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Mechanism of Action of the Novel Anticancer Agent 6-Fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinolinecarboxylic Acid Sodium Salt (NSC 368390): Inhibition of *de Novo* Pyrimidine Nucleotide Biosynthesis

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ABSTRACT

Exposure of cultured clone A human colon tumor cells to 25 to 75 μM of NSC 368390 [6-fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinolinecarboxylic acid sodium salt, DuP 785] for 48 to 72 h resulted in a 99.9% cell kill as determined by clonogenic assay. Cells exposed to NSC 368390 became depleted in intracellular pools of uridine 5′-triphosphate and cytidine 5′-triphosphate. Both uridine 5′-triphosphate and cytidine 5′-triphosphate were decreased to 50% of levels in control cells at 3 h and were undetectable at 15 h after addition of 25 μM of NSC 368390 to the cultures. Similar effects were observed in L1210 leukemia cells. Addition of 0.1 mM of uridine or cytidine restored intracellular pools of uridine 5′-triphosphate and cytidine 5′-triphosphate to control levels and rescued clone A cells from NSC 368390 cytotoxicity. Addition of uridine circumvented NSC 368390 cytotoxicity in L1210 cells, but addition of cytidine did not. This result is consistent with the fact that L1210 cells lack cytidine deaminase and thus cannot form uridine or its anabolites from cytidine. These results indicated that NSC 368390 inhibits a step in the *de novo* biosynthetic pathway leading to uridine 5′-monophosphate. Therefore, the effects of NSC 368390 on the six enzymes that comprise the *de novo* pathway leading to the formation of uridine 5′-monophosphate were examined. The results showed that NSC 368390 was a potent inhibitor of dihydroorotate dehydrogenase, the fourth enzyme in the pathway; thus, this study demonstrates that NSC 368390 exerts its tumoricidal effect by inhibiting a step in *de novo* pyrimidine biosynthesis resulting in the depletion of critical precursors for RNA and DNA synthesis.

INTRODUCTION

The novel anticancer agent NSC 368390[2] (Fig. 1) inhibits the growth of a broad spectrum of human solid tumors implanted in nude mice; the water soluble compound is equally efficacious whether administered p.o. or parenterally (1). Because of its activity against experimental tumors, NSC 368390 has recently been entered into phase 1 clinical trials. The structural novelty of the compound as an antitumor agent has precluded any *a priori* prediction of its mode of action; therefore, studies were conducted to elucidate the mechanism of action of NSC 368390. Our results demonstrate that NSC 368390 exerts its tumoricidal effect by inhibiting dihydroorotate dehydrogenase, an enzyme in the *de novo* pyrimidine nucleotide biosynthetic pathway. Portions of this work have been presented in a preliminary form (2).

MATERIALS AND METHODS

Materials and Chemicals. NSC 368390 was synthesized by the Medicinal Chemistry Section, Pharmaceuticals Division, Du Pont Biomedical Products Department. The reagents for tissue culture were purchased from GIBCO, Grand Island, NY. These included RPMI 1640 medium, fetal bovine serum, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, tricine buffer, sodium bicarbonate, fungizone, antipleuropneumonia-like organism agent, penicillin-streptomycin, trypsin, and trypan blue. Gentamicin was purchased from Valley Biologicals, State College, PA. Glycerol, dimethyl sulfoxide, NaF, and HPLC grade potassium phosphate monobasic were obtained from Fisher Scientific Co., King of Prussia, PA. DEAE-Sephadex A25 was purchased from Pharmacia, Piscataway, NJ. Acetaldehyde was obtained from Aldrich Chemical Co., Milwaukee, WI. Whatman DE81 and 3 MM chromatography papers were purchased from VWR Scientific, Philadelphia, PA. Kodak X-Omat AR film was obtained from PX Imaging Inc., Plymouth Meeting, PA. BALB/c × DBA/2 F_1 (hereafter called $CD2F_1$) mice were purchased from Charles River, Wilmington, MA. Dowex 50 W (8X), pyruvate kinase (rabbit muscle), ornithine transcarbamylase (*Streptococcus faecalis*), dihydroorotate dehydrogenase (*Zymobacterium oroticum*), alcohol dehydrogenase (equine liver), and all other chemicals were purchased from Sigma Chemical Company, St. Louis, MO. En[3] Hance spray, Biofluor, L-[U-^{14}C]aspartic acid (specific activity, 231.0 mCi/mmol), [*carboxyl*-^{14}C]orotic acid (specific activity, 60 mCi/mmol), and [^{14}C]sodium bicarbonate (specific activity, 51.5 mCi/mmol) were purchased from New England Nuclear Research Products, Boston, MA.

Synthesis and Purification of L-[*carboxyl*-^{14}C]Dihydroorotate. L-[*carboxyl*-^{14}C]Dihydroorotate was synthesized enzymatically from [*carboxyl*-^{14}C]orotate, and purified from other radiolabeled by-products using a DEAE-Sephadex A25 column (16 x 330 mm) eluted with 0.2 M ammonium formate (pH 7.0) (3). The fractions containing L-[*carboxyl*-^{14}C]dihydroorotate were pooled and concentrated to dryness by lyophilization. The residue was reconstituted in water and applied to a Dowex 50 W (8X) column (25 x 140 mm), which was eluted with glass distilled water. The radioactive fractions were pooled and concentrated again. The purified L-[*carboxyl*-^{14}C]dihydroorotate was reconstituted with glass distilled water and stored at −20°C. Assuming its specific activity to be the same as that of the starting [*carboxyl*-^{14}C]orotate, the concentration of L-[*carboxyl*-^{14}C]dihydroorotate was determined from its radioactivity. L-[*carboxyl*-^{14}C]Dihydroorotate was identified by its mobility on DE81 chromatographic paper as compared to a nonradioactive standard as well as by its ability to be converted enzymatically to orotate.

