Schedule Optimization of Hydroxyurea and 1-β-D-Arabinofuranosylcytosine in Sarcoma 180 in Vitro


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ABSTRACT

The lethal and sublethal effects of 1-β-D-arabinofuranosylcytosine (ara-C) were studied in Sarcoma 180 in vitro in relation to drug concentration and drug exposure duration. Drug lethality was found to be dependent on both drug concentration and drug exposure duration. These studies confirmed that radioautographic grain counts reflect relative rates of DNA synthesis in individual cells. When the radioautographic data and flow cytometry data are considered together, it is apparent that ara-C blocked cells at multiple points in the cell cycle. However, blockade was not complete; low levels of DNA synthesis persisted, even during prolonged drug exposure. The time course of recovery of rapid DNA synthesis following exposure to ara-C was delayed in comparison with that observed following exposure to hydroxyurea. The optimal time interval between two split 1-hr exposures to ara-C or two split 1-hr exposures to hydroxyurea coincided with the time that cells recruited into rapid cycle by the first dose were passing through mid-S and synthesizing DNA at high rates. These findings suggest that the concept of graded DNA synthesis rate-dependent drug lethality may be preferable to the all-or-none concept of S-phase specificity. The optimum interval between split dosages of ara-C was not related to intracellular levels of 1-β-D-arabinofuranosylcytosine 5'-triphosphate as a function of time after exposure to the first dose. Overall, the dependence of ara-C lethality on drug concentration, drug exposure duration, and the rate of cellular DNA synthesis are most consistent with the premise that the amount of ara-CTP incorporated into DNA is a major determinant of drug lethality.

INTRODUCTION

Studies of the kinetic characteristics of Sarcoma 180 cells grown in vitro have been reported previously (18, 19). In these studies, it was found that radioautographic labeling intensity varied systematically during the cell cycle. Nuclear grain counts were lowest early and late in the cell cycle and maximal in mid-S. Studies with HU demonstrated that drug-induced changes in radioautographic labeling intensity reflected changes in the rates of DNA synthesis in individual cells as determined independently by serial flow cytometry measurements (7).

In this paper, we report on the postperturbation effects of ara-C in Sarcoma 180 cells in vitro. As in our earlier studies with HU, ara-C-induced changes in radioautographic labeling intensity were also reflected in serial changes in the DNA histogram. However, the time course of drug-induced changes was more protracted after brief exposure to ara-C than after brief exposure to HU at drug concentrations of comparable lethality. The patterns of kinetic perturbation following HU or ara-C were distinctive and highly reproducible.

The kinetic effects of ara-C and their relationship to the effectiveness of split-dose schedules have been studied in a variety of experimental tumor systems (1, 2, 6, 23). An optimum interval between ara-C doses has been found which generally ranges from 6 to 16 hr. However, the relation between this optimum interval and known pharmacological effects of ara-C is not entirely clear. In this paper, we report our findings on the optimal intervals between 2 doses of HU and between 2 doses of ara-C in Sarcoma 180 cells in vitro. Our findings in this cell system suggest that there is a relationship between the lethality of HU or ara-C and the rate of DNA synthesis in individual cells at the time of drug exposure. These findings, taken together with the dependence of ara-C lethality on drug concentration and drug exposure duration, support the premise that incorporation of ara-C into DNA is a major mechanism underlying its lethal effects.

MATERIALS AND METHODS

All studies were carried out in Sarcoma 180 (Foley strain CCRFI1, supplied by American Type Culture Collection, Rockville, Md.) grown in vitro in Earle’s Medium 199 (Flow Laboratories, Rockville, Md.) supplemented with 10% fetal bovine serum, glutamine (2 mmol/ml), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cultures were grown at 37° in a 5% CO2 atmosphere. Cells were grown in monolayer in 250-ml plastic tissue culture flasks (Falcon Plastics, Oxnard, Calif.) containing 10 ml of medium, at an initial concentration of 1 x 10⁶ cells/ml. ara-C and HU were supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute.

Cloning Studies. Two-day-old log-phase cultures were incubated with ara-C at final concentrations ranging from 0.5 to 500 μM for either 1, 2, 4, or 12 hr. At intervals, cells were removed by trypsinization and cloned in soft agar using methods described previously (7). Cell viability was defined by the ability to form colonies in the cell-cloning studies. Total cell counts and viable cell counts were obtained at each time point, as described previously (7). All cloning experiments involving single drug exposures were carried out in triplicate, and the data reported represent the log means for normalized data from the 3 experiments.

