SENSITIVITY TO METHOTREXATE AND NOVEL ANTIFOLATES IN HUMAN SQUAMOUS CARCINOMA CELL LINES OF THE HEAD AND NECK

B.F.A.M. van der Laan

SENSITIVITY TO METHOTREXATE AND NOVEL ANTIFOLATES IN HUMAN SQUAMOUS CARCINOMA CELL LINES OF THE HEAD AND NECK

aan mijn moeder en josephine,
jullie wil om te leven was mijn motivatie.

SENSITIVITY TO METHOTREXATE AND NOVEL ANTIFOLATES IN HUMAN SQUAMOUS CARCINOMA CELL LINES OF THE HEAD AND NECK

DE GEVOELIGHEID VOOR METHOTREXAAT EN NIEUWE ANTIFOLATEN
IN HUMANE PLAVEISELCELCARCINOOM-CELLIJNEN
UIT HET HOOFD-HALSGEBIED
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Rijksuniversiteit te Utrecht op gezag van de Rector Magnificus, Prof. Dr. J.A. van Ginkel, ingevolge het besluit van het College van Dekanen in het openbaar te verdedigen op woensdag 11 december 1991 des voormiddags te 10.30 uur

door

BERNARDUS FRANCISCUS AUGUSTINUS MARIA VAN DER LAAN

geboren op 23 mei 1959 te Laren, N.H.

promotor:

Prof. Dr. G.J. Hordijk,
verbonden aan de Faculteit der Geneeskunde van de
Rijksuniversiteit te Utrecht

co-promotoren:

Dr. J.H. Schornagel,
verbonden aan het Nederlands Kanker Instituut,
Antoni van Leeuwenhoek Ziekenhuis te Amsterdam

Dr. G. Jansen,
verbonden aan de afdeling Oncologie van het
Academisch Ziekenhuis Vrije Universiteit te Amsterdam

The work presented in this thesis was performed at the anti-folate laboratory, Department of Internal Medicine, Oncology Unit, University Hospital, Utrecht, which was later transferred to the Academic Hospital Free University, Amsterdam, in collaboration with the Department of Otorhinolaryngology Head and Neck Surgery, Academic Hospital, Utrecht, The Netherlands.

This study was supported by grants from the Dutch Cancer Society (UUKC 89-06 and IKA 89-34).

Publication of this thesis was partially enabled by gifts from Entermed B.V., SmithKline Beecham, Glaxo B.V. and Pharmachemie B.V. Haarlem, The Netherlands.

CONTENTS

LIST OF ABBREVIATIONS

AICAR-TFase	aminoimidazole carboxamide ribonucleotide transformylase
CB3717	N^{10}-propargyl-5,8-dideazafolic acid
DDATHF	5,10-dideazatetrahydrofolic acid
DHFR	dihydrofolate reductase
FCS	fetal calf serum
FH_2	dihydrofolate
FH_4	(THF) tetrahydrofolate
FPGH	folylpolyglutamate hydrolase
FPGS	folylpolyglutamate synthase
FUdR	5-fluoro-2-deoxyuridine
GAR-TFase	glycinamide ribonucleotide transformylase
Glu	glutamate
HNSCC	squamous cell carcinoma of the head and neck
IC_{50}	concentration of drug required to inhibit cell growth by 50%, as compared to controls
ICI-198,583	2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid
mFBP	membrane-associated folate binding protein
MTX	methotrexate
NHS-MTX	N-hydroxysuccimide ester of methotrexate
THF	(FH_4) tetrahydrofolate
TMQ	trimetrexate
TS	thymidylate synthase
5-formyl-THF	(leucovorin) d,l-5-formyltetrahydrofolate
5-FU	5-fluorouracil
10-EdAM	10-ethyl-10-deazaaminopterin

Chapter 1

GENERAL INTRODUCTION

INTRODUCTION

Squamous cell carcinomas arising in the upper aerodigestive tract account for 4-5% of all cancers. Due to its region of occurrence these tumours have an enormous impact upon the patients affected. The tumour itself or the applied treatment, particularly surgery, may result in disfigurement and loss of function with inevitable psychological implications.

Surgery and radiotherapy are still the best local and regional treatments for head and neck cancer. It appears, however, that the cure rate of surgery and radiotherapy have reached a plateau [1]. Although local invasion and regional lymph node metastases remain the primary causes for patients morbidity and mortality, distant metastatic disease is not an infrequent problem. Up to 25% of patients with advanced disease will relapse at distant sites [2]. Moreover, autopsy studies suggest that as many as 50% of patients who die of squamous cell carcinomas of the head and neck (HNSCC) harbour clinical or occult distant metastasis [3,4].

These data indicate that apart from local and regional treatment, advanced squamous cell carcinomas of the head and neck will need systemic therapy, such as chemotherapy, either as a primary or as an adjunctive treatment. Although many tumours are initially responsive to chemotherapy, the duration of response is usually short. So far, chemotherapy has failed to increase the overall survival of patients with head and neck cancer [2,5]. This may be due to natural or acquired drug resistance.

Research on mechanisms of cellular resistance to chemotherapy and on identifying new drugs will probably provide the key to more successful drug treatment. Thus optimization of chemotherapy is still a very important goal in improving the treatment of head and neck cancer.

REFERENCES

1. Snow GB. Evaluation of new treatment methods for head and neck cancer: a challenge. *Acta Otolaryngol* 1989, **107**, 352-356.

2. Clark JR, Frei III E. Chemotherapy for head and neck cancer: progress and controversy in the management of patients with M_0 disease. *Seminars in Oncology* 1989, **16**, suppl 6, 44-57.

3. Hoye RC, Herrold KM, Smith RR, Thomas LB. A clinicopathological study of epidermoid carcinoma of the head and neck. *Cancer* 1962, **15**, 741-749.

4. Dennington ML, Carter DR, Meyers AD. Distant metastases in head and neck epidermoid carcinoma. *Laryngoscope* 1980, **90**, 196-201.

5. Al-Sarraf M. Head and Neck Cancer: Chemotherapy Concepts. *Seminars in Oncology* 1988, **15**, 70-85.

OBJECTIVES OF THIS THESIS:

1. To elucidate the role of membrane transport in methotrexate (MTX) cytotoxicity to HNSCC. For this purpose HNSCC cell lines were adapted to growth in culture medium containing folate concentrations within the physiological range, to clarify the role of two different (anti-)folate transport systems: (a) the classical reduced folate/MTX carrier and (b) a membrane-associated folate binding protein (mFBP).

2. To investigate the mechanisms of acquired resistance to MTX in HNSCC. For this purpose a HNSCC cell line was exposed to pulse doses of MTX to imitate clinical drug schedules.

3. To analyze the occurrence and mechanisms of natural resistance to MTX in HNSCC. For this purpose the growth inhibitory effect of MTX was determined in seven different HNSCC cell lines following short-term drug exposures to mimic a more clinical situation.

4. To evaluate the sensitivity of HNSCC to novel antifolates. For this purpose MTX-sensitive and MTX-resistant HNSCC cell lines with inherent or acquired resistance to MTX were exposed to novel antifolates such as trimetrexate, 10-EdAM, ICI-198,583 and DDATHF.

INTRODUCTION

Surgery and radiotherapy are still the best local and regional treatment for head and neck cancer. It appears, however, that the cure rate of surgery and radiotherapy have reached a plateau [1]. Although these regimens are curative, and lymph node metastases remain the primary cause for curative treatment, and mortality, distant metastatic disease is not an infrequent problem. Up to 25% of patients with advanced disease will relapse at distant sites [2]. Moreover, distant metastasis [3,4].

These data indicate that apart from local and regional treatment, advanced of response is usually short. So far, chemotherapy has not improved the overall survival of patients with head and neck cancer [2,5]. This may be due to natural or acquired drug resistance.

Research on mechanisms of cellular resistance to chemotherapy and on identifying new drugs will probably provide the key to more successful drug treatment. Thus optimization of chemotherapy is still a very important goal in improving the treatment of head and neck cancer.

REFERENCES

1. Snow GB. Evaluation of new treatment methods for head and neck cancer: a challenge. *Acta Otolaryngol* 1989, 107, 352-356.

2. Clark JR, Frei III E. Chemotherapy for head and neck cancer: progress and controversy in the management of patients with M_1 disease. *Seminars in Oncology* 1989, 16, suppl 6, 44-57.

3. Hoye RC, Herrold KM, Smith RR, Thomas LB. A clinicopathological study of epidermoid carcinoma of the head and neck. *Cancer* 1962, 15, 741-749.

4. Dennington ML, Carter DR, Meyers AD. Distant metastases in head and neck epidermoid carcinoma. *Laryngoscope* 1980, 90, 196-201.

5. Al-Sarraf M. Head and Neck Cancer: Chemotherapy Concepts. *Seminars in Oncology* 1988, 15, 70-85.

CHAPTER 2

AN INTRODUCTION TO FOLATE ANALOGUES, IN PARTICULAR THE ANTIFOLATE METHOTREXATE.

INTRODUCTION

Antimetabolites are analogues of normal metabolites which may inhibit metabolic pathways or may be mistaken for normal metabolites during synthesis of macromolecules such as DNA or RNA. Antimetabolites can be divided in two major classes; (a) the purine and pyrimidine analogues, and (b) the folate analogues. Reduced folates play an important role as C_1 donor in several biosynthetic reactions, including the *de novo* synthesis of amino acids, purines and thymidylate (Table 1).

The recognition of reduced folate compounds as important coenzymes in *de novo* synthesis of purines, thymidylate and, ultimately, DNA soon led to the development of folate analogues as potential antineoplastic drugs [1]. The folate analogue methotrexate (MTX) is by now the most commonly used and most thoroughly studied antineoplastic drug. MTX has an established role in the treatment of acute lymphocytic leukaemia, non-Hogkin's lymphoma, osteosarcoma, choriocarcinoma,
breast cancer, head and neck cancer and other tumours [2,3]. MTX (4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid) is a structural analogue of folic acid (Fig. 1), in which the hydroxyl-group at the N^4 position of the pteridine ring has been replaced by an amino group, and an additional methyl group is located at the N^{10} position.

CELL MEMBRANE TRANSPORT

MTX is a divalent anion on basis of the negatively charged carboxyl groups of the glutamate side chain (Fig. 1). Therefore, this drug cannot simply pass the plasma membrane by passive diffusion. Several transport routes of cellular MTX entry have been identified, which shall be discussed hereafter.

Table 1. Some biosynthetic reactions in which folate coenzymes serve as a one-carbon donor.

Coenzyme	Reaction
5-methyl-THF	homocysteine ----- methionine
5,10-methylene-THF	glycine ----- serine
5,10-methylene-THF	dUMP ----- dTMP
10-formyl-THF	GAR ----- formyl-GAR
10-formyl-THF	AICAR ----- formyl-AICAR

THF: tetrahydrofolate, dUMP: deoxyuridine monophosphate, dTMP: deoxythymine monophosphate, GAR: glycinamide ribonucleotide, AICAR: aminoimidazole carboxamide ribonucleotide.

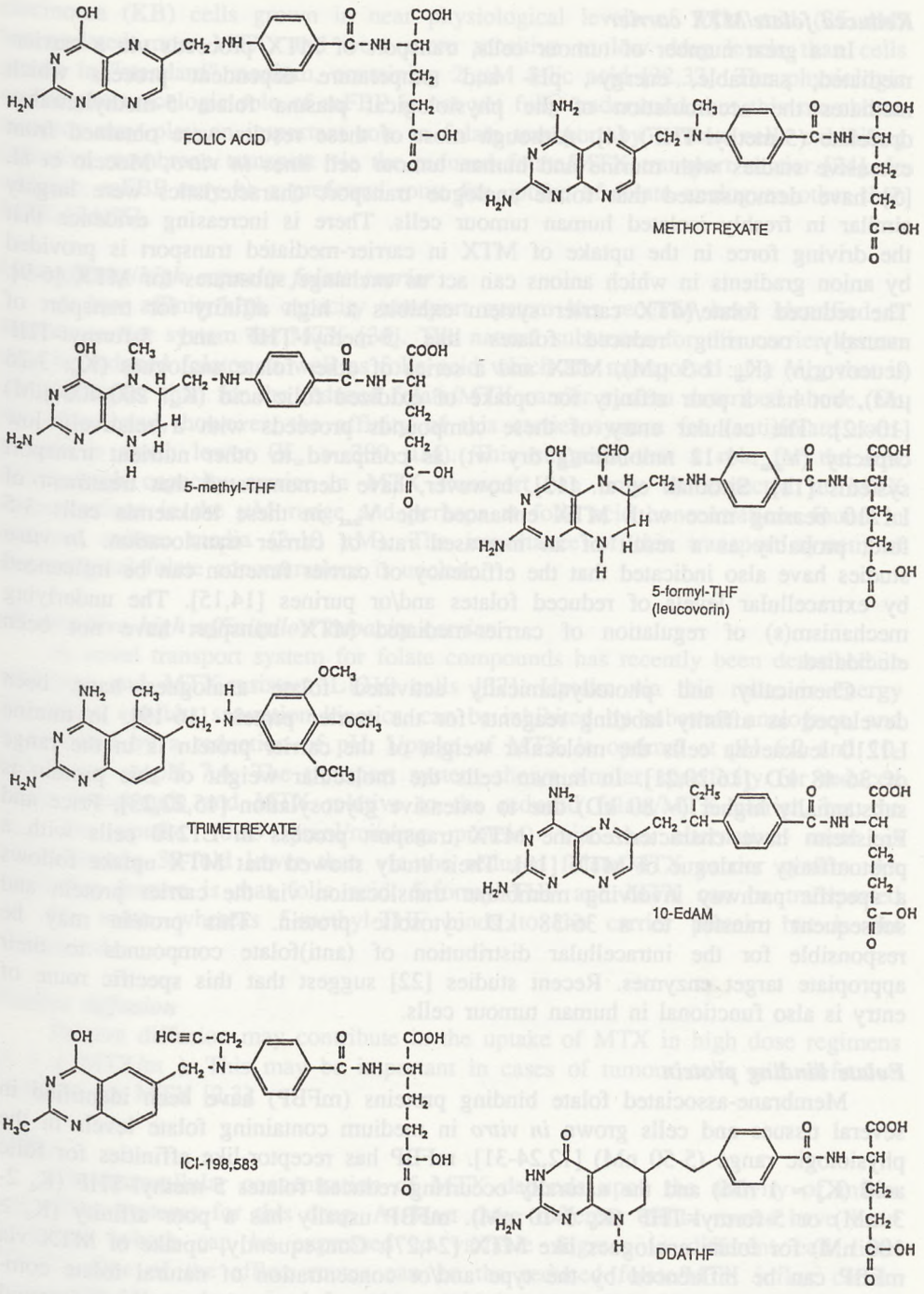

Fig. 1. The chemical structures of folic acid, MTX, reduced folates and some novel antifolates.

Reduced folate/MTX carrier

In a great number of tumour cells, transport of MTX proceeds via a carrier-mediated, saturable, energy-, pH- and temperature dependent process which mediates the accumulation of the physiological plasma folate 5-methyltetrahydrofolate (5-methyl-THF) [4]. Although most of these results were obtained from extensive studies with murine and human tumour cell lines *in vitro*, Moccio et al. [5] have demonstrated that folate analogue transport characteristics were largely similar in freshly isolated human tumour cells. There is increasing evidence that the driving force in the uptake of MTX in carrier-mediated transport is provided by anion gradients in which anions can act as exchange substrates for MTX [6-9]. The reduced folate/MTX carrier system exhibits a high affinity for transport of naturally occurring reduced folates like 5-methyl-THF and 5-formyl-THF (leucovorin) (K_m: 1-3 μM), MTX and a series of other folate analogues (K_m: 3-26 μM), but has a poor affinity for uptake of oxidized folic acid (K_m: 200-400 μM) [10-12]. The cellular entry of these compounds proceeds with a relatively low capacity (V_{max}: 1-12 nmol/min/g dry wt) as compared to other nutrient transport systems [11]. Sirotnak et al. [13], however, have demonstrated that treatment of L1210 bearing mice with MTX enhanced the V_{max} in these leukaemia cells 3-5 fold, probably as a result of an increased rate of carrier translocation. *In vitro* studies have also indicated that the efficiency of carrier function can be influenced by extracellular levels of reduced folates and/or purines [14,15]. The underlying mechanism(s) of regulation of carrier-mediated MTX transport have not been elucidated.

Chemically and photodynamically activated folate analogues have been developed as affinity labeling reagents for the carrier protein [16-19]. In murine L1210 leukaemia cells the molecular weight of the carrier protein is in the range of 36-48 kD [16,20,21]. In human cells the molecular weight of this protein is substantially higher (≈ 80 kD) due to extensive glycosylation [15,22,23]. Price and Freisheim have characterized the MTX transport process in L1210 cells with a photoaffinity analogue of MTX [18]. Their study showed that MTX uptake follows a specific pathway involving membrane translocation via the carrier protein and subsequent transfer to a 36-38 kD cytosolic protein. This protein may be responsible for the intracellular distribution of (anti)folate compounds to their appropiate target enzymes. Recent studies [22] suggest that this specific route of entry is also functional in human tumour cells.

Folate binding protein

Membrane-associated folate binding proteins (mFBP) have been identified in several tissues and cells grown *in vitro* in medium containing folate levels in the physiologic range (5-50 nM) [12,24-31]. mFBP has receptor-like affinities for folic acid ($K_d \approx 1$ nM) and the naturally occurring reduced folates 5-methyl-THF (K_d 2-3 nM) or 5-formyl-THF (K_d 5-10 nM). mFBP usually has a poor affinity ($K_d \geq 100$ nM) for folate analogues like MTX [24,27]. Consequently, uptake of MTX via mFBP can be influenced by the type and/or concentration of natural folate compounds present in the cell culture medium. Human nasopharyngeal epidermoid

carcinoma (KB) cells grown in near physiological levels of folic acid (25 nM) accumulated more MTX, and were more sensitive to low drug levels than cells grown in "standard" medium, containing 2 μM folic acid [32,33]. The physiologic and/or pharmacologic role of mFBP is not yet fully understood, but this membrane receptor may play an important role in folate transport by tumour cells exhibiting defective membrane transport via the reduced folate/MTX transport carrier [24]. In addition, mFBP may be a preferred route for uptake of folate analogues other than MTX [34,35].

Low affinity/high capacity folate carrier

A low affinity/high capacity transport system has recently been identified as another carrier system for MTX [36]. The natural substrates for this carrier system may be reduced folates as well as folic acid which are transported at a V_{max} that is 20-fold higher than for the reduced folate/MTX carrier system described above. On the other hand, however, the affinity of this carrier system for (anti)folate compounds is much lower (K_m > 300 μM). This suggests that a role for the low affinity/high capacity carrier in MTX transport can only be expected at MTX concentrations in the μM range and perhaps at folic acid concentrations found in standard culture media (2-10 μM). The importance of this transport system at physiological folate concentrations is unclear.

pH sensitive high affinity/low capacity carrier

A novel transport system for folate compounds has recently been described in wild-type and MTX-resistant L1210 cells [37]. Uptake via this route is energy dependent, exhibits saturation kinetics, can be inhibited by substrate analogues, and is activated by a reduction of pH. Uptake of MTX is optimal at pH 6.2 and 10-fold lower at pH 7.4. The transport system shows similar specificity for reduced folate compounds and MTX relative to the reduced folate/MTX carrier, but the V_{max} for uptake (0.55 pmol/min/mg protein) via the alternative route is approximately 30-fold lower than via the reduced folate/MTX carrier system. An interesting feature is that folic acid, 5-formyl-THF and MTX can be transported via this route, whereas 5-methyl-THF binds to the carrier protein but is not transported.

Passive diffusion

Passive diffusion may contribute in the uptake of MTX in high dose regimens (> 1 g MTX/m). This may be important in cases of tumour cells with defective transport for MTX [2,3].

Efflux

The intracellular concentration of MTX depends upon the activity of influx and efflux systems for this drug. At least three different efflux routes have been described, which can be expressed to variable degrees in different cell lines [38-41]. One of the efflux routes can be the reduced folate/MTX influx carrier system [38,42] as determined from its sensitivity to an N-hydroxysuccinimide ester

of MTX, which is a specific and irreversible inhibitor of this influx system [43]. This efflux route is predominantly active in ATP-depleted cells [38,41,42,44]. Under physiological conditions, with normal levels of ATP, two other routes play a role in mediating efflux of MTX. These efflux systems, which are both energy (ATP/glucose) dependent, have been identified by their sensitivities to inhibition by the organic anions bromosulfophtalein (BSP) and probenecid [38,42,44,45]. The BSP-sensitive efflux route for MTX can also be inhibited by metabolic inhibitors and compounds like prostaglandin A_1, vincristine and verapamil [40,41]. Although this latter compound is a well known potent inhibitor of ATP-dependent P-glycoprotein-mediated drug efflux in multidrug resistant cells [46], there is no direct evidence that these systems are related. Rather, Henderson and Tsjui [40] observed some common features of the BSP-sensitive efflux route and the efflux route for cyclic nucleotides like 3',5'-cyclic AMP.

Thus far, the characterisation of efflux routes has not obtained as much attention as influx routes, but further studies are warranted since the activity of efflux routes can be directly related to tumour cell sensitivity to MTX.

INTRACELLULAR METABOLISM

Inhibition of DHFR

The most important intracellular target of MTX is dihydrofolate reductase (DHFR) (see Fig. 2). MTX is, with respect to dihydrofolate (FH_2), a competitive inhibitor of DHFR with an extremely tight binding to the enzyme [47,48].

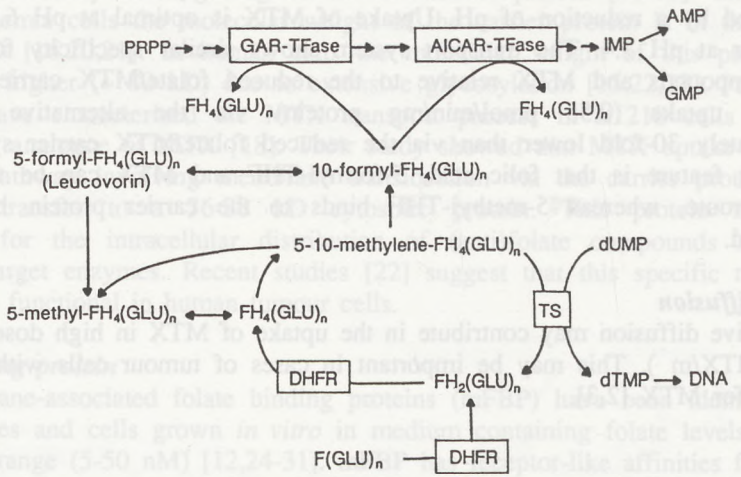

Fig. 2. Folate metabolic pathways and major target enzymes for folate antagonists. Target enzymes are: DHFR: dihydrofolate reductase, TS: thymidylate synthase, GAR-TFase: glycinamide ribonucleotide transformylase, AICAR-TFase: aminoimidazole carboxamide ribonucleotide transformylase. FH_2: dihydrofolate, FH_4: tetrahydrofolate, $(GLU)_n$: glutamate residues, PRPP: phosphoribosylpyrophosphate, IMP: inosine monophosphate, AMP: adenosine monophosphate, GMP: guanine monophosphate, dUMP: deoxyuridine monophosphate, dTMP: deoxythimine monophosphate

The binding affinity of DHFR for MTX is higher than for its natural substrate dihydrofolate (FH_2) [49]. At pH 6.0, the binding of MTX to DHFR is stoichiometric [50,51], which means that each molecule of drug binds to one molecule of enzyme. Only small amounts of DHFR activity are required to maintain the tetrahydrofolate (FH_4) pools. Therefore, at least 95% of DHFR needs to be inhibited before perturbations in cellular growth rate can be observed [52].

Polyglutamylation

Once inside the cell, the naturally occurring folates and folate analogues like MTX are extensively metabolized to polyglutamate dervatives [53-61]. Folylpolyglutamate synthase (FPGS) catalyzes the reaction by which multiple glutamate moieties are sequentially added to the γ-carboxyl group of the glutamate side chain of the (anti)folate molecule (Fig. 3). Reduced folate compounds are good substrates for this enzyme, the substrate affinity for MTX is slightly lower (K_m: 100-140 μM) [62-67]. Polyglutamylation is a time-, dose- and cell type-dependent process [68,69], and the activity of FPGS is enhanced by low intracellular folate pools [62-64,70] and high rates of cell growth [63,70,71]. Polyglutamate derivatives of

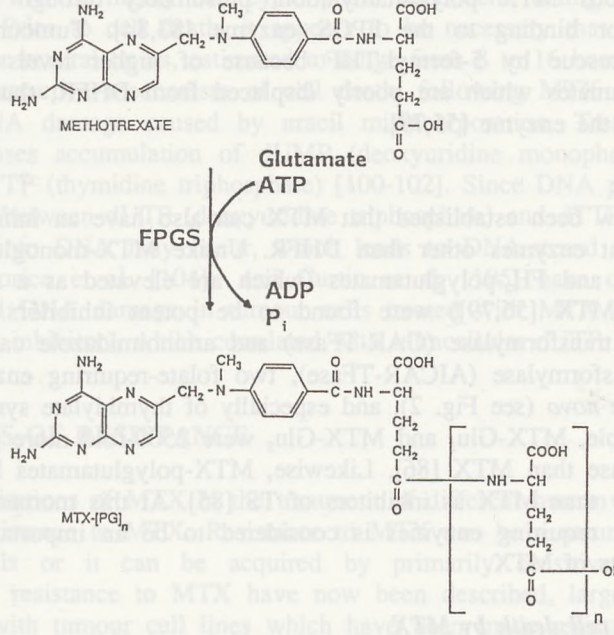

Fig. 3. Reaction scheme for the polyglutamamylation of MTX via folylpolyglutamate synthase. FPGS: folylpolyglutamate synthase, P_i: inorganic phosphate, MTX-$[PG]_n$: polyglutamate derivative of MTX, n: represents the total glutamate residues in γ-linkage.

naturally occurring folates form an intracellular depot. Their retention is significantly increased by the elongation of the glutamate side chain, with additional negatively charged glutamate residues, which prevents a rapid efflux from the cell. MTX-polyglutamates, in particular the tetra- and penta-glutamate forms, are also selectively retained by the cell [53,72-74]. MTX-polyglutamates are at least as potent as MTX-monoglutamates in inhibiting DHFR. Furthermore, MTX-polyglutamates dissociate from DHFR with a much slower rate than monoglutamates [55,68,75,76]. These effects may prolong the cytotoxic action of MTX. The importance of MTX-polyglutamylation is further illustrated by the observation of a significantly lower cytotoxic effect (in short term exposure) of fluoro-MTX, a MTX derivate without any FPGS substrate activity [77].

