Anti-proliferative and anti-tumor effects of antisense oligonucleotide GTI-2601 targeted against human thioredoxin

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Human thioredoxin has been implicated in cancer as a growth stimulator through regulation of DNA replication and growth factor activity, as a modulator of transcription factor activity, and as an inhibitor of apoptosis. In the present study, the steady-state level of thioredoxin protein was examined in a number of cancer cell lines. Interestingly, thioredoxin expression is elevated in a variety of human tumor cell lines compared with normal cell lines. The altered expression of thioredoxin in tumor cells suggests it may be a target in the development of novel therapeutic agents for the treatment and prevention of cancer. Further to this possibility, 26 phosphorothioate antisense oligodeoxynucleotides (PS-AS-ODNs) were evaluated for the ability to inhibit thioredoxin expression in cell culture. One PS-AS-ODN, GTI-2601, specifically reduced the levels of thioredoxin mRNA and protein, exhibited potent anti-proliferative effects on colony formation *in vitro*, and had anti-tumor effects in human tumor xenograft mouse models *in vivo*. Sequence-specific decreases in thioredoxin expression levels were accompanied by significant suppression of tumor growth in mice. Taken together, these data suggest that thioredoxin may be a useful target for developing PS-AS-ODNs as drug candidates against human cancer. *Anti-Cancer Drugs* 17:143–154 © 2006 Lippincott Williams & Wilkins.

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Introduction

Thioredoxin is a small ubiquitous redox protein (M_r 11 500 in humans) [1–4], originally identified as a reducing cofactor for ribonucleotide reductase – an enzyme that is essential for DNA synthesis [5]. This enzyme is a part of the thioredoxin system, which includes thioredoxin reductase. Thioredoxin reductase uses NADPH as a proton donor to reduce thioredoxin, which in turn reduces ribonucleotide reductase. In addition, the thioredoxin system is responsible for a large part of intracellular reducing capacity [6]. In recent years, mammalian thioredoxin has been implicated in a variety of biochemical pathways [1,2,7]. It modulates redox properties of transcription factors by dithiol/disulfide exchanges, thus altering their DNA-binding characteristics. Transcription factors such as NF-κB [8], glucocorticoid receptor [9], BZLFI [10] and TFIIIC [11] are directly regulated by thioredoxin, while AP-1 activation is mediated indirectly through the nuclear redox factor ref-1, which is further reduced by thioredoxin [12]. AP-2, the estrogen receptor, PEBP2 and HIF-1α are also regulated by thioredoxin [1]. Finally, overexpression of thioredoxin has been found to enhance p53-dependent expression of p21 via an increase in ref-1-mediated activation of p53 [13].

Cloned human thioredoxin was shown to be identical to a growth factor termed adult T cell leukemia-derived factor that is released by human T cell leukemia virus 1-transformed T cells [9]. It has also been identified as an interleukin (IL)-1-like cytokine produced by Epstein–Barr virus-infected B-lymphoblastoid cells, IL-2 receptor-inducing factor and early pregnancy factor [14]. It utilizes a leaderless pathway for secretion, and stimulates the proliferation of normal fibroblasts, lymphoid cells and a number of human solid tumor cell lines [15,16]. Growth stimulation by thioredoxin appears to be induced indirectly by sensitizing cells to growth factors such as IL-2 and basic fibroblast growth factor, but not insulin or epidermal growth factor [17]. In addition, redox-inactive forms have been used to show that growth stimulation requires functional redox activity of thioredoxin [15]. In addition to sensitizing cells to growth factor stimulation, thioredoxin modulates the expression of a number of cytokines [IL-1, IL-2, IL-6, IL-8 and tumor necrosis factor (TNF)-α] [1]. Finally, transfection of thioredoxin into human breast cancer cells results in increased expression of CYP1B1 [18]. CYP1B1 is aberrantly expressed in a number of human cancers and is involved in the production of estrogen metabolites that are implicated in the development of cancer [18].

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There is growing correlative evidence that suggests thioredoxin overexpression may be involved in tumor formation and growth [1]. Thioredoxin is overexpressed in a number of human malignancies, including lung, colorectal, cervical, hepatic, colon, liver and pancreatic cancer. In some examples, elevated expression of thioredoxin is associated with aggressive tumor growth, inhibition of apoptosis and poor patient prognosis [19–31]. Human breast cancer cells transfected with wild-type thioredoxin cDNA exhibit increased tumor cell growth and decreased spontaneous apoptosis *in vitro* [32]. In contrast, cells transfected with a dominant-negative, redox-inactive mutant thioredoxin showed reduced anchorage-independent growth *in vitro* and marked inhibition of tumor growth *in vivo* [32]. Overexpression of thioredoxin was also found to inhibit apoptosis in WEHI7.2 thymoma cells *in vitro* and *in vivo* [33]. Recently, breast cancer cells overexpressing thioredoxin were found to have increased NF-κB activity. A dominant-negative IκBαM expressed in these cells did not decrease either anchorage-independent or -dependent growth, suggesting thioredoxin confers a growth advantage to cancer cells via another pathway, possibly AP-1 [34]. When inoculated into SCID mice, thioredoxin-transfected cells formed tumors that grew more rapidly than vector-transfected cells, implicating thioredoxin in tumor progression [35]. Thioredoxin does not need to be expressed endogenously by cancer cells to have an effect on growth and apoptosis. Recombinant human thioredoxin added to B-CLL (chronic lymphocytic leukemia) cells derived from patients increased viability, maintained Bcl-2 levels, increased TNF-α release and reduced apoptosis [36]. The exact mechanism by which thioredoxin prevents apoptosis is not understood at this time, but may be the result of thioredoxin binding to apoptosis signal-regulating kinase (ASK)-1 and modulating TNF-α-induced apoptosis [37]. An alternative/parallel mechanism by which thioredoxin may inhibit apoptosis is intracellularly via thioredoxin peroxidase [38]. Cells transfected with thioredoxin were shown to upregulate thioredoxin peroxidase-1 expression. Thioredoxin peroxidase-1 overexpression in turn can prevent H_2O_2 induced-apoptosis, suggesting that at least one mechanism by which thioredoxin can act is via a peroxide-scavenging pathway [38]. In addition to cell growth and inhibition of apoptosis, thioredoxin appears to be involved in stimulating the expression of vascular endothelial growth factor and increasing tumor angiogenesis [39]. Farina *et al.* demonstrated that thioredoxin inhibited tissue inhibitor of metalloproteinase (TIMP)-1, thus altering the MMP-2/MMP-9 balance, leading to increased invasive potential of SK-N-SH cells *in vitro* [40].

