In Vitro and in Vivo Cytotoxicity of Rhodamine 123 Combined with Hyperthermia

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ABSTRACT

Because both Rhodamine 123 (R123) and hyperthermia have been shown to be cytotoxic, we examined their effect, independently and in combination, on five different human malignant cell lines in vitro and on cultured melanoma cells grown intradermally in nude mice. The cell lines examined include two human melanomas, UCLA-SO-M14 and UCLA-SO-M21, the breast cancer cell line HT29, the human lung cancer cell line P3, and the human breast cancer cell line B231. R123 and hyperthermia, when used in combination, were found to be cytotoxic for these five different human malignant cell lines in vitro. The two agents together appear to enhance the cytotoxic effect of each alone, as documented by synergistic ratios ranging from 2.31 to 45 for the different cell lines. In the "nude" mouse model, animals were treated with a combination of R123 and hyperthermia (43°C for 90 min). A statistically significant (P = 0.04) decrease in tumor growth rate was observed when compared with the rate of tumor growth in untreated animals. The results suggest a potential role for R123 in combination with hyperthermia in the treatment of malignant cells.

INTRODUCTION

R123 is a cationic fluorescent molecule that has been used as a tool in studying the morphology and localization of mitochondria in living cells (1). As a consequence of the high transmembrane potential of the mitochondrion, the R123 molecule, which is lipophilic and positively charged at physiological pH, appears to be specifically concentrated in the mitochondrial matrix. It was discovered, fortuitously, that R123 was retained for longer periods of time by the mitochondria of many carcinoma cells when compared with normal epithelial cells (2). When tested under conditions of prolonged exposure, R123 proved to be selectively toxic for cancer cells in vitro, although prolonged dye retention appeared not to be the sole mechanism of the selective inhibitory effects of R123 (3). These findings suggested a possible role for the molecule in the treatment of cancer. Several in vitro and in vivo studies indicated that the mechanism of toxicity involves the inhibition of mitochondrial energy production (4-6).

The selective toxicity of hyperthermia against cancer cells is well documented (7, 8). Despite this selective toxicity, the utility of hyperthermia in the treatment of human malignancy remains limited. The mechanism(s) of cytotoxicity of hyperthermia is still the subject of much speculation and remains controversial. A number of morphological abnormalities including subtle changes in DNA structure, damage to cell membrane, and subcellular organelles have been found following exposure to heat. Additionally, hyperthermia has been shown to affect a number of cellular functions including metabolism, macromolecular synthesis, membrane function, and the integrity of the cytoskeleton. However, the responsible cytotoxic events remain to be clearly defined. For a review of the mechanisms of cellular damage caused by heat, see Refs. 9 and 10.

Hahn (11) demonstrated that the cytotoxic effect of a number of currently available anticancer drugs was augmented by elevated temperatures. Because R123 had been shown to have significant antitumor activity in mice bearing Ehrlich ascites tumor and MB49 mouse bladder carcinoma (4) and had been shown to have significantly more cytotoxicity at elevated temperatures (12), we initiated studies of hyperthermia in the treatment of human cancer, using R123. This paper describes the effects of R123, hyperthermia, and R123 and hyperthermia in combination on five human tumor cell lines in culture and demonstrates the potential of combined R123 and hyperthermia in controlling the growth of a human melanoma in vivo.

MATERIALS AND METHODS

Solutions and Drugs. Culture medium (Solution E) consisted of VAS medium (KC Biological, Lenexa, KS) with 200 mm l-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Fetal bovine serum (Flow Laboratories, McLean, VA) was added to achieve a final concentration of 10%. R123, laser grade purity (Kodak, Rochester, NY), was prepared as a 1-mg/ml stock solution in distilled water. Further dilutions were made in culture medium. For the in vitro experiments, all the solutions were prequilihibrated to 37°C in a 5% CO2 incubator for 24 h.

Cells. Five different cell lines derived from human cancers were used for the in vitro studies. Two of the cell lines, UCLA-SO-M14 and UCLA-SO-M20, were derived from human melanomas. HT29 is a human colon cancer cell line, P3 a human lung cancer cell line, and B231 is a human breast cancer cell line. Four of the cell lines (M14, M20, HT29, and P3) were selected for study because of the current lack of effective systemic therapy for these histological types of cancer, and the fifth cell line (B231) because previous studies in our laboratory had shown this to be relatively thermal sensitive.

