Neoplasia • Vol. 3, No. 3, 2001, pp. 227–235 227
www.nature.com/neo

Acute Metabolic Alkalosis Enhances Response of C3H Mouse Mammary Tumors to the Weak Base Mitoxantrone

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Abstract

Uptake of weak acid and weak base chemotherapeutic drugs by tumors is greatly influenced by the tumor extracellular/interstitial pH (pH_e), the intracellular pH (pH_i) maintained by the tumor cells, and by the ionization properties of the drug itself. The acid-outside plasmalemmal pH gradient in tumors acts to exclude weak base drugs like the anthracyclines, anthraquinones, and vinca alkaloids from the cells, leading to a substantial degree of "physiological drug resistance" in tumors. We have induced acute metabolic alkalosis in C3H tumor-bearing C3H/hen mice, by gavage and by intraperitoneal (i.p.) administration of $NaHCO_3$. ^{31}P magnetic resonance spectroscopic measurements of 3-aminopropylphosphonate show increases of up to 0.6 pH units in tumor pH_e, and 0.2 to 0.3 pH units in hind leg tissue pH_e, within 2 hours of i.p. administration of $NaHCO_3$. Theoretical calculations of mitoxantrone uptake into tumor and normal (hind leg) tissue at the measured pH_e and pH_i values indicate that a gain in therapeutic index of up to 3.3-fold is possible with $NaHCO_3$ pretreatment. Treatment of C3H tumor-bearing mice with 12 mg/kg mitoxantrone resulted in a tumor growth delay of 9 days, whereas combined $NaHCO_3$–mitoxantrone therapy resulted in an enhancement of the TGD to 16 days. *Neoplasia* (2001) **3,** 227–235.

Keywords: tumor pH, drug resistance, sodium bicarbonate, weak base, weak acid.

Introduction

The extracellular pH (pH_e) and intracellular pH (pH_i) of tumors influence the effectiveness of chemotherapy by influencing drug uptake kinetics and the ionic equilibrium of weak acid and weak base drug molecules. Weak base drug molecules like mitoxantrone will tend to be retained in the more acidic compartments within a tumor, whereas weak acid species like chlorambucil will tend to concentrate in the more alkaline compartments [1,2]. Electrode measurements in humans and animals reveal tumor extracellular/interstitial pH to be generally lower than the pH of normal tissue [3], but pooled measurements of pH of normal and tumor tissue made by various investigators show some overlap [4]. In a recent review, Gerweck [4] has argued that this apparent overlap results from differences in technical variables associated with electrode measurements of tissue pH by different investigators, as well as physiological and metabolic differences between patients at the time of examination, and has resulted in an underexploitation of the pH difference between normal and tumor tissue for therapeutic purposes. Noninvasive ^{31}P magnetic resonance spectroscopy (MRS) allows the simultaneous investigation of tissue pH_i from the chemical shift of inorganic phosphate (P_i) [5] and pH_e from the chemical shift of an extracellular pH probe such as 3-aminopropylphosphonate (3-APP) [6]. MRS measurements of tumor pH_i and pH_e in various human tumor xenografts in mice indicate that tumor pH_i is similar to or more alkaline than in normal tissue, whereas tumor pH_e is more acidic than in normal tissues [7,8], leading to an acid-outside plasmalemmal pH gradient in tumors.

We have recently demonstrated that this acid-outside plasmalemmal pH gradient can be abolished, even reversed, in experimental tumor xenografts by chronic *ad libitum* administration of 200 mM sodium bicarbonate to the host mice [9]. Further, we have demonstrated that the antitumor activity of doxorubicin in an SCID mouse model of MCF-7 human breast carcinoma is enhanced by $NaHCO_3$-induced tumor alkalinization [9]. Chronic administration of $NaHCO_3$ to mice is undesirable due to potential hypernatremia and other metabolic disorders, and is also not translatable to humans. We have investigated the feasibility of inducing acute alkalinization of tumors in a C3H mouse model by i.p. and gavage administration of $NaHCO_3$ to the host animals. We have also measured tumor and normal tissue pH_e and pH_i in mice after oral administration of NH_4Cl to the host animals to induce acidosis. The influence of acute alkalinization of tumors on tumor response to a weak base drug, mitoxantrone, has been investigated. Mitoxantrone was chosen over doxorubicin because of its greater octanol–water partition coefficient, and because of its dual positive ionization status

Abbreviations: 3-APP, 3-aminopropylphosphonate; MRS, magnetic resonance spectroscopy; NTP, nucleoside triphosphates; PCr, phosphocreatine; pH_e, extracellular/interstitial pH; pH_i, intracellular pH; P_i, inorganic phosphate; TGD, tumor growth delay

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Received 6 February 2001; Accepted 2 March 2001.

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at physiological pH compared to the singly charged doxorubicin.

Materials and Methods

Cells and Animals Used

C3H murine mammary carcinoma tumor fragments were implanted in the mammary fat pads of 5- to 6-week-old female C3H/Hen mice by means of a 12G trocar. Tumor volumes were calculated from orthogonal measurements of external dimensions as $(\text{width})^2 \times (\text{length})/2$. MRS was performed on the mice once the tumors had grown to $> 300\ mm^3$.

Gavage Administration of $NaHCO_3/NH_4Cl$

To induce acute metabolic acidosis or alkalosis by the oral route, mice were administered either 0.7 ml 1 M NH_4Cl or 0.7 ml 1 M $NaHCO_3$, respectively, by gavage. Anesthetization immediately following NH_4Cl gavage resulted in the deaths of all three mice tested. For this reason, for MRS measurements of mice that had been administered either NH_4Cl or $NaHCO_3$ by gavage, mice were anesthetized 2 hours following gavage. Control and $NaHCO_3$-treated mice were anesthetized using a mix of Ketamine, Xylazine and Acepromazine (72/6/6 mg/kg), whereas mice that had been administered NH_4Cl by gavage were anesthetized with 75% of this dose, due to reduced tolerance of these mice to one or more of the components even 2 hours after the gavage. NH_4Cl- and $NaHCO_3$-treated mice were denied access to normal *ad libitum* water for 2 hours following gavage, at which point they were anesthetized and prepared for ^{31}P MRS of either tumor or hind leg tissue. pH measurements were also made on untreated control mice for comparison. Despite the reduced dose of anesthetic, the duration of anesthesia and recovery time were greater in NH_4Cl-treated mice than in control mice that had been given the full anesthetic dose. Recovery from anesthesia was also somewhat prolonged in $NaHCO_3$-treated mice compared with control mice.

