**Effects of Combinations of Drugs Having Different Modes of Action at the Ribonucleotide Reductase Site on Growth of L1210 Cells in Culture**

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**ABSTRACT**

Combinations of inhibitors directed at the individual components of ribonucleotide reductase were studied for their effects on L1210 cell growth in culture. The combinations included pyrazolomimidazole (IMPY) plus deoxyadenosine and hydroxyurea plus deoxyadenosine. Modulators were utilized to potentiate the effects of hydroxyurea, IMPY, or deoxyadenosine. Desferal was used to modulate the activity of hydroxyurea and IMPY while erythoro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was used as the modulator of deoxyadenosine metabolism. While the combinations of deoxyadenosine-EHNA, hydroxyurea-Desferal, or IMPY-Desferal caused increased growth inhibition of L1210 cells at high drug concentrations, combinations which consisted of deoxyadenosine-EHNA-IMPY-Desferal or deoxyadenosine-EHNA-hydroxyurea-Desferal gave strong synergistic inhibition of L1210 cell growth in culture at concentrations of each of the drugs which alone had minimal inhibitory effects on tumor cell growth. The four-drug combination was clearly more effective than any three-drug combination in terms of inhibition of tumor cell growth. It was also observed that the concentrations of the modulators (Desferal or EHNA) were as critical as the concentrations of hydroxyurea, IMPY, or deoxyadenosine in establishing an effective drug combination.

**INTRODUCTION**

Ribonucleotide reductase offers a unique metabolic site at which combination chemotherapy can be directed. It has been shown that mammalian ribonucleotide reductase is composed of 2 nonidentical protein components consisting of a subunit containing a nonheme iron and a subunit containing the effector-binding site(s) (6, 11, 24). These components can be specifically and independently inhibited (5). Utilizing combinations of inhibitors directed at each component (e.g., IMPY-dATP) synergistic inhibition of ribonucleotide reductase activity was observed (20). Other studies had shown that iron-chelating agents (e.g., Desferal) would potentiate the inhibition of reductase activity by hydroxyurea, guanazole, and IMPY (5, 12). To extend these studies with the highly purified ribonucleotide reductase to tumor cells growing in culture, combinations of deoxyadenosine and IMPY or hydroxyurea were utilized. However, because of the rapid deamination of deoxyadenosine by the cells, EHNA was used to modulate the metabolism of deoxyadenosine. EHNA has been shown to be a potent inhibitor of adenosine deaminase (1, 30).

In this report, we present data to show that combination of deoxyadenosine-EHNA and IMPY-Desferal or hydroxyurea-Desferal, which specifically interact with the individual components of ribonucleotide reductase, will give synergistic inhibition of L1210 cell growth in culture.

**MATERIALS AND METHODS**

**Growth of L1210 Cells in Culture.** The L1210 cells were grown in suspension in Roswell Park Memorial Institute Culture Medium 1640 medium supplemented with 10% horse serum, sodium bicarbonate (2 g/liter), and gentamicin sulfate (50 mg/liter). Cells were grown at 37°C. Each group, control or experimental, was set up as triplicate cultures. Aliquots (1 ml) were removed at daily intervals. The cells were pelleted by centrifugation, and the cells were resuspended in filtered phosphate-buffered saline (NaCl, 140 mm-KCl, 2.7 mm-NaH2PO4, 8.1 mm-KH2PO4, 1.47 mm-CaCl2, 0.9 mm-MgCl2, 0.49 mm-K, pH 7.4). Each cell sample was counted in triplicate in a Biophysics Cytograf, Model 6300. The experiments were initiated by seeding the culture flasks with L1210 cells at an initial concentration of 2 x 10^5 cells/ml. The drugs alone or in combination were added at Time zero. The L1210 cell line was obtained from Dr. Richard G. Moran, Children's Hospital of Los Angeles, Calif.

**Chemicals.** Hydroxyurea, deoxyadenosine, and gentamicin were purchased from Sigma Chemical Company, St. Louis, Mo. IMPY was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, through the assistance of Dr. Ven L. Narayan. Desferal was a gift from Ciba-Geigy Corp., Summit, N. J. EHNA was obtained from Dr. Ray Olsson who had received the sample from Burroughs-Wellcome, Research Triangle, N.C. Roswell Park Memorial Institute Culture Medium 1640, sodium bicarbonate, and horse serum were purchased from Grand Island Biological Company, Grand Island, N. Y.