Cell Cultures. Clone A human colon cancer cells were isolated from the heterogeneous DLD-1 colon tumor line established from a surgical specimen of primary colon adenocarcinoma (4). Clone A cells are hyperploid, highly clonogenic in soft agar, and produce poorly differentiated carcinomas when injected s.c. into nude mice (5). The colon cancer cells were grown in 60-mm tissue culture dishes (Falcon Plastics, Oxnard, CA) in RPMI-C as reported previously (5); cells were passaged by trypsin treatment. Murine leukemia L1210 cells were cultured in RPMI-L. Both cell lines were incubated at 37°C in a humidified atmosphere of 5% CO_2-95% air. For routine maintenance, the cells were subcultured every 4 days. Cell numbers and viability were determined by trypan blue dye exclusion using a hemocytometer.

Clonogenic Assay. Exponentially growing cultured clone A cells were incubated with graded concentrations of NSC 368390 for different time intervals. The cells were then harvested with trypsin (0.033%) and centrifuged. The cell pellets were resuspended in RPMI-C and the cell concentrations were adjusted for appropriate seeding density (depending on NSC 368390 concentration and exposure time). The cells were

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[2] The abbreviations used are: NSC 368390, 6-fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinolinecarboxylic acid sodium salt, DuP 785; DTT, DL-dithiothreitol; PALA, *N*-(phosphonacetyl)-L-aspartate; HPLC, high-performance liquid chromatography; RPMI-C, RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, buffers, and antibiotics; RPMI-L, RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, gentamicin (25 μg/ml), and 55 μM 2-mercaptoethanol.

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then replated in fresh RPMI-C and after 7 days incubation at 37°C, the cultures were washed and stained with crystal violet and the number of colonies with more than 50 cells/colony was counted. The plating efficiency was defined as the percentage of colonies formed from the total number of cells seeded. Cell survival was calculated as the ratio of plating efficiency of NSC 368390 treated cells *versus* that of control cells × 100% (6).

Rescue of Cultured Tumor Cells from NSC 368390 Cytotoxicity. Clone A cells (2.5 x 10^5) were seeded on day 0 in 60-mm culture dishes. On day 1, growth medium was removed and fresh RPMI-C with or without NSC 368390, or NSC 368390 plus various compounds, was added. After incubation at 37°C for 3 days, cell numbers and viability were determined by trypan blue dye exclusion in a hemocytometer.

L1210 cells (1 x 10^3 cells in 0.1 ml) were seeded on day 0 in a 96-well microtiter plate. On day 1, 0.1-ml aliquot of RPMI-L containing graded concentrations of NSC 368390, or NSC 368390 with uridine or cytidine, was added to the initial volume. After incubation at 37°C for 3 days, cell growth was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction method (7). Briefly, cell cultures were incubated with 50 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (1 mg/ml in Dulbecco's phosphate buffered saline) for 4 h at 37°C. The resulting purple formazan precipitate was solubilized with 100 µl of 0.04 N HCl in isopropyl alcohol. Absorbance was read in a Titertek[R] Multiskan MCC scanning well spectrophotometer (Flow Laboratories, Inc., McLean, VA) at a test wavelength of 570 nm and a reference wavelength of 630 nm. Cell growth is directly proportional to absorbance.

HPLC Analysis of Acid-Soluble Nucleotides. Clone A cells were harvested by trypsin treatment. Clone A or L1210 cells (1 to 4 x 10^7 cells) were centrifuged at 1000 × *g* for 6 min and were washed once with 5 ml of cold 0.9% NaCl. Cold perchloric acid (4%; 0.45 ml) was added to the pellets, and the tubes were vortexed vigorously. The cold perchloric acid mixture was placed in ice for 20 min and was vortexed occasionally. The precipitated protein and nucleic acids were removed by centrifugation at 3000 x *g* for 20 min at 4°C. The supernatant fluids (4°C) were then neutralized to pH 6.5 to 7.5 by 5 N KOH, the precipitated potassium perchlorate was removed by centrifugation, and the supernatant fluids were frozen for HPLC analysis.

HPLC analysis was performed on a Gilson HPLC equipped with two Model 302 pumps, Rheodyne injector 7161, and two Du Pont UV detectors. The gradient and flow rate were controlled, and data analyses were performed on a Gilson 704 HPLC System Manager using an Apple II Plus computer. A standard HPLC procedure (8) was used to analyze acid-soluble nucleotides. The separation was achieved by a linear gradient elution of potassium phosphate buffer pH 4.5 (10 to 500 mM) on a Whatman strong anion exchange column, Partisil 10 SAX.

Partial Purification of Multienzyme Complex of Carbamyl Phosphate Synthetase: Aspartate Carbamyltransferase: Dihydroorotase. The procedure used was that described previously (9) with some modifications. L1210 cells passaged in $CD2F_1$ mice were harvested (2 x 10^9 cells) from the peritoneal cavity and were washed twice with cold 0.9% NaCl. The cell pellets were disrupted by sonication in 8 ml of Buffer 1 containing 50 mM potassium phosphate, 30% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 3 mM glutamine, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride, pH 7.0 (9). The sonicated cells were centrifuged at 20,000 x *g* for 15 min and the supernatant fluids were treated with 5.2 ml of cold saturated $(NH_4)_2SO_4$ solution. The precipitated proteins were homogenized in 1.2 ml of Buffer 2 (2 mM potassium phosphate-30% dimethyl sulfoxide-5% glycerol-0.5 mM EDTA-1 mM DTT, pH 7.0) and centrifuged at 105,000 x *g* for 1 h. The partially purified enzyme complex was obtained by treating the supernatant solution with 0.78 ml of cold saturated $(NH_4)_2SO_4$ solution and dissolving the precipitate in Buffer 2 to give a 7.2-mg protein/ml solution. The enzyme complex solution was kept at −70°C and was stable for over 4 months without losing activity.

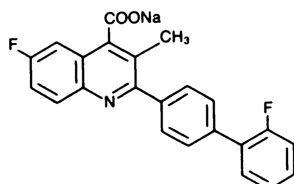

Fig. 1. Chemical structure of NSC 368390.

Preparation of Intact Mitochondria from L1210 Cells. L1210 cells harvested from the peritoneal cavity of $CD2F_1$ mice were washed twice with 0.9% NaCl and then incubated at 37°C overnight in RPMI-L. The cells were then collected from the culture medium and homogenized using a Dounce tissue grinder in 9 volumes of 0.25 M sucrose. Intact mitochondria were isolated according to a previously reported procedure (10).

