A Cell Fractionation Approach for the Quantitative Analysis of Subcellular Drug Disposition

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Purpose. The purpose of this work was to develop and validate a method that can be used to quantify drugs associated with intracellular compartments.

Methods. The human leukemic cell line U-937 was used to evaluate the distribution of model compounds with known and different subcellular distribution profiles. Lysotracker Red is a lysosomal vital stain and doxorubicin is an anticancer agent with a strong propensity for nuclear accumulation in U-937 cells. After incubation with compounds, cells were separated into fractions containing nuclei, cytosol, and cytoplasmic organelles (lysosomes, mitochondria, Golgi apparatus). Compounds contained within isolated fractions were subsequently extracted and analyzed by high-performance liquid chromatography. Diffusion of compounds from isolated organelles was also investigated.

Results. Using this approach we have shown that the model compounds Lysotracker Red and doxorubicin preferentially accumulated within lysosomes and nuclei, respectively. We have reproducibly determined concentrations of these compounds in each of the cellular fractions. We have also shown that diffusion of these compounds from isolated cellular compartments was minimal during the time required to complete the experimental procedure.

Conclusions. The analytical approach described in this manuscript yielded reproducible quantitative data regarding the intracellular distribution of model compounds in U-937 cells. With the aid of a relatively sensitive analytical assay, this technique should be useful for most drugs that have a specific concentrative mechanism for organelle accumulation similar to Dox and LTR.

KEY WORDS: organelle; drug partitioning; cell fractionation; drug transport; bioanalysis.

INTRODUCTION

Knowledge regarding the distribution of a drug within a cell is fundamentally important to drug delivery research where the ultimate goal is to maximize the interaction of a drug with its target. Drug targets are often inside cells or, more specifically, within cellular organelles. Preferential localization of a drug within a subcellular compartment housing its target would be expected to improve potency and reduce side effects; accumulation in extraneous sites would be expected to reduce the drug's therapeutic potential. Considering the fact that the majority of the space in a cell is comprised of organelles, the potential barriers to drug delivery are significant and often overlooked. Distinct populations of organelles have unique intralumenal pH values (1), resident molecules (2), electronic potential (3), lipid-bilayer composition, and membrane-bound proteins (4). These properties can significantly influence drug partitioning behavior which can result in preferential accumulation in selected organelles (5).

Using fluorescence microscopy, several research groups have visualized the subcellular distribution of fluorescent compounds in cultured cells. Hydrophobic cationic compounds have been shown to selectively accumulate in mitochondria resulting from the net negative membrane potential associated with this organelle (6). Weakly basic drugs (i.e., lysosomotropic agents) have been shown to accumulate in acidic organelles such as the lysosome through an ion-trapping mechanism (7,8). The localization of molecules within nuclei has been directly correlated with DNA binding capacity (9). Furthermore, the subcellular localization pattern of drugs can change when drug sensitive cancer cells become multidrug resistant (10,11). These studies have been informative and have suggested that drugs do not distribute evenly throughout the cell; however, there are many limitations with the microscopic approaches used in these studies. An obvious disadvantage is that only compounds with sufficient fluorescence can be analyzed. Even when fluorescent compounds are used it is difficult to quantitate drugs in cellular compartments based on the fluorescence emission. Quantum yields and maximum excitation and emission wavelengths of fluorescent molecules are often extremely sensitive to environmental factors such as pH, ionic strength and noncovalent interactions. Millot and coworkers have shown that the fluorescence of aclacinomycin A was quenched 200-fold upon binding to DNA (12). Furthermore, subcellular spaces with particularly high concentrations of fluorescent molecules may exhibit little or no observable fluorescence emissions because of self-quenching reactions (13). Metabolism of the fluorescent compound within the cell can also result in complete loss or significant changes in fluorescence properties. Taken alone or together, these limitations can result in significant misinterpretation of cellular distribution data.

