A robust method to identify compounds that undergo intracellular lysosomal sequestration

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INTRODUCTION

Lysosomes are acidic organelles (pH 4-5) that play a key role in various metabolic processes such as the turnover of phospholipids, the breakdown of waste products (including bacteria and viruses) and apoptosis. Lipophilic and amphiphilic drugs (a.k.a. cationic amphiphilic amines or CADs) with ionizable amines (pKa >6) can accumulate in lysosomes (a process known as lysosomal trapping), which contributes to presystemic clearance in lysosome-rich organs (such as liver and lung) and, together with the binding of lipophilic amines to phospholipids, is associated with a large volume of distribution of numerous cardiovascular and CNS drugs (MacIntyre and Cutler, 1988; Daniel and Wojcikowski, 1999; Houston and Hallifax, 2007). The prolonged accumulation of lipophilic amines in lysosomes and their binding to phospholipids, both of which disrupt lysosomal function, have been implicated as the major cause of phospholipidosis, where an excessive accumulation of phospholipids occurs in various tissues (Hanumegowda *et al.*, 2010). Furthermore, elevated levels of CADs in lysosomes can lead to high organ to blood ratios of drugs that can be mistaken for active drug transport.

In the present study, we describe an in vitro fluorescence-based method to identify lysosomotropic agents. A diverse set of compounds with various physicochemical properties were tested, such as acids, lipophilic amines and zwitterions. In brief, the method involves dosing immortalized human hepatocytes (Fa2N-4 cells) with the test drug and LysoTracker Red®, a lysosome-specific fluorescent probe, and determining the inhibition of LysoTracker Red® uptake. The Fa2N-4 cells are SV40 virus large T antigen transformed human hepatocytes that do not express constitutive androstane receptor (CAR) and do not retain significant transporter activity (Hariparsad *et al.*, 2008). While fluorescence-based methods to evaluate lysosomotropism have been used in other cell lines (Nadanaciva *et al.*, 2011; Duvvuri *et al.*, 2005, Lemieux *et al.*, 2004), this study is the first evaluation of immortalized hepatocytes as a possible test system to evaluate lysosomal trapping.

METHODS

Chemicals

Acetaminophen, ammonium chloride, astemizole, chloroquine, dextromethorphan, diclofenac, fluoxetine, imipramine, ketoprofen, labetalol, monensin, nifedipine, nigericin, paroxetine, pravastatin, propranolol, and raclopride were purchased from Sigma-Aldrich (St. Louis, MO); atorvastatin, cetirizine, desipramine, erlotinib, fluconazole, fluvastatin, gefitinib, ketorolac, lapatinib, rosuvastatin, and tenoxicam were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada); amodiaquine was purchased from US Pharmacopeia (Rockville, MD); LysoTracker Red® was purchased from Invitrogen (Eugene, OR). The sources of the other reagents used in this study have been described elsewhere (Paris *et al.*, 2009).

Test system

Fa2N-4 cells, MFE plating media, MFE support media were prepared; and human hepatocytes from non-transplantable livers were isolated and cryopreserved at XenoTech, LLC (Lenexa, KS).

Epifluorescence microscopy

Epifluorescence microscopy was conducted at the University of Kansas, Department of Pharmaceutical Chemistry (Lawrence, KS) as described previously (Lemieux *et al.*, 2004; Duvvuri *et al.*, 2004). In brief, cryopreserved human hepatocytes or Fa2N-4 cells were thawed and diluted to 1 x 10^6 cells and incubated with 200 nM of LysoTracker Red® for two hours. Controls included incubations with nigercin and monensin, which are ionophores that uncouple the proton gradient present in lysosomes. Ammonium chloride (at 10 mM), which raises the pH of lysosomal compartment, was also used as a control. The cells were subsequently washed and then visualized with a fluorescence microscope at the excitation/emission wavelengths for LysoTracker Red® assessment (530/590 nm).

Assessment of lysosomal trapping in Fa2N-4 cells using LysoTracker Red®

Fa2N-4 cells (50,000 cells/well) were plated in collagen-coated black-clear bottom 96-well microtiter plates (Corning Incorporated, Corning, NY). Cells were maintained at 37°C with 95% humidity and 5% CO_2. After cell attachment (approximately three hours), the MFE plating media was removed and the wells were rinsed twice with 1xPBS. Stock solutions of each test drug were initially prepared in methanol and diluted with MCM+ such that the final incubation concentrations of each drug were 1, 5, 10, 50, 100 and 500 µM. LysoTracker Red® was added to each test drug sub-stock solution such that the final incubation concentration of LysoTracker Red® was 50 nM. Incubations with the cells were performed in triplicate for 30 min at 37°C with 95% humidity and 5% CO_2. The incubation matrix was then aspirated (and kept for LDH release analysis) and the plate was rinsed twice with 1xPBS prior to solubilizing the cells with 100 µL of acetonitrile. Samples were then analyzed for residual fluorescence with a BioTek Synergy plate reader (BioTek, Winooski, VT) at the excitation/emission wavelengths for LysoTracker Red® assessment (530/590 nm).

