Biochemical Pharmacology, Vol. 51, pp. 1349–1355, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00057-3

Resistance to Tomudex (ZD1694): Multifactorial in Human Breast and Colon Carcinoma Cell Lines

James C. Drake, Carmen J. Allegra,**
Richard G. Moran† and Patrick G. Johnston‡*

*NCI-NAVY Medical Oncology Branch, National Cancer Institute, Bethesda, MD 20889; and †Massey Cancer Center, Medical College of Virginia, Richmond, VA, U.S.A.

ABSTRACT. ZD1694 (Tomudex; TDX) is a quinazoline antifolate that, when polyglutamated, is a potent inhibitor of thymidylate synthase (TS), the enzyme that converts dUMP to dTMP. Continuous exposure of MCF-7 breast and NCI H630 colon cells to TDX, with stepwise increases in TDX up to 2.0 μM, resulted in stably resistant cell lines (MCF_{TDX} and $H630_{TDX}$) that were highly resistant to TDX. Initial studies revealed 34-fold increase in TS protein levels in MCF_{TDX} and a 52-fold increase in TS levels in $H630_{TDX}$ cell lines. Despite continued exposure of these cells to 2.0 μM TDX, TS protein and TS mRNA expression decreased to parental levels in $H630_{TDX}$ cells, whereas in MCF_{TDX} cells TS mRNA expression and TS protein levels remained elevated. Southern blot analysis revealed a 20-fold TS gene amplification in the MCF_{TDX} cell line. TDX uptake was 2-fold higher in resistant MCF_{TDX} cells than in parental MCF-7 cells, whereas in $H630_{TDX}$ cells TDX uptake was 50-fold less than that observed in parental H630 cells. In contrast, no change in the transport of either leucovorin or methotrexate into $H630_{TDX}$ cells was noted when compared with the H630 parental cells. In $H630_{TDX}$ cells, folylpolyglutamate synthetase (FPGS) activity was 48-fold less compared to parent H630 cells; however, FPGS mRNA expression was similar in both lines. $H630_{TDX}$ cells were also highly resistant to ZD9331, a novel quinazoline TS inhibitor that does not require polyglutamation, suggesting that defective transport by the reduced folate carrier was also an important mechanism of resistance in these cells. In MCF_{TDX} and $H630_{TDX}$ resistant cells, several mechanisms of resistance are apparent: one increased TS expression; the others evolved over time from increased TS expression to decreased FPGS levels and decreased TDX transport. BIOCHEM PHARMACOL 51;10:1349–1355, 1996.

KEY WORDS. resistance; Tomudex; thymidylate synthase; folylpolyglutamate synthetase; colon cancer; breast cancer

TS§ (EC 2.1.1.45) catalyzes the methylation of dUMP to dTMP an essential step in DNA biosynthesis [1, 2]. TS is also a critical target for the fluoropyrimidines, an important group of antineoplastic drugs that are widely used in the treatment of gastrointestinal tumors, breast tumors, and epithelial tumors of the upper aerodigestive tract [1, 2].

Due to the clinical relevance of inhibiting TS, a new generation of quinazoline antifolate inhibitors of TS has been developed. CB 3717 was the first of these inhibitors to be tested in patients; however, due to its limited solubility in water, this drug was found to produce severe and unpredictable nephrotoxicity, which ultimately resulted in a decision to discontinue its further development [3]. ZD1694 (TDX, Tomudex) is a more water-soluble analog of CB 3717 designed to circumvent unpredictable nephrotoxicity [4]. TDX structurally resembles the physiologic folates and, hence, requires an active transmembrane transport system to gain intracellular access. The antifolate also undergoes metabolism to the polyglutamated forms, which are approximately 100-fold more potent as inhibitors of TS when compared with the unmetabolized monoglutamated compound [4].

