Quantitative Differential Effects of Rhodamine 123 on Normal Cells and Human Colon Cancer Cells by Magnetic Resonance Spectroscopy

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ABSTRACT

Rhodamine 123 is a lipophilic cationic compound that is selectively taken up by cancer cell mitochondria. This compound is toxic to epithelial cancer cells in vitro and displays significant anticancer activity in vivo. However, the mechanism of action of rhodamine 123 in intact, actively metabolizing cell preparations is unknown. We have used 31P- and 13C-nuclear magnetic resonance spectroscopy to quantitatively characterize how rhodamine 123 affects the energetics of human colon cancer cells (HCT-116) and spontaneously immortalized normal epithelial cells (CV-1). Rhodamine 123 differentially altered the phosphorus and glucose metabolism of HCT-116 and CV-1 cells. 31P-nuclear magnetic resonance detected mitochondrial poisoning in the HCT-116 human colon cancer cell line in its early stages after selective uptake of rhodamine 123. When we compared administration of rhodamine 123 and [1-13C]glucose to administration of [1-13C]glucose alone in the HCT-116 cells, we noted a marked decrease in intracellular pH to 6.7 ± 0.06 (mean ± SD) units, a 2.2-fold increase in lactate production, and a 1.8-fold increase in glucose consumption after 10 h. In addition, we found a 2-fold rise in intracellular free magnesium 12 h after rhodamine 123 administration. These results suggest that when rhodamine 123 inhibits mitochondrial ATP production, it initially stimulates cytoplasmic glycolysis in an attempt to maintain cellular energy demands. The marked fall in intracellular pH and rise in intracellular free magnesium after administration of rhodamine 123 may inhibit activity of several glycolytic enzymes: this effect would inhibit cytoplasmic ATP generation and interfere with multiple cell enzymatic processes, leading to cell death. The CV-1 cells showed no change in intracellular pH, intracellular free magnesium, or magnesium-bound ATP levels over the 24-h period following rhodamine 123 administration. Rhodamine 123 also failed to alter glucose utilization and lactate production levels significantly in the CV-1 cells. These results prove the usefulness of 31P- and 13C-nuclear magnetic resonance spectroscopy for quantifying differing effects of rhodamine 123 on the high energy phosphate metabolism and glucose metabolism of HCT-116 and CV-1 cells.

INTRODUCTION

Epithelial carcinomas, particularly those of breast, colon, lung, and prostate, are the leading causes of death due to cancer in the United States. Most of the established cancer drugs used clinically are directed toward DNA-related cellular metabolic processes (1, 2). They exploit the faster growth rate of cancer cells relative to normal cells and are often cell cycle dependent. Since these drugs are often much less effective against epithelial cancers of breast, colon, lung, and prostate origin (2), there is a need to develop new and more selective drugs against these cancers.

Lipophilic cationic dyes, such as rhodamine 123, are a class of compounds recently shown to exhibit selective antimitochondrial activity for cancer cells of epithelial origin (3). We also know that rhodamine 123 can stain the mitochondria in living cells (4, 5). A wide variety of epithelial carcinoma cells accumulates and retains these compounds, in contrast to untransformed epithelial cells (6). The basis for this selective uptake may be due to the high mitochondrial membrane potential of many carcinoma cell lines (7, 8). Rhodamine 123 is selectively toxic to cancer cells in vitro (1, 9, 10) and displays significant anticarcinoma activity in vivo (1). The higher levels of rhodamine 123 accumulated in the mitochondria of carcinoma cells not only may interfere with mitochondrial metabolism but also may lead to the slow release of rhodamine 123 into the cytoplasm, eventually leading to cell death. The cytotoxicity of rhodamine 123 directly correlates with the intracellular accumulation of rhodamine 123 (10). Lipophilic cationic dyes such as rhodamine 123 may thus be prototypes for a new class of anticancer drugs aimed at the mitochondria of epithelial carcinomas. Studies of isolated mitochondria suggest that cell killing by these cationic compounds involves the inhibition of mitochondrial F1F0 ATPase (11, 12), mitochondrial protein synthesis (13), and mitochondrial transhydrogenase (14). Other studies suggest that rhodamine 123 may inhibit import of precursors to carbamyl phosphate synthetase I and ornithine transcarbamylase into mitochondria of rat hepatocytes (15). Data from Abou et al. (16) suggest that rhodamine 123 might be acting through an uncoupler-like mechanism.

