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# Effect of Hammerhead Ribozyme against Human Thymidylate Synthase on the Cytotoxicity of Thymidylate Synthase Inhibitors

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**One of the resistance mechanisms to folate-based thymidylate synthase (TS) inhibitors is the increase in TS activity in tumor cells. Human B lymphoblastoid cell line (W1L2) was made resistant to a lipophilic non-polyglutamatable TS inhibitor (ZM249148), and the subline (W1L2:R179) showed a 20-fold increase in TS enzyme activity with concomitant overexpression of *TS* mRNA. To overcome the resistance, we designed a ribozyme that can cleave the CUC sequences in a triple tandemly repeated sequence of *TS* mRNA. Expression of this ribozyme in W1L2:R179 cells transfected with Epstein Barr virus-based expression vector resulted in sensitization to TS inhibitors concomitantly with a decrease of *TS* expression. The ribozyme expressed in transfectants was shown to be functional in cleaving artificial *TS* RNA *in vitro*.**

Key words: Hammerhead ribozyme — Thymidylate synthase inhibitor — Drug resistance

TS[5] (EC 2.1.1.45) is a critical enzyme in the synthesis of DNA and an important target in cancer chemotherapy. Recently, a series of TS inhibitors have been developed and some of these drugs have shown promising results in clinical trials.[1)] The acquisition of resistance to TS inhibitors in tumor cells limits the use of these drugs as cytotoxic agents. Resistance to folate-based TS inhibitors could occur as a result of one or more of the following mechanisms: (a) *TS* gene amplification and/or overexpression with subsequent elevation in TS enzyme activity[2–4)]; (b) alteration of the enzyme with reduced affinity for the inhibitor; (c) impaired uptake or reduced accumulation of the inhibitor in the cells[5)]; (d) alteration in FPGS (EC 6.3.2.17) activity.[6,7)] In an attempt to overcome drug resistance resulting from *TS* overexpression, we designed a hammerhead ribozyme that can cleave the CUC sequences in a triple tandemly repeated sequence located in the 5′-untranslated region of *TS* mRNA. In this study, we examined the efficacy of the ribozyme for *TS* mRNA suppression and subsequent reversal of resistance in a TS-overproducing human B lymphoblastoid cell line.

The parental W1L2 human B lymphoblastoid cell line was maintained in RPMI-1640 (GIBCO, Grand Island, NY) containing 10% FBS (GIBCO) and fed twice a week with fresh medium. The TS inhibitors ZM249148, ZD9331, ZD1694 and CB3717 were generously provided by Zeneca Pharmaceuticals, Cheshire, UK. TMQ acetate was kindly supplied by Dr. T. Ohnuma, Mount Sinai School of Medicine, New York. MTX was purchased from Japan Lederle Pharmaceuticals, Tokyo. For the establishment of the resistant W1L2 subline to ZM-249148, a newly developed lipophilic non-polyglutamatable quinazoline-based TS inhibitor, W1L2 cells were initially exposed to 0.55 $\mu M$ ZM249148, which is nearly equal to the $IC_{50}$ value. The drug concentration was escalated approximately every two weeks up to 5 $\mu M$, and thereafter the resistant cells were maintained in the culture medium containing 5 $\mu M$ ZM249148. The resistant cells were kept drug-free for 2 weeks before an experiment. The resistant subline (W1L2:R179) thus established showed a 20-fold increase in TS activity (26 $\pm$6.8 nmol/h/$10^6$ cells, determined by the method described previously[8)]) compared to the parental cell line (1.3 nmol/h/$10^6$ cells)[9)] and concomitant overexpression of *TS* mRNA was demonstrated by Northern blot analysis (Fig. 1, lanes 1 and 2). As shown in Table I, the resistances to all of ZD9331, ZD1694 and CB3717,

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[5] The abbreviations used are: CB3717, $N^{10}$-propargyl-5,8-dideazafolic acid; DHFR, dihydrofolate reductase; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FPGS, folylpolyglutamate synthetase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; $IC_{50}$, drug concentration producing 50% inhibition of cell growth; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; MTX, methotrexate; PCR, polymerase chain reaction; TMQ, trimetrexate; TS, thymidylate synthase.

which are potent TS inhibitors but have different biochemical properties in drug uptake and cellular metabolism, were confirmatory of TS overproduction as a major mechanism of the resistance in W1L2:R179 cells.

