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13. ABSTRACT (Maximum 200) The pH of tumors plays an important role in the efficacy of chemotherapy and radiotherapy and may have relevance to carcinogenesis itself. During the past year, we have generated a quantitative model and empirical evidence to support a role for the recycling of endocytic vesicles in the resistance to weakly basic chemotherapeutic drugs (Project 1). A spinoff of this project is the isolation of these acidic vesicles and characterization of their activity. A technique has been developed to simultaneously measure the extracellular and intracellular pH of tumors using magnetic resonance spectroscopy (MRS, project 2). This technique has been refined to accurately report the entire extracellular pH range within an entire tumor. This is significant because acidic pH is both clastogenic and mutagenic. As a result of these measurements we have been able to design and monitor protocols to chronically raise the extracellular pH of tumors (Project 3). The effect of these treatments on chemosensitivity is being tested. Finally, because extracellular pH in tumors is expected to be heterogenous, we have begun developing methods to measure pHe using 1-H MRS of exogenous imidazoles (project 4). With this method, we will be able to generate maps of pHe in living tumors.				
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FOREWORD

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INTRODUCTION

The pH of tumors plays an important role in the efficacy of chemotherapy and radiotherapy, and may have relevance to carcinogenesis itself.

Design of therapy using weakly basic chemotherapeutic agents, such as doxorubicin, requires accurate knowledge of the extracellular pH (pHe) range within tumors. Resistance to weakly basic chemotherapeutic drugs (WBDR) can be augmented by acidic pHe. A quantitative model has been developed to demonstrate that the turnover of acidic vesicles within cells can also contribute to WBDR. During the past year, we have supplied empirical evidence in support of this model and a manuscript has been submitted (Project 1). A spinoff of this project is the isolation of these acidic vesicles and characterization of their activity.

A technique has been developed to simultaneously measure the pHe and the intracellular pH (pHi) of tumors in vivo using magnetic Resonance Spectroscopy (MRS). During the past year, this technique has been refined to accurately report the entire pHe distribution within the tumor (Project 2). This is significant since, at the low extremes, acidic pH has been reported to be mutagenic and clastogenic. These techniques have been used to monitor the response of tumor pH to exogenous bicarbonate. These treatments chronically raise the extracellular pH of tumor tissue, which should increase the efficacy of weakly basic chemotherapeutic drugs (Project 3).

Because the extracellular pH in tumors is heterogeneous, we have begun developing methods to measure pHe using 1-H MRS of exogenous imidazoles (project 4). With this method, we will potentially be able to obtain pH measurements from volumes as small as 1 mm³.

BODY

Project 1. Turnover of Acidic Vesicles and Multidrug Resistance.

A major barrier to successful chemotherapeutic treatment of cancer is the development of drug resistance by cells in tumors. Cells acquiring resistance to one type of drug almost always show resistance to other chemically unrelated drugs. Hence, the phenomenon is known as multidrug resistance (MDR). The most well-studied mechanism of multidrug resistance involves the MDR-1 gene product, P-glycoprotein (P-gp), a drug pump which also transports protons (Thiebaut et al., 1990; Boscoboinik et al., 1990). However, P-gp accounts for only 50% of the observed MDR phenotypes (Gottesman and Pastan, 1993). Tumor pH can contribute to the resistance to weakly basic drugs, and this can be attributed to the phenomenon of "ion trapping", wherein the drugs are accumulated in acidic compartments and excluded from alkaline compartments (Tannock and Rotin, 1989; Jahde et al., 1990; Roepe et al., 1993; Simon et al., 1994). Weakly basic drug resistance (WBDR) is not classically MDR, since the drugs are structurally related. However WBDR is clinically important since weak base chemotherapeutic drugs, such as mitoxantrone and doxorubicin (adriamycin), are commonly used to treat cancer.

Ion trapping is relevant to WBDR not only because of the acidic tumor environment (see above), but also by sequestration of drugs into acidic vesicles. Intracellular compartments are acidified through the action of V-ATPases, and there is considerable evidence that these pumps can be involved in multidrug resistance. Sehested et al. (1987) observed enhanced rates of exocytosis in multidrug resistant EAT cells, compared to their drug-sensitive counterparts. Dubowchik et al. (1994) have shown that lysosomotropic weak bases raise intravesicular pH as well as reverse doxorubicin resistance in NCT116-VM46 colon carcinoma cells. Similar results were presented by Sehested et al. (1988).