There is increasing evidence that the selectivity of antitumour action by MTX is related to differences in polyglutamylation between normal (e.g. intestinal mucosa or marrow progenitor cells) and neoplastic cells [56,72,74,78-80]. In concert with these data is the observation of Koizumi et al. [54] that a 2-fold higher intracellular MTX polyglutamate level resulted in a 30-fold increased cytotoxic effect of MTX in HL-60 leukaemia cells compared to marrow progenitor cells. Differences in polyglutamylation may also form the basis for the selectivity of leucovorin (5-formyl-THF) rescue of normal cells [56,79-82]. In clinical practice 5-Formyl-THF is used to rescue normal cells from MTX cytotoxicity [2,3]. 5-Formyl-THF inhibits MTX polyglutamylation, presumably through competition for transport and/or binding to the FPGS enzyme [83,84]. Tumour cells are less sensitive for rescue by 5-formyl-THF because of higher levels of intracellular MTX-polyglutamates which are poorly displaced from DHFR, thus sustaining the inactivation of the enzyme [55,79].

Indirect effects

It has now been established that MTX can also have an inhibitory effect on folate-dependent enzymes other than DHFR. Unlike MTX-monoglutamates, MTX-polyglutamates and FH_2-polyglutamates (which are elevated as a result of DHFR inhibition by MTX [56,79]) were found to be potent inhibitors of glycinamide ribonucleotide transformylase (GAR-TFase) and aminoimidazole carboxamide ribonucleotide transformylase (AICAR-TFase), two folate-requiring enzymes in purine biosynthesis *de novo* (see Fig. 2), and especially of thymidylate synthase (TS) [85-87]. For example, MTX-Glu_3 and MTX-Glu_5 were 2500-fold more potent inhibitors of AICAR-TFase than MTX [86]. Likewise, MTX-polyglutamates had 75-300 fold greater potency than MTX as inhibitors of TS [85]. At this moment, the inhibition of these folate requiring enzymes is considered to be an important factor in the cytotoxic effects of MTX.

Mechanism of cell death by MTX

It has been traditionally assumed that DHFR inhibition by MTX initiates a series of intracellular events that culminate in impaired *de novo* synthesis of thymidylate and purines as a result of a depletion of FH_4 pools. Although a reduction and changes in the distribution of folate-pools has been observed after tumour

cell exposure to antifolates [56,88-91], it is believed that these changes are not sufficient to explain the cytotoxic effects of MTX. In addition, experimental studies and network thermodynamic computer modeling by Seither et al. [92,93] and Trent et al. [94] suggest that TS inhibition by MTX- and FH_2-polyglutamates only slows down but does not abolish the rate of interconversion of FH_4 cofactors. Hence, inhibition of folate-requiring enzymes per se cannot explain the cytotoxic effects of MTX. These authors raise the hypothesis that only a fraction of cellular tetrahydrofolates are available for *de novo* purine and thymidylate synthesis, and that a (large) fraction is in a biochemical form, in a physical compartment (e.g. mitochondria), or bound to cellular constituents which make them unavailable for oxidation to dihydrofolate. In conclusion, the metabolic inhibition by MTX is likely to be a multifactorial event that includes folate substrate depletion and direct inhibition of several critical folate-dependent enzymes by MTX- and FH_2-polyglutamates.

In mammalian cells antifolate-induced cytotoxicity is associated with unbalanced growth [95,96]. Unbalanced growth is a state in which synthesis of RNA and proteins can continue, but DNA synthesis, and consequently cell division, are blocked. This suggests that the effect on the nucleotide synthesis following inhibition of TS is a more important factor in MTX cytotoxicity than inhibition of purine and pyrimidine synthesis. Since TS is an S-phase specific enzyme, MTX will be highly cell cycle phase dependent, acting primarily in S-phase [97,98]. Prior to cell death, it appears to be necessary that the unbalanced state persists for several hours, estimated to range from 8 to 16 hours [97,99].

A more attractive mechanism of cell death, following MTX exposure, is an irreversible DNA damage caused by uracil misincorporation. Treatment of cells with MTX causes accumulation of dUMP (deoxyuridine monophosphate), and a depletion of dTTP (thymidine triphosphate) [100-102]. Since DNA polymerases can not distinguish between dUTP (deoxyuridine triphosphate) and dTTP, misincorporation of uracil into DNA may occur, which leads to DNA-strand brakes and cell death [103]. Lorico et al. [104] and Curtin et al. [105] have observed single/double stranded DNA damage in tumour cells treated with MTX and CB3717 (a folate-based TS inhibitor), which correlated with intracellular dUTP levels.

MECHANISMS OF RESISTANCE

One of the limitations of MTX in the treatment of different human malignancies is tumour cell resistance to MTX. Resistance to MTX can be a natural characteristic of tumour cells or it can be acquired by primarily sensitive cells. Several mechanisms of resistance to MTX have now been described, largely on basis of investigations with tumour cell lines which have been made resistant to MTX by continuous exposure to progressively increasing concentrations of MTX. The most important mechanisms of tumour cell resistance against MTX are described below. MTX-resistance may develop as a consequence of either one or a combination of these mechanisms [106-108].

DHFR gene amplification

One of the most common mechanisms of acquired MTX resistance is an increased activity of DHFR resulting from DHFR gene amplification [109,110]. The step-wise increase in drug concentration in the cell culture medium may lead to as many as 100-1000 copies of the DHFR gene, and to high levels of drug resistance. It has been demonstrated that gene amplification may be a determinant in clinical drug resistance as well [111,112]. Human tumour cells can also promptly increase DHFR levels when exposed to MTX, even without evidence for gene amplification [113]. The rapid elevation of the enzyme appears to be mediated at the level of protein transcription or translation.

altered DHFR

Point mutations may result in the synthesis of variant forms of DHFR characterized by a (1) decreased affinity for MTX or (2) by altered kinetic properties relative to dihydrofolate.

ad 1): Altered forms of DHFR have been found to retain adequate function for reduction of their normal substrate, dihydrofolate, but have a greatly decreased affinity for MTX [114-116]. Haber has found that, in mouse 3T6 fibroblasts, amplification is associated with an altered DHFR that has a decreased affinity (by 270-fold) and V_{max} (by 20%) for MTX [117].

ad 2): Studies with a MTX-resistant human promyelocytic leukaemia cell line HL-60 suggest that the mechanism of resistance was due to an altered DHFR exhibiting a 20-fold increase in catalytic activity as compared to the enzyme from wild-type cells [118,119]. The altered DHFR was further characterized as having a higher affinity constant (K_m) for dihydrofolate and a higher V_{max} when compared to the DHFR from the parent cell line. Interestingly, the inhibition constant (IC_{50}) for inhibition of the altered enzyme by MTX was identical to that for the wild-type DHFR [119].

Impaired MTX transport

Impaired transport manifested as quantitative (reduction of influx V_{max}) and/or qualitative (increased influx K_m) alterations of the reduced folate/MTX carrier system has been associated with resistance to MTX *in vitro* and *in vivo* [11,24, 120-126]. A decreased V_{max} for influx may be related to a functional defect in the translocation of the reduced folate/MTX carrier [127,128]. Transport-related resistance to MTX can also be due to the expression of folate uptake systems, other than the reduced folate/MTX carrier, which have a low affinity for MTX relative to naturally occurring folates [24,27,33].

Decreased MTX polyglutamylation

A defect in the ability to polyglutamylate MTX has first been described by Cowan and Jolivet [129] in an MTX-resistant human breast cancer cell line. Although this cell line had an additional decrease in MTX uptake, the 1000-fold resistance could not be explained by this mechanism alone since the cells remained resistant in the presence of intracellular drug levels above those found in sensitive

cells. Decreased polyglutamylation was also found in a transport-deficient human squamous carcinoma cell line of the head and neck in culture, which had normal DHFR activity [130].

Pizzorno et al. were able to select a cell line which displays acquired resistance to MTX which was solely due to defective MTX polyglutamate synthesis [131]. Two MTX resistant sublines were isolated from human CCRF-CEM leukaemia cells after short-term, high-dose treatment with this drug. Although the resistant sublines were able to form substantial amounts of folate polyglutamates when measured with [^{3}H]-folic acid, the level of polyglutamates formed was decreased to about 50% of that formed by the parent cell line.

Inherent resistance to MTX was observed in two out of three human squamous cell carcinoma cell lines following short term exposure to MTX [132]. The basis for the natural resistance to MTX correlated with the different amounts of MTX-polyglutamates synthesized by these cell lines.

The intracellular levels of MTX-polyglutamates is dependent upon the relative activity of FPGS and FPGH (folylpolyglutamate hydrolase), the enzyme that is responsible for the breakdown of polyglutamates. Samuels et al. [133] observed that levels of FPGH activity were considerably greater (10-20 fold) in normal tissues than in tumour cells. Whitehead et al. [134] reported higher activities of FPGH in myeloblasts from patients with acute non-lymphoblastic leukaemia compared to lymphoblasts from patients with acute lymphoblastic leukaemia (ALL). The difference in FPGH activity correlated with higher levels of long chain MTX-polyglutamates in ALL cells.

Decreased TS activity

Low TS activity may play a role in MTX resistance due to a decreased rate of thymidylate synthesis. The reduced thymidylate synthesis will slow down the depletion of the reduced folate pools and, as a consequence, the inhibition of DHFR by MTX can be enhanced without any damage to the cell [135,136]. From studies in eight patient-derived small cell lung carcinoma cell lines, Curt et al. [137] concluded that the rate of polyglutamylation was the most critical determinant in MTX sensitivity. Their results also suggested that low TS activity contributed to MTX resistance in two of these cell lines. In an other study, Rodenhuis et al. [106] showed that MTX resistance in fresh human leukemic cells was based upon both a transport defect and low TS activity. The role of TS activity in the resistance to MTX, however, is questionable, since levels of TS may be 4-5 fold higher in cells with a logarithmic phase of growth as compared to cells in a plateau phase, low levels of TS may reflect low cell growth activity [138].

OTHER ANTIFOLATES

Several novel antifolates have got an increased cytotoxicity to tumour cells. Novel antifolates used in this study are discussed below. The chemical structures of these compounds are shown in Fig. 1.

DHFR inhibitors

10-Ethyl-10-deaza-aminopterin (10-EdAM) is a DHFR inhibitor with increased cytotoxic effects, as compared to MTX [139,140]. 10-EdAM exhibits marked potential for improved antitumour selectivity at the level of two biochemical properties which are believed to be crucial to the cytotoxic action of antifolate compounds. This drug is transported and polyglutamylated with greater efficiency than MTX (Table 2). It was found to have an enhanced activity against a variety of solid tumours [141]. At this moment 10-EdAM is tested in a clinical phase III trial for advanced squamous cell carcinomas of the head and neck [142].

In the search to develop new antifolates to circumvent resistance due to decreased drug transport, the lipophilic antifolates have shown promising features [146]. At this moment trimetrexate (TMQ), a 2,4-diaminoquinazoline derivative, is one of the best studied non-classical antifolate compounds. TMQ is an inhibitor of DHFR like MTX, but cannot be polyglutamylated because of the lack of a glutamate residue (Fig. 1) [147-149]. Membrane transport of TMQ is not mediated by a specific membrane protein such as for MTX, but the compound may enter the cell by passive/facilitated diffusion [147]. Transport kinetic studies showed uptake of TMQ to be proportional to extracellular drug concentrations between 10^{-9} and 10^{-4} M [148]. Kamen et al. demonstrated that the uptake of TMQ is as rapid by MTX-sensitive and MTX-resistant cells. TMQ may therefore be active against tumours which are resistant to MTX on basis of altered transport. Preclinical studies were promosing, but in clinical trials, however, TMQ was only modestly active as an anticancer agent.

Table 2. Affinity of the reduced folate carrier and FPGS for MTX and novel antifolates.

Folate antagonist	Target enzyme (K_m) (μM)	Affinity RF-carrier (K_m) (μM)	Affinity FPGS
MTX	DHFR	5-10	100-140
10-EdAM	DHFR	2-3	30
TMQ	DHFR	none	none
ICI-198,583	TS	2-3	40
DDATHF	GAR-TFase	2-3	8

Data obtained from references [34,60,67,143-145].

TS inhibitors

In an effort to circumvent MTX resistance due to altered or amplified DHFR, new antifolate compounds, N^{10}-propargyl-5,8-dideazafolic acid (CB3717) and 2-desamino-2-methyl-N^{10}-propargyl-5,8,-dideazafolic acid (ICI-198,583), have been synthesized. These compounds act through potent inhibition of TS, and CB3717 was found to have antitumour activity in both preclinical and clinical studies [150-153]. Transport of these antifolates can proceed via the reduced folate/MTX carrier, but is also mediated by mFBP which has a high affinity for these compounds [34]. Once inside the cell the folate based TS inhibitors are rapidly converted to polyglutamate metabolites [154]. The polyglutamate forms of CB3717 were significantly more potent competitive inhibitors of TS as the length of the polyglutamate chain increased [154]. Unfortunately, in large phase I studies CB3717 appeared to be too toxic for further clinical evaluation [155].

GAR-TFase inhibitors

5,10-Dideazatetrahydrofolate (DDATHF) is another antimetabolite which inhibits folate metabolism at sites other than DHFR. DDATHF is a potent folate analogue which suppresses purine synthesis through direct or indirect inhibition of glycinamide ribonucleotide transformylase (GAR-TFase) [156]. In preclinical studies DDATHF was, as a single agent, not as potent as MTX, but low doses of DHFR-inhibitors and DDATHF together may have synergistic effects [157,158]. Multiple membrane-transport routes may be involved in the cellular uptake of DDATHF. These routes include the classic reduced folate carrier, and the membrane associated mFBP [35]. FPGS has the highest substrate affinity for DDATHF (Table 2), as compared to other antifolates [156].

REFERENCES

1. Jukes ThJ. Searching for magic bullets: early approaches to chemotherapy - antifolates, methotrexate - the Bruce F. Cain memorial award lecture. *Cancer Res* 1987, **47**, 5528-5536.

2. Schornagel JH, McVie JG. The clinical pharmacology of methotrexate, a review. *Cancer Treat Rev* 1983, **10**, 53-75.

3. Ackland SP, Schilsky RL. High-dose methotrexate: a critical reappraisal. *J Clin Oncol* 1987, **5**, 2017-2031.

4. Henderson GB, Tsuji JM, Kumar HP. Transport of folate compounds by leukemic cells. Evidence for a single influx carrier for methotrexate, 5-methyltetrahydrofolate, and folate in CCRF-CEM human lymfoblasts. *Biochem Pharmacol* 1987, **36**, 3007-3014.

5. Moccio DM, Sirotnak FM, Samuels LL, Ahmed T, Yagoda A, DeGraw JI, Piper JR. Similar specificity of membrane transport for folate analogues and their metabolites by murine and human tumor cells: a clinically directed laboratory study. *Cancer Res* 1984, **44**, 352-357.

6. Goldman ID. The characteristics of membrane transport of amethopterin and naturally occuring folates. *Ann N Y Acad Sci* 1971, **186**, 400-422.

7. Henderson GB. Transport of folate compounds into cells. In: Blakley RL, Whitehead VM, eds. *Folates and Pterins*. New York, John Whiley & Sons, 1986, 207-250.

8. Henderson GB, Zevely EM. Functional correlations between the methotrexate and general anion transport systems of L1210 cells. *Biochem Int* 1982, **4**, 493-502.

9. Henderson GB, Zevely EM. Intracellular phosphate and its possible role as an exchange anion for active transport of methotrexate in L1210 cells. *Biochem Biophys Res Commun* 1982, **104**, 474-478.

10. Sirotnak FM. Correlates of folate analog transport pharmacokinetics and selective antitumor action. *Pharmac Ther* 1980, **8**, 71-103.

11. Sirotnak FM. Obligate genetic expression in tumor cells of a fetal membrane property mediating "folate" transport: Biological significance and implications for improved therapy of human cancer. *Cancer Res* 1985, **45**, 3992-4000.

12. Ratnam M, Freisheim JH. Proteins involved in the transport of folates and antifolates by normal and neoplastic cells. In: *Folic acid metabolism in health and disease*. Wiley-liss, Inc., 1990, 91-120.

13. Sirotnak FM, Poser RE, Barrueco JR. Enhancement of folate analogue transport inward in L1210 cells during methotrexate therapy of leukemic mice: Evidence of the nature of the effect, possible host mediation, and pharmacokinetic significance. *Cancer Res* 1987, **47**, 5334-5339.

14. 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.

15. Matherly LH, Czajkowski CA, Angeles SM. Identification of a highly glycosylated methotrexate membrane carrier in K562 human erythroleukemia cells up-regulated for tetrahydrofolate cofactor and methotrexate transport. *Cancer Res* 1991, **51**, 3420-3426.

16. Henderson GB, Zevely EM. Affinity labeling of the 5-methyltetrahydrofolate/methotrexate transport protein of L1210 cells by treatment with an N-hydroxysuccimide ester of [^{3}H]methotrexate. *J Biol Chem* 1984, **259**, 4558-4562.

17. Price EM, Sams L, Harprring KM, Kempton RJ, Freisheim JH. Photoaffinity analogues of methotrexate as probes for dihydrofolate reductase structure and function. *Biochem Pharmacol* 1986, **35**, 4341-4343.

18. Price EM, Ratnam M, Rodeman KM, Freisheim JH. Characterization of the methotrexate transport pathway in murine L1210 leukemia cells: Involvement of a membrane receptor and a cytosolic protein. *Biochemistry* 1988, **27**, 7853-7858.

19. Fan J, Pope LE, Vitols KS, Huennekens FM. Affinity labeling of folate transport proteins with the N-hydroxysuccimide ester of the γ-isomer of fluorescein-methotrexate. *Biochemistry* 1991, **30**, 4573-4580.

20. Yang CH, Sirotnak FM, Mines LS. Further studies on a novel class of genetic variants of the L1210 cell with increased folate analogue transport inward. *J Biol Chem* 1988, **263**, 9703-9709.

21. Price EM, Freisheim JH. Photoaffinity analogues of methotrexate as folate antagonist binding probes. 2. Transport studies, photoaffinity labeling, and identification of the membrane carrier protein for methotrexate from murine L1210 cells. *Biochemistry* 1987, **26**, 4757-4763.

22. Jansen G, Westerhof GR, McAlinden TP, Schornagel JH, Freisheim JH. Molecular events in membrane transport of methotrexate in human CCRF-CEM leukemia cell lines. *Proc Am Assoc Cancer Res* 1991, **32**, 323.

23. Yang CH, Pain J, Sirotnak FM. Molecular properties of the one-carbon, reduced folate transporter in HL-60 cells and evidence for down-regulation of this synthesis during differentiation. *Proc Am Assoc Cancer Res* 1991, **32**, 325.

24. 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.

25. Jansen G, Kathmann I, Rademaker BC, Braakhuis BJM, Westerhof GR, Rijksen G, Schornagel JH. Expression of a folate binding protein in L1210 cells grown in low folate medium. *Cancer Res* 1989, **49**, 1959-1963.

26. Kamen BA, Capdevila A. Receptor-mediated folate accumulation is regulated by the cellular folate content. *Proc Natl Acad Sci USA* 1986, **83**, 5983-5987.

27. 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.

28. Ratnam M, Marquardt H, Duhring JL, Freisheim JH. Homologous membrane folate binding proteins in human placenta: Cloning and sequence of a cDNA. *Biochemistry* 1989, **28**, 8249-8254.

29. Kane MA, Waxman S. Role of Folate Binding Proteins in folate metabolism. *Lab Invest* 1989, **60**, 737-746.

30. Elwood PC, Kane MA, Portillo RM, Kolhouse JF. The isolation, characterisation, and comparison of the membrane-associated and soluble folate-binding proteins from human KB Cells. *J Biol Chem* 1986, **261**, 15416-15423.

31. Henderson GB. Folate-binding proteins. *Ann Rev Nutr* 1990, **10**, 319-335.

32. Kane MA, Portillo RM, Elwood PC, Antony AC, Kalhouse JF. The influence of extracellular folate concentration on methotrexate uptake by human KB cells. *J Biol Chem* 1986, **261**, 44-49.

33. Deutsch JC, Elwood PC, Portillo RM, Macey MG, Kolhouse JF. Role of the membrane-associated folate binding protein (folate receptor) in methotrexate transport by human KB cells. *Arch Biochem Biophys* 1989, **274**, 327-337.

34. 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.

35. 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.

36. Sirotnak FM, Goutas LJ, Jacobsen DM, Mines LS, Barrueco JR, Gaumont Y, Kisliuk RL. Carrier-mediated transport of folate compounds in L1210 cells. *Biochem Pharmacol* 1987, **36**, 1659-1667.

37. Henderson GB, Strauss BP. Characteristics of a novel transport system for folate compounds in wild-type and methotrexate-resistant L1210 cells. *Cancer Res* 1990, **50**, 1709-1714.

38. Henderson GB, Zevely EM. Transport routes utilized by L1210 cells for the influx and efflux of methotrexate. *J Biol Chem* 1984, **259**, 1526-1531.

39. Henderson GB, Tsuji JM, Kumar HP. Characterisation of the individual transport routes that mediate the influx and efflux of methotrexate in CCRF-CEM human lymfoblastic cells. *Cancer Res* 1986, **46**, 1633-1638.

40. Henderson GB, Tsuji JM. Identification of the bromosulfophthaleinsensitive efflux route for methotrexate as the site of action of vincristine-dependent enhancement of methotrexate uptake in L1210 cells. *Cancer Res* 1988, **48**, 5995-6001.

41. Sirotnak FM, O'Leary DF. The issues of transport multiplicity and energetics pertaining to methotrexate efflux in L1210 cells addressed by an analysis of cis and trans effects of inhibitors. *Cancer Res* 1991, **51**, 1412-1417.

42. Henderson GB, Tsuji JM. Methotrexate efflux in L1210 cells. Kinetic and specificity properties of the efflux system sensitive to bromosulfophtalein and its possible identity with a system which mediates the efflux of 3',5'-cyclic-AMP. *J Biol Chem* 1987, **262**, 13571-13578.

43. Henderson GB, Montague-Wilkie B. Irreversible inhibitors of methotrexate transport in L1210 Cells. Characteristics of inhibition by an N-hydroxysuccimide ester of methotrexate. *Biochim Biophys Acta* 1983, **735**, 123-130.

44. Dembo M, Sirotnak FM, Moccio DM. Effects of metabolic deprivation on methotrexate transport in L1210 leukemia cells: further evidence for separate influx and efflux systems with different energetic requirements. *J Membrane Biol* 1984, **78**, 9-17.

45. Sirotnak FM, Moccio DM, Young CW. Increased accumulation of methotrexate by murine tumour cells *in vitro* in the presence of probenecid is mediated by a preferential inhibition of efflux. *Cancer Res* 1981, **41**, 966-670.

46. Endicott JA, Ling V. The biochemistry of P-glycoprotein-mediated multidrug resistance. *Ann Rev Biochem* 1989, **58**, 137-171.

47. Jackson RC. Biological effects of folic acid antagonists with antineoplastic activity. *Pharmac Ther* 1984, **25**, 61-82.

48. Waltham MC, Holland JW, Robinson SC, Winzor DJ, Nixon PF. Direct experimental evidence for competitive inhibition of dihydrofolate reductase by methotrexate. *Biochem Pharmacol* 1988, **37**, 535-539.

49. Ozaki Y, King RW, Carey PR. Methotrexate and folate binding to dihydrofolate reductase: separate characterization of the pteridine and p-amino-benzoyl binding sites by resonance Raman spectroscopy. *Biochemistry* 1981, **20**, 3219-3225.

50. Bertino JR. Folate antagonists. In: Sartorelli AC, Johns DG, eds. *Antineoplastic and immunosuppressive agents II*. New York, Springer Verlag, 1975, 468.

51. Werkheiser WC. Specific binding of 4-amino folic acid analogues by folic acid reductase. *J Biol Chem* 1961, **237**, 888-893.

52. Jackson RC, Harrap KR. Studies with a mathematical model of folate metabolism. *Arch Biomed Biophys* 1973, **158**, 827-841.

53. Chabner BA, Allegra CJ, Curt GA, Clendinn NJ, Baram J, Koizumi S, Drake JC, Jolivet J. Polyglutamation of methotrexate. Is methotrexate a prodrug? *J Clin Invest* 1985, **76**, 907-912.

54. Koizumi S, Curt GA, Fine RL, Griffin JD, Chabner BA. Formation of methotrexate polyglutamates in purified myeloid precursor cells from normal human bone marrow. *J Clin Invest* 1985, **75**, 1008-1014.