This article describes a cellular fractionation approach that was used to quantitatively evaluate subcellular localization of small molecule compounds independent of their fluorescent properties. After a brief incubation with drug, cells were separated into fractions containing cell cytosol, nuclei, and cytoplasmic organelles (assayed for lysosomes, endosomes, endoplasmic reticulum, Golgi apparatus, and mitochondria). Drug was subsequently extracted from the fractions and analyzed by high-performance liquid chromatography (HPLC). To demonstrate the feasibility of this approach, we used two compounds that have been previously evaluated with regard to their subcellular distribution by fluorescence microscopy. Both of these compounds were shown to localize to their respective compartment following our fractionation approach. Diffusion of the drugs from the isolated organelle was also estimated and appeared to be negligible. Our ability to reproducibly quantitate the distribution of these model compounds will allow us to evaluate the subcellular distribution of both fluorescent and nonfluorescent drugs. This will improve our ability to investigate how changes in drug structure influence drug partitioning within selected cellular compartments. An understanding of these structure transport relationships could lead to novel drug delivery strategies within a cell.

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MATERIALS AND METHODS

Isolation of Cytoplasmic Organelles

All experiments with cells were performed using the human myeloid leukemia cell line U-937 (American Type Culture Collection, Rockville, MD, USA) grown in RPMI-1640 cell culture medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1% penicillin, and 0.1% streptomycin at 37°C/5% CO_2, grown to a density of 1.5×10^6 cells/ml before experimentation. After a 2-h incubation with 0.5 μM of either doxorubicin (Dox, Alexis Chemicals, San Diego, CA, USA) or Lysotracker Red DND-99 (LTR, Molecular Probes, Eugene, OR, USA), 225×10^6 cells were pelleted (500 g, 5 min) and washed twice with 50 ml of ice-cold phosphate-buffered saline (PBS) to remove unincorporated compound. Cells were resuspended in 2.1 ml of solution 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 phenylmethyl sulfonyl fluoride, 5 mg DNase I, and 1 μg/ml each of aprotin, leupeptin, and pepstatin. After incubating the cells on ice for 10 min, they were homogenized in a Dounce homogenizer (20 strokes using Pestle B). To the homogenate, 0.5 ml of solution B (375 mM potassium chloride, 22.5 mM magnesium acetate, 1 mM dithiothreitol, and 220 mM HEPES, pH 7.4) was added. Unbroken cells and nuclei were pelleted by centrifugation at 700 g for 10 min at 4°C to generate the postnuclear supernatant (PNS). A mixture of 1.5 ml of PNS and 5 mL of 2.5 M sucrose was layered beneath a sucrose step gradient (top: 13 ml of 15% w/v sucrose; bottom: 13 ml 60% w/v sucrose) in a 36-ml (25 × 89 mm) centrifuge tube. All the sucrose solutions were buffered to pH 7.4 (3 mM Tris) and contained 1 μg/ml each of aprotin, leupeptin, and pepstatin. The gradient was spun at 4000 g (4°C) in a Beckman SW28 (swinging bucket) rotor for 3 h Gradient fractions of 1 ml each were collected from the top of the tube using a piston gradient fractionator (Biocomp Instruments Inc., Frederictown, Canada). Compounds were extracted and quantified from each fraction using the following procedures.

LTR Extraction from Cytoplasmic Organelles

To a 0.75-ml aliquot of a gradient fraction, 20 μl of 5% w/v Triton X-100, 10 μl of 10 mg/ml proteinase K, and 20 μl of 1 mg/ml DNase I were added. The sample was vortexed for 10 s and incubated on ice for 10 min. This was followed by treatment with 250 μl of saturated ammonium sulfate solution and additional 10 min ice incubation. The sample was heated at 65°C for 5 min and centrifuged at 16000 g for 5 min. A 1-ml portion of the resulting supernatant was vortexed with 2 ml of ethyl acetate for 20 s and centrifuged at 500 g for 3 min to separate the two phases. The organic phase was removed and evaporated to dryness. The residue was dissolved in 100 μl of mobile phase and analyzed by HPLC. The HPLC system was comprised of a Waters 600E system controller, 616 pump, 717 plus autosampler and 474 fluorescence detector (Waters Corp., Milford, MA, USA). HPLC mobile phase consisted of 35% acetonitrile and 65% buffered H_2O (10 mM ammonium acetate, pH 8.2). The excitation and emission wavelengths used for detecting LTR were 577 and 590 nm, respectively.