Intraperitoneal Administration of $NaHCO_3$

For experiments involving i.p. administration of $NaHCO_3$, mice were anesthetized as described earlier and administered 3-APP (i.p., 0.4 ml×0.24 M, pH 7.4 at room temperature). A 3/4-in., 24G catheter (Elf Sanofi Inc., Overland Park, KS), connected through a three-way valve to two 60-cm-long, 1.58-mm-ID polyethylene tubes (Becton Dickinson, Parsippany, NJ), was then inserted into the i.p. cavity of the anesthetized mouse. This set-up permitted delivery of $NaHCO_3$ (0.7 ml×1 M) at the appropriate time, or delivery of a "booster" dose of 3-APP (0.2 ml×0.24 M, if required) without moving the animal out of the magnet. The combined dead volume of the i.p. catheter and three-way valve was 0.2 ml, meaning that delivery of a booster dose of 3-APP resulted in the delivery of an additional 0.2 ml of $NaHCO_3$ as well. The mouse was immobilized on a home-built MRS solenoid coil and centered in the magnet. $NaHCO_3$ was tolerated by anesthetized and alert C3H mice up to the maximum tested dose of 1 ml×1 M. It was found that i.p. $NaHCO_3$ enhanced the duration of the anesthesia and also increased the recovery time of C3H mice. Intraperitoneal administration of NH_4Cl was not tolerated by anesthetized mice, although alert mice tolerated up to 1 ml×1 M NH_4Cl without any fatalities ($n=4$).

Localized In Vivo ^{31}P MR Spectroscopy

All *in vivo* measurements were performed at 4.7 T on a Bruker Biospec spectrometer/imager equipped with a 14 G/cm self-shielded gradient insert, using home-built solenoid coils of suitable diameter. Image-guided volume-selective ^{31}P MR spectra of tumors or hind leg tissue in anesthetized mice were acquired using the PRESS sequence [10]. pH_e and pH_i were measured from the chemical shifts of exogenous 3-APP and endogenous P_i, respectively, as described earlier [8]. For spectroscopy of tumors, 0.4 ml of 0.24 M 3-APP was administered i.p. to anesthetized mice just before their immobilization on the MRS coil. This procedure did not result in sufficient loading of hind leg (mostly muscle) tissue with 3-APP. Hence, for spectroscopy of hind legs, 0.6 ml of 0.24 M 3-APP was administered (i.p.) to alert mice 2 hours before anesthetization. Immediately following anesthetization, a further 0.4 ml of 3-APP was injected (i.p.) and the mouse prepared for ^{31}P MRS as before. This procedure resulted in usably high 3-APP signals in the ^{31}P MR spectra of 16 of 19 mice tested.

Chemotherapy

Two groups of 6-week-old female C3H/Hen mice ($n=4$, each) bearing C3H tumors in the mammary fat pad (approximately 250 mm^3) were used. One group was administered a single dose of 0.7 ml×1 M $NaHCO_3$ (i.p.) and denied access to drinking water for the next 4 hours, whereas the other group did not receive any treatment and had uninterrupted access to *ad libitum* water. The two groups were compared for changes in tumor growth rates to assess the influence of $NaHCO_3$ on tumor growth. In a separate experiment, four groups of C3H/Hen mice ($n=4$, each) bearing C3H tumors in the mammary fat pad were used. Groups A and B were administered two doses of mitoxantrone (i.v., 6 mg/kg) given 7 days apart, whereas groups C and D were administered a single dose of mitoxantrone (i.v., 12 mg/kg). Groups B and D were also administered 0.7 ml×1 M $NaHCO_3$ (i.p.) 2 hours before each mitoxantrone injection. $NaHCO_3$-treated mice were denied access to *ad libitum* water for 4 hours starting with administration of the $NaHCO_3$, but were allowed continuous access to solid food. Access to drinking water was restored 2 hours after drug administration. The toxicity of mitoxantrone to mice has been reported to have a marked circadian dependence, with the lowest toxicity being observed at 11 to 15 hours after light onset [11]. For this reason, mitoxantrone was administered 12 hours after light onset in all experiments.

Results

^{31}P MR spectra of tumor and (normal) hind leg tissue were acquired before and immediately following i.p. administration of $NaHCO_3$ to anesthetized mice placed in the magnet. Chemical shifts were calibrated against the α-NTP resonance, which was set to -10.05 ppm in all spectra. Tissue pH_e and pH_i were calculated from the chemical shifts of 3-APP and P_i, respectively, as explained elsewhere [8]. The 3-APP resonance shifts upfield (to lower parts per million (ppm) values) on the ^{31}P MR spectrum with increasing pH, whereas the opposite is true of the P_i resonance. Figure 1 shows a series of ^{31}P MR spectra obtained from a C3H tumor before and following $NaHCO_3$ administration. A slow shift over 2 hours of the P_i resonance to higher chemical shifts is visible, indicating an alkalinization of tumor pH_i. It can also be seen that the 3-APP resonance shifts to lower chemical shifts, indicating an alkalinization of tumor pH_e. The 3-APP resonance tended to broaden immediately following $NaHCO_3$ administration. This indicates a greater heterogeneity of tumor pH_e induced by $NaHCO_3$ administration, and suggests the presence of compartments within the tumor exhibiting differing kinetics of alkalinization in response to the $NaHCO_3$. The intensities of the NTP resonances, an indicator of the energetic status of the tumors, were not significantly altered by the $NaHCO_3$ administration. The significant increase in the phosphocreatine resonance seen in Figure 1 was not consistently observed in all tumors. The pH_i and pH_e corresponding to these spectra are plotted in Figure 3*B*.

Figure 2 shows a series of ^{31}P MR spectra acquired from the normal hind leg tissue of a tumor-bearing C3H mouse. A shift of the 3-APP peak to lower ppm values is visible 45 minutes after i.p. delivery of $NaHCO_3$ to the mouse, indicating an increase in tissue pH_e that persists through 2 hours postinjection. A small shift of the P_i peak to higher ppm values is also visible by 45 minutes postinjection, indicating an increase in tissue pH_i. Significant changes in the NTP peaks or in the large phosphocreatine peak (≈ -2.5 ppm) were not observed subsequent to the $NaHCO_3$ injection. The pH_e and pH_i values corresponding to this experiment are plotted in the inset in Figure 2. It can be seen that $NaHCO_3$ administration produces a small but persistent alkalinization of both pH_e and pH_i in hind leg tissue through at least 2 hours postinjection.

