Biological Activity and Intracellular Metabolism of ZD1694 in Human Leukemia Cell Lines with Different Resistance Mechanisms to Antifolate Drugs

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The biological activity and cellular metabolism of ZD1694, a novel folate-based thymidylate synthase (TS) inhibitor, were analyzed in a human leukemia cell line, MOLT-3, and its antifolate-resistant sublines with different mechanisms of resistance to methotrexate (MTX), trimetrexate (TMQ) and N^{10}-propargyl-5,8-dideazafolic acid (CB3717). MOLT-3/CB3717$_{40}$, which was selected for CB3717 resistance, demonstrated impaired membrane drug transport via reduced folate carrier (RFC) and lower accumulation of [^{3}H]ZD1694-polyglutamates in the cells with a shift in the polyglutamate distribution profile to shorter chain length polyglutamates, indicating an alteration in polyglutamation capacity in this subline. Impaired RFC and reduced rate of polyglutamation could explain the cross-resistance (12-fold) of this subline to ZD1694. On the other hand, there was little or no cross-resistance to this drug in a subline (MOLT-3/TMQ$_{800}$) reportedly resistant to TMQ through impaired membrane transport for TMQ and an increase in dihydrofolate reductase (DHFR) activity. Total amount of ZD1694 polyglutamated to a level higher than diglutamate was approximately 1.7-fold higher in the TMQ-resistant cells than that in the parent cells, but a low degree of increase in TS activity in the cells counteracted the supposed increase in sensitivity to ZD1694. MOLT-3/TMQ$_{800}$-MTX$_{10000}$ cells, which were established by sequential exposure of the TMQ-resistant cells to MTX and were previously shown to amplify mutated DHFR with low affinity for MTX, showed a decreased accumulation of polyglutamated ZD1694 as compared with the parent line and this was consistent with cross-resistance to ZD1694 in this subline. Overproduction of variant DHFR scarcely influenced the sensitivity to this drug. These results indicate that ZD1694 could overcome antifolate resistance through a mechanism such as amplified DHFR activity, and the biological activity of this drug against the cells paralleled the amount of polyglutamated drug inside the cells. Determination of polyglutamation capacity in tumor cells may allow prediction of sensitivity to this drug.

Key words: ZD1694 — Thymidylate synthase — Polyglutamation — Antifolate resistance

DHFR[5] and TS are key enzymes in the thymidylate cycle and are good targets for cancer chemotherapeutic agents.[1, 2] MTX, a classical antifolate drug, is a potent inhibitor of DHFR *in vitro* and *in vivo*, but prolonged treatment with this drug may result in the acquisition of resistance by tumor cells, by virtue of impaired membrane transport for the drug (e.g., alteration of RFC), an increase in DHFR activity, induction of mutated DHFR with low affinity for MTX, or diminished polyglutamation of the drug.[3] In addition, MTX may not be active against certain tumors even in the initial treatment with the drug, and this inherent (natural) resistance may result from one of the above mechanisms alone, or a combination of them. Recent studies have demonstrated the significance of impaired polyglutamation not only in the inherent MTX resistance in soft tissue sarcoma cell lines[4, 5] and in acute non-lymphocytic leukemia cells,[6] but also in the acquired resistance to MTX in leukemia cell lines.[7, 8]

Another enzyme critical to the *de novo* synthesis of thymidine 5′-monophosphate is TS, which is an attractive target for the development of anticancer therapeutic agents. Direct inhibition of TS may be achieved by an active metabolite of 5-FU, 5-fluorodeoxyuridine monophosphate (FdUMP), but 5-FU resistance is frequently accompanied by deletion or diminished activity of vari-

