Decreased Folylpolyglutamate Synthetase Activity as a Mechanism of Methotrexate Resistance in CCRF-CEM Human Leukemia Sublines*

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Determinants of methotrexate (MTX) resistance in cell lines resistant to short, but not continuous, MTX exposure were investigated since such lines may have relevance to clinical resistance. CCRF-CEM R30dm (R30dm), cloned from CCRF-CEM R30/6 (a MTX-resistant subline of the CCRF-CEM human leukemia cell line), had growth characteristics similar to CCRF-CEM. R30dm was resistant to a 24-h exposure to levels as high as 300 μM MTX but was as sensitive as CCRF-CEM to continuous MTX exposure. MTX resistance of R30dm was stable for >68 weeks in the absence of selective pressure. Initial velocities of MTX transport were comparable for R30dm and CCRF-CEM, as were dihydrofolate reductase specific activity and MTX binding. A 2-fold thymidylate synthase activity decrease for R30dm from that of CCRF-CEM was not a significant factor in R30dm MTX resistance. Decreased MTX poly(γ-glutamate) synthesis resulted in lower levels of drug accumulation by R30dm. Decreased polyglutamylation was attributable to folylpolyglutamate synthetase (FPGS) activity in R30dm extracts which was 1, 2, and ≤10% of CCRF-CEM extracts with the substrates MTX, aminopterin, and naturally occurring folic acids, respectively. Comparison of cell lines with varying levels of resistance to short term MTX exposure indicated that the extent of MTX resistance was proportional to the reduction of FPGS activity. The evidence supported decreased FPGS activity as the mechanism of resistance to short MTX exposure in the cell lines investigated.

The antifolate MTX is an anticancer agent used clinically for treatment of leukemia, breast cancer, and choriocarcinoma as well as other cancers (2, 3). One factor limiting its effectiveness is resistance to the drug which is often developed by patients during treatment (4). To circumvent problems of resistance it is desirable to understand the causative mechanisms. Although several mechanisms of MTX resistance have been identified in cell lines developed in laboratory settings (5) a clear correlation has not been established between these mechanisms and clinical MTX resistance (4). This discrepancy may be related to the methods used to develop MTX-resistant cell lines. Traditional laboratory methods utilize continuous exposure to successively higher concentrations of drug which often far exceed those used in the clinic (3) whereas clinical protocols usually involve repeated, intermittent exposures to a single drug dose. A selection method designed to mimic the clinical protocol was employed recently by Pizzorno et al. (6) to develop MTX-resistant cell lines from the human leukemia cell line CCRF-CEM. MTX resistance was developed through repeated short term (24-h) exposure of CCRF-CEM cells to MTX concentrations corresponding to moderate to high clinical levels of the drug. Two cell lines selected for resistance to 24-h exposure to 3 μM (R3/7) and 30 μM (R30/6) MTX were shown to be resistant solely as a result of impaired polyglutamylation of MTX (6). The source of the impaired polyglutamylation was not determined. However, we now report further characterization of these resistant cell lines and R30dm, a clone of R30/6 derived under selective pressure. The results establish that decreased activity of the enzyme responsible for synthesis of folate and MTX polyglutamates, folylpolyglutamate synthetase, is responsible for the observed MTX resistance.

MATERIALS AND METHODS

RESULTS

Characterization of R30dm—The R30dm cell line was derived from CCRF-CEM R30/6 (6) by cloning under selective

The abbreviations used are: MTX, methotrexate (4-smino-N10-carboxyl), methylpteroylgulamic acid; 4-NH-C=CH2-PteGlu, pterolylglutamate derivatives of MTX in which the subscript n is total glutamates in the linkages; L-[14C]Glu, L-[5,6-3H]glutamic acid; AMT, aminopterin (N(N)-aminopterinylglutamic acid); PteGlu, pteroylgulamic acid, folic acid; H2PteGlu, tetrahydrofolic acid; H2PteGlu, PteGlu, 7,8-dihydrofolic acid; H2PteGlu, 5,6,7,8-tetrahydrofolic acid; 5-HCO3-, 5,10-CH2-, 5,10-CH2-, 5-formyl-, 5,10-methylen-, and 5-methyltetrahydrofolic acid, respectively; FPGS, folylpolyglutamate synthetase; R30dm, CCRF-CEM R30dm; HPLC, high-performance liquid chromatography.

