pH and drug resistance in tumors

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Abstract Uptake of weakly ionizing drugs by tumours is greatly influenced by the interstitial and intracellular pH, and the ionization properties of the drug. Extracellular pH in tumors is acidic, while the intracellular pH is in the neutral-to-alkaline range. Tumors of the bladder, kidney and gastrointestinal system in particular are exposed to extremes of pH. Strategies for exhancing and exploiting acid-outside plasmalemmal pH gradients to drive the uptake of weak acid drugs into tumors are discussed, as are techniques for alkalinizing tissues to improve response to weak base drugs. The participation of acidic intracellular vesicles in non-specific drug resistance is explored. © 2000 Harcourt Publishers Ltd

INTRODUCTION

Otto Warburg's early observations that tumors have a high rate of lactic acid production led him to postulate that tumors are acidic tissues.[1] Microelectrode measurements of acid pH in a variety of solid tumors by numerous investigators have subsequently strengthened this belief.[2] It is assumed that microelectrode measurements primarily interrogate only extracellular (interstitial) volumes.[31] P magnetic resonance spectroscopy (MRS) can be used to non-invasively measure intracellular pH (pH_i) from the chemical shift of the pH-sensitive resonance of endogenous inorganic phosphate (P_i). In vivo ^{31}P MRS measurements show a tumor pH_i which is neutral-to-alkaline.[3,4] Thus, the apparent acidity of the extracellular (interstitial) pH (pH_e) does not lead to an acidic pH_i. Recently, ^{31}P MRS methods have been developed to simultaneously and non-invasively measure the pH_i and pH_e of tumors using endogenous P_i and exogenous 3-aminopropylphosphonate (3-APP), respectively.[5] This method has clearly shown, in a number of solid tumor xenografts, that the pH_i of tumor cells is neutral-to-alkaline while the pH_e of the same tumors is acidic.[5-7] While MRS can measure a volume-averaged pH_i and pH_e over tumors, the new technique of magnetic resonance spectroscopic imaging (MRSI) permits the 'imaging' of pH_e through the use of appropriately localized exogenous pH markers. MRSI has been used to assess the spatial distribution of pH_e in experimental tumors, revealing the presence of large regions of very acidic pH_e.[8] Available evidence thus points to the presence of substantial acid-outside plasmalemmal pH gradients in tumors.

Modern cancer chemotherapy employs a variety of agents, classified variously as alkylating agents, microtubule disruptors, antimetabolies, DNA intercalators, topoisomerase inhibitors, hormones and other antimitotic agents.[9] Classification of these agents can also be accomplished on a more chemical basis, according to ionization status under physiological conditions. Most combination chemotherapy regimens employ at least one partly ionizable species. Weak acid drugs ionize in solution into negatively charged, deprotonated species which exist in equilibrium with the uncharged,
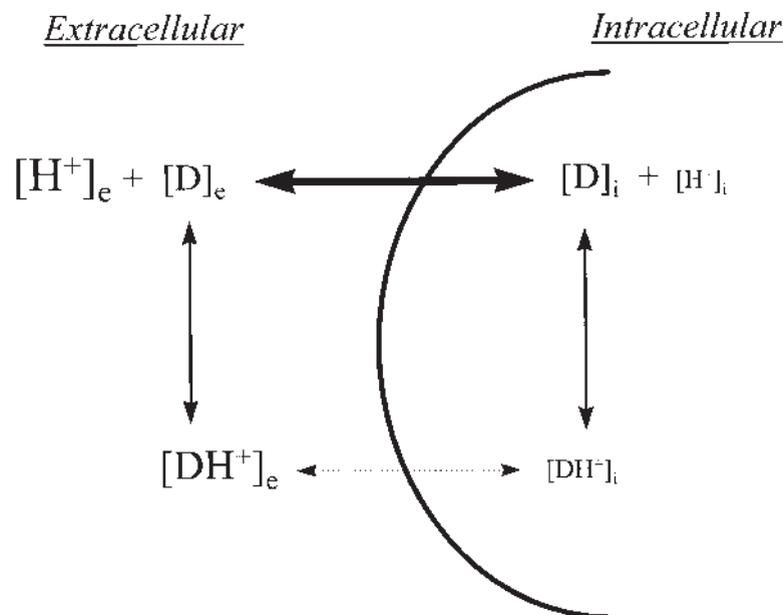

Fig. 1 **The 'ion trapping' hypothesis. In this example, [H+]$_e$> [H+]$_i$ (i.e. pH$_e$ < pH$_i$). The weak base drug (uncharged D + charged DH$^+$) tends to be excluded from the cell. Type size is a qualitative indicator of the concentration of a species in that compartment. Conversely, a weak acid will tend to concentrate in the more alkaline compartment, i.e. the intracellular compartment.**

