Thymidine Phosphorylase Moderates Thymidine-dependent Rescue after Exposure to the Thymidylate Synthase Inhibitor ZD1694 (Tomudex) in Vitro

Adam V. Patterson, Denis C. Talbot, Ian J. Stratford, and Adrian L. Harris

Abstract

The inhibition of de novo thymidine (dThd) synthesis by the novel folate-based thymidylate synthase (TS) inhibitor ZD1694 (Tomudex) can achieve tumor cell-specific cytotoxicity in vivo. However, nucleosides in the surrounding microenvironment of tumors may be used by the salvage pathway to regenerate any depleted pools, thus providing an efficient mechanism through which to circumvent the ZD1694-dependent toxicity. Anabolism of dThd to dTMP by dThd kinase (TK) is the first committed step in the dThd salvage pathway. However, dThd phosphorylase (dThdPase) can compete with TK by catalyzing the reversible phosphorolytic cleavage of dThd to thymine and deoxyribose 1-phosphate and rendering the salvaged dThd metabolically unavailable. Both TK and dThdPase are up-regulated in some tumors, and their relative importance is not fully defined. We have studied the influence of dThdPase expression on the capacity of exogenous dThd to reverse ZD1694-dependent growth inhibition and have shown that both intra- and extracellular dThdPase activity can effectively moderate dThd-rescue. This suggests that tumor levels of dThdPase may be an important factor in the outcome of ZD1694 therapy.

Introduction

ZD1694 (Tomudex) is a quinazoline antifolate TS2 inhibitor (1). It is extensively polyglutamated by folypolyglutamate synthase resulting in enhanced intracellular retention. Polyglutamation also serves to increase its potent inhibitory activity against TS and, thus, compromises de novo dThd synthesis. In the absence of salvageable extracellular dThd, a “dThd-less” state arises; the concurrent perturbations of the nucleotide triphosphate pools (2) are considered the dominant mechanism by which ZD1694 exerts its cytotoxic effects. However, preformed dThd is potentially bioavailable within a tumor mass from both the vascular supply and that released by dying cells. The high levels of dThd kinase in tumor compared with normal tissues (3) imply that the salvage pathway is the preferred source of dThd for subsequent anabolism and DNA synthesis. Cellular uptake readily occurs via: (a) S-(p-nitrobenzyl)-6-thioinosine-sensitive and/or -in insensitive cell-surface equilibrated nucleoside transporters (4); (b) a Na+-associated concentrative transport process (5, 6); or (c) nonfacilitated diffusion. It has been demonstrated that nucleoside transport activity can be up-regulated 22- to 39-fold as a consequence of acute TS inhibition in the human bladder cancer cell line MGH-U1 (7). Such elevations in nucleoside transport function may be highly relevant when dThd availability is the potentially rate-limiting step in the salvage pathway. Thus, facilitated and nonfacilitated uptake processes could provide an effective intracellular source of dThd for the salvage pathway that could prove sufficient to circumvent the cytotoxic impact of ZD1694-mediated TS inhibition.

Materials and Methods

Chemicals. ZD1694, a gift from Zeneca (Alderley Edge, United Kingdom) was dissolved in 0.4 m sodium bicarbonate and stored in aliquots at 4°C, protected from light. Cell culture-grade dThd, E. coli dThdPase, and all of the other chemicals were purchased from Sigma (Poole, United Kingdom).

Cell Lines. Human MCF-7 breast cell lines (passage 52–75) and the clone TP-4 were grown in DMEM. (All of the media were prepared at Imperial Cancer Research Fund Laboratories, Clare Hall, United Kingdom.) The medium was supplemented with 10% (v/v) FCS and 2 mM glutamine. Cells were grown at 37°C in a 100%-humidified incubator with a gas phase of 5% CO2. Cultures were routinely screened as negative for Mycoplasma.

Transfection of dThdPase cDNA into MCF-7 Cells. Transfection and isolation of dThdPase clones have been described elsewhere (14). Briefly, pS152 plasmid vector containing full-length dThdPase DNA was introduced into MCF-7 cells by electroporation. Stable transfectants were selected by long-term incubation in Geneticin.

