Cytokinetic and Cytotoxic Effects of Antimetabolites on 9L Rat Brain Tumor Cells in Vitro

Michael Weizsaecker, Takao Hoshino, Dennis F. Deen, and Shu Kobayashi

Brain Tumor Research Center of the Department of Neurological Surgery, School of Medicine, University of California, San Francisco, California 94143

ABSTRACT

Treatment of exponentially growing 9L monolayer cells with graded concentrations of methotrexate or 5-fluorouracil (5-FUra) resulted in approximately 95% cell kill, corresponding to the percentage of cells in this system labeled by autoradiography (98%). Additional cell kill could be obtained if 5-FUra was administered at a high concentration (7.7 \times 10^{-4} \text{ M}) and could be partly reversed in the presence of 8.2 \times 10^{-5} \text{ M} uridine.

When cell kill was measured as a function of time of exposure, a 3-hr exposure to 2.2 \times 10^{-4} \text{ M} methotrexate or 7.7 \times 10^{-4} \text{ M} or 7.7 \times 10^{-4} \text{ M} 5-FUra killed 30 to 50% of the cell population, which is equivalent to the percentage of the S-phase cells of the system. With both agents, additional cell kill was offset by division of unaffected G2 cells and a drug-induced block of G1 cells at the G1-S border, and it depended on long drug exposure.

Nontoxic concentrations of 5-FUra could synchronize and increase the fraction of cells in the drug-sensitive S phase.

INTRODUCTION

Cell cycle phase-specific agents are used primarily against systemic cancers in which the growth fraction is relatively high, but studies of cell recruitment suggest that these agents may also have therapeutic benefit against tumors with low growth fractions (11, 14). Most phase-specific agents interfere with DNA synthesis, and cells not in S phase may be spared from their cytotoxic action. These agents may have phase-limiting cytotoxic activity because blocking effects on the progression of G1 cells into S phase have been observed (2, 16). We have studied the cytokinetic and cytotoxic effects of MTX4 and 5-FUra, 2 agents of major interest for brain tumor chemotherapy, with 9L rat brain tumor cells in vitro.

MATERIALS AND METHODS

Cell Culture Conditions. 9L rat brain tumor cells (1 \times 10^6) were seeded into 75-cm culture flasks (Falcon Plastics, Oxnard, Calif.) and grown in complete medium consisting of MEM supplemented with nonessential amino acids, 10% fetal bovine serum, and gentamicin (50 \mu g/ml). After 48 hr, drugs were added to exponentially growing cells. Cells were screened routinely to assure the absence of Mycoplasma. Cell cycle parameters of the untreated exponentially growing monolayer culture were: G1, 8.5 hr; S, 8.2 hr; G2, 3.2 hr; M, 0.5 hr; and cell cycle time, 19.5 to 20 hr (8).

TREATMENT. Dilutions of 5-FUra (injectable; Roche Laboratories, Nutley, N. J.), MTX (parenteral; Lederle Laboratories, Pearl River, N. Y.), or uridine (Sigma Chemical Co., St. Louis, Mo.) were made in 1.5 ml of MEM, which was added to 13.5 ml (or 12 ml if 2 agents were added simultaneously) of medium in the culture flask to achieve the final dilution. For autoradiography, [\textsuperscript{3}H]dThd (New England Nuclear, Boston, Mass.; specific activity, 2 Ci/mmol) was added to the flasks to achieve a final concentration of 0.1 \mu Ci/ml. Cells were maintained at 35 to 37° and pH 7.2 to 7.4. If the drug exposure exceeded 24 hr, the drug-containing medium was renewed every 24 hr. After a time interval indicated for each experiment, the drug-containing medium was deanted, and the monolayer cells were washed twice with MEM and trypsinized. For growth delay, cell counts were performed in a Royco cell counter (Mountain View, Calif.). Single cells were suspended in complete medium and processed for colony formation, autoradiography, or flow cytometry.

