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2-5A-DNA conjugate inhibition of respiratory syncytial virus replication: effects of oligonucleotide structure modifications and RNA target site selection

D.L. Barnard [a], R.W. Sidwell [a], W. Xiao [b,1], M.R. Player [b,2], S.A. Adah [b], P.F. Torrence [b,*]

[a] *Institute For Antiviral Research, Utah State University, 5600 Old Main Hill, Logan, UT 84322-5600, USA*
[b] *Section on Biomedical Chemistry, Laboratory of Medicinal Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bldg 8 Rm B2A02, Bethesda, MD 20892-0805, USA*

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Abstract

To define more fully the conditions for 2-5A-antisense inhibition of respiratory syncytial virus (RSV), relationships between 2-5A antisense oligonucleotide structure and the choice of RNA target sites to inhibition of RSV replication have been explored. The lead 2-5A-antisense chimera for this study was the previously reported NIH8281 that targets the RSV M2 RNA. We have confirmed and extended the earlier study by showing that NIH8281 inhibited RSV strain A2 replication in a variety of antiviral assays, including virus yield reduction assays performed in monkey ($EC_{90} = 0.02$ μM) and human cells ($EC_{90} = 1$ μM). This 2-5A-antisense chimera also inhibited other A strains, B strains and bovine RSV in cytopathic effect inhibition and Neutral Red Assays (EC_{50} values = 0.1–1.6 μM). The 2′-*O*-methylation modification of NIH8281 to increase affinity for the complementary RNA and provide nuclease resistance, the introduction of phosphothioate groups in the antisense backbone to enhance resistance to exo- and endonucleases, and the addition of cholesterol to the 3′-terminus of the antisense oligonucleotide to increase cellular uptake, all resulted in loss of activity. Of the antisense chimeras targeting other RSV mRNAs (NS1, NS2, P, M. G, F, and L), only those complementary to L mRNA were inhibitory. These results suggest that lower abundance mRNAs may be the best targets for 2-5A-antisense; moreover, the active 2-5A antisense chimeras in this study may serve as useful guides for the development of compounds with improved stability, uptake and anti-RSV activity.

Keywords: Respiratory syncytial virus; Oligonucleotides; Antiviral; 2-5A-antisense; RNase L

* Corresponding author. Tel.: +1-301-4962653; fax: +1-301-4020589.
E-mail address: torrence@helix.nih.gov (P.F. Torrence)
[1] Present address: Computer Data Systems, Inc., 4 Choke Cherry Road, First Floor A172, Rockville, MD 20850, USA.
[2] Present address: 3-Dimensional Pharmaceuticals, Inc., Eagleview Corporate Center, 605 Stockton Drive, Suite 104, Exton, PA 19341, USA.

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1. Introduction

Respiratory syncytial virus (RSV) is a negative-strand RNA virus responsible for serious morbidity and mortality among newborn and infant children (Collins et al., 1996). In 1986, the FDA approved treatment of RSV infection using aerosolized ribavirin, the efficacy of which is still debated (Carmack and Prober, 1995; De Clercq, 1996). Thus, additional antivirals are needed for this disease (Wyde, 1998).

Increasingly, RNA has become a significant target for medicinal chemical intervention in human disease (Zamecnik, 1997). In theory, RNA processing might be interfered with at the stages of transcription, capping, polyadenylation, splicing, transport, translation, and/or degradation. Use of antisense, particularly antisense oligonucelotides to inhibit virus replication, is an active arena of research that capitalizes on the ability of such molecules to arrest or impede the above processes through mechanisms that may include steric blocking or RNase H-catalyzed degradation of antisense oligonucleotide-RNA hybrids (Neckers and Whitesell, 1993; Torrence et al., 1994a,b). Recently, the antisense drug Vitravene™ (formivirisen) (Azad et al., 1993, 1995; Anderson et al., 1996; Leeds et al., 1997), for treatment of cytomegalovirus-induced retinitis in AIDS patients, has completed Phase III clinical trials and has been approved by the FDA (Anonymous, 1998). Application of the antisense strategy to RSV has been reported by Jairath et al. (1997) wherein phosphothioate antisense oligonucleotides inhibited the replication of RSV in vitro.

Recently, the enzyme, 2-5A-dependent RNase L, (Silverman, 1997; Player and Torrence, 1998) has been exploited to widen the spectrum of mechanisms available for targeting RNA (Torrence et al., 1993, 1997). When 2-5A moieties are covalently linked to an antisense molecule complementary to a target sequence, the chimera binds and the 2-5A moiety activates RNase L, which subsequently degrades the targeted RNA to which the 2-5A chimera is bound. 2-5A-antisense oligonucleotides have been reported to elicit selective RNA cleavage in cell-free systems as well as in intact cells (Torrence et al., 1993; Maran et al., 1994; Maitra et al., 1995; Cirino et al., 1997; Xiao et al., 1997; Player et al., 1998a). The recent description of a 2-5A-antisense chimeras inhibiting RSV replication (Cirino et al., 1997) prompted the present studies to attempt to more fully define the conditions for and spectrum of 2-5A-antisense anti-RSV activity and to relate 2-5A-antisense oligonucleotide structure and RNA target site to antiviral activity.

2. Materials and methods

2.1. Chemistry

2.1.1. Synthesis of 2-5A-antisense chimeric oligonucleotides

The following generic oligonucleotide structural types were synthesized for this study.

