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A Novel Assay Reveals That Weakly Basic Model Compounds Concentrate in Lysosomes to an Extent Greater Than pH-Partitioning Theory Would Predict

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Abstract: Many weakly basic drugs incubated with cells have been shown to specifically accumulate in lysosomes. The mechanistic basis and substrate specificity for this sequestration have not been rigorously evaluated; however, conditions are favorable for a pH-partitioning type accumulation. In some circumstances, this compartmentalization can be very extensive, which can impact the therapeutic efficacy of a drug. Despite the pharmaceutical importance, direct quantitative assessments of drug accumulation in lysosomes have not been previously described. We report here a novel magnetic capture technique that allows for quick and efficient isolation of lysosomes from cultured HL-60 cells that have been preincubated with model compounds. The amount of compound associated with the isolated fraction is determined by HPLC. Extensive biochemical and morphological characterizations of isolated lysosomes, together with HPLC data, allowed for estimates to be made regarding the concentration of model compounds in lysosomes. The corresponding theoretically determined concentration values, based on pH-partitioning theory, were also calculated for comparison purposes. Interestingly, experimentally determined values were approximately 3−15 times higher than theoretically predicted values. This finding suggests that mechanisms, in addition to pH-partitioning, may play a significant role in the accumulation of drugs in lysosomes.

Keywords: Lysosomes; drug sequestration; pH-partitioning; dextran; lysosomotropic; quinacrine; LysoTracker Red

Introduction

Scientists engaged in drug delivery research are becoming increasingly concerned with how drugs distribute within cells. Significant accumulation of drug within a cell containing target molecules is a necessary but not sufficient step in successful drug delivery. The interior of a cell is a highly compartmentalized and dynamic environment, and rarely are drug targets evenly distributed throughout this space; rather, they are often localized to a specific intracellular domain. Thus, for effective intracellular delivery, a drug must sufficiently concentrate in the compartment/organelle housing the intended target molecule.

A number of organelles have been shown to specifically accumulate a variety of small molecule compounds according to different mechanisms.[1] Lysosomes, in particular, have been shown to be involved in the sequestration of a variety of weakly basic molecules.[2] This was initially demonstrated with cells in culture that were incubated with high concentrations of weakly basic molecules that caused the lysosomes to expand and take on a vacuolar appearance.[3−5] More

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recently, a number of fluorescent weakly basic molecules have been shown to concentrate in lysosomes using fluorescence microscopy.[6,7] There are therapeutic consequences related to this sequestration event since a number of weakly basic drugs have also been shown to accumulate in lysosomes. For example, we and others have demonstrated that a number of anthracycline anticancer agents are sequestered in lysosomes of multidrug resistant (MDR) cancer cell lines.[8,9] The lysosomal sequestration limits the drug's cytotoxicity since the target molecules (i.e., DNA and topoisomerase II) are located within the nucleus away from the sequestration site.[10]

Weakly basic molecules can be expected to accumulate in acidic organelles such as lysosomes (pH ~5) according to a pH-partitioning type mechanism. Generally, significant accumulations can be expected if (1) the neutral form of a compound can efficiently cross both the plasma and lysosomal membranes, (2) the protonated form of the molecule is relatively membrane impermeable, and (3) the pH inside the lysosomes is considerably lower than that of the cell cytosol. An elegant theoretical model describing the accumulation of weakly basic molecules in lysosomes has been developed by de Duve and co-workers.[11] Their model predicts that a molecule's physicochemical properties and the pH values associated with the cell will both influence the degree of lysosomal sequestration. However, considering the complexity of cellular systems and the potential for multiple sequestration mechanisms, such a model cannot be relied on without experimental verification. In fact, qualitative evaluations have suggested that some weakly basic molecules are not sequestered into lysosomes even though conditions are favorable for pH-partitioning.[5] The opposite scenario has also been proposed for the weakly basic drug chloroquine, which was proposed to accumulate in lysosomes by mechanisms in addition to pH-partitioning.[12]

The ability to rationally design or modify drugs to optimize their intracellular disposition would be highly desirable. In order to make progress in this new area of drug delivery research we must improve our understanding regarding drug sequestration mechanisms within cells. A major hurdle to this area of research is a lack of suitable assays for accurately measuring the lysosomal accumulation of drugs. In earlier attempts, indirect assays were employed.[2,13] These approaches, however, do not allow for quantitative estimations. We describe here a novel magnetic capture technique that can be used to experimentally estimate the lysosomal concentrations of weakly basic compounds incubated with cells in culture. A preliminary study with two model compounds establishes the utility of the assay and also suggests that mechanisms in addition to pH-partitioning may play a significant role in the accumulation of drugs in lysosomes.

