Evaluating the Roles of Autophagy and Lysosomal Trafficking Defects in Intracellular Distribution-Based Drug–Drug Interactions Involving Lysosomes

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ABSTRACT: Many currently approved drugs possess weakly basic properties that make them substrates for extensive sequestration in acidic intracellular compartments such as lysosomes through an ion trapping-type mechanism. Lysosomotropic drugs often have unique pharmacokinetic properties that stem from the extensive entrapment in lysosomes, including an extremely large volume of distribution and a long half-life. Accordingly, pharmacokinetic drug–drug interactions can occur when one drug modifies lysosomal volume such that the degree of lysosomal sequestration of secondarily administered drugs is significantly altered. In this work, we have investigated potential mechanisms for drug-induced alterations in lysosomal volume that give rise to drug–drug interactions involving lysosomes. We show that eight hydrophobic amines, previously characterized as perpetrators in this type of drug–drug interaction, cause a significant expansion in lysosomal volume that was correlated with both the induction of autophagy and with decreases in the efficiency of lysosomal egress. We also show that well-known chemical inducers of autophagy caused an increase in apparent lysosomal volume and an increase in secondarily administered lysosomotropic drugs without negatively impacting vesicle-mediated lysosomal egress. These results could help rationalize how the induction of autophagy could cause variability in the pharmacokinetic properties of lysosomotropic drugs. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

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INTRODUCTION

A large number of drugs currently approved for use in humans possess weakly basic properties that make them lysosomotropic, meaning they are substrates for extensive ion trapping-based entrapment in acidic intracellular organelles such as lysosomes.[1,2] Several high-throughput assays have been recently developed aiding in the identification of lysosomotropic drugs.[3,4] Drug accumulation in lysosomes is driven by the large pH gradient that exists across the lumen of the organelle and the cell cytosol. Inhibitors of the lysosomal vacuolar proton ATPase, a protein complex that is responsible for acidification of lysosomes, have been shown to abrogate lysosomal trapping of drugs.[5] There are two drug-associated properties possessed by weak bases that can theoretically influence the extent of lysosomal trapping, which include the acid dissociation constant for the conjugate acid of the weak base (p*K*a) and the permeability properties of the drug in its ionized versus unionized forms (i.e., alpha).[1,6,7] For many lysosomotropic drugs, the majority of total cellular accumulation can be attributed to lysosomal ion trapping. We have experimentally shown that lysosomal concentrations of the lysosomotropic drug quinacrine are approximately 750 times greater than extracellular concentrations.[8]

The lysosomal sequestration of drugs is a very important clinical consideration, influencing activity, toxicity, and macroscopic pharmacokinetic distribution parameters. On a cellular level, changes in the extent of lysosomal sequestration can directly influence activity by altering the availability of the drug to bind with intended intracellular targets. Consistent with this notion, we have previously shown that relatively small changes in lysosomal sequestration can result in substantial changes in drug activity, both in cultured cells and *in vivo*.[9,10] In addition, some cancer cells have been shown to acquire an enhanced capacity to sequester anticancer drugs in their lysosomes, and this is considered to be a mechanism for multidrug resistance.[11–13] Drug accumulation in lysosomes also has potential toxicological ramifications. The extensive lysosomal accumulation of a number of cationic amphiphilic drugs has been associated with the development of drug-induced phospholipidosis; the long-term consequences of this, if any, are controversial and not completely understood.[14] Finally, lysosomotropic drugs typically have unique pharmacokinetic properties that result from their extensive accumulation in lysosomes. Specifically, substrates for lysosomal trapping typically have a very large volume of distribution and half-life.[2]

Inspired by the clinical ramifications of lysosomal trapping, we are interested in revealing drugs that can significantly alter the extent of drug accumulation within lysosomes at clinically relevant concentrations. We reasoned that such changes in lysosomal trapping could provide the basis for variability in drug pharmacokinetics and activity. We have recently demonstrated that a group of hydrophobic amines can dramatically increase the cellular accumulation of secondarily administered drugs and this provided the basis for intracellular distribution-based drug–drug interactions involving lysosomes.[15] In this work, we have demonstrated that the perpetrators of the interaction caused an expansion in aqueous volume of lysosomes without significantly altering the pH of the lysosomes.

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The goal of the work presented here is to further explore the mechanisms by which previously identified perpetrators of the drug–drug interaction pathway were able to expand the volume of lysosomes. We specifically examined the role of autophagy. This work demonstrated that previously identified hydrophobic amine-containing perpetrators modulated lysosomal volume, both through the induction of autophagy and through inhibition of lysosomal egress efficiency. We also show that well-known chemical inducers of autophagy can be classified as perpetrators of drug–drug interactions involving lysosomes. The therapeutic implications of these findings as they relate to variability in drug activity and pharmacokinetics are discussed.

METHODS

Cell Lines and Reagents

Wild-type (WT) human fibroblasts (catalogue #CRL-2076) were purchased from ATCC (Manassas, Virginia). All cells were cultured in glutamine-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM sodium pyruvate, and 2 mM Glutamax and maintained at 37°C and 5% CO2. Cells were routinely subcultured to maintain 50%–90% confluency. Experiments were carried out within 10 passages following removal from cryopreservation.

