LITERATURE REVIEW:
INTRACELLULAR DISTRIBUTION OF DRUGS

EXPERIMENTAL WORK:
SUBCELLULAR FRACTIONATION PROTOCOL TO ISOLATE PLASMA MEMBRANE AND CYTOPLASM OF MDCKII CELLS

Karoliina Huusko
University of Helsinki
Faculty of Pharmacy
Division of Biopharmaceutics and Pharmacokinetics

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Tiedekunta - Fakultet– Faculty	Osasto-Sektion-Department
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Tekijä/Författare – Author
Karoliina Huusko

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Tiivistelmä/Referat – Abstract
Intracellular drug sequestration is useful to understand when designing new drugs with intracellular targets. The knowledge of the intracellular distribution can also help to understand the side effects and pharmacokinetics of a drug, as well as the lack of response in e.g. some multidrug resistant cancer cells. Intracellular concentrations are also important to know when predicting the role of active transport in the overall transport process when binding site of the transporter is intracellular. The literature review describes the mechanisms causing intracellular drug sequestration along with the consequences of intracellular drug sequestration and methods that are used to study it. Alterations of intracellular distribution of anticancer drugs in multidrug resistant cancer cells are also described as an example of the many factors affecting the distribution pattern of the drugs inside cells. Understanding these mechanisms is valuable when designing strategies to overcome the multidrug resistance.

The most commonly applied methods for studying intracellular concentrations of drugs are based on fluorescence microscopy. In experimental work, subcellular fractionation protocol is introduced and applied to determine the concentration of CDCF, clotrimazole and celiprolol *in vitro* in the plasma membrane and cytoplasm of MDCKII cells. CDFC and celiprolol are substrates of the MDR1 transporter and clotrimazole is an inhibitor. Concentrations in the fractions were measured in wild type cells and in MDR1-transfected cells with and without MDR1 inhibitor verapamil to see if the transporter had an effect on the concentrations. Also the effect of lipophilicity of the drug on partition between plasma membrane and cytoplasm was reviewed. Celiprolol showed a typical behaviour of the MDR1 substrate whereas CDCF and clotrimazole did not. Clotrimazole as a lipophilic compound was accumulated more to the plasma membrane than less lipophilic CDCF and celiprolol. Lipophilicity affected also to the ratio of K_m (or K_i)(determined from the concentration in extracellular fluid) and K_m (or K_i)(membrane) (determined from the plasma membrane concentration) values, with clotrimazole K_i(membrane) value being larger than respective K_i value, and CDCF and celiprolol K_m(membrane) values being smaller than their respective K_m values.

Avainsanat – Nyckelord – Keywords
Intracellular distribution, drug sequestration, subcellular fractionation, MDR1, MDCKII

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Tekijä/Författare – Author

Karoliina Huusko

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Kirjallisuuskatsaus: Lääkeaineiden solunsisäinen jakautuminen
Erikoistyö: Solunosien fraktionaatioon perustuva menetelmä plasma membraanin ja sytoplasman eristykseen MDCKII -soluista

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Tiivistelmä/Referat – Abstract

Lääkeaineiden solunsisäinen jakautuminen on hyödyllistä ymmärtää kehitettäessä uusia lääkkeitä, joiden vaikutuskohteet ovat solunsisäisiä. Tieto solunsisäisestä jakautumisesta voi auttaa myös ymmärtämään lääkkeiden haittavaikutuksia ja farmakokinetiikkaa, sekä tehon puutetta esim. lääkeaineille vastustuskykyisissä syöpäsoluissa. Solunsisäiset pitoisuudet ovat myös tärkeitä ennustettaessa aktiivisen kuljetuksen osuutta koko lääkeaineen kulkeutumisprosessissa, silloin kun kuljettajaproteiinin sitoutumiskohta on solun sisällä. Kirjallisuuskatsauksessa tarkastellaan lääkeaineen solunsisäisen jakautumisen mekanismeja, seurauksia joita jakautumisella on ja tapoja joita käytetään sen tutkimiseen. Osassa lääkkeille vastustuskykyisistä syöpäsoluista lääkeaineen solunsisäinen jakautuminen on muuttunut ja kirjallisuuskatsauksen lopussa tarkastellaan näitä tapauksia esimerkkeinä havainnollistamaan monia jakautumiseen vaikuttavia tekijöitä. Näiden tekijöiden ja mekanismien ymmärtäminen on tärkeää suunniteltaessa tapoja vastustuskykyisten syöpäsolujen tuhoamiseen.

Yleisimmin käytetyt tavat tutkia lääkkeiden solunsisäisiä pitoisuuksia perustuvat fluoresenssimikroskopiaan. Erikoistyössä esitellään menetelmä, jossa eri sentrifugaationopeuksia käyttäen erotellaan solunosia toisistaan. Menetelmää hyväksikäyttäen määritettiin CDCF:n, klotrimatsolin ja seliprololin pitoisuudet *in vitro* MDCKII -solujen plasma membraanissa ja sytoplasmassa. CDCF ja seliprololi ovat MDR1-proteiinin substraatteja ja klotrimatsoli on sen inhibiittori. Osioiden pitoisuudet mitattiin villityypin soluissa ja MDR1-proteiinia ilmentävissä soluissa MDR1-inhibiittori verapamiilin kanssa ja ilman sitä, jotta nähtiin onko kuljettajaproteiinilla vaikutusta pitoisuuksiin. Myös rasvaliukoisuuden vaikutusta lääkeaineiden jakautumiseen plasma membraanin ja sytoplasman välillä tutkittiin. Seliprololi käyttäytyi kokeissa kuten MDR1-substraatin oletetaan käyttätyvän, mutta CDCF ja klotrimatsoli eivät. Rasvaliukoisena yhdisteenä klotrimatsoli kertyi enemmän plasma membraaniin kuin vähemmän rasvaliukoiset CDCF ja seliprololi. Rasvaliukoisuus vaikutti myös K_m (tai K_i)(määritetty solunulkoisen nesteen pitoisuuksista) ja K_m (tai K_i)(membraani) (määritetty pitoisuuksista plasma membraanissa) suhteeseen, siten että rasvaliukoisella klotrimatsolilla K_i(membraani) oli suurempi kuin vastaava K_i ja vähemmän rasvaliukoisilla CDCF:llä ja seliprololilla K_m(membraani) oli pienempi kuin vastaava K_m.

Avainsanat – Nyckelord – Keywords

Solunsisäinen jakautuminen, lääkeaineiden jakautuminen, solunosien fraktiointi, MDR1, MDCKII

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CONTENTS

1 INTRODUCTION

For a drug to have a pharmacological response, it has to first absorb to the systemic circulation, partition into tissues and bind to its target. For some targets, such as receptors on the plasma membrane, binding takes place from the extracellular fluids. However, there are plenty of intracellular drug targets and for drug to reach them it has to also permeate through the plasma membrane and partition into the subcellular location in question. Approximately half of all known drug targets are intracellular. Examples of intracellular targets of drugs include nucleus for many anticancer drugs and gene or antisense therapy, lysosomes for drugs in lysosomal storage disease, mitochondria for antiapoptotic or some anticancer drugs or gene therapy, and plasma membrane for HIV fusion inhibitors (Breunig et al. 2008, Torchilin 2006). Number of compounds reported to have an intracellular target and the localization of the target are listed in Table 1. For these drugs, reaching the systemic circulation is not adequate, but they have to also permeate through the plasma membrane and preferably sequester into a certain subcellular location to induce the pharmacological action. Intracellular distribution of these drugs is useful to know in order to evaluate the concentration at the site of action. The concentration of the drug in that specific intracellular compartment determines the therapeutic effect of a drug while accumulation into another, non-target, compartment can result in side effects and lack of response.

Table 1. Number of compounds reported to have an intracellular target and the localization of these targets (Zheng et al. 2011) .

Localization	No. of compounds	%	No. of references
total	967	100	448
endolysosomes	226	23	96
mitochondria	259	27	136
nucleus	123	13	67
plasma membrane	162	17	75
endoplasmic reticulum and Golgi apparatus	37	4	26
cytosol	59	6	36
multiple sites	101	10	71

The knowledge of intracellular drug distribution and factors affecting it can also be utilized in drug development: drugs can be designed to target certain intracellular compartments or avoid them. Intracellular targeting strategies are just in its infancy and currently the drugs with intracellular targets are usually designed to be plasma membrane permeable i.e. they pervade the entire cell and not just the specific subcellular compartment (Rajendran et al. 2010).

Some drugs are substrates to transporters whose binding sites are inside the cell (Duvvuri, Krise 2005). In this case, the knowledge of drug distribution can be utilized when predicting the role of active transport in the overall transport process. If the binding site of a transporter is inside a certain cellular compartment, the rate of transport depends on the concentration of the drug in that cellular compartment. One example is an efflux transporter P-glycoprotein (MDR1, ABCB1) whose binding site locates in the plasma membrane of cells (Raub 2006).

Drug sequestration into organelles not containing the target of the drug is also suggested to be one mechanism causing multidrug resistance in some cancer cells (Duvvuri, Krise 2005). Lysosomal accumulation of weakly basic anticancer drugs seems to play a major role in multidrug resistance of cells, which do not express common efflux transporters, such as MDR1, on their plasma membrane (Duvvuri et al. 2005). Accumulation of drugs into non-target compartments results in decreased concentrations in the nucleus, which is the site of action for many anticancer drugs. Understanding the mechanisms behind drug sequestration in multidrug cancer cells is valuable when designing strategies to overcome the multidrug resistance.

Intracellular distribution of small molecule drugs is not commonly studied (Duvvuri, Krise 2005). Perhaps the most frequently used methods are those based on fluorescent microscopy so the knowledge of intracellular distribution is somewhat restricted to fluorescent compounds (Duvvuri et al. 2004a). Subcellular fractionation is also used, but the problem with that method seems to be the diffusion of drug between compartments during the fractionation. Despite the lack of research in the field of intracellular distribution, the mechanisms that affect the distribution and sequestration

of drugs inside cells are quite well understood. Determination of intracellular drug concentrations is not routinely demanded when new drugs are being developed, but e.g. with certain anti-infective agents, FDA recommends determining the degree of intracellular penetration (Food and Drug Administration 1998).

In this literature review the mechanisms causing intracellular drug sequestration are described along with the consequences of intracellular drug sequestration and methods that are used to study it. At first some background information about cell biology and intracellular trafficking of molecules are introduced. In the end of this review the alterations of intracellular distribution of the anticancer drugs in multidrug resistant cancer cells are described as an example of the many factors affecting the distribution pattern of drugs inside the cells.

2 CELL

The cell consists of distinct membrane-enclosed compartments, i.e. organelles, and cytosol (Alberts 2002a). The cytosol occupies over half of the total cell volume in eukaryotic cells (e.g. 54 % in hepatocytes) and the rest is occupied by organelles. Each organelle has its own properties, function and protein composition which can all affect the sequestration of drugs into these organelles. Lipid bilayers isolate organelles from their environment, and enable specific characteristics of the organelle to be maintained.

The pH values of the organelles vary from the basic mitochondria to the acidic lysosomes (Figure 1). Cytosolic and endoplasmic reticulum pH is near neutral and about 7.2 (Alberts 2002b). The acidic compartments of the cell include lysosomes, recycling endosomes, Golgi network and secretory vesicles (Larsen et al. 2000, Demaurex 2002). The most basic compartment is mitochondria, whose pH is about 8 (Llopis et al. 1998). An optimal pH is maintained by the balance between active and passive proton transport (Demaurex 2002). Diseases or pharmacological compounds can disrupt this balance thus resulting in changes in organelles function.

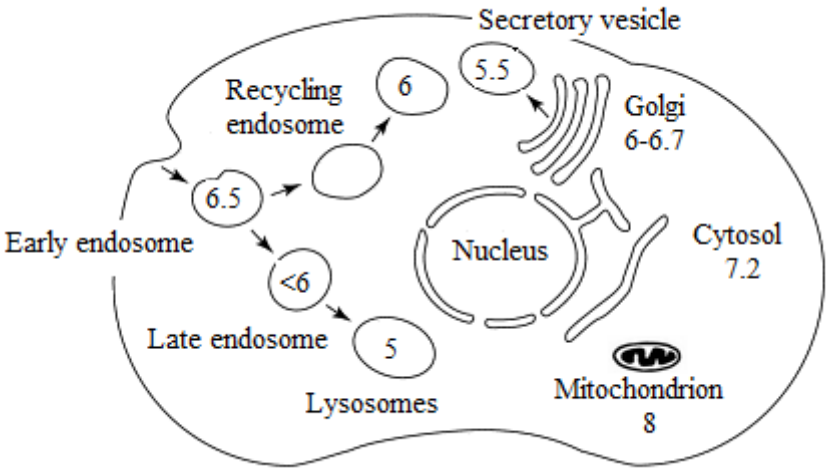

Figure 1. Cell organelles and their pH. Modified from Demaurex (2002).

2.1 Membrane trafficking and subcellular organization in cells

Cells are constantly recycling their constituents and newly synthetized material is imported to the right destination according to sorting signals included in its structure (Rajendran et al. 2010). The same sorting machinery is involved in trafficking of proteins from plasma membrane to subcellular compartments through endocytosis. Early endosomes act as a sorting station for endocytosed material and depending on the membrane interaction and components, which are involved in endosome formation, endocytosed material is imported to specific subcellular location. Pathogens, such as viruses or toxins, use these same mechanisms to enter subcellular locations and drugs can be targeted to certain subcellular compartment by linking a specific sorting moiety into its structure.

2.2 Permeation of drugs through the lipid bilayers

A drug has to be able to permeate through the lipid bilayer plasma membrane to get into the cell (Duvvuri, Krise 2005). When inside the cell, it has to also permeate through

organelle membrane if the target of the drug is inside the organelle. Lipid bilayers consist mainly of phospholipids and cholesterol (Krämer et al. 2009). Hydrophobic carbon chains of the phospholipids are organized towards inside of the bilayer and hydrophilic head groups form the outside of the bilayer. The hydrophobic core is the main barrier for the permeation of hydrophilic compounds.

Three main pathways of permeation are simple diffusion, carrier-mediated diffusion and active transport (Thomae 2007). Simple and carrier-mediated diffusion are passive processes which do not require energy and take place along concentration gradient. Active transport requires energy and the drug can be transported also against concentration gradient. Drugs can also be taken into the cell by endocytosis.

2.2.1 Simple diffusion

Krämer et al, 2009 reviewed two models for simple diffusion of drugs through the lipid bilayer: diffusion model and flip-flop model (Figure 2). In the diffusion model, the bilayer is assumed to be homogenous lipophilic solvent in which the drug diffuses according to Fick's first law:

$$J = \frac{D*P*(C_d - C_r)}{h}$$

, where D is the diffusion coefficient, P is the partition coefficient between bilayer and aqueous compartment, h is the thickness of the bilayer and C_d and C_r are concentrations in donor and receiver compartments, respectively.

However, in some cases permeation cannot be explained with this simple model and a better fit is achieved with the flip-flop model. Amphiphilic drugs are observed to arrange themselves like phospholipids in bilayers, hydrophobic region facing inside and hydrophilic outside (Siarheyeva et al. 2006). It is also observed, that polar head groups of the phospholipids can have electrostatic interactions with charged drug molecules and thus partitioning of the charged form of the drug can be higher than partitioning of the neutral form (Lombardi et al. 2009). In the flip-flop model a drug can translocate itself between two lipid leaflets, "flip" from the outer leaflet to the inner leaflet or

opposite, its hydrophobic moiety facing inside of the bilayer and hydrophilic outside. Depending on the kinetics of the translocation and partitioning into the membrane, diffusion can be described with either of the models. If the rate of translocation is much higher than the rate of partitioning, diffusion is better described with the flip-flop model and opposite holds true for the diffusion model.

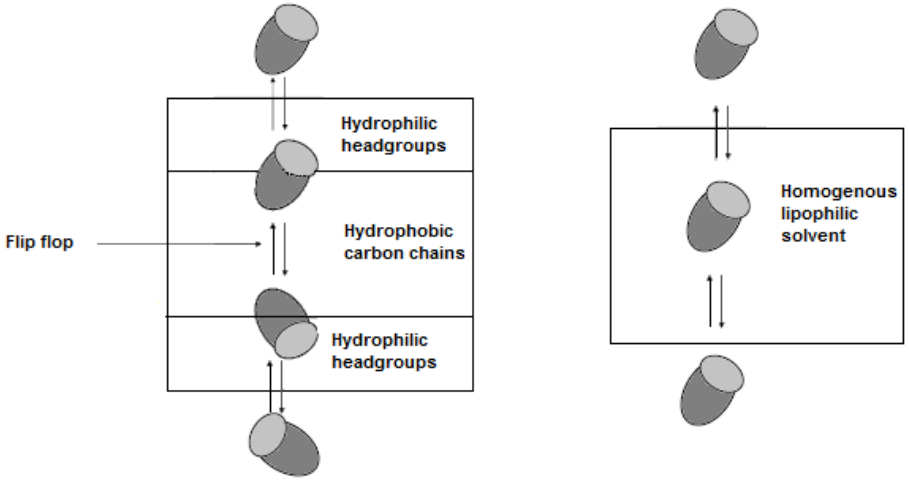

Figure 2. Flip flop model (on the left) and diffusion model (on the right) for permeation of drugs through the lipid bilayer. In the flip flop model, the hydrophilic moiety of the amphiphilic drug (light grey) is oriented towards the hydrophilic head groups of phospholipids and the hydrophobic moiety (dark grey) towards the hydrophobic carbon chains. Drug can translocate itself from the one lipid leaflet to the other and partition between lipid leaflets and aqueous phase. In the diffusion model, the molecule partitions into lipid bilayer, which is assumed to be homogenous lipophilic solvent.

According to the diffusion model, uncharged drugs are assumed to cross the bilayer more easily than charged ones, since they have higher partitioning in lipophilic core of the bilayer (Krämer et al. 2009). In the flip-flop model, the probability of flip-flop event determines the permeation rate and high dissolution of charged lipophilic drugs into polar head groups of the phospholipids is possible, thus enabling also charged species to permeate. Size of the drug affects as well, smaller molecules have better permeability than larger ones. For example molecules larger than 1 kDa do not permeate through the plasma membrane (Bareford, Swaan 2007).

Passive diffusion depends also on the organelle in question, since the characteristics of the organelle membranes are different. For example nucleus contains pores, which allows compounds smaller than 40 kDa to permeate (Keminer, Peters 1999).

2.2.2 Carrier-mediated and active transport

Drugs can also cross the lipid bilayer by transporter proteins or carriers. Transport can be either passive or active and it is saturable unlike transport by simple diffusion. Often transport is partly diffusional and partly mediated by carrier or active transporter. Energy for active transport can be derived from ATP (primary active) or from ion gradients (secondary active) (Hediger et al. 2004). Two widely studied transporter families are SLC (solute carrier) and ABC (ATP-binding cassette) families. Almost all passive transporters, secondary-active transporters and exchangers belong to the SLC family, and primary active transporters belong to the ABC family (Huang, Sadée 2006).

The members of the SLC family carry many natural cellular substances such as amino acids, sugars, peptides and organic anions and cations into the cell or out of the organelles (Dobson, Kell 2008). Some of them are known to be involved also in the drug transportation. For example some antibiotics and cardiac glycosides use carriers from the SLC family to entry the cell. Members of the SLC family are also found in the mitochondrial membrane and in the membranes of intracellular vesicles (Figure 3) (Hediger et al. 2004).

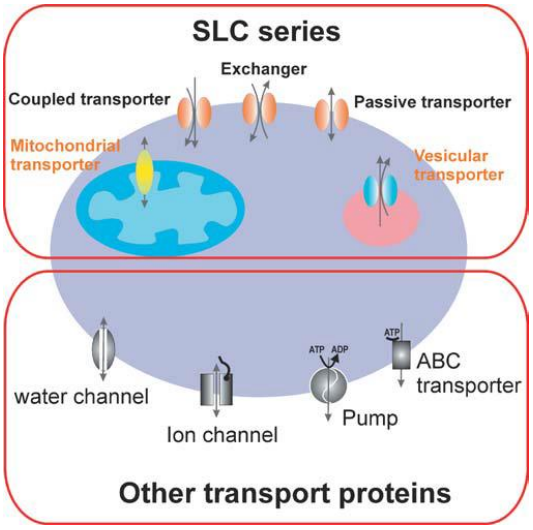

Figure 3. Solute carrier (SLC)- and other transporter proteins involved in the permeation of drugs or endogenous substances through the lipid bilayers of the cell (Hediger et al. 2004). Non-SLC transporters can also locate in the membranes of intracellular compartments.

Members of the ABC family transport ions, carbohydrates and drugs out of the cell or into cellular organelles. Subcellular locations of ABC transporters are plasma membrane, lysosomes, mitochondria, endoplasmic reticulum and peroxisomes (Borst, Elferink 2002). The effect of the intracellular ABC transporters on the drug sequestration especially in multidrug resistant cancer cells has been widely studied and is discussed later in this review.

2.2.3 Endocytosis

If the drug is large or very polar, it can be taken into cell by endocytosis. Endosomes formed by the plasma membrane and ingested contents will mature into acidic vesicles which can fuse with lysosomes where the molecule is decomposed (Bareford, Swaan 2007). Fusion with lysosomes can also be avoided depending on the membrane interaction and components which are involved in the vesicle formation. This will result in accumulation of endocytosed molecule into endosomes from where it can be transported to subcellular (non-lysosomal) compartments. Knowledge of the endocytic

mechanism can be exploited when developing drugs that are targeted to endosomes or lysosomes or designed to avoid them.

Endocytosis can be clathrin independent or dependent (Figure 4) (Tarragó-Trani, Storrie 2007). Clathrin-dependent endocytosis (Figure 4B) is more widely studied and better understood. Clathrin is a cytosolic protein which coats the vesicles formed when material is endocytosed. The content of clathrin-coated vesicles is then transported to early endosomes and after that to late endosomes and lysosomes or Golgi apparatus.

Clathrin-independent endocytosis can be caveolar or non-caveolar (Tarragó-Trani, Storrie 2007). In non-caveolar endocytosis, the lipid raft (plasma membrane microdomain) surrounds the endocytosed material which is transported to the early endosome for sorting. Caveolae are uncoated cell surface invaginations, a subtype of lipid rafts (Figure 4C). Caveosome can transport its contents directly to the endoplasmic reticulum or Golgi network, bypassing the early endosome. Alternatively, it can transport it contents to early endosomes. Other examples of clathrin-independent endocytic pathways are macropinocytosis (Figure 4A) and phagocytosis.

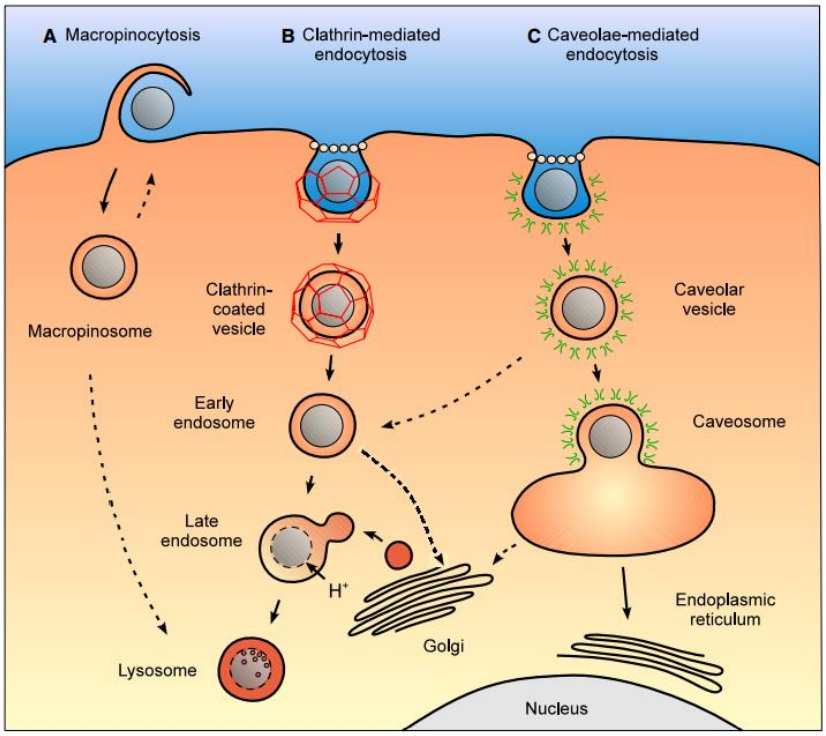

Figure 4. Intracellular trafficking after endocytosis by different mechanisms. A) Macropinocytosed material form macropinosome which can be fused with lysosome or recycle its content to the cell surface. B) In clathrin-mediated endocytosis material is first transported to early endosome where it can be delivered to lysosomal pathway or to Golgi apparatus. C) In caveloae-mediated endocytosis caveosomes are formed from which endocytosed material can be delivered to endoplasmic reticulum or Golgi apparatus or to early endosomes. Modified from Hillaireau, Couvreur (2009).

