Temperature Effect on Mitoxantrone Cytotoxicity in Chinese Hamster Cells in Vitro

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ABSTRACT

The effect of heat on 1,4-dihydroxy-5,8-bis[2-(2-hydroxyethyl)amino]-9,10-anthracenedione dihydrochloride (DHAD; mitoxantrone, NSC 301739) cytotoxicity was studied in V79 Chinese hamster cells. An overnight exposure to the drug at 40°C enhanced drug damage in chromosome aberrations, culture growth, and cellular reproductive integrity. Preincubation of cells overnight in medium containing no drug at this temperature also showed some enhancement in subsequent DHAD lethality (at 37°C as well as 43°C). Short exposures (1 h) to DHAD at 43°C was more damaging than were exposures at 37°C. This was also true for cells in the plateau phase of culture growth. As compared with exponentially growing cells, plateau-phase cells were more resistant to DHAD.

INTRODUCTION

There has been increasing interest in the role of various response modifiers, notably hyperthermia and misonidazole, as related to chemotherapeutic agents. Elevated temperatures during a short pulse of drug exposure potentiate the lethal effect of compounds such as Adriamycin, bleomycin, 1,3-bis(2-chloroethyl)-1-nitrosourea, and cis-diamminedichloroplatinum [see review (7)]. On the other hand, prolonged treatment at moderately high temperatures or short pulses at high temperatures induce heat tolerance (9, 10, 12, 15) and in some cases drug tolerance as well (3, 8). Relatively little attention has been focused on heat at temperatures below 41°C. Recently, Jung (12) examined the interaction induced by heat at 40°C and 43°C; Nielsen et al. (15) analyzed the kinetics of heat killing at temperatures including 40.5°C. Although temperatures of 40°C and 40.5°C may or may not be lethal, they do exert profound effects on cell response to subsequent insults (10, 12, 15). The temperature level of 40°C is of particular interest in whole-body heating, since patients can tolerate such levels for several hours or even a few days. Thus, we became interested in studying the effect of 40°C heat on the cytotoxicity of DHAD.2

DHAD (mitoxantrone, NSC 301739) is a newly synthesized anthracenedione derivative showing improved antitumor activity and reduced cardiotoxicity in experimental animals as compared to other commonly used anthracyclines, e.g., Adriamycin (2, 6, 17). It is currently being investigated in Phase I and II clinical trials. This report presents our studies of the effect of DHAD at the elevated temperatures of 40°C and 43°C on cultured Chinese hamster cells.

1 To whom requests for reprints should be addressed, at Department of Radiotherapy, Methodist Hospital, 506 Sixth Street, Brooklyn, NY 11215.
2 The abbreviations used are: DHAD, 1,4-dihydroxy-5,8-bis[2-(2-hydroxyethyl)amino]-9,10-anthracenedione dihydrochloride (NSC 301739); DHAQ, the free base of 1,4-dihydroxy-5,8-bis[2-(2-hydroxyethyl)amino]-9,10-anthracenedione dihydrochloride (NSC 301739).

RESULTS

Overnight DHAD Exposure

Chromosomal Aberrations. Chart 1 and Table 1 show the extent of chromosome aberrations induced by 24 h contact with DHAD as a function of drug concentration and temperature during exposure. Cells were scored for the type and number of aberrations. Some cells had chromosomes so severely fragmented beyond recognition that it was impossible to score the
number of breaks. These cells were not included in calculating the average number of aberrations per cell (metaphase); their frequency was listed in Table 1, Column F. Heat alone for 24 h at 40°C did not induce chromosomal or chromatid aberrations.

Cells exposed to DHAD at 40°C had substantially more chromosome breakages with a steeper rise in frequency as drug concentration increased when compared to control cells treated at 37°C. The excess was in both chromosomal and chromatid breaks, with a sharper increase in the latter. Both simple breaks and exchanges increased with the rise in drug concentration, the rate for the latter being more dramatic. As seen in Chart 1 and Table 1, the rise of total aberrations per cell followed closely that of exchanges, while contributions due to simple breaks remained low.

**Growth Inhibition.** In addition to chromosome damage, the enhancing effect of heat at 40°C on DHAD damage was shown by changes in the rate of culture growth (Table 2). Overnight incubation at 40°C (no drug) yielded similar cell numbers per dish as controls at 37°C, an approximate 3-fold increase over the initial numbers. Incubation at 25°C yielded very little increase. In the presence of drug, culture growth at 37°C was reduced; this effect was enhanced at 40°C. For example, at DHAD concentrations of both 0.008 and 0.01 μg/ml, the increase in cell number at 37°C was 60 to 75% of the values noted in drug-free medium as compared to 40 to 50% at 40°C. In order to find out whether disintegration of drug-damaged cells (rather than growth inhibition) during exposure contributed significantly to the reduced growth rate observed, we pooled and counted cells floated off in medium and in rinsing solutions prior to enzymatic removal of adhered cells. Various drug concentrations, up to 0.04 μg DHAD/ml, were examined. The proportion of dislodged cells, whether partially disintegrated or intact, remained below 2% of the total population, similar to that commonly found in controls.

