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Enhancement of Doxorubicin Content by the Antitumor Drug Lonidamine in Resistant Ehrlich Ascites Tumor Cells through Modulation of Energy Metabolism

Aristide Floridi,†§ Tiziana Bruno,† Stefania Miccadei,† Maurizio Fanciulli,† Antonio Federico‡ and Marco G. Paggi†*

*Department of Experimental Medicine, University of L'Aquila, 67100 L'Aquila; †Laboratory of Cell Metabolism and Pharmacokinetics and ‡Laboratory of Biochemistry, Center for Experimental Research, Regina Elena Cancer Institute, Rome, Italy

ABSTRACT. The effect of the antitumor drug lonidamine (LND) on respiration, aerobic glycolysis, adenylate pool, doxorubicin (DOX) uptake, and efflux in DOX-resistant and DOX-sensitive Ehrlich tumor cells was investigated. The results may be summarized as follows: 1) In both types of cells, LND inhibited both respiration and glycolysis in a dose-dependent manner and lowered the ATP concentration. The effect was more marked in cells incubated in glucose-free medium; 2) LND raised, to a remarkable extent, the intracellular content of DOX in resistant and sensitive cells respiring on endogenous substrates because of reduced ATP availability, whereas in glucose-supplemented medium, where both respiration and glycolysis contributed to ATP synthesis, the increase was lower; and 3) when LND was added to DOX-loaded cells, it failed to significantly inhibit DOX efflux because of time-dependent phenomena. These findings indicated that LND, a drug currently employed in tumor therapy, might also be useful in reducing or overcoming multidrug resistance (MDR) of those cells with a reduced ability to accumulate and retain antitumor drugs. BIOCHEM PHARMACOL **56**;7:841–849, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. multidrug resistance; doxorubicin; lonidamine; energy metabolism; Ehrlich tumor cells

The efficacy of cancer therapy is often limited by the presence of drug-resistant cell clones within the tumor. Some tumors are initially resistant, whereas others, initially responsive to cytotoxic agents, become resistant and do not respond to subsequent chemotherapy courses. Moreover, once exposed to a given chemotherapeutic drug, tumors become resistant to a range of chemically and functionally unrelated agents. This phenomenon, named MDR¶ has been ascribed to biochemical properties intrinsic to the genotype of the tumor cells or to mutation caused by the antitumor agent administered [1].

One of the major changes in the phenotype of MDR cells is a decreased accumulation of cytotoxic drugs, mainly due to overexpression of a 170-kDa membrane glycoprotein, called P-170, which uses ATP to extrude chemotherapeutic agents from the cells [1, 2]. Such an energy-dependent process may require a high energy demand with an enhancement of energy-yielding pathways [3, 4].

LND, 1-(2,4 dichlorobenzyl)-H-indazole-3-carboxylic acid, is a relatively new, nonconventional anticancer agent that inhibits cell survival by affecting energy metabolism in a very peculiar way. LND decreases oxygen consumption both in normal and neoplastic cells [5] by inhibiting electron transport between FAD-linked dehydrogenases and the respiratory chain [6]. LND stimulates aerobic glycolysis in normal differentiated cells, but decreases that of neoplastic cells by affecting mitochondria-bound hexokinase (EC 2.7.1.1) [7], usually absent in normal cells [8]. The effect of LND does not depend either on the nature or the origin of neoplastic cells, but is related to their metabolic capacity: the more a tumor is able to sustain high energy metabolism, the more LND lowers it. Moreover, LND strongly potentiates the therapeutic efficacy, both *in vitro* and *in vivo*, of other antineoplastic drugs, e.g. *cis*-platin [9], DOX [10], alkylating agents [11] as well as that of other antitumor agents such as radiation and/or hyperthermia [12–16]. Yet, LND raises intracellular DOX content in DOX-sensitive EH-WT cells by affecting both oxidative and glycolytic metabolism [17].

In a previous report [18], we have shown that DOX-resistant Ehrlich ascites tumor cells, termed here EH-DOX

§ Corresponding author: Prof. Aristide Floridi, Department of Experimental Medicine, University of L'Aquila, Via Vetoio, Coppito 2, 67100, L'Aquila, Italy. Tel. +39–862-433521; FAX +39–862-433523.

¶ *Abbreviations:* DOX, doxorubicin; EH-DOX cells, Ehrlich ascites tumor cells resistant to doxorubicin; EH-WT, Ehrlich ascites tumor cells sensitive to doxorubicin; LND, lonidamine; MDR, multidrug resistance; and NKT medium, 10^5 mM NaCl, 5 mM KCl, 50 mM *N*-tris[hydroxymethyl]-2-aminoethanesulfonic acid.

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cells, show a modified energy metabolism which reflects metabolic adaptations associated with the development of MDR. EH-DOX cells, when compared to sensitive cells, exhibit a lower DOX content and enhanced rate of oxygen consumption as well as of ATP and aerobic lactate production. Furthermore, the activities of some regulatory enzymes of both the glycolysis and Krebs cycle are remarkably higher in EH-DOX cells than in sensitive cells. Therefore, EH-DOX cells should be more sensitive to energy-depleting agents so that LND, that selectively inhibits energy metabolism of neoplastic cells, might constitute an effective measure to reverse the MDR phenotype.

This paper reports data on the effect of LND on respiration, aerobic glycolysis, hexokinase activity, adenine nucleotide content, and DOX uptake and efflux in EH-DOX cells. The results showed that the increased intracellular amount of DOX in LND-treated EH-DOX cells was mainly due to energy depletion

MATERIALS AND METHODS

Chemicals

[^{14}C]DOX (51 mCi/mmol) was purchased from Amersham and Soluene 350 from Packard Instruments Italia. The reagents for the enzymatic assay were obtained from Boehringer Mannheim Italia, while HEPES and TES (*N*-tris[hydroxymethyl]-2-aminoethanesulfonic acid) were from Sigma Italia. Aquassure scintillation liquid from New England Nuclear and DOX from Farmitalia. LND (doridamine) was obtained from the F. Angelini Research Institute, Pomezia. All of the other reagents were of analytical grade or better and were purchased from BDH Italia.

