Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 66 (2003) 1207–1218
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Tumor acidity, ion trapping and chemotherapeutics I. Acid pH affects the distribution of chemotherapeutic agents *in vitro*

Brent P. Mahoney, Natarajan Raghunand*, Brenda Baggett, Robert J. Gillies

Department of Biochemistry and Cancer Biology Program, Arizona Cancer Center, University of Arizona Health Sciences Center, Tucson, AZ 85724-5024, USA

Received 24 January 2003; accepted 13 June 2003

Abstract

Resistance to anti-cancer chemotherapies often leads to regional failure, and can be caused by biochemical and/or physiological mechanisms. Biochemical mechanisms include the overexpression of resistance-conferring proteins. In contrast, physiological resistance involves the tumor microenvironment, and can be caused by poor perfusion, hypoxia and/or acidity. This communication investigates the role of tumor acidity in resistance to a panel of chemotherapeutic agents commonly used against breast cancer, such as anthracyclines, taxanes, anti-metabolites and alkylating agents. The effects of pH on the cytotoxicity of these agents were determined, and ion trapping was confirmed by monitoring the effect of pH on the cellular uptake of radiolabeled anthracyclines. Furthermore, pH-dependent cytotoxicity and uptake were compared between parental drug sensitive MCF-7 cells and variants overexpressing p-glycoprotein (MDR-1) and Breast Cancer Resistance Protein. These data indicate that the magnitude of physiological resistance from pH-dependent ion trapping is comparable to biochemical resistance caused by overexpression of drug efflux pumps. Hence, microenvironment-based ion trapping is a significant barrier to anthracycline-based chemotherapy and can itself be a therapeutic target to enhance the efficacy of existing chemotherapies.

Keywords: Multidrug resistance; Acid–base; Weak acid; Weak base; Chemotherapy

1. Introduction

The NCI estimates that 182,800 new cases will occur and about 41,200 deaths will result from breast cancer in the United States this year. The 5-year relative survival rate for localized breast cancer is approximately 96%. However, if the cancer has spread regionally, the 5-year survival rate drops to 77%, and for women with distant metastases the rate drops further to 21%. For localized disease, standard treatment includes surgical resection and removal of regional lymph nodes, often in combination with radiotherapy [1–4]. For disseminated disease, chemotherapy and/or hormone therapy are widely used [3,5]. Mortality often results from distant metastases and their failure to respond to therapy. Resistance to therapy can involve both biochemical and microenvironmental factors. *Biochemical resistance* includes up-regulation of drug efflux and metabolizing pathways such as MDR-1 (p-glycoprotein) or Breast Cancer Resistance Protein (BCRP) [6]. *Physiological resistance*, on the other hand, is a consequence of poor perfusion, hypoxia and/or acidity in the tumor microenvironment [7,8].

Human tumors have long been considered acidic based on microelectrode measurements [9]. Non-invasive measurements of tumor pH in animal tumor models by magnetic resonance spectroscopy (MRS) using 3-aminopropylphosphonate and ZK-150471 reveal that it is the extracellular pH (pHe) in tumors which is acidic while the intracellular pH (pHi) is neutral-to-alkaline [10,11]. Hence, tumors contain regions with large, acid-outside plasmalemmal pH gradients, while normal tissues generally have alkaline-outside pH gradients [12].^{1}H magnetic resonance spectroscopic imaging (MRSI) of a pH-sensitive imidazole, IEPA, has shown that tumor pHe is locally variable, with a range of greater than $\pm$0.3 pH unit over 8–10 mm [13–15]. The causes for the acidic pH in tumors are not well-defined, but may include deficiencies in tumor perfusion, metabolic

* Corresponding author. Tel.: +1-520-626-6041; fax: +1-520-626-5051.
E-mail address: raghunan@U.Arizona.Edu (N. Raghunand).

Abbreviations: 3-APP, 3-aminopropylphosphonate; 5-FU, 5-fluorouracil; BCRP, Breast Cancer Resistance Protein; FBS, fetal bovine serum; LRP, lung resistance protein; MRS, magnetic resonance spectroscopy; MRSI, magnetic resonance spectroscopic imaging; pHe, extracellular pH; pHi, intracellular pH; SCID, severe combined immunodeficient.

0006-2952/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0006-2952(03)00467-2

abnormalities associated with transformation, and an increased capacity for transmembrane pH regulation. In human tumors, including breast cancers, pHe has only been measured by microelectrodes and these data are in concordance with the values observed in animal systems, i.e. that the pHe is significantly acidic, in the range from 6.2 to 7.0 [9]. *In vivo* ^{31}P MRS of human tumors reveal that the pHi is neutral to alkaline, in the range 7.0–7.4, as measured by endogenous inorganic phosphate [16]. Hence, the acid-outside pH gradient observed in animal tumors is a good model for many human tumors, including carcinoma of the breast.

The low pHe in tumors can reduce the effectiveness of some chemotherapies. A number of mechanisms have been postulated to be responsible for this effect, including reduction in cycling cell fraction [17], selection for apoptosis-resistant phenotypes [18], and direct effect of ion gradients on drug distribution or *ion trapping* ([8], and references therein). The ion trapping model predicts that weakly basic chemotherapeutic drugs such as anthracyclines, anthraquinones and vinca alkaloids will concentrate in more acidic compartments. The acid pHe of tumors will therefore effectively hinder weakly basic drugs from reaching their intracellular target, thereby reducing cytotoxicity. The ion trapping model also predicts that the acid pHe of tumors will improve uptake of weak acids such as chlorambucil into the relatively neutral intracellular space. Clinically, breast carcinoma is commonly treated with doxorubicin, paclitaxel, cyclophosphamide, and 5-flourouracil [19,20]. Vincristine, vinblastine, mitoxantrone, daunorubicin and chlorambucil are also used to various degrees [19–21]. With the exception of paclitaxel, all of these clinically useful drugs are ionizable and hence their distribution will be affected by the microenvironmental pHe.

The present study investigates the pH-dependent behavior of these drugs *in vitro*. While some of these drugs have been investigated previously, others have not, and no studies exist wherein this panel of anti-breast cancer drugs was investigated by the same methods. For this work, the well-characterized MCF-7/s cell line was selected to model a locally invasive breast carcinoma *in vitro*. Two drug-resistant variants of this cell line, MCF-7/D40 and MCF-7/mitox, were also used. These cell lines were created by exposure to increasing concentrations of doxorubicin or mitoxantrone, respectively, over many months [22]. MCF-7/D40 cells are p-glycoprotein positive and exhibit a 40-fold greater IC_{50} of doxorubicin compared to the parental MCF-7/s cells. MCF-7/mitox cells overexpress the ATP-binding-cassette containing multi-drug transporter, BCRP [23]. These cells tolerate an 80-fold greater mitoxantrone concentration than do the parental MCF-7/s cells.

Results of the present study indicate that ion trapping can significantly modulate chemotherapy. The activities of the weak bases mitoxantrone, doxorubicin and daunorubicin are all inhibited by low pHe. Conversely, the actions of the weak acids cyclophosphamide, 5-fluorouracil and chlorambucil are all enhanced by low pHe. In the case of the anthracyclines these effects are mirrored in altered drug accumulation, confirming the ion trapping mechanism. Furthermore, the magnitude of this physiological resistance is comparable to the better-characterized biochemical resistance induced by overexpression of MDR-1 or BCRP.

2. Description of drugs

2.1. Doxorubicin

Doxorubicin is an antibiotic isolated from *Streptomyces peucetius var. caesius* [24]. It consists of a weakly basic amino sugar, daunosamine, linked *via* a glycosidic bond to the red-pigmented tetracyclic moiety, adriamycinone. Doxorubicin (Adriamycin®) has been used clinically against cancer since 1969, and displays an extremely broad spectrum of activity both in experimental tumor models and in human malignancy. The anthracycline portion of doxorubicin intercalates between DNA base pairs, and the sugar moiety is absolutely required for DNA intercalation and anti-tumor activity [25]. Intercalation of doxorubicin into cellular DNA results in inhibition of both DNA-directed DNA and RNA synthesis [26]. Although the cytotoxicity of doxorubicin is not cell-cycle specific, it does have increased activity during late S phase of the cell cycle [27]. Doxorubicin is also a topoisomerase type II inhibitor. Anthracycline cytotoxicity is largely dependent on inhibition of topoisomerase type II activity, resulting in strand breaks (Fig. 1) [28].