Experimental Section

Cell Line and Reagents. The doxorubicin-selected multidrug resistant human acute promyeloid leukemia cell line HL-60 was employed in these studies and was a gift from Dr. Yueshang Zhang (Arizona Cancer Center, University of Arizona). Cells were grown in RPMI 1640 medium supplemented with 10% FCS, 10 mM Hepes, 1 mM sodium pyruvate, and 0.1% penicillin−streptomycin and were maintained at a density of 1×10^5 to 1×10^6 cells/mL at 37 °C in a humidified 5% CO_2 atmosphere. Quinacrine (QNC) was purchased from Sigma (St. Louis, MO). LysoTracker Red DND-99 (LTR) was obtained from Molecular Probes (Eugene, OR). Oregon green conjugated dextran (10 kDa) was a gift from the Microscopy and Analytical Imaging Laboratory (The University of Kansas, Lawrence).

Fluorescence Microscopy. Cells were grown to a density of 1×10^6 cells/mL prior to experimentation and incubated with LTR or QNC (1 μM) for 2 h under normal growth conditions. Following incubation, cells were pelleted (500*g*, 5 min) and washed twice with ice-cold phosphate buffered saline pH 7.4 (PBS) to remove unincorporated compound. Cells were viewed with a microscope (Leica Diaplan, Leitz Weltzar, Germany) equipped for epifluorescence with a 100× objective, and images were captured using an Orca ER camera (Hamamatsu Corp.) controlled by SimplePCI imaging

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software (Compix Inc.). Texas Red and fluorescein filter sets were employed for LTR and QNC, respectively.

Lysosome Isolation. Isolation of lysosomes was carried out using a magnetic chromatography procedure originally described by Diettrich and co-workers with modifications.[14] Iron−dextran particles (FeDex) were prepared as described in the above reference. Cells (200×10^6) were incubated with FeDex at a concentration of 2 mg/mL for 1 h at 37 °C in PBS to allow for endocytic uptake. Following this, cells were washed four times with PBS and incubated in FeDex-free culture medium at 37 °C for a 24 h chase period to allow for specific lysosomal accumulation of FeDex. At the end of the chase, cells were washed twice with ice-cold PBS and homogenized in 2 mL of buffer A (15 mM potassium chloride, 1.5 mM magnesium acetate, 1 mM dithiothreitol, and 10 mM Hepes; pH 7.4) supplemented with 0.1 mM PMSF (phenyl-methyl sulfonyl fluoride), 0.5 mg/mL DNase I, and 1 μg/mL each of aprotin, leupeptin, and pepstatin. Cells were homogenized in a dounce homogenizer (15 strokes) using the tight fitting pestle (pestle B). To the homogenate was added 0.5 mL of buffer B (375 mM potassium chloride, 22.5 mM magnesium acetate, 1 mM dithiothreitol, and 220 mM Hepes; pH 7.4), and the mixture was centrifuged at 700*g* for 10 min to pellet nuclei and unbroken cells. The postnuclear supernatant (PNS) thus obtained was passed through a miniMacs column (Miltenyi Biotec, CA) contained in a magnetic sleeve. The column was pre-equilibrated with 500 μL of 0.5% bovine serum albumin in PBS to prevent nonspecific binding of organelles to the column. Subsequently, two column volumes of PBS containing 0.5 mg/mL DNase I was passed through the column and the column was allowed to stand in the magnet for 10 min. This was done to break down any organelle agglomerates that may have formed during homogenization. The column was then washed with two volumes of 500 μL of ice-cold PBS. The column contents were eluted by removal of the column from the magnet followed by the addition of PBS containing 0.5% Triton X-100.

Assessment of Lysosomal Purity and Recovery. Aliquots of PNS, column flow through and the fraction retained in the magnetic column, containing 10 μg of total protein each, were analyzed using a 7.5% SDS−PAGE. Quantitation of protein content in the samples was carried out by the method of Bradford using bovine serum albumin as a standard. Purity of the isolated lysosomal fraction was assessed by Western blotting using mouse monoclonal antibodies specific to the following organelle-associated proteins: EEA1 (early endosomes), Lamp-1 (lysosomes), Hsp60 (mitochondria), Golgin-84 (the Golgi apparatus), and Bip/GRP-78 (endoplasmic reticulum, ER). Integrity of isolated lysosomes was determined by Western blotting for the soluble intralysosomal protease, cathepsin D. Following electrophoretic separation, proteins were transferred onto a nitrocellulose membrane (0.45 μm pore size). Transfer conditions were 100 V for 2 h for EEA1, Lamp-1, and Golgin-84; and 100 V for 30 min for Hsp60, Bip/GRP-78, and cathepsin D. The nitrocellulose blot was then blocked with 5% milk (nonfat) in Tris-buffered saline (pH 8.0 containing 0.05% Tween 20). Blots were then incubated with one of the following antibody cocktails: cocktail 1 contained antibodies against EEA1, Lamp-1, and Golgin-84 while cocktail 2 contained antibodies against Hsp60, Bip/GRP-78, and cathepsin D. The antibodies were prepared in the following dilutions in 5% milk (nonfat) in Tris-buffered saline (pH 8.0): Lamp-1 (1:100), BiP/GRP-78 (1:250), Golgin-84 (1:250), EEA1 (1:250), Hsp60 (1:10000), and cathepsin D (1:1000). This was followed by incubation with a horseradish peroxidase (HRP) conjugated secondary antibody (goat-anti-mouse). The secondary antibody was diluted 1:1000 in the aforementioned solution. Protein bands were detected by chemiluminescence, and images were captured on a Kodak Biomax MR Scientific Imaging film.

