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1. Title Page

Title:

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

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2. Running Title Page

Running Title:

In vitro Assays and *In silico* Predictions of Lysosomal Trapping

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- Number of words in the Introduction: 670
- Number of words in the Discussion: 1452

List of nonstandard abbreviations:

LTR - LysoTracker™ Red DND-99

ITC - International Transporter Consortium

DAPI - 4′,6-diamidino-2-phenylindole

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3. Abstract

Lysosomal sequestration may affect the pharmacokinetics, efficacy and safety of new basic lipophilic drug candidates potentially impacting their intracellular concentrations and tissue distribution. It may also be involved in drug-drug interactions, drug resistance or phospholipidosis. Currently, however, there are no assays to evaluate the lysosomotropic behaviour of compounds in a setting fully meeting the needs of drug discovery. We have therefore integrated a set of methods to reliably rank order, quantify and calculate the extent of lysosomal sequestration in rat hepatocytes. An indirect fluorescence-based assay monitors the displacement of the fluorescence probe LysoTracker™ Red by test compounds. Using a lysosomal specific evaluation algorithm allows to generate IC_{50} values at lower than previously reported concentrations. The concentration range directly agrees with the concentration dependency of the lysosomal drug content itself directly quantified by LC-MS/MS and thus permits a quantitative link between the indirect and the direct trapping assay. Furthermore, we have determined the full pH profile and corresponding volume fractions of the endo-/lysosomal system in plated rat hepatocytes, enabling a more accurate *in silico* prediction of the extent of lysosomal trapping based only on pK_a values as input allowing early predictions even prior to chemical synthesis. The concentration dependency, i.e. the saturability of the trapping can then be determined by the IC_{50} values generated *in vitro*. Thereby, a more quantitative assessment of the susceptibility of basic lipophilic compounds for lysosomal trapping is possible.

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4. Introduction

Many drugs show lipophilic properties in combination with moderate to high basicity. This property combination is often associated with pH-driven lysosomal sequestration which can have a strong impact on the distribution, efficacy and safety of such drugs as it may lead to high concentrations in lysosome-rich tissues such as lung, liver, kidney or spleen (MacIntyre and Cutler, 1988a; Ndolo et al., 2012). In order to undergo lysosomal sequestration a compound needs to (I) be membrane permeable, and (II) have a basic moiety that is ionisable at acidic pH. Indeed, the major driving force into the lysosome is the pH gradient between the neutral cytosol (pH 7.0 – 7.2) (Berezhkovskiy, 2011; Hallifax and Houston, 2012; Poulin et al., 2012) and the acidic lysosomal matrix (pH 4.5 - 5) (Feng and Forgac, 1992; Kornhuber et al., 2010; Mindell, 2012). While being able to enter the lysosome via passive diffusion, the acidic environment within the lysosome causes a protonation of basic groups. This ionisation converts the formerly neutral molecule into a charged molecule which significantly reduces its permeability across the lipid bilayer resulting in a “trapping” of the cationic form (). Besides the lysosome, any cellular compartment with a lower pH than the cytosol (e.g. early and late endosomes) is in principle eliciting this trapping mechanism. For simplification, all acidic cellular compartments are herein after referred to as lysosomes. Despite the typically minor volume of < 2 % of the cellular volume (with exceptions in certain cell types, e.g. macrophages of up to 8% (Ufuk et al., 2015)), the contribution to cellular drug uptake can be significant as trapping can theoretically lead up to 160,000x fold higher drug concentrations within the lysosome relative to the cytosol (MacIntyre and Cutler, 1988a; MacIntyre and Cutler, 1988b). Besides contributing to distribution into lysosome-rich tissues, lysosomal sequestration also affects the intracellular localisation of drugs. This may be beneficial if the therapeutic target is inside of the lysosome, but in most cases the drug is actually drawn away if the site of action is in the cytosol or cell nucleus, thereby influencing its efficacy. In addition, excessive lysosomal accumulation may affect drug safety by impairing the normal physiological function of lysosomes. Indeed, this is one of the main mechanisms of drug-induced phospholipidosis (Reasor et al., 2006), Furthermore, lysosomal

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trapping has also been implicated in drug-drug interactions. Due to the limited trapping capacity of lysosomes, two co-administered lysosomotropic drugs may compete for lysosomal accumulation which in turn could lead to elevated cytosolic concentrations and hence may elicit adverse drug effects, however, thus far, this was seen only in preclinical studies and at rather high doses (Daniel and Wojcikowski, 1999b; Funk and Krise, 2012). Finally, lysosomal trapping has also been associated with cancer drug resistance. Sunitinib for instance can cause augmented lysosome biogenesis in certain cancer cells. This in turn may lead to increased lysosomal sequestration which then may result in inadequate target exposure after continuous treatment with lysosomotropic anticancer drugs. This has been reported as mechanism for cancer resistance for several approved drugs and is also implicated to result in a cross-resistance of lysosomotropic agents (Gotink et al., 2015; Zhitomirsky and Assaraf, 2016). Looking at the potential impact of these implications, early information on lysosomal trapping in drug discovery will increase our understanding of drug distribution both at the level of body tissues and the subcellular disposition, thereby supporting the selection of new drug candidates with improved efficacy and safety profile in relation to their intracellular distribution.

In this paper we present a set of methods that were established to assess the lysosomotropic properties of basic compounds in drug discovery with particular suitability for lead optimisation and candidate selection and profiling. We have developed two complementary experimental methods an indirect fluorescence-based method that utilises the fluorescent probe LysoTracker™ Red DND-99 (LTR) to screen and rank order compounds for lysosomal trapping, and a direct quantification method using LC-MS/MS to quantify the intralysosomal versus cellular drug content. We have furthermore derived a quantitative link between both methods. Together with an in-depth characterisation of the endo-/lysosomal system in cultivated rat hepatocytes we were able to accurately predict the hepatocellular drug distribution to lysosomes.

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5. Materials & Methods

Chemicals and Reagents

Ammoniochloride, Chloroquine, diclofenac, fluoxetine, monensin sodium and imipramine were purchased from Sigma-Aldrich (St. Louis, MO) and propranolol was purchased from Research Biochemical International (Natick, MA). Olaparib was obtained from Active Biochem LTD (Hong Kong, China). LysoTracker™ Red DND-99, Lysosensor™ Yellow/Blue DND-160 and Hoechst 33342 were purchased from Life Technologies (Carlsbad, CA). Sodium chloride, potassium chloride, HEPES, EGTA, calcium chloride were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile and methanol were purchased from Honeywell Specialty Chemicals Seelze GmbH, Seelze, Germany.

Isolation of rat hepatocytes

Fresh hepatocytes were isolated from male Han:Wistar rats (Janvier Labs, Le Genest-Saint-Isle, France). Animals were anesthetized via i.p. injection of xylazine/ketamine (1:1). The liver was perfused in situ with 200 mL buffer 1 (4.2 % NaCl, 0.3 % KCl and 1.2 % HEPES and 0.19 % EGTA in bidistilled water., pH 7.2) and subsequently with 180 mL buffer 2 (3.9 % NaCl, 0.5 % KCl, 0.7 % $CaCl_2$, 2.4 % HEPES in bidestilled water, pH 7.2) containing 13500 U of collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ) via the portal vein. After perfusion the liver was excised, and the tissue was disassociated by scraping with forceps. The hepatocytes were transferred into hepatocyte medium (20 mL glutamine (Gibco, Grand Island, NY), 500 µL insulin (Sigma-Aldrich, St. Louis, MO), 20 µL glucagon (Sigma-Aldrich, St. Louis, MO) in 500 mL William's Medium E (Sigma-Aldrich, St. Louis, MO) and purified via Percoll™ (Sigma-Aldrich, St. Louis, MO) centrifugation according to Kreamer et al. (1986) with hepatocyte medium as the washing solution. In the final step the purified hepatocytes were diluted in hepatocyte medium containing 5% fetal calf serum (Sigma-Aldrich, St. Louis, MO).