Monolayers of all cell lines were maintained at 37°C in a 5% CO2 incubator in the culture medium described previously. The cells were passaged several days before the experiment to 25-cm2 culture flasks. All studies were done with cells in a logarithmic growth phase. Prior to transplantation to nude mice, cells were maintained in 150-cm2 culture flasks. The cells were trypsinized and washed 3 times in the culture medium prior to s.c. inoculation.

Treatment of Cell Lines. Thirteen 25-cm2 culture flasks were segregated into three groups containing five, four, and four flasks, respectively. The medium in Group 1 was replaced with fresh prequilihibrated medium containing 10 μg/ml of R123. These flasks were subsequently incubated at 37°C and served as the rhodamine treatment group. The medium in Group 2 served as the hyperthermia treatment group. The media in these flasks were replaced with fresh media and subsequently heated to 43°C for varying periods of time, as defined in the treatment protocol. The medium in Group 3 was replaced, in a similar fashion to Group 1, with fresh medium containing 10 μg/ml of R123. These flasks were heated to 43°C simultaneously with those of the second group and served as the group treated with R123 and hyperthermia.

Hyperthermia was administered by immersing the sealed flasks of Groups 2 and 3 in an insulated water bath. Water temperature in the bath was controlled to 43°C ± 0.05°C by a TU-15 thermoregulator (Techne Incorporated, Princeton, NJ), and temperature was verified by
an ASTM-calibrated thermometer. At 30, 60, 90, and 120 min the flasks were removed from their respective environments, and the treatments were terminated by aspirating the medium.

Survival. Following treatment, the cells were trypsinized, suspended in culture medium, and plated on a feeding layer as described previously (13). Briefly, Chee’s modification of Eagle’s medium was warmed to 50°C, after which 3% prewarmed agar was added. The solution was rapidly mixed and dispersed on to plates. Serial dilutions of the cell suspension containing between 20,000 and 90,000 cells were utilized. The plates were kept at 37°C in a CO₂ incubator for 7 days. We have previously demonstrated that cell cultures have a rapid doubling time and that maximal colony growth was obtained between 7 and 10 days, in contrast to cells obtained from fresh biopsy specimens in which the growth rate is substantially lower (14). For this reason, colony numbers were determined on Day 7 with an Omnicon image analysis system. Colony image was enhanced by adding 0.5 ml of tetrazolium violet, a vital dye, to each well 1 day prior to counting.

Each survival value was determined following cell plating in triplicate. Because different cell densities resulted in a variable number of colonies, we determined a plating efficiency utilizing the following formula.

\[
\text{Plating efficiency} = \frac{\text{no. of colonies}}{\text{no. of cells plated}}
\]

The percentage of survival of cells was calculated by determining the optimal plating efficiency of the control cells and comparing this to the plating efficiency of the cells which had undergone R123, hyperthermia, or hyperthermia and R123 treatment as previously described at the identical cell density as the control cells which had achieved optimal plating efficiency.

Mice. Five-wk-old, female, athymic (nude) mice were obtained (Siemens Laboratories, Inc., Gilroy, CA) and individually labeled. Ten million cells in 0.2 ml of Solution E were injected s.c. on the backs of 20 mice. After approximately 2 wk tumors became measurable. We calculated tumor volumes every 2 days by measuring three dimensions of the tumor (length, width, and height) utilizing vernier calipers. The tumor volume was calculated using the following formula (15).

\[
\text{Tumor volume} = \frac{\pi \times L \times W \times H}{6}
\]

where \(\pi = 3.1416\), \(L\) is length, \(W\) is width, and \(H\) is height.

When the tumors were approximately 0.5 ml in volume (range, 0.37 to 0.57 ml), the mice were randomly assigned to one of four treatment groups: (a) control; (b) R123 alone; (c) hyperthermia alone; or (d) R123 + hyperthermia. Following treatment, tumor volumes were recorded for 24 days.