Comparable studies on intact actively metabolizing cancer cells have not been conducted. Investigations based solely on using isolated mitochondria have limited value: in vivo studies have shown that many factors in the cytoplasm (17) seem to influence the mitochondria. Mitochondria that act normally in vitro could behave differently in an environment that had aberrant concentrations of calcium, protons, pyruvate, lactate, glutamine, ATP, and oxygen. We have utilized and perfected a technique that enables us to assess cancer cell metabolism in actively metabolizing cell preparations in a noninvasive and nonperturbing manner. We have maintained anchorage-dependent carcinoma cells and normal epithelial cells in a state of exponential growth in a NMR3 magnet for over 1 week and have shown that our particular microcarrier perfusion system serves as an excellent model system for emulating tumor metabolism in vivo (18). A perfused cell culture preparation has the advantages of allowing for a more uniform delivery of oxygen and thus allows us to investigate the effects of rhodamine 123 on cellular metabolism independent of potential hypoxia that can develop in large in vivo tumors.

This study describes experiments using 31P- and 13C-NMR spectroscopy to investigate the mechanism of cytotoxicity of rhodamine 123 in intact human colon cancer cells by comparing its effects on the energetics of a colon cancer cell line (HCT-116) and a normal epithelial cell line (CV-1). We report specifically on the early metabolic effects of rhodamine 123 on glycolysis, intracellular pH, intracellular magnesium, and high energy phosphate metabolism.

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3 The abbreviations used are: NMR, nuclear magnetic resonance; RD, repetition delay; NA, number of acquisitions; PCr, phosphocreatine; Pi, inorganic phosphate; pHNMR, pH determined by NMR.
MATERIALS AND METHODS

Cell Cultures. We obtained HCT-116 cells, a moderately differentiated line of colon cancer cells, and CV-1 cells, immortalized normal epithelial cells from African green monkey, from American Type Culture Collection (Rockville, MD). Prior to use in our preparations, we periodically tested the cells (Flow Laboratories, Inc., McLean, VA) for Mycoplasma infection and found them to be negative. We grew all cells without antibiotics in T-flasks (175 cm²) at 37°C in L15 media (Gibco) and supplemented them with 10% calf serum in 100% air. When they were 80-90% confluent, we either harvested the cells with a trypsin/EDTA solution (10:1) and used them to seed the microcarriers (described below), or diluted them 1:10 and re inoculated them into new T-flasks. We performed all cell counts on a Coulter Counter. Cell viability was determined by trypan blue exclusion using a hemocytometer.

Collagen-coated Cytodex III beads (170-220 μm diameter) obtained from Pharmacia (Piscataway, NJ) were used for microcarrier culture. We began the seeding process for microcarrier culture by placing 3-4 x 10⁶ cells (HCT-116 or CV-1) on the beads in a Belco spinner flask and growing them until the 100-ml stainless steel culture contained about 1 x 10⁸ cells. Then we placed 5 ml of beads with the attached cells in a specially designed 20-mm diameter bioreactor. We immediately subjected the cells to continuous sterile perfusion with oxygenated 40% O₂ + 0.04% CO₂ (concentration of CO₂ in air), L15 + 10% calf serum, pH 7.3-7.4, at a rate of 4.4 ml/min (18). All cultures were placed in the NMR bioreactor 4 days prior to NMR acquisition with continuous perfusion using galactose-free L15 medium + 10 mM glucose + 10% calf serum. Oxygen tension measurements in a sample of media from around the cells were measured at 26% oxygen with 2.5 x 10⁷ HCT-116 cells perfused under the conditions above. We determined the cell number per volume of bead by removing a known volume of beads, trypsinizing the cells from the beads, and counting them with a hemocytometer. At the time of NMR study, the cells were actively proliferating on the beads with a cell number doubling time of 12 h. The average total cell count in the NMR bioreactor at the time of study was 2-3 x 10⁸ cells.

