Interaction of Methotrexate Polyglutamates and Dihydrofolate Rescue in a Human Breast Cancer Cell Line (MCF-7)

Carmen J. Allegra and Donna Boarman

ABSTRACT

Previous investigations have suggested that high-dose methotrexate with leucovorin rescue is a potentially useful strategy for overcoming antifolate resistance. Interactions between methotrexate (MTX) and leucovorin and their respective metabolites appear to occur at multiple intracellular sites, including dihydrofolate reductase (MTX/MTX polyglutamates versus dihydrofolate) and other folate-dependent enzymes (MTX polyglutamates versus reduced folate substrates). The present studies were designed to test the ability of dihydrofolate to compete with methotrexate and methotrexate polyglutamates for dihydrofolate reductase activity using an intact human breast carcinoma cell line (MCF-7) as the model system. Exposure of the breast cells to methotrexate for 24 h resulted in a concentration-dependent formation of methotrexate polyglutamates that markedly exceeded the dihydrofolate reductase-binding capacity for up to 24 h after the removal of drug from the growth media. Under these conditions of dihydrofolate reductase inhibition, we found that tritium-labeled dihydrofolate was capable of competing with methotrexate and its metabolites for dihydrofolate reductase activity as evidenced by the appearance of tritium-labeled reduced folates in the treated cells. We found the interaction between dihydrofolate and methotrexate to be dependent on the exposure concentrations of both methotrexate and dihydrofolate. These studies provide direct evidence that competition during leucovorin rescue occurs at the level of dihydrofolate reductase between methotrexate polyglutamates and dihydrofolate polyglutamates in intact human cells.

INTRODUCTION

MTX\(^2\) has been in clinical use for the treatment of neoplastic disorders for more than four decades. While many MTX analogs have been tested in attempts to identify new agents capable of circumventing known mechanisms of resistance to MTX, none have yet supplanted MTX in clinical usage. High-dose MTX with leucovorin (citrovorum factor; 5-formyltetrahydrofolate) rescue has been shown to enhance the therapeutic activity of MTX in preclinical animal models (1) and has the theoretical ability to overcome many of the known mechanisms by which neoplastic cells become resistant to MTX. Unfortunately, the clinical utility of high-dose MTX therapy has met with only modest success (2, 3). MTX is a potent inhibitor of DHFR (EC 1.3.1.5). While MTX polyglutamates retain a potent ability to inhibit DHFR, they are also potent inhibitors of several folate-dependent enzymes, including thymidylate synthase and the enzymes of de novo purine synthesis (4–7).

Recent investigations concerning the mechanism of action of MTX have indicated that metabolic inhibition is a multifactorial event that includes folate substrate depletion and direct inhibition of several critical folate-dependent enzymes by MTX and dihydrofolate polyglutamates (8–12). The occurrence of such inhibition may be partially responsible for the competitive

Received 9/28/89; revised 2/26/90.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1To whom requests for reprints should be addressed, at Building 10, Room 12N226, National Cancer Institute, Bethesda, MD 20892.

2The abbreviations used are: MTX, methotrexate (4-amino-10-methylpteroylglutamate); DHFR, dihydrofolate reductase; H\(_4\)PteGlu, tetrahydrofolate; H\(_3\)PteGlu, dihydrofolate; HPLC, high-pressure liquid chromatography.

MATERIALS AND METHODS

Materials. MTX and MTX polyglutamates were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). [3',5',7',9-\(^3\)H]MTX (specific activity, 18 Ci/mmol) and [3',5',7',9-\(^3\)H]folic acid (specific activity, 40 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). RPMI 1640, glutamine, phosphate-buffered saline, and fetal calf serum were purchased from Biofluids (Rockville, MD). Dialyzed fetal calf serum was purchased from Gibco (Grand Island, NY). Sep-Pak C-18 cartridges and Pic A were purchased from Waters Associates (Milford, MA). Calcium leucovorin was obtained from Burroughs Wellcome Co. (Research Triangle Park, NC). H\(_3\)PteGlu, H\(_4\)PteGlu, folic acid, 2-mercaptopethanol, and albumin (Fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol was purchased from J. T. Baker Inc. (Phillipsburg, NJ). Acetonitrile and sodium dithionite were purchased from Fisher Scientific (Fair Lawn, NJ). All other reagents were purchased from either the NIH supply store (Bethesda, MD) or Sigma Chemical Co.

