Energy Metabolism of Human LoVo Colon Carcinoma Cells: Correlation to Drug Resistance and Influence of Lonidamine

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ABSTRACT

The relationship between modification of energy metabolism and extent of drug resistance was investigated in two sublines (LoVoDX and LoVoDX10) from human LoVo colon carcinoma cells that exhibit different degrees of resistance to doxorubicin. Results indicated that the extent of alteration in energy metabolism strictly correlated with degree of resistance. In LoVoDX cells, only 14CO2 production was enhanced, whereas in the more resistant LoVoDX10 cells, both 14CO2 and aerobic lactate production were stimulated. The basal and glucose-supported efflux rate and the amount of drug extruded by LoVoDX10 cells were significantly higher than in the resistant LoVoDX cells. Because the expression of surface P-170 glycoprotein was similar in both cell lines, this phenomenon was attributed to increased efflux pump activity resulting from greater ATP availability. Inhibition of 14CO2 production, aerobic glycolysis, and clonogenic activity by lonidamine (LND) increased with enhancement of the energy metabolism. Moreover, LND, by affecting energy-yielding processes, reduced intracellular ATP content, lowered the energy supply to the ATP-driven efflux pump, and inhibited, almost completely, doxorubicin extrusion by resistant LoVo cells. These findings strongly suggest that LND, currently used in tumor therapy, reduces drug resistance by restoring the capacity to accumulate and retain drug of cells with the MDR phenotype that overexpress P-170.

INTRODUCTION

The major obstacle to effectiveness in cancer chemotherapy is the development of drug resistance, which remains one of the main reasons of treatment failure. Some tumors are inherently resistant to cytotoxic drugs; others initially respond, but become resistant during treatment as a consequence of the selection of preexisting resistant cell population and/or drug-induced mutations. The diminished sensitivity to the original drug is often associated with a cross-resistance to other drugs, diverse in their chemical structure and targets, and this phenomenon is referred to as MDR1 (1).

One of the most important mechanisms of MDR involves a decrease of drug content within the cell resulting from an increased expression of a M, 170,000 membrane glycoprotein, termed P-170 (2–5). This protein, a product of the MDR-1 gene, acts as an energy-dependent efflux pump that reduces the intracellular concentration of antitumor drugs by exporting them across the plasma membrane.

Neoplastic transformation modifies cellular energy metabolism (6, 7). In normal differentiated cells, oxidative phosphorylation is the major metabolic pathway for ATP synthesis. In cancer cells, even in the presence of oxygen, glucose catabolism is elevated with associated higher lactate production, which increases with malignancy (8–11). Elevate aerobic glycolysis raises the concentration of glucose 6-phosphate, which not only provides a cellular supply of ATP but also produces high levels of metabolites for lipid, protein, and nucleic acid biosynthesis, all of which are necessary for cell growth and replication (12).

The development of MDR generally correlates with enhanced energy metabolism, consistent with a higher energy requirement for drug efflux at the expense of ATP hydrolysis by P-170. However, in a variety of doxorubicin-resistant cell lines, there does not appear to be a single or common metabolic alteration, with resistant cell lines developing metabolic alterations on the basis of histogenetic derivation and/or pattern of malignancy (13–18).

The current study was undertaken to examine the relationship between the modification of energy metabolism and extent of drug resistance in two sublines from human LoVo colon carcinoma cells that exhibit different degrees of resistance to doxorubicin. Moreover, the sensitivity to energy-depleting agents of cells with the MDR phenotype, due to an overexpression of P-170, would be expected to increase as a function of the energy metabolism. Therefore, the effect of LND, which inhibits both energy-yielding processes (19–21), was also evaluated.

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3 The abbreviations used are: MDR, multidrug resistance; LND, lonidamine.
MATERIALS AND METHODS

Chemicals. d-[6-14C] glucose was purchased from Amersham Pharmacia Biotech; ATP and reagents for enzymatic assay were obtained from Roche Molecular Biochemicals. N-Tris(hydroxymethyl)-2-aminoethanesulfonic acid was purchased from Sigma. Ham’s F-12 culture medium, FCS, and glutamine were purchased from BioWhittaker. Doxorubicin was furnished by Farmitalia. Hyamine was purchased from Packard Instruments Italia, and Aquassure scintillation liquid was purchased from NEN Life Science Products. All other reagents were of analytical grade and purchased from BDH Italia.