NMR Measurements. NMR measurements were performed at 8.4 T using a spectrometer built at the Francis Bitter Magnet Laboratory, Massachusetts Institute of Technology. The specially designed large-volume NMR cell bioreactor, used with a commercial broadband observe/proton decouple probe for carbon (90.4 MHz) and phosphorus (145.6 MHz) NMR, was described previously (18). With this system, we were able to obtain spectra with a signal-to-noise ratio of ≥10 in 5 min (RD = 10 s, 90° pulse) for γ-NTP when the average total cell count in the NMR bioreactor at the time of study was 2-3 x 10⁸ cells. Baseline serial 31P-NMR spectra were recorded at 145.6 MHz by applying a 90° pulse with a 10-s repetition delay. Time domain data were collected as 1024 data points using a spectral sweep width of 10 kHz. Line broadening of 20 Hz was used for spectral processing. Using NMR1 software, we obtained peak areas relative to a methylphosphonoacetic acid reference in a 1-mm sealed capillary that we had placed in the bioreactor.

For the 31P-NMR studies, a series of baseline 20-min fully relaxed spectra (RD = 10, NA = 128) were obtained over a 4-h period. Rhodamine 123 was dissolved in medium and diluted in the perfusion media to a final concentration of 50 μg/ml. The rhodamine was added to the perfusate at time 0, and 20-min spectra were accumulated sequentially for 24 h. After 24 h of NMR observations, cell culture samples were taken for determination of cell number and cell viability by trypan blue. At the time of NMR study, the cells were 80-90% confluent, and we harvested the cells with a trypsin/EDTA solution (10:1) and used them to seed the microcarriers (described below), or diluted them 1:10 and re-inoculated them into new T-flasks. We performed all cell counts on a Coulter Counter. Cell viability was determined by trypan blue exclusion using a hemocytometer.

We recorded 13C-NMR spectra at 90.4 MHz by applying a 60° pulse with a RD time of 6 s. A WALTZ-16 proton decoupling sequence (19), using 4 W of radiofrequency power, was applied for 1.5 s during both the interpulse delay and data acquisition. Time domain data were collected as 4096 data points using a spectral sweep width of 24 kHz. Zero filling to 8192 data points with a line broadening of 10 Hz was applied for all 13C-NMR spectral processing. We used NMR1 software to obtain peak areas relative to a [1-13C]mannose reference placed in the above-mentioned capillary tube. The temperature in the bioreactor during proton decoupling was determined as described previously (18). We adjusted the air flow rate to maintain the cells and perfusate at 37°C during the acquisition of proton-decoupled 13C-NMR spectra. Resonance assignments for the 13C-NMR spectra were made by comparing them to spectra of pure metabolites in the L15 + 10% calf serum medium as well as the spectra of published chemical shift data (20, 21). We obtained the content of the 13C-labeled metabolites by peak area integration using NMR1 software and scaled it to the signal area of the [1-13C]mannose resonance.

Our protocol for the 13C-NMR rhodamine studies of glucose metabolism started by obtaining serial 13C-NMR baseline spectra for 2 h for a cell culture perfused with carbohydrate-free L15 + 10 calf serum containing unlabeled [12C]glucose added at 1 mg/ml (5 mM). We then acquired serial 13C-NMR baseline spectra for a 2-h period under the same perfusion conditions. [1-13C] Glucose was added at 1 mg/ml (5 mM) to the perfusate of carbohydrate-free L15 + 10% calf serum at time 0. Serial 15-min 13C-NMR spectra were collected sequentially for a 12-h period following administration of labeled glucose alone. Before initiating the next time course experiment on the same cell culture, we washed the 13C label by perfusing the cells with unlabeled [12C]glucose added at 1 mg/ml (5 mM) to the perfusate of carbohydrate-free L15 + 10% calf serum for 2 h. Following this washout period, 13C-NMR serial 20-min fully relaxed spectra (RD = 10, NA = 128) were obtained over a 2-h period to verify the healthy state of cells prior to proceeding with rhodamine administration to the same cell culture. We then acquired serial 13C-NMR baseline spectra for a 2-h period under the same perfusion conditions. [1-13C] Glucose at 1 mg/ml (5 mM) along with rhodamine 123 at 50 μg/ml were added to the perfusate of carbohydrate-free L15 + 10% calf serum at time 0. Serial 15-min 13C-NMR spectra were collected sequentially for a 12-14-h period following administration of labeled glucose and rhodamine 123. This was followed by recording 31P-NMR follow-up spectra for 2-4 h to assess changes in phosphate metabolism in the culture 16 h after rhodamine 123 administration.

