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Molecular mechanisms of resistance to antifolates, a review*

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Methotrexate (MTX) is a clinically important antifolate that has been used in combination with other chemotherapeutic agents in the treatment of malignancies including acute lymphocytic leukemia, osteosarcoma, carcinomas of the breast, head and neck, choriocarcinoma and non-Hodgkin's lymphoma. The primary target of MTX is the enzyme dihydrofolate reductase (DHFR) which catalyzes the reduction of folate and 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. Understanding of MTX action has revealed how cells acquire resistance to this drug. The four known mechanisms of MTX resistance are a decrease in the uptake of the drug, a decrease in the retention of the drug due to defective polyglutamylation or an increase in polyglutamate breakdown, an increase in the enzyme activity and a decrease in the binding of MTX to DHFR. The molecular basis for some of these mechanisms has been elucidated in MTX resistant cell lines; in particular the occurrence of gene amplification resulting in increased DHFR and point mutations resulting in altered DHFR with reduced affinity for MTX. Cloning of the human folylpolyglutamate synthase gene and the reduced folate transport gene have been reported recently and should facilitate the identification of the molecular basis of these resistant phenotypes. DHFR protein has been shown to regulate its synthesis by exerting an inhibitory influence on its own translation. Addition of MTX relieves this inhibition thus providing a possible molecular explanation for the rapid rise in DHFR activity noted in some cells after MTX administration. Alterations in genes involved in regulating the cell cycle such as cyclin D1 and the retinoblastoma (Rb) gene have also been shown to influence cellular response to MTX. Overexpression of cyclin D1 in HT1080, a human fibrosarcoma cell line, results in decreased MTX sensitivity. The molecular basis of this observation is under investigation. Abnormalities in the Rb gene may also have profound effects on MTX sensitivity. Rb interacts with the family of transcription factors called E2F reducing transcription of genes that contain E2F binding sites in the promoter regions e.g. DHFR. When Rb is deleted or rendered nonfunctional levels of "free" or unbound

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Abbreviations: AICAR, aminoimidazole carboxamide ribonucleotide trnasformylase; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthase; GAR transformylase, 5'-phosphoribosylglicineamide transformylase; GGH, gamma glutamylhydrolase; LV, leucovorin; MTX, methotrexate; RB, retinoblastoma; RFC, reduced folate carrier; pRb, retinoblastoma protein; TMTX, trimetrexate; TS, thymidylate synthase.

E2F are high resulting in enhanced transcription of genes such as DHFR. This results in increased DHFR protein and may lead to MTX resistance. As the knowledge regarding mechanisms of resistance increases newer approaches to circumvent such resistance or to target resistant cells can be undertaken.

Methotrexate (MTX), in combination with other chemotherapeutic agents, has been successfully used in the clinic for the treatment of malignancies that include acute lymphoblastic leukemia (ALL), non-Hodgkin's lymphoma, osteogenic sarcoma, choriocarcinoma and carcinomas of breast, head and neck. In addition to the use of MTX for treatment of malignancies, it is also used for treatment of nonmalignant conditions such as rheumatoid arthritis, psoriasis and in the prevention of graft *versus* host disease [1]. Although response to treatment is observed in patients, many of them relapse due to development of resistance. The underlying mechanisms responsible for resistance to MTX have been the subject of intense scrutiny for the past decade. In order to understand the mechanisms of resistance to MTX we must first understand the mode of action of the drug. The primary cellular target of MTX is the enzyme dihydrofolate reductase (DHFR) which catalyzes the reduction of folate and 7,8--dihydrofolate to 5,6,7,8-tetrahydrofolate [2]. To act on this intracellular target MTX must first enter the cell. It is known that MTX enters the cell primarily by an active carrier transport mechanism also shared by the reduced folates and mediated by the reduced folate carrier (RFC) [3]. Once inside the cell, MTX is modified so that it is retained in the cell. This is accomplished by addition of glutamate residues (upto 5) catalyzed by the enzyme folylpolyglutamate synthase (FPGS) [4, 5]. MTX and polyglutamylated forms of MTX are tight binding inhibitors of DHFR and interfere with pyrimidine *viz.* thymidylate biosynthesis. MTX polyglutamates as well as dihydrofolate polyglutamates are also inhibitors of enzymes involved in purine biosynthesis including 5'-phosphoribosylglycinamide transformylase (GAR transformylase) and aminoimidazole carboxamide ribonucleotide transformylase (AICAR transformylase) [6–8]. Enzymatic removal of glutamyl groups from polyglutamylated forms of both folates and MTX is catalyzed by gamma glutamyl hydrolase (GGH). This enzyme maintains a balance between the various glutamylated forms in concert with FPGS [9–12].

