27. Etievant C, Kruczynski A, Pauwels O, Kiss R. The combination of the tetrazolium derivative reduction (MTT) and digital cell image analysis to monitor in vitro the cytotoxicity of anti-neoplastic drugs. *Anticancer Res* 1991, **11**, 305–312.
28. Kiss R, de Launoit Y, Danguy A, Paridaens R, Pasteels JL. Influence of pituitary grafts or prolactin administrations on the hormone sensitivity of ovarian hormone-independent mouse mammary MXT tumors. *Cancer Res* 1989, **49**, 2945–2951.
29. Weinstock J, Baldwin GS. Binding of gastrin 17 to human gastric carcinoma cell lines. *Cancer Res* 1988, **48**, 932–937.
30. Kolori O, Vaillot MT, Martin F. Growth responses of rat stomach cancer cells to gastro-entero-pancreatic hormones. *Int J Cancer* 1982, **30**, 65–67.
31. Rentis A, Tsuchiya R, Miyamoto T, Harado N, Eto T, Kittas C. Influence of sex-hormones on serum gastrin and antral G-cell population in guinea pigs. *Hormone Metabol Res* 1986, **18**, 423–425.
32. Eidne KA, Flanagan CA, Harris NS, Millar RP. Gonadotropin-releasing hormone (GnRH)-binding sites in human breast cancer cell lines and inhibitory effects of GnRH antagonists. *J Clin Endocrinol Metab* 1987, **64**, 425–432.
33. Giacchetti S, Gauville C, de Cremoux P, *et al.* Characterization, in some human breast cancer cell lines, of gastrin-releasing peptide-like receptors which are absent in normal breast epithelial cells. *Int J Cancer* 1990, **46**, 293–298.

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Mechanisms of Acquired Resistance to Methotrexate in a Human Squamous Carcinoma Cell Line of the Head and Neck, Exposed to Different Treatment Schedules

Bernard F.A.M. van der Laan, Gerrit Jansen, Ietje Kathmann, Jan H. Schornagel and Gerrit Jan Hordijk

Mechanisms of acquired resistance to methotrexate (MTX) were evaluated in HNSCC-11B cells which were made resistant to methotrexate either by continuous (11B-MTX-C) or by pulse exposure (11B-MTX-P) to the drug. 11B-MTX-C cells were 91-fold resistant to methotrexate and 30-fold or 49-fold crossresistant to trimetrexate and 10-EdAM, respectively. Dihydrofolate reductase (DHFR) activity was increased 63-fold in 11B-MTX-C cells together with a decrease in [^{3}H]-methotrexate transport and folylpolyglutamate synthase (FPGS) activity (2.5-fold and 3.8-fold, respectively). Against two novel antifolates targetting enzymes other than DHFR, minor crossresistance was observed for ICI-198, 583, but full sensitivity was retained for DDATHF. 11B-MTX-P cells were 46-fold resistant to methotrexate and 47-fold crossresistant to ICI-198,583 in short-term drug exposure, but showed only minor changes in methotrexate sensitivity following prolonged drug exposure. The resistant phenotype in 11B-MTX-P cells were characterised by a 5.6-fold decrease in FPGS activity. These results suggest that different mechanisms of methotrexate resistance in HNSCC cells *in vitro* can be obtained dependent on the schedule of exposure to the drug.
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INTRODUCTION

SURGERY AND RADIOTHERAPY are the primary modalities of treatment in patients with squamous cell carcinomas of the head and neck (HNSCC) [1]. Chemotherapy is most commonly reserved for end-stage disease. Active drugs for the treatment of head and neck carcinomas include methotrexate, cisplatin, bleomycin and 5-fluorouracil (5-FU). The overall response rate to each of these four agents has ranged from 15% to 30% [2].

The antimetabolites remain among the most effective drugs in HNSCC, with methotrexate as its most used agent [3]. Although methotrexate is considered to be an "active" drug, only one third of patients will have an objective but transient response. This may be due to either inherent or acquired cellular resistance to methotrexate. The mechanisms by which neoplastic cells become resistant have been the subject of intense research efforts and a variety of such mechanisms have now been identified [4]. Four mechanisms of resistance to methotrexate have been extensively studied *in vitro* [5], and are of significant importance for resistance in HNSCC cell lines [6–9]. These mechanisms include: (a) increase of the intracellular level of dihydrofolate reductase (DHFR), the target enzyme of methotrexate, usually as a result of DHFR gene amplification; (b) alteration of DHFR, with decreased affinity for methotrexate; (c) decrease of methotrexate transport into the cell; and (d) decrease of intracellular polyglutamylation of methotrexate.