Dox Extraction from Cytoplasmic Organelles

The extraction procedure for isolating Dox from cytoplasmic organelles was identical to that described for the LTR except that 75 μl of 1.5 M Tris-HCl was added instead of ammonium sulfate (without heating to 65°C). HPLC mobile phase consisted of 20% acetonitrile, 80% buffered H_2O (10 mM ammonium formate, pH 3.0). The excitation and emission wavelengths were 470 and 550 nm, respectively.

Cytosol Isolation

The procedure for isolation of cytosol was identical to that for the isolation of cytoplasmic organelles, except for the following. PNS was generated from 75×10^6 cells. A mixture of 0.5 ml of ice-cold and 0.8 ml of the PNS was layered on top of a sucrose step gradient (top 1.4 ml of 18% w/v; bottom 0.5 ml of 86% w/v) in a 3.2 ml (13 × 56 mm) centrifuge tube. The tube was then centrifuged at 260000 g in a Beckman Optima TLX centrifuge using a Beckman TLA100.4 fixed angle rotor for 3 h at 4°C. Fractions of 300 μl were collected from the top of the tube for further analysis.

LTR and Dox Extraction from Cytosol Fraction

The extraction and quantification procedures for LTR and Dox from cytosol fractions were identical to those described for extraction from cytoplasmic organelles; however, all reagent additions were scaled down in a 7 to 3 ratio.

Isolating Nuclei

The isolation procedure of intact nuclei from U-937 cells was carried out by a slightly modified procedure of Hurwitz and coworkers (7). Briefly, 125×10^6 cells incubated with either Dox or LTR were resuspended in 10 ml of 0.25 M sucrose in TKMC buffer (50 mM Tris, 25 mM potassium chloride, 5 mM magnesium chloride, pH 7.0). Cells were homogenized in a Dounce homogenizer with 20 strokes using the tight fitting B pestle. To the homogenate, 10 ml of 2.3 M sucrose in TKMC buffer (containing 0.75% Triton X-100) was added. The homogenate was then placed on top of 2.3 M sucrose in TKMC buffer in a 25 × 89 mm centrifuge tube and centrifuged at 40500 g in a Beckman SW28 (swinging bucket) rotor for 70 min at 4°C to pellet the nuclei. The recovery was estimated by resuspending the nuclei in ice-cold PBS and counting nuclei using a hemacytometer after treatment with trypan blue.

Extraction of LTR and Dox from Isolated Nuclei

The isolated nuclei pellet was resuspended in 750 μl of ice-cold PBS. The extraction procedure for LTR from nuclei was identical to the previously described method for extraction and quantification of LTR from cytoplasmic organelles. The extraction and quantification of Dox was achieved by suspending isolated nuclei in 750 μl of acetonitrile followed by a 15-min sonication. The suspension was centrifuged at 16000 g for 10 min. A 500-μl portion of the supernatant was evaporated to dryness. The residue was dissolved in 50 μl of HPLC mobile phase for Dox analysis by HPLC as previously described.

Accumulation of Compounds in Intact Cells

U-937 cells (50×10^6) were incubated with either 0.5 μM LTR or Dox for 2 h at 37°C and washed twice with ice cold PBS. The cells were resuspended in 750 μl of PBS and the compounds were extracted. The extraction and quantification procedures for LTR and Dox from intact cells were identical to those described for extraction from cytoplasmic organelles.

Isopycnic Separation of Cytoplasmic Organelles

A 1.5 ml portion of PNS, generated as described previously for isolation of cytoplasmic organelles, was loaded on top of 34 ml of a 40% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in 0.25 M sucrose (pH 7.4) in a 36.2 ml (25 × 86 mm) centrifuge tube. The tube was centrifuged at 100000 g for 2 h at 4°C in a Beckman VTi50 (vertical) rotor. The gradient was separated into 1 ml fractions (collected from the top of the tube) and assayed for cytoplasmic organelles by Western blot. LTR extraction and quantification was conducted as previously described for cytoplasmic organelles.

Extraction Efficiencies and Standard Curves

Extraction efficiencies and standard curves were determined by spiking known amounts or either Dox or LTR (five different amounts representing high, medium, and low concentrations) into blank cytoplasmic organelle, nuclear or cytosolic fractions and carrying out the respective extraction procedures. Standard curves were linear with $r^2 > 0.98$. Extraction efficiencies for LTR and Dox were 29.5 ± 3.2% and 20 ± 6.7%, respectively.

