Anti-Cancer Drugs 1999, **10**, pp. 677–683

Preclinical study

Variable expression of the folylpolyglutamate synthetase gene at the level of mRNA transcription in human leukemia cell lines sensitive, or made resistant, to various antifolate drugs

Yuzuru Takemura,[1,3] Hiroyuki Kobayashi[1,4] and Hayato Miyachi[2]

[1]Department of Laboratory Medicine, National Defense Medical College, Tokorozawa, Saitama 359-8513, Japan. [2]Department of Clinical Pathology, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan. [3]Present address: Department of Pathology and Information Technology Program, Baylor College of Medicine, Texas Medical Center, Houston, TX 77030-3498, USA. [4]Present address: Department of Pharmacology, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan.

We investigated the expression of the folylpolyglutamate synthetase (FPGS) gene at the mRNA level in MOLT-3 and K562 human leukemia cell lines sensitive, or made resistant, to methotrexate (MTX) and/or trimetrexate (TMQ), or raltitrexed (ZD1694). Northern blot analysis demonstrated approximately 3-fold higher FPGS mRNA expression in K562 cells than that in MOLT-3 cells, being consistent with graded polyglutamation capacities of these cell lines. A slight increase in the expression of the FPGS gene was observed in the TMQ-resistant MOLT-3 cells (MOLT-3/TMQ_{800}); moreover, sequential development of MTX resistance in the TMQ-resistant cells (MOLT-3/TMQ_{800}–$MTX_{10\,000}$) resulted in a further enhancement of FPGS mRNA expression despite of decreased polyglutamation capacity in this subline. Another MTX-resistant subline with impaired reduced folate carrier (MOLT-3/$MTX_{10\,000}$) also showed overexpression of FPGS mRNA. Conversely, both raltitrexed-resistant sublines (MOLT-3/ZD1694·C and K562/ZD1694·C) displayed a moderately decreased expression of FPGS mRNA. These findings did not correspond to the virtual absence of ZD1694 polyglutamates inside the former cells nor to possibly intact polyglutamation capacity in the latter cells. These results indicate that FPGS mRNA expression may predict cellular ability to produce polyglutamate metabolites of antifolate drugs in the sensitive cells, but does not necessarily reflect FPGS function at the enzyme level in the antifolate-resistant tumor cells. [© 1999 Lippincott Williams & Wilkins.]

Key words: Antifolate resistance, folylpolyglutamate synthetase, leukemia cells, mRNA transcription, polyglutamation capacity.

Introduction

Folylpolyglutamate synthetase (FPGS) catalyzes the addition of glutamate residues to physiological folates and glutamate-containing antifolate compounds such as methotrexate (MTX) and raltitrexed (ZD1694; Tomudex). Elongation of the glutamate chain of physiological folates is significant for two reasons: (i) intracellular retention of folates as polyglutamate forms and (ii) increase in cofactor activity of 5,10-methylene tetrahydrofolate on thymidylate synthase (TS).[1] Like physiological folates, polyglutamatable antifolate drugs are retained inside cells as polyglutamate metabolites and continue to exert their cytotoxic activity after removal of the drug from the extracellular circumstances. Folate compounds have different substrate activities for FPGS,[2,3] e.g. raltitrexed, a novel folate-based TS inhibitor, is the best known substrate for isolated FPGS and is metabolized to polyglutamate derivatives much faster than MTX.[3,4] Polyglutamates of raltitrexed progressively enhance their potency toward TS as the glutamate chain is elongated.[5,6]

Recent studies have demonstrated that diminished cellular polyglutamation significantly contributed to the acquired resistance to polyglutamatable antifolate drugs.[6–12] In fact, leukemia cells resistant to antifolates by virtue of diminished polyglutamation showed an extremely high magnitude of resistance to raltitrexed.[11,12] MTX is markedly less active against

This work was supported in part by a grant from the Japan Research Foundation for Clinical Pharmacology.