2.3 Cytoplasm as a diffusion barrier for macromolecules

Cytoplasm is an aqueous compartment, crowded with solutes, soluble macromolecules, skeletal proteins and membranes (Verkman 2002). It represents a major barrier for diffusion, especially for large compounds such as DNA. For small molecules, e.g. fluorescent probe BCECF with molecular weight less than 1 kDa, diffusion in cytoplasm was approximately 4 times slower than in water. The main reason for slower diffusion turned out to be the probe collisions with intracellular components (molecular crowding). For larger compounds, e.g. FITC-dextran with molecular weight of 2000 kDA, diffusion in cytoplasm was approximately 50 times slower than in water. In

addition to molecular crowding, also e.g. binding to intracellular components decreases the diffusion rate of DNA and proteins in cytoplasm.

3 INTRACELLULAR SEQUESTRATION MECHANISMS OF DRUGS

After permeation through the membrane, a drug can accumulate into specific intracellular compartments due to pH partitioning, electrochemical gradient, macromolecular binding or active transport (Duvvuri, Krise 2005). The drug can also partition into the lipid bilayer. If the drug is taken into the cell by endocytosis, it can accumulate in endosomes or lysosomes and possibly be transferred into other organelles of the recycling pathway. The mechanisms of intracellular drug sequestration are presented in Figure 5. Mechanisms, along with some examples of drugs which are utilizing these mechanisms and organelles most likely involved are presented in Table 2.

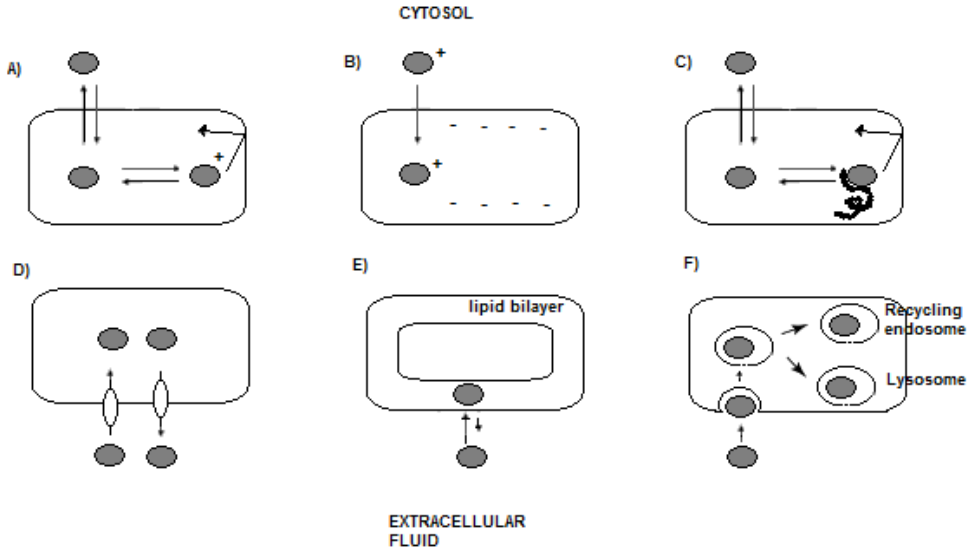

Figure 5. Intracellular sequestration mechanisms of drugs. A) In pH partitioning, a drug (grey oval) ionizes due to different pH of the organelle and cytosol and gets trapped inside the organelle. B) In electrochemical accumulation, positively charged drug accumulates into the organelle due to electrochemical gradient caused by negative charge inside organelle. C) In macromolecular binding, a drug binds to e.g. proteins and the complex is impermeable and gets trapped into the organelle. D) In active transport, a drug binds to the transporter and can be transported into or out from the cell or organelle. E) Lipophilic drugs can partition into lipid bilayer. F) In endosomal sequestration, after endocytosis, early endosome can fuse with lysosomes or develop into recycling endosome. Some toxins can avoid lysosomal pathway after endocytosis and get transported to the Golgi apparatus or endoplasmic reticulum instead. Figures A-C represent organelles and figures E-F whole cells. Figure D can represent both, the whole cell or the organelle.

Table 2. Intracellular sequestration mechanisms of drugs

Mechanism	**Drugs using this mechanism**	**Organelles which are most likely involved**	**References**
pH partitioning	Weakly basic drugs with pK_a near neutrality, e.g. daunorubicin	Acidic vesicles such as lysosomes, recycling endosomes and multivesicular bodies	(Gong et al. 2003, Altan et al. 1998, Chen et al. 2006)
Electrochemical accumulation	Lipophilic cationic compounds, e.g. TPP linked antioxidants	Mitochondria	(Brown et al. 2007, Kelso et al. 2001)
Macromolecular binding	Drugs with affinity to the nuclear or mitochondrial DNA, e.g. doxorubicin Drugs with affinity to cytoskeleton e.g. paclitaxel	Nuclei, mitochondria, cytosol	(De Beer et al. 2001, Lansiaux et al. 2002, Ashley, Poulton 2009, Jordan, Wilson 2004)
Transport processes	Substrates of transporters e.g. daunorubicin as a MDR1 substrate or doxorubicin as a MRP1 substrate	Cytoplasmic vesicles, such as lysosomes and multivesicular bodies	(Shapiro et al. 1998, Meschini et al. 2000, Gong et al. 2003, Van Luyn et al. 1998, Chapuy et al. 2008)
Partitioning into lipid bilayers	Highly lipophilic drugs	Plasma membrane, organelle membranes	(Wils et al. 1994)
Endosomal sequestration	Toxins, e.g. Shiga toxin Folic acid, cholesterol Drug carrier systems taken into cell by endocytosis, e.g. nanoparticles	Endosomes, lysosomes, Golgi apparatus, endoplasmic reticulum	(Tarragó-Trani, Storrie 2007, Bareford, Swaan 2007, Hillaireau, Couvreur 2009)

3.1 pH partitioning

pH partitioning, or ion trapping, is based on the different permeability of unionized and ionized form of the molecule through the lipid bilayer restricting two compartments with different pH (Duvvuri, Krise 2005). The ionization degree of weakly acidic drug in certain pH can be calculated with Henderson-Hasselbalch equation:

$$pH = pK_a + \log(\frac{[A^-]}{[HA]})$$

, where pK_a is the logarithmic acid dissociation constant of the drug, and $[A^-]$ and [HA] are concentrations of ionized and molecular form of the drug, respectively. As can be seen from the Henderson-Hasselbalch equation, the pKa of the drug and pH difference of the compartments dictate the ionization degree. For pH partitioning to occur, drug must have pK_a close to pH values of the compartments and permeability of ionized and unionized form must be clearly different.

pH partitioning is most likely to happen with weakly basic compounds between lysosomes and cytosol, because of the large pH difference between them (Duvvuri, Krise 2005). Since pH in the lysosomes can be as low as 4.5-5 (Mellman et al. 1986), weakly basic compounds can ionize and accumulate there. Also other acidic vesicles, such as endosomes, can sequester drugs. For weakly acidic compounds, accumulation can occur into mitochondria, but the degree of accumulation is not expected to be as large since the pH difference of mitochondria and cytosol is smaller, only about 0.8 whereas the difference between lysosomes and cytosol can be 2 or larger.

Lysosomal sequestration is a quite common phenomena, since many drugs marketed today are weakly basic amines, containing at least one basic nitrogen (Kaufmann, Krise 2006). In malaria and Q-fever, the target of the drug is inside lysosomes and this accumulation is favorable. However, more common is the reduced interaction with the target causing e.g. multidrug resistance in some cancer cells. For example, daunorubicin nuclear concentration in multidrug resistant human U-937 myeloid leukemia cells was 2.5-fold to threefold smaller than in drug sensitive counterparts, due to daunorubicin pH-partitioning into lysosomes (Hurwitz et al. 1997).

Amine-containing drugs can accumulate into lysosomes by passive diffusion or active transport from the cytosol (Kaufmann, Krise 2006). They can also undergo autophagocytosis, where autophagic vacuoles (cytolysosomes) are formed and fused with lysosomes or endocytosis from extracellular fluid. The accumulation degree by active transport, endocytosis or autophagocytosis is usually not that large, since there are passive mechanisms which balance them. One example of a compound accumulating into lysosomes is weakly basic lysotracker red, which concentration in lysosomes was about 20-fold larger than in extracellular medium (Duvvuri et al. 2004a). In addition to pH-partitioning, there could also be some other mechanisms responsible for accumulation into lysosomes, since experimentally observed concentrations are 3-15 times higher than the pH-partitioning theory predicts (Duvvuri, Jeffrey 2005).

At least two important parameters affecting drug sequestration into lysosomes can be named: pKa and permeability parameter called α (Kaufmann, Krise 2006). The higher the pKa of a compound, the greater is its accumulation into lysosomes if permeability through lipid bilayer is similar. Duvvuri et al. (2005) studied the accumulation of weakly basic model compounds (aminoquinoline isomers) to lysosomes in human leukemic cell line (HL-60) and observed lysosome/cytosol concentration ratios of about 4, 20, 50 and 60 when pK_a values of a compound were 5, 6, 7.4 and 9, respectively. The parameter α can be calculated by dividing the intrinsic octanol-water partition coefficient of a fully ionized base by that of unionized base. If α is 1, the partition coefficient of ionized and unionized form is the same, and thus the permeability can be assumed to be equal. The higher the value of α, the lower is the accumulation into lysosomes. Duvvuri et al. (2004b) studied the effect of α to lysosome/cytosol amount ratio of compounds in HL-60 cells and observed ratios of approximately 40, 5, 0.7 and 0.1 when α-values were 0.004, 0.02, 1 and 1.4, respectively. These effects of pKa and α are reasonable, since when pKa of a basic drug is increased, its ionized fraction in acidic lysosomes is increased, and if the ionized fraction cannot cross lipid bilayers, it accumulates into lysosomes. However, when permeability of the ionized and unionized form of the drug is similar (α is near 1), the ionized form cannot accumulate into lysosomes.

Another example of acidic vesicles presumably able to sequester drugs by pH-partitioning is multivesicular bodies (MVB) (Chen et al. 2006). Chen et al. used human erythroleukemic cells (K562) to study doxorubicin vesicular transport. K562 cells have MVB which are involved in endocytic trafficking and release their contents to extracellular medium after fusion with plasma membrane. MVBs are acidic vesicles to which drugs can accumulate due to pH-partitioning or membrane binding and doxorubicin efflux from the cell is at least partly mediated by MVBs.

3.2 Electrochemical accumulation

Accumulation due to electrochemical gradient can occur into mitochondria, since there is a net negative membrane potential in the inner membrane of mitochondria (Alberts 2002a). Cationic molecules can therefore accumulate into mitochondria, if they are lipophilic enough to cross the membrane.

The membrane potential of mitochondria is about 130-150 mV in living cells with inner side being negative (Murphy, Smith 2000). Mitochondrial sequestration can be calculated with Nernst equation:

$$Membrane\ potential\ (mV) = \frac{RT}{zF} * log\frac{[cation]_{in}}{[cation]_{out}}$$

, where R is the universal gas constant, T is the temperature, z is the number of electrons transferred in the reaction, F is the Faraday constant and $[cation]_{in}$ and $[cation]_{out}$ are concentrations of cations inside and outside of the mitochondria, respectively. In physiological temperature 37 °C, constant $\frac{RT}{zF} = 61.5\,mV$ and concentration inside mitochondria can be 100-fold or higher.

Mitochondrial accumulation of lipophilic cations can be utilized when developing drugs targeted to mitochondria. One widely used cation known to accumulate into mitochondria is triphenylphosphonium cation (TPP) (Murphy 2008). Brown et al. (2007) targeted antioxidant lipoic acid (LA) to mitochondria by conjugating it to TPP. Study was done with isolated rat liver mitochondria and accumulation into mitochondria was

several hundred-fold, consistent with the Nernst equation. Also Kelso et al. (2001) used TPP to target antioxidant ubiquinone to mitochondria of human osteosarcoma 143B cells. Uptake of ubiquinone-TPP complex (mitoQ) was decreased about 50 % by disrupting mitochondrial membrane potential with carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) indicating the importance of the membrane potential in mitochondrial accumulation.

Measuring mitochondrial accumulation in whole cells is challenging, since fractionation of cell depolarizes mitochondria and drug can be rapidly released (Murphy 2008). This release can be minimized using 4-iodobutyltriphenylphosphonium (IBTP), a TPP linked iodoalkyl system, which forms a stable thioether linkage with proteins. With IBTP it has been shown that almost all IBTP within cells is located in the mitochondria. Another challenge in whole cell experiments is that accumulation of lipophilic cations can disrupt mitochondrial function which sets the limits to the concentrations that can be used in experiments. Naturally, also when considering the drug therapy, the drug has to be potent enough to have the pharmacological effect with a concentration that is small enough not to disrupt mitochondrial function.

3.3 Macromolecular binding

Macromolecular binding can affect accumulation if the drug-macromolecule complex is membrane impermeable or if the permeability of the complex is reduced compared to the free drug (Duvvuri, Krise 2005). The binding of drugs to macromolecules can improve therapeutic effect if macromolecule is the target, however this is not always the case and therapeutic response can also decrease due to undesirable binding to non-target macromolecules. Only unbound drug can bind to its target and achieve the required response.

One example of macromolecular binding is the accumulation of DNA-binding drugs, such as doxorubicin, to nuclei (De Beer et al. 2001, Lansiaux et al. 2002). For example, 95 % of the total doxorubicin was bound to DNA in tumor tissue (Laginha et al. 2005). Reversible drug-DNA-interactions can be divided into three major classes: electrostatic

interactions, intercalation or groove-binding (Reddy et al. 1999). Drugs can also irreversibly bind to DNA. Knowledge of the interaction mechanisms has improved the possibilities to target e.g. anticancer drugs to the nucleus. Introduction of a positive charge to improve electrostatic interaction or linkage of DNA targeting ligands, such as intercalators or DNA-groove binders, into drugs are examples of strategies used to target DNA (Zutphen, Reedijk 2005).

Recently, it was shown that anticancer drugs, such as doxorubicin, bind also to mitochondrial DNA in addition to nuclear DNA (Ashley, Poulton 2009). The authors observed that doxorubicin concentration-dependently quenched fluorescence of DNA-binding dye PicoGreen in mitochondria of HCA2 human fibroblasts, possibly by displacing PicoGreen from the mitochondrial DNA. Fluorescence decreased approximately 50 % when doxorubicin concentration was 0.7 μM and 75 % when concentration was 3.5 μM compared to 100 % fluorescence when PicoGreen was incubated without doxorubicin.

Drugs can also bind to proteins in other organelles, to cytoskeleton in cytosol or to phospholipids in the plasma membrane, endoplasmic reticulum or Golgi apparatus (Zheng et al. 2011). Again, an example is found among the anticancer agents. *Vinca* alkaloids and paclitaxel are bound to microtubule in cytoskeleton disrupting the mitosis of cancer cells (Jordan, Wilson 2004).

Melanin pigment is a polymer found in hair, skin and eyes of the animals and it has been demonstrated to bind to many drugs both *in vitro* and *in vivo* (Karlsson, Lindquist 2013). Melanin is also found in the pigmented part of the brains. Many basic drugs such as chloroquine, amphetamine and atropine bind to melanin and may be accumulated into tissues containing melanin. Organic amines and metallic ions show usually the highest affinity to melanin. Accumulation of drugs into tissues containing melanin has been observed to lead to ocular toxicity and skin pigmentation and its possible role in neurodegeneration has also been studied. Intracellular organelles responsible for the synthesis of melanin are melanosomes and drug sequestration to these organelles because of melanin binding can be one factor decreasing the cytotoxic effect of anticancer drugs doxorubicin and daunorubicin on melanocytes (Svensson et al. 2003).

3.4 Transport processes

Accumulation due to active transport can occur for substrates of transport proteins. Transport proteins are located in the plasma membrane and in the membranes of intracellular compartments and use energy from e.g. ATP hydrolysis or ion gradient to transport substrates against concentration gradient (Alberts 2002b). Efflux transporters, such as MDR1 or multidrug resistant protein 1 (MRP1), carry substrates out of the cell and uptake transporters, such as organic anion transporter, carry substrates into the cell. These transporters are widely studied for their ability to either enhance drug uptake into cells or inhibit it when localized in the plasma membrane of the cells.

Studies have shown the existence of transporter proteins also in intracellular compartments, but it is unclear whether these proteins are functional there or if they are just on their way to the plasma membrane after biosynthesis (Duvvuri, Krise 2005). Ferrao et al. (2001) found out that MDR1 inhibitors could alter daunorubicin intracellular distribution in acute myeloid leukemia cells, and that total MDR1 (intracellular and surface) correlated better with the efflux than only surface MDR1. Based on these results, the authors suggested that the intracellular MDR1 is functional. Shapiro et al. (1998) made the same conclusion by demonstrating that daunorubicin accumulated in cytoplasmic, non-endocytic vesicles of multidrug resistant Chinese hamster ovary CHrC5 cells can be released with MDR1 inhibitor. Active transporters are suggested to play a role in drug distribution in nucleus, lysosomes, endosomes and mitochondria (Zheng et al. 2011).

3.5 Partitioning into lipid bilayers

If a drug has very high lipophilicity it can partition into lipid bilayers i.e. membranes that restrict organelles (Zheng et al. 2011). Sawada et al. (1999) studied the permeation of pyrrolopyrimidine antioxidants with different lipophilicity through Madin-Darby

canine kidney (MDCK) cell monolayers. Different lipophilicities were obtained by placing hydrophobic or hydrophilic groups, or groups capable of hydrogen bonding, to the structure. Compounds with more hydrophobic structure had decreased permeability compared to compounds with more hydrophilic groups and more hydrogen bonding groups, when considering both disappearance of the compound from donor side and its appearance on the receiver side. For example, when calculated log P values were increased from 4 to 6 to 8, the apparent permeability coefficients were decreased from 11 to 5 to 1 $x10^{-6}$ cm/s, respectively. The authors suggested this to be the consequence of increased cell partitioning of lipophilic compounds.

Wils et al. 1994 (Wils et al. 1994) reported similar results. They studied the permeation of drugs and chemicals with different lipophilicity through cell monolayers and found a cut-off value for log D of a compound to be 3.5 and for drugs with log D higher than 3.5 (lipophilicity increases as log D increases) permeability decreased with increasing lipophilicity.

However, studies with very lipophilic drugs are not that easy to execute, and drug adsorption into plastic devices and low solubility of the highly lipophilic compounds can complicate the interpretation of results, especially in permeation studies.

3.6 Membrane trafficking and endosomal sequestration

Retrograde trafficking pathway from endosomes to Golgi and ER has been largely studied with toxins and research of the utilization of these mechanisms in drug transport is also underway (Tarragó-Trani, Storrie 2007). If the drug is taken into cell by endocytosis it can be delivered progressively to early endosomes, late endosomes and lysosomes, where it is degraded but this path can also be avoided or minimized (Figure 4). Some toxins, such as Shiga toxin, are taken into the cell in clathrin-coated pits and can be transported into Golgi apparatus bypassing the lysosomal path and degradation. Degradation in lysosomes is also avoided by some toxins using caveolar uptake to gain entry to the smooth endoplasmic reticulum or Golgi apparatus. In theory, proteins from

these toxins could be used as a carrier for drugs targeting the Golgi apparatus and endoplasmic reticulum.

Nanoparticles can also be taken into the cell by endocytosis (Hillaireau, Couvreur 2009). Depending on the physicochemical properties of the drug and carrier, the target of the drug can be e.g. cytosol or lysosomes. Endocytosis can occur by clathrin-mediated- or caveolar pathway or by macropinocytosis (Figure 4). If endocytosis occurs by clathrin-mediated pathway or macropinocytosis, the target is usually lysosomes or cytosol. When using caveolar pathway, the target can be the Golgi apparatus or endoplasmic reticulum. Folic acid, albumin and cholesterol are examples of ligands internalized by caveolar endocytosis (Bareford, Swaan 2007). Internalization pathway can be controlled by physicochemical properties of the drug-carrier complex, such as the size and surface charge of the nanoparticle or by attaching targeting ligands into the surface of the nanoparticle (Hillaireau, Couvreur 2009).

4 CONSEQUENCES OF DRUG SEQUESTRATION INTO ORGANELLES

Intracellular sequestration of drugs can affect pharmacological action, metabolism and excretion if the drug is sequestered away from the site of action. The fraction available for these processes decreases which can cause lack of effect or decreased metabolism and excretion. Also other pharmacokinetic parameters of a drug can be altered due to sequestration into organelles. Accumulation of drugs into non-target organelles and their toxicity to these organelles can cause side-effects. Also phospholipidosis, induced by accumulation, can cause side-effects or drug-drug interactions as discussed later in this chapter. Finally, efficacy of transporters depends on the substrate concentration at the binding site, which can be altered due to accumulation into the organelle. Sequestration into organelles should be taken into account when discrepancies in behavior of drug are observed.

4.1 Alterations in pharmacokinetics

Apparent volume of distribution is usually very large if drug accumulates into organelles (Funk, Krise 2012). By definition, apparent volume of distribution can be calculated with equation: $V_d = \frac{A}{C}$, where A is the amount of the drug in the body and C is the concentration of the drug in plasma. Since the accumulation of drug into intracellular sites decreases the plasma concentration, volume of distribution increases.

Partition coefficient K_p, calculated from total intracellular and extracellular concentrations, increases because of intracellular sequestration. If the drug is taken into the cell by passive diffusion and no transport or metabolism processes are involved, K_p is assumed to be 1 i.e. concentration of unbound drug is the same in extracellular and intracellular spaces (Chu et al. 2013). However, sequestration caused by e.g. partitioning of drug into lipid membranes causes K_p values greater than unity, since the total intracellular concentration increases. If this deviation from unity is not taken into account, it can complicate e.g. the prediction of drug effects or transporter activity. However, it should be noted that under steady-state conditions intracellular sequestration does not change the unbound concentration in the cytosol, only total intracellular concentration is changed.

Lag-times observed with some drugs can be a consequence of sequestration. Some sequestration processes can be saturable. For example, pH-partitioning into lysosomes depends on the pH-gradient between lysosomes and cytosol. This pH-gradient is maintained by membrane bound ATPases and if buffering capacity of these ATPases is exceeded, it may cause saturable sequestration of lipophilic amines into lysosomes (Hallifax, Houston 2007). Saturable sequestration into organelles has been suggested to cause delayed response to drug therapy if sequestration site is not the site of action (Kaufmann, Krise 2006). This mechanism may cause lag-time observed with e.g. antidepressants, since saturation of lysosomal sequestration takes time, and only after this saturation, plasma concentration of antidepressants rises to therapeutic level.

Sequestration can also increase the half-life of the drug in the body (Kaufmann, Krise 2006). Sequestered drug is not available for metabolism and can act as a reservoir, from where the drug is slowly released.

