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Effects on dihydrofolate reductase of methotrexate metabolites and intracellular folates formed following methotrexate exposure of human breast cancer cells

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Dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase; EC 1.5.1.3) is the primary intracellular enzyme responsible for maintaining a pool of reduced folates. These folates serve as coenzymes in a number of one-carbon transfer reactions for the intracellular production of purines, thymidylate, and certain amino acids. The mechanism of action of methotrexate (MTX), a clinically important drug in the treatment of a variety of neoplasms, appears to be multifaceted. MTX is capable of potent inhibition of mammalian DHFR with a binding affinity estimated to lie between 1 nM and 1 pM [1]. Exposure of human breast cancer cells to MTX has been shown to lead to a rapid intracellular accumulation of dihydrofolate (FH_2) with the subsequent appearance of formyl-dihydrofolate (formyl-FH_2) [2]. The latter compound is presumed to be formed via a direct formylation of FH_2 as the amount of these two compounds follows an inverse relationship with a constant sum. This compound has been shown to be a weak substrate for chicken liver DHFR [3]. Its effect on other folate-requiring enzymes has not been reported. MTX undergoes the process of intracellular polyglutamation in breast cells with up to a total of five glutamate residues appended to the para-aminobenzoate moiety of the parent compound. This process imparts several peculiar properties to the MTX polyglutamates (PGs) that parallel those of the naturally occurring folate PGs and include an intracellular half-life that is inversely proportional to the PG tail length and a higher affinity (and, therefore, greater inhibitory potential) for many of the folate-requiring enzymes, including AICAR transformylase and thymidylate synthase [4, 5].

Several investigators have shown that MTX is metabolized in human liver to its 7-hydroxy form [6–8]. This form has also been found to undergo intracellular polyglutamation to metabolites that have been reported to have an enhanced affinity for DHFR [9, 10].

It is the purpose of this report to measure the interactions and potential inhibitory effects on human DHFR—the target enzyme for the parent compound—of the substances that accumulate in a cell following MTX exposure.

Materials and methods

Methotrexate and methotrexate-Glu_{1-4}, as well as folic acid-Glu_5, were obtained from the Drug Synthesis and Development Branch of the National Cancer Institute (Bethesda, MD). 7-Hydroxy-MTX and 7-hydroxy-MTX-Glu_4 were gifts from Drs. I. David Goldman and Richard Seither (Medical College of Virginia, Richmond, VA). Folic acid and sodium dithionite were purchased from the Sigma Chemical Co. (St. Louis, MO). 10-Formyl-FH_2 and 10-formyl-FH_2-Glu_5 were prepared by chemically formylating folic acid or folic acid-Glu_5 using the method of Blakely [11] followed by reduction to the final product using sodium dithionite [3].

Human DHFR (sp. act. 27.3 μmol/min/mg at 37°) was purified from cultured MCF-7 breast cancer cells as previously described [12].

Measurement of DHFR catalyzing activity. The catalytic activity of human DHFR was followed spectrophotometrically according to the method of Bertino and Fischer [13]. Each 1-ml reaction cuvette contained 1 pmol of enzyme, 2 μmol of NADPH, and various concentrations of substrate/inhibitors in 160 mM KCl and 160 mM Tris, pH 7.4. After a 10-min temperature equilibration period (37°), the reaction was initiated with the addition of 0.1 μmol of FH_2. Reactions were followed for 10 min, and the reaction velocities, were determined using an extinction coefficient for the reaction of 12×10^3. Formyl FH_2 was assayed as a substrate for DHFR using an identical method but using an extinction coefficient for the reaction of 21×10^3 at 270 nm (unpublished observations). All kinetic constants were calculated using standard Lineweaver–Burk plots. MTX and MTX-PGs were treated as slow tight-binding inhibitors. The calculation of kinetic constants was simplified by preincubation of the enzyme with the inhibitor to measure the effects of the inhibitors on the catalytic reaction at a time of steady-state interaction with the enzyme, i.e. after the time-dependent slow-binding phase of the inhibitor–enzyme interaction.

Results

Substrate kinetics. Table 1 illustrates the kinetic measurements for FH_2 mono- and pentaglutamates when used as substrates for the reaction catalyzed by human reductase. The Michaelis–Menten constants (K_m) for each substrate in either parent or polyglutamated form were equivalent (1.1. to 1.5 μM).

