The Role of Lysosomes in Limiting Drug Toxicity in Mice^S

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ABSTRACT

The distribution behavior of a drug within a cell is an important, yet often overlooked, variable in both activity and differential selectivity. In normal cells, drugs with weakly basic properties are known to be extensively compartmentalized in acidic organelles such as lysosomes via ion trapping. Several cancer cell lines have been shown to have defective acidification of endocytic organelles and therefore have a diminished capacity to sequester such lysosomotropic agents. In this study, we tested the hypothesis that the low lysosomal pH of normal cells plays an important role in protecting normal tissues from the toxic effects of lysosomotropic anticancer drugs. The influence of lysosomal pH status on the toxicity of inhibitors of the molecular chaperone Hsp90 that did or did not possess lysosomotropic properties was evaluated in mice. Toxicity of Hsp90 inhibitors was evaluated in normal mice

and in mice treated with chloroquine to elevate lysosomal pH by assessing morbidity and utilizing biochemical assays to diagnose hepatic and renal toxicity. Toxicity of the lysosomotropic inhibitor 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG) was significantly enhanced in mice with elevated lysosomal pH relative to mice with normal lysosomal pH. In contrast, elevation of lysosomal pH had no significant impact on toxicity of the nonlysosomotropic inhibitor geldanamycin. These results support the notion that the low lysosomal pH of normal cells plays an important role in protecting these cells from the toxic effects of anticancer agents with lysosomotropic properties and has implications for the design/selection of anticancer drugs with improved safety and differential selectivity.

Under the most ideal circumstances, anticancer agents should be minimally toxic to normal cells and maximally noxious to cancer cells. Unfortunately, an optimal degree of selectivity is not typically achieved, and chemotherapy is often prematurely stopped due to potentially life-threatening damage to normal tissues and organs. The inherent selectivity of a cancer drug toward tumors typically results from exploiting biochemical and/or metabolic differences between the cell types (Dutcher et al., 2000a,b; Kroemer and Pouyssegur, 2008). Theoretically, the observed selectivity of an anticancer agent can be further enhanced using a variety of drug delivery strategies. Broadly speaking, these approaches have been centered around Ehrlich's proposed "magic bullet" concept (Houshmand and Zlotnik, 2003; Imai and Takaoka, 2006). Accordingly, these strategies share a common requirement in that the active cytotoxic agent is expected to accumulate to a greater extent in or around transformed cells

relative to normal cells. Although numerous creative strategies have been examined (i.e., prodrug strategies, antibody-drug conjugates), their therapeutic usefulness has been somewhat limited. Indeed, computational modeling experiments have illustrated a number of theoretical limitations to achieving site-specific drug delivery of traditional small molecule drugs (Stella and Himmelstein, 1980).

In a previous publication, Duvvuri et al. (2006) propose and investigate a different approach to enhance the differential selectivity of anticancer agents toward tumor cells. This approach differs from previous ones in that active drug molecules are not preferentially localized in cancer cells but are allowed to permeate into both normal and transformed cells to an equal extent. In this approach, selectivity is achieved based on the fact that anticancer agents with optimized physicochemical properties can distribute differently in normal versus cancer cells, resulting in differences in drug-target interactions and ultimately, differences in drug response.

The mechanism for altered intracellular distribution of drugs in normal versus cancer cells originates from differences in intracellular pH gradients. Normal cells have lysosomes that are very acidic relative to the cell cytosol. This pH gradient facilitates the sequestration of weakly basic small molecular weight compounds in lysosomes via ion trapping

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ABBREVIATIONS: GDA, geldanamycin; 17-DMAG, 17-dimethylaminoethylamino-17-demethoxy-geldanamycin; CQ, chloroquine; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; NiK-12192, 4-(5,6-dichloro-1*H*-indol-2-yl)-3-ethoxy-*N*-(2,2,6,6-tetramethyl-piperidin-4-yl) benzamide.

(de Duve et al., 1974). Although normal cells typically have a low lysosomal pH, a number of cancer cell lines have been shown to have defective acidification of lysosomes (Jiang et al., 1990; Altan et al., 1998; Belhoussine et al., 1999; Gong et al., 2003; Kokkonen et al., 2004). Defective acidification of lysosomes in cancer cells dramatically reduces the lysosome to cytosol pH gradient and therefore decreases the propensity for sequestration of lysosomotropic agents (Gong et al., 2003). As a result, these compounds will be extensively concentrated in lysosomes of normal cells; however, in cancer cells (with elevated lysosomal pH), they will have a greater tendency to accumulate in extra-lysosomal compartments of cells. Because anticancer drug targets are not typically localized within lysosomes, this difference in distribution would promote drug-target interactions in cancer cells while limiting them in normal cells, resulting in enhanced drug selectivity. This concept was previously illustrated in cultured cells using a series of Hsp90 inhibitors with variable physicochemical and lysosomotropic properties (Duvvuri et al., 2006).

