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Laboratory Investigation

Mechanism of action of lonidamine in the 9L brain tumor model involves inhibition of lactate efflux and intracellular acidification

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Summary

Malignant gliomas have been associated with a high rate of glycolytic activity which is believed necessary to sustain cellular function and integrity. Since lonidamine (LND) is believed to reduce tumor glucose utilization by inhibition of the mitochondrially-bound glycolytic enzyme hexokinase (HK), ^{31}P magnetic resonance spectroscopy (MRS) was used to noninvasively follow the effects of LND on both tumor pH and the high-energy phosphate metabolites; ATP, phosphocreatine (PCr) and inorganic phosphate (P_i) in subcutaneous rat 9L gliosarcomas. ^{31}P tumor spectra acquired in 5 min intervals pre- and post LND administration of 50 and 100 mg/kg, i.p. revealed an acidotic pH shift of – 0.25 and – 0.45 pH units, respectively within 30 min post administration. The ATP/P_i ratio of 9L tumors decreased to 40% of control and P_i levels increased to 280% of control over a 3 hr period. LND exerted no effect on tumor blood flow and mean arterial blood pressure. Brain and muscle metabolite levels and pH were also unaffected by LND. *In vitro* measurements of cultured 9L tumor cell intra- and extracellular lactate, pentose phosphate pathway (PPP) and hexokinase (HK) activities suggest that the mode of action of LND involves inhibition of lactate efflux and intracellular acidification. The selective reduction of tumor energy metabolites and pH by LND may be exploitable for sensitizing gliomas to radiation, chemotherapy or hyperthermia.

Abbreviations: pH_e – extracellular pH; GC/MS – gas chromatography/mass spectrometry; LND – lonidamine; HK – hexokinase, pH_i – intracellular pH; KRB – Krebs ringer bicarbonate buffer; MABP – mean arterial blood pressure, PPP – pentose phosphate pathway; PCr – phosphocreatine; ^{31}P MRS – phosphorus-31 magnetic resonance spectroscopy; SIM – selected ion monitoring

Introduction

Malignant gliomas remain refractory to treatment despite the development of new antineoplastic strategies. The rapid cellular proliferation and resistance to therapy exhibited by malignant gliomas is, in part, due to the ability to maintain essential energy metabolite levels through abnormally high rates of glucose utilization and aerobic glycolysis [1, 2]. In highly glycolytic tumor cells, glucose is committed to metabolism by hexokinase (HK)[1], which catalyses the phosphorylation of glucose at the expense of mitochondrially-produced ATP. A number of workers have shown that tumor cells exhibit a marked elevation in HK activity [3] with reduced sensitivity to product inhibition by glucose 6-phosphate [4]. Moreover, subfractionation studies have shown that HK in neoplastic cells is preferentially

bound to the outer surface of the mitochondrial membrane [5, 6] and it has been suggested that mitochondrially-bound HK has preferred accesses to ATP generated by oxidative phosphorylation [7]. These studies, taken together, may provide the metabolic basis for the high rates of glucose 6-phosphate formation and aerobic glycolysis in malignant tumors.

It has been suggested that the significantly large differences in cytosolic versus mitochondrial-bound hexokinase between normal and malignant tissue offers an intriguing target for selective metabolic manipulation [6]. The rationale of using LND in the sensitization of neoplastic cells relies on the supposition that its mechanism of action involves interference with mitochondrially-bound hexokinase and aerobic glycolysis [8, 9]. The interference of glycolysis would result in decreased cellular energy status thereby making tumor cells more susceptible to damage by radiation or hyperthermia treatments. Indeed, several studies have suggested that LND is beneficial in reducing cell growth and recovery from damage induced by adjunctive therapeutic modalities including radiation [10–12], hyperthermia [13, 14] and chemotherapy [15, 16]. Other studies have reported other LND-induced effects such as ultrastructural changes in the plasma membrane [17] and cytoskeleton [18] and intracellular calcium elevation [19]. More recently, using a perfused human breast cancer cell line, investigators have shown that exposure of tumor cells to LND inhibits lactate efflux leading to intracellular acidification [20].

