Biochemical Pharmacology, Vol. 45, No. 5, pp. 1157–1164, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00
Pergamon Press Ltd

ISOLATION AND CHARACTERIZATION OF A HUMAN ILEOCECAL CARCINOMA CELL LINE (HCT-8) SUBCLONE RESISTANT TO FLUORODEOXYURIDINE

ZHENG-GANG ZHANG, MARTIN MALMBERG,* M.-B. YIN, HARRY K. SLOCUM and YOUCEF M. RUSTUM†

Grace Cancer Drug Center and Department of Experimental Therapeutics, Roswell Park Cancer Institute, Buffalo, NY 14263, U.S.A.

(*Received* 6 *July* 1992; *accepted* 2 *November* 1992)

Abstract—A 5-fluoro-2′-deoxyuridine (FdUrd)-resistant subclone (Fd9XR) of HCT-8 (human ileocecal carcinoma) cells was established by two schedules of drug exposure. Initially, cells were exposed to short-term (3 hr) 100 nM FdUrd repeatedly (9 cycles over 8 months), and cells were then exposed to 10 nM FdUrd continuously. During this latter stage, a colony (Fd9XR) with fast growth rate was isolated, expanded, and characterized with respect to mechanisms of resistance to FdUrd and cross-resistance to other chemotherapeutic agents. Fd9XR cells were 1000-fold resistant to FdUrd, but 3-fold more sensitive to 5-fluorouracil (FUra) than HCT-8 cells. After a 3-hr treatment with FdUrd, Fd9XR cells accumulated 6630-, 69-, and 3.7-fold less fluorodeoxyuridylate (FdUMP), fluorouridine triphosphate (FUTP) and acid-insoluble materials, respectively, than HCT-8 cells. However, when FUra was substituted for FdUrd, Fd9XR cells accumulated 9.2-, 3.1-, and 2.3-fold more FdUMP, FUTP and acid-insoluble materials, respectively, than HCT-8 cells. Fd9XR and HCT-8 were similar in their growth rates, combined pools of 5,10-methylenetetrahydrofolates (5,10-$CH_2H_4PteGlu_n$) and tetrahydrofolates ($H_4PTeGlu_n$), thymidine phosphorylase (TP) activity, and level and activity of thymidylate synthase (TS). In contrast, thymidine kinase (TK) activity of Fd9XR was 0.23 and 0.35% of that of HCT-8, for thymidine (dThd) and FdUrd as substrates, respectively. Furthermore, Fd9XR cells exhibited greater sensitivity to the antifolate TS inhibitor ICI D1694 and to methotrexate (MTX) than HCT-8 cells. In addition, dThd alone and in combination with hypoxanthine did not offer any protection against the cytotoxic effect of ICI D1694 in Fd9XR cells. These results indicate that in Fd9XR cells (1) TK deficiency is the primary mechanism of resistance to FdUrd; (2) the greater sensitivity to FUra was associated with higher pools of FdUMP and FUTP with a subsequently higher level of incorporation into cellular RNA; and (3) antifolate compounds, e.g. ICI D1694 and MTX, could be useful agents in the treatment of FdUrd-resistant tumors associated with decreased TK activity and decreased capacity of utilizing dThd.

Although the mechanisms of action of the fluoropyrimidines 5-fluoro-2′-deoxyuridine (FdUrd)‡ and 5-fluorouracil (FUra) are multifactorial, the determinants associated with therapeutic activity of these drugs include inhibition of thymidylate synthase (TS), incorporation into cellular RNA and DNA, [1, 2] and DNA damage [3, 4]. The predominant mechanism may vary depending on tumor type and metabolic state which include the activities of metabolic enzymes, levels of competing substrates, availability of cofactors, and schedule of drug administration. Inhibition of TS by FdUrd and FUra is mediated by the formation of a stable ternary complex between the active anabolite 5-fluoro-2′-deoxyuridylate (FdUMP), the target enzyme TS, and the cofactor 5,10-methylenetetrahydrofolates (5,10-$CH_2H_4PteGlu_n$) [5, 6]. Since the levels of 5,10-$CH_2H_4PteGlu_n$ in some tumors are insufficient for promoting complete ternary complex formation [7, 8], a reduced folate derivative, 5-formyltetrahydrofolate (5-$HCOH_4PteGlu$), is frequently used in combinations with FUra or FdUrd in the treatment of solid tumors [9–11]. Although the response rates of these tumors to FUra or FdUrd in combination with 5-$HCOH_4PteGlu$ have increased significantly, tumor resistance still

* Present address: Department of Surgery, Helsingborg Hospital, S25187 Helsingborg, Sweden.

† Corresponding author: Dr. Youcef M. Rustum, Grace Cancer Drug Center and Department of Experimental Therapeutics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263. Tel. (716) 845-4532; FAX (716) 845-8857.

‡ Abbreviations: FdUrd, 5-fluoro-2′-deoxyuridine; FUra, 5-fluorouracil; FdUMP, 5-fluoro-2′-deoxyridylate; FdUDP, 5-fluoro-2′-deoxyuridine diphosphate; FdUTP, 5-fluoro-2′-deoxyuridine triphosphate; FUTP, 5-fluorouridine triphosphate; FUMP, 5-fluorouridylate; FUDP, 5-fluorouridine diphosphate; dUMP, 2′-deoxyuridylate; 5-$HCOH_4PteGlu$, 5-formyltetrahydrofolate or leucovorin; 5,10-$CH_2H_4PteGlu_n$ and $H_4PteGlu_n$, 5,10-methylenetetrahydrofolates and tetrahydrofolates containing n glutamate residues in γ-linkage; dThd, thymidine; TS, thymidylate synthase; TK, thymidine kinase; TP, thymidine phosphorylase; DTT, dithiothreitol; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; MTX, methotrexate; ICI D1694, *N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazoline-6-ylmethyl)-*N*-methylaminol-2-thenoyl)-1-glutamic acid; PBS, phosphate-buffered saline; Fd9X, a subpopulation of HCT-8 cells surviving 9 cycles of 3-hr exposure to 100 nM FdUrd; and Fd9XR, a subclone of Fd9X whose growth rate was among the fastest of all Fd9X cells.