We have investigated the role of pH in WBDR using a cell line, MCF/7-mitox, which displays an MDR phenotype, yet does not possess P-gp (Taylor et al., 1991). MCF/7-mitox cells treated with

mitoxantrone sequester it into subcellular vesicles (Willingham et al., 1986). We hypothesize that overexpressed V-ATPase activity at the plasma membrane (pmV-ATPase) may be involved in the MDR phenotype of these cells (e.g. Marquardt and Center, 1991). Both MCF/7-mitox as well as P-gp-positive MCF/7-dox cells contain pmV-ATPase activity, while parental MCF/7 cells do not (**Figure 1**).

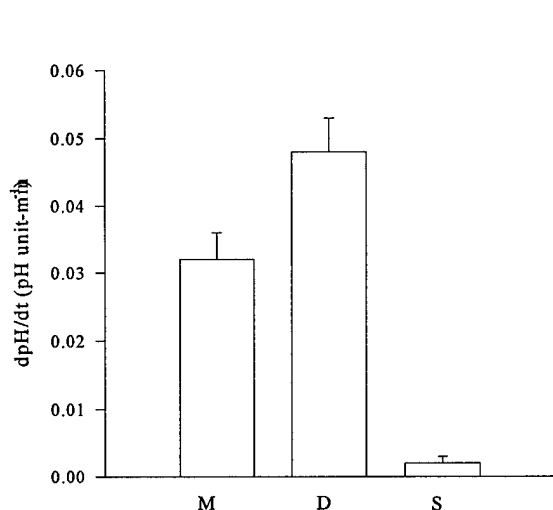


Figure 1. pmV-ATPase and drug resistance. pmV-ATPase activity in MCF/7, MCF/7-mitox and MCF/7-dox cells. Cells were acidified with a pre-pulse of NH_4Cl and then perfused with buffer at pH 8.0 lacking Na^+ and HCO_3^- . Recovery is due to pmV-ATPase and is inhibitable with bafilomycin.

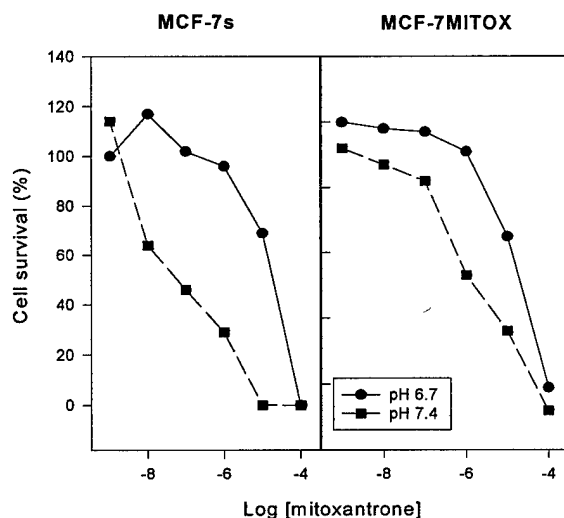


Figure 2. Low pHe induces drug resistance in vitro. Cells used were MCF-7S and MCF-7MITOX cultured in 96-well plates. Mitoxantrone was added to the indicated concentrations and, cell number determined 96 hours later, as described in text. Tests were performed at pHe 6.7 and 7.4

We have written a manuscript which details the effect of acidic vesicle recycling on resistance to common, weakly basic chemotherapeutic drugs, such as mitoxantrone and doxorubicin (adriamycin). Using conservative estimates for parameters of vesicle size, number, pH and turnover rate, it can be clearly demonstrated that: 1) vesicle movement contributes greatly to a multidrug-resistant phenotype, 2) activity of drug pumps (i.e. P-gp) that are statically resident on the plasma membrane would be insufficient to confer drug resistance, and 3) the activity of V-ATPase combined with a drug/ H^+ exchange activity would be kinetically identical to the activity of p-glycoprotein.

