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pH and Drug Resistance. II. Turnover of Acidic Vesicles and Resistance to Weakly Basic Chemotherapeutic Drugs

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ABSTRACT. Resistance to chemotherapeutic agents is a major cause of treatment failure in patients with cancer. The primary mechanism leading to a multidrug-resistant phenotype is assumed to be plasma-membrane localized overexpression of drug efflux transporters, such as P-glycoprotein (P-gp). However, acidic intracellular organelles can also participate in resistance to chemotherapeutic drugs. In this study, we investigated, both experimentally and theoretically, the effect of acidic vesicle turnover on drug resistance. We have developed a general model to account for multiple mechanisms of resistance to weakly basic organic cations, e.g. anthracyclines and *Vinca* alkaloids. The model predicts that lower cytosolic concentrations of drugs can be achieved through a combination of high endosomal turnover rates, a low endosomal pH, and an alkaline-inside pH gradient between cytosol and the extracellular fluid. Measured values for these parameters have been inserted into the model. Computations using conservative values of all parameters indicate that turnover of acidic vesicles can be an important contributor to the drug-resistant phenotype, especially if vesicles contain an active uptake system, such as H^+/cation exchange. Even conservative estimates of organic cation-proton antiport activity would be sufficient to make endosomal drug extrusion a potent mechanism of resistance to weakly basic drugs. The effectiveness of such a drug export mechanism would be comparable to drug extrusion via drug pumps such as P-gp. Thus, turnover of acidic vesicles can be an important factor in chemoresistance, especially in cells that do not overexpress plasma membrane-bound drug pumps like P-glycoprotein. BIOCHEM PHARMACOL **57**;9: 1047–1058, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. multidrug resistance; mathematical model; compartmentation; organic cation transport; exocytosis; vacuolar-type H^+-ATPase

Acquired resistance to chemotherapeutic agents is a major cause of treatment failure in patients with cancer [1]. In classic multidrug resistance, cells exhibit resistance to a wide range of structurally unrelated cytostatic drugs of primarily natural origin, such as anthracyclines, *Vinca* alkaloids, and epipodophyllotoxins [2]. MDR‡ cell lines have been obtained either from resistant tumors or from drug-sensitive cell lines that have been adapted in culture to progressively higher concentrations of the drug [3]. A majority of drug-resistant cells overexpress a variety of membrane proteins, the commonest among them being P-gp, a 170- to 180-kDa plasma membrane glycoprotein [4]. In addition, an MRP has been cloned from a multidrug-resistant, doxorubicin-selected cell line that did not overexpress P-gp [5]. Transfection with cDNA encoding P-gp [6, 7] or MRP [8] induces multidrug resistance in drug-sensitive cell lines. Drug-resistant cells also have been reported to overexpress vesicular proteins, such as vacuolar H^+-ATPase [9, 10] and LRP [11, 12].

To be multidrug-resistant, cells are thought to have one or both of the following features: (i) lower intracellular drug concentration [1], possibly in conjunction with compartmentation of the drug away from the site of drug action (the nucleus) [13], and/or (ii) altered susceptibility to the drug and increased repair mechanisms [14, 15]. A lower intracellular concentration of drug may be obtained by a decreased rate of uptake of drug [16, 17] or an enhanced efflux of drug from the cell. In the most widely accepted hypothesis for MDR, P-gp acts as a plasmalemmal ATP-dependent "drug pump" to extrude drug molecules from the cell (reviewed in Ref. 4). P-gp-mediated drug transport is saturable [18] and capable of acting against a concentration gradient of its substrate [19]. Molinari *et al.* [20] have

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‡*Abbreviations:* 3-APP, 3-aminopropylphosphonate; LRP, lung resistance-Associated protein; MDR, multidrug resistance/resistant; MR, magnetic resonance; MRP, multidrug resistance-associated protein; P-gp, P-glycoprotein; pH_e, extracellular pH; pH_i, cytosolic pH; pH_v, endosomal/lysosomal pH; and SNARF-1, carboxy-seminaphthorhodafluor-1.

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presented evidence that, in addition to being present on the plasma membrane, P-gp is also present in the Golgi apparatus of several MDR cells. There have also been an increasing number of reports of MDR cells that do not overexpress P-gp [21–23]. Daunorubicin efflux against a concentration gradient has been observed in non-small-cell lung carcinoma cells that were multidrug-resistant, but that did not overexpress P-gp, suggesting that a multidrug transporter different from P-gp is functional in these cells [24]. One such transporter could be the MRP, an ATP-binding cassette-containing transporter overexpressed in some MDR cell lines. MRP confers MDR properties on cells transfected with MRP expression vectors [25]. However, MRP is thought to be an anion transporter [25], and drugs such as anthracyclines and *Vinca* alkaloids are cations.

An alternative, yet non-exclusive, explanation for achieving lowered intracellular drug concentrations involves drug molecules being transported to and trapped in acidic vesicles, from where they are extruded from the cell by exocytosis. Sehested *et al.* [26] have reported observing greatly enhanced rates of endocytosis in Ehrlich ascites tumor cells resistant to daunorubicin, compared with their drug-sensitive parent cell line. The same authors report a 3- to 4-fold increase in endosomal volume, endosomal surface area, and number of endosomes in daunorubicin-resistant P388 leukemic cells, as compared with drug-sensitive P388 cells [27]. Sognier *et al.* [28] reported the development of a Chinese hamster cell line highly resistant to doxorubicin whose increased drug resistance seems to result not from increased P-gp expression or activity, but from a greatly increased number of endocytic vesicles as compared with the parent LZ-8 cell line. Seidel *et al.* [29] have studied the subcellular localization of daunorubicin in drug-sensitive and drug-resistant EPG85–257 gastric carcinoma cells and have found that while the drug rapidly accumulates in the nuclei of the sensitive cells, resistant cells redistribute the drug from the nucleus to perinuclear vesicles, which subsequently move to the cell periphery. The major vault protein LRP has also been hypothesized to be involved in vesicle-related extrusion of drug molecules away from cell nuclei [12].

Other evidence for the involvement of acidic vesicles in the phenomenon of MDR links vesicle alkalinization with diminished resistance to drugs. Dubowchik *et al.* [30] have found that some imidazole compounds, in addition to raising lysosomal pH, also reversed drug resistance in a doxorubicin-resistant human colon carcinoma cell line, HCT116-VM46. Sehested *et al.* [31] have employed the carboxylic ionophores monensin and nigericin, as well as exogenous amines, to raise intravesicular pH and disrupt vesicular traffic, and have found a concomitant inhibition of the MDR phenotype in daunorubicin-resistant Ehrlich ascites tumor cells. Schindler and co-workers [32] have found a similar re-sensitization of drug-resistant MCF-7adr cells upon disruption of vesicular acidification with nigericin and monensin. In this context, it is interesting that some MDR cells overexpress a subunit of the vacuolar-type H^+-ATPase (V-ATPase) [9], the proton pump responsible for vesicle acidification. In a companion manuscript [10], we report that plasma-membrane V-ATPase activity and increased rates of endosomal turnover occurred in MDR human breast carcinoma cells.

It is therefore likely that some MDR cell lines transport drug molecules from their cytosol into acidic vesicles and expel the drug by exocytosis. There is also evidence that these vesicles may be predominantly perinuclear in location, possibly affording greater protection to the nucleus [33]. Drug uptake into these vesicles can be achieved either with the activity of P-gp [34], or by the combined activity of V-type H^+-ATPase-driven acidification and an organic cation/proton antiporter. The low pH in the vesicles would cause the typical weak-base drug molecule to exist in its charged, protonated form, possibly causing it to be "trapped" in the vesicle due to the low permeability of the charged molecule to the vesicular membrane [35]. In this study, we investigated, mathematically, the efficacy of these proposed mechanisms of MDR.

MATERIALS AND METHODS

Cell Culture

Primary human tumorigenic and non-tumor cells were obtained from the Arizona Cancer Center. These have been characterized previously with respect to colony formation in soft agar, lactic acid production rates, karyotype, P-gp content, invasiveness, and expression of plasmalemmal V-type H^+-ATPase activity [36]. Established cell lines were obtained from the American Type Culture Collection or the Michigan Cancer Foundation. MCF-7 cells resistant to mitoxantrone (MCF-7/MITOX) and doxorubicin (MCF-7/D40) were generated by sequential culturing in increased concentrations of mitoxantrone and doxorubicin, respectively [21]. MCF-7/D40 cells overexpress P-gp, whereas MCF-7/MITOX cells do not [21]. Human primary cells from ACC included non-tumor fibroblasts from esophagus and foreskin. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% Nu-serum (Collaborative Research) in a 5% CO_2 atmosphere at 37°. Cells were grown in T-75 tissue culture flasks and passed bi-weekly at an inoculation density of 2×10^5 cells/flask. For fluorescence measurements, cells were inoculated at 5×10^4 cells/60-mm Petri dish containing sterile glass coverslips (9×22 mm) and grown to subconfluency.