Doxorubicin has a primary amine with a basic pK_a, and has been previously shown to undergo ion trapping and sequestration into acidic vesicles within the cytoplasm that have been associated with drug resistance ([8], and references therein). Although this sequestration into acidic vesicles may be the result of passive ion trapping, it is also possible that as-yet unidentified active proton-dependent transporters are responsible [6,29,30]. A number of agents have been used to inhibit vesicle acidification, causing release of doxorubicin into the cytoplasm and subsequent enhancement of cytotoxicity [31,32].

Although the ability of doxorubicin to intercalate into DNA is pH independent, topoisomerase type II activity increases with alkaline pHi [28]. Cytosolic pH can also affect the electrostatic interactions of doxorubicin with proteins and lipids, which constitute the third localization compartment for doxorubicin *in vivo* [33,34]. Doxorubicin is a substrate for multiple drug export proteins including p-glycoprotein (MDR-1) and the lung resistance protein (LRP), which may or may not be affected by pH [35].

2.2. Daunorubicin

Daunomycin (Rubidomycin®, Cerubidine®) is an anthracycline-derived antibiotic that was first isolated from a unique strain of *Streptomyces*. It consists of a weakly

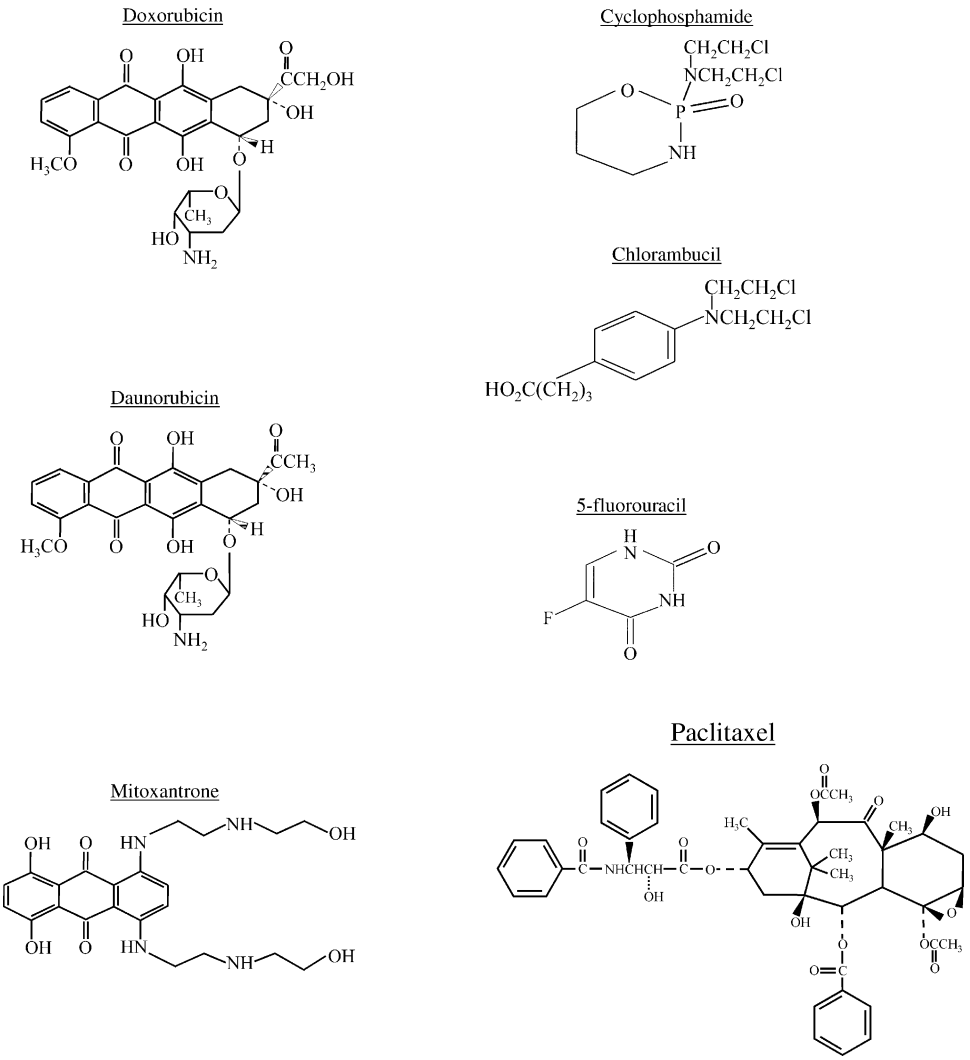

Fig. 1. Drug molecules used in this study.

basic amino sugar, daunosamine, linked *via* a glycosidic bond to the red-pigmented tetracyclic moiety daunomycinone. Daunorubicin is structurally identical to doxorubicin, except for the presence of hydrogen in place of a hydroxyl group at the 14-carbon position in the anthracycline ring. Clinically, daunorubicin has a much more limited spectrum of anti-tumor activity than doxorubicin and is used primarily in the treatment of leukemias. Daunorubicin has the same primary amine with the same pK_a as doxorubicin, and has been demonstrated to have increased anti-tumor activity as well as increased intracellular accumulation at alkaline pHe [36]. Like doxorubicin, daunorubicin is sequestered into acidic intracellular organelles in a pH dependent manner [37]. Daunorubicin is also a substrate for p-glycoprotein and LRP [38].

2.3. Mitoxantrone

Mitoxantrone (Novantrone®) is a synthetically derived anthracycline analog and member of the anthracenedione family. It contains a planar polycyclic aromatic ring structure, as in doxorubicin, but lacks a sugar moiety. Mitoxantrone has two polar side-chains attached to the aromatic rings which render the molecule water-soluble [39]. Clinically, mitoxantrone is used primarily in the treatment of leukemias, lymphomas and advanced breast cancers [40]. Mitoxantrone is a DNA intercalating agent and topoisomerase type II inhibitor that causes DNA strand breaks [41]. Like anthracyclines, mitoxantrone is cytotoxic to cells throughout the cell cycle but is more potent to cells in late S phase [39]. Mitoxantrone has been reported to undergo ion trapping similar to anthracyclines [42,43]. Mitoxantrone has also been reported to be more toxic at alkaline pH independent of intracellular drug concentration, and this is in part mediated by cell cycle alterations [44]. At alkaline pH mitoxantrone can complex with iron or copper cations, resulting in the generation of free radicals [45]. Mitoxantrone is readily bound by proteins and lipids in the body in a pH-dependent manner [46]. As in the case of anthracyclines, mitoxantrone's anti-tumor activity is dependent on topoisomerase type II activity, which has an alkaline pH optimum [28].

2.4. Paclitaxel

Paclitaxel (Taxol®) is an extract from the Pacific Yew, *Taxus brevifolia* [47]. Paclitaxel consists of an eight-member taxane ring with a four-member oxetane ring and a carboxyl side-chain located at the C-13 position. Paclitaxel is a mitotic spindle poison that stabilizes microtubules and inhibits their depolymerization [48]. Paclitaxel has been reported to have significant activity against ovarian carcinoma, breast carcinoma, small cell lung carcinoma, and Kaposi's sarcoma [49]. Paclitaxel is highly lipophilic, and does not have any ionizable groups with pK_a values in the physiological range. However, pH-dependent alterations in the cell cycle can alter the cytotoxicity of taxol [44]. In tissue extracts alkaline pH favors the assembly of microtubules [50], and this may indirectly affect the anti-tumor activity of the taxoids. However, since the intracellular concentrations of paclitaxel are not expected to be affected by changes in pH, it is a useful control in the current study.

2.5. Cyclophosphamide

Cyclophosphamide (Cytoxan®, Neosar®) is a rationally designed nitrogen mustard analog first synthesized in 1958 [51]. Cyclophosphamide is a pro-drug that requires enzymatic activation to a phosphoramide mustard for biological activity. *In vivo*, cyclophosphamide is activated both in the liver by mixed function oxidases, and in tissues by various constitutive and inducible p-450 enzymes which have been shown to be overexpressed in tumors [52]. Cyclophosphamide is a bi-functional alkylating agent, and causes both single-stranded DNA breaks and DNA cross-linkages that are cytotoxic to dividing cells. It is somewhat more toxic to cells in S phase of the cell cycle. The *in vivo* anti-tumor activity of cyclophosphamide inversely correlates with aldehyde dehydrogenase activities [53].

Cyclophosphamide and its 4-hydroxylated metabolite enter the cell *via* both passive diffusion and active transport, and may therefore be substrates for ion trapping. However, the influence of pH on cyclophosphamide is complex because all of the metabolic activating and inactivating processes are influenced by pH. Cyclophosphamide metabolites have a 200-fold enhancement in cytotoxicity at an pHe of 6.2 *in vitro*, which is similar to other alkylating anti-tumor agents such as chlorambucil [54]. Lower pH accelerates the rate of conversion of 4-hydroxycyclophosphamide into its active carbocation [55]. Acidic pH also enhances the cytotoxicity of the four most prevalent urine metabolites of cyclophosphamide. Bicarbonate inhibits these reactions directly by facilitating the metabolic conversion of the more mutagenic metabolites into less mutagenic ones [56]. These metabolites of cyclophosphamide have pK_a values in the physiological range, and thus pH can be expected to influence their membrane permeabilities.