Drug sensitivity in vitro. At appropriate concentrations, chemotherapeutic drugs added to cultured cells will induce apoptosis. The resulting reductions in cell number can be monitored in a 96-well assay by staining the cells 3-4 days following treatment with vital dyes, such as MTT, or chromatin-specific metachromatic dyes, such as crystal violet (Gillies et al., 1986). The resulting optical densities are then plotted as a function of drug concentration to yield cell survival curves. **Figure 2** illustrates the survival of human MCF-7 breast cancer cells following treatment with mitoxantrone, a weak base chemotherapeutic agent. As shown in this figure, at a medium pH of 7.4 (squares), drug sensitive cells (MCF-7S) are killed at lower concentrations of mitoxantrone than are multidrug resistant MCF-7MITOX cells. At a medium pH of 6.7 (circles), the MCF-7S cells exhibit a sensitivity similar to that found in the drug-resistant cells. At pHe 6.7, there is a greater pH gradient (pHi-pHe) in both cell lines (data not shown). Thus, as predicted, low pHe (and a greater pH gradient) reduces the effectiveness of weak base chemotherapeutics.

Project 2. Measurement of Interstitial pH of Tumors by ^{31}P MRS.

Introduction. Extracellular pH in tumors has been found to have a bearing on the effectiveness of different treatment strategies. Increasing tumor acidity increases thermal sensitivity of tumors and may also play a role in the effectiveness of chemotherapeutic agents, many of which are weak bases (Tannock and Rotin, 1989). The partition of weak base molecules across the cell membrane is dependent on intra- and extracellular pH. Knowledge of pH distribution within tumors thus has clinical relevance. The chemical shift of the ^{31}P nucleus in 3-aminopropylphosphonate (3-APP), a cell-impermeant molecule, is pH-sensitive, making it a good indicator of extracellular pH (Gillies et al., 1994). A robust calibration equation for the chemical shift of 3-APP has been reported earlier by us which can be used to calculate the pH distribution *in vivo*. However, the lineshape of the 3-APP peak is dependent not just on the pH distribution within the sample, but is also broadened by T_2^* effects.

Over the past few years, we have developed the application of 3-aminopropylphosphonate (3-APP) as a ^{31}P labeled indicator of *in vivo* extracellular pH (Gillies et al., 1994). Under the auspices of the current grant, we have developed a method to deconvolve the T_2^* component from the 3-APP resonance to yield estimates of the *in situ* pHe distribution throughout tumors. This has been described in a peer-reviewed abstract (Raghunand et al., 1997a), and is in the process of being written up as a full manuscript (Raghunand et al., 1997b). Deconvolution is obtained by determining the T_2^* of 3-APP using a pH-insensitive resonance (e.g. the H-2 of 3-APP), then removing this contribution from the 3-APP resonance, leaving the chemical shift dispersion arising solely from the heterogeneity of pHe. Results of this analysis are shown in **figure 3**. Note that the deconvolved resonances are much narrower than the native. The calculated pHe distributions is shown as an inset.