In this article, 5,10-$CH_2H_4PteGlu_n$ and $H_4PteGlu_n$ refer to the natural diastereoisomers of the compounds. 5-$HCOH_4PteGlu$ refers to the natural [6*S*] diastereoisomer in a 1:1 mixture of the natural and unnatural [6*R*,*S*] isomers of the compound, or the pure [6*S*] isomer.

represents a major obstacle to curative chemotherapy [9].

To study mechanisms of tumor cell resistance to fluoropyrimidines, various cell lines resistant to FdUrd and/or FUra have been isolated [12–19]. The procedures of isolation usually involve exposure of cells to stepwise increasing concentrations of the drug, or to a single dose of a mutagenic agent [20]. The surviving cells are then cloned under the selective pressure of the drug. Under these conditions, high degrees of cellular resistance are not guaranteed because the growth rates of the selected cells are unknown at the time of isolation. It has also been suggested that repeated exposure to a constant concentration of a cytotoxic agent, which resembles clinical chemotherapeutic regimes, may give rise to resistant cells with unique biochemical mechanisms which are more relevant clinically [21].

We describe here a new approach of recognizing high-resistant colonies by monitoring their growth rates with a computerized image analysis system [22] after repeated exposure of cells to FdUrd. Using this system, we isolated an FdUrd-resistant subclone of HCT-8 (human ileocecal carcinoma) cells. The primary mechanism of resistance of this subclone was investigated by evaluating multiple enzymes involved in the metabolic pathways of FdUrd and FUra and parameters associated with response to the drugs. In addition, potential approaches to circumvent resistance were also explored.

MATERIALS AND METHODS

Chemicals, medium, cultureware, and cell line. [6-^{3}H]FdUrd (20 Ci/mmol), [6-^{3}H]FUra (20 Ci/mmol), [6-^{3}H]FdUMP (20 Ci/mmol) and [5-^{3}H]2′-deoxyuridylate (dUMP) (22 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), FdUrd and FUra were purchased from the Sigma Chemical Co. (St. Louis, MO). Ecoscint A liquid scintillation fluid was obtained from National Diagnostics (Manville, NJ). RPMI 1640 medium, sodium pyruvate, dialyzed horse serum, and trypsin (0.05% trypsin and 0.53 mM EDTA) were purchased from GIBCO (Grand Island, NY). A Mycoplasma T.C. Rapid Detection System was purchased from Gen-Probe Inc. (San Diego, CA), and seaplaque agarose from FMC Bioproducts (Rockland, ME). Falcon No. 3046 6-well plates, Falcon No. 3084 75-cm^2 flasks, and Corning No. 25860 96-well plates were used. A human ileocecal carcinoma cell line, HCT-8, was obtained from the American Type Culture Collection (Rockville, MD).

Cell culture. HCT-8 and its subclones were maintained as monolayer cultures in 75-cm^2 flasks, in RPMI 1640 medium supplemented with 1 mM sodium pyruvate and 10% dialyzed horse serum, in a 37° incubator with 5% CO_2. Cells were subcultured by trypsinization (at 37° for 5 min), and dilution at 1:20 every 4–5 days. The cultures were tested with a Mycoplasma T.C. Rapid Detection System every 2 months and consistently found to be free from Mycoplasma contamination.

Isolation of an FdUrd-resistant subclone. HCT-8 cells were treated repeatedly with FdUrd according to the following procedure: cells were seeded in a 75-cm^2 flask at 1×10^6 cells in 20 mL medium, and on the next day exposed to 100 nM (50% inhibition concentration) FdUrd for 3 hr. Cells were then allowed to grow in FdUrd-free medium to confluency (10 days). Then cells were subcultured in FdUrd-free medium at 1:10 dilution for 3 passages. After 9 cycles of treatment the surviving cell population was designated Fd9X. The Fd9X cells were further cloned using a method described previously [22]. Briefly, 1.4×10^3 cells in 0.2 mL of 0.3% agarose were seeded at 37° over a lower layer of 1.8 mL solidified 1% agarose in medium with 10 nM FdUrd in a 6-well plate. Cells were observed at daily intervals for 6 days, observations digitally recorded and area measurements made. These measurements were matched with colony identities using custom software, and a simple exponential growth model was fit to the data for individual colonies as previously described [22]. One of the colonies with a growth rate similar to controls was recognized, carefully aspirated with a 30-gauge needle attached to a 1-mL syringe under a stereomicroscope (Olympus, model SZH) and transferred into 10 μL medium in a 96-well culture plate, expanded in culture, and designated as Fd9XR.

Growth rates of cells in monolayer culture. HCT-8 and Fd9XR cells were seeded in 96-well plates at 600 cells/well in 0.1 mL medium on day 0. On days 1 through 6 after seeding, the viable cell mass in one of the plates was determined by an MTT colorimetric assay described previously [23]. A simple exponential growth model was fit to the data and the doubling time estimated using Microsoft Excel software.

