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IMPORTANCE OF PHARMACODYNAMICS IN THE *IN VITRO* ANTIPROLIFERATIVE ACTIVITY OF THE ANTIFOLATES METHOTREXATE AND 10-ETHYL-10-DEAZAAMINOPTERIN AGAINST HUMAN HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Abstract—The pharmacodynamic profiles of methotrexate (MTX) and 10-ethyl-10-deazaaminopterin (10-EdAM) were determined in three head and neck squamous cell carcinoma (HNSCC) cell lines. Cell growth inhibition was tested using a semi-automated 96-well based proliferation assay, the sulforhodamine B (SRB) assay. Drug concentrations ranged from 10^{-5} to 10^{-9} M, with exposure periods of 4, 24, 48, 72 and 96 hr. The SRB-test was performed after each of these periods of continuous exposure and after an additional period of 24 and 48 hr in drug-free medium. Without a drug-free period the IC_{50} values strongly depended on the time of exposure. For example, with respect to MTX, IC_{50} values at 24 hr ranged from 2.9 (UM-SCC-14C) to over 10 μM (UM-SCC-22B and -11B), but when exposed continuously for 96 hr, IC_{50} values varied between 0.039 and 0.1 μM. 10-EdAM followed a similar sensitivity pattern with 5–20-fold lower IC_{50} values. The minimal time to achieve significant growth inhibition varied between the cell lines, <24 hr for UM-SCC-14C, >24 and >48 hr for UM-SCC-11B and -22B, respectively. The cell lines also varied with respect to growth behaviour when placed in drug-free medium for an additional period. Growth of UM-SCC-14C cells was recovered significantly after removing the drug, whereas UM-SCC-22B showed a different pattern: when cultured for over 48 hr, cell growth was strongly inhibited, independent of the drug being removed. This variable pattern of sensitivity could be correlated with the capacity of the cells to form polyglutamate derivatives. After 24 hr, drug accumulation was at least three times lower in UM-SCC-14C than in both other cell lines. The low level of antifolate accumulation in UM-SCC-14C is in line with the recovery from growth inhibition at culture in drug-free medium, while the persistent growth inhibition observed in UM-SCC-22B agrees with the intracellular accumulation of higher polyglutamates. In conclusion, these experiments show that the pharmacodynamic profile varies between HNSCC cell lines and plays an important role in the growth inhibition by antifolates. Both exposure time and the intrinsic capacity to synthesize polyglutamates are important factors in the sensitivity of HNSCC to antifolate drugs.

The efficiency with which surgery or radiotherapy controls locoregional head and neck squamous cell carcinoma (HNSCC‖) is well established, especially in the early stages of disease. Patients with advanced disease, however, still have a poor prognosis [1]. Over the last two decades chemotherapy has been integrated into combined modality treatment for progressive cancer. However, despite good initial responses to chemotherapeutic treatment the survival of patients is not enhanced [2, 3].

The antifolate drugs methotrexate (MTX) and 10-ethyl-10-deazaaminopterin (10-EdAM) can be considered as two of the most active drugs against HNSCC. Responses, mostly partial remissions, are observed in 15–40% of the patients [4, 5]. Mechanisms limiting the antiproliferative effect of these drugs include defective membrane transport [6], decreased levels of intracellular dihydrofolate reductase (DHFR) [7] and a low capacity to form and retain polyglutamates [8–10]. 10-EdAM is an antifolate with better transport properties and a higher affinity to folylpolyglutamate synthetase (FPGS) than MTX [11]. Against metastatic murine tumors 10-EdAM was found to be more effective than MTX [12].

It is conceivable that the therapeutic efficacy of antifolates can be increased by optimizing the schedule of administration. An optimal schedule can be defined as that with the largest possible difference in drug accumulation between neoplastic and normal cells. The cytotoxicity of MTX appears to be determined by the extent by which a time threshold, rather than a concentration, is exceeded [13].