Measurement of Endosomal/Lysosomal and Cytosolic pH by Fluorescence

Endosomal and cytosolic pH were measured simultaneously using the fluorescence of 7-hydroxycoumarin conjugated to 70-kDa dextran (coumarin-dextran) and SNARF-1, respectively. Both dyes were obtained from Molecular Probes. Cultures on coverslips were incubated with 0.05 mg/mL of coumarin-dextran for 12–16 hr, after which time cells were

loaded with a 0.007 mM concentration of the acetoxymethyl ester form of SNARF-1 [SNARF-1(AM)] for 30 min, followed by a 30-min incubation in dye-free buffer to complete ester hydrolysis at 37°. Under these loading conditions, the coumarin-dextran is localized to the endosomes and lysosomes (the E–L compartment), and the de-esterified SNARF-1 is localized to the cytosol [36].

Fluorescence measurements were carried out in a temperature-controlled coverslip perfusion unit housed in an SLM-8000C spectrofluorometer (SLM Instruments) using 4-nm slits and an external rhodamine standard. Cells were perfused with Hanks' balanced salt solution supplemented with 1 mM glutamine and 2 mM glucose (HBSS-G). For simultaneous measurement of cytosolic and endosomal/lysosomal pH, data were collected by sequential acquisition at excitation wavelengths of 352, 370, and 402 nm at an emission wavelength of 448 nm (coumarin-dextran conditions), followed by excitation at 534 nm, collected at emissions of 584, 600, and 644 nm (SNARF-1 conditions). Cycles were repeated every 13.3 sec. For coumarin-dextran, the ratio of fluorescence at excitations of 352 and 402 nm is pH-sensitive, whereas fluorescence at 370 is not (isoexcitation point). For SNARF-1, the ratio of fluorescence at emissions of 584 and 644 nm is pH-sensitive, whereas fluorescence at 600 nm is not (isoemissive point). These ratio values were converted to pH values using standard calibration curves, as described by Martínez–Zaguilán *et al.* [36].

Cells and Animals Used for Tumor Growth

Severe combined immune deficient (SCID) mice were obtained from the University of Arizona SCID mouse resource. MCF-7/MITOX cells were implanted in the mammary fat pads of 6- to 7-week-old female SCID mice as a suspension of 2×10^6 cells in a total volume of 0.05 mL of Matrigel (Collaborative Research) diluted to 50% with Hanks' balanced salt solution. 17β-Estradiol pellets (0.72 mg, 60-day release, Innovative Research of America) were implanted subcutaneously on the backs of the mice by means of a 12-gauge trochar (Innovative Research) 2 days prior to tumor inoculation.

Preparation of Mice for NMR Spectroscopy

Tumors were allowed to grow for 3–8 weeks to volumes of 150-1500 mm^3, as estimated by external morphometry. Prior to MR spectroscopy, the mice were anesthetized with a combination of ketamine (72 mg/kg), xylazine (6 mg/kg), and acepromazine (6 mg/kg). A 3/4 inch, 24-G catheter (Elf Sanofi Inc.) connected to a 1 m long, 1.58 mm i.d. polyethylene tube (Becton Dickinson) was inserted into the intraperitoneal cavity of the anesthetized animal. The mouse was then immobilized on a home-built probe with a coil tunable to ^1H or ^{31}P. A solution of 3-APP (0.1 to 0.3 mL, 128 mg/mL, pH 7.4) could be injected into the mouse at the appropriate time via the i.p. catheter. This permitted the acquisition of spectra from the tumor before and after administration of 3-APP without changing the position of the animal within the magnet.

Localized In Vivo *MR Spectroscopy*

All *in vivo* measurements were performed at 4.7 T on a Bruker Biospec MR spectrometer/imager with a maximum gradient strength of 14 G/cm, using a home-built probe and solenoid coil tunable to ^1H or ^{31}P. Unlocalized ^{31}P spectra were acquired using 20–45° pulses with repetition times of 500-1000 msec. Volume-selective ^{31}P MR spectra were acquired using either VSEL, an implementation of the PRESS sequence provided by Bruker Medizintechnik, or the ISIS sequence, as previously described [37]. VSEL spectra were acquired using 764-μsec slice-selective Hemetian radiofrequency (rf) pulses (corresponding to 80 ppm in the ^{31}P spectrum), an echo time of 11.0 msec, and a repetition time of 1200 msec. ISIS spectra were acquired with adiabatic slice-selective and excitation pulses repeated every 10–12 sec. In all cases, a dwell time of 62.5 μsec was employed, and 8192 data points were collected. Transients were averaged for 10–30 min. Time-domain data were processed by exponential multiplication with a line broadening factor of 5–15 Hz, followed by Fourier transformation. The chemical shifts of 3-APP and inorganic phosphate were used to measure the extracellular and cytosolic pH values, respectively, as previously described [37–39].

Modeling the Effect of Vesicle Turnover on Drug Resistance

Our model is based upon drug molecules partitioned between four compartments: the extracellular medium, the cytosol, the perinuclear region and nucleus, and the endosomal/exocytotic vesicles (Fig. 1). Equations, discussed below, describe the flux of drug molecules between these pools. Calculations are made assuming that: (i) all cells are perfect spheres of identical geometry, (ii) endosomes are clustered in the perinuclear region, (iii) diffusional movement of the uncharged drug across a membrane is described by Fick's Law, and (iv) diffusional movement of the charged drug across a membrane is described by the constant electric field (Goldman) equation [40].

Binding of drug molecules to membranes or macromolecules is not taken into account. It is assumed that the equilibrium between free drug and drug bound with DNA and lipids is fast, and concentration of bound drug can be fully explained by partition with free drug.

FLUX OF DRUG INTO THE CYTOSOL. The flux of uncharged molecules entering the cytosol via the plasma membrane is described as a Fickian diffusion process:

$$J_{D,1} = P_{D,c}(C_{De} - C_{D1})S_{cell} \quad (1)$$

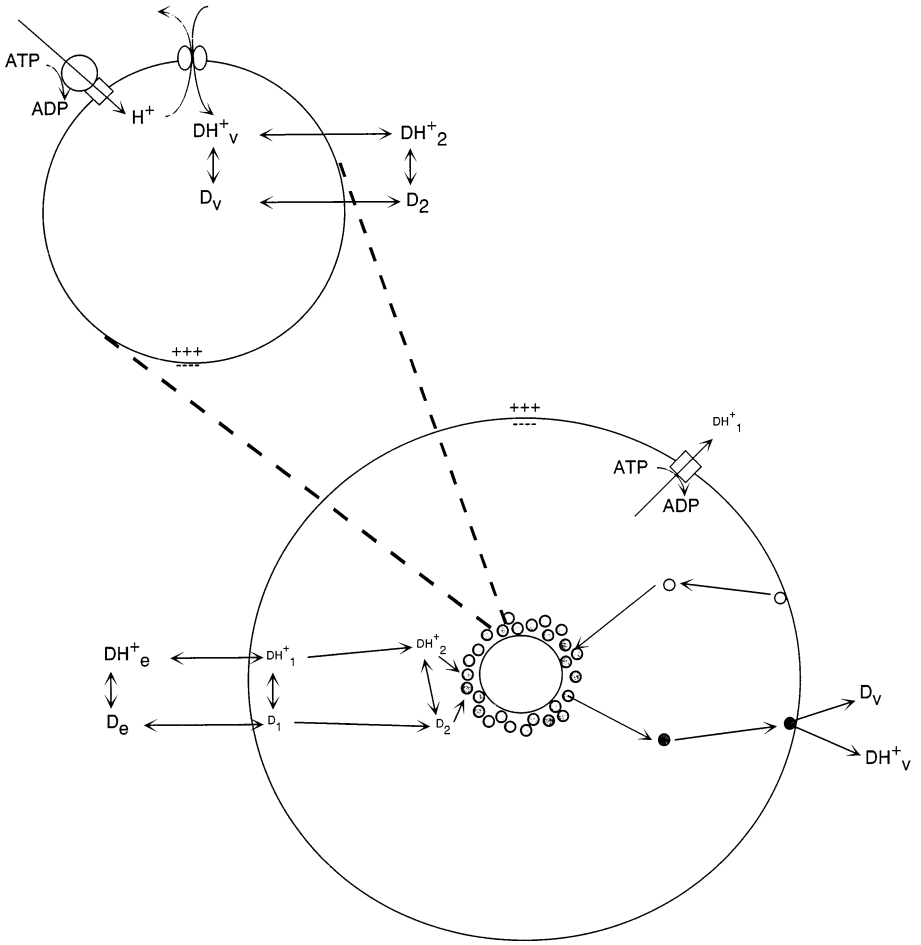

FIG. 1. Four-compartment model of drug distribution in the cell. Compartments include extracellular medium, cytosol, perinuclear region and nucleus, and endosomal/exocytotic vesicles. P-gp-mediated active transport of the drug may occur at the plasma membrane. In addition, protonated drug molecules may also be transported into endosomes by an organic cation/H^+ antiporter.

