Cell-Kill Kinetics of Several S-Phase-specific Drugs

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SUMMARY

The drugs [1-β-D-arabinofuranosylcytosine (ara-C), hydroxyurea (HU), 5-hydroxy-2-formylpyridine thiosemicarbazone (5-HP), and camptothecin sodium salt (camptothecin)] considered in this paper markedly inhibit DNA synthesis and are maximally cytotoxic to cells in S phase. In these studies, high-specific-activity thymidine-3H (HSA-TdR-3H) was used as a control compound which killed cells in S but which did not affect the progression of cells into S. The cell-kill kinetics indicated that ara-C, HU, and 5-HP, unlike camptothecin, blocked the L1210 cells from progressing into S in the presence of the drug. We found that L1210 cells that were blocked in G1 by HU started moving into S immediately after the drug was removed. Therefore, the time interval between two doses of HU that gave maximal cell kill was the same as that for HSA-TdR-3H. However, L1210 cells exposed to ara-C and 5-HP took about 2 hr to recover from the effect of the drug and then progress into S. Therefore, the time interval (between two doses of either ara-C or 5-HP) that gave maximal cell kill was longer than that needed for HSA-TdR-3H. Camptothecin did not block L1210 cells from moving into S and, therefore, the cell-kill kinetics with camptothecin were the same as those with HSA-TdR-3H.

The time for maximal recovery of DNA synthesis by L1210 cells after a single exposure to the drugs was determined. The time for maximal recovery of DNA synthesis correlated well with the interval required for maximal cell kill.

INTRODUCTION

A phase-specific agent will be maximally effective only if it allows cycling cells to enter the cytotoxic phase. Thus, an S-phase-specific agent that blocks the progression of G1 cells into S will kill only those cells that are in S at the time the drug is added. Sinclair (14, 15) showed that HU has such an effect; namely, the cells in S are killed, while the non-S-phase cells accumulate at the G1-S boundary. When HU is removed, the accumulated cells proceed synchronously through the cell cycle. ara-C has been shown to have similar effects (7). The protective effect of such self-limiting compounds can be overcome if multiple doses are scheduled so that the cells can recover from drug effects and enter S during the drug-free interval.

The purpose of our studies was to determine the cell-kill kinetics of ara-C, HU, HSA-TdR-3H, 5-HP, and camptothecin with the use of L1210 cells. These drugs were chosen because all of them markedly inhibit DNA synthesis (3), all are maximally cytotoxic to cells in S phase (3), and all (except HSA-TdR-3H) are being studied for efficacy in cancer chemotherapy. L1210 cells were chosen because they are widely used by Drug Research and Development, National Cancer Institute, in screening for antileukemic drugs. Also, the use of L1210 cells enabled us to correlate our in vitro data to data obtained in vivo. We also determined the optimal time interval between 2 doses of the drug required to give maximal cell kill in vitro. The optimal time interval between 2 doses of the drug that gave the maximal increase in life-span of L1210 leukemic mice is reported in a companion paper (13).