Fold-enrichment and recovery of lysosomes in the cellular fractions were quantitatively evaluated by measuring activity of the lysosomal enzyme β-hexosaminidase using *p*-nitrophenyl-*N*-acetyl-β-D-glucosamidine (PNPG) as substrate. Aliquots of each fraction containing 0.1 mg of total protein (determined by the method of Bradford) were incubated with the assay mixture containing 0.5 mM PNPG in 0.1 M citrate buffer (pH 4.5) for 90 min at 37 °C to allow for enzymatic conversion of PNPG to *p*-nitrophenol (PNP). The reaction was then stopped by addition of 0.8 mL of 0.1 M sodium bicarbonate buffer (pH 10.0). This mixture was centrifuged at 16000*g* for 2 min, and the absorbance of the supernatant was read at 405 nm to determine the concentration of PNP formed. Using the concentration of PNP and sample volume, amount of PNP (nanomoles) formed during the reaction was obtained. β-Hexosaminidase activity in a given cellular fraction was determined as the number of nanomoles of PNP formed per microgram of protein in that fraction. Enzyme activity in lysosomal fraction was divided by that in the PNS to obtain fold-enrichment of lysosomes. Total enzyme activity in the lysosomal fraction, obtained as the product of activity per microgram of protein and total protein content in this fraction, was divided by the total activity in PNS to determine percent recovery of lysosomes.

Number of lysosomes per cell was estimated using confocal fluorescence microscopy. Cells were incubated with 5 mg/mL Oregon green conjugated dextran for 1 h (in PBS) under normal culture conditions. Subsequent wash and chase conditions were identical to those employed with FeDex. Cells were then fixed with 3% paraformaldehyde for 30 min on ice and viewed under a Zeiss Meta 510 confocal microscope (Thornwood, NY). Cells were exposed to an argon laser at 514 nm, and an LP 530 filter set was employed to view Oregon green fluorescence. Images were captured using a Hamamatsu Orca ER camera. A total of 25 slices of 0.5 μm thickness (*Z*-stacks) were obtained for each individual cell that was visualized. Twenty-five slices were found to

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be adequate to capture fluorescence signals from an entire cell (size of the cell ~10 μm). Images were analyzed using an Image J software version 1.33u (National Institutes of Health). With the appropriate threshold conditions, the "Analyze Particles" option was used to count total number of lysosomes per slice. Total number of fluorescently labeled lysosomes per cell was estimated by adding the counts obtained from each of the 25 slices. Fluorescence signals that were duplicated between slices were removed from total counts. The above procedure was repeated with 10 different cells, and an average number for lysosomes per cell was obtained.

Assessment of Lysosomal Morphology. For transmission electron microscopy experiments, cells were incubated with FeDex particles, which were allowed to specifically accumulate within lysosomes as described under Lysosome Isolation. Cells were subsequently washed twice with ice-cold PBS and fixed with 3% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.4) containing 8% sucrose for 1 h at 4 °C. Fixed cells were washed twice with 50 mM sodium cacodylate buffer containing 8% sucrose. For routine TEM processing, cells were pelleted at 500*g* for 5 min and postfixed in 1% osmium tetroxide in sodium cacodylate buffer (pH 7.4) for 2.25 h (on ice). This was followed by several rinses in deionized water and dehydration with ethanol. Following three changes in the intermediate solvent acetone, the pellets were infiltrated for several hours in various mixtures of acetone and EMBed-812 (Electron Microscopy Sciences, Fort Washington, PA). The pellets were infiltrated overnight in 100% EMBed-812 and polymerized at 60 °C for 24 h. Ultrathin sections of the pellet were mounted on copper grids and stained with uranyl acetate and lead citrate. All samples prepared for TEM were viewed using a JEOL 1200 EXII transmission electron microscope. Digital images were acquired using a Soft Imaging System Mega View II camera and software (Soft Imaging System Corp., Lakewood, CO). Micrographs were analyzed morphometrically using MetaMorph version 6.2 (Universal Imaging Corp., PA) to determine average lysosome diameter. A total of 60 lysosomes were individually analyzed. Since the vesicles were not exactly spherical, an average of two diameters measured perpendicular to each other was obtained for each lysosome.

