started producing viral protein products. When the treatment was started at a later time point, i.e. 4 h after infection, virus-infected cells had already accumulated an abundance of viral messenger and anti-genomic RNAs. RBI034 introduced at this time can still induce RSV genomic RNA cleavage. However, it will have no effect on newly made copies of anti-genomic RNA. Therefore, genomic RNAs might be rapidly regenerated by the viral RNA-dependent RNA polymerase using its anti-genome as template for transcription. Another explanation might be that the genomic RNA at this time of infection had already been packaged into newly formed viral particles, ready to be released from the infected cell. Therefore, RBI034 may only be able to act on the next cycle of infection after newly assembled viral particles have entered additional uninfected cells. This may offer an explanation for the reduction of viral RNA levels seen when analyzed at a later time, i.e. 48 h after initiation of infection.

At suboptimal concentrations, RBI034 and ribavirin combination treatment provided a potent protection against RSV infection in cell culture, much greater than either treatment alone (Fig. 4). At this time, it is not clear whether the activity resulting from combination treatment is additive or synergistic. When we applied our data to the classical formula (Hallander et al., 1982), the result was inconclusive, largely due to the limited number of dilutions used. Therefore, additional studies will be necessary to determine if a combination treatment has a synergistic effect. Nevertheless, the enhanced activity of RBI034 and ribavirin combination treatment offers hope for a possible new treatment regiment against RSV infections in humans. Ribavirin is the only approved drug for the treatment of RSV infections. However, its clinical efficacy is controversial and it is only recommended for use in severely ill infants and children. Administration of ribavirin by aerosolization is expensive, and effective dosages are difficult to maintain in the lower respiratory tract. As a result, ribavirin therapy in RSV patients only showed effectiveness in studies with high-dose, multiple short-duration administrations (Englund et al., 1990, 1994). By administering ribavirin in combination with RBI034, it

might be possible to achieve markedly enhanced activity at significantly reduced dosages.

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