In the schedule optimization studies, the first drug was removed after 1 hr by rinsing each flask 3 times with Hanks’ balanced salt solution. Cultures were then refed with Medium 199. At intervals, a second dose of drug was added for 1 hr followed by 3 rinses with Hanks’ balanced salt solution. Aliquots of cells were then taken for counting and for cloning studies. At each interval, aliquots of cells exposed to the first drug only were rinsed, counted, cloned, and also

1 Excerpted in Cell Kinetics (20).
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3 The abbreviations used are: HU, hydroxyurea; ara-C, 1-β-D-arabinofuranosylcytosine; dThd, thymidine; ara-CTP, 1-β-D-arabinofuranosylcytosine 5'-triphosphate; PCA, perchloric acid.

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fixed for flow cytometry. Each schedule optimization cloning study was repeated at least 5 times.

Radioautographic Studies. Two-day-old log-phase cell cultures were incubated with ara-C at a final concentration of 50 μM for either 2 or 12 hr and were harvested at intervals. Cultures were exposed to [³H]dThd (specific activity, 6.7 Ci/mmol; New England Nuclear, Boston, Mass.) at a final concentration of 0.05 µCi/ml during the 30 min prior to sample collection. Radioautograms were prepared and analyzed as described previously (19). The emulsion exposure duration was 4 days. Nuclear grain counts, corrected for local background, were obtained for 250 to 500 cells at each time point. The average background grain count was less than 1 grain/cell.

[³H]dThd Incorporation Studies. Cells were exposed to [³H]dThd (6.7 Ci/mmol) at final concentrations of 2.0 µCi/ml for 30 min. Cells were washed 3 times with 10 ml Hanks’ balanced salt solution, harvested by trypsinization, and counted. A 1-ml aliquot was centrifuged at 400 x g for 15 min and resuspended in 10 ml 10% trichloroacetic acid. Cell precipitates were collected on a 0.45-μm Millipore filter with extensive trichloroacetic acid washing and placed in counting vials to which 17 ml of Aquasol (New England Nuclear) were added for scintillation counting. [³H]dThd counts were normalized to dpm/10⁶ cells. Each study was performed at least twice, and the reported results represent the means of the replicate data at each time point.

DNA Content Distribution Studies. Cells exposed to 50 μM ara-C or 5 mM HU for 2 hr were harvested at intervals. Cells from 2 replicate flasks were pooled, fixed in 70% ethanol, and stained with mithramycin at a final concentration of 100 μg/ml and a final cell concentration of 1 x 10⁵ cells/ml in each sample. Nuclear fluorescence was measured with a Los Alamos cell sorter. Data were recorded, stored, and analyzed on a DEC 11/40 computer system, using software developed at the Los Alamos Scientific Laboratory, Los Alamos, N. M. All DNA content distributions were obtained at constant photomultiplier voltage and amplifier gain settings. At least 10,000 cells were measured in each sample. All DNA content distributions were normalized with respect to total cells analyzed per sample for display purposes. The fraction of cells in S was computed by the method of Jett (11).

ara-CTP Analysis. Two-day-old log-phase cells were incubated with ara-C, 50 or 5 μM, for 1 to 4 hr. Drug-containing medium was removed at the end of exposure period and flasks were washed twice with Hanks’ balanced salt solution and refed with drug-free medium. At various times after drug washout, cells were then detached by trypsinization and centrifuged for 4 min at 400 x g at 4°; the supernatant was removed, and the cell pellet was extracted with ice-cold 0.8 M PCA and centrifuged at 1600 x g for 15 min at 4°.

PCA-soluble extracts were neutralized with 1 M KOH, and the resulting KCIO₃ was removed by centrifugation. Nucleotides were separated on a Whatman Partisil PXS 10-25 SAX column using a Waters high-pressure liquid chromatographic system, pump Model 6000A and injector Model U6K (Waters Associates, Inc., Milford, Mass.). Aliquots (100 μl) of PCA-soluble extracts were applied to the column and were eluted isocratically with a 0.4 to 0.1 M KH₂PO₄ gradient (pH 3.1) at a flow rate of 1 ml/min. Under these conditions, ara-CTP was separated from other known ara-C metabolites, including 1-β-D-arabinofuranosyluridine 5'-triphosphate. One-min fractions were collected, and absorbance was recorded at 254 nm. The amount of ara-CTP was determined by counting the radioactivity of the samples eluting with a nonradiolabeled ara-CTP standard.