Diffusion Experiments

Diffusion of LTR from isolated cytoplasmic organelles was assessed by obtaining PNS from three sets of cells (1.5 ml each) incubated with 0.5 μM LTR. These samples were each diluted with 5 ml of 2.5 M sucrose and allowed to remain on ice for 0, 3, or 6 h Cytoplasmic organelles were then isolated and LTR was quantified as previously described. Evaluation of Dox diffusion from nuclei was carried out using nuclei isolated from three sets of cells previously incubated with 0.5 μM Dox. The nuclei were incubated in 5 ml of PBS (on ice) for 0, 1.5, or 3 h before the nuclei were pelleted and assayed for drug as previously described.

Fluorescence Microscopy

U-937 cells were suspended in RPMI medium containing 0.5 μM of Dox or LTR and incubated under growth conditions for 2 h Cells were pelleted at 1000 rpm for 5 min and washed twice with ice-cold PBS. Dox or LTR fluorescence was examined under a Diaplan fluorescence microscope (Leitz Weltzar, Germany) and the images were captured using a Hamamatsu ORCA-ER digital camera (Hamamatsu, Japan).

Miscellaneous Procedures

All refractive index measurements were made using a refractometer (Spectronic Instruments, Rochester, NY, USA). Total protein was determined according to the method of Bradford using bovine serum albumin as a standard (14). Western blot analysis was used to detect organelle-associated proteins using an antibody cocktail containing Lamp-1 (1:50), BiP/GRP78 (1:50), Golgin-84 (1:50), EEA1 (1:1000), Hsp 60 (1:1000) and Nucleoporin p62 (1:1000). Antibodies were diluted in 5% milk in Tris buffered saline (pH 8.0) containing 0.05% Tween 20.

RESULTS

Cytosol Isolation

Centrifugation of the PNS–PBS mixture on top of 18% w/v sucrose at 260000 g allowed concentration of the cytoplasmic organelles in the sucrose phase. Western blot analysis of organelle-associated proteins confirmed that the top fraction was free of organelles (see Fig. 1) and this fraction was used as the source of cytosol for extraction and quantification of model compounds.

The two phases were of sufficiently different viscosities as to minimize convective mixing of the phases and dissolved solutes contained within them. This was visually confirmed by observing the retention of dissolved bromophenol blue in the upper phase following routine experimental manipulation including centrifugation.

Cytoplasmic Organelle Isolation

The density of cytoplasmic organelles in sucrose range from 1.13 to 1.23 g/ml (15); therefore, we chose to concentrate organelles at the interface of a 15% and 60% sucrose step gradient. As is shown in Fig. 2B, the experimentally determined density of sucrose at this interface was sufficiently broad to concentrate organelles. After centrifugation, intact organelles (contained in the PNS) floated to the interface of the step gradient. This was confirmed by Western blot analysis of proteins associated with common cytoplasmic organelles (Fig. 2A). The organelle-enriched fractions (13 through 16) were collected and pooled. Comparison of Western blot exposures of the lysosomal protein Lamp-1 from pooled fractions to that from whole cell lysate allowed us to estimate lysosomal recovery for the procedure. Densitometric comparisons of these blots revealed that our recovery was approximately 15% (data not shown).

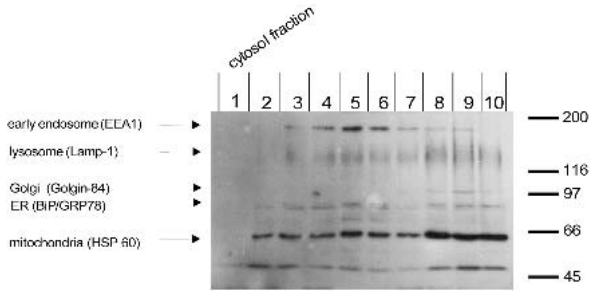

Fig. 1. Western blot analysis of 300-μl fractions taken from the top of a sucrose step gradient which was used to separate cytoplasmic organelles from cytosol. The first fraction is free of significant amounts of contamination from organelles and is used as a sample of cell cytosol. The cytoplasmic organelles examined have migrated into the second step of the gradient.