In addition to increased growth and decreased apoptosis, overexpression of thioredoxin has also been associated with drug resistance [1]. A correlation has been made between cisplatin resistance and thioredoxin levels in colon, gastric and 11 ovarian cancer cell lines [41]. Increased thioredoxin expression, either endogenously expressed, selected by culturing in drug containing media or by transfection of thioredoxin cDNA, has been shown to increase resistance of a number of different cancer cell lines to cisplatin, doxorubicin and mitoxantrone [42–45]. The corollary to this is that decreasing thioredoxin expression in a cisplatin-resistant cell line using an antisense plasmid results in increased sensitivity not only to cisplatin, but also to a number of other drugs [43]. Moreover, expression of a dominant-negative thioredoxin in murine leukemic cells increased sensitivity of these cells to apoptosis-inducing drugs such as doxorubicin, dexamethasone, staurosporine, thapsigargin and etoposide [46].

Due to its involvement in cell proliferation and tumor formation, we extended the expression study of thioredoxin to various cancer cell lines. We demonstrate that thioredoxin is overexpressed to varying degrees in all human cancer cell lines tested. As a result we chose thioredoxin as a target to identify potential phosphorothioate antisense oligodeoxynucleotides (PS-AS-ODNs) as inhibitors of tumor cell proliferation. One of several antisense compounds tested, GTI-2601, displayed potent anti-tumor effects *in vivo* and anti-tumor activity correlated with the sequence-specific inhibition of thioredoxin gene expression.

Materials and methods

Oligonucleotides

A total of 26 different PS-AS-ODNs, complementary to various parts of thioredoxin mRNA, were synthesized at Dalton Chemical Laboratories (North York, Ontario, Canada). GTI-2601 hybridizes to the 5′-untranslated region (5′-UTR) of thioredoxin mRNA and its sequence is composed of 5′-TCC AAA GCA CCA AAC AGA GC-3′ (see Fig. 5 below). A mismatched control analog of GTI-2601 named GTI-2601-MIS8 contains 8 base changes or mismatches located in the middle of the GTI-2601 sequence and has the same base-composition ratio as GTI-2601 (5′-TCC AAA AAC AAG CCC AGA GC-3′). The large-scale preparations (30–50 mg) of PS-AS-ODNs for use in animal experiments were made at TriLink Biotechnologies (San Diego, California, USA).

Cell lines and treatments with antisense ODNs

Human normal embryonic lung cell line (WI-38), human umbilical vein endothelial cells (HUVECs) and six different human cancer cell lines, including breast adenocarcinoma (MDA-MB-231), colon adenocarcinoma (HT-29), renal carcinoma (A498, Caki-1) and melanoma (A2058), were maintained in α-MEM medium (Gibco/BRL, Gaithersburg, Maryland, USA) supplemented with 10% FBS. Aliquots of cell suspension were seeded into 60- or 100-mm tissue culture dishes and grown to subconfluency (70–80%). Cells were washed once with

PBS and treated with 200 nmol/l (except where indicated otherwise) of PS-AS-ODNs in the presence of cationic lipid (Lipofectin reagent, final concentration, 5 μg/ml; Gibco/BRL) in Opti-MEM (Gibco/BRL) for 4 h. The media containing PS-AS-ODNs was removed, and cells were washed once with PBS and cultured in α-MEM + 10% FBS medium for 16–20 h.

Cell-free transcription/translation

Full-length thioredoxin cDNA including the 5′-UTR and 3′-UTR regions was PCR amplified from a human colon adenocarcinoma cDNA library (5′ StretchPLUS; Clontech, Palo Alto, California, USA) using Taq polymerase (Amersham, Baie d'Urfe, Quebec, Canada) and temperature gradient PCR screening (TGradient, Biometra, Montreal Biotech, Kirkland, Quebec, Canada). From the initial temperature condition screen an amplified product of the correct length was identified and re-amplified with Expand polymerase (Roche Boehringer Mannheim, Laval, Quebec, Canada). The PCR primers used were as follows: 5′-UTR - 5′ ACC GTT GTA TAC **CTT GAA GCT CTG TTT GGT GC** 3′ and 3′-UTR - 5′ TCA ACG GAG CTC **CAT TTC ACA TTT ATT TTG AAA GCT** 3′ (Sigma Biosys, Oakville, Ontario, Canada). The thioredoxin-specific sequences are indicated in bold face and the restriction sites for subsequent cloning are underlined. The PCR product was purified by a commercially available kit (QIAquick; Qiagen, Mississauga, Ontario, Canada), and cloned into the *Acc*I and *Sac*I restriction sites in the multiple cloning region of pSP64polyA (Promega, Madison, Wisconsin, USA, FisherScientific, Nepean, Ontario, Canada and NEB, Mississauga, Ontario, Canada). The resultant construct was oriented such that thioredoxin transcripts could be produced from the SP6 promoter. Two clones that were correct by restriction mapping were sent for DNA sequencing (York University Core Molecular Biology Facility, North York, Ontario, Canada). With the exception of two bases in the 3′-UTR, the sequence matched that of thioredoxin (GenBank accession no. X77584.1 [9], sequence available from the authors by request). The differences were in the 3′-UTR, and as such should not interfere with the antisense targeting of the 5′-UTR and coding region. Thioredoxin and luciferase control (SP6 linear control DNA; Promega) uncapped transcripts were produced with the riboMAX (Promega) in-vitro transcription kit according to the manufacturer's protocol. Full-length transcript yield was determined by agarose gel electrophoresis. Rabbit reticulocyte lysate (RRL) cell-free translation reactions were performed according to the manufacturer's protocol (Promega). Proteins were labeled with [^{35}S]methionine (Redivue L-[^{35}S]methionine; Amersham) during the translation reaction and visualized by autoradiography after resolution by SDS–PAGE. Oligonucleotides were mixed with transcript prior to addition to the translation reaction mix in the amounts indicated in the legends. GTI-2040, an oligonucleotide specific for the small subunit of ribonucleotide reductase, was used as a non-specific control. Between 1/3 and 1/10 molar amounts of luciferase transcript were included in each reaction as an internal control. Where indicated, RNase H (USB, Lake Placid, New York. USA) was added to the transcript mixture at a final concentration of 20 U/ml and incubated for 45 min at 37°C prior to addition to reticulocyte lysate reactions.

Northern and Western blot analysis

To measure the effects of PS-AS-ODNs on thioredoxin mRNA levels, Northern blot analysis was performed as previously described [47] with minor modifications. Briefly, at the indicated times, total cellular RNA was prepared from cells or excised tumors using TRIzol reagent (Gibco/BRL). RNA (10–20 μg) was fractionated on 1.5% formaldehyde gels, transferred to nylon membranes and UV crosslinked. The blots were hybridized with α-^{32}P-labeled 300-bp PCR fragments synthesized using the forward primer (5′-CAG ATC GAG AGC AAG ACT G-3′), the reverse primer (5′-TTC ATT AAT GGT GGC TTC AA-3′) and a human liver 5′ StretchPLUS cDNA library (Clontech) as the template. The thioredoxin nucleotide sequence information was obtained from GenBank. Human *Trx* mRNA, expressed as a 520-bp transcript [48], was visualized and quantified using autoradiography or phosphorimager (Molecular Dynamics, Sunnyvale, California, USA). *GAPDH* mRNA or ribosomal RNA levels were simultaneously probed or stained with methylene blue, respectively, for RNA loading controls. The ^{32}P-labeled 308-bp *GAPDH* DNA probe was generated from the same cDNA library described above by PCR using forward (5′-CGC GGG GCT CTC CAG AAC AT-3′) and reverse (5′-GCA ATG CCA GCC CCA GCG TC-3′) primers.