Cells

EH-DOX Ehrlich ascites tumor cells (kindly provided by Prof. T. Skovsgaard, Department of Oncology, R. Herlev Hospital, University of Copenhagen, Denmark) and EH-WT cells were grown in 2-month-old first generation hybrids of female random-bred Swiss mice and male inbred DBA/2 mice by weekly transplantation. The resistant line was maintained by daily treatment for 4 days in each passage with LD10 dose of DOX. No drug treatment was given in the last passage before use. The cells were withdrawn from the killed animals and suspended in a medium containing a final concentration of 105 mM NaCl, 5 mM KCl, 50 mM TES, pH 7.4 (NKT). The cells were centrifuged at 100 *g* for 10 min at room temperature, washed with NKT medium until free of red blood cells, counted (Coulter Counter, mod ZM) and resuspended in the same medium at a concentration of 1×10^8 cells/mL. The viability was always 95–98% as indicated by phase-contact microscopy in the presence of the trypan blue.

Assay of Respiration and Glycolysis

Respiration was measured with a Clark oxygen electrode (Yellow Springs Instrument Company) at 37°; the concentration of dissolved oxygen was taken to be 406 ng/atoms O mL^{-1} when the medium was air-equilibrated at 760 Torr (≈10,080 Pa) [19]. Reaction rates were determined by adding 0.2 mL of cell suspension (2×10^7 cells) to 1.8 mL of air-equilibrated NKT medium in a closed glass chamber of 2-mL capacity (Gilson Medical Electronics). The final volume was always 2 mL. The measurements of aerobic glycolysis were carried out in 25-mL Erlenmeyer flasks at 37°. The complete reaction medium contained NKT medium, pH 7.4, and 0.2 mL of cellular suspension (2×10^7 cells) and established LND concentration. The final volume was always 3.0 mL. After 10 min of preincubation, the reaction was started by the addition of glucose at a final concentration of 6 mM and was allowed to proceed for a further 30 min. At the end of incubation, the flasks were immersed in an ice bath for 2 min and the cells sedimented by centrifugation at 3,000 *g* for 3 min at 4°. The amount of L(-)-lactate was evaluated on supernatants according to Hohorst [20].

Assay of Hexokinase Activity

Control and LND-treated cells were incubated as for the glycolysis. At the end of incubation, cells were collected by centrifugation at 4°, washed twice in ice-cold NKT medium, and resuspended in 7 mL of ice-cold H medium (220 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.4), disrupted with digitonin and homogenized according to [21]. The mitochondria fraction of the enzyme was prepared as previously reported [22], and the activity was measured according to Parry and Pedersen [21], except that lubrol was omitted from the incubation mixture. The formation of NADPH was recorded at 37° for 10 min at 340 nm with a Beckman DU7 spectrophotometer equipped with a thermospacer apparatus. The reaction was linear for the first 5 min. Protein content was evaluated by means of bicinchoninic acid [23].

Assay of the Adenine Nucleotide Pool

Control and LND-treated cells were incubated as for the glycolysis, collected by centrifugation at 1800 *g* for 3 min at 4°, and deproteinized with 1.25 mL of ice-cold 0.5 M KOH. After 5 min standing in ice, 1 mL of ice-cold NKT medium was added and the solution transferred to a Centricon 30 (Amicon) and centrifuged for 90 min at 6,000 *g* at 0° in a Beckman J21 centrifuge using a JA-20 rotor. One milliliter of the clear ultrafiltered solution was taken up and the pH adjusted to 6.5 by adding 0.4–0.5 mL of 1 M KH_2PO_4 solution. The determination of adenine nucleotides (ATP, ADP, AMP) was performed by reverse-phase HPLC according to Stocchi *et al.*, [24] with an LKB 2150 apparatus equipped with a RoSil 5-μm C18 column (BioRad). The

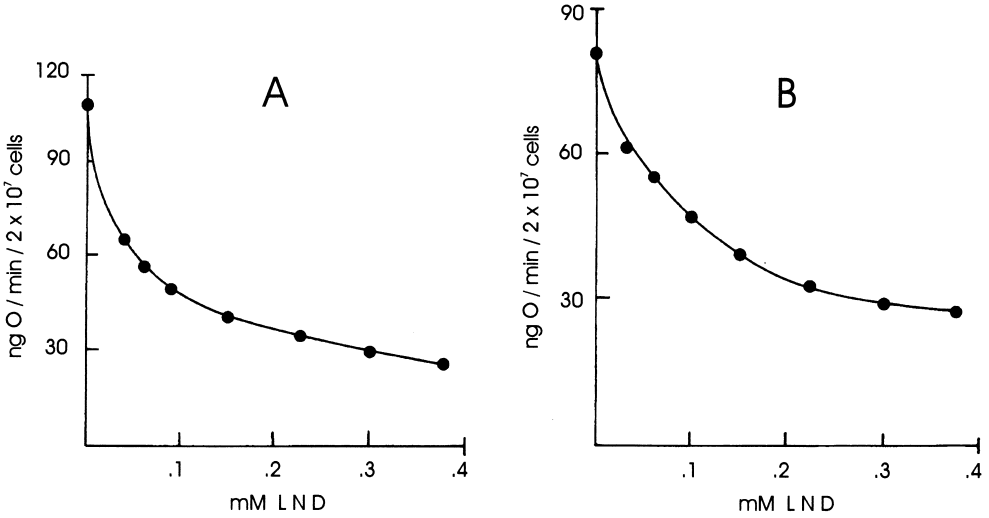

FIG. 1. (A) Dependence of oxygen consumption of EH-DOX cells on LND concentration. The reaction was started by adding 0.2 mL of cell suspension (2×10^7) to 1.8 mL of NKT medium, and the basal rate of oxygen consumption was recorded for 2 min. Then LND, at established concentrations, was added and the rate of oxygen uptake recorded for a further 3 min. The rates of oxygen consumption (ng of atoms O/min/2×10^7 cells) were calculated from the linear portion of the curves. Each point was averaged from five different experiments. Error bars within the symbols. (B) Dependence of oxygen consumption of EH-WT cells on LND concentration. Each point was averaged from seven different experiments. Error bars within the symbols. Other experimental conditions as in (A).

apparatus was connected to a computer through a Nelson Analytical series 900 interface (Cupertino). The analysis of chromatograms, as well as the integration of peak areas, was performed with Nelson Analytical Chromatographic Software, version 3.6. Quantitative measurements were carried out by injection of standard solutions of known concentrations.