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‖ Abbreviations: DHFR, dihydrofolate reductase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; FPGS, folylpolyglutamate synthetase; HBSS, Hank's buffered salt solution; HNSCC, head and neck squamous cell carcinoma; MTX, methotrexate; PG, polyglutamate; SRB, sulforhodamine B; TS, thymidylate synthase; 10-EdAM, 10-ethyl-10-deazaaminopterin.

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Although MTX has been in use for four decades, the optimal duration of the administration has not been assessed [4, 14]. 10-EdAM has shown significant antitumor activity in HNSCC [15], although it is not clear whether the optimal schedule and dose have been used. To improve the efficacy of antifolate chemotherapy, knowledge of the pharmacodynamics, defined as the relationship between therapeutic and toxic response and drug concentration [16] needs to be expanded. To define the relevance of pharmacodynamic parameters of antifolates in HNSCC, we have investigated the effect of 10-EdAM and MTX on three HNSCC cell lines. Inhibition of cell growth was related to (1) the duration of drug exposure, (2) the effect of a drug-free period and (3) the capacity to form polyglutamates.

MATERIALS AND METHODS

Chemicals. MTX and [3′,5′,7,9-^{3}H]MTX (20 mCi/μmol) were purchased from Lederle (Etten-Leur, the Netherlands) and Moravek Biochemicals (Brea, CA, U.S.A.), respectively. 10-EdAM (Edatrexate) was a gift from Ciba-Geigy (Arnhem, the Netherlands). [^{3}H]EdAM was obtained from Moravek Biochemicals. MTX-polyglutamate standards, PG_1–PG_5, were purchased from B. Schircks Laboratories (Jona, Switzerland).

Cell lines. HNSCC cell lines were obtained from Dr T. E. Carey (Ann Arbor, MI, U.S.A.) and are described elsewhere [17]. UM-SCC-14C originated from a local recurrence of the floor of the mouth, UM-SCC-11B from a laryngeal and UM-SCC-22B from a hypopharyngeal tumor. Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS). Cellular doubling times were 26 hr for UM-SCC-14C, 28 hr for UM-SCC-11B and 34 hr for the UM-SCC-22B cell line.

Cell growth inhibition studies. Inhibitory effects on growth of HNSCC cell lines were determined by applying the "SRB-assay" [18, 19]. Cells were cultured in 96-well plates and growth was measured at 540 nm using a micro-plate reader, after staining the cellular proteins with sulforhodamine B (SRB, the Sigma Chemical Co., St Louis, MO, U.S.A.). Cells were plated at a concentration of 1500 cells/well in 150 μL DMEM and 5% FCS, and were allowed to attach and grow for a lag-phase of 72 hr. Subsequently, MTX or 10-EdAM was added in 50 μL medium resulting in a final concentration that varied between 10^{-5} and 10^{-9} M. Cell growth was logarithmic up to 96 hr. Exposure for less than 24 hr did not show any reduction of cell growth. After the lag-phase, it was found that control (only incubated with culture medium) cell growth was logarithmic for a period up to 96 hr. Regrowth experiments were performed for both drugs and the three cell lines after a continuous exposure period of 4, 24 and 48 hr after which the cells were washed twice with fresh medium and allowed to grow for an additional drug-free period of 24 and 48 hr. IC_{50} values were estimated based on the absorption values and defined as the concentration that corresponded to a reduction of cellular growth by 50% as compared to values of untreated control cells.

