Published in final edited form as:
Eur J Pharmacol. 2009 March 15; 606(1-3): 38–44. doi:10.1016/j.ejphar.2009.01.030.

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

Michael Fisher[†], **Mikhail Abramov**[*], **Arthur Van Aerschot**[*], **Jef Rozenski**[*], **Vidula Dixit**[†], **Rudy L. Juliano**[†,#], and **Piet Herdewijn**[*]

[*] *Laboratory of Medicinal Chemistry, Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium*

[†] *Department of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill NC 27599, USA, Ph 919 9664383, Fax 919 966 5640, arjay@med.unc.edu*

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.

Index words

siRNA; hexitol; altritol; chemical modification; B-Raf; stability

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).

#corresponding author.

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, 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, Lafyette, 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 h 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 per 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). BRAF 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 dilution of 1:1000 followed by peroxidase-conjugated goat anti-mouse IgG antibody (Calbiochem, San Diego, CA) at a dilution of 1:5000. Signals were detected by enhanced chemiluminescence (ECL kit, Amersham Biosciences Inc). Image analysis was performed with Scion Imaging Software (Fredrick, MD).

2.8. RT-PCR

RNA was isolated at times indicated using TRI REAGENT (Molecular Research Inc, Cincinnati, OH) as per manufacturer's instructions. cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. The B-Raf signal was normalized using human actin as an internal control. Primers were purchased

from the Oligonucleotide Synthesis Core Facility, University of North Carolina. B-Raf forward and reverse primer sequences were 5′CGAATGAAAACACTTGGTAGACGG3′ and 5′ CCCTGTTGTTGATGTTTGAATAAGG respectively. Actin forward and reverse primers were 5′GCTCGTCGTCGACAACGGCT3′ and 5′ CAAACATGATCTGGGTCATCTTCTC3′.

2.9. Nuclease stability of hexitol and altritol modified siRNAs

Standard B-Raf siRNA and modified siRNA's were exposed to 10% fetal bovine serum at 37° C for various times and degradation was monitored by 3% agarose gel electrophoresis with ethidium bromide staining.

3. Results

The altritol and hexitol modified oligonucleotides used in this study are described in Table 1 and the structures of the nucleotides are given in Fig 1A. As indicated, modifications in sense and antisense strands were placed at various positions particularly at or near the 3′ end or in the middle of the sequence near the presumed cleavage site; in some cases modifications were placed at the 5′ end of the sense strand. No modifications of the 5′ end of the antisense strand were used since previous work had shown them to be inactive (Fisher et al., 2007).

In order to rapidly screen the various chemically modified oligonucleotides for biological activity, we adopted a convenient assay for the initiation of programmed cell death based on the binding of FITC- tagged annexin V to the surface of apoptotic cells; the amount of binding is then quantitated by flow cytometry (Steensma et al., 2003). This screening approach was chosen because inhibition of B-Raf is known to trigger apoptosis in melanoma cells (Boisvert-Adamo and Aplin, 2006; Karasarides et al., 2004). This simple initial screen can then be followed by more detailed analysis of promising candidates. As illustrated in the flow cytometry histograms of Fig 1B, non–targeted siRNA produced only a slight increase over control in terms of the number of apoptotic cells. By contrast, both unmodified B-Raf siRNA, and a B-Raf siRNA that was hexitol modified in both strands, produced significant increases in apoptosis, with the modified material displaying greater activity than the unmodified oligonucleotide. Using this assay we generated dose-response curves for the same pair of unmodified or hexitol -modified siRNAs and one additional hexitol duplex. As seen in Fig 1C, significant effects on annexin V binding were seen at 50nM siRNA; however the dose-response curve rose progressively to 200nM oligonucleotides. At all doses the hexitol-modified duplexes tested were more active than the unmodified B-Raf siRNA. Relatively high concentrations of siRNAs were needed to attain robust effects in terms of cytotoxicity. We believe that this is due to the fact that a very substantial reduction in B-Raf levels is required to fully inhibit mitogenic signaling pathways (Boisvert-Adamo and Aplin, 2006; Karasarides et al., 2004). The hexitol duplexes utilized in Figs 1B, C were chosen based on preliminary screening similar to that more fully illustrated in Fig 2 below.

Based on the findings of Figs 1, we screened all of the hexitol- and altritol -modified oligonucleotides in Table 1 using the annexin V assay and treating cells at a concentration of 200nM. The siRNA duplexes tested contained either one modified (hexitol or altritol) strand hybridized to an unmodified strand, or were composed of two modified strands. Fig 2A illustrates the results for modifications of a single strand and Fig 2B for the cases where both strands were modified. As indicated, many of the hexitol modified siRNAs were as or more active than the corresponding unmodified BRAF 461 or BRAF 600 siRNAs. In some cases the modified duplexes were substantially more active. For example, several hexitol duplexes targeting the B-Raf 600 site were approximately twice as effective in inducing apoptosis as the unmodified BRAF600 (2495/2497, 2595/2498, 2496/2497, 2496/2498). This overall observation was repeated in several independent experiments. Interestingly, these siRNAs had

at least one hexitol modification near the presumed site for cleavage of the mRNA target. In this assay the altritol modifications seemed somewhat less helpful than the hexitol modifications, with most of the altritol duplexes showing effects similar or slightly less than those of the unmodified BRAF600 or BRAF461 siRNAs. However, none of the modifications abrogated siRNA activity and thus it is clear that altritol and hexitol modifications at various sites are well tolerated. In addition to the siRNAs illustrated in Fig 2, we also screened several oligomers (# 2681-2688) with a single modification at the 3′ end of the sense or antisense strand, since this type of modification seemed promising in previous work on another gene target (Fisher et al., 2007). However, none of the singly modified oligonucleotides were as effective as some of the doubly or multiply modified compounds illustrated in Fig 2 (data not shown).