Inhibition of human DHFR. The inhibition of DHFR by MTX, MTX-PGs, and its metabolites 7-OH-MTX and 7-OH-MTX-Glu_4 is shown in Table 2. For each inhibitor, the K_i value was determined using both the mono- and polyglutamated FH_2 as the substrate for the reaction. Formyl-FH_2 and 7-OH-MTX acted as competitive inhibitors. MTX and MTX-PGs were considered tight-binding inhibitors. In parallel with the effect of polyglutamation on the affinity of the folate co-substrates for DHFR, the polyglutamation of MTX was found to have little effect on the inhibitory potential of this compound. With respect to FH_2-monoglutamate, the higher MTX-PGs were less than 2-fold more potent than the parent inhibitor. This minimal increase in potency of the MTX-PGs was not apparent when FH_2-Glu_5 was used as the substrate for the reaction. By contrast, the inhibitory potential of 7-OH-MTX ($K_i = 2.6–7.4 \times 10^{-8}$ M) was increased by the tetraglutamate such that this compound was 10-fold more potent than the parent metabolite when competing with the monoglutamated FH_2 substrate; like MTX, this increment in

Table 1. Kinetic constants of dihydrofolate and dihydrofolate pentaglutamate as substrates for human dihydrofolate reductase

Substrate	K_m (μM)	V_{max} (nmol/mg/min)	Relative specificity constant† (V_{max}/K_m)
FH_2	1.1 ± 0.5*	177 ± 103*	1.0
FH_2–Glu_5	1.5 ± 0.8	353 ± 135	1.5

* Mean ± SEM, N ≥ 3.
† V_{max}/K_m for FH_2 = 1.0.

potency was decreased to less than 3-fold when competing with the pentaglutamated FH_2 substrate.

10-Formyl-FH_2 as an inhibitor of DHFR. 10-Formyl-FH_2 was found to be a poor substrate for mammalian DHFR, with a relative specificity constant (a measure of substrate preference by the enzyme) that was 0.24% compared to FH_2. However, this compound was found to be a highly potent naturally occurring inhibitor of the reaction catalyzed by human DHFR with an inhibition potency similar to that of 7-OH-MTX. The inhibition constants for the monoglutamated form of the inhibitor were found to be 17 and 36 nM when facing FH_2–Glu_1 and Glu_5 respectively. Formyl-FH_2–Glu_5 was found to be 3- and 6-fold more potent than the monoglutamated inhibitor with respect to FH_2–Glu_1 and FH_2–Glu_5. Like MTX and 7-OH-MTX, formyl-FH_2 demonstrated competitive inhibition kinetics.

Discussion

This report suggests that the inhibition of DHFR following MTX exposure is a multifaceted event. Inhibition of DHFR would be initiated by MTX and enhanced by the subsequent generation of the non-exchangeable MTX-PGs, 7-OH-MTX and its PGs and, finally, formyl-FH_2 and its PGs. The contribution of each of these inhibitors in the overall continued inhibition of DHFR following MTX exposure would depend on the concentration of each inhibitor at a given time as well as their relative inhibition constants. MTX and MTX-PGs were the most potent inhibitors, with inhibition constants of 1.7 to 0.5 × 10^{-10} M as measured in the catalytic reaction, whereas 7-OH-MTX along with formyl-FH_2 and their respective PGs had equivalent K_i values 100- to 500-fold higher than MTX (7.4 to 0.6 × 10^{-8} M). Published reports suggest that micromolar concentrations of formyl-FH_2 may occur in cells following clinically achievable MTX exposures and that the serum concentrations of 7-OH-MTX in humans treated with high doses of MTX far exceed a 1 μM concentration.

Unlike the marked effects of polyglutamation of folate substrates and inhibitors on their binding affinities for several other folate-requiring enzymes, including thymidylate synthase [5], AICAR transformylase [14], and methylene tetrahydrofolate reductase [14], the effects of polyglutamation on both substrate affinity and inhibitor potency for DHFR appear to be minimal. The MTX-PGs and 7-OH-MTX-PGs have modestly enhanced inhibitory potencies as compared to their monoglutamated counterparts, but this increment was negated when pentaglutamated FH_2 was used as the substrate. Polyglutamation of formyl-FH_2 significantly enhanced its inhibition capacity, and this was preserved even when tested against the pentaglutamated substrate. The change in an inhibitor's K_i as a function of substrate glutamylated state is not a novel finding but has been reported previously for the competitive inhibition of AICAR transformylase by MTX-PGs and FH_2-PGs when tested against its mono- and pentaglutamated folate substrate, 10-formyltetrahydrofolate [4].