Measurement of Intracellular pH_{NMR} and Intracellular Magnesium. The PCr to Pi chemical shift was used to estimate the intracellular pH for the cells in vitro (22). We calculated the intracellular free magnesium concentration using the method described by Mosher et al. (23), which accounts for the influence of pH on the chemical shift separation of α- and β-phosphorus resonances of ATP.

Cell Extracts. HCT-116 cells and CV-1 cells on microcarriers were placed in solutions of cold 7% (w:v) perchloric acid containing a volume ratio of beads to extraction solution of 1:8. We homogenized the suspension at 4°C with a Dounce homogenizer, neutralized with 3 mM KOH to pH 7.4, and centrifuged for 10 min at 5000 rpm. The extract’s supernatant was removed and stored at -80°C until the time for NMR spectral analysis, when it was lyophilized and reconstituted with 1.5 ml H₂O containing 20% (v:v) D₂O.

Creatine Kinase Assay. We separated the isoenzymes of creatine kinase according to their electrophoretic mobility on agarose gel at 100 V for 20 min. After separation, the agarose gel plate was incubated with Titan gel creatine kinase isoenzyme reagent at 45°C for 10 min, dried, visually inspected for bands with a UV lamp source, and photographed.

RESULTS

Fig. 1 shows fully relaxed 31P-NMR spectra of HCT-116 cells and CV-1 cells on microcarriers being perfused at 37°C with culture medium. When shimming on protons, with the bioreactor loaded with cells, it was possible to routinely obtain line widths of 15-20 Hz. The 11 observed phosphorous peaks, i.e., phosphoethanolamine, phosphocholine, P_i, glycerophosphoethanolamine, glycerophosphocholine, PCR, adenosine triphosphate (γ-, α-, and β-ATP), nicotinamide adenine dinucleotide, and uridine diphosphoglucone, are labeled in Fig. 1. All chemical shifts are referenced to that of PCr at -2.52 ppm with respect to phosphoric acid at 0 ppm. We identified the peaks by analyzing the pH dependence of chemical shifts of perchloric acid extracts prepared at pH 7, pH 8.5, and pH 10 and by addition of known compounds to these cell extracts as well as from published phosphorus chemical shift data. The single inorganic phosphate peak observed was roughly 66% from intracellular inorganic phosphate as estimated from studies which rapidly change the pC0₂ (and thus pH) of the external media. The pH of the culture medium, as derived from NMR measurement, was 7.45 ± 0.04 and agrees with the value measured using a pH electrode. The absence of a separate, clearly discernible Pi peak starting from the intracellular compartment is indicative of a high
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Fig. 1. A, 31P-NMR spectra of 3 × 10^4 HCT-116 cells at 37°C; 90° pulse, RD = 10 s, NA = 256, line broadening of 10 Hz. B, 31P-NMR spectra of 2.5 × 10^8 CV-1 cells at 37°C; 90° pulse, RD = 10 s, NA = 512, line broadening of 10 Hz. PE, phosphoethanolamine; PC, phosphocholine; GPE, glycerophosphoethanolamine; GPC, glycerophosphocholine; NAD, nicotinamide adenine dinucleotide; UDPG, uridine diphosphoglucose.

Table 1

<table>
<thead>
<tr>
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<th>CV-1 in vitro (microcarriers)</th>
<th>HCT-116 in vitro (microcarriers)</th>
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<tbody>
<tr>
<td>PCr/γ-NTP</td>
<td>0.09 ± 0.02</td>
<td>0.86 ± 0.05</td>
</tr>
<tr>
<td>PC^α-γ-NTP</td>
<td>1.5 ± 0.18</td>
<td>0.79 ± 0.12</td>
</tr>
<tr>
<td>PE/γ-NTP</td>
<td>0.95 ± 0.18</td>
<td>0.99 ± 0.23</td>
</tr>
<tr>
<td>GPE/γ-NTP</td>
<td>0.18 ± 0.03</td>
<td>ND</td>
</tr>
<tr>
<td>GPC/γ-NTP</td>
<td>0.38 ± 0.11</td>
<td>ND</td>
</tr>
<tr>
<td>Intracellular pH</td>
<td>7.40 ± 0.04</td>
<td>7.46 ± 0.04</td>
</tr>
<tr>
<td>Intracellular Mg^2+ (mm)</td>
<td>0.230 ± 0.023</td>
<td>0.245 ± 0.038</td>
</tr>
</tbody>
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*p, phosphocholine; PE, phosphoethanolamine; GPE, glycerophosphoethanolamine; GPC, glycerophosphocholine; ND, not detectable.

