BIOCHEMICAL PHARMACOLOGY 72 (2006) 256–266

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Leucovorin-induced resistance against FDH growth suppressor effects occurs through DHFR up-regulation

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ARTICLE INFO

Article history:
Received 18 February 2006
Accepted 6 April 2006

Keywords:
FDH
Folate
DHFR
Leucovorin
Enzyme product inhibition
Growth suppressor
A549 cells
Resistance mechanism

Abbreviations:
AICARFT, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase
DDF, 5,8-dideazafolate
DHFR, dihydrofolate reductase
FDH, 10-formyltetrahydrofolate dehydrogenase
GARFT, glycinamide ribonucleotide formyltransferase
MTX, methotrexate
PI, propidium iodide
THF, tetrahydrofolate

ABSTRACT

10-Formyltetrahydrofolate dehydrogenase (FDH) converts 10-formyltetrahydrofolate to tetrahydrofolate (THF). Expression of the enzyme in FDH-deficient cancer cells induces cytotoxicity that can be reversed by supplementation with high concentrations of a reduced folate, 5-formyl-THF (leucovorin). In contrast, non-tumor cells are resistant to FDH. The present study was undertaken to investigate mechanisms that could protect cells against FDH suppressor effects. Using 10 μM leucovorin supplementation of FDH-sensitive A549 cells transfected for FDH expression, we selected clones that have acquired resistance against FDH. Resistant cells expressed high levels of FDH and were capable of growing after withdrawal of leucovorin. These cells, however, have increased doubling time due to prolonged S phase. They also have significantly increased levels of total folate pool and THF/5,10-methylene-THF pool while the level of 10-formyl-THF was two-fold lower than in parental FDH-sensitive cells. We have shown that the FDH-catalyzed reaction proceeds at about a three-fold slower rate at the ratio of 10-formyl-THF/THF corresponding to the resistant cells than at the ratio corresponding to parental sensitive cells, due to product inhibition (K_I is 2.35 μM). FDH-resistant cells have strongly up-regulated dihydrofolate reductase (DHFR) that is proposed to be a mechanism for the alteration of folate pools and a key component of the acquired resistance. Elevation of DHFR in A549 cells by transient transfection decreased sensitivity to FDH toxicity and allowed selection of FDH-resistant clones. DHFR-induced repression of FDH catalysis could be an S phase-related metabolic adjustment that provides protection against FDH suppressor effects.

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doi:10.1016/j.bcp.2006.04.005

1. Introduction

Folate coenzymes function as acceptors and donors of one-carbon groups and are crucial for cellular homeostasis because of participation in nucleotide biosynthesis and amino acid metabolism [1]. The intracellular folate pool consists of several major forms of the coenzyme that are interconvertible through multiple reactions catalyzed by more than a dozen enzymes [1,2]. Intracellular folate pathways relevant to the present studies are summarized in Fig. 1. Distribution of the total intracellular folate pool between different forms of folate coenzymes apparently depends on the relative levels of the corresponding enzymes and the availability of metabolites involved in folate-required pathways [3]. Folate is an essential nutrient since higher animals are unable to synthesize it and depend on the diet to provide folate for cellular metabolism [4]. Epidemiological studies have linked a low folate status, or a low folate intake, with increased risk for several types of cancer, neural tube defects, and cardiovascular diseases [5]. At present, it is generally accepted that increased folate intake decreases the risk of these diseases in humans [6]. Cells obtain folate as naturally occurring coenzyme form (food folate) which is predominantly 5-methyltetrahydrofolate (5-methyl-THF) or as the synthetic form which is folic acid [7]. Another folate coenzyme often used in folate supplementation studies is 5-formyl-THF (known therapeutically as leucovorin or folinic acid) [8]. It is also widely used in chemotherapy to reduce cytotoxic side effects of MTX [9], a potent inhibitor of several folate enzymes [10].

One of the folate coenzymes, 10-formyltetrahydrofolate (10-formyl-THF), is an essential substrate for two reactions of de novo purine biosynthesis (Fig. 1) [1], which are crucial for cellular proliferation. This was demonstrated in experiments with inhibitors of GARFT, the first folate dependent enzyme in the de novo purine pathway: these inhibitors result in severe depletion of intracellular purine nucleotides followed by suppressed cellular proliferation and cytotoxicity [11,12]. 10-Formyl-THF can be also metabolized by another enzyme, 10-formyl-THF dehydrogenase (FDH). FDH catalyzes the $NADP^+$-dependent irreversible conversion of 10-formyl-THF to THF and CO_2 [2,13]. Although the physiological significance of this reaction, apparently controlling intracellular levels of 10-formyl-THF, is not clear, it could be a regulator of de novo purine synthesis and could potentially restrict this pathway by depleting intracellular 10-formyl-THF [14]. FDH is an abundant enzyme in several tissues with highest levels observed in liver and kidney [15]. In liver, for example, it comprises about 1.2% of the total cytosolic protein [2,16]. In contrast to normal tissues, the levels of this enzyme are ubiquitously low in a number of human tumor tissues and cancer cell lines [15]. De novo purine biosynthesis is especially active in rapidly proliferating cells, like cancer cells, where it supplies a substantial portion of purine nucleotides required for enhanced cellular proliferation [17]. Therefore, down-regulation of FDH could be one of the mechanisms to support the increased purine biosynthesis, by maintaining higher intracellular concentrations of 10-formyl-THF.

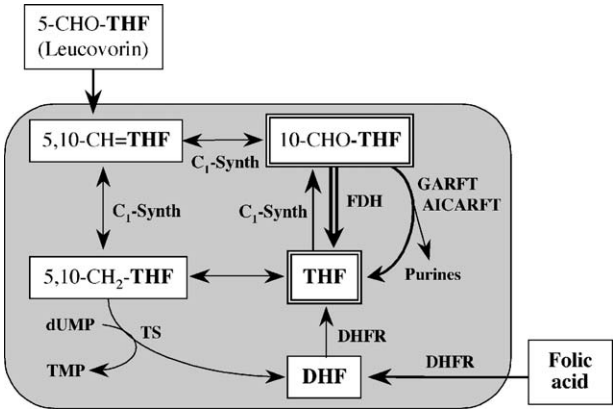


Fig. 1 – Intracellular pathways involving conversion of 10-formyl-THF. For simplicity, only pathways directly relevant to the present work and corresponding enzymes are depicted. Abbreviations: TS, thymidylate synthase; THF, tetrahydrofolate; CH_2, methylene; CH, methenyl. Enzymes directly involved in 10-formyl-THF metabolism are: FDH; C_1-Synth, C_1-synthase; enzymes of de novo purine biosynthesis, GARFT and AICARFT. Two reactions catalyzed by DHFR are shown.