Correspondence to Y Takemura, Pathology/Information Technology Program, Baylor College of Medicine, One Baylor Plaza, Room 126E, Texas Medical Center, Houston, TX 77030-3498, USA.
Tel: (+1) 713 799-7105; Fax: (+1) 713 790-7052;
E-mail: takemura@bcm.tmc.edu

polyglutamation-defective cells when the cells were cultured in short-term drug exposure conditions, in which drug retention is critically important for cytotoxic effect of MTX.[12,13] MTX polyglutamates, unlike raltitrexed, are not significantly more potent as inhibitors of the target enzyme, dihydrofolate reductase (DHFR), than the parent drug (monoglutamate),[14] and thus the importance of their formation is limited to their drug-retentive properties. On the other hand, cytotoxic activity of non-polyglutamatable antifolate drugs such as trimetrexate (TMQ) may not be influenced by the cellular activity of FPGS and, in fact, TMQ is active against antifolate-resistant cells with defective polyglutamation.[11,12,15]

Cellular ability to produce polyglutamate forms of folate compounds was also reported to relate to the sensitivity or inherent (natural) resistance of tumor cells to polyglutamatable antifolate drugs. Inherent resistance to MTX was associated with reduced cellular polyglutamation capacity in soft tissue sarcoma cell lines.[16,17] K562 chronic myelogenous leukemia cell line showed a 3-fold higher sensitivity to raltitrexed than MOLT-3, an acute lymphoblastic leukemia (ALL) cell line, being consistent with a much higher polyglutamation rate in the former cells.[15] In addition, the inherent resistance of acute myeloid leukemia (AML) cells to MTX is considered to result from the weaker polyglutamation ability of these cells as compared with that of ALL cells.[18,19]

Our previous studies demonstrated altered polyglutamation capacity in various antifolate-resistant leukemia cells.[12,15,20] MOLT-3 cells made resistant to raltitrexed revealed extremely diminished polyglutamation of the drug with a high magnitude of resistance irrespective of methods used to establish resistant cells.[12] In contrast, the MOLT-3 cells selected for resistance to TMQ were accompanied with an accelerated polyglutamation rate of raltitrexed as compared with the parent MOLT-3 cells.[20] However, further acquisition of MTX resistance in the TMQ-resistant cells resulted in a reduced polyglutamation rate in the doubly-resistant cells.[20] K562 cells acquired resistance to raltitrexed through an extremely impaired reduced folate carrier (RFC) function but not through diminished polyglutamation.[15] In the present study, we investigated FPGS gene expression at the mRNA level in these MTX- and/or TMQ-, or raltitrexed-resistant MOLT-3 and K562 sublines and observed some unforeseen results of FPGS gene expression in these cells. We provide some evidence for whether gene expression at the level of mRNA transcription can predict cellular polyglutamation capacity and consequent drug cytotoxic efficacy in the sensitive and resistant tumor cells to antifolate drugs, or not.

Materials and methods

Biological and pharmacological properties of MOLT-3 and K562 cell lines sensitive or resistant to various antifolate drugs are summarized in Table 1. Selection methods for, and cellular properties of, these resistant cell lines were described in detail elsewhere.[12,15,20–24]

Figure 1 illustrates cellular ability of these cell lines to produce polyglutamate metabolites, re-constituting data taken from our previous works[12,15,20,25] and unpublished experimental results. Although we have already demonstrated an increased or decreased expression of FPGS mRNA in MOLT-3/MTX_{10000} or MOLT-3/ZD1694-C cells, respectively,[12] we used these

Table 1. Biological and pharmacological properties of MOLT-3 and K562 sublines resistant to TMQ and/or MTX, or raltitrexed

Cell line	Population doubling time (h)	Relative resistance (-fold)[a]			Known mechanism(s) for antifolate resistance
		MTX	TMQ	Raltitrexed	
MOLT-3	18	1	1	1	–
MOLT-3/TMQ_{800}	22	7.0	300	1.2	Impaired membrane transport for TMQ; increase in DHFR activity[21]
MOLT-3/TMQ_{800}–MTX_{10000}	35	11 000	>2500	6.8	Amplification of mutated DHFR gene; diminished polyglutamation[20,22,23]
MOLT-3/MTX_{10000}	32	7800	0.88	1400	Impaired RFC; amplification of DHFR gene[24]
MOLT-3/ZD1694-C	26	0.53[b]	0.033	1600	Diminished polyglutamation[12]
K562	22	1	1	1	–
K562/ZD1694-C	24	170	1.6	4200	Impaired RFC[15]

[a]Relative resistance is given by: IC_{50} values of the resistant cells/IC_{50} value of the parent cells, when cells were cultured for 72 h with a drug.
[b]Relative resistance was 120-fold when cells were exposed to MTX for 24 h.

