Tumor pH controls the *in vivo* efficacy of weak acid and base chemotherapeutics

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Abstract

The extracellular pH of tumor tissue is significantly lower than the extracellular pH of normal tissue, whereas the intracellular pH of both tissues is similar. In principle, extracellular acidity may be expected to enhance the intracellular uptake and cytotoxicity of weak acid chemotherapeutics that are membrane permeable in their uncharged state and inhibit the efficacy of weak bases. However, procedures for assessing the role of the gradient as a determinant of drug efficacy *in vivo* by altering the pH gradient may also alter drug availability and thus mask or exaggerate the effect of the gradient change. In the present study, we have altered the extracellular pH of tumors and compared the effect of the resultant pH gradient change on the efficacy of a weak acid versus a weak base. This experimental design gives rise to a change in the ratio of chlorambucil- to doxorubicin-induced tumor growth delay, independent of possible changes in drug availability. The extracellular pH of the 54A human tumor in NCr/Sed/*nu/nu* mice was altered by administration of 5 mg/g i.v. glucose. The resultant 0.2 pH unit increase in the tumor cell pH gradient gives rise to a predicted 2.3-fold increase in the ratio of chlorambucil to doxorubicin growth delay. The experimentally measured change in the growth delay ratio was 2.1. The results provide compelling evidence that the pH gradient in a determinant of the efficacy of weak electrolytes in the complex *in vivo* environment and may be exploited for the treatment of cancer. [Mol Cancer Ther 2006;5(5):1275–9]

Introduction

Tumors exhibit a substantially lower extracellular pH than normal tissues, whereas the intracellular pH of both tissues is similar (1–4). As a consequence, a naturally occurring and significant intracellular-extracellular pH gradient difference exists between these tissues (1). It has been suggested that this pH gradient difference may be exploitable for the treatment of cancer by weak acid drugs that are membrane permeable in their uncharged state (1, 5, 6). In an acidic extracellular environment, the nonionized fraction of a weak acid increases, allowing more drug to diffuse through the cell membrane into the relatively basic intracellular compartment where the ionized fraction increases, resulting in an increased intracellular drug concentration (7, 8).

Numerous *in vitro* studies are consistent with or have confirmed the pH gradient–dependent partitioning and cytotoxicity of weak electrolytes both in artificial lipid vesicles and in living cells under defined conditions (6, 9–16). In contrast, the role of the gradient as a determinant of cytotoxicity in the complex and dynamic *in vivo* environment has received less attention and is substantially more difficult to assess (15, 17, 18). In principle, the intracellular-extracellular pH gradient does not alter the quantity of drug delivered to tissue, but rather the intracellular-extracellular distribution of the drug. Procedures for altering the cell pH gradient of tissues for evaluation of the influence of the gradient on drug efficacy may affect the systemic biodistribution of the drug, and alter normal tissue pH gradients and systemic or local tissue physiology (17). These effects may affect the quantity of duration of drug available to tumor tissue and thereby mask or exaggerate any apparent pH gradient–dependent changes in intracellular drug concentration. Additionally, drugs such as doxorubicin adhere to numerous intravascular and interstitial matrix components (19–21), further limiting the usefulness of direct assessments of total tissue drug concentration as an index of intracellular drug concentration. The present study examines the role of the pH gradient as a determinant of the efficacy of weak electrolytes using a procedure that minimizes or reduces these potential artifacts.

We have determined the effect of pH gradient modulation on the tumor response to a weak acid versus a weak base chemotherapeutic following the same method of pH gradient alteration in the same tumor model. If the pH gradient is an operative determinant of the drug efficacy *in vivo*, altering the magnitude of the gradient will result in theoretically predicted changes in the transmembrane distribution and resulting efficacy of these drugs independent of changes in drug availability. This is because changes in the ratio of the efficacy of a weak acid versus a weak base following pH gradient manipulation is independent of the quantity of drug delivered to the tumor cells, or other unknown effects of pH manipulation on tumor growth. Additionally, the choice of the drugs to evaluate the effect of pH gradient manipulation should

Received 1/16/06; revised 2/28/06; accepted 3/24/06.

Grant support: NIH grant RO1 CA092366.