Determination of Accumulation and Efflux of DOX

EH-DOX or EH-WT cells (2×10^7) were incubated in 3 mL of NKT buffer at 37° in a Dubnoff metabolic shaker in the presence of 0.5 μCi of [^{14}C]labeled DOX. The final concentration of the drug, adjusted with unlabeled drug, was 13 μM whereas that of LND and oligomycin was 0.225 mM and 1 μg/mL, respectively. After 10 min of preincubation, glucose, when present, was added at a final concentration of 6 mM and the incubation was allowed to proceed for a further 50 min. At the end of incubation, 1 mL of cell suspension was withdrawn with a Gilson pipette and centrifuged for 20 sec in an Eppendorf microfuge. The cells were washed twice with ice-cold NKT, solubilized with 1 mL of Soluene and assayed for the radioactivity (time 0). The remaining 2 mL of cell suspension were transferred to

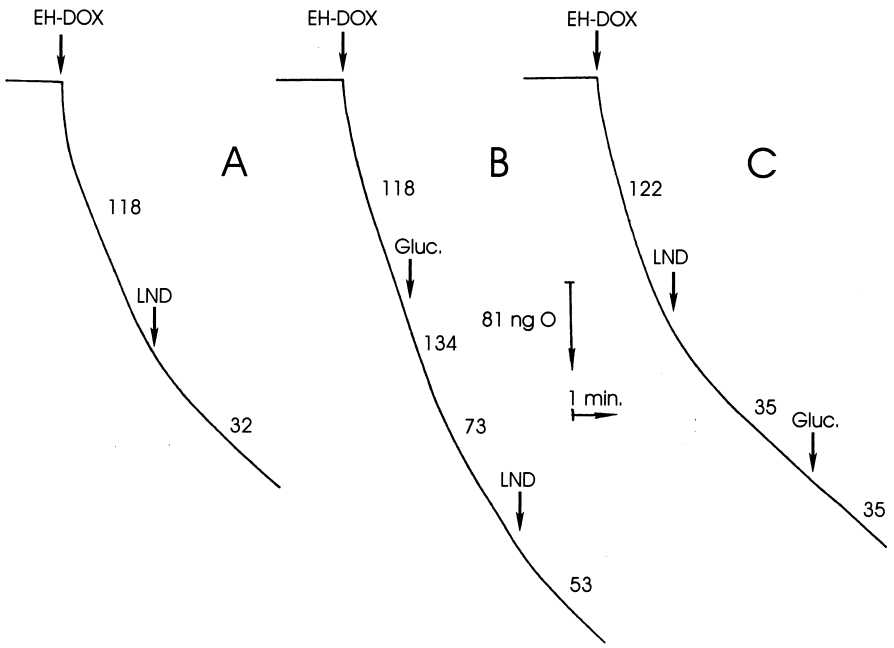

FIG. 2. Typical polarographic traces showing the kinetics of oxygen consumption by EH-DOX ascites tumor cells. The reaction was started by adding 0.2 mL of cellular suspension (2×10^7 cells) to 1.8 mL of NKT medium. LND (0.225 mM) and glucose (6 mM) were added at the points indicated. The numbers along the traces are the rates of oxygen consumption (ng of atoms O/min/2×10^7 cells). The temperature was 37°. The experiments were repeated five times and yielded reproducible results (±4%).

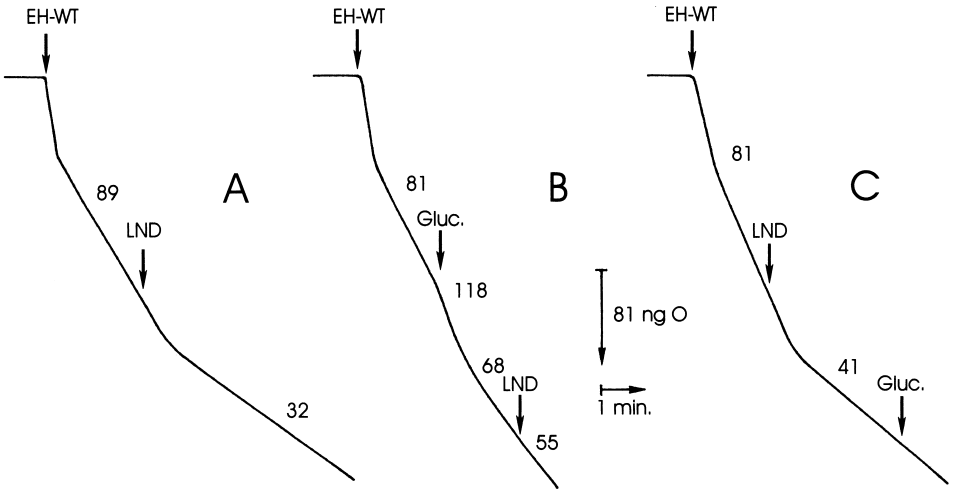

FIG. 3. Typical polarographic traces showing the kinetics of oxygen consumption by EH-WT ascites tumor cells. The numbers along the traces are the rates of oxygen consumption (ng atoms O/min/2 × 10^7 cells). The temperature was 37°. The experiments were repeated four times and yielded reproducible results (±5%). LND and glucose (Gluc) were added at points indicated at a final concentration of 0.225 mM and 6 mM, respectively.

ice-cold tubes and centrifuged at 800 *g* for 3 min at 4°. The cells were washed with ice-cold NKT medium, resuspended with 1 mL of NKT and then transferred to Erlenmeyer flasks containing 2 mL of DOX-free NKT medium. The incubation was allowed to proceed for 1 hr at 37° in a Dubnoff metabolic shaker. At the end of incubation, the cells were recovered by centrifugation at 800 *g* for 3 min at 4°, washed twice with NKT medium, and solubilized with 1 mL of Soluene (time 60 min). The radioactivity was assayed with a Beckman LS 6000 SC liquid scintillation spectrometer at an efficiency of 85%.

RESULTS

Effect of LND on Respiration, Glycolysis and Mitochondria-bound Hexokinase

Figure 1A shows the rate of oxygen consumption of EH-DOX cells as function of LND concentration. The rate of oxygen consumption decreased exponentially as LND concentration increased with maximal inhibition being reached at 0.4 mM LND. Higher drug concentrations, e.g. 0.8 mM LND, did not increase its inhibitory effect (data not shown). Half-maximal inhibition took place at 0.06 mM LND. The concentration-dependent effect of LND on the oxygen uptake of EH-WT cells is shown in Fig. 1B. The basal rate of respiration was lower, but the extent of inhibition at the highest drug concentration was quite similar to that found for EH-DOX cells, i.e. 66% vs 74%. The concentration of LND needed to achieve half-maximal inhibition was 0.135 mM.

