Effect of Dihydroxyanthraquinone and Radiation on $G_2$ Progression

Bruce F. Kimler

Radiation Biology Laboratory, Department of Radiation Therapy, University of Kansas Medical Center, Kansas City, Kansas 66103

ABSTRACT

The effect of dihydroxyanthraquinone (DHAQ), a potential anticancer chemotherapeutic agent, on the progression of Chinese hamster ovary cells into mitosis and on the division delay induced by ionizing radiation was studied using the mitotic selection procedure for cell cycle analysis.

Following the addition of DHAQ, the number of mitotic cells selected from an asynchronous population remained unaltered for a refractory period and then decreased. This effect was concentration dependent with transition points between the $S$/$G_2$ boundary at $10^{-4}$ $\mu g$/ml and the $G_2$/$M$ boundary at $\geq 10^2$ $\mu g$/ml. The duration of the transient division delay was dependent upon the concentration of drug used and the duration of pulse exposure.

When cells were treated with pulses of DHAQ in addition to X-irradiation, there was no change in the location of the radiation transition point. There was an increase in the duration of division delay compared to that produced by X-ray alone that was dependent upon the concentration and duration of drug treatment.

The effect of DHAQ is similar to that of other cancer chemotherapeutic agents (Adriamycin, bleomycin, and lucanthone), and the same cautions should therefore be considered when combining DHAQ and radiation for clinical use.

INTRODUCTION

DHAQ$^2$ (NSC 279836) is a newly synthesized compound that has been shown to be effective in preliminary antitumor testing (8). At present, the drug is entering Phase I clinical investigations. Since the compound is structurally related to a family of intercalating antibiotics that includes Adriamycin, actinomycin D, and lucanthone, care must be taken to avoid any significant problem with normal tissue damage. Preliminary data suggest that this compound has much less cardiotoxicity and nephrotoxicity than do the conventional antibiotics. It seems likely that DHAQ will one day be utilized with radiation therapy in a combined modality approach. Therefore, it seems prudent to establish some guidelines by which to estimate the potential for synergistic action of the modalities. The mitotic selection procedure for cell cycle analysis (6) was used to investigate the effects of DHAQ on cell cycle progression, the concentration-dependent blockade in the $G_2$ phase, and the interaction of DHAQ and radiation-induced division delay. It is hoped that these results will assist the oncologist in the design of combined modality therapeutic schedules using radiation therapy and DHAQ so as to avoid the severe complications that resulted from the early use of actinomycin D and Adriamycin in combination with radiation therapy.

MATERIALS AND METHODS

Cell and Culture Conditions. Chinese hamster ovary cells were maintained in exponential growth as monolayer cultures at 37° in 6% CO$_2$. McCoy's modified Medium 5A was supplemented with 10% fetal calf serum and penicillin plus streptomycin (KC Biological, Lenexa, Kans.). Under these conditions, the cells displayed a doubling time of approximately 14 hr with $G_1 = 5$ hr, $S = 7$ hr, $G_2 = 1.5$ hr, and $M = 0.7$ hr as determined by autoradiographic studies of synchronous cells. Cells were monitored periodically with a fluorescent stain technique (1) to assure the absence of Mycoplasma contamination.

Mitotic Selection Procedure. The mitotic selection procedure for cell cycle analysis (6) was utilized to monitor the perturbatory effects of DHAQ upon cell cycle progression. Briefly, ten 75-sq cm tissue culture flask containing approximately $10^7$ asynchronous cells in exponential growth were shaken for 20 sec every 10 min on a horizontal shaker. The medium, now containing the detached, rounded-up cells in late mitosis, was collected on ice and replaced with 10 ml of fresh medium. The cell suspension was counted using a Coulter counter coupled to a frequency size analyzer to verify that the collected cells were predominately mitotic cells with a volume twice that of the smallest ($G_1$) cells. The mitotic index, as determined by fixing and staining with acetoorcein, was customarily greater than 90 to 95%. This technique resulted in a record of the number of cells that moved into a narrow region of mitosis, approximately 4 to 22 min prior to cytokinesis (6), i.e., the selection window, every 10 min (the mitotic rate).