In all of these studies, however, the development of methotrexate resistance *in vitro* was induced by stepwise increasing concentrations of methotrexate to the cell culture. Recently Pizzorno *et al.* [10] have shown for leukaemia cells *in vitro* that pulse doses of methotrexate, in an attempt to mimic clinical

conditions, preferentially resulted in impaired polyglutamylation as the mechanism of methotrexate resistance.

In this study we investigated the mechanism(s) of acquired resistance to methotrexate for a HNSCC cell line which was made resistant in two different ways, either by continuous (11B-MTX-C), or by 24 hour pulse (11B-MTX-P) exposures to methotrexate.

During the development of methotrexate resistance, the HNSCC cell lines were grown in "folate-conditioned" medium, containing near physiological concentrations of natural folates (≈5 nmol/l) rather than the high levels of folic acid (≈2 μmol/l) which are usually present in cell culture media. The rationale for growing cells in "folate-conditioned" medium is that recent studies by our laboratory and others have shown that an up-regulation in [^{3}H]-methotrexate membrane transport may occur via the classical reduced folate/methotrexate carrier system [11–13]. Another rationale is that the expression of a membrane-associated folate-binding protein (mFBP) can only be established in "folate-conditioned" medium [14–17]. An mFBP can serve as an alternative folate transport system in tumour cells with a non-functional reduced folate carrier system [17], or may be functional together with the reduced folate carrier system [15, 16].

The level of resistance to methotrexate in 11B-MTX-C and 11B-MTX-P cells were analysed in long-term (7 days) and short-term (24 hour) drug exposure. In order to establish whether there is crossresistance or sensitivity to other antifolates, the cytotoxicity experiments were also done with two other DHFR inhibitors: trimetrexate and 10-ethyl-10-deazaaminopterin (10-EdAM), and with two novel folate analogues which are inhibitors of thymidylate synthase (TS): 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid (ICI-198,583), or glycinamide ribonucleotide transformylase (GAR-TFase): 5,10-dideazatetrahydrofolic acid (DDATHF).

The results demonstrate that resistance to methotrexate in 11B-MTX-C cells was mainly due to increased DHFR levels and to a minor extent as a result of reduced methotrexate transport and decreased FPGS activity. On the other hand, in 11B-MTX-P cells the major defect was found to be a significant decline in FPGS activity. It is also demonstrated that methotrexate-resistant 11B-MTX-P cells were concomitantly crossresistant to ICI-198,583, but retained full sensitivity to DDATHF.

MATERIALS AND METHODS

Chemicals

Folate-free RPMI-1640 medium and dialysed fetal calf serum (FCS) were obtained from Gibco. d,l-5-Formyltetrahydrofolate (5-formyl-THF), folic acid, 5-FU and 5-fluoro-2-deoxyuridine (FUdR) were purchased from Sigma and methotrexate was a gift from Pharmachemie, Haarlem, The Netherlands. Trimetrexate glucuronate salt was obtained from Warner Lambert/Park Davis, Ann Arbor, USA. 10-EdAM was a gift from Ciba Geigy, Basel; DDATHF was a generous gift of Dr G. B. Grindey, Lilly Research Laboratories, Indianapolis, USA. ICI-198,583 was provided by ICI Pharmaceuticals, Macclesfield, UK. [^{3}H]-methotrexate (740 GBq/mmol) was obtained from Moravek Biochemical, Brea, USA, and was purified prior to use by thin-layer chromatography as described before [16–18]. Radiochemical purity of the labelled compound was more than 99% after rechromatography. [^{3}H]-glutamate (925 GBq/mmol) was obtained from NEN Research, Boston, USA. All other reagents were of the highest grade of purity available.

