Use of the Proton Pump Inhibitor Pantoprazole to Modify the Distribution and Activity of Doxorubicin: A Potential Strategy to Improve the Therapy of Solid Tumors

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Abstract

Purpose: Limited drug distribution within solid tumors is an important cause of drug resistance. Basic drugs (e.g., doxorubicin) may be sequestered in acidic organelles, thereby limiting drug distribution to distal cells and diverting drugs from their target DNA. Here we investigate the effects of pantoprazole, a proton pump inhibitor, on doxorubicin uptake, and doxorubicin distribution and activity using in vitro and murine models.

Experimental Design: Murine EMT-6 and human MCF-7 cells were treated with pantoprazole to evaluate changes in endosomal pH using fluorescence spectroscopy, and uptake of doxorubicin using flow cytometry. Effects of pantoprazole on tissue penetration of doxorubicin were evaluated in multilayered cell cultures (MCC), and in solid tumors using immunohistochemistry. Effects of pantoprazole to influence tumor growth delay and toxicity because of doxorubicin were evaluated in mice.

Results: Pantoprazole (>200 μmol/L) increased endosomal pH in cells, and also increased nuclear uptake of doxorubicin. Pretreatment with pantoprazole increased tissue penetration of doxorubicin in MCCs. Pantoprazole improved doxorubicin distribution from blood vessels in solid tumors. Pantoprazole given before doxorubicin led to increased growth delay when given as single or multiple doses to mice bearing MCF7 xenografts.

Conclusions: Use of pantoprazole to enhance the distribution and cytotoxicity of anticancer drugs in solid tumors might be a novel treatment strategy to improve their therapeutic index.

Introduction

Drug resistance limits treatment of cancer by chemotherapy. For a tumor to respond to chemotherapy, a drug must leave tumor blood vessels efficiently and distribute throughout tumor tissue to reach all cancer cells in concentrations that will lead to cytotoxicity (1, 2). The distribution of anticancer drugs such as doxorubicin is limited in solid tumors and this limited distribution may be an important mechanism of drug resistance (3–9).

Many solid tumors develop regions of extracellular acidity because of production and poor clearance of carbonic and lactic acid (10–13). The pH gradient between an acidic extracellular environment and a neutral–alkaline intracellular pH may influence drug uptake and activity. Also, cells contain acidic organelles such as lysosomes and endosomes (14–16). Because many chemotherapeutic drugs such as anthracyclines and vinca alkaloids are weak lipophilic bases, they readily enter acidic organelles where they are protonated and sequestered (17).

Vacuolar-H+-ATPases (V-H+-ATPase) transport H+ ions across the membranes of a wide array of intracellular compartments and are the major mechanism for regulation of endosomal pH (18). Agents that disrupt the pH gradient between the cytoplasm and endosomes in tumors might decrease the sequestration of basic anticancer drugs and render cells more sensitive to the drug. A class of H+-ATPase inhibitors called proton pump inhibitors (PPI) inhibit acidification of cells in the wall of the stomach and are used clinically for treating patients with peptic ulcer disease. These agents also inhibit V-H+-ATPase albeit at somewhat higher concentrations then is required to inhibit acidification in the stomach (19). PPIs accumulate selectively in acidic spaces and with the inhibition of V-H+-ATPase activity, increase both extracellular pH and the pH of acidic organelles (20). Pretreatment with a PPI might alter intracellular drug distribution by inhibiting drug sequestration, thereby allowing more drug to enter the nucleus and cause cytotoxicity, and to exit the cell and be taken up by cells.
Translational Relevance
The tumor microenvironment is likely to play an important role in drug resistance. Our group and others have shown that limited drug distribution may influence the therapeutic efficacy of anticancer drugs. This article examines the use of the proton pump inhibitor pantoprazole in combination with doxorubicin in in vitro and in vivo model systems. Pantoprazole was found to increase doxorubicin uptake and penetration through tissue in in vitro studies. In solid tumors, pantoprazole was found to improve doxorubicin distribution and combined treatment increased tumor growth delay in mice. This study suggests that pantoprazole may improve the therapeutic efficacy of doxorubicin by altering intracellular and extracellular drug distribution.