In this study, *in vivo* ^{31}P NMR spectroscopy was used to noninvasively monitor the effects of LND on the energy state and tumor pH in subcutaneous 9L gliosarcomas in the rat. To date, no reports have been published demonstrating direct evidence that LND actually impairs *in vivo* tumor glucose metabolism with subsequent impairment of cellular energy metabolism. We have also performed *in vitro* studies to determine whether inhibition of lactate efflux plays a role in the mediation of LND-induced impairment of glucose consumption as was previously reported in breast tumor cells [20]. Our results demonstrate that LND indeed causes an impairment of tumor energy state and pH homeostasis and that these effects appear to be associated, at least in part, with disruption of lactate transport. The loss of high-energy phosphate stores and decline in intracellular pH may provide a selective approach for sensitizing gliomas to adjunctive therapeutic modalities. Furthermore, ^{31}P NMR spectroscopy allows the effects of LND to be monitored dynamically *in vivo* and thereby provides a means for dosage optimization and timing of subsequent adjunctive therapies.

Methods

Tumor implantation

Rat 9L tumor cells were grown in MEM containing 10% fetal bovine serum and antibiotics. Cells were harvested by trypsinization, counted and suspended in serum-free MEM at 10^6 cells/0.1 ml. Subcutaneous tumors were induced by injection of 0.2 ml 9L tumor cell suspension in the right thigh muscle of male Fischer-344 rats under isofluorane anesthesia. Tumors were allowed grow to approximately 2.5 cm^3 for these studies.

In vivo *^{31}P NMR spectroscopy*

For all *in vivo* studies, rats were intubated and ventilated with a 1 to 1.25% isofluorane/air mixture and kept at 37° C using a water-jacketed blanket. Isofluorane was used for *in vivo* studies since we found that animals anesthetized with sodium pentobarbital or a ketamine/xlyazine mixture were not able to tolerate the 100 mg/kg dose of LND. Cannulation of a femoral vein was accomplished in order to obtain blood samples necessary for adjusting the ventilation rate to maintain normal blood pH, PO_2 and PCO_2 throughout the experimental period. A Radiometer ABL-300 blood gas analyzer was used for blood analysis. The tumor was positioned adjacent to the surface coil probe and the entire apparatus was placed in the bore of the magnet. ^{31}P NMR experiments were accomplished at 121.4 MHz on a Varian Imaging system equipped with an 18.3 cm horizontal bore 7 Tesla magnet. ^{31}P NMR spectra

were acquired using a 1 cm diameter surface coil which was switchable to the ^{1}H resonance frequency (300 MHz) for shimming purposes. ^{31}P spectra were acquired using the following parameters: 4K data points per FID, spectral width of 8,000 Hz, repetition time of 2.3 sec, 132 signal averages per spectrum (5 min total accumulation time), and a pulse length of 18 μs. A minimum of three control ^{31}P spectra were obtained before administration of LND through an indwelling i.p. cannula. ^{31}P NMR spectra were apodized with a 35 Hz exponential line broadening function prior to Fourier transformation. Spectral baselines were corrected using a cubic spline method. Quantification of ^{31}P resonance areas was accomplished using a spectral deconvolution routine provided with the Varian MR system. Intracellular pH (pH_{MRS}) was calculated from the chemical shift of P_i relative to PCr.

For the ^{31}P NMR studies, a total of 9 tumor-bearing rats were divided evenly between control (buffer only), 50 and 100 mg/kg LND. The buffer used for dissolving LND consisted of trizma base (12 g) and glycine (57.6 g) in 500 ml sterile water (final pH = 8.3). LND (22 mg) was dissolved in 1 ml buffer solution and vortexed until the solution was clear.

Laser Doppler tumor blood flow measurements

In a separate series of experiments, a laser Doppler blood perfusion monitor (Vasamedics Inc., Model No. BPM 403) was used to monitor changes in microvascular blood flow in subcutaneous 9L gliosarcomas before and following administration of LND. The laser emits an infrared light at 760–800 nm which is transmitted into the tissue through a right angle probe (Model No. P-430) containing an optical fiber. The probe was placed on the surface of the tumor following resection of a small area of skin over the tumor to allow light penetration. MABP and rectal temperature were continuously monitored using a Gould ACQ4600 acquisition recording system interfaced to a desktop computer. Before administration of LND was accomplished, a minimum of 10 minutes of stable blood flow measurements were recorded. Three rats were injected with buffer alone (1 ml) and 3 rats were injected with buffer containing LND (100 mg/kg, i.p.).

