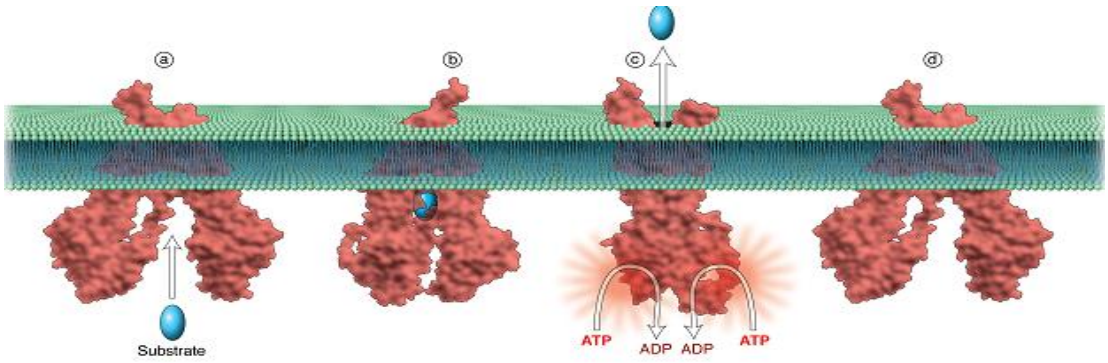
# Partial characterization of predicted ABCC5 inhibitors by the aid of human erythrocyte inside-out vesicles

Master of Science thesis

By

Elin Øien Ørvoll

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# Abstract

ABCC5 is a member of the superfamily of ABC-transporters, and it has been identified as an efflux transporter of cGMP. This transporter is also involved in export of antibiotic and cytostatic drugs from target cells, and as such represents a challenge in treatment of cancer and infectious diseases. In order to find inhibitors to ABCC5 mediated drug efflux, compounds predicted as potent inhibitors by virtual ligand screening (VLS) were chosen for in-vitro studies by the use of human erythrocyte inside-out vesicles (IOV). The procedure for IOV preparation was improved, and transport assays were performed where the inhibiting effects of the various compounds on transport of cGMP into inside-out vesicles were measured. Several of these compounds showed a potent inhibiting effect on cGMP transport, and the few that were chosen for further characterization showed more potent inhibition of ABCC5 than the known ABCC5 and PDE5 inhibitor sildenafil.

# Abbreviations

| | |
|---|---|
| ABC | ATP-binding cassette |
| ANP | Atrial natriuretic peptide |
| Asn | Asparagine |
| ATCC | Acetylthiocholine-chloride |
| ATP | Adenosine 5’-triphosphate |
| BSA | Bovine serum albumin |
| cAMP | Cyclic adenosine monophosphate |
| CFTR | Cystic fibroses transmembrane conductance regulator |
| cGMP | Guanosine 3’:5’-cyclic monophosphate |
| GC | Guanylate cyclase |
| GTP | Guanosine-5’-triphosphate |
| IOV | Inside-out vesicle |
| MDR | Multi drug resistance |
| MOAT | Multispecific organic anion transporter |
| MRP | Multidrug resistance protein |
| NBD | Nucleotide binding domain |
| NO | Nitric oxide |
| PDE | Phosphodiesterase |

| | |
|---|---|
| RBC | Red blood cell |
| ROV | Rightside-out vesicle |
| Ser | Serine |
| SUR | Sulfonylurea receptors |
| Thr | Threonine |
| TMD | Transmembrane domain |
| VLS | Virtual ligand screening |

# Contents

# 1. INTRODUCTION

## 1.1 Brief introduction to pharmacology

The word pharmacology is derived from two Greek words, *pharmakon* meaning ‘drug’, ‘poison’ or ‘medicine’ and *logos* being equivalent to ‘study’. In broad terms a drug may be defined as any chemical other than food that has effect on living organisms, but in a medical sense the term drug is used to describe any chemical agent used to treat, prevent, cure or diagnose a disease. Pharmacology may be explained as the unified study of the property of chemicals and the property of living organisms and all aspects of the way they interact with each other. Common further characterization of pharmacology is to divide the study into pharmacodynamics and pharmacokinetics. Pharmacodynamics may be defined as the study of the biochemical and physiological effect of a chemical at all levels of organization to a living organism; or in short, what a chemical does to a living organism. Pharmacokinetics may be called the study of the factors that influence the amount of a chemical at the sites of biological effects in a living organism; in short, what a living organism does to the chemical. These factors include the rate of absorption of the chemical, the rate of distribution to the site of action, the rate of metabolism by the organism, and the rate of excretion.

A chemical or drug has to react with a binding site on a cell or tissue to produce an effect. These binding sites are often referred to as drug targets, and most drug targets are proteins. In a pharmacological perspective the regulatory proteins that function as drug targets may with a few exceptions be divided into four groups:

- Enzymes
- Receptors
- Ion channels
- Carrier molecules (transporters)

### 1.1.1 Enzymes

With the exception of some catalytic RNA molecules, enzymes are highly specialized proteins that function as a catalyst in order to increase the rate of a reaction. The reaction rate is enhanced by lowering activation energy.

An enzyme-catalyzed reaction takes place in an enzymes active site. The surface of this active site is lined with amino acids containing groups that have the ability to bind a molecule (substrate) that is specific to a special enzyme.

When an enzyme is the target for a drug molecule, the drug molecule is often a substrate analogue that acts as a competitor to the endogenous substrate and by its interaction with the enzyme inhibits a normal reaction. The drug molecule may also act as a false substrate and the metabolite produced will be abnormal. An enzyme may also create an active drug through a reaction with an inactive drug molecule (pro-drug).

### 1.1.2 Receptors

Receptors are protein structures situated on a tissue or cell, with the ability to recognize and bind to endogenous or exogenous substances at the site of action. They play an important part in the chemical communication system that regulates the function of all the different cells in the body.

When a drug molecule binds to a receptor, it may or may not result in an activation of the receptor leading to a response in a cell or tissue. A drug that binds to a receptor may also be called a ligand, and when this binding leads to a response, the ligand is called an agonist. A ligand that binds to a receptor and thereby prevents the effect of an agonist is called a receptor antagonist.

### 1.1.3 Ion channels

Ion channels are proteins integrated in a cell membrane forming pores that regulate the flow of ions into or out of the cell or cell organelles. Some channels allow passage of ions based on their charge, while others are gated.

The opening or closing of gated channels is governed by voltage (membrane potential), ligand binding or other chemical signals.

Drugs can either interact with ion channels by plugging the channel physically and thus block the permeability to ions, or the drug may modulate the channel directly with binding to the channel itself or indirectly by involving intermediaries, both resulting in increased or decreased transport through the ion channel.

### 1.1.4 Transporters

Transmembrane transport processes are usually mediated by protein structures that are integrated within the membrane. Synonyms used for these transport proteins are transporter, porter, transport systems, permeases, or permease systems.

Transport systems are very important to the cell. These systems allow the entry of essential nutrients into the cell, first to the cytoplasmic compartment and then into the different cell organelles. Transporters also contribute in the regulation of metabolite concentrations with excretion of end products from metabolic pathways both from cell organelles and the cytoplasmic compartment of the cell. They also play an important role in maintaining the membrane potential by mediating efflux and uptake of ionic species to create a milieu that differs between the inner and the outer side of the membrane. Another very important role is the active extrusion of drugs and other toxic compounds from cell's compartments or the plasma membrane (Saier 2000).

A compound that is sufficiently lipid soluble is able to penetrate the cell membrane by itself. This is called passive diffusion, and the rate of diffusion is dependent on the compound's degree of lipid solubility. Ions and other small molecules are often too polar to do the same and thus require a transport protein to get across a cell membrane.

## *1.2 Classifications of transporters (TC-system)*

A classification system for transporters based on function and phylogeny was published in 2000 (Saier 2000). In this system transmembrane solute transporters are divided into 6 different categories, but in the overview presented in Fig 1, only carriers are shown in some detail.

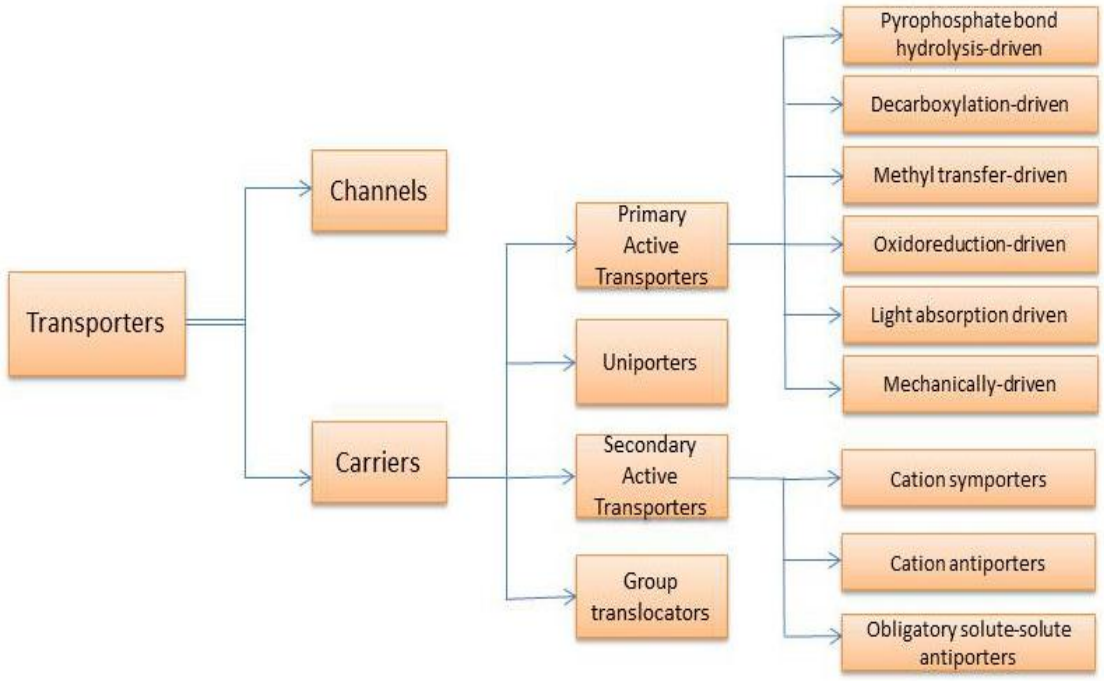


**Fig 1**: Scheme illustrating selected transporters of the currently recognized membrane solute transporters found in nature. Transporter proteins are divided into channels and carriers. The subdivision of channels is not shown here. Carriers are subdivided into four groups; primary active carriers, secondary active carriers, uniporters and group translocators. Primary active transport can be driven by chemical energy produced in i. e. ATP hydrolysis, decarboxylation, methyl transfer, oxidation-reduction reaction, light absorption or mechanically forces. Secondary active transport is driven by ions and is able to transport a variety of cations across the cell membrane.

*Channels* function as pores allowing movement of a solute down an electrochemical gradient after being opened by an electrochemical or chemical stimulus. This type of transport requires no additional energy source.

*Active carriers* transport a solute against a concentration gradient, and this translocation requires coupling of another energy producing process to the transporter in order to move the solute across a biological membrane.

Transport of a solute by an active carrier may take place by a “symporter” (cotransporter) that translocates two or more solutes in the same direction, or an antiporter (exchange transporter) that catalyzes the translocation of one or more solutes in opposite directions of each other. Uniporters transport one solute down the concentration gradient and are as such facilitated diffusion carriers. Both symporters and antiporters belong to the porter group called “Secondary Active Transporter” which uses the movement of one molecular species down the concentration gradient to drive the transport of another molecular species across the membrane. When a primary energy source like a chemical reaction, electron flow or light absorption is coupled to the translocation of a solute across a membrane, the transporter protein is considered a primary active transporter.

## *1.3 ABC-transporters*

The ABC (ATP-binding cassette) family of transporters has 49 human members (Loo and Clarke 2008) and they are classified into seven subfamilies. The subfamily classification is as follows, with the number of members of each family in parentheses:
ABCA (12), ABCB (11), ABCC (13), ABCD (4), ABCE (1), ABCF (3), ABCG (5). The ABCB subfamily is usually referred to as “MDR-ABC transporters” and the ABCC subfamily can be referred to as the “MRP-ABC transporters”.

Many of these transporters have been cloned, and studies have identified their substrates and their expression in different tissues. This knowledge has made significant progress in understanding the various transport mechanisms for both endogenous solutes as well as xenobiotics in and out of cells (Choudhuri and Klaassen 2006).

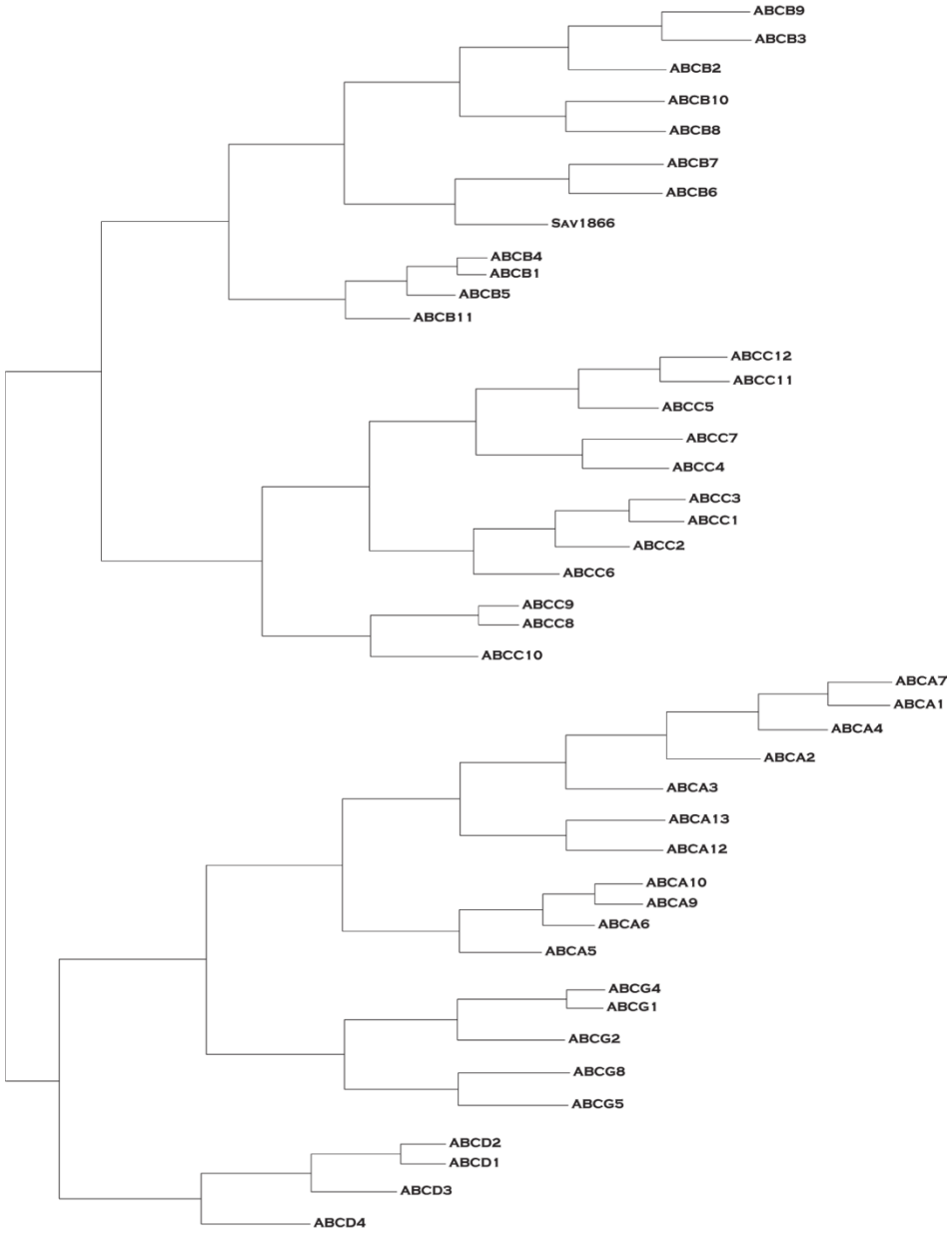


**Fig 2**: Phylogenic classification tree for ABC transporters

The general structure of ABC transporters is based on the study of the best characterized member which is ABCB1 (MDR1/P-glycoprotein). A typical structure consists of two parts that have a high degree of similarity in respect to amino acid sequence. Each part has a hydrophobic transmembrane domain (TMD) that typically contains six transmembrane segments and a hydrophilic nucleotide-binding domain (NBD) that are situated on the intracellular compartment of the cell. Each NBD contains 3 distinct motifs that are called Walker A, Walker B and ABC signature (C). Motif C is located

upstream to the Walker B motif.

