Lonidamine induced selective acidification of DB-1 human melanoma xenografts enhances tumor response to doxorubicin

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Introduction: Accumulation of weakly basic or weakly acidic drugs inside the tumor cell depends upon the transmembrane pH-gradient producing differences in the permeability of protonated and unprotonated forms of the drug. The inverted pH gradient between the inside and outside of cells that is observed in tumors presents both obstacles and opportunities for enhancement of drug activity**.** As a consequence of their high levels of aerobic glycolysis (1), tumors generally exhibit an acidic extracellular pH (pHe) and a neutral to alkaline intracellular pH (pHi) leading to an acid-outside/neutral to mildly alkaline inside plasmalemmal pH gradient (2). This gradient (dysregulated pHi/pHe) also impacts tumor response to certain chemotherapeutic agents (3) as well as to hyperthermia or high-intensity focused ultrasound (4). Our aim was to decrease the pHi in order to increase the intracellular activity of doxorubicin against DB-1 melanoma xenografts. We accomplished this by administering an inhibitor of the monocarboxylate transporter (MCT), lonidamine (LND), that blocks cellular export of lactic acid and also inhibits transport of pyruvate into mitochondria, thereby simultaneously inhibiting tumor energy production and the rate of oxygen consumption.

Material and Methods: Human melanoma xenografts development and pH measurement in tumors and in normal tissues (liver, brain and muscle) have been described in a previous publication (5). Four cohorts of five age- and weight-matched animals were randomized to the following treatment groups: cohort 1 (sham treated control) was infused intravenously (i.v.) with PBS and given appropriate sham intraperitoneal (i.p.) injections of tris/glycine buffer; cohort 2 was infused i.v. with PBS 40 minutes after LND administration i.p. (100 mg/kg); cohort 3 was injected i.p. with tris/glycine buffer and infused i.v. with doxorubicin (10 mg/kg delivered in ~10 sec) in PBS; cohort 4 was injected i.p. with LND (100 mg/kg) and after 40 minutes (determined by ^{31}P MRS), doxorubicin (10 mg/kg) was infused i.v. During the treatment and sham-treatment procedures, all animals were anesthetized with ketamine hydrochloride and acepromazine with additional anesthesia being readministered as needed approximately every 45-60 min. Animals were placed on a water pad heater to maintain body temperature during anesthesia. While the animals were held in a restrainer, tail vein catheters filled with heparin were placed to prevent blood clotting. The catheters were removed and animals allowed to recover. For the first five days post-treatment, tumor volume (caliper measurements) and body weight were measured daily and then every other day.. Statistical analysis of pHi, pHe, NTP/Pi at various time points following LND administration were compared using ANOVA with Bonferroni corrections and Tukey multiple comparison tests.. To assess the significance of treatment effects, we fit spline models to the longitudinal tumor growth data (6). The models described log tumor volume as linear throughout the period of observation in the control arm and prior to treatment in the active arms, and as a quadratic spline in the period immediately after treatment in the active arms. We evaluated significance of the treatment effects by likelihood ratio tests involving the spline coefficients. Other analyses were performed in Microsoft Excel 2010.

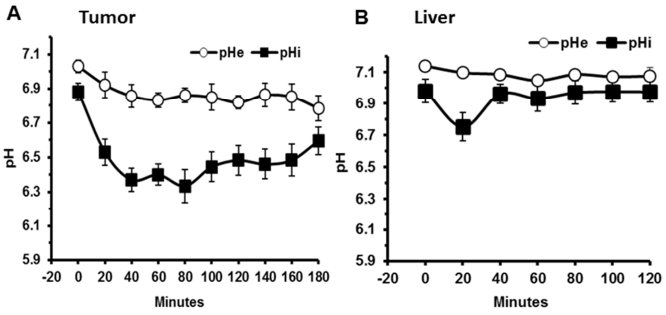

Fig. 1. The intracellular pH (pHi) and extracellular pH (pHe) profile (**A**) human melanoma xenografts **(B)** Liver, relative to baseline as a function of time of in response to LND (100 mg/kg; i.p.) administration at time zero. The values are presented as mean ± S.E.M. When not displayed, S.E.M. values were smaller than the symbol size.