Determination of intracellular pH in vitro

Measurements of pH_i *in vitro* were accomplished using the pH-sensitive fluorescence dye BCECF as previously described [21]. Briefly, cultured 9L gliosarcoma cells were trypsinized and dispersed to single cells, resuspended in Tris-buffered RPMI 1640 at pH 7.4 and incubated for 3 hr at 37° C. The cells (10^7 cells/ml) were then loaded with 5 μg/ml BCECF-AM in DMSO and incubated for 30 min at 37° C to allow permeation of the dye and subsequent deesterification by endogenous esterases. After loading with BCECF the cells were centrifuged and resuspended in Tris-buffered RPMI 1640 pH 7.4 or Mes/Mops buffered media at pH 6.6 at 2×10^5 cells/ml in 1.5 ml micro centrifuge tubes in the presence or absence of 100 μM LND for up to 120 min. Aliquots were read in a Hitachi F-2000 fluorometer using excitation wavelengths of 440 and 500 nm, emission wavelength of 530 nm and slit width of 10 nm. The fluorescence ratio was then converted to actual pH values using a calibration curve obtained from titration of the cell suspension to known pH values. Control of intracellular pH was achieved in a high K^+ buffer (124 mM) containing 10 μg/ml of the ionophore nigericin, which would allow intra- and extracellular pH to be equalized.

Determination of intra- and extracellular lactate in vitro

Rat 9L gliosarcoma cells were grown to confluence in 6-well flat bottom sterile tissue culture plates. The cells were rinsed 3 times with KRB, pH = 7.4, and exposed for 60 min to KRB containing 100 μM LND and 5.5 mM (1,6-$^{13}C_2$,6,6-$^{2}H_2$) glucose for quantitation of both lactate and PPP activity (described below). Following exposure to LND, the media was collected for analysis of released lactate and the plates washed 3 times with cold KRB. Plates were kept on ice while the tumor cells were re-

moved using a rubber policeman after addition of 0.6 M perchloric acid to the wells. The cells were then homogenized manually and sonicated for 30 sec using a Branson sonifier (Model # 250) equipped with a micro-tip. The homogenates were neutralized with equivolumes of $KHCO_3$ centrifuged at 1000 × g for 10 min and the supernatant collected for analysis of intracellular lactate. Lactate levels were determined by GC/MS as previously described [22]. In brief, samples were lyophilized to dryness and derivatized with bis(trimethylsilyl)trifluoroacetamide. A Hewlett Packard 5890/5971 GC/MS system was used for analysis of lactate. SIM was performed on the m/z 219, 220, 221 and 222 ions which correspond to the loss of a methyl group from the trimethylsilyl derivative of lactic acid.

Measurements of pentose phosphate pathway activity

The method for determination of PPP activity was described in detail elsewhere [22, 23]. This method relies on the differential labeling of released lactate following metabolism of (1,6-$^{13}C_2$,6,6-$^{2}H_2$)glucose. Metabolism of this isotopically substituted glucose molecule through glycolysis produces (3-^{13}C)lactate, originating from the C1 position of glucose, and (3-^{13}C,3,3-$^{2}H_2$)lactate, originating from the C6 position of glucose. In contrast, metabolism of the labeled glucose through the PPP produces (3-^{13}C,3,3-$^{2}H_2$)lactate from the C6 position and unlabeled lactate from the C1 position since the label in the C1 position is lost as $^{13}CO_2$ by the action of the PPP enzyme phosphogluconate dehydrogenase. These different lactate isotopomers can be easily resolved and quantitated by GC/MS and the percentage of glucose metabolized through the PPP relative to glycolysis calculated from the amounts of each of these lactate isotopomers in the extracted ion chromatogram. Total lactate production was determined from the sum of the four lactate ion species.

