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Preclinical report

Modulation of dihydrofolate reductase gene expression in methotrexate-resistant human leukemia CCRF-CEM/E cells by antisense oligonucleotides

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An increase in the cellular levels of dihydrofolate reductase (DHFR) is one of the most common mechanisms of tumor resistance to methotrexate (MTX), an antimetabolite that is widely used in the treatment of a variety of human malignancies. The MTX-resistant phenotype generally occurs as a consequence of DHFR gene amplification which in turn is responsible for DHFR gene overexpression. We have designed antisense oligodeoxynucleotides (aODNs) against the DHFR mRNA and tested their *in vitro* effect on human leukemia CCRF-CEM/E cells, overexpressing the DHFR gene about 20-fold in comparison with the CCRF-CEM/S parental cell line. An aODN complementary to a region encompassing the AUG translation start (DHFR1) of DHFR mRNA and a mixture of two aODNs complementary to the 5′ untranslated region (DHFR2+DHFR3) have been used. A DHFR1 scrambled-sequence ODN and a fully degenerated ODN were the controls. All ODNs had a phosphodiester backbone. DHFR1 and the relevant scrambled ODN were also capped with two phosphorothioate derivatives at both the 5′ and 3′ ends in order to increase ODN stability against serum nucleases. ODNs were vehiculated with a cationic lipid, *N*-[1-(dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulfate (DOTAP), known to enhance ODN cell uptake and biological activity. The effects of ODNs on DHFR gene expression were studied after a 4 day treatment by measuring both DHFR mRNA levels, using a semi-quantitative reverse transcription polymerase chain reaction method, and DHFR protein levels by flow cytometry. A marked reduction in DHFR mRNA levels (79.7 and 74.2%, respectively) was observed with both DHFR1 and DHFR2+DHFR3 aODNs, associated with a lower decrease in DHFR enzyme (44.8 and 61%, respectively). aODN effects on MTX cytotoxicity in CCRF-CEM/E cells were also assessed. No marked enhancement of *in vitro* MTX cytotoxicity was observed following co-exposure of cells with aODNs and the tested concentrations of the antifol (0.05 and 0.5 μM), indicating that no substantial reversal of the MTX-resistant phenotype was induced by the study aODNs. [© 2000 Lippincott Williams & Wilkins.]

Key words: Antisense, DHFR mRNA, flow cytometry, methotrexate resistance, oligodeoxynucleotides, reverse transcription polymerase chain reaction.

Introduction

Methotrexate (MTX) is a classic pteridine derivative of folic acid that interferes with folate-dependent metabolic processes essential to the synthesis of nucleic acids and is therefore widely used as an antineoplastic agent.[1] Either as a single agent or, more frequently, in combination chemotherapy, MTX has been clinically successful in the treatment of many human tumors including gestational choriocarcinoma, acute lymphocytic leukemia, Burkitt's lymphoma and non-Hodgkin's lymphomas, contributing often to cure. Other tumors, such as osteogenic sarcoma, breast, head and neck, gastric and bladder carcinoma, often respond, at least temporarily, providing transitory complete remissions.[2] The major obstacle to complete therapeutic success with MTX, as well as with other antineoplastic agents currently in use, is the occurrence of intrinsic or acquired drug resistance of tumor cells.[2]

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The pharmacological determinants of MTX are well known. MTX enters cells primarily through an active transport mechanism mediated by a membrane carrier (RFC) endowed with a high affinity for reduced folates,[3] but also via interaction with membrane receptors endowed with a high affinity for folic acid (MFR).[4] Inside the cell MTX undergoes a polyglutamylation process that leads to increased cytotoxicity.[5] The antifolate, both in the monoglutamate and in the polyglutamate forms, potently inhibits dihydrofolate reductase (DHFR), a key enzyme in folate-dependent metabolism.[1] Both in tumor models and in the clinical setting, cell resistance to MTX has been described to occur at many levels of pharmacological interaction (i.e. intracellular transport, drug activation, interaction with the target enzyme).[6–11] However, an increase in the levels of DHFR, mostly due to gene amplification, appears to be one of the most common mechanisms of tumor resistance.[12–16]

One of the most innovative and potentially useful strategies for specifically inhibiting expression of genes responsible for this phenomenon is based on antisense oligonucleotides (aODNs) designed to complement the mRNA of the target gene, and thus induce its degradation and/or inhibiting translation.

The antisense strategy aimed at inhibiting tumor cell growth has recently provoked more interest in oncology, as a consequence of a number of positive results obtained both in *in vitro* and *in vivo* experimental models by targeting oncogenes involved in neoplastic transformation, progression and apoptotic pathway.[17] The results of preliminary clinical studies[18–23] with antisense therapy have already built a large basis of knowledge of pharmacodynamics and toxicology that warrants further clinical experience with this approach.

The reversal of multidrug-resistant tumor phenotypes has been described in *in vitro* model systems by inhibiting the expression of the responsible *mdr1* gene with antisense oligomers.[24–26]

The antisense approach has also been employed to modulate expression of the DHFR gene, both by using antimessenger oligonucleotides[27] and ribozymes[28] inhibiting DHFR mRNA translation or with antigene ODNs inhibiting DHFR gene transcription by triple helix formation.[29,30]

In the attempt to lower DHFR gene overexpression responsible for MTX resistance of human leukemia CCRF-CEM/E cell line,[31] we have treated this cell line *in vitro* with different concentrations of three aODNs directed against DHFR mRNA for 4 days in the present study. Following treatments, aODN biological effects on DHFR expression were determined by quantitating DHFR mRNA and protein intracellular levels, and their effectiveness in reversing the MTX-resistant phenotype was evaluated by measuring cell growth following combined aODN and MTX *in vitro* treatment.