To measure the effects of PS-AS-ODNs on thioredoxin protein levels, Western blot analysis was performed as previously described [49,50] with minor modifications. Briefly, whole-cell protein extracts were prepared at the indicated times in 50–150 μl of 2 × sample loading buffer (100 mmol/l Tris–HCl, pH 6.8, 0.2 mol/l DTT, 4% SDS, 20% glycerol and 0.015% bromophenol blue) for cell culture studies or in 0.5 ml of RIPA extraction buffer (50 mmol/l Tris–HCl, pH 7.5, 150 mmol/l NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.02% NaN_3 1 mmol/l PMSF and 10 μmol/l leupeptin) by rapid homogenization for in-vivo studies. The protein extracts (10–20 μg) were fractionated by 15% SDS–PAGE, transferred to nitrocellulose membranes and visualized by India ink staining. The expression of thioredoxin was detected with anti-thioredoxin antibody (0.2–1 μg/ml) (American Diagnostics, Greenwich, Connecticut, USA) followed by horseradish peroxidase-conjugated anti-goat IgG (Sigma, St Louis, Missouri, USA) at a dilution of 1:8000. A protein of approximately 12 kDa was visualized by ECL (Amersham, Arlington Heights, Illinois, USA).

Colony-forming assay

The colony-forming ability of cells treated with PS-AS-ODNs was evaluated as previously described [49]. Aliquots of cell suspension were seeded into 60-mm tissue culture dishes at a density of 1×10^4 cells and incubated overnight at 37°C in α-MEM medium supplemented with 10% FBS. Cells were washed once in 5 ml of PBS and treated with 0.2 μmol/l of ODNs in the presence of cationic lipid (Lipofectin reagent, final concentration, 5 μg/ml; Gibco/BRL) for 4 h. The PS-AS-ODNs were removed by washing the cells once with PBS and the cells were cultured in growth medium (α-MEM + 10% FBS) for 7–10 days at 37°C. Colonies were stained with methylene blue and scored by direct counting as previously described [49,50,51].

In-vivo treatment with antisense ODNs

CD-1 athymic female nude mice were purchased from Charles River Laboratories (Montreal, Quebec, Canada), and all animal experimentation was performed following the National Institutes of Health, Sunnybrook and Women College Health Science Center and Lorus Therapeutics Inc. animal care and use guidelines. Experiments were initiated when the mice were 6–7 weeks old. The human colon cancer cell line HT-29 was grown in α-MEM medium supplemented with 10% FBS and 3×10^6 HT-29 cells in 100 μl PBS were s.c. injected into the right flank of each mouse. Each experimental group included five mice randomized into groups so as to provide a similar average tumor volume prior to start of treatment. Once tumors had reached an approximate volume of 50 mm^3, typically 5 days post-tumor cell injection, PS-AS-ODNs were administered by bolus infusion into the tail vein every other day at 10 mg/kg. Treatment lasted 10–14 days thereafter. During treatment, anti-tumor activity was evaluated by calculating tumor volume based on caliper measurements taken at 2- to 3-day intervals. At the end of the treatment (approximately 24 h after the last treatment with PS-AS-ODNs), the animals were sacrificed, and tumor, spleen, liver and body weights were measured. To measure the changes in the expression of thioredoxin protein in tumors, excised tumor fragments of similar size were cut in half, and immediately collected into RIPA extraction buffer for protein preparation and Western blot analysis following the procedure as indicated above.

Densitometry

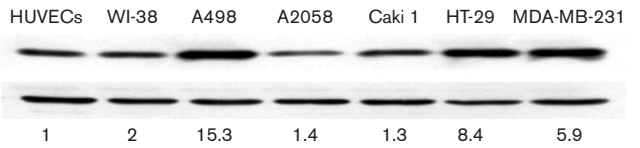
Results were quantified using the GelDoc System and Quantity One quantitation software (version 4.3.0; Bio-Rad, Hercules, California, USA).

Results

Overexpression of thioredoxin in human cancer cell lines

A number of primary tumor tissues have been shown to have markedly elevated levels of thioredoxin mRNA and protein expression. To date, only relative expression levels among different human tumor cell lines have been quantified to define the extent of variation in thioredoxin expression [20]. We extended these studies by comparing the protein expression profiles of five different human tumor cell lines to that of two normal cell lines, HUVECs and WI-38, by Western blot analyses. As illustrated in Fig. 1, thioredoxin protein expression in each of the different tumor cell lines was higher than in HUVECs or in WI-38. Expression varied among the different cell lines, ranging from 1.3- to 15.3-fold relative to HUVEC samples and half that in comparison to WI-38 samples (all samples were normalized to the GAPDH loading control). Elevated expression of thioredoxin was also evident in other tumor cell lines tested, i.e. HT-1080, A549, SK-OV-3, Hep G2, AsPC-1 and C8161, with expression levels ranging from 1.5- to 4.4-fold relative to WI-38 (not shown).

