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ACS chemical biology

A New Approach for Enhancing Differential Selectivity of Drugs to Cancer Cells

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ABSTRACT The degree to which anticancer agents selectively target cancer cells is a key determinant in successful therapeutic outcomes. Inhibitors of the Hsp90 molecular chaperone represent an important new class of anticancer agents. We propose here a novel mechanism by which physiochemical properties of Hsp90 inhibitors can be optimized to increase selectivity towards cancer cells. The basis for this approach relies on differential intracellular pH gradients that have been shown to exist between normal and transformed cells. Five Hsp90 inhibitors containing basic or neutral properties were evaluated in antiproliferation assays using cells with variable lysosomal pH. Inhibitors with basic functionalities had reduced activity in cells with normal (low) lysosomal pH but showed significantly greater activity in cells with abnormally elevated lysosomal pH (similar to what has been recorded in many types of cancer cells). Conversely, such selectivity enhancement was not observed for neutral inhibitors. The mechanistic basis for the observed selectivity was demonstrated quantitatively by determining the concentration of inhibitors within relevant intracellular compartments. Collectively, these findings suggest that Hsp90 inhibitors with optimal basicity and physicochemical properties have enhanced selectivity toward cancer cells than their neutral counterparts. It is anticipated that these findings may be applicable to other classes of anticancer agents for improvement of differential selectivity.

A fundamental goal in the battle against cancer is to design drugs that selectively target tumor cells but have limited effects against the same machinery in nontransformed cells. Many investigators have attempted to direct anticancer agents toward cancer cells by taking advantage of specific receptors or enzymes that are known to be overexpressed on the plasma membrane and thereby increase the concentration of drugs in the vicinity of transformed cells (*1*). Although conceptually appealing, no such approaches have proven to be highly efficacious. Unfortunately, most conventional anticancer agents have sufficient membrane permeability to penetrate both normal and cancer cells to a similar extent.

We describe herein an innovative strategy by which the selective toxicity of drugs toward cancer cells can be rationally enhanced. We propose that drugs, with optimized physicochemical characteristics, can have considerably different intracellular distribution patterns in normal versus transformed cells and can provide a basis for enhancing selectivity toward malignant cells. Specifically, it is proposed that inhibitors can accumulate differentially in desirable intracellular locations (*i.e.*, a site containing the drug target) depending on the pH status of the cell.

The basis for altered intracellular distribution of inhibitors in normal versus cancer cells relies on differences in intracellular pH gradients that have been previously shown to exist. Normal cells have acidic lysosomes in the pH range of 4–5, whereas the cytosol is approximately neutral. This lysosome-to-cytosol pH gradient provides a driving force for accumulation of weakly basic compounds into the acidic organelles. In the cytosol, many weakly basic drugs (depending on their pK_a value) exist predominantly in a unionized state and can readily permeate lipid bilayers. When such bases cross the lysosomal lipid bilayer, they readily

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Received for review March 15, 2006 and accepted May 12, 2006.

