Amine-Containing Molecules and the Induction of an Expanded Lysosomal Volume Phenotype: A Structure–Activity Relationship Study

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Received 30 January 2013; revised 26 February 2014; accepted 28 February 2014

Published online 19 March 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.23949

ABSTRACT: Many weakly basic amine-containing compounds have a strong propensity to become highly concentrated in lysosomes by virtue of an ion-trapping-type mechanism; the substrates for this are referred to as lysosomotropic. We have previously shown that many lysosomotropic drugs can produce a significant expansion in the apparent volume of lysosomes, which can ultimately result in an intracellular distribution-based drug–drug interaction. In this study, we have systematically evaluated the physicochemical and structural features of weakly basic molecules that correlate with their ability to induce an expanded lysosomal volume phenotype (ELVP) in cultured human fibroblasts. By quantitatively evaluating the cellular accumulation of Lysotracker Red, a fluorescent lysosomotropic probe, the volume of the lysosomal compartment was determined. We specifically explored the influence that lysosomotropism, molecular size, and amphiphilicity had on a molecule's ability to induce an ELVP. The capacity of these molecules to intercalation into biological membranes was also evaluated using a red blood cell hemolysis assay. The present results suggest that a molecule's potency in eliciting an ELVP is influenced by lysosomotropism, amphiphilicity, and its ability to intercalate into biological membranes. Despite being highly lysosomotropic, low-molecular-weight, nonaromatic amines failed to cause an ELVP at all concentrations evaluated. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:1572–1580, 2014

Keywords: cell biology; drug interactions; distribution; structure–activity relationship (SAR); physicochemical properties; cell culture; log *P*; membrane transport; pharmacokinetics; partition coefficient

INTRODUCTION

Drugs and compounds that possess a weakly basic amine are often considered to be lysosomotropic, meaning they are substrates for ion-trapping-based accumulation within the acidic lysosomes of cells.[1] Simply stated, weakly basic substrates for ion trapping are preferentially protonated and ionized within the lumen of acidic lysosomes, which limits their passive permeability back out of the membrane-enclosed compartment. Through this process, weakly basic drugs can become highly concentrated within lysosomes. In experiments with cells exposed to lysosomotropic drugs in culture, we and others have shown that drug concentrations inside lysosomes can reach levels 1000 times greater than the concentration of drug in the cell culture medium.[2,3]

Ion-trapping-based accumulation of weakly basic drugs can theoretically occur in any organelle that has a lower luminal pH than the extracellular pH.[1] For example, the Golgi apparatus, early endosomes, and late endosomes are relatively acidic (~pH 6.4, 6.2, 5.5, respectively)[4–6] and would theoretically accumulate weakly basic compounds. In relation to these organelles however, the lysosomes would be subjected to the greatest ion-trapping-based accumulation of weakly basic compounds by virtue of their vastly more acidic luminal pH (4.0–4.5).[7,8] This hyperaccumulation of drugs within lysosomes can make their resident proteins, enzymes, and lipid bilayers particularly susceptible to drug-induced alterations. Studies examining the effects of lysosomotropic drugs on lysosome structure and function have mainly been conducted using cells grown in culture. The concentrations of drugs employed and the length of incubation can determine the type of effect as well as the potential clinical relevance of the observation.

For several decades, it has been known that lysosomotropic compounds can cause a transient increase in the luminal pH of lysosomes,[3] which could conceivably decrease the activity of lysosomal enzymes that have optimal activity at low pH. Typically, this has been shown with cells grown in culture that are exposed to supratherapeutic concentrations (i.e., low millimolar) for a short period of time (i.e., minutes). It has been proposed that the pH increase is caused by leakage of the protonated base out of lysosomes, thereby short circuiting the lysosomal vacuolar proton ATPase that is responsible for maintaining the acidity of this compartment.[9] In addition, it has been shown that the osmotic influx of water resulting from the high concentration of lysosomotropic agents can cause lysosomes to take on a vacuolar appearance that is often visible with light microscopy.[10–12] However, like drug-induced pH alterations, the therapeutic relevance of such observations is questionable because of the high concentrations of base required to produce these effects. Several hydrophobic amine-containing drugs have been shown to have generalized effects on lipid metabolism and/or transport associated with lysosomes that can potentially occur at therapeutic concentrations (i.e., high nanomolar to low micromolar). Specifically, the term "drug-induced phospholipidosis" has been attributed to the lysosomal accumulation of weakly basic drugs that cause the hyperaccumulation of phospholipids and cholesterol in lysosomes.[13] There exists some controversy regarding the mechanism for

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Journal of Pharmaceutical Sciences, Vol. 103, 1572–1580 (2014)

drug-induced phospholipidosis. It has been postulated that the amines can directly interfere with lysosomal enzyme function,[14] whereas other reports suggest that lysosomal enzymes can become destabilized in the presence of amines, and this can lead to an enhancement of their proteolytic degradation within the lysosomes.[15,16]