Colony Formation Assay. The assay has been described (7). In brief, single-cell suspensions from treated and untreated cultures were plated in 60-mm Petri dishes (Falcon Plastics). Irradiated (4000 rads) 9L feeder cells were added 24 hr before plating. The number of feeder cells and the time of incubation were optimized for untreated and treated cells to assure maximum cell survival: 2 \times 10^6 feeder cells were added to the Petri dishes for control and 5-FUra-treated cells; while 5 \times 10^6 feeder cells were added when control and MTX-treated cells were tested (18). At least 2 sets of experiments were performed using 10 Petri dishes at each cell dilution. Control yields equal PE for both 5 \times 10^6 and 2 \times 10^6 cells. Plated cells were incubated for 12 to 14 days in a humidified 95% air-5% CO2 environment at 37° and pH 7.2 to 7.4.

PE's for untreated and treated cells were calculated as the percentage of cells able to form colonies. Surviving fractions were calculated as the ratio of the PE of treated cells to the PE of untreated cells. The PE of untreated 9L cells is 70 to 80%.

 Autoradiography. After a 24-hr exposure to [\textsuperscript{3}H]dThd with or without simultaneous treatment with drugs, cells were harvested, centrifuged at 1000 rpm for 5 min, fixed with 70% ethanol, and spread on slides. The slides were dipped in 75% NTB-2 nuclear emulsion (Kodak, Rochester, N. Y.) and exposed for 10 days at 4°. The autoradiographs were developed in Kodak D-19 for 4 min at 18°, fixed in Kodak Rapid Fixer, rinsed, dehydrated, and stained with hematoxylin. The labeled population was determined by scoring 1000 cells from each slide. Labeled cells were defined as those containing more than 4 grains/nucleus.

FCM Analysis. Single-cell suspensions from untreated and treated cultures were centrifuged at 1000 rpm for 5 min and fixed with 70% ethanol. After centrifugation, cells were stained with chromomycin A\textsubscript{3} (Calbiochem-Behring, La Jolla, Calif.) for 20 min at room temperature, centrifuged again, and resuspended in distilled water for FCM analysis. Approximately 1 \times 10^6 cells of each specimen were analyzed at 457 nm using a FACS III flow cytometer (Becton-Dickinson, Mountain View, Calif.) (5). Quantitative information from the DNA distributions was obtained by using Dean's computer program (6).

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**RESULTS**

**Dose Survival Curves.** The survival curves had similar shapes when 9L cells were treated with MTX or 5-FUra for 24 hr (Chart 1). MTX decreased cell survival between concentrations of $2.2 \times 10^{-8}$ M [cell survival, $86.7\% \pm 5.4$ (S.E.))] and $2.2 \times 10^{-1}$ M (cell survival, $8.1 \pm 0.1\%$), while 5-FUra decreased cell survival between concentrations of $7.7 \times 10^{-7}$ M (cell survival, $94.4 \pm 5.6\%$) and $1.9 \times 10^{-4}$ M (cell survival, $2.3 \pm 0.7\%$). The shape of the survival curve of cells treated with 5-FUra depended on the optimization of the colony formation assay (18). Under optimum conditions, a plateau occurred for both drugs when cell survival had decreased to approximately 5%.

**Time Survival Curves.** Cell survival as a function of exposure time was determined for 2 concentrations of each drug, one taken from the steep and the other from the plateau region of the dose survival curves. Cells survived an exposure to $1.1 \times 10^{-7}$ M MTX for up to 12 hr (cell survival, $98.8 \pm 4.9\%$) (Chart 2A). Thereafter, the survival curve decreased continuously to a plateau at 48 and 72 hr exposure, where $3.5 \pm 0.2\%$ or $7.6 \pm 0.7\%$ of the cells survived. After treatment with $2.2 \times 10^{-4}$ M MTX, cell survival dropped to $55.1 \pm 3.7\%$ by 3 hr, decreased more slowly thereafter, and also reached a plateau of approximately 8% by 24 hr.

