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Intracellular unbound drug concentrations

Methodology and application for understanding cellular
drug exposure

ANDRÉ MATEUS

Doutoramento em Farmácia
(Especialidade: Biofarmácia e Farmacocinética)

Tese elaborada em regime de co-tutela entre a Universidade de Uppsala e a Universidade de Lisboa. Tese orientada pelo Professor Doutor Per Artursson e, coorientada pelos Doutor Pär Matsson e Professor Doutor José Morais, especialmente elaborada para a obtenção do grau de doutor.



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Abstract

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Most known drug targets and metabolizing enzymes are located inside cells. Interactions with these proteins are determined by intracellular unbound drug concentrations. Assessing intracellular drug exposure is technically challenging, but essential for predicting pharmacokinetic, pharmacological, and toxicological profiles of new drugs.

This thesis aims at establishing and applying a straightforward methodology to measure intracellular unbound drug concentrations. This was achieved by separately measuring cellular drug binding ($f_{u,cell}$), and total intracellular drug accumulation (K_p). This allowed the calculation of intracellular drug bioavailability (F_{ic}), which represents the fraction of the concentration added to the cells that is unbound in the cell interior.

The methodology was initially developed in HEK293 cells, where the F_{ic} of 189 drug-like compounds was measured. Binding to HEK293 cells was governed by compound lipophilicity and was correlated with binding to more complex systems, such as hepatocytes and brain. Due to negligible expression of drug transporters, F_{ic} in this cell line was consistent with pH-dependent subcellular sequestration of lipophilic cations in low pH compartments.

The methodology was then applied to study the effects of drug transporters on F_{ic} . The uptake transporter OATP1B1 increased the F_{ic} of its substrates in a concentration-dependent manner. In contrast, the F_{ic} of P-gp substrates was decreased when P-gp was present. In human hepatocytes, the methodology allowed the determination of F_{ic} without prior knowledge of transporter mechanisms or metabolic activity.

Finally, the methodology was applied to measure the impact of F_{ic} on target binding and cellular drug response. Intracellular concentrations of active metabolites of pro-drugs targeting the intracellular target thymidylate synthase were in agreement with the level of binding to this target. Further, high F_{ic} was generally required for kinase and protease inhibitors to be active in cellular assays.

In conclusion, the methodology can be used to predict if new drug candidates reach their intracellular targets in sufficient amounts. Furthermore, the methodology can improve *in vitro* predictions of drug clearance and drug-drug interactions, by measuring the drug available for intracellular enzymes. Finally, this work can be expanded to other xenobiotics, e.g., to predict their intracellular toxicity.

Keywords: intracellular unbound drug concentrations, free drug, drug binding, drug transport, drug accumulation, cellular drug response, drug target engagement

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In the face of overwhelming odds, I'm left with only one option: I'm gonna have to science the hell out of this.

Mark Watney
(in *The Martian*)

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I Mateus, A., Matsson, P., Artursson, P. (2013) Rapid measurement of intracellular unbound drug concentrations. *Molecular Pharmaceutics*, 10(6):2467–2478
- II Mateus, A., Matsson, P., Artursson, P. (2014) A high-throughput cell-based method to predict the unbound drug fraction in the brain. *Journal of Medicinal Chemistry*, 57(7):3005–3010
- III Mateus, A., Treyer, A., Karlgren, M., Matsson, P., Artursson, P. Impact of drug-transporters on intracellular drug concentrations. *In manuscript*
- IV Almqvist, H., Axelsson, H., Jafari, R., Dan, C., Mateus, A., Haraldsson, M., Larsson, A., Martinez-Molina, D., Artursson, P., Lundbäck, T., Nordlund, P. CETSA screening identifies known and novel thymidylate synthase inhibitors and slow intracellular activation of 5-FU. *Nature Communications (submitted)*
- V Mateus, A., Gordon, L. J., Wayne, G. J., Almqvist, H., Axelsson, H., Seashore-Ludlow, B., Treyer, A., Matsson, P., Lundbäck, T., West, A., Hann, M. M., Artursson, P. Impact of intracellular drug bioavailability on cellular drug response. *In manuscript*

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Abbreviations

ABC	ATP-binding cassette
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
C_{cell}	Total intracellular drug concentration
CETSA	Cellular thermal shift assay
C_{medium}	Total extracellular drug concentration
$C_{\text{u,cell}}$	Intracellular unbound drug concentration
$C_{\text{u,medium}}$	Extracellular unbound drug concentration
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
EC_{50}	Half maximal effective concentration
EdU	Ethynyldeoxyuridine
EdUMP	Ethynyldeoxyuridine-monophosphate
FBS	Fetal bovine serum
FdUMP	Fluorodeoxyuridine-monophosphate
F_{ic}	Intracellular drug bioavailability
$f_{\text{u,brain}}$	Fraction unbound in brain
$f_{\text{u,cell}}$	Intracellular fraction unbound
$f_{\text{u,hom}}$	Fraction unbound in cell homogenate
$f_{\text{u,medium}}$	Extracellular fraction unbound
$f_{\text{u,microsomes}}$	Fraction unbound in microsomes
$f_{\text{u,plasma}}$	Fraction unbound in plasma
GI_{50}	Half maximal growth inhibitory concentration
GST	Glutathione-S-transferase
HEK293	Human embryonic kidney cells
IC_{50}	Half maximal inhibitory concentration
ITDRF	Isothermal dose-response fingerprint
K_{m}	Michaelis-Menten constant
Kp	Total drug accumulation ratio
$K_{\text{p,uu}}$	Unbound drug accumulation ratio
$\log D_{7.4}$	Octanol-water partition coefficient at pH 7.4
$\log P$	Octanol-water partition coefficient
MALDI	Matrix-assisted laser desorption ionization
MWt	Molecular weight
NCI	National cancer institute
OATP	Organic anion-transporting polypeptide

PBMC	Peripheral blood mononuclear cells
P-gp	P-glycoprotein
PKIS	Published kinase inhibitor set
PSA	Polar surface area
r_s	Spearman's rank correlation coefficient
SIMS	Secondary ion mass spectrometry
SLC	Solute carrier
SULT	Sulfotransferase
TFT	Trifluorodeoxythymidine
TFTMP	Trifluorodeoxythymidine-monophosphate
UGT	Uridine-diphosphate-glucuronosyltransferase
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
V_{\max}	Maximal uptake rate

Introduction

The cell interior harbors a large number of proteins of pharmacological interest.¹ These include not only proteins through which drugs exert their effects or toxicity, but also proteins responsible for the elimination of drug molecules. The interaction with these proteins is determined by the intracellular levels of the drug. More specifically, it is only the unbound drug at the site of action that is able to engage the target and affect the biological activity.²

Historically, unbound drug concentrations in cells or tissues have been assumed to be equal to those in plasma.³ However, these concentrations are not always identical.⁴⁻¹⁰ Overlooking this imbalance has resulted in poor predictions of *in vivo* drug clearance and drug-drug interactions in *in vitro* experiments.¹¹⁻¹⁶ In addition, recent analyses have shown that uncertainty of drug exposure at the target is one of the major causes for clinical failure of drug candidates.¹⁷⁻¹⁹ Therefore, methods that provide measurements of intracellular unbound drug concentrations have the potential to improve predictions of pharmacokinetics and to reduce attrition in drug development.^{20, 21} Previous research in brain, liver, and lung tissues has provided an important framework on how to determine unbound drug concentrations in tissues.²²⁻²⁶ In this thesis, that work was expanded to develop a methodology to measure unbound drug concentrations in cell cultures. In addition, the developed methodology was applied to study the factors affecting intracellular drug concentrations and to measure the impact of these on the cellular pharmacological response.

Factors affecting cellular disposition of drugs

Intracellular drug disposition is affected by the physicochemical characteristics of the drug molecule, and by the structure of the cell and its surroundings (Figure 1). Transport mechanisms across the membrane and extracellular binding to proteins affect how much drug reaches the cell interior, whereas elimination by metabolic processes reduces the amount of intracellular drug. Furthermore, intracellular accumulation of drug can occur when it binds to cellular structures, or when it is sequestered into subcellular compartments. The sections below present the factors influencing drug disposition in the cell in more detail.

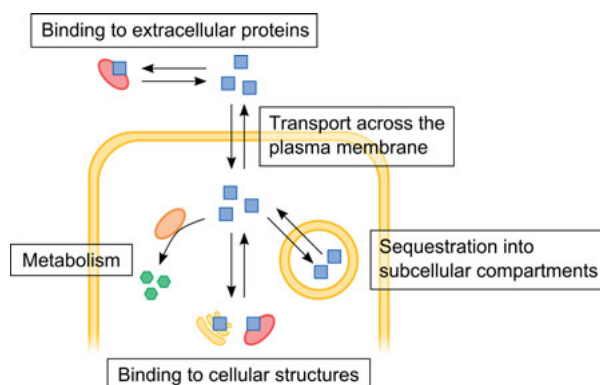


Figure 1. Factors affecting cellular disposition of drugs. Transport mechanisms across the membrane and extracellular binding to proteins affect how much drug reaches the cell interior, whereas elimination by metabolic processes reduces the amount of intracellular drug. Furthermore, drug can accumulate intracellularly when it binds to cellular structures or is sequestered into subcellular compartments.

Transport of drugs across the plasma membrane

To access the cell interior, drug molecules must cross the lipid bilayer that constitutes the plasma membrane. This is achieved by passive lipoidal transmembrane diffusion or by carrier-mediated transport (Figure 2).

Passive lipoidal transmembrane diffusion of drugs

Low molecular weight lipophilic molecules enter the cell via passive lipoidal diffusion (Figure 2).^{27, 28} This process does not require energy consumption, as it is driven by the concentration gradient across the membrane (i.e., molecules spontaneously move to the side of the membrane where their concentration is lower).²⁹ Equilibrium is reached when the intracellular unbound drug concentration equals that of the extracellular space.² Therefore, passive transmembrane diffusion does not contribute to an accumulation of unbound drug in the cell interior. However, if lipoidal diffusion happens at a slow rate, it can limit the amount of drug that reaches the cell interior.^{30, 31} This parameter is routinely screened in drug discovery to predict drug absorption.

Carrier-mediated transport of drugs

Drug transport across the membrane can also be assisted by transport proteins (i.e., carriers).⁵⁻⁷ These proteins are categorized as either uptake or efflux transporters, depending on the direction in which they transport molecules through the membrane. When carrier-mediated transport occurs in favor of a concentration gradient, it does not expend energy and is named facilitated diffusion (Figure 2). Conversely, transport against a concentration gradient requires energy consumption and is termed active transport (Figure 2).

The human genome encodes for more than 400 membrane transporters.⁵ These belong to two major superfamilies: the ATP-binding cassette (ABC) and the solute carrier (SLC) families.^{5, 6} Transporters belonging to the ABC family bind and hydrolyze ATP as their energy source.^{32, 33} Conversely, members of the SLC family that perform active transport use ion gradients generated by ATP-dependent primary transporters.^{34, 35}

Active transport can result in significant drug accumulation in the intracellular space, when mediated by uptake transporters⁸⁻¹⁰, or in limited cellular exposure, when mediated by efflux transporters.^{36, 37} This has implications in drug discovery, because:

1. Transporter expression varies for the different cell types in the organism. Some transporters are tissue-specific (e.g., the organic anion-transporting polypeptide 1B1 (OATP1B1/*SLCO1B1*) is only expressed in hepatocytes^{38, 39}), whereas others are expressed in multiple organs (e.g., P-glycoprotein (P-gp/*ABCB1*) is expressed in the liver, small intestine, kidney, blood brain barrier, and other tissues⁴⁰);
2. Transporter function and expression can be inhibited or enhanced by other xenobiotics, resulting in drug–drug interactions (e.g., OATP1B1 is inhibited by cyclosporine A, resulting in reduced elimination of its substrates, such as atorvastatin^{41, 42}), or drug–food interactions (e.g., naringenin, present in grape-fruit juice, reduces the uptake of OATP1B1 substrates⁴³);
3. Variations in drug transporter genes (polymorphism) can result in altered expression or function of transporters.⁴⁴

Some researchers have hypothesized that carrier-mediated transport is the sole mechanism of transport across the membrane.⁴⁵⁻⁴⁸ Still, it appears unlikely that transporter capacity is sufficient to support this hypothesis.^{27, 28, 49, 50}

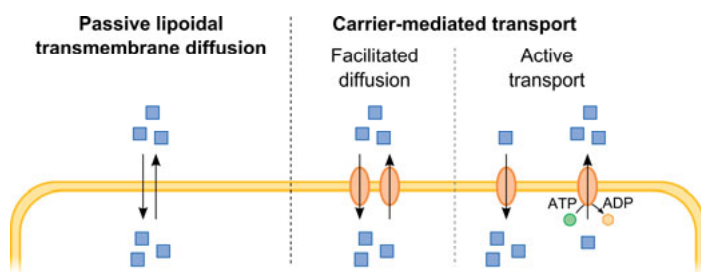


Figure 2. Transport mechanisms for translocation of drugs across the plasma membrane. Drugs can enter the cell via passive lipoidal transmembrane diffusion or via carrier-mediated transport. If membrane proteins transport the drug in favor of the concentration gradient, without energy consumption, the process is named facilitated diffusion. Conversely, transport against the concentration gradient, expending energy, is termed active transport.

Elimination of drugs from the cell interior by metabolism

When drug molecules reach the cell interior, their intracellular levels can be affected by metabolizing enzymes (Figure 1). These proteins generally inactivate drug molecules by hydrolysis, oxidation or reduction (Phase I metabolism), or by conjugation with polar or reactive groups (Phase II metabolism).⁵¹ In humans, the major superfamilies of drug-metabolizing enzymes are: the cytochrome P450 (CYP), UDP-glucuronosyltransferase (UGT), glutathione-S-transferase (GST), and sulfotransferase (SULT) families. Many other minor families exist, such as serum and tissue proteases that also play an important role in the elimination of peptide and protein drugs.^{52, 53}

Drug metabolism contributes to a decrease in intracellular drug concentration. This is particularly noticeable when the metabolic rate is much higher than the uptake rate of the drug into the cell.^{31, 54} The interplay between metabolizing enzymes and membrane transporters is thus recognized as a key aspect defining drug distribution.^{54, 55} Importantly, the substrate specificity of some transporters and enzymes significantly overlaps (e.g., many compounds are substrates of both CYP3A4 and P-gp⁵⁶). In addition, transporters play an important role in removing polar metabolites from the cell interior.⁵⁷

As with membrane transporters, multiple aspects of drug-metabolizing enzymes are important to consider in drug discovery:

1. Expression of these enzymes is not ubiquitous in the organism;⁵⁸ most of the ones involved in drug metabolism are expressed in the intestinal wall and in the liver, with some isoforms being expressed in other tissues, such as the kidney, lung, or blood brain barrier;
2. Drug metabolism can be inhibited or enhanced by other xenobiotics;⁵²
3. Genetic polymorphism can alter the expression or function of metabolizing enzymes (e.g., CYP2D6 gene duplication leads to more rapid metabolism of its substrates⁵⁹).