Since it is unwise to depend on a single assay to evaluate cytotoxic effects on cells, we also examined several other parameters. Fig 3A displays effects of several siRNAs on DNA synthesis monitored using a BrdU incorporation assay; as indicated, the B-Raf targeted siRNAs reduced BrdU incorporation while the control siRNAs did not. A doubly modified hexitol siRNA displayed slightly greater activity than the corresponding unmodified version but the result was not statistically significant in this case. In a soft agar colony formation assay (Fig 3B), the doubly modified 2495/2497 duplex produced a major reduction of cell viability, significantly greater than that attained by the BRAF600 unmodified version. Thus several assays indicate that B-Raf siRNAs (but not control siRNAs) caused reductions in cell viability and that in most cases hexitol -modified siRNAs were somewhat more effective.

We have also evaluated effects of the B-Raf siRNAs at the biochemical level. As indicated in Fig 4A, the B-Raf 600 siRNAs caused a substantial reduction in the amount of B-Raf protein as detected by western blotting. Once again a hexitol modified duplex (2495/2497) had a greater effect than the unmodified siRNA, although both produced a marked reduction in B-Raf protein levels without affecting levels of a control protein (tubulin). Interestingly, significant ‘knock down’ of B-Raf was attained with as little as 25nM siRNA; however, substantially higher concentrations were needed to attain strong effects on apoptosis or reduction of colony formation (see above). This indicates that the melanoma cells can still function with a reduced level of B-Raf and that rather profound ‘knock down’ or pharmacological inhibition is needed to attain therapeutic effects, in agreement with other studies (Boisvert-Adamo and Aplin, 2006;Karasarides et al., 2004). In Fig 4B we demonstrate that siRNAs targeting the B-Raf 461 site can also effectively reduce levels of B-Raf protein without affecting tubulin; however, in this case little difference was noted between the hexitol modified (2489/2493) and unmodified siRNAs. We have also examined effects at the mRNA level using semi-quantitative RT-PCR. As seen in Fig 5, B-Raf 600 siRNAs caused a significant dose-dependent reduction in B-Raf message without affecting levels of actin message.

An important issue concerns the duration of action of siRNAs. To address this we performed a time-course analysis comparing effects of an hexitol modified duplex with that of unmodified B-Raf siRNA. As seen in Fig 6, both modified and unmodified B-Raf 600 siRNAs produced substantial reductions in B-Raf protein levels at 72h, with a somewhat greater effect for the hexitol compound. However, between 96h and 120h the cells treated with the unmodified siRNA almost completely regained their pre-exposure levels of B-Raf, while the cells treated with the hexitol modified siRNA displayed much lower levels. Recovery of the cells treated with hexitol modified B-Raf siRNA was not complete until 160h. Thus the hexitol modified siRNA seemed to have a substantially increased duration of action as compared to unmodified B-Raf siRNA.

The basis for the increased effectiveness and duration of action of the hexitol modified siRNA is not clear. One contribution may come from increased nuclease stability. Thus, as shown in

Fig 7, a hexitol modified duplex (2495/2497) displayed increased stability to serum nucleases as compared to an unmodified siRNA of the same sequence. However, other, as yet to be determined, aspects of the modified siRNAs may also contribute to their effectiveness.

4. Discussion

There is increasing interest in pharmacological manipulation of Raf kinases for cancer therapeutics, with both conventional small molecule and siRNA approaches being actively pursued (Gollob et al., 2006; Schreck and Rapp, 2006). Obviously it would be advantageous to be able to produce very active siRNAs able to selectively target mutant B-Raf. In this report we have demonstrated that some hexitol modifications can increase the efficacy of of B-Raf siRNA by a factor of two or more. This report also represents the first time that the hexitol modification has been used in an siRNA. We have employed a rapid annexin V-based screening approach to identify modified siRNAs with a strong biological effect (apoptosis). While we believe that much of the apoptotic effect is due to 'knock down' of the B-Raf target, we cannot completely exclude other possibilities. Thus, it is well known that siRNAs can have off-target effects due both to induction of interferon pathways (Schlee et al., 2006) and to miRNA effects related to partial sequence matches (Birmingham et al., 2006). Thus some promising candidates in the apoptosis assay were further studied using more direct biochemical assays such as western blotting for B-Raf.

A variety of chemical modifications have been employed to improve the characteristics of siRNA (De Paula et al., 2007; Manoharan, 2004). Phosphorothioate modifications of the backbone at the ends of strands have proven useful for stabilization against nuclease degradation. Incorporation of 2′- F residues is well tolerated and can increase hybridization, while several 2′-O methyl modifications are also usually well tolerated although full modification can lead to loss of activity. LNA modifications provide increased duplex stability, but only a small number of positions can tolerate such modifications. Replacing the pentose ring with a six-membered ring as in hexitol, altritol and cyclohexenyl oligonucleotides allows formation of A-type duplexes with RNA targets (Fisher et al., 2007; Nauwelaerts et al., 2007), a key point for siRNA effectiveness. We have previously shown that such modifications are well tolerated at 3′ positions and at some interior positions, but not at the 5′ position of the antisense strand (probably because of the inability of the modified 5′ moiety to be phosphorylated by cellular kinases) (Fisher et al., 2007). In some cases altritol and cyclohexenyl modifications demonstrated substantially improved efficacy over unmodified siRNA, while in the current study hexitol modifications provided improved results. The basis of these effects is not clear at present; in addition to nuclease stability, one possibility is that the six-membered ring modification assists in efficient loading of the RISC complex, but this remains to be determined.