MECHANISMS OF RESISTANCE

In experimental cell culture model systems four common mechanisms of resistance to MTX have been identified; a decrease in uptake of the drug, a decrease in retention of the drug due to defective polyglutamylation or increased polyglutamate breakdown, an increase in DHFR activity as well as a decrease in the binding of MTX to DHFR [13].

TRANSPORT

The most common mechanism of resistance to MTX is due to defective transport of the drug. This is true for cell culture model systems as well as leukemic blast cells from patients with ALL at the time of relapse after MTX treatment. This is an example of acquired resistance to MTX after acute or chronic exposure to MTX [14–16]. With the recent cloning of the gene encoding the mouse as well as the human RFC it will now be possible to determine the molecular basis of resistance in cells that have to have impaired MTX uptake [16–19]. Using a complementation assay a mouse cDNA clone has been identified that partially restores the transport defect in a human breast cancer cell line ZR75 [18]. This gene codes for a protein of 58 kDa and has structural similarity with the human glucose transporter GLUT1. Hydropathy analysis has revealed that it contains 12 transmembrane domains and belongs to the superfamily of transporter molecules collectively referred to as the major facilitator superfamily members of which carry out sugar transport in mammalian cells [20]. The human cDNA clone that complement transport defect in a Chinese hamster ovary cell line encodes a putative RFC of 64 kDa with approximately 50% homology to the hamster and mouse RFCs at the amino-acid level [19]. Currently we are working on the development of screening

methods for identification of mutations in RFC in ALL patients at relapse.

POLYGLUTAMYLATION

MTX, upon entry into the cell, is modified by the sequential addition of glutamate residues catalyzed by the enzyme FPGS. It has been observed that this ability to form long chain polyglutamates of MTX correlates well with the therapeutic outcome of several leukemias. Adult T and B lineage ALL have been found to accumulate lower amounts of long chain polyglutamates as compared to pediatric B lineage ALL and this correlates with poorer prognosis for the former [21–24]. Pediatric T lineage ALL was also seen to accumulate less long chain polyglutamates as compared to its B lineage counterpart [24]. Nonhyperdiploid B lineage blasts were also found to accumulate significantly less long chain polyglutamates once again correlating with outcome [23]. The ability to polyglutamylate has now been accepted to be an important determinant of outcome of leukemias, most likely through predicting response to MTX. It is worthwhile to mention that the monoblastic subset of acute myeloid leukemia (AML) FAB classification-M5 subtype was found to form polyglutamates of MTX as efficiently as childhood ALL blasts [25]. MTX may be an effective therapy for this subtype and may have been overlooked because other subtypes of AML are intrinsically resistant to MTX. Lack of accumulation of MTX polyglutamates may be due to lack of formation of long chain polyglutamates, due to impaired activity of FPGS or due to enhanced breakdown of the formed long chain polyglutamates due to increased activity of GGH [26]. The relative contribution of FPGS and GGH to overall MTX resistance remains an area of active investigation. Mammalian FPGS has molecular mass of 60 kDa and catalyzes the addition of glutamates to all naturally occurring folates as well some folate analogs such as MTX. Characterization of alterations in FPGS at the molecular level will now be possible with the recent cloning of the cDNA for human FPGS [27]. Using sequence information made available recently a quantitative polymerase chain reaction (PCR) method has been developed to measure FPGS mRNA levels in leukemic blasts. FPGS mRNA expression using this method correlated well with the enzyme activity levels [28]. Relatively little is known about the enzyme GGH as compared to FPGS. The hydrolases can be classified broadly into two types; lysosomal type with pH optimum around 4.4 and cytoplasmic type with a more alkaline pH optimum. It appears that the lysosomal hydrolase is more important in regulation of polyglutamate chain length. The bovine hepatic enzyme remains the most well characterized lysosomal hydrolase to date. This enzyme has a molecular mass of 108 kDa and contains highly reactive sulfhydryl groups. Several laboratories including our own are engaged in the purification and further molecular characterization of GGH from tumor cells in an attempt to understand the role of GGH in intrinsic and acquired resistance to MTX [29, 30].

