Schedule Dependence of Vincristine Lethality in Sarcoma 180 Cells following Partial Synchronization with Hydroxyurea

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ABSTRACT

The effects of exposure to 0.1, 0.5, or 2 \( \mu \)M vincristine for 4 hr were studied in Sarcoma 180 cells at various times after synchronization with 5 \( \mu \)M hydroxyurea for 1 hr. Maximum sensitivity to the lethal effects of vincristine was observed at 10 to 14 hr after hydroxyurea exposure at the higher vincristine concentrations, compared to a period of a maximum sensitivity to a second dose of hydroxyurea at 8 to 12 hr. Serial flow cytometry studies indicated that the apparent decrease in sensitivity to vincristine at 14 to 18 hr was due to the division of cells in the leading segment of the synchronized wave and their entry into the relatively resistant G\(_2\) phase prior to vincristine exposure. Synchronized cells that had not divided at the time of vincristine exposure were blocked transiently in G\(_2\). Serial metaphase index studies suggested that the G\(_2\) cells closest to the end of the cell cycle at the time of vincristine exposure were likely to exhibit the greatest degree of mitotic disorganization when they overcame the G\(_2\) block and entered metaphase. The present studies suggest that sensitivity to vincristine increases progressively as cells approach mitosis. The molecular mechanisms underlying this phenomenon are considered in relation to the increase in cell tubulin content during the course of cell cycle progression.

INTRODUCTION

The Vinca alkaloids, VCR\(^2\) and vinblastine, are cytotoxic tubulin-binding agents that are effective in the treatment of leukemias, lymphomas, and a variety of solid tumors in animals and in humans.

Early studies of the Vinca alkaloids suggested that their lethal effects were cell-cycle phase-specific (8, 11, 14, 24). These agents were shown to disrupt the mitotic spindle (8, 11, 14), and their lethality was attributed largely to their effects on cells in mitosis. However, in other studies, the Vinca alkaloids were found to exert their lethal effects on interphase cells (5, 9, 10, 12, 13, 25). When synchronized cells were exposed to VCR, a nadir in clonogenic cell survival was observed during S phase (9, 13) or during late S and G\(_2\). In autoradiographic studies of cells doubly labeled with \( \text{[}^3\text{H}\text{]}\text{dThd}\) and \( \text{[}^14\text{C}\text{]}\text{dThd}\), it was shown that cells exposed to VCR during S and G\(_2\) were later arrested in mitosis and subsequently underwent necrosis (5, 10).

In our own studies on the effects of VCR in asynchronously growing Sarcoma 180 cells (16), VCR produced a transient block of interphase cells in G\(_2\). We also found that not all cells that were lethally damaged by VCR accumulated in mitosis, and that many of the cells that did accumulate in mitosis did not become necrotic but went on to become polyploid cells.

In order to explore these observations in greater detail, we have studied the effects of VCR on cell survival, cell cycle progression, DNA synthesis, and metaphase accumulation in Sarcoma 180 cells that were synchronized by prior exposure to hydroxyurea for 1 hr. The results of these studies are described in this paper. We have found that the lethal effects of VCR became more pronounced as cells progressed through the cell cycle and approached mitosis. The mechanism underlying this observation may be related to the replication of cell tubulin content late in the cell cycle.

MATERIALS AND METHODS

All studies were carried out in Sarcoma 180 (Foley strain CCFR11; supplied by American Tissue Type Culture, Rockville, Md.) grown in vitro in Earle’s Medium 199 (Flow Laboratories, Rockville, Md.), which was supplemented with 10% fetal bovine serum, 2 \( \mu \)M l-glutamine, 100 units per ml of penicillin per ml, and 100 \( \mu \)g of streptomycin per ml. Cultures were grown in monolayer in 250-ml plastic tissue culture flasks (growth surface area, 75 sq cm) (Costar, Cambridge, Mass.) containing 10 ml of medium. Cells were plated at an initial concentration of \( \times 10^5\) cells/ml. Medium was changed on Days 2 and 4, and cells were subcultured on Day 5.