In this study we have shown that hexitol and altritol modifications of B-Raf siRNAs are well tolerated at 3′ sites of sense and antisense strands, as expected, but also at sites adjacent to the position on the antisense strand where mRNA cleavage takes place (for example 2493,2518,2503,2498,2508). This suggests that the hexitol or altritol modifications can be used relatively freely in siRNAs. While some modifications, particularly hexitols, provided substantial improvements in effect over unmodified B-Raf siRNAs, this was not true of all of the substitutions. Somewhat to our surprise, the altritol modifications were not particularly beneficial in this system although they did not reduce activity to any great degree. This contrasts with our previous study using altritol -modified siRNAs against another gene target (Fisher et al., 2007) where improved effectiveness was noted. Thus chemical modification strategies with these moieties may need to be carefully optimized for particular siRNA targets rather than being universally applicable. An important finding in this study is the increased duration of action of hexitol modified siRNA; this could have interesting ramifications for therapeutic use

of this type of oligonucleotide. In summary, since these six-membered ring entities provide both good thermodynamic stability and partial protection against nuclease cleavage (Allart et al., 1999; Froeyen et al., 2000) (Nauwelaerts et al., 2007), the hexitol and altritol chemistries may prove to be a useful addition to strategies for improving siRNA effectiveness.

Acknowledgements

This work was supported by NIH grant PO1 GM59299 (to RLJ) and by KULeuven (GOA) (to PH). We thank Betsy Clarke for excellent editorial assistance.

References

Allart B, Khan K, Rosemeyer H, Schepers G, Hendrix C, Rothenbacher K, Seela F, Van Aerschot A, Herdewijn P. Properties of oligonucleotides with six membered carbohydrate mimics and a 1,4-relationship between the base moity and the hydroxmethyl group. Chem Eur J 1999;5:2424–2431.

Birmingham A, Anderson EM, Reynolds A, Ilsley-Tyree D, Leake D, Fedorov Y, Baskerville S, Maksimova E, Robinson K, Karpilow J, Marshall WS, Khvorova A. 3′ UTR seed matches, but not overall identity, are associated with RNAi off-targets. Nature Methods 2006;3:199–204. [PubMed: 16489337]

Boisvert-Adamo K, Aplin AE. B-RAF and PI-3 kinase signaling protect melanoma cells from anoikis. Oncogene 2006;25:4848–4856. [PubMed: 16547495]

Corey DR. RNA learns from antisense. Nat Chem Biol 2007;3:8–11. [PubMed: 17173018]

Czauderna F, Fechtner M, Dames S, Aygun H, Klippel A, Pronk GJ, Giese K, Kaufmann J. Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. Nucleic Acids Res 2003;31:2705–2716. [PubMed: 12771196]

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flaltritolgan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA. Mutations of the BRAF gene in human cancer. Nature 2002;417:949–954. [PubMed: 12068308]

de Fougerolles A, Vornlocher HP, Maraganore J, Lieberman J. Interfering with disease: a progress report on siRNA-based therapeutics. Nat Rev Drug Discov 2007;6:443–453. [PubMed: 17541417]

De Paula D, Bentley MV, Mahato RI. Hydrophobization and bioconjugation for enhanced siRNA delivery and targeting. Rna 2007;13:431–456. [PubMed: 17329355]

Fisher M, Abramov M, Van Aerschot A, Xu D, Juliano RL, Herdewijn P. Inhibition of MDR1 expression with altritol-modified siRNAs. Nucleic Acids Res 2007;35:1064–1074. [PubMed: 17264131]

Froeyen M, Wroblowski B, Esnouf R, De Winter H, Allart B, Lescrinier E, Herdewijn P. Molecular dynamics studies of single stranded hexitol, altritol, mannitol, and ribose nucleic acides (HEXITOL, MNA, ALTRITOL, and RNA, resp.) and of the stability of HND, RNA, ALTRITOL-RNA and MNA-RNA duplexes. Helv Chim Acta 2000;83:2153–2182.

Gollob JA, Wilhelm S, Carter C, Kelley SL. Role of Raf kinase in cancer: therapeutic potential of targeting the Raf/MEK/ERK signal transduction pathway. Semin Oncol 2006;33:392–406. [PubMed: 16890795]

Karasarides M, Chiloeches A, Hayward R, Niculescu-Duvaz D, Scanlon I, Friedlos F, Ogilvie L, Hedley D, Martin J, Marshall CJ, Springer CJ, Marais R. B-RAF is a therapeutic target in melanoma. Oncogene 2004;23:6292–6298. [PubMed: 15208680]

Kurreck J. Antisense technologies. Improvement through novel chemical modifications. Eur J Biochem 2003;270:1628–1644. [PubMed: 12694176]

Manoharan M. RNA interference and chemically modified small interfering RNAs. Curr Opin Chem Biol 2004;8:570–579. [PubMed: 15556399]

Nauwelaerts K, Fisher M, Froeyen M, Lescrinier E, Aerschot AV, Xu D, DeLong R, Kang H, Juliano RL, Herdewijn P. Structural characterization and biological evaluation of small interfering RNAs containing cyclohexenyl nucleosides. J Am Chem Soc 2007;129:9340–9348. [PubMed: 17616127]

Peek AS, Behlke MA. Design of active small interfering RNAs. Curr Opin Mol Ther 2007;9:110–118. [PubMed: 17458163]

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. Rational siRNA design for RNA interference. Nat Biotechnol 2004;22:326–330. [PubMed: 14758366]