We have previously demonstrated that elevating FDH in FDH-deficient cancer cells inhibits proliferation and induces cytotoxicity [15], which is consistent with observations of low FDH in cancer cells. These results raised the question of why FDH is not toxic to normal cells in contrast to cancer cells. One explanation could be that FDH effects are associated with the proliferation stage in general and therefore slowly proliferating cells are insensitive to elevated FDH. However, the finding of high FDH in regenerating liver, as well as in some non-cancer cell lines [15], rather suggests that normal cells have mechanisms that could protect them from FDH-induced cytotoxicity, and that in cancer cells these mechanisms are inactive. We have previously shown that the cytotoxic effects of FDH can be partially reversed by supplementation with high concentrations of leucovorin [15]. Thus, while FDH is a cytotoxic factor for cancer cells, leucovorin is a rescue factor that alleviates the growth suppressor effects of FDH. In the present study, using leucovorin supplementation as a protective factor against FDH suppressor effects, we have generated FDH-resistant stable clones of the A549 cell line and evaluated potential mechanisms of such resistance.

2. Materials and methods

2.1. *Reagents and cell culture*

Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified. Cell media and reagents were purchased from Invitrogen Inc. (Carlsbad, CA) unless otherwise indicated. Human A549 lung non-small carcinoma cells were obtained from American Type Culture Collection (Manassas, VA). Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), L-glutamine (2 mM) and sodium pyruvate (1 mM). Media were changed every 2 days.

2.2. Generation of DHFR expression vector

The translated portion of human DHFR cDNA (NCBI access number NM_000791) was PCR amplified from the total cDNA pool of A549 cells (generated with Advantage RT-for-PCR Kit, Clontech) using primers 5′-ATGGTTGGTTCGCTAAACTGC-3′ and 5′-CCTTCATGTTAATCATTCTTCTC-3′. The amplified 564 bp fragment was cloned into pCR2.1 vector using TA-cloning kit (Invitrogen). The DHFR cDNA was then excised from the construct by subsequent treatment with *Xho*I and *Hind*III restriction enzymes; the *Xho*I end was blunted by treatment with *Pfu* Turbo DNA polymerase before restriction the plasmid with *Hind*III. This fragment was cloned into pcDNA3.1 plasmid through a blunt end created by treatment with *Afl*II/*Pfu* Turbo, and *Hind*III site. The entire DHFR coding region of the construct was sequenced to ensure absence of mutations. Sequencing has been performed by the MUSC Nucleic Acid Analysis Facility.

2.3. Transfection

Cells at about 50% confluence were transfected with the pcDNA3.1/FDH construct (A549 cells) or pcDNA3.1/DHFR construct (A549 cells, clone ATG10.26 [18]) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's directions. Antibiotic G418 (Sigma) was added to the culture medium at a concentration of 600 μg/ml 48 h later to allow selection of transfected cells. Every 24 h cells were detached by treatment with trypsin and counted with a hemocytometer. Efficiency of transfection estimated by co-transfection with GFP-containing vector was about 35%. Experiments were performed in triplicate and data expressed as the mean of the triplicates. Protein expression was detected by immunobloting analysis.

2.4. Analysis of DNA content

Cells (6×10^6) were labeled with BrdU for 45 min using the In Situ Proliferation Kit, FLUOS (Roche Applied Science, Indianapolis, IN), rinsed twice with PBS, detached by trypsin and fixed with mixture of absolute ethanol and 50 mM glycine, pH 2.0 at a ratio of 7:3, overnight at 4 °C. Fixed cells were denatured with 4 M HCl for 20 min at room temperature and incubated in blocking solution for 10 min to prevent non-specific binding. Cells were incubated with FITC-labeled anti-BrdU antibodies for 45 min at 37 °C in a humidified chamber. Then the sample was labeled with propidium iodide (PI) using Cellular DNA Flow Cytometric Analysis Kit (Roche Applied Science) according to the manufacturer's protocol. BrdU and PI staining was analyzed by cell flow cytometry. Flow cytometry analysis was carried out in the Hollings Cancer Center core facility on a Becton Dickinson FACSCalibur. Data analysis was performed using CellQuest and Mod Fit software (Becton Dickinson, Mountain View, CA).

2.5. Evaluation of cell growth characteristics

Actual doubling time of resistant and non-resistant A549 cells has been determined from regression analysis of cell number as a function of time [19]. Cells were harvested with 1-day interval, counted by Trypan Blue Exclusion assay, and replated. The first order growth rate constant (K_g) was calculated using the following equation: $C_{(t)} = C_0 \times e^{K_g \times t}$, where $C_{(t)}$ is the number of viable cells at specific time and C_0 is the number of viable cells at a time of cell plating [19]. The actual doubling time (T_d) has been estimated as $T_d = 0.693/K_g$. Cell growth rates were evaluated from the double cell staining by PI and pulse-labeling with BrdU according to the published report [19]. Cells incubated with BrdU for 1, 3 and 5 h were harvested, fixed, and stained with FITC-conjugated anti-BrdU monoclonal antibody as described previously [18]. For staining total DNA, cells were incubated with PI and analyzed by FACS [18]. BrdU labeled cells were considered to consist of two populations: labeled dividing and labeled undividing cells. The percentage of cells in each population was determined from BrdU/PI labeling using IsoContour software (Verity Software House, Topsham, ME).

2.6. Assays of reduced folate pools

Approximately 5×10^6 cells were collected and rapidly washed three times with ice-cold PBS. The cell pellet was resuspended in 50 mM Tris–HCl buffer, pH 7.4, containing 50 mM sodium ascorbate. Cells were lysed by heating for 3 min in a boiling water bath. Cell lysates were chilled on ice and centrifuged for 5 min at 17,000 × *g* at 4 °C. Folate pools were measured in cell lysates by the ternary complex assay method as described [20]. Folate levels were calculated per mg of cellular protein measured by Bradford assay.