55. Matherly LH, Fry DW, Goldman ID. Role of methotrexate polyglutamation and cellular energy metabolism in inhibition of methotrexate binding to dihydrofolate reductase by 5-formyltetrahydrofolate in ehrlich ascites tumor cells *in vitro*. *Cancer Res* 1983, **43**, 2694-2699.

56. Matherly LH, Barlowe CK, Goldman ID. Antifolate polyglutamylation and competative drug displacement at dihydrofolate reductase as important elements in leucovorin rescue in L1210 cells. *Cancer Res* 1986, **46**, 588-593.

57. Johnson TB, Nair MG, Galivan J. Role of folylpolyglutamate synthetase in the regulation of methotrexate polyglutamate formation in H35 hepatoma cells. *Cancer Res* 1989, **48**, 2426-2431.

58. Schoo MM, Pristupa ZB, Vickers PJ, Schrimgeour KG. Folate analogues as substrates of mammalion folylpolyglutamate synthetase. *Cancer Res* 1985, **45**, 3034-3041.

59. Schirch V, Strong W. Interaction of folylpolyglutamates with enzymes in one-carbon metabolism. *Arch Biochem Biophys* 1989, **269**, 371-380.

60. 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.

61. Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA. The pharmacology and clinical use of methotrexate. *N Engl J Med* 1983, **309**, 1094-1104.

62. Galivan J, Nimec Z. Effects of folinic acid on hepatoma cells containing methotrexate polyglutamates. *Cancer Res* 1983, **43**, 551-555.

63. Nimec Z, Galivan J. Regulatory aspects of the glutamylation of methotrexate in cultured hepatoma cells. *Arch Biomed Biophys* 1983, **226**, 671-680.

64. Galivan J, Nimec Z, Baliska M. Regulation of methotrexate polyglutamate accumulation *in vitro*: effects of cellular folate contents. *Biochem Pharmacol* 1983, **32**, 3244-3247.

65. McGuire JJ, Hsieh P, Coward JK, Bertino JR. Enzymatic synthesis of folylpolyglutomates. *J Biol Chem* 1980, **225**, 5776-5788.

66. 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.

67. Moran RG, Colman PD, Rosowsky A. Structural requirements for the activity of antifolates as substrates for mammalian folylpolyglutamate synthetase. *NCI Monogr* 1987, **5**, 133-138.

68. Fry DW, Yalowich JC, Goldman ID. Rapid formation of poly-γ-glutamyl derivatives of methotrexate and their association with dihydrofolate reductase as assessed by high-pressure liquid chomatograpy in Ehrlich ascitis tumor cell *in vitro*. *J Biol Chem* 1982, **257**, 1890-1896.

69. Fry DW, Yalowich JC, Goldman ID. Augmentation of the intracellular levels of polyglutamyl derivatives of methotrexate by vincristine and probenecid in Ehrlich ascites tumor cells. *Cancer Res* 1982, **42**, 2532-2536.

70. Sirotnak FM, Johnson TB, Samuels LL, Galivan J. Proliveration-dependency of folylpolyglutamyl synthetase activity in maturating luminal epithelial cells of mouse small intestine. *Biochem Pharmacol* 1988, **37**, 4239-4241.

71. Johnson TB, Nair MG, Galivan J. Role of folylpolyglutamate synthetase in the regulation of methotrexate polyglutamate formation in H35 hepatoma cells. *Cancer Res* 1988, **48**, 2426-2431.

72. Poser RG, Sirotnak FM, Chello PL. Differential synthesis of methotrexate polyglutamates in normal proliferative and neoplastic mouse tissues *in vivo*. *Cancer Res* 1981, **41**, 4441-4446.

73. Jolivet J, Schilsky RL, Bailey BD, Drake JC, Chabner BA. Synthesis, retension, and biological activity of methotrexate polyglutamates in cultured human breast cancer cells. *J Clin Invest* 1982, **70**, 351-360.

74. Fry DW, Anderson LA, Borst M, Goldman ID. Analisis of the role of membrane transport and polyglutamation of methotrexate in gut and the Ehrlich tumor *in vivo* as factors in drug sensitivity and selectivity. *Cancer Res* 1983, **43**, 1087-1092.

75. Jolivet J, Chabner BA. Intracellular pharmacokinetics of methotrexate polyglutamates in human breast cancer cells: selective retension and dissociable binding of 4-NH2-10-CH3-pteroylglutamate4 and 4-NH2-10-CH3-pteroylglutamate5 to dihydrofolate reductase. *J Clin Invest* 1983, **72**, 773-778.

76. Drake JC, Allegra CJ, Baram J, Kaufman BT, Chabner BA. Effects on dihydrofolate reductase of methotrexate metabolites and intracellular folates formed following methotrexate exposure of human breast cancer cells. *Biochem Pharmacol* 1987, **36**, 2416-2418.

77. McGuire JJ, Piper JR, Coward JK, Galivan J. Folate analog nonsubstrates and inhibitors of folylpolyglutamate synthetase as potential cancer chemotherapy drugs. *NCI Monogr* 1987, **5**, 139-144.

78. Fabre I, Fabre G, Goldman ID. Polyglutamylation, an important element in methotrexate cytotoxicity and selectivity in tumor versus murine granulocytic progenitor cells *in vitro*. *Cancer Res* 1984, **44**, 3190-3195.

79. Matherly LH, Barlowe CK, Phillips VM, Goldman ID. The effects on 4-aminoantifolates on 5-formyltetrahydrofolate metabolism in L1210 cells (a biochemical basis of the selectivity of leucovorin rescue). *J Biol Chem* 1987, **262**, 710-717.

80. Goldman ID, Matherly LH. Biochemical factors in the selectivity of leucovorin rescue: Selective inhibition of leucovorin reactivation of dihydrofolate reductase and leucovorin utilization in purine and pyrimidine biosynthesis by methotrexate and dihydrofolate polyglutamates. *NCI Monogr* 1987, **5**, 17-26.

81. Allegra CJ, Boarman D. Interaction of methotrexate polyglutamates and dihydrofolate during leucovorin rescue in a human breast cancer cell line (MCF-7). *Cancer Res* 1990, **50**, 3574-3578.

82. Boarman DM, Baram J, Allegra CJ. Mechanism of leucovorin reversal of methotrexate cytotoxicity in human MCF-7 breast cancer cells. *Biochem Pharmacol* 1990, **40**, 2651-2660.

83. Balinska M, Nimec Z, Galivan J. Characteristics of methotrexate polyglutamation formation in cultured hepatic cells. *Arch Biomed Biophys* 1982, **216**, 466-476.

84. Rosenblatt DS, Whitehead VM, Matiaszuk NV, Pottier A, Vuchich M-J, Beaulieu O. Differential effects of folinic acid and glycine, adenosine, and thymidine as rescue agents in methotrexate-treated human cells in relation to the accumulation of methotrexate polyglutamates. *Mol Pharmacol* 1982, **21**, 718-722.

85. Allegra CJ, Chabner BA, Drake JC, Lutz R, Rodbard D, Jolivet J. Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates. *J Biol Chem* 1985, **260**, 9720-9726.

86. Allegra CJ, Drake JC, Jolivet J, Chabner BA. Inhibition of phosphoribosyaminoimidazole-carboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. *Proc Natl Acad Sci USA* 1985, **82**, 4881-4885.

87. Allegra CJ, Fine RL, Drake JC, Chabner BA. The effect of methotrexate on intracellular folate pools in human MCF-7 breast cancer cells: evidence for direct inhibition of purine synthesis. *J Biol Chem* 1986, **261**, 6478-6485.

88. Bunni M, Doig MT, Donato H, Kesavan V, Priest DG. Role of methylenetetrahydrofolate depletion in methotrexate-mediated intracellular thymidylate synthesis inhibition in cultured L1210 cells. *Cancer Res* 1988, **48**, 3398-3404.

89. Priest DG, Bunni M, Sirotnak FM. Relationship of reduced folate changes to inhibition of DNA synthesis induced by methotrexate in L1210 cells *in vivo*. *Cancer Res* 1989, **49**, 4204-4209.

90. Galivan J, Nimec Z, Rotundo R. The characteristics and consequences of folate depletion in hepatoma cells *in vitro* by inhibition of dihydrofolate reductase. *Adv Enzyme Regul* 1988, **27**, 209-217.

91. Galivan J, Rhee MS, Johnson TB, Dilwith R, Nair MG, Bunni M, Priest DG. The role of cellular folates in the enhancement of activity of the thymidylate synthase inhibitor 10-propargyl-5,8-dideazafolate against hepatoma cells *in vitro* by inhibitors of dihydrofolate reductase. *J Biol Chem* 1989, **264**, 10685-10692.

92. Seither RL, Trent DF, Mikulecky DC, Rape TJ, Goldman ID. Folate-pool interconversions and inhibition of biosynthetic processes after exposure of L1210 leukemia cells to antifolates. Experimental and network thermodynamic analyses of the role of dihydrofolate polyglutamylates in antifolate action in cells. *J Biol Chem* 1989, **264**, 17016-17023.

93. Seither RL, Trent DF, Mikulecky DC, Rape TJ, Goldman ID. Effect of direct suppression of thymidylate synthase at the 5,10-methylenetetrahydrofolate binding site on the interconversion of tetrahydrofolate cofactors to dihydrofolate by antifolates. *J Biol Chem* 1991, **266**, 4112-4118.

94. Trent DF, Seither RL, Goldman ID. Rate and extent of interconversion of tetrahydrofolate cofactors to dihydrofolate after cessation of dihydrofolate reductase activity in stationary versus log phase L1210 leukemia cells. *J Biol Chem* 1991, **266**, 5445-5449.

95. Taylor IW, Slowiaczek P, Francis PR, Tattersall MHN. Purine modulation of methotrexate cytotoxicity in mammalian cell lines. *Cancer Res* 1982, **42**, 5159-5164.

96. Hoffbrand AV, Tripp E. Unbalanced deoxyribonucleotide synthesis caused by methotrexate. *Br Med J* 1972, **2**, 140-142.

97. Ernst P, Killmann S-A. Perturbation of generation cycle of human leukemic myeloblasts *in vivo* by methotrexate. *Blood* 1971, **38**, 689-705.

98. Goldman ID. Effects of methotrexate on cellular metabolism: some critical elements in drug-cell interaction. *Cancer Treat Rep* 1977, **61**, 549-558.

99. Rueckert RR, Mueller GC. studies on unbalanced growth in tissue culture: Induction and consequences of thymidine deficiency. *Cancer Res* 1960, **20**, 1584-1591.

100. Tattersall MHN, Jackson RC, Connors TA, Harrap KR. Combination chemotherapy: The interaction of methotrexate and 5-fluorouracil. *Eur J Cancer* 1973, **9**, 733-739.

101. Li JC, Kaminskas E. Accumulation of DNA strand breaks and methotrexate cytotoxicity. *Proc Natl Acad Sci USA* 1984, **81**, 5694-5698.

102. Hori T, Ayusawa D, Shimizu K, Koyoma H, Seno T. Chromosome breakage induced by thymidylate stress in thymidilate synthase-negative mutants of mouse FM3A cells. *Cancer Res* 1984, **44**, 703-709.

103. Richards RG, Brown OE, Gillison ML, Sedwick WD. Drug concentration-dependent DNA lesions are induced by lipid-soluble antifolate piritrexim (BW3010). *Mol Pharmacol* 1986, **30**, 651-658.

104. Lorico A, Toffoli G, Boicchi M, Erba E, Broggini M. Rappa G, D'Incalci M. Accumulation of DNA strand breaks in cells exposed to methotrexate or N^{10}-propargyl-5,8-dideazafolic acid. *Cancer Res* 1988, **48**, 2036-2041.

105. Curtin NJ, Harris AL, Aherne GW. Mechanism of cell death following thymidylate synthase inhibition: 2'-deoxyuridine-5'-triphosphate accumulation, DNA damage, and growth inhibition following exposure to CB3717 and Dipyridamole. *Cancer Res* 1991, **51**, 2346-2352.

106. Rodenhuis S, McGuire JJ, Norayaman R, Bertino JR. Development of an assay sysytem for the detection and classification of methtrexate resistance in fresh human leukemic cells. *Cancer Res* 1986, **46**, 6513-6519.

107. 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.

108. Rosowsky A, Wright JE, Cucchi CA, Lippke JA, Tantravahi R, Ervin TJ, Frei III E. Phenotypic heterogeneity in cultured human head and neck squamous cell carcinoma lines with low level methotrexate resistance. *Cancer Res* 1985, **45**, 6205-6212.

109. Schimke RT. Gene amplification, drug resistance, and cancer. *Cancer Res* 1984, **44**, 1735-1742.

110. Bernson RJ, Francke U, Dolnick BJ, Bertino JR. Karyotype analysis of methotrexate-resistant sensitive mouse L5178Y cells. *Cytogenet Cell Genet* 1981, **29**, 143-147.

111. Curt GA, Carney DN, Cowan KH, Jolivet J, Bailey BD, Drake JC, Kao-shan CS, Minna JD, Chabner BA. Unstable methotrexate resistance in human small-cell carcinoma associated with double-minute chromosomes. *N Engl J Med* 1983, **308**, 199-202.

112. Carman MD, Schornagel JH, Rivest RS, Srimatkandada S, Portlock CS, Duffy T, Bertino JR. Resistance to methotrexate due to gene amplification in a patient with acute leukemia. *J Clin Oncol* 1984, **2**, 16-20.

113. Domin BA, Grill SP, Bastow KF, Cheng Y. Effect of methotrexate on dihydrofolate reductase activity in methotrexate-resistant human KB cells. *Mol Pharmacol* 1982, **21**, 478-482.

114. Goldie JH, Krystal G, Hartley D, Gudauskas G, Dedhar S. A methotrexate insensitive variant of folate reductase present in two lines of methotrexate-resistant L5178Y cells. *Eur J Cancer* 1980, **16**, 1539-1546.

115. Dedhar S, Goldie JH. Overproduction of two antigenically distinct forms of dihydrofolate reductase in a highly methotrexate-resistant mouse leukemia cell line. *Cancer Res* 1983, **43**, 4863-4871.

116. Thillet J, Absil J, Stone SR, Pictet R. Site-directed mutagenesis of mouse dihydrofolate reductase. Mutants with increased resistance to methotrexate and trimethoprim. *J Biol Chem* 1988, **263**, 12500-12508.

117. Haber DA, Schimke RT. Unstable amplification of an altered dihydrofolate reductase gene associated with double-minute chromosomes. *Cell* 1981, **26**, 355-362.

118. Dedhar S, Hartley D, Goldie JH. Increased dihydrofolate reductase activity in methotrexate-resistant human promyelocytic-leukemia (HL-60) cells. *Biochem J* 1985, **225**, 609-617.

119. Dedhar S, Goldie JH. Methotrexate-resistant human promyelocitic leukemia (HL-60) cells express a dihydrofolate reductase with altered properties associated with increased enzyme activity. *Biochem Biophys Res Commun* 1985, **129**, 536-545.

120. Galivan J. Transport and metabolism of methotrexate in normal and resistant rat hepatoma cells. *Cancer Res* 1979, **39**, 735-743.

121. Galivan J. 5-Methyltetrahydrofolate transport by hepatoma cells and methotrexate-resistant sublines in culture. *Cancer Res* 1981, **41**, 1757-1762.

122. Hill BT, Bailey BD, White JC, Goldman ID. Characteristics of transport of 4-amino antifolates and folate compounds by two lines of L51178Y lymfoblasts, one with impaired transport of methotrexate. *Cancer Res* 1979, **39**, 2440-2446.

123. Rosowsky A, Lazarus H, Yuan GC, Beltz WR, Mangini L, Abelson HT, Modest EJ, Frei III E. Effects of methotrexate esters and other lipophilic antifolates on methotrexate-resistant human leukemic lymfoblasts. *Biochem Pharmacol* 1980, **29**, 648-652.

124. McCormick JI, Susten SS, Freisheim JH. Characterisation of the methotrexate transport defect in a resistant L1210 lymphoma cell line. *Arch Biochem Biophys* 1981, **212**, 311-318.

125. Sirotnak FM, Moccio DM, Kelleher LE, Goutas LJ. Relative frequency and kinetic properties of transport-defective phenotypes among methotrexate-resistant L1210 clonal cell lines derived *in vivo*. *Cancer Res* 1981, **41**, 4447-4452.

126. Niethammer D, Jackson RC. Changes in the molecular properties associated with the development of resistace against methotrexate in human lymphoblastoid cells. *Eur J Cancer* 1975, **11**, 845-854.

127. Schuetz JD, Matherly LH, Westin EH, Goldman ID. Evidence for a funcional defect in the translocation of the methotrexate transport carrier in a methotrexate-resistant murine L1210 leukemia cell line. *J Biol Chem* 1988, **263**, 9840-9847.

128. Schuetz JD, Westin EH, Matherly LH, Pincus R, Swerdlow PS, Goldman ID. Membrane protein changes in an L1210 leukemia cell line with a translocation defect in the methotrexate-tetrahydrofolate cofactor transport carrier. *J Biol Chem* 1989, **264**, 16261-16267.

129. Cowan KH, Jolivet J. A methotrexate-resistant human breast cancer cell line with multiple defects, including diminished formation of methotrexate polyglutamates. *J Biol Chem* 1984, **259**, 10793-10800.

130. Frei III E, Rosowsky A, Wright JE, Cucchi CA, Lippke JA, Ervin TJ, Jolivet J, Haseltine WA. 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.

131. Pizzorno G, Mini E, Corronnello M, McGuire JJ, Moroson BA, Cashmore AR, Dreyer RH, Lin JT, Mazzei T, Periti P. Bertino JR. 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.

132. 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.

133. Samuels LL, Goutas LJ, Priest DG, Piper JR, Sirotnak FM. Hydrolytic cleavage of methotrexate γ-polyglutamates by folylpolyglutamyl hydrolase derived from various tumors and normal tissues of the mouse. *Cancer Res* 1986, **46**, 2230-2235.

134. Whitehead VM, Vuchich M-J, Payment C, Hucal S, Kalman TI. Study of methotrexate polyglutamate formation in cultured leukemic cells. In: Curtius H-Ch, Ghisla S, Blau N, eds. *Chemistry and Biology of Pteridines 1989*. Berlin, New York, Walter de Gruyter, 1990, 1166-1169.

135. White JR, Goldman ID. Methotrexate resistance in an L1210 cell line resulting from increased dihydrofolate reductase, decreased thymidylate synthase activity and normal membrane transport. *J Biol Chem* 1981, **256**, 5722-5727.

136. Moran RG, Mulkins M, Heidelberger C. Role of thymidylate synthase activity in development of methotrexate cytotoxicity. *Proc Natl Acad Sci USA* 1979, **76**, 5924-5928.

137. Curt GA, Jolivet J, Carney DN, Bailey BD, Drake JC, Clendeninn NJ, Chabner BA. Determinants of the sensitivity of human small-cell lung cancer cell lines to methotrexate. *J Clin Invest* 1985, **76**, 1323-1329.

138. Cadman E, Heimer R. Levels of thymidylate synthetase during normal culture growth of L1210 cells. *Cancer Res* 1986, **46**, 1195-1198.

139. Sirotnak FM, DeGraw JI, Schmid FA, Goutas LJ, Moccio DM. New antifolate analogs of the 10-deaza-aminopterin series: further evidence for markedly increased antitumor efficacy compared with methotrexate in ascitic and solid tumor models. *Cancer Chemother Pharmacol* 1984, **12**, 26-30.

140. Brown DH, Braakhuis BJM, van Dongen GAMS, Snow GB. Comparative study of the sensitivity of head and neck cell lines to methotrexate (MTX) and the analog 10-ethyl-10-deaza-aminopterin (10EdAM). *Otolaryngol Head Neck Surg* 1990, **102**, 20-25.

141. Schmid FA, Sirotnak FM, Otter GM, DeGraw JI. New folate analogues of 10-deaza-aminopterin series: markedly increased anti-tumor activity of the 10-ethyl analogue compared to the parent compound and methotrexate against some human tumor xenografts in nude mice. *Cancer Treat Rep* 1985, **69**, 551-553.

142. Schornagel JH, Cappelaere P, Verwey J, Cognetti F, de Mulder PHM, Clavel M, Vermorken JB, Snow GB. A randomized phase II study of 10-ethyl-10-deaza-aminopterin (10-EdAM) and methotrexate (MTX) in advanced head and neck squamous cell cancer (AHNC), an EORTC study. *Proc Am Assoc Clin Oncol* 1989, **8**, 174. (manuscript in preparation)

143. Moran RG, Baldwin SW, Taylor EC, Shih C. The 6S- and 6R-diastereomers of 5,10-dideaza-5,6,7,8,-tetrahydrofolate are equiactive inhibitors of *de novo* purine synthesis. *J Biol Chem* 1989, **264**, 21047-21051.

144. 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.

145. 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, New York, Walter de Gruyter, 1990, 1031-1034.

146. Lin JT, Bertino JR. Trimetrexate: a second generation folate antagonist in clinical trial. *J Clin Oncol* 1987, **5**, 2032-2040.

147. 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.

148. Kamen BA, Eibl B, Cashmore AR, Bertino JR. Uptake and efficacy of trimetrexate (TMQ, 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl] quinazoline), a non-classical antifolate in methotrexate-resistant leukemia cells *in vitro*. *Biochem Pharmacol* 1984, **33**, 1697-1699.

149. Bertino JR. Folate antagonists: toward improving the therapeutic index and development of new analogs. *J Clin Pharmacol* 1990, **30**, 291-295.

150. Bassendine MF, Curtin NJ, Loose H, Harris AL, James OFW. Induction of remission in hepatocellular carcinoma with a new thymidylate synthetase inhibitor CB3717: A phase II study. *J Hepatol* 1987, **4**, 349-356.

151. Cantwell BMJ, Macaulay V, Harris AL, Kaye SB, Smith IE, Milstead RAV, Calvert AH. Phase II study of the antifolate N^{10}-propargyl-5,8-dideazafolic acid (CB3717) in advanced breast cancer. *Eur J Cancer* 1988, **24**, 733-736.

152. Vest S, Bork E, Hansen HH. A phase 1 evaluation of N^{10}-propargyl-5,8-dideazafolic acid. *Eur J Cancer* 1988, **24**, 201-204.

153. Jackman AL, Newell DR, Jodrell DI, Taylor GA, Bishop JAM, Hughes LR, Calvert AH. *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, New York, Walter de Gruyter, 1990, 1023-1026.

154. Sikora E, Jackman AL, Newell DR, Calvert AH. Formation and retention and biological activity of N^{10}-propargyl-5,8-dideazafolic acid (CB3717) polyglutamates in L1210 cells *in vitro*. *Biochem Pharmacol* 1988, **37**, 4047-4054.

155. Harrap KR, Jackman AL, Newell DR, Taylor GA, Hughes LR, Calvert AH. Thymidylate synthase: a target for anticancer drug design. *Adv Enzyme Regul* 1989, **29**, 161-179.

156. 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.

157. Ferguson K, Boschelli D, Hoffman P, Oronsky AL, Whiteley J, Webber S, Galivan J, Freisheim JH, Hynes J, Kewar SS. Synergy between 5,10-dideaza-5,6,7,8-tetrahydrofolic acid and methotrexate in mice bearing L1210 tumors. *Cancer Chemother Pharmacol* 1989, **25**, 173-176.

158. Galivan J, Nimec Z, Rhee M, Boschelli D, Oronsky AL, Kewar SS. Antifolate drug interactions: Enhancement of growth inhibition due to the antipurine 5,10-dideazatetrafolic acid by the lipophilic dihydrofolate reductase inhibitors metoprine and trimetrexate. *Cancer Res* 1988, **48**, 2421-2425.

Chapter 3

MEMBRANE TRANSPORT OF METHOTREXATE IN A SQUAMOUS CARCINOMA CELL LINE ADAPTED TO LOW FOLATE CONCENTRATIONS

Published in *Anticancer Res* 1991, **11**, 1265-1268

Authors: B.F.A.M. van der Laan, G. Jansen, J.A. van Gestel, J.H. Schornagel and G.J. Hordijk

ABSTRACT

Membrane transport characteristics of the folate analogue methotrexate (MTX) were studied in a human squamous carcinoma cell line of the head and neck (HNSCC) adapted to grow in tissue culture media with nanomolar reduced folate concentrations (SCC-11B-LF), as compared to SCC-11B cells grown in standard medium containing high folate concentrations.

We observed that SCC-11B-LF cells exhibited a 10.5-fold increased uptake of [^{3}H]-MTX via the reduced folate/MTX carrier system compared to SCC-11B cells. Affinity labelling of the reduced folate/MTX carrier system suggests that the up-regulation of [^{3}H]-MTX transport mainly results from an increased rate of carrier translocation, and only to a minor extent (15-20%) from an increased amount of carrier protein. The up-regulation of MTX transport resulted in a 2.4-fold increased growth inhibitory effect by MTX.

These results suggest that membrane transport may play a more important role in MTX-cytotoxicity when SCC-11B cells *in vitro* are grown in more physiological folate concentrations.

INTRODUCTION

Patients with squamous cell carcinomas of the head and neck (HNSCC) presenting with an early localized disease are usually curable with surgery or radiotherapy alone, whereas advanced stages of disease can be cured with a combination of surgery and radiotherapy [1]. In patients with recurrent and/or systemic disease, however, adequate tumour control cannot be provided by surgery and radiotherapy alone. The role of chemotherapy in these circumstances is still disappointing [2]. Squamous cell carcinomas are nevertheless responsive to a variety of antineoplastic agents. Active drugs for the treatment of head and neck carcinomas include methotrexate (MTX), cisplatin, bleomycin, and 5-fluorouracil [3].