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ous activating enzymes.[9] Furthermore, 5-FU is extensively metabolized in the cells to several anabolites other than FdUMP, so that incorporation of 5-FU into RNA and possibly DNA may be important additional determinants of its antitumor activity and toxicity.[10] The additional effects of 5-FU on RNA and DNA synthesis suggested that a more selective TS inhibitor would be highly desirable. The problems associated with incorporation into nucleic acids could be overcome by the design of analogues of the folate cofactor rather than the pyrimidine substrate.[10] Such compounds may be expected to have superior activity in tumors resistant to MTX by virtue of increased activity in DHFR and should also be less toxic to the host, since the synthesis of purines would not be affected.[11] In a series of efforts to find such compounds, introduction of a propargyl group at the N^{10} position of 2-amino-4-hydroxypteroyl glutamate was shown to enhance TS inhibitory action,[11] but clinical application of that compound, designated as CB3717, the prototype folate-based TS inhibitor, was unsuccessful because of unpredictable and life-threatening nephrotoxicity caused by poor water solubility of the drug.[12, 13] This precipitated a further search for water-soluble, nonnephrotoxic analogues, and the result was the development of ZD1694 (Tomudex; Fig. 1).[14] Following preclinical evaluation, ZD1694 entered clinical trials in
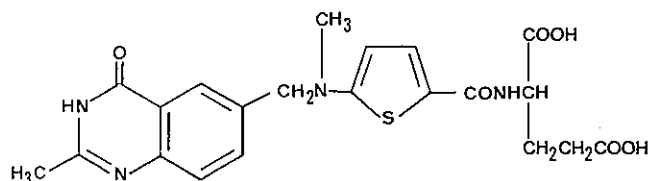
Europe and the USA, and has completed phase I and phase II evaluation, showing activity in several tumor types, particularly colorectal cancer with a 26% objective response rate. Currently a phase III study is in progress, using a randomized design against 5-FU with leucovorin.[15]

In the present study, we tested the biological activity of ZD1694 against antifolate-resistant human leukemia cell lines with different resistance mechanisms. The cellular pharmacokinetics of this drug was also analyzed in order to understand the pharmacological basis of its activity against resistant cells.

MATERIALS AND METHODS

Drugs and radioactive materials 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, USA. MTX was purchased from Lederle Japan, Tokyo. Drug solutions were prepared as previously described.[16, 17]

[^{3}H]ZD1694 used for the drug uptake study was synthesized by Amersham International plc (Buckinghamshire, UK) at a specific activity of 11.3 Ci/mmol; its radiochemical properties and purification were previously described.[17] Another radioactive preparation of the drug ([5-^{3}H]ZD1694)[18] was synthesized by ICI Cambridge Research Biochemicals Ltd. (Billingham, UK) at a specific activity of 10.8 Ci/mmol, and was used for the polyglutamate formation study. [3′,5′,7-^{3}H]MTX sodium salt (14.0 Ci/mmol) was purchased from Amersham and used in the drug uptake study.

Cell culture and the antifolate-resistant MOLT-3 sublines MOLT-3, a human acute lymphoblastic leukemia cell line,[19] and its antifolate-resistant sublines were maintained in RPMI-1640 medium (GIBCO, Grand Island,

Fig. 1. Chemical structure of ZD1694.

Table I. IC$_{50}$ Values of Antifolates against Sensitive and Resistant MOLT-3 Sublines and Known Mechanism(s) of Resistance to Antifolates

| Cell line | IC$_{50}$ value (μM)[a] (Relative resistance)[b] | | | | Known mechanism(s) for antifolate resistance |
	MTX	TMQ	CB3717	ZD1694	
MOLT-3	0.0090	0.0040	1.1	0.0038	
MOLT-3/CB3717$_{40}$	0.020 (2.2)	0.00024 (0.06)	33 (30)	0.047 (12)	
MOLT-3/TMQ$_{800}$	0.063 (7.0)	1.2 (300)	0.88 (0.80)	0.0046 (1.2)	impaired membrane transport for TMQ; increased DHFR activity[20]
MOLT-3/TMQ$_{800}$-MTX$_{10000}$	100 (11000)	>10 (>2500)	2.0 (1.9)	0.026 (6.8)	amplification of mutated DHFR with low affinity for MTX[21, 22]