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doi:10.1158/1535-7163.MCT-06-0024

exhibit the following characteristics: pK_a values in the physiologic pH range, an intracellular site of action and entry to the site of action by passive diffusion through the cell membrane, equivalent cytotoxicity under oxygenated and hypoxic conditions, and stability under relevant pH conditions. The weak acid chlorambucil and weak base doxorubicin meet these criteria (6, 12, 22, 23).

Materials and Methods

Mice and Tumors

Twenty-four hours before tumor transplantation, 8- to 10-week-old NCr/Sed/*nu/nu* male mice (bred and maintained in our defined flora and specific pathogen-free colony) were ear-tagged and 5 Gy whole body irradiated to further depress the immune system. Third- to sixth-generation human small-cell lung carcinoma 54A (18) source tumors were excised, cut into ~2-mm chunks, and s.c. transplanted into the right hind leg at the level of the gastrocnemius. Following transplantation, the mice were periodically examined until tumors approached a volume of 100 mm^3, at which time the tumors were scored daily. Upon reaching volume of 150 mm^3, the mice were randomly assigned to the various treatment groups. Greater than 95% of chunk transfers gave rise to tumors.

Measurement of Tumor pH

Extracellular and intracellular tumor pH were measured as described previously (18). Mice were anesthetized with 50 mg/kg i.p. pentobarbital before pH analyses. ^{31}P-magnetic resonance spectroscopy estimated tumor pH along with the energy status parameters, nucleotide triphosphate/inorganic phosphate and phosphocreatine/inorganic phosphate, and tumor pO_2 are similar in conscious nonanesthetized versus pentobarbital anesthetized mice for s.c. transplanted to the hind limb and of the size used in this study (24, 25). For measurement of extracellular pH, the mice were anesthetized and placed on a heating pad to maintain core body temperature. A 0.65-mm-diameter glass microelectrode probe (Microelectrodes, Inc., Londonderry, NH) was inserted to a depth of 2 to 4 mm through a small skin puncture overlaying the tumor and a reference microelectrode was inserted in nearby s.c. tissue overlying the hip or thigh. Glucose (5 mg/g body weight) was injected i.v., and the pH was continuously monitored. Monitoring was discontinued after ~100 minutes when the extracellular pH began to increase. Intracellular pH was evaluated by ^{31}P-magnetic resonance spectroscopy with a 7 T horizontal magnet and Brucker Biospec console with a 10-mm-diameter surface coil. Following the acquisition of an initial spectrum, the mouse and probe were removed from the magnet, and then the mouse was i.v. injected with glucose and reinserted into the magnet. Spectra were acquired at 14-minute intervals (512 scans in 5 minutes followed by a 9-minute interval). pH was determined from the chemical shift of inorganic phosphate.

Drugs and Treatment Protocol

The weak acid chlorambucil, pK_a = 5.8 (Sigma Chemical Co., St. Louis, MO; ref. 6) was dissolved in DMSO, and 0.05 to 0.07 mL of the solution was injected i.p. to yield a final concentration of 26 mg chlorambucil/kg body weight. The weak base doxorubicin (pK_a = 8.2; Bedford Labs, Bedford, OH; ref. 12) was dissolved in saline and administered via the lateral tail vein in 0.15 to 0.18 mL volumes to achieve a final concentration of 12 mg/kg body weight. Administration of DMSO or saline alone was without affect on the normal increase in body weight or tumor (data not shown). The concentrations of chlorambucil and doxorubicin used in this study were near the maximum that could be administered without significant mortality, which is apparent within 24 hours following chlorambucil, or substantial weight loss that progressively develops for up to 30 days following doxorubicin administration (data not shown). For the doses used, mortality figures were <5% of treated animals for all causes over the course of the experiments and was unaffected by the addition of glucose. Aqueous glucose (25% solution) was administered by bolus injection via a lateral tail vein to yield a final concentration of 5 mg glucose/kg body weight. Glucose alone was without effect on tumor growth (18).