We designed a hammerhead ribozyme that can cleave the triple tandemly repeated CUC sequences in the 5′-untranslated region of *TS* gene (Fig. 2A). Since the region in *TS* mRNA to be targeted by the ribozyme is assumed to have loop structures,[11–13] which the ribozyme molecules can easily access, the ribozyme was expected to cleave efficiently at least one of the target sites (Fig. 2B). The TS ribozyme used for transfection study was designed as described below. Two single-stranded oligodeoxynucleotides were synthesized such that the 40-base hammerhead ribozyme contained flanking *Kpn*I and *Bam*HI restriction sites on both ends and were 5′-phosphorylated by T4 polynucleotide kinase to afford 5′-pCGGGGGACGCTGATGAGGCCGAAAGGCCGAAAGGCAGGCG-3′, and 5′-pGATCCGCCTGCCTTTCGGCCTTTCGGCCTCATCAGCGTCCCCCGGTAC-3′. This ribozyme was cloned into the vector pCEP4™ (Invitrogen, San Diego, CA). The sequence and orientation of the ribozyme in the vector were confirmed by DNA sequencing using a DNA sequencer, Model 373A (Perkin Elmer Japan, Chiba). For the transfection procedure, cells in the exponential growth phase were washed twice with RPMI-1640 medium and resuspended at a final concentration of $4\times10^6$ cells/500 $\mu$l in RPMI-1640 medium without FBS. Twenty $\mu$g of plasmid DNA was added to the cell suspension and the whole was incubated on ice for 10 min. The cell-DNA mixture was subjected to electroporation at 330 V with a capacitance of 1000 $\mu$F using an Electroporator II (Invitrogen). After a further 10 min incubation at room temperature, cells were diluted with 10 ml of RPMI-1640 medium containing 10% FBS. The medium was changed 24 h later. Three days after transfection, hygromycin B was added at a concentration of 800 $\mu$g/ml. Following culture for 3 to 4 weeks, hygromycin B-resistant cells were pooled, and drug sensitivities to folate analogues were measured by MTT assay,[14] then mRNA was extracted. The doubling time of W1L2, W1L2:R179 and the transfectants was not significantly changed (16–18 h). As shown in Table I, the W1L2:R179 cells transfected with *TS* ribozyme became sensitive to TS inhibitors such as ZD9331, ZD1694 and CB3717, while the drug sensitivities of W1L2:R179 cells transfected with the vector alone did not alter. Moreover, the collateral sensitivity to DHFR (EC 1.5.1.3) inhibitors seen in W1L2:R179 cells was obscured after the suppression of *TS* expression by the ribozyme. Northern blot analysis demonstrated a remarkable decrease of *TS* mRNA expression in W1L2:R179 cells transfected with *TS* ribozyme as compared with W1L2:R179 cells and W1L2:R179 cells transfected with the vector alone (Fig.