TDX has demonstrated activity in a variety of preclinical animal models and has been administered safely to patients in a series of phase I and II trials [5–7]. Two phase I trials using a 15-min infusion given every 21 days have been completed [5, 6]. In 1992, a large phase II international study was begun with the objective of defining the response rate to TDX in various malignancies [7]. Preliminary data from these studies suggest that TDX is an active drug in

‡ Corresponding author: Patrick G. Johnston, M.D., Ph.D., NCI-Navy Medical Oncology Branch, Naval Hospital Bethesda, 8901 Wisconsin Ave., Bldg. 8, Rm. 5101, Bethesda, MD 20889-5105. Tel. (301) 496-0901; FAX (301) 496-0047.

§*Abbreviations:* TS, thymidylate synthase; TDX, Tomudex; FdUMP, fluorodeoxyuridine monophosphate; FPGS, folylpolyglutamate synthetase; SSC, 0.15 M sodium chloride + 0.015 M sodium citrate; LCV, leucovorin; MTX, methotrexate; DDATHF, 5,10-dideazatetrahydrofolic acid; and HSR, homogeneously staining region.

Received 23 August 1995; accepted 12 December 1995.

several solid tumors including colorectal, breast, ovarian, and non-small cell lung cancer. A major limitation to the use of chemotherapeutic agents has been the development of clinical resistance. Since these initial clinical studies suggest that TDX is an active agent in the treatment of human colon and breast cancer, we wished to determine the potential mechanisms of resistance to TDX in human breast and colon cell lines.

MATERIALS AND METHODS

Cell Culture

The characteristics of the human colon cancer NCI H630 cell line and the human breast cancer MCF-7 cell line have been described previously [8, 9]. All cells were maintained in RPMI-1640 (Biofluids, Inc., Rockville, MD) with 10% dialyzed fetal bovine serum (Gibco, Inc., Grand Island, NY) plus 2 mM glutamine and were grown in 75 cm^2 plastic culture flasks (Falcon Labware, Oxnard, CA). The resistant colon sublines NCI $H630_{TDX}$ and MCF_{TDX} were selected for resistance to TDX *in vitro* by continuous stepwise increases in TDX exposures beginning with 1 nM and increased every 4 weeks over a 3-month-period. Both cell lines were eventually maintained in 2 μM TDX.

TDX Growth Inhibition Studies

An equal number of cells (5×10^4 cells/mL) from each cell line were plated onto 25 cm^2 flasks (Falcon Labware) and incubated at 37° in RPMI-1640 with 10% dialyzed fetal bovine serum. After 24 hr, various concentrations of TDX were added to each flask. PBS was added to control flasks. The cells were incubated with TDX for 72 hr at 37° and subsequently trypsinized and counted using a Coulter Counter (Coulter Electronics, Hialeah, FL). The TDX IC_{50} values were determined for each cell line using a curve of cell number versus log of drug concentration.

Western Blot Analysis

An equal amount of protein (200 μg) from each cell line was resolved by polyacrylamide gel electrophoresis using 12.5% acrylamide, according to the method of Laemmli [10]. The gels were transferred onto a nitrocellulose membrane in transfer buffer (48 mM Tris, 39 mM glycine, 0.5M EDTA in 20% methanol) for 2 hr. The nitrocellulose blots were incubated at room temperature with blocking solution (Blotto: 5% nonfat milk, 10 mM Tris, 0.01% Thimerosal) for 45 min. After washing with PBS-Tween (PBS with 0.1% Tween 20), primary antibody (TS106, ascitic fluid, 1:100 in Blotto) was applied for 90 min. After four washes with PBS-Tween and two washes with Blotto, secondary antibody (goat anti-mouse horseradish peroxidase, Bio-Rad, 1:2000 in Blotto) was applied for 1 hr. After another four washes with PBS-Tween, the chemiluminescent substrate (luminol, plus enhancer, according to the ECL method of Amersham) was applied for 1 min. Blots were then air-dried, covered by a plastic wrap, and exposed to film (Kodak, X-OmatAR) for 5 min. Densitometry scanning of the film was performed using a Hewlett Packard Scan Jet Plus and analyzed using an image analysis software program (NIH IMAGE v.1.38; provided by Wayne Rasband, NIMH, NIH).