The pH of NMR and healthy state of the cells. The ADP level was not detectable by NMR when measured as the difference between the combined intensity of the γ-ATP and β-ADP signals minus the β-ATP signal. The majority of ADP is protein bound and may be NMR “invisible” so the lack of a difference between the γ- and β-ATP molecules is difficult to interpret. Comparison of the CV-1 and HCT-116 in vitro cell spectra (Fig. 1) reveals that the HCT-116 cells have 7-fold higher levels of PCr than CV-1 (P = 0.0001) cells but lower levels of phosphocholine, glycerophosphoethanolamine, and glycerophosphocholine (P = 0.01). Table 1 shows the resulting phosphate metabolite ratios, pH_{NMR}, and intracellular free magnesium concentrations that were obtained from the HCT-116 cells and the CV-1 cells. Agarose gel electrophoresis detected the presence of large quantities of creatine kinase BB isoenzyme that was not detected in the CV-1 cells.

Effect of Rhodamine 123 on Phosphorus Metabolism. Fig. 2 shows the effects of 50 μg/ml of rhodamine 123 on the phosphorus metabolism of 3 × 10^6 perfused HCT-116 cells on microcarrier beads. Under baseline conditions (spectrum at t = 0), the cells had a high PCr/β-ATP ratio of 0.91, and a single inorganic phosphate peak corresponding to pHE = 7.4 was measured. In the spectrum acquired 2 h after administration of 50 μg/ml of rhodamine 123, there was a roughly 10% increase in β-ATP, a 10% increase in PCr, and an unchanged level of P1. Ten h after administration of rhodamine 123, the PCr resonance disappeared and the β-ATP peak area was reduced to 75% of the baseline level. The inorganic phosphate resonance was then split into 2 peaks, corresponding to extracellular pH 7.3 and intracellular pH_{NMR} of 6.8. At 20 h post-rhodamine 123 administration, there was no detectable PCr and ATP was reduced to 20% of baseline levels. The intracellular P1 peak had increased by 230%, and intracellular pH_{NMR} decreased further to 6.0. We repeated the above experiment on 5 separate HCT-116 cell preparations, each containing between 2 × 10^8 to 3 × 10^8 cells, with similar results. Fig. 3 summarizes the average effects of 50 μg/ml of rhodamine 123 on the intracellular pH_{NMR}, PCr, ATP, and P1 levels in these 5 experiments. There was a rapid fall in PCr to an undetectable level by 10 h (P = 0.0001, n = 5). The β-ATP peak increased by 21% ± 7% (mean ± SD; P = 0.022, n = 5) at 2 h and then steadily declined to 83% ± 10% (P = 0.04, n = 5) of baseline levels at 10 h and to 16% ± 10% (P = 0.0001, n = 5) of baseline ATP level at 24 h. Inorganic phosphate rose steadily, paralleling the fall in high energy phosphates, and by 24 h it had risen to 240% ± 22% (P = 0.0001) above baseline levels. The phosphomonoester levels remained relatively constant over a 24-h period following rhodamine 123 administration. The mean intracellular pH at baseline was 7.45 ± 0.04 (n = 5). The pH values...
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then fell steadily to 7.2 ± 0.04, 6.7 ± 0.06, and 6.2 ± 0.06 at 4, 10, and 16 h, respectively. At the end of the 24-h run, the HCT-116 cells remained viable (94%) by trypan blue staining; they became trypan blue positive within a 1–2-day period following the NMR experiment.

Fig. 4 summarizes the effects of rhodamine 123 on CV-1 cells. We doubled the concentration of rhodamine 123 in these experiments to 100 µg/ml. The CV-1 cell line, in contrast to HCT-116, showed no detectable changes (Fig. 4) in ATP or phosphomonoester levels (+ 5%, P > 0.05), and intracellular pH remained constant over the entire 24-h period after administration of 100 µg/ml of rhodamine 123.

