

# Multiple Mechanisms of Resistance to Polyglutamatable and Lipophilic Antifolates in Mammalian Cells: Role of Increased Folylpolyglutamylation, Expanded Folate Pools, and Intralysosomal Drug Sequestration

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Received September 11, 1998; accepted January 4, 1999

This paper is available online at <http://www.molpharm.org>

## ABSTRACT

Chinese hamster ovary  $\text{Pyr}^{\text{R100}}$  cells display more than 1000-fold resistance to pyrimethamine (Pyr), a lipophilic antifolate inhibitor of dihydrofolate reductase.  $\text{Pyr}^{\text{R100}}$  cells had wild-type DHFR activity, lost folate exporter activity, and had a 4-fold increased activity of a low pH folic acid transporter. Here we report on the marked alterations identified in  $\text{Pyr}^{\text{R100}}$  cells compared with parental cells: 1) ~100-fold decreased folic acid growth requirement; 2) a 25-fold higher glucose growth requirement in Pyr-containing medium; 3) a 2.5- to 4.1-fold increase in folypolyglutamate synthetase activity; 4) a 3-fold increase in the accumulation of [ $^3\text{H}$ ]folic acid and a 3-fold expansion of the intracellular folate pools; 5) a 4-fold increase in the activity of the lysosomal marker  $\beta$ -hexoseaminidase, suggesting an increased lysosome number/ $\text{Pyr}^{\text{R100}}$  cell; and 6) a small reduction in the steady-state accumulation of [ $^3\text{H}$ ]Pyr and no evi-

dence of catabolism or modification of cellular [ $^3\text{H}$ ]Pyr. Consequently,  $\text{Pyr}^{\text{R100}}$  cells were markedly resistant to the lipophilic antifolates trimetrexate (40-fold) and AG377 (30-fold) and to the polyglutamatable antifolates 5,10-Dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF) (26-fold) and AG2034 (14-fold). Resistance to these drugs was reversed in  $\text{Pyr}^{\text{R100}}$  cells transferred into folate-depleted medium. In conclusion, these multiple resistance factors collectively result in a prominent increase in folate accumulation, an expansion of the intracellular folypolyglutamate pool, and abolishment of the cytotoxic activity of polyglutamatable and lipophilic antifolates. The role of increased lysosome number per cell in sequestration of hydrophobic weak base drugs such as Pyr is also discussed as a novel mechanism of drug resistance.

Lipophilic antifolate inhibitors of dihydrofolate reductase (DHFR), including piritrexim, trimetrexate (TMQ), pyrimethamine (Pyr), and trimethoprim, find widespread use in the treatment of fungal, protozoal, and bacterial infections (Allegra et al., 1987; Borst and Ouelette, 1995). Because lipophilic antifolates can enter cells and tissues by passive or facilitated diffusion, they have been exploited as anticancer agents to circumvent transport-related resistance to classic antifolate drugs such as methotrexate (MTX) (Bertino, 1993). MTX is a divalent anion, which therefore depends on specific membrane transport systems for its cellular entry. At least three membrane transport systems have been shown to me-

diate the cellular uptake of MTX: 1) the major route is the reduced folate carrier (RFC) system (Goldman and Matherly, 1985; Sirotnak, 1985; Jansen, 1998a), 2) membrane folate receptors (Antony, 1992; Jansen, 1998a), and 3) a transporter that displays optimal activity at low pH (pH 5–6) (Sierra and Goldman, 1998; Assaraf et al., 1998). These transporters can operate exclusively or simultaneously in mammalian cells (Westerhof et al., 1995a, b).

Resistance to lipophilic drugs may involve multiple mechanisms, including decreased transport, increased efflux via the multidrug resistance gene product P-glycoprotein, and/or elevated levels of the target enzyme DHFR as a result of DHFR gene amplification (Borst and Ouelette, 1995; Assaraf et al., 1989a, b). Workers at our laboratory have isolated a Chinese hamster ovary (CHO)  $\text{Pyr}^{\text{R100}}$  cell line that dis-

This study was supported by grants from the Dutch Cancer Society (NKB-VU-96-1260) (G.J.) and Chemotech Technologies Ltd. (Y.G.A.).

**ABBREVIATIONS:** MTX, methotrexate; RFC, reduced folate carrier; TMQ, trimetrexate; Pyr, pyrimethamine; HF, high folate; LF, low folate; CHO, Chinese hamster ovary; FPGS, folypolyglutamate synthetase; DHFR, dihydrofolate reductase; TS, thymidylate synthase; GARFT, glycinamide ribonucleotide formyltransferase; THF, tetrahydrofolate; DHF, dihydrofolate; LV, leucovorin/5-formyltetrahydrofolate; 10-CHOTHF, 10-formyltetrahydrofolate; DDATHF, 5-10-Dideaza-5,6,7,8-tetrahydrofolic acid.

played more than 1000-fold resistance to Pyr (Assaraf and Slotky, 1993). These cells lacked P-glycoprotein expression, had wild-type DHFR activity levels, retained parental sensitivity to MTX, but were markedly cross-resistant to other lipophilic antifolate inhibitors of DHFR such as TMQ and piritrexim (Assaraf and Slotky, 1993). Recently, we found that Pyr<sup>R100</sup> cells lost folate exporter activity (Assaraf and Goldman, 1997) and had a 4-fold increased activity of a low pH folate transporter (Assaraf et al., 1998). These findings suggested that alterations in folate metabolism/homeostasis could play a contributing role in the antifolate-resistance phenotype of Pyr<sup>R100</sup> cells. Expanding on these recent studies, we here find that the 1000-fold Pyr resistance phenotype is accompanied by a 100-fold decreased folic acid growth requirement. Delineation of the multiple factors contributing to this antifolate resistance phenotype revealed 1) an increased folylpolyglutamate synthetase (FPGS) activity, 2) expansion of intracellular folate pools that undergo longer-chain polyglutamylation and thereby effectively abolish the cytotoxicity of various antifolates, and 3) an apparent increase in the number of lysosomes per Pyr<sup>R100</sup> cell, thereby suggesting an irreversible intralysosomal sequestration and accumulation to high concentrations of the hydrophobic weak base Pyr, rendering it less accessible to the target enzyme DHFR.

## Materials and Methods

**Chemicals.** RPMI-1640 cell culture medium with and without 2.3  $\mu$ M folic acid and (dialyzed) fetal calf serum (FCS) were obtained from GIBCO (Grand Island, NY). [3',5',7',9-<sup>3</sup>H]Folic acid (25–35 Ci/mmol) and [3',5',7-<sup>3</sup>H]MTX (15–25 Ci/mmol) were obtained from Moravsek Biochemicals (Brea, CA). These radiochemicals were purified before use by thin-layer chromatography as described previously (Westerhof et al., 1995a, b). [2,3-<sup>3</sup>H]-L-Glutamic acid (20–30 Ci/mmol, NET-395), formulated in 0.01 N HCl, was purchased from New England Nuclear (Boston, MA). [<sup>3</sup>H]Pyr (10 Ci/mmol) was kindly synthesized by Prof. E. Keinan (Department of Chemistry, Technion, Israel Institute of Technology, Haifa, Israel).

Folic acid, 5-formyltetrahydrofolate (leucovorin), and Pyr were obtained from Sigma Chemical Co. (St. Louis, MO). MTX-Glu<sub>2-6</sub> was purchased from Schircks Laboratories (Jona, Switzerland). The antifolate drugs used in this study were gifts from various institutions or persons as described previously (Westerhof et al., 1995b; Jansen et al., 1998). All other chemicals were of the highest grade of purity available.

