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Functional aspects of membrane folate receptors in human breast cancer cells with transport-related resistance to methotrexate

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Abstract Two methotrexate (MTX)-resistant human breast-cancer cell lines with impaired transport via the reduced folate carrier (RFC), one established in vitro (MTX^R-ZR-75-1) and another inherently resistant (MDA-231), were adapted to grow in medium containing 2 n*M* folic acid. This induced the expression of pre viously undetectable membrane folate receptors (MFR) to levels of 8.2 and 2.3 pmol/10^7 cells, respectively. Polymerase chain reaction (PCR) quantitation revealed that MFR messenger-RNA levels of the isoform first described in human nasopharyngeal carcinoma KB cells (MFR-α) were increased in low-folate-adapted MTX^R-ZR-75-1 cells, whereas placental transcripts (MFR-β) coincided with MFR-α expression in low-folate (LF)-adapted MDA-231 cells. These cell lines were used to study the role of MFR in the uptake and growth-inhibitory effects of five different antifolates with varying affinities for MFR: N^{10}-propargyl-5, 8-dideazafolic acid (CB3717) > 5,10-dideazatetrahydrofolic acid (DDATHF) > N-{5- [*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-methyl)-N-methylamino] -2-theonyl}-glutamic acid (ZD1694) ⩾ MTX > edatrexate (EDX). Expression of MFR only slightly decreased the resistant phenotype for MTX, EDX, and ZD1694, suggesting that these drugs are not transported intracellularly to cytotoxic concentrations at these levels of MFR expression. On the other hand, both cell lines became from at least 180- to 400-fold more sensitive to growth inhibition by CB3717 and DDATHF, which may be correlated with their high affinity for MFR. These sensitivity/resistance profiles were largely similar following cell culture in medium containing 1 n*M* L-leucovorin, a folate with an affinity for MFR 10-fold lower than that of folic acid, the one exception being the increased sensitivity for ZD1694 seen in the LF-adapted cells with the highest level of MFR expression (MTX^R-ZR-75-1). These results illustrate that the efficacy of MFR in mediating antifolate transport and cytotoxicity depends on their affinity for the folate antagonist, their degree of expression, and the levels of competing folates.

Key words Membrane folate receptors · Methotrexate · Sensitivity/resistance profiles · Antifolates · Human breast cancer cells

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Introduction

The folate analogue methotrexate (MTX) is a commonly used anticancer agent that accumulates in cells as polyglutamate metabolites and exerts its cytotoxic effect by inhibiting the enzyme dihydrofolate reductase [1]. At least two transport systems are potentially involved in MTX uptake. The drug preferentially enters cells via the reduced folate carrier (RFC), a membrane anion-exchange transport protein with a high turnover rate and greater affinity for reduced folates and MTX

($K_i \approx 1$ μM) as compared with folic acid [2, 3]. New antifolates such as 10-ethyl-10-deazaaminopterin (EDX) show even greater affinity for this transporter relative to MTX [4]. The RFC, a glycosylated protein, has a reported molecular weight of 80–100 kDa in human cell lines [5, 6]. A cDNA encoding for a ≈65-kDa membrane protein that restores RFC activity to two RFC-deficient cell lines has recently been cloned [7–9]. MTX can also be internalized by another transport system involving the membrane folate receptors (MFR) [10]. This family of receptors has greater affinity for folic acid ($K_D \approx 1$ n*M*) and 5-methyltetrahydrofolate as compared with MTX but has lower turnover rates than the RFC [11]. New antifolates such as N^{10}-propargyl-5,8-dideazafolic acid (CB3717), a thymidylate synthase inhibitor [12], enter cells preferentially via the MFR, whereas others, including 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF), a de novo purine synthesis GAR transformylase inhibitor [13], and *N*-{5- [*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-methyl)-*N*-methyl-amino]-2-theonyl}-glutamic acid (ZD1694), a thymidylate synthase inhibitor [14], can be internalized via both the RFC and the MFR [15, 16]. Folate receptors have been found in milk, placenta, and a number of cultured cell lines, including human nasopharyngeal epidermoid carcinoma KB cells, where they are expressed to high levels [10]. They are glycosylated and have an apparent molecular weight of 40 kDa, and at least three different MFR cDNAs, one from human placenta designated herein as MFR-β [17–20], another from KB cells designated as MFR-α [17, 18, 21], and (recently) a third from human leukemic cells (MFR-γ) [22], have been cloned. Drug transport is a critical determinant of antifolate cytotoxicity, and impaired MTX intracellular accumulation is frequently encountered in drug-resistant cell lines [1, 15, 23, 24].

