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Antisense-induced down-regulation of thymidylate synthase and enhanced cytotoxicity of 5-FUdR in 5-FUdR-resistant HeLa cells

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1 Thymidylate synthase (TS) is a target for several anticancer drugs. We previously showed that an antisense oligodeoxynucleotide (ODN) directed against TS mRNA down-regulated TS protein and enhanced cytotoxicity of TS-targeting drugs [including 5-fluorodeoxyuridine (5-FUdR)] in HeLa cells. Patient tumours with increased TS expression are resistant to TS-targeting drugs. It was hypothesized that TS mRNA and consequently TS protein could be down-regulated in 5-FUdR-resistant cells that overexpress TS, sensitizing them to 5-FUdR cytotoxicity. In this study we assessed the capacity of an anti-TS antisense ODN to circumvent resistance dependent on TS overexpression.

2 Variant HeLa clones exhibiting 2–20 fold resistance to 5-FUdR were selected by exposing cultured cells to drug. Clones FUdR-5a, -25b, and -50a expressed TS protein levels 10 fold, 10 fold, and 17 fold higher (respectively) than parental cells. Cells were treated with antisense ODN 83 (a 2′-methoxy-ethoxylated, phosphorothioated 20-mer, complementary to a portion of the 3′-untranslated region of TS mRNA), or ODN 32 (a control ODN with the same base composition as ODN 83, but in randomized order). Twenty-four and 48 h following transfection (50–100 nMPCR) and protein levels (by radiolabelled 5-FUdR-monophosphate binding) were decreased by at least 60% in ODN 83-treated cells compared with control ODN 32-treated cells. ODN 83 enhanced the cytotoxicity of 5-FUdR by up to 85% in both parental and 5-FUdR-resistant cell lines.

3 Antisense ODN can be used to down-regulate TS and attenuate drug resistance in TS-overexpressing cells.

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Abbreviations: D-MEM, Dulbecco's modified Eagle's medium; 5-FdUMP, 5-fluorodeoxyuridine monophosphate; 5-FU, 5-fluorouracil; 5-FUdR, 5-fluorodeoxyuridine; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; IC_{50} value, concentration of drug that inhibited proliferation by 50%; LFA-2K, Lipofectamine 2000®; Me-FH_4, 5,10-methylene-tetrahydrofolate; ODN, oligodeoxynucleotide; PBS, phosphate-buffered saline (0.15 M NaCl + 0.67 mM KH_2PO_4, pH 7.4); RT–PCR, reverse transcriptase-polymerase chain reaction; TS, thymidylate synthase (5,10-methylenetetrahydrofolate : dUMP *C*-methyltransferase; EC 2.1.1.45); UTR, untranslated region

Introduction

Thymidylate synthase (5,10-methylenetetrahydrofolate:dUMP *C*-methyltransferase; EC 2.1.1.45) (TS) is important in DNA precursor synthesis and repair, and is an important target for anticancer chemotherapy. The enzyme is a highly conserved homodimer of 35 kDa subunits, and catalyses the synthesis of thymidylate from deoxyuridylate and 5,10-methylene-tetrahydrofolate (Me-FH_4) (Chu & Allegra, 1996; Danenberg, 1977). The TS protein exerts tight control over its own synthesis during cell cycle by binding to TS mRNA both at the translational start site (TSS) and at a specific coding region, thereby regulating translational processing of the message (Johnson, 1994; Chu *et al*., 1991; 1993b). TS protein has been reported to bind to mRNAs of at least nine other proteins important in cell cycling and resistance to toxicity, including p53 (Chu *et al*., 1996) and c-*myc* (Chu *et al*., 1994), and modulates the translatability of p53 mRNA *in vitro* (Chu *et al*., 1999; Ju *et al*., 1999). Direct TS-targeting drugs also arrest cells *in vitro* in early S phase (Inaba & Mitsuhashi, 1994; Matsui *et al*., 1996; Yin *et al*., 1999). Therefore, altering the level of active TS protein changes the ability of cells to replicate DNA and progress through the cell cycle.

Direct inhibitors of the TS enzyme include the nucleoside analogue 5-fluorodeoxyuridine monophosphate (5-FdUMP) [a metabolite of 5-fluorouracil (5-FU) and 5-fluorodeoxyuridine (5-FUdR) produced within mammalian cells] and folate analogues including N-(5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl-N-methylamino]-2-thenoyl)-L-glutamic acid (raltitrexed, Tomudex, ICID 1694) (Jackman *et al*., 1991). In cell lines selected for resistance to drugs of both these types, a common mechanism of resistance is increased cellular expression of TS (Zhang *et al*., 1992), possibly resulting directly from increased TS mRNA (Murakami *et al*., 2000; Kitchens *et al*., 1999; Shibata *et al*., 1998). In

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patient tumours, therefore, preferential survival of variant tumour cells with increased TS levels in the presence of drug, or induction of TS gene transcription leading to increased TS levels, is a potential impediment to antitumour drug treatment. In addition, a novel drug-dependent 'translational release' leading to transiently increased TS levels has been described. Raltitrexed, 5-FU or 5-FUdR, and Me-FH_4 are all able to relieve the repression of translation caused by binding of TS protein to TS mRNA (Chu & Allegra, 1996; Chu *et al*., 1991; Lin *et al*., 2000; Mader *et al*., 1997; Parr *et al*., 1998; Peters *et al*., 2000; Welsh *et al*., 2000). This results in a transient increase in TS mRNA translation and TS protein levels, a phenomenon observed *in vitro* (Chu *et al*., 1990; 1991; 1993a; Keyomarsi *et al*., 1993; Mader *et al*., 1997; Parr *et al*., 1998; Van der Wilt *et al*., 1992), and in animals (Van der Wilt *et al*., 1992) and patients (Peters *et al*., 1994; Swain *et al*., 1989). This induction of TS synthesis [upwards of 3–5 fold (Chu *et al*., 1990; 1993a; Van der Wilt *et al*., 1992)] would serve to at least partially circumvent the cytotoxic effect of the drug (Berne *et al*., 1986). Conversely, a favourable response by colon carcinomas to treatment with 5-FU correlates directly with lower levels of TS in patient tumours (Edler *et al*., 2000; Mini *et al*., 1999; Peters *et al*., 1994; Salonga *et al*., 2000; Van Triest & Peters, 1999).