1 Portions of this paper (including "Materials and Methods" and Figs. 5–7) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Decreased FPGS Activity as a Mechanism of MTX Resistance

### Growth characteristics of R30dm and CCRF-CEM cell lines

The level of MTX resistance of R30dm was first assessed by monitoring the time for return to logarithmic growth after a 24-h exposure to MTX (Fig. 1). The times for R30dm cells to double the initial seeding density (N) after treatment with 30, 100, and 300 μM MTX were 76 ± 7 h (n = 11), 82 ± 5 h (n = 3), and 88 h, respectively (Fig. 1; data for treatment with 300 μM MTX not shown). Once the 2N level was achieved, growth was logarthmic for each treatment level (average generation time = 22 h) and paralleled that of untreated cells. CCRF-CEM cells were unable to double over the 260 h of the experiment even at 30 μM MTX. Stability of R30dm MTX resistance was also assessed by this method (see "Stability of the Resistance Phenotype," below).

### MTX Transport—Diminished ability to transport MTX

The average initial velocity of [3H]MTX uptake and the time course of intracellular [3H]MTX accumulation were measured for whole cells of CCRF-CEM and R30dm after treatment with either 2 or 5 μM MTX (cf. Fig. 3; data for 5 μM MTX not shown). Average initial velocities were 1.2 and 2.2 pmol/min/10^7 cells for CCRF-CEM, and 1.1 and 1.9 pmol/min/10^7 cells for R30dm after 2 and 5 μM MTX treatment, respectively. The average accumulation of total intracellular [3H]MTX at 30 min was 18.9 and 35.1 pmol/10^7 cells for CCRF-CEM and 14.4 and 25.5 pmol/10^7 cells for R30dm after 2 and 5 μM MTX treatment, respectively. At both MTX treatment levels, the amount of total intracellular drug had reached a plateau after 20 min for R30dm but was increasing at 30 min for CCRF-CEM.

### Dihydrofolate Reductase Activity—Increases in dihydrofolate reductase levels or activity as well as alterations in MTX binding by dihydrofolate reductase are also known mechanisms of resistance to MTX (3). The average dihydrofolate...
reductase specific activity in crude extracts was 9.7 × 10⁻³ and 7.4 × 10⁻³ pmol/min/mg of protein (8.0 × 10⁻³ and 7.7 × 10⁻³ pmol/min/10⁷ cells) for CCRF-CEM and R30dm, respectively. Mixing experiments indicated that no soluble activators or inhibitors of dihydrofolate reductase were present in either extract (data not shown). There was essentially no difference in MTX Iₐₜ values between CCRF-CEM (0.82 × 10⁻⁵ m) and R30dm (1.0 × 10⁻⁵ m). Based on analysis of these data (see "Materials and Methods") the dihydrofolate reductase level was 8.2 and 7.7 pmol/10⁷ cells in CCRF-CEM and R30dm, respectively. Dihydrofolate reductase binding capacity was also assessed after treatment of whole cells with either 2 (Fig. 3, inset) or 5 μM MTX. The average amounts of [³H]MTX bound at 30 min, a time point at which the dihydrofolate reductase was saturated (as indicated by the plateau of binding [³H]MTX, Fig. 3, inset), were 5.1 and 5.0 pmol/10⁷ cells for CCRF-CEM and R30dm cells, respectively, after 2 μM MTX treatment and 6.3 pmol/10⁷ cells in each cell line after 5 μM MTX treatment.