Cell Lines. Doxorubicin-sensitive and -resistant human LoVo colon carcinoma cells were kindly supplied by Dr. M. Colombo (Istituto Nazionale Tumori, Milano, Italy). The doxorubicin-sensitive cell line (LoVo) was propagated as a monolayer culture in Ham’s F-12 medium supplemented with 10% FCS, vitamins, antibiotics, and glutamine. The doxorubicin-resistant line (LoVoDX) were grown in the same culture medium supplemented with 1.2 μg/ml doxorubicin. The resistant subclone LoVoDXH10 was induced as described above by Yang and Trujillo (22) by exposing LoVoDX cells to 1 pulse of doxorubicin (10 μg/ml). For each pulse treatment, LoVoDX cells (2 × 10^5) were plated on a 25 cm² Corning flask. At exponential growth phase, cells were exposed to 10 μg/ml doxorubicin for 1 h and immediately replated at a density of 500 cells/60-mm Petri dish in doxorubicin-free growth medium. After a 2-week incubation at 37°C, colonies were isolated by trypsinization and grown to confluence prior to further pulse treatment. The LoVoDXH10 subclone was established 7 months following initial treatment. LoVoDXH10 cells were routinely grown in Ham’s F-12 medium supplemented with 10 μg/ml doxorubicin. Prior to experiment, cells were grown in drug-free medium for 2 weeks.

Drug sensitivity of LoVo, LoVoDX, and LoVoDXH10 was evaluated by calculating the drug dose responsible for 50% cell killing (IC50 value). Briefly, cells plated in triplicate at a density of 2 × 10^5 cells/60-mm-diameter tissue culture dish were incubated for 24 h in Ham’s F-12 medium supplemented with 10% FCS, and the appropriate doxorubicin concentrations were added. After 8 days, dishes were washed with PBS, and cells were detached by trypsinization, stained with trypan blue, and counted in a Fuchs-Rosenthal chamber. All cell lines were examined simultaneously, and all experiments were performed at least four times.

Drug. LND, obtained from the F. Angelini Research Institute, was dissolved in DMSO at a concentration of 10 mg/ml (31.1 mM) immediately prior to use and sterilized by filtration through a 0.22 μm Millex GV filter (Millipore).

Determination of P-170 Glycoprotein by Flow Cytometry. The monoclonal antibody Mab57, kindly provided by Dr. M. Cianfriglia (Istituto Superiore di Sanità, Rome, Italy), which recognizes an external domain of P-170 (23), was used to detect the P-170 expression as reported previously (24). Briefly, 5 μl of propidium iodide (1 mg/ml) was added to each sample prior to fluorescence-activated cell sorting analysis to exclude nonviable cells. Samples were analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Logarithmic green fluorescence of FITC-bound Mab57 was collected after filtration through a BP 530/15 filter, and the linear red propidium iodide fluorescence was collected after filtration through an LP 620 filter. Ten thousand duplicated events for each sample were accumulated and data were analyzed using Becton Dickinson LYSIS II C-32 software.

Assay of 14CO2 Production, Aerobic Glycolysis, ATP Content, and Enzyme Activities. Doxorubicin-sensitive and -resistant subclones of human LoVo colon carcinoma in exponential growth (5th day of culture) were detached by EDTA (0.02% in Ham’s F-12 medium for 5 min), recovered by centrifugation (600 × g for 5 min), washed twice in NKT buffer (105 mM NaCl, 5 mM KCl, 50 mM N-tris(hydroxymethyl)-2-aminoethanesulfonic acid, pH 7.40), counted in a Coulter counter (model ZM, Coulter Electronics), and resuspended in NKT medium at a concentration of 5 × 10^5 cells/ml. Determination of 14CO2 production from [6,14C]glucose, aerobic glycolysis, ATP content, and enzyme activity were performed as described previously (16, 17).