HPLC Setup and Conditions. The HPLC system was composed of a Waters 600E system controller, 616 pump, 717 plus autosampler, and 474 fluorescence detector (Waters Corp., MA). A Waters Xterra MS C_{18} column (2.1 × 50 mm; 3.5 μm particle size) was used. The mobile phase contained 25% and 28% acetonitrile in 10 mM ammonium formate (pH 4.0) for QNC and LTR, respectively. The excitation and emission wavelengths for each of the compounds were as follows: QNC (λ_{ex} = 463 nm; λ_{em} = 500 nm), and LTR (λ_{ex} = 577 nm; λ_{em} = 590 nm).

Estimating Lysosomal Concentrations of LTR and QNC. Lysosomes were isolated from cells previously incubated with either LTR or QNC at a concentration of 1 μM for 12 h. Isolated fractions were vortexed for 30 s with an equal volume of acetonitrile and centrifuged at 16000*g* for 5 min. Supernatant was evaporated to dryness in a SpeedVac concentrator (Thermo Electron Corp., MA), and the residue was dissolved in 100 μL of the appropriate HPLC mobile phase and analyzed. Total volume of the isolated lysosomes, estimated as the product of average lysosomal volume (TEM experiments) and number of lysosomes per cell (confocal microscopy), was used to convert amount of drug recovered to its lysosomal concentration. Extraction efficiencies and standard curves were used in all our calculations and were determined by spiking known amounts of each test compound (5 different amounts, representing high, medium, and low concentrations) into blank lysosomal fractions and carrying out the extraction procedure described above. Standard curves, constructed by plotting amount of drug spiked into blank fractions against amount of drug recovered by the extraction procedure, were linear with $r^2 > 0.98$. Extraction efficiencies for the test compounds ranged from 63% to 95%.

Theoretical Calculations of Lysosomal Concentrations. The theoretical basis for pH-partition driven accumulations of weakly basic compounds in lysosomes of cells has been previously described.[11] From this work, the ratio of drug concentration in lysosomes to that in the extracellular medium at steady state is given by

$$\frac{[\text{lysosome}]}{[\text{extracellular}]} = \frac{(\alpha[H^+]_E + K_a)}{([H^+]_E + K_a)} \frac{([H^+]_L + K_a)}{(\alpha[H^+]_L + K_a)}$$

The weak base dissociation constant is denoted as K_a, and $[H^+]$ is the proton concentration (subscript E represents extracellular, and L represents lysosomal). The ratio of permeabilities of the ionized base to that of the un-ionized base in the lysosomal lipid bilayer is denoted by the α term. This is experimentally determined by measuring octanol/water partition coefficients for the ionized and free base forms of the compound and taking the ratio of these values as previously described. We have previously estimated α values for LTR and QNC, which are 0.017 and 0.004, respectively.[15] From the same report, the pK_a values of LTR and QNC are 7.5 and 8.0, respectively. The pH value associated with lysosomes of the HL-60 cell line employed in this study was previously shown to be 5.1.[8] The pH of the extracellular medium was found to be 7.48 using a pH meter. Using these values, the ratios of lysosomal to extracellular concentrations of LTR and QNC are 24 and 48, respectively. Since the extracellular concentration of molecules used in this study was 1 μM, the lysosomal concentrations for LTR and QNC are predicted to be 24 and 48 μM, respectively.

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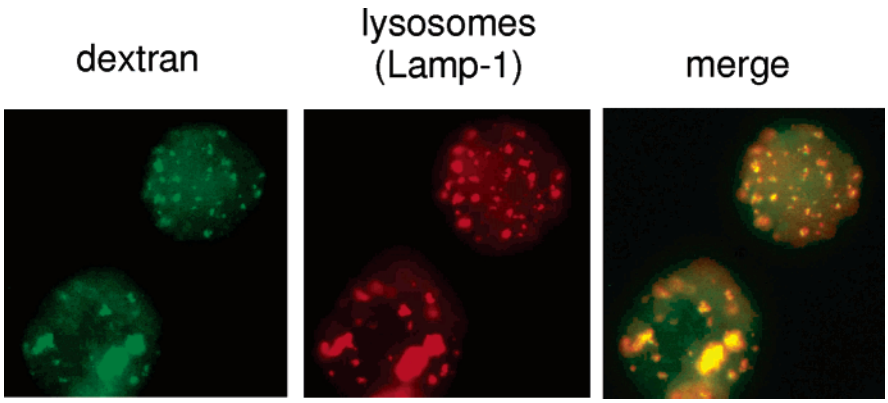

Figure 1. Colocalization of endocytosed dextran particles with lysosomes in HL-60 cells. The left panel reveals the intracellular distribution of FITC-labeled dextran following the pulse chase protocol described in the Experimental Section. The middle panel indicates immunofluorescence staining of the lysosomal protein Lamp-1 in the same cells. The panel on the right is a merge of the previously described images.

Results

Lysosome Isolation. Lysosomes were isolated from cells using a modified magnetic capture technique originally described by Diettrich and co-workers.[14] The procedure involves incubation of cells with iron-coated dextran particles (FeDex), which are taken up into cells by fluid phase endocytosis. After the initial uptake period, cells are placed in cell culture medium devoid of the dextran for 24 h to allow lysosomal-specific accumulation of FeDex. To isolate lysosomes, cells were homogenized and passed through a magnetic column, which retained the organelles containing FeDex.