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Indirect fluorescence-based assay for lysosomotropism and cytotoxicity assessment

Drug stock solutions for the assay were prepared in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) from which the loading solutions were diluted with William's medium E in a concentration range of 0.5 - 100 µM for each drug. LysoTracker™ Red DND-99 to stain lysosomes and Hoechst 33342 to assess cytotoxic drug effects were added to each loading solution in final concentrations of 50 nM and 4 µg/mL, respectively. Negative control contained no drug and positive control contained 25 mM NH_4Cl to elevate lysosomal pH, abolishing the pH gradient and thereby the accumulation of LTR. As the cell system, freshly isolated rat hepatocytes were seeded (200 000 cells/well) in clear 24-well Biocoat Collagen I plates (Corning Incorporated, Corning, NY) and allowed to adhere for 24 h at 37°C, 5% CO_2. Each well was washed twice with prewarmed PBS (Gibco, Grand Island, NY) and cells were subsequently incubated with 300 µL loading solution for 40 min² at 37°C, 5% CO_2. The loading solution was removed and replaced with 500 µL of fresh William's Medium E. Fluorescence was then immediately captured with an Axiovert 200 (Zeiss, Jena, Germany) equipped with an AxioCam MRm. Image acquisition was performed at six random locations within each well in the Rhodamine and the DAPI channel. The used microscope settings are specified in Table 1. The lysosomotropic character of each drug was determined over the whole concentration range in duplicates. The assay was replicated twice in a reduced form for concentrations relevant for lysosomal trapping, resulting in n = 6 measurements.

Data analysis and evaluation

Fluorescence images were analysed with Axiovision 4.8.2. The cell viability was evaluated by counting cell nuclei in the DAPI channel, with a decreasing number of nuclei indicating cytotoxic drug effects. The assessment of lysosomal drug sequestration by LTR fluorescence reduction was analysed in the rhodamine channel. In the fluorescence images each pixel has a possible intensity from 0 to 4096. The brightness of lysosomes was manually identified and two thresholds were applied.[1] The entire lysosomal system was analysed by measuring any fluorescence intensity ≥ 500, discarding the background/cytosol and serving as a surrogate for the cell number in the images. For lysosomal peak intensities the second threshold of ≥

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1000 was used. visualizes LTR intensities with applied thresholds of a control cell and a schematic visualization of applies thresholds. Due to the variability of the number of cells per image the proportion ($p_{lyso.}$) of lysosomal peak intensities ($\sum I_{\geq 1000}$) in the lysosomal system ($\sum I_{\geq 500}$) was calculated for every image. To detect alterations in the lysosomal LTR fluorescence it was normalized to the $p_{lyso.}$ of the respective negative controls ($\bar{p}_{lyso.neg.}$):

$$p_{lyso./norm.} = \frac{\sum I_{\geq 1000}}{\sum I_{\geq 500}} * \bar{p}_{lyso.neg.}{}^{-1} \quad (1)$$

Results of all measurements were pooled and a logistic regression on $p_{lyso./norm.}$ against compound concentration was performed in Origin® 2018 or Sigmaplot® 13 to generate IC_{50} values.

Direct quantification of lysosomal drug content

To identify the relation between LTR displacement and lysosomal drug accumulation, propranolol and imipramine accumulation was measured (I) in control hepatocytes and (II) hepatocytes with inactive lysosomes as well as (III) in hepatocytes additionally treated with LTR to evaluate any interaction on drug accumulation. Each compound was tested three times in hepatocytes of three different rats over a concentration range of 1-100 µM (I) containing the test compound only , additionally containing (II) either 25 µM Monensin or (III) 50 nM LTR . Freshly isolated rat hepatocytes were cultured and plated as described above. The plates were washed twice with prewarmed PBS and cells were incubated with 300 µL loading solution (t_0) for 40 min at 37°C, 5% CO_2. After incubation the overlaying medium (t_{40}) was sampled. The cells were washed twice with ice cold PBS. Methanol containing internal standard (0.4 µM) was added to each well and the sealed plate was shaken for 3 min, at 750 rpm to lyse the cells. The lysate was transferred to a 96-well analytical plate, in which t_0 and t_{40} were diluted in methanol containing internal standard. The analytical plate was shaken for 15 min at 750 rpm and subsequently centrifuged for 15 min at 3700 rpm, 4 °C for protein precipitation. Samples were then analysed by LC-MS/MS.

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To determine the number of cells in the assay an additional 24-well plate was seeded and cultured as described above. The plate was washed twice with prewarmed PBS and hepatocyte medium containing 4 µg/mL Hoechst 33342 was added to the wells. The fluorescence was measured after 10 min incubation with an ImageXpress Micro (Molecular Devices, San Jose, CA) using a 10x objective and a DAPI filter cube. In each well 16 images were acquired. Cell nuclei were counted using ImageJ (Version 1.43u).

Analytical method

Samples were analysed with liquid chromatography-tandem mass spectrometry using an Agilent 1290 Infinity System comprising a G4220A binary pump, a G1316C column compartment and G7167B multisampler linked to an AB Sciex API4000 mass spectrometer with electrospray ionization. All compounds were detected in positive MRM mode with N-(4-chlorophenyl)-2-[(4-pyridinylmethyl)amino]-benzamide (Sigma-Aldrich, St. Louis, MO) as an internal standard (IS). An Ascentis® Express C18 column (30 x 2.1 mm, 2.7 µm particle size; Sigma-Aldrich, St. Louis, MO) was used with mobile phases (A) water with 0.1 % acetic acid and (B) acetonitrile with 0.1 % acetic acid and a linear rising gradient from 10 % to 95 % B over 0.6 min with a flow of 1 ml/min, followed by a washback with a flow of 1.2 mL/min to 10% B over 0.2 min and 0.4 min of equilibration with a flow of 1 mL/min. MRM transitions for propranolol, imipramine were 260.3/116.2 and 281.3/86.1 with elution times of approximately 0.37 min and 0.42 min, respectively. The internal standard eluted after 0.48 min with a MRM transition of 337.8/210.9.

Data evaluation

LC-MS quantification results were corrected for recovery and the counted number of cells in the assay under the assumption of a similar lysosomotropic character of metabolites and a linear relation between cell number and accumulated drug. The accumulated amount of drug in lysosomes ($A_{lyso.}$) was calculated from the difference in accumulation in control cells ($A_{control}$) vs. lysosome inactivated cells ($A_{lyso.inact.}$) according to:

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$$A_{lyso.} = \left(\frac{A_{control}}{Rec_{control}} - \frac{A_{lyso.inact.}}{Rec_{lyso.inact.}}\right) * \frac{f_{cell\ no.}}{N_{nuc.}} \quad (2)$$

where $Rec_{control}$ and $Rec_{lyso.inact.}$ are the drug recovery at the end of each experiment respectively. $N_{nuc.}$ is the average number of counted cell nuclei per image on the simultaneously handled reference plate. The amount in lysosomes was scaled to 200,000 cells per well using $f_{cell\ no.}$= 805.14, that relies on the size of the acquired images vs. the well size and an average of 1.27 nuclei per hepatocyte (determined separately, data not shown).