Binding of drugs to extracellular proteins

Binding to extracellular proteins limits the amount of drug that reaches the cell interior, since only the unbound drug is available to enter the cell (Figure 1).^{2, 60} Protein binding is generally reversible, arising from electrostatic/hydrophobic interactions, or hydrogen bonding.⁶⁰ *In vivo*, drug molecules bind to plasma proteins, such as albumin and α 1-acid glycoprotein.⁶¹ *In vitro*, some assays are run in the presence of protein to support cell cultures.

Protein binding has direct implications on the administered drug dose.² Therefore, this parameter is routinely measured during drug discovery, using equilibrium dialysis, ultrafiltration, or ultracentrifugation.⁶⁰

Binding of drugs to cellular structures

Drug molecules can bind cellular constituents (e.g., proteins or nucleic acids) or partition into membranes (Figure 3). Binding is generally non-specific, as drugs interact with the cell proteome,^{62, 63} or partition into membranes via hydrophobic interactions.⁶⁴⁻⁶⁶ Non-specific binding is largely associated with drug lipophilicity.^{8, 67, 68} Binding to specific cellular structures only impacts drug accumulation in rare cases and is generally associated with the drug effect (e.g., anthracyclines bind strongly to DNA, which results in high tissue concentrations^{69, 70}; Figure 3).

As a consequence of high levels of cellular binding, the intracellular concentration of some drugs can be several orders of magnitude higher than the extracellular one.⁷¹ However, this only reflects an increase in total drug concentration, and does not necessarily translate into a higher amount available to bind the target.

The ability of a drug to bind to cellular and tissue components can be measured using equilibrium dialysis^{22, 72} or immobilized membrane fractions^{66, 73}, or it can be predicted from the physicochemical characteristics of the drug.^{8, 67, 74, 75}

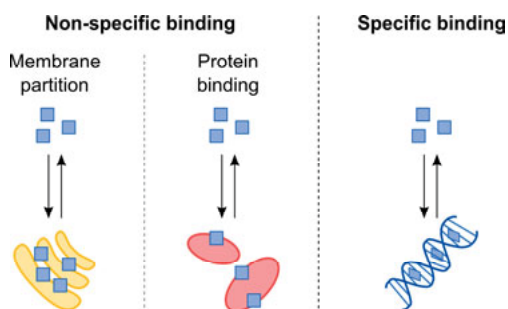


Figure 3. Binding of drugs to cellular structures. Drugs bind non-specifically to cellular structures, such as membranes and proteins. In rare cases, drugs can bind to a specific cellular structure to a great extent, influencing the cellular distribution.

Sequestration of drugs into subcellular compartments

Subcellular distribution of drugs occurs as a result of: 1) pH and electrochemical gradients established across organelle membranes; 2) specific interactions with cellular components; 3) active transport of the drug molecule into a specific organelle (Figure 4). The distribution is largely influenced by the physicochemical characteristics of the drug molecule.⁷⁶ Sequestration of the drug in subcellular compartments can increase or decrease drug effects or toxicity, depending on whether the target or off-targets are localized in the interior of the organelle where the compound accumulates.⁷⁷

Lysosomal accumulation of drugs

Low-molecular weight lipophilic molecules with weakly basic properties accumulate in lysosomes, late endosomes, the Golgi apparatus, and secretory vesicles, due to the low intraorganelle pH (generally one to two units below cytosolic pH).⁷⁸ Upon entering the organelle, these drugs become predominantly ionized and less likely to diffuse out, resulting in pronounced accumulation (Figure 4).^{22, 78-84}

Large, charged molecules can also accumulate in lysosomes as a result of entering the cell through endocytosis or pinocytosis. These compounds are retained in the organelle by being intrinsically membrane impermeant, requiring drug delivery strategies to escape the endosomal pathway.^{76, 85}

Apart from potentially affecting the efficacy of drugs, lysosomal accumulation can lead to alterations in lysosomal pH, volume, or lipid content.⁷⁸ These changes might affect the distribution of concomitantly-administered drugs.^{77, 84}

Mitochondrial accumulation of drugs

In mitochondria, the inner membrane maintains a potential difference of approximately -180 mV.⁸⁶ The high negative potential of the mitochondria interior leads to a charge-gradient-driven accumulation of positively-charged compounds (Figure 4).⁸⁷⁻⁸⁹ This knowledge has been used to design mitochondria-targeted therapeutics.^{90, 91}

Nuclear accumulation of drugs

Nuclear accumulation of flat and rigid small molecules occurs when these intercalate between the DNA bases (Figure 4).^{69, 70, 76} Intercalation also leads to alterations in the DNA structure that are used as therapeutic strategies.⁹²

Subcellular carrier-mediated transport of drugs

Subcellular distribution of compounds might also be affected by active uptake and efflux transporters located in the membranes of cellular organelles, e.g., lysosomes and mitochondria (Figure 4). This mechanism contributes to the toxicity of some compounds, e.g., fialuridine, an antiviral drug that produces mitochondrial toxicity after being transported into the organelle by the equilibrative nucleoside transporter 1 (ENT1; *SLC29A1*).^{93, 94} Other members of the ABC^{95, 96} and SLC^{97, 98} families, capable of transporting drug-like compounds,^{99, 100} are expressed in the lysosomal membrane and influence the subcellular distribution of drugs.

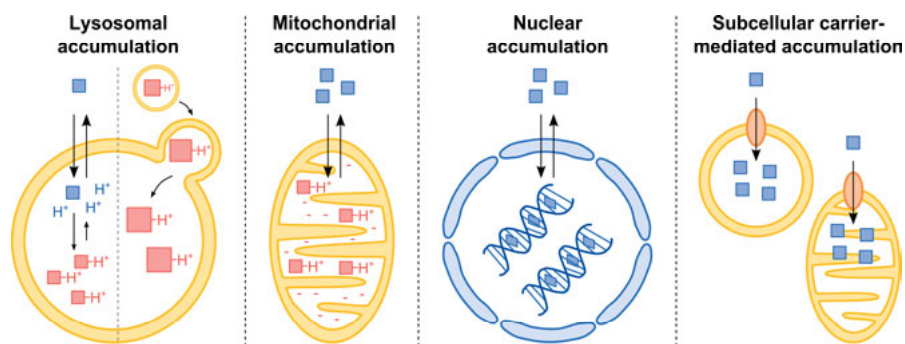


Figure 4. Sequestration of drugs into subcellular compartments. Lipophilic bases accumulate in lysosomes by becoming predominantly charged in the acidic environment of the organelle. Large, charged molecules are retained in the lysosome after entering the cell via endocytosis. Positively-charged drugs accumulate in the mitochondrion due to the high negative potential in the organelle interior. Flat and rigid small molecules intercalate between the DNA bases and, therefore, accumulate in the nucleus. Drug molecules might also be transported into and out of organelles via transporters.

Methods to measure cellular concentrations of drugs

Measuring drug concentrations in the cell interior is technically challenging.^{7, 20, 21} The low cellular volume (0.1–10 picoliter)¹⁰¹ precludes direct measurements, and the low number of drug molecules per cell requires highly sensitive methods (at 1 μM there are only 10^5 – 10^6 molecules/cell). Nevertheless, multiple approaches have been developed and are reviewed in the following sections, with a summary of their advantages and limitations (Table 1).

Indirect determination of intracellular concentrations of drugs

A simple approach to estimate the cellular levels of a drug is to measure a response elicited from an intracellular target. This response is measured in two setups: 1) with a recombinantly expressed target, or cell lysate (generally termed biochemical assay); 2) with live cells (generally designated cellular assay). In the former case, all compound added to the assay can directly bind the target, whereas in the latter, the compound must permeate the membrane to reach the target. A comparison of the measurements from these two setups provides an estimate of how much drug is bioavailable in the cell interior (Figure 5).

This comparison can be made by directly measuring the engagement of the drug onto a target protein,¹⁰²⁻¹⁰⁵ or by measuring downstream events (e.g., consumption of a co-factor, phosphorylation of another protein, gene expression, or cell growth inhibition).¹⁰⁶

Table 1. *Methods to measure cellular drug concentrations.*

Method	Advantages	Limitations
Indirect methods		
<u>Drug target engagement</u> (displacement assays)	<ul style="list-style-type: none"> • No drug labeling required • Amenable to high-throughput formats • Measurement of only bioavailable drug 	<ul style="list-style-type: none"> • Requires specific ligand to target of interest • Drug tested must bind the same binding site as the ligand • Target can be stabilized by other compounds
<u>Drug target engagement</u> (thermal shift assays)	<ul style="list-style-type: none"> • No drug labeling required • No specific ligand required • Amenable to high-throughput formats • Possible to detect target engagement across the proteome • Measurement of only bioavailable drug 	<ul style="list-style-type: none"> • Only for soluble targets that show a thermal shift when stabilized • Requires method to measure a specific protein • Target can be stabilized by other compounds
<u>Biochemical and cellular assays</u>	<ul style="list-style-type: none"> • No drug labeling required • High-throughput format • Measurement of only bioavailable drug 	<ul style="list-style-type: none"> • Only for targets with unique and measurable activity • Response can be caused by activity on other targets (if using far-from-target activity)
Imaging methods		
<u>Microautoradiography imaging</u>	<ul style="list-style-type: none"> • Radiolabeling has low impact on physicochemical characteristics of the drug 	<ul style="list-style-type: none"> • Requires labeling • Drug and metabolites cannot be distinguished • Low spatial and temporal resolution • Measurement of total intracellular drug
<u>Fluorescence microscopy</u> (including super-resolution microscopy)	<ul style="list-style-type: none"> • Visualization of subcellular drug distribution (high spatial and temporal resolution) 	<ul style="list-style-type: none"> • Requires labeling (for most drugs) <ul style="list-style-type: none"> ◦ Labeling prior to incubation: can alter physicochemical properties of the drug ◦ Labeling after incubation: requires fixation and permeabilization of cells • Drug and metabolites cannot be distinguished • Measurement of total intracellular drug (except fluorescence anisotropy)

Method	Advantages	Limitations
<u>Raman microscopy</u>	<ul style="list-style-type: none"> • No drug labeling required • High spatial and temporal resolution (newer techniques) • High specificity 	<ul style="list-style-type: none"> • Requires unique vibrational spectra fingerprint • Low sensitivity (millimolar range) • Measurement of total intracellular drug
<u>Mass spectrometry imaging (MALDI)</u>	<ul style="list-style-type: none"> • No drug labeling required • High specificity 	<ul style="list-style-type: none"> • Low spatial resolution • Sample preparation can alter drug distribution • Measurement of total intracellular drug
<u>Mass spectrometry imaging (SIMS)</u>	<ul style="list-style-type: none"> • No drug labeling required • High specificity • High spatial resolution 	<ul style="list-style-type: none"> • Sample preparation can alter drug distribution • Measurement of total intracellular drug
Bulk analysis methods		
<u>Scintillation counting</u>	<ul style="list-style-type: none"> • High sensitivity 	<ul style="list-style-type: none"> • Requires labeling • Drug and metabolites cannot be distinguished • No information on subcellular distribution (possible to obtain some information using mathematical modeling or parallel experiments) • Measurement of total intracellular drug (possible to obtain bioavailable concentration using mathematical modeling or parallel experiments)
<u>Mass spectrometry</u>	<ul style="list-style-type: none"> • No drug labeling required • High sensitivity • High specificity • Amenable to high-throughput formats 	<ul style="list-style-type: none"> • No information on subcellular distribution (possible to obtain some information using mathematical modeling or parallel experiments) • Measurement of total intracellular drug (possible to obtain bioavailable concentration using mathematical modeling or parallel experiments)

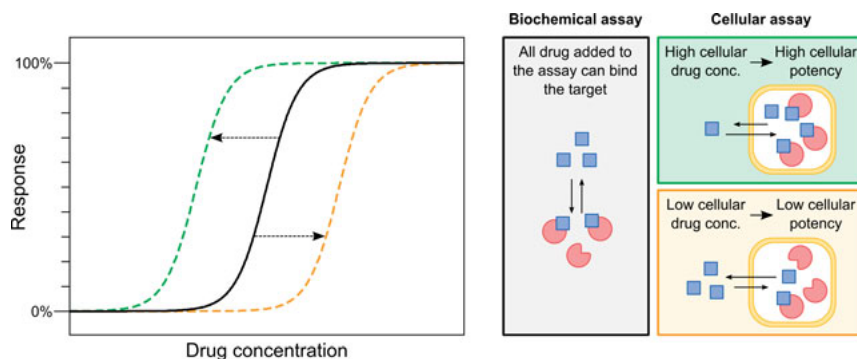


Figure 5. Indirect determination of intracellular drug concentrations. Intracellular drug levels can be estimated from the potency of compounds in a biochemical assay (black line) and a cellular assay (green or yellow lines). In a biochemical assay, all drug added to the assay can directly bind the target, whereas in a cellular assay it must first permeate the membrane. If the compound accumulates in the cell, it will appear to be more potent in the cellular assay (green line). If the compound has restricted access to the cell interior, it will appear to be less potent in the cellular assay (yellow line). The magnitude of the shift will correspond to the fold-difference in intracellular drug concentration in relation to the extracellular drug concentration (i.e., how much higher or lower the intracellular drug concentration is than the extracellular one).

Measuring drug target engagement

Drug target engagement is one of the keystones to ensure success in discovery programs.¹⁷⁻¹⁹ Target engagement can be measured by using displacement assays^{102, 104, 105} or thermal shift assays.^{62, 63, 103, 107}

In displacement assays, a known ligand of the target competes with the drug of interest for available binding sites. To monitor the amount of ligand bound to the target, this probe is labelled with a fluorescent marker. When the probe is bound to the target, its rotational Brownian motion slows down and this results in fluorescence anisotropy (i.e., there is an unequal intensity of light emitted by the probe along two different polarization planes; the amount of light emitted along each polarization plane can be monitored to determine how much probe is bound to the target).^{102, 108} Another alternative to monitor the amount of bound probe is by fusing the target with a luciferase. Photons emitted by the fluorophore of the probe are captured by the luciferase, resulting in luminescence.¹⁰⁴ An advantage of displacement assays is that they do not require labeling of the drug of interest. However, they require a specific ligand of the target (which is not always available) and that the drug tested engages the same binding site as the probe (Table 1).