a) IC$_{50}$ values were obtained from dose-response curves of each cell line after exposure of the cells for 72 h to a drug. Each point was determined from at least two quadruplicate experiments.
b) Relative resistance is given by; IC$_{50}$ values of the resistant cells/IC$_{50}$ value of the parent cells.
MTX, methotrexate; TMQ, trimetrexate; CB3717, N^{10}-propargyl-5,8-dideazafolic acid; DHFR, dihydrofolate reductase.

NY) supplemented with 10 % (v/v) fetal bovine serum (GIBCO).

MOLT-3/CB3717$_{40}$ was established by continuously exposing MOLT-3 cells initially to 1 μM CB3717 and then to gradually increasing drug concentrations. A 40-fold resistant clone to CB3717 was selected through stepwise dose-escalation up to 30 μM. This subline showed a stable resistance for >6 months in drug-free medium. The cellular properties and antifolate-resistance mechanism(s) of MOLT-3/TMQ$_{800}$ and MOLT-3/TMQ$_{800}$-MTX$_{10000}$ (the latter was selected by sequential exposure of the former cells to MTX with stepwise escalation of the drug concentration up to 50 μM) were defined in detail[20-22] and are summarized in Table I. Since the MTX resistance of MOLT-3/TMQ$_{800}$-MTX$_{10000}$ cells was unstable in the drug-free medium, the cells were periodically exposed to 50 μM MTX at 1–2 month intervals, while MOLT-3/TMQ$_{800}$ cells retained the original TMQ resistance for more than 12 months in the medium devoid of the drug.

Drug sensitivity studies The *in vitro* sensitivity of the parent and resistant MOLT-3 cells to antifolates was determined by using the MTT assay with minor modifications.[23, 24] The cells were incubated at 37°C in a humidified 5% CO_2/95% air atmosphere for 72 h with continuous drug-exposure, and then harvested for the MTT assay.

Enzyme assay TS activity was measured by ^{3}H release assay as previously described.[2] Briefly, exponentially growing cells (3–5 × 10^5 cells/ml) were washed in PBS, resuspended in 0.125 M potassium phosphate buffer (pH 7.4) containing 3 mM dithiothreitol at a concentration of 1 × 10^7 cells/ml and then sonicated at 20 kHz for 10 s 3 times in ice using an MSE Soniprep 150 ultrasonicator (MSE, Crawley, UK). Following centrifugation at 50,000g for 1 h at 4°C, the supernatants were harvested for the measurement of enzyme activity.

DHFR activity was assayed spectrophotometrically as described by Jackson et al.,[25] except that 0.15 M potassium phosphate buffer (pH 7.0) was used instead of 0.05 M Tris.

Initial uptakes of [^{3}H]ZD1694 and [^{3}H]MTX by the sensitive and resistant MOLT-3 cells The Vortex-Finnpipette procedure[26] was used to measure the initial uptake of ^{3}H-labeled drugs by MOLT-3 and its antifolate-resistant sublines. Aliquots of 0.4 ml [^{3}H]ZD1694 (11.3 μCi/ml, 1μM) or [^{3}H]MTX (14.0 μCi/ml, 1 μM) were added to 3.6 ml of concentrated cell suspension (1 × 10^6 viable cells/ml) in HBSS (GIBCO), and the mixture was incubated at 37°C or 2°C. At specified time intervals, 0.5 ml of the reaction mixture was removed and layered on a mixture of mineral oil (Sigma, St. Louis, MO) and silicon oil (Fluka Japan, Tokyo), of which the final density was adjusted to 1.031 g/ml. This was

followed immediately by centrifugation at 12,000 g for 1 min using a microcentrifuge to separate the cells from the supernatant under the oil layer. After removal of the supernatant and most of the oil layer, the cell pellet was solubilized by incubation with Solvable (New England Nuclear, Boston, MA) at 50°C for 3 h, then the solution was transferred to a counting vial. Radioactivity was measured with a liquid scintillation counter after the addition of 10 ml of aqueous counting scintillant (ACS-II, Amersham). The results were calculated as net uptake by subtracting the diffusion value at 2°C from the total radioactivity at 37°C for each incubation period.