Fig. 1

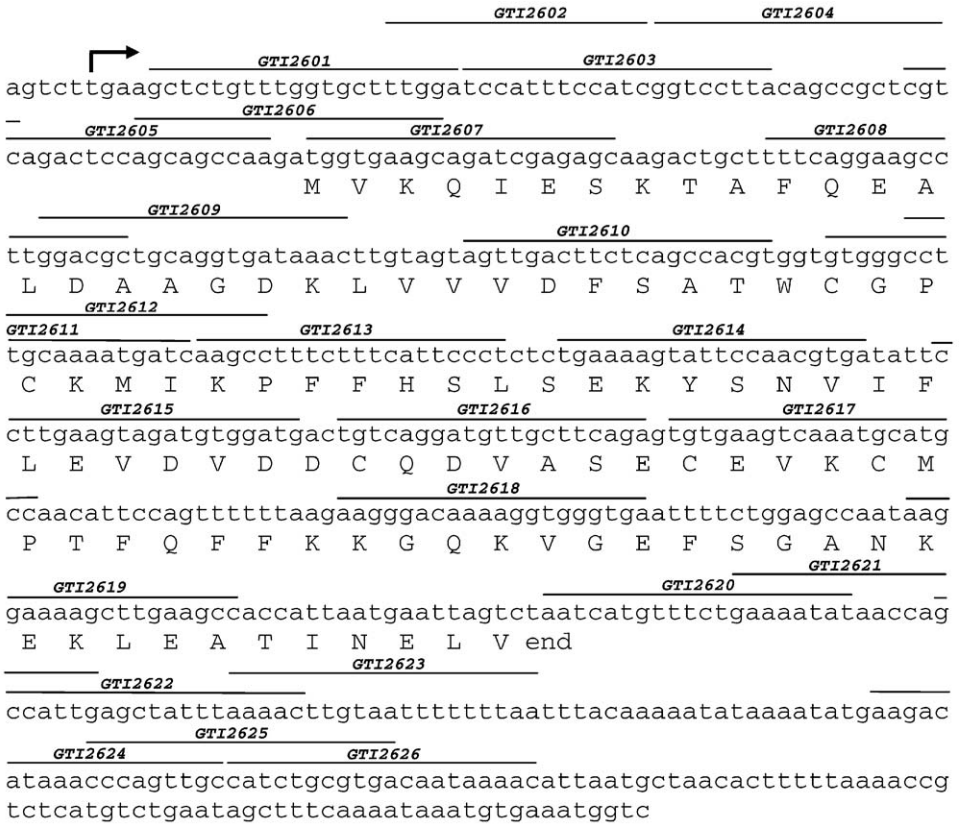


Overexpression of thioredoxin protein in a variety of human tumor cell lines. Human normal and tumor cell lines were cultured to subconfluency, and thioredoxin protein expression levels were determined by Western blot analysis (described in Materials and Methods). Thioredoxin protein expression in human tumor cell lines was compared to that in two normal cell lines (HUVECs and WI-38). Thioredoxin protein levels were quantified and normalized to *GAPDH* protein levels. Relative expression level of thioredoxin protein compared to that of HUVECs is indicated below each cell line.

Selection of PS-AS-ODNs as effective inhibitors of thioredoxin expression

In order to identify an antisense drug candidate that has the ability to perturb thioredoxin expression and thus potentially reduce tumor growth, a library of 26 PS-AS-ODNs ranging from 17 to 20 nucleotides was designed (Fig. 2). The antisense oligonucleotides were selected from the sequence complementary to the thioredoxin mRNA that exhibits the least potential for duplex formation, hairpin formation and homooligomers/sequence repeats, while maintaining a high potential for binding to the thioredoxin mRNA sequence. In addition, false priming to other frequently occurring or repetitive sequences in the human and mouse genomes was eliminated. The above properties were determined using the OLIGO primer analysis software (version 5.0; distributed by National Biosciences, Plymouth, Minnesota, USA). In total, 26 PS-AS-ODNs were selected with five PS-AS-ODNs (GTI-2601 to -2605) targeting the 5′-UTR, two PS-AS-ODNs (GTI-2606 and -2607) designed around the translation initiation site, 12 PS-AS-ODNs

Fig. 2



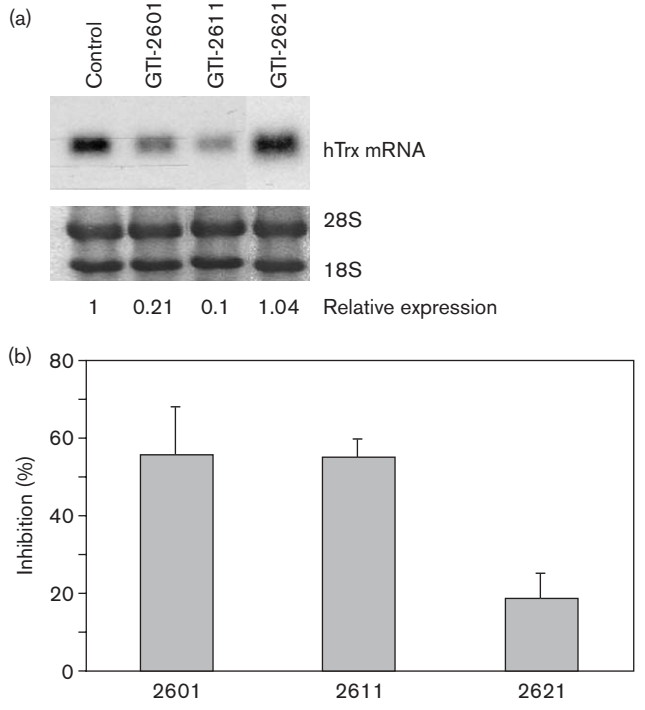
Antisense oligonucleotide sequences designed to target thioredoxin mRNA. Hybridization sites to which 26 different antisense ODNs (GTI-2601 to GTI-2626) anneal are indicated on the human thioredoxin cDNA sequence obtained from GenBank accession no. X77585. Target sites are shown with bars above the sequence and the transcription initiation site is indicated with an arrow. Amino acid sequences are also indicated to distinguish the coding region from the 5′-UTR and 3′-UTR.

(GTI-2608 to -2619) targeting the coding region and seven PS-AS-ODNs (GTI-2620 to -2626) targeting the 3′-UTR (Fig. 2).

All 26 antisense PS-AS-ODNs were subjected to in-vitro screening by Northern blot analysis to identify potential candidates which reduce thioredoxin mRNA levels by possibly eliciting RNase H-dependent cleavage of the mRNA/PS-AS-ODN hybrids [52]. The HT-29 human colon tumor cell line was chosen for these experiments since the level of thioredoxin expression was consistently high in these cells in both Western (see Fig. 1) and Northern blot (not shown) analyses. GTI-2601 and -2611, targeting the 5′-UTR and coding region of thioredoxin mRNA, respectively, exerted potent inhibitory effects (greater then 75%) on the steady-state level of thioredoxin mRNA (a representative Northern result is shown in Fig. 3a). The majority of the other antisense PS-AS-ODNs exhibited moderate inhibition of thioredoxin mRNA (not shown). A few PS-AS-ODNs, including GTI-2621, did not have an inhibitory effect on thioredoxin mRNA expression (Fig. 2). Thioredoxin mRNA levels in two other cell lines (MDA-MB-231 and Hep G2) followed a similar overall pattern of inhibition with only minor variations (data not shown), suggesting that the sites recognized by GTI-2601 and -2611 are highly accessible, and are likely to be sensitive to RNase H-mediated cleavage.

To determine whether GTI-2601- and -2611-mediated inhibition of thioredoxin gene expression leads to inhibition of cell proliferation, the colony-forming ability of HT-29 cells was estimated. HT-29 cells treated with GTI-2601 and -2611 were significantly retarded in their cell growth and colony-forming ability *in vitro*, with the degree of inhibition being greater then 50% in both cases (Fig. 3b). Under the same conditions, GTI-2621 had significantly lower anti-proliferative activity (less then 20%) (Fig. 3b). These results indicate a correlation between inhibition of thioredoxin expression and anti-proliferative activity. The minor reduction in colony formation observed with treatment with GTI-2621 may be due in part to non-specific effects of the phosphorothioated backbone of the ODN.