Given the importance of regulation of mRNA in TS production, we hypothesized that the use of an antisense strategy that targeted TS mRNA in combination with drugs targeting TS protein would be effective in wholly or partially circumventing resistance to treatment.

A number of antisense oligodeoxynucleotides (ODNs) and vectors are reported to enhance the cytotoxicity of various drugs, by targeting modulators of drug activity and cell response as opposed to the drug target *per se*, with *mdr1* and *bcl-2* being the most common targets of antisense (Cucco & Calabretta, 1996; Kitada *et al*., 1994; Li *et al*., 1997; Luo *et al*., 1999; Miayake *et al*., 2000; Quattrone *et al*., 1994; Thierry *et al*., 1993). Based on the success of *in vivo* screening of antisense ODNs, at least seven of these agents are currently in clinical trial for anti-cancer treatment, with targets including bcl-2 (Chen *et al*., 2000; Scher *et al*., 2000; Waters *et al*., 2000) and p53 (Bishop *et al*., 1996). The success of antisense strategies to reduce specific cellular proteins and improve disease therapies led us to generate a protocol to reduce the cellular levels of TS mRNA through the use of specific antisense ODNs. An antisense ODN was designed to anneal with a 20-base region in the 3′-untranslated region (UTR) of TS mRNA and induce RNase H-dependent cleavage (Bennett, 1998; Binder *et al*., 1994; Stein *et al*., 1988; Stewart *et al*., 1996). The ODN used in these experiments is a phosphorothioated oligomer of 20 bases, modified by methoxyethoxylation at the 2′-positions of the six sugars on either end of the ODN. This structure is very resistant to nuclease digestion (Dean *et al*., 1994; 1996; Shaw *et al*., 1991; Stein & Cheng, 1993; Stein *et al*., 1988).

We previously demonstrated that antisense-induced decreases in cellular TS mRNA in HeLa cells: (a) reduced the cellular content of TS protein; (b) inhibited cellular proliferation; and (c) enhanced the cytotoxicity of TS inhibitors (Ferguson *et al*., 1999). However, elevated TS levels expressed in some patient tumours of various tissue etiologies (Mini *et al*., 1999; Peters *et al*., 1994; Suzuki *et al*., 1999; Takenoue *et al*., 2000) might make them resistant to antisense therapy. To determine the potential for anti-TS antisense therapy to overcome drug resistance associated with TS, it was necessary to determine whether the antisense protocol used against parental HeLa cells, unselected for resistance to TS-targeting drugs, could abrogate resistance due to TS overexpression. Therefore, a series of cell lines was selected for increasing resistance to 5-FUdR in order to establish a cell culture model in which the ability to down-regulate TS-overexpression could be tested. We report here that, in these TS-overexpressing, 5-FUdR-resistant cell lines, an antisense ODN against TS mRNA was able to decrease the amount of TS enzyme and increase the sensitivity of the cells to 5-FUdR.

Methods

Antisense oligonucleotides

Fully phosphorothioated 20-base ODNs were synthesized by ISIS Pharmaceuticals (Carlsbad, CA, U.S.A.) as described previously (Dean *et al*., 1996). The six nucleotides on either end of the ODN were methoxyethoxylated in the 2′-position to enhance hybridization to TS mRNA and ODN resistance to exonuclease digestion (Dean *et al*., 1994). The central eight nucleotides were left without methoxyethoxylation to allow access by RNase H and degradation of mRNA hybridized to the ODN (Dean *et al*., 1994). ODN 83 is complementary to TS mRNA, starting from a position 136 bases downstream of the translational stop site (5′-GCCAGTGGCAACATCCT-TAA-3′). ODN 32 is a randomized sequence of ODN 83 (5′-ATGCGCCAACGGTTCCTAAA-3′), with the same base constituents in random order. A search of available mRNA sequences using the NCBI BLAST search tool, assuming a requirement for 10 or more hybridizable bases (adjacent or not) to constitute complementarity, revealed that ODN 83 was complementary only to human TS mRNA, and ODN 32 was not complementary to any known mRNA.

Radioisotopes

[6-^{3}H]5-FdUMP (specific activity 18.6 Ci $mmol^{-1}$) was purchased from Moravek Biochemicals (Brea, CA, U.S.A.). This isotope was 99.98% pure upon initial production, with a degradation rate of 0.5–1% per month at −20°C. It was used within 6 months of manufacture.

Chemotherapy reagent

5-FUdR was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Other supplies

Cell culture chemicals and nutrients were obtained from Canadian Life Technologies (CLT/GIBCO/BRL) (Burlington, ON, Canada). Oligonucleotides for use as polymerase chain reaction primers were synthesized at the LRCC Cancer Research Laboratories Core DNA Synthesis facility, using a Beckman Oligo 1000 M DNA Synthesizer and cyanethyl-phosphoramidite chemistry. All other chemicals were obtained from commercial sources. Plasticware was purchased

from Canadian Life Technologies (NUNC), VWR Canlab (Mississauga, ON, Canada) and Fisher Scientific (Uniondale, ON, Canada).