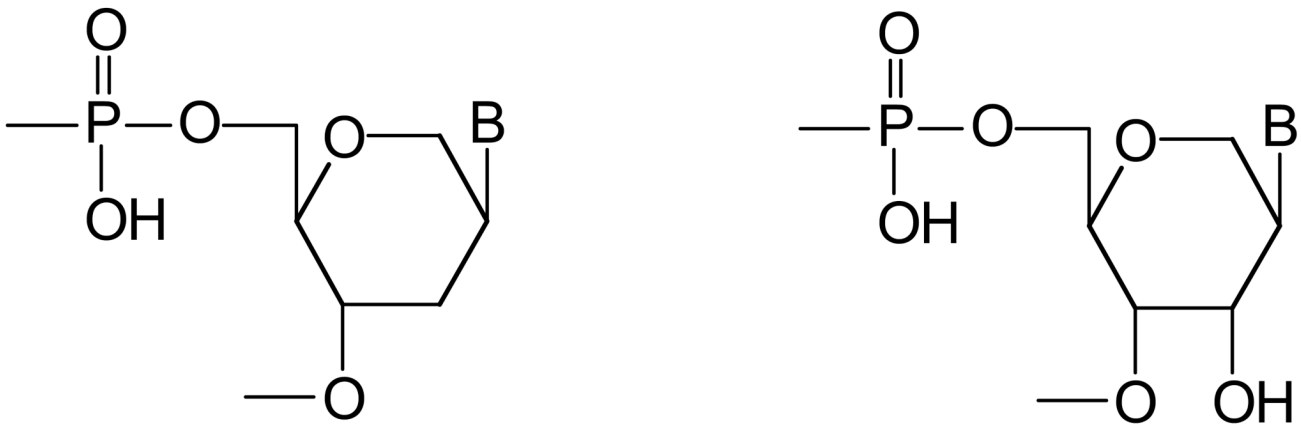
Schlee M, Hornung V, Hartmann G. siRNA and isRNA: two edges of one sword. Mol Ther 2006;14:463–470. [PubMed: 16877044]

Schreck R, Rapp UR. Raf kinases: oncogenesis and drug discovery. Int J Cancer 2006;119:2261–2271. [PubMed: 16894562]

Steensma DP, Timm M, Witzig TE. Flow cytometric methods for detection and quantification of apoptosis. Methods Mol Med 2003;85:323–332. [PubMed: 12710217]

Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R. Control of translation and mRNA degradation by miRNAs and siRNAs. Genes Dev 2006;20:515–524. [PubMed: 16510870]