GENE AMPLIFICATION

As intracellular MTX levels are limited by a saturable uptake process and MTX is a tight binding inhibitor of the enzyme DHFR, an increase in the level of intracellular DHFR would cause MTX resistance. It has been demonstrated that exposure of tumor cells to increasing doses of MTX results in amplification of the DHFR gene. In fact four case reports have been published indicating that low level gene amplification occurs in tumor cells from patients treated with MTX [31–34]. This agrees well with the concept that low level amplification of the DHFR gene is sufficient to cause clinical resistance to MTX. In a recent study reported from this laboratory approx. 30% of relapsed ALL patients (9/29) had low level DHFR gene amplification (2 to 4 copies of the gene) as compared to only 10% (4/38) of newly diagnosed ALL patients. This study confirmed that low level DHFR gene amplification is a mechanism of acquired resistance to MTX [35].

MUTATIONS IN DHFR

Alterations in DHFR with reduced affinity for MTX have been observed in cell lines that were exposed to increasing doses of the drug [36–41]. In order to examine whether mutations in the DHFR occur in patient samples we have tested

blast samples from over twenty patients for decreased binding of MTX to DHFR; in addition we have analyzed the complete DHFR cDNA sequence of 8 patients; 6 ALL (4 relapsed and 2 untreated) and 2 patients with untreated AML. We have not found any evidence for decreased binding at the enzymatic level or alterations in the coding region of the DHFR cDNA from these patient samples. Thus, it seems unlikely that mutations in DHFR are a major mechanism of acquired resistance in patients exposed to MTX. In contrast, mutations leading to a decreased affinity of DHFR for the antifolate pyrimethamine, used in the treatment of malaria, is the major cause of resistance to this drug [42].

TUMOR SUPPRESSOR GENES

Loss of functional retinoblastoma protein (pRb) may also contribute to antimetabolite resistance as cells lacking pRb may have increased levels of enzymes associated with proliferation (e.g. DHFR, thymidylate synthase (TS)) as a consequence of increased levels of free E2F, a transcription factor that is normally quenched by hypophosporylated pRb. When cells begin to move out of G1 and into S phase pRb becomes hyperphosphorylated and releases the bound E2F which then enhances the transcription of genes invovled in DNA synthesis [43]. We have observed that a human osteosarcoma cell line SaOs2 that lacks pRb is intrinsically resistant to MTX as compared to lines with pRb present. This cell line has a higher level of DHFR and the increase in this activity is attributable to increased trancriptional activity of the DHFR gene. When the cDNA encoding pRb is reintroduced into this cell line, sensitivity to MTX is restored. There are other human sarcoma cell lines established in the laboratory which lack pRb and these cell lines also show a similar resistance to MTX [44].

Since free or unbound E2F levels increase when pRb is hyperphosphorylated we reasoned that activation of regulators of pRb phosphorylation like the cyclin-dependent kinase system (the cyclin D1-CDK4) may also cause an increase in DHFR transcription and hence MTX resistance. Transfection of cyclin D1 into HT-1080 human sarcoma cell line resulted in an increase in MTX resistance in clones that expressed high levels but not in clones that expressed low levels of the gene suggesting a direct relationship between the level of cyclin D1 expression and DHFR transcription (Hochhauser, D. *et al.*, unpublished results from our laboratory). Since certain tumors have been shown to express high levels of cyclin D1 it will be interesting to examine the relationship between overexpression of cyclin D1, DHFR transcription and MTX sensitivity in these tumors. It is becoming increasingly clear that deregulation of cell cycle genes has a profound effect on antimetabolite resistance.