In the present study, two human breast-cancer cell lines with impaired RFC transport were used. MTX^R-ZR-75-1 cells were selected in vitro and are more than 1,000-fold resistant to MTX relative to parental ZR-75-1 cells [25–27]. The MDA-231 cell line was isolated directly from the malignant pleural effusion of a patient who had received a single course of MTX-containing combination chemotherapy [28, 29]. The two cell lines were adapted to grow in medium containing nanomolar concentrations of folic acid as the folate source. Both cell lines thereafter expressed MFR, allowing us to study a number of functional aspects of the MFR with respect to antifolate transport and cytotoxicity and to examine some of the underlying mechanisms of increased expression.

Materials and methods

Chemicals

Unlabeled MTX, folic acid, and L-leucovorin (6S-5-formyltetrahydrofolate) were purchased from Sigma Chemical Co. (St. Louis, Mo.) and B. Schircks Laboratories (Jona, Switzerland), whereas EDX was provided by Ciba-Geigy Ltd. (Basel, Switzerland). ZD1694 and CB3717 were gifts from Dr. A.L. Jackman (Institute for Cancer Research, UK). DDATHF was gift from the late Dr. G.B. Grindey (Eli Lilly Research Laboratories, Indianapolis, Ind.) 3′,5′,7,9- [^{3}H]-Folic acid (29 Ci/mmol) was purchased from Moravek Biochemicals (Brea, Calif.), purified by thin-layer chromatography [11, 30], and kept at − 80°C prior to use. All other chemicals were obtained from Fisher Scientific Co. (Pittsburg, Pa.) or Sigma.

Cell lines and cell culture

The MTX-resistant MTX^R-ZR-75-1 subline [25, 27] was previously selected in vitro by subculture of the drug-sensitive wild-type ZR-75-1 human breast-carcinoma cell line for over 1 year in the presence of stepwise increasing concentrations of MTX. MTX^R-ZR-75-1 cells are > 1,000-fold less sensitive to MTX as compared with wild-type ZR-75-1 cells [25–27]. Both the sensitive line and the MTX-resistant MTX^R-ZR-75-1 subline were obtained from the National Cancer Institute (Bethesda, Md.). The MDA 231 human breast-cancer cell line [28] was purchased from the American Type Culture Collection (Rockville, Md.). MDA-231 cells were isolated directly from the malignant pleural effusion of a patient who had received a single course of MTX-containing combination chemotherapy [28, 29]. MD-231 cells were resistant to MTX with an IC_{50} (298 μM) following 3-h incubations that was 20-fold higher than that found for wild-type ZR-75-1 and MCF-7 breast cancer cells [31]. In both resistant cell lines, dihydrofolate reductase levels are unchanged [25, 29] but polyglutamate metabolism is secondarily impaired [25, 31].

The three cell lines were routinely grown as monolayers in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), penicillin (200 μg/ml), and streptomycin (200 μg/ml) at 37°C under 5% CO_2 in a humidified atmosphere. This medium contains 2.2 μM folic acid and the suffix "high folate" (HF) is hereafter added to the line numbers of cells grown under these conditions. MTX^R-ZR-75-1 and MDA-231 cells were adapted to grow in low-folate medium in folate-free RPMI-1640 supplemented with 10% dialyzed FCS, 2 m*M* glutamine, penicillin (200 μg/ml), and streptomycin (200 μg/ml) and stepwise decreasing concentrations of folic acid until they could be maintained in 2 n*M* folic acid. The suffix "low folate" (LF) is hereafter added to the line numbers of these cells.

Cytotoxicity assays

Antifolate cytotoxicity curves were obtained as previously described [32, 33]. Cells were plated in the individual wells of a 24-well tissue-culture plate at a density of 1×10^4 cells/cm^3. Drugs were added 1 day after plating and incubations were continued until control cells reached near confluence (3–4 doubling times). At the end of the incubation period, cells were washed twice with phosphate-buffered saline (PBS), trypsinized [0.25% trypsin/0.05% ethylenediaminetetraacetic acid (EDTA) in PBS], and counted with a Sysmex CC-110 cell counter.

