

Down-Regulation of Thiamine Transporter *THTR2* Gene Expression in Breast Cancer and Its Association With Resistance to Apoptosis

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Abstract

The recent molecular identification of two thiamine transporters, SLC19A2 (*THTR1*) and SLC19A3 (*THTR2*), has provided the opportunity to study thiamine transporter gene expression in human malignancies. We compared RNA levels of both *THTR1* and *THTR2* in a panel of human breast tumors and corresponding normal tissues. *THTR2* RNA levels were down-regulated in breast cancer to 14% of the level found in corresponding normal tissues, while *THTR1* levels were unchanged. Both thiamine transport genes were cloned and expressed in a breast cancer cell line to examine the impact of reconstituted thiamine transport gene expression on drug and radiation sensitivity and on resistance to apoptosis. *THTR2*-transfected breast cancer cells showed a 2.5-fold increase in specific *THTR2* activity and a 3-fold increase in cytotoxicity against a bromoacetyl ester derivative of thiamine. Surprisingly, these cells also showed a 3-fold increase in sensitivity to doxorubicin and an increase in sensitivity to ionizing radiation, but no change in sensitivity to methotrexate or paclitaxel. TUNEL assays demonstrate an increase in apoptosis in *THTR2*-transfected cells exposed to doxorubicin and radiation, and Western blot analysis suggests that apoptosis associated with these cytotoxic stresses is mediated at least in part by a caspase-3-dependent pathway. Therefore, thiamine transporter *THTR2* gene expression is down-regulated in breast cancer, which may contribute to resistance to apoptosis in these tumors.

Introduction

Thiamine, like folate and other essential nutrients, uses specific carrier mechanisms for transport into cells. However, unlike folate, the amount of thiamine stored in the body is usually very small. When ingested in excess of the body's

needs, this water-soluble vitamin is readily excreted in the urine, and therefore it must be continually supplied in the diet. Thiamine deficiency is most often associated with severe malnutrition, alcoholism, and fad diets (1), and clinically results in beriberi and Wernicke-Korsakoff syndromes (2).

The gene for a thiamine transport protein, *THTR1*, was isolated by gene mapping strategies which identified it as the cause of the thiamine-responsive megaloblastic anemia syndrome (TRMA), an autosomal-recessive disorder of megaloblastic anemia, diabetes mellitus, and sensorineural deafness (3–5). *THTR1* has been shown to carry mutations in affected individuals and to encode a high-affinity thiamine transporter (3–6).

THTR1 is a member of the SLC19 family of vitamin transporters (SLC19A2), which includes the reduced folate carrier *RFC1* (SLC19A1). Recently, another member of this family of genes, SLC19A3, was identified (7) and characterized as a thiamine transporter (8). To determine the potential role of thiamine transport in cancer therapy, we measured the level of expression of both thiamine transporters in a panel of human tumors and corresponding normal tissues. The down-regulation of *THTR2* in breast cancer led us to investigate the potential role of thiamine transport gene expression in the development of these tumors.

Results

THTR1 and *THTR2* Expression in a Tissue Array Panel

We originally hypothesized that increased tumor cell metabolic requirements would lead to an increase in expression of thiamine transporter gene expression in tumors compared to corresponding normal tissues. Initial preliminary data had demonstrated an increase in thiamine transporter gene expression in two of six melanoma specimens (data not shown). Therefore, a cDNA microarray of 50 breast tumors and corresponding normal tissues was probed for *THTR1*, *THTR2*, and ubiquitin as a control (Fig. 1). Boxes around three specimens indicate two tumor specimens from the same individual, with the corresponding single specimen of normal tissue. The signal strength for each specimen was quantitated for each probe using a phosphorimager software, and the *THTR1* and *THTR2* values were normalized with the strength of the ubiquitin signal for each specimen. By this quantitative analysis, average *THTR1* levels were unchanged in the 50 specimens, with the average *THTR1* signal in tumors averaging 101% of the control tissue. However, the *THTR2* levels averaged 14% of control levels, or an ~7-fold decrease in tumor levels versus corresponding normal tissues.

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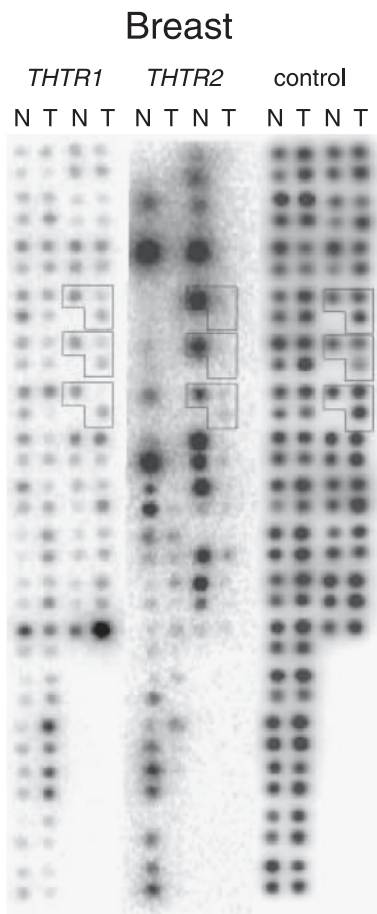


FIGURE 1. cDNA array of paired breast normal tissues (N) and tumors (T) probed for *THTR1* (first panel) and *THTR2* (second panel) expression. A probe for ubiquitin was used to control for equivalent cDNA loading.