**Thymidylate Synthase Activity**—Reduced thymidylate synthase activity is another mechanism of MTX resistance (3). In whole cells, rates of [³H]dUMP release during the thymidylate synthase-mediated conversion of [⁵⁻³H]dUrd to thymidylate were 5.3 × 10⁻⁴ and 5.0 × 10⁻⁴ pmol/min/10⁷ cells for CCRF-CEM and R30dm, respectively. In crude extracts, the thymidylate synthase activity of CCRF-CEM (6.5 ± 0.8 × 10⁻⁴ pmol/min/mg of protein; 5.0 ± 0.6 × 10⁻⁴ pmol/min/10⁷ cells, n = 3) was twice that of R30dm (3.0 ± 0.3 × 10⁻⁴ pmol/min/mg of protein; 3.0 ± 0.4 × 10⁻⁴ pmol/min/10⁷ cells, n = 3). Mixing experiments indicated that no soluble inhibitors or activators of thymidylate synthase were present in either extract (data not shown). To assist in judging whether the 2-fold reduction in thymidylate synthase activity was a factor in R30dm MTX resistance, growth inhibition by other thymidylate synthase and dihydrofolate reductase inhibitors (under conditions of 120-h continuous exposure) was assessed. Average EDb₀ values for two thymidylate synthase inhibitors were as follows against CCRF-CEM and R30dm, respectively; 5-fluorouracil (0.8, 1.8 nM), and 5-fluorouracil (2.2, 1.6 nM). Trimetrexate (8) was the dihydrofolate reductase inhibitor tested; the EDb₀ values were 2.3 and 0.6 nM for CCRF-CEM and R30dm, respectively.

**MTX Polyglutamylation**—Polyglutamylation is a determinant of cellular retention of MTX (9). The ability of CCRF-CEM and R30dm to metabolize intracellular MTX to polyglutamate forms was assessed. With either 2 or 5 μM MTX at 4 min there was little or no MTX polyglutamylation in either cell line (data not shown). At 30 min, the amounts of unmetabolized MTX were similar, but CCRF-CEM displayed a significant accumulation of 4-NH₂-10-CH₃-PteGlu₄-aₜ and 4-NH₂-10-CH₃-PteGlu₄, whereas only a small amount of 4-NH₂-10-CH₃-PteGlu₄-aₜ was present in R30dm (Table I (2 μM extracellular MTX)). Neither N-(a-methylation)glutamic acid nor N-(a-methylation)benzoyl]glutamic acid, two degradation products of MTX, was detected in any HPLC analysis.

Both cell lines displayed similar levels of unmetabolized MTX in whole cells extracted after a 24-h incubation with 10 μM MTX, but there was a marked difference in the MTX polyglutamates formed (Table I (10 μM extracellular MTX)). 4-NH₂-10-CH₃-PteGlu₄-aₜ constituted 52% of intracellular MTX. The uncloned CCRF-CEM R30/6 line, from which R30dm was derived, displayed FPGS specific activity (5 ± 20 pmol/ h/mg of protein, n = 3) 4% of that of CCRF-CEM. CCRF-CEM R3/7, also developed by "pulse" exposure but at 3 μM instead of 30 μM MTX (6), showed 25% of the activity (30 ± 40 pmol/ h/mg of protein, n = 3) of CCRF-CEM. In all cases, mixing experiments combining crude extracts of the resistant lines with either crude extracts of the wild-type CCRF-CEM or with partially purified CCRF-CEM FPGS indicated that no soluble activators or inhibitors of FPGS were present in any extracts (data not shown).

**Stability of the Resistance Phenotype**—R30dm cells were routinely cultured in MTX-free medium; their outgrowth behavior after a 24-h exposure to 30 μM MTX was used to assess stability of MTX resistance in the absence of selection pressure. The time of recovery to 2N and 4N levels of growth (Fig. 4) and the EDb₀ values for a 14.5-h MTX exposure (not shown) remained constant for R30dm cells over a 68-week period (approximately 570 generations). Growth characteristics of untreated cells of both lines remained unchanged over this period as did the inability of CCRF-CEM to recover from 30 μM MTX treatment. R30dm FPGS specific activity in fresh cell extracts, with MTX and AMT as substrates, remained at low levels throughout a 71-week period (Fig. 4). CCRF-CEM FPGS activity assayed at each time point was

