

Sub-cellular sequestration of alkaline drugs in lysosomes: new insights for pharmaceutical development of lysosomal fluid

Malaz Yousef^{1,2}, Tyson S. Le¹, Jieyu Zuo¹, Chulhun Park³, Nadia Bou Chacra⁴,
Neal M. Davies^{1,*}, and Raimar Löbenberg^{1,*}

¹Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada.

²Faculty of Pharmacy, University of Khartoum, Khartoum, Sudan.

³College of Pharmacy, Jeju National University, Jeju 63243, South Korea.

⁴Faculty of Pharmaceutical Sciences, University of Sao Paulo, Sao Paulo, Brazil.

Abstract

Background and purpose: Lysosomal-targeted drug delivery can open a new strategy for drug therapy. However, there is currently no universally accepted simulated or artificial lysosomal fluid utilized in the pharmaceutical industry or recognized by the United States Pharmacopeia (USP).

Experimental approach: We prepared a simulated lysosomal fluid (SLYF) and compared its composition to a commercial artificial counterpart. The developed fluid was used to test the dissolution of a commercial product (Robitussin[®]) of a lysosomotropic drug (dextromethorphan) and to investigate *in-vitro* lysosomal trapping of two model drugs (dextromethorphan and (+/-) chloroquine).

Findings/Results: The laboratory-prepared fluid or SLYF contained the essential components for the lysosomal function in concentrations reflective of the physiological values, unlike the commercial product. Robitussin[®] passed the acceptance criteria for the dissolution of dextromethorphan in 0.1 N HCl medium (97.7% in less than 45 min) but not in the SLYF or the phosphate buffer media (72.6% and 32.2% within 45 min, respectively). Racemic chloroquine showed higher lysosomal trapping (51.9%) in the *in-vitro* model than dextromethorphan (28.3%) in a behavior supporting *in-vivo* findings and based on the molecular descriptors and the lysosomal sequestration potential of both.

Conclusion and implication: A standardized lysosomal fluid was reported and developed for *in-vitro* investigations of lysosomotropic drugs and formulations.

Keywords: Biological fluids; CADs; Lysosomal trapping; Simulated lysosomal fluid.

INTRODUCTION

Intracellular sequestration of drug molecules by various organelles has differential effects on the disposition of xenobiotics which can impart significant alterations to their distribution, efficacy, and safety (1,2). Moreover, this phenomenon can also yield unintentional targets for molecules which may help in repurposing them for indications that involve initially an off-target site of action (3-5). Previous studies listed lysosomes, mitochondria, and Golgi apparatus as intracellular sites for various drug entities (1,6,7). In these investigations we highlight lysosomal trapping, focusing on the structure and function of the lysosomes and the

mechanism of “trapping” potential drugs in addition to the therapeutic implications of this phenomenon. We will also detail the various physicochemical and structural properties that candidate molecules possess to be subjected to sub-cellular sequestration in lysosomes and the models used to predict this occurrence.

Lysosomes structure and function

Lysosomes are acidic membrane-enclosed organelles (pH 4 - 5.5) (8,9). They are ubiquitous in cells, yet predominant in the liver, kidney, spleen, and lung (10). Their size can differ in the various tissues, ranging from 0.1 to 1.2 μm (11).

*Corresponding authors:

N.M. Davies, Tel: +1-7802210828, Fax: +1-7804921217

Email: ndavies@ualberta.ca

R. Löbenberg, Tel: +1-7804921255, Fax: +1-7804921217

Email: raimar@ualberta.ca

Access this article online



Website: <http://rps.mui.ac.ir>

DOI: 10.4103/1735-5362.363591

Lysosomes are essential in certain metabolic processes including the digestion of macromolecules and cell fragments, phospholipid turnover, and destroying invading bacteria and viruses (1,12,13). Lysosomes play this key role through their array of hydrolytic enzymes (> 60) that function within the acidic environment of the lysosomes, which protects other cell molecules and organelles from being destroyed if these enzymes were able to leak into the cytosol (10,14).

To maintain their acidic pH, lysosomes depend on proton-pumping V-type ATPase complexes that utilize the energy of ATP hydrolysis in transporting the protons from cytosol into lysosomes (15). As the parallel influx of anions is essential for electro-neutrality, chloride proton antiporter, known as chloride channel-7 or ClC-7, moves chloride ions into lysosomes to dissipate the transmembrane voltage (15,16). Cation efflux into the cytosol has also been reported to play a role in preserving a steady-state pH inside the lysosomes (16).

Mechanism of lysosomal trapping

In 1974 Duve *et al.* first described drug sequestration in the acidic lysosomal vicinity *via* a process known as lysosomal trapping, lysosomal sequestration, lysosomotropism, acid trapping, or proton pump effect (13,17,18).

Lipophilic basic drugs were suggested to be the targets for this process (18,19). However, not all basic drugs have the requisite characteristics to be possible candidates for lysosomal trapping. It is the physicochemical properties of the drugs that determine the accumulation propensity in the lysosomal organelles. Lysosomotropic drugs are termed

cationic amphiphilic drugs, referring to their nature being relatively lipophilic ($\log P \sim 2 - 6$) and can bear a positive charge ($pK_a > 6$) (13).

The pH gradient between cytosol (pH 7 - 7.2) and lysosomes represents the driving force for the candidate drugs' deposition into lysosomes (1). Uncharged basic molecules can passively diffuse into lysosomes and their basic group(s) are protonated in the acidic lysosomal vicinity. Ionized charged molecules cannot easily permeate biological membranes, thus the acquired charges reduce the permeability of the molecules across the lipid bilayer lysosomal membrane back to the cytosol. The equilibrium between the protonated and the neutral forms favors the former and consequently leads to a high fraction of the drug being "trapped" as depicted in Fig. 1 (1,2,10). The percentage of drugs in the lysosomes can reach up to 70% of the total amount of the drug in the intracellular vicinity (3).

Effects of lysosomal trapping

Drug action and disposition implications of lysosomal trapping mark both the pharmacodynamics and pharmacokinetic properties of drugs (2,20). For certain molecules, it can be beneficial if the drug target is sequestered inside the lysosomes. Trapping of the antimalarial drug, chloroquine, inside the parasites' lysosome (food vacuole) is fortuitous as this drug binds the heme part of the hemoglobin which is usually metabolized by the parasite to the non-toxic form, hemozoin. Prevention of this process by chloroquine would lead to the death of the parasite in its own waste from the hemoglobin metabolism (21-23).

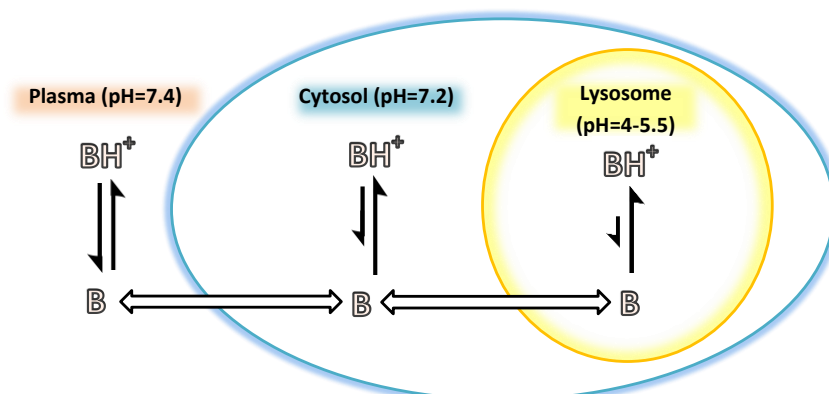


Fig. 1. Illustration showing the lysosomal trapping of basic drug molecules.

Additionally, inhibition of the intralysosomal enzyme acid sphingomyelinase is one way through which certain antidepressants elicit their pharmacological action. Thus, lysosomal trapping of fluoxetine and amitriptyline which happens because of their physicochemical properties aids in their pharmacological action as well (24,25). Lysosomal trapping can also prolong the availability of the drug and in doing so potentiate its activity as seen with the anticancer drug palbociclib and others (5,26). Palbociclib is an anticancer drug with a mechanism of action that relies primarily on inducing cell-cycle arrest and senescence (27). The process of lysosomal sequestration and release of palbociclib facilitates an increase in the exposure time of the drug and extends the duration of the effect. In addition, the lysosomal trapping of palbociclib can occur in both responsive and resistant cancer cells, the latter effect, however, was found to accumulate and release the drug inducing its paracrine senescence effect (a stable arrest mediated by secreted factors). Moreover, the displacement of palbociclib by other lysosomotropic drugs into the cytosol potentiates its action on cytosolic and nuclear targets (26).