Treatment (9 treatment flasks and 1 control flask) was accomplished by either irradiating the flask or replacing the control medium with medium containing the desired concentration of drug for the required number of shakes.

X-Irradiation. A Norelco 300 kvp X-ray unit was operated at 10 ma with 1.2 mm aluminum additional filtration (half-value layer, 1.0 cm aluminum), producing a dose rate of 1.2 Gy$/min$ at 45 cm. The irradiation procedure necessitated the flasks being out of the incubator at room temperature for 4 min, but this did not cause any significant perturbation of mitotic rate.

Drug Treatment. DHAQ was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. The drug was dissolved in balanced salt solution, sterilized by filtration, and stored as a concentrated solution in the freezer until needed. Prior to treatment, the concentrated solution was diluted in complete medium. The drug solutions were protected from light so as to prevent photodegradation. Drug-containing medium at the appropriate concentrations was added to the flasks for the desired number of shakes before control medium was once again used.

RESULTS

The effect of a 60-min exposure to various concentrations of DHAQ ($10^{-3}$ to $10^2$ $\mu g$/ml) on the selection of mitotic Chinese

---

1 Supported in part by Mid-America Cancer Center Program, National Cancer Institute Grant 5 P30 CA15080-04.
2 The abbreviation used is DHAQ, dihydroxyanthraquinone.

Received April 30, 1979; accepted October 4, 1979.
hamster ovary cells is shown in Chart 1. For comparison, the effect of X-irradiation (2.0 Gy) on the induction of a progression blockade (midpoint, 40 min) and the subsequent period of division delay (206 min) is also shown. Mitotic cells continued to enter the selection window at the control rate for a period of 40 to 80 min after initiation of drug treatment and then declined to a concentration-dependent minimum. The midpoint of progression blockade (the time from initiation of treatment to the time at which the mitotic rate is 50% of the control; Ref. 5) decreased with increasing concentration of DHAQ. The increasing concentration also resulted in a progressive shortening of the time that cells were selected at the control rate. Concentrations of $10^{-3}$ and $10^{-2}$ μg/ml did not completely reduce the number of cells selected per shake to the constant background level of nonmitotic cells and debris over the duration of this experiment. In other experiments, longer treatment times produced the same lower plateau region with no recovery as long as the drug was present.

The midpoint of progression blockade as a function of the logarithm of DHAQ concentration is plotted in Chart 2. The composite curve from 14 experiments was described by a straight line since the midpoint occurred closer to mitosis with increasing drug concentration. Identical midpoints were obtained for pulse exposures (10 to 60 min) as for continuous treatment, indicating that the response is indeed the result of different points of action in G2 and is not due to different rates of drug diffusion, uptake, or intercalation. A plateau was attained where the midpoint did not decrease further with increasing concentration. This minimum value is coincident with the value obtained for radiation-induced blockade, as has been shown for a number of cancer chemotherapeutic agents (5). The mean value from 14 experiments for the X-ray transition point (36 min) was used to define $T_0$ (see below). It was often difficult to obtain data for concentrations above $10^{-1}$ μg/ml due to the increased selection of nonmitotic cells. However, in no experiment was there a drug transition point less than the X-ray transition point, confirming the concept of a minimum transition point. The linear portion of the curve was calculated by least-squares regression analysis from $10^{-4}$ to $10^{-2}$ μg/ml to fit the equation

$$T = T_0 + m \log(C_0/C)$$

where $T$ is the midpoint value in min prior to the selection window, $T_0$ is the minimum transition point attained (36 min), $m$ is the slope constant to be derived, $C_0$ is the concentration at which $T_0$ is obtained ($10^2$ μg/ml; $2.2 \times 10^2$ μM), and $C$ is the independent variable concentration. The value of 10 for the slope constant is significantly lower than are the values for actinomycin D, Adriamycin, lucanthone, mitomycin C, and bleomycin (a range of 25 to 68) that have been previously reported (5). This difference cannot be due to the choice of a value of $10^2$ μg/ml for $C_0$ since $C_0$ would have to change by several orders of magnitude to appreciably alter the value of the slope constant. The low value for $m$ indicates that the effectiveness of DHAQ for inducing a concentration-dependent blockade of
G2 progression is less than that of the other agents mentioned. Moreover, the concentration required to produce the minimum midpoint of blockade, i.e., C0, was greater for DHAQ than for the others (5), indicating that the absolute molar effectiveness of DHAQ for G2 blockade was also less.

