Hyperthermic Enhancement of Rhodamine 123 Cytotoxicity in B16 Mouse Melanoma Cells in Vitro

David N. Krag,1 Alain P. Theon, and Lydia Gan

Department of Surgery, Division of Surgical Oncology, University of California, Davis, Sacramento, California 95817

ABSTRACT

The effect of elevated temperature on cytotoxicity of rhodamine 123 (R123) was tested in vitro on B16 mouse melanoma cells. Simultaneous exposure to R123 and hyperthermia (43°C for 1 h) resulted in marked enhancement of R123 cytotoxicity. Thermal enhancement of R123 cytotoxicity occurred at temperatures as low as 38°C. Heat treatment (43°C for 1 h) given immediately before or after R123 exposure (37°C for 1 h) yielded no significant increase in cytotoxicity over that expected for strict additivity. The effects of heat on two mechanisms reported to be associated with R123 cytotoxicity were evaluated: (a) target inactivation by R123; and (b) R123 intracellular accumulation. Hyperthermia caused an increased rate of target inactivation by R123 and also caused an increased net intracellular accumulation of R123. This indicates that at least two mechanisms are responsible for the synergistic cytotoxicity of R123 and hyperthermia.

INTRODUCTION

R123 is a cationic lipophilic fluorescent chemical that accumulates specifically in mitochondria of living cells (1–4). The uptake of R123 by mitochondria appears to depend on elevated mitochondrial and plasma transmembrane potentials (2, 5–10). Some epithelial cancer cell lines accumulate and retain this dye more than normal epithelial cells (11, 12). Preliminary data from animal experiments have shown that R123 is taken up preferentially in malignant tissue versus surrounding normal tissue (13). As a result of prolonged retention, R123 is selectively cytotoxic to some carcinoma cell lines in vitro and in vivo (2, 14–16). R123 resistance in Friend leukemia and B16 mouse melanoma cells is related to decreased retention and appears to be the result of an efflux mechanism absent in R123-sensitive cells (17–19).

Hyperthermia increases R123 cytotoxicity of HeLa cells in glucose-deprived media (19, 20). Short hyperthermic exposures (90–120 min) at 43°C also increase R123 cytotoxicity in M14 human malignant melanoma cells in vitro and in vivo (21, 22). In addition, a longer exposure (24 hours) at lower temperatures (38°C–41°C) is also effective in increasing the cytotoxicity of R123 on M14 cells in vitro (23). We have determined in vitro that there is greater thermal enhancement of R123 cytotoxicity in two malignant than in three nonmalignant cell lines (24) which support the concept that R123 and hyperthermia may be selectively cytotoxic for cancer tissues in vivo. In view of the clinical potential of rhodamine 123 combined with hyperthermia, we investigated the mechanisms of thermal potentiation of R123 cytotoxicity in vitro.

MATERIALS AND METHODS

Cell Cultures. B16 mouse melanoma cells were maintained in RPMI 1640 supplemented with 2 mM glutamine (both from Sigma Chemical Company, St. Louis, MO), 100 units of penicillin, 100 μg/ml of streptomycin (Grand Island Biological Company, Grand Island, NY), and 10% calf serum (HyClone, Logan, UT). The cells were maintained as monolayers in 25-cm² culture flasks and passed twice weekly. Cell cultures were kept at 37°C in a humidified incubator with a mixture of 95% air and 5% CO₂. All experiments were performed with logarithmic phase cells, obtained by incubation of 8–10 × 10⁶ cells in the culture flasks for 48 h.

Drug Exposure. Rhodamine 123 (laser grade) was kindly provided by Eastman Kodak Company (Rochester, NY). The stock solution (0.1 mg/ml) was kept in the dark at 4°C and the desired concentrations were made in complete medium. Prior to all experiments, the medium with or without R123 was replaced in the flasks with fresh medium adjusted to temperature, pH, and CO₂ level. For survival studies, cells were rinsed 5 times after drug exposure with phosphate-buffered 0.9% saline and then either overlaid with fresh, drug-free medium for subsequent hyperthermia or assayed for survival. For efflux studies the cells were preloaded with R123 (60 μg/ml) for 30 min, rinsed 3 times with phosphate-buffered 0.9% saline, and overlaid with medium free of R123. Uptake studies were performed by adding R123 in medium at time 0.