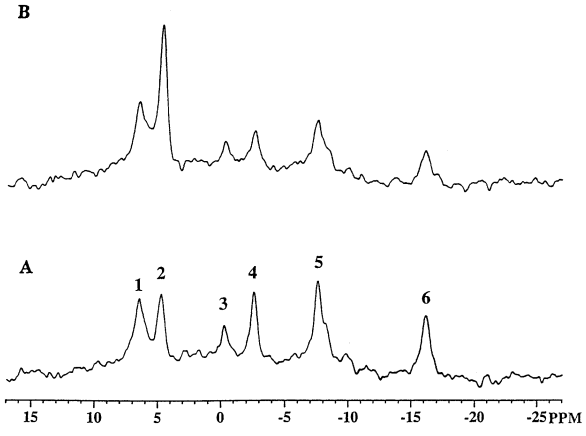

Figure 1. In vivo ^{31}P MR spectra of a subcutaneous 9L tumor (A) pre- and (B) 3 hr post-administration of LND (100 mg/kg, i.p.). Resonance assignments are as follows: 1, phosphomonoesters (PME); 2, inorganic phosphate (P_i); 3, phosphocreatine (PCr); 4, γ-ATP and β-ADP; 5, α-ATP, α-ADP; and 6, β-ATP. Note the decline in ATP levels and the corresponding increase in P_i following LND administration (Spectrum B) indicating impaired energy metabolism.

Assay of hexokinase activity

Hexokinase was assayed spectrophotometrically as previously described [24]. Pelleted 9L gliosarcoma cells were washed and resuspended in 0.25 M sucrose, and lysed by brief sonication. The lysate was centrifuged at 1000 × g for 10 minutes; all hexokinase activity remained in the supernatant. The latter was then centrifuged at 43,500 × g for 20 minutes. This supernatant contained 93% of the initial total activity. The pellet was resuspended in 0.25 M sucrose, assayed, and found to contain 6% of the initial total activity (total recovery, 99% of initial total activity). The particulate enzyme presumably represents mitochondrially bound hexokinase, reported to occur in many normal tissues as well as tumors. Both soluble and particulate hexokinase fractions were preincubated in assay medium minus substrate ATP, and 68 μM LND for 10 min. After the preincubation, the reaction was started by addition of ATP. Control samples contained only the Tris-glycine buffer used as solvent for LND. The enzyme preparations were preincubated with LND (25 μM up to 1.25 mM), with or without glucose present during the incubation with LND.

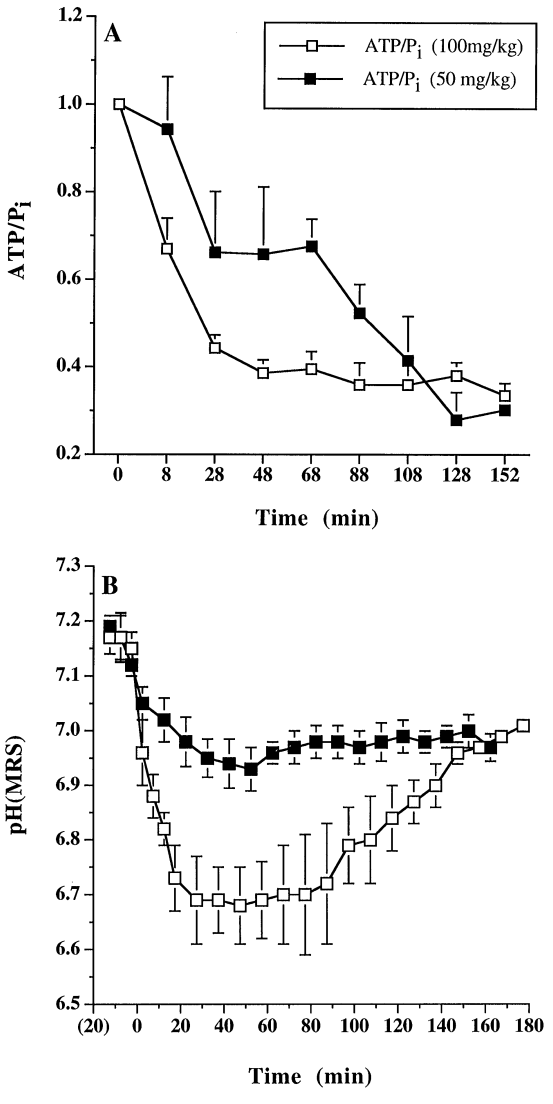

Figure 2. The effects of LND on the ATP/P_i ratio (A) and tumor pH (B) versus time in subcutaneous 9L gliomas following a bolus i.p. injection of 50 (filled squares) and 100 mg/kg (open squares) LND (n = 3 ± S.E.).