In cell lines with mutated p53, a tumor suppressor gene, amplification of the target gene has been observed after antimetabolite exposure [45, 46]. In a study carried out in this laboratory it was reported that mutations in p53 gene were seen in 7 of 9 ALL blast samples with DHFR amplification. In contrast, only 2 of 26 ALL blast samples without DHFR gene amplification had p53 mutations [35].

TRANSLATIONAL AUTOREGULATION

Previous studies have shown that administration of MTX to patients leads to an increase in the level of DHFR protein in both normal and leukemic leukocytes as well as in erythrocytes within hours to days [47]. Studies carried out *in vitro* using a lymphoblastoid cell line showed that an increase in DHFR protein is not transcriptionally mediated but was abrogated by cycloheximide treatment, suggesting that new protein synthesis was involved. The rapid increase in DHFR observed after MTX treatment could have been due to either protection of DHFR from degradation by bound MTX and / or dihydrofolate, or to an increase in translation of this enzyme [48]. An increase in TS activity has been reported after 5-FU treatment, and it has been suggested that this increase may be due to regulation at the translational level [49]. Using a rabbit reticulocyte translation system we have found that DHFR protein inhibited its own synthesis. The reversal of this inhibition by MTX or dihydrofolate may explain in part the observed induction of DHFR activity in normal and leukemic cells after MTX treatment [50]. In order to locate the region of DHFR mRNA involved in binding to the DHFR protein we have been using a system where ^{32}P-labelled *in vitro*

transcribed RNA from different regions of the coding sequence of DHFR mRNA is UV cross linked to DHFR protein and electrophoresed after RNAse treatment. Competition experiments with molar excess of cold RNA fragments have revealed that a small region toward the 3′ end may be involved in the binding (Ercikan-Abali, E. *et al.*, unpublished results from our laboratory).

bcl-2 AND RESISTANCE

In experimental systems that aim to determine cytotoxicity of anticancer agents against tumor cells, the end point usually measured is cell death. Many types of cancer cells undergo apoptosis or programmed cell death when exposed to a wide variety of chemotherapeutic agents. Thus, interference with induction of the apoptotic process in response to chemotherapy will result in resistance to the particular drug. It has been shown previously that the *bcl-2* gene encodes a 26-kDa mitochondrial protein that contributes to neoplastic cell growth mainly by enhancing cell survival through inhibition of the apoptotic pathway [51]. Transfection of the *bcl-2* gene in a human pre-B-cell leukemia line 697 resulted in an increase in cell survival and greatly reduced DNA fragmentation when grown in the presence of antineoplastic agents including MTX [52]. It was found that although the drugs were able to suppress the proliferation of these *bcl-2* transfectants, re-initiation of cell growth occurred upon withdrawal of drug. Although resistance is not limited to any particular agent, the overall resistance phenotype imparted by *bcl-2* can have direct relevance to clinical correlation between poor prognosis and *bcl-2* gene rearrangements or translocations [53]. If further clinical studies indeed point to a clear association between *bcl-2* and drug resistance it will suggest that mechanisms of drug resistance may not be limited to alterations in specific intracellular targets.

STRATEGIES TO OVERCOME OR EXPLOIT MTX RESISTANCE

The observation that defective transport was a common mechanism of resistance to MTX in ALL has prompted experimentation with lipophilic antifolates such as trimetrexate (TMTX) which do not use the reduced folate carrier to enter cells. Phase II clinical trials with TMTX in relapsed ALL were not encouraging owing to dose limiting severe mucositis. TMTX has now been approved for use against *Pneumocystis carinii* infections in acquired immunodeficiency syndrome (AIDS) patients without severe side effects because of the simultaneous administration of 5-formyltetrahydrofolate (leucovorin, LV). The combination of TMTX and LV rescues the host cells from TMTX toxicity but is toxic to the parasite cells as they have no reduced folate carrier and hence cannot take up LV. This selectivity has encouraged us to target transport deficient MTX resistant cells with the TMTX -LV combination. Initial *in vitro* studies with CCRF-CEM cells have shown that cells transport deficient MTX resistant cells are sensitive to TMTX while the MTX sensitive cells can be protected by LV. In order to take this one step further we have treated SCID mice bearing tumors from transport deficient MTX resistant CCRF-CEM ALL cells with the TMTX-LV combination. This treatment led to tumor regression without any significant toxicity [54]. We plan to treat relapsed ALL patients with the TMTX-LV combination with the hope that a similar selectivity will be observed for tumor cells while the normal cells will be protected.