MATERIALS AND METHODS

L1210 Cell Methods. L1210 cells were maintained in culture in RPMI Medium 1634 (Grand Island Biological Company, Grand Island, N. Y.) supplemented with fetal calf serum (5%), NaHCO3 (0.075%, w/v), penicillin (0.1 mg/ml), and streptomycin (0.05 mg/ml). The medium was obtained as a freeze-dried powder (without antibiotics, NaHCO3, or serum). The tube dilution assay used to determine cytotoxicity of agents has been described by Buskirk (6). Cell survival after drug treatment was determined by the cloning method of Himmelfarb et al. (8). Drug-treated cells were centrifuged at 500 X g, and the cells were washed with medium and resuspended at 10^6 cells/ml. The cells were serially diluted in medium, and the final dilution was made in RPMI Medium 1634 containing 20% serum and 0.15% agar. To prepare this soft-agar medium, we autoclaved 1.5% Noble agar (Difco Laboratories, Inc., Detroit, Mich.) in 0.9% NaCl solution. The agar was cooled to 45°, and an aliquot was added to RPMI Medium 1634 containing 20% serum at 45°, to give 0.15% agar. Five ml of the soft agar medium containing suspended cells were dispensed into test tubes and incubated in a humid atmosphere of 8% CO2 and 92% air at 37°. Each sample was pipetted into 6 tubes for a determination of percentage of cell survival. Colonies were visually counted after 8 to 10 days of incubation. The plating efficiency for L1210 cells was about 50%. In the calculation of percentage survivals, the control (no drug treatment) samples were normalized to 100% survival. The coefficient of variation in determining cell survival was about 15%, the coefficient of variation being the standard deviation expressed as a percentage of the mean.
**DON Cell Methods.** DON cells, a Chinese hamster fibroblast line (American Type Culture Collection No. CCL16), were grown at 37°C in McCoy Medium 5A supplemented with lactalbumin hydrolysate (0.8 g/liter) and fetal calf serum (200 ml/liter). Details of culture maintenance, cell synchronization, and determination of cell survival have been described (3). The plating efficiencies were about 50% for synchronous cells and 70 to 80% for asynchronous cells. In the calculation of survival percentages, the control samples were normalized to 100% survival. The coefficient of variation in determining cell survival was about 15%. Almost all cell-survival experiments were repeated, and the results were found to be reproducible.

**Drug Samples.** ara-C (Cytosar; cytarabine) was supplied by the Upjohn Company, Kalamazoo, Mich. Camptothecin and 5-HP were obtained from Drug Research and Development, National Cancer Institute, Bethesda, Md. The method for preparing solutions of these drugs has been described (3). HSA-TdR-3H, 6.7 Ci/m mole, was obtained from New England Nuclear, Boston, Mass.

**Determination of Macromolecular Synthesis in L1210 Cells.** Two-ml samples of L1210 cells (about 5 X 10^5/ml in RPMI Medium 1634) were preincubated at 37°C for 1 hr with various concentrations of the agents studied. Radioactive precursors were then added at the following concentrations: TdR-3H, 2.56 μCi/0.385 μg/ml; uridine-UR-3H, 1.3 μCi/0.13 μg/ml; DL-valine-14C, 0.26 μCi/0.73 μg/ml. After 60 min of incubation, radioactive precursor incorporation was stopped by the addition of excess unlabeled precursor. The amount of precursor incorporation was determined as described previously (3), except that Whatman No. 1 paper discs were used.

**DNA Synthesis Recovery Experiments.** L1210 cells were subcultured 16 hr prior to use and had reached a cell concentration of approximately 3 X 10^6/ml at the beginning of the experiment. Fifty-ml portions of cells were added to conditioned culture bottles (i.e., bottles in which L1210 cells had been growing). Agents were added at zero time (Chart 6). Prior to the addition of the agents, a 2.0-ml aliquot of cells was removed and added to 0.1 ml TdR-3H (final concentration, 2 μCi/0.55 μg/ml). After 60 min of incubation, the incorporation of TdR-3H into DNA was determined as described above. This value was taken as the zero-time value equal to 100% of the control DNA synthesis. Sixty min after the addition of agents, DNA synthesis was determined in another 2.0-ml aliquot. At 62 min, 40 ml of the cell suspension were removed and centrifuged (700 X g for 2 min at room temperature). Medium that contained drug was removed by aspiration. The cells were washed twice with 15 ml of warm (37°C), conditioned medium (i.e., obtained by centrifugation of parallel non-drug-treated cultures) and finally were resuspended in 40 ml conditioned medium, and the incubation was continued. The washing procedure took approximately 20 to 30 min. Cell counts were obtained at various intervals, subsequently. TdR-3H incorporation was determined as described above at various times after the cells were washed free of drug.

**RESULTS**

**Cytotoxicity and Stability of the Drugs.** The concentrations needed for 50 and 90% inhibition of L1210 cell growth after 3-day exposure to drug are shown in Table 1. The order of cytotoxicity of the drugs was as follows: camptothecin > ara-C > 5-HP > HU. Only camptothecin was somewhat unstable in medium at 37°C.

