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Effect of combinations of deoxyguanosine and 8-aminoguanosine with 2,3-dihydro-1*H*-imidazo[1,2-*b*]pyrazole on L1210 cell crowth in culture

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Since ribonucleotide reductase catalyzes the rate-limiting step in the formation of the 2′-deoxyribonucleoside 5′-triphosphate precursors of DNA, this pathway represents a controlling step in DNA replication. Lowe and Grindley [1] reported that deoxyguanosine inhibits the growth rate of L1210 cells in culture and that this inhibition of cell growth is correlated with decreased intracellular levels of dTTP and dCTP. While deoxyguanosine has been shown to be toxic to cells, high concentrations are required because of the rapid phosphorylysis of deoxyguanosine by purine nucleoside phosphorylase [2]. Kazmers *et al.* [3] showed that, by selective inhibition of purine nucleoside phosphorylase with 8-aminoguanosine in intact cells, the toxicity of deoxyguanosine in T lymphoblasts is greatly enhanced. This is correlated with a marked increase in the dGTP levels in these cells. B lymphoblasts which are not sensitive to the combination of deoxyguanosine plus 8-aminoguanosine do not show elevations in intracellular dGTP. Stoeckler *et al.* [4] showed that 8-aminoguanine and 8-aminoguanosine are very effective inhibitors of purine nucleoside phosphorylase and that in combination with guanosine or deoxyguanosine lead to increased intracellular pools of GTP or dGTP respectively.

We have shown previously that mammalian ribonucleotide reductase consists of two non-identical protein components [5], each of which can be specifically and independently inhibited [6]. Studies with combinations consisting of agents directed at the individual subunits of tumor cell ribonucleotide reductase indicate the feasibility of such an approach to combination chemotherapy [7]. It has been shown that deoxyadenosine (presumably through the accumulation of dATP) in combination with hydroxyurea or 2,3-dihydro-1*H*-imidazo[1,2-*b*]pyrazole (IMPY) result in synergistic inhibition of L1210 cell growth in culture provided that the deamination of deoxyadenosine is decreased markedly with the aid of an adenosine deaminase inhibitor such as erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) [8].

In the current studies, experiments were carried out determine if combinations of drugs which included deoxyguanosine and IMPY could be generated which synergistically inhibited L1210 cell growth in culture. It was anticipated that 8-aminoguanosine would be needed to protect deoxyguanosine from degradation. The rationale for these combinations was that dGTP would inhibit the effector-binding subunit of ribonucleotide reductase while IMPY would inhibit the non-heme iron subunit.

Materials and methods

The L1210 cells were grown in suspension in RPMI 1640 medium supplemented with sodium bicarbonate (2 g/l), gentamicin sulfate (50 mg/l) and 10% horse serum. The cells were grown at 37°. Each group, control or drug-treated, was set up in triplicate. The flasks were seeded at day-zero with 0.15×10^6 cells/ml. At daily intervals, aliquots (1 ml) were removed for cell counts. Each cell sample was counted in duplicate in a model ZBI, Coulter Counter. The L1210 cell cultures were tested for mycoplasma contamination by the method of Schneider *et al.* [9].

8-Aminoguanosine was synthesized from 8-bromoguanosine by the method of Holmes and Robins [10]. The 8-aminoguanosine was characterized by its u.v. spectrum and analyzed for purity by thin-layer chromatography and high performance liquid chromatography (HPLC). Finally, it was tested and found to be a potent inhibitor of purine nucleoside phosphorylase [4]. 8-Bromoguanosine was purchased from the United States Biochemical Corp., Cleveland, OH. IMPY was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, through the assistance of Dr. Ven L. Narayanan. Desferal was a gift of the Ciba-Geigy Corp., Summit, NJ. Deoxyguanosine and gentamicin were purchased from the Sigma Chemical Co., St. Louis, MO. The horse serum, RPMI 1640 culture medium, and the sodium bicarbonate were purchased from the Grand Island Biological Co., Grand Island, NY.

Results and discussion

Deoxyguanosine is toxic to cells due to the buildup of dGTP which, in turn, presumably inhibits ribonucleotide reductase activity [1, 3, 5, 11–14]. Normal cells have high concentrations of purine nucleoside phosphorylase which degrades deoxyguanosine and prevents the accumulation

of dGTP [2]. In cells lacking purine nucleoside phosphorylase, deoxyguanosine becomes very toxic at low concentrations. This is seen in cells taken from immunodeficient patients [15] and in specifically generated mutant cell lines [13]. 8-Aminoguanosine, an inhibitor of purine nucleoside phosphorylase [3, 4], was shown to increase the cytotoxicity of deoxyguanosine in cells having normal levels of purine nucleoside phosphorylase [3, 16]. 8-Aminoguanine and 8-aminoguanosine are, themselves, substrates for purine nucleoside phosphorylase while also serving to inhibit the activity of the enzyme toward its natural substrates [16].