To confirm that the dextran particles were specifically localized to lysosomes, the experimental protocol was repeated using fluorescent dextran instead of FeDex that could be visualized using a fluorescence microscope and examined for colocalization with the lysosome specific protein Lamp-1. Accordingly, Figure 1 reveals that fluorescent dextran molecules colocalize with lysosomes.

Assessment of Lysosomal Purity and Intactness. Isolated lysosomes obtained from this procedure were characterized for their purity using two separate techniques. The first employed Western blotting with antibodies to organelle specific proteins illustrated in Figure 2. The analyzed fractions were derived from the starting postnuclear supernatant (PNS), PNS that passed through the magnetic column (flow through), and the material retained in the magnetic column (retained fraction). In the PNS fraction, all organelle-specific proteins were visible. The column flow through fraction had noticeably reduced quantities of Lamp-1 whereas the retained fraction had high levels of this protein.

For the assay to be suitable for the intended purpose of determining concentrations of molecules contained within the organelle, it is important that the vesicles remain intact during the isolation procedure. Also shown in Figure 2, the soluble intralysosomal protease, cathepsin D, was highly enriched in the fraction retained in the column. This observation is consistent with the vesicles remaining intact; otherwise a significant fraction of the protease would be present in the column flow through.

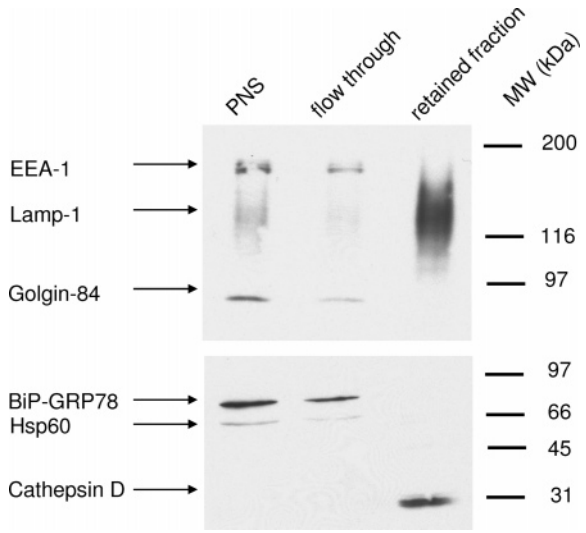

Figure 2. Western blot analysis of organelle-associated proteins in samples obtained from the lysosome isolation procedure. Samples containing equal amount of total protein (10 μg) from the starting PNS fraction (left lane), column flow through (middle lane), and the fraction retained on the column (right lane) are represented on the blot. Specific organelle-associated proteins were probed for using primary antibodies specific to the following proteins: EEA-1 (early endosomes); Lamp-1 (lysosome); Golgin-84 (Golgi apparatus); Bip-GRP78 (endoplasmic reticulum); Hsp60 (mitochondria); and cathepsin D (intralysosomal protease). The location of MW markers is indicated on the right-hand side of each blot.

Lysosomal recovery and purity were more quantitatively evaluated using an activity assay for the lysosomal enzyme β-hexosaminidase. Table 1 lists the activity of this enzyme on a per microgram total protein basis from column flow through and the retained fraction. From these results the isolation efficiency of lysosomes from the PNS was determined to be 20%. Additionally, the procedure resulted in a 10-fold enrichment of the lysosomal enzyme from the PNS.

We next examined if the isolation procedure could specifically capture compounds known to accumulate in lysosomes of intact cells. Prior to isolating lysosomes, cells

Table 1. Activity of the Lysosomal Marker in Isolated Fractions and Recovery of Lysosomes Relative to Levels Contained within the Starting PNS Fraction

fraction	β-hexosaminidase activity[a,b]	% recovery[a]
flow through	0.48 ± 0.01	69.2 ± 2.57
retained fraction	5.7 ± 0.06	20.3 ± 0.57

[a] Values are the means of three determinations ± SD. See Experimental Section for details. [b] Values represent nanomoles of PNP formed per microgram of total protein.