Published online June 2, 2006

10.1021/cb6001202 CCC: $33.50

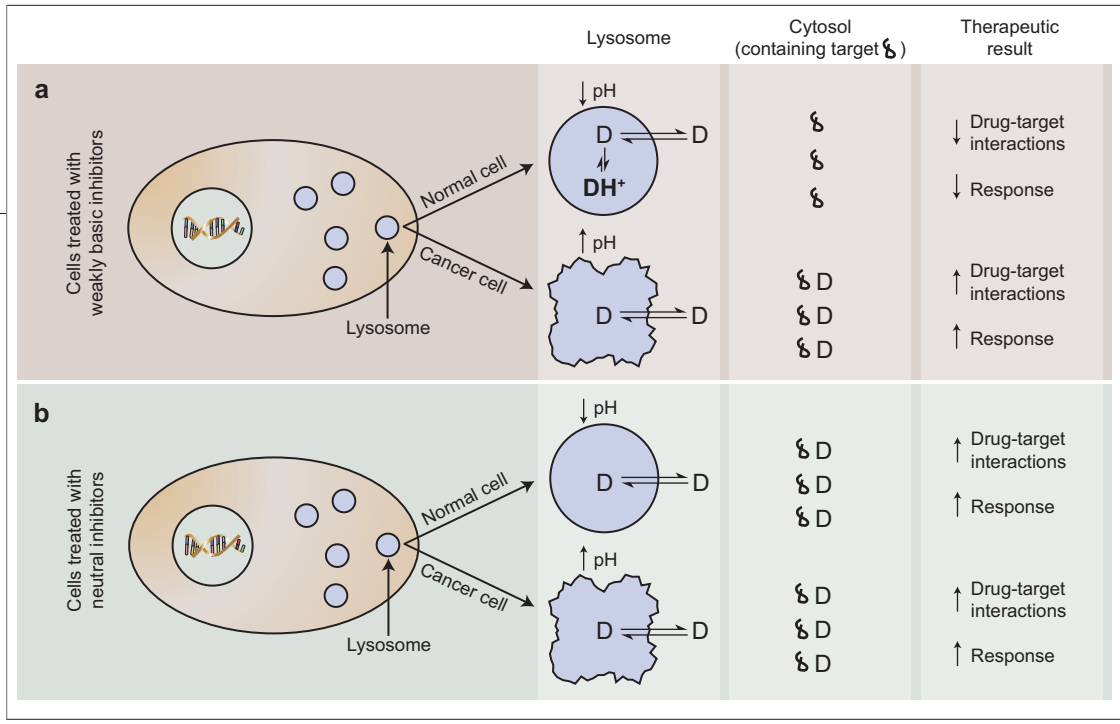

Figure 1. Schematic illustrating the mechanism for improved selectivity of weakly basic inhibitors toward cancer cells proposed in this study. a) Cells treated with weakly basic inhibitors have different intracellular distribution in normal cells (having low lysosomal pH) compared to transformed cells (having defective lysosomal acidification and higher lysosomal pH). Weakly basic drugs (represented as D and DH^+ for the free base and ionized species, respectively) are extensively compartmentalized in lysosomes within normal cells according to an ion trapping mechanism. This results in reduced drug available to interact with cytosolic target molecules and therefore reduced response. Alternatively, cancer cells with elevated lysosomal pH have a reduced capacity to sequester drugs in the lysosomes. This increases the availability of the drug to interact with cytosolic targets and leads to a greater potential for therapeutic response. b) Cells treated with neutral inhibitors (represented as D) do not have differential intracellular distribution in normal cells *versus* cancer cells, since these compounds are not subject to ion trapping in lysosomes and are not influenced by differences in lysosomal pH existing between the cell types. Similar amounts of drug are available to interact with cytosolic target molecules in both normal and cancer cells, and therefore, similar therapeutic responses can be expected.

ionize into their ammonium salts, which are typically unable to diffuse back into the cytosol. This sequestration process has been referred to as ion trapping, as well as pH-partitioning, and the theoretical basis for this phenomenon has been previously reviewed (*2*).

Although the precise mechanism is undetermined, it is clear that numerous cancer cell lines have defective acidification of lysosomes (*3*–*5*). For example, the lysosomal pH of the human leukemic cell line HL-60 is 6.5, whereas the pH of cytosol is near neutral (*6*). Consequently, there is very little driving force for the accumulation of weakly basic drugs into lysosomes in these cells. As a result, weakly basic molecules have a greater propensity to accumulate in the cytosol of such cells (similar to how a neutral molecule may distribute).

Methods to quantitate the accumulation of drugs in lysosomes and the cytosol, as well as other intracellular compartments, have recently been reported (*7*, *8*). Specifically, structure–localization relationships that correlate weak-base permeability characteristics and pK_a values with lysosomal sequestration tendency in cells with normally acidified lysosomes have been established (*9*, *10*). Collectively, these evaluations have shown that lysosomes exhibit an extremely high capacity for sequestering weakly basic molecules possessing optimal characteristics. For example, the weakly basic molecule quinacrine has been shown to achieve concentrations inside lysosomes which are in excess of 750 times the concentration contained in the cell culture medium (*8*). Therefore, extensive lysosomal trapping of drugs can result in dramatically reduced cytosolic concentrations.