Type I: sp5′A2′p(5′A2′p)$_3$-[O(CH$_2$)$_4$Op]$_2$-5′dN3′p(5′dN3′p)$_n$5′dN3′p-3′dN5′

Type II: sp5′A2′p(5′A2′p)$_3$-[O(CH$_2$)$_4$Op]$_2$-5′dN3′ps(5′dN3′ps)$_n$5′dN

Type III: sp5′A2′p(5′A2′p)$_3$-[O(CH$_2$)$_4$Op]$_2$-5′dN3′p(5′dN3′p)$_x$(5′dN3′ps)3dN

Type IV: sp5′A2′p(5′A2′p)$_3$-[O(CH$_2$)$_4$Op]$_2$-5′dN3′p(5′dN3′p)$_m$5′dNp[CH$_2$-CH(OH)CH$_2$NH$_2$]

Type V: 2-5A-antisense chimera was a cholesterol-linked congener (Fig. 1)

Type VI: sp5′A2′p(5′A2′p)$_3$-[O(CH$_2$)$_4$Op]$_2$-5′dN$_m$3′p(5′dN$_m$3′p)$_m$5′dN$_m$

The following procedures were used for synthesizing 2-5A-antisense chimeric oligonucleotides of the general structures shown above with varying antisense cassettes targeted to various regions of RSV mRNAs, generally following the synthetic strategy developed by Lesiak et al. (1993), Xiao et al. (1996), Li et al. (1997).

2.1.2. Reagents and chemicals

All DNA synthesis reagents, phosphoramidites, and long chain alkylamino-controlled pore glass solid supports (3′-lcaa′CPG 500) were purchased

from Applied Biosystems (Foster City, CA). To synthesize oligonucleotides with the reversed polarity 3′→3′ phosphodiester bond, 3′-*O*-dimethoxytritylthymidine-5′-lcaa-CPG solid supports (1 μmol size) were obtained from Glen Research (Sterling, VA). For elongation of the DNA antisense chain of the oligonucleotides, the following phosphoramidites were used: 5′-*O*-dimethoxytrityl-N^6-benzoyl-2′-deoxyadenosine-3′-(2-cyanoethyl-*N*,*N*-diiso-propyl)phosphoramidite, 5′-*O*-dimethoxytrityl-N^4-benzoyl-2′-deoxycytidine-3′-(2′-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite, 5′-*O*-dimethoxytrityl-N^2-isobutyryl-2′-deoxyguanosine-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite, and 5′-*O*-dimethoxytrityl-2′-deoxythymidine-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (Applied Biosystems). Synthesis of the 2′,5′-oligoadenylate domain of a chimera employed 5′-*O*-dimethoxytrityl-N^6-benzoyl-3′-*O*-*t*-butyldimethylsilyladenosine-2′-*N*,*N*-di-isopropylcyanoethylphosphoramidite. The phosphorylation reagent for the 5′-terminus of the 2′,5′-oligoadenylate domain of a chimera was 2-[2-*O*-(4,4′-dimethoxytrityl)ethylsulfonyl]ethyl-2′-(cyanoethyl-*N*,*N*-diisopropyl)-phosphoramidite (Glen Research, Sterling, VA). To join chimeric domains, 2-cyanoethyl-*N*,*N*-diisopropyl)-[4-*O*-(4,4′-dimethoxytrityl)butyl]-phosphoramidite was used and was synthesized by a modification of the method of Lesiak et al. (1993).

2.1.3. Synthesis and purification

Conditions for synthesis and deprotection of oligonucleotides were as previously described (Lesiak et al., 1993; Xiao et al., 1994, 1996; Li et al., 1997). Phosphorothioate-modified 2-5A-antisense chimera syntheses were also done as previously described with modifications in the sulfurization step shown below. The 2-5A-antisense chimeras were automatically synthesized with an ABI 392 DNA/RNA synthesizer using multiple cycle phosphoramidite chemistry. The four different domains present within 2-5A-antisense oligonucleotides, each of which required different coupling wait times, were: antisense DNA, 1,4-butanediol linker, 2′,5′-oligoadenylate and 5′-monophosphate. The corresponding coupling wait times were: 15, 300, 660 and 60 s, respectively. Sulfurization employed 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent, Iyer et al., 1990) with a 30 s delivery time and a 10 s wait time which yielded the phosphorothioate residue(s) as in Type II and Type III chimeras.

For oligonucleotide purification, a PRP-1 HPLC column (300 × 7 mm) was used with the following elution protocol: solvent A was 10 mM tetrabutylammonium dihydrogenphosphate (TBAP), pH 7.5 in water; solvent B was 10 mM TBAP, pH 7.5 in acetonitrile/water (8:2 v/v); elution with a convex gradient of 5–80% solvent B in A in 45 min at a flow rate of 1.5 ml/min. Fractions containing desired oligonucleotide were pooled and evaporated to about 1–2 ml and desalted by a C-18 SepPak cartridge. The oligo-TBA salt was converted into its sodium salt from using Dowex 50W ion exchange resin (Na^+ form). The oligonucleotide was dialyzed against 0.02 M NaCl first for 4–6 h and then against water for 48 h. Finally, after dialysis, the product was sterilized using a 0.22 μm Millex-GV filter unit (Millipore, Bedford, MA). The resulting solution was quantitated as A_{260} by UV spectrophotometry. The purities of (2′,5′)-oligoadenylate/antisense chimeras were checked by Dionex HPLC and/or capillary gel electrophoresis using conditions already published (Xiao et al., 1996; Li et al., 1997; Player et al., 1998b,c).

2.1.4. Oligonucleotide characterization

For HPLC analysis, an HP1050 instrument with Chemstation software and data analysis was used throughout. Four different columns and methods were used for analytical and preparative evaluations. The ion-pair method utilized a Hamilton polystyrene reverse phase (PRP-1) 300 × 7 mm column with a flow rate of 1.5 ml/min running a convex gradient of 5–90% mobile phase B in A during 60 min, where A was 10 mM tetrabutyl ammonium phosphate (TBAP) solution, pH 7.5, and B was 10 mM TBAP, pH 7.5, in acetonitrile/water (8:2, v/v) solution. The method (Xiao et al., 1996; Li et al., 1997) used to analyze snake venom digestion digests employed a Beckman Ultrasphere ODS (4.6 × 250 mm) column (Beckman, Fullerton, CA) with a flow rate of 0.5 ml/min running 2% B isocratically for 20 min,

then a linear gradient of 2–45% B in A during 15 min, followed by isocratic conditions for another 10 min where A was 100 mM ammonium phosphate, pH 5.5 and B was methanol/water (1:1, v/v). The ion-exchange method made use of Dionex PA-100 (4×250 mm) ion-exchange column (Dionex, Sunnyvale, CA) at a flow rate of 1.0 ml/min using a linear gradient of 10–90% solvent B in A for 25 min, followed by isocratic conditions at 25 mM Tris–HCl, 1 M ammonium chloride, pH 7.0, in MeCN/H2O (1:200, v/v).