Preparation of Cytosolic Extracts for Orotate Phosphoribosyltransferase and Orotidylate Decarboxylase Assay. Clone A cells (2.5 x 10^8) were harvested, centrifuged, and the cell pellets were disrupted by sonication in 3 volumes of 40 mM Tris-HCl buffer (pH 8.0) containing 1 mM DTT. The cytosolic extracts were prepared by centrifugation at 105,000 x *g* for 30 min and were used for enzyme assays on the same day.

Enzyme Assays. The activity of carbamyl phosphate synthetase was determined by a radioactive coupled assay which measured the conversion of $NaHCO_3$ to acid-stable citrulline (11).

The enzyme activities of orotate phosphoribosyltransferase and orotidylate decarboxylase were determined by measuring the release of $[^{14}C]O_2$ from [*carboxyl*-^{14}C]orotate (12).

The activities of aspartate carbamyltransferase, dihydroorotase, and dihydroorotate dehydrogenase were determined by a previously described combined assay method (11) with modifications. A reaction mixture (50 µl) containing 50 mM potassium-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 6.8), 10% glycerol, 2 mM DTT, 1 mM carbamyl phosphate, 30 µg of multienzyme complex of carbamyl phosphate synthetase, aspartate carbamyltransferase and dihydroorotase with addition of intact L1210 mitochondria, and 86.6 µM of L-[*U*-^{14}C]aspartic acid was incubated at room temperature. At indicated time intervals, 10 µl of reaction mixture were withdrawn and spotted on Whatman DE81 chromatography paper. The origin of the paper was pretreated with 20 µl of 0.46 N formic acid and air dried. This pretreatment caused the enzymatic reaction to be stopped instantly when a sample was applied. The chromatograms were developed with 0.46 N formic acid in an ascending system for approximately 5 h, air dried, sprayed with En^{3}Hance spray, and air dried again. The spots containing radioactivity were detected by autoradiography using Kodak X-Omat AR film and cut out; the amounts of radioactivity were determined in Biofluor using a Packard Tricarb scintillation counter. This chromatographic system provided excellent separation for the following compounds: aspartate (R_f, 0.80); *N*-carbamyl aspartate (R_f, 0.58); dihydroorotate (R_f, 0.40); and orotate (R_f, 0.11).

The activity of dihydroorotate dehydrogenase was also determined by the direct conversion of L-[*carboxyl*-^{14}C]dihydroorotate to [*carboxyl*-^{14}C]orotate. The reaction mixture containing 67 mM Tris-HCl buffer (pH 7.4), 5 mM KCN, 600 µM ubiquinone (Q30), and 4 µg of lubrol-solubilized L1210 mitochondria (1 mg protein:0.3 mg lubrol) with or without NSC 368390 in a total volume of 80 µl was incubated at room temperature for 30 min. When 20 µl of L-[*carboxyl*-^{14}C]dihydroorotate (final concentration 10 µM) was added, the rate of orotate formation was determined over a period of 8 min using the chromatographic system described above.

RESULTS

Tumoricidal Action of NSC 368390. The cytocidal effects of graded concentrations of NSC 368390 on clone A cells are shown in Fig. 2. Approximately 50% of the cells were killed after a 24-h exposure to NSC 368390 at concentrations greater than 2.5 µM. No increase in cell kill was observed when the concentration was increased from 2.5 to 250 µM for the 24-h period; however, greater cell kill (>99.9%) was achieved when exposure of cells to NSC 368390 was extended to 48 and 72 h. Maximal cell kill was achieved at 25 to 75 µM of NSC 368390.

A decrease rather than an increase in cytotoxicity was ob-

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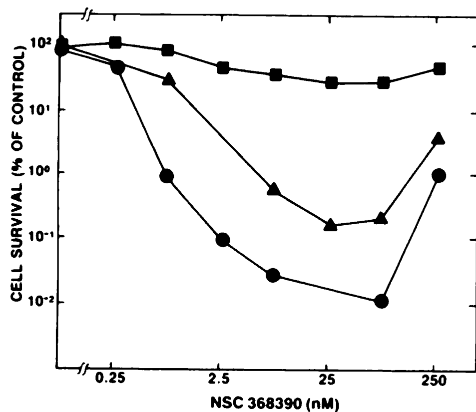

Fig. 2. Clonogenic survival of clone A cells treated with NSC 368390. Clone A cells grown in monolayer culture were incubated with NSC 368390 (0 to 250 μM) at 37°C for 24 (■), 48 (▲) and 72 (●) h. Cell survival was determined as the ratio of plating efficiency of NSC 368390 treated cells *versus* that of control cells × 100%. The plating efficiency of control cells was 58.5 ± 2.7%.

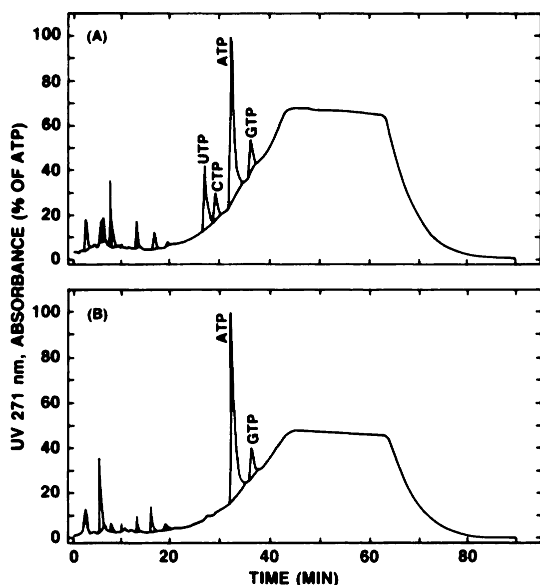

Fig. 3. HPLC chromatography of acid-soluble nucleotides of clone A cells. Clone A cells were incubated with RPMI-C (control) (*A*) and RPMI-C containing 25 μM of NSC 368390 (*B*) for 24 h. Acid-soluble nucleotides were prepared and analyzed by HPLC as described in "Materials and Methods." *Peaks* were identified by direct comparison with standards. Intracellular UTP, CTP, ATP, and GTP concentrations of control cells were similar to those reported in Table 3.

served with a 48 to 72 h exposure to 250 μM of NSC 368390 (Fig. 2). HPLC analysis of a 250 μM NSC 368390 solution in RPMI-C showed that its concentration remained at ~250 μM for 3 days; this indicates that the decrease in the cidal effect of NSC 368390 was not due to the precipitation of the agent from the medium at this concentration. The cause of this unusual phenomenon is currently under investigation.