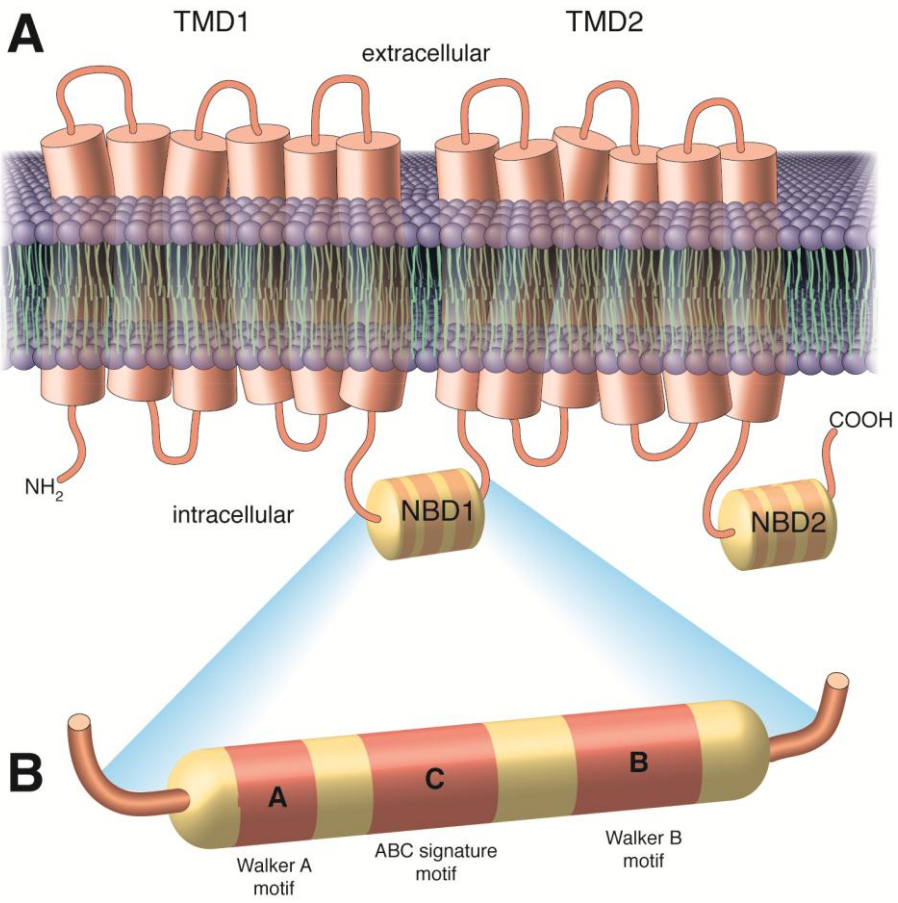


**Fig 3**: **A**. General structure of ABCC 4,5,11 and 12 embedded in the lipid bilayer. The protein contains two transmembrane domains each with 6 membrane spanning helices, and two nucleotide (ATP) binding domains. **B**. The nucleotide binding domain of ABC proteins all contains the Walker A and the Walker B motifs in addition to the ABC signature motif C.

The identification and characterization of the multidrug resistance protein (ABCC, MRP) family as drug and conjugate efflux pumps started with the cloning of ABCC1 (MRP1) by Cole in 1992 (Cole et al. 1992). It was later functionally characterized as an anionic efflux pump depending on hydrolysis of ATP as an energy source (Jedlitschky et al. 1994; Leier et al. 1994; Saier 2000). The terminology of multi-drug resistance protein or multidrug resistance-associated protein came into use when cells overexpressing ABCC1 showed increased resistance to drugs.

## *1.4 ABCC5*

ABCC5 (MRP5) is a member of the C subfamily of ABC-transporters, and it is classified as a pyrophosphate bond hydrolysis-driven primary active transporter in the transporter classification system. This subfamily contains 13 members, and nine of them; ABCC (1-6) and ABCC (10-12) are multidrug resistance protein related. The other 3 are the cystic fibroses transmembrane conductance regulator (ABCC7/CFTR) and the two sulfonylurea receptors (ABCC8/SUR1 and ABCC9/SUR2). Primary structures and the transmembrane domains differ among ABC transporters and these differences are recognized as the main determinants of substrate specificity among the different transporters. The ATP binding cassette, containing Walker A, Walker B, and Signature C and being essential for the binding of ATP, share an overall sequence identity of approximately 30% among the different members of the family.

ABCC5 (previously termed MRP5 and MOAT-C) is identified as a transporter of cGMP (Jedlitschky et al. 2000). Different cells have the ability to transport cGMP, and ABCC5 is expressed in most tissues in the human body. Intermediate levels are found in heart, brain and testis, while the highest levels are found in skeletal muscle. In liver and lung the levels found are barely detectable. ABCC5 is also localized in vascular smooth muscle cells, in smooth muscle cells of the corpus cavernosum, ureter and bladder, in pyramidal neurons and astrocytes and in placenta. ABCC5 is also identified as responsible for the high affinity transport of cGMP in erythrocytes.

Multidrug resistance transporter proteins are a part of a system most likely evolved to protect the cell from cytotoxicity either from xenobiotics or endogenous solutes in concentrations above the normal level. However, this efflux system presents a challenge in the treatment of cancer and infectious diseases in addition to difficulties in the development of new therapeutics (Chang and Roth 2001). To overcome this problem, more knowledge about the structure and function of ABCC is required.

## *1.5 cGMP*

Guanosine 3'-5' cyclic monophosphate (cGMP) was first discovered in rat urine by Ashman et al. in 1963, and that led to a search for this cyclic nucleotide's biological role. Goldberg and his group (Goldberg et al. 1969) established that cGMP was a natural constituent in mammalian tissues, and they were able to quantify this compound in milligram amounts in biological samples from liver, kidney, and brain as well as from urine samples.

Since 1977 several reports about plasma and urine cGMP concentrations in relation to both pathological and physiological conditions were published. cGMP was proposed as a biomarker for various kinds of cancers, including cancers in the breast, ovaries, lung, colon, uterine cervix, liver and in leukemia. Reports were also given about cGMP related to diseases in lung, liver, and the cardiovascular system (Dazert et al. 2003; Miller and Yan 2010) as well as of its usefulness as a biomarker of both normal and pathological pregnancies (Francoual et al. 1995a; Francoual et al. 1995b; Grunewald et al. 1994).

In approximately the same period it became clear that cGMP was transported out of cells by an energy dependent process, and several membrane transporter proteins have been suggested as the cGMP pumps.

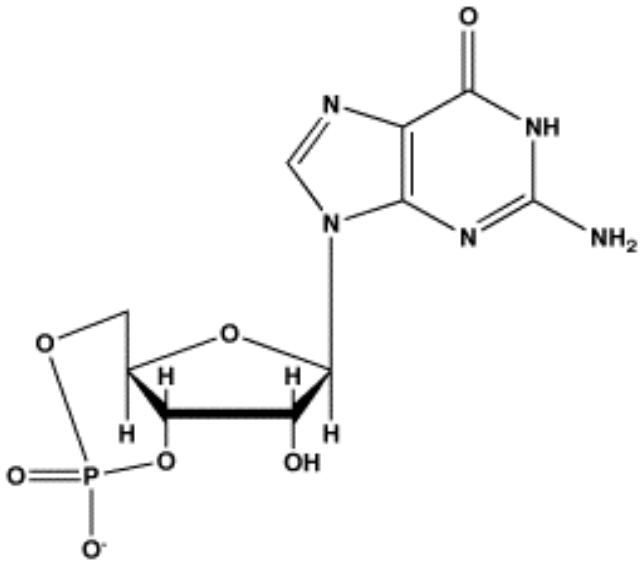


**Fig 4**: Structure formula of cGMP

### 1.5.1 Synthesis of cGMP

Some of the synthesis of cGMP is activated by endogenous nitric oxide (NO), a free radical gas that in biological systems is formed when nitric oxide synthase catalyzes the reaction between molecular oxygen and L-arginine. Among NO's several postulated physiological roles, which include reducing peripheral vascular resistance that decreases systemic blood pressure, inhibition of platelet aggregation, and effects in both the central and peripheral nervous system, it is the endogenous activator of soluble guanylate cyclases (sGC). This part of the cGMP production is initiated by NO, and as a consequence cGMP is regarded as a second messenger to NO.

Membrane bound GCs also called particulate GC (pGC)are divided into different classes due to structural similarity and tissues they are found in. All pGCs share a structure that consists of an intracellular region where a domain with similar properties to a protein kinase and a catalytic cyclic domain are found, in addition to a transmembrane domain and an extracellular ligand binding domain (Yang et al. 1995). The different pGCs are activated by peptide hormones like the atrial natriuretic peptide (ANP) and they are named with a letter in addition to GC, with different letters according to the tissues

where they are found.

GC-A is found in kidney, testis and retina, and this isoform is activated by ANP. ANP is produced in the walls of the heart as a response to high pressure, and after transport to the kidney, a resulting effect is increased cGMP concentration which leads to increased efflux of $Na^+$, diuretic effect and reduced blood pressure (Nelson 2005).

GC-B is found in fibroblasts and the central nervous system, and the enzyme is activated by a peptide called brain natriuretic peptide (BNP).

GC-C, GC-D, GC-E and GC-F are also classified with special functions related to nervous tissues.

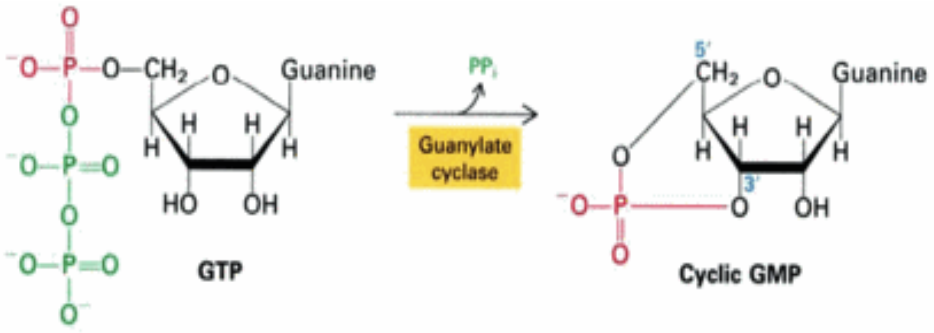


**Fig 5**: Synthesis of cGMP

http://openwetware.org/wiki/BIO254:2ndMessenger

Guanylate cyclases catalyze the reaction where guanosine-5'-triphosphate (GTP) is converted to cGMP

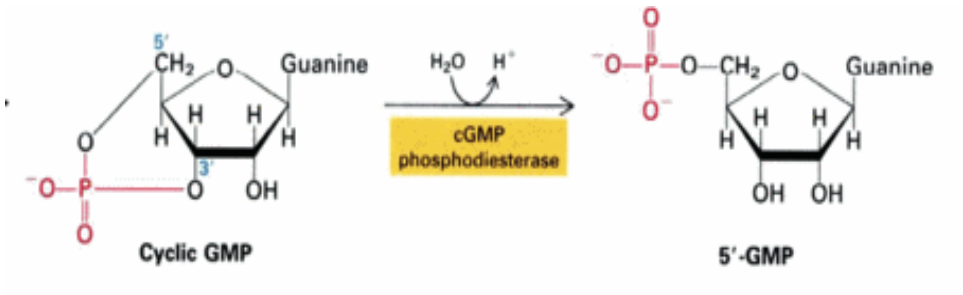


**Fig 6**: Degradation of cGMP

http://openwetware.org/wiki/BIO254:2ndMessenger

### 1.5.2 Degradation of cGMP

The degradation of cGMP is catalyzed by cGMP phosphodiesterases (PDE) which hydrolyze cGMP into 5'-GMP. Several isoforms of PDE have been identified, and more than 60 (encoded for by 22 different genes) have been grouped into 11 families. PDE1, 2 and 3 are able to hydrolyse both cAMP and cGMP, but PDE5 specifically hydrolyses cGMP. Sildenafil, zaprinast and dipyramidole are specific PDE5 inhibitors, and these inhibitors have also proved to be potent inhibitors of cGMP transport into inside-out vesicles (IOV)(Hagmann et al. 2009) (Saier 2000; Sundkvist et al. 2002). The respective $K_i$-values found by the last group were 3.6 μM (sildenafil) and 0.35 μM (zaprinast and dipyramidole).

### 1.5.3 Cellular export

Intracellular levels of cGMP reflect the balance between the rate of synthesis and the rate of elimination. The activity of PDEs provides an effective mechanism for reducing cGMP levels, but efflux pathways also contribute to the control of intracellular cGMP concentrations. ABCC5 has been identified as a cGMP transporter (Jedlitschky et al. 2000; Wielinga et al. 2003). Elevated levels of cGMP have been shown to induce apoptosis (Tinsley et al.

2011), and reducing cellular efflux may become a new mechanism in cancer treatment. Cancer cells may have an increased expression of ABCC5 gene products, causing increased efflux of cGMP and various types of cytostatica exported by ABCC5 transporters(Hagmann et al. 2009).

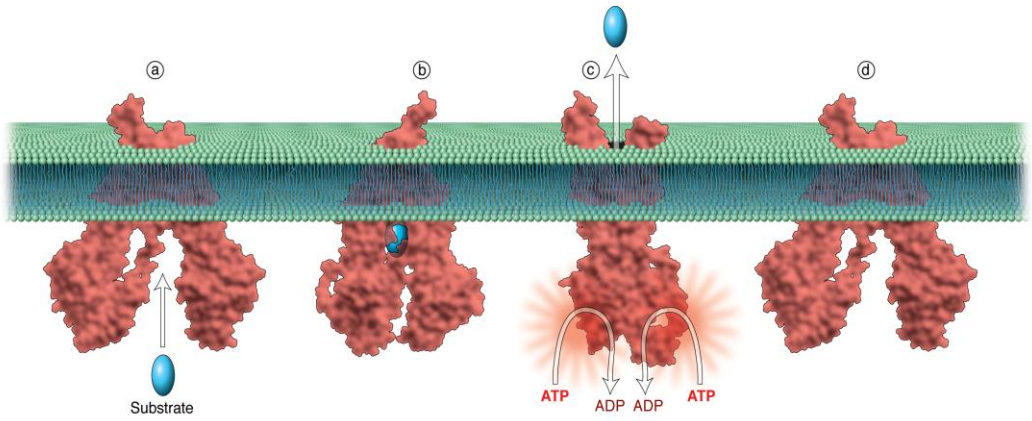


**Fig 7**: Transport cycle of ABCC5 showing: a) Substrate (e.g. cGMP) is recognized by the transporter, b) Substrate binds to the transporter's binding site, and the transporter closes towards the cytoplasma. c) The ATPases are stimulated, and ATP is hydrolysed to ADP. The chemical energy released in this process will open the transporter toward the extracellular space and the substrate is transported out of the cell. d) The transporter returns to its inward facing position and is ready for a new transport cycle.

## *1.6 BIOLOGICAL MEMRANES AND TRANSPORT*

### 1.6.1 Membrane composition

In a biological membrane the components are proteins, lipids and carbohydrates. Proteins and lipids account for most of the mass of biological membranes, while carbohydrates are present as parts of glycolipids and glycoproteins.

The composition of proteins and lipids varies with the type of membrane, thus reflecting functional specialization in different cell types and cell organelles. In an erythrocyte's plasma membrane about 20 different prominent types of proteins were found (in addition to some that were less prominent) by Steck in 1971 (Steck et al. 1971). Many of these proteins are transporters that move a specific solute across the plasma membrane.

Many membrane proteins are found linked to complex arrays of carbohydrates. In the erythrocyte plasma membrane the glycoprotein called glycophorin owes 60 % of its mass to complex oligosaccharide units covalently bound to specific amino acid residues. Residues like Ser, Thr, and Asn are the most common point of attachment to oligosaccharides. These sugar moieties of surface glycoproteins influence the stability, folding, and intracellular destination of proteins and specific binding of ligands to glycoprotein surface receptors (Nelson 2005).