Quantification of Drug Sensitivity. The MTT-proliferation assay was used to determine the dose-response curves of the parental and clonal cell lines as described previously (15). The cells were seeded at an initial density of 104

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2 The abbreviations used are: TS, thymidylate synthase; dThd, thymidine; dThdPase, dThd phosphorylase; 5-FU, 5-fluorouracil.
cells/well. IC50 values were determined relative to the control wells, which contained no drug. After initial evaluation, all of the ZD1694 exposures were for 48 h. Exogenous dThd or dThdPase additions were varied as described in the “Results” section. Cells were allowed to grow for a total of 12 days.

Preparation of Cell Lysates. Cell lines were harvested, washed, and sonicated using a MSE Soniprep 150 three times for 5 s (nominal frequency of 23 KHz and an oscillation amplitude of 5–10 μm). Sonication was performed in 50 mM Tris-HCl and 0.15 M NaCl buffer (pH 7.4) at 4°C. Suspension was centrifuged at 10,000 x g for 15 min (4°C). Supernatants were stored in liquid N2 until required.

dThdPase Activity Determinations. Enzyme activity determinations were conducted for the parental MCF-7 and cloned TP-4 line in vitro. The assay conditions have been described previously (16). Briefly, lysates were incubated for 16 h at 37°C in 10 mM dThd and 50 mM K3PO4 (pH 7.4). The reaction was terminated by the addition of 0.7 ml of ice-cold 0.5 M NaOH to 0.3 ml of reaction mixture to produce a final solution pH of 13.3, and the conversion of dThd to thymine was monitored spectrophotometrically at 300 nm. Optical densities were related to the standard plots for known thymine concentrations. The protein content of the cell lysates were determined using the Bio-Rad protein dye-binding assay and quantified against a high-grade BSA protein standard. dThdPase activity is expressed as nmol substrate converted/mg total cytosolic protein/h.

Influence of ZD1694 Exposure on [3H]dThd Uptake. Subconfluent monolayers of MCF-7 parental and TP-4 cells were incubated for 4 h with or without ZD1694 (10–100 nm) in serum-free medium; 0.3 μM [3H]dThd (20 μCi/nmol) was added for 30 min, after which the medium was rapidly aspirated. Cells were washed three times in ice-cold calcium- and magnesium-free phosphate buffered saline-A, harvested by trypsinization, and pelleted (4°C). The supernatant was discarded and 0.5 ml of ice-cold 0.4 M perchloric acid was added to the pellet and mixed thoroughly. After standing on ice for 30 min, the acid-insoluble material was pelleted at 1600 x g for 20 min (4°C). Supernatant, containing the acid-soluble nucleotide pool, was spotted on silica gel 60 TLC plates, and dThd and thymine were separated in chloroform:acetone:methanol (17:3:1). The solvent front was run to the top of the TLC sheet, and spots corresponding to dThd and thymine were visualized under UV illumination (254 nm), recovered, and dispensed into scintillation vials for counting. The acid-insoluble pellet was washed twice with 0.2 M perchloric acid and solubilized in 0.5 ml of 0.3 M KOH before transfer to scintillation vials.

Results

dThdPase Activity Determinations. The dThdPase activities of the parental and TP-4 MCF-7 cell lines and their relationship to activities found in primary breast biopsies have been reported previously (13, 14). Basal activity of the parental MCF-7 cell line was 38.2 ± 5.9 nmol thymine released/h/mg lysate protein (37°C) for the TP-4 clonal line. This represents an 88-fold elevation in dThdPase activity. Doubling times for the two cell lines were not significantly different in vitro.

Optimization of dThd Rescue in the MCF-7 Wild-Type Cell Line. Initial experiments were conducted on the parental line to determine the most effective schedule of dThd (1.0 μM) rescue during and/or after the 48-h exposure to ZD1694. These data are summarized in Table 1. The addition of dThd during the 48-h ZD1694 exposure was essential for effective rescue. If the rescue was initiated only on the removal of the drug and continued for the remaining 10 days of growth, a modest shift in IC50 from 6.0 to 9.6 nm could be achieved. However, if a delay of 24 h was introduced before the dThd addition, rescue was totally ineffective. Thus, the ability of exogenous dThd to rescue the

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* All of the plates were treated with 1–10,000 nm ZD1694 for an initial 48-h period.
* IC50 (where determinable) is the mean of three independent experiments.
* +, 24-h treatment with 1.0 μM dThd.