protonated form of the drug. Conversely, weak base drugs ionize in solution into positively charged, protonated species which exist in equilibrium with the uncharged, unprotonated form of the drug. The uncharged forms of ionizable drugs typically cross the plasma membrane of cells fairly readily, this being a requirement for an effective drug. However, physiological membranes are less permeable to the charged forms of these drugs.[10] This leads to the phenomenon of 'ion-trapping', wherein weak bases partition into and are sequestered by acidic compartments, and weak acids are sequestered in alkaline compartments.[11] As depicted in Fig. 1, the relatively high permeability of the plasma membrane to uncharged drug leads to equilibration of uncharged drug on both sides of the membrane, while charged molecules are not free to equilibrate across the membrane. In the case of weak bases, the charged form of the drug is the protonated form, and it concentrates on the more acid side of the membrane, leading to greater total drug on the acidic side of the membrane. Conversely, in the case of weak acids, the charged form of the drug is the deprotonated form, and it concentrates on the more alkaline side of the membrane, leading to greater total drug on the more alkaline side of the membrane. The extent of partitioning of weak-base or weak-acid drug molecules across the plasma membrane of a tumor cell is therefore dependent upon the pK$_a$ of the drug as well as the intracellular and extracellular pH. Thus, the large acid-outside pH gradient in tumors can exert a protective effect upon the cell from weak-base drugs such as anthracyclines and vinca alkaloids, as well as act to potentiate the action of weak acid drugs like chlorambucil.[12–14]

INFLUENCE OF TUMOR pH ON DRUG UPTAKE AND ACTIVATION

Figures 2A and B illustrate the theoretically calculated dependence of the intracellular-to-extracellular drug partition ratio on pH$_e$ and pH$_i$ for a weak base drug with pK$_a$ 8.25 and a weak acid drug with pK$_a$ 6.0, respectively. It can be seen from Figure 2A that at typical values for tumor pH$_e$ of 6.7 and pH$_i$ of 7.1,[7] the predicted intracellular concentration of a weak base drug of pK$_a$ 8.25 is only 40% of the extracellular drug concentration. Thus, a substantial degree of 'physiological drug resistance' is conferred upon cells in acidic regions of tumors. Conversely, we see from Figure 2B that at pH$_e$ 6.7 and pH$_i$ 7.1, a weak acid drug with pK$_a$ 6.0 will be concentrated inside the cell at 2.4-fold the extracellular drug concentration. Thus, weak acid drugs are well-suited for treatment of tumors with large acidic regions. Table 1 lists some commonly used weak base and weak acid drugs, and the most relevant pK$_a$ values for them.

Acid-adapted cells have been shown in vitro to better resist acute changes in pH$_i$ in response to acidification of pH$_e$, resulting in increased acid-outside plasmalemmal pH gradients in such cells. Kozin and Gerweck[25] have shown that this increased pH gradient results in up to a 14-fold decrease in sensitivity of acid-adapted cells to the weak base drug mitoxantrone, and up to a 6-fold increase in sensitivity to the weak acid chlorambucil. The presence of two equally ionizable groups on mitoxantrone, as opposed to only one on chlorambucil, has been postulated to result in the roughly 2-fold greater pH-sensitivity of the cytotoxicity of mitoxantrone
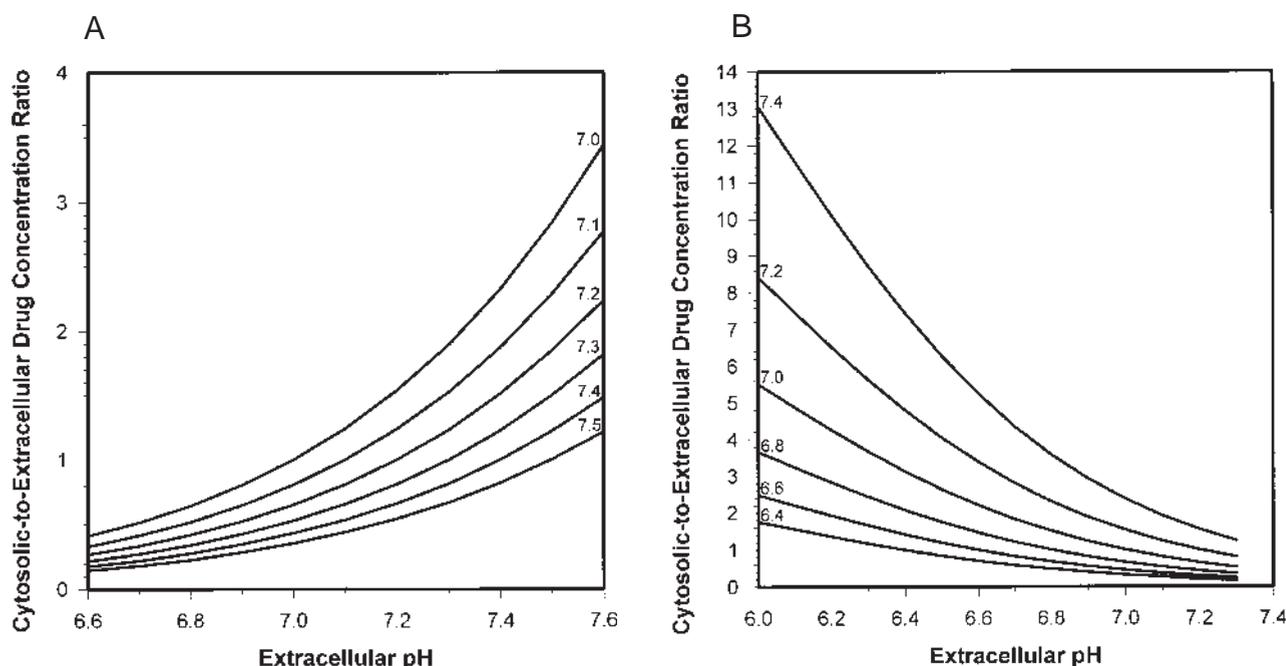