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Exposure time</th>
<th>Intracellular 4-NH₂-10-CH₃-PteGlu₄, forms</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 μM MTX</td>
<td>h</td>
<td>pmol/10⁷ cells</td>
</tr>
<tr>
<td>CEM</td>
<td>0.5</td>
<td>11.5 3.7 0.2 0.2 15.6</td>
</tr>
<tr>
<td>R30dm</td>
<td>0.5</td>
<td>12.0 0.1 12.1</td>
</tr>
<tr>
<td>10 μM MTX</td>
<td>h</td>
<td>61.8 23.4 29.2 11.4 2.7 0.8 129.3</td>
</tr>
<tr>
<td>CEM</td>
<td>24</td>
<td>51.0 0.8 0.4 52.2</td>
</tr>
<tr>
<td>R30dm</td>
<td>24</td>
<td>51.0 0.8 0.4 52.2</td>
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Fig. 4. Stability of MTX resistance over time as measured by recovery of outgrowth of R30dm cells and R30dm FPGS activity. The time required for R30dm cells to multiply to twice (2N; •) and four times (4N; ○) the initial seeding density after 24-h exposure (starting at t = 0) to 30 μM MTX was measured as described under "Materials and Methods," at intervals over a 71-week period. FPGS activity with MTX (■) and AMT (□) substrates was assayed in crude extracts of R30dm cells, as described under "Materials and Methods," at intervals over a 71-week period. FPGS activity data are reported as the mean ± S.E. (n ≥ 5).
1,280 ± 190 pmol/h/mg of protein (MTX, n = 4) and 2,240 ± 570 pmol/h/mg of protein (AMT, n = 6).

Characterization of R30dm FPGS—Dependence of FPGS activity on pH, ATP, L-Glu, KCl, and 2-mercaptoethanol was similar for CCRF-CEM and R30dm, and the requirements for optimal activity were the same for the two synthetases (data not shown). Substrate dependence curves of FPGS activity were generated for CCRF-CEM and R30dm for the substrates PteGlu, H₄PteGlu, (dl)-H₄PteGlu, (dl)-5,10-CH₂-H₄PteGlu, (dl)-5-HCO-H₄PteGlu, (dl)-5-CHO-H₄PteGlu, MTX, and AMT (Figs. 5-7). R30dm FPGS activity was substantially lower than that of CCRF-CEM at all concentrations of each substrate. The maximum specific activity achieved in each cell line and the ratio of maximum FPGS activity of R30dm to that of CCRF-CEM are shown in Table II. Of the folates tested, H₄PteGlu produced the highest activity with the synthetases of both cell lines and, at 0.10, the highest ratio of R30dm FPGS activity to that of CCRF-CEM. For the antifolates MTX and AMT, the relative FPGS activity of R30dm to CCRF-CEM was 0.01 and 0.02, respectively, lower than the ratio for any folate tested. Substrate concentrations required for maximum activity were higher in most cases for R30dm than for CCRF-CEM, but increases in activity above those at lower concentrations were small. With H₄PteGlu, (dl)-H₄PteGlu, and (dl)-5,10-CH₂-H₄PteGlu, substrate inhibition was noted for CCRF-CEM FPGS (Figs. 5B and 6, A and B). No substrate inhibition was noted with R30dm FPGS with any of the substrates. Because conditions of time and enzyme linearity could not be established for R30dm FPGS with the substrate (dl)-10-HCO-H₄PteGlu, results of assays with that substrate are not reported, but its measured activity was also low with R30dm.

The ratios of CCRF-CEM R3/7 FPGS activity in crude extracts to that of CCRF-CEM were 0.19, 0.20, and 0.26 with the substrates H₄PteGlu, AMT, and MTX, respectively. Substrate dependence curves of FPGS activity for these substrates with CCRF-CEM R3/7 were similar in shape to those for CCRF-CEM (data not shown).

**DISCUSSION**

These results indicated that the R30dm cell line was resistant to short term exposure to MTX, similar to the R30/6 line from which it was derived (6). In addition, R30dm cells were shown to be resistant to at least 500 μM MTX for 24 h, a level 10-fold higher than the selecting concentration. The lower level of R30dm MTX resistance indicated by comparison of ED₅₀ values for a 14.5-h exposure to MTX for R30dm and the wild-type CCRF-CEM is explained by the fact that the time required for R30dm cells to recover to logarithmic growth after MTX exposure (Fig. 1) was a major portion of the 120-h time period of the ED₅₀ experiments.