Cell Survival Studies. Two-day-old log-phase cell cultures were incubated at 37\(^\circ\) with 5 \( \mu \)M of HU for 1 hr. At the end of the drug exposure period, the medium containing drug was removed, and the cells were rinsed 3 times with 5 ml of HBSS. Cells were then resuspended in Medium 199. A drug-free control flask was included and was treated identically in each experiment. At various intervals after HU pretreatment, VCR was added at final concentrations of 0.1, 0.5, or 2 \( \mu \)M for 4 hr. For reference purposes, time intervals between drugs are reported from the onset of exposure to each drug.

At the end of the VCR exposure period, the medium was removed, and the cells were washed 4 times with HBSS. The cells were then harvested by incubation (37\(^\circ\)) with 0.25% trypsin for 8 min. Controls treated with HU alone were also obtained at each time point. Total cell counts in each flask were determined using a Coulter Counter (Coulter Electronics, Hialeah, Fla.). Cell suspensions were diluted with Medium 199, and known numbers of cells were cloned in soft agar. Nine-day-old colonies were fixed, stained with Giemsa stain, and counted visually. Clonogenic cell yield for each flask, defined as the number of colonies enumerated divided by the number of cells plated, was averaged for 5 replicate flasks at each drug concentration. The viable cell count per flask was calculated as the total cell number per flask multiplied by the clonogenic cell yield at a given time point. The surviving viable cell fraction following VCR exposure was calculated as the number of viable HU-treated cells per flask divided by the number of viable HU-treated control cells per flask, harvested at the same time, Thus, the reported...
surviving cell fractions after VCR exposure reflected the lethal effects produced by VCR alone. Each experiment was performed at least 3 times, and the reported results represented the log means of replicate studies.

For comparison, flasks of cells exposed initially to 5 mM HU for 1 hr were exposed to a second dose of HU at the same concentration for 1 hr at the same time intervals at which VCR exposures were initiated.

Metaphase Index Studies. Serial metaphase indices were obtained at intervals during and after HU and HU plus VCR exposure. Aiquots of cells were cyto centrifuged on glass slides and stained with acetic orcein. Only those cells containing condensed, discrete chromosomes in the absence of a nuclear membrane were included in the numerator of the metaphase index. Metaphases were further classified on the basis of the degree of disruption of normal metaphase chromosome patterns. Metaphases in which the chromosomes were organized in a single ring (or band-like structure, when viewed on edge) were considered to have intact kinetochore microtubules (2) and were classified as organized for our purposes. Metaphases in which chromosomes were spread randomly throughout the cell, and/or in which there were multiple-ring structures, each involving 2 to 3 chromosomes, were classified as disorganized metaphases. Two thousand cells were counted at each time point. Each experiment was performed 3 times, and reported results represent the means of replicate studies.

Flow Cytometry Studies. Serial DNA histograms were obtained before, during, and after exposure to HU and HU plus VCR. Cells were washed with HBSS, harvested by trypsinization, fixed in cold 70% ethanol, and stained with mithramycin (100 μg/ml) in 15 mM MgCl₂ and 0.15 M NaCl at a final concentration of 1 × 10⁶ cells/ml. 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. At least 20,000 cells were measured in each sample. All DNA histograms were normalized with respect to total cells analyzed per sample for display purposes.

Prior to the measurement of each drug-treated sample, a sample of untreated control cells was run, and amplifier gain settings were adjusted so that the G₁ peak of the control cells appeared in the same reference channel (Channel 60 of 256). Immediately after the measurement of each drug-treated sample, the position of the G₁ peak of control cells was rechecked to ensure that it had not drifted during the course of the measurement.

DNA Histogram Analysis. Because of the presence of large numbers of nuclear fragments and polyplody cells at various times after VCR exposure (see Chart 4, and Ref. 15), conventional methods of DNA histogram analysis could not be used. In order to quantitate population shifts among the cell cycle phases, and in order to quantitate the development of nuclear fragmentation and polyplodicy during and at various times after drug exposure, the DNA histogram was analyzed by a modification of the method of Alabaster and Cassidy (1). The DNA histogram was divided into bounded regions as follows: pre-G₁; Channels <50; G₁, Channels 50 to 70; S, Channels 71 to 99; G₂, Channels 100 to 140; and post-G₂-M, Channels >140. The fraction of cells in each region was calculated for each histogram. Because the gain settings were adjusted to an external reference standard within one channel of 60 for each sample, valid comparisons could be made among sequential histograms, and corresponding data from multiple experiments could be grouped for analysis. The reported results represent the means of 3 experiments. Although by this method the cell fraction in each region was contaminated to some extent by cells from adjacent regions, this proved to be of little practical consequence. Major shifts of cells from one region of the histogram to another that were apparent on visual inspection were faithfully reflected in the processed data.