Cell culture

UM-SCC-11B cells, a cell line originated from a moderately differentiated human squamous cell carcinoma of the larynx, were kindly provided by Dr T. E. Carey, University of Michigan, Ann Arbor, USA. Parental cells (further referred to as SCC-11B cells) were adapted to nanomolar concentrations of 5-formyl-THF as described before [12]. Cells were grown as a monolayer at 37°C in a 5% CO_2 humidified atmosphere in "folate-conditioned" RPMI-1640 medium supplemented with 10% dialysed FCS, 5 nmol/l 5-formyl-THF, 5 nmol/l folic acid, 2 nmol/l glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml).

Development of resistance

11B-MTX-C. The isolation of cells which were made resistant by continuous exposure to methotrexate was established by growing SCC-11B cells in folate-conditioned medium supplemented with stepwise increasing concentrations of the drug from 10 to 400 nmol/l over 12 months. Cell doubling times at that time were similar to those of control cells.

11B-MTX-P. In other experiments, SCC-11B cells were made resistant to pulse exposures of methotrexate by adding 2 μmol/l methotrexate to cells 3 days after trypsinisation. 24 h later, cells were washed three times with drug-free medium and allowed to recover in folate-conditioned RPMI-1640 medium. At confluency, cells were trypsinised and seeded to a density of 1×10^4 cells/cm^2 in folate-conditioned medium. After eleven series of 24 h pulses of 2 μmol/l methotrexate, 11B-MTX-P cells had established the same doubling time as control cells. The resistant phenotype in 11B-MTX-P cells were found to be stable for at least four passages without addition of short-term methotrexate exposures (results not shown).

Cytotoxicity assay

Cells in folate-conditioned medium were plated in the individual well of a 24-well tissue culture plate at a density of 1×10^4 cells/cm^2. Appropriate dosages of 5-formyl-THF and the antifolate drugs were added 24 h later. In cytotoxicity assays drug exposure to the cells was maintained either for a period of 7 days (long-term exposure) or for 24 h (short-term exposure). In the latter case, the drug containing medium was removed after 24 h, cells were washed three times with drug-free medium then incubated for 7 days in folate-conditioned medium. After this time, cells were washed twice with phosphate-buffered saline solution (PBS) to remove non-viable cells, trypsinised (0.25% trypsin/0.05% EDTA in PBS) and counted by a Sysmex CC-110 cell counter. The IC_{50} is defined as the concentration of drug required to inhibit cell growth by 50%, compared to controls.

Drugs

Methotrexate and 10-EdAM are classical folate-based inhibitors of DHFR. Trimetrexate is also a potent inhibitor of DHFR but unlike methotrexate or 10-EdAM does not require a specific carrier system for cellular uptake [19]. Growth inhibition exper-

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iments for methotrexate and trimetrexate were either done in the absence or presence of an additional 20 nmol/l 5-formyl-THF in order to establish the role of the (anti)folate membrane transport system in the cytotoxicity of trimetrexate and methotrexate. Parental and resistant SCC-11B cells were also tested for growth inhibition by two novel antifolates which have target enzymes other than DHFR. DDATHF is a potent folate-based inhibitor of GAR-TFase, one of the folate-dependent key enzymes in purine biosynthesis *de novo* [20]. ICI-198, 583 is a folate-based inhibitor of TS [21]. FUdR and 5-FU, after conversion to the active metabolite 5-fluoro-2'de-oxyuridine-5'monophosphate (FdUMP), can also have an inhibitory effect on TS [22]. These two drugs were used as a control for ICI-198, 583 cytotoxicity.

[^{3}H]-methotrexate uptake

HNSCC cells in the mid-log phase of growth were harvested by trypsinisation, washed with folate-free RPMI-1640 medium supplemented with 10% dialysed FCS, and resuspended to a single cell suspension in 1 ml of Hepes-buffered saline solution (HBSS buffer) [13], containing 107 mmol/l NaCl, 20 mmol/l Hepes, 26.2 mmol/l $NaHCO_3$, 5.3 mmol/l KCl, 1.9 mmol/l $CaCl_2$, 1.0 mmol/l $MgCl_2$, 7 mmol/l D-glucose, pH 7.4 with NaOH. Influx of [^{3}H]-methotrexate was determined over a period of 2.5 min at 37°C at an extracellular concentration of 2 μmol/l [^{3}H]-methotrexate (specific activity 500 cpm/pmol). Uptake of [^{3}H]-methotrexate at 4°C served as a control. [^{3}H]-methotrexate uptake was stopped by the addition of 9 vol ice-cold transport buffer. Cells were centrifuged for 5 min at 800 *g*, and washed once more with 10 ml ice-cold transport buffer. The final pellet was resuspended in 0.5 ml water and analysed for ^{3}H-radioactivity in Optifluor scintillation fluid (United Technologies Packard, Brussels) with the use of an Isocap/300 (Searle Nuclear, Chicago) scintillation counter with a counting efficiency for ^{3}H of 51%.