**RESULTS**

**Modulation of Inhibition of L1210 Cell Growth by Hydroxyurea with Desferal.** Hydroxyurea at high concentrations will inhibit the growth of L1210 cells in culture. As seen in Chart 1A, hydroxyurea at a final concentration of 200 μM caused essentially complete inhibition of cell growth. Hydroxyurea at concentrations of 50 or 100 μM had much less effect on cell growth. When Desferal was added at a final concentration of 20 μM in combination with hydroxyurea (50 μM), essentially complete inhibition of tumor cell growth was observed (Chart 1D). In effect, the presence of Desferal (100 μM) increased the effects of hydroxyurea on tumor cell growth by at least 4-fold. By comparing the cell growth curves in Chart 1, C and D, it is observed that the concentration of Desferal is an important factor in the drug combination.

**Modulation of Inhibition of L1210 Cell Growth by IMPY with Desferal.** IMPY at a concentration of either 100 μM or 250...
μM had very little effect on L1210 cell growth in culture. Whereas 20 μM Desferal in combination with IMPY (250 μM) caused little increase in the inhibition of cell growth, the combination of IMPY (250 μM) and Desferal (100 μM) caused essentially complete inhibition of tumor cell growth. These data are shown in Chart 2. As seen by the comparison of Chart 2, Panels A, B, and C, the synergistic combination was dependent on the concentration of both IMPY and Desferal.

Modulation of Inhibition of L1210 Cell Growth by Deoxyadenosine with EHNA. Deoxyadenosine has been shown to be toxic to cells. However, the rapid deamination of deoxyadenosine by the cells has required the use of adenosine deaminase inhibitors to protect deoxyadenosine from deamination, thereby allowing the build up of dATP in the cells. The effects of combinations of deoxyadenosine and EHNA at various concentrations of each on L1210 cell growth were studied. These results are shown in Chart 3. Concentrations of deoxyadenosine as high as 150 μM were not inhibitory to cell growth in the absence of EHNA. However, as the concentration of EHNA was increased, the concentration of deoxyadenosine required to inhibit cell growth decreased markedly.

Effect of Combinations of Hydroxyurea and Deoxyadenosine in the Presence of Desferal and EHNA on L1210 Cell Growth. The growth of L1210 cells in culture in the presence of hydroxyurea, deoxyadenosine, Desferal, and EHNA, alone and in combination, was studied. As seen in Chart 4, at the concentrations of drugs used, the 4-drug combination consisting of hydroxyurea, Desferal, deoxyadenosine, and EHNA (Chart 4B) was far more inhibitory than the combinations consisting of 2 drugs (hydroxyurea/Desferal, deoxyadenosine-EHNA, or hydroxyurea-deoxyadenosine) (Chart 4A) or 3 drugs (hydroxyurea-Desferal-deoxyadenosine or hydroxyurea-deoxyadenosine-EHNA) (Chart 4B). Essentially complete inhibition of cell growth was obtained with the combination consisting of hydroxyurea (50 μM), Desferal (20 μM), deoxyadenosine (15 μM) and EHNA (5 μM). Reducing the concentration of either the reductase inhibitors or the modulators lowered the effectiveness of this 4-drug combination on tumor cell growth (data not shown).

Effect of Combinations of IMPY and Deoxyadenosine in the Presence of Desferal and EHNA on L1210 Cell Growth. The effects of IMPY, deoxyadenosine, and the modulators Desferal and EHNA on L1210 cell growth were determined using each drug alone and in various combinations. From the data shown in Chart 5, it is seen that, at the concentrations used, the combinations deoxyadenosine-IMPY, deoxyadenosine-EHNA, and IMPY-Desferal had relatively little effect on L1210 cell growth (Panel A). The combination of deoxyadenosine-EHNA-IMPY was more effective in inhibiting tumor cell
growth than was the combination of deoxyadenosine-IMPY-Desferal (Chart 5, Panel B) or the 2-drug combinations. However, when all 4 drugs were added, essentially complete inhibition of cell growth was observed (Chart 5, Panel B). These data indicate that the modulators play an important role in producing an effective drug combination at these low concentrations of agents.

**DISCUSSION**

The mammalian ribonucleotide reductase catalyzes the reaction unique to the synthesis of the deoxyribonucleotide precursors of DNA. This enzyme catalyzes the reduction of ribonucleoside diphosphates to the corresponding 2'-deoxyribonucleoside diphosphates. The mammalian enzyme (like the enzyme from *Escherichia coli*) consists of 2 nonidentical components which are both required for enzyme activity (6, 11, 24). One component contains a nonheme iron while the other component contains the effector-binding site(s). This enzyme is a key enzyme and possibly the rate-limiting step in the formation of DNA. Several important aspects of this enzyme are the facts that: its activity is extremely low in resting cells (31); the level of reductase activity correlates with the growth rate of the cell population (17); the activity of this enzyme is very strongly allosterically regulated (7, 13, 25); and the ratio of the nonidentical components making up the active enzyme does not remain constant during the cell cycle (10, 19). These factors lead to the conclusion that this enzyme has many properties which might be exploited in the development of specific antitumor agents. Furthermore, since the enzyme is a multicomponent enzyme consisting of a nonheme iron subunit and an effector-binding subunit and each of the components can be specifically and individually inhibited/inactivated, it was possible that this metabolic site (ribonucleotide reductase) could serve as a target for combination chemotherapy (8).

