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Biological effects of hexitol and altritol-modified siRNAs targeting B-Raf

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ABSTRACT

Increasing the effectiveness of siRNAs through chemical modification is an important task. Here we describe altritol and hexitol modified oligonucleotides targeting the B-Raf oncogene that is critical for the growth and survival of melanoma cells. Using assays for apoptosis, DNA synthesis, colony formation and B-Raf protein and message levels, we demonstrate that certain hexitol modifications can improve the effectiveness of B-Raf siRNAs and also increase duration of action. Altritol modified siRNAs were similar to or slightly less effective than unmodified B-Raf siRNA. Modifications at the 3′ or 5′ end of the sense strand, at the 3′ end of the antisense strand, or within either strand were well tolerated. The basis for the increased effectiveness of the hexitol-modified siRNAs is not fully understood but may be partly due to increased stability to nucleases.

1. Introduction

There is currently substantial interest in the potential therapeutic utilization of RNA interference (RNAi) (de Fougerolles et al., 2007). In order to therapeutically exploit RNAi in humans it will be essential to develop siRNAs that are specific, potent, persistent in their action, and suitable for effective *in vivo* delivery. Many of these issues can be addressed through various chemical modification approaches. A variety of strategies for siRNA modification have been pursued including alterations in the backbone chemistry, 2′-sugar modifications, nucleobase modifications and others, as recently reviewed (Corey, 2007; De Paula et al., 2007; Kurreck, 2003; Manoharan, 2004; Peek and Behlke, 2007). One approach that we have extensively employed is to replace the pentose ring of RNA with six carbon moieties forming altritol, cyclohexenyl, or hexitol nucleic acids (Allart et al., 1999; Froeyen et al., 2000). We have recently demonstrated that atritol-modified siRNAs targeting the mRNA for *MDR1* (a drug resistance gene) had a stronger and more persistent effect than unmodified siRNAs; this was particularly true in cases where altritol modifications were at the 3′ ends of the sense or antisense strands (Fisher et al., 2007). Incorporation of cyclohexenyl moieties into selected positions of MDR1 siRNA also increased activity, possibly due to an increase in nuclease stability (Nauwelaerts et al., 2007).

The mechanism of targeted mRNA degradation by siRNA is complex and not yet fully understood. It involves the formation of an RNA-induced silencing complex (RISC) that contains the Argonaute 2 protein and that specifically cleaves mRNAs complementary to the antisense (guide) strand of the siRNA (Valencia-Sanchez et al., 2006). When designing modified siRNAs, some key parameters should be considered. Effective siRNA duplexes display reduced thermodynamic stability at the 5′-end of the antisense siRNA relative to the 3′-end (Reynolds et al., 2004). RISC cleaves the target mRNA near the middle of the complementary region ten nucleotides upstream of the nucleotide at the 5′-end of the guide strand yielding 5′-phosphate and 3′-hydroxyl termini. Based on these observations, modifications at the 5′-end of the antisense strand that increase 5′ thermodynamic stability, or impede 5′-O-phosphorylation, as well as modifications in the middle of the duplex that interfere with RNase cleavage, are likely to reduce siRNA activity. However, it has been demonstrated that some modifications, such as 2′-OMe and 2′-F modified nucleoside residues, can be incorporated at the cleavage site without interfering with nucleolytic activity (Czauderna et al., 2003; De Paula et al., 2007).