Measurement of intracellular ZD1694-polyglutamates Sensitive or resistant MOLT-3 cells in the exponential growth phase (3–5 × 10^5 cells/ml) were incubated with 0.1 μM [5-^{3}H]ZD1694 at 37°C for 24 h. After incubation, the cells were washed twice with ice-cold PBS and the final cell pellets were resuspended in 0.7 ml of ice-cold deionized water, then sonicated using an MSE Soniprep 150 ultrasonicator for 30 s (70 kHz, probe amplitude 70 μm). ZD1694 and its polyglutamates were extracted and measured by ion-pair HPLC as previously described[18] with synthetic ZD1694-polyglutamate standards (di- to hexaglutamate).[27]

RESULTS

***In vitro* sensitivity of antifolate-resistant MOLT-3 sublines to ZD1694 and cellular enzyme activities** The IC$_{50}$ values of ZD1694 against MOLT-3 sublines with different resistance mechanisms to antifolates are shown in Table I. MOLT-3/CB3717$_{40}$ cells displayed a cross-resistance to ZD1694 (12-fold), whereas the cells showed a remarkable collateral sensitivity to TMQ. Only a slight cross-resistance to MTX was observed in this subline. MOLT-3/TMQ$_{800}$ cells which were selected for resistance to TMQ and possessed impaired membrane transport for TMQ and an increase in DHFR activity as resistance mechanisms[20] exhibited a cross-resistance to MTX (7-fold); however, these mechanisms of resistance had little effect on the sensitivities to the TS inhibitors used (0.8- and 1.2-fold for CB3717 and ZD1694, respectively). MOLT-3/TMQ$_{800}$-MTX$_{10000}$ cells, which were made more resistant to MTX by continuously exposing MOLT-3/TMQ$_{800}$ cells to MTX and were shown to express mutated DHFR with low affinity for MTX as the resistance mechanism,[21, 22] displayed some cross-resistance to ZD1694 (6.8-fold).

DHFR and TS activities in the sensitive and resistant cells are shown in Table II. Approximately 2-fold and 15-fold increases in DHFR activity were observed in MOLT-3/TMQ$_{800}$ and MOLT-3/TMQ$_{800}$-MTX$_{10000}$ cells, respectively. A slight increase in TS activity was also observed in MOLT-3/TMQ$_{800}$.

Table II. Enzyme Activities in the Sensitive and Resistant MOLT-3 Sublines

Cell line	DHFR activity (nmol product/min/10^6cells)	TS activity (nmol product/h/10^6cells)
MOLT-3	0.15	1.8
MOLT-3/CB3717$_{40}$	0.16 (1.1)	1.7 (0.94)
MOLT-3/TMQ$_{800}$	0.29 (1.9)	3.2 (1.8)
MOLT-3/TMQ$_{800}$-MTX$_{10000}$	2.3 (15)	2.3 (1.3)·