Chart 3 shows the effect of a 10-min pulse of 10^-2 µg DHAQ per ml on the division delay produced by 2.0 Gy X-ray. These conditions (10 min and 10^-2 µg/ml) were chosen (see below) since they produced quantifiable effects with a minimum of complications, e.g., midpoints close to the X-ray midpoint, long delay times, etc. DHAQ alone produced a moderate depression of the mitotic rate to about 30% of the control value at the nadir. The midpoint of progression blockade is 78 min prior to selection, in agreement with Charts 1 and 2. After a period of time, the mitotic rate increased as cells recovered from a transient division delay. The combination of DHAQ and X-ray produced a mitotic rate curve that was identical to the curve produced by X-ray alone (dashed line) over the descending portion of the curve. The midpoint of progression blockade for both treatments was 37 min prior to selection. However, the recovery and eventual progression of cells into the selection window occurred later after the combined treatment than after X-ray alone. The midpoint division delay (the time between the midpoints of the descending and ascending portions of the mitotic rate curve) was 237 min for the combined treatment compared to 207 min for X-ray only. The plateau region of mitotic rate following recovery from the division delay induced by DHAQ plus X-ray was higher than the mitotic rate following recovery from the delay induced by the 10-min pulse of DHAQ alone. This is similar to the 'overshoot' that occurs following radiation-induced division delay, wherein the mitotic rate from an irradiated flask is greater than that from a control flask (see Chart 1).

The effect of various concentrations of DHAQ on the midpoint division delay produced by radiation is shown in Chart 4. To compensate for variation between experiments (not needed within experiments) in the absolute value of the midpoint division delay induced by 2.0 Gy X-ray, the values are expressed as relative to the delay produced by X-ray only within each experiment (average midpoint division delay induced by 2.0 Gy X-ray, 204 min). There was still considerable variation by this method as evidenced by the large error bars; however, in each experiment there was a consistent trend of increasing division delay with increasing concentration. There was no change in the midpoint division delay induced by radiation if given in combination with concentrations of 10^-3 or 10^-2 µg DHAQ per ml. Above these concentrations, the division delay increased as a linear function of the logarithm of the concentration. Thus, irradiated cells that experienced a progression blockade were delayed for a longer period before they eventually appeared in
the selection window. Also shown in Chart 4 is the midpoint division delay for cells treated with DHAQ only. The slope of this line is similar to the slope of the line calculated for the X-ray plus DHAQ division delays.

Chart 5 shows the effect of pulse treatments of various durations on the values of midpoint of progression blockade and midpoint division delay observed for either nonirradiated or irradiated cells. The location of the midpoint of progression blockade for either DHAQ alone or DHAQ plus X-ray was independent of the duration of the DHAQ pulse, being 72 and 37 min prior to selection, respectively. The plot of midpoint division delay (expressed as relative to the delay produced by 2.0 Gy X-ray alone) for cells that received either DHAQ only or DHAQ plus X-ray shows a linear response as a function of the duration of DHAQ treatment. The data are consistent with the 2 lines possessing equal slopes and being merely displaced one from the other by a constant factor.

Chart 6 shows the effect of various doses of X-ray on the response of cells either nontreated or treated with DHAQ. The midpoint of progression blockade for either X-ray alone or X-ray plus DHAQ was independent of the dose of X-ray received, the value being 37 min. Radiation-induced division delay as measured by midpoint division delay is seen as a linear function of dose for both X-ray and X-ray plus DHAQ. Extrapolation yields a displacement between the 2 responses of approximately 40 min at zero dose. For both conditions, the slope of the line indicates a dose-dependent, radiation-induced division delay of approximately 1 hr/Gy.

