Int. J. Cancer: **85,** 683–690 (2000)

Publication of the International Union Against Cancer

RESISTANCE TO METHOTREXATE IN SKOV-3 CELL LINES AFTER CHRONIC EXPOSURE TO CARBAMAZEPINE IS ASSOCIATED WITH A DECREASED EXPRESSION OF FOLATE RECEPTOR

Giuseppe TOFFOLI[1], Giuseppe CORONA[1], Barbara TOLUSSO[1], Franca SARTOR[1], Roberto SORIO[2], Enrico MINI[3] and Mauro BOIOCCHI[1]*

[1]*Experimental Oncology 1, Centro di Riferimento Oncologico, National Cancer Institute, Aviano, Italy*
[2]*Medical Oncology, Centro di Riferimento Oncologico, National Cancer Institute, Aviano, Italy*
[3]*Department of Preclinical and Clinical Pharmacology University of Florence, Florence, Italy*

The detrimental effect of chronic administration of carbamazepine (CBZ) on serum and erythrocyte folates of patients has been extensively analyzed. However, at present, no data have been reported on the effect of CBZ on the intracellular content and activity of antimetabolite cytotoxic agents that can be used together with CBZ in the treatment of cancer patients. To investigate this issue, we chronically exposed *in vitro* the human ovarian cancer cell line SKOV-3 (grown under physiological folate concentrations) to CBZ, thus selecting SKOV-CBZ clones (SKOV-CBZ-50–2, SKOV-CBZ-50–5 and SKOV-CBZ-100–2) able to grow in the continuous presence of the antiepileptic drug. All of the SKOV-CBZ clones were more resistant to methotrexate (MTX) than the parental cells. MTX resistance index, as determined by the ratio between MTX concentrations inhibiting cell growth by 50% (MTX IC_{50}) in SKOV-CBZ clones and in the parental cells, ranged between 3- and 5-fold. This resistance was related to a reduced intracellular content of MTX. No alteration activity of the cellular enzymes directly involved in MTX cytotoxicity (dihydrofolate reductase, thymidylate synthase [TS] and folylpolyglutamate synthetase) was observed. SKOV-CBZ clones were cross-resistant to the TS inhibitors tomudex and CB3717, but not to the TS inhibitor 5-fluorodeoxy uridine and other antineoplastic drugs (cisplatin, doxorubicin, vincristine and taxol), whose cellular uptake is derived from transmembrane transport mechanisms different from folate receptor α (FRα) or reduced folate carrier (RFC). FRα mRNA and protein levels were significantly lower in SKOV-CBZ clones than in the parental cells. The reduced level of FRα in SKOV-CBZ clones was associated with a decreased binding capacity of folic acid. No variation of mRNA RFC expression in the SKOV-CBZ clones as compared to the parental cells was observed. Thus, after chronic exposure to CBZ, SKOV-CBZ clones develop resistance towards MTX due to defective MTX uptake. *Int. J. Cancer* 85: 683–690, 2000.

Carbamazepine (CBZ) is an antiepileptic drug currently used in the treatment of patients with epilepsy. CBZ produces a voltage-dependent block of sodium channels that can be removed by hyperpolarization. It is not clear whether CBZ binds to a receptor near or at the sodium channel (Macdonald and Kelly, 1995). CBZ is also important in the management of trigeminal neuralgia and chronic pain syndromes. Pain is the most common complaint of cancer patients and CBZ has been used in the treatment of neuropathic pain syndromes due to tumor infiltration, seizures, muscle cramps, or for symptom control in geriatric patients with terminal cancer.

Several studies have shown subnormal levels of serum folate in patients who had received prolonged administration of antiepileptic drugs such as CBZ and valproic acid (Kishi *et al.*, 1997), and an association between antiepileptic drug-induced folate deficiency and teratogenicity or adverse pregnancy outcomes has been postulated (Lewis *et al.*, 1998). At present, the biochemical mechanisms responsible for folate depletion after CBZ administration are not clear. Several mechanisms have been proposed: induction of liver enzymes that catabolize the folate or divert reducing equivalent away from folate metabolism (Lucock *et al.*, 1994), impairment of folate absorption and increased demand for folate as a cofactor in the hydroxylation of the anticonvulsant (Kishi *et al.*, 1997). It has been also hypothesized that CBZ may interact with the methylenetetrahydrofolate reductase, an enzyme involved in the intracellular folate pathway (Lucock *et al.*, 1994) and crucial for regulating serum homocysteine. Antiepileptic drugs may also interfere with intracellular folate transport (Finnell *et al.*, 1997).

Methotrexate (MTX) is an anticancer drug used widely in neoplastic and non- neoplastic disorders. MTX and folates have common metabolic pathways. Dihydrofolate reductase (DHFR) (Bertino, 1993) is the major target enzyme for MTX activity and other key enzymes of folate metabolism, such as thymidylate synthase (TS), glycinamide ribonucleotide transformylase (GARTF) and folylpolyglutamate synthetase (FPGS), have been recognized as being involved in MTX cytotoxicity. MTX can interact with the transport mechanism of folates. Therefore, transport of folates and antifolates can play a role in regulating their intracellular pool (Joel *et al.*, 1996). Two different transport systems have been described: the folate receptor (FR) and the reduced folate carrier (RFC). Three isoforms of FR, α, β and γ, have been described in humans. *FRα*, the gene believed to be homologous to the murine folate receptor FBP1, has a 10- to 90-fold higher binding affinity than FRβ for natural folates, as well as for folate analogues (Wang *et al.*, 1992). FRs have a relatively higher affinity for folic acid (FA) than for reduced folates, such as folinic acid and MTX. In contrast, RFC is characterized by a relatively high affinity for N_5-substituted reduced folates (Joel *et al.*, 1996). However, in the absence of RFC activity, the level of FRα activity and the folate composition of the medium may influence MTX sensitivity (Jansen *et al.*, 1989). In addition, other MTX transport systems with similar affinities for FA and folinic acid have been described (Henderson and Strauss, 1990).

At present, no data on the effect of chronic administration of CBZ on MTX cellular uptake and activity have been reported. To investigate this issue, we chronically exposed *in vitro* the human ovarian cancer cell line SKOV-3 (grown in conditioned medium without FA) to CBZ, thus selecting SKOV-CBZ clones able to grow in the continuous presence of the antiepileptic drug. These clones were then analyzed for the effect of chronic CBZ exposure on the cellular uptake and cytotoxic activity of MTX.

MATERIAL AND METHODS

Chemicals

[^{3}H]FA (33 Ci/mmol), [^{3}H]MTX (16.7 Ci/mmol), [^{3}H]vincristine ([^{3}H]VCR) (6.6 Ci/mmol), [^{3}H]glutamate (46 Ci/mmol), deoxy-5'[^{3}H]-uridine monophosphate (17.8 Ci/mmol) and 5-methyl-[^{14}C]-tetrahydrofolic acid (5CH_3-[^{14}C]-H_4THF) (54 mCi/

Grant sponsor: Ministero della Sanità;, Ricerca Finalizzata 1998, Italy.