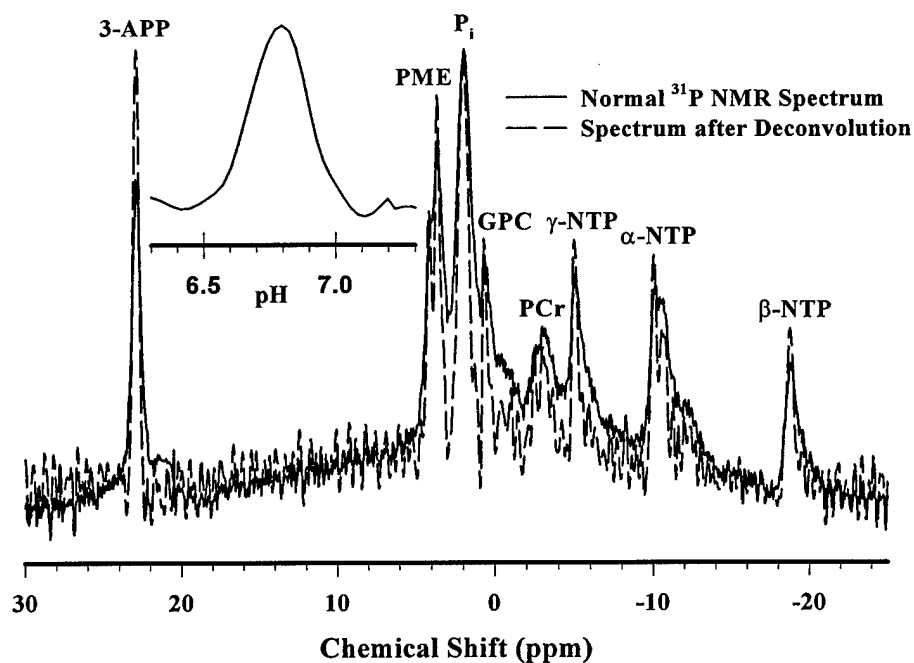


Figure 3. ^{31}P MRS spectrum of a 0.9 cc MCF-7/Mitox tumor. Inset shows the pHe distribution obtained by deconvolution of the 3-APP peak.

Although the mean pHe decreases with increasing tumor size, the range of values is consistently ca. + 0.3 pH unit about the mean at all tumor sizes (figure 4). These measurements are important because they will define the lowest pHe faced by a sub-population of cells within tumors. If low pHe does enhance development of later stages of carcinogenesis, it would be more enhanced in the small fraction of cells residing in the lowest pHe environment.

Project 3. pH and Drug Resistance in Vivo.

The first two projects show that chemotherapeutic efficacy is reduced by low pH in vitro and that the pH of tumors is low, establishing an alkaline-inside pH gradient which increases as tumors get larger. As shown below, this pH gradient is larger in tumors of drug-resistant cells. In this project, we will ask the question whether reducing the pH gradient can have a beneficial effect on chemotherapy in vivo. In order to accomplish this, reliable methods to manipulate extracellular pH are required.

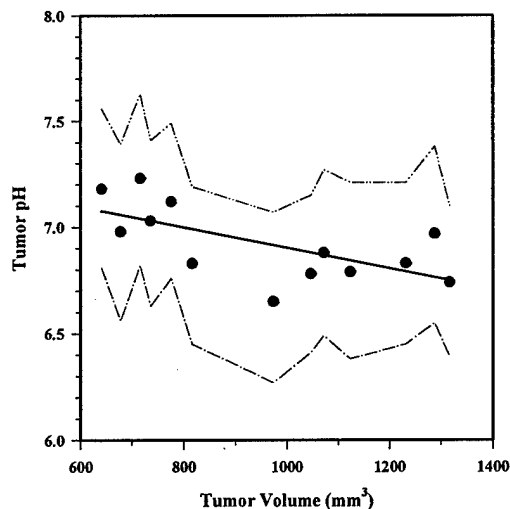


Figure 4. pHe as a function of tumor size. MCF-MITOX tumors were grown to different sizes and the pHe range determined by deconvolution of the 3-APP resonance.

Tumor pH

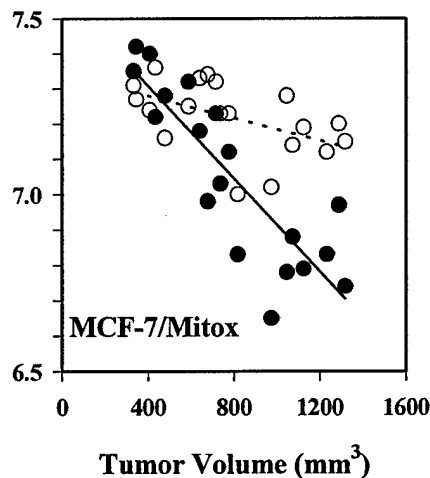
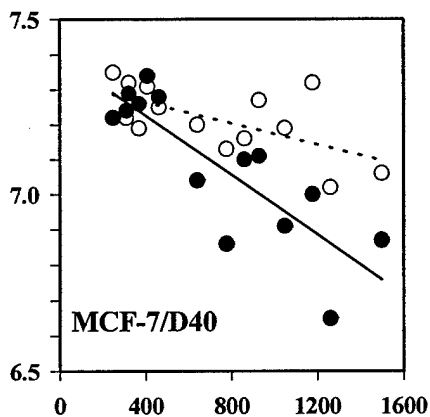
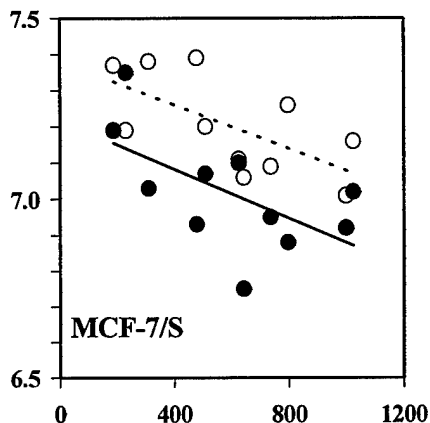