If the anticancer drug target resides in the cytosol, it is proposed that the difference in lysosomal pH between normal and malignant cells will provide a basis for the enhancement of differential selectivity. Therefore, drugs with optimized physicochemical properties that promote lysosomal sequestration will have lower cytosolic concentrations in normal cells (*i.e.*, those with low lysosomal pH) compared to transformed cells. A schematic illustration of the proposed mechanism for enhanced selectivity towards cancer cells is illustrated (Figure 1). To demonstrate this novel selectivity principle, we chose to focus our investigation on Hsp90 inhibitors. This class of anticancer agents was particularly suitable since (1) there are many inhibitors in the class that have variable physicochemical properties yet similar binding affinity to Hsp90 *in vitro* (*11*); and (2) Hsp90, the molecular target of these inhibitors, is localized in the cell cytosol (*12*) and would therefore be sensitive to changes in drug sequestration in lysosomes.

RESULTS AND DISCUSSION

Comparative Cytotoxicity of Hsp90 Inhibitors. The antiproliferative activities of five Hsp90 inhibitors of

varying neutral or weakly basic character were determined. The structures of these compounds are shown (Figure 2). GDA and RAD are neutral molecules, whereas 17-DMAG, 17-DMAP, and 17-AEP are weakly basic (see Table 1 for pK_a values). These compounds were evaluated in the human leukemic cell line HL-60 and its multidrug-resistant (MDR) variant. This pair of cell lines was chosen because the HL-60 strain has a high lysosomal pH (6.5) and the MDR strain mimics cells with normal lysosomal acidification, which was measured to be pH 5.1 (*6*). The IC_{50} values obtained for these inhibitors are reported (Table 1). To illustrate the selectivity for cells with high versus low lysosomal pH, we divided the IC_{50} value obtained for a given inhibitor in the MDR cell line by the IC_{50} for the inhibitor in the parent cell line. High IC_{50} ratios indicate inhibitors with greater selectivity toward cancer cells, or any cell line with abnormally high lysosomal pH. The ratio of the IC_{50} values is relatively small for neutral inhibitors (Figure 3, panel a), suggesting they have little differential intracellular distribution and selectivity in cells with normal lysosomal pH compared to those that have elevated lysosomal pH. As proposed, weakly basic inhibitors (*i.e.*, 17-DMAG, 17-AEP, and 17-DMAP) had significantly enhanced IC_{50} ratios that result from the differential lysosomal pH observed in these cell lines. Since we are reporting ratios in Figure 3, the observed trend could possibly occur if weakly basic compounds were progressively accumulating to a greater degree in the MDR cell line relative to the drug-sensitive cell line. Although we have not quantitatively evaluated the total cell concentrations for all compounds examined, we have done so for the neutral inhibitor GDA and the basic inhibitor 17-DMAG.

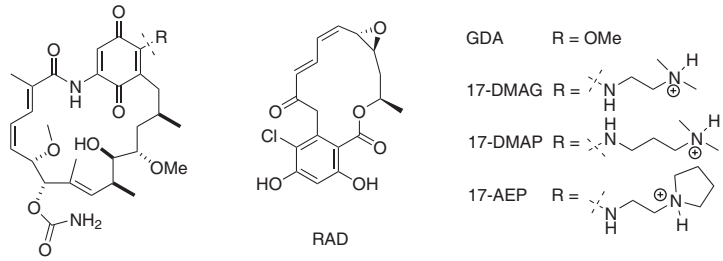

Figure 2. Structures of Hsp90 inhibitors evaluated. GDA and RAD are neutral compounds; the rest (17-DMAG, 17-AEP, and 17-DMAP) are weak bases (pK_a values are reported in Table 1).