Materials and methods

Chemicals

Media and sera for tissue culture were purchased from Gibco European Division (Milan, Italy) and antibiotics were from Sigma (St Louis, MO). Plasticware was obtained from Nunc (Roskilde, Denmark). MTX calcium salt was obtained from Cyanamid Italia (Catania, Italy). Fluorescein–MTX triammonium salt (F-MTX) was purchased from Molecular Probes (Eugene, OR). F-MTX (1 mg) was dissolved in 0.1 ml of 50 mM NaOH, adjusted to 1 mM with sterile distilled water, aliquoted and stored at $-20^{\circ}C$ protected from light. The cationic lipid vector (DOTAP) was from Boehringer Mannheim (Mannheim, Germany). RNAzol B was from Cinna Biotecx (Houston, TX). RNAguard, RNase inhibitor and reverse transcription polymerase chain reaction (RT-PCR) amplimers were from Pharmacia Biotech (Uppsala, Sweden). DNase, Mo-MLV reverse transcriptase and ampliTaq polymerase were purchased from Promega (Madison, WI). Oligomers were purchased from TIB MOLBIOL (Berlin, Germany). All the other reagents were of the highest purity available.

Cell lines

The parental cell line CCRF-CEM/S was sensitive to MTX, while the MTX-resistant subline CCRF-CEM/E was characterized by an about 20-fold increase in DHFR enzyme activity since it carried 18 copies of the DHFR gene.[31] Cells were grown in RPMI 1640 medium supplemented with 10% horse serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at $37^{\circ}C$ in a 5% CO_2 atmosphere, and subcultured twice weekly. The CCRF-CEM/E subline was grown in the presence of 0.2 μM MTX. Under these conditions the doubling times of exponentially growing sensitive and resistant cells were 24 and 26 h, respectively. Prior to the experiments the resistant cells were maintained for at least 10 days in MTX-free medium.

Design and vehiculation of oligonucleotides

Three antisense ODNs (DHFR1, DHFR2 and DHFR3 aODN) were used in our experiments (Table 1). We have designed DHFR1 18mer aODN that targets a segment encompassing the AUG translation start site of

Table 1. ODNs used as antimessenger agents

Name	Sequence	DHFR mRNA residues	Orientation
DHFR1	TAG CGA ACC AAC CAT GAC	−3 to +15	antisense
DHFR2	AGC AGC GGG AGG AC	−17 to −4	antisense
DHFR3	CTC CGA GCC CGC TC	−31 to −18	antisense
SCRM	TCA AGG CTG AAC ACC ACA		scrambled
FD	NNN NNN NNN NNN NN		degenerate

the human DHFR mRNA,[32] i.e. the same site successfully targeted by other authors with aODNs against other genes.[33–35] The mixture of DHFR2 and DHFR3 14mer aODNs targeted the adjacent upstream segment in the 5′-untranslated region of the DHFR mRNA have already been successfully employed by others to inhibit DHFR translation in a 'cell-free' system.[27] A scrambled (SCRM) 18mer relative to the DHFR1 aODN and a fully degenerated (FD) 14mer relative to DHFR2 and DHFR3 aODNs were used as controls for evaluating aspecific ODN effects. DHFR1 and SCRM 18mer oligonucleotides were capped with two phosphorothioate residues at both 5′ and 3′ ends; DHFR2, DHFR3 and FD were 14mer phosphodiester oligonucleotides. Each ODN was Sephadex G-25 gel-purified. In order to enhance both uptake and stability ODNs were vehiculated by the cationic lipid DOTAP according to our previous works.[36,37] DHFR1 aODN or an equimolar mixture of DHFR2+DHFR3 aODNs were preincubated at 37°C for 15 min with 1.3 mM DOTAP and added to the cells at the final experimental concentration.

Determination of cytotoxicity and ODN treatment duration

Exponentially growing cells (CCRF-CEM/E, 5×10^4 cells/ml) were seeded in duplicate in 1 ml wells in RPMI 1640 medium supplemented with 10% horse serum previously treated at 56°C for 30 min in order to inactivate serum nucleases. On the first day of the experiment, DOTAP vehiculated ODNs were added to the wells at concentrations varying from 0.1 to 30 μM and at one-half the initial concentration on the following treatment days according to the ODN half-life in the medium (i.e. every 24 and 48 h for phosphodiester and phosphorothioate ODNs, respectively).[36] At the end of the exposure to ODNs (96 h), the total cell number was counted using a Coulter Counter model D (Coulter Electronics, Hialeh, FL) and cell viability was tested by staining with the Trypan blue exclusion test. Cell growth of ODN-treated samples was expressed as percentage of total cell divisions of untreated controls, as described by Mini *et al.*[38]

The DHFR protein level is cell cycle dependent and in rapidly dividing cells undergoes an intracellular turnover resulting in a half-life of about 20 h.[39] Therefore, a 96 h continuous exposure to DOTAP-vehiculated aODNs applied to the resistant cell line has been considered a sufficient time to obtain a remarkable reduction in the DHFR protein intracellular level and, consequently, a substantial reversion of MTX-resistance.

DHFR mRNA quantitation by RT-PCR

Basal and post-treatment DHFR mRNA levels were determined by an internal standard-based semi-quantitative RT-PCR assay.[40] β-Actin was chosen as internal standard gene. Briefly, total cellular RNA[41] was extracted from 10^6 cells treated with DNase and reversely transcribed to cDNA. The RT mixture (100 μl) contained 20 μg of total RNA, 0.7 U/μl RNAguard RNase inhibitor, bovine serum albumin (0.2 μg/μl), dNTP (1 nM), random hexamers (0.045 μg/μl), Tris–HCl (50 mM), pH 8.3, KCl (75 μM), $MgCl_2$ (3 mM) and 10% glycerol. After heating at 60°C for 5 min and rapidly cooling in order to allow hexamer annealing, the reaction was started by the addition of 20 U/μl of Mo-MLV reverse transcriptase, performed at 38°C for 60 min and blocked by heating at 95°C for 15 min. PCR was carried out by separately amplifying either DHFR or β-actin segments using serial dilutions of cDNA in a DNA thermocycler 480 (Perkin-Elmer, Norwalk, CT). An aliquot of 50 μl of PCR mixture contained serial dilutions of cDNA, 0.4 μM primers, 200 μM dNTP, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl, pH 9, 1% Triton X-100 and 2.5 U of Taq polymerase. DHFR cDNA amplimers were 5′-TATTTCCAGAGAGAATGACCA-3′ and 5′-AGGCATCATCTAGACTTCTGG-3′, positions 97–118 and 272–292, respectively, according to the sequence reported by Masters and Attardi.[32] β-Actin cDNA amplimers were 5′-GCGGGAAATCGTGCGTGACATT-3′ and 5′-GATGGAGTTGAAGGTAGTTTCGTG-3′, positions 2104–2127 and 2409–2432, respectively, according to the sequence of Ng *et al.*[42] The chosen primers were identical to those published by Horikoshi *et al.*[40] For both genes, PCR cycles were: 7 min 94°C, 1 min 58°C and 1 min 72°C for 1 cycle; 40 s 94°C, 1 min 58°C and 1 min 72°C for 31 cycles. Negative controls were obtained by omitting the Mo-