Cytotoxicity assessment

The incubation matrices from the aforementioned Fa2N-4 lysosomal trapping assessment were analyzed for lactate dehydrogenase (LDH) release, a marker of drug induced cytotoxicity, using the cytotoxicity detection kit (LDH) purchased from Roche Diagnostics (Indianapolis, IN).

Data analysis

Physicochemical information (see **Table 1**) for each compound was predicted using the ChEMBL database (https://www.ebi.ac.uk/chembldb/) or chemicalize.org (http://www.chemicalize.org). LysoTracker Red® IC_{50} values were calculated using GraFit 4.0.21 (Erithacus Software Ltd., Horley, Surrey, UK).

RESULTS

Figure 1 depicts the mechanism by which cationic amphiphilic amines (CADs) become sequestered in lysosomes. Drugs with an ionizable amine groups with pKa values near plasma and cytosolic pH will largely exist as neutral species (R-NH_2). In this form, drugs can undergo rapid passive diffusion across the plasma membrane as well as the lysosomal membrane. Once neutral drugs enter the acid environment of the lysosome, they become ionized and exist predominantly in their protonated state (R-NH_3^+). The charged species are not readily membrane permeable and are unable to cross back across the lysosomal membrane into the intracellular space, thus becoming sequestered in the lysosomal compartment (Kaufmann and Krise, 2006).

As shown in **Figure 2**, epifluorescence experiments showed that human hepatocytes maintain functional lysosomes by virtue of LysoTracker Red® accumulation in lysosomal compartments (panel A) that could be disrupted by the ionophores nigericin and monensin (panel B). Immortalized hepatocytes (Fa2N-4 cells) were also shown to maintain functional lysosomes (panel C), as LysoTracker Red® was sequestered in the lysosomes of these cells and was disrupted upon treatment with ammonium chloride (panel D), which raises lysosomal pH.

The ability of various compounds to inhibit LysoTracker Red® accumulation into lysosomes of Fa2N-4 cells was tested (as summarized in **Table 1**). These compounds spanned a diverse set of therapeutic indications as well as physicochemical properties. Of the compounds tested, those that were acids (diclofenac, ibuprofen, ketoprofen, ketorolac, atorvastatin, fluvastatin, pravastatin, and rosuvastatin) showed no evidence for lysosomal trapping.

Most drugs that were bases and known lysosomotropics (fluoxetine, paroxetine, desipramine, imipramine, chloroquine, and propranolol) showed inhibition of LysoTracker Red® uptake by giving measurable LysoTracker Red® IC_{50} values (as depicted in **Figure 3**). The basic drugs amodiaquine, dextromethorphan and labetalol did not give a measurable LysoTracker Red® IC_{50} value within the concentration range tested, however began to exhibit a trend of inhibition at the highest concentration tested, indicating that these compounds may be trapped in lysosomes at higher concentrations.

Of the drugs that are neutral at plasma pH, gefitinib inhibited LysoTracker Red® uptake and gave measurable IC_{50} values. None of the zwitterions tested inhibited LysoTracker Red® uptake. Both astemizole (base) and lapatinib (neutral) also inhibited LysoTracker Red® uptake, however these compounds also exhibited cytotoxicity at the same concentrations, thus their propensity to be trapped in lysosomes, while possible, could not be determined.

Figure 1

Lysosomal trapping of lipophilic amines

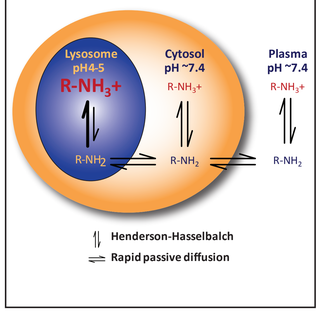

Figure 2

Lysosomal trapping of LysoTracker Red® in human hepatocytes and Fa2N-4 cells as determined by epifluorescence microscopy

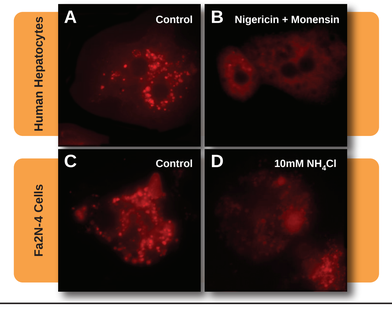

Figure 3

The effect of known lysosomotropics towards LysoTracker Red® inhibition in Fa2N-4 cells