We found that the HL-60 cells consistently accumulated about twice the amount of inhibitors relative to the MDR HL-60 cell line (*i.e.*, 1.9- and 1.7-fold increases for 17-DMAG and GDA, respectively). Considering the IC_{50} ratios were not consistently 2-fold, we do not believe this to be the major contributor of the observed trend

Although MDR HL-60 cells have lower lysosomal pH relative to drug-naïve HL-60 cells, it could be argued that the differences observed in these cell lines could be attributed to other factors such as the expression of drug transporters on the plasma membrane of MDR cells that may preferentially efflux weakly basic molecules more readily than neutral molecules (*13*). However, this is not perceived to be a significant concern with these cells, since we have previously shown that the MDR HL-60 cells do not express transporters typically associated with drug efflux at the plasma membrane (*6*). Nevertheless, we determined the IC_{50} values for these compounds in a different cell line, namely, RPTE cells. To mimic the defective acidification that occurs when cells undergo transformation, we pretreated

TABLE 1. Physicochemical and cytotoxicity values for Hsp90 inhibitors

				IC_{50} values (μM)[b]			
				HL-60		RPTEC	
Inhibitor	Classification	pK_a[a]	α	HL-60	MDR HL-60	+ NH_4Cl	− NH_4Cl
GDA	neutral	NA[c]	NA[c]	0.9 ± 0.5	4.7 ± 0.2	0.1 ± 0.1	0.05 ± 0.02
RAD	neutral	NA[c]	NA[c]	8.3 ± 0.1	6.0 ± 1.7	1.7 ± 1.0	3.0 ± 0.1
17-DMAG	weak base	7.6	0.010 ± 0.003	0.1 ± 0.02	2.2 ± 0.6	0.1 ± 0.02	0.4 ± 0.2
17-AEP	weak base	8.1	0.005 ± 0.002	0.05 ± 0.01	2.9 ± 0.1	0.04 ± 0.01	1.4 ± 0.7
17-DMAP	weak base	8.1	0.010 ± 0.007	0.08 ± 0.02	8.8 ± 0.3	0.1 ± 0.04	5.5 ± 0.1

[a]Obtained by curve-fitting the pH-partition plots in Figure 5 (see Methods for procedure). [b]Mean ± standard deviation (n = 3). [c]NA, not applicable.

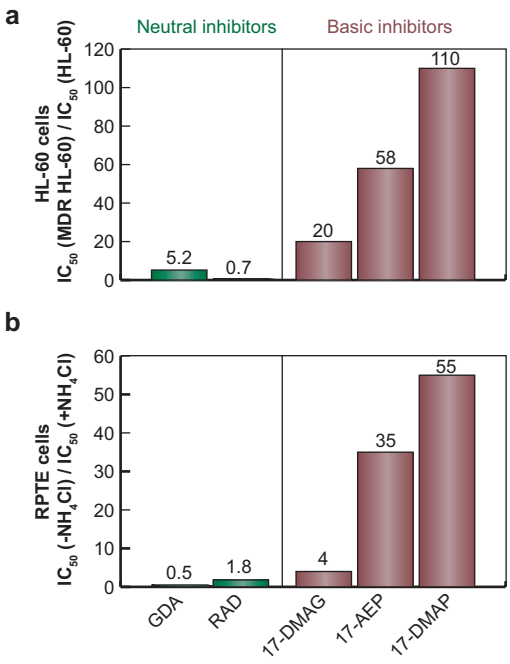

Figure 3. Inhibitors of Hsp90 that have weakly basic functionalities have greater activity in cells with elevated lysosomal pH relative to cells with low lysosomal pH, which is not the case for neutral Hsp90 inhibitors. a) Weakly basic inhibitors have greater differential selectivity (IC_{50} ratios) in cells with high (HL-60) *versus* low (MDR HL-60) lysosomal pH. Neutral inhibitors do not show such differential selectivity. b) Weakly basic inhibitors have greater differential selectivity in RPTE cells treated with NH_4Cl (inducing increased lysosomal pH) compared to untreated cells with low and normal lysosomal pH. Neutral inhibitors do not show such differential selectivity. The bars in panels a and b represent the ratio of IC_{50} values for compounds in cells with low lysosomal pH over the IC_{50} value in cells with elevated lysosomal pH. Refer to Table 1 for individual IC_{50} values and associated experimental errors.