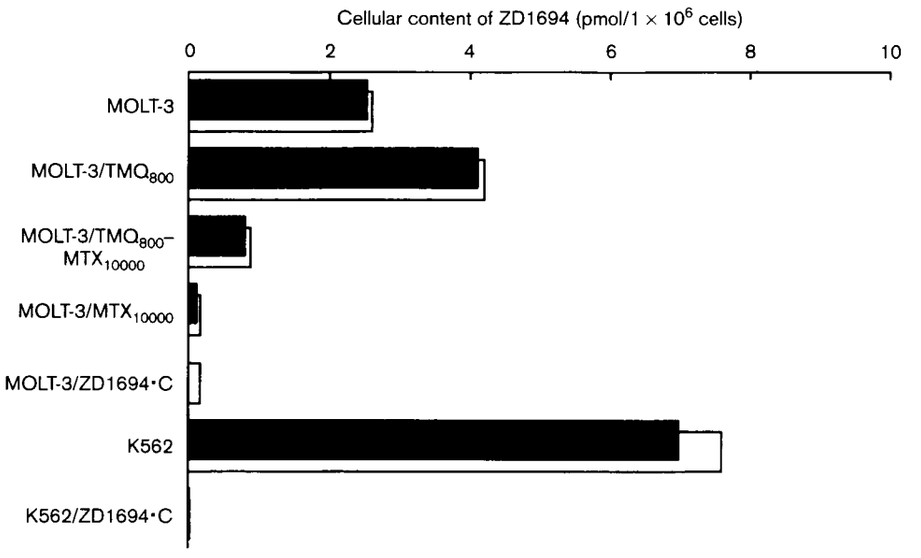

Figure 1. Polyglutamation capacity of the sensitive and resistant MOLT-3 and K562 cell lines to TMQ and/or MTX, or raltitrexed (ZD1694). Cells were incubated with 0.1 μM [5-^{3}H]ZD1694 at 37°C for 24 h, then harvested for measurement of intracellular ZD1694 polyglutamate formation. Cellular content of ZD1694 was measured by the ion-pairing HPLC method using synthetic ZD1694 polyglutamate standards.[12] White columns represent total cellular contents of ZD1694 accumulated and black columns overlapped represent total amounts of triglutamate or longer chain length polyglutamates produced. Total amount of tri- to hexa-glutamate in K562/ZD1694-C composed 95% of total cellular [^{3}H]ZD1694 with the same distribution profile as that of the parent cells.[15] Presenting data were reconstituted from Takemura *et al.*[12,15,20] and unpublished raw data.

sublines again in this study to serve as control cells over- or under-expressing FPGS mRNA. Cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (Gibco) at 37°C and fed with fresh medium twice a week. Resistance to raltitrexed and TMQ in the respective resistant subline was stable for more than 12 months in the culture medium devoid of the drug. In contrast, MTX resistance in MOLT-3/MTX_{10000} and MOLT-3/TMQ_{800}-MTX_{10000} was unstable in the medium without the drug, so that these two sublines had been maintained in the medium containing 50 μM of MTX up to 2 or 3 weeks before experiments. The magnitude of resistance to respective drug was periodically checked by using the MTT assay.[26]

The full-length cDNA for the human FPGS gene was a generous gift from Dr B Shane (University of California, Berkeley, CA). The FPGS probe (2.2 kb fragment) was prepared by *Eco*RI digestion from a pTZ18U plasmid.[27] The cDNA for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (pHcGAP, *Pst*I-*Xba*I-digested 1.4 kb fragment) was obtained from the ATCC (Rockville, MD). Total cellular RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method.[28] Following quantification of RNA by a spectrophotometer at a wavelength of 260 nm, 5 μg total RNA per lane was size-fractionated by electrophoresis in 0.41 M formaldehyde/1% agarose gels. RNA was transferred onto Maximum Strength Nytran nylon membranes using Turboblotter Rapid Downward Transfer Systems (Schleicher and Schuell, Keene, NH) as described by Chomczynski.[29] The membranes were hybridized with ^{32}P-labeled cDNA probes. Specific hybridization signals were visualized by autoradiography using Konica SR-G film (Konica, Tokyo, Japan) between two intensifying screens at −80°C for 6–24 h.