where $J_{D,1}$ stands for the molar diffusional flux of uncharged drug entering the cytosol from the extracellular medium, $P_{D,c}$ stands for the permeability of the uncharged drug to the plasma membrane, C_{De} and $C_{D,1}$ stand for the concentration of the uncharged molecule at the external and internal faces of the plasma membrane, respectively, and S_{cell} is the surface area of the plasma membrane. The amount of drug entering the cell by fluid phase endocytosis would be negligible compared with that entering by diffusion across the plasma membrane (data not shown). This term was therefore neglected in the model.

Charged drug molecules are taken to cross the plasma membrane passively, and by active transport of charged drug molecules out of the cytosol by a pump or exchanger. The passive flux of charged molecules entering the cytosol via the plasma membrane is described by the constant electric-field equation [40], whereas active transport out of the cytosol was taken to occur as per the kinetics described by Spoelstra *et al.* [41]. Thus, an equation of the following form describes the *net* movement of charged drug molecules into the cytosol:

$$J_{DH,1} = P_{DH,c}\left(\frac{\Delta\psi_{mc}F}{RT}\right) \cdot \left(\frac{C_{DHe} - C_{DH1}\exp\left(\frac{\Delta\psi_{mc}F}{RT}\right)}{\exp\left(\frac{\Delta\psi_{mc}F}{RT}\right) - 1}\right)S_{\text{cell}} - \frac{V_P[C_{DH1}]^h}{K_m^h + [C_{DH1}]^h} \quad (2)$$

where $J_{DH,1}$ represents the net molar flux of protonated drug entering the cytosol through the plasma membrane: positive for net inward flux, and negative for net outward flux. $P_{DH,c}$ is the permeability of the charged drug molecule to the plasma membrane; $\Delta\psi_{mc}$ is the plasma membrane potential ($\psi_{\text{in}} - \psi_{\text{out}}$); F is Faraday's constant; R is the gas constant; T is the absolute temperature (Kelvin); C_{DHe} and C_{DH1} are the concentrations of the protonated drug at the external and internal faces of the plasma membrane, respectively; V_P is the maximal velocity of the plasmalemmal drug efflux pump (mol/sec/cell); K_m is the Michaelis–

Menten constant for this process; and h is the Hill coefficient for binding of drug molecules to the transporter.

FREE DIFFUSION OF DRUG MOLECULES ACROSS THE CYTOSOL. In our model, drug molecules need not be homogeneously distributed in the cytosol. Instead, there can be an inwardly-directed concentration gradient from the plasma membrane toward the perinuclear region. $J_{D,2}$ and $J_{DH,2}$, the fluxes across the cytosol of the unprotonated and protonated drug molecules, respectively, represent a process of free diffusion (see Appendix):

$$J_{DH,2} = \frac{4\pi\, D_{DH}\, R_c R_P (C_{DH1} - C_{DH2})}{R_c - R_P} \tag{3}$$

$$J_{D,2} = \frac{4\pi\, D_D R_c R_P (C_{D1} - C_{D2})}{R_c - R_P} \tag{4}$$

Here, R_c is the radius of the cell; R_P is the radius of the perinuclear region; C_{D2} and C_{DH2} are the concentrations of unprotonated and protonated drug molecules at the radius R_P; and D_D and D_{DH} are the diffusivities of unprotonated and protonated drug molecules in the cytosol.

DIFFUSION OF DRUG MOLECULES INTO THE PERINUCLEAR REGION. There have been numerous reports in the literature of MDR cells displaying diminished nuclear drug accumulation [13, 42, 43], and drug sequestration in perinuclear vesicles with very little nuclear drug accumulation [33]. We have therefore modeled a situation where most of the endosomes are clustered in the perinuclear region. Drug molecules are transported from the perinuclear region into vesicles, which then move to the plasma membrane and expel the drug by exocytosis. Since the nuclear drug concentration is set by the concentration of drug around the nucleus, this transport of drug out of the perinuclear region would result in lowered nuclear drug levels at steady state. Because the positions of the endosomes in the perinuclear region will change continuously over time, the concentration of drug at any point in the region, even at steady state, will be changing constantly. The volume-averaged concentration of drug in the region, however, will not change at steady state. We have therefore modeled the perinuclear region as a region of uniform drug distribution. Thus, at steady state, the concentration of unprotonated and protonated drug molecules in the nucleus and perinuclear region will equal their concentrations at radius $r = R_P$, C_{D2} and C_{DH2}.

PASSIVE FLUX OF DRUG (MOLES/TIME) INTO ENDOSOMES. Passive movement of uncharged molecules into endosomes can be described by:

$$J_{D,3} = P_{D,v}\,(C_{D1} - C_{Dv}) S_{\text{endo}} N_{\text{endo}} \tag{5}$$

where $J_{D,3}$ is the net molar flux of uncharged drug into all the endosomes from the perinuclear region; $P_{D,v}$ stands for the permeability of the uncharged drug molecule to the endosomal membrane; C_{Dv} is the concentration of the uncharged molecule in the endosomes; S_{endo} is the surface area of a single endosome; and N_{endo} is the total number of endosomes per cell.

TRANSPORT OF PROTONATED DRUG INTO ENDOSOMES. Protonated drug may be transported across the endosomal membrane either along its electrochemical gradient, or by active transport. This active transport could be ATP dependent, as is the case with P-gp [44]. Izquierdo *et al*. [12] have hypothesized that LRP, the major vault protein, may have a transport function and may be involved in drug resistance by vesicular extrusion of drug molecules from the cell. Active transport of organic cations into endosomes may also be driven by the proton-gradient across the endosomal membrane [45, 46]. This is also an energy-dependent mechanism for drug transport into the endosomes, since the H^+ gradient across the endosomal membrane is maintained by V-type H^+-ATPases [47]. The characteristics of endosomal organic cation/H^+ exchange in the kidney and liver are the same as those measured in plasma membrane vesicles from these tissues [48–52]. Based on these studies, we have assumed the following equation to describe the dependence of transporter kinetics on $C_{DH,2}$ and vesicular pH:

$$v_{DH,v} = \frac{V_T\,[C_{DH2}][H^+]_v}{(k_{DH} + [C_{DH2}])(k_H + [H^+]_v)} \tag{6}$$

where V_T = the maximum transport rate (mol DH^+ transported/time/cell); k_{DH} = the apparent Michaelis constant of the transporter for protonated drug; k_H = the apparent Michaelis constant of the transporter for protons; and $[H^+]_v$ = the free H^+ concentration in the endosomes.

Combined with diffusive flux, the movement of charged molecules into endosomes is described by:

$$J_{DH,3} = P_{DH,v}\left(\frac{\Delta\psi_{mv}F}{RT}\right) \cdot \left(\frac{C_{DHv} - C_{DH2}\exp\left(\frac{-\Delta\psi_{mv}F}{RT}\right)}{\exp\left(\frac{-\Delta\psi_{mv}F}{RT}\right) - 1}\right) S_{\text{endo}} N_{\text{endo}} + \frac{V_T\,[C_{DH2}][10^{-pH_v}]}{k_{DH} + [C_{DH2}](k_H + [10^{-pH_v}])} \tag{7}$$

where $J_{DH,3}$ is the net molar flux of protonated drug entering all the endosomes; $P_{DH,v}$ stands for the permeability of the charged drug molecule to the endosomal membrane; $\Delta\psi_{mv}$ is the endosomal membrane potential ($\psi_{\text{in}} - \psi_{\text{out}}$); and C_{DHv} is the concentration of the protonated drug in the endosomes.