The effect of LND on the kinetics of oxygen consumption by EH-DOX cells is shown in Fig. 2. Trace A was a typical polarographic recording of the oxygen utilization by cells respiring on endogenous substrates. The addition of partially anaerobic 2 × 10^7 cells caused a downward deflection of the trace followed by a steady-state oxidation rate of 118 ng of O/min/2 × 10^7 cells. The addition of 0.225 mM LND, after a lag period of approximately 1 min, lowered the oxygen uptake rate to 32 ng of O/min/2 × 10^7 cells ($\Delta\% = -73$); the rate remained almost constant until the dissolved oxygen was exhausted (data not shown).

Addition of 6 mM glucose (trace B) resulted in an immediate stimulation of the rate of oxygen consumption for *ca.* 30 sec followed by a new steady-state oxidation rate of 73 ng of O/min/2 × 10^7 cells. The addition of 0.225 mM LND further decreased the respiration rate, but the extent of the inhibition was lower than in the absence of glucose. The LND-inhibited oxygen uptake rate was not modified by the glucose addition (trace C). The effect of LND and glucose on the kinetics of oxygen uptake was qualitatively similar to that observed with EH-DOX cells (Fig. 3).

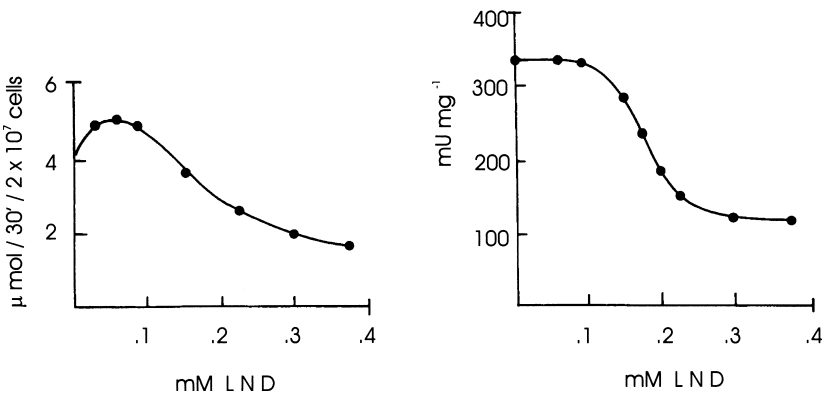

FIG. 4. (Left) Dependence of the aerobic lactate production by EH-DOX ascites tumor cells on LND concentration. The cells (2 × 10^7 cells) were added to 2.8 mL of NKT medium and preincubated for 10 min with LND, followed by the addition of 6 mM glucose. Each point was averaged from five different experiments. Error bars within the symbols. (Right) Dependence of mitochondria-bound hexokinase activity on LND concentration. Each point was averaged from five different experiments. Error bars within the symbols.

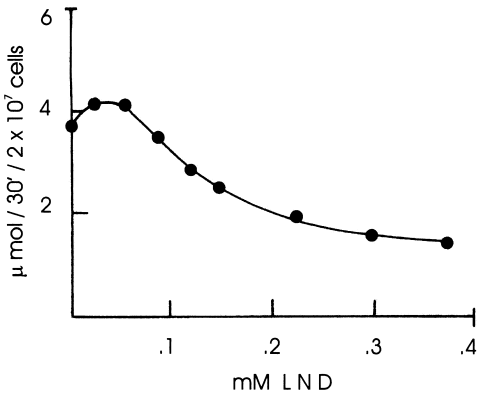

FIG. 5. Dependence of the aerobic lactate production by EH-WT ascites tumor cells on LND concentration. The cells (2×10^7 cells) were added to 2.8 mL of NKT medium and preincubated for 10 min with LND, followed by the addition of 6 mM glucose. Each point was averaged from four different experiments. Error bars within the symbols.

Figure 4 (left) shows the effect of LND concentration on the aerobic rate of lactate production of EH-DOX cells. The drug, up to 0.09 mM, slightly stimulated the aerobic glycolysis ($\Delta\% = 25$). Further increases in LND concentration resulted in an inhibition of lactate production. The half-maximal inhibition was achieved by 0.29 mM LND, whereas the maximum effect took place at 0.37–0.40 mM.

Because it has been demonstrated that the impairment of glycolysis by LND in tumor cells is mainly due to an inhibition of mitochondria-bound hexokinase [6], the effect of LND concentration on the activity of this enzyme was evaluated (Fig. 3, right). The drug, up to 0.1 mM, did not affect at all the bound enzyme activity that, on the contrary, was strongly inhibited by higher LND concentrations. At 0.37 mM LND, enzymatic activity was reduced by 61%, a value close to that found for aerobic glycolysis, thus confirming that in EH-DOX cells as well LND inhibited glycolysis by affecting mitochondria-bound hexokinase.

Aerobic lactate production by EH-WT cells as affected by increasing LND concentrations is shown in Fig. 5. As for DOX-resistant cells, aerobic glycolysis was stimulated by low and inhibited by higher LND concentrations. Half-maximal inhibition was achieved by 0.25 mM LND. The activity of mitochondria-bound hexokinase in EH-WT cells was lower than in EH-DOX cells, 150 mU/mg of protein vs 325 mU/mg of protein, respectively, and the treatment with 0.37 mM LND inhibited enzyme activity by 54%, a value overlapping that found for aerobic glycolysis.

Effect of LND on Adenylate Pool, Accumulation and Efflux of DOX

The data reported above clearly show that LND inhibited both respiration and glycolysis in EH-DOX cells, so that a decrease in ATP content and/or supply would be expected. Therefore, the content of adenylate, i.e. ATP, ADP, and AMP, was evaluated by reverse phase HPLC both in control and LND-treated cells in the presence and the absence of glucose.

The quantitative analysis of chromatograms (Table 1) pointed out that in control cells incubated in glucose-supplemented medium the greatest part of the adenine nucleotide pool was present as ATP, 12.51 nmol/10^7 cells, i.e. 3.52 mM with a cell volume of 3.55 μL [25]. LND lowered ATP but raised ADP and AMP content. When EH-DOX cells were incubated in glucose-deprived medium, ATP was remarkably lower than in the presence of glucose, i.e. 7.56 nmol/10^7 cells vs 12.51 nmol/10^7 cells ($\Delta\% = -40$), whereas the amount of ADP and AMP was noticeably higher ($\Delta\% = 110$ and 343 respectively). Yet LND, in the absence of glucose, was more effective in reducing the adenylate pool. ATP was lowered from 7.56 nmol/10^7 cells to 1.47 nmol/10^7 cells ($\Delta\% = -81$). ADP content was also decreased by 65% whereas the amount of AMP was raised by 30%.