Panels B to D of Figure 3 depict pH_e and pH_i changes in response to i.p. administration of $NaHCO_3$ in large C3H tumors, whereas panel A depicts the pH response of a smaller tumor. It can be seen from Figure 3*A* that both pH_e and pH_i in this smaller C3H tumor are neutral to alkaline, and do not increase further upon i.p. administration of $NaHCO_3$. As seen in Figure 3$B-D$, resting pH_e in large C3H tumors ranges from 6.1 to 6.7, and inversely corresponds to tumor size. It can also be seen that pH_e in these acidic tumors increases in response to i.p. administration of $NaHCO_3$. Resting pH_i in the tumors shown in Figure 3$B-D$, was neutral to acidic (6.75 to 7.05), and increased in response to $NaHCO_3$ administration. Unlike the 3-APP resonance, which is purely extracellular in origin, the P_i resonance includes the contributions of both extracellular and intracellular P_i. As

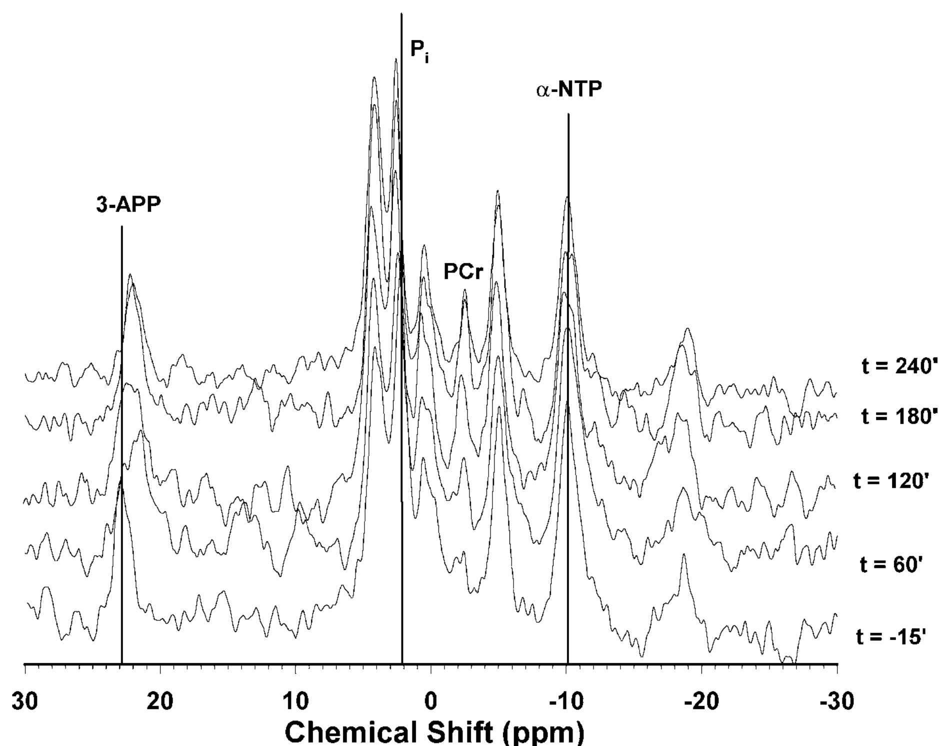


Figure 1. *A series of ^{31}P MR spectra were obtained from a 975-mm^3 C3H tumor before and after i.p. administration of 0.7 ml$\times$1 M $NaHCO_3$ to the mouse at t=0 minutes. Chemical shifts are calibrated against the α-NTP resonance, which is set to -10.05 ppm. By 1 hour postinjection, the pH_e of the tumor is substantially raised, as indicated by the shift of the 3-APP resonance upfield to lower ppm values, and this increase persists through 4 hours postinjection. Tumor pH_i is increased to a small extent by 4 hours postinjection as well, as indicated by the shift to higher ppm values of the P_i resonance.*