With exposure to $7.7 \times 10^{-6}$ M 5-FUra, cell survival dropped to $79.0 \pm 3.4\%$ by 3 hr, dropped to $68.2 \pm 1.2\%$ by 6 hr, and then plateaued from 6 to 12 hr (Chart 2B). Thereafter, cell survival decreased to $12.5 \pm 0.9\%$ by 23 hr, and to $3.0 \pm 0.2\%$ by 72 hr. When cells were exposed to $7.7 \times 10^{-4}$ M 5-FUra, cell survival dropped to $56.7 \pm 1.8\%$ by 3 hr and to $40.4 \pm 2.0\%$ by 6 hr. No additional cell kill was observed between 6 and 9 hr, but thereafter cell survival decreased to $3.4 \pm 0.1\%$ at 23 hr and to $0.05 \pm 0.03\%$ at 72 hr. In a separate internally controlled experiment, surviving fractions at exposure times of 24 hr could be increased when $8.2 \times 10^{-5}$ M uridine was present during treatment with $7.7 \times 10^{-4}$ M 5-FUra (Chart 3). Uridine was nontoxic to 9L cells at the administered concentration (data not shown).

**Growth Rate.** The number of treated cells increased parallel to the control culture (doubling time, 16 to 18 hr) up to 6 hr after administration of both MTX and 5-FUra at 3 drug concentrations (50 to 60% actual increase in cell number); decelerated growth was observed at 12 hr after treatment (approximately 70% of control cell number), except for the cells treated with $1.1 \times 10^{-8}$ M MTX, which continued to grow almost parallel to control cells for 24 hr, after which they plateaued. Cells treated with $7.7 \times 10^{-7}$ M 5-FUra stopped growing 12 hr after treatment, and the number remained unchanged for up to 72 hr, whereas the number of cells treated with higher concentrations began to decline 24 hr after treatments (Table 1).

**Cell Progression Analysis.** After treatment with $1.1 \times 10^{-8}$ M MTX, cells accumulated initially in G1 but were released into S phase after about 12 hr of exposure (Charts 4A and 5A); after 24 hr of exposure, the same percentage of untreated and treated cells were labeled with $[^{3}H]$dThd (Table 2). Cells treated with $1.1 \times 10^{-7}$ M MTX showed an increase in the G1 population by 6 hr, which persisted up to 24 hr, while the proportion of S-phase cells did not change significantly; the G2-M population decreased to below 5% by 6 hr and remained there for
Antimetabolites and 9L Cells

Table 1
Percentage of surviving cells treated with MTX and 5-FUra compared to control cells

<table>
<thead>
<tr>
<th>Drug concentration (M)</th>
<th>MTX</th>
<th>6 hr</th>
<th>12 hr</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
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<tbody>
<tr>
<td>1.1 x 10^-8</td>
<td>98.3 ± 1.9</td>
<td>98.9 ± 4.7</td>
<td>90.3 ± 4.3</td>
<td>58.5 ± 2.6</td>
<td>38.4 ± 1.2</td>
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</tr>
<tr>
<td>1.1 x 10^-7</td>
<td>85.7 ± 5.3</td>
<td>92.2 ± 3.6</td>
<td>36.2 ± 1.2</td>
<td>16.3 ± 0.4</td>
<td>4.5 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>2.2 x 10^-4</td>
<td>92.6 ± 5.6</td>
<td>73.9 ± 3.4</td>
<td>33.3 ± 0.9</td>
<td>14.3 ± 0.5</td>
<td>5.0 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

5-FUra

<table>
<thead>
<tr>
<th>Drug concentration (M)</th>
<th>7.7 x 10^-7</th>
<th>101.0 ± 3.6</th>
<th>72.8 ± 0.4</th>
<th>20.1 ± 1.0</th>
<th>10.8 ± 0.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.7 x 10^-6</td>
<td>107.0 ± 5.5</td>
<td>76.9 ± 3.3</td>
<td>55.8 ± 2.6</td>
<td>20.3 ± 1.5</td>
<td>10.9 ± 0.4</td>
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Mean ± S.D.