**Survival.** In addition to cytotoxicity as expressed in chromosome breakage and growth inhibition, we assayed single-cell survival as defined by cloning ability which has special relevance to tumors and to tumor control. Chart 2 shows survival as a function of drug concentration and incubation temperatures during a 16- to 20-h drug contact, with no difference identifiable among exposure times of 16, 18, or 20 h. Heat alone at 40°C for an overnight incubation did not affect subsequent cloning ability, with plating efficiency remaining around 85%, similar to controls at 37°C. On the other hand, heating at 40°C during DHAD exposure enhanced the drug damage compared to its effect at 37°C. Lowering temperature to 25°C during DHAD treatment reduced lethality (Chart 2).

**Preincubation at 40°C**

Since a 50-min exposure to 43°C was found to reduce cellular sensitivity to subsequent Adriamycin treatment (8), we wondered whether a prolonged exposure to 40°C would also alter responses to DHAD. Cultures were transferred to a 40°C CO₂ incubator 16 h prior to testing of subsequent response to drug. Heat tolerance was induced following 16 h of preincubation at 40°C; this is demonstrated in survivals after 1 h subsequent heating at 43°C (Chart 3, Curves A and B).

Whether preheated (40°C overnight) cells became tolerant to DHAD damage was tested by 1 h of exposure to drug at 37°C and at 43°C. No reduction in sensitivity to DHAD was detected whether preheated cells were exposed to the drug at 37°C or at 43°C. As drug concentration increased, survival of the 40°C preincubated cells (Chart 3, Curves D and F) was further reduced below that of control cells preincubated at the normal temperature of 37°C (Chart 3, Curves C and E).

<table>
<thead>
<tr>
<th>Concentration</th>
<th>No. of cells</th>
<th>No. of chromosomal aberrations</th>
<th>No. of chromatid aberrations</th>
<th>Breaks/cell</th>
<th>No. fragmented F</th>
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<td>12,24</td>
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* A, simple breaks; B, exchanges; C, average breaks per cell due to simple chromosomal and chromatid breaks; D, average breaks per cell due to chromosomal and chromatid exchanges; E, average breaks per cell due to all chromosomal and chromatid aberrations; F, number of unscorable cells with extensively fragmented chromosomes.

![Chart 1. Average aberrations per cell following 24 h exposure to DHAD.](image-url)
Table 2
Number of cells per dish following an overnight incubation in varying concentrations of DHAD at 25°C, 37°C, and 40°C, from 4 separate experiments

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>37°C Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>Exp. 4</th>
<th>40°C Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>Exp. 4</th>
<th>25°C (Exp. 4)</th>
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<td>0.7</td>
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* —, not done.

Plateau Phase

Chart 4 shows the response of plateau-phase cells to 1 h exposure of DHAD and the influence of temperature during exposure. As compared with exponentially growing cultures (Chart 4, Curve D), these cells were less sensitive to DHAD (Chart 4, Curves A to C). The survival curve followed a steep slope initially but tended to level off gradually as drug concentration increased. The plating efficiency of cells in normal medium without drug was reduced following 1 h incubation at 43°C to 55% of controls at 37°C. Exposures to DHAD at 43°C brought survival to lower levels than expected from the sum of damages by separately applied heat and DHAD as evident by the divergence of Curves B and C. DHAD treatment at a room temperature of 23°C was much less toxic as seen from the minimized slope of the survival curve (Chart 4, Curve A), while plating efficiency remained similar to that of controls at 37°C.

DISCUSSION

There has been considerable interest in heat-potentiated cytotoxicity of chemotherapeutic agents (7). Certain compounds interact with hyperthermia synergistically, others interact mildly so, and still others show no interaction. DHAD is one of the active anthraquinone derivatives synthesized in attempt to produce a new drug similar to Adriamycin but with reduced cardiotoxicity. It is a promising compound as indicated by its significant cytotoxic activity and well-tolerated toxicity (2, 5, 6, 17, 18). DHAQ, the free base of DHAD, was found to potentiate the lethal damage of radiation (14). The present work reports modifications of DHAD damage by heat.
This study showed enhanced DHAD cytotoxicity to cells treated at elevated temperatures; mild heat at 40°C during an overnight treatment with DHAD effectively enhanced drug damage as measured by chromosome breakage, growth retardation, and cell survival (Charts 1 and 2; Tables 1 and 2). Similar with observations on other drugs such as Adriamycin and cis-diaminedichloroplatinum (11), DHAD produced less damage at room temperatures (Charts 2 and 4). The study of the thermopotentiating effect of cytotoxic drugs has concentrated on temperatures higher than 42°C (7). Its use in the clinics is limited to localized hyperthermia. For maximum interaction with a given drug, a short duration of intense heat is applied at precise times, relative to drug administration, as dictated by the pharmacokinetic behavior of the drug. When routine pharmacokinetic monitoring is not feasible in the face of appreciable variation among patients, it would be anyone's guess as to the optimal time for heat administration. Under such circumstances, prolonged heating at more tolerable temperatures of below 42°C may be preferable, provided that drug clearance from serum and tissues is slow relative to the duration of hyperthermia. Furthermore, mild heat can be used for both systemic and localized hyperthermia. Comparing DHAD cytotoxicity at 40°C and at 43°C, it is evident that further increase of temperature to 43°C during a shorter drug exposure of 1 h also accentuated lethal effect of DHAD. From our observations, we conclude that the thermopotentiating effect on DHAD toxicity for a prolonged exposure at 40°C is at least equal to that of a short exposure at 43°C.

