Experimental Combination Chemotherapy with Thymidylate Synthetase and Ribonucleotide Reductase Inhibitors

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Abstract: The synergistic cytotoxic effects on exponentially growing 9L rat brain tumor cells of several inhibitors of thymidylate synthetase (TS) and ribonucleotide reductase (RNR) used in combination were investigated using a colony forming efficiency assay as the experimental endpoint. A 24 h treatment with nontoxic (0.1 µg/ml) or low (1.0 µg/ml) doses of 5-fluorouracil (5-FUra), 5-fluoro-2-deoxyuridine, 5,8-dideazafolic acid, or 2′-deoxy-2-fluoro-ara-uracil markedly enhanced cell kill caused by subsequent administration of 100 µg/ml hydroxyurea (HU) for 6 h. When a similar dose of HU or 1-formyl-5-isouquinoline thiosemicarbazone was administered for 6 h immediately after a 24 h treatment with either a 0.1 µg/ml or 1.0 µg/ml dose of 5-FUra, a cell kill of approximately 1 log in addition to that caused by each drug alone was obtained. Thus a synergistic cell kill was consistently obtained when a low dose of TS inhibitors was administered 24 h before a 6 h treatment with another low dose of agents that act as RNR inhibitors. This synergism was not observed when FUra-treated cells were treated with methotrexate, 6-mercaptopurine, vincristine, or 1,3-bis(2-chloroethyl)-1-nitrosourea. Similarly, a 6 h treatment with 1 µg/ml of FUra of cells that had been treated for various periods with 100 µg/ml of HU did not increase cell kill more than that obtained with HU alone (30 % cell kill).

Patients harboring malignant brain tumors are often treated on chemotherapeutic regimens that use various combinations of drugs that have different modes of action. Combinations are selected to obtain an enhanced cell kill by metabolic interaction of two agents, to take advantage of cytokinetic perturbations induced in tumor cells treated with different agents, or to decrease drug-induced side effects. Because of the limited number of available, efficacious chemotherapeutic agents, use of these agents in combination provides a reasonable approach to the treatment of cancer patients. Even though biological and/or biochemical mechanisms are not well understood, there are many combinations of drugs that enhance cytotoxicity in experimental settings.

5-Fluorouracil (FUra) has been used extensively for the treatment of various neoplasms and, because it crosses the blood-brain barrier, has been used in combination with other drugs for treatment of malignant brain tumors (1). We have shown (2) that treatment of exponentially growing 9L rat brain tumor cells with low nontoxic doses of FUra resulted in the accumulation of cells in S-phase. Treatment of such cells with hydroxyurea (HU) resulted in a greatly enhanced cell kill (3). Because cells blocked at the G1/S border by moderately toxic doses of FUra also showed enhanced sensitivity towards HU, this synergism is not simply a result of cytokinetic perturbations induced by FUra nor of the phase specificity of HU.

In this report, we describe experiments in which inhibitors of thymidylate synthetase (TS), which are more specific than FUra, and more potent inhibitors of ribonuclease reductase (RNR) than HU were used in combination against 9L rat brain tumor cells in vitro. Results obtained support the hypothesis that synergism is the result of a blockade of TS followed by inhibition of RNR.

Materials and Methods

9L Cell Culture

9L rat brain tumor cells (1 to 2 x 106 cells) were seeded into 75 cm2 tissue culture flasks and grown in 16 ml of Eagle’s minimum essential medium (MEM) supplemented with 10% newborn calf serum, nonessential amino acids, and gentamicin (50 µg/ml) (CMEM). Before treatment, cells were incubated for approximately 24 h at 37°C in a humidified % CO2: 95% air atmosphere to establish early log phase growth. Cell survival was determined with a colony forming efficiency (CFE) assay (2, 4). Surviving fractions (SFs) were calculated as the ratio of the CFE’s of treated cells to the CFE’s of control cells.

Drugs and Treatment

FUra (fluorouricil injectable, Roche Laboratories, Nutley, NJ), 5-fluoro-2-deoxyuridine (FdUrd, Sigma, St. Louis, MO), 5,8-dideazafolic acid (H-338, kindly supplied by Dr. John B. Hynes, Department of Pharmaceutical Chemistry, Medical University of South Carolina), 2′-deoxy-2′-fluoro-5-fluoro-ara-uracil (FFdA1, kindly supplied by Dr. K. A. Watanabe, Walker Laboratory of the Memorial Sloan-Kettering Cancer Center, New York), HU (Calbiochem-Behring, La Jolla, CA), 1-formylisoquinoline thiosemicarbazone (IQ-1, a gift of Dr. A. Sartorelli), methotrexate (MTX, Lederle Laboratories, Pearl River, NY), 6-mercaptopurine (6-MP, 3

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Calbiochem), vincristine (Oncovin, Eli Lilly, Indianapolis, IN), and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, National Cancer Institute, Bethesda, MD) were dissolved in MEM to give stock solutions that were added to exponentially growing cells in different volumes to achieve the desired final concentration. For combination treatments, medium that contained the first drug was decanted, cells were rinsed twice with prewarmed MEM, and then refed with CMEM that contained the second drug. When two drugs were combined, the expected additive cell kill was estimated using the method of Valeriote et al. (5), as the product of the SF’s of each drug acting alone.

Results

Figure 1 shows dose-survival curves for 9L cells treated with FUra, FdUrd, FFdAU, and H-338 alone for 24 h, and for pretreatment with these drugs followed by treatment with HU for 6 h. Under the experimental conditions, HU alone produced a slight (22%) cell kill. In each instance, the combined regimen produced a marked increase in cell kill.

Figure 2 shows survival curves for 9L cells pretreated with 0.1 and 1.0 μg/ml of FUra for a 24 h period and, after removal of the drug containing medium, treated with HU or IQ-1 for 6 h. As found for HU treatment, IQ-1 produced less than a 20% cell kill when used alone, but showed enhanced cytotoxicity in combination with FUra.

The extent of the synergism varied with the combination and with the concentration of inhibitors used; in many instances, the increased cell kill caused