RESULTS

ara-C Cell-Cloning Studies. The pretreatment viable cell fraction (viable cells/total cells, and viability as defined in “Materials and Methods”) ranged from 0.6 to 0.8. The effects of ara-C concentration and drug exposure duration on cell survival over time are shown in Chart 1. In general, the surviving viable cell fraction (expressed as viable cells at time t divided by viable cells at Time zero) decreased with increasing drug concentration. For example, when cells were exposed to ara-C at a concentration of 0.5 μM, the surviving viable cell fraction

Chart 1. Effects of ara-C on total cells and cell viability as a function of time in relation to drug concentration and drug exposure duration. A₁, relative total cell number as a function of time in control cultures (□) and in those exposed to 0.5 μM (△), 5 μM (●), and 50 μM (○) ara-C for 1 hr. B₁, 2-hr exposure data; C₁, 4-hr exposure data; D₁, 12-hr exposure data. Corresponding surviving viable cell fraction as determined by colony-forming ability, are shown in A₂, B₂, C₂, and D₂. Bars, S.E. For discussion, see text.
was not significantly different from control viable cells over the course of the observation period regardless of the duration of drug exposure (Charts 1, A to D). When cells were exposed to ara-C concentrations of 50 or 500 μM, the surviving viable cell fraction decreased and remained below control values over the entire course of the observation period regardless of the duration of drug exposure. For ara-C concentrations of 50 μM or greater, the differences in cell survival between drug-exposed and control viable cells were statistically significant (p < 0.05) at every time point.

Total cell counts were affected by increasing concentrations of ara-C but to a much lesser degree than viable cells (Chart 1, A to D). Even at the highest drug concentrations and the longest drug exposure times, there was little decrease in total cells with time in comparison with controls at Time zero. Comparisons of total and viable cells indicate that nonviable cells persisted intact throughout the period of observation.

In order to compare the relative importance of the effects of drug concentration and drug exposure duration on cell survival in quantitative terms, the time of termination of drug exposure was taken as a reference point for drug schedule comparison. The results are shown in Chart 2. At this reference point, significant cell killing (p < 0.05) was observed consistently only at drug concentrations of 50 μM or higher. When different drug schedules were compared at the same drug concentrations, the 12-hr schedule produced significantly lower surviving cell fractions than did drug schedules with shorter drug exposure times at drug concentrations of 50 μM or higher (p < 0.05).

From the results shown in Chart 2, there does not appear to be a simple relationship between cell survival and the product of drug concentration and drug exposure duration (C × T). Thus, for example, there was greater cell killing after 12 hr of exposure to 50 μM ara-C than after 4 hr of exposure to 500 μM ara-C, although C × T was less for the former schedule than for the latter (12 hr × 50 μM = 600 μM-hr versus 4 hr × 500 μM = 2000 μM-hr).

When Charts 1 and 2 are considered together, it is apparent that factors other than cell killing may contribute to the overall effect of a given drug schedule. For example, following a 1-hr exposure to a concentration of ara-C of 50 μM or higher, the surviving cell fraction remained depressed, and relative total cell number remained static for at least 12 hr after the termination of drug exposure. It is because of the absence of population regrowth during this 12-hr interval that the additive lethality of 2 separate 1-hr drug exposures within 12 hr of one another can approach that of a single 12-hr exposure at the same drug concentration under optimum conditions (see below).

Radioautographic Studies. Cells exposed to ara-C at a concentration of 50 μM for 2 hr were pulse labeled with [3H]-dThd at intervals and processed for radioautography. Multithresholded labeling indices as a function of time are shown in Chart 3A. The labeling indices at all but the lowest-grain count thresholds fell rapidly after drug exposure. It is because of the absence of population regrowth during this 12-hr interval that the additive lethality of 2 separate 1-hr drug exposures within 12 hr of one another can approach that of a single 12-hr exposure at the same drug concentration under optimum conditions (see below).

Labeling patterns during and after 12-hr exposure to ara-C at a concentration of 50 μM are shown in Chart 3B. There was a rapid reduction in [3H]-dThd incorporation which was sustained throughout the period of drug exposure and for up to 12 hr thereafter. The degree of inhibition of DNA synthesis was considerable, with less than 1% of the cells exhibiting more than 5 grains/cell by the end of the 12-hr exposure period. Since 25 to 30% of the cells remained viable by the cell-cloning assay (Chart 1D), it is clear that many cells that underwent a drug-induced reduction in the rate of DNA synthesis recovered without lasting effects. Thus, the prolonged inhibition of DNA synthesis per se was not sufficient to account for ara-C lethality. It is also apparent that the radioautographic labeling was reduced but not abolished. During the course of the 12-hr exposure period, 10 to 30% of the cells exhibited grain counts in the range of 2 to 5 grains/cell (Chart 3B). Since the background grain count was less than one grain/cell and since the data were corrected for local background grain counts in any case, it is likely that the low grain counts observed during the course of prolonged drug exposure reflected bona fide [3H]-dThd incorporation into DNA at low levels in the presence of drug.

Flow Cytometry Studies. The effects of exposure to ara-C at 5 and 50 μM for 2 hr on the DNA content distribution are shown in Chart 4. Following exposure to 5 μM ara-C, there was produced a comparable cell kill (7).
Chart 3. Multithresholded labeling indices as a function of time after exposure to ara-C, 50 μM. A, 2-hr ara-C exposure; B, 12-hr ara-C exposure. For discussion, see text.