Table 2. Inhibition constants (K_i) of intracellular metabolites formed following methotrexate exposure of the human breast cancer cell line MCF-7

Substrate	Inhibitor	K_i (nM)
FH_2	MTX	0.11 ± 0.03
	MTX–Glu_2	0.17 ± 0.05
	MTX–Glu_3	0.08 ± 0.03
	MTX–Glu_5	0.08 ± 0.01
	7-OH-MTX	74.0 ± 2.0
	7-OH-MTX–Glu_4	8.4 ± 1.7
	Formyl-FH_2	17.0 ± 1.0
	Formyl-FH_2–Glu_5	6.4 ± 0.1
FH_2–Glu_5	MTX	0.055 ± 0.001
	MTX–Glu_2	0.11 ± 0.01
	MTX–Glu_3	0.048 ± 0.002
	MTX–Glu_5	0.051 ± 0.002
	7-OH-MTX	26.0 ± 11.0
	7-OH-MTX–Glu_4	9.2 ± 0.4
	Formyl-FH_2	36.0 ± 3.0
	Formyl-FH_2–Glu_5	6.2 ± 0.3

* Each value is the mean ± SEM, N ≥ 3.

These results suggest that the inhibition of DHFR may be initiated and prolonged by a variety of inhibitors that are formed within the cell at various times after exposure to MTX and that inhibition by these metabolites may sustain DHFR inhibition. The accumulation of formyl-FH_2 has been found in normal myeloid precursor cells as well as the MCF-7 breast cell line. The extent to which these inhibitors, particularly the polyglutamates of MTX and 7-OH-MTX, are formed in malignant versus normal cells may explain some of the selective cytotoxic effects of MTX against malignant cells.

In summary, MTX, a potent antineoplastic agent, is a potent inhibitor of DHFR. Once inside the cell, MTX is converted to its polyglutamated forms (MTX–Glu_n) with 1 to 4 additional glutamates in γ linkage. MTX is also metabolized by the liver to 7-OH-MTX, which may also undergo intracellular polyglutamation. Exposure of breast cancer cells to MTX leads to the intracellular accumulation of FH_2, formyl-FH_2 and their polyglutamates. We have examined the inhibition of DHFR by MTX, MTX–Glu_n, 7-OH-MTX and its tetraglutamated metabolite, and formyl-FH_2 mono- and pentaglutamate using both FH_2–Glu_1 and FH_2–Glu_5 as substrates for DHFR. The K_m for FH_2–Glu_1 and FH_2–Glu_5 was 1 μM. The potency of inhibition of DHFR by MTX and formyl-FH_2 was found to be 1.1 × 10^{-10} and 1.7 × 10^{-8} M, respectively, and was not increased by the addition of glutamyl groups, whereas 7-OH-MTX-Glu_4 (K_i = 8.4 × 10^{-9} M) was 7- to 8-fold more potent than 7-OH-MTX. Although formyl-FH_2 and 7-OH-MTX were 100-fold less potent than MTX as inhibitors of DHFR, consideration of their respective pool sizes following cellular exposure to MTX will be necessary to determine their relative importance.

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Secretion and properties of a polypeptide factor generated by phorbol ester stimulation of human blood platelets

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12-Deoxyphorbolphenylacetate (DOPP) was shown to be the most potent of a range of hydroxy- and deoxy-phorbol esters in terms of induction of human platelet aggregation [1, 2]. This compound is also a known tumour promoting agent [3] *in vivo* and will activate the phospholipid and calcium-dependent protein kinase C [4] in a cell free assay. Protein kinase C is believed to be the phorbol ester receptor site [5] and one member of this series of compounds, tetradecanoylphorbolacetate (TPA), has been shown to induce platelet aggregation which may be associated with myosin light chain phosphorylation [6].