Together with the endosomes (membrane-formed-vacuoles), lysosomes play a major role in viral replication (3). Therefore, lysosomotropic drugs have also been investigated to be potentially repurposed and therapeutically utilized for their antiviral properties (28). Several research investigations support the use of lysosomotropic drugs as antiviral agents as demonstrated through

in-vitro, *in-vivo*, and clinical data. Ebola, Zika, influenza, and even coronavirus are examples of the targets for which lysosomotropic drugs are being investigated (3,4,28-30). Table 1 lists examples of different lysosomotropic drugs which have been reported to have antiviral activity.

Nevertheless, lysosomal trapping can also have negative effects on the efficacy of drugs by impeding and decreasing their therapeutic concentration in their intended sites of action. Such effects have been linked to cross-resistance of lysosomotropic drugs and might even result in drug resistance as in the case of some anticancer drugs (41-43). An example of lysosomotropic drug-induced resistance has been reported with sunitinib (13). This cytotoxic agent can trigger lysosomal biogenesis which can result in increased lysosomal sequestration and inadequate target exposure following treatment with lysosomotropic anticancer agents including sunitinib (44).

Excessive accumulation of phospholipids as a result of drug hindrance of their degradation is known as drug-induced phospholipidosis (45). Most of the drugs that have been reported to cause this pathological implication (> 50) are lysosomotropic drugs (cationic amphiphilic drugs) (46). Examples of which are collated in Table 2. These molecules can be protonated to yield cationic species that when bonded with the phospholipid bilayer of the lysosomal membrane alter its surface charge and subsequently lead to the slowing of phospholipid degradation, which results in their lysosomal accumulation (46,53).

Table 1. Examples of some antiviral lysosomotropic agents from various drug classes.

Therapeutic categories	Compounds	References for potential repurposing of the lysosomotropic drug to an antiviral
Anti-malarial	(+/-) Chloroquine	(29)
	(+/-) Hydroxychloroquine	(29)
	(+/-) Mefloquine	(31)
	(+/-) Amodiaquine	(32)
	(+/-) Quinacrine	(4)
Psychoactive	Chlorpromazine	(33)
	Clomipramine	(34)
	(+/-) Fluoxetine	(35)
Cardiovascular	Amiodarone	(36)
	(+/-) Verapamil	(36)
Antibiotic	Azithromycin	(37)
Estrogen receptor modulator	Tamoxifen	(38)
	Toremifene	(39)
	Raloxifene	(40)

Table 2. Examples of phospholipidosis-inducing lysosomotropic drugs.

Therapeutic categories	Compounds	References
Anti-malarial	(+/-) Chloroquine	(47)
	Chlorpromazine	(48)
Psychoactive	Clomipramine	(49)
	(+/-) Fluoxetine	(48)
	Amitriptyline	(50)
Cardiovascular	Amiodarone	(51)
	(+/-) Propranolol	(52)
Antibiotic	Erythromycin	(48)
	Gentamycin	(47)
Antifungal	(+/-) Ketoconazole	(48)
	Perhexiline	(48)
Estrogen receptor modulator	Tamoxifen	(49)

Models for lysosomal trapping

Models describing the intracellular localization of drugs are not only helpful when it comes to interpreting experiments; they are also useful in rational drug design. Approaches that consider quantitative structure-activity relationships (QSAR) have been widely used to forecast the movement of molecules within the different intracellular compartments including lysosomes (1). Due to increased interest in lysosomal trapping, several *in-silico* and *in-vitro* models have been proposed.

Fluorescent lipophilic amines such as LysoTracker Red were used to evaluate the lysosomal sequestration in *in-vitro* cell lines including rat and human hepatocytes (2,13). The compounds that inhibit the fluorescence of these marker compounds in a concentration-dependent manner are considered lysosomotropics (2,54). This method succeeded in predicting many possible lysosomotropic agents yet failed at determining known lysosomotropic drugs like dextromethorphan and labetalol (13).

Nigericin, monensin, and ammonium chloride are known as inhibitors of lysosomal trapping (54). The first two inhibitors interfere with the proton pump present in the lysosomes, thus disrupting the pH of lysosomal fluid and ammonium chloride achieves a similar effect by increasing the pH *via* buffering (13). If the cellular partitioning of the tested compounds decreases with the addition of inhibitors, this indirectly confirms categorizing the investigated drugs under lysosomotropic as reported in some papers (13,20,55).

Effects of drug trapping on the intralysosomal processes have also been used to predict possible lysosomotropic drugs. The

degree of phospholipidosis induction was strongly correlated with lysosomal trapping through a study that involved 47 potential compounds. A similar correlation was undertaken for the phenomenon of lysosomal swelling (56). Inhibition of autophagy can similarly result from lysosomal trapping as previously mentioned and was reported to correlate with the concentrations of drugs accumulating in the lysosomes. However, the study was conducted on a smaller group of drugs including lysosomotropic drugs such as astemizole, chlorpromazine, and (+/-) chloroquine among others (3,56-58).

Trapp *et al.* designed an *in-silico* cell model to predict the distribution of weakly basic drug moieties inside the cell. The model is composed of a cell comprising cytosol, lysosome, and mitochondria; each is contained by a membrane and has both an aqueous and a lipid portion. The Fick-Nernst-Planck equation was utilized to calculate the chemical potential-driven flux of the molecules across the electrically charged membranes. It evaluated the ionization status of the different molecules tested *via* this model. Neutral and ionic molecules across the membrane model were examined for ten model antimalarial drugs whose simulation results anticipated that six of them are optimum for the lysosomal trapping and three were close to the optimum. The results suggested specific physicochemical criteria for potential excellent candidates for lysosomal trapping. These properties included a pK_a range of (6 - 10), with a $\log P$ of (0 - 3) for monovalent bases, whereas for the bivalent bases, pK_a values of below 10 for the higher pK_a (pK_{a1}) and above 4 for the lower pK_a (pK_{a2}) and a $\log P$ of 3 - 6 are optimum for lysosomotropism (1).

Another *in-silico* tool that could potentially predict lysosomal trapping is Membrane Plus™ which is a software developed by Simulations Plus, Inc. (California, USA) for modeling *in-vitro* drugs permeability using different cell types (*e.g.* human colorectal adenocarcinoma cells (CaCo-2), Madin-Darby canine kidney cells (MDCK)) and parallel artificial membrane permeability assay (PAMPA). As there is no reported data on lysosomal trapping using Membrane Plus™, it was used to estimate the lysosomal trapping of the lysosomotropic drugs listed in Tables 1 and 2 in addition to other molecules mentioned throughout the paper (the method is detailed in the Materials and Methods section). The obtained data is depicted in Fig. 2. Some drugs, namely, the four quinolines; (+/-) chloroquine, (+/-) hydroxychloroquine, (+/-) amodiaquine (+/-), and quinacrine had high lysosomal trapping percentages predicted (88.6, 85.4, 74.7, and 88.6, respectively) while the highest percentage for other drugs was 7% for astemizole. Some other well-known

lysosomotropics like (+/-) fluoxetine and (+/-) propranolol were not predicted to be trapped in lysosomes to a significant extent and had only 1.7% and 1.9% trapped predicted within lysosomes, respectively. In our attempts to modify the main input parameters (pKa and log P) for the lysosomotropic drug, dextromethorphan, that has been used in some of the reported experiments in this work we could only obtain the percentage of 4.9% predicted by this modeling methodology.

Generally, lysosomes are fragile organelles, which can make it challenging to quantify the compounds trapped inside them (10). The *in-vitro* cellular models avoid the poor recovery challenge and the drug diffusion during sample preparation (59).

They are also superior to the *in-silico* models based on their better resemblance to the *in-vivo* conditions. However, all models have so far been unable to accurately predict every lysosomotropic drug as they depend on indirect methods to quantify the lysosomotropic agents.