Additionally, the percentage of accumulated drug in lysosomes ($p_{acc.}$) of total drug applied in the medium (A_{total}) was calculated, as well as the percentage of total cellular content corrected for recovery. The statistical significance of reduced accumulation in lysosome inactivated cells compared to control cells was tested with a one-sided t-test.

Characterization of rat hepatocyte lysosomes

Freshly isolated rat hepatocytes were plated (36 000 cells/well) in black 96-well Biocoat Collagen I plates (Corning Incorporated, Corning, NY) and allowed to adhere for 24h at 37°C, 5% CO_2. Cells were washed twice with prewarmed PBS. Williams Medium E was added to each well containing 3 µM Lysosensor™ Yellow/Blue DND-160. After one minute of incubation a ratiometric imaging of fluorescence using a Fura-2 filter set on an ImageXpress Micro with 1000 ms of exposure was performed. The calibration was done according to (Wang et al., 2016) from pH 4.5 to pH 6. Images were analysed in ImageJ (Version 1.43u). In short, background was subtracted, regions of interest were identified by finding circular spots with the size of lysosomes, the fluorescence intensity was measured in both channels and the ratio of corresponding regions of interests was calculated. The ratio was transformed into a pH value using the calibration curve. The experiment was conducted with hepatocytes from 6 different rats with a total of >350,000 lysosomes being analysed.

Prediction of lysosomal drug sequestration

The intracellular distribution of drugs to lysosomes was calculated based on the Henderson-Hasselbalch equation, experimentally determined hepatocyte specific parameters, and *in*

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silico predicted compound properties. The extent of lysosomal sequestration L was calculated by integrating the partitioning of a drug over the whole range of possible intracellular pH values, see eq. 4 and a simplified schematic in . To weight the contribution of a pH value to the overall accumulation, we adjusted the volume of the lysosomal compartment at a given pH $V(pH)$ based on the experimentally determined pH distribution in hepatocytes, the average lysosomal volume V_{lyso} and the total number of lysosomes per cell N_{lyso} by:

$$V(pH) = \frac{p(pH)}{\int_{pH_{min}}^{pH_{cyto.}} p(x)\, dx} \cdot N_{lyso} \cdot V_{lyso} \tag{3}$$

where $p(\cdot)$ is the probability density function represented by a normal distribution with the mean $\overline{pH}$ and variance σ^2. A constant pH of 7.2 in the non-acidic compartment $pH_{cyto.}$ is assumed (Hallifax and Houston, 2012) and a minimum possible pH_{min} of the lysosome of 4. Hepatocyte specific parameters used for calculations are listed in Table 2. The Henderson-Hasselbalch based concentration ratio K_L of mono-/dibasic compounds between lysosomes and the surrounding medium is stated by MacIntyre and Cutler (1988a). We incorporate K_L to our equation to calculate a lysosomal sequestration extent L for the entire lysosomal system in relation to the cytosolic drug content by:

$$L = \int_{pH_{min}}^{pH_{cyto.}} \frac{V(pH) \cdot K_L\left(pH, pK_{a,1}, pK_{a,2}\right)}{V_B}\, dpH \tag{4}$$

with V_B as the volume of the non-acidic compartment in a hepatocyte. The acid dissociation constants of basic drug moieties used to derive K_L were predicted *in silico* using ADMET Predictor™ (Simulations Plus Inc.). A ready-to-use Excel file to predict the lysosomal trapping by the aforementioned equations and only requiring the pK_a values of compounds can be found in the supplement.

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6. Results

Indirect fluorescence-based assay for lysosomotropism

We chose a fluorescence-based method which uses the lysosomotropic dye LysoTracker™ Red DND-99 to stain lysosomes, and Hoechst 33342 to stain cell nuclei in order to avoid misinterpretations due to cytotoxicity in plated rat hepatocytes as the cell system. The final LTR concentration of 50 nM was chosen as it gives a strong signal without overloading the cells (pilot experiments, data not shown). The plated rat hepatocytes showed well defined fluorescent spots which was abolished by co-incubation with 25 mM NH_4Cl indicating a functioning proton gradient in the lysosomes, thus an intact lysosomal system (). To establish the assay six well known reference compounds were chosen, including four lysosomotropic compounds chloroquine, imipramine, fluoxetine and propranolol and two non-lysosomotropic compounds diclofenac and olaparib. The compounds were selected based on their known lysosomotropic behaviour (Lemieux et al., 2004; Nadanaciva et al., 2011; Kazmi et al., 2013) and their physico-chemical properties with basic pK_a values spanning from low to high. Additionally, with a $logD_{pH7.5}$ between 1.6 – 3.2, all reference compounds can be expected to passively cross the lysosomal membrane (Table 3). For developing the evaluation method of the assay, the compounds were measured in a concentration range of 0.5 µM – 100 µM. The fluorescence threshold of ≥ 500 (intensity scale 0 – 4096) was identified to omit the background and cytosolic fluorescence allowing the lysosome specific analysis of LTR, which is different to previously published assays (Kazmi et al., 2013). Due to random attachment of hepatocytes on the plates, the number of cells and therefore the size of the lysosomal system per image differs. To compensate for this, we calculated the cell number independent parameter $p_{lyso./norm.}$ as the lysosomal fluorescence (). The concentration dependency of this lysosomal fluorescence was used as the basis for IC_{50} calculations as a measure of lysosomal trapping. None of the compounds had cytotoxic effects over the concentration range tested. Propranolol, imipramine, chloroquine and fluoxetine all caused a significant reduction in LTR fluorescence in lysosomes with IC_{50} values of 15 ± 2.1 µM, 4.8 ± 1.2 µM, 3.9

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± 0.5 µM and 8.0 ± 2.4 µM, respectively. The negative reference compounds diclofenac and olaparib both did not alter lysosomal fluorescence significantly. The strength of the lysosome specific fluorescence assessment results in very sensitive, i.e. lower IC_{50} and a strong resolution.

Direct quantification of lysosomal drug content

Additional investigations were performed with the aim to establish a quantitative link between fluorescence based IC_{50} values and the extent of lysosomal drug sequestration. We have quantified the intracellular content of selected compounds via LC-MS/MS in control hepatocytes, hepatocytes with inactive lysosomes (+ 25 µM monensin), and after co-incubation with LTR. The results indicate that there is no interference by the presence of 50 nM LTR on the lysosomal drug content as shown for propranolol and imipramine (A/D). In cells with inactive lysosomes the amount of drug was markedly decreased, showing an effective lysosome inactivation by 25 µM monensin. The reduction is significant ($P < 0.05$) for imipramine and highly significant ($P < 0.01$) for propranolol at every concentration tested (A/D). Assuming complete lysosomal inactivation, the difference between the cellular drug accumulation of control cells and cells with inactive lysosomes represents the extent of sequestration by lysosomes, i.e. the lysosomal drug content. The lysosomal content reaches a plateau at 0.77 and 0.57 nmol per 200,000 cells for propranolol and imipramine, respectively, indicating saturation. Notably, this saturation occurs at concentrations beyond which the cellular drug content continues to rise. While the plateau is similar, the saturation concentrations differ between the compounds with imipramine being more sensitive (B/E). Furthermore, the distribution to lysosomes can be determined with our assay by expressing the lysosomal drug content relative to the drug in control cells. At 1 µM the endo-/lysosomal system holds about 56% of the cellular propranolol and about 50 % of the imipramine, meaning most of the cellular propranolol and imipramine is located in the endo-/lysosomal system. As concentrations increase, lysosomal but not the cellular content becomes saturated causing the relative drug content to decline to 34 % and 19 % at 100 µM, respectively (C/F).