Growth inhibition assay. Cells were seeded in 96-well plates at 1200–1500 cell/well in 50 μL of medium. At 21 hr after seeding, 50 μL of 2× final concentrations of drug of interest was added. At 3 hr after the addition of the drug the drug/medium was aspirated, and 10 μL of drug-free medium was added. Cells were allowed to grow for an additional 4 days. Cell growth was measured by the MTT colorimetric assay. The concentrations that cause 50% inhibition (IC_{50}) values were determined graphically from the concentration-response curves.

Accumulation and metabolism of FdUrd and FUra. Logarithmically growing cells in a 75-cm^2 flask were incubated with 127 nM [6-^{3}H]FUra or 86 nM [6-^{3}H]-FdUrd for 3 hr at 37°. The medium was then removed and the cells were washed twice with phosphate-buffered saline (PBS). The cells were detached by trypsinization and suspended in 10 mL PBS. A 0.5-mL sample of the suspension was taken for cell count on a model ZBI Coulter counter (Coulter Electronics, Inc., Hialeah, FL). The rest of the suspension was centrifuged at 500 *g* for 5 min at 4°. The cell pellet was then solubilized with 70–80 μL of 1 M perchloric acid in 0.1 M ammonium formate buffer, pH 2. The contents were mixed and incubated on ice for 5 min for precipitation. The tube was centrifuged at 1600 *g* for 5 min at 4°. The supernatant was carefully collected and neutralized to pH 7 with

2 N potassium hydroxide. Aliquots of the supernatant were used for the determination of drug uptake into the acid-soluble compartment and distribution of mono-, di- and triphosphates by HPLC. The HPLC analysis was performed on a Spherisorb S5 SAX ion-exchange column, 25 cm × 4.6 mm i.d. (Phase Separations Inc., Norwalk, CT), with a gradient mobile phase driven by a model 2152 LC controller with a dual model 2150 pump set (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) at 1 mL/min. The mobile phase was composed of Buffer A (50 mM ammonium phosphate, pH 3.5) and Buffer B (500 mM ammonium phosphate, pH 2.9). The column was equilibrated with Buffer A before sample injection. At 30 min after the injection of a sample, the percentage of Buffer B started to increase linearly and reached 100% at 60 min (end of analysis). Absorbance of the eluent at 260 nm was monitored with a model 481 UV detector (Waters Chromatography Division, Millipore Corp., Milford, MA). Fractions of 1 mL were collected and mixed with 5 mL of Ecoscint A liquid scintillation fluid and counted for radioactivity in a model LS1701 counter (Beckman Instruments, Fullerton, CA). The acid-insoluble pellet was washed twice with 10 mL of 1 M perchloric acid, and the radioactivity incorporated into the acid-insoluble compartment was determined by liquid scintillation counting as described above.

5,10-CH_2H_4PteGlu$_n$ level and polyglutamate distribution assay. The method of Priest and Doig [24] was adopted to determine the total intracellular combined pools of 5,10-CH_2H_4PteGlu$_n$ and tetrahydrofolates (H_4PteGlu$_n$), and polyglutamate distribution as described previously [23].

Preparation of cell extracts for various enzyme assays. The method used for the preparation of cell extracts for various enzyme assays was described previously [25]. Briefly, cells were suspended in extraction buffer (the components of the buffer vary according to the type of enzyme assay). A sample of the cell suspension was taken for cell number determination of a Coulter Counter. The rest of the cells were lysed by agitating on a vortex mixer briefly, freezing in a dry-ice/ethanol bath for 5 min and thawing in a 37° water bath for 2 min alternately for three times each. The lysates were centrifuged at 8800 *g* for 15 min in 4°. The supernatants were transferred into new tubes, kept on ice, to be used as the cell extracts.

TS assays. The TS content assay was based on the stoichiometric binding of TS to [6-^{3}H]FdUMP and 5,10-CH_2H_4PteGlu. The TS activity assay was based on the [^{3}H]water release from [5-^{3}H]dUMP. These assays were conducted as described previously [25].

Thymidine kinase (TK) assay. The method used was modified from that of Cheng [26].

Thymidine phosphorylase (TP) assay. The method used was modified from that of Houghton *et al.* [19]. Extraction buffer (50 mM Na_2HPO_4, 1 mM EDTA, 5 mM DTT, pH 6.4) was added to the cell pellets at