[^{3}H]-folic acid binding

[^{3}H]-folic acid binding studies were carried out as described previously [17]. In short, HNSCC cells (10^7) were harvested as described above and suspended in 1 ml ice-cold HBSS buffer. Cells were then incubated for 10 min at 4°C with 100 pmol [^{3}H]-folic acid (specific activity 37 GBq/mmol), followed by centrifugation in an Eppendorf minicentrifuge (13 000 *g*, 1 min). The supernatant was removed by suction and residual fluid was removed by cotton tissues. Cell pellets were resuspended in water and analysed for radioactivity. Non-specific binding of radiolabel was determined by measuring radioactivity in the presence of unlabelled folic acid.

Other methods

Dihydrofolate reductase (DHFR) activity was determined according to the method described by Mini *et al.* [23]. Folylpolyglutamate synthase activity was analysed as described by McGuire *et al.* [24] using 250 μmol/l methotrexate as substrate for FPGS. Protein concentrations were determined according to Bradford [25].

RESULTS

Cytotoxicity experiments

Compared to parental cells 11B-MTX-C cells were found to be 91-fold resistant to methotrexate (Fig. 1a) in growth inhibition experiments with long-term drug exposure. In addition, these cells were 30-fold and 49-fold more crossresistant to trimetrexate and 10-EdAM, respectively. 11B-MTX-C cells exhibited minor crossresistance to ICI-198,583 (5.9-fold), but retained full sensitivity to DDATHF. Following a short-term drug exposure (Table 1) 11B-MTX-C cells were highly (42 000-fold) resistant to methotrexate as compared to parental cells (IC_{50}: 7.4 mmol/l vs. 177 nmol/l, respectively). In similar experiments a 13.5-fold crossresistance was also noticed for 11B-MTX-C cells during a short exposure to ICI-198,583.

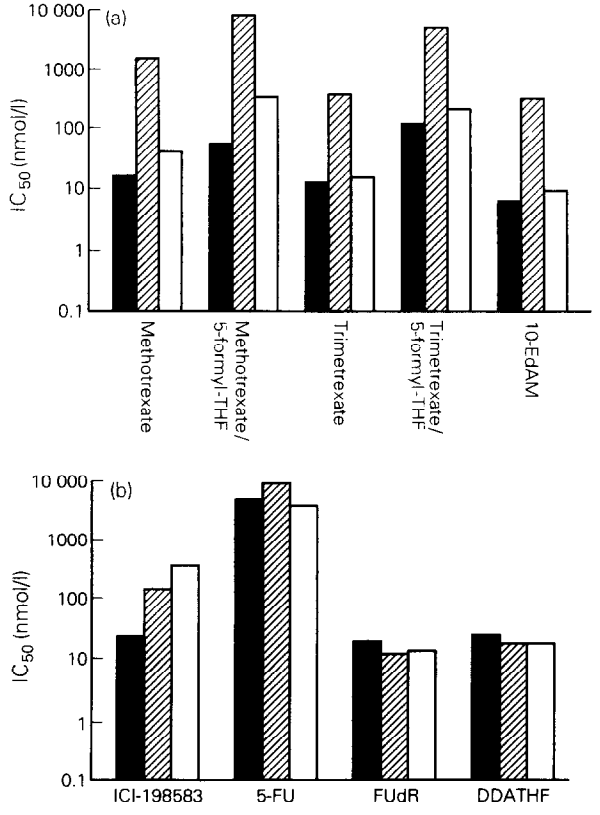

Fig. 1. Growth inhibition of parental SCC-11B (■), 11B-MTX-C (▨) and 11B-MTX-P (□) cells by (a) DHFR inhibitors in the absence or presence of 20 nmol/l 5-formyl-THF or (b) non-DHFR targetting folate analogues. Drug exposure: 7 days. Results are expressed as the mean of at least 5 separate experiments.