Polyglutamate measurements. Cells were plated in 9-cm petridishes at an initial density of 3×10^4 cells/cm^2. Cells at 80% confluence were incubated in medium without folic acid, supplemented with 10% dialysed FCS with 50 μM [^{3}H]MTX (sp. act. of 100 cpm/mol) or 1 μM [^{3}H]10-EdAM (sp. act. of 100 cpm/mol) over a time period of 24 hr, followed by three washes at 4° with Hank's buffered salt solution (HBSS). Cells were then trypsinized at 25°, washed with HBSS at 4° and frozen at −20° until analysis. Intracellular folates and their polyglutamate forms (PGs) were extracted from the cells by resuspending cell pellets in 150 μL ice-cold phosphate-buffered saline (PBS) supplemented with 50 μL TCA-solution [40% (w/v) trichloroacetic acid in water]. After 20 min incubation on ice, samples were centrifuged for 5 min at 12,000 *g* at 4°. To the supernatant 400 μL trioctylamine/trifluoro trichloroethane (1:4, v/v) was added and mixed well. Samples were again centrifuged at 12,000 *g* for 1 min at 4°.

MTX and 10-EdAM PGs were measured by HPLC anion exchange chromatography, according to Nelson *et al.* [20]. Separation was achieved using a PartiSphere Sax Column (internal diameter 4.70 mm; length 110 mm; pore size 5 μm) purchased from Whatman Intern Ltd (Maidstone, U.K.). The HPLC system consisted of a Perkin Elmer Series 2 gradient system connected to a UV-detector (set at 309 nm) and a flow-through radioactivity detector [Flo One Beta (A200), Radiometic Instruments and Chemical Co. Inc., Tampa, FL, U.S.A.]. MTX and 10-EdAM were separated from their PGs using gradient elution, with a flow rate of 1.0 mL/min. After injection of the samples onto the column (150 μL injection volume) a 4 min isocratic elution with 98% buffer A and 2% buffer B was followed by a linear gradient (of 16 min) to 1% buffer A and 99% buffer B. Buffer A consisted of 60 mM $NH_4H_2PO_4$ with 20% (v/v) methanol, pH 4.9 and buffer B of 600 mM $NH_4H_2PO_4$ with 20% (v/v) methanol, pH 4.9. The retention times of MTX and 10-EdAM were 3.8 and 3.0 min, respectively. Retention times of the PGs determined using the commercially available MTX PG standards were found to be 3.8 min for MTX-PG_1, 7.9, 11.3, 12.9 and 15.5 min for MTX-PG_2, -PG_3, -PG_4 and -PG_5, respectively. The standards for the 10-EdAM PGs were not available and so their retention profile was based on that observed with MTX.

Activities of folate-dependent enzymes. DHFR activity was assayed spectrophotometrically [10] and the activity of FPGS assayed using either MTX [21] or aminopterin [22] as the folate substrate. Both methods used tritiated glutamate as the glutamate donor. Thymidylate synthase (TS) was measured in cell extracts using the tritium release with 10 μM [5-^{3}H]dUMP as the substrate [23].

RESULTS

Continuous drug exposure

It was found that the growth inhibition strongly depended on the time of exposure (Fig. 1), as indicated by the much lower IC_{50} values observed for 72 and 96 hr, when compared with the shorter

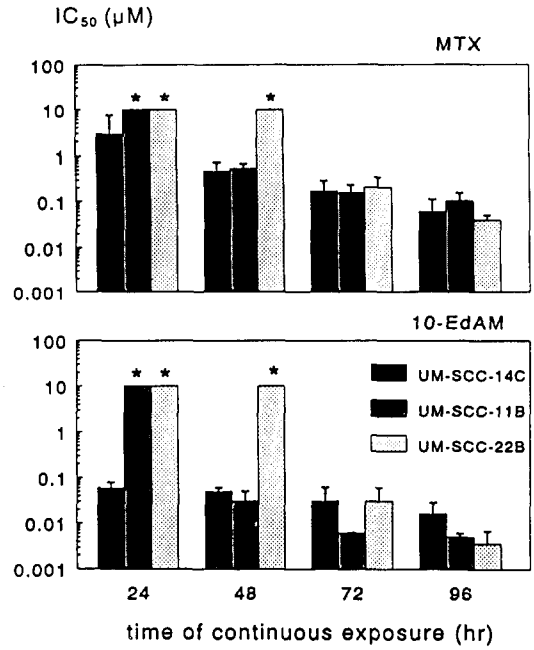

Fig. 1. Growth inhibition after continuous exposure to MTX and 10-EdAM. Each IC_{50} value represents the mean ± SD of three separate experiments. *IC_{50} was over 10 μM.