2.7. Assay of FDH activity and determination of kinetic parameters

FDH activity at different 10-formyl-THF/THF ratios was measured as we previously described [21]. The reaction mixture contained 0.05 M Tris–HCl, pH 7.8, 100 mM 2-ME, 100 μM $NADP^+$ and different concentrations of substrate, 10-formyl-5,8-dideazafolate, and product, 5,8-dideazafolate (DDF). The reaction was started by the addition of FDH (10 μg) in a final volume of 1.0 ml and read against a blank cuvette containing all components except FDH. Increase in concentration of product, DDF, was measured at 295 nm using a molar extinction coefficient of 18.9×10^3. K_m and V_{max} were determined from double reciprocal plots and K_I was determined by plotting the apparent K_m versus inhibitor concentration as described elsewhere [22]. Initial velocity of FDH-catalyzed reaction in resistant and sensitive cells was calculated from the modified Michaelis-Menten equation, $V_0 = V_{max}[S]/((1 + ([I]/K_I))K_m + [S])$ [22].

2.8. Immunoblot techniques

Attached cells (about 1×10^6) were washed with PBS and lysed by adding 50 mM Tris–HCl buffer, pH 8.0 containing 0.15 M NaCl, 2 mM EDTA, 1% Triton X-100 and protease inhibitors (Sigma). Cell lysates were subjected to SDS-PAGE followed by immunobloting with the corresponding antibodies. FDH was detected using FDH-specific polyclonal antiserum [15]; DHFR was detected using commercial polyclonal antibodies (Oncogene, San Diego, CA); C_1-

synthase, GARFT and AICARFT were detected using corresponding polyclonal antibodies generated against recombinant proteins. In all cases immunobloting procedures were carried out using an ECL kit and Hybond-C nitrocellulose membranes (both from Amersham Bioscience Corp., Piscataway, NJ) according to the manufacturer's protocol.

2.9. Clonogenic assay

ATG10.26 cells capable of induced FDH expression [18] were transfected with pcDNA3.1 vector bearing DHFR cDNA. In control plates, cells were mock-transfected by "empty" pcDNA3.1 plasmid. Forty-eight hours after transfection, doxycycline (2.5 μg/ml) was added into both the control and experimental plates to induce FDH expression. Formation of clones under constant FDH induction was detected 14 days post-transfection by staining with crystal violet [15]. The medium was removed from the plates, the cells were rinsed with PBS, fixed with 4% formaldehyde for 1 h, stained with 0.2% crystal violet for 20 min and clones were counted. In a parallel experiment, clones were examined for FDH and DHFR levels by immunoblot with corresponding specific polyclonal antibodies.

2.10. Statistical analysis

Statistical analysis of the data was performed using Student's *t*-test. *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Generation of FDH-resistant A549 cells

We have previously shown that expression of FDH in FDH-deficient A549 cells induces strong cytotoxicity while addition of leucovorin to these cells protects them from FDH suppressor effects [15]. Using high (10 μM) leucovorin supplementation of A549 cells transfected with the pcDNA3.1/FDH vector, we have selected clones that stably expressed FDH. In these experiments transfected cells were selected by growing in the presence of antibiotic, G418: non-transfected cells did not survive due to lack of antibiotic resistance. Three weeks post-transfection 13 clones that sustained elevated levels of FDH in the presence of high leucovorin were obtained. Selected clones were then grown on regular medium (2.2 μM folic acid, no leucovorin) with the anticipation that after withdrawal of leucovorin proliferation would be inhibited and cells would die, as was observed in experiments with transient transfection [15] and with Tet-On A549 stable cells capable of regulated FDH expression [18]. Surprisingly, we observed that these cells did not show signs of cytotoxicity although they grew slower than the original A549 cells (Fig. 2A). Immunoblot assays confirmed that these cells express FDH at a level that would be toxic to the parental A549 cells (Fig. 2A, inset). Growth characteristics of two more clones were examined in the absence of leucovorin and they also demonstrated resistance to elevated FDH (Fig. 2B). Thus, in these experiments we have generated A549 cells (designated as A549/FDH-r) with the FDH resistant phenotype.

3.2. FDH-resistant cells have prolonged S phase and increased doubling time

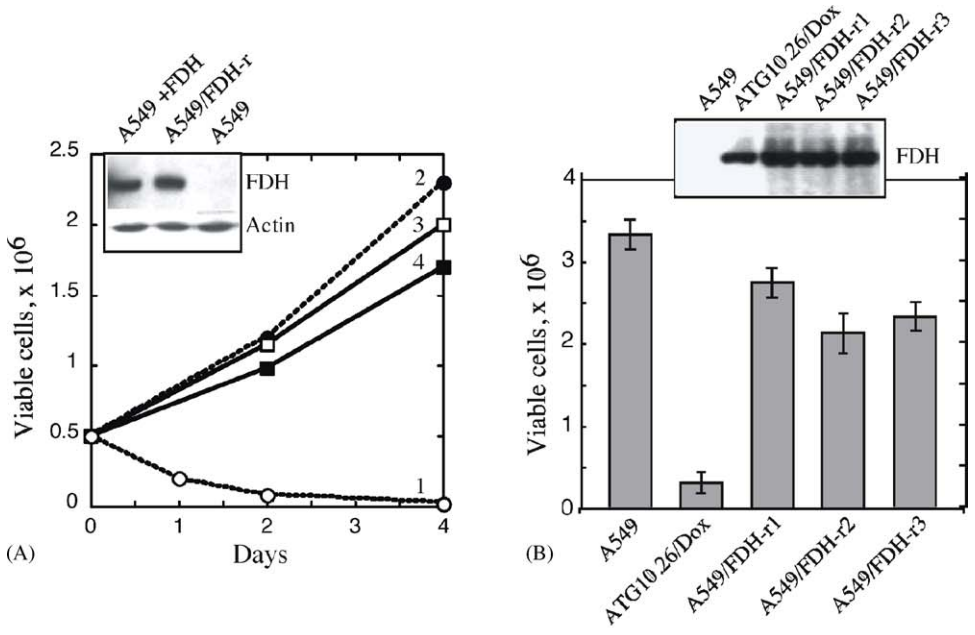
FDH-resistant cells proliferate notably slower than the original A549 cells with the doubling times of 26.0 and 20.5 h, correspondingly. Using bivariate BrdU-DNA analysis, we have estimated times required for the resistant cells to traverse different phases of the cell cycle. We have observed a significant increase (40%) in S phase duration in A549 resistant cells compared to the original cells (9.5 ± 0.87 and 6.8 ± 0.53 h, correspondingly) while durations of other phases were only slightly increased (Fig. 2C). Thus, the increase of the doubling time in A549/FDH-r cells can be attributed mainly to the prolonged S phase. The difference between the actual doubling time and the calculated doubling time observed in our experiments for both the parental cell line and FDH resistant cell line should be attributed to the cell loss that is a well known common feature of a growing cell population [23] and is not associated with FDH elevation. In agreement with the increased S phase duration, A549/FDH-r cells had higher population of cells in S phase compared to the control non-resistant cells (Fig. 2D). Distribution of cells between the cell cycle phases (Fig. 2D) further confirmed that slower proliferation of the resistant cells was associated with slower traversing S phase but not with G_0/G_1 or G_2/M arrest.

