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The telomerase enzyme consists of an RNA template and a reverse transcriptase which ensures replication of the termini of chromosomes. Normal somatic cells do not express telomerase and progressively lose telomeric repeat units at each cell division which ultimately results in celluarl senescence. Rapidly growing tumor cells overcome this handicap by reexpressiong the telomerase enzyme making it a potential target for therapy. We have demonstrated using antisense oligonuclotides that targeting the RNA component of telomerase (hTR) for degradation commits tumor cells to rapid apoptosisi in vitro and in vivo. Normal cells are unaffected a tumor cells treated with oligonucleotides carrying mismateched bases do not illicit this response. The same apoptotic response was induced in breast cancer cells in vitro using small interfering RNA molecules (siRNA) targeting hTR. In these approaches only a single treatment over a seven day period was sufficient to reduce cell viability significantly. This is in contrast to the need for daily administration of the antisense molecules. These observations provide strong support for the possibility that targeting the telomerase RNA could be developed as a rational therapeutic approach if limitations of delivery and specificity can be overcome.

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Targeted Therapy of Human Breast Cancer By 2-5A-Antisense Directed Against Telomerase RNA

INTRODUCTION.

Telomerase is the riboprotein enzyme complex which prevents the ends of chromosomes from shortening below a critical length in cancer cells which would otherwise result in cell death. This enzyme is normally not expressed in the majority of human cells after an early point in embryonic development but is reactivated in the vast majority (95%) of highly malignant cancer cells. It is thought to be an essential requirement for the maintenance of cell viability in cancer cells which express it. We have investigated whether inactivating telomerase in cancer cells using both antisense and small interfering RNA oligonucleotide approaches targeting the RNA component of the enzyme will result in cell death. The oligonucleotides used carry a 2-5A moiety attached to the antisense molecule. 2-5A activates endogenous RNAseL which is normally found as an inactive monomer in the cytoplasm in most cells. In the presence of 2-5A the monomer dimerizes and become a potent RNAse. Thus, the antisense molecule targets a specific RNA and the recruitment of RNAseL then selectively degrades the target. The overall aim of the project, therefore, is to determine whether inactivating telomerase can be developed as a viable form of anti cancer therapy for breast tumors.

BODY.

Design of the antisense oligonucleotide.

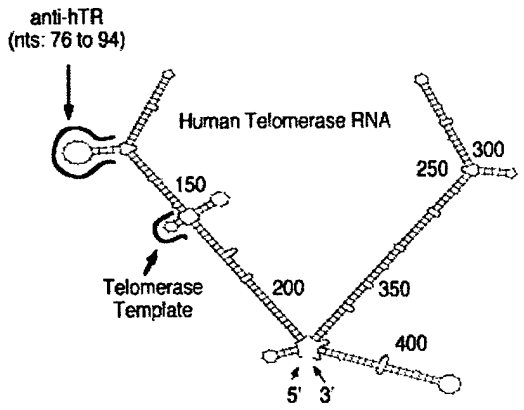

Figure 1. MFOLD prediction of the folding structure of the RNA component of the telomerase enzyme.

For successful application of this antisense technology, it was essential to construct antisense oligonucleotides against an "open" part of the RNA molecule to ensure the maximum likelihood of achieving homologous binding. To determine the best target sequence, therefore, the telomerase RNA (hTR) structure was analyzed using the MFOLD program. The predicted structure of the telomerase RNA showed that this molecule had very tight secondary folding which would make the binding of small oligonucleotides difficult (Figure 1). The most "open" part of the molecule is seen between residues 76 and 94, 20 nucleotides 3' of the telomerase template sequence. Therefore, we designed the 2-5A antisense oligonucleotide against the predicted loop comprising nucleotides 76-94. BLASTN searches of available databases for this nucleotide sequence only revealed homologies to the human hTR and a DNA sequence from H. influenzae. To investigate the effect of 2-5A antisense against hTR on malignant breast cells,

Table 1. Nomenclature and sequences of oligonucleotides complementary to human telomerase RNA nucleotides 76 to 94.

Oligonucleotide:	Sequence:
spA_4-anti-hTR	Sp5'A(2'p5'A)$_3$-Bu$_2$-5'GCG CGG GGA GCA AAA GCA C3'-3'T5'
spA_2-anti-hTR	Sp5'A2'p5'A-Bu$_2$-5'GCG CGG GGA GCA AAA GCA C3'-3'T5'
spA_4-anti-(M6)hTR	Sp5'A(2'p5'A)$_3$-Bu$_2$-5'GC<u>C</u> CG<u>C</u> GG<u>T</u> GC<u>T</u> AA<u>T</u> GC<u>T</u> C3'-3'T5'
Bu - butanediol linkers; underline indicates mismatched nucleotides.	

we synthesized spA$_4$-anti-hTR, with complete homology to the targeted sequence, as well as two control oligonucleotides; spA$_2$-anti-hTR, and spA$_4$-anti-(M6)hTR (Table 1). To establish the contribution of the 2-5A moiety of the chimeras to the anti-telomerase effects, spA$_2$-anti-hTR contains only two 2', 5'-linked adenylyl residues instead of the usual four (Table 1). Chains of three or more 2', 5'-linked adenylyl residues are absolutely required for RNase L activation, and so dimeric forms of 2-5A are inactive. Another control oligonucleotide, spA$_4$-anti-(M6)hTR, contains functional tetrameric 2-5A, but there are six mismatched nucleotides in the antisense cassette of the chimera which would reduce or prevent binding with the telomerase RNA. All three oligonucleotides contained stabilizing modifications at both termini. The 5' termini contain a thiophosphate to protect against phosphatase activity and the opposite termini contain a 3', 3' inverted linkage to inhibit 3' and 5' exonuclease activities.

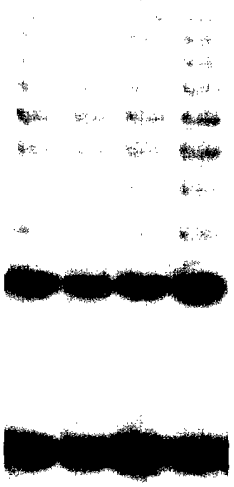

Figure 2. Examples of the TRAP assay from extracts of breast cancer cell lines demonstrated the progressive laddering resulting from the sequential addition of template repeats by telomerase in the cell extracts.

In vitro analysis of targeting telomerase RNA.