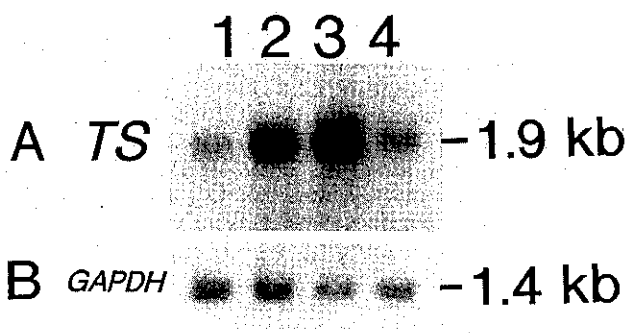


Fig. 1. Northern blot analysis of W1L2 sublines. Messenger RNA was extracted from these cells by using a Micro-Fast Track mRNA Isolation Kit (Invitrogen), and 0.25 $\mu$g of mRNA was used for Northern blot analysis. As probes, the cDNA for *TS* (pcHTS-7, *Xho*I digestion, kindly provided by Dr. T. Seno of Saitama Cancer Center Research Institute, Saitama)[10] and the cDNA for human *GAPDH* (pHcGAP, *Pst*I-*Xba*I fragment, obtained from ATCC) were used. Samples in lanes: 1, W1L2; 2, W1L2:R179; 3, W1L2:R179 transfected with pCEP4; 4, W1L2:R179 transfected with pCEP4 containing *TS* ribozyme.

Table I. Sensitivities of W1L2 Sublines to Various Antifolates

| Cell line | $IC_{50}$[a]/(Relative resistance)[b] | | | | |
|---|---|---|---|---|---|
| | ZD9331 (n*M*) | ZD1694 (n*M*) | CB3717 ($\mu$*M*) | MTX (n*M*) | TMQ (n*M*) |
| W1L2 | 1 | 2.3 | 0.3 | 11.8 | 0.09 |
| W1L2:R179 | 250 (×250) | 27 (×11.7) | 30 (×100) | 4.7 (×0.4) | 0.03 (×0.33) |
| W1L2:R179 with *TS* ribozyme | 25 (×25) | 6 (×2.6) | 2.1 (×7) | 7.5 (×0.64) | 0.05 (×0.56) |
| W1L2:R179 with pCEP4 | 240 (×240) | 25 (×10.9) | 29.5 (×98.3) | 4.8 (×0.41) | 0.03 (×0.33) |

*a*) For the determination of antifolate sensitivities, $2\times10^3$ cells were inoculated in 200 $\mu$l 96-well plates in the presence of graded concentrations of drugs. After 96-h incubation at 37°C in a 5% $CO_2$/95% air atmosphere, growth-inhibitory effects were measured by MTT assay.[14] $IC_{50}$ values were obtained from dose-response curves of each cell line. Each $IC_{50}$ value represents the mean of at least two independent quadruplicate experiments. All experimental data points were within 15% of the mean.
*b*) $IC_{50}$ value of the resistant cells/$IC_{50}$ value of the parent cells.

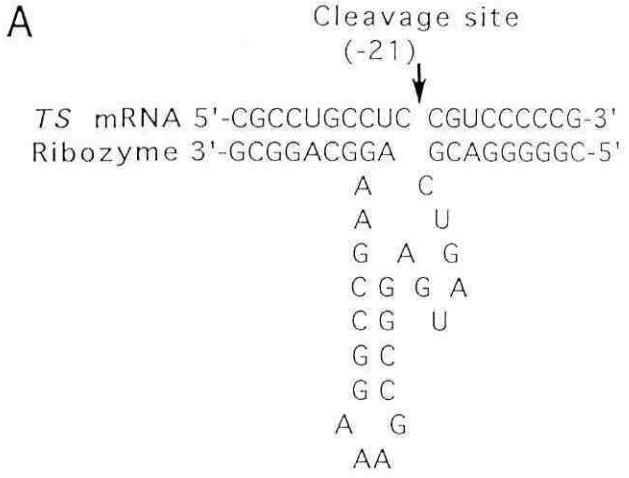


Fig. 2. A. Design of *TS* ribozyme. The number of nucleotides is based on the reported cDNA sequence of human *TS* gene.[11] B. Three possible interconvertible stem-loop structures in the 5′-untranslated region of human *TS* mRNA. The inverted repeat sequence is indicated by thick arrows (nucleotide number −71 to −65, −43 to −37 and −9 to −3). Because of the presence of this inverted repeat sequence, three possible stem structures could be formed. The sites cleaved by the designed ribozyme are indicated by arrows (nucleotide numbers −77, −49 and −21). An ATG initiation codon is indicated by an open box.