Dose-dependent Proliferation Analysis for LND Cytotoxicity. For these experiments, 1 × 10^5 cells were plated onto 25-cm² culture flasks (Corning). On the 5th day of culture, during exponential growth, freshly prepared drug was added to the flasks, which were then incubated for 24 h. Appropriate DMSO controls evaluated under the identical experimental conditions exhibited no toxicity. Following incubation, medium was discarded, and cells were detached with 0.02% EDTA for 1 min and counted in a Coulter Counter. Aliquots of cell suspension of known concentration were then dispersed into 80-mm plastic Petri dishes (five dishes for each point), and colonies were allowed to grow for 15–18 days. In each experiment plating efficiency of at least five controls was assayed simultaneously. The proliferating fractions for different drug concentrations were normalized with respect to the individual control for each experiment. All experiments were performed at least three times, with five samples for each drug concentration. The fitting and analysis of curves was as reported previously (25, 26).

Analysis of Doxorubicin Distribution and Efflux. The intrinsic fluorescence emission of doxorubicin, when exited at 488 nm, was used to monitor intracellular drug distribution and to measure drug efflux kinetics in drug-sensitive and -resistant human LoVo colon cancer cells. For this purpose, cells were plated onto 35-mm dishes and used on the 5th day of culture. Cells were rinsed with fresh medium and incubated with 20 μM doxorubicin, in the presence or absence of 0.2 mM LND, for 1 h at 37°C in 5% CO2. Cells were then washed twice with NKT buffer and mounted on the stage of a Zeiss Axioskop upright microscope interfaced with a real time laser confocal fluorescence microscope (Osydsee, Noran Instruments, Redwood City, CA) equipped with an Argon laser. Cells were visualized by a × 40 water immersion objective (Zeiss, numerical aperture = 0.75) and continuously perfused with NKT buffer, with or without 6 mM glucose, with a glass pipette positioned close to the cell field and connected to a gravity-driven perfusion system. The effect of glucose on drug extrusion kinetics was studied by switching from control to the glucose-containing reservoir. Image acquisition and analysis were performed using IMAGE-1 software (Universal Imaging Corp.). Cells were exited at 488 nm, and fluorescence emission was monitored at λ > 515 nm with a confocal slit of 100 nm. The laser beam was set at
60–90% intensity. Fluorescence was monitored for 50 min at one digitized image/min to minimize doxorubicin bleaching and irradiation toxicity by a customized protocol within Image-1. An averaged image \((256 \times 256\) pixels) from 100 images, taken by exposing the cells to light for 4 s, was stored on the computer hard disc. In some experiments real time acquisition (video rate, 25 Hz) was used to better resolve the drug extrusion kinetics. In this case, images were stored on a video recorder (Sony VO 9600P, Tokyo, Japan). Both sets of images were analyzed off-line, extracting fluorescence values in the function of time from manually positioned areas on nuclear and cytoplasmatic regions of each cell, using the brightness versus time function of IMAGE-1. Data were displayed as space averaged fluorescence intensity. At least 20 cells were measured for each set of experimental data, and mean values were plotted and normalized with respect to the first determination.

RESULTS

Resistance Profile of LoVo Subclones. The \(IC_{50}\) values for sensitive cells (LoVo) and two doxorubicin-resistant subclones (LoVoDX and LoVoDX\(_{10}\)) were 0.05, 2.10, and 10.20 \(\mu\)M, respectively, following 8 days of doxorubicin treatment (Table 1). These values indicated that LoVoDX and LoVoDX\(_{10}\) were 42 and 204 times more resistant to doxorubicin, respectively, than the parental LoVo cell line.

To establish whether the greater resistance of LoVoDX\(_{10}\) could be attributed to increased expression of P-170, flow cytometric analysis of cell surface P-170 expression was performed in sensitive and doxorubicin-resistant LoVo cells (Fig. 1). High P-170 expression was common to both resistant subclones, in contrast to the doxorubicin-resistant line, which did not bind Mab57.