TABLE 1. Parameters used in model calculations

Symbol	Parameter	High	Low	References
pH_e	Extracellular pH	7.5	6.5	38
pH_i	Cytosolic pH	6.5	7.5	
pH_v	Endosomal /lysosomal pH	4.5	6.5	10, 46, 53, 54
T_{vc}	Rate of exocytosis (% endosomal vol.min^{-1})	10	0	10, 26
pK_a	Acid dissociation constant of the drug	9.5	7.5	55, 56
P_D	Permeability of uncharged drug ($\times 10^{-7}$ m/sec)	2.5	7.5	41, 57, 58
P_{DH}	Permeability of charged drug (rel. to P_D)	0.001	0.005	
D_D, D_{DH}	Diffusion coefficients of drug ($\times 10^{-10}$ m^2/sec)	1	5	59, 60
$\Delta\psi_{mc}$	Plasma membrane potential (mV)	−30	−80	61
$\Delta\psi_{mv}$	Endosomal membrane potential (mV)	+10	+100	
N_{endo}	Number of endosomes	50	200	27
d_{cell}	Diameter of the cell (μm)	20	10	
$V_{endo} \cdot N_{endo}$	Total endosomal volume, relative to cell volume	0.02	0.005	27
	Ratio, (perinuclear + nuclear)/cell volume	0.1	0.25	
k_{DH}	K_d of drug from cation/H^+ antiporter (μM)	1	10	49, 51
k_H	K_d of protons from cation/H^+ antiporter (M)	10^{-8}	10^{-7}	48
C_T	Initial extracellular drug concentration (μM)	1	10	21, 62
V_T	V_{max} cation/H^+ antiporter (10^{-18} mol/cell/sec)	7.5	1.0	48–52
V_P	V_{max}, P-glycoprotein (10^{-18} mol/cell/sec)	3.5	0	41
K_m	Michaelis-Menten constant for P-gp (μM)	1	5	41, 63
h	Hill coefficient for binding of drug to P-gp	1	2	41, 63

STEADY–STATE REQUIREMENTS. All parameters are modeled to determine steady-state values. Therefore, we have the following flux balance equations:

$$J_{D,1} + J_{DH,1} = J_{D,2} + J_{DH,2} \quad (8)$$

$$J_{D,2} + J_{DH,2} = J_{D,3} + J_{DH,3} \quad (9)$$

The flux into the endosomes = flux of drug out of the cell by exocytosis. Therefore,

$$J_{D,3} + J_{DH,3} = (C_{Dv} + C_{DHv})T_{vc}V_{endo}N_{endo} \quad (10)$$

where V_{endo} is the volume of a single endosome, and T_{vc} is the fractional endosomal volume exocytosed per unit time (the "turnover rate").

EQUILIBRIUM REQUIREMENTS. The equilibrium between unprotonated and protonated drug molecules is taken to be instantaneous. We therefore have the following equations:

$$C_{De} = C_{DHe} \cdot 10^{(pH_e - pK_a)} \quad (11)$$

$$C_{D1} = C_{DH1} \cdot 10^{(pH_c - pK_a)} \quad (12)$$

$$C_{D2} = C_{DH2} \cdot 10^{(pH_c - pK_a)} \quad (13)$$

$$C_{Dv} = C_{DHv} \cdot 10^{(pH_v - pK_a)} \quad (14)$$

OVERALL CONSERVATION OF DRUG. There must be a conservation of drug mass over the extracellular medium, the cytosol, and the endosomes. Since the extracellular space dominates all other volumes, we have:

$$C_{De} + C_{DHe} \approx C_T \quad (15)$$

where C_T is the total extracellular concentration of the drug. Equations 1–5 and 7–15 may be solved simultaneously once we have set values for all the parameters involved. Solutions to the model were calculated using Mathcad 5.0 (Mathsoft Inc.), and parameter values are shown in Table 1.

RESULTS

Selection of Parameters

The model describing the role of endosomal turnover in drug resistance includes several parameters, listed in Table 1. Physiologically reasonable ranges for these parameters are available in the literature, and the extremes of these ranges are presented in the table as either "low" or "high." Low values represent those extremes that contribute the least toward active efflux of the drug from the cell. This is an important point because, with so many parameters, it would be simple to choose high parameter combinations that yield maximal drug resistance yet are physiologically unreasonable. Hence, the most rigorous test of the model would employ only low values. To determine which parameters are most important to the model, we calculated the steady-state nuclear drug concentration (relative to the extracellular) in cells with all parameters set to their low values, and then iteratively determined the effects of altering single parameters. In this way, the principal components of the model could be readily determined.

One of the most important effects of pH on drug concentration ratio is the intra- to extracellular pH gradient. It can be shown that weakly basic chemotherapeutic agents will partition away from the cytosol [35, 64] simply

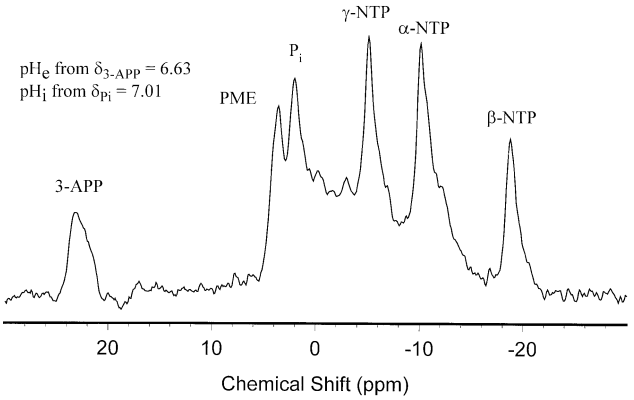

FIG. 2. ^{31}P MR spectrum acquired from a 974-mm^3 tumor of MCF-7/MITOX cells growing in the mammary fat pad of a severe combined immune deficient (SCID) mouse. An exponential line-broadening factor of 15 Hz was applied to the time-domain signal prior to Fourier transformation. P$_i$, inorganic phosphate; NTP, nucleoside triphosphates; PME, phosphomonoesters. The extracellular and cytosolic pH values reported in the figure were determined from the chemical shift of 3-APP and P$_i$, respectively.

because the extracellular pH of tumors is acidic [38, 39, 65, 66]. The extracellular pH in solid tumors is typically 0.3 to 0.4 pH units more acidic than the cytoplasmic pH. Fig. 2 illustrates a ^{31}P MR spectrum of MCF-7/MITOX breast carcinoma cells, which are idiotypically resistant, *in vivo* and *in vitro*, to mitoxantrone, a weak base chemotherapeutic drug. As shown in this spectrum, the extracellular pH (pH$_e$) is 6.63 and the cytosolic pH (pH$_i$) is 7.01, as measured from the resonance frequencies of exogenous 3-APP and endogenous inorganic phosphate, respectively. These data are similar to those measured in other tumors of similar size [38, 39, 65, 66] and indicate that ΔpH values of

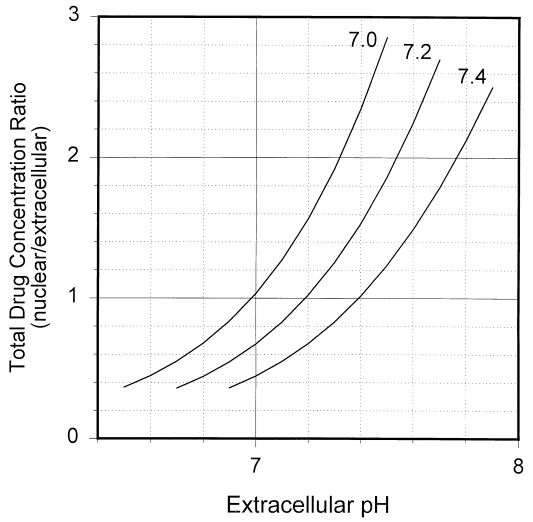

FIG. 3. Drug ratios affected by extracellular pH. Predicted nuclear/extracellular drug concentrations are shown at three different cytosolic pH values (7.0, 7.2, and 7.4) as a function of extracellular pH, for a drug of pK$_a$ 8.25. These data were calculated according to coupled equations, as described in the text, in the absence of endosomal turnover.