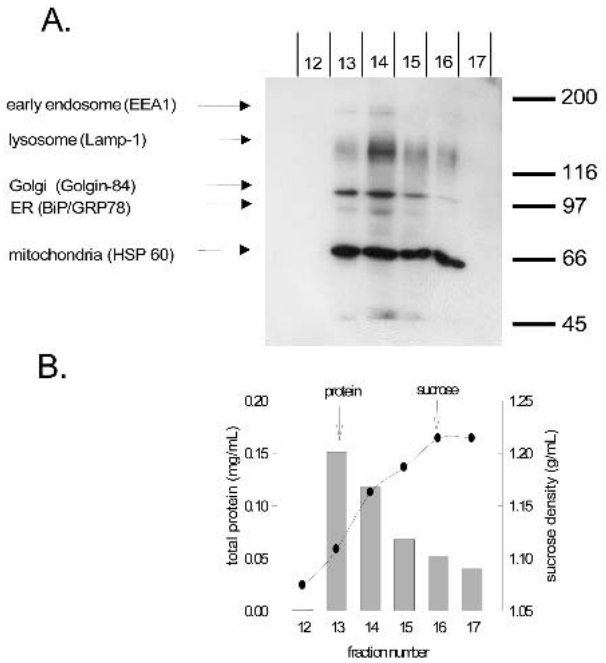

Fig. 2. A, Western blot of fractions 12 through 17 (1 ml each) obtained from a two step sucrose flotation gradient. Assayed organelle-associated proteins accumulated at the interface of the two-step gradient (fractions 13 through 16) with the exception of nuclei, which were pelleted in making the PNS. B, Total protein (bars) and experimentally determined sucrose density (solid points) of fractions 12 through 17.

Isolation of Nuclei

Nuclei were prepared as described in materials and methods. A phase contrast image of isolated intact nuclei is shown in Fig. 3A. Using a hemacytometer we calculated a 38% recovery of intact nuclei based on the number of cells started with. The purity of the nuclear fraction was further assessed by Western blotting for proteins specifically associated with organelles. A comparison of these blots from whole cell lysate and isolated nuclei are shown in Fig. 3B. Our initial observations using an antibody cocktail which contained the antibody for detecting nuclei (+ nucleoporin p62) revealed a band at approximately 60 kD that could either indicate contamination from mitochondria (Hsp 60) or represent a fragment of the nucleoporin protein. To resolve this, we used a modified antibody cocktail in which the antibody for nuclei was left out (-nucleoporin p62). The disappearance of the 60-kD band suggests that this was not representative of a mitochondrial contamination and that the fraction is indeed free of significant contamination from other organelles.

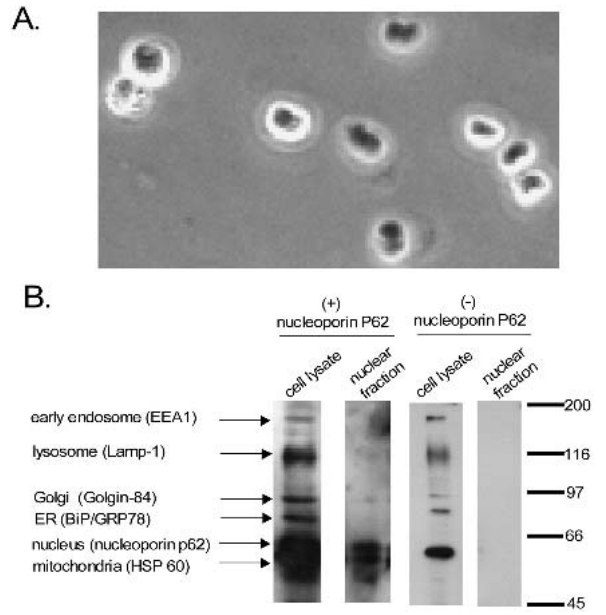

Fig. 3. A, Phase contrast image of isolated nuclei. B, Western blot of purified nuclear fraction with entire antibody cocktail (+nucleoporin p62) or with modified cocktail that is devoid of the antibody for nucleus associated protein Nucleoporin p62 (–nucleoporin p62).