Fig. 2 **(A) Predicted intracellular-extracellular partitioning of a weak base drug with pK$_a$ = 8.25, at various extracellular and intracellular pH. (B) Predicted partitioning of a weak acid drug with pK$_a$ = 6.0, at various pH$_e$ and pH$_i$. Numbers on top of each curve refer to the intracellular pH. The theoretical calculations were made as in ref 15.**

compared to chlorambucil.[25] Tumor pH can also influence the uptake of drugs such as melphalan which are actively transported into the cell. As such, the cytotoxicity of melphalan has been reported to be enhanced in some cell types by acidification of tumor pH_i, despite the fact that it is a weak acid.[23,26] Tumor pH can also have indirect effects on cell sensitivity to drugs. Chronic exposure to acid pH is reported to increase expression of heat shock protein HSP27 levels in tumor cells, leading to resistance to cisplatin.[27] These in vitro findings are relevant to the situation in vivo, since large regions of experimental tumors have been shown by pH_e-imaging to be chronically acidic.[8] While a neutral-to-alkaline pH_i is more typical in tumors,[3,4,7] severely hypoxic regions in tumors can also have reduced pH_i, and this has been implicated in the accumulation of tumor cells in G_1 phase with consequent reduced sensitivity to topotecan and the zwitterionic drug paclitaxel.[28] Complicating the effect of tumor pH on the activity of topotecan, however, the fraction of topotecan existing in the uncharged and more permeant lactone form will increase at acid pH_e, thereby potentially improving drug uptake.[28] Acid tumor pH_e has also been shown to enhance the cytotoxicity of Mitomycin C on tumor cells in vitro.[24] Thus, tumor pH_e and pH_i exert a powerful influence on the cytotoxicities of a variety of commonly used chemotherapeutic drugs.

INHIBITORS OF pH_i REGULATION AS CYTOTOXIC AGENTS

Since pH_e in tumors tends to be substantially acidic, drugs which cause a systemic equilibration of pH_e and pH_i may have a cytotoxic effect that is selective for cells in the tumor. Pursuing that logic, Yamagata and Tannock[29] have treated KHT fibrosarcoma-bearing Balb/c mice by continuous infusion with the ionophore nigericin, which abolishes the plasmalemmal pH gradient. The mice were able to tolerate a cumulative dose of up to 22 mg/Kg nigericin, with a surviving cell fraction in the tumor of 0.1–0.2 at the end of a 72 h infusion. The two primary mechanisms of cellular pH regulation are the amiloride-inhibitable Na^+/H^+ antiporter[30] and the stilbene-inhibitable Na^+-dependent HCO_3^-/Cl^- exchanger.[31] Inhibition of these two pH_i recovery mechanisms, combined with reduction of the plasmalemmal pH gradient by means of nigericin, can potentially further enhance cell kill in acidic tumors. Furthermore, even greater acidification of the tumor can be achieved by reduction in blood flow to tumors relative to normal tissues. The vasodilator hydralazine is thought to act on the arteriolar smooth muscle, and since a large proportion of tumor blood vessels lack smooth muscle, they do not respond to the drug. Bhujwalla et al.[32] have argued that this leads to a redistribution of cardiac output away from the tumor, thus selectively reducing tumor blood flow, with a consequent reduction in tumor pH. They have shown a 0.4 pH unit drop in tumor pH_i 20–40 min following intravenous administration of hydralazine to RIF-1 tumor-bearing mice.[32] Yamagata and Tannock have shown that KHT and EMT-6 tumor bearing Balb/c mice are able to tolerate continuous 72 h infusions of nigericin (7.2 mg/Kg), the stilbene derivative DIDS (36 mg/Kg), and hydralazine (45 mg/Kg), with no animal deaths up to 2 weeks post-treatment. This protocol

Table I **Commonly used weak acid and weak base chemotherapeutic drugs and their most physiologically relevant pK_a values**

Weak base drugs	pKa	Reference
Bleomycin	7.4	16
Doxorubicin	8.34	17
Daunorubicin	8.46	17
Epirubicin	8.08	17
Idarubicin	8.4	18
Mitoxantrone	8.13	19
Vinca alkaloids	7.4–8.8	20
Weak acid drugs	**pK_a**	**Reference**
Chlorambucil	5.8	21
5-Fluorouracil	8.0*,#	22
Melphalan	*,#	23
Mitomycin C	#	24

* Actively transported into cell: drug uptake is not a function of pH_e, pH_i and pK_a alone.
\# Cytotoxicity is known to be potentiated by low pH_e.