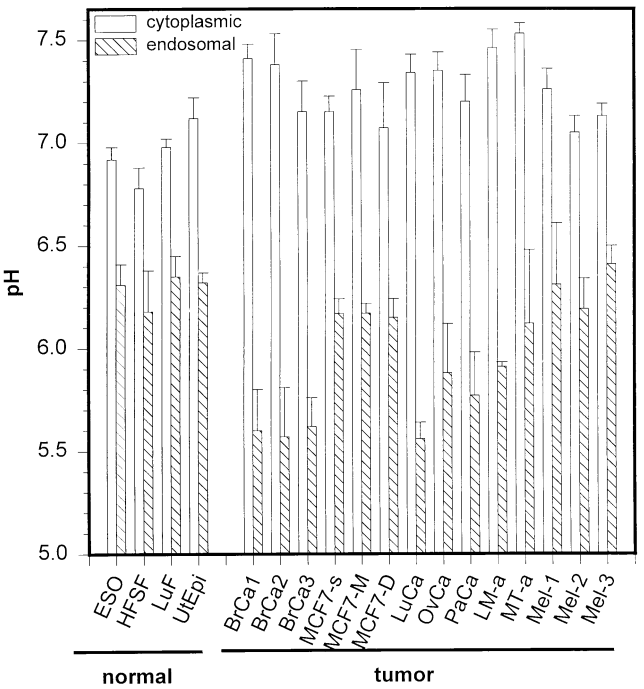

FIG. 4. Endosomal/lysosomal and cytosolic pH in human normal and tumor cells. Compartment pH values were measured using the pH-dependent fluorescent dyes coumarin-dextran and SNARF-1, respectively. Normal cells include esophageal (ESO), foreskin (HFSF), and lung (LuF) fibroblasts and uterine epithelial (UtEpi) cells. Tumor cells include breast (BrCa), lung (LuCa), ovarian (OvCa), and pancreatic (PaCa) carcinomas, leiomyo (LM) and mesothelial (MT) sarcomas, and melanomas (Mel). Data represent means ± SEM with a minimum of five independent measurements.

up to +0.38 are observed in tumors *in vivo*. Fig. 3 illustrates the effect of varying extracellular pH on the nuclear-to-extracellular drug ratio with all other parameters set to their low values, including no endosomal turnover. As expected, the steady-state drug distribution followed the cytosolic-extracellular pH gradient (ΔpH), with a ratio of ~1.0 at a ΔpH of 0.0, and a ratio of 0.35 to 0.4 at a ΔpH of +0.5 (alkaline inside). Thus, a ΔpH of +0.38 would lead to a nuclear-to-extracellular drug ratio of *ca.* 0.4, even in the absence of endosomal turnover. This phenomenon of "physiological resistance" will be additive to the effects of endosomal sequestration and turnover investigated in the current study, since it will reduce the amount of drug presented to the surface of the endosome.

Two additional parameters that most markedly affect the efficacy of endosome-mediated drug extrusion are the endosomal pH and turnover rates. These have been measured in breast cancer cells with fluorescence [10]. To assess the generality of these values, we have extended these measurements to other cell lines. Fig. 4 illustrates the pH$_v$ and pH$_i$ values in a series of primary and established human tumor cell lines measured using the pH-dependent fluorescent dyes, coumarin-dextran and SNARF-1, respectively. As shown in this figure, the pH$_v$ of all carcinomas taken together (x-Ca) was 5.72 ± 0.04. Interestingly, the pH$_v$ of

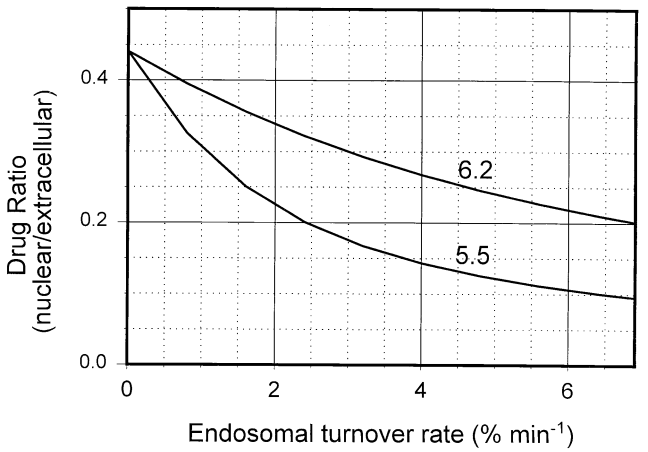

FIG. 5. Effects of endosomal turnover rates and endosomal pH on the nuclear drug concentration. Predicted nuclear/extracellular drug concentrations are shown at two endosomal pH values (5.5 and 6.2) as a function of endosomal turnover rate. These data were calculated according to coupled equations, as described in the text, with the "low" values for parameters from Table 1 except for: $pH_e = 6.7$, $pH_i = 7.1$, and drug $pK_a = 8.25$.

the MCF-7 series of breast cancer cells studied in the companion manuscript [10] was higher than that of the other breast cancer cells in this study (cf. BrCa vs MCF-7). These differences are correlated with metastatic potential, since the other breast cancer cell lines were highly metastatic, whereas MCF-7 cells are non-metastatic. The pH_v of the melanoma cells (Mel) was higher, 6.28 ± 0.12, and that of the sarcomas (LM-a, MT-a) was intermediate, 6.01 ± 0.09. The pH_v of normal cells was similar to that of melanomas, i.e. 6.27 ± 0.06. Other researchers have measured endosomal and late endosomal pH values of 4.5 to 6.5 [46, 53, 54]. Thus, the pH_v of 5.5 to 6.2 seen here is a physiologically reasonable range.

Endosomal turnover rates in the MCF-7 breast cancer cells of the companion study [10] were 0.59 to 1.58% min^{-1}. Endosomal turnover rates have been measured in other cell lines by other methods. For instance, labeling cells *in situ* with antibody against an extracellular epitope of V-ATPase yielded uptake time constants of 6.7% min^{-1} for C8161 melanoma cells (data not shown). Other researchers have measured values for endosomal turnover of up to 10% min^{-1} [26]. Thus, a physiologically reasonable range of values for endosomal turnover rates is 0.6 to 6.0% min^{-1}.

Figure 5 shows the effects of endosomal pH and endosomal turnover rate on the drug ratios, using a drug pK_a = 8.25 (daunorubicin), a pH_e of 6.7, a pH_i of 7.1, and low values for all remaining parameters. These data show that, at physiological endosomal pH values (5.5 to 6.2) and endosomal turnover rates (0.6 to 6.0% min^{-1}), the drug concentration in the cytoplasm will be 10–45% that of the extracellular environment. Thus, turnover of acidic vesicles can contribute to a significant lowering of steady-state concentrations of weakly basic drugs.

Drug pK_a and Mediated Transport of Drug into Endosomes

Two other interrelated parameters were revealed to be important in the endosome-mediated drug export during analyses: the activity of a mediated drug transport system and the drug pK_a. The rates of the organic cation/H^+ antiporter activity used in this simulation are modeled after those found in kidney brush border membrane vesicles and are conservatively within the physiologically reasonable range [48, 49, 51]. It is assumed that the driving force for this reaction is the activity of intravesicular H^+, provided by V-type H^+-ATPase. Since these antiporters act on the charged form of the drug, the pK_a of the drug molecule will affect the efficiency of the transport. Fig. 6 illustrates the effects of drug pK_a and transporter activity (V_T) on the perinuclear drug concentration. In Fig. 6A, these were calculated with the values $pH_e = 6.7$, $pH_i = 7.1$, $pH_v = 5.75$, endosomal turnover rate of 5.0%/min, and low values for all other parameters. It can be seen that this mechanism of drug efflux from the cell is strongly influenced by the acid dissociation constant of the drug, because a higher proportion is present in its charged form. Fig. 6B illustrates the influence of endosomal turnover rate on this carrier-mediated process with values for parameters $pH_e = 6.7$, $pH_i = 7.1$, $pH_v = 5.75$, $pK_a = 8.25$, and low values for other parameters. It is noteworthy that, if the transporter is inactive, there is no reduction in the perinuclear drug concentration even at the highest rates of endosomal turnover. This is seen from similar values of the y-intercept for all turnover rates. This illustrates that an endosomal pH gradient alone is not sufficient to make increased rates of exocytosis an effective mechanism of drug resistance. Equally, even at the highest transporter activity, there is no reduction in perinuclear drug concentration in the absence of vesicular turnover. Thus, both transport of drug into endosomes and turnover of the vesicles are required for reducing perinuclear concentrations of the drug. Note that, even with modest transport rates and rates of endosomal turnover, there are significant decreases in the perinuclear drug concentration.