The theoretical basis governing lysosomal trapping of weak bases has been reviewed by de Duve et al. (1974). Weakly basic compounds that are sequestered in lysosomes are often referred to as "lysosomotropic agents," and in this article, we use this term to designate any weakly basic compound that has a propensity to accumulate in lysosomes via ion trapping.

In the present work, we sought to evaluate this drug selectivity platform in vivo using mice. Specifically, we examined whether lysosomotropic anticancer agents were relatively less toxic to mice with normal lysosomal compared with mice with elevated lysosomal pH, due to their propensity to be extensively sequestered in lysosomes, away from target sites. If this is the case, raising the lysosomal pH of mice should cause a redistribution of the drug from lysosomes, which would allow the drug to interact with the intended target molecules and exert its toxic effects to a greater degree. In contrast, given that the intracellular distribution of nonlysosomotropic compounds is not influenced by the lysosome to cytosol pH gradient, the toxicity of such drugs should not be affected by changes in lysosomal pH. Therefore, we also evaluated the impact of lysosomal pH changes on the toxicity of a nonlysosomotropic anticancer agent.

Materials and Methods

Animals

The present study was performed with approval from the University of Kansas Institutional Animal Care and Use Committee. Male BALB/c mice (10–12 weeks old) were obtained from the Charles River Laboratories (Wilmington, MA). Animals were housed under standard conditions in a 12-h light/dark cycle and with free access to commercial food pellets and water.

Chemicals

Geldanamycin (GDA) was obtained from LC Laboratories (Woburn, MA), and 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG) was synthesized and characterized according to a previously published method (Tian et al., 2004). Structures of these compounds are shown in Supplemental Fig. 1. All other reagents were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

Drug Treatments and Morbidity Evaluations in Mice

Dosing protocols for the Hsp90 inhibitors, GDA and 17-DMAG, were experimentally accomplished by determining a regimen that resulted in symptoms of acute toxicity (morbidity) in approximately 20% of animals in a treatment group ($n = 10$). Accordingly, 17-DMAG was administered intraperitoneally at a dose of 75 mg/kg on days 1 and 2 and 30 mg/kg on day 3. GDA was administered at a dose of 3.5 mg/kg i.p. on days 1 through 4 and 7 mg/kg on days 5 through 9. To elevate lysosomal pH in mice, indicated groups of mice were pretreated with 50 mg/kg/day chloroquine (CQ) by intraperitoneal administration for 5 days before and concurrent with the dosing of the Hsp90 inhibitors at the aforementioned doses. During concurrent dosing of CQ and Hsp90 inhibitors, CQ was administered 2 to 3 h before Hsp90 inhibitor treatment. CQ and 17-DMAG were reconstituted in normal saline (200 μ l), and GDA was dissolved in neat DMSO (30 μ l/dose) for intraperitoneal administrations. The presence of morbid symptoms was determined by an experienced observer with no prior information regarding the drug treatments. Animals were considered morbid if they were severely immobile, hunched in posture, experiencing severe diarrhea and hypothermia, and/or unresponsive to noise. At the conclusion of treatments, or after signs of morbidity were detected, mice were euthanized via cardiac puncture and exsanguinations.

Elevation and Measurement of Lysosomal pH in Mice

To elevate lysosomal pH in mice, intraperitoneal injections of 50 mg/kg/day CQ diphosphate were given for 5 days (untreated group received intraperitoneal injections of saline). To evaluate the effect of CQ treatment on lysosomal pH, mice were dosed via tail vein injection with 100 μ l of a 5 mg/ml solution of the pH-responsive dye Oregon Green 488 conjugated to dextran (molecular weight of 70,000; Invitrogen, Carlsbad, CA). Dextran polymers of this molecular size are known to extensively localize in the liver shortly after administration (Mehvar et al., 1994). To determine lysosomal pH, hepatocytes were isolated from mice dosed with Oregon Green dextran 6 h after dosing using a previously published technique (Lees and Stephen, 1985) with modifications. After sacrifice via cardiac puncture and exsanguination, mouse organs were perfused through an incision in the left ventricle at a flow rate of 7 ml/min using the following buffers and times of perfusion: 1) perfusion buffer A, containing 142 mM NaCl, 6.7 mM KCl, 25 mM NaHCO_3 (adjusted to pH 7.4 with 1 N HCl) for 5 min; 2) perfusion buffer A to which 0.5 mM EGTA was added for 5 min; 3) perfusion buffer A for 3 min; and 4) perfusion buffer A containing 0.05% collagenase/dispase (Roche Diagnostics, Indianapolis, IN) and 5 mM CaCl_2 for 5 min. During the procedure, perfusate was drained via an incision in the right atrium of the heart. Livers were excised and collected into the solution containing perfusion buffer A with 0.05% collagenase and 5 mM CaCl_2 . Livers were subsequently minced under sterile conditions using a scalpel and incubated at 37°C for 10 min with occasional agitation. The cell suspension was filtered through a 100 μ m cell strainer (Fisher, Woburn, MA). The filtrate was then centrifuged at 50g for 5 min and washed twice in buffer B containing 142 mM NaCl, 6.7 mM KCl, 1.2 mM CaCl_2 , and 10 mM HEPES, pH 7.4. Lysosomal pH was then measured using a previously published technique (Ohkuma and Poole, 1978; Jiang et al., 1990; Gong et al., 2003). In brief, freshly isolated hepatocytes were resuspended in pH 7.4 buffer containing 150 mM NaCl, 20 mM MES, 5 mM KCl, and 1 mM MgSO_4 . Cells were placed onto a microscope slide, sealed with a coverslip, and the 525-nm emission intensity of Oregon Green was measured at 495- and 450-nm excitation wavelengths, respectively, through a 525/10-nm bandpass filter mounted on a microscope equipped with a Ratiometer spectrofluorimeter (PTI, Trenton, NJ) with a photomultiplier tube detector. The ratio of 495/450-nm excitation was calculated, and converted into pH using a calibration curve. To create a calibration curve for pH determination (Supplemental Fig. 2), isolated hepatocytes were separately resuspended in pH 4, 5, 5.5, 6, or