Data are the mean values from two experiments carried out on separate days.
Figures in parentheses indicate relative increases in enzyme activities in the resistant cells as compared to that in MOLT-3 cells.
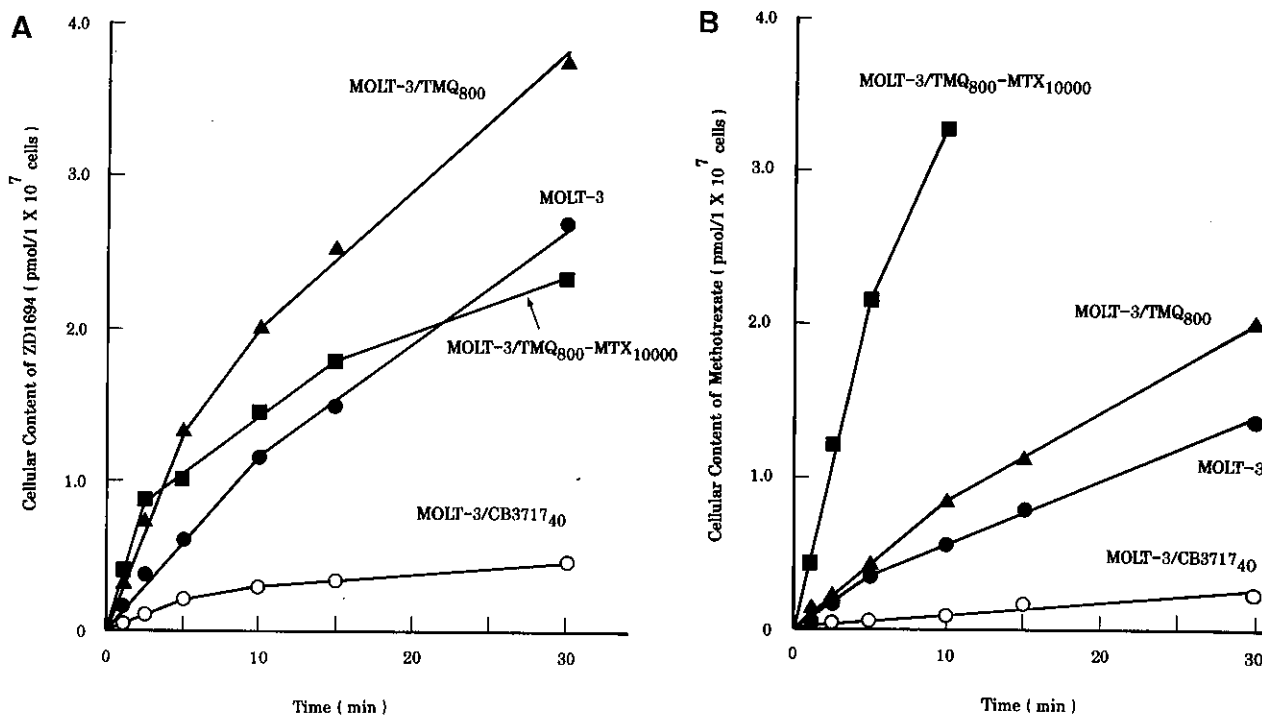DHFR, dihydrofolate reductase; TS, thymidylate synthase.

Fig. 2. Initial uptake and subsequent accumulation of [^{3}H]ZD1694 (A) and [^{3}H]methotrexate (MTX) (B) in sensitive and antifolate-resistant MOLT-3 cells. MOLT-3 (●), MOLT-3/CB3717$_{40}$ (○), MOLT-3/TMQ$_{800}$ (▲) and MOLT-3/TMQ$_{800}$-MTX$_{10000}$ cells (■) were incubated with 0.1 μM [^{3}H]ZD1694 (11.3 μCi/ml) or 0.1 μM [^{3}H]MTX (14.0 μCi/ml) at 37°C or 2°C for the time periods indicated and then harvested for the measurement of radioactivity in the cells. Data shown are from one representative experiment.