On the basis of quantitative data, the adenylate energy charge, i.e. the proportion of adenine nucleotide in high form, was evaluated (Table 1). The adenylate energy charge of cells incubated in glucose-supplemented medium was remarkably higher than in its absence, 0.93 vs 0.77, and was slightly affected by LND ($\Delta\% = -13$) whereas, in glucose-deprived medium, the inhibition was remarkably higher, the adenylate energy charge being lowered from 0.77 to 0.47 ($\Delta\% = -39$).

The effect of LND on the adenylate concentration of EH-WT cells is shown in Table 2. As for EH-DOX cells, the greatest part in the glucose-supplemented medium was present as ATP. LND treatment lowered its content ($\Delta\% = 45$), whereas the concentration of ADP and AMP was raised by 58% and 172%, respectively, thus decreasing the adenylate energy charge from 0.92 to 0.76.

TABLE 1. Effect of LND on the adenylate concentration in EH-DOX cells

Glucose	LND	ATP	ADP	AMP	AEC
+	−	12.51 ± 0.13	1.25 ± 0.10	0.30 ± 0.04	0.93
+	+	6.44 ± 0.15	1.70 ± 0.12	0.78 ± 0.09	0.82
−	−	7.56 ± 0.10	2.63 ± 0.11	1.33 ± 0.12	0.77
−	+	1.47 ± 0.08	0.92 ± 0.08	1.73 ± 0.15	0.47

The adenine nucleotide content was expressed as nmoles/10^7 cells. Each value ± SD was averaged from three different experiments performed in duplicate. The final concentration of glucose and LND was 6 mM and 0.225 mM, respectively. The treatment was performed as described in Methods. ACE: adenylate energy charge.

TABLE 2. Effect of LND on the adenylate concentration in EH-WT cells

Glucose	LND	ATP	ADP	AMP	AEC
+	−	10.58 ± 0.16	1.20 ± 0.14	0.32 ± 0.04	0.92
+	+	5.80 ± 0.12	1.90 ± 0.12	0.87 ± 0.10	0.76
−	−	6.04 ± 0.11	3.26 ± 0.19	1.38 ± 0.15	0.71
−	+	0.95 ± 0.08	0.72 ± 0.07	2.72 ± 0.20	0.30

The adenine nucleotide content was expressed as nmols/10^7 cells. Each value ±SD was averaged from three different experiments performed in duplicate. The final concentration of glucose and LND was 6 mM and 0.225 mM, respectively. The treatment was performed as described in Methods. AEC: adenylate energy charge.

When EH-WT cells were incubated in glucose-depleted medium, the intracellular ATP content was decreased from 10.58 nmol/10^7 cells to 6.04 nmol/10^7 cells, whereas that of ADP and AMP was noticeably higher. LND strongly affected ATP and ADP content, but increased the concentration of AMP. The adenylate energy charge was lowered from 0.71 to 0.30.

DOX efflux is felt to occur via an active transport system [2, 26], so that the decreased ATP content elicited by LND in EH-DOX cells should result in an increased intracellular DOX content.

Table 3 shows the effect of LND on DOX uptake and efflux by resistant cells. The cells were loaded with DOX for 1 hr in the presence of 0.225 mM LND, then washed twice with ice-cold NKT medium and incubated for a further hour, always in the presence of LND, in DOX-free medium. At the end of incubation in the absence of glucose, i.e. when respiration was the only energy-yielding process, the intracellular DOX content of the control cells was lowered from 1.15 nmol/10^6 cells to 0.61 nmol/10^6 cells, indicating that *ca.* 50% of the drug was released into the medium. LND raised drug accumulation and reduced the efflux as shown by the fact that, after incubation in DOX-free medium, 78% of the drug was still retained by the cells. On the contrary, when LND was added to DOX-loaded cells, the amount of the drug released into the medium was quite similar to that of control cells.

The glycolysis in Ehrlich ascites tumor cells, both wild-type and DOX-resistant, occurs only when exogenous glucose is present [18]. Accordingly, to ascertain its role in providing energy requirements for the outward transport of DOX, experiments in glucose-supplemented medium were carried out.

DOX accumulation by resistant cells, when both glycolysis and oxidative phosphorylation contributed to ATP synthesis, was significantly lower than in the absence of glucose, 0.79 nmol/10^6 cells vs 1.15 nmol/10^6 cells ($\Delta\% = -32$), even if the percent amount released in the medium after 1 hr was quite similar to that observed in the cells respiring on endogenous substrates. LND increased intracellular DOX content from 0.79 nmol/10^6 cells to 1.16 nmol/10^6 cells ($\Delta\% = 47$), but inhibited outward transport to a lesser extent since only 63% of the DOX was retained by the cells. In the presence of glucose, when LND was added to DOX-loaded cells, the amount of the drug released did not differ significantly from the control. It should be noted that in LND-treated cells, both in the presence and absence of glucose, the amount of DOX, after 60 min of incubation in DOX-free medium, was quite similar to that found at time 0 in the control cells.

LND is a lipotropic agent which induces deep ultrastructural and functional modifications in cell membranes [27, 28], so that the increased DOX uptake in LND-treated EH-DOX cells might be not only due to an ATP depletion, but also to a modified membrane-associated function. Therefore, the effect of oligomycin, which does not alter membrane permeability, was evaluated on DOX accumulation. The cells were incubated in glucose-free medium so that oxidative phosphorylation was the only source of ATP for DOX efflux.

The intracellular amount of DOX was similar both in LND- and oligomycin-treated EH-DOX cells, 1.33 nmol/10^6 cells ± 0.03 vs 1.30 ± 0.04 nmol/10^6 cells, thus suggesting that in EH-DOX cells the increased intracellular drug content could be mainly ascribed to a reduced ATP availability.