To investigate the response of breast cancer cells to treatment with 2-5A anti-hTR we selected three different breast cancer cell lines, MDA468, MCF7 and T47D. The activity of telomerase can be assayed using the TRAP procedure. In this assay an oligonucleotide 6 bp template is combined with extracts from cells to be analyzed. If telomerase activity is present the 6-mer template will be amplified producing a ladder of molecules on an acrylamide gel (figure 2). Using this TRAP assay clearly demonstrated strong telomerase activity in all of these cell lines. This activity could be heat inhibited by incubating the cell extracts for 10 minutes at 80^{o}C. In contrast, telomerase activity was not detected in either fibroblast cell lines MRC5 and WI38, or primary cultures of human vesicular endothelial cells or human corneal epithelial cells. Since the 2-5A-antisense system depends on recruiting RNAse L to the molecule targeted for degradation, we investigated the levels of RNAseL expression using western blotting. All breast cancer cell lines demonstrated the presence of cellular RNase L using a monoclonal anti-human RNase L antibody (figure 3). Amplification of RNase L mRNA using RT-PCR also revealed high levels of gene expression in all breast cancer cells.

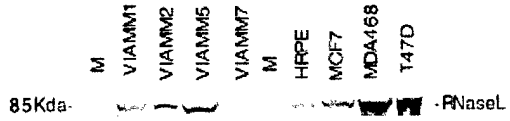

Figure 3. Western blot analysis of RNAseL expression in breast cancer cell lines and controls.

We next treated cell lines T47D, MCF7 and MDA468 with the 2-5A-anti-telomerase oligonucleotides (spA4-anti-hTR) and representative results are shown in figure 4 for the MDA 468 cell line. In all cases rapid cell death was induced in vitro following only 4-7 days of treatment. When we treat the same cells with a control oligonucleotide which contains an active

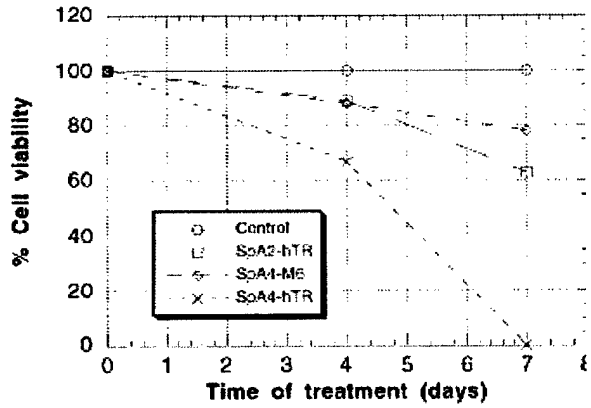

Figure 4. Typical kill curve for MCF7 cells treated with the functional 2-5A shows complete death after 7 days. Cells treated with the non-functional 2-5A or the mismatch oligo have a much less dramatic effect on growth.

2-5A, but which carries mismatches within the targeting oligonucleotide (spA4-anti-hTR(M6)), no significant cell death is seen. When we treat cells with a perfect match oligonucleotide which is attached to a defective 2-5A moiety

(spA2-anti-hTR), there is no significant cell death either. These experiments demonstrate that both the correct antisense targeting molecule as well as an active 2-5 moiety are required for cell death. Although it has been difficult to formally prove that cell death results from selective targeting of telomerase it is assumed that, because the controls have no effect on cell growth, the toxicity of the test compound is due to the disruption of enzyme function. To demonstrate that the oligonucleotides actually enter the cells we constructed a fluorescence tagged spA4-anti-hTR oligonucleotide and transfected it into breast cancer cells. This analysis demonstrated that large intracellular concentrations of oligos are achieved, although there is heterogeneity from cell to cell which may account for the differential response of cells during the 8 day treatment period.

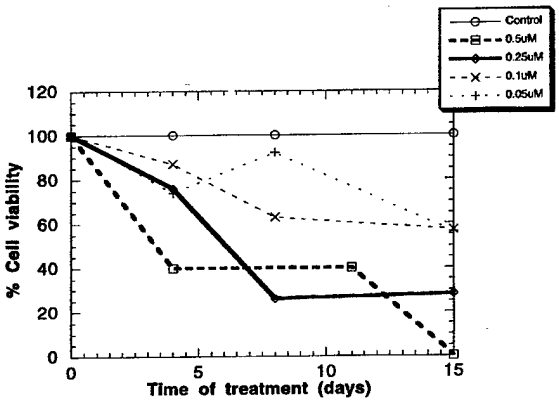

Figure 5. Viability curve of MDA468 cells treated with various concentrations of the spA4-anti-hTR oligo.

Since the dose of oligonucleotides will clearly be important in the overall cellular response we titrated the cellular toxicity of the test compound between a concentration range of 0.1-5 uM. All oligos were administered using a cationic liposome (lipofectamine) to facilitate entry of the molecules into the cell. These experiments demonstrated a peak response at approximately 0.5 uM (figure 5). We then investigated the dose response of MDA468 cells in the range 0.1-0.5 uM. As shown in figure 5 significant cell death was only achieved using oligo concentrations of 0.5 uM. This clearly demonstrated that there is a lower, effective concentration that must be used to achieve cell death. For all further experiments, therefore, we standardized the treatment using 0.5 uM oligos at a ratio of 1:4, DNA:lipofectamine. Since the initial treatments were every 12 hours we next investigated whether reducing the frequency of treatment could still achieve the same toxicity. When cells were treated either every 24 or 48 hours the same results were obtained. Treating every 72 hours resulted in initial toxicity followed by recovery of the cells. We therefore standardized the frequency of treatment to every 24 hours for all subsequent experiments.

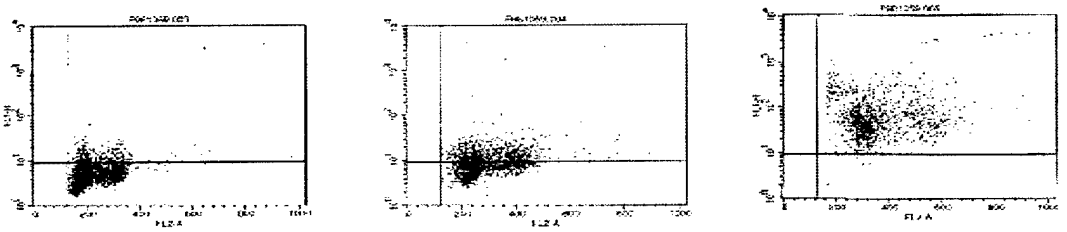

Figure 6. Treatment of MCF7 cells for one (left), two (center) and three (right) days with the functional 2-5A oligo shows increasing presence of cells in the apoptotic quadrant.