Biological membranes are formed by a bilayer of phospholipids. The nonpolar lipid regions of both layers point inward toward the core of the layers, and the polar heads of the phospholipids face outward toward the aqueous phases on the intracellular and extracellular side of the membrane. Proteins are situated in the bilayer in an asymmetric way that gives the membrane "sidedness". This sidedness may be explained by the functional asymmetry of the two sides of the membrane, causing the protein domains exposed on one side of the membrane to be different from those exposed on the other side. The way

the various proteins are situated in the lipid bilayer may be compared to a mosaic, but as this mosaic is a fluid, different protein and lipid molecules are free to move laterally in the membrane.

### 1.6.2 IOV Membrane theory

In a sealed inside-out vesicle, solutes that are normally exported from the cell will be transported into the vesicle. And as the vesicles are sealed and impermeable to solutes that need a transporter system to cross the membrane, what has been transported in will stay inside until the vesicles are disrupted. Vesicles prepared with the external side facing inward may be used to test what is usually efflux from the cell, or when vesicles are prepared with the external side facing outwards, the vesicles may be used to test import to the cell. For regulation purposes, the cell may have a transporter that is able to transport certain solutes in both directions (Cropp et al. 2008), but so far a transporter like this has not been verified in erythrocytes.

# 2 BACKGROUND FOR THE STUDY

ABCC5 has been identified as a cGMP efflux transporter, and as ABCC5 is also involved in efflux of cytostatic drugs, interest is growing in finding molecular compounds that can inhibit this efflux pump.

Knowledge about the 3D structure of members of the ABCC is important to understand the molecular mechanism involved in transport and substrate specificity. Molecular modelling has proved a useful tool in this approach and is based on homology between the known template structure and the protein structure modelled. To be able to construct a realistic molecular model of an ABC transporter (target) by homology modelling, the sequence identity between the target and the template used (known X-ray structure) ought to be relatively high. The alignment between the two structures also should identify positions that correspond to each other in the target and the template.

Virtual ligand screening (VLS) is a method where chemical compounds can be tested for affinity to drug targets by the use of a computer program. The chemical compound sildenafil has been proven an inhibitor of cGMP transport and an inhibitor to PDE5, both effects being elevated intracellular concentration of cGMP. The ICM Pocket Finder software has been used to search for potential ABCC5 inhibitors with structural similarity to sildenafil, and by the use of an ABCC5 homology model as target, 11 compounds predicted to be potential inhibitors of ABCC5 were chosen for in vitro testing by the use of IOV. These compounds were selected based on score by VLS and drug likeness.

# 3 AIM/HYPOTHESIS

Multidrug resistance is an obstacle to cancer chemotherapy and antibiotic treatment that constitutes a serious problem throughout the world. One of the reasons may be increased excretion of drug molecules from their target cells. The ABCC5 transporter has been identified as an efflux pump of chemotherapeutic compounds (Hagmann et al. 2010; Kruth et al. 2001; Wielinga et al. 2003) and organic anions.

In order to test “hits” from VLS, inside-out membrane vesicles (IOV) prepared from erythrocytes constitute a suitable system for biochemical and pharmacological characterization of the cGMP transport. This method has been used at the Department of Medical Pharmacology and Toxicology at UIT since 1996 (Sager 2004; Sager et al. 1996; Schultz et al. 1998; Sundkvist et al. 2000; Sundkvist et al. 2002; Vaskinn et al. 1999). On IOV, both the nucleotide (ATP) binding domain of the transporter and its site for substrate binding activity (translocation) normally situated on the cytoplasmic side of the plasma membrane are found on the outer side of the membrane. Molecules normally transported out of the cell will be transported into IOV and accumulated here.

Based on molecular modeling and VLS, 11 substances were tested for their ability to inhibit cGMP extrusion from RBC by the aid of inside-out vesicles.

Aims of this study:

1) Establish a refined IOV based method in order to test the effect of possible inhibitors on cGMP transported by ABCC5.
2) By experimental studies test the inhibiting effect on ABCC5 transported cGMP by 11 selected compounds postulated as potential inhibitors by the use of VLS.

# 4 MATHERIALS AND METHODS

## *4.1 Reagents and solutions*

### 4.1.1 Reagents

Table 1. Overview of reagents used in the different methods

| Reagent | Generic name or chemical name | Producer/Distributer | Catalog number |
|---|---|---|---|
| Bovine serum albumin | | Sigma Aldrich | A 7511 |
| Tris•HCl | Tris(hydroxymethyl) aminomethan hydrochloride | Merck | 1.08219.1000 |
| KCl | Potassium chloride | Merck | 1.04936.1000 |
| $KH_2PO_4$ | Potassium dihydrogen phosphate | Merck | 1.04873.1000 |
| $K_2HPO_4•3H_20$ | di-Potassium hydrogen phosphate trihydrate | Merck | 1.05099.1000 |
| EGTA | Ethyleneglycol-bis(2-aminoethylether)-N,N,N', N'-tetraacetic acid | Sigma Aldrich | E3889 |
| Histodenz | | Sigma Aldrich | D2158 |
| $MgCl_2$ | Magnesium chloride | Merck | 5833.0250 |
| ATP-magnesiumsalt | Adenosine 5'-triphosphate magnesium salt | Sigma Aldrich | A9187-1G |
| cGMP | Guanosine 3':5'-cyclic monophosphate | Sigma Aldrich | G-6129 |
| [$^3$H]-cGMP | Guanosine 3',5'-cyclic phosphate, ammonium salt, [8-$^3$H]- | Perkin Elmer | NET337001MC |
| Dithiothreitol | 44,5-dihydroxy-1,2-dithiane | Fluka | 43819 |
| Acetylthiocholinechloride | | Sigma, St. Louis, USA | A5626 |
| 5,5'-Dithiobis-(2-nitrobenzoic acid) | | Sigma, St. Louis, USA | D21.820-0 |
| Ultima Gold XR scintillation Solution | (chemical composition see Dianu et al., 2007) | Perkin Elmer. Inc, USA | 6013110 |
| DMSO | Dimethyl sulfoxide | Calbiochem | 317275 |
| Potential inhibitors | | Ambinter (www.ambinter.com) | |

### 4.1.2 Solutions

## 4.1.2.1 Solutions used for preparing “inside-out” vesicles

1. Solution for washing red blood cells (RBC), preparation buffer A,
   5 mM Tris•HCl, 113 mM KCl, pH 8.1

2. Solution for hemolyzing RBC, preparation buffer B,
   5 mM Tris•HCl, 0.5 mM EGTA, 4 mM KCl, pH 8.1

3. Solution for vesicles to form, preparation buffer C,
   500 nM Tris•HCl, pH 8.2

4. Phosphate buffered saline potassium version (KPBS),
   1.47 mM $KH_2PO_4$ , 81 mM $K_2HPO_4$. $3H_2O$ and 140 mM KCl, pH

## 4.1.2.2 Solutions used for preparing separation gradients

1. Solution for preparing gradients – preparation buffer D, 5 mM Tris·HCl, 0.3 mM EGTA, 172 mM KCl, pH 8.1 Density = 1.00 g/ml

2. Preparation buffer with 27.6% Histodenz – preparation buffer E, 5 mM Tris·HCl, 0.3 mM EGTA, 336 mM Histodenz, 3.0 mM KCl, pH 8.1. Density 1.146 g/ml

### 4.1.2.3 Solutions used for transport assay

1) Solution 1 for incubation (with ATP) – 34.0 mM Tris, 13.6 mM $MgCl_2$, 1.7 mM EGTA, 3.00 µM [$^1$H]-cGMP, 0.334 µM [$^3$H]-cGMP, 148.3 mM KCl, 3.34 mM ATP
2) Solution 2 for incubation (without ATP) – 33.3 mM Tris, 16.7 mM $MgCl_2$, 1.7 mM EGTA, 3.00 µM [$^1$H]-cGMP, 0.334 µM [$^3$H]-cGMP, 148.4 mM KCl
3) Solution used to stop transport reaction and washing filters was KPBS
4) Ethylacetate was used to completely dissolve the filters
5) Ultima Gold XR scintillation solution
6) Modulation compounds/potential inhibitors

   These compounds were hydrophobic. With the exception of sildenafil, inhibitor I (INI), and inhibitor L (INL) these were difficult to dissolve in water. Dimethyl sulfoxide (DMSO) was used to dissolve these compounds initially, but the solutions were not stable and precipitation was seen for some of the compounds some minutes or hours after they were dissolved. See Fig 8.

### 4.1.2.4 Other equipment

1. Filters for vacuum manifold:
   Nitrocellulose membrane, 0.22um GSWP, Millipore, cat.no GSWP02500
2. Incubation vial:
3. Centrifuge tube:
   a. Sorvall centrifuge tube, TUBE, PC-F 50ML, cat.no 03146
   b. Beckman Ultra clear centrifuge tube, 25x89mm, cat.no 344058
   c. Beckman Ultra clear centrifuge tube, 14x95mm, cat.no 344060

Centrifuges

Vacuum manifold

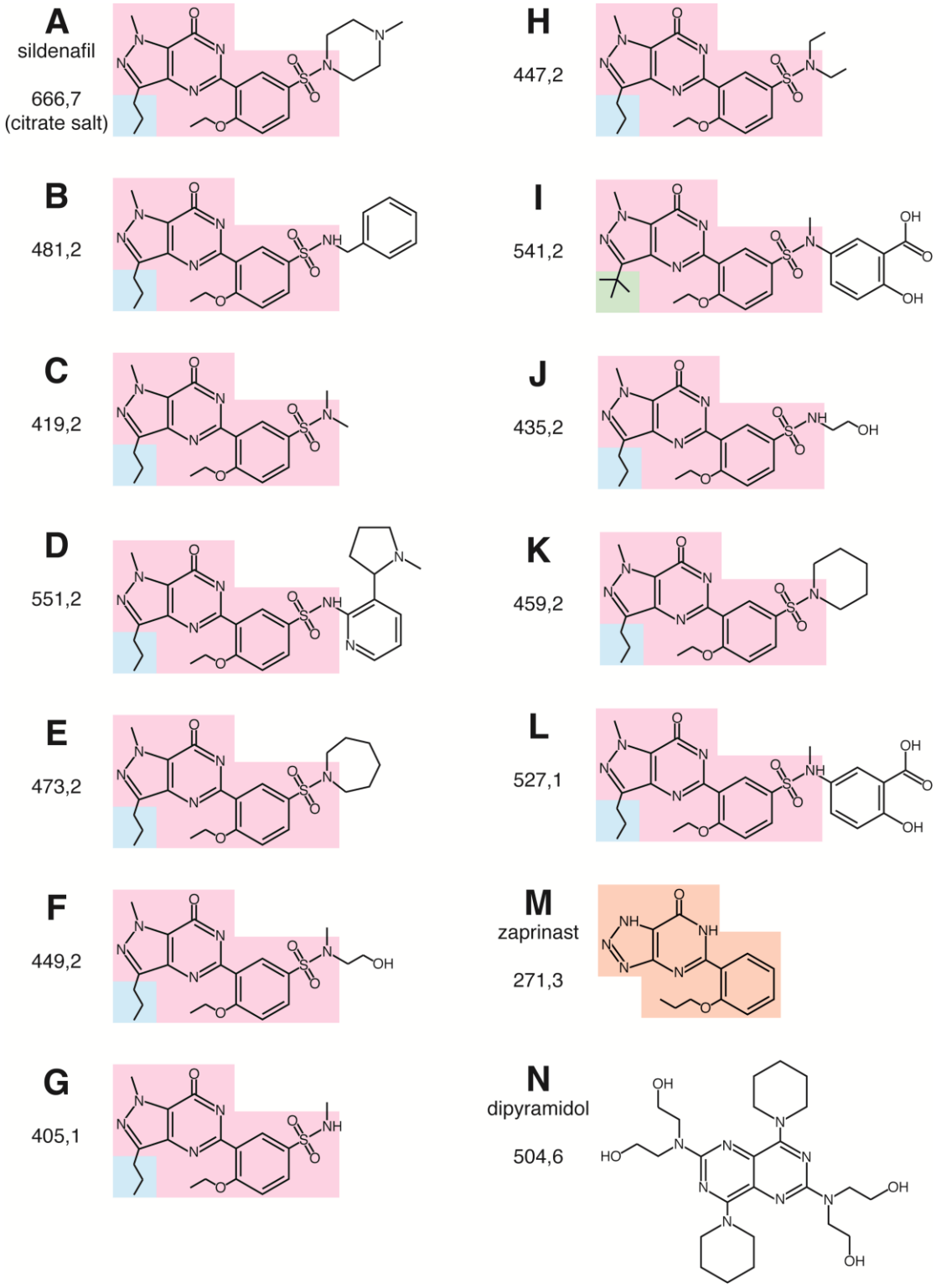


**Fig 8**: Colored areas denote structures that are common for all compounds. M and N are not sildenafil derivates. The blue color marks propyl groups, while the green shows an isobutyl group. The letters to the left were given to each compound to make a simple distinction between them given the lack of generic names.

## *4.2 Method*

### 4.2.1 Preparation of inside-out vesicles

#### 4.2.1.1 General principles

A method for preparing “inside-out” vesicles from human erythrocytes was published by Steck and Kant in 1974 (Steck and Kant 1974). This method was later modified by various groups, i.e. Kondo and his colleges in 1980 (Kondo et al. 1980)), and Sager and his group in 1996 (Sager et al. 1996). The procedure used today in the department of Medical Pharmacology and Toxicology, in the Faculty of Health and Science at the University of Tromsø is a further modification of the methods mentioned above.

A cell membrane is organized with an asymmetrical distribution of functions on the inner and outer surfaces. By preparing closed membrane sacs (sealed vesicles), each side of the membrane can be chemically probed. Sealed membrane sacs with same sidedness as original in cells are called right-side out vesicles, and if the sealed membrane sacs have the opposite sidedness with inner membrane leaflet facing outwards they are called inside-out vesicles. In a normal erythrocyte, the membrane will be found with the outer side facing outwards, but after lysis and the loss of most of its cytoplasm, the resulting ghost may be resealed to form impermeable vesicles. When unsealed ghosts are incubated in a chilled slightly alkaline solution with very low ionic strength and without any divalent cations present, most vesicles formed tend to be of the inside-out type (Steck and Kant 1974).

#### 4.2.1.2 Preparing vesicles from human erythrocytes

Fresh human blood with anticoagulant EDTA was used to produce inside-out vesicles from human erythrocytes. Typically suspensions of 10 ml red blood cells (RBC) x 2 were used in each preparation. All further volumes were adjusted to one vial of 10 ml red blood cells suspension and all steps after collecting the blood were performed at 0-4°C. RBCs were sedimented in a swinging bucket-rotor 2300 x g (Kubota, 2200 rpm for 15 minutes), plasma

and buffy coat were carefully aspirated from the vial, and RBCs were washed three times in 5 volumes of buffer A by centrifugation for 10 minutes in a Beckman Coulter centrifuge at 1000 x g (2875 rpm), using rotor JA-25.50 with slow brake before removing supernatant and carefully aspirating buffy coat each time from the surface of the RBCs.

Lysis of the RBCs was initiated by adding and thoroughly mixing approximately 80 ml of buffer B with the cell pellet. The large volume used made the solution less viscous and sedimentation of membranes more visible. The resulting membranous ghosts were sedimented by centrifugation at 20.000x g for 20 minutes without brake (Beckman refrigerated centrifuge, 12859 rpm in rotor JA 25.50). Large volume of supernatant was removed by the aid of an electrical pipette followed by a plastic pasteur pipette for small volumes in order to avoid disturbing the fluffy ghost pellet. After the first centrifugation, the ghost pellet was moved into a new vial. This had to be done carefully to avoid the transfer of a relatively solid pellet-like collection of proteases in the bottom of the centrifuged vial. Collecting and discarding the proteases by this method made the presence of protease inhibitors unnecessary throughout the preparation. The supernatant will still be relatively red due to the presence of hemoglobin in centrifugation tubes, and the procedure of resuspending, lysis, centrifugation and removing of supernatant needed to be repeated until the supernatant appeared colorless as water and the ghosts were milky-white.