The antifolate methotrexate [4] is one of the most widely used chemotherapeutics in recurrent or inoperable squamous cell carcinomas [3]. In this regard, HNSCC cell lines have been used as *in vitro* model systems for screening sensitivity and/or resistance to antifolate compounds [5-10].

Usually, however, these type of studies are carried out with HNSCC cells grown in standard tissue culture media containing 2-10 μM of folic acid. This concentration has to be considered as supraphysiological because circulating plasma reduced folate levels vary between 5-50 nM [11].

In this study membrane transport characteristics of MTX were compared for a human HNSCC cell line grown in standard high folate medium, and for the same cells adapted to grow at nanomolar reduced folate concentrations.

MATERIALS AND METHODS

Chemicals

Standard RPMI-1640 medium with 2.2 μM folic acid, folate-free RPMI-1640 medium, fetal calf and dialysed fetal calf serum were obtained from Gibco, Grand Island, NY, U.S.A. d,l-5-Formyltetrahydrofolate (5-formyl-THF), folic acid, and N-hydroxysuccimide were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Methotrexate was a gift from Pharmachemie, Haarlem, The Netherlands. Trimetrexate (TMQ) glucuronate-salt was obtained from Warner Lambert/ Park Davis, Ann Arbor, MI, U.S.A. [^{3}H]-MTX (20 Ci/mmol) and [^{3}H]-folic acid (35 Ci/mmol) were obtained from Moravek Biochemical, Brea, CA, U.S.A. Radiolabels were purified prior to use by thin-layer chromatography as described before [12-15]. Radiochemical purity of the labelled compounds was more than 99% after rechromatography.

Cell culture

UM-SCC-11B cells, a cell line that 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, U.S.A. Parental (SCC-11B) cells were grown as a monolayer at 37°C in a 5% CO_2 humidified atmosphere in RPMI-1640 medium (with 2,2 μM folic acid) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, penicillin (100 Units/ml) and streptomycin (100 μg/ml), further referred to as "standard" medium.

SCC-11B-Low Folate (SCC-11B-LF) cells, adapted to low reduced folate concentrations, were obtained by growth of parental cells in folate-free RPMI-1640 medium (supplemented with 10% dialysed FCS, glutamine and antibiotics as described above), containing a starting concentration of 10 nM 5-formyl-THF as sole reduced folate source. 5-Formyl-THF was used as a model for the circulating plasma folate 5-methyltetrahydrofolate, which was found to be unstable in culture medium used during growth experiments [16,17]. Cells were transferred in serial passages in stepwise decreasing concentrations of 5-formyl-THF. After 6 months, SCC-11B-LF cells were maintained in medium containing 0.25 nM 5-formyl-THF (further referred to as "low folate" medium). Growth rate of SCC-11B-LF cells under these conditions was identical to that of SCC-11B cells grown in standard (high folate) medium.

Cytotoxicity assay

Cells were plated in the individual well of a 24-well tissue culture plate at a density of 1 x 10^4 cells/cm^2. Appropriate dosages of 5-formyl-THF and the antifolate drugs were added 24 hours later. Following incubation for 7-10 days, cells were washed twice with phosphate buffered saline solution (PBS) to remove non-viable cells, trypsinized (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.

[^{3}H]-MTX uptake

HNSCC cells in the mid-log phase of growth were harvested by trypsinization, 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) [18], containing :107 mM NaCl, 20 mM Hepes, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1.0 mM $MgCl_2$, 7 mM D-glucose, pH 7.4 with NaOH. Uptake of MTX was followed for 10 minutes at 37°C at an extracelluar concentration of 2 μM [^{3}H]-MTX (specific activity 500 cpm/ pmol). At selected intervals the uptake was stopped by the addition of 9 volumes ice-cold transport buffer. Cells were centrifuged for 5 minutes 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 analyzed for ^{3}H-radioactivity as described before [14] in Optifluor scintillation fluid (United Technologies Packard, Brussels, Belgium) with the use of an Isocap/300 (Searle, Nuclear Chicago) scintillation counter with a counting efficiency for ^{3}H of 51%. Experiments in which uptake of [^{3}H]-MTX was analyzed for non-trypsinized HNSCC-11B cells, or for CCRF-CEM leukaemia cells incubated with 0.25% trypsin, demonstrated that trypsinization had no effect on the uptake of [^{3}H]-MTX in these cells.

[^{3}H]-MTX labelling

An N-hydroxysuccimide ester of [^{3}H]-MTX (NHS-[^{3}H]-MTX) [19] was used as an affinity label for the carrier protein in HNSCC cells. NHS-[^{3}H]-MTX was synthesized as described previously [14,19]. SCC-11B(-LF) cells were grown as a monolayer in 10 cm^2 culture dishes. Cells were washed twice with HBSS buffer and 150 pmol of NHS-[^{3}H]-MTX (in 1.5 ml HBSS) was added to the culture dish. Labelling in the presence of 1 mM unlabelled MTX served as control for specificity. After 5 min incubation at 25°C, cells were washed twice with HBSS after which 1.5 ml 1% (v/v) Triton X-100 in HBSS was added to the culture dish. After 1 hour the cell homogenate was transferred to an Eppendorf tube and centrifuged for 1 min at 13,000 *g*. The supernatant was diluted 1:1 with methanol and kept for 1 hour at -20°C. The precipitated protein was recovered by centrifugation, dissolved in 10 mM sodium phosphate buffer (pH 7.5) containing 1% sodium dodecyl sulphate, and analyzed for ^{3}H-radioactivity. The incorporation of [^{3}H]-MTX is expressed as pmol/mg protein.

DHFR assay

Dihydrofolate reductase (DHFR) activity was determined according to the method described by Mini et al. [20].

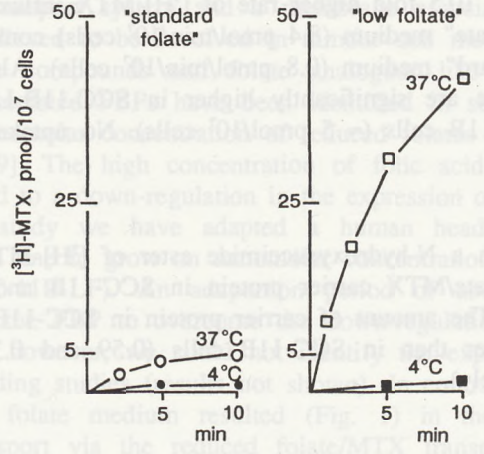

Fig. 1. Time course of accumulation of [^{3}H]-MTX by SCC-11B cells grown at "standard" folate (left hand panel) and SCC-11B-LF cells grown at "low folate" concentrations (right hand panel). The results are the mean of three experiments, S.D. less than 19%. Uptake buffer; HBSS pH 7.4, extracelluar concentration of [^{3}H]-MTX: 2 μM.

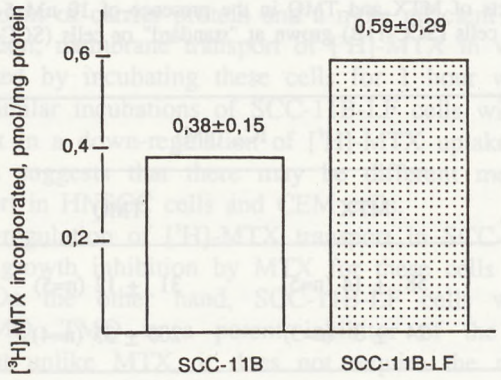

Fig. 2. The specific incorporation of [^{3}H]-MTX after incubating SCC-11B and SCC-11B-LF cells with a N-hydroxysuccimide ester of [^{3}H]-MTX. Details of NHS-[^{3}H]-MTX labelling are described in "Materials and Methods".

RESULTS

[^{3}H]-MTX uptake

Fig. 1 demonstrates a 10.5 fold higher rate of [^{3}H]-MTX influx by SCC-11B-LF cells grown at "low folate" medium (8.4 pmol/min/10^7 cells) compared to SCC-11B cells grown at "standard" medium (0.8 pmol/min/10^7 cells). Also steady state levels of [^{3}H]-MTX uptake are significantly higher in SCC-11B-LF cells (≈ 50 pmol/10^7 cells) than SCC-11B cells (≈ 5 pmol/10^7 cells). No uptake of [^{3}H]-MTX is observed at 4°C.

NHS-[^{3}H]-MTX labelling

Affinity labelling with a N-hydroxysuccimide ester of [^{3}H]-MTX to estimate the amount of reduced folate/MTX carrier protein in SCC-11B and SCC-11B-LF cells, is shown in Fig. 2. The amount of carrier protein in SCC-11B-LF cells was found to be 1.6-fold higher than in SCC-11B cells (0.59 and 0.38 pmol [^{3}H]-MTX/mg protein, respectively).

Cytotoxicity

Growth inhibitory effects of MTX and TMQ for human squamous cell carcinoma cell lines of the head and neck (SCC-11B) and (SCC-11B-LF) grown in respectively "standard" or "low folate" medium are shown in Table 1. In the presence of 10 nM 5-formyl-THF as folate source, SCC-11B-LF cells are a 2.4 fold more sensitive to MTX than SCC-11B cells grown in "standard" medium (IC_{50}: 24 nM vs 58 nM, respectively). On the other hand SCC-11B-LF cells were 6.6 fold less sensitive to TMQ as compared to SCC-11B cells (IC_{50}: 205 nM vs 31 nM, respectively).

Table 1. Growth inhibitory effects of MTX and TMQ in the presence of 10 nM 5-formyl-THF for human squamous cell carcinoma cells (SCC-11B) grown at "standard" or cells (SCC-11B-LF) grown at "low folate" concentrations.

MEDIUM	IC_{50} (nM)	
	MTX	TMQ
standard [A]	58 ± 14 (n=5)	31 ± 12 (n=5)
low folate [B]	24 ± 3 (n=3)	205 ± 35 (n=4)

[A] SCC-11B cells were grown in RPMI-1640 medium supplemented with 2.2 μM folic acid and 10% fetal calf serum.

[B] SCC-11B-LF cells were grown in folate free RPMI-1640 medium supplemented with 10% dialysed fetal calf serum and 0.25 nM 5-formyl-THF as the sole folate source.

DISCUSSION

At present two different transport routes, a carrier-mediated process via the reduced folate/MTX transport system and a membrane associated folate binding protein (FBP), are believed to be involved in tumour cell membrane transport of natural reduced folate compounds and folate analogues like MTX [12,14,15,17,21-28]. Membrane associated FBPs have been identified in several established cell lines adapted to nanomolar concentration of reduced folates in the cell culture medium [13,14,23,26,29]. The high concentration of folic acid in commercially available media can lead to a down-regulation in the expression of FBP [13].

In this study we have adapted a human head and neck squamous cell carcinoma cell line to grow on nanomolar concentrations of reduced folates in the medium (SCC-11B-LF). An adaptation period of about six months is usually necessary for the FBP to overcome the down-regulation [13,14,26]. In the SCC-11B-LF cells, however, we could not identify the expression of a FBP by [^{3}H]-folic acid binding studies (results not shown). In contrast, growth of SCC-11B-LF cells in low folate medium resulted (Fig. 1) in the up-regulation of carrier-mediated transport via the reduced folate/MTX transport system. This transport system has also been identified in other HNSCC cell lines [5,7-9]. Affinity labelling of SCC-11B-LF cells with NHS-[^{3}H]-MTX [19,30], suggests that the up regulation of [^{3}H]-MTX transport was associated only to a minor extent with an increase in the amount of carrier protein. The major part in the up-regulation of membrane transport must therefore be ascribed to a more efficient carrier function. This finding is analogous to recent studies with murine L1210 and human CCRF-CEM leukaemia cells exhibiting an up-regulation of reduced folate/MTX transport following adaptation of cells to grow at low medium folate concentrations [18,27,31]. In the L1210 variants [18,31] the increased membrane transport of [^{3}H]-MTX correlated with a similar increase in the amount of carrier protein, whereas an up-regulated variant of CCRF-CEM [27] was characterized by both an increased amount of carrier protein and a more efficient carrier function.

In addition, membrane transport of [^{3}H]-MTX in variant CEM cells could be down regulated by incubating these cells for 1 hour with 5-25 nM of 5-formyl-THF [27]. Similar incubations of SCC-11B-LF cells with 5-formyl-THF, however, did not result in a down-regulation of [^{3}H]-MTX uptake in these cells (results not shown). This suggests that there may be different mechanisms of regulation of MTX transport in HNSCC cells and CEM cells.

The up-regulation of [^{3}H]-MTX transport in SCC-11B-LF cells resulted in a more potent growth inhibition by MTX for these cells compared to parental cells (Table 1). On the other hand, SCC-11B-LF cells were less sensitive to the antifolate TMQ. TMQ is a potent inhibitor of the intracellular dihydrofolate reductase, but unlike MTX, it does not require the reduced folate/MTX carrier system for cell entry [32,33]. Because there was no change in DHFR activity (results not shown), it is reasonable to assume that a more efficient uptake of 5-formyl-THF, which can protect cells from TMQ cytotoxicity [34], accounts for the decreased TMQ sensitivity in SCC-11B-LF cells compared to parental SCC-11B

cells.

In summary, a human head and neck squamous cell carcinoma cell line, grown in cell culture medium containing "low-folate" concentrations, exhibited an up-regulation of (anti)folate transport via the reduced folate carrier system. This suggests that in HNSCC cells grown in folate-conditioned medium, membrane transport could play a more important role in MTX-cytotoxicity than was anticipated from studies with HNSCC cell lines grown at "standard" conditions [5,7-9]. Studies are now in progress to test this hypothesis *in vivo* using SCC-11B(-LF) cells as xenografts in athymic nude mice [35,36].

ACKNOWLEDGEMENTS

This study was supported by the Dutch Cancer Society (Grant UUKC 89-06).
Ms Ietje Kathmann is thanked for her excellent technical assistance.

REFERENCES

1. Snow GB. Evaluation of new treatment methods for head and neck cancer: a challenge. *Acta Otolaryngol* 1989, **107**, 352-356.

2. Tannock IF, Browman G. Lack of evidence for a role of chemotherapy in the routine management of locally advanced head and neck cancer. *J Clin Oncol* 1986, **4**, 1121-1126.

3. Al-Sarraf M. Head and Neck Cancer: Chemotherapy Concepts. *Seminars in Oncology* 1988, **15**, 70-85.

4. Schornagel JH, McVie JG. The clinical pharmacology of methotrexate, a review. *Cancer Treat Rev* 1983, **10**, 53-75.

5. Frei III E, Rosowsky A, Wright JE, Cucchi CA, Lippke JA, Ervin TJ, Jolivet J, Haseltine WA. 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.

6. 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.

7. Rosowsky A, Wright JE, Cucchi CA, Lippke JA, Tantravahi R, Ervin TJ, Frei III E. Phenotypic heterogeneity in cultured human head and neck squamous cell carcinoma lines with low level methotrexate resistance. *Cancer Res* 1985, **45**, 6205-6212.

8. Rosowsky A, Wright JE, Cucchi CA, Flatow Jl, Trites DH, Teicher BA, Frei III E. Collateral methotrexate resistance in cultured human head and neck carcinoma cells selected for resistance to cis-diamminedichloroplatinum (II). *Cancer Res* 1987, **47**, 5913-5918.

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. Brown DH, Braakhuis BJM, van Dongen GAMS, Snow GB. Comparative study of the sensitivity of head and neck cell lines to methotrexate (MTX) and the analog 10-ethyl-10-deaza-aminopterin (10EdAM). *Otolaryngol Head Neck Surg* 1990, **102**, 20-25.

11. Beck WS. Folic acid deficiency. In: Williams WJ, Beutler E, Erslev AJ, Rundles RW, eds. *Hematology*. Cambridge, MA:MIT Press, 1977, 334-350.

12. Henderson GB, Tsuji JM, Kumar HP. Characterisation of the individual transport routes that mediate the influx and efflux of methotrexate in CCRF-CEM human lymfoblastic cells. *Cancer Res* 1986, **46**, 1633-1638.

13. Jansen G, Kathmann I, Rademaker BC, Braakhuis BJM, Westerhof GR, Rijksen G, Schornagel JH. Expression of a folate binding protein in L1210 cells grown in low folate medium. *Cancer Res* 1989, **49**, 1959-1963.

14. 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.

15. 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. *Leukemia Research* 1989, **13**, 981-987.

16. Sirotnak FM, Goutas LJ, Mines LS. Extent of the requirement for folate transport by L1210 cells for growth and leukemogenesis *in vivo*. *Cancer Res* 1985, **45**, 4732-4734.

17. Sirotnak FM. Obligate genetic expression in tumor cells of a fetal membrane property mediating "folate" transport: Biological significance and implications for improved therapy of human cancer. *Cancer Res* 1985, **45**, 3992-4000.

18. 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.

19. Henderson GB, Zevely EM. Affinity labeling of the 5-methyltetrahydrofolate/methotrexate transport protein of L1210 cells by treatment with an N-hydroxysuccimide ester of [^{3}H]methotrexate. *J Biol Chem* 1984, **259**, 4558-4562.

20. 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.

21. Hill BT, Bailey BD, White JC, Goldman ID. Characteristics of transport of 4-amino antifolates and folate compounds by two lines of L51178Y lymfoblasts, one with impaired transport of methotrexate. *Cancer Res* 1979, **39**, 2440-2446.

22. Galivan J. 5-Methyltetrahydrofolate transport by hepatoma cells and methotrexate-resistant sublines in culture. *Cancer Res* 1981, **41**, 1757-1762.

23. 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.

24. Henderson GB, Tsuji JM, Kumar HP. Transport of folate compounds by leukemic cells. Evidence for a single influx carrier for methotrexate, 5-methyltetrahydrofolate, and folate in CCRF-CEM human lymfoblasts. *Biochem Pharmacol* 1987, **36**, 3007-3014.

25. Price EM, Freisheim JH. Photoaffinity analogues of methotrexate as folate antagonist binding probes. 2. Transport studies, photoaffinity labeling, and identification of the membrane carrier protein for methotrexate from murine L1210 cells. *Biochemistry* 1987, **26**, 4757-4763.

26. 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.

27. 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.

28. 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.

29. Kamen BA, Capdevila A. Receptor-mediated folate accumulation is regulated by the cellular folate content. *Proc Natl Acad Sci USA* 1986, **83**, 5983-5987.

30. Jansen G, Westerhof GR, Rijksen G, Schornagel JH. Interaction of N-Hydroxy(sulfo)succimide active esters with the reduced folate/methotrexate transport system from human leukemic CCRF-CEM cells. *Biochim Biophys Acta* 1989, **985**, 266-270.

31. Yang CH, Sirotnak FM, Mines LS. Further studies on a novel class of genetic variants of the L1210 cell with increased folate analogue transport inward. *J Biol Chem* 1988, **263**, 9703-9709.

32. Kamen BA, Eibl B, Cashmore AR, Bertino JR. Uptake and efficacy of trimetrexate (TMQ, 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl] quinazoline), a non-classical antifolate in methotrexate-resistant leukemia cells *in vitro*. *Biochem Pharmacol* 1984, **33**, 1697-1699.

33. 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.

34. Matherly LH, Barlowe CK, Phillips VM, Goldman ID. The effects on 4-aminoantifolates on 5-formyltetrahydrofolate metabolism in L1210 cells (a biochemical basis of the selectivity of leucovorin rescue). *J Biol Chem* 1987, **262**, 710-717.

35. Braakhuis BJM, Schoevers EJ, Heinerman ECM, Sneeuwloper G, Snow GB. Chemotherapy of human head and neck cancer xenografts with three clinically active drugs: cis-platinum, bleiomycine and methotrexate. *Br J Cancer* 1983, **48**, 711-716.

36. Braakhuis BJM, Leyva A, Schoevers EJ, Boerrigter GH, Schornagel JH, Snow GB. Lack of effect of methotrexate on human head and neck tumors transplanted in athymic nude mice. *Acta Otolaryngol* 1985, **99**, 208-213.

Chapter 4

MECHANISMS OF ACQUIRED RESISTANCE TO METHOTREXATE IN A HUMAN SQUAMOUS CARCINOMA CELL LINE OF THE HEAD AND NECK, EXPOSED TO DIFFERENT METHOTREXATE TREATMENT SCHEDULES

Published in *Eur J Cancer* 1991, **27**, 1274-1278

Authors: B.F.A.M. van der Laan, G. Jansen, G.A.M. Kathmann, J.H. Schornagel and G.J. Hordijk.

ABSTRACT

Mechanisms of acquired resistance to methotrexate (MTX) were evaluated in HNSCC-11B cells which were made resistant to MTX 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 MTX and 30-fold or 49-fold cross-resistant to trimetrexate (TMQ) and 10-EdAM, respectively. Dihydrofolate reductase (DHFR) activity was increased 63-fold in 11B-MTX-C cells together with a decrease in [^{3}H]-MTX transport and folylpolyglutamate synthase (FPGS) activity (2.5- and 3.8-fold, respectively). Against two novel antifolates targetting enzymes other than DHFR, minor cross-resistance was observed for ICI-198.583, but full sensitivity was retained for DDATHF.

11B-MTX-P cells were 46-fold resistant to MTX and 47-fold cross-resistant to ICI-198,583 in short-term drug exposure, but showed only minor changes in MTX sensitivity following prolonged drug exposure. The resistant phenotype in 11B-MTX-P cells was characterised by a 5.6-fold decrease in FPGS activity.

These results suggests that different mechanisms of MTX-resistance in HNSCC cells *in vitro* can be obtained dependent on the schedule of exposure to MTX.

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 (MTX), 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 MTX as its most used agent [3]. Although MTX 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 MTX. 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 MTX 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 DHFR, the target enzyme of MTX, usually as a result of DHFR gene amplification; (b) alteration of DHFR, with decreased affinity for MTX; (c) decrease of MTX transport into the cell; and (d) decrease of intracellular polyglutamylation of MTX.

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

impaired polyglutamylation as the mechanism of MTX-resistance.

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

During the development of MTX-resistance, the HNSCC cell lines were grown in "folate-conditioned" medium, containing near physiological concentrations of natural folates (≈ 5 nM) rather than the high levels of folic acid (≈ 2 μM) 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]-MTX membrane transport may occur via the classical reduced folate/MTX 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 MTX in 11B-MTX-C and 11B-MTX-P cells were analysed in long-term (7 days) and in short-term (24 hours) drug exposure. In order to establish whether there is cross-resistance or sensitivity to other antifolates, the cytotoxicity experiments were also done with two other DHFR inhibitors: TMQ 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 MTX in 11B-MTX-C cells was mainly due to increased DHFR levels and to a minor extent as a result of reduced MTX-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 MTX resistant 11B-MTX-P cells were concomitantly cross-resistant 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, Grand Island, NY, USA. d,l-5-Formyltetrahydrofolate (5-formyl-THF), folic acid, 5-fluorouracil (5-FU) and 5-fluoro-2-deoxyuridine (FUdR) were purchased from Sigma Chemical Co., St. Louis, MO, USA. MTX was a gift from Pharmachemie, Haarlem, The Netherlands. Trimetrexate (TMQ) glucuronate-salt was obtained from Warner Lambert/ Park Davis, Ann Arbor, MI, USA. 10-Ethyl-10-deazaaminopterin (10-EdAM) was a gift from Ciba Geigy, Basel, Switzerland. 5,10-Dideazatetrahydrofolic acid (DDATHF) was a generous gift of Dr G.B. Grindey, Lilly Research Laboratories, Indianapolis, USA. 2-Desamino-2-methyl-

N^{10}-propargyl-5,8-dideazafolic acid (ICI-198,583) was provided by ICI-Pharmaceutical Division, Alderly Park, Macclesfield, Cheshire, United Kingdom. [^{3}H]-MTX (20 Ci/mmol) was obtained from Moravek Biochemical, Brea, CA, 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 re-chromatography. [^{3}H]-glutamate (25.0 Ci/mmol) was obtained from NEN Research Products, Boston, MA, 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 nM 5-formyl-THF, 5 nM folic acid, 2 mM glutamine, penicillin (100 Units/ml) and streptomycin (100 µg/ml).

Development of resistance

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

11B-MTX-P: In other experiments, SCC-11B cells were made resistant to pulse exposures of MTX by adding 2 µM MTX to cells three days after trypsinisation. 24 hours 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 x 10^4 cells/cm^2 in "folate-conditioned" medium. After eleven series of 24 hour pulses of 2 µM MTX, 11B-MTX-P cells had established the same doubling time as control cells. The resistant phenotype in 11B-MTX-P cells was found to be stable for at least four passages without addition of short-term MTX 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 x 10^4 cells/cm^2. Appropriate dosages of 5-formyl-THF and the antifolate drugs were added 24 hours later. In cytotoxicity assays drug exposure to the cells was maintained either for a period of 7 days (long-term exposure) or for 24 hours (short-term exposure). In the latter case, the drug containing medium was removed after 24 hours, 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

MTX and 10-EdAM are classical folate-based inhibitors of DHFR. TMQ is also a potent inhibitor of DHFR but unlike MTX or 10-EdAM does not require a specific carrier system for cellular uptake [19]. Growth inhibition experiments for MTX and TMQ were either done in the absence or presence of an additional 20 nM 5-formyl-THF in order to establish the role of the (anti)folate membrane transport system in the cytotoxicity of TMQ and MTX. 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 glycinamide ribonucleotide 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'deoxyuridine-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]-MTX 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 mM NaCl, 20 mM Hepes, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1.0 mM $MgCl_2$, 7 mM D-glucose, pH 7.4 with NaOH. Influx of [^{3}H]-MTX was determined over a period of 2.5 min at 37°C at an extracelluar concentration of 2 µM [^{3}H]-MTX (specific activity 500 cpm/pmol). Uptake of [^{3}H]-MTX at 4°C served as a control. [^{3}H]-MTX uptake was stopped by the addition of 9 volumes 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, Belgium) 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 minutes at 4°C with 100 pmol [^{3}H]-folic acid (specific activity 1.0 Ci/mmol), followed by centrifugation in an Eppendorf minicentrifuge (13000 g, 1 min). The supernatant was removed by suction and residual fluid was removed by cotton tissues. Cell pellets were resuspended in water and analyzed 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 (FPGS) activity was analyzed as described by McGuire et al. [24] using 250 μM MTX as substrate for FPGS. Protein concentrations were determined according to Bradford [25].

