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Tumor acidity, ion trapping and chemotherapeutics II. pH-dependent partition coefficients predict importance of ion trapping on pharmacokinetics of weakly basic chemotherapeutic agents

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Abstract

Ion-trapping theory predicts that alkalinization of tumor extracellular pH will enhance the anti-tumor activity of weak-base chemotherapeutics. We have previously demonstrated that chronic and acute treatment of tumor-bearing mice with sodium bicarbonate results in tumor-specific alkalinization of extracellular pH. Furthermore, bicarbonate pretreatment enhances the anti-tumor activity of doxorubicin and mitoxantrone in two different mouse tumor models. Previous work has indicated subtle, yet significant differences between the pH sensitivities of the biodistribution and anti-tumor efficacies of doxorubicin and mitoxantrone *in vitro*. The present study demonstrates that systemic alkalinization selectively enhances tumor uptake of radiolabeled mitoxantrone, but not doxorubicin. Results using these two drugs are quantitatively and qualitatively very different, and can be explained on the basis of differences in the octanol–water partition coefficients of their charged forms. These results suggest that inducing metabolic alkalosis in patients would have a positive effect on response to mitoxantrone therapy. However, the therapeutic index would not increase if sodium bicarbonate also caused increased retention of mitoxantrone in susceptible normal tissues in the host. The major dose-limiting organ systems for mitoxantrone are heart, liver, bone marrow, spleen and blood cells. Bicarbonate was found to have no significant effect on the distribution of mitoxantrone to any of these tissues except for spleen. However, neither spleen weights nor lymphocyte counts were adversely affected by $NaHCO_3$ pretreatment, indicating that this co-therapy does not enhance myelosuppression due to mitoxantrone therapy. These findings suggest that metabolic alkalosis would produce a net gain in mitoxantrone therapeutic index.

Keywords: Mitoxantrone; Doxorubicin; Alkalosis; Radio-label; Partition coefficient

1. Introduction

Previous work has indicated subtle, yet significant differences between the pH sensitivities of the anti-tumor efficacies of doxorubicin and mitoxantrone *in vitro* [1]. Ion-trapping theory predicts that alkalinization of tumor extracellular pH (pHe) would enhance the anti-tumor activity of these weakly basic drugs. In agreement with this prediction, more of these drugs are taken up in cells cultured at pH 7.4 than at pH 6.6 *in vitro*. Furthermore, both of these drugs are more cytotoxic at pH 7.4 than at pH 6.6 [1]. However, in both cytotoxicity and distribution studies, mitoxantrone was consistently more pH-sensitive than doxorubicin. *In vivo*, chronic and acute treatment of tumor-bearing mice with sodium bicarbonate results in tumor-specific alkalinization of pHe, and bicarbonate pretreatment enhances the anti-tumor activity of doxorubicin and mitoxantrone in two different tumor models [2,3]. However, similar to the *in vitro* results, these studies also showed greater pH sensitivity of the anti-tumor activity of mitoxantrone than doxorubicin. The purpose of the current study is to further examine the difference in pH-dependent activities of these weakly basic chemotherapeutics, in order to better understand the contribution of ion trapping to physiological drug resistance.

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Abbreviations: AUC, area-under-the-curve; FBS, fetal bovine serum; i.p., intraperitoneal; i.v., intravenous; pHe, extracellular pH; SCID, severe combined immunodeficient.

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Both mitoxantrone and doxorubicin are weakly basic chemotherapeutics that intercalate into DNA, inhibit topoisomerase II and generate reactive oxygen species [4–7]. They are, however, chemically distinct. Doxorubicin consists of an amino sugar, daunosamine, linked *via* a glycosidic bond to the planar tricyclic, adriamycinone. Mitoxantrone also has a planar polycyclic aromatic ring structure, but lacks a sugar moiety. It has two polar side chains attached to the aromatic rings, which render the molecule water-soluble [8]. Doxorubicin has a single ionizable amine with a pK_a of approximately 8.3, and mitoxantrone has two ionizable amines with pK_a values of 8.3–8.6. Previous work has shown that the uptake and cytotoxicity of these drugs is enhanced at elevated pHe, with the effects being greater for mitoxantrone compared to doxorubicin [1]. Subtle differences were observed in the kinetics of uptake of the radiolabeled drugs. Although the uptake of both drugs were enhanced at elevated pHe, the differences were not as significant for doxorubicin as might be expected on the basis of published pK_a values. *In vivo*, we have found that tumor alkalinization significantly enhances mitoxantrone efficacy in the C3H tumor/C3H mouse model [3], and also enhances doxorubicin efficacy in the MCF-7 tumor/SCID mouse model, albeit to a lesser extent [2]. The current investigation examines the differences between mitoxantrone and doxorubicin in greater detail by determining the *in vivo* pharmacokinetic consequences of systemic metabolic alkalosis induced in the host animal by bolus or chronic administration of $NaHCO_3$. These data confirm the previous *in vitro* observations, i.e. that only modest improvements in doxorubicin efficacy can be expected with tumor alkalinization and that these effects may be independent of ion trapping. On the other hand, mitoxantrone showed a large and robust enhancement in both tumor uptake and response following systemic metabolic alkalosis, suggesting that this approach may improve response to mitoxantrone in the clinic. The differences in the behavior of these two weakly basic drugs could be predicted by differences in their pH-dependent partition coefficients, and such a model may be useful for further drug design.

Gains in therapeutic index would require that the bicarbonate pretreatment protocol not have an adverse effect on drug distribution to normal tissues. In the case of doxorubicin the dose-limiting toxicity is cardiomyopathy [9]. The dose-limiting toxicity associated with mitoxantrone is bone marrow suppression, typically observed 7–14 days after treatment [10]. In studies using radiolabeled doxorubicin, no increase in drug distribution to the heart was observed following bicarbonate pretreatment. In studies with radiolabeled mitoxantrone, there was a slight (14%) increase in the spleen drug accumulation following bicarbonate pretreatment. However, follow-up studies showed no effect of bicarbonate on the effects of mitoxantrone on spleen weights or lymphocyte counts, suggesting that this co-therapy does not introduce additional myelosuppression. Furthermore, bicarbonate pretreatment had no effect on the LD_{50} values for normal mice. These control studies indicate that alkalinization *via* bicarbonate pretreatment will effectively increase the therapeutic index for mitoxantrone.