Thermal shift assays are based on the principle that a higher energy is required to denature a protein when a ligand is bound to it.¹⁰⁹ In practice, the amount of soluble protein is measured after a heat challenge at several temperatures in the absence or presence of the drug of interest. If the drug binds the target, there will be a shift in the temperature necessary to unfold the

protein. The assay can also be run at a constant temperature, but varying drug concentration, to estimate target affinity.¹⁰³ Thermal shift assays do not require a specific probe for the target, or labeling of the tested drug. Therefore this type of assay is amenable to high-throughput formats.¹⁰⁷ However, they can only be applied to proteins that are stabilized by ligands and require a specific detection method for those proteins (e.g., antibodies^{103, 107} or mass spectrometry^{62, 63, 110}). In addition, it is not possible to assure that thermal stabilization is a consequence of engagement of the target by the tested drug. For example, other compounds, such as drug metabolites, or endogenous ligands whose levels are increased by the drug, might instead stabilize the target (Table 1).

Measuring events downstream of target engagement (biochemical and cellular assays)

The amount of compound that binds a target can be inferred by monitoring an event related to the activity of that protein (e.g., when studying a kinase inhibitor, levels of a downstream phosphorylated protein or ATP levels can be measured¹¹¹). Assays based on this principle can process a large number of compounds, since they do not require drug labeling. However, monitoring the unique activity of a protein in a cellular context is generally difficult, due to redundancy of cellular processes. Therefore, it is rarely possible to assure that the response to the drug is caused only by the target of interest (Table 1).

Imaging methods to measure intracellular concentrations of drugs

Microscopy techniques allow not only the determination of levels of intracellular drug, but also the visualization of subcellular distribution. Despite providing information about the organelles in which the compound might accumulate, these techniques measure only total drug concentrations (and not the fraction of compound available to bind the target). Furthermore, some of these techniques require labeling of the compound, which limits them to low-throughput formats (Table 1).

Microautoradiography imaging

Autoradiography provides an image of cellular distribution by revealing a pattern of decay emissions from a radioisotope.¹¹² A very limited number of drug molecules include radioisotopes in their structure, and, consequently, this technique requires the radiolabeling of drug molecules.^{112, 113} This can be achieved by using the radioisotope carbon-14, with a low impact on the physicochemical characteristics of the drug. However, it is not possible to ensure that the drug is not metabolized and that the signal originates from the

original drug molecule. In addition, the low spatial resolution of this technique (generally on the multicellular level) limits its application (Table 1).

Fluorescence microscopy

Fluorescence imaging techniques with good spatial and temporal resolution have emerged in the last decades.^{114, 115} These provide an important platform to study subcellular drug distribution. However, only a restricted number of drugs display levels of autofluorescence that allow their direct visualization in a cellular environment.^{116, 117} More commonly, the drug molecules must be labelled with a fluorescent marker for this purpose.^{102, 118-122} This marker can be added to the drug prior to^{102, 118} or after^{121, 122} incubation with the cells. When added prior to the incubation, distribution can be observed in real time, but the presence of the label might affect the physicochemical characteristics of the drug. Adding the marker after the incubation (e.g., using click chemistry^{121, 122}) requires fixation and permeabilization of the cells. Therefore, both approaches are prone to alter the cellular distribution of the studied drug. In addition, it is not possible to ensure that the drug is not metabolized with the result that the marker is labeling a metabolite (Table 1).

Raman microscopy

Raman spectroscopy provides information on vibrational and rotational states of a molecule.¹²³ Coupled to a microscope, it allows the visualization of distribution of drug molecules. Confocal Raman microscopy provides subcellular spatial resolution, but limited sensitivity and temporal resolution.^{124, 125} New technologies, such as hyperspectral stimulated Raman scattering (hsSRS), overcome some of these limitations.¹²⁶ In contrast to microautoradiography and fluorescence microscopy, Raman microscopy does not require labeling of the drug.¹²⁷ Instead, it relies on the unique vibrational spectral fingerprints of each molecule.¹²³ However, when spectra fingerprints overlap with those of cellular components, sensitivity can be low due to high background noise (Table 1).¹²⁶

Mass spectrometry imaging

Mass spectrometry allows high-sensitivity quantification of molecules due to its high specificity. This level of specificity is attained by precisely monitoring the mass or the fragmentation pattern of a drug molecule (not requiring labeling).¹²⁸ For this, mass spectrometry detectors require that drug molecules are ionized prior to analysis. Two major ionization strategies are employed in mass spectrometry imaging: matrix-assisted laser desorption ionization (MALDI),¹²⁹ and secondary ion mass spectrometry (SIMS).^{130, 131}

As stated in the name, MALDI requires the addition of a matrix to extract and crystallize the analytes, which are then ionized by absorption of energy from a laser source.^{128, 129} Matrix deposition can alter the distribution of compounds as they are extracted from the cell interior to the surface. In addi-

tion, this technique provides low spatial resolution (usually multicellular) (Table 1).

SIMS uses a high energy ion beam that releases and ionizes analytes from the surface of the sample.^{128, 130, 131} This technique provides very high spatial resolution (including z-axis resolution). However, sample preparation involves fixation or freezing of samples, which can affect the cellular distribution of drugs (Table 1).

Bulk analysis methods to measure intracellular concentrations of drugs

Bulk analysis methods rely on the lysis of a large number of cells to increase the number of drug molecules to be analyzed. In this way, it is possible to overcome the detection limits of methods commonly used for the analysis of drugs (e.g., scintillation counting or mass spectrometry). The major drawbacks of this approach are: 1) the loss of information about subcellular distribution of the drug; and 2) that it only provides a measure of total drug concentrations (Table 1). However, these limitations can be partially overcome.

To overcome the first limitation, and understand in which organelles the drug is located, it is possible to use mathematical modeling,^{22, 80, 82, 89} chemical inhibitors,^{22, 88, 132, 133} or subcellular fractionation.^{88, 134, 135} Mathematical modeling is based on the pH and electrochemical gradients established across certain organelle membranes. pH partition theory^{22, 80, 82} or the Nernst equation⁸⁹ can be used to predict the fraction of compound trapped in a certain organelle (e.g., lysosome or mitochondrion). In addition, inhibitors of transporters that are responsible for the maintenance of these gradients can be used to gain knowledge on organelle trapping (i.e., by comparing the cellular uptake of the compound in the presence and absence of inhibitor).^{22, 88, 133} However, these approaches are limited to subcellular compartments where these gradients exist or are measurable (e.g., it is difficult to model the distribution into the endoplasmic reticulum). An approach that can be applied to a larger number of subcellular compartments is subcellular fractionation. This involves differential centrifugation of organelles. Fractionation can be performed prior to^{88, 134} or after¹³⁵ incubation with the drug. In both approaches, compound distribution might be altered by the organelle not being in its native environment, or by the compound redistributing during the fractionation procedure. In addition, pure organelle fractions are difficult to obtain,¹³⁶ and contamination from organelles with high drug accumulation can contribute to large errors. The low yields obtained from some fractions can also bias the results.¹³⁷

To overcome the second limitation, and be able to measure the intracellular unbound concentration of compound, it is possible to use mathematical

modeling,^{8-10, 138, 139} or to measure binding of drug to the cell.^{22, 139-141} Mathematical modeling involves a mechanistic approach where the kinetic parameters of drug distribution are fitted to experimental data.^{8-10, 138, 139} To obtain reliable results, this approach demands a large number of experiments (at multiple time points and concentrations), restricting it to low throughput formats. The other approach to determine the intracellular unbound drug concentration is to measure drug binding in a separate experiment and to subtract it from the total uptake of drug into the cell. Binding can be estimated by inhibiting all active processes,¹³⁹⁻¹⁴¹ or by homogenizing the cells and separating the unbound drug using equilibrium dialysis.²² Inhibition of active processes can be done using chemical inhibitors,^{140, 141} or a reduced temperature.¹³⁹ The use of chemical inhibitors requires that all transport and metabolism mechanisms for a drug are known and can be inhibited. This limits the applicability of this methodology to only drugs for which all the mechanisms involved are known. Alternatively, lowering the temperature to halt active processes might also affect membrane fluidity and compound binding.¹⁴² The approach involving cell homogenization and dialysis to measure compound binding²² assumes that the destruction of the cellular context does not affect drug binding. In addition, the dialysis process usually requires long incubation times to achieve equilibrium.¹⁴³

Scintillation counting

To determine intracellular drug concentrations, the decay emissions from a radioisotope can be measured using liquid scintillation counting.^{89, 144, 145} In this technique, the radiation emitted by the radioisotope (α or β particles) excites the electrons of an aromatic compound added during sample preparation.¹⁴⁶ The electrons release energy when they return to the ground state. This energy is captured by another molecule called scintillator, which emits light that can be detected by a luminometer. As with microautoradiography imaging, this sensitive technique requires radiolabeling of the drug molecule (Table 1).

Mass spectrometry

Mass spectrometry is used for drug quantification by monitoring the precise mass of a drug molecule, or its fragmentation pattern. This technique requires molecules to be ionized prior to entering the analyzer.¹⁴⁷ The type of ionization used depends on the type of sample being analyzed, e.g., electrospray ionization is one of the most commonly used ionization sources for small drug-like molecules.¹⁴⁷ The ions are then conveyed to the mass analyzer using magnetic or electric fields. The most common types of mass analyzers in use today are time-of-flight (TOF) instruments, quadrupole mass filters, ion traps, or combinations of these.¹⁴⁸

TOF instruments operate under the principle that the velocity of ions with the same charge, and identical kinetic energy depends only on their mass.¹⁴⁹

The time necessary to reach the detector is measured and the mass-to-charge ratio (m/z) of the ion can be determined. This type of instrument has high mass accuracy and can be used to determine the precise mass of a drug molecule.

Quadrupole mass filters consist of four parallel rods, with each opposing rod pair acting together. An alternating current (AC) voltage is applied to all rods, with a direct current (DC) offset voltage applied between the rod pairs.¹⁵⁰ This affects the trajectory of ions travelling through the quadrupole. For given AC and DC voltages, only ions of certain m/z pass through the analyzer and reach the detector.¹⁵⁰ These voltages can be rapidly changed to monitor ions with different m/z . To achieve high sensitivity, these analyzers are generally operated with low mass resolution settings, i.e., in a way where it is not possible to distinguish ions of slightly different m/z .¹⁴⁸ To increase their specificity, quadrupole mass filters are usually used in tandem (generally, in a geometry comprising a linear series of three quadrupoles, known as a triple quadrupole mass spectrometer). In the first quadrupole the mass of the analyte (e.g., a drug) is selected (this is named the parent ion). In the second quadrupole, argon, neon, or nitrogen gas is used to promote the collision and fragmentation of ions. This quadrupole operates only under AC voltage (not as a mass filter) and is usually called a collision cell. In the third quadrupole, one of the fragment ions generated in the collision cell is selected (this is named the daughter ion). By monitoring a specific parent-to-daughter ion transition, it is possible to increase the specificity of the analyzer, since there is a low probability that two analytes with the same mass generate the same fragments.

Ion traps accumulate ions in their interior prior to passing them to the detector. Different geometries of ion traps exist, but the most commonly used are the quadrupole ion trap and the orbitrap. The quadrupole ion trap is used to increase the sensitivity of quadrupole mass filters. The operating principles are similar to those for a quadrupole, but they include an additional AC voltage at the two ends of the quadrupole, effectively trapping ions in this field.¹⁵¹ Orbitraps consist of a spindle-shaped electrode around which ions are trapped due to a balance of electrostatic attraction and centrifugal forces.¹⁵² By measuring the frequency of harmonic ion oscillations on an outer electrode that encloses the central spindle, the mass spectrum of the trapped ions can be obtained using fast Fourier transforms.¹⁵² As with TOF instruments, this type of analyzer has high mass accuracy.

Mass spectrometry detectors are generally coupled to liquid chromatography to isolate and concentrate the analytes, and thus increase sensitivity.¹⁵³ Since compound labeling is not required, mass spectrometry is one of the most versatile and frequently used techniques for the quantification of intracellular drug concentrations.^{8-10, 22, 71, 135, 138-141}

Aims of the thesis

The overall aim of this thesis was to establish a general and straightforward methodology to measure intracellular unbound drug concentrations.

The specific aims were:

- to develop a small scale methodology for measuring intracellular unbound drug concentrations (Papers I and II)
- to compare cellular drug binding to binding in more complex cell-based systems, such as tissues (Papers I and II)
- to assess the impact of drug transporters on intracellular unbound drug concentrations (Paper III)
- to investigate the relationship between intracellular levels of active metabolites of pro-drugs and target engagement (Paper IV)
- to investigate the relationship between intracellular unbound drug concentrations and the pharmacological response *in vitro* (Paper V)

Methods

Compound selection

This thesis focused on establishing a general method to measure intracellular unbound concentrations of compounds. To ensure the applicability of this technique to all drug-like molecules, 189 compounds with a wide range of physicochemical characteristics were included.

In Papers I and III, compounds were selected to cover the drug chemical space, and to overlap with previous studies measuring intracellular unbound drug concentrations.^{8-10, 68, 71, 140} To study transporter effects in Paper III, a large number of the included compounds were previously reported to be OATP1B1 (n = 11)³⁹ and/or P-gp substrates (n = 22).^{154, 155}

In Paper II, data on brain binding ($f_{u, \text{brain}}$) for 174 compounds were collected from the literature.^{22, 156-158} A subset of compounds (n = 46) was selected, ensuring that the physicochemical characteristics of these compounds were similar to those of a dataset of more than 800 approved drug molecules.¹⁵⁹

In Paper IV, the hit compounds from a target engagement screen against thymidylate synthase were selected for the study of the intracellular kinetics of their metabolites.

In Paper V, three sets of discovery compounds were included. These compounds belonged to the published kinase inhibitor set (PKIS),^{160, 161} or were known inhibitors of β -secretase (BACE-1) or mitogen-activated protein kinase 14 (p38 α). Biochemical and cellular potencies were available for these compounds, and a large number of them displayed ‘cell drop-off’ (i.e., lower potency in the cellular assay than in the biochemical assay).²¹

Determination of unbound drug accumulation ratio (Kp_{uu}) or intracellular drug bioavailability (F_{ic})

The unbound drug accumulation ratio (Kp_{uu}) was calculated as the ratio of intracellular unbound drug concentration ($C_{u, \text{cell}}$) and extracellular unbound drug concentration ($C_{u, \text{medium}}$), according to:

$$Kp_{uu} = \frac{f_{u, \text{cell}}}{f_{u, \text{medium}}} \cdot Kp \quad (1)$$

where $f_{u,cell}$ and $f_{u,medium}$ correspond to the intracellular and medium unbound drug fractions, respectively, and Kp corresponds to the ratio of total intracellular (C_{cell}) and extracellular drug concentrations (C_{medium}). Binding in the extracellular space was considered to be negligible ($f_{u,medium} = 1$) for incubations performed in buffer.