4.2 Phospholipidosis

The function and structure of the organelle can change if there is extensive accumulation of drugs into it (Funk, Krise 2012). One extensively studied alteration is phospholipidosis. In drug-induced phospholipidosis, a cationic amphiphilic drug (CAD) that accumulates into lysosomes inhibits lipid metabolism there, resulting in accumulation of phospholipids into lysosomes and consequently increase in the volume of the lysosomes. The increase in lysosomal volume causes the increase in cellular uptake of compounds that are sequestered into lysosomes and drug-drug interactions or toxicity may follow.

Phospholipidosis occurs most commonly in lungs and liver, since they have the highest abundance of lysosomes, but there is no clinical evidence that CAD-induced phopholipidosis is detrimental to organs (Reasor, Kacew 2001).

4.3 Effect to transport processes

Intracellular sequestration is important to consider also when interpreting the results of transport experiments across monolayers with efflux transporters (Chu et al. 2013). Affinity of a drug to efflux transporter can be described with parameter K_m. K_m values calculated using extracellular concentrations can vary greatly depending on experimental conditions and transporter expression. If K_m values are calculated using intracellular concentrations, i.e. concentration in the site of action, more consistent results are obtained.

It is assumed that MDR1 substrate binding takes place in plasma membrane of cells (Hennessy, Spiers 2007). Consequently, partitioning of drugs into lipid membranes can improve the efficiency of MDR1 since the concentration of substrate in the site of action is increased. Inversely, if drug partitioning into lipid bilayers is scarce, efflux by MDR1 transporter can be assumed to be less efficient. Hence, the knowledge of the intracellular distribution of drugs is useful to know in order to predict the role of active transport in the overall transport process.

5 METHODS FOR STUDYING INTRACELLULAR DRUG DISTRIBUTION

5.1 Microscopic imaging methods

Fluorescence microscopy is commonly applied method when studying intracellular distribution of fluorescent compounds in cultured cells (Duvvuri et al. 2004a). It is a relatively easy method and it can be done with living cells, enabling one to study the kinetics of the drugs. However, there are some limitations. The compounds studied must have sufficient fluorescence, quantification is difficult and assay is sensitive to changes in pH, ionic strength and noncovalent interactions. Fluorescence can also be lost due to metabolism of the compound or self-quenching reactions. Also, when using fluorescence labels, labeling can alter the distribution of drug. For example, lysosomal sequestration of drugs in whole cells or in isolated lysosomes, have been studied with methods based on fluorescence (Kaufmann, Krise 2006).

Confocal Raman microscopy and secondary ion mass spectroscopy are more sensitive and general imaging methods, which are independent of fluorescence (Zheng et al. 2011). However, their use in intracellular drug distribution studies is still quite scarce.

5.2 Subcellular fractionation

Subcellular fractionation can separate the organelles based on their different physical properties (Pasquali et al. 1999). It consists of two steps: homogenization of cells and fractionation of the cell homogenate by centrifugation steps. Limitations in this approach are that it is rather low-throughput method and similar properties of different organelles, such as similar density of plasma membranes, Golgi membranes and endoplasmic reticulum, can complicate fractionation. Also different cytoskeletal organization in tissue cultured cells commonly used in research makes fractionation challenging. Efforts are made to overcome these challenges by e.g. density shift methods, immunoisolation or flow electrophoresis. Diffusion of the drug out from the organelle during isolation procedure can also be a problem with fractionation based methods. The advantage of subcellular fractionation methods is that analysis of non-fluorescent compounds is also possible. Quantification can be done with e.g. HPLC or mass spectrometry.

5.2.1 Classical subcellular fractionation

Classical subcellular fractionation involves homogenization of cells and fractionation of homogenate using sucrose density gradient (Figure 6) (Pasquali et al. 1999). Homogenization is important step and efforts should be made to prepare an ideal homogenate with no aggregates which can be lost during initial centrifugation steps for removal of nuclei and intact cells. Homogenization should also be mild enough to preserve the functionality of proteins and enzymes in the organelle. Postnuclear supernatant is centrifuged with sucrose density gradient. Membranes from different organelles have different density based on their protein to lipid ratio and composition. Gradient medium affects the degree of separation and most commonly used medium is sucrose. Separation can be based on velocity centrifugation, where the particle size and density affects the time it takes to pellet them, or equilibrium centrifugation, where particle moves into certain position in the gradient determined by its density. Validation

of fractionation protocol can be done with Western blotting, where antibodies against established organelle markers are used.

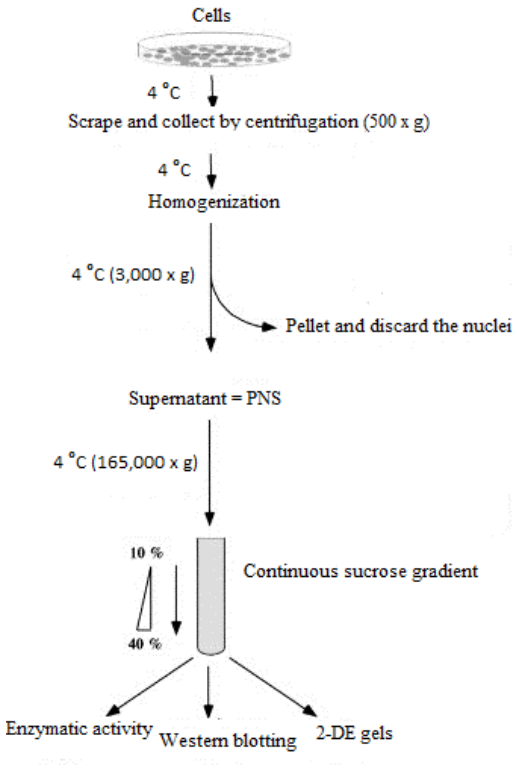

Figure 6. Classical subcellular fractionation using sucrose gradient. Cells are scraped and homogenized and after that, nuclei were removed by centrifugation. Postnuclear supernatant (PNS) is loaded on top of a sucrose density gradient to separate subcellular organelles. Modeled from Pasquali et al. (1999).

One study utilizing density gradient centrifugation is conducted by Duvvuri et al. (2004a), who incubated human leukemic U-937 cells with 5 μM lysotracker red or 0.5 μM doxorubicin for 2 hours and homogenized and fractionated cells into cytosolic, nuclear and lysosomal fractions. The concentration of drugs in fractions was measured by HPLC with fluorescence detection. They found out that lysotracker red was predominantly associated with lysosomes (concentration in lysosomes about 11 μM vs. in cytosol and nucleus up to 1 μM) while doxorubicin predominantly associated with nucleus (concentration in nucleus about 1.5 μM vs. in cytosol about 0.5 μM and in lysosomes about 0.25 μM).

5.2.2 Magnetic capture technique

Duvvuri and Jeffrey (2005) developed a method for studying the lysosomal sequestration of non-fluorescent compounds known to be accumulated into lysosomes: quinacrine and lysotracker red. At first, they incubated 1 μM compounds with multidrug resistant human acute promyeloid leukemia (HL-60) cells. Then they isolated lysosomes using iron-dextran particles which were taken up in the cell by endocytosis and thus concentrating in the lysosomes. Cells were homogenized, nuclei were pelleted and magnetic chromatography was used to capture iron-containing lysosomes from post-nuclear supernatant. Analysis was done with HPLC and the concentrations in lysosomes were 766 ± 69 μM for quinacrine and 60 ± 1.8 μM for lysotracker red.

5.2.3 Immunoisolation

Immunoisolation technique uses antibodies to fractionate organelles (Pasquali et al. 1999). Antibodies against selected antigens in organelles are used to capture the organelles. If antigen can be found in several organelles, pre-fractionation can be used for initial separation of organelles. There are different solid supports for immunoisolation, with magnetic beads being the most commonly used. Antibody is bound to the solid support and the complex is used to retrieve organelle of interest. The isolation of plasma membranes (Figure 7) (Lawson et al. 2006), mitochondria (Hornig-Do et al. 2009) and peroxisomes (Wang et al. 2012) are examples of immunoisolation with magnetic beads. With immunoisolation, very high purity of fractions can be achieved but the high cost and availability of specific antibodies can limit its use.

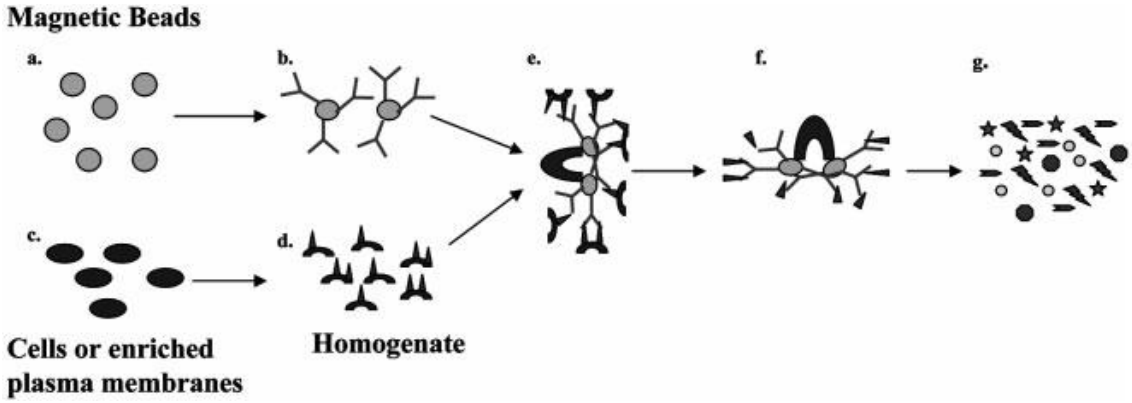

Figure 7.Isolation of plasma membranes using immunoisolation with magnetic beads (Lawson et al. 2006). (a) Magnetic beads with immobilized protein. (b) Magnetic beads are incubated with antibody against protein. (c) Cells or tissue. (d) Plasma membrane vesicles are recovered after homogenization. (e) Magnetic beads with immobilized antibody bind to proteins in plasma membrane vesicles and magnet is applied to isolate these complexes. (f) Membrane proteins are removed from complex by selectively solubilizing them. (g) Solubilized plasma membrane proteins are available for further analysis.

5.2.4 Electromigration

Electromigration is based on different charge of membrane vesicles from different organelles. This difference in charge results in different electrophoretic mobility (Pasquali et al. 1999).

In free flow electrophoresis (FFE), cell homogenate or fractions can be injected into a buffer between anode and cathode and membrane particles migrate in the electric field according to their membrane potential (Pasquali et al. 1999). FFE enables large amounts of material purified with high speed but it is usually not possible to use it as one-step technique for purification. FFE have been used in purification of plasma membrane vesicles and endosomes, most commonly used in combination with other methods. Quite recently, also purification of peroxisomes (Islinger et al. 2009) and mitochondria (Zischka et al. 2006) have been conducted with FFE.

Extended applications of FFE include density gradient electrophoresis (DGE) and immune free flow electrophoresis (IFFE) (Pasquali et al. 1999). In density gradient electrophoresis (DGE), homogenate is layered within sucrose gradient and the gradient

is separated by anode and cathode, resulting in the migration of membrane vesicles based on their density and membrane potential. DGE has been used to purify organelles belonging to lysosomal/endosomal pathway (Tulp et al. 1998). Limitations with DGE include long separation times and quite poor resolution. In immune free flow electrophoresis (IFFE) homogenate is incubated with antibodies against organelle markers prior FFE, resulting in reduced electrophoretic mobility of antigen-antibody complex. IFFE has been used to isolate rat hepatic peroxisomes (Völkl et al. 1997). As with other techniques using antibodies, the cost can limit IFFE's large scale use in purification of organelles.

5.2.5 Fluorescent activated organelle sorting

Fluorescent activated organelle sorting (FAOS) combines the labeling of organelles with fluorescent probes and separation of them on the basis of that (Böck et al. 1997). Usually the separation is done on the basis of physical properties, e.g. the density of intracellular membranes, and so the organelles with similar physical properties are hard to distinguish. In FAOS a fluorescent dye which accumulates in the organelle of interest is first incubated with cells which are then homogenized and subcellular fractionation is done by centrifugation and sucrose gradient step. After that, fractions are sorted in a flow cytometer (Figure 8), in which droplets of liquid to be tested are analyzed and if they are fluorescent, they are charged positively or negatively. The sorting is done electrically and it will further improve the purity of the fraction. In addition to purifying endosomes, FAOS has been used to purify secretory granules from mouse anterior pituitary cells (Gauthier et al. 2008). Sensitivity can be a problem, since small organelles can contain only small amounts of fluorescent probes to be detected by the device.

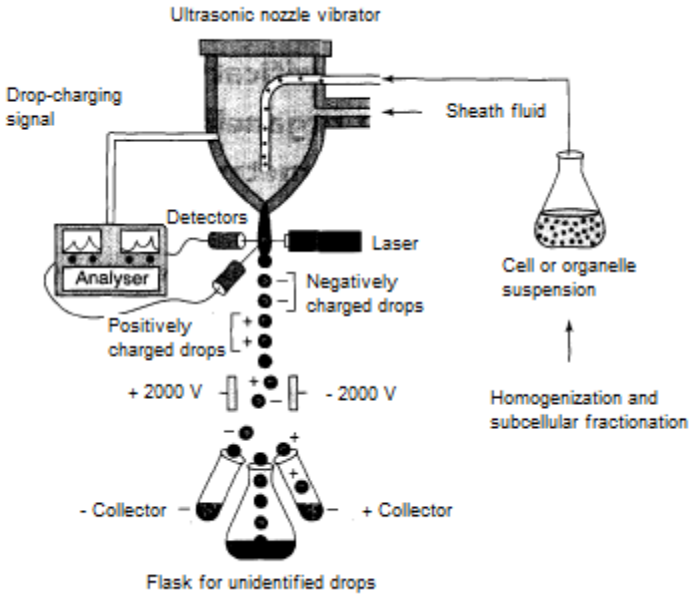

Figure 8. Flow cytometer sorts the droplets of test suspension into those containing fluorescent dye molecules and those not containing. Modeled from Böck et al. (1997).

5.3 Computational models

Computational models for predicting intracellular drug distribution can be divided into statistically based regression models and mechanism-based physiological models (Zheng et al. 2011).

Statistically based regression models, such as quantitative structure-activity relationship (QSAR) models, are used to predict quantitatively and qualitatively the existence of a drug in certain subcellular organelle (Zheng et al. 2011). Training set is used to provide the distribution pattern of drugs based on the physicochemical properties of the drugs. This model can then be used with different test set by using their physicochemical properties as input parameters.

Mechanism based physiological models predict distribution according to mass transfer calculations using Fick's law of diffusion or Nernst-Planck equation (Zheng et al. 2011).

Reliability and accuracy of a model depends largely on the quality of the input data and different experimental approaches can result in large differences in that (Zheng et al. 2011). Therefore universal models are hard to develop.

6 ALTERATIONS IN DRUG DISTRIBUTION IN RESISTANT CANCER CELL LINES

Understanding the mechanisms responsible for drug resistance in cancer cells can help to develop drugs that are not prone to resistance. Many cancer cells are more sensitive to anticancer drugs than normal cells, which enables drug therapy to destroy tumors but not normal cells (Simon et al. 1994). However, cancer cells can develop resistance and become less sensitive to anticancer drugs. Multidrug resistance is most likely multifactorial (Duvvuri, Krise 2005, Gong et al. 2003). There are many suggested characteristics causing multidrug resistance, such as decreased accumulation of drug, increased DNA repair and apoptosis defects. Also intracellular distribution differences between sensitive and resistant cells are suggested to cause multidrug resistance and the reasons for these differences are discussed in this chapter. Examples of hypothesized drug sequestering organelles in different multidrug resistant cells are provided in Table 3.

Table 3. Hypothesized drug sequestering organelles in different multidrug resistant cell lines (Duvvuri, Krise 2005).

Cell line	Sequestering organelles
NIH/3T3	lysosomes, Golgi, ER
DKLP	cytoplasmic vesicles
CHO	lysosomes
MCF-7	Golgi, lysosomes, recycling endosomes
V-79	cytoplasmic vesicles
HL-60	Golgi, mitochondria, lysosomes
P388	Golgi, secretory vesicles
KB3-1	Golgi, lysosomes
U 937	lysosomes
EPG85	secretory vesicles
SW-1573	cytoplasmic vesicles
AUXB1	cytoplasmic vesicles
K562	lysosomes, mitochondria, Golgi
LoVo	Golgi
CEM	lysosomes, Golgi
A2780	Golgi
PKSV-PR	lysosomes
MES-SA	lysosomes
Bone Marrow	cytoplasmic organelles
AML cells	Golgi

6.1 Difference in cytoplasmic pH

Many cancer cells have lower cytoplasmic pH than normal cells (Larsen et al. 2000). Altan et al. (1998) measured pH in the cytosol of human MCF-7 breast cancer cells and multidrug resistance MCF-7/ADR cells and observed the cytosolic pH in drug sensitive MCF-7 cells to be 6.75±0.3 whereas in resistant MCF-7/ADR cells it was 7.15±0.1. The acidification of cytosol in cancer cells improves the accumulation of weakly basic anticancer drugs to the cell, if ionized form of the drug is less permeable. If this acidification is abolished, like it is in resistant cells, accumulation decreases and this in turn can affect the saturable processes possibly dictating the intracellular distribution of drugs. With MCF-7 cells, accumulation of adriamycin into drug sensitive cells was about 2.5-fold greater than into resistant ones. Since intracellular concentrations are higher in sensitive cells, capacity of saturable processes such as vesicular transport may

be exceeded and concentration in cytoplasm increases. Cytosolic pH affects also the pH gradients between cytosol and organelles, which is important factor in intracellular distribution of anticancer drugs.

Changes in cytoplasmic pH can affect also vesicular function. Cosson et al. (1989) studied the effect of low cytoplasmic pH on the endocytosis and recycling of transferrin in hamster lung fibroblast (CCL39) cell line. Transferrin was used since it is a marker of receptor–mediated endocytosis using clathrin-coated pits. Researchers found out that the rate of transferrin accumulation in acidified mutated cells lacking Na^+/H^+ exchange activity was decreased 80 % compared to mutant cells that were not acidified. Also recycling of the internalized transferrin from the Golgi apparatus to the cell membrane was inhibited in acidified cells. They suggested the reason to be the slower withdrawal of clathrin-coated pits from the cell surface or organelle membranes in acid environment. Hansen et al. (1993) reported similar results with human Hep-2 carcinoma cells and transferrin and deduced that acidification of cytoplasm paralyzes clathrin to the membrane-bound state. If cytoplasm of drug sensitive cancer cells is more acidic, vesicular transport of drug out from the cell could be decreased compared to resistant cells and this can be one contributor to the multidrug resistance.

6.2 Difference in lysosomal pH

Many anticancer drugs are weakly basic with pKs between 7 and 9 (Larsen et al. 2000). Consequently, they can accumulate into acidic organelles because of the pH partitioning. The unionized fraction of the drug molecules can permeate through the membranes of cell and intracellular organelles. After permeation into acidic organelle, the molecule ionizes and accumulates there, since the permeability of the ionized form is lower than the unionized form. In drug sensitive cancer cell lines, the pH of lysosomes is disrupted and near to cytosolic pH whereas in resistant cell lines the pH of lysosomes is re-established to normal acidic pH (Table 4) (Duvvuri et al. 2005). Weakly basic anticancer drugs, such as daunorubicin, accumulate into acidic lysosomes in resistant

cells and concentration in target-site (nucleus) is decreased. This mechanism also protects the normal non-transformed cells from the cytotoxic effects of anticancer drugs.

Table 4. Intracellular pH gradients in multidrug resistant and sensitive cancer cells (Duvvuri, Krise 2005).

	Lysosomes	Cytosol	Lysosome to cytosol pH differential
Non-transformed cells			
3T3 fibroblasts	<5,0	7,40	> 2,4
Transformed cells (drug sensitive[a] and MDR[b])			
HL-60[a]	6,44 ± 0,17	6,96 ± 0,17	0,52
HL-60/ADR[b]	5,17 ± 0,14	7,12 ± 0,04	1,95
MCF-7[a]	>5,8	6,75 ± 0.3	<0,85
MCF-7/ADR[b]	5,1 ± 0,1	7,15 ± 0,1	2,05

Gong et al. (2003) detected a different intracellular distribution of daunorubicin in sensitive and resistant human acute promyeloid leukemia (HL-60) cells. In resistant cells, daunorubicin was sequestered into lysosomes whereas in sensitive cells, it was more evenly distributed throughout the whole cell. They measured the pH difference between lysosomes and cytosol, and detected that pH difference was 0.52 in sensitive cells and 1.95 in resistant cells. When abolishing cellular endomembrane pH gradients of resistant cells, sequestration of daunorubicin into lysosomes was abolished and intracellular distribution was similar with sensitive cells.

This pH difference results in large differences in intracellular distribution of drugs in resistant and sensitive cancer cells. For example concentration of daunorubicin in the nucleus of drug sensitive HL-60 cells was approximately 85-fold larger than in resistant HL-60 cells (Duvvuri et al. 2005).

6.3 Difference in pH of the recycling endosomes

In addition to lysosomes, drugs can accumulate into other acidic compartments in the cells, such as recycling endosomes. Altan et al. (1998) measured the pH within the recycling endosomes of human MCF-7 breast cancer cells and multidrug resistance MCF-7/ADR cells and observed the endosome pH in drug sensitive MCF-7 cells to be 6.6±0.1 whereas in resistant MCF-7/ADR cells it was 6.1±0.1. That can cause the sequestration of weakly basic anticancer drugs to the endosomes and away from their site of action.

6.4 Changes in vesicular architecture and localization

The distribution of lysosomes, trans-Golgi network and endosome compartment is different in resistant and sensitive cells. Altan et al. (1998) studied the distribution of these acidic compartments in human MCF-7 breast cancer cells and multidrug resistance MCF-7/ADR cells by labeling with specific fluorescent probes against these compartments. The authors observed that in drug sensitive cells these compartments are distributed throughout the cytoplasm whereas in resistant cells they are localized to one side of the nucleus. That could enhance the redistribution of drug into these vesicles away from the nucleus and thus protect the nucleus from anticancer drugs in resistant cells (Raghunand et al. 1999).

Changes in number, volume and membrane area of the endosomes can also alter the distribution of drugs in resistant cancer cells. Sehested et al. (1987) found out that doxorubicin-resistant murine P388 cells had increased volume (0 vs 4.7-7.8 μm^3), number (0 vs. 139-205 endosomes/cell) and membrane area (14.1-17.5 vs. 73.6-95.5 μm^2) of the endosome compartment compared to sensitive cells. In sensitive cells, the endosomal compartment was so small that median values were below detection limit in some cases (zero values), but as can be seen from the original article, the endosomal compartment was still present also in sensitive cells. Hurwitz et al. (1997) studied the architecture of human U-937 myeloid leukemia cell line and its doxorubicin-selected

variant U-A10 and found expanded acidic vesicles belonging to lysosomal compartment in resistant cells compared to sensitive cells. In resistant cells, daunorubicin was linearly accumulated into these vesicles, whereas in sensitive cells daunorubicin accumulation to these vesicles was nonlinear and saturated with larger daunorubicin incubation concentrations. Consequently, accumulation into these vesicles in resistant cells with larger, but still pharmacologically relevant, incubation concentration (500 ng/mL of daunorubicin for 1 hour) was 2-2.5-fold greater than in sensitive cells causing decreased concentrations of doxorubicin in the nucleus (Figure 9).