**Inhibition of Macromolecule Synthesis in L1210 Cells.** The effect of different doses of drug on the inhibition of DNA synthesis is shown in Chart 1. These data were used to select drug concentrations that gave maximal inhibition of DNA synthesis. The inhibition of DNA, RNA, and protein synthesis at the selected drug concentrations is shown in Table 2. The drugs were used at these concentrations in experiments described below.

**L1210 Cell Survival on Short-Term Exposure to Drug.** Chart 2 shows the percentage survival of L1210 cells exposed to different levels of drug for 1 hr. In all cases, the slope of the dose-survival curves changed at a certain concentration, indicating that the population consisted of cells with different sensitivities to the drug. With HU, ara-C, and 5-HP, the dose-survival curve decreased to a constant saturation value, indicating that the remaining population was insensitive to the drug (4). With camptothecin, increasing concentrations of the drug killed increasing proportions of the less sensitive population. These agents are maximally cytotoxic to cells in the S phase (3). Therefore, if we correlate the percentage cell survival to the percentage of cells in S (which is about 65% for L1210 cells in culture), we find that (a) ara-C, 5-HP, and HU killed cells probably only in the S phase, i.e., about 65% of the cells, and (b) high levels of camptothecin killed cells in phases

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Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (μCi)</th>
<th>Growth inhibition&lt;sup&gt;a&lt;/sup&gt; (μmole/ml)</th>
<th>Drug left in medium&lt;sup&gt;b&lt;/sup&gt; (%) at 2.5 hr</th>
<th>Drug left in medium&lt;sup&gt;b&lt;/sup&gt; (%) at 4 hr</th>
<th>Drug left in medium&lt;sup&gt;b&lt;/sup&gt; (%) at 7 hr</th>
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<td>Camptothecin</td>
<td>0.008</td>
<td>1.8 X 10&lt;sup&gt;-5&lt;/sup&gt;</td>
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<td>75</td>
<td>60</td>
</tr>
<tr>
<td>ara-C</td>
<td>0.007</td>
<td>2.8 X 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>98</td>
<td>93</td>
<td>80</td>
</tr>
<tr>
<td>5-HP</td>
<td>0.56</td>
<td>2.9 X 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>107</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>HU</td>
<td>2.3</td>
<td>3 X 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>81</td>
<td>80</td>
<td>82</td>
</tr>
</tbody>
</table>

<sup>a</sup> L1210 cells were incubated with the drugs for three days, following which growth inhibition of drug-treated cells was compared to the controls (6).

<sup>b</sup> Drug was incubated in RPMI Medium 1634 for different periods at 37°C. The amount of drug left in the medium was determined by tube-dilution assay (6). Similar results were obtained when L1210 cells in RPMI Medium 1634 were used instead of medium alone.

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Chart 1. Inhibition of DNA synthesis by L1210 cells with different levels of drugs. The protocol has been described in "Materials and Methods." Cells were incubated with drug and TdR-3H for 1 hr.

Table 2

Inhibition of macromolecule synthesis by L1210 cells

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (µg/ml)</th>
<th>Inhibition of synthesis (%)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>DNA</td>
</tr>
<tr>
<td>ara-C</td>
<td>5</td>
<td>97</td>
</tr>
<tr>
<td>HU</td>
<td>30</td>
<td>92</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>10</td>
<td>82</td>
</tr>
<tr>
<td>5-HP</td>
<td>24</td>
<td>96</td>
</tr>
</tbody>
</table>

other than S, since more than 65% of the cells were killed.

L1210 Cell Survival on Long-Term Exposure to Drug. Chart 3 shows the percentage survival of L1210 cells after exposure to drugs for up to 12 hr. The drug concentrations used were such that an increase in concentration did not result in markedly increased cell kill (Chart 2). The results obtained are compared with the survival of cells exposed to HSA-TdR-3H (6.7 Ci/mmole, 10 µCi/ml). TdR-3H kills S-phase cells subsequent to its incorporation into DNA (5) and presumably does not affect the passage of cells into S at the concentration used. The results indicate the following. (a) During the 1st hr, HSA-TdR-3H, HU, ara-C, and 5-HP killed about the same percentage (60 to 70%) of cells (probably the cells in S). In the 1st hr, camptothecin killed more than just S-phase cells. (b) Between 1 hr and 4 to 5 hr, the rate of cell kill with ara-C, 5-HP, and HU was much lower than that with HSA-TdR-3H. Thus, at the end of 4 hr, only 2.3% of the cells exposed to HSA-TdR-3H survived, compared with 20 to 30% survival of the cells exposed to HU, ara-C, and 5-HP. After 4 hr, there was