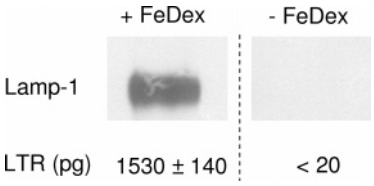

Figure 3. The retention of lysosomes and LysoTracker Red on the magnetic column are specific to cells incubated with FeDex. The results in the left-hand column are from cells incubated with FeDex (+ FeDex). The results in the right-hand column are obtained from cells incubated with unconjugated dextran as a control (− FeDex). The upper panels illustrate Western blot analysis results from proteins retained on the magnetic column using a primary antibody specific to the lysosomal membrane protein Lamp-1. The lower panels illustrate levels of the lysosomotropic agent LTR associated with the retained fraction determined by HPLC. The quantitation of LTR represents the mean ± SD for three experiments. LTR was always below the limit of detection (20 pg) in cells incubated with unconjugated dextran.

were incubated with LysoTracker Red (LTR), a commercially available lysosomal probe that is known to be sequestered into lysosomes according to a pH-partitioning mechanism. Following the incubation, the isolated lysosomal fraction was evaluated for LTR by HPLC and Lamp-1 by Western blotting. As shown in Figure 3 (+ FeDex), this fraction contained 1530 pg of LTR as well as significant amounts of Lamp-1. To evaluate the potential for nonspecific retention of LTR on the magnetic column, the experiment was repeated in cells that were preincubated with unconjugated dextran instead of FeDex (− FeDex, Figure 3). In these cells, the fraction retained in the column was devoid of both LTR and Lamp-1, indicating that retention of LTR was due to FeDex incorporation as opposed to nonspecific association with the column.

The Influence of FeDex Particles on Drug Accumulation in Lysosomes. FeDex particles contained in lysosomes could potentially interfere with the accumulation of drugs within the organelle and confound interpretations. To test for this possibility, we visualized the lysosomal accumulation of LTR in cells with and without FeDex treatment and observed no obvious change in apparent uptake or cellular distribution (Figure 4, panel A). To address this more quantitatively we made the assumption that all LTR in the cell was contained within lysosomes. This approximation is consistent with fluorescence micrographs in Figure 4. Thus, measuring whole cell amounts of LTR in cells by HPLC,

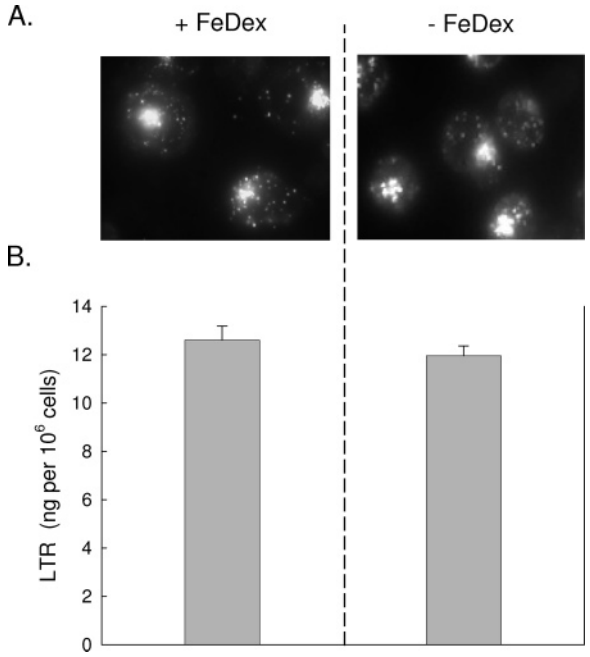

Figure 4. The influence of FeDex on the degree of LTR accumulation in cells: (A) representative fluorescence micrographs illustrating the subcellular distribution LTR in cells with and without FeDex treatment; (B) whole cell accumulation of LTR in cells with or without FeDex particles. Results are expressed as mean ± SD ($n = 3$).

with and without FeDex pretreatment, should provide a more quantitative evaluation of the effect of FeDex on LTR sequestration in lysosomes. As is shown in Figure 4 (panel B), cells preincubated with FeDex accumulated similar amounts of LTR as those without FeDex, suggesting that these particles did not significantly influence the extent of lysosomal accumulation of LTR.

Characterization of Lysosomes. As previously stated, the amount of compound associated with isolated lysosomal fraction was obtained using HPLC. In order to convert this amount to a concentration estimate in an isolated lysosome, more extensive characterizations of the isolated organelles were required.

The average volume of lysosomes was assessed using transmission electron microscopy (TEM) of intact cells incubated with FeDex. In Figure 5, representative TEM images of lysosomes loaded with FeDex are shown. Using these micrographs, plus additional micrographs (not shown), the mean diameter from a total of 60 lysosomes was found to be 184 nm. Thus, assuming a spherical lysosomal structure, the average volume of an individual lysosome is 3.26×10^{-6} pL.

The total number of lysosomes in the intact cell was estimated using confocal fluorescence microscopy with cells that have taken up fluorescently labeled dextran into the lysosomal compartment (data not shown). By taking stepwise visual slices (500 nm each) we were able to visualize the contents of the entire cell. Using this approach the average number of punctate fluorescent spots per cell was determined to be 180 ± 15 (average from a total of 10 cells).