RPTE cells with 5 mM NH_4Cl, which is a well-known reagent used to elevate lysosomal pH (*14*). The IC_{50} values of inhibitors in these cells were determined in the presence and absence of NH_4Cl pretreatment. The results from these studies are presented (Table 1). These results (Figure 3, panel b) parallel those obtained in HL-60 cells (Figure 3, panel a). Once again, inhibitors with weakly basic functional groups had greater activity against cells with high lysosomal pH (*i.e.*, those treated with NH_4Cl). Conversely, raising the lysosomal pH with NH_4Cl had very little effect on differential selectivity of neutral inhibitors. As a control, we had shown that treatment of cells with 5 mM NH_4Cl did not influence cell viability during the time course of the experiment (data not shown).

Together, the general trends represented in Figure 3 strongly suggest that weakly basic anticancer agents that have a cytosolic target and physicochemical characteristics that favor sequestration in lysosomes with low pH values (*i.e.*, 17-DMAG, 17-AEP, and 17-DMAP) have preferential activity in cells with higher lysosomal pH (*i.e.*, transformed cells) compared to normal cells with low lysosomal pH.

Intracellular Distribution of Hsp90 Inhibitors. In an effort to provide mechanistic support to the previously stated theoretical basis for differential selectivity, we quantitatively investigated the intracellular distribution differences of two Hsp90 inhibitors in drug-sensitive and MDR HL-60 cells that have differential lysosomal acidification, as previously stated. From the set of five inhibitors previously evaluated for cytotoxicity, we selected GDA and 17-DMAG as representatives of neutral and basic inhibitors, respectively. Lysosome and cytosol concentrations of GDA and 17-DMAG were evaluated in cells according to our previously reported procedure (*9*). Results from these studies are shown (Figure 4). These data clearly show that neither compound is extensively sequestered into the lysosomes of drug-naïve HL-60 cells, which is consistent with the fact that lysosomal pH is elevated in this cell line to 6.5, which is near cytosolic pH. This translates to relatively low lysosome-to-cytosol concentration ratios for GDA (~1) and 17-DMAG (~16). In contrast, this ratio is ~175 for 17-DMAG and ~2 for the neutral compound, GDA, in MDR HL-60 cells that have low lysosomal pH. These data provide quantitative support for the selective cytotoxicity (Figure 3).

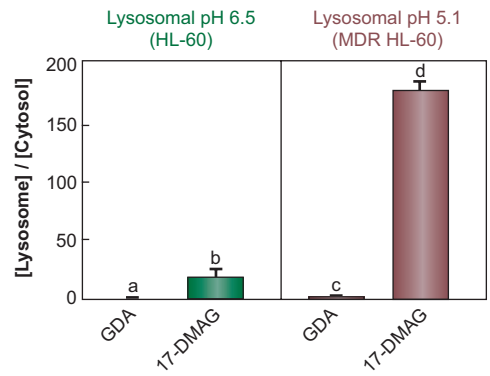

Figure 4. Quantitative evaluations of the intracellular distribution of Hsp90 inhibitors support the observed cytotoxicity differences between neutral and weakly basic inhibitors shown in Figure 3. The weakly basic Hsp90 inhibitor 17-DMAG extensively concentrates in lysosomes in a pH-dependent manner in HL-60 cells. The neutral inhibitor, GDA, does not significantly concentrate in lysosomes regardless of lysosomal pH. Bars represent the experimentally determined concentration of the respective inhibitor obtained from purified lysosomes divided by the concentration in cytosol. Mean ± standard deviations from three experiments are shown ($a \neq b$, $P < 0.1$; $c \neq d$, $P < 0.0001$).