Binding/transport studies

[^{3}H] -Folic acid binding studies were carried out essentially as previously described [11]. Monolayer cells were brought into suspension after incubation with PBS containing 2 m*M* EDTA. Then, 50 pmol [^{3}H] -folic acid (spec. act. 1,000 cpm/pmol) was added to 5×10^6 cells in 1 ml ice-cold PBS. After 10 min of incubation, cells were centrifuged in an Eppendorf microcentrifuge (1 min; 13,000 *g*). The supernatant was removed by suction and residual fluid, by cotton tissues. Cell pellets were resuspended in water and analyzed

for radioactivity. Nonspecific binding of radiolabel was determined by measuring radioactivity in the presence of a 1,000-fold molar excess of unlabeled folic acid. [^{3}H] -MTX uptake in MDA-231 cells was carried out essentially as described elsewhere [27].

MFR fluorescence-activated cell-sorter analysis

ZR-75-1 (HF), MTX^R-ZR-75-1 (HF and LF), and MDA-231 (HF and LF) cells were tested for reactivity with monoclonal antibodies MOv18 and MOv19 (provided by Dr. S.O. Warnaar, Centocor Inc, Leiden, The Netherlands) essentially as described previously [34]. Briefly, cells were brought into suspension by incubation with 2 m*M* EDTA in PBS and then incubated with antibody (30 min, 4°C), which was followed by detection of bound antibody with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and analysis of fluorescence staining with a FACSCAN cytofluorometer (Becton and Dickinson, Mountain View, Calif.).

MFR mRNA quantitation by polymerase chain reaction

Polymerase chain reaction (PCR) quantitation of MFR-α and -β mRNAs were performed as recently described [35].

Results

MTX transport in MDA-231 and MTX^R-*ZR*-75 *cells*

Figure 1 shows a time course for the uptake of [^{3}H]-MTX by MDA-231 cells [29, 31]. The component of uptake of [^{3}H] -MTX appears to be unrelated to the RFC since the uptake profiles obtained in the absence or presence of an excess of 1 m*M* L-leucovorin were indistinguishable. Using the same experimental conditions, we previously demonstrated that impaired transport via the RFC was the main mechanism of MTX resistance in MTX^R-ZR-75-1 cells [27].

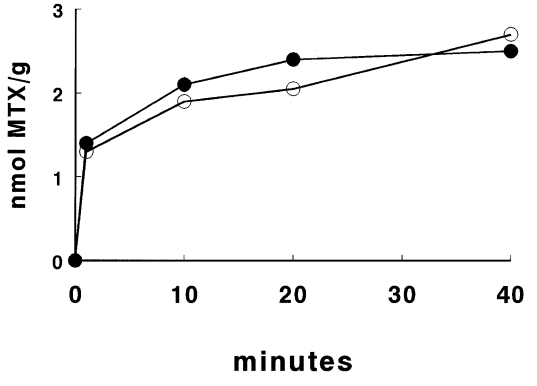

Fig. 1 MTX uptake in MDA-231 human breast cancer cells. MDA-231 human breast cancer cells were exposed to 1 μM [^{3}H] -MTX and total cellular uptake was measured at various intervals over 40 min. Cells were incubated either in control medium (*white circles*) or in the presence of 1 m*M* L-leucovorin (*black circles*). Results represent mean values for duplicate experiments

Table 1 Expression and characterization of MFR in MTX^R-ZR-75-1 (LF) and MDA-231 (LF) cells

	MTX^R-ZR-75-1 (LF)	MDA-231 (LF)
[^{3}H] -Folic acid binding (pmol/10^7 cells)	8.2	2.3
Fluorescence index[a]:		
MOv18	32	3
MOv19	71	18
Relative affinity of MFR for folates and antifolates[b]:		
Folic acid	1.0	1.0
L-Leucovorin	0.15	0.08
MTX	0.06	0.03
EDX	0.005	0.004
CB3717	2.0	1.18
ZD1694	0.47	0.28
DDATHF	1.65	1.04

[a]Fluorescence index = (mean fluorescence MOv18-mean fluorescence control IgG) /(mean fluorescence control IgG)
[b]Relative affinity of MFR for folates and antifolates is expressed as the inverse molar ratio of compound required to displace 50% of [^{3}H] -folic acid from the receptor. The relative affinity of MFR for folic acid is set to 1