**Cells and Tissue Cultures.** Unless otherwise indicated, wild-type CHO AA8 cells and their 1000-fold Pyr-resistant subline (Pyr<sup>R100</sup>) were grown in RPMI-1640 medium [containing 2.3  $\mu$ M folic acid, further referred to as "high folate" (HF) medium] supplemented with 10% FCS, 2 mM L-glutamine, and 100 units/ml of both penicillin and streptomycin. The cell culture medium for Pyr<sup>R100</sup> cells also included 100  $\mu$ M Pyr. Before experiments, Pyr<sup>R100</sup> cells were transferred at least three passages without Pyr. In other experiments, AA8 and Pyr<sup>R100</sup> cells were maintained in folate-deplete RPMI medium consisting of folate-free RPMI-1640 medium, 10% dialyzed FCS, 2 mM L-glutamine, and 100 units/ml of both penicillin and streptomycin. Folic acid was added to the cell culture medium at a minimal concentration that still allowed optimal growth (i.e., 100 nM folic acid for AA8 cells and 5 nM folic acid for Pyr<sup>R100</sup> cells). These conditions are further referred to a "low folate" (LF) medium. The monolayer cells were passaged twice a week by trypsinization (0.25% trypsin/0.05% EDTA in PBS). Cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

**Growth Inhibition Assay.** Cells were plated at an initial density of  $1 \times 10^4$  cells/cm<sup>2</sup> in individual wells of a 24-well tissue culture

plate. Appropriate concentrations of folic acid were added at the time of plating, and drugs were added 24 h later. After 72 h of drug exposure, cells were trypsinized, and viability counts were determined by trypan blue exclusion (Westerhof et al., 1995a). IC<sub>50</sub> values are depicted as the drug concentration at which cell growth is inhibited by 50% compared with controls that received no drug.

**[<sup>3</sup>H]Folic Acid/[<sup>3</sup>H]MTX Accumulation and Polyglutamylation.** For accumulation studies, AA8 and Pyr<sup>R100</sup> cells were plated onto 80-cm<sup>2</sup> tissue culture flasks and allowed to grow to approximately 70% confluency. At this stage, the culture medium was aspirated and replaced by medium containing 2  $\mu$ M [<sup>3</sup>H]folic acid or 1  $\mu$ M [<sup>3</sup>H]MTX (specific activity, 1.0 Ci/mmol). After 1, 4, and 24 h, the medium was aspirated, and the cells were washed twice with ice-cold HEPES-buffered saline, pH 7.4, and collected by trypsinization. Extraction of [<sup>3</sup>H]MTX-polyglutamates and their separation by HPLC were carried out as described previously (Westerhof et al., 1995a; Jansen et al., 1998b).

**Transport of [<sup>3</sup>H]Pyr in AA8 and Pyr<sup>R100</sup> Cells.** Transport of Pyr was measured by uptake of [<sup>3</sup>H]Pyr into monolayer cells. Cells ( $5 \times 10^4$  to  $1.0 \times 10^5$ ) were plated onto 24-well plates in normal growth medium. Before (48 h) transport assays, the drug-containing medium of Pyr<sup>R100</sup> cells was aspirated and replaced by 0.5 ml of  $\alpha$ -minimum essential medium lacking sodium bicarbonate. Aliquots of drug-containing medium were added such that the final volume was 1 ml, and the Pyr concentration was 1 to 50  $\mu$ M, depending on the experiment. The experiment was performed in a 37°C bath. At each time point, the medium was aspirated, and cells were washed twice with 5 ml of ice-cold PBS. Monolayer cells were lysed in 0.5 ml of a buffer containing 1% *n*-octylglucoside, 5 mM EDTA, 5 mM EGTA, and 10 mM Tris, pH 7.4. The cell lysate was quantitatively transferred into scintillation vials and counted for radioactivity. For efflux assays, cells were exposed to 1  $\mu$ M [<sup>3</sup>H]Pyr for 3 h, the medium was aspirated, and the cells were washed quickly with PBS and incubated in 1 ml drug-free medium. Washes, lysis, and scintillation counting were performed in the same manner as in uptake assays.

**Catabolism of Pyr in AA8 and Pyr<sup>R100</sup> Cells.** Potential cellular modification or degradation of Pyr was examined by HPLC profiles from cellular lysates using a method modified from Roberts et al. (1995). Cells ( $10^7$ ) in 100-mm petri dishes were incubated with 1  $\mu$ M [<sup>3</sup>H]Pyr for 3 h, washed twice with ice-cold PBS, and lysed in 3% perchloric acid. After protein precipitation and centrifugation for 5 min at 12,000g, the supernatant was recovered and analyzed by HPLC. The HPLC profile was compared with that of nonradioactive Pyr and [<sup>3</sup>H]Pyr standards that were run under identical conditions.

**Enzyme Assays.** FPGS activity, with 250  $\mu$ M MTX as a substrate, was determined according to procedures described previously (Jansen et al., 1992, 1998b). FPGH activity, with 100  $\mu$ M MTX-Glu<sub>2</sub> as a substrate, was measured essentially as described by O'Connor et al. (1991) and Jansen et al. (1998b).

Latent activity of enzymes located specifically in lysosomes was used as an indicator of an apparent increase in the number of lysosomes, using a method modified from Hall et al. (1978). Cells were washed twice in homogenization buffer (1 mM EDTA, 250 mM sucrose, pH 7.2), and lysates were prepared by homogenization on ice using a 5-ml Dounce homogenizer, until 70% disruption was achieved, as judged by light microscopy. Postnuclear fractions were prepared by centrifugation at 900g, 4°C, and collection of the supernatant, which was stored in aliquots at –80°C.

$\beta$ -Hexosaminidase activity was determined by incubation of cell lysates with 4.8 mM *p*-nitrophenyl- $\beta$ -*N*-glucosaminide, 100 mM sodium acetate, pH 5.0, and 0.1% Triton X-100 for 1 h at 37°C in a total reaction volume of 100  $\mu$ l. The reaction was stopped by the addition of 200  $\mu$ l of 50 mM NaOH. The reactions were carried out on 96-well microtiter plates. Enzyme activity was determined by measuring the product formed, as determined spectrophotometrically at 405 nm in an Anthos 2001 ELISA reader. Protein concentration was assayed according to the method of Bradford (1976).

**Analysis of Intracellular Folate Pools.** The measurement of reduced folate cofactors was made using procedures described previously (Bunni et al., 1994; Jansen et al., 1998b). Appropriate recovery experiments indicated that Pyr did not interfere with the assay (not shown).

## Results

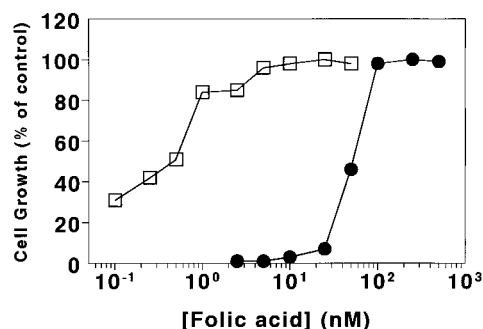
### Folate Growth Dependence of AA8 and Pyr<sup>R100</sup> Cells.