Many compounds have been developed which are targeted at ribonucleotide reductase. These include hydroxyurea (32), guanazole (4), benzohydroxamic acid analogs (18), IMPY (3, 9), the thiosemicarbazones (28), and the diahydroxides of inosine and 5'-deoxyinosine (3, 9). We have been able to categorize some of these compounds with respect to whether they are inhibitors of the nonheme iron component or the effector-binding component (16). Hydroxyurea, guanazole, IMPY, MAIQ, and IQ inhibit the nonheme iron component, while Inox, 5'-dlnox, dATP, dGTP, and dtTP inhibit the effector-binding component of CDP reductase (16). All of these compounds have been shown to inhibit DNA synthesis and tumor cell growth in culture. In clinical trials, hydroxyurea, guanazole, IMPY, MAIQ, and IQ have not lived up to expectations, failing to give the anticipated responses.

In the studies presented, the combinations of hydroxyurea-deoxyadenosine and IMPY-deoxyadenosine were studied to determine if combinations of drugs directed at the individual components of ribonucleotide reductase would offer an approach that could lead to synergistic inhibition of tumor cell growth. The combinations of hydroxyurea-deoxyadenosine or IMPY-deoxyadenosine were not especially good combinations except at high concentrations of each (data not shown). However, when these combinations were supplemented with Desferal to potentiate the action of hydroxyurea and IMPY and with EHNA to decrease the deamination of deoxyadenosine, effective drugs combinations were generated which blocked tumor cell growth in a synergistic manner (Charts 4 and 5). By
including the modulators Desferal and EHNA in the protocol, the effective concentrations of hydroxyurea, IMPY, and deoxyadenosine could be significantly reduced. The 3-drug combinations were not as effective as all 4 drugs combined at the noninhibitory concentrations used. The 3-drug combinations which included deoxyadenosine-EHNA-hydroxyurea or deoxyadenosine-EHNA-IMPY were more effective inhibitors of L1210 cell growth than were the combinations deoxyadenosine-hydroxyurea-Desferal or deoxyadenosine-IMPY-Desferal. It was more important to protect deoxyadenosine from deamination than to modulate the effects of hydroxyurea or IMPY. The presence of EHNA in combination with deoxyadenosine has been shown to lead to the accumulation of dATP in cells (23, 33) and to inhibit ribonucleotide reductase activity (23). Three-drug combinations (deoxyadenosine-EHNA-hydroxyurea or deoxyadenosine-EHNA-IMPY) would result in complete inhibition of cell growth if the concentration of deoxyadenosine and the concentrations of hydroxyurea or IMPY were both increased (data not shown).

It is possible that the synergistic inhibition of cell growth is not due entirely to the effects of these agents on the ribonucleotide reductase site. EHNA is known, for example, to interfere with purine metabolism in intact cells (20) and to retard cells in the G₂ + M phase of the cell cycle (23). However, at the concentration of EHNA (5 μM) used in the 4-drug combinations, it would be expected that the side effects of EHNA would be minimal. Furthermore, deoxyadenosine toxicity of nondividing human lymphoid cells has been reported to occur by a mechanism which does not involve the inhibition of reductase by dATP (22). Other sites of action have also been suggested for hydroxyurea. These include the inhibition of deoxyoctonucleotide kinase (27) and thymidylate synthase (2) and the degradation of DNA by a "reactive" derivative of hydroxyurea (21). IMPY has been shown to cause hemolysis. It is possible that the reactions which IMPY or its derivatives promote in causing RBC lysis (21) are also cytotoxic to other cells as well (26). Studies are in progress to determine directly the effects of these combinations on cellular reductase activity and dNTP levels in the intact cell. Our data to date strongly support the hypothesis that successful combination chemotherapeutic regimens which result in synergistic or additive inhibition of tumor cell growth can be designed which are directed at the ribonucleotide reductase site. Our studies involving the combination chemotherapy directed at the individual components or ribonucleotide reductase have indicated that this approach can be extended beyond the highly purified reductase to studies in intact tumor cells in culture. These studies will provide the basic information required to better utilize compounds such as hydroxyurea, IMPY, MAIQ, and IQ in combination with other agents in the successful treatment of human tumors.

REFERENCES

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Drug Combinations Directed at Reductase


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