Deoxyguanosine (dGuo) alone had relatively little effect on the growth of L1210 cells (Fig. 1). While dGuo at 0.4 mM inhibited L1210 cell growth for the first 2 days, by day-5 of the growth curve the total cell number had increased significantly. 8-Aminoguanosine (8-AGuo), alone, had no effect on L1210 cell growth at concentrations as high as 0.2 mM (data not shown). When 8-AGuo was added in combination with dGuo, L1210 cell growth was synergistically decreased. In the presence of the combination dGuo (0.3 mM) and 8-AGuo (0.2 mM), there was essentially no cell growth. Lowering the concentration of dGuo to 0.2 mM in the presence of 8-AGuo (0.2 mM) still inhibited cell growth for 3 days, but by 5 days the cells were growing rapidly. When the concentration of dGuo was held constant (0.3 mM) and the concentration of 8-AGuo varied, it was observed that, as the concentration of 8-AGuo was lowered, the overall effect on L1210 cell growth was decreased markedly.

Since the lower concentrations of deoxyguanosine and 8-AGuo inhibited cell growth for only 2 or 3 days, experiments were carried out to determine if supplementation of the culture medium with either dGuo or 8-AGuo would extend the inhibition of cell growth. As seen in Fig. 2, when 25 μM 8-AGuo was added on day-zero and again on day-2 in combination with dGuo (0.2 mM), the inhibition of L1210 cell growth was extended to the full 5 days of the experiment. This was equivalent to having added 100 μM 8-AGuo on day-zero. On the other hand, the addition of dGuo at day-zero and day-2 in combination with 8-AGuo did not provide inhibition over significantly longer periods of time. This was surprising in view of the fact that the degradation of 8-aminoguanosine would lead to the for-

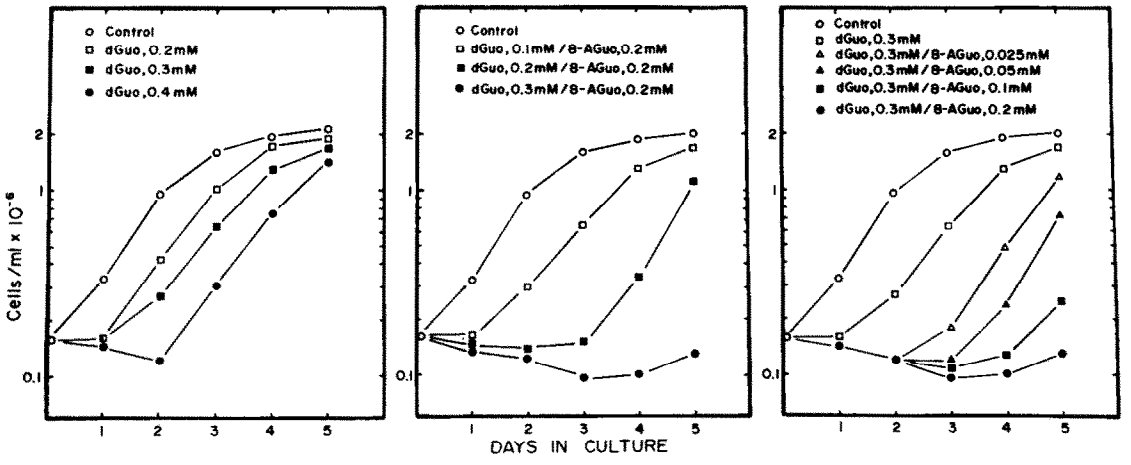

Fig. 1. Effect of deoxyguanosine and 8-aminoguanosine on L1210 cell growth. Deoxyguanosine (dGuo) and 8-aminoguanosine (8-AGuo) were added at the concentrations and in the combinations indicated at day-zero (the day the culture flasks were seeded with 0.15×10^6 L1210 cells/ml). All cultures were set up in triplicate.