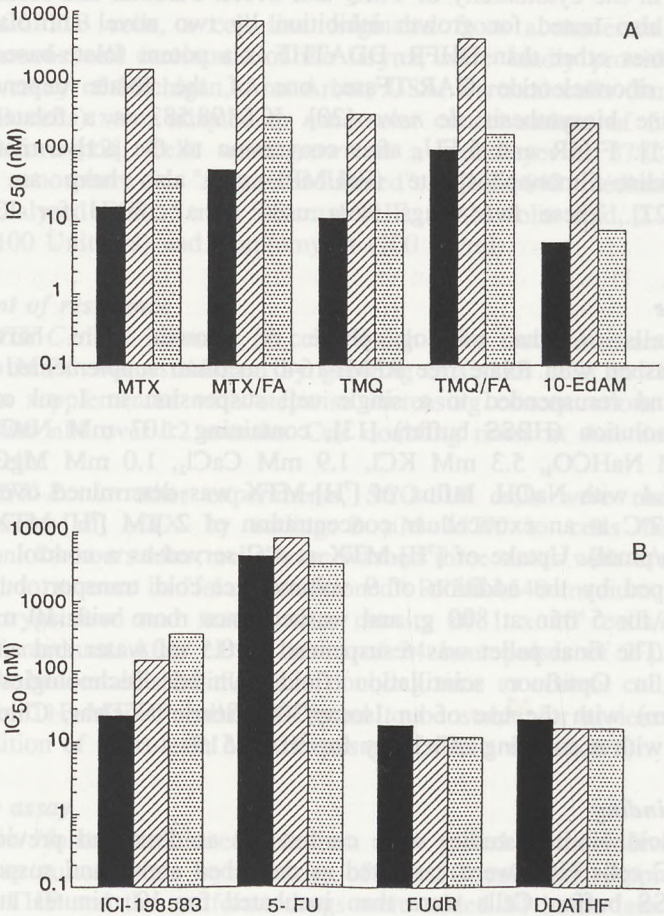

Fig. 1. Growth inhibition of parental SCC-11B (■), 11B-MTX-C (▨) and 11B-MTX-P (▩) cells by: **(A)** DHFR inhibitors in the absence or presence of 20 nM 5-formyl-THF (FA) or **(B)** non-DHFR targetting folate analogues. Drug exposure: 7 days. Results are expressed as the mean of at least 5 separate experiments.

RESULTS

Cytotoxicity experiments

Compared to parental cells 11B-MTX-C cells were found to be 91-fold resistant to MTX (Fig. 1A) in growth inhibition experiments with long-term drug exposure. In addition, these cells were 30-fold and 49-fold cross-resistant to TMQ and 10-EdAM, respectively. 11B-MTX-C cells exhibited minor cross-resistance 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 (42000-fold) resistant to MTX as compared to parental cells (IC_{50}: 7.4 mM vs 177 nM, respectively). In similar experiments also a 13.5-fold cross-resistance was noticed for 11B-MTX-C cells during a short-term exposure with ICI-198,583.

Table 1 shows that 11B-MTX-P cells, compared to parental cells, were 46-fold less sensitive to growth inhibition by MTX following a 24 hours exposure to the drug (IC_{50}: 8.1 μM vs 177 nM, respectively). However, during long-term drug exposure (Fig. 1A) 11B-MTX-P cells showed only a 2.5-fold diminished sensitivity for MTX, 1.5-fold decreased sensitivity for 10-EdAM, but full sensitivity for TMQ. 11B-MTX-P cells demonstrated substantial cross-resistance to ICI-198,583 both in short-term (Table 1) as well as to 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.

MTX-transport

The presence of multiple transport routes for MTX in parental and resistant cells was determined via [^{3}H]-MTX influx and [^{3}H]-folic acid binding studies, and indirectly via the protective effect by 5-formyl-THF upon growth inhibition by

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

	IC_{50}		
DRUGS	SCC-11B	11B-MTX-C	11B-MTX-P
MTX	177 ± 95 nM	7.4 ± 3.5 mM	8.1 ± 4.4 μM
ICI-198,583	43 nM	580 nM	2 μM

Results are expressed as the mean ± S.D. of at least four experiments (MTX) and the mean of two experiments (ICI-198,583).

MTX and TMQ. [^{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 nM) 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 MTX and TMQ cytotoxicity by 5-formyl-THF is characteristic feature indicative for the role of the reduced folate carrier system in MTX/5-formyl-THF transport rather than mFBP [18].

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

Table 2. [^{3}H]-MTX 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/hr /mg protein)	0.012 ± 0.005 (n=4)	0.758 ± 0.162 (n=3)	0.014 ± 0.008 (n=4)
MTX-uptake (pmol [^{3}H]-MTX /min/10^7cells)	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/ hr/mg protein)	1575 ± 602 (n=4)	407 ± 152 (n=3)	281 ± 80 (n=5)

Results are expressed as the mean ± S.D.

DISCUSSION

This study describes the mechanisms of resistance to MTX for a human HNSCC cell line in which MTX 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/MTX 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 MTX 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]-MTX transport and decreased FPGS activity. Cross-resistance of 11B-MTX-C cells to TMQ and 10-EdAM suggest that the increased level of DHFR is the major factor for the MTX-resistance in these cells. Whether, and to what extent, the diminished [^{3}H]-MTX transport and FPGS activity contributed in the resistance to MTX is not clear. The fact that full sensitivity is retained for DDATHF, an antifolate which can also be transported via the reduced folate/MTX 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, cross-resistance 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 cross-resistance 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 cross-resistance to ICI-198,583. It has been reported that MTX and ICI-198,583 are relatively poor substrates for FPGS ($K_m \approx 100$ μM and 40 μM, respectively) [29-31]. This is in contrast to DDATHF, which is an efficient substrate for FPGS (K_m: < 10 μM) [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 MTX 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 MTX 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 can be associated with *intrinsic* resistance of HNSCC cells to MTX. In the present study we describe that also *acquired* resistance to MTX related to decreased FPGS activity can be of importance as mechanism of resistance to MTX in HNSCC cells. Similar as was shown for 11B-MTX-C cells, 11B-MTX-P cells were cross-resistant to ICI-198,583 but not to DDATHF. This result suggest that although SCC-11B cells were exposed to serial pulses of a single agent like MTX, 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 cross-resistance of 11B-MTX-P to ICI-198,583. On the other hand, the highly efficient transport of DDATHF via the reduced folate/MTX carrier system [27,32], together with its excellent substrate affinity for FPGS, may be the determining factor in the sensitivity of (MTX-resistant) SCC-11B cells to DDATHF.

These experiments indicate that different mechanisms of resistance to MTX and cross-resistance to other antifolates can be acquired by schedule dependent treatment of HNSCC cells with MTX. 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.

ACKNOWLEDGEMENTS

This study was supported by the Dutch Cancer Society (Grant IKA 89-34).

REFERENCES

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. *Seminars in Oncology* 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, New York, Walter de Gruyter, 1990, 1089-1099.

6. Frei III E, Rosowsky A, Wright JE, Cucchi CA, Lippke JA, Ervin TJ, Jolivet J, Haseltine. 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, Lippke JA, Tantravahi R, Ervin TJ, Frei III E. 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, McGuire JJ, Moroson BA, Cashmore AR, Dreyer RH, Lin JT, Mazzei T, Periti P. Bertino JR. 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* 1991, **11**, 1265-1268.

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, Braakhuis BJM, Westerhof GR, Rijksen G. Schornagel JH. 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. *Leukemia Research* 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, Taylor GA, Bishop JAM, Hughes LR, Calvert AH. *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, New York, 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, New York, Walter de Gruyter, 1990, 1031-1034.

Chapter 5

IN VITRO ACTIVITY OF NOVEL ANTIFOLATES AGAINST HUMAN SQUAMOUS CARCINOMA CELL LINES OF THE HEAD AND NECK WITH INHERENT RESISTANCE TO METHOTREXATE

Submitted for publication in *Int J Cancer*

Authors: B.F.A.M. van der Laan, G. Jansen, G.A.M. Kathmann, G.R. Westerhof, J.H. Schornagel and G.J. Hordijk.

ABSTRACT

A series of seven human squamous carcinoma cell lines of the head and neck (HNSCC), grown in "folate-conditioned" medium, were all found to be sensitive (IC_{50}: ≤50 nM) in growth inhibition studies by methotrexate (MTX) following long-term drug exposure for 7 days. However, when MTX exposure is limited to 24 hours, only two out of seven HNSCC cell lines were sensitive to MTX (IC_{50}: <500 nM), two cell lines were moderately sensitive (IC_{50}: 1-2 μM), and three cell lines exhibited inherent resistance to MTX (IC_{50}: > 250 μM). In these three cell lines, the mechanism of resistance was not correlated with an altered membrane transport of MTX or changes in dihydrofolate reductase (DHFR) activity, but was rather associated with a 3-fold lower activity of intracellular folylpolyglutamate synthase (FPGS) activity compared to MTX-sensitive HNSCC cells.

The three cell lines exhibiting inherent resistance to MTX, however, did not show inherent cross-resistance in 24 hours exposure to one or more of three novel antifolate compounds. These compounds, which appear to be more efficiently transported and polyglutamylated than MTX, include: 10-ethyl-10-deazaaminopterin (10-EdAM), 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid (ICI-198,583), and 5,10-dideazatetrahydrofolic acid (DDATHF).

These results indicate that antifolate membrane transport and intracellular FPGS activity are important factors in determining sensitivity or resistance of HNSCC cells to short-term antifolate compound exposures.

INTRODUCTION

Most of the patients with circumscribed squamous cell carcinomas of the head and neck can be cured with either surgery, radiotherapy, or with a combination of these regimens [1]. Systemic chemotherapy is usually accepted as the standard treatment for palliation in patients with recurrent and metastatic disease who have failed the definitive therapy of surgery and/or radiotherapy. Antimetabolites have shown to be among the most effective drugs in HNSCC [2]. In particular, the folate analogue methotrexate (MTX) [3] is one of the most used antimetabolites, although the overall response rates are rather low. This may be either due to inherent resistance, or after an initial good response, due to the development of an acquired cellular resistance to MTX.

Thus far, *in vitro* studies have indicated that at least four different mechanisms may be of importance in acquired resistance of HNSCC cells to MTX [4-6]. These mechanisms include [7]: (a) increase of the intracellular level of dihydrofolate reductase (DHFR), usually as a result of DHFR gene amplification, (b) a decreased binding affinity of DHFR for MTX, (c) impaired MTX transport into the cell, and (d) decrease of intracellular polyglutamylation of MTX. Recently Pizzorno et al. [8] have shown that previously untreated HNSCC cells *in vitro* can exhibit inherent or "natural" resistance to MTX when drug exposure is limited to a short-term period (≤ 24 hours). Impaired polyglutamylation was found to be the major

factor in resistance of these cells.

In order to circumvent the problem of inherent or acquired resistance to MTX, new antifolate compounds have been developed in recent years which either have better membrane transport or polyglutamylation characteristics, and/or have target enzymes in folate metabolism other than DHFR [9-12]. In this study we have used seven human squamous carcinoma cell lines to evaluate the *in vitro* activity of MTX and two other inhibitors of DHFR (10-EdAM and trimetrexate (TMQ), a lipophilic folate analogue), as well as two novel antifolate compounds: ICI-198,583 a folate-based inhibitor of thymidylate synthase (TS) [13,14] or DDATHF a folate based inhibitor of glycinamide ribonucleotide transformylase (GAR-TFase) [15]. The growth inhibitory effects by MTX and other folate analogues were determined after long-term (7 days) or short-term (24 hours) drug exposure to HNSCC cells. For these studies HNSCC cell lines were maintained in cell culture media that traditionally contain high levels of folic acid ($\approx$ 2 μM) as folate source or, alternatively, adapted to grow in "folate-conditioned" medium containing near physiological concentrations of natural folates ($\approx$ 5 nM).

The results demonstrate inherent resistance to MTX in three out of seven HNSCC cell lines following short-term (24 hours) drug exposure, supposedly to a low intracellular FPGS activity, compared to the MTX-sensitive HNSCC cell lines. The cell lines with inherent MTX-resistance, however, retained *in vitro* sensitivity to one or more of novel antifolates 10-EdAM, ICI-198,583 or DDATHF.

MATERIALS AND METHODS

Chemicals

RPMI-1640, folate-free RPMI-1640 medium and (dialysed-) fetal calf serum (FCS) were obtained from Gibco, Grand Island, NY, USA. d,l-5-Formyltetrahydrofolate (5-formyl-THF), and folic acid were purchased from Sigma Chemical Co., St. Louis, MO, USA. MTX was a gift from Pharmachemie, Haarlem, The Netherlands. Trimetrexate (TMQ) glucuronate-salt was obtained from Warner Lambert/Park Davis, Ann Arbor, MI, USA. 10-Ethyl-10-deazaaminopterin (10-EdAM) was a gift from Ciba Geigy, Basel, Switzerland. 5,10-Dideazatetrahydrofolic acid (DDATHF) was a generous gift of Dr G.B. Grindey, Lilly Research Laboratories, Indianapolis, USA. 2-Desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid (ICI-198,583) was provided by ICI-Pharmaceutical Division, Alderly Park, Macclesfield, Cheshire, United Kingdom. [^{3}H]-MTX (20 Ci/mmol) and [^{3}H]-folic acid (35 Ci/mmol) were obtained from Moravek Biochemical, Brea, CA, USA, and were purified prior to use by thin-layer chromatography as described before [16-19]. Radiochemical purity of the labelled compounds were more than 99% after rechromatography. [^{3}H]-glutamate (25 Ci/mmol) was obtained from NEN Research Products, Boston, MA, USA. All other reagents were of the highest grade of purity available.

Cell culture

A series of seven HNSCC cell lines: UM-SCC-2 (alveolar carcinoma), -10A (larynx tumour), -10B (neck metastasis), -11B (supraglottic larynx tumour), -14A (floor of the mouth), -14C (neck metastasis), and -22B (neck metastasis of hypopharynx carcinoma) cells were kindly provided by Dr. T.E. Carey, University of Michigan, Ann Arbor, USA. Further characteristics of these cell lines are described elsewhere [20]. The cells were grown as a monolayer culture at 37°C in a 5% CO_2 humidified atmosphere in "standard" RPMI-1640 medium (with 2 µM folic acid) containing 10% FCS and supplemented with 2 mM glutamine, penicillin (100 Units/ml) and streptomycin (100 µg/ml). In other experiments, cells were adapted to grow in medium containing nanomolar concentrations of 5-formyl-THF as described before [21]. In short, HNSCC cells were transferred to RPMI-1640 medium (without folic acid) containing 10% dialysed FCS, glutamine and antibiotics as above, and 10 nM 5-formyl-THF as sole folate source. The concentration of 5-formyl-THF in the cell culture medium was gradually decreased until the final concentration of 5-formyl-THF in the medium was 0.5 - 1.0 nM (further referred to as "folate-conditioned" medium). Cell growth under these conditions was similar as for control cells grown in "standard" medium.

Cytotoxicity assay

Parental cells and cells in "folate-conditioned" medium were plated in the individual well of a 24-well tissue culture plate at a density of 1 x 10^4 cells/cm^2. Cells were incubated with the drugs either for a period of 7 days (long-term drug exposure) or for 24 hours (short-term drug exposure).

Long-term drug exposure: Appropriate dosages of 5-formyl-THF and the antifolate drugs were added the day following cell plating. After seven days, cells were washed twice with phosphate buffered saline solution (PBS) to remove non-viable cells, trypsinized (0.25% trypsin/0.05% EDTA in PBS) and counted by a Sysmex CC-110 cell counter.

Short-term drug exposure: The antifolate drugs were added three days after seeding the cells. Then the drug containing medium was removed 24 hours later, cells were washed three times with drug free medium, and incubated for another 7 days in drug free medium. After this time cells were washed, trypsinized, and counted as above. During short-term drug exposures the medium was supplemented with 5 nM 5-formyl-THF and 5 nM folic acid.

The IC_{50} is defined as the concentration of drug required to inhibit cell growth by 50%, compared to controls.

Drugs used

MTX and 10-EdAM [11,22] were included as folate-based inhibitors of DHFR. TMQ was used as a potent inhibitor of DHFR, which, unlike MTX or 10-EdAM, does not require a specific carrier system for cellular uptake [23]. Growth inhibition experiments for MTX and TMQ were either done in the absence or presence of an additional 20 nM 5-formyl-THF in order to establish the role of the (anti-)folate membrane transport system in the cytotoxicity of TMQ and MTX. ICI-

198,583 was selected as a folate-based inhibitor of TS [13]. DDATHF is a potent folate-based inhibitor of GAR-TFase, one of the folate dependent key-enzymes in purine biosynthesis *de novo* [10].

[^{3}H]-MTX uptake

HNSCC cells in the mid-log phase of growth were harvested by trypsinization, 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) [24], containing :107 mM NaCl, 20 mM Hepes, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1.0 mM $MgCl_2$, 7 mM D-glucose, pH 7.4 with NaOH. Influx of [^{3}H]-MTX was determined over a period of 2.5 minutes at 37°C at an extracelluar concentration of 2 μM [^{3}H]-MTX (specific activity 500 cpm/pmol). Uptake of [^{3}H]-MTX at 4°C served as a control. [^{3}H]-MTX uptake was stopped by the addition of 9 volumes ice-cold transport buffer. Cells were centrifuged for 5 minutes at 800 x g, and washed once more with 10 ml ice cold transport buffer. The final pellet was resuspended in 0.5 ml water and analyzed for ^{3}H-radioactivity in Optifluor scintillation fluid (United Technologies Packard, Brussels, Belgium) with the use of an Isocap/300 (Searle, Nuclear Chicago) scintillation counter with a counting efficiency for ^{3}H of 51%.

Other methods

Dihydrofolate reductase (DHFR) activity was determined according to the method described by Mini et al. [25]. Folylpolyglutamate synthase (FPGS) activity was analyzed as described by McGuire et al. [26] using 250 μM MTX as substrate for FPGS. [^{3}H]-folic acid binding was determined as described previously [18]. Protein concentrations were determined according to Bradford [27]. Statistical significance was evaluated by student t-test for paired observation.

RESULTS

Cytotoxicity experiments

Long-term drug exposure: All seven human squamous carcinoma cell lines grown in "standard" RPMI-1640 medium with 10% FCS were sensitive (IC_{50} < 90 nM) to inhibitors of DHFR (MTX, TMQ and 10-EdAM) in growth inhibition experiments with a long-term drug exposure (Fig. 1A). For all of the cell lines 10-EdAM was approximately a 10-fold better growth inhibitor than MTX (IC_{50} < 7.5 nM). Fig. 1B shows that against HNSCC cells grown in "folate-conditioned" medium, the growth inhibitory effects were greater for all the DHFR inhibitors: MTX (2.1- to 7.3-fold), 10-EdAM (1.7- to 3.8-fold) and TMQ (1.4- to 17-fold).

For non-DHFR inhibitors (Fig. 2A), five out of seven HNSCC cell lines were sensitive to growth inhibition by ICI-198,583 (IC_{50}: 3.6-56 nM). Two cell lines, SCC-10A and SCC- 14A, were less sensitive to growth inhibition by ICI-198,583 (IC_{50}: 140 nM and 180-840 nM, respectively). The inhibitory activity of ICI-198,583 against HNSCC cells grown in "folate-conditioned" medium was 1.3- to

3.8-fold greater than for parental cells grown in standard medium (Fig. 2B).

In four out of seven cell lines (SCC-10A, -10B, -11B, and -14C) no growth inhibitory effect could be observed by DDATHF (IC_{50}: ≥ 10 μM) when cells were grown in "standard" RPMI-1640 medium supplemented with 10% FCS (Fig. 2A). However, when the FCS concentration in the cell culture medium was reduced from 10% to 5%, DDATHF inhibits cell growth at concentrations < 50 nM (results not shown). It is reasonable to assume that this effect is related to high levels of reversing agents like hypoxanthine in undialysed serum [10,28]. For HNSCC cells grown in "folate-conditioned" medium (supplemented with dialysed FCS) half-maximal growth inhibition by DDATHF was observed at < 8 nM for all cell lines (Fig. 2B).

Short-term drug exposure: Fig. 3 shows the growth inhibitory effect of MTX against seven HNSCC cell lines following a short-term (24 hours) exposure to this drug. Two cell lines (SCC-11B and -22B) were found to be sensitive to MTX (IC_{50}: <500 nM), two cell lines (SCC-2 and -10B) were moderately sensitive (IC_{50}: 1-2 μM), whereas three cell lines (SCC-10A, -14A and -14C) were resistant to short-term MTX exposure (IC_{50}: > 500 μM). The same pattern of MTX-sensitivity

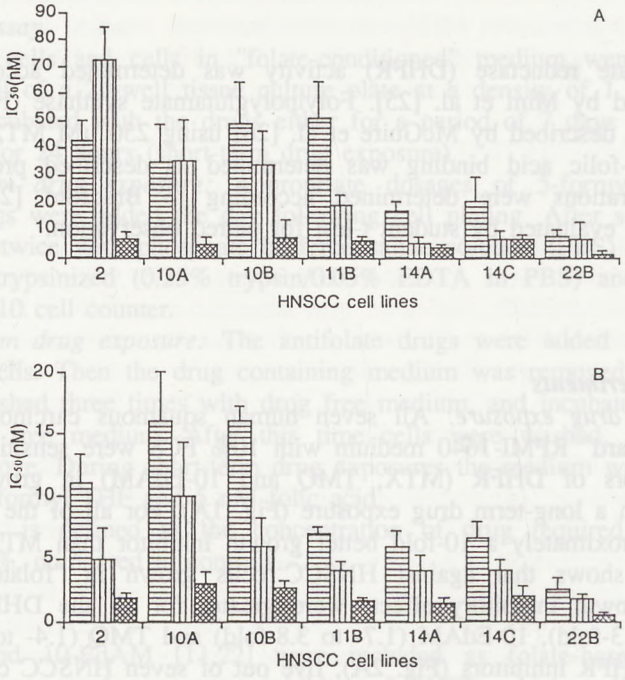

Fig. 1. Growth inhibition of human HNSCC cells by DHFR inhibitors following long-term (7 days) drug exposure. MTX (≡), TMQ (|||||), and 10-EdAM (▒). **1A:** "Standard" cell culture medium. **1B:** "Folate-conditioned" cell culture medium. Results are presented as the mean ± S.D. of at least four separate experiments.

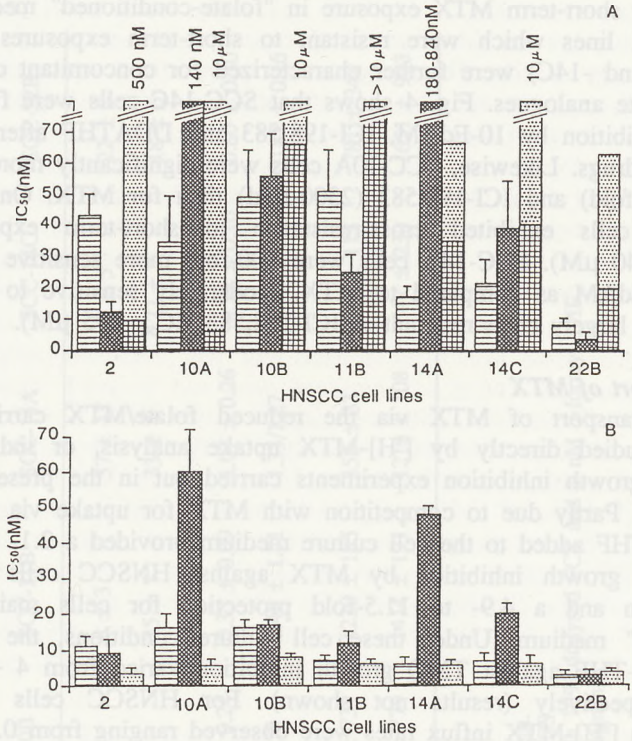

Fig. 2. Growth inhibition of human HNSCC cells by MTX (▤), and non-DHFR inhibitors: ICI-198,583 (▩), and DDATHF (▨) following 7 days drug exposure. **2A:** "Standard" cell culture medium. **2B:** "Folate-conditioned" cell culture medium. Results are presented as the mean ± S.D. of at least four separate experiments, with the exception that IC_{50} values for DDATHF in Figure 2A are given in a variable range: lowest range (▦), highest range (▨), (see results).