Figure 5. Intra- and extracellular pH of drug-sensitive and drug resistant breast cancer cells in vivo as measured by MRS.

pH of drug-resistant tumors in vivo.

The pHi and pHe of tumors from MCF-7S, MCF-7MITOX and MCF-7DOX cells grown in SCID mice have been determined as a function of tumor growth. pHi and pHe values were determined from the chemical shifts of Pi and 3-APP, respectively. As shown in **figure 5**, the pHe of all three tumors are not significantly different, and all decrease with increasing tumor size. In contrast, the pHi values of the drug-resistant cells remain high, while the pHi of the drug-sensitive cells drops along with the decrease in pHe. We believe that these differences in the pH gradient may be significant contributors to weak base chemotherapeutic resistance.

Manipulation of interstitial pH of tumors

There are some literature data on the ability to acutely manipulate pH up and down with bicarbonate, and ammonium chloride, respectively (e.g., Whitford and Angmar-Mansson, 1995). We therefore adopted these methods by adding bicarbonate and ammonium chloride in increasing concentrations to the ad lib drinking water. Water intake, animal weight and tumor size were all monitored for each individual animal in this trial. 16 animals were involved: 8 on bicarbonate and 8 on ammonium chloride. After the initial day, all animals consumed an adequate amount of liquid. These data also show that there were no significant effects of ammonium chloride or bicarbonate on animal weight or tumor growth rate. Thus, animals can be chronically maintained on ammonium chloride or bicarbonate up to 250 mM in concentration.

At the end of this trial, the extracellular pH values for each animal were determined. These data showed that ammonium chloride had no effect on the extracellular pH, presumably due to the up-regulation of renal Na/H exchange (R. Alpern, personal communication). On the other hand, bicarbonate has a significant effect on increasing the extracellular pH of tumors. These experiments were repeated by treating the animals for 2 weeks with 250 mM bicarbonate drinking water. Once again, after the initial day, there were no significant differences in fluid intake compared to water controls. As shown in **figure 6**, the animals on bicarbonate maintained an extracellular pH more alkaline than the intracellular pH. Thus, bicarbonate not only raised the extracellular pH, it reversed the pH gradient.

We are in the process of testing the effects of these conditions on the sensitivity of tumors to chemotherapy in vivo and predict that bicarbonate will be an effective chemosensitizer.

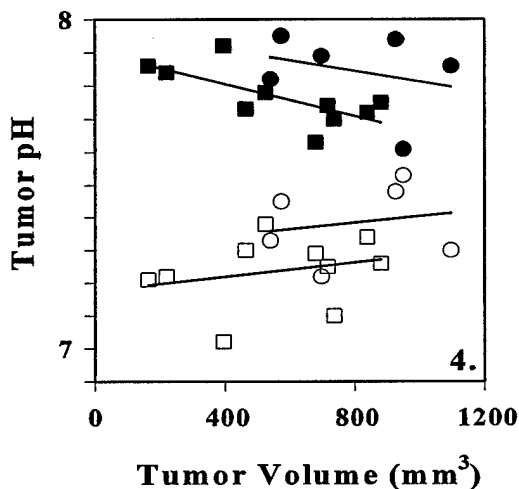


Figure 6. pHi (open symbols) and pHe (closed) of MCF-7S (squares) and MFC-7-mitox (circles) tumors in SCID mice after 2 weeks of bicarbonate therapy.