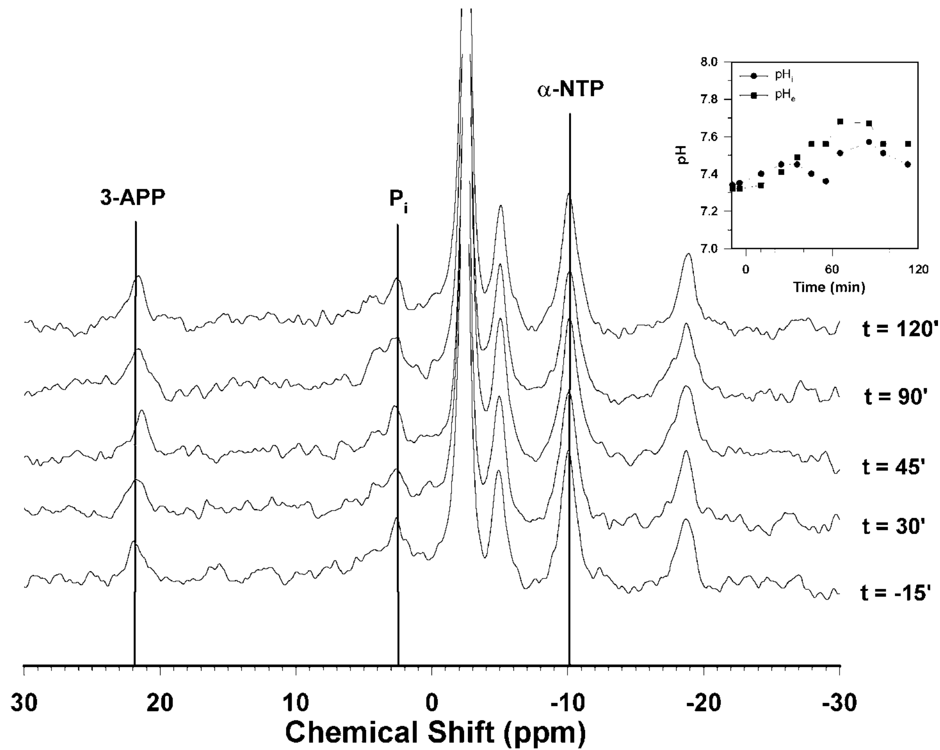


Figure 2. *A series of ^{31}P MR spectra were obtained from the hind leg of a C3H/Hen mouse before and after i.p. administration of 0.7 ml×1 M $NaHCO_3$ to the mouse at t=0 minutes. Signal intensities of both the 3-APP and P_i peaks were low compared with spectra obtained from tumors. A small shift of the 3-APP peak to lower ppm values is visible by 45 minutes postinjection, indicating alkalinization of tissue pH_e. A small shift of the P_i resonance to higher ppm values is also visible, indicating alkalinization of pH_i. The intensities of the phosphocreatine peak (≈ -2.5 ppm) and the NTP peaks ≈ -5, -10.05, and -18.5 ppm) did not change significantly during the course of MRS experiment. The inset shows the calculated pH_e and pH_i versus time in hind leg tissue from these spectra. Both pH_e and pH_i are seen to increase slightly following i.p. administration of 0.7 ml×1 M $NaHCO_3$.*

such, the increase in pH reported by the P_i resonance is partly due to the extracellular alkalinization reported independently by the 3-APP resonance. The increases in pH indicated by the P_i resonance in these tumors following $NaHCO_3$ administration is thus an overestimate of any increases in tumor pH_i.

pH_e and pH_i of C3H tumors were also measured in mice that had been administered either $NaHCO_3$ or NH_4Cl by gavage, as well as in control mice, as explained earlier. To further investigate a possible relationship between tumor size and tumor pH_e (Figure 3), tumors from mice in the control group are presented in three size categories in Figure 4. pH values for tumors in all groups of mice were analyzed by the Student's *t* test or by ANOVA. Although there is a downward trend to the mean, neither pH_e nor pH_i was statistically significantly affected by tumor size for tumors in the control group, for the tumor size ranges shown in Figure 4 ($P > .2$). Three hours after $NaHCO_3$ administration by

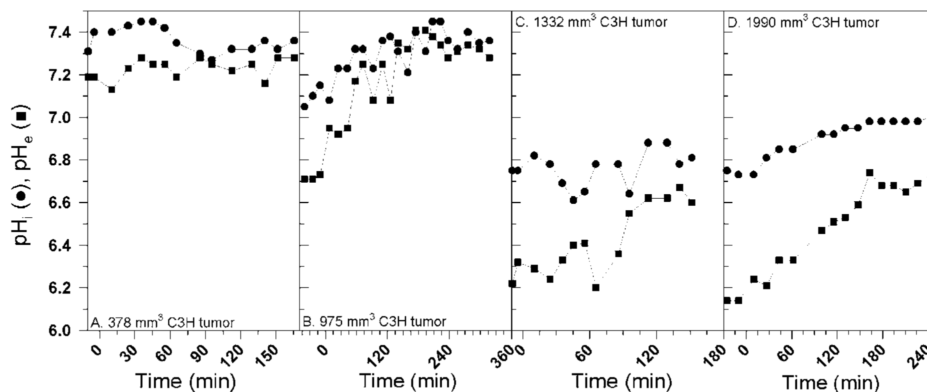


Figure 3. *pH_e and pH_i versus time in C3H tumors from four different animals that were administered 0.7 ml×1 M $NaHCO_3$ (i.p.) at t=0 minutes. The larger tumors in panels B to D can be seen to have more acidic pH_e and pH_i before $NaHCO_3$ administration than the smaller tumor shown in panel A. The more acidic tumors in B to D show increases of both pH_e and pH_i by 2 hours postinjection of the $NaHCO_3$, whereas the neutral tumor in A did not alkalinize in response to the $NaHCO_3$ bolus.*

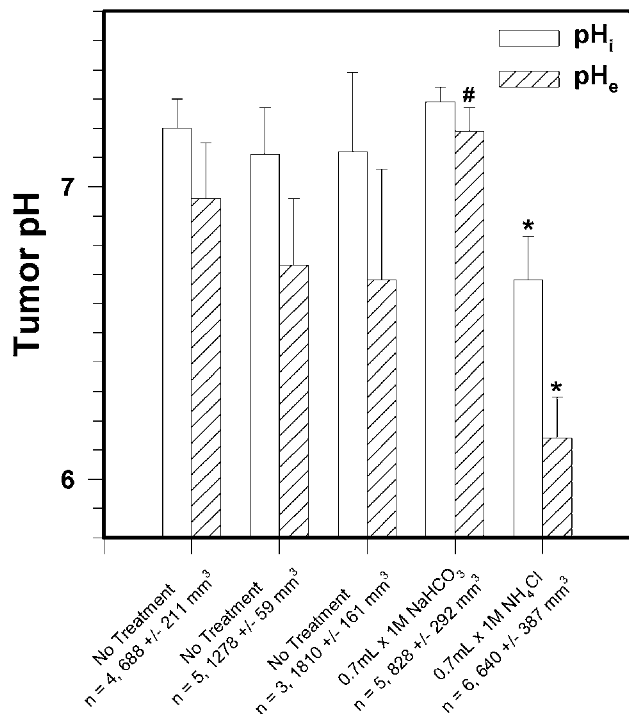
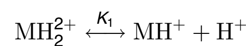


Figure 4. *Average pH$_e$ and pH$_i$ values (±SD) from C3H tumors in control mice, and in mice that were administered either NaHCO$_3$ or NH$_4$Cl (0.7 ml×1 M) by gavage. Tumors from control mice were separated into three categories based on size. Neither pH$_e$ nor pH$_i$ was significantly affected by tumor size for tumors in the control group (P > .2). Tumor pH$_i$ in NaHCO$_3$-treated mice was not significantly different from tumor pH$_i$ in control animals (P > .05 against tumors in all three control groups). #Tumor pH$_e$ was significantly higher in NaHCO$_3$-treated mice compared to tumors in untreated control mice in the two larger size categories (P < .05). Tumor pH$_e$ in NaHCO$_3$-treated mice was marginally higher than tumor pH$_e$ in the smallest group of tumors (688±211 mm^3) in control mice (P = .06). *Both tumor pH$_i$ and tumor pH$_e$ were significantly lower in NH$_4$Cl-treated mice than in control mice regardless of tumor size (P < .03 in all cases).*