THTR1 and THTR2 Expression in Breast Cancer Cell Lines

A scattergram showing RNA levels measured by reverse transcription (RT)-PCR in breast cancer cell lines is shown in Fig. 2. *THTR1* levels did not vary markedly among breast cancer cell lines, and did not vary markedly from the mean of 50 cell lines. There was much more variation in breast cancer cell lines with regard to *THTR2* levels. The breast cancer cell line HS578T had RNA levels 4-fold higher than the mean of 50 cancer cell lines, and the breast cancer cell line BT549 showed *THTR2* levels 2-fold higher than the mean. In contrast, the other four cell lines we looked at (MCF-7, NCI/ADR, MDA231, and T47-D) all had *THTR2* levels less than one-fourth of the mean.

Functional Characterization of THTR1- and THTR2-Transfected Cells

To study potential reasons for down-regulation of thiamine transporter gene expression in breast cancer, we cloned the open reading frames of *THTR1* and *THTR2* into the episomal expression vector pREP4 and transfected them into a folate-transport-deficient, MTX-resistant ZR-75 human breast cancer cell line (MTX^R ZR75). The cells were selected for resistance

to hygromycin, and individual clones were selected by limiting dilution for further study. As shown in Fig. 3, MTX^R ZR75 cells transfected with the cDNAs for *THTR1* and *THTR2* both showed an increase in initial thiamine uptake relative to the control cell line. *THTR2*-transfected cells accumulated thiamine more rapidly than *THTR1*-transfected cells. The linear range of initial uptake appeared to be ~1 min. The *THTR2* transgene increased the V_{max} for thiamine 2.5-fold (2 versus 5 pmol/min/mg protein in the control cells versus *THTR2*-transfected cells, respectively).

Because *RFC1* (SLC19A1), *THTR1* (SLC19A2), and *THTR2* (SLC19A3) share significant peptide homology, we determined whether the two transporters shared common substrates. We could detect no incremental increase in thiamine uptake in *RFC1*-transfected cells compared to controls (data not shown), consistent with data previously reported by Zhao *et al.* (9, 10). We also could not find any evidence that there was any functional cooperation between *RFC1* and *THTR1* in the uptake of thiamine in cells transduced with both genes. Similarly, MTX^R ZR75 cells transfected with *THTR1* or *THTR2* showed no significant incremental increase in the rate of initial uptake of folic acid, folinic acid, or methotrexate (data not shown), also consistent with the findings of Rajgopal *et al.* (8).

Increased THTR2 Expression and Cytotoxicity of a Thiamine Analogue

To potentially target thiamine transport for cancer therapy, we constructed several thiamine analogues. One of these drugs, a bromoacetyl derivative of thiamine (Fig. 4A), inhibited thiamine uptake (40 nM) to 25% of control values when present in 1000-fold excess. We therefore examined the cytotoxicity of this thiamine analogue in *THTR2*-transfected cells. As shown in Fig. 4B, the 2.5-fold increase in V_{max} for thiamine in these cells correlated with a 3-fold increase in the sensitivity of the transfected cells for the bromoacetyl ester derivative of thiamine. To determine whether the increase in cytotoxicity of bromoacetylthiamine in *THTR2*-transfected cells was due to increased activation of apoptotic pathways, we examined apoptosis by TUNEL assay (Fig. 4C) and found that the increase in bromoacetylthiamine cytotoxicity in *THTR2*-transfected cells correlated with an increased percentage of TUNEL-positive cells after exposure to the drug. These studies further demonstrate the presence of increased thiamine uptake activity in the *THTR2*-transfected MTX^R ZR75 cells and the feasibility of targeting thiamine transport and thiamine-dependent pathways for cancer therapy.

Thiamine Transport and Resistance to Chemotherapy

We investigated the possibility that down-regulation of thiamine transport in breast cancer might play a role in oncogenesis through increased resistance to apoptosis. First, we examined cytotoxicity of three standard agents used in breast cancer treatment, paclitaxel, methotrexate, and doxorubicin, in *THTR2*-transfected cell lines. As can be seen in Fig. 5, there was no difference among the cell lines in sensitivity to methotrexate or paclitaxel, but there was a 3-fold increase in sensitivity of the thiamine-transfected cell

lines to doxorubicin (Fig. 6A). Because there is no obvious interaction between doxorubicin and thiamine transport, we examined whether the increased sensitivity to doxorubicin in thiamine-transfected cells was associated with an increase in apoptosis after doxorubicin exposure. As shown in Fig. 6B, there was an increase in the number of cells undergoing apoptosis among cells transfected with *THTR2* compared with controls at increasing concentrations of doxorubicin, suggesting that increased sensitivity to doxorubicin was associated with increased sensitivity to doxorubicin-induced apoptosis.

Increased *THTR2* Expression and Radiation Sensitivity

We also examined whether *THTR2* gene expression altered cellular sensitivity to radiation and to radiation-induced apoptosis. As shown in Fig. 7A, *THTR2*-transfected cells were more sensitive to ionizing irradiation than control cells. For the control cells, the SF₂ was 0.35 and the D₀ was 128.8 cGy. For the *THTR2*-transfected cells, the SF₂ was 0.12 and the D₀ was 81 cGy. Once again, this increase in sensitivity was associated with an increased number of cells undergoing apoptosis (Fig. 7B), suggesting that increased *THTR2* expression increased the propensity of cells to undergo programmed cell death under certain conditions.