*Correspondence to: Experimental Oncology 1, Centro di Riferimento Oncologico, via Pedemontana Occidentale 12, I-33081 Aviano (PN) Italy. Fax: +39 0434 659428. E-mail: mboiocchi@ets.it

Received 5 July 1999; Revised 25 August 1999

mmol) were obtained from Amersham (Aylesbury, UK). 6S-5CH_3-[^{3}H]tetrahydrofolic acid (6S-5CH_3-[^{3}H]THF) (30 Ci/mmol) was obtained from Moravek (Brea, CA). NADPH, ATP and FAD from Fluka (Buchs, Switzerland). Sodium fluoride, dithiothreitol, charcoal, BSA, dimedione, menadione and CBZ were obtained from Sigma (St. Louis, MO), and dextran T10 from Pharmacia (Uppsala, Sweden). Stock solution of 100 mg/ml CBZ was obtained dissolved in DMSO and stored at -30°C, no cytotoxicity due to DMSO alone was observed. Unlabeled FA, 5CH_3-THF and tetrahydrofolic acid (THF) were obtained from Schricks Laboratories (Jona, Switzerland), MTX from Lederle (Catania, Italy), tomudex from Zeneca (Milan, Italy), fluoro-deoxy uridine (FUDR) from Hoffman-Roche (Nutley, NJ), doxorubicin (DOX) from Pharmacia-Upjohn (Milan, Italy), VCR from Eli-Lilly (Florence, Italy), cisplatin (CDDP) and taxol from Bristol-Meyer-Squibb (Latina, Italy). CB3717 (N^{10}-propargyl-5,8-dideazafolic acid) was kindly provided by Dr. A.L. Jackman (Sutton, UK). SepPak C18 solid phase extraction columns were purchased from Waters (Milford, MA). The Bradford protein assay was performed using the Pierce (Rockford, IL) kit. All other chemicals were reagent grade. RPMI 1640 medium with FA was obtained from Bio Whittaker (Verviers, Belgium), RPMI 1640 cell culture medium without FA from Euroclone (Paignton, UK) and dialyzed FBS from Sigma.

Cell lines and CBZ-resistant clones

The human ovarian carcinoma cell line SKOV-3 (ATCC, Rockville, MD) was grown for more than 6 months as a monolayer in FA-free RPMI 1640 medium supplemented with glutamine (200 mM), streptomycin (100 μg/ml), penicillin (100 U/ml), 10% dialyzed FBS supplemented with 2 nM folinic acid. The CBZ clones were obtained from a single-step prolonged exposure of the parental cell line (SKOV-3 grown in conditioned medium) to 50 and 100 μg/ml CBZ concentrations. After 2 months of continuous exposure to CBZ, different resistant clones were selected. The cloning efficiency was about 4×10^{-5} and 1.5×10^{-5} for SKOV-3 cells selected with 50 and 100 μg/ml of CBZ, respectively. The doubling time of each clone was analyzed and only the cell clones with a doubling time similar to that of the parental cell line (*i.e.*, about 25–30 hr) were considered for this study: SKOV-CBZ-50–2, SKOV-CBZ-50–5 selected at 50 μg/ml CBZ and SKOV-CBZ-100–2 selected at 100 μg/ml CBZ.

Cytotoxicity studies

Cytotoxic effects were assessed as previously described (Toffoli *et al.*, 1994). Cells were plated in 6-well tissue culture plates (Costar, Cambridge, MA) in FA-free RPMI 1640 medium with 10% dialyzed FBS, 2 nM folinic acid and incubated in the presence of cytotoxic agents for about 3 doubling times (72 hr). Previous data (Stander *et al.*, 1998) indicate that the anticonvulsants are stable for 72 hr. Cells were then washed with saline, trypsinized and counted with a ZM counter (Coulter, Luton, UK). Results are reported as the extracellular drug concentration inhibiting cell growth by 50% (IC_{50}). IC_{50} values were extrapolated from regression analysis of cell growth inhibition curves. Drug IC_{50} values obtained by this assay were not different from those obtained by clonogenic assay in liquid medium (Toffoli *et al.*, 1994).

Crude cell extract

Cytosolic extracts were obtained from the cellular pellet of approximately 30×10^6 cells harvested at about 80% confluence. The cells were suspended with 1 ml of lysis buffer (50 mM Tris-HCl, 20 nM KCl, 10 mM $MgCl_2$ and 5 mM dithiothreitol, pH 7.6) and sonicated 4 times for 5 sec with 1 min intervals at 4°C and 50% power. The cell extract was then centrifuged at 40,000 g for 1 hr at 4°C. The supernatant (cytosolic fraction) was aliquoted and stored at -80°C until enzyme analysis.

DHFR assay

DHFR levels were evaluated using the [^{3}H]MTX binding assay, as described by Cowan *et al.* (1982). Briefly, 10 pmol of [^{3}H]MTX was added to different amounts of cell extract volume in the presence of 10 mM NADPH in 400 μl of 0.15 M potassium phosphate buffer, pH 5.3. After 30 min incubation at 4°C, unbound [^{3}H]MTX was eliminated by adding to the reaction mixture 50 μl of 10% charcoal suspension, 2.5% BSA. The reaction mixture was then mixed and spun at 10,000 g for 5 min and 200 μl of supernatant was analyzed for radioactivity.The DHFR levels are expressed as pmol of [^{3}H]MTX/mg of total protein.

TS assay

TS was determined by the tritium release assay, as described by Roberts (1966). The reaction mixture in a final volume of 50 μl contained 50 μM deoxy-5'[^{3}H]-uridine monophosphate, 100 μM THF, 10 mM dithiothreitol, 0.05% formaldehyde and a dilution of cellular extract. The mixture was incubated for 15 and 30 min. The reaction was terminated by addition of 200 μl of charcoal suspension in 5% TCA. The suspension was centrifuged at 10,000 g for 5 min and 100 μl of supernatant was counted for radioactivity. Results are expressed as nmol/hr/mg of protein of cellular extract.

MTHFR assay

MTHFR activity was evaluated using a modified method of Kutzbach and Stokstad (1967). The reaction mixture in a final volume of 600 μl contained 50 μM FAD, 20 μM 5CH_3-[^{14}C]-H_4THF, 3.5 mM menadione bisulfite, 10 mM ascorbate, 1 mM EDTA and 50–100 μl of cell extract in 0.2 M phosphate buffer (pH 6.3). The mixture was incubated for 30 min at 37°C. The reaction was terminated by adding 200 μl of 50 μM dimedione in 50% ethanol solution and 100 μl 3 M sodium acetate (pH 3.0) and heated at 95°C for 15 min. The [^{14}C]CH_2O formed was extracted with 3 ml toluene. After centrifugation, 2 ml of organic phase was counted for radioactivity. Enzyme activity was expressed as nmol/hr/mg of protein of cellular extract.

FPGS assay

FPGS activity was measured as reported by Jansen *et al.* (1992) with few modifications. The assay mixture in a final volume of 250 μl contained 100 mM tris-HCl, pH 8.85, 20 mM $MgCl_2$, 10 mM KCl, 10 mM dithiothreitol, 10 mM ATP, 4 mM [^{3}H]glutamate, 250 μM MTX and 200 μl of cell extract. The enzyme activity was measured after a 4-hr incubation at 37°C. The reaction was stopped by the addition of 1 ml of ice-cold 5 mM glutamate. Separation of the MTX-unbound [^{3}H]glutamate from the MTX-[^{3}H]di-glutamate was performed by solid phase extraction using a SepPak C18 (500 mg) column (Waters). The MTX-[^{3}H]di-glutamate was eluted with 3 ml of methanol and radioactivity content was measured by liquid scintillation counting. The results are expressed as pmol/hr/mg of protein cellular extract.

mRNA extraction and cDNA synthesis

Total cellular RNA was extracted from log phase cells by treating the cells directly on the plate with trizol, according to the Gibco (Grand Island, NY) kit. For cDNA synthesis, 0.5–1 μg of total RNA was retrotranscribed using 10 pmol of oligo-d$(T)_{16}$ primer with AMV reverse transcriptase (Promega, Madison, WI), according to the manufacturer's instructions.

Quantification of cellular expression of FRα and RFC mRNA

Molecular analysis and quantification of FRα and RFC were performed using competitive PCR, as described (Corona *et al.*, 1998; Miotti *et al.*, 1995). In brief, a fixed amount of target cDNA was mixed with increasing amounts of the competitor template and amplified with the specific primers for FRα or RFC in 50 μl of reaction solution, using 1.25 U of Taq polymerase (Promega). The amplification products were resolved by electrophoresis in 4% agarose gel in TBE and stained with ethidium bromide. The bands of the competitor and the target cDNA sequence were quantified by densitometric scanning. The number of the molecules of the competitor added to the sample before starting amplification was plotted against the competitor/target template ratio and the number of template copies of the target sequence was extrapolated for a ratio value of 1. Equivalence in the amounts of starting RNA as

well in the reverse transcription efficiency among samples was evaluated by comparison with expression of the S14 ribosomal subunit protein. The results are expressed as the number of template copies per microgram of total RNA.