Fig 1A



HNA ANA

Fig 1B

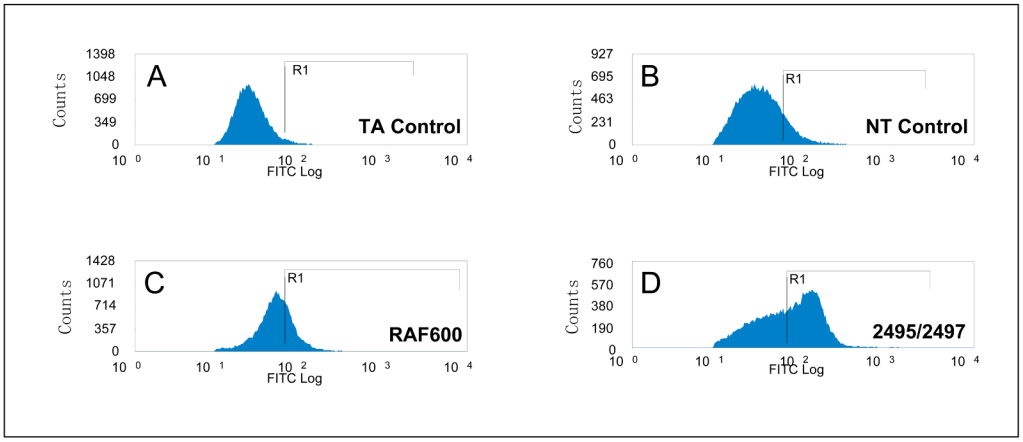


Fig 1C

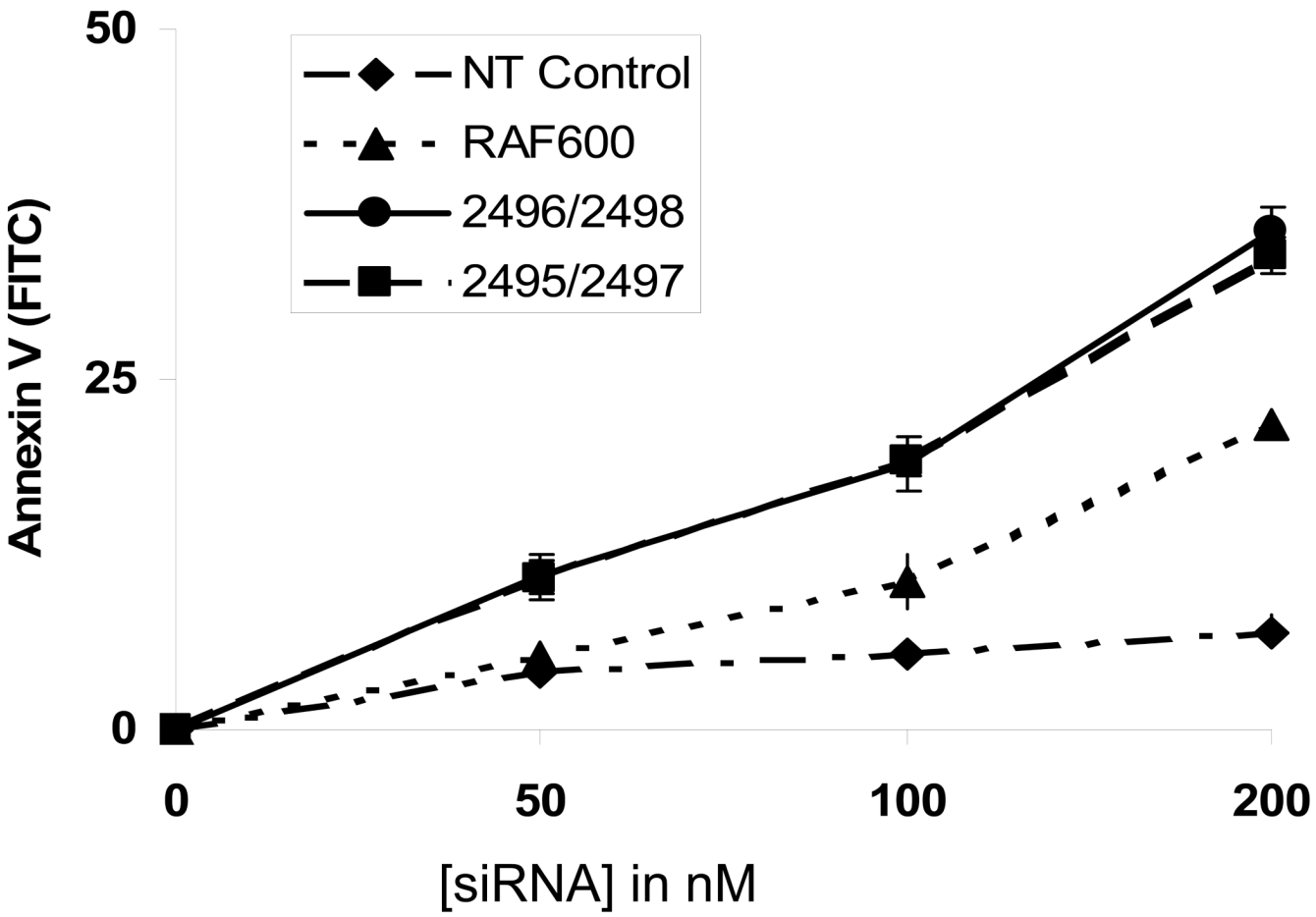


Fig 1.

Fig 1A. *Structures of the modified HNA and ANA nucleotides as repeating units of an oligonucleotide*.

Fig 1B. *FACS analysis after staining with Annexin-V to detect apoptotic cells*. A375 cells were treated for 3 days with Dharmafect 1 transfection agent alone (TA), 200 nM non-targeting (NT) siRNA's, 200 nM conventional unmodified B-Raf siRNA (RAF600), or 200 nM duplexed hexitol modified B-Raf siRNA (2495/2497). Apoptotic cells were estimated by FITC-annexin V binding and flow cytometry. The abscissa represents the relative fluorescence, and the ordinate the number of cells. A window of annexin V binding (R1) was set to exclude all but ~3% of the TA control population. The number of apoptotic cells due to the other treatments was then estimated as the percentage of cells in the R1 window.

Fig 1C. *Dose–response curves for hexitol modified siRNAs*. A375 cells were treated with various concentrations of non-targeting (NT) siRNA's, conventional unmodified siRNA (RAF600) or duplexed hexitol modified siRNAs (2495/2497, or 2496/2498) for 3 days at 37° C in complete medium. Cells were monitored for Annexin V-FITC expression by flow cytometry. Results are the means and standard errors of triplicate determinations.

FIG 2A

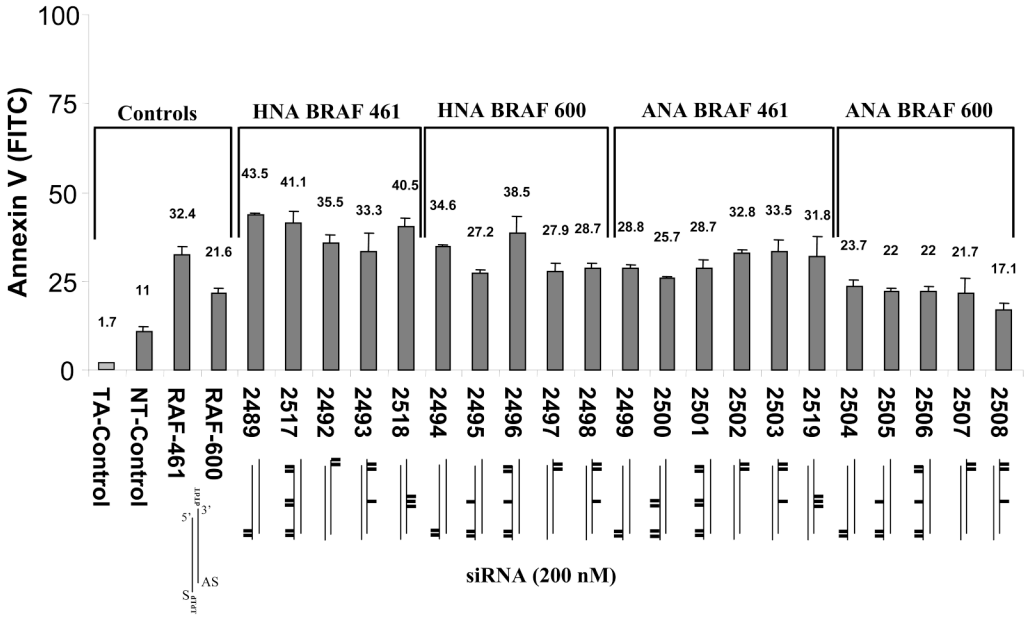


FIG 2B

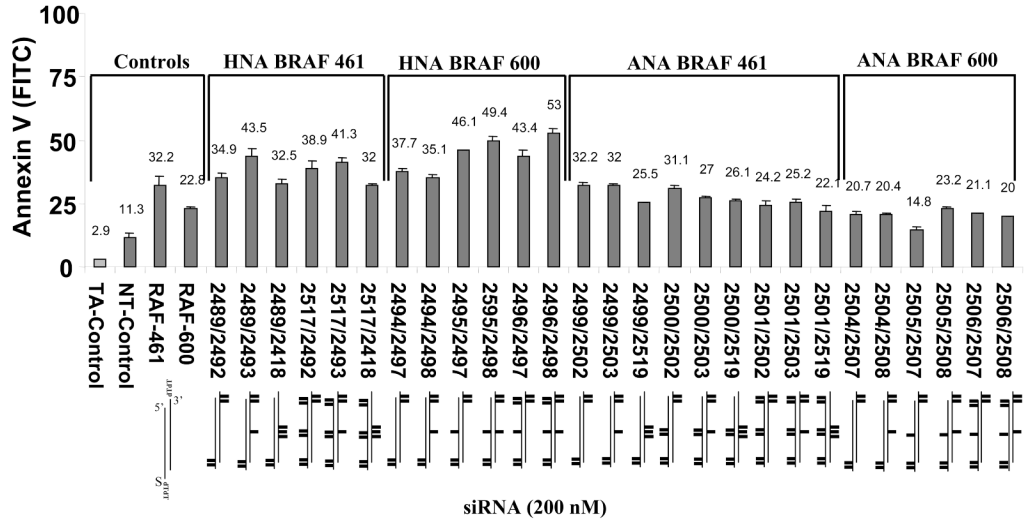


Fig 2.

