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Drug resistance and cellular adaptation to tumor acidic pH microenvironment

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Abstract

Despite advances in developing novel therapeutic strategies, a major factor underlying cancer related death remains resistance to therapy. In addition to *biochemical* resistance, mediated by xenobiotic transporters or binding site mutations, resistance can be *physiological;* emerging as a consequence of the tumor's physical microenvironment. This review focuses on extracellular acidosis, an end result of high glycolytic flux and poor vascular perfusion. Low extracellular pH, pHe, forms a physiological drug barrier described by an "ion trapping" phenomenon. We describe how the acid-outside plasmalemmal pH gradient negatively impacts drug efficacy of weak base chemotherapies but is better suited for weakly acidic therapeutics. We will also explore the physiologic changes tumor cells undergo in response to extracellular acidosis which contribute to drug resistance including reduced apoptotic potential, genetic alterations, and elevated activity of a multidrug transporter, p-glycoprotein, pGP. Since low pHe is a hallmark of solid tumors, therapeutic strategies designed to overcome or exploit this condition can be developed.

Keywords

Microenvironment; Acidosis; Ion Trapping; Drug resistance

Introduction

A major obstacle to overcome during the treatment of solid tumors is resistance to therapy [1, 2]. One factor contributing to this problem is the physical tumor microenvironment (pO_2 and pH) and its impact on therapeutic efficacy [3–5]. Hypoxia (Figure 1) and high glycolytic activity are common characteristics of solid tumors leading to increased production and secretion of lactate and H+ to the extracellular space. The culmination of elevated glycolysis coupled with poor vascular perfusion is an acidic extracellular space [6–8]. Non-invasive measurements have shown that pHe ranges from 6.5 to 6.9 while intracellular pH, pHi, remains neutral to alkaline [7, 9] creating an acid-outside pH gradient typically not observed in normal tissue [10].

Tumor cells exposed to these harsh intratumoral physical conditions undergo many changes and it is becoming increasingly evident that acidosis plays an important role in the somatic

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evolution and progression of cancer from pre-invasive to malignant disease [6, 11–13]. Early studies by Morita et al. described the clastogenic properties of low pHe on mammalian cell lines *in-vitro* [14–17]. Other early studies by LeBoeuf observed that low pHe inhibits gap junctions, which are classified as tumor suppressors [18]. These alterations may contribute to the observation that low pHe can promote the transformation of normal cells to a neoplastic phenotype [19]. Additional studies show that a low extracellular pH increases the expression of vascular endothelial growth factor (VEGF), carbonic anhydrase, interlukin-8, cathepsin B, and matrix metalloproteinases- 2 and -9, all of which are associated with increased tumor cell survival, migration and invasion [20–23].

A low extracellular pH also contributes to drug resistance both *in-vitro* and *in-vivo*. The acid-outside pH gradient generated between intra- and extracellular space affects the distribution and uptake of select weak base chemotherapeutic drugs resulting in physiological drug resistance [24–27]. Tumors cells adapted to low pHe *in-vitro* harbor p53 mutations and have elevated activity of p-glycoprotein both of which can contribute to drug resistance [28–30]. In addition, chronically adapted low pHe cells are radio-insensitive *in-vitro* [31].

This review will focus on drug resistance and the extracellular acidic microenvironment. It will begin by discussing “ion trapping”, a phenomenon that describes how low pHe negatively impacts the uptake of weak base chemotherapeutics followed by the use of strategies to alkalinize tumor pH in order to increase therapeutic efficacy. We will conclude this review with a section on cellular adaptation and responses to acidosis that may contribute to drug resistance.

Low pH and physiological drug resistance

The cell membrane functions as a semi-permeable structure between the intra- and extracellular microenvironment. Small-uncharged molecules readily diffuse across the phospholipid portions of membranes while charged species tend to remain impermeable. Because of this characteristic, the acidic extracellular space of solid tumors creates a physiological barrier for the cellular uptake of weak bases [3]. This phenomenon is termed “ion trapping” (Figure 2). Ion trapping occurs when there is a large permeability difference between ionized (impermeant) and non-ionized (permeant) species of a drug. On each side of the membrane, an equilibrium between ionized and non-ionized forms of the drug are established according to a Henderson-Hasselbach relationship. For a weak base, the ratio of ionized BH+ to non-ionized B is $10^{-(pH-pK)}$. Thus, if the pKa is 8.3, the ratio will be ~10:1 at pH 7.3 (typical for pHi) and ~100:1 at a pH of 6.3 (lower range of pHe). As the non-ionized form of the drug equi-distributes on both sides of the membrane, more drug is sequestered in the lower pH of the extracellular environment, reducing therapeutic efficacy [32].