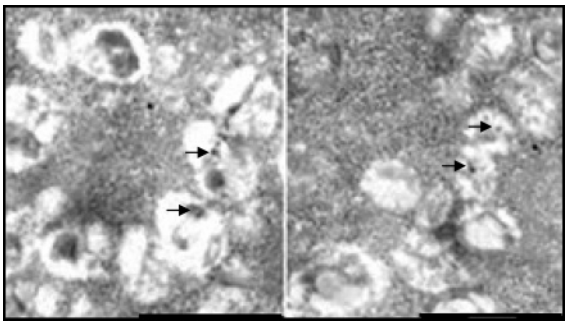

Figure 5. Transmission electron micrographs (TEM) of lysosomes in intact HL-60 cells. Arrows indicate instances where FeDex particles were visible inside lysosomes. The bar represents 0.6 μm.

Estimating Concentrations of Model Compounds in Isolated Lysosomes. Using our estimations for the total number of lysosomes per cell (180) and the average volume of a lysosome (3.26×10^{-6} pL), we estimated that the total volume of a cell occupied by lysosomes is 5.86×10^{-4} pL. We started with 200×10^6 cells in our protocol, and we have previously shown that our lysosomal recovery from the starting cell lysates is 20% (see Table 1). Therefore, a final estimate of the volume of lysosomes obtained from the magnetic capture technique is approximately 23.4 nL. The amount of drug contained within isolated lysosomes (as determined by HPLC analysis) was subsequently divided by this lysosomal volume to obtain a final estimate for lysosomal concentration.

For validation purposes, the lysosomal concentration of two fluorescent model compounds (QNC and LTR) was evaluated in cells incubated in media containing 1 μM compound for 12 h. Fluorescence micrographs of cells incubated with these compounds are shown in Figure 6A and demonstrate the large degree of lysosomal sequestration. Our estimates for lysosomal concentrations for QNC and LTR were 766 μM and 60 μM, respectively (Figure 6B).

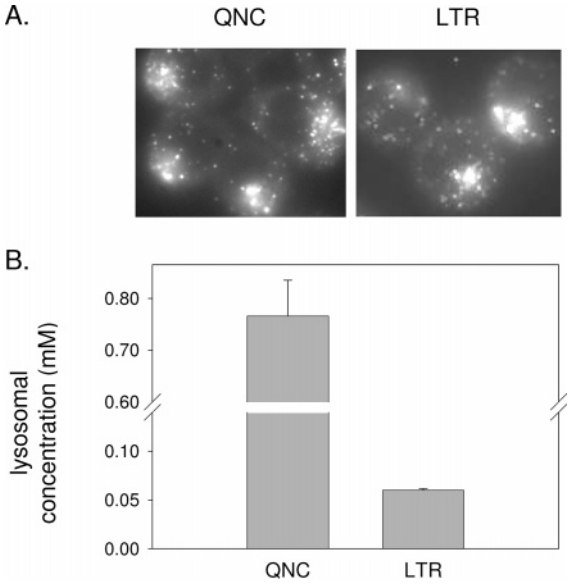

Figure 6. Qualitative and quantitative assessments of the lysosomal sequestration of QNC and LTR in HL-60 cells: (A) representative fluorescence micrographs of cells incubated with 1 μM of QNC (left column) or LTR (right column) revealing the punctate intracellular accumulation; (B) estimates of lysosomal concentrations of QNC and LTR using the magnetic capture technique. Results are expressed as mean $\pm$ SD ($n = 3$).

Discussion

There has been much recent interest in evaluating the intracellular distribution of drugs considering the potential impact on drug activity.[16] Specifically, the sequestration of drugs in lysosomes has received much attention. For example, lysosomal sequestration of anticancer agents has been proposed to play a role in drug resistance in cancer cells.[17] Moreover, this sequestration has been proposed to influence whole body distribution patterns of drugs.[18−20] Despite the pharmaceutical implications, quantitative assays used to directly evaluate drug sequestration in lysosomes have not been previously described.

We illustrated here a novel magnetic capture technique to isolate lysosomes that can be used to assess lysosomal accumulation of drugs. Alternative techniques for isolating lysosomes from cells, for other purposes, have been previously described. For example, multistep density gradient based approaches have been routinely employed for this purpose.[21,22] However, problems with reproducibility, excessive time required for the procedure, and insufficient purity prompted us seek alternative approaches. Immunoisolation techniques, on the other hand, have been shown to be quite rapid and efficient.[23] The routine use of this approach,

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however, would inevitably require large quantities of antibodies, which would be prohibitively expensive. The magnetic capture technique described here is particularly well suited for our purpose. We have demonstrated it to be fast and reproducible and to yield good purity. Control experiments described in this manuscript support this claim.

The isolation technique, together with HPLC based analysis, allowed us to quantitate the amount of model compounds contained within lysosomes from cells that have been exposed to these agents in culture. Drug diffusion represents a potential limitation for any cell fractionation based approach to assess the intracellular distribution of drugs. It may be anticipated that drugs would significantly leach out of organelles during the isolation procedure. In a previous report we have explored the general feasibility of cell fractionation based approaches.[24] In this report we used a density gradient based approach to obtain a single fraction that contained all cytoplasmic organelles, not just lysosomes as was described here. For compounds known to be sequestered in lysosomes (i.e., LTR) we had shown that the amount of compound associated with isolated organelles remained unchanged, even after hours of incubation in drug-free buffer. These results suggest that diffusion may not be a limiting factor for those drugs that are highly concentrated in lysosomes by mechanisms that are not disrupted by the isolation procedure.