RESULTS

Cell Survival Studies. Chart 1 shows the effects on cell survival of VCR exposure for 4 hr, commencing at various time intervals after an exposure for 1 hr to 5 mM HU. The effects of exposure to a second dose of HU are shown for comparison. Since the data are normalized with respect to time-matched controls treated with HU alone (see "Materials and Methods"), they reflect the effects of administration of the second drug only on cell survival.

When cells were exposed to 0.1 μM VCR for 4 hr, starting at 2 to 10 hr after exposure to HU, the surviving clonogenic cell fraction ranged from 0.70 to 0.75 (Chart 1). When cells were exposed to 0.5 μM VCR at 12 to 14 hr after exposure to HU, the surviving cell fraction fell to 0.5. Exposure to 0.1 μM VCR at 16 and 18 hr produced surviving cell fractions of 0.6 and 0.75, respectively.

When cells were exposed to 0.5 μM VCR at various intervals after exposure to HU, the schedule-dependent effects on cell survival were more pronounced. The nadir in the surviving cell fraction (0.25 to 0.31) was observed at 12 to 14 hr (Chart 1). Cell killing was also greater at 6 to 10 hr with 0.5 μM VCR than with 0.1 μM VCR. The effects of 2 μM VCR were similar to those observed at 0.5 μM VCR. The nadir in the surviving cell fraction (0.22 to 0.27) also occurred at 12 to 14 hr. The decrease in surviving cell fraction at 10 hr was greater with exposure to 2 μM VCR than with 0.5 μM VCR.

By comparison, the nadir in the surviving cell fraction following exposure to a second dose of HU was observed between 6 and 12 hr and ranged from 0.45 to 0.55 (see Chart 1, shaded region). While there was extensive overlap in the intervals of maximum sensitivity to a second dose of HU and to VCR, respectively, the interval of maximum sensitivity to VCR extended 2 to 4 hr later than that for a second dose of HU, and the nadir was lower at
VCR concentrations of 0.5 μM or greater.

In earlier radioautographic studies of the effects of exposure of 5 mM HU for 1 hr, peak rates of DNA synthesis during the posttreatment period were observed at 6 to 10 hr. Concomitantly, a wave of recruited cells was shown by flow cytometry to move through early S at 6 hr, mid-S at 8 to 10 hr, and late S-early G2 at 12 hr (6). Preliminary studies of changes in the metaphase index following exposure to 5 μM HU for 1 hr indicated a decrease in the metaphase index during the first 10 to 12 hr, and a rise at 14 to 18 hr. That is, exposure to HU for 1 hr was followed by the appearance of a cohort of rapidly cycling, synchronized cells that moved through mid-S at 8 to 10 hr, traversed late S and G2 at 12 to 14 hr, and underwent mitosis between 14 and 18 hr. Thus, one interpretation of the cell survival studies shown in Chart 1 might be that susceptibility to VCR peaked in late S and early G2 and then decreased late in G2. Alternatively, it may be that cells in late G2 were no less susceptible to VCR effects than were cells in late S and early G2, and that the apparent decrease in drug effectiveness at 14 to 18 hr was due to the vanguard of the partially synchronized wave of cells dividing and entering a relatively resistant G1 phase at this time. In order to distinguish between these 2 possibilities, we chose the 10- and 14-hr time points after HU exposure for more detailed study by means of flow cytometry.