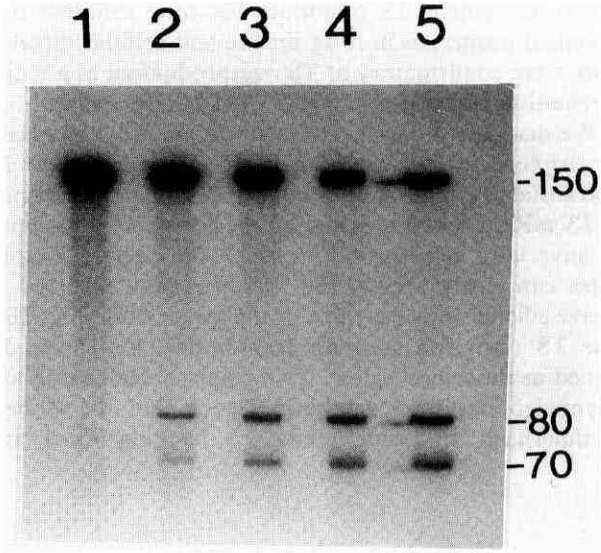


Fig. 3. Cleavage reaction with mRNA from hygromycin-resistant cells. Polyadenylated RNA from ribozyme-expressing, hygromycin-resistant W1L2:R179 cells was examined in the cell-free cleavage assay. Artificial *TS* RNA (150 bases) was cleaved by the ribozyme to generate 80- and 70-base fragments. Samples in lanes: 1, radiolabeled *TS* substrate only; 2–5, cleavage reaction with 0.5, 1, 2 and 3 μg, respectively, of mRNA from ribozyme-expressing cells.

1 lanes 2, 3 and 4), indicating that *TS* ribozyme efficiently suppressed the overexpression of *TS* mRNA.

At the next step, we examined whether the *TS* ribozyme expressed in the transfectants retained its cleavage activity *in vitro* in the following manner. For the preparation of the ribozyme substrate containing the cleavage site, mRNA was extracted from W1L2:R179 cells. For the reverse transcription-PCR (RT-PCR) of transcripts, cDNA was synthesized by using Superscript II reverse transcriptase (GIBCO) with the following specific antisense primer. Based on the reported full-length cDNA sequence for human *TS*[11] the portion from −36 to +55 was amplified with the primers, 5′-CCACTTCGCCTG-CCTCCGTC-3′ (sense primer) and 5′-CCTGTGCGG-CGGGGGGCAA-3′ (antisense primer). The distinct 91-bp product obtained from the RT-PCR was ligated directly to pT7 BlueT-vector (Novagen, Madison, WI). After transformation of *Escherichia coli* (Novablue, Novagen), white colonies were selected and screened by PCR for orientation using the antisense primer shown above and the T7 promoter primer. Plasmid DNA was prepared by using a Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany), and the sequence was confirmed by using a Taq Dye Deoxy Terminator Cycle Sequencing kit (Perkin Elmer Japan). The clones that could produce the sense transcript were chosen, and *in vitro* transcription was carried out. The transcription reaction mixture contained 5 μg of linearized plasmid DNA cut with *Bam*HI, 0.5 U/μl T7 RNA polymerase (New England Biolabs, Beverly, MA), 40 m*M* Tris-HCl (pH 7.9), 6 m*M* $MgCl_2$, 10 m*M* NaCl, 2 m*M* spermidine, 10 m*M* DTT, 0.5 m*M* ATP, GTP and UTP, 0.1 m*M* CTP, 50 μCi of [$\alpha$-$^{32}$P]CTP (sp. act. 800 Ci/mmol; DuPont, Boston, MA), and 1 U/μl recombinant ribonuclease inhibitor