Treatment of Mice. Prior to treatment, all mice were anesthetized with Nembutal (0.4 to 0.5 ml of a 5-mg/ml solution) delivered by i.p. injection. This anesthetic dose kept the mice anesthetized for approximately 90 min. Mice randomized to the R123 alone and R123 + heat groups were weighed and given 15 mg/kg of R123 by i.p. injection. Mice assigned to the R123 + heat group were heated immediately following injection.

A special water bath allowing immersion of nodular tumors without immersion of mice was used (Fig. 1). Water temperature was controlled to 43°C ± 0.05°C with a TU-15 thermoregulator as described previously. At the time of treatment, tumor and rectal temperature probes were placed in the mice assigned to the heat alone and R123 + heat groups. An external fan was used to control core temperature during the 90-min treatment period.

RESULTS

Sensitivity of Cell Lines to R123 or Heat. Fig. 2A summarizes the sensitivity of the five human tumor cell lines to R123. Only the cell line P3 showed some degree of cytotoxicity at 120 min. The remaining 4 cell lines tested in the in vitro experiments did not exhibit any cytotoxicity following exposure to 10 µg/ml of R123 alone for up to 120 min. However, all cell lines were sensitive to R123 following 24-h exposure with 0% survival (data not shown). Each cell line demonstrated progressive sensitivity to 43°C hyperthermia over the 120-min exposure period. At the 30-, 60-, 90-, and 120-min time points, a progressive and characteristic decrease in cell survival was observed with only 6% of M14 cells, 10% of M20 cells, 5% of P3 cells, and 1% of B231 cells surviving after 120 min. The colon cancer cell line demonstrated a greater degree of heat resistance, with 31% survival after 120 min of exposure to 43°C (Fig. 2B).

Fig. 1. Specially constructed apparatus used to hold mice during hyperthermia treatment in water bath. Inset shows a side view of a mouse in the apparatus. Note the nodular tumor protruding through a hole in the tray and the tumor temperature and rectal temperature probes.

Fig. 2. In A, five different malignant human cell lines were exposed to R123 (10 µg/ml) for up to 24 h. Little toxicity was observed with this dose of R123 for up to 120 min of exposure in all but the cell line P3. All cell lines were sensitive to R123 following 24-h exposure with 0% survival (data not shown). In B, each of the cell lines demonstrated progressive thermal sensitivity with a decrease in survival over the 120-min exposure period. The thermal sensitivity varied markedly among different cell lines. The colon cancer cell line HT29 demonstrated the greatest degree of heat resistance. Points, mean; bars, SD.
Exposure to R123 and Heat in Combination. With the exception of the cell line P3 at 120 min of R123 and heat, survival of each of the cell lines following exposure to R123 and heat was less, at each of the time points, than that observed following exposure to heat alone (Fig. 3). Because the experimental design allowed exposure of the cells to each of these agents individually, it was possible, using the following equation

\[ SE = S_R \times S_H \]

where \( S_R \) is survival after exposure to R123 and \( S_H \) is survival after exposure to hyperthermia to calculate an expected survival \( (SE) \) to the combination of hyperthermia and rhodamine at each experimental time interval. This equation assumes that R123 and heat act as independent variables. When the observed survival \( (S_{obs}) \) was less than \( SE \), synergism between R123 and the heat was indicated. The synergistic ratio was determined as follows.

\[ R = \frac{S_E}{S_{obs}} \]

The \( R \) values ranged from 2.31 to 45 for the different cell lines at the point of maximum synergy. This generally occurred between 60 and 120 min.

While some degree of synergy between R123 and heat was observed in each cell line, the heterogeneity of response was striking. The two melanoma cell lines (Fig. 3, A and B) had similar response with \( r \) values of 5 to 8 from 60 to 120 min. The parallel slopes of the heat alone and R123 + heat curves indicate that the degree of synergy was constant with time. By contrast, the P3 lung cancer line evidenced only slight synergy at 90 min (\( r = 2.3 \)) and a less than additive effect at 120 min. The breast cell line B231 was the most sensitive to R123 + heat as well as to heat alone. \( r \) values of 20 were evident at 20 and 90 min. The HT29 colon carcinoma line was the most resistant to heat alone and had the highest \( r \) value (\( r = 45 \) at 120 min) for R123 + heat. The diverging slopes indicated that synergy between R123 and heat was clearly time dependent for HT29 cells.