Cell Line. An early-passage human breast cancer cell line, MCF-7, was used for these experiments. The cells were grown as a monolayer in 75-cm\(^2\) tissue culture flasks (Falcon Labware, Oxnard, CA) with RPMI 1640 media supplemented with 10% dialyzed fetal calf serum and 2 \(\mu\)g/ml of glutamine. The cells were used for the experiments when they had reached 60–70% confluency (~96 h after plating).

\(^{[\text{H}]}\)H\(_3\)PteGlu was prepared by a modified procedure based on the methodology described by Blakely (21). Six nm of \(^{[\text{H}]}\)PteGlu were placed in a small conical bottom glass tube and concentrated 10-fold by evaporation under a steady stream of nitrogen. Two \(\mu\)mol of unlabeled folic acid and 20 \(\mu\)l of a 10% ascorbate solution, pH 6.0, were added to the labeled folic acid. The pH was adjusted to 6.0 and followed by the addition of 10 mg sodium dithionite. The mixture was stirred contin-

Downloaded from cancerres.aacrjournals.org on January 5, 2018. © 1990 American Association for Cancer Research.
usually for 25 min at room temperature. The solution was then placed in an ice bath, and 0.1 N HCl was added at the rate of 20 μl/min with continuous stirring until the pH fell to 2.5–2.8. The solution was stirred for an additional 5–8 min to allow complete precipitation of the H3PteGlu. The precipitate was recovered by centrifugation for 5 min at 14,000 × g. The supernatant was discarded, and the pellet was washed with 400 μl 1 N HCl and centrifuged again. The recovered pellet was resuspended in 250 μl of ascorbate buffer, pH 6.0. Purity of the labeled H3PteGlu was determined by HPLC using previously described methods (8). The labeled and unlabeled folate acid used for the synthesis was >98% pure. We found that the recovered compound contained 70–90% H3PteGlu and 10–30% unreacted folic acid. No reduced folates were detectable, and all the radioactivity coeluted with either H2PteGlu or folic acid. The compound was stored at −80°C in 10% ascorbate buffer, pH 6.0, and 100 mM 2-mercaptoethanol. While H3PteGlu could have been further purified by a second crystallization, we elected to use a single crystallization, as the yield was markedly diminished by recrystallization; the only contaminating folate was unreduced folic acid that would not adversely affect the intended experiments.

Stability of Dihydrofolate. The stability of dihydrofolate was determined by incubating the compound at 37°C for various time intervals up to 48 h and using HPLC to quantitate the remaining dihydrofolate. Dihydrofolate at a concentration of 10 μM was incubated in the absence or presence of various concentrations of mercaptoethanol (0.05–10 mM). H3PteGlu was quantitated from aliquots removed at 0-, 2-, 6- and 24-h intervals.

Cell Growth Experiments. Growth inhibitory effects of mercaptoethanol on MCF-7 breast carcinoma cells were also determined. Approximately 105 cells were plated in 25-cm2 tissue culture flasks with RPMI 1640 media supplemented with 10% dialyzed fetal calf serum and 2 mM glutamine. After 24 h, various concentrations of mercaptoethanol, 0.05–10 mM, were added to the flasks. A flask containing no mercaptoethanol was used as a control. After 72 h, an aliquot from each flask was removed and the number of cells determined by a cell counter (Coulter Counter, Model ZB, Hialeah, FL). The number of cells/ml was calculated and the percentage of growth inhibition for the flasks treated with mercaptoethanol was determined by comparing the number of cells/ml for each treatment to that of the control.