2.1.5. Base composition analyses of 2-5A-antisense chimeras

The nucleotide compositions of the Type I chimeras were analyzed by digestion with snake venom phosphodiesterase (*Crotallus adamanteus*) (Amersham Pharmacia Biotech, Piscataway, NJ) (Xiao et al., 1996; Li et al., 1997). This procedure gives information about the DNA antisense composition and the presence of requisite amounts of butanediol phosphate linker, and 2′,5′-linked AMP residues. Typically, the HPLC of digestion products revealed a total of seven major peaks (depending on the composition of the chimera). Six of these had retention times of 10.8, 29.2, 32.59, 33.4, 39.3 and 41.5 min, corresponding to dCMP, dTMP, dGMP, rAMP, pA2′pO$(CH_2)_4$OpO$(CH_2)_4$OH, and dAMP, respectively (Lesiak et al., 1993; Xiao et al., 1996). A seventh peak had a retention time of 40.2 min and corresponded to the last two nucleotides at the 3′-terminus of the chimera as a 3′-3′-phosphodiester-linked dinucleotide 5′-monophosphate (Li et al., 1997) of Type I chimeras. This compound survived the snake venom phosphodiesterase digestion because of resistance of the 3′-3′-linkage to degradation. Thus, each oligonucleotide that was modified at the 3′-terminus with a terminal nucleotide joined to the antisense chain with a 3′-3′-phosphodiester bond (Type I chimeras) gave a characteristic 3′-3′-linked dinucleotide product upon digestion. For the series of 2-5A-antisense chimeras that terminated with a thymidine nucleotide, there were four possible products, depending on the nature of the penultimate nucleotide. For each of these four products, their specific retention times (R_t) and retention times relative to 5′-AMP (R_t[AMP]) were: p5′dT3′p3′dT ($R_t = 54.1$ min, R_t[AMP] = 1.67); p5′dC3′p3′dT ($R_t = 49.8$ min, R_t[AMP] = 1.47); p5′dG3′p5′dT ($R_t = 51.8$ min, R_t[AMP] = 1.53); p5′dA3′p5′($R_t = 53.4$ min, R_t[AMP] = 1.89). The 3′-3′ dinucleotides were assigned structures by comparing their UV spectra with calculated spectra from 1:1 summation of constituent mononucleotides.

2.2. Virology

2.2.1. Cells and Viruses

Embryonic African green monkey kidney cells (MA-104) were obtained from BioWhittaker (Walkersville, MD) and were grown in minimum essential medium (MEM, GIBCO-BRL, Gaithersburg, MD) supplemented with 0.1% $NaHCO_3$ and 9% fetal bovine serum (FBS, Hyclone, Logan, UT). Human larynx epidermoid carcinoma cells (HEp-2) and embryonic bovine trachea cells (EBTr) were from American Type Culture Collection (ATCC, Manassas, VA) and were grown in Dulbecco's MEM (DMEM, GIBCO-BRL) with 0.1% $NaHCO_3$ and 10% FBS. For antiviral tests, the serum was reduced to 2% in media containing 50 μg/ml gentamicin (Sigma, St. Louis, MO).

RSV strains A2, Long and 9320 (subgroup A; ATCC) and 18357 (subgroup B; Lee Biomolecular, San Diego, CA) and subgroup B strain 393 (Richard Weltzin, OraVax, Cambridge, MA) were propagated in HEp-2 and MA-104 cells. Bovine respiratory syncytial virus (BRSV, strain 375, ATCC) was grown in EBTr cells.

2.3. Virucidal assay

The method of Barnard et al. (1992) was used. Each test compound was diluted to various concentrations and each concentration or MEM as a control were mixed with an equal volume of virus having a titer of 1×10^5 plaque-forming units/ml and incubated at 37°C for 1 h. Surviving virus was assayed by cytopathic effect (CPE) assay using a monolayer of the appropriate susceptible cells. Each concentration of compound was assayed in quadruplicate.

2.3.1. Cytopathic effect (CPE) inhibition assay

CPE inhibition assays used in this study were done as described by Barnard et al. (1997) with slight modifications. Test compounds at varying concentrations and virus at a multiplicity of infection (MOI) = 0.001 were added to near-confluent cell monolayers and incubated at 37°C. Once or twice a day, depending on the assay, for up to 4 days post infection, the medium was replaced with fresh medium containing compound. The assay was continued until the cells in the control wells showed complete viral CPE as observed by light microscopy (usually 6 d). Each concentration of drug was assayed for virus inhibition in quadruplicate and for cytotoxicity in duplicate. Four wells were set aside as uninfected, untreated cell controls per test and four wells per test compound received virus only and represented positive controls for virus replication. This format allowed two compounds to be evaluated for antiviral activity per 96-well plate. For all CPE-based assays, the 50% effective concentrations (EC_{50}) were calculated by regression analysis (RA) of the means of the CPE ratings expressed as percentages of untreated, uninfected controls for each concentration.

Morphological changes resulting from compound cytotoxicity were graded on a scale of 0–5 with 5 being defined as complete cytotoxicity. The 50% cytotoxic doses (IC_{50}) were calculated by RA and a selectivity index (SI) was calculated using the formula: $SI = IC_{50}/EC_{50}$.