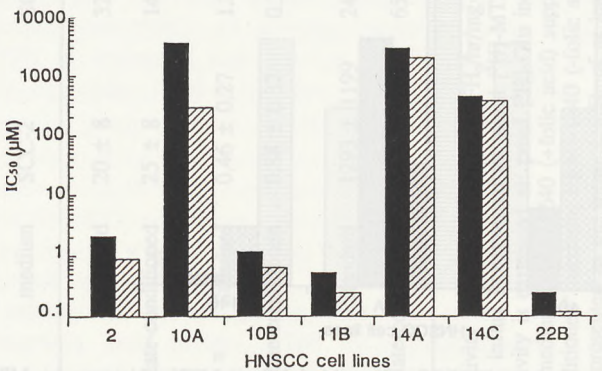

Fig. 3. Growth inhibition of human HNSCC cells by MTX after short-term (24 hours) drug exposure in "standard" (■) "Standard" cell culture medium and in "folate-conditioned" (▨) cell culture medium. Results are presented as the mean of at least four separate experiments.

was observed after short-term MTX exposure in "folate-conditioned" medium (Fig. 3). The three cell lines which were resistant to short-term exposures of MTX (SCC-10A, -14A and -14C) were further characterized for concomitant cross-resistance to other folate analogues. Fig. 4 shows that SCC-14C cells were fully sensitive to growth inhibition by 10-EdAM, ICI-198,583 and DDATHF after 24 hours exposure to these drugs. Likewise, SCC-10A cells were significantly more sensitive for 10-EdAM (75-fold) and ICI-198,583 (2300-fold) than for MTX. On the other hand, SCC-10A cells exhibited cross-resistance to short-term exposures of DDATHF (IC_{50}: 440 μM). SCC-14A cells were 400-fold more sensitive to growth inhibition by 10-EdAM as compared to MTX, moderately sensitive to DDATHF (IC_{50}: 46 μM), but largely cross-resistant to ICI-198,583 (IC_{50}: 495 μM).

Membrane transport of MTX

Membrane transport of MTX via the reduced folate/MTX carrier system [16,19,29] was studied directly by [^{3}H]-MTX uptake analysis, or indirectly via MTX and TMQ growth inhibition experiments carried out in the presence of 20 nM 5-formyl-THF. Partly due to competition with MTX for uptake via the carrier system, 5-formyl-THF added to the cell culture medium provided a 2.1- to 7.3-fold protection against growth inhibition by MTX against HNSCC cells grown in "standard" medium and a 4.9- to 11.5-fold protection for cells maintained in "folate-conditioned" medium. Under these cell culture conditions, the protection factor of 5-formyl-THF against TMQ growth inhibition varied from 4 - 14.8 and 16.2 - 34.8, respectively (results not shown). For HNSCC cells grown in "standard" medium [^{3}H]-MTX influx rates were observed ranging from 0.35 to 1.28 pmol/min/10^7 cells (Table 1). For two cell lines grown in "folate-conditioned" medium (SCC-11B and -14C) a significant increase in [^{3}H]-MTX influx (7-fold and

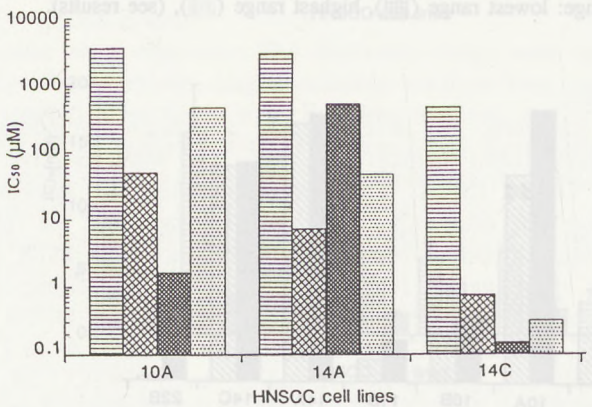

Fig. 4. Growth inhibition of human HNSCC cells with inherent MTX-resistance by MTX (☰), and the novel-antifolates: 10-EdAM (▨), ICI-198,583 (▩), and DDATHF (▤) during short-term (24 hours) drug exposure in "folate-conditioned" cell culture medium. Results are presented as the mean of at least four separate experiments.

Table 1. DHFR activity, [^{3}H]-MTX influx and FPGS activity in HNSCC cell lines grown in "standard" or "folate-conditioned" medium.

assay	medium	Cell line						
		SCC-2	SCC-10A	SCC-10B	SCC-11B	SCC-14A	SCC-14C	SCC-22B
DHFR [1]	Standard	20 ± 8	32 ± 12	5 ± 2	8 ± 3	37 ± 17	9 ± 5	22 ± 3
	Folate-conditioned	25 ± 8	14 ± 8	4 ± 3	25 ± 7	33 ± 4	27 ± 2	18 ± 6
MTX-influx [2]	Standard	0.46 ± 0.27	1.28 ± 0.52	0.76 ± 0.24	0.35 ± 0.09	1.07 ± 0.26	0.39 ± 0.16	0.80 ± 0.30
	Folate-conditioned	0.88 ± 0.32	0.33 ± 0.11	0.53/0.73	2.46 ± 1.71	1.33/1.27	1.48 ± 0.37	0.82 ± 0.16
FPGS [3]	Standard	1293 ± 1199	249 ± 103	1305 ± 73	1230 ± 185	358 ± 33	335 ± 112	781 ± 348
	Folate-conditioned	1241 ± 522	656 ± 99	1360 ± 779	1414 ± 191	1212 ± 108	547 ± 230	1651 ± 903

[1] DHFR activity is expressed as: nmol FH_2/hr/mg protein;
[2] [^{3}H]-MTX influx is expressed as: pmol [^{3}H]-MTX/min/10^7cells;
[3] FPGS activity is expressed as: pmol [^{3}H]-Glu incorporated/hr/mg protein.
"Standard" medium: RPMI-1640 (+folic acid) supplemented with 10% FCS.
"Folate-conditioned" medium: RPMI-1640 (-folic acid) supplemented with 10% dialysed FCS and 1 nM 5-formyl-THF.
Results are presented as the mean ± S.D. of at least three separate experiments.

3.8-fold, respectively) was noted. In SCC-10A cells influx of [^{3}H]-MTX was decreased 4-fold, whereas for the other cell lines influx rates were only marginally higher than for parental cells. There appears to be no direct correlation between the level of MTX-transport and the growth inhibitory effects of MTX against HNSCC cells following a 24 hours exposure to this drug (Fig. 3).

In a variety of other human cell lines grown in "folate-conditioned" medium, a membrane-associated folate binding protein (mFBP) has been identified as an alternative/additional transport route for (anti-)folate compounds [17,18,30-32]. However, by [^{3}H]-folic acid binding studies no detectable levels of mFBP expression were observed in one of the seven HNSCC cell lines (results not shown).

DHFR activity

Table 1 shows the specific activity of DHFR in the HNSCC cell lines grown in "standard" or "folate-conditioned" medium. DHFR activity in the cell lines grown in "standard" medium varied between 5 to 37 nmol FH_2/hr/mg protein. For two cell lines (SCC-11B and -14C) a 3-fold increase in DHFR activity was found after adapting the cell lines to grow in "folate-conditioned" medium. For one cell line (SCC-10A) DHFR activity was decreased 2-fold, and for the remainder of the cell lines no significant changes in DHFR activity were noted. Differences in MTX-sensitivity of HNSCC cells for short-term drug exposures (Fig. 3) could not be correlated with DHFR reductase activities in the different HNSCC cell lines.

FPGS activity

The specific activity of FPGS in HNSCC cell lines grown in "standard" and "folate-conditioned" medium is shown in Table 1. In the HNSCC cell lines with inherent resistance to MTX during short-term drug exposure (Fig. 3: SCC-10A, -14A, and -14C), FPGS activity was significantly lower (> 3-fold) as compared to HNSCC cells which were (moderately) sensitive to MTX: SCC-2 ($p < 0.4$), SCC-10B ($p < 0.001$), SCC-11B ($p < 0.01$) and SCC-22B ($p < 0.2$). Except for a 2-fold increase in SCC-22B cells, no significant changes in FPGS activity was observed in these latter cell lines after prolonged growth in "folate-conditioned" medium. Under these conditions, however, FPGS activity was increased in SCC-10A (2.6-fold), SCC-14A (3.4-fold) and SCC-14C cells (1.6-fold), although resistance to short-term MTX exposure is retained (Fig. 3).

DISCUSSION

Inherent resistance to short-term exposure of MTX has recently been reported by Pizzorno et al. [8] within a series of human squamous cell carcinomas of the head and neck. Impaired polyglutamylation of MTX, rather than changes in DHFR levels or alterations in membrane transport properties, was found to be the major defect in the resistant cells. In this study we have extended the studies by Pizzorno et al. by evaluating, in seven HNSCC cell lines, not only the sensitivity for MTX,

but for four other novel antifolates as well. In preclinical models, 10-EdAM has been shown to be superior to MTX as a result of enhanced uptake and polyglutamylation [22]. TMQ is potentially effective against MTX-transport defective cells since its transport is independent of the reduced folate/MTX carrier system [23,33]. In addition to DHFR, also TS and GAR-TFase are recognized as important target enzymes for folate antagonists. ICI-198,583 and DDATHF represent a new class of folate-based inhibitors of TS and GAR-TFase, respectively. The potency of both compounds has been correlated with their effective transport and polyglutamylation [10,13-15].

HNSCC cells in this study have been grown in "folate-conditioned" medium in order to mimic near physiological conditions with respect to extracelluar folate concentrations. However, also two other aspects have been considered in following this approach. First, it has been reported [24,29,34] that media containing high folate levels can have a down-regulatory effect on carrier-mediated transport of MTX, thus underestimating the role of this carrier system in the cytotoxic events of MTX. Secondly, prolonged culture of cells in media containing high folate concentrations may result in a down-regulation of synthesis of another class of (anti-)folate transport proteins, i.e. membrane-associated folate binding proteins (mFBP) [17,18,31,35]. mFBP expression in tumour cells can be of interest since it may be a very effective route for the uptake of folate-based TS and GAR-TFase inhibitors [36,37]. One particular example of mFBP expression in a cell line originated from the head and neck are KB cells, an epidermoid nasopharyngeal carcinoma cell line [30]. With respect to possible alternative/additional transport routes for folate antagonists, mFBP expression could not be observed in this series of HNSCC cell lines. Rather, in two cell lines (SCC-11B and -14C) a significant up-regulation of MTX-transport via the RF-carrier was noted, an effect which has also been reported previously for human and murine leukaemia cells grown in "folate-conditioned" medium [29,38]. We have recently reported that the increased [^{3}H]-MTX uptake in SCC-11B cells is due to a more efficient carrier functioning (increased rate of carrier-translocation) rather than an increased expression of carrier protein [21]. Antifolate drugs showed greater activity against HNSCC cells grown in "folate-conditioned" medium than for cells maintained in "standard" medium. This is likely due to the absence of medium/serum folates which can compete with antifolates for transport and intracellular target enzymes [39].

Among the group of seven HNSCC cell lines, three were identified exhibiting resistance to short-term (24 hours) incubations of MTX, but all were sensitive after long-term (seven days) drug exposure. There was no correlation with two possible determinants in MTX-resistance, altered membrane transport or changes in DHFR activity. The results shown in Table 1 suggest that a low FPGS activity may be involved in the resistant phenotype of SCC-10A, -14A and -14C cells. Impaired polyglutamylation of MTX has been recognized as a mechanism of acquired resistance to this drug *in vitro* [40]. Recent studies have indicated that MTX-resistance due to a low FPGS-activity may also be of importance in clinical resistance, particularly since this type of resistance has rapidly developed through short courses of repeated short-term exposure of different tumour cells to MTX

[6,9,41-43].

In order to improve selectivity of antitumour action and to challenge the problem of acquired or intrinsic resistance to MTX, drug research has focused on designing novel antifolates which are more efficiently transported and polyglutamylated or which have different target enzymes in folate metabolism [44-46]. The novel antifolates evaluated in this study (10-EdAM, ICI-198,583 and DDATHF) fulfil one or more of these criteria [9,11,15,47,48]. The results presented here (Fig. 4) suggest that, in short-term drug exposure and relatively low FPGS activity, these novel folate analogues can have an enhanced potency over MTX against HNSCC cells. These drugs may therefore be considered for further evaluation in the treatment of head and neck squamous cell carcinomas, either for first line therapy or in cases of intrinsic or acquired resistance to MTX. Studies by Galivan et al. [49,50] support the further testing of antifolate drug combinations, because they showed a synergistic growth inhibitory effect when a folate-based TS inhibitor was combined with a DHFR inhibitor. At this moment 10-EdAM has entered a phase II/III clinical trial for the treatment of advanced head and neck cancer [51]. Preliminary results suggest that this compound is at least as effective as MTX. DDATHF [52] and folate-based TS inhibitors [13,53,54] are also being tested in clinical trials, but their usefulness in the treatment of Head and Neck Cancer remains to be established.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Dutch Cancer Society (IKA 89-34).

REFERENCES

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. *Seminars in Oncology* 1988, **15**, 70-85.

3. Schornagel JH, McVie JG. The clinical pharmacology of methotrexate, a review. *Cancer Treat Rev* 1983, **10**, 53-75.

4. Frei III E, Rosowsky A, Wright JE, Cucchi CA, Lippke JA, Ervin TJ, Jolivet J, Haseltine WA. 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.

5. Rosowsky A, Wright JE, Cucchi CA, Lippke JA, Tantravahi R, Ervin TJ, Frei III E. Phenotypic heterogeneity in cultured human head and neck squamous cell carcinoma lines with low level methotrexate resistance. *Cancer Res* 1985, **45**, 6205-6212.

6. van der Laan BFAM, Jansen G, Kathmann I, Schornagel JH, Hordijk GJ. Mechanisms of acquired methotrexate resistance in a human squamous carcinoma cell line of the head and neck, grown in folate-conditioned medium. Effects of continuous and pulse exposure to methotrexate. *Eur J Cancer* 1991, **27**, 1274-1278.

7. 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, New York, Walter de Gruyter, 1990, 1089-1099.

8. 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.

9. 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.

10. 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.

11. Sirotnak FM, Schmid FA, Samuels LL, DeGraw JI. 10-Ethyl-10-deaza-aminopterin: structural design and biochemical, pharmacologic, and antitumor properties. *NCI Monogr* 1987, **5**, 127-131.

12. Harrap KR, Jackman AL, Newell DR, Taylor GA, Hughes LR, Calvert AH. Thymidylate synthase: a target for anticancer drug design. *Adv Enzyme Regul* 1989, **29**, 161-179.

13. Jackman AL, Newell DR, Jodrell DI, Taylor GA, Bishop JAM, Hughes LR, Calvert AH. *In vitro* and *in vivo* studies with 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolate (ICI-198,583), an inhibitor of thymidylate synthase. In: Curtius H-Ch, Ghisla S, Blau N, eds. *Chemistry and Biology of Pteridines 1989*. Berlin, New York, Walter de Gruyter, 1990, 1023-1026.

14. 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.

15. Moran RG, Baldwin SW, Taylor EC, Shih C. The 6S- and 6R-diastereomers of 5,10-dideaza-5,6,7,8,-tetrahydrofolate are equiactive inhibitors of *de novo* purine synthesis. *J Biol Chem* 1989, **264**, 21047-21051.

16. Henderson GB, Tsuji JM, Kumar HP. Characterisation of the individual transport routes that mediate the influx and efflux of methotrexate in CCRF-CEM human lymfoblastic cells. *Cancer Res* 1986, **46**, 1633-1638.

17. Jansen G, Kathmann I, Rademaker BC, Braakhuis BJM, Westerhof GR, Rijksen G, Schornagel JH. Expression of a folate binding protein in L1210 cells grown in low folate medium. *Cancer Res* 1989, **49**, 1959-1963.

18. 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.

19. 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. *Leukemia Research* 1989, **13**, 981-987.

20. Grénman R, Carey TE, McClatchey KD, Wagner JG, Pekkola-Heino K, Schwartz DR, Wolf GT, Lacivita LP, Ho L, Baker SR, Krause CJ, Lichter AS. *In vitro* radiation resistance among cell lines established from patients with squamous cell carcinoma of the head and neck. *Cancer* 1991, **67**, 2741-2747.

21. 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* 1991, **11**, 1265-1268.

22. Schmid FA, Sirotnak FM, Otter GM, DeGraw JI. New folate analogues of 10-deaza-aminopterin series: markedly increased anti-tumor activity of the 10-ethyl analogue compared to the parent compound and methotrexate against some human tumor xenografts in nude mice. *Cancer Treat Rep* 1985, **69**, 551-553.

23. 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.

24. 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.

25. 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.

26. 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.

27. 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.

28. Sobrero AF, Bertino JR. Endogenous thymidine and hypoxantine are a source of error in evaluating methotrexate cytotoxicity by clonogenic assays using undialyzed fetal bovine serum. *Int J Cell Cloning* 1986, **4**, 51-62.

29. 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.

30. Elwood PC, Kane MA, Portillo RM, Kolhouse JF. The isolation, characterisation, and comparison of the membrane-associated and soluble folate-binding proteins from human KB Cells. *J Biol Chem* 1986, **261**, 15416-15423.

31. 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.

32. Kamen BA, Capdevila A. Receptor-mediated folate accumulation is regulated by the cellular folate content. *Proc Natl Acad Sci USA* 1986, **83**, 5983-5987.

33. Lin JT, Bertino JR. Trimetrexate: a second generation folate antagonist in clinical trial. *J Clin Oncol* 1987, **5**, 2032-2040.

34. Sirotnak FM, Poser RE, Barrueco JR. Enhancement of folate analogue transport inward in L1210 cells during methotrexate therapy of leukemic mice: Evidence of the nature of the effect, possible host mediation, and pharmacokinetic significance. *Cancer Res* 1987, **47**, 5334-5339.

35. Kane MA, Elwood PC, Portillo RM, Antony AC, Najfeld V, Finley A, Waxman S, Kolhouse JF. Influence on immunoreactive folate-binding proteins of extracellular folate concentration in cultured human cells. *J Clin Invest* 1988, **81**, 1398-1406.

36. 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.

37. 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.

38. Yang CH, Sirotnak FM, Mines LS. Further studies on a novel class of genetic variants of the L1210 cell with increased folate analogue transport inward. *J Biol Chem* 1988, **263**, 9703-9709.

39. Galivan J, Nimec Z, Rotundo R. The characteristics and consequences of folate depletion in hepatoma cells *in vitro* by inhibition of dihydrofolate reductase. *Adv Enzyme Regul* 1988, **27**, 209-217.

40. Cowan KH, Jolivet J. A methotrexate-resistant human breast cancer cell line with multiple defects, including diminished formation of methotrexate polyglutamates. *J Biol Chem* 1984, **259**, 10793-10800.

41. Pizzorno G, Mini E, Corronnello M, McGuire JJ, Moroson BA, Cashmore AR, Dreyer RH, Lin JT, Mazzei T, Periti P, Bertino JR. 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.

42. Rumberger BG, Schmid FA, Otter GM, Sirotnak FM. Preferential selection drug therapy *in vivo* by edatrexate compared to methotrexate of resistant L1210 cell variants with decreased folylpolyglutamate synthetase activity. *Cancer Com* 1990, **2**, 305-310.

43. McCloskey DE, McGuire JJ, Russell CA, Rowan BG, Bertino JR, Pizzorno G, Mini E. Decreased folylpolyglutamate synthetase activity as a mechanism of methotrexate resistance in CCRF-CEM human leukemia sublines. *J Biol Chem* 1991, **266**, 6181-6187.

44. Sirotnak FM. Determinants of resistance to antifolates: biochemical phenotypes, their frequency of occurence and circumvention. *NCI Monogr* 1987, **5**, 27-35.

45. Bertino JR, Sobrero AF, Mini E, Moroson BA, Cashmore AR. Design and rationale for novel antifolates. *NCI Monogr* 1987, **5**, 87-91.

46. Moran RG, Colman PD, Rosowsky A. Structural requirements for the activity of antifolates as substrates for mammalian folylpolyglutamate synthetase. *NCI Monogr* 1987, **5**, 133-138.

47. Ratnam M, Marquardt H, Duhring JL, Freisheim JH. Homologous membrane folate binding proteins in human placenta: Cloning and sequence of a cDNA. *Biochemistry* 1989, **28**, 8249-8254.

48. Poser RG, Sirotnak FM, Chello PL. Differential synthesis of methotrexate polyglutamates in normal proliferative and neoplastic mouse tissues *in vivo*. *Cancer Res* 1981, **41**, 4441-4446.

49. Galivan J, Nimec Z, Rhee M. Synergistic growth inhibition of rat hepatoma cells exposed *in vitro* to N^{10}-propargyl-5,8-dideazafolate with methotrexate or the lipophilic antifolates trimetrexate or metoprine. *Cancer Res* 1987, **47**, 5256-5260.

50. Galivan J, Rhee MS, Johnson TB, Dilwith R, Nair MG, Bunni M, Priest DG. The role of cellular folates in the enhancement of activity of the thymidylate synthase inhibitor 10-propargyl-5,8-dideazafolate against hepatoma cells *in vitro* by inhibitors of dihydrofolate reductase. *J Biol Chem* 1989, **264**, 10685-10692.

51. Schornagel JH, Cappelaere P, Verwey J, Cognetti F, de Mulder PHM, Clavel M, Vermorken JB, Snow GB. A randomized phase II study of 10-ethyl-10-deaza-aminopterin (10-EdAM) and methotrexate (MTX) in advanced head and neck squamous cell cancer (AHNC), an EORTC study. *Proc Am Assoc Clin Oncol* 1989, **8**, 174.

52. Muggia F, Martin T, Ray M, Leichman CG, Grunberg S, Gill I, Moran RG, Dyke R, Grindey GB. Phase I study of weekly 5,10-dideazatetrahydrofolate (LY-264618, DDATHF-B). *Proc Am Assoc Clin Oncol* 1990, **9**, 74.

53. Vest S, Bork E, Hansen HH. A phase 1 evaluation of N^{10}-propargyl-5,8-dideazafolic acid. *Eur J Cancer* 1988, **24**, 201-204.

54. Cantwell BMJ, Macaulay V, Harris AL, Kane SB, Smith IE, Milstead RAV, Calvert AH. Phase II study of the antifolate N^{10}-propargyl-5,8-dideazafolic acid (CB3717) in advanced breast cancer. *Eur J Cancer* 1988, **24**, 733-736.

Chapter 6

EVALUATION OF A NEW ASSAY FOR MEASUREMENT OF FOLYLPOLYGLUTAMATE SYNTHASE ACTIVITY

Submitted for publication in *Cancer Lett*

Authors: B.F.A.M. van der Laan, G.R. Westerhof, G.A.M. Kathmann, G.J. Hordijk, J.H. Schornagel and G. Jansen.

ABSTRACT

Activity of folylpolyglutamate synthase (FPGS) was measured in extracts of a head and neck squamous cell carcinoma cell line. [^{3}H]-glutamate incorporated into methotrexate (MTX) as a substrate for FPGS was separated from free [^{3}H]-glutamate following an established procedure of DE-52 anion exchange chromatography and via a new method including a reversed phase Sep-Pack C_{18} cartridge. A good correlation was observed between both methods, but the Sep-Pack C_{18} assay has the advantage that it can be accomplished in less than 5 min, whereas the DE-52 procedure requires about 2 hours.

INTRODUCTION

Polyglutamylation plays an important role in folate metabolism and is also a determining factor in the effectiveness of folate analogues as cancer chemotherapeutic agents [1,2]. Naturally occuring folates and antifolate compounds like methotrexate (MTX) are converted intracellularly to polyglutamate forms. The enzyme responsible for this reaction, folylpolyglutamate synthase (FPGS), catalyzes in an ATP-dependent reaction the addition of multiple glutamyl residues in a γ-linkeage to the glutamate side chain of the (anti)folate molecule [3-6];

$$H_4PteGlu_n + \text{glutamate} + ATP \text{ ----> } H_4PteGlu_{n+1} + ADP + P_i$$

or

$$MTX\text{-}Glu_n + \text{glutamate} + ATP \text{ ----> } MTX\text{-}Glu_{n+1} + ADP + P_i$$

The elongation of the glutamate side chain with negatively charged glutamyl residues prevents a rapid efflux of (anti)folate compounds from the cell, therefore, polyglutamylation mediates the cellular retention of natural and antifolate compounds [2-6]. In addition, polyglutamate derivatives of folate analogues can be more potent inhibitors of key-enzymes in folate metabolism than monoglutamate forms [7-9]. In this regard, high cellular levels of FPGS have been correlated with tumour cell sensitivity to MTX, whereas impaired polyglutamylation due to low FPGS activity can result in resistance to this drug [2,10-13].

At this moment a number of assays for measuring FPGS activity have been described. The assays are based on the separation of free [^{3}H]-glutamate from [^{3}H]-glutamate incorporated into an (anti)folate substrate for FPGS via charcoal adsorption [6,14] or anion exchange chromatography [3-5]. Alternatively, Antonsson et al. [15] have developed an assay system that is based on the entrapment of FH_4-[^{3}H]-diglutamate in a macromolecular complex with fluorodeoxyuridylate and pure thymidylate synthase. We have evaluated a new procedure for determining FPGS activity using reverse phase chromatography with Sep-Pack C_{18} cartridges. These cartridges have been applied previously to clean-up methotrexate containing plasma

samples prior to HPLC analysis of this drug [16]. The results of this assay procedure were compared with one of the "standard" methods (DE-52 anion exchange chromatography).

MATERIALS AND METHODS

Chemicals

L-[2,3-^{3}H]-glutamic acid (25 Ci/mmol) was obtained from New England Nuclear (NET-395). Adenosine-5'-triphosphate was purchased from Boehringer Mannheim, FRG. DE-52 anion exchange cellulose was obtained from Whatman Biosystems Ltd, UK., and prepared according to the manufacturers' instructions. Sep-Pack C_{18} cartridges were from Millipore Waters Associates, Etten-Leur, The Netherlands. MTX was a gift from Pharmachemie, Haarlem, The Netherlands. RPMI-1640 medium and fetal calf serum were from Gibco, NY. All other chemicals were of the highest grade of purity available.

