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Targeted Therapy of Respiratory Syncytial Virus in African Green Monkeys by Intranasally Administered 2-5A Antisense

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Respiratory syncytial virus (RSV) is a leading cause of respiratory disease in infants, young children, immunocompromised patients, and the institutionalized elderly. Previous work had shown that RNase L, an antiviral enzyme of the interferon system, could be recruited to cleave RSV genomic RNA by attaching tetrameric 2′-5′-linked oligoadenylates (2-5A) to an oligonucleotide complementary to repetitive gene-start sequences within the RSV genome (2-5A antisense). A 2′-*O*-methyl RNA-modified analog of the lead 2-5A anti-RSV chimera is shown here to have enhanced antiviral activity in cell culture studies while also cleaving RSV genomic RNA in an RNase L- and sequence-specific manner. When administered intranasally to RSV-infected African green monkeys, this chimera reduced nasal RSV replication by up to four $\log_{10}$ units in a dose- and time-dependent manner.

Key Words: ribonuclease L; 2′-*O*-methyl antisense.

INTRODUCTION

2′,5′-oligoadenylates (2-5A) are produced by cells following exposure to interferon and double-stranded RNA (dsRNA), a common intermediate in viral replicative cycles (Player and Torrence, 1998). The only well-established function of 2-5A is activation of a ubiquitous cellular enzyme, ribonuclease L (RNase L), resulting in degradation of single-stranded RNAs (Silverman, 1997). Although RNase L cleaves RNA nonspecifically, it has been harnessed to selectively degrade RNA targets by coupling a 2-5A activator moiety to antisense oligonucleotides (Torrence *et al.,* 1993, 1997; Maran *et al.,* 1994; Silverman *et al.,* 1997). 2-5A antisense compounds associate with complementary RNA sequences within cells and activate latent RNase L to cleave proximal RNA. Recruitment and activation of RNase L by 2-5A antisense increases the ability of certain antisense compounds to cause degradation of the RNA target and thus promises to increase the impact of antisense drugs on disease. An important advantage of the 2-5A-antisense strategy is that 2-5A is bioactive for RNase L when attached to antisense with advanced chemistries, including peptide nucleic acids and 2′-*O*-methyl antisense (Cramer *et al.,* 1999; Verheijen *et al.,* 1999).

Respiratory syncytial virus (RSV) is a nonsegmented, negative-strand RNA virus belonging to the pneumovirus subfamily of the family Paramyxoviridae (Collins *et al.,* 1996). RSV is the most common cause of viral bronchiolitis and pneumonia in children, infecting infants from 6 weeks to 6 months of age, where it usually causes upper respiratory infection, but in 10–50% of the cases lower respiratory symptoms occur (La Via *et al.,* 1992; Wyde, 1998). Outbreaks in the United States frequently reach epidemic proportions during the winter months, leading to roughly 500 deaths per year (Shay *et al.,* 2001). Among viral respiratory infections, RSV accounts for 40–50% of hospitalizations for bronchiolitis, and 25% for pneumonia (La Via *et al.,* 1992). RSV is also a major cause of nosocomial infections, and severe pneumonia can occur in the elderly (Wyde, 1998). Although pharmacological agents have been approved for the treatment (Ribavirin) or prevention (Respigam, Synagis) of RSV infection in high-risk individuals, the continued prevalence of RSV outbreaks demonstrates a strong need for effective RSV therapeutics.

The RSV virion consists of a helical nucleocapsid within a lipid envelope with a single strand of negative-sense genomic RNA that is divided into 10 nonoverlapping genes (Collins *et al.,* 1996). Transcription of each gene begins at the first nucleotide of a highly conserved signal (consensus = 3′-CCCCGUUUA-5′) found at gene-