2.6. Chlorambucil

Chlorambucil (Leukeran®) is a nitrogen mustard analog similar to cyclophosphamide that is primarily used to treat leukemias [57]. Cellular uptake of chlorambucil occurs by simple diffusion, and it is extensively metabolized. Beta-oxidation of the butyric side chain in chlorambucil results in the formation of its major metabolite, phenyl acetic mustard, which retains anti-tumor activity [58]. Like other clinically useful nitrogen mustards, chlorambucil is bi-functional and can cross-link DNA.

Chlorambucil is not a substrate for p-glycoprotein. Resistance to chlorambucil has been associated with increased glutathione and glutathione-*S*-transferase levels [59]. Additionally, up-regulation of DNA excision repair enzymes is also associated with chlorambucil resistance, as are mutations in p53 [60]. Chlorambucil is a weak acid with a pK_a of 5.8, and is thus expected to be a substrate for ion trapping [61,62]. A number of different mechanisms have been successfully used to increase tumor acidity and subsequently increase chlorambucil anti-tumor activity [63,64]. Additionally, alterations of pH within the physiological range increase the rate of mono-alkalinization but do not affect the formation of DNA-cross-linkages [65].

2.7. 5-Flourouracil

5-Flourouracil (5-FU, Adrucil®) is a fluorinated pyrimidine antagonist. 5-FU is clinically used in the treatment of GI cancers, breast cancer, skin cancer and cervical cancer [66]. 5-FU is an inactive prodrug with several active metabolites. A major metabolic product is 5-flouro-2′-deoxyuridine-5′-monophosphate, an inhibitor of thymidylate synthase. Alternatively, 5-FU can be incorporated into the DNA and RNA of tumor cells, resulting in chain termination or base mismatch.

Resistance to 5-FU is often mediated by deficiencies in both the quantity and quality of metabolizing enzymes [67]. 5-FU is a weak acid with a pK_a of 7.6, due to the electron-withdrawing properties of fluorine [68]. In DNA, ionization of N-3 causes incorrect base pairing with guanine, resulting in a high frequency of mismatch [69]. 5-FU may also participate in pH dependent ion trapping. However, it is unclear how 5-FU is taken up by cells. Simple diffusion, passive diffusion and active transport have all been reported as transport mechanisms. Acidic pHe has been shown to result in elevated intracellular concentrations of 5-FU *in vitro* and *in vivo* [70,71].

3. Methods

3.1. Cell and tumor growth

MCF-7/s, MCF-7/D40 and MCF-7/mitox cells were cultured in 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/s 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 (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.

3.2. Magnetic resonance spectroscopy (MRS)

Mice were anaesthetized with a combination of ketamine (72 mg kg^{-1}), xylazine (6 mg kg^{-1}), and acepromazine (6 mg kg^{-1}) and immobilized on a home-built probe with a solenoid coil tunable to ^1H or ^{31}P. 3-Aminopropylphosphonate (3-APP, 0.15–0.3 mL, 128 mg mL^{-1}, pH 7.4) could be injected into the mouse during the experiment *via* an intraperitoneal catheter. Volume-selective PRESS or ISIS ^{31}P spectra were acquired at 4.7 T on a Bruker Biospec as previously described [10,72].

3.3. Measurement of intracellular pH in vitro

pHi was measured *in vitro* using the fluorescent dye SNARF-1, as previously described [30]. Briefly, cells were grown onto 9 mm × 22 mm glass cover slips, washed three times with buffer A (1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 5.4 mM KCl, 0.44 mM KH_2PO_4, 110 mM NaCl, 0.35 mM NaH_2PO_4, 5 mM glucose, 2 mM glutamine, 5 mM HEPES, 5 mM MES, 10 mM $NaHCO_3$) at a pH of 7.15 at 37° and incubated for 30 min at 37° in a 5% carbon dioxide atmosphere with 3 mL of buffer A containing 10 μM acetoxymethylester SNARF-1 (Molecular Probes). This was followed by a second incubation in buffer A for 45 min to allow for complete hydrolysis of the dye. Coverslips were then placed in a holder/perfusion device and inserted into a fluorometer cuvette and fluorescence measurements were acquired at an excitation of 534 nm with the emission sequentially sampled at 584 and 644 nm in an SLM8000C (SLM). The ratio (R) of fluorescence intensities of emissions at 584 and 644 nm was converted to pHi values using the equation: $pH = 7.38 + \log_{10}0.822 + \log_{10}[(R - 0.458)/(1.928 - R)]$. Data are presented as mean ± SEM of six independent measurements.

3.4. Drug uptake

Cells were grown to confluence in 6-well plates, at which time the cells were incubated in DMEM/F12 containing 20 mM HEPES, 20 mM MES, 10% FBS and 0.208 μCi per well of ^{14}C doxorubicin (Amersham) for 30 min at 37° in a 5% carbon dioxide atmosphere at pH of 6.8 and 7.4, respectively. After the incubation, the plates were placed on ice and washed five times with ice-cold HBSS, followed by extraction with 1.0 mL of 0.1 N NaOH for 1 hr at 4°. Samples were divided into equal aliquots for determination of protein content using the Bradford assay (www.bio-rad.com) and radioactivity using liquid scintillation counting.

3.5. Drug cytotoxicity

Cytotoxicity was determined as described previously [73]. Briefly, cells were grown to log phase in 96-well plates and the medium was exchanged for one at either pH 6.8 or 7.4 containing the indicated concentrations of drug. Medium was buffered using non-volatile buffers (10 mM MES, 20 mM HEPES, and 10 mM tricine) in combination with bicarbonate concentrations that were adjusted to be in equilibrium with 5% ambient carbon dioxide. Twenty-four hours later the growth medium was replaced with drug-free growth medium at pH 7.1, and cells were allowed to grow the subsequent 72 hr, after which time they were fixed and stained with 0.1% crystal violet. The dye was then solubilized with acetic acid, and absorbance was used for the determination of cell number.

3.6. Calculation of theoretical drug distributions

Cytoplasmic-extracellular drug ratios were calculated at the steady state as described previously [6]. Briefly, the ratio of protonated to deprotonated drug was calculated from the Henderson–Hasselbach equation in both intracellular and extracellular compartments for a given pHi, pHe, and pK_a of the drug. The concentration of the uncharged species was set to 1.0 on both sides of the membrane and the total concentration was calculated as the sum of charged + uncharged. Data were expressed as a ratio of intracellular to extracellular concentrations. Enhancement was calculated as the ratio at high pH relative to ratio at low pH.

4. Results

Figure 2 illustrates a ^{31}P MR spectrum of an MCF-7/s tumor growing in the mammary fat pad of a SCID mouse. The pHe of MCF-7/s xenografts was measured from the chemical shift of exogenously administered 3-APP, and the pHi was measured from the chemical shift of endogenous inorganic phosphate. Both pHe and pHi of MCF-7/s xenografts are tumor size-dependent, but the pHi − pHe difference is not significantly correlated with tumor size [10]. For tumors between 150 and 1200 mm^3, tumor pHe varied between 6.56 and 7.35 and tumor pHi varied between 7.01 and 7.39, while the pHi − pHe difference was 0.19 ± 0.17 (N = 13). Measurement of tumor pH has been recently reviewed [7] and these numbers are entirely consistent with measurements made by other groups in other tumor sys-

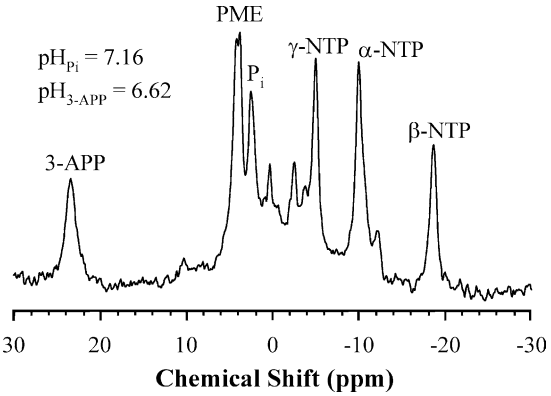

Fig. 2. ^{31}P MR spectrum of a 1020 mm^3 MCF-7/s tumor xenograft. pHe is calculated from the chemical shift of 3-aminopropylphosphonate (3-APP) and pHi from the chemical shift of inorganic phosphate (P_i). PME: phosphomonoesters; NTP: nucleoside triphosphates.

tems. As discussed previously, these results are also consistent with measurements made in human tumors, including breast cancers [9,16].