Chart 2. Dose response of L1210 cells to drugs. L1210 cells were exposed to different concentrations of drugs for 1 hr, after which the drug was removed by centrifugation and by washing of the cells. Cell survival was determined as described in "Materials and Methods."

Chart 3. Survival of L1210 cells after exposure to drugs for different periods. After different periods of exposure, aliquots of cells were removed and cell survival was determined. TdR-3H was added to give 20 µCi/ml (6.7 Ci/µmole). ara-C, HU, 5-HP, and camptothecin were present at 5, 300, 30, and 1 µg/ml, respectively. The results shown are the average of 3 to 4 experiments. The mean deviations for ara-C and TdR-3H are shown as examples of mean deviations in these experiments.
a marked increase in the rate of cell kill with cultures exposed to ara-C, 5-HP, and HU. (c) The behavior of camptothecin was different from that of the 3 drugs mentioned above. The rate of cell kill with camptothecin paralleled that seen with HSA-TdR-3H.

These experiments suggested that, unlike camptothecin, HU, ara-C, and 5-HP delay the entry of non-S-phase cells into the drug-sensitive S phase. A similar plateau in the survival curve of mammalian cells exposed to HU and ara-C was previously reported (1, 7, 10) and was explained as indicating a reduced rate of flow of G1 cells into the drug-sensitive S phase. In order to determine directly the effect of HU, 5-HP, ara-C, and camptothecin on the rate of entry of cells into S, we performed the following experiments. Since a synchronized population of L1210 cells was not available, synchronized DON cells were used.

Studies with Synchronized DON Cells. These experiments were based on the following facts: (a) Mitotic DON cells stay in G1 for about 2 hr, and (b) all of the drugs studied are most cytotoxic to cells in the S phase (3).

In the 1st series, the drug was added to the mitotic cells immediately after the cells were planted, and the drug was left in contact with the cells for the total period of the experiment. If the drug inhibited the entry of cells into S, then the cells would not be killed even after exposure to the drug for 4 to 5 hr, i.e., by the time that the cells would normally be in S.

In the 2nd series, the cells were exposed to the drug for 1 hr at different times after they were planted. When the drug was added to cells in G1, a small proportion of the cells were killed, partly due to contamination of G1 cells with S cells. However, if the drug was added to cells after they entered S, then marked cell kill was seen with these S-phase-specific agents.

Therefore, a difference in cell survival between mitotic cells exposed to the drug continuously (1st series) and S-phase cells exposed to the drug (2nd series) would indicate that the drug affects the rate of entry of cells into S phase.

The results shown in Chart 4 indicate that ara-C, 5-HP, and HU (in contrast to HSA-TdR-3H and camptothecin) inhibit the entry of cells into S. HSA-TdR-3H was used as a control compound which kills cells only in the S phase and which does not affect the passage of cells into S. HSA-TdR-3H killed about 18% of the cells during the 1st hr, indicating partial contamination of the mitotic cells with S-phase cells. By 2 hr, cells had started entering S and, by 4 hr, about 90% of the cells were in S and were killed. The percentage cell survival was the same when cells were continuously exposed to HSA-TdR-3H from zero time as when HSA-TdR-3H was added for 1-hr periods at different times after planting. Such a result would be expected for an agent that did not affect the entry of G1 cells into S. Camptothecin behaved in a manner similar to that of HSA-TdR-3H, indicating that this drug did not affect the rate of entry of G1 cells into S.