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TABLE 1

CPE Reduction Data Using 2-5A Antisense Chimeras—Three Treatments

Chimera	Structure/sequence	Chimera description	Representative[a] EC_{50}	Representative[a] IC_{50}	Relative[b] activity (Mean ± SD)	Significance[c] level
RBI034	spA_4-Bu_2-(AsAsAsAAUGGGGCAAAsUsAsA)$_m$	Lead chimera	0.10	>50	1	
NIH351	spA_4-Bu_2-d(AsAsAsAATGGGGCAAAsTsAsA)	DNA version	0.26	>10	1.5 ± 0.8	$P < 0.6$
RBI066	spA_4-Bu_2-(**G**sAs**U**sA**GAAAUAG**AAs**G**s**C**sA)$_m$	Scrambled sequence	>3.3	>10	>23	$P < 0.0001$
RBI065	(AsAsAsAAUGGGGCAAAsUsAsA)$_m$	Non-2-5A	0.75	>10	7.5 ± 2.8	$P < 0.01$
RBI064	spA_2-Bu_2-(AsAsAsAAUGGGGCAAAsUsAsA)$_m$	Defective 2-5A	0.9	>10	7.5 ± 2.5	$P < 0.01$
RBI062	spA_4-Bu_2-(AAAAAUGGGGCAAAUAA)$_m$	No PS	0.36	>10	3.7 ± 1.3	$P < 0.1$
RBI063	(AAAAAUGGGGCAAAUAA)$_m$	No PS, No 2-5A	>3.3	>10	>23	$P < 0.001$
RBI045	spA_4-Bu_2-(AAAAAUGGGGCAAAsUsAsA)$_m$	3′-only PS	0.38	>10	4.7 ± 2.5	$P < 0.1$
RBI035	spA_4-Bu_2-(AsAsAsAAUGGGGCsAsAsA)$_m$	Shortened antisense	0.17	>10	1.4 ± 0.2	$P < 0.6$

Note. Oligonucleotides were added to cells 1 h after exposure to virus and again 25 and 49 h postinfection. The assay was stopped on day 4 or 5, before all of the cells in the virus-infected untreated control wells exhibited signs of virus cytopathicity. Antisense moieties followed by a lowercase, subscripted *m* represent 2′-*O*-methyl-modified chimeras. A lowercase d preceding the antisense moiety designates deoxyribonucleotides. Bolded nucleotides represent mismatched bases.

[a] Representative EC_{50} values (in μM) are the concentration of oligonucleotide that reduced virus replication by 50%. Representative IC_{50} values (in μM) are the concentration of oligonucleotide that reduced uninfected cell monolayer density by 50%. The EC_{50} and IC_{50} values presented are from a single, representative experiment.

[b] Average relative activity of each chimera compared to RBI034; based on the results of four independent experiments ($n = 4$). Relative activity of each compound was calculated as follows: The EC_{50} was determined for each chimera. The EC_{50} value obtained for RBI034 was arbitrarily set at 1 and the activities of other chimeras relative to RBI034 were transformed to a ratio between the two (e.g., a relative activity of 2 indicates that the EC_{50} of second chimera was twice that of RBI034). Relative activities for each chimera were averaged from four independent experiments and the standard deviation (SD) calculated.

[c] Paired Student's *t* test, two-tailed, versus RBI034.

start, intergenic, and gene-end signals of the RSV genome (Mink *et al.,* 1991; Jairaith *et al.,* 1997; Player *et al.,* 1998a). Previous cell culture antiviral studies have targeted these sequences with antisense to inhibit RSV replication (Jairaith *et al.,* 1997; Player *et al.,* 1998a). Thus, the 2-5A antisense chimera NIH351, containing core sequences complementary to the conserved nine-nucleotide gene-start sequence, effectively inhibited RSV replication when administered to cells in a single dose just prior to, or 1 h following, virus absorption (Player *et al.,* 1998a).

To provide greater *in vivo* stability and higher affinity for target sequences, 2′-*O*-methyl-modified chimeric 2-5A anti-RSV compounds were synthesized. 2′-*O*-methyl antisense moieties confer enhanced nuclease resistance and exclude any contribution of RNase H (Inoue *et al.,* 1987; Lesnik and Frier, 1995; Cramer *et al.,* 1999). Our results provide the first cell-based demonstration of RNase H-independent cleavage of targeted RNA by a 2-5A antisense chimera and represent the first report in which 2-5A antisense effectively suppressed RSV replication in a primate.