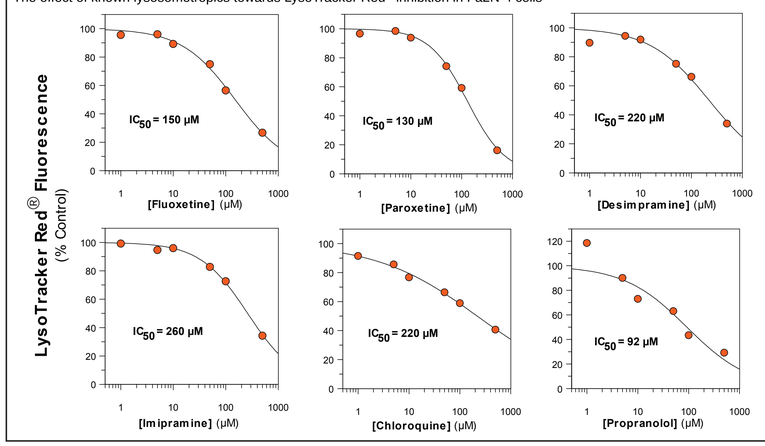

Table 1

Predicted physicochemical properties and experimentally-determined LysoTracker Red® IC_{50} values for 27 compounds

Drug	Drug Class	Species	LogP	$LogD_{pH\ 7.4}$	Polar Surface Area (Å^2)	Acidic pKa	Basic pKa	LysoTracker Red® IC_{50} (µM)	Cytotoxicity (LDH release)	Evidence for Lysosomal trapping
Diclofenac	NSAID	Acid	4.55	1.44	49.33	4.20	-	>500	Little or none	None
Ibuprofen	NSAID	Acid	3.50	0.58	37.30	4.41	-	>500	Little or none	None
Ketoprofen	NSAID	Acid	2.91	-0.16	54.37	4.23	-	>500	Little or none	None
Ketorolac	NSAID	Acid	2.68	-0.34	59.30	4.29	-	>500	Little or none	None
Atorvastatin	Statin	Acid	3.85	0.74	111.79	4.29	0.39	>500	Little or none	None
Fluvastatin	Statin	Acid	4.57	1.46	82.69	4.27	-	>500	Little or none	None
Pravastatin	Statin	Acid	2.21	-0.88	124.29	4.31	-	>500	Little or none	None
Rosuvastatin	Statin	Acid	0.89	-2.24	140.92	4.25	-	>500	Little or none	None
Fluoxetine	Antidepressant (SSRI)	Base	3.93	1.41	21.26	-	10.06	150	Little or none	Yes
Paroxetine	Antidepressant (SSRI)	Base	3.70	1.48	39.72	-	9.68	130	Little or none	Yes
Desipramine	Antidepressant (Tricyclic)	Base	3.97	1.27	15.27	-	10.40	220	Little or none	Yes
Imipramine	Antidepressant (Tricyclic)	Base	4.36	2.35	6.48	-	9.49	260	Little or none	Yes
Astemizole	Antihistamine	Base	5.52	4.13	42.32	-	8.68	110	Yes	Possible
Amodiaquine	Antimalarial	Base	3.13	0.95	48.39	9.43	5.62	>500	Little or none	Possible
Chloroquine	Antimalarial	Base	4.41	1.59	28.16	-	10.47	220	Little or none	Yes
Dextromethorphan	Antitussive	Base	3.89	2.17	12.47	-	9.13	>500	Little or none	Possible
Labetalol	Beta blocker	Base	2.72	0.83	95.58	8.21	9.30	>500	Little or none	Possible
Propranolol	Beta blocker	Base	2.90	0.79	41.49	13.84	9.50	92	Little or none	Yes
Acetaminophen	Analgesic	Neutral	0.48	0.47	49.33	9.86	1.72	>500	Little or none	None
Erlotinib	Anticancer	Neutral	3.03	3.03	74.73	-	5.32	>500	Little or none	None
Gefitinib	Anticancer	Neutral	2.70	2.56	68.74	-	7.00	77	Little or none	Yes
Lapatinib	Anticancer	Neutral	6.30	6.26	106.35	-	6.34	380	Yes	Possible
Fluconazole	Antifungal antibiotic	Neutral	0.45	0.45	81.65	11.01	2.94	>500	Little or none	None
Nifedipine	Calcium channel blocker	Neutral	3.58	3.58	110.45	-	2.69	>500	Little or none	None
Cetirizine	Antihistamine	Zwitterion	1.62	-1.45	53.01	3.46	6.71	>500	Little or none	None
Raclopride	Antipsychotic	Zwitterion	3.58	1.12	61.80	5.93	8.97	>500	Little or none	None
Tenoxicam	NSAID	Zwitterion	0.34	-0.71	99.60	1.07	5.34	>500	Little or none	None

LDH: lactate dehydrogenase
SSRI: selective serotonin reuptake inhibitor
NSAID: non-steroidal anti-inflammatory drug

CONCLUSIONS

- The Fa2N-4 cell line (immortalized hepatocytes) were shown to have functional lysosomes, similar to that of native human hepatocytes.
- LysoTracker Red® uptake inhibition in Fa2N-4 cells correctly identified known lysosomotropics (compounds that are lipophilic amines with logP >1 and basic pKa values > 6).
- The Fa2N-4 cell line, in conjunction with LysoTracker Red® inhibition, can be used as a robust test system to determine the potential for drugs to be trapped in lysosomes.

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