To examine whether AA8 and Pyr<sup>R100</sup> cells exhibit differences in folate metabolism/utilization, folic acid growth requirement was determined in a nonclonogenic growth assay. Figure 1 shows that Pyr<sup>R100</sup> cells have a ~100-fold decreased folic acid requirement for half-optimal growth compared with parental AA8 cells ( $EC_{50}$  = 0.5 versus 55 nM, respectively).

**Accumulation of [<sup>3</sup>H]Folic Acid and [<sup>3</sup>H]MTX in AA8 and Pyr<sup>R100</sup> Cells.** We subsequently examined whether the ability of Pyr<sup>R100</sup> cells to grow on lower concentrations of folic acid is related to increased cellular uptake. Figure 2A shows the accumulation of 1  $\mu$ M [<sup>3</sup>H]folic acid in AA8 and Pyr<sup>R100</sup> cells over a 4- to 24-h period. At 24 h, Pyr<sup>R100</sup> cells had accumulated 3-fold more [<sup>3</sup>H]folates than AA8 cells (302 versus 101 pmol/10<sup>7</sup> cells). In addition, we analyzed the accumulation of the antifolate [<sup>3</sup>H]MTX in AA8 and Pyr<sup>R100</sup> cells over a 4- to 24-h period (Fig. 2B). The 24-h accumulation of 1  $\mu$ M [<sup>3</sup>H]MTX was 9-fold higher in Pyr<sup>R100</sup> cells than in parental AA8 cells (306 versus 38 pmol/10<sup>7</sup> cells).

### Polyglutamylation of MTX in AA8 and Pyr<sup>R100</sup> Cells.

To explore the efficiency of [<sup>3</sup>H]MTX polyglutamylation in AA8 and Pyr<sup>R100</sup> cells, the formation of MTX polyglutamates was determined after 4- and 24-h exposure to 1  $\mu$ M [<sup>3</sup>H]MTX. Figure 3 shows that after 4 h of drug exposure in AA8 cells, MTX was largely present as the monoglutamate (69%), whereas 25% and 6% were present as the diglutamate and triglutamate, respectively. In Pyr<sup>R100</sup> cells, after 4-h exposure, 56% of MTX was present as the monoglutamate form, and 22% was present in each of the diglutamate and triglutamate forms. A marked shift in MTX-polyglutamylation was noted after 24 h of drug exposure. In AA8 cells, 52% was present as the monoglutamate form, and 20% and 28% were converted to diglutamates and triglutamates, respectively. In contrast, in Pyr<sup>R100</sup> cells, only a minor portion of MTX was present as monoglutamate and diglutamate (21% together),

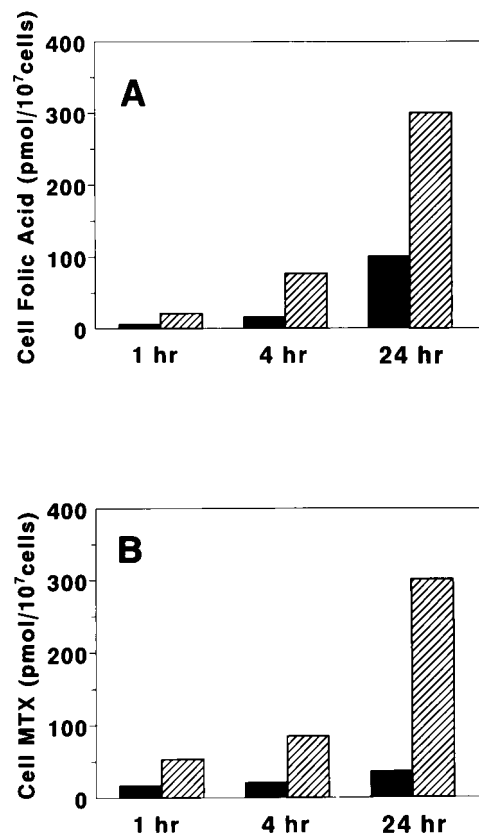


**Fig. 1.** Folic acid growth dependence of AA8 and Pyr<sup>R100</sup> cells. AA8 cells (●) and Pyr<sup>R100</sup> cells (□) were plated onto individual wells of a 24-well plate at an initial inoculum of  $1 \times 10^4$  cells/cm<sup>2</sup> in folate-free RPMI medium with 10% dialyzed FCS. Folic acid was added to the wells at a concentration range of 0 to 500 nM, whereas controls serving as 100% normal growth were added with 2.3  $\mu$ M folic acid, the concentration present in the RPMI medium. After 72 h, cell growth was determined by cell counting after trypsinization as described in *Materials and Methods*.

whereas the major and predominant metabolite species were the triglutamates (45%) and tetraglutamates (34%).

To investigate whether the formation of longer-chain polyglutamate species in Pyr<sup>R100</sup> cells was related to differences in levels of the enzyme catalyzing the formation of MTX-polyglutamates (i.e., FPGS), we directly measured the activity of FPGS in AA8 and Pyr<sup>R100</sup> cells (Table 1). FPGS activity in cell extracts of AA8 and Pyr<sup>R100</sup> cells grown in standard cell culture medium (containing 2.3  $\mu$ M folic acid) showed a 2.4-fold higher FPGS activity in Pyr<sup>R100</sup> cells as compared with AA8 cells. Furthermore, we measured FPGS activity in AA8 cells and Pyr<sup>R100</sup> cells grown under folate-restricted conditions of 100 and 5 nM folic acid, respectively. These folic acid concentrations were selected as the minimal necessary concentrations allowing optimal growth of these cell lines (Fig. 1). Under these folate-restricted conditions, FPGS activity in Pyr<sup>R100</sup> cells was 4.1-fold higher than that in AA8 cells.

To exclude the possibility that a decreased FPGH activity could underlie the formation of longer-chain polyglutamates in Pyr<sup>R100</sup> cells, the activity of FPGH was directly measured;



**Fig. 2.** Accumulation of [<sup>3</sup>H]folic acid (A) and [<sup>3</sup>H]MTX (B) by AA8 and Pyr<sup>R100</sup> cells. A, AA8 cells (black bars) and Pyr<sup>R100</sup> cells (dashed bars) were plated in 80-cm<sup>2</sup> tissue culture flasks and allowed to grow to approximately 70% confluency. At this stage, the RPMI medium was aspirated and replaced by folate-free RPMI-medium supplemented with 2  $\mu$ M [<sup>3</sup>H]folic acid (specific activity, 1 Ci/mmol). B, AA8 cells (black bars) and Pyr<sup>R100</sup> cells (dashed bars) were plated in 80-cm<sup>2</sup> tissue culture flasks and allowed to grow in RPMI medium (containing 2.3  $\mu$ M folic acid) to approximately 70% confluency. At this stage, [<sup>3</sup>H]MTX was added to a final concentration of 1  $\mu$ M (specific activity, 1 Ci/mmol). At the indicated time intervals of [<sup>3</sup>H]folic acid and [<sup>3</sup>H]MTX incubation, the medium was aspirated, and the cells were washed twice with ice-cold HEPES-buffered saline, pH 7.4, collected by trypsinization, and counted for radioactivity as described in *Materials and Methods*.