Chart 4. Effects of a 2-hr exposure to ara-C on the DNA content distribution. A1, to A6, control cultures (change of medium at 2 hr). B1, to B6, exposure to ara-C, 5 μM, for 2 hr. C1, to C6, exposure to ara-C, 50 μM, for 2 hr. For discussion, see text.

no discernible change in the DNA content distribution at 2 and 4 hr (Chart 4, B1 and B2). At 6 hr, there was a broadening of the postmitotic peak, suggesting the entry of a cohort of recruited cells into early S, as well as a more diffuse increase in the fraction of cells throughout the S region. At 8 hr (Chart 4B4), the postmitotic peak broadened further, and the cohort of cells previously in early S appears to have progressed further into the S region of the DNA content distribution. Overall, the predominant change was that of a further diffuse increase in the fraction of cells throughout the S region of the DNA content distribution. By 12 hr (Chart 4B8), a narrow, distinct, and somewhat higher postmitotic peak was reestablished, indicating at least a transient increase in the rate of cell division by 12 hr. At 12 hr, the increased fraction of cells in S was more prominent in the late-S region of the DNA content distribution. At 16 hr (Chart 4B9), there was a residual increase in late S and G2M cell fraction, and by 24 hr (Chart 4B7) a normal DNA content distribution pattern was reestablished (compare Chart 4, A7 and B7).

After a 2-hr exposure to 50 μM ara-C, there were no discernible changes in the DNA content distribution during the first 8 hr (Chart 4, C1 to C4). Since the radioautographic labeling index and labeling intensity were depressed during this period (Chart 3A), the failure to deplete cells from the G2M region or accumulate cells in the postmitotic region of the DNA content distribution (Chart 4, C1 to C4) imply that drug-induced inhibition or retardation of cell cycle progression must have occurred not only in S but at multiple points in the cell cycle.

At 12 hr (Chart 4C5), there was a broad wave of cells in the early-S region of the DNA content distribution, which moved into the mid- and late-S region by 16 hr (Chart 4C6). By 24 hr, the majority of these cells were found in the late-S and G2M region (Chart 4C7). Thus, after exposure to a concentration of 50 μM ara-C, the wave of cells recruited into rapid cycle was more distinct than at 5 μM, and its onset was delayed up to 6 hr.

It is apparent from a comparison of Chart 3A and Chart 4, C1 to C7, that the recovery in cell-labeling intensity after exposure to a concentration of 50 μM ara-C occurred at about the time that the majority of recruited cells were passing through the mid-S region of the DNA content distribution. From Chart 4, C5 to C7, passage of the majority of recruited cells through mid-S can be estimated to have occurred at some time between 12 and 16 hr after drug exposure. From additional studies reported below (Chart 8, B5 to B6), this can be localized further to between 13 and 16 hr after drug exposure.

The temporal correlation of rapid passage of cells through the S region of the DNA content distribution with increased cell radioautographic labeling intensity during recovery indicates that postperturbation changes in cell-labeling intensity following exposure to ara-C reflected bona fide changes in the rate of DNA synthesis in individual cells.

During continuous exposure to ara-C for 12 hr, there were no discernible changes in the DNA content distribution at drug
concentrations of 5 or 50 μM (Chart 5). Following drug removal, a wave of recruited cells was apparent in late S at 24 hr at the lower drug concentration but not at the higher one. Recovery patterns after a 12-hr exposure to ara-C were studied separately in greater detail (Chart 6). The onset of recovery following a 12-hr exposure to 5 μM ara-C occurred 4 to 6 hr after drug removal, and the wave of recruited cells passed through the mid-S region at approximately 8 hr after drug removal. After a 12-hr exposure to 50 μM ara-C, the onset of recruitment was delayed until 8 to 10 hr after drug removal, and the recruited cell cohort passed through the mid-S region 12 to 16 hr after drug removal. The intervals between drug removal and the recovery in DNA synthesis were comparable to those seen after 2 hr of drug exposure at both drug concentrations, respectively (Chart 4). Thus, the duration of ara-C-induced suppression of DNA synthesis is drug concentration dependent but, unlike ara-C lethality, does not appear to involve cumulative effects that are dependent on drug exposure duration.

When Chart 5, C1 to C5, and Chart 3B are considered together, it is apparent that prolonged suppression of [3H]dThd uptake is correlated with the absence of discernible changes in the DNA content of individual cells not only throughout the S region but in the postmitotic and premitotic-mitotic regions as well. As with brief drug exposures, this would imply an inhibition of cell progression at multiple points in the cell cycle.