Using this standard treatment protocol we next investigated the mechanism of cell death. Early indications were that cells were dying through apoptosis rather than necrosis. Using the ApoTag apoptosis kit and FACS analysis it was possible to demonstrate that, within 5 days of treatment, all cells were being committed to apoptosis when treated with the 2-5A-anti-telomerase (figure 6). In contrast, very little apoptosis was seen when cells were treated with either the mismatch control or the 2-5A defective control oligos (figure 7). These results demonstrated that active cell death was being induced by targeting the RNA component of telomerase, further supporting a causal role for the oligo treatment in the observed toxicity.

We have clearly established that tumor cells can be killed by using 2-5A-antisense against telomerase RNA in vitro. The next question was whether normal cells which do not express telomerase are affected or not. It has not been possible to establish progressively growing cultures of normal breast epithelial cells. We have therefore addressed this question indirectly and investigated normal human fibroblasts, WI38 and MRC5, and vascular and corneal endothelial cells. In all these 3 cases, normal cells which do not express telomerase, were

relatively unaffected by the treatment. This demonstrates that there is unlikely to be toxicity of normal cells following treatment with 2-5A antisense.

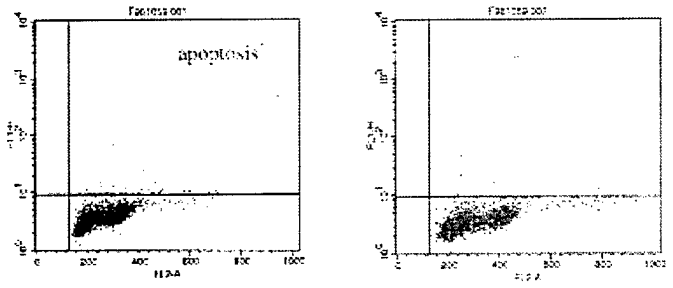

Figure 7. Apoptosis assays for MCF7 cells treated with lipofectamine (left) or the mismatch oligo for three days (right). In both cases the number of cells in the apoptosis quadrant is minimal.

In vivo analysis of targeting telomerase RNA.

To investigate whether tumor cells responded to antisense treatment in vivo, breast cancer cells were subcutaneously inoculated into nude mice and, after 8 days, tumors from the MDA468 cells reached a mean volume of 29.6mm3, (SD=3.9); MCF7 tumors reached a mean volume of 32.4mm3, (SD=10.2), and the T47D tumors reached a mean volume of 44.8mm^3, (SD=11.7). Six tumors were used for each treatment arm which involved daily intratumoral injections of the appropriate oligos. Tumor volume was then measured at even time points during the 10 day treatment period. For MDA468, treated with SpA4-hTR, the average size of the tumors was reduced to a mean volume of 27.7mm3, (SD=13), whereas tumors treated with spA4-hTR(M6) grew to an average 40.8mm3, (SD=9.6). The p value from the T-test = < 0.18 which was not statistically significant. In contrast, the average size of the MCF7 tumors after the 10 day treatment with SpA4-hTR was 15.2mm3 (SD=4.2), and for SpA4-hTR (M6) treated tumors was 36mm3, (SD=4). This difference is statistically significant, the p value from T-test being <0.001. The same was true for tumors from T47D cells where, after 10 days treatment with SpA4-anti-hTR the average size was 18.7mm3, (SD=2.5), whereas tumors treated with SpA4-hTR (M6) were 43.5mm3, (SD=12.6). This difference is also statistically significant, with a p <0.01. Thus, for two cell lines treatment with the spA4-anti-hTR oligo reduced tumor volume by approximately 50% whereas the mismatch control did not affect tumor size (figure 8).

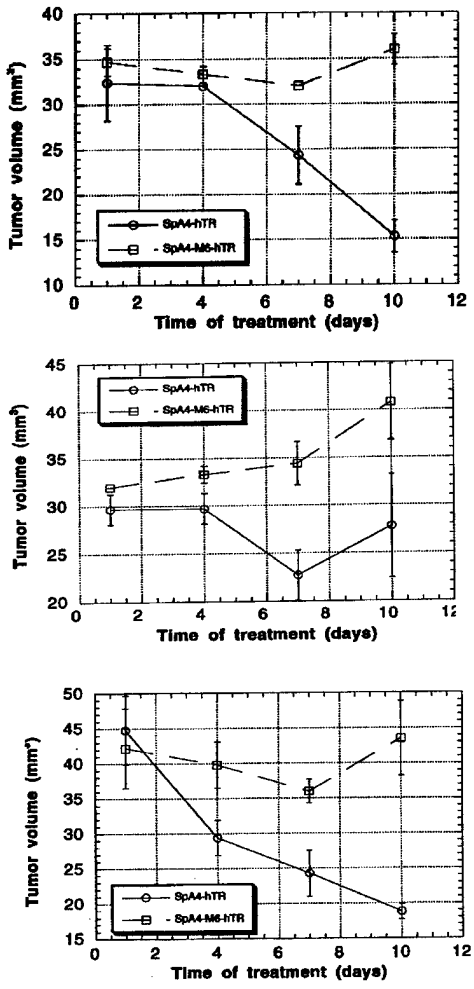

Figure 8. Analysis of tumor growth rate in vivo for (A) MCF7, (B) T47D and (C) MBA 231 following daily treatment with either the functional 2-5A oligo or the mismatch oligo.

To establish whether the rapid induction of apoptosis is due to a by-stander effect due to some signal secreted by cells undergoing apoptosis in the cultures, we transferred the tissue culture medium from cells treated with spA4-anti-hTR treatment to untreated cells daily. In these experiments no effect was seen on the cells which had never been challenged by anti-hTR oligos. The morphology of the cells also showed no differences compared with cultures grown in fresh medium. It appears, therefore, that the rapid cell death seen in anti-hTR treated cultures is due to the induction of apoptosis within the individual cells.

To summarize the work so far, we have clearly demonstrated that the 2-5A-anti-hTR oligonucleotide H1 could induce apoptosis in all breast cancer cell lines tested. A scrambled oligonucleotide did not produce this response and neither did the oligo which carried a defective 2-5A moiety. Since we designed the original oligo against the most open region of the hTR RNA (figure 1) we interpret these results to mean that accessibility of the antisense was determining the specificity.