In the present report we have extended our studies of chemically modified siRNAs to examine hexitol and altritol modifications placed at the 3′ ends of the sense or antisense strands, at the 5′ end of sense strands, or adjacent to the site of endonucleolytic cleavage. We have designed siRNAs that target the message for human B-Raf, a member of the Raf family of serine/threonine kinases that are key elements in the Erk MAP Kinase (Extracellular Signal Regulated Kinase, Mitogen Activated Protein Kinase) signalling pathway that is essential for mitogenesis and survival. Activating B-Raf mutations occur in approximately 50% of human melanomas, with the most common form being the V600E mutation (Davies et al., 2002; Schreck and Rapp,

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2006). Melanoma cells are highly dependent on B-Raf activity for growth and survival as demonstrated by increased apoptosis and reduced cell growth rates subsequent to treatment with pharmacological inhibitors of Raf kinases, or by siRNA-mediated inhibition of B-Raf (Boisvert-Adamo and Aplin, 2006; Karasarides et al., 2004), or specifically of B-Raf V600E. In the current study we have utilized 20-mer hexitol or altritol modified siRNAs targeted to sites spanning the coding sequences flanking amino acids 461 or 600. After transfection of the various B-Raf targeted siRNAs into A375 human melanoma cells, we have evaluated effects on apoptosis, DNA synthesis, colony forming ability and levels of B-Raf protein and message. Our results indicate that hexitol and altritol modifications are well tolerated and that some hexitol modifications lead to significant increases in activity of B-Raf directed siRNAs, as well as to increased duration of action.

2. Materials and methods

2.1. Synthesis and characterization of oligonucleotides

Altritol and hexitol modified 20-mer oligonucleotides were synthesized using classical phosphoramidite chemistry on solid supports as previously described (Allart et al., 1999; Froeyen et al., 2000) and were confirmed by mass spectrometry. The unmodified siRNA oligonucleotides and non-targeting siRNA oligonucleotide (cat# D0001210-01-20, 5′-UAGCGACUAAACACAUCAAUU 3′) were made by Dharmacon.

2.2. Cell culture

A375 human melanoma cells were obtained from E. Sharpless (UNC) and were grown in DMEM-H medium containing 10% FBS. The cell line was grown in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.3. Treatment of cells with siRNA oligonucleotides

A375 cells were cultured as described above. Hybridization of the siRNA strands was done in Dharmacon universal buffer by heating the solutions to 90 °C in a Perkin Elmer PCR machine for 1.5 min, then gradually cooling to 30 °C for 30 min. Transfection of siRNA using Dharmafect 1 (Dharmacon,Lafayette, CO) was done according to the manufacturer's standard procedure. The oligonucleotides bound to Dharmafect 1 were mixed in 10% FBS/DMEM-H and incubated with cells at 37 °C for 72 h. Cells were either plated in 6 well or 12 well plates depending on experiment. All experiments were done with siRNA sequences termed BRAF461 (antisense sequence 5′-AUGAUCCAGAUCCAAUUCUdTdT-3′) or BRAF600 (antisense sequence 5′-AUCGAGAUUUCUCUGUAGC dTdT-3′); these sequences span the codons for amino acids 461 or 600 of the B-Raf protein.

2.4. Apoptosis and DNA synthesis assays

Apoptosis was measured using an Annexin V staining kit (Trivigen, Gaithersberg, MD) followed by flow cytometry, usually at 3 days post transfection. Briefly, cells were harvested with typsin EDTA, suspended in complete medium and washed once. Cells (2.5×10^5) were incubated in the presence of Annexin V (diluted 1/250) for 30′ and then processed by flow cytometry. DNA synthesis was measured by a Cell Proliferation BrdU ELISA assay (Roche Diagnostics, Mannheim, Germany). Briefly, 1.25×10^4 transfected cells were plated in 96 well plates for 72 h. The BrdU ELISA was performed according to the manufacturer's standard procedure. The ELISA was read at 405 nM on a Bio-Tek El_x800 microplate reader.

2.5. Cell growth assay

A375 cells were treated with Dharmafect 1 complexes of standard or chemically modified siRNA oligos as described above. Cells were then washed with PBS, harvested with trypsin-EDTA and resuspended in 1 ml of a complete medium. The final cell number was enumerated by using an electronic particle counter (Particle Data, Elmhurst, IL).