Western Blot Analysis

To begin to determine apoptosis pathways affected by *THTR2* activity, we analyzed protein expression in *THTR2*-transfected cells and control cells (transfected with the empty pREP4 expression vector alone) after exposure to doxorubicin (Fig. 8A) and radiation (Fig. 8B). The results shown are representative of two independent experiments. Both vector- and *THTR2*-transfected cells showed induction of p53 protein

at 6 h of doxorubicin treatment. No changes were observed in bcl-2 kinetics before and after treatment in both transfected cells. Interestingly, a significant decrease in the inactive form of caspase-3 was observed in *THTR2*-transfected cells but not in the vector-transfected cells in response to both doxorubicin and radiation, suggesting that there is an induction of cell death programs at the molecular level in *THTR2*-transfected cells but not in vector-transfected cells in response to these cytotoxic stresses.

Discussion

These studies demonstrate that *THTR2* mRNA levels are down-regulated in breast cancer and suggest that this down-regulation may be associated with the development of increased resistance to apoptosis in these tumors. Furthermore, the increase in sensitivity of *THTR2*-transfected cells to doxorubicin and ionizing radiation, but not to paclitaxel or methotrexate, also suggests an interaction between *THTR2* and the mechanisms of apoptosis. It should be noted, however, that many mechanisms in addition to dysregulation of apoptosis may result in resistance to radiation and doxorubicin, and changes in thiamine metabolism may affect multiple intracellular pathways.

The association of certain vitamin deficiencies and cancer is well known, and usually associated with environmental factors under conditions that affect all tissues. In particular, one study has drawn association between thiamine status and breast cancer (11). In addition, cell- or tissue-specific vitamin deficiencies have been associated with increased cancer risk. The pathogenesis of acute promyelocytic leukemia involves the characteristic translocation and super-repression of the retinoic acid α receptor, making these cells functionally vitamin A-deficient and unable to undergo terminal differentiation (12). In

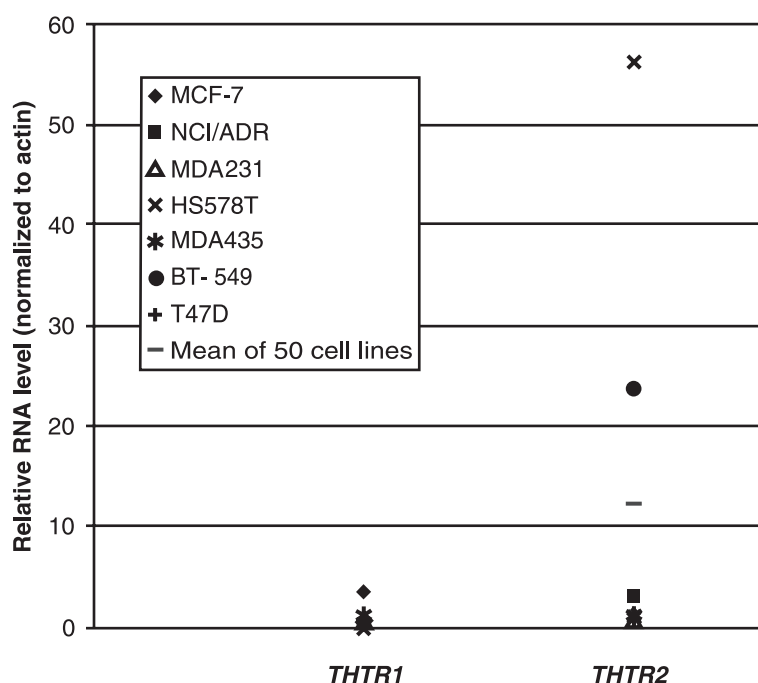


FIGURE 2. Scattergram illustrating relative *THTR1* and *THTR2* RNA levels in breast cancer cell lines compared to the mean levels in 50 cell lines. *THTR2* RNA levels in two breast cancer cell lines were 2- and 4-fold greater than the mean, whereas the other cell lines were all at least 4-fold lower than the mean.