Most chemotherapeutic drugs have ionizable species under physiological conditions that may enhance or hinder their ability to cross membranes. Uptake and efficacy of weak base chemotherapeutics with a dissociation constant of 7.5–9.5 such as anthracyclines, anthraquinones, and vinca alkaloids are reduced by the acid-outside pH gradient of solid tumors, as shown by *in-vitro* and *in-vivo* studies [10, 24–27, 33].

Figure 3A illustrates *in-vitro* plasmalemmal pH gradients in MCF-7 cells as a function of the extracellular pH. MCF-7 cells cultured at a pHe of 6.8 and 7.4 had a pHi of 7.05 and 7.2 respectively generating both acid-outside and alkaline-outside plasmalemmal pH gradients. Doxorubicin is an anthracycline consisting of an ionizable primary amine with a basic pKa of 8.3. Doxorubicin has been previously shown to undergo ion trapping[3] in acid conditions and is a substrate for p-glycoprotein, a drug exporter with enhanced activity in acidic

environments[34]. Intracellular accumulation of ^{14}C-labeled doxorubicin was greater in MCF-7 cells cultured at a pHe of 7.4 (~168 pmol/mg/protein^{-1}) than that of cells cultured at a pHe 6.8 (65 pmol/mg/protein^{-1}) increasing *in-vitro* toxicity (Figure 3B and 3C). Table 1 is a list of additional weak base and weak acid chemotherapeutics and their respective pKas plus their LD_{50} against MCF-7 cells cultured at a pHe of 6.8 or 7.4 [25].

Conversely, if weak bases are protonated and trapped extracellularly in acidic environments, then uptake of weak acidic chemotherapeutics such as chlorambucil should be enhanced under similar acid-outside pH conditions. Chlorambucil, with a dissociation constant of 5.78, readily crosses the plasma membrane of cells cultured at a low pHe. *In-vivo* experimental acidosis following a bolus injection of glucose resulted in a 2.3 fold increase in the efficacy of chlorambucil compared to weak base doxorubicin [24]. Intratumoral alkalization with sodium bicarbonate ($NaHCO_3$) greatly reduced chlorambucil efficacy both in in-vitro and *in-vivo* studies (to be discussed in the next section). Friberg and Moan showed similar effects with the photosensitizing agent Hematoporphyrin IX (HpIX). Uptake of HpIX was increased in T-47D cells cultured under acidic conditions compared to neutral conditions [35] implying that the “ion trapping” phenomenon must be taken into consideration while designing and implementing all therapeutic strategies in addition to chemotherapy.

Melphalan is a weak acid chemotherapeutic compound with pKa values of 1.83 and 9.13 at pH 7.4 [36] and is approved clinically for treatment of multiple myeloma and ovarian cancer [37]. Conforming with the “ion trapping” hypothesis, increased cellular uptake of melphalan is observed in cells cultured at low pHe [38, 39] and the anti-tumoral effect of melphalan is enhanced by low pHe across many tumor xenograft models [40–42]. Melphalan is one such compound that may benefit from a therapeutic approach that takes the “ion trapping” hypothesis into consideration. Melphalan is used in isolated limb perfusion and infusion models both pre-clincally and clinically for the treatment of melanoma [43, 44]. Isolation of the limb temporarily halts blood circulation to the extremity resulting in local hypoxia and acidosis. Delivery of melphalan directly into the isolated limbs dramatically increases the compounds efficacy prolonging patient survival and reducing the number of limb amputees [45–51]. These results suggest that inclusion of “ion trapping” in further studies may prove to be a viable therapeutic strategy.

Paclitaxel is commonly used in the clinic to treat early stage breast cancer and has been used *in-vitro* to induce cell death in MCF-7 cells [52, 53]. Paclitaxel is not ionizable and drug distribution should not be affected by extracellular pH. The effect of pH on paclitaxel efficacy determined in-vitro (Figure 4) showed no significant differences in toxicity in MCF-7 cells cultured at a pHe of 6.8 or 7.4 [26]. In addition, paclitaxel treatment in combination with sodium bicarbonate did not alter tumor growth rates suggesting the increased therapeutic benefit stemming from extracellular alkalinization by sodium bicarbonate may be drug selective. These results confirm that not all chemotherapeutics are ionizable under physiological conditions and are therefore not candidates for “ion trapping” [26].