3.3. Levels of reduced folate pools in FDH-resistant cells

To evaluate whether resistance against FDH could be caused by changes in levels of reduced folates the following pools were measured in A549/FDH-r and in the parental A549 cells: combined THF and 5,10-methylene-THF; 10-formyl-THF; 5-methyl-THF, combined folate and dihydrofolate. These coenzymes represent the major intracellular reduced folate pools and the sum of their concentrations was considered as the total folate pool. As expected based on the FDH-catalyzed reaction, strong depletion of 10-formyl-THF in A549/FDH-r compared to original A549 cells was observed (Fig. 3). At the same time, the combined THF/5,10-methylene-THF pool was significantly increased. Thus, the former was decreased almost two-fold (from 14.2 to 7.4 pmol/mg of cellular protein) while the latter was increased by 23 pmol/mg of protein (about 2.5-fold). Two other pools, 5-methyl-THF and combined folate/dihydrofolate, which are not directly related to FDH activity, were also changed. While 5-methyl-THF was just slightly decreased (from 8.8 to 7.4 pmol/mg of protein), combined folate/dihydrofolate pool was increased more than twice (from 2.2 to 4.6 pmol/mg of protein). Overall, the total folate pool was notably increased, by about 45%, in the resistant cells compared to A549 cells (Fig. 3). Since intracellular folates were measured long after leucovorin has been withdrawn as a supplement, the observed folate levels are likely an intrinsic property of the resistant cells.

3.4. Product inhibition of FDH

Assays of FDH activity in the presence of increased concentrations of the reaction product, DDF, demonstrated strong product inhibition (Fig. 4A). Monitoring of the initial velocity in the presence of different concentrations of the product as a



Estimated duration (h) of the cell cycle phases and the doubling time

Cell type	T_{G1}	T_S	$T_{G2/M}$	T_C	T_d
A549	3.2 ± 0.9	6.8 ± 0.5	4.0 ± 0.4	14.0 ± 1.2	20.5 ± 1.5
A549/FDH-r	3.5 ± 0.9	9.5 ± 0.9	4.3 ± 0.5	17.3 ± 1.4	26.0 ± 1.8

(C)

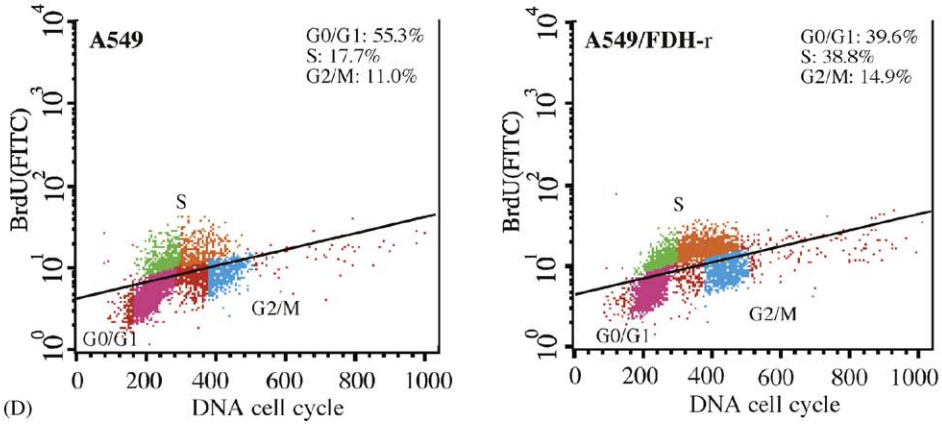


Fig. 2 – Proliferation characteristics of FDH resistant A549 cells. (A) Proliferation of the regular A549 cells transiently transfected for FDH expression (curve 1) or mock-transfected with pcDNA3.1 (curve 2); proliferation of FDH-resistant (A549/FDH-r) cells grown on leucovorin supplemented media (2.2 μM folic acid, 10 μM leucovorin) (curve 3) and regular media (2.2 μM folic acid, no leucovorin) (curve 4); inset shows levels of FDH in A549 cells 48 h post-transfection and in A549/FDH-r cells. (B) Growth inhibitory effects of FDH on A549 cells (clone ATG10.26 [18]) compared to its effects on the resistant A549 clones. Cells (5×10^5) were seeded in six-well plates; viable cells were counted by Trypan Blue Exclusion assay 3 days later. FDH in ATG10.26 cells was induced by 2.5 μg/ml doxycycline added after cells were attached. Mean ± S.E. of measurements performed in triplicate are shown. Inset shows levels of FDH in the resistant clones, regular A549 cells and in A549 cells (clone ATG10.26 [18]) induced for FDH expression. (C) Duration of the cell cycle phases (h) of A549 cells and A549/FDH-r cells. T_d, actual doubling time; T_S, DNA synthesis time; T_{G1} and $T_{G2/M}$, duration of G1 and G2/M phases, correspondingly; T_C, the total cell cycle time. Means ± S.E. are shown with exception of T_d which was derived from regression analysis using mean values of cell numbers. (D) Bivariate distribution of cells between cell cycle phases evaluated by PI labeling and BrdU incorporation for unsynchronized cell population. Magenta, G0/G1; blue, G2/M; S phase is represented by dividing (green) and undividing (orange) cells.