resulted in cell surviving fractions in treated tumors of 0.02–0.03.[29] The addition of EIPA, a derivative of amiloride, to this protocol resulted in the deaths of EMT-6 tumor bearing Balb/c mice, but not of KHT tumor bearing C3H mice. In the latter case, the cell surviving fraction in tumors of treated mice was reduced to approximately 0.002–0.003.[29] EIPA alone was not found to be toxic to the animals, and it is unclear whether the toxicity of EIPA when it is part of the whole cocktail is related to its ability to inhibit Na^+/H^+ antiport or some other effect. A more potent and specific inhibitor of Na^+/H^+ antiport, cariporide (HOE 642), is currently in phase II/III clinical trials for evaluating its efficacy in preventing reperfusion injury following myocardial ischemia.[33] Given the promising results of Tannock and colleagues, systemic toxicity and tumor cell kill efficacy testing of cariporide in combination with nigericin, DIDS and hydralazine in animal models are warranted.

ACIDIFICATION OF TUMOR pH_E TO ENHANCE WEAK ACID DRUGS

A metabolic characteristic of tumor cells which distinguishes them from normal cells is the phenomenon of aerobic glycolysis. Induced hyperglycemia has been successfully employed to accentuate the pH differences between tumor and normal tissues in animal models.[34] However, the extreme hyperglycemia (plasma glucose levels ~ 30 mM) required to achieve tumor-selective acidification is accompanied by systemic hemoconcentration and increased red blood cell rigidity, resulting in reduced tumor blood flow and reduced drug delivery to tumor tissue.[35] Kuin et al.[35] report that intraperitoneal administration of m-iodobenzylguanidine (MIBG), an inhibitor of mitochondrial respiration, to RIF-1 tumor bearing mice reduces the degree of hyperglycemia required

(plasma glucose levels ~ 14 mM) to produce tumor-specific acidification of pH_e. While combined treatment with MIBG and glucose produced reductions of tumor pH_e (estimated using microelectrodes) on the order of 0.7 pH unit, this regimen did not produce significant changes in tumor pH_i (measured by ^{31}P MRS). As can be seen from Figure 2B, such a striking increase in the (pH_i–pH_e) gradient can substantially enhance the cellular uptake of weak acid drugs like chlorambucil, a result that has been verified in vitro in more than one cell type.[25,36] Recently, Kuin et al.[37] have shown that oral administration of MIBG to mice results in therapeutically meaningful bioavailability with acceptably low renal and gastrointestinal toxicities. Intravenous MIBG and glucose are already used clinically, and this should facilitate the translation of these techniques for tumor-selective acidification to humans.

ACIDIFICATION OF TUMOR pH_I TO ENHANCE DRUG ACTIVITY

The previous two sections have discussed the use of drugs that disrupt pH_i regulation in tumors for therapeutic purposes, as well as techniques to acidify tumor pH_e in order to enhance the efficacy of weak acid drugs. These two approaches can be combined in order to acidify tumor pH_i. The cytotoxicities of melphalan, mitomycin C and paclitaxel are potentiated in vitro by acidification of pH_i in the target cells.[23,24,28] Wood et al.[26] report that the ionophore nigericin lowered tumor pH_i and induced a significant increase in growth delay of RIF-1 tumors over that effected by treatment with melphalan alone. However, this effect was tumor dependent and nigericin did not enhance melphalan-induced cell killing in two other tumor models. Kuin et al.[36] have treated C3H mice bearing RIF-1 tumors with either MIBG plus glucose, or benzylguanidine plus glucose, in order to reduce tumor pH_e. Following this treatment, amiloride and DIDS were administered to inhibit pH_i normalization. The resulting acidification of tumor pH_i resulted in a significant increase in tumor response to melphalan, although the enhancement of tumor response to mitomycin C was seen only with tumors larger than 5 mm.[36] MIBG, amiloride and glucose are already used clinically. There are no literature reports on the clinical use of the bicarbonate/chloride exchange inhibitor DIDS to this point, but it is promising that animals are able to tolerate therapeutically meaningful doses of this stilbene.

ALKALINIZATION OF TUMOR pH_E TO ENHANCE WEAK BASE DRUGS

The acidic pH_e that is characteristic of many tumors will act to protect tumor cells from weak base drugs, as seen in Figure 2A. Gerweck and colleagues have demonstrated that low pH adapted CHO cells maintain a larger acid-outside plasmalemmal pH gradient than normal CHO cells.[25,38] They have also demonstrated a clear relationship between increasing medium pH and increasing cytotoxicity of doxorubicin to cultured CHO cells.[38] Thus, one potential means of sensitizing cells in acidic tumors to weak base drugs like mitoxantrone and doxorubicin would be to selectively alkalinize tumor tissue. Sodium bicarbonate has long been used to treat