Flow Cytometry Studies. Chart 2 shows the effects on the fractions of cells in different regions of the DNA histogram of a 4-hr exposure to VCR that was initiated at 10 hr after HU exposure. The effects of VCR concentrations of 0.1, 0.5, and 2 μM are shown in comparison with the effects of initial exposure to HU alone. The presence of VCR did not prevent the continued progression of the wave of synchronized cells through the S region and into the G2-M region of the DNA histogram of any of the VCR concentrations studied (Chart 2C, shaded region). At all 3 VCR concentrations, cells accumulated in the G2-M region in the presence of drug through the 14-hr time point (Chart 2D, shaded region), and the fraction of cells in the G2-M region remained elevated or continued to rise for at least 2 hr after drug removal. Peak fractions of VCR-treated cells in the G2-M region ranged from 0.8 to 0.9. However, concomitant metaphase index studies showed that the fraction of cells in metaphase at 10 to 16 hr was less than 0.05 at all drug concentrations (see below and Chart 4 and associated text). Thus, the cells that accumulated in the G2-M region of the DNA histogram between 12 and 18 hr were almost all premitotic cells in G2. A similar VCR-induced G2 block was observed in asynchronously growing sarcoma 180 cells as well (16).

The G2 block induced by VCR was transient, and its duration was dependent on VCR concentration. Following exposure to 0.1 μM VCR, the fraction of cells in G2 fell to control values by 22 hr (Chart 2D). Following exposure to 0.5 and 2 μM VCR, a significant decrease in the G2 fraction was observed at 22 hr, but return to control values did not occur until 26 hr.

The progressive and sustained increase in the G2 cell fraction of 0.2 to 0.3 above the HU-treated controls between 12 and 18 hr at VCR concentrations of 0.5 and 2 μM (Chart 2D) would suggest that VCR induced a G2 block, not only in all cells that were in G2 at the time of drug exposure, but also in S-phase cells that were within 6 to 8 hr of mitosis at the time of initial drug exposure. This is supported by the observations that VCR-treated cells continue to empty out of the S region at up to 18 hr (Chart 2C) but do not proceed from the G2-M region into the G1 region until after 18 hr (Chart 2B, 0.5 and 2 μM VCR). This would suggest that the wave of cells recruited by HU that was passing through G2 at the time of VCR exposure had a trailing segment that extended well back into S and included approximately 20 to 30% of the population, i.e., that, initially, HU-induced only partial synchronization of these cells.

Cells arrested in G2 by VCR underwent one of several subsequent fates. A small fraction of the cells was arrested transiently in metaphase at 18 and 22 hr (see below); presumably, these metaphases were included in the descending limb of the wave of cells in the G2-M region of the histogram (Chart 2D). Some cells resumed cycling and reappeared in the G1 region of the histogram. A larger proportion of cells exposed to 0.1 μM VCR recovered than after exposure to 0.5 or 2 μM VCR, and recovery occurred more rapidly at the lowest drug concentration (Chart 2B). Some G2-arrested cells subsequently underwent fragmentation and appeared in the pre-G1 region of the histogram (Chart 2A), while others went on to become polyploid cells that appeared in the post-G2-M region of the histogram (Chart 2E).

Chart 3 shows the effects on the fractions of cells in different regions of the DNA histogram of a 4-hr exposure to VCR initiated at 14 hr after HU exposure. The patterns are somewhat more complex, due to the fact that cells in the leading segment of the HU-recruited wave had divided and reappeared in G1 between 12 and 14 hr, just prior to VCR exposure (Chart 3B), while cells in the broader trailing segment of the HU-recruited wave had not divided yet when VCR was introduced. While VCR promptly blocked the entry of new cells into G1, it did not prevent the 20% of the total population that had already reentered G1 from progressing out of the G1 region between 14 and 22 hr (Chart 3B, shaded region and beyond). This subgroup of recruited cells that had escaped the effects of VCR could be seen passing through and leaving the S region between 16 and 24 hr (Chart 3C). Presumably, it was this subgroup of recruited, partially synchro-
observed at 4 to 8 hr after the removal of VCR.

When Chart 4 is considered together with Charts 2D and 3D, it becomes apparent that premitotic cells were blocked transiently in G2 during VCR exposure and for 2 to 4 hr after drug removal. Thus, the delayed appearance of the peaks in the metaphase index until 4 to 8 hr after drug removal, regardless of the time of VCR exposure (Chart 4), is directly attributable to prior VCR-induced G2 block.