MLV enzyme in the RT mixture. PCR products were analyzed by agarose gel electophoresis and stained with ethidium bromide. The gel photographs were analyzed densitometrically by the BioRad system GS-670 imaging densitometer (BioRad, Milan, Italy). The linear amplification ranges of the DHFR and β-actin genes were found by plotting the intensity of the bands against the cDNA volumes added to the PCR reaction. The ratio between the slopes in the linear amplification regions of DHFR and β-actin genes was calculated for each sample.

DHFR protein analysis by flow cytometry

Cellular levels of DHFR protein were determined by flow cytometry analysis of labeled cells with F-MTX.[43] Exponentially growing CCRF-CEM/E cells were treated with ODNs (10 and 30 μM) for 96 h and, during the last 14 h, 10 μM F-MTX was added. Thereafter cells were washed twice with PBS and placed on ice protected from light. Aliquots of 1×10^6 cells were then immediately analyzed with a FACStar cell sorter (Becton Dickinson, Mountain View, CA) equipped with an argon ion laser (Model Innova 90; Coherent, Palo Alto, CA) operating at 500 mW output at 488 nm. The green fluorescence emitted by F-MTX bound to DHFR, was collected by a 530/30 nm double filter and recorded as a measure of DHFR content at the single-cell level. Green fluorescence was displayed on a logarithmic scale and at least 20 000 cells were analyzed with Consort 30 software (Becton Dickinson) for each histogram. DHFR levels determined by fluorescence intensity of samples treated with ODNs were expressed as percentage of fluorescent mean channel of the untreated cells. The autofluorescence level was measured in cells incubated in F-MTX-free medium.

Determination of aODN cytotoxicity and MTX used in combination

After a 24 h pre-treatment with 30 μM ODNs alone, CCRF-CEM/E cells were co-exposed to 0.05 μM (corresponding to an IC_{50} of CCRF-CEM/S but no cytotoxic dose for CCRF-CEM/E cells) or 0.5 μM (IC_{25} and IC_{75} of CCRF-CEM/E and CCRF-CEM/S cells, respectively) MTX concentrations and ODNs for 72 h. At the end of this treatment, cells were counted, analyzed for viability and cell growth was expressed as previously described.[38]

Statistical analysis

Data were expressed as means $\pm$ SD of at least two determinations, and statistical analysis was performed employing Student's *t*-test and analysis of variance for non-parametric data (Kruskal–Wallis test), when appropriate.

Fluorescence histograms were subjected to statistical analysis using the non-parametric Kolmogorov–Smirnow test.[44] The Kolmogorov–Smirnow two-sample test was applied to sets of data comprising ODN-treated and -untreated samples to objectively determine the decrease in DHFR protein.

Results

Effects of anti-DHFR aODN treatment

Cytotoxicity of DOTAP-combined aODN treatment was evaluated by quantifying both cell growth and viability. Within the range of aODN concentrations we used (0.1–30 μM) cell viability was always greater than 90%, while the inhibition of total cell division (TCD) versus controls ranged from 10 to 20%, independently of the ODN concentration (Figure 1). Administration of 10 μg/ml DOTAP alone was responsible for a slight cytotoxic effect (10% cell growth inhibition) (data not shown). No significant difference between control ODN and aODN cytotoxic effects was noted.

Effects of anti-DHFR aODN treatment on DHFR mRNA levels

The DHFR mRNA was quantitated by RT-PCR both in untreated MTX-sensitive parental CCRF-CEM/S cells and in MTX-resistant CCRF-CEM/E cells before and after 96 h treatment with 30 μM aODNs (Figure 2). As compared to the untreated control, the DHFR mRNA levels of CCRF-CEM/E cells treated with either DHFR1 aODN or with the equimolar mixture of DHFR2+DHFR3 aODNs were significantly lower (79.7 and 74.2%, respectively). The DHFR mRNA levels in MTX-resistant cells following treatment with DHFR1 and DHFR2+DHFR3 were, however, 3.2- and 4-fold higher, respectively, than those of CCRF-CEM/S parental cells. DHFR1 control ODN (SCRM) did not significantly affect DHFR mRNA, while DHFR2+DHFR3 control ODN (FD) was responsible for lowering DHFR mRNA levels by about 27% compared to untreated control cells. However, the difference in decreased DHFR mRNA levels observed between aODN-treated and FD ODN-treated cells was significant ($p < 0.01$). No substantial changes in DHFR gene expression were obtained with 10 μM aODNs (data not shown).