Cell culture

Cell lines Human cervical carcinoma HeLa cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) plus 10% foetal bovine serum and penicillin (50 units ml^{-1})/streptomycin (50 $\mu g\ ml^{-1}$) (growth medium). Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Rapidly proliferating cells were utilized for establishing cultures of experimental cells, which were allowed to incubate overnight prior to manipulation.

Establishment of 5-FUdR-resistant lines 5-FUdR-resistant variants were selected by growing HeLa cells in the continuous presence of 5, 10 or 15 nM FUdR for 3 weeks. Medium was replaced weekly with growth medium containing fresh FUdR. Colonies propagated from single cells were selected and expanded in medium containing drug. Cells from one clone (HeLa/FUdR-5a) were used as starting material for selection of variants resistant to higher drug concentrations, by propagation in the presence of 25, 50, 100 or 200 nM FUdR.

Transfection of ODNs Cultures for experimentation were established from rapidly proliferating populations of cells 24 h prior to manipulation. Resistant cell lines were cultured for at least 4 days in the absence of drug prior to experiments. Transfection was performed using Lipofectamine 2000 (LFA-2K) [CLT/GIBCO, Burlington, ON, Canada], a polycationic liposome formulation. Cells were transfected for 4 h with 50–100 nM ODN in the appropriate concentration of LFA-2K, in growth medium. For proliferation experiments, the starting cell number was between 0.6 and 1×10^5 cells per 25-cm^2 flask, and LFA-2K was used at 0.5 $\mu g\ ml^{-1}$. For cells in 75-cm^2 flasks, to be harvested and extracted for assay of mRNA or TS content, the starting cell number was approximately $8-10 \times 10^5$, and the LFA-2K concentration was 2 $\mu g\ ml^{-1}$. The 4-h incubation of the cells with ODN/LFA-2K was followed immediately by dilution by addition of one volume of growth medium without ODN/LFA-2K. Cells assessed for relative TS mRNA levels and TS protein levels were harvested 24 and 48 h after transfection. In cultures that were subsequently exposed to 5-FUdR, drug exposure was initiated by addition of 0.25-volume of growth medium containing the drug at five times the final concentration. Cell numbers were determined after 4 days of growth in medium with or without chemotherapeutic drug, by enumerating with a particle counter (Coulter Electronics, Hialeah, FL, U.S.A.). The proliferation of drug-treated cells (fold-increase in cell number) was calculated as a percentage of that of the control cells grown in the absence of 5-FUdR. IC_{50} values (concentration of drug that inhibited proliferation by 50%) were determined by interpolation of plotted data.

Reverse transcriptase–polymerase chain reaction (RT–PCR) to measure TS mRNA

Measurement of TS and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA in the same ODN-transfected cell populations was conducted using RT–PCR, as previously described (Ferguson *et al*., 1999). RNA was isolated from transfected cells using Trizol® (CLT/GIBCO/BRL). PCR products were separated on a 1.0% agarose gel, and visualized by ethidium bromide staining, using an ImageMaster VDS gel documentation system and LISCAP Capture Utility software (Amersham Pharmacia Biotech). The intensity of staining of individual bands was quantitated using ImageQuant software (Molecular Dynamics/Amersham Pharmacia).

TS binding assay

Cellular content of TS was assayed by binding of [6-^{3}H]5-FdUMP, according to previous methodology (Spears & Gustavsson, 1988), with slight modifications in the final steps of removing activated charcoal (Ferguson *et al*., 1999). This method lables total TS unless the cells are pretreated with 5-FU or 5-FUdR (Chu *et al*., 1990), and correlates well with *in situ* activity assays (Ju *et al*., 1998; van Triest *et al*., 1999) and Western blots (Kitchens *et al*., 1999; van Triest *et al*., 2000).

Statistical analysis

Data for cell proliferation after treatment with ODNs alone, or in combination with 5-FUdR, are presented as the mean ± standard deviation, and assayed for significance using Student's *t*-test. For determinations of [6-^{3}H]5-FdUMP binding, differences between paired samples from cells transfected with different ODNs were assessed using a paired *t*-test. In all cases, significance was chosen *a priori* to be indicated by differences at a confidence level of $P \leqslant 0.05$.

Results

Establishment of FUdR-resistant variants of HeLa

It was the intent of this study to determine whether an antisense ODN targeted against TS could downregulate this enzyme in cells that overexpress it as a consequence of selection for growth in TS-targeting drugs. Based on the previous finding of enhancement of cytotoxicity of TS inhibitors in HeLa cells, it was expected that drug resistance could be circumvented in TS-overexpressing cells using an antisense ODN. To this end, it was necessary to establish a model system in which cell lines overexpressed TS to various degrees, to determine the magnitude of resistance that could be overcome. The variants of HeLa that were established by continuous exposure to 5-FUdR are listed in Table 1. Three cell lines (FUdR-5a, -10g, and -15a) were propagated from colonies that grew among HeLa cells cultured in the presence of 5-FUdR at the respective concentrations (5, 10 and 15 nM). The remaining four cell lines were each selected from FUdR-5a by a single selection step, in the presence of the indicated concentrations of 5-FUdR. The first three cell lines displayed a similar level of resistance to 5-FUdR, but the TS protein level in the FUdR-5a line was approximately six times greater than that measured in FUdR-10g or -15a. The lines selected from FUdR-5a had at least a 10 fold increase in TS protein level compared to parental HeLa cells, accom-

panied by a slight increase in relative TS mRNA concentration.