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Linking the indirect to the direct assay

As depicted in A/C the reduction of lysosomal fluorescence is mirrored by a rising lysosomal drug content for imipramine and propranolol, respectively. While the lysosomal content rises to a maximum, the lysosomal fluorescence approaches a minimum fluorescence level. When the lysosomal drug content is expressed as the proportion of applied drug, it completely overlays with the lysosomal fluorescence reduction (B/D). Consequently, the IC_{50} values of the both assays are similar, with 4.8 ± 1.2 µM vs. 4.9 ± 0.9 µM for imipramine and 15 ± 2.1 µM vs 9.5 ± 0.9 µM for propranolol. In addition, the very similar Hill slopes indicate a direct relation of lysosomal drug content and displaced LTR fluorescence over the whole concentration range. Therefore, an estimation of lysosomal drug accumulation and its concentration dependence can be derived from the fluorescence data.

Lysosome characterisation in cultured rat hepatocyte

The endo-/lysosomal volume and the pH are the most important cellular parameters for lysosomal sequestration. Therefore, we determined both parameters experimentally for the hepatocytes used in our assays. The size of hepatocytes was determined with brightfield microscopy (465 analysed) and for the endo-/lysosomal volume we carried out LTR staining followed by counting of fluorescence spots (5143 counted), determining their area (3200 analysed) to calculate a spherical volume (data not shown). The proportion of the endo-/lysosomal system in plated rat hepatocytes is 1.49 ± 0.08 % of the cellular volume. The lysosomal pH was determined with the pH sensitive dye Lysosensor Yellow/Blue DND-160 which changes its fluorescence spectrum depending on the surrounding pH. A calibration curve was constructed from pH 4.5 – 6.0 and extrapolated for pH values outside of these margins. Hepatocytes from six individual rats were subject to investigation with >350,000 endo-/lysosomes analysed in total for sufficient statistical power. The endo-/lysosomal pH values found cover a wide range from pH 4 up to 7.2 with different frequencies of occurrence that follow a Gaussian distribution with a mean at pH 5.53 (). See supplement for detailed distribution.

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In silico prediction of lysosomal sequestration

In addition to the experimental data, the lysosomal accumulation was calculated according to eq. 4 for propranolol and imipramine. Both compounds are strong bases, with a pK_a of 9.4 for propranolol and two basic pK_a values of 9 and 2.2 for imipramine. The second basic pK_a of imipramine however does not have a significant influence on the pH driven accumulation, thus it effectively behaves monobasic. The calculated amount in the lysosome (L) for propranolol and imipramine are 1.15 and 1.14 times the amount in the cytosol respectively, i.e. the endo-/lysosomal system holds about 53 % of the intracellular drug content (see supplement). This prediction matches perfectly with the direct quantification assay at low concentrations where the lysosomes are not saturated (see horizontal line in C/F).

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7. Discussion

Understanding intracellular distribution and intracellular concentrations of drugs is a topic of growing interest, as highlighted in by the ITC White Paper of Guo et al. (2018). The implications of intracellular pharmacokinetics in safety and efficacy makes early information crucial for the selection of new drug candidates. The sub-cellular distribution to lysosomes plays a major role for basic lipophilic compounds. While there are some assay formats to assess lysosomotropism, none of them completely fulfils the requirements needed in drug discovery. For instance, the assay by Kazmi et al. (2013) requires compounds concentrations up to 500 µM, which most often cannot be achieved in the lead optimisation phase. Nadanaciva et al. (2011) determines lysosomal trapping in cell line that seems overly sensitive to cytotoxicity, thereby biasing the results. The fluorescence assay presented herein overcomes these shortcomings and can also be quantitatively linked to the lysosomal drug content. As cell system we have used freshly isolated rat hepatocytes which are rich in lysosomes and are routinely available in DMPK departments (Reichel and Lienau, 2016). The assay principle, however is also applicable to other palatable cells.

Indirect fluorescence-based assay for lysosomotropism

In analogy to previously published assays (Lemieux et al., 2004; 2011; Kazmi et al., 2013) we have used LysoTracker™ Red as a fluorescence marker, however modifying the analysis by specifically quantifying the fluorescence associated with lysosomes fluorescence. This is the major difference to Kazmi et al. (2013) who measured the fluorescence irrespective of its cellular location. Using a set of reference compounds we have shown that this modification reduces the IC_{50} values and therefore the concentration range to be tested, e.g. for chloroquine from 220 µM to 4 µM, propranolol from 80 µM to 15 µM or imipramine from 260 to 5 µM compared to Kazmi et al. (2013). The more sensitive method now allows to measure also low soluble discovery compounds. When measured on an automatic microscope as used by Nadanaciva et al. (2011) this method is amenable for higher throughput.

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In order to avoid misinterpretations, we have simultaneously monitored cell viability in the assay. None of the compounds showed cytotoxic effects at any concentration tested, attesting the suitability of rat hepatocytes.

Direct quantification of lysosomal drug content

In order to get a better understanding of the meaning and implications of the IC_{50} values from fluorescence assay we have directly determined the drug content in the lysosomes. To quantify the lysosomal drug content, we have directly measured the drugs via LC-MS/MS in hepatocytes with intact and inactive lysosomes which allows to quantify both the lysosomal and the total drug content in the cells. To abolish lysosomal trapping, we have used the ionophore monensin which collapses the pH (proton) gradient between the lysosome and cytosol. It therefore specifically disables the pH driven part of drug accumulation leaving other distribution processes such as lipid and protein binding intact.

At low compound concentrations lysosomes contain the highest fraction of total intracellular drug with about 50 % and 56 % for imipramine and propranolol, respectively. This is comparable to data by Kazmi et al. (2013) despite using different cells and ammonium chloride to inhibit lysosomotropism. Whereas the extent of lysosomal trapping of both compounds is very similar their IC_{50} values differ by a factor of three (see above). This suggests they carry information that is more related to the concentration dependence of the lysosomal trapping rather than the extent.