Two-Dose Schedule Optimization Studies. Since the lethality of 5 mM HU (7) and 50 μM ara-C (Charts 1 and 2) following short-term exposures were comparable while the time course of recovery in the rate of DNA synthesis differed for the 2 drugs, these 2 drug concentrations were chosen to study the relation between drug lethality and the rate of DNA synthesis in individual cells. At various time intervals after a 1-hr exposure to 5 mM HU, cells were reexposed to 5 mM HU for 1 hr and cloned in soft agar. Cells exposed only to HU initially were also cloned at these time intervals, and the effect of the second dose of drug was expressed as the fraction of cells surviving the second dose at a given time divided by the fraction of cells surviving the first dose at that time. The results are shown in Chart 7A. Each data point represents the mean of 6 experiments. Differences among the time points were evaluated in pairs by Student's t test. It is apparent from Chart 7A that, following brief initial exposure to HU, the lethality of a second dose of drug was greatest when the second dose was given at an interval of 8 or 10 hr. The differences between surviving cell fractions at either of these 2 scheduling intervals for the second dose and the surviving cell fractions at intervals other than 8 or 10 hr for the second dose were statistically significant (p < 0.005). A statistically significant decrease in the effectiveness of the second dose of HU was observed when the interval between doses was increased from 10 to 13 hr or longer (p < 0.005).

Following an initial 1-hr exposure to 50 μM ara-C, a second dose of ara-C was found to be maximally effective between 5 and 13 hr (Chart 7B). Data points represent the means of 5 experiments. The differences between surviving cell fractions at either of these 2 scheduling intervals for the second dose and the surviving cell fractions at intervals other than 8 or 10 hr for the second dose were statistically significant (p < 0.005). Unlike the 2-dose HU schedule, a second dose of ara-C was as effective at 13 hr as it was at 10 hr (Chart 7B); a statistically significant decrease in effectiveness of the second dose of ara-C was observed when the interval between doses was increased from 13 to 18 hr (p < 0.05).

During the course of these studies, aliquots of cells that were...
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**ara-CTP Studies.** In order to determine if there is a relationship between intracellular concentrations of ara-CTP following exposure to an initial dose of ara-C and schedule-dependent susceptibility to a second dose of ara-C, intracellular ara-CTP levels were determined during and after exposure to 50 µM ara-C for 1 hr. The results are shown in Chart 10 (●). Intracellular ara-CTP levels during and after exposure to 5 µM ara-C for 1 hr, a nonlethal dose (Chart 1), are also shown for comparison (○). During exposure to 50 µM ara-C, intracellular ara-CTP levels rose rapidly, peaking at 110 pmol/10^6 cells at 1 hr. With the removal of ara-C from the medium, intracellular ara-CTP fell nearly 10-fold to 17 pmol/10^6 cells at 2 hr.

Parallel changes were observed during and after exposure to 5 µM ara-C. Intracellular ara-CTP concentrations were approximately one-tenth those observed with 50 µM ara-C. At 1 hr, ara-CTP levels peaked at 14 pmol/10^6 cells, falling to 1.8 pmol/10^6 cells at 2 hr and 0.1 pmol/10^6 cells at 6 hr.

The data suggest that intracellular ara-CTP levels following initial exposure to ara-C per se cannot account for the ineffec-

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**Chart 7.** Effect of a second dose of drug on cell survival as a function of time after administration of the first dose. A, HU, 5 mM, given first for 1 hr, followed by a second dose of HU, 5 mM, given at various intervals after the first dose. Surviving cell fraction reflects the effect of the second dose, having been normalized with respect to time-matched single-dose controls in order to eliminate confounding effects of population regrowth on later time points. B, ara-C, 50 µM, given first for 1 hr followed by a second dose of ara-C, 50 µM, given at various intervals after the first dose. Surviving cell fraction reflects the effect of the second dose only, having been normalized with respect to time-matched single-dose controls. For discussion, see text.

Exposed only to the initial dose of drug were fixed in ethanol at intervals for flow cytometry. Following HU exposure, the recruited cohort was found to traverse the mid-S region of the DNA content distribution between 8 and 10 hr after drug exposure (Chart 8, A4 to A6). The computed fraction of cells in S remained essentially unchanged initially and peaked at 10 hr (Chart 8A4). Following exposure to ara-C, the recruited cell cohort traversed the mid-S region between 13 and 16 hr (Chart 8, B5 to B6), and the fraction of cells in S peaked at about 13 hr (Chart 8B5).

The behavior of the radioautographic labeling index at low-grain count thresholds was similar to that of the fraction of cells in S as determined by flow cytometry. The labeling index was essentially unchanged for several hr during and after HU exposure and rose at 8 hr (Chart 9A3). No distinct trend in the labeling index could be discerned during the first 12 hr after exposure to ara-C. However, it is clear that the labeling index was not elevated at 8 to 10 hr and that the rise in labeling index above 0.9 occurred after 12 hr (Chart 9B3).