Three 5-FUdR-resistant lines representative of different levels of TS overexpression, and three levels of resistance to 5-FUdR, were chosen for further study: FUdR-5a, -25b, and -50a. The effectiveness of anti-TS antisense ODN 83 in downregulating TS and overcoming resistance to 5-FUdR was assessed in these three lines. Optimal conditions were established within which antisense ODN treatment alone significantly decreased TS protein levels, but did not inhibit proliferation more than 30%. This permitted sufficient remaining growth potential to clearly measure the effect of subsequent 5-FUdR treatment on proliferation. Transfection conditions were also adjusted to minimize non-specific short-term toxicity, depending on whether cells were transfected at low density ($0.6-1\times10^5$ cells per 25-cm^2 flask) for assays of proliferation, or higher density ($8-10\times10^5$) for assays of protein or RNA content. These conditions were: for low cell density, 0.5 μg ml^{-1} LFA-2K for all lines (except FUdR-50a and -100a, where 0.25 μg ml^{-1} LFA-2K/ml was used), plus 50 nM ODN; for high cell density, 2 μg ml^{-1} LFA-2K, plus 100 nM ODN.

Effect of antisense ODN on TS mRNA and protein expression

It was expected that anti-TS antisense ODN 83 would decrease TS protein levels by specifically interacting with TS mRNA to promote degradation through the action of RNase H. Therefore, ODN-treated cells were assayed for TS mRNA content, relative to control GAPDH mRNA levels, 1 and 2 days following treatment. Total cellular RNA was isolated, reverse-transcribed, and selected regions of TS and control GAPDH cDNA products amplified by PCR and characterized by agarose gel electrophoresis. Figure 1 is representative of assays performed on extracts from three different sets of ODN-treated cultures, and clearly demonstrates the specific decrease in TS mRNA. RNA from three independent antisense transfection experiments was quantitated using image analysis software, the results of which are summarized in Figure 2. The decrease in TS mRNA level (greatest on day 2 after ODN transfection) was mirrored by the decreased level of TS protein (measured by [^{3}H]FdUMP-binding). (In this set of experiments, the relative TS protein content of the FUdR-5a line was less than that presented in Table 1, probably as a result of incubation of the cell line in the absence of drug during the period of these experiments. Cultures were maintained drug-free prior to experiments to avoid interference of 5-FUdR and its metabolites in assays of TS protein and of inhibition of proliferation by exogenous 5-FUdR. To enable comparison of the antisense ODN effect between experiments, the *relative* amounts of TS mRNA and TS protein were determined within each experiment.) Under these treatment conditions, proliferation was inhibited to varying degrees depending on the cell line being treated. In two experiments in which proliferation was evaluated over 2 days following transfection, relative proliferation of antisense-treated cells (compared with control ODN 32) was, respectively, $33.5\pm21.2\%$, $41.0\pm1.1\%$, $52.8\pm15.2\%$, and $-17\pm15.3\%$ (a negative value indicating a drop in the number of cells below the starting number) for HeLa, FUdR-5a, FUdR-25b, and FUdR-50a.

Effect of antisense ODN on sensitivity of 5-FUdR-resistant cell lines to 5-FUdR

Since antisense ODN 83 was able to downregulate the intracellular content of TS, it was expected that this treatment would enhance sensitivity of the cells to 5-FUdR. As demonstrated by the representative experiment presented in Figure 3, the antisense treatment enhanced drug sensitivity, at concentrations of ODN 83 that inhibited proliferation by only 10–20% on their own. (At 100 nM ODN 83 plus 2 μg ml^{-1} LFA-2K, proliferation of all the cell lines was inhibited by approximately 50% (data not shown).) The proliferation rates shown specifically reveal the enhancing effect of antisense ODN 83 on drug sensitivity, and exclude the effect of ODN 83 alone on cell proliferation. Cells treated with ODN 83 or ODN 32 were assigned a proliferation value of 100% and all data points showing proliferation in the

Table 1 Characteristics of FUdR-Resistant variants of HeLa

Cell line[a]	*Resistance index*[b]	*Relative thymidylate synthase protein level* (fold × HeLa, Ave ± s.d.)[c]	*Relative thymidylate synthase mRNA level* (fold × HeLa)[d]
HeLa	1.0	1.000 ± 0.101 (4)	1.00
FUdR-5a	2.03 ± 1.23 (6)	10.69 ± 0.52 (3)	2.80
FUdR-10g	2.66 ± 1.06 (6)	1.606 ± 0.085 (3)	2.89
FUdR-15a	2.33 ± 0.76 (6)	1.361 ± 0.158 (3)	2.99
FUdR-25b	21.6 ± 9.2 (3)	10.34 ± 0.142 (3)	4.90
FUdR-50a	10.6 ± 6.3 (5)	16.68 ± 0.95 (3)	6.77
FUdR-100a	61.9 ± 81.9 (6)	10.99 ± 0.39 (3)	4.81
FUdR-200b	68.1 ± 65.2 (6)	27.46 ± 1.27 (3)	6.39

[a]The numerical value of the cell lines indicates the concentration of FUdR in which the cells were cultured for 3 weeks, and variants derived. Colonies, which grew from single cells, were removed with a pipet and propagated. [b]Resistance index was calculated as a ratio of the IC_{50} values between the resistant line and the parent HeLa line. The IC_{50} of FUdR in the parental HeLa line was 6.55 ± 1.95 nM ($n=7$). The resistance level varied depending on the length of time the cells were grown in the absence of FUdR. The resistance indices for FUdR-100a ranged from 12.5 to 64, and for FUdR-200a form 38 to 80. [c]TS protein level was assayed by [^{3}H]FdUMP-binding. The TS level in the parental HeLa cells within this set of experiments was 0.963 ± 0.097 pmol mg^{-1} ($n=4$). The relative amounts were calculated within individual assays, and then averaged. Numbers in parentheses, number of determinations. These values were determined shortly after the cell lines were established. All values are statistically significantly different from that of HeLa [$P<0.001$, except for FUdR-10g ($P<0.001$) and FUdR-15a ($P<0.05$)]. [d]TS mRNA level was assayed by RT–PCR, and quantitated relative to GAPDH.