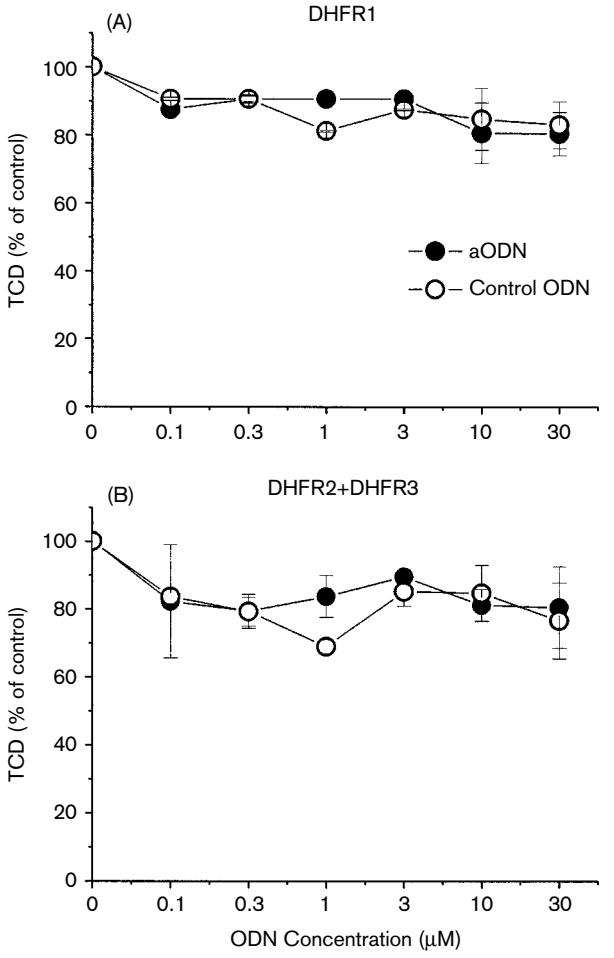

Figure 1. Inhibitory effects of anti-DHFR aODNs (0.1–30 μM) combined with DOTAP 10 μg/ml on the growth of CCRF-CEM/E MTX-resistant cells. The cells were treated for 96 h with the phosphorothioate aODN DHFR1 (A) or an equimolar mixture of the phosphodiesters aODNs (DHFR2+DHFR3) (B) and with the respective control ODNs (SCRM and FD). Results are expressed as percent of total number of viable cell divisions (TCD) compared to the untreated cells (mean $\pm$ SD of four determinations).

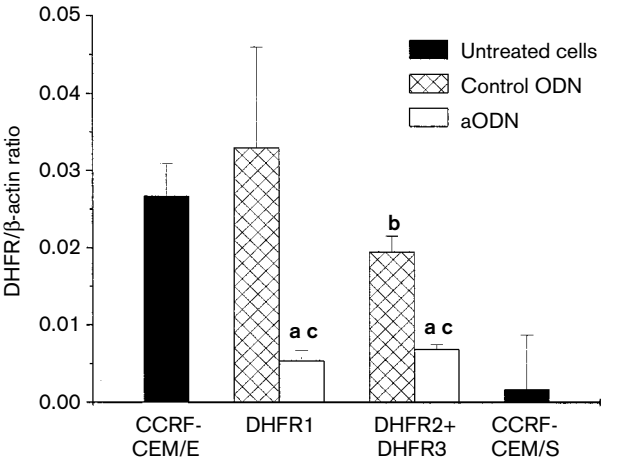

Figure 2. Effects of anti-DHFR aODNs on specific mRNA levels in CCRF-CEM/E MTX-resistant cells. The cells were treated for 96 h with an equimolar mix of DHFR2+DHFR3 aODN phosphodiesters or DHFR1 phosphorothioate and the respective control ODNs (FD ODN, SCRM ODN) in combination with DOTAP 10 μg/ml. The results are expressed as ratio of the mRNA of the DHFR gene to β-actin internal standard (means $\pm$ SD of five determinations). The mRNA levels of the DHFR gene in the parental MTX-sensitive cells (CCRF-CEM/S) are also reported. Statistical analysis: ODN-treated versus untreated cells, $^{a}p<0.01$; control ODN-treated versus untreated cells, $^{b}p<0.05$; ODN-treated versus control ODN-treated cells, $^{c}p<0.01$.

Effects of anti-DHFR aODNs treatment on DHFR protein level

The effects of the anti-DHFR aODN treatment on DHFR gene expression were further evaluated by quantitating the cell levels of DHFR protein. As reported in Figure 3, a 96 h treatment of CCRF-CEM/E cells with DHFR1 and DHFR2+DHFR3 aODNs (30 μM) (Figure 3E and F) reduced DHFR protein content to a significantly lower level as compared to the untreated CCRF-CEM/E cells (Figure 3A) (55.2 and 39%, respectively). This level was, however, higher than that observed in parental CCRF-CEM/S (Figure 3B). A significant lowering of DHFR protein level was also observed following exposure of cells with control ODNs (Figure 3C and D). The results obtained with the Kolmogorov–Smirnov test showed that the histograms of aODN-treated cells were significantly different from histograms of untreated and control ODN treated cells ($D > D_{crit}$ at α=0.001).

The lower dose of aODNs (10 μM) did not affect the level of DHFR protein of cells exposed as above (data not shown).

Effects of aODNs on the MTX-resistant cell phenotype

We investigated the inhibition of CCRF-CEM/E cell growth induced by MTX 0.05 μM (corresponding to an IC_{50} of CCRF-CEM/S but no cytotoxic dose for CCRF-CEM/E cells) and 0.5 μM (IC_{25} and IC_{75} of CCRF-CEM/E and CCRF-CEM/S cells, respectively) after a 96 h exposure to 30 μM aODNs combined with DOTAP. A moderate increase (about 25%, $p<0.01$) in cytotoxicity induced by MTX was observed at the lower concentration (0.05 μM) after combined treatment with both DHFR1 and DHFR2+DHFR3 aODNs; at