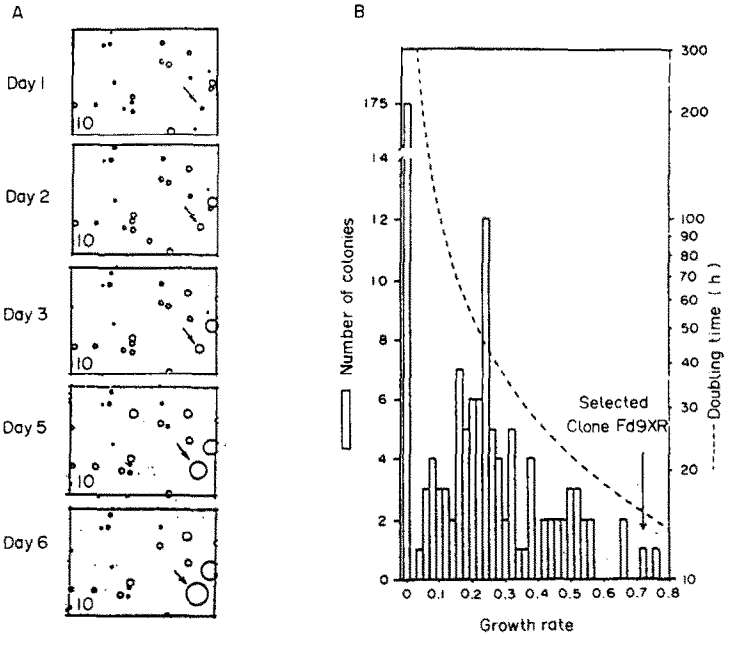

Fig. 1. Image analysis for the isolation of Fd9XR cells. Fd9X cells, obtained from HCT-9 cells pretreated 9 times with 100 nM FdUrd for 3 hr, were grown on semisolid agarose medium in the presence of 10 nM FdUrd in a 6-well culture plate. Images of 16 fields of view (total area 40 mm^2) were analyzed by an image analyzer on days 1, 2, 3, 5, and 6. The area of each colony was calculated and maps of the positions and sizes of all colonies were generated. Panel A shows the maps of field 10 which contained the Fd9XR colony (arrows). The growth rate of each colony was calculated based on area. The histogram of the growth rate distribution and the corresponding volume doubling time of all colonies are shown in Panel B.

0.4 mL/10^7 cells. The substrate solution contained 150 μM (0.2 Ci/mmol) [^{3}H]Thd in the extraction buffer. The reaction was started by mixing 50 μL of the substrate solution and 100 μL of cell extract (both were prewarmed at 37° for 3 min). The mixture were agitated briefly and incubated in a 37° water bath. At 30, 60 and 90 min, 40-μL aliquots of the reaction mixture were taken and put into a tube containing 12 μL of 1 M (6%, w/w) perchloric acid in 0.1 M ammonium formate buffer, pH 2. The contents were mixed well, incubated on ice for 5 min and then neutralized to pH 7 with 2 N potassium hydroxide. The tubes were centrifuged at 1700 *g* for 5 min at 4° and the supernatants were analyzed by HPLC. The HPLC system included 2 serially connected Spherisorb S3 ODS2 columns (15 cm + 10 cm, 4.6 mm i.d., Phase Separations Inc.) and a mobile phase containing 2.5 mM ammonium acetate and 1.25% methanol, pH 5.0, with a flow rate of 1 mL/min. Aliquots (20 μL) were loaded on the columns. Eluted fractions of 1 mL were collected and counted for radioactivity in 7-mL scintillation vials with 5 mL of liquid scintillation fluid (Ecoscint A). The retention times for dThd and thymine were 13 and 45 min, respectively.

RESULTS

Growth rate distribution of colonies under selective pressure and isolation of an FdUrd-resistant subclone. HCT-8 cells were treated with 9 cycles of short-term (3 hr) exposure to 100 μM FdUrd, and recovery, as described in Materials and Methods. The surviving population (Fd9X) was seeded in semisolid medium under continuous selection pressure (10 nM FdUrd) and the growth rate of each colony derived from a single cell within a defined area of the culture plate was determined. Figure 1A demonstrates a series of graphical representations of the sizes and positions over time of colonies in a field of view containing the colony isolated (arrows). Figure 1B is the frequency distribution of the growth rate of all individual colonies monitored; there was considerable heterogeneity in the growth rates (0.04 to 0.76). The mean area growth rate ± SD of all growing colonies was 0.273 ± 0.159, representing a volume doubling time of 40.6 hr employing a spherical colony model (see Ref. 22). One of the fastest growing colonies (Fd9XR), whose growth rate was 0.705 (corresponding to a volume doubling time of 15.7 hr), was successfully expanded. The growth rate of Fd9XR in monolayer culture with drug-free medium was similar to that of HCT-8 with doubling times of 18.9 and 19.2 hr, respectively.

Growth inhibition by FdUrd, FUra, ICI D1684 and methotrexate (MTX). Growth inhibition of HCT-8 and Fd9XR cells exposed for 3 hr to FdUrd or FUra was evaluated (Table 1). Compared to the parent HCT-8 cells, the Fd9XR cells were about 1000-fold more resistant to FdUrd. However, the Fd9XR cells were 3-fold more sensitive to FUra, 2-fold more sensitive to ICI D1694 and 3-fold more sensitive to MTX than the parent line. The drug sensitivity characteristics of Fd9XR cells were stable for at least 6 months in FdUrd-free medium (not shown).

Table 1. IC_{50} values of several anticancer agents in HCT-8 and Fd9XR cells

Cells	IC_{50} (μM)			
	FdUrd	FUra	ICI D1694	MTX
HCT-8	0.1	160	0.03	0.6
Fd9XR	100	50	0.015	0.2
Fold resistance	1000	0.3	0.5	0.3

HCT-8 and Fd9XR cells growing in standard medium were treated for 3 hr with various concentrations of the agents indicated. After treatment, cells were allowed to grow for an additional 4 days. Cell growth was determined by the MTT assay. The IC_{50} values were estimated as described in Materials and Methods.

Table 2. FdUrd and FUra metabolite pools in HCT-8 and Fd9XR cells

Metabolites	Cell line			
	HCT-8		Fd9XR	
	FdUrd	FUra	FdUrd	FUra
Total uptake	13,400	31.2	262	87.4
FdUMP	12,000	0.72	1.81	6.55
FUTP	155	12.9	2.24	39.4
Acid-insoluble	952	13.1	254	29.4

HCT-8 and Fd9XR cells were exposed to 86 nM [6-^{3}H]-FdUrd or 127 nM [6-^{3}H]FUra for 3 hr. Intracellular drug metabolite pools (fmol/10^6 cells) were measured as described in Materials and Methods.