Drug cellular uptake

The parental cell line SKOV-3 grown in conditioned medium and the SKOV-CBZ clones were seeded at a density of 0.5–1 $\times$ 10^6 cells in 6-cm Petri dishes in folate-free RPMI with 10% dialyzed FCS. After 24 hr, the cells were exposed to [^{3}H]MTX, 5-CH_3-[^{3}H]THF, [^{3}H]FA and [3H]VCR, respectively, for the specified period in folate-free RPMI supplemented with 20 mM HEPES, pH 7.2, at 37°C. The cells were then chilled on ice for 3 min and washed 3 times with 10 ml of ice-cold PBS (pH 7.4). To strip off membrane FR-bound folate, cells were washed for 60 sec with 4 ml of ice-cold acidic saline buffer (pH 3.5 by acetic acid) and then washed with ice-cold PBS (pH 7.4). Subsequently, the cells were harvested with 2 ml of 2% trypsin solution and counted by a hemocytometer; the intracellular content of the drug was evaluated by liquid scintillation counting. The unspecific cellular drug uptake was evaluated for each drug by determining the drug uptake in the presence of a large excess (1,000-fold) of unlabeled drug. All the results represent specific drug accumulations obtained by subtracting total drug uptake from unspecific drug uptake. The results are expressed as pmol $\times$ 10^6 cells. Competition studies were performed by determining the MTX cellular uptake when cells were incubated at 20 nM and 1 μM MTX in the presence of CBZ (200 and 400 μM), FA (range, 20 nM–2 μM) or 5-formyl-THF acid (leucovorin, LV) (range, 20 nM–2 μM). Cell volumes of SKOV-3 parental cells and SKOV-CBZ clones, as determined with the ZM counter, were not significantly different.

CBZ cellular uptake

For CBZ uptake, the cells were incubated with 100 μg/ml (approximately 200 μM) CBZ for 4 hr at 37°C in medium RPMI supplemented with 10% FCS. The cells were then processed as reported for MTX, 5CH_3-THF, FA and VCR, with the difference that cellular CBZ content was evaluated by an HPLC method. Trypsin cell suspension was treated with 100 μl of NaOH 1 M, and CBZ was extracted with 8 ml of chloroform for 1 hr under slow agitation in the dark. The organic phase was then saved and dried under vacuum. The residue was dissolved in 100 μl of acetonitrile and 10 μl of this solution was injected in a C18 NovaPak 300 $\times$ 3.9-mm column equilibrated with 50 mM ammonium acetate, pH 4.0, and 15% acetonitrile. Flow rate was 1 ml/min and the UV detector was set up at 254 nm. The retention time of CBZ under these conditions was 6.8 min. The drug cellular concentration was obtained from the peak area data by the external method using a calibration curve ranging from 1 to 100 ng/ml. The results are expressed as pmol CBZ/10^6 cells.

FA binding assay

Cellular FA binding capacity was determined as reported by Corona *et al.* (1998). Briefly, cells in the growth log phase were chilled and washed with ice-cold physiological solution. Cells were then incubated on ice with 20 nM [^{3}H]FA in 20 mM HBSS buffer containing 20 mM HEPES, 10^7 mM NaCl, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1 mM $MgCl_2$ and 7 mM glucose (pH 7.4). After 30 min of incubation, the cells were washed 4 times with ice-cold physiological solution and then treated with HBSS (pH 3.5) for 60 sec to release cellular FR-bound [^{3}H]FA. The acidic radioactive solution was evaluated by liquid scintillation counting. Results were expressed as pmol FA/10^6 cells.

Cytoimmunofluorescence analysis

Cells were incubated with the monoclonal antibody (MAb) MOv18 (1:40) (kindly provided by Dr. Miotti, Milan, Italy) or the isotopic control mouse (IgG_1) (1:20) (Becton Dickinson, Mountain View, CA) in a total volume of 200 μl for 1 hr at 4°C. The primary antibody was eliminated by centrifugation and the FITC-conjugated sheep antimouse IgG_1 (Sigma) was added for 15 min at a dilution of 1:20. The suspension was filtered through 50-μm nylon filters and analyzed by a FACScan flow cytometer. The FRα fluorescence index (FI) was defined as the mean of FRα-associated fluorescence divided by isotopic control fluorescence.

Statistical analysis

The non-parametric Mann-Whitney test was used to assess statistical significance for unpaired data and non-parametric Wilcoxon signed rank test for paired data. A p value $\leq$ 0.05 was considered significant.

RESULTS

SKOV-CBZ clones and drug cytotoxicity

Growth inhibitory effects (IC_{50} and resistance index [RI]) of MTX and other antineoplastic drugs against the SKOV-3 parental cells (grown in conditioned medium without FA) and the SKOV-CBZ clones are shown in Table I. All the selected clones were more resistant to CBZ than the parental cells and were cross-resistant to MTX, tomudex and CB3717, but not to FUDR, VCR, taxol and CDDP. The RI, determined by the ratio between MTX IC_{50} in CBZ clones and MTX IC_{50} in parental cells, was 5.0, 3.3 and 3.0 for SKOV-CBZ-50–5, SKOV-CBZ-50–2 and SKOV-CBZ-100–2, respectively, and it did not appear to be related to the concentration of CBZ used for clone selection.

To evaluate whether folates may protect against growth inhibitory effects of MTX according to their differential affinity for FRα and RFC, growth inhibition experiments were carried out using conditioned medium supplemented with LV or FA, respectively. The SKOV-3 parental cells and SKOV-CBZ-50–5 clone were investigated. It can be anticipated that LV will compete for

TABLE I – DRUG CYTOTOXICITY IN THE PARENTAL SKOV-3 CELL LINE AND IN SELECTED SKOV-CBZ CLONES[1]

Drugs	SKOV-3	Drug IC_{50}					
		SKOV-CBZ-50-2	RI	SKOV-CBZ-50-5	RI	SKOV-CBZ-100-2	RI
CBZ	280.6 ± 102.4	792.5 ± 104.3[2]	(2.8)	1,043.2 ± 142.4[2]	(3.7)	740.2 ± 177.8[2]	(2.6)
MTX	2.1 ± 0.6	6.9 ± 2.2[2]	(3.3)	10.5 ± 2.3[2]	(5.0)	6.3 ± 1.0[2]	(3.0)
Tomudex	0.6 ± 0.2	2.1 ± 0.6[2]	(3.5)	2.3 ± 0.6[2]	(3.8)	2.2 ± 0.7[2]	(3.7)
CB3717	86.3 ± 30.2	209.7 ± 68.1[2]	(2.4)	255.4 ± 38.5[2]	(3.0)	224.3 ± 65.5[2]	(2.6)
FUDR	4,618.2 ± 163.3	4,996.3 ± 1,226.4	(1.1)	3,781.9 ± 1,281.9	(0.8)	4,223.4 ± 817.7	(0.9)
VCR	11.2 ± 2.8	7.7 ± 6.3	(0.7)	12.7 ± 4.2	(1.1)	9.3 ± 2.7	(0.8)
Taxol	4.1 ± 1.5	4.7 ± 0.2	(1.1)	3.8 ± 1.0	(0.9)	5.3 ± 2.1	(1.3)
DOX	16.9 ± 0.7	14.3 ± 5.9	(0.8)	12.9 ± 6.4	(0.8)	18.4 ± 5.6	(1.1)
CDDP	1,255.4 ± 456.6	1,222.5 ± 466.2	(1.0)	1,549.1 ± 555.5	(1.2)	1,050.3 ± 168.0	(0.8)

[1]Cells were exposed to the indicated drug for 72 hr in folate medium without FA. IC_{50} data (mean ± SD) are expressed as nM except for CBZ (μM). Data were obtained from at least triplicate experiments. In parentheses is reported the -fold increase of drug IC_{50} compared to the parent cell line. MTX IC_{50} of SKOV-3 cells cultured in the presence of 2 μM FA and exposed to the drug in folates contained medium was 10.7 ± 2.3 μM.–[2]$p \leq 0.01$, significance compared to the SKOV-3 parental cell line by Mann-Whitney test.