The amount of ghost pellet may vary from one preparation to another, and at this point in the procedure the volume of ghosts achieved was measured. To initiate vesiculation, the ghosts were resuspended in 39 volumes of buffer C. After 2 – 18 hours (it may be left overnight) on ice the suspension was pelleted at 100.000 x g (23700 rpm) for 20 minutes in Beckman Optima LE-80K ultracentrifuge, rotor SW28). Supernatant was carefully removed by suction with a glass Pasteur pipette.

Collected pellets were resuspended in a suitable volume of buffer C (usually

300 µl/vial). To enhance further vesiculation, vesicles and unsealed ghosts were passed five times through a No. 27G ¾, 0,4 x 19mm cannula on a 1 ml syringe. The solution was adjusted to 2000 µl in buffer C for each gradient vial used in the preparation.

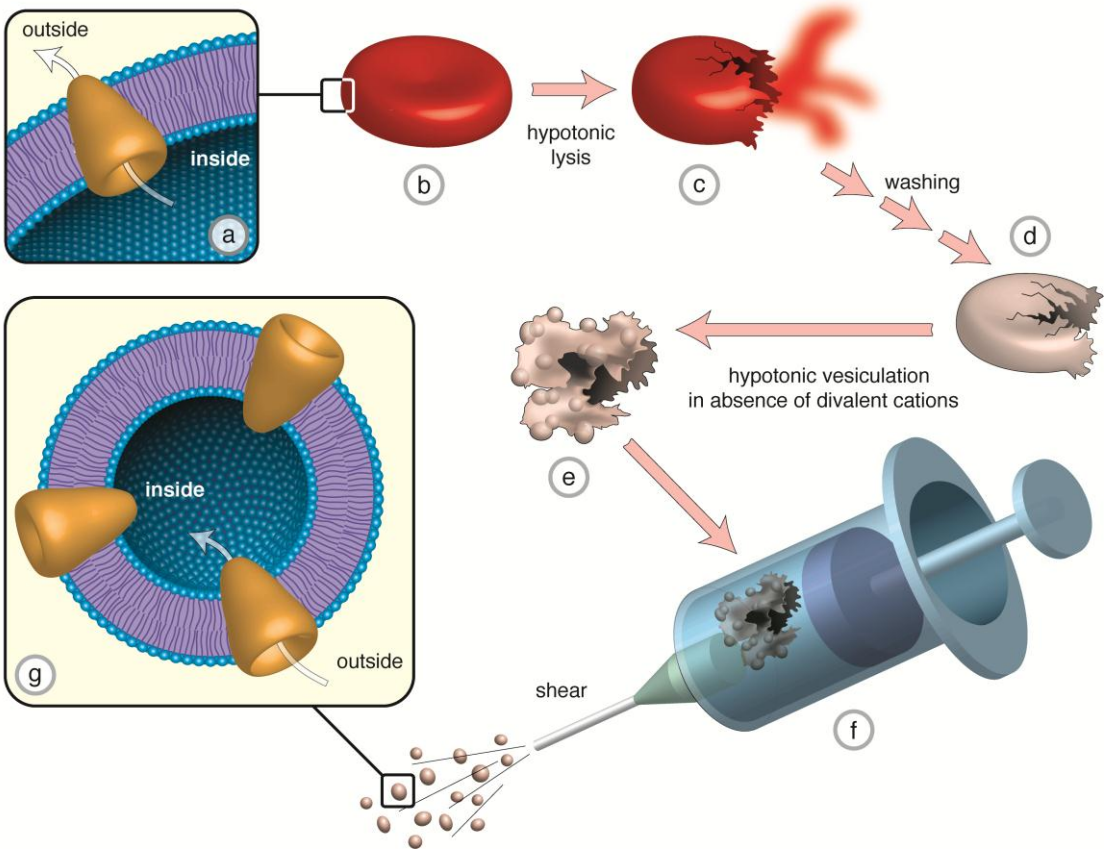


**Fig 9**: Illustration of the different steps in an inside-out vesicle (IOV) preparation. a) section of a normal erythrocyte showing direction of transporter, b) normal shape of erythrocyte, c) lysis of cell membrane by adding hypotonic solution, d) after repeated washing steps all intracellular contents and hemoglobin were removed, e) incubation in hypotonic solution in the absence of divalent cations initiate vesicle formation, f) vesicles separated from unsealed ghosts by passing solution through thin cannula 5 times, g) enlarged view of IOV with transporter pointed inwards.

### 4.2.1.3 Separation of inside-out vesicles from unsealed ghosts

Inside-out vesicles, rightside-out visicles and unsealed vesicles and ghosts were separated by centrifugation using a density gradient. The particles put on top of the gradient will during the centrifugation migrate towards higher density until the density of the particle (vesicle or ghost) and the gradient fluid surrounding it is equal. Unsealed ghosts may form sealed vesicles of both types of sidedness after migrating in the density gradients. These vesicles will contain some density gradient, and stop migrating at a higher density lower in the tube than vesicles already sealed before centrifugation.

Density gradients were made using three different densities, each prepared using preparation buffer E diluted with preparation buffer D:

| Dilution | Density | % w/v Histodenz | mM Histodenz | Volume in vial |
|---|---|---|---|---|
| 1:2 (E+D) | 1,048 g/ml | 9,2 | 112 | 2ml |
| 2:1(E+D) | 1,097 g/ml | 18,4 | 224 | 6 ml |
| Undiluted E | 1,146 g/ml | 27,6 | 336 | 1,5 ml |

Gradients were placed carefully in a Beckman ultracentrifugation vial size 13 ml with the highest density at the bottom of the vial and the lowest density on top, without mixing the gradients. Approximately 2 ml of vesicle solution were put on top, before ultracentrifugation at 100.000 x g (Beckman Optima LE-80K ultracentrifuge, rotor SW40Ti; 23700 rpm) typically overnight without brake. Two hours centrifugation proved to be sufficient, but 12 – 16 hours was mostly used of practical reasons.

After centrifugation two or three bands can be seen in the vial, and the uppermost band was collected with the aid of a syringe by entering the band with its cannula through the vial wall. The yield was 1-2 ml per vial, and the vesicles were transferred to a Sorvall 50 ml centrifuge vial. After adding 40 ml KPBS in order to remove Histodenz from the previous step in the procedure, the solution was centrifuged in a Beckman Coulter, Rotor JA-25.50 at 28.000

x g for 30 minutes (15.215 rpm).

A pellet containing a high percentage of inside-out vesicles was resuspended in a suitable volume of KPBS, typically between 700 and 1000 μl.

### 4.2.2 Sidedness test using acetylcholinesterase accessibility

## 4.2.2.1 Principle of method

A cell membrane is organized with an asymmetrical distribution of functions between the two surfaces. The sidedness test used will establish which face of the membrane is facing outwards on most vesicles prepared.

The enzyme acetylcholinesterase (AChE) is anchored to the extracellular side of the cell membrane. For inside-out vesicles this enzyme will not be accessible from the outside of the vesicle. Adding the detergent Triton-X100 to the vesicles will make the membrane leaky, and in the presence of this detergent, substrate surrounding the vesicles will become accessible to the intravesicular located enzymes. By measuring enzyme activities in vesicles untreated with detergent and comparing with enzyme activity in vesicles treated with detergent, the relative percentage of ROV and IOV can be determined, see procedure below.

Reagents used in this test are 5 mM phosphate buffer with or without 0,2% Triton –X100 (called FX and FO respectively), incubation solution for sidedness containing 0,7 mM 5,5'-Dithiobis-(2-nitrobenzoic acid) and 100 mM phosphate, and 12,5 mM acetylthiocholine-chloride (ATCC).

The method used is based on following reactions:
Adding the substrate acethylthiocholine to the enzyme acethylcholinesterase will produce thiocholine + acetate in the first reaction. Thiocholine and 5,5'-Dithiobis-(2-nitrobenzoic acid) will in the coupled second reaction form the yellow thionitrobenzoic acid with strong absorbance in the 412 nm region of

visible light.  See fig 10.

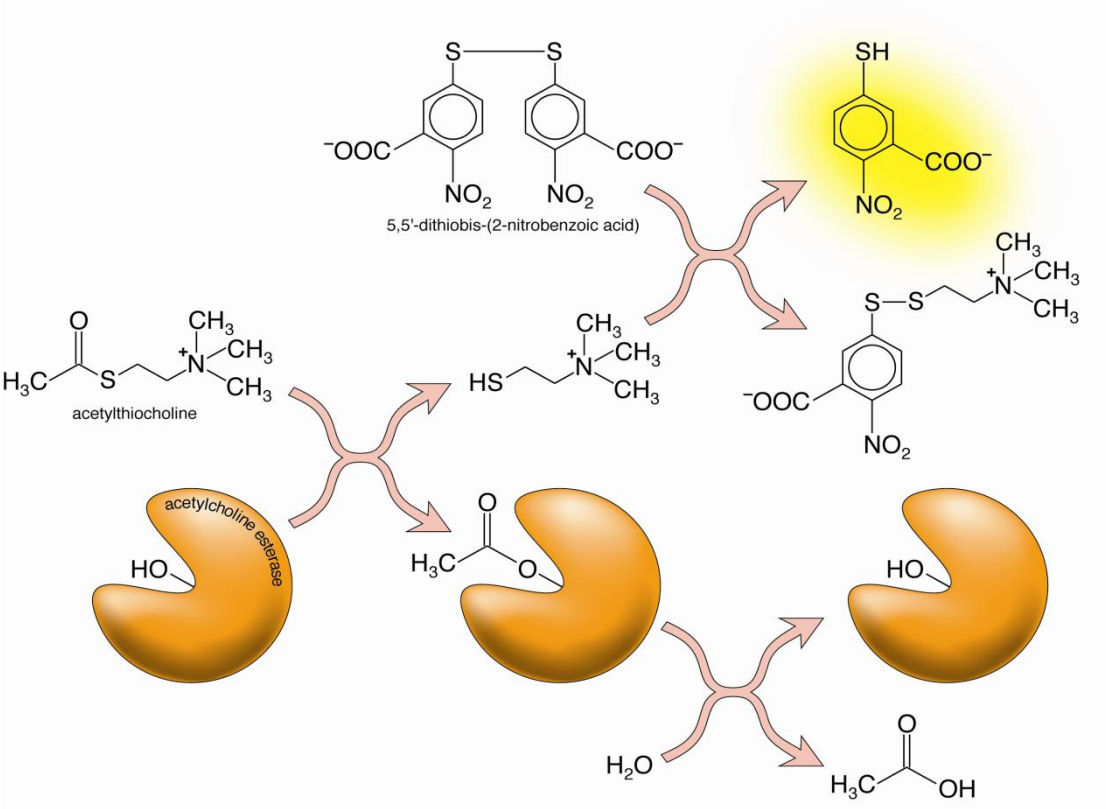


**Fig 10**: Acetylcholine esterase dissociates acetylthiocholine into acetate and thiocholine. The latter reacts non-enzymatically with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and yields the yellow color product thionitrobenzoic acid which exhibits strong absorbance at 412 nm.

### 4.2.2.2 Procedure

40ul of vesicle solution was mixed with equal volume of F0 = sample IV0, and the same was done with vesicle solution and FX = sample IVX.

In a 1 ml optical cell, 920 µl incubation solution, 20 µl sample (IV0 or IVX) and 63 µl ATCC (which starts the reaction) were mixed. The absorbance at 412 nm was set to zero as the measurements started, and the enzymatic reaction was monitored by measuring the increase in absorbance; 1 minute and 3 minutes after reaction started for 3 parallels of IVO and 3 parallels of IVX with the aid of a spectrophotometer.

Definition: $\Delta abs = abs(t = 3minutes) - abs(t = 1minute)$

Acetyl esterase activity ≡ AE

The number of IOVs, $n_{ROV}$, is proportional to the acetyl esterase activity, $AE_{ROV}$, on the surface of the ROVs, which must be proportional to the difference in the measured absorbance, $\Delta abs_{ROV,}$ or:

$n_{ROV} \propto AE_{ROV} \propto \Delta abs_{ROV}$

or short:

$n_{ROV} \propto \Delta abs_{ROV}$

when $AE_{ROV+IOV} = AE_{ROV} + AE_{IOV}$:

$n_{ROV + IOV} \propto AE_{ROV+IOV} \propto \Delta abs_{ROV+IOV}$

or short:

$n_{ROV + IOV} \propto \Delta abs_{ROV+IOV}$

this means that:

$\%ROV = \frac{n_{ROV}}{n_{ROV+IOV}} = \frac{\Delta \mathrm{abs}_{ROV}}{\Delta \mathrm{abs}_{ROV+IOV}}$

Calculating percentage of IOV:

$$\% \text{ ROV} = \frac{\Delta abs_{withoutTriton}}{\Delta abs_{withTriton}} x100\% = \frac{0.067 - 0.020}{0.280 - 0.085} x100\% = 24.10$$

% IOV: 100 – 24.10 = 75,89 ≈ 76%

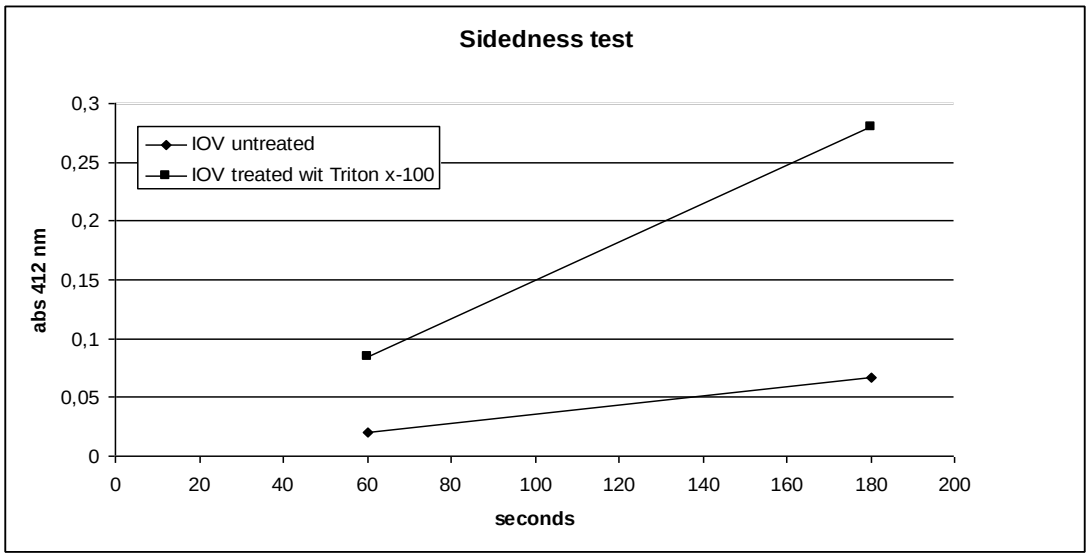


**Fig 11**: example of sidedness test

Previous repeated studies have shown that the increase in absorbance during the first minutes is linear.

### 4.2.3 Protein determination

## 4.2.3.1 Principle of method

The BCA (bicinchoninic acid) Protein Assay (Pierce Protein Research Products, Thermo Scientific) was used to determine the protein concentration of vesicles used in the different experiments. This method combines the reduction of $Cu^{2+}$ to $Cu^{1+}$ by the oxidation of certain groups in the peptide backbone with the colorimetric detection of the cuprous cation ($Cu^{1+}$) by the use of bicinchoninic acid. The first step of the color development reaction is $Cu^{2+}$ chelating with protein in an alkaline environment and a light blue complex is formed. This reaction is known as the biuret reaction, in which cupric ions form a weakly colored chelate with certain amino acids (tyrosine,

tryptophan, and cysteine or cystine) caused by the presence of sodium potassium tartrate. In the second step, bicinchoninic acid (BCA) chelates with the $Cu^{1+}$ that was formed in the previous step (the biuret reaction), and an intense purple color is produced by the reaction when two molecules of BCA form a chelating product with one cuprous ion. The BCA/Copper chelating product formed exhibits a strong linear absorbance at 562 nm with increasing concentrations of protein. Adding the second step of the reaction makes the sensitivity of the method (lower limit of detection) approximately 100 times stronger than the blue color produced in the first step of the reaction. The intensity of the purple color will relate to the concentration of protein in the sample and linearity of this method using bovine serum albumin (BSA) as standard range from 20 to 2000 μg/ml. The reaction is not an end point reaction and will continue until no more cuprous cations are available.