2.4. Neutral red (NR) uptake assay

The incubation of cells, virus and test compounds was stopped when the untreated, virus-infected control cells showed 100% CPE including cell lysis. A modified method of Cavenaugh et al. (1990) then was used to evaluate neutral red uptake. Medium was removed from each well of a plate used for the CPE inhibition assay and 0.2 ml of neutral red (NR, 0.034% in physiological saline) was added to the wells of the plate and incubated for 2 h at 37°C in the dark. The NR solution was then removed from the wells and the wells rinsed two times with phosphate-buffered saline (pH 7.4). Equal volumes (0.1 ml) of absolute ethanol and Sorenson citrate buffer (0.1 M sodium citrate, 0.1 M HCl, pH 4.2) were added to the wells. Plates were incubated in the dark for 30 min at room temperature to solubilize the dye. The plates were then gently mixed on a 96-well plate-adapted vortexer for 1 min. Absorbance at 540 and 450 nm was read with a microplate reader (Bio-Tek EL 1309, Bio-Tek, Winooski, VT). All compound concentrations of each compound were assayed in quadruplicate. Absorbance values were expressed as percents of untreated, uninfected controls. EC_{50} and IC_{50} values were calculated by RA.

2.4.1. Virus yield reduction assay

To confirm the antiviral activity detected in the CPE inhibition and NR assays, virus yields for each active compound (SI > 10) from a second CPE inhibition assay were titered by CPE assay. After the CPE was scored as described above, each plate was frozen at −80°C and thawed. Samples from each concentration of compound tested were pooled and diluted using a 10-fold dilution series. Each dilution was plated in triplicate on monolayers of susceptible cells. After absorption (1 h) the sample was removed, maintenance medium added, and the plates incubated at 37°C until the virus control wells showed 4+ CPE.

A 90% decrease in virus yield was calculated by RA. This represents a 1 $\log_{10}$ inhibition of titer compared to the untreated virus controls. The SI value was calculated using the formula: $SI = IC_{50}/EC_{90}$, the IC_{50} derived from log phase cell yield assays.

2.4.2. Multiplicity of infection (MOI) assay

Virus at different MOIs (Table 2) and compound were added to cell monolayers as described above. The medium from each well was removed twice a day for 4 days and replaced with fresh medium with or without compound. The assay continued until the untreated, virus-infected control wells showed 100% virus CPE. After the addition of NR, the uptake into cells was measured by monitoring absorbance at 540/450 nm in a spectrophotometric microplate reader. All concentrations of the compound were assayed in

Table 1
Inhibition of RSV strain A2 by NIH8281 in HEp-2 and MA-104 cells

Compound[a]	Cytopathic effect inhibition assay			Neutral red uptake assay			Virus yield reduction assay		
	EC_{50} (μM)	IC_{50}[b] (μM)	SI	EC_{50} (μM)	IC_{50} (μM)	SI	EC_{90} (μM)	IC_{50}[c] (μM)	SI
Hep-2 cells									
NIH8281	3	>10[d]	>3	1	>10	>10	1	>10	>10
Ribavirin	12	>410	>34	4	>410	>103	3	>410	>137
MA-104 cells									
NIH8281	5	>10	>2	0.3	>10	>33	0.02	>10	>500
Ribavirin	40	330	9	30	300	10	20	500	26

[a] Compound was added immediately after virus exposure to cells. Medium was removed twice a day and replaced with medium containing fresh compound for a total of 4 consecutive days.
[b] Derived from toxicity control in the CPE reduction assay in resting cells.
[c] Derived from a cell yield toxicity assay done with actively growing cells.
[d] Concentrations greater than indicated were not tested.

quadruplicate. Absorbance values were expressed as percentages of untreated controls and EC_{50} values were calculated by RA.

2.4.3. Cell yield assay

Cytotoxicity in rapidly dividing cells was evaluated by determining the total number of cells after a 3 day exposure to several concentrations of compound. Ninety-six well tissue culture plates were seeded with 5×10^4 cells suspended in growth medium. After 4 h at 37°C, the cells were approximately 20% confluent; the medium was then replaced with growth medium containing varying concentrations of test compound as in the antiviral experiments. The cells were then incubated at 37°C for 72 h, at which time the medium was removed and 0.2 ml of NR was added each well and the plates incubated for 2 h at 37°C in the dark. The plates were treated as described above for the NR assay. Absorbance values were expressed as percentages of untreated controls and IC_{50} values were calculated by RA.

3. Results

3.1. Characterization of the inhibition of RSV by NIH8281

For further evaluation of NIH8281 ([spA4-Bu2-d(ATG GTT ATT TGG GTT GTT3′3′T], we used an embryonic African green monkey cell line, MA-104, because of its exquisite sensitivity to RSV cytopathic effects. However, since the RNase L of monkey cells has not been well-characterized and since the mechanism of action of 2-5A-antisense depends upon specific structure-activity relationships for RNase L, we also employed the human cell line Hep-2. The RNase L of human cells has been extensivley characterized (Player and Torrence, 1998). NIH8281 inhibited RSV replication in MA-104 cells and in Hep-2 cells in CPE inhibition and Neutral Red Assays (Table 1). The compound was extremely inhibitory to the production of new virus by the virus yield reduction assay, with $EC_{90} = 20$ nM in monkey cells and 1 μM in human cells. Continued replenishment of the 2-5A-antisense chimera NIH8281 in fresh medium approximately every 12 h for a total of 4 days was necessary to achieve this effect. However, the minimum dosing regimen to significantly inhibit viral replication was twice a day for 2 days (data not shown). As with some other antivirally active drugs, the potency of NIH8281 was viral load dependent. At MOIs greater than 0.02, no inhibition of virus replication was observed (Table 2). At lower MOIs the compound inhibited at similar concentrations reported in the initial experiments where the

Table 2
Effects of MOI on the sensitivity of RSV strain A2 replication in MA-104 cells to inhibition by NIH8281 or ribavirin

Assay length[a]	MOI	CPE inhibition assay		Neutral red uptake assay	
		NIH8281	Ribavirin	NIH8281	Ribavirin
		EC_{50} (μM)	EC_{50} (μM)	EC50 (μM)	EC_{50} (μM)
5 days	0.1	>5.7[b]	4	>5.7	4
5 days	0.02	>5.7	<4[c]	>5.7	<4
6 days	0.01	5.7	<4	5.7	<4
7 days	0.005	1.8	<4	1.0	<4
7 days	0.002	1.8	<4	1.0	<4

[a] Compounds were added immediately after virus exposure to cells. Medium was removed twice a day and replaced with medium containing fresh compound for 4 days.
[b] >Concentration higher than indicated was not evaluated.
[c] <Concentration lower than indicated was not evaluated.