Results

Figure 2(A) shows the expression of FPGS mRNA by Northern blot analysis in the MOLT-3 sublines sensitive and resistant to TMQ and/or MTX. A slight increase in FPGS mRNA expression was observed in MOLT-3/TMQ_{800} cells (Figure 2A, lane 2) as compared with the parent cells (Figure 2A, lane 1), corresponding to an increase in polyglutamation rate of raltitrexed (Figure 1). The gene expression was obviously increased (Figure 2A, lane 3) when the TMQ-resistant cells were sequentially made resistant to MTX, although polyglutamation capacity was conversely decreased in the

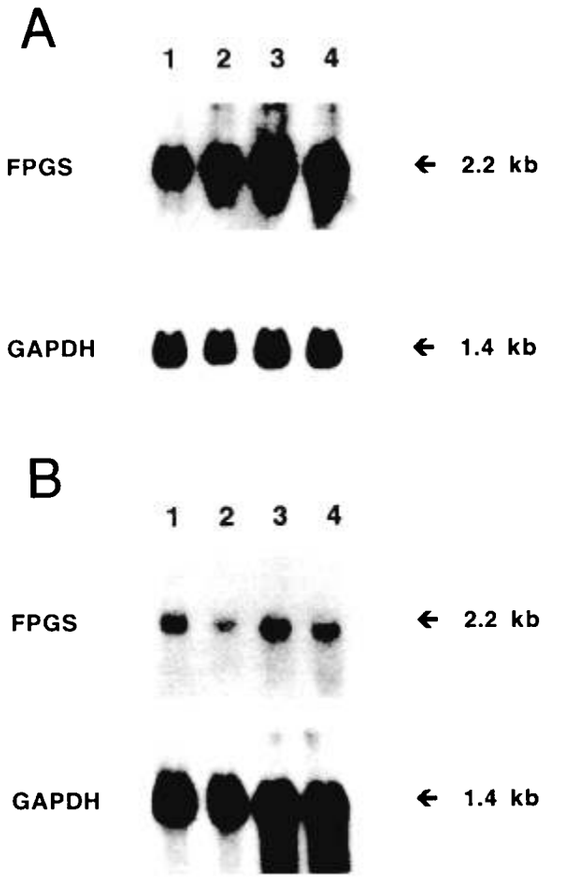

Figure 2. Expression of the FPGS gene at the mRNA level in MOLT-3 and K562 cells sensitive and resistant to TMQ and/or MTX, or raltitrexed (ZD1694). Total RNA (5 μg) was applied to each lane. All lanes were equally loaded, as determined with a GAPDH probe. The results were reproducible in two to four experiments. (A) Lane 1, MOLT-3; lane 2, MOLT-3/TMQ_{800}; lane 3, MOLT-3/TMQ_{800}–MTX_{10000}; lane 4, MOLT-3/MTX_{10000}. (B) Lane 1, MOLT-3; lane 2, MOLT-3/ZD1694-C; lane 3, K562; lane 4, K562/ZD1694-C.

MOLT-3/TMQ_{800}-MTX_{10000} cells as compared with MOLT-3 and MOLT-3/TMQ_{800} cells. As demonstrated in our previous study,[12] another MTX-resistant MOLT-3 subline (MOLT-3/MTX_{10000}) also showed enhanced FPGS mRNA expression (Figure 2A, lane 4) despite of the shift to shorter chain length metabolites in the polyglutamate production profile.

FPGS mRNA expression in the MOLT-3 and K562 cell lines sensitive or resistant to raltitrexed was compared in Figure 2B. The sensitive K562 cells (Figure 2B, lane 3) expressed approximately 3-fold higher FPGS mRNA than the MOLT-3 cells (Figure 2B, lane 1) when band intensities were quantified by a densitometer, and this virtually corresponded to the 2.5-fold higher sensitivity to, and 3-fold greater cellular accumulation of raltitrexed in K562 cells than those in MOLT-3 cells.[15] Signal intensity from MOLT-3/ZD1694-C (Figure 2B, lane 2) was approximately 40% of that from MOLT-3 (Figure 2B, lane 1). However, this was not consistent with the virtual absence of tri- to hexa-glutamate metabolites of raltitrexed in this resistant MOLT-3 subline (Figure 1). K562/ZD1694-C (Figure 2B, lane 4) displayed a significant decrease in FPGS mRNA transcription as well. Total cellular drug content accumulated in the resistant K562 cells was less than 1% of that in the parent K562 cells following 24 h incubation with 0.1 μM raltitrexed, resulting from extremely impaired RFC function,[15,30] but there was no change in the raltitrexed-polyglutamate distribution profile as compared with that seen in the parent cells.[15]