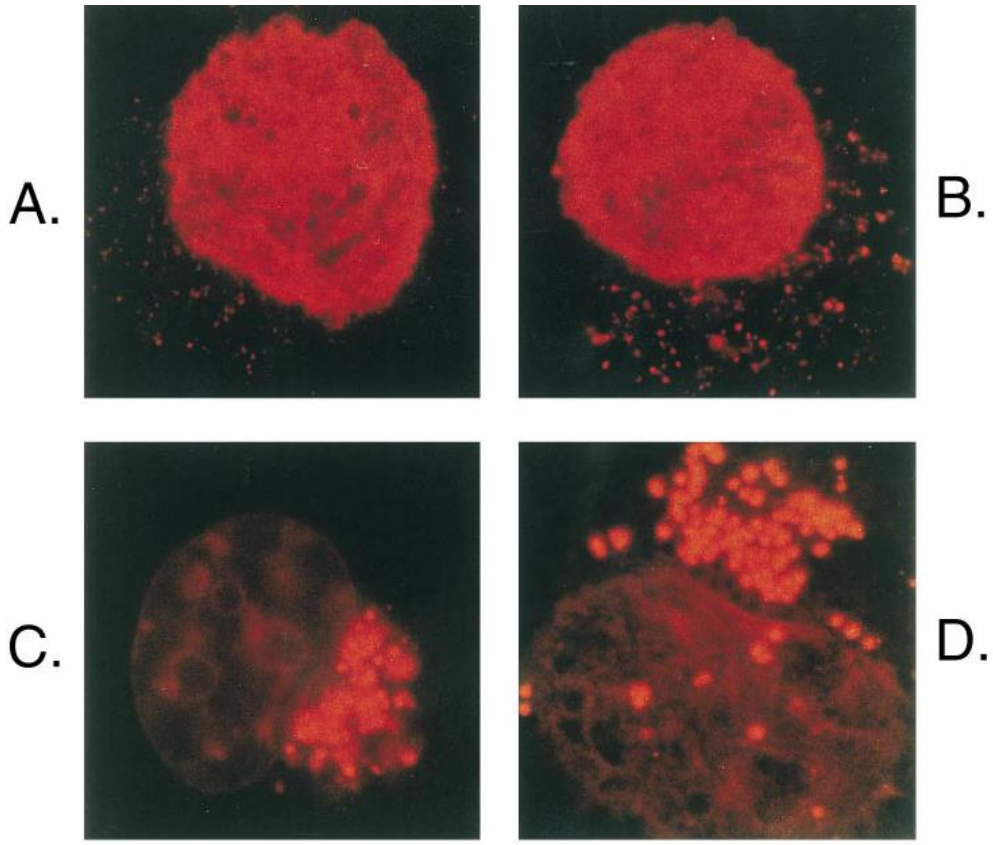

Figure 9. Daunorubicin distribution visualized by fluorescence microscopy in U-937 (A, B) and U-A10 cells (C, D) (Hurwitz et al. 1997). In drug sensitive cells (A, B) daunorubicin is mainly concentrated in the nucleus whereas in resistant cells (C,D) daunorubicin is also distributed to expanded acidic vesicles decreasing the concentration in the nucleus.

Enhanced turnover of acidic vesicles i.e. enhanced endocytosis/exocytosis have been observed in resistant cells (Martínez-Zaguilán et al. 1999). The authors observed that the release rate of endosomally trapped coumarin-dextran was higher in drug resistant MCF-7 cells (about 1.5 % min^{-1}) than in parental drug-sensitive cells (about 0.5 % min^{-1}). Consequently, the authors suggested that the reason for drug resistance could be enhanced exocytosis of drug out from the cell in addition to its accumulation into acidic vesicles. The same group investigated the effect of enhanced turnover on drug resistance in cells not overexpressing MDR1 by modeling drug partition between four compartments: extracellular medium, cytosol, perinuclear region and nucleus, and ensomal/exocytotic vesicles (Raghunand et al. 1999). They named three major parameters reducing perinuclear concentrations in resistant cells to be low endosomal pH, high endosomal turnover rate and active transport of drug into endosomes. In their model both active transport of drug into endosomes by e.g. organic cation/H^+ antiporter and high endosomal turnover rate were required to lower the perinuclear concentration enough to have a therapeutic relevance, either of them alone was not sufficient to do so. Also, the relevance was seen only when MDR1 was not considered i.e in resistant cells not overexpressing MDR1.

Enhanced turnover of acidic vesicles was also observed by Seidel et al. (1995), who studied the intracellular pathway of daunorubicin by fluorescence microscopy in resistant and sensitive human gastric carcinoma cells EPG85. The authors observed the formation of fluorescent vesicles in resistant cells, starting from the perinuclear region after 6 hours incubation. Daunorubicin was increasingly concentrated into these vesicles and the amount in nucleus was decreased to a baseline after 48 h. Vesicles were moved from the perinuclear region to the cell periphery.

6.5 Increase in transporter expression

Different protein expression of drug sensitive and resistant cancer cell lines can also cause variation in distribution of drugs (Duvvuri, Krise 2005). Many efflux proteins such as MDR1, MRP1 and breast cancer resistance protein (BCRP) are overexpressed

in resistant cancer cell lines and decrease intracellular concentrations of their substrates. But as mentioned earlier, it is questionable if these transporters have a role in intracellular sequestration of drugs. Another protein suggested to participate in drug distribution in resistant cancer cell lines is lung resistance-associated protein (LRP) (Meschini et al. 2002), but more studies are needed to confirm its role.

Perhaps most widely studied efflux transporters are MDR1 and MRP1. MDR1 and MRP1 are located in the plasma membrane of cells, where they pump drugs out from the cell, thus decreasing intracellular concentrations. MDR1 binds its substrates from the inner leaflet of plasma membrane, whereas MRP1 binds its substrates from the cytoplasm (Larsen et al. 2000).

Efflux transporters are also found in vesicular membranes of resistant cancer cells, where they may have a role in pumping drugs from the cytosol to the vesicles and thus increasing the drug accumulation into vesicles (Van Luyn et al. 1998). The authors studied microscopically the localization of the MRP1 protein in human small cell lung carcinoma (GLC_4) cell line as well as in its multidrug resistant subline GLC_4-ADR. MRP1 expression was slightly positive in plasma membrane of GLC_4-cells and strongly positive in plasma membrane of GLC_4-ADR-cells. Expression was positive also in dense spots near the nucleus in GLC_4-ADR-cells. Highly water-soluble glutathione conjugate known to be transported by MRP1 was found inside these vesicles in resistant cells which led the authors to conclusion that MRP1 is active also inside cell in the membrane of these vesicles. Also doxorubicin, known to be transported by MRP1, accumulated into these vesicles in the GLC_4-ADR-cells.

Another study concerning intracellular MRP1 was conducted by Gong et al. (2003), who, in addition to studying daunorubicin distribution in sensitive and resistant HL-60 cells, studied also another compound, sulforhodamine101 (SR101), which was noticed to sequester into the Golgi apparatus in resistant cells. Unlike with daunorubicin, disruption of pH difference in cellular endomembranes did not alter the distribution of SR101 in resistant cells and authors suggested alternative mechanism for sequestration to be transport proteins. Overexpression of MRP1 was detected in resistant cells compared to sensitive cells and MRP1 was localized to the Golgi apparatus using immunofluorescence protocol. When preincubating resistant cells with MRP1 inhibitor,

sequestration of SR101 into the Golgi apparatus was abolished. MRP1 inhibitor didn't have an effect to daunorubicin distribution and expression of MDR1 in both cells was negligible.

Also MDR1 has been detected in intracellular sites of multidrug resistant cells. Meschini et al. (2000) observed that cyclosporine A, a known MDR1 modulator, significantly increased doxorubicin accumulation into resistant human colon adenocarcinoma (Lovo 7) cells (mean fluorescence channel was about 25 units larger with cyclosporine A than without it). Lovo 7 cells do not express MDR1, or two other major efflux proteins (LRP or MRP1), in their cell membrane but intracellular MDR1 was demonstrated. Intracellular MDR1 could sequester doxorubicin away from the nucleus in resistant cells and inhibition of MDR1 by e.g. cyclosporine A could improve doxorubicin performance in anticancer therapy.

However, there are also opposite results about the activity and existence of intracellular transporter proteins and these seem to be dependent on the cell line in question. For example human ovarian carcinoma (A2780) and its multidrug resistant subline A2780AD did not express MRP1 in their plasma membranes or intracellular compartments (Van Luyn et al. 1998). With human U-937 myeloid leukemia cell line and its doxorubicin-selected variant U-A10, MDR1 and MRP was colocalized with plasma membrane, but not lysosomes or other intracellular compartments (Hurwitz et al. 1997). Even if these transporters are found in intracellular compartments, it is challenging to prove whether they are active there or just on their way to the plasma membrane after synthesis.

There is also some speculation that the overexpression of ABC transporters may affect the pH of the intracellular compartments, which affects the sequestration of drugs into these compartments (Larsen et al. 2000). However, the evidence for that is still quite scarce.

7 CONCLUSIONS

Although many of the examples discussed in this literature review concern anticancer drug therapy, intracellular distribution is important to consider with other drugs also. It affects the efficacy of the drug, it can explain the side-effects and the knowledge of the intracellular sequestration mechanisms can be utilized when designing new drugs. The importance amplifies especially with potent drugs having narrow therapeutic window, since even small changes in concentrations can have a large effect on the response to the drug therapy. New methods available for studying intracellular drug distribution have expanded our understanding about it, but there is still work to do in the development of isolation and purification methods for organelles. Intracellular sequestration of drugs can also partly explain multidrug resistance in cancer drug therapy and knowledge of the mechanisms can be utilized when designing strategies to overcome the resistance.

8 REFERENCES

Alberts B: Intracellular compartments and protein sorting. In book Molecular biology of the cell, pp. 695-749, 4th ed. Garland, New York 2002a.

Alberts B: Membrane transport of small molecules and the electrical properties of the membranes. In book Molecular biology of the cell, pp. 615-659, 4th ed. Garland, New York 2002b.

Altan N, Chen Y, Schindler M, Simon S M: Defective acidification in human breast tumor cells and implications for chemotherapy. J Exp Med 187: 1583-1598, 1998.

Ashley N, Poulton J: Mitochondrial DNA is a direct target of anti-cancer anthracycline drugs. Biochem Biophys Res Commun 378: 450-455, 2009.

Bareford L M, Swaan P W: Endocytic mechanisms for targeted drug delivery. Adv Drug Deliv Rev 59: 748-758, 2007.

Böck G, Steinlein P, Huber L A: Cell biologists sort things out: analysis and purification of intracellular organelles by flow cytometry. Trends Cell Biol 7: 499-503, 1997.

Borst P, Elferink R O: Mammalian ABC transporters in health and disease. Annu Rev Biochem 71: 537-592, 2002.

Breunig M, Bauer S, Goepferich A: Polymers and nanoparticles: Intelligent tools for intracellular targeting? Eur J Pharm Biopharm 68: 112-128, 2008.

Brown S E, Ross M F, Sanjuan-Pla A, Manas A B, Smith R A, Murphy M P: Targeting lipoic acid to mitochondria: synthesis and characterization of a triphenylphosphonium-conjugated α-lipoyl derivative. Free Radic Biol Med 42: 1766-1780, 2007.

Chapuy B, Koch R, Radunski U, Corsham S, Cheong N, Inagaki N, Ban N, Wenzel D, Reinhardt D, Zapf A: Intracellular ABC transporter A3 confers multidrug resistance in leukemia cells by lysosomal drug sequestration. Leukemia 22: 1576-1586, 2008.

Chen V Y, Posada M M, Blazer L L, Zhao T, Rosania G R: The role of the VPS4A-exosome pathway in the intrinsic egress route of a DNA-binding anticancer drug. Pharm Res 23: 1687-1695, 2006.

Chu X, Korzekwa K, Elsby R, Fenner K, Galetin A, Lai Y, Matsson P, Moss A, Nagar S, Rosania G R: Intracellular Drug Concentrations and Transporters: Measurement, Modeling and Implications in the Liver. Clin Pharmacol Ther 94(1):126-41, 2013.

Cosson P, De Curtis I, Pouysségur J, Griffiths G, Davoust J: Low cytoplasmic pH inhibits endocytosis and transport from the trans-Golgi network to the cell surface. J Cell Biol 108: 377-387, 1989.

De Beer E L, Bottone A E, Voest E E: Doxorubicin and mechanical performance of cardiac trabeculae after acute and chronic treatment: a review. Eur J Pharmacol 415: 1-11, 2001.

Demaurex N: pH Homeostasis of cellular organelles. Physiology 17: 1-5, 2002.

Dobson P D, Kell D B: Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? Nat Rev Drug Discov 7: 205-220, 2008.

Duvvuri M, Jeffrey P: A novel assay reveals that weakly basic model compounds concentrate in lysosomes to an extent greater than pH-partitioning theory would predict. Mol Pharm 2: 440-448, 2005.

Duvvuri M, Krise J P: Intracellular drug sequestration events associated with the emergence of multidrug resistance: a mechanistic review. Front Biosci 10: 1499-1509, 2005.

Duvvuri M, Feng W, Mathis A, Krise J P: A cell fractionation approach for the quantitative analysis of subcellular drug disposition. Pharm Res 21: 26-32, 2004a.

Duvvuri M, Gong Y, Chatterji D, Krise J P: Weak base permeability characteristics influence the intracellular sequestration site in the multidrug-resistant human leukemic cell line HL-60. J Biol Chem 279: 32367-32372, 2004b.

Duvvuri M, Konkar S, Funk R S, Joanna M, Jeffrey P: A chemical strategy to manipulate the intracellular localization of drugs in resistant cancer cells. Biochemistry (N.Y.) 44: 15743-15749, 2005.

Ferrao P, Sincock P, Cole S, Ashman L: Intracellular P-gp contributes to functional drug efflux and resistance in acute myeloid leukaemia. Leuk Res 25: 395-405, 2001.

Food and Drug Administration: Guidance for Industry Developing Antimicrobial Drugs: General Considerations for Clinical Trials, 1998. From the internet 21.9.2013: http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm070983.pdf.

Funk R S, Krise J P: Cationic amphiphilic drugs cause a marked expansion of apparent lysosomal volume: implications for an intracellular distribution-based drug interaction. Mol Pharm 9: 1384-1395, 2012.

Gauthier D J, Sobota J A, Ferraro F, Mains R E, Lazure C: Flow cytometry-assisted purification and proteomic analysis of the corticotropes dense-core secretory granules. Proteomics 8: 3848-3861, 2008.

Gong Y, Duvvuri M, Krise J P: Separate roles for the Golgi apparatus and lysosomes in the sequestration of drugs in the multidrug-resistant human leukemic cell line HL-60. J Biol Chem 278: 50234-50239, 2003.

Hallifax D, Houston J B: Saturable uptake of lipophilic amine drugs into isolated hepatocytes: mechanisms and consequences for quantitative clearance prediction. Drug Metab Disposition 35: 1325-1332, 2007.

Hansen S H, Sandvig K, Van Deurs B: Clathrin and HA2 adaptors: effects of potassium depletion, hypertonic medium, and cytosol acidification. J Cell Biol 121: 61-72, 1993.

Hediger M A, Romero M F, Peng J, Rolfs A, Takanaga H, Bruford E A: The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteins. Pflügers Archiv 447: 465-468, 2004.

Hennessy M, Spiers J: A primer on the mechanics of P-glycoprotein the multidrug transporter. Pharmacol Res. 55: 1-15, 2007.

Hillaireau H, Couvreur P: Nanocarriers' entry into the cell: relevance to drug delivery. Cell Mol Life Sci 66: 2873-2896, 2009.

Hornig-Do H, Günther G, Bust M, Lehnartz P, Bosio A, Wiesner R J: Isolation of functional pure mitochondria by superparamagnetic microbeads. Anal Biochem 389: 1-5, 2009.

Huang Y, Sadée W: Membrane transporters and channels in chemoresistance and -sensitivity of tumor cells. Cancer Lett 239: 168-182, 2006.

Hurwitz S J, Terashima M, Mizunuma N, Slapak C A: Vesicular anthracycline accumulation in doxorubicin-selected U-937 cells: participation of lysosomes. Blood 89: 3745-3754, 1997.

Islinger M, Li K W, Loos M, Liebler S, Angermüller S, Eckerskorn C, Weber G, Abdolzade A, Völkl A: Peroxisomes from the heavy mitochondrial fraction: isolation by zonal free flow electrophoresis and quantitative mass spectrometrical characterization. J Proteome Res 9: 113-124, 2009.

Jordan M A, Wilson L: Microtubules as a target for anticancer drugs. Nat. Rev. Cancer 4: 253-265, 2004.

Karlsson O, Lindquist N G: Melanin affinity and its possible role in neurodegeneration. J Neural Transm 1-8, 2013.

Kaufmann A M, Krise J P: Lysosomal sequestration of amine-containing drugs: Analysis and therapeutic implications. J Pharm Sci 96: 729-746, 2006.

Kelso G F, Porteous C M, Coulter C V, Hughes G, Porteous W K, Ledgerwood E C, Smith R A, Murphy M P: Selective Targeting of a Redox-active Ubiquinone to Mitochondria within Cells antioxidant and antiapoptotic properties. J Biol Chem 276: 4588-4596, 2001.

Keminer O, Peters R: Permeability of single nuclear pores. Biophys J. 77: 217-228, 1999.

Krämer S D, Lombardi D, Primorac A, Thomae A V, Wunderli-Allenspach H: Lipid-Bilayer Permeation of Drug-Like Compounds. Chem Biodiversity 6: 1900-1916, 2009.

Laginha K M, Verwoert S, Charrois G J, Allen T M: Determination of doxorubicin levels in whole tumor and tumor nuclei in murine breast cancer tumors. Clin Cancer Res 11: 6944-6949, 2005.

Lansiaux A, Tanious F, Mishal Z, Dassonneville L, Kumar A, Stephens C E, Hu Q, Wilson W D, Boykin D W, Bailly C: Distribution of Furamidine Analogues in Tumor Cells Targeting of the Nucleus or Mitochondria Depending on the Amidine Substitution. Cancer Res 62: 7219-7229, 2002.

Larsen A K, Escargueil A E, Skladanowski A: Resistance mechanisms associated with altered intracellular distribution of anticancer agents. Pharmacol Ther 85: 217-229, 2000.

Lawson E L, Clifton J G, Huang F, Li X, Hixson D C, Josic D: Use of magnetic beads with immobilized monoclonal antibodies for isolation of highly pure plasma membranes. Electrophoresis 27: 2747-2758, 2006.

Llopis J, McCaffery J M, Miyawaki A, Farquhar M G, Tsien R Y: Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. Proc Nat Acad Sci 95: 6803-6808, 1998.

Lombardi D, Cuenoud B, Wunderli-Allenspach H, Krämer S D: Interaction kinetics of salmeterol with egg phosphatidylcholine liposomes by surface plasmon resonance. Anal Biochem 385: 215-223, 2009.

Martínez-Zaguilán R, Raghunand N, Lynch R M, Bellamy W, Martinez G M, Rojas B, Smith D, Dalton W S, Gillies R J: pH and drug resistance. I. Functional expression of plasmalemmal V-type H -ATPase in drug-resistant human breast carcinoma cell lines. Biochem Pharmacol 57: 1037-1046, 1999.

Mellman I, Fuchs R, Helenius A: Acidification of the endocytic and exocytic pathways. Annu Rev Biochem 55: 663-700, 1986.

Meschini S, Calcabrini A, Monti E, Del Bufalo D, Stringaro A, Dolfini E, Arancia G: Intracellular P-glycoprotein expression is associated with the intrinsic multidrug resistance phenotype in human colon adenocarcinoma cells. Int J Cancer 87: 615-628, 2000.

Meschini S, Marra M, Calcabrini A, Monti E, Gariboldi M, Dolfini E, Arancia G: Role of the lung resistance-related protein (LRP) in the drug sensitivity of cultured tumor cells. Toxicol In vitro 16: 389-398, 2002.

Murphy M P: Targeting lipophilic cations to mitochondria. Biochim Biophys Acta (BBA)-Bioenergetics 1777: 1028-1031, 2008.

Murphy M P, Smith R A: Drug delivery to mitochondria: the key to mitochondrial medicine. Adv Drug Deliv Rev 41: 235-250, 2000.

Pasquali C, Fialka I, Huber L A: Subcellular fractionation, electromigration analysis and mapping of organelles. Journal of Chromatography B: Biomed Sci Appl 722: 89-102, 1999.

Raghunand N, Martínez–Zaguilán R, Wright S H, Gillies R J: pH and drug resistance. II. Turnover of acidic vesicles and resistance to weakly basic chemotherapeutic drugs. Biochem Pharmacol 57: 1047-1058, 1999.

Rajendran L, Knölker H, Simons K: Subcellular targeting strategies for drug design and delivery. Nat Rev Drug Discov 9: 29-42, 2010.

Raub T J: P-glycoprotein recognition of substrates and circumvention through rational drug design. Mol Pharm 3: 3-25, 2006.

Reasor M J, Kacew S: Drug-induced phospholipidosis: are there functional consequences? Exp Biol Med 226: 825-830, 2001.

Reddy B, Sondhi S M, Lown J W: Synthetic DNA minor groove-binding drugs. Pharmacol Ther 84: 1-111, 1999.

Sawada G A, Barsuhn C L, Lutzke B S, Houghton M E, Padbury G E, Ho N F, Raub T J: Increased lipophilicity and subsequent cell partitioning decrease passive transcellular diffusion of novel, highly lipophilic antioxidants. J Pharmacol Exp Ther 288: 1317-1326, 1999.

Sehested M, Skovsgaard T, Van Deurs B, Winther-Nielsen H: Increased plasma membrane traffic in daunorubicin resistant P388 leukaemic cells. Effect of daunorubicin and verapamil. Br J Cancer 56: 747, 1987.

Seidel A, Bunge A, Schaefer B, Herzig I, Steidtmann K, Dietel M, Hasmann M, Löser R: Intracellular localization, vesicular accumulation and kinetics of daunorubicin in sensitive and multidrug-resistant gastric carcinoma EPG85-257 cells. Virchows Archiv 426: 249-256, 1995.

Shapiro A B, Fox K, Lee P, Yang Y D, Ling V: Functional intracellular P-glycoprotein. Int J Cancer 76: 857-864, 1998.

Siarheyeva A, Lopez J J, Glaubitz C: Localization of multidrug transporter substrates within model membranes. Biochemistry (N.Y.) 45: 6203-6211, 2006.

Simon S, RoY D, Schindler M: Intracellular pH and the control of multidrug resistance. Proc Nat Acad Sci 91: 1128-1132, 1994.

Svensson S P, Lindgren S, Powell W, Green H: Melanin inhibits cytotoxic effects of doxorubicin and daunorubicin in MOLT 4 cells. Pigment Cell Res 16: 351-354, 2003.

Tarragó-Trani M T, Storrie B: Alternate routes for drug delivery to the cell interior: pathways to the Golgi apparatus and endoplasmic reticulum. Adv Drug Deliv Rev 59: 782-797, 2007.

Thomae A V: *Experimental and theoretical investigations on lipid bilayer permeation.* Swiss Federal Institute of Technology Zurich, 2007.

Torchilin V P: Recent approaches to intracellular delivery of drugs and DNA and organelle targeting. Annu Rev Biomed Eng 8: 343-375, 2006.

Tulp A, Verwoerd D, Benham A, Jalink K, Sier C, Neefies J: High performance density gradient electrophoresis of subcellular organelles, protein complexes and proteins. Electrophoresis 19: 1171-1178, 1998.

Van Luyn M J, Müller M, Renes J, Meijer C, Scheper R J, Nienhuis E F, Mulder N H, Jansen P L, De Vries E G: Transport of glutathione conjugates into secretory vesicles is mediated by the multidrug-resistance protein 1. Int J Cancer 76: 55-62, 1998.

Verkman A S: Solute and macromolecule diffusion in cellular aqueous compartments. Trends Biochem.Sci 27: 27-33, 2002.

Völkl A, Mohr H, Weber G, Fahimi H D: Isolation of rat hepatic peroxisomes by means of immune free flow electrophoresis. Electrophoresis 18: 774-780, 1997.

Wang Y, Taylor T H, Arriaga E A: Analysis of the bioactivity of magnetically immunoisolated peroxisomes. Anal Bioanal Chem 402: 41-49, 2012.

Wils P, Warnery A, Phung-Ba V, Legrain S, Scherman D: High lipophilicity decreases drug transport across intestinal epithelial cells. J Pharmacol Exp Ther 269: 654-658, 1994.