RFC-mediated drug uptake, whereas FA will be more effective in competing for FRα binding and drug uptake. For both the folates, the protective effect was dose-dependent. LV had a substantially higher protective effect against growth inhibition than FA, both in SKOV-3 cells and in the SKOV-CBZ-50–5 clone, at all the extracellular concentrations used (Table II). The protective effect of LV was already clear at 20 nM both in SKOV-3 parental cells and in the SKOV-CBZ-50–5 clone. At LV concentrations ≥200 nM, which probably are sufficient to block both RFC and FRα, MTX IC_{50} in parental cells approached that of the SKOV-50–5 clone (Table II). Conversely, the FA protective effect was higher in SKOV-3 parental cells than in the SKOV-CBZ-50–5 clone. In SKOV-3 parental cells, the protective effect was already evident at 20 nM FA, whereas in the SKOV-CBZ-50–5 clone it was significant ($p < 0.01$) at FA concentrations greater than 200 nM (Table II).

DHFR, FPGH, TS and MTHFR activity

To ascertain whether the differences in MTX cytotoxicity between the SKOV-3 parental cells and SKOV-CBZ-selected clones were due to differences in the cellular enzymes involved in the mechanism of action of MTX, we assessed the intracellular levels of DHFR, TS, FPGS and MTHFR. The levels of DHFR evaluated by the MTX binding assay were homogeneous in the SKOV-CBZ-selected clones and not significantly different (p = NS) from those of the parental cell line. Analogously, the activities of FPGS, involved in the polyglutammation process of MTX, and TS were not significantly (p = NS) different in the SKOV-3-CBZ clones compared with the parental cell line (Fig. 1). In contrast, the activity of MTHFR in the SKOV-CBZ-50–5 and SKOV-CBZ-100–2 clones was 1.6 and 2.0-fold higher, respectively, than in the SKOV-3 parental cell line. The activity of MTHFR was 33.1 ± 12.6, 53.6 ± 11.9 and 64.7 ± 9.5 nmol/hr/mg of protein in the SKOV-3 parental cells, SKOV-CBZ-50–5 clone, and SKOV-CBZ-100–2 clone, respectively ($p < 0.05$). No significant differences were observed between the SKOV-3 parental cells and the SKOV-CBZ-50–2 cell clone (Fig. 1).

FRα and RFC expression

The expression levels of FRα and RFC in the 3 SKOV-CBZ clones and in the SKOV-3 parental cell line were analyzed by quantitative reverse transcriptase (RT)-PCR. FRα mRNA was 3,120 ± 1,770 copies of template/μg of total RNA in SKOV-3 parental cells cultured in FA-conditioned medium, whereas it was 850 ± 165, 1,100 ± 305 and 936 ± 288 copies of template/μg of total RNA in the SKOV-CBZ-50–5, SKOV-CBZ-50–2 and SKOV-CBZ-100–2 cell clones, respectively. Compared to the parental cells, FRα mRNA decreased by 3.7-, 2.8-, and 3.3-fold in SKOV-CBZ-50–5, SKOV-CBZ-50–2 and SKOV-CBZ-100–2 cell clones, respectively (Fig. 2, Table III).

FRα protein level and activity were analyzed in the SKOV-3 parental cells and SKOV-CBZ-50–5 clone. FACScan analysis (Fig. 3) using a specific MAb (MOv18) indicated that the decreased expression of FRα mRNA in the SKOV-CBZ-50–5 clone was associated with a decreased level of the FRα gene product.

TABLE II – PROTECTIVE EFFECT OF FA AND LV AGAINST MTX CYTOTOXICITY[1]

Drug	MTX IC_{50} (nM)		Ratio[2]
	SKOV-3	SKOV-CBZ-50-5	
MTX	2.2 ± 0.6	8.6 ± 1.8	3.9
MTX + FA (20 nM)	3.3 ± 0.6[3]	10.0 ± 2.6	3.0
MTX + FA (200 nM)	6.4 ± 1.6[4]	11.4 ± 2.0	1.7
MTX + FA (2,000 nM)	11.2 ± 2.4[4]	16.6 ± 3.1[4]	1.5
MTX + LV (20 nM)	18.9 ± 5.7[4]	37.8 ± 8.6[4]	2.0
MTX + LV (200 nM)	359.0 ± 181.3[4]	607.3 ± 140.2[4]	1.7
MTX + LV (2,000 nM)	1,569.5 ± 428.2[4]	1,837.2 ± 381.3[4]	1.2

[1]Data are the mean ± SD of at least quadriplicate experiments. Cell incubation was for 72 hr in the presence of FA or LV at the indicated medium concentrations.–[2]Ratio between the MTX IC_{50} of SKOV-CBZ-50-5 clone and MTX IC_{50} of SKOV-3 parental cells.–[3]$p \leq 0.05$.–[4]$p \leq 0.01$ compared to the treatment with MTX alone, Mann-Whitney test.

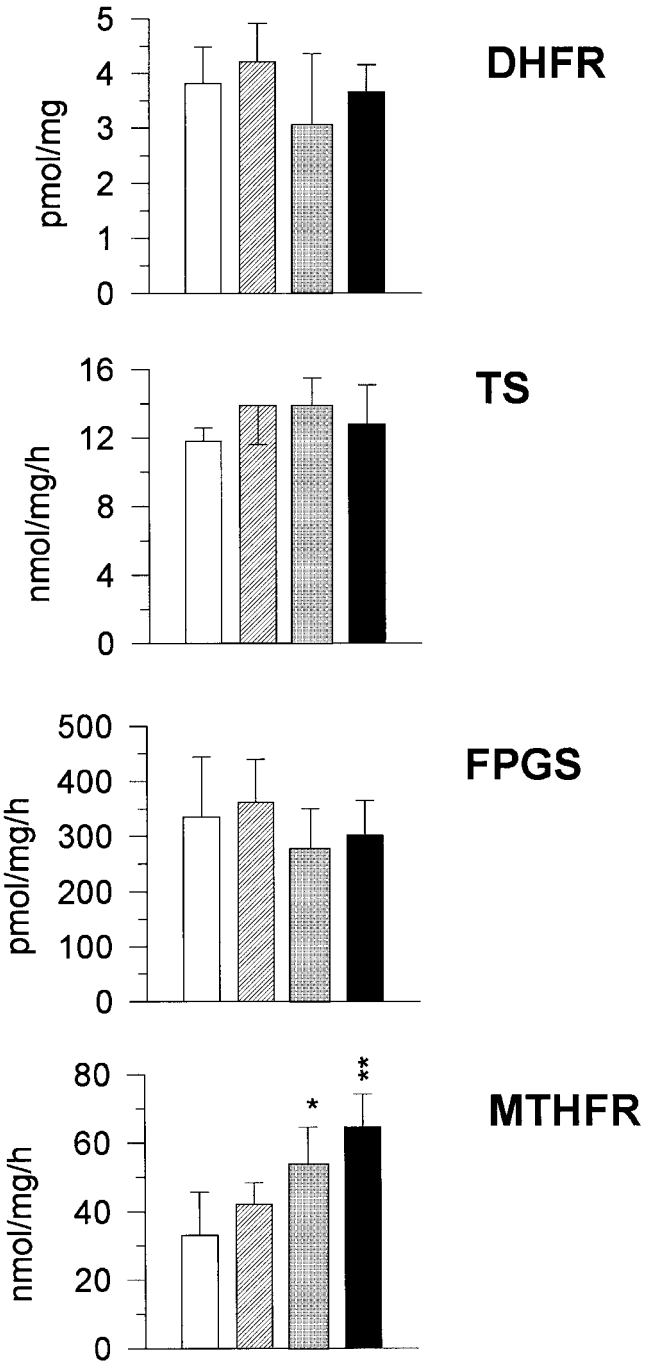

FIGURE 1 – Cellular levels of DHFR, TS, FPGS and MTHFR folate-dependent enzymes in the SKOV-3 parental cell line (open bars) and SKOV-CBZ-selected clones (SKOV-CBZ-50–2 [diagonal bars], SKOV-CBZ-50–5 [stippled bars], SKOV-CBZ-100–2 [solid black bars]). Bars, ±SD. *$p \leq 0.05$, evaluated by the Mann-Whitney test.