Effects of NSC 368390 on Acid-soluble Nucleotides of Clone A Cells. The separation of acid-soluble purine and pyrimidine nucleotides of control and NSC 368390 treated clone A cells is shown in Fig. 3; intracellular UTP and CTP concentrations were depleted to undetectable levels (<0.3 pmol) after 24 h treatment with 25 μM of NSC 368390. The time-dependent effects of NSC 368390 on intracellular UTP, CTP, ATP, and GTP levels in clone A cells are shown in Fig. 4. UTP and CTP levels were reduced to about 50% of control after 3 h and to about 10% of control after 6 h of incubation with 25 μM of NSC 368390. Both UTP and CTP concentrations were reduced to undetectable levels after 15 h. In contrast, there was a 20 to 50% increase in intracellular ATP and GTP levels associated with the depletion of UTP and CTP.

Rescue of Clone A Cells from NSC 368390 Cytotoxicity by Uridine or Cytidine. The cytotoxicity of NSC 368390 against clone A cells can be circumvented by the concurrent addition of 0.1 mM uridine or cytidine (Table 1). Uridine or cytidine had little effect on cell growth in the absence of NSC 368390. After 72 h exposure, NSC 368390 (25 μM) alone reduced the growth of clone A cells to about 20% of the control cells; however, when either uridine or cytidine was added concurrently with NSC 368390, cell growth in the rescued cultures was similar to that in the control cultures. Other purine and pyrimidine bases (adenine, guanine, hypoxanthine, uracil, cytosine, thymine), and their respective ribonucleosides and 2′-deoxyribonucleosides were also tested to determine their ability to prevent the cytotoxicity of NSC 368390 against clone A cells. None of these compounds circumvents the cytotoxicity of NSC 368390 (data not shown).

Rescue of L1210 Cells from NSC 368390 Cytotoxicity by Uridine But Not by Cytidine. In clone A cells, both uridine and cytidine prevented the cytotoxicity of NSC 368390; however, in L1210 cells, cytotoxicity was only circumvented by uridine and not by cytidine (Table 2). Uridine or cytidine (0.1 mM) slightly stimulated the growth of L1210 cells in the absence of NSC 368390. NSC 368390 (25 and 75 μM) reduced the growth of L1210 cells to about 10% of that of control cultures. As was seen with clone A cells, concurrent addition of uridine com-

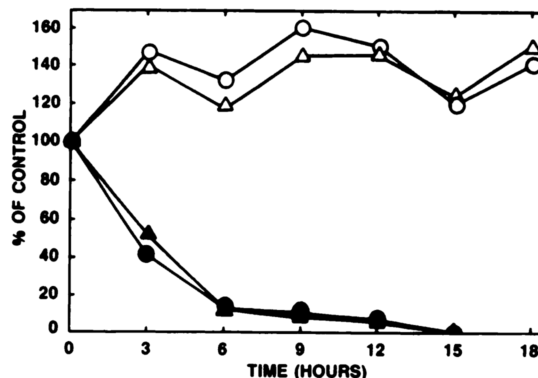

Fig. 4. Time-dependent effect of NSC 368390 on acid-soluble nucleotides of clone A cells. Clone A cells were incubated with 25 μM of NSC 368390 at 37°C, and at the indicated time intervals, acid-soluble nucleotides were prepared and analyzed by HPLC. Intracellular ATP (○), GTP (△), UTP (●), and CTP (▲) (pmol per 10^6 cells) were quantitated by comparing the peak area to that of the known concentration of standard. The concentrations of the corresponding nucleotides were expressed as the percentage of those in untreated control cells. Intracellular UTP, CTP, ATP, and GTP concentrations of control cells were similar to those reported in Table 3.

Table 1 *Effects of uridine or cytidine on NSC 368390 cytotoxicity in clone A cells*

Clone A cells (2.5×10^5) were seeded on day 0 in 60-mm culture dishes. On day 1, growth medium was removed and fresh medium with or without NSC 368390 or NSC 368390 plus uridine or cytidine (0.1 mM) was added. The cultures were then incubated at 37°C for 3 days. The cells were harvested and the total number of viable cells was determined by hemocytometer.

NSC 368390 (μM)	Nucleoside added	Total viable cells (% of control)[a]
0		100 ± 18.1[b]
0	Uridine	102 ± 15.3
0	Cytidine	98 ± 14.5
25		20 ± 2.5
25	Uridine	95 ± 17.6
25	Cytidine	92 ± 12.7

[a] Total number of viable cells in the control was $(2.2 \pm 0.4) \times 10^6$ cells.
[b] Mean ± SD (average of three determinations).

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pletely abrogated the cytotoxicity of NSC 368390 against L1210 cells; however, addition of cytidine did not protect the cells. The failure of cytidine to rescue these cells can be explained by the lack of cytidine deaminase in L1210 cells (13); thus, cytidine cannot be converted to uridine required to form UMP (Fig. 5).

Utilization of Uridine or Cytidine by Clone A Cells During NSC 368390 Treatment. The conversion of uridine or cytidine to UTP and CTP by clone A cells exposed to 25 μM of NSC 368390 was determined by HPLC (Table 3). Four nucleoside triphosphates (ATP, GTP, UTP, and CTP) were detected in control clone A cells; however, neither UTP nor CTP was detected in NSC 368390 treated cells; these results are similar to those shown in Fig. 3. In contrast, when uridine or cytidine was added to cells treated with NSC 368390, both intracellular UTP and CTP were found to be present at levels approximating those in untreated control cells.