Accumulation and metabolism of FdUrd and FUra. The metabolites derived from [^{3}H]FdUrd or [^{3}H]-FUra in a 3-hr exposure of HCT-8 and Fd9XR cells were quantitated (Fig. 2 and Table 2). The data in Fig. 2 demonstrate the separation by HPLC of various fluorinated (F) nucleotides. The data in Fig. 2 indicate that F-ribonucleotides, FUMP, FUDP and FUTP, can be separated from each other and from their corresponding F-deoxyribonucleotides, FdUMP, FdUDP and FdUTP. In a separate experiment, normal uridine ribonucleotides or deoxyribonucleotides could not be separated from the corresponding F-nucleotides. The retention time for FUMP was 4.9 min; FdUDP, 14 min; FUTP, 23 min; and FdUTP, 24 min. Thus, with this method unambiguous separation of F-ribonucleotides from F-deoxyribonucleotides can be achieved. Using this method, the ribo- and deoxyribonucleotides derived from [^{3}H]FUra and [^{3}H]FdUrd can be quantitated reproducibly.

A summary of the total accumulation and distribution of F-nucleotides is given in Table 2. The total accumulation of acid-soluble pools derived from [^{3}H]GdUrd in HCT-8 cells was 51-fold higher than that achieved in Fd9XR. The largest difference between the two cell lines was in the extent of

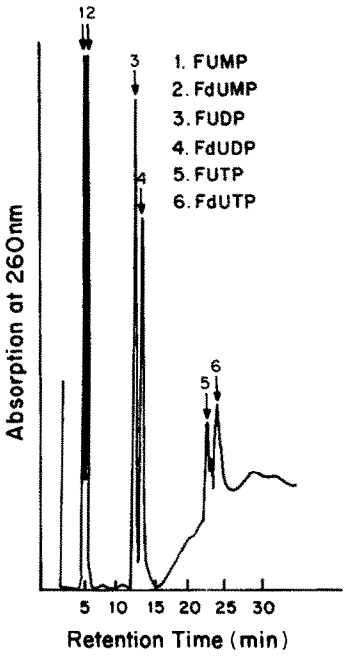

Fig. 2. HPLC separation of a standard mixture of F-nucleotides. Conditions are described in Materials and Methods. Twenty microliters of a mixture of nucleotides (10 μg/mL each) was injected on the HPLC column and eluted at a flow rate of 1 mL/min.

metabolism of FdUrd to FdUMP. HCT-8 cells accumulated 6630-fold more FdUMP than did Fd9XR cells. HCT-8 also accumulated 69-fold more FUTP, and incorporated 3.7-fold more into the acid-insoluble compartment than did Fd9XR. The acid-insoluble compartment includes drug incorporated into or bound to cellular RNA, DNA and protein. In contrast, the total accumulation of [^{3}H]FUra by Fd9XR was 2.8-fold higher than that by HCT-8. The FdUMP, FUTP, and the acid-insoluble pools after FUra exposure were each higher in Fd9XR than in HCT-8 (9.2-, 3.1-, and 2.3-fold, respectively; Table 2). This is consistent with the higher sensitivity of Fd9XR cells to FUra.

TK, TP, TS and 5,10-CH_2H_4*PteGlu*$_n$. To evaluate further mechanisms associated with resistance to FdUrd in Fd9XR cells, additional relevant parameters were evaluated (Table 3). Except for TK activity, all other parameters evaluated, including the activity of the catabolic enzyme TP, target enzyme TS content and activity, and cofactor pools of 5,10-CH_2H_4PteGlu$_n$, were similar in both HCT-8 and Fd9XR cells. The activity of TK, the enzyme responsible for the conversion of FdUrd and thymidine to FdUMP and dTMP, respectively, in Fd9XR cells was less than 0.5% of that in HCT-8 cells. Thus, the lower accumulation of FdUrd (and hence resistance to the drug) was due primarily to the low level of TK activity in Fd9XR cells.

*Growth inhibition by MTX and ICI D*1694, and protection by dThd and hypoxanthine. Since TK activity was low and the salvage pathway for the

Table 3. Combined pools of 5,10-CH_2H_4PTeGlu$_n$ and H_4PTeGlu$_n$, TS, TP and TK in HCT-8 and Fd9XR cells

Parameter	HCT-8	Fd9XR
Combined 5,10-CH_2H_4PTeGlu$_n$ and H_4PTeGlu$_n$ pools*	6.55	6.65
TS content*	0.228	0.176
TS activity†	67.8	45.2
TP activity†	1.28	1.49
TK-dThd†	641	1.45
TK-FdUrd†	183	0.635

The parameters in logarithmically growing HCT-8 and Fd9XR cells were determined as described in Materials and Methods. Values were calculated from the means of duplicate measurements in one experiment. Coefficients of variation were <10% in all cases.

* Units of measure: pmol/10^6 cells.

† Units of measure: pmol/min/mg protein.

biosynthesis of thymine nucleotides was inoperative, Fd9XR cells were expected to be sensitive to specific TS inhibitors, which do not require activation by TK, and antifolates that are inhibitors of the *de novo* thymidylate synthetic pathway. To test this hypothesis, the growth inhibitory activities of ICI D1694, a new antifolate TS inhibitor [27], and a classical antifolate, MTX, in Fd9XR and HCT-8 cells were investigated.

The sensitivities of Fd9XR and HCT-8 to ICI D1694 were evaluated in the absence or presence of 30 μM dThd (Fig. 3). The growth inhibition of Fd9XR cells by ICI D1694 was 2-fold greater than of HCT-8. A 30 μM concentration of dThd completely protected HCT-8 cells from ICI D1694 growth inhibition. In contrast, dThd had no significant effect on ICI D1694 cytotoxicity in Fd9XR cells (Fig. 3, left panel).