The differences in the ranges of peak values of the metaphase index in Chart 4, A and B, are attributable to differences in the proportions of cells in the population that were susceptible to VCR-induced metaphase arrest in the 2 drug schedules. When VCR was introduced at 10 hr after HU exposure, the HU-recruited wave was just entering G2 (Chart 2D); when VCR was introduced at 14 hr, many of the HU-recruited cells had already divided and entered G1 (Chart 3B).

It is clear that, of all the cells that were blocked transiently in G2 by VCR, only a small proportion were subsequently arrested in metaphase after 4 hr of exposure to the drug. To explore the possibility that the cells that were closest to mitosis at the time of VCR addition were those that were more likely to undergo metaphase arrest once the G2 block was overcome, metaphases were subclassified with respect to the degree of disruption of spatial organization of the chromosomes in the metaphase figure (see "Materials and Methods"). Organized and disorganized metaphases were then scored separately. The total metaphase index and organized metaphase index are shown in Chart 5 for each VCR concentration and drug schedule. At concentrations of 0.1 μM VCR, nearly all metaphases were organized, whether the drug was administered at 10 or 14 hr after HU (Chart 5, A and B, respectively). At the higher drug concentrations, disorganized metaphases first appeared at the end of the 4-hr drug exposure period and peaked at 4 hr after the termination of drug exposure, regardless of drug schedule (Chart 5, A2, A3, B2, and B3). In contrast, at 8 hr after the termination of drug exposure, the metaphase peaks consisted almost entirely of organized metaphases. These data are consistent with the premise that the cells closest to mitosis were the first to overcome VCR-induced G2 block and, subsequently, exhibited the greatest degree of disruption of metaphase organization. Presumably, cells that were 2 to 4 hr away from mitosis at the time of VCR exposure overcame VCR-induced G2 block at 4 to 8 hr after

Metaphase Index Studies. During the course of the flow cytometry studies, aliquots of cells were also taken for parallel determinations of the metaphase index at each time point. The results are shown in Chart 4. Whether VCR was added at 10 hr after HU (Chart 4A) or at 14 hr after HU (Chart 4B), there were only minimal changes in the metaphase index after 4 hr of drug exposure, and for 2 hr thereafter. Increases in the metaphase index above 0.1 were observed only in cells exposed to 0.5 and 2 μM VCR at 10 hr after HU exposure (Chart 4A); peak metaphase indices ranging from 0.10 to 0.15 were seen at 4 to 8 hr after VCR removal. When 0.5 and 2 μM VCR were added at 14 hr after HU, there were modest rises in the metaphase index above control values; peak values ranging from 0.05 to 0.075 were
DISCUSSION

Effects of VCR in Relation to the Cell Cycle. Published studies on the cell cycle-dependent lethality of VCR have yielded seemingly conflicting results. Madoc-Jones and Mauro (13) and Hill and Whelan (9) have claimed that VCR is S-phase-specific on the basis of their studies in HU-synchronized and mitotically selected cells, respectively. That is, VCR sensitivity was thought to peak during S and decline thereafter as cells approached G2, at the time of VCR exposure, were later arrested in metaphase, and subsequently went on to become necrotic (5, 10). In the present study, there were several other indications that cells approaching mitosis were especially sensitive to the effects of VCR. The transient blockade of cells in G2 (Charts 2D and 3D) is itself a manifestation of this phenomenon. Our metaphase index data are also consistent with the premise that the G2 cells closest to the end of the cell cycle at the time of initial exposure to VCR were the cells that were likely to exhibit the greatest degree of mitotic disorganization when they overcame the G2 block and entered metaphase (Chart 5).

Mechanisms of VCR Lethality. The finding that sensitivity to VCR lethality increases progressively as cells traverse the late portion of the cell cycle may have important implications with regard to understanding the relation between known molecular mechanisms of VCR action and drug lethality. VCR is known to bind specifically to tubulin (17–19), a major component of intracellular microtubular structures that are essential for a variety of normal cell functions, including cell division. The lethal effects of the Vinca alkaloids are commonly attributed to their tubulin-binding properties (23). Historically, the primary target of tubulin-binding agents was thought to be the mitotic spindle, the disruption of which prevents centriole migration (2, 8, 21). However, VCR-induced G2 block observed in these and other published studies itself can lead to erroneous conclusions regarding the phase specificity of drug lethality. For example, the mitotic shake technique might select for the most rapidly proliferating component of a population, and this selected cohort might traverse the cell cycle more rapidly than expected. On the other hand, synchronizing agents such as HU are cytotoxic and might introduce unwanted retardant effects on cell cycle progression. Regardless of which method is chosen, the degree of synchronization can be expected to diminish progressively as cells traverse the cell cycle (22), and this, too, can affect the interpretation of cell survival studies.