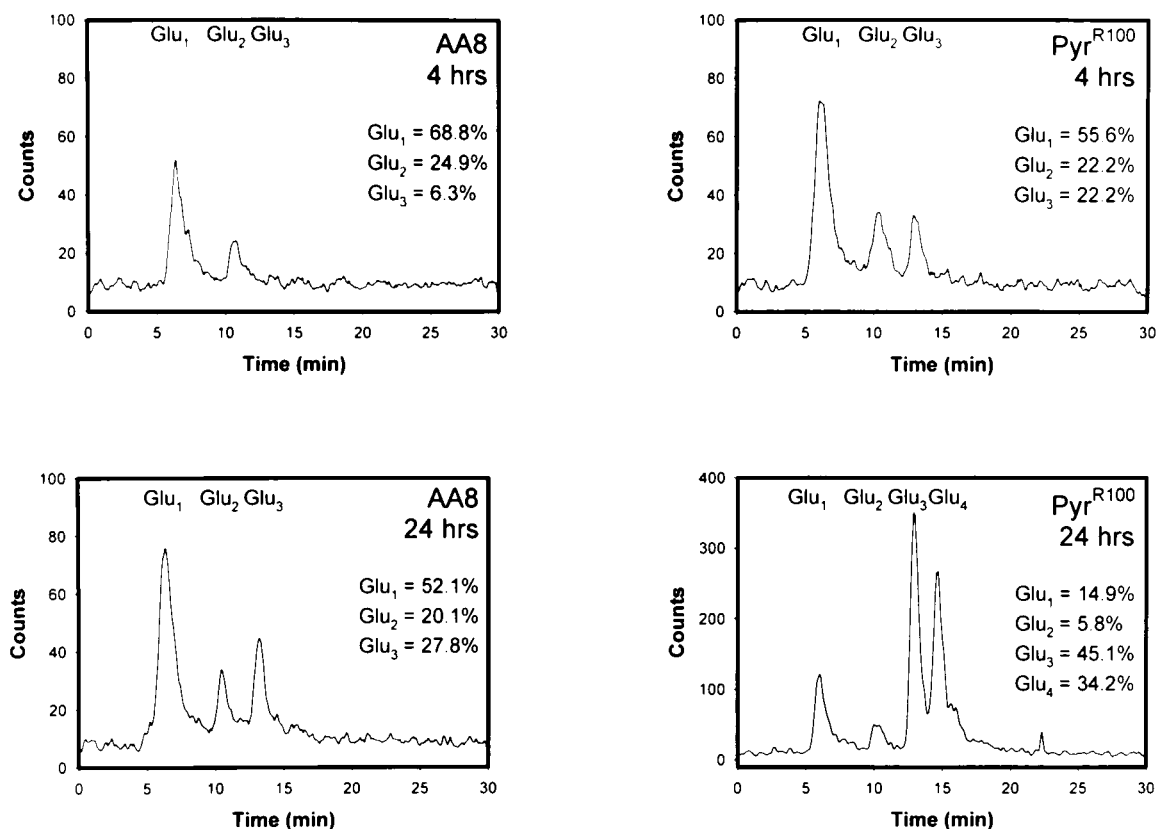
however, both AA8 and Pyr<sup>R100</sup> cells had undetectable enzyme levels, both in cell extracts and, as a possible secreted form, in the cell culture medium (results not shown).

**Intracellular Folate Pools in AA8 and Pyr<sup>R100</sup> Cells.** Given the increased accumulation of [<sup>3</sup>H]folic acid (Fig. 2A), the augmented activity of FPGS (Table 1), and the lack of FPGH activity in Pyr<sup>R100</sup> cells, we subsequently analyzed the total folate pools and the levels of the individual reduced folate in Pyr<sup>R100</sup> and AA8 cells. When grown in medium containing 2.3  $\mu$ M folic acid, the total folate pool in Pyr<sup>R100</sup> cells was 3-fold higher than that in AA8 cells. In both cell lines, 10-formyltetrahydrofolate (10-CHOTHF) was the major reduced folate metabolite, accounting for approximately 50% of the total folate pool. 5,10-methylenetetrahydrofolate + tetrahydrofolate (THF) and 5-methyltetrahydrofolate pools accounted for 28% and 19% of the total pools in AA8 cells, respectively. In Pyr<sup>R100</sup> cells, these metabolites accounted for 14% and 34%, respectively, of the total pool. In both AA8 and Pyr<sup>R100</sup> cells, DHF pools were a minor component (3–6%) of the total folate pool. On cell growth in medium containing folic acid that is sufficient to allow optimal growth of AA8 cells and Pyr<sup>R100</sup> cells (100 and 5 nM folic acid, respectively), the total folate pool dropped 6-fold for AA8 cells and 21-fold for Pyr<sup>R100</sup> cells, to nearly the same level of 15 pmol/mg protein.

To examine whether DHF pools in AA8 and Pyr<sup>R100</sup> cells would rise on inhibition of DHFR, cells were incubated with the lipophilic DHFR inhibitors Pyr or TMQ (Fig. 4). In wild-

type AA8 cells, a 1-h exposure to 0.3  $\mu$ M Pyr raised DHF levels from 6% in the antifolate-untreated cells (control) to 21% of the total folate pool. On exposure to 0.5  $\mu$ M TMQ, DHF levels accounted for 59% of the total folate pool. As anticipated, in Pyr<sup>R100</sup> cells, a much higher Pyr concentration of 150  $\mu$ M was required to raise DHF levels from 3% in untreated cells to 13% of the total pool. Exposure of Pyr<sup>R100</sup> cells to 2.5  $\mu$ M TMQ increased DHF levels to 52% of the total folate pool.

**Antifolate Growth Inhibition of AA8 and Pyr<sup>R100</sup> Cells.** Finally, we investigated to what extent expanded intracellular folate pools, along with an increase FPGS activity in Pyr<sup>R100</sup> cells, had an effect on the growth-inhibitory potential of a series of (non)polyglutamatable and lipophilic antifolates targeted to DHFR, TS, or glycinamide ribonucleotide formyltransferase (GARFT) (Table 3). Furthermore, these growth-inhibitory effects were determined for AA8 and Pyr<sup>R100</sup> cells under folate-restricted (LF) cell culture conditions. Regarding antifolate DHFR inhibitors, AA8 cells grown at 2.3  $\mu$ M folic acid (HF) display sensitivity to the polyglutamatable compounds MTX and EDX (Sirotnak et al., 1987; Bertino, 1993), the nonpolyglutamatable compound PT523 (Rhee et al., 1994), and the lipophilic compounds TMQ (Bertino, 1993) and AG377 (Jones et al., 1992). Pyr is 21-fold less potent than TMQ against AA8 (HF) cells. Pyr<sup>R100</sup> (HF) cells retain sensitivity (IC<sub>50</sub> < 100 nM) for MTX, EDX, and PT523, although a low level of cross-resistance is observed for the latter two compounds. A marked level of cross-resistance



**Fig. 3.** Polyglutamylation of [<sup>3</sup>H]MTX by AA8 and Pyr<sup>R100</sup> cells. Cells were plated in 80-cm<sup>2</sup> tissue culture flasks and allowed to grow in RPMI medium (with 2.3  $\mu$ M folic acid) and 10% FCS to approximately 70% confluency. At this stage, [<sup>3</sup>H]MTX was added to a final concentration of 1  $\mu$ M (specific activity, 1 Ci/mmol). After 4 and 24 h, the medium was aspirated, and the cells were washed twice with ice-cold HEPES-buffered saline, pH 7.4, and collected by trypsinization. An aliquot of the cells was counted for viability, and the remaining portion was extracted for analysis of [<sup>3</sup>H]MTX polyglutamates by HPLC as described in *Materials and Methods*.

is noted for AG377 (30-fold) and TMQ (40-fold). Growth of AA8 cells in LF-medium increased their sensitivity for the DHFR inhibitors from 2.4-fold (for EDX) to 21-fold (for Pyr). Likewise, Pyr<sup>R100</sup> (LF) cells showed greater sensitivity to DHFR inhibitors than Pyr<sup>R100</sup> (HF) cells; with the exception of Pyr, drug sensitivities approximated those of wild-type cells.