Expression of MFR in MTX^R-ZR-75-1 and MDA-231 cells grown in LF-containing medium

Over a period of 2 months, MTX^R-ZR-75-1 cells and MDA-231 cells were adapted to grow in medium containing 2 n*M* folic acid as the folate source rather than the 2 μM folic acid that is normally present in the cell-culture medium. MTX^R-ZR-75-1 (LF) and MDA-231 cells (LF) expressed MFRs to levels of 8.2 and 2.3 pmol/10^7 cells, respectively, as determined by [^{3}H]-folic acid binding, the results being confirmed by fluorescence-activated cell-sorter (FACS) analysis with the monoclonal antibodies MOv18 and MOv19 as shown in Table 1. MFR expression in ZR-75-1 (HF), MTX^R-ZR-75-1 (HF), and MDA-231 (HF) cells was below the limit of detection (fluorescence index $<$ 1.0). The relative affinities of the MFR for various folates and antagonists in the LF sublines were then determined (Table 1). CB3717, DDATHF, and folic acid had the highest affinities, followed by ZD1694 and, more than 1 order of magnitude below, by L-leucovorin, MTX, and EDX.

MFR mRNA quantitation in human breast-cancer cell lines

PCR quantitation assays were next performed on cDNAs prepared from the different human breast-cancer cell lines to examine the mechanisms underlying enhanced MFR expression. The results are shown in Fig. 2. Wild-type MTX-sensitive ZR-75-1 cells (lane 4) had a barely detectable MFR-α message, with a ratio of 0.23 relative to the internal standard, a coamplified

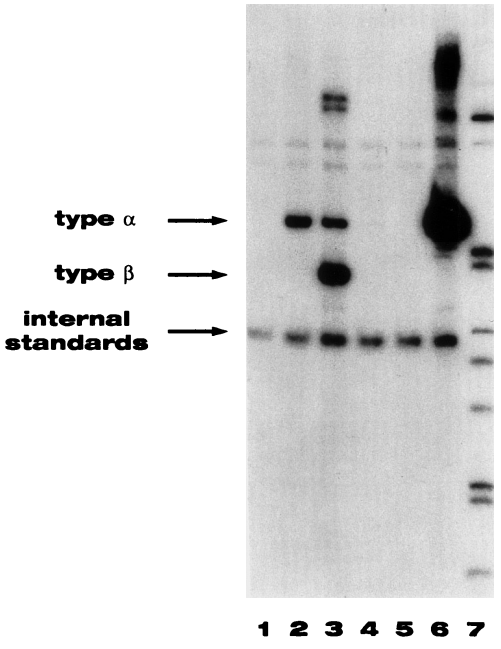

Fig. 2 MFR mRNA quantitation in human breast-cancer cell lines. cDNAs prepared from wild-type MTX-sensitive ZR-75-1 cells, MTX^R-ZR-75-1 HF and LF cells, and MDA-231 HF and LF human breast cancer cells were assayed by quantitative PCR for type α and β MFR mRNA. A 100-bp deleted MFR-β cDNA was used as an internal standard in each sample. Results are expressed as a cpm-to-cpm ratio of each band, excised from the gel, to the internal standard. A DNA ladder was used as a size standard (*lane* 7). Amplification without prior use of reverse transcriptase was performed for each cell line [as shown in lane 1 for the MDA-231 (HF) cell line]. cDNA amplification was performed for the following cell lines: MDA-231 (HF) *lane* 2, MDA-231 (LF) *lane* 3, ZR-75-1 (HF) *lane* 4, MTX^R-ZR-75-1 (HF) *lane* 5, and MTX^R-ZR-75-1 (LF) *lane* 6

100-bp deleted MFR-β cDNA. No MFR-α message could be detected in MTX^R-ZR-75-1 (HF) cells (lane 5), but LF adaptation markedly increased its expression to a ratio of 16.8 (lane 6). MDA-231 (HF) cells (lane 2) expressed only low levels (1.39 ratio) of MFR-α message and, although these remained low (0.84 ratio) in the LF subline (lane 3), the latter then expressed MFR-β (2.58 ratio), the only subline in which this MFR message was identified.