Table 1 Parental MCF-7 cell line

Fig. 1. Representative ZD1694 dose-response curves for the MCF-7 parental cell line (A) and the TP-4 clonal line (B), which expresses an 88-fold elevation of dThdPase activity relative to the parental line. All of the ZD1694 treatments were for 48 h in the presence of dThd, and dThd was present for an additional 48 h after the removal of the drug. Concentrations of exogenously added dThd are (O) 0 μM, (C) 0.3 μM, (△) 1.0 μM, and (Θ) 3.0 μM. Cells were allowed to grow for an additional 8 days after the removal of dThd (total growth time, 12 days).
Fig. 2. The influence of ZD1694 exposure on [3H]dThd uptake in MCF-7 parental cells and TP-4 cells. Cultures were incubated for 4 h with or without ZD1694 (3 and 30 nm, respectively) in serum-free medium. Then 0.3 µM [3H]dThd (20 µCi/nmol) was added for 30 min, after which the medium was rapidly aspirated. Cells were harvested (4°C) and [3H]dThd (clear bars) and [3H]thymine (hatched bars) were separated by TLC and counted.

Influence of Elevated Intracellular dThdPase Activity on dThd Rescue. In contrast, experiments conducted using the clonal line TP-4 demonstrated that the presence of elevated dThdPase activity could very significantly modulate this "dThd rescue" phenomenon. By using the previously optimized schedule of drug exposure (48 h) and dThd rescue (96 h), MCF-7 wild-type and TP-4 cell lines were rescued with various concentrations of exogenous dThd (0.3–3.0 µM).

In the absence of dThd, dose-response curves were similar for the two cell lines, with IC50 values in the low nm range (Fig. 1A and B). The TP-4 line was modestly but consistently (P = 0.027) more sensitive than the parental line (3.5 and 6.0 nm, respectively) under these conditions, perhaps indicating the presence of low levels of dThd in the PCS medium supplement.

The addition of 0.3 µM dThd (96 h) resulted in a significant modulation of the dose-response curve for the parental line, such that an IC50 value was no longer determinable (Fig. 1A). In contrast, 0.3 µM dThd rescue in the TP-4 cell line only shifted the IC50 value from 3.5 to 8.0 nm (Fig. 1B). Increasing concentrations of exogenous dThd (1.0 and 3.0 µM) were more effective at reversing the ZD1694-dependent toxicity in both cell lines. However, the rescue was far superior in the parental line. Indeed, no significant inhibition of growth could be achieved in the parental line at either 1.0 or 3.0 µM dThd, whereas IC50 values for the TP-4 line were 26 and 88 nm, respectively. This represents a very significant modulation of rescue at physiologically relevant dThd concentrations and suggests that, within this in vitro model, elevated dThdPase activity need only be present during the initial period of TS inhibition to compromise any dThd-dependent rescue phenomena.

Uptake and Metabolism of [3H]dThd in ZD1694-Treated Cells. After a 4-h treatment at 3 or 30 nm ZD1694, parental and TP-4 cells were pulsed with 0.3 µM [3H]dThd for 30 min before harvesting and acid extraction. Total uptake of [3H]-label was similar in both cell lines under comparable conditions. Consistent with the impact of exogenous dThd on the modulation of the dose-response curves of the parental (but not the TP-4 cell line), a greater proportion of the initial dThd uptake was metabolized to thymine. At 30 nm ZD1694 pretreatment, 29% (0.57 pmol/106 cells) of the total salvaged dThd was rendered metabolically unavailable to relieve the acute TS inhibition in the TP-4 cells, compared with just 3.6% (0.11 pmol/106 cells) in the parental cell line (Fig. 2). Therefore, the presence of intracellular dThdPase activity could modify the extent to which dThd was available via the nucleotide salvage pathway.