AA8 (HF) cells displayed a wide range of sensitivities for a group of five folate-based TS inhibitors. ZD1694 (Jackman et al., 1991) and LY231514 (MTA) (Shih et al., 1997) were potent growth inhibitors; GW1843 (Duch et al., 1993) was moderately cytotoxic; and a poor sensitivity was noted for the nonpolyglutamatable compound ZD9331 (Jackman et al., 1997) and the lipophilic TS inhibitor AG337 (Webber et al., 1996). Pyr<sup>R100</sup> (HF) cells were collaterally sensitive to ZD9331 and GW1843, retained wild-type sensitivity for ZD1694, and displayed a low level of resistance to LY231514 (MTA) (2.5-fold) and AG337 (3-fold). Under LF conditions, AA8 cells were markedly more sensitive to all TS inhibitors, in particular, ZD9331 (29-fold) and AG337 (20-fold), compared with HF conditions. For Pyr<sup>R100</sup> (LF) cells, an even greater increase in sensitivity was noted for GW1843 (56-fold) and ZD9331 (90-fold).

Finally, three polyglutamatable GARFT inhibitors (Beardsley et al., 1989; Boritzki et al., 1996) showed a growth-inhibitory potency in the low nanomolar range against AA8 (HF) cells. Pyr<sup>R100</sup> (HF) cells retained wild-type sensitivity for AG2032 but were prominently cross-resistant to AG2034 (14-fold) and 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF) (26-fold). This resistance profile was no longer observed in Pyr<sup>R100</sup> (LF) cells

because drug sensitivity increased by 155-fold for AG2034 and 533-fold for DDATHF, thus resuming wild-type AA8 (LF) sensitivity levels.

**Influx and Efflux of [<sup>3</sup>H]Pyr in AA8 and Pyr<sup>R100</sup> Cells.** Because unlike other antifolates Pyr<sup>R100</sup> (LF) cells retained a marked resistance to Pyr, we investigated whether additional factors, including reduced influx or increased efflux of Pyr, contributed to the antifolate resistant phenotype. For this purpose, we determined the influx and efflux activities of [<sup>3</sup>H]Pyr in Pyr<sup>R100</sup> cells versus AA8 cells. Figure 5A shows that the cellular steady-state accumulation of [<sup>3</sup>H]Pyr was slightly lower in Pyr<sup>R100</sup> cells compared with AA8 cells. Furthermore, examination of the rate of efflux of [<sup>3</sup>H]Pyr from cells that had been loaded with [<sup>3</sup>H]Pyr for 3 h also showed a small increase in efflux in Pyr<sup>R100</sup> cells compared with AA8 cells (Fig. 5B).

**Catabolism of [<sup>3</sup>H]Pyr in AA8 and Pyr<sup>R100</sup> Cells.** Although Fig. 5 shows cell association and penetration of [<sup>3</sup>H]Pyr into AA8 and Pyr<sup>R100</sup> cells, these transport experiments did not address whether Pyr undergoes cellular catabolism or inactivation by modification. To examine whether a catabolized form of Pyr may be preferentially accumulating in drug-resistant cells, AA8 and Pyr<sup>R100</sup> cells were loaded with 1  $\mu$ M [<sup>3</sup>H]Pyr for 3 h, and the acid-soluble fraction of cell lysates was analyzed by HPLC. These profiles of AA8 and Pyr<sup>R100</sup>, as measured by scintillation counting, were identically superimposable and coincided with a Pyr standard (data not shown). The superimposable HPLC profiles of AA8 and Pyr<sup>R100</sup> indicate that the internalized radioactive drug accumulating in both cell lines has not undergone any structural alteration capable of causing inactivation.

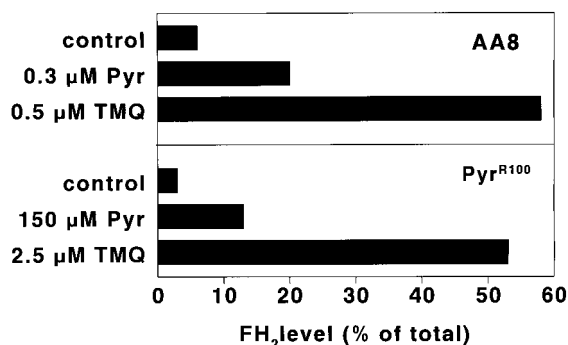
**Pyr Inhibits a Lysosomal Enzyme.** The increased numbers of intracellular vesicles observed in electron micrographs of Pyr<sup>R100</sup> cells appeared to indicate an increased number of lysosomes, presumably as a means of increasing the volume of the acidic vesicular subcellular compartment (Sprecher et al., 1995). An increased number of lysosomes per Pyr<sup>R100</sup> cell should also be detected as an increased activity of lysosomal enzymes per cell (Warren et al., 1991). Determination of  $\beta$ -hexoseaminidase activity in postnuclear cell lysates from three independent preparations showed an average increase of 4-fold in Pyr<sup>R100</sup> cells (2.3 versus 8.7  $\mu$ mol/mg/h for AA8 and Pyr<sup>R100</sup> cells, respectively). Because increased number of lysosomes and consequent selective overexpression of  $\beta$ -hexoseaminidase could be a result of an inhibitory activity of Pyr, particularly at high concentrations, we performed  $\beta$ -hexoseaminidase activity assays in the presence of increasing concentrations of Pyr. These studies (Fig. 6A) reveal that 50% inhibition of the activity from AA8 cell lysates is achieved with 20  $\mu$ M Pyr, whereas a similar inhibition requires 70  $\mu$ M when a similar amount of protein from Pyr<sup>R100</sup> is used; this indicates a 3.5-fold increased  $\beta$ -hexoseaminidase activity in Pyr<sup>R100</sup> cells, thus being consistent with the 4-fold increased enzyme activity in Pyr<sup>R100</sup> cells. To characterize the type of inhibition exerted by Pyr on  $\beta$ -hexoseaminidase, a double-reciprocal plot analysis was undertaken (Fig. 6D). The competitive inhibition as seen in the Lineweaver-Burk plot has a  $K_i$  value of 25  $\mu$ M, and the substrate used,  $\beta$ -*n*-acetyl-glucosamine, has a  $K_m$  value of 4.5  $\mu$ M, in Pyr<sup>R100</sup> cells. Furthermore, the inhibition by Pyr is specific compared with other antifolates, such as MTX (not shown) and metoprine (Mtp) (Fig. 6B), which failed to inhibit

TABLE 1

Folypolyglutamate synthetase activity in parental AA8 and Pyr<sup>R100</sup> cells grown under different folic acid concentrations

| Cell Line           | Medium Folate Concentration | FPGS Activity <sup>a</sup> |
|---------------------|-----------------------------|----------------------------|
|                     | $\mu$ M folic acid          |                            |
| AA8                 | 2.3                         | 1276 $\pm$ 84              |
| AA8                 | 0.1                         | 552 $\pm$ 131              |
| Pyr <sup>R100</sup> | 2.3                         | 3011 $\pm$ 271             |
| Pyr <sup>R100</sup> | 0.005                       | 2255 $\pm$ 382             |

<sup>a</sup> FPGS activity is expressed as pmol of [<sup>3</sup>H]Glutamate incorporated/h/mg protein using MTX as a substrate at a concentration of 250  $\mu$ M. Results are the mean  $\pm$  S.D. of three or four separate experiments.