7 buffers containing the ionophores nigericin (10 μ M) and monensin (20 μ M) as described previously (Ohkuma and Poole, 1978; Bach et al., 1999). Excitation spectra of Oregon Green-labeled hepatocytes treated with the ionophores exhibit pH-dependent emission at 525 nm (Supplemental Fig. 3). To confirm whether the Oregon Green-labeled dextran administered to mice was localized in lysosomes 6 h after intravenous injection in mice, freshly isolated hepatocytes were incubated with 50 nM LysoTracker Red (Invitrogen) for 30 min at 37°C, washed twice with PBS, and viewed with an Olympus spinning disk confocal microscope using the appropriate filter sets to visualize Oregon Green 488 and LysoTracker Red.

Biochemical Assays of Serum Arginase Activity and Serum Creatinine

At the time of euthanasia, blood was collected into heparinized microcentrifuge tubes and centrifuged in a Mini Spin Plus Eppendorf Centrifuge (Eppendorf AG, Hamburg, Germany) for 10 min at 4000 rpm. Plasma was immediately collected and stored at -80°C until assays to evaluate hepatic and renal toxicity were performed. To comparatively assess hepatic toxicity, a commercially available colorimetric arginase activity kit (BioAssay Systems, Hayward, CA) was used according to the manufacturer's instructions. Samples were desalted before analysis using desalting spin columns (Pierce, Rockford, IL). To assess renal toxicity, a commercial creatinine assay kit (BioVision, Mountain View, CA) was used according to the manufacturer's instructions. Before analysis, samples were depleted of protein using a molecular weight 10,000 cut-off filter (Millipore Corporation, Billerica, MA).

Analysis of Tissue/Plasma Drug Concentrations

Drug Treatments. To determine drug concentrations in tissue and plasma, mice were dosed twice with 50 mg/kg/day 17-DMAG and 15 mg/kg/day GDA. Mice pretreated with chloroquine to elevate lysosomal pH received 50 mg/kg chloroquine diphosphate for 5 days before and concurrent with dosing with Hsp90 inhibitors. Mice were sacrificed via cardiac puncture and exsanguination for 15 min and 3 h, respectively, after administration of the second dose of Hsp90 inhibitors. Plasma samples were collected as described previously and stored at -80°C until analysis of drug concentration was performed. Before organ collection, organs were perfused, as described previously, with PBS at a flow rate of 8 ml/min for 5 min. Liver, kidneys, heart, lungs, and spleen were harvested and stored at -80°C.

Sample Preparation and High-Performance Liquid Chromatography Analysis. *N*-Phenyl-1-naphthylamine was used as an internal standard. Before drug extraction, tissue samples were weighed and homogenized in 0.5 to 1 ml of PBS containing internal standard using a Sonic Dismembrator model 500 (Fisher Scientific, Pittsburgh, PA) tissue homogenizer. Samples were mixed with acetonitrile or ethyl acetate (1:1 v/v) for extraction of 17-DMAG and GDA, respectively. Samples were vortexed for 15 s each and then centrifuged at 16,100g for 10 min. The organic phases were removed and evaporated to dryness under vacuum centrifugation. Samples were reconstituted in initial mobile phase and analyzed using an Agilent Series 1200 high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA) equipped with a binary pump, autosampler, and variable wavelength detector. For analysis of 17-DMAG, mobile phase A consisted of 50 mM acetic acid containing 10 mM triethylamine and mobile phase B consisted of methanol containing 10 mM triethylamine. UV absorbance was monitored at 332 nm. For analysis of GDA, mobile phase A consisted of 2% methanol in 0.1% formic acid in H₂O, and mobile phase B consisted of 95% methanol in 0.1% formic acid in H₂O. UV absorbance was monitored at 308 nm. Standard curves were constructed by plotting the peak area ratio of 17-DMAG and GDA to that of the internal standard against concentration and were linear in the range studied. Linear regression was used to determine the equation of line of best fit. The

result of the regression analysis was used to determine analyte concentrations in tissue and plasma samples.

