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EFFECT OF HYDROXYUREA ON 2',3'-DIDEOXYCYTIDINE ACTIVATION

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The effect of hydroxyurea (HU) on cell growth, cell cycle progression, and the intracellular accumulation of 2',3'-dideoxycytidine (ddC) and ddCTP formation was examined. Twenty-four-hour exposure of H9 cells (human lymphocytic cell line) to 500 μM HU significantly increased the cells in the G1 phase and inhibited cell growth to 33.3$\pm$10.6% of control. The total intracellular ddC and ddCTP accumulation in HU-treated cells increased to 232.3% and 310% of control, respectively.

2',3'-Dideoxycytidine (ddC), a dC analog, is the most potent inhibitor of HIV replication (1). However, the drug itself is inactive and exerts its anti-HIV effect through its 5'-triphosphate metabolite, ddCTP, which inhibits viral DNA synthesis by competing with dCTP for viral reverse transcriptase and entering the DNA molecule causing chain termination (2-4). As a consequence, any strategy which increases ddCTP formation may enhance the efficacy of ddC chemotherapy and reduce dose requirement and associated drug toxicity (5).

Recently, we have shown that acivicin, a glutamine antagonist, significantly increased ddCTP synthesis from ddC (6). We have also shown that HU, an inhibitor of the enzyme ribonucleoside diphosphate reductase (7), increased the formation of ara-CTP from ara-C (8,9). Since ddC and ara-C follow the same metabolic path to form their triphosphates, the results of a study investigating the effect of HU on ddCTP formation are presented in this communication.

MATERIALS AND METHODS

Chemicals and Reagents: [5,6-^{3}H]-dideoxycytidine (5 Ci/mmole) was obtained from Moravek Biochemicals Inc. (Brea, CA). HU was purchased from Sigma Chemical Co. (St. Louis, MO).

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Abbreviations: ara-C, arabinosylcytosine; dC , 2'-deoxycytidine; ddC, 2',3'-dideoxycytidine; ddCMP, ddCDP and ddCTP, 5'-mono-,di-,and tri-phosphates of ddC; HPLC, high-performance liquid chromatography; HIV,human immunodeficiency virus; HU, hydroxyurea; PBS, phosphate-buffered saline.

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Cytotoxicity and ddC Metabolism: H9 cells (human lymphocytic cell line) were grown in RPMI 1640 medium containing heat inactivated calf-serum, streptomycin, and penicillin as described earlier (10). Triplicate cell suspensions (0.5-1.0 x10^6 cells/mL) were incubated with 500 μM HU for 15 min prior to the addition of 25 μM [^{3}H]-ddC (1μCi/mL). Incubation was continued for 24 hr at 37^{0}C. Following incubation aliquots were used for flow cytometry, cell count, and the determination of intracellular ddC nucleotide levels as described earlier (6). In brief, following a 24 hr incubation, the cells were collected by centrifugation, washed twice with PBS, and extracted overnight at -20^{0}C with 1 mL of 65% (v/v) methanol. Aliquots of the extracts were counted directly or analyzed by HPLC.

HPLC Analysis: To identify ddC metabolites, aliquots of the methanol extracts were dried in a Speed-Vac and reconstituted in 0.1 mL of HPLC water. Fifty microliter samples were injected into an HPLC (Bio-Rad) equipped with a Partisil-10-SAX column (4.5 X 250 mm), equilibrated and developed for 5 min at a flow rate of 1.5 mL/min with solution A (0.06 M $NH_4H_2PO_4$ containing 10% acetonitrile, v/v), followed by a linear gradient of 80% of solution B (0.75 M $NH_4H_2PO_4$ containing 10% acetonitrile) over the next 20 min and maintained at 80% for 5 min. The eluate was monitored at 254 nm and 1.5 mL samples were collected and counted for radioactivity.

Data Analysis: The data was analyzed by the Student's t-test. The values of $p<0.05$ were considered significant.

RESULTS

The effect of HU and ddC on cell cycle distribution is shown in Figure 1. In ddC treated cells, the percentage of cells in the S and G2+M phases was slightly increased (about 8%) and