(Promega, Madison, WI) in a 50-$\mu$l volume. The reactions were carried out at 30°C for 3 h and the products were treated with RQ1 RNase-free DNase (Promega) followed by phenol-chloroform extraction and ammonium acetate ethanol precipitation. The 150-base RNA obtained was used for the cleavage reaction in a cell-free system. The mRNA of W1L2:R179 cells transfected with *TS* ribozyme and *in vitro*-transcribed substrate RNA were mixed in a 10-$\mu$l volume of 50 m*M* Tris-HCl (pH 7.4) and 1 m*M* EDTA. The reaction mixture was heated at 95°C for 2 min to denature the RNAs then quickly chilled on ice, and the reaction was initiated by addition of $MgCl_2$. The reaction mixtures were incubated at 37°C for 3 h in the presence of 10 m*M* $MgCl_2$, followed by the addition of an equal volume of reaction-stop solution (95% deionized formamide, 20 m*M* EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), then the mixtures were heated at 80°C for 5 min, and analyzed in 8% polyacrylamide-7 *M* urea gels in 1× Tris-borate-EDTA buffer. The gels were dried and subjected to autoradiography. The autoradiogram showed that the *TS* ribozyme expressed in the transfectants cleaved the artificial substrate containing one cleavage site into 80-base and 70-base fragments in a dose-dependent manner *in vitro* (Fig. 3), indicating that *TS* ribozyme expressed in the transfectants retained sufficient cleavage activity. Although this result cannot be extrapolated to predict which site might actually be cleaved in cells, because the *in vitro* annealing conditions are not encountered in cells, this experiment strongly suggests that the ribozyme expressed is functional *in vitro* and, hence, very likely also functional in cells.

We showed that the introduction of the *TS* ribozyme into W1L2:R179 cells overexpressing *TS* mRNA resulted in the restoration of the sensitivity to TS inhibitors. In the present study, the pooled transfectants were used for experiments such as the drug sensitivity assay and Northern blot analysis instead of clones selected by G418 and limiting dilution, in contrast to the previous study with hammerhead ribozymes against human *MDR1* for the reversal of P-glycoprotein-mediated multidrug resistance.[15] The adequate suppression of *TS* mRNA and successful reversal of resistance in the pooled transfectants may be related to the use of pCEP4, a mammalian episomal expression vector that can replicate extrachromosomally in the W1L2 human B lymphoblastoid cell line, and this vector appears to contribute to the production of homogenous transfectants expressing almost the same amounts of *TS* ribozyme after hygromycin selection. When the DNA was extracted from W1L2:R179 cells transfected with pCEP4 by the alkaline lysis minipreparation method,[16] the original vector was recovered as extrachromosomal DNA (data not shown). These data suggested that an Epstein Barr virus-based expression vector may have a superior efficacy for transfection in the cell lines of B cell origin used in this study. For the efficacious suppression of the target mRNA by hammerhead ribozymes,[17,18] another important factor is the choice of the target site. In general, the regions around translation initiation sites or splicing sites, and large loop structures are good candidates as target sites. Therefore, we chose loop structures to which ribozyme molecules could have easy access. Although the 5′-untranslated region of human *TS* mRNA is highly GC-rich (80%),[11] our study demonstrated partial but successful reversal of the resistance phenotype by the ribozyme used.

The W1L2:R179 cells showed collateral sensitivity to MTX and TMQ (Table I). In fact, the positive correlation between TS activity and MTX sensitivity has been reported in a variety of cell lines.[19–23] TS binds a polyglutamyl cofactor, polyglutamyl 5,10-methylenetetrahydrofolate, which serves as a carbon donor. It is likely that TS-overproducing cells may have a greater demand for reduced folates than cells with a normal level of TS, resulting from intracellular depletion of the cofactor. This is a possible explanation for the increased sensitivity of the resistant W1L2 cells to DHFR inhibitors. As expected, this phenomenon was obscured when sensitivity to TS inhibitors was restored by the suppression of *TS* using the ribozyme.

Since the *TS* gene has a putative AP-1 binding site sequence upstream from the translational initiation site,[13] *TS* expression can be modulated by suppressing the transcriptional activation. Indeed, Scanlon *et al.* designed a c-*fos* ribozyme that could efficiently suppress the expression of *TS*.[24] Both our and their experimental results demonstrate that *TS* expression can be suppressed directly or indirectly by ribozymes. The *TS* ribozyme designed here may be universally applicable to overcome resistance caused by TS overproduction. Our results imply that ribozymes may be efficacious tools to suppress gene expression.

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