With 5-HP, there was little cell kill during the 1st 2 hr after mitotic cells were planted (i.e., of the cells in G1). When the drug was added for 1 hr to S-phase cells (i.e., 4 hr after planting), 88% of the cells were killed. However, when the drug was added to cells at zero hr and were left in contact with the cells for 5 hr, only 30% of the cells were killed. This suggests that 5-HP inhibited the entry of cells into S. Similar results were obtained with HU and ara-C, and similar results were obtained if, instead of adding the drugs to mitotic cells, we added the drugs shortly before the cells were due to enter S. This indicated that the drugs were not acting by blocking mitosis or lengthening the G1 phase.

Effect on L1210 Cells of Intermittent Exposure to Drug. Previous experiments showed that, in the presence of ara-C, HU, and 5-HP, the progression of G1 cells (both DON and L1210) into S was inhibited. Thus, the non-S-phase cells were protected from the cytotoxic effects of the drug. This protective effect can be overcome by giving the drug in multiple doses, with the intervals designed to give enough time for the non-S-phase cells to recover and enter S during the drug-free period. The experiments reported here (Charts 5 and 6) show the cell kill obtained after 2 exposures (each 1 hr in duration), with the exposures separated by different time intervals.

When the agents were given in 2 divided doses, the time...
Chart 5. Survival of L1210 cells when the cells were exposed to HSA-TdR-3H continuously (—) or to 2 divided doses added at different intervals (····). ····, HSA-TdR-3H (10 µCi/ml, 6.7 Ci/mmmole) was added to L1210 cells in suspension. Aliquots were taken at different times, and thymidine (10 µg/ml) was added to dilute the HSA-TdR-3H. Cells were centrifuged, washed, diluted in medium, and planted to determine cell survival. ····, cells were exposed to HSA-TdR-3H (10 µCi/ml, 6.7 Ci/mmmole) for 1 hr, following which thymidine (10 µg/ml) was added. The cells were centrifuged, washed, and replanted at approximately the same cell concentration in fresh medium. The cells were exposed to HSA-TdR-3H (10 µCi/ml, 6.7 Ci/mmmole) for a 2nd 1-hr period at various times after the 1st dose. Then the cells were washed and cell survival was determined. Time scale, time between the beginning of the initial drug exposure to the middle of the 2nd drug exposure.

intervals for maximal cell kill were HSA-TdR-3H, 5.5 and 14.5 hr; HU, 5.5 hr; camptothecin, 5.5 and 15.5 hr; ara-C, 7.5 and 18.5 hr; and 5-HP, 7.5 and 17.5 hr. The time intervals for minimal cell kill were: HSA-TdR-3H, 10.5 hr; HU, 10.5 hr; camptothecin, 9.5 hr; ara-C, 12.5 hr; and 5-HP, 13.5 hr. With all of the drugs (except camptothecin), 2 exposures of 1-hr duration, separated by the optimal time interval, killed more cells than did continuous exposure to the drug for 6 hr. Thus, 6 hr of continuous exposure to ara-C, HU, or 5-HP killed about 90% of the cells, compared to greater than 99% cell kill achieved with 2 doses, given at the optimal interval (compare Charts 3 and 6).

Chart 6 also shows the time taken for L1210 cells to recover their capacity to synthesize DNA after a single dose of each drug. Immediately after 1 dose of each drug, DNA synthesis is almost completely inhibited. After removal of the drug, TdR-3H is incorporated into (a) the cells in S at the time of exposure to drug and (b) the cells entering S from G1. Therefore, the time for maximal recovery of DNA synthesis indicates (in a very approximate manner) the time taken for non-S-phase cells to enter S after removal of the drug. This should be the optimal time for a 2nd dose of an S-phase-specific drug to obtain maximal cell kill.

The interval between two 1-hr exposures to the agent, for optimal recovery of DNA synthetic capacity, corresponded well with the interval for maximal cell kill. With camptothecin and 5-HP, DNA synthetic capacity reached levels greater than those observed with control samples.

DISCUSSION

Previous studies with synchronous DON cells showed that only ara-C, HU, and 5-HP were specifically cytotoxic to cells in S, while camptothecin also killed cells in G1 and G2 at high concentrations (3, 12). The dose-survival curves (Chart 1) with L1210 cells indicated that ara-C, HU, and 5-HP were specifically cytotoxic to cells in a certain phase (probably S).
of the cell cycle, while camptothecin killed cells in other phases.