Our laboratory studies the influence of lysosomotropic drugs on lysosomal trafficking and subsequent volume regulation. We have previously shown that weakly basic amines can increase the apparent steady-state volume of lysosomes in a time- and concentration-dependent manner.[17] We have also shown that the apparent lysosomal volume is affected by drugs and/or disease states that influence the efficiencies of vesicle-mediated trafficking to and away from lysosomes.[18] Throughout many of our studies, we have extensively used Lysotracker Red (LTR), a fluorescent lysosomotropic probe, as a surrogate marker for lysosomal volume.[19,20] The rationale for measuring LTR cellular accumulation as a surrogate measurement for lysosomal volume is based on the notion that the net cellular accumulation of LTR is dictated by the luminal pH and the volume of the lysosomes, and we have shown that these drug-induced lysosomal volume increases occur at drug concentrations well below those required to cause perturbations in lysosomal pH. Accordingly, alterations in LTR cellular accumulation can, most appropriately, be reflective of alterations in lysosomal volume.

The purpose of the current manuscript is to probe the structure–activity relationship of small molecules and their ability to cause perturbations in lysosomal volume. By measuring the cellular accumulation of LTR, we systematically evaluated how various structural and physicochemical properties influenced a molecule's ability to cause an expansion in lysosomal volume. We have limited our analysis to concentrations of compounds that would be considered to be nontoxic and achievable under normal therapeutic dosing. As will be discussed, our finding shed new light on the potential mechanism for drug-induced perturbations of lysosomes and how this knowledge can ultimately be used by medicinal chemists to rationally design drugs with a reduced potential to cause lysosomal perturbation.

METHODS

Cell Lines

Wild-type (WT) human fibroblasts (catalog #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% CO_2. Cells were routinely subcultured to maintain 50%–90% confluency. Experiments were carried out within 10 passages following removal from cryopreservation.

Reagents

Dulbecco's phosphate-buffered saline (D-PBS), DMEM, HEPES, sodium pyruvate, Glutamax, and LTR DND-99 were purchased from Invitrogen (Carlsbad, California). FBS was purchased from Atlanta Biologicals (Lawrenceville, Georgia). DL-Propranolol hydrochloride, lidocaine monohydrate, tertiarybutylamine, tri-isopropylamine, benzylamine, 2-phenylethanamine, 3-phenyl-1-propylamine, 4-phenylbutylamine, 1-naphthylmethylamine, 3,3-diphenylpropylamine, 9-(methylaminomethyl)anthracene, heprin sodium salt, and methylamine were purchased from Sigma–Aldrich (St. Louis, Missouri). Chlorpromazine hydrochloride and imipramine hydrochloride were purchased from MP Biomedicals Inc. (Solon, Ohio). Pierce bicinchoninic acid assay (BCA) protein assay kit was ordered from ThermoScientific (Rockford, Illinois).

LTR Cellular Accumulation

The cellular accumulation of LTR was measured as previously described.[19] Briefly stated, WT human fibroblasts were grown in plastic 12-well culture plates at a seeding density of 80,000 cells per well. The cells were exposed to vehicle alone or test condition for 48 h. At the end of the 48-h pretreatment period, LTR was spiked into the existing media to a concentration of 200 nM, and the cells were incubated at 37°C 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 titrated to 7.4 with HCl) for 30 min at 37°C. The lysate was recovered from each well by aspiration using a standard pipette. The abundance of LTR was determined by measuring fluorescent signal in relative fluorescence units (RFU) using a Bio-Tek FL600 microplate fluorescence reader. Background signal contributed from nonspecific binding of LTR to the plate surface was subtracted from each measurement. 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.

We have chosen this quantitative plate-based assay because of its advantages over traditional fluorescence imaging. This quantitative plate-based assay has the advantage of unbiasedly capturing the average apparent lysosomal volume of many thousands of cells compared with imaging approaches that are typically restricted to a much smaller number of cells. For this reason, we consider the values obtained from the plate-based assay to be a more accurate method for approximating the influence that test compounds have on lysosomal volume.

Red Blood Cell Hemolysis Assay

Red blood cells (RBCs) were isolated from whole blood that was obtained from a male subject using standard venipuncture technique. Heparin was spiked directly into the whole blood (20 U/mL) immediately after venipuncture was completed. The whole blood was then centrifuged at 1500*g* for 30 min at 4°C. The blood plasma and white blood cells were removed. The remaining RBCs were washed twice by resuspending the RBCs in 4°C D-PBS to 5% hematocrit followed by centrifuging at 1500*g* for 30 min at 4°C and then removing the D-PBS supernatant. RBCs were resuspended to 5% hematocrit in 4°C D-PBS and aliquoted into 2 mL microcentrifuge tubes. After warming the RBC aliquots to 37°C in a water bath for 5 min, the RBCs were exposed to various drugs at multiple concentrations by spiking drug directly into the RBC/D-PBS aliquots. Following 2 h of incubation in a 37°C water bath, the samples were analyzed for RBC hemolysis. Each sample was centrifuged at 1500*g* for 10 min at 4°C. The supernatant was removed and the optical density was measured at 540 nm using a MultiSkan MCC microplate photometer (ThermoScientific). The O.D. representing