Antifolate cytotoxicity against ZR-75-1, MTX^R-ZR-75-1 (HF/LF), and MDA-231 (HF/LF) cells

The growth-inhibitory effect of different antifolates was next examined (Table 2). ZR-75-1 cells were sensitive to growth inhibition by MTX, EDX, ZD1694, and DDATHF, which is consistent with efficient transport of these drugs via the RFC [4, 14–16, 36], and were relatively resistant to CB3717, reflecting the absence of MFR and poor transport of this drug via the RFC [11]. Substantial cross-resistance of MTX^R-ZR-75-1 (HF) cells and MDA-231 (HF) cells against MTX, EDX, ZD1694, and DDATHF pointed to defective RFC-mediated drug transport in these cells. Expression of MFR in MTX^R-ZR-75-1 (LF) and MDA-231 (LF) cells had a relatively minor effect on the resistance factor for MTX (decrease by 7- and 1.1-fold, respectively), EDX (decrease by 3.6- and 1.7-fold, respectively), and ZD1694 (decrease by 2- and 2.1-fold, respectively). On the other hand, MTX^R-ZR-75-1 (LF) and MDA-231 (LF) cells became significantly more sensitive to growth inhibition by CB3717 (decrease in resistance factor of 85- and 59-fold, respectively) and DDATHF (decrease in resistance factor of 20- and 21-fold, respectively), the two agents with the highest MFR affinities of the compounds tested. To determine if the continued resistance of the LF cell lines to MTX, EDX, and ZD1694 could be due to the (2 n*M*) folic acid in the medium preventing binding of these poor substrates to the MFR, LF cells were removed from folic acid and grown for approximately 1 week in 1 n*M* L-leucovorin

Table 2 Growth inhibition of ZR-75-1, MTX^R-ZR-75-1 (HF/LF), and MDA-231 (HF/LF) cells by folate antagonists[a] (*FA* Folic acid, *LV* leucovorin)

Folate analogue	Cell line						
	ZR-75-1	MTX^R-ZR-75-1	MTX^R-ZR-75-1	MTX^R-ZR-75-1	MDA-231	MDA-231	MDA-231
	10% FCS 2 μ*M* FA	10% FCS 2 μ*M* FA	10% dFCS 2 n*M* FA	10% dFCS 1 n*M* LV[b]	10% FCS 2 μ*M* FA	10% dFCS 2 n*M* FA	10% dFCS 1 n*M* LV[b]
MTX	6.7 ± 1.2	18, 060 ± 600	2, 530 ± 260	6, 300 ± 1, 410	432 ± 140	378 ± 105	407 ± 58
MTX (20 n*M* FA)			4, 440 ± 1, 040	9, 600 ± 910		344 ± 163	363 ± 55
10-EdAM	1.8 ± 0.1	7, 380 ± 385	2, 040 ± 1, 050	2, 240 ± 1, 130	1, 060 ± 98	610 ± 262	401 ± 108
CB3717	1, 240 ± 470	4, 380 ± 650	51.5 ± 33.0	23.8 ± 14.7	2, 875 ± 490	49 ± 24	9.6 ± 5.8
CB3717 (20 n*M* FA)			366 ± 170	1, 510 ± 510		835 ± 184	938 ± 540
ZD1694	15.8 ± 9.3	1, 100 ± 204	523 ± 100	16.8 ± 9.2	825 ± 70	390 ± 184	364 ± 104
DDATHF	35.9 ± 6.8	4, 810 ± 1, 325	242 ± 197	5.3 ± 2.5	1, 770 ± 100	84 ± 32	4.5 ± 2.0

[a]Cells were exposed to folate antagonists as described in Materials and methods. Results are expressed as drug concentrations (in n*M*) inhibiting growth by 50% (IC_{50}) as the mean value ± SD for at least 3 separate experiments