2. Materials and methods

2.1. Cells and tumor

MCF-7 cells were cultured in RPMI-1640 or Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone). For *in vivo* culturing, a suspension of 5×10^6 MCF-7 cells in 0.05 mL of Matrigel were implanted in the mammary fat pads of 6- to 7-week-old female severe combined immunodeficient (SCID) mice. Since MCF-7 cells are estrogen-dependent, 17β-estradiol pellets (0.72 mg, 60-day release; Innovative Research of America) were subcutaneously implanted in the shoulder region of the mice by means of a 12-gauge trocar (Innovative Research) 2 days prior to tumor inoculation. For C3H tumors, 5- to 6-week-old C3H/Hen mice were obtained from Abora. All mice were housed in microisolator cages, four mice per cage, in a pathogen-free environment. The ventral surface of each mouse was depilated with Nair® 2 days prior to tumor implantation. Frozen C3H mammary carcinoma tumor fragments of approximately 100 mg were thawed immediately prior to tumor implantation. Tumor fragments were implanted with a 12-gauge stainless steel trocar (Innovative Research) within the mammary fat pad of each C3H/Hen mouse.

2.2. Measurement of partition coefficients

The partition coefficients for ^{14}C labeled doxorubicin and mitoxantrone were obtained by the addition of 100 μL of dilute ^{14}C labeled drugs into 15 mL tubes that contained 4.9 mL of PBS at various pH. Following pH measurement, 5 mL 1-octanol was added to each tube. All tubes were vortexed three times for 1 min and allowed to settle overnight. One hundred microliters of samples from both the 1-octanol and PBS layers were taken and counted using a Beckman Liquid Scintillation Counter. Because the ^{14}C label on doxorubicin was on the sugar moiety which could potentially be hydrolyzed from the adriamycinone, the partition coefficient for this drug was also measured with absorbance. Briefly, 200 μL samples from both the 1-octanol and PBS layers were taken and added to separate wells in a 96-well plate and optical density was measured at 480 nm.

2.3. Tumor growth statistics

Tumor volumes were measured with calipers and calculated as $[(\text{width})^2 \times \text{length}]/2$. Data were linearized by converting volumes (in mm^3) to their cube roots. The least squares regression lines of the cube root volumes from

day 9 onward were fit for each mouse. Three-way ANOVA were used to examine the effects of different treatments, injection (drug vs. saline) and pH (water vs. sodium bicarbonate). Tumor cell log kill was calculated from the equation $[(T - C)/3.32T_d]$, where T and C are the number of days it took for treated and control groups, respectively, to reach 1000 mm^3. T_d is the exponential doubling time of the treated groups [11]. *T/C* was calculated as the ratio of the median volumes of treated and control groups on day 20. *T/C* <44% is considered significant by the Division of Cancer Treatment (National Cancer Institute), while a *T/C* value of 10% (DN-2 level activity) is considered highly significant.

2.4. Biodistribution of radiolabeled drugs

The pH-dependent pharmacokinetics of radiolabeled mitoxantrone and doxorubicin were determined in MCF-7 tumors grown in SCID mice and C3H tumors grown in C3H/Hen mice. In the first experiment the pharmacokinetics of doxorubicin were studied in SCID mice. Ninety-two mice were inoculated with MCF-7 cells, and those with the six largest tumors and the 14 smallest tumors were excluded on the day of the study in order to reduce the range of the tumor volumes, resulting in N = 72. These animals were then randomized by size into nine groups (N = 8 per group) representing three treatment arms (control, chronic *ad libitum* $NaHCO_3$, and acute $NaHCO_3$) and three time points of 2, 6 and 18 hr post-injection. The average tumor volume in each group on the day of experiment was 1286 ± 89 mm^3. To investigate the pharmacokinetics of doxorubicin in C3H/Hen mice, 34 mice were inoculated with C3H tumor fragments, and those with the four largest and the six smallest tumors were excluded on the day of the study, resulting in N = 24. These remaining animals were randomized by tumor size into three groups (N = 8 per group) representing three treatment arms (control, chronic *ad libitum* $NaHCO_3$, and acute $NaHCO_3$) and a single time point of 3 hr post-injection. The average tumor volume per group on day 0 was 1566 ± 198 mm^3. To investigate the pharmacokinetics of mitoxantrone in C3H mice, 54 mice were inoculated with C3H tumor fragments, and those with the two largest and the four smallest tumors were excluded on the day of the study, resulting in N = 48. These were randomized for tumor size into six groups representing two treatment arms (control and acute $NaHCO_3$) and three time points of 2, 6 and 18 hr post-injection. The average tumor volume on the day of the experiment was 1982 ± 242 mm^3.

Chronic alkalinization was achieved by replacing the water supply of the mice with a 200 mM sodium bicarbonate solution starting 2 days prior to doxorubicin administration, as described previously [2]. The acutely alkalinized group of mice received a 0.5 mL intraperitoneal (i.p.) injection of 1 M sodium bicarbonate 2 hr prior to drug injection, as described previously [3]. The control mice received no pretreatment prior to receiving drug.

For the doxorubicin experiments, mice received a tail-vein injection containing 0.069 μCi ^{14}C in a total dose (labeled + unlabeled) of 2 mg/kg doxorubicin. For the mitoxantrone experiments, mice received a tail-vein injection containing 0.158 μCi ^{14}C in a total dose of 9 mg/kg mitoxantrone. Mice were sacrificed at the indicated time points and the tissues immediately harvested and weighed. Tissues collected included tumor, blood, spleen, liver, kidneys, heart, lung, muscle, and GI tract. Tissues were solubilized for 7 days in 4 mL NSC-II tissue solubilizer (Amersham Life Science Inc.). Fifteen milliliters of NBS-204 liquid scintillation fluid (Amersham Life Science Inc.) was then added and the samples were assayed by liquid scintillation counting.

2.5. Chemotherapy

In order to investigate the influence of $NaHCO_3$ on tumor growth, two groups of 6-week-old female C3H/Hen mice (N = 4) bearing approximately 250 mm^3 C3H tumors in the mammary fat pad were treated as follows. 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 hr, while the other group received a saline injection and had uninterrupted access to *ad libitum* water. The two groups were compared over 2 weeks for differences in tumor growth rate. In a separate experiment, four groups of C3H/Hen mice (N = 4 per group) 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, while 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 hr prior to each mitoxantrone injection. $NaHCO_3$-treated mice were denied access to *ad libitum* water for 4 hr starting with administration of the $NaHCO_3$, but were allowed continuous access to solid food. Access to drinking water was restored 2 hr 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 11–15 hr after light onset [12]. For this reason, mitoxantrone was administered 12 hr after light onset in all experiments.