Despite the very strong biological indication that targeting the RNA component of the human telomerase enzyme results in rapid and almost complete death of breast cancer cells in vitro and reduces the growth of tumors in the flanks of nude mice, we have not proven a mechanism behind the observed cell death. This has been a tricky technique and requires demonstrating that indeed the 2-5A anti-hTR specifically cleaves the hTR molecule. The main problem with this approach has been that the induction of apoptosis is not an all-or-nothing event and cells die over a 5-6 day period which is concomitant with the daily addition of the antisense molecule. Thus, although partial degradation of the target can be demonstrated, for some reason not all cells are equally affected in the heterogeneous culture, and so at any given time during the treatment there are always cells which are still expressing the target. This has been a frustrating aspect of the research since we clearly have an important biological effect but no clear mechanism. The same problem has also been repeatedly demonstrated using the apoptosis assays. Flow sorting of cells during treatment with the 2-5A anti-hTR oligo results in a clear demonstration of the increasing commitment of cells to apoptosis until they finally die out. However, since not all cells are responding to the treatment simultaneously we cannot easily quantify biochemical parameters associated with apoptosis such as annexin 5 release or cleavage of caspases. The same is true for the function of telomerase using the TRAP assay where, for the most part, even if there are 10% of cells expressing telomerase at any one time, because the assay is so sensitive, activity is demonstrable although reduced.

To establish whether there was a cell-cycle specific stage at which cells became susceptible to the 2-5A anti-hTR treatment we undertook cell cycle assays during the treatment period. There was apparently no change in the passage of the cells through the cell cycle during the treatment arguing against a susceptible phase which led to apoptosis. Synchronizing cells using blocking agents is difficult in cancer cell lines where the cell cycle is so disregulated. We have, however, achieved 60% sychronization using G1 arresting agents but, when these cells are released from the block in the presence of 2-5A anti-hTR, cell death occurred at the same rate seen in the parental culture. This observation argues that the response to 2-5A anti-hTR is not dependent on the stage of the cell cycle. We are still unable, therefore, to account for why different cells in a given culture are responding differently to the same treatment, although we expect that it is a consequence of the short half-life of the oligo and the differential uptake using the lipofectamine approach that creates different intracellular concentrations in different cells.

Analysis of cell viability using different targets to hTR.

The original H1 oligonucleotide was designed based on the rational interpretation of the MFOLD structure of telomerase RNA (figure 1) and corresponded to the most open part of the structure. To investigate whether there were better regions within the target for inducing cell death we designed several additional 2-5A-linked oligos from throughout the sequence and used these in cytotoxicity assays using the MCF7 cell line. The results obtained are shown in figure 9. A duplicate of H1 was

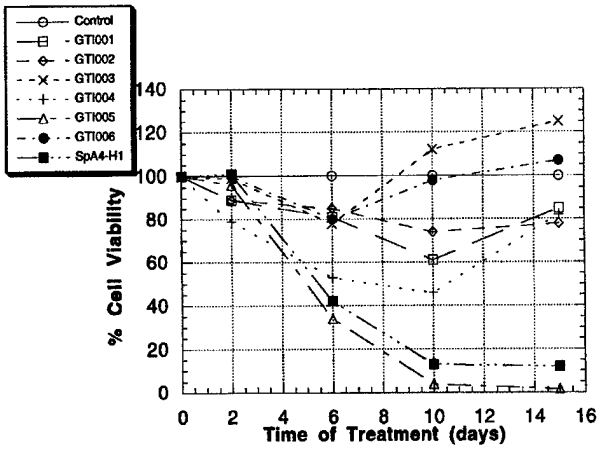

Figure 9. Viability curves of MCF7A cells treated with 2-5A-linked oligos targeting different regions of the telomerase RNA 9 see table2).

synthesized and coded within this series and the code was only broken after the in vitro studies were completed (table 2). Oligo GTI001 was designed from within the promoter region of hTR and GTI002 was at the initiation of

transcription site. It can clearly be seen from the results in figure 9 that only one of the new oligos (GTI005) performed as well as H1. In fact, when decoded this was the duplicate of H1. These results had important implications to the validity of our observations since we could demonstrate that cell death induced by H1 was not due to oligo toxicity otherwise the other oligos would have produced similar effects. A second oligo (GTI004) showed a significant effect over the extended 16 day period but none of the other molecules showed any killing effect at all. From this limited survey we conclude that the original design of the oligonucleotide was still the most efficient. This observation was important firstly to demonstrate that the killing effect is not due simply to oligonucleotide toxicity, since if this were the case then all of the oligos should have had the same effect. Secondly, in a subsequent series of experiments (see below) we began to investigate the effects of modifying the oligos to make them more stable and because of the high costs of synthesis we needed to be sure which was the best antisense molecule.

gagtgactct cacgagagcc gcgagagtca gcttggccaa tccgtgcggt cggcggccgc
GTI001
tccctttata agccgactcg cccggcagcg_caccgggttg cffagggtgg gcctgggagg
GTI002
ggtggtggcc atttttttgtc taaccctaac tgagaagggc gtaggcgccg tgcttttgct
H1
ccccgcgcgc tgtttttctc gctgactttc agcgggcgga aaagcctcgg cctgccgcct
GTI003
tccaccgttc attctagagc aaacaaaaaa tgtcagctgc tggcccgttc gcccctcccg

gggacctgcg gcgggtcgcc tgcccagccc ccgaaccccg cctggaggcc gcggtcggcc

cggggcttct ccggaggcac ccactgccac cgcgaagagt tgggctctgt cagccgcggg
GTI004
tctctcgggg gcgagggcga ggttcaggcc tttcaggccg caggaagagg aacggagcga

gtccccgcgc gcggcgcgat tccctgagct gtgggacgtg cacccaggac tcggctcaca

catgc.

Table 2. DNA Sequence of the hTR gene. The location of the primers used in in vitro assays are underlined.

Analysis of modified oligonucleotides in viability tests of breast cancer cells.