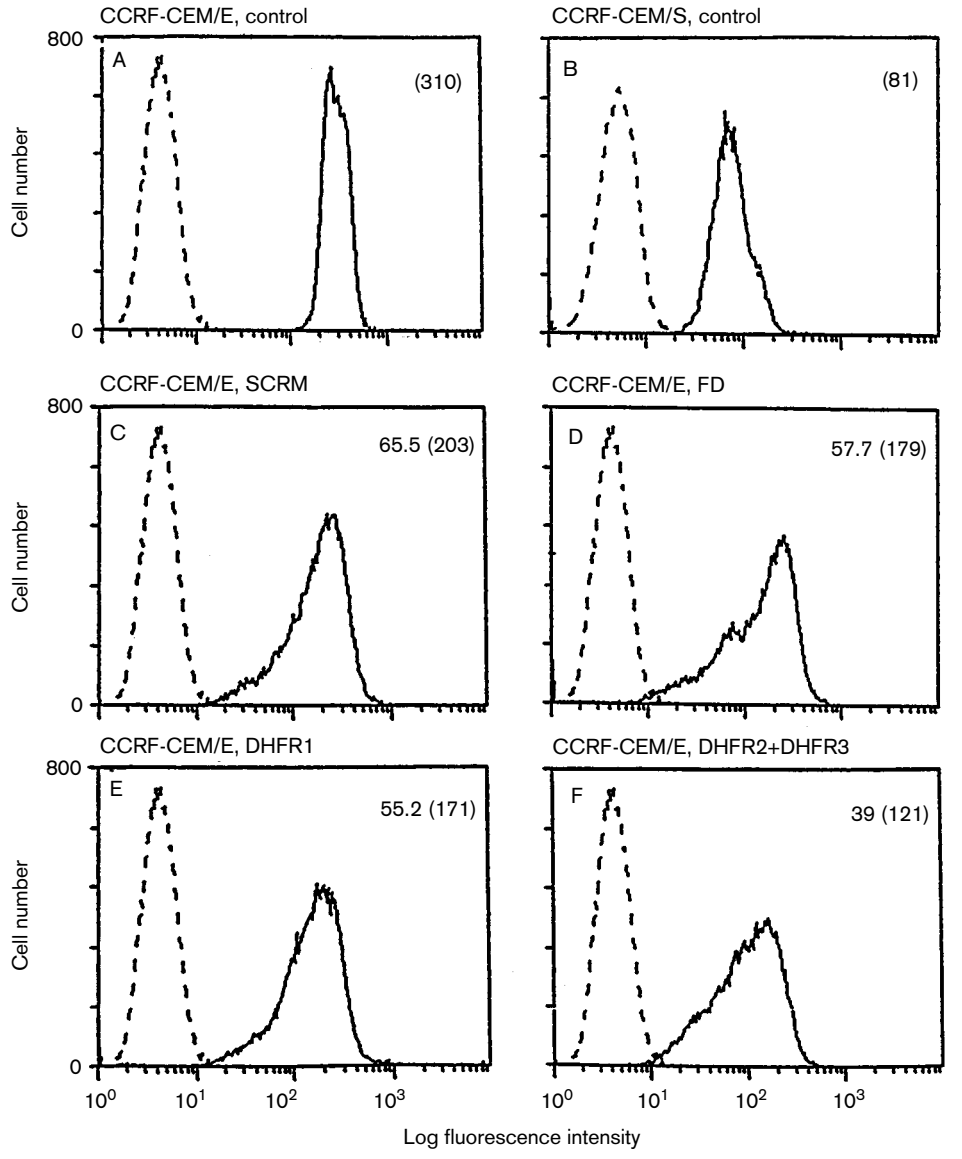

Figure 3. Lowering of DHFR protein level in CCRF-CEM/E cells after culture in the presence of 30 μM aODNs (E and F: DHFR1 and DHFR2+DHFR3, respectively) as compared to untreated (A) or control ODN-treated cells (C and D: SCRM and FD, respectively). The DHFR content on CCRF-CEM/S is also reported (B). Dotted line, cells not exposed to F-MTX; solid line, green fluorescence after 14 h exposure to 10 μM F-MTX as described in Materials and methods In parentheses: mean channel fluorescence intensity/cell; the number in the upper right corner represents the value of mean fluorescence expressed as percentage of the untreated cells (A) which have a mean fluorescence of 310 fluorescein units/cell.

the higher MTX concentration tested (0.5 μM) an increase in cytotoxicity (about 20%, $p<0.01$) was obtained with DHFR2+DHFR3 aODNs treatment and no substantial (less than 10%) but statistically significant ($p<0.05$) increase in cytotoxicity was observed with DHFR1 aODN treatment (Figure 4). Similar effects were also obtained with combined control ODNs and the same concentrations of MTX.

Discussion

MTX-resistant tumor clones may be selected by MTX-based chemotherapy, often endowed with DHFR mRNA and protein overproduction consequent to DHFR gene amplification.[15,16]

We evaluated the possibility of overcoming DHFR gene amplification-related MTX-resistance of tumor cells by using aODNs targeting the DHFR mRNA.