Results

In vivo *^{31}P MRS studies*

Shown in Figure 1 are representative ^{31}P MRS spectra of a rat subcutaneous 9L tumor (A) before and (B) 3 hr following i.p. administration of 100 mg/kg LND. These spectra reveal a dramatic decline in ATP with a reciprocal increase in P_i levels. Minimal

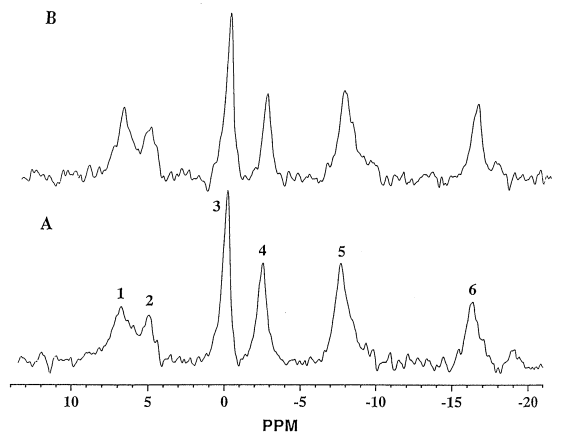

Figure 3. In vivo ^{31}P MR spectra of the brain (A) pre- and (B) 3 hr post-administration of LND (100 mg/kg, i.p.). Resonance assignments are as described in Figure 1.

changes in intensity of the PCr resonance were observed thus it was assumed that this resonance was derived primarily from the overlying skin as previously reported [25]. Injecting rats with drug vehicle alone (glycine buffer) resulted in no detectable changes in phosphorus metabolites in the ^{31}P spectrum (not shown) indicating that LND is responsible for the deleterious effects on tumor energy metabolism. The decrease in ATP and the increase in P_i were observed almost immediately following LND administration. As shown in Figure 2A, the β-ATP/P_i ratio decreased to about 63% and 40% of control with 50 and 100 mg/ml LND, respectively, in the first 30 min of the experiments and remained low throughout the experimental period. Even more dramatic was the effect of LND on intracellular pH, as determined from the chemical shift difference between the P_i and PCr resonances in the ^{31}P spectrum. Shown in Figure 2B, is the time course of the average intracellular pH of the tumor pre- and post-administration of 50 and 100 mg/kg LND. Tumor pH declined approximately 0.45 pH units within the first 30 min (with 100 mg/kg) and slowly returned to within 0.15 pH units 3 hr post-LND. Although less pronounced, the lower dose of 50 mg/kg LND also resulted in a similar pattern of intracellular acidification.

The deleterious effects of LND appeared to be most specific to the tumor since no significant

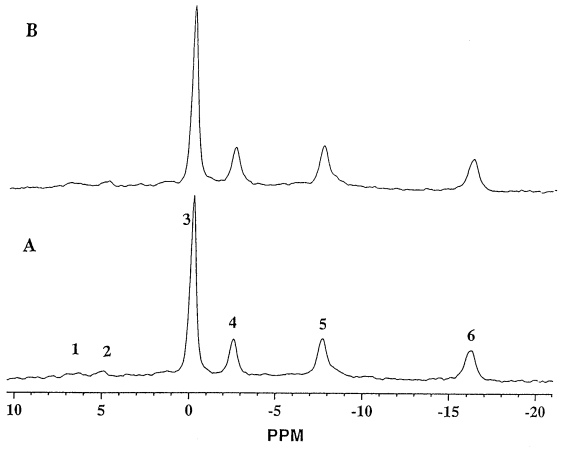

Figure 4. In vivo ^{31}P MR spectra of the hind leg muscle (A) pre- and (B) 3 hr post-administration of LND (100 mg/kg, i.p.). Resonance assignments are as described in Figure 1.

change in energy metabolism or intracellular pH was observed in brain (Figure 3) or skeletal muscle (Figure 4) following administration of 100 mg/kg LND. In addition, LND (100 mg/kg) had no appreciable effect on tumor blood flow or MABP as shown in Table 1. Blood pH, PO_2, PCO_2 and body temperature also remained within normal parameters (not shown).