Histopathology

Tissue samples from euthanized mice were preserved in 10% neutral buffered formalin at the time of sacrifice. Samples were processed for routine automated paraffin infiltration and embedding. Paraffin blocks were sectioned at 5 μ m and stained with hematoxylin and eosin. Sections were processed and evaluated by a board-certified veterinary pathologist who interpreted the specimens without information regarding treatment protocol (i.e., a blinded study). Histological changes were scored for severity of change on a scale of 1 to 5 after a preliminary review of all slides to determine the range of lesions. Microscopic descriptions and diagnoses then were determined.

Statistical Analysis

Data presentation and statistical analysis was carried out using Sigma Plot 10.0 (SPSS, Inc., Chicago, IL). Data are represented as means with standard deviation where applicable. Statistical analyses of significance (*p* values) were derived from one-tailed *t*-tests.

Results

Lysosomal pH Elevation in Mice. According to our proposed drug selectivity platform, the low lysosomal pH of normal cells plays an important role in protecting tissues from the harmful effects of anticancer agents with lysosomotropic properties due to their extensive sequestration in this compartment. To test this theory in vivo requires a procedure to significantly raise the lysosomal pH in the cells of mice to levels that have been previously observed for cancer cells with defective acidification in vitro (approximately pH 6), as was measured for the MCF-7 cell line (Altan et al., 1998). Elevating lysosomal pH in cultured cells is routinely done using a number of different approaches; however, to our knowledge, such approaches have not been previously established in vivo. Inhibitors of the vacuolar-H⁺-ATPase, such as concanamycin A, are effective agents in raising lysosomal pH (Temesvari et al., 1996); however, their use in animals has not been previously established. Alternatively, the antimalarial drug CQ is known to be very well tolerated in both humans and animals and is known to raise lysosomal pH in cultured cells (Poole and Ohkuma, 1981). To examine whether this compound altered lysosomal pH in vivo, we utilized the pH-sensitive dye (Oregon Green) attached to high molecular weight dextran polymers to determine lysosomal pH. We and others have found that several hours of chase (i.e., incubation time in dextran-free medium) is a sufficient amount of time for the endocytosed dextran molecules to traverse through the early and late endocytic compartments and predominantly localize within lysosomes (Poole and Ohkuma, 1981). Mehvar et al. (1994) have previously shown that dextran polymers, with a molecular weight of 70,000, localize predominantly in the liver shortly after a tail vein injection in mice, and their concentration in this organ remains virtually unchanged for up to 48 h afterward. Therefore, we injected Oregon Green-labeled dextran (molecular weight of 70,000) into the tail vein of mice and waited 6 h to allow the dextran that accumulated in the liver to reach terminal lysosomes. To confirm whether the Oregon Green-labeled dextran was localized in lysosomes before determination of lysosomal pH, isolated hepatocytes were labeled with the lysosomal vital stain LysoTracker Red. Oregon Green

Fig. 1. Intravenously delivered Oregon Green 488-conjugated dextran, with a molecular weight of 70,000, localizes predominantly in lysosomes after 6 h. Mice were dosed with 0.5 mg Oregon Green dextran via the tail vein, and hepatocytes were isolated 6 h later. To determine the intracellular localization of dextran, hepatocytes were labeled with the lysosome vital stain LysoTracker Red. Dextran (green) colocalizes significantly with the lysosome stain (red), which indicates that the dextran-conjugated pH probe was localized in lysosomes 6 h after intravenous injection. Hepatocytes were isolated using collagenase digestion (see *Materials and Methods*), labeled with 50 nM LysoTracker Red for 30 min at 37°C, and viewed using confocal microscopy.

dextran was found to significantly colocalize with LysoTracker Red, which suggests that the dextran was predominantly localized within lysosomes (Fig. 1). Evaluating ratios of the fluorescence intensities at different wavelengths allowed us to estimate liver cell lysosomal pH in untreated mice to be 4.2 ± 0.2 ($n = 3$), which is in close agreement with previous reports on normal lysosomal pH (Bach et al., 1999; Nilsson et al., 2003). Dosing mice intraperitoneally with CQ (50 mg/kg/day for 5 days) resulted in a significant elevation in lysosomal pH to a value of 5.6 ± 0.3 ($n = 3$). Although these treatments with CQ are higher than typical therapeutic doses administered to mice, which is typically 10 mg/kg i.p. for 3 to 4 days (Falanga et al., 1984), there were no visible side effects or toxicities in mice (data not shown). CQ was subsequently used to examine the influence of elevated lysosomal pH on anticancer drug toxicity in mice in the remainder of the studies.