Since the technique of mitotic selection does not provide sufficient numbers of cells for analysis by flow cytometry, we used HU synchronization in order to examine directly the behavior of synchronized sarcoma 180 cells before, during, and after exposure to VCR for 4 hr. Although the cell survival patterns superficially resembled those that might be expected for a cycle phase-specific drug (Chart 1), a more detailed analysis of the data showed that the sensitivity of cells to the lethal effects of VCR increased progressively as they traversed S and G2. It was apparent from our serial flow cytometry studies that the HU-synchronized cohort of cells actually traversed the late portion of cell cycle in a broad wave with a relatively long trailing segment (Charts 2 and 3). The apparent decrease in VCR lethality between 12 and 18 hr after exposure to HU (Chart 1) could be attributed to the division of an increasingly larger proportion of cells in the HU-recruited cohort and their entry into a relatively resistant G1 phase during this period (Charts 2B and 3B). There were striking differences in the subsequent behavior of cells that had just divided prior to VCR exposure and cells that were approaching mitosis but had not yet divided at the time of VCR addition. VCR had no effect on the movement out of G0 of cells that had already divided (Chart 3B) and had no effect on their subsequent movement through S (Chart 2C). However, the entry of new cells into G1 was halted rapidly (Charts 2B and 3B), and cells began to accumulate in the G1-M region of the histogram (Charts 2D and 3D) soon after the introduction of VCR.

In the present study, there were several other indications that cells approaching mitosis were especially sensitive to the effects of VCR. The transient blockade of cells in G2 (Charts 2D and 3D) is itself a manifestation of this phenomenon. Our metaphase index data are also consistent with the premise that the G2 cells closest to the end of the cell cycle at the time of initial exposure to VCR were the cells that were likely to exhibit the greatest degree of mitotic disorganization when they overcame the G2 block and entered metaphase (Chart 5).

Cell survival studies in synchronized cells must be interpreted with care. Artifacts associated with the synchronization procedure and care during treatment and drug removal, and their metaphases exhibited a greater degree of organization.
studies (25) may reflect the disruption or prevention of formation of microtubular structures associated with very early stages of mitosis. Microtubules are also involved in the maintenance of cell shape and locomotion; disruption of the cell cytoskeleton may account for reported observations of interphase cell lysis by tubulin-binding agents (12, 13, 20).

Total cell tubulin content normally doubles between cell divisions, increasing in sigmoidal fashion as cells progress through the cell cycle (4, 7). Peak rates of tubulin synthesis occur during a phase of the cell cycle that overlaps S and G2 (4). Using fluorescent antitubulin antibody to determine cell tubulin content, we have shown by flow cytometry that the cell tubulin content distribution in Sarcoma 180 cells spans a 2-fold range and exhibits a prominent postmitotic peak and a saddle shape, much like the DNA histogram (15). This would imply that cell tubulin content increases in parallel with cell DNA content as cells progress through the cell cycle; like cell DNA content, cell tubulin content doubles between cell divisions and halves at mitosis.

It is conceivable that the increase in sensitivity to the lethal effects of VCR during the course of cell cycle progression in Sarcoma 180 cells might be related to the increase in cell tubulin content during the course of cell cycle progression. That is, cells exposed to VCR before they have synthesized a full complement of tubulin might, after VCR removal, go on to synthesize a sufficient amount of new, VCR-free tubulin to form the microtubular structures that are required for mitosis. On the other hand, cells in which tubulin synthesis had nearly been completed at the time of VCR exposure would not synthesize enough new tubulin for normal cell division, even if the drug were removed prior to mitosis. Additional studies will be required to explore this possibility.

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