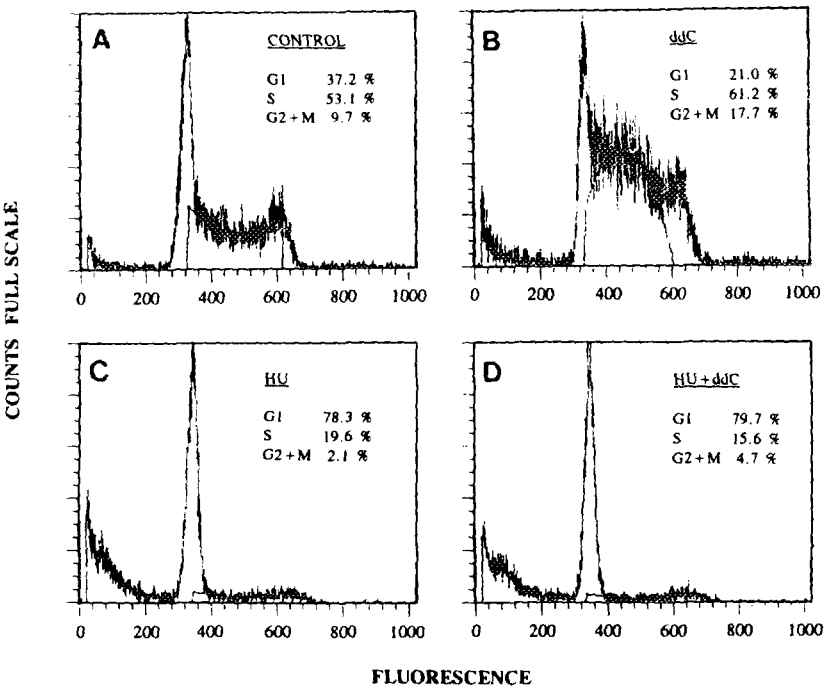

Figure 1. Histograms of DNA distribution in H9 cells. Following 24 hr incubation with ddC (25 μM), HU (500 μM) or combination of ddC and HU, the cells were analyzed by flow cytometery.

in G1 phase decreased (16%) (Figure 1A,B). HU, on the other hand, significantly increased the G1 phase (about 41%) and decreased S and G2+M phases (Fig. 1C). ddC failed to reverse the HU effect on the cell cycle (Fig. 1D).

Twenty four hour exposure of H9 cells to 25μM ddC, 500 μM HU, and their combinations inhibited their growth to 76.7$\pm$10.6%, 33.3$\pm$10.6% and 46.8$\pm$3.9% of control ($p<0.05$), respectively (Figure 2). ddC may have slightly reversed the HU cytotoxicity but the difference was insignificant.

The values of total ddC incorporation of 215$\pm$18 picomoles/10^6 cells in the control were consistent with the values reported earlier (6). HU (500 μM) significantly increased ddC incorporation to 505$\pm$116 picomoles/10^6 cells, i.e., 232.3$\pm$61.9% of control (Figure 3). Most of the incorporated ddC (>98%) was found in the methanol "soluble" fractions. The "insoluble" fractions which had <2% of radioactivity were not analyzed further. Chromatography of the "soluble" fractions on HPLC resolved into 3 major peaks of radioactivity, ddC+ddCMP, ddCDP and ddCTP (Figure 4). The increases in ddC+ddCMP, ddCDP and ddCTP in HU treated cells were 2.5, 3.3, and 3.1 fold, respectively (Table 1). From the mean ddCTP values of 82.4$\pm$12.1

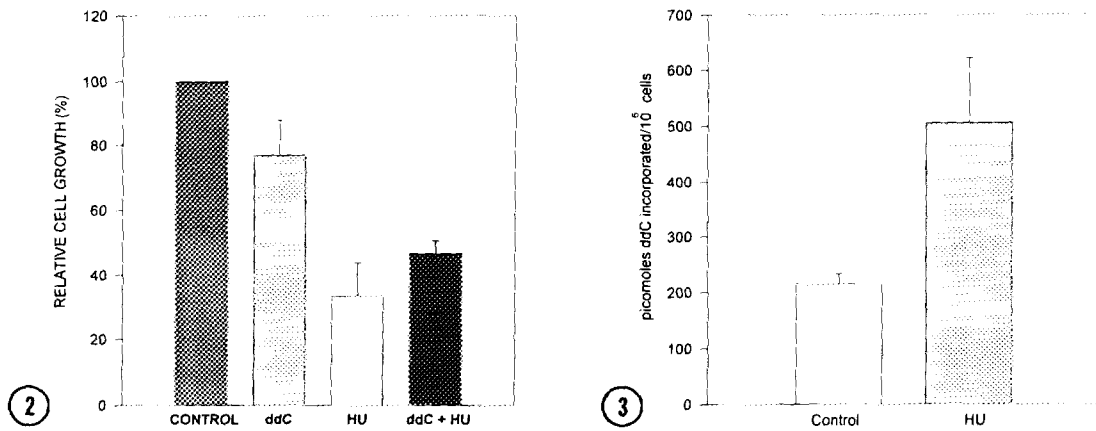

Figure 2. Effect of hydroxyurea and dideoxycytidine on the growth of H9 cells in the absence and presence of 25 μM ddC. Triplicate 2.0 mL logarithmically growing cell suspensions (0.5-1.0 x 10^6cell/mL) in RPMI 1640 growth medium were with PBS (control), HU (500 μM), ddC (25 μM) and HU plus ddC. Following incubation for 24 hr at 37^{0}C the trypan blue excluding cells were counted by hemocytometer. The values are mean $\pm$ s.d. of 3-4 experiments.