Project 4. Measurement of Interstitial pH by 1-H MRS: A Prelude to pH Imaging

Imidazoles as pHe indicators. In order to improve the spatio-temporal resolution of our pHe measurements, we have begun investigating the use of derivatized imidazoles as 1-H labeled extracellular pH markers (Gillies et al., 1997). In these studies, we have examined 8 imidazole derivatives produced by Paloma Ballesteros and her colleagues (Gil et al., 1992, 1994; Zaderenko et al., 1994; Lopez et al. 1996). We have determined the toxicity of these compounds both in vivo and in vitro, the membrane transport kinetics, the pharmacodynamics, and the effects of pH on the 1-H chemical shifts. The most promising compound to date is (+)2-imidazol-1-yl-3-ethoxycarbonyl propionic acid (IEPA, **figure 7**). This compound is not toxic up to 20 mM in vitro, and up to 3 g/kg in rats in vivo. The H-2 of this compound (i.e. between the imidazole nitrogens) resonates between 7.79 (deprotonated) and 8.89 (protonated) ppm, relative to TSP (**figure 8**).

MRSI of IEPA. IEPA has been injected i.p. into anesthetized mice bearing RIF-1 tumors and 2D MRSI data were obtained using BASSALE, an MRSI routine which includes outer volume lipid suppression and CHESS water suppression (Shungu and Glickson, 1993, 1994). The routines typically contained 16 phase-encoding steps in orthogonal directions, yielding a 16 x 16 matrix of 2 x 2 x 4 mm voxel spectra in 34 min. on a GE Signa 4.7 T imaging spectrometer. Preliminary results from tumors yields three observations:

1. The distribution of IEPA is heterogeneous within the tumor. It is not known if this reflects regional differences in perfusion or extracellular volume fractions.
2. The pHe distribution within the tumor is also heterogeneous. Virtually every voxel contains a complex peak with resonances corresponding to a range of pHe values. The ranges of pHe observed are ca. 0.2 pH unit in each voxel. Note that this is less than the pHe range reported by 3-APP.
3. IEPA is cleared rapidly. This is an unfortunate aspect of this indicator. From these preliminary experiments, we have estimated that it is available in the tumor for less than 40 min. Higher resolution pharmacodynamic studies are underway and improvements are designed to increase the retention.

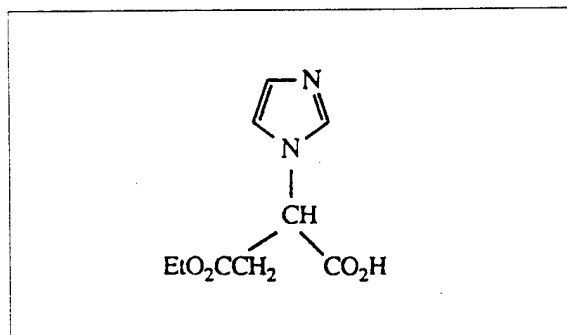


Figure 7. Chemical formula for IEPA (+2, imidazol-1-yl-3-ethoxy carbonylpropionic acid).

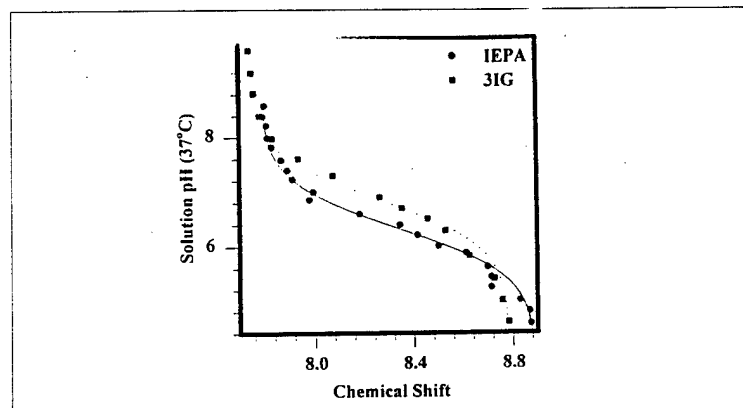


Figure 8. 1-H NMR titration curve of IEPA and 3IG. Imidazoles IEPA and 3IG were independently titrated at 37 °C in physiological buffer (PBS + 10% serum). Data are relative to TSP at 0 ppm.