The temporal patterns of [3H]dTthd uptake as determined by liquid scintillation counting following HU or ara-C are shown in Chart 9, A2 and B2, respectively. Each point represents the mean of 2 experiments. In both instances, a rapid fall in [3H]dTthd uptake was observed following exposure to drug, and a rise was observed during recovery. However, the pattern of increased [3H]dTthd uptake was too diffuse to identify the optimum interval between drug doses. Following exposure to HU, peak [3H]dTthd uptake by the liquid scintillation counting method occurred at 12 hr (Chart 9A3). At this time, radioautographic labeling intensity was declining (7), and the recruited cell cohort had already traversed mid-S (Chart 8, A4 to A6). By 13 hr, there was a clear diminution in the effectiveness of the second dose of drug (Chart 7A). Following ara-C exposure, there was a diffuse increase in [3H]dTthd uptake as determined by liquid scintillation counting between 8 and 18 hr, with peak [3H]dTthd uptake at 18 hr (Chart 9B4). However, by 18 hr, the recruited cell cohort had already traversed mid-S (Chart 8B4), and the optimum time interval between doses had been passed (Chart 7B).
Schedule Optimization of HU or ara-C

Radioautographic Labeling Intensity and the Rate of DNA Synthesis. The present studies demonstrate that changes in radioautographic labeling intensity following exposure to ara-C are correlated with changes in the rate of increase of DNA content of individual cells. These findings suggest that radioautographic labeling intensity reflects the rate of DNA synthesis in individual cells. These findings confirm the results of our earlier studies with HU (7) and are similar to the findings reported for ara-C by Aglietta and Colly (1).

DNA Synthesis Rate-dependent Drug Lethality versus Cycle Stage Specificity. The lethal effects of ara-C were shown to be dependent not only on drug concentration and drug exposure duration but also on the rate of DNA synthesis in individual cells at the time of drug exposure. Following exposure to ara-C or HU, radioautographic labeling intensity and 

With prolongation of exposure to ara-C, intracellular ara-CTP levels continued to rise but at a lower rate. After 4 hr of exposure to ara-C, 50 μM, the intracellular ara-CTP concentration was 296 pmol/10^6 cells (data not shown). Again, intracellular ara-CTP levels fell rapidly after drug removal.

DISCUSSION

Radioautographic Labeling Intensity and the Rate of DNA Synthesis. The present studies demonstrate that changes in radioautographic labeling intensity following exposure to ara-C are correlated with changes in the rate of DNA content of individual cells. These findings suggest that radioautographic labeling intensity reflects the rate of DNA synthesis in individual cells. These findings confirm the results of our earlier studies with HU (7) and are similar to the findings reported for ara-C by Aglietta and Colly (1).

DNA Synthesis Rate-dependent Drug Lethality versus Cycle Stage Specificity. The lethal effects of ara-C were shown to be dependent not only on drug concentration and drug exposure duration but also on the rate of DNA synthesis in individual cells at the time of drug exposure. Following exposure to ara-C or HU, radioautographic labeling intensity and [3H]Thd uptake by liquid scintillation counting fell immediately, but neither the labeling index nor the fraction of labeled cells by flow cytometry changed appreciably. That is, in the immediate postperturbation period, the population consisted largely of lightly labeled cells (Chart 3A; Ref. 7) that were relatively resistant to a second exposure to either drug (Chart 7). Maximum responsiveness to a second dose of HU occurred at 8 to 10 hr, when the heavily labeled fraction of cells in S had increased to its maximum value during the recovery period (Chart 7A; Ref. 7). The lowest surviving cell fraction following a second dose of ara-C was also observed when the rate of DNA synthesis was maximal during recovery, i.e., at 13 hr (Charts 3A and 7B). In the case of ara-C, the pattern of schedule-dependent lethality was more diffuse than in the case of HU, and the differences in surviving fraction following second doses of ara-C given at intervals of 5 to 13 hr were not significantly different from one another. Nonetheless, it is clear that the time course of recovery in DNA synthesis following an initial dose of drug differed for ara-C and HU and that for each drug the period of maximum susceptibility to a second dose of drug bracketed the period of recovery of DNA synthesis for that drug.

Aglietta and Colly (1) also studied the optimum interval between 2 doses of ara-C in relation to cell-labeling index and cell-labeling intensity in a rat leukemia in vivo. They also found that the maximum effect of the second dose occurred between 5 and 15 hr, at a time when the majority of recruited cells were passing through mid-S and had high radioautographic grain counts. Bhuyan et al. (2) studied the schedule dependence of 2 doses of HU or ara-C in mouse L1210 cells in vitro and found that the greatest decrease in cell survival occurred at a time when cells recruited by the first dose exhibited maximal incorporation of [3H]Thd. They also found that the optimum interval between 2 doses of ara-C was several hr longer than the optimum interval between 2 doses of HU.