Figure 3. Effect of hydroxyurea on dideoxycytidine accumulation in H9 cells. The cells were treated as described in Figure 2 and aliquots were extracted with 65% methanol as described in "Materials and Methods." Twenty-five microliter samples were counted for radioactivity. The amounts of ddC were calculated from the radioactivity. The values are the mean $\pm$ s.d. of 3 experiments.

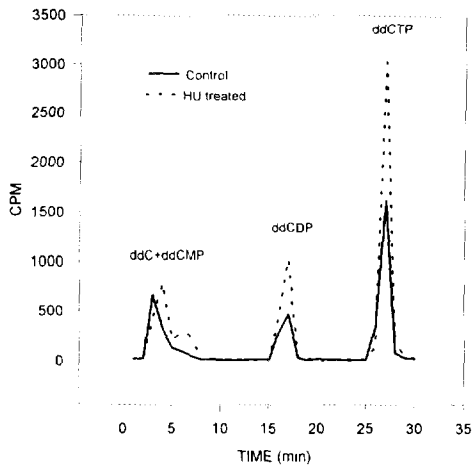

Figure 4. HPLC profile of the methanol "soluble" fractions from control and hydroxyurea treated cells. The analysis was performed as described in the "Materials and Methods" section.

and 255.6±37.1 picomoles/10^6 cells and the value of water content of 1.16 μL/10^6 H9 cells (10), the ddCTP concentrations were calculated to be 71 and 220 μM in the control and HU treated cells, respectively.

DISCUSSION

The results of these studies confirm the hypothesis that HU increases the total accumulation and concentration of ddCTP in H9 cells. The increases were 2.3 and 3.1 fold in the ddC accumulation and ddCTP concentrations.

A number of mechanisms might explain HU induced increase in ddCTP concentrations including: (i) increased ddC transport into the cell and its phosphorylation to ddCTP; (ii) a decrease in ddC degradation by cytidine deaminase; (iii) decreased incorporation of ddCTP into

Table 1: Distribution of dideoxycytidine in the nucleotide pool of H9 cells

	ddC+ddCMP	ddCDP	ddCTP
	picomoles/10^6 H9 cells (mean ± s.d)		
Control (n=4)	65.3 ± 12.4	53.2 ± 24.5	70.9 ± 25.1
HU (500 μM) (n=3)	153.3 ± 18.28*	149.3 ± 18.5*	255.6 ± 37.1*

*Significantly different from control; $p < 0.05$.

DNA; and (iv) decreased breakdown of ddCTP. However, some of these mechanisms may easily be ruled out. For example, dideoxynucleosides enter cells through simple diffusion. Secondly, deamination of ddC by cytidine deaminase is very poor (3,9,11); additionally, the activity of cytidine deaminase is higher in cells in G1 phase (12), the state induced by HU. Therefore, the effect of HU on ddC transport and deamination seems to be an unlikely mechanism.

A decrease in ddCTP incorporation into DNA due to the accumulation of cells in G1 phase by HU (a non DNA synthesizing phase) may have resulted in the observed increase in cellular ddCTP concentration. However, a very low recovery (<2%) of radioactivity in methanol "insoluble" fractions indicated that the contribution from ddCTP incorporation into DNA was negligible. And the ddCTP concentrations (70-220 μM) achieved in control and HU treated cells were much higher than the relative extent of ddCTP incorporated into DNA. Therefore, the decreased incorporation of ddCTP as a mechanism of HU induced increase in ddCTP levels is unlikely. Whether hydroxyurea affected the degradation of ddCTP has not been tested.

The first and the rate limiting step in the sequential phosphorylation of ddC to ddCTP (ddC $\rightarrow$ ddCMP $\rightarrow$ ddCDP $\rightarrow$ ddCTP) is catalyzed by the enzyme dCKinase (13). The activity of this enzyme is cell cycle dependent (low in G1 phase and elevated in S phase) and inhibited by dCTP (12,13) and increased by dTTP through a complex regulation of the enzyme (14). Our earlier studies have shown that whereas HU had no effect on dCKinase activity in a cell free system, it altered the kinetics of the enzyme in intact L1210 cells (9). Since the enzyme is inhibited by dCTP, the concentration of this inhibitor might have been depleted due to the inhibition of ribonucleoside diphosphate reductase by HU (7). However, the reports on HU induced changes in cellular dCTP concentrations are conflicting, some showing a decrease and others an increase (15,16). Another possibility is HU-induced changes in dTTP levels which have been reported to be consistently increased by HU (15). dTTP is an efficient phosphate donor for dCKinase and has been shown to reverse the inhibitory effect of dCTP in a complex manner (14,17). It is possible that HU affected the cellular dTTP levels and increased ddC phosphorylation.

Further work is underway to delineate the mechanism of HU induced ddC metabolism. However, irrespective of the mechanism, the present findings underscore the importance of the interaction of HU in ddC metabolism and activation of this important antiviral agent.

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