RESULTS AND DISCUSSION

Cell-based analysis of a 2′-*O*-methyl-stabilized 2-5A anti-RSV chimera

To improve the stability of the 2-5A anti-RSV chimera NIH351 (Player *et al.,* 1998a), a 2′-*O*-methyl-modified version of the oligonucleotide was synthesized (Irribarren *et al.,* 1990; Cramer *et al.,* 1999). The resulting second-generation chimera, RBI034, included the same 3′- and 5′-terminal internucleotide phosphorothioate (PS) linkages for enhanced exonuclease resistance, and a 5′-thiophosphate modification to prevent phosphatidic cleavage of the critical 5′-monophosphate residue (Xiao *et al.,* 1998). When applied to cells daily for 3 days (starting 1 h after infection), RBI034 was ~50% more effective than NIH351 in preventing RSV replication as determined by using a cytopathic effects (CPE)-reduction assay (Table 1) or virus yield reduction assay (Table 2). Similar results were obtained when cells received only one or two treatments over the same time frame (Table 3). RBI034 was not cytotoxic at doses up to 50 μM, the highest dose tested (Table 1). Sequence specificity was confirmed by using a sequence-scrambled control chimera, RBI066, which had EC_{50}s greater than 3.3 μM (Tables 1–4). RNase L activation was required for full activity as demonstrated by several compounds, notably RBI065, which had an antisense moiety identical to RBI034 but lacked the 2-5A attachment. RBI034 was seven- to eightfold more active than RBI065, regardless of treatment protocol (Tables 1–3). Similar results were obtained with RBI064, which had a fully complementary antisense portion, but only a dimeric 2-5A attachment that was incapable of activating RNase L (Table 1). RBI034 added to cells 3 h postinfection inhibited viral replication as effectively as when added 1 h postinfec-

TABLE 2

Virus Yield Reduction Data Using 2-5A Antisense Chimeras

Oligonucleotide	Average EC_{50}[a] (μM)	Relative[b] activity (±SD)	Significance[c] level
RBI034	0.03	1	
NIH351	0.04	1.6 ± 0.8	$P < 0.4$
RBI066	>3.3	>156	$P < 0.0001$
RBI065	0.28	8.7 ± 4.3	$P < 0.04$

Note. Oligonucleotides were added to cells 1 h following virus exposure.

[a] Average EC_{50}: concentration of oligonucleotide (μM) that reduced virus replication by 50%. Average of four independent experiments.

[b] Relative activity indicates the average activity (±SD) relative to RBI034 for each compound.

[c] Paired Student's *t* test, two tailed, versus RBI034.

TABLE 4

2-5A Antisense Activity against Different RSV Subtypes—EC_{50}s (μM)

Oligonucleotide	Virus strain/type (HEp-2 cells) 9320/A	393/B	CH18537/B
RBI034	0.2	0.75	0.5
RBI066	5.0	>7.5	7.5

Note. Oligonucleotides were added to cells immediately following virus exposure. EC_{50} values are the concentration of oligonucleotide (μM) required to reduce virus replication by 50%, and the values presented are from a single experiment. RSV A2, CH18537 (B), and 9320 (A) were acquired from ATCC. Strain 393 (B) was acquired from Richard Weltzin (Oravax, Inc.; Cambridge, MA).

tion, and RBI034 added 6–8 h after infection was only threefold less active (data not shown).

To assess the contributions of oligonucleotide length and stabilizing PS linkages on chimera biological activity, a series of modified chimeras were tested in CPE reduction studies. RBI035 was shortened by the three 3′-terminal nucleotides, but had roughly the same activity as the longer RBI034 (Table 1). When either the 5′-proximal PS linkages alone (RBI045) or all of the PS linkages (RBI062) were removed, the resulting compounds had less antiviral activity (4.8- and 3.8-fold, respectively) but were still more active than similar non-2-5A controls (compare to RBI063, Table 1). The slightly reduced activity of RBI045 and RBI062 as compared to RBI034 may result from increased sensitivity to intracellular nucleases.

None of the compounds tested were cytotoxic within the effective dose ranges. All chimeras had IC_{50}s (the concentration required to reduce cell viability by 50%) greater than 10 μM (the highest dose tested for most—Table 1). The IC_{50} for RBI034 was greater than 50 μM. Together these results indicated that RBI034 was a highly effective, nontoxic anti-RSV compound that exhibited both sequence- and 2-5A-specific effects.