**DISCUSSION**

The action of DHAQ to induce a progression blockade of G2 cells towards mitosis is qualitatively similar to that observed for Adriamycin (3, 5) and several other cancer chemotherapeutic agents (2, 5). The location of the transition point in G2 is concentration dependent, near the S/G2 boundary (located approximately 120 min prior to selection) at low concentrations, and at the G2/M boundary (approximately 40 min prior to selection) at high concentrations. A minimum transition point is also produced that is located at or near the X-ray transition point. This implies the completion of some process or structure at this time, 36 min prior to selection, such that cells beyond that point are refractory to delay produced by either X-ray or DHAQ and proceed into mitosis at the control rate. As suggested previously (5), the linear nature of concentration-dependent transition points past the last point in the cell cycle at which metabolic synthesis is required for progression and the existence of a common minimum transition point argue for a physical mechanism of action. This could occur by intercalation of the drug into DNA causing perturbation of the chromatin structure or the division apparatus. It is not surprising that DHAQ would be a functional analog of Adriamycin since by at least 2 criteria it is a structural analog. First, both Adriamycin and DHAQ are substituted anthraquinones. The planar 3-ring structure has been postulated to be the active structure that confers cytotoxic properties to the molecule (8). Adriamycin possesses a glycosidic linkage whereas DHAQ is bisubstituted with aminoalkylamino chains. Secondly, there is a particular triangular arrangement of 2 oxygen atoms and one nitrogen atom that has also been implicated as the functional moiety of various cytotoxic compounds (9). The only difference is that DHAQ possesses 2 such triangular arrangements whereas Adriamycin has only one. This has been used to explain the higher level of cytotoxic activity observed when DHAQ is compared to Adriamycin.
As with Adriamycin (3) and bleomycin (2), DHAQ does not alter the location of the radiation transition point. This is consistent with the minimum transition point for radiotherapy and DHAQ (at high concentrations) being the same. Thus, any cells refractory to radiation-induced blockade are also refractory to DHAQ-induced blockade. The effect of DHAQ and radiation on division delay appears to be additive. That is, the presence of DHAQ does not prevent the repair of that damage associated with radiation-induced division delay. Consequently, once a cell recovers from DHAQ-induced division delay it will appear with radiation-induced division delay. As seen in Charts 5 and 6, the cell recovers from DHAQ-induced division delay it will appear in the selection window, having already recovered from radiation-induced division delay. The additional 40 mm of delay could be explained by the reversion of those cells located between the DHAQ and radiation transition points, i.e., those cells between 78 and 36 min prior to selection, so that when they once again resume progression they do so from the DHAQ transition point. This, the transition point following combined treatment would be identical with the transition point following radiation only, but the time of appearance of cells in the selection window would be delayed by that additional 40 min. A similar phenomenon has been observed with radiation and Adriamycin (3) but not with radiation and either actinomycin D or lucanthone (7). Overall, it appears that the action of DHAQ and radiation is at best additive for this particular 40-min-wide region of cells. For long delays, i.e., those produced by large X-ray doses, high concentrations of DHAQ, and long pulse treatments, the effect may be subadditive. Overall, there is no synergistic action to increase the duration of radiation-induced division delay beyond this factor of 40 min.

This report has demonstrated the close similarity of Adriamycin and DHAQ in regard to their effects on cell cycle progression blockades in the G2 phase and on the duration of radiation-induced division delay. Based on this information, it is suggested that a prudent approach to combining DHAQ and radiation therapy for clinical use would take into account the at least additive nature of the interaction of these 2 modalities. While effects on progression blockade do not prove that there will be a clinical problem as was produced by radiation therapy and Adriamycin, the similarity between DHAQ and Adriamycin and between their effects in vitro do suggest caution. There is still an obvious need for studies to compare the effects of DHAQ and radiation on the clonogenic potential of mammalian cells to the effects of Adriamycin and radiation (4). These investigations are currently under way in this laboratory and will be reported at a later date.

ACKNOWLEDGMENTS

The author would like to thank the staff of the Department of Radiation Therapy for their support. The technical assistance of Iris L. Abrahams, Mary L. Barnes, and Patrick W. Gunn is acknowledged with gratitude. Special thanks are extended to Dr. C. C. Cheng for his introduction to DHAQ and for many helpful conversations.

REFERENCES

Effect of Dihydroxyanthraquinone and Radiation on G₂ Progression

Bruce F. Kimler


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/40/1/42

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.