MTX inhibits both *de novo* syntheses of purine and thymidylate. Sensitivities of Fd9XR and HCT-8 to MTX were evaluated in the absence or presence of dThd and hypoxanthine (Fig. 3, right panel). In the absence of dThd and hypoxanthine, Fd9XR cells were 3-fold more sensitive to MTX than HCT-8 cells. In the presence of 30 μM dThd and 100 μM hypoxanthine, HCT-8 cells were protected completely from growth inhibition by MTX. In contrast, hypoxanthine and dThd were significantly less effective in protecting Fd9XR cells from MTX growth inhibition. In brief, Fd9XR cells exhibited collateral *in vitro* sensitivity to MTX and ICI D1694 and to FUra (Table 1).

DISCUSSION

Cell lines resistant to anticancer agents have been established by various approaches to facilitate studies of mechanisms of resistance and improvement of clinical anticancer chemotherapy. Various methods have been used for the selection of resistant cells. One of the approaches is selection of outgrown colonies after a single exposure of the drug to the cells [16, 20]. This simple method can select intrinsic resistant cells, but its clinical relevance is unclear

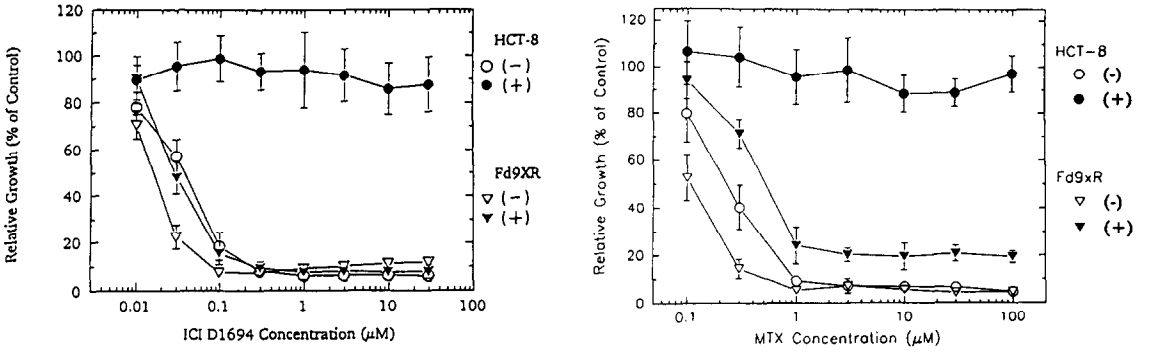

Fig. 3. Growth inhibition of HCT-8 and Fd9XR cells by ICI D1694 or MTX. (Left panel) HCT-8 and Fd9XR cells were treated with various concentrations of ICI D1694 or 3 hr in the presence (●, ▼) or absence (○, ▽) of 30 μM dThd. After treatment cells were allowed to grow in ICI D1694-free medium in the presence or absence of dThd of the same concentration for an additional 4 days. Cell growth was determined by the MTT colorimetric assay. The data points and error bars are the means and SD of 6 replicates. (Right panel) HCT-8 and F39XR cells were treated with various concentrations of MTX for 3 hr in the presence (●, ▼) or absence (○, ▽) of 30 μM dThd and 100 μM hypoxanthine. After treatment, cells were allowed to grow in MTX-free medium in the presence or absence of dThd and hypoxanthine of the same concentrations for an additional 4 days. Cell growth was determined by the MTT colorimetric assay. The data points and error bars are the means and SD of 6 replicates.

[21]. A multistep method involves exposure of increasing concentrations of anticancer agents to cells [17, 19, 28]. Although the yield of this method is relatively high, the concentrations of the drugs used are often much higher than those achieved clinically. Some investigators therefore use multiple short-term exposures of a fixed concentration of the drugs to mimic clinical situations in the selection of resistant cells [21].

In this study, we applied a multiple short-term drug exposure method in the initial steps of selection for FdUrd-resistant cells, followed by a cloning step in the presence of a FdUrd concentration, which was lower than that used in the initial steps. After the initial short-term exposures to FdUrd, the surviving cell population (designated Fd9X) was only slightly more resistant to FdUrd than the parent line (data not shown). However, since FdUrd has been shown to be mutagenic [29], pretreatment with FdUrd may induce gene mutations resulting in resistant cells. Since the surviving population is heterogeneous, the resistant mutant phenotype could be masked by those of other cells so that the overall resistance is not high. After the final cloning step, we obtained a clone (Fd9XR) that was 1000-fold resistant to FdUrd. This cloning step was carried out under conditions of continuous exposure to FdUrd. A clone obtained with an identical cloning procedure from the parent line without prior exposures to FdUrd had an equal sensitivity to FdUrd as Fd9X cells (data not shown), suggesting that the pretreatment with FdUrd increased the probability for selecting highly resistant clones.

Conventional methods of isolation of drug-resistant clones are usually based on estimation of the size of the colonies by eye at the time of isolation. In contrast, the cloning method used in this study utilized a computerized image analysis system which allowed us to follow the growth kinetics of individual colonies in agarose while simultaneously evaluating the sensitivity of the drug. At the time of isolation, the growth rate of the selected cells in the presence of the drug had been identified. With this approach clones displaying different growth rates during drug exposure can be chosen directly. It is possible to select a highly resistant clone, and it may also be possible to select colonies with differential degrees of resistance based on the growth rates of the individual colony in agarose. Therefore, this approach could be a powerful tool in the studies of drug resistance.