In vitro *studies*

The effects of LND on pH_i were also studied in suspensions of 9L cells by fluorescence spectroscopy. Cells incubated with 100 μM LND had a rapid decline in pH_i of 0.20 to 0.25 pH units, which was followed by a partial recovery over 120 min (Figure 5). In an acidic environment, i.e. when medium pH was kept at 6.6, exposure to LND caused a substantially larger decline in pH_i (e.g. 0.35–0.40 pH units) (Figure 5).

The effects of LND on intra- and extracellular lactate levels following a 1 hr exposure of 9L culturres to 100 μM LND was evaluated. In these experiments, lactate was quantitated from GC/MS analysis of the media and PCA extracts. LND induced a pronounced decrease in the extracellular lactate concentration to 23 ± 11% (± S.D., n = 4) of control levels while intracellular lactate accumulated more

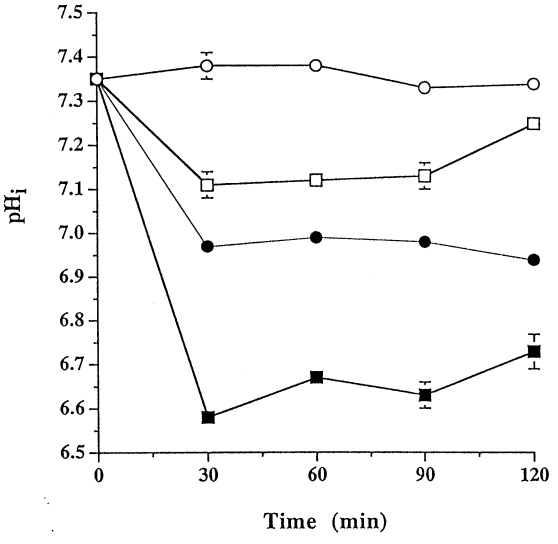

Figure 5. Fluorescence measurements of pH_i versus time from suspensions of 9L cells under control conditions at pH_e 7.4 (○) and pH_e 6.6 (●), and in the presence of 100 μM LND at pH_e 7.4 (□) and at pH_e 6.6 (■) (n = 3 ± SD).

than 5-fold (526 ± 56%) indicating an inhibition of lactate transport.

The effects of LND on cellular PPP activity was also examined. Since the cytotoxic effects of LND in a variety of tumor cell types have been ascribed to inhibition of HK bound to the outer surface of the mitochondrial membrane [9, 26], we have measured PPP activity in order to determine whether such inhibition indeed occurs in 9L tumor cells *in situ*. The rational behind these measurements was that HK inhibition by LND would be expected to result in a net reduction in glucose 6-phosphate formation available for consumption by the PPP. PPP

Table 1. Effects of LND administration (100 mg/kg, body weight, i.p.) on rat 9L tumor blood flow and mean arterial blood pressure (MABP)

Time (min)	TBF (normalized)	MABP (mmHg)
0	100	88.8 ± 4.4
10	99.2 ± 11.1	82.4 ± 8.9
20	96.6 ± 14.7	81.0 ± 5.2
30	100.2 ± 6.4	86.5 ± 10.6

Values for TBF were obtained from laser Doppler measurements and are expressed as percent of control. All values are expressed as means ± SD (n = 6).

activity (defined as the amount of glucose metabolized to lactate through the PPP versus glycolysis) in 9L cultures in the absence and presence of 100 μM LND was found to be 10.7 ± 0.8% and 11.0 ± 1.4%, respectively. Exposure of 9L cells to 100 μM LND caused no detectable change in PPP activity suggesting that LND exerts minimal inhibition of HK.

Discussion

The ^{31}P MRS data clearly demonstrate that LND (50–100 mg/kg) exerts marked effects on energy metabolism and intracellular pH in rat subcutaneous 9L tumors *in vivo*. The rapid and pronounced intracellular acidification of approximately 0.45 pH units observed with 100 mg/kg LND could not be attributed to LND-induced changes in physiological parameters such as MABP, tumor blood flow or blood chemistry since these remained within normal physiological limits. Furthermore, since a similar LND-induced decrease in pH_i was also observed in cultured 9L cells, the effect of LND on *in vivo* tumor energy metabolism is most likely due to a direct action on the tumor rather than an indirect effect on the host animal.