Zheng N, Tsai H N, Zhang X, Rosania G R: The subcellular distribution of small molecules: from pharmacokinetics to synthetic biology. Mol Pharm 8: 1619-1628, 2011.

Zischka H, Braun R J, Marantidis E P, Büringer D, Bornhövd C, Hauck S M, Demmer O, Gloeckner C J, Reichert A S, Madeo F: Differential analysis of Saccharomyces cerevisiae mitochondria by free flow electrophoresis. Mol Cell Proteomics 5: 2185-2200, 2006.

Zutphen S v, Reedijk J: Targeting platinum anti-tumour drugs: Overview of strategies employed to reduce systemic toxicity. Coord Chem Rev 249: 2845-2853, 2005.

EXPERIMENTAL WORK:

SUBCELLULAR FRACTIONATION PROTOCOL TO ISOLATE PLASMA MEMBRANE AND CYTOPLASM OF MDCKII CELLS

Karoliina Huusko
University of Helsinki
Faculty of Pharmacy
Division of Biopharmaceutics and Pharmacokinetics

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CONTENTS

APPENDIXES

1 INTRODUCTION

Knowledge of the intracellular distribution of drugs is necessary to predict the effect of drugs on intracellular targets. Also drug clearance and transporter binding sites are often intracellular. In many cases, total plasma concentrations are used to predict the disposition and function of the drug, which can lead to false conclusions. Knowing the unbound concentration in the site of action would greatly improve correlation between in vitro experiments and in vivo parameters (in vitro-in vivo correlation, IVIVC).

Intracellular distribution of small molecule drugs is not commonly studied (Duvvuri, Krise 2005). Perhaps the most frequently used methods are those based on fluorescent microscopy so the knowledge of intracellular distribution is somewhat restricted to fluorescent compounds (Duvvuri et al. 2004). However, the importance of the intracellular concentration of drugs is recognized and new experimental and computational methods for studying it are being developed.

The multidrug resistance 1 (MDR1) transporter (ABCB1, P-glycoprotein) is a member of the ABC transporter family. It uses energy from ATP hydrolysis to export substrates out of the cell through the apical plasma membrane of the cell (Alberts 2002b). MDR1 is expressed in the small intestine, blood-brain barrier, liver and kidneys and it exports a wide selection of usually hydrophobic and cationic molecules out of the cells (Giacomini et al. 2010). Expression and functionality of MDR1 is susceptible to induction and inhibition which can affect ADME-properties of MDR1 substrates and cause drug-drug interactions. Passive diffusion through the plasma membrane has to be taken into account since it affects the clinical significance of these changes.

The main mechanism of MDR1 efflux is most likely influx hindrance in which the substrate partitions into the plasma membrane and binds directly to MDR1 without partitioning into intracellular fluids (Raub 2006). Therefore unbound concentration of MDR1 substrate in the binding site (plasma membrane) is useful to know in order to better predict the role of active transport in overall transport process. Many models for MDR1 function and substrate binding have been suggested but two of them are more studied than others: hydrophobic vacuum cleaner - and flippase model. In both of them

the binding of substrates takes place in the inner leaflet of the plasma membrane and thus substrates have to first partition into the lipid bilayer in order to interact with transporter (Hennessy, Spiers 2007).

Three compounds were used in our experiments: celiprolol, clotrimazole and 5(6)-carboxy-2',7'-dichlorofluorescein (CDCF). The drugs selected have different lipophilicities, and celiprolol and CDCF are known MDR1 substrates.

2 AIMS OF THE STUDY

The goal of this work is to study partitioning of celiprolol, clotrimazole and CDCF between plasma membrane and cytoplasm *in vitro* in MDCKII-wt and MDCKII-MDR1 cells. The aims of the study were to (1) validate the isolation protocol for plasma membrane and cytoplasm and (2) measure the concentrations of three drugs selected in plasma membrane, cytoplasm and cell lysate.

Isolation of plasma membrane is done by a fractionation protocol based on different centrifugation speeds to pellet different cellular compartments. The concentrations of drugs are measured in these cell compartments and in cell lysate after validation of the protocol. Experiments with MDCKII-MDR1 cells are done with and without MDR1-inhibitor verapamil. Kinetics of the partitioning of CDCF to the plasma membrane is also studied. Vesicle assay is done for clotrimazole to study if it is a MDR1 substrate and/or inhibitor. The drugs selected have different lipophilicity (different log P or log D) and celiprolol and CDCF are known substrates of efflux transporter MDR1.

Considering the drug uptake experiments, the hypothesis was that the concentration in wild type cells is similar as in MDR1 transfected cells when inhibiting MDR1 efflux by verapamil. Also, according to the hypothesis, concentrations in plasma membrane of MDCKII-MDR1 cells should be smaller than in wild type cells if the drug is a MDR1 substrate. Partition of drugs to cellular fractions was also compared within cell types to

see if partitioning was similar in smaller and higher concentrations or is it e.g. saturable. Finally, K_m (or K_i for clotrimazole) concentrations from literature were used to determine the corresponding concentration in plasma membrane, K_m(membrane), which could be more useful in predicting transporter contribution to transport process and not that prone to variation between different experimental design.

3 MATERIALS AND METHODS

3.1 Test compounds

Celiprolol is beta-blocking agent used in the treatment of high blood pressure and coronary artery disease. It blocks selectively beta-1-receptors. The usual dose is 200 mg and it can be doubled to 400 mg if needed. It is mainly (95 %) excreted unchanged through kidneys and bile and only about 25 % is bound to plasma proteins. Half-life of celiprolol is 5-8 h. It is actively excreted back to intestines after oral absorption and bioavailability increases when dose is increased. It is available with multiple brand names in Europe, e.g. Selectol and Celiprolol Vitabalans (Duodecim Oy 2013). Physicochemical parameters of celiprolol are presented in Table 1. Celiprolol is a substrate of MDR1 with K_m value of 1 mM and V_{max}, of 113 ± 11 pmol/10^6 cells/ min (Karlsson et al. 1993). It is also suggested to be a substrate of organic anion transporting polypeptide (OATP-1A2) influx transporter (Kato et al. 2009).

Clotrimazole is a broad-spectrum antifungal medicine used in local treatment of vaginal yeast infection as a cream (10-20 mg/g) or vaginal tablets (200 or 500 mg). It can also be used to treat yeast infections in the skin or mouth. It inhibits ergosterol synthesis of the yeast. Only 3-10 % is absorbed systemically after local treatment. Clotrimazole undergoes hepatic metabolism and inhibits CYP3A4 and CYP2C9 enzymes which can cause drug-drug interactions (Duodecim Oy 2013). Clotrimazole is poorly absorbed orally and has a half-life of 2 h. Clotrimazole is available as a cream or vaginal tablets

with brand name Canesten in Europe. In US Clotrimazole is available as oral topical tablets with the brand name Mycelex Troche and as topical preparation with brand names Lotrimin, Gyne-Lotrimin, Canesten and Mycelex (Drugsite Trust 2013). Physicochemical parameters of clotrimazole are presented in Table 1. Clotrimazole is an inhibitor of MDR1 with K_i value of 44 μM (Yasuda et al. 2002). It may also have an influence on multidrug resistance proteins 1 and 2 (MRP1 and MRP2) (Wishart research group, 2013).

5(6)-Carboxy-2',7'-dichlorofluorescein (CDCF) is a fluorescent compound extensively used in research. The hydrolyzed form of CDCF can pass the cell membrane by active efflux mediated by MRP2, MRP3, MRP5, and perhaps also by MRP1 (Vellonen et al. 2010). It has also proven to be MDR1 substrate in membrane vesicle assay, with K_m of 2.73 μM and V_{max} of 1.97 pmol/min/mg tot.prot. (results from Sf9-MDR1 membrane vesicle assay, Nora Sjöstedt, personal communication). Physicochemical parameters of CDCF are presented in Table 1.

Verapamil is a calcium-channel blocker used as anti-arrhythmia agent. It is commonly used as an inhibitor of MDR1 in experiments and its IC_{50} values are 10.7-33.5 μM depending on the substrate used (Rautio et al. 2006) and Ki value is 15.1 μM (Tang et al. 2002). Commonly used verapamil concentration in inhibitor experiments is 200 μM.

Table 1. Physicochemical parameters of test drugs and their interaction with MDR1.

Physicochemical parameters for clotrimazole, celiprolol and CDCF			
	clotrimazole	celiprolol (HCl)	CDCF
Molecular mass (g/mol)[1]	344.84	415.95	445.21
log P [1]	5.4	1.9	2.6
log D (pH 7.4) [1]	5.4	0.1	-0.5
pKa	6.6 [2]	9.7 [3]	5.1 [4]
MDR1 substrate	-	yes[5]	yes[7]
Km (µM)	-	1000 [5]	3 [7]
MDR1 inhibitor	yes[6]	-	-
Ki (µM)	44 [6]	-	-

[1] Royal Society of Chemistry, 2013,calculated by ACDlabs

[2] Wishart research group, 2013

[3] Pharma Professional Services, 2013

[4] Sigma-aldrich, 2013

[5] Karlsson et al. 1993

[6] Yasuda, Lan et al. 2002

[7] Results from Sf9-MDR1 membrane vesicle assay, Nora Sjöstedt, personal communication

3.2 Materials

Materials used in cell culturing were Dulbecco's modified eagles medium D-MEM (low glucose (1 g/l D-glucose), L-glutamine, pyruvate, Gibco, 31885-023), Fetal bovine serum and Penicillin-Streptomycin antibiotic solution (10 000 IU/ml penicillin, 10 000 µg/ml streptomycin, Gibco, 15140-122). Washing solution was Dulbecco's phosphate buffered saline (DPBS, - CaCl, -$MgCl_2$, Gibco 14200-067). The solution used to detach the adherent cells was 0.5% trypsin-EDTA (Gibco, 15400-054) or Tryple express (Gibco 12604-021).

Celiprolol hycdrochloride was supplied by Santa Cruz Biotech (USA), clotrimazole by MP Biomedicals (France), and 5(6)-Carboxy-2',7'-dichlorofluorescein (CDCF) and verapamil were supplied by Sigma Aldrich (USA). Stock solutions were made in dimethyl sulfoxide (DMSO, Sigma Aldrich, France) and stored at -20 ºC. Stock solutions were diluted with DMSO and added to buffer in order to get the desired concentration in the experiments. Buffer used was Hanks' Balanced Salt Solution

(HBSS, 14025-050, Gibco) supplemented with 10 mM Hepes. Final concentration of DMSO in experiments was 2 % of the total reaction volume (v/v).

3.3 Cell culture

Madin-Darby canine kidney II wild type (MDCKII-wt) renal epithelial cells were received from University of Tokyo. Madin-Darby canine kidney II cells expressing human MDR1 (MDCKII-MDR1) cells were received from Netherlands Cancer Institute. The growth medium used was D-MEM supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin antibiotic solution. MDCKII-wt and MDCKII-MDR1 cells were split twice a week by washing the confluent cell monolayer with DPBS, detaching the cells by incubating with 0.5% trypsin-EDTA for 5 minutes at 37 °C and splitting 1:9. Cells were kept at 37 °C in 5 % CO_2.

Working stocks of both MDCKII-wt and MDCKII-MDR1 cells were cryopreserved in liquid nitrogen which enabled the use of cells with similar passage numbers in all the drug uptake experiments. Freezing medium used was growth medium supplemented with 10 % DMSO and 50 % Fetal bovine Serum. Cells were first kept in isopropyl alcohol container at -80 °C overnight and then placed to liquid nitrogen until use. Successful freezing was ensured microscopically. Passage numbers used in drug uptake experiments were 30-33 for wild type cells and 11-16 for MDR1-transfected cells. Passage number of cells used in validation of isolation protocol was 30 and validation was done with wild type cells.

3.4 Validation of isolation protocol

Isolation protocol used in these experiments is based on different sedimentation rate of cell organelles at different centrifugal force, and the main interest was to isolate the plasma membrane and cytoplasm. The protocol is described in Appendix 1. Shortly, MDCKII-wt cells were harvested, homogenized and centrifuged at different centrifugal

forces. Centrifugation steps are described in Figure 1. Four samples were collected: sample A containing cell lysate; sample B mitochondria, peroxisomes and lysosomes; sample C plasma membrane and sample D cytoplasm. Determination of the protein concentration in samples was done by Bio-Rad protein assay to evaluate the need for sample dilution to SDS-page. Bio-Rad protein assay is based on a color change of a dye Coomassie blue (Bio-Rad laboratories, Finland, 500-0006) in response to different protein concentrations. Visual quantification showed that the protein concentration in sample A was larger than in other samples, so sample A was diluted 1:2 with milliQ water prior to SDS-page.

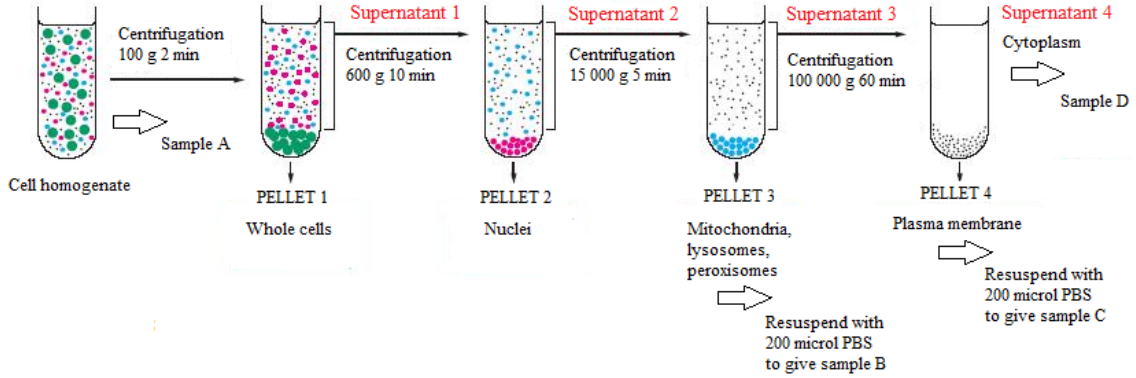


Figure 1. Isolation protocol for plasma membrane and cytoplasm. In validation of protocol samples A-D were collected. When studying drug distribution, only samples A, C and D were collected.

Western Blot was done to validate the protocol. Proteins were separated according to their molecular weights on 10 % SDS-polyacrylamide gel (456-1033, Bio-Rad Laboratories, USA) and electroblotted onto a nitrocellulose membrane (162-0145, Bio-Rad Laboratories, Germany) to which antibodies were added. Primary antibodies were anti UGP2 (Aviva systems biology, San Diego, ARP48303_P050) as a marker of cytoplasm, anti G6PC (Aviva systems biology, San Diego, ARP44224_P050) as a marker of endoplasmic reticulum, anti-COX (antibodies-online.com, Germany, ABIN401531) as a marker of mitochondria and anti CD73 (antibodies-online.com, Germany, ABIN739353) as a marker of plasma membrane. Secondary antibody was Anti-Goat (Millipore, USA, AP106P) against anti-COX and Anti-Rabbit (Millipore,

USA, AP307P) against the other antibodies. Used antibody dilutions were 1:1000 for all primary antibodies, 1:5000 for Anti-Goat secondary antibody and 1:2000 for Anti-Rabbit secondary antibody. UGP2 (UDP-glucose pyrophosphorylase 2) is an enzyme involved in mammalian carbohydrate inter-conversions with molecular weight of 57 kDa. G6PC (glucose-6-phosphatase, catalytic subunit) is an enzyme functioning in gluconeogenesis and glycogenolysis in endoplasmic reticulum with molecular weight of 41 kDa. COX (cytochrome c-oxidase) is an enzyme involved in mitochondrial electron transport chain with molecular weight of 17 kDa. CD73 (5'-nucleotidase, ecto, NT5E) is an enzyme which catalyses the conversion of extracellular nucleotides to membrane-permeable nucleosides in plasma membrane, with molecular weight of 65 kDa. Low protein marker (Prestained SDS-PAGE standards, 161-0305, Bio-Rad Laboratories, USA), containing six proteins with known molecular weights, was used to estimate the size of the proteins in samples A-D (Figure 2). Detection was done with ECL detection system (GE Healthcare Amersham, Germany, ECL Western Blotting Detection reagents, RPN 2106) and films were exposed 30 min.

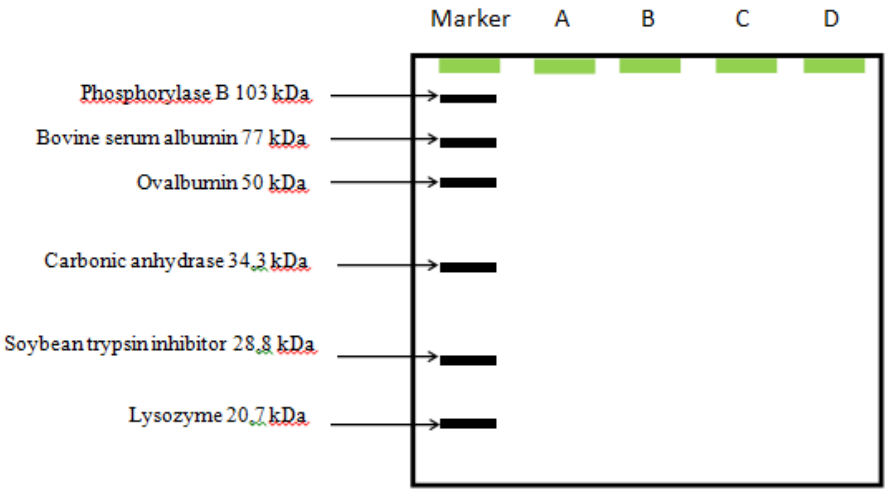


Figure 2. Low protein marker including six reference bands with known molecular weights

3.5 Analysis method

CDCF concentrations in isolated cell fractions were measured with Varioskan Flash spectrofluorometer (Thermo Scientific, Finland) using excitation and emission wavelengths 510 nm and 535 nm, respectively. Standard curve to calculate concentrations is presented in Appendix 2. Celiprolol and clotrimazole concentrations were measured using UPLC-MS technique. ULPC (Acquity, Waters, USA) column used was UPLC HSS T3 with dimension 2.1 x 100 mm and particle size 1.8 µm (Waters, USA). Flow rate was 0.3 ml/min and injection volume 0.5 µl. Mass spectrometry used was triple quadrupole mass spectrometer (Waters TQ-S, Waters, USA) with electrospray ionization on positive mode. Propranolol was used as an internal standard. Prior to analysis, samples were centrifuged at 9600 g for 5 min and 25 µl internal standard (propranolol 0.25 mg/ml) was added to 100 µl of supernatant.

3.6 Uptake kinetics of CDCF

Different incubation times were tested to see how long it takes for CDCF to achieve steady state in MDCK-MDR1 cell lysates and whether verapamil as an MDR1 inhibitor affects that time or not. Incubation times were 1, 5, 10, 30, 60, 90 and 120 minutes for CDCF 100 µM and 5, 10, 30, 60 and 90 minutes for CDCF 100 µM with verapamil 200 µM. Since the interest was only to evaluate uptake kinetics of drugs, the whole isolation protocol was not done, instead only sample A was collected.

3.7 Vesicle study for clotrimazole

Vesicle study was done to determine if clotrimazole is a substrate and/or an inhibitor of MDR1. The use of inside-out vesicles enables the measurement of drug uptake into vesicles and evaluation of the effect of efflux transporter MDR1 in the overall transport

process. Since MDR1 uses energy from ATP to transport its substrates, the efflux does not work in the absence of ATP.

Vesicle assay steps and plate setup are described in detail in Appendix 3. In the substrate test, inside-out vesicles prepared from MDR1-transfected *Spodoptera frugiperda* (Sf9) insect cells were incubated in 96-well plate with clotrimazole 1, 10 and 100 µM in the presence and absence of ATP. In the inhibitor test, CDCF, a known substrate of MDR1, was incubated with vesicles with clotrimazole 5 µM, clotrimazole 50 µM and control (DMSO) to see if clotrimazole inhibits the transport of CDCF by MDR1. Uptake of clotrimazole to the vesicles was measured by mass spectrometry and uptake of CDCF by spectrofluorometer.

3.8 Drug uptake experiments

Experiments were done with MDCKII-wt cells and MDCKII-MDR1 cells. All the experiments were done in duplicates and some of them were repeated in order to see if there is day-to-day variation in experiments. Concentrations of drugs were 30 µM, 100 µM and 200 µM for CDCF and clotrimazole and 100 µM, 300 µM and 500 µM for celiprolol. MDCKII-MDR1 cell experiments were done in the presence and absence of MDR1 inhibitor verapamil 200 µM. The concentration of DMSO was kept under 2 % in order not to harm cells.

Culture media from MDCKII-wt or MDCKII-MDR1 cells was first removed and cells were washed with 5 ml HBSS/Hepes 10 mM solution and incubated with 5 ml of HBSS/Hepes for 10 minutes at 37 °C. Drugs in DMSO were added to HBSS/Hepes solution and incubated with cells for 60 minutes at 37 °C with agitation.

After 60 minutes incubation, reaction was stopped by removing drug-buffer solution and by washing cells three times with 10 ml of ice cold PBS. Isolation of cell organelles was done as previously described (Appendix 1, Figure 1), except that sample B, containing mitochondria, was not collected since the interest was to measure

concentrations in plasma membrane and cytoplasm. The experiments after drug incubation were done on ice and centrifugations at 4 °C.

3.9 Data analysis

Cell count was done by Cedex XS (Switzerland) using Cedex Smart Slides (05650801001, Roche, Germany). Cells were stained with Tryptan Blue Stain (0.4 %) (Gibco, USA, 15250-061) and the amount and diameter of cells were measured before and after homogenization of cells.

Concentrations of test drug in lysate, plasma membrane and cytoplasm were calculated with equation

$$c_1 \times V_1 = c_2 \times V_2 \qquad \text{Equation 1}$$

, where c_1 is concentration of test drug in certain fraction (lysate, plasma membrane or cytoplasm), V_1 is volume of that fraction, c_2 is measured concentration of test drug gained from mass spectrometry or spectrofluorometer and V_2 is the volume from which the sample was taken.

When calculating lysate concentration, volume of cell lysate V_1, can be calculated knowing the amount (n) and radius of cells (r) before homogenization ($V_1 = n\frac{4}{3}\pi r^3$) and V_2 is the volume of cell homogenate (5 ml).

When calculating plasma membrane concentration, volume of plasma membrane V_1, can be calculated knowing the amount of broken cells (n_{broken}), radius of the cells (r) and thickness of plasma membrane (d=4.7 nm) in MDCK cells (Lärmer et al. 1997). $V_1 = n_{broken\ x}(\frac{4}{3}\pi r^3 - \frac{4}{3}\pi(r-d)^3)$. V_2 is the volume to which the plasma membrane pellet has been resuspended (200 µl).

When calculating cytoplasm concentration, volume of cytoplasm V_1, can be calculated knowing the amount of broken cells (n_{broken}), radius of the cells and knowing that

cytoplasm occupies 54 % of the total cell volume (Alberts 2002a). $V_1 = 0.54 \times n_{broken} \frac{4}{3} \pi r^3$. V_2 is the volume of supernatant after last centrifugation (5 ml).

K_m(membrane) for CDCF and celiprolol and K_i(membrane) for clotrimazole is determined as the concentration in plasma membrane of MDCKII-MDR1 cells without verapamil when incubation concentration is set to be K_m (or K_i) value of the compound.

4 RESULTS

4.1 Validation of isolation protocol

Four gels were prepared for four primary antibodies: (1) anti UGP2 (marker of cytoplasm), (2) anti G6PC (marker of endoplasmic reticulum), (3) anti-COX (marker of mitochondria) and (4) anti CD73 (marker of plasma membrane). With anti-COX, there were some technical problems due to e.g. unspecific binding and blotting was unsuccessful with that antibody. Films from gels 1, 2 and 4 along with clarifying drafts are presented in Figure 3, Figure 4 and Figure 5, respectively.