MOI = 0.001. The antisense chimera also potently inhibited other RSV strains, including bovine RSV, with EC_{50} values less than 1 μM (Table 3). NIH8281 was not virucidal at the concentrations tested ($EC_{90} > 10$ μM, data not shown).

In the previous assays, compound was added to the culture medium followed by the addition of virus within 2 min. When NIH 8281 was added to the culture medium at times later than 2 h post infection, it was no longer inhibitory (Table 4). Ribavirin was very inhibitory even up to 12 h post infection.

3.2. Structure activity relationships: anti-RSV activity of congeners of NIH8281

The original 2-5A-antisense chimera, NIH8281 (Cirino et al., 1997) had a 3′-3′-terminal phosphodiester bond to lessen serum/cell exonuclease degradation of the chimera (Li et al., 1997). Without this modification, no anti-RSV activity was observed in human tracheal endothelial cell culture (P. F. Torrence, G. Li, W. Xiao, N. Cirino and R. H. Silverman, unpublished observations). Thus, several congeners of NIH8281 were synthesized to determine if other exo- and endonuclease-stabilizing modifications would be compatible with anti-RSV activity. We also wished to ascertain if the addition of a 5′-monothiophosphate moiety to reduce phosphate inactivation of 2-5A-antisense (Xiao et al., 1994) was required for optimal anti-RSV activity.

3.2.1. NIH207

NIH207 was a 3′-cholesterol-modified analogue of NIH8281. The 2-5A-antisense chimera NIH207 contained the parent NIH8281 structure tethered from the 3′-terminus through a 3′-phosphate and a seven atom spacer to the 1-hydroxyl function of cholesterol (Fig. 1). This product showed significant antiviral activity (Table 5), but was not selective due to cytotoxicity (SI = 1).

3.2.2. NIH208

When NIH8281 was modified to an all phosphorothioate antisense domain (NIH208), the $EC_{50} = 0.5$ μM. However, NIH208 was also very cytotoxic ($IC_{50} = 1$ μM); SI = 2 (Table 5).

3.2.3. NIH280

To reduce the toxicity of NIH208, but to retain the endo- and exonuclease resistance of the phosphorothioate linkage, an analogue, NIH280, was generated. This congener contained phosphorothioate internucleotide bonds only at the last three 3′-terminal positions of the antisense domain of the chimera. NIH280 was not inhibitory to RSV replication (Table 5).

3.2.4. NIH302

The introduction of 2′-*O*-methylribonucleotides into the chain of 2-5A-antisense chimeras was done for two reasons: First, the 2′-*O*-methyloligoribonucleotide has a higher affinity than a corre-

Table 3
Inhibition of RSV strains by NIH8281[a]

	Cytopathic effect inhibition assay						Neutral red uptake assay					
	NIH8281			Ribavirin			NIH8281			Ribavirin		
Virus (type or subgroup)	EC_{50} (μM)	IC_{50} (μM)	SI	EC_{50} (μM)	IC_{50} (μM)	SI	EC_{50} (μM)	IC_{50} (μM)	SI	EC_{50} (μM)	IC_{50} (μM)	SI
9320 (A)	1	>10	>10	20	100	5	0.4	>10	24	20	>100	>5
Long (A)	0.3	>3.2	>10	12	205	17	0.4	2.0	5	12	280	23
CH 18537 (B)	0.1	>3.2	>32	4	208	52	0.1	2.4	24	12	233	19
393 (B)	0.3	>3.2	>10	4	96	24	1.6	>3.2	>2	4	96	24
Bovine RSV	1	>10	>10	50	100	2	1	>10	>10	20	100	5

[A] Compound was replenished once a day for 2 days. All assays were done using Hep-2 cells except for the bovine RSV assay that used EBTr cells.

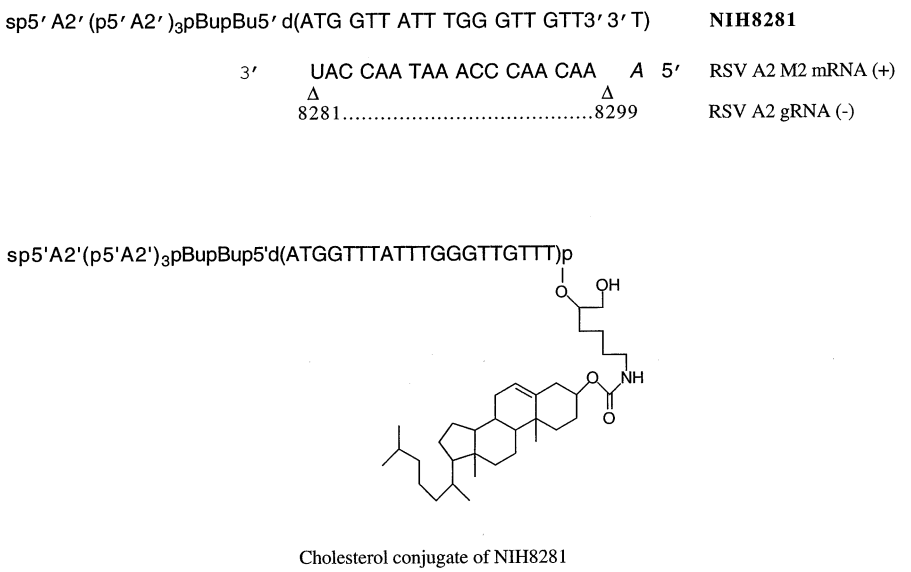

Fig. 1. Structure of NIH8281 and cholesterol-conjugated 2-5A-antisense chimera analogue of NIH8281. The antisense domain of NIH8281 is shown juxtaposed to the complementary M2 mRNA of RSV strain A2. The nucleotide numbering scheme corresponds to the genomic (g)RNA of RSV strain A2 derived from GenBank (accession no. M74568). The 5′ most A in the RSV mRNA is shown in italics as it is most probably not incorporated into the double-helical complex formed with.