The primary biochemical mechanism of resistance of cells to FdUrd has been investigated and reported to be due to increased contents of TS [12], altered TS so that the affinities of the enzyme for FdUMP and/or 5,10-$CH_2H_4PteGlu_n$ were decreased [13, 14], decreased transport of the drug into the cells [15], increased phosphatase activity resulting in decreased accumulation and retention of FdUMP [16], altered folylpolyglutamylation [25, 30], and decreased activity of TK [17–19]. The mechanism of resistance of the Fd9XR selected clone is associated with a decreased TK activity, as supported not only by TK activity measurements but also by the inability of dThd to protect cells from MTX and ICI D1694 growth inhibition and by the low FdUMP pool achieved after FdUrd treatment. The possibility still exists, however, that this clone could also be deficient in the transport of FdUrd since the total nucleotide pools derived from FdUrd in Fd9XR cells was only about 2% of those found in HCT-8 cells (Table 2).

TK is the enzyme responsible for converting FdUrd to the active form, FdUMP. It is also the enzyme that converts preformed dThd to thymidylate, providing the salvage source of dThd nucleotides for DNA synthesis. If a tumor has heterogeneous cellular components including a subpopulation that has intrinsic or acquired TK

deficiency, following repeated treatments with FdUrd, this subpopulation which is resistant to the therapy may outgrow other cellular components. In such a case where TK deficiency represents part of the overall tumor resistance to FdUrd chemotherapy, the use of thymidylate *de novo* synthesis inhibitors that do not require activation by TK, such as ICI D1694 or MTX, could selectively inhibit the growth of those tumor cells, resulting in a greater therapeutic index, since these tumor cells are unable to salvage preformed dThd.

Several possible explanations may be offered as to why the Fd9XR cells display collateral sensitivity to FUra: (1) the amount of total F-nucleotide pools derived from FUra in Fd9XR cells is about 3-fold higher (Table 2) resulting in a 3-fold increase in FUTP and a 9-fold increase in FdUMP pools. Thus, the observed increases in these pools were proportional to the increase in total intracellular drug accumulation and sensitivity to FUra; (2) since the Fd9XR cells are unable to salvage preformed dThd, the pools of dThd deoxyribonucleotide are likely to be lower and, consequently, the feed-back inhibition at the level of ribonucleotide reductase by these pools is reduced. Under these conditions, FdUMP formation is primarily via the ribonucleotide reductase pathway. The observed increases in FdUMP (Table 2) and TS inhibition (data not shown) are consistent with this hypothesis; and (3) the inability to salvage dThd reduces the chance of dThd to bypass TS inhibition and reverse growth inhibition by FdUMP derived from FUra in the Fd9XR cells.

The data in Table 2 also indicate that, after FdUrd treatment, although the pool of FdUMP in the Fd9XR was about 6600-fold less than that found in HCT-8 cells, the amount of drug incorporated into the acid-insoluble fractions was only 4-fold less in the Fd9XR cells. Some possible explanations may be offered: (1) a non-linear relationship exists between acid-soluble and acid-insoluble compartments, and (2) the amount of drug incorporated into the acid insoluble compartment represents a contribution of the amount of drug incorporated into cellular RNA, DNA and protein (TS-complex). Indeed, and in particular with FdUrd in Fd9XR cells, a significant fraction of the drug in the acid-insoluble fraction may in fact represent drug incorporation into RNA and DNA rather than complex with TS, since one of the substrates for ternary complex formation, FdUMP, was decreased markedly. Studies are underway to separate these fractions and define the contributions of each to drug metabolism.

In brief, the studies outlined herein offer several unique features: (1) FdUrd, a clinically active chemotherapeutic agent, was used to clone and isolate a human cell line highly resistant to FdUrd; (2) the clone was selected based upon its growth rate using an image analysis system; (3) the selective pressure by FdUrd yielded a cell line resistant to FdUrd but not to FUra, MTX and ICI D1694; (4) multiple parameters associated with response to FdUrd were evaluated; and (5) ways of circumventing the resistance were explored.

Acknowledgements—The authors wish to thank Denice Buscaglia, Cheri Frank, Robert Maue and Carol Wrzosek for their excellent technical assistance. This work was supported by National Cancer Institute Grants CA18420, CA16056 and CA21071.