[b]LF cells were removed from folic acid and growth for approximately 1 week in 1 n*M* leucovorin prior to repetition of the cytotoxicity experiments in this new medium

prior to repetition of the cytotoxicity experiments. Under these conditions, in medium containing a folate with 10-fold lower affinity for MFR, the IC_{50} values were not lowered for MTX and EDX but decreased 77- and 18.7-fold for DDATHF and 2.2- and 5.1-fold for CB3717 against MTX^R-ZR-75-1 (LF) and MDA-231 (LF) cells, respectively. The IC_{50} value for ZD1694 was decreased only for MTX^R-ZR-75-1 (LF) cells (31-fold) but not for MDA-231 (LF) cells. The drug sensitivity profiles of the MTX^R-ZR-75-1 (LF) and MDA-231 (LF) cells were thus closely correlated with the high (CB3717 and DDATHF), moderate (ZD1694), and poor (MTX, EDX) affinity of these drugs for the MFR (Table 1). Further evidence for the role of MFR in transport of antifolates with a high binding affinity for MFR was illustrated by a marked protective effect (7- to 97-fold) of 20 n*M* folic acid against growth inhibition for MTX^R-ZR-75-1 (LF) and MDA-231 (LF) cells by CB3717, whereas folic acid had no effect (0.9- to 1.8-fold) on growth inhibition by MTX. The growth-inhibitory effect of CB3717 was not related to blocking of the receptor to allow entry of folates required for cell growth since thymidine, for which MFR has no affinity, completely protected the cells against growth inhibition by CB3717 (results not shown).

Discussion

MTX-resistant MDA-231 human breast cancer cells share many antifolate sensitivity parameters with the in vitro-derived MTX^R-ZR-75-1 cell line: impaired drug uptake via the RFC associated with decreased drug metabolism to polyglutamates, and no immunologically detectable MFR. The MDA-231 cell line was established from the malignant pleural effusion of a patient who had received MTX-containing combination chemotherapy only 3 weeks before the effusion was drawn for culture [28], suggesting that this MTX-resistance mechanism could occur in vivo. Furthermore, the survival of these cell lines in vitro, seemingly without any folate transporter, suggests the possibility of other routes of folate transport at high (μM) medium-folate concentrations [37, 38].

As previously shown in leukemia cells [11, 39], growth adaptation of MDA-231 and MTX^R-ZR-75-1 cells to low folic acid concentrations markedly enhanced the expression of MFRs. They are structurally different from RFC in their mode of membrane anchoring via a glycosylphosphatidyl inositol (GPI) anchor [40] and in folate and antifolate substrate specificity [11, 16, 20, 36, 41]. At least three isoforms of MFRs have been identified in normal and malignant tissues and in established cell lines [18, 19, 22, 35]. One isoform (type β) was originally identified in human placenta [17]; another (type α) is also expressed in placenta but is the predominant isoform in human nasopharyngeal KB cells [21], whereas a third (type γ) has recently been cloned from leukemic cells [22]. Although there is more than 70% sequence homology between the three isoforms, significant differences have been observed in the binding characteristics of stereoisomers of reduced folates and antifolates between MFR-α and -β [20, 41]. Stimulation of MFR expression by LF growth adaptation has been correlated with increased type-α mRNA levels in KB cells, attributed partly to increased mRNA stability [42]. Our studies (Fig. 2) also demonstrate a markedly increased expression of MFR-α in MTX^R-ZR-75-1 (LF) cells. LF-selective pressure for MDA-231 cells, however, did not change the level of the MFR-α transcript but resulted in increased expression of the MFR-β message. These results suggest that the same stimulus can lead to enhanced production of MFR in different cell lines that are the product of independent genes [43].

There is now substantial evidence that MFRs may play a role in the uptake of natural reduced folates required for cell growth as illustrated by the observation that several cell lines transfected with the cDNA for MFR were capable of surviving at low concentrations of folates in vitro [44–48]. Likewise, a similar role for MFR has been suggested in vivo [49]. Results from the present study support these observations, both MFR expressing MTX^R-ZR-75-1 (LF) and MDA-231 (LF) cells could survive in vitro at nanomolar concentrations of folates. The role of MFR, as compared with RFC, in relation to the transport of folate antagonists remains controversial. Since antifolate transport activity via RFC is severely impaired in MTX^R-ZR-75-1 and MDA-231 cells, we were capable of addressing the role of MFR more specifically. In this study we mimicked the potential transport activity of MFR by analyzing the growth-inhibitory effects of classic and novel folate antagonists for which MFR has poor (MTX, EDX), moderate (ZD1694), or high affinity (CB3717, DDATHF). These appeared to be a close correlation between the degree of growth inhibition and the affinity of the folate antagonists for MFR, as CB3717 and DDATHF were potent growth inhibitors of MTX^R-ZR-75-1 (LF) and MDA-231 (LF) cells. On the other hand, small concentrations of folic acid (20 n*M*) provided significant protection against growth inhibition by CB3717. Folic acid was selected because it has a greater stability in cell culture than does the natural reduced folate cofactor 5-methyltetrahydrofolate [50]. Furthermore, since the binding affinity of MFR for folic acid is close to that of 5-methyltetrahydrofolate [16, 20, 41], the results shown in Table 2 may predict that at physiological concentrations (5–50 n*M*) of 5-methyltetrahydrofolate, receptor activity may not always be sufficient to achieve cytotoxic intracellular drug levels. The level of MFR protein also appeared to be an important factor determining cytotoxicity, since MTX^R-ZR-75-1 (LF) cells (8.2 pmol receptor protein/10^7 cells) were sensitive to ZD1694, whereas MDA-231 (LF) cells (2.3 pmol receptor protein/10^7 cells) were insensitive to ZD1694.