2.6. Colony-formation assay

Seventy-two hours after transfection, 100 A375 cells from each treatment group were replated in 6 well plates containing a mixture 1% low gelling temperature agarose (SeaKem, Rockland, ME) and complete DMEM-H medium with 10% FBS. After 14 days, surviving colonies larger than 25 cells were counted. Survival was expressed as total colonies/well.

2.7. Measurement of B-Raf expression by western blotting

A375 cells were transfected with various siRNAs for 3 days. Cells were lysed with modified RIPA buffer and equal amounts of protein were resolved on 10% SDS-polyacrylamide gels and blotted onto polyvinyl difluoride membranes (Millipore, Bedford, MA). B-Raf was detected using mouse monoclonal anti-B-Raf antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1000 and tubulin was detected using mouse monoclonal anti-tubulin antibody (Sigma) at a

Table 1
Hexitol and altritol modified RNAs

Sense strand (5′→3′)							
Hexitol modified targeting AA 461							
2489	AGA	AUU	GGA	UCU	GGA	U**CA**	UdTdT
2517	**AG**A	AUU	GG**A**	**U**CU	GGA	U**CA**	UdTdT
2685	AGA	AUU	GGA	UCU	GGA	UC**A**	UdTdT
Altritol modified targeting AA 461							
2499	AGA	AUU	GGA	UCU	GGA	U**CA**	UdTdT
2500	AGA	AUU	GG**A**	**U**CU	GGA	U**CA**	UdTdT
2501	**AG**A	AUU	GG**A**	**U**CU	GGA	U**CA**	UdTdT
2681	AGA	AUU	GGA	UCU	GGA	UC**A**	UdTdT
Antisense strand (5′→3′)							
Hexitol modified targeting AA 461							
2492	AUG	AUC	CAG	AUC	CAA	U**UC**	UdTdT
2493	AUG	AUC	CAG	**A**UC	CAA	U**UC**	UdTdT
2518	AUG	AUC	CA**G**	**AU**C	CAA	UUC	UdTdT
2686	AUG	AUC	CAG	AUC	CAA	UU**C**	UdTdT
Altritol modified targeting AA 461							
2502	AUG	AUC	CAG	AUC	CAA	U**UC**	UdTdT
2503	AUG	AUC	CAG	**A**UC	CAA	U**UC**	UdTdT
2519	AUG	AUC	CA**G**	**AU**C	CAA	UUC	UdTdT
2682	AUG	AUC	CAG	AUC	CAA	UU**C**	UdTdT
Sense strand (5′→3′)							
Hexitol modified targeting AA 600							
2494	GCU	ACA	GAG	AAA	UCU	C**GA**	UdTdT
2495	GCU	ACA	GA**G**	AAA	UCU	C**GA**	UdTdT
2496	**GC**U	ACA	G**AG**	AAA	UCU	C**GA**	UdTdT
2687	GCU	ACA	GAG	AAA	UCU	CG**A**	UdTdT
Altritol modified targeting AA 600							
2504	GCU	ACA	GAG	AAA	UCU	C**GA**	UdTdT
2505	GCU	ACA	G**AG**	AAA	UCU	C**GA**	UdTdT
2506	**GC**U	ACA	G**AG**	AAA	UCU	C**GA**	UdTdT
2683	GCU	ACA	GAG	AAA	UCU	CG**A**	UdTdT
Antisense strand (5′→3′)							
Hexitol modified targeting AA 600							
2497	AUC	GAG	AUU	UCU	CUG	U**AG**	CdTdT
2498	AUC	GAG	AUU	UC**U***	CUG	U**AG**	CdTdT
2688	AUC	GAG	AUU	UCU	CUG	UA**G***	CdTdT
Altritol modified targeting AA 600							
2507	AUC	GAG	AUU	UCU	CUG	U**AG**	CdTdT
2508	AUC	GAG	AUU	UC**U**	CUG	U**AG**	CdTdT

Modified nucleosides are shown in **bold**.