metabolic acidosis in humans resulting from renal failure,[39] and to mitigate exercise-induced acidosis.[40] Tumor interstitial fluid has a reduced buffering capacity compared to normal tissue, and in combination with poor perfusion of tumors this leads to acidification in response to increased lactic acid secretion by tumor cells.[41] Sodium bicarbonate could potentially selectively alkalinize tumors by increasing the buffering capacity of tumor interstitial fluid, with only minor effects on normal tissue pH_e. We have employed ^{31}P MRS to measure pH_e and pH_i in MCF-7 human breast carcinoma xenografts in SCID mice which were chronically treated with ad lib water containing 200 mM $NaHCO_3$. Tumors in control mice had a pH_e of 7.02±0.16 and pH_i of 7.20±0.13 while tumors in $NaHCO_3$-treated mice had pH_e of 7.85±0.13 and pH_i of 7.38±0.12.[42] ^{31}P MRS of control hind leg muscle tissue showed only a minor alkalinization of pH_e in bicarbonate-treated animals. This selective alkalinization of tumor tissue resulted in a 2-fold enhancement of the tumor growth delay induced by doxorubicin in MCF-7 tumors.[42] The lifetime of free drug in mice following intravenous administration is on the order of only one hour for drugs like mitoxantrone,[43] and for the purposes of enhancement of uptake of weak base drugs, acute alkalinization lasting 1–2 h may suffice. Acute administration of sodium bicarbonate is also more easily translated to the clinic than chronic bicarbonate treatment. Carbicarb, an equimolar mixture of sodium bicarbonate and sodium carbonate, has been reported to be suitable for intravenous administration and capable of inducing acute systemic metabolic alkalosis without the major changes in arterial Pco_2 and intracellular acidification that is sometimes associated with sodium bicarbonate therapy.[44] Leung et al.[45] have determined that intravenously administered carbicarb is as effective as sodium bicarbonate in correcting metabolic acidosis in humans, and produces a 0.05 pH unit increase in blood pH. A similar magnitude of alkalinization of arterial blood has been reported by Van de Ven et al.[46] following the use of furosemide, a diuretic which causes 'anion-gap' related alkalinization of blood and other fluids. These results are consistent with the small alkalinization of pH_e in normal hind leg tissue which we have found in mice treated with orally administered sodium bicarbonate. However, the alkalinization of tumor pH_e was significantly larger, thus providing a gain in therapeutic index.[42] Application of carbicarb or furosemide therapy to enhance the chemotherapeutic efficacy of weak base drugs awaits confirmation of the tumor-selective nature of the resulting alkalinization. Thus far, clinical measurements of response to sodium bicarbonate, carbicarb and furosemide therapy have focused on changes in blood and urine pH. But non-invasive MRS techniques for measuring tissue pH_e are now becoming available,[5,8] and it should soon be possible to follow tumor pH changes in humans consequent to systemic alkalinization therapies.

CHEMOTHERAPY OF CANCERS IN NATURALLY ACIDIC TISSUES

Tissue pH is a potentially important parameter affecting the chemotherapy of bladder carcinoma, some renal carcinomas, and gastrointestinal cancers. Superficial bladder cancer is commonly treated with transurethral resection of the tumor

followed by intravesical instillation of a combination of drugs to reduce the incidence of disease recurrence. Chemotherapy may include drugs like 5-fluorouracil, which would be potentiated by the acid pH of urine. Many regimens also include weak bases like the anthracyclines[47] which would be inhibited by urine pH_e. Intravesical pharmacotherapy maximizes drug delivery to the tumor and its immediate surroundings, while minimizing systemic exposure to the drug.[48] Wientjes et al.[49] have proposed the alkalinization of urine prior to intravesical chemotherapy, in order to preserve the activity of the acid-degradable mitomycin C. However, the activity of mitomycin C is greater under acidic conditions,[24] and the urine pH that optimizes the balance between stability and activity of mitomycin C needs to be identified. Since mitomycin C and doxorubicin are often used together in intravesical chemotherapy for bladder cancer,[47] alkalinization of urine in order to reduce degradation of mitomycin C should enhance tumor uptake of doxorubicin as well. Ekstrom et al.[50] have determined that alkalinization of the instillate from pH 5 to 9.2 resulted in a 3-fold increase in drug retained in the bladder muscle layer in rabbits treated with terodiline, as well as a 3-fold increase in the serum concentration of terodiline. However, there was a 100-fold difference in drug concentration between the bladder muscle layer and serum regardless of the pH of the instillate.[50] Thus, one may expect alkalinization of the contents of the bladder prior to intravesical instillation of a weak base drug to not only increase uptake of drug into the bladder wall, but also drive more drug into the systemic circulation. This protocol should therefore only be used with drugs that have poor systemic penetration from the bladder.