Materials and Methods

Drugs and reagents
Doxorubicin (Pharmacia) was purchased from the hospital pharmacy as a solution at a concentration of 2 mg/mL. Purified rat anti-mouse CD31 (platelet/endothelial adhesion molecule 1) monoclonal antibody was purchased from BD PharmMingen, and Cy3-conjugated goat anti-rat IgG secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. Pantoprazole (Nycomed) was purchased from the hospital pharmacy as a lyophilized powder and dissolved in 0.9% saline. Radiolabeled doxorubicin was obtained from Amersham Life Sciences. Lansoprazole was purchased from Sigma and dissolved in ethanol.

Cell lines
The parental mouse mammary sarcoma EMT-6 was provided originally by Dr. Peter Twentyman, Cambridge, United Kingdom. The human breast cancer cell line MCF-7 and the human vulvar epidermoid carcinoma cell line A431 were obtained from the American Type Culture Collection. EMT-6 and MCF-7 cells were maintained as monolayers in α-MEM media, supplemented with 10% FBS (Hyclone).

A431 cells were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% FBS. All media were obtained from the hospital media facility. Cells were grown in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Routine tests to exclude mycoplasma were performed.

Tumors were generated by subcutaneous injection of 1 to 5 × 10⁶ exponentially growing cells into the left and right flank regions of female athymic nude mice (MCF7 and A431) or syngeneic Balb/C mice (EMT-6). Estrogen pellets were implanted into the nude mice one day before injection of MCF-7 tumor cells. All procedures were carried out following approval of the Institutional Animal Care Committee.

Measurement of endosomal pH
To measure endosomal pH, EMT6 cells (10⁶/mL) were treated with varying concentrations of lansoprazole, pantoprazole, or tamoxifen (included because of prior reports of effects to raise endosomal pH; ref. 25), and MCF7 cells were treated with varying concentration of pantoprazole in the presence or absence of doxorubicin (1.8 μmol/L). They were incubated for 3 hours with dextran–fluorescein–tetramethylrhodamine 10,000 MW, anionic (FITC/TMR-dextran; Molecular Probes, Inc.), which is taken up into endosomes, followed by exposure to media for 2 hours. Fluorescence was measured using a Coulter Epics Elite flow cytometer (Beckman Coulter) equipped with an argon laser emitting at 488 nm. The argon laser was used to excite FITC and TMR with emission evaluated at 525 nm (pH-dependent) and 575 nm (pH-independent), respectively. Calibration of fluorescence measurements was performed using the ionophore nigericin (Sigma) in buffers of known pH (26).

Doxorubicin uptake and distribution in cells
Doxorubicin distribution in cells was evaluated by fluorescence microscopy. Cells attached to a chambered cover glass were pretreated with pantoprazole (1 mmol/L) for 2 hours and then incubated in media containing 2 μg/mL doxorubicin for 1 hour. The drugs were washed out and the fluorescence signal was recorded with excitation at 514 nm and emission at 488 nm using a Zeiss Axiovert 200 M fluorescence inverted microscope and the fluorescence signals were captured with a Roper Scientific CoolSnap HQ CCD camera. To visualize endosomes, the cells were exposed to the pH-sensitive endosomal dye, LysoSensor Yellow/Blue DND-160 (Molecular Probe, Inc.) at a concentration of 5 μmol/L for 15 minutes. The fluorescent signal was measured with excitation at 360 nm and emission at 420 nm.

To determine net uptake of doxorubicin in EMT-6, MCF-7, and A431 cell lines, 10⁵ cells were treated in vials with either saline or pantoprazole (1 mmol/L). After 2 hours, doxorubicin (1.8 μmol/L) was added to each vial and incubated for 1 hour and then washed twice with PBS. Mean doxorubicin fluorescence was measured using the Becton Dickinson FACScan and Cell Quest software. The
FL1 filter (530 nm) was used to detect doxorubicin fluorescence.