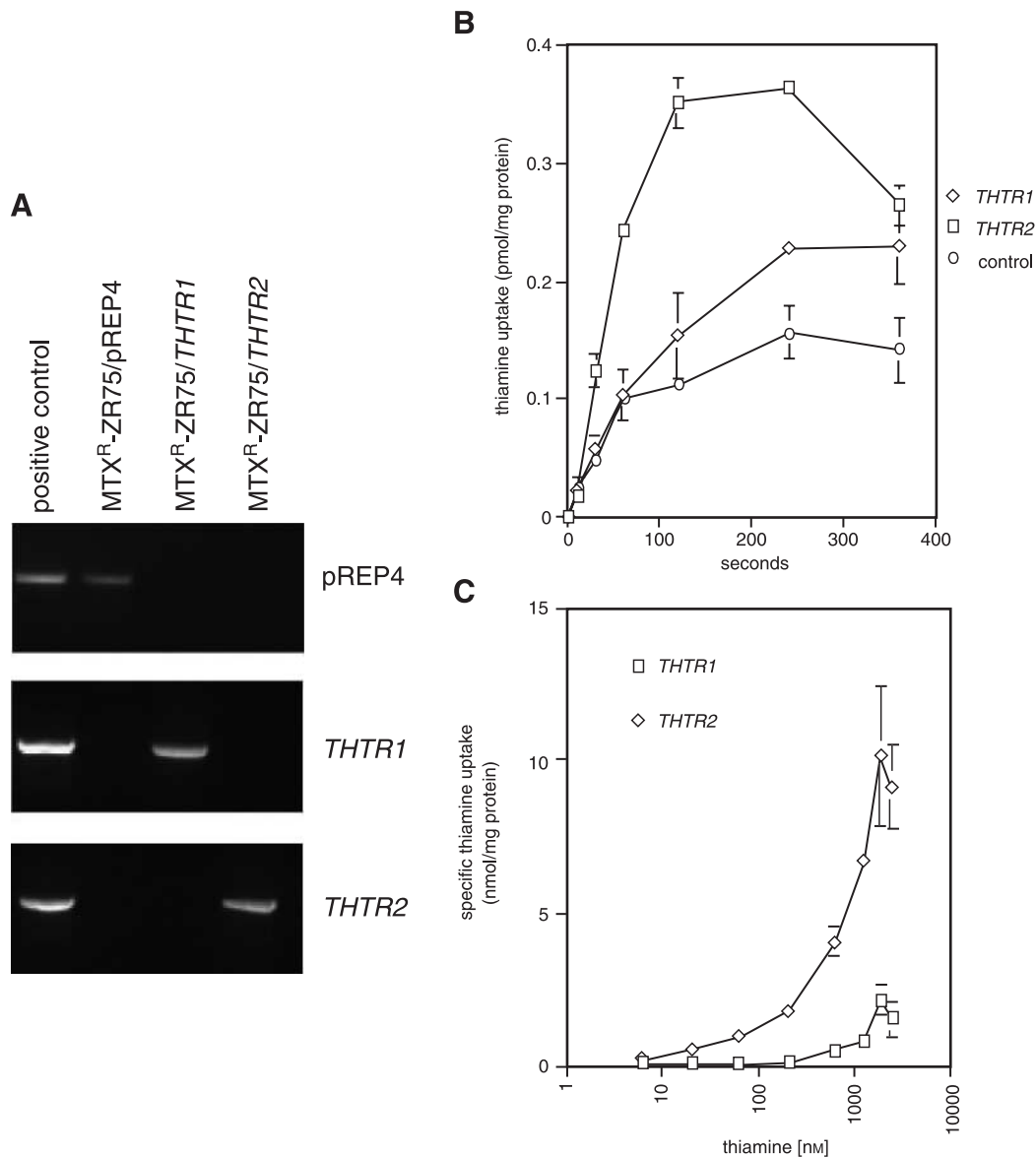


FIGURE 3. **A.** PCR reactions demonstrating presence of *THTR1* and *THTR2* transgenes in MTX^R ZR75 cells transfected with the thiamine transporter expression vectors. **B.** Thiamine uptake in *THTR1*- and *THTR2*-transfected MTX^R ZR75 cells. Cells were exposed to transport medium containing 20 nmol thiamine for the times indicated. **C.** Specific thiamine uptake attributable to the transfected thiamine transporter gene in the transfected cell lines.

the case of folic acid, epidemiologic studies have established that the high-functioning allelic variant of the methylenetetrahydrofolate reductase (MTHFR) gene, which shunts intracellular folate away from DNA synthesis pathways, is associated with an increased risk of acute leukemia, breast cancer, and colon cancer (13, 14).

Severe thiamine deficiencies result in tissue damage and an increase in apoptosis (15, 16), and *THTR1* mutations in cells lacking other saturable route of thiamine uptake also result in severe thiamine deficiency and apoptotic cell death (16). However, the natural protective mechanisms of cells to the stress of mild thiamine deficiency, by decreasing the rate of cell turnover (17), may also set the stage for further cellular changes in a multi-step model of oncogenesis.

The analysis of the cytotoxic effects of a bromoacetyl ester analogue of thiamine demonstrates that increased expression of the thiamine transporter can make cells more sensitive to thiamine analogue compounds. Presumably, this compound acts as an alkylating agent, although it may also be an inhibitor of thiamine-dependent pathways. Because thiamine utilization is increased in tumor cells (18) and, like folate, thiamine supplementation increases tumor cell proliferation (19, 20), anti-thiamine drugs may prove to be useful in cancer therapy. However, the clinical utility of thiamine analogue compounds remains to be determined.

Perhaps most surprising is the effect of *THTR2* expression on toxicity of ionizing radiation. Ruth and Roninson (21) reported that the *MDR1* gene, which belongs to a different class