Film from anti UGP2 (Figure 3) shows that antibody identifies cytoplasmic markers in sample A (lysate) and sample D (cytoplasm). Molecular weights of the identified proteins are approximately 34.3 kDa and 20.7 kDa in lysate, and 34.3 kDa in cytoplasm and there are several bands in both of the samples. Molecular weight of the UGP2 protein is 57 kDa.

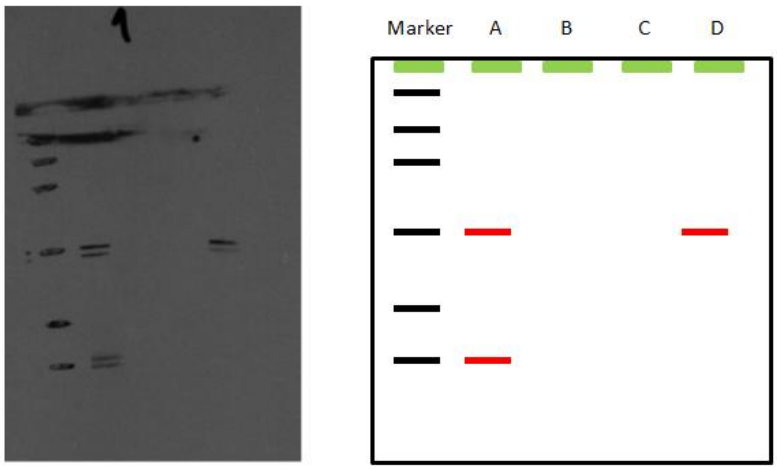


Figure 3. Anti UGP2 antibody identifies cytoplasmic markers in samples A and D.

Film from anti G6PC (Figure 4) shows that antibody identifies endoplasmic reticulum markers in sample A (lysate) and sample C (plasma membrane). Molecular weights of the identified proteins are approximately 23 kDa in lysate (several bands) and 90 kDa in plasma membrane. Molecular weight of the G6PC is 41 kDa.

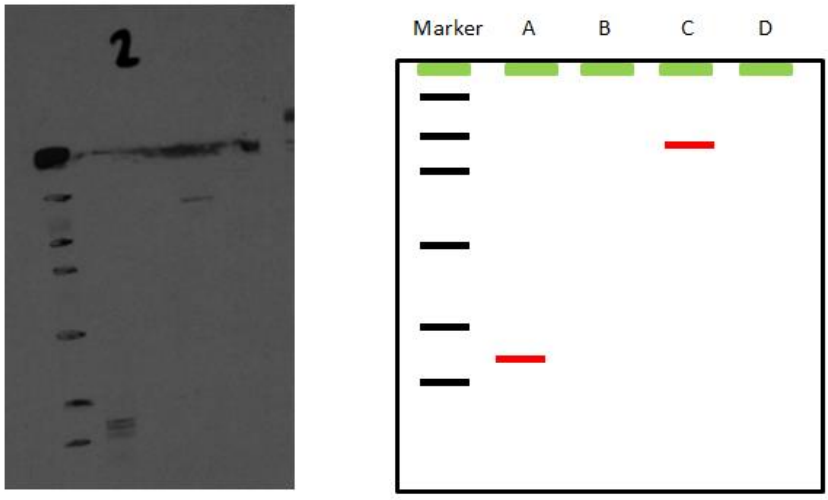


Figure 4. Anti G6PC antibody identifies endoplasmic reticulum markers in samples A and C.

Film from anti CD73 (Figure 5) shows that antibody identifies plasma membrane markers in sample A (lysate), sample C (plasma membrane) and sample D (cytoplasm).

Molecular weights of the identified proteins are 34.3 kDa and less than 20.7 kDa in lysate and plasma membrane, and less than 20.7 kDa in cytoplasm. There are several bands in all of the three samples. Molecular weight of CD73 is 65 kDa.

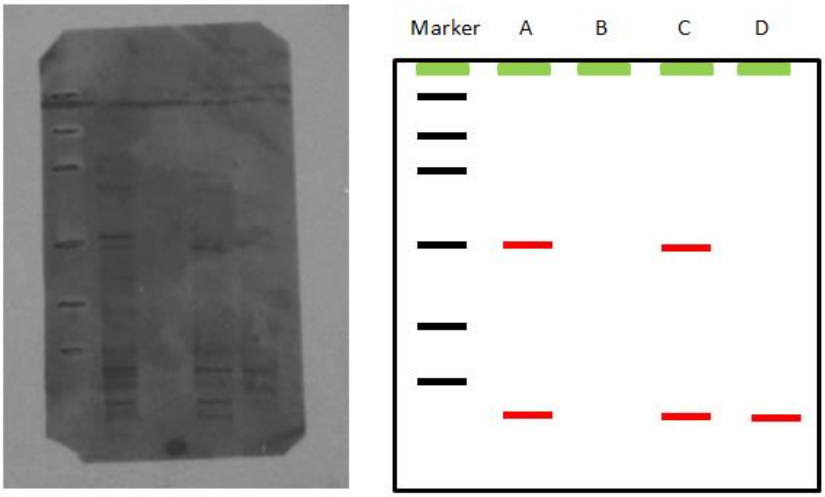


Figure 5. Anti CD73 antibody identifies plasma membrane markers in samples A, C and D.

4.2 Uptake kinetics of CDCF

Concentration of CDCF with incubation concentration 100 µM in MDCKII-MDR1 cell lysate after different incubation times is presented in Figure 6. The experiments were done with and without MDR1 inhibitor verapamil 200 µM.

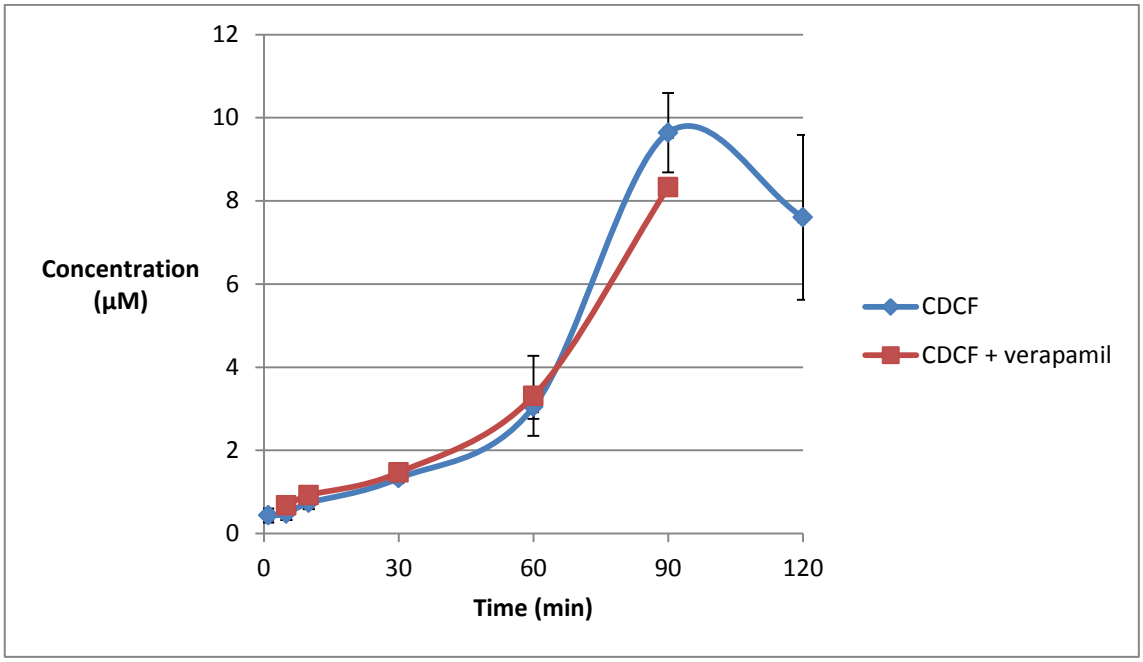


Figure 6. Average concentration of CDCF in cell lysate ± standard deviation after different incubation times (n=2).

4.3 Vesicle study for clotrimazole

Average concentrations of clotrimazole (substrate study) and average fluorescence of CDCF (inhibitor study) in vesicles in the presence and absence of ATP are presented in Figure 7 and Figure 8.

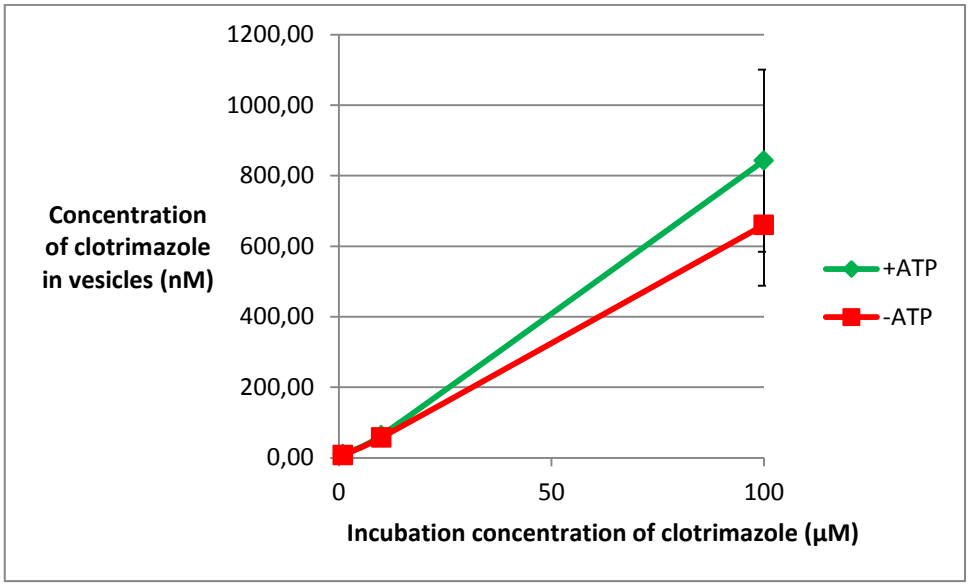


Figure 7. Average concentration of clotrimazole in vesicles ± standard deviation after different incubation concentrations with and without ATP (n=3).

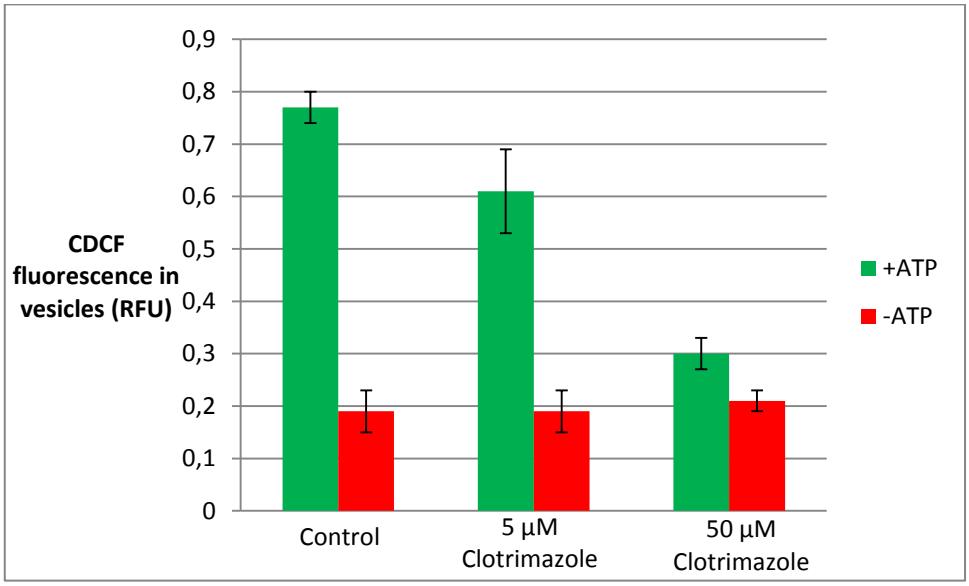


Figure 8. Average fluorescence of CDCF in vesicles ± standard deviation when incubating with clotrimazole or control with and without ATP (n=3).

4.4 Drug uptake experiments

Concentrations of the drugs tested in lysate, plasma membrane and cytoplasm of MDCKII-MDR1 cells with and without verapamil and in MDCKII-wt cells are presented in Appendix 4.

4.4.1 CDCF

Concentrations of CDCF in lysate, plasma membrane and cytoplasm fractions of MDCKII-MDR1 cells with and without verapamil and in MDCKII-wt cells with incubation concentrations 30, 100 and 200 µM are presented in Figure 9-Figure 11.

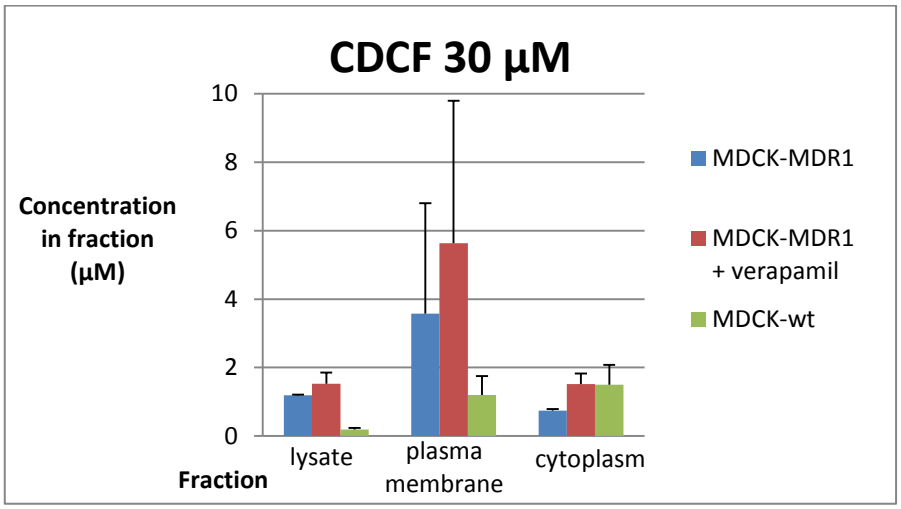


Figure 9. Mean concentrations ± standard deviation (n=2) of CDCF in lysate, plasma membrane and cytoplasm of MDCKII-MDR1 cells with and without verapamil and in MDCKII-wt cells when incubation concentrations of CDCF is 30 µM.

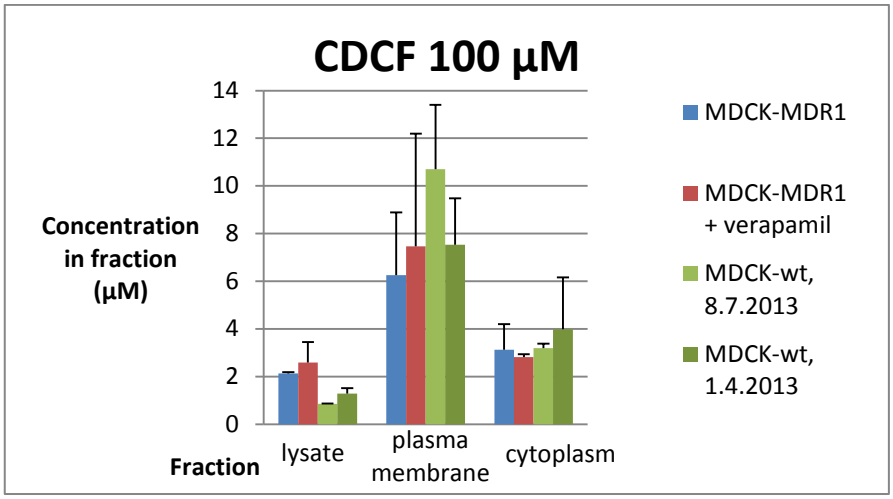


Figure 10. Mean concentrations ± standard deviation (n=2) of CDCF in lysate, plasma membrane and cytoplasm of MDCKII-MDR1 cells with and without verapamil and in MDCKII-wt cells when incubation concentrations of CDCF is 100 µM.

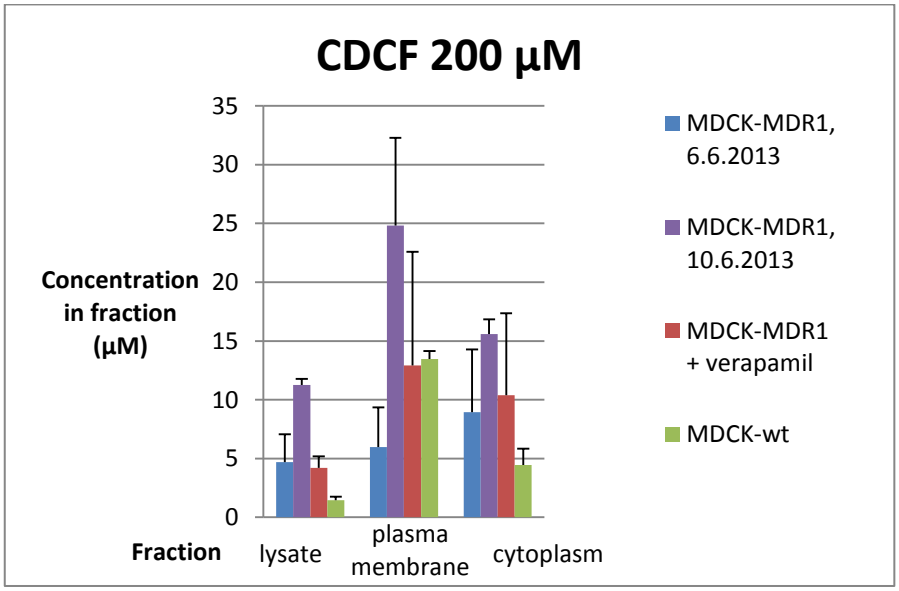


Figure 11. Mean concentrations ± standard deviation (n=2) of CDCF in lysate, plasma membrane and cytoplasm of MDCKII-MDR1 cells with and without verapamil and in MDCKII-wt cells when incubation concentrations of CDCF is 200 µM.

Uptake profiles of CDCF in MDCKII-MDR1 cells with and without verapamil and in MDCKII-wt cells are presented in Figure 12. Shown are concentrations in plasma

membrane, cytoplasm and lysate ($C_{fraction}$) when incubation concentration is increased from 30 to 200 µM.

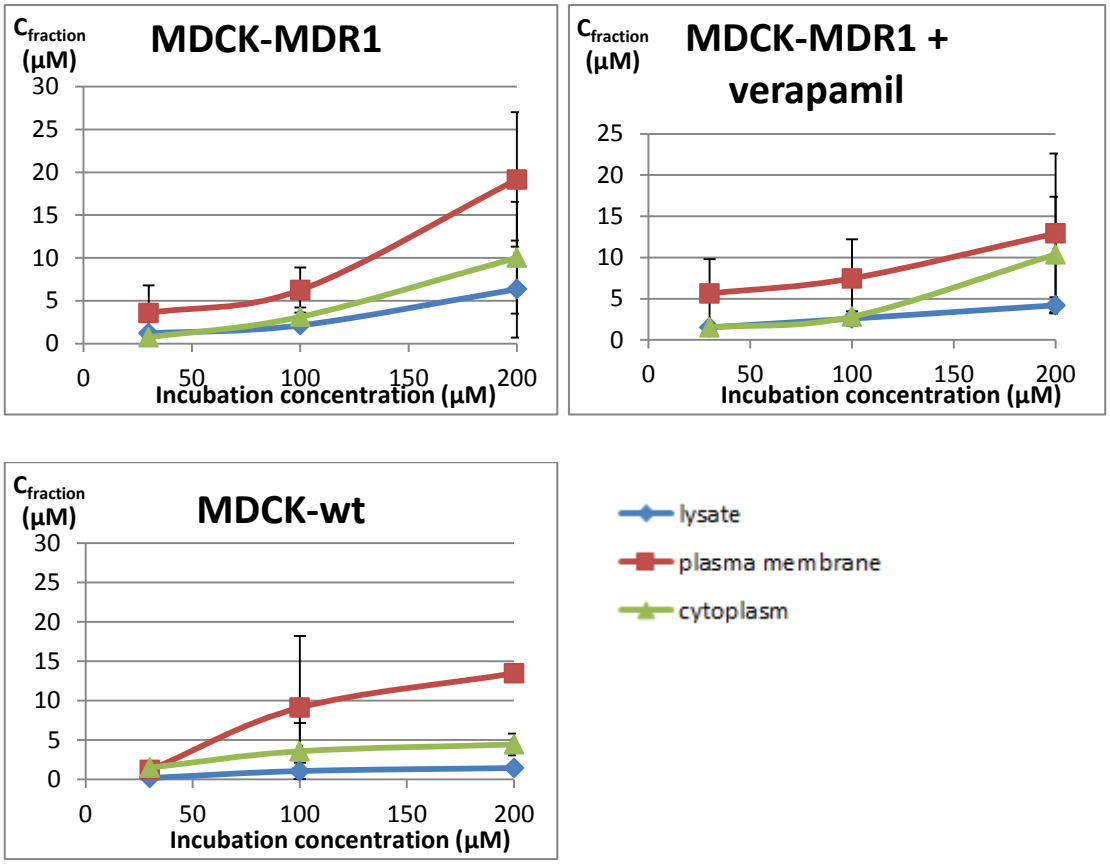


Figure 12. Uptake profile of CDCF. Mean concentration ± standard deviation (n=2) of CDCF in the fractions ($C_{fraction}$) after different incubation concentrations in MDR1 transfected cells with and without verapamil and in wild type cells.

Determination of the K_m(membrane) value can be done by determining the concentration in the plasma membrane of MDR1 cells when extracellular concentration is K_m (3 µM for CDCF). With the drug uptake experiments, the extracellular concentration is assumed to be approximately the same as the incubation concentration and the K_m(membrane) can be determined from the uptake profile. This requires data to be extrapolated, since the incubation concentration as low as 3 µM could not be tested because of detection limits. Value for K_m(membrane) calculated from this extrapolated curve is 0.3 µM (data not shown).

4.4.2 Clotrimazole

Concentrations of clotrimazole in lysate, plasma membrane and cytoplasm fractions of MDCKII-MDR1 cells with and without verapamil and in MDCKII-wt cells with incubation concentrations 30, 100 and 200 µM are presented in Figure 13-Figure 15.

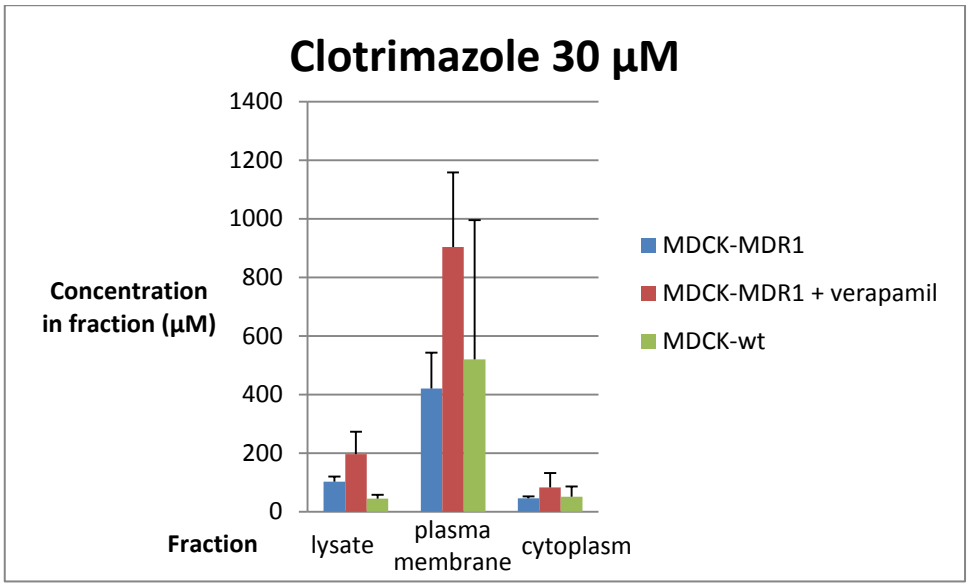


Figure 13. Mean concentrations ± standard deviation (n=2) of clotrimazole in lysate, plasma membrane and cytoplasm of MDCKII-MDR1 cells with and without verapamil and in MDCKII-wt cells when incubation concentrations of clotrimazole is 30 µM.