TS Binding Assay

The assay to determine TS levels was performed in a total volume of 200 μL containing 50 μL of cell lysate, 75 μM $CH_2N_4PteGlu$, 3 pmol [6-^{3}H]FdUMP, 100 μM 2-mercaptoethanol and 50 μM KH_2PO_4, pH 7.4. Samples were incubated at 37° for 30 min, and subsequently 1 mL of an albumin-coated charcoal slurry (pH 7.2; prepared by mixing 10 g of acid-washed activated charcoal with 2.5 g of bovine albumin, 0.25 g of dextran and 100 mL of ice-cold water) was added. The mixture was vortexed, allowed to stand at room temperature for 10 min, and then centrifuged for 30 min at 3000 *g*. The residual radioactivity representing enzyme bound FdUMP in the supernatant was counted by liquid scintography [11].

TS Catalytic Activity

Cell lysates were prepared as described above. The assay was performed in a total volume of 200 μL containing 50 μL of cell lysate, 10^{-5} M [5-^{3}H]dUMP, 100 μM 2-mercaptoethanol, 75 μM $CH_2H_4PteGlu$, and 50 μM KH_2PO_4, pH 7.4, using a modification of the tritium release assay [12]. Protein levels were determined by the method of Bradford [13].

FPGS Activity Assays

Cells were removed from 150-mm dishes, washed with PBS, and sonicated in HEPES buffer containing several protease inhibitors. Desalted high-speed supernatants were incubated with [^{3}H] glutamic acid and 6S-tetrahydrofolate for varying times. The [^{3}H]tetrahydrofolate diglutamate formed was trapped as a ternary complex with bacterial thymidylate synthase and fluorodeoxyuridylate in formaldehyde containing buffer and assayed as previously described [14].

Northern Blot Analysis

Total RNA was prepared from mid-log cultures by a minor modification of the method of Chomczynski and Sacchi [15]. Aliquots (20 μg/lane) were applied to a 1% agarose gel following denaturation in glyoxal/dimethyl sulfoxide, transferred to a Biotrans nylon membrane by capillary action in 20× SSC, and cross-linked by UV irradiation. The blots were then probed for human TS and FPGS with a ^{32}P-radiolabeled cDNA insert probe for human TS and FPGS, respectively. Blots were washed to a stringency of 2× SSC at 37° and exposed to film for 6–16 hr. Each of the northern blots were stripped and reprobed for β-actin. Densitometry

scanning of the blots was performed as described for western blot analysis.

Southern Analysis

Cellular DNA from H630 and MCF-7 cells was isolated as previously described [16]. Southern analysis was performed by digestion of 20 μg of genomic DNA with *Hind* III and restriction endonuclease fractionation of the DNA fragments on a 0.5% agarose gel followed by transfer onto a nytran filter membrane. The membrane was hybridized to a ^{32}P-radiolabeled cDNA probe for human TS and processed as previously described for RNA. Densitometry scanning of Southern blots was performed as described for western blot analysis.

Uptake Studies

An equal number of cells (2.5×10^5 cells) from each cell line (MCF-7, MCF_{TDX}, H630, and $H630_{TDX}$) were plated onto 6-well plates (Falcon Labware) and incubated at 37° in RPMI-1640 with dialyzed fetal bovine serum. After 72 hr, the medium was removed, and cells were washed with PBS. Subsequently, cells were incubated in Minimum Essential Medium that did not contain folates or dialyzed fetal bovine serum. Radiolabeled drug was added at time point zero. At various time points thereafter (30 sec, 1 min, 10 min, 30 min, 1 hr, 2 hr, and 4 hr), the labeled medium was removed and cells were washed with ice-cold PBS. Cells were lysed subsequently in 1 mL of 1 N NaOH, and a 100-μL aliquot was counted by liquid scintography.