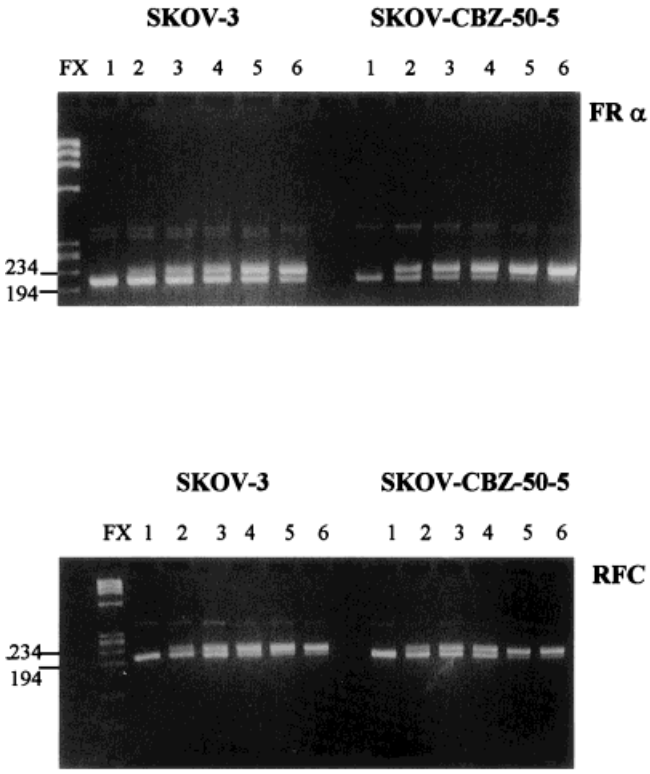

FIGURE 2 – Representative results of quantification of FRα and RFC expression by competitive RT-PCR in SKOV-3 parental cells and in a representative SKOV-CBZ clone (SKOV-CBZ-50–5). For FRα, lanes 1–6, 2 μl of cDNA obtained from 1 μg of total mRNA was amplified in the presence of 0, 50, 100, 200, 400 and 800 copies of the FRα competitor. For RFC, lanes 1–6 correspond to cDNA amplifications run in the presence of 0, 5, 10, 20, 40 and 80 copies of the RFC competitors. The amplification products were resolved in 2% agarose gel in TBE and the quantification process was performed as reported in Material and Methods.

Cellular [^{3}H]FA binding capacity well agreed with the levels of FRα mRNA expression and protein level as determined by cytofluorimetric studies. After exposure to 20 nM [^{3}H]FA, binding was 0.60 ± 0.17 pmol/10^6 cells in the SKOV-3 parental cell line and 0.22 ± 0.09 pmol/10^6 cells in the SKOV-CBZ-50–5 clone ($p = 0.02$, n = 4). Unlike FRα, RFC mRNA level was quite similar in the SKOV-3 parental cell line and SKOV-3-CBZ clones (Fig. 2, Table III).

As previously reported (Miotti *et al.*, 1995), the SKOV-3 cell line cultured in conditioned medium expressed higher (about 2 to 4-fold) FRα mRNA than the SKOV-3 cell line cultured in medium supplemented with 2 μM FA; conversely, no variations were observed in RFC mRNA expression between the 2 cell lines grown in different FA concentrations (data not shown). It must be considered that SKOV-CBZ clones had FRα mRNA levels similar to the SKOV-3 cells growing in medium containing 2 μM FA and had similar sensitivity to MTX (Table 1, legend).

Cellular drug accumulation

To ascertain whether MTX resistance was due to an impairment in drug intracellular content, we studied [^{3}H]MTX uptake in parental cells and SKOV-CBZ clones. Uptake of MTX was compared to [^{3}H]FA and 5CH_3[^{3}H]-THF cellular uptakes to investigate whether decreased expression of FRα could contribute to the resistance of SKOV-CBZ clones and to distinguish between FRα and RFC drug transport route. The uptake of radiolabeled compounds was measured at 20 nM extracellular concentrations, approximating Kd values for binding to FRα, and at 2 μM extracellular concentrations, approximating Km values for a putative carrier-mediated transport process. Incubation time was 4 hr. As previously reported, at that time, the uptake of the folates/antifolate was approximately linear (Corona *et al.*, 1998; Westerhof *et al.*, 1995*a*; data not shown). After incubation with 20 nM [^{3}H]MTX extracellular concentration, [^{3}H]MTX intracellular content in the SKOV-3 parental cell line was 0.56 ± 0.22 pmol/10^6cells, whereas it was 0.27 ± 0.07 pmol/10^6 cells, 0.32 ± 0.06 pmol/10^6 cells and 0.27 ± 0.08 pmol/10^6 cells in SKOV-CBZ-50–5, SKOV-CBZ-50–2 and SKOV-CBZ-100–2 cells, respectively. At the extracellular concentration of 2 μM [^{3}H]MTX, the intracellular content of MTX in SKOV-3 parental cells was 5.99 ± 1.34 pmol/10^6 cells and it was about 2-fold lower in the SKOV-CBZ clones (Fig. 4). [^{3}H]MTX binding capacity was close to that of [^{3}H]FA. After exposure to 20 nM [^{3}H]MTX, the drug binding capacity was 0.47 ± 0.11 pmol/10^6 cells and 0.25 ± 0.07 pmol/10^6 cells in the SKOV-3 parental cell line and in the SKOV-CBZ-50–5 clone, respectively.

Figure 4 shows the intracellular content of [^{3}H]FA and 5-CH_3-[^{3}H]THF at extracellular concentrations of 20 nM and 2 μM over a 4 hr incubation period. At nanomolar concentrations, the intracellular content of 5-CH_3-[^{3}H]THF was decreased by 1.8- and 1.6-fold in the SKOV-CBZ-50–5 and SKOV-CBZ-50–2 clones, respectively, compared with the parental cells ($p < 0.05$). A decreasing trend (1.4-fold) that failed to reach significance was observed for the SKOV-CBZ-100–2 clone. After cell exposure to 2 μM 5-CH3-[^{3}H]THF, the intracellular drug content in all the SKOV-CBZ clones was significantly ($p < 0.05$) decreased by about 2-fold (Fig. 4).

FA intracellular content was significantly ($p < 0.05$) decreased in SKOV-CBZ clones as compared to the parental cell line when cells were exposed to extracellular FA concentrations of 20 nM and 2 μM [^{3}H]FA (Fig. 4).