Fig. 3



Inhibition of thioredoxin mRNA expression in HT-29 cells by antisense oligonucleotides corresponds to inhibition of colony formation. (a) HT-29 cells were transfected with 200 nmol/l of PS-AS-ODNs GTI-2601, -2611 or -2621 in the presence of Lipofectin reagent (Invitrogen) as described in Materials and methods, and total RNA was prepared 20 h post-transfection for Northern blot analysis. Control samples were also prepared in which case HT-29 cells were treated with Lipofectin alone. Human thioredoxin mRNA levels were determined by Northern blot analysis as described in Materials and methods. Ribosomal RNA markers (28S and 18S) are shown in the lower panel to indicate loading consistency. Thioredoxin mRNA levels were quantified and normalized to 28S RNA as described in Materials and methods. (b) Anti-proliferative effects of GTI-2601, -2611 and -2621 in HT-29 human tumor cells were determined using a colony-forming assay. HT-29 cells were transfected with PS-AS-ODNs as indicated and colony numbers were determined as described in Materials and methods. Percent inhibition was calculated by comparing the number of colonies present in cultures grown in the absence of PS-AS-ODNs with that of cultures transfected with GTI-2601, -2611 or -2621. All experiments were performed in quadruplicate.

Anti-tumor activity of GTI-2601 and -2611 *in vivo*

In-vitro studies discussed above indicate that GTI-2601 and -2611 are potent inhibitors of thioredoxin expression and cell proliferation, and are therefore interesting candidates for further study. To determine whether GTI-2601 and -2611 can act as effective antisense compounds that decrease tumor growth *in vivo*, we tested both PS-AS-ODNs on nude mice bearing s.c. grown HT-29 tumor xenografts. When administered i.v. as a bolus injection every other day at a concentration of 10 mg/kg for 10 days, GTI-2601 showed significant inhibitory effects on tumor growth as measured by changes in tumor volume and weight (Fig. 4a and b). Compared with saline, GTI-2601 displayed an almost complete inhibition (greater then 80%) of HT-29 tumor growth in mice over the 10-day treatment period. Although GTI-2611 also produced a significant reduction of thioredoxin mRNA and cell proliferation *in vitro*, the effect of GTI-2611 treatment *in vivo* was less significant then that of GTI-2601. Compared with saline, inhibition of tumor growth with GTI-2611 treatment was approximately 50% (Fig. 4b).

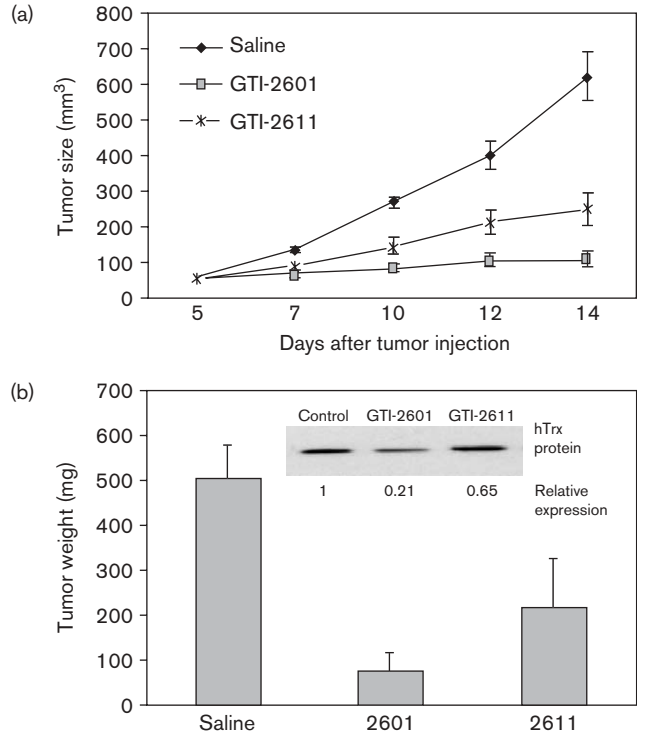
We characterized the effects of GTI-2601 and -2611 on thioredoxin protein expression to determine whether the observed anti-tumor effects may indeed occur through an antisense mechanism of action *in vivo* and to determine whether the observed differences in anti-tumor activity could be correlated to target downregulation. Tumors were isolated from saline control and PS-AS-ODN-treated mice as described in Materials and methods, and thioredoxin protein levels were determined by Western blot analysis. Reduced levels of thioredoxin protein (Fig. 4b, inset) were observed in the tumors isolated from mice treated with GTI-2601. The level of thioredoxin protein in tumors treated with GTI-2611 (that had lower anti-tumor activity) was higher than that of GTI-2601, suggesting that the observed anti-tumor activity is related to target downregulation (Fig. 4b). The contrast between in-vitro and in-vivo results obtained for treatment with GTI-2611 highlights the potential contributions of pharmacokinetic properties and cellular uptake rates on the in-vivo efficacy of antisense compounds.

Sequence specificity of GTI-2601 *in vitro*

To examine whether GTI-2601-mediated effects on thioredoxin gene expression were sequence specific, an 8-bp mismatched analog of GTI-2601 (GTI-2601-MIS8) was synthesized (Fig. 5a). GTI-2601-MIS8 was used to treat HT-29 cells, and its effects on thioredoxin mRNA and protein levels were determined. As displayed in Fig. 5b, the thioredoxin mRNA level in cells treated with GTI-2601-MIS8 was similar to that of control cells. Again, significant reduction of thioredoxin mRNA was evident in the cells treated with GTI-2601. Furthermore, as illustrated in Fig. 5b, GTI-2601-mediated reduction of thioredoxin mRNA was dose-dependent, whereas an inhibition of thioredoxin mRNA level was not evident in cells treated with GTI-2601-MIS8 even at the maximum concentration tested of 500 nmol/l. Inhibition of thioredoxin protein level was also observed for HT-29 cells treated with GTI-2601, but not for cells treated with GTI-2601-MIS8 (Fig. 5c). Taken together, these results suggest that the inhibitory effects of GTI-2601 are likely due to its ability to bind specifically to its intended target and invoke downregulation of the target mRNA, thereby producing fewer transcripts for translation into protein.