### 4.2.3.2 Procedure

The manufacturer of the BCA Protein Assay specifies a lower limit of sensitivity for this method to be 5 μg/ml. Bovine serum albumin (BSA) was used to prepare standard solution for this method. From a stock solution of 1mg/ml dilutions with concentrations of 2, 4, 8, 16, 32, 64, 128, and 256 μg/ml were made.

Solutions used in the assay were BCA Protein Assay reagent A (1,0 g $Na_2$-Bicinchoninat·x$H_2$0, 2,0 g $Na_2CO_3$, 0,16 g Na-tartrat, 0,40 g NaOH, 0,95 g $NaHCO_3$ in 100 ml $H_2$0) and BCA Protein Assay reagent B (0,40 g $CuSO_4 \cdot 5H_2O$ in 10 ml $H_2O$). Working reagents in the procedure were made from 50 parts of BCA Protein Assay reagent A and 1 part of Protein Assay reagent B, forming the solution called Protein Assay reagent C.

In each tube were added 1 ml of Protein Assay reagent C and 50 μl of standard or sample. After appropriate mixing the samples were incubated for 30 minutes at 37°C and then absorbance at 562 nm was measured by the aid of a spectrophotometer.

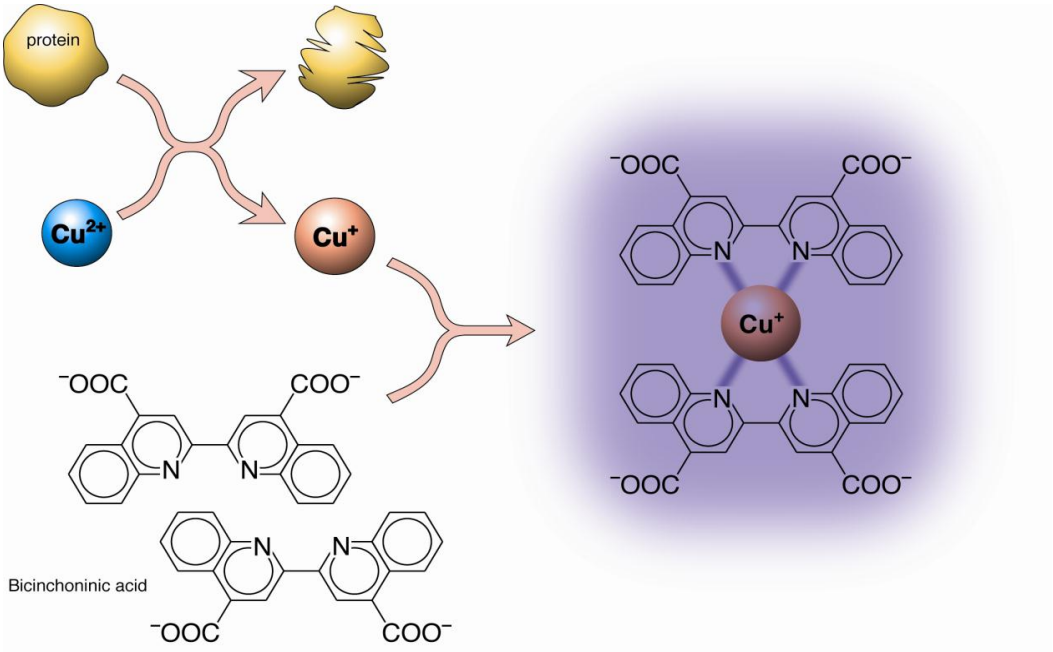


**Fig 12**: Copper is reduced from $Cu^{2+}$ to $Cu^{1+}$ as $Cu^{2+}$ form a chelate with certain amino acids in an alkaline environment caused by sodium potassium tartrate. Two molecules of bicinchoninic acid (BCA) bind to each $Cu^{1+}$ and an intense purple color is produced.

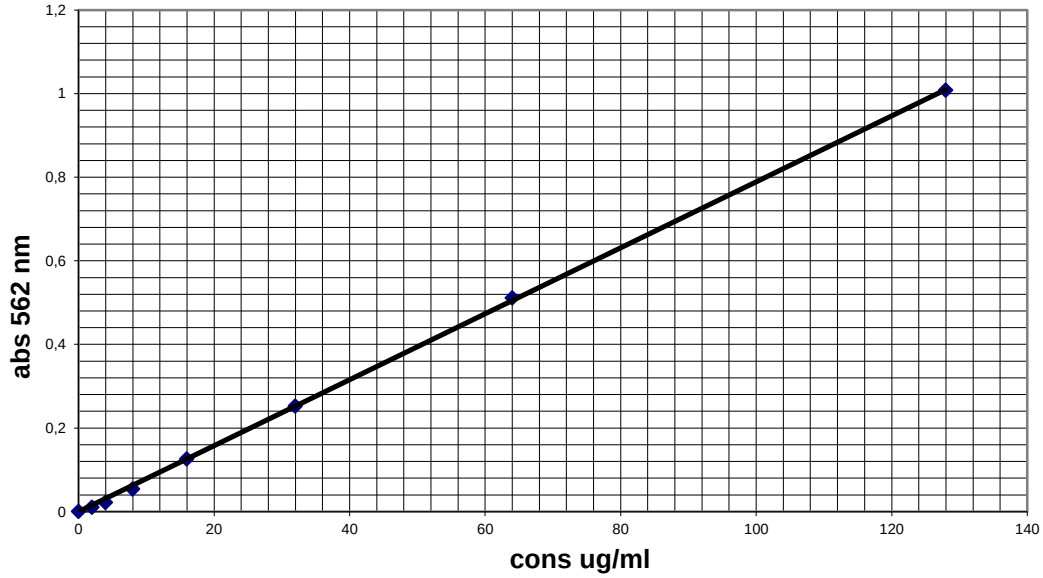


**Fig 13**: Typical protein assay standard curve

### 4.2.4 Test of potential inhibitors for cyclic GMP transport

## 4.2.4.1 General principles

Studies have shown that the multidrug resistance protein isoform MRP5 (gene symbol ABCC5) is involved in the ATP-dependent cellular export of 3',5'-cyclic GMP. Jedlitschky et al. (Jedlitschky et al. 2000) reported in 2000 that MRP5 transfected hamster cells had a fourfold higher ATP-dependent transport of cGMP than in untransfected control cells. In a normal cell, transport of cGMP will be out of cells, but in our model system, the compounds that will normally be exported will be transported into IOVs where they accumulate.

Previous studies have also shown that Sildenafil, Probenecid, Zaprinast, and other amphiphilic anions are able to inhibit MRP5-mediated cGMP transport into inside-out vesicles (Jedlitschky et al. 2000)

In this procedure 11 new compounds were tested for the first time in addition to Sildenafil, Zaprinast and Dipyramidol. As the three last mentioned had

shown inhibiting effect earlier, they were used as positive controls.

The 11 compounds were chosen due to 1) their structure similarity to Sildenafil, a well known Phosphodiesterase 5 inhibitor and an inhibitor of cGMP transport, and 2) their predicted affinity to the ABCC5 transporter protein by Virtual Ligand Screening (VLS).

For all compounds an invariable number of moles was weighed out. This number was the same for all compounds. They were all initially dissolved in 50 µl DMSO to obtain solutions with free compounds. Further dilution in water was done to achieve a concentration of $10^{-4,5}$M (0,03162 mM) in the final incubation reaction, and the concentration of DMSO in the final incubate was 68 mM for all compounds. Concentration of DMSO was kept as low as possible and was related to the minimal volume needed to initially dissolve the different compounds. It was important to keep the concentration of DMSO as low as 68 mM as higher concentrations have proved to significantly inhibit the transport of cGMP into vesicles (Schultz et al. 1998).

### 4.2.4.2 Weighing procedure

An approximate amount of the different compounds used in the test was weighed using a milligram scales. The compounds were put into the cap of a thrombosis test (TT) vial turned upside down when weighed, and the appropriate amount of the compound was immediately dissolved in the corresponding volume of DMSO added directly into the TT-vial cap. The cap was put on the table, and a TT-vial was placed on top of the cap. The vial was kept upside-down like this until centrifugation by 1000 x g for 30 seconds in order to place the solution in the bottom of the vial. The right volume of water to create the predetermined concentration could now be added. The solution was mixed well, and as this solution was over its saturation point, the solution had to be used immediately to avoid precipitation. Test compound A (Sildenafil) with and without the content of DMSO and water with and without

DMSO were included as controls in order to make sure effects seen were not simply due to an inhibiting effect of DMSO.

#### 4.2.4.3 Calculation of [$^3$H]-cGMP concentration

Radioactive decay is the process by which an atomic nucleus of an unstable atom loses energy by spontaneously emitting particles. Radioactive decay happens due to a process confined to the nucleus of the unstable atom and without any interaction with another particle from outside the atom. Radioactive decay is entirely random, i.e, it is impossible to predict when a given single unstable atom will decay. However, the decay rate for a large number of identical unstable atoms (radionuclides) is predictable. The decay results when an atom with one type of nucleus, called the parent radionuclide, transforms to an atom with a nucleus in a different state, or a different nucleus, either of which is named the daughter nuclide. In an example of this, an hydrogen isotope, $^3$H, decays into the daughter $^3$He, with the release of one beta-particle and one undetectable antineutrino:

$$^3_1H \rightarrow ^3_2He^+ + e^- + \bar{\nu}_e$$

The unit of radioactivity is becquerel (Bq). One Bq is defined as one decaying radionuclide per second. Another unit is Ci (Curie) which is equal to $3{,}7 \cdot 10^{10}$ Bq.

In a sample of a particular radioisotope, the number of decay events, i.e the number of atoms of this radioisotope that disappear, $-dN$ , in a small interval of time, *dt,* is proportional to the amount N of atoms of the radioisotope present at any time, or:

$$-\frac{dN}{dt} = k \cdot N \qquad (I)$$

where k is the decay constant.

Rearrangement yields:

$$\left(-\frac{dN}{N}\right) = k \cdot dt$$

This first-order differential equation is solved by integration on both sides, from the initial amount $N_0$ at time 0, to the remaining amount N at time t.

This yields:

$$N = N_0 \cdot e^{-kt} \qquad (II)$$

The half-life is the time taken for half of the radionuclides to decay, i.e, the time $t_{½}$ that passes until $N = ½ N_0$:

$$\frac{1}{2} N_0 = N_0 \cdot e^{-kt_{\frac{1}{2}}}$$

$$\Downarrow$$

$$\ln 2 - \ln 1 = k \cdot t_{\frac{1}{2}}$$

$$\Downarrow$$

$$t_{\frac{1}{2}} = \frac{\ln 2}{k}$$

The half-life $t_{½}$ of an amount of $^3H$ atoms has been proved to be 12,32 years, or $388{,}5 \cdot 10^6$ seconds.
Thus, the decay constant for $^3H$ is calculated to be: $k = \ln 2/t_{½} = 1{,}784 \cdot 10^{-9}\ s^{-1}$.
The specific activity of 1 mol of pure $^3H$, which consists of A = Avogadros number = $6{,}0221 \cdot 10^{23}$ of atoms, is given by equation ( I ):

$$\frac{dN}{dt} = k \cdot N = k \cdot A =$$

$1{,}784 \cdot 10^{-9}\ s^{-1} \cdot 6{,}022 \cdot 10^{23}$ atoms = $1{,}066 \cdot 10^{15}$ atoms/s,

which pr def equals $1{,}066 \cdot 10^{15}$ Bq/mol, or 28800 Ci/mol.

Example:
Remaining activity when storing an initial amount of molecules containing one $^{3}H$ per molecule for 3 months, or 8035200 seconds, can be calculated.
Equation (II) gives the fraction of remaining activity:

$$\frac{N}{N_0} = e^{-kt} = e^{-(1{,}784 \cdot 10^{-9}\, s^{-1} \cdot 8035200)} = 0{,}986 = 98{,}6\%$$

Thus 98,6% of the initial amount $^{3}H$ remains after 3 months, i.e 1,4% of the initial amount has decayed.
If every single molecule of an initial amount of a compound is “labelled” with one $^{3}H$, the specific activity of the compound would be 28800 Ci/mol, as shown above. In this example both the specific activity and the radioactive concentration was given on the label of the bottle containing the compound, and was less; 13500 mCi/mmol, and 1mCi/ml, respectively.
This means that only a fraction of the compound is $^{3}H$ labelled.
The concentration of $^{3}H$-“labelled” compound can be calculated:

$$C_{^3H} = \frac{(radioactive\ conc)}{(specific\ activity)} \cdot (fraction\ of\ remaining\ act) =$$

$$\frac{1\frac{mCi}{ml}}{13500\frac{mCi}{mmol}} \cdot 0{,}986 = 0{,}000073\frac{mmol}{ml} = 0{,}073\frac{\mu mol}{ml} = 73\mu M$$

The fraction of the total amount of the compound which is “labelled” with $^{3}H$ can be approximated:

$$fraction\ of\ ^{3}H\ labelled\ compound = \frac{(specific\ activity\ of\ ^{3}H-labelled\ compund)}{(specific\ activity\ of\ pure\ ^{3}H)} = \frac{13500\frac{mCi}{mmol}}{28800\frac{mCi}{mmol}} = 0{,}469 = 47\%$$

The total amount of both $^{3}H$ and $^{1}H$-labelled compound is then 73 μM/0,469 = 155μM.

Calculating concentration needed of [3H]-cGMP as test parameter:

The purchased $^{3}H$-labeled cGMP was specified to be 1mCi in a 1ml volume, and the specific activity given per mole was 6,5 Ci/mmole. Calculated chemical concentration of cGMP (containing both [$^{3}H$]-cGMP and [$^{1}H$]-cGMP) was 153,7μM. Specific activity for pure tritium, or the case when 100% of 1mmole of cGMP consists of [$^{3}H$]-cGMP is 28,7 Ci/mmole.

Calculated percentage of [$^{3}H$]-cGMP in this batch of cGMP will be:
(6,5Ci/mmole)/(28,7Ci/mmole) = 0,22648 = 22,65%,

which means that the concentration of [$^{3}H$]-cGMP = 0,2265 • 153,7 μM = 34,8 μM.

Calculated percentage of [$^{1}H$]-cGMP in present batch will be (100-22,64)% = 77,36%,

which means that the concentration of [$^{1}H$]-cGMP = 0,7736 • 153,7 μM = 118,9 μM.

Concentration of [$^{3}H$]-cGMP wanted during incubation of vesicles for ABCC5 activity accessment is 2 μM.

With a total amount of 153,7 μM in 1 ml, total volume of reaction solution with the desired chemical concentration of cGMP will only be (153,7 μM • 1 ml/ 2 μM =) 76,85 ml.

Considering a volume of 250 μL in each incubation, the volume of incubation solution containing cGMP will only give 307 incubations. This meant that the purchased [$^{3}H$]-cGMP would only last through a few experiments.

To solve this problem, [$^{1}$H]-cGMP was added to achieve a ratio between [$^{3}$H]-cGMP and [$^{1}$H]-cGMP that equals 1: 9 (10% [$^{3}$H]-cGMP) for high percentage of IOVs and a ratio of 1:4 for low percentage of IOVs in the various IOV-preparations. The resulting concentrations in each incubate would give a total concentration of 2,0 μM cGMP .

#### 4.2.4.4 Assay procedure

All steps in the assay procedure were performed in a refrigerated room or the samples were kept on ice except for the incubation at 37°C.

Previous studies have shown that

1) optimal pH for uptake of cGMP into inside-out vesicles is pH 8,0(Sundkvist et al. 2000),
2) optimal concentration of magnesium is 10 mM (Vaskinn et al. 1999) and
3) optimal concentration of ATP is 2 mM (Vaskinn et al. 1999).
4) It has also been shown that Calcium has an inhibiting effect on transport of cGMP into inside-out vesicles (Sundkvist et al. 2000).
5) EGTA has the abilty to chelate divalent cations, showing more affinity to $Ca^{2+}$ than $Mg^{2+}$ . As a consequence 1 mM EGTA was added to the reaction solution. Remaining $Ca^{2+}$ will be chelated while free $Mg^{2+}$ will still be available during the incubation as [$Mg^{2+}$] exceeds [EGTA].
6) As $Na^{+}$ hypothetically could have a positive effect on non-ATP-dependent transport or association to inside-out vesicles, this cation was not included in any part of the preparation of vesicles or assay procedures.