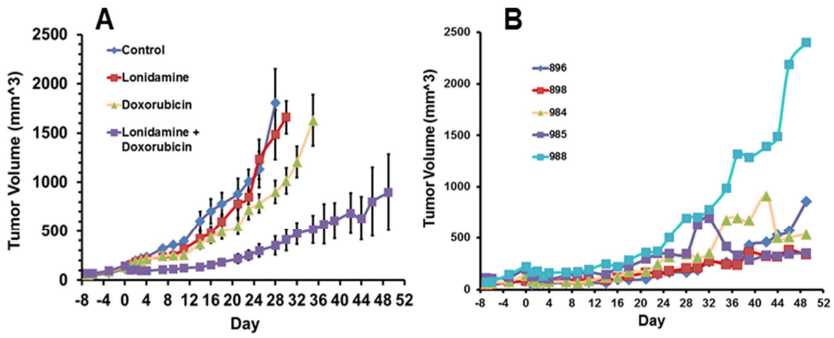

Fig. 2. **(A)** Growth delay experiments performed on DB-1 human melanoma xenografts in nude mice treated with 100mg/kg LND or 10 mg/kg Doxorubicin, mean ± SEM of each cohort. **(B)** Individual tumor regrowth curves of the five animals treated with LND and Doxorubicin combined. The values are presented as mean ± S.E.M. When not displayed, S.E.M. values were smaller than the symbol size

Results: The pHi significantly decreased 20-180 min after the administration of LND; the maximum decrease in pHi, 0.6 ± 0.1 ($p < 0.001$) occurred 80 min post-LND administration (Fig. 1, A). However, pHe exhibited a smaller decrease of 0.20 ± 0.07 ($p = 0.085$) (Fig. 1, A). Liver exhibited a small transient intracellular acidification by 0.2 ± 0.1 pH units ($p = 0.027$) at 20 min post-LND (Fig. 1, B); there were no significant changes in hepatic pHe. The effect of treatment with LND plus doxorubicin were evaluated by tumor growth delay experiments (Fig. 2). LND + doxorubicin produced effects significantly different from those induced by placebo, LND alone, and doxorubicin alone. Tumor growth delay was determined by calculating the time in days between logarithmic re-growth regions of the curves of treated tumors and saline treated controls. Tumor doubling times were estimated from the slopes of the log-linear portions of the tumor regrowth curves determined by linear regression analysis. Graphical analysis then yielded the results of treatment when LND and Doxorubicin were combined were dramatic (Fig. 2B). One of the five tumor bearing animals exhibited a tumor growth delay very similar to the five in the Doxorubicin group. The other four tumor bearing animals were still in remission at 50 d post-treatment. One of those four tumors showed evidence of potential recurrence at 50 days.

Discussion: Weak electrolytes are usually membrane permeable in their uncharged state and impermeable in their ionized state; their intracellular to extracellular distributions are governed by the pKa of the electrolyte and the pH gradient across the membrane barrier. With a normal pH gradient in normal cells, weakly basic drugs (e.g. doxorubicin) accumulate at higher concentrations inside cells than in the extracellular space. When the pH gradient is inverted, such as in cancer cells, the concentration of weakly basic drugs can be much higher in the extracellular space than inside cells. After injection of LND, pHi significantly decreased (pH=6.33), but pHe diminished much less (pH=6.8) compared to baseline. Under these conditions, doxorubicin becomes more protonated inside the tumor cell compared to outside the cell. Only the neutral form of the drug crosses the membrane. Since the ratio of charged to uncharged species increases with lower pH, more of the drug accumulates in acidic compartments. Doxorubicin intercalates into DNA, inhibits topoisomerase II and combines with iron to generate reactive oxygen species affecting DNA and cell membranes (7). This anthracycline has a single ionizable amino group with a pKa of 8.2. Since acute acidification has been reported to enhance the activity of platinum compounds (8) and alkylating agents such as nitrogen (N)-mustards (5, 9-12), we have evaluated the effect of LND-induced acidification on, doxorubicin, one of the most potent commonly used antineoplastic agent. We found that while LND alone and doxorubicin alone had minimal effects on melanoma growth, the combination of these agents administered according to a schedule based on NMR measurement of tumor pHi, substantially decreased the growth rate of this highly malignant tumor. This could provide a method for systemic therapy of this deadly cancer. **Acknowledgements:** This study is supported by grant 1-R01-CA-129544-01A2.

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