Experimental Alkalization of pHe

Experimental and mathematical models demonstrate that it is possible to raise extracellular pH of tumors using systemic buffers [54–57]. An *in-silico* tumor model developed by Silva *et al.* determined that the buffer best suited to raise intratumoral pH should have a pKa of ~7.0 [57]. As stated by Silva, candidate buffers cholamine chloride (pKa, 7.1), BES (pKa, 7.15), TES (pKa, 7.5), and HEPES (pKa, 7.55) are available, but the effects of these buffers *in-vivo* need additional testing [58]. Sodium bicarbonate is a physiological buffer with a pKa of 6.1 that regulates the pH in blood and tissue [59]. Chronic administration of sodium

bicarbonate increased the pHe of MCF-7 mammary fat pad tumors with little detectable effect on pHi (Figure 5A). These values were determined using ^{31}P MR spectra to measure the chemical shift of exogenously added 3-APP (pHe) and endogenous inorganic phosphates (pHi). Notice that the pHe and pHi differed between two sets of control tumors grouped by size, but an acid-outside membrane gradient was present in both sets [33].

Although it affected the pHe, treatment with sodium bicarbonate alone had no effect on growth of primary tumors. However, combining sodium bicarbonate with doxorubicin reduced tumor volume and delayed growth compared to doxorubicin alone suggesting that alkalinization by sodium bicarbonate may enhance doxorubicin uptake (Figure 5B). These data support the *in-vitro* data of MCF-7 cells cultured at a pHe of 7.4 have increased doxorubicin uptake and sensitivity to treatment (Figure 3B and 3C). Even more striking results have been observed using mitoxantrone [60, 61], and a generalized model has been developed that uses the pH-dependent partition coefficients to predict the severity of ion trapping in drug distribution [25, 26].

Epirubicin, also a weak base with a pKa of 8.1 [62], is an anthracycline that inhibits DNA and RNA synthesis. Epirubicin is used clinically to treat breast cancer and has been investigated as a treatment for superficial bladder cancer via intravesical delivery [63, 64]. *In-vitro* studies show that epirubicin exhibits increased efficacy against human bladder cancer cells [65, 66] and chineese hamster ovary cells cultured under alkaline conditions [67]. Clinically, issues may arise during intravesical delivery of epirubicin directly into the bladder since the patient urine may be acidic potentially decreasing cellular uptake of epirubicin. Buffering the pH of the bladder or alkalinizing the pH of epirubicin prior to delivery may have a beneficial impact on the therapeutic efficacy [65, 66], however, this has yet to be investigated.

Maintaining an alkaline intracellular environment is critical for cell survival. Cells maintain an intracellular alkaline environment by transporting intracellular H+ to the extracellular space via a number of mechanisms, including vacuolar-ATPase, Na+/H+ exchanger (NHE-1), carbonic anhydrases (CA-IX) and anion exchangers [68–72]. Due to elevated glycolytic activity of tumor cells, dependence on these mechanisms for survival is critical. Vacuolar-ATPase located at the plasma membrane through membrane recycling has elevated expression and activity in metastatic tumors [73]. Na+/H+ exchange expression correlates with hypoxic/necrotic regions of an *in-vitro* tumor spheroid [12]. Carbonic anhydrases reversibly convert carbon dioxide and water to bicarbonate and a proton. Inhibition of CA-IX reduces tumor acidity and pH heterogeneity [74, 75]. The end result is acidification of the extracellular space. Proton pump inhibitors (PPIs) are a selective class of vacuolar-ATPase inhibitors that are commonly used to treat patients with gastric disease [76]. PPIs reduce the outward flux of H+ raising the pH of the extracellular environment [76]. Some efficacy of PPIs has been observed in solid tumor models and *in-vitro* against melanoma cells. Luciani et al. utilized PPI omeprazole to reduce v-H+ -ATPase activity and to breakdown the acid-outside physiological barrier [77]. The result was alkalization of both extracellular pH and intracellular vacuoles. They showed that pre-treatment with PPIs increased the uptake and efficacy of compounds that were under normal tumor conditions excluded from intracellular compartments [77].

Cellular adaptations to low pHe

The tumor physical microenvironment is composed of low oxygen tension and high acidity. These conditions lead exposed cells to physiological changes as well as to selective pressures. Physiological changes include changes in gene expression [78], apoptotic potential [31], autophagy [79], as well as drug resistance [3]. Because acidity may cause p53-dependent apoptosis, selection of p53-mutant cells may occur [30]. This loss of apoptotic

potential and other adaptive changes are likely driven by microenvironment-induced genomic instability and inhibition of DNA repair [15, 80, 81].