Linking the indirect to the direct assay

With the possibility of drug-drug interactions involving lysosomes (Daniel and Wojcikowski, 1999b; Daniel and Wojcikowski, 1999a; Daniel, 2003), we first ensured that LTR at the concentration used is not affecting the sequestration of test drugs in the fluorescence assay. The results shown in A/D confirm that the presence of LTR does not affect lysosomal accumulation of test drugs, a prerequisite for a quantitative link between the assays. A/C clearly suggests a relationship between the two assays, i.e. the declining lysosomal fluorescence with increasing drug concentrations is directly mirrored by the rising lysosomal

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drug content with both readouts approaching a plateau at high concentrations. Expressing the lysosomal drug content in relation to the amount of drug applied in the assay, a direct relationship with the fluorescence assay is obtained (B/D). Remarkably, there is a complete overlay of the data points from the two assays for both drugs, including Hill slopes and IC_{50} values. This implies a quantitative displacement of LTR by both drugs and therefore the reduction of lysosomal fluorescence can be used to describe their concentration dependent lysosomal accumulation. This thus allows to reduce the number of measurements over the whole concentration range in the direct assay down to just the highest compound concentration as anchor point. This simplification is possible only through the lysosome specific evaluation of the fluorescence in the indirect assay, so that both assays can now operate in the same concentration range.

***In silico* prediction of the extent of lysosomal trapping**

For an accurate *in silico* prediction of lysosomal trapping comprehensive information on the acidity and the fractional volume of the endo/lysosomal system is needed due to tremendous variations between cell types ranging from 0.23 % up to 7.8 % of the cellular volume (de Duve et al., 1974; Blouin, 1977; MacIntyre and Cutler, 1988a; Ufuk et al., 2015) with pH values reported in the range of 4.65 – 5.18 (Regec et al., 1989; Tietz et al., 1990; Kharbanda et al., 1997). We have for the first time determined a full profile of the pH values and corresponding fractional volumes of the endo-/lysosomal system in rat hepatocytes (). Simply using a mean lysosomal pH would underpredict the extent of lysosomal sequestration because the lysosome/cytosol concentration ratio (K_L) rises exponentially with decreasing lysosomal pH (). However, due to insufficient knowledge on the pH distribution of lysosomes this oversimplification was frequent practice until now. The improvement in the predictivity by using the full pH profile is shown with the following examples. For two monobasic drugs with a pK_a of 7 and 9 the predicted extent is 31 % instead of 23 %, and 53 % instead of 43 % calculated by eq. 4 (see supplement) versus the mean lysosomal pH (Table 2), respectively. The superiority of the prediction is confirmed by the experimental data of imipramine and propranolol (both $pk_a \approx 9$) resulting in values of 50 % and 56 %, respectively.

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However, it needs to be kept in mind that these predictions are valid only for low concentrations where lysosomal trapping is not saturated, because this may change the lysosomal pH profile or volume. The concentrations at which saturation will occur have to be determined experimentally, e.g. via the IC_{50} values of the indirect assay. The *in silico* predictions for a given drug are only valid up to concentrations of its lysosomal IC_{50}. If information on the extent of trapping beyond these concentrations is needed this can be obtained by the direct assay (C/F).

Application in Drug Discovery

Lysosomal drug accumulation, although likely to occur for most basic lipophilic drugs, is not regarded as a property requiring routine screening in drug discovery. It is rather a subject of special investigation that is indicated by certain trigger points. For example I) to elucidate unusual compound accumulation in (lysosomal rich) tissues observed in preclinical animal species requiring a more mechanistic understanding of the mechanism and/or a handle for optimisation, II) to direct the distribution of compounds whose on-targets or off-targets are within lysosomes in or out of the organelle, III) to specifically modify the lysosomotropism of compounds in differentiation to a front-runner project or competitor drugs, or IV) to reduce the propensity of compounds to induce lysosomal sequestration-related phospholipidosis, V) to examine whether a saturable uptake of a basic compound in hepatocytes is due to saturation of an uptake transporter or just a result of lysosomal trapping that may also get saturated, to name but a few. While some of these questions require mechanistic studies to identify whether or not lysosomal trapping is involved, other questions may need compound screening, rank-ordering and optimisation at a higher throughput. The set of assays proposed herein is principally able to meet these requirements allowing a tailored application in the project work.

In conclusion, we have established an indirect fluorescence and a direct quantification assay set that can be quantitatively linked to assess both the susceptibility, the concentration dependency and the maximal extent of lysosomal trapping in a drug discovery context

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suitable for either screening or more mechanistic examinations. In addition, for the first time we have determined the full endo-/lysosomal pH profile of cultured rat hepatocytes now allowing a more accurate *in silico* prediction of the extent of lysosomal trapping of basic lipophilic compounds.

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8. Acknowledgments

We would like to thank Stephan Menz for his assistance in mathematical questions and Daniel Trost and Martin Klein for their support in hepatocyte isolation and LC-MS/MS quantification.

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9. Authorship Contributions

Participated in research design: MVS, PL, AR, GF

Conducted experiments: MVS

Performed data analysis: MVS, PL

Discussed results: MVS, PL, AR, GF

Drafted manuscript: MVS, AR, PL

Commented on and approved manuscript: MVS, PL, AR, GF

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11. Footnotes

* This work was financed by Bayer AG, Germany.

[1]Thresholds need to be adjusted individually for different setups.

[2] to accommodate both rapidly and slowly permeating compounds. Incubation time may have to be adjusted for other cell types.

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12. Legends for Figures

Figure 1: Mechanism of pH driven partitioning. Lipophilic basic compounds (B) can freely diffuse across the cell membrane in their unionized form to enter the cell and the lysosome. The acidic environment within the lysosome (pH 4-5) causes a drastic shift of the Henderson-Hasselbalch equilibrium towards the ionized form (BH^+), which has a markedly reduces permeability across lipid bilayers. Unable to diffuse back to the cytosol, the drug gets "trapped" in the lysosome.

Figure 2: 3D fluorescence intensity plot of rat hepatocyte in rhodamine channel stained with LTR. Image analysis focused on the reduction of lysosomal peak intensities (dark red) by compounds. Due to a variable quantity of cells per fluorescence image, the entire lysosomal system (dark + light red) served as a surrogate for the number of cells. Background and cytosolic fluorescence (blue) were discarded in all analysis.

Figure 3: Simplified schematic of the model to calculate the pH driven distribution within the cell. It utilizes the in vitro conditions of the pH distribution of plated rat hepatocytes to adjust the contribution in accumulation of a given pH depending on the abundance of lysosomes at this pH. The entire pH driven accumulation of the lysosomal system was calculated from pH 4 to pH 7.2.

Figure 4: Fluorescence microscopy (20x Objective) of plated rat hepatocytes treated with 50 nM LTR. Hepatocytes treated only with LTR showed selective accumulation with well-defined bright spots indicating functional lysosomes (left). Co-incubation with 25 mM NH_4Cl (lysosomal inhibitor) attenuates lysosomal fluorescence markedly (right).

Figure 5: Effect on Lysotracker Red® DND-99 fluorescence of lysosomes (closed circles) and cell viability (open circles) of reference compounds chloroquine, imipramine, diclofenac, propranolol, fluoxetine and olaparib in plated rat hepatocytes after 40 min co-incubation. Data expressed as mean ± SEM relative to control cells. Data was obtained from hepatocytes of three different rats, each measured on two separate occasions with 6 fluorescence measurements (n = 36). Corresponding IC50 values are summarized in Table 3**.**

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Figure 6: Concentration dependent cellular accumulation of propranolol (A, B, C) and imipramine (D, E, F) in control cells, with co-incubation of 50 nM LTR or 25 µM monensin (A, D). LTR did not reduce the amount of compound taken up by the cells, while the lysosomal inhibitor monensin restricted cellular accumulation (*P < 0.05, **P < 0.01, ***P < 0.001). The amount of drug in lysosomes (closed squares) was calculated by the difference in accumulation in control cells and lysosome inactivated cells (+ monensin). For both drugs a maximum was reached indicating a limited capacity of lysosomes, while cellular concentrations linearly rise after saturation (B, E). Intracellular distribution of propranolol and imipramine to lysosomes (closed triangles) is maximal at low concentrations. Due to lysosomal saturation the proportion of drug in lysosomes is decreased at higher concentrations. The horizontal line marks the predicted (eq. 4) distribution to lysosomes. (C, F). Experiments (three replicates) were performed with freshly prepared hepatocytes originating from three individual rats. Data is expressed as mean ± SEM of n= 3 rats.