Influence of Lysosomal pH on Drug-Induced Morbidity in Mice. The selectivity platform examined in this work relies on the assumption that weakly basic lysosomotropic anticancer agents will distribute extensively into lysosomes of normal cells, which will diminish their ability to interact with target molecules that are localized outside of this compartment. For Hsp90 inhibitors, the target molecules (Hsp90) are thought to be primarily localized in the cell cytosol (Young et al., 2001). 17-DMAG is weakly basic with a pKa value of 7.6 and has been previously shown to localize in lysosomes of cells with normal pH regulation (Duvvuri et al., 2006). Accordingly, mice pretreated with CQ to elevate lysosomal pH should experience redistribution of the lysosomotropic inhibitor from lysosomes to the cytosol, which should enhance interactions with Hsp90 and increase the effectiveness (toxicity) of the drug. To test this, we established a dose of 17-DMAG that caused morbidity in approximately 20% of normal mice. Subsequently, the change in the extent of morbidity in mice following pretreatment with CQ was evaluated (Fig. 2). Morbidity assessments were carried out as outlined under *Materials and Methods* by an experienced observer who was blinded to the experimental treatments. The number of morbid animals in each group was counted and represented as a percentage of the total number of mice per treatment group. Consistent with our hypothesis, mice with elevated lysosomal pH experienced significantly greater morbidity compared with those with normal lysosomal pH.

Despite the fact that neither the drug vehicles (normal saline or DMSO) nor CQ treatment alone resulted in any morbidity to mice at the doses employed (data not shown), it

Fig. 2. Elevations in lysosomal pH enhance drug-induced morbidity in mice treated with the lysosomotropic inhibitor 17-DMAG but not when treated with the neutral inhibitor GDA. The percentage of mice determined to show symptoms of morbidity for each of the indicated treatment groups is shown ($n = 10$ per indicated group). Mice treated with CQ and 17-DMAG experienced greater incidences of morbidity compared with mice receiving equivalent doses of 17-DMAG alone. No significant changes in morbidity were observed for mice receiving GDA with CQ pretreatment. 17-DMAG was administered intraperitoneally at a dose of 75 mg/kg on days 1 and 2 and 30 mg/kg on day 3. GDA was administered intraperitoneally at a dose of 3.5 mg/kg on days 1 through 4 and 7 mg/kg on days 5 through 9. Where indicated, CQ was administered at a dose of 50 mg/kg/day i.p. for 5 days before and concurrent with the indicated Hsp90 inhibitor treatments. Morbidity was evaluated by a blinded observer during Hsp90 inhibitor dosing, as described under *Materials and Methods*.

is possible that the CQ treatment could cause some additive toxicity unrelated to the changes in lysosomal pH when co-administered with 17-DMAG. To address this, we additionally examined the impact of CQ pretreatment on morbidity in mice dosed with the neutral, nonlysosomotropic inhibitor GDA. CQ pretreatment had no impact on morbidity in mice dosed with the neutral, nonlysosomotropic inhibitor GDA. As with 17-DMAG, we accomplished a dosing regimen of GDA that caused approximately 20% of the group to show signs of morbidity and subsequently examined the influence of CQ pretreatment on GDA-induced toxicity. CQ-pretreated mice experienced no significant increase in morbidity when dosed with GDA (Fig. 2).

Influence of Lysosomal pH on Drug-Induced Changes in Liver and Kidney Function. To quantitatively assess the trends observed with the previously described morbidity evaluations, biochemical assays of liver and kidney function were performed on plasma samples from mice in all treat-

ment groups. The toxicity of Hsp90 inhibitors has previously been shown to be primarily associated with liver and kidneys (Glaze et al., 2005). Therefore, the effect of treatment on the function or integrity of these organs was comparatively assessed for each drug treatment with or without CQ pretreatment. Serum arginase levels were measured as a specific diagnostic of liver integrity. Arginase I has been evaluated as a highly specific hepatotoxicity marker, and its activity is found to be elevated in the serum of animals as a result of liver damage or injury (Ozer et al., 2008). Consistent with previous morbidity results, mice treated with 17-DMAG alone had relatively low serum arginase activities, whereas CQ pretreatment before 17-DMAG treatment resulted in significantly elevated arginase activity levels that are consistent with increased liver damage (Fig. 3). Conversely, the CQ pretreatments resulted in no significant change in serum arginase levels ($p > 0.01$) in mice treated with the nonlysosomotropic inhibitor GDA (Fig. 3).

Creatinine levels in serum are routinely used as an indicator of renal function. An increase in serum creatinine indicates defective renal function that may be caused by drug toxicity. Therefore, we measured the levels of creatinine in plasma of all drug treatment and control groups. We observed no statistically significant differences in serum creatinine levels between control and CQ pretreated mice receiving 17-DMAG, which could be attributed to high levels of animal-to-animal variability in creatinine levels (Fig. 4). Serum creatinine levels in GDA-treated mice were significantly higher than control ($p < 0.01$), but as expected, there was no difference in serum creatinine levels with or without CQ pretreatment. Compared with control mice, there was no significant change in serum arginase activity and creatinine levels ($p > 0.01$) in mice dosed with CQ alone, GDA vehicle (DMSO) alone, or CQ and DMSO together (Figs. 2 and 3).