Intracellular Folate Pool Quantitation. MCF-7 cells were exposed to 1, 5, and 10 μM MTX for 24 h. At the end of the 24-h period the cells were washed two times with phosphate-buffered saline. Various concentrations of labeled dihydrofolate were added along with new MTX-free media for an additional 24-h period. 2-Mercaptoethanol was added to give a final concentration of 0.1 mM. After the second 24-h period the cells were again washed two times with ice-cold phosphate-buffered saline and harvested in 1 ml of saline with the aid of a rubber cell scraper. A 100-μl aliquot was removed for protein analysis, and the folates were extracted from the remaining cell suspension according to previously published methods (8).

The labeled folates were separated by HPLC using a Waters Model 510 pump and a Waters Model 440 UV absorption detector with a fixed detection wavelength of 256 nm. A C-8 μBondapak column was developed using a flow rate of 2 ml/min under isocratic conditions. The mobile phase consisted of 75% Pic Reagent A, pH 5.5, and 25% methanol. The separated labeled folate pools were quantitated using an in-line liquid scintillation counter (Model Flo-One Beta; Radiomatic Instruments, Tampa, FL). The identity of the various physiological folates was authenticated by coelution with unlabeled standard folates injected with each chromatographic assay. Folates were further identified by acting as substrates for specific enzymatic reactions as previously detailed (8). The recovery rate for the various folates ranged from 60 to 70%. No correction for recovery was applied as all comparisons were made to control cells processed under identical conditions and at the same time as the treated cells. The retention times for the folates were as follow: paraaminobenzoate, 5 min; paraaminobenzoyl glutamate, 7.5 min; 10-formyl-H3PteGlu, 10 min; H2PteGlu, 12.5 min; 5-formyl-H3PteGlu, 15 min; dihydrofolate, 18.5 min; and 5-methyl-H3PteGlu, 26 min.

Intracellular MTX Polyglutamate Measurements. MCF-7 cells were exposed to 1 and 10 μM [3H]MTX with final specific activities of 0.5 and 0.1 Ci/mmol, respectively, for 24 h. At the end of this period the cells were washed two times with phosphate-buffered saline, and new MTX-free medium was added. After an additional 24-h period the cells were again washed two times with ice-cold phosphate-buffered saline and harvested in 1800 μl of saline with the aid of a rubber cell scraper. The cell lysate was transferred to a test tube containing 200 μl of 100% trichloroacetic acid for a final concentration of 10%. The precipitated protein was centrifuged for 15 min at 10,000 × g. The supernatant was reserved, and the pellet was dissolved in 1 ml of 1 N NaOH for protein quantitation. The MTX polyglutamates were extracted from the supernatant according to published methods (22) and separated by HPLC using a 22-min linear gradient from 20 to 35% acetonitrile in Pic A (pH 5.5). The separated polyglutamates were quantitated by an in-line liquid scintillation counter. The identity of the various polyglutamates was authenticated by coelution with MTX and MTX polyglutamate standards.

Intracellular Dihydrofolate Reductase Measurements. The measurement of DHFR was based on a modification of the protein-binding assay described by Myers et al. (23). MCF-7 cells were exposed to 10 μM MTX for 24 and 48 h or no MTX (control cells). The cells were harvested from 75-cm2 tissue culture flasks by the addition of 4 ml of 0.05% trypsin/0.02% versene in Hank’s balanced salt solution. The cells were pelleted by centrifugation at 700 × g for 15 min. The pellet cells were resuspended in 500 μl of 100 mM Tris-HCl buffer (pH 7.5), and then sonicated with a 20-s burst from a Branson Model 350 sonicator. The cell mixture was then centrifuged at 10,000 × g for 10 min. The cell supernatants were then dialyzed against 4 liters of 50 mM Tris/HCl buffer (pH 8.5) for a 24-h period to remove any MTX bound to DHFR. Control cells were exposed to 10 μM MTX for only 1 h in parallel to demonstrate the efficiency of free enzyme recovery. We found >95% recovery of free enzyme compared to control cells not exposed to MTX and not subjected to dialysis. Following dialysis, 0.12 mg of cytosolic protein was added to a reaction tube containing 100 mM Tris-HCl buffer (pH 7.5) and 0.15 μCi [3H]MTX in a total volume of 200 μl and incubated at 37°C for 3 h. The extended incubation with [3H]MTX allowed for complete exchange to take place with any remaining unlabeled drug bound to DHFR. For the binding assay, 50 mM β-nicotinamide adenine dinucleotide phosphate, reduced form, was added to the reaction mixture, and the tubes were vortexed and allowed to equilibrate for 10 min at room temperature. After ternary complex formation, the unbound drug was removed by the addition of 50 μl of charcoal slurry (10 g activated charcoal, 2.5 g bovine serum albumin, fraction V, and 0.1 g of high molecular weight dextran in 100 ml H2O), followed by vortexing and immediate centrifugation at 10,000 × g for 20 min. A 150-μl aliquot of the supernatant was counted in a Packard scintillation counter after the addition of 10 ml of liquid scintillation cocktail.