RELATIONSHIP TO ORIGINAL STATEMENT OF WORK

Task 1a, measurement of pH_i as a function of pH_e by fluorescence in complete buffer, using all four cell lines, was completed, as per our previous report. *A manuscript describing our results is in preparation.*

Task 1b-d, Titrations of two cell lines using MRS of cells cultured in bioreactors was postponed to year 04 due to late availability of suitable bioreactors.

Tasks 2a-d, determination of pH regulatory mechanisms, have been completed. *A manuscript describing our results is in preparation.*

Tasks 3a-d, experiments on measurement of tumor pH as a function of tumor size *in vivo*, are proceeding ahead of schedule. We have completed the pH studies for three cell lines. *A manuscript describing our results is also being written.* In addition, we have added new experiments to improve the sensitivity of pH measurement techniques to allow *pH imaging*.

Tasks 4a-b, effects of altering blood flow on extracellular and intracellular pH, and analysis of data, has been replaced by the manipulation of pH using bicarbonate. The effects of these treatments on the response to chemotherapeutic agents is being tested.

Additionally, we have added experiments to test the involvement of acidic vesicles in WBDR. *A manuscript describing this study has been submitted.*

CONCLUSIONS

- There is empirical and theoretical evidence that the turnover of acidic vesicles is involved in the resistance of cells to chemotherapeutic agents.
- pH measurements with 3-APP continue to be robust, and can be used to determine the pH range within a tumor.
- The environmental pH of tumors can be manipulated with bicarbonate. Preliminary *in vitro* and model data predict that this will enhance the efficacy of chemotherapeutic agents.
- Preliminary evidence shows that pH imaging is possible.

REFERENCES

- Boscoboinik D, Gupta RS and Epanand RM (1990) Investigation of the relationship between altered intracellular pH and multidrug resistance in mammalian cells, *Br. J. Cancer* **61**: 568-572.
- Dubowchik GM, Padilla L, Edinger K and Firestone RA (1994) Reversal of doxorubicin resistance and catalytic neutralization of lysosomes by a lipophilic imidazole, *Biochim. Biophys. Acta* **1191**: 103-108.
- Gil MS, Zaderenko P, Cruz F, Cerdán S and Ballesteros P (1994) Imidazol-1-ylalkanoic acids as extrinsic ¹H NMR probes for the determination of intracellular pH, extracellular pH and cellular volume. *Bioorg. Med. Chem.* **2**:305-314.
- Gillies RJ, Raghunand N, Bhujwala ZM, Ballesteros P, Alvarez J, Cerdan S. (1997) Measurement of extracellular pH in tumors by ¹H MRSI. *Proc Int Soc Magn Reson Med* **5**:1099(Abstract)
- Gillies RJ, Liu Z and Bhujwala Z (1994) ³¹P-MRS measurements of extracellular pH of tumors using 3-aminopropylphosphonate, *Am. J. Physiol.* **267**: C195-C203.
- Gotessman MM and Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter, *Ann. Rev. Biochem.* **62**: 385-427.
- Graham RA, Taylor AH and Brown TR (1994) A method for calculating the distribution of pH in tissues and a new source of pH error from the ³¹P-NMR spectrum, *Am. J. Physiol.* **266**: R638-R645.
- Hurd RE, and Freeman D (1991) Proton editing and imaging of lactate, *NMR in Biomed.* **4**: 73-80.
- Jahde E, Glusenkamp KH and Rajewsky MF (1990) Protection of cultured malignant cells