Alternatively, it is possible that the binding of GTI-2601 to the 5′-UTR may interfere with the translation processes (initiation, elongation or termination) and this may partially be responsible for the decrease in protein

Fig. 4



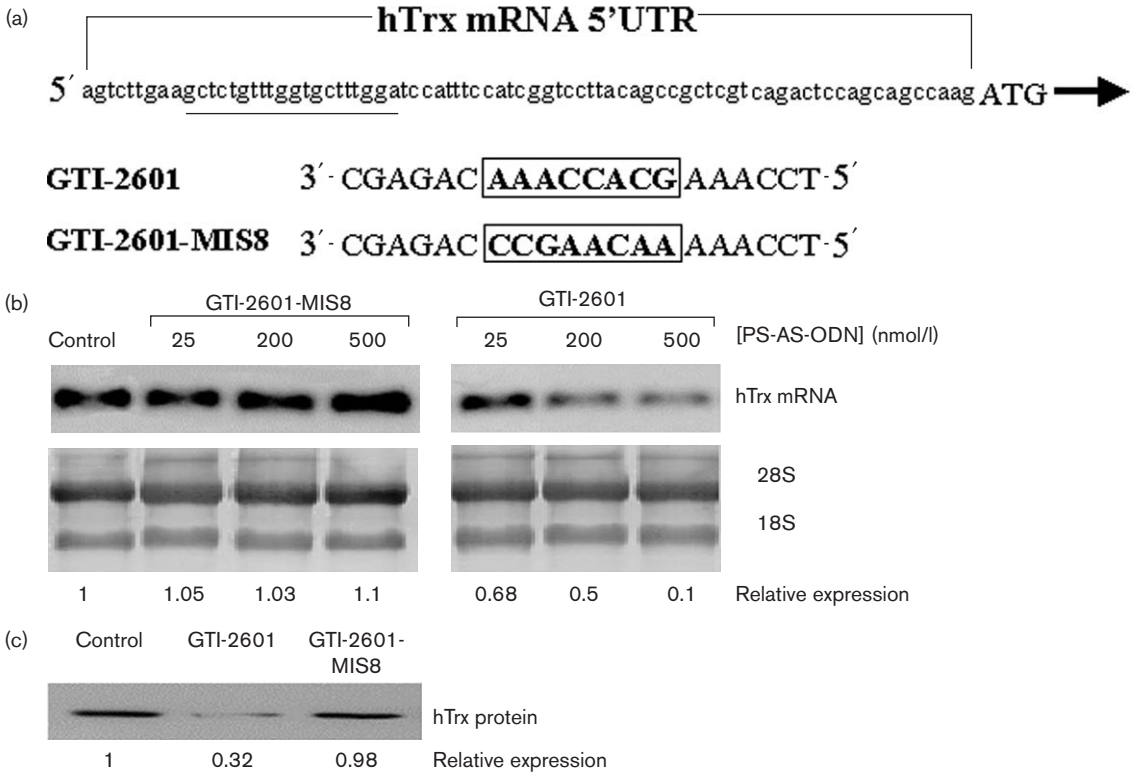
Effects of antisense PS-AS-ODNs on tumor growth and thioredoxin mRNA protein levels. HT-29 human colon cancer cells were s.c. injected into CD-1 athymic female nude mice (3×10^6 cells injected). After the size of the tumor reached an approximate volume of 50 mm^3, 5 days post-tumor cell injection, antisense PS-AS-ODNs GTI-2601 or -2611 were administered by bolus infusion into the tail vein every other day at 10 mg/kg. The control or saline group received PBS alone every other day. (a) Tumor volumes were calculated from caliper measurements taken at 2- to 3-day intervals. Mean tumor volume and SEM are shown for each experimental group. (b) At the end of the experiment tumors were excised from animals treated with either saline alone or PS-AS-ODNs as indicated. Mean tumor weight and SEM are shown for each experimental group. Excised tumor fragments of similar size were immediately collected into RIPA extraction buffer and rapidly homogenized for protein preparation. To measure the effects of PS-AS-ODNs on thioredoxin protein levels, Western blot analysis was performed as described in Materials and methods. Relative expression level (based on equal protein loading) of thioredoxin protein in PS-AS -ODN treatment groups compared to the expression level in the control or saline group is indicated (see inset of graph).

levels. This mechanism of action by antisense ODNs is restricted to ODNs directed against the 5′-UTR and starting AUG as the fully assembled 80S ribosome has unwinding activity that would resolve RNA/DNA duplexes as translation proceeds [53,54]. In order to determine whether GTI-2601 can inhibit translation of thioredoxin mRNA in the absence of RNase H activity, cell-free translation of *in vitro* synthesized thioredoxin transcripts was analyzed in RRL. Previous studies have demonstrated that RRL has no intrinsic RNase H activity [55]. Full-length thioredoxin cDNA was amplified out of a colon cDNA library and subcloned into a pSP64polyA – a vector that supports cell-free SP6 RNA polymerase synthesis of polyadenylated RNA transcripts. Plasmid containing luciferase cDNA was used to produce internal control transcripts. Transcript mixtures, containing thioredoxin and luciferase mRNA, were incubated with RRL in the presence of [^{35}S]methionine and proteins resolved by SDS–PAGE. Figure 6 represents results of typical translation reactions. GTI-2601MIS8 is the 8-base mismatch analog of GTI-2601 (Fig. 5a). GTI-2040 is an antisense compound specific for the small subunit of ribonucleotide reductase. Each reaction contained the same thioredoxin:luciferase transcript mixture so that the resulting thioredoxin translation products could be normalized to luciferase. Pre-incubation of transcript mixtures with oligonucleotides resulted in decreased expression of thioredoxin in RRL in a non-specific manner, suggesting a general suppression of translation occurs. Addition of RNase H to the transcript:ODN mix and incubation for 45 min prior to RRL translation resulted in an inhibition pattern consistent with GTI-2601 having sequence-specific effects on thioredoxin translation (*cf.* 25 and 17% for GTI-2601-MIS8 and -2040, respectively, to 8% for GTI-2601). These results suggest that the major pathway of antisense inhibition in cell-free translation systems is RNase H mediated. The observed inhibition of thioredoxin expression by the control oligonucleotides is consistent with a number of reports that demonstrate that PS-ODNs can produce non-specific inhibition of translation in cell-free systems over a broad concentration range (reviewed extensively in [56]). This inhibition may be due to a number of factors including RNase H cleavage of non-targeted RNA (i.e. limited sequence specificity at elevated concentrations) or, alternatively, the PS backbone may have a non-specific effect on translation due to its high protein binding properties (reviewed in [56]). Another cell-free translation system, wheat germ lysate (WGL), has been shown to have measurable RNase H activity [55]. In WGL translation reactions GTI-2601 inhibited thioredoxin translation to a greater extent than either control oligonucleotide, consistent with RNase H-mediated downregulation by GTI-2601 (data not shown).