Table 1 shows that 11B-MTX-P cells, compared to parental cells, were 46-fold less sensitive to growth inhibition by methotrexate following a 24-h exposure to the drug (IC_{50}: 8.1 μmol/l vs. 177 nmol/l, respectively). However, during long-term drug exposure (Fig. 1a) 11B-MTX-P cells showed only a 2.5-fold diminished sensitivity for methotrexate, 1.5-fold decreased sensitivity for 10-EdAM, but full sensitivity for trimetrexate. 11B-MTX-P cells demonstrated substantial crossresistance to ICI-

Table 1. Growth inhibition of parental and resistant HNSCC-11B cells after short-term (24 h) drug exposure

	IC_{50}		
Drugs	SCC-11B	11B-MTX-C	11B-MTX-P
Methotrexate	177 (95) nmol/l	7.4 (3.5) mmol/l	8.1 (4.4) μmol/l
ICI-198,583	43 nmol/l	580 nmol/l	2 μmol/l

Mean (S.D.) of at least four experiments (methotrexate) and mean of two experiments (ICI-198,583).

Table 2. [^3H]-methotrexate membrane transport, DHFR and FPGS-specific activity in parental cells and resistant HNSCC-11B cells

	SCC-11B	11B-MTX-C	11B-MTX-P
DHFR activity (μmol FH_2/h/mg protein)	0.012 (0.005) (n=4)	0.758 (0.162) (n=3)	0.014 (0.008) (n=4)
Methotrexate uptake (pmol [^{3}H]-methotrexate/min/10^7 cells)	0.71 (0.25) (n=4)	0.29 (0.11) (n=3)	0.57 (0.15) (n=3)
FPGS activity (pmol [^{3}H]-Glu incorporated/h/mg protein)	1575 (602) (n=4)	407 (152) (n=3)	281 (80) (n=5)

Mean (S.D.)

198,583 both in short-term (Table 1) as well as in long-term exposure (Fig. 1b) to this drug (47-fold and 14-fold, respectively). No difference in growth inhibition by 5-FU and FUdR was observed for parental and resistant cells (Fig. 1b). 11B-MTX-C cells, 11B-MTX-P cells and parental cells were equally sensitive to growth inhibition by DDATHF.

Methotrexate transport

The presence of multiple transport routes for methotrexate in parental and resistant cells was determined via [^{3}H]-methotrexate influx and [^{3}H]-folic acid binding studies, and indirectly via the protective effect by 5-formyl-THF upon growth inhibition by methotrexate and trimetrexate. [^{3}H]-folic acid binding studies failed to detect the expression of mFBP in parental and/or resistant cells (results not shown). Other observations also suggest that mFBP is not involved in (anti)folate uptake in these cells. Cells expressing mFBP are usually very sensitive (IC_{50}<2 nmol/l) to growth inhibition by ICI-198,583 [26] or DDATHF [27], which is based upon the high affinity of mFBP for these compounds. In contrast, 11B-MTX-C and 11B-MTX-P cells exhibit partial resistance to ICI-198,583 (Fig. 1b). Furthermore, the significant protection against methotrexate and trimetrexate cytotoxicity by 5-formyl-THF is characteristic feature indicative for the role of the reduced folate carrier system in methotrexate/5-formyl-THF transport rather than mFBP [18].

Methotrexate transport studies (Table 2) demonstrate that [^{3}H]-methotrexate influx in 11B-MTX-C cells is approximately 60% lower than in parental cells. In 11B-MTX-P cells [^{3}H]-methotrexate influx is reduced by approximately 20%.

DHFR and FPGS activity

As shown in Table 2, DHFR activity in 11B-MTX-C cells was significantly increased (63-fold) compared to parental cells. No significant changes in DHFR activity were observed in 11B-MTX-P cells.

FPGS-activity was found to be significantly decreased in 11B-MTX-P cells (5.6-fold) as well as in 11B-MTX-C cells (3.8-fold).