**Fig. 4.** Induction of dihydrofolate formation in AA8 and Pyr<sup>R100</sup> cells after exposure to Pyr or TMQ. Cells were plated in 80-cm<sup>2</sup> tissue culture flasks and allowed to grow in RPMI medium (with 2.3  $\mu$ M folic acid) and 10% FCS to approximately 70% confluency. Then, Pyr or TMQ was added at the indicated final concentrations. After 1-h drug exposure, the medium was aspirated, and cells were washed twice with ice-cold HEPES-buffered saline, pH 7.4, collected by trypsinization, and analyzed for reduced folate metabolites as described in *Materials and Methods*.

this enzyme. The structural 2,4-diamino-pyrimidine analog trimethoprim (Tnp) was slightly inhibitory at a 0.1 to 1.0 mM range (Fig. 6C).

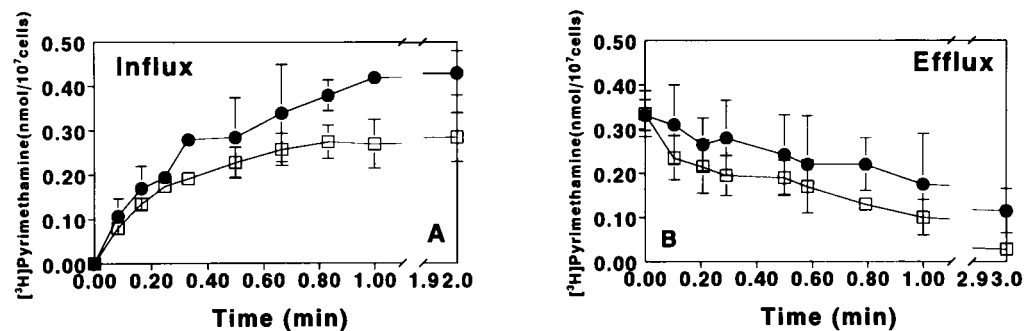
**Glucose Consumption by AA8 and Pyr<sup>R100</sup> Cells.** Pyr<sup>R100</sup> cells grown continuously in the presence of 100  $\mu$ M Pyr have been shown to be exposed to a respiratory stress, which is reflected by impaired oxidative phosphorylation and an increased lactate production, indicative of increased glycolytic activity (Sprecher et al., 1995). A high rate of glycolysis would also necessitate a high rate of glucose consumption, which will make Pyr<sup>R100</sup> more dependent on an adequate supply of glucose in the growth medium. By limiting the concentration of glucose in the medium of a clonogenic assay, we noted that parental AA8 cells were capable of full growth at glucose concentrations of 0.25 mM or higher (Fig. 7). Similarly, when Pyr<sup>R100</sup> cells were grown in medium lacking Pyr, they displayed a profile similar to that of parental AA8 cells, with a 50% plating efficiency in 50  $\mu$ M glucose. The presence of 100  $\mu$ M Pyr in the glucose-restricted medium drastically reduced the plating efficiency of Pyr<sup>R100</sup> cells. Fifty percent growth of Pyr<sup>R100</sup> cells is achieved at a glucose concentration 25-fold greater than that in medium lacking Pyr. As opposed to parental AA8 cells, in the presence of Pyr,

Pyr<sup>R100</sup> cells achieved maximum viability at or near the glucose concentration (5 mM) present in the growth medium.

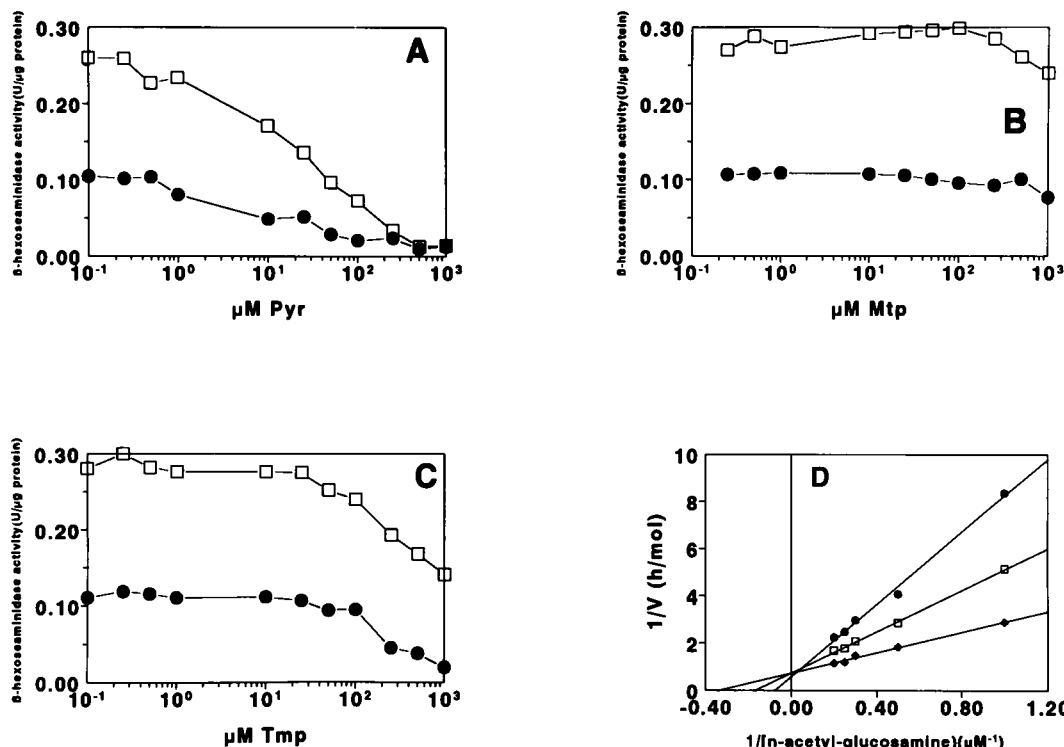
## Discussion

The aim of the present study was to further identify the mechanistic basis for the high level of resistance displayed by Pyr<sup>R100</sup> cells to lipophilic antifolates (Assaraf and Slotky, 1993; Assaraf and Goldman, 1997; Assaraf et al., 1998). We investigated how an increased activity of FPGS, as well as folate-unrelated alterations (i.e., increased lysosome number per cell), contributes to alterations in the cellular metabolism of natural reduced folate cofactors to confer a novel mechanism of resistance to lipophilic antifolates and some (non)-polyglutamatable antifolates.

The dramatically reduced folic acid growth requirement (Fig. 1) indicates that Pyr<sup>R100</sup> cells have an increased capacity to accumulate and retain folate cofactors. Indeed, Fig. 2A and Table 2 illustrate that [<sup>3</sup>H]folates are accumulated in Pyr<sup>R100</sup> cells to a 3-fold higher level than in wild-type AA8 cells. Part of the augmented folate accumulation can be accounted for by an increased ratio of folate influx to folate efflux capacity in Pyr<sup>R100</sup> cells as a result of the 4-fold in-



**Fig. 5.** Time course of [<sup>3</sup>H]Pyr accumulation in AA8 and Pyr<sup>R100</sup> cells. [<sup>3</sup>H]Pyr transport assay of influx (A) and efflux (B) in AA8 cells (●) and Pyr<sup>R100</sup> cells (□). Results are presented as the mean  $\pm$  S.D. of 3 independent experiments. Experimental details are described in *Materials and Methods*.