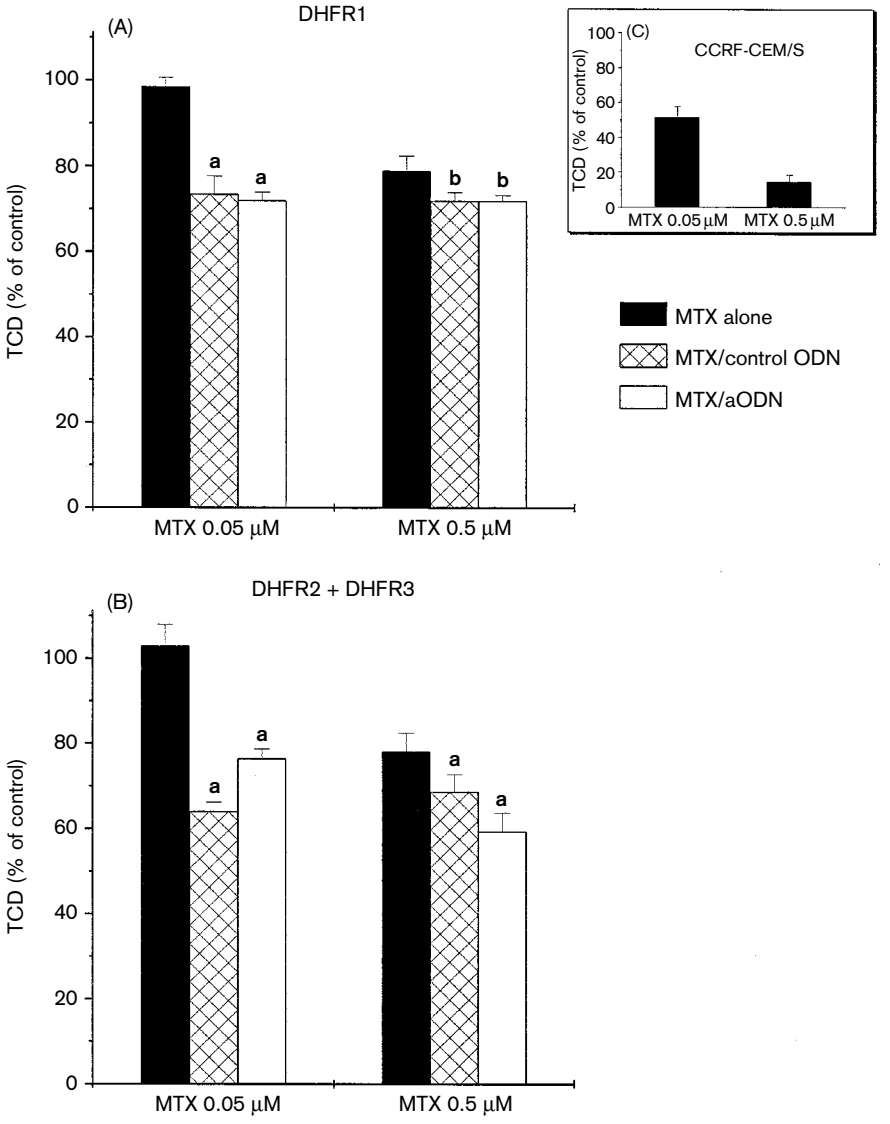

Figure 4. Growth inhibition by MTX of CCRF-CEM/E cells after culture with aODNs DHFR1 (A) and DHFR2+DHFR3 (B) targeted DHFR gene, with relevant control ODNs, SCRM and FD, respectively. Cells were preincubated for 24 h with 30 μM ODNs; 0.05 or 0.5 μM MTX was added in the next 72 h. Results were expressed as percentage of the number of total viable cell divisions compared to untreated controls (means $\pm$ SD of two determinations). Results of parental CCRF-CEM/S sensitive cells are also reported (C). $^{a}p<0.01$, $^{b}p<0.05$, MTX/ODN-treated samples versus MTX alone-treated samples.

Antisense oligomers are known to specifically inhibit gene expression both by forming a RNA/DNA heteroduplex responsible for activating a mRNA-cleaving RNase H[45] and/or by sterically inhibiting mRNA translation.[46]

An 18mer phosphorothioate-capped aODN (original sequence) targeting the start site of DHFR mRNA (DHFR1) or two 14mer unmodified phosphodiester contiguous ODNs targeting the adjacent upstream segment located in the 5′ untranslated region (DHFR2+DHFR3) (sequences published by Maher and Dolnick[27]) were administered to the human leukemia CCRF-CEM/E cell line overexpressing the DHFR gene about 20-fold in comparison with the CCRF-CEM parental cell line.[31]

Treatment with anti-DHFR aODNs following a 4 day exposure of MTX-resistant CCRF-CEM/E cells to 30 μM aODNs induced a significant lowering of DHFR mRNA levels but only a slight lowering of the relevant protein in the CCRF-CEM/E MTX-resistant subline. However, both DHFR mRNA and protein levels of aODN-treated cells remained higher than the levels of the parental

cells. Our findings could be explained by lack of aODN effects on DHFR translational or post-translational regulation events[47] which may consist of an increase in enzymatic activity of DHFR or in its half-life. On the other hand, the inhibition of DHFR gene expression observed by other investigators, using anti-gene ODNs[30] or anti-mRNA ODNs corresponding to those used in this investigation as a mixture,[27] occurred in cell-free systems, which lack cell compensatory mechanisms.

Although activity of lipid-vehiculated aODNs at lower concentrations has been reported for other molecular targets,[17] our results are not at odds with those obtained with aODNs directed toward other genes responsible for tumor drug resistance showing substantial *in vitro* biological effects only at high concentrations (10 μM or above).[24,35,48]

Plasma concentrations in the 10–100 μM range can also be reached at peak in animal models after single administration of various aODNs at doses ranging from 20 to 100 mg/kg [e.g. MT-AS, a DNA-methyltransferase aODN; CGP 69846A (ISIS 5132), a C-RAF-1 kinase aODN[49–51]]. These concentrations could be maintained with repeated dosing of phosphorothioate aODNs. Since maximum-tolerated doses in humans may well be within the therapeutic range observed in animal models,[19] it is conceivable that plasma concentrations similar to those achievable in *in vivo* and *in vitro* systems may be also obtained in the clinic.

The percent of cell growth inhibition induced by MTX at doses of 0.05 and 0.5 μM in MTX-resistant cells after aODNs treatment was markedly lower in comparison with that obtained in sensitive CCRF-CEM cells treated with the same concentrations of MTX alone (i.e. 50 and 75%, respectively).

The moderate increase of MTX cytotoxicity observed with ODN combined treatments could not be explained as a real, although limited, enhancement of antifol cytotoxicity since ODNs alone were partly responsible for reduced cell proliferation and the apparent reversion of the MTX-resistant phenotype obtained with aODN combined treatments was similar to that obtained with control ODNs.

Our results demonstrate that the use of DHFR aODNs targeting the AUG translation start and the 5′-untranslated region at relatively high concentration are effective in consequently inducing a substantial but not optimum lowering of specific mRNA in the MTX-resistant tumor phenotype. In fact, the level of DHFR protein remains almost double than that of MTX-sensitive cells and may therefore be enough to maintain MTX resistance. In order to reach an optimal degree of cytotoxicity with the antifol, an inhibition of DHFR activity higher than 95% must be obtained in MTX-sensitive tumor cells.[52]

In CCRF-CEM/E cells characterized by an about 20-fold enzyme activity elevation as compared to parental CCRF-CEM cells, the threshold value of DHFR activity inhibition necessary to confer MTX cytotoxicity as in CCRF-CEM-sensitive cells may be theoretically even greater than 99.5%. Furthermore intracellular aODN degradation by endonucleases[53] could cause the release of thymidine and a probable salvage from MTX cytotoxicity.[54]

Finally, we cannot exclude that longer-term exposure to aODNs may provide more effective protein inhibition levels.

Conclusion

In conclusion, plans for future treatment approaches will therefore comprise long-term cell exposure experiments, the use of second-generation aODNs (e.g. 2′-*O*-alkyl and morpholino derivatives) and the design of new aODNs targeted against additional DHFR mRNA regions, with the aim of developing a more effective antisense strategy combined with traditional chemotherapy.