**Doxorubicin penetration in MCCs**

Drug penetration was evaluated in an *in vitro* tumor-like environment using MCCs (27, 28). Approximately 2 × 10⁵ cells were seeded on collagen-coated microporous Teflon membranes (Millipore) and given 4 to 6 hours to attach. Experiments using MCCs were carried out using EMT-6 and MCF-7 cells. A431-derived MCCs were not used because cells did not grow well in these conditions. The membranes were immersed in α-MEM media in a large vessel with continuous stirring and allowed to incubate at 37 °C for approximately 6 to 8 days; this led to the formation of MCCs containing approximately 5 × 10⁶ cells. The MCCs were incubated in either media alone or media containing 1 mmol/L pantoprazole for 2 hours. The penetration experiments were performed at 37 °C in an atmosphere of 95% air and 5% CO₂. To evaluate doxorubicin penetration, solutions containing 10 μmol/L radiolabeled doxorubicin were prepared in 2 × α-MEM (without FBS) and mixed in a 1:1 ratio with 1% agar solution; the agar is added to prevent convection. A volume of 0.5 mL of this mixture was added to one side of the MCC. The membranes were then floated in glass polyshell vials containing 18 mL of α-MEM media. A cell-free membrane insert was included in all experiments as a control. The drug, after penetrating through the MCC, appeared in the compartment below and 150 minutes and 3 hours after administration (6). Also, removing the tumor 10 minutes after doxorubicin administration ensures an adequate concentration of pantoprazole in the plasma. The tissues were embedded immediately in OCT compound, frozen in liquid nitrogen, and stored at −70 °C. Cryostat sections 10-μm-thick were cut at 3 levels approximately 100 μm apart from each tumor, mounted on glass slides.

**Toxicity studies in mice**

Mice were treated with either PBS containing CaCl₂ and MgCl₂ alone, doxorubicin alone (8 mg/kg), or pantoprazole at each of the following doses: 100, 150, 200, 250, 300 mg/kg, alone and 2 hours before doxorubicin. The body weight of the mice was recorded every other day.

**Plasma concentrations of pantoprazole in mice**

At various times after mice were treated with pantoprazole (200 mg/kg), blood was collected using cardiac puncture in heparin-coated tubes on ice, after sample collection the mice were killed. Plasma was isolated and frozen by centrifugation. Pantoprazole concentration was determined using high-performance liquid chromatography (HPLC) using a protocol similar to that described by Peres and colleagues (29). Shimadzu SIL-20AC auto-injector and Shimadzu-20AD pumps were used for HPLC-MS/MS analysis and Applied Biosystem MDS Sciex API3200 tandem mass spectrometry equipped with a TurboIonSpray interface was used subsequently. The data were processed using Analyst 1.4.2 software (Applied Biosystem MDS Sciex).

**Doxorubicin distribution in solid tumors**

Mice bearing EMT-6 or MCF-7 subcutaneous tumors in both flank regions were divided randomly into groups of 5 and were treated when the mean tumor diameter was in the range of 8 to 12 mm. Animals were treated with PBS containing CaCl₂ and MgCl₂, doxorubicin alone, or pantoprazole before doxorubicin. Doxorubicin was given intravenously at a dose of 25 mg/kg to facilitate detection and quantification of drug auto-fluorescence. Pantoprazole was administered intraperitoneally 2 hours before doxorubicin treatment at a dose of 200 mg/kg. To detect hypoxia, EF5 was injected intraperitoneally approximately 2 hours before killing the mice (0.2 mL of a 10 mmol/L stock per mouse), and to detect functional blood vessels the perfusion marker DiOC7 (1 mg/kg) was injected intravenously 1 minute before killing the mice. Mice were killed 10 minutes after doxorubicin injection and the tumors were excised. Previous studies in our laboratory have showed that doxorubicin is maximally distributed in solid tumors between 10 minutes and 3 hours after administration (6). Also, removing the tumor 10 minutes after doxorubicin administration ensures an adequate concentration of pantoprazole in the plasma. The tissues were embedded immediately in OCT compound, frozen in liquid nitrogen, and stored at −70 °C. Cryostat sections 10-μm-thick were cut at 3 levels approximately 100 μm apart from each tumor, mounted on glass slides.

Doxorubicin fluorescence was quantified with an Olympus BX50 microscope with a 100 W HBO mercury light source equipped with 530 to 560 nm excitation/573 to 647 nm emission filter sets. Tissue sections were imaged with a Photometrics CoolSNAP HQ2 (monochrome for fluorescence imaging) camera and tiled using a motorized stage so that the distribution of doxorubicin was obtained for the entire tissue section. All images were captured in 8-bit signal depth and subsequently pseudo-colored.