presence of drug after ODN treatment are relative to that 100% value. The results of three independent experiments are summarized in Figure 4. The 70–80% enhancement of 5-FUdR cytotoxicity was significant ($P<0.001$) for all cell lines. There was no significant difference in the degree of enhancement of sensitivity to FUdR by anti-TS antisense ODN 83 between any TS-overexpressing, FUdR-resistant cell line and the parental, FUdR-sensitive HeLa cells with low TS levels.

Discussion

The amount of anticancer drug administered to patients is limited by debilitating, dose-dependent side effects, including potentially fatal toxicity. Therefore, the acquisition of some form of resistance by a tumour, including low level, incremental decreases in drug sensitivity, is a major obstacle to the success of treatment. A minor increase in the cellular content of a target of a specific drug could impede the ability of that drug to lethally damage tumour cells. Therefore, it would be of benefit to chemotherapeutic outcome if a drug target could be down-regulated in tumour cells in order to increase the effective drug : target ratio. The first step toward specifically decreasing drug-target levels in tumour cells has been the development of antisense technology. Antisense ODNs of 15–21 bases in length that are complementary to various regions of a particular mRNA can effectively and specifically downregulate a selected gene product (Bennett, 1998). Although the use of antisense RNA expression vectors have been tested as a strategy to generate a prolonged antisense effect on a specific mRNA target (Dean *et al*., 1994; 1996), the stability of phosphorothioated ODNs, and their capacity to be taken up into animal-borne tumours without a requirement for transfection agents, has resulted in their superseding antisense RNA vectors as the agents of choice in targeting specific mRNAs *in vivo* (Dean *et al*., 1994; 1996; Shaw *et al*., 1991; Stein & Cheng, 1993; Stein *et al*., 1988).

Many proteins have been down-regulated by antisense with resulting enhancement in drug toxicity. Of those, however, only tubulin (Kyu-Ho Han *et al*., 2000; Kavallaris *et al*., 1999) and TS have been tested as targets for both antisense nucleic acids (at the mRNA level) and traditional drug (at the protein level). We previously demonstrated that a 20-mer ODN antisense to the 3′UTR of TS mRNA (ODN 83) specifically down-regulated

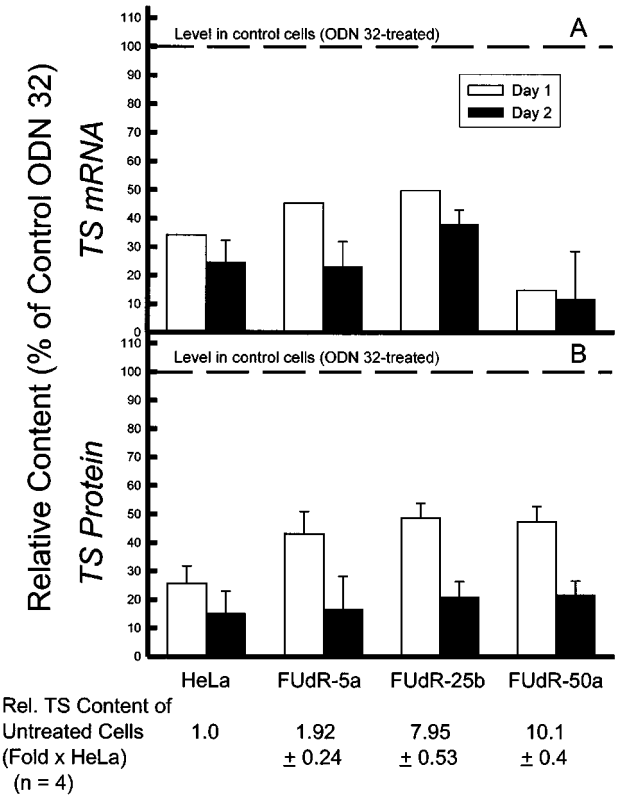


Figure 2 Reduction in thymidylate synthase mRNA and protein levels by treatment of HeLa cells and FUdR-resistant variants with antisense ODN 83. Following a 4 h transfection of cells with ODN at the concentration indicated, one volume of medium was added, and cells were further incubated in ODN at 50% of the original concentration. Cells were harvested 1 and 2 days following initiation of exposure to ODN. (A) relative TS mRNA was assayed by RT–PCR, and compared with the level of the housekeeping gene product GAPDH. Data are summarized from the analysis of two separate determinations for day 1 and 3 for day 2, as represented by Figure 1. (B) TS protein level was measured by [^{3}H]-FdUMP-binding. The TS content in the ODN 32-treated parental HeLa line was 0.983 ± 0.132 pmol mg^{-1} ($n=5$) on Day 1, and 0.792 ± 0.232 pmol mg^{-1} ($n=5$) on Day 2. All values presented are significantly different from the respective ODN 32-treated control ($P<0.01$).