Dulbecco's phosphate-buffered saline (D-PBS), DMEM, HEPES, sodium pyruvate, glutamax, and LysoTracker Red (LTR) DND-99 were purchased from Invitrogen (Carlsbad, California). FBS was purchased from Atlanta Biologicals (Lawrenceville, Georgia). Carbamazepine, rapamycin, valproic acid, halofantrine, propranolol, U18666A, lidocaine, imipramine, haloperidol, chloroquine, bupivacaine, quinacrine, Laemmli buffer, and sodium deoxycholate were purchased from Sigma–Aldrich (St. Louis, Missouri). (S)-naproxen was purchased from Cayman Chemical Company (Ann Arbor, Michigan). Pierce BCA protein assay kit was ordered from ThermoScientific (Rockford, Illinois). Anti-LCB3I/II antibody (catalogue #ab51520) was purchased from AbCam (Cambridge, United Kingdom). Anti-beta-actin antibody (catalogue #8H10D10) was purchased from Cell Signaling Technology (Danvers, Massachusetts). 3[H]-dextran 70,000 MW, 3[H]-halofantrine, and 3[H]-propranolol were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, Missouri).

LTR Accumulation Assay

Wild-type human fibroblasts were grown in plastic 12-well culture plates (Corning Life Sciences, Tewksbury, MA) at a seeding density of approximately 75,000 cells per well. Following a 48-h pretreatment with various drugs (concentrations for each drug is stated in the figure legend), LTR was spiked into the growth media to a concentration of 200 nM and the cells were incubated for 1 h. Cells were then rapidly washed twice with 4°C D-PBS. Cells were lysed with lysis buffer (50 mM tris base, 150 mM NaCl, 1% NP40, pH 7.4). The quantity of LTR was determined by fluorescent signal in relative fluorescence units (RFU) using a Bio-Tek (Winooski, VT) FL600 microplate fluorescence reader. Protein abundance was measured for each sample using the BCA method. Measured LTR signal (RFU) was then normalized to protein. These normalized values were then compared with the control condition (vehicle treated) and depicted as a percentage of the control.

Drug Accumulation Assays

Following a 48-h pretreatment with rapamycin (200 nM) or vehicle alone, cells were exposed to propranolol or halofantrine for 1 h. Naproxen accumulation (5 μM) was conducted concurrently with the pretreatment of rapamycin (200 nM) or vehicle alone for 48 h. The concentrations chosen for these compounds (200 nM propranolol, 200 nM halofantrine, and 5 μM naproxen) were determined as the necessary concentrations to yield adequate signal while also being incapable of inducing a lysosomal volume expansion. Cells were then washed twice with 4°C D-PBS rapidly to prevent diffusion of cell-associated drug. The cells were then lysed using lysis buffer (50 mM tris base, 150 mM NaCl, 1% NP40, pH 7.4). The quantity of tritium-labeled drug (propranolol and halofantrine) was measured in disintegrations per minute (DPM) using a Beckman LS 60001C liquid scintillation counter, and naproxen was measured by liquid chromatography–mass spectrometry (LC–MS/MS) using the method stated in *LC–MS/MS Quantification of Naproxen*. Background signal contributed from nonspecific binding to the plate surface was subtracted from each measurement. Protein abundance was measured for each sample using the BCA method. All measured quantities of drug accumulation were then normalized to protein. These normalized values were then compared with the corresponding control condition (vehicle treated) and depicted as a percentage of the control.

LC-MS/MS Quantification of Naproxen

To measure naproxen cellular accumulation, a drug extraction was performed to recover naproxen from the cell lysate using the following technique. Methyl *tert*-butyl ether was combined with the cell lysates in a 5:1 ratio (v/v). The samples were vortexed for 10 min and then centrifuged at 15,000*g* for 10 min. The top 95% of the supernatant was removed and the process was repeated. The supernatant was evaporated, and the remaining solid particulates were then reconstituted in mobile phase prior to injection. An integrated Agilent 1100 series liquid chromatography system with an API 2000 (Applied Biosciences, Framingham, MA) triple quadrupole mass spectrometer was employed to quantitate drug extracted from cell lysates. Liquid chromatography was conducted on an Agilent Zorbax Eclipse XDB-C18 column (2.1 × 150 mm^2 3.5 μm particle size) (Santa Clara, California). All samples were analyzed with an 8-μL sample injection. Mobile phase A consisted of 10 mM ammonium acetate at pH 8.0. Mobile phase B consisted of acetonitrile. Using 150 μL/min flow rate, the first minute of elution was an isocratic mixture of 82% A followed by a linear gradient to 5% A for 3 min. A linear gradient was then executed to 82% A over 0.5 min and maintained at 82% A for an additional 3.5 min. The first 1.8 min of eluted sample was diverted to waste in order to avoid nonvolatile material from entering the mass spectrometer.

The mass spectrometer was equipped with an atmospheric ionization source and operated in the negative mode. Detection was by multiple reaction monitoring. The specific parameters for the mass spectrometer can be found in Supplemental Data S1.