Physicochemical Evaluations of Hsp90 Inhibitors. To optimize the design of inhibitors with enhanced selectivity toward cancer cells based on the lysosome sequestration mechanism presented in this work, it is important to focus on the differential selectivity trends shown for weakly basic inhibitors (Figure 3). Specifically, 17-DMAP produced higher differential selectivity than 17-AEP, which was more selective than 17-DMAG.

In an attempt to rationalize this trend, the physicochemical properties of these weakly basic inhibitors were investigated. Two key parameters appear to be important for lysosomal sequestration. The first has to do with permeability of the base in the ionized state (referred to as α (*10*)) and the second is the pK_a value of the base (*9*). Briefly, α is an indicator of the propensity of the weakly basic molecule to diffuse out of the lysosomal lumen in its ionized state. Obviously, the degree to which this occurs will decrease lysosomal retention. Theoretically, α values can range from 0 to 1. When α is near zero, the base is assumed to be completely membrane-impermeable in the ionized state and will therefore significantly accumulate in lysosomes by ion trapping (*i.e.*, it cannot readily reestablish the equilibrium with the ionized inhibitor contained in the cytosol). Alternatively, if the α value is near 1, the base can freely diffuse out of the lysosomes in the ionized state, will behave similar to a neutral molecule, and will not be significantly sequestered into this acidic organelle. The α values were experimentally determined using data generated from octanol/buffer partitioning behavior as a function of pH (Figure 5). Calculated α values from plots shown (Figure 5) are reported (Table 1). The α value for 17-DMAG is low (0.01) and similar to the values obtained for the other weakly basic inhibitors evaluated in this study. Considering these findings, we do not believe that differences in α are playing a significant role in the aforementioned selectivity trend shown (Figure 3).

From the plots shown (Figure 5), we were also able to extrapolate the pK_a value for each base, which, as previously mentioned, is another important factor in lysosomal sequestration. In a previous report, we have quantitatively evaluated the influence of pK_a values for model compounds in cells with low lysosomal pH and have shown that molecules with higher pK_a values accumulated in lysosomes to a greater extent than compounds with lower pK_a (*9*).

The calculated pK_a values for the bases evaluated in this work are reported (Table 1). The fact that both 17-AEP and 17-DMAP have significantly higher pK_a values relative to 17-DMAG is consistent with greater lysosomal sequestration and is consistent with the observed higher selectivity for these compounds. 17-DMAP appears to have greater differential selectivity than 17-AEP. Although 17-DMAP has a slightly higher pK_a value than 17-AEP, the difference is not significant enough to explain the observed differences in selectivity between these two inhibitors.

The findings presented here suggest that the differential selectivity can be further enhanced by taking into account the physicochemical properties of the inhibitor and the pH status of the cancer cell. Although investigations presented in this work focused on inhibitors of Hsp90, the results presented here are expected to have widespread application in the design, selection, or modification of future anticancer agents with improved differential selectivity. Future studies with additional pairs of cell lines and different classes of drugs will be essential in defining the ultimate scope of the work presented here.

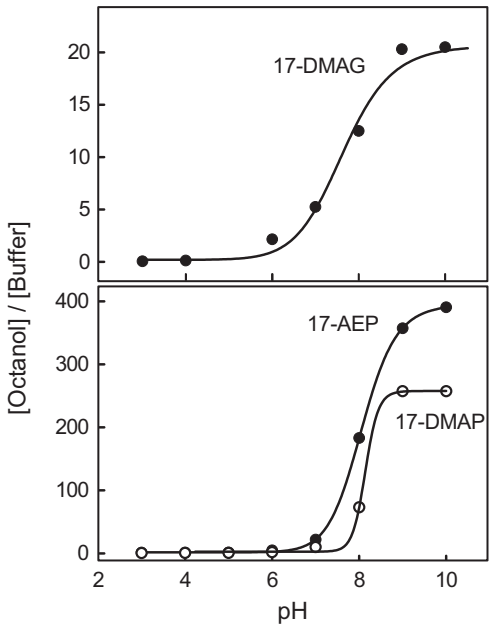

Figure 5. Partition coefficients (octanol/buffer) as a function of pH for weakly basic Hsp90 inhibitors. The above plots are used to determine pK_a and α values, both of which influence lysosomal sequestration tendency and therefore selectivity of the agents toward cancer cells. The α and pK_a values generated from these plots are reported (Table 1).