It is currently hypothesized that a major mechanism of LND action is the inhibition of mitochondrially-bound HK and subsequently of aerobic glycolysis. Floridi et al. [9] have reported 66% inhibition of mitochondrial HK with 5 μM LND with very little effect on cytoplasmic HK. To test whether this hypothesis is applicable to 9L gliosarcoma cells, sub-cellular fractionation of 9L cells was accomplished. An assay for HK activity in the supernatant and pellet was then done (Wilson J.E., personal communication). The protocol we used was similar to one previously described [9], and the concentration of LND was well above the levels (2–5 μM) reported by these authors which they reported caused marked inhibition of the mitochondrially-bound enzyme. The supernatant obtained from 9L cells was found to contain 93% of the initial total HK activity whereas the pellet which contained mitochondrially bound HK contained 6% of the total activity. Preincubation of both the soluble and particulate HK 9L cell fractions in medium containing from 25 μM up to 1.25 mM LND for 10 min resulted in no detectable inhibition of HK in either sample fraction. Thus, we find no support for the suggestion that LND is a selective inhibitor of the mitochondrial form of hexokinase. In fact, we have been unable to demonstrate any inhibition at all, of either soluble or mitochondrial form. Moreover, if LND was an inhibitor of HK, it would be anticipated that such inhibition would result in a net decrease in glucose 6-phosphate formation with a subsequent decrease in basal activity of the PPP. This however was not the case, as PPP activity remained at approximately 10% of glucose metabolized through the PPP relative to glycolysis in the presence and absence of LND. It is also interesting to note that although subcutaneous tumor pH tended to recover during the 3 hr experimental time period, ATP/P_i levels did not recover. This is possibly due to the fact that inhibition of lactate transport by LND occurred at the lactate transporter site in the cell membrane resulting in a new, lower rate of glucose to lactate production. However, pH regulatory mechanisms remain intact thereby allowing the cells to neutralize their pH over time.

Interestingly, the decrease in pH_i was dependent on extracellular pH with a much higher LND-induced pH_i shift observed at $[pH]_e$ of 6.6. This data suggested that a possible mechanism of action of LND involved inhibition of the pH-dependent [27] lactate efflux across the plasma membrane. LND exposure resulted in a 5-fold increase in intracellular lactate accumulation accompanied by a decreased in lactate release to about 20% of control values. The lack of effects of LND on HK and PPP activities, the exacerbated LND-induced reduction in pH_i under extracellular acidic conditions and the LND-induced intracellular accumulation of lactate suggest that the mechanism of LND action does not involve HK inhibition but rather inhibition of lactate efflux and intracellular acidification. This is also in agreement with Ben-Horin et al. [20] who reported a more than 3-fold increase in intracellular lactate content in MCF-7 human breast cancer cells exposed to LND using ^{13}C MRS. Furthermore, in an elegant ^{31}P MRS study, these workers have also shown that LND had no effect on the buildup of 2-deoxyglucose 6-phosphate compared with 2-deox-

yglucose alone. 2-Deoxyglucose is phosphorylated by HK to form 2-deoxyglucose 6-phosphate, which is not further metabolized thus inhibiting glycolysis. Since treating the cells with a combination of 2-deoxyglucose and LND did not result in a reduction in 2-deoxyglucose 6-phosphate accumulation, these authors concluded that LND did not act as a significant HK inhibitor in intact perfused tumor cells.

In conclusion, this study demonstrated a significant reduction in rat 9L gliosarcoma pH_i and ATP occurred *in vivo* following administration of LND which is likely attributable to an inhibition of lactate efflux rather than by a direct inhibition of HK. Since lowering pH_i has been shown to sensitize tumor cells to various therapies such as hyperthermia [28], the relative selectivity of LND for reducing 9L tumor pH_i and ATP levels should prove to be beneficial. However, further work is needed to determine if the effects of LND on subcutaneous glioma pH and ATP levels can be reproduced on intracerebral gliomas. Providing that intracerebral studies are positive, studies combining the use of LND and radiation and/or hyperthermia would be warranted. Finally, the use of *in vivo* ^{31}P NMR to dynamically follow the changes in tumor pH and ATP levels should provide a valuable method for optimizing both the dose of LND as well as the timing of subsequent adjunctive therapy following LND administration.

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