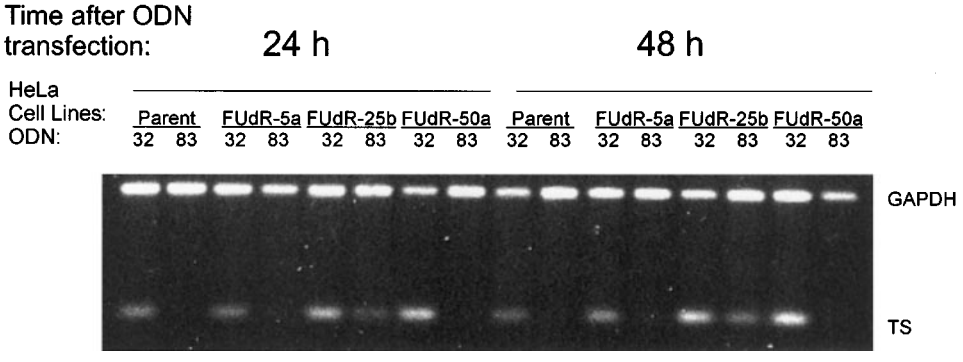


Figure 1 Reduction of cellular levels of TS mRNA in HeLa cells and FUdR-resistant variants following treatment with antisense ODN 83. Following administration of ODNs to cultures, cells were harvested on days 1 and 2. Relative TS mRNA was visualized by RT–PCR, using GAPDH as a control for RNA integrity. This image is typical and representative of assays of cells harvested from three separate experiments.

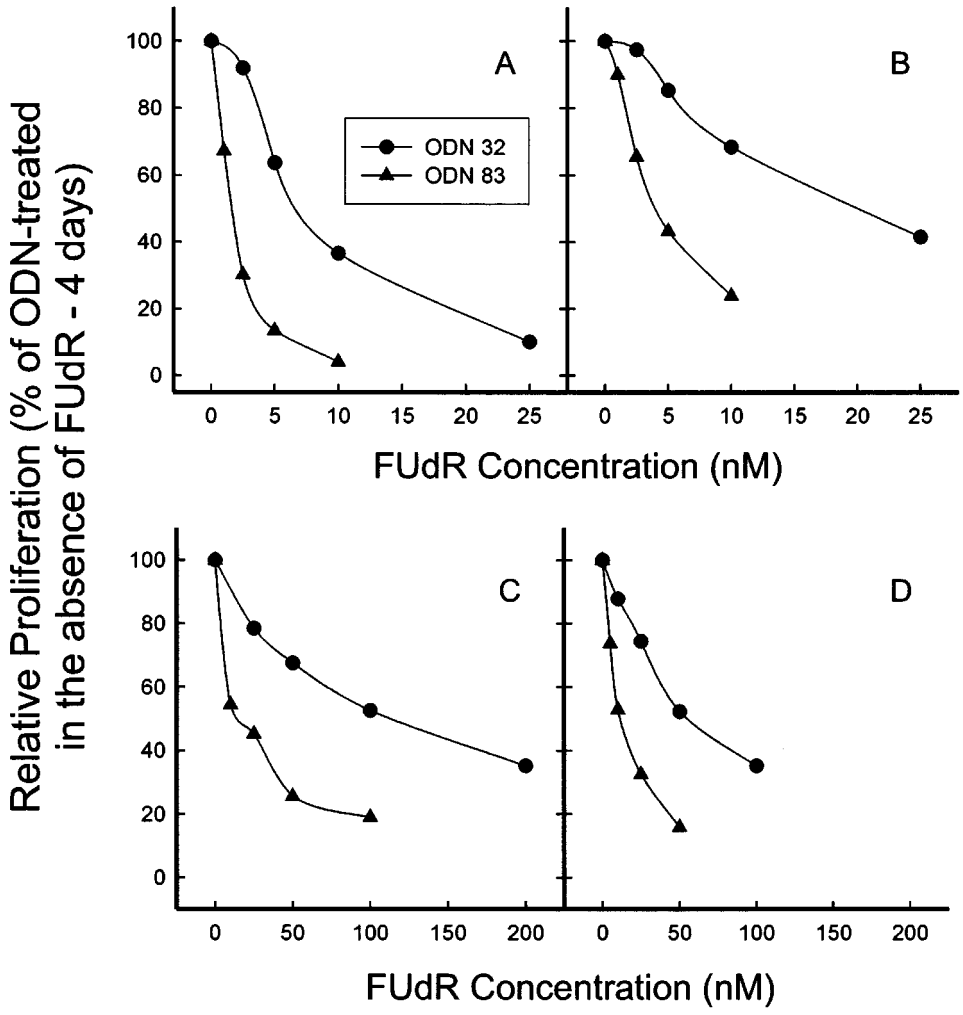
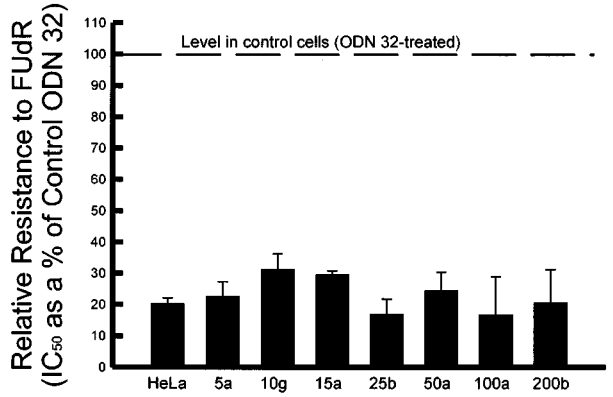


Figure 3 Enhancement of FUdR cytotoxicity in HeLa cells and FUdR-resistant variants by treatment with antisense ODN 83. Cells were treated with ODNs for 4 h, followed by administration of FUdR without changing the medium. Cell number was determined after 4 days, and proliferation calculated as a percentage of that of control ODN-treated cells. (A) HeLa; (B) FUdR-5a; (C) FUdR-25b; (D) FUdR-50a. Error bars are smaller than the symbols.