In order to convert the amount of drug contained within an isolated fraction of lysosomes into concentration terms, we extensively characterized the isolated lysosomal fraction. Few previous reports have evaluated parameters such as the number of lysosomes in a cell or the average volume of a lysosome. The work described here represents the first characterizations in HL-60 cells, to our knowledge. In studies with human hepatocytes, however, it has been reported that there are 200−300 lysosomes per cell.[25] This is in reasonable agreement with our estimate of 180 considering the differences in cell types. The size and shape of lysosomes have been shown to be quite heterogeneous.[26] The average diameter of the organelle has been reported to vary between 50 and 300 nM. Our estimate of 184 nm is within this range.

In order to evaluate the reproducibility and accuracy of the method, we estimated the lysosomal concentrations of two test compounds (QNC and LTR). These compounds were present in the cell culture media at a concentration of 1 μM for 12 h prior to lysosome isolation. Our estimates for lysosomal concentrations for QNC and LTR were 766 $\pm$ 69 μM and 60 $\pm$ 1.8 μM, respectively. Importantly, the relative standard deviations for our measurements are on the order of 3−9%, which illustrates that the assay is quite reproducible. It is likely that some lysosomes rupture and reseal during the cell homogenization procedure, thus releasing some of the entrapped drug. Therefore it is possible that our concentration estimates are below the actual concentrations found in lysosomes in an intact cell. However, we do not believe that this occurs extensively since the soluble lysosomal enzyme cathepsin D appears to be significantly retained in isolated lysosomes and not visibly contained within the column flow through (see Figure 2).

This is the first time that lysosomal concentrations of these compounds have been quantitatively evaluated; therefore, direct experimentally based comparisons are not possible. However, Bulychev and co-workers have incubated cultured fibroblasts with high concentrations of the weakly basic dye neutral red (130 μM), which they had shown to specifically localize in lysosomes using microscopic methods.[27] The authors reported that, following the incubation, cells contained 180 nmol of the molecule per milligram of cellular protein. Assuming that all the drug was associated with lysosomes, the authors proposed that lysosomal concentrations might have exceeded 150 mM. This work illustrates the extraordinary concentrative capacity of the organelle and suggests that our estimates are reasonable in magnitude.

It has been assumed that pH-partitioning provides a major driving force for the accumulation of weakly basic drugs into lysosomes. Considering this assumption to be true, we calculated the predicted lysosomal concentrations of the two model compounds used in this study. Such values can be obtained using theoretical models of pH-partitioning described by de Duve.[11] The theoretically based estimates for QNC and LTR are 48 μM and 24 μM, respectively (for a full description of these calculations see the Experimental Section).

The trend of our experimental results is consistent with theoretical predictions in that QNC concentrated in lysosomes to a greater extent than LTR. This is expected since QNC has both a slightly higher pK_a than LTR and it is less permeable in its ionized state, both of which are expected to result in enhanced lysosomal accumulation capacity.

Both experimentally determined concentrations, however, are considerably higher than would be predicted on the basis of pH-partitioning alone. There are numerous possible explanations for this discrepancy. One likely possibility is that other factors are contributing to lysosomal sequestration in addition to pH-partitioning. Ionic association of weakly basic compounds with negatively charged groups comprising lipid bilayers of lysosomes has been proposed to significantly contribute to lysosomal associations.[27,28] Specifically, acidic polysaccharides and acidic glycolipids, both of which are

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highly abundant in lysosomes, have been implicated.[29] This explanation is also consistent with the work of Colombo and Bertini, who have proposed that the weakly basic drug chloroquine has multiple binding sites on lysosomes that would cause it to accumulate to an extent greater than pH-partitioning theory would predict.[12] As it was designed, the assay described in this work cannot differentiate between intralysosomal versus membrane associated drug. Further evaluations with isolated lysosomes will be needed to assign the relative contributions of each of these pathways.

This method presented here represents a significant advancement in the ability to quantitatively assess the sequestration of drugs in lysosomes of cells. There has been much recent interest in correlating intracellular distribution of molecules with structural attributes because of the implications in drug activity.[16] In previous efforts to establish such relationships, the intracellular distribution of libraries of fluorescent compounds has been investigated using a fluorescent microscope.[7,30,31] Despite the usefulness of these approaches in establishing structure−localization type relationships, they are limited to fluorescent molecules. The approach described here should be useful for all drugs regardless of fluorescent properties. Preliminary studies with two model compounds described here suggested that multiple mechanisms might contribute to the sequestration of drugs in lysosomes. This approach should facilitate future studies that aim to more thoroughly elucidate these contributions.

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