The thermoenhancement of DHAD cytotoxicity is demonstrated not only in cell survival but also in chromosome aberrations and culture growth. As simple breaks and exchanges are scored and tabulated separately, it can be seen that cells treated by DHAD at 40°C may have a faster rise in exchange per cell with increasing drug concentration than do cells at 37°C (Chart 1; Table 1). However, a closer examination comparing similar levels of damage reveals similar distribution of simple breaks versus exchanges in the 2 groups exposed to drug at different temperatures. The apparent high proportion of exchanges in the high-temperature group is due to increased overall damage (average break per metaphase).

In the work of Au et al. (1) on chromosome aberrations in Chinese hamster ovary cells exposed to DHAQ, a higher range of drug concentration for a shorter exposure time of 5 h was used. The extent of chromosome breakage at comparable concentrations were similar in both studies.

In his study of DHAQ-induced G2 blockade in Chinese hamster ovary cells, Kimler (13) found sustained low-level mitotic activity as long as the drug was not removed. At comparable concentrations, our V79 cells appeared to be less sensitive than did their Chinese hamster ovary cells to G2 arrest as evident from considerable overnight growth in drug. However, drug treatment at 40°C retarded culture growth markedly (Table 2).

The thermopenhancement of DHAD cytotoxicity was also shown in cells in the plateau phase of growth. Drug exposures of these cells at room temperature resulted in minimal cell kill (Chart 4). In contrast with LoVo cells which yielded the same exponential killing after 1 h treatment by DHAD regardless of phases of culture growth (4), our cells exhibited definite differences between plateau phase and exponential phase, with the former being more resistant. It is possible that the higher serum content in medium that we used for plateau- than for exponential-phase cultures may have caused more serum binding with resultant less drug available to the cells, and/or better nutrient status than that of the exponentially growing cells. However, an examination of data (not shown) of experiments where exponential cultures in 10 versus 15% fetal bovine serum-containing medium were grown and exposed to 1 h DHAD in parallel revealed no significant difference in survival between the 2 groups, both similar with that shown in Chart 3, Curve C. Thus, the differential sensitivity to DHAD between exponential- and plateau-phase cells cannot be explained by serum content. In discussing the potential effectiveness of mitoxantrone in proliferating and nonproliferating cells, Duris (5) presented survival curves of WiDr human colon carcinoma cells displaying a much shallower slope for plateau than for "growth" phase. It is not certain whether the discrepancy on observations of these cells from that of the LoVo cell lines is due primarily to the behavior of specific cells and techniques involved. Nor is it clear whether the resistance of plateau-phase cells that we observed is due entirely to cellular changes inherent to the stationary growth phase or is also related to density effect as observed in responses of exponentially growing cells to spirogermanium (19). In either case, response differences related to proliferation state is a factor not to be ignored in clinical settings.

The modification of cellular response after preheating at 40°C was addressed. Our results indicated that cells incubated overnight in drug-free medium at 40°C become resistant to subsequent 43°C heating (Chart 3, Curves A and B). While this phenomenon is widely reported, details for development of thermotolerance vary. For instance, Nielsen et al. (15) reported its occurrence only when a period of incubation at 37°C is allowed to elapse (6 h in the case of 40.5°C heating), Henle et al. (10) observed resistance expressed by increased Dq (quasithreshold dose), and our results suggested increases of both Dq and Do.
(inactivation constant). However, these preheated cells became more sensitive to DHAD exposure at both 37°C and 43°C (Chart 3, Curves C to F). This differs from the transient resistance to Adriamycin found by Hahn and Strande (8) following short preconditioning exposures of 50 to 120 min at 43°C. In a clinical setting where drug dosing is repeated with or without heating, it should be of particular interest that, during the unheated sessions, DHAD damage will be enhanced in the preconditioned cell population in addition to the expected cytotoxic effect on fresh cell population. The gain during heated sessions will be noticeably modified, particularly when the drug persists in the body as shown by Savaraj et al. (16), who monitored 10 patients following DHAQ administration and found slow plasma clearance with terminal $t_{1/2}$ ranging from 50 h to 7 days, low cumulative urinary excretion of less than 9% in 96 h, and in one case low biliary excretion of less than 3% in 96 h.

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