Intracellular Free Magnesium. The baseline intracellular free Mg²⁺ level for the HCT-116 cells was calculated to be 0.245 ± 0.038 mM. Fig. 5 shows the change in intracellular free magnesium level, corrected for pH, as the pH dropped from 7.45 to 6.2 over the first 16 h after rhodamine 123 was administered. The free magnesium level in the HCT-116 cells increased 1.9-fold at 8 h post-rhodamine 123, reaching an average level of 0.465 ± 0.141 mM (n = 4, P = 0.006). At 14 h post-rhodamine 123 administration to HCT-116 cells, the intracellular free magnesium increased 3.4-fold over baseline values to an average level of 0.824 ± 0.255 mM (n = 4, P = 0.0001).

Effects of Rhodamine 123 on Carbon Metabolism. To evaluate the biochemical effects of rhodamine 123 on glycolysis, we administered [1-¹³C]glucose to the HCT-116 cells, both with and without rhodamine 123, in identical cell preparations. Fig. 6 compares the course of [1-¹³C]glucose utilization and [3-¹³C]lactate production between these preparations: we evaluated cells that we perfused with 5 mM [1-¹³C]glucose against those perfused with both 5 mM [1-¹³C]-glucose and 50 µg/ml of rhodamine 123. Estimates in fmol/cell/h were calculated for 4 different cultures by determining the slope of the linear best fit for [3-¹³C]lactate and [1-¹³C]glucose levels 2 to 10 h after label administration. During this 8-h period following administration of 5 mM [1-¹³C]glucose alone, the mean glucose utilization and lactate production rates were 271 ± 27 fmol/cell/h and 150 ± 25 fmol/cell/h, respectively; 10 h after administration of 5 mM [1-¹³C]-glucose plus 50 µg/ml of rhodamine 123, the mean glucose utilization and lactate production rates were 488 ± 47 fmol/cell/h and 333 ± 32 fmol/cell/h, respectively. The addition of the 50 µg/ml of rhodamine 123 in these cells thus yields a 1.8-fold increase in [1-¹³C]glucose alone, the mean glucose utilization and lactate production rates were 271 ± 27 fmol/cell/h and 150 ± 25 fmol/cell/h, respectively; 10 h after administration of 5 mM [1-¹³C]-glucose plus 50 µg/ml of rhodamine 123, the mean glucose utilization and lactate production rates were 488 ± 47 fmol/cell/h and 333 ± 32 fmol/cell/h, respectively. The addition of the 50 µg/ml of rhodamine 123 were administered, we noted that after 10 h, 68% ± 2% of the glucose that was utilized was released as [3-¹³C]lactate in the presence of oxygen. When rhodamine 123 was absent, we found that 55% ± 4% (n = 4, P = 0.03) of the glucose utilized had been released as (3-¹³C) lactate while oxygen was present.

Studies of the impact of rhodamine 123 upon glucose and lactate levels in CV-1 cells led to results different from those of our HCT-116 research. Fig. 7 compares [1-¹³C]glucose utilization and [3-¹³C]lac-
This 8-h period after administration of 5 mM [1-13C]glucose alone, the rhodamine 123 were coadministered with 5 mM [1-13C]glucose under administration of the antimitochondrial agent rhodamine 123. We found that 76% ± 6% (n = 4) of the glucose utilized was released as [3-13C]lactate 10 h after labeled glucose administration in the absence of rhodamine 123: in the studies of coadministration of rhodamine 123 and glucose, 72% ± 8% (P > 0.05) of the glucose utilized was released as [3-13C]lactate 10 h after initial label administration. Thus, rhodamine 123 administration failed to cause significant increases in either glucose utilization or lactate production in these normal epithelial cells.