METHODS

Cell Lines and Reagents. The human acute promyelocytic leukemia cell line HL-60 and the doxorubicin-selected, drug-resistant MDR HL-60 cell lines were kindly provided by Dr. Yueshang Zhang (Arizona Cancer Center, University of Arizona). Renal proximal tubule epithelial (RPTE) cells were purchased from Cambrex Bio Science, and MC/CAR cell line (CRL-8083) was purchased from American Type Culture Collection. Geldanamycin (GDA) was obtained from the Developmental Therapeutics Program (National Institutes of Health). 17-(Dimethylamino)-17-demethoxygeldanamycin (17-DMAG) was synthesized according to a procedure described by Tian and co-workers (*15*). 17-(Dimethylaminopropylamino)-17-demethoxygeldanamycin (17-DMAP) and 17-[2-(pyrrolidin-1-yl)ethyl]amino-17-demethoxygeldanamycin (17-AEP) were purchased from Invivogen, and radicicol (RAD) was purchased from Sigma-Aldrich.

Determination of Octanol/Buffer Partition Coefficients. The partition coefficients for Hsp90 inhibitors were determined as a function of pH using a previously described shake-flask method (*10*). For 17-DMAG, 17-DMAP, and 17-AEP, partition coefficients of ionized (PC_i) and unionized base (PC_u) and the pK_a values were obtained by curve-fitting the pH-partition profile with the Hill equation (4 parameters) using SigmaPlot 2001 (SPSS, Inc.). The ratio of PC_i to PC_u was defined as the α value for a given compound.

Cytotoxicity Evaluations. Cell sensitivity to compounds was determined using WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) as the cell proliferation reagent, and the assays were performed according to the manufacturer's instructions (Roche Applied Science). Briefly, cells were seeded in triplicate in 96-well plates at a density of 5000 cells per well in culture medium and were incubated with increasing concentrations of drug for a period of 72 h (RPTE cells were allowed to adhere to the wells overnight prior to addition of drug). At the end of the incubation period, 10 μL of a 5 mg mL^{-1} solution of WST-1 in phosphate-buffered saline (PBS) was added to each well, and the plates were returned to the incubator for an additional 2 h. The absorbance was measured at a wavelength of 450 nm in a Multiskan (model MCC/340) microplate reader (Thermo Electron Corp.). IC_{50} was defined as the concentration of drug causing 50% inhibition of cell growth as compared with untreated control (in the absence of drug). Designated cells ($+NH_4Cl$) were incubated with 5 mM ammonium chloride for 1 h prior to co-incubation with drug for 72 h. All assays were done in triplicate.

Quantitating Drug in Subcellular Compartments. Cells (200×10^6) were incubated with 5 μM compound for 2 h. Subsequently, intact lysosomes were isolated from cells using a magnetic chromatographic approach previously described by our laboratory (*10*). Isolated lysosomal fraction was acidified with 0.1% formic acid, vortexed with 600 μL of acetonitrile for 20 s, and centrifuged at 16 000*g* for 5 min. The supernatant was evaporated to dryness, and the residue was analyzed by HPLC to quantitate the amount of compound extracted. This amount was divided by the total lysosomal volume in the isolated fraction to obtain the lysosomal concentration of the compound. We have previously determined the total volume of isolated lysosomes using this procedure in MDR HL-60 cells to be 32.2 nL (*8*). For HL-60 and MC/CAR cells, the total volume of isolated lysosomes was estimated based on the total protein content of the isolated lysosomal fraction from each of these cell lines relative to that obtained from MDR HL-60 cells, assuming that the protein content of lysosomes remains constant between cell lines. Using this assumption, we determined the values for total volume of lysosomes in the isolated fraction from HL-60 and MC/CAR cells used in our calculations to be 124.8 and 40.7 nL, respectively. Drug concentrations in cell cytosol were determined as previously described (*7*). To evaluate the influence of heat shock on the intracellular drug distribution, cells were incubated at 42 °C for 15 min and allowed to recuperate at 37 °C for 5 h prior to incubation with drug.