Figure 7: Comparison of the reduction of lysosomal fluorescence (closed circles) with the absolute lysosomal drug content (closed squares) of propranolol (A) and imipramine (C). With increasing amounts of drug in lysosomes the fluorescence is equivalently reduced to a minimum when reaching lysosomal saturation. The overlay of the lysosomal fluorescence and the lysosomal drug content expressed as the proportion of applied drug (open triangles), shows the coherent relationship of both parameters with comparable IC_{50} values and hillslopes for propranolol (B) and imipramine (D), respectively.

Figure 8: pH profile of the endo-/lysosomal system in plated rat hepatocytes. Lysosomes were stained with 3 µM Lysosensor Yellow/Blue DND-160 and fluorescence was immediately measured with a Fura-2 Filter set. Single lysosomes were then identified, and pH was determined against a calibration curve. Data represents the mean ± SD abundance of lysosomes from hepatocytes of n = 6 different rats with a total of > 350,000 analysed lysosomes. The calculated concentration ratio KL (MacIntyre and Cutler, 1988a) for a basic drug (pKa=9) rises exponentially with decreasing pH, showing the potential contribution to the sequestration of lysosomes below pH = 5 , despite their relatively low abundance.

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13. Tables

Table 1: Microscope settings of fluorescence imaging

Microscope	LTR	Hoechst 33342
Filter	Rhodamine	DAPI
Objective	LD Plan-Neofluar 20x	A-Plan 10x
Exposure time	1000 ms	100 ms

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Table 2: Hepatocyte characteristic parameter used for predicting lysosomal accumulation

Parameter	Value
V_B	5.54 ± 0.07 pL
V_{lyso}	0.539 ± 0.001 fL
N_{lyso}	156 ± 4.6
$f_{V,\mathrm{lyso.}}$	1.49 ± 0.08 %
$\overline{\mathrm{pH}}_{lyso.}$	5.53
σ^2	0.1849
pH_{min}	4
$pH_{cyto.}$	7.2

V_B: Volume of non-acidic compartment; V_{lyso}: Volume of single lysosome; N_{lyso}: Number of lysosomes per cell; $f_{V,\mathrm{lyso.}}$: lysosomal fraction of cellular volume; $\overline{\mathrm{pH}}_{\mathrm{lyso.}}$: mean lysosomal pH; σ^2: variance of mean lysosomal pH; pH_{min}: minimum pH in lysosomes; pH_{cyto}: cytosolic pH; pL: picoliter; fL: femtoliter

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Table 3: Lysosomal trapping of reference compounds in rat hepatocytes. Data expressed as mean ± SE of duplicate measurements from hepatocytes of three individual rats. Physicochemical data ($logD_{7.5}$, basic pKa values) were calculated by ADMET Predictor™.

Compound	$logD_{pH7.5}$	Highest basic pk_a	2nd highest basic pk_a	Lysosomal trapping IC_{50} [µM]	Cytotoxicity IC_{50} [µM]
Chloroquine	2.4	9.9	7.3	3.9 ± 0.5	> 100
Diclofenac	2.4	-	-	> 100	> 100
Fluoxetine	3.2	9.8	-	8.0 ± 2.4	> 100
Imipramine	2.1	9	2.2	4.8 ± 1.2	> 100
Olaparib	1.6	0.2	-	> 100	> 100
Propranolol	1.8	9.4	-	15 ± 2.1	> 100

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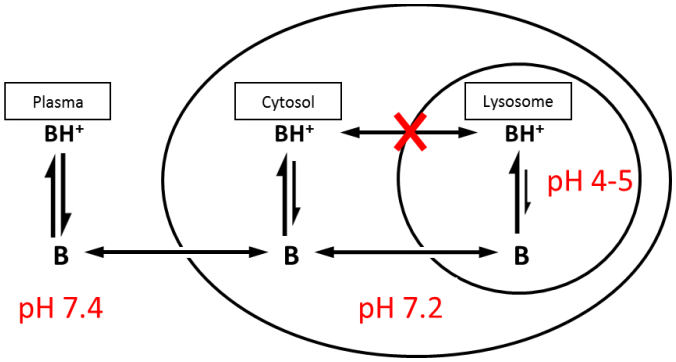
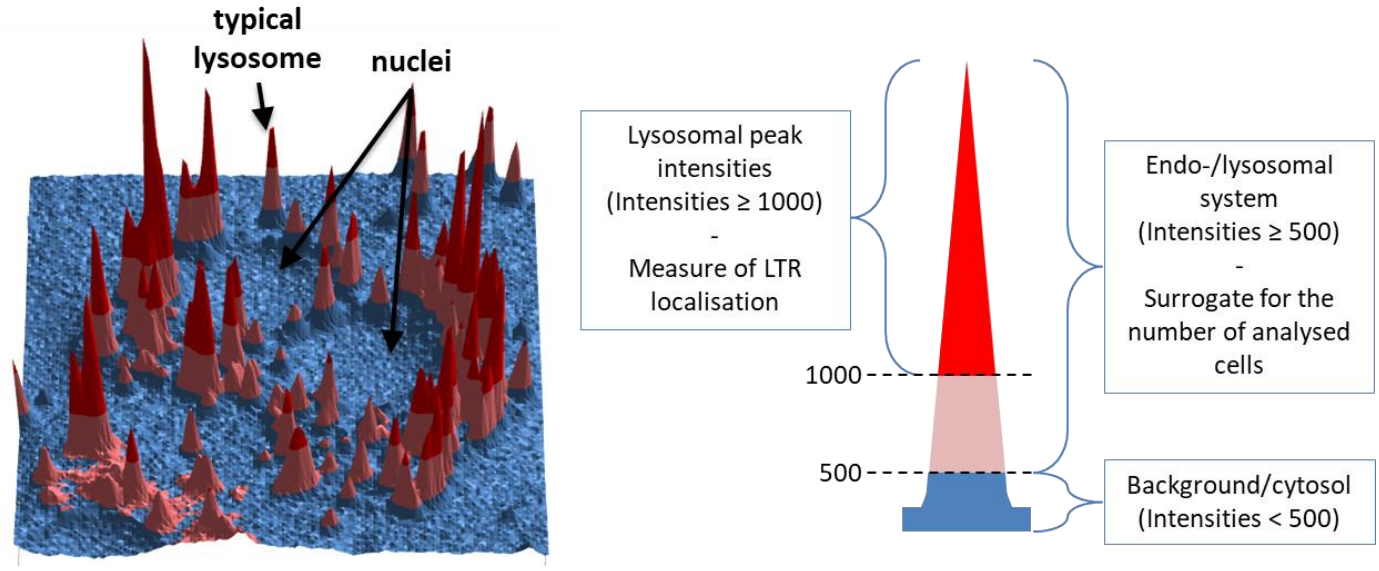
14. Figures