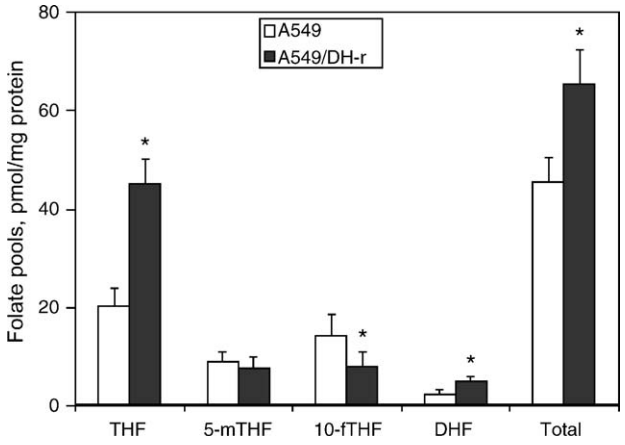


Fig. 3 – Intracellular levels of reduced folate pools in A549 cells and derived A549/FDH-r cells resistant to FDH. THF, combined THF and 5,10-methenyl-THF pools; 5-mTHF, 5-methyl-THF; 10-fTHF, 10-formyl-THF; DHF, combined folate and dihydrofolate pool; total, sum of the all shown pools. Levels of folate were determined in cells grown on 2.2 μM folic acid in the absence of leucovorin. $^{*}P < 0.05$.

function of the substrate concentration further confirmed inhibitory effects of DDF (Fig. 4B). Double reciprocal plots (Fig. 4C) derived from these data have clearly shown that the observed inhibition is competitive. The calculated K_I for the FDH reaction was 2.35 μM in the case of the synthetic substrate and product, 10-formyl-DDF/DDF. In the case of natural substrate and product, 10-formyl-THF and THF, K_I was 1.92 μM.

3.5. *Rate of FDH-catalyzed reaction at conditions of FDH-sensitive and FDH-resistant cells*

Based on the measurements of 10-formyl-THF and THF/5,10-methylene-THF pools we have calculated their concentrations in FDH-sensitive A549 cells and in FDH-resistant A549/FDH-r cells. The volume of A549 cell (1.7 μl corresponding to 10^6 cells) was taken from [24]. We have also made an assumption that cytosol comprises approximately 50% of the total cell volume [25]. Thus, in A549 cells concentrations were about 2 μM each for 10-formyl-THF and THF/5,10-methylene-THF, while in A549/FDH-r cells their concentrations were about 1 and 5 μM, correspondingly. It has been reported that concentrations of THF in cells are 1.5–3-fold higher than concentrations of 5,10-methylene-THF [26,27]. Accordingly, THF levels were estimated to be in the range of 1.2–1.6 μM in A549 cells and 3.0–3.8 μM in the resistant cells. The calculated initial velocity of FDH-catalyzed reaction was 0.083 μmol/min per mg for FDH-sensitive A549 cells and 0.028 μmol/min per mg for FDH-resistant A549 cells suggesting that in the resistant cells FDH-catalyzed reaction occurs at much slower rate.

We have also assayed FDH activity in an in vitro model system in which ratios of 10-formyl-THF/THF corresponded to those found in resistant and sensitive cells. In these studies we used stable synthetic analogs of 10-formyl-THF and THF, 10-formyl-DDF and DDF. We have previously shown that 10-formyl-DDF is an equally good substrate for FDH [28]. Results

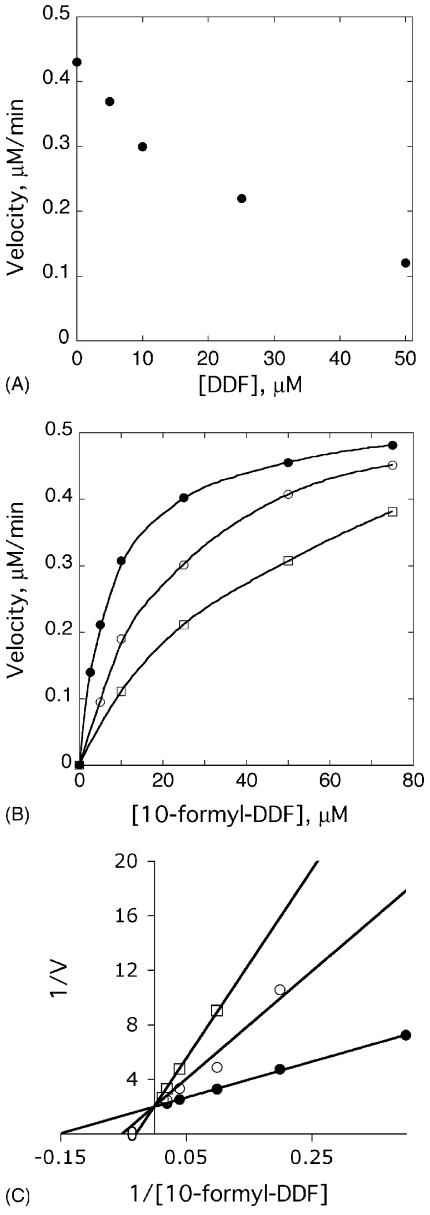


Fig. 4 – Product inhibition of FDH catalysis. (A) FDH activity in the presence of 50 μM of substrate plotted as a function of increased concentrations of the reaction product, DDF. (B) FDH activity plotted as a function of substrate concentration in the presence of a fixed concentration (closed circles, 0 μM; open circles, 5 μM; open squares, 10 μM) of product. (C) Double reciprocal plots derived from (B). About 10 μg/ml FDH was used in these assays.

of these experiments corresponded well to the calculated velocities giving further support for the hypothesis that in the resistant cells FDH catalysis is inhibited due to an increased concentration of THF (Fig. 5). Indeed, at conditions of these cells, decreased concentrations of THF analog, DDF, resulted in a significantly activated catalysis (Fig. 5, compare curves 1, 2 and 3). In contrast, at conditions of FDH-sensitive cells either omitting of DDF or increasing its concentration by two-fold did not result in a critical shift in the reaction rate (Fig. 5, compare curves 4, 5, and 6).