Drug resistance is a major adaptive change in aggressive cancers and is a confounding factor during treatment. This may arise due to the chronic exposure to an acidic microenvironment. A major mechanism of drug resistance involves the activity or expression of the multidrug transporter, p-glycoprotein (pGP) [28, 29]. pGP, encoded by the MDR1 gene, actively pumps cytotoxins, such as doxorubicin and paclitaxel, out of the cell [82]. Although mRNA levels are not changed during acidosis, the activity of pGP is increased, and this effect is amplified by hypoxia [28]. The localization of pGP is also crucial, and has been reported to change after induction of selective pressures [83]. The changes in pGP activity during acidosis is accompanied with changes in intracellular pH, which may decrease the effectiveness of chemotherapeutics [84, 85], or the capacity of drugs to be pumped out of the cell [86].

Conclusions

We described mechanisms by which low pHe contributes to chemotherapy resistance. Since maintained acidification of the extracellular space is a hallmark of solid tumors, novel methods are needed to overcome low pHe drug resistance in order to improve therapeutic efficacy of current and future compounds. One approach is to alkalinize the microenvironment through the use of systemic buffers. While sodium bicarbonate successfully increased the efficacy of weak base chemotherapies *in-vivo*, a systemic buffer with a pKa of ~7.0 is predicted to be more effective. The opposite approach is to take advantage of low pHe through increased use and design of weak acid compounds. Many groups have developed low pH activated micelle systems that are designed to enter the core of solid tumors followed by the release of toxins within the acidic microenvironment, however additional *in-vivo* studies are required to determine their effectiveness [87]. Although periods of hypoxia can be transient [88, 89], acidification of the extracellular microenvironment likely remains constant due to aerobic glycolysis. Because acidosis provides a modality for selection and for drug resistance, new techniques and pharmacological agents must be developed to adress tumor acidification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS

pHi	intracellular pH
pHe	extracellular pH
VEGF	vascular endothelial growth factor
pGP	p-glycoprotein
$NaHCO_3$	sodium bicarbonate
3-APP	3-aminopropyl phosphonate
NHE1	Na+/H+ exchanger
CA-IX	carbonic anhydrase 9
PPI	proton pump inhibitor

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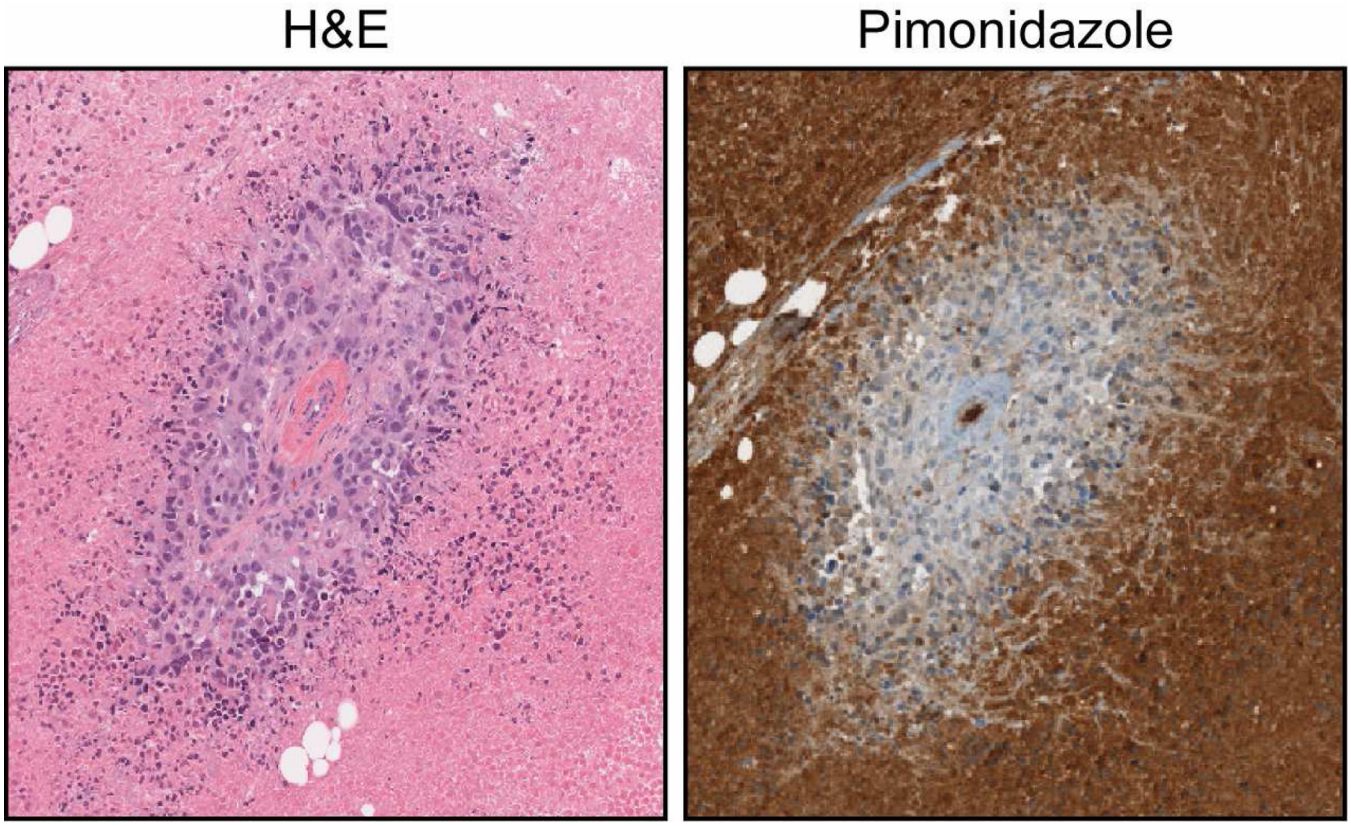