Heating. Heat exposures were performed by immersing the paraffin-sealed culture flasks in a water bath. Temperature of the bath was controlled to within ±0.03°C (Tempunit TU-16A; Techne). All heat treatments were for 1 h.

Survival Assay. Treated cells were trypsinized, collected, and counted with a Coulter Counter. Cells were then plated in triplicate for colony-forming ability according to the technique of Puck and Marcus (25). All experiments were performed at least three times.

R123 Extraction. At completion of exposure to R123, cells were rinsed 5 times with 2 ml ice-cold phosphate-buffered 0.9% saline and then trypsinized with 1 ml 0.25% trypsin. A 10-μl aliquot of the cell suspension was removed for cell counting with a Coulter Counter. The cell suspension was transferred to a 15-ml centrifuge tube containing 3 ml of extraction solution (50% ethanol and 3 N HCl) and centrifuged at 2000 rpm for 10 min. Emission fluorescence of the supernatant (emission, 525 nm; excitation, 510 nm) was measured on a spectrofluorometer (Varian model SF-330). The single-step extraction procedure yielded greater than 95% total cellular R123 as verified by repeated extractions and measurement of R123 in residual pellet. The emission value of the extracted R123 was compared to a known standard curve to determine the R123 content per million cells. All reported values are the mean of triplicate measurements.

RESULTS

The effects of a 1-h exposure of graded concentrations of R123 (10–60 μg/ml) at 37–43°C on B16 cells are shown in Fig. 1. The R123 dose-survival curve at 37°C showed minimal cytotoxicity. The slopes of the R123 dose-response curves increased steadily from 37–43°C. Drug enhancement factors, listed in Table 1, were calculated as the dose increment (ΔD) of R123 needed to yield a 1/e survival fraction at 37°C divided by the ΔD at temperatures above 37°C. The value of the drug enhancement factor increased almost 10 times as temperature increased from 37°C to 43°C. The sequence effects of heat and R123 exposure on R123 dose-response curves are shown in Fig. 2. Enhanced R123 cytotoxicity by hyperthermia was sequence dependent. Hyperthermia (43°C for 1 h) administered either before or after 1 h R123 exposure at 37°C had no...
significant effect on R123 cytotoxicity. Hyperthermic potentiation of R123 cytotoxicity occurred only with simultaneous exposure.

The survival fraction of B16 cells exposed to 0, 5, and 20 μg/ml R123 was determined at 37–45°C (Fig. 3). At lower temperatures exposure times to R123 were increased up to 4 h to decrease survival to a factor of e for the Arrhenius plot. The Welch-Aspin t test (26) was used to determine the significance of the difference in slopes of time-dependent survival curves at 41–45°C for 0 and 5 μg/ml (Fig. 3, A and B). There was a significant difference in slopes at 41°C at the 5% level. There was no significant difference at 42–45°C. The data was analyzed in terms of an Arrhenius plot (27). The inactivation rate constants, k, were calculated by linear regression analysis and plotted on Arrhenius coordinates. The points were fitted by straight lines and activation energies were determined from the slopes of each line by linear regression analysis. The Arrhenius plot for 20 μg/ml R123 (Fig. 3) was linear in the range of 37–45°C (r = 0.93, P = 0.008). The corresponding activation energy was 87.5 kcal/mol. The curves for 5 μg/ml R123 and for heat alone indicated a discontinuity between 42 and 43°C (Fig. 4). The activation energies calculated for 5 μg/ml R123 below 42°C and above 43°C were 83.8 and 177.9 kcal/mol. The activation energy for heat alone above 43°C was 202.2 kcal/mol. In the range of 39–42°C, the average ratio of inactivation...
rate constants for 5 and 20 µg/ml R123 (determined as the ratio of the intercepts of the two parallel lines on Arrhenius coordinates) was equal to approximately 10. The rate of cell inactivation increased by a factor of 10 when R123 doses were increased by a factor of 4. Thus, the inactivation rate constants were not proportional to R123 dose.