The direct aggregating effect of DOPP on platelets is followed by a small release reaction from dense granules together with the release of a transferable aggregating substance (TAS) [7, 8]. As part of our investigations into phorbol ester induction of platelet aggregation we have investigated the production and properties of TAS following platelet aggregation induced by DOPP.

Materials and methods

Reagents. Pinane thromboxane A_2 was provided by Dr J. B. Smith (Philadelphia, PA), prostacyclin (PGI_2) by Dr J. E. Pike (Upjohn Co., MI) and β-γ-methylene ADP, platelet aggregating factor (PAF) and phenidone by Dr J. Westwick (London, U.K.). Verapamil was purchased from Roche (Basle, Switzerland), EDTA and trypsin from Sigma (U.K.), *p*-bromophenacylbromide from BDH (Poole, U.K.), trifluoperazine from Smith, Kline & French (U.K.), indomethacin from Merck, Sharp and Dohme (Rathway, U.S.A.) and promethazine from May and Baker (Dagenham, U.K.).

12-Deoxyphorbolphenylacetate (DOPP) was isolated from the fresh latex of *Euphorbia poissonii* [9]: human venous blood was collected from healthy male donors, platelet-poor plasma (PPP) and platelet-rich plasma (PRP) were prepared as previously described [8]. Rabbit blood was collected from the marginal ear vein of New Zealand White laboratory animals and PPP and PRP prepared as above.

Secretion of TAS. A Born Mk. III aggregometer was used to monitor the platelet aggregations. Acetone was used as the solvent for DOPP and this solvent had no effect upon platelets at a maximum concentration of 0.5% in PRP. Aggregation was quantified by determining the maximum aggregation obtained within 4 min. A dose–response curve was obtained for DOPP induced aggregation and the minimum dose of DOPP that induced aggregation was found to be 0.09 μM. DOPP (0.43 or 0.86 μM) was added to 500 μl of PRP in an aggregometer cuvette and 100 μl of PRP was removed at intervals from 30 sec. to 1 hr and added to 400 μl of fresh recipient PRP. The degree of aggregation of the recipient PRP was determined as before.

Inhibition of TAS production. Phenidone (0.5 mM in ethanol) or trypsin (20 μl of 0.1 mg/ml in phosphate buffer saline at pH 7.4) were added to 500 μl of PRP 1 min before the addition of 0.86 μm DOPP. After 4 min 100 μl of PRP was transferred to recipient PRP and aggregation monitored as above.

Inhibition of TAS induced aggregation. TAS was produced by the addition of either 0.43 or 0.86 μM of DOPP to PRP. To the recipient platelets inhibitors dissolved in ethanol or acetone were added 1 min before the introduction of 100 μl of donor PRP. The concentrations of inhibitors used is given in Table 1.

Response of stored platelets. PRP was stored for 24 hr at −4°, and DOPP-induced aggregation carried out as before.

Storage of TAS. 500 μl of PRP subsequent to DOPP-induced aggregation (0.86 μM) was centrifuged at 2700 *g* for 30 min and the supernatant plasma was stored at −4° for 24 hr. Quantities of plasma (100 μl) were used to induce aggregation in 400 μl of recipient platelets as before.

Washed platelet system. Whole human blood (50 ml) was mixed with 7.5 ml of ACD (1.4% citric acid, 2.5% trisodium citrate and 2% glucose). The pH was adjusted to 6.5 with sodium hydroxide, centrifuged at 650 *g* for 10 min and the pH of the PRP adjusted to 6.5 with 110 mM citric acid. EDTA (100 μl, 1 mM per 10 ml) was added. The suspension was recentrifuged at 2500 *g* for 10 min and the platelets suspended in citric acid buffer pH 6.5; EDTA (1 mM) and PGE_1 (7 ng/ml) were added. The suspension was recentrifuged for 7.5 min and the pellet resuspended as before. This suspension was again centrifuged and resuspended in a solution of KCl (0.0373%), NaCl (0.671%), $CaCl_2$ (0.0294%), $MgCl_2$ (0.0203%) and Tris (0.375%). To this was added 90 mg/100 ml of glucose and the pH adjusted to 7.4. The suspended washed platelets were used for aggregation studies (500 μl) as previously described for PRP. These aggregations were repeated upon the addition of 10 μl of 1.01% $MgCl_2$, 100 mM $CaCl_2$ and 50 mg/ml of fibrinogen to the agregometer cuvette.