Two incubation solutions were prepared, incubation solution 1 (with ATP) and incubation solution 2 (without ATP). Both solution gave a final incubation consisting of 20.0 mM Tris, 13.6 mM $MgCl_2$, 1.0 mM EGTA, 1.6 μM [1H]-cGMP, 0.40 μM [3H]-cGMP, 148.3 mM KCl, and 2.0 mM ATP (in solution 1) or no ATP (in solution 2), pH 8.0.

Total volume of test sample:

50 μl incubation solution 1 or incubation solution 2

150 μl inhibitor solution to final incubate concentration of $10^{-4,5}$M

50 μl vesicles (dissolved in KPBS)

Incubation solutions and inhibitor solution were put into the test vial while the vials were placed on ice. The series with ATP contained 3 parallels while the series without ATP contained 2 parallels for each concentration of inhibitor tested.

Every vial was pre incubated for 5 minutes in a water bath at 37°C before adding the vesicles and thereby starting the transport. Vials were incubated for 90 minutes. The reaction was stopped by adding 3 ml of ice cold KPBS to the test tubes, as previous studies have shown that the transport of cGMP into IOVs will stop at temperatures lower than 15ºC (Sundkvist et al. 2000).

### 4.2.4.5 Filter procedure

Before starting the transport assay, filter manifolds were prepared with a nitrocellulose filter size 0,22 μm in each well. The manifolds were pre chilled in a refrigerated room. Transport reaction had been stopped by adding 3 ml ice cold KPBS to the test tube. The solution was applied on to the filter well. An additional volume of 3 ml ice cold KPBS was used to flush out any possible vesicles remaining in the incubation tube, and this solution was applied on to the filter as soon as any visible liquid on top of the filter had disappeared. Each filter was then rinsed 3 times with 2 ml ice cold KPBS. After the filtering process, filters were removed from the manifold and put into scintillation vials.

## 4.2.4.6 Scintillation procedure

### *4.2.4.6.1 General principles*

An element with an unstable nucleus is subject to radioactive decay which means that nucleus emits particles or electromagnetic radiation. Beta decay results from a transformation of a neutron into a proton or a proton into a neutron. The electron or positron emissions are called beta particles. The isotope used in our experiments is tritium and the radioactivity measured by liquid scintillation is beta particles.

In this test the isotope (on the filter) is transferred to a vial and mixed with ethyl acetate. This process will dissolve the filter and thus prevent quenching (emitted particles not detected) by the nitrocellulose filter which acts like a barrier against β-radiation. When the scintillation liquid is added, certain component molecules in the scintillation liquid will accept the energy from the emitted β-particles, and the molecule will achieve a higher energy level. When it returns to a stable level, it will free a photon (light). The intensity of emitted light, i.e. the amount of photons per time unit is proportional to the amount of emitted β-particles per time unit, which is proportional to the accumulated [$^3$H]-cGMP inside the IOV. (Sjoeberg)

### *4.2.4.6.2 Procedure*

When all filters corresponding to the test tubes were transferred to scintillation vials, 2 ml of ethyl acetate were added to the vials in order to dissolve the filters. 10 ml of scintillation liquid were then added to the vials before thoroughly mixing followed by scintillation counting.

## 4.2.5 Transport assay to find $IC_{50}$ value

$IC_{50}$ (inhibitory concentration) corresponds to the inhibitor concentration needed to reduce the transport activity to 50% of maximum transport. Maximum transport is transport activity without any inhibition. To determine a

compound's $IC_{50}$ value is useful to evaluate the suitability and performance early in a drug discovery process. In this study finding inhibitor concentrations that reduced the transport into IOV with 50% for each of the compounds was of special interest.

The compounds showing the most promising results from the test of possible inhibitor of cGMP transport to inside-out vesicles also considering the compounds solubility, were chosen for testing their IC50 values. Sildenafil (Inhibitor A – INA) was used as a positive control, while the inhibitor compounds with the short names inhibitor D (IND), inhibitor I (INI) and inhibitor L (INL) to our knowledge have not been tested for IC50 values relative to cGMP transport before. IND was difficult to dissolve in water, and DMSO had to be used in the same volume/concentration as for the previous test of all the possible inhibiting compounds mentioned above. For the same reason, the highest possible concentration achieved was $10^{-4,5}$M, resulting in testing this compound in a concentration range between $10^{-4,5}$ to $10^{-\infty}$M for IND. INA, INI and INL were more soluble in water, and the concentration range tested for these compounds was $10^{-3}$ to $10^{-\infty}$M.

Two incubation solutions were prepared, one incubation solution containing ATP (1) and the other without ATP(2), both with this concentration of solutes in the final incubation: 20.6 mM Tris·HCl, 13.4mM Tris, 10.0 mM MgCl, 1.0 mM EGTA, 1.8 μM [1H]-cGMP, 0.2 μM [3H]-cGMP, 148.2 mM KCl, and 2.0 or no ATP, pH 8.0. There were four parallels for each concentration of inhibitor with ATP and 3 parallels for each concentration of inhibitor without ATP.

Total incubation volume per test vial:

150 μl incubation solution 1 or 2
50 μl inhibitor solution
50 μl vesicle solution

The rest of the procedure was performed the same way as described above.

### 4.2.6 Displacement assay

This procedure was done to test whether the concentration of [1H]-cGMP would attenuate the transport of [3H]-cGMP by displacement when added in increasing concentrations. The concentration of [3H]-cGMP was kept the same in all vials (0.4524 μM), while the concentration of [1H]-cGMP ranged from 1.55 to 143 μM in 8 different concentrations added to the test vials. The ratio between [1H]-cGMP: [3H]-cGMP was from ($10^{0,53}$ :1) to ($10^{2,50}$ :1) which equals (3,39 :1) to (316 :1) from the lowest to the highest concentration of [1H]-cGMP in the final test suspension.

All incubations with ATP were performed with 4 parallels while incubations without ATP were performed with 3 parallels. Both incubation solutions had the same composition as in previous test except for the concentration of [3H]-cGMP which was adjusted to 0,45 μM. This was the highest concentration of [3H]-cGMP achievable with the batch of isotope purchased.

Total incubation volume per test vial:

50 μl of incubation solution 1 (with ATP) and 2 (without ATP)
150 μl of the different displacement concentrations
50 μl vesicles

The rest of the procedure was done as described for the test of potential inhibitors of the transport of cGMP.

### 4.2.7 Test of the influence of magnesium concentration on cyclic GMP transport

#### 4.2.7.1 General principles

Previous studies have shown that magnesium has an effect on the activity of different transporters. Doige et al., (Doige et al. 1992) demonstrated that high concentrations of $Mg^{2+}$ had an inhibiting effect on the activity of P-glycoprotein, and as ABCC5 has certain similarities to P-glycoprotein, it is possible that high concentration of this cation may also influence the activity of MRP5. Sundkvist et al. tested different $Mg^{2+}$ concentrations on the transport of cGMP into inside-out vesicles, and found that concentrations above 10 mM had an inhibiting effect on the transport. (Sundkvist et al. 2000)In the present study the effect of $Mg^{2+}$ in combination with INI was investigated. A plateau was observed in the $IC_{50}$ curve for concentrations between $10^{-7}$ to $10^{-9}$ M for this compound, and from a hypothesis that it could be chelating with a cation present in the incubation solution resulting in reduction in the free concentration of this compound, it was chosen to be tested in combination with different $Mg^{2+}$ concentrations. Two different concentrations of $Mg^{2+}$ (1 mM and 10 mM) were combined with 5 different concentrations of INI. The concentrations of inhibitor tested ranged from $10^{-3}$ to $10^{-8}$ M. The ATP used in this study is Mg-ATP, and this magnesium also contributes to the total concentration of magnesium. As a result the concentration of Mg-ATP was reduced to 1 mM in this test.

### 4.2.7.2 Assay procedure

The same composition of incubation solutions was used as in the previous transport assays except for $Mg^{2+}$, which were in the concentrations 1 mM or 10 mM.

Total incubation volume per test vial:

50 μl of incubation solution 1 or incubation solution 2
150 μl of the different inhibitor solutions
50 μl vesicles

The reaction was performed as previously described, but the reaction was stopped after 60 minutes incubation. The rest of the procedure was done as previously described.

## 4.2.8 Thin layer chromatography

### 4.2.8.1 General principles

To find out whether the radio labeled cGMP used in the assays mentioned above was of good quality, the purity of the radiochemical was assessed by thin layer chromatography. If the total amount of radioactivity can be experimentally verified to be confined to one chemical structure only, then the radiochemical purity of the tested compound is excellent, and it may be concluded that no degradation of the compound has occurred. With a non-degraded compound, what was measured in the transport assays were [3H]-cGMP and not a degradation product.

### 4.2.8.2 Test procedure

A sheet covered with silica was used in this experiment, and in two different areas, one tiny volume of [3H]-cGMP(0.154 mM) and one equal volume of [1H]-cGMP (87.1 mM) were applied approximately 1,5 cm from the bottom of the sheet. Before adding the samples, a vertical line was made dividing the sheet in two separate lanes, and horizontal lines spaced 4 mm were drawn, in order to be able to evaluate the movement of the two compounds in the mobile phase correctly. (See fig 14.) The sheet was placed in a glass container where mobile phase consisting of 2 parts 1 M ammonium acetate and 5 parts ethanol had been added in approximately 0.5 cm height. When the mobile phase migration was almost at its end, the sheet was removed from the glass container and left to dry. The lane with non-radio labeled cGMP was made visible by staining with potassium permanganate for 0,5 minutes and the remaining permanganate was then removed by flushing with running water until the pink color had disappeared.

Staining solution consists of 2g $KMnO_4$, 13,3g $K_2CO_3$, 0,19g NaOH dissolved in 200 ml water.

Potassium permanganate is dissolved in this manner:

$$KMnO_{4(s)} + K^+_{(aq)} + MnO^-_{4(aq)}$$

Under alkaline conditions, this red-ox reaction will occur:

$$MnO^-_{4(aq)} + H_2O + 3e- \longrightarrow MnO_{2(s)} + 4OH^-_{(aq)}$$

The electrons in the equation are donated by the organic molecule which is oxidized. The brown mangane dioxide precipitates and will create a visible brown spot at the exact place as the organic compound is situated on the silica plate.

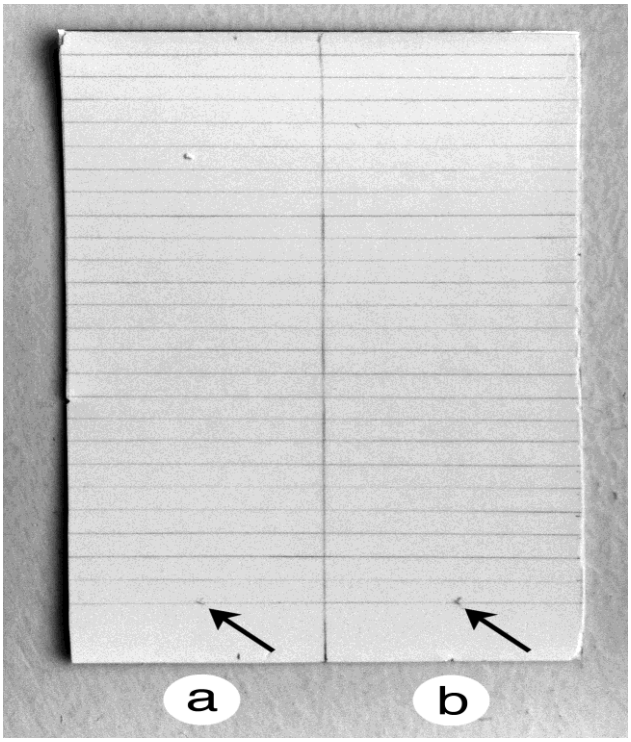


**Fig 14**: Labeled cGMP was applied to lane a) and non-labeled cGMp was applied to lane b). Arrows point to the sites where applications were made. Horisontal lines were 4 mm apart and was later used as a guide for cutting and scintillation counting.

A brown spot left on the rinsed silica showed where the non-radio labeled cGMP was found on the silica sheet, and the radio labeled cGMP could be expected to be found in the corresponding site on the other plate. The part with [3H]-cGMP was carefully cut into pieces following the horizontal pencil lines, and each piece was put into 1.5 ml eppendorf tubes in the same order as they appeared in the sheet. 1.4 ml water was added and the tubes were sonicated (≥ 4 minutes) until the silica was released from the metal, followed by 30 second of centrifugation at 16.000 x g (Heraeus Biofuge pico). 200 µl supernatant containing cGMP were transferred to scintillation tubes, and scintillation liquid was added to the vials before scintillation counting was done in a Packard 1900 TR Liquid Scintillation analyzer.

# 5 RESULTS

## *5.1 Results regarding preparation of inside-out vesicles*

### 5.1.1 Improvements to increase protein concentration yield

1. Increased volume of buffer B (from 20ml) to 80 ml made the solution less viscous and the membranous ghosts more visible.
2. Sedimentation of ghosts was apparantly not completed when centrifugation was done with slow brake, and centrifugation without brake resulted in 4-5 times higher yield of ghosts at this step in the procedure.

### 5.1.2 Improvements to increase the inside-out yield of vesicles

3. Measuring the volume of ghost achieved and adding exactly 39 volumes of the hypotonic buffer C, reduced the influence of the isotonic buffer B from the previous step.

## *5.2 Result of test of potential inhibitors for cyclic GMP transport*

Eleven “new” compounds from a database were chosen as potential inhibitors of cGMP transport by ABCC5, due to their structural similarity to Sildenafil and their predicted high affinity to the ABCC5 transporter by VLS. Sildenafil, Zaprinast and Dipyramidol had shown inhibiting effect on cGMP transport earlier, and were added to the test as positive controls. Water containing 68 mM DMSO and water without DMSO were used as negative controls, to find the maximum transport (100%) of cGMP and to investigate the effect DMSO had as a potential inhibitor compound. The water solubility of these compounds varied. Sildenafil, (INA), INI, and INL were relatively soluble in water, while other potential inhibitors were more hydrophobic and had to be dissolved in DMSO before water was added to give a solution of free inhibitor.

In this test, the concentration of test compounds in final incubation reaction was $10^{-4,5}$M (0,03162 mM) and the concentration of DMSO in the final

incubate was 68 mM for all compounds. It was noted that some of the compounds (INB, INC, INE, ING and INK) precipitated during incubation, and thus the free solute concentration in these compounds would be lower than calculated. All compounds were tested in triplicate with 3 parallels containing ATP and 2 parallels without ATP present in the sample. All results showed below relates to the active transport (results with ATP minus results without ATP = active transport). See fig 15.

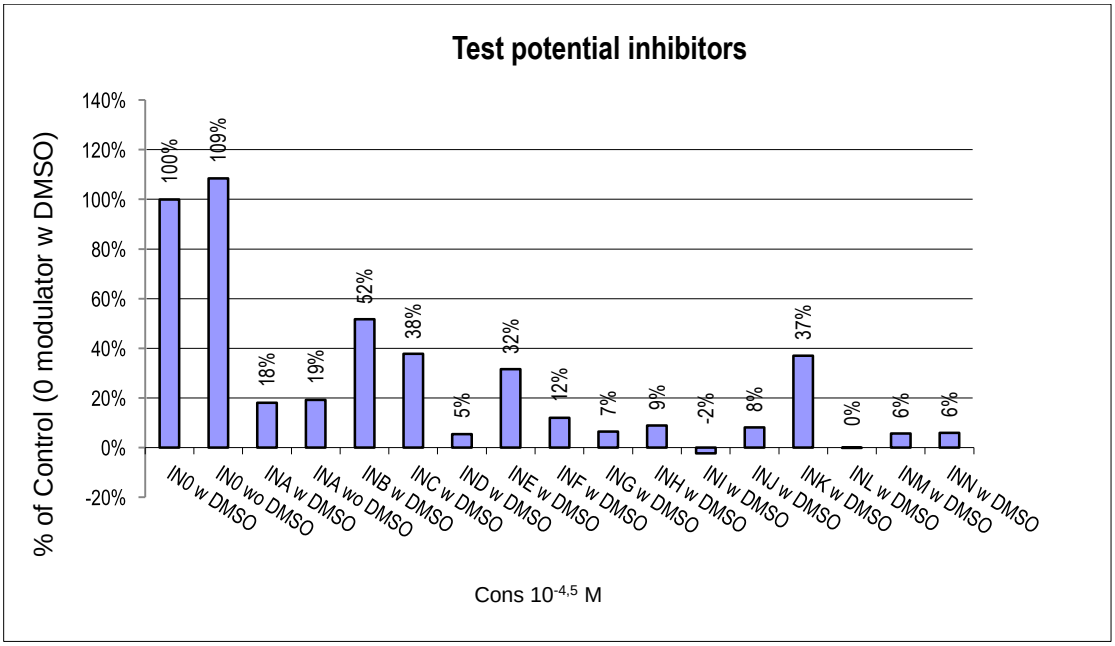


**Fig 15**: Results for the different potentilal inhibitors relative to the maximum transport of GMP into IOV vesicles. Only differences between cGMP accumulation with and without ATP are shown.