Systemic chemotherapy regimens involving the use of weak bases like doxorubicin and vinblastine have also made a substantial impact on muscle-invasive and metastatic bladder cancer.[51] When administered intravenously in dogs, up to 38% of vinblastine and 13% of vincristine have been reported to be secreted unmetabolized into urine in a single pass, via a P-glycoprotein-mediated process.[52] In humans, 6% of the total dose of doxorubicin and epirubicin administered intravenously are excreted unmetabolized into urine within 48 h post-injection.[53] This unmetabolized pool of excreted drug can be recruited to enhance the therapy of cancers of the urinary tract. Krarup-Hansen et al. report that oral ingestion of 2.5 g $NaHCO_3$ three times daily, beginning 2 days prior to doxorubicin infusion and ending 2 days after doxorubicin infusion, did not alter the plasma pharmacokinetics of the drug but did raise the urine pH to 8.0 in human patients.[54] The orally administered diuretic acetazolamide, an inhibitor of carbonic anhydrase, is also known to achieve significant alkalinization of urine in humans.[55] Given the high amounts of unmetabolized vinca and anthracycline drug found in urine after intravenous administration, serious consideration should be given to augmenting the systemic chemotherapy of muscle-invasive and metastatic bladder cancer with oral administration of $NaHCO_3$ and/or acetazolamide. Drug-partition calculations similar to those shown in Figure 2A indicate that alkalinization of urine from pH 6 to pH 8 can result in a greater than 10-fold enhancement of uptake of vinca and anthracycline drugs into tumor cells facing the bladder lining. The resulting increase in tumor response would be desirable,

given the moderately high post-resection recurrence of the tumor at the primary site in disseminated bladder cancers.[51] One concern with systemic administration of sodium bicarbonate to alkalinize urine might be that the resulting metabolic alkalosis could adversely alter the pharmacokinetics of the drug and reduce drug activity against the disseminated disease. The results of Krarup-Hansen et al.[54] indicate that oral ingestion of sodium bicarbonate in quantities that cause substantial alkalinization of urine do not, however, significantly alter the pharmacokinetics of doxorubicin in humans. Another concern might be that the ingested sodium bicarbonate could increase blood pH and drive more drug into normal tissues, thus increasing toxicity to non-target tissues. Krarup-Hansen report that their protocol produced a less than 0.05 pH unit increase in arterial blood pH,[54] an increase that is unlikely to significantly alter drug partitioning to normal tissues.

Renal cell carcinoma is considered a drug-resistant tumor, with low response rates to standard cytotoxic agents including paclitaxel and 5-fluorouracil. Combination chemotherapy with the weak base drugs vinblastine and doxorubicin has been reported to achieve a modest 14% response rate, and the addition of modulators of P-glycoprotein-mediated drug resistance such as cyclosporine A and quinidine do not result in enhanced antitumor activity [reviewed in 56]. Thus, the drug resistance exhibited by renal cell carcinomas, which primarily arise from renal proximal tubular epithelium, appears to have components which are not P-glycoprotein-mediated.[56] It is conceivable that some of this resistance to vinca and anthracycline drugs arises from the partitioning of these weak base drugs into the acidic post-glomerular filtrate. As with bladder cancers, alkalinization of post-glomerular filtrate by oral administration of $NaHCO_3$ or acetazolamide prior to administration of vinblastine and doxorubicin, can yield a significant increase in uptake of these drugs into the renal carcinoma cells, potentially chemo-sensitizing this highly resistant tumor. It remains to be determined whether such alkalinization therapies will increase toxicities of these chemotherapeutic drugs to normal renal tissue as well.

Gastric cancer is a fairly chemosensitive adenocarcinoma which has a high incidence in East Asia, South America and Eastern Europe. While surgical resection remains the primary treatment, chemotherapy is beneficial in patients with advanced gastric carcinoma.[57] While chemotherapy by intravenous drug administration remains common, patient convenience and cost-containment issues have led to the development of oral agents like UFT, a 4:1 molar ratio mix of uracil and tegafur [reviewed in 58]. Tegafur spontaneously degrades to yield 5-fluorouracil, and can also be converted to 5-fluorouracil by thymidine phosphorylase in the tumor tissue and by the cytochrome p-450 enzyme system in the liver.[58] Based on its structure, tegafur appears to be a weak acid, and its entry into tumor cells would be facilitated by the very low pH characteristic of the stomach. Thus, from the standpoint of local pH_e, UFT is a well-designed oral agent for treatment of gastric carcinoma. Oral UFT is gaining use in the treatment of colonic neoplasms,[59] and it is worth investigating whether a reduction in colonic pH by dietary manipulation, concomitant with ingestion of UFT, will enhance the effectiveness of UFT against colon cancer.