Anti-tumor activity of GTI-2601 relative to GTI-2601-MIS8 *in vivo*

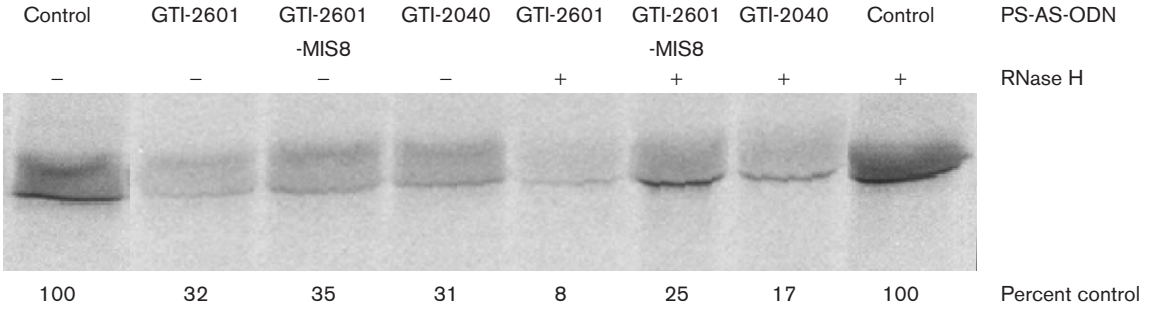
To further characterize the sequence specificity of GTI-2601, the anti-tumor activity of GTI-2601-MIS8 was assessed. Tumor weights obtained from mice treated with saline and those from mice treated with GTI-2601-MIS8 were not significantly different ($P = 0.17$; Fig. 7a), whereas tumors from mice treated with GTI-2601 showed a significant reduction in size ($P = 0.01$; Fig. 7a). The relatively small reduction in the mean tumor weight of GTI-2601-MIS8-treated mice may be attributed to the non-sequence-specific growth-inhibitory effects often experienced when using PS-AS-ODNs as therapeutic agents [57]. The significant anti-tumor effects of GTI-2601 along with the lack of the

Fig. 5



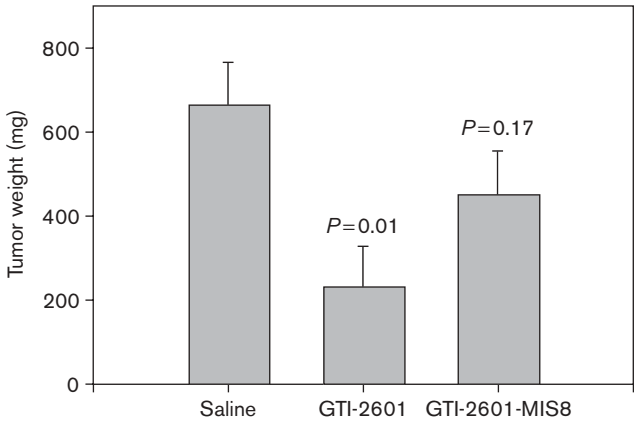
Sequence-specific and dose-dependent inhibition of thioredoxin expression by GTI-2601. (a) The target site in the 5′-UTR to which GTI-2601 anneals is underlined on the thioredoxin cDNA sequence obtained from [9]. The translation initiation codon ATG is also indicated. Shown is the sequence for PS-AS-ODNs GTI-2601 and -2601-MIS8. GTI-2601-MIS8 contains 8 base changes in the middle of the GTI-2601 sequence (see boxed sequence). (b) HT-29 cells were transfected with increasing concentrations (25–500 nmol/l) of GTI-2601 or -2601-MIS8 in the presence of Lipofectin reagent and thioredoxin mRNA levels were analyzed by Northern blot analysis. Control cells were treated with Lipofectin alone. Ribosomal RNA markers (28S and 18S) are shown below to indicate loading consistency. Thioredoxin mRNA levels were quantified and normalized to 28S RNA. Relative expression level of thioredoxin mRNA in treated samples compared to the control or untreated sample is indicated. (c) HT-29 cells were transfected with 200 nmol/l of GTI-2601 or -2601-MIS8 in the presence of Lipofectin and thioredoxin protein levels were determined by Western blot analysis. Relative expression of treated samples in comparison to the control sample was determined (based on equal protein loading).

Fig. 6



GTI-2601 mediated inhibition of thioredoxin mRNA translation in the RRL cell-free system. Rabbit reticulocyte translations containing 4 μCi Redivue L-[^{35}S]methionine (Amersham) were performed using micrococcal nuclease-treated RRL prepared from New Zealand white rabbits (Promega). An aliquot of 0.5 μl of transcript mixture containing thioredoxin transcript, luciferase transcript and, where indicated, PS-AS-ODN (right hand panel) was added to 9.5 μl of the RRL translation mixture. The samples were incubated at 30°C for 1 h. Where indicated, the PS-AS-ODN:transcript mixture was treated with RNase H prior to translation. Translation reactions were diluted in 1 × SDS sample buffer, proteins resolved by SDS–PAGE, and gels stained with Coomassie blue (Bio-Rad) in a 10% acetic acid/20% methanol solution. Following staining, gels were destained with 10% acetic acid/20% methanol, dried and autoradiographed on Kodak X-OMAT film with a Transcreen LE intensifying screen (Kodak). The results were quantitated using Quantity One software (Bio-Rad). Below each lane is the percent thioredoxin translation product corrected for loading using the luciferase band at 60 kDa.

Fig. 7



Effects of GTI-2601 and -2601-MIS8 on HT-29 tumor growth in nude mice. Experiments were carried out essentially as described in Fig. 4 except PS-AS-ODNs GTI-2601 and -2601-MIS8 were administered seven times. At the end of the experiment tumors were excised from all animals within each experimental group and tumor weight measured. Mean tumor weights and SEMs are shown with *P* values (above error bars) for the PS-AS-ODN-treated groups in comparison to the saline group.

sequence-specific anti-tumor effects exhibited with GTI-2601-MIS8 suggest that GTI-2601 exerts its effects through an antisense mechanism of action *in vivo*. The anti-tumor effects of GTI-2601 were also observed in mice bearing Hep G2 hepatocellular carcinoma (data not shown). In addition to anti-tumor activity, GTI-2601 was tested in an experimental model of metastasis. Preliminary studies indicated that treatment of human melanoma cells with GTI-2601 prior to injection into the tail vein of mice prevented metastasis to the lung (data not shown).

Discussion

Thioredoxin plays a critical role in a number of cellular processes, demonstrating an involvement in DNA synthesis, inhibition of apoptosis, and stimulation of angiogenesis, cell proliferation and transcription factor activity. Thioredoxin is reportedly overexpressed in a number of primary tumors such as colon, non-small cell lung carcinomas, cervical, gastric, breast, hepatocellular carcinoma, squamous cell carcinoma, myeloma, non-Hodgkin's lymphoma, mesothelioma and acute lymphocytic leukemia [19–30,57]. Furthermore, there is a demonstrated correlation between thioredoxin expression, aggressive tumor growth and poorer patient prognosis. In certain cancers thioredoxin also confers drug resistance to chemotherapeutic agents [21,26,28,42–45]. Taken together, these studies indicate that thioredoxin is an important target for development of new drugs to treat human cancer.