The pH values measured *in vivo* can be recapitulated *in vitro*, as shown in Fig. 3. In this figure the pHi of MCF-7/s cells is shown at pHe values of 6.8 and 7.4. Note that, at a medium pHe of 7.4, the pHi is 7.2, providing an alkaline-outside chemical gradient for protons. Conversely, at a medium pHe of 6.8 (similar to that found in tumors) the pHi of 7.05 provides an acid-outside chemical gradient. Models have been generated to predict the effect of incubation pH on drug uptake, if ion trapping were the dominant mechanism mediating uptake [6]. An acid-outside environment will tend to drive the uptake of weak acids into cells, where they will ionize and be trapped because of a lower permeability for the charged species. Conversely, an alkaline outside environment will tend to exclude weak acids from cells. The opposites are true for weak bases.

Many chemotherapeutic drugs used in the treatment of breast cancers are weak acids or bases. These and other

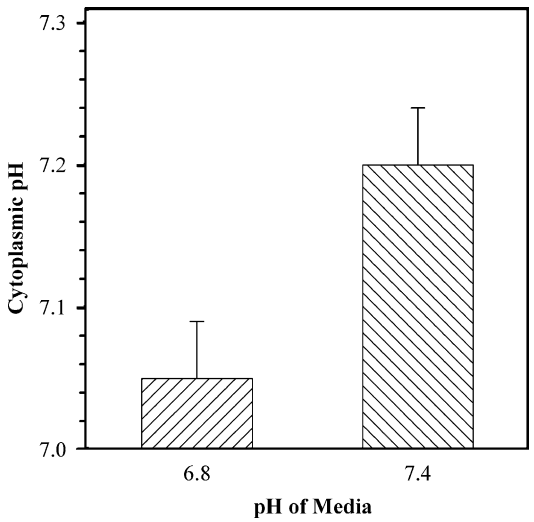

Fig. 3. Cytosolic pH (pHi) of MCF-7/s cells as a function of medium pH (pHe).

Table 1
Drugs used in this study

Drug	Mechanism	Ionization behavior
Daunorubicin	Anthracycline: Topo II inhibitor	Weak base (pK_a = 8.3)
Doxorubicin	Anthracycline: Topo II inhibitor	Weak base (pK_a = 8.3)
Mitoxantrone	Anthraquinone: Topo II inhibitor	Weak base (pK_a = 8.3)
Paclitaxel	Taxane: stabilizes microtubules	Zwitterionic
Chlorambucil	DNA alkylating agent	Weak acid (pK_a = 5.8)
Cyclophosphamide	DNA alkylating agent pro-drug	Weak acid (pK_a = 6.0)
5-Fluorouracil	Antimetabolite pro-drug	Weak acid (pK_a = 7.6)

drugs used in this study are listed in Table 1, and include anthracyclines and an anthraquinone (doxorubicin, daunorubicin, and mitoxantrone), cyclophosphamide, chlorambucil, 5-fluorouracil and paclitaxel. These were chosen because they are commonly used in the treatment of breast cancer and they represent a variety of mechanisms and ionization behaviors. Anthracyclines and vinca alkaloids are weak bases; chlorambucil, cyclophosphamide and 5-fluorouracil are weak acids; and paclitaxel is non-ionizable in the physiological pH range. The effects of pH on the activity of cyclophosphamide and 5-fluorouracil are complex, presumably because they are pro-drugs, and the conversion reactions exhibit pH-dependency [74].

The predicted behaviors for two representative drugs under our experimental conditions are shown in Fig. 4. Figure 4A shows the predicted effects of pHe on the distribution of mitoxantrone at two different pHi values, 7.05 and 7.20. The pHi in MCF-7/s cells is 7.2 at a pHe of 7.4 (Figs. 2 and 3), for which the predicted intracellular-to-extracellular drug distribution ratio is approximately 1.8, shown as a filled circle in Fig. 4A. At a pHe of 6.8, the pHi in MCF-7/s cells is 7.05 (Figs. 2 and 3), for which the predicted drug distribution ratio is approximately 0.6, shown as an open circle in Fig. 4A. Hence, raising the pHe would be expected to enhance mitoxantrone accumulation into cells 3-fold, assuming constant extracellular drug concentration. The converse is true for weak acids such as chlorambucil, as shown in Fig. 4B. In this case, the cytoplasmic/interstitium drug ratio is approximately 1.8 at a pHe of 6.8, and approximately 0.7 at a pHe of 7.4. Hence, theoretical distribution of chlorambucil into tumor cells is expected to be enhanced approximately 3-fold by the acid-outside plasmalemmal pH gradient.

To test these predictions, MCF-7/s cells were exposed to chemotherapeutic drugs at different pHe values, as described in Section 3. Since the pHe was altered for periods up to 24 hr, an important control was the effect of this treatment on the growth of cells in the absence of drug. Under conditions of this study, there were no differences in the final cell number following treatment of the

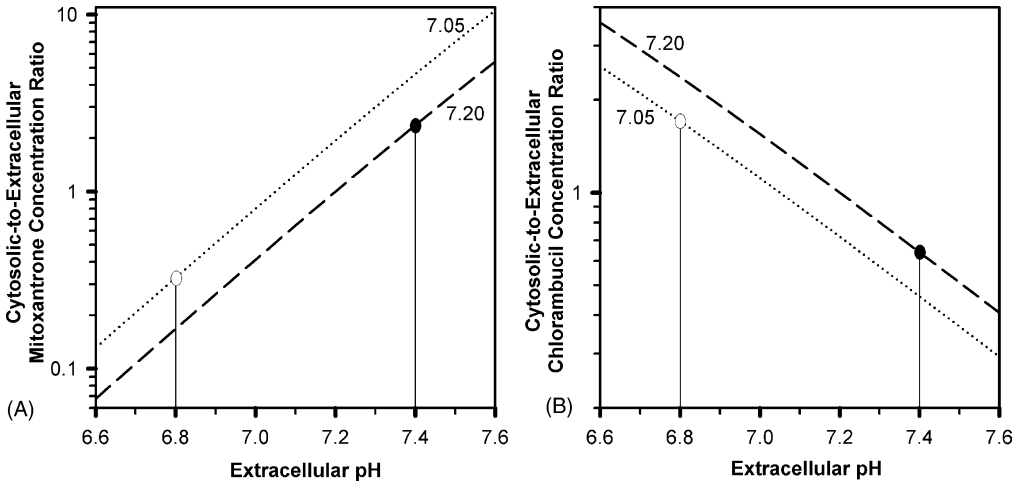

Fig. 4. Predicted transplasmalemmal partitioning of (A) mitoxantrone, and (B) chlorambucil.

cells for 24 hr at pH 6.8 or 7.4 (Fig. 5). An example of the effects of pHe on cell survival following drug treatment is depicted in Fig. 6, which shows the effect of increasing mitoxantrone concentration on cell number at an incubation pH of 6.8 or 7.4. These data show that the IC_{50} decreases from 13×10^{-7} M at pHe 6.8 to 3.4×10^{-7} M at pHe 7.4. These and results from various drugs are presented in Table 2A. Note that weak acids had consistently lower IC_{50} values at the lower pHe, that weak bases had consistently lower IC_{50} values at the higher pHe, and that the IC_{50} values of paclitaxel were not affected by pH. These results indicate that there is a direct effect of pH on drug sensitivity. Furthermore, the effect of pH on drug sensitivity was not simply due to altered cell cycle kinetics at lower pH values.

This physiological resistance induced by low pHe on weak bases can be significant, even compared to biochemical resistance exhibited by p-glycoprotein or the BCRP. This is illustrated in the data from drug-resistant cells presented in Table 2B and C. As expected, at both pH values, the IC_{50} values for doxorubicin and mitoxantrone are considerably higher in the drug-resistant cells (Table 2B and C) compared to the parental MCF-7/s cells (Table 2A). Nonetheless, the IC_{50} values for both drugs are significantly lower at pHe 7.4, compared to pHe 6.8. The enhancement in cytotoxicity of mitoxantrone resulting from a raising of pH from 6.8 to 7.4 was 3.8-, 7.8- and 8-fold for the MCF-7/s, MCF-7/D40 and MCF-7/mitox cells, respectively. The enhancements for doxorubicin were consistently lower, at 2.7-, 5.8- and 2.4-fold, respectively. Notably, for a given drug, the degree of enhancement caused by raising pHe was similar across all cell lines. This can be interpreted to indicate that the *physiological* resistance imposed by ion trapping can significantly compound a *biochemical* resistance caused by overexpression of drug resistance proteins.