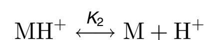
gavage, tumor pH$_i$ in NaHCO$_3$-treated mice was not significantly different from tumor pH$_i$ in control animals ($P>.05$ against tumors in all three control groups). However, tumor pH$_e$ was significantly higher in NaHCO$_3$-treated mice compared to tumors in untreated control mice in the two larger size categories ($P<.05$). Tumor pH$_e$ in NaHCO$_3$-treated mice was marginally higher than tumor pH$_e$ in the smallest group of tumors (688±211 mm^3) in control mice ($P=.06$). Both tumor pH$_i$ and tumor pH$_e$ were significantly lower in NH$_4$Cl-treated mice than in control mice regardless of tumor size ($P<.03$ in all cases).

pH$_e$ and pH$_i$ of normal hind leg tissue were measured in mice 3 hours after they were treated by gavage administration of either NaHCO$_3$ or NH$_4$Cl, and in control mice (Figure 5). pH values for the three groups were analyzed by the Student's *t* test. Gavage administration of NaHCO$_3$ to mice did not significantly affect pH$_i$ ($P=.17$) or pH$_e$ ($P=.5$) of hind leg tissue compared to control animals. However, gavage administration of NH$_4$Cl to mice did significantly lower both pH$_e$ ($P=.003$) as well as pH$_i$ ($P=.04$) compared with control animals.

We have previously mathematically modeled the partitioning of singly charged weak base and weak acid drugs into cells at various pH$_i$ and pH$_e$ [12]. For a doubly charged weak base drug molecule M, the following equilibria are expected for the various charged species:



$$MH_2^{2+} \overset{K_1}{\longleftrightarrow} MH^+ + H^+$$



$$MH^+ \overset{K_2}{\longleftrightarrow} M + H^+$$

Actually, there will be two distinct singly charged intermediates MH$^+$ in the above equilibrium, depending on which proton on MH$_2$$^{2+}$ comes off first. But the relevant protons are symmetrically located on mitoxantrone, and we have therefore shown both singly charged species as being identical in the above chemical equations. This simplification is justifiable in our situation because, at physiological pH, a drug with pK_{a1} of 8.25 (such as mitoxantrone) exists mostly in the charged forms, and the expression for intracellular-to-extracellular drug ratio (Equation 1, below) is insensitive to the value of the acid dissociation constants for the two singly charged species. Making the assumption that the cell membrane is essentially impermeable to the charged species MH$^+$ and MH$_2$$^{2+}$, and that there is a fast equilibration of the uncharged species across the cell membrane [12], we can compute the following relationship for the intracellular-to-extracellular ratio of total drug:

$$\frac{[M]_i+[MH^+]_i+[MH_2^{2+}]_i}{[M]_e+[MH^+]_e+[MH_2^{2+}]_e} = \frac{1+10^{(pK_2-pH_i)}+10^{(pK_1+pK_2-2pH_i)}}{1+10^{(pK_2-pH_e)}+10^{(pK_1+pK_2-2pH_e)}} \quad (1)$$

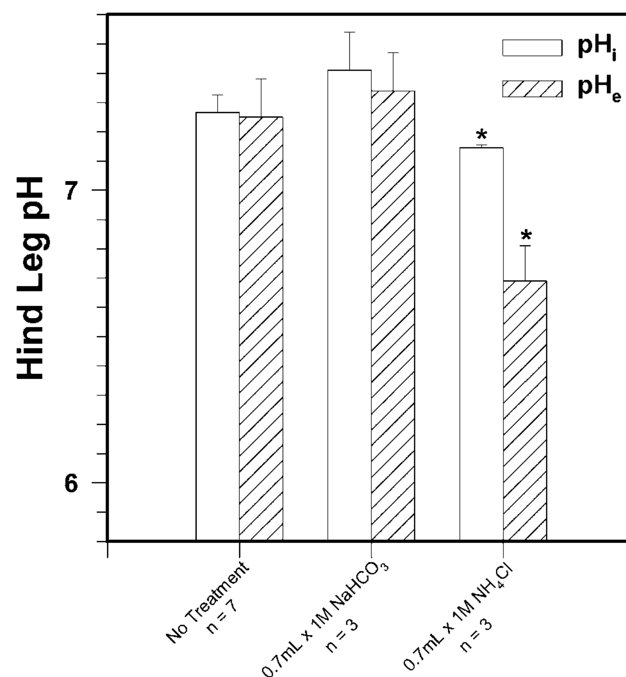
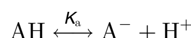


Figure 5. *Average pH$_e$ and pH$_i$ values (±SD) from normal hind leg tissue in control mice, and in mice that were administered either NaHCO$_3$ or NH$_4$Cl (0.7 ml×1 M) by gavage. Gavage administration of NaHCO$_3$ did not result in significant changes in either pH$_e$ (P=.5) or pH$_i$ (P=.17). *Gavage administration of NH$_4$Cl resulted in a considerable drop in pH$_e$ (P=.003) as well as pH$_i$ (P=.04) of hind leg tissue in treated mice compared with control mice.*

Analogous calculations can be performed for a singly charged weak acid drug AH, for which the following equilibrium can be expected:



$$AH \overset{K_a}{\longleftrightarrow} A^- + H^+$$

and the following relationship can be derived for the intracellular-to-extracellular drug ratio:

$$\frac{[A^-]_i+[AH]_i}{[A^-]_e+[AH]_e} = \frac{1+10^{(pH_i-pK_a)}}{1+10^{(pH_e-pK_a)}} \quad (2)$$