incubation periods. For example, with respect to MTX, IC_{50} values at 24 hr ranged from 2.9 (UM-SCC-14C) to over 10 μM (UM-SCC-22B and UM-SCC-11B), but when exposed for 96 hr, IC_{50} values varied between 0.039 (UM-SCC-22B) and 0.06 (UM-SCC-14C) to 0.1 μM (UM-SCC-11B). The minimal exposure time to achieve significant growth inhibition varied between the cell lines, less than 24 hr for UM-SCC-14C, and over 24 and 48 hr for UM-SCC-11B and UM-SCC-22B, respectively. 10-EdAM followed a similar sensitivity pattern with 5–20-fold lower IC_{50} values. Exposure for less than 24 hr did not show any reduction of cell growth.

Effect of drug-free period

The cell lines varied considerably with respect to growth behavior when the period of continuous exposure was followed by an additional drug-free period (Table 1, Fig. 2). When exposed for 4 hr followed by a 24 or 48 hr drug-free period all cell lines continued to grow at control levels (data not shown). At removal of the drug after the longer exposure times of 24 or 48 hr, the UM-SCC-14C cell line resumed growth to a significant extent, as shown by a very low ratio between the IC_{50} values after 24 hr exposure and 24 hr exposure followed by 48 hr drug-free period (24/24 + 48 ratio; Table 1). For instance, at 48 hr exposure to MTX, the IC_{50} values did not change after the additional drug free period of 48 hr (as indicated by a 48/48 + 48 ratio of 1.14, as explained in Table 1), again giving evidence that a significant proportion of cells resumed growth. The UM-SCC-11B cell line showed a similar response to MTX or 10-EdAM as UM-SCC-14C, although recovery was somewhat less, as indicated by a higher 24/24 + 48 ratio. In contrast, UM-SCC-22B showed a completely different behavior. Growth inhibition induced by MTX was retained after removal of the drug; this effect was more pronounced for 10-EdAM, the IC_{50} values were almost independent of removal of the drug, as indicated by a 96/48 + 48 ratio of 0.72. In general, growth inhibition was better retained with 10-EdAM than with MTX, for almost all conditions tested (Table 1).

Polyglutamation

This variable pattern of sensitivity was correlated with the capacity of the cells to form PGs. As shown previously, FPGS levels measured with MTX or aminopterin as a substrate (Table 2), revealed higher levels for UM-SCC-11B cells. Accumulation of polyglutamated forms of both MTX and 10-EdAM were determined after 24 hr exposure to 50 and 1 μM, respectively (Table 3). In UM-SCC-14C cells the lowest level of folate uptake was observed, values of 10-EdAM being comparable to those of MTX. However, more 10-EdAM than MTX was accumulated in the UM-SCC-11B and -22B cell lines. After a 24 hr exposure period, PG_4 and PG_5 were the predominant polyglutamylated species of 10-EdAM in the UM-SCC-11B and -22B cell lines. The concentration of the various MTX PGs was within the same range in UM-SCC-22B, while in UM-SCC-11B PG_3 was the predominant form.

Folate-dependent enzymes

TS is a target for inhibition by MTX PGs [24] and by polyglutamates of dihydrofolate which accumulate after MTX treatment. A low activity of TS, however, may play a role in the intrinsic resistance to antifolates, since at a low activity of TS 5,10-methylene tetrahydrofolate, the substrate for this reaction, will not be used, preventing a depletion of intracellular folates and bypassing the folate-depleting effects of DHFR inhibition [25, 26]. UM-SCC-14C had the lowest and UM-SCC-11B the highest TS activity of the tested cell lines, measured as the catalytic activity (Table 2), while UM-SCC-22B levels were intermediate. DHFR activities were almost similar in all three cell lines (Table 2).