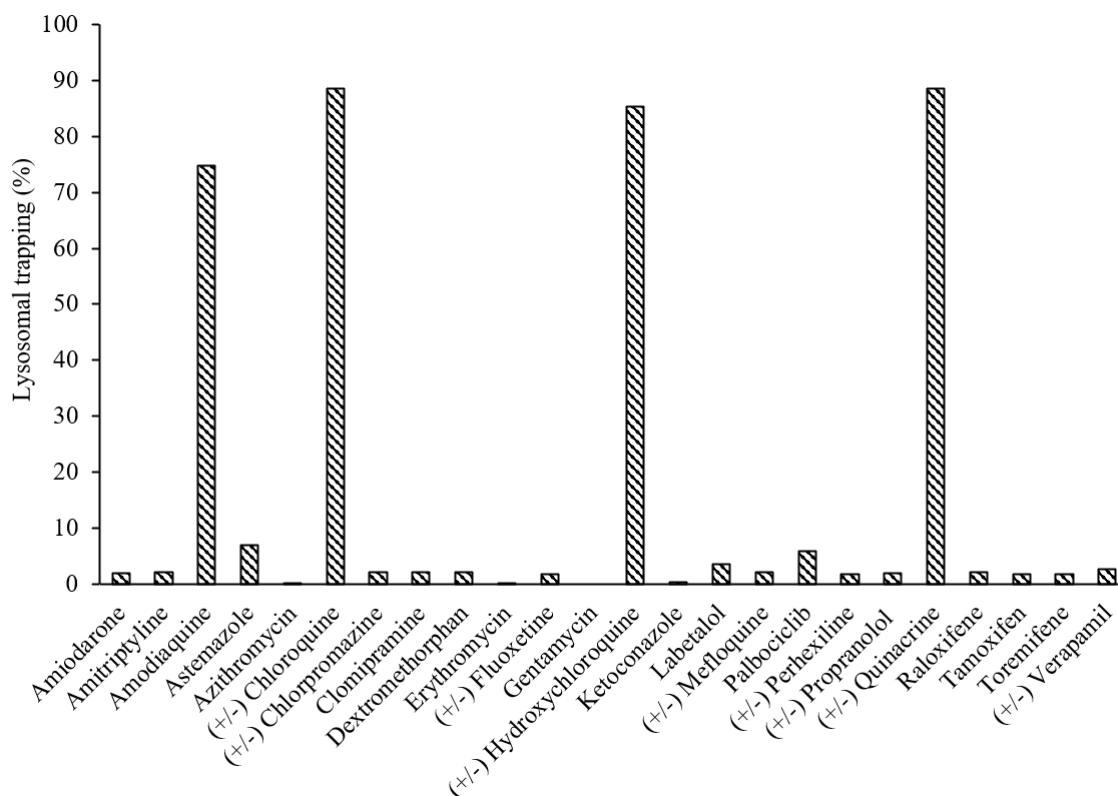


Fig. 2. Percentage of lysosomal trapping of different lysosomotropic drugs (100 μ M) predicted by Membrane Plus™ 2.0 (Simulations Plus, Inc., California, USA) in Caco-2 cells. Default values for the cell model were used with a cytosol pH of 7.22 and lysosomes pH of 4. All properties were calculated by ADMET Predictor 8.1 (Simulations Plus, Inc., California, USA).

The *in-silico* models are at times limited by being incapable of fully replicating what is occurring biologically in the living cells, which is sometimes due to the uncertainty related to the accuracy of the underlying model parameter inputs. Additionally, such models might not consider non-linear processes such as transporter saturation. The effects of the trapped drugs on the lysosome's membranes, intracellular, and intra-organelle environment properties need to be more holistically integrated into the algorithms or the differential equations that underlie the *in-silico* models. The ionic strength effect on pKa has not been considered in some *in-silico* models and this may affect the accuracy of an important molecular descriptor that determines the lysosomal trapping tendency.

Lysosomal fluid

In the field of pharmaceutical sciences, lysosomal targeted drug delivery could be designed *ab initio* for drug therapy and this approach has potential pharmacokinetic and therapeutic benefits. However, there currently is no universally accepted simulated or artificial lysosomal fluid utilized in the pharmaceutical industry or published by the United States Pharmacopeia. There is at least one commercially available artificial lysosomal fluid sold by Biochemazone (www.biochemazone.com). Biochemazone produces commercially available artificial lysosomal fluid BZ257. This formulation is proprietary although it has been communicated

that the use of HCl/acetic acid is employed to maintain acidic pH.

The importance of standardized and physiologically relevant simulated fluids for use in biopharmaceutics and dissolution has been highlighted in many excellent articles and reviews (60,61). However, there is no mention of a general or optimal lysosomal fluid in these publications for pharmaceutical investigations. The reported lysosomal fluids (artificial lysosomal fluid and phagolysosomal fluid) are categorized under simulated lung fluids (62-66). The former simulated conditions occurring in conjunction with phagocytosis by cells (62), while the latter is a potassium hydrogen phthalate buffered solution that represents the medium inside the phagolysosomes (compartments form after fusion of alveolar macrophages and lysosomes when encountering a foreign particle) (63). Table 3 details the composition of each fluid.

Apart from inhalable formulations investigated with the reported lysosomal fluids, the bioaccessibility of lysosomotropic drugs in preparations intended for other routes of administration must also be considered. Lysosomal sequestration, in general, can demonstrate various therapeutic advantages as illustrated in the earlier section-- "Effects of lysosomal trapping". Additionally, enterocyte and hepatocyte lysosomal trapping can have a profound impact on the pharmacokinetics of a lysosomotropic compound, resulting in a delay in drug input into the systemic vasculature and thus retarded appearance in the plasma (67).

Table 3. The concentration of various constituents in artificial lysosomal fluid is reported in the biomedical literature.

Compounds	Artificial lysosomal fluid composition (g/L) (62,64-66)	Phagolysosomal fluid composition (g/L) (63,66)
Sodium chloride	3.21	6.65
Sodium hydroxide	6.0	
Sodium hydrogen phosphate dibasic (anhydrous)	0.071	0.142
Calcium chloride dihydrate	0.128	0.029
Citric acid	20.8	
Trisodium citrate dihydrate	0.077	0.45
Glycine	0.059	0.071
Sodium sulfate	0.039	-
Magnesium chloride	0.05	-
Disodium tartrate sodium tartrate (dihydrate)	0.09	-
Sodium lactate	0.085	-
Sodium pyruvate	0.086*	-
Potassium hydrogen phthalate	-	4.085
pH	4.5-5	4.55

*The sodium pyruvate composition is 0.086 g/L according to references No. 64-66 but 0.172 g/L according to reference No. 62. All other ingredients have similar composition across the different references.

Furthermore, ophthalmological lysosomal trapping within the retinal blood barrier has been suggested to be a part of novel transport systems that could safely and effectively deliver candidate drugs to treat retinal dysfunction (68). Development and standardization of a simulated lysosomal fluid (SLYF) would be an essential contribution to pharmaceutical drug and formulation development and aid standardization of *in-vitro* studies for lysosomotropic drugs and their formulations. Based on literature reports on the principal ions responsible for lysosomal hemostasis and their concentrations (69), a facile SLYF was developed for routine pharmaceutical use. Selected representative drugs that trap within lysosomes were identified using *in-silico* modeling and a review of the literature and solubility and dissolution were examined.

MATERIALS AND METHODS

Materials

Sodium Chloride (NaCl, CAS: 7647-15-5), potassium chloride (KCl, CAS: 7447-40-7) and sodium acetate (CH₃COONa, CAS: 127-09-3)

were obtained from Caledon Laboratories (Ontario, Canada) while the calcium chloride dihydrate (CaCl₂·2H₂O, CAS: 10035-04-8) was from Sigma (Steinheim, Germany) and the acetic acid (CH₃COOH, CAS:64-19-7) was purchased from Fischer Scientific, USA. Distilled water was used for dissolving the reagents. Dextromethorphan hydrobromide (CAS: 6700-34-1) and chloroquine diphosphate (CAS: 50-63-5) were obtained from Sigma-Aldrich Co. (Missouri, USA) whereas the dextromethorphan soft gel capsules (Robitussin[®]) were a product of Pfizer (Ontario, Canada) from the lot: DP5155 (Expiration Date: 02/2023).