We compared the time course of cell kill with ara-C, HU, and 5-HP to that of HSA-TdR-3H (Chart 2). HSA-TdR-3H was chosen as a reference S-phase-specific agent that does not block the progression of non-S-phase cells into S. The following discussion justifies this choice.

The cell-cycle times and percentage of cells in each phase for L1210 cells in culture were found to be as follows: G1, 1.6 hr and 17% of total cells; S, 8.2 hr and 69%; G2, 1.9 hr and 11%; and M, 0.5 hr and 3%. If we assume that all S-phase cells are killed by a short exposure to HSA-TdR-3H and that HSA-TdR-3H does not affect the progression of G1 cells into S, then we can calculate the cell kill that can be expected after increasing periods of exposure to TdR-3H. The expected percentage of cell kill would be approximately equal to 100 \times \left(\frac{T_S + \text{length of exposure time}}{T_C}\right), where T_S is the S time period and T_C is the cell-cycle time. If we also take into account the increase in cell number that occurs as the viable G2 and M cells divide, then the percentage cell kill after 1, 2, 3, and 4 hr of exposure to TdR-3H would be 75, 80.5, 88.2, and 98.2, respectively. These calculated values correspond closely with the observed values (Chart 3). This justifies our use of HSA-TdR-3H as an S-phase-specific agent that does not block the progression of G1 cells into S.

We found that the continuous presence of ara-C, HU, and 5-HP delayed the entry of non-S-phase cells into the drug-sensitive S phase (Charts 3 and 4). Other workers using L-cells also observed a rapid initial cell kill by HU (1) and by ara-C (7), followed by a plateau phase with a slow rate of cell kill. Similar results were also obtained by Kim et al. (10) when they exposed HeLa cells to HU. The plateau in the survival curves of cells exposed to HU and ara-C resulted from a reduced rate of flow of G1 cells into the drug-sensitive S phase (1, 7, 10). Sinclair (14, 15) also suggested that cells not in S phase at the time of HU addition are prevented from entering S, due to a lack of DNA synthesis, and are thereby protected from the lethal effects of the drug. Bertalanffy and Lindsay-Gibson (2) showed that 4 daily injections of 12.5 mg of ara-C per kg produced partial synchronization of B16 melanoma and Ehrlich ascites tumor cells in vivo. Such synchrony would be expected if the cells were blocked at the end of G1 in the cell cycle and were released in unison when the drug was metabolized and removed from the body fluids. Also, Tobey and Crissman (19) showed that G1-arrested cells released from the isoleucine-mediated arrest start entering S 4 hr after isoleucine addition. However, similar cells treated for 10 hr with HU (10^{-3} M) or ara-C (5 \mu g/ml) contained the amount of DNA expected of G1 cells. These and other studies (18) led these authors to conclude that, although neither ara-C nor HU completely prevented cells from initiating DNA synthesis, they grossly decreased the progression of cells from G1 to S.

Skipper et al. (16) found that ara-C (15 to 20 mg/kg), given at 3-hr intervals on Days 2, 6, 10, and 14, cured a substantial number of leukemic mice. This is apparently contradictory to the protective effect (by blocking non-S-phase cells from entering S) of ara-C discussed above. However, this can be explained by the decrease in ara-C level in the plasma, with a half-life of about 15 min (16), to about 1 \mu g/ml 1 hr after injection. Karon and Shirakawa (9) have shown that the transit rate of cells from G1 to S or from G2 to G1 was not affected when DON cells were exposed to 1 \mu g ara-C per ml for 1 hr. Therefore, it is possible that L1210 cells exposed to about 1 \mu g ara-C per ml for 1 hr would be able to progress into S and would be killed.

In contrast to the self-limiting effect of ara-C, HU, and 5-HP, camptothecin did not affect the progression of G1 cells into S. Tobey (17) showed that initiation of genome replication could occur in the presence of this drug.