Studies in Athymic Mice. These in vitro results provided the rationale for the in vivo studies of combined R123 and hyperthermia. The nude mouse model was chosen because the human cell lines tested in vitro could be used and the experimental design used in vitro could be approximated in vivo in terms of treatment time and R123 dosage. Injection of 10⁸ M14 melanoma cells s.c. on the backs of nude mice yielded reproducible nodular tumor growths within 2 to 3 wk. When the volumes of these tumors were followed for 24 days after reaching a predefined range, progressive growth was observed in the untreated controls. Growth rates of tumors in the untreated animals were uniform and led to an approximate linear increase in tumor volume with time.

Hyperthermia was achieved in vivo in all tumors of the combination- and hyperthermia alone-treated groups. Internal tumor temperatures reached at least 42°C for a minimum of 80 min.

In contrast to the tumor growth characteristics of the untreated controls, the tumor growth of the group treated with R123 + heat was significantly affected by the combined treatment. Within 2 days of treatment, tumor growth in this group slowed dramatically, and the growth rate remained low until 14 to 16 days after treatment, at which time the rate of tumor growth increased. During this "slow growth phase" the tumors in the combined treatment group grew at a rate of 0.0084 ml/day compared with a growth rate of 0.0675 ml/day in the untreated controls. This represented an 8-fold decrease in growth rate and led to smaller overall tumor volumes in the R123 + heat group (Fig. 4). When the areas under the growth curves (Days 0 to 24) for the untreated controls were compared

![Fig. 3. Five malignant human cell lines were exposed to R123 (10 μg/ml) alone, heat (43°C) alone, or R123 + heat for up to 120 min. Survival after exposure to R123 alone for 90 min was 100% for all the cell lines. Except for the P3 cell line at 120 min, the sensitivity of the cells to hyperthermia was increased in the presence of R123: A, M14, malignant melanoma; B, M20, malignant melanoma; C, P3, lung carcinoma; D, B231, breast carcinoma; and E, HT29, colon carcinoma. The synergistic ratio varied from 2.31 to 45. Points, mean; bars, SD.](image-url)
with those for the R123 + heat-treated mice, the single-tailed $t$ test yielded a significant difference ($P = 0.04$). The rate of growth and subsequent smaller overall tumor volumes in mice receiving R123 alone or heat alone were less than those of controls and greater than the R123 + hyperthermia-treated group, but these differences were not statistically significant.

**DISCUSSION**

In this study, human tumor cells were exposed to R123 and hyperthermia (42°C) alone and in combination in order to evaluate the potential synergistic effect of the cationic fluorescent molecule with hyperthermia on survival of human cancer cells *in vitro*. The results suggest that R123, in combination with hyperthermia, has a synergistic cytotoxic effect *in vitro*. Further studies of the effect of R123 and hyperthermia on established melanoma tumors in nude mice indicate a similar synergistic effect *in vivo*.

R123 is recognized to be selectively toxic toward certain carcinoma cells (3). The selective toxicity of R123 appears to be in part due to differences in the mitochondria with respect to both membrane potential and sensitivity of F$_0$F$_1$-ATPase to R123 (16). However, the mechanism of R123-induced toxicity has not been completely elucidated. It probably involves inhibition of mitochondrial metabolic activity (17, 18). R123 in high doses has been observed to inhibit oxidative phosphorylation in isolated mitochondria (5) and therefore could deprive cells of ATP and other molecules essential for function and replication. In this study, cell lines were treated with 10 $\mu$g/ml of R123. This dose had previously been shown to be cytotoxic to tumor cells *in vitro* (3). R123 at this concentration demonstrated little cytotoxicity for up to 120 min of exposure in four of the five cell lines studied (M14, M20, HT29, and B231) but had uniform cytotoxicity at 24 h in all five cell lines. There appears to be an important time dependence of the cytotoxic effect of R123, a finding consistent with that of Bernal (3).