In Papers III and V, Kp_{uu} was renamed to intracellular drug bioavailability (F_{ic}), as a more general and descriptive term. Furthermore, the term F_{ic} does not necessarily assume that compound in the medium is unbound. In this way, F_{ic} represents the fraction of the concentration added to the cells that is unbound in the cell interior (e.g., if $F_{ic} = 0.1$ and cells are incubated with a 2 μ M solution of drug, the intracellular unbound drug concentration will be 0.2 μ M). F_{ic} was calculated as:

$$F_{ic} = f_{u,cell} \cdot Kp \quad (2)$$

Measurement of intracellular drug binding ($f_{u,cell}$)

The fraction of drug unbound in cells ($f_{u,cell}$) was measured using equilibrium dialysis (Figure 6). Cells were homogenized using an ultrasonic processor. Compounds were added to the cell homogenate individually (Paper I) or in cassettes (Papers II, III, and V). Homogenates were dialyzed against Hank's buffered salt solution (HBSS) in a Rapid Equilibrium Dialysis device. The dialysis unit was incubated on an orbital shaker for 4 h at 37 °C. At the end of the incubation, uniform sample matrices were obtained by addition of blank homogenate to samples from the buffer chamber and by addition of blank buffer to samples from the homogenate chamber. After protein precipitation, samples were analyzed with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

The fraction of drug unbound in the cell homogenate ($f_{u,hom}$) was calculated as:

$$f_{u,hom} = \frac{C_{buffer}}{C_{hom}} \quad (3)$$

where C_{buffer} is the concentration of compound in the buffer chamber and C_{hom} is the concentration of compound in the homogenate chamber. The $f_{u,cell}$ was calculated after correcting for homogenate dilution (D)⁷² according to:

$$f_{u,cell} = \frac{1}{D \cdot (1/f_{u,hom} - 1) + 1} \quad (4)$$

D was calculated as $1/(V_{\text{cell}} \cdot P_{\text{hom}})$, where V_{cell} is the cellular volume (6.5 $\mu\text{l}/\text{mg}$ protein¹⁶²), and P_{hom} is the protein concentration of the cell homogenate (in $\text{mg}/\mu\text{l}$).

Measurement of extracellular drug binding ($f_{\text{u,medium}}$)

The fraction of extracellular unbound drug ($f_{\text{u,medium}}$) was measured in a similar manner to that for the intracellular drug binding. Compounds were added to cell culture medium, and this solution was dialyzed against HBSS in a Rapid Equilibrium Dialysis device. At the end of a 4 h incubation at 37°C, uniform sample matrices were obtained by addition of blank cell culture medium to samples from the buffer chamber and by addition of blank buffer to samples from the cell culture medium chamber. After protein precipitation and quantification of drugs using LC-MS/MS, $f_{\text{u,medium}}$ was calculated as:

$$f_{\text{u,medium}} = \frac{C_{\text{buffer}}}{C_{\text{medium}}} \quad (5)$$

where C_{buffer} is the concentration of compound in the buffer chamber and C_{medium} is the concentration of compound in the cell culture medium chamber.

Measurement of intracellular drug accumulation (Kp)

Cellular drug uptake was measured by incubating cells with compound solutions (Figure 6). For steady-state uptake measurements, cells were incubated for 45 min. At the end of the incubation, a medium sample was collected and cells were washed to remove any compound not associated with the cells. The cells were lysed to extract intracellular compound. The amount of compound in cells (A_{cell}) and the compound concentration in extracellular space (C_{medium}) were quantified using LC-MS/MS.

The ratio between total compound concentration in cells and in the extracellular space (Kp) was calculated as:

$$Kp = \frac{A_{\text{cell}}/(V_{\text{cell}} \cdot P_{\text{cell}})}{C_{\text{medium}}} \quad (6)$$

where V_{cell} is the cellular volume (6.5 $\mu\text{l}/\text{mg}$ protein¹⁶²), and P_{cell} is the protein amount in the cells.

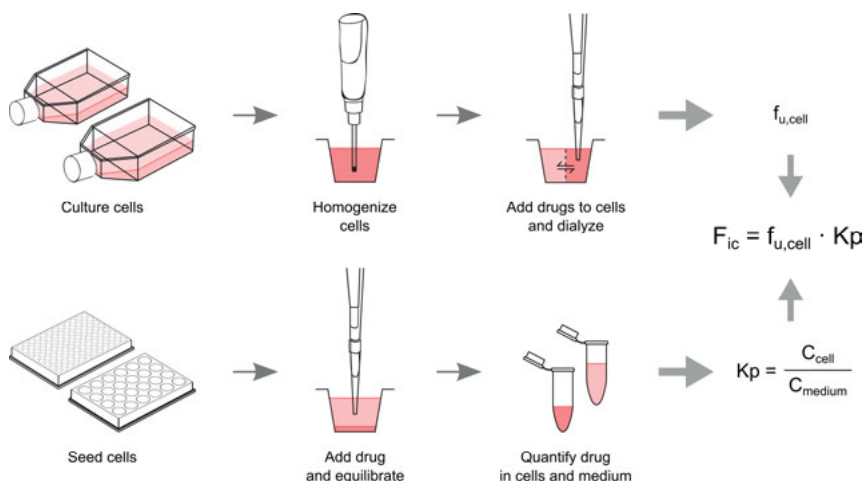


Figure 6. Overview of determination of intracellular drug bioavailability via parallel measurements of intracellular drug binding ($f_{u,cell}$) and intracellular drug accumulation (Kp). To measure $f_{u,cell}$, cells are homogenized and drugs are added to the cell homogenate. After equilibrium dialysis of the cell homogenate, $f_{u,cell}$ is determined. To measure Kp , drug is added to intact cells. After equilibration, the drug is quantified in the cells and in the medium to calculate Kp .

Analytical procedures

Drug quantification using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

Drugs were quantified using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). For most drugs, a generic 2 min gradient elution was used for chromatographic separation. Compounds were then analyzed using tandem mass spectrometry with electrospray ionization. Two unique parent-to-daughter transitions were monitored for each drug, and the transition with the highest signal-to-noise ratio was used for compound quantification.

Protein quantification using the bicinchoninic acid (BCA) assay

To estimate cellular volume, the Pierce bicinchoninic acid (BCA) protein assay reagent kit was used according to the manufacturer's instructions to determine the protein concentration of cell homogenates ($f_{u,cell}$ measurements) and seeded cells (Kp measurements). Briefly, the BCA solution was added to cell lysates and incubated for 30 min at 37 °C. After incubation, absorbance was read at 562 nm.

Results and discussion

Development of a methodology to measure intracellular unbound drug concentrations (Papers I and II)

This work started with the development of a simple experimental methodology to measure intracellular unbound drug concentrations. The approach was analogous to previous work done in brain tissue,^{22, 23} and consisted of a parallel measurement of the unbound drug fraction in cells ($f_{u,cell}$) and total intracellular drug concentrations (C_{cell}) (Figure 6). These two measurements were combined and normalized by the extracellular drug concentration (C_{medium}) to obtain the intracellular drug bioavailability (F_{ic} ; previously named unbound drug accumulation ratio (Kp_{uu})). Drug quantification was performed using tandem mass spectrometry.

The initial part of this work was performed in HEK293 cells. This cell line was used because of its negligible expression of drug transporters and drug-metabolizing enzymes,¹⁶³ which allowed the study of F_{ic} without these confounding factors. Furthermore, incubations were performed in the absence of extracellular proteins, so that all added compound was available to enter the cells. In this way, the only factors affecting cellular drug disposition were intracellular binding and potential sequestration into subcellular compartments (Figure 1).

Throughout this thesis, the F_{ic} of 189 molecules was measured in these cells. The physicochemical properties of these compounds largely overlapped with those of currently approved drugs (Figure 7),¹⁵⁹ ensuring that the methodology is generally applicable to new low-molecular weight chemical entities. In the following sections, the development of the different components of the methodology is described in more detail.

Unbound drug fraction in cells ($f_{u,cell}$)

The unbound drug fraction in cells ($f_{u,cell}$) was measured in cell homogenates with equilibrium dialysis. Despite the long time required to reach equilibrium, the Rapid Equilibrium Dialysis apparatus used in this thesis allowed parallel processing of large numbers of compounds with minimal effort.¹⁴³ This apparatus allows for full automation of binding measurements. Additionally, the cassette-mode approach developed in Paper II further increased compound throughput.

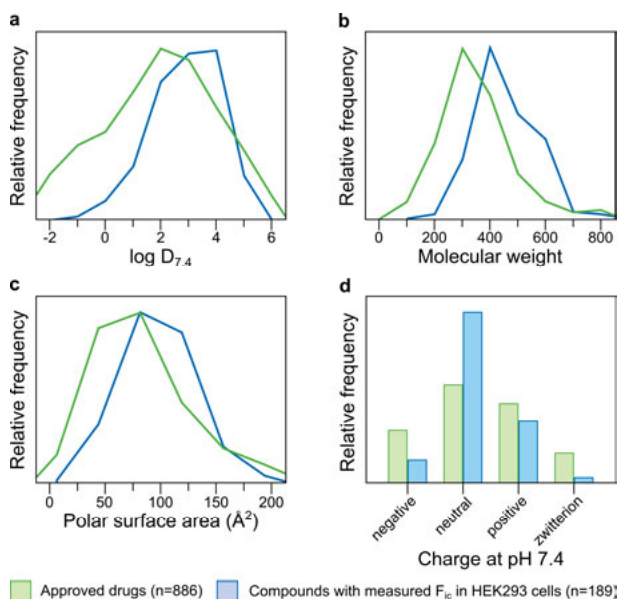


Figure 7. Relative frequency distribution of physicochemical properties of approved drugs¹⁵⁹ (green) and compounds for which F_{ic} was measured in HEK293 cells throughout this thesis (blue). (a) $\log D$ at pH 7.4, predicted with ADMET Predictor v7.0 (SimulationsPlus); (b) molecular weight; (c) polar surface area; (d) major charge species at pH 7.4, predicted with ADMET Predictor v7.0.

Concentration dependence of intracellular drug binding

A requirement for using a single binding constant to measure intracellular unbound drug concentrations is that binding is not saturable. Saturation occurs when the number of drug molecules exceeds the number of available binding sites.¹⁶⁴ To ensure that intracellular binding was not saturable, the $f_{u,cell}$ of nine drugs was measured across a range of concentrations typically found *in vivo* in the cell interior. The compounds were chosen to cover a range of physicochemical properties typical of approved drugs (Figure 7). Within the range tested, $f_{u,cell}$ was independent of compound concentration (Figure 8; $p > 0.2$ – 0.7 , depending on the compound, based on a Kruskal-Wallis test). This result supported the use of a single $f_{u,cell}$ measurement per compound throughout the rest of this work.

Measurement of intracellular drug binding in cassette-mode

Throughput of $f_{u,cell}$ measurements was increased by combining multiple drugs into a single dialysis device (cassette-mode). This was deemed reasonable, since $f_{u,cell}$ was measured at submicromolar concentrations and binding did not appear to be saturable even at high compound concentrations (100 μM ; Figure 8). Furthermore, the $f_{u,cell}$ of a subset of 14 drugs was not significantly different for the incubations with individual compounds and those in cassette-mode (Figure 9a; $r_s = 0.99$).

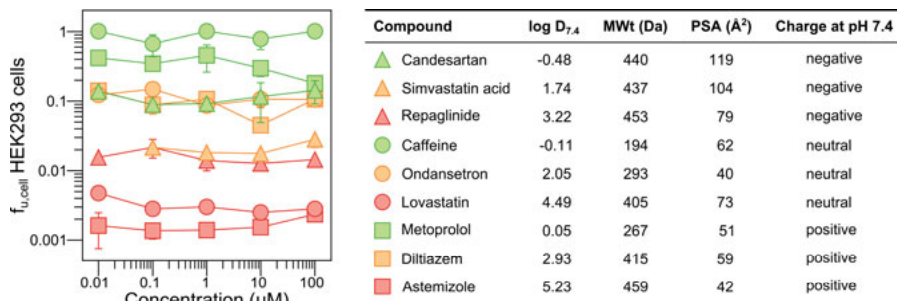


Figure 8. Concentration dependence of intracellular drug binding ($f_{u,cell}$) in HEK293 cells. Negatively charged compounds at pH 7.4 are represented by triangles, neutral compounds by circles, and positively charged compounds by squares. Compounds were color coded by lipophilicity (low lipophilicity ($\log D_{7.4} \leq 0$) in green; intermediate lipophilicity ($\log D_{7.4}$: 1–3) in orange; high lipophilicity ($\log D_{7.4} > 3$) in red).

For all subsequent measurements of intracellular drug binding, compounds were randomly assigned to at least three different cassettes, with a total of six compounds per cassette. This minimized the risk of potential interactions between pairs of compounds, due to the low probability of two interacting compounds being assigned to the same three cassettes. To further ensure the quality of $f_{u,cell}$ measurements, atorvastatin and lopinavir were added to all cassettes as controls. The $f_{u,cell}$ of these drugs did not differ significantly each time it was measured (Figure 9b; $p = 0.79$ based on a Kruskal-Wallis test; average deviation from median $f_{u,cell}$ was 1.3-fold for both atorvastatin and lopinavir), indicating that the assay was stable over time.

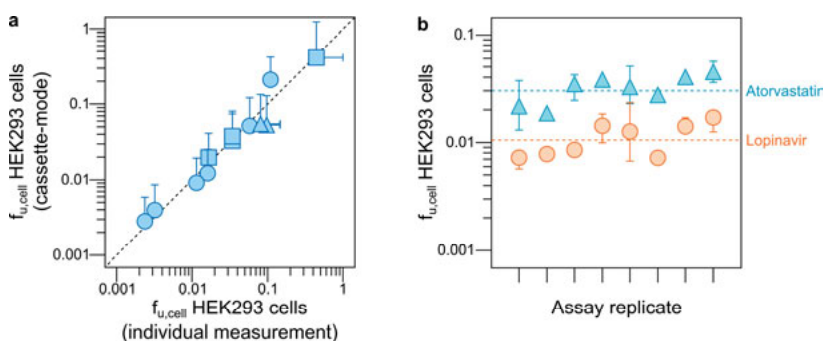


Figure 9. Cassette-mode measurement of intracellular drug binding ($f_{u,cell}$) in HEK293 cells. **(a)** Comparison between drug binding measured in incubations with individual compounds and in cassette-mode. Negatively charged compounds at pH 7.4 are represented by triangles, neutral compounds by circles, and positively charged compounds by squares. **(b)** $f_{u,cell}$ of atorvastatin (blue) and lopinavir (orange) measured in multiple cassettes over time. Dashed line represents median $f_{u,cell}$ of all measurements.