3. Results

3.1. $NaHCO_3$ enhances anti-tumor effects of anthracyclines but not taxol

Sodium bicarbonate has been previously shown to increase the pHe of tumors, whether delivered *ad libitum* chronically [2] or acutely by gavage or i.p. injection [3]. The increase in pHe relative to the corresponding tissues in untreated mice is greater in tumors than in normal tissues. This is possibly due to the low pHe in tumors in control

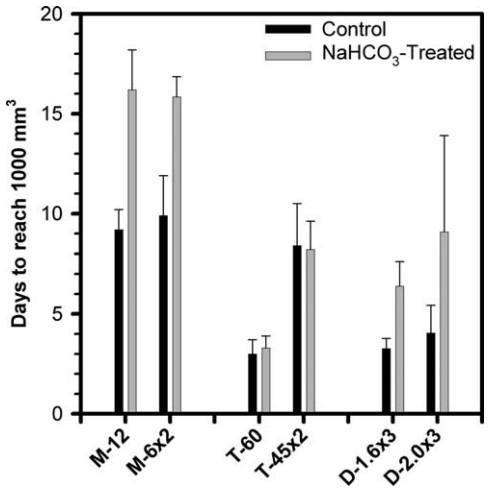

Fig. 1. Effect of $NaHCO_3$ on tumor growth. Animals bearing tumors were treated in four groups: saline, bicarbonate, drug, and drug + bicarbonate. Doses were as indicated, in mg/kg. M: mitoxantrone, T: taxol, D: doxorubicin. Tumor volumes were measured 3× per week and, from each individual growth curve, the time it took to reach 1.0 mL was scored. Bicarbonate alone had no significant effect on tumor growth rate (not shown).

mice which reduces the buffering power of endogenous bicarbonate. These treatments would be expected to increase the chemotherapeutic efficacy of weakly basic agents such as doxorubicin and mitoxantrone, but have no significant effect on zwitterionic agents such as paclitaxel. Figure 1 shows the effect of bicarbonate-induced alkalinization on the chemotherapeutic efficacy for these three drugs in mouse models of breast cancer. As shown in this figure, the tumor growth delay following mitoxantrone treatment in a C3H tumor model is almost doubled if the drug is administered following treatment of the tumor-bearing mice with sodium bicarbonate. A similar enhancement of MCF-7 tumor response to doxorubicin was found in SCID mice chronically treated with $NaHCO_3$, albeit with reduced statistical significance. Acute pretreatment of tumor-bearing mice with $NaHCO_3$ was found to have no significant effect on the therapeutic efficacy of paclitaxel in this study. This disagrees to some extent with a published report of lowered cell-cycle dependent cytotoxicity at acidic pH [13]. In the present system, however, neither acute nor chronic $NaHCO_3$ alone altered tumor growth rates, indicating little, if any, cell-cycle effects of bicarbonate on the cytotoxicity of paclitaxel in our tumor models.

3.2. *Systemic alkalinization affects biodistribution and pharmacokinetics of mitoxantrone to a greater degree than doxorubicin*

Selective tumor alkalinization by $NaHCO_3$ treatment should increase the biodistribution of doxorubicin and mitoxantrone into tumors if they are substrates for ion trapping. To test this hypothesis, the biodistributions and pharmacokinetics of radiolabeled drugs were determined in animals bearing tumors that were treated acutely or chronically with bicarbonate. Figure 2 shows typical pharmacokinetic profiles for mitoxantrone and doxorubicin in tumor and liver tissues taken from C3H and SCID mice. Figure 2A shows a consistently higher uptake of mitoxantrone into C3H tumors from mice that were pretreated with $NaHCO_3$ compared to tumors from control mice. The differences are apparent at all time points, but only become statistically significant after 6 hr. Figure 2B shows the pharmacokinetics in livers of the same animals. In the alkalinized animals the washout kinetics are significantly slower than in the controls, and this is observed in a number of organ systems. Hence, part of the enhanced chemotherapeutic effectiveness in the alkalinized animals may be due to prolonged pharmacokinetics. Pharmacokinetic profiles from MCF-7 tumor and liver of doxorubicin treated SCID mice are shown in Fig. 2C and D. No significant or apparent differences were observed between $NaHCO_3$-treated animals and controls. Doxorubicin levels in the SCID livers are approximately 2-fold higher than in the tumors, in contrast to the order of magnitude difference evidenced with mitoxantrone in the C3H model. In no case are the drugs retained in the liver, which is interesting as bile is a major clearance pathway for both drugs. These mitoxantrone and doxorubicin experiments were carried out in different tumor/mouse systems. To permit direct comparisons, the doxorubicin experiments were also repeated at a single time point in the C3H/Hen system. In these experiments, $NaHCO_3$-pretreatment was found to have no significant effects on the uptake of radiolabeled doxorubicin into tumors (*vide infra*).

Data from all experiments and all organs are presented in Tables 1–3. For these analyses, both peak concentrations (in ng/g) and the area-under-the-curve (AUC) were determined. Acute metabolic alkalosis resulted in altered mitoxantrone biodistribution (Table 1). Significant and marginally significant differences are highlighted with bold text in Table 1. Acute $NaHCO_3$-pretreatment caused drug to accumulate more in tumors, compared to controls. The AUC was 45% greater ($P < 0.01$) and the peak drug concentration was 75% greater in the alkalotic mice ($P < 0.01$). This result is consistent with the hypothesis that ion trapping is a significant modulator of mitoxantrone biodistribution. Additionally, the mitoxantrone levels in the tumor continued to rise, such that the difference between the saline and the bicarbonate groups increased with time, indicating enhanced re-distribution of drug to tumor in the alkalotic animals (Fig. 2A). This indicates that metabolic alkalosis achieved part of its anti-tumor effect by altering mitoxantrone re-distribution.

In contrast to the mitoxantrone results, neither acute nor chronic alkalosis has a significant effect on the biodistribution of doxorubicin to MCF-7 mammary tumors in SCID mice (Table 2) or C3H tumors in C3H/Hen mice (Table 3). Sodium bicarbonate pretreatment did lead to decreased doxorubicin distribution to heart in both SCID (Table 2) and C3H mice (Table 3), to as low as 37% of control levels.