HPLC Analysis. An integrated Agilent 1100 series capillary liquid chromatography system comprised of pump, in-line degasser, column thermostat, and autosampler was used. A Waters Xterra MS C_{18} column (100 mm $\times$ 1 mm; 3.5 μm particle size) was employed and maintained at 30 °C. Injection volumes were 5 μL for all samples.

The mobile phase was comprised of methanol and 0.1% formic acid. Solvents A and B contained 2% and 95% methanol, respectively. A gradient elution method (25 μL min^{-1}) was used for 17-DMAG. For the initial 5 min, the mobile phase composition was 100% A, followed by a linear change to 90% A and 10% B in 1 min, then to 10% A and 90% B over 1 min, and maintained at 90% B for 5 min. Subsequently, the mobile phase composition was changed linearly to the initial conditions over 1 min, and the column was equilibrated for 7 min. For GDA, an isocratic elution method (25 μL min^{-1}) was used with a mobile phase composition of 77% B and 23% A. The duration of runs for GDA and 17-DMAG were 10 and 20 min, respectively.

An API 2000 (Applied Biosciences) triple quadrupole mass spectrometer equipped with a Turbo Ionspray ionization source was employed for detecting 17-DMAG and operated in the positive mode. The transition from 617.1 to 58.1 amu was monitored with a declustering potential of 21 V and collision energy of 53 V. For GDA, an Agilent 1100 series variable wavelength detector was employed, and the column eluent was monitored at 308 nm.

Western Blot Analysis. MC/CAR cells (4×10^7) were cultured in 10 mL media and allowed to incubate for 24 h at 37 °C under 5% CO_2. The cells were heat-shocked at 42 °C for 15 min and then incubated at 37 °C for 5 h, before the cells were harvested by washing with PBS and pelleted (500*g* for 5 min at 4 °C). PBS was removed, and the pellets were resuspended in lysis buffer (150 μL, 50 mM Tris, pH 7.5, 1% NP-40, 150 mM NaCl, 2.5 mM Na_3VO_4, 10 mM PMSF, 10 μM aprotinin, 10 μM leupeptin, and 10 μM soybean trypsin inhibitor) and transferred to a 1.5 mL centrifuge tube. Samples were incubated at 4 °C for 1 h with intermittent agitation. Lysates were cleared by centrifugation at 21 000*g* for 10 min at 4 °C. The supernatants were collected and comprised the experimental samples. The protein concentration of each sample was determined by a BSA assay (Pierce). Equal amount of protein was resolved on a 9% polyacrylamide gel (100 V, 100 mA) and transferred to a nitrocellulose membrane (30 V, 10 mA). Bands were visualized with Ponceau S to confirm protein transfer. Blots were blocked with 5% nonfat milk in PBST (3×20 mL), incubated with primary antibodies (Hsp90 α/β (H-114), sc-7947, lot no. J0504, rabbit polyclonal IgG, Santa Cruz Biotechnology, and actin (I-19)-R, sc1616-R, lot no. B1204, rabbit polyclonal IgG, Santa Crutz Biotechnology) for 2.5 h. The blots were washed (3×10 mL) with 5% nonfat milk in PBST, incubated with a horseradish peroxidase-conjugated secondary antibody (Amersham; 1 h), and washed with 5% nonfat milk in PBST (1×10 mL), followed by PBST (2×10 mL). Protein bands were visualized by chemiluminescence using the ECL detection reagents (Amersham).

Acknowledgment: This work was supported by a grant from the National Cancer Institute, Grant No. CA106655 (to J.P.K.).

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