Molecular determinants of intracellular drug binding

To investigate the molecular determinants of $f_{u,cell}$ in HEK293 cells, a multivariate structure-property relationship was developed using partial least-squares projection.¹⁶⁵ $f_{u,cell}$ was predicted from molecular descriptors with an average 3.5-fold error ($r_S = 0.75$; Figure 10a). In agreement with previous observations,⁸ lipophilicity ($\log P$ and $\log D_{7.4}$) correlated strongly with $f_{u,cell}$, with higher binding observed for the more lipophilic compounds (Figure 10b). Furthermore, binding also appeared to be higher for molecules with a large surface for interaction, i.e., rigid molecules stretched out in two dimensions (Balaban distance connectivity index J , number of unsaturated bonds). Conversely, negative charge and aromaticity (number of distinct π systems, π partial atomic charges) were associated with decreased binding (Figure 10). Altogether, this suggested that $f_{u,cell}$ may be driven mainly by partitioning to cellular membranes. This is supported by the higher binding of lipophilic compounds with a large surface for interaction (with higher affinity to membrane lipids). The lower binding of negatively-charged and aromatic compounds is possibly due to electrostatic repulsions between the electron-rich groups in the drug molecules and the negatively-charged membrane lipids. Although intracellular protein binding might also partly contribute to $f_{u,cell}$, recent studies^{62, 63} have shown that drugs bind only a small fraction of the cell proteome (e.g., staurosporine, a promiscuous kinase inhibitor, with high lipophilicity, was bound to only 0.8% of the analyzed proteins).⁶²

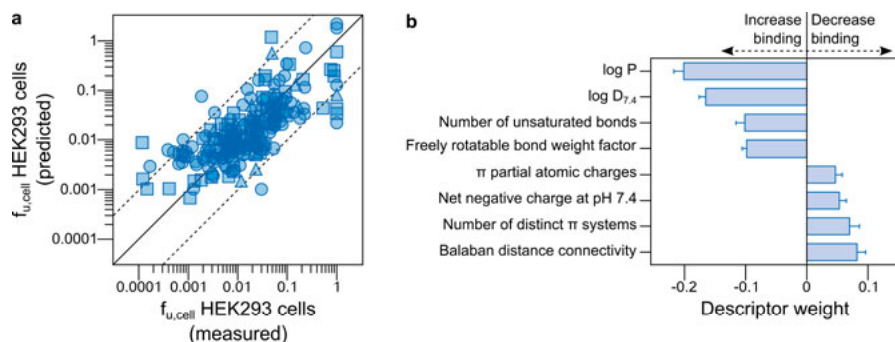


Figure 10. Molecular determinants of intracellular drug binding ($f_{u,cell}$) in HEK293 cells. **(a)** Predictions of $f_{u,cell}$ from a multivariate structure-property relationship using partial least-squares projection. Negatively charged compounds at pH 7.4 are represented by triangles, neutral or zwitterionic compounds by circles, and positively charged compounds by squares. Solid line represents a perfect prediction; dashed lines represent a ten-fold error in prediction. **(b)** The most influential descriptors in the multivariate prediction. Descriptors with negative coefficients had higher values in drugs with high binding, and descriptors with positive coefficients had higher values in drugs with low binding.

Comparison of intracellular drug binding to binding in other systems

Drug binding to HEK293 cells was compared to binding in other systems of varying complexity.

Comparison to plasma protein binding

First, $f_{u,cell}$ was compared with plasma protein binding ($f_{u,plasma}$),¹⁶⁶ a parameter regularly measured in the early stages of drug discovery. There was a poor correlation between these two parameters (Figure 11a; $r_S = 0.51$, $n = 58$), with most compounds binding more extensively to cellular structures than to plasma proteins. These differences were expected, since binding in plasma is mainly driven by interactions with proteins,⁶¹ while HEK293 cells are composed of a mixture of all cell constituents including proteins, membranes, and DNA.

Comparison to binding in liver-derived systems

Next, $f_{u,cell}$ was compared with binding to other cell-based systems, such as liver microsomes and hepatocytes. Binding to liver microsomes ($f_{u,microsomes}$),^{30, 68, 140, 167-175} a purified fraction of liver homogenates, strongly correlated with $f_{u,cell}$ in HEK293 cells (Figure 11b; $r_S = 0.88$, $n = 14$). Similarly, binding to hepatocytes ($f_{u,cell\ hepatocytes}$)^{30, 68, 71, 138, 140, 144, 145, 175-177} correlated with binding to HEK293 cells (Figure 11c; $r_S = 0.74$, $n = 43$). However, binding was more extensive in the liver-derived systems. In microsomes, binding was on average 4.9-fold higher, whereas in hepatocytes, binding was on average 2.5-fold higher. These differences can be attributed to the higher protein and lipid levels present in hepatocytes, which are approximately 2- to 2.5-fold higher than in embryonic kidney cells, based on a comparison of the tissues of origin of the two cell types: liver (for hepatocytes) and embryonic kidney (for HEK293 cells).¹⁷⁸

Comparison to binding to brain

Finally, $f_{u,cell}$ was compared with brain binding ($f_{u,brain}$).^{22, 156-158} Surprisingly, despite the higher levels of lipids present in the brain (six-fold higher than in the embryonic kidney), $f_{u,brain}$ values were similar to those of $f_{u,cell}$ (Figure 11d; $r_S = 0.91$, $n = 46$). This might have arisen as a consequence of comparing two systems at different levels: organ (brain) and cell (HEK293 cells). The extracellular space constitutes approximately 20% of the brain volume,¹⁷⁹ and contributes to a dilution of the homogenate used for $f_{u,brain}$ measurements. Nevertheless, the strong correlation between these two systems can be used to predict $f_{u,brain}$ in an inexpensive way without the use of experimental animals.

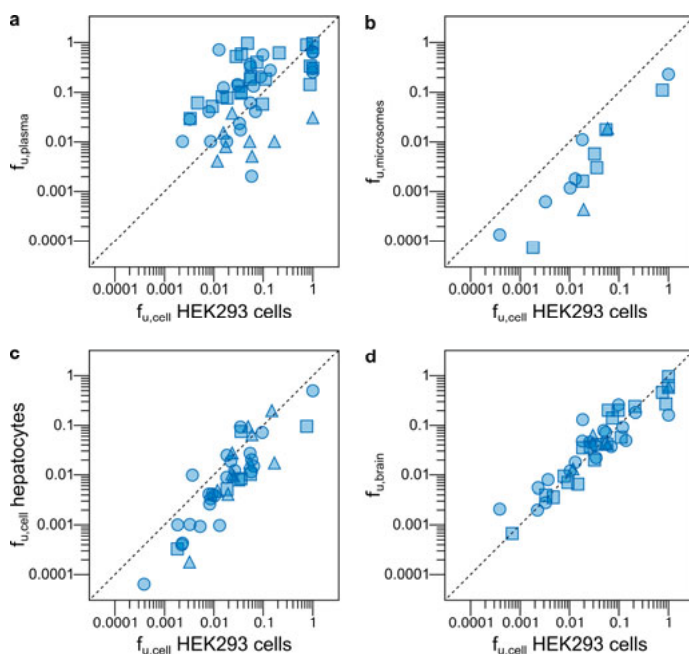


Figure 11. Intracellular drug binding ($f_{u,cell}$) in HEK293 cells and binding in other systems. Comparison between $f_{u,cell}$ in HEK293 cells and: **(a)** plasma protein binding (collected from literature¹⁶⁶); **(b)** binding to liver microsomes, corrected for the dilution that occurs during the preparation of microsomes from liver tissue (collected from literature^{30, 68, 140, 167-175}); **(c)** binding to human and rat hepatocytes (measured in-house for human hepatocytes, and collected from literature for rat hepatocytes^{30, 68, 71, 138, 140, 144, 145, 175-177}); **(d)** binding to brain (collected from literature^{22, 156-158}). Negatively charged compounds at pH 7.4 are represented by triangles, neutral or zwitterionic compounds by circles, and positively charged compounds by squares.

Intracellular drug accumulation ratio (Kp)

The intracellular drug accumulation ratio (Kp) was measured after incubation of the cells with drug. To determine the time required to achieve steady-state accumulation, uptake experiments with nine compounds, covering a wide range of physicochemical properties, were stopped at different time points (the compounds used were the same as the ones in ‘Concentration dependence of intracellular drug binding’ experiments). High lipophilicity and the presence of basic groups increased the time required for equilibrium (Figure 12). Nevertheless, 45 min appeared to be sufficient to reach equilibrium for all compounds (Figure 12).

As with cellular drug binding experiments, atorvastatin and lopinavir were used as controls to ensure the quality of Kp measurements. These compounds were run in parallel with the other assayed compounds. The Kp of these drugs did not differ significantly each time it was measured (Figure 13; $p = 0.97$ based on a Kruskal-Wallis test; average deviation from median Kp was 1.6-fold for atorvastatin and 1.3-fold for lopinavir).

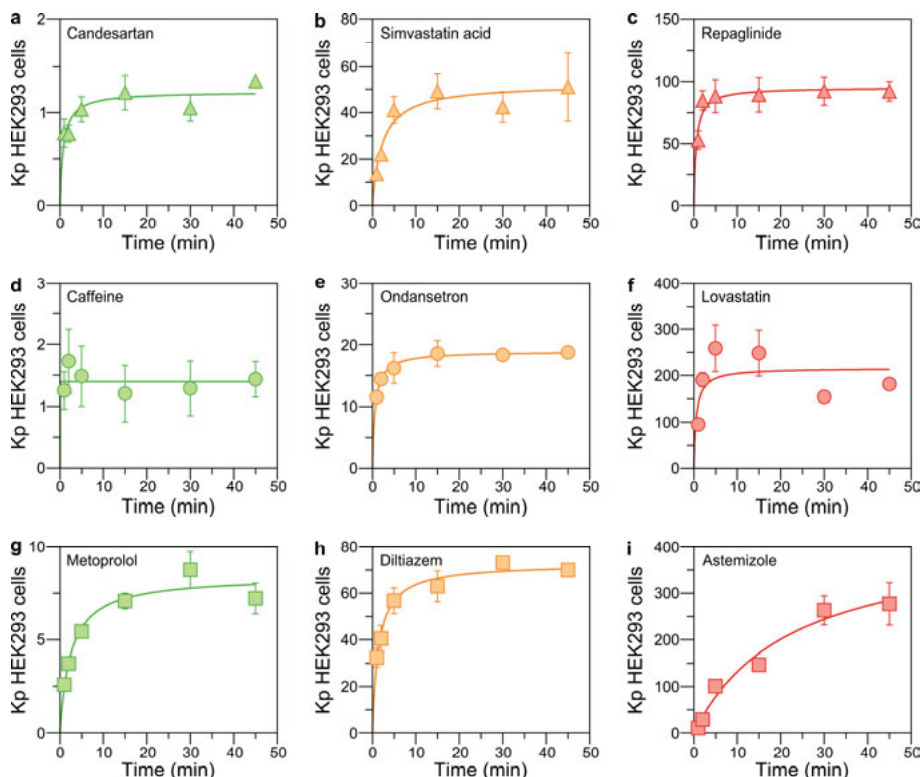


Figure 12. Time dependence of intracellular drug accumulation (Kp) in HEK293 cells for (a) candesartan, (b) simvastatin acid, (c) repaglinide, (d) caffeine, (e) ondansetron, (f) lovastatin, (g) metoprolol, (h) diltiazem, and (i) astemizole. Negatively charged compounds at pH 7.4 are represented by triangles, neutral compounds by circles, and positively charged compounds by squares. Compounds are color coded by lipophilicity (low lipophilicity ($\log D_{7.4} \leq 0$) in green; intermediate lipophilicity ($\log D_{7.4}$: 1–3) in orange; high lipophilicity ($\log D_{7.4} > 3$) in red). The lines represent the fit to a one-site binding model.

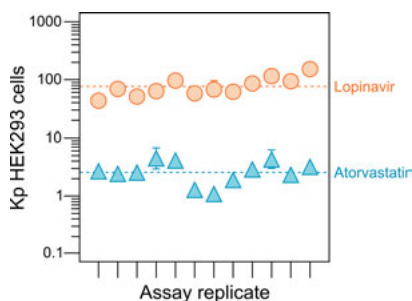


Figure 13. Controls for intracellular drug accumulation (Kp) experiments in HEK293 cells. Kp of atorvastatin (blue) and lopinavir (orange) measured in parallel to other compounds. Dashed line represents median Kp of all measurements.

Intracellular drug bioavailability (F_{ic})

After determination of the intracellular drug binding ($f_{u,cell}$) and accumulation (K_p), intracellular bioavailability (F_{ic}) was calculated as the product of these parameters. F_{ic} was related to the charge of the compound, with negatively-charged compounds displaying a median F_{ic} of 0.43, neutral compounds of 1.0, and positively-charged of 3.4 (Figure 14a; $p < 0.0001$ based on a Kruskal-Wallis test; a post hoc analysis using Dunnett's test found significant differences between all groups). The higher value for the basic drugs is consistent with the trapping of charged species in acidic subcellular organelles, such as endosomes and lysosomes, while the lower-than-unity value for acids is to be expected because of the pH gradient across the plasma membrane (Figure 14b). Since HEK293 cells express negligible amounts of drug transporters and metabolizing enzymes,¹⁶³ it was possible to accurately predict the median F_{ic} of each charge category using pH partition theory (Figure 14a).

In the following sections, the methodology was applied to other cell types: first to evaluate the impact of drug transporters on F_{ic} , and then to assess the impact of F_{ic} on drug target engagement and cellular pharmacological responses.