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References

1. Chu E, Allegra JC. Antifolates. In: Chabner BA, Longo DL, eds. *Cancer chemotherapy and biotherapy: principles and practice*. Philadelphia, PA: Lippincott-Raven 1996: 109–48.
2. Bertino JR. Karnofsky memorial lecture. Ode to methotrexate. *J Clin Oncol* 1993; **11**: 5–14.
3. Dixon KH, Lanpher BC, Chiu J, Kelley K, Cowan KH. A novel cDNA restores reduced folate carrier activity and methotrexate sensitivity to transport deficient cells. *J Biol Chem* 1994; **269**: 17–20.
4. Ross JF, Prabir BS, Chaudhury K, Ratnam M. Differential regulation of folate receptor isoform in normal and malignant tissues *in vivo* and in established cell lines. Physiologic and clinical implications. *Cancer* 1994; **73**: 2432–43.
5. McGuire JJ, Mini E, Hsieh P, Bertino JR. Role of methotrexate polyglutamates in methotrexate- and sequential methotrexate–5-fluorouracil-mediated cell kill. *Cancer Res* 1985; **45**: 6395–400.

6. Dedhar S, Hartley D, Fitz-Gibbons D, Phillips G, Goldie JH. Heterogeneity in the specific activity and methotrexate sensitivity of dihydrofolate reductase from blast cells of acute myelogenous leukemia patients. *J Clin Oncol* 1985; **3**: 1545–52.
7. Göker E, Lin JT, Trippett T, *et al*. Decreased polyglutamylation of a methotrexate in acute lymphoblastic leukemia blast in adults compared to children with this disease. *Leukemia* 1993; **7**: 1000–4.
8. Saikawa Y, Knight CB, Saikawa T, Page ST, Chabner BA, Elwood PC. Decreased expression of the human folate receptor mediates transport defective methotrexate resistance in KB cells. *J Biol Chem* 1993; **268**: 5293–301.
9. Rhee MS, Wang Y, Nair MG, Galivan J. Acquisition of resistance to antifolates caused by enhanced γ-glutamyl hydrolase activity. *Cancer Res* 1993; **53**: 2227–30.
10. Masson E, Relling MV, Synold TW, *et al*. Accumulation of methotrexate polyglutamates in lymphoblasts is a determinant of antileukemic effects *in vivo*. *J Clin Invest* 1996; **97**: 73–80.
11. Gorlick R, Göker E, Trippett T, *et al*. Defective transport is a common mechanism of acquired methotrexate resistance in acute lymphocytic leukemia and is associated with decreased reduced folate carrier expression. *Blood* 1997; **89**: 1013–8.
12. Carman MD, Schornagel JH, Rivest RS, *et al*. Resistance to methotrexate due to gene amplification in patient with acute leukemia. *J Clin Oncol* 1984; **2**: 16–20.
13. Horns Jr RC, Dower WJ, Schimke RT. Gene amplificaton in a leukemic patient treated with methotrexate. *J Clin Oncol* 1984; **2**: 2–7.
14. Trent JM, Buick RN, Olson S, Horns Jr RC, Schimke RT. Cytologic evidence for gene amplification in methotrexate-resistant cells obtained from a patient with ovarian adenocarcinoma. *J Clin Oncol* 1984; **2**: 8–15.
15. Göker E, Waltham M, Kheradpour A, *et al*. Amplification of the dihydrofolate reductase gene is a mechanism of acquired resistance to methotrexate in patients with acute lymphoblastic leukemia and is correlated with p53 gene mutations. *Blood* 1995; **86**: 677–84.
16. Matherly LH, Taub JW, Wong SC, *et al*. Increased frequency of expression of elevated dihydrofolate reductase in T-cell versus B-precursor acute lymphoblastic leukemia in children. *Blood* 1997; **90**: 578–89.
17 Ho PT, Parkinson DR. Antisense oligonucleotides as therapeutics for malignant diseases. *Semin Oncol* 1997; **24**: 187–202.
18. Bishop MR, Iversen PL, Bayever E, *et al*. Phase I trial of an antisense oligonucleotide OL (1) p53 in hematologic malignancies. *J Clin Oncol* 1996; **14**: 1320–6.
19. Webb A, Cunningham D, Cotter F, *et al*. Bcl-2 antisense therapy in patients with non- Hodgkin lymphoma. *Lancet* 1997; **349**: 1137–41.
20. de Fabritiis P, Petti MC, Montefusco E, *et al*. BCR-ABL antisense oligodeoxynucleotide *in vitro* purging and autologous bone marrow transplantation for patients with chronic myelogenous leukemia in advanced phase. *Blood* 1998; **91**: 3156–62.
21. Gewirtz AM, Sokol DL, Ratajczak MZ. Nucleic acid therapeutics: state of the art and future prospects. *Blood* 1998; **92**: 712–36.
22. Kuss B, Cotter F. Antisense—time to shoot the messenger. *Ann Oncol* 1999; **10**: 495–503.
23. Nemunaitis J, Holmlund JT, Kraynak M, *et al*. Phase I evaluation of ISIS 3521, an antisense oligodeoxynucleotide to protein kinase C-alpha, in patients with advanced cancer. *J Clin Oncol* 1999; **17**: 3586–95.
24. Quattrone A, Papucci L, Morganti M, *et al*. Inhibition of MDR1 gene expression by antimessenger oligonucleotides lowers multiple drug resistance. *Oncol Res* 1994; **6**: 311–20.
25. Bertram J, Palfner K, Killian M, *et al*. Reversal of multiple drug resistance *in vitro* by phosphorothioate oligonucleotides and ribozymes. *Anti-Cancer Drugs* 1995; **6**: 124–34.
26. Li X, Smyth AP, Barrett DJ, Ivy SP, von Hofe E. Sensitization of multidrug-resistant human leukemia cells with MDR-targeted antisense and inhibition of drug-mediated MDR1 induction. *Leukemia* 1997; **11**: 950–7.
27. Maher LJ III, Dolnick BJ. Specific hybridization arrest of dihydrofolate reductase mRNA *in vitro* using antisense RNA or antisense oligonucleotides. *Arch Biochem Biophys* 1987; **253**: 214–2.
28. Kobayashi H, Kim N, Halatsch ME, Ohnuma T. Specificity of ribozyme designed for mutated DHFR mRNA. *Biochem Pharmacol* 1994; **47**: 1607–13.
29. Blume SW, Gee JE, Shrestha K, Miller DM. Triple helix formation by purine-rich oligonucleotides targeted to the human dihydrofolate reductase promoter. *Nucleic Acids Res* 1992; **20**: 1777–84.
30. Gee JE, Blume S, Snyder RC, Ray R, Miller DM. Triplex formation prevents Sp1 binding to the dihydrofolate reductase promoter. *J Biol Chem* 1992; **267**: 11163–7.
31. Mini E, Srimatkandada S, Medina WD, Moroson BA, Carman MD, Bertino JR. Molecular and kariological analysis of methotrexate-resistant and -sensitive human leukemic CCRF-CEM cells. *Cancer Res* 1985; **45**: 317–24.
32. Masters JN, Attardi G. The nucleotide sequence of the cDNA coding for human dihydrofolic reductase. *Gene* 1983; **21**: 59–63.
33. Morelli S, Delia D, Capaccioli S, *et al*. The antisense *bcl-2*-IGH transcript is an optimal target for synthetic oligonucleotides. *Proc Natl Acad Sci USA* 1997; **94**: 8150–5.
34. Schmitz JC, Agrawal S, Chu E. Repression of human thymidylate synthase mRNA translation by antisense 2′-*O*-methyl oligoribonucleotides. *Antisense Nucleic Acid Drug Dev* 1998; **8**: 371–8.
35. Ju J, Kane SE, Lenz HJ, Danenberg KD, Chu E, Danenberg PV. Desensitization and sensitization of cells to fluoropyrimidines with different antisenses directed against thymidylate synthase messenger RNA. *Clin Cancer Res* 1998; **4**: 2229–36.
36. Capaccioli S, Di Pasquale G, Mini E, Mazzei T, Quattrone A. Cationic lipids improve antisense oligonucleotide uptake and prevent degradation in cultured cells and in human serum. *Biochem Biophys Res Commun* 1993; **197**: 818–25.
37 Quattrone A, Papucci L, Schiavone N, Mini E, Capaccioli S. Intracellular enhancement of intact antisense oligonucleotide steady-state levels by cationic lipids. *Anti-Cancer Drug Des* 1994; **9**: 549–53.
38. Mini E, Moroson BA, Bertino JR. Cytotoxicity of floxuridine and 5-fluorouracil in human T-lymphoblast leukemia cells: enhancement by leucovorin. *Cancer Treat Rep* 1987; **71**: 381–9.