From a functional therapeutic standpoint, then, the concept of DNA synthesis rate-dependent lethality would appear to be...
of greater practical usefulness than that of S-phase specificity. The term S-phase specificity distinguishes between labeled and unlabeled cells but does not distinguish between lightly labeled and heavily labeled S cells. On the other hand, the concept of DNA synthesis rate-dependent lethality would assign greater drug susceptibility following brief exposure to the more heavily labeled cells and less to the lightly labeled cells, in keeping with our findings and those of others (1, 2).

**Monitoring of Drug Perturbation Kinetics.** Radioautographic labeling intensity and DNA content distribution patterns obtained by flow cytometry were useful in monitoring the kinetics of drug perturbation and in identifying the optimal treatment intervals. The fraction of cells in S obtained by flow cytometry and the radioautographic labeling index were not as helpful. Although the S fraction and radioautographic labeling index peaked at the time that the recruited cohort passed through mid-S, these parameters did not reflect the early fall in the rate of DNA synthesis and the concomitant relative resistance to treatment with a second dose of drug. In contrast, \(^{3}H\)dThd uptake by the liquid scintillation counting method reflected the early fall in the rate of DNA synthesis but was not as useful in pinpointing the optimum treatment interval during recovery.

Bhuyan et al. (2) found that the time of maximum \(^{3}H\)dThd incorporation by liquid scintillation counting did correspond with the time of maximum effectiveness of a second dose of ara-C in L1210 leukemia in vivo. This may be attributable to the relatively high degree of kinetic homogeneity of L1210 cells in tissue culture.

Straus and Moran (23) carried out studies similar to ours using ara-C and HU in L1210 leukemia and in Sarcoma 180 in vivo. As in our studies, both drugs inhibited DNA synthesis rapidly, and recovery from a single dose occurred more quickly after HU than after ara-C. Cell-labeling intensity fell immediately after drug exposure but, in contrast to our findings, pronounced decreases in the labeling index were also observed. This difference may be due to radioautographic technical factors. When most cells are lightly labeled, small differences either in the degree of drug-induced inhibition of \(^{3}H\)dThd incorporation or in the efficiency of radioautographic detection of residual low rates of DNA synthesis may determine whether these lightly labeled cells will fall above or below the radioautographic background threshold.

**Kinetics of Drug Perturbation: Inhibition of Cell Cycle Progression.** ara-C was the first drug shown by Skipper et al. (22) to produce schedule-dependent cures in large inocula of murine L1210 leukemia. Skipper et al. postulated that cells not in S phase proceed unimpeded through the remainder of the cell cycle and are killed as they proceed through S. This has served as the rationale for prolonged infusions and frequent multiple-dose schedules in the treatment of adult acute leukemia in humans (8).

Our studies do not support this view. Drug-induced inhibition of DNA synthesis occurred rapidly and this inhibition persisted for over 10 hr, even after brief drug exposure (Chart 3). This precluded the normal entry of cells into S or their normal transit through S during and after drug exposure. If the inhibition of cell progression were limited to cells that had been actively synthesizing DNA, one would expect to observe a progressive accumulation of the remaining cells in the postmitotic or early-S region of the DNA content distribution during prolonged drug exposures. No such accumulations were seen during the experimental observation period (Chart 5), indicating that there was inhibition of cell progression in G1 and G2 as well. Others have also found an inhibitory effect of ara-C on the cell cycle progression of cells in G1, (2, 24, 25) and G2 (24).

The persistence of some light labeling above background even during prolonged drug exposure (Chart 3) indicates that the inhibition of DNA synthesis was not complete. Yataganas et al. (24) have demonstrated the persistence of residual low rates of DNA synthesis even after 48 hr of continuous exposure in vitro to concentrations of ara-C as high as 1000 \(\mu M\).

**Mechanisms of ara-C Lethality.** Although our studies and those of others (1, 2) suggest that the rate of DNA synthesis in individual cells is a major determinant of ara-C lethality following brief drug exposure, it is clear that other factors are also important. For example, there is progressive cell killing during prolonged exposure to ara-C at high drug concentrations (Chart 1D2) in the face of persistent low rates of DNA synthesis (Chart 3B). The interrelationships among these factors might best be understood in terms of the basic mechanisms that underly ara-C lethality.

The pharmacological mechanisms of action of ara-C have been reviewed by Cohen (5). ara-C is rapidly phosphorylated to its mono-, di-, and triphosphates. The last of these, ara-CTP, is considered to be the active form of the drug. The intracellular retention of high levels of ara-CTP in human leukemic cells over time has been correlated with clinical response to therapy (16). ara-CTP inhibits mammalian DNA polymerases (9, 10, 17) and is also incorporated into DNA (4, 10, 12, 14, 15, 21).