Comparative Roles of Intra- and Extracellular dThdPase Activity. To examine whether the presence of extracellular dThdPase activity would be sufficient to mimic this effect seen for intracellular expression, increasing quantities of E. coli dThdPase were added exog-
enously. Extracellular addition of dThdPase at a total activity like that found in 10^4 TP-4 cells (20.6 nmol thymine released/h at 37°C) was sufficient to modulate the salvage capacity of 0.3 μM exogenous dThd in the parental cell line, whereas dThdPase activity equivalent to 10^7 cells (206 nmol/h) ablated rescue by 3 μM dThd in vitro. The addition of E. coli dThdPase in the absence of exogenous dThd had only a modest influence on ZD1694 toxicity, consistent with the presence of some dThd in the undialyzed FCS supplement. Representative dose-response curves are shown in Fig. 3, A and B (compare with Fig. 1A).

These data suggest that the delivery of catalytically active dThdPase to the cell surface may enhance the efficacy of ZD1694 in vivo and may, therefore, be an appropriate enzyme to consider for antibody-directed therapy in this context.

**Western Blotting Analysis of Intra- versus Extracellular dThdPase Protein in Conditioned Medium.** Analysis of the relative proportions of dThdPase protein in the intra- and extracellular fractions after 24-h medium conditioning was conducted by Western blotting (Fig. 4). Densitometric analysis of total dThdPase protein in cell extracts versus conditioned medium that was concentrated 100-fold by Macrostep centrifugal concentration (10 kDa cutoff) confirmed the presence of at least a 10,000-fold excess of intracellular dThdPase protein in the TP-4 cell line. This suggests that the background levels of extracellular dThdPase are not contributing significantly to the metabolism of exogenous dThd, and, therefore, in the absence of exogenously added E. Coli dThdPase, intracellular metabolism dominates.

**Discussion**

The observation that ZD1694 toxicity is totally ablated by the presence of salvageable dThd is consistent with the primary mode of action being TS-inhibition. Thus, despite a complete block in de novo dThd synthesis, dThd in the surrounding environment can be used by the salvage pathways to regenerate the depleted nucleotide pools, ensuring continued DNA synthesis and repair. Critically, nucleosides are present in human plasma at concentrations sufficient to fulfill a salvage function and may also be available locally from the nucleic acids of dying cells within a tumor mass (17). Considering this together with evidence that the contribution of salvage flux is greater than that of de novo synthesis in neoplastic tissues and that the inhibition of de novo synthesis leads to even higher salvage activity (7), one might speculate that any approach that modulates the availability of exogenous pyrimidine nucleotides should significantly enhance ZD1694 toxicity. Consistent with this proposal, we have demonstrated that the presence of elevated dThdPase activity, the first enzyme in the dThd salvage pathway, can markedly compromise the intracellular bioavailability of salvaged dThd.

Significant evidence has accumulated that dThdPase is markedly elevated in many solid tumors including breast, esophageal, pancreatic, lung, bladder, ovarian, gastric, and colorectal cancers (12). However, the analysis of dThdPase activities in primary biopsy samples and/or the immunostaining of tumor sections have demonstrated a significant intertumoral variability. Our in vitro data suggest that this heterogeneity may be a factor in the efficacy of ZD1694 treatment and, therefore, imply that approaches aimed at rational patient selection based on "enzyme-profiling" of tumor tissues could target those individuals most likely to benefit from treatment with ZD1694. Several studies have indicated that high dThdPase expression is associated with poor prognosis (12, 16, and references therein). We have reported recently that patients treated with adjuvant cyclophosphamide, methotrexate, and 5-FU for node-positive breast cancer had a better prognosis if dThdPase was elevated in their tumors (18). Methotrexate cytotoxicity, and perhaps 5-FU cytotoxicity, can be potentiated by preventing dThd-rescue (17), which suggests the influence of dThdPase on therapy with cyclophosphamide, methotrexate, and 5-FU reflects a potential for manipulation of the salvage pathway in vivo. Perhaps therapies that are aimed at exploiting this prognostically unfavorable tumor characteristic may prove to be of value. Alternatively, approaches that are aimed at inducing tumor dThdPase activity, such as IFN-α and IFN-γ treatment (11), may enhance the response to ZD1694 treatment.

**References**

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