Fig 2A. *Hexitol / altritol single modified siRNAs-annexin V assay*. A375 cells were treated for 3 days with Dharmafect 1 alone (TA), non-targeting (NT) siRNA's, conventional unmodified siRNA, or modified siRNA's, all at 200 nM, as indicated in the figure. Cells were harvested and were monitored for Annexin V-FITC binding by flow cytometry as described in Fig 1. Results are means and standard errors of triplicate determinations.

Fig 2B. *Hexitol / altritol doubly modified siRNAs-annexinV assay*. The same procedure was used to test doubly modified siRNA duplexes, as indicated in the figure. Results are means and standard errors of triplicate determinations.

Fig 3A

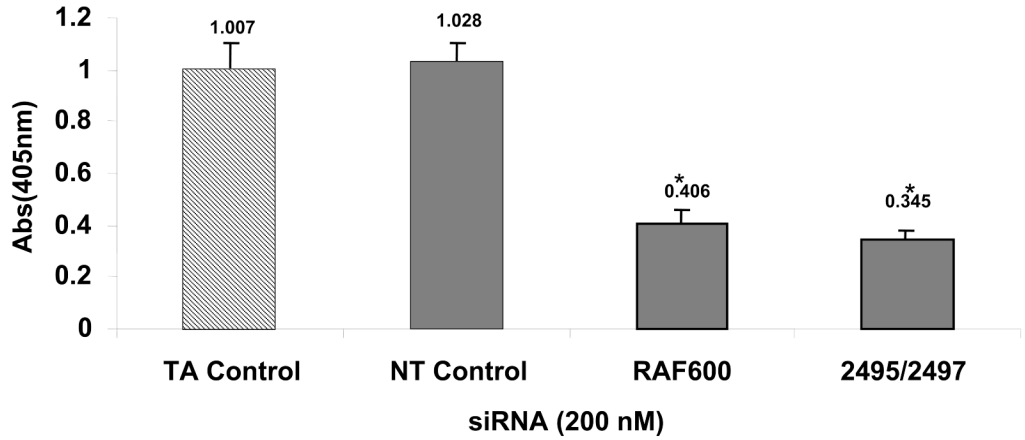


Fig 3B

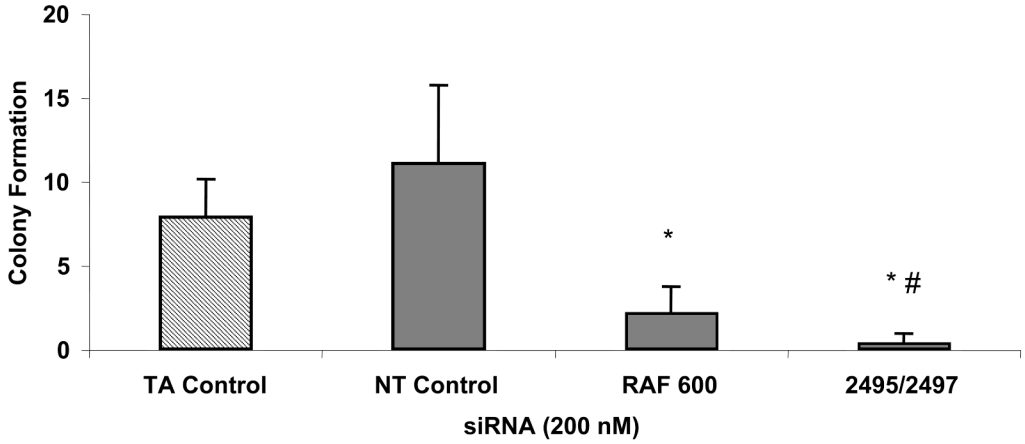


Fig 3.

Fig 3A. *Effects of siRNAs on DNA synthesis*. A375 cells were plated in 96 well plates at 6.25 × 10^3 cells per plate The following day the A375 cells were transfected with Dharmafect 1 alone (TA), non-targeting (NT) siRNA's, conventional unmodified siRNA(RAF600), or duplexed HNA modified siRNA (2495/2497), all at 200 nM. Following a 3 day transfection, a BudR ELISA assay was performed according to the manufacturer's standard procedure. Results are means and standard deviations taken from 24 determinations. The TA and NT values differ from both the RAF 600 and 2495/2497 values (*) with a statistical significance of P<0.0001 using the Student's t-test.

Fig 3B. *Soft agar colony formation*. A375 cells were transfected with Dharmafect 1 alone (TA), non-targeting (NT) siRNA's, conventional unmodified siRNA, or duplexed HNA modified siRNA for 3 days. Cells were then counted and were replated in soft agar. Colony formation was determined 14 days later as described in Methods. Results are expressed as colonies (>25 cells) per 100 cells plated. Data represents means and standard deviations from 12 determinations. The TA and NT controls differ from both the RAF600 and 2495/2497 values (*) with a statistical significance of p<0.0001 using the Student's t-test. The RAF 600 and 2495/2497 values are different (#) with a statistical significance of P<0.002.