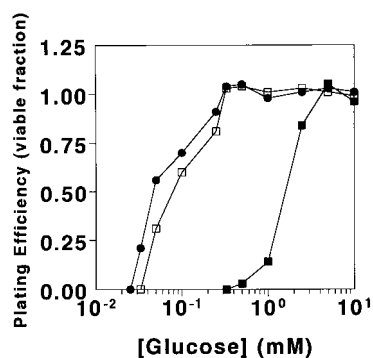
**Fig. 6.** Inhibition of  $\beta$ -hexosaminidase by 2,4-diaminopyrimidine antifolates. Activity of  $\beta$ -hexosaminidase extracted from AA8 and Pyr<sup>R100</sup> in the presence of Pyr (A), Mtp (B), and Tnp (C). Lineweaver-Burk plot (D) of  $\beta$ -hexosaminidase inhibition by Pyr, in a Pyr<sup>R100</sup> extract; concentrations of Pyr: (bottom to top) were 0, 15, and 30  $\mu$ M. Results presented are from a representative experiment of two independent experiments.

creased activity of a low pH folic acid influx transporter (Assaraf et al., 1998) and the loss of folate exporter activity (Assaraf and Goldman, 1997). Subsequently, the significantly higher (2.4–4.1-fold) FPGS activity in Pyr<sup>R100</sup> cells compared with AA8 cells will contribute to the enhanced intracellular retention of reduced folate cofactors (McGuire et al., 1980; Osborne et al., 1993). The expanded folate pools and elevated FPGS activity can provide a mechanistic basis for the resistance to lipophilic antifolates. First, the increased reduced folate pools may be more effective in bypassing a folate-depleting effect that arises from inhibition of DHFR. Second, lipophilic antifolate inhibitors of DHFR increase the levels of DHF (Fig. 4), the latter of which is an excellent substrate for FPGS (Chen et al., 1996). Given the elevated activity of FPGS in Pyr<sup>R100</sup> cells, DHF-polyglutamates will readily displace lipophilic antifolates from DHFR, especially those with lower affinities like Pyr and the 2,4-diaminopyrimidines, thereby restoring, at least partially, DHFR enzyme activity. Figure 4 demonstrates that TMQ markedly raises DHF levels at lower concentrations than Pyr, which is consistent with TMQ being a 33-fold more potent inhibitor of CHO DHFR than Pyr (Assaraf and Slotky, 1993). The sensitivity of AA8 and Pyr<sup>R100</sup> cells for lipophilic antifolate DHFR inhibitors is markedly influenced by changes in the intracellular folate pool (Tables 2 and 3). After growth of Pyr<sup>R100</sup> cells at LF, the growth-inhibitory potency of TMQ increased 138-fold and approximated parental sensitivity. Likewise, the sensitivity of Pyr<sup>R100</sup> cells to Pyr in-

creased (25-fold) on cell culturing under LF conditions but did not reach parental sensitivity.

The additional novel features of elevated FPGS activity and expanded folate pools in Pyr<sup>R100</sup> cells prompted us to investigate the implications for the drug sensitivity of other (non)polyglutamatable and lipophilic antifolates (Table 3). The drug sensitivity profile of parental AA8 (HF) cells for (non)polyglutamatable antifolates is largely consistent with their differential uptake efficiency via the RFC (Westerhof et al., 1995b). GW1843 is relatively poorly transported via RFC from rodent cells (Duch et al., 1993; Westerhof et al., 1995b), which is reflected by a diminished growth-inhibitory potency. Because the low pH folate transporter displays a marked uptake of folic acid and MTX at physiological pH in Pyr<sup>R100</sup> cells (Assaraf et al., 1998) and the latter cell line showed an increased sensitivity to ZD9331 (6-fold) and GW1843 (13-fold) under LF conditions, relative to parental cells (Table 3), it is possible that the low pH transporter plays an important uptake role of these new-generation antifolates. The results from Tables 2 and 3 indicate that an expanded folate pool is also the major factor mediating resistance to some hydrophilic antifolates. In particular, Pyr<sup>R100</sup> cells (HF) display resistance to the GARFT inhibitors DDATHF and AG2034. Because these compounds heavily rely on polyglutamylation for their pharmacological activity (Westerhof et al., 1995b; Boritzki et al., 1996), it is conceivable that resistance is achieved by a direct competition of 10-CHOTHF (the reduced folate cofactor in the GARFT reaction) and these antifolates on polyglutamylation by FPGS. Indeed, Table 2 demonstrates the markedly expanded 10-CHOTHF pools in Pyr<sup>R100</sup> cells (HF). Besides competition at the level of polyglutamylation, it is conceivable that expanded polyglutamate pools of the natural folate cofactor substrate may also affect competitive or mixed noncompetitive inhibition of target enzymes by nonpolyglutamatable antifolates (e.g., ZD9331) (Jackman et al., 1997). Regardless of competition at the level of polyglutamylation or target enzyme, it is evident that under LF circumstances, antifolate resistance in Pyr<sup>R100</sup> (HF) cells can be reversed to wild-type sensitivity for the TS and GARFT inhibitors.

Recently, workers at our laboratory reported on the role of expanded folate pools in conferring resistance to lipophilic and polyglutamatable antifolates in a variant human CEM leukemia line expressing a structurally altered RFC protein that displayed a highly increased affinity for folic acid transport (Jansen et al., 1997, 1998b). Consequently, the intracellular folate pool was increased 7-fold (500 pmol/mg protein)



**Fig. 7.** Glucose growth dependence of AA8 and Pyr<sup>R100</sup> cells. AA8 (●), Pyr<sup>R100</sup> cells growing in drug-free medium (□), or Pyr<sup>R100</sup> cells growing in 100  $\mu$ M Pyr-containing medium (■) were plated onto 60-mm petri dishes in the presence of the various concentrations of glucose as the sole sugar source. Results of the clonogenic assay are presented as a representative experiment of three performed with a variation of less than 15%.

TABLE 2

Cellular folate changes in response to alterations of folic acid in the growth medium of AA8 and Pyr<sup>R100</sup> cells

| Cell Line           | Medium Folate Concentration | CH <sub>2</sub> THF + THF | Reduced Folates <sup>a</sup> |                       | 10-CHOTHF                | Total            |
|---------------------|-----------------------------|---------------------------|------------------------------|-----------------------|--------------------------|------------------|
|                     |                             |                           | 5-CH <sub>3</sub> THF        | DHF                   |                          |                  |
|                     | $\mu$ M                     |                           | (pmol/mg protein)            |                       |                          |                  |
| AA8                 | 2.3                         | 27.1 $\pm$ 1.6<br>(28)    | 19.1 $\pm$ 4.0<br>(19)       | 6.0 $\pm$ 0.6<br>(6)  | 46.5 $\pm$ 6.4<br>(47)   | 98.6 $\pm$ 10.0  |
| AA8                 | 0.1                         | 4.6 $\pm$ 0.7<br>(28)     | 1.7 $\pm$ 0.3<br>(10)        | 0.6 $\pm$ 0.3<br>(4)  | 9.8 $\pm$ 0.5<br>(58)    | 16.7 $\pm$ 1.1   |
| Pyr <sup>R100</sup> | 2.3                         | 41.6 $\pm$ 6.4<br>(14)    | 100.1 $\pm$ 9.3<br>(34)      | 8.3 $\pm$ 3.2<br>(3)  | 143.0 $\pm$ 12.7<br>(49) | 293.0 $\pm$ 22.7 |
| Pyr <sup>R100</sup> | 0.005                       | 2.3 $\pm$ 0.3<br>(16)     | 2.9 $\pm$ 0.5<br>(21)        | 5.2 $\pm$ 0.7<br>(37) | 3.7 $\pm$ 0.9<br>(26)    | 14.1 $\pm$ 0.9   |

<sup>a</sup> Values represent the mean  $\pm$  S.E.M. from at least six independent determinations. Values in parentheses represent the percentage of the individual folate intermediate of the total pool.