REFERENCES

1. Parker WB and Cheng Y-C, Metabolism and mechanism of action of 5-fluorouracil. *Pharmacol Ther* **48**: 381–395, 1990.
2. Heidelberger C, Dannenberg PV and Moran RG, Fluorinated pyrimidines and their nucleosides. *Adv Enzymol* **54**: 57–119, 1983.
3. Yin M-B and Rustum YM, Comparative DNA strand breakage induced by FUra and FdUrd in human ileocecal adenocarcinoma (HCT-8) cells: Relevance to cell growth inhibition. *Cancer Commun* **3**: 45–51, 1991.
4. Lonn U and Lonn S, DNA lesions in human neoplastic cells and cytotoxicity of 5-fluoropyrimidines. *Cancer Res* **46**: 3866–3970, 1986.
5. Santi DV, McHenry CS and Sommer H, Mechanism of interaction of thymidylate synthetase with 5-fluorodeoxyuridylate. *Biochemistry* **13**: 471–481, 1974.
6. Danenberg PV and Lockshin A, Fluorinated pyrimidines as tight-binding inhibitors of thymidylate synthetase. *Pharmacol Ther* **13**: 69–90, 1981.
7. Yin M-B, Zakrzewski SF and Hakala MT, Relationship of cellular folate cofactor pools to the activity of 5-fluorouracil. *Mol Pharmacol* **23**: 190–197, 1982.
8. Houghton JA and Houghton PJ, Basis for the interaction of 5-fluorouracil and leucovorin in colon adenocarcinoma. In: *The Current Status of 5-Fluorouracil Calcium-Leucovorin Combination* (Eds. Bruckner HW and Rustum YM), pp. 23–32. John Wiley, New York, 1984.
9. Mini E, Trave F, Rustum YM and Bertino JR, Enhancement of the antitumor effects of 5-fluorouracil by folinic acid. *Pharmacol Ther* **47**: 1–19, 1990.
10. Levin RD, Gordon JH, Simonich W, Mellijor A, Sanchez R and Williams RM, Phase I clinical trial with floxuridine and high-dose continuous infusion of leucovorin calcium. *J Clin Oncol* **9**: 94–99, 1991.
11. Kemeny N, Cohen A, Bertino JR, Sigurdson ER, Botet J and Oderman P, Continuous intrahepatic infusion of floxuridine and leucovorin through an implantable pump for the treatment of hepatic metastases from colorectal carcinoma. *Cancer* **65**: 2446–2450, 1990.
12. Berger SH, Jenh CH, Johnson LF and Berger FG, Thymidylate synthase overproduction and gene amplification in fluorodeoxyuridine-resistant human cells. *Mol Pharmacol* **28**: 461–467, 1985.
13. Bapat AR, Zarow C and Danenberg PV, Human leukemic cells resistant to 5-fluoro-2′-deoxyuridine contain a thymidylate synthetase with lower affinity for nucleotides. *J Biol Chem* **258**: 4130–4136, 1983.
14. Berger SH, Barbour KW and Berger F, A naturally occurring variation in thymidylate synthase structure is associated with a reduced response to 5-fluoro-2′-deoxyuridine in a human colon tumor cell line. *Mol Pharmacol* **34**: 480–484, 1988.
15. Fry DW and Jackson RC, Membrane transport alterations as a mechanism of resistance to anticancer agents. *Cancer Surv* **5**: 47–79, 1986.
16. Fernandes DJ and Cranford SK, Resistance of CCRF-CEM cloned sublines to 5-fluorodeoxyuridine associated with enhanced phosphatase activities. *Biochem Pharmacol* **34**: 125–132, 1985.
17. Jastreboff MM and Zielinska ZM, Development and characteristics of a subline of Ehrlich ascites carcinoma cells persistently resistant to 5-fluoro-2′-deoxyuridine. *Acta Biochim Pol* **30**: 185–191, 1983.
18. Kanzawa F, Matsushima Y, Ishihara J, Hoshi A, Ohba

T and Watanabe K, Antitumor activity of alkylesters of 5-fluoro-23′-deoxyuridine 5′-monophosphate (FdUMP) against murine lymphoma L5178Y resistant to 5-fluoro-2′-deoxyuridine. *J Pharmacobiodyn* **9**: 688–693, 1986.

19. Houghton PJ, Houghton JA, Germain G and Torrance PM, Development and characterization of a human colon adenocarcinoma xenograft deficient in thymidine salvage. *Cancer Res* **47**: 2117–2122, 1987.
20. Mulkins MA and Heidelberger C, Isolation of fluoropyrimidine-resistant murine leukemic cell lines by one-step mutation and selection. *Cancer Res* **42**: 956–964, 1982.
21. Pizzorno G, Mini E, Coronnello M, McGuire JJ, Moroson BA, Cashmore AR, Dreyer BN, Lin JT, Mazzei T, Periti P and Bertino JR, Impaired polyglutamylation of methotrexate as a cause of resistance in CCRF-CEM cells after short-term, high-dose treatment with this drug. *Cancer Res* **48**: 2149–2155, 1988.
22. Slocum HK, Malmberg M, Greco WR, Parsons JC and Rustum YM, The determination of growth rates of individual colonies in agarose using high-resolution automated image analysis. *Cytometry* **11**: 793–804, 1990.
23. Zhang ZG and Rustum YM, Effects of diastereoisomers of 5-formyltetrahydrofolate on cellular growth, sensitivity to 5-fluoro-2′-deoxyuridine, and methylenetetrahydrofolate polyglutamate levels in HCT-8 cells. *Cancer Res* **51**: 3476–3481, 1991.
24. Priest DG and Doig MT, Tissue folate polyglutamate chain length determination by electrophoresis as thymidylate synthase-fluorodeoxyuridylate ternary complexes. *Methods Enzymol* **122**: 313–319, 1986.
25. Zhang ZG, Harstrick A and Rustum YM, Mechanisms of resistance to fluoropyrimidines. *Semin Oncol* **19**: 4–9, 1992.
26. Cheng Y-C, Thymidine kinase from blast cells of myelocytic leukemia. *Methods Enzymol* **51**: 365–371, 1978.
27. Jackman AL, Taylor GA, Gibson W, Kimbell R, Brown M, Calvert AH, Judson IR and Hughes LR, ICI D1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumor cell growth *in vitro* and *in vivo*: A new agent for clinical study. *Cancer Res* **51**: 5579–5586, 1991.
28. Srimatkandada S, Medina WD, Cashmore AR, Whyte W, Engel D, Moroson BA, Franco CT, Dube SK and Bertino JR, Amplification and organization of dihydrofolate reductase genes in a human leukemic cell line, K-562, resistant to methotrexate. *Biochemistry* **22**: 5774–5781, 1983.
29. Sobrero AF, Moir RD, Bertino JR and Handschumacher RE, Defective facilitated diffusion of nucleosides, a primary mechanism of resistance to 5-fluoro-2′-deoxyuridine in the HCT-8 human carcinoma line. *Cancer Res* **45**: 3155–3160, 1985.
30. Romanini A, Lin JT, Niedzwiecki D, Bunni M, Priest DG and Bertino JR, Role of folylpolyglutamates in biochemical modulation of fluoropyrimidines by leucovorin. *Cancer Res* **51**: 789–793, 1991.