Discussion

In this study, we demonstrated variable expression of the FPGS gene at the mRNA level in MTX- and/or TMQ-, or raltitrexed-resistant leukemia cell lines. Interestingly, the expression did not necessarily correspond to cellular polyglutamation capacity in these cell lines. Since none of our resistant MOLT-3 and K562 sublines used for experiments demonstrated a significant increase in γ-glutamyl hydrolase activity (Takemura *et al.*[12,31] and unpublished data), decreases in polyglutamation capacity measured were attributable to quantitative or qualitative alteration of FPGS in the cells.

MOLT-3/TMQ_{800} cells which were selected for the resistance to TMQ, a non-polyglutamatable DHFR inhibitor, showed a higher polyglutamation rate and an increase in FPGS mRNA expression in parallel. In fact, an increase in FPGS activity concomitant with enhanced mRNA expression was also reported in the L1210 cells made resistant to another lipophilic DHFR inhibitor, metoprine.[32] Sequential development of MTX resistance in the TMQ-resistant cells (MOLT-3/TMQ_{800}-MTX_{10000}) resulted in a reduced cellular ability of polyglutamation, but in further enhancement of FPGS mRNA transcription. Another MTX-resistant MOLT-3 subline (MOLT-3/MTX_{10000}) showed the up-regulation of FPGS mRNA transcription as well, despite of the shift to shorter chain length metabolites in the polyglutamate production profile.[12] On the other hand, both raltitrexed-resistant sublines (MOLT-3/ZD1694-C and K562/ZD1694-C) displayed a decreased FPGS mRNA expression as compared with their parent cell lines. A moderate decrease in FPGS mRNA expression could not explain the virtual absence of cellular polyglutamate metabolites of

raltitrexed in MOLT-3/ZD1694·C cells. In addition, down-regulation of FPGS mRNA transcription in K562/ZD1694·C cells was also an unforeseen finding contrary to the intact ability to produce polyglutamate metabolites in this subline.[15] These results suggest that FPGS mRNA expression levels may not reflect the actual cellular ability of polyglutamation nor predict cytotoxic efficacy of polyglutamatable antifolate drugs in some antifolate-resistant tumor cells.

Cell multiplication of two MTX-resistant sublines was much slower than the parent MOLT-3 cells (Table 1) and this seems to result from intracellular folate deficiency through an extremely impaired RFC function (MOLT-3/MTX_{10000}) or through a diminished polyglutamation of folate compounds (MOLT-3/TMQ_{800}-MTX_{10000}). In fact, Kano *et al.*[33] demonstrated incremental requirements not only of reduced folates but also of pteroylglutamate (oxidized folate) for optimal growth of the former cells. The MTX-resistant cells with impaired RFC showed a collateral sensitivity to TMQ which does not utilize RFC nor membrane-associated folate binding protein for cell entry. Relative resistance of MOLT-3/MTX_{10000} to TMQ (0.88-fold; Table 1) might be a big value of collateral sensitivity if considered a >10-fold increase of DHFR activity in this subline.[23,24] The increased sensitivity of the transport-defective MTX-resistant cells to lipophilic antifolates is probably attributable to relatively low levels of intracellular folates by virtue of impaired transport of folates.[34] Likewise, the raltitrexed-resistant MOLT-3 cells with extremely diminished polyglutamation displayed a remarkable collateral sensitivity to TMQ,[12] suggesting the intracellular folate deficiency caused by dispersion of non-polyglutamated folates outside cells. In the aspect of intracellular folate homeostasis, therefore, it is likely that our MTX- and TMQ/MTX-resistant sublines, if not having the same resistance mechanism, may up-regulate FPGS gene transcription to retain much more folates inside cells as polyglutamate forms or to compensate for folate deficiency.