Tissue/Plasma Drug Concentrations. The difference in toxicity observed for 17-DMAG upon CQ pretreatment could theoretically result from CQ-induced alterations in tissue

Fig. 3. Elevations in lysosomal pH induced by CQ treatment enhance drug-induced liver toxicity in mice treated with the lysosomotropic Hsp90 inhibitor 17-DMAG but not when treated with the neutral inhibitor GDA. Arginase I activity was measured in the plasma of control mice (no treatment), CQ-treated mice, mice receiving GDA vehicle (DMSO), mice receiving both DMSO and CQ, and mice treated with the indicated Hsp90 inhibitors with or without CQ pretreatment. Dosing regimens are described in the legend of Fig. 2. Bars, means $\pm$ S.D. for the indicated Hsp90 inhibitor treatments with or without CQ treatment ($n = 6$) and for control and CQ-treated groups of mice ($n = 4$). *, $p < 0.01$; **, $p > 0.05$.

Fig. 4. CQ-induced elevations in lysosomal pH have no significant impact on renal toxicity induced by indicated Hsp90 inhibitor treatments. Creatinine levels were measured in serum of control (no treatment), CQ-treated mice, mice receiving GDA vehicle (DMSO), mice receiving DMSO and CQ, and mice treated with indicated Hsp90 inhibitors with or without CQ pretreatment. Dosing regimens are described in the legend of Fig. 2. Bars, means $\pm$ S.D. for the indicated Hsp90 inhibitor treatments ($n = 6$) and for control and CQ-alone-treated groups ($n = 4$).

distribution or pharmacokinetics of Hsp90 inhibitors. Accordingly, to evaluate this possibility, the tissue and plasma drug concentrations of 17-DMAG and GDA with and without CQ pretreatment were measured. Plasma, liver, kidneys, spleen, lungs, and heart were evaluated for drug 15 min (estimated peak time) and 3 h after administration of Hsp90 inhibitors. These time points were selected based on previously published pharmacokinetic profiles of 17-DMAG (Glaze et al., 2005) and GDA (Supko et al., 1995). We found no significant impact of CQ treatment on tissue/plasma drug concentration ratios of 17-DMAG in all of the organs evaluated (Fig. 5). Likewise, tissue/plasma concentrations obtained for GDA were not found to be significantly influenced by CQ pretreatment (Supplemental Fig. 4). Collectively, these results suggest that the enhanced 17-DMAG-induced toxicity found to occur after CQ treatment probably resulted from changes in intracellular distribution of 17-DMAG and can not be attributed to an increased overall exposure of the organs to the drug.

Tissue Histopathology Evaluations. To further characterize results obtained from analyzing morbidity, organs were examined for the presence of drug-induced lesions or injury. Lung, liver, spleen, and kidney histological examinations were performed on hematoxylin and eosin-stained sections from each treatment group using light microscopy. All sections were reviewed and scored for severity of morphological changes (scale 1–5, with 5 being the worst), and an overall diagnosis was determined by a veterinary pathologist. Of all of the organs evaluated, only the liver showed consistent and significant histological changes upon different treatment protocols examined in this study. Shown are representative liver specimens from each treatment group (Fig. 5). A summary of liver diagnosis and hepatic necrosis severity scores is listed in Table 1. Histological sections of livers from saline-treated (control) mice were populated with normal hepatocytes having intact nuclei and cytoplasm (Fig. 5A). Sections obtained from mice treated with CQ alone (Fig. 5B) were not visibly different from the untreated control group (Fig. 5A). Histological sections obtained from mice with unaltered (normal) lysosomal pH that were dosed with

Fig. 5. CQ pretreatment has no significant impact on 17-DMAG tissue uptake and distribution in mice. Tissue/plasma drug concentration ratios in the indicated tissues are shown for mice at 15 min (A) and 3 h (B) after intraperitoneal administration of 17-DMAG. Mice were treated with 50 mg/kg/day 17-DMAG for 2 days and sacrificed 15 min and 3 h, respectively, after the last dose of 17-DMAG. Where indicated, CQ was administered intraperitoneally as described in the legend of Fig. 2. Bars, means $\pm$ S.D. for Hsp90 inhibitor concentration with or without CQ treatment. *, $p < 0.01$.