Cellular content of R123/10^6 cells exposed to R123 5 µg/ml (within coordinates) was equal to approximately 10. The rate of cell ratio of the intercepts of the two parallel lines on Arrhenius rate constants for 5 and 20 ng/ml R123 (determined as the cellular content of R123 at all time points compared to cells treated at 37°C. Fig. 6 shows the efflux of R123 from cells preloaded with R123 at 37°C and then placed in R123-free media at 37 or 43°C. There was minimal efflux of R123 at 37°C but a marked and rapid efflux at 43°C.

DISCUSSION

R123 cytotoxicity has been shown to relate to target inactivation and to cellular uptake. The target of R123 cytotoxicity is likely to be in the mitochondria. R123 is cationic and is driven inside the mitochondria by the mitochondrial transmembrane electrochemical gradient (1). Prior to other discernible cell changes, R123 (>10 µg/ml) causes distinct morphological alterations of the mitochondria including swelling, disorganization of cristae, and loss of matrix (28, 29). Mitochondrial swelling induced by R123 is consistent with inhibition of bioenergetic function leading to diminished ion pumping and loss of volume regulation (30). R123 has been shown to disrupt mitochondrial electron transport-coupled ATP synthesis (10, 31, 32). FoF1-ATPase at the inner membrane of the mitochondria has been identified as a potential biochemical target for R123 (10). Cytotoxicity of R123 has been reported to correlate with cellular content of R123 in several different cell types (12, 18, 19). R123 is selectively cytotoxic to cells that retain it for longer periods of time (1, 6, 11, 14).

Our results show that mild elevation of temperature (38–43°C) increases the cytotoxicity of R123. The drug enhancement factor increases almost 10-fold as temperature increases from 37 to 43°C (Table 1). In order to evaluate the effects of heat on R123 cytotoxicity, we investigated the effects of heat on two mechanisms associated with normothermic R123 cytotoxicity: (a) target inactivation by R123; and (b) R123 intracellular accumulation.

Concomitant with cell death is rapid loss of R123 from the mitochondria and from the cell; this phenomenon has been reported to be useful as an indicator of cell death from chemotherapeutic agents (33). As cell death occurred from exposure to R123 and heat, R123 was observed by fluorescence microscopy to be lost from the cell after transient staining of the cytoplasm (24). Rapid loss of R123 from the cell limits the ability to extend cellular uptake studies of R123 to the identical treatment conditions that lead to substantial cell death. To avoid the artifact of altered cellular R123 levels associated with cell death, cellular pharmacokinetics of R123 was determined at 37 and 43°C prior to substantial cell death by using low dose R123 (5 µg/ml) over 0–60 min (see Fig. 5).

The Arrhenius data were consistent with the existence of a critical target for R123. In the range of 39–42°C, cytotoxicity of 5 and 20 µg/ml R123 yielded similar activation energies and, thus, most likely involved a common chemical process. The Arrhenius data revealed that the cytotoxicity of R123 at elevated temperatures was thermodynamically distinct from thermal denaturation. The data indicated also that the inactivation rate by R123 was both temperature and dose dependent. When cells were exposed to 20 µg/ml R123 there was no change in activation energy between 42 and 43°C. The temperature at which there was a break in the Arrhenius curve for heat and 5 µg/ml R123 (between 42 and 43°C) corresponded to a critical temperature common with the hyperthermic inactivation process. Under 42°C, the activation energy for 5 µg/ml was similar to that for cells exposed to 20 µg/ml. However, under 42°C the activation energies for 0 and 5 µg/ml R123 were significantly