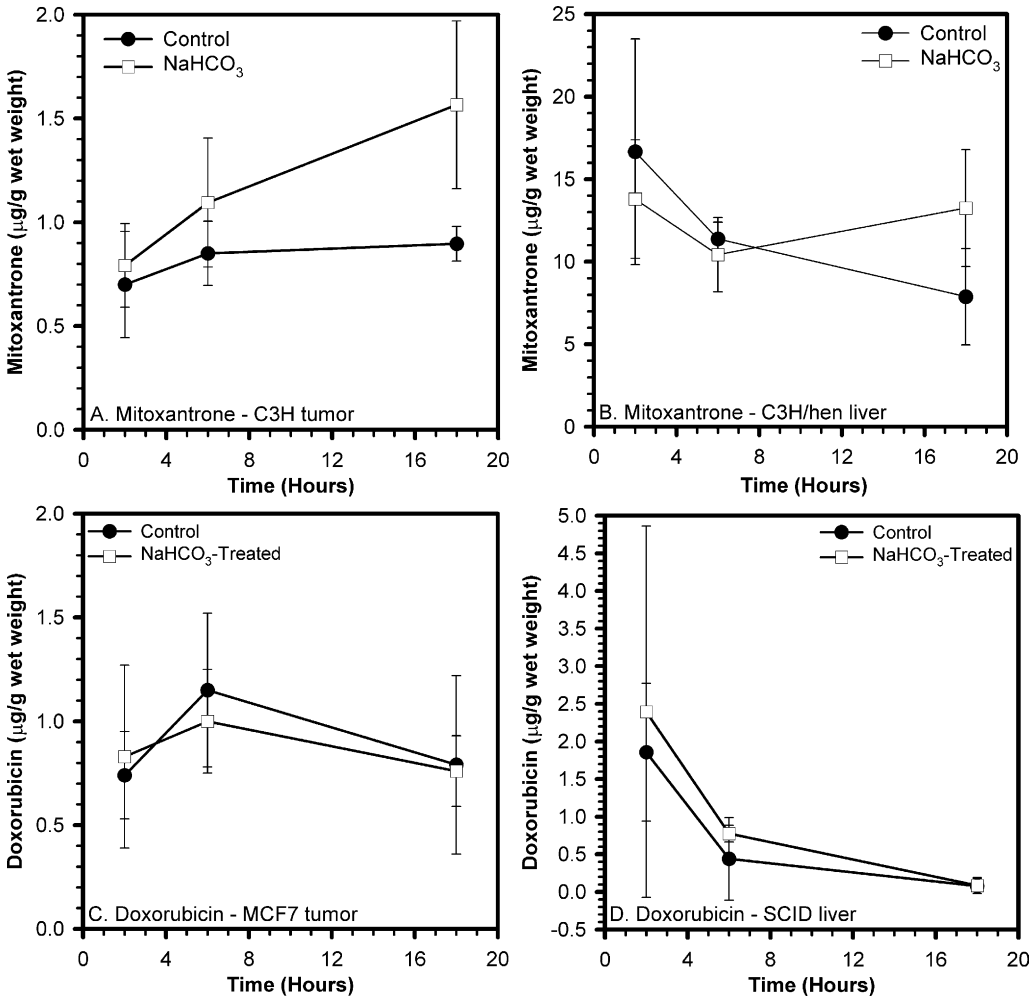

Fig. 2. Systemic alkalinization affects biodistribution and pharmacokinetics of mitoxantrone to a greater degree than doxorubicin. (A) Mitoxantrone concentration vs. time in C3H tumor tissue. Mitoxantrone content was assayed at 2, 6, and 18 hr and the values ± SD are plotted (N = 8) as a function of time following the administration of intravenous mitoxantrone. (B) Mitoxantrone concentration vs. time in the livers of C3H/Hen mice. Mitoxantrone content was assayed at 2, 6, and 18 hr and the values ± SD are plotted (N = 8) as a function of time following the administration of intravenous mitoxantrone. (C) Doxorubicin concentration vs. time in the MCF-7 human adenocarcinoma tumor tissue of SCID mice. Doxorubicin content was assayed at 2, 6, and 18 hr and the values ± SD are plotted (N = 8) as a function of time following the administration of intravenous doxorubicin. (D) Doxorubicin concentration vs. time in the liver of MCF-7 tumor bearing SCID mice. Doxorubicin content was assayed at 2, 6, and 18 hr and the values ± SD are plotted (N = 8) as a function of time following the administration of intravenous doxorubicin.

However, these differences did not achieve statistical significance. Nonetheless, this effect may have clinical importance, as cardiomyopathy is the dose-limiting toxicity for doxorubicin therapy [9]. Surprisingly, acute alkalosis also resulted in a significant reduction in the peak doxorubicin concentration found in the lungs. Specifically, a 46% reduction in the doxorubicin concentration in the lung was observed in acutely alkalotic SCID mice (Table 2, $P = 0.03$), and a 47% reduction in the drug concentration was observed in lungs of the acutely alkalotic C3H mice, although this change was of borderline significance (Table 3, $P = 0.07$). The mechanism underlying these differences are unclear, although the direction of the effect and the lack of effects in other tissues argues against ion-trapping as a mechanism. Most likely, this effect involves alkalosis-induced alveolar shunting, which would reduce exposure of the alveolar epithelium to drug [14–16].

The AUC of mitoxantrone in the spleen and kidneys of C3H mice increased significantly, but only by 14 and 9%, respectively (Table 1). Moreover, the thigh muscle in these mice showed significant decreases in the peak mitoxantrone concentration in alkalotic mice at the 2 hr time point. This result is consistent with the thigh muscle being a slow clearance tissue [17–20]. Metabolic alkalosis both theoretically and empirically results in more rapid plasma clearance and distribution of weakly basic drugs [21–23]. At later time points (6 and 18 hr), there were no significant differences between groups for thigh muscle drug levels, suggesting that the additional drug present in $NaHCO_3$-treated C3H mice at the 2 hr time point had cleared by the 6 hr time point.