DRUG RESISTANCE AND ACIDIC INTRACELLULAR VESICLES

Classical multidrug resistance is associated with the overexpresion of drug pumps like P-glycoprotein (P-gp), a 170–180 kD plasma membrane glycoprotein[60] or the multidrug resistance-associated protein (MRP) which has been cloned from a multidrug resistant, doxorubicin-selected cell line that did not overexpress P-glycoprotein.[61] More recently, a breast cancer resistance protein (BCRP) which confers an ATP-dependent resistance to anthracycline and anthraquinone drugs has been described.[62] And, as discussed by us so far, cells can also be conferred a 'physiological drug resistance' by the presence of a large acid-outside plasmalemmal pH gradient which acts to keep out weakly basic drugs. This manner of 'ion trapping' is also implicated in the transport to and trapping in acidic vesicles of weak base drug molecules from the cytosol. There is also evidence that suggests that the sequestered drug may be continuously extruded from the cell by exocytosis, in what could effectively function as a 'drug pump' for a class of drugs, the lipophilic weak bases. For instance, Sehested et al.[63] have reported observing greatly enhanced rates of endocytosis, as well as a 3–4 fold increase in endosomal volume, endosomal surface area and number of endosomes in drug resistant P388 leukemic cells, as compared to their drug-sensitive parent cells. Seidel et al.[64] 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 moved to the cell periphery. The lung resistance protein (LRP) has also been hypothesized to be involved in vesicle-related extrusion of drug molecules away from cell nuclei.[65] Other indirect evidence for the involvement of acidic vesicles in the phenomenon of MDR links vesicle alkalinization with diminished resistance to drugs. For example, Dubowchik et al.[66] have found that some imidazole compounds, in addition to rasing lysosomal pH, also reversed drug resistance in a doxorubicin-resistant human colon carcinoma cell line. Sehested et al.[67] 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 EAT cells. We have found that drug-resistant MCF-7 human breast carcinoma cells exhibit V-type H^+-ATPase activity at the plasma membrane, as well as increased rates of endosomal turnover as compared to the drug-sensitive parent MCF-7 cells.[68] There is also evidence that these vesicles may be predominantly perinuclear in location, possibly affording greater protection to the nucleus.[69] Theoretical calculations by us, however, indicate that passive drug sequestration by ion trapping alone will not result in sufficient concentration of drugs like the anthracyclines in vesicles to meaningfully reduce steady state cytosolic drug levels at realistic rates of exocytosis.[15] Model calculations suggest that active transport of drug molecules from the cytosol into acidic exosomes will be required for sufficient reductions in steady state cytosolic drug concentrations by exocytosis.[15]

Recently, Hogue et al.[70] have reported that the mouse transporter protein (MTP), a highly conserved transmembrane protein, confers drug resistance upon expression in intracellular organelles in a drug-sensitive strain of *Saccharomyces cerevisiae*. MTP was found to regulate the subcellular localization of a variety of molecules, including cationic lipophilic drugs such as anthracyclines. MTP has been shown to be localized to late endosomes and lysosomes in mammalian cells, and is thought to be involved in transport of small amphiphilic and hydrophobic molecules across endosomal and lysosomal membranes.[71]

There are thus strong indications, but not direct proof, that V-type H^+-ATPases, endosomes, and endosomal/lysosomal transporters like MTP are involved in the phenomenon of multidrug resistance. Attempts to reverse this putative acidic vesicle-mediated drug resistance are currently only being made in cultured cells in vitro, and are being carried out at two levels-by interfering with the acidification of the vesicles, and by interfering in the endocytotic/exocytotic fluid-phase trafficking of the vesicles. Alkalinization of intracellular endocytic vesicles can be accomplished either by the use of lysosomotropic agents or through the use of drugs which have been reported to interfere with vacuolar acidification. Bafilomycin A1 and concanamycin A, members of the macrolide family of antibiotics, are well known specific inhibitors of vacuolar-type H^+-ATPase, and have been demonstrated to reverse drug resistance in MCF-7 human breast cancer cells in vitro.[72] The anticancer drug lonidamine has been reported to increase the proton permeability of endosomal-lysosomal membranes, thereby inhibiting ATP-driven acidification of these vesicles.[73] Lonidamine has been reported to significantly enhance the chemotherapeutic efficacy of epirubicin[74] and moderately enhance the activity of doxorubicin[75] in breast cancer patients. Tamoxifen, an anti-estrogen stilbene which has been extensively used in the treatment of estrogen-dependent breast cancers in humans, has been reported to inhibit the acidification of endosomes and lysosomes in cancer cells in vitro, without affecting cytosolic pH.[76] Monensin, in addition to alkalinizing normally acidic intracellular vesicles, also disrupts vesicular trafficking.[67] Thus, a small but growing number of drugs which inhibit vesicular acidification and trafficking are becoming available. The non-specific inhibitors of vesicular acidification, lonidamine and tamoxifen, are already in use in humans for their other therapeutic effects, while cost considerations have so far restricted the in vivo testing of bafilomycin and concanamycin. Direct proof of the involvement of acidic vesicles in drug resistance, as well as detailed studies in whole animals of the above mentioned inhibitors of vesicular traffic and acidification are required, before they can be translated to the clinic.

CONCLUSIONS AND FUTURE DIRECTIONS

Uptake of weak acid and weak base chemotherapeutic drugs by tumors is greatly influenced by the extracellular (interstitial) pH impinging on the cells in the tumor, the intracellular pH maintained by the cells, and by the ionization properties of the drug itself. Non-invasive pH measurement and pH-imaging modalities reveal the presence of large regions of acidic extracellular pH in tumors, with the intracellular pH