Maximal cell kill was obtained when 2 doses of TdR-3H were given 5 or 14 hr apart, and minimal cell kill resulted when the doses were 10 hr apart. If we assume that the L1210 cell population is homogeneous with respect to cell-cycle times and the lengths of various phases, the expected interval for maximal and minimal cell kill can be calculated. Since the S cells are killed by the 1st dose, we need only consider the position of the non-S (G1 + M + G2) phase cells at different times after the 1st dose. Since G1 + M + G2 is 4 hr (see the cell-cycle times given previously), all of the G1 + M + G2 cells will be in S between 4 and 8.2 hr after the 1st dose and will be killed by the 2nd dose. Between 8.2 and 12.2 hr after the 1st dose, the G1 + M + G2 cells will be leaving S and will be insensitive to the 2nd dose. Between 12.2 and 16.2 hr, all of the G1 + M + G2 cells will be entering S and will be sensitive to the 2nd dose. These calculated values compare quite well with the observed values for HSA-TdR-3H, if one considers that the cells are not completely homogeneous with respect to cell-cycle times and with the time taken to wash off HSA-TdR-3H. Intervals for maximal and minimal cell kill with HU and camptothecin were similar to those with TdR-3H.

Thus, although cells are blocked from progressing into S in the presence of HU, once the HU is removed, G1 + M + G2 cells immediately start entering S. However, with ara-C and 5-HP, G1 + M + G2 cells are slowed down in their entry into S, even after the drug is removed. Therefore, our results indicate that, after removal of the 1st dose of ara-C and 5-HP, it takes 2 hr longer for G1 + M + G2 cells to enter S than after removal of HU, camptothecin, or HSA-TdR-3H. Karon and Shirakawa (9) found that after exposure of DON cells to 10 \mu g ara-C per ml for 1 hr, the subsequent transit rate of G1 cells into S, and of S cells to G2 was decreased, although the progression of G2 cells to mitosis was not affected. A block in the progression of S cells to G2 would affect the cell kill with the 2nd dose, since the cells in S would already be killed by the 1st dose.

These results with cell-kill experiments were supported by the measurements of the time course of recovery of DNA synthesis in cells exposed to the drugs. These measurements indicate (although in a very approximate manner) the time taken for non-S-phase cells to enter S after the drug is removed. The amount of TdR-3H incorporated following the removal of the drug consists of (a) incorporation by the cells (in S) "injured" by the S-phase-specific drugs and (b) the incorporation by cells entering S. Regarding the 1st point, Sinclair (15) showed that S-phase cells, destined to die after exposure to HU, resynthesized DNA after the drug was removed. The rate of recovery of DNA synthesis by the injured cells may vary depending on their position in S and the period of recovery. Also, the rate of DNA synthesis in the cells entering S will vary depending on the position of these cells in
S at different times after drug is removed. However, in spite of these factors, there is a reasonable correlation between the optimal dose interval and the interval for maximal recovery of DNA synthesis.

We have shown that 2 doses of an S-phase-specific drug given at proper intervals are more cytotoxic toward L1210 cells in culture than the same dose maintained continuously for approximately 8 hr (compare Charts 3 and 6). This is particularly true if the cells are blocked from entering the sensitive phase in the presence of the drug. However, it is important to note that, during continuous exposure to these latter agents, the rate of cell kill increases rapidly after the 1st 4 to 6 hr of exposure. Indeed, it is possible to achieve greater cell kill with long exposures (i.e., more than 10 to 12 hr) than it is with intermittent exposures. These results taken together would suggest that in the clinical treatment of leukemia with ara-C, for example, properly spaced intermittent treatment would be superior to short-term infusions but would be inferior to long-term infusions. The clinical data bearing on this point is quite ambiguous (11). No one regimen was shown to be clearly superior. It is, of course, impossible to directly extrapolate “therapeutic regimens” in cell culture experiments to the clinical situation. The effectiveness and, in particular, the therapeutic ‘index of any dosage regimen in vivo will depend on a number of additional factors, not the least of these factors, there is a reasonable correlation between the optimal dose interval and the interval for maximal recovery of DNA synthesis.

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