Fig 4A

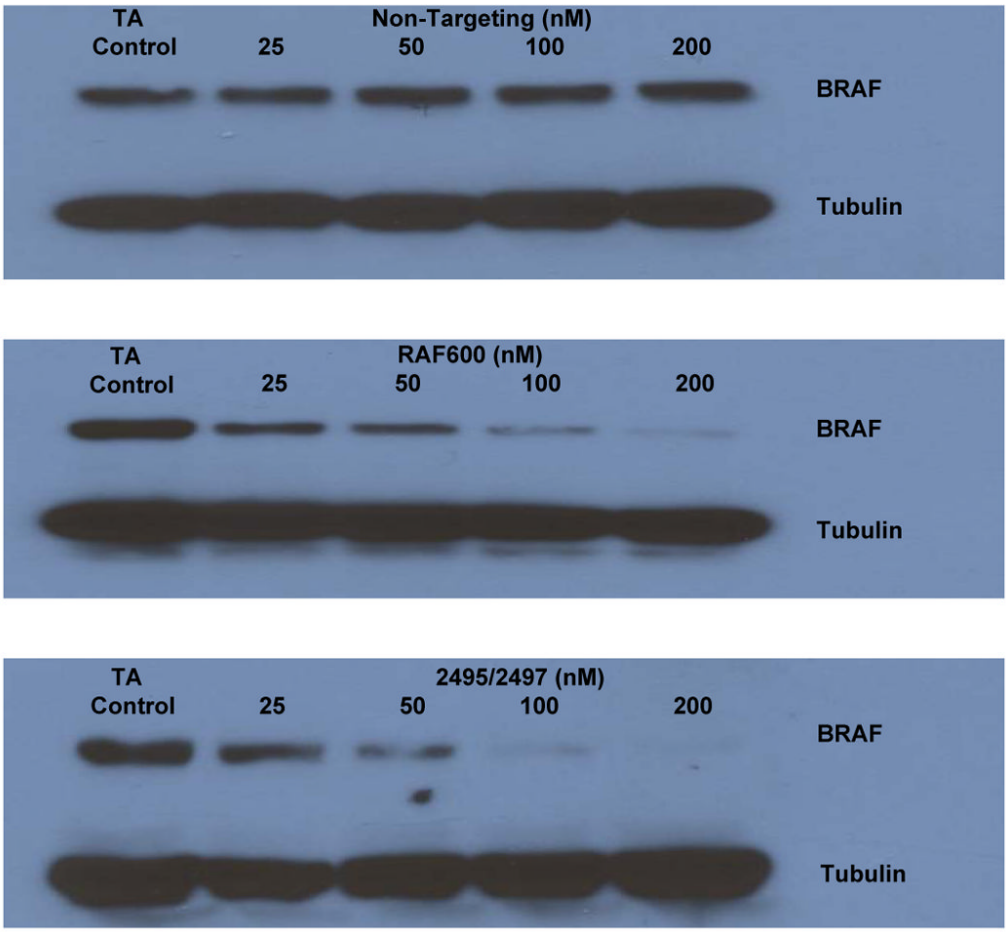


Fig 4B

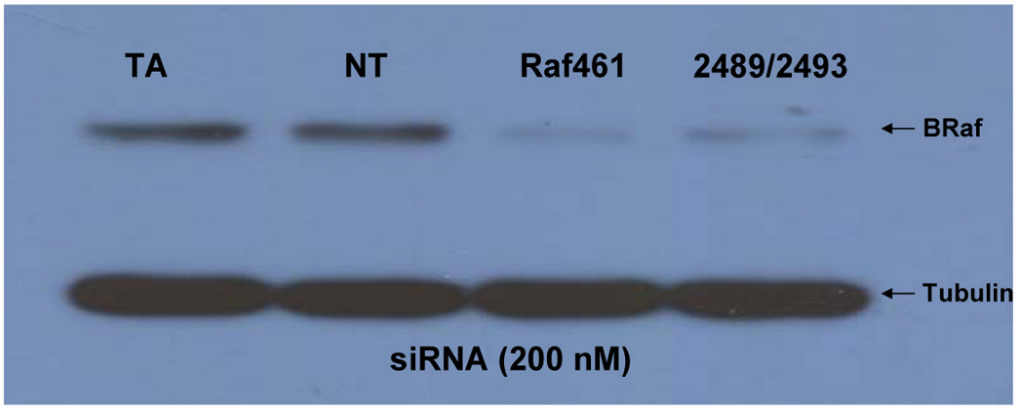


Fig 4. BRAF expression by western blotting

(A) A375 cells were treated with Dharmafect 1 alone (TA), non-targeting (NT) siRNA's, conventional B-Raf 600 unmodified siRNA or hexitol modified 2495/2497 siRNA at the indicted concentrations for 3 days. Cells were lysed with modified RIPA buffer and equal amounts of protein were resolved on 10% SDS-polyacrylamide gels, transblotted, and probed

using mouse monoclonal anti-B-Raf antibody. Tubulin was detected using mouse monoclonal anti-tubulin antibody Signals were detected by enhanced chemiluminescence. (B) A similar experiment was performed with unmodified or hexitol modified (2489/2493) B-Raf 461 siRNA used at 200 nM.