Quantitation of Model Compounds in Isolated Cellular Fractions

To evaluate our ability to quantitate drugs in subcellular organelles we used two model compounds with known subcellular distribution. Dox is an anticancer drug that has a predominantly nuclear distribution (11). LTR is a probe for lysosomes in mammalian cells (16). Each of these compounds is fluorescent, which allows one to visualize their distribution using a fluorescent microscope. The visual distributions of LTR and Dox in the U-937 cell line are shown in Fig. 4A and B. To quantitatively evaluate the concentration of these drugs in their respective sites we need an estimate for volume of these compartments along with our calculated recoveries. Oehler and coworkers have estimated the total cell volume of the U-937 cell line to be 1 pL (17). Estimates for the percentage of the total cell volume that are represented by the cytosol, lysosomes, and nuclei are 54, 1, and 6%, respectively (18). These estimates are from a human hepatocyte and may be considerably different from those of the U-937 cell type based on morphological comparisons. This may lead to slight discrepancies in our absolute concentration estimates; however, this inaccuracy will not affect relative comparisons of the accumulation of different compounds within these subcellular

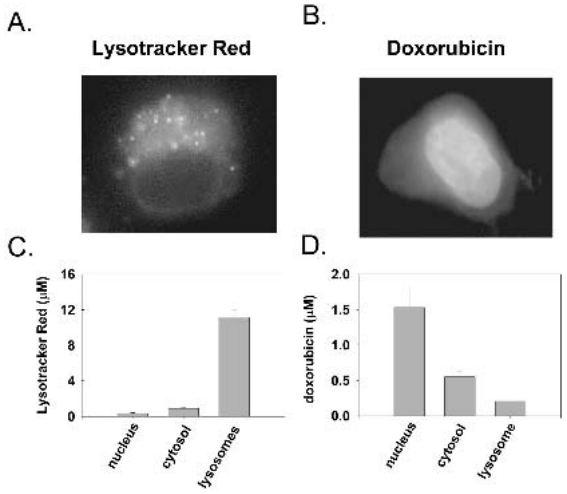

Fig. 4. Fluorescent micrograph of U-937 cells incubated with 5 μM LTR (A) or Dox (B) for 2 h Quantitation of LTR (C) or Dox (D) in isolated cellular fractions following the incubations described in A and B.

compartments. Estimates of total concentrations in the cellular compartments are represented graphically in Fig. 4C and D. Consistent with visual observations in Fig. 4A and B, Dox is predominantly associated with the nucleus and LTR is concentrated in the cytoplasmic organelles. In order to estimate a concentration of LTR in the cytoplasmic organelles we needed to confirm that LTR was predominantly associated with lysosomes and not with other cytoplasmic organelles. For this purpose we used an isopycnic separation of lysosomes in a self-forming gradient of Percoll. This method allowed for a broad separation of the organelle populations based upon their density. Using this approach we observed colocalization of LTR with the lysosomes. This was evaluated by comparing the location of Lamp-1 (by Western blot analysis) with LTR, which was analyzed by HPLC (see Fig. 5). Assuming that the lysosomes were the only organelle housing this compound, we estimated the lysosomal concentration to be approximately 11 μM.

Loss of Compounds from Isolated Cell Fractions

A significant limitation of this approach is the potential diffusion of drug from isolated organelles during the isolation procedure. We investigated this diffusion for times exceeding the time required for isolation and extraction of drug. In order to get accurate estimates for the first order rate of diffusion of drug from organelles, they must be suspended in medium not containing any drug and of sufficient volume to achieve sink conditions. This was experimentally achievable for nuclei but not for the cytoplasmic organelles. Nevertheless, these estimations provided good estimates of drug loss from the organelle during the experimental procedure. Surprisingly, we observed neither loss of LTR from isolated lysosomes nor any loss of Dox from isolated nuclei. In fact, we observed a slight increase in concentration as a function of time (see Fig. 6). We suspect that the extraction efficiency may have improved slightly as a function of time although this finding has not been experimentally confirmed.