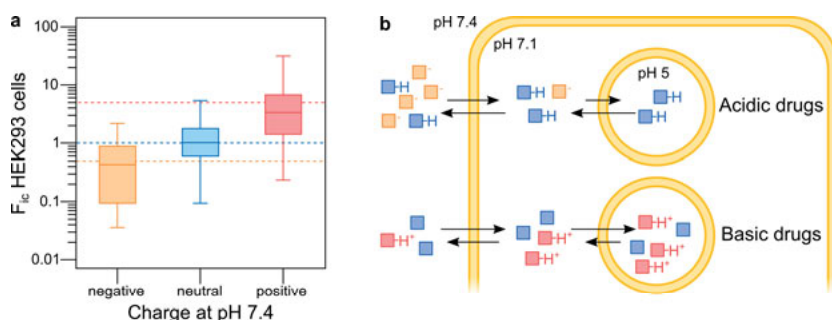


Figure 14. Impact of charge on intracellular drug bioavailability (F_{ic}) in HEK293 cells. **(a)** Distribution of F_{ic} for negatively charged compounds at pH 7.4 (orange; $n = 17$), neutral and zwitterionic (blue; $n = 127$), and positively charged compounds (red; $n = 45$). The line in the boxes represents the median; edges of the boxes represent the first and third quartiles; and whiskers represent the 5th and 95th percentile. Dashed lines represent predictions from a pH partition model. **(b)** Schematic representation of cellular distribution for acidic and basic drugs, taking into consideration that the permeability of charged species is lower than the uncharged species. Low pH subcellular compartments (pH 5) represent endosomes and lysosomes.

Impact of drug transporters on intracellular unbound drug concentrations (Paper III)

The impact of drug transporters on intracellular unbound drug concentrations was studied by first measuring F_{ic} in cell lines expressing the organic anion-transporting polypeptide 1B1 (OATP1B1; *SLCO1B1*—an uptake transporter), or P-glycoprotein (P-gp; *ABCB1*—an efflux transporter). Next, the impact of multiple transporters was assessed by measuring F_{ic} in a more complex system—freshly isolated human hepatocytes. These studies used a compound set of physicochemically-diverse molecules and included compounds previously reported to be OATP1B1 ($n = 11$)³⁹ and/or P-gp substrates ($n = 22$)^{154, 155}.

Impact of OATP1B1 on F_{ic}

To exemplify the influence of an uptake transporter on intracellular unbound drug concentrations, F_{ic} in HEK293 cells transfected with OATP1B1 was compared to that in HEK293 cells transfected with an empty vector (Figure 15a). Overall, the F_{ic} of OATP1B1 substrates was on average 2.9-fold higher in the OATP1B1-transfected cells (range: 1.1 – 9.8-fold; $p = 0.001$ based on a Wilcoxon matched-pairs signed rank test; Figure 15b). Conversely, non-substrates of OATP1B1 showed a similar F_{ic} in both cell lines (range: 0.57 – 1.9-fold difference between OATP1B1- and mock-transfected cells; $p = 0.06$ based on a Wilcoxon matched-pairs signed rank test). The impact of the uptake transporter decreased with increasing compound lipophilicity ($r_s = -0.63$), likely reflecting the increase in transmembrane diffusion with increased lipophilicity.^{27, 180, 181}

To investigate if the differences observed were caused by an active uptake mediated by OATP1B1, the F_{ic} of four substrates (atorvastatin, pitavastatin, fluvastatin, and simvastatin acid) was measured across a range of concentrations (Figure 15c-f). In mock-transfected cells, F_{ic} was independent of compound concentration for all substrates. This indicated that passive permeability was the main uptake mechanism in these cells. In contrast, F_{ic} decreased in the OATP1B1-transfected cells as the compound concentration increased, approaching levels in mock-transfected cells at higher concentrations. The concentrations at which F_{ic} was half of its maximal value were close to previously reported K_m values for OATP1B1-mediated transport of the respective substrates,¹⁸²⁻¹⁸⁴ indicating transporter saturation and an ensuing dominance of a passive mechanism.

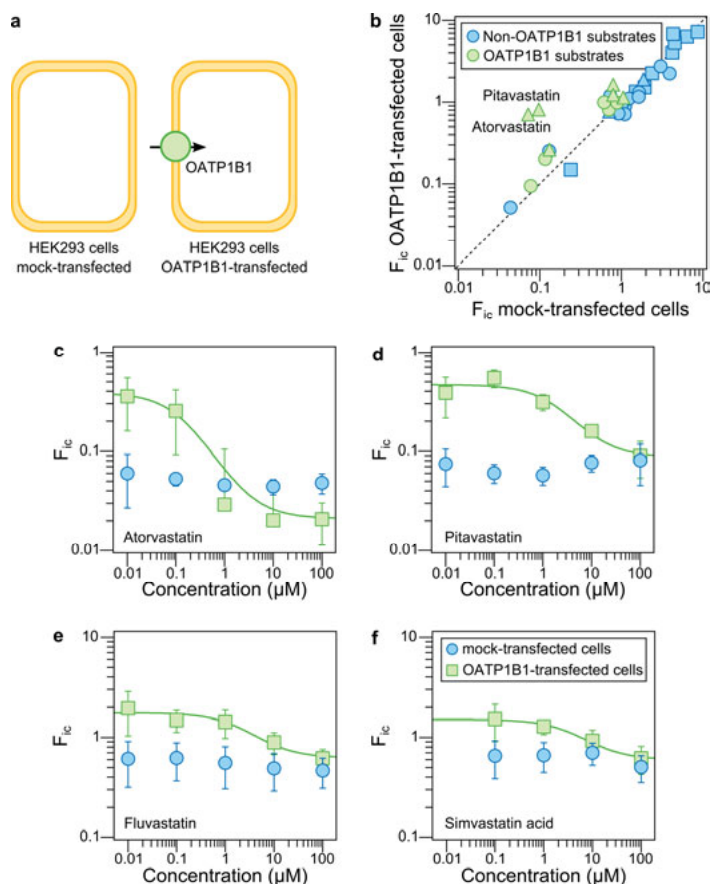


Figure 15. Impact of the uptake transporter OATP1B1 on intracellular bioavailability (F_{ic}). **(a)** Schematic representation of cell types used: mock-transfected HEK293 cells express negligible levels of relevant drug transporters¹⁶³; OATP1B1-transfected HEK293 cells express OATP1B1. **(b)** Comparison between F_{ic} in mock-transfected and OATP1B1-transfected HEK293 cells at 0.1 μM compound concentration. Substrates of OATP1B1 are highlighted in green. Negatively charged compounds at pH 7.4 are represented by triangles, neutral or zwitterionic compounds by circles, and positively charged compounds by squares. **(c-f)** Concentration-dependence of F_{ic} in OATP1B1-transfected HEK293 cells (green squares), fitted with a sigmoidal model (green line), and mock-transfected HEK293 cells (blue circles) for atorvastatin **(c)**, pitavastatin **(d)**, fluvastatin **(e)**, and simvastatin acid **(f)**.

Impact of P-gp on F_{ic}

Next, the effect of an efflux transporter on F_{ic} was evaluated in MDCK cells transfected with human P-gp and in wild-type MDCK cells where background endogenous canine P-gp was knocked-out with CRISPR/Cas9 technology (cMdr1-KO; Figure 16a).¹⁸⁵ F_{ic} of P-gp substrates was, on average, 2-fold lower in P-gp-expressing than in cMdr1-KO cells (range: 0.94 – 20-fold lower in P-gp transfected than cMdr1-KO cells; $p < 0.001$ based on a

Wilcoxon matched-pairs signed rank test; Figure 16b). For compounds not previously described as substrates, F_{ic} was more similar in the two cell lines (range: 1.1 – 3.0-fold difference between cMdr1-KO cells and P-gp transfected cells). Overall, lipophilic molecules showed lower F_{ic} in P-gp transfected cells ($r_s = -0.60$), independently of being previously described as substrates or not. This possibly reflects that compounds bind P-gp from within the inner leaflet of the plasma membrane,^{186, 187} and suggests that additional compounds in the dataset may be substrates of P-gp.

As for OATP1B1 substrates, the concentration-dependence of F_{ic} of simvastatin acid was measured. As expected, F_{ic} in P-gp-expressing cells was lower than that in cMdr1-KO cells at low concentrations and approached it at higher concentrations (Figure 16c). Surprisingly, F_{ic} decreased with increasing compound concentration in both the P-gp-transfected and cMdr1-KO cells, suggesting that there are additional active uptake mechanisms present in these cells. To better understand these observations, cellular drug accumulation was simulated using two simple kinetic models: 1) including both active uptake and active efflux mechanisms (corresponding to P-gp-transfected cells); and 2) including the uptake mechanisms only (corresponding to cMdr1-KO cells; Figure 16a). The simulations were in agreement with the experimental observations for a compound with high passive permeability, medium affinity ($K_m = 10 \mu\text{M}$) for an uptake transporter, and lower affinity ($K_m = 100 \mu\text{M}$) for an efflux transporter, with both transporters having comparable (high) transport capacities (V_{max}) (Figure 16d). This matched previous observations for simvastatin acid, which has a high passive permeability, a high affinity for uptake transporters, and a low affinity for efflux transporters.¹⁸⁸⁻¹⁹⁰

F_{ic} in primary human hepatocytes

Finally, the impact of multiple transporters and metabolizing enzymes on F_{ic} was studied in human hepatocytes in two different culturing conditions (Figure 17a): 1) freshly isolated cells (in suspension) that express similar levels of transporters and metabolizing enzymes as those in the human liver; and 2) cells cultured for 24 h (in monolayer format) that show a significant down-regulation of many important transporters and enzymes due to cell dedifferentiation.¹⁹¹⁻¹⁹⁴ On average, F_{ic} was 5.7-fold lower in freshly isolated suspension hepatocytes than in monolayer hepatocytes cultivated for 24 h (Figure 17b; $p = 0.0002$ based on a Wilcoxon matched-pairs signed rank test). This was in agreement with a higher gene expression of efflux transporters and enzymes in suspension hepatocytes.¹⁹¹⁻¹⁹⁴ These proteins are associated with the removal of compounds from the cell interior, which leads to a lower F_{ic} . This was particularly evident for atorvastatin, cerivastatin, fluvastatin, imipramine, propranolol, ritonavir, and simvastatin acid, which showed 4.9 – 15-fold lower F_{ic} in suspension hepatocytes.

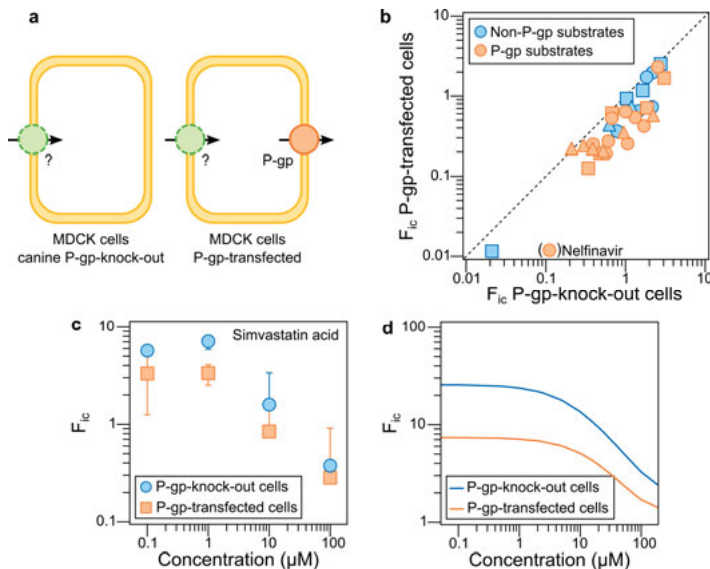


Figure 16. Impact of the efflux transporter P-gp on intracellular bioavailability (F_{ic}). (a) Schematic representation of cell types used: MDCK cells where canine P-gp has been knocked-out using CRISPR/Cas9; MDCK cells expressing canine and human P-gp. (b) Comparison between F_{ic} in P-gp-knockout MDCK cells and in P-gp-transfected MDCK cells at $0.5 \mu\text{M}$ compound concentration. Negatively charged compounds at pH 7.4 are represented by triangles, neutral or zwitterionic compounds by circles, and positively charged compounds by squares. Substrates of P-gp are highlighted in orange. (c) Concentration-dependence of F_{ic} in P-gp-transfected (orange squares), and P-gp-knock-out MDCK cells (blue circles). (d) Kinetic cell model simulations that best described experimental observations of concentration-dependence F_{ic} for simvastatin acid (conditions: passive permeability = $10 \times 10^{-6} \text{ cm/s}$; $V_{\text{max,uptake}} = 1000 \text{ pmol/min/mg protein}$; $K_{\text{m,uptake}} = 10 \mu\text{M}$; $V_{\text{max,efflux}} = 1000 \text{ pmol/min/mg protein}$; $K_{\text{m,efflux}} = 100 \mu\text{M}$).

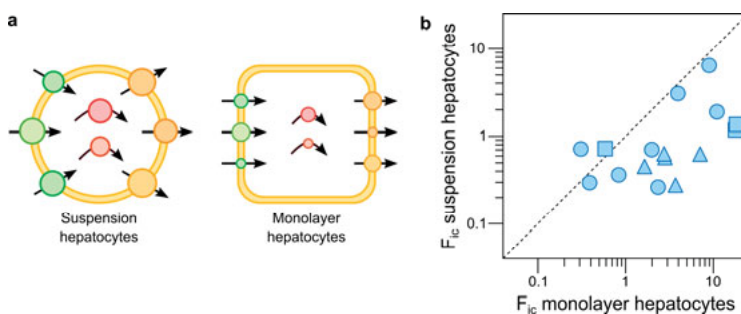


Figure 17. Impact of multiple transporters and metabolizing enzymes on intracellular bioavailability (F_{ic}). (a) Schematic representation of hepatocyte cultures used: freshly-isolated human hepatocytes in suspension express higher levels of drug transporters and metabolizing enzymes; hepatocytes cultured for 24 h in monolayer format show downregulation of most drug transporters and metabolizing enzymes.^{191, 192} (b) Comparison of F_{ic} in suspension hepatocytes and in hepatocytes cultured in monolayer format. Negatively charged compounds at pH 7.4 are represented by triangles, neutral compounds by circles, and positively charged compounds by squares.

Influence of intracellular drug concentrations on target engagement (Paper IV)

The methodology developed in this thesis was used to evaluate the impact of intracellular drug levels on target engagement. First, a compound library ($n = 10,928$) was screened for the capacity to bind thymidylate synthase in K562 cells, using the cellular thermal shift assay (CETSA).^{103, 107} Among the hits were pyrimidine-based nucleosides and their analogs, such as 5-fluorouracil, floxuridine, trifluorodeoxythymidine (TFT), and ethynyldeoxyuridine (EdU). These compounds are known inhibitors of thymidylate synthase that require intracellular activation to the monophosphate form prior to interaction with the enzyme (Figure 18a).^{195, 196} To study the kinetics of activation of the four hit compounds, CETSA was then performed after various pre-incubation times. In parallel, the intracellular levels of the hit compounds and their metabolites were monitored.