Glucose Metabolism, ATP Content, Enzyme Activities, and Clonogenic Activity. It has been demonstrated that the appearance of the MDR phenotype is associated with major modifications in energy metabolism (13–18). To investigate whether enhancement of energy-yielding processes correlated with the extent of drug resistance, oxidative and glycolytic glucose metabolism and the effect of LND were evaluated. Table 2 shows \(^{14}\)CO\(_2\) production by LoVo cells from [6-\(^{14}\)C]glucose was very low (369 cpm/2 \(\times\) 10\(^7\) cells/h) and unaffected by LND (307 cpm/2 \(\times\) 10\(^7\) cells/h). \(^{14}\)CO\(_2\) production by resistant LoVoDX cells was significantly higher (2852 cpm/2 \(\times\) 10\(^7\) cells/h) and was dramatically reduced by LND (1692 cpm/2 \(\times\) 10\(^7\) cells/h). Oxidative degradation of glucose and the effect of LND increased as cells became more resistant (5832 \(\times\) 2,349 cpm/2 \(\times\) 10\(^7\) cells/h).

The rate of aerobic lactate production was similar for both sensitive and LoVoDX cells and was inhibited by approximately 40% in the presence of LND. Aerobic glycolysis by LoVoDX\(_{10}\) cells was, in contrast, significantly higher than that of drug-sensitive and drug-resistant LoVoDX cells (\(\Delta = 94\) and 65%, respectively) and was inhibited by LND to an extent similar to that of glucose oxidation (\(\Delta = -56\%\)).

The enhancement of energy-yielding pathways in resistant cells should theoretically correlate with increased levels of intracellular ATP. In LoVoDX and LoVoDX\(_{10}\) cells, ATP content was 26 and 46% higher than in drug-sensitive cells, respectively. LND did not significantly affect ATP concentration in LoVo-sensitive cells, but inhibition increased with resistance. ATP content was reduced by 22 and 66% in LoVoDX and in LoVoDX\(_{10}\) cells, respectively.

Table 3 shows the activity of hexokinase, isocitric dehydrogenase, and citrate synthase in sensitive and resistant human LoVo carcinoma cells. No differences in hexokinase activity were observed in LoVo and LoVoDX cells. In LoVoDX\(_{10}\) cells, which exhibited higher lactate production, hexokinase activity was markedly increased (\(\Delta = 110\%\)). Higher glucose utilization in resistant cells via tricarboxylic acid cycle was confirmed by enhanced enzymatic activities of two regulatory enzymes. The activity of isocitric dehydrogenase was elevated from 5 to 12 and 38 nmol/min/mg of protein, and the activity of citrate synthase increased from 78 to 115 and 474 nmol/min/mg of protein in LoVoDX and LoVoDX\(_{10}\) cells, respectively.

Fig. 2 shows the clonogenic activity curves of sensitive and resistant LoVo cells, treated for 24 h with different LND con-

### Table 1: Doxorubicin sensitivity of LoVo, LoVoDX, and LoVoDX\(_{10}\) carcinoma cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>(IC_{50}) ((\mu)M)</th>
<th>Resistance over LoVo</th>
</tr>
</thead>
<tbody>
<tr>
<td>LoVo</td>
<td>0.05 (\pm) 0.01</td>
<td></td>
</tr>
<tr>
<td>LoVoDX</td>
<td>2.10 (\pm) 0.04</td>
<td>42</td>
</tr>
<tr>
<td>LoVoDX(_{10})</td>
<td>10.20 (\pm) 0.10</td>
<td>204</td>
</tr>
</tbody>
</table>

Fig. 1 Flow cytometric analysis of P-170 expression in the sensitive LoVo cell line and the doxorubicin-resistant LoVoDX and LoVoDX\(_{10}\) cell lines.
Table 2  Effect of LND on $^{14}$CO$_2$ and aerobic lactate production and ATP content by sensitive and doxorubicin-resistant (DX and DX$_{10}$) LoVo carcinoma cells

The cells (2 × 10$^7$) were incubated at 37°C for 1 h with or without 0.2 mM LND. The final concentration of glucose was 6 mM. $^{14}$CO$_2$, aerobic lactate production, and ATP content are expressed as cpm/2 × 10$^7$ cells/h, μmol/2 × 10$^7$ cells/h, and nmol/2 × 10$^7$ cells, respectively. Mean values ± SD from seven different experiments performed in duplicate are presented.