Cytogenetic Analysis

Cytogenetic metaphase spread analysis was performed by H&W Cytogenetic Services (Lovetsville, VA). Chromosome spreads were prepared and G-banded according to the method of Modi *et al.* [17].

RESULTS

TDX-resistant NCI H630 colon and MCF-7 breast cancer cells were developed by exposing cells to stepwise increasing concentrations of TDX. After 4 months, $H630_{TDX}$ and MCF_{TDX} resistant cell lines were isolated and were maintained in TDX (2 μM). The TDX IC_{50} values were 50 and 30 μM in the $H630_{TDX}$ and MCF_{TDX} cells compared with 1 and 2 nM in the H630 and MCF-7 parental cell lines, respectively. The stability of the TDX-resistant phenotype remained unchanged in both cell lines after they had been out of TDX for at least 30 passages (6 months).

Activity of TS

We next examined the intracellular TS protein level in both TDX-resistant and parent cell lines. Using both radioenzymatic FdUMP and dUMP assays, MCF_{TDX} cells had a 34-fold increase in total TS levels and a 45-fold increase in TS catalytic activity (Table 1). Similarly, the $H630_{TDX}$ cell line had a 52-fold increase in total TS levels and a 49-fold increase in TS catalytic activity (Table 1). TS levels in cytosolic extracts, determined by western immunoblot, also were increased markedly in both the MCF_{TDX} (35-fold) and $H630_{TDX}$ (45-fold) cell lines (Fig. 1A). These initial studies suggested that increased TS expression was the major determinant of TDX resistance in these early passage (passage number $p < 10$) resistant MCF_{TDX} and $H630_{TDX}$ cells. After an initial 3 months in 2 μM TDX (passage number $p > 10$), continued exposure of NCI $H630_{TDX}$ cells to the antifolate resulted in a decrease in intracellular TS levels to parental levels (Table 1). The decrease in intracellular TS levels in these late passage NCI $H630_{TDX}$ cells was also noted on western immunoblot analysis (Fig. 1A). Despite the decrease in TS mRNA expression and TS protein to levels seen in the parental H630 cells, the TDX IC_{50} value remained unchanged in late passage $H630_{TDX}$ cells. In late passage MCF_{TDX} cells, no decrease in TS protein levels could be detected. Northern blot analysis of TS mRNA expression in late passage $H630_{TDX}$ cells revealed that TS mRNA expression was similar to that in parental H630 cells, whereas in MCF_{TDX} early and late passage cells TS mRNA was 30-fold higher than in parental MCF-7 cells (Fig. 1B). All northern blots were stripped and reprobed for β-actin mRNA expression, which was similar in all cell

TABLE 1. FdUMP binding and dUMP TS catalytic activity in early and late passage NCI H630, $H630_{TDX}$, MCF-7, and MCF_{TDX} cells

Cell line	TS FdUMP binding (pmol/mg protein)	TS dUMP catalytic activity (pmol/mg/min)
MCF-7	5.1 ± 0.7	8.3 ± 0.1
MCF_{TDX} (p* < 10)	174 ± 9.6	373 ± 15
MCF_{TDX} (p > 10)	181 ± 18	351 ± 15
H630	2.7 ± 0.5	3.8 ± 1.5
$H630_{TDX}$ (p < 10)	140 ± 19.5	187 ± 11.3
$H630_{TDX}$ (p > 10)	8.6 ± 1.5	3.0 ± 0.3

The total TS protein and the TS catalytic activity were analyzed as outlined in Materials and Methods. These results are the means ± SEM of at least 5 separate experiments.

* Passage number.