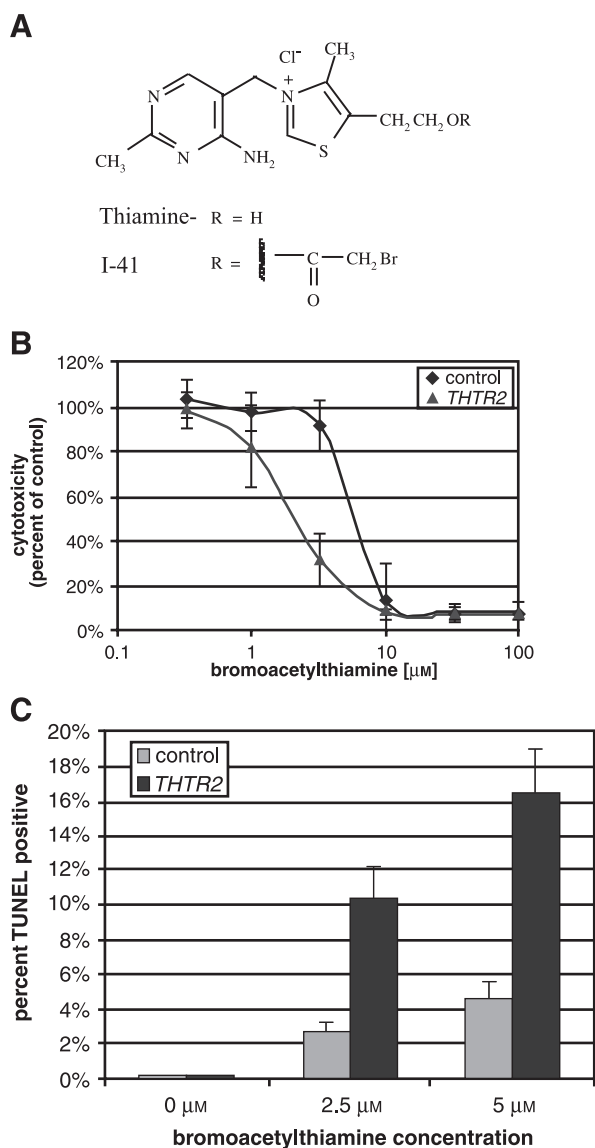


FIGURE 4. **A.** Structure of compound I-41, a bromoacetyl ester derivative of thiamine as described in "Materials and Methods." **B.** Cytotoxicity assay of I-41 in MTX^R ZR75 cells transfected with either SLC19A3 (*THTR2*) or empty vector (pREP4) control. The graph shows the results \pm range of three independent experiments performed in triplicate. **C.** TUNEL assay of *THTR2*-transfected cells versus control cells at different concentrations of bromoacetylthiamine, demonstrating increased apoptosis associated with increased cytotoxicity. The results are the average \pm range of two independent determinations. At 2.5 μ M, $P = 0.04$, and at 5 μ M, $P = 0.03$.

of membrane transporters, inhibited radiation-induced apoptosis, but did not change the overall sensitivity of cells to radiation. Johnstone *et al.* (22) also demonstrated increased resistance of cells expressing *MDR1* to UV irradiation, and further demonstrated a more general resistance to caspase-dependent mechanisms of cell death. Our studies with *THTR2* demonstrate the opposite effect, in which increased expression of a transporter gene results in increased sensitivity to radiation. Western blot studies suggest that *THTR2* expression influences

the activity of the caspase-3-dependent pathway in response to cytotoxic stresses of radiation and doxorubicin exposure. Future studies will define how *THTR2* interacts with caspase-dependent pathways and whether its effect on cellular resistance results as well from other determinants of cellular response to cytotoxic stress.

In addition, it is unclear whether this biological activity is caused by the transporter itself or its substrates. Once inside the cell, thiamine quickly becomes phosphorylated to thiamine PP_i, a key cofactor in several critical cellular pathways, including the key respiratory reactions involving the oxidation of pyruvate to acetyl CoA and α -ketoglutarate to succinyl CoA, both of which reduce NAD to NADH₂, along with the synthesis of glyceraldehyde-3-phosphate in the pentose phosphate pathway and generation of NADPH (23). Thus, a potential role for thiamine as a radiation sensitizer is not easily seen, as it serves to enhance the reducing environment in cells, which should in turn enhance rather than inhibit recovery from oxidative stress (24, 25). However, studies of vitamin deprivation in the mouse hybridoma cell line TB/C3 revealed that thiamine deprivation improved cell viability and reduced apoptosis, suggesting that thiamine deprivation may induce a cellular stress response that may be protective (17). Further studies will determine the role of thiamine and thiamine antagonists, such as oxythiamine, on cellular sensitivity to doxorubicin and ionizing radiation.

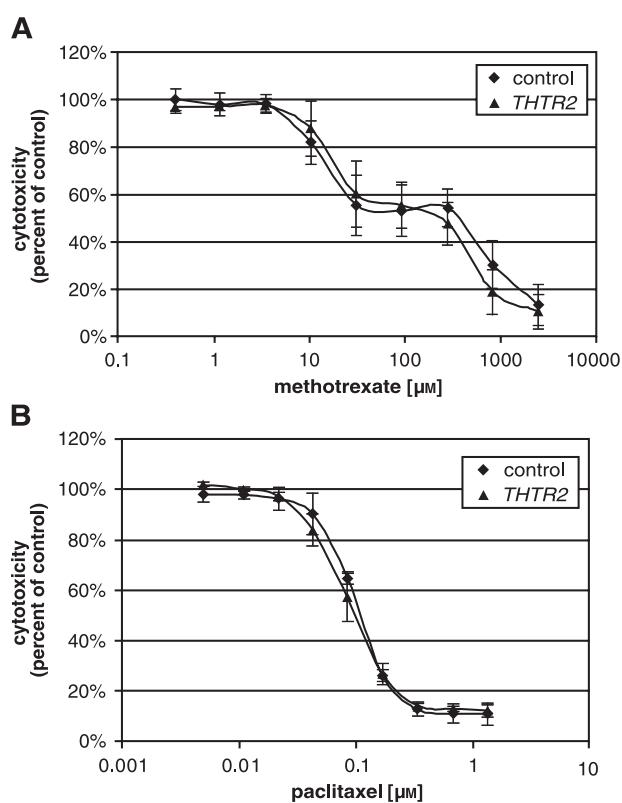


FIGURE 5. Cytotoxicity assays of *THTR2*-transfected MTX^R ZR75 cells and control cells against methotrexate (**A**) and paclitaxel (**B**).