## *5.3 Resuløt of transport assay to find $IC_{50}$ value*

The compounds chosen for testing had the highest inhibiting effect on cGMP transport into IOV vesicles (lowest % transport of cGMP) in the previous test, in addition to the required solubility in water. INA (Sildenafil) was tested as a control compound, and INI, INL and IND were tested as possible ABCC5 inhibitors. These new compounds chosen all appeared to be better inhibitors of the cGMP efflux than Sildenafil.

INA, INI and INL were readily dissolved in water only, at pH 8.2, but IND had

to be dissolved in DMSO before it was diluted to the wanted concentrations. The same concentration of DMSO was kept for all measurements.

### 5.3.1 Calculation of $IC_{50}$ value

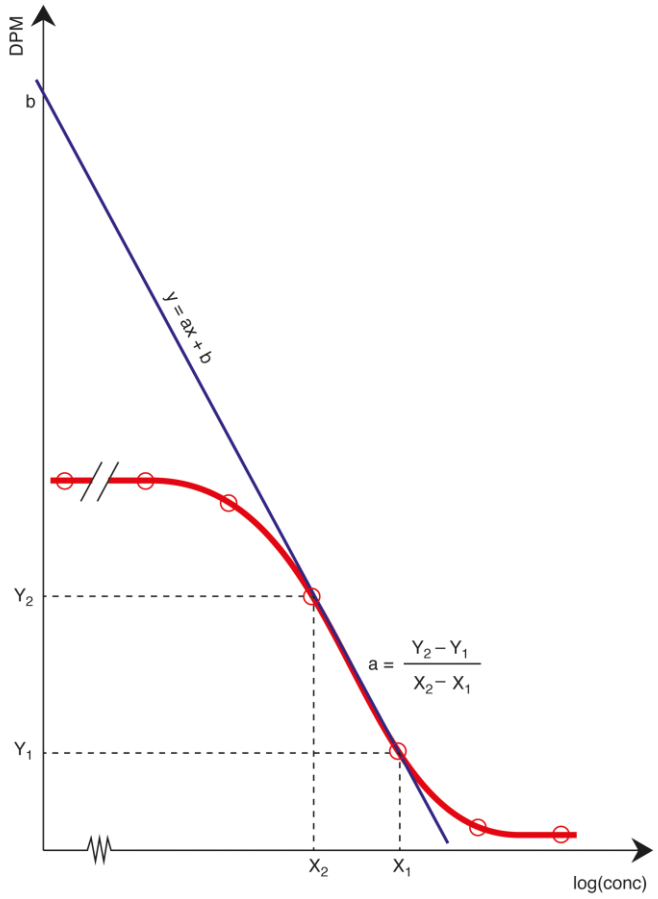


**Fig 16**: Sketch showing how to find the slope of a curve in order to calculate an $IC_{50}$ -value.

Equation for a straight line is: $y = ax + b$

b = intersection between line and second axis

a = slope

What is the value of x when y = 50%?

$$x = \frac{y - b}{a}$$

$IC_{50}$-values for the different compounds was calculated with data from each single test, and the 3 values obtained were used to calculate the mean $IC_{50}$-value and the standard deviation for each compound. Charts presented below represent data from 3 different tests for each predicted inhibitor, but they were not used for calculation.

### 5.3.2 $IC_{50}$ values for Inhibitor A

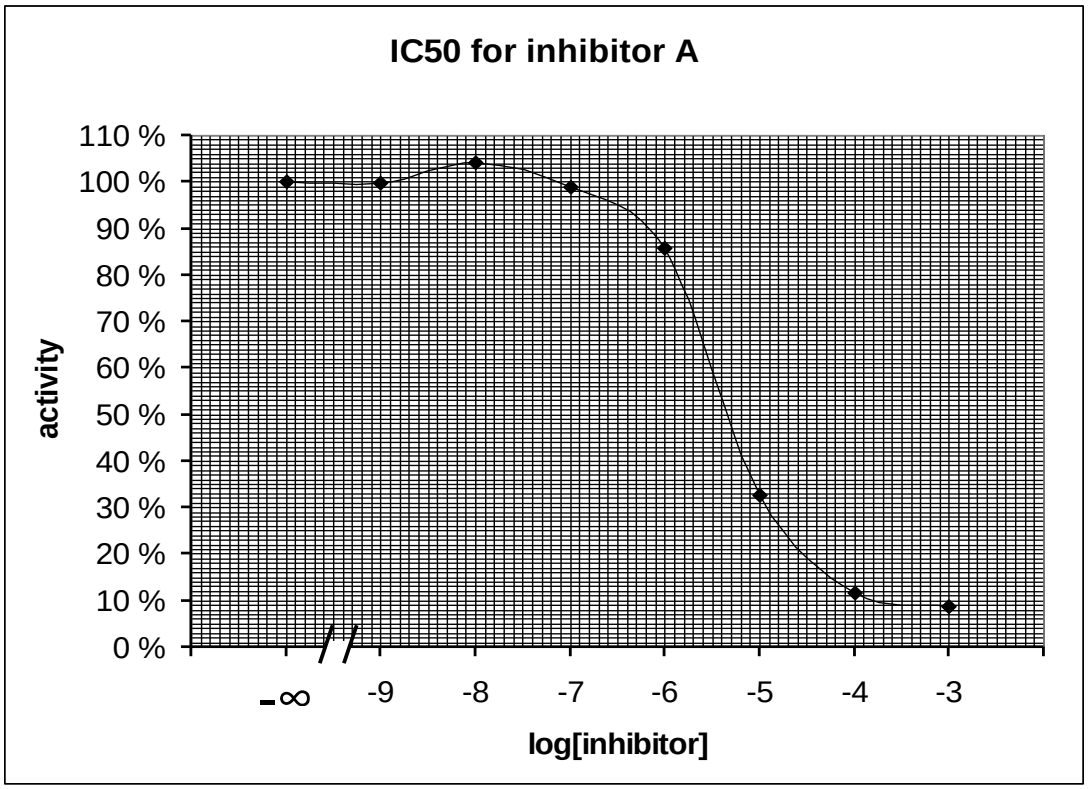


**Fig 17**: $IC_{50}$-curve for INA. The data used derived from 3 separate tests fitted together in one curve. The activity presented is desintegrations per minute (dpm) calculated to percentage, and inibitor concentrations are in the range between $10^{-3}$ and $10^{-9}$M. $10^{-\infty}$M had no inhibitor added.

The concentration at which 50% of the transport was inhibited was calculated to 5.3 μM. Standard deviation ± 2.4 μM.

### 5.3.3 $IC_{50}$ values for Inhibitor D

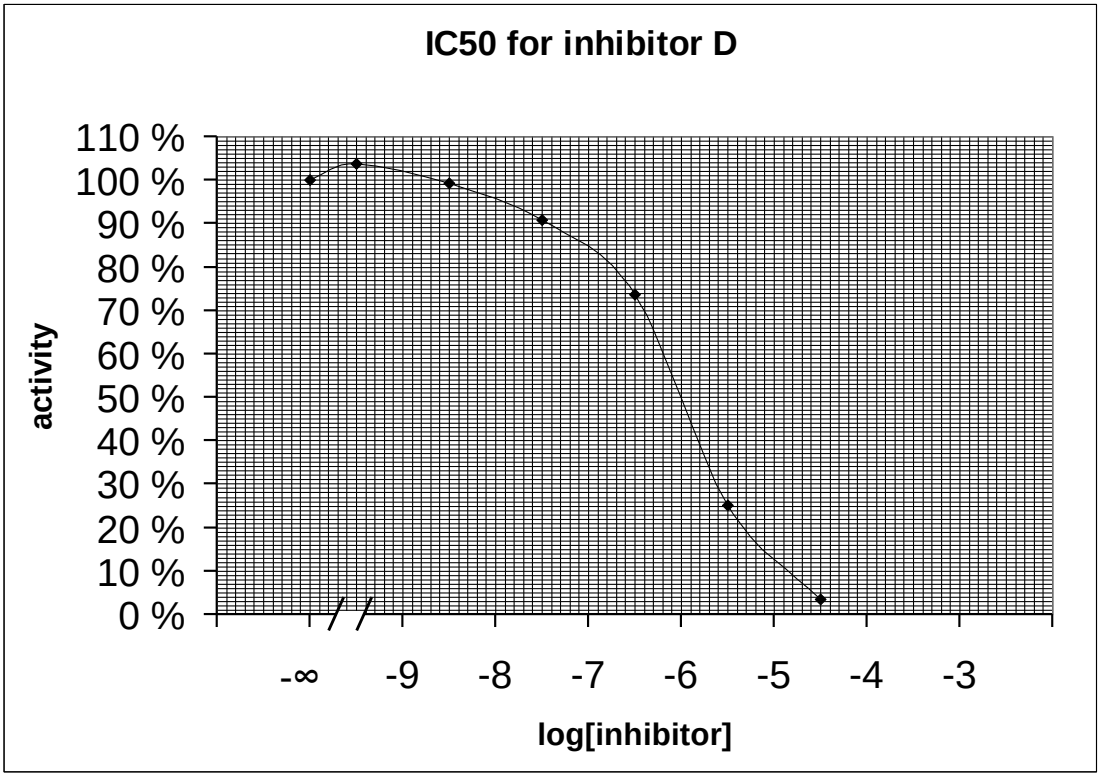


**Fig 18**: Curve for the $IC_{50}$ tests of compound IND in this study. The data used derived from 3 separate tests fitted together in one curve. The activity presented is desintegrations per minute (dpm) calculated to percentage, and inibitor concentrations are in the range between $10^{4,5}$ and $10^{-9,5}$M. $10^{-\infty}$M had no inhibitor added.

There was no visible precipitation of IND in any of the triplicate tests, and the concentration needed to inhibit 50% of the cGMP transport into IOV was calculated to 1.2 μM. Standard deviation ± 0,5 μM.

### 5.3.4 $IC_{50}$ values for Inhibitor I

This potential inhibitor was dissolved in water adjusted to pH 8.3 with KOH and no DMSO was added in this test. The $IC_{50}$ curves for this inhibitor showed a plateau between [$10^{-7}$] and [$10^{-9}$] M. The calculated value for inhibiting 50% of the influx of cGMP into IOV was 0.75 μM. Standard deviation ± 0.15 μM.

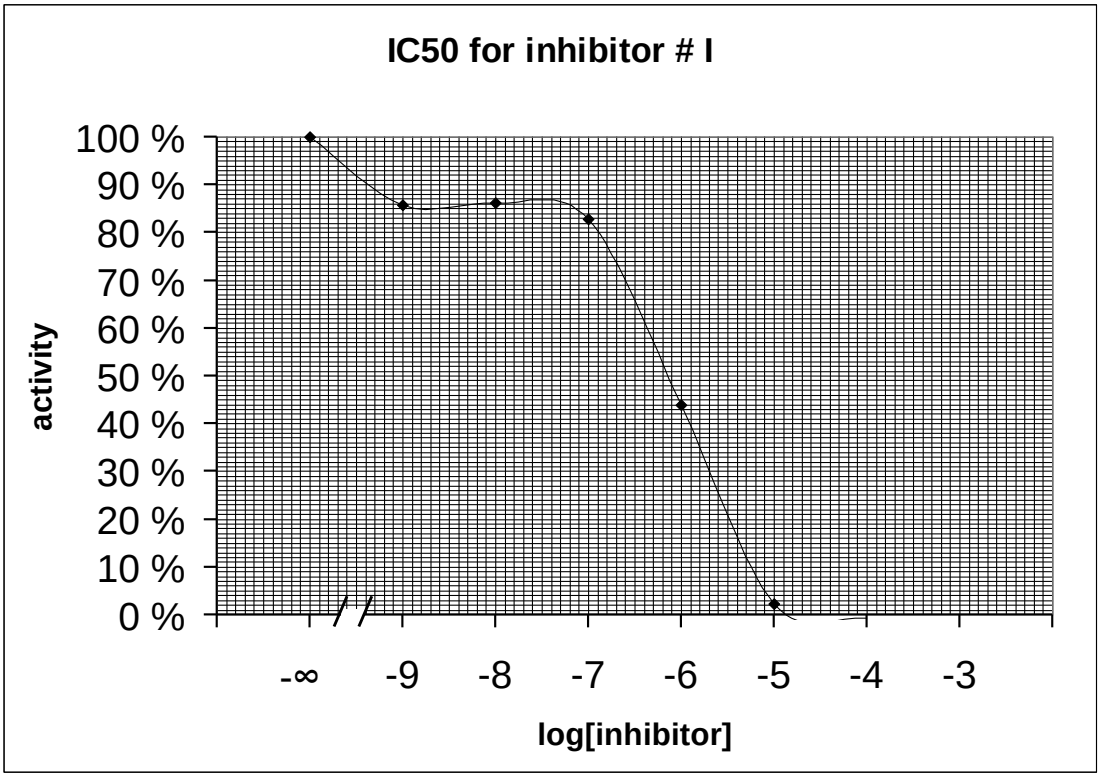


**Fig 19**: Chart with combined values from three separate tests for compound INI.

### 5.3.5 $IC_{50}$ values for Inhibitor L

INI and INL are structurally very similar, but the $IC_{50}$ curves for this compound did not show any plateau for the concentrations mentioned for INI. The $IC_{50}$ value for this inhibitor is calculated to be 0.29μM with SD $\pm$ 0.08 μM. This inhibitor is also relatively soluble in water pH 8.3 with KOH, and no precipitation was observed for this compound even over days.

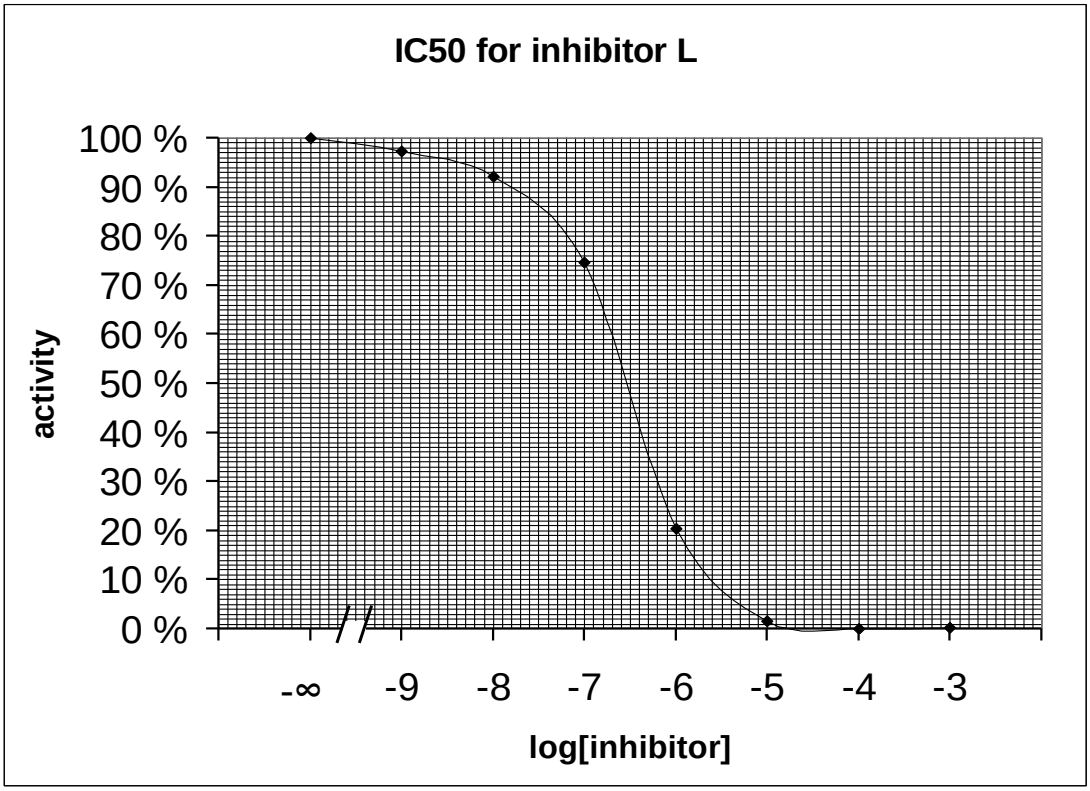


**Fig 19**: This chart presents data from 3 parallelle tests for INL.

## *5.4 Results of saturation study*

This test was done to find out whether the concentration [$^{1}$H]-cGMP in increasing concentrations would diminish the transport [$^{3}$H]-cGMP when this concentration was kept the same in all vials (0,4524 μM). The concentration of [$^{1}$H]-cGMP ranged from 1,55 to 143 μM in 8 different concentrations added to test vials. The incubations with ATP present were performed with 4 parallels and the incubations without ATP present were performed with 3 parallels for every [$^{1}$H]-cGMP concentration, and the test was done in triplicate.