The possibility existed that uridine or cytidine reacted directly with NSC 368390 to prevent its cytotoxicity. To determine if this was the case, clone A cells were first incubated with 25 μM of NSC 368390 for 24 h to deplete intracellular UTP and CTP. The cells were then incubated with NSC 368390 and uridine or NSC 368390 and cytidine for an additional 24 h, and intracellular nucleotide levels were determined. These results were similar to those shown in Table 3 (data not shown); both uridine and cytidine were incorporated into UTP and CTP in the presence of NSC 368390; thus, it was concluded that the cells were rescued from the cytotoxicity of NSC 368390 not by a direct reaction of uridine or cytidine with this agent but by a conversion of uridine and cytidine to UMP in metabolic steps not susceptible to inhibition by NSC 368390 (Fig. 5).

Table 2 *Effects of uridine or cytidine on NSC 368390 cytotoxicity in L1210 cells*

L1210 cells (1 × 10^3 cells in 0.1 ml) were seeded on day 0 in 96-well microtiter plates. On day 1, 0.1 ml of medium containing graded concentrations of NSC 368390 or NSC 368390 and uridine/cytidine was added to the initial volume. The final concentration of uridine or cytidine was 0.1 mM. After incubation at 37°C for 3 days, the growth of cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction method.

NSC 368390 (μM)	Nucleoside added	Cell growth (% of control)[a]
0		100.0 ± 11.7[b]
0	Uridine	110.0 ± 15.1
0	Cytidine	128.1 ± 16.5
25		10.7 ± 2.4
75		12.0 ± 2.0
25	Uridine	118.4 ± 18.4
75	Uridine	96.5 ± 15.5
25	Cytidine	8.8 ± 1.4
75	Cytidine	7.3 ± 1.2

[a] The absorbance of the control was 0.623 ± 0.073.
[b] Mean ± SD (average of four determinations).

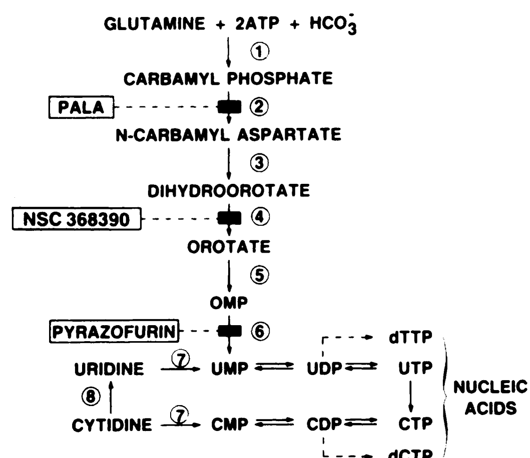

Fig. 5. Schematic drawing of the *de novo* pyrimidine biosynthetic pathway showing the inhibition sites of PALA, NSC 368390, and pyrazofurin. The enzymes catalyzing the above reactions are: ① carbamyl phosphate synthetase, EC 6.3.5.5; ② aspartate transcarbamylase, EC 2.1.3.2; ③ dihydroorotase, EC 3.5.2.3; ④ dihydroorotate dehydrogenase, EC 1.3.3.1; ⑤ orotate phosphoribosyltransferase, EC 2.4.2.10; ⑥ orotidylate decarboxylase, EC 4.1.1.23; ⑦ uridine-cytidine kinase, EC 2.7.1.48; ⑧ cytidine deaminase, EC 3.5.4.5. *OMP*, orotidine 5′-monophosphate.

Table 3 *Incorporation of uridine or cytidine into acid-soluble nucleotides of clone A cells treated with NSC 368390*

Clone A cells were incubated under various conditions at 37°C for 24 h. The cells were harvested, and acid-soluble nucleotides were prepared for analysis by HPLC as described in "Materials and Methods." Acid-soluble nucleotides, UTP, CTP, ATP, and GTP (pmol per 10^6 cells) were quantitated by comparing peak areas to those of the known concentrations of standards.

Treatment	Acid-soluble nucleotide concentration (pmol/10^6 cells)			
	UTP	CTP	ATP	GTP
None (control)	7,941[a]	2,719	13,797	3,665
NSC 368390 (25 μM)	Not detected[b]	Not detected	18,603	4,160
NSC 368390 (25 μM) + cytidine (0.1 mM)	6,745	3,256	14,882	4,072
NSC 368390 (25 μM) + uridine (0.1 mM)	6,956	3,376	15,129	4,454

[a] Averages of duplicate determinations and agree within 10% between the two separate experiments.
[b] The minimum detectable limit is 0.3 pmol.

Table 4 *Incorporation of uridine or cytidine into acid-soluble nucleotides of L1210 cells treated with NSC 368390*

L1210 cells were incubated under various conditions at 37°C for 24 h. The cells were harvested, and acid-soluble nucleotides were prepared for analysis by HPLC as described in "Materials and Methods." Acid-soluble nucleotides, UTP, CTP, ATP, and GTP (pmol per 10^6 cells) were quantitated by comparing peak areas to those of the known concentrations of standards.

Treatment	Acid-soluble nucleotide concentration (pmol/10^6 cells)			
	UTP	CTP	ATP	GTP
None (control)	599[a]	209	932	267
NSC 368390 (25 μM)	Not detected[b]	Not detected	1056	328
NSC 368390 (25 μM) + cytidine (0.1 mM)	Not detected	742	681	184
NSC 368390 (25 μM) + uridine (0.1 mM)	804	306	881	268

[a] Averages of duplicate determinations and agree within 10% between the two separate experiments.
[b] The minimum detectable limit is 0.3 pmol.

Utilization of Uridine or Cytidine by L1210 Cells During NSC 368390 Treatment. All four nucleoside triphosphates (ATP, GTP, UTP, and CTP) were detected in control L1210 cells (Table 4). UTP and CTP were depleted during NSC 368390 treatment. Similar to results with clone A cells, uridine added to L1210 cells in the presence of NSC 368390 was converted to UMP and then to UTP and CTP; however, when cytidine was added, it was anabolized only to CTP in L1210 cells and no UTP was detected. This finding is again consistent with the finding that L1210 cells lack cytidine deaminase and thus are unable to form UTP from cytidine (13).

Effects of NSC 368390 on the Activities of the First Six Enzymes of *de Novo* Pyrimidine Nucleotide Biosynthesis. The results shown above suggest that NSC 368390 interferes with a step(s) in the *de novo* biosynthetic pathway leading to the formation of UMP; therefore, the effects of NSC 368390 on the activities of all six enzymes in this pathway were examined. NSC 368390 had no effect on the activity of the first enzyme, carbamyl phosphate synthetase. It also had no effect on the activities of fifth and sixth enzymes, orotate phosphoribosyltransferase and orotidylate decarboxylase (data not shown).