sponding deoxyribooligonucleotide for this complimentary RNA, giving rise to a duplex with a higher T_m (Inoue et al., 1987); secondly, the 2′-*O*-methyl modification leads to complete resistance to endonucleases such a pancreatic RNase A (Iribarren et al., 1990). In spite of these attributes, the analogue NIH302, containing an antisense domain with all 2′-*O*-methylribonucleotides, was devoid of anti-RSV activity (Table 5).

Table 4
Effects of time of addition of compound on the replication of RSV strain A2 in Hep-2 cells[a]

Time of addition of compound post virus exposure (h)	NIH 8281 EC_{50} (μM)	Ribavirin EC_{50} (μM)
0	3	30
1	3	30
2	3	30
4	>10	30
8	>10	30
12	>10	>100

[a] Virus (MOI = 0.001) was added to near-confluent cell monolayers. Test compounds, at varying concentrations, were then added to the infected cells at various times post exposure to virus and incubated at 37°C. The medium was then replaced with fresh medium containing compound twice a day, beginning 24 h post virus exposure, for 4 days thereafter.

3.2.5. NIH301

When NIH8281 was modified at the 3′ terminus in which 3-amino-1,2-propanediol was linked through a 3′ phosphoryl group to the oligonucleotide antisense domain (NIH301), the 2-5A-antisense chimera also was inactive against RSV (Table 5). This 3′-terminal phosphoropropylamino moiety has been shown to slow the attack of 3′-exonucleases and previously had been demonstrated to increase both in vitro and in vivo stabilities and lifetimes of oligonucleotides (Zendegui et al., 1992; Tam et al., 1994).

3.2.6. NIH500

The structure and sequence of NIH500 was identical to the lead oligonucleotide NIH8281, except that the 5′-monothiophosphate of NIH8281 was converted to a 5′-monophosphate moiety to determine the necessity of thiophos-

Table 5
Inhibition of RSV strain A2 by congeners of NIH8281[a]

Compound	Description	Chimera sequence	Neutral Red Assay		
			EC_{50} (μM)	IC_{50} (μM)	SI
NIH8281	Lead oligonucleotide	spA$_4$-Bu$_2$-d(ATG GTT ATT TGG GTT GTT3′3′T)	1.0	>10	>10
NIH207	Cholesterol derivative	spA$_4$-Bu$_2$-d(ATG GTT ATT TGG GTT GTT3′-cholesterol)	0.3	0.4	1
NIH280	All phosphorothioate antisense domain phosphothiorate modifications at termini only	spA$_4$-Bu$_2$-d(AsTsGs GsTsTs AsTsTs TsGsGs)	0.5	1.0	2
		spA$_4$-Bu$_2$-d(ATG GTT ATT TGG GTT GsTsTs T)3′			
NIH301	Aminopropyl end modification	spA$_4$-Bu$_2$-d(ATG ATT TGG GTT GTT T)3′-aminopropyl	>10	>10	–
NIH302	All 2′(ribose) *O*-methyl groups, no 3′ tail	spA$_4$-Bu$_2$-d(A$_m$T$_m$G$_m$ A$_m$T$_m$Tm T$_m$G$_m$G$_m$- G$_m$T$_m$T$_m$ G$_m$T$_m$T$_m$T$_m$)3′	>10	>10	–
NIH500	Monophosphate	pA$_4$-Bu$_2$-d(ATG GTT ATT TGG GTT GTT3′-3′T)			

[a] Compound was added twice daily for 4 days.

phate for activity. NIH500 was without anti-RSV activity (Table 5).

3.3. Targeting other sequences

3.3.1. Oligonucleotide design based on predicted mRNA secondary structure

The anti-RSV activities of the Type I 2-5A-antisense chimera generated from targeting secondary structure of various mRNAs of RSV are listed in Table 6. Of the 25 2-5A-antisense chimeric oligonucleotides evaluated, only four possessed significant, selective anti-RSV activity. These included NIH8841, NIH8861, NIH9030 and NIH10180, all of which targeted the L polymerase mRNA.

4. Discussion

The present study had three primary goals. The first goal was to define more completely the characteristics of anti-RSV activity of the lead 2-5A-antisense chimera, NIH8281, that had been reported previously (Cirino et al., 1997). Such studies included assaying for virucidal activity, determining anti-RSV activity in different cell lines, defining EC_{50} values by CPE inhibition and NR assays and EC_{90} values by virus yield reduction assay, and determining the cytotoxicity of the chimera in actively growing cells. The second objective was to examine modifications to the lead NIH8281 2-5A-antisense oligonucleotide to determine how structure would affect anti-RSV activity. The third goal was to explore whether other sequences within the RSV mRNAs or within the RSV RNA genome itself could be targets for 2-5A-antisense inhibition of RSV.