Protein Measurement. A 100-μl aliquot of cell suspension was sonicated with five 3-s bursts using a Branson Model 350 sonicator equipped with a microtip. The cell debris was pelleted by centrifugation at 10,000 × g for 10 min, and the protein in the supernatant was quantitated using the method of Bradford (24).

Calculations. Disintegration/min obtained by counting an aliquot of the extracted labeled folates were converted to total intracellular folate content in pmol/mg protein by dividing total dpm/flask of cells by total protein/flask of cells and then dividing by the specific activity of the labeled dihydrofolate.

\[
\text{pmol folate/mg protein} = \frac{(\text{dpm/flask}) + (\text{mg protein/flask}) + (\text{dpm/pmol folate})}{\text{protein measurement}}
\]

RESULTS

Stability of Dihydrofolate. Because of the inherent susceptibility of H3PteGlu to oxidation, we investigated the use of 2-mercaptoethanol to stabilize the compound during the cell exposures. Since 2-mercaptoethanol is toxic to the breast carcinoma cells, we first determined the maximum concentration of the compound that would not produce toxicity over the 24-h incubation periods. We used cell growth as a measure of...
toxicity and found that a 2-mercaptopoethanol dose of 1 mM resulted in a 50% decrease in the growth of the breast cells over a 72-h exposure period. A dose of 0.1 mM was chosen for inclusion in the 24-h dihydrofolate experiments as this dose resulted in <5% decrease in the growth of the cells over a 72-h period of continuous exposure. Using this dose of 2-mercaptopoethanol, we found that the half-life of H₂PteGlu incubated at 37°C in phosphate-buffered saline, pH 7.4, was 24 h. The half-life of the compound in the absence of 2-mercaptopoethanol was 4 h.

Intracellular Folate Pool Measurements. Using the human breast carcinoma cell line MCF-7 as the model system, we examined the intracellular folate pools in MTX-treated cells using labeled dihydrofolate as a tracer. For these experiments, cells were treated with no MTX or with a 24-h preexposure to 0.5, 1.0, and 10 μM MTX, which resulted in 65, 80, and 90% lethality, respectively. This treatment was followed by a 24-h exposure to various concentrations of dihydrofolate. Dihydrofolate was found only to be toxic at high concentrations such that 50 and 100 μM exposures resulted in 15 and 44% cytotoxicity, respectively. As illustrated in Fig. 1, we found that the total intracellular folate pool increased in direct proportion to the dihydrofolate concentration in which the cells were treated. The total amount of folate was essentially the same with each MTX concentration including cells that had not been treated with MTX. Upon fractionation of the various folate pools, we found that cells not exposed to MTX had detectable dihydrofolate levels only at the highest dihydrofolate exposures consistent with a rapid reduction of this folate in cells with active DHFR (Fig. 2). However, in cells pretreated with MTX, the concentration of dihydrofolate was directly proportional to both the dihydrofolate and MTX exposure concentrations. Those cells pretreated with 10 μM MTX had the highest intracellular dihydrofolate levels at any given dihydrofolate exposure concentration. When we examined the reduced folate pools (Fig. 3), we found that reduced folates were present in all cells regardless of the MTX treatment concentration. Thus, dihydrofolate was able to compete for DHFR activity, even in cells exposed to up to 10 μM MTX. In contrast to the intracellular dihydrofolate accumulation, the reduced folate accumulation was inversely related to the MTX exposure concentration. Therefore, while the highest levels of dihydrofolate were found in cells pretreated with 10 μM MTX (Fig. 3D), the levels of reduced folates were lower compared to 0.5 and 1.0 μM MTX-treated cells (Fig. 3, B and C).