Tumor Growth Delay Assay

Upon reaching a volume of ~100 mm^3, all tumors were measured daily and treated at an average volume of ~150 mm^3. Volumes were calculated as $(a \times b^2)\pi/6$, where a is the larger, and b the smaller, of two orthogonal diameters. Mice were randomly assigned to one of three treatment groups: control, drug, and glucose+ drug. Following treatment, tumor volumes were measured at 1- to 2-day intervals. The pooled data from control tumors exhibited exponential growth and were fit to the equation $V = Voe^{mt}$,

Table 1. Changes in the extracellular and intracellular pH of tumors following i.v. glucose

	Control	Minutes following glucose injection				
		15 min	30 min	45 min	70 min	95 min
pHe	6.77 ± 0.03*	6.66 ± 0.03	6.57 ± 0.06	6.50 ± 0.05	6.46 ± 0.05	6.50 ± 0.05
pHi	7.17 ± 0.06	7.08 ± 0.05	7.07 ± 0.06	7.11 ± 0.05	7.07 ± 0.05	7.07 ± 0.05
pHe-pHi	−0.38 ± 0.07	−0.42 ± 0.06	−0.50 ± 0.08	−0.61 ± 0.07	−0.61 ± 0.07	−0.57 ± 0.07

Abbreviations: pHe, extracellular pH; pHi, intracellular pH.
*SE.

where Vo is the initial volume at the onset of treatment, V is the volume at time t, and m is the tumor growth constant. For mice receiving drug alone or glucose + drug, an initial growth delay was followed by exponential growth. The growth data for drug-treated mice were fit to the exponential function over a volume range of about two to four times the initial treatment volume. For each curve, the SE of the time to achieve $3Vo$ (i.e., SEt_{3Vo}) was estimated as $\pm[\ln SE_{Vo} / (\ln 3)^2 + (SE_m / m)^2]^{1/2}$, where $\ln SE_{Vo}$ and SE_m are the SE values of the intercept and the slope of the best-fit exponential. The unpaired t test was used to determine if the time to achieve $3Vo$ significantly differed between the treatments, i.e., $(t3Vo_1 - t3Vo_2) / [SEtVo_1^2 + SEtVo_2^2]^{1/2}$ with $(n_1 + n_2) - 4$ degrees of freedom, where n_1 and n_2 equals the number of animal and Vo_1 and Vo_2 are the volumes of the two treatment groups being compared.

Results

The effect of 5 mg/g i.v. glucose on the extracellular and intracellular pH of s.c. 54A tumors in the leg of NCr/Sed/*nu/nu* mice is shown in Table 1. These previously published data show that i.v. glucose minimally affects intracellular pH but induces significant changes in extracellular pH (18). Extracellular pH decreases from pH ~6.8 before glucose administration to pH 6.5 at 45 minutes following glucose, and then remains unchanged for an additional 45 minutes before slowly increasing. The net effect is to increase the pH gradient (extracellular pH-intracellular pH) from −0.38 ± 0.07 units to approximately −0.60 ± 0.07 units at 45 through 90 minutes after glucose administration. Based on these results, drugs were administered at 45 minutes following i.v. glucose administration.

Figure 1 shows the growth of control and drug-treated tumors following administration of doxorubicin (A) or chlorambucil (B). Growth curves of control and drug-treated tumors are indicated by closed squares and circles, respectively; growth curves of tumors administered drug following i.v. glucose is indicated by dashed curves. The growth curves of control tumors in Fig. 1 were obtained by an exponential fit to all tumor volume data from the initiation of treatment to 600 mm^3. Growth curves following treatment were fit to the data over the volume range beginning at 300 mm^3 in Fig. 1A and 250 mm^3 in Fig. 1B. This was judged to be the time points at which exponential growth was initiated following treatment-induced growth delay, and increasing or decreasing the range of the fit tumor volumes by 50 mm^3 did not significantly affect the results. The slopes of the regrowth curves in control versus treated tumors do not significantly differ ($P = 0.15$, Fig. 1A; $P = 0.3$, Fig. 1B), whereas the intercepts substantially and significantly differ ($P < 0.01$). As predicted, increasing the magnitude of the pH gradient (increased extracellular acidity) by ~0.2 pH units resulted in a modest but significant decrease in the efficacy of the weak base doxorubicin and increase in the efficacy of the weak acid chlorambucil. The best-fitting curves to the six