GENE THERAPY USING DRUG RESISTANCE GENES

Introduction and efficient expression of drug resistant genes such as mutant DHFR cDNAs into bone marrow progenitor cells should allow further dose intensification and has the potential to increase the cure rate for those tumors in which a steep dose response curve is present. Using retroviral constructs bearing a mutant human DHFR cDNA (the Ser31 mutant) we have infected mouse marrow progenitor cells and have transplanted irradiated mice with these transduced cells. Mice receiving transduced marrow progenitor cells were protected from the lethal effects of MTX while the controls died. We have recently initiated studies to transduce human peripheral blood and cord blood CD34+ stem cells *in vitro* with retroviral contructs bearing the Ser31 mutant human DHFR cDNA. Our results indicate that

these transduced CD34+ stem cells are less sensitivite to MTX than mock transduced cells [55]. We plan to exploit this system for cotransduction and ultimate *in vivo* selection of nonselectable genes [56].

REFERENCES

1. Bertino, J.R. (1993) Karnofsky Memorial Lecture. Ode to methotrexate. *J. Clin. Oncol.* **11**, 5–14.
2. Blakley, R.L. (1984) Dihydrofolate reductase; in *Folates and Pterins. Chemistry and Biochemistry of Folates. Dihydrofolate reductase* (Blakley, R.L. & Benkovic, S.J., eds.) vol 1, pp. 191–251, Wiley, New York.
3. Goldman, I.D. & Matherly, L.H. (1986) The cellular pharmacology of methotrexate; in *Membrane Transport of Antineoplastic Agents. International Encyclopedia of Pharmacology and Therapeutics* (section 118) (Goldman, I.D., ed.) p. 283, Pergamon, Oxford, U.K.
4. McGuire, J.J., Hsieh, P., Coward, J.K. & Bertino, J.R. (1980) Enzymatic synthesis of folylpolyglutamates. Characterization of the reaction and its products. *J. Biol. Chem.* **255**, 5776–5788.
5. Whitehead, V.M., Rosenblatt, D., Vuchich, M.J., Shuster, J.J., Witte, A., Beaulieu, D. (1990) Methotrexate and methotrexate polyglutamates in lymphoblasts at diagnosis of childhood ALL: A pilot prognostic factor analysis. *Blood* **76**, 44–50.
6. Allegra, C.J., Chabner, B.A., Drake, J.C., Lutz, R., Rodbard, D. & Jolivet, J. (1985) Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates. *J. Biol. Chem.* **260**, 9720–9726.
7. Allegra, C.J., Drake, J.C., Chabner, B.A. (1985) Inhibition of phosphoribosyl-aminoimidazolecarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamate. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4881–4885.
8. Jolivet, J., Cowan, K.H., Curt, G.A. & Chabner, B.A. (1983) The pharmacology and clinical use of methotrexate. *N. Engl. J. Med.* **309**, 1094–1103.
9. Silnik, M., Reddel, R., Bethel, M. & Rowe, P.B. (1975) Gamma-glutamyl hydrolase (conjugase) purification and properties of the bovine hepatic enzyme. *J. Biol. Chem.* **250**, 5982–5994.