TABLE 1

Summary of liver section diagnosis following the indicated treatments

Treatment	Liver Diagnosis	Severity Scores
Saline	Normal appearance	0, 0
DMSO	Normal appearance	0, 0
CQ	Normal appearance	0, 0
DMSO + CQ	Normal appearance	0, 0
DMAG	Normal appearance	0, 0
DMAG + CQ	Moderate to severe hepatic necrosis	4, 3
GDA	Moderate to severe hepatic necrosis	4, 3
GDA + CQ	Mild to severe hepatic necrosis	4, 2

17-DMAG (Fig. 5C) also appeared similar to control sections (Fig. 5A). In contrast, liver sections from mice with elevated lysosomal pH (CQ-treated) and subsequently dosed with 17-DMAG were characterized as having many dead cells devoid of nuclei or cells with fragmented nuclei as well as pale-staining cytoplasm, all features characteristic of hepatic necrosis (Fig. 5D). Moderate to severe hepatic necrosis was diagnosed in all sections examined in this treatment group (Table 1). Sections of mice receiving DMSO (Fig. 5E) and both DMSO and CQ (Fig. 5F) were similar to sections of mice receiving saline only (Fig. 5A). Both groups of GDA-treated mice, with normal (Fig. 5G) and elevated lysosomal pH (Fig. 5H), had signs of hepatic necrosis. Histological sections in these groups were significantly different from those of control mice and were characterized as having mild to severe hepatic necrosis (Table 1).

Discussion

A great deal of anticancer research is directed toward the development of agents that have potent cytotoxic or antiproliferative effects on a wide range of cancer cells. However, very few studies have focused on systematically evaluating the factors that can potentially diminish the "effectiveness" of such anticancer agents in normal cells, which would result

in the identification of safer and more selective chemotherapeutics. Because the overall efficacy of any chemotherapeutic agent is determined by the difference in the degree of cytotoxicity between normal and transformed cells, we argue that research in the latter should be viewed as equally important.

We have previously shown that the sequestration of weakly basic drugs in lysosomes via ion trapping can profoundly affect drug activity in cells. In this article, we tested the hypothesis that sequestration of anticancer drugs in lysosomes of normal cells plays an important role in limiting their toxic effects in vivo using mice. Our previous evaluations using cultured cells have shown that anticancer agents with lysosomotropic properties can distribute differently in normal cells compared with cells with impaired lysosomal acidification, a trait common to several types of cancer cells (Altan et al., 1998; Belhoussine et al., 1999). Specifically, our results suggested that anticancer agents with lysosomotropic properties are extensively compartmentalized in lysosomes of normal cells, thereby diminishing their availability to interact with extra-lysosomal target sites. Therefore, by virtue of their compartmentalization in lysosomes, anticancer agents with lysosomotropic properties should have greater safety in normal tissues relative to cancer cells with defective acidification.

To test this mechanism in vivo required us to modulate lysosomal pH in mice and compare the toxicity of the lysosomotropic Hsp90 inhibitor 17-DMAG. Elevation of lysosomal pH in the livers of mice was accomplished using multiday administrations of CQ as described under *Materials and Methods* (Fig. 1). To our knowledge, this work represents the first time that quantitative elevations of lysosomal pH were evaluated in animals. Raghunand et al. (2003) have shown that the addition of NaHCO_3 to the drinking water of mice for several days increased the extracellular and cytosolic pH of MCF-7 human breast cancer xenografts in mice; however, the pH of lysosomes was not measured. Petrangolini and

colleagues have previously evaluated an inhibitor of the vacuolar- H^+ -ATPase named NiK-12192 in mice (Supino et al., 2008). The authors did show, for cells grown in culture, that this inhibitor altered the fraction of acridine orange that yielded red versus green intracellular fluorescence, which is used to indicate the degree of acidity in cells; however, no such confirmation of pH changes was reported when the compound was administered orally in mice. Interestingly, and relevant to this work, the authors found that when NiK-12192 was administered with the weakly basic anticancer agent topotecan, the combination caused enhanced generalized toxicity in mice as was evidenced by increased weight loss and, in one case, death. It is noteworthy that the weight loss observed when these compounds were co-administered was significantly greater than the sum of the values obtained when treatments were administered separately. This synergistic effect is analogous to the results

observed when 17-DMAG and CQ were coadministered to mice in Fig. 1.

Consistent with our hypothesis, we have demonstrated that mice with elevated lysosomal pH experienced a higher incidence of drug-induced morbidity compared with mice with normal lysosomal pH when an anticancer agent with lysosomotropic properties was administered. Moreover, serum arginase levels (Fig. 3) and histological evaluations of livers (Fig. 6) both indicate an increase in liver damage when lysosomotropic inhibitors are administered in mice with elevated lysosomal pH. Such changes were not observed with the neutral Hsp90 inhibitor GDA.

It is important to point out an apparent discrepancy associated with our findings that occurs when one attempts to compare GDA and 17-DMAG-induced morbidity (Fig. 2) and liver toxicity (Fig. 3). Specifically, morbidity in mice receiving GDA (with or without CQ pretreatment) was found to be