The effect of LND on DOX uptake and efflux in EH-WT cells was shown in Table 4. DOX uptake by the cells incubated in glucose-free medium was higher than in EH-DOX cells ($\Delta\% = 71$). After 1 hr of incubation in

TABLE 3. Effect of LND on the uptake and efflux of doxorubicin in EH-DOX cells

Minutes	Glucose	DOX	Δ%	DOX+LND	Δ%	DOX>LND	Δ%
0	−	1.15 ± 0.05		1.28 ± 0.02		1.14 ± 0.07	
60	−	0.61 ± 0.03	−47	1.00 ± 0.03	−22	0.71 ± 0.02	−38
0	+	0.79 ± 0.04		1.16 ± 0.02		0.78 ± 0.06	
60	+	0.39 ± 0.06	−51	0.73 ± 0.06	−37	0.44 ± 0.04	−44

The values are expressed as nmol/10^6 cells. Each value ±SD was averaged from five different experiments performed in duplicate. The final concentration of DOX, LND and glucose was 13 μM (SA: 24,927 cpm/nmol), 0.225 mM and 6 mM, respectively. **DOX+LND**: the cells were loaded with DOX for 1 hr in the presence of 0.225 mM LND, washed twice and then incubated for 1 hr in NKT medium DOX-free medium, always in the presence of LND. **DOX>LND**: LND was added to DOX-loaded cells.

TABLE 4. Effect of LND on the uptake and efflux of doxorubicin in EH-WT cells

Minutes	Glucose	DOX	Δ%	DOX+LND	Δ%	DOX>LND	Δ%
0	−	1.97 ± 0.06		2.53 ± 0.05		2.02 ± 0.06	
60	−	0.92 ± 0.04	−53	2.19 ± 0.02	−13	1.30 ± 0.04	−36
0	+	1.54 ± 0.03		1.87 ± 0.05		1.60 ± 0.06	
60	+	0.78 ± 0.08	−49	1.30 ± 0.06	−30	0.94 ± 0.04	−41

The values are expressed as nmol/10^6 cells. Each value ±SD was averaged from three different experiments performed in duplicate. The final concentration of DOX, LND and glucose was 13 μM (SA: 27,246 cpm/nmol), 0.225 mM and 6 mM, respectively. **DOX+LND**: the cells were loaded with DOX for 1 hr in the presence of 0.225 mM LND, washed twice and then incubated for 1 hr in NKT DOX-free medium, always in the presence of LND. **DOX>LND**: LND was added to DOX-loaded cells.

DOX-free medium, the intracellular drug amount was lowered from 1.97 nmol/10^6 cells to 0.92 nmol/10^6 cells.

LND raised the DOX uptake in EH-WT cells by *ca.* 30%, but its most remarkable effect was on the efflux as only 13% of the drug was released into the medium. On the contrary, when LND was added to DOX-loaded cells the outward transport was three times higher. In glucose-supplemented medium, the intracellular DOX content, although lower (Δ% = −22), was twice that found in EH-DOX cells (Table 2). The amount of DOX extruded was quite similar to that in the absence of glucose.

When LND was added to glucose-supplemented medium, the intracellular drug content overlapped that found in glucose-free medium, 1.87 nmol/10^6 cells vs 1.97 nmol/10^6 cells. LND also reduced the DOX efflux in the presence of glucose as shown by the fact that 70% was still retained by the cells.

DISCUSSION

The data reported herein demonstrate that LND affected the energy metabolism of EH-DOX cells in a manner almost similar to that observed for sensitive cells. As far as the oxygen consumption is concerned, EH-DOX cells were more susceptible to the effect of LND because of the lower drug concentration required to give half-maximal inhibition, i.e. 0.06 mM vs 0.135 mM LND. This higher sensitivity might be related to the increased rate of oxygen consumption associated with the appearance of the multidrug resistant phenotype [18].

It has been shown that to impair oxygen consumption LND needs mitochondria with electron carriers in a relatively oxidized state [5]. Indeed, LND affects only state 3 respiration, whereas it is ineffective on state 4 respiration. This was confirmed by the fact that in the presence of glucose, LND was much less effective in decreasing the rate of oxygen consumption (Fig. 2). Glucose affects the steady state of the respiratory components of ascites tumor cells with a biphasic response [29] (Fig. 2). Soon after glucose addition, a transition from the endogenous state 1 to an active state 3 occurs and a more oxidized state of pyridine nucleotides as well as of cytochrome b and cytochrome c + c_1 can be observed. After approximately 1 min, there is a second transition from the active state of rapid respiration, i.e. state 3, to an inhibited state 4. The cytochrome b becomes more reduced, and a further oxidation of cytochrome c + c_1 takes place. Therefore, the low respiration rate and the more reduced state of the carriers of the first two energy-conserving sites of the respiratory chain caused the cells, both DOX-resistant and DOX-sensitive, to be less affected by LND.

The rate of aerobic lactate production was strictly correlated to the mitochondria-bound hexokinase activity [8], so that the reduced lactate production by EH-WT cells (Fig. 5) may be ascribed to the low amount of enzyme associated with the outer mitochondria membrane. LND inhibited the aerobic glycolysis of EH-DOX (Fig. 4 left) and EH-WT cells (Fig. 5) with a similar, but not identical, pattern to that observed for the oxygen consumption. The most remarkable difference was that the drug was less effective in reducing aerobic glycolysis. This can be easily understood considering that higher drug concentrations were required to affect enzyme activity (Fig. 4, right).

Nevertheless, Ben-Horin *et al.* [30], using ^{31}P and ^{13}C NMR spectroscopy, concluded that the lower extracellular content of lactate in LND-treated DOX-sensitive MCF-7 cells depends on an impairment of lactate transport, resulting in an accumulation and intracellular acidification responsible for LND toxicity. However, further data of Fanciulli *et al.* [3] argue strongly against inhibited lactate transport and confirm, once more, that the inhibition of aerobic glycolysis by LND must be ascribed to an impairment of mitochondria-bound hexokinase, as also demonstrated by Oudard *et al.* on human gliomas transplanted in nude mice [31]. This discrepancy can be ascribed to the higher LND concentration used in NMR experiments, 0.623 mM vs 0.155 mM of Fanciulli *et al.* [3]. Indeed, it has been shown that LND induces a remarkable concentration-dependent effect on membrane ultrastructure and composition [32, 33]. At LND concentrations higher than 0.3 mM, e.g. 0.4–0.8 mM, there is a massive loss of phospholipids, so that a concentration as high as 0.623 mM might impair not only bound hexokinase but also carrier-mediated lactate transport.