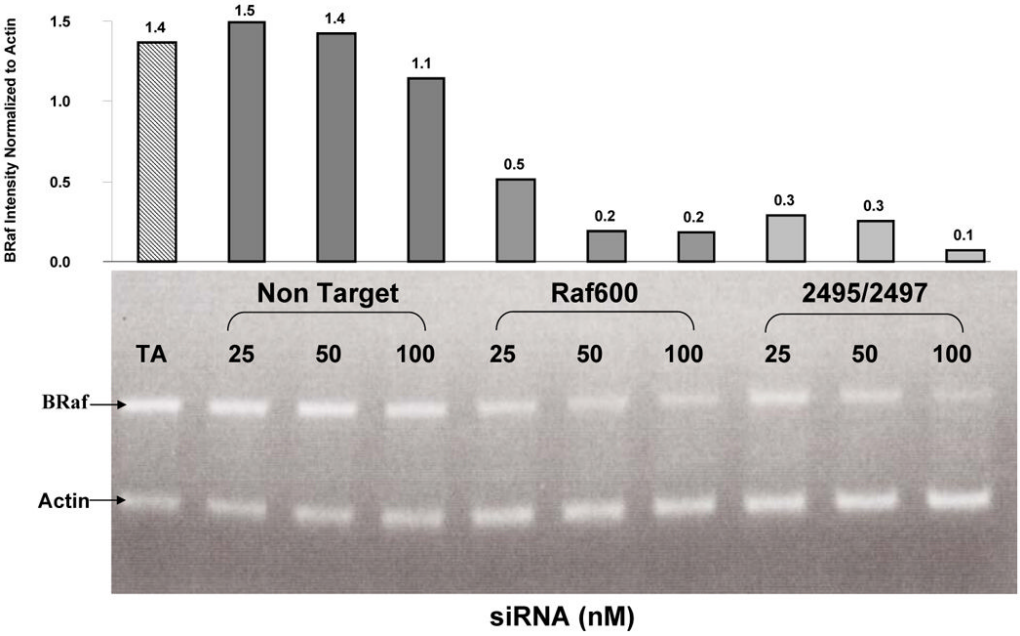


Fig 5. B-Raf mRNA levels by RT-PCR

A375 cells were treated with increasing concentrations of siRNA for 72 h and cellular mRNA levels were analyzed by RT-PCR as described in methods. The bar graph represents the ratio of BRaf/Actin signals as analyzed by Scion Analysis software. Results are typical of several experiments.

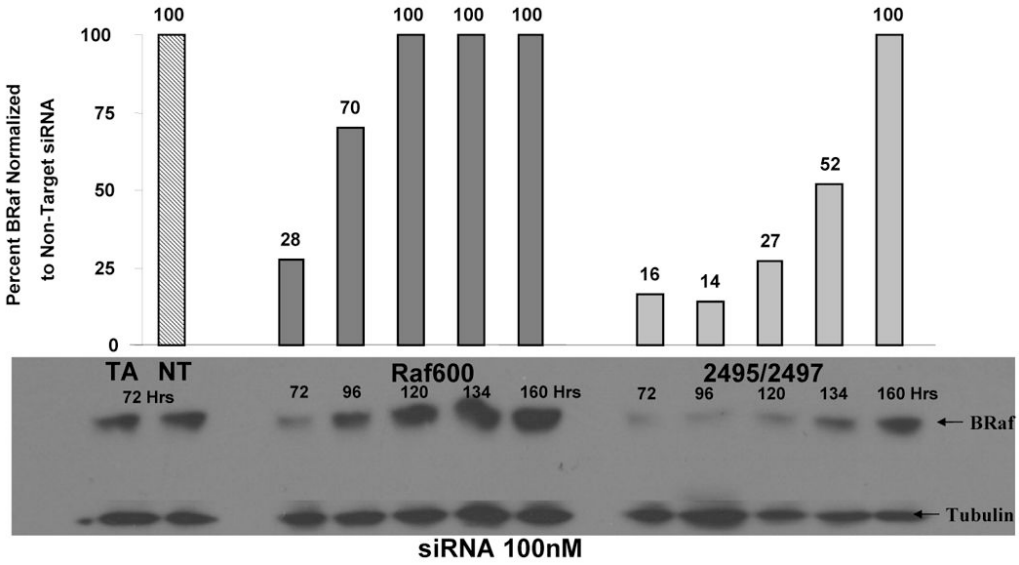


Fig 6. Persistence of effect of hexitol -siRNA

A375 cells were treated with 100 nM hexitol modified (2495/2497) or conventional B-Raf siRNA or with a non-targeted siRNA as a control. Cells were recovered on days 3-7 after siRNA treatment and monitored for B-Raf expression by western blot. In the bar graph B-Raf expression was normalized to the corresponding tubulin expression using Scion Image software; then the B-Raf expression level at 72h in cells treated with the non-targeted (NT) siRNA was taken as 100% and results for subsequent days for B-Raf siRNA treated cells were compared to that value. The result presented is typical of several individual experiments.

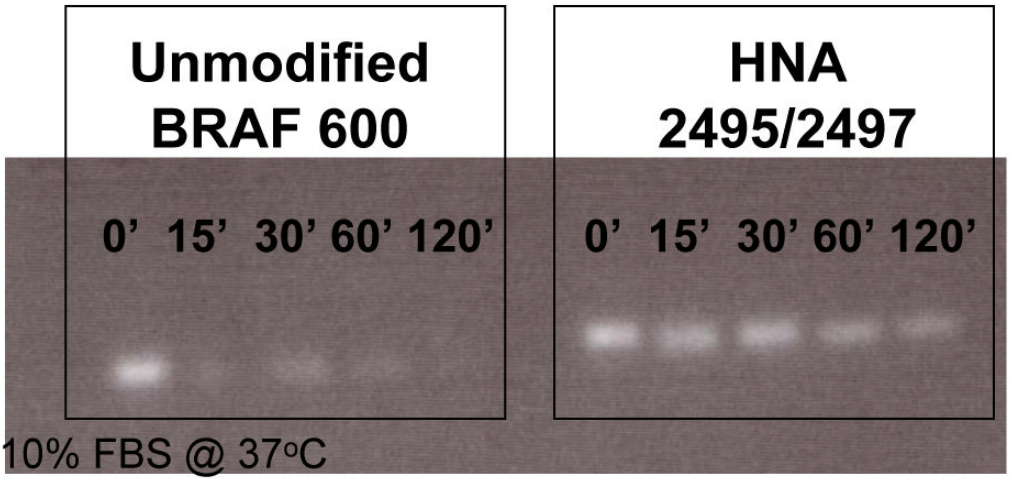


Fig 7. Nuclease stability of hexitol modified siRNAs

BRAF siRNAs were exposed to 10% fetal bovine serum at 37°C for different times and monitored by 3% agarose gel electrophoresis with ethidium bromide staining.

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**A**G	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**A**G	AAA	UCU	C**GA**	UdTdT
2506	**GC**U	ACA	G**A**G	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**