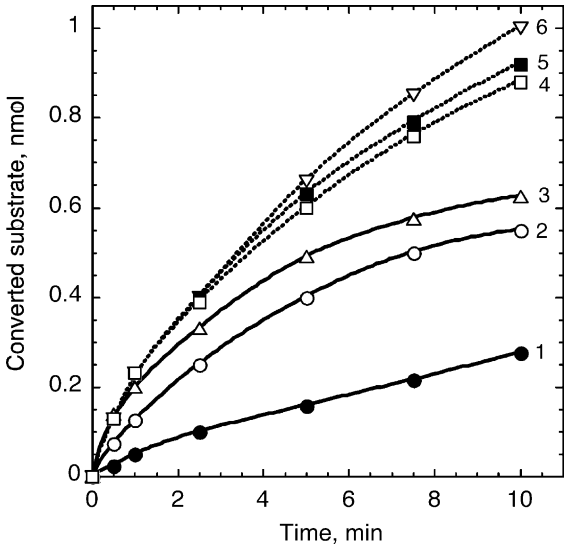


Fig. 5 – In vitro FDH activity at different initial ratios of substrate and product. Activity was assayed at initial concentrations of substrate, 10-formyl-DDF, either 2.0 μM (dotted curves) or 1.0 μM (solid curves). Concentrations of product, DDF, were 0 μM (curves 3 and 6); 1.0 μM (curves 2 and 5); 2.0 μM (curve 4); 3.5 μM (curve 6). Curve 1 (closed circles) models activity at conditions of resistant A549 cells. Curve 5 (closed squares) models activity at conditions of sensitive A549 cells. About 10 μg/ml FDH was used in these assays.

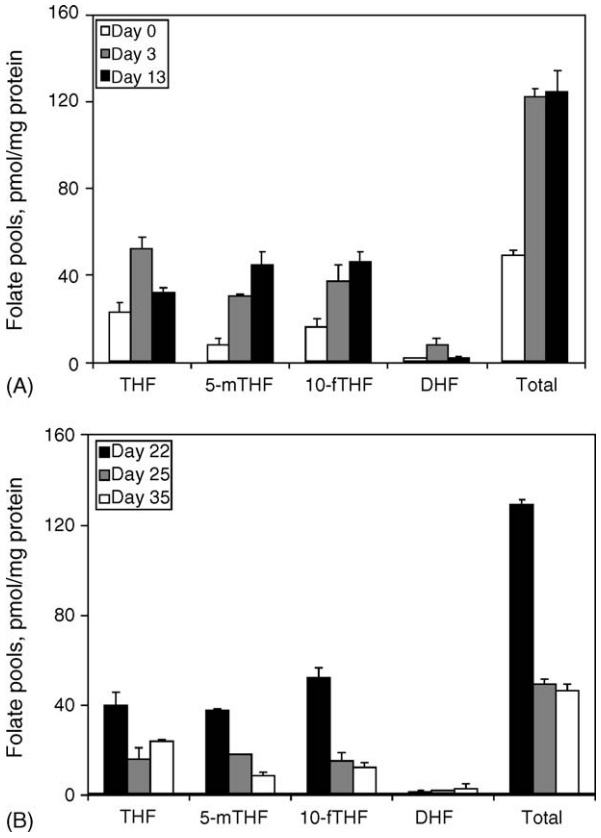


Fig. 6 – Intracellular levels of reduced folate pools in A549 cells grown on regular media (2.2 μM folic acid) supplemented with 10 μM leucovorin. (A) Folate pools in the cells supplemented with high leucovorin measured immediately before supplementation (day 0) and 3 and 13 days after beginning of supplementation. (B) Folate pools in the cells measured immediately before leucovorin withdrawal (day 22) and 3 and 13 days after leucovorin withdrawal (days 25 and 35, correspondingly). THF, combined THF and 5,10-methenyl-THF pools; 5-mTHF, 5-methyl-THF; 10-fTHF, 10-formyl-THF; DHF, combined folate and dihydrofolate pool; total, sum of the all shown pools.

3.6. *Levels of reduced folate pools in A549 cells grown on leucovorin*

After supplementation of A549 cells with high leucovorin concentrations (10 μM), a significant increase in all intracellular folate pools was observed (Fig. 6A). Most of the increase occurred by day 3 of the supplementation and was not changed significantly at later times. Interestingly, dihydrofolate pool was strongly increased (four-fold) at day 3 after beginning leucovorin supplementation but dropped back to normal level at later times (Fig. 6A). Three days after withdrawal of leucovorin from the medium intracellular folate concentrations were decreased essentially to the levels observed in the cells before leucovorin supplementation (Fig. 6B, leucovorin supplementation has been withdrawn at day 22).

3.7. *Expression pattern of folate enzymes in FDH-resistant cells*

We suggest that up-regulation of some folate enzymes could contribute to FDH-resistance. Moreover, it is likely that the changes in reduced folates in A549/FDH-r cells were caused by altered levels of other folate enzymes in addition to FDH. We have evaluated levels of C_1-synthase, the only mammalian enzyme involved in 10-formyl-THF biosynthesis, and levels of GARFT and AICARFT, the enzymes that utilize 10-formyl-THF as a substrate in the de novo purine pathway [1]. We have also evaluated levels of DHFR, one of the key folate enzymes. Increased DHFR levels are often associated with resistance against antifolates [29]. Levels of AICARFT were only slightly increased in the resistant cells compared to A549 cells while levels of GARFT and C_1-synthase were unchanged (Fig. 7). In contrast, DHFR levels were highly increased indicating strong overexpression of this enzyme in the resistant cells (Fig. 7). In the parental non-resistant A549 cells elevation of DHFR was also observed after growing cells on high leucovorin (Fig. 7). There were no changes in C_1-synthase levels in A549 cells at these conditions and changes of GARFT/AICARFT were rather marginal (Fig. 7).

3.8. *Effect of DHFR overexpression on sensitivity of A549 cells to FDH*

To test whether DHFR up-regulation could be a mechanism of FDH resistance, we have evaluated the influence of FDH on the proliferation of cells with elevated DHFR. We have elevated

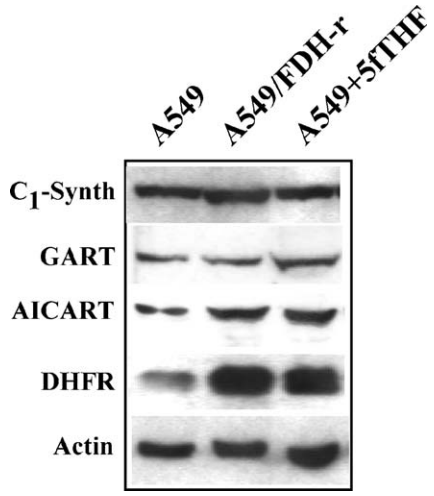


Fig. 7 – Intracellular levels of enzymes related to 10-formyl-THF metabolism. A549, original cell line; A549/FDH-r, FDH-resistant A549 clones selected on high folate supplementation; A549 + 5fTHF, A549 cells grown for 3 weeks on high (10 μM) leucovorin supplementation.