Cleavage of RSV genomic RNA

To assess the effects of 2-5A anti-RSV compounds on the targeted RSV genomic RNA, reverse transcriptase coupled polymerase chain reaction (RT-PCR) was used to amplify two of the more highly conserved RBI034 target regions within the genome, the NS1/NS2 gene start site and the NS2/N gene start site (Fig. 1A). The primer used to reverse transcribe the genomic RNA was 3′ of the leader/NS1 start site, which contains another canonical target sequence. Thus, cleavage in this region would reduce further the amount of cDNA template available for subsequent PCR amplifications. These primer sets could not amplify RSV mRNAs since the RT primer was specific to the genome and because the PCR primer sets were located in different transcripts. Treatment of cells with 500 nM NIH351 or RBI034 led to a significant (>90%) decrease in the RNA representing the assessed subgenomic regions. The control compound RBI066 had no effect and RBI065 had intermediate effects (Fig. 1B). The modest reduction (~30%) in RSV genomic RNA by RBI065 could result from inhibition of replication rather than RNA decay. To control for nonspecific cleavage of cellular transcripts, glyceraldehyde-3-phosphate dehy-

TABLE 3

CPE Reduction Data Using 2-5A Antisense—Various Treatment Regimens

Oligo	Average EC_{50} (n) Coadmin., 1 trt	*t* test	1 h p.i., 1 trt	*t* test	1 h p.i., 2 trt	*t* test
RBI034	0.03 (7)		0.22 (5)		0.21 (4)	
NIH351	0.14 (5)	$P < 0.2$	0.34 (4)	$P < 0.2$	0.35 (4)	$P < 0.4$
RBI066	>3.3 (5)	$P < 0.001$	>3.3 (5)	$P < 0.0001$	>3.3 (3)	$P < 0.0001$
RBI065	0.25 (7)	$P < 0.001$	1.1 (5)	$P < 0.2$	1.59 (3)	$P < 0.2$

Note. The indicated oligonucleotides were added to cells either with virus (coadministration), 1 h postinfection (p.i.), or 1 h p.i. and again 24 h later (2 trt). Four or five days later the cells were stained, syncytia were counted, and the EC_{50} (μM) was calculated by regression analysis as described under Materials and Methods. Numbers in parentheses indicate the number of independent experiments for each compound. Paired Student's *t* test, two-tailed, determined the level of significance versus RBI034.