The conventional 2-5A antisense oligonucleotides are only protected against rapid degradation by intracellular and extracellular nucleases by the addition of a phosphothioated moiety at the 5' end and a 3'3' inversion of the nucleotide at the 3' end. These modifications make the oligonucleotides resistant to rapid degradation by nucleases but it has been estimated that their activity falls off after approximately six hours in vitro. Thus, to develop these drugs for systemic treatment in the future it would be necessary to develop a more stable oligonucleotide. Oligonucleotides can be modified in a number of ways which makes them more resistant to nuclease digestion. One modification is to replace the phosphodiester linkages in the backbone with sulfur

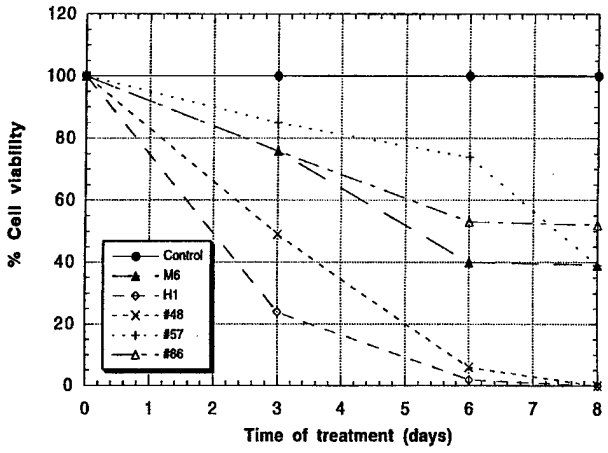

Figure 10. Preliminary studies of the effect on cell viability in breast cancer cells using modified oligonucleotides.

linkages. Although complete replacement of the phosphodiester backbone has been one strategy, it is often possible to achieve greater stability by substituting only those nucleotides at the ends of the oligonucleotides.

The other modification is to substitute 2'-O-methyl groups for the phosphodiester groups which is a modification seen in tRNA molecules and which makes them naturally resistant to nuclease digestion within the cell. To investigate whether any of these modifications to the 2-5A-anti-hTR affected their ability to inhibit cell growth we created a series of novel oligonucleotides and used them in the in vitro assays for several cell lines.

The first oligonucleotide tested (#48) carries a phosphothioate substitution of the last nucleotides at the 5' end of the H1 oligo. As can be seen in figure 10 these oligos were effective in killing MDA468 cells over the six days treatment although these oligos were not as effective as the original unsubstituted anti-hTR oligonucleotide (H1). Oligos substituted with 2'-O-methyl groups throughout the oligonucleotides (#86) were much less efficient in killing MDA468 cells (figure 10). From these experiments it was shown that the original H1 oligonucleotide was still the most efficient for cell killing in vitro. We extended this series of oligos using a variety of different combinations of modifications as shown in table 2. These analyses demonstrate again that the phosphothioate substituted oligo (102) has no effect on cell growth in either treatment. The same was true for oligonucleotides where the 2'-O-methyl nucleotide residues were used with (#106) or without (#104) phosphothioate substitutions as well. The oligo (#108) with 2'-O-methyl substitutions at all of the last three nucleotides, however, appeared to have a greater killing effect but again not as efficient as the original H1 oligo. The issue was, however, whether the oligos would be more stable and so to investigate this we treated cells with the various test oligos at 48 hour intervals rather than 24 hour intervals. When the cells were treated every 72 hours there was no net effect on cell viability for any of the oligos suggesting that the population of cells after an initial response can then recover indicating that none of the oligos can persist for the 72 hour period. In summary, therefore, these results show that modifications of the oligos either abolished their effectiveness or gave no appreciable advantage over the original H1 oligonucleotide. These small scale preparations of very specific oligos involved a very lengthy preparative procedure and were also very expensive to produce. Given that they showed no appreciable increase in effectiveness we decided not to continue to test further modifications especially since the H1 molecule was so efficient and we have obtained industrial qualities of this reagent.

H1	spA4-d(GCGCGGGGAGGGGGCAAAAGCA-3'-3'T5')
102	spA4-d(GCGCGGGGAGGGGGCAAAAGsCsAs-3'-3'T5')
104	spA4-d(GCGCGGGGAGGGGGCAAAAGCA-3'-3'T5')2'O-Me
106	spA4-d(GCGCGGGGAGGGGGCAAAAGsCsAs-3'-3'T5')2'O-Me
108	spA4-d(GCGCGGGGAGGGGGCAAAAGmCmAm-3'-3'T5')
GTI013	spA4-d(AGTGGGTGCCTCCGGAGA3'-3'5)
GTI116	spA4-d(CAGuuAGGGUUAG3'-3"T5')2'O-Me

Table 3: Sequences for oligonuclotides and modifications used in cytotoxicity experiments. S = phosphothioate substitutions. M = 2'-O-methyl substitutions.

The original strategy for targeting the open loop using 2-5A linked oligos was that the specific target within the sequence is not important per se but rather that the oligo can bind. Once bound, RNAse L is recruited to the target to degrade the RNA molecule. In a more traditional antisense approach the oligo must bind to a functional region of the template to interfere with transcription or function. The main functional component of the hTR RNA is the template region which is used by the telomerase holoenzyme to preserve the length of human telomeres. To test whether targeting the template region would have any effect on cell viability we constructed an oligonucleotide, GTI116 (table 3), which carries antisense to the template region. The results of

this assay in MCF7 cells is seen in figure 11. Clearly GTI116 has little effect on cell survival (figure 11) probably because the template region of hTR is only open over the 6 critical base pair region and so may not allow access of the oligo to the template. Again this result supported our plan to pursue modifications of the original H1 oligo.

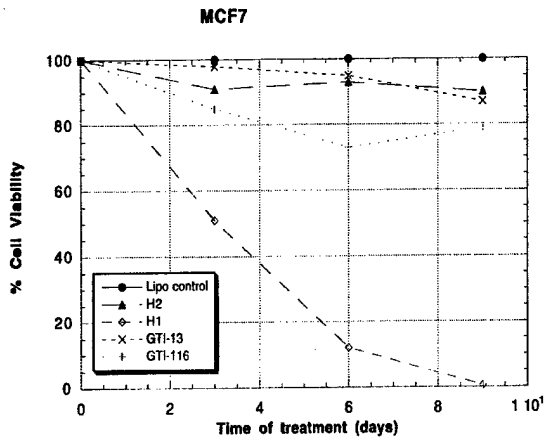

Figure 11. Effect of cell viability in MCF7 cells using an oligonucleotide (GTI-116) targeting the template region of the telomerase RNA.

Analysis of the role of BCL2 in the apoptotic response.