DHFR by transient transfection in A549 cells capable of inducible FDH expression (clone ATG10.26 [18]). Significant elevation of DHFR protein was achieved in these experiments 48 h post-transfection (Fig. 8A). We have observed that similar to A549/FDH-r cells DHFR-expressing cells were less sensitive to FDH antiproliferative effects (Fig. 8B). Thus, 4 days after induction of FDH expression about 3.9 times more viable cells were seen in the presence of elevated DHFR than at lower DHFR levels. Likewise, colony growth assay revealed that prolonged culturing of cells with elevated DHFR under constant FDH induction (these cells have high levels of both DHFR and FDH) allows selection of FDH-resistant clones (Fig. 8C).

4. Discussion

FDH, a folate-metabolizing enzyme, induces cytotoxicity upon expression in FDH-deficient cancer cells [15]. In our studies leucovorin rescued cells from FDH toxicity, resulting in selection of a FDH-resistant phenotype. The progression of tumors to malignancy depends on their ability to evolve into heterogeneous phenotypes, a process that appears to involve mainly quantitative changes in gene expression [30]. Heterogeneous clones can also be a major contributor in the emergence of refractory tumors [31]. Therefore, it was important to understand metabolic alterations that are responsible for the resistant phenotype in our studies.

Leucovorin is used clinically to elevate intracellular folate levels [32]. In the cell, excess leucovorin is rapidly converted to 5,10-methenyl-THF and then to 5,10-methylene-THF [32]. Alterations in intracellular reduced folate pools in response to leucovorin supplementation are one of the mechanisms of rescue from MTX toxicity [10,33,34]. On the other side, elevation of intracellular 5,10-methylene-THF is the basis for the application of leucovorin in combination with 5-fluorouracil, an inhibitor of thymidylate synthase, in cancer chemotherapy. Thus, increased 5,10-methenyl-THF results in stabilization of the ternary complex between thymidylate synthase and the active 5-fluorouracil metabolite (5-fluorodeoxyuridylate) enhancing the enzyme inhibition [35]. In agreement with the effect of leucovorin supplementation, the combined THF/5,10-methylene-THF pool was strongly increased in the resistant cells as well as the total intracellular folate pool. However, elevated THF/5,10-methylene-THF and the total folate pool in the resistant cells was maintained after leucovorin supplementation was cancelled, suggesting that if this mechanism took place during the earlier stage of clonal selection, it was not directly responsible for the long-term increase in total folate. Indeed, evaluation of the intracellular folates in regular A549 cells supplemented with leucovorin has demonstrated that though an increase in intracellular folates took place, it affected all folate pools and folate concentrations rapidly returned to normal levels after the supplementation was withdrawn.

Supplementation with leucovorin resulted in an increase in DHFR protein levels in A549 cells. The rescue of FDH-expressing cells after elevation of DHFR by transient transfection supports a role for the enzyme in the FDH resistant phenotype. In the cell, DHFR reduces dihydrofolate, produced in the reaction of thymidylate biosynthesis, to THF [1]. The enzyme is also capable of reducing folic acid to dihydrofolate [1]. Mechanistically, if extracellular folate is freely available in the form of folic acid, and if reduced folates can be retained in the cell, the elevation of DHFR would result in an increase in the total intracellular THF pool through dihydrofolate as an intermediate step. Thus, increased levels of DHFR and THF as well as dihydrofolate in A549/FDH-r cells implies enhanced incorporation of folic acid into the reduced folate pool catalyzed by DHFR. Elevated DHFR, together with elevated FDH, can also contribute to the total folate increase by providing more folate binding sites. It has been proposed that most intracellular folate is protein-bound and the concentration of free coenzymes is very low [36,37]. Together with the fact that cellular folate levels are saturable, this has led to conclusion that the accumulation of cellular folate is limited by the folate binding capacity of the cell [4]. Accordingly, an increase in the number of folate-binding sites should allow the retention of more intracellular folate resulting in its accumulation.

FDH possesses high binding affinity to THF [16] and it has been suggested that FDH activity is regulated by the ratio of 10-formyl-THF to THF in liver [13]. Thus, direct inhibition of the FDH-catalyzed reaction, through tight binding in the FDH catalytic center, could be a mechanism by which elevated THF overcomes the suppressor effects of FDH. Our kinetic studies have demonstrated that the product of the FDH-catalyzed reaction is indeed a strong competitive inhibitor of the enzyme. Therefore, the ratio of 10-formyl-THF/THF shifted towards THF is unfavorable for the FDH-catalyzed reaction. Our results indicate that the initial rate of the FDH-catalyzed reaction is about three-fold slower in the resistant cells than in the parental sensitive cells. Further evaluation of FDH enzymatic activity in a model system confirmed that at the substrate/product ratios observed in the resistant cells, FDH catalysis is significantly inhibited. Thus, FDH is