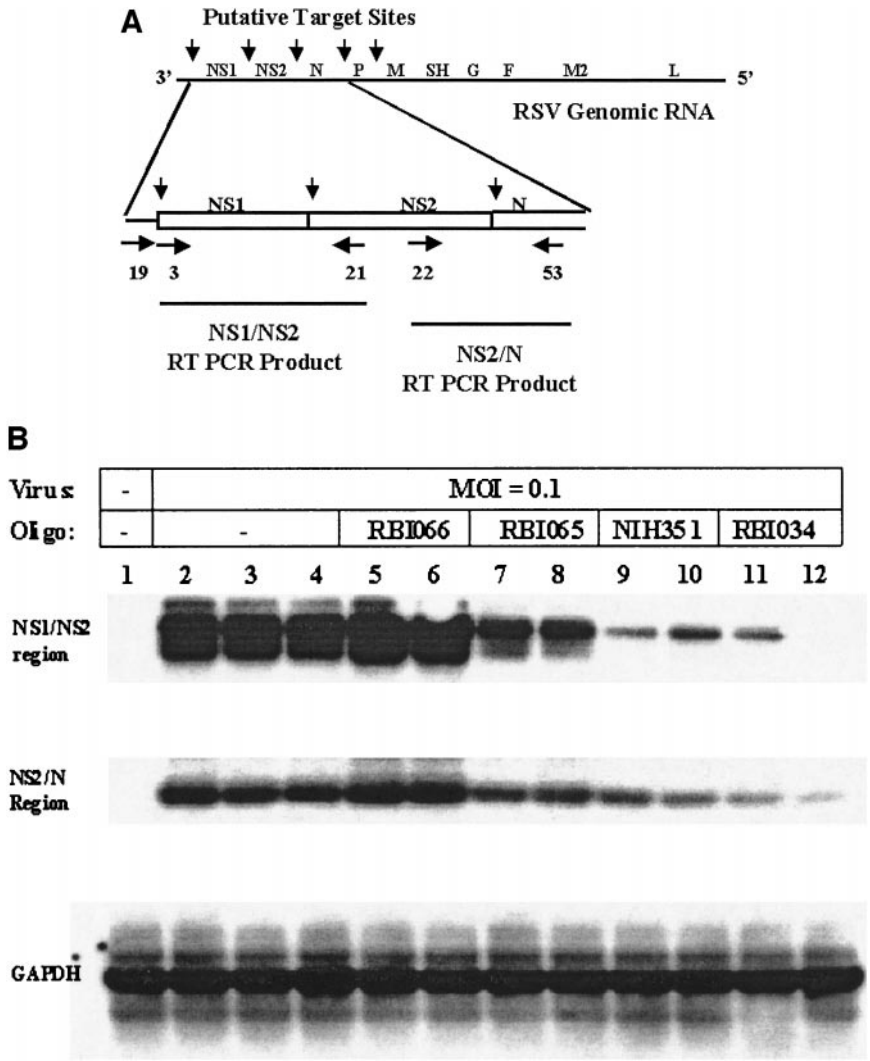

FIG. 1. RT-PCR analysis of RSV genomic RNA fragments following treatment of infected cells with 2-5A anti-RSV chimeras. (A) Schematic diagram of the negative-stranded RSV genome showing the organization of genes. The relative locations of specific primers used to reverse transcribe the genomic RNA into cDNA (primer 19) and for PCR amplifying specific subgenomic fragments (primers 3, 21, 22, and 53) are shown. Vertical arrows above the schematized genome (which is presented in a 3′-to-5′ orientation) represent putative intergenic target sequences most highly conserved with the RBI034 oligomer. Locations within the genome of additional, less highly conserved intergenic target sequences are described in Player *et al.* (1998a). (B) RT-PCR amplification of RSV subgenomic fragments. Cells were either left uninfected (lane 1) or were infected with RSV at an m.o.i. = 0.1 (lanes 2–12). Infected cells were then left untreated (lanes 2–4) or were treated in duplicate with 2-5A anti-RSV chimeras within 5 min of infection (lanes 5–12). Total cellular RNA was isolated 18 h later. RT-PCR amplification products were separated on a 1% agarose gel, transferred to nylon membrane, and hybridized with RSV-specific (top two panels) or GAPDH-specific (bottom panel) probes. The top panel represents PCR products amplified by primers 3 and 21, and the middle panel represents products obtained with primers 22 and 53. GAPDH was RT-PCR amplified separately from the same RNA.

drogenase (GAPDH; Fig. 1B) and double-stranded RNA-dependent protein kinase (PKR) (data not shown) mRNA levels were examined by RT-PCR and found to be unaffected by treatment with any of the chimeras tested.

Antiviral specificity and activity in monkey cells

RBI034 was effective against both A and B subtypes of RSV, although it was slightly more potent in inhibiting A strains (Table 4), confirming that RBI034 has broad specificity against different RSV strains. When tested against another paramyxovirus family member, human parainfluenza virus-3 (HPIV3), RBI034 had no effect on replication (data not shown). This was consistent with the absence of the target sequence for RBI034 in HPIV3. It was critical to evaluate the activity of RBI034 in African green monkey cells in preparation for subsequent *in vivo* antiviral testing since the 2-5A structural elements required for optimal RNase L activation varies among mammals (Krause *et al.,* 1986). RBI034 inhibited RSV replication in African green monkey MA104 cells at concentrations comparable to those observed in human cells (Table 5; compare to results in Table 1).

TABLE 5

2-5A Antisense Activity in African Green Monkey Cells—EC_{50}s (μM)

	MA-104 cells (RSV A2)
RBI034	0.04 ± 0.002
RBI066	>3.3
RBI065	0.38 ± 0.13

Note. Oligonucleotides were added to cells immediately following virus exposure. EC_{50} values are the concentration of oligonucleotide (μM) required to reduce virus replication by 50%, and the values presented are from three independent experiments (±SD).