Comparison with P-gp Activity at the Plasma Membrane

P-gp is thought to act at the plasma membrane, although it has also been detected in intracellular organelles [20]. Figure 7 shows the effect of increasing P-gp activity (at the plasma membrane) upon the cytosolic drug concentration, for drugs of different pK_a values. These calculations were made with $pH_e = 6.7$, $pH_i = 7.1$, $pH_v = 5.75$, endosomal turnover rate of 5.0%/min, $V_T = 0.0$, Hill coefficient h = 1, and low values for all other parameters. V_P, the plasma-membrane pump activity, was chosen to be in the same range of values as V_T in Fig. 6A. A comparison with Fig. 6A revealed that for equal maximal activities, a drug pump located at the plasma membrane was slightly more effective

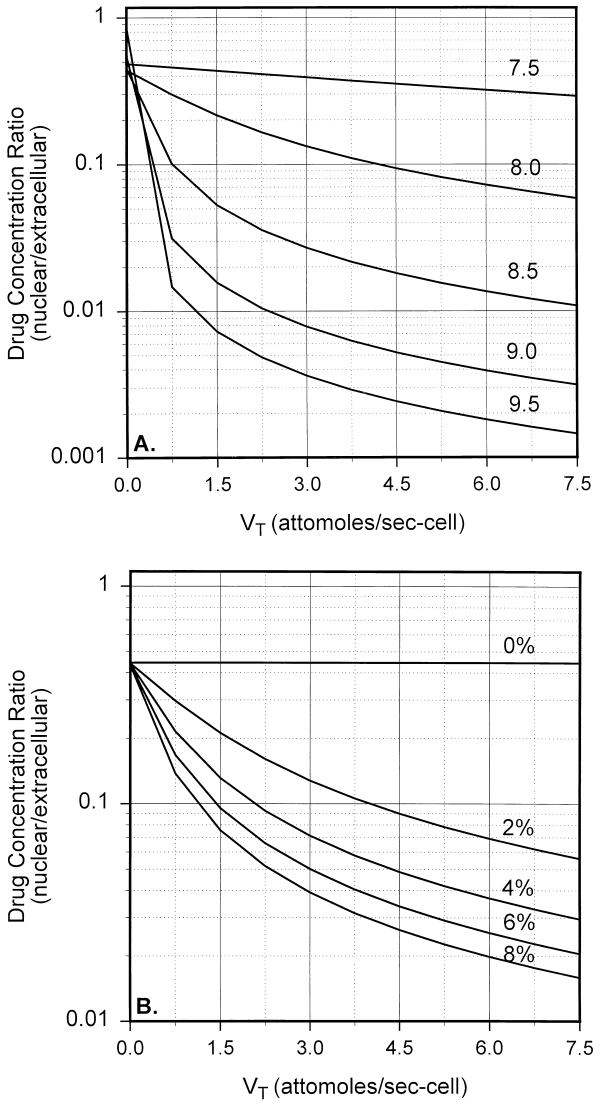

FIG. 6. Effects of endosomal transporter activity (V_T) on drug ratios. (A) Effects of drug pK_a and endosomal organic cation/H^+ antiporter activity (V_T) on the nuclear drug concentration. Model calculations were made with pH_e = 6.7, pH_i = 7.1, pH_v = 5.75, endosomal turnover rate = 5%/min, and "low" values for other parameters from Table 1. (B) Effects of endosomal turnover rate and endosomal organic cation/H^+ antiporter activity (V_T) on the nuclear drug concentration. Model calculations were made with pH_e = 6.7, pH_i = 7.1, pH_v = 5.75, drug pK_a = 8.25, and "low" values For other parameters from Table 1.

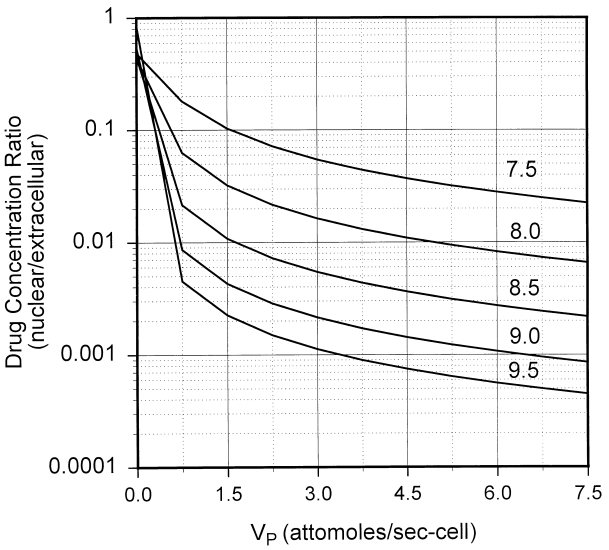

FIG. 7. Effects of plasmalemmal transporter activity (V_P) on drug ratios. Effects of drug pK_a and plasmalemmal drug pump activity (V_P) on the nuclear drug concentration. Model calculations were made with pH_e = 6.7, pH_i = 7.1, pH_v = 5.75, endosomal turnover rate = 5%/min, V_T = 0, and "low" values for other parameters from Table 1.

at reducing the nuclear concentration of a weak-base drug than a pump located at the endosomal membrane. However, this was only true if the drug did not display cooperative binding with the plasmalemmal drug transporter ($h = 1$). We also determined that for more basic drug molecules (higher pK_a), the difference in efficiencies between the two mechanisms narrowed considerably. Furthermore, it can be seen that in conjunction with high rates of endosomal turnover, an endosomal drug transporter can produce reductions in nuclear drug concentrations comparable to those produced by a plasma-membrane bound drug transporter. Indeed, with a conservative value of $h = 2$, the effectiveness of the plasmalemmal transporter at maintaining a low nuclear drug concentration was inferior to that of an endosomal drug transporter (calculations not shown).

Membrane Potentials

A correlation has been made by some groups between a less negative plasma membrane potential and increased drug resistance [61, 67, 68]. Agents that reverse MDR, e.g. verapamil and cyclosporin A, have been shown to reduce the plasma membrane potential of the resistant cells [67]. In our model, a more negative plasma membrane potential resulted in a small lowering of the perinuclear-to-extracellular drug ratio, but a more positive endosomal membrane potential did not have a noticeable effect on the drug ratio, for values above 10 mV.

Endosomal Clustering in the Perinuclear Region

When the perinuclear region was modeled to include the entire cell, the perinuclear-to-extracellular drug concentration ratios were 10–15% higher than the drug ratios resulting with endosomal clustering in a perinuclear region comprising only 25% of the cell volume. Thus, clustering of the endosomes in the perinuclear region results in an enhancement of their protective effect. The values assumed for diffusivities in the cytosol of the charged and uncharged drug molecules (Table 1) were at the high end of values reported for other molecules in the cytosol [59, 60]. Lower values of diffusivities would enhance the protective effect of

drug-sequestering acidic vesicles clustered around the nucleus.

DISCUSSION

Our data, both theoretical and experimental, indicate that a combination of low endosomal pH, high endosomal turnover rate, and active transport of drug molecules into endosomes, can effectively reduce perinuclear drug concentrations, yielding a drug-resistant phenotype. The activity of endosome-mediated drug resistance is slightly lower than that produced by equal activities of a plasmalemmal drug pump, but the difference narrows for weak-base drugs with more alkaline acid-dissociation constants or with cooperative binding of drug to the plasmalemmal transporter.

Demant *et al.* [69] have published a mathematical model simulating the effects of plasma membrane P-gp activity, transmembrane pH gradient, and endosomal turnover on the cytosolic concentration of drug molecules, and have concluded that for exocytotic extrusion of drug molecules to be an effective mechanism of MDR, unrealistically high rates of endosomal turnover and/or unrealistically low endosomal pH values would be needed. They have concluded therefore that endosomal turnover-mediated drug extrusion does not contribute to multidrug resistance. In their model, only the uncharged form of the drug molecule was taken to cross (passively or actively) any membrane. Our data are in complete agreement. The y-intercepts of panels A and B of Fig. 6 show that, in the absence of active transport of charged species into the endosomes, there is only a modest reduction of 40–50% in the levels of drug in the nucleus. In this study, we have chosen to also consider the movements of charged drug molecules, and have determined that this makes a significant difference. Moreover, we have chosen to consider vesicles that are located primarily in the perinuclear region.

It appears from our model that some form of active transport is required for a reduction in the cytosolic/perinuclear drug concentration with respect to the extracellular drug concentration at steady state. Simple alterations in cytosolic pH or number and volume of endosomes, in the absence of active transport, do not lower the nuclear drug concentration to therapeutically significant levels (i.e. $< 10\%$). Model calculations reveal that for cells that do not overexpress P-gp, an organic cation/H^+ antiporter located at the endosomal membrane can, in conjunction with physiologically reasonable rates of turnover of the acidic vesicles, substantially reduce the drug concentration in the perinuclear region. Computations using physiologically reasonable values for all model parameters illustrate that cells can lower cytosolic concentrations of weak base drugs through a combination of mechanisms including: (i) active transport of the drug into the endosomes, (ii) increased endosomal turnover rates, (iii) decreased endosomal pH, and (iv) increased plasma membrane pH gradient. Such a mechanism of drug export could be important to multidrug-resistant cells not overexpressing plasma membrane-bound drug pumps like P-gp.