Cells

UM-SCC-11B cells, a cell line originated from a moderately differentiated human squamous cell carcinoma of the larynx, was kindly provided by Dr T.E. Carey, University of Michigan, Ann Arbor, U.S.A. The cells were grown as a monolayer at 37°C in a 5% CO_2 humidified atmosphere in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

FPGS activity was measured in cells which were harvested at sub-confluency by trypsinisation (0.25% trypsin/0.05% EDTA in phosphate buffered saline).

Enzyme assays

Crude extracts were prepared from a cell pellet of 15 x 10^6 UM-SCC-11B cells by suspending in 0.5 ml ice-cold extraction buffer (50 mM Tris-HCl, 20 mM KCl, 10 mM $MgCl_2$, 5 mM dithiotreitol, pH 7.6). Following sonication (3 x 5 sec with 30 sec intervals), a clear supernatant was obtained after centrifugation (4°C) at 50,000 *g* for 30 minutes. Protein content in this fraction was determined according to Bradford [17].

The FPGS assay mixture was prepared according to McGuire et al. [3,4] containing, in a final volume of 250 µl and at pH 8.4, 100 mM Tris-HCl (pH 8.85), 10 mM ATP (neutralized), 20 mM $MgCl_2$, 20 mM KCl, 10 mM dithiotreitol, 4 mM L-[^{3}H]-glutamate and 250 µM MTX as substrate for FPGS. One volume of [^{3}H]-glutamate (in 0.01 N HCl) was neutralized by one half volume of 150 mM Na-glutamate (pH 9.0) and then further diluted with 50 mM Na-glutamate (pH 7.5) to a specific acitivity of 5.5 cpm/pmol. Protein concentrations in the reaction mixture were 100-400 µg, and incubation times varied from 1h - 3h. The reactions were terminated by the addition of 1 ml ice-cold 5 mM L-glutamate (pH 7.5). Appropiate blanks were included consisting of reaction mixtures lacking MTX.

DE-52 anion exchange chromatography: Anion exchange chromatography to separate free [^{3}H]-glutamate from MTX-[^{3}H]-diglutamate was carried out essentially as described by McGuire et al. [3,4]. In short, a pasteur pipet is packed to 6 cm with DE-52 and equilibrated with column buffer (10 mM HCl pH 7.5, 110 mM NaCl and 2.5 mM dithiotreitol). After loading the sample, the column is washed with 20 ml column buffer (flow rate 15 ml/h). MTX-[^{3}H]-diglutamates were then eluted with 3 ml 0.1 N HCl.

Sep-Pack C_{18} cartridge: A 10 ml syringe with Luer end fitting is placed on top of a Sep-Pack C_{18} cartridge. Prior to sample application, the cartridge is pretreated with 10 ml of methanol and 10 ml 0.2 M NaAc (pH 5.5), respectively (flow rate: 20-30 ml/min) After loading of the sample solution the cartridge is washed successively with 10 ml 0.2 M NaAc (pH 5.5), 2.5 ml 0.1N NaOH and 3 ml 0.2 M NaAc (pH 5.5) (flow rate: 20-30 ml/min) to remove free [^{3}H]-glutamate. The FPGS reaction product MTX-[^{3}H]-diglutamate is then eluted with 3 ml of methanol.

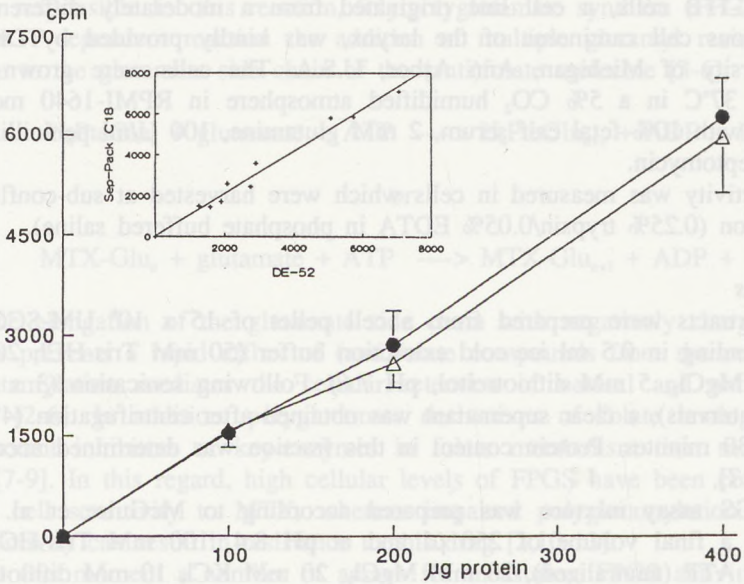

Fig. 1. DE-52 (-Δ-) and Sep-Pack (-•-) procedure for the assay of FPGS activity in extracts of UM-SCC-11B cells. FPGS activity is depicted as cpm of product formed as a function of sample protein. Incubation time: 2 hours. Inset: comparison of DE-52 assay and Sep-Pack assay. Parameters of linear regression analysis were: slope = 1.04 ± 0.05 (SE), intercept = 36, correlation coefficient = 0.99, n = 12.

RESULTS AND DISCUSSION

Extracts of a human head and neck squamous cell carcinoma cell line were incubated in duplicate and analyzed for FPGS activity via DE-52 anion exchange chromatography and Sep-Pack C_{18}. Fig. 1 shows for both methods a linear relationship for the incorporation of [^{3}H]-glutamate into the substrate MTX as a function the protein concentration in the assay mixture. The inset of Fig. 1 demonstrates a good correlation between the DE-52 and Sep-Pack C_{18} procedure. Linear regression analysis (using Statgraph computer program) indicates a slope of 1.04 ± 0.05 (SE) and a correlation coefficient of 0.99.

Linearity of [^{3}H]-glutamate incorporation was also observed by both methods as a function of incubation time (1-3 hours) (Fig. 2). Linear regression analysis of the correlation curve (inset Fig. 2) showed: slope = 0.93 ± 0.15 (SE), correlation coefficient = 0.89.

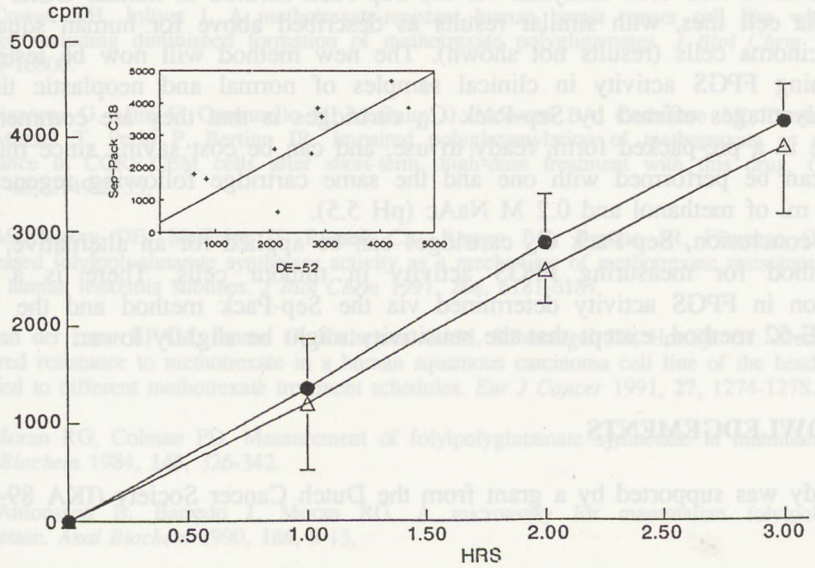

Fig. 2. DE-52 (-Δ-) and Sep-Pack (-•-) procedure for the assay of FPGS activity in extracts of UM-SCC-11B cells. FPGS activity is shown as cpm of product formed as a function of time. Sample protein content is 200 µg. Inset: comparison of DE-52 assay and Sep-Pack assay. Parameters for linear regression analysis were: slope = 0.93 ± 0.15 (SE), intercept = 306, correlation coefficient = 0.89, n = 12.

Sep-Pack C_{18} cartridges have been applied as a simple and rapid procedure to extract MTX from patients' serum samples during high dose infusions with this drug [16]. In this report we have evaluated whether the hydrophobic packing of the Sep-Pack C_{18} cartridge could be exploited to develop an alternative, simple and rapid assay for FPGS. A slight modification in the experimental conditions described by Buice et al. [16] allowed a selective retention of the (anti)folate substrate for FPGS on the Sep-Pack C_{18} cartridge whereas (hydrophilic) [^{3}H]-glutamate is rapidly eluted. The results were compared with the DE-52 anion exchange chromatography described by McGuire et al. [3,4], which is considered to be one of the standard methods for measuring FPGS activity. Fig. 1 and 2 indicate that there is a good correlation between the two methods. It should be noted, however, that the assay time for Sep-Pack procedure is much shorter (< 5 min) than for the DE-52 method (≈ 2 hours). In the 3 ml elution, the nonenzymatic background radioactivity observed after DE-52 chromatography was lower than for the Sep-Pack assay (315 ± 129 (SD) cpm/ml and 575 ± 119 (SD) cpm/ml, respectively). The slightly higher background as compared to DE-52 may limit the sensitivy of the Sep-Pack method to measure very low levels of FPGS activity, but for FPGS activity levels observed in the UM-SCC-11B cell line, the Sep-Pack method can be very useful. FPGS activities have also been analyzed via the Sep-Pack method in human CCRF-CEM leukaemia cell ines, with similar results as described above for human squamous cell carcinoma cells (results not shown). The new method will now be tested for determining FPGS activity in clinical samples of normal and neoplastic tissues. Other advantages offered by Sep-Pack C_{18} cartridges is that they are commercially available in a pre-packed form, ready to use, and can be cost saving since multiple assays can be performed with one and the same cartridge following regeneration with 10 ml of methanol and 0.2 M NaAc (pH 5.5).

In conclusion, Sep-Pack C_{18} cartridges can be applied for an alternative, rapid test method for measuring FPGS activity in tumour cells. There is a good correlation in FPGS activity determined via the Sep-Pack method and the "standard" DE-52 method, except that the sensitivity might be slightly lower.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Dutch Cancer Society (IKA 89-34).

REFERENCES

1. Schirch V, Strong WB. Interaction of folylpolyglutamates with enzymes in one-carbon metabolism. *Arch Biochem Biophys* 1989, **269,** 371-380.

2. Chabner BA, Allegra CJ, Curt GA, Clendeninn NJ, Baram J, Koizumi S, Drake JC, Jolivet J. Polyglutamylation of methotrexate. Is methotrexate a prodrug? *J Clin Invest* 1985, **76,** 907-912.

3. McGuire JJ, Hseih P, Coward JK, Bertino JR. Enzymatic synthesis of folylpolyglutamates. Characterization of the reaction and its products. *J Biol Chem* 1980, **255,** 5776-5788.

4. McGuire JJ, Hseih P, Coward JK, Bertino JR. *In vitro* methotrexate polyglutamate synthesis by rat liver polyglutamte synthetase and inhibition by bromosulfophtalein. In: Goldman ID, Chabner BA, Bertino JR, eds. *Folyl and antifolyl polyglutamates*. New York, London, Plenum press 1983, 199-214.

5. Johnson TB, Nair MG, Galivan J. Role of folylpolyglutamate synthetase in the regulation of methotrexate polyglutamate formation in H35 hepatoma cells. *Cancer Res* 1988, **48,** 2426-2431.

6. 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* 1985, **27,** 156-166.

7. Allegra CJ, Drake JC, Jolivet J, Chabner BA. Inhibition of phophoribosylaminoimidazole-carboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. *Proc Natl Acad Sci USA* 1985, **82,** 4881-4885.

8. Sikora E, Jackman AL, Newell DR, Calvert AH. Formation and retention and biological activity of N^{10}-propargyl-5,8-dideazafolic acid (CB3717) polyglutamates in L1210 cells *in vitro*. *Biochem Pharmacol* 1988, **37**, 4047-4054.

9. Pizzorno G, Sokoloski JA, Cashmore AR, Moroson BA, Cross AD, Beardsley GP. Intracellular metabolism of 5,10-dideazatetrahydrofolic acid in human leukemia cell lines. *Mol Pharmacol* 1991, **39**, 85-89.

10. Cowan KH, Jolivet J. A methotrexate-resistant human breast cancer cell line with multiple defects, including diminished formation of methotrexate polyglutamates. *J Biol Chem* 1984, **259**, 10793-10800.

11. Pizzorno G, Mini E, Coronnello M, McGuire JJ, Moroson BA, Cashmore AR, Dreyer RN, Lin JT, Mazzei T, Periti P, Bertino JR. 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.

12. McKloskey DE, McGuire JJ, Russell CA, Rowan BG, Bertino JR, Pizzorno G, Mini E. Decreased folylpolyglutamte synthetase activity as a mechanism of methotrexate resistance in CCRF-CEM human leukemia sublines. *J Biol Chem* 1991, **266**, 6181-6187.

13. van der Laan BFAM, Jansen G, Kathmann GAM, Schornagel JH, Hordijk GJ. Mechanisms of acquired resistance to methotrexate in a human squamous carcinoma cell line of the head and neck, exposed to different methotrexate treatment schedules. *Eur J Cancer* 1991, **27**, 1274-1278.

14. Moran RG, Colman PD. Measurement of folylpolyglutamate synthetase in mammalian tissues. *Anal Biochem* 1984, **140**, 326-342.

15. Antonsson B, Barredo J, Moran RG. A microassay for mammalian folylpolyglutamate synthetase. *Anal Biochem* 1990, **186**, 8-13.

16. Buice RG, Evans WE, Karas J, Nicholas CA, Sidhu P, Straughn AB, Meyer MC, Crom WR. Evaluation of enzyme immunoassay, radioassay, and radioimmunoassay of serum methotrexate, as compared with liquid chromatography. *Clin Chem* 1980, **26**, 1902-1904.

17. 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.

Chapter 7

GENERAL DISCUSSION AND CONCLUSIONS

CLINICAL ASPECTS

Cancer of the head and neck is, with respect to other tumours, a relatively rare disease and includes diseases of the oral cavity, pharynx, larynx, ear, salivary glands, nose and the paranasal sinuses. Over 95% of head and neck cancers are squamous cell carcinomas, principal exceptions are tumours of the salivary glands. Salivary gland tumours include pleomorphic adenomas, acinic cell tumours, mucoepidermoid carcinomas, adenoid cystic carcinomas and adenocarcinomas. Other histologic categories that occasionally pertain to the head and neck include sarcomas, melanomas, plasmacytomas and lymphomas.

Malignant tumours of the head and neck often present in middle-aged patients and the incidence increases with age, being maximal between the age of 70-80 years. For larynx carcinomas the maximum incidence is found between 60-70 years. The incidence of head and neck cancer in Europe is approximately 35 per 100.000 males and 5 per 100.000 females. In contrast, the incidence of lung cancer is 130 per 100.000 males and for malignant breast tumours 75 per 100.000 females [1].

One third of the patients with squamous cell carcinomas of the head and neck presents with localized disease which is curable by surgery or radiotherapy in the majority of cases. Survival rates are compromised, however, by the age and general condition of the patients and by the frequent occurrence of a second primairy malignancy, mostly carcinomas of the lung. Unfortunately, however, more than 60% of the patients presents with advanced disease [2]. The overall five year survival rate for this group of patients is still 25% or less. Although combining surgery and radiotherapy for treatment of stage III and IV disease has produced improvements in local and regional control [3,4], there is still an unacceptably high rate of local and distant failure [2]. Local recurrences occur in up to 60% of these patients and distant metastases develop in 20 to 30% [5]. The prognosis for patients with locally recurrent or metastatic head and neck cancer is very poor with a median survival of less than 6 months [6]. It would appear that the 5-year relative survival rate for larynx carcinomas in the southeast part of the Netherlands improved during the period 1975-1986 as compared with the period 1956-1958 (71% vs 50% respectively) [7]. Stage-adjusted survival rates, however, for T_3 laryngeal cancer in the Netherlands did not improve during the period 1975-1984 [8]. This means that the survival time of the patients did not increase, if the survival rate is corrected for stage of disease. Each year in the USA, among both sexes, 32% of patients diagnosed with head and neck cancer eventually die [9].

Surgery and radiotherapy are still the primary modalities of treatment for patients with head and neck cancer. However, it appears that this combination has reached a plateau. To improve the results of surgery and radiotherapy, combined approaches with chemotherapy or immunotherapy have been further explored. In contrast to the good results of chemotherapy in treatment of some other cancers such as lymphomas or testicular cancer, in which chemotherapy alone can frequently lead to cure, the addition of chemotherapy to surgery and radiotherapy failed to increase the five year survival rate in patients with head and neck cancer.

In fact the role of chemotherapy in the treatment of patients with head and neck cancer is still modest, at best [10]. This can only partly be explained by extensive previous treatment and/or declining physical and nutritional status, since squamous cell carcinomas of the head and neck usually have an initial good response to a variety of antineoplastic agents [5]. The most active drugs, used as monochemotherapy, are methotrexate (MTX), bleomycin and cisplatin. Since the initial response is good, systemic chemotherapy is an accepted treatment for palliation in many patients with locally recurrent disease or distant metastases. Chemotherapy is not only used as palliation in end-stage disease, but as neo-adjuvant or induction chemotherapy as well, by giving initial chemotherapy prior to surgery and/or radiotherapy. Unfortunately, however, there is no evidence that induction chemotherapy will improve the survival rate or sterilize peripheral margins of the tumour, which means that an inoperable patient will not become operable with neo-adjuvant chemotherapy [11]. More benefit might be expected from induction chemotherapy combined with radiotherapy [12], since a decrease in tumour load by induction chemotherapy should enhance the effect of radiotherapy. Stell and Rawson, however, were not able to find prolonged survival in a large group of patients treated with neo-adjuvant chemotherapy and radiotherapy [13]. In fact, the patients who received chemotherapy prior to radiotherapy fared somewhat worse than those treated with radiotherapy alone. Another possibility is adjuvant chemotherapy, where chemotherapy is used after local therapy to destroy the micro-metastases. Preliminary results are suggestive of a beneficial effect [11], but have to be confirmed with randomized prospective studies.

Systemic chemotherapy can be given as a single agent (monochemotherapy) or as multiple drug regimens (combination chemotherapy). The advantage of combination chemotherapy is that the combination of effective cytostatics, each with its specific mechanism of action, can increase the effect on tumour cells and thus delay the development of acquired resistance or overcome inherent drug resistance. A wide range of combination chemotherapy regimens has been used in patients with recurrent head and neck cancer [2]. The overall response rates (36-57%) appear to be higher than those for single agent chemotherapy (15-31%), but the differences in complete response were not statistically significant [5]. The duration of response and survival did not improve and none of the combination drug programs has yet proved to be superior to single agent chemotherapy in any randomized trial [14,15].

MTX is the drug with which most experience has been accumulated, but controversy still exists concerning the optimal dosage and schedule of administration. Initial reports suggested increased benefit, with overall remission percentages up to 60%, from a high dose (1.5 gr/m^2) continuous infusion of MTX during 6-24 hours, followed by leucovorin-rescue [16]. Studies with a randomized comparison between high dose and low dose MTX, however, never suggested that high dose methotrexate infusion had a better effect than the more conventional weekly standard dose of MTX [17-19]. A weekly intravenous schedule of 40-60 mg MTX/m^2 can produce average response rates of 40-50% without major toxicity [20]. Therefore, monochemotherapy with conventional low-dose methotrexate con-

tinues to be the standard treatment against which results with new agents and combinations should be compared.

PRECLINICAL ASPECTS

Although MTX has gained widespread acceptance as a potent antineoplastic drug, it is generally believed that the activity of folate antagonists in cancer chemotherapy can be further improved [21]. Important aspects in improving the selectivity of folate antagonists for tumour cells are optimal exploitation of the differences in membrane transport and in intracellular metabolism between normal and tumour cells. In a great number of tumour cells uptake of folates and antifolates proceeds via the high affinity/low capacity reduced folate carrier system, but in normal cells several alternative uptake routes may be taken (Chapter 2). A better understanding of the transport kinetic properties of the different folate transport proteins in normal and neoplastic cells or tissues may allow the development of strategies to manipulate these pathways for therapeutic purposes. The intracellular metabolization of folate antagonists to polyglutamate derivatives plays a critical role in the cytotoxicity and selectivity of antifolate compounds (Chapter 2). Polyglutamate forms, as compared to monoglutamate forms, have a prolonged intracellular retention and are also substantially better inhibitors of their target enzymes. Differences in polyglutamylation between normal and tumour cells may underly the selective rescue of normal cells by 5-formyl-THF [22]. It has been observed that MTX-polyglutamates are accumulated in higher quantities in tumour cells than in normal cells. The competitive displacement of MTX-polyglutamates from dihydrofolate reductase (DHFR) following 5-formyl-THF administration, resulting in a reactivation of the enzyme, will therefore be less in tumour cells than in normal cells.

A major problem that has limited the clinical use of folate antagonists is drug resistance. Neoplastic cells can exhibit natural resistance to MTX, or acquired resistance to MTX can be developed by primarily sensitive cells. The mechanism(s) involved in natural and acquired MTX resistance have been thoroughly studied in experimental tumour cell systems *in vitro* (Chapter 2). Some of these mechanisms were found to play a role in MTX-resistance *in vivo* as well. Drug resistant cell lines have been exploited as important tools in the development of novel antifolates. New drug development has included the synthesis of antifolates that were (a) more efficiently transported into the cell than MTX, (b) better substrates for folylpolyglutamate synthase (FPGS) than MTX, or (c) selected as inhibitors of enzymes other than DHFR. For example, in order to circumvent transport-related MTX resistance, lipophilic folate antagonists like TMQ have been designed that do not require a specific transport system for cell entry. Preclinical studies were promising, but in clinical trials, however, TMQ was only modestly active as an anticancer agent. Novel antifolates with better substrate affinities for FPGS may also improve therapeutic effectiveness. Besides DHFR, enzymes like TS and GAR-TFase are considered to be attractive targets for folate antagonists. At

this moment, for each of these enzymes, specific inhibitors are available which can meet one or more of the criteria described above [23-26].

An important topic throughout the studies was that HNSCC cells were grown in folate-conditioned media containing nanomolar concentrations of folates in order to mimic a more physiological situation. Under these conditions membrane transport of MTX was significantly increased in two HNSCC cell lines (Chapter 5). For one of these lines (SCC-11B), it could be demonstrated that an increased rate of carrier translocation is probably responsible for the up-regulation of transport (Chapter 3). These results indicate that for some cell lines membrane transport could play a more important role in MTX-cytotoxicity than could be anticipated from cell lines which are maintained in high folate-containing media. Although carrier-mediated transport is one of the most important routes for the uptake of antifolate drugs, studies by our laboratory, and that of others, have shown that a membrane-associated folate binding protein (mFBP) may be functional as an alternative transport route, either simultaneously with the carrier system, or as a major route when carrier-mediated transport is impaired or absent. mFBP is expressed in high quantities in a nasopharyngeal epidermoid carcinoma cell line (KB), so it was reasonable to assume that mFBP would also be expressed in other cell lines originating from squamous cell carcinomas of the head and neck. Despite the negative results for identifying mFBP in the seven HNSCC lines studied in chapter 5, or in MTX-resistant SCC-11B cells studied in chapter 4, the possibility of multiple transport routes for folate antagonists should be taken into consideration in future studies.

In chapters 4 and 5 we described studies on intrinsic and acquired MTX-resistance in HNSCC cells and determined to what extent novel antifolates were effective against resistant cells. The classical procedure for developing MTX resistance in tumour cells has always been via a continuous exposure to steadily increasing concentrations of MTX. Mostly, resistant cells obtained via this procedure are characterized by elevated levels of DHFR (Chapter 4). In contrast, a stable resistant phenotype could be obtained from SCC-11B cells following 11 cycles of short term exposure to 2 μM MTX (Chapter 4). The MTX concentration following pulse doses may be representative for plasma levels achieved in therapeutic schedules. Houwever, it should be realized that a 46-fold resistance to MTX (which was observed in this cell line) will be more than sufficient to allow recurrent tumour growth in a clinical setting. In other words, it is possible that a lower resistance factor *in vivo*, obtained after fewer MTX exposures, can still be of clinical importance. The fact that acquired resistance to MTX, as a result of a decreased activity of FPGS, has now been observed in human leukaemia cells and human HNSCC cells (Chapter 4), strongly suggests that this phenomenon could be an important mechanism in clinical resistance when patients are treated with short-term exposures to MTX. This hypothesis is further supported by studies described in chapter 5. Low levels of FPGS activity in three HNSCC cell lines could be correlated with inherent MTX resistance in short-term exposure schedules. It should be noted that during long-term MTX exposures FPGS activity is of lesser importance since all seven HNSCC lines (Chapter 5) and 11B-MTX-P cells

(Chapter 4) were sensitive to MTX following a 7 day exposure with this drug.

Of considerable interest is the observation that some of the novel antifolates, like 10-EdAM, ICI-198,583 and DDATHF, demonstrate good activity against HNSCC cells and MTX-resistant SCC-11B cells, both after short-term and after long-term exposure to these drugs. The sensitivity, however, is variable and is dependent on the drug exposure time. These results indicate that these novel antifolates can be of great interest in the treatment of patients with recurrent tumour growth due to resistance to MTX. Another use can be the combination of novel antifolates with MTX to minimize the formation of cellular drug resistance in the future.