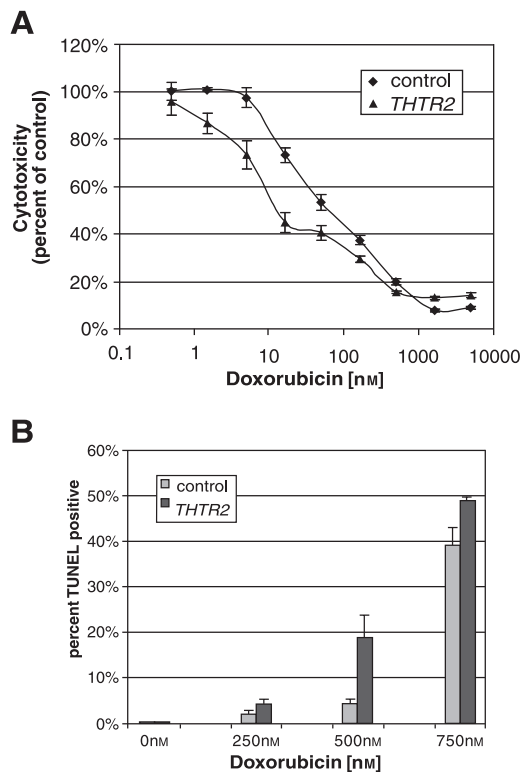


FIGURE 6. **A.** Cytotoxicity assays of *THTR2*-transfected MTX^R ZR75 cells and control cells against doxorubicin. **B.** TUNEL assay of the same cells exposed to increasing concentrations of doxorubicin. At 250 nM, control versus *THTR2*, $n = 3$, $P < 0.01$; at 500 nM, $n = 3$, $P = 0.01$; at 750 nM, $n = 3$, $P = 0.02$.

Materials and Methods

Human Tissue cDNA Array

A filter array containing normalized cDNA from 50 breast cancers and corresponding normal tissues (Cancer Profiling Array, Clontech, Palo Alto, CA) was probed in succession with ³²P-labeled cDNA probes for *THTR1*, *THTR2*, and ubiquitin (control) according to the methods recommended by the manufacturer. The cDNA samples were generated using SMART (Switch Mechanism At the 5' end of RNA Transcript) technology and represent the entire mRNA population from its original sample according to the manufacturer (www.bdbiosciences.com). Of the 50 breast cancer samples, 33 carried the diagnosis of infiltrating ductal carcinoma, 10 were diagnosed as lobular carcinoma, and the remaining 7 breast tumors were assigned miscellaneous diagnoses. The precise histological description of each sample can be downloaded from the manufacturer's website, www.clontech.com/techinfo/manuals/index.shtml#C. *THTR1* and *THTR2* cDNA probes were amplified by PCR using pREP4-*THTR1* and pREP4-*THTR2* plasmid DNA as a template, respectively. The PCR was performed with a 30-ng template DNA in a final volume of 50 μ l and 0.3 μ M of each specific primer. (For *THTR1*: THTR1-F 5'-CTTCTTTACCACATTCCTTCTAC-3' and THTR1-B 5'-GCTTTTACATTTCTTCATAACAC-3'. For *THTR2*: THTR2-F 5'-AGGGTCGGTGCTGGCTCAACTCTT-3' and THTR2-B 5'-ATAGCCAGCATAGCACGCCAGAT-3'.) The

reaction was carried out with 0.5 μ l of Taq DNA polymerase (Gibco, Rockville, MD) in a thermal cycler programmed as follows: initial step for denaturation of DNA fragments, 5 min at 94°C; 40 cycles, 30 s at 94°C for denaturation; 30 s at 62°C for annealing; 30 s at 72°C for polymerization, followed by the final elongation step, 5 min at 72°C. The PCR products were purified from agarose gel using QIAquick gel extraction kit (Qiagen, Chatsworth, CA) and labeled with [³²P]dCTP using the Random Primer DNA Labeling System (Promega, Madison, WI). Image acquisition and densitometry were performed using a phosphorimager.

Expression of *THTR1* and *THTR2* in Breast Cancer Cell Lines

Quantitative RT-PCR for measuring RNA levels was performed by using a Roche LightCycler, which uses real-time fluorescence detection for quantitative measurement of PCR products. Total RNA isolated from 50 human cancer cell lines from the NCI Drug Screen panel of cell lines was provided by the Developmental Therapeutics Program, NCI, as we have previously described (26) and includes six leukemia, five lung, seven colon, five central nervous system, six ovarian, six renal, two prostate, and seven breast cancer cell lines. All primers

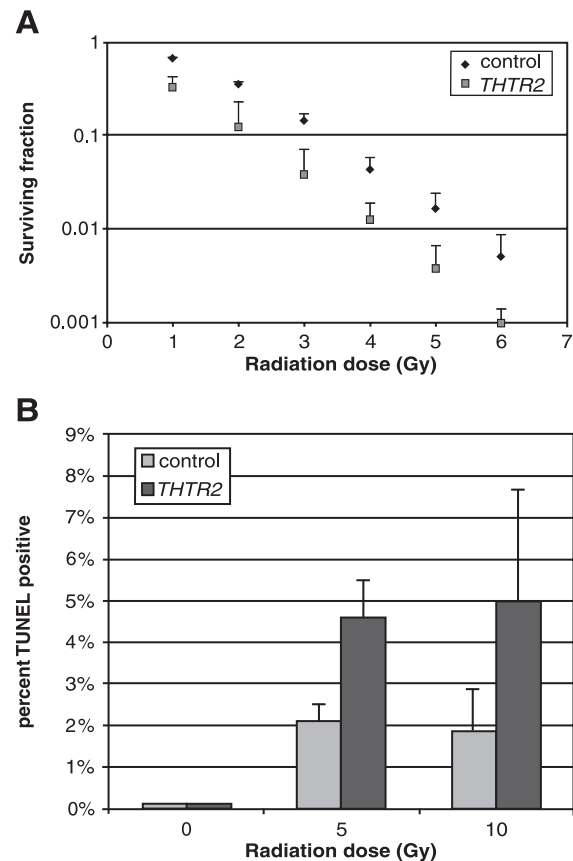


FIGURE 7. **A.** Colony forming assay of cytotoxicity of ionizing radiation in *THTR2*-transfected MTX^R ZR75 cells and control cells. **B.** TUNEL assay of radiation-induced apoptosis in *THTR2*-transfected MTX^R ZR75 cells. At 5 Gy, control versus *THTR2*, $n = 3$, $P = 0.02$; at 10 Gy, $n = 5$, $P = 0.04$.