Equation 1 predicts that alkalinization of tumor pH_e will increase weak base uptake into tumor cells, whereas Equation 2 predicts that acidification of tumor pH_e will increase weak acid uptake into tumor cells. However, concomitant alkalinization (or acidification) of normal tissue pH_e in the host animal would drive more drug into those tissues as well. We have made pH measurements in both tumor and hind leg to calculate the relative change in drug uptake into tumors. For the pH_e and pH_i values observed in tumor and normal (hind leg) tissue before and 2 hours after alkalinization by i.p. administration of $NaHCO_3$ (Figure 3*B* and inset in Figure 2), we predict from Equation 1 that the partitioning of mitoxantrone will increase by 1.5-fold in normal tissue and five-fold in tumor tissue, and this is depicted in Figure 6. Thus, a net therapeutic gain of 3.3-fold is predicted when chemotherapy with mitoxantrone is preceded by $NaHCO_3$-induced alkalinization of the host animal. Calculations for a hypothetical weak acid with pK_a 6.0 predict that the acidosis induced by gavage administration of NH_4Cl (Figures 4 and 5) will drive two-fold more drug into cells of hind leg tissue, but only 1.2-fold more drug into cells in the tumor (Figure 6). Thus, acidosis induced in the host animal by NH_4Cl gavage may actually reduce the effectiveness of a weak acid drug. Of course, drug dosage is usually limited by cardiac and bone-marrow toxicities and not toxicity to muscle, but reliable MRS measurements of pH_e in these organs could not be obtained. Tumor acidification by gavage administration of NH_4Cl appears to be not tumor-selective and will not result in any gain in therapeutic index for treatment with weak acid drugs. Kuin et al. [13] report that induction of moderate degrees of hyperglycemia combined with i.p. administration of *m*-iodobenzylguanidine (MIBG), an inhibitor of mitochondrial respiration, lowers tumor pH_e but not pH_i in RIF-1 tumors in C3H mice. Their protocol seeks to enhance the degree of aerobic glycolysis exhibited by tumor cells and may offer greater potential to selectively acidify tumors.

The influence of $NaHCO_3$-induced acute metabolic alkalosis itself on tumor growth, as well as on tumor response to mitoxantrone therapy, are shown in Figure 7*A* – *C*. In an initial experiment, a group of four mice bearing C3H tumors that were 200 to 300 mm^3 in volume was administered a single i.p. injection of 0.7 ml×1 M $NaHCO_3$ and denied access to *ad libitum* water for the following 4 hours. The mean tumor volumes of the $NaHCO_3$-treated group and an untreated control group are compared in Figure 7*A*. No significant change in tumor growth rates were observed as a result of the $NaHCO_3$ treatment. To test the influence of $NaHCO_3$-induced alkalosis on tumor response to mitoxantrone therapy, four groups of four C3H tumor-bearing mice each were treated with either two doses of 6 mg/kg mitoxantrone (groups A and B) or a single dose of 12 mg/kg mitoxantrone (groups C and D). Groups B and D were also administered 0.7 ml×1 M $NaHCO_3$ (i.p.) 2 hours before each mitoxantrone injection. The kinetics of tumor alkalinization observed in Figure 3*B* – *D* indicate that significant tumor alkalinization occurs a minimum of 2 hours following $NaHCO_3$ administration, and this was the reason for the delay of 2 hours between $NaHCO_3$ and mitoxantrone injections administered to groups B and D. When the two doses of 6 mg/kg were administered 4 days apart to $NaHCO_3$-treated mice, a significant enhancement of tumor response to mitoxantrone was observed, but this was accompanied by substantial loss of body weight in the mice and increased rates of mortality compared to mice treated with mitoxantrone alone (data not shown). Intraperitoneal

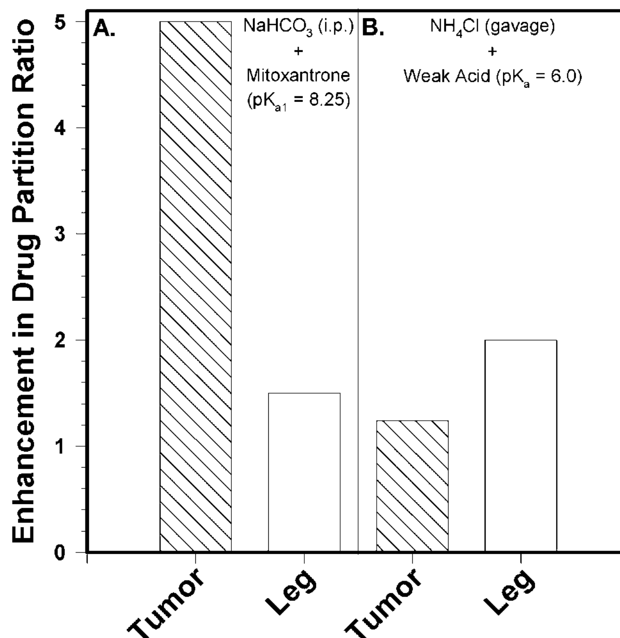


Figure 6. *Theoretically calculated tissue–blood drug partition ratios: (A) from Equation 1, for combined $NaHCO_3$ (i.p.) and mitoxantrone therapy, and (B) from Equation 2, for combined therapy using NH_4Cl (gavage) and a hypothetical singly charged weak acid drug of pK_a=6.0. For mitoxantrone a pK_{a2} of 12 was assumed, because Equation 1 is insensitive to pK_{a2} for physiological values of pH_i and pH_e. The following representative values of pH_e and pH_i in control/$NaHCO_3$-treated/NH_4Cl-treated animals, respectively, were obtained from the data reported in Figures 2–5 — tumor pH_i 7.05/7.3/6.7, tumor pH_e 6.7/7.3/6.16, hind leg pH_i 7.33/7.5/7.15, and hind leg pH_e 7.35/7.6/6.8 — and inserted into Equations 1 and 2 to obtain the drug partition ratios shown here. Pretreatment with $NaHCO_3$ (i.p.) is predicted to result in a five-fold enhancement in uptake of mitoxantrone into tumor tissue compared to tumor tissue in an untreated host, but only a 1.5-fold increase in uptake of drug into normal tissue in the $NaHCO_3$-treated animal compared with normal tissue in an untreated animal. Thus, the chemotherapeutic index of mitoxantrone can potentially be increased 3.3-fold by $NaHCO_3$ pretreatment of the host animal. However, acidosis induced by gavage administration of NH_4Cl to the host animal is not tumor specific, resulting in a two-fold increase in uptake of a weak acid drug of pK_a 6 into normal tissue and only a 1.2-fold increase in uptake into tumor tissue. Thus, the chemotherapeutic index of a weak acid drug is predicted to be reduced by gavage administration of NH_4Cl.*