Significant changes occurred in the peak distribution of both mitoxantrone and doxorubicin to the small intestines and colon in both SCID and C3H mice as a result of acute

Table 1
Mitoxantrone biodistribution in C3H mice

Tissue	AUC or peak[a]	Mitoxantrone group[b]	Mitoxantrone + $NaHCO_3$ group[b]	*t*-test	Percent change[c]
C3H mammary tumor	AUC	13577 ± 517	19744 ± 1339	**$P < 0.01$**	**45**
	Peak	897 ± 39	1566 ± 143	**$P < 0.01$**	**75**
Blood	AUC	1248 ± 76	1152 ± 45	$P = 0.28$	−8
	Peak	157 ± 19	133 ± 13	$P = 0.29$	−16
Liver	AUC	171588 ± 8374	190424 ± 10158	$P = 0.15$	11
	Peak	16661 ± 2418	13790 ± 1268	$P = 0.29$	−17
Heart	AUC	75763 ± 2145	74139 ± 2616	$P = 0.63$	−2
	Peak	6652 ± 911	5095 ± 392	$P = 0.12$	−23
Spleen	AUC	101562 ± 4545	115496 ± 4121	**$P = 0.02$**	**14**
	Peak	7661 ± 663	8796 ± 474	$P = 0.16$	14
Thigh muscle	AUC	20509 ± 1590	18276 ± 1010	$P = 0.24$	−11
	Peak	2485 ± 331	1251 ± 160	**$P < 0.01$**	**50**
Kidneys	AUC	404618 ± 10609	439507 ± 10628	**$P = 0.02$**	**9**
	Peak	27681 ± 2500	30996 ± 1623	$P = 0.27$	12
Small intestine	AUC	144470 ± 11628	195190 ± 24748	**$P = 0.06$**	**36**
	Peak	13883 ± 1599	33217 ± 7870	**$P = 0.02$**	**139**
Colon	AUC	456090 ± 71217	335733 ± 40648	$P = 0.14$	−27
	Peak	50914 ± 8744	31394 ± 4888	**$P = 0.05$**	**−38**

[a] AUC or peak tissue concentration.
[b] Mitoxantrone (ng)/gram tissue wet weight × hours ± SE or mitoxantrone (ng)/gram tissue wet weight ± SE.
[c] Percent change with $NaHCO_3$ pretreatment.

metabolic alkalosis (Tables 1–3). It should be noted that the bowels were assayed along with their contents, and that biliary excretion is the major route of both mitoxantrone and doxorubicin clearance [20,24–28]. Significant differences in peak drug concentrations disappeared at longer time points, suggesting that the radioactive drug was located in the mobile contents. The acute, but not the chronic, metabolic alkalosis protocol resulted in a significant effect on drug distribution within the bowels. This short-term effect is consistent with inhibition of peristalsis being the most likely explanation for our results.

3.3. Mitoxantrone and doxorubicin exhibit different pH-dependent partition coefficients

The clear conclusions from the above biodistribution study are that pretreatment with $NaHCO_3$ significantly increases the distribution of mitoxantrone to tumor, yet is without significant effect on the distribution of doxorubicin to tumor. Hence, mitoxantrone is a much better substrate for ion trapping than doxorubicin. To examine the physico-chemical basis for this difference, the pH-dependent octanol–water partition coefficients of the drugs were

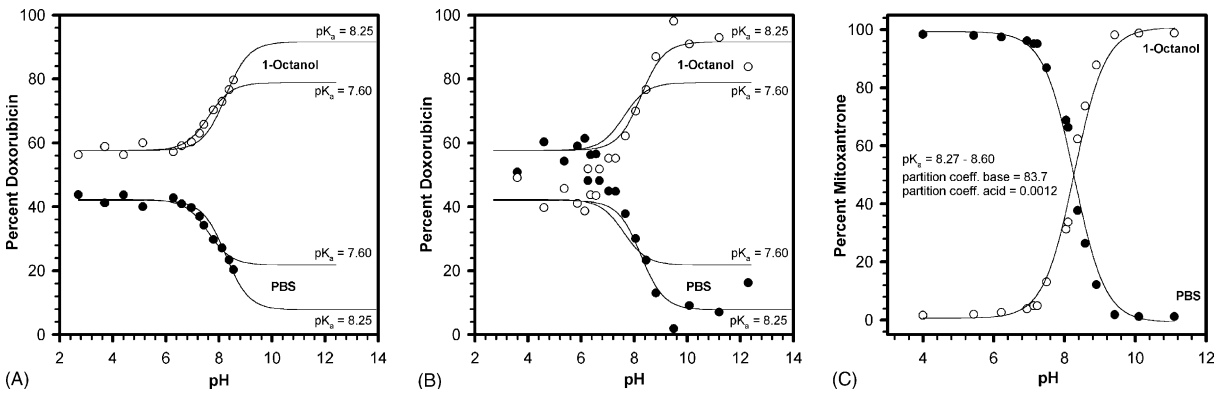

Fig. 3. The difference between mitoxantrone and doxorubicin may be explained by differences in their pH-dependent partition coefficients. Open circles: 1-octanol; closed circles: PBS. (A) ^{14}C labeled doxorubicin partitioning across a 1-octanol–aqueous interface as a function of the pH of the aqueous phase. The data are fit to two sets of curves representing the published range of pK_a values for doxorubicin. (B) Fluorescence measurement of doxorubicin's partition coefficient. Partitioning of doxorubicin across a 1-octanol–aqueous interface as a function of the pH of the aqueous phase. The data are fit to two sets of curves representing the published range of pK_a values for doxorubicin. (C) ^{14}C labeled mitoxantrone partitioning across a 1-octanol–aqueous interface as a function of the pH of the aqueous phase.