MTX Polyglutamate Formation. The MCF-7 breast carcinoma cell has been previously shown to avidly polyglutamate MTX. In the present experiments we wanted to precisely quantify the MTX polyglutamate levels under the same conditions and at the same time that the intracellular folate pools were measured. We measured the MTX polyglutamates 48 h from the start of MTX exposure (24 h after MTX was taken out of the cell culture medium). We also measured DHFR levels for comparison to the MTX polyglutamate levels. We found the DHFR level to be 3 ± 1.5 (SE) pmol/g cytosolic protein in untreated cells and 3.1 ± 1.2 pmol/g cytosolic protein after a 48-h exposure to 10 μM MTX. As illustrated in Table 1, almost all MTX present after 24 h in drug-free media was in the form of polyglutamates. The absolute level of the polyglutamates was a direct function of the MTX exposure concentration. At 1 and 10 μM MTX, the polyglutamate levels exceeded the DHFR level by 7- and 14-fold, respectively.

Since the folates and MTX share a common transmembrane transport system, we defined the effect of adding exogenous dihydrofolate on the formed intracellular MTX polyglutamates.

**Fig. 1.** Total labeled intracellular folates in MCF-7 breast carcinoma cells treated with sequential MTX and dihydrofolate. Breast carcinoma cells were plated onto 75-cm² tissue culture flasks at an initial density of 1 × 10⁶ cells. After 96 h of growth (60–70% confluency) in RPMI 1640 media supplemented with 10% dialyzed fetal bovine serum and 2 mM glutamine, the cells were treated with 0.5 MM MTX (●), 1 MM MTX (□), and no MTX (○) for 24 h; washed, and then placed in drug-free media containing various amounts of [³H]dihydrofolate. After an additional 24 h, the cells were washed and harvested, and the intracellular dihydrofolate was separated by HPLC. The dihydrofolate pool was quantitated using an in-line scintillation counter. Points, means of 4–6 independent experiments; bars, SE.

**Fig. 2.** Intracellular dihydrofolate pools in MCF-7 cells treated with sequential MTX and dihydrofolate. MCF-7 cells were plated onto 75-cm² tissue culture flasks and grown to 60–70% confluency in RPMI 1640 media containing 10% dialyzed fetal calf serum and 2 mM glutamine. The cells were treated with 0.5 μM MTX (●), 1 μM MTX (□), 10 μM MTX (●), and no MTX (○) for 24 h; washed, and then placed in drug-free media containing various concentrations of [³H] dihydrofolate. After an additional 24 h, the cells were washed and harvested, and the intracellular dihydrofolate was separated by HPLC. The dihydrofolate pool was quantitated using an in-line scintillation counter. Points, means of 4–6 independent experiments; bars, SE.
METHOTREXATE AND DIHYDROFOLATE INTERACTIONS

Fig. 3. Reduced folate pools in MCF-7 cells treated with sequential MTX and dihydrofolate. MCF-7 cells were grown and treated under conditions identical to those shown in Figs. 1 and 2. MTX exposures included no MTX (A), 0.5 μM MTX (B), 1 μM MTX (C), and 10 μM MTX (D). Following MTX exposure, the cells were incubated in various concentrations of [3H]dihydrofolate for 24 h and washed, and the folate pools were separated and quantitated by HPLC and liquid scintography, respectively. Points, means of 4–6 separate experiments; bars, SE.