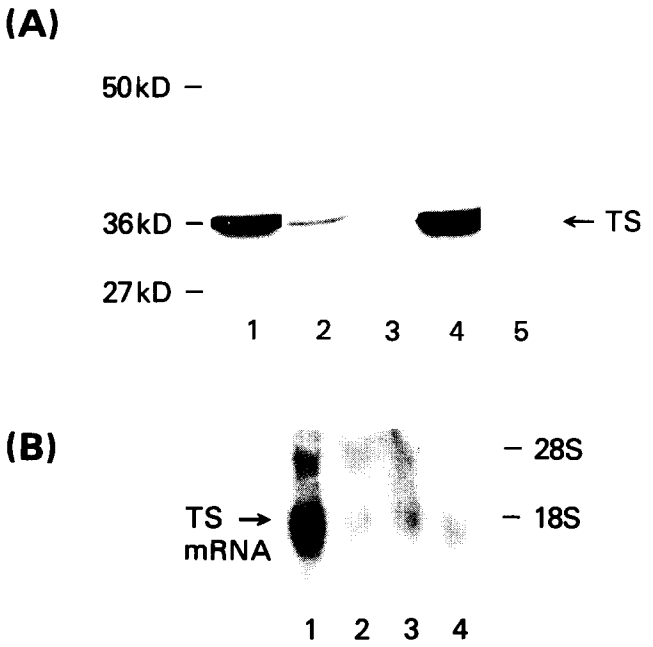

FIG. 1. (A) TS western immunoblot of NCI H630 and $H630_{TDX}$ early and late passage cells and MCF-7 and MCF_{TDX} cells. Cell lysates were used from (lane 1) $H630_{TDX}$ < p 10, (lane 2) $H630_{TDX}$ > p 10, (lane 3) H630 parent cells, (lane 4) MCF_{TDX} cells, and (lane 5) MCF parent cells. Western blotting was performed as outlined in Materials and Methods. (B) TS northern blot of NCI H630 and $H630_{TDX}$ late passage cells and MCF-7 and MCF_{TDX} cells. Key: TS mRNA expression in (lane 1) MCF_{TDX} cells, (lane 2) MCF parent cells, (lane 3) H630 parent cells, and (lane 4) $H630_{TDX}$ p > 10. Northern blotting was performed as outlined in Materials and Methods.

lines. To determine whether the increased TS expression was the result of amplified TS-specific sequences, a Southern blot hybridization analysis was performed. This analysis demonstrated a 20-fold increase in the intensity of the TS genomic band in the MCF_{TDX} cells compared to the parental MCF-7 cells, confirming TS gene amplification. No evidence of TS amplification was detected in the H630 cells.

Cytogenetic Analysis

Cytogenetic analyses of metaphase spreads from parental MCF-7 and NCI H630 cells and early and late passage NCI $H630_{TDX}$ and MCF_{TDX} cells were performed. Compared to parental cells, MCF_{TDX} chromosomes demonstrated multiple HSRs, whereas in early passage $H630_{TDX}$ cells with elevated TS levels double minute chromosomes were noted. In MCF_{TDX} cells, HSRs were located on chromosome 13q and at the telomeric end of chromosome 5q. The short arm of chromosome 18, which is the location of TS, was deleted in MCF_{TDX} cells; however, a small piece of the 18p was attached to the telomeric end of chromosome 5q containing the HSR region. Cytogenetic analysis of late passage $H630_{TDX}$ cells in which TS levels had returned to normal demonstrated that the double minute chromosomes were absent; however, in MCF_{TDX} cells, the HSRs persisted.

TDX Uptake Studies

In an effort to determine the mechanism responsible for TDX resistance in NCI $H630_{TDX}$ cells with normal TS levels, we next measured TDX uptake in both $H630_{TDX}$ and MCF_{TDX} cells using radioactively labeled TDX. In MCF-7 and MCF_{TDX} cells, TDX was efficiently transported; moreover, TDX transport in the MCF_{TDX} resistant line was approximately 2-fold greater than that observed in the parent line (Fig. 2, left panel). However, in the NCI $H630_{TDX}$ resistant cells, TDX uptake was only 4% of that observed in H630 parent cells and reached a plateau after 1 min (Fig. 2, left panel). Thus, the TDX-resistant NCI $H630_{TDX}$ cells had developed an impediment to intracellular transport or accumulation of TDX. We also examined the ability of these cells to transport MTX and LCV (Fig. 2, center and right panels). Both MCF_{TDX} and $H630_{TDX}$ resistant cells efficiently transported MTX and LCV intracellularly, and no significant alteration in MTX or LCV transport was noted in MCF_{TDX} or NCI $H630_{TDX}$ cell lines compared to their respective parental cell lines (Fig. 2, center and right panels).