Initial uptake and subsequent intracellular accumulation of [^{3}H]ZD1694 and [^{3}H]MTX in the sensitive and resistant cells Initial uptake and subsequent accumulation of [^{3}H]ZD1694 in the cells were determined by measuring cellular ^{3}H up to 30 min and are illustrated in Fig. 2A. Since ZD1694 is an excellent substrate for FPGS[28], intracellular drug contents after 10 min represent not only cellular drug uptake, but also accumulated polyglutamates, so we also measured cellular uptake of MTX, which is polyglutamated much more slowly than ZD1694 (Fig. 2B). The parent MOLT-3 cells showed a linear uptake of [^{3}H]ZD1694 up to 10 min and a slight decline thereafter. Decreases in cellular uptakes of both ^{3}H-labeled ZD1694 and MTX seen in MOLT-3/CB3717$_{40}$ indicated the impairment of RFC in this subline. On the other hand, the initial ZD1694 uptake was higher, though not with statistical significance, in MOLT-3/TMQ$_{800}$ and MOLT-3/TMQ$_{800}$-MTX$_{10000}$ cells than in the
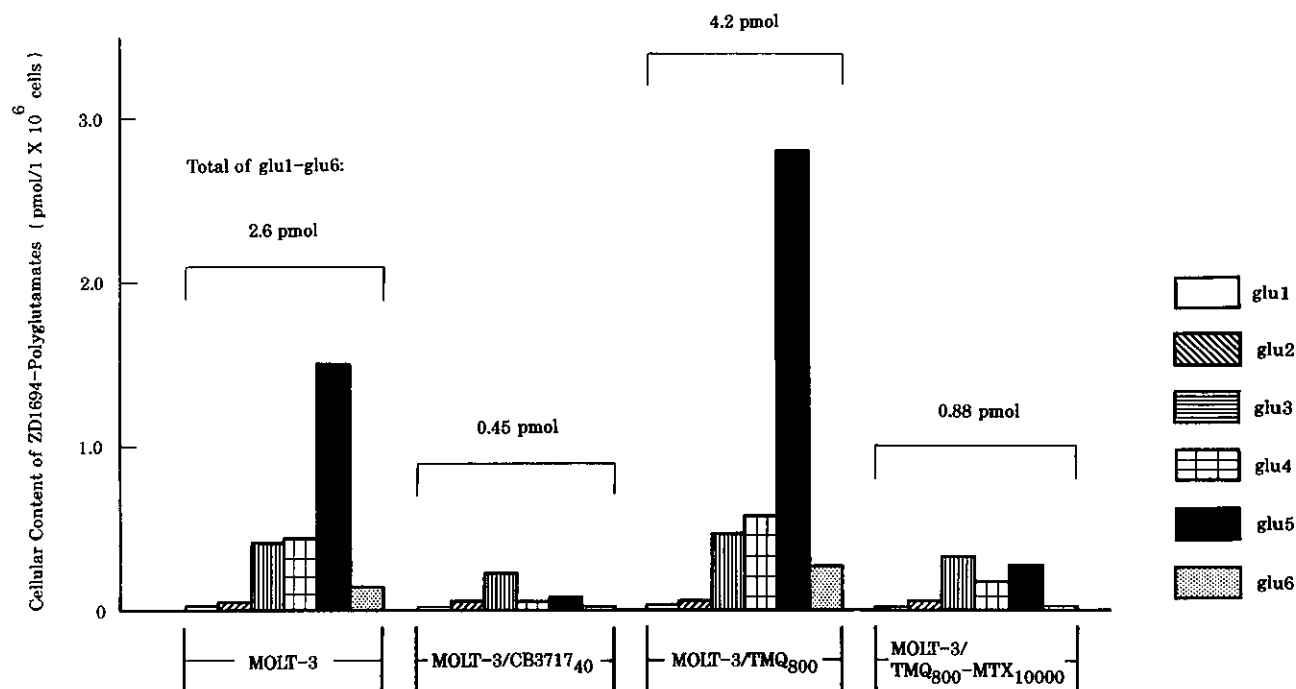
Fig. 3. ZD1694-polyglutamate distribution profiles in the sensitive and resistant MOLT-3 cells. Cells were exposed to 0.1 μM [^{3}H]ZD1694 at 37°C for 24 h, then harvested for HPLC analysis as described in "Materials and Methods." Bars indicate mean values of cellular contents of ZD1694-polyglutamates in a representative duplicate experiment.