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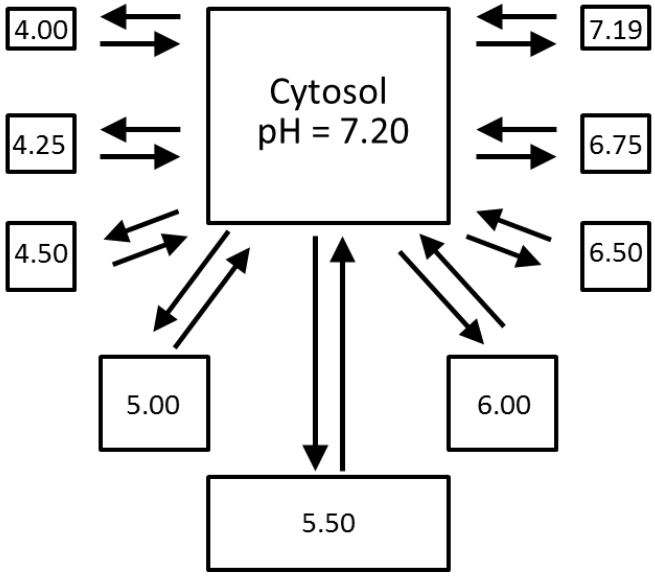

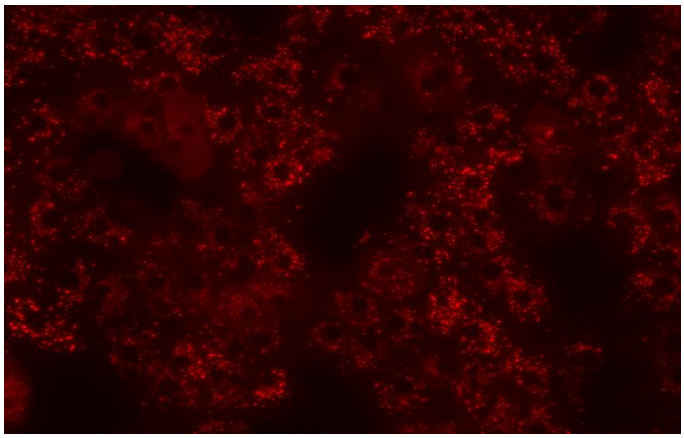

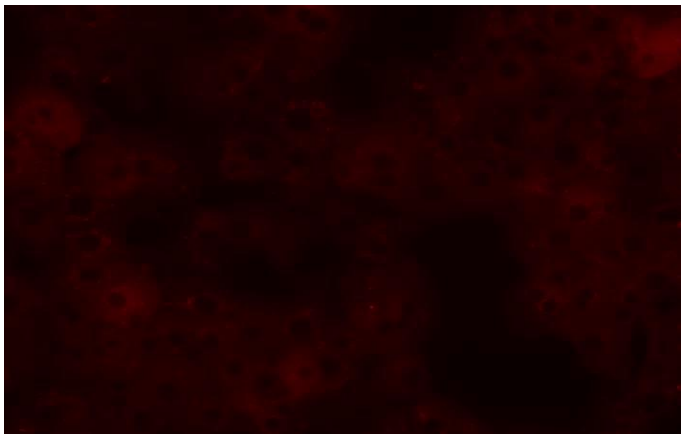

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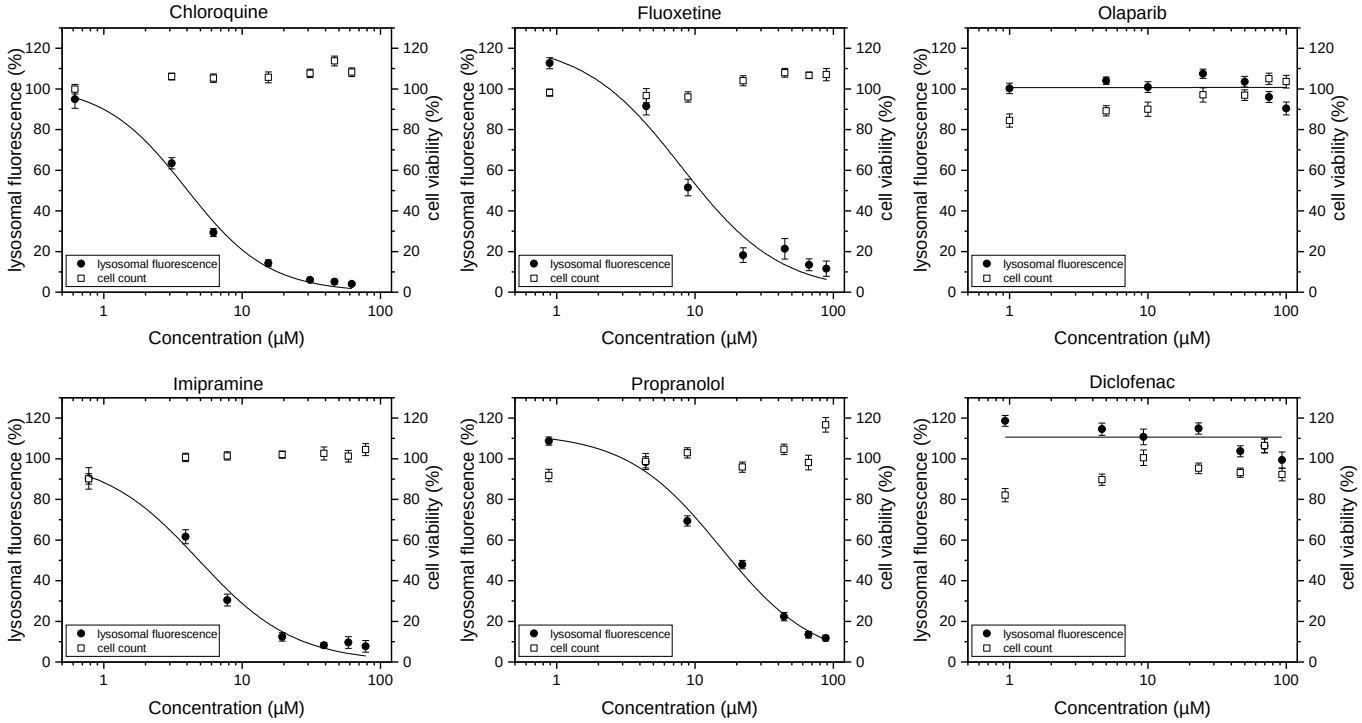