Using Membrane Plus for lysosomal trapping via CaCo-2 permeability model

Membrane Plus[™] 2.0 from Simulations Plus, Inc. (California, USA) was used to estimate the lysosomal trapping of the potential lysosomotropic. The molecular structure of these drugs was imported into the Caco-2 12-well model. Properties of drugs were predicted by absorption, distribution, metabolism, excretion, and toxicity (ADMET) Predictor 8.1 (Table 4).

Table 4. List of the tested drugs *via* Membrane Plus[™] with their log P and PKa values as predicted by ADMET Predictor 8.1 and used by Membrane Plus[™] 2.0 (Simulations Plus, Inc., California, USA).

Drugs	% Of lysosomal trapping	Log P	PKa
Amiodarone	2	6.83	8.96
Amitriptyline	2.1	4.97	8.93
Amodiaquine	74.7	4.95	6.25, 7.95, 10.29
Astemizole	7	5.54	7.97
Azithromycin	0.1	3.39	7.63, 8.72
(+/-) Chloroquine	88.6	5.11	7.25, 9.86
Chlorpromazine	2.1	5.31	8.87
Clomipramine	2.1	5.7	8.87
Dextromethorphan	2.2	3.81	8.91
Erythromycin	0.1	2.3	8.63
(+/-) Fluoxetine	1.7	4.39	9.82
Gentamycin	0	-1.79	7.29, 8.08, 8.9, 9.96
(+/-) Hydroxychloroquine	85.4	3.94	7.19, 9.17
(+/-) Ketoconazole	0.4	3.74	6.15
(+/-) Labetalol	3.6	2.54	8.44, 9.66, 11.72
(+/-) Mefloquine	2.1	3.81	8.52
Palbociclib	5.8	1.87	8.96
(+/-) Perhexiline	1.8	6.69	10.1
(+/-) Propranolol	1.9	2.89	9.48
(+/-) Quinacrine	88.6	6.16	7.88, 9.8
Raloxifene	2.2	5.39	8.14, 9.4, 10.08
Tamoxifen	1.8	6.64	8.48
Toremifene	1.8	6.73	8.42
(+/-) Verapamil	2.6	4.45	8.46

Table 5. Simulated lysosomal fluid composition for pharmaceutical investigations.

Reagents	Amount per 100 mL ($\pm 0.1\%$)
NaCl	0.321 g
KCl	0.0224 g
CaCl ₂ .2H ₂ O	0.0119 g
Na acetate	0.4046 g
Acetic acid*	0.5 ml
HCl	q.s to adjust the pH (4-5.5)

*Acetic acid volume can be adjusted to be only part of the buffering system and to use the HCl to adjust the pH to the required values.

The apical compartment of the model was chosen to be filled with 0.5 mL of the drug solution (100 μ M, pH = 7.4) while the basolateral one was filled with 1.5 mL of buffer solution (pH = 7.4). The default data for the membrane thickness and pore size were used at a shaking rate of 100. Sampling was done at 0.25, 0.5, 1, 1.5, and 2 h. Default values for the cell model were considered with a cytosol pH of 7.22 and lysosomal pH of 4. The obtained values for the lysosomal trapping percentages for the tested drugs were noted.

Preparation of the SLYF

The fluid was prepared with the components listed in Table 5. Components were added successively and dissolved in 70 mL water, and then HCl was used to adjust the pH before completing the volume to 100 mL.

Analysis of the developed lysosomal fluid and a commercial artificial fluid

The laboratory-prepared lysosomal fluid (SLYF) and the commercial artificial fluid (BZ257, Biochemazone, Alberta, Canada) were examined for their composition and pH as follows.

pH determination

The pH of the fluids was determined *via* Fischer Scientific XL20 pH/conductivity meter (Massachusetts, USA).

Chemical analysis

The lysosomal fluids were chemically investigated using Medica's EasyRA[®] analyzer (Massachusetts, USA). They were analyzed for their content of potassium (K⁺), sodium (Na⁺), calcium (Ca²⁺), and chloride (Cl⁻) ions, in addition to lithium (Li⁺), magnesium (Mg⁺⁺), and total protein. After checking and calibrating the system, the probe and the ion-selective electrode were cleaned and the latter was calibrated. A properly calibrated reagent for

each component was put with a blank (high-performance liquid chromatography (HPLC)-grade water) in the sample holder with 2000 μ L samples of the SLYF and the commercial artificial fluids. The required components were selected and the analysis was run for their concentration in blank and fluids samples.

Measuring the solubility of dextromethorphan in SLYF and the commercial artificial fluid

The solubility of the model lysosomotropic dextromethorphan was tested in both fluids using the shake-flask method (70). In brief, the excess drug was weighed into 2 mL samples of SLYF and the commercial lysosomal fluid, then they were shaken at room temperature for 24 h. Subsequently, samples from the supernatant were filtered through 0.45 μ m nylon filters before being analyzed at 230 and 280 nm using HPLC C18 (4.6 \times 250 mm \times 5 μ m) column that utilized 60:40 methanol and phosphate buffer (pH = 5.5) as a mobile phase at a rate of 0.8 mL/min for a run time of 10 min.

Measuring the dissolution and release profile of dextromethorphan from the commercial product Robitussin[®]:

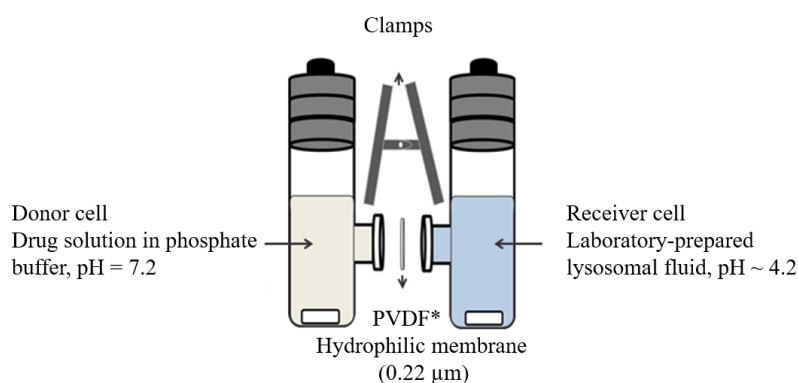
Table 6 lists the main components in the liquid-filled dextromethorphan capsules (Robitussin DM Coughgels). *In-vitro* dissolution profiles for this were conducted *via* VK 7000, USP 711 apparatus by VanKel[®] (Varian Inc, USA) in 900 mL of 0.1 N HCl, SLYF fluid and 0.05 M phosphate buffer (pH = 7.4), using a USP type I basket method dissolution apparatus. A temperature of 37 \pm 0.5 $^{\circ}$ C and a rotation speed of 100 rpm were maintained according to USP dissolution methods (USP34NF29). Three mL samples were withdrawn over a period of 60 min at predetermined time intervals (15, 30, 45, and 60 min). The filtered samples were suitably analyzed using the HPLC procedure described in the previous section.

Table 6. Summary of Robitussin drug product information obtained from: [https:// www.medsafe.govt.nz/Profes/Datasheet/t/robitussinDryCoughcap.pdf](https://www.medsafe.govt.nz/Profes/Datasheet/t/robitussinDryCoughcap.pdf)

Drug	Inactive ingredients
Dextromethorphan HBr-15 mg	Allura red AC, brilliant blue FCF, gelatin, macrogol 400, opacode white NSP-78-18022, povidone, propyl gallate, purified water, sorbitol special glycerin blend A810.

Table 7. Results of Analysis of the simulated lysosomal fluid and a commercial artificial lysosomal fluid using Medica's EasyRA[®] Analyzer (Massachusetts, USA) with the reference values of each component as adopted from literature.