TS expression and inhibited HeLa cell proliferation (Ferguson *et al*., 1999). In addition, ODN 83 specifically enhanced cell sensitivity to drugs that target TS (raltitrexed, 5-FU, and 5-FUdR). This has since been extended to animal studies, in which the same anti-TS antisense ODN inhibits human colon tumour growth in nude mice (Berg *et al*., 2001).

To date there have been few attempts to sensitize cells to TS inhibitors using antisense technology, and those reported in the literature describe only limited success. Transfection of an anti-TS antisense expression vector sensitized cells to 5-FUdR (Ju *et al*., 1998). However, an antisense ODN against TS induced resistance to 5-FUdR, possibly due to a rebound in TS expression following an initial transient decrease in TS mRNA (Ju *et al*., 1998). Antisense against a number of other mRNAs has been used to alter cytotoxicity to TS inhibitors. Sensitivity to 5-FU and/or 5-FUdR was enhanced following treatment of cultured cells with an antisense ODN against c-*jun* (Kakutani *et al*., 1998) and antisense vectors against cyclin D1 (Kornmann *et al*., 1999) and proline-directed protein kinase-FA (Yang *et al*., 2000). In contrast, antisense against thymidine phosphorylase or uridine phosphorylase antagonizes cytotoxicity of 5-FU (Mader *et al*., 1997), as does pre-administration of antisense ODN against TGFα mRNA (De Luca *et al*., 1997), although the latter has no effect if administered after the drug (De Luca *et al*., 1997). Antisense ODN against c-*myc* (Mizutani *et al*., 1994) and antisense vectors against heat shock protein 27 (Garrido *et al*., 1996) or the antimetastatic protein nm23-H1 (Iizuka *et al*., 1999) did not affect 5-FU cytotoxicity.

The level of TS in tumour cells correlates well with sensitivity to cytotoxicity of TS-inhibitory drugs *in vitro* and *in vivo*. A low basal level of TS has been shown to yield greater sensitivity of cultured cells to TS inhibitors (Nita *et al*., 1998; van Triest *et al*., 1999), and a more favourable response of patient tumours to 5-FU (Edler *et al*., 2000; Mini *et al*., 1999; Peters *et al*., 1994; Salonga *et al*., 2000; van Triest & Peters, 1999). Therefore, down-regulation of basal levels of TS could lead to improved responses in drug-naïve tumours. However, antisense could also be used to down-regulate TS that for some reason becomes overexpressed in tumour cells. The etiology of such altered expression can be



	HeLa	5a	10g	15a	25b	50a	100a	200b
Relative Resistance (fold X HeLa)[a]	1.0	2.03	2.66	2.33	21.6	10.6	61.9	68.1
Rel. Proliferation in Absence of 5-FUdR (% of Control, n = 3)[b]	82.2 ±11.7	89.3 ±17.1	96.8 ±4.9	87.7 ±18.2	100.4 ±9.7	106 ±25	91.9 ±12.4	87.2 ±4.5

Figure 4 Enhancement of FUdR cytotoxicity in HeLa cells and in FUdR-resistant variants by antisense ODN 83. Cells were treated with antisense ODN 83 or scrambled control ODN 32 for 4 h, followed immediately by coincubation with FUdR for 4 days. Proliferation was determined by increase in cell number. IC_{50} values were interpolated from plotted data, from a series of experiments represented by Figure 3. The value presented is the IC_{50} of the ODN 83-treated cells as a percentage of that of the control ODN 32-treated cells, calculated within individual experiments. All values for relative resistance are significantly different from that of control ODN-treated cells, $P<0.001$. [a]This value is reproduced from Table 1 (see table for s.d. and *n* values for these averages). Resistance index was calculated by dividing the IC_{50} of the FUdR-resistant line by that of the parent HeLa cell line, within each individual experiment. [b]Relative proliferation is the relative increase in cell number, as a percentage of the control ODN-treated cells, of the cultures treated with antisense ODN 83 alone.

considered as: (1) responsive, or epigenetic, resistance; or (2) acquired, or mutational, resistance.

Responsive, or epigenetic, resistance to TS inhibitors

TS regulates its own production by binding to its own mRNA to inhibit translation (Kaneda *et al*., 1987; Keyomarsi *et al*., 1993). Removal of TS protein from TS mRNA, possibly triggered by an increase in the pool of substrates of TS (Chu & Allegra, 1996), or by binding of inhibitors of TS enzyme activity (Chu *et al*., 1990; 1991; 1993a; Keyomarsi *et al*., 1993; Lin *et al*., 2000; Mader *et al*., 1997; Parr *et al*., 1998; Peters *et al*., 2000; Van der Wilt *et al*., 1992; Welsh *et al*., 2000), can restore TS protein synthesis from the mRNA template. This drug-induced increase in TS protein has been observed to enhance resistance to 5-FU therapy in mice (Van der Wilt *et al*., 1992), and has been reported following treatment with TS-targeted chemotherapeutics in cancer patients (Peters *et al*., 1994; Swain *et al*., 1989).

Acquired, or mutational, resistance

As in the case of resistance involving other enzyme drug targets, cell lines selected for resistance to 5-FU or 5-FUdR have elevated levels of TS mRNA, usually due to gene amplification (Kitchens *et al*., 1999), and subsequently increased TS protein (Kitchens *et al*., 1999; Murakami *et al*., 2000; Shibata *et al*., 1998). However, other mechanisms of resistance are possible, and may or may not accompany TS overexpression. These are: (a) acquisition of a mutant TS with reduced affinity for drug, but having decreased stability (Kitchens *et al*., 1999); or (b) an increase in the half-life of the wild-type TS, putatively due to a mutated or adapted cellular component that stabilizes TS or is responsible for degrading it (Kitchens *et al*., 1999). TS overexpression due to any of the above phenomena presents a potentially important target for use of an anti-TS antisense ODN to enhance the ability of TS inhibitors to kill tumour cells.