DISCUSSION

Sensitivity of HNSCC cells to antifolates appeared to be very dependent on the time of exposure. This is in agreement with the concept of Powis [16], which assumes that antifolates display a time rather than a concentration-dependent antiproliferative effect. This time threshold could have important implications: for certain HNSCC tumors a drug exposure of over 48 hr may give a better therapeutic index. In this respect this assumption is supported by data on the toxic effects of antifolates in normal tissue. It has been estimated that the toxic concentration and the time threshold of methotrexate for bone marrow and gastrointestinal epithelium were 2×10^{-8} M and 42 hr, respectively [13]. Considering the toxicity of normal epidermal cells, it has been determined *in vitro* that these cells are less sensitive to a 48 hr exposure to MTX (IC_{50} of over 1 μM) than

Table 1. Pharmacodynamic profile of antifolates

Cell line	Drug	Ratio* 24/24 + 48 A	72/24 + 48 B	48/48 + 48 C	96/48 + 48 D
UM-SCC-14C	MTX	<0.02	<0.017	1.14	0.14
UM-SCC-14C	10-EdAM	0.12	0.06	0.8	0.27
UM-SCC-11B	MTX	>12.3	0.18	1.47	0.28
UM-SCC-11B	10-EdAM	>200	0.12	2.7	0.45
UM-SCC-22B	MTX	NE	<0.02	1.4	0.28
UM-SCC-22B	10-EdAM	NE	0.003	>2000	0.72

* The ratio is calculated based on the growth inhibition studies and expresses the IC_{50} values of the continuous exposure for an indicated time period, divided by the IC_{50} values obtained by a certain exposure period but followed by an additional time period of drug-free culture. Absolute IC_{50} values are shown in Figs 1 and 2. So 24/24 + 48 = the IC_{50} value corresponding to a 24 hr continuous exposure period divided by the value obtained by a 24 hr continuous exposure but followed by a 48 hr drug free period.

For columns A and C the lower the ratio the better the cells are able to recover from the drug effect during the drug-free period. For columns B and D, the higher the ratio, the better the growth inhibiting effect of the drug is retained during the drug-free period. The means of three separate experiments are shown.

NE = not evaluable, because numerator and denominator are over 10 μM.