10. Elsenhans, B., Ahmad, O. & Rosenberg, I. (1984) Isolation and characterization of pteroylglutamate hydrolase from rat intestinal mucosa. *J. Biol. Chem.* **259**, 6364–6368.
11. Chandler, C., Wang, T. & Halsted, C. (1986) Pteroylglutamate hydrolase from human jejunal brush borders: purification and characterization. *J. Biol. Chem.* **261**, 928–933.
12. Wang, T., Chandler, C. & Halsted, C. (1986) Intracellular pteroylglutamate hydrolase from human jejunal mucosa: isolation and characterization. *J. Biol. Chem.* **261**, 13551–13555.
13. Schweitzer, B.I., Dicker, A.P. & Bertino, J.R. (1990) Dihydrofolate reductase as a therapeutic target. *FASEB J.* **4**, 2441–2452.
14. Trippett, T., Schlemmer, S., Elisseyeff, Y., Goker, E., Wachter, M., Steinherz, P., Tan, C., Berman, E., Wright, J.S., Rosowsky, A., Schweitzer, B.I. & Bertino, J.R. (1992) Defective transport as a mechanism of acquired resistance to methotrexate in patients with acute leukemia. *Blood* **80**, 1158–1162.
15. Matherly, L.H., Angeles, S.M. & Czajkowski, C.A. (1992) Characterization of transport mediated methotrexate resistance in human tumor cells with antibodies to the membrane carrier for methotrexate and tetrahydrofolate cofactors. *J. Biol. Chem.* **267**, 23253–23260.
16. Matherly, L.H., Taub, J.W., Ravindranath, Y. Proefke, S., Wong, S.C., Gimotty, P., Buck, S., Wright, J.S. & Rosowsky, A. (1995) Elevated dihydrofolate reductase and impaired methotrexate transport as elements in methotrexate resistance in childhood acute lymphoblastic leukemia. *Blood* **85**, 500–509.
17. Williams, F.M., Murray, R.C., Underhill, T.M. & Flintoff, W.F. (1994) Isolation of a hamster cDNA clone coding for a function involved in methotrexate uptake. *J. Biol. Chem.* **269**, 5810–5816.
18. Dixon, K.H., Lanpher, B.C., Chiu, J., Kelley, K. & Cowan, K.H. (1994) A novel cDNA restores reduced folate carrier activity and methotrexate sensitivity to transport deficient cells. *J. Biol. Chem.* **269**, 17–20.
19. Williams, F.M. & Flintoff, W.F. (1995) Isolation of a human cDNA that complements a mutant hamster cell defective in methotrexate uptake. *J. Biol. Chem.* **270**, 2987–2992.
20. Marger, M.D. & Saier, M.H., Jr. (1993) A major superfamily of transmembrane facilitators that catalyze uniport, symport and antiport. *Trends Biochem. Sci.* **18**, 13–20.
21. Goker, E., Lin, J.T., Trippett, T., Elisseyeff, Y., Tong, W.P., Niedzwiecki, D., Tan, C., Steinherz, P., Schweitzer, B.I. & Bertino, J.R. (1993) Decreased polyglutamylation of methotrexate in acute lymphoblastic leukemia blasts in adults compared to children with this disease. *Leukemia* **7**, 1000–1004.
22. Lin, J.T., Tong, W.P., Trippett, T., Niedzwiecki, D., Tao, Y., Tan, C., Steinherz, P., Schweitzer, B.I.