- from mitoxantrone cytotoxicity by low extracellular pH: a possible mechanism for chemoresistance *in vivo*, *Eur. J. Cancer* **26**: 101-106.
- López P, Zaderenko P, Balcazar JL, Fonseca I, Cano FH, Ballesteros P (1996) The zwitterion structure of imidazol-1-ylacetic acids in the solid state and in solution. *J. Mol. Struct.* **377**:105-112.
- Marquardt D and Center MS (1991) Involvement of vacuolar H⁺-Adenosine triphosphatase activity in multidrug resistance in HL60 cells, *J. Nat. Cancer Inst* **83**: 1098-1102.
- Mason RP, Cha GH, Gorrie GH, Babcock EE and Antich PP (1993) Glutathione in whole blood: a novel determination using double quantum coherence transfer proton NMR spectroscopy, *FEBS Lett.* **318**: 30-34.
- Roepe PD, Wei LY, Cruz J and Carlson D (1993) Lower electrical membrane potential and altered pH homeostasis in multidrug-resistant (MDR) cells: further characterization of a series of MDR cell lines expressing different levels of P-glycoprotein, *Biochemistry* **32**: 11042-56.
- Sehested M, Skovsgaard T, van Deurs B, and Winther-Neilson H (1987) Increased plasma membrane traffic in daunorubicin resistant P388 leukemic cells: effect of daunorubicin and verapamil, *Br. J. Cancer* **56**: 747-751.
- Sehested M, Skovsgaard T and Roed H (1988) 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.
- Shungu DC, Glickson JD (1993): Sensitivity and localization enhancement in multinuclear *in vivo* NMR spectroscopy by outer volume presaturation. *Magn. Reson. Med.* **30**:661-71.
- Shungu DC, Glickson JD (1994): Band-selective spin echoes for *in vivo* localized ¹H NMR spectroscopy *Magn. Reson. Med.* **32**:277-84.
- Simon S, Roy D and Schindler M (1994) Intracellular pH and the control of multidrug resistance, *Proc. Nat. Acad. Sci. USA* **91**: 1128-1132.
- Tannock IF and Rotin D (1989) Acid pH in tumors and its potential for therapeutic exploitation, *Cancer Res.* **49**: 4373-84.
- Taylor CW, Dalton WS, Parrish PR, Gleason MC, Bellany WT, Thompson FH, Roe DJ and Trent JM (1991) 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.
- Thiebaut F, Currier SJ, Whitaker J, Haugland RP, Gottesman MM, Pastan I and Willingham MC (1990) Activity of the multidrug transporter results in alkalization of the cytosol: measurement of cytosolic pH by microinjection of a pH-sensitive dye, *J. Histochem. Cytochem.* **38**: 685-90.
- Whitford GM and Angmar-Mansson B (1995) Fluorosis-like effects of acidosis, but not NH₄⁺, on rat incisor enamel, *Caries Res.* **29**: 20-25.
- Willingham MC, Cornwell MM, Cardarelli CO, Gottesman MM and Pastan I (1986) Single cell analysis of daunomycin uptake and efflux in multidrug-resistant and -sensitive KB cells: effects of verapamil and other drugs, *Canc. Res.* **46**: 5941-5946.
- Zaderenko P, Gil MS, Ballesteros P and Cerdán S (1994) Synthesis and regioselective hydrolysis of 2-imidazol-1-yl-succinic esters. *J. Org. Chem.* **59**:6268-6273.

Additional references:

Gil MS, Cruz F, Cerdan S and Ballesteros P (1992) Imidazol-1-ylalkanoate esters and their corresponding acids. A novel series of extrinsic ^1H NMR probes for intracellular pH. *Bioorg. Med. Chem. Lett* 2:1717-1722.

Gillies, R J, N Didier and M Denton (1986). "Determination of cell number in monolayer cultures." *Analytical Biochemistry* 159(1): 109-13.

Raghunand, N, Wright, S H, Smith, D S, Fehr, C N, Dalton, W S, and Gillies, R J, Turnover of Acidic Vesicles and Resistance to Weakly Basic Chemotherapeutics: a Quantitative Model, *Cancer Res*, 1997. (Submitted)

Raghunand, N, Altbach, M I, Aiken, N R, Bhujwala, Z M, Stubbs, M, and Gillies R J, Measurement of Extracellular pH Excursions in Tumors by ^{31}P MRS of 3-aminopropylphosphonate: deconvolution of T_2^* and pH-heterogeneity effects, *NMR in Biomed*, 1997. (Submitted)

Raghunand, N, Altbach, M I, Bhujwala, Z M and Gillies R J (1997) Extracellular pH Excursions in Drug-Sensitive and Drug-Resistant MCF-7 Human Breast Carcinoma Cells: a ^{31}P MRS Study *Proc Intl Soc Magn Reson Med* 5, 1096.