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References

1. Nielsen D and Skovsgaard T, P-glycoprotein as multidrug transporter: A critical review of current multidrug resistant cell lines. *Biochim Biophys Acta* **1139:** 169–183, 1992.
2. Dalton WS, Is p-glycoprotein a potential target for reversing clinical drug resistance? *Curr Opin Oncol* **6:** 595–600, 1994.
3. Clynes M, Cellular models for multiple drug resistance in cancer. *In Vitro Cell Dev Biol* **29A:** 171–179, 1993.
4. Gottesman MM and Pastan I, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62:** 385–427, 1993.
5. Cole SPC, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AMV and Deeley RG, Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* **258:** 1650–1658, 1992.
6. Pastan I, Gottesman MM, Ueda K, Lovelace E, Rutherford AV and Willingham MC, A retrovirus carrying an MDR1 cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells. *Proc Natl Acad Sci USA* **85:** 4486–4498, 1988.
7. Ueda K, Cardarelli C, Gottesman MM and Pastan I, Expression of a full-length cDNA for the human "*MDR1*" gene confers resistance to colchicine, doxorubicin and vinblastine. *Proc Natl Acad Sci USA* **84:** 3004–3008, 1987.
8. Grant CE, Valdimarsson G, Hipfner DR, Almquist KC, Cole SPC and Deeley RG, Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. *Cancer Res* **54:** 357–361, 1994.
9. Ma L and Center MS, The gene encoding vacuolar H^+-ATPase subunit C is overexpressed in multidrug-resistant HL60 cells. *Biochem Biophys Res Commun* **182:** 675–681, 1992.
10. Martínez-Zaguilán R, Raghunand N, Lynch RM, Bellamy W, Martinez GM, Rojas B, Smith D, Dalton WS and 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* **57:** 1037–1046, 1999.
11. Scheper RJ, Broxterman HJ, Sceffer GL, Kaaijk P, Dalton WS, van Heijningen THM, van Kalken CK, Slovak ML, de Vries EGE, van der Valk P, Meijer CJLM and Pinedo HM, Overexpression of a M_r 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. *Cancer Res* **53:** 1475–1479, 1993.
12. Izquierdo MA, Scheffer GL, Flens MJ, Schroeijers AB, van der Valk P and Scheper RJ, Major vault protein LRP-related multidrug resistance. *Eur J Cancer* **32A:** 979–984, 1996.
13. Schuurhuis GJ, Broxterman HJ, de Lange JHM, Pinedo HM, van Heijningen TMH, Kuiper CM, Scheffer GL, Scheper RJ, van Kalken CK, Baak JPA and Lankelma J, Early multidrug resistance, defined by changes in intracellular doxorubicin distribution, independent of P-glycoprotein. *Br J Cancer* **64:** 857–861, 1991.
14. De Isabella P, Capranico G and Zunino F, The role of topoisomerase II in drug resistance. *Life Sci* **48:** 2195–2205, 1991.
15. Klohs WD, Steinkampf RW, Leopold WR and Fry DW,

Potentiation of adriamycin, trimetrexate and an AMSA analog (CI-921) cytotoxicities by amiodarone and reserpine in multidrug resistant P388 cells. *Proc Am Assoc Cancer Res* **28:** 298–302, 1987.
16. Klohs WD, Steinkampf RW, Havlick MJ and Jackson RJ, Resistance to anthrapyrazoles and anthracyclines in multidrug-resistant P388 murine leukemia cells: Reversal by calcium channel blockers and calmodulin antagonists. *Cancer Res* **46:** 4352–4356, 1986.
17. Klohs WD, Steinkampf RW, Besserer JA and Fry DW, Cross resistance of pleiotropically drug resistant P388 leukemia cells to the lipophilic antifolates trimetrexate and BW 301U. *Cancer Lett* **31:** 253–260, 1986.
18. Spoelstra EC, Dekker H, Schuurhuis GJ, Broxterman HJ and Lankelma J, P-glycoprotein drug efflux pump involved in the mechanisms of intrinsic drug resistance in various colon cancer cell lines. *Biochem Pharmacol* **41:** 349–359, 1991.
19. Ruetz S and Gros P, A mechanism For P-glycoprotein action in multidrug resistance: Are we there yet? *Trends Pharmacol Sci* **15:** 260–263, 1994.
20. Molinari A, Cianfriglia M, Meschini S, Calcabrini A and Arancia G, P-glycoprotein expression in the Golgi apparatus of multidrug-resistant cells. *Int J Cancer* **59:** 789–795, 1994.
21. Taylor CW, Dalton WS, Parrish PR, Gleason MC, Bellamy WT, Thompson FH, Roe DJ and Trent JM, Different mechanisms of decreased drug accumulation in doxorubicin and mitoxantrone resistant variants of the MCF7 human breast cancer cell line. *Br J Cancer* **63:** 923–929, 1991.
22. Dalton WS, Cress AE, Alberts DS and Trent JM, Cytogenetic and phenotypic analysis of a human colon carcinoma cell line resistant to mitoxantrone. *Cancer Res* **48:** 1882–1888, 1988.
23. Harker WG, Slade DL, Dalton WS, Meltzer PS and Trent JM, Multidrug resistance in mitoxantrone-selected HL-60 leukemia cells in the absence of P-glycoprotein overexpression. *Cancer Res* **49:** 4542–4549, 1989.
24. Mulder HS, Lankelma J, Dekker H, Broxterman HJ and Pinedo HM, Daunorubicin efflux against a concentration gradient in non-P-glycoprotein multidrug-resistant lung-cancer cells. *Int J Cancer* **59:** 275–281, 1994.
25. Leier I, Jedlitschky G, Buchholz U, Cole SPC, Deeley RG and Keppler D, The *MRP* gene encodes an ATP-dependent export pump for leukotriene C_4 and structurally related conjugates. *J Biol Chem* **269:** 27807–27810, 1994.
26. Sehested M, Skovsgaard T, van Deurs B and Winther-Neilsen H, Increase in nonspecific adsorptive endocytosis in anthracycline- and vinca alkaloid-resistant Ehrlich ascites tumor cell lines. *J Natl Cancer Inst* **78:** 171–179, 1987.
27. Sehested M, Skovsgaard T, van Deurs B and Winther-Neilson H, Increased plasma membrane traffic in daunorubicin resistant P388 leukemic cells: Effect of daunorubicin and verapamil. *Br J Cancer* **56:** 747–751, 1987.
28. Sognier MA, Zhang Y, Eberle RL, Sweet KM, Altenberg GA and Belli JA, Sequestration of doxorubicin in vesicles in a multidrug-resistant cell line (LZ-100). *Biochem Pharmacol* **48:** 391–401, 1994.
29. Seidel A, Hasmann M, Loser R, Bunge A, Schaefer B, Herzig I, Steidtmann K and Dietel M, Intracellular localization, vesicular accumulation and kinetics of daunorubicin in sensitive and drug-resistant gastric carcinoma EPG85–257 cells. *Virchows Arch* **426:** 249–256, 1995.
30. Dubowchik GM, Padilla L, Edinger K and Firestone RA, Reversal of doxorubicin resistance and catalytic neutralization of lysosomes by a lipophilic imidazole. *Biochim Biophys Acta* **1191:** 103–108, 1994.
31. Sehested M, Skovsgaard T and Roed H, The carboxylic ionophore monensin inhibits active drug efflux and modulates *in vitro* resistance in daunorubicin resistant Ehrlich ascites tumor cells. *Biochem Pharmacol* **37:** 3305–3310, 1988.
32. Schindler M, Grabski S, Hoff E and Simon SM, Defective pH regulation of acidic compartments in human breast cancer cells (MCF-7) is normalized in adriamycin-resistant cells (MCF-7adr). *Biochemistry* **35:** 2811–2817, 1996.
33. Gervasoni JE Jr, Fields SZ, Krishna S, Baker MA, Rosado M, Thuraisamy K, Hindenburg AA and Taub RN, Subcellular distribution of daunorubicin in P-glycoprotein-positive and -negative drug-resistant cell lines using laser-assisted confocal microscopy. *Cancer Res* **51:** 4955–4963, 1991.
34. Klohs WD and Steinkampf RW, The effect of lysosomatropic agents and secretory inhibitors on anthracycline retention and activity in multiple drug-resistant cells. *Mol Pharmacol* **34:** 180–185, 1988.
35. Simon SM and Schindler M, Cell biological mechanisms of multidrug resistance in tumors. *Proc Natl Acad Sci USA* **91:** 3497–3504, 1994.
36. Martínez-Zaguilán R, Lynch RM, Martinez GM and Gillies RJ, Vacuolar-type H^+-ATPases are functionally expressed in plasma membranes of human tumor cells. *Am J Physiol* **265:** C1015–C1029, 1993.
37. Raghunand N, Altbach MI, van Sluis R, Baggett B, Taylor CW, Bhujwalla ZM and Gillies RJ, Plasmalemmal pH-gradients in drug-sensitive and drug-resistant MCF-7 human breast carcinoma xenografts measured by ^{31}P magnetic resonance spectroscopy. *Biochem Pharmacol* **57**: 309–312, 1999.
38. Gillies RJ, Liu Z and Bhujwalla Z, ^{31}P-MRS measurements of extracellular pH of tumors using 3-aminopropylphosphonate. *Am J Physiol* **267:** C195–C203, 1994.
39. McCoy CL, Parkins CS, Chaplin DJ, Griffiths JR, Rodrigues LM and Stubbs M, The effect of blood flow modification on intra- and extracellular pH measured by ^{31}P magnetic resonance spectroscopy in murine tumors. *Br J Cancer* **72:** 905–911, 1995.
40. Goldman DE, Potential, impedance, and rectification in membranes. *J Gen Physiol* **27:** 37–60, 1943.
41. Spoelstra EC, Westerhoff HV, Dekker H and Lankelma J, Kinetics of daunorubicin transport by P-glycoprotein of intact cancer cells. *Eur J Biochem* **207:** 567–579, 1992.
42. Toffoli G, Simone F, Gigante M and Boiocchi M, Comparison of mechanisms responsible for resistance to idarubicin and daunorubicin in multidrug resistant LoVo cell lines. *Biochem Pharmacol* **48:** 1871–1881, 1994.
43. Slapak CA, Mizunuma N and Kufe DW, Expression of the multidrug resistance associated protein and P-glycoprotein in doxorubicin-selected human myeloid leukemia cells. *Blood* **84:** 3113–3121, 1994.
44. Schlemmer SR and Sirotnak FM, Functional studies of P-glycoprotein in inside-out plasma membrane vesicles derived from murine erythroleukemia cells overexpressing MDR3. *J Biol Chem* **269:** 31059–31066, 1994.
45. Pritchard JB, Sykes DB, Walden R and Miller DS, ATP-dependent transport of tetraethylammonium by endosomes isolated from rat renal cortex. *Am J Physiol* **266:** F966–F976, 1994.
46. Van Dyke RW, Faber ED and Meijer DKF, Sequestration of organic cations by acidified hepatic endocytic vesicles and implications for biliary excretion. *J Pharmacol Exp Ther* **261:** 1–11, 1992.
47. Forgac M, Structure, function and regulation of the coated vesicle V-ATPase. *J Exp Biol* **172:** 155–169, 1992.
48. Wright SH and Wunz TM, Mechanism of *cis-* and *trans-*substrate interactions at the tetraethylammonium/H^+ exchanger of rabbit renal brush-border membrane vesicles. *J Biol Chem* **263:** 19494–19497, 1988.
49. Ayer Lazaruk KD and Wright SH, MPP^+ is transported by the TEA^+-H^+ exchanger of renal brush-border membrane vesicles. *Am J Physiol* **258:** F597–F605, 1990.
50. Moseley RH, Jarose SM and Permoad P, Organic cation