Table 1 MTX polyglutamate formation in human breast carcinoma cells (MCF-7)

<table>
<thead>
<tr>
<th>MTX preexposure (μM)</th>
<th>Dihydrofolate exposure (μM)</th>
<th>Total MTX (pmol/mg)</th>
<th>Higher MTX polyglutamatesa (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>23 ± 5.2</td>
<td>22.4 ± 7.0</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>15.6 ± 0.0</td>
<td>14.3 ± 2.7</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>12.2 ± 4.0</td>
<td>10.9 ± 2.7</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>12.1 ± 3.2</td>
<td>9.6 ± 2.6</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>49.1 ± 9.3</td>
<td>43.1 ± 5.2</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>31.9 ± 6.2</td>
<td>28.6 ± 7.1</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>31.3 ± 12.3</td>
<td>25.2 ± 8.2</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>35.2 ± 12.0</td>
<td>27.4 ± 12.1</td>
</tr>
</tbody>
</table>

a Higher polyglutamates = MTX-Glu3-MTX-Glu5.

As shown in Table 1, the addition of dihydrofolate at 1, 10, and 50 μM resulted in a decrease in MTX polyglutamates by approximately 35, 40, and 50%, respectively, compared to cells placed into drug-free medium without the addition of the folates.

DISCUSSION

This report describes the ability of dihydrofolate to compete with MTX and MTX polyglutamates for DHFR in intact human breast carcinoma cells. While such an interaction has been postulated to be an important element of leucovorin rescue, these data provide direct evidence for the competition in intact human cells as evidenced by the reduction of labeled dihydrofolate in the presence of DHFR-saturating amounts of MTX. The occurrence of competition in the presence of MTX polyglutamates is of central importance. The breast carcinoma cells readily polyglutamate MTX such that concentrations of these metabolites are in excess of the DHFR-binding capacity throughout the 48-h experimental period. The inverse relationship between the MTX treatment concentration and the reduced folate pools resulting from the various dihydrofolate exposures supports competition between substrate and inhibitor at the level of DHFR. This is further supported by the proportional increase in intracellular dihydrofolate with respect to MTX levels. This competition provides at least one explanation for the competitive nature of leucovorin rescue that has been described in several well-studied in vitro experimental systems (13–16). Previous studies from our laboratory have supported a critical role of dihydrofolate in the process of leucovorin rescue and have suggested that rescue from the cytotoxicity of MTX occurs coincident with the generation of competitive intracellular levels of dihydrofolate.

While high-dose MTX with leucovorin rescue has the theoretical ability to overcome many of the known mechanisms by which neoplastic cells become resistant to MTX, these studies provide a plausible explanation for the less than optimal clinical results using this strategy. The relatively greater polyglutamation of MTX by malignant versus normal cells has been considered to be a critical factor in the selectivity of leucovorin rescue. While polyglutamation may provide some measure of selectivity, clearly, even cells that extensively polyglutamate MTX can be rescued by leucovorin through the generation of dihydrofolate. It is possible that the dose and/or schedule of leucovorin rescue may be optimized to allow a more effective application of this promising strategy.

The ability of dihydrofolate to diminish the intracellular concentration of MTX and MTX polyglutamates is of interest in that it suggests an additional mechanism by which rescue is achieved. This process most likely represents a heteroexchange of reduced folates and the antifolate, as both share a common transport mechanism (25–29). In addition, both compounds compete with folylpolyglutamyl synthetase for polyglutamation...
METHOTREXATE AND DIHYDROFOLATE INTERACTIONS

(30–33). In general, the folates are 10- to 50-fold more avid for the enzyme and may, therefore, be expected to effectively compete for enzymatic activity at the expense of the antifolate whose mono- and diglutamated forms are readily effluxable. The competition between MTX and leucovorin in the course of rescue appears to be based on interactions between dihydrofolate and MTX polyglutamates at the level of DHFR and between reduced folate and dihydrofolate/MTX polyglutamates at certain folate-dependent enzymes other than DHFR. The central role of dihydrofolate in these interactions provides a basis on which more selective therapeutic strategies may be incorporated into the use of high-dose MTX therapy.

ACKNOWLEDGMENTS

The authors wish to thank Kathy Moore for her editorial assistance in the preparation of this manuscript and Dr. Bruce Chabner for his instructive criticisms of the manuscript.

REFERENCES

Interaction of Methotrexate Polyglutamates and Dihydrofolate during Leucovorin Rescue in a Human Breast Cancer Cell Line (MCF-7)

Carmen J. Allegra and Donna Boarman


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/50/12/3574

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/50/12/3574. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.