Ions	Amount in the prepared lysosomal fluid (mM)	Amount in the commercial lysosomal fluid (mM \pm 5%)	Optimum concentration for lysosomal homeostasis (mM) (69)
Na+	59.08 \pm 2.0 \checkmark	< 10 X	20-140
K+	3.09 \pm 0.06 \checkmark	4.24 \checkmark	2-50
Ca ²⁺	0.46 \pm 0.02 \checkmark	1.65 X	~0.5
Cl-	59.1 \pm 5.0 \checkmark	90.8 X	< 80
pH	4.21 \checkmark	7.51 X	4-5.5 (1)

**Fig. 3.** Schematic representation of the developed lysosomal trapping apparatus. *PVDF, Polyvinylidene fluoride.

Using the developed fluid (SLYF) in predicting lysosomal trapping

Hydrophilic synthetic 0.22 μ m polyvinylidene fluoride membranes were first immersed in the developed lysosomal fluid (SLYF) for 10 min and the excess media was removed using absorbent tissues. The membrane was then placed between the two compartments of a horizontal Franz-cell (Fig. 3). The receptor compartment was filled with 20 mL of the SLYF (pH = 4.21), whereas the donor compartment was filled with 20 mL of the drug solution (1 mg/mL) in phosphate buffer (pH = 7.2). Both compartments were magnetically stirred at 600 rpm throughout the experiment. At time points (1, 2, 3, 4, and 24 h), 400 μ L aliquots were withdrawn from the receiver compartment and replaced immediately with an equal volume of fresh receptor medium to maintain a constant volume

of the receiving solution. Analysis of both drugs was done using the same HPLC system. For chloroquine, the detection was done at 342 nm using a mobile phase of 60% methanol and 40% acetate buffer (pH = 5.8) that was run at the rate of 1 mL/min for 10 min.

RESULTS

The pH of the commercial artificial lysosomal fluid was determined to be 7.51, while that of SLYF fluid was 4.2. The chemical analysis of the constituents of both SLYF and the commercial lysosomal fluid for the essential ions for lysosomal function are depicted in Table 7 along with the reported reference values of the tested components. The commercial artificial lysosomal fluid contained only the potassium concentration within the optimum range, while the sodium, calcium, and

chloride were all out of range. Moreover, similar to the SLYF fluid, the commercially purchased fluid had no lithium yet contained 1.09 mg/dL of magnesium and a total protein of 2 g/L.

The solubility testing of dextromethorphan in both fluids yielded a clear solution with the SLYF, while the artificial commercial fluid produced a turbid sample with a precipitate (Fig. 4). The average solubility values of the dextromethorphan in the commercial artificial fluid and the SLYF were 1.3 mg/mL and 4.9 mg/mL, respectively (Fig. 5). Figure 6 depicts the drug's rate and extent of release was

different in the three dissolution media; 0.1 N HCl, SLYF, and phosphate buffer (pH = 7.4). The highest release values were in 0.1 N HCl (> 97%) followed by SLYF (72.6%) and then the phosphate buffer in which only about 32% of the drug was released into.

Using SLYF in the developed model to assess the lysosomal trapping of both chloroquine and dextromethorphan revealed that throughout the experiment the rate and extent of chloroquine release into the lysosomal compartment were higher than the other one as seen in Fig. 7.



Fig. 4. Images of samples of the solubility test of the model drug dextromethorphan in the laboratory prepared lysosomal fluid (SLYF) where it fully dissolved (right) and in the commercial artificial counterpart where the fluid was turbid with a precipitate formed (left).

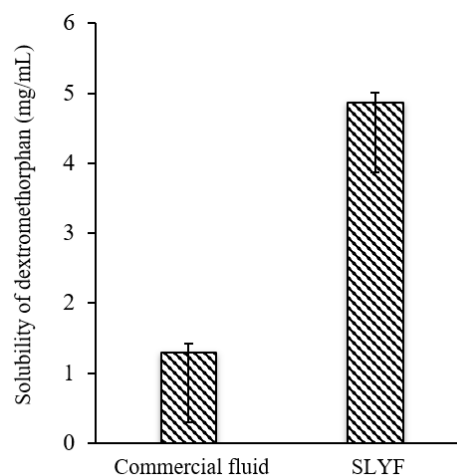


Fig. 5. Solubility of dextromethorphan in SLYF and in the commercial artificial fluid. Data are presented as mean \pm SEM, n = 3. SLYF, laboratory-prepared simulated lysosomal fluid

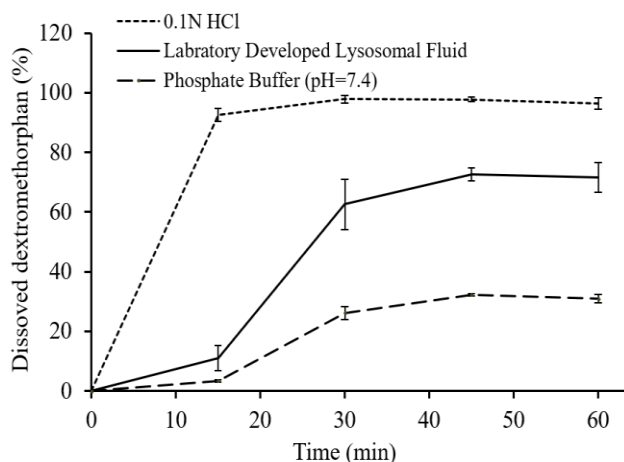


Fig. 6. Dissolution profile of dextromethorphan performed on the commercial product robitussin in 0.1 N HCl, developed simulated lysosomal fluid (SLYF), and phosphate buffer (pH = 7.4). Data are presented as mean \pm SEM, n = 3.

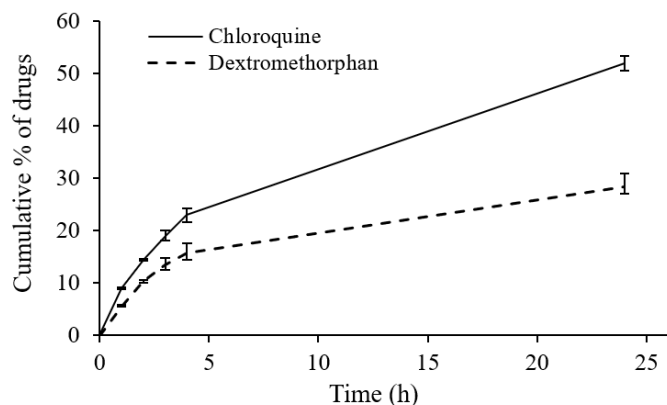


Fig. 7. Cumulative percentage of tested lysosmotropics ((+/-) chloroquine and dextromethorphan) in the receiver compartment of the model using the developed simulated lysosomal fluid (SLYF). Data are presented as mean \pm SEM, n = 3.

DISCUSSION

Lysosomal ionic hemostasis is crucial for its function. Sodium (Na^+) and potassium (K^+) ions are essential for determining lysosomal membrane potential that in turn affect the movement of the different ions across the lysosomal membrane and regulate lysosomal acidification. Calcium ions (Ca^{2+}) efflux also contributes to lysosomal acidification and is linked to the fusion of lysosomes with other cellular parts. Chloride ions (Cl^-) are critical counter ions for the chloride proton antiporter which is the main mean that maintains the acidic environment inside lysosomes. The optimal range of these ions inside lysosomes is listed in Table 7. In addition to the mentioned ions, micromolar deposits of other ions including iron (Fe^{3+} and Fe^{2+}), zinc (Zn^{2+}), and copper (Cu^{2+}) are also found in lysosomes to be provided to the cell when required (69).

For SLYF preparation, only ions that are essential for the lysosomal internal hemostasis were selected. Sodium, potassium, and calcium chlorides were used to provide their corresponding chosen ions (Na^+ , K^+ , Ca^{2+} , and Cl^-). The equipped buffering system contained acetate and acetic acid (buffering range = 3.5-5.5) (71). In addition to the acetic acid playing a part in decreasing the pH, HCl was used to adjust the pH to the required value.

The pH of the commercial fluid does not reflect the pH inside the lysosomes that should be maintained within 4 - 5.5 (1). Yet, the pH of SLYF does reside within the range.

Additionally, the results showed that concentrations of all SLYF components complied with reported data in the literature, unlike the commercial fluid in which only potassium values were optimized.