<table>
<thead>
<tr>
<th>Cells</th>
<th>LND</th>
<th>$^{14}$CO$_2$</th>
<th>Δ (%)</th>
<th>Lactate</th>
<th>Δ (%)</th>
<th>ATP</th>
<th>Δ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LoVo</td>
<td>–</td>
<td>369 ± 56</td>
<td></td>
<td>3.4 ± 0.6</td>
<td></td>
<td>42.4 ± 4.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>307 ± 24 (NS)$^c$</td>
<td>−17</td>
<td>2.1 ± 0.5$^a$</td>
<td>−38</td>
<td>36.9 ± 4.8 (NS)$^c$</td>
<td>−13</td>
</tr>
<tr>
<td>LoVoDX</td>
<td>–</td>
<td>2852 ± 193</td>
<td></td>
<td>4.0 ± 0.3</td>
<td></td>
<td>53.4 ± 3.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1692 ± 100$^a$</td>
<td>−41</td>
<td>2.4 ± 0.2$^a$</td>
<td>−40</td>
<td>41.4 ± 2.7$^a$</td>
<td>−22</td>
</tr>
<tr>
<td>LoVoDX$_{10}$</td>
<td>–</td>
<td>5832 ± 535</td>
<td></td>
<td>6.6 ± 0.6</td>
<td></td>
<td>61.8 ± 2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2349 ± 249$^c$</td>
<td>−60</td>
<td>2.9 ± 0.4$^b$</td>
<td>−56</td>
<td>21.3 ± 1.8$^b$</td>
<td>−66</td>
</tr>
</tbody>
</table>

$^a$ P < 0.05 vs. control.  
$^b$ P < 0.001 vs. sensitive cells.  
$^c$ NS, not significant.

Table 3  Activities of hexokinase, isocitric dehydrogenase and citrate synthase of sensitive and doxorubicin-resistant (DX and DX$_{10}$) LoVo carcinoma cells

Values ± SD are expressed as nmol/min/mg of protein. Mean value, averaged from seven different cell preparations, is presented.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>LoVo</th>
<th>LoVoDX</th>
<th>LoVoDX$_{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase</td>
<td>42 ± 3</td>
<td>44 ± 4 (NS)$^a$</td>
<td>84 ± 6$^a$</td>
</tr>
<tr>
<td>Isocitric dehydrogenase</td>
<td>5 ± 1</td>
<td>12 ± 3$^b$</td>
<td>38 ± 5$^b$</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>78 ± 4</td>
<td>115 ± 7$^c$</td>
<td>474 ± 9$^b$</td>
</tr>
</tbody>
</table>

$^a$ NS, not significant.  
$^b$ P < 0.0001 vs. sensitive cells.  
$^c$ P < 0.005 vs. sensitive cells.

Concentration in the exponential phase of growth. The colony- 
forming ability of LoVo and LoVoDX cells was poorly affected 
by LND, with both curves decreasing exponentially and D$_0$ 
values of 522 and 360 μM obtained, respectively. The effect 
of LND on clonogenic activity of LoVoDX$_{10}$ cells was far more 
evident. The curve had a shoulder (n = 2.2), prior to an 
exponential decrease (D$_0$ = 110 μM). The presence of a shoulder 
directed that 24 h treatment with LND at concentrations up 
to 75 μM did not affect proliferation.

Doxorubicin Distribution and Efflux. The data we 
report indicate that extent of resistance correlates with enhanced 
energy metabolism. Doxorubicin efflux is energy-dependent (4). 
Because LoVoDX$_{10}$ should extrude drug to a greater extent 
and/or rate than LoVoDX cells, the kinetics of drug efflux and 
intracellular distribution were evaluated.

Sensitive and resistant cells were loaded with 20 μM doxorubicin, 
washe to remove the uninternalized drug, mounted on 
a microscope stage, and perfused at a constant rate with glucose- 
free NKT medium. Images were acquired for 10 min, and then 
perfusion was switched to glucose-supplemented NKT medium. 
Calibration of cellular doxorubicin concentration was not possible 
under our experimental conditions. Therefore, the variation 
of intracellular fluorescence over time with respect to fluorescence 
at the beginning of each experiment (F/F$_n$) was evaluated. 
F/F$_n$ was measured in nucleus and cytoplasm of each cell and 
mean results plotted against time. Each data set represents the 
mean of at least 20 cells ± SD.