DISCUSSION

In this study, we have identified several early biochemical changes in intact, actively metabolizing cancer cell preparations following administration of the antimitochondrial agent rhodamine 123. We have used 31P- and 13C-NMR spectroscopy to quantify alterations in high energy phosphate metabolism, intracellular pH, intracellular free magnesium, and glucose catabolism after administering rhodamine 123 to 2 cell lines: the HCT-116 line of human colon cancer cells and the normal epithelial cell line CV-1. Rhodamine 123 caused significant biochemical changes in the HCT-116 cell line that were not evident in the CV-1 cell line. Just 2 h after administration of rhodamine 123 to the HCT-116 cells, there was a 20% rise in ATP levels concurrent with a 6% decrease in PCr level, a 30% increase in inorganic phosphate levels, and no significant change in pHNMR. This initial rise in ATP of 20% although small represents a statistically significant increase in ATP levels at 2 h from baseline (P = 0.02). This significant increase in ATP along with the 6% decrease (minimal) change in PCr levels suggests a 26% increase in NMR visible ADP assuming no change in the creatine kinase equilibrium constant. Thus, the apparent rise in NMR visible ATP, ADP, and Pi levels with only a small decrease in PCr level suggests that a portion of the mitochondrial bound ATP and ADP in the untreated state may be NMR invisible. Following addition of rhodamine 123, early changes in mitochondrial ATP/ADP translocase or FoF1 ATPase kinetics may result in the increased release of mitochondrial ADP or protein bound ADP which was previously NMR invisible to the cytoplasmic compartment. This would have the net effect of adding additional NMR visible phosphate to the system and thus explain the rise in ATP, ADP, and Pi that was found in the absence of a corresponding decrease of the NMR visible phosphorus compounds measured.

The higher ADP levels drive cytoplasmic glycolysis in the colon cancer cells under study. We note as evidence that in the [1-13C]-glucose-labeling experiments we found that over the first 10 h following rhodamine 123 administration to HCT-116 cells, there was a 2.2-fold increase in [3-13C]lactate production compared to the control where rhodamine 123 was absent. With this large increase in lactate production, intracellular pH begins to fall after 2 h.

At 6 h, PCr levels are reduced to 40% of baseline and ATP levels are essentially unchanged. Intracellular pH has fallen about 0.4 pH unit. This fall would shift the creatine kinase equilibrium from PCr to ATP, accounting for the fall in PCr levels and stable ATP levels. At 8 h, the intracellular pH has fallen to 6.9, with a 2-fold increase in intracellular free magnesium, to an average level of 0.465 ± 0.141 mM, and ATP levels are 90% of their baseline value. By 10 h PCr is not detectable by NMR. The cells, having depleted their energy reserves in the form of PCr, have ATP levels that are about 75% of their baseline; 16 to 24 h after rhodamine 123 administration, the HCT-116...
The rise in intracellular free magnesium may be due to 2 possible sources: (a) either inhibition of magnesium efflux across the plasma membrane or stimulation of magnesium influx across the plasma membrane; or (b) release of magnesium from an intracellular compartment such as mitochondria. Influx across the plasma membrane is sensitive to extracellular pH and to ionophores that alter the intracellular pH or transmembrane pH gradient (30). Mitochondrial magnesium levels are well below electrochemical equilibrium with passive influx and active efflux.

The large size of the mitochondrial pool of magnesium could be important in controlling cytoplasmic magnesium concentration. Recent work in isolated heart mitochondria has suggested that the rapid respiration-dependent uptake of magnesium and the slower respiration-dependent efflux of mitochondrial magnesium probably occur through different pathways (31). Magnesium uptake seems to occur in response to the mitochondrial membrane potential. In contrast, the efflux of magnesium from mitochondria is consistent with the presence of a pH gradient-dependent Mg²⁺/H⁺ antiport pathway for magnesium extrusion. Uncoupling agents such as fluoroacetyl-cyamidophenylhydrazone, which cause the collapse of both the proton and membrane potential gradient, can bring about the release of accumulated magnesium from mitochondria (32).

In the present study, we see a 2-fold increase in intracellular free magnesium as early as 8 h after rhodamine administration and a 3.4-fold increase in free magnesium after 14 h of rhodamine exposure. In vitro studies have shown that 6–8 h after continuous rhodamine exposure there is a loss of mitochondrial fluorescence with continued cytoplasmic fluorescence for as long as 24 h (9). The redistribution of rhodamine 123 in the cell implies dissipation in the mitochondrial membrane potential after 6–8 h of exposure. This change may account for the increased levels of cytoplasmic free magnesium levels, since a fall in membrane potential should inhibit the influx of magnesium through the membrane. The resulting high cytoplasmic free magnesium level would in turn inhibit hexokinase, severely restricting the cell’s ability to use glucose for production of ATP. The only other potential source of an increased intracellular free magnesium level would be a decrease in ATP level to maintain equilibrium. However, we feel that this contribution to the increased intracellular free magnesium is small since at 8 h a 2-fold increase in magnesium level is seen with only a 10% decrease in ATP level.