parent cells; there was progressive accumulation of intracellular [^{3}H]ZD1694 in the former cells, while the cellular contents of the drug remarkably declined in the latter cells after 15 min. The much greater uptake of [^{3}H]MTX in the MOLT-3/TMQ$_{800}$-MTX$_{10000}$ cells may be attributed to the overproduction of variant DHFR in this subline, even though its enzyme affinity for MTX was lower than that in the wild-type cells.

Polyglutamation capacity in the sensitive and resistant cells Cellular metabolism of ZD1694 to polyglutamate forms was determined after 24-h incubation of the cells with 0.1 μM [^{3}H]ZD1694 and is illustrated in Fig. 3. The cellular total-drug accumulation pattern was similar to that in the cellular uptake study at 30 min (Fig. 2A), except for the lesser increment in drug accumulation in MOLT-3/TMQ$_{800}$-MTX$_{10000}$ cells. The pentaglutamate, which was the predominant metabolite in the parent cells, accounted for 66% of the total cellular drug in MOLT-3/TMQ$_{800}$ cells and was approximately 2-fold higher than in the parent MOLT-3 cells. In contrast, total cellular drug content was much lower in MOLT-3/ CB3717$_{40}$ and this was accompanied by a shift in the polyglutamate distribution profile to shorter chain length polyglutamates, indicating an alteration in polyglutamation capacity in this subline. Approximately 3-fold lesser

accumulation of tri- to hexaglutamates of ZD1694 was observed in MOLT-3/TMQ$_{800}$-MTX$_{10000}$ cells as compared with MOLT-3 cells, and this appears to result in the cross-resistance to ZD1694 in this subline.

DISCUSSION

In the present study, we tested the activity of ZD1694 against antifolate-resistant MOLT-3 cells with various mechanisms of resistance and analyzed the cellular metabolism of this novel folate-based TS inhibitor in the resistant cells to obtain an understanding of the pharmacological basis of the biological activity of the drug. The results indicated that the biological activity of ZD1694 ran in parallel with intracellular accumulation of the drug as polyglutamate forms.

ZD1694 utilizes the RFC to enter cells[14] and is rapidly metabolized to polyglutamate forms by FPGS inside the cells.[28] Since ZD1694 shows greatly enhanced potency for TS inhibition as the glutamate chain is elongated,[29] the ability of the cells to produce polyglutamate forms of the drug and to accumulate them inside cells must affect the biological efficacy of this drug. In fact, our previous studies demonstrated that extensive polyglutamation of the drug in K562 leukemia cells could account for the

greater sensitivity of the cells to ZD1694 than that of MOLT-3,[17] and cells with defective polyglutamation displayed extremely high resistance to this drug.[30, 31] In contrast, MTX is polyglutamated much more slowly in the cells and MTX-polyglutamates are retained inside cells but, unlike ZD1694 polyglutamates, are not significantly more potent as inhibitors of the target enzyme, DHFR, than the parent monoglutamate,[32] thus the lack of MTX-polyglutamates in cells with defective polyglutamation does not contribute to MTX resistance under continuous drug-exposure conditions. Defective polyglutamation would have significance for MTX resistance only under short-term drug-exposure conditions, when the cellular retention of the drug as polyglutamate forms is critical for the cytotoxic efficacy.[31] Resistance to TMQ, which is a non-polyglutamatable DHFR inhibitor and does not use RFC to enter cells, in MOLT-3/TMQ$_{800}$ cells had little or no influence on the biological activity of ZD1694, but the cells showed a higher polyglutamation activity than the parent cells (Fig. 3). On the other hand, sequential enhancement of MTX resistance in the TMQ-resistant cells resulted in lower accumulation of polyglutamated drug in the cells. The defective polyglutamation in MOLT-3/TMQ$_{800}$-MTX$_{10000}$ is substantiated by the observation that the level of ZD1694-polyglutamates formed (tri- to hexa-) was decreased to about one-third of that in the parent cells, with a shift in the polyglutamate distribution profile to shorter chain length polyglutamates. It is likely that the higher substrate activity of ZD1694 for FPGS than that of MTX, which was used for the selection of the resistant clone, allows the formation of polyglutamates of the former drug to some extent in this subline. The observation that cellular accumulation of [^{3}H]ZD1694 reached a plateau at 30 min in this subline (Fig. 2A) was similar to our previous finding with polyglutamation-defective cell lines[31] and supports the existence of diminished polyglutamation in this subline. Further work will be required to elucidate the exact mechanism of defective polyglutamation in this subline, including measurement of FPGS and γ-glutamyl hydrolase activities (the latter is responsible for the degradation of polyglutamates of folates to monoglutamate).