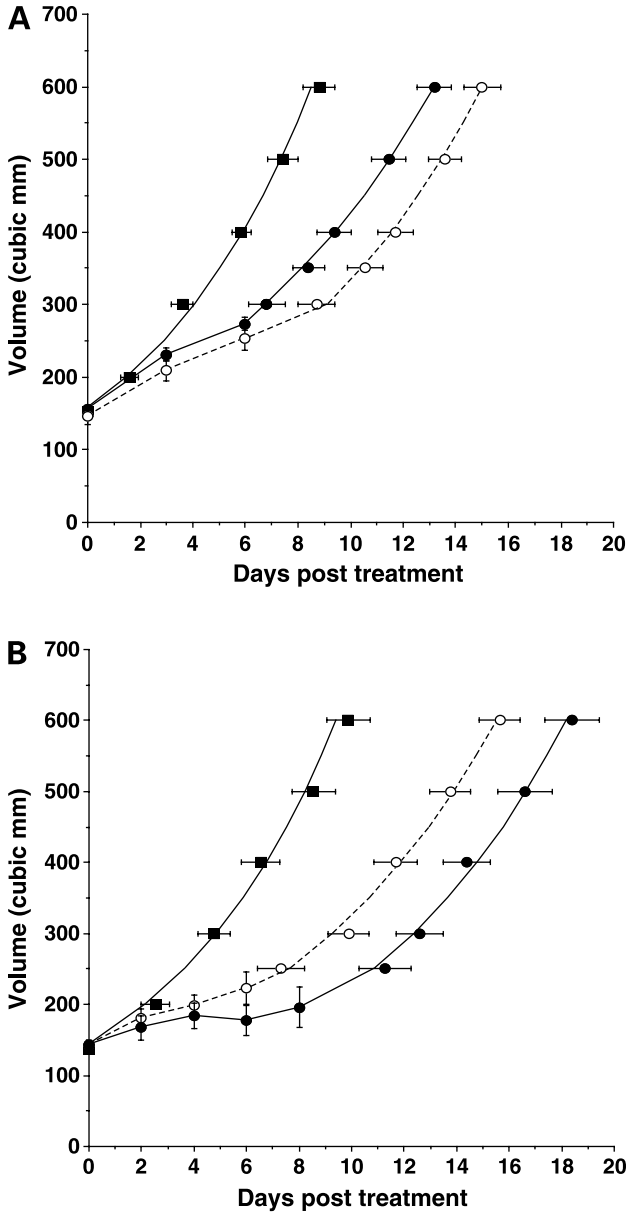

Figure 1. The growth of control and drug-treated tumors in the absence of or following glucose administration. **A**, chlorambucil. **B**, doxorubicin. ■, control tumors; ●, growth following drug treatment alone; ○ (*dashed line*), growth of tumors receiving drugs at 45 min following i.v. glucose. Confidence intervals are SEs.

sets of data were solved for the time required for the tumors to triple their initial volumes from the time of treatment. The results are shown in Table 2. Control tumors tripled their initial volumes of ~150 mm^3 in 6.8 days and doxorubicin-treated tumors required 12.4 days. Administration of glucose before doxorubicin resulted in a modest reduction in the time required for a 3-fold increase in tumor volume from 12.4 to 10.9 days. The same glucose-induced pH gradient alteration increased the time of chlorambucil-treated tumors to triple their initial volume

from 12.9 to 15.4 days. For both doxorubicin and chlorambucil, the 0.2 pH gradient change resulted in opposite and significant changes in the time for tumors to triple their volume ($P < 0.05$).

Discussion

Theory predicts that for weak electrolytes that are membrane permeable in their nonionized state and impermeable in their ionized state, their intracellular to extracellular distribution is governed by pK_a of the electrolyte and the pH gradient across the membrane barrier. For weak acids, the intracellular-extracellular distribution (C_i/C_e) is computed as follows:

$$C_i/C_e = (1 + 10^{pH_i - pK_a})/(1 + 10^{pH_e - pK_a}) \quad (A)$$

where pH_i and pH_e are the intracellular and extracellular pH values, respectively. For a weak base, the expression is similar (7):