The improved solubility of the selected model drug in the SLYF could be attributed to the pH of the fluid (4.21) and the pKa of the drug (9.8) (72). However, the discrepancy in the solubility data poses a challenge when considering pharmaceutical or biochemical tests using simulated or artificial biological fluids such as the lysosomal fluid. It is also of special importance for the optimal dissolution conditions of lysosmotropic drug entities and their formulations.

Dextromethorphan's rate and extent of release were different in the three dissolution media examined. The pH can be one factor to the faster release of the basic drug (pKa 9.85) in the 0.1 N HCl (pH = 1) than the SLYF fluid (pH = 4.2) and the phosphate buffer (pH = 7.4). Fluid composition could also be another factor affecting dissolution. However, even after 45 min, it was only the 0.1 N HCl medium that passed the acceptance criteria for the dextromethorphan dissolution testing of not less than 75% in 45 min in the specified dissolution medium. Other reports indicated the success of the different dextromethorphan dosage forms including capsules to pass the dissolution test in various media *e.g.* 0.1 N HCl, acetate buffer (pH= 4.5), and simulated intestinal fluid (SIF, pH = 6.8) (73,74).

The developed lysosomal fluid also showed its usefulness in the model used to predict the lysosomal trapping of the two lysosomotropic; (+/-) chloroquine and dextromethorphan. The model was designed in a way to replicate the movement of drugs from the cytosol to lysosomes by having two compartments filled with representative media; a donor compartment in which the drug was dissolved in a phosphate buffer with a pH of 7.2 and a receiver one containing the simulated lysosomal fluid (pH = 4.21). The release profile into the receiver compartment which is an indicator for the lysosomal trapping demonstrated that chloroquine appeared in the SLYF at *e.g.* a greater rate and extent than dextromethorphan. That observation is reflected by physicochemical molecular descriptors of both drugs, namely the pKa and the log P which would favor chloroquine (pKa = 7.29 and 10.32 and LogP = 3.93) lysosomal trapping over that of the dextromethorphan (pKa = 9.85 and Log P = 3.49) (17). Using this developed SLYF fluid as an *in-vitro* model to evaluate additional lysosomotropic or potentially lysosomotropic drugs.

CONCLUSION

The importance of lysosomes to drug action and disposition is well known. Several *in-vitro* models have been developed to assess lysosomal trapping and quantify the proportion of potential candidates that will be sequestered inside lysosomes. However, there is no standardized composition for lysosomal fluid. Therefore, we developed a lysosomal fluid with the basic components required for lysosomal hemostasis (SLYF fluid) and compared it to a commercial artificial lysosomal fluid. The laboratory-prepared fluid (SLYF fluid) complied with the reported data on the pH and the concentration of the lysosomal components. The obtained result helps fill the void with a lysosomal fluid that can be used to examine various factors related to pharmaceutical product performance including dissolution, solubility, and disposition which are relevant and necessary parts of drug and product development. Preliminary data from the developed fluid and model with

dextromethorphan and (+/-) chloroquine suggest the pharmaceutical utility of this developed methodology. The obtained results and model system could be adapted to enable investigating the sequestration of drugs in other cellular organelles using a similarly developed experimental design approach.

Acknowledgments

This work was part of M. Yousef's Ph.D. project funded by MITACS.

Conflict of interest statement

The authors declared no conflict of interest in this study.

Author's contribution

M. Yousef carried out the experiments with the help of T.S. Le, J. Zuo, and C. Park; N.B. Chacra, N.M. Davies and R. Löbenberg conceived the study and were in charge of work direction, experimental planning, and manuscript revisions and suggestions. The finalized article was approved by all authors.

REFERENCES

1. Trapp S, Rosania GR, Horobin RW, Kornhuber J. Quantitative modeling of selective lysosomal targeting for drug design. *Eur Biophys J.* 2008;37(8):1317-1328. DOI: 10.1007/s00249-008-0338-4.
2. Schmitt MV, Lienau P, Fricker G, Reichel A. Quantitation of lysosomal trapping of basic lipophilic compounds using *in-vitro* assays and *in-silico* predictions based on the determination of the full pH profile of the endo-/lysosomal system in rat hepatocytes. *Drug Metab Dispos.* 2019;47(1):49-57. DOI: 10.1124/dmd.118.084541.
3. Norinder U, Tuck A, Norgren K, Kos VM. Existing highly accumulating lysosomotropic drugs with potential for repurposing to target COVID-19. *Biomed Pharmacother.* 2020;130:110582,1-7. DOI: 10.1016/j.biopha.2020.110582.
4. Lane TR, Dyall J, Mercer L, Goodin C, Foil DH, Zhou H, *et al.* Repurposing Pyramax® for the treatment of ebola virus disease: additivity of the lysosomotropic pyronaridine and non-lysosomotropic artesunate. *BioRxiv.* 2020;1-34. DOI: 10.1101/2020.04.25.061333.
5. Hu M, Carraway KL III. Repurposing cationic amphiphilic drugs and derivatives to engage lysosomal cell death in cancer treatment. *Front Oncol.* 2020;10:605361,1-7. DOI: 10.3389/fonc.2020.605361.