Previously, Cirino et al. (1997) had shown the specificity and antisense mode of inhibition of RSV replication by NIH8281. In our studies it was most active in a virus yield reduction assay (Table 1) and inhibitory only when added within 2 h of virus infection (Table 4). The antiviral activity was also MOI-dependent (Table 2), which is not surprising, since its putative mode of inhibition of virus replication may be to bind to sequences of RSV RNA responsible for replication via the antisense portion of the chimera to allow the 2′5′-linked oligoadenylate (2-5A) portion of the molecule to activate RNase L. The activated

Table 6
Inhibition of RSV strain A2 by 2-5A-antisense chimeras targeting RSV mRNAs[a]

Oligo-nucleotide	mRNA target	RSV GenBank nt sequence numbers[b]	Secondary structure motif targeted[c]	Antisense sequence in 2-5A-antisense chimera[d]	Neutral Red Assay		
					EC_{50} (μM)	IC_{50} (μM)	SI
NIH321	NS1	321–330	HPL	5′ cat tgt tgt gaa att gaa	>10	6	<1
NIH721	NS2	721–738	5′HPL	5′ tgt tat gat gtc tct	>10	>10	–
NIH1721	NP	1721–1738	HPL	5′ cct ttg taa cgt ttc	3	6	2
NIH2491	P	2491–2508	5′HPL	5′ ggc ttt ctt tgg tta ctt	>10	8	–
NIH3225	P	3225–3242	HPL	5′ tta tat caa tgt ttt ttt	>10	>10	–
NIH3797	M	3797–3814	5′HPL	5′ tca gtg gtt gtt ata	>10	8	1
NIH3833	M	3833–3850	3′HPL	5′ taa ggg atg att ttt cga	>10	>10	–
NIH5071	G	5071–5088	SSR	5′ ttg gtc ttg act gtt gtg	4	6	2
NIH5157	G	8157–5174	SSR	5′ aat cat tat tgg gtt tgc	5	5	1
NIH5241	G	5241–5258	5′	5′ ttg gat ttc ttt tgc agg	>10	8	–
NIH5272	G	5272–5289	3′	5′ ttg gta gtg gtt ttc ttt	>10	7	–
NIH5381	G	5381–5398	SSR	5′ gtt gat ggt tgg ctc ttc	>10	7	–
NIH5413	G	5413–5430	SSR	5′ agt agt gta gtt atg atg	10	>10	>1
NIH6331	Fo	6331–6448	5′HPL	5′ tgt tgt tct ttt gtt	>10	8	<1
NIH8670	L	8670–8687	SSR	5′ gtt tct tta gat tca tgt	>10	7	<1
NIH8687	L	8687–8704	SSR	5′ gga ctg tgt tat att tag	>10	>10	–
NIH8819	L	8819–8836	5′ HPL	5′ tat tat ctt ttt aag taa	>10	>10	–
NIH8841	L	8841–8858	HPL	5′ cac tta ttt cat tag ctc	3	8	3
NIH8861	L	8861–8878	3′ HPL	5′ tat agc ata gac ttt gac	3	>10	>3
NIH9030	L	9030–9047	SL	5′ tgt ctt ttt gtt ttg tag	2	>10	>5
NIH10180	L	10180–10197	5′ HPL	5′ tat ata tag ttt tgt atg	2	>10	>5
NIH12541	L	12541–12558	5′ HPL	5′ ggt att gat gca ggg aat	>10	4	<1
NIH12564	L	12564–12581	HPL	5′ gat aat ttg ttg ttc tat	>10	7	<1
NIH12587	L	12587–12604	3′ HPL	5′ att aat agg gct agt gtc	>10	10	<1

[a] Compound was added immediately after virus exposure to cells. Medium was removed twice a day and replaced with medium containing fresh compound for 4 days.

[b] Numbering refers to nucleotide numbers in the RSV strain A2 15222 bp mRNAs (GenBank accession no. M74568). The 2-5A-antisense sequences are complementary to the indicated sequences in the respective RSV mRNA.

[c] Abbreviations for secondary structure motifs derived from MFOLD calculations: SSR, single-strand region; 5′HPL, HPL, 3′HPL correspond to 5′-most sequence of hairpin loop, middle sequences of hairpin loop, and 3′-most sequence of hairpin loop, respectively; SL, stem-loop.

[d] This is the antisense domain of the 2-5A-antisense chimera of general structure: ps5′A2′$(p5'A2')_3(pBu)_2$-$(p5'dN3')_n$3′pdT.

enzyme presumably then degrades the virus RNA (Torrence et al., 1993). Cirino et al. (1997) provided evidence for this in the case of RSV inhibition by NIH8281. Since this inhibition is dependent on an enzymatic reaction, we presume it is sensitive to substrate (homologous virus RNA) concentration. The inhibitory activity was also species specific; NIH8281 did not inhibit measles or parainfluenza viruses (data not shown), also members of the *Paramyxoviridae* family. However, the material was equally inhibitory to other RSV strains, including a clinical isolate, suggesting that a conserved RNA sequence in these strains was being targeted (Table 3).

The present results do not necessarily imply that antisense binding to specific virus sequences with the subsequent RNase L-catalyzed mRNA degradation was the mode of inhibition of RSV replication. Other possible modes of inhibition could have been the prevention of virus binding (Gao et al., 1990) and inhibition of viral polymerase (Ojwang et al., 1995). Our data suggest that NIH8281 does not inhibit virus binding, since it is still potently inhibitory even when added 2 h post virus exposure. However, we cannot yet rule out polymerase inhibition. In support of an RNase L-catalyzed degradation of RSV mRNA, earlier experiments showed a diminution in levels of the targeted M2 mRNA by NIH8281, with little, if any effect on non-targeted P and N mRNAs (Cirino et al., 1997)

Surprisingly, NIH280 did not possess significant anti-RSV activity (Table 5). The compound was designed with phosphorothioate internucleotide bonds only at the last three 3′-terminal positions of the antisense domain of the chimera to decrease the toxicity of NIH208. An extensive literature exists describing the biological activities of phosphorothioate oligonucleotides. This finding of toxicity is consistent with other reports that phosphorothioate oligonucleotides possess a variety of non-specific effects (Stein et al., 1991a; Yaswen et al., 1993; Ramanathon et al., 1994) that can lead to toxic responses. Reduction of the phosphorothioate content of oligonucelotides diminishes both non-specific effects as well as toxicity (Ghosh et al., 1993). Moreover, substantial exonuclease resistance and increased biological half-life have been associated with minimal phosphorothioate substitution at the 3′-(and/or 5′-) termini of oligonucleotides (Stein et al., 1988; Majumdar et al., 1989; Shaw et al., 1991; Ghosh et al., 1993; Peyman and Uhlmann, 1996).