Although the data to this point suggest that changes in the pH gradient are driving altered uptake of drug, it could also be the case that the intracellular targets (e.g. topoi-

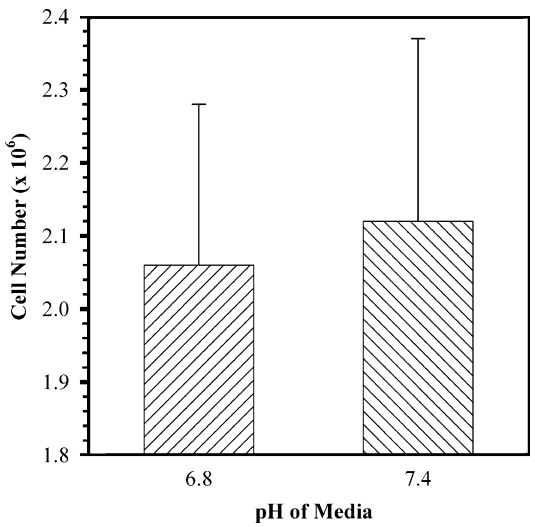

Fig. 5. MCF-7/s cell density following 24 hr exposure to medium at pH 6.8 or 7.4. No significant differences were found.

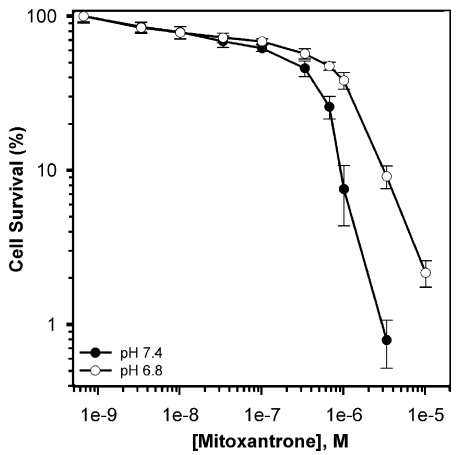

Fig. 6. A typical cytotoxicity curve showing enhanced cytotoxicity of mitoxantrone to MCF-7/s cells at higher pHe.

Table 2
pH-dependent cytotoxicity of drugs to MCF-7/s, MCF-7/D40 and MCF-7/mitox cells

Drug	Drug type	pK_a	IC_{50} (M) pH 7.4	IC_{50} (M) pH 6.8	*P*-value (*t*-test)
(A) MCF-7/s cells					
Mitoxantrone	Weak base	8.3	$3.4 \pm 0.6 \times 10^{-7}$	$13 \pm 9 \times 10^{-7}$	<0.002
Doxorubicin	Weak base	8.3	$9.6 \pm 4.9 \times 10^{-6}$	$26 \pm 14 \times 10^{-6}$	<0.03
Daunorubicin	Weak base	8.3	$17 \pm 1.4 \times 10^{-8}$	$32 \pm 6 \times 10^{-8}$	<0.0001
Paclitaxel	Zwitterionic	NA	$3.5 \pm 1.6 \times 10^{-9}$	$5.7 \pm 2.1 \times 10^{-9}$	<0.17
Cyclophosphamide	Weak acid	6.0	$66 \pm 28 \times 10^{-5}$	$16 \pm 9 \times 10^{-5}$	<0.02
5-Flourouracil	Weak acid	7.6	$28 \pm 9 \times 10^{-6}$	$13 \pm 7 \times 10^{-6}$	<0.004
Chlorambucil	Weak acid	5.8	$34 \pm 4.3 \times 10^{-5}$	$8 \pm 2.1 \times 10^{-5}$	<0.015
(B) MCF-7/D40 cells					
Doxorubicin	Weak base	8.3	$15 \pm 3 \times 10^{-6}$	$87 \pm 24 \times 10^{-6}$	<0.0001
Mitoxantrone	Weak base	8.3	$14 \pm 2 \times 10^{-6}$	$11 \pm 3 \times 10^{-5}$	<0.0001
Chlorambucil	Weak acid	5.8	$9.8 \pm 1.1 \times 10^{-5}$	$7.1 \pm 1.2 \times 10^{-5}$	<0.01
(C) MCF-7/mitox cells					
Doxorubicin	Weak base	8.3	$16 \pm 1 \times 10^{-7}$	$39 \pm 2 \times 10^{-7}$	<0.0003
Mitoxantrone	Weak base	8.3	$25 \pm 2 \times 10^{-6}$	$20 \pm 2 \times 10^{-5}$	<0.0003
Chlorambucil	Weak acid	5.8	$23 \pm 2 \times 10^{-6}$	$14 \pm 1 \times 10^{-6}$	<0.0001

somerase II) are more sensitive at a pHi of 7.2 (pHe = 7.4) compared to 7.05 (pHe = 6.8). Therefore, the accumulation of radiolabeled drugs was determined at both these pH values. Typical uptake curves are shown in Fig. 7, showing the time dependent accumulation of ^{14}C-labeled mitoxantrone and doxorubicin into MCF-7/s cells at pHe values of 6.8 and 7.4. There is clearly an increased accumulation at pH 7.4, compared to 6.8, indicating that the enhanced drug toxicity observed above was due to increased drug accumulation. Data for doxorubicin and mitoxantrone in all cell types are presented in Fig. 8, which show the uptake at pHe 6.8 and 7.4 for both drugs in all three cell lines. Note that the ratio of uptake at pHe 7.4 to pHe 6.8 is clearly greater than 1.0 in five out of six data sets, indicating an increased accumulation of drug at the higher pH values. Note also that, except for mitoxantrone accumulation in MCF-7/s cells, the ratio of enhanced uptake is not as high as the ratio of enhanced cytotoxicity (Cf Table 2).

5. Discussion

This work shows that, in a controlled *in vitro* breast carcinoma model system, the effect of pHe on anti-tumor chemotherapeutic drug response was largely predictable and consistent with an ion-trapping model. Moreover, the results obtained are consistent with other findings in the literature for all of the drugs tested. Thus, the weak bases mitoxantrone, daunorubicin and doxorubicin all showed enhanced cytotoxicity at high pHe values. Conversely, the weak acids chlorambucil, cyclophosphamide and 5-fluorouracil all showed enhanced cytotoxicity at low pHe values. This difference between weak acids and weak bases suggests that the effect of pHe is not simply an effect on cell cycle or DNA repair machinery. This is also supported by the observation that taxol toxicity showed no dependence on pHe. Hence, it is tempting to conclude that the effects of pHe on the actions of these drugs are

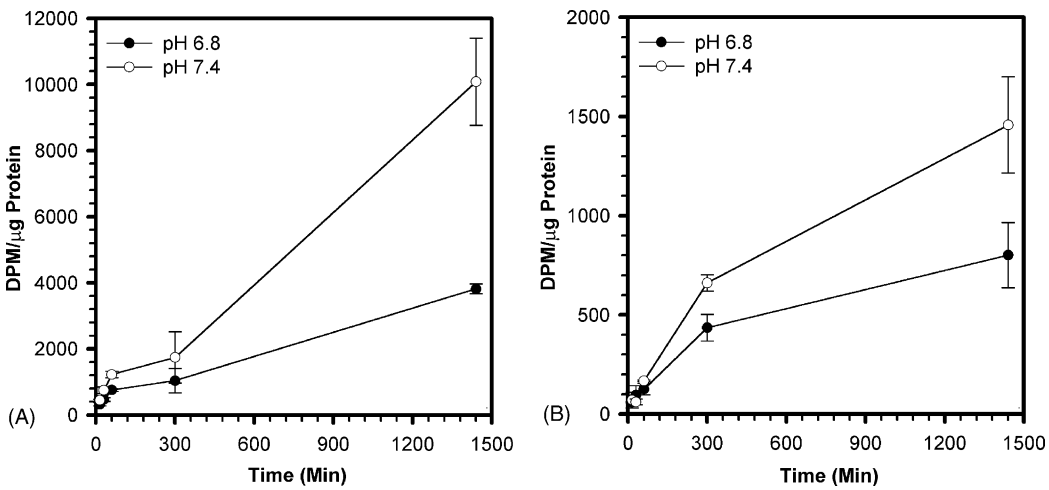

Fig. 7. Typical uptake curves for (A) mitoxantrone, and (B) doxorubicin, in MCF-7/s cells.