In LoVo-sensitive cells, perfusion with glucose-free and 
glucose-supplemented medium did not modify cytoplasmic or 
nuclear fluorescence. This indicated that all drug was retained 
by the cells over the time course (Fig. 3, A–C). The intracellular 
distribution of doxorubicin was similar in both nucleus and 
cytoplasm (Fig. 3, B and C), as confirmed by the ratio of nuclear 
to cytoplasmatic fluorescence (F$_n$/F$_c$), which, when plotted 
against time, remained constant (Fig. 3A, inset). The values of 
F$_n$/F$_c$ calculated from 120 cells, at the beginning of each 
experiment, were plotted in a histogram of the number of the cells 
over F$_n$/F$_c$ and peaked at 1.061 ± 0.028 (not shown).

Resistant LoVo cells exhibited a markedly different behavior. 
Fig. 3, D–F, shows doxorubicin efflux and distribution in 
LoVoDX cells. Cells perfused with glucose-free medium elimi-
nated up to 20% of the doxorubicin into the medium. After 2–3 
min of perfusion with glucose, the rate of outward transport 
increased to a plateau, i.e., the same efflux rate as without 
glucose after 25–30 min. At the end of the experiment, approxi-
mately 40% of the drug had been retained by cells. The con-
centration of doxorubicin in the cytoplasm was higher than in 
the nucleus (Fig. 3E). A histogram of F$_n$/F$_c$, calculated from 110
cells in multiple experiments, was symmetrical around a mean value of 0.616 ± 0.018 (not shown). The decrease of nuclear doxorubicin was less than that of cytoplasmatic decrease as demonstrated by the ratio \( F_n/F_c \), which increased with time (Fig. 3D, inset).

In LoVoDX10 cells, perfused without glucose, outward drug transport was 2-fold greater than that observed in LoVoDX cells. The efflux rate increased dramatically when perfusion was switched to glucose-supplemented medium, and after 45 min, the cytoplasmatic drug fraction was almost completely (91%) released into the medium. The release of doxorubicin accumulated within the nucleus proceeded at the same rate of cytoplasmatic release for the first 20 min, slowing thereafter, resulting in an increased ratio of nuclear to cytoplasmatic fluorescence (Fig. 3G, inset). At the end of the experiment, 80% of nuclear doxorubicin had been released into the medium. As for LoVoDX cells, the concentration of doxorubicin was higher in the cytoplasm, and the \( F_n/F_c \) ratio calculated from 110 cells in several experiments was symmetrical around a mean value (± SD) of 0.620 ± 0.022 (not shown).

These data indicate a relationship between drug extrusion and energy metabolism. Therefore, the effect of LND, which inhibits both oxidative and glycolytic metabolism (19–21, 27), on drug efflux and distribution was evaluated. Fig. 4A shows that in sensitive cells, LND did not affect intracellular doxorubicin concentration but modified its distribution, resulting in an elevated cytoplasmatic concentration (Fig. 4, A–C).

LND had a dramatic effect on the efflux of doxorubicin from resistant cells. Both basal and glucose-supported drug extrusion were almost completely inhibited. Indeed, after 50
min, 100 and 85% of the drug was still retained by LoVoDX (Fig. 4D) and LoVoDX_{10} cells (Fig. 4G), respectively. The nuclear drug concentration in LoVoDX (Fig. 4D, inset, E, and F) and LoVoDX_{10} cells (Fig. 4G, inset, H, and I) was higher than that of cytoplasm. The F_{n}/F_{c} ratio, evaluated from 130 cells pooled from several experiments, indicated a largely symmetrical distribution peaking around a mean ± SD of 1.27 ± 0.06 (not shown), i.e., a value quite similar to that found for the sensitive cells.