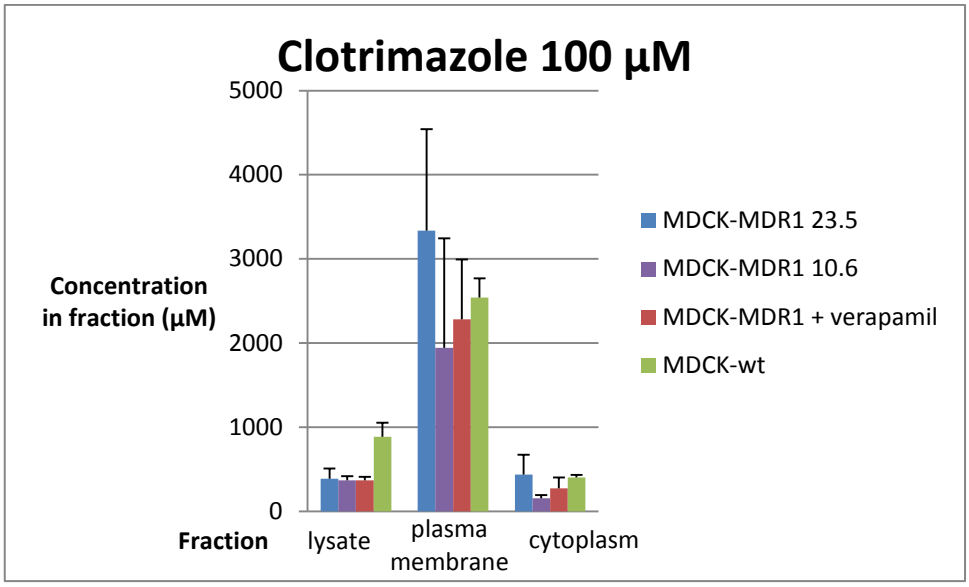


Figure 14. Mean concentrations ± standard deviation (n=2) of clotrimazole in lysate, plasma membrane and cytoplasm of MDCKII-MDR1 cells with and without verapamil and in MDCKII-wt cells when incubation concentrations of clotrimazole is 100 µM.

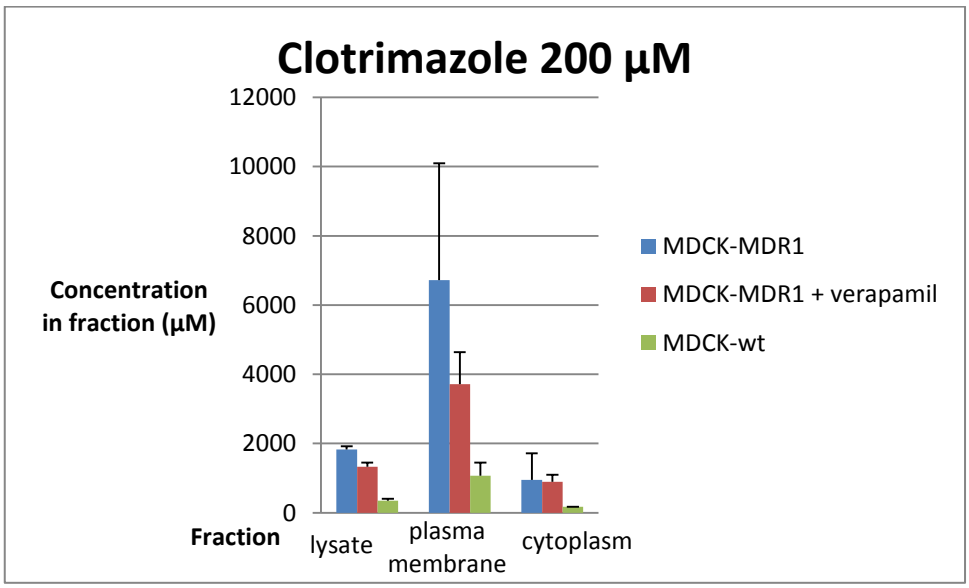


Figure 15. Mean concentrations ± standard deviation (n=2) of clotrimazole in lysate, plasma membrane and cytoplasm of MDCKII-MDR1 cells with and without verapamil and in MDCKII-wt cells when incubation concentrations of clotrimazole is 200 µM.

Uptake profiles of clotrimazole in MDCKII-MDR1 cells with and without verapamil and in MDCKII-wt cells are presented in Figure 16. Shown are concentrations in plasma membrane, cytoplasm and lysate ($C_{fraction}$) when incubation concentration is increased from 30 to 200 µM.

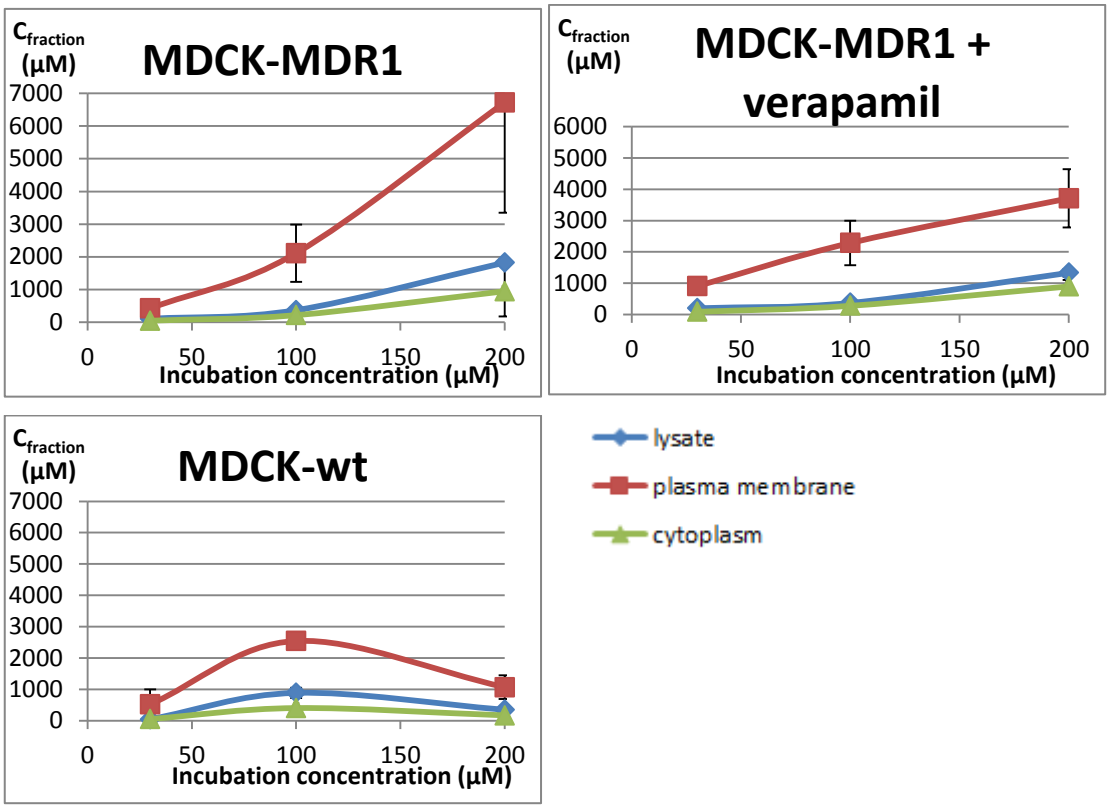


Figure 16. Uptake profile of clotrimazole. Mean concentration ± standard deviation (n=2) of clotrimazole in the fractions (y-axis) after different incubation concentrations (x-axis) in MDR1 transfected cells with and without verapamil and in wild type cells.

Inhibitory affinity of clotrimazole to MDR1 is K_i=44 µM, so K_i(membrane) value for clotrimazole in plasma membrane of MDR1 cells can be determined from the Figure 16. By definition, Ki(membrane) is the corresponding concentration in plasma membrane, when incubation concentration is 44 µM, so K_i(membrane)=597 µM.

4.4.3 Celiprolol

Concentrations of celiprolol in lysate, plasma membrane and cytoplasm fractions of MDCKII-MDR1 cells with and without verapamil and in MDCKII-wt cells with incubation concentrations 100 and 300 and 500 µM are presented in Figure 17-Figure 19.

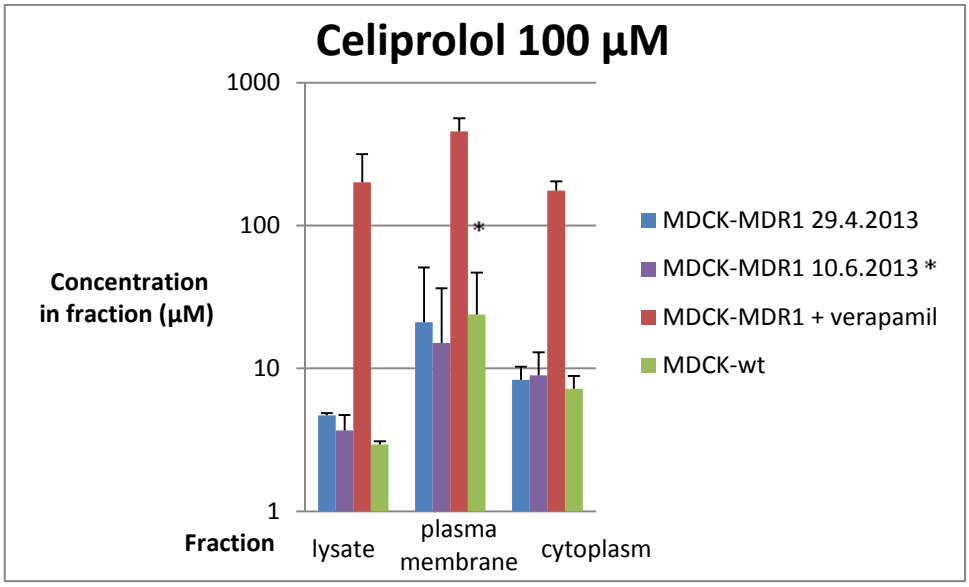


Figure 17. Logarithm of mean concentrations ± standard deviation (n=2) of celiprolol in lysate, plasma membrane and cytoplasm of MDCKII-MDR1 cells with and without verapamil and in MDCKII-wt cells when incubation concentrations of celiprolol is 100 µM. (* value extrapolated from calibration curve)

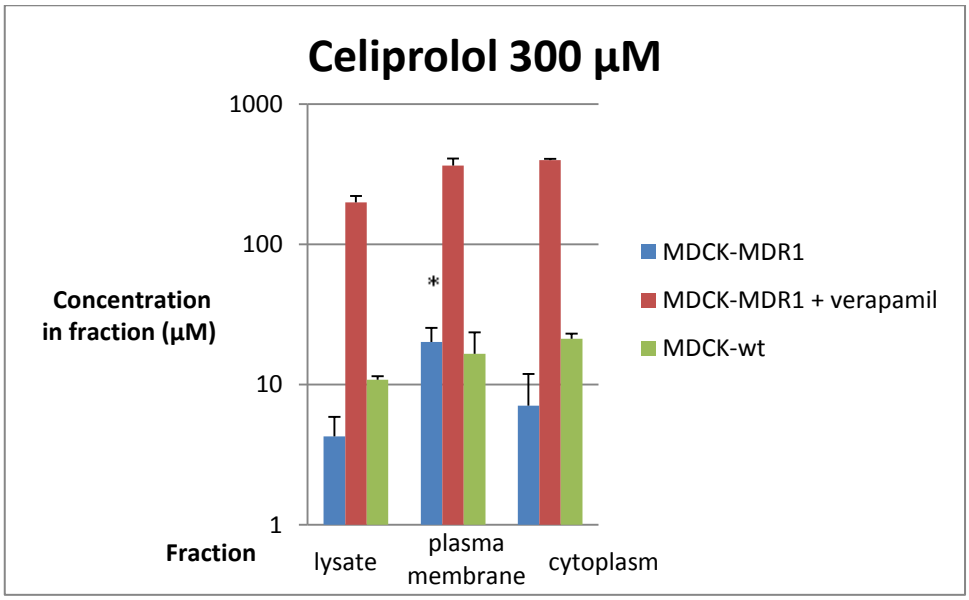


Figure 18. Logarithm of mean concentrations ± standard deviation (n=2) of celiprolol in lysate, plasma membrane and cytoplasm of MDCKII-MDR1 cells with and without verapamil and in MDCKII-wt cells when incubation concentrations of celiprolol is 300 µM. (*value extrapolated from calibration curve)

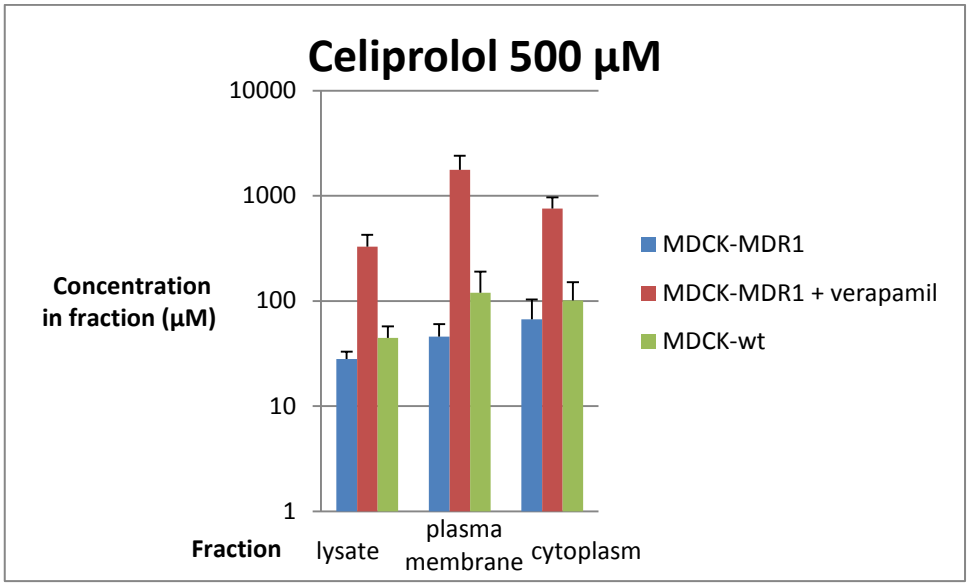


Figure 19. Logarithm of mean concentrations ± standard deviation (n=2) of celiprolol in lysate, plasma membrane and cytoplasm of MDCKII-MDR1 cells with and without verapamil and in MDCKII-wt cells when incubation concentrations of celiprolol is 500 µM.

Uptake profiles of celiprolol in MDCKII-MDR1 cells with and without verapamil and in MDCKII-wt cells are presented in Figure 20. Shown are concentrations in plasma membrane, cytoplasm and lysate ($C_{fraction}$) when incubation concentration is increased from 100 to 500 µM.

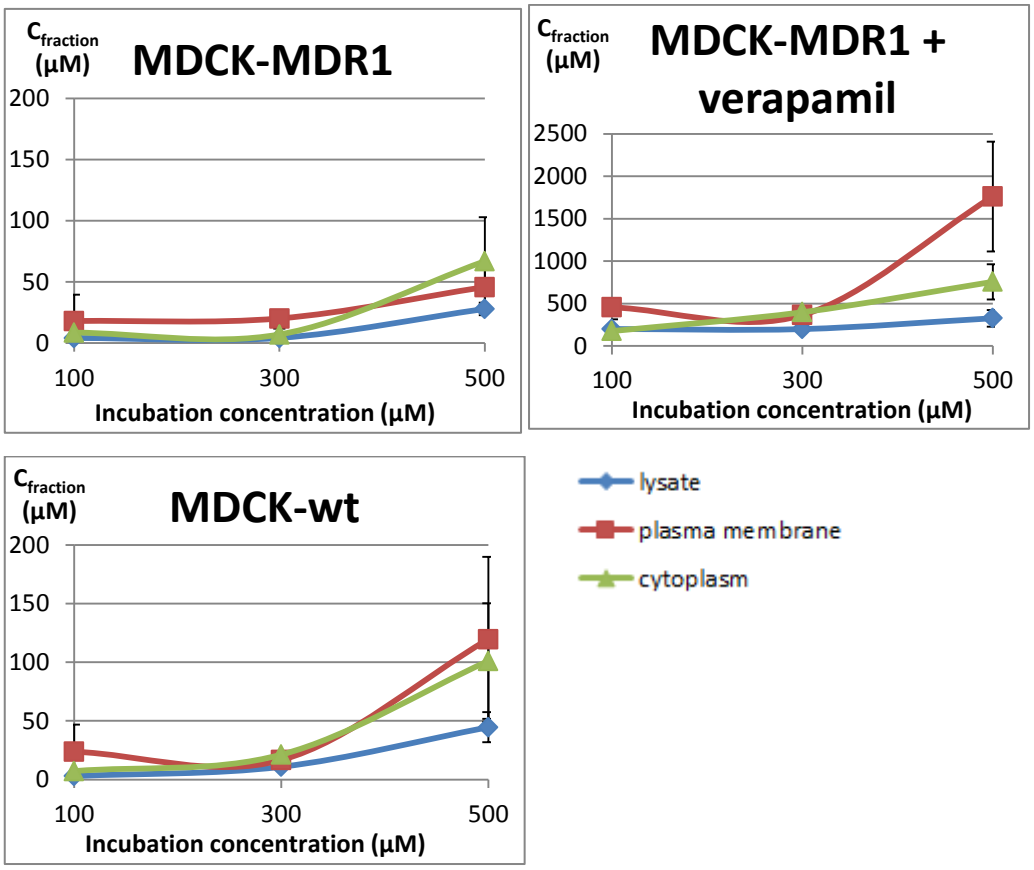


Figure 20. Uptake profile of celiprolol. Mean concentration ± standard deviation (n=2) of celiprolol in the fractions (y-axis) after different incubation concentrations (x-axis) in MDR1 transfected cells with and without verapamil and in wild type cells.

Determination of the K_m(membrane) value for celiprolol in plasma membrane of MDR1 cells when incubation concentration is K_m=1 mM, requires data to be extrapolated. Value for K_m(membrane) calculated from this extrapolated curve is 77 µM (data not shown).

5 DISCUSSION

5.1 Validation of isolation protocol

Immunoblotting with mitochondrial antibody was not successful. There were some technical problems due to e.g. unspecific binding. Secondary antibody from different batch was also tested but despite several attempts made, the immunoblotting was not successful. Validation of isolation protocol showed that in sample A (lysate) markers from cytoplasm, endoplasmic reticulum and plasma membrane was identified by antibodies, which is expected since lysate contains the broken cells and so all organelles of the cells should be found in that fraction. In sample B (mitochondria) no markers were identified and since immunoblotting was not successful with mitochondrial antibody, the content of the sample B remains to be confirmed. In sample C (plasma membrane) antibody identified plasma membrane and endoplasmic reticulum markers. In sample D (cytoplasm) markers from cytoplasm were identified as well as some weak bands from plasma membrane markers. Since the bands from the plasma membrane markers (gel 4) were stronger in lysate and plasma membrane, and all the other antibodies were found in their designated samples, it can be assumed that separation of the plasma membrane and cytoplasm was successful. The molecular weights of the identified proteins deviate from the ones expected. However, this is not uncommon and the reason could be e.g. degradation of the proteins by proteases, since protease inhibitors were not added. Antibody may also bind to other proteins in samples which can explain the different size of the proteins identified. Degradation and/or different glycosylation can also explain several bands shown in gels.

5.2 Uptake kinetics of CDCF

Uptake kinetics studies reveal that concentration in MDCKII-MDR1 cell lysates is increasing up to 90 minutes and after that the steady state seems to be achieved. Increase is linear up to 60 minutes and from 60 to 90 minutes, the concentration in cell

lysate is increased more strongly. The reason for this strong increase could be e.g. deterioration of cells. There seems to be no distinction between incubation with CDCF alone or with verapamil. Longer times were not studied since it cannot be confirmed that the cells maintain their functionality after long incubations without their growth medium and consequently the reliability of the results would deteriorate. The initial purpose of the uptake kinetics studies was to evaluate the incubation time needed after which steady state would be reached. Since necessary incubation time to achieve steady state might cause the cells to deteriorate, we ended up for 60 minutes incubation time, which should be short enough for the cells not to weaken and still long enough to achieve the concentrations in the fractions high enough to analyze.

5.3 Vesicle study for clotrimazole

Considering standard deviations, there is no difference in clotrimazole uptake into vesicles with and without ATP (Figure 7). Based on that it appears that clotrimazole is not a MDR1 substrate. However, since clotrimazole has high lipophilicity (log D (7.4) =4.9), it can permeate through vesicle membrane easily and passive diffusion has a large role in the permeation process. So even if it was a substrate of MDR1, the transporter should function very effectively to overcome passive diffusion. Inhibitor test reveals that clotrimazole lowers the concentration of CDCF in vesicles in the presence of ATP, whereas in the absence of ATP similar lowering does not occur (Figure 8). Consequently, it can be deduced that clotrimazole is an inhibitor of MDR1.

There has been some disagreement whether clotrimazole is a MDR1 substrate or not (Takano et al. 2006, Xue et al. 2004, Crivori et al. 2006). Possible interaction with MDR1 was further studied by drug uptake experiments, measuring clotrimazole uptake into MDCKII-MDR1 cells in the presence and absence of MDR1 inhibitor verapamil to see if inhibitor increases the concentrations in the cells.

5.4 Drug uptake experiments

5.4.1 CDCF

Concentrations of CDCF in fractions and between cell lines were quite similar in all cases (Figure 9-Figure 11). Only difference can be detected in lysate concentrations: Somewhat smaller concentrations can be seen in lysates of wild type cells when compared to MDR1 transfected cells.

In our experiments, CDCF did not show the expected behavior of a MDR1 substrate. There was no difference in the concentrations of fractions in MDR1 transfected cells with or without verapamil. Also, concentration in the plasma membrane of MDR1 transfected cells was not smaller than in wild type cells. One reason for these results may be that CDCF has high affinity to MDR1 (K_m value of 3 μM) and quite low V_{max} so with higher concentrations active transport is saturated and passive uptake dominates, and the concentrations in fractions become similar with and without inhibitor. Smaller incubation concentrations could not be tested, since the sensitivity of the analysis method was not high enough.

The reason for smaller concentration in lysates of wild type cells may be that overexpression of MDR1 protein can change the characteristic of plasma membrane and affect the drug permeation through it. Also different passage numbers can cause differences between wild type and MDR1 transfected cells, since the passage numbers used were 30-33 for wild type cells and 11-16 for MDR1 transfected cells.

No difference can be seen between uptake profiles of MDR1 transfected cells and wild type cells (Figure 12). Average concentrations in plasma membrane are similar or up to 3-fold larger than concentration in cytoplasm.

5.4.2 Clotrimazole

Focusing on MDR1 transfected cells, the difference with and without verapamil can only be seen when incubation concentration of clotrimazole is 30 μM (Figure 13). In that case, concentrations in lysate and plasma membrane of MDR1 transfected cells with verapamil are larger than without verapamil. With incubation concentrations 100 μM and 200 μM, concentrations in fractions are quite similar (Figure 14 and Figure 15). Some differences can be seen between wild type cells and MDR1 transfected cells: with incubation concentration 100 μM, concentrations are somewhat larger in lysates of wild type cells, and with incubation concentrations 30 and 200 μM, concentrations are smaller in wild type cells.

There has been some inconsistent information about whether or not clotrimazole is a substrate of MDR1. In our experiments, clotrimazole did not show the typical behavior of the MDR1 substrate in uptake experiments, except with the smallest concentration tested, where verapamil seemed to increase concentrations of clotrimazole in the fractions. Also, concentration in wild type cells was not the same as in MDR1 cells with verapamil. However, as discussed earlier, clotrimazole has high lipophilicity (log D (7.4) =4.9) so even if it was a substrate of MDR1, the transporter should function very effectively to overcome passive diffusion which has a large role in the permeation of highly lipophilic compounds. Minor difference can be seen with the smallest concentration 30 μM, and perhaps smaller concentrations should be tested to draw conclusions about clotrimazole interaction with MDR1, although detection limits can cause problems.