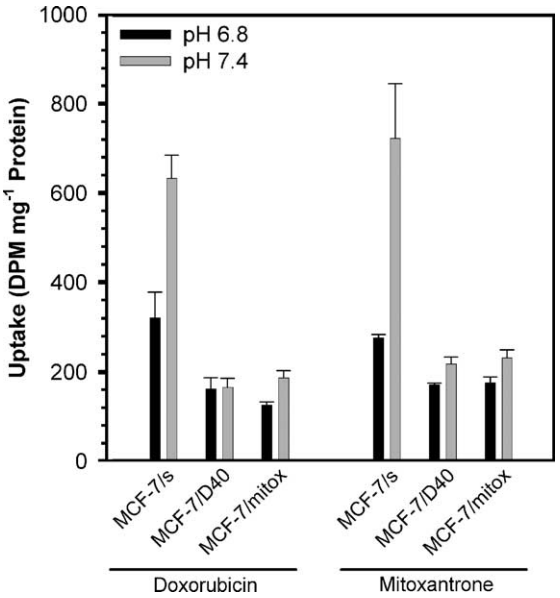

Fig. 8. Uptake of radiolabeled doxorubicin and mitoxantrone in drug-sensitive (MCF-7/s) and drug-resistant (MCF-7/D40, MCF-7/mitox) cell lines at low (■) and high (▨) pH.

dominated by ion trapping. However, there were subtle, yet significant differences between mitoxantrone and doxorubicin that confound this conclusion.

Mitoxantrone is weakly basic with two ionizable groups with pK_a values of 8.3. It is theoretically predicted that mitoxantrone's intracellular drug concentration will be 3.1-fold higher at a pHe of 7.4, compared to 6.8. Radiolabeled drug showed a 2.6-fold enhancement of uptake at pH 7.4 compared to pH 6.8 ($P < 0.05$). Hence, the distribution of mitoxantrone in MCF-7 cells appears to be dominated by ion trapping. Ion trapping in the case of doxorubicin is less clear. The radiolabeled drug accumulation and cytotoxicity data do indicate that pH affects doxorubicin behavior. However, the lack of significant difference in the uptake of doxorubicin between low and high pH at early time points (<30 min) suggests that the pH dependent effect on doxorubicin may not be mediated simply by ion trapping. Low signal in the rapidly changing system at the early time-points may also have contributed to the lack of a clear difference at the two pHe. The effect of increasing pHe on doxorubicin toxicity may also be mediated through increased pHi, which will decrease the charge on the molecule and render it more lipophilic. A similar secondary binding could also be evident for mitoxantrone, despite the existence of two ionizable groups.

A significant, 1.3-fold, enhancement in mitoxantrone accumulation was observed for the MDR-1 overexpressing cell line, MCF-7/D40, at the 1-hr time point ($P < 0.05$). MCF-7/mitox cells also exhibited a significant 1.3-fold enhancement in drug uptake following 24 hr of exposure to mitoxantrone ($P < 0.05$). Notably, the accumulation of mitoxantrone at high pHe in the drug-resistant cells was comparable to that in the drug-sensitive parent cells at low pHe (Fig. 8). Accumulation in MCF-7/s cells at pH 6.8 was 295 DPM min^{-1} mg^{-1}, compared to 240 and 248 DPM min^{-1} mg^{-1}, respectively, in the MCF-7/D40 and MCF-7/mitox lines at pHe 7.4. Hence, at least in the case of mitoxantrone, physiological resistance mediated by ion trapping is comparable to biochemical resistance mediated by upregulation of drug efflux pumps.

The effects of pHe on the uptake and toxicity of doxorubicin in the drug-resistant cell lines were more modest, compared to mitoxantrone. In the case of the MCF-7/D40 cell lines, pH did not affect uptake, although there was a significant effect of pHe on cytotoxicity in this cell line. Uptake and cytotoxicity were both enhanced at high pH in the MCF-7/mitox cell lines, albeit to a lesser degree when compared to mitoxantrone. Although the anti-tumor activity for doxorubicin may be improved with selective tumor alkalinization, the modest effect of pHe on the uptake and cytotoxicity of this drug indicates that ion trapping is less of a factor than with mitoxantrone.

Chlorambucil, a weakly acidic anti-neoplastic chemotherapeutic, was also utilized to test the prediction that weakly acidic drugs will reach higher intracellular concentrations when cells are in an acidic medium and that these higher intracellular concentrations will translate into an increased cytotoxicity at acidic pHe. In all three drug-sensitive and drug-resistant cell lines, chlorambucil was more cytotoxic at acidic pHe. Interventions leading to tumor acidification have been demonstrated in several different *in vivo* model systems (reviewed in Ref. [8]), and these should enhance the anti-tumor activity of chlorambucil.

Results from the other two weakly acidic chemotherapeutic drugs utilized in these studies, 5-flourouracil and cyclophosphamide, have caveats that should be noted. First, in the case of 5-flourouracil, acidic pH does enhance its anti-tumor activity (*vide supra*). However, it is unclear whether or not 5-flourouracil undergoes the phenomenon of ion trapping since its mechanism of transport is controversial [74]. Ion trapping would only be responsible for the increased cytotoxicity of 5-flourouracil if the drug entered the cell *via* passive diffusion.

In the case of cyclophosphamide, acidic pH does enhance the anti-tumor activity of this drug and this is due to ion trapping *in vitro*. However, these observations may not have direct *in vivo* application because hepatic enzymes are thought to be the predominant mode of activation of prodrug *in vivo*. Hepatic metabolism of cyclophosphamide is pH-sensitive and hence, systemic acidification may result in the generation of multiple active metabolic products [65]. Rodrigues *et al.* [75] have reported increased uptake and cytotoxicity of ifosfamide upon air + 5% CO_2 and carbogen (95% O_2 + 5% CO_2) breathing, although this effect is thought to be more a function of blood supply to tumor which is altered by the hypercapnia. Ifosfamide behaves as a weak base with estimated pK_a of 3.5–4, and is therefore not expected to participate in ion-trapping.

Paclitaxel demonstrated an insignificant enhancement in IC_{50} drug sensitivity with alkaline pH. Vukovic and Tannock previously reported that paclitaxel sensitivity increased with alkaline pH, possibly due to cell cycle effects [44]. Paclitaxel is not a substrate for ion trapping, but its cytotoxic effects may be sensitive to increased pHi. The current system was limited to acute alterations in pHe, while Tannock's design involved chronic alterations in pHe and hence would have had a greater effect on the cell cycle distribution. Moreover, Tannock and co-workers used nigericin, a K^+/H^+ ionophore, to equilibrate pHi and pHe, creating sub-physiologic pHi in the acid-treatment group. Thus, although paclitaxel may be slightly more active in alkaline conditions, the magnitude of this enhancement is minor and not statistically significant in our system. Neither our work nor that of Tannock suggests that paclitaxel undergoes ion trapping or is sensitive to transplasmalemmal pH gradients.

We hypothesized that a change in the pHe of tumor cells in culture will result in predictable changes in cytotoxicity based on differential ion trapping of the drug by the cells. The data presented here are consistent with this hypothesis in the case of mitoxantrone. The effects of doxorubicin are milder and suggest that the pH-dependent effects of this drug are only partly described by ion trapping. Nonetheless, these results consistently show that selective tumor alkalinization *in vivo* is likely to result in an enhancement in the anti-tumor activity of weakly basic chemotherapeutic drugs. Likewise, pH dependent effects on chlorambucil activity are consistent with ion trapping being a dominant mechanism. A similar case can be made for 5-fluoruracil and cyclophosphamide, yet *in vivo* metabolism of these drugs makes it difficult to extrapolate these results to the clinical situation. Further studies are needed to determine whether selective acidification in patients will result in the enhancement in the anti-tumor activity of these weakly acidic chemotherapeutic drugs.

Acknowledgments

National Institutes of Health grant R01 CA77575.

References

[1] Veronesi U, Banfi A, Salvadori B, Luini A, Saccozzi R, Zucali R, Marubini E, Del Vecchio M, Boracchi P, Marchini S. Breast conservation is the treatment of choice in small breast cancer: long-term results of a randomized trial. Eur J Cancer 1990;26:668–70.

[2] Veronesi U, Salvadori B, Luini A, Greco M, Saccozzi R, Del Vecchio M, Mariani L, Zurrida S, Rilke F. Breast conservation is a safe method in patients with small cancer of the breast: long-term results of three randomized trials on 1973 patients. Eur J Cancer 1995;31A:1574–9.

[3] Diab SG, Hilsenbeck SG, de Moor C, Clark GM, Osborne CK, Ravdin PM, Elledge RM. Radiation therapy and survival in breast cancer patients with 10 or more positive axillary lymph nodes treated with mastectomy. J Clin Oncol 1998;16:1655–60.