6. Gong Y, Duvvuri M, Krise JP. Separate roles for the Golgi apparatus and lysosomes in the sequestration of drugs in the multidrug-resistant human leukemic cell line HL-60. *J Biol Chem.* 2003;278(50):50234-50239.
DOI: 10.1074/jbc.M306606200.
7. Trapp S, Horobin RW. A predictive model for the selective accumulation of chemicals in tumor cells. *Eur Biophys J.* 2005;34(7):959-966.
DOI: 10.1007/s00249-005-0472-1.
8. Baltazar GC, Guha S, Lu W, Lim J, Boesze-Battaglia K, Laties AM, *et al.* Acidic nanoparticles are trafficked to lysosomes and restore an acidic lysosomal pH and degradative function to compromised ARPE-19 cells. *PloS One.* 2012;7(12):e49635,1-10.
DOI: 10.1371/journal.pone.0049635.
9. Xue F, Wei P, Ge X, Zhong Y, Cao C, Yu D, *et al.* A pH-responsive organic photosensitizer specifically activated by cancer lysosomes. *Dyes Pigm.* 2018;156:285-290.
DOI: 10.1016/j.dyepig.2018.04.008.
10. Macintyre AC, Cutler DJ. The potential role of lysosomes in tissue distribution of weak bases. *Biopharm Drug Dispos.* 1988;9(6):513-526.
DOI: 10.1002/bod.2510090602.
11. Radisavljevic Z. Lysosome activates AKT inducing cancer and metastasis. *J Cell Biochem.* 2019;120(8):12123-12127.
DOI: 10.1002/jcb.28752.
12. Saftig P, Klumperman J. Lysosome biogenesis and lysosomal membrane proteins:trafficking meets function. *Nat Rev Mol Cell Biol.* 2009;10(9):623-635.
DOI: 10.1038/nrm2745.
13. Kazmi F, Hensley T, Pope C, Funk RS, Loewen GJ, Buckley DB, *et al.* Lysosomal sequestration (trapping) of lipophilic amine (cationic amphiphilic) drugs in immortalized human hepatocytes (Fa2N-4 cells). *Drug Metab Dispos.* 2013;41(4):897-905.
DOI: 10.1124/dmd.112.050054.
14. Govindaraj J, Govindaraj K, Padmavathy K, Jayesh R, Mathangi, Vidyarekha U. Review On lysosomal enzymes. *Eur J Mol Clin Med.* 2020;7(8):1688-1692.
15. Christensen KA, Myers JT, Swanson JA. pH-dependent regulation of lysosomal calcium in macrophages. *J Cell Sci.* 2002;115(3):599-607.
DOI: 10.1242/jcs.115.3.599.
16. DiCiccio JE, Steinberg BE. Lysosomal pH and analysis of the counter ion pathways that support acidification. *J Gen Physiol.* 2011;137(4):385-390.
DOI: 10.1085/jgp.201110596.
17. De Duve C, De Barsey T, Poole B, Trouet A, Tulkens P, Van Hoof F. Commentary. Lysosomotropic agents. *Biochem Pharmacol.* 1974;23(18):2495-2531.
DOI: 10.1016/0006-2952(74)90174-9.
18. Ndolo RA, Luan Y, Duan S, Forrest ML, Krise JP. Lysosomotropic properties of weakly basic anticancer agents promote cancer cell selectivity *in vitro*. *PloS One.* 2012;7(11):e49366,1-9.
DOI: 10.1371/journal.pone.0049366.
19. Marceau F, Bawolak MT, Lodge R, Bouthillier J, Gagné-Henley A, Gaudreault RC, *et al.* Cation trapping by cellular acidic compartments:beyond the concept of lysosomotropic drugs. *Toxicol Appl Pharmacol.* 2012;259(1):1-12.
DOI: 10.1016/j.taap.2011.12.004.
20. Daniel WA, Wójcikowski J. Contribution of lysosomal trapping to the total tissue uptake of psychotropic drugs. *Pharmacol Toxicol.* 1997;80(2):62-68.
DOI: 10.1111/j.1600-0773.1997.tb00285.x.
21. Homewood CA, Warhurst DC, Peters W, Baggaley VC. Lysosomes, pH and the anti-malarial action of chloroquine. *Nature.* 1972;235(5332):50-52.
DOI: 10.1038/235050a0.
22. Daniel WA, Bickel MH, Honegger UE. The contribution of lysosomal trapping in the uptake of desipramine and chloroquine by different tissues. *Pharmacol Toxicol.* 1995;7(6):402-406.
DOI: 10.1111/j.1600-0773.1995.tb01050.x.
23. Fong KY, Wright DW. Hemozoin and antimalarial drug discovery. *Future Med Chem.* 2013;5(12):1437-1450.
DOI: 10.4155/fmc.13.113.
24. Kornhuber J, Reichel M, Tripal P, Groemer TW, Henkel AW, Mühle C, *et al.* The role of ceramide in major depressive disorder. *Eur Arch Psychiatry Clin Neurosci.* 2009;259(Suppl 2):S199-S204.
DOI: 10.1007/s00406-009-0061-x.
25. Beckmann N, Sharma D, Gulbins E, Becker KA, Edelmann B. Inhibition of acid sphingomyelinase by tricyclic antidepressants and analogons. *Front Physiol.* 2014;5:331,1-15.
DOI: 10.3389/fphys.2014.00331.
26. Llanos S, Megias D, Blanco-Aparicio C, Hernández-Encinas E, Rovira M, Pietrocola F, *et al.* Lysosomal trapping of palbociclib and its functional implications. *Oncogene.* 2019;38(20):3886-3902.
DOI: 10.1038/s41388-019-0695-8.
27. Dhillon S. Palbociclib:first global approval. *Drugs.* 2015;75(5):543-551.
DOI: 10.1007/s40265-015-0379-9.
28. Salata C, Calistri A, Parolin C, Baritussio A, Palù G. Antiviral activity of cationic amphiphilic drugs. *Expert Rev Anti Infect Ther.* 2017;15(5):483-492.
DOI: 10.1080/14787210.2017.1305888.
29. Naghipour S, Ghodousi M, Rahsepar S, Elyasi S. Repurposing of well-known medications as antivirals: hydroxychloroquine and chloroquine-from HIV-1 infection to COVID-19. *Expert Rev Anti Infect Ther.* 2020;18(11):1119-1133.
DOI: 10.1080/14787210.2020.1792291.
30. Vaugeois JM. Psychotropics drugs with cationic amphiphilic properties may afford some protection against SARS-CoV-2: a mechanistic hypothesis. *Psychiatry Res.* 2020;291:113220,1-2.
DOI: 10.1016/j.psychres.2020.113220.
31. Sacramento CQ, Fintelman-Rodrigues N, Dias SSG, Temerozo JR, Da Silva APD, da Silva CS, *et al.* Unlike chloroquine, mefloquine inhibits SARS-CoV-2 infection in physiologically relevant cells and does not induce viral variants. *BioRxiv.* 2021;1-25

- DOI: 10.1101/2021.07.21.451321.
32. DeWald LE, Johnson JC, Gerhardt DM, Torzewski LM, Postnikova E, Honko AN, *et al.* *In vivo* activity of amodiaquine against Ebola virus infection. *Sci Rep.* 2019;9(1):20199,1-12.
DOI: 10.1038/s41598-019-56481-0.
 33. Vela JM. Repurposing sigma-1 receptor ligands for COVID-19 therapy? *Front Pharmacol.* 2020;11:582310,1-23.
DOI: 10.3389/fphar.2020.582310.
 34. Nobile B, Durand M, Olié E, Guillaume S, Molès JP, Haffen E, *et al.* The anti-inflammatory effect of the tricyclic antidepressant clomipramine and its high penetration in the brain might be useful to prevent the psychiatric consequences of SARS-CoV-2 infection. *Front Pharmacol.* 2021;12:615695,1-10.
DOI: 10.3389/fphar.2021.615695.
 35. Schafer A, Xiong R, Cooper L, Nowar R, Lee H, Li Y, *et al.* Evidence for distinct mechanisms of small molecule inhibitors of filovirus entry. *PLoS Pathog.* 2021;17(2):e1009312,1-21.
DOI: 10.1371/journal.ppat.1009312.
 36. Gunesch AP, Zapatero-Belinchón FJ, Pinkert L, Steinmann E, Manns MP, Schneider G, *et al.* Filovirus antiviral activity of cationic amphiphilic drugs is associated with lipophilicity and ability to induce phospholipidosis. *Antimicrob Agents Chemother.* 2020;64(8):e00143-20,1-39.
DOI: 10.1128/AAC.00143-20.
 37. Oliver ME, Hinks TSC. Azithromycin in viral infections. *Rev Med Virol.* 2021;31(2):e2163,1-13.
DOI: 10.1002/rmv.2163.
 38. Bogush TA, Polezhaev BB, Mamichev IA, Bogush EA, Polotsky BE, Tjulandin SA, *et al.* Tamoxifen never ceases to amaze: new findings on non-estrogen receptor molecular targets and mediated effects. *Cancer Invest.* 2018;36(4):211-220.
DOI: 10.1080/07357907.2018.1453933.
 39. Johansen LM, Brannan JM, Delos SE, Shoemaker CJ, Stossel A, Lear C, *et al.* FDA-approved selective estrogen receptor modulators inhibit Ebola virus infection. *Sci Transl Med.* 2013;5(190):190ra79,1-14.
DOI: 10.1126/scitranslmed.3005471.
 40. Allegretti M, Cesta MC, Zippoli M, Beccari A, Talarico C, Mantelli F, *et al.* Repurposing the estrogen receptor modulator raloxifene to treat SARS-CoV-2 infection. *Cell Death Differ.* 2022;29(1):156-166.
DOI: 10.1038/s41418-021-00844-6.
 41. Zhitomirsky B, Assaraf YG. Lysosomes as mediators of drug resistance in cancer. *Drug Resist Updat.* 2016;24:23-33.
DOI: 10.1016/j.drup.2015.11.004.
 42. Halaby R. Influence of lysosomal sequestration on multidrug resistance in cancer cells. *Cancer Drug Resist.* 2019;2(1):31-42.
DOI: 10.20517/cdr.2018.23.
 43. Logan R, Funk RS, Axcell E, Krise JP. Drug-drug interactions involving lysosomes: mechanisms and potential clinical implications. *Expert Opin Drug Metab Toxicol.* 2012;8(8):943-958.
DOI: 10.1517/17425255.2012.691165.
 44. Azijli K, Gotink KJ, Verheul HM. The potential role of lysosomal sequestration in sunitinib resistance of renal cell cancer. *J Kidney Cancer VHL.* 2015;2(4):195-203.
DOI: 10.15586/jkcvhl.2015.44.
 45. Anderson N, Borlak J. Drug-induced phospholipidosis. *FEBS Lett.* 2006;580(23):5533-5540.
DOI: 10.1016/j.febslet.2006.08.061.
 46. Breiden B, Sandhoff K. Emerging mechanisms of drug-induced phospholipidosis. *Biol Chem.* 2019;401(1):31-46.
DOI: 10.1515/hsz-2019-0270.
 47. Müller-Höcker J, Schmid H, Weiss M, Dendorfer U, Braun GS. Chloroquine-induced phospholipidosis of the kidney mimicking Fabry's disease: case report and review of the literature. *Hum Pathol.* 2003;34(3):285-289.
DOI: 10.1053/hupa.2003.36.
 48. Fischer H, Atzpodien EA, Csato M, Doessegger L, Lenz B, Schmitt G, *et al.* *In silico* assay for assessing phospholipidosis potential of small druglike molecules: training, validation, and refinement using several data sets. *J Med Chem.* 2012;55(1):126-139.
DOI: 10.1021/jm201082a.
 49. Sawada H, Takami K, Asahi S. A toxicogenomic approach to drug-induced phospholipidosis: analysis of its induction mechanism and establishment of a novel *in vitro* screening system. *Toxicol Sci.* 2005;83(2):282-292.
DOI: 10.1093/toxsci/kfh264.
 50. Vitovic P, Alakoskela JM, Kinnunen PKJ. Assessment of drug-lipid complex formation by a high-throughput Langmuir-balance and correlation to phospholipidosis. *J Med Chem.* 2008;51(6):1842-1848.
DOI: 10.1021/jm7013953.
 51. Hanumegowda UM, Wenke G, Regueiro-Ren A, Yordanova R, Corradi JP, Adams SP. Phospholipidosis as a function of basicity, lipophilicity, and volume of distribution of compounds. *Chem Res Toxicol.* 2010;23(4):749-755.
DOI: 10.1021/tx9003825.
 52. Bauch C, Bevan S, Woodhouse H, Dilworth C, Walker P. Predicting *in vivo* phospholipidosis-inducing potential of drugs by a combined high content screening and *in silico* modelling approach. *Toxicol In Vitro.* 2015;29(3):621-630.
DOI: 10.1016/j.tiv.2015.01.014.
 53. Shayman JA, Abe A. Drug induced phospholipidosis: an acquired lysosomal storage disorder. *Biochim Biophys Acta.* 2013;1831(3):602-611.
DOI: 10.1016/j.bbalip.2012.08.013.
 54. Kazmi F, Funk R, Pope C, Czerwinski M, Yerino P, Bolliger P, *et al.* A robust method to identify compounds that undergo intracellular lysosomal sequestration. *Drug Metab Rev.* 2011;43:136-137.
 55. Ufuk A, Assmus F, Francis L, Plumb J, Damian V, Gertz M, *et al.* *In vitro* and *in silico* tools to assess extent of cellular uptake and lysosomal sequestration