**Tumor sections**

Tumor sections were first imaged for doxorubicin and the perfusion marker DiOC7 using a TRITC and FITC filter set, respectively. Sections were then stained for blood vessels using antibodies specific for the endothelial cell marker CD31 [rat anti-CD31 primary antibody (1:100); BD Biosciences; and C73-conjugated goat anti-rat IgG secondary antibody (1:400)]. Hypoxic regions were identified using a Cy5-conjugated mouse anti-EF5 antibody (1:50). Tumor sections were imaged for CD31 using the Cy3 (530–560 nm excitation/573–647 nm emission) filter set, and EF5 using the Cy5 far-red filter set. Image analysis was performed as previously described in our laboratory (30), where doxorubicin 8-bit grayscale images were overlaid with binary CD31 images. Nonfunctional vessels from the CD31 image were determined by comparing the image with a binarized...
DiOC7 image and these vessels were removed. The overlaid images were then run through a customized algorithm to generate drug-intensity distributions in relation to distance from the nearest blood vessel.

Tumor vasculature was quantified using Media Cybernetics Image Pro PLUS Software. The total number of blood vessels was measured by setting a threshold for CD31 positive pixel intensity and minimum blood vessel area (62 μm²) and counting the number of objects within these settings. Objects above this threshold range but below the minimum area were removed as artifacts (31). The tumor area was recorded and areas of necrosis and artifact were excluded. The mean number of total blood vessels per tumor area was determined. The number of functional blood vessels per tumor area was calculated in a similar manner using DiOC7 positive pixels.

Growth delay studies

Two perpendicular diameters of tumors growing in the flanks of mice were measured with a caliper, and treatment began once tumors reached a diameter of 5 to 8 mm. Tumor volume was estimated using the formula: 0.5(ab²), where a is the longest diameter and b is the shortest diameter.

To determine the effects of pantoprazole, mice bearing either MCF-7 or A431 tumors were divided into 4 groups of 4 to 5 mice each and treated with either saline, doxorubicin alone (8 mg/kg i.v.), pantoprazole alone (200 mg/kg, i.p.), or pantoprazole 2 hours before doxorubicin (200 mg/kg + 8 mg/kg). For evaluation of growth delay following multiple doses of drugs, mice were treated with either doxorubicin alone (6 mg/kg i.v.), pantoprazole alone (200 mg/kg i.p.), or pantoprazole combined with doxorubicin (200 mg/kg + 6 mg/kg), once a week for 3 weeks. Every 2 to 3 days, the tumor volume and body weight were measured. Measurements were taken until tumors reached a maximum diameter of 1.2 cm or began to ulcerate, when mice were killed humanely. All mice were ear tagged and randomized to avoid bias with measurements. Growth delay experiments were also done in EMT-6 tumors but because of the fast-growing nature of these tumors, ulcerations and overgrown tumors prevented data from being collected beyond 8 days.

Statistical analysis

A one-way ANOVA, followed by a post hoc t test were performed to determine statistical differences between treatment groups. For drug uptake using flow cytometry and quantification of functional tumor vasculature and hypoxia, t tests were performed to determine significant differences between treatment groups. P < 0.05 was used to indicate statistical significance.

Results

PPiS increase endosomal pH in tumor cells

Pantoprazole, lansoprazole, or tamoxifen led to concentration-dependent increases in endosomal pH in EMT-6 cells; increases in endosomal pH were observed with concentrations of tamoxifen above 10 μmol/L, and with lan-

Pantoprazole pretreatment influences doxorubicin uptake in tumor cells in culture

Photomicrographs of fluorescence in MCF-7 and EMT-6 (not shown) cells exposed to doxorubicin alone show the drug to be present in the nucleus and in punctuate compartments within the cytoplasm; and localized staining of doxorubicin with lysosensor gives evidence of endosomal sequestration (Fig. 2A). Treatment with pantoprazole reduces the amount of doxorubicin fluorescence in the cytoplasm whereas it is retained within the nucleus (Fig. 2A, panel iii).