transport by rat liver plasma membrane vesicles: Studies With tetraethylammonium. *Am J Physiol* **263:** G775–G785, 1992.
51. Wright SH, Wunz TM and Wunz TP, Structure and interaction of inhibitors with the TEA/H^+ exchanger of rabbit renal brush border membranes. *Pflugers Arch* **429:** 313–324, 1995.
52. Moseley RH and Van Dyke RW, Organic cation transport by rat liver lysosomes. *Am J Physiol* **268:** G480–G486, 1995.
53. Murphy RF, Powers S and Cantor CR, Endosome pH measured in single cells by dual fluorescence flow cytometry: Rapid acidification of insulin to pH 6. *J Cell Biol* **98:** 1757–1762, 1984.
54. Fuchs R, Male P and Mellman I, Acidification and ion permeabilities of highly purified rat liver endosomes. *J Biol Chem* **264:** 2212–2220, 1989.
55. Skovsgaard T and Nissen NI, Membrane transport of anthracyclines. *Pharmacol Ther* **18:** 293–311, 1982.
56. Dalmark M, Characteristics of doxorubicin transport in human red blood cells. *Scand J Clin Lab Invest* **41:** 633–639, 1981.
57. Dalmark M and Hoffman EK, Doxorubicin (Adriamycin) transport in Ehrlich ascites tumour cells: Comparison with transport in human red blood cells. *Scand J Clin Lab Invest* **43:** 241–248, 1983.
58. Dordal MS, Winter JN and Atkinson AJ Jr, Kinetic analysis of P-glycoprotein-mediated doxorubicin efflux. *J Pharmacol Exp Ther* **263:** 762–766, 1992.
59. Hubley MJ, Rosanske RC and Moerland TS, Diffusion coefficients of ATP and creatine phosphate in isolated muscle: Pulsed gradient ^{31}P NMR of small biological samples. *NMR Biomed* **8:** 72–78, 1995.
60. Tanner JE, Intracellular diffusion of water. *Arch Biochem Biophys* **224:** 416–428, 1993.
61. Roepe PD, Wei LY, Cruz J and Carlson D, Lower electrical membrane potential and altered pH_i homeostasis in multidrug-resistant (MDR) cells: Further characterization of a series of MDR cell lines expressing different levels of P-glycoprotein. *Biochemistry* **32:** 11042–11056, 1993.
62. Awasthi S, Sharma R, Awasthi YC, Belli JA and Frenkel EP, The relationship of doxorubicin binding to membrane lipids with drug resistance. *Cancer Lett* **63:** 109–116, 1992.
63. Guiral M, Viratelle O, Westerhoff HV and Lankelma J, Cooperative P-glycoprotein mediated daunorubicin transport into DNA-loaded plasma membrane vesicles. *FEBS Lett* **346:** 141–145, 1994.
64. Wadkins RM and Roepe PD, Biophysical aspects of P-glycoprotein-mediated multidrug resistance. *Int Rev Cytol* **171:** 121–165, 1997.
65. Bhujwalla ZM, Shungu DC, Chatham JC, Wehrle JP and Glickson JD, Glucose metabolism in RIF-1 tumors After reduction in blood flow: An *In vivo* ^{13}C and ^{31}P NMR study. *Magn Reson Med* **32:** 303–309, 1994.
66. Stubbs M, Rodrigues L, Howe FA, Wang J, Jeong KS, Veech RL and Griffiths JR, Metabolic consequences of a reversed pH gradient in rat tumors. *Cancer Res* **54:** 4011–4016, 1994.
67. Vayuvegula B, Slater L, Meador J and Gupta S, Correction of altered plasma membrane potentials: A possible mechanism of cyclosporin A and verapamil reversal of pleiotropic drug resistance in neoplasia. *Cancer Chemother Pharmacol* **22:** 163–168, 1988.
68. Hasmann M, Valet GK, Tapiero H, Treverrow K and Lampidis T, Membrane potential differences between Adriamycin-sensitive and -resistant cells as measured by flow cytometry. *Biochem Pharmacol* **38:** 305–312, 1989.
69. Demant EJF, Sehested M and Jensen PB, A model for computer simulation of P-glycoprotein and transmembrane ΔpH-mediated anthracycline transport in multidrug-resistant tumor cells. *Biochim Biophys Acta* **1055:** 117–125, 1990.

APPENDIX

Diffusion of drug molecules across the cytosol from radius $(r+\delta r)$ to radius r is described by Fick's law:

$$Ji = Di\left(\frac{\partial Ci(r)}{\partial r}\right)(4\pi r^2)$$

where J_i is the steady-state flux of species *i* (unprotonated or protonated drug); D_i is the cytosolic diffusion coefficient of species *i*; and $C_i(r)$ is the radius-dependent steady-state concentration of species *i* in the cytosol. Integrating both sides of the above equation, we have:

$$\int_{R_c}^{R_P} \frac{dr}{r^2} = \frac{4\pi Di}{J} \int_{C_{i,r=R_c}}^{C_{i,r=R_P}} dCi$$

i.e. the flux of species *i* from radius R_c to radius R_P is:

$$J_i = \frac{4\pi D_i R_c R_P (C_{i,r=R_c} - C_{i,r=R_P})}{R_c - R_P}$$