Diminished polyglutamation was also observed in MOLT-3/CB3717$_{40}$ which was made resistant to CB3717, a prototype folate-based TS inhibitor. This subline also showed impaired membrane transport for both ZD1694 and MTX (Fig. 2A, B), suggesting an alteration in the drug transport system via RFC. This finding supports multiple transport pathways for CB3717, as reported by Jansen et al.[33] Cross-resistance to ZD1694 in this subline, therefore, resulted from at least two mechanisms of resistance to ZD1694, impaired RFC and diminished polyglutamation. The cells displayed enhanced sensitivity (collateral sensitivity) to TMQ (Table I). This

phenomenon had been demonstrated in MTX-resistant cell lines with impaired RFC,[34, 35] and recently in cells with defective polyglutamation,[31] which resulted in a reduced retention of folates in the cells. Although MOLT-3/TMQ$_{800}$-MTX$_{10000}$ cells should also show collateral sensitivity to TMQ because of a polyglutamation defect in this subline, the remarkable increase in DHFR activity may have negated the increase in sensitivity to TMQ, while the TMQ-CB3717 doubly resistant cells (MOLT-3/TMQ$_{200}$-CB3717$_{30}$) with no change in DHFR, but with supposedly defective polyglutamation, showed a collateral sensitivity to TMQ.[36]

Increases in DHFR activity in MOLT-3/TMQ$_{800}$ (2-fold) and MOLT-3/TMQ$_{800}$-MTX$_{10000}$ cells (15-fold) are unlikely to influence ZD1694 resistance, whereas a slight increase in TS activity seen in the former cells may contribute to the resistance. Indeed, an increase in TS activity was reported to be one of the major mechanisms of resistance to folate-based TS inhibitors[30, 37] and those drugs were not active against TS-overproducing cell lines.[38, 39] A potential increase in the sensitivity to ZD1694 in MOLT-3/TMQ$_{800}$ cells by virtue of a higher rate of polyglutamation appears to be counteracted by the increase in TS activity in this subline.

In conclusion, ZD1694 could overcome antifolate resistance through a mechanism such as DHFR overproduction irrespective of normal or altered gene expression, but must undergo polyglutamation inside the cells to exert its maximal biological activity. ZD1694 was less potent in cells with a low rate of polyglutamation and, therefore, cells that had acquired diminished polyglutamation as a resistance mechanism were cross-resistant to this drug. Furthermore, differences in rate of polyglutamation between tissues may be related to the toxic effects of ZD1694; the high rate of polyglutamation in normal gut mucosal cells in mouse is supposed to be responsible for the severe intestinal toxicity of this drug.[40] The critical role of polyglutamation of this drug on its biological activity or toxicity implies that the cytotoxic effect of ZD1694 may be predictable through determination of the ability of the cells to produce polyglutamates, that is, by assay of FPGS activity in the cells.

ACKNOWLEDGMENTS

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