Doxorubicin uptake within cells pretreated with either saline or pantoprazole 1 mmol/L was measured using flow cytometry. In EMT-6 cells, doxorubicin fluorescence significantly increased with pantoprazole pretreatment (P < 0.05). In contrast, MCF-7 and A431 (not shown) cells showed a decrease in doxorubicin fluorescence by 24%
(P < 0.05) and 36% (P < 0.05), respectively, with pantoprazole pretreatment (Fig. 2B). When cells were treated with a lower concentration of pantoprazole (100 μmol/L), similar changes in distribution were observed, but these results were not significant (data not shown).

Doxorubicin penetration in MCCs pretreated with pantoprazole

When MCCs were pretreated with 1 mmol/L pantoprazole, there was a greater than 2-fold increase in doxorubicin penetration for those grown from EMT-6 cells (P < 0.05; Fig. 3A) and ~1.3-fold increase through those grown from MCF-7 cells (P < 0.02; Fig. 3B). Internal standards indicated by 3H-sucrose penetration were used to control for variations in thickness of the cell cultures, and showed <10% variation.

Photomicrographs of MCCs derived from MCF-7 cells indicate an increase in doxorubicin fluorescence in cells more distal from the source of drug that are pretreated with pantoprazole compared with control MCCs at 2 hours after the start of doxorubicin exposure (Fig. 3C and D). Similar changes in fluorescence were observed in MCCs derived from EMT-6 cells (photomicrographs not shown).

Plasma concentration of pantoprazole and toxicity in mice

After intraperitoneal injections of 200 mg/kg pantoprazole in mice, the peak plasma concentration was ~300 μmol/L within the first hour after administration, and ~150 μmol/L after 2 hours. After 5 hours, the concentration in the plasma decreased to less than 1% of the maximum concentration in the blood and by 24 hours less than 0.01 μmol/L of pantoprazole was detectable (Fig. 4).

Toxicity studies in mice

Mice treated with saline alone showed a gradual increase in body weight over more than 20 days. Mice treated with doxorubicin alone (8 mg/kg i.v.) or pantoprazole alone (up to 300 mg/kg) showed minimal increase in body weight. Combined treatment led to no change in body weight at pantoprazole doses of 100 or 150 mg/kg, but at 200 mg/kg mice showed a temporary decrease in body weight (~15%) within the first 5 to 8 days after treatment followed by rapid recovery to their original body weight. Mice treated with 250 or 300 mg/kg of pantoprazole and then doxorubicin showed continual loss in body weight beyond 20 days (data not shown). The maximum tolerated...
dose was determined to be 200 mg/kg when combined with doxorubicin.

**Effect of pantoprazole on distribution of doxorubicin in tumors**

Photomicrographs taken from MCF-7 tumors show substantial increases in doxorubicin fluorescence in tumors in mice pretreated with pantoprazole, compared with mice treated with doxorubicin alone (Fig. 5A and B). Doxorubicin distribution was quantified in EMT-6 and MCF-7 tumors in mice at 10 minutes after injection. Doxorubicin fluorescence intensity was determined in relation to distance to the nearest functional blood vessel in areas of interest with similar numbers of functional vessels. In all tumors, there were steep gradients of decreasing doxorubicin fluorescence in relation to distance from the nearest functional blood vessel in the section. In MCF-7 tumors, there was a shallower gradient of decrease in doxorubicin distribution in the tumors pretreated with pantoprazole compared with the tumors treated with doxorubicin alone \( (P < 0.05; \text{Fig. 5C}) \). However, in EMT-6 tumors there was no
significant difference in drug distribution between treatment groups (data not shown).

**Pantoprazole pretreatment increases growth delay in MCF-7 tumors**

Doxorubicin alone led to growth delay of MCF-7 tumors ($P < 0.05$), and there was no effect of pantoprazole alone (Fig. 6A). Mice treated with pantoprazole before a single dose of doxorubicin significantly increased tumor growth delay compared with the other treatment groups ($P < 0.05$), with minimal tumor growth out to 42 days; because of ulceration, mice were killed thereafter. Studies were also carried out in a second human xenograft model that was derived from the epidermoid carcinoma cell line A431: this tumor was resistant to doxorubicin but pretreatment with pantoprazole led to a small increase in growth delay compared with groups treated with the control ($P = 0.06$) or doxorubicin alone ($P = 0.05$; Fig. 6B). There was no substantial loss in body weight after treatment (data not shown).

