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Inhibition of human dihydrofolate reductase by antifolyl polyglutamates

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Methotrexate (MTX*), 10-deazaaminopterin (10-DAM), 10-ethyl-10-deazaaminopterin (10-EDAM) and aminopterin (AMT) are potent inhibitors of dihydrofolate reductase (DHFR; EC 1.5.1.3) [1, 2]. Polyglutamate derivatives of these analogs have a prolonged intracellular half-life [3, 4] and have an increased affinity for folate requiring enzymes such as thymidylate synthase (EC 2.1.1.45) [5] and aminoimidazolecarboxamide ribonucleotide formyltransferase (EC 2.1.2.3) [6]. Folate substrates also exist intracellularly as polyglutamates, and many folate enzymes show a higher affinity for folate polyglutamate substrates than for their monoglutamate counterparts [5]. While binding studies in cell extracts indicate that MTX polyglutamates have an affinity for DHFR as high [3, 7, 8] or higher [9, 10] than MTX, the effect of antifolylpolyglutamates on mammalian DHFR activity has not been studied extensively. However, increasing inhibition of DHFR from sheep, beef and chicken liver is observed as the MTX glutamate chain is lengthened [11]. Another indication that polyglutamate chain length is a factor in DHFR activity is that, when $H_2PteGlu_5$ is substituted for $H_2PteGlu_1$ as substrate, the inhibitory potency of MTX derivatives is decreased [11]. The effect of polyglutamylation on the inhibitory potency of MTX is much less pronounced for *Lactobacillus casei* DHFR [12] than for animal DHFRs [11].

We report here on the inhibitory potency of polyglutamates of MTX, 10-DAM, 10-EDAM and AMT for pure human DHFR using $H_2PteGlu_1$ or $H_2PteGlu_5$ as substrate. We show that the polyglutamate chain length of both inhibitor and substrate are determinants of human DHFR inhibition.

Materials and methods

Poly-gamma-glutamyl derivates of folate and folate analogs were synthesized by the solid phase method [13]. All were analyzed by HPLC [11] to verify their authenticity and purity. None of the compounds showed significant contamination. $H_2PteGlu_5$ was prepared from $PteGlu_5$ by dithionite reduction [14]. The concentrations of $H_2PteGlu_1$ and $H_2PteGlu_5$ were determined by their absorbance at 282 nm in 0.2 M 2-mercaptoethanol using a molar absorbance coefficient of 19,000 [15]. Both substrates were stoichiometrically reduced to tetrahydro forms in the presence of DHFR. The concentrations of antifolates and antifolate polyglutamates were determined by their absorbance at 256 nm in 0.1 N KOH. The molar absorbance coefficients used were 23,000, 28,500, 33,000 and 31,000 for MTX [15], AMT [15], 10-DAM [13] and 10-EDAM [13] respectively.

The DHFR reaction was monitored in a Gilford model 250 spectrophotometer at 30° using the decrease in absorbance at 340 nm that occurs when NADPH and $H_2PteGlu_1$ are converted to $NADP^+$ and $H_4PteGlu_1$. The incubation mixture contained: NADPH, 0.14 mM; $H_2PteGlu_1$ or $H_2PteGlu_5$, 0.1 mM; Tris–HCl buffer, pH 7.2, 50 mM. The reactions were initiated by addition of an amount of enzyme sufficient to obtain a change of absorbance of 0.027/min. NADPH, $H_2PteGlu_1$ and $H_2PteGlu_5$ were present at saturating concentrations. Control rates obtained with $H_2PteGlu_1$ and $H_2PteGlu_5$ were the same. The reaction velocities obtained at various inhibitor concentrations were plotted as described [11] to determine the IC_{50} values.

Human DHFR was obtained by recombinant DNA techniques using an overproducing plasmid vector carried by *Escherichia coli* [16]. The K_m values for NADPH and dihydrofolate, the isoelectric point, and the N-terminal amino acid sequence for this preparation were in close agreement with those of the enzyme isolated from WIL2/M4 human lymphoblastoid cells [17].