Table 2
Doxorubicin biodistribution in SCID mice

Organ	Group	AUC or peak[a]	Value[b]	*t*-test[c]
Blood	Control	AUC	2.596 ± 0.341	
	Chronic	AUC	2.627 ± 0.198	
	Acute	AUC	2.463 ± 0.209	
	Control	Peak	0.181 ± 0.019	
	Chronic	Peak	0.280 ± 0.052	$P = 0.07$ vs. control
	Acute	Peak	0.177 ± 0.024	$P = 0.07$ vs. chronic
Tumor	Control	AUC	15.494 ± 1.453	
	Chronic	AUC	15.000 ± 0.839	
	Acute	AUC	14.215 ± 0.858	
	Control	Peak	1.155 ± 0.132	
	Chronic	Peak	1.038 ± 0.081	
	Acute	Peak	1.001 ± 0.092	
Spleen	Control	AUC	312.68 ± 63.59	
	Chronic	AUC	436.79 ± 184.87	
	Acute	AUC	426.55 ± 145.95	
	Control	Peak	29.18 ± 10.26	
	Chronic	Peak	50.89 ± 30.56	
	Acute	Peak	42.13 ± 18.18	
Liver	Control	AUC	7.69 ± 1.69	
	Chronic	AUC	38.57 ± 31.46	
	Acute	AUC	11.47 ± 3.58	
	Control	Peak	1.85 ± 0.91	
	Chronic	Peak	5.44 ± 13.86	
	Acute	Peak	2.39 ± 1.10	
Kidney	Control	AUC	102.15 ± 4.57	
	Chronic	AUC	105.59 ± 10.26	
	Acute	AUC	125.44 ± 23.24	
	Control	Peak	8.77 ± 2.5	
	Chronic	Peak	10.00 ± 1.70	
	Acute	Peak	9.34 ± 7.08	
Colon	Control	AUC	161.13 ± 2.19	
	Chronic	AUC	153.24 ± 9.26	
	Acute	AUC	112.25 ± 10.05	$P = 0.01$ vs. control; $P = 0.06$ vs. chronic
	Control	Peak	16.60 ± 0.040	
	Chronic	Peak	12.90 ± 2.26	
	Acute	Peak	9.30 ± 2.61	$P < 0.01$ vs. control
Small intestine	Control	AUC	42.09 ± 6.91	
	Chronic	AUC	29.44 ± 3.61	$P = 0.07$ vs. control
	Acute	AUC	19.90 ± 2.45	$P < 0.01$ vs. control; $P = 0.07$ vs. chronic
	Control	Peak	4.71 ± 2.32	
	Chronic	Peak	3.21 ± 1.21	
	Acute	Peak	2.81 ± 1.78	$P = 0.07$ vs. control
Thigh muscle	Control	AUC	239.71 ± 37.06	
	Chronic	AUC	168.79 ± 18.99	
	Acute	AUC	139.03 ± 20.18	
	Control	Peak	19.60 ± 9.80	
	Chronic	Peak	14.79 ± 4.48	
	Acute	Peak	11.29 ± 4.77	
Heart	Control	AUC	215.37 ± 91.56	
	Chronic	AUC	82.63 ± 11.08	
	Acute	AUC	80.60 ± 8.41	
	Control	Peak	22.48 ± 32.34	
	Chronic	Peak	6.61 ± 3.77	
	Acute	Peak	6.08 ± 2.21	
Lungs	Control	AUC	131.56 ± 8.79	
	Chronic	AUC	101.32 ± 7.87	
	Acute	AUC	89.07 ± 8.11	$P = 0.06$ vs. control
	Control	Peak	12.17 ± 6.89	
	Chronic	Peak	11.41 ± 6.13	
	Acute	Peak	6.56 ± 1.82	$P = 0.03$ vs. control; $P = 0.03$ vs. chronic

[a] AUC or peak tissue concentration.
[b] Doxorubicin (ng)/gram tissue wet weight × hours ± SE or doxorubicin (ng)/gram tissue wet weight ± SE.
[c] Only significant and marginally-significant changes are noted.

Table 3
Doxorubicin biodistribution in C3H mice

Organ	Group	Concentration[a]	*t*-test[b]
Blood	Control	796.8 ± 357.3	
	Chronic	3.21	
	Acute	61.35 ± 101.3	$P < 0.01$ vs. control
Tumor	Control	606.0 ± 109.4	
	Chronic	651.7 ± 169.1	
	Acute	578.4 ± 119.8	
Spleen	Control	1551 ± 302	
	Chronic	2684 ± 829	$P = 0.01$ vs. control
	Acute	2634 ± 376	$P < 0.01$ vs. control
Liver	Control	1700 ± 275	
	Chronic	2765 ± 903	$P < 0.01$ vs. control
	Acute	5189 ± 1318	$P < 0.01$ vs. control; $P < 0.01$ vs. chronic
Kidney	Control	8075 ± 7675	
	Chronic	3040 ± 730	
	Acute	5411 ± 1747	$P < 0.01$ vs. chronic
Colon	Control	3188 ± 2329	
	Chronic	5525 ± 1670	$P = 0.07$ vs. control
	Acute	1536 ± 742	$P < 0.01$ vs. chronic
Small intestine	Control	1673 ± 1450	
	Chronic	2755 ± 1221	
	Acute	5558 ± 2044	$P < 0.01$ vs. control; $P < 0.01$ vs. chronic
Thigh muscle	Control	4492.6 ± 2630	
	Chronic	849.8 ± 311	$P < 0.01$ vs. control
	Acute	764.2 ± 285	$P < 0.01$ vs. control
Heart	Control	2977 ± 1571	
	Chronic	1838 ± 614.9	
	Acute	1391 ± 301.5	
Lungs	Control	3268.3 ± 1894	
	Chronic	1573.8 ± 326	
	Acute	1734 ± 245	$P = 0.07$ vs. control; $P = 0.08$ vs. chronic

[a] Doxorubicin (ng)/gram tissue wet weight ± SE.
[b] Only significant and marginally-significant changes are noted.