Mice treated with multiple doses of pantoprazole and doxorubicin (once a week for 3 weeks), showed even greater growth delay of MCF-7 xenografts compared with the single-dose combination (Fig. 6C). In A431 xenografts however, multiple doses of pantoprazole and doxorubicin did not lead to significant growth delay (Fig. 6D).

**Discussion**

The acidic microenvironment in solid tumors may cause resistance to some anticancer drugs. The pH gradient between the cytoplasm and intracellular organelles may be modified in cancer cells and there may be sequestration of basic drugs in acidic organelles (16). PPIs increase pH in acidic vesicles such as endosomes (22), thereby inhibiting the accumulation of basic cytotoxic agents in acidic organelles (32) and they may lead to apoptosis of cancer cells (33).

In this study, we investigated the effect of pantoprazole on endosomal pH of cancer cells. We showed that pantoprazole increased endosomal pH at concentrations of 200
μmol/L or higher, and the effect of pantoprazole to raise endosomal pH was enhanced in the presence of (basic) doxorubicin. In addition to pantoprazole, lansoprazole, another type of PPI, and tamoxifen also showed an increase in endosomal pH likely caused by blocking acidification in these intracellular compartments.

We observed increased uptake of doxorubicin in EMT-6 cells with pantoprazole pretreatment in contrast to decreased uptake with the pretreatment in MCF-7 and A431 cells. It is likely that EMT-6 cells have slightly higher levels of P-glycoprotein (PgP) compared with MCF-7 cells (34, 35). Similar patterns of doxorubicin uptake were seen in EMT-6 cells and PgP overexpressing AR1 and NCI cells (Supplementary A). This would explain the low uptake in EMT-6 cells treated with doxorubicin alone compared with that in MCF-7 and A431 cells. It has been shown that PPIs, including pantoprazole, inhibit PgP (36), and this may contribute to the increase in drug uptake after pantoprazole pretreatment of EMT6 cells. There is evidence that pantoprazole may enhance uptake of PgP substrates across the blood–brain barrier that expresses PgP: for example, there was increased penetration of imatinib in the brain with pantoprazole pretreatment (37). We did not measure doxorubicin concentration in mouse brain with and without co-administration of pantoprazole, but we did not observe neurotoxicity at the doses used in our experiments.

PPIs have been shown to increase the sensitivity of resistant tumor cells to the cytotoxic effects of several drugs, including cisplatin, 5-flurouracil, and vinblastine, and increase the cytotoxicity of these agents in cells (22–24). In the MCF-7 cells, we observed consistently decreased doxorubicin uptake with pantoprazole pretreatment. In these cells, the nucleus may be saturated with drug and excess concentrations are likely stored in acidic vesicles, whereas in PPI-treated cells this sequestration will be reduced leading to a decreased net uptake of drug. With decreased uptake of drug, there is more drug available to distribute to cells distal from blood vessels. Our in vitro data show an increase in doxorubicin penetration in pantoprazole-treated MCCs.

If pantoprazole decreases net drug uptake in cells close to blood vessels, there is likely to be increased doxorubicin distribution to more distant cells and the gradient of decreasing doxorubicin intensity will be significantly shallower. In MCF-7 xenografts, we observed a significant increase in doxorubicin distribution in relation to the nearest blood vessel following treatment with pantoprazole, but this effect was not observed in EMT-6 tumors. This may again be attributed to slightly higher levels of PgP in
EMT-6 cells. Previous studies in our laboratory have shown that Pgp inhibitors may decrease doxorubicin distribution in solid tumors derived from Pgp-expressing cells, most likely because they increase drug uptake in cells close to blood vessels, such that there is less drug available to penetrate to distal cells (30, 38).

Causes of drug resistance in tumors are both multifactorial and interconnected such that overcoming one mechanism may not improve drug resistance. An effective strategy is likely to influence several processes that contribute to resistance to give an overall therapeutic benefit. The effect of pantoprazole on MCF-7 cells and xenografts may improve therapeutic efficacy by influencing the net effect of different mechanisms of resistance.