$$C_i/C_e = (1 + 10^{pK_a - pH_i})/(1 + 10^{pK_a - pH_e}) \quad (B)$$

Both predicted drug concentration ratios (i.e., C_i/C_e for chlorambucil and C_i/C_e for doxorubicin) are independent of the extracellular drug concentrations (C_e). Similarly, the chlorambucil/doxorubicin intracellular concentration ratios before glucose $[(C_i/C_e)_{chlorambucil}]/[(C_i/C_e)_{doxorubicin}]$, as well as after glucose, are independent of C_e. This pertains regardless of whether glucose increases or decreases drug availability. Substitution of extracellular pH and intracellular pH values from Table 1, and the drug pK_a values into Eqs. A and B, predicts that the glucose-mediated 0.2 pH gradient increase will increase the ratio of intracellular chlorambucil to doxorubicin by a factor of 2.3. As seen in Table 2, glucose increased the ratio of chlorambucil-to-doxorubicin–induced tumor growth delay by a factor of 2.1 from 5.63:5.54 days to 8.49:4.07 days. The measured 2.1-fold change in the ratio is in close agreement with the predicted 2.3-fold change in the ratio. It is to be noted that the above analysis is based on the assumption that a linear increase in intracellular drug concentration results in an exponential decrease in the tumor cell surviving fraction, and that surviving tumor cells grow exponentially as shown in Fig. 1A and B. A linear-log relationship between cell drug concentration and cell surviving fraction assessed by colony formation assays has previously been shown by ourselves and others for doxorubicin and chlorambucil over a substantial drug concentration range (13, 14, 22, 23, 26).

Table 2. Effect of drug treatment on tumor growth

	Days to 3 × initial volume	Tumor growth delay
Doxorubicin		
Control	6.83 ± 0.38* (n = 6)†	—
Doxorubicin alone	12.37 ± 0.48‡ (n = 7)	5.54 ± 0.61
Glucose + doxorubicin	10.90 ± 0.40* (n = 6)	4.07 ± 0.55
Chlorambucil		
Control	6.90 ± 0.36 (n = 8)	—
Chlorambucil alone	12.53 ± 0.79* (n = 8)	5.63 ± 0.87
Glucose + chlorambucil	15.39 ± 0.92* (n = 8)	8.49 ± 0.99

*SE.
†Number of tumor-bearing animals.
‡$P < 0.05$ for drug alone versus glucose + drug.

The results obtained in the present study are qualitatively and quantitatively similar to our previous *in vitro* studies (13, 14). In these *in vitro* studies, chlorambucil and doxorubicin cytotoxicity and intracellular doxorubicin accumulation were evaluated in cells that were cultured under normal physiologic pH conditions (pH 7.4) as well as in the same cells cultured at low extracellular pH (pH 6.8; i.e., pH condition that mimics the tumor microenvironment). An increase in the cell pH gradient by 0.2 pH unit results in an ~2-fold change in the chlorambucil-to-doxorubicin cytotoxicity ratio *in vitro* and *in vivo*. Additionally, the measured changes in the *in vitro* cytotoxicity ratios are consistent with the *in vitro* measured change in intracellular accumulation of doxorubicin (14) and chlorambucil (6) for a 0.2 pH unit change.

In related studies, Raghunand et al. (15, 17) evaluated the effect of pH on the uptake, cytotoxicity, and tumor growth delay induced by the weak bases doxorubicin and mitoxantrone *in vitro* and *in vivo*. Modulation of the pH gradient by extracellular alkalinization enhanced the *in vitro* cellular drug uptake and cytotoxicity as predicted by theory (15). Systemic alkalinization of tumor-bearing mice increased the efficacy of both bases, although the pH-mediated change in gross tumor doxorubicin concentration was not statistically significant (17). In studies by Kozin et al. (18), chlorambucil-induced growth delay was more pronounced in tumors following prior tumor irradiation, and this effect was further enhanced by the administration of glucose before chlorambucil. As both pO_2 and pH, and likely glucose, decrease with increasing radial distance from microvessels (27–29), these results suggest that the influence of the pH gradient and glucose-induced gradient modulation is more pronounced in hypoxic tumor regions distal from functioning vasculature. The present and other studies in rodent, canine, and humans show the utility of i.v. glucose for altering tumor pH (30–33), and various methods for enhancing glucose-mediated pH changes have been investigated (32, 34). Elevating blood glucose levels result in little or no change in the intracellular pH of normal tissue and tumor tissue, but selectively decreases the extracellular pH of tumor tissue (35). Finally, it is to be noted that the naturally occurring extracellular acidity of tumors may be directly exploitable independent of the pH gradient difference between tumor and normal tissue (36–38). For example, extracellular tumor acidity has been shown to significantly influence the stability and efficacy of drugs, such as camptothecin and analogues (36).

To summarize, a modest increase in the intracellular-extracellular pH gradient increases tumor growth delay induced by the weak acid chlorambucil and decreases growth delay induced by the weak base doxorubicin. These results indicate that the pH gradient difference between tumor and normal tissue may be targeted for the treatment of cancer by the development of weak acid chemotherapeutics with appropriate pK_a values.

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