To investigate whether selection of SKOV-3 cells by CBZ modulated the intracellular content of the antiepileptic drug, intracellular CBZ content was investigated. After exposure to 100 μg/ml CBZ for 4 hr, the intracellular content of CBZ was 1,414.8 ± 430 pmol/10^6 cells, 1,105.4 ± 256.2 pmol/10^6 cells, and 938.5 ± 332.8 pmol/10^6 cells in the SKOV-CBZ-50–5, SKOV-CBZ-50–2, and SKOV-CBZ-100–2 clones, respectively. Intracellular drug content was not significantly (p = NS) different from that observed in SKOV-3 parental cells (1,275.2 ± 154.3 pmol/10^6 cells). Analogously, no variations in the intracellular content were observed between SKOV-3 parental cells and SKOV-CBZ clones when cells were exposed for 4 hr to [^{3}H]VCR, an antineoplastic drug whose cellular uptake is not related to FRα or RFC activity (Fig. 4).

LV had a similar inhibitory effect on [^{3}H]MTX cellular uptake both in the SKOV-3 parental cell line and in the SKOV-CBZ-50–5 clone. Two hundred nanomolar LV decreased 20 nM [^{3}H]MTX uptake over a 4 hr incubation by 2.0- and 2.1-fold in SKOV-3 parental cells and in the SKOV-CBZ-50–5 clone, respectively (Fig. 5). A dichotomic picture was observed for FA. Concentrations of FA ≥ 200 μM inhibited 20 nM [^{3}H]MTX uptake by about 30% in SKOV-3 parental cells; conversely, no inhibitory effect of FA was observed in the SKOV-CBZ-50–5 clone, even at 2 μM FA concentrations (Fig. 5).

No competitive effect of CBZ on MTX and FA uptake was observed, even at molar 1,000-fold excess (data not shown).

DISCUSSION

Several studies have focused on the detrimental effect of CBZ on serum or erythrocyte folate concentration, but at present it is not clear whether CBZ administration also reduces folates in other tissue cells. It has been reported that following teratogenic exposure to the antiepileptic valproic acid in a mouse model, the expression of the folate transport gene *FBP-1* was decreased in the

TABLE III – MRNA OF FRA AND RFC IN SKOV-3 PARENTAL CELL LINE AND IN SELECTED SKOV-CBZ CLONES[1]

mRNA (number of copies mRNA/ total RNA)	SKOV-3	SKOV-CBZ-50-5	SKOV-CBZ-50-2	SKOV-CBZ-100-2
FRα	3,120 ± 1,770	850 ± 165 (3.7)	1,100 ± 305 (2.8)	936 ± 288 (3.3)
RFC	125 ± 38	130 ± 75 (0.96)	172 ± 96 (0.73)	95 ± 43 (1.3)

[1]Data are mean ± SD of at least 3 experiments. In parentheses is reported the -fold decrease compared to the SKOV-3 parental cells grown in folate conditioned medium.

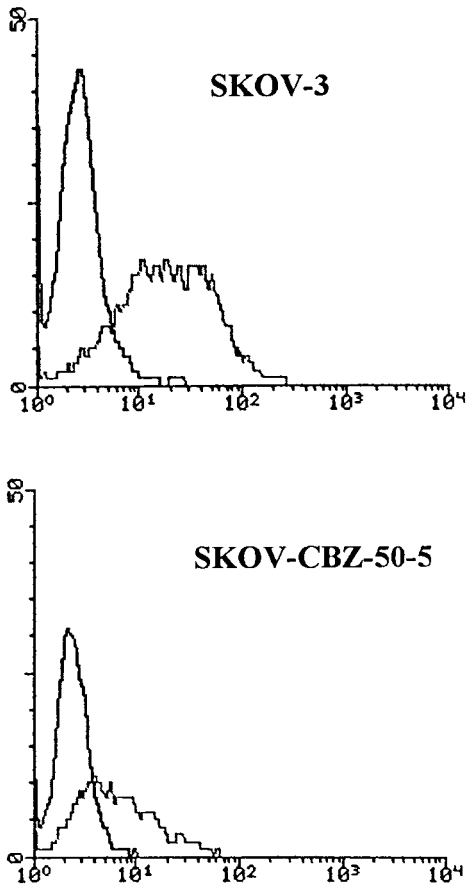

FIGURE 3 – Relative content of FRα in the SKOV-CBZ-50–5 clone and in SKOV-3 parental cells as determined by cytofluorimetric analysis using the MOv18 plus the FITC-goat antimouse IgG. Negative controls obtained by non-immune IgG are also shown.

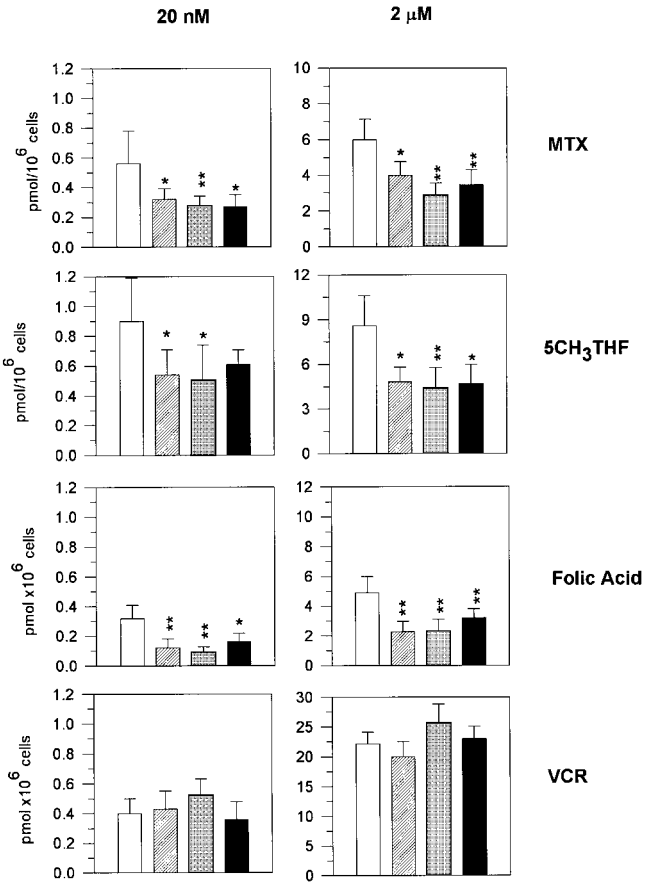

FIGURE 4 – Cellular drug uptake of [^{3}H]MTX, [^{3}H]FA, 5CH$_3$-[^{3}H]-THF and [^{3}H]VCR. Intracellular drug content in the SKOV-3 parental cell line (open bars) and SKOV-CBZ-selected clones (SKOV-CBZ-50–2 [diagonal bars], SKOV-CBZ-50–5 [stippled bars], SKOV-CBZ-100–2 [solid black bars]) was evaluated after 4 hr of incubation at 37°C at 20 nM and 2 μM extracellular drug concentrations. Bars, ±SD. * $p \leq 0.05$, ** $p \leq 0.01$, Mann-Whitney test.

neural tube, suggesting a folate defect due to an impairment in cellular folate transport. Since folates and antifolates have common cellular transport mechanism(s), it could be expected that antiepileptic drugs also affect intracellular content of antifolates. At present, no data have been reported on the effect of CBZ on the intracellular content of antimetabolite cytotoxic agents that can be used together with CBZ in the treatment of cancer patients.

Previous results on depletion of folates after CBZ administration have pointed out that depletion occurs only after chronic exposure to the antiepileptic and that, for serum and erythrocyte folates, it is dose-dependent (Lewis *et al.*, 1998). In our *in vitro* experimental model, to intensify the biochemical mechanisms associated with chronic CBZ treatment, we exposed SKOV-3 human ovarian cancer cells to toxic concentrations of CBZ, thus selecting clones able to grow in the presence of the antiepileptic drug without dramatic perturbations in cell growth. Selection of SKOV-CBZ clones was obtained in conditioned medium containing nanomolar folate concentrations, to approach the physiological conditions in the plasma of patients, in which only small amounts (about 5–50 nM) of reduced folates are present.