FPGS Analysis

We next assayed the activity of FPGS, the enzyme responsible for the intracellular polyglutamation of TDX in the TDX-resistant and -sensitive cell lines. In the H630 parental cells, the specific activity of FPGS was 0.48 nmol/hr/mg compared with 0.01 nmol/hr/mg in the $H630_{TDX}$ resistant cells. Despite this 48-fold reduction in the specific activity of FPGS in $H630_{TDX}$ cells, FPGS mRNA expression was comparable to that of the parent H630 cell line (Fig. 3). No difference in FPGS activity was noted between the MCF-7 parent (58 pmol/hr/mg) and TDX-resistant (78 pmol/hr/mg) cells.

To determine whether TDX resistance in $H630_{TDX}$ cells was due solely to decreased FPGS activity, we tested the cytotoxicity of the more recently developed quinazoline TS inhibitor, ZD 9331; which does not require polyglutamation in order to inhibit TS enzyme. Competition experiments with leucovorin indicated that ZD 9331 was transported by the reduced folate carrier (data not shown) in the H630 parent cells. In the H630 parent cells, the ZD 9331 IC_{50} was 10 nM, while in $H630_{TDX}$ cells the ZD 9331 IC_{50} was 30 μM, similar to the TDX IC_{50} of 50 μM. This suggested that in addition to decreased FPGS activity defective transport by the reduced folate carrier was also an important mechanism of resistance to the quinazolines.

DISCUSSION

ZD1694 is a novel antifolate TS inhibitor that has entered clinical trials recently and has shown promising activity in