& Bertino, J.R. (1991) Basis for natural resistance to methotrexate in human acute non-lymphocytic leukemia. *Leukemia Res.* **15**, 1191–1196.

23. Synold, T.W., Relling, M.V. & Boyett, J.M. (1994) Blast cell methotrexate polyglutamate accumulation *in vivo* differs by lineage, ploidy, and methotrexate dose in acute lymphoblastic leukemia. *J. Clin. Invest.* **94**, 1996–2001.
24. Barredo, J.C., Synold, T.W. & Laver, J. (1994) Differences in constitutive and post-methotrexate folylpolyglutamate synthetase activity in B-lineage and T-lineage leukemia. *Blood* **84**, 564–569.
25. Goker, E., Kheradpour, A., Waltham, M.C., Banerjee, D., Tong, W.P., Elisseyeff, Y. & Bertino, J.R. (1995) Acute monocytic leukemia: a myeloid subset that may be sensitive to methotrexate. *Leukemia* **9**, 274–276.
26. Galivan, J., Johnson, T., Rhee, M., McGuire, J.J., Priest, D.G. & Kesevan, V. (1987) The role of folylpolyglutamate synthetase and gamma--glutamylhydrolase in altering cellular folyl- and antifolyl-polyglutamates. *Adv. Enz. Reg.* **26**, 147–155.
27. Garrow, T.A., Admon, A. & Shane, B. (1992) Expression cloning of a human cDNA encoding folylpoly(-glutamate) synthetase and determination of its primary structure. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9151–9155.
28. Lenz, H.J., Danenberg, K., Schnieders, B., Goker, E., Peters, G.J., Garrow, T., Shane, B., Bertino, J.R. & Danenberg, P.V. (1994) Quantitative analysis of folylpolyglutamate synthetase gene expression in tumor tissues by the polymerase chain reaction: marked variation of expression among leukemia patients. *Oncol. Res.* **6**, 329–335.
29. Samuels, L.L., Goutas, L.J., Priest, D.G., Piper, J.R. & Sirotnak, F.M. (1986) Hydrolytic cleavage of methotrexate-polyglutamates by folylpolyglutamyl hydrolase derived from various tumors and normal tissues of the mouse. *Cancer Res.* **46**, 2230–2235.
30. Rhee, M.S., Wang, Y., Gopal Nair, M. & Galivan, J. (1993) Acquisition of resistance to antifolates caused by enhanced gamma-glutamyl hydrolase activity. *Cancer Res.* **53**, 2227–2230.
31. Carman, M.D., Schornagel, J.H., Rivest, R.S., Srimatkandada, S., Portlock, C.S., Duffy, T. & Bertino, J.R. (1984) Resistance to methotrexate due to gene amplification in a patient with acute leukemia. *J. Clin. Oncol.* **2**, 16–20.
32. Horns, R.C., Dower, W.J. & Schimke, R.T. (1984) Gene amplification in a leukemia patient treated with methotrexate. *J. Clin. Oncol.* **2**, 2–5.
33. Trent, J.M., Buick, R.N., Olson, S., Horns, R.C. & Schimke, R.T. (1984) Cytologic evidence for gene amplification in methotrexate resistant cells obtained from a patient with ovarian adenocarcinoma. *J. Clin. Oncol.* **2**, 8–12.
34. Curt, G.A., Corney, D.N., Cowan, K.H., Jolivet, J., Bailey, B.D., Drake, J.C., Kao-Shan, C.S., Minna, J. & Chabner, B.A. (1983) Unstable methotrexate resistance in human small cell lung cancer associated with double minute chromosomes. *New Engl. J. Med.* **308**, 99–104.
35. Goker, E., Waltham, M.C., Kheradpour, A., Trippett, T., Majumdar, M., Elisseyeff, Y., Schnieders, B., Steinherz, P., Tan, C., Berman, E. & Bertino, J.R. (1995) Amplification of the dihydrofolate reductase gene is a mechanism of acquired resistance to methotrexate in patients with acute lymphoblastic leukemia and is correlated with p53 gene mutations. *Blood* **86**, 677–684.
36. Simonsen, C.C. & Levinson, A.D. (1983) Isolation and expression of an altered dihydrofolate reductase cDNA. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2495–2499.
37. Haber, D.A., Beverly, S.M., Kiely, M.L. & Schimke, R.T. (1981) Properties of an altered dihydrofolate reductase encoded by amplified genes in cultured mouse fibroblasts. *J. Biol. Chem.* **256**, 9501–9510.
38. Srimatkandada, S., Schweitzer, B.I., Moroson, B.A., Dube, S. & Bertino, J.R. (1989) Amplification of a polymorphic dihydrofolate reductase gene expressing an enzyme with a decreased binding to MTX in a human colon carcinoma cell line, HCT-8R4 resistant to this drug. *J. Biol. Chem.* **264**, 3524–3528.
39. Melera, P.W., Davide, J.P., Hession, C.A. & Scotto, K.W. (1984) Phenotypic expression in *Escherichia coli* and sequence of two Chinese hamster lung cDNA encoding different dihydrofolate reductase. *Mol. Cell. Biol.* **4**, 38–48.
40. Dicker, A.P., Volkenandt, M.V., Schweitzer, B.I., Banerjee, D. & Bertino, J.R. (1990) Identification and characterization of a mutation in the dihydrofolate reductase gene from methotrexate resistant CHO cell line Pro-3 MTX RIII. *J. Biol. Chem.* **265**, 8317–8321.
41. McIvor, R.S. & Simonsen, C.C. (1990) Isolation and characterization of a variant dihydrofolate reductase cDNA from the methotrexate resistant murine L5178Y cells. *Nucleic Acids Res.* **18**, 7025–7032.
42. Hyde, J.E. (1990) The dihydrofolate reductase--thymidylate synthase gene in the drug resistance of malaria parasites. *Pharmacol. Ther.* **48**, 45–59.