On the other hand, if LND acted only on lactate transport and did not inhibit glycolysis, a greater intracellular content of lactate with a higher acidification should have been observed in LND-treated DOX-resistant MCF-7 cells due to their enhanced glycolysis [3, 34]. Instead, NMR studies on these cells, performed by the same group, show a very low intracellular lactate accumulation, with only slight intracellular acidification when compared to LND-treated

sensitive cells [35]. Moreover, at LND concentrations ranging from 0.15 and 0.30 mM, an effect on lactate transport should be excluded, because the drug stimulates aerobic lactate production in normal as well as in low malignant tumors [6, 36].

The ability of LND to impair both energy-yielding processes resulted in a reduction of ATP production which was less marked in the presence of glucose (Table 1) because of the contribution of glycolysis and the reduced inhibition of oxidative metabolism. Therefore, the higher intracellular content of DOX in LND-treated EH-DOX cells, both in the presence and the absence of glucose, may be ascribed to a decreased efflux more than to an enhanced influx as suggested by the results with oligomycin. When oligomycin, which specifically inhibits oxidative ATP production, was added to EH-DOX cells incubated in glucose-free medium, there was an increase in DOX uptake similar to that found with LND.

DOX efflux is an energy-requiring process, and EH-DOX, as sensitive Ehrlich ascites tumor cells can use glycolysis, respiration or both as energy sources for DOX outward transport. In the absence of glucose, i.e. when oxidative phosphorylation was the only energy-yielding process, EH-DOX cells showed an intracellular DOX content remarkably higher than in the presence of glucose (Table 2). Moreover, because of the very low ATP concentration (1.47 nmol/10^7 cells; Table 1) in LND-treated cells, the greatest amount of the drug was retained by the cells. In glucose-supplemented medium, both glycolysis and respiration contributed to ATP synthesis ([18] and Table 1); however, although LND inhibited both these pathways, the ATP concentration was still high enough, 6.44 nmol/10^7 cells; Table 1, to extrude a greater amount of DOX.

LND does not affect soluble enzyme, but inhibits only membrane-bound enzymes [5], so that to display its effect on energy metabolism it must be associated to mitochondria membranes, a process which takes 1–2 min [37] (Fig. 2, traces A and C; Fig. 3, traces A and C). On the other hand, in anthracycline-resistant cells, *ca.* 50% of the intracellular drug is released into the medium within the first 4–5 min [38]. These observations, taken together, explain the reduced effect of LND on the efflux when added to DOX-loaded cells.

In conclusion, our data demonstrate that the increased DOX content in Ehrlich ascites tumor cells elicited by LND depend on an inhibition of respiration and glycolysis with a remarkable decrease in ATP availability. Therefore, LND, currently employed in tumor therapy, might also be useful in reducing or reversing drug resistance in those cells in which the overexpression of P-170 glycoprotein impairs the ability to accumulate and retain the drug [3, 39]. This possibility is a promising one and surely worthy of further investigation. Nevertheless, it should be kept in mind that these results were obtained *in vitro* with isolated cells and at an LND concentration (0.225 mM, i.e. 70 (μg/mL) higher than peak plasma concentrations achievable in humans. Moreover, the amount of drug needed to obtain the same effect *in vivo* depends on several factors such as drug distribution, drug metabolism, and time-dependent phenomena which may vastly alter the situation *in vivo* compared to *in vitro*. However, the levels of concentration required to obtain these effects *in vitro* may provide an initial estimate of concentrations which would be required to obtain comparable effects *in vivo*.

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References

1. Simon SM and Schindler M, Cell biologic mechanisms of multidrug resistance in tumors. *Proc Natl Acad Sci* **91:** 3497–3504, 1994.
2. Gottesman MM and Pastan I, Biochemistry of multidrug resistance mediated by the multidrug transporters. *Ann Rev Biochem* **62:** 385–427, 1993.
3. Fanciulli M, Valentini A, Bruno T, Citro G, Zupi G and Floridi A, Effect of the antitumor drug lonidamine on glucose metabolism of adriamycin-sensitive and -resistant human breast cancer cells. *Oncol Res* **8:** 111–120, 1996.
4. Fanciulli M, Bruno T, Castiglione S, Del Carlo C, Paggi MG and Floridi A, Glucose metabolism in doxorubicin-sensitive and -resistant LoVo human colon carcinoma cells. *Oncol Res* **5:** 357–362, 1993.
5. Floridi A and Lehninger AL, Action of the antitumor and antispermatogenic agent lonidamine on electron transport in Ehrlich ascites tumor mitochondria. *Arch Biochem Biophys* **226:** 73–83, 1983.
6. Floridi A, Paggi MG, Marcante ML, Silvestrini B, Caputo A and De Martino C, Lonidamine, a selective inhibitor of aerobic glycolysis of murine tumor cells. *J Natl Cancer Inst* **66:** 497–499, 1981.
7. Floridi A, Paggi MG, D'Atri S, De Martino C, Marcante ML, Silvestrini B and Caputo A, Effect of lonidamine on the energy metabolism of Ehrlich ascites tumor cells. *Cancer Res* **41:** 4661–4666, 1981.
8. Pedersen PL, Tumor mitochondria and bioenergetics of cancer cell. *Prog Exp Tumor Res* **22:** 190–274, 1978.
9. Raaphorst GP, Feeley MM, Heller DP, Danjoux CE, Martin L, Maroun JA and De Santis AJ, Lonidamine can enhance the cytotoxic effect of *cis*-platin in human and rodent cells. *Anticancer Res* **10:** 923–928, 1990.
10. Zupi G, Greco C, Laudonio N, Benassi M, Silvestrini B and Caputo A, *In vitro* and *in vivo* potentiation of the antitumor effect of adriamycin. *Anticancer Res* **6:** 1245–1250, 1986.
11. Rosbe KW, Brann TW, Holden SA, Teicher BA and Frei E III, Effect of lonidamine on the cytotoxicity of four alkylating agents *in vitro*. *Cancer Chemother and Pharmacol* **25:** 32–36, 1989.
12. Kim JH, Alfieri AA, Kim SH and Young CW, Potentiation of radiation effects of two murine tumors by lonidamine. *Cancer Res* **46:** 1120–1123, 1986.
13. Raaphorst GP, Feeley MM, Danjoux CE, Martin L, Mariun J and De Santis J, The effect of lonidamine (LND) on radiation and thermal responses of human and rodent cell lines. *Int J Radiat Oncol Biol Phys* **20:** 509–515, 1991.
14. Raaphorst GP, Feeley MM, Martin L, Danjoux CE, Mariun, J De Santis J and Ko D, Enhancement of sensitivity to