Figure 1. Tumor Microenvironment

This is an immunohistochemical example of intratumoral diffusion limited hypoxia of MDA-MB-231 mammary fat pad tumors using pimonidazole to detect hypoxic tissue. Pimonidazole is a nitromidazole that binds to thiol groups at oxygen levels below 1%. The H&E stain identifies a vascular cross section surrounded by a population of well-oxygenated cells. Diffusion limited hypoxia (pimonidazole stain) surrounding patent vasculature is common in solid tumors where tumor growth extends beyond the oxygen diffusion limit (~200 μM). Due to significant changes in metabolism, hypoxic regions (pimonidazole positive) are most likely acidic generating an acid-outside pH gradient.

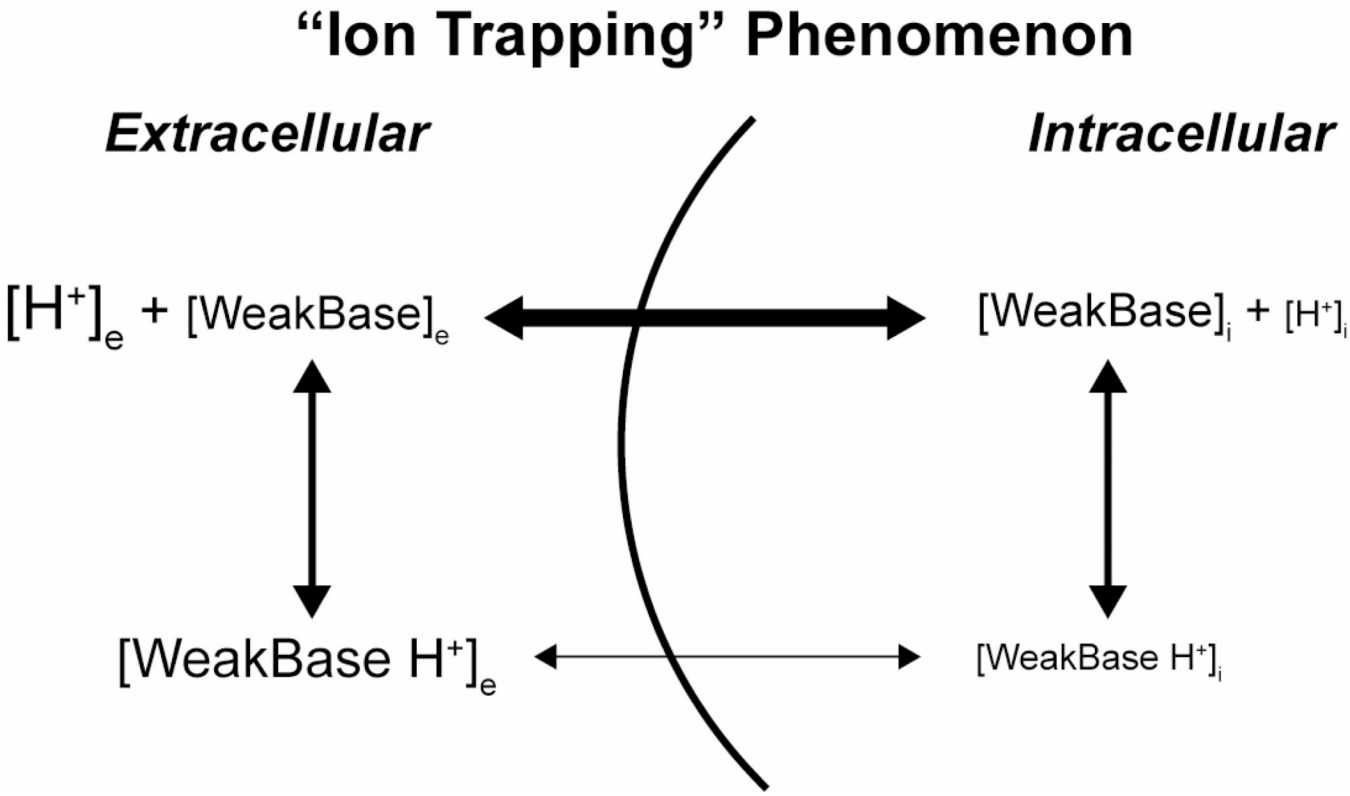

Figure 2. "Ion Trapping" phenomenon

This example assumes the extracellular H^+ concentration is greater than the intracellular H^+ concentration (i.e. pHe < pHi). Uncharged ionizable weak bases such as doxorubicin freely permeate membranes [WeakBase]. However, in acidic solutions, weak bases are ionized becoming positively charged protonated species [WeakBase H^+] reducing cell permeability. Therefore, positively charged weak bases become trapped in extracellular compartments reducing cellular uptake and efficacy. Weak acids tend to concentrate in more alkaline environments such as intracellular compartments. (Adapted from Raghunand and Gillies, Drug. Resist Update [3])