Antiviral activity in an African green monkey model

The cell culture based studies were extended to an *in vivo* model of RSV replication in African green monkeys. RBI034 was delivered at varying doses (0.1–50 mg/kg) by nasal instillation to groups of two to three animals 30 min after intranasal administration of about 10^6 $TCID_{50}$ of RSV-A2 strain and twice daily thereafter for five additional days (Weltzin *et al.,* 1996). Beginning on day 1 postinfection, nasal swabs were taken each morning prior to the first chimera administration. A single swab of both nostrils was placed in 1 ml of medium, which was applied in half-log_{10} dilutions (from 10^{-1} to 10^{-7}) to HEp-2 indicator cells for CPE analyses. Titers are expressed as the greatest dilution showing viral cytopathology. The two highest doses tested, 10 and 50 mg/kg, provided significant reduction in nasal viral titers over the treatment period (Fig. 2A). The lowest doses (0.1 and 1.0 mg/kg) had no significant effect on virus shedding, although they did delay the onset of virus detection by about 1 day (data not shown). The antiviral effects were dose-dependent, and at the 50 mg/kg body weight dose, viral titers were ~10,000-fold lower than observed in saline-treated animals on day 6 p.i. (Fig. 2B). Only after oligonucleotide administration ended (on day 5, see arrow) did viral titers rise in the animals receiving the higher doses, and they did not reach the maximal titers observed earlier in the untreated animals (Fig. 2A).

The results presented here provide the first cell- and animal-based demonstrations that 2-5A antisense chimeras can elicit RNase H-independent effects, and the first evidence that an oligonucleotide therapeutic agent can effectively suppress RSV replication in a primate. The development of antisense strategies that do not rely on RNase H activation, such as the 2-5A antisense approach, may provide a means to utilize advanced base chemistries that afford higher affinity, greater *in vivo* stability, and thus specific and longer acting drugs (Nyce and Metzger, 1997; Player *et al.,* 1998b). Moreover, the linkage of 2-5A enhances the efficacy of antisense, presumably by harnessing the action of RNase L. Future studies are required to address issues of delivery to the lower pulmonary tract where advanced RSV infections reside. Aerosolized delivery of oligonucleotides to the lungs has been performed successfully (Nyce and Metzger, 1997), and surfactants present in the pulmonary tract may assist in oligonucleotide uptake into the cells. Therefore, while the highly potent and minimally toxic profile of 2-5A antisense makes it an attractive experimental therapeutic agent, efforts aimed at directing the drug to the lower respiratory tract can be expected to improve further its efficacy for the treatment of RSV.

MATERIALS AND METHODS

2-5A antigenome chimera synthesis

Chimeric oligodeoxyribonucleotides were synthesized by using solid-phase phosphoramidite chemistry as described previously (Xiao *et al.,* 1996; Player *et al.,* 1998b; Leaman and Cramer, 1999). Chimeric 2′-*O*-methyl oligonucleotides were synthesized and purified analogously by replacing deoxyphosphoramidites with 2′-*O*-methyl phosphoramidites. Phosphorothioate linkages were introduced by oxidizing with 0.05 M [3H]1,2-benzodithiol-3-