DISCUSSION

This study describes the mechanisms of resistance to methotrexate for a human HNSCC cell line in which resistance was developed either by continuous exposure to stepwise increasing drug concentrations or by serial pulse exposures. All these experiments were carried out in "folate-conditioned" medium containing nanomolar concentrations of folic acid and 5-formyl-THF as folate source. Recent data have shown that adaptation of cells to folate concentrations approaching near physiological levels in the culture medium, can result in either a more efficient function of the classical reduced folate/methotrexate carrier system [11–13] or in an up-regulation of the expression of an mFBP [15–17, 28]. In the present study, however, we have not found any evidence for the expression of mFBP, neither in parental nor in resistant 11B-MTX-C and 11B-MTX-P cells.

The mechanisms of resistance to methotrexate observed for SCC-11B cells, grown in folate-conditioned medium and exposed to progressively increasing concentrations of the drug (11B-MTX-C), were not significantly different from mechanisms of resistance in HNSCC cells grown in "standard" folate medium as reported by others [6, 8, 9]. Multiple defects in resistant 11B-MTX-C cells could be identified, including a significantly increased activity of DHFR, diminished [^{3}H]-methotrexate transport and decreased FPGS activity. Crossresistance of 11B-MTX-C cells to trimetrexate and 10-EdAM suggest that the increased level of DHFR is the major factor for the methotrexate resistance in these cells. Whether, and to what extent, the diminished [^{3}H]-methotrexate transport and FPGS activity contributed in the resistance to methotrexate is not clear. The fact that full sensitivity is retained for DDATHF, an antifolate which can also be transported via the reduced folate/methotrexate carrier system [27], suggests that the decreased membrane transport capacity is of lesser importance in long term cytotoxicity experiments. In this regard, however, it should be noted that the folate-based TS inhibitor ICI-198, 583 is a high affinity substrate for the reduced folate carrier system as well [26]. Nevertheless, crossresistance to ICI-198,583 was observed for 11B-MTX-C cells in cytotoxicity experiments with short as well as in long-term drug exposures.

It has been shown that 5-FU can interfere with the translocation of nuclear RNA to the cytoplasm, although it is not clear whether the effect of 5-FU is RNA or DNA directed [22]. It is generally assumed that FUdR acts as an inhibitor of TS [22]. The action of 5-FU and FUdR is mediated by their metabolite 5-fluoro-2'deoxy-5'monophosphate (FdUMP). The unchanged sensitivities of 11B-MTX-C and 11B-MTX-P cells for 5-FU and FUdR (Fig. 1b) therefore suggest that elevated levels of TS are not involved in the crossresistance to ICI-198,583.

There is reason to believe that the decreased activity of FPGS in these cells could be of importance in explaining the crossresistance to ICI-198,583. It has been reported that methotrexate and ICI-198,583 are relatively poor substrates for FPGS ($K_m \approx 100$ μmol/l and 40 μmol/l, respectively) [29–31]. This is in contrast to DDATHF, which is an efficient substrate for FPGS (K_m: <10 μmol/l) [20]. The differences in the affinities of the antifolates for FPGS in combination with a 3.8-fold decreased FPGS activity are in concert with the observed cytotoxic effects of ICI-198,583 and DDATHF.

Resistance to methotrexate in 11B-MTX-P cells can be ascribed to a significantly reduced FPGS activity and a slightly reduced alteration in membrane transport. Recently, Pizzorno *et al.* [10] have described that human leukaemia cells treated with a similar regimen of methotrexate pulse exposures also developed resistance to the drug which was based upon decreased FPGS activity. Another study by Pizzorno *et al.* [9] indicated that a diminished FPGS activity could be associated with intrinsic resistance of HNSCC cells to methotrexate. In the present study we describe that also acquired resistance to

methotrexate related to decreased FPGS activity can be of importance as mechanism of resistance to methotrexate in HNSCC cells. Similarly as was shown for 11B-MTX-C cells, 11B-MTX-P cells were crossresistant to ICI-198,583 but not to DDATHF. This result suggests that although SCC-11B cells were exposed to serial pulses of a single agent like methotrexate, a decreased FPGS activity can also lead to resistance to antifolates which have target enzymes other than DHFR. In case of ICI-198,583, the relatively poor affinity for FPGS is likely to be an important factor in the crossresistance of 11B-MTX-P to ICI-198,583. On the other hand, the highly efficient transport of DDATHF via the reduced folate/methotrexate carrier system [27, 32], together with its excellent substrate affinity for FPGS, may be the determining factor in the sensitivity of (methotrexate-resistant) SCC-11B cells to DDATHF.