43. Nevins, J.R. (1992) E2F; a link between the Rb tumor suppressor protein and viral oncoproteins. *Science* **258**, 424–427.

44. Li, W.W., Fan, J., Hochhauser, D., Banerjee, D., Zielinski, Z., Almasan, A., Yin, Y., Kelly, R., Wahl, G.M. & Bertino, J.R. (1995) Lack of functional retinoblastoma protein mediates increased resistance to antimetabolites in human sarcoma cell lines. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10436–10440.

45. Livingstone, L.R., White, A., Sprouse, J., Livanos, E., Jacks, T. & Tlsty, T. (1992) Altered cell cycle arrest and gene amplification potential accompany loss of wild type p53. *Cell* **70**, 923–930.

46. Yin, Y., Tainsky, M.A., Bischoff, F.Z., Strong, L.C. & Wahl, G.M. (1992) Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. *Cell* **70**, 937–944.

47. Bertino, J.R., Donohue, D.M., Simmons, B., Gabrio, B.W., Silber, R. & Huennekens, F.M. (1963) The induction of dihydrofolate reductase in leukocytes and erythrocytes of patients treated with methotrexate. *J. Clin. Invest.* **423**, 466–475.

48. Hillcoat, B.L., Swett, V. & Bertino, J.R. (1967) Increase of dihydrofolate reductase activity in cultured mammalian cells after exposure to methotrexate. *Proc. Natl. Acad. Sci. U.S.A.* **58**, 1632–1637.

49. Chu, E., Koeller, D.M., Casey, J.L., Drake, J.C., Chabner, B.A., Elwood, P.C., Zinn, S. & Allegra, C.A. (1991) Autoregulation of human thymidylate synthase messenger RNA translation by thymidylate. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8977–8981.

50. Ercikan, E., Banerjee, D., Waltham, M.C., Schnieders, B., Scotto, K.W. & Bertino, J.R. (1993) Translational regulation of the synthesis of dihydrofolate reductase; in *Chemistry and Biology of Pteridines and Folates* (Ayling, J.E., ed.) pp. 537–540, Plenum Press.

51. Korsmeyer, S.J. (1992) Bcl-2 initiates a new category of oncogenes: regulators of cell death. *Blood* **80**, 879–886.

52. Miyashita, T. & Reed, J.C. (1993) Bcl-2 oncoprotein blocks chemotherapy induced apoptosis in a human leukemia cell line. *Blood* **81**, 151–156.

53. Delia, D., Aiello, A., Soligo, D., Fontanell, E., Melani, C., Pezzella, F., Pierotti, M.A. & Della Porta, G. (1992) Bcl-2 proto-oncogene expression in normal and neoplastic human myeloid cells. *Blood* **79**, 1291–1295.

54. Lacerda, J.F., Goker, E., Kheradpour, A., Dennig, D., Elisseyeff, Y., Jagiello, C., O'Reilly, R.J. & Bertino, J.R. (1995) Selective treatment of SCID mice bearing methotrexate transport resistant human ALL tumors with trimetrexate and leucovorin. *Blood* **85**, 2675–2679.

55. Flasshove, M., Banerjee, D., Mineishi, S., Li, M.-X., Bertino, J.R. & Moore, M.A.S. (1995) *Ex vivo* expansion and selection of human CD34+ peripheral blood progenitor cells after introduction of a mutated dihydrofolate reductase cDNA *via* retroviral gene transfer. *Blood* **85**, 566–574.

56. Banerjee, D., Zhao, S.C., Li, M.-X., Schweitzer, B.I., Mineishi, S. & Bertino, J.R. (1994) Gene therapy utilizing drug resistance genes: a review. *Stem Cells* **12**, 378–385.