An alternative possible explanation for the paradoxical enhancement of FPGS mRNA expression in our TMQ/MTX doubly-resistant cells is the induction of mutated FPGS mRNA which does not encode functional enzyme. This hypothesis is analogous to our previous observation that the doubly-resistant subline amplified and overexpressed a variant DHFR gene with two critical point mutations which resulted in the low affinity of the enzyme for MTX.[22,23] Recent studies demonstrated a dissociation between the FPGS mRNA levels and the polyglutamation rates in the antifolate-resistant human and murine cell lines with defective polyglutamation,[12,35,36] indicating that cells resistant to antifolates by virtue of decreased FPGS activity do not necessarily have reduced FPGS mRNA expression. Indeed, in one of the above studies, edatrexate-resistant L1210 cells were shown to express a variant FPGS mRNA with significantly lower efficiency in mediating formation of the FPGS peptide product in a manner correlating with FPGS activity.[36] Furthermore, there are several different splice forms of FPGS mRNA and variable expression of the different forms in different cells was demonstrated.[37–39] Some of the spliced mRNA do not encode functional FPGS, associating with acquired resistance to antifolates (B Shane, pers commun). Paradoxical overexpression of FPGS gene contrary to the reduced capacity of polyglutamation in the MOLT-3/TMQ_{800}-MTX_{10000} cells may suggest such an alteration in the FPGS gene and consequent production of functionally defective FPGS.

FPGS mRNA expression in MOLT-3/ZD1694·C was approximately 40% of that in MOLT-3; however, it is difficult to reconcile the moderate decrease in FPGS mRNA expression with the virtual absence of tri- to hexa-glutamates of raltitrexed in the cells and high magnitude of resistance to this drug (Figure 1 and Table 1). In addition, despite the extremely defective polyglutamation of raltitrexed used for the selection of the resistance, the growth of the resistant MOLT-3 cells was only minimally affected,[12] suggesting that physiological folates essential for cell survival and proliferation could be effectively polyglutamated. The dissociation between defective polyglutamation and cell multiplication rate was also observed in the raltitrexed-resistant L1210 cells.[11] These observations may support the induction of mutated FPGS being responsible for decreased enzyme affinity only for the drug(s). Finally, the raltitrexed-resistant K562 cells with extremely impaired RFC (K562/ZD1694·C) displayed an unforeseen but significant decrease in FPGS mRNA transcription as well, although there was no change in the polyglutamate distribution profile.[15] This finding with K562/ZD1694·C supported the observation by Roy *et al.* of a transport-defective, MTX-resistant L1210 variant which had decreased FPGS mRNA transcription,[32] but was not consistent with our experimental result with the MTX-resistant MOLT-3 cells (MOLT-3/MTX_{10000}). Regulation of FPGS might be different in cells with different biological characteristics.

In conclusion, there may be variable expression in FPGS mRNA in cells resistant to antifolate drugs, and the expression would not be predictive for polyglutamation ability of the cells and subsequent cytotoxic efficacy of polyglutamatable antifolates, at least in the resistant cells. Although exact mechanism(s) to explain the difference in FPGS regulation in different

antifolate-resistant cells remain(s) to be clarified, cellular biological dispositions and biochemical properties of antifolate drugs used for the establishment of resistance seem to be involved in the intricate regulation.

Acknowledgments

The authors thank Dr Barry Shane for providing cDNA for the human FPGS gene and valuable suggestions for this study.