hyperthermia by lonidamine in human cancer cells. *Int J Hyperthermia* **7:** 763–772, 1991.
15. Cividalli A, Gentile Fp, Alonzi A, Beanssi M, Mauro F and Floridi A, *In vivo* control of tumor growth by lonidamine and radiations: influence of the timing and sequence of drug administration and irradiation treatment. *Int J Oncology* **1:** 561–565, 1992.
16. Floridi A, Gentile FP, Bruno T, Delpino A, Iacobini C, Paggi MG, Castiglione S and Benassi M, Thermal behavior of a human cell line and its response to combinations of hyperthermia and lonidamine. *Oncol Res* **5:** 1–10, 1993.
17. Floridi A, Gambacurta A, Bagnato A, Bianchi C, Paggi MG, Silvestrini B and Caputo A, Modulation of adriamycin uptake by lonidamine in Ehrlich ascites tumor cells. *Exp Mol Pathol* **49:** 421–431, 1988.
18. Miccadei S, Fanciulli M, Bruno T, Paggi MG and Floridi A, Energy metabolism of adriamycin-sensitive and -resistant Ehrlich ascites tumor cells. *Oncol Res* **8:** 27–35, 1996.
19. Reynafarje B, Costa L and Lehninger AL, O_2 solubility in aqueous media determined by kinetic method. *Anal Biochem* **45:** 430–450, 1985.
20. Hohorst HJ, Determination with lactic-dehydrogenase and DPN. In: *Methods of Enzymatic Analysis* (Ed. Bergmeyer HU), pp. 266–270. Academic Press, New York, 1965.
21. Parry DM and Pedersen PL, Intracellular localization and properties of particulate hexokinase in the Novikoff ascites tumor. *J Biol Chem* **258:** 10904–10912, 1983.
22. Floridi A, Barbieri R, Pulselli R, Fanciulli M and Arcuri E, Effect of the local anesthetic bupivacaine on the energy metabolism of Ehrlich ascites tumor cells. *Oncol Res* **6:** 593–601, 1994.
23. Kaushal V and Barnes LD, Effect of zwitterionic buffers on measurement of small masses of protein with bicinchoninic acid. *Anal Biochem* **157:** 291–294, 1986.
24. Stocchi V, Cucchiarini L, Magnani M, Chiarantin L, Palma P and Crescentini G, Simultaneous extraction and reverse-phase high-performance liquid chromatographic determination of adenine and pyridine nucleotides in human red blood cells. *Anal Biochem* **146:** 118–124, 1985.
25. Levinson C, Regulatory volume increase in Ehrlich ascites tumor cells. *Biochem Biophys Acta* **1021:** 1–8, 1990.
26. Nielsen D, Maare C and Skovsgaard T, Influx of daunorubicin in multidrug resistant Ehrlich ascites tumour cells: Correlation to expression of P-glycoprotein and efflux. Influence of verapamil. *Biochem Pharmacol* **50:** 443–450, 1995.
27. Malorni W, Arancia G, De Martino C, Nista A, Accinni L, Masella R and Cantafora A, On the mechanism of action of lonidamine: A study on human erythrocyte membrane. *Exp Mol Pathol* **49:** 361–372, 1988.
28. Santini MT, Cametti C, Indovina PL, Malorni W, Benassi M, Gentile Fp, Castiglione S and Floridi A, Effects of lonidamine on the membrane electrical properties of Ehrlich ascites tumor cells. *FEBS Lett* **291:** 196–172, 1991.
29. Chance B and Hess B, Metabolic control mechanism. IV. The effect of glucose upon the steady-state of respiratory enzymes in the ascites cells. *J Biol Chem* **234:** 2421–2427, 1959.
30. Ben-Horin H, Tassini M, Vivi A, Navon G and Kaplan O, Mechanism of action of the antineoplastic drug lonidamine: ^{31}P and ^{13}C nuclear magnetic resonance studies. *Cancer Res* **55:** 2814–2821, 1995.
31. Oudard S, Poirson F, Miccoli L, Bourgeois Y, Vassault A, Poisson M, Magdelenat H, Dutrillaux B and Poupon MF, Mitochondria-bound hexokinase as target for therapy of malignant gliomas. *Int J Cancer* **62:** 216–222, 1995.
32. Malorni W, Arancia G, De Martino C, Nista A, Accinni L, Masella R and Cantafora A, On the mechanism of action of lonidamine: A study on the human erythrocyte membrane. *Exp Mol Pathol* **49:** 361–372, 1988.
33. Arancia G, Malorni W, Crateri Trovalusci P, Isacchi G, Giannella G and De Martino C, Differential effect of lonidamine on the plasma membrane ultrastructure of normal and leukemic human lymphocytes. *Exp Mol Pathol* **48:** 37–47, 1988.
34. Lyon RC, Cohen JS, Faustino PJ, Megnin F and Myers CE, Glucose metabolism in drug-sensitive and drug-resistant human breast cancer cells monitored by magnetic resonance spectroscopy. *Cancer Res* **48:** 870–877, 1988.
35. Vivi A, Tassini M, Navon G and Kaplan O, Comparison of action of anti-neoplastic drug lonidamine between drug sensitive and multidrug resistant MCF-7 human breast cancer cells. Proceeding of the Third International Congress of the Magnetic Resonance Society, pp. 1699–1699. Nice, France, August 1995.
36. Paggi MG and Caputo A, Clinical significance of a rearranged hexokinase isozyme expression in neoplastic phenotype: The astrocytoma model. In: *Human Tumor Markers* (Eds. Klavins JV, Plimentel E and Salvatore F), pp. 869–881. Walter De Gruyter & Co, Berlin, 1987.
37. Floridi A, D'Atri S, Paggi MG, Marcante ML, De Martino C, Silvestrini B, Caputo A, and Lehninger AL, Binding of lonidamine to Ehrlich ascites tumor and liver mitochondria. In: *Membranes and Tumor Growth* (Eds. Galeotti T, Cittadini A, Neri G and Papa S), pp. 559–565. Elsevier Biomedical Press, Amsterdam, 1982.
38. Bates DA and Mackillop WJ, Hyperthermia, adriamycin transport and cytotoxicity in drug-sensitive and -resistant Chinese hamster ovary cells. *Cancer Res* **46:** 5477–5481, 1986.
39. Citro G, Cucco C, Verdina A and Zupi G, Reversal of adriamycin resistance by lonidamine in a human breast cancer cell line. *Br J Cancer* **64:** 534–536, 1991.