39. Jackson RC, Huennekens FM. Turnover of dihydrofolate reductase in rapidly dividing cells. *Arch Biochem Biophys* 1973; **154**: 192–8.
40. Horikoshi T, Danenberg KD, Stadlbauer THW. Quantitation of thymidylate synthase, dihydrofolate reductase and DT-diaphorase gene expression in human tumors using the polymerase chain reaction. *Cancer Res* 1992; **52**: 108–16.
41. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 1987; **162**: 156–9.
42. Ng SY, Gunning P, Eddy R, *et al*. Evolution of the functional human β-actin gene and its multipseudogene family: conservation of noncoding regions and chromosomal dispersion of pseudogenes. *Mol Cell Biol* 1985; **5**: 2720–32.
43. Assaraf YG, Seamer LC, Schimke RT. Characterization by flow cytometry of fluorescein–methotrexate transport in Chinese hamster ovary cells. *Cytometry* 1989; **10**: 50–5.
44. Ian TY. Proof without prejudice: use of the Kolmogorov–Smirnow test for the analysis of histograms from flow systems and other sources. *J Histochem Cytochem* 1977; **25**: 935–41.
45. Tidd DM. Ribonuclease H-mediated antisense effects in intact human leukemic cells. *Biochem Soc Trans* 1996; **24**: 619–23.
46. Pierga JY, Magdelenat H. Applications of antisense oligonucleotides in oncology. *Cell Mol Biol* 1994; **40**: 237–61.
47. Li XK, Kobayashi H, Holland JF, Ohnuma T. Expression of dihydrofolate reductase and multidrug resistance genes in trimetrexate resistant human leukemia cell lines. *Leuk Res* 1993; **17**: 483–90.
48. Cucco C, Calabretta B. *In vitro* and *in vivo* reversal of multidrug resistance in a human leukemia-resistant cell line by *mdr1* antisense oligodeoxynucleotides. *Cancer Res* 1996; **56**: 4332–7.
49. Geary RS, Leed JM, Fitchett J, *et al*. Pharmacokinetics and metabolism in mice of a phosphorothioate oligonucleotide antisense inhibitor of C-RAF-1 kinase expression. *Drug Metab Disp* 1997; **25**: 1272–81.
50. Phillips JA, Craig SJ, Bayley D, Christian RA, Geary R, Nicklin PL. Pharmacokinetics, metabolism, and elimination of 20-mer phosphorothioate oligodeoxynucleotide (CGP 69846A) after intravenous and subcutaneous administration. *Biochem Pharmacol* 1997; **54**: 657–68.
51. Qian M, Chen SH, von Hofe E, Gallo JM. Pharmacokinetics and tissue distribution of a DNA-methyltransferase antisense (MT-AS) oligonucleotide and its catabolites in tumor-bearing nude mice. *J Pharmacol Exp Ther* 1997; **282**: 663–70.
52. Goldman ID, Matherly LH. Biochemical factors in the selectivity of leucovorin rescue: selective inhibition of leucovorin reactivation of dihydrofolate reductase and leucovorin utilization in purine and pyrimidine biosynthesis by methotrexate and dihydrofolate polyglutamates. *NCI Monogr* 1987; **5**: 17–26.
53. Uhlmann E, Ryte A, Peyman A. Studies on the mechanism of stabilization of partially phosphorothioated oligonucleotides against nucleolytic degradation. *Antisense Nucleic Acid Drug Dev* 1997; **7**: 345–50.
54. Taylor IW, Slowiaczek P, Francis PR, Tattersall MH. Biochemical and cell cycle perturbations in methotrexate-treated cells. *Mol Pharmacol* 1982; **21**: 204–10.

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