Our HPLC data show that pantoprazole concentrations decrease exponentially after 2 to 3 hours. By administering pantoprazole 2 hours before doxorubicin, we hypothesized that pantoprazole would be in sufficient concentration to increase pH within intracellular organelles and inhibit doxorubicin sequestration into these organelles. However, our HPLC data indicate maximum levels of pantoprazole in the plasma of mice that are lower than the concentration of pantoprazole required to raise endosome pH in the in vitro experiments. High concentrations of pantoprazole may account for increases in doxorubicin penetration in MCCs, but in drug-sensitive xenografts, lower in vivo concentrations of pantoprazole in combination with doxorubicin can still alter drug distribution. After 2 hours, plasma concentrations of pantoprazole are around 200 \( \mu \text{mol/L} \). In drug uptake experiments using flow cytometry, we observed that pantoprazole alters doxorubicin uptake into cells at 1 \( \mu \text{mol/L} \) and at 100 \( \mu \text{mol/L} \), albeit to a lesser extent. The finding that lower doses of pantoprazole are effective in vivo might be because of the role of the microenvironment and its effects on ion gradients between intracellular compartments and between intracellular and extracellular pH. The extracellular pH is known to be low in tumor regions distant from functional blood vessels, an effect that will inhibit cellular uptake of basic drugs (11); the pH gradient between the cytoplasm and the extracellular fluid may be inhibited by Pgps, leading to improved uptake of basic drugs such as doxorubicin, although this effect is likely to have limited therapeutic benefit if the concentration of doxorubicin in such regions is low.

In addition to contributing to drug sequestration, acidic endosomes have been shown to be important in the process of autophagy (39). Autophagy may be a survival mechanism of nutrient-deprived cells (40, 41). Our laboratory has preliminary data to indicate that pantoprazole is an inhibitor of autophagy because of its role in altering the pH of acidic compartments; this may be an additional mechanism through which Pgps may affect therapeutic efficacy (42).

The strategies of altering drug sequestration to improve drug distribution in tumors, and of inhibiting autophagy may overcome drug resistance. Our studies of tumor growth delay in mice bearing MCF-7 xenografts indicate a therapeutic advantage of using pantoprazole in combination with doxorubicin. Multiple-dose treatments showed even greater effects to delay growth of MCF-7 tumors. These experiments were repeated in another xenograft model using A431 tumors. A431 xenografts were resistant to doxorubicin and pantoprazole pretreatment had only minimal effects to improve growth delay. Preliminary studies in our laboratory indicate that docetaxel has much greater effects to delay growth of this tumor and using biomarkers to demonstrate the distribution of (nonfluorescent) docetaxel (43), we have shown recently that pantoprazole pretreatment enhances both drug distribution and antitumor effects considerably (44). These studies indicate that pantoprazole in combination with some chemotherapeutic agents may have important antitumor effects.

Conclusions

Our findings suggest that pantoprazole increases endosomal pH in tumor cells and may increase nuclear uptake of doxorubicin within these cells. Pantoprazole increases tissue penetration of doxorubicin in MCCs and improves doxorubicin distribution from blood vessels in solid tumors. Pantoprazole given before doxorubicin increases growth delay when given as single or multiple doses to mice bearing MCF-7 xenografts.

Our data, and those of others (22–24), suggest that pretreatment with pantoprazole may be an effective strategy to improve the therapeutic efficacy of chemotherapy in some solid tumors. Increased drug sequestration and limited drug distribution may have important roles in multifactorial drug resistance and must be considered when developing novel therapeutics treatment strategies.

The preclinical results described in this report led to funding by the Komen Foundation of a phase I trial of escalating doses of pantoprazole given before doxorubicin; this trial has shown that high-dose pantoprazole (240 mg) can be given intravenously before standard dose doxorubicin without apparent increase in toxicity, and that high serum levels of pantoprazole (~100 \( \mu \text{mol/L} \)) can be achieved in patients. Although the main goal of a phase I trial is to evaluate tolerance and toxicity in patients who have exhausted standard therapy, responses were seen in that trial. A phase II trial using pantoprazole with chemotherapy has been initiated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: K.J. Patel, I.F. Tannock
Development of methodology: K.J. Patel, I.F. Tannock
 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.J. Patel, C. Lee
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.J. Patel, C. Lee, Q. Tan, I.F. Tannock
Writing, review, and/or revision of the manuscript: K.J. Patel, I.F. Tannock
Study supervision: I.F. Tannock

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