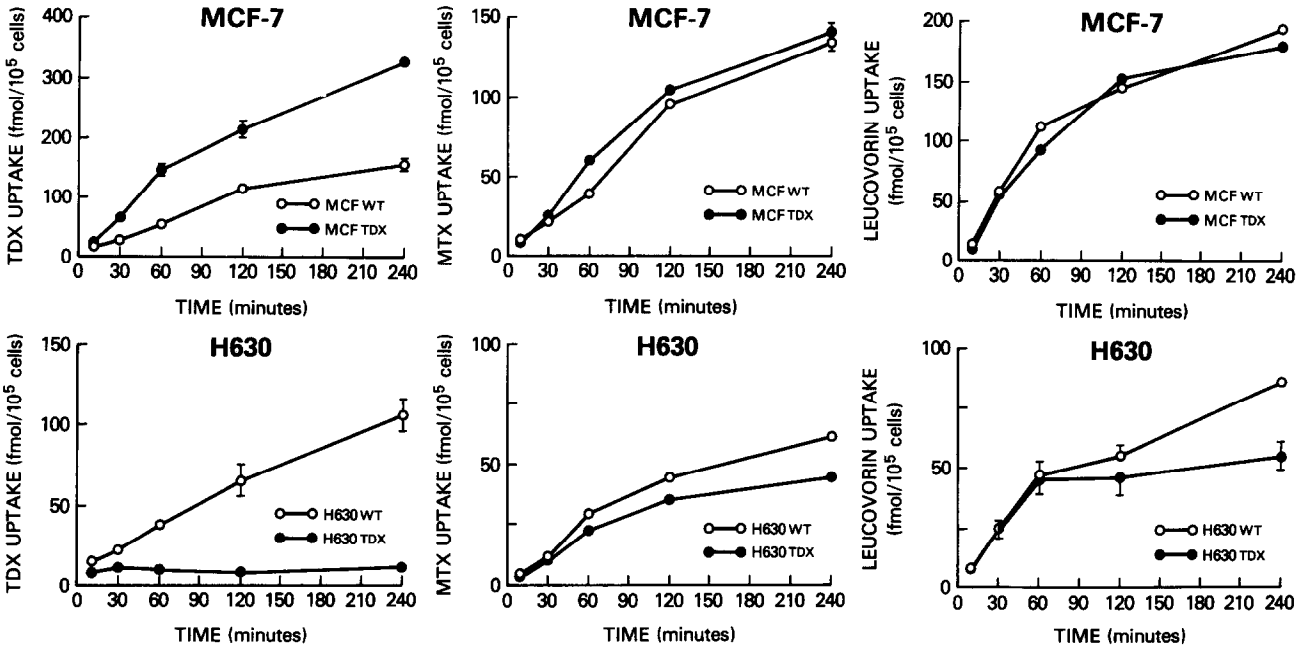

FIG. 2. Radiolabeled Tomudex (left), MTX (center), and LCV (right) uptake in NCI H630 and $H630_{TDX}$ cells and MCF-7 and MCF_{TDX} cells at various time points (30 sec, 1 min, 10 min, 30 min, 1 hr, 2 hr, and 4 hr) after labeled drug was added. Results are the means ± SD of at least 5 separate experiments.

human breast and colon carcinoma. In this study, we have described the development and characterization of TDX-resistant human breast and colon cell lines.

MCF_{TDX} cells had a 30-fold increase in TS mRNA expression and a 34-fold increase in TS protein levels, suggesting that TS overproduction was a major mechanism of resistance to TDX in these cells. When MCF_{TDX} cells were taken out of drug and grown in drug-free medium for 60 passages, no change in TS mRNA or protein levels was noted and cells remained resistant to TDX. Cytogenetic analysis of early and late passage MCF_{TDX} cells revealed the presence of HSRs. Moreover, transport studies revealed that TDX was efficiently transported into MCF_{TDX} cells. Early passage $H630_{TDX}$-resistant cells exhibited a 52-fold increase in TS protein levels compared to the parental cells; however, in late passages of $H630_{TDX}$ cells, decreased expression in TS protein and mRNA expression comparable to that of the parental cells was noted despite the persistence of resistance to TDX. Cytogenetic analysis of early passage $H630_{TDX}$ cells revealed the presence of double minute chromosomes that were not detectable in the late passage $H630_{TDX}$ cells with normal TS expression, suggesting that this initial resistance mechanism was unstable and due to the presence of double minute chromosomes.

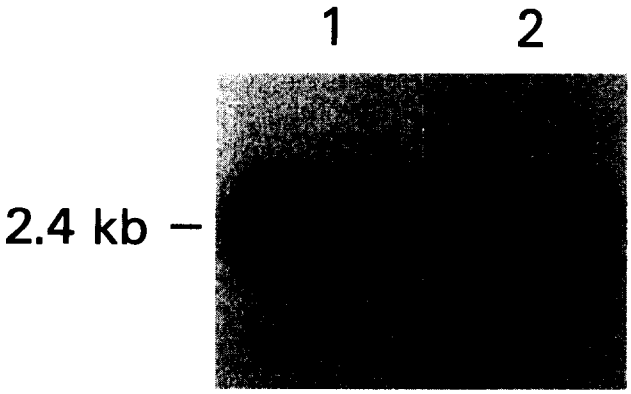

FIG. 3. Northern blot of FPGS mRNA expression in (1) H630 parent and (2) $H630_{TDX}$ cells. Northern blotting was performed as outlined in Materials and Methods.