References

1. Shane B. Folylpolyglutamate synthesis and role in the regulation of one-carbon metabolism. *Vit Horm* 1989; **45**: 263–335.
2. George S, Cichowicz DJ, Shane B. Mammalian folylpoly-γ-glutamate synthetase. 3. Specificity for folate analogues. *Biochemistry* 1987; **26**: 522–9.
3. Habeck LL, Mendelsohn LG, Shih C, *et al.* Substrate specificity of mammalian folylpolyglutamate synthetase for 5,10-dideazatetrahydrofolate analogs. *Mol Pharmacol* 1995; **48**: 326–33.
4. Jackman AL, Marsham PR, Moran RG, *et al.* Thymidylate synthase inhibitors: the *in vitro* activity of a series of heterocyclic benzoyl ring modified 2-desamino-2-methyl-N^{10}-substituted-5,8-dideazafolates. *Adv Enz Reg* 1991; **31**: 13–27.
5. Ward WHJ, Kimbell R, Jackman AL. Kinetic characteristics of ICI D1694: a quinazoline antifolate which inhibits thymidylate synthase. *Biochem Pharmacol* 1992; **43**: 2029–31.
6. Takemura Y, Jackman AL. Folate-based thymidylate synthase inhibitors in cancer chemotherapy. *Anti-Cancer Drugs* 1997; **8**: 3–16.
7. Jackman AL, Farrugia DC, Gibson W, *et al.* ZD1694 (Tomudex): a new thymidylate synthase inhibitor with activity in colorectal cancer. *Eur J Cancer* 1995; **31A**: 1277–82.
8. Bertino JR, Srimatkandada S, Carman MD, *et al.* Drug resistance: new approaches to treatment. In: Chandra P, ed. *New experimental modalities in the control of neoplasia*. New York: Plenum 1986: 183–93.
9. McCloskey DE, McGuire JJ, Russell CA, *et al.* Decreased folylpolyglutamate synthetase activity as a mechanism of methotrexate resistance in CCRF-CEM human leukemia sublines. *J Biol Chem* 1991; **266**: 6181–7.
10. McGuire JJ, Haile WH, Russell CA, Galvin JM, Shane B. Evolution of drug resistance in CCRF-CEM human leukemia cells selected by intermittent methotrexate exposure. *Oncol Res* 1995; **7**: 535–43.
11. Jackman AL, Kelland LR, Kimbell R, *et al.* Mechanisms of acquired resistance to the quinazoline thymidylate synthase inhibitor ZD1694 (Tomudex) in one mouse and three human cell lines. *Br J Cancer* 1995; **71**: 914–24.
12. Takemura Y, Kobayashi H, Gibson W, Kimbell R, Miyachi H, Jackman AL. The influence of drug-exposure conditions on the development of resistance to methotrexate or ZD1694 in cultured human leukaemia cells. *Int J Cancer* 1996; **66**: 29–36.
13. Pizzorno G, Mini E, Coronnello M, *et al.* Impaired polyglutamylation of methotrexate as a cause of resistance in CCRF-CEM cells after short-term, high-dose treatment with this drug. *Cancer Res* 1988; **48**: 2149–55.
14. Chabner BA, Allegra CJ, Curt GA, *et al.* Polyglutamation of methotrexate. *J Clin Invest* 1985; **76**: 907–12.
15. Takemura Y, Gibson W, Kimbell R, Kobayashi H, Miyachi H, Jackman AL. Cellular pharmacokinetics of ZD1694 in cultured human leukaemia cells sensitive, or made resistant, to this drug. *J Cancer Res Clin Oncol* 1996; **122**: 109–17.
16. Li WW, Lin JT, Tong WP, Trippett TM, Brennan MF, Bertino JR. Mechanisms of natural resistance to antifolates in human soft tissue sarcomas. *Cancer Res* 1992; **52**: 1434–8.
17. Li WW, Lin JT, Schweitzer BI, Tong WP, Niedzwiecki D, Bertino JR. Intrinsic resistance to methotrexate in human soft tissue sarcoma cell lines. *Cancer Res* 1992; **52**: 3908–13.
18. Lin JT, Tong WP, Trippett TM, *et al.* Basis for natural resistance to methotrexate in human acute non-lymphocytic leukemia. *Leuk Res* 1991; **15**: 1191–6.
19. Gorlick R, Goker E, Trippett T, Waltham M, Banerjee D, Bertino JR. Intrinsic and acquired resistance to methotrexate in acute leukemia. *N Engl J Med* 1996; **335**: 1041–8.
20. Takemura Y, Kobayashi H, Miyachi H, Gibson W, Kimbell R, Jackman AL. Biological activity and intracellular metabolism of ZD1694 in human leukemia cell lines with different resistance mechanisms to antifolate drugs. *Jpn J Cancer Res* 1996; **87**: 773–80.
21. Arkin H, Ohnuma T, Kamen BA, Holland JF, Vallabhajosula S. Multidrug resistance in a human leukemic cell line selected for resistance to trimetrexate. *Cancer Res* 1989; **49**: 6556–61.
22. Kobayashi H, Kim N, Halatsch ME, Ohnuma T. Specificity of ribozyme designed for mutated *DHFR* mRNA. *Biochem Pharmacol* 1994; **47**: 1607–13.
23. Miyachi H, Takemura Y, Kobayashi H, Ando K, Ando Y. Expression of variant dihydrofolate reductase with decreased binding affinity to antifolates in MOLT-3 human leukemia cell lines resistant to trimetrexate. *Cancer Lett* 1995; **88**: 93–9.
24. Ohnuma T, Lo RJ, Scanlon KJ, *et al.* Evolution of methotrexate resistance of human acute lymphoblastic leukemia cells *in vitro*. *Cancer Res* 1985; **45**: 1815–22.
25. Takemura Y, Kobayashi H, Miyachi H. Cellular and molecular mechanisms of resistance to antifolate drugs: new analogues and approaches to overcome the resistance. *Int J Hematol* 1997; **66**: 459–77.
26. Takemura Y, Kobayashi H, Miyachi H, Hayashi K, Sekiguchi S, Ohnuma T. The influence of tumor cell density on cellular accumulation of doxorubicin or cisplatin *in vitro*. *Cancer Chemother Pharmacol* 1991; **27**: 417–22.
27. Garrow TA, Admon A, Shane B. Expression cloning of a human cDNA encoding folylpoly (γ-glutamate) synthetase and determination of its primary structure. *Proc Natl Acad Sci USA* 1992; **89**: 9151–5.
28. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid-guanidinium-thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156–9.
29. Chomczynski P. One-hour downward alkaline capillary transfer for blotting of DNA and RNA. *Anal Biochem* 1992; **201**: 134–9.