being maintained in the neutral-to-alkaline range. Agents like hydralazine, which reduces tumor blood flow, MIBG, which inhibits mitochondrial respiration, and glucose, which enhances aerobic glycolysis in tumor cells, have been demonstrated to selectively lower tumor pH_e. This selectively enhanced tumor acidity may itself be used for targeting cells in a tumor, through the use of ionophores like nigericin which tend to equilibrate pH_e and pH_i, and inhibitors of pH_i regulatory mechanisms like EIPA and DIDS. Tannock and colleagues have achieved mixed results in tumor-bearing mice with this treatment protocol, which was fatal in one mouse system, but reduced the tumor cell survival fraction to 0.002–0.003 in another. Cariporide (HOE 642), a new inhibitor of Na^+/H^+ antiport which is more potent and specific than amiloride derivatives, may be useful in lowering the toxicity of this treatment protocol to acceptable levels. However, it is likely that the toxicity of this protocol results from the combined toxicities of each of the drugs in the treatment regimen, and this may not be altered by the use of cariporide in place of EIPA. A reduction in the dose of EIPA or one or more of the other components of the combination may yet yield meaningful tumor cell kills with acceptable toxicity. This acid-outside plasmalemmal pH gradient in tumors can also be enhanced and exploited to drive the uptake of weak acid drugs into tumors. Induced hyperglycemia, combined with inhibition of mitochondrial respiration by MIBG, has been demonstrated to significantly lower pH_e but not pH_i in tumors, creating an increase in the (pH_i – pH_e) gradient which would substantially enhance the uptake of weak acid drugs like chlorambucil. Oral administration of MIBG has been shown to result in meaningful bioavailability and low renal and gastrointestinal toxicities in mice. With intravenous MIBG and glucose already in use in humans, this protocol shows promise for translation to humans for enhancing the efficacy of weak acid anti-cancer drugs. Combination therapy with MIBG, glucose, EIPA and DIDS has been shown to lower tumor pH_i in mouse models of cancer, thereby enhancing tumor response to drugs like melphalan and mitomycin C which are activated under acidic conditions. While MIBG, glucose and EIPA are already in use in humans, reports of the use of DIDS in humans are hard to find. Nonetheless, DIDS is well tolerated by mice and rats, and tumor pH_i acidification to enhance drugs like mitomycin C and melphalan appears feasible in humans.

The acid-outside plasmalemmal pH gradient also acts to exclude weak base drugs like the anthracyclines, anthraquinones and vinca alkaloids from the cells. A substantial degree of 'physiological drug resistance' to weak base drugs may thus exist in tumors. Induction of metabolic alkalosis by oral administration of sodium bicarbonate has been shown to reverse the direction of the plasmalemmal pH gradient in experimental tumors in mice, yielding a 2-fold enhancement in doxorubicin effectiveness. Protocols for manipulation of systemic pH in humans by oral and intravenous administration of sodium bicarbonate, carbicarb, or diuretics like furosemide are described in the literature, and significant alkalinization of urine and moderate alkalinization of blood have been reported. In order for these alkalinization protocols to yield increases in the chemotherapeutic indices of weak base drugs, the resulting increase in tumor pH_e would need to be greater than any increase in blood pH. Chronic oral administration of sodium bicarbonate to mice has been reported to produce significant alkalinization of tumor pH_e but only minor changes in the pH_e of normal hind leg tissue and the pH_i of normal and tumor tissue. Such a tumor-selective alkalinization of pH_e, if reproduced in humans, would provide a mechanism for enhancing tumor response to weak base drugs.

The native extracellular pH becomes especially important in the therapy of bladder, renal and esophago-gastric cancers where extreme acidity of the extracellular milieu is the norm. A significant fraction of intravenously administered weak base drugs like vinblastine and doxorubicin is rapidly excreted into the urine by the kidneys. Oral ingestion of sodium bicarbonate and acetazolamide have both been shown to significantly increase urine pH in humans. Such an alkalinization, if induced concomitant with drug administration, can potentially yield a greater than 10-fold enhancement of uptake of drugs like doxorubicin and vinblastine from the urine (or post-glomerular filtrate) by bladder (or renal) cancer cells. It may also drive more weak base drugs into normal tissues, potentially increasing their toxicities. However, sodium bicarbonate and acetazolamide produce significantly greater alkalinization of urine (~2 pH units) than blood (~0.05 pH units), and a gain in chemotherapeutic indices of weak base drugs directed against renal and bladder cancers is a real possibility. This would be significant, given the innate drug-resistance of renal cell carcinoma, and the recurrence of treated bladder cancers at the primary site. The acid pH of the stomach will enhance the uptake of weak acid drugs like tegafur, making it an excellent choice for the therapy of esophago-gastric cancers. Oral acidification regimens may also enhance tumor response if used as an adjunct to therapy of intestinal cancers with orally administered weak acid drugs like tegafur and 5-fluorouracil.

Acid pH is also implicated in a different mechanism of drug resistance in cancers – the transport from the cytosol and sequestration in acidic intracellular vesicles of weak base drugs, for possible extrusion from the tumor cell by exocytosis. Such 'ion trapping' of weak base drugs in these vesicles appears to be enabled by the proton gradient at the vesicular membranes, as well as by the action of transporters like the mouse transporter protein. The steady-state extrusion of sequestered drug from the cell by exocytosis would constitute a non-specific mechanism for reduction of the cytosolic drug concentration, although this has yet to be conclusively proven. Inhibitors of vesicular trafficking and acidification have been demonstrated in vitro, but further investigation of vesicle-mediated mechanisms of drug resistance is required before such drugs can be tested in vivo for toxicity and efficacy.

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