- of respiratory drugs in human alveolar macrophages. *Mol Pharm*. 2017;14(4):1033-1046.
DOI: 10.1021/acs.molpharmaceut.6b00908.
56. Easwaranathan A, Inci B, Ulrich S, Brunken L, Nikiforova V, Norinder U, *et al*. Quantification of intracellular accumulation and retention of lysosomotropic macrocyclic compounds by high-throughput imaging of lysosomal changes. *J Pharm Sci*. 2019;108(1):652-660.
DOI: 10.1016/j.xphs.2018.11.001.
 57. Garcia DS, Sjödin M, Hellstrandh M, Norinder U, Nikiforova V, Lindberg J, *et al*. Cellular accumulation and lipid binding of perfluorinated alkylated substances (PFASs)-A comparison with lysosomotropic drugs. *Chem Biol Interact*. 2018;281:1-10.
DOI: 10.1016/j.cbi.2017.12.021.
 58. Ashoor R, Yafawi R, Jessen B, Lu S. The contribution of lysosomotropism to autophagy perturbation. *PloS One*. 2013;8(11):e82481,1-15.
DOI: 10.1371/journal.pone.0082481.
 59. Ufuk A, Somers G, Houston JB, Galetin A. *In vitro* assessment of uptake and lysosomal sequestration of respiratory drugs in alveolar macrophage cell line NR8383. *Pharm Res*. 2015;32(12):3937-3951.
DOI: 10.1007/s11095-015-1753-8.
 60. Marques MRC, Löbenberg R, Almukainzi M. Simulated biological fluids with possible application in dissolution testing. *Dissolution Technol*. 2011;18(3):15-28.
DOI: 10.14227/DT180311P15.
 61. Innes E, Yiu HHP, McLean P, Brown W, Boyles M. Simulated biological fluids-a systematic review of their biological relevance and use in relation to inhalation toxicology of particles and fibres. *Crit Rev Toxicol*. 2021;51(3):217-248.
DOI: 10.1080/10408444.2021.1903386.
 62. Pelfrène A, Cave MR, Wragg J, Douay F. *In vitro* investigations of human bioaccessibility from reference materials using simulated lung fluids. *Int J Environ Res Public Health*. 2017;14(2):112,1-15.
DOI: 10.3390/ijerph14020112.
 63. Stefaniak AB, Guilmette RA, Day GA, Hoover MD, Breyse PN, Scripsick RC. Characterization of phagolysosomal simulant fluid for study of beryllium aerosol particle dissolution. *Toxicol In Vitro*. 2005;19(1):123-134.
DOI: 10.1016/j.tiv.2004.08.001.
 64. Colombo C, Monhemius AJ, PlantJA. Platinum, palladium and rhodium release from vehicle exhaust catalysts and road dust exposed to simulated lung fluids. *Ecotoxicol Environ Saf*. 2008;71(3):722-730.
DOI: 10.1016/j.ecoenv.2007.11.011.
 65. Midander K, Pan J, Wallinder IO, Leygraf C. Metal release from stainless steel particles *in vitro*-influence of particle size. *J Environ Monit*. 2007;9(1):74-81.
DOI: 10.1039/B613919A.
 66. Kastury F, Smith E, Karna RR, Scheckel KG, Juhasz AL. Methodological factors influencing inhalation bioaccessibility of metal(loid)s in PM_{2.5} using simulated lung fluid. *Environ Pollut*. 2018;241:930-937.
DOI: 10.1016/j.envpol.2018.05.094.
 67. Bolger MB, Macwan JS, Sarfraz M, Almukainzi M, Löbenberg R. The irrelevance of *in vitro* dissolution in setting product specifications for drugs like dextromethorphan that are subject to lysosomal trapping. *J Pharm Sci*. 2019;108(1):268-278.
DOI: 10.1016/j.xphs.2018.09.036.
 68. Kubo Y, Yamada M, Konakawa S, Akanuma SI, Hosoya KI. Uptake study in lysosome-enriched fraction: critical involvement of lysosomal trapping in quinacrine uptake but not fluorescence-labeled verapamil transport at blood-retinal Barrier. *Pharmaceutics*. 2020;12(8):747,1-13.
DOI: 10.3390/pharmaceutics12080747.
 69. Xu H, RenD. Lysosomal physiology. *Annu Rev Physiol*. 2015;77:57-80.
DOI: 10.1146/annurev-physiol-021014-071649.
 70. Souza JB, Castro LML, Siqueira MF, Savedra RML, Silva-Barcellos NM. Evaluation of the losartan solubility in the biowaiver context by shake-flask method and intrinsic dissolution. *Pharm Dev Technol*. 2019;24(3):283-292.
DOI: 10.1080/10837450.2018.1472610.
 71. Bezzina JP, Ogden MD, Moon EM, Soldenhoff KL. REE behavior and sorption on weak acid resins from buffered media. *J Ind Eng Chem*. 2018;59:440-455.
DOI: 10.1016/j.jiec.2017.11.005.
 72. Davidson AG, Mkoji LM. The simultaneous assay of triprolidine, pseudoephedrine and dextromethorphan in combined preparations by derivative-difference spectrophotometry. *J Pharm Biomed Anal*. 1988;6(5):449-460.
DOI: 10.1016/0731-7085(88)80012-8.
 73. Gao Z, Yu L, Clark S, Trehy M, Moore T, Westenberger B, *et al*. Dissolution testing for bioavailability of over-the-counter (OTC) drugs-a technical note. *AAPS Pharm Sci Tech*. 2015;16(5):1227-1233.
DOI: 10.1208/s12249-015-0297-x.
 74. Mostafa HF, Ibrahim MA, Sakr A. Development and optimization of dextromethorphan hydrobromide oral disintegrating tablets:effect of formulation and process variables. *Pharm Dev Technol*. 2013;18(2):454-463.
DOI: 10.3109/10837450.2012.710237.