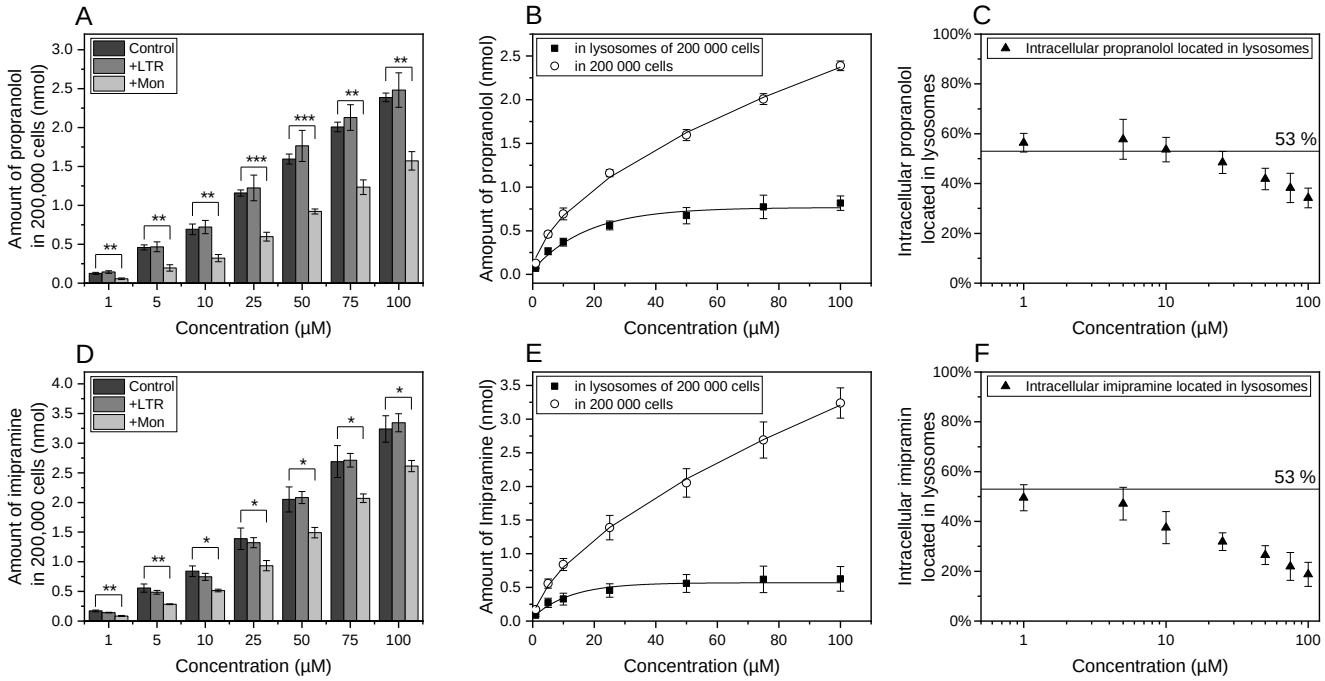

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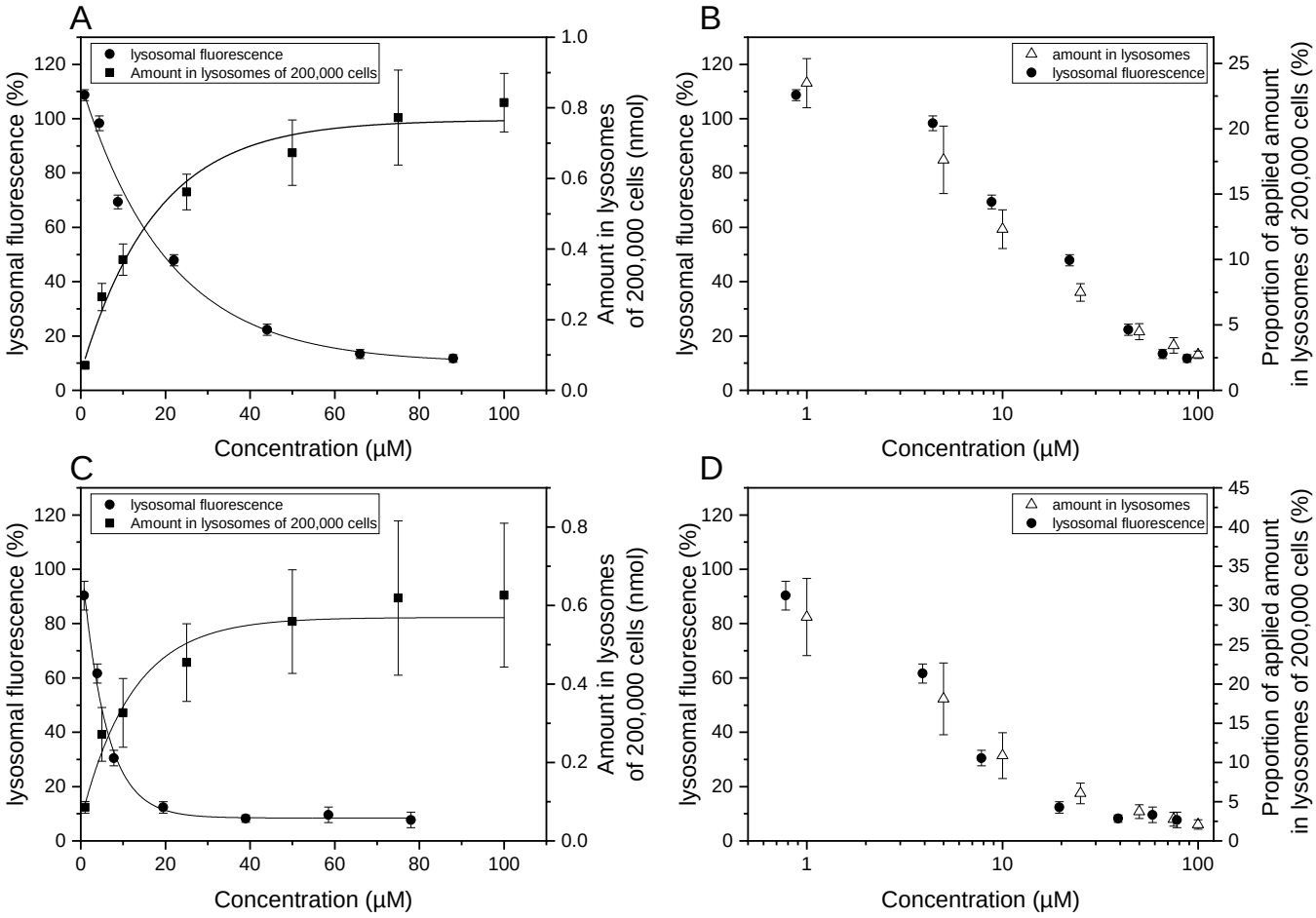

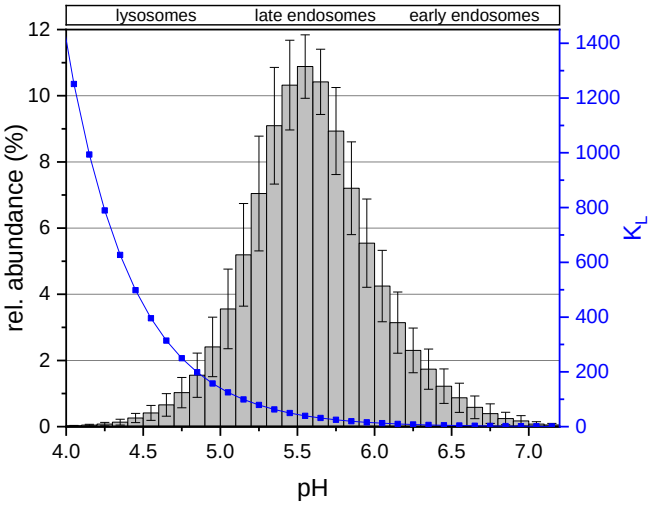

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