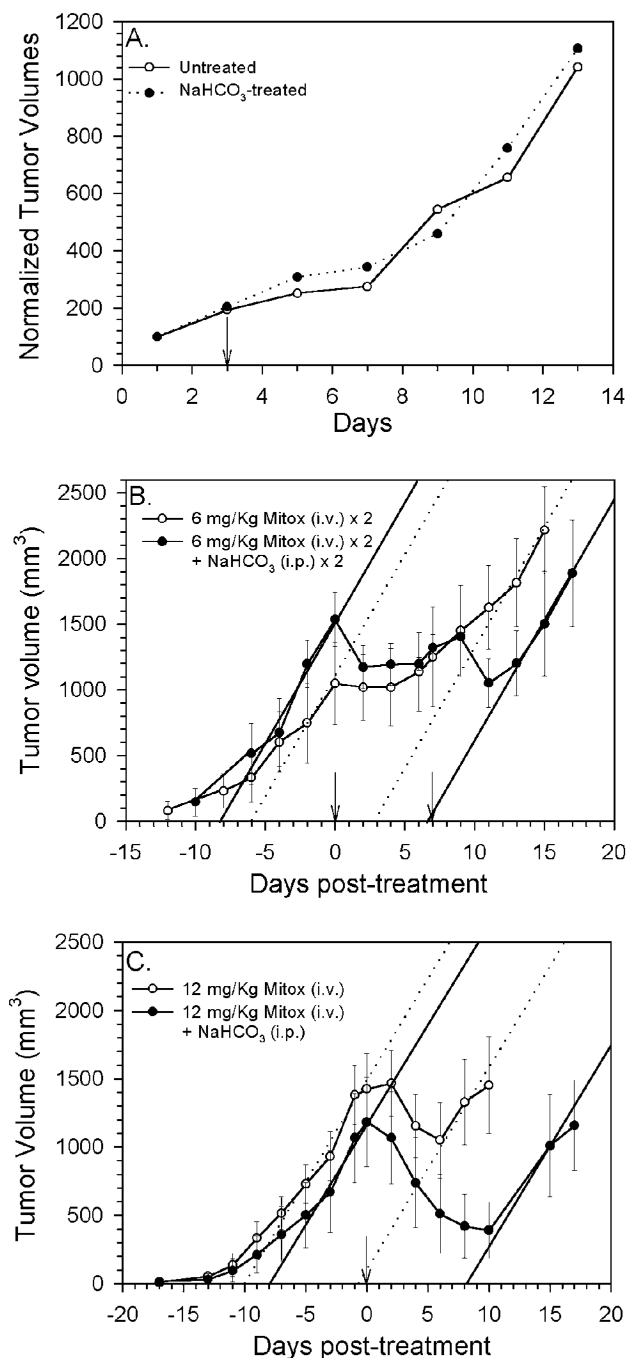


Figure 7. *Growth curves for C3H tumors in (A) untreated mice and $NaHCO_3$ (0.7 ml×1 M, i.p.)-treated mice; (B) mitoxantrone (6 mg/kg×2)-treated mice and mitoxantrone (6 mg/kg×2) plus $NaHCO_3$ (0.7 ml×1 M×2, i.p.)-treated mice; (C) mitoxantrone (12 mg/kg)-treated mice and mitoxantrone (12 mg/kg) plus $NaHCO_3$ (0.7 ml×1 M, i.p.)-treated mice. Each point in panels A to C represents the mean tumor volume from cohorts of four mice per treatment group. Error bars in panels B and C represent standard deviations. $NaHCO_3$ treatment alone was found to not affect the growth rate of C3H tumors (panel A). Treatment with mitoxantrone alone resulted in a TGD of 9 days, regardless of whether the 12 mg/kg dose was fractionated into two doses (panel B) or given as a single bolus (panel C). Combined treatment with mitoxantrone and $NaHCO_3$ resulted in a TGD of 15 to 16 days, regardless of dose fractionation. Pretreatment with $NaHCO_3$ resulted in an increase in log_{10} cell kill from 0.9 to 1.56, corresponding to a greater than 4.5-fold increase in cell kill.*

administration of $NaHCO_3$ did not, however, alter the LD_{50} of mitoxantrone when it was administered as a single dose (data not shown). A gap of 7 days between successive doses of 6 mg/kg mitoxantrone was well tolerated by $NaHCO_3$-treated mice, and this was used in subsequent experiments. Figure 7*B* shows the tumor growth curves of mice in groups A and B. The arrows in Figure 7*B* and *C* indicate the days of treatment. The error bars (standard deviation) in Figure 7*B* and *C* reflect not just the heterogeneity in response to treatment within each group, but also the pretreatment heterogeneity in tumor volumes within each treatment group. No deaths were recorded in any of the 4 groups during the course of the experiment. A tumor growth delay (TGD) of 9 days was calculated for group A, whereas a TGD of 15 days was calculated for tumors in $NaHCO_3$-treated mice from group B (Figure 7*B*). Group C mice were treated with a single 12 mg/kg dose of mitoxantrone resulting in a TGD of 9 days, whereas a TGD of 16 days was calculated for the mice in group D that were cotreated with 12 mg/kg mitoxantrone and $NaHCO_3$ (Figure 7*C*). Thus, cotreatment with $NaHCO_3$ produced a significant enhancement of TGD following mitoxantrone therapy when it was given either as a single dose or as two fractionated doses separated by 7 days. A comparison of the TGDs shown in Figure 7*B* and *C* indicates that the TGD induced by mitoxantrone therapy is not significantly altered by dose fractionation, regardless of whether the therapy also included $NaHCO_3$ treatment or not. Log_{10} cell kills [14] were calculated for groups C and D: mitoxantrone therapy in the absence of $NaHCO_3$ produced a 0.9 log_{10} cell kill, whereas combined $NaHCO_3$–mitoxantrone therapy results in a log_{10} cell kill of 1.56, indicating a greater than 4.5-fold increase in cell kill effected by treatment with $NaHCO_3$ before treatment with mitoxantrone in this tumor model.