Results and discussion

Polyglutamylation of MTX, 10-DAM, 10-EDAM and AMT resulted in a progressive decrease in IC_{50} values (Fig. 1) with $H_2PteGlu_1$ as substrate. For example, the IC_{50} values decreased 2-,2-,3- and 2-fold when comparing the Glu_1 forms to the Glu_4 forms for MTX, 10-DAM, 10-EDAM and AMT respectively. The largest overall effect was seen in the 10-DAM series where the IC_{50} decreased 5-fold

* Abbreviations: MTX, methotrexate; DHFR, dihydrofolate reductase; AMT, aminopterin; 10-DAM, 10-deazaaminopterin; 10-EDAM, 10-ethyl-10-deazaaminopterin; and $PteGu_1$, folic acid with subscript numeral indicating the total number of Glu residues.

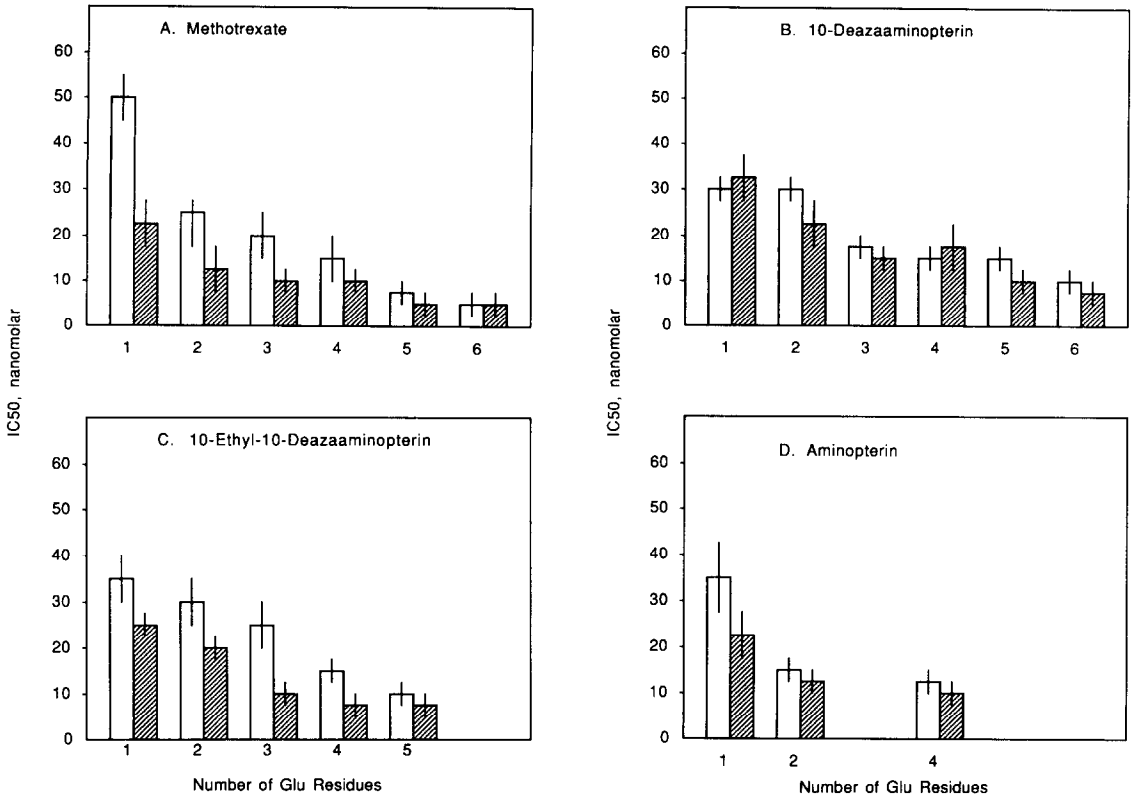

Fig. 1. Inhibition of human dihydrofolate reductase by polyglutamyl derivatives of (A) methotrexate, (B) 10-deazaaminopterin, (C) 10-ethyl-10-deazaaminopterin and (D) aminopterin. The hatched bars indicate values obtained with $H_2PteGlu_1$ as substrate and the open bars indicate values obtained with $H_2PteGlu_5$ as substrate. The solid vertical bars indicate the standard deviation of three trials.

between 10-DAM and the derivative with six Glu residues (Fig. 1B).