Competition experiments using LCV and folic acid in both MCF-7 and H630 cells had revealed previously that TDX was specifically transported via the reduced folate carrier (data not shown). In late passage $H630_{TDX}$ cells, TDX uptake was impaired markedly; however, both LCV and MTX uptake into $H630_{TDX}$ cells was normal. These data suggest that the transport defect is specific for the quinazoline antifolate and does not affect intracellular transport of either MTX or LCV. We also noted that the FPGS activity was 48-fold less in $H630_{TDX}$ cells, whereas FPGS mRNA expression was similar to parental levels suggesting that $H630_{TDX}$ cells may contain a qualitatively altered FPGS enzyme. Thus, in $H630_{TDX}$ cells the mechanism of resistance to TDX appears to have evolved from increased TS expression to decreased TDX transport and decreased FPGS activity. Elevated levels of TS demonstrated in early passage cells was lost when the selective pressure on TS was decreased in late passage $H630_{TDX}$ cells due to the inability to transport and polyglutamate TDX. These $H630_{TDX}$ cells were also noted to be 10,000-fold resistant to the more recently developed quinazoline TS inhibitor ZD 9331, which does not require polyglutamation

to inhibit TS enzyme, consistent with defective transport by the reduced folate carrier as an important mechanism of resistance to the quinazolines in these cells.

The potential mechanisms of resistance to folate TS inhibitors would include altered TS enzyme with reduced affinity for the folate inhibitor, impaired transport or uptake, and altered FPGS. Resistance to earlier folate based TS inhibitors such as CB 3717 and ICI 198583 had been described previously in mouse L1210 cells and human lymphoblastoid cells, respectively, and appeared to be due to TS overexpression [18, 19]. More recent studies have demonstrated that increased TS expression and decreased FPGS activity appear to be the major determinants of TDX response in human tumor cell lines [20–22]. In this study, the major mechanism of resistance to TDX in MCF-7 and early passage NCI $H630_{TDX}$ cells was increased TS expression; however, in late passage $H630_{TDX}$ cells altered TDX transport and decreased FPGS activity were the predominant mechanisms of resistance. Human cells are thought to possess two major folate transport systems. The first system is a low affinity, high capacity, reduced folate carrier that transports reduced folates such as LCV and antifolates such as MTX with affinity constants in the micromolar range. This system transports folic acid much less efficiently than reduced folates. The second transport system is a family of high affinity, membrane associated folate binding proteins with affinity constants for folic acid in the nanomolar range [23, 24]. Despite the fact that MTX, LCV, and TDX share the same reduced folate carrier, $H630_{TDX}$ cells readily transport both MTX and LCV, consistent with a specific alteration in the transport protein that decreases TDX affinity but maintains affinity for MTX and LCV.

The finding of decreased FPGS has been described in cells resistant to MTX and 5-fluorouracil and more recently to DDATHF, a GAR transformylase inhibitor [25–27]. While the majority of MTX-resistant cells exhibit other mechanisms of resistance in addition to decreased FPGS, one study of MTX resistance in human lymphoblastoid CCRF-CEM cells has described decreased FPGS levels as the sole mechanism of MTX resistance [25]. Pizzorno and colleagues have described a resistant DDATHF CCRF-CEM cell line in which the intracellular level of polyglutamated forms of the drug were decreased by 2- to 3-fold. The major reason for development of DDATHF resistance was felt to be the decrease in FPGS activity; however, a 2- to 3-fold increase in γ-glutamylhydrolase activity, the enzyme responsible for folate polyglutamate breakdown, was also detected [26]. A study by Wang and colleagues [27] detected a 75% decrease in FPGS expression in an HCT-8 colon cancer cell line selected for resistance to low dose 5-fluorouracil, resulting in an inability to form polyglutamated reduced folates. The inability to form polyglutamated reduced folates may result in decreased TS inhibition due to a reduction in TS ternary complex stability and formation. The finding of decreased FPGS activity in TDX-resistant cells has also been demonstrated by Jackman *et al.* [21] and Lu *et al.* [22] and suggests that the ability to inhibit TS catalytic activity plays a critical role in the development of resistance to TDX in human tumor cell lines [21, 22].

Further study will be needed to determine if decreased FPGS activity is a result of decreased FPGS protein or decreased activity due to either a mutation or post-translational modification. Moreover, it will also be important to determine the effects of altered FPGS substrate specificity on folate levels and metabolism in these TDX-resistant cells and whether specific alterations exist within the transport protein.

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