Results from the combined assay which measured the effects of NSC 368390 on conversion of L-[*U*-^{14}C]aspartate, via *N*-

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carbamyl aspartate and dihydroorotate to orotate by aspartate carbamyltransferase, dihydroorotase, and dihydroorotate dehydrogenase, the second, third, and fourth enzymes, are shown in Fig. 6. The depletion of substrate, aspartate, was not affected by the addition of 25 μM NSC 368390 and the formation of the intermediates, *N*-carbamyl aspartate and dihydroorotate, was increased by 20 and 44%, respectively, with the addition of this concentration of the agent; however, the formation of the final product, orotate, was inhibited >90% by 25 μM of NSC 368390. These data demonstrate that dihydroorotate dehydrogenase activity was inhibited by NSC 368390, which caused a reduction in the formation of orotate with concomitant accumulations of dihydroorotate and *N*-carbamyl aspartate.

The inhibition of dihydroorotate dehydrogenase activity by NSC 368390 was also determined by its effect on the direct conversion of L-[*carboxyl*-^{14}C]dihydroorotate to L-[*carboxyl*-^{14}C]orotate. It was found by direct assay that the NSC 368390 concentration required to inhibit the enzyme activity was lower than 25 μM. Over a range of NSC 368390 concentrations from 15 to 105 nM, progressive inhibition of dihydroorotate dehydrogenase activity was observed. A Dixon plot (the plot of 1/rate of orotate formation *versus* NSC 368390 concentration) was constructed from these data; the intersection point with the *X*-axis allowed estimation by linear regression of an apparent K_i value of 23.5 + 1.3 (SD) nM (Fig. 7); thus, NSC 368390 is a potent inhibitor of dihydroorotate dehydrogenase.

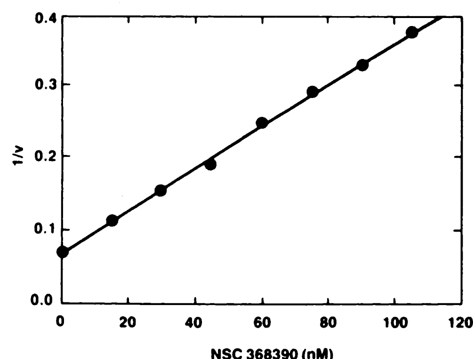

Fig. 7. Dixon plot of the effect of the NSC 368390 concentration on the activity of dihydroorotate dehydrogenase. The concentration of the substrate, dihydroorotate, used in this assay was 10 μM. $1/v$ = (nmol per min per mg protein)$^{-1}$. The apparent K_i value was estimated from the *intersection point* with the *X*-axis.

DISCUSSION

Our study has demonstrated that the novel anticancer drug candidate NSC 368390 inhibits dihydroorotate dehydrogenase, the fourth enzyme in the *de novo* pyrimidine biosynthetic pathway leading to the formation of UMP. This inhibition causes a depletion of pyrimidine nucleotide (and presumably deoxynucleotide) pools, with cell death most likely due to an inability to synthesize RNA and/or DNA.

A systematic approach was conducted to elucidate the mode of action of the structurally novel agent NSC 368390. HPLC analysis of nucleotide levels in NSC 368390 treated cells showed that UTP and CTP pools were depleted within 15 h following exposure to the agent. This result suggests that NSC 368390 interferes with pyrimidine nucleotide biosynthesis.

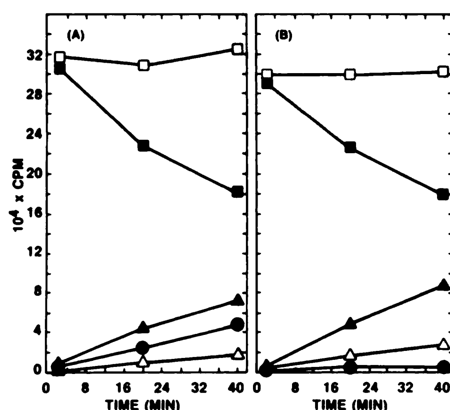

Fig. 6. Effects of NSC 368390 on aspartate carbamyltransferase, dihydroorotase, and dihydroorotate dehydrogenase activities. The reaction mixture containing a multienzyme complex of carbamyl phosphate synthetase, aspartate carbamyltransferase, and dihydroorotase with mitochondria enrichment was incubated with 0 μM (*A*) and 25 μM (*B*) of NSC 368390 at room temperature for 15 min, and 86.6 μM of L-[*U*-^{14}C]aspartic acid was added. The radioactivity in aspartate (■), *N*-carbamyl aspartate (▲), dihydroorotate (△), and orotate (●) was determined by the combined assay described in "Materials and Methods." Total radioactivity (□) is the sum of radioactivity at each *time point*.

Concurrent or delayed addition of uridine or cytidine with NSC 368390 not only rescued clone A cells from NSC 368390 cytotoxicity but also restored intracellular UTP and CTP levels. Similar results were obtained with L1210 cells when uridine was added concurrently with NSC 368390; however, addition of cytidine restored only intracellular CTP but not UTP; thus, cytidine was not able to rescue L1210 cells from NSC 368390 cytotoxicity. This is consistent with the fact that L1210 cells lack cytidine deaminase and thus cannot form UMP from cytidine (13). Overall, the results indicated that NSC 368390 inhibits a step(s) in *de novo* pyrimidine nucleotide biosynthesis prior to the formation of UMP. Analysis of all six enzymes in the pathway subsequently demonstrated that only the mitochondrial enzyme dihydroorotate dehydrogenase is inhibited by NSC 368390.