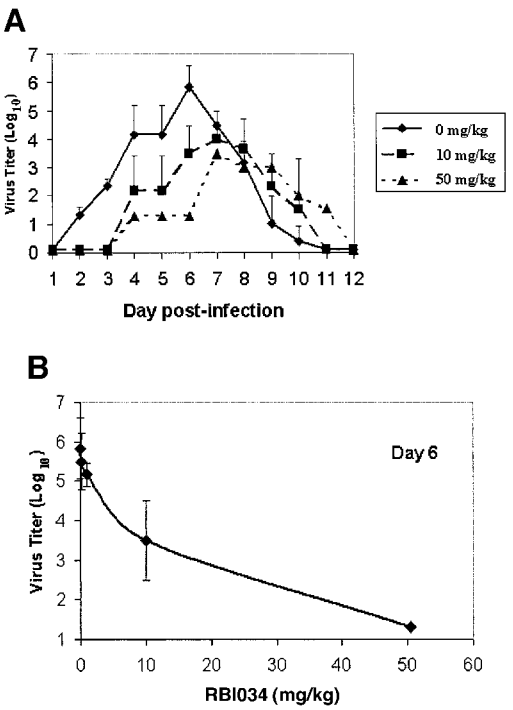

FIG. 2. Effect of RBI034 on nasal RSV titers in African green monkeys. (A) Virus titers in the nasal passages of African green monkeys left untreated (0 mg/kg; $n = 3$) or treated with 10 mg/kg ($n = 3$) or 50 mg/kg ($n = 1$) body weight RBI034 twice daily were analyzed on indicator HEp-2 cells. Treatment continued for 5 days p.i. (6 days total—see arrow), and nasal swabs were collected prior to the first daily treatment. Values represent the dilution at which cell lysis was no longer detectable and the *y*-axis is a log_{10} scale and reflects the average titer in swabs of all animals in the treatment group (±SD). (B) Dose dependence of RBI034's effects. Average viral titers (Log_{10}) in nasal passages on day 6 p.i. were plotted against treatment dose. Values represent the average titers obtained in nasal swabs of three animals per group (±SD) except for the 50 mg/kg group that included only one animal.

one 1,1-dioxide (Beaucage Reagent) (Glen Research) or 0.025 M 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH) (Perseptive Biosystems) in acetonitrile. Sulfurization reactions were prolonged from 60 to 300 s for 2′-*O*-methyl internucleotide phosphorothioate linkages.

The 2-5A antigenome compounds have the general formula sp5′A2′(p5′A2′)$_3$pO(CH$_2$)$_4$OpO(CH$_2$)$_4$O(p5′N3′)$_n$, where N is either a deoxy- or 2′-*O*-methyl nucleotide, and the italicized, subscripted *n* is the number of bases in the antisense cassette (binding domain). The 2′-*O*-methyl compounds are represented as spA$_4$-Bu$_2$-(N)$_m$, where the subscripted *m* designates the 2′-*O*-methyl modification. The 5′-terminus of the 2-5A moiety bears a monothiophosphoryl group and the antisense cassette is of varying nucleotide composition and may include limited numbers of PS internucleotide linkages (see Table 1).

All 2-5A-anti-RSV chimeras were analyzed by HPLC. Due to the G-quartet and the high purine content, the lead compound RBI034 tended to aggregate. Therefore, a new anion exchange method using a PA-100 (250 × 4 mm) column (Dionex) was developed. A linear gradient was run from 1 to 95% mobile phase B in A within 60 min, where A was 6 M urea in acetonitrile/water (30:70, v/v) and B was 395 mM sodium perchlorate, 6 M urea in acetonitrile/water (30:70, v/v). A flow rate of 1.5 ml/min was used. The chimera needed for primate studies was purchased from Hybridon, Inc. (Milford, MA).

Cytopathic effects reduction assay

CPE reduction assays were performed as described by Sidwell and Huffman (1971) with slight modifications. HEp-2 cells (ATCC) were infected with RSV strain A2 (ATCC) at a multiplicity of infection (m.o.i.) = 0.01, and each of the test compounds were provided to duplicate wells at final concentrations ranging from 3.3 to 0.015 μM using 1/3-log$_{10}$ stepwise dilutions. For each compound, two wells were set aside as uninfected, untreated cell controls and two wells per test compound received virus only as a control for virus replication. The assay was stopped after 4–5 days, before all of the cells in the virus-infected untreated control wells exhibited signs of virus cytopathology (giant cell formation, syncytia, cell rounding, etc.). Cells were fixed with methanol (10 min) and then stained with crystal violet (0.1% w/v, 1 min). Darkly stained syncytia in each well were counted and the 50% effective concentration (EC$_{50}$) was calculated by using regression analysis, based on the mean numbers of syncytia at each concentration of compound. Virus yield reduction assays were performed essentially as described by Cirino *et al.* (1997), except that human HEp-2 cells were used as indicator cells, the starting viral titer for the experiments was m.o.i. = 0.01, and the compounds were added directly following virus infection. The neutral red uptake assay (Cavenaugh *et al.,* 1990) was used to assess cytotoxic effects of the test compounds on cultured cells using the modifications described in Player *et al.* (1998a), with the exception that the treatments were carried out for a minimum of 1 week.