Lincoln et al. (13) proposed a cell kinetic-pharmacokinetic model for ara-C in which ara-CTP incorporation into DNA is considered the primary lethal process. One might expect that ara-CTP incorporation into DNA would depend on at least 3 factors, namely, the rate of DNA synthesis in individual cells, the intracellular concentration of ara-CTP, and the duration of intracellular ara-CTP availability. Thus, according to the model of Lincoln et al. (13) persistent inhibition of DNA polymerase would afford relative protection from the lethal effects of subsequent drug exposures because of the reduced rate of incorporation of ara-CTP into DNA. However, one might also expect that the relative protection afforded by reduced rates of DNA synthesis might be overcome in the presence of persistently high intracellular levels of ara-CTP.

Our studies are consistent with this formulation. In our studies and in those of others (3), intracellular levels of ara-CTP alone did not account for patterns of schedule-dependent drug susceptibility or drug resistance following brief drug exposure. However, experimental conditions which favored ara-CTP incorporation into DNA, i.e., increased rates of DNA synthesis during recovery, were associated with increased cell killing. Conversely, depressed rates of DNA synthesis immediately following pulse drug exposure were associated with a decrease in the lethality of a second dose. The progressive cell killing during prolonged exposure to high drug concentrations (Chart 1D2) can be explained by continued ara-CTP incorporation at low rates of DNA synthesis in the presence of increased intracellular ara-CTP concentrations for prolonged periods. Indeed, it has been shown recently both in L1210 leukemia (12) and in human myeloblasts in vitro (14) that there is a highly significant correlation between the amount of ara-CTP incorporated into DNA and clonogenic cell survival over a broad range of drug...
schedules and $C \times T$ values. These studies indicated a progressive increase in ara-CTP incorporation into DNA with increasing duration of drug exposure (12, 14).

Drug Schedule Optimization: Steady-State Kinetic Characteristics versus Perturbation Effects. It has generally been assumed that the effects of ara-C given by prolonged infusion and those obtained in frequent multiple-dose regimens are similar and that both are related primarily to pretreatment population kinetic characteristics (8, 21). The present study and those of others suggest that drug-specific perturbation effects and cell population-dependent perturbation and recovery kinetics may be of greater importance than pretreatment population kinetics in determining the effectiveness of multidose drug schedules and prolonged infusions.

In the present study, the time to recovery after brief exposure to a single dose of ara-C was found to be drug concentration dependent (Chart 4). Similarly, Edelson et al. (6) found that the optimal interval between 2 doses of ara-C in L1210 leukemia in vivo was dependent on the magnitude of the initial dose. The present study and those of others (2, 23) have shown that the time to recovery after a dose of ara-C differed from that of HU at drug concentrations of comparable lethality. Corresponding differences in optimal 2-dose schedules were observed both in the present study and in that of Bhuyan et al. (2).

It is also apparent from the present study that the kinetic and pharmacokinetic circumstances that are associated with a split-dose schedule may not be comparable to those associated with prolonged drug exposures. It would appear that there is a complex interplay among a variety of kinetic and pharmacokinetic factors which, under certain circumstances, might produce similar therapeutic results. Thus, for example, a 1-hr exposure to 50 $\mu$M ara-C reduced the surviving cell fraction to 0.7 (Chart 2). Intracellular ara-CTP concentrations fell to low levels within 1 hr of drug removal (Chart 10), but the inhibition of DNA synthesis persisted for several hr longer (Charts 3, 4, and 8). When a second dose of ara-C was given 5 to 13 hr after the first at a time when the recruited cell cohort was synthesizing DNA at increased rates, an additional 50% reduction of the surviving cell fraction was observed (Chart 7B). Since there was no population regrowth during the interval between doses (Chart 1), the net surviving cell fraction for the 2-dose schedule was 0.7 $\times$ 0.5, or 0.35. In contrast, with increasing drug exposure duration, intracellular levels of ara-CTP rose progressively, while the rate of DNA synthesis fell rapidly and remained depressed during prolonged drug exposure (Chart 3). In our studies, a 12-hr exposure to 50 $\mu$M ara-C produced a surviving cell fraction of approximately 0.3 (Chart 2). This is comparable to the overall surviving cell fraction achieved by 2 optimally spaced 1-hr exposures to 50 $\mu$M ara-C (see above) despite major differences in kinetic and pharmacokinetic conditions and despite a 6-fold difference in $C \times T$ values for the 2-drug schedules.

These considerations may prove to be of value in the optimization of more complex schedules of ara-C alone and in the rational development of schedules which use ara-C or HU in combination with other drugs.

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Schedule Optimization of Hydroxyurea and 1-β-d-Arabinofuranosylcytosine in Sarcoma 180 in Vitro


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