This finding is somewhat surprising, since the structure of NSC 368390 does not resemble that of the known inhibitors of this enzyme. There are three general classes of compounds that have been reported to inhibit the activity of mammalian mitochondrial dihydroorotate dehydrogenase (14). The first class of inhibitors is orotate (dihydroorotate dehydrogenase is subject to product inhibition) and analogues of either dihydroorotate or orotate (15–17). This type of inhibitor may compete with the substrate or product for the active site of the enzyme. The second class of inhibitors consists of naphthoquinone analogues that are structural analogues of ubiquinone, a cofactor required in the electron transport system and for the activity of mammalian dihydroorotate dehydrogenase (18–21). Many naphthoquinones interfere with electron transport and act as respiratory poisons (22, 23); however, Bennett *et al.* (24) have reported that dichloroallyl lawsone, a naphthoquinone analogue and a known respiratory poison, is a potent uncompetitive inhibitor of dihydroorotate dehydrogenase. The study showed that the primary antitumor effect of dichloroallyl lawsone was due to the inhibition of dihydroorotate dehydrogenase and that interference with electron transport by this agent was only a secondary effect. The last class of inhibitors includes agents that are also inhibitors of electron transfer such as cyanide, thenoyltrifluoroacetone, antimycin, and 2,4-dinitrophenol, but which interfere with dihydroorotate dehydrogenase nonspecifically (15, 19, 20). Experiments are now in progress to determine the inhibition kinetics of dihydroorotate dehydrogenase with NSC 368390 and also to determine whether the agent interferes with electron transport.

The depletion of intracellular UTP and CTP by NSC 368390 is similar to the effects on levels of these nucleotides by dichloroallyl lawsone and by two other known inhibitors of *de*

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novo pyrimidine biosynthesis, PALA and pyrazofurin (24–26). In addition, uridine has been shown to rescue tumor cells from dichloroallyl lawsone, PALA, and pyrazofurin cytotoxicity (24, 27–29), similar to our findings with NSC 368390. PALA is a transition-state inhibitor of aspartate transcarbamylase, the second of the six enzymes catalyzing reactions leading to UMP (30). Pyrazofurin inhibits the sixth enzyme, orotidylate decarboxylase, in the *de novo* pathway (31, 32); thus, inhibitors of several of the enzymes responsible for the biosynthesis of UMP can have similar effects on intracellular nucleotide pools.

Our data on uridine rescue of cultured cancer cells from the tumoricidal action of NSC 368390 suggest that uridine or cytidine might be utilized as rescue agents in NSC 368390 chemotherapy should patient toxicity become difficult to manage. There is much clinical experience with protocols utilizing substances such as nucleosides or folates to circumvent antimetabolite-induced patient toxicity (33, 34). Studies are now in progress to determine the effects of uridine on NSC 368390 toxicity and efficacy in experimental tumor models.

In summary, the clinical drug candidate NSC 368390 has a unique cellular target distinct from those of other clinically used cancer chemotherapeutic agents: the enzyme dihydroorotate dehydrogenase. Because of its enzyme target, NSC 368390 may be useful as a research tool to study various aspects of *de novo* pyrimidine biosynthesis; moreover, the lack of structural similarity between the agent and either the substrate, the product, or the cofactor of the dihydroorotate dehydrogenase-catalyzed reaction raises intriguing questions as to the nature of the inhibition of this enzyme by NSC 368390. Finally, our finding that NSC 368390 interferes with a critical step in *de novo* pyrimidine biosynthesis will be helpful in designing clinical protocols possibly including regimens utilizing rescue agents.