Several known determinants of MTX resistance (3) were ruled out as causes of the R30dm MTX resistance. In crude extracts, dihydrofolate reductase activity, dihydrofolate reductase inhibition by MTX, and binding of MTX by dihydrofolate reductase did not differ appreciably between the R30dm and sensitive cell lines. Thymidylate synthase specific activity in crude extracts was half as high for R30dm as CCRF-CEM. However, decreases of thymidylate synthase activity large enough to cause MTX resistance would result (12) in increased sensitivity to direct thymidylate synthase inhibitors and decreased sensitivity to other dihydrofolate reductase inhibitors (as a result of slower depletion of reduced folate pools). The continuous exposure ED₅₀ values for the two cell lines with 5-fluorouracil were similar. R30dm was actually cross-resistant to 5-fluoro-uracyl and collaterally sensitive to the lipid-soluble dihydrofolate reductase inhibitor trimethopterin under these conditions. Thus, the lower thymidylate synthase activity in R30dm apparently did not contribute to its MTX resistance. The paradoxical effects of 5-fluoro-uracyl and trimethopterin on R30dm may be a result of decreased folate pools secondary to the FPGS deficiency (13, 14). Effects of these inhibitors under conditions of 24-h "pulse" exposure will be examined in order to extend these data. Finally, decreased MTX uptake was eliminated as a mechanism of R30dm MTX resistance since the initial velocity of MTX uptake into whole cells was similar for CCRF-CEM and R30dm.

Despite similar uptake kinetics, there was a distinct difference in the ability of CCRF-CEM and R30dm to accumulate MTX at times >5 min. Decreased intracellular drug accumulation in R30dm was accounted for by reduction in levels of MTX polyglutamate formation (Table I). This indicated that resistance of R30dm to short exposures of MTX could be caused by decreased cellular retention of the drug resulting from reduced polyglutamylation as compared with the MTX-sensitive CCRF-CEM cells.

FPGS, the enzyme that catalyzes formation of polyglutamates of MTX and biologically important folates, was investigated as a source of the decrease in MTX polyglutamate synthesis observed in R30dm (Table I) and in R30/6 and R3/7 (6). Each MTX-resistant cell line developed by intermittent exposure to MTX exhibited decreased FPGS activity as compared with wild-type cells, with both folate and antifolate substrates. The magnitude of the FPGS activity decrease was directly proportional to the concentration of MTX used to select for resistance or the number of cycles of selection at one concentration. Therefore, a direct correlation was established between the reduced ability for polyglutamylation as a result of decreased FPGS activity and the degree of MTX resistance manifested by these cell lines.

The FPGS from R30dm and CCRF-CEM were characterized to determine the source of the reduced activity of R30dm. Mixing experiments showed that soluble activators and inhibitors were not involved. The concentrations of ATP and L-Glu giving optimal activity with both FPGS were identical, suggesting that increased Kₐ values for these substrates were not a factor; other reaction conditions were also comparable. Large increases in pteroyl substrate concentrations (up to 200 μM for the folates and 400 μM for MTX) resulted in only small increases in R30dm FPGS activity, suggesting that its decreased activity relative to CCRF-CEM was an effect on its apparent Vₘₐₓ and was not the result of increased Kₐ values for pteroyl substrates. However, because of the low activities observed with R30dm FPGS, actual determination of Kₐ

<table>
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<tr>
<th>Substrate</th>
<th>Maximum FPGS specific activity</th>
<th>Ratio of R30dm to CCRF-CEM</th>
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<tbody>
<tr>
<td>PteGlu</td>
<td>1,040 ± 80 40 ± 20</td>
<td>0.04</td>
</tr>
<tr>
<td>H₄PteGlu</td>
<td>1,230 ± 160 200 ± 30</td>
<td>0.10</td>
</tr>
<tr>
<td>(dl)-H₄PteGlu</td>
<td>1,570 ± 100 80 ± 20</td>
<td>0.04</td>
</tr>
<tr>
<td>(dl)-5,10-CHO-H₄PteGlu</td>
<td>1,590 ± 29 80 ± 15</td>
<td>0.05</td>
</tr>
<tr>
<td>(dl)-5-HCO-H₄PteGlu</td>
<td>770 ± 60 30 ± 10</td>
<td>0.04</td>
</tr>
<tr>
<td>(dl)-5-CHO-H₄PteGlu</td>
<td>900 ± 100 50 ± 5</td>
<td>0.04</td>
</tr>
<tr>
<td>MTX</td>
<td>1,180 ± 140 10 ± 10</td>
<td>0.01</td>
</tr>
<tr>
<td>AMT</td>
<td>2,170 ± 520 50 ± 25</td>
<td>0.02</td>
</tr>
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values was not possible. Further investigation is needed to determine whether the decreased activity is a result of decreased FPGS protein and/or decreased catalytic activity resulting from either a mutation in the FPGS gene or aberrant regulation. The greater decrease of R30dm FPGS activity with antifolates as compared with folates suggests an alteration of enzyme specificity in at least this case.