Under folate-depleted conditions, Pyr<sup>R100</sup> cells resumed wild-type sensitivity (and sometimes increased sensitivity; Table 3) to various antifolates while surprisingly retaining a high level of resistance to Pyr, the antifolate used in the stepwise selection protocol to establish this cell line (Assaraf and Slotky, 1993). This unexpected finding prompted us to investigate the folate metabolism-independent mechanisms that may underlie this major component of persistent Pyr resistance. In this respect, the small changes in Pyr uptake and efflux (Fig. 5), and the lack of cellular [<sup>3</sup>H]Pyr drug inactivation cannot explain the 1000-fold resistance to Pyr in Pyr<sup>R100</sup> cells. Pyr is a highly lipophilic drug with a *log P* value of 2.54 that is reflected in its rapid membrane penetration and cellular uptake (Fig. 5), followed by a fast association with the target enzyme DHFR in parental AA8 cells (Assaraf and Slotky, 1993). Importantly, like the 2,4-diaminopyrimidines, Pyr has a *pK<sub>a</sub>* value close to the physiological pH, rendering it a weak base (Cavallito et al., 1978). These weak bases (especially with two aromatic amino groups like in Pyr) that are completely uncharged at physiological pH can become irreversibly protonated in an acidic compartment such as lysosomes, thus accumulating to millimolar concentrations in this compartment (Tragano and Darzynkiewicz, 1994). Based on this lipophilicity and weak base properties,

Pyr could easily traverse the membrane of lysosomes and endosomes, after which it could undergo double-protonation, irreversible entrapment, and marked sequestration within these acidic organelles, thereby markedly decreasing its accessibility to the cytosolic target enzyme, DHFR. Indeed, a concentration as high as 150  $\mu\text{M}$  Pyr was required to significantly raise DHF levels in Pyr<sup>R100</sup> cells. More importantly, these Pyr-loaded acidic vesicles could empty their contents by endosome-mediated exocytosis. According to this paradigm, an increase in the number of lysosomes per Pyr<sup>R100</sup> cell could provide a mechanistic explanation to the persistent Pyr-resistance observed under folate-depleted conditions. Thus, we explored this possibility by a direct measurement of  $\beta$ -hexosaminidase activity, a well established lysosomal marker reflecting lysosome number per cell (Warren et al., 1991); enzyme activity was elevated by 4-fold in Pyr<sup>R100</sup> cells. Consistently, we further found that Pyr was a potent and competitive inhibitor of  $\beta$ -hexosaminidase ( $K_i = 25 \mu\text{M}$ ). These results are in good agreement with our previous studies in which electron microscopy of Pyr<sup>R100</sup> cells identified an increased number of intracellular vesicles (Sprecher et al., 1995). It is therefore likely that during the prolonged step-wise selection with Pyr, an increased inhibition of lysosomal  $\beta$ -hexosaminidase activity by the increasing concentration and sequestration of Pyr in the lysosomes occurred, thereby providing the possible basis for the compensatory increase in the number of lysosomes per Pyr<sup>R100</sup> cell. Hence, Pyr<sup>R100</sup> cells provide an excellent example for a novel component of lipophilic antifolate resistance that is potentially based on hydrophobic weak base concentration within lysosomes and potentially rapid and efficient drug extrusion, presumably by energy-dependent endosome-mediated exocytosis. Indeed, in the presence of Pyr, Pyr<sup>R100</sup> cells required 25-fold more glucose for their survival; it is therefore likely that these cells use a significant fraction of their metabolic energy resources to support this energy-costly drug exocytotic pathway. Clearly, this lysosome sequestration-based mechanism of lipophilic antifolate resistance should persist even under folate-depleted conditions. Indeed, Pyr resistance (unlike all other antifolates) was highly retained under folate-depleted growth conditions.

| Antifolate   | Target  | Polyglutamylation | IC <sub>50</sub>   |                    |                                    |                                  |
|--------------|---------|-------------------|--------------------|--------------------|------------------------------------|----------------------------------|
|              |         |                   | AA8<br>(2.3 μM FA) | AA8<br>(100 nM FA) | Pyr <sup>R100</sup><br>(2.3 μM FA) | Pyr <sup>R100</sup><br>(5 nM FA) |
|              |         |                   | <i>nM</i>          |                    |                                    |                                  |
| MTX          | DHFR    | +                 | 23 ± 1             | 3.4 ± 0.7          | 79 ± 24                            | 4.9 ± 1.6                        |
| EDX          | DHFR    | +                 | 1.7 ± 0.7          | 0.7 ± 0.2          | 12 ± 3                             | 1.3 ± 0.8                        |
| PT523        | DHFR    | −                 | 1.8 ± 0.2          | 0.5 ± 0.2          | 20 ± 2                             | 1.0 ± 0.5                        |
| TMQ          | DHFR    | −                 | 10 ± 4             | 0.4 ± 0.3          | 401 ± 39                           | 2.9 ± 1.5                        |
| Pyr          | DHFR    | −                 | 214 ± 43           | 10 ± 6             | 150,000                            | 5875 ± 1305                      |
| AG377        | DHFR/TS | −                 | 27 ± 14            | 2.5 ± 1.5          | 820 ± 100                          | 16 ± 6                           |
| ZD1694       | TS      | +                 | 17 ± 3             | 3.2 ± 0.8          | 21 ± 8                             | 1.5 ± 1.0                        |
| GW1843       | TS      | +                 | 253 ± 11           | 33 ± 14            | 141 ± 51                           | 2.5 ± 2.2                        |
| LY231514/MTA | TS      | +                 | 43 ± 9             | 6.9 ± 0.9          | 109 ± 40                           | 3.8 ± 2.5                        |
| ZD9331       | TS      | −                 | 1665 ± 390         | 58 ± 35            | 900 ± 185                          | 10 ± 8                           |
| AG337        | TS      | −                 | 20,220 ± 6750      | 1030 ± 560         | 62,280 ± 14,820                    | 2385 ± 1315                      |
| DDATHF       | GARFT   | +                 | 62 ± 36            | 6.2 ± 1.1          | 1600 ± 1065                        | 3.0 ± 1.9                        |
| AG2032       | GARFT   | +                 | 81 ± 16            | 5.3 ± 2.2          | 96 ± 33                            | 5.5 ± 2.0                        |
| AG2034       | GARFT   | +                 | 28 ± 11            | 3.2 ± 0.8          | 402 ± 222                          | 2.6 ± 1.2                        |

The 50% growth-inhibitory concentration (IC<sub>50</sub> in nM) was determined after 72-h drug exposure. Results are the mean ± S.D. of three to six separate experiments.



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