Discussion

Previously, we have reported that chronic supplementation of *ad libitum* water provided to MCF-7 tumor-bearing SCID mice with 200 mM $NaHCO_3$ resulted in alkalinization of tumor pH_e by 0.8 pH units whereas increasing tumor pH_i by only 0.2 pH units [9]. Chronic sodium bicarbonate therapy is undesirable because it presents a substantial sodium load to the animal, and is also impractical from the standpoint of translation of this protocol to humans. Acute administration of $NaHCO_3$ by the oral and intravenous routes have been reported to cause a small but significant increase in blood pH in humans [15,16], although measurements of tissue pH in humans following $NaHCO_3$ administration are not found in the literature. In our C3H tumor model, i.p. administration of 2.3 to 2.9 g/kg $NaHCO_3$ to tumor-bearing C3H mice resulted in increases of 0.4 to 0.6 pH units in tumor pH_e and 0.1 to 0.25 pH units in tumor pH_i for periods of more than 3 to 6 hours, with only small changes in pH_e and pH_i of control normal hind leg tissue. Theoretical calculations of mitoxantrone uptake into cells at these measured pH_e and pH_i values indicate that a gain in therapeutic index of up to 3.3-fold is possible with $NaHCO_3$ pretreatment. Treatment of C3H

tumors with 12 mg/kg mitoxantrone results in a $\log_{10}$ cell kill of 0.9, whereas combined $NaHCO_3$–mitoxantrone therapy results in a $\log_{10}$ cell kill of 1.56. Kozin and Gerweck [17] have shown that acid-adapted Chinese hamster ovary (CHO) cells cultured at pH 6.8 maintain a pH_i similar to that of normal CHO cells cultured at pH 7.4. Acid-adapted CHO cells were found to be 14-fold more resistant to mitoxantrone than normal CHO cells. Theoretical calculations predict that the increased ($pH_i - pH_e$) gradient in acid-adapted CHO cells will result in a two-fold decrease in cellular mitoxantrone uptake, which is significantly less than the 14-fold reduction in mitoxantrone toxicity to these cells, suggesting that other pH-sensitive mechanisms also play a role in potentiating mitoxantrone action on these cells. Thus, in our *in vivo* experiments, although increased uptake of mitoxantrone by tumor cells in $NaHCO_3$-treated mice due to elimination of the acid-outside plasmalemmal pH gradient would appear to be the primary mechanism by which tumor response to mitoxantrone is enhanced, other explanations cannot be ruled out. In our protocol, treatment with hyperosmolal $NaHCO_3$ was accompanied by denial of *ad libitum* water to the mice for 4 hours and this could conceivably have reduced renal/biliary clearance of mitoxantrone from the mice, thereby increasing drug retention in the entire animal. This possible mechanism is currently under investigation. An alternate explanation for the pH dependencies of the toxicities of various ionic and nonionic drugs to tumor cells has been proposed by Vukovic and Tannock [18]. They have observed that a lower proportion of cells with acidic pH_i are in G_1 phase than at alkaline pH_i, leading to diminished sensitivity to the drugs mitoxantrone (a weak base) and paclitaxel (a zwitterion) in acidic cells [18]. In our experiments, $NaHCO_3$ treatment increased not only tumor pH_e, but also tumor pH_i. Tumor pH_e measurements using MRS imaging (MRSI) reveal a heterogeneity in pH_e [19] that might conceivably lead to heterogeneity in tumor pH_i as well. Thus, although the volume-averaged pH_i values in Figure 3*B* – *D* range from 6.75 to 7.05, there might exist pockets of cells in these tumors that chronically experience even lower pH_i. Cells in such pockets may be quiescent, making them resistant to antimitotic therapies, and alkalinization using $NaHCO_3$ may bring them into G_1 phase and sensitize them to drug bound to their DNA. In this context, it is relevant to note that although free mitoxantrone disappears from the blood in mice within 1 hour of intravenous administration, drug has been reported to remain bound to intracellular targets beyond 48 hours [20]. For such a cell cycle–related mechanism of drug enhancement to be active, tumor pH_e would need to remain higher than normal for several hours following $NaHCO_3$ administration. We have observed that anesthetized mice remain alkalotic for at least up to 6 hours following $NaHCO_3$ administration. The kinetics of recovery from $NaHCO_3$ loading are likely different in alert mice, but some effects of the $NaHCO_3$ are present even 24 hours later: we have observed that 24 hours following a single i.p. dose of $NaHCO_3$, mice exhibit delayed recovery from the ketamine/xylazine/acepromazine anesthetic compared with untreated control mice. We have not made pH measurements on mice 24 hours after $NaHCO_3$ administration, but if tissue alkalinization does indeed persist out to 24 h, this may be long enough to cause quiescent tumor cells to re-enter G_1 phase and become susceptible to bound drug that may have been administered 24 hours earlier. Acute treatment with $NaHCO_3$ did not, however, alter the tumor growth rate in this model.

In conclusion, oral administration of NH_4Cl resulted in a significant acidification of both pH_e and pH_i in tumors as well as in control hind leg tissue in C3H mice. Theoretical calculations of weak acid drug partitioning into tumor and normal tissue cells show that the non–tumor-specific nature of NH_4Cl-induced acidosis may actually reduce the chemotherapeutic index of a weak acid drug. However, pretreatment of tumor-bearing mice with $NaHCO_3$ results in a significant enhancement of tumor response to mitoxantrone, although the exact mechanism by which this occurs remains to be elucidated. Also requiring further investigation is the mechanism of increased toxicity of mitoxantrone to $NaHCO_3$-treated C3H mice when the drug is administered as two doses of 6 mg/kg given 4 days apart. This increased toxicity to $NaHCO_3$-treated mice, *vis-a-vis* mice treated with mitoxantrone alone, was not observed when the two doses of 6 mg/kg mitoxantrone were separated by 7 days, or when the drug was administered as a single dose of 12 mg/kg. Intraperitoneally administered $NaHCO_3$ did not alter the LD_{50} of mitoxantrone to C3H mice when it was administered as a single dose. Metabolic alkalosis can be induced in humans as well, and small increases in blood pH have been reported following oral administration of $NaHCO_3$ [16], intravenous $NaHCO_3$ [15], intravenous Carbicarb (an equimolar mix of Na_2CO_3 and $NaHCO_3$) [15], and oral administration of the loop diuretic furosemide [21]. Additionally, oral administration of $NaHCO_3$ [22] or the carbonic anhydrase inhibitor acetazolamide [23] has also been reported to achieve significant alkalinization of urine in humans, and this may be exploited for enhancement of therapies of certain bladder and renal tumors that are treated using drug cocktails containing vinca and anthracycline weak base drugs [12]. Techniques such as MRS and MRSI make it possible to measure tissue pH noninvasively, allowing for a determination of the effects of agents like $NaHCO_3$, carbicarb and furosemide on tumor pH in humans. Demonstrable tumor-selective alkalinization by one or more of these agents may permit their use in humans to enhance tumor response to weak base drugs.

Acknowledgements

This work was made possible by funding from the National Institutes of Health (CA77575) to R. J. G.

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