For floxuridine, CETSA data showed target stabilization at low nanomolar concentrations after only 10 minutes of pre-incubation (Figure 18b). The potency increased during the first 2 hours and persisted throughout the experiment. This was consistent with the intracellular appearance of the active species fluorodeoxyuridine-monophosphate (FdUMP), which was measurable after 10 minutes and increased during the first hours of incubation (Figure 18c). In contrast, the CETSA response of 5-fluorouracil (a pro-drug of floxuridine) increased slowly during the first 6 hours (Figure 18b), with undetectable intracellular levels of FdUMP, revealing that the enzymatic conversion of 5-fluorouracil to FdUMP is much slower than for floxuridine.

The intracellular metabolism of TFT was similar to that observed for floxuridine (Figure 18d). This was consistent with a build-up of trifluorodeoxythymidine-monophosphate (TFTMP) and binding to thymidylate synthase in the first hours. Both TFTMP and FdUMP form a covalent complex with thymidylate synthase,^{195, 197-199} in line with a persistent target engagement even after a decrease in their intracellular levels (Figure 18c and d).

EdU behaved differently, with a maximal CETSA response at the earliest time-point. The decrease of CETSA response with time was accompanied by a decrease of intracellular levels of the active form ethynyldeoxyuridine-monophosphate (EdUMP) (Figure 18e). This profile might have arisen as a consequence of EdUMP forming a non-covalent complex with thymidylate synthase (i.e., target stabilization decreased with decreased intracellular concentrations of EdUMP due to further metabolism; Figure 18a).²⁰⁰

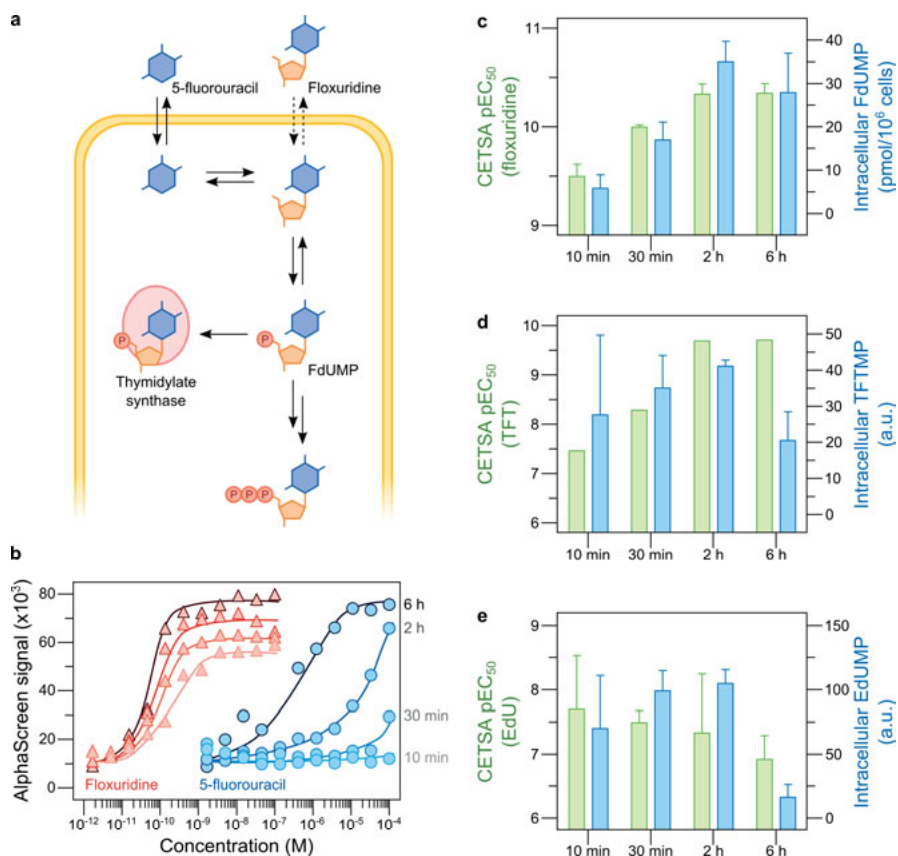


Figure 18. Impact of intracellular concentrations of the active metabolites of prodrugs on target engagement. **(a)** Schematic representation of activation of 5-fluorouracil. Prior to binding to thymidylate synthase, 5-fluorouracil is converted to floxuridine by thymidine phosphorylase, which is then phosphorylated by thymidine kinase to floxuridine monophosphate (FdUMP).¹⁹⁵ Alternatively, floxuridine can be directly administered to avoid the first metabolic step. **(b)** Thymidylate synthase engagement (CETSA) for floxuridine (red triangles) and 5-fluorouracil (blue circles), measured at different incubation times (from 10 min (lighter colors) to 6 h (darker colors)). **(c-e)** Comparison of CETSA potency (green) and intracellular levels of active metabolites of thymidylate synthase inhibitors (blue) at different incubation times: **(c)** incubation with floxuridine and intracellular levels of FdUMP; **(d)** incubation with trifluorodeoxythymidine (TFT) and intracellular levels of trifluorodeoxythymidine-monophosphate (TFTMP); **(e)** incubation with ethynyldeoxyuridine (EdU) and intracellular levels of ethynyldeoxyuridine-monophosphate (EdUMP).

Impact of F_{ic} on cellular pharmacological response (Paper V)

The impact of intracellular unbound drug concentrations on the cellular pharmacological response was evaluated with three compound sets: 1) a subset of compounds from the published kinase inhibitor set (PKIS),^{160, 161} 2) a set of β -secretase 1 (BACE-1) inhibitors; and 3) a set of mitogen-activated protein kinase 14 (MAPK14 or p38 α) inhibitors.

Published kinase inhibitor set (PKIS)

The published kinase inhibitor set (PKIS) includes 367 compounds that have been screened in the Nanosyn kinase panel (biochemical screen) and in the NCI-60 cell panel (cellular screen; Figure 19a).¹⁶¹ A subset of compounds with potential to inhibit cell growth was selected from this library. For this, compounds were grouped based on their biochemical affinity profiles, using hierarchical clustering with complete linkage (Figure 19a).²⁰¹ Then, clusters were selected that contained at least one active compound ($pGI_{50} > 6$) in more than half of the NCI-60 cell lines. This resulted in three clusters of molecules originally targeting intracellular kinases involved in the cell cycle, such as cyclin-dependent kinase 2 (CDK2),²⁰² glycogen synthase kinase 3 (GSK3), or polo-like kinase 1 (PLK1).²⁰³ Despite showing similar biochemical inhibition profiles, compounds within each cluster displayed very different cellular potencies (Figure 19a). To assess if these discrepancies were caused by differences in the levels of compound available at the target, F_{ic} was measured in HEK293 cells. This was a cell line not included in the NCI-60 panel, but with a gene expression profile close to that of an average cell line from this panel, allowing the use of the average of pGI_{50} across the 60 cell lines as a measure of compound potency.

F_{ic} was correlated with potency, in that cellular potency increased with a higher F_{ic} ($r_S=0.64$; Figure 19b). To help explain the cellular activity of compounds, a classification model was established (Figure 19c). In this model, compounds were classified based on their F_{ic} and cellular potency. The F_{ic} and cellular potency thresholds can be adjusted to reflect the tolerated F_{ic} and the desired potency of the discovery program (for this set, they were fixed at $F_{ic} = 1$ and $pGI_{50} = 6$). Class 1 compounds were active in cellular assays and their concentrations at the target were equal to or higher than the nominal concentration added to the cells; class 2 compounds were active in cells, despite their access to the target being restricted; class 3 compounds were inactive in cells, even though they had high F_{ic} ; and class 4 compounds were not active in cells and displayed low F_{ic} .

For this compound set, most compounds (71%) belonged to either class 1 (23%) or class 4 (48%) (Figure 19c). Compounds in class 1 are active because of their high F_{ic} , while compounds in class 4 are inactive because their

access to the target is limited. Furthermore, only 18% of compounds in this set were in class 2 and 11% were in class 3. Compounds appear in class 2 if they are promiscuous, or if they have high biochemical potency.²⁰⁴ Class 3 compounds have either low biochemical potency, or their target protein is not relevant for the cellular response being studied.²⁰⁵

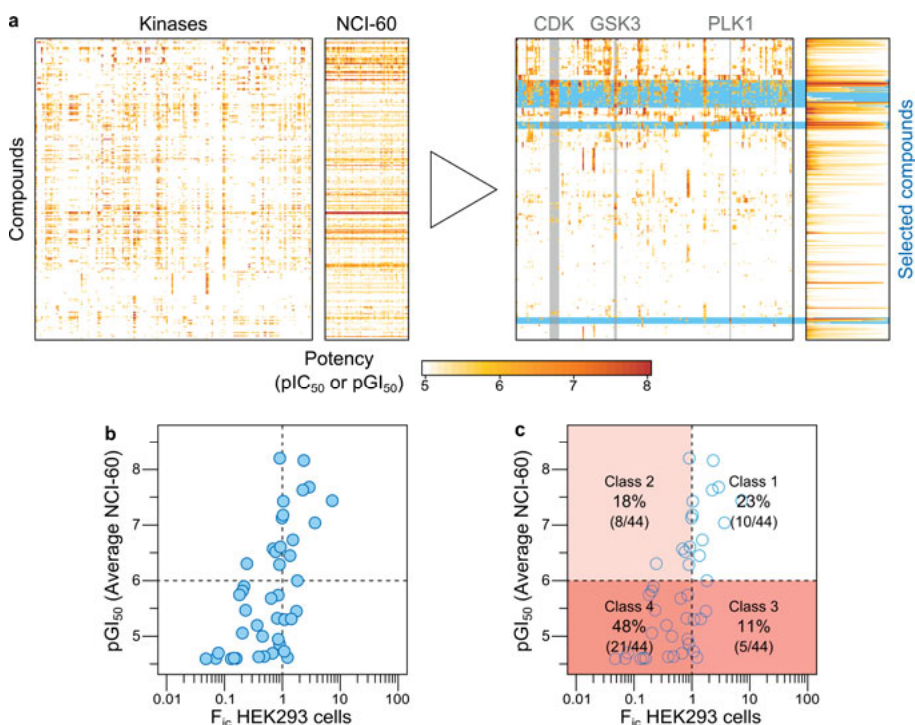


Figure 19. Intracellular bioavailability (F_{ic}) of compounds from the published kinase inhibitor set (PKIS). **(a)** Biochemical and cellular data for PKIS. Each row represents one compound and each column represents one kinase of the Nanosyn panel or one cell line of the NCI-60 panel. Colors represent the potency of the compounds (pIC₅₀ for kinases and pGI₅₀ for cell lines). In the left panel, compounds, kinases and cell lines are sorted alphabetically. In the right panel, compounds were clustered based on their biochemical data and cell lines were sorted from the most potent to the least potent for each compound for better visualization of the cell growth inhibition potential of each molecule. Selected compounds are highlighted in blue and targets for which these compounds were initially developed are highlighted in grey. **(b)** Relationship between average pGI₅₀ in the NCI-60 panel and F_{ic} in HEK293 cells. **(c)** Classification model based on pGI₅₀ and F_{ic} , with thresholds at $F_{ic} = 1$ and pGI₅₀ = 6.

β -secretase 1 (BACE-1) inhibitors

A set of 25 BACE-1 inhibitors was used to further study the impact of F_{ic} on cellular drug response. This set displayed strong ‘cell drop-off’,²¹ i.e., compounds showed on average one order of magnitude lower potency in a cellular screen than in a biochemical assay.

F_{ic} of these compounds was measured in SH-SY5Y cells using a similar setup as in the cellular potency assay. As expected, these compounds displayed low F_{ic} (median = 0.27, interquartile range: 0.15 – 0.68). Using the classification model introduced for the PKIS dataset (with thresholds set at $F_{ic} = 1$ and cellular $pIC_{50} = 6$), most of these substances belonged to class 2 (56%; low F_{ic} , high cellular pIC_{50}) and class 4 (24%; low F_{ic} , low cellular pIC_{50}). The unexpectedly high number of class 2 compounds was justified by the high biochemical potency of these compounds, i.e., in spite of limited access to the target, the compounds were still active in cells due to their high biochemical potency. In fact, 7 of the 14 compounds in this category were among the strongest BACE-1 inhibitors, with a $pIC_{50} \geq 8$, two orders of magnitude higher than the cell potency cut-off.

Mitogen-activated protein kinase 14 (p38 α) inhibitors

A set of 35 p38 α inhibitors was used to explore the reasons for the low F_{ic} of some of the compounds. Similarly to the BACE-1 inhibitors, these compounds displayed ‘cell drop-off’.²¹

F_{ic} of these compounds was measured in peripheral blood mononuclear cells (PBMC), analogous to the setup of the cellular screen. As anticipated from the generalized presence of ‘cell drop-off’, all compounds displayed low F_{ic} (median = 0.088, interquartile range: 0.069 – 0.19) and belonged to classes 2 (74%) and 4 (26%). Class 2 compounds were strong inhibitors of p38 α (pIC_{50} in biochemical assay ≥ 7 , two orders of magnitude more potent than in the cellular assay). Further studies showed that the F_{ic} and cellular potency of these compounds was hampered by the presence of extracellular proteins in the cell culture medium (Figure 1), and by active transport mechanisms (Figure 2).

Conclusions

In this thesis, a novel methodology was developed for the measurement of intracellular unbound drug concentrations. This methodology was applied to nearly 200 drug-like compounds in multiple cell types (including primary liver cells, and blood cells). Cellular drug-binding was correlated across multiple cell systems (including tissues). Furthermore, intracellular unbound drug concentrations were affected by drug transporters. More specifically, OATP1B1 increased the cellular levels of its substrates, whereas P-gp contributed to a decrease of intracellular concentrations of its substrates. Additionally, target engagement was correlated with intracellular concentrations of active metabolites of pro-drugs targeting the intracellular target thymidylate synthase. Finally, low intracellular unbound concentrations provided an explanation for the lack of cellular activity of drugs targeting intracellular proteins. In summary, the methodology developed here can be applied in drug discovery to predict if compounds reach their intracellular targets. Additionally, by providing information on the amount of drug available to be metabolized or excreted, this methodology provides important information for *in vitro* predictions of drug elimination.