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REFERENCES

1. Dexter, D. L., Hesson, D. P., Ardecky, R. J., Rao, G. V., Tippett, D. L., Dusak, B. A., Paull, K. D., Plowman, J., DeLarco, B. M., Narayanan, V. L., and Forbes, M. Activity of a novel 4-quinolinecarboxylic acid, NSC 368390 [6-fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinolinecarboxylic acid sodium salt], against experimental tumors. Cancer Res., *45:* 5563–5568, 1985.
2. Chen, S. F., Ruben, R. L., and Dexter, D. L. Effects of a novel antitumor agent, DuP-785 (6-fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinolinecarboxylic acid sodium salt) on *de novo* pyrimidine biosynthesis. Proc. Am. Assoc. Cancer Res., *27:* 298, 1986.
3. Smithers, G. W., Gero, A. M., and O'Sullivan, W. J. A simple radioassay for dihydroorotate dehydrogenase. Anal. Biochem., *88:* 93–103, 1978.
4. Dexter, D. L., Barbosa, J. A., and Calabresi, P. *N,N*-Dimethylformamide-induced alteration of cell culture characteristics and loss of tumorigenicity in cultured human colon carcinoma cells. Cancer Res., *39:* 1020–1025, 1979.
5. Dexter, D. L., Spremulli, E. N., Fligiel, A., Barbosa, J. A., Vogel, R., Van Voorhees, A., and Calabresi, P. Heterogeneity of cancer cells from a single human colon carcinoma. Am. J. Med., *71:* 949–956, 1981.
6. Leith, J. T., Bliven, S. F., Lee, E. S., Glicksman, A. S., and Dexter, D. L. Intrinsic and extrinsic heterogeneity in the responses of parent and clonal human colon carcinoma xenografts to photon irradiation. Cancer Res., *44:* 3757–3762, 1984.
7. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods, *65:* 55–63, 1983.
8. Crabtree, G. W., Agarwal, R. P., Parks Jr., R. E., Lewis, A. F., Wotring, L. L., and Townsend L. B. *N*-Methylformycins. Reactivity with adenosine deaminase, incorporation into intracellular nucleotides of human erythrocytes and L1210 cells and cytotoxicity to L1210 cells. Biochem. Pharmacol., *28:* 1491–1500, 1979.
9. Mori, M., and Tatibana, M. A multienzyme complex of carbamoyl-1-phosphate synthase (glutamine): aspartate carbamoyltransferase: dihydroorotase (rat ascites hepatoma cells and rat liver). *In:* P. A. Hoffee and M. E. Jones (eds.), Methods in Enzymology, Purine and Pyrimidine Nucleotide Metabolism, Vol. 51, pp. 111–121. New York: Academic Press, Inc., 1978.
10. Dileepan, K. N., and Kennedy, J. Rapid conversion of newly-synthesized orotate to uridine-5-monophosphate by rat liver cytosolic enzymes. FEBS Lett., *153:* 1–5, 1983.
11. Christopherson, R. I., Yu, M-L., and Jones, M. E. An overall radioassay for the first three reactions of *de novo* pyrimidine biosynthesis. Anal. Biochem., *111:* 240–249, 1981.
12. Jones, M. E., Kavipurapu, P. R., and Traut, T. W. Orotate phosphoribosyltransferase: orotidylate decarboxylase (Ehrlich ascites cell). *In:* P. A. Hoffee and M. E. Jones (eds.), Methods in Enzymology, Purine and Pyrimidine Nucleotide Metabolism, Vol. 51, pp. 155–167. New York: Academic Press, Inc., 1978.
13. Boytek, P., Beisler, J. A., Abbasi, M. M., and Wolpert-DeFilippes, M. K. Comparative studies of the cytostatic action and metabolism of 5-azacytidine and 5,6-dihydro-5-azacytidine. Cancer Res., *37:* 1956–1961, 1977.
14. Kensler, T. W., and Cooney, D. A. Chemotherapeutic inhibitors of the enzymes of the *de novo* pyrimidine pathway. *In:* S. Garattini, A. Goldin, F. Hawking, and I. J. Kopin (eds.), Advances in Pharmacology and Chemotherapy, Vol. 18, pp. 274–352. New York: Academic Press, Inc., 1981.
15. Chen, J. J., and Jones, M. E. The cellular location of dihydroorotate dehydrogenase: relation to *de novo* biosynthesis of pyrimidine. Arch. Biochem. Biophys., *176:* 82–90, 1976.
16. Wuu, K. D., and Krooth, R. S. Dihydroorotic acid dehydrogenase activity of human diploid cell strains. Science (Wash. DC), *160:* 539–541, 1968.
17. Potvin, B. W., Stern, H. J., May, S. R., Lam, G. F., and Krooth, R. S. Inhibition by barbituric acid and its derivatives of the enzymes in rat brain which participate in the synthesis of pyrimidine ribotides. Biochem. Pharmacol., *27:* 655–666, 1978.
18. Miller, R. W., Kerr, C. T., and Curry, J. R. Mammalian dihydroorotate-ubiquinone reductase complex. Can. J. Biochem., *46:* 1099–1106, 1968.
19. Miller, R. W., and Curry, J. R. Mammalian dihydroorotate-ubiquinone reductase complex. II. Correlation with cytochrome oxidase, mode of linkage with the cytochrome chain, and general properties. Can. J. Biochem., *47:* 725–734, 1969.
20. Forman, H. J., and Kennedy, J. Superoxide production and electron transport in mitochondrial oxidation of dihydroorotic acid. J. Biol. Chem., *250:* 4322–4326, 1975.
21. Forman, H. J., and Kennedy, J. Dihydroorotate-dependent superoxide production in rat brain and liver, a function of the primary dehydrogenase. Arch. Biochem. Biophys., *173:* 219–224, 1976.
22. Ball, E. G., Anfinsen, C. B., and Cooper, O. The inhibitory action of naphthoquinone on respiratory processes. J. Biol. Chem., *168:* 257–270, 1947.
23. Gosalvez, M., Garcia-Canero, R., Blanco, M., and Gurucharri-Lloyd, C. Effects and specificity of anticancer agents on the respiration and energy metabolism of tumor cells. Cancer Treat. Rep., *60:* 1–8, 1976.
24. Bennett, Jr., L. L., Smithers, D., Rose, L. M., Adamson, D. J., and Thomas, H. J. Inhibition of synthesis of pyrimidine nucleotides by 2-hydroxy-3-(3,3-dichloroallyl)-1,4-naphthoquinone. Cancer Res., *39:* 4868–4874, 1979.
25. Moyer, J. D., and Handschumacher, R. E. Selective inhibition of pyrimidine synthesis and depletion of nucleotide pools by *N*-(phosphonacetyl)-L-aspartate. Cancer Res., *39:* 3089–3094, 1979.
26. Moyer, J. D., Smith, P. A., Levy, E. J., and Handschumacher, R. E. Kinetics of *N*-(phosphonacetyl)-L-aspartate and pyrazofurin depletion of pyrimidine ribonucleotide and deoxyribonucleotide pools and their relationship to nucleic acid synthesis in intact and permeabilized cells. Cancer Res., *42:* 4525–4531, 1982.
27. Johnson, R. K. Reversal of toxicity and antitumor activity of *N*-(phosphonacetyl)-L-aspartate by uridine or carbamyl-DL-aspartate *in vivo*. Biochem. Pharmacol., *26:* 81–84, 1977.
28. Cadman, E. C., Dix, D. E., and Handschumacher, R. E. Clinical, biological and biochemical effects of pyrazofurin. Cancer Res., *38:* 682–688, 1978.
29. Cadman, E., and Benz, C. Uridine and cytidine metabolism following inhibition of *de novo* pyrimidine synthesis by pyrazofurin. Biochem. Biophys. Acta., *609:* 371–382, 1980.
30. Collins, K. D., and Stark, G. R. PALA—Inhibitor of aspartate transcarbamylase. J. Biol. Chem., *264:* 6599–6605, 1971.
31. Dix, D. E., Lehman, C. P., Jakubowski, A., Moyer, J. D., and Handschumacher, R. E. Pyrazofurin metabolism, enzyme inhibition and resistance in L5178Y cells. Cancer Res., *39:* 4485–4490, 1979.
32. Gutowski, G. E., Sweeney, M. J., Delong, D. C., Hamill, R. L., and Gerzon, K. Biochemistry and biological effects of the pyrazofurins (pyrazomycins): initial clinical trial. Ann. NY Acad. Sci., *255:* 544–551, 1975.
33. Frei, E., III, Blum, R. H., Pitman, S. W., Kirkwood, J. M., Henderson, I. C., Skarin, A. T., Mayer, R. J., Bast, R. C., Garnick, M. B., Parker, L. M., and Canellos, G. P. High dose methotrexate with leucovorin rescue: rationale and spectrum of antitumor activity. Am. J. Med., *68:* 370–376, 1980.
34. Abelson, H. T., Fosburg, M. T., Beardsley, G. P., Gourin, A. M., Gorka, C., Link, M., and Link, D. Methotrexate-induced renal impairment: clinical studies and rescue from systemic toxicity with high-dose leucovorin and thymidine. J. Clin. Oncol., *1:* 208–216, 1983.

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