Decreased MTX polyglutamylation was reported previously in a few MTX-resistant cell lines; however, other mechanisms of resistance were also present in those lines (15–17). FPGS activity in cell extracts was assayed in one of those studies (15) and found to be equivalent to that of the sensitive line. The CCRF-CEM R30/6, R3/7, and R30dm cell lines are thus the first in which reduced polyglutamylation resulting from decreased FPGS activity was the only mechanism of MTX resistance identified. Although it is possible that heretofore undescribed factors are contributing, other currently known determinants of MTX resistance have been ruled out.

These resistant lines offer insights into the cellular requirements for folypolyglutamate synthesis. The level of FPGS activity required to maintain normal cell growth in CCRF-CEM must be significantly below the levels observed in wild-type CCRF-CEM since R30dm cells have similar growth characteristics but greatly reduced FPGS activity. A similar conclusion regarding the level of FPGS required for optimal growth was reached using Chinese hamster ovary cells containing graded levels of FPGS activity (18). The R30dm cell line provides an opportunity to investigate cellular requirements for FPGS activity and the effects of altered FPGS substrate specificity and decreased FPGS activity on folate levels and metabolism. These results also suggest that attempts to use FPGS inhibitors (19, 20) as single chemotherapeutic agents to disrupt folate metabolism and inhibit cell growth might be unsuccessful unless tight-binding inhibitors that lead to complete inhibition of the FPGS enzyme are developed.

Since these resistant lines were selected by a protocol that mimics the clinical use of MTX, their phenotype may be more relevant to clinical resistance than previously described mechanisms (3). The stability of this resistance phenotype and the occurrence of clinical resistance. If studies of clinical samples confirm that this mechanism occurs at a significant frequency, then these lines will offer a unique opportunity for discovering ways to circumvent this particular mode of resistance.

REFERENCES

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Diluted FPG Activity as a Mechanism of RTX Resistance

Supplemental Material to Increased Polyphosphate Synthesis Activity as a Mechanism of RTX Resistance in CRF CRN Nucleus Labeled Subunits

Deanna L. McCuskey, John J. McIsaac, Cynthia A. Russell, Brian G. Young, Jonathan A. Benton, Giuseppe Fierro, and Don C. Reddy

Diluted polyphosphate (polyP) is a potent virulence factor in certain pathogens, including Yersinia pseudotuberculosis. In previous studies, we demonstrated that polyP synthesis is upregulated in response to nutrient limitation, such as carbon source deprivation, and plays a role in bacterial virulence. We hypothesized that increased polyP synthesis might be a mechanism of resistance to rifampin (RTX), a common anti-TB drug, in Y. pseudotuberculosis. To test this hypothesis, we analyzed polyP synthesis in RTX-resistant strains and compared it to wild-type strains. Our results showed that polyP synthesis is increased in RTX-resistant strains and is correlated with reduced RTX sensitivity. These findings suggest that polyP synthesis may be a mechanism of RTX resistance in Y. pseudotuberculosis and provide new insights into the role of polyP in bacterial virulence. Future studies are needed to further investigate the relationship between polyP synthesis and RTX resistance in Y. pseudotuberculosis.
Decreased FPGS Activity as a Mechanism of MTX Resistance

Figure 6: Dependence of FPGS activity on A) d-Glu, B) (d)-5,10-DG-
HyProA, C) (dL)-5,10-DG-HyProA, and D) (dL)-5,10-DG-HyProA, at varying concentrations.
Standard conditions were used with the concentrations of both substrates varied as indicated to assay FPGS activity in crude extracts of NIH-3T3 (●) and KB/DS (■) cells.

Figure 7: Dependence of FPGS activity on MTX and AMF concentrations.
Standard conditions were used with the concentrations of MTX and AMF varied as indicated to assay FPGS activity in crude extracts of NIH-3T3 (●) and KB/DS (■) cells.