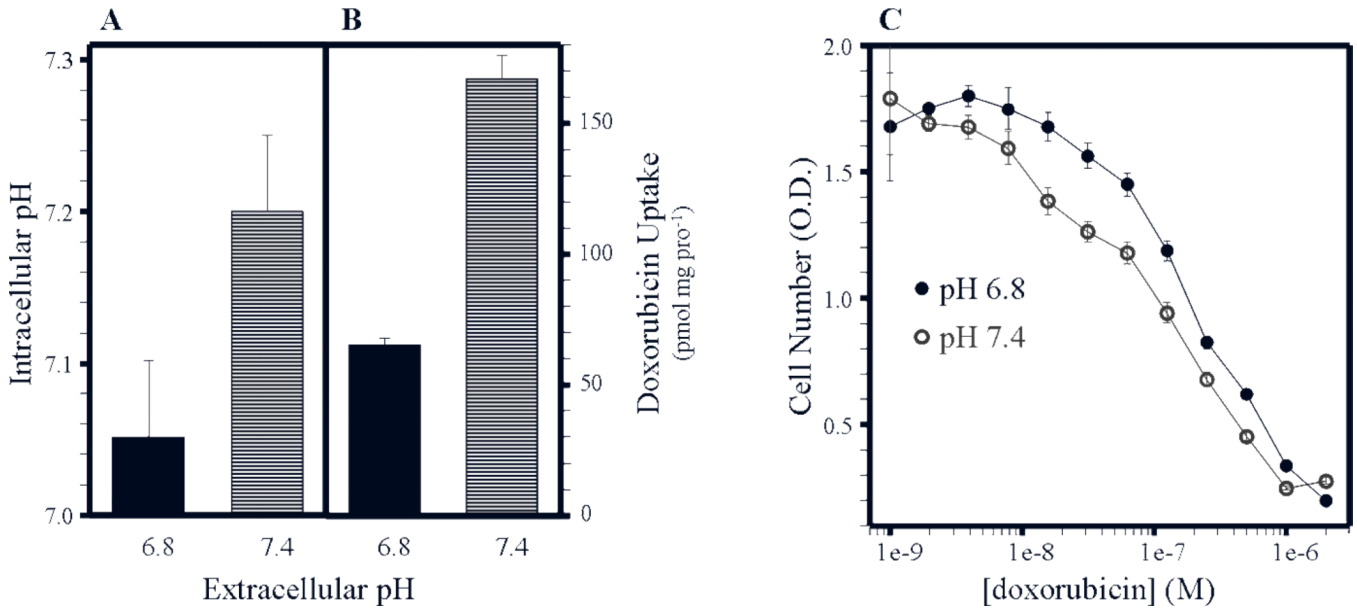

Figure 3. Increased doxorubicin uptake and efficacy under alkaline conditions

(A) Intracellular pH measurements and **(B)** doxorubicin uptake were determined as a function of extracellular medium pH in MCF-7 cells. **(C)** The effect of doxorubicin toxicity on MCF-7 cells *in-vitro* as a