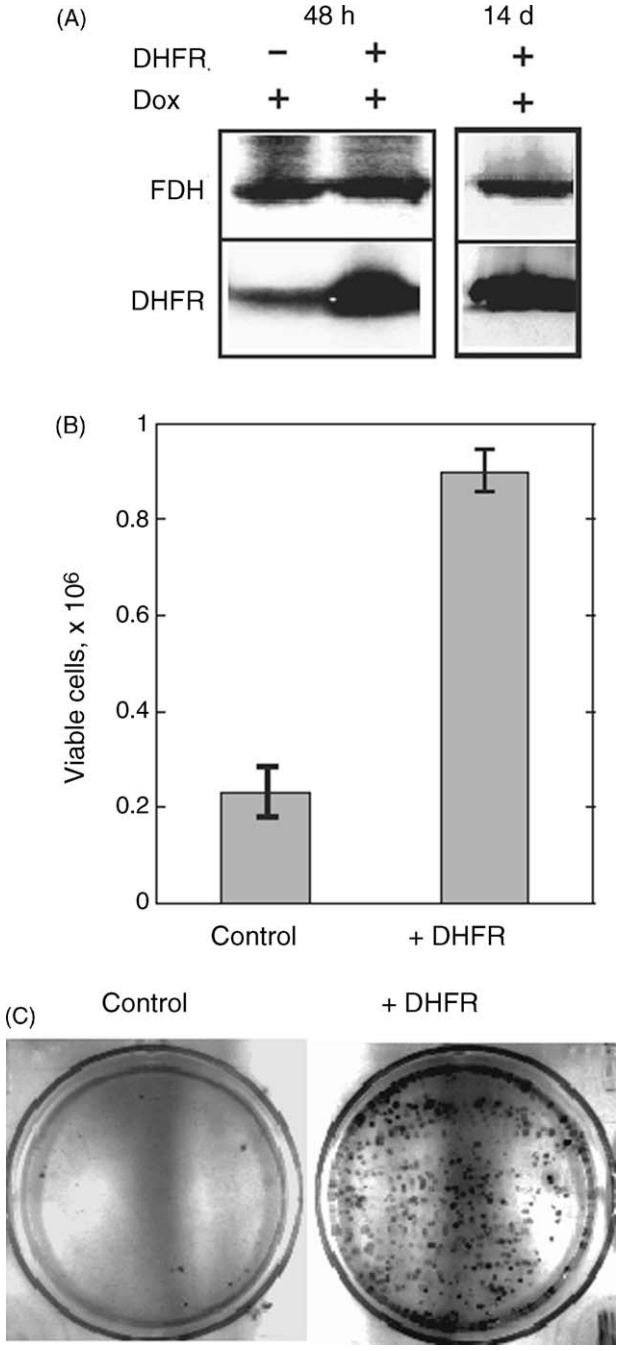


Fig. 8 – Effect of FDH on cells expressing elevated levels of DHFR. (A) Left panel, levels of FDH and DHFR in A549 cells (clone ATG10.26 [18]) (−) and in the same cells transiently transfected with DHFR-expressing vector (+). In both cases cells were treated with doxycycline to induce FDH expression. Right panel shows levels of the enzymes in stable clones (panel C) expressing DHFR (14 days DHFR post-transfection). (B) Growth inhibitory effects of FDH on ATG10.26 cells with (+DHFR) and without (Control) elevated DHFR. Viable cells were counted by Trypan Blue exclusion assay 4 days after transfection with DHFR-expressing vector. FDH expression was induced by addition of 2.5 μg/ml doxycycline 12 h after transient transfection with DHFR expressing vector. Mean ± S.E. of two independent experiments performed in triplicate are shown. (C) Clonal growth assay of ATG10.26 cells transfected with DHFR-expressing vector (+DHFR) and with the "empty" vector (Control). During the clonal selection the cells were permanently induced with doxycycline for FDH expression. Clones were stained 14 days after transfection.

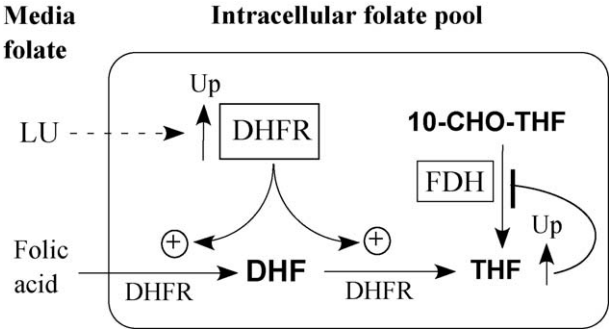


Fig. 9 – Schematic depicting leucovorin-induced mechanism of FDH resistance. Abbreviations: LU, leucovorin; THF, tetrahydrofolate; 10-CHO-THF, 10-formyl-THF; DHF, dihydrofolate. The proposed mechanism of FDH resistance includes three steps: (1) LU induces DHFR elevation; (2) elevated DHFR increases rate of conversion of folic acid, absorbed from the media, to THF; (3) increased THF inhibits FDH thus blocking conversion of 10-formyl-THF to THF and preserving 10-formyl-THF pool.

not fully functional in the resistant cells due to product inhibition.

An interesting question is, what is the mechanism of DHFR elevation in A549/FDH-r cells? Intracellular DHFR concentrations are regulated at transcriptional [38], translational [39,40] and protein [41] levels. It is possible that the mechanism of DHFR up-regulation in FDH-resistant cells is related to an increase of intracellular dihydrofolate and is similar to translational up-regulation of DHFR by MTX [42]. It has been demonstrated that translation of DHFR mRNA is repressed by the binding of DHFR protein [29]. Binding of MTX as well as dihydrofolate to the enzyme results in the release of mRNA from this complex and activates translation [29]. Numerous studies have shown nearly quantitative conversion of leucovorin to dihydrofolate (see [10] and references therein). In agreement with this mechanism, a strong elevation of the dihydrofolate pool and DHFR levels was seen in our studies following the beginning of leucovorin supplementation. Alternatively, well-known DHFR gene amplification, which can support elevated DHFR for many cell generations [10], could not be ruled out as a mechanism of DHFR elevation in FDH-resistant cells.

Overall, our studies demonstrate that DHFR up-regulation in A549 cells causes acquired resistance against FDH antiproliferative effects. The down-stream event is a significant elevation of intracellular THF (Fig. 9) resulting in FDH inhibition. Importantly, this mechanism can be activated by leucovorin. Leucovorin is often used in combination with MTX or 5-fluorouracil in chemoprevention treatments [9,37]. Our studies

indicate that leucovorin itself selects for an abnormal phenotype that could potentially result in more refractory tumors. It is unclear at present to what extent the DHFR-induced mechanism of FDH resistance is relevant to normal cells. With regard to this question, it should be noted that A549/FDH-r cells require longer time to traverse S phase, which implies that most of the FDH suppressor effects are associated with this phase of the cell cycle. DHFR, an S phase enzyme, is strongly activated during transition from G_1 to S phase when the cell initiates S phase-related synthetic processes to prepare for division [38]. Therefore, DHFR-induced repression of FDH catalysis could be an S phase-related metabolic adjustment that protects cells against FDH suppressor effects.

Acknowledgements

This work was supported by the National Institute of Health Grants DK54388 and CA95030 (to S.A.K.), and by the Abney Foundation Research Fellowship (to S.N.R.). The authors thank Dr. David Priest for help with setting up folate assays and for productive discussion, Dr. David Goldman and members of his lab for critical reading of the manuscript, and Ms. Angela Shepherd for technical assistance.

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