These experiments indicate that different mechanisms of resistance to methotrexate and crossresistance to other antifolates can be acquired by schedule dependent treatment of HNSCC cells with methotrexate. This finding can be of importance in further research for mechanisms of resistance to antifolate drugs, which is considered to be one of the most important factors in the low response rates to antifolates in the clinic.

1. Snow GB. Evaluation of new treatment methods for head and neck cancer: a challenge. *Acta Otolaryngol* 1989, **107**, 352–356.
2. Al-Sarraf M. Head and neck cancer: chemotherapy concepts. *Semin Oncol* 1988, **15**, 70–85.
3. Schornagel JH, McVie JG. The clinical pharmacology of methotrexate, a review. *Cancer Treat Rev* 1983, **10**, 53–75.
4. Curt GA, Clendeninn NJ, Chabner BA. Drug resistance in cancer. *Cancer Treat Rep* 1984, **68**, 87–99.
5. Bertino JR, Romanini A, Dicker A, Volkenandt M, Lin JT, Schweitzer B. Resistance to methotrexate in experimental models and in patients. In: Curtius H-Ch, Ghisla S, Blau N, eds. *Chemistry and Biology of Pteridines 1989*. Berlin, Walter de Gruyter, 1990, 1089–1099.
6. Frei III E, Rosowsky A, Wright JE, *et al.* Development of methotrexate resistance in a human squamous cell carcinoma of the head and neck in culture. *Proc Natl Acad Sci USA* 1984, **81**, 2873–2877.
7. Spiegel J, Carey TE, Shimoura S, Krause CJ. *In vitro* sensitivity and resistance of cultured human squamous carcinoma cells to cis-platinum and methotrexate. *Otolaryngol Head Neck Surg* 1984, **92**, 524–531.
8. Rosowsky A, Wright JE, Cucchi CA, *et al.* Phenotypic heterogeneity in cultured human head and neck squamous cell carcinoma lines with low level methotrexate resistance. *Cancer Res* 1985, **45**, 6205–6212.
9. Pizzorno G, Chang Y, McGuire JJ, Bertino JR. Inherent resistance of human squamous carcinoma cell lines to methotrexate as a result of decreased polyglutamylation of this drug. *Cancer Res* 1989, **49**, 5275–5280.
10. Pizzorno G, Mini E, Corronnello 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**, 2149–2155.
11. Jansen G, Westerhof GR, Jarmuszewski MJA, Kathmann I, Rijksen G, Schornagel JH. Methotrexate transport in variant human CCRF-CEM leukemia cells with elevated levels of the reduced folate carrier. Selective effect on carrier-mediated transport of physiological concentrations of reduced folates. *J Biol Chem* 1990, **265**, 18272–18277.
12. van der Laan BFAM, Jansen G, van Gestel JA, Schornagel JH, Hordijk GJ. Membrane transport of methotrexate in a human squamous cell carcinoma cell line adapted to low folate concentrations in the culture medium. *Anticancer Res* (in press).
13. Sirotnak FM, Moccio DM, Yang CH. A novel class of genetic variants of the L1210 cell up-regulated for folate analogue transport inward. *J Biol Chem* 1984, **259**, 13139–13144.
14. Antony AC, Kane MA, Portillo RM, Elwood PC, Kolhouse JF. Studies of the role of a particulate folate binding protein in the uptake of 5-methyltetrahydrofolate by cultured human KB cells. *J Biol Chem* 1985, **260**, 14911–14917.
15. Henderson GB, Tsuji JM, Kumar HP. Mediated uptake of folate by a high-affinity binding protein in sublines of L1210 cells adapted to nanomolar concentrations of folate. *J Membrane Biol* 1988, **101**, 247–258.
16. Jansen G, Kathmann I, Rademaker BC, *et al.* Expression of a folate binding protein in L1210 cells grown in low folate medium. *Cancer Res* 1989, **49**, 1959–1963.
17. Jansen G, Westerhof GR, Kathmann I, Rademaker BC, Rijksen G, Schornagel JH. Identification of a membrane-associated folate-binding protein in human leukemic CCRF-CEM cells with transport related methotrexate resistance. *Cancer Res* 1989, **49**, 2455–2459.