<table>
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<tr>
<th>Temperature</th>
<th>D0 ± SEM* (µg/ml)</th>
<th>Drug enhancement factora</th>
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<tbody>
<tr>
<td>37°C</td>
<td>92.0 ± 12</td>
<td>1.0</td>
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<tr>
<td>38</td>
<td>24.6 ± 0.9</td>
<td>3.7</td>
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<tr>
<td>39</td>
<td>18.7 ± 0.5</td>
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<tr>
<td>40</td>
<td>14.1 ± 1.2</td>
<td>6.5</td>
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<tr>
<td>41</td>
<td>14.3 ± 1.5</td>
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<tr>
<td>42</td>
<td>12.3 ± 0.5</td>
<td>7.5</td>
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<tr>
<td>43</td>
<td>9.7 ± 0.6</td>
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*Calculated from data of Fig. 1.

aDrug enhancement factor = D0 (37°C)/D0 (treatment temperature).
different at the 5% level. Above 43°C, the activation energy for 5 μg/ml R123 (177.9 kcal/mol) was in the range of that for heat alone and suggested that thermal denaturation was the predominant reaction.

The lack of proportionality between inactivation rate constants and concentration of R123 seen with the Arrhenius data may reflect an alteration of intracellular accumulation of R123 with heat. The ratio of intra- and extracellular concentration of R123 may be a temperature-dependent phenomenon. Our data show that hyperthermia increases intracellular accumulation of R123 which correlates with cytotoxicity. The mechanism of increased R123 uptake with heat may be related to changes in fluidity or charge of plasma and mitochondrial membranes. Hyperthermia has been shown to increase the fluidity of biological membranes (34). This may be responsible for increased uptake of R123 inasmuch as it has been shown for Adriamycin (35), a drug which shares cross-resistance with R123 (18, 19).

Hyperthermia has also been shown to alter transmembrane potentials (36). The plasma membrane electrochemical potential has been reported to preconcentrate lipophilic cations relative to the extracellular medium, thus increasing the amount available for accumulation by the mitochondria (7). As temperature is increased from 37 to 42°C, reversible hyperpolarization of the membrane potential of tumor cells may occur (36) which could contribute to an increased intracellular accumulation of R123. R123 uptake has been related to mitochondrial membrane potential (6) and is currently used as a sensitive indicator for mitochondrial status under a variety of conditions (3, 37-39). By altering mitochondrial enzyme activities heat may directly increase the inner electronegative charge of the mitochondria. Increased electronegativity of the mitochondrial membrane would result in an increased accumulation of R123. Although heat (43°C) caused a rapid efflux of R123 from the cell it caused a greater influx of R123 when present in the medium at the time of heating. In R123-free media heated to 43°C, the amount of R123 in the cell rapidly decreased to nontoxic levels (Fig. 6). A similar phenomenon has been observed with Adriamycin over the temperature range of 37-45°C (35). At the time of heating, influx of R123 predominated over efflux only if an adequate amount of R123 was present in the medium. This may explain why simultaneous treatment of R123 and heat was needed to achieve synergistic cytotoxicity.

The design of in vivo tests of R123 and hyperthermia will be guided by an understanding of the mechanisms of R123 and heat synergistic cytotoxicity. Our data indicate that both an increased net cellular content of R123 and an increased rate of inactivation contribute to synergistic cytotoxicity of R123 and hyperthermia. The R123 uptake data indicate that heat will probably increase the net cellular content of R123 in malignant cells in vivo only if R123 is present in adequate levels outside the cell at the time of heating. The Arrhenius data show that any elevation of temperature above 37°C would contribute to increased cytotoxicity and the higher the temperature the greater the inactivation of the target of R123 in the cell.

Our in vitro data show that R123 cytotoxicity is increased at temperature clinically achievable during systemic (total body) hyperthermia (≤41.8°C). In order to achieve greater influx than efflux with hyperthermia, the timing and method of in vivo R123 administration will be aimed to maximize the amount of R123 outside malignant cells at the time of heating. Further research must be performed in vivo to determine the clinical potential of R123 because it may be a candidate for use in combination with systemic hyperthermia in cancer treatment.

REFERENCES


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