RNA analyses

The reverse transcriptase coupled polymerase chain reaction was used to evaluate the effects of the 2-5A chimeras on RSV genomic RNA. HEp-2 cells (3×10^5 cells/35 mm^2) were infected with RSV (strain A2) at an m.o.i. = 0.1 in 1 ml of 2% FCS MEM. Antigenome chimeras were added within 5 min. Total cellular RNA was isolated after 18 h by using Trizol reagent (Life Technologies). RSV genomic RNA was reverse transcribed from 2 μg of total cellular RNA by using a RSV-specific primer (primer 19: TGCGTACAACAAACTTGCGT, Fig. 1A) in a 20 μl RT reaction (Promega). RSV subgenomic fragments were PCR amplified (24 cycles; 94°C, 1 min; 65°C, 1 min; 72°C, 2 min; 25 μl final volume) from 2 μl of the RT reaction by using primer sets shown in Fig. 1A (primers 3: GGCAGCAATTCATTGAGTATG; 21: TGTTATGATGTCTGGTTAG; 22: GTTCTTAGAATGCATTGGC; 53: AGTGTATCATTCAACTTGAC). Under these conditions, no amplification products were visible when separated on an ethidium bromide stained gel. Therefore, 15 μl of each PCR reaction was separated on 1% agarose gel and transferred to nylon membrane (Hybond N+, Amersham) for Southern blot analysis. A RSV genome-specific probe encompassing the NS1, NS2, and N genes (amplified by using primers 19 and 53, Fig. 1A) was utilized. The GAPDH probe encompassed sequences 273-1035 of the GAPDH transcript (gb:M33197). Gel-purified RSV genomic cDNA and GAPDH cDNA inserts were labeled by using the AlkPhos nonradioactive detection kit (Amersham). After hybridization at 50°C overnight, the blots were washed, incubated with the supplied chemiluminescent reagent for 5 min, and then exposed to X ray film (XAR, Kodak).

Animal protocols

All animal protocols were approved by the IACUC at the Tulane Regional Primate Research Center. Fourteen African green monkeys from the island of St. Kitts were quarantined for 6 weeks, during which time they were bled and tested for the absence of prior antibody to RSV. On day 0 of the experimental protocol, animals were weighed and 3 ml of blood was drawn as a baseline serum sample. While sedated (Ketamine), they were inoculated with RSV-A2 strain by instillation of 0.25 ml/nostril of an inoculum containing 10^6 TCID$_{50}$ per ml (Weltzin *et al.,* 1996). Thirty minutes after inoculation, the first dose of freshly prepared RBI034 chimera was administered to groups of three monkeys at doses of 0, 0.1, 1.0, and 10 mg/kg. One group of two monkeys received a dose of 50 mg/kg. The doses were given in a volume of 0.5 ml by administration of 0.25 ml per nostril. The monkeys were placed on their backs for 15 min before re-

turning to their cages. Treatments were repeated at 12 h intervals for 6 days (day 0–5). Beginning on day 1 and continuing through day 12, nasal swabs were taken each morning (before compound administration on treatment days). Calcium alginate fiber-tipped swabs on aluminum wires were used to swab both nostrils and then were placed in 1 ml of complete medium (5% FCS MEM plus antibiotics). Half-$\log_{10}$ dilutions of this stock were prepared in 2% FCS MEM and applied to HEp-2 cells plated 48 h earlier on 24-well plates. The plates were incubated for 7 days when titers were determined by microscopic examination of the cultures for viral cytopathology. The titers were expressed as the greatest dilution producing viral cytopathic effects. Based on previous data (Kakuk *et al.,* 1993; Weltzin *et al.,* 1996), a reduction of titer of 1.0 $\log_{10}$ or greater reflects an antiviral effect. Blood samples were drawn on days 14 and 21 postinfection to confirm the presence of antibodies to RSV using a neutralization assay. Antibodies to RSV developed in all animals (data not shown).

With one exception, all monkeys fared well through the 21 day experiment with no signs of toxicity associated with the treatment. One animal receiving 50 mg/kg of chimera died as a result of aspiration pneumonia associated with intubation problems encountered during the performance of bronchoalveolar lavage sampling on day 3. Necropsy confirmed the presence of lung hemorrhage that was determined to be unrelated to the compound administration. No weight loss or clinical symptoms of RSV infection were observed in the other animals.

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