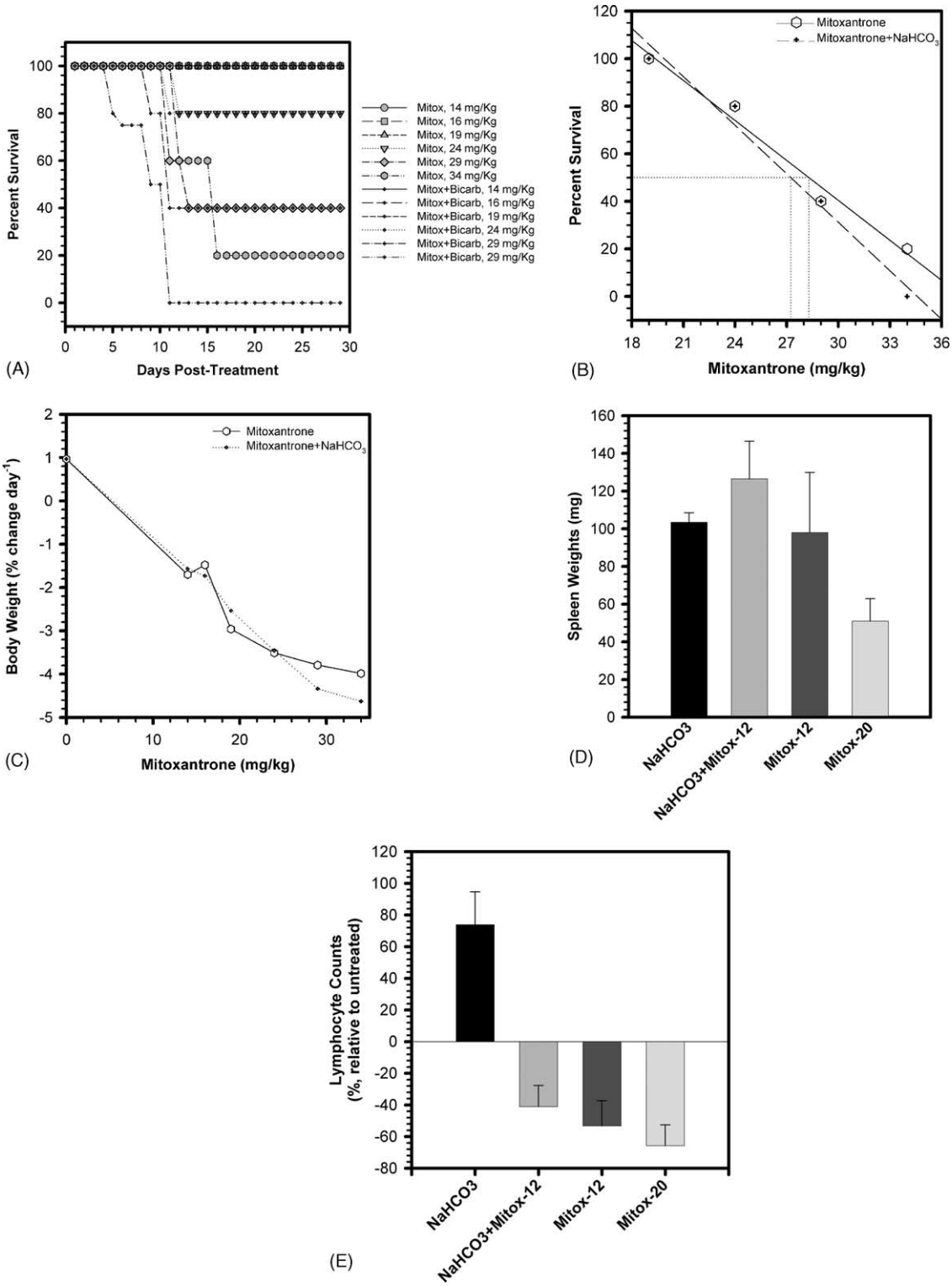

Fig. 4. Alkalinization does not affect the dose-limiting toxicities of mitoxantrone. (A) C3H/Hen mouse survival. Survival of C3H/Hen mice treated with increasing concentrations of mitoxantrone at 14, 16, 19, 24, 29, 34 mg/kg, with and without $NaHCO_3$ (0.7 mL × 1 M, i.p. 2 hr prior to drug administration). A single dose of mitoxantrone was administered on day 1 and the percent of mice alive in each group (N = 5 per group) is plotted against time in days. (B) LD_{50} of mitoxantrone with and without bicarbonate. The survival of C3H/Hen mice administered increasing concentrations of mitoxantrone (19, 24, 29, 34 mg/kg) with and without $NaHCO_3$ (0.7 mL × 1 M, i.p. 2 hr prior to drug administration). A single dose of mitoxantrone was administered on day 1 and the percent of mice alive after 30 days is plotted against drug concentration (N = 5 for each group). The diagonal lines are independent linear regressions of the data. The vertical lines represent the LD_{50} determination for mitoxantrone in C3H/Hen mice with and without $NaHCO_3$, which are 27.2 and 28.3 mg/kg, respectively. (C) Change in body weight for C3H/Hen mice following either mitoxantrone or mitoxantrone + bicarbonate. The rate of weight loss is plotted as a function of mitoxantrone dosage. The weight loss of C3H/Hen mice following administration of a single dosage of mitoxantrone (14, 16, 19, 24, 29, 34 mg/kg) with and without $NaHCO_3$ (0.7 mL × 1 M, i.p. 2 hr prior to drug administration). The mean weight loss/mouse over the first 10 days was used to calculate the ordinate

determined. Figure 3A shows the partition coefficient for doxorubicin as a function of pH. In this case, the drug concentrations were determined using a ^{14}C label on the daunosamine sugar. These data were fit using pK_a values of 7.6 and 8.25 to represent the range of values available in the literature. Three observations come from these data. First, the higher pK_a value provides a better fit, as it accounts for increased octanol partitioning above a pH of 8.0. Second, data could not be obtained above a pH of 8.4 because the molecule is hydrolyzed at high pH, consistent with destabilization of the deprotonated form. This was confirmed with thin layer chromatography (data not shown). Third, and most significant, is that doxorubicin partitions well into octanol ($P = 1.36$) even in its fully charged form. Consequently, the protonated form of doxorubicin would be expected to have high membrane permeability, making doxorubicin a poor candidate for ion trapping. Because of the observed instability of the radiolabeled drug we also used visible absorbance to quantify the distribution of the pigmented tetracyclic adriamycinone moiety, as shown in Fig. 3B. These data essentially agree with the data in Fig. 3A in that the pK_a of 8.25 provides a better fit and that doxorubicin partitions well into octanol ($P = 0.74$) at acid pH. In contrast to the behavior of doxorubicin, the partitioning of mitoxantrone is much more affected by pH (Fig. 3C). The data in Fig. 3C were fit using pK_a values of 8.27 and 8.60 for the two ionizable groups. At low pH (fully protonated) mitoxantrone has a partition coefficient of 83.7, while at high pH (fully deprotonated) mitoxantrone has a partition coefficient of 0.0012. Thus, the ratio of the octanol–water partition coefficients of protonated/deprotonated mitoxantrone is approximately 70,000. On these theoretical grounds, $NaHCO_3$ pretreatment is expected to yield a 13-fold enhancement of the anti-tumor activity of mitoxantrone, and a 2-fold enhancement of the anti-tumor activity of doxorubicin [2,3,29]. However, only a approximately 2-fold decrease in tumor growth rate by $NaHCO_3$-pretreatment was observed for both drugs in animal models (Fig. 1). It is possible that the expected enhancement for mitoxantrone is blunted *in vivo* by antagonistic factors which are also enhanced by alkalosis.