The cell culture model used in this study is comprised of TS-overexpressing cell lines selected in one or two steps of exposure to 5-FUdR. The FUdR-5a, -10g and -15a lines all express a similar level of resistance to 5-FUdR, and a similar level of TS mRNA. However, FUdR-5a has 10 fold more TS protein than parental HeLa cells compared to a maximum 2 fold elevation in TS protein in FUdR-10g and -15a. Upon a second selection step, the increased level of TS protein expressed by FUdR-5a was, at a minimum, maintained in the derived variants, and increased by 0.6–1.7-fold in the FUdR-50a and -200b lines, respectively. Conversely, resistance to 5-FUdR increased substantially in the FUdR-25b and -100a lines without a significant increase in TS protein. The disparity in the ratio of TS mRNA : TS protein among these drug-resistant cell lines suggests that, in addition to increased TS mRNA levels, other changes (including regulation of translation of TS mRNA; stability/drug affinity of TS protein; altered intracellular drug availability; or other events not directly related to regulation of TS (Kitchens *et al*., 1999)) may be involved in mediating resistance to one or more of the 5-FUdR-resistant HeLa lines.

Because of the different levels of TS overexpression and of resistance displayed by FUdR-5a, -25b and -50a (including the potential for differences in the overall mechanisms mediating 5-FUdR resistance among them), these lines were chosen as representative of tumours in which different mechanisms of 5-FUdR resistance might be encountered, and as models to test the potential clinical use of anti-TS antisense ODNs against TS-overexpressing tumours. In the parental HeLa cells, sensitivity to several TS inhibitors was enhanced up to 70% by treatment with antisense ODN 83 (Ferguson *et al*., 1999). In the present study, an 80% enhancement of 5-FUdR toxicity rendered the 5-FUdR-resistant FUdR-5a, -10g and -15a cell lines even more sensitive than the non-ODN-treated, parental HeLa cells. Therefore, human tumours that had acquired 2–3-fold resistance imparted by an incremental increase in TS content could potentially be sensitized by antisense treatment to a sufficient degree to completely abolish resistance.

In cell lines with higher levels of 5-FUdR-resistance, the antisense ODN enhanced sensitivity to 5-FUdR to the extent that resistance of the FUdR-25b and -50a lines (relative to parental cells) was reduced from between 10–20-fold to between 2–3-fold. This suggests that, for human tumours expressing a high level of resistance, it may be possible to achieve a lethal combination of antisense and drug. Under the transfection conditions used in these experiments, the cells were sensitized using a dose of ODN 83 that did not substantially slow proliferation on its own. However, as described above, antisense ODN 83 alone (at appropriate concentrations) can inhibit proliferation. Thus, the use of TS inhibitors together with higher concentrations of ODN 83

would be expected to inhibit proliferation due to a combined effect of antisense alone plus antisense-mediated enhancement of drug sensitivity, as previously reported in the parent HeLa line under these conditions (Ferguson *et al*., 1999). Direct measurement of this combination effect *in vitro* is hindered by the non-specific toxicity of higher concentrations of ODN and LFA-2K against the 5-FUdR-resistant cell lines.

The degree of enhancement of sensitivity of cells to FUdR by ODN 83 (70–80% reduction in IC50) was similar in all 5-FUdR-resistant cell lines, regardless of the level of resistance. This is likely due to the fact that, in the presence of 5-FUdR, the rate of proliferation is due to the remaining amount of active TS, and this amount, for a given rate of proliferation, is the same regardless of the original level of resistance. Therefore, since the antisense ODN causes a similar relative decrease in TS mRNA and protein among the parental and drug-resistant cell lines (Figure 2), the relative reduction in active TS is also the same among the different lines. A given percentage reduction in active TS requires approximately the same percentage less 5-FUdR to achieve a cytotoxic effect, regardless of the absolute, initial amount of TS in the cell. The question remains why the antisense ODN can reduce the TS mRNA by 80% in both a highly-overexpressing cell line and a similar amount in the parental cell line. This is possibly due to the kinetics of ODN binding to mRNA and of RNase enzyme action.

The consequence of the anti-TS ODN treatment may potentially extend beyond the immediate effect on drug cytotoxicity. In fact, we have reported that antisense ODN 83 induces a block at G2/M in HeLa cells, without appreciable apoptosis (Berg *et al*., 2001). In addition, TS protein has been reported to bind to, and inhibit translation of, p53 mRNA *in vitro* (Ju *et al*., 1999; Chu *et al*., 1999), and antisense downregulation of TS protein resulted in a 7 fold increase in p53 protein in a human colon cancer cell line (Schmitz *et al*., 2001). This increased p53 expression could permit easier induction of cell death by cytotoxic agents. Altered expression of proteins capable of mediating cell cycling and apoptosis in response to antisense targeting of TS mRNA warrants further investigation.

In summary, use of an anti-TS antisense ODN targeting TS mRNA, in combination with a TS inhibitor targeting TS protein, effectively enhanced the cytotoxicity of the protein-targeting drug in cells with low TS levels and in cells selected for TS overexpression by selection for growth in drug-containing media. Furthermore, enhancement of cytotoxicity was effective in drug-resistant cell lines that appear to employ more than one mechanism to mediate 5-FUdR resistance. These data indicate that use of anti-TS antisense reagents against tumours that overexpress TS may ultimately be of clinical utility, particularly as an adjunct to enhance the effectiveness of drugs targeting TS protein.

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