A significant concern when performing these studies is that the majority of compound contained within a cell may be lost during cell homogenization and/or subsequent cellular isolation procedures. This being the case, it is possible that we could be measuring only a small fraction of drug that may be tightly bound to isolated cellular fractions and this drug would not be representative of the entire amount of drug contained within the intact cell. To evaluate this, we have separately measured the amount Dox and LTR contained within whole cell homogenates and compared this to the sum of drug contained within isolated cellular fractions. The amount of Dox and LTR per million cells contained within whole cell homogenates was found to be 390 ± 20 and 470 ± 35 pg respectively. The sum of the amounts of Dox and LTR isolated from nuclei, cytoplasmic organelles and cytoplasm were found to be 240 ± 30 and 250 ± 28 pg for Dox and LTR respectively (per 1 million cells). From these values the percentage compound recovered in isolated fractions relative to whole cell amounts was calculated. The recoveries were 61.5% and 53.2% for Dox and LTR, respectively. Although these recoveries are not quantitative they are reasonable considering the fact that organelle isolations involve multiple steps and transfers, which would be expected to result in significant drug loss. On the other hand, the acquisition of whole cell homogenates is a one step process. Taken together, these results are consistent with the assumption that the majority of compound contained within intact cells is indeed recovered in our isolated cellular fractions.

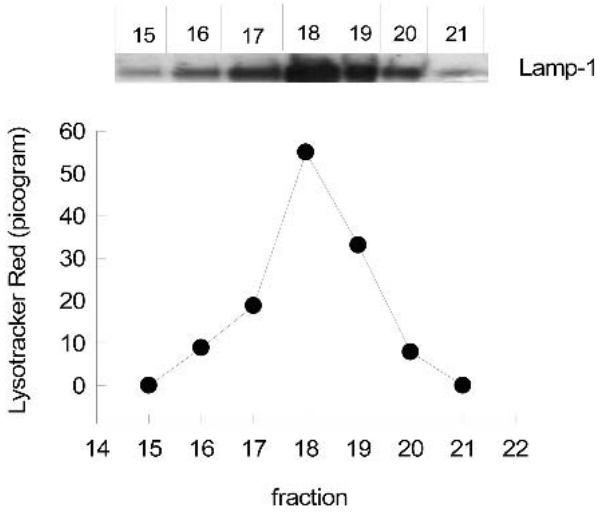

Fig. 5. Colocalization of Lamp-1 (Western blot analysis, upper panel) with LTR (analyzed by HPLC, lower graph) in fractions 15 through 21 from a linear gradient of Percoll.

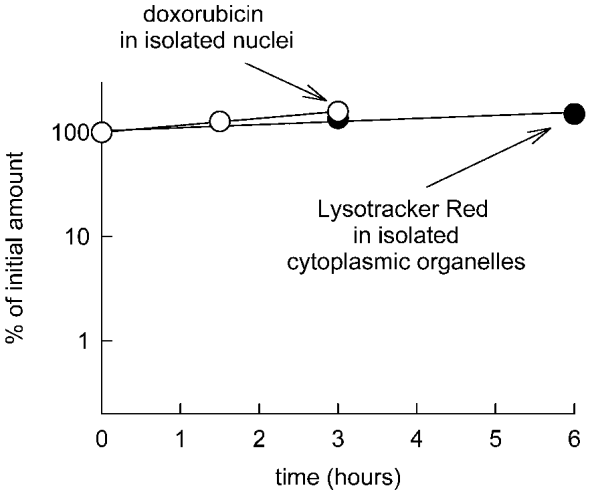

Fig. 6. Diffusion of Dox out of isolated Nuclei (○) and diffusion of LTR out of isolated cytoplasmic organelles (●) as a function of time.

DISCUSSION

Information regarding the subcellular distribution of drugs must be reliable and quantitative if it is to be used in the rational design or modification of drugs in order to achieve optimized intracellular delivery. Furthermore, the method should not be limited to only a small subset of pharmaceutically relevant structures.