Fig. 6. Elevations in lysosomal pH, caused by CQ treatment, enhance drug-induced liver damage in mice treated with the lysosomotropic inhibitor 17-DMAG but not when treated with the neutral inhibitor GDA. Shown are photomicrographs of hematoxylin and eosin-stained liver sections from control mice and from those treated with Hsp90 inhibitors with or without CQ pretreatment. Representative photomicrographs are from control mice (A), mice treated with CQ alone (B), mice treated with 17-DMAG alone (C), mice treated with 17-DMAG after CQ pretreatment (D), mice treated with DMSO (E), mice treated with DMSO and CQ (F), mice treated with GDA alone (G), and mice treated with GDA after CQ pretreatment (H). A, B, C, E, and F illustrate hepatocytes with intact nuclei and cytoplasm with no diagnosis of necrosis reported (Table 1). D, H, and G show hepatocytes with condensed chromatin or lacking nuclei and pale staining cytoplasm characteristic of advanced necrosis (Table 1). Dosing regimens are described in the legend of Fig. 2. Tissues were prepared and examined as described under *Materials and Methods*.

relatively low (~20%), yet the liver toxicity assessments associated with these mice were similar to those of mice treated with 17-DMAG and CQ that were approximately 100% morbid. This observation suggests that these two drugs have different organ-associated toxicity profiles that ultimately lead to signs of morbidity. We hypothesize that this has to do with differences in the tissue distribution profiles for the two drugs. Using the data generated for Fig. 5, we have plotted the overall molar accumulation of the two drugs in all organs evaluated per gram of tissue (see Supplemental Fig. 5). From these data, one can see that GDA preferentially accumulates to a significantly greater extent in the liver relative to other organs evaluated (kidney, spleen, lungs, and heart). On the contrary, 17-DMAG accumulates to approximately the same degree in both liver and kidneys and also has reasonably high levels in the remaining organs evaluated. Based on these findings, it is likely that 17-DMAG-induced morbidity results from cumulative low-level insult to many organs, whereas GDA has the majority of its toxic effects associated with the liver, and this alone does not cause overt signs of morbidity at the doses of GDA examined here. It is not clear what causes GDA to distribute in tissues differently from 17-DMAG. It is possible that factors such as differences in protein binding could contribute to this difference.

A significant concern with the experimental design of this work stems from the possibility that CQ treatment enhances the toxic effects of lysosomotropic Hsp90 inhibitors through pathways unrelated to lysosomal pH modulation. Our results that showed that CQ pretreatment caused no increase in morbidity or organ toxicity of GDA suggest that CQ does not generally augment the pharmacological activity of all Hsp90 inhibitors, rather it is specific to those with lysosomotropic properties. It is also possible that CQ pretreatment could selectively promote enhanced tissue uptake and retention of 17-DMAG. This could be the case if CQ inhibited an efflux transporter that was specific for 17-DMAG but not GDA. If this were the case, we would expect that CQ pretreatment would cause a significant elevation of the tissue/plasma concentration ratio of 17-DMAG. This was not found to be the case for all of the organs evaluated (see Fig. 5). Consequently, considering that CQ pretreatment does not appear to have any significant impact on GDA toxicity and that CQ pretreatment did not influence tissue distribution and pharmacokinetics of 17-DMAG, we concluded that the enhanced toxicity observed for 17-DMAG in CQ-pretreated mice was due to alterations in the drug's intracellular distribution as a result of the change in lysosomal pH and not due to CQ modulating relevant pathways influencing its *in vivo* activity.

Taken together, the current results are consistent with the perception that anticancer agents with optimal lysosomotropic characteristics will be safer in normal tissues due to their extensive compartmentalization in lysosomes. Our previously published results established that weakly basic Hsp90 inhibitors had increased selectivity toward cancer cells with elevated pH *in vitro* (Duvvuri et al., 2006). Collectively, these evaluations significantly enhance our understanding of the selectivity platform described in this work; however, further *in vivo* studies using tumor-bearing mice will be required to fully examine its feasibility and/or limitations. For example, one significant potential obstacle to the usefulness of this approach stems from the fact that the microenvironment surrounding solid tumors is known to be

acidic relative to normal tissue. As a result, the pH gradient existing between the extracellular microenvironment and the cell cytosol is expanded in some solid tumors relative to the pH gradient in normal tissues. According to pH partitioning theory, the steady-state accumulation of weak electrolytes that are membrane-permeable in their un-ionized state and membrane-impermeable in their ionized state will be determined by differences in the pH gradient existing between the cell cytosol and the extracellular space. Specifically, weakly basic drugs are predicted to accumulate to a lesser degree inside cancer cells at steady state when extracellular pH is reduced. This has been shown to occur both *in vitro* and *in vivo* (Mahoney et al., 2003; Gerweck et al., 2006). It is clear that such issues would represent an important consideration when treating many solid tumors that have acidic extracellular pH; however, it should not be a concern for all cancer types since many tumors have been shown to have normal extracellular pH (Ng et al., 1989). An obvious example would be hematological cancers. Moreover, it is possible that the favorable intracellular distribution of weakly basic drugs inside cancer cells with defective lysosomal acidification could offset the aforementioned unfavorable accumulation differences that may exist. Further *in vivo* investigations will be required to address these questions.

It is clear that the physicochemical properties of drugs can have profound effects on drug distribution and activity. This work marks an important advance toward enhancing our understanding regarding the optimal properties of anticancer drugs that could lead to the discovery of new agents with significantly improved therapeutic indices.

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