The inhibitory effects of elevated temperatures on cell growth in culture are well documented (7). Despite the selective heat sensitivity of cancer cells, it is apparent that the effect is not uniform and that heat-resistant cells can be developed (7, 19). Since both R123 and hyperthermia are independently cytotoxic, establishing that the combination of R123 and hyperthermia results in an additive or synergistic cytotoxic effect is difficult. Dewey (20) defined a synergistic effect of two independently cytotoxic agents when the slope of the dose-response curve of the two agents together was greater than the slope of either agent alone. We performed our experiments on the plateau of the dose-response curve of the R123 (up to 120 min). This definition of synergy did not seem directly applicable to our *in vitro* model. Instead, we chose to define synergy as a measured effect that was greater than the additive effects of each agent independently.

Our results demonstrate a clear time-dependent response to both hyperthermia and R123. The cytotoxicity resulting from exposure to R123 was manifested only after 120 min in all but one cell line. The hyperthermia-associated cytotoxicity appeared to be more pronounced and occurred after a shorter exposure. Additionally, the hyperthermia-induced cytotoxicity varied considerably among cell lines. It is apparent in this setting that the greater the cytotoxic effect of hyperthermia alone, the more difficult to demonstrate any degree of synergy. For this reason, the more heat-sensitive cell lines M14, B231, and P3 demonstrated little synergy (M14) or no synergy (B231 and P3) with R123 after 120 min of hyperthermia, because of the steepness of the hyperthermia dose-response curve. However, at 90 min, the cytotoxicity of R123 + hyperthermia together appeared to be greater than their additive effect, suggesting a degree of synergy. In contrast, thermally resistant cell lines exhibit cytotoxicity greatly in excess of the apparent additive effect of each agent alone. These results suggest that relatively thermoresistant malignancies might be more effectively treated with a combination of R123 and hyperthermia than with either modality alone.

The mechanism(s) of synergy between R123 and hyperthermia is not clear. Potentially, R123 could sensitize the cells to the cytotoxic effects of hyperthermia. Alternatively hyperthermia might enhance the intracellular incorporation of R123 resulting in increased cytotoxicity, or the combination of R123 and hyperthermia might result in an entirely different mechanism of cytotoxicity, distinct from that postulated for the two modalities alone. However, our experimental design does not allow us to define the potential mechanism(s) of increased cytotoxicity observed *in vitro* with R123 and hyperthermia.

The *in vivo* findings with respect to an apparent hyperthermic enhancement of any R123-induced growth reduction were consistent with our *in vitro* findings. We were unable to demonstrate a significant growth difference in tumors implanted on nude mice treated with a single dose of R123 alone. Our experiments were designed to determine the antitumor effects of R123, with and without heat, on established tumors. In order to determine this, we utilized a formula to calculate tumor volumes based on the assumption that the tumor on the back of a nude mouse has characteristics similar to but not identical to a sphere. Because the tumor radius is difficult to determine, we based our volume on measurements of the length, width, and height of the tumor on the assumption that half these measurements would approximate the radius of a sphere. Monitoring of R123 uptake in a murine renal adenocarcinoma by flow cytometry (21) has demonstrated prolonged retention in this tumor model with an associated antitumor effect. In order to minimize this cytotoxic effect, we chose to treat the tumors shortly after the i.p. injection of R123. R123 uptake in culture has been shown to be extremely rapid (6). However, the pharmacokinetics of tumor uptake of R123, administered i.p., has not completely been elucidated. Although the tumor uptake of R123 might have increased over time, perhaps augmenting the response we observed, mice treated with a combination of R123 and hyperthermia in the treatment protocol we designed demonstrated a significant inhibition in the rate of tumor growth within 2 days of treatment when
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compared with control animals. Tumor growth was effectively controlled for 16 days following a single treatment with R123 and hyperthermia. This finding suggests the possibility of prolonging the slow tumor growth phase or even of obtaining tumor regressions with multiple combined treatments of R123 and hyperthermia given during this 14-day interval. The optimal timing, route of administration, and dose of R123 in nude mice remain to be determined.

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