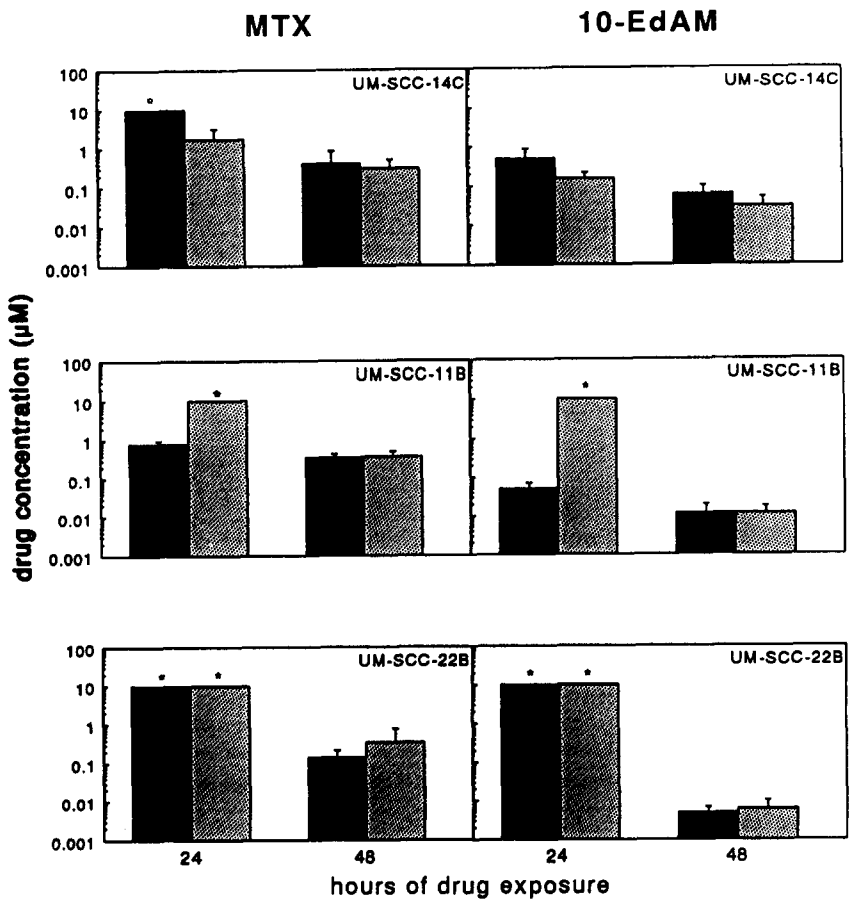

Fig. 2. IC_{50} values after 24 and 48 hr of continuous exposure followed by an additional period of 24 (■) and 48 hr (▨) incubation without drug. Each IC_{50} value represents the mean ± SD of three separate experiments. *IC_{50} was over 10 μM.

Table 2. Analysis of folate-dependent enzymes

Cell line	TS-activity* (pmol/hr/10^6 cells)	FPGS-activity† (pmol [^{3}H]glutamate/hr/10^6 cells) According to Ref. 22	FPGS-activity† (pmol [^{3}H]glutamate/hr/10^6 cells) According to Ref. 21	DHFR-activity‡ (nmol FH_2/hr/10^6 cells)
UM-SCC-14C	57 ± 37	113 ± 7	40.4 ± 13.5	1.08 ± 0.6
UM-SCC-11B	368 ± 225	233 ± 20.3	135.2 ± 20.3	0.88 ± 0.33
UM-SCC-22B	158 ± 16	141 ± 41	39.1 ± 17.4	1.1 ± 0.06

Results are expressed as mean from three separate experiments ± standard deviations.

* Results are from Van der Wilt *et al.* [27].

† FPGS was measured by two methods. In method 1 aminopterin was used as the substrate [22], while in method 2 MTX was used [21].

‡ The results are from Van der Laan *et al.* [10].

Table 3. MTX and 10-EdAM polyglutamate formation in HNSCC cell lines

Cell line	Drug	Intracellular polyglutamate concentration* (pmol/10^6 cells) Total	PG_2	PG_2	PG_3	PG_4	PG_5
UM-SCC-14C	MTX	10.2 ± 6.3	5.5 ± 0.7	1.3 ± 0.2	1.3 ± 0.3	1.4 ± 0.2	0.7 ± 0.4
UM-SCC-14C	10-EdAM	10.3 ± 1.8	2.1 ± 0.7	1.6 ± 0.1	2.7 ± 2.2	2.0 ± 0.4	1.8 ± 0.5
UM-SCC-11B	MTX	58.4 ± 5.7	11.4 ± 1.4	8.4 ± 0.7	17.0 ± 1.5	13.1 ± 0.8	8.6 ± 1.9
UM-SCC-11B	10-EdAM	172.8 ± 6	6.0 ± 0.7	9.6 ± 1.4	44.1 ± 1.7	50.7 ± 10.4	62.4 ± 6.9
UM-SCC-22B	MTX	26.7 ± 5.3	5.7 ± 1.1	3.6 ± 0.7	4.7 ± 0.5	6.0 ± 1.4	6.7 ± 1.5
UM-SCC-22B	10-EdAM	101.1 ± 13.6	4.3 ± 1.3	5.6 ± 0.5	14.0 ± 0.7	23.7 ± 0.9	53.5 ± 3.5

Drug exposure time was 24 hr, extracellular concentration [^{3}H]MTX: 50 μM, extracellular concentration of [^{3}H]10-EdAM: 1 μM. Results are expressed as mean from three separate experiments ± SD.