function of extracellular medium pH. (Adapted from Mahoney et al., Biochemical Pharmacology [25])

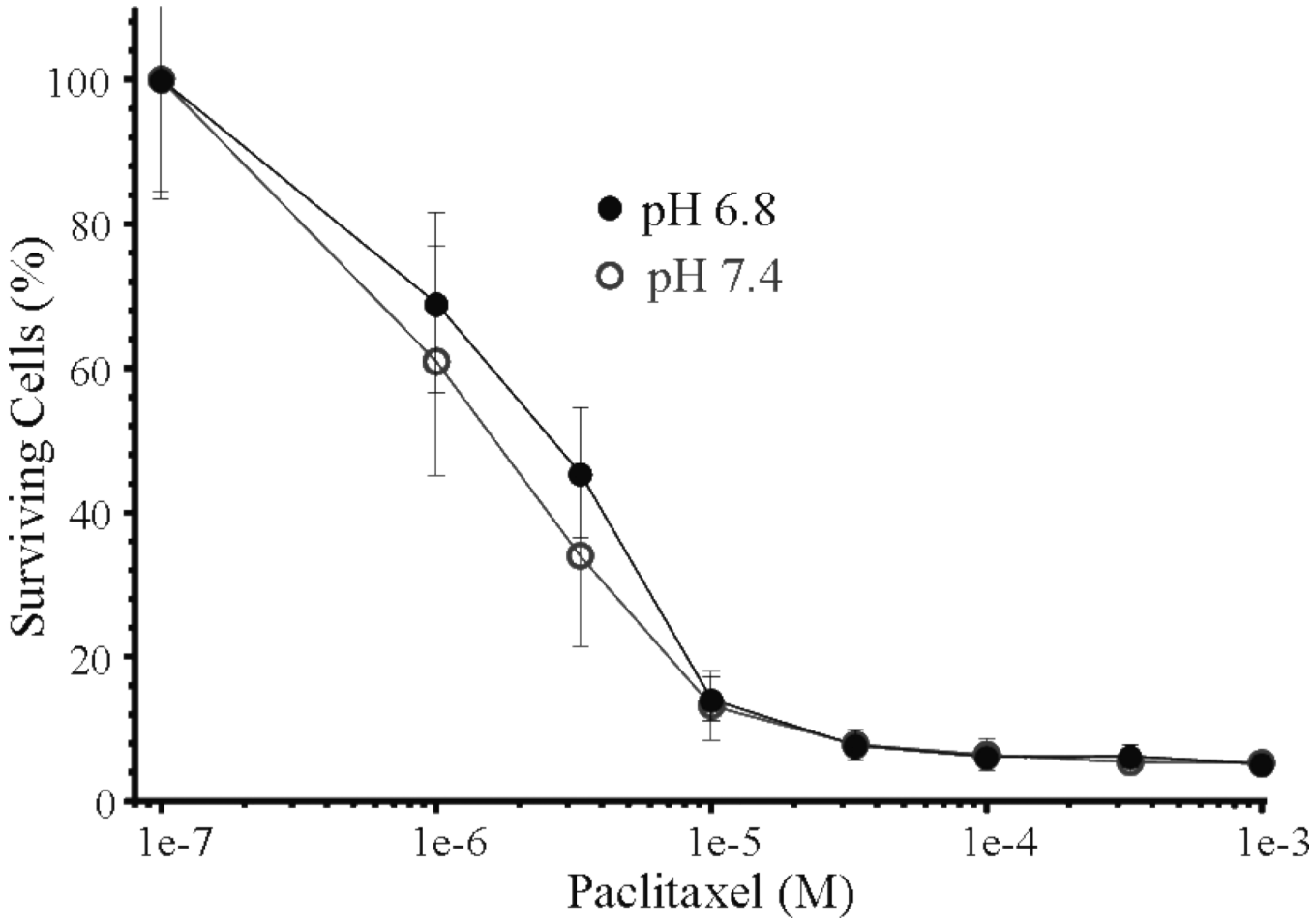

Figure 4. Extracellular pH has no effect on paclitaxel cytotoxicity

The effect of taxol cytotoxicity as a function of extracellular medium pH (Adapted from Raghunand et al. Br. J Cancer [26])