FUTURE STUDIES

In this thesis initial studies have been described investigating the activity of novel folate antagonists on MTX-sensitive and -resistant HNSCC cell lines *in vitro*. Special attention was paid to folate conditions of the medium and the role of membrane transport and polyglutamylation. Future studies must be designed to confirm the *in vitro* results with HNSCC cells grown as xenografts in athymic nude mice. So far, however, studies by Braakhuis et al. [27,28], have failed to demonstrate any effect of MTX on HNSCC xenografts, but antitumour activity could be observed with 10-EdAM [29]. Studies on xenografts including HNSCC cells grown in folate-conditioned medium which are treated with different schedules of ICI-198,583 or DDATHF, can provide new insight into the possible therapeutic effectiveness of these new drugs in head and neck cancer. In this respect it may also be worthwhile to study the effect of antifolate drug combinations. Galivan et al. [30,31] have recently provided a biochemical basis for synergistic growth inhibitory effects with a combination of a folate-based TS inhibitor and DHFR inhibitors (MTX or TMQ). Likewise, Ferguson et al. [32] demonstrated synergy between DDATHF and MTX in the treatment of mice inoculated with L1210 leukaemia cells. These drug combinations may also be of great importance in circumventing either acquired or intrinsic MTX resistance, as suggested by the results in chapter 5.

Future studies should particularly focus on the identification of factors known to determine tumour cell sensitivity or resistance to folate antagonists in biopsy material from patients with advanced squamous cell carcinomas of the head and neck. Although the enzymatic activity of FPGS (Chapter 6), TS and DHFR can be determined in extracts of HNSCC cell lines, more sensitive analysis techniques will be required to detect low levels of activity in tumour biopsies. Polymerase chain reaction assays (PCR) might be helpful for these studies. Dolnick et al. [33] have very recently described a PCR assay system for the detection of TS- and DHFR-mRNA levels in colorectal cells and KB cells which may be applicable for HNSCC cells as well. Similar techniques may soon become available for FPGS which has recently been cloned [34]. Unfortunately, at this moment, no molecular probes are available for detection of the reduced folate transport protein.

CONCLUSIONS

1. Membrane transport of (anti-)folates in HNSCC cell lines mainly proceeds via the reduced folate/MTX carrier system. mFBP as an alternative folate transport route was not detectable in seven HNSCC cell lines adapted to grow in folate-conditioned medium, or in HNSCC cell lines with acquired resistance to MTX.

2. In HNSCC cell lines with acquired MTX-resistance following MTX pulse exposure schedules, low FPGS activity was found to be the main mechanism of resistance to MTX. In contrast, increased DHFR levels were found in HNSCC cell lines made resistant to MTX after gradually increasing the drug concentration in the culture medium.

3. After short-term MTX exposure, inherent resistance to MTX was detected in three out of seven HNSCC cell lines.

4. HNSCC cell lines with inherent and/or acquired resistance to MTX were more sensitive to novel antifolates, e.g.: 10-EdAM, ICI-198,583 and DDATHF, with another target enzyme, better membane transport and/or better FPGS affinities.

REFERENCES

1. de Boer J, Derom F, Gruwez JA, Kuijjer PJ, den Otter G, Zwaveling A, eds. *Leerboek chirurgie*. Utrecht/Antwerpen, Bohn, Scheltema & Holkema, 1988.

2. Glick JH, Zehngebot LM, Taylor SG. Chemotherapy for squamous cell carcinoma of the head and neck: a progress report. *Am J Otolaryngol* 1980, **1**, 306-323.

3. Arriagda R, Eschwege F, Cachin Y, Richard JM. The value of combining radiotherapy with surgery in the treatment of hypopharyngeal and laryngeal cancers. *Cancer* 1983, **51**, 1819-1825.

4. Terhaard CHJ, Wiggenraad RG, Hordijk GJ, Ravasz LA. Regression after 50 Gy as a selection for therapy in advanced laryngeal cancer. *Int J Radiation Oncol Biol Phys* 1988, **15**, 591-597.

5. Al-Sarraf M. Head and Neck Cancer: Chemotherapy Concepts. *Seminars in Oncology* 1988, **15**, 70-85.

6. Stell PM. Survival times in end-stage head and neck cancer. *Eur J Surg Oncol* 1989, **15**, 407-410.

7. Coebergh JWW, Verhagen-Teurlings MTh, Crommelin MA, van der Heijden LH, Hop WCJ. De overlevingskansen bij patiënten met kanker gediagnostiseerd in 1975-1985 in Zuidoost-Noord-Brabant en Noord-Limburg. *Ned Tijdschr Geneeskd* 1991, **135**, 938-943.

8. Terhaard CHJ, Hordijk GJ, van der Broek P, de Jong PC, Snow GB, Keus RB, Annyas AA, Tjho-Heslinga RE, de Jong JMA. T_3 laryngeal cancer: a retrospective study of the dutch head and neck oncology cooperative group: study design and general results. (Submitted for publication)

9. Blitzer PH. Epidemiology of head and neck cancer. *Seminars in Oncology* 1988, **15**, 2-9.

10. Tannock IF, Browman G. Lack of evidence for a role of chemotherapy in the routine management of locally advanced head and neck cancer. *J Clin Oncol* 1986, **4**, 1121-1126.

11. Schuller DE. Do otolaryngologist-head and neck surgeons and/or chemotherapy have a role in the treatment of head and neck cancer? *Arch Otolaryngol Head Neck Surg* 1991, **117**, 498-501.

12. Snow GB, Vermorken JB. Neo-adjuvant chemotherapy in head and neck cancer: state of the art, 1988. *Clin Otolaryngol* 1989, **14**, 371-375.

13. Stell PM, Rawson NSB. Adjuvant chemotherapy in head and neck cancer. *Br J Cancer* 1990, **61**, 779-787.

14. Vogl SE, Schoenfeld DA, Kaplan BH, Lerner HJ, Engstrom PF, Horton J. A randomized prospective comparison of methotrexate with a combination of methorexate, bleomycin and cisplatin in head and neck cancer. *Cancer* 1985, **56**, 432-442.

15. Williams SD, Velez-Garcia E, Essessee I, Ratkin G, Birch R, Einhorn LH. Chemotherapy for head and neck cancer. Comparison of cisplatin + vinblastine + bleomycin versus methotrexate. *Cancer* 1986, **57**, 18-23.

16. Levitt M, Mosher MB, DeConti RC, Farber LR, Skeel RT, Marsh JC, Mitchell MS, Papac RJ, Thomas ED, Bertino JR. Improved therapeutic index of methotrexate with "leucovorin rescue". *Cancer Res* 1973, **33**, 1729-1734.

17. DeConti RC, Schoenfeld D. A randomized prospective comparison of intermittent methotrexate, methotrexate with leucovorin and a methotrexate combination in head and neck cancer. *Cancer* 1981, **48**, 1061-1072.

18. Woods RL, Fox RM, Tattersall MHN. Methotrexate treatment of squamous-cell head and neck cancers: dose-response evaluation. *Br Med J* 1981, **282**, 600-602.

19. Taylor SG, McGuire WP, Hauck WW, Showel JL, Lad TE. A randomized comparison of high-dose infusion methotrexate versus standard-dose weekly therapy in head and neck cancer. *J Clin Oncol* 1984, **2**, 1006-1011.

20. Hong WK, Bromer R. Chemotherapy in head and neck cancer. *N Engl J Med* 1983, **308**, 75-79.

21. Bertino JR. Folate antagonists: toward improving the therapeutic index and development of new analogs. *J Clin Pharmacol* 1990, **30**, 291-295.

22. Matherly LH, Barlowe CK, Phillips VM, Goldman ID. The effects on 4-aminoantifolates on 5-formyltetrahydrofolate metabolism in L1210 cells (a biochemical basis of the selectivity of leucovorin rescue). *J Biol Chem* 1987, **262**, 710-717.

23. Bertino JR, Sobrero AF, Mini E, Moroson BA, Cashmore AR. Design and rationale for novel antifolates. *NCI Monogr* 1987, **5**, 87-91.

24. Sirotnak FM. Determinants of resistance to antifolates: biochemical phenotypes, their frequency of occurence and circumvention. *NCI Monogr* 1987, **5**, 27-35.

25. Huennekens FM, Duffy TH, Vitols KS. Folic acid metabolism and its disruption by pharmacologic agents. *NCI Monogr* 1987, **5**, 1-8.

26. Baldwin SW, Tse A, Gossett LS, Taylor EC, Rosowsky A, Shih C, Moran RG. Structural features of 5,10-dideaza-5,6,7,8-tetrahydrofolate that determine inhibition of mammalian glycinamide ribonucleotide formyltransferase. *Biochemistry* 1991, **30**, 1997-2006.

27. Braakhuis BJM, Schoevers EJ, Heinerman ECM, Sneeuwloper G, Snow GB. Chemotherapy of human head and neck cancer xenografts with three clinically active drugs: cis-platinum, bleiomycine and methotrexate. *Br J Cancer* 1983, **48**, 711-716.

28. Braakhuis BJM, Leyva A, Schoevers EJ, Boerrigter GH, Schornagel JH, Snow GB. Lack of effect of methotrexate on human head and neck tumors transplanted in athymic nude mice. *Acta Otolaryngol* 1985, **99**, 208-213.

29. Brown DH, Braakhuis BJM, van Dongen GAMS, van Walsum M, Bagnay M, Snow GB. Activity of the folate analog 10-ethyl,10-deaza-aminopterin (10-EdAM) against human head and neck cancer xenografts. *Anticancer Res* 1989, **9**, 1549-1552.

30. Galivan J, Nimec Z, Rhee M. Synergistic growth inhibition of rat hepatoma cells exposed *in vitro* to N^{10}-propargyl-5,8-dideazafolate with methotrexate or the lipophilic antifolates trimetrexate or metoprine. *Cancer Res* 1987, **47**, 5256-5260.

31. Galivan J, Rhee MS, Johnson TB, Dilwith R, Nair MG, Bunni M, Priest DG. The role of cellular folates in the enhancement of activity of the thymidylate synthase inhibitor 10-propargyl-5,8-dideazafolate against hepatoma cells *in vitro* by inhibitors of dihydrofolate reductase. *J Biol Chem* 1989, **264**, 10685-10692.

32. Ferguson K, Boschelli D, Hoffman P, Oronsky AL, Whiteley J, Webber S, Galivan J, Freisheim JH, Hynes J, Kewar SS. Synergy between 5,10-dideaza-5,6,7,8-tetrahydrofolic acid and methotrexate in mice bearing L1210 tumors. *Cancer Chemother Pharmacol* 1989, **25**, 173-176.

33. Dolnick BJ, Zhang ZG, Rustum Y. Quantitative polymerase chain reaction assays for human thymidylate synthase and dihyrofolate reductase mRNA. *Proc Am Assoc Cancer Res* 1991, **32**, 327.

34. Kan JL, Baldwin SW, Taylor SM, Moran RG. Molecular cloning of mouse glycinamide ribonucleotide formyltransferase (GART). *Proc Am Assoc Cancer Res* 1991, **32**, 325.

SUMMARY

This thesis describes a study of the mechanisms of cellular resistance to the folate analogue methotrexate (MTX) and the sensitivity to novel antifolates in human squamous carcinoma cell lines of the head and neck (HNSCC). The aim of this study has been to elucidate the role of cellular membrane transport in MTX cytotoxicity, to investigate the mechanisms of acquired and/or inherent resistance to MTX and to evaluate the sensitivity of HNSCC cell lines to novel antifolates. For this purpose seven human squamous carcinoma cell lines originating from different tumours of the head and neck were studied. An important aspect of these *in vitro* studies was the attempt to mimic a clinical situation by: (a) adapting HNSCC cell lines to grow in a culture medium with a reduced folate concentration within the physiological plasma concentration range and (b) exposing HNSCC cell lines to MTX pulse schedules rather than to continuous drug exposures.

Chapter 2 gives an overview of the literature concerning possible routes for membrane transport of MTX, the intracellular metabolism of MTX and the different mechanisms of cellular resistance to MTX. In addition, membrane transport and intracellular metabolism of some of the novel antifolates used in this study are discussed. These novel antifolates include either inhibitors of dihydrofolate reductase (DHFR), such as MTX, or inhibitors of other key-enzymes in folate metabolism.

In Chapter 3, membrane transport characteristics of the folate analogue MTX were studied in an HNSCC cell line adapted to grow in tissue culture media containing nanomolar concentrations reduced folates. Usually, studies of MTX membrane transport are carried out in standard tissue culture media containing 2-10 μM of folic acid, whereas plasma reduced folate levels vary between 5-50 nM. The "low folate" HNSCC cell line exhibited a 10.5-fold increase of (anti)-folate transport via the reduced folate/MTX carrier system, which was mainly due to more efficient carrier function and only to a minor extent (15-20%) due to an increase in the amount of carrier protein.

Chapter 4 describes the mechanisms of acquired resistance to MTX in an HNSCC cell line exposed to different MTX treatment schedules. Cells which were grown in continuously increasing MTX concentration (11B-MTX-C cells) were 91-fold resistant to MTX and 30-fold or 49-fold cross-resistant to trimetrexate (TMQ) and 10-EdAM, respectively. DHFR activity was increased 63-fold in 11B-MTX-C cells, together with a decrease in [^{3}H]-MTX transport and folylpolyglutamate synthase (FPGS) activity (2.5- and 3.8-fold, respectively). Against two novel anti-folates, targetting enzymes other than DHFR, minor cross-resistance was observed for ICI-198,583, but full sensitivity was retained for DDATHF.

If cells were made resistant by MTX pulse exposures (11B-MTX-P cells), they were 46-fold resistant to MTX and 47-fold cross-resistant to ICI-198,583 after short-term drug exposure, but showed only minor changes in MTX sensitivity following prolonged drug exposure. The resistant 11B-MTX-P cells were characterized by a 5.6-fold decrease in FPGS activity.

Chapter 5 describes the occurence of inherent resistance to MTX in three out of seven HNSCC cell lines when MTX exposure is limited to 24 hours instead of seven days. The mechanism of inherent resistance was associated with a 3-fold reduction in intracellular FPGS activity in comparison with MTX sensitive HNSCC cells. The three cell lines exhibiting inherent resistance to MTX, however, retained sensitivity to one or more of the three novel antifolates (10-EdAM, ICI-198,583 and DDATHF) after short term drug exposure.

In Chapter 6 the activity of FPGS was measured in extracts of an HNSCC cell line. Two different methods were compared, an established procedure of DE-52 anion exchange chromatography and a new method consisting of a reversed phase chromatography via a Sep-Pack C_{18} cartridge. Good correlation was observed between both methods, but the Sep-Pack C_{18} assay has the advantage of being accomplished in less than 5 min, whereas the DE-52 procedure requires about 2 hours.

Finally, in Chapter 7 clinical and preclinical aspects of HNSCC sensitivity or resistance to antifolates are discussed. To increase the effectiveness of chemotherapy in patients with HNSCC, some suggestions are given for future studies of mechanisms of tumour cell resistance to antifolates *in vivo*.

SAMENVATTING

In dit proefschrift wordt een onderzoek besproken naar de mechanismen van cellulaire resistentie tegen het antifolaat methotrexaat (MTX) en naar de gevoeligheid voor nieuwe antifolaten in humane plaveiselcelcarcinoom-cellijnen uit het hoofd-halsgebied (HNSCC). De bedoeling van dit onderzoek was om: (1) de rol van cellulair membraantransport bij MTX cytotoxiciteit te belichten, (2) de mechanismen van verworven en/of natuurlijke resistentie tegen MTX te onderzoeken en (3) de gevoeligheid van HNSCC cellijnen voor nieuwe antifolaten te bestuderen.

Met dit doel werden zeven humane plaveiselcelcarcinoom-cellijnen bestudeerd, die oorspronkelijk afkomstig waren uit verschillende tumoren in het hoofd-halsgebied. Een belangrijk uitgangspunt in deze *in vitro* onderzoeken was om een meer klinische situatie na te bootsen door: (a) HNSCC cellijnen langzaam aan te laten passen aan een groeimedium met een lage folaatconcentratie, die de fysiologische plasma concentratie benadert, (b) HNSCC cellijnen bloot te stellen aan een gepulseerde toediening van MTX, in plaats van aan een continue aanwezigheid van MTX, in het groeimedium.

In hoofdstuk 2 wordt een overzicht gegeven van de literatuur omtrent de mogelijke routes van membraantransport van MTX, de intracellulaire stofwisseling van MTX en de verschillende mechanismen van cellulaire resistentie tegen MTX. Vervolgens worden membraantransport en intracellulaire stofwisseling besproken van enkele nieuwe antifolaten die gebruikt zijn in dit onderzoek. Nieuwe antifolaten zoals trimetrexaat (TMQ) en 10-EdAM blokkeren, net als MTX, het enzym dihydrofolaat reductase (DHFR), maar ICI-198,583 en DDATHF blokkeren andere sleutel-enzymen binnen de folaatstofwisseling.

In hoofdstuk 3 worden membraantransport karakteristieken bestudeerd van MTX in een HNSCC cellijn die langzaam aan gewend is te groeien in een kweekmedium met nanomolair gereduceerde-folaatconcentraties. Gewoonlijk worden proeven voor MTX membraantransport uitgevoerd in standaard groeimedia die 2-10 µM foliumzuur bevatten, gereduceerde folaatconcentraties in plasma daarentegen variëren tussen de 5-50 nM. De "laag-folaat" HNSCC cellijn liet een tienvoudige toename van (anti)-folaat transport via het gereduceerde folaat/MTX transportsysteem zien, hetgeen hoofdzakelijk veroorzaakt werd door een efficiënter transport en in mindere mate (15-20%) door een toename van de hoeveelheid transporteiwit.

In hoofdstuk 4 worden de mechanismen beschreven van verworven resistentie tegen MTX in een HNSCC cellijn wanneer die is blootgesteld aan verschillende MTX behandelschema's. In cellen die gekweekt werden in een continu toenemende MTX concentratie (11B-MTX-C cellen) werd de resistentie tegen MTX 91 maal zo groot, de kruisresistentie tegen TMQ en 10 EdAM werd 30- respectievelijk 49 maal zo groot. De DHFR activiteit in 11B-MTX-C cellen was 63 maal toegenomen, terwijl er tevens een afname in [3H]-MTX transport en de folylpolyglutamaat synthase (FPGS) activiteit optrad van (respectievelijk 2.5- en 3.8

maal). Bij 2 nieuwe antifolaten, die andere doel-enzymen hebben dan DHFR, werd een lichte kruisresistentie waargenomen tegen ICI-198,583, maar de 11B-MTX-C cellen bleven volledig gevoelig voor DDATHF.

Als cellen resistent werden gemaakt door gepulseerde MTX toediening (11B-MTX-P cellen), bleken zij 46 maal resistent tegen MTX en 47 maal kruisresistent tegen ICI-198,583 bij kortdurende toediening van deze middelen, maar lieten slechts een kleine verandering in de MTX-gevoeligheid zien bij langdurige toediening. De resistente 11B-MTX-P cellen werden gekarakteriseerd door een afname in FPGS activiteit (5.6 maal) ten opzichte van de moedercellijn.

Hoofdstuk 5 beschrijft het voorkomen van natuurlijke resistentie tegen MTX in drie van de zeven HNSCC cellijnen, wanneer de duur van de MTX toediening wordt beperkt tot 24 uur (in plaats van 168 uur). Het mechanisme van de natuurlijke resistentie was gerelateerd aan een drievoudige verlaging van de intracellulaire FPGS activiteit. De drie cellijnen met een natuurlijke resistentie tegen MTX behielden echter de gevoeligheid voor één of meer van de drie nieuwe antifolaten (10-EdAM, ICI-198,583 en DDATHF) bij kortdurende toediening van deze middelen.

In hoofdstuk 6 worden de meetresultaten belicht van de activiteit van FPGS gemeten in een HNSCC cellijn. Twee verschillende meetmethoden worden vergeleken, een bestaande methode met DE-52 anion uitwisseling chromatografie en een nieuwe methode met een Sep-Pack C_{18} patroon, die berust op omgekeerde fase chromatografie. Er werd een goede correlatie geconstateerd tussen de twee methoden, waarbij de Sep-Pack C_{18} test het voordeel heeft, dat de analysetijd minder dan 5 minuten duurt, dit in tegenstelling tot de DE-52 procedure die ongeveer 2 uur in beslag neemt.

Tot slot worden in hoofdstuk 7 de klinische en pré-klinische aspecten van de gevoeligheid vóór en de resistentie tegen antifolaten in HNSCC besproken. Om in de toekomst de effectiviteit van chemotherapie bij de behandeling van patiënten met HNSCC te kunnen vergroten, worden enige suggesties gedaan voor vervolgstudies naar cellulaire resistentievorming tegen antifolaten *in vivo*.

DANKWOORD

Een proefschrift komt mede tot stand door de morele steun van vele mensen in de naaste omgeving van de promovendus. Allen die op enigerlei wijze hebben bijgedragen aan de totstandkoming van dit proefschrift wil ik bij deze hartelijk danken. Graag wil ik mijn waardering uitspreken voor enkele personen in het bijzonder:

Prof. Dr. G.J. Hordijk, uw enthousiasme en heldere inbreng zijn een grote stimulans voor mij geweest. De besprekingen die wij voerden over de resultaten van het onderzoek waren steeds de drijfveer om mijn daden om te zetten in woorden. Alle stukken tekst werden door u aandachtig bestudeerd en gecorrigeerd, ook al was mijn opbrengst in het begin niet meer dan een alinea per bijeenkomst.

Dr. J.H. Schornagel, beste Jan, jouw klinische inbreng en je ervaring met de chemotherapie waren onmisbaar voor de voortgang van dit onderzoek. Zonder jouw buitengewone kennis van de engelse taal was dit proefschrift slechts een "tante Betje" verhaal geworden.

Dr. G. Jansen, beste Gerrit, je gedegen kennis van de biochemie en in het bijzonder van het folaat metabolisme was essentieel voor de vormgeving van dit onderzoek. Ik bewonder je fenomenaal geheugen en je vermogen om te allen tijde zowel kwalitatief als kwantitatief een goed stuk werk te leveren; tevens ben ik je erkentelijk voor de manier waarop je mijn onderzoek hebt gestuurd en begeleid.

Ietje Kathmann, van je nauwgezette kweektechnieken heb ik veel geleerd, maar zonder jouw "gouden handjes" en je liefde voor de cellijnen had ik mijn laboratoriumwerk niet kunnen afwisselen met het werk in de kliniek.

Robbin Westerhof, je humor (o ké) en je bereidwillige assistentie in moeilijke tijden waren een stimulans. "We werkten ons te pletter samen"!

Hans Claessen, de prettige sfeer gedurende de gezamenlijke zaaltijd was het begin van onze vriendschap. De interesse die je altijd had voor het wel en wee in zowel de werk- als de persoonlijke sfeer is voor mij met name de laatste maanden van grote betekenis geweest.

Jacobien, het is niets nieuws dat we weinig samen hebben kunnen ondernemen dit laatste jaar, want dat overkomt iedere partner vlak voor een promotie. Wat ik zo in je heb gewaardeerd was het geduld en je gave om me telkens maar weer te stimuleren om door te gaan. Ik ben je daar zeer dankbaar voor.

CURRICULUM VITAE

Bernardus Franciscus Augustinus Maria van der Laan werd geboren op 23 mei 1959 te Laren (N.H.). In 1978 behaalde hij zijn eindexamen Atheneum B aan het St. Vitus college te Bussum. In datzelfde jaar begon hij met zijn studie geneeskunde aan de Rijksuniversiteit Utrecht, waar hij in 1985 zijn artsexamen behaalde.

Van mei 1986 tot eind maart 1987 was hij werkzaam als arts-assistent in het Antoni van Leeuwenhoek Ziekenhuis te Amsterdam, op de afdelingen hoofd-hals oncologie en buikchirurgie.

In april 1987 begon hij zijn opleiding tot Keel-, Neus- en Oorarts in het Academisch Ziekenhuis Utrecht (Opleiders: Prof. Dr. E.H. Huizing en Prof. Dr. G.J. Hordijk). Tijdens deze opleiding heeft hij als analist gewerkt, onder leiding van Dr. G. Jansen, op het anti-folaat laboratorium (Hoofd: Dr. J.H. Schornagel) van de afdeling Interne Geneeskunde, werkgroep Oncologie, Academisch Ziekenhuis Utrecht, wat uiteindelijk heeft geleid tot dit proefschrift. Tijdens het onderzoek werd het anti-folaat laboratorium verplaatst naar het Academisch Ziekenhuis der Vrije Universiteit Amsterdam.

Op 1 april 1992 zal hij de opleiding tot Keel-, Neus- en Oorarts voltooien. Daarna is hij voor een periode van twee jaar houder van een klinisch fellowship van het Koningin Wilhelmina Fonds om zich te bekwamen in de hoofd-hals oncologie. De auteur is getrouwd met Jacobien Bresser.

Patient characteristics, tumour site, and histologic characterisation of the specimens used to establish the SCC cell lines.

Cell line	Sex/Age	Prior therapy	Specimen site	Type of lesion	Histologic Characterisation
UM-SCC-2	F/64	Surgery	Alveolus	Locally recurrence	Well differentiated SCC
UM-SCC-10A	M/57	None	Larynx	Primary tumour	Mod. to well diff. SCC
-10B		None	Neck	Metastasis	Mod. to well diff. SCC
UM-SCC-11B	M/65	Chemotherapy	Supraglottic larynx	Primary tumour	SCC
UM-SCC-14A	F/58	Surgery/Radiation	Floor of mouth	Locally recurrence	Poorly to mod. well diff.
-14C		Surg/Rad/Chemo	Floor of mouth	Locally recurrence	Poorly diff. SCC
UM-SCC-22B	F/59	None	Neck	Metastasis	Mod. to well diff. SCC

SCC: squamous cell carcinoma; Mod: moderately; Diff: differentiated. (Table is reprinted from: Grénman et al. *Cancer* 1991, **67**, 2741-2747)