The original experiments clearly showed that the response of the cells to H1 treatment was apoptosis and so active cell death was occurring in response to targeting hTR. To investigate this effect further we treated cells with the H1 oligonucleotide in the presence of caspase inhibitors. Indeed, in these experiments (data not shown) the cytotoxicity was reduced by up to 40% in the cells treated with inhibitors further demonstrating that apoptosis is the mechanisms by which cells are killed. The BCL-2 gene is also an inhibitor of the apoptotic pathway and so we tested whether induction of high levels of BCL2 could prevent the cellular response to anti-hTR treatment. A HeLa cell line (kind gift of Dr A. Almasan) which contains a tet-inducible BCL2 gene was treated with the H1 and control oligonucleotides and the cell viability was measured in the presence of tetracycline. In these experiments HeLa cells were as responsive to anti-hTR treatment in the presence of tetracycline (and so increased levels of BCL2) as they were in the absence of tet. These experiments suggest that BCL2 is not involved in the cellular response to anti-HTR. To establish whether any significant difference in response could be seen with reduced concentrations of oligonucleotides we treated HeLa cells in the presence of Tetracycline with varying concentrations of 2-5A anti-hTR. The cell killing effect was reduced according to the concentration of oligonucleotides used. This was the same response that was seen in the absence of tetracycline and consistent with our earlier studies which show that 0.5 uM is the effective killing dose of 2-5A antisense.

Through this very extensive series of experiments we have demonstrated further the specificity of the H1 oligonucleotide in causing apoptosis in breast cancer cell lines. None of the other targets with hTR were as effective. Modification of the oligos to make them more stable had the unexpected consequence of reducing or ablating the efficiency of the H1and other oligos to cause apoptosis in breast cancer cells. We are still unclear about the exact mechanism of induction of apoptosis in breast cancer cells but it is clearly not due to decreasing the expression of BCL2.

Microarray analysis of gene expression changes during ablation of hTR.

Formal demonstration of the mechanism of 2-5A anti-hTR action has been difficult. It is also been a problem in predicting the pathways that lead to apoptosis. We anticipate that disrupting the telomerase function may expose DNA damage resulting from incomplete replication of the ends of chromosomes. However, since some of the cell lines we are using are p53 deficient it is clear that the apoptosis pathway is p53 independent. Although the existence of such pathways have been suggested from data from many different systems no specific pathways have been described. It is also of interest that the T47D cells are deficient in caspase 3 activity which is the major effector of apoptosis and so the mechanism in these cells presumably involves other caspases. The other approach we took was to use Affymetrix GeneChip experiments to survey gene expression change in cells treated with the 2-5A anti-hTR versus the same cells treated with the mismatch oligonucleotide, which does not produce a biological consequence of apoptosis. We treated cells in the standard way using 2-5A anti-hTR and then prepared RNA from cells after 8 hours and 24 hours and compared the gene expression

profile using the Affymetrix HUGFL Chips which carry 6800 genes with that from cells treated with lipofectamine alone in the first instance.

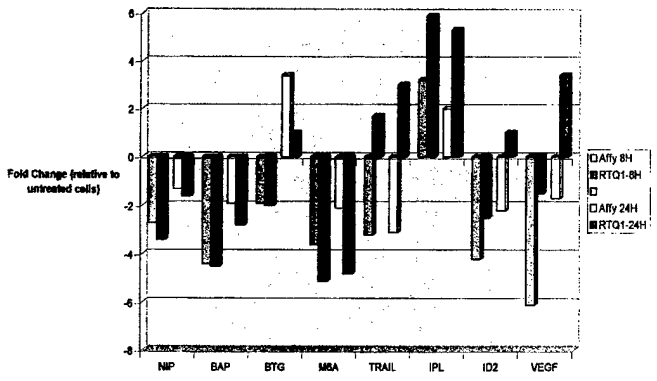

Figure 12. Comparison between fold change for the 10 differentially expressed genes using quantitative rt-PCR versus Affymetrix chip analysis.

In this experiment we clearly saw gene changes which were present in the 8 hour treatment as well as in the 24 hour treatment, we also saw gene changes that were present in the 8 hour treatment but which returned to normal after 24 hours, as well as changes in the 24 hour treatment but which were not seen after only 8 hours. From the list of gene expression changes, a sublist was compiled based on subjective interest level (i.e. possible functional significance) and examination of the data points on the actual genechip. From these genes, 10 were selected for verification of the results using real time quantitative PCR (RTQ). These genes included 3 genes associated with apoptosis: NIP, TRAIL and IPL; several growth factor related genes: BAP (Btk assoc'd tyr kinase); IGFBP5 and VEGF; a transcription factor: ID; an antiproliferative gene: BTG; and two genes for membrane bound proteins with unknown relevance: M6A and tissue factor (TF). While the observed fold change using rt-PCR did not always match the predicted GeneChip value exactly, the trends were typically the same. The exceptions were TRAIL at 8h and 24h, ID2 and VEGF at 24h (Figure 12). Because of the exquisite sensitivity and logarithmic nature of the assay, fold change values normally vary between +/-2 fold of the observed value which could explain some of the variability. For 2 genes: TF and IGFBP5, the RTQ fold change values were significantly greater compared to those predicted by Affymetrix GeneChip, e.g. 10-50X than expected (Figure 13). These elevated values were found reproducibly over several experiments. It is not clear whether these represent variation between different cDNA samples and/or genechip limitations/effects.

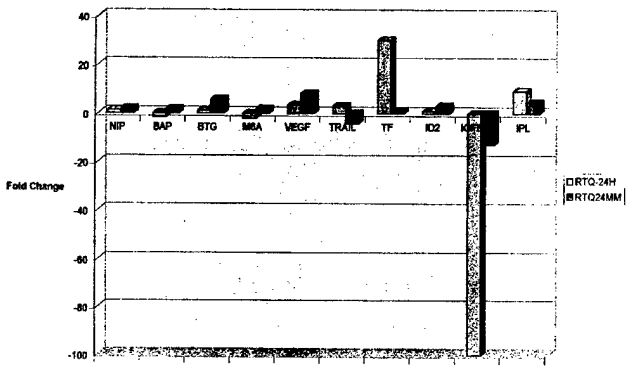

Figure 13. Comparison of gene expression levels between cells treated with the active oligonucleotide versus the mismatch oligonucleotide.

To determine whether any of the observed gene changes was linked to the induction of apoptosis in the treated cells, we next compared cDNAs from mismatch, antisense-treated cells with the same set of 10 gene primers. As shown in Figure 13, there were only small differences, if any, between the specific antisense and mismatch treated controls, typically within the 2-fold normal variation. Three genes, TF, IPL (implicated in fast pathways) and IGFBP5 had a reproducible induction of expression compared to mismatch controls. Tissue factor (TF) was not examined further since it was known to be IFN induced (a possible consequence of the 2-5A moiety). For IPL and IGFBP5, multiple cell lines were then tested to see if a similar induction of expression was observed correlating with the similar apoptotic responses. Figure 14 shows the results for these two genes on MDA468, U373 and HK cell lines treated for 24 hrs with either the antisense or mismatch control oligonucleotide-2'5'A hybrid. The results showed that, there was no consistent induction of expression for either gene in the 3 different tumor cell lines examined even though they all responded to treatment by undergoing apoptosis.