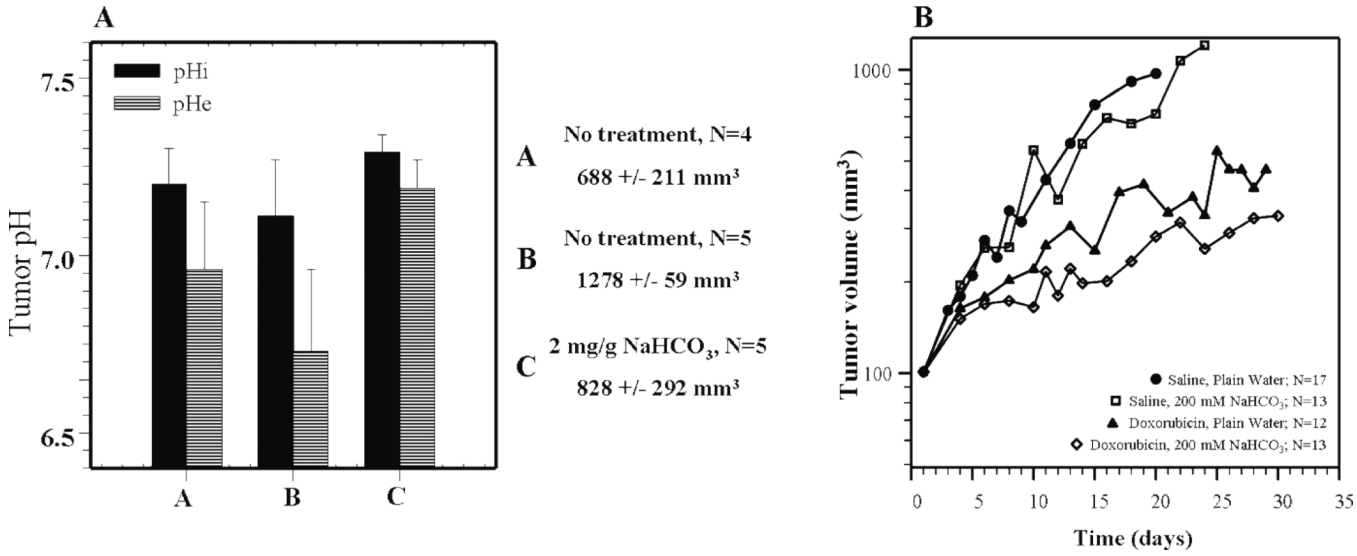

Figure 5. Sodium bicarbonate significantly increases extracellular pH of tumors *in-vivo*

(A) Intracellular pH (pHi) and extracellular pH (pHe) measurements of untreated MCF-7 tumors (A and B) of varying sizes. Notice that pHe is acidic irrespective of tumor size. Administration of sodium bicarbonate significantly alkalinizes the extracellular pH. Tumor pHe and pHi were measured by ^{31}P MRS. **(C)** *In-vivo* MCF-7 tumor volume measurements from mice either treated with 200 mM sodium bicarbonate (cyan), 2.0 mg/kg doxorubicin (red), or co-administration of 200 mM sodium bicarbonate and 2.0 mg/kg doxorubicin (purple). Administration of doxorubicin alone reduced tumor volume, but a greater reduction of tumor size was observed with co-administration of sodium bicarbonate. (Adapted from Raghunand et al. Br. J Cancer [33])

Table 1

Summary of weak base and weak acid chemotherapeutic pKa values [25] and LD50 against MCF-7 cells cultured at a pHe of 6.8 and 7.4 [25, 26].

	pKa	LD_{50} pHe 6.8	LD_{50} pHe 7.4
Weak Bases			
Doxorubicin	8.30	312 ± 29 (nM)	176 ± 33 (nM)
Daunorubicin	8.30	384 ± 61 (nM)	158 ± 37 (nM)
Mitoxantrone	7.6 – 8.2	703 ± 62 (nM)	262 ± 46 (nM)
Weak Acids			
Chlorambucil	5.8	14.3 ± 3 (μM)	22 ± 4 (μM)
5-Fluorouracil	7.6	29 ± 13 (μM)	27 ± 8 (μM)