From the work presented herein, it can be concluded that:

- the methodology developed can be used for drug-like compounds in a wide range of cell types to provide information about the amount of drug bioavailable in the cell interior
- a commonly used cell line, such as HEK293 cells, can be used to predict binding in more complex cell-based systems
- drug transporters can affect intracellular unbound drug concentrations and hence drug activity or elimination
- the amount of unbound drug that reaches intracellular targets influences target engagement and the subsequent response

Future perspectives

This thesis describes the development of a new methodology to measure intracellular unbound drug concentrations. In this work, the methodology was applied to small molecule drugs, generally within the boundaries of Lipinski's rule-of-five. However, it should be possible to extend its application to other xenobiotics, such as environmental toxins and pollutants, to predict their intracellular toxicity. This technique might also be applied to larger compounds, such as peptides or macrocycles. These compounds generally have low intracellular bioavailability and require formulation strategies to reach the interior of the cell. The methodology presented here could act as a simple tool for the screen of effective formulations.

The results in this thesis suggest that $f_{u,cell}$ is mainly driven by membrane partition, however further studies on which cellular structures drugs bind to need to be performed. Such studies could provide an explanation for the observed correlation between binding to a simple cell line and binding to more complex systems, e.g., tissues. Furthermore, it could open the possibility for accurate predictions of drug binding to any cell-based system of known composition, resulting in a reduction of experimental efforts and an increased throughput of determination of intracellular unbound drug concentrations. To achieve this goal, the composition of different cell types can be studied together with measurements of cellular drug binding in those cells. Alternatively, it is also possible to measure binding to isolated cell components (e.g., phospholipids, proteins, and nucleic acids). Further, technologies combining the cellular thermal shift assay (CETSA) with mass spectrometry-based proteomics could be used to provide information on which proteins drugs are bound to.

In parallel to the measurement of intracellular drug binding, the methodology developed in this thesis requires the measurement of total intracellular drug concentrations. Since the technique used relies on bulk analysis, information about the precise location of the drug in the cell interior is lost. Methods that provide this information can be used together with the methodology developed in this thesis to understand where the drug is located. These include, for example, imaging methods with high spatial resolution, such as super-resolution fluorescence microscopy or secondary ion mass spectrometry imaging (SIMS). If these techniques could be used on large datasets, it could be possible to find the molecular properties that determine the subcellular distribution of drugs to individual organelles. This could allow the de-

sign of drugs that accumulate in compartments where their target is located, while avoiding accumulation in other organelles.

The increased knowledge on protein expression made possible by advances in mass spectrometry-based proteomics can also further improve the understanding of the cellular drug disposition and response. The quantification of drug transporters and metabolizing enzymes is useful for cell kinetic models. These models can be further developed by adding target expression and affinity data. This systems biology approach will facilitate the design of effective drugs that show minimal toxicity (i.e., drugs that are co-localized with the target, and that do not engage off-target proteins). The methodology presented in this study facilitates the validation of these models.

Svensk populärvetenskaplig sammanfattning

De flesta läkemedel utövar sin effekt genom att binda till proteiner. Dessa så kallade målproteiner finns oftast inuti de celler man vill behandla. Där finns också de flesta proteiner som eliminerar läkemedel från kroppen genom att bryta ned dem, så kallade metaboliska enzymer. Dessutom binds läkemedlet ospecifikt till andra komponenter i cellen, t.ex. fettmolekyler i cellens membran. Det är bara den fraktion av läkemedelsmolekylen som inte är uppbunden som kan binda till målproteinet. Denna fria fraktion varierar mellan olika läkemedelsmolekyler.

Historiskt har man ansett att den dos läkemedel som återfinns i fri form i blodet också kommer att nå sitt målprotein inne i cellen, något som senare visat sig vara felaktigt då läkemedelskoncentrationen i blodet inte sällan skiljer sig från den i cellen. Resultatet av detta kan bland annat vara att läkemedlets effekt och eliminering från kroppen sker annorlunda än beräknat. Eftersom brist på effekt är den vanligaste orsaken till misslyckade kliniska läkemedelsprövningar är det viktigt att kunna bestämma den testade läkemedelsmolekylens fria fraktion inne i målcellen. Det är dock mycket svårt och inte etiskt försvarbart att utföra sådana tester rutinmässigt eftersom de skulle kräva att man tar vävnadsprover från friska organ hos försökspersonerna.

I avhandlingen utvecklas en ny, enkel och småskalig provrörsmetod för att mäta koncentrationen av fritt läkemedel inuti målcellen. Inte oväntat visade det sig att mer fettlösliga, lipofila, läkemedel i större utsträckning fångades upp och band till olika cellkomponenter. I dessa studier användes en lättodlad cellinje som ofta används i laboratorieforskning. Resultaten överensstämde väl med tidigare studier i mera komplexa system, såsom leverceller eller hjärnvävnad. Den nya provrörsmetoden har redan fått stort genomslag och används nu rutinmässigt av flera läkemedelsföretag i tidig läkemedelsforskning istället för djurvävnader.

I nästa steg undersöktes hur så kallade transportproteiner påverkar läkemedelsupptaget i cellen. Transportproteiner återfinns i cellernas membran och kan förflytta både kroppsegna ämnen och läkemedel in i och/eller ut ur cellerna. I denna studie användes transportören OATP1B1, som transporterar bland annat vanliga kolesterolsänkande läkemedel såsom statiner in i levercellerna där de har sin effekt. Denna transportör bidrog till en ökad läkemedelskoncentration i cellen. Omvänt bidrog transportören P-gp, som transporterar kroppsegna ämnen och läkemedel ut ur cellerna till att läkemedelskon-

centrationen i cellen minskade. Olika transportörer hade därmed olika påverkan på koncentrationen av fria läkemedelsmolekyler inne i cellerna.

I sista delen av avhandlingen studerades hur den uppmätta mängden fritt läkemedel inne i cellerna korrelerade till såväl läkemedlets bindning till målproteinet som dess effekt. Här användes olika substanser som hämmar tymidylatsyntas, ett viktigt målprotein inom cancerterapi. Försöken visade att ju mer fritt läkemedel som fanns inne i cellen, desto mer läkemedel kunde också binda sitt målprotein. Därefter studerades hur cellen i sig påverkas av upptagna läkemedelskandidater som visat sig vara aktiva (så kallade hits) i olika screeningskampanjer där 100 000-tals molekyler testas mot ett visst målprotein hos läkemedelsindustrin. Här inkluderades substanser för behandling av cancer, Alzheimers sjukdom och inflammation. Det visade sig att substanser som hade en hög fri koncentration inne i cellerna hade den starkaste effekten, medan de som hade en låg fri koncentration inne i cellen var ineffektiva.

Sammanfattningsvis kan den nya enkla metoden bidra till att välja ut de läkemedelsmolekyler som har störst sannolikhet att vara effektiva inne i målcellerna och det är just därför som den omedelbart blivit attraktiv för läkemedelsindustrin. Dessutom kan man på ett tillförlitligt sätt förutse hur summan av olika transportproteiner och läkemedelsmetaboliserande enzymer bidrar till läkemedelskoncentrationen i olika målceller.

Sumário em Português

O interior da célula contém um grande número de proteínas com interesse farmacológico. Estas proteínas incluem não só alvos terapêuticos, mas também proteínas responsáveis pela eliminação de fármacos. É a fração livre de fármaco dentro da célula que está disponível para interações com essas proteínas.

No passado, a concentração livre intracelular tem sido considerada igual à concentração de fármaco livre no plasma. Contudo, estudos recentes mostraram que vários fatores podem levar a que estas concentrações não sejam sempre idênticas. Ignorar esta diferença resulta numa má previsão da eliminação de fármacos do organismo a partir de experiências *in vitro*. Além disso, o desconhecimento da quantidade de fármaco que chega ao seu alvo terapêutico foi apontado como uma das principais causas para a falha clínica de fármacos. Por estas razões, métodos que possibilitem a medição de níveis intracelulares de fármaco livre têm o potencial de melhorar as previsões da farmacocinética e aumentar o número de fármacos que chega ao mercado.

Esta tese descreve o desenvolvimento de um método simples e geral para a medição da concentração de fármaco livre no interior de células. O método desenvolvido baseou-se na determinação em paralelo da fração de fármaco livre intracelular ($f_{u,cell}$) e da concentração intracelular total de fármaco (K_p). Estes dois parâmetros foram combinados para calcular a biodisponibilidade de fármaco dentro da célula (F_{ic}). Para a determinação da $f_{u,cell}$ foi utilizada uma técnica miniaturizada de diálise. Os níveis intracelulares de fármaco foram medidos através da incubação de células com o composto até se atingir um equilíbrio. A quantificação de fármacos foi feita com recurso a cromatografia líquida acoplada a espectrometria de massa em tandem. Este método foi posteriormente aplicado em diversas moléculas e linhas celulares para estudar o efeito de transportadores de membrana nos níveis de fármaco disponíveis no interior da célula. Foi também estudado o efeito dos níveis intracelulares de fármaco na ligação a alvos terapêuticos e na resposta celular induzida pelo fármaco.

A primeira parte da tese (Papers I e II) foca-se no desenvolvimento dos componentes individuais da metodologia numa linha celular simples (células HEK293). Em primeiro lugar, foi demonstrado ser possível utilizar apenas um valor de $f_{u,cell}$ para cada fármaco, uma vez que a ligação a estruturas celulares demonstrou ser independente da concentração de fármaco utilizada. Este resultado permitiu também avaliar a possibilidade de combinar vários

fármacos aquando da determinação da $f_{u,cell}$. Uma vez que os valores da $f_{u,cell}$ não foram afetados quando medidos na presença de até oito compostos diferentes, foi possível acelerar o processo de medição e reduzir a carga experimental. De seguida, as propriedades moleculares que determinam a $f_{u,cell}$ foram estudadas. Fármacos lipofílicos e com uma elevada superfície disponível para interações apresentaram maior ligação a componentes celulares, enquanto fármacos com carga negativa e aromaticidade demonstraram menor ligação a elementos da célula. No conjunto, estes resultados sugerem que a $f_{u,cell}$ é o resultado da interação com membranas fosfolipídicas. Estes resultados foram também confirmados pela má correlação entre a $f_{u,cell}$ em células HEK293 e a ligação de fármacos às proteínas plasmáticas (este último sistema constituído principalmente por proteínas), mas boa correlação com a ligação a outros sistemas, como microsomas hepáticos, hepatócitos, ou cérebro (estruturas que incluem também lípidos na sua constituição). Após a medição da $f_{u,cell}$, foi determinado que a maioria dos fármacos requerem 45 minutos para atingir um equilíbrio de acumulação (K_p). Finalmente, foi determinada a F_{ic} , utilizando os valores da $f_{u,cell}$ e da K_p . Nas células HEK293, que expressam baixos níveis de transportadores de membrana e de enzimas, a F_{ic} esteve principalmente correlacionada com a ionização dos fármacos. Fármacos com características básicas acumularam-se em compartimentos celulares com um pH baixo, como por exemplo lisosomas, e fármacos com características ácidas tiveram acesso limitado ao interior da célula devido ao baixo pH no interior comparado com o exterior.

Na segunda parte da tese (Paper III), a metodologia desenvolvida foi aplicada ao estudo do efeito de transportadores de membrana na F_{ic} . Para isso, células HEK293 foram transfetadas com o transportador de influxo “organic anion-transporting polypeptide 1B1” (OATP1B1). Nestas células, os substratos desta proteína demonstraram níveis mais elevados de F_{ic} . Foi também demonstrado que este efeito depende da concentração de fármaco, já que a concentrações mais elevadas o transportador foi saturado. De seguida, o efeito contrário foi demonstrado para o transportador de efluxo “P-glycoprotein” (P-gp), i.e., que a F_{ic} de fármacos foi reduzida por este transportador. Finalmente, a metodologia foi utilizada em hepatócitos humanos mantidos em diferentes condições de cultura, de modo a alterar a expressão de transportadores. Foi observado que a F_{ic} variou na direção prevista de acordo com a expressão de transportadores e das características do fármaco. Este resultado demonstrou que esta metodologia pode ser utilizada para medir o impacto de transportadores de membrana na F_{ic} .

A terceira parte da tese (Paper IV) mostrou a importância da concentração intracelular de fármaco para os níveis de ligação ao alvo terapêutico. Para o efeito, foram seleccionados compostos que demonstraram ligar-se à timidilato sintase num ensaio biofísico denominado “cellular thermal shift assay” (CETSA). Os fármacos seleccionados necessitam de ser ativados antes de se

ligarem à enzima, i.e. pró-fármacos. Os níveis intracelulares destes fármacos e respectivos metabolitos foram medidos em função do tempo. Foi observada uma correlação entre os níveis de metabolito ativo e os níveis de estabilização do alvo terapêutico. Estes resultados demonstraram que a metodologia desenvolvida pode ser usada para estudar a cinética de distribuição e metabolismo intracelular de fármacos, sendo possível prever a extensão de ligação ao alvo terapêutico.

Na quarta parte desta tese (Paper V), demonstrou-se a importância da F_{ic} na resposta farmacológica celular. Para este efeito, foram utilizados três conjuntos de compostos. O primeiro conjunto foi baseado numa biblioteca pública de inibidores de cinases. Desta biblioteca foram selecionadas moléculas com potencial para inibir o crescimento de células e cujos alvos terapêuticos eram cinases localizadas no interior da célula. Foi observado que a F_{ic} estava correlacionada com a potência destes compostos em células. Este resultado demonstrou que estes fármacos necessitam de chegar ao seu alvo terapêutico para exercerem a sua atividade, i.e., fármacos que não atingem concentrações suficientemente elevadas dentro da célula não demonstraram atividade. De seguida foi utilizado um grupo de inibidores potentes da β -secretase 1 (BACE-1) com fraca resposta em células SH-SY5Y. Uma baixa F_{ic} destes compostos nas células explicou a ineficiência destas moléculas. Finalmente, estes resultados foram confirmados com um grupo de inibidores da “mitogen-activated protein kinase 14” (p38 α). Tal como os inibidores da BACE-1, também estes compostos demonstraram F_{ic} limitada em células mononucleares do sangue periférico e, por essa razão, baixa atividade nessas células. As razões para uma baixa atividade foram exploradas, tendo sido demonstrado que a presença de soro fetal bovino no meio de cultura limita a quantidade de fármaco disponível para entrar na célula.

Em suma, a metodologia apresentada nesta tese é simples de utilizar e aplicável a diversos fármacos e a diversas linhas celulares. Esta técnica pode ser utilizada durante o processo de desenvolvimento de fármacos para garantir que os compostos atingem os seus alvos terapêuticos. Para além de permitir determinar a quantidade de fármaco disponível para interação com os alvos terapêuticos, a metodologia desenvolvida fornece informação importante para previsões de eliminação feitas a partir de experiências *in vitro*. No futuro, esta metodologia poderá ser expandida para incluir outros xenobióticos, como toxinas ambientais.

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