An interesting observation was that both MTX^R-ZR-75-1 (LF) and MDA-231 (LF) cells largely retained the resistance phenotype for MTX and EDX observed in their respective (HF) cells. This is in agreement with data reported by Dixon et al. [46] for MTX^R-ZR-75-1 cells transfected with MFR. It is reasonable to assume that these results are related to the poor affinity of the MFR-α and -β isoforms for these compounds (K_i 0.11 and 1.9 μM, respectively) [11, 20, 41]. Exceptions to this concept have been reported for leukemia [39] and nasopharyngeal KB cells [51], in which MFR transport activity is sufficient to achieve significant MTX cytotoxicity in the absence of competing folates. However, it should be noted that these cells express substantially higher levels of MFR (50–500 pmol/10^7 cells) than do MDA-231 (LF) or MTX^R-ZR-75-1 (LF) cells. Thus, it could be speculated that under in vitro conditions a threshold level of > 10 pmol receptor protein/10^7 cells may be required to achieve the internalization of cytotoxic concentrations of MTX.

Two processes have been described for MFR-mediated folate uptake; one occurs via the classic receptor-mediated endocytosis route via clathrin-coated pits [52–54], and the other has been designated as potocytosis [55]. The latter proposes clustering of receptor molecules in specialized microdomains on the plasma membrane (caveolae) having caveolin as the coat-protein. Following binding of folates or antagonists to the receptor, the caveolae temporarily close but do not pinch off from the membrane. Acidification of the lumen of the caveolae results in dissociation of the ligand from the MFR, after which the ligand is translocated across the membrane via a specific carrier protein, putatively the RFC [56]. Which mechanism is operative for (anti) folate uptake via MFR in MTX^R-ZR-75-1 (LF) and MDA-231 (LF) cells has not been established, except that a coupled process of initial binding of ligand to the receptor and internalization via RFC is not likely because of impaired RFC transport in these cells. The growth-inhibitory effects of CB3717 and DDATHF against MTX^R-ZR-75-1 (LF) and MDA-231 (LF) cells support a role for MFR in transport of these compounds independent of RFC, which is consistent with previous reports on L1210 leukemia cells expressing both transport proteins [11]. Rather than the potocytosis process, it has been shown in KB cells that the classic receptor-mediated endocytosis pathway can be operative for the cellular uptake of folates [53, 54, 57, 58]. The extent to which the binding affinity of MFR for folates and antifolates will determine the efficiency of delivery of these compounds to intracellular compartments for polyglutamylation [59], their storage [60], and/or binding to the target enzymes is presently unknown. The outcome of these studies will further establish the functional role of MFRs in antifolate uptake, retention of polyglutamate forms [61], and growth-inhibitory potential.

In summary, we characterized the role of MFR in the uptake growth-inhibitory effects of classic and novel folate antagonists in vitro in two human breast-cancer cell lines with impaired RFC transport-related resistance to MTX. Cellular MFR levels of ≥ 2 pmol/10^7 cells were sufficient to internalize cytotoxic concentrations of folate antagonists for which MFR has a high (CB3717, DDATHF) affinity. Higher receptor protein levels may be required for moderate affinity (ZD1694) and low-affinity binders (MTX, EDX). Since MFR expression in various normal and neoplastic tissues has been documented to be in this range [35, 62], these results may be of significance in predicting drug sensitivity to tumor cells and drug-related toxicity to normal cells.

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