We report here an increased MTX resistance in the cell clones selected after chronic exposure to CBZ. This resistance was related to a reduced intracellular content of MTX, whereas no alteration activity of the cellular enzymes directly involved in MTX cytotoxicity (DHFR, TS and FPGS) was observed. The impairment in MTX uptake in SKOV-CBZ clones was associated with cross-resistance to the TS inhibitors tomudex and CB3717, which exhibit common transmembrane transport mechanism(s) (FRα and/or RFC). Conversely, resistant clones were collaterally sensitive to the TS inhibitor 5-FUDR and other antineoplastic drugs (CDDP, DOX, VCR and taxol), whose cellular uptake is derived from transmembrane transport mechanisms different from FRα or RFC.

FRα mRNA and protein levels were significantly decreased in SKOV-CBZ clones compared to the parental cells. The reduced

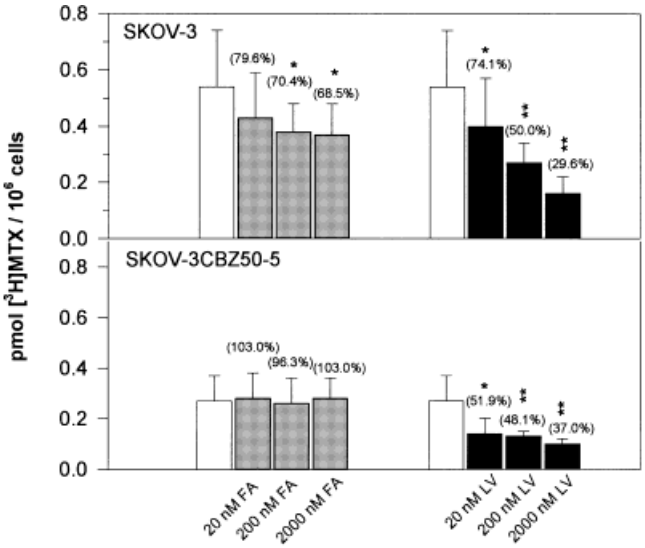

FIGURE 5 – Inhibition of 20 nM [^{3}H]MTX uptake by CBZ, FA and LV in SKOV-3 parental cells and the SKOV-CBZ-50–5 clone. The SKOV-3 cell line and SKOV-CBZ-50–5 clone were incubated for 4 hr at 37°C at 20 nM extracellular MTX concentrations in the absence (open bars) and in the presence of FA (grey bars) and LV (black bars) at indicated concentrations. Results are expressed as mean ± SD of at least 3 independent experiments. In parentheses, percent MTX uptake as compared to the control cells (100%). *$p \leq 0.05$, **$p \leq 0.01$, Wilcoxon matched pair test.

level of FRα in SKOV-CBZ clones was associated with a decreased binding capacity of FA. In contrast, analysis of mRNA RFC expression in the SKOV-CBZ clones showed no variation.

At present, we cannot conclude that the reduced FRα levels are selectively responsible for the reduced MTX uptake and cytotoxicity. Generally, cells resistant to MTX because of defective intracellular MTX content exhibit lower expression of RFC than FRα (Moscow, 1998) and, although we did not find significant variations in RFC expression levels between the parental cell line and SKOV-CBZ clones, we cannot exclude variations in RFC activity (Roy *et al.*, 1998; Tse *et al.*, 1998). Moreover, SKOV-CBZ clones exhibited an RI towards CB3717 in the range observed for MTX, even if CB3717 has a higher affinity for FRα than MTX (Westerhof *et al.*, 1995*b*), and it is expected that cells expressing low levels of FRα could be more resistant to CB3717. Nevertheless, we believe that involvement, at least partial, of FRα in MTX resistance of SKOV-CBZ clones should be considered. It has been previously demonstrated that overexpression of FRα could contribute to the transport of MTX, especially when experiments were performed in culture medium containing physiological folate concentrations (Jansen *et al.*, 1989; Chung *et al.*, 1993; Moscow, 1998). There is evidence that a high binding affinity of FRα for antifolates does not necessarily imply a better transport efficiency. The latter is also influenced by the recycling rates of FRα in the cells and by the dissociation of the ligand from the receptor cells (Jansen, 1999). Tight binding, such as that between CB3717 and FRα, could hamper the dissociation of the ligand from the receptor. This could not occur with MTX, for which FRα has a lower affinity. Efficiency of polyglutamylation may be another rate-limiting factor in FRα-mediated (anti) folate uptake (Spinella *et al.*, 1996). On these grounds, MTX may well be a better substrate for FPGS than CB3717. Finally, due to its high affinity for FRα, FA competes for MTX cytotoxicity and uptake, better in SKOV-3 parental cells than in the SKOV-CBZ-50–5 clone. In contrast, after competitive experiments with 2 μM LV, which can be anticipated to interfere with both RFC and FRα activity (Westerhof *et al.*, 1995*a*), MTX IC_{50} in SKOV-3 parental cells approached that of the SKOV-CBZ-50–5 clone. Further linking between FRα expression and MTX sensitivity is also suggested by the absence of discernible differences in FRα mRNA level and MTX cytotoxicity between SKOV-CBZ clones and SKOV-3 cells growing in medium containing 2 μM FA.

There is a wealth of clinical and experimental data that link the teratogenic effects of antiepileptic drugs, such as valproic acid and CBZ, to altered folate levels in serum. However, little is known about the biochemical mechanisms by which this occurs. We have demonstrated that chronic exposure to CBZ determines a reduced expression of FRα in SKOV cells, but the underlying biochemical mechanism remains unknown. It is also not clear whether reduced FR expression is a primary or secondary effect of CBZ resistance since pleiotropic effects could be provoked during the development of CBZ resistance in SKOV-3 cells. CBZ does not appear to be a substrate for folate or antifolate transporters (FRα or RFC), since the intracellular content of CBZ in the SKOV-CBZ clones was not significantly different from that of the parental cell line, and no competitive effect for transport was observed between CBZ and MTX or FA. It has been suggested that anticonvulsant drugs might affect the enzymes involved in folate metabolism, thus influencing antifolate transport and cytotoxicity. Jansen *et al.* (1997) showed that RFC-mediated transport of MTX can be regulated by changes in cellular folate, purine, and possibly methylation status, although no definitive conclusions have been proposed for the exact mechanism(s) of down-regulation of folate and antifolate transport. The pattern of *MTHFR* gene expression may be modulated by antiepileptic drugs, resulting in altered intracellular concentrations of 5- and 10-formyl-THF, as well as by 5-CH_3-THF metabolites, which may interfere with folate/antifolate uptake and purine or methionine biosynthesis (Kim *et al.*, 1993; Westerhof *et al.*, 1995*a*; Finnell *et al.*, 1997). On these grounds, we observed an increased activity of MTHFR in the SKOV-CBZ-50–5 and SKOV-CBZ-50–2 clones compared with the parental cell line. These data well agree with those of Finnell *et al.* (1997) who described that, after exposure of inbred mouse to valproic acid, the expression of FBP-1 mRNA in the neural tube was decreased, whereas MTHFR was increased, in the SWV strain susceptible to the valproic acid-induced neural tube defect.

In conclusion, our results support the hypothesis that CBZ may alter the intracellular transport of folates and give new insight into the modulation of MTX uptake by CBZ. We have indeed demonstrated that, after chronic exposure to CBZ, the selected cell clones were resistant towards MTX due to a defective MTX uptake.

ACKNOWLEDGEMENT

We thank Dr. P. Tonel for help with the manuscript.

REFERENCES

BERTINO, J.R., Ode to methotrexate. *J. clin. Oncol.*, **11**, 5–14 (1993).

CHUNG, K., SAIKAWA, Y., PAIK, T., DIXON, K.H., MULLIGAN, T., COWAN, K.H. and ELWOOD, P.C., Stable transfectants of human MCF-7 breast cancer cells with increased levels of the human folate receptor exhibit an increased sensitivity to antifolates. *J. clin. Invest.*, **91**, 1289–1294 (1993).