3.4. Effect of alkalinization on mitoxantrone toxicity

The above results indicate that mitoxantrone is a strong candidate for ion trapping, likely by virtue of its two ionizable groups. Consequently, raising tumor pHe by treatment of the host with sodium bicarbonate should improve chemotherapeutic response. But this would be true only if $NaHCO_3$ did not exacerbate acute myelosuppression, which is the dose limiting toxicity [10]. Data in Table 1 indicate that $NaHCO_3$ pretreatment causes a slight (14%) but significant elevation of mitoxantrone distribution to spleen in C3H mice. Hence, potential toxicities of $NaHCO_3$ pretreatments were further examined. Figure 4A is a Kaplan–Meier plot from cohorts of mice treated with 14–34 mg/kg mitoxantrone administered as a single dose, with or without sodium bicarbonate pretreatment. As shown in this figure, there are no differences between control and $NaHCO_3$-treated mice at mitoxantrone doses of up to 29 mg/kg. At the highest tested dose of 34 mg/kg all five $NaHCO_3$-treated mice died and 4/5 of the non-$NaHCO_3$-treated mice died. These data were used to determine an LD_{50} for mitoxantrone in the presence or absence of $NaHCO_3$ (Fig. 4B). No significant effect of $NaHCO_3$ pretreatment on the calculated LD_{50} of mitoxantrone was found. Mitoxantrone therapy is also invariably accompanied by weight loss, which is a measure of toxicity. As shown in Fig. 4C, mitoxantrone caused weight loss, yet the rate was not significantly different between animals treated with mitoxantrone alone and those treated with mitoxantrone and $NaHCO_3$. The acute, dose-limiting toxicity associated with mitoxantrone is bone marrow suppression, typically observed 7–14 days after treatment [10]. Because of this, potentially more subtle effects of $NaHCO_3$ were examined. As shown in Fig. 4D, 20 mg/kg mitoxantrone causes a significant reduction in spleen weights 8 days after injection, relative to treatment with 12 mg/kg mitoxantrone. Pretreatment with $NaHCO_3$ had no significant effect on spleen weight at 12 mg/kg mitoxantrone. These effects are mirrored in the lymphocyte counts 8 days post-treatment (Fig. 4E). As expected, even at a low dose of 12 mg/kg, mitoxantrone causes significant reductions in the number of circulating lymphocytes relative to treatment with $NaHCO_3$ alone. Pretreatment with $NaHCO_3$ prior to 12 mg/kg mitoxantrone does not significantly affect this decline. The apparent increase in circulating lymphocyte levels in the bicarbonate-only group reflects normal immune development in these 6-week-old juvenile mice.

4. Discussion

In a companion manuscript, the effect of ion trapping on a series of ionizable chemotherapeutic drugs was examined [1]. These results identified a subtle, yet significant, difference between the pH sensitivities of the anti-tumor activities of doxorubicin and mitoxantrone *in vitro* [1].

($N = 5$ for each group). (D) Median spleen weights 8 days post-treatment. The median spleen weights of C3H/Hen mice 8 days following administration of either 0.7 mL i.p. 1 M $NaHCO_3$, 0.7 mL i.p. 1 M $NaHCO_3$ followed 2 hr later by 12 mg/kg i.v. mitoxantrone, 12 mg/kg i.v. mitoxantrone without $NaHCO_3$, or 20 mg/kg i.v. mitoxantrone. The data indicate that $NaHCO_3$ pretreatment does not result in increased myelosuppression. (E) Median lymphocyte count 8 days post-treatment. The median circulating lymphocyte count of C3H/Hen mice 8 days following administration of either 0.7 mL i.p. 1 M $NaHCO_3$, 0.7 mL i.p. 1 M $NaHCO_3$ followed 2 hr later by 12 mg/kg i.v. mitoxantrone, 12 mg/kg i.v. mitoxantrone without $NaHCO_3$, or 20 mg/kg i.v. mitoxantrone. The data indicate that $NaHCO_3$ pretreatment does not result in increased immune suppression.

The current work was designed to further explore the potential mechanisms underlying these differences *in vivo*.

Sodium bicarbonate causes alkalinization of tumors in SCID and C3H/Hen model systems [2,3,29]. Because tumors have an acidic pretreatment pHe relative to normal tissues, $NaHCO_3$-induced alkalinization has a greater effect on tumors than on normal tissues. $NaHCO_3$ treatment alone has no effect on the growth rates of either C3H or MCF-7 tumors. This suggests that metabolic alkalosis will result in the enhancement of anti-tumor activity for weakly basic chemotherapeutic drugs, while not enhancing the toxicity to the host. There was no significant effect of $NaHCO_3$ pretreatment on the LD_{50} or weight loss in response to mitoxantrone, further indicating that pretreatment with $NaHCO_3$ will not affect normal tissues in a dose-limiting fashion.

$NaHCO_3$-induced alkalization enhances mitoxantrone therapy in the C3H/Hen system. In the bicarbonate-treated groups, tumors underwent greater regression and experienced longer tumor growth delays compared to controls that were treated with mitoxantrone alone. $NaHCO_3$-induced alkalinization also enhances doxorubicin therapy in both C3H/C3H and MCF-7/SCID systems. *In vitro* studies indicate that mitoxantrone undergoes classic and substantial ion-trapping, while results with doxorubicin were equivocal [1]. $NaHCO_3$ induced a significant redistribution of mitoxantrone to tumors, whereas it had no significant effect on the tumor uptake of doxorubicin. Hence, the enhancement of the anti-tumor activity of doxorubicin that we have observed ([2], Fig. 1) must be due to a mechanism other than increased drug retention in the tumor. Perhaps, as observed *in vitro*, the subcellular distribution of doxorubicin or the activity of topoisomerase are affected by pHe alterations.

These results suggest that inducing metabolic alkalosis in patients would have a positive effect on response to mitoxantrone therapy. However, the therapeutic index would not increase if sodium bicarbonate also caused increased retention of mitoxantrone in susceptible normal tissues in the host. The major dose-limiting organ systems for mitoxantrone are heart, liver, bone marrow, spleen and blood cells. $NaHCO_3$ was found to have no significant effect on the distribution of mitoxantrone to any of these tissues except for spleen, which was increased by only 14%. However, neither spleen weights nor lymphocyte counts in SCID and C3H mice were adversely affected by $NaHCO_3$ pretreatment, indicating that this co-therapy does not enhance myelosuppression due to mitoxantrone therapy. These findings indicate that metabolic alkalosis would produce a net gain in mitoxantrone therapeutic index.

In a clinical setting mitoxantrone could be given following acute metabolic alkalosis, although it remains to be seen if sodium bicarbonate treatment can alter tumor pHe in humans. Microelectrode data indicate that pHe values of human tumors, including breast, are acidic [30–34] and that the intracellular pH is neutral-to-alkaline [34–36]. In animal systems, significant acute alkalization was achieved by i.p. injection of $NaHCO_3$. In clinical protocols sodium bicarbonate is infused intravenously, most commonly to reverse metabolic acidosis. $NaHCO_3$ has also been utilized in conjunction with anti-tumor chemotherapy, specifically in conjunction with cyclophosphamide, in an attempt to limit the kidney damage caused by the drug [37]. Accepted protocols for sodium bicarbonate induced alkalinization are therefore available for humans.

Acknowledgments

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