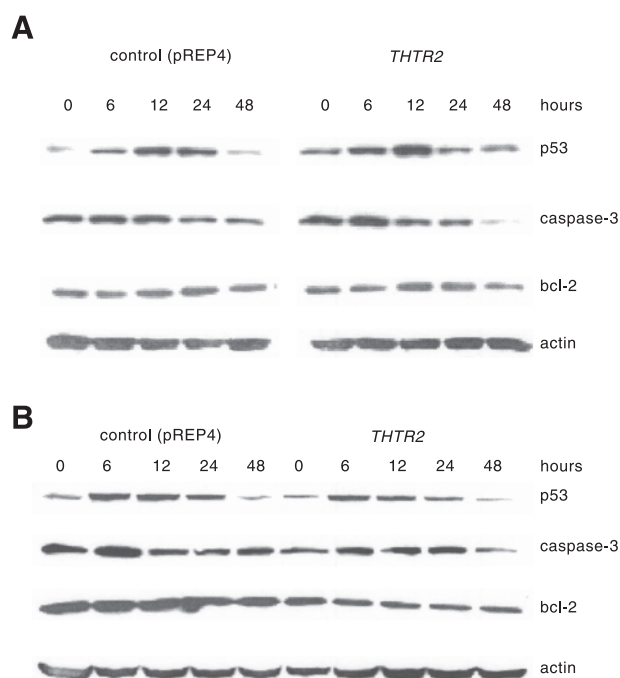


FIGURE 8. Protein kinetics of p53, bcl-2, and caspase-3 analyzed by Western blot analysis of breast cancer cells that were transfected with vector alone (*control*) or *THTR2* cDNA treated with doxorubicin (200 nM) (**A**) and radiation (10 Gy) (**B**). The lysates from untreated and doxorubicin-treated cells were isolated at indicated time points. Whole cell protein extracts were subjected to Western blot analysis using anti-p53, anti-caspase-3, and anti-BCL-2 antibodies. Anti- β -actin antibody was used as an internal loading control.

were designed with Oligo 4.0 software and purchased from Integrated DNA Technologies, Inc. (Coralville, IA). First, cDNA was synthesized from 2 μ g of total RNA extracted from cell lines using SuperScript First-Strand Synthesis System (Life Technologies, Inc., Gaithersburg, MD) in a 20- μ l volume following the instructions supplied by the manufacturer. The cDNA was treated with RNase H for 20 min at 37°C and two reactions for each cell line were mixed together, divided into small aliquots, and stored at -20°C. Then, 2 μ l of cDNA reaction were amplified in a standard PCR reaction condition, using 0.3 μ M primer concentration, with the addition of SYBR Green I Dye. After 30 s denaturation at 95°C, the amplification reaction proceeded through 45–50 cycles of 95°C denaturation for 0 s, 62–65°C annealing for 10 s, and a 72°C extension for 40 s, with slopes of 20°C/s, 20°C/s, and 2°C/s, respectively. Quantification was performed using the LightCycler analysis software. The quantitative measurement of each gene in each cell line was normalized to the relative amount of actin RNA in each cell line, as a control for equivalent cDNA loading in each sample.

Cloning and Expressing *THTR1* and *THTR2*

The characteristics of *THTR1* and *THTR2* were defined in the background of MTX^R ZR75 cells, because these cells have no detectable specific RFC activity (27, 28) which can transport thiamine monophosphate and thiamine PP_i (9, 10). First, the

THTR1 cDNA was cloned by RT-PCR using skeletal muscle RNA as a template, with primers which span the putative open reading frame: *THTR1*-U1 5'-CAGTTGGCGGAGGAGGA-GAAGGAAG-3' and at the 3' end *THTR1*-L2 5'-AAGGTAT-TAGTCAAGTGGCTGCTGT-3'. For *THTR2*, the cDNA was cloned by RT-PCR using placenta RNA as a template, with the primers which span the putative open reading frame. The primers used were: *THTR2*-U1 5'-GGG GTA CCT AGT GAG CGA TTT GGT GAA CAG AC-3' and *THTR2*-L2 5'-CCG CTC GAG TAT GCC ACC CAT CTC AAA ATC TTT-3'. Unique restriction enzyme sites for directional cloning into the multiple cloning site of the expression vector pREP4 were added to the 5' ends of these primers. MTX^R ZR75 cells transfected with pREP4/*THTR1* and pREP4/*THTR2* and pREP4 alone were selected in hygromycin. The presence of the transgene in the surviving clones was determined by PCR. *THTR1* and *THTR2* cDNA were isolated from transfected cells using QIAprep spin plasmid kit (Qiagen). The PCR was performed with a 30-ng template DNA in a final volume of 50 μ l and 0.3 μ M of each specific primer. Each PCR reaction contained a 5' vector-specific primer. For pREP4 reaction: pREP4A 5'-TTAGGAAGGCAACAGACAGG-3' and pREP4B 5'-TAAAAAACCTCCCCACCTCC-3'. For *THTR1* vector: pREP4F 5'-GGAGGGGGAAATGTAGTCTTATGC-3' and *THTR1*-B2 5'-CAGGTAGAAGGAATGTGGTG-3'. For *THTR2* vector: pREP4F 5'-GGAGGGGGAAATGTAGTCT-TATGC-3' and *THTR2*-B2 5'-CTGATGTGGGTTTCTGC-TCT-3'. The reaction was carried out with 0.5 μ l of Taq DNA polymerase (Gibco) in a thermal cycler programmed as follows: initial step for denaturation of DNA fragments, 5 min at 94°C; 35 cycles, 30 s at 94°C for denaturation; 45 s at 64°C for annealing; 45 s at 72°C for polymerization, followed by the final elongation step, 5 min at 72°C.