The marked fall in intracellular pH following rhodamine administration seems related to several factors. (a) There is a 2-fold increase in lactic acid production (0–10 h) as a result of increased glycolysis. (b) ATP limitation (12–24 h) may diminish the Na⁺/H⁺ antiport activity. (c) Hydrolysis of ATP that produces hydrogen ions may contribute to the fall in pH. The fall in intracellular pH acts to suppress DNA synthesis and cell proliferation (33): it also interferes with multiple cellular enzymatic functions.

Previous studies with isolated mitochondria have suggested that the basis for cell killing upon prolonged exposure to rhodamine 123 depends upon inhibition of mitochondrial function: rhodamine 123 inhibits mitochondrial F₁Fₒ ATPase (11, 12), mitochondrial protein synthesis (13), mitochondrial transhydrogenase, and mitochondrial import of both carbamyl phosphate synthetase I and ornithine transcarbamylase (14, 15). Others have found that glycolysis alone can provide tumor cells with enough energy and substrates for survival and growth (34). Hence, inhibition of mitochondrial ATP production is insufficient by itself to mediate cellular toxicity. From the present work, we postulate that the rise in intracellular free magnesium and fall in intracellular pH between 4 and 16 h after rhodamine 123 administration may together be sufficient to disrupt cytoplasmic energy generation and enzymatic processes.

![Fig. 7. [1-13C]Glucose utilization and [3-13C]lactate production in CV-1 cells with and without rhodamine 123, following administration of 1 mg/ml [1-13C]glucose to the per- fusate. Note the break in the ordinate scale.](image-url)
The present study suggests that rhodamine 123 produces an early decrease in pH and rise in intracellular magnesium levels in intact cancer cells while causing a marked increase in lactate production. These findings support the idea that inhibition of mitochondrial ATP production by rhodamine initially stimulates cytoplasmic glycolysis. As free intracellular magnesium increases, it would impair glycolysis, leading to a further deficit of ATP as well as impaired ATP utilization. Thus, the alterations in intracellular free magnesium and intracellular pH brought about by rhodamine 123 may eventually disrupt cytoplasmic glycolysis. The selective uptake of rhodamine 123 and its poisoning of the mitochondria in the HCT-116 line of human colon cancer cells can be detected sensitively in their early stages by 31P- and 13C-NMR.

The selective toxicity of rhodamine 123 to the human colon carcinoma cell HCT-116 compared to that for CV-1 is proven by the fact that 31P-NMR reveals no change in ATP levels, intracellular pH, and magnesium levels over a 24-h period despite doubling of the dose of rhodamine 123 (100 μg/ml) administered to the CV-1 cells. In addition, we observed no significant change in glucose utilization or lactate production following rhodamine 123 in the CV-1 cell line. Thus, rhodamine 123 has a differential effect on the high energy phosphate and glucose metabolism of the HCT-116 line of colon cancer cells compared to that of the normal epithelial CV-1 cells.

This study shows the potential for 31P- and 13C-NMR to predict the response of carcinoma cells to antimitochondrial agents such as rhodamine 123. Since NMR can monitor changes in intracellular metabolites, pH, and magnesium levels continuously in actively metabolizing cell preparations, we have a better understanding of the events mediating cellular toxicity for rhodamine 123. Such studies may lead to the design of new and effective lipophilic cationic agents. This research may also suggest new ways of altering cellular metabolism to improve cancer cell kill rates with these novel agents. Knowledge of cellular biochemistry and energetics would serve as the foundation for this work, in contrast to the empirical trials that currently serve as the standard for drug efficacy.

REFERENCES

Quantitative Differential Effects of Rhodamine 123 on Normal Cells and Human Colon Cancer Cells by Magnetic Resonance Spectroscopy