[4] Fisher B, Anderson S, Redmond CK. Reanalysis and results after 12 years of follow-up in a randomized clinical trial comparing total mastectomy with lumpectomy with or without irradiation in the treatment of breast cancer. New Engl J Med 1995;333:1456–61.

[5] Senn HJ, Maibach R, Castiglione M, Jungi WF, Cavalli F, Leyvraz S, Obrecht JP, Schildknecht O, Siegenthaler P. Adjuvant chemotherapy in operable breast cancer: cyclophosphamide, methotrexate, and fluorouracil versus chlorambucil, methotrexate, and fluorouracil—11-year results of Swiss Group for Chinese Cancer Research Trials SAKK 27/82. J Clin Oncol 1997;15:2502–9.

[6] Raghunand N, Martinez-Zaguilan R, Wright SH, Gillies RJ. pH and drug resistance. II. Turnover of acidic vesicles and resistance to weakly basic chemotherapeutic drugs. Biochem Pharmacol 1999;57:1047–58.

[7] Gillies RJ, Raghunand N, Karczmar GA, Bhujwalla ZM. MR Imaging of the tumor microenvironment. J Magn Reson Imaging 2002;16:430–50.

[8] Raghunand N, Gillies RJ. pH and drug resistance in tumors. Drug Resist Updates 2002;3:39–47.

[9] Wike-Hooley JL, Haven J, Reinhold HS. The relevance of tumour pH to the treatment of malignant disease. Radiother Oncol 1984;2:343–66.

[10] Raghunand N, Altbach MI, Van Sluis R, Baggett B, Taylor CW, Bhujwalla ZM, Gillies RJ. Plasmalemmal pH gradients in drug-sensitive and drug-resistant MCF-7 human breast carcinoma xenografts measured by ^{31}P MR spectroscopy. Biochem Pharmacol 1999;57:309–12.

[11] Ojugo AS, McSheehy PM, McIntyre DJ, McCoy C, Stubbs M, Leach MO, Judson IR, Griffiths JR. Measurement of the extracellular pH of solid tumours in mice by magnetic resonance spectroscopy: a comparison of exogenous ^{19}F and ^{31}P probes. NMR Biomed 1999;12:495–504.

[12] Stubbs M, Rodrigues L, Howe FA, Wang J, Jeong KS, Veech RL, Griffiths JR. Metabolic consequences of a reversed pH gradient in rat tumours. Cancer Res 1994;54:4011–6.

[13] Van Sluis R, Bhujwalla ZM, Raghunand N, Ballesteros P, Alvarez J, Cerdan S, Galons JP, Gillies RJ. In vivo imaging of extracellular pH using ^{1}H MRSI. Magn Reson Med 1999;41:743–50.

[14] Garcia-Martin ML, Herigault G, Remy C, Farion R, Ballesteros P, Coles JA, Cerdan S, Ziegler A. Mapping extracellular pH in rat brain gliomas in vivo by H-1 magnetic resonance spectroscopic imaging: comparison with maps of metabolites. Cancer Res 2001;61:6524–31.

[15] Bhujwalla ZM, Artemov D, Ballesteros P, Cerdan S, Gillies RJ, Solaiyappan M. Combined vascular and extracellular pH imaging of solid tumors. NMR Biomed 2002;15:114–9.

[16] Negendank W. Studies of human tumors by MRS: a review. NMR Biomed 1992;5:303–24.

[17] Newell K, Wood P, Stratford I, Tannock I. Effects of agents which inhibit the regulation of intracellular pH on murine solid tumours. Br J Cancer 1992;66:311–7.

[18] Ohtsubo T, Igawa H, Saito T, Matsumoto H, Park HJ, Song CW, Kano E, Saito H. Acidic environment modifies heat- or radiation-induced apoptosis in human maxillary cancer cells. Int J Radiat Oncol Biol Phys 2001;49:1391–8.

[19] Smalley RV, Lefante J, Bartolucci A, Carpenter J, Vogel C, Krauss S. A comparison of cyclophosphamide, adriamycin, and 5-fluorouracil (CAF) and cyclophosphamide, methotrexate, vincristine and prednisone (CMFVP) in patients with advanced breast cancer: a southeastern cancer study group project. Breast Cancer Res Treat 1983;3:209–20.

[20] Taylor CW, Dalton WS, Mosley K, Dorr RT, Salmon SE. Combination chemotherapy with cyclophosphamide, vincristine, adriamycin, and dexamethasone (CVAD) plus oral quinine and verapamil in patients with advanced breast cancer. Breast Cancer Res Treat 1997;42:7–14.

[21] Burghouts JT. Mitoxantrone, methotrexate and chlorambucil in metastatic breast cancer, a combination with relatively low subjective toxicity. Neth J Med 1990;36:43–5.