Antisense technology has been widely adopted not only as a useful research tool [58], but also as a rational approach to develop new therapeutic compounds for the treatment of many human diseases including cancer [59–63]. AS-ODNs specifically hybridize to mRNA sequences and inhibit expression of proteins that are important in initiation and/or progression of human cancer. Several AS-ODN compounds are in various developmental stages clinically for a wide range of human diseases [62–64]. One antisense compound has recently been approved for the treatment of cytomegalovirus retinitis in AIDS patients [65]. Although AS-ODN compounds targeting thioredoxin have not been developed for the treatment of cancer, one study has demonstrated that an expression vector harboring the thioredoxin gene inserted in an antisense orientation can reduce the cellular level of thioredoxin when transfected into human tumor cells [43]. These thioredoxin antisense transfectants became more sensitive to various chemotherapeutic drugs, including cisplatin, doxorubicin, mitomycin C and etoposide [43]. Additional support for targeting of the thioredoxin/thioredoxin reductase redox system comes from studies in which disulfide inhibitors of these enzymes display anti-tumor efficacy [66]. Finally, several small-molecule inhibitors of thioredoxin and thioredoxin reductase are being developed with anti-proliferative activities against human cancer cell lines [1,67–70], including, PX-12, NSC 131233, motexafin gadolinium, organotellurium compounds, naphthaquinone spiroketal compounds and platinum complexes [1], some of which are currently being assessed in clinical trials.

In this study we show that thioredoxin is overexpressed in a variety of human tumor cell lines that are routinely used for in-vivo animal studies, when compared with two normal cell lines. The highest expression was observed with colon, lung, liver and breast cancer cell lines, correlating well with previous reports from primary tumors from patients [21,23–30]. These and other studies showing growth-promoting activities of thioredoxin suggest that thioredoxin may play an important role in tumor cell growth. In the present study we have utilized antisense technology not only to further characterize thioredoxin's involvement in cancer, but also to validate thioredoxin as a potential target for anti-cancer therapy and characterize a possible antisense drug candidate. Current therapeutic approaches to inhibit the thioredoxin system focus on small-molecule inhibitors of thioredoxin and thioredoxin reductase. One clear advantage of antisense therapeutics over conventional drugs is the level of specificity that can be achieved. As a result of this high specificity, antisense drugs are very well tolerated both in animal Good Laboratory Practice toxicology studies and in clinical trials [61,62].

One PS-AS-ODN, GTI-2601, selected based on its potent inhibitory activity on thioredoxin expression, targets the 5′-UTR of thioredoxin mRNA. Treatment

with GTI-2601 resulted in significant reduction of thioredoxin mRNA and protein levels in a sequence-specific and dose-dependent manner. This led to a marked reduction in cell proliferation as estimated by the ability to form colonies in cell culture or by inhibition of tumor growth in human carcinoma xenograft mouse models. It is interesting to note that one PS-AS-ODN, GTI-2611, displayed antisense activity *in vitro*, in terms of decreasing mRNA and decreasing cell proliferation, but was not as effective (relative to GTI-2601) *in vivo*. One possible explanation for this discrepancy is that the mechanism by which ODNs are taken up by cells *in vivo* is not as controlled as it is *in vitro*. As a result there may be differences in the rates at which different ODNs are taken up by tumor cells. Alternatively, the half-life of ODNs may vary *in vivo* and only the more stable remain at an effective concentration once injected into animals. Finally, it is possible that mRNA/ODN duplexes are differentially recognized by RNase H. As a result, different concentrations of each ODN may be required for optimal antisense activity *in vivo*. Taken together, these data suggest that a specific decrease in thioredoxin gene expression by GTI-2601 may be a direct cause of growth arrest observed *in vitro* and *in vivo*.

An issue that must inevitably be addressed when developing antisense therapeutics is whether the compound produces non-specific immune stimulation that is not a result of target sequence interactions [57]. Immune stimulation can be the result of two properties of AS-ODN – one sequence specific and one backbone specific [57]. Unmethylated CpG dinucleotides, usually present in bacterial DNA, stimulate innate immune responses in vertebrates, and can further augment acquired immune responses to both pathogens and tumor cells. The presence of unmethylated CpGs in an oligonucleotide can have the same effect if in an optimal sequence context. In addition, the PS backbone, used in first-generation antisense compounds, has been found to be immune stimulatory in a sequence-independent manner [57]. GTI-2601 does not contain a CpG dinucleotide and yet demonstrates significant anti-tumor activity. Both GTI-2611 and -2601MIS8 have the same backbone as GTI-2601, but demonstrate less anti-tumor activity (especially in the case of GTI-2601-MIS8), suggesting that the effect of GTI-2601 is sequence specific. The small, anti-tumor activity with GTI-2601MIS8 and -2611 may be a reflection of non-specific immune-stimulatory activity.

The specific mechanism by which GTI-2601 inhibits tumor cell proliferation is not yet clear. Downregulation of thioredoxin in human cancer cells by GTI-2601 may interfere with a variety of biological processes that are important for cell survival. The activity of ribonucleotide reductase, which is directly regulated by thioredoxin [5], can be profoundly affected by a decrease in thioredoxin level. Deregulation of ribonucleotide reductase could lead to the attenuation of, or arrest in, DNA replication, leading to the inhibition in cell proliferation. Another mechanism by which tumor cell growth may be affected is that the autocrine growth factor activity associated with extracellular thioredoxin [9,15,16] is reduced by the inhibition of thioredoxin production by GTI-2601 treatment. Alternatively, it has been shown that a mutation in the thioredoxin could reverse the transformed phenotype by inducing spontaneous or drug-induced apoptosis [32]. Antisense-mediated inhibition of thioredoxin expression by GTI-2601 may confer similar properties to cells by rendering the treated cells more susceptible to spontaneous or drug-induced apoptosis. In addition, the activities of transcription factor protooncogenes are modulated by the reducing capacity of thioredoxin [13,71], hence downregulated thioredoxin may interfere with transcriptional activation of genes important in oncogenesis. These possibilities are currently being explored in detail to elucidate the mechanisms underlying the growth inhibitory function of GTI-2601.

A number of recent reports demonstrated that changes in cellular levels of thioredoxin modulate the sensitivity of human cancer cells to various chemotherapeutic drugs, and suggested that upregulated thioredoxin may be responsible for the acquired resistance to cisplatin and other superoxide-generating agents [1]. Therefore, a downregulation of thioredoxin may render tumors more susceptible to conventional anti-neoplastic drugs, and opens the possibility of developing combination therapies involving GTI-2601 and existing chemotherapeutic drugs. This approach may improve the therapeutic efficacy of chemotherapeutic drugs in clinical use by exerting additive and/or synergistic effects. Studies to evaluate the anti-tumor efficacy of GTI-2601 in combination therapy are ongoing.

In conclusion, thioredoxin, which is widely overexpressed in human cancer, appears to play an important role in tumor growth, and specific inhibition of thioredoxin expression by the PS-AS-ODN GTI-2601 resulted in potent antiproliferative and anti-tumor activities. The ability of GTI-2601 to specifically inhibit tumor cell growth opens up the exciting possibility of further developing GTI-2601 as a novel anti-tumor therapeutic compound. The effects of thioredoxin downregulation on a variety of downstream cellular targets are currently being assessed in order to elucidate the biochemical mechanisms of tumor suppression.

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