30. Kobayashi H, Takemura Y, Ohnuma T. Variable expression of *RFC1* in human leukemia cell lines resistant to antifolates. *Cancer Lett* 1998; **124**: 135–42.
31. Takemura Y, Kobayashi H, Miyachi H, Sekiguchi S. Down-regulation of γ-glutamyl hydrolase activity in the human leukemia cell lines made resistant to methotrexate or ZD1694. *Proc Am Ass Cancer Res* 1996; **37**: 381.
32. Roy K, Mitsugi K, Sirlin S, Shane B, Sirotnak FM. Different antifolate-resistant L1210 cell variants with either increased or decreased folylpolyglutamate synthetase gene expression at the level of mRNA transcription. *J Biol Chem* 1995; **270**: 26918–22.
33. Kano Y, Ohnuma T, Holland JF. Folate requirements of methotrexate-resistant human acute lymphoblastic leukemia cell lines. *Blood* 1986; **68**: 586–91.
34. Sirotnak FM, Moccio DM, Goutas LJ, Kelleher LE, Montgomery JA. Biochemical correlates of responsiveness and collateral sensitivity of some methotrexate-resistant murine tumors to the lipophilic antifolate, metoprine. *Cancer Res* 1982; **42**: 924–30.
35. Lu K, Yin MB, McGuire JJ, Bonmassar E, Rustum YM. Mechanisms of resistance to *N*-[5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazoline-6-yl-methyl)-*N*-methyl-amino]-2-thenoyl]-L-glutamic acid (ZD1694), a folate-based thymidylate synthase inhibitor, in the HCT-8 human ileocecal adenocarcinoma cell line. *Biochem Pharmacol* 1995; **50**: 391–8.
36. Roy K, Egan MG, Sirlin S, Sirotnak FM. Posttranscriptionally mediated decreases in folylpolyglutamate synthetase gene expression in some folate analogue-resistant variants of the L1210 cell. *J Biol Chem* 1997; **272**: 6903–8.
37. Chen L, Qi H, Korenberg J, Garrow TA, Choi YJ, Shane B. Purification and properties of human cytosolic folylpoly-γ-glutamate synthetase and organization, localization, and differential splicing of its gene. *J Biol Chem* 1996; **271**: 13077–87.
38. Roy K, Mitsugi K, Sirotnak FM. Organization and alternate splicing of the murine folylpolyglutamate synthetase gene. *J Biol Chem* 1996; **271**: 23820–7.
39. Roy K, Mitsugi K, Sirotnak FM. Additional organizational features of the murine folylpolyglutamate synthetase gene. *J Biol Chem* 1997; **272**: 5587–93.

(Received 27 May 1999; accepted 8 June 1999)