Other methods of achieving exonuclease resistance include the addition of 2′-*O*-methyl groups to the nucleotides of the antisense portion of the chimera. This change in chemical structure also increases affinity for the target RNA. Therefore, extensive modification of the antisense cassette with a 2′-*O*-methyl substitutions (NIH302) should have led to complete resistance to endonucleases (Iribarren et al., 1990); and thus inhibition of virus replication, but NIH302 was totally inactive (Table 5). Perhaps extensive substitution with 2′-*O*-methylribonucleotides hindered the kinetics of base pairing to render this antisense chimera inactive. Another factor may be that ribose 2′-*O*-methylation can block or slow endonucleolytic attack on the oligo, but this modification has relatively little effect on the rate of exonuclease degradation.

NIH207 (Fig. 1) was a 3′-cholesterol-modified analogue of NIH8281, synthesized to increase oligonucleotide stability toward nucleases and to increase cellular uptake (Letsinger et al., 1989; Boutorine et al., 1992; Boutorine and Kostina, 1993; Zelphati et al., 1994). Such compounds may be taken up by the LDL receptor. The 2-5A-antisense chimera NIH207 contained the parent NIH8281 structure tethered from the 3′-terminus through a 3′-phosphate and a seven atom spacer to the 1-hydroxyl function of cholesterol (Fig. 1). Although NIH207 inhibited RSV replication, it was not selective due to cytotoxicity (SI = 1) (Table 5). Various reports have noted both sequence non-specific (Letsinger et al., 1989) and toxic effects of cholesterol conjugated oligonucleotides (Reed et al., 1995). These effects have been attributed to increased hydrophobicity of the tethered cholesterol, leading to association with important cellular or viral proteins (Shea et al., 1990; Stein et al., 1991b).NIH500 was constructed to determine the need for 5′-phosphate sulfurization. The lack of activity (Table 5) of this chimera implied that the 5′-monothiophosphate of NIH8281 was required for anti-RSV activity,

most probably because the thioated monophosphate is resistant to the action of serum and cellular phosphates which can convert the chimera to the inactive 5′-unphosphorylated 'core' oligonucleotide. Previous studies have demonstrated convincingly that 5′-unphosphorylated 2′, 5′-oligoriboadenlyates possess a dramatically reduced ability to activate RNase L (Torrence et al., 1984; Player et al., 1998c) and that the biological activity of 2-5A-antisense chimeras also is dependent on the presence of the 5′monophosphate group (Torrence et al., 1993; Maran et al., 1994; Maitra et al., 1995; Cirino et al., 1997). In addition, when the 5′-monophosphate of 2-5A trimer was converted to the corresponding 5′-monothiophosphate, the resultant 2′, 5′-oligoriboadenylate was just as efficient an activator of RNase L as the parent 5′-monophosphate, but was extremely resistant to phosphatases (Xiao et al., 1994). This latter observation has been the rationale for the construction of 5′-monothiophosphoryated 2-5A-antisense chimeras whenever they are to be used in cell culture (Cirino et al., 1997).

The third objective of this research was to determine if other sequences within the RSV genome or within the various mRNAs would be suitable antiviral targets for inhibition by antisense chimeras. Determination of RNA secondary structure as an aid in the design of efficacious antisense oligonucleotides can be approached in several ways, including thermodynamic stability, phylogenetic comparison, chemical and enzymatic probing, and NMR (Chastin and Tinoco, 1993). The difficulties associated with these and other approaches have been reviewed (Chastin and Tinoco, 1993; Ecker, 1993; Freier, 1993; Milligan et al., 1993). Previously, we used computer-assisted analysis of RSV RNA secondary structure as a guide for the generation of potential anti-RSV 2-5A-antisense chimeras. In that study, the most active anti-RSV 2-5A-antisense chimeras were directed toward a large loop structure, as predicted by RNA structure analysis with the MFOLD program, in the M2 RSV mRNA (Cirino et al., 1997). Therefore, we subjected all the mRNAs of RSV to the same MFOLD program analysis, which determines the secondary structures of minimum free energy for RNA molecules based on published values of stacking and loop destabilizing energies. For the largest loops generated by this algorithm, 2-5A-antisense binding sites were selected in each the RSV mRNAs, except for the previously targeted M2 mRNA.

The RSV inhibitory chimeras, NIH8841, NIH8861, NIH9030, and NIH10180, all targeted the L mRNA, although NIH8841 may be of limited usefulness because of its toxicity limit (Table 6). It is well established that RSV genomic RNA is transcribed to yield the 5′-leader RNA and the 10 viral mRNAs in decreasing abundance with the first transcribed mRNAs (NS1 and NS2) being the most abundant and the last transcribed mRNA (L) being the least abundant (Collins et al., 1996). The greatest number of active 2-5A-antisense chimeras targeted sequences in the least abundant RSV transcript, which is consistent with the tenet that the mRNAs most susceptible to a successful antisense approach would be those of lowest abundance (Bischofberger and Wagner, 1992). Moreover, since there appears to be a direct correlation of RSV protein levels with mRNA abundance (Collins et al., 1996), a significant reduction in the M2 or L mRNAs would be expected to affect profoundly virus replication. Three other active 2-5A-antisense chimeras, including NIH8281, directed against the second least abundant M2 mRNA, have been reported (Cirino et al., 1997).

Most compounds targeting secondary structures in RSV mRNAs were not active (Table 6). Other investigations have also failed to discern a relationship between predicted RNA secondary structure and antisense oligonucleotide efficacy (Stull et al., 1992).

The active 2-5A-antisense chimeras found in this study may provide useful leads to develop additional compounds with enhanced anti-RSV activity by further chemical modifications that will improve oligonucleotide stability, uptake and subcellular distribution. Meanwhile, the synthesized 2-5A-antisense chimeras that lacked anti-RSV activity have pointed the way to molecular modifications with significant potential as RSV antivirals (Player et al., 1998a).

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