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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 (FUra), 5-fluorodeoxyuridine, 5,8-dideazaisofolic 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-formylisoquinoline thiosemicarbazone was administered for 6 h immediately after a 24 h treatment with either a 0.1 μg/ml or 1.0 μg/ml of 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).

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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 G_1/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 10^6 cells) were seeded into 75 cm^2 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 5 % CO_2 : 95 % air atmosphere to establish early log phase growth. Cell survival was determined with a colony forming efficiency (CFE) assay (2, 4). Surviving fractions (SF's) were calculated as the ratio of the CFE's of treated cells to the CFE's of control cells.

Drugs and Treatment

FUra (fluorouracil injectable, Roche Laboratories, Nutley, NJ), 5-fluorodeoxyuridine (FdUrd, Sigma, St. Louis, MO), 5,8-dideazaisofolic 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 (FFdAU, 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,

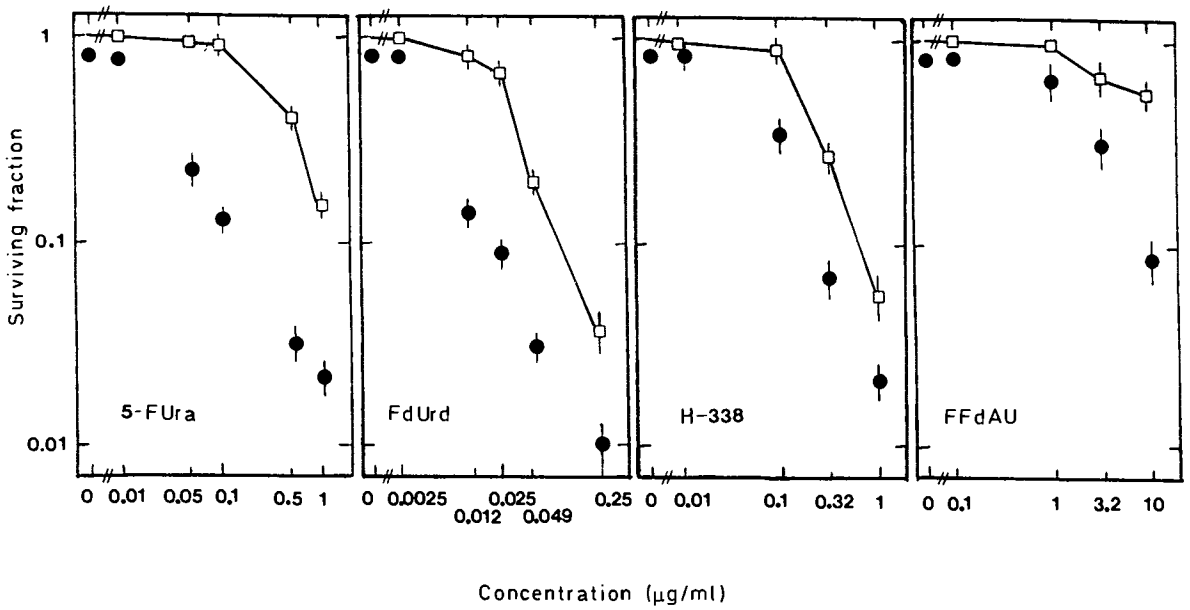

Fig. 1 Dose response curves for 9L cells (□) treated with FUra, FdUrd, H-338, or FFdAU for 24 h are shown. The combination treatment in which HU (100 μg/ml, 6 h, 20 % cell kill) was administered to 9L cells pretreated for 24 h with FUra, FdUrd, H-338, or FFdAU gave the values indicated by closed circles (•) (shown at the point at which HU was administered). (Mean ± S. E. of at least four samples from at least two separate experiments.)