This prompted us to investigate an approach involving subcellular fractionation and subsequent extraction and quantitation of drug contained within them. We used the human leukemic cell line U-937 as model cell line to evaluate the potential usefulness and limitations of this approach. Cells were separated into three fractions consisting of the nucleus, cell cytosol and cytoplasmic organelles using density gradient

centrifugation steps. As shown in Figs. 1 through 3, we were able to achieve these isolations with minimal contamination from separated fractions. We have investigated the distribution of two model compounds with known and different subcellular distribution. We chose compounds that were fluorescent and had a well-characterized distribution in cultured cells. Dox is an anticancer drug that localizes predominantly within the nucleus of the wild-type U-937 cell type. The selective accumulation of Dox in the nuclei of cells is driven by its high binding affinity with nuclear DNA. The subcellular distribution of Dox has been a topic of many publications that attempt to understand a unique drug resistance mechanism whereby Dox distribution shifts from a predominantly nuclear fluorescence in drug sensitive cells to a predominantly lysosomal accumulation in the corresponding drug resistant cell type. Our fluorescent microscopic images of cells incubated with Dox are consistent with other reports that demonstrated a preferential nuclear accumulation (11). Our estimation for the concentration within the nucleus was 1.5 μM after a 2-h incubation with 0.5 μM Dox. To our knowledge, this is the first report estimating the nuclear concentration of this drug in the U-937 cell line; however, Gigli and coworkers have estimated it in the human leukemic cell line K-562 using a microspectrofluorometric approach (19). They reported a nuclear concentration of approximately 65 μM following a 2-h incubation with 1 μM Dox. If the apparent higher nuclear concentrations in K-562 cells were indeed real then we would expect at least a 20-fold difference in IC50 values since Dox has its mechanism of action inside the nucleus. The IC_{50} values for Dox in U-937 and K-562 cells are 0.6 ± 0.07 μM (unpublished results) and 1.02 ± 0.71 μM (20), respectively. Considering the fact that these values are not dramatically different we believe that the discrepancy in nuclear accumulation data is most likely due to differences in the analytical methods used to determine nuclear concentrations as well as inaccurate estimates of nuclear volume.

LTR is a weakly basic commercially available fluorescent probe that selectively stains acidic organelles such as lysosomes (16). To confirm that this organelle was principally involved in the sequestration of LTR, we have demonstrated the colocalization of LTR and lysosomes in a continuous gradient of Percoll. The mechanism for the accumulation in lysosomes is thought to occur through a pH trapping mechanism. It is well known that many weak bases can readily diffuse across biological membranes in unionized form but have very limited diffusion in their ionized state. The lysosomal lumen has a relatively low pH (approximately pH 5) and is surrounded by the more neutral cell cytoplasm. Weak bases (unionized form) can penetrate into the organelle but have a reduced capacity to diffuse back out due to the greater extent of ionization. Theoretically, this can lead to significant accumulation within this organelle. Weak bases that demonstrate this behavior are referred to as lysosomotropic agents (7). To our knowledge no one has quantified the concentration of lysosomotropic compounds such as LTR in lysosomes; however, theoretical predictions suggest that the maximum concentration ratio achievable (at steady state) is equal to the ratio of hydrogen ion concentration in the lysosomes to that in the extracellular medium (approximately 250-fold assuming pH values of 5 and 7.4 for lysosomes and extracellular medium respectively). Our quantification of LTR in the lysosomes yielded over a 20-fold concentration in lysosomes relative to extracellular medium. It is likely that our short incubation times did not allow steady-state concentrations to be achieved and the concentration in lysosomes may have continued to increase if given additional incubation time.

Compounds were evaluated by HPLC with fluorescence detection. We are confident that this assay could be equally useful for non-fluorescent compounds using mass spectrometric detection. Mass spectrometry would also provide a better opportunity to detect metabolites and degradation products, which would provide valuable insight into identifying intracellular sites of drug metabolism.

For both compounds the cellular localization observed with the fluorescent microscope was confirmed with our fractionation approach. We feel that this correlation justifies the use of this assay to investigate the subcellular distribution of both fluorescent and non-fluorescent compounds that specifically concentrate into subcellular compartments. It is important to note that such an approach would most likely not be useful for those drugs that partition into cellular spaces and compartments solely through a passive diffusion type mechanism driven by concentration gradient alone. Diffusion of such drugs from isolated cellular compartments would be expected to be problematic. Despite this fact we think that this approach should be useful for a large percentage of drugs. Most drugs currently on the market can be classified as weak bases. As previously mentioned weakly basic drugs have the propensity to specifically accumulate into acidic organelles according to a pH partitioning type mechanism similar to LTR. Considering this it is expected that such quantitative assays should find great utility in further characterization of drug sequestration mechanisms.

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