[22] Taylor CW, Dalton WS, Parrish PR, Gleason MC, Bellamy WT, Thompson FH, Roe DJ, Trent JM. Different mechanisms of decreased accumulation in doxorubicin and mitoxantrone resistant variants of the MCF-7 human breast cancer cell line. Br J Cancer 1991;63:923–9.
[23] Scheffer GL, Maliepaard M, Pijnenborg AC, van Gastelen MA, de Jong MC, Schroeijers AB, van der Kolk DM, Allen JD, Ross DD, van der Valk P, Dalton WS, Schellens JH, Scheper RJ. Breast cancer resistance protein is localized at the plasma membrane in mitoxantrone- and topotecan-resistant cell lines. Cancer Res 2000;60:2589–93.
[24] Arcamone F, Franceschi G, Penco S, Selvia A. Adriamycin 14-hydroxydaunomycin: a novel antitumor antibiotic. Tetrahedron Lett 1969;13:1007–16.
[25] Harris PA, Gross JF. Preliminary pharmacokinetic model for adriamycin (NSC-123127). Cancer Ther Rep 1975;59:819–25.
[26] Painter RB. Inhibition of DNA replicon initiation by 4-nitroquinoline-1-oxide, adriamycin, and ethyleneimine. Cancer Res 1978;38:4445–9.
[27] Kim SH, Kim JH. Lethal effect of adriamycin on the division of HeLa cells. Cancer Res 1972;32:323–5.
[28] Gieseler F, Glasmacher A, Kampfe D, Wandt H, Nuessler V, Valsamas S, Kunze J, Wilms K. Topoisomerase II activities in AML and their correlation with cellular sensitivity to anthracyclines and epipodophyllotoxins. Leukemia 1996;10:1177–80.
[29] Moriyama Y, Manabe T, Yoshimori T, Tashiro Y, Futai M. ATP-dependent uptake of anti-neoplastic agents by acidic organelles. J Biochem 1994;115:213–8.
[30] Martinez-Zaguilan R, Raghunand N, Lynch RM, Bellamy W, Martinez GM, Rojas B, Smith D, Dalton WS, Gillies RJ. pH and drug resistance. I. Functional expression of plasmalemmal V-type H^+-ATPase in drug-resistant human breast carcinoma cell lines. Biochem Pharmacol 1999;57:1037–46.
[31] Dubowchik GM, Padilla L, Edinger K, Firestone RA. Reversal of doxorubicin resistance and catalytic neutralization of lysosomes by a lipophilic imidazole. Biochim Biophys Acta 1994;1191:103–8.
[32] Altan N, Chen Y, Schindler M, Simon SM. Tamoxifen inhibits acidification in cells independent of the estrogen receptor. Proc Natl Acad Sci USA 1999;96:4432–7.
[33] Burke TG, Sartorelli AC, Tritton TR. Selectivity of the anthracyclines for negatively charged model membranes: role of the amino group. Cancer Chemother Pharmacol 1988;21:274–80.
[34] Demant EJ, Norskov-Lauritsen N. Binding of transferrin-iron by adriamycin at acidic pH. FEBS Lett 1986;196:321–4.
[35] Goda K, Balkay L, Marian T, Tron L, Aszalos A, Szabo GJ. Intracellular pH does not affect drug extrusion by P-glycoprotein. J Photochem Photobiol 1996;34:177–82.
[36] Skovsgaard T. Transport and binding of daunorubicin, adriamycin, and rubidazone in Ehrlich ascites tumour cells. Biochem Pharmacol 1977;26:215–22.
[37] Gallois L, Fiallo M, Garnier-Suillerot A. Comparison of the interaction of doxorubicin, daunorubicin, idarubicin and idarubicinol with large unilamellar vesicles. Biochim Biophys Acta 1998;1370:31–40.
[38] Frezard F, Pereira-Maia E, Quidu P, Priebe W, Garnier-Suillerot A. P-glycoprotein preferentially effluxes anthracyclines containing free basic versus charged amine. Eur J Biochem 2001;268:1561–7.
[39] Faulds D, Balfour JA, Chrisp P, Langtry HD. Mitoxantrone: a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in the chemotherapy of cancer. Drugs 1991;41:400–49.
[40] Saletan S. Mitoxantrone: an active, new antitumor agent with an improved therapeutic index. Cancer Treat Rev 1987;14:297–303.
[41] Pommier Y, Schwartz RE, Zwelling LA, Kohn KW. Effects of DNA intercalating agents on topoisomerase II induced DNA strand cleavage in isolated mammalian cell nuclei. Biochemistry 1985;24:6406–10.
[42] Jahde E, Glusenkamp KH, Rajewsky MF. Protection of cultured malignant cells from mitoxantrone cytotoxicity by low extracellular pH: a possible mechanism for chemoresistance in vivo. Eur J Cancer 1990;26:101–6.
[43] Kozin SV, Shkarin P, Gerweck LE. The cell transmembrane pH gradient in tumors enhances cytotoxicity of specific weak acid chemotherapeutics. Cancer Res 2001;61:4740–3.
[44] Vukovic V, Tannock IF. Influence of low pH on cytotoxicity of paclitaxel mitoxantrone and topotecan. Br J Cancer 1997;75:1167–72.
[45] Reszka KJ, Chignell CF. Acid-catalyzed oxidation of the anticancer agent mitoxantrone by nitrite ions. Mol Pharmacol 1996;50:1612–8.
[46] Rentsch KM, Schwendener RA, Hanseler E. Determination of mitoxantrone in mouse whole blood and different tissues by high-performance liquid chromatography. J Chromatogr Biomed Appl 1996;679:185–92.
[47] Wall ME, Wani MC. Camptothecin and taxol: discovery to clinic. Cancer Res 1995;55:753–60.
[48] Schiff PB, Hortwitz SB. Taxol stabilizes microtubules in mouse fibroblast cells. Proc Natl Acad Sci USA 1980;77:1561–5.
[49] Pazdur R, Kudelka AP, Kavanagh JJ, Cohen PR, Raber MN. The taxoids: paclitaxel (Taxol) and docetaxel (Taxotere). Cancer Treat Rev 1993;19:351–86.
[50] Donato R. Calcium-independent, pH-regulated effects of S-100 proteins on assembly-disassembly of brain microtubule protein in vitro. J Biol Chem 1988;263:106–10.
[51] Arnold H, Bourseaux F, Brock N, Asaumi J, Kawasaki S, Nishikawa K, Kuroda M, Hiraki Y. Chemotherapeutic action of cyclic nitrogen mustard phosphamide ester (B518-ASTA) in experimental tumours of the rat. Influence of the extracellular pH. Nature 1958;181:931–4.
[52] Fenselau C, Kan MN, Rao SS, Myles A, Friedman OM, Colvin M. Identification of aldophosphamide as a metabolite of cyclophosphamide in vitro and in vivo in humans. Cancer Res 1977;37:2538–43.
[53] Sladek NE. Influence of aldehyde dehydrogenase activity on the sensitivity of lymphocytes and other blood cells to oxazaphosphorines. Methods Findings Exp Clin Pharmacol 1987;9:617–26.
[54] Jahde E, Glusenkamp KH, Rajewsky MF. Increased drug cytotoxicity at reduced pH counteracts cyclophosphamide resistance in cultured rat mammary carcinoma cells. Int J Cancer 1989;44:1082–7.
[55] Engle TW, Zon G, Egan W. ^{31}P NMR investigations of phosphoramide mustard: evaluation of pH control over the rate of intramolecular cyclization to an aziridinium ion and the hydrolysis of this reactive alkylator. J Med Chem 1979;22:897–9.
[56] Balbinder E, Reich CI, Shugarts D, Keogh J, Fibiger R, Jones T, Banks A. Relative mutagenicity of some urinary metabolites of the antitumor drug cyclophosphamide. Cancer Res 1981;41:2967–72.
[57] Portlock CS, Fischer DS, Cadman E, Lundberg WB, Levy A, Bobrow S, Bertino JR, Farber L. High-dose pulse chlorambucil in advanced, low-grade non-Hodgkin's lymphoma. Cancer Treat Rep 1987;71: 1029–31.
[58] McLean A, Newell D, Baker G, Connors T. The metabolism of chlorambucil. Biochem Pharmacol 1980;29:2039–47.
[59] Miyamoto K, Wakabayashi D, Minamino T, Nomura M. Glutathione-*S*-transferase P-form dependent chlorambucil resistance to Yoshida rat ascites hepatoma cell lines. Cancer Lett 1994;78:77–83.
[60] Jiang BZ, Bank BB, Hsiang YH, Shen T, Potmesil M, Silber R. Lack of drug-induced DNA cross-links in chlorambucil-resistance Chinese hamster ovary cells. Cancer Res 1989;49:5514–7.
[61] Mikkelsen RB, Asher C, Hicks T. Extracellular pH, transmembrane distribution and cytotoxicity of chlorambucil. Biochem Pharmacol 1985;34:2531–4.
[62] Skarsgard LD, Chaplin DJ, Wilson DJ, Skwarchuk MW, Vinczan A, Kristl J. The effect of hypoxia and low pH on the cytotoxicity of chlorambucil. Int J Radiat Oncol Biol Phys 1992;22:737–41.
[63] Kuin A, Aalders M, Lamfers M, van Zuidam DJ, Essers M, Beijnen JH, Smets LA. Potentiation of anti-cancer drug activity at low intratumoral pH induced by the mitochondrial inhibitor *m*-iodobenzylguanidine (MIBG) and its analogue benzylguanidine (BG). Br J Cancer 1999;79:793–801.
[64] Parkins CS, Chadwick JA, Chaplin DJ. Inhibition of intracellular pH control and relationship to cytotoxicity of chlorambucil and vinblastine. Br J Cancer Suppl 1996;27:s75–7.

[65] Jahde E, Glusenkamp KH, Klunder I, Hulser DF, Tietze LF, Rajewsky MF. Hydrogen ion-mediated enhancement of cytotoxicity of bis-chloroethylating drugs in rat mammary carcinoma cells in vitro. Cancer Res 1989;49:2965–72.

[66] Pinedo HM, Peters GF. Fluorouracil: biochemistry and pharmacology. J Clin Oncol 1988;6:1653–64.

[67] Peters GJ, Laurensse E, Leyva A, Lankelma J, Pinedo HM. Sensitivity of human, murine, and rat cells to 5-fluorouracil and 5′-deoxy-5-fluorouridine in relation to drug-metabolizing enzymes. Cancer Res 1986;46:20–8.

[68] Trouet A, Massoulie J, Michelson AM, Pochon F. Polynucleotide analogues. VI. Physical studies on 5-substituted pyrimidine polynucleotides. Biochim Biophys Acta 1966;114:16–26.

[69] Schuetz JD, Wallace HJ, Diasio RB. 5-Fluorouracil incorporation into DNA of CF-1 mouse bone marrow cells as a possible mechanism of toxicity. Cancer Res 1984;44:1358–63.

[70] Ojugo AS, McSheehy PM, Stubbs M, Alder G, Bashford CL, Maxwell RJ, Leach MO, Judson IR, Griffiths JR. Influence of pH on the uptake of 5-fluorouracil into isolated tumour cells. Br J Cancer 1998;77: 873–9.

[71] McSheehy PM, Robinson SP, Ojugo AS, Aboagye EO, Cannell MB, Leach MO, Judson IR, Griffiths JR. Carbogen breathing increases 5-fluorouracil uptake and cytotoxicity in hypoxic murine RIF-1 tumors: a magnetic resonance study in vivo. Cancer Res 1998;58:1185–94.

[72] Raghunand N, He X, van Sluis R, Mahoney B, Baggett B, Taylor CW, Paine-Murrieta G, Roe D, Bhujwalla ZM, Gillies RJ. Enhancement of chemotherapy by manipulation of tumour pH. Br J Cancer 1999; 80:1005–11.

[73] Gillies RJ, Didier N, Denton M. Determination of cell number in monolayer cultures. Anal Biochem 1986;159:109–13.

[74] McSheehy PM, Stubbs M, Griffiths JR. Role of pH in tumor-trapping of the anticancer drug 5-fluorouracil. Adv Enzyme Regul 2000;40:63–80.

[75] Rodrigues LM, Robinson SP, McSheehy PMJ, Stubbs M, Griffiths JR. Enhanced uptake of ifosfamide into GH3 prolactinomas with hypercapnic hyperoxic gases monitored in vivo by ^{31}P MRS. Neoplasia 2002;4:539–43.