With $H_2PteGlu_5$ as substrate, a progressive decrease in IC_{50} values was also seen as Glu residues were added to the inhibitors (Fig. 1); however, the IC_{50} values were increased up to 2.5-fold higher than with $H_2PteGlu_1$ as substrate. The 10-DAM series differed from this general pattern in that the IC_{50} values were similar with $H_2PteGlu_1$ or $H_2PteGlu_5$ as substrate. Comparing the IC_{50} values of the Glu_1 forms of the inhibitors with the Glu_4 forms, as above, the values decreased 3-,2-,2- and 3-fold for MTX, 10-DAM, 10-EDAM and AMT respectively. Thus, changing the substrate from $H_2PteGlu_1$ to $H_2PteGlu_5$ did not alter greatly the relative inhibitions obtained as the inhibitor Glu chain was lengthened.

We conclude that polyglutamylation of MTX, 10-DAM, 10-EDAM and AMT made them better inhibitors of human DHFR. Conversely, polyglutamylation of the substrate $H_2PteGlu_1$ decreased inhibitory potency, except for the 10-DAM series. Our results show that polyglutamate chain length of inhibitor and substrate can be important variables in the inhibition of human DHFR.

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Differential formation of dinitrate metabolites from glyceryl trinitrate in subcellular fractions of rabbit liver

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Glyceryl trinitrate (GTN) was first synthesized in the 19th century. Since then, it has been the most commonly used organic nitrate for the treatment of angina pectoris. The mechanism of action of GTN and other organic nitrates is coupled to the metabolism of the organic nitrates *in vivo*, as defined by the *S*-nitrosothiol hypothesis of Ignarro *et al.* [1]. The first step in the scheme, i.e. formation of glyceryl dinitrate metabolites (1,2-GDN and 1,3-GDN) and an inorganic nitrite ion, is crucial for the production of the pharmacological effect.

It has been widely accepted that the metabolic conversion from GTN to GDNs is mediated by the enzyme glutathione *S*-transferase. Yet, when homogenates of different organs are incubated with GTN, different ratios of the GDNs are formed [2–5]. Previous work from our laboratory [6] has also shown that ratios of GDNs differ following various routes of administration to humans. Although these data may be explained by the presence of isozymes of glutathione *S*-transferases in different organs, it is equally possible that other pathways, both enzymatic and non-enzymatic, can be responsible for GTN metabolism. This study was designed to investigate the patterns of GTN metabolism in cytosolic and microsomal fractions of rabbit livers, to probe whether such alternate pathways may be present.

Methods

New Zealand White rabbits (2–3 kg) were killed by decapitation. The liver of each animal was immediately perfused with ice-cold buffer (0.05 M Tris-Cl–0.15 M KCl, pH 7.4) to clear out the remaining blood in the organ. The organ was then mixed with 2 vol. of buffer, minced, and homogenized. Using differential ultra-centrifugation, the 105,000 *g* supernatant fraction and pellet were obtained.

In each incubation, 2.0 mg/ml of protein from either fraction was used, with protein content determined by the method of Lowry *et al.* [7]. Glutathione (2 mM) was added to the buffered sample solutions to avoid co-factor depletion. GTN, at a starting concentration of 20 ng/ml, was added to the 37° incubation, and 500-μl samples were drawn at 0.5, 5, 10, 15, 30, 60, 90 and 120 min. Further reaction was stopped by immersing the samples into a mixture of dry ice and methanol.

Concentrations of GTN and GDNs were measured simultaneously using our capillary gas-chromatographic assay [8] with one minor change. Instead of a mixture of pentane and methylene chloride, 3 × 10 ml mixtures of pentane and methyl-*t*-butyl ether (80%:20%) were used to extract the samples. 2,6-Dinitrotoluene (10 ng/500 μl sample) was used as the internal standard. A clean baseline was obtained, with the peaks for 1,3-GDN, 1,2-GDN, and GTN clearly separated. Linearity was observed between the range of 0.25 and 20 ng/ml.

Results and discussion

GTN was metabolized in both subcellular fractions. However, the first-order degradation rate of GTN was 2- to 4-fold more rapid in the cytosolic fractions than in the microsomal fractions in each of the preparations from four animals. Moreover, the patterns of metabolite formation were different in the two subcellular fractions.

The formation of GDN metabolites in the cytosolic fraction is shown in Fig. 1a, where preferential formation of