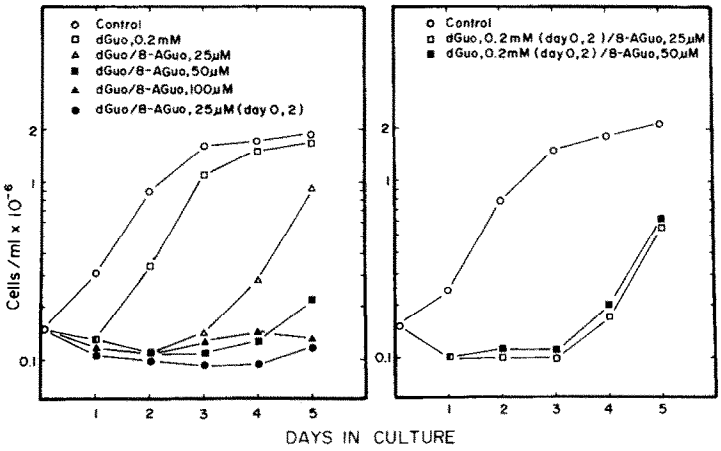

Fig. 2. Effect of supplementation with deoxyguanosine or 8-aminoguanosine on L1210 cell growth. After deoxyguanosine (dGuo) and 8-aminoguanosine (8-AGuo) were added on day-zero, 8-aminoguanosine (●) was again added to the cultures on day-2, or additional deoxyguanosine (□ or ■) was added to the cultures at day-2. All cultures were set up in triplicate.

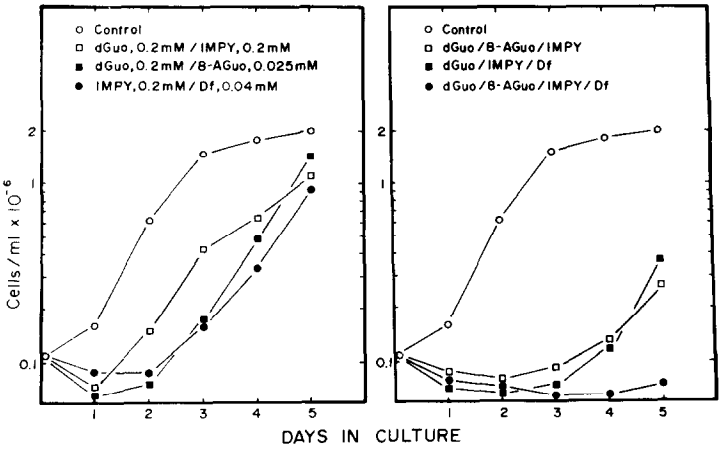

Fig. 3. Effect of combinations of deoxyguanosine and IMPY on L1210 cell growth. Deoxyguanosine (dGuo), 8-aminoguanosine (8-AGuo), IMPY and Desferal (Df) were added at the concentrations and in the combinations indicated at day-zero. The concentrations of drugs in the right-hand panel are the same as indicated in the left-hand panel. All cultures were set up in triplicate.

mation of 8-aminoguanine, an even more effective inhibitor of purine nucleoside phosphorylase [16]. This may be related to the fact that 8-aminoguanosine inhibits the uptake of deoxyguanosine (J. G. Cory, unpublished data).

The combinations of dGuo and IMPY were studied for their effects on the growth of L1210 cells in culture. As seen in Fig. 3, the two-drug combinations consisting of dGuo/IMPY, dGuo/8-AGuo and IMPY/Desferal had essentially the same effects on L1210 cell growth at the concentrations used. Drug combinations which included agents directed at both subunits of ribonucleotide reductase could be utilized to effectively prevent tumor cell growth if modulators were added. Three-drug combinations which included deoxyguanosine/8-aminoguanosine/IMPY or deoxyguanosine/IMPY/Desferal provided better inhibition of tumor cell growth. Desferal has been shown to potentiate the inhibition of ribonucleotide reductase and tumor cell growth by hydroxyurea and IMPY [8, 17, 18]. The four-drug combination consisting of deoxyguanosine plus 8-aminoguanosine (to inhibit purine nucleoside phosphorylase activity) and IMPY plus Desferal was most effective. This combination of agents synergistically inhibited L1210 cell growth over the entire period studied. Notably, the concentrations of deoxyguanosine and 8-aminoguanosine could be reduced significantly in the presence of IMPY/Desferal.

These results indicate that combination chemotherapy directed at a multi-component enzyme can provide synergistic inhibition of cell growth. It is an approach which has now been demonstrated for the ribonucleotide reductase sites utilizing deoxyadenosine/EHNA [8] and deoxyguanosine/8-aminoguanosine targeted for the effector-binding subunit. As more effective inhibitors of purine nucleoside phosphorylase are developed, it may be possible to decrease further the concentrations of these agents that are required.

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