18. van der Veer LJ, Westerhof GR, Rijksen G, Schornagel JH, Jansen G. Cytotoxicity of methotrexate and trimetrexate and its reversal by folinic acid in human leukemic CCRF-CEM cells with carrier-mediated and receptor-mediated folate uptake. *Leuk Res* 1989, **13**, 981–987.
19. Fry DW, Besserer JA. Characterisation of trimetrexate transport in human lymphoblastoid cells and development of impaired influx as a mechanism of resistance to lipophilic antifolates. *Cancer Res* 1988, **48**, 6986–6991.
20. Beardsley GP, Moroson BA, Taylor EC, Moran RG. A new folate antimetabolite 5,10-dideaza-5,6,7,8-tetrahydrofolate is a potent inhibitor of de novo purine synthesis. *J Biol Chem* 1989, **264**, 328–333.
21. Jackman AL, Newell DR, Jodrell DI, *et al.* *In vitro* and *in vivo* studies with 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolate (ICI 198583), an inhibitor of thymidylate synthase. In: Curtius H-Ch, Ghisla S, Blau N, eds. *Chemistry and Biology of Pteridines 1989*. Berlin, Walter de Gruyter, 1990, 1023–1026.
22. Pinedo HM; Peters GJ. Fluorouracil: biochemistry and pharmacology. *J Clin Oncol* 1988, **6**, 1653–1664.
23. Mini E, Moroson BA, Franco CT, Bertino JR. Cytotoxic effects of folate antagonists against methotrexate-resistant human leukemic lymphoblast CCRF-CEM cell lines. *Cancer Res* 1985, **45**, 325–330.
24. McGuire JJ; Mini E, Hsieh P, Bertino JR. Role of methotrexate polyglutamates in methotrexate- and sequential methotrexate-5-fluorouracil-mediated cell kill. *Cancer Res* 1985, **45**, 6395–6400.
25. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976, **72**, 248–254.
26. Jansen G, Schornagel JH, Westerhof GR, Rijksen G, Newell DR, Jackman AL. Multiple membrane transport systems for the uptake of folate-based thymidylate synthase inhibitors. *Cancer Res* 1990, **50**, 7544–7548.
27. Jansen G, Westerhof GR, Kathmann I, Rijksen G, Schornagel JH. Growth inhibitory effects of 5,10-dideazatetrahydrofolic acid on variant murine L1210 and human CCRF-CEM leukemia cells with different membrane-transport characteristics for (anti)folate compounds. *Cancer Chemother Pharmacol* 1991, **28**, 115–117.
28. Kamen BA, Capdevila A. Receptor-mediated folate accumulation is regulated by the cellular folate content. *Proc Natl Acad Sci USA* 1986, **83**, 5983–5987.
29. Moran RG, Colman PD, Rosowsky A, Forsch RA, Chan KK. Structural features of 4-amino antifolates required for substrate activity with mammalian folylpolyglutamate synthetase. *Mol Pharmacol* 1984, **27**, 156–166.
30. Jackman AL, Taylor GA, O'Connor BM, Bishop JAM, Moran RG, Calvert AH. Activity of the thymidylate synthase inhibitor 2-desamino-N^{10}-propargyl-5,8-dideazafolic acid and related compounds in murine (L1210) and human (W1L2) systems *in vitro* and in L1210 *in vivo*. *Cancer Res* 1990, **50**, 5212–5218.
31. Rumberger BG, Barrueco JR, Sirotnak FM. Differing specificities for 4-aminofolate analogues of folylpolyglutamyl synthetase from tumors and proliferative intestinal epithelium of the mouse with significance for selective antitumor action. *Cancer Res* 1990, **50**, 4639–4643.
32. Pizzorno, G, Moroson BA, Cashmore AR, Cross AD, Beardsley GP. Transport of 5,10-dideazatetrahydrofolic acid (DDATHF) in CCRF-CEM sensitive and methotrexate resistant cell lines. In: Curtius H-Ch, Ghisla S, Blau N, eds. *Chemistry and Biology of Pteridines 1989*. Berlin, Walter de Gruyter, 1990, 1031–1034.

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