To more quickly identify those genes which were distinctly induced/repressed in response to antisense hTR but not in the mismatch control, another GeneChip experiment was performed using only antisense hTR

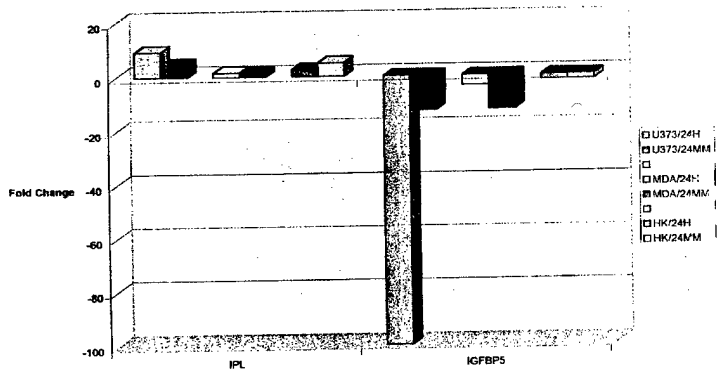

Figure 14. Comparative gene expression changes for IPL and IGFBP5 between treated with the active 2-5A oligo in different cell lines which show the same phenotypic response to treatment (apoptosis).

treated and mismatch hTR treated (at 8h post treated) as the RNA sources. Only 92 genes showed increased expression between the two of which only 15 had a sort score of $\geq$0.5 (the standard significance cut-off commonly use in these experiments). These 92 genes were then matched to the list of genes previously identified in the treated vs. untreated (lipofectamine) comparison described above. Only 10 genes were changed in BOTH experimental comparisons, e.g., treated vs. untreated AND treated vs. mismatch. Data from the individual tiles on the chip, 4 of these 10 were eliminated due to artifact (dust, scratches) and 2 due to lack of signal leaving: BDP (Ca++ regulator), EPCR (centrosome assoc'd), GGF (heregulin/neu) and RAB8 (GTPase).

Real time quantitative PCR was performed for 6 genes on one or more of the cell lines (MDA468, MCF 7, HK, U373) comparing treated and mismatch treated at various time points. Unfortunately, no consistent change was detected in all 3 cell lines relative to the mismatch. The inability to identify differences in gene expression between the authentic antisense treated and mismatch treated may have been partly due to the early time point utilized. However, we feel that the gene expression changes are probably reflecting a stress response to the presence of high levels of oligonuclotides inside the cell rather than identifying pathways involved in the response to hTR poisoning. The other persistent problem is again that only small percentages of cells are induced to undergo apoptosis and so the gene expression changes that may be occurring in these cells is masked by the gene expression levels seen in the majority of the cells. At this point we feel that, although targeting telomerase offers great promise in the treatment of breast cancer, we need to explore other approaches of targeting hTR which are more robust and controllable.

Another limitation of these GeneChip experiments was that they only carry 7000 genes so it is possible that we simply missed the critical players in the response to telomerase damage. There are now chips available which carry a more comprehensive set of human genes and might represent a future direction although since the primary confounding issue is the heterogeneous response of the breast cancer cells to 2-5A anti-hTR treatment which is an issue that should be clarified before committing to even more extensive analysis.

Application of siRNA technology to the inactivation of telomerse RNA.

Clearly, we have induced a profound biological response by targeting the RNA component of telomerase which should lead to a therapeutic option if a better understanding of the mechanism and a more controllable system can be established. With this in mind we changed our strategy to investigate the possibility of using RNA-interference approaches to target telomerase as an alternative approach.

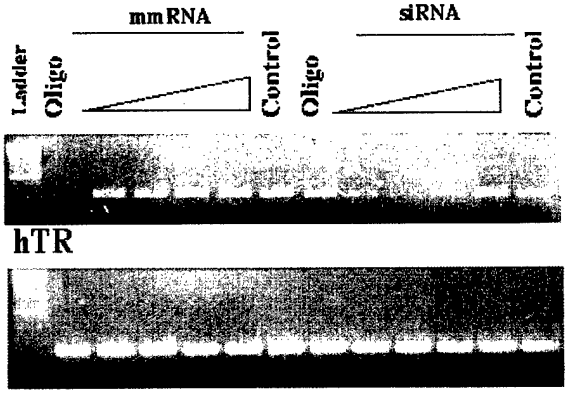

Figure 15. RT-PCR analysis of hTR expression in MCF7 cells treated with a mismatch (MM) siRNA at varying concentrations (40-400 pMol) and the perfect match siRNA. The mismatch has no effect on RNA levels whereas in the midrange of concentration the perfect match show almost complete ablation of the message.

RNA inference (RNAi) is a phenomenon where specific double-stranded RNA molecules can selectively bind to the homologous target RNA and illicit degradation of the target. In this process an endogenous RNAse cleaves the double stranded RNA into small

single stranded, small, interfering RNAs (siRNA) which mediate the degradation of the target, thus eliminating the function of that gene in the cells.

We have designed an siRNA directed against the telomerase RNA template (hTR) from nucleotides 76-94 (the same ones used for the 2-5A targeting) in an effort to determine the effects of eliminating the RNA component required for telomerase activity (see figure 15). The siRNA duplex was chemically synthesized by Dharmacon Research Inc. Transient transfections were performed using either the complementary siRNA (hTR) or an siRNA that contains several mismatches (mismatch) using the MCF7 cell line, which possesses an elevated endogenous level of telomerase activity.

The transfection protocol was: Cells were seeded on a 6-well plates 24 hours prior to transfection in DMEM containing 10% FBS without antibiotics. Transfections were done at approximately 70% confluency. Two amounts of siRNA (for timecourse expt) were utilized, 80 pmoles or 240 pmoles as per recommended protocol (Dharmacon). SiRNA was incubated in Opti-mem (200ul) for approximately 10 minutes. Oligofectamine (6 ul) was incubated in Opti-mem (54 ul) for approximately 10 minutes. SiRNA and oligofectamine tubes were mixed gently and incubated (at RT) for 25 minutes. Following incubation, an additional 150 ul of Opti-mem was added. Cells were washed with PBS 1x after removal of media, and replaced with siRNA/oligofectamine mixture and placed in 37° incubator. Approximately 8 hours later, 1ml of DMEM (+10%FBS) was added to each well. Cells were counted and harvested at 48-96 hours post-transfection.