* Our highest glutamate standard during the HPLC analysis was MTX-PG_5. It is reasonable to assume that the PG_5 fraction may also contain higher glutamate forms.

squamous carcinoma cells (IC_{50} values of about 0.01 μM) [28]. Since it is not known for how long the administration of an antifolate drug to a patient should last, to obtain an optimal therapeutic index [4, 14], an increase of the infusion time to over 48 hr may be a way to improve therapy that needs further investigation. It can be anticipated that leucovorin rescue will play a role in such a setting.

The difference in persistence of the inhibitory effect is probably mainly due to the ability of the tumor cells to form polyglutamated forms of MTX and 10-EdAM, confirming that polyglutamylation is an important determinant in antifolate cytotoxicity [8, 10]. Although significantly inhibited already after a short period, the low intracellular drug levels and the limited capacity to form glutamated forms of the UM-SCC-14C line corresponds with the ability of the cells to recover quickly. The UM-SCC-22B cell line is certainly the antipode cell line, being persistently affected after a certain threshold time is passed. In comparison with UM-SCC-14C, in UM-SCC-22B polyglutamylated drug (with more of the higher glutamated forms, especially for 10-EdAM) was accumulated after 24 hr exposure to 50 μM MTX or 1 μM 10-EdAM. Jolivet *et al.* [29] recently studied the accumulation and retention of glutamates in MTX or 10-EdAM treated cells of a number of cell lines, including the ones tested in the present study. After an exposure time of 3 hr, these authors observed an impaired retention of both MTX and 10-EdAM in UM-SCC-14C cells, whereas a good retention was observed in the UM-SCC-22B cell line. These data are consistent with our data and the observed kinetics of the antiproliferative effects.

Continued culture in drug free medium revealed a rapid recovery of UM-SCC-14C cells, while further inhibition of growth was observed with UM-SCC-22B cells. Possibly due to the relatively low FPGS activity, accumulation of glutamates in UM-SCC-22B was initially low, especially when compared with UM-SCC-11B, but antifolate glutamates continued to accumulate.

All tested cell lines have a more or less similar DHFR activity, most likely excluding this factor as being responsible for the observed differences in sensitivity. In addition to the already discussed differences in polyglutamylation, the differences in TS activity might contribute to observed differences in growth inhibition. Due to the lower TS activity in UM-SCC-14C and UM-SCC-22B, these cells have a lower capacity than UM-SCC-11B cells to use 5,10 methylene tetrahydrofolate, the substrate for TS,

and deplete intracellular reduced folates. Thus, at a low rate of TS, reduced folates remain available for other cellular processes [25, 26]. After a prolonged exposure to either MTX or 10-EdAM, this intracellular folate pool will, however, not be sufficient to provide for the cellular folate requirements, leading to growth inhibition.

Our data confirm that 10-EdAM is a very active drug against HNSCC cell lines [10, 30]. Its activity greatly depends on the time of exposure, enabling even a good sensitivity in cell lines with an initially low capacity to form PGs. The growth inhibitory effect and in particular the retention of growth inhibition could very well be correlated with accumulation of PGs. The growth inhibition studies as described here could conveniently be performed by using the SRB-assay which proved to be a rapid and reproducible test, enabling us to study recovery from or persistence of a drug effect. This type of experiment offers the possibility to achieve insight into the total intracellular metabolism of folates and PG turnover in relation to cytotoxic and cytostatic effects. These data also help to design proper schedule for drug administration in order to determine the schedules with the best therapeutic efficacy.

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