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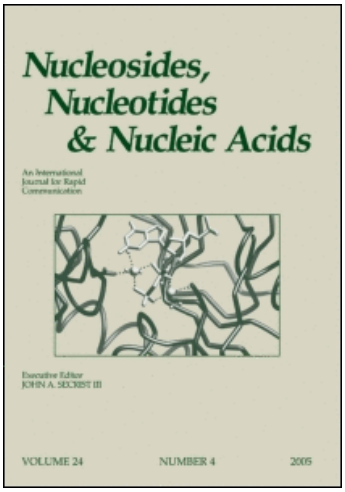

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Inhibition of Respiratory Syncytial Virus by Double Termini-Protected 2-5A Antisense Chimeras

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INHIBITION OF RESPIRATORY SYNCYTIAL VIRUS BY DOUBLE TERMINI-PROTECTED 2-5A ANTISENSE CHIMERAS

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ABSTRACT: Respiratory syncytial virus (RSV) replication was reduced by greater than 90% after treatment of infected human tracheal epithelial cell line, 9HTE, with double termini-protected 2-5 A antisense chimeras.

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Respiratory Syncytial Virus (RSV), a non-segmented, negative-stranded RNA virus in the pneumovirus family, is a widespread human pathogen accounting for over 1 million deaths per year worldwide. There is currently only one approved treatment for RSV, aerosolized ribavirin which has several limitations including minimal efficacy in clinical use, the potential to clog ventilating units etc.. Significant side effects and the high cost of ribavirin restrict its use for the majority of infected individuals.

"2-5A antisense" represents a unique class of chimeric oligonucleotides in which a small activator (2-5A) of the endoribonuclease RNase L is covalently coupled through linkers to antisense DNA directed against a targeted RNA[1-3]. Compared to antisense oligonucleotides currently being pursued as antiviral agents (e.g. to inhibit HIV replication), 2-5A antisense involves an alternative and potent mechanism of action in which the 2-5A activates the catalytic function of RNase L

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while the antisense domain serves as a specific high affinity binding site for the target RNA[4]. The result is selective cleavage of the target mRNA.

The 2-5 A antisense chimeras were synthesized through the covalent linkage of a 3',5'-antisense oligodeoxyribonucleotide and a 2',5'-oligoadenylate activator of the 2-5A-dependent RNase through a linker. To enhance resistance of the chimera to degradation by nuclease, we synthesized various of modified chimeras. Modification of the 3'-terminus with a 3'-3' phosphodiester linkage to the penultimate deoxynucleotide slowed 3'-exonuclease digestion. In addition, a 5'-thiophosphate was used to the 2-5 A domain of the chimera against phosphatase[5]. It was found that the double termini-blocked chimera was resistant to both 3'-exonuclease in and phosphatase. Such chemical modifications showed a substantially enhanced anti-RSV activity.

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double termini-blocked anti-RSV/8490-8509 chimera

ps5'A2'(p5'A2')$_3$-pBu-pBu-d(5'AATGGGATCCATTTTGTC3'-***3'C)***

To develop 2-5 A antisense chimeras with the potential to block RSV replication, we first select an oligonucleotide binding site in the viral RNA polymerase (RSV L) mRNA (binding site 8490-8509), which encodes a low abundance message that is absolutely required for RSV replication. 9HTE cells were infected with RSV and subsequently treated three times (2, 12 and 24 hours) with three concentrations of oligonucleotides and virus was harvested after 36 hours. Chimeric antisense lacking only the 5'-phosphate deficient in the ability to activate RNase L, was used as a control and showed only minimal anti-RSV activity. The "tailed" pA$_4$-5'antiRSV3'-3'C/(8490-8509) produced 1.6-fold enhanced antiviral activity at 6.6 mM compared with the unmodified chimera, pA$_4$-5'antiRSV3'/8490-8509). Alternately, a 5'-thiophosphated 2-5A were previously shown to be fully capable of activating RNase L[3] and spA$_4$-5'antiRSV3'/8490-8509) showed a substantially increase antiRSV effect with 71%

and 94% inhibition of viral growth at treatment concentrations of 6.6 and 9.9 mM respectively.

To further improve the anti-RSV activity of the 2-5A antisense chimeras, the double termini-blocked chimera was used for increasing the stability of the chimera in the cell lines. Meanwhile, a computer-assisted analysis of the secondary structure of RSV mRNA was performed to identify single-stranded region as oligonucleotide binding sites. Computer prediction of the secondary structure of RSV RNA nucleotides 7900-9080, including a 3' portion of the M2 gene, encoding a viral envelope protein, and a 5' region of the L gene, was performed using the program FoldRNA which finds a secondary structure of minimum free energy for an RNA molecule based on published values of stacking and loop destabilizing energies. This loop was present in a 90 codon open reading frame of unknown function downstream (3') of the major M2 open reading frame. Three chimeric compounds were synthesized which were complementary to sequences in the loop, spA_4-antiRSV3'-3'A/(8251-8270), spA_4-antiRSV3'-3'T/(8261-8279) and spA_4-antiRSV3'-3'T/(8281-8299). In addition, three oligonucleotides were synthesized to other regions in RNA that included a bulge, a hair-pin and a small loop respectively. When added to the infected 9HTE cells at concentrations of 3.3 mM, the three oligonucleotides directed to the large loop had the greatest level of antiviral activity (>90%). The chimera with the greatest anti-RSV effect was spA_4-antiRSV3'-3'T/(8281-8299).

Several conclusions are apparent based on the above result: (1) anti-RSV activity of 2-5A antisense can be greatly improved by double termini-protection of the chimeras; (2) the effective 2-5A antisense can be designed based on the computer-assisted analysis of secondary structure of RSV mRNA which the single-stranded large loop region as binding site; (3). the specific 2-5A antisense functions as a very effective anti-RSV agents and have the potential to be developed as agents for the treatment of active RSV infection in humans.

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