Different profile is observed when comparing concentrations in the fractions of wild type cells and MDR1 transfected cells (Figure 16). When incubation concentration increases, concentration in the fractions increases in MDR1 transfected cells. In wild type cells, some saturation can be seen when incubation concentration is increased from 100 μM to 200 μM and with 200 μM concentration in fractions are even smaller than with 100 μM. However, reason for this could be e.g. incomplete dissolution of

clotrimazole with concentration of 200 µM. Average concentrations in plasma membrane are significantly larger (4- to 10-fold) than in cytoplasm.

5.4.3 Celiprolol

With all the incubation concentrations, it can be seen that concentrations in fractions are greatest in MDR1 transfected cells with verapamil and quite similar in wild type- and MDR1 transfected cells (Figure 17- Figure 19).

Celiprolol shows the behavior expected from MDR1 substrates. When MDR1 is inhibited with verapamil, concentrations in fractions are more than 10-fold larger than without verapamil. However, concentrations in wild type cells are not similar as concentrations in MDR1 transfected cells with verapamil. The reason for this may be changed permeability of plasma membrane due to overexpression of MDR1 or different passage numbers between wild type and MDR1 transfected cells as discussed earlier. Also, there could be some constitutional transporters in MDCKII cells, which are also inhibited by verapamil and that could explain the difference between MDCKII-wt and MDCKII-MDR1 cells with verapamil.

Uptake profile in MDR1 cells with verapamil deviates from others (Figure 20). When MDR1 is inhibited with verapamil, the concentration in plasma membrane of MDR1 cells is larger than in cytoplasm with the largest incubation concentration 500 µM. With smaller concentrations and in other cell types, the concentrations are similar between different fractions. This is quite unexpected, since verapamil should increase the concentration in all fractions in a similar manner. Perhaps there could be some competitive processes involved in the partitioning of celiprolol to the plasma membrane, but further studies need to be conducted to clarify the mechanisms responsible for this. If incubation concentration is increased further, the effect of the transporter to concentrations is expected to increase, however this was not tested because of the quite large amounts of celiprolol needed for that. With incubation concentrations 100 and 300 µM, average concentrations in plasma membrane are similar or up to 3-fold larger than in cytoplasm.

5.4.4 Effect of lipophilicity to drug distribution

Comparing partitioning of drugs between plasma membrane and cytoplasm, it can be seen that the more lipophilic the compound, the larger is its concentration in plasma membrane compared to the cytoplasm. Clotrimazole as a lipophilic compound (log D (7.4)=4.9) has concentrations in plasma membrane that are 4-10-fold larger than in cytoplasm. On the other hand, celiprolol (log D (7.4)=0.1) and CDCF (log D (7.4)=-0.5) as less lipohilic compounds have concentrations in plasma membrane similar or up to 3-fold larger than in cytoplasm.

This effect of lipophilicity can also be seen in K_m(membrane) or K_i(membrane) values. K_i(membrane) for lipophilic clotrimazole is 597 µM, more than 13-fold larger than its K_i=44 µM. For celiprolol K_m(membrane) is only 77 µM, almost 13-fold smaller, than its K_m=1 mM and for CDCF K_m(membrane) is 0.3 µM, 10-fold smaller than its K_m=3 µM. So with lipophilic compounds, affinity to the MDR1 inside plasma membrane seems to be weaker (K_m is larger) than expected when K_m is determined from the concentrations in extracellular fluids. Opposite can be assumed to hold true for hydrophilic compounds. Also it can be assumed that because of the low affinity with hydrophobic compounds, binding to MDR1 is quite unspecific and perhaps multiple binding sites are involved whereas with hydrophilic compounds, the binding is more specific. So when predicting the role of active transport in the overall transport process, using K_m values determined from extracellular fluids can cause overestimation of the role with lipophilic compounds and underestimation with hydrophilic compounds. These results are in line with previous work by Clay and Sharom, who determined K_{Mlip} value (comparable to K_m(membrane)) for two lipophilic (calculated log P values log P=1.28 for LDS-751 and log P=2.96 for Hoechst 33342 (Royal Society of Chemistry 2013)) MDR1 substrates to be in the millimolar range (1.5-8.8 mM) whereas the affinity determined from the extracellular fluid K_m is in the micromolar range (0.8-2.8 µM) (Clay, Sharom 2013). Also Meier et al. (2006) reported K_m(membrane) values of millimolar range. Although as large difference of the K_m and K_m(membrane) values as was not observed in our work, the trend is similar. The reason for different results could be the use of phospholipids

monolayers instead of the whole cells used in our experiments. Clay and Sharom suggested that total binding affinity of the substrate to MDR1 is determined by both the substrate-lipid bilayer-interactions, and the substrate-MDR1-interactions. Our results are also in line with that assumption, since lipophilic clotrimazole has most likely larger affinity to lipids than less lipophilic celiprolol and CDCF.

5.4.5 Other studies

Quantitative studies determining the intracellular concentrations in plasma membrane and cytoplasm are still quite rare, so comparison of our results to others is challenging. Campoli et al. measured the concentration of posaconazole in plasma membrane and cytosol of pulmonary epithelial cells (A549) (Campoli et al. 2011). The absolute concentrations were not determined, but instead the concentrations were normalized to protein amounts of the samples. Concentration in plasma membrane was approximately 10-fold larger than cytosolic concentration, similar magnitude as our results with clotrimazole, which is expected since posaconazole is also a lipophilic compound (log P >3, (Courtney et al. 2004).

5.5 The applicability, challenges and further improvement of isolation protocol

The protocol for plasma membrane isolation introduced here can be used to study concentration in plasma membrane and cytoplasm of cells. One great advantage of this protocol is that the intracellular concentrations of non-fluorescent compounds can be determined, unlike with the most frequently used methods utilizing fluorescent microscopy. For drugs with intracellular targets, knowing the concentration of a drug in the site of action greatly improves the predictability of the pharmacological response. The same concerns also drug-transporter interaction and the effect of the active transport can be more accurately predicted knowing the concentrations in the transporter binding site. This protocol is not suitable for high-throughput measurements since the

volumes needed are large. The protocol is also quite laborious, including many steps, and is more valuable as a research tool to measure intracellular concentrations and to study partition of drugs between plasma membrane and cytoplasm.

One limiting factor in this protocol is the sensitivity of the analysis method. Experiments have to be conducted in T75 flasks, since with smaller culture bottles or well plates, the amount of plasma membrane and consequently concentration of drug in plasma membrane fraction becomes too small to detect. The large deviation seen especially with concentration in plasma membrane is most likely due to difficulties in re-suspending the pellet after the last centrifugation, since the pellet was so scarce and almost nonvisible in some experiments. There was also quite large deviation in the amount of cells in different flasks which is unexpected, since all the flasks are treated similarly and should contain approximately the same amount of cells. There could be many reasons for this, for example mixing could have been insufficient before dividing cells to new flasks or before taking sample to cell count. This deviation in the cell amounts reflects also to concentrations calculated based on cell count.

The protocol can be improved further. Homogenization can be improved e.g. by adding ammonium hydroxide or other detergent to cell suspension before homogenization. This pretreatment lyses the cells and can increase the fraction of the broken cells. The fraction of broken cells after homogenization was 70-85 % in most of the experiments but fractions as low as 45 % were measured. Since the amount of the broken cells was taken into account when calculating concentrations and the fraction of the broken cells in most of the cases was quite large, addition of the detergent was not essential. Also, detergent addition may result in detachment of the drug from the plasma membrane. However, if the concentration in the plasma membrane fraction is near analysis detection limit, the addition of the detergent after plasma membrane isolation can improve the reliability of the results. Cell count can be determined also by other methods. One option to determine cell count is to measure the protein concentration of the samples e.g. by Bradford method and proportion protein concentration to cell amount. This could be done after isolation of the fractions and the exact amount of protein in the samples could be determined. In our experiments, cell count was determined before and after homogenization but the amount of broken cells in fractions

can be smaller if some material gets lost during the isolation protocol. However, cell count by Cedex XS was fast and easy and it seemed more reasonable to count cells directly than determine the cell count indirectly from protein concentrations. Diffusion of the drug can be a problem in fractionation based methods of isolation. Whether or not working on the ice is sufficient to inhibit the diffusion should perhaps be examined for this protocol.

6 CONCLUSIONS

The protocol for isolation of plasma membrane and cytoplasm introduced in this work can be used to study intracellular partition of drugs. It is most valuable as a research tool in the later phases of the drug development, when the lead compound is already selected, since the protocol is quite laborious and not applicable for high throughput screening. In this work, it was used to study the plasma membrane and cytoplasm concentrations of three drugs with different lipophilicity and MDR1 interaction. Celiprolol concentrations in the fractions followed the trend expected for MDR1 substrates but CDCF and clotrimazole concentrations did not. It was found out that as expected, the partitioning of lipophilic compound (clotrimazole) into plasma membrane is larger than partition of hydrophilic compound (CDCF and celiprolol). Lipophilicity affected also to the K_m(membrane) or K_i(membrane) values determined for compounds, with clotrimazole K_i(membrane) value being larger than respective K_i value, and CDCF and celiprolol K_m(membrane) being smaller than their respective K_m values.

7 REFERENCES

Alberts B: Intracellular compartments and protein sorting. In book Molecular biology of the cell, pp. 695-749, 4th ed. Garland, New York 2002a.

Alberts B: Membrane transport of small molecules and the electrical properties of the membranes. In book Molecular biology of the cell, pp. 615-659, 4th ed. Garland, New York 2002b.

Campoli P, Al Abdallah Q, Robitaille R, Solis N, Fielhaber J, Kristof A, Laverdiere M, Filler S, Sheppard D: Concentration of antifungal agents within host cell membranes: a new paradigm governing the efficacy of prophylaxis. Antimicrob Agents Chemother 55: 5732-5739, 2011.

Clay A T, Sharom F J: Lipid bilayer properties control membrane partitioning, binding, and transport of p-glycoprotein substrates. Biochemistry (N.Y.) 52: 343-354, 2013.

Courtney R, Wexler D, Radwanski E, Lim J, Laughlin M: Effect of food on the relative bioavailability of two oral formulations of posaconazole in healthy adults. Br J Clin Pharmacol 57: 218-222, 2004.

Crivori P, Reinach B, Pezzetta D, Poggesi I: Computational models for identifying potential P-glycoprotein substrates and inhibitors. Mol Pharm 3: 33-44, 2006.

Drugsite Trust: Drugs.com -database 2013. From the internet 19.07.2013: http://www.drugs.com/.

Duodecim Oy: Duodecim lääketietokanta 2013. From the internet 19.07.2013: http://www.terveysportti.fi/terveysportti/dlr_laake.koti.

Duvvuri M, Krise J P: Intracellular drug sequestration events associated with the emergence of multidrug resistance: a mechanistic review. Front Biosci 10: 1499-1509, 2005.

Duvvuri M, Feng W, Mathis A, Krise J P: A cell fractionation approach for the quantitative analysis of subcellular drug disposition. Pharm Res 21: 26-32, 2004.

Giacomini K M, Huang S, Tweedie D J, Benet L Z, Brouwer K L, Chu X, Dahlin A, Evers R, Fischer V, Hillgren K M: Membrane transporters in drug development. Nat Rev Drug Disc 9: 215-236, 2010.

Hennessy M, Spiers J: A primer on the mechanics of P-glycoprotein the multidrug transporter. Pharmacol Res 55: 1-15, 2007.

Karlsson J, Kuo S, Ziemniak J, Artursson P: Transport of celiprolol across human intestinal epithelial (Caco-2) cells: mediation of secretion by multiple transporters including P-glycoprotein. Br J Pharmacol 110: 1009-1016, 1993.

Kato Y, Miyazaki T, Kano T, Sugiura T, Kubo Y, Tsuji A: Involvement of influx and efflux transport systems in gastrointestinal absorption of celiprolol. J Pharm Sci 98: 2529-2539, 2009.

Lärmer J, Schneider S, Danker T, Schwab A, Oberleithner H: Imaging excised apical plasma membrane patches of MDCK cells in physiological conditions with atomic force microscopy. Pflügers Archiv 434: 254-260, 1997.

Meier M, Blatter X L, Seelig A, Seelig J: Interaction of verapamil with lipid membranes and P-glycoprotein: connecting thermodynamics and membrane structure with functional activity. Biophys J 91: 2943-2955, 2006.

Pharma Professional Services: Online Drug Information System database 2013. From the internet 19.07.2013: http://www.druginfosys.com/.

Raub T J: P-glycoprotein recognition of substrates and circumvention through rational drug design. Mol Pharm 3: 3-25, 2006.

Rautio J, Humphreys J E, Webster L O, Balakrishnan A, Keogh J P, Kunta J R, Serabjit-Singh C J, Polli J W: In vitro p-glycoprotein inhibition assays for assessment of clinical drug interaction potential of new drug candidates: a recommendation for probe substrates. Drug Metab Disposition 34: 786-792, 2006.

Royal Society of Chemistry: ChemSpider database, 2013. From the internet 19.07.2013: http://www.chemspider.com/.

Sigma-Aldrich: Homepage 2013. From the internet 19.07.2013: http://www.sigmaaldrich.com/united-states.html.

Takano M, Yumoto R, Murakami T: Expression and function of efflux drug transporters in the intestine. Pharmacol Ther 109: 137-161, 2006.

Tang F, Horie K, Borchardt R T: Are MDCK cells transfected with the human MDR1 gene a good model of the human intestinal mucosa? Pharm Res 19: 765-772, 2002.

Vellonen K, Mannermaa E, Turner H, Häkli M, Wolosin J M, Tervo T, Honkakoski P, Urtti A: Effluxing ABC transporters in human corneal epithelium. J Pharm Sci 99: 1087-1098, 2010.

Wishart research group: DrugBank database 2013. From the internet 07.08.2013: http://www.drugbank.ca/.

Xue Y, Yap C W, Sun L Z, Cao Z W, Wang J, Chen Y Z: Prediction of P-glycoprotein substrates by a support vector machine approach. J Chem Inf Comput Sci 44: 1497-1505, 2004.

Yasuda K, Lan L, Sanglard D, Furuya K, Schuetz J D, Schuetz E G: Interaction of cytochrome P450 3A inhibitors with P-glycoprotein. J Pharmacol Exp Ther 303: 323-332, 2002.

APPENDIX 1

Protocol for isolation of cell organelles

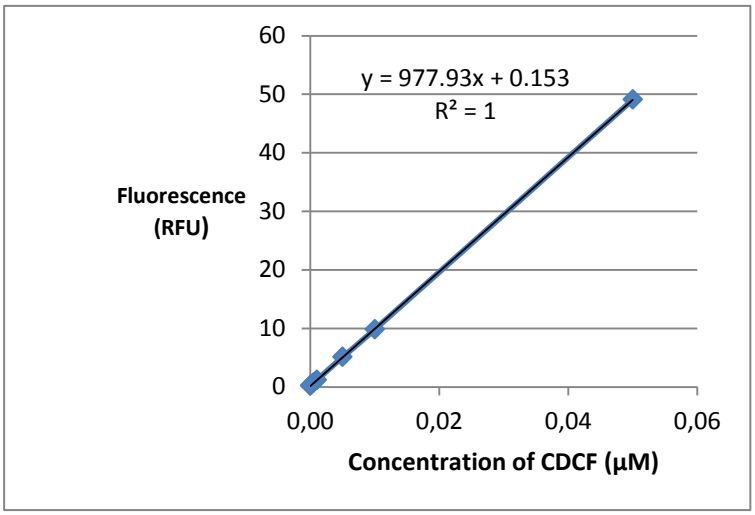
1. Detach cells from the bottom of the culture flask by cell scraper, centrifuge at 400g for 5 min at 4 °C. Resuspend pellet with 10 ml of cold PBS. Centrifuge suspension at 400g for 5 min at 4 °C.
2. Resuspend pellet with 5 ml of cold PBS and homogenize using Dounce homogenisator and 40 up and down strokes. Incubate for 1 hour on ice and take sample A (150 microl).
3. Centrifuge at 100 g for 2 min at 4 °C to remove unbroken cells and 600g for 10 min at 4 °C to remove nuclei.
4. Centrifuge at 15 000 g for 5 min at 4 °C to remove mitochondria, lysosomes and peroxisomes. Resuspend pellet with 200 µl of PBS (sample B).
5. Centrifuge supernatant at 100 000g for 1 hour at 4 °C to remove plasma membrane. Resuspend pellet with 200 µl of PBS (sample C). Supernatant is sample D (cytoplasm).

APPENDIX 2.

Standard curve for CDCF

Standard curve for CDCF 22.3.2013

Concentration of CDCF (µM)	Fluorescence (RFU)			Average	SD	SD (%)
0.05	50.6400	49.1500	47.4100	49.0667	1.6166	3.2947
0.01	9.8360	9.5650	10.0300	9.8103	0.2336	2.3808
0.005	5.2840	4.9590	5.0900	5.1110	0.1635	3.1993
0.001	1.1640	1.0410	1.1640	1.1230	0.0710	6.3236
0.0005	0.6682	0.5897	0.7375	0.6651	0.0739	11.1177
0.0001	0.2480	0.2882	0.2657	0.2673	0.0201	7.5375
0	0.1588	0.1468	0.1657	0.1571	0.0096	6.0878



APPENDIX 3

Assay steps in vesicle study

1. Mix 1000 µl of membrane suspension with 3925 µl Assay mix. Add 75 µl 50 µM CDCF. Mix well, but gently! Add 50 µl of the suspension to each well on a standard 96-well plate.

2. Add test drugs (in 0.75 µl DMSO) and DMSO as indicated on the plate setup.

3. Mix 90 µl of Mg-ATP with 1410 µl assay mix.

4. Preincubate the plate, 1500 µl Mg-ATP solution and assay mix for 10 min at 37 °C.

5. Start the reaction by adding 25 µl of the MgATP solution or Assay mix to appropriate wells as indicated on the plate setup. Incubate the plate at 37 °C for 30 min.

6. Wet the filters with 100 µl of distilled water per well and set up the filtering apparatus.

7. Stop the reaction by adding 200 µl of ice-cold Washing mix to the wells. Transfer the samples from the 96-well plate to the filter plate and filter.

8. Wash the wells 5 times with 200 µl washing mix (should be done in 2 minutes from stopping the reaction).

9. Dry the filters.

10. Add 100 µl of 0.1 M NaOH to each well and incubate for 10 min. Transfer the liquid under vacuum to a clear, flat-bottom 96-well plate.

11. Measure fluorescence at Ex: 510 nm and Em: 535 nm. (bandwith 12 nm + scan 450-510 nm to pdf)

	+ ATP			- ATP		
	7	8	9	10	11	12
A	Clotrimazole 1 µM					
B	Clotrimazole 10 µM					
C	Clotrimazole 100 µM					
D	0.5 µM CDCF + 5 µM Clotrimazole					
E	0.5 µM CDCF + 50 µM Clotrimazole					
F	0.5 µM CDCF + 100 % DMSO					

APPENDIX 4

Concentrations in drug uptake experiments

CDCF 30 µM

MDR1, 27.5

	lysate	membrane	cytoplasm
average	1.193	3.579	0.736
std.dev	0.012	3.222	0.048

wt 8.7

	lysate	membrane	cytoplasm
average	0.184	1.197	1.498
std.dev	0.055	0.550	0.583

+ verapamil, MDR1, 27.5

	lysate	membrane	cytoplasm
average	1.526	5.632	1.518
std.dev	0.326	4.164	0.312

CDCF 100 µM

MDR1, 25.4&29.4

	lysate	membrane	cytoplasm
average	2.130	6.249	3.122
std.dev	0.049	2.636	1.081

wt 8.7

	lysate	membrane	cytoplasm
average	0.852	10.705	3.185
std.dev	0.012	2.691	0.199

+ verapamil, MDR1, 25.4&29.4

	lysate	membrane	cytoplasm
average	2.585	7.469	2.816
std.dev	0.866	4.721	0.119

wt 1.4

	lysate	membrane	cytoplasm
average	1.286	7.534	3.986
std.dev	0.221	1.947	2.178

CDCF 200 µM

MDR1, 6.6

	lysate	membrane	cytoplasm
average	4.689	5.965	8.945
std.dev	2.380	3.383	5.344

MDR1, 10.6

	lysate	membrane	cytoplasm
average	11.245	24.820	15.599
std.dev	0.529	7.458	1.233

+ verapamil, MDR1, 6.6

	lysate	membrane	cytoplasm
average	4.207	12.918	10.376
std.dev	0.968	9.683	6.976

wt 8.7

	lysate	membrane	cytoplasm
average	1.456	13.477	4.446
std.dev	0.305	0.673	1.389

Clotrimazole 30 µM

MDR1, 23.5

	lysate	membrane	cytoplasm
average	102.759	420.561	45.389
std.dev	16.659	122.502	6.163

wt, 17.6

	lysate	membrane	cytoplasm
average	44.898	520.424	50.6211
std.dev	12.189	475.488	35.612

+ verapamil, MDR1, 30.5

	lysate	membrane	cytoplasm
average	196.829	903.220	83.155
std.dev	76.308	255.264	48.373

Clotrimazole 100 µM

MDR1, 23.5

	lysate	membrane	cytoplasm
average	387.3813	3335.589	436.884
std.dev	120.114	1207.367	236.909

MDR1, 10.6

	lysate	membrane	cytoplasm
average	367.457	1941.091	155.712
std.dev	50.414	1302.649	35.3608

+ verapamil, MDR1, 23.5

	lysate	membrane	cytoplasm
average	366.680	2283.280	274.100
std.dev	41.262	709.193	127.681

wt, 8.4

	lysate	membrane	cytoplasm
average	885.107	2541.881	400.709
std.dev	166.973	226.941	32.835

Clotrimazole 200 µM

MDR1, 6.6

	lysate	membrane	cytoplasm
average	1824.378	6725.089	946.363
std.dev	95.599	3375.062	772.654

wt 17.6

	lysate	membrane	cytoplasm
average	345.894	1067.423	167.932
std.dev	56.133	377.553	7.958

+ verapamil, MDR1, 3.6

	lysate	membrane	cytoplasm
average	1329.633	3710.589	894.360
std.dev	122.319	928.677	204.253

Celiprolol 100 µM

MDR1, 29.4

	lysate	membrane	cytoplasm
average	4.694	21.063	8.327
std.dev	0.161	29.788	1.927

MDR1, uusinta, 10.6

	lysate	membrane	cytoplasm
average	3.685	15.077	8.956
std.dev	1.025	21.322	3.976

+ verapamil, MDR1, 29.4

	lysate	membrane	cytoplasm
average	200.397	456.229	175.765
std.dev	115.315	105.843	28.304

wt, 24.6

	lysate	membrane	cytoplasm
average	2.933	23.762	7.179
std.dev	0.154	23.033	1.663

Celiprolol 300 µM

MDR1, 30.5

	lysate	membrane	cytoplasm
average	4.274	20.180	7.085
std.dev	1.601	5.273	4.808

wt 24.6

	lysate	membrane	cytoplasm
average	10.859	16.614	21.209
std.dev	0.650	7.019	1.841

+ verapamil, MDR1, 30.5

	lysate	membrane	cytoplasm
average	199.349	364.133	397.919
std.dev	22.025	45.852	10.010

Celiprolol 500 µM

MDR1, 3.6

	lysate	membrane	cytoplasm
average	27.960	45.827	66.905
std.dev	5.063	14.290	36.009

wt 24.6

	lysate	membrane	cytoplasm
average	44.603	119.688	101.111
std.dev	12.772	70.247	49.347

+ verapamil, MDR1, 3.6

	lysate	membrane	cytoplasm
average	327.133	1762.075	756.772
std.dev	98.182	646.507	208.636

extrapolated from calibration curve