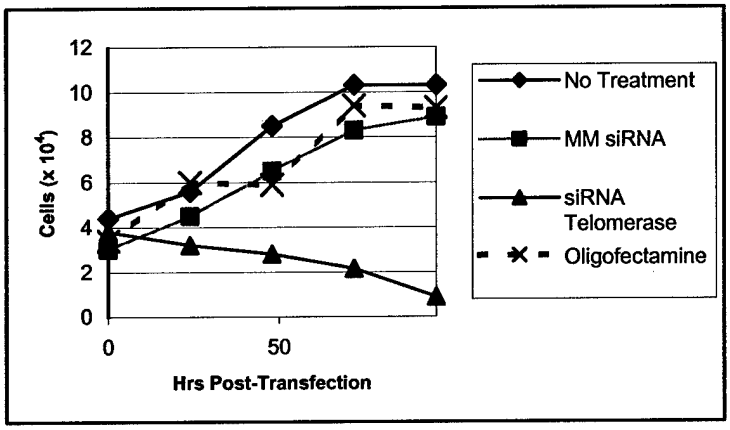

Figure 16 Cell viability assays for MCF7 cells treated with either teh mismatch siRNA or the perfect match siRNA against hTR. Only cells treated with the perfect match reagent show progressive cell death.

The results from these pilot experiments were very encouraging. Semi-quantitative analysis of cell survival suggests that while cells treated with a scrambled siRNA molecule show no change in growth rate, cells treated with the siRNA targeting molecule cause an approximately 60% reduction in cell numbers over a 4 day period (figure 16). A control cell line, MRC5 which does not express hTR or telomerase, was completely unaffected by the siRNA (data not shown) strongly suggesting that we are seeing a specific response to the treatment. We are currently performing apoptosis assays to determine whether this is the cause of death. Analysis of the hTR RNA in MCF-7 cells using RT-PCR demonstrated complete absence after 2 days (figure 15) and furthermore, that this loss of the transcript was maintained for several days only reappearing after 7 days following the single initial treatment. Control PCR reactions performed simultaneously using primers designed against GAPDH showed no changes. Thus, these experiments demonstrate that it is possible to 'knock-down' hTR transcripts in breast cancer cells lines and that this effect is stable over a 6 day period with only a single treatment. Furthermore, the consequence appears to be cell death. This approach has obvious advantages over the need to treat every day with oligonucleotides, especially since the mechanism of siRNA action has been well studied.

siRNA duplex: GUG CUU UUG CUC CCC GCG CdTdT
dTdT CAC GAA AAC GAG GGG CGC G

mRNA target (5'-3'): GUG CUU UUG CUC CCC GCG C

siRNA scrambled duplex: GUG CUG UCG CUA CCA GCG CdTdT
dTdT CAC GAC AGC GAU GGU CGC G

Figure 17. Sequences used to create hairpin siRNAs in the p Silencer vector.

A major drawback in using synthetic siRNAs, however, is that the silencing effect is sustained for only a brief period of time, maximally two weeks. In order to generate longer term silencing

effects, many companies have recently developed plasmid based expression systems that utilize an RNA polymerase III promoter to drive the expression of hairpin-structured RNA sequences. The plasmids also contain an antibiotic selectable marker in order to provide a means for generating stable cell lines expressing the siRNA. In order to generate a specific siRNA against a target sequence, the message sequence is scanned for AA dinucleotides starting 75 bases downstream from the start AUG codon. This is to ensure that any critical regulatory region in the promoter area that may be involved in protein binding is not utilized as a design target. AA dinucleotides are located in the message sequence, the subsequent 19 nucleotides downstream are recorded, and the G/C content is calculated. G/C content is recommended to be between 30-50% for optimal siRNA function. Additionally, recent data suggest that targeting areas lacking secondary structure has been shown to improve siRNA accessibility to the target mRNA. Finally, the siRNA sequences are compared with the human genome database using the BLAST program to ensure specificity against only a single mRNA. The pSilencer plasmid requires the cloning of an annealed pair of oligonucleotides containing the sense and antisense sequences against the target mRNA. The sequences are flanked by overhangs containing HindIII and BamHI cloning sites and a transcription termination sequence of 6 thymidines. The 19 nt-sense and antisense sequences are separated by a loop sequence of 9 nucleotides. The vector is supplied linearized by restriction digestion with HindIII and BamHI and purified to increase the percentage of positive clones upon ligation. After selection of positive clones, large quantities of vector can be produced for use in transfections and to generate stable cell lines. The sequences we have generated as the final part of this project are shown in figure 17. We have transfected these constructs into a series of different cell lines and placed them under selection. At the present time we have not been able to develop a breast cancer cell line stably expressing the siRNA. The response of the cells in all cases has been massive cell death which may be related to knockdown of hTR or may be a toxic response to the siRNA. We are currently preparing inducible pSilencer constructs to investigate this. We were however able to generate a HeLa cell line which stably expressed the hairpin siRNA and this resulted in 50% knockdown of hTR but there was no apoptotic response by these cells. Presumably the incomplete eradiction of hTR allows for cell survival.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstration that targeting the RNA component of human telomerase with an antisense oligonucleotide can result in rapid apoptosis in breast cancer cell lines in vitro.
- Demonstration that targeting the RNA component of human telomerase with an antisense oligonucleotide can result in apoptosis in breast cancer cell lines in vivo.
- Normal cells which do not express telomerase are not susceptible to the induction of apoptosis.
- Specific regions of the hTR RNA molecule are responsible for the apoptotic response to targeting hTR.
- Modification of the oligonucleotides to increase stability does not improve their efficacy.
- The apoptotic response is p53 independent.
- SiRNA induces the same apoptotic response in breast cancer cell lines with only single treatments.

CONCUSIONS:

We have clearly demonstrated that targeting hTR in breast cancer cells using two different approaches can result in the rapid induction of apoptosis in vitro. Direct injection of antisense oligonucleotides into breast tumors in vivo results in a reduction in their growth rate and size. It has become clear that only certain sequences within the hTR molecule are susceptible to targeting. The use of oligonucleotides and antisense has limitations in terms of specificity and delivery to solid tumors remains a problem. Our demonstration that siRNA targeting hTR can induce the same apoptotic response in breast cancer cells although because of time limitations we have not been able to pursue this to a more extensive analysis. As the use of siRNA becomes more extensive in therapeutic protocols the background studies we have undertaken will allow us to adapt these new protocols to the treatment of breast tumors in vivo.

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