Table 4 shows the effect of LND exposure on doxorubicin sensitivity of LoVo-resistant cells. In LoVoDX cells, 8 days of treatment with 50 μM LND, i.e., a concentration that by itself was unable to affect cell survival, lowered the IC_{50} value from 2.1 to 0.5 μM doxorubicin. LoVoDX_{10} cells were more sensitive to LND; therefore, to avoid any effect on cell survival, a concentration 8 times lower (6.25 μM) was used. Nevertheless, despite this low concentration, LND was still able to reduce IC_{50} concentration from the 10.2 to 2.5 μM.

**DISCUSSION**

The data reported in this communication provide, to our knowledge, the first experimental evidence that the extent of alterations in energy metabolism strictly correlate with degree of drug resistance.

In doxorubicin-sensitive cells, glycolysis was the main energy-yielding process, as indicated by the very low amount of glucose metabolized via the Krebs cycle. In resistant LoVoDX cells, the most relevant metabolic modification was a marked increase in $^{14}$CO$_2$ production from [6-$^{14}$C]glucose (Table 2), which releases $^{14}$CO$_2$ only at the level of the Krebs cycle, whereas aerobic glycolysis remained essentially unmodified. In
The marked inhibition of LoVoDX10 cell proliferation by LND can be attributed mainly to the decrease in ATP content, leading to an altered functional cellular state (28–30). Such alterations can be reversed although ATP has been low for several hours but becomes irreversible only after 18–24 h (31).

Low ATP content was also responsible for the increased doxorubicin content in LND-treated resistant LoVo cells. Decreased ATP availability may affect the P-170 activity by two mechanisms. In the first, reduced ATP production would not supply sufficient energy for extrusion by molecular pump. In the second, P-170 function may be affected by reduced phosphorylation, a critical factor for MDR. Indeed, human SW 620 colon carcinoma cells treated with sodium butyrate show decreased P-170 phosphorylation tightly correlated to reduced drug efflux. Decrease in P-170 phosphorylation is observed after 12 h treatment and further decreases at 24, 36, and 48 h (32).

Because drug efflux by LoVo-resistant cells was already inhibited after 1 h of exposure to LND, an effect on P-170 phosphorylation may be excluded. Thus, the inability of LND-treated LoVo-resistant cells to extrude doxorubicin was likely to depend upon reduced energy supply to the ATP-driven efflux pump resulting from inhibition of glycolysis and respiration (Table 2).

An alternative pathway by which LND can increase the intracellular doxorubicin concentration in LoVo-resistant cells may be an acidic shift of cytosolic pH (33), resulting from lactate accumulation. Indeed, Ben-Horin et al. (34) and Vivi et al. (35, 36) report that the lower extracellular content of lactate in LND-treated adriamycin-sensitive and -resistant MCF-7 cells is dependent upon impaired outward lactate transport. However, such a mechanism may be excluded because low extracellular lactate in LND-treated cells is primarily attributed to inhibition of glycolysis by impairment of mitochondria-bound hexokinase (16, 20, 37), confirmed in human gliomas transplanted in nude mice by Oudard et al. (38), with extent of inhibition of tumor growth rate by LND strongly correlated with mitochondria-bound hexokinase activity. Moreover, LND decreases the acidity of vacuolar system organelles due to a decreased ATP content and increased ion membrane permeability (39).

In conclusion, although our results neither prove nor disprove the existence of alternative pathways for MDR (e.g., a passive-trapping mechanism), they clearly demonstrated that inhibition of doxorubicin extrusion by LND in LoVo-resistant cells can be attributed to an effect on oxidative and glycolytic metabolism that results in reduced intracellular ATP content, thus lowering the energy supply to the ATP-driven efflux pump.

There are a number of potential therapeutic implications from this work. If MDR is associated with P-170 overexpression, then LND, currently used in tumor therapy, may reduce or reverse drug resistance by restoring the capacity to accumulate and retain drug (40). It is noteworthy that in LoVo cells, reduction of resistance (Table 4) was obtained with an LND concentration similar to (LoVoDX) or remarkably lower than (LoVoDX10) the peak plasma concentration achievable in humans (18–40 μg/ml).

Moreover, LND, because of its effect on the energy-yielding processes, potentiates the therapeutic efficacy of other antineoplastic drugs or agents, including radiation and/or hyperthermia, while reducing toxic side effects (41–45).
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