Cell Culture

MTX^R ZR75 cells transfected with *THTR1*, *THTR2*, or the empty pREP4 plasmid were maintained and grown at 37°C, 5% CO₂ in Improved MEM Zinc Option (IMEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 250 μ g/ml hygromycin B.

Kinetic Studies

Thiamine uptake studies were performed in a manner similar to previous studies of MTX uptake (29). ³[H]-labeled thiamine was obtained from American Radiolabeled Chemicals (St. Louis, MO) and its purity was assayed by the manufacturer. For drug uptake studies, cells were plated at a density of 1 \times 10⁵ in six-well Linbro dishes in medium containing 10% FBS. After 48–72 h of growth, cells were washed three times in transport medium (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, and 25 mM HEPES, pH 7.4) without ³[H]-labeled thiamine and then exposed to 20 nM ³[H]-labeled thiamine in transport medium. At specified intervals, the transport medium was aspirated and the plates immersed in three successive washes of ice-cold 1 \times Dulbecco's PBS containing Mg²⁺ and Ca²⁺ (D-PBS). The cells were then solubilized by overnight incubation in 0.2 N NaOH, neutralized with 0.2 N HCl, and the radioactivity was determined by liquid

scintillation counting as previously described (30). Protein concentrations were determined by Bradford assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA) using a spectrophotometer (Beckman, Fullerton, CA). Non-specific binding of drug was determined by exposure of cells to transport medium for less than 5 s, and was subtracted from measured values to indicate specific uptake.

Cytotoxicity Assays

A chemically reactive bromoacetyl ester derivative of thiamine was prepared by the straightforward reaction of acyl chlorides of the organic acids with thiamine. For cytotoxicity studies, cells transfected with the expression vector containing the *THTR2* cDNA were plated in triplicate in 96-well microtiter plates in medium containing 5% FBS at densities of ~1000 cells/well. The control cell line was transfected with an empty expression vector. After 24 h, serial dilutions of drugs were added to the cells. After 6 days, the cells were fixed in 10% tricarboxylic acid, rinsed with water, and dried. The cells were then be stained with sulforhodamine in 1% acetic acid, washed in 1% acetic acid, and dried again as previously described (29, 30). The stained cells were solubilized in 1 M Tris base (pH 10.5) and the absorbance at 570 nm determined on a microplate reader. The IC_{50} value was calculated from the dose-response curve as the concentration of drug that produced a 50% decrease in the mean absorbance compared to the untreated wells and reported as the average of at least three independent determinations performed in triplicate.

Ionizing Radiation

All experiments were irradiated using a 100-kV industrial X-ray machine (Phillips, Netherlands) at room temperature. The dose rate with a 2 mm Al plus 1 mm Be filter was ~2.64 Gy/min at a focus-surface distance of 10 cm. For colony forming assays, cells were seeded in T25 flasks at two different concentrations in quadruplet sets for each radiation dose. Cells were left untreated or exposed to 1–6 Gy dose of radiation. After incubation for 15–20 days, colonies of at least 50 cells were scored as surviving cells. The results were plotted using X-Y log scatter (Delta Graph 4.0) and the formula of the single hit multi-target model was used to calculate D_0 (the dose required to reduce the fraction of cells to 37% indicative of single-event killing) and SF_2 (the survival fraction of exponentially growing cells at the clinically relevant dose of 2 Gy) as described by Chendil *et al.* (31).

Quantification of Apoptosis

Apoptotic cells were quantified by the terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) assay using the ApopTag peroxidase *in situ* apoptosis detection kit (Intergen Company, Purchase, NY). In this assay, the fragmented DNA in individual cells was end-labeled using digoxigenin-labeled nucleotides at strand breaks with terminal deoxynucleotidyl transferase. Procedures were followed according to the instruction of the kit. Briefly, cells were plated in chamber slides and followed by treatment with radiation or doxorubicin (Sigma, St. Louis, MO). After incubation for 48 h at 37°C, the cells were fixed in 1% paraformaldehyde in PBS,

pH 7.4. The DNA was tailed with digoxigenin-dNTP and conjugated with an anti-digoxigenin peroxidase. The cells were stained with peroxidase and methyl green. For quantitation, ~1000 cells were counted using light microscopy in each experiment, and four experiments were performed. Statistical difference between the means was analyzed with a Student *t* test.

Western Blot Analysis

Cells were treated with 200 nM doxorubicin or 10 Gy radiation, and harvested 0, 6, 12, 24, and 48 h after treatment. Cell pellets were resuspended in M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). Lysed cells were centrifuged to remove cellular debris. Protein (30 µg) from each cellular lysate was size fractionated on 10% or 12.5% precast SDS-polyacrylamide gels (Bio-Rad) and electroblotted onto nitrocellulose membranes. The membrane was blocked with 5% milk in Tris-buffered saline [10 mM Tris (pH 8.0), 0.01% Tween 20] and probed with specific primary antibodies. Antibodies against bcl2, p53, and β-actin were purchased from Sigma, and antibody against caspase-3 was purchased from BD Biosciences (San Diego, CA). The membrane was washed with Tris-buffered saline and then incubated with anti-mouse IgG peroxidase conjugate (Sigma), washed with Tris-buffered saline again, and developed using a chemiluminescence detection kit according to the manufacturer's instructions (ECL-plus kit, Amersham-Pharmacia, Arlington Heights, IL). Each experiment was performed at least twice.

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