CORONA, G., GIANNINI, F., FABRIS, M., TOFFOLI, G. and BOIOCCHI, M., Role of folate receptor and reduced folate carrier in the transport of 5-methyltetrahydrofolic acid in human ovarian carcinoma cells. *Int. J. Cancer*, **75**, 125–133 (1998).

COWAN, K.H., GOLDSMITH, M.E., LEVINE, R.M., AITKEN, S.C., DOUGLASS, E., CLENDENINN, N., NIENHUIS, A.W. and LIPPMAN, M.E., Dihydrofolate reductase gene amplification and possible rearrangement in estrogen-responsive methotrexate-resistant human breast cancer cells. *J. biol. Chem.*, **257**, 15079–15086 (1982).

FINNELL, R.H., WLODARCZYK, B.C., CRAIG, J.C., PIEDRAHITA, J.A. and BENNETT, G.D., Strain-dependent alterations in the expression of folate pathway genes following teratogenic exposure to valproic acid in a mouse model. *Amer. J. Med. Genet.,* **70**, 303–311 (1997).

HENDERSON, G.B. and STRAUSS, B.P., Characteristics of a novel transport system for folate compounds in wild-type and methotrexate-resistant l1210 cells. *Cancer Res.,* **50**, 1709–1714 (1990).

JANSEN, G., Receptor- and carrier-mediated transport systems for folates and antifolates. *In:* A.N. Jackman (ed.), *Receptor- and carrier-mediated transport systems for folates and antifolates,* Human Press, Totowa, NJ (1999).

JANSEN, G., MAURITZ, R.M., ASSARAF, Y.G., SPRECHER, H., DRORI, S., KATHMANN, I., WESTERHOF, G.R., PRIEST, D.G., BUNNI, M., PINEDO, H.M., SCHORNAGEL, J.H. and PETERS, G.J., Regulation of carrier-mediated transport of folates and antifolates in methotrexate-sensitive and -resistant leukemia cells. *Adv. Enzyme Regul.,* **37**, 59–76 (1997).

JANSEN, G., SCHORNAGEL, J.H., KATHMANN, I., WESTERHOF, G.R., HORDIJK, G. and VAN DER LAAN, B.F.A.M., Measurement of folylpolyglutamate synthetase activity in head and neck squamous carcinoma cell lines and clinical samples using a new rapid separation procedure. *Oncol. Res.,* **4**, 299–306 (1992).

JANSEN, G., WESTERHOF, G.R., KATHMANN, I., RADEMAKER, B.C., RIJKSEN, G. and SCHORNAGEL, J.H., Identification of a membrane-associated folate-binding protein in human leukemic Ccrf-Cem cells with transport-related methotrexate resistance. *Cancer Res.,* **49**, 2455–2459 (1989).

JOEL, S.P., SHAH, R., CLARK, P.I. and SLEVIN, M.L., Predicting etoposide toxicity: relationship to organ function and protein binding. *J. clin. Oncol.,* **1**, 257–267 (1996).

KIM, J.S., LOWE, K.E. and SHANE, B., Regulation of folate and one-carbon metabolism in mammalian cells. iv. Role of folylpoly-gamma-glutamate synthetase in methotrexate metabolism and cytotoxicity. *J. biol. Chem.,* **268**, 21680–21685 (1993).

KISHI, T., FUJITA, N., EGUCHI, T. and UEDA, K., Mechanism for reduction of serum folate by antiepileptic drugs during prolonged therapy. *J. neurol. Sci.,* **145**, 109–112 (1997).

KUTZBACH, C. and STOKSTAD, E.L.R., Feedback inhibition of methylene-tetrahydrofolate reductase in rat liver by S-adenosylmethionine. *Biochim. biophys. Acta,* **139**, 217–220 (1967).

LEWIS, D.P., VAN DYKE, D.C., STUMBO, P.J. and BERG, M.J., Drug and environmental factors associated with adverse pregnancy outcomes. Part II: Improvement with folic acid. *Ann. Pharmacother.,* **32**, 947–961 (1998).

LUCOCK, M.D., WILD, J., SCHORAH, C.J., LEVENE, M.I. and HARTLEY, R., The methylfolate axis in neural tube defects: *in vitro* characterisation and clinical investigation. *Biochem. Med. Metab. Biol.,* **52**, 101–114 (1994).

MACDONALD, R.L. and KELLY, K.M., Antiepileptic drug mechanisms of action. *Epilepsia,* **36**, S2–12 (1995).

MIOTTI, S., FACHERIS, P., TOMASSETTI, A., BOTTERO, F., BOTTINI, C., OTTONE, F., COLNAGHI, M.I., BUNNI, M.A., PRIEST, D.G. and CANEVARI, S., Growth of ovarian-carcinoma cell lines at physiological folate concentration: effect on folate-binding protein expression *in vitro* and *in vivo*. *Int. J. Cancer,* **63**, 395–401 (1995).

MOSCOW, J.A., Methotrexate transport and resistance. *Leukemia Lymphoma,* **30**, 215–224 (1998).

ROBERTS, D., An isotopic assay for thymidylate synthetase. *Biochemistry,* **5**, 3546–3548 (1966).

ROY, K., TOLNER, B., CHIAO, J.H. and SIROTNAK, F.M., A single amino acid difference within the folate transporter encoded by the murine RFC-1 gene selectively alters its interaction with folate analogues. Implications for intrinsic antifolate resistance and directional orientation of the transporter within the plasma membrane of tumor cells. *J. biol. Chem.,* **273**, 2526–2531 (1998).

SPINELLA, M.J., BRIGLE, K.E., FREEMANTLE, S.J., SIERRA, E.E. and GOLDMAN, I.D., Comparison of methotrexate polyglutamylation in l1210 leukemia cells when influx is mediated by the reduced folate carrier or the folate receptor: lack of evidence for influx route-specific effects. Proc. *Ann. Meet. Amer. Assoc. Cancer Res.,* **37**, A2607 (1996).

STANDER, M., DICHGANS, J. and WELLER, M., Anticonvulsant drugs fail to modulate chemotherapy-induced cytotoxicity and growth inhibition of human malignant glioma cells. *J. Neuro-Oncol.,* **3**, 191–198 (1998).

TOFFOLI, G., SIMONE, F., GIGANTE, M. and BOIOCCHI, M., Comparison of mechanisms responsible for resistance to idarubicin and daunorubicin in multidrug resistant lovo cell lines. *Biochem. Pharmacol.,* **48**, 1871–1881 (1994).

TSE, A., BRIGLE, K., TAYLOR, S.M. and MORAN, R.G., Mutations in the reduced folate carrier gene which confer dominant resistance to 5,10-dideazatetrahydrofolate. *J. biol. Chem.,* **273**, 25953–25960 (1998).

WANG, X., SHEN, F., FREISHEIM, J.H.,GENTRY, L.E. and RATNAM, M., Differential stereospecificities and affinities of folate receptor isoforms for folate compounds and antifolates. *Biochem. Pharmacol.,* **44**,1898–1901 (1992).

WESTERHOF, G.R., RIJNBOUTT, S., SCHORNAGEL, J.H., PINEDO, H.M., PETERS, G.J. and JANSEN, G., Functional activity of the reduced folate carrier in KB, MA104, and IGROV-1 cells expressing folate-binding protein. *Cancer Res.,* **17**, 3795–3802 (1995*a*).

WESTERHOF, G.R., SCHORNAGEL, J.H., KATHMANN, I., JACKMAN, A.L., ROSOWSKY, A., FORSCH, R.A., HYNES, J.B., BOYLE, F.T., PETERS, G.J., PINEDO, H.M. and JANSEN, G., Carrier- and receptor-mediated transport of folate antagonists targeting folate-dependent enzymes: correlates of molecular-structure and biological activity. *Mol. Pharmacol.,* **48**, 459–471 (1995*b*).