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DISCUSSION

The toxicity of MTX and 5-FUra to 9L cells is influenced by the proliferative behavior of the culture system and kinetic perturbations induced by these agents. Except for cells treated with 7.7 x 10^-4 M 5-FUra, survival curves for cells treated with both agents up to 72 hr plateaued at 5% cell survival (Charts 1 and 2). In exponentially growing 9L monolayer cultures, this survival level is related to the percentage of cells that are not labeled after long exposures to [3H]dThd and may represent the noncycling population of the system (Table 2). However, survival of cells treated with 7.7 x 10^-4 M 5-FUra decreased below the 5% level. This additional toxicity may be the result of the interference of 5-FUra with the RNA synthesis of noncycling cells because uridine present during 5-FUra exposure partly rescued cells (Chart 3) (12). Also, 5-FUra that has penetrated into noncycling cells may remain an active compound intracellularly and, when these cells are forced to proliferate, become cytotoxic (4). Additionally, 5-FUra is transferred to Petri dishes with dead cells that, on lysis, release it into the dishes where it may interfere with the colony formation of as yet unaffected cells. This inherent bias of the colony formation assay has been observed for cells treated with toxic doses of [3H]dThd (19).
Exposure to $1.1 \times 10^{-4}$ M MTX and $7.7 \times 10^{-6}$ or $7.7 \times 10^{-4}$ M 5-FUra resulted in a 30 to 50% cell kill during the first 6 hr of exposure, followed by a plateau in cell survival of different times for each agent and concentration (Chart 2). While all cells in S phase (30 to 50% of exponentially growing 9L monolayers) can be killed by a short drug exposure, the survival plateau may be caused by G₀ cells that divide and replace G₁ cells entering S phase, and/or by the block of G₁ cells at the G₁-S border induced by MTX or 5-FUra as shown by FCM analysis (Charts 4 to 7, B and C). The presence of a G₁-S block is reflected in decreasing percentages of cells labeled with $[^{3}H]$dThd after exposure to increasing drug concentrations (Table 2). Such "self-limiting" activity has been attributed to several S-phase-specific agents (1, 2, 9, 10). Nevertheless, the percentage of 9L cells labeled during exposure to MTX or 5-FUra for 24 hr always exceeded the number of S-phase cells at the beginning of treatment. Cell growth continued up to 12 hr after treatment, and some cells in late S phase could complete mitosis. This may explain, in part, the discrepancy between the observed labeling index and the fractions of G₁- and S-phase cells computed from FCM profiles. Another possible explanation for the discrepancy is that, because DNA polymerase is not affected by MTX or 5-FUra, cells in very early S phase that would not be detected by FCM may incorporate $[^{3}H]$dThd to become labeled cells.

Sinclair has related the death of Chinese hamster cells blocked at the G₁-S border by treatment with hydroxyurea to “unbalanced growth,” in which RNA and protein levels reached the stage when division should occur but DNA synthesis was incomplete (15, 16). For 9L cells, the mechanisms of cell kill induced by MTX or 5-FUra may be the same for S-phase cells and for cells that enter S phase during treatment; the length of drug exposure is critical for survival because the progression from G₁ into S phase is slowed substantially. Therefore, the use of nontoxic concentrations of 5-FUra to synchronize cells in S phase before the administration of toxic concentrations may be a promising therapeutic approach. 5-FUra perturbed the cycle progression of 9L cells at concentrations that did not affect cell survival. After a 24-hr exposure to $7.7 \times 10^{-7}$ M 5-FUra, 70% of the cell population was in S phase, while less than 6% was killed (Charts 1, 64, and 7A). Similarly, a transient G₁-S block and partial synchronization has been shown for L1210 cells in vivo after treatment with low doses of 5-FUra (3). Multiple, spaced toxic drug doses were found to be more effective than a single dose in experimental regimens with S-phase-specific agents (2, 13, 17). Nontoxic drug doses that induce cell kinetic perturbations may achieve additional therapeutic benefit.

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REFERENCES


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