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

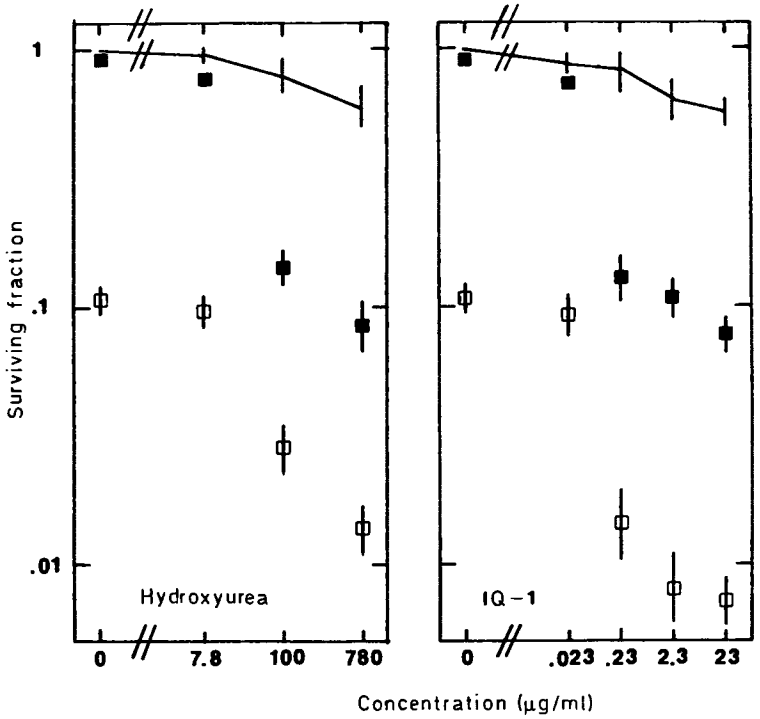

Fig. 2 Dose response curves for 9L cells (–) treated with HU or IQ-1 for 6 h are shown. The combination treatment in which HU or IQ-1 was administered to 9L cells pretreated with 0.1 μg/ml (■) or 1 μg/ml (□) of FUra for 24 h also produced enhanced cell kill. (Mean ± S. E. of at least four samples from at least two separate experiments.)

by synergistic effects approached 1 log. In contrast, when 9L cells were pretreated with both concentrations of FUra for 24 h and then treated for 6 h with the cytotoxic agents MTX (0.45 to 136 μg/ml), 6-MP (0.1 to 100 μg/ml), vincristine (0.01 to 10 μg/ml), or for 2 h with BCNU (1.5 or 3.0 μg/ml), cell kill obtained was approximately equal to the calculated, purely additive values, and no synergism was apparent. When 100 μg/ml of HU was administered as a first agent for 6 to 48 h followed by a 6 h treatment with 1.0 μg/ml of FUra, the increased cell kill was no more than was obtained by treatment with that dose of HU alone.

Discussion

We have shown that pretreatment of 9L cells with nontoxic (0.1 μg/ml) or moderately toxic (1.0 μg/ml) doses of FUra greatly enhances the cytotoxicity of subsequently administered HU (3). An attractive general hypothesis for the mechanism of this synergistic effect is that the lowered deoxythymidine monophosphate (dTMP) levels that would be the result of a blockade of TS might enhance the imbalance of deoxyribonucleotide triphosphates caused by inhibition of RNR, and might lead to defective DNA synthesis and/or repair. However, because FUra and HU have effects other than inhibition of TS and RNR, respectively (6–12), other mechanisms could be responsible for the observed synergism.

To determine whether a blockade of TS was involved in this synergism, 9L cells were pretreated with compounds, including the nucleoside analogs FdUrd (7, 12) and FFdAU (13), and the quinazoline analog H-338 (14), that are known to be more specific inhibitors of this enzyme than FUra in cultured cells. FdUrd is directly converted to FdUMP, a potent inhibitor of dTMP synthetase, in a number of cell lines (7, 12). Except for cells that have relatively high levels of pyrimidine nucleoside phosphorylases, there is little catabolism of FdUrd to FUra (15). Furthermore, because the concentration of FdUrd required for cytotoxicity in 9L cells is so much lower than that of FUra, the latter cannot be responsible for the effects of FdUrd. FFdAU is also converted to its 5'-nucleotide, which is a specific inhibitor of TS *in vitro* (16). Although FFdAU is not as potent an inhibitor as FdUrd, the glycosidic bond is metabolically stable toward pyrimidine nucleoside phosphorylases, which precludes any possible catabolism to FUra (17). H-338 is a quinazoline analog of folic acid that has also been shown to be a potent inhibitor of TS in mammalian cells (18). As found for treatment of 9L cells with FUra (3), subsequent treatment with HU produced enhanced cell kill when 9L cells were treated with 0.1 μg/ml or 1.0 μg/ml of these inhibitors for 24 h. These results and the fact that the synergism of FUra and HU can be reversed by addition of thymidine (3) lead to the reasonable conclusion that the synergistic effects described here were the result, at least in part, of a blockade of TS.

To demonstrate that RNR was the target for HU in the synergistic combination with FUra, the effects of another inhibitor of RNR was studied in 9L cells pretreated with 0.1 μg/ml or 1.0 μg/ml of FUra. We found that low concentrations of IQ-1, a potent and specific inhibitor of RNR (18), has a synergistic effect similar to that observed with HU. In contrast, the cytotoxic agents MTX, 6-MP, vincristine, and BCNU, which do not inhibit RNR, did not enhance the cytotoxicity against 9L cells pretreated with FUra. Often this kind of synergism is cell line dependent; however, similar effects have been found in L 1210 cells (20). In addition, the sequence of administration is important because either HU administered during treatment with FUra (3) or the reverse sequence of treatment did not produce much synergism.

The results reported here provide evidence that a blockade of TS for a period of approximately 24 h, followed by inhibition of RNR produces a synergistic effect on cell kill. Although the mechanism that causes this synergism has not been defined, these results suggest a possible clinical use for combination protocols such as those described above. Studies of metabolic perturbations and of possible biochemical mechanisms that may be responsible for the results obtained in these studies are in progress.

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