It is known that cGMP is transported by ABCC5, and as the two compounds used in this study are almost identical, [$^{1}$H]-cGMP is expected to have the same affinity to the transporter as [$^{3}$H]-cGMP. As a result, one may expect to see a decrease in the transport of [$^{3}$H]-cGMP into IOVs caused by the competition of the much higher concentration of [$^{1}$H]-cGMP. The results below

shows that this is only the case to a certain extent, and even when the ratio between [$^{1}$H]-cGMP and [$^{3}$H]-cGMP was at its highest, the transport of [$^{3}$H]-cGMP was only reduced to
approximately 61%.

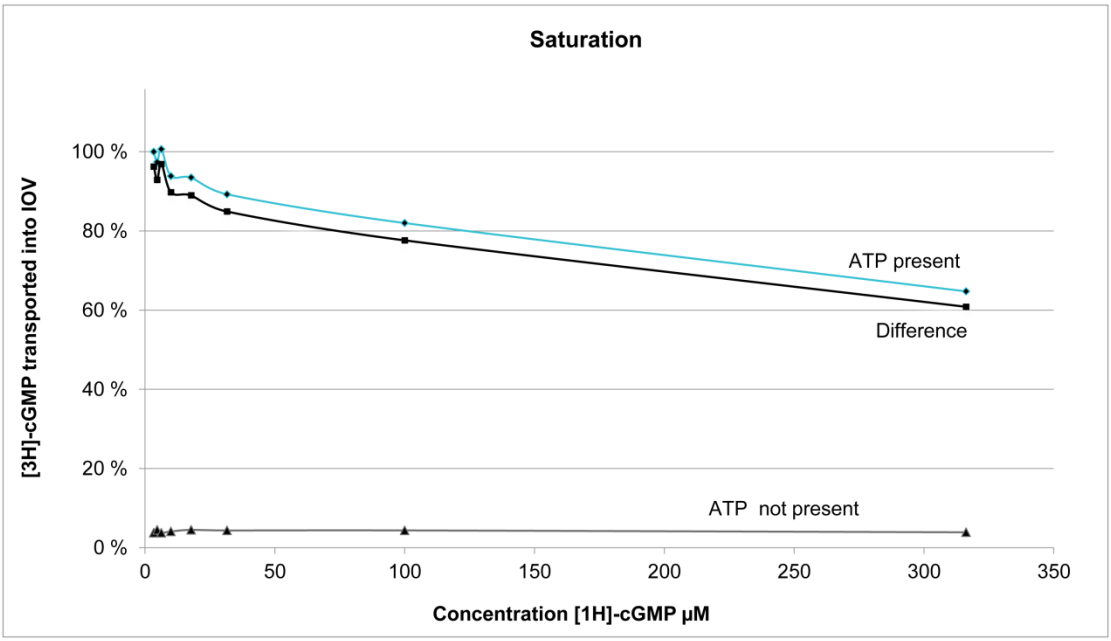


**Fig 20**: Graph showing saturation curve when [$^{3}$H]-cGMP was kept in a concentration of 0,4524 µM and [$^{1}$H]-cGMP ranged from 1,55 to 143 µM.

## *5.5 Results for the test on the influence of magnesium concentration on cyclic GMP transport*

Previous studies have shown that magnesium concentration has an effect on the activity of different transporters. High concentrations had an inhibiting effect on P-glycoprotein (Doige et al. 1992), and it may have similar effects on ABCC5 as the two transporters share some common features. In the $IC_{50}$ study of INI, it was found relatively low transport of cGMP into IOV in the concentration range $10^{-7}$ to $10^{-9}$ M. Magnesium concentrations above 10 mM had an inhibiting effect on cGMP transport into IOV(Vaskinn et al. 1999)but 10 mM was an optimal concentration for the transport. Calcium ions have shown inhibitory effect to the uptake of cGMP into IOV (ref.) and to prevent this effect, 1 mM EGTA was added to the incubation solutions. EGTA has the ability to chelate divalent cations, but with a much higher affinity to $Ca^{2+}$ than to $Mg^{2+}$ (Sigma) approximately all $Ca^{2+}$ will be chelated while $Mg^{2+}$ will still be present in the incubation solutions. The observed presumably lower free concentration of INI than could be expected in the above mentioned concentration range in the $IC_{50}$ study of INI, made it interesting to test whether this was a result of interaction between the magnesium ions and the inhibitor. This inhibitor contains a negatively charged carboxyl group with a potential to form a complex with divalent cations. As a result, concentrations of magnesium and INI were combined covering the concentration range from $10^{-6}$ to $10^{-\infty}$ M for INI and magnesium concentrations of 1 mM and 10 mM in the incubation solutions, and all series were done with and without the presence of ATP.

The results show ( see figure T) that the transport of cGMP when 1 mM $Mg^{++}$ is present is highest. With 10 mM Mg present in the incubation solutions, little additional cGMP is transported into IOV for inhibitor concentrations lower than $10^{-7}$M. When the magnesium concentration used was 1 mM, the percentage of cGMP transport into IOV increased until INI was as low as $10^{-8}$M. The result shows that a magnesium concentration of 10 mM has an inhibiting

effect on cGMP transportation, but no conclusion on the basis for this effect can be explained from the data presented below.

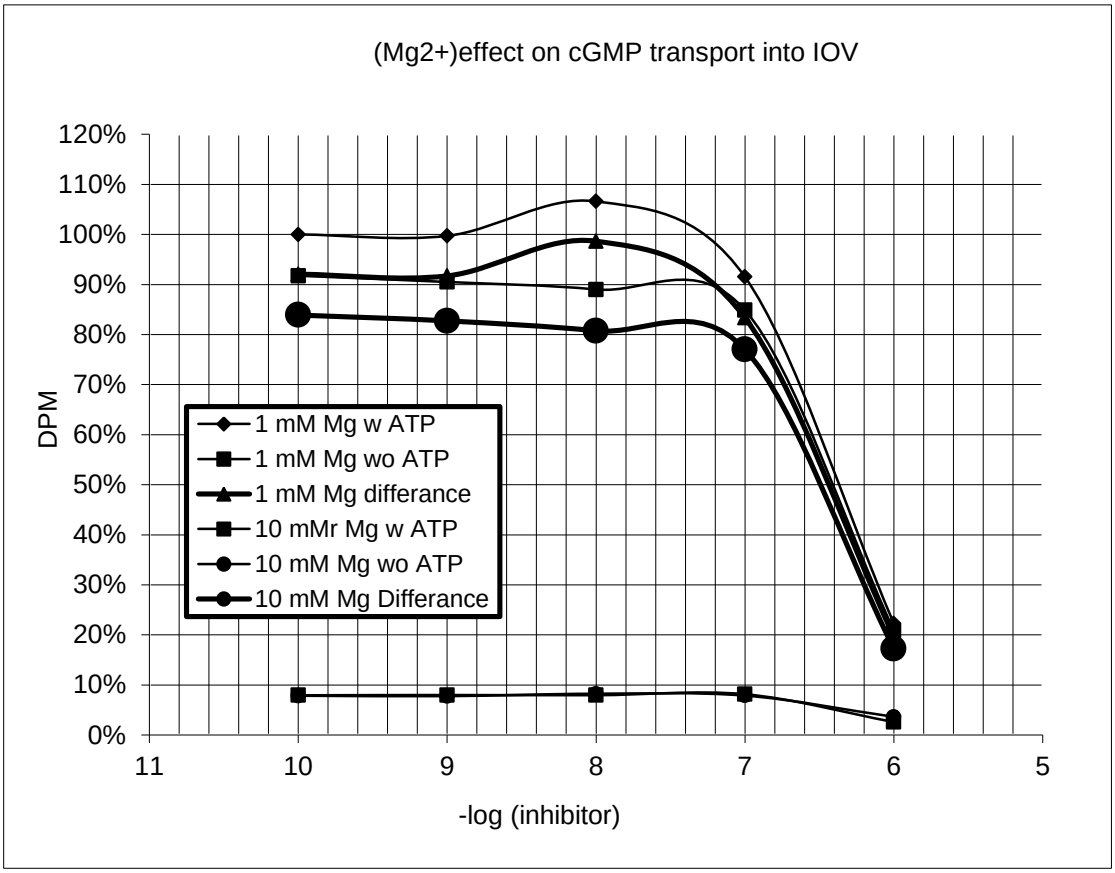


**Fig 21**: Effect of two $Mg^{2+}$ concentrations in combination with inhibitor I on the ATP dependent uptake of cGMP. ATP-independent uptake for the two $Mg^{2+}$ concentrations shows equal values for all inhibitor concentrations except $10^{-6}$M and appear as one single line for the other concentrations tested.

## *5.6 Results from thin layer chromatography*

Thin layer chromatography was performed to investigate the quality of the [$^3$H]-cGMP used in the experiments, and the results in Fig 23 show that the [$^3$H]-cGMP used was not degraded.

Almost all (a negligible amount was found above) was found in band 18. For comparison, [$^1$H]-cGMP was tested simultaneously, and this compound was visualized by permanganate staining within the 4 millimeters space equal to band 17. This small discrepancy may be a result of an uneven movement of the mobil phase. Besides, a much higher concentration of [$^1$H]-cGMP just below the saturation of the silica was applied to lane b compared to the very limited amount of [$^3$H]-cGMP applied to lane a. A large amount is needed to visualize the compound by the use of permanganate. The large amount of cGMP that acts like a buffer per se may have altered the pH level of the mobile phase locally, and thus changed the surface charges on the silica that influences the retardation of cGMP.

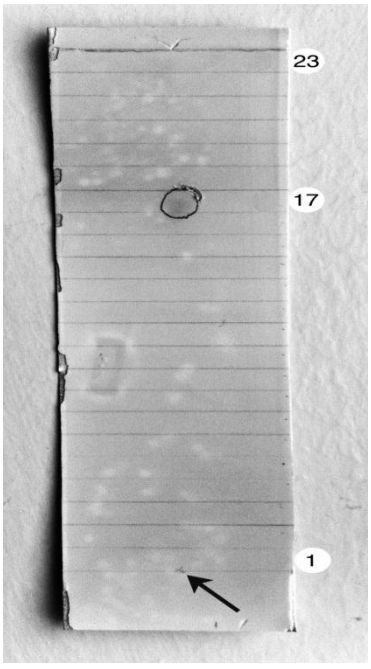


**Fig 22**: Silica sheet showing permanganate stained [$^1$H]-cGMP visible in band 17.

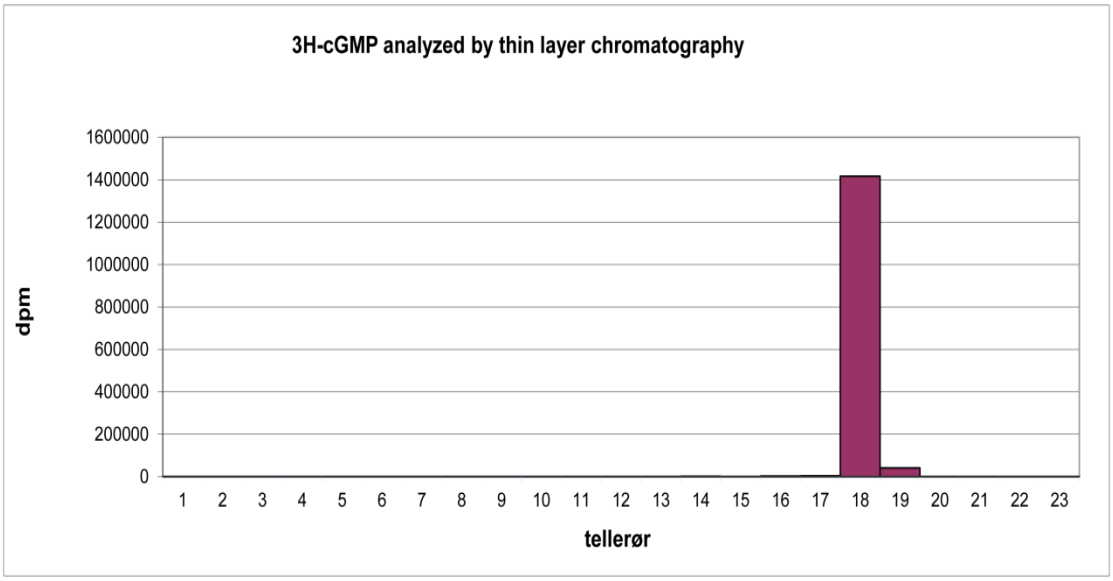


**Fig 23**: Chart showing disintegration per minute for all vials equal to lanes cut out from the radio-labeled cGMP tested by thin layer chromatography.

# 6 DISCUSSION

Multidrug resistance is a major obstacle to cancer chemotherapy as well as antibiotic treatment. One of the mechanisms involved is increased excretion from the cell and its expected site of action. The drug molecules are often ions or hydrophilic molecules that are not able to penetrate the cell membrane by passive diffusion and thus require a transport protein to cross the cell membrane. ABCC transporters have the ability to efflux anions, while e.g. ABCB1(p-glycoprotein) is involved in efflux of lipophilic and neutral solutes. The ATP Binding Cassette (ABC) transporter ABCC5 has been identified as a transporter taking part in efflux of different drugs molecules (Pratt et al. 2005). To prevent the efflux of drugs from the target cells, development of inhibitors is of increasing interest as a supplement to drug therapy.

Inside-out vesicles have proved a good system to test ABCC5 mediated transport as well as inhibition of this transport in cells where this transporter is expressed. The main aim of this study is to try to confirm by in vitro studies the binding/inhibiting properties predicted by molecular modeling for the ABCC5 transporter, and as a consequence it was necessary to verify that the method used to prepare IOVs was reliable concerning yield of IOV.

## *6.1 Preparation of inside-out vesicles*

The preparation of inside-out vesicles from human erythrocytes is a procedure that has been in use since it was published by Steck and Kant in 1970. Different groups have made slight modification to this procedure, as is also the case at the department of Medical Pharmacology and Toxicology, in the Faculty of Health and Science at UIT today (Sager et al. 1996).

Initial tests proved that the yield of IOV, both regarding percentage of inside-out facing vesicles and protein concentration, varied from one preparation to another. A certain amount of IOV is needed to perform a transport study, and a consequence of the various yield in the different preparations, made it difficult to plan assays including several test vials in advance. Thus it became

clear that some improvement to the present method in order to both increase and stabilize the yield of IOV would be an advantage.

### 6.1.1 Improvements to increase protein concentration yield

The first washing steps, where plasma and buffy coat were removed, usually gave 4 ml of packed RBCs from a 10 ml EDTA vial of full blood. When lysis was initiated by adding the previously recommended volume of buffer B (Solution for hemolyzing RBC, 5 mM Tris•HCl, 0.5 mM EGTA, 4 mM KCl, pH 8.1), the solution became too viscous to achieve an effective sedimentation during centrifugation. It also appeared difficult to verify the sedimentation of membranous ghosts. The typically yield of membranous ghost collected after these washing steps, where intracellular solutes and hemoglobin were removed, was approximately 1 ml.

#### 6.1.1.1 Increasing volume of buffer B

An attempt to solve this problem was done by increasing the volume of buffer B to 80 ml per 4 ml packed RBC. The result was a less viscous solution and after the following centrifugation it became obvious that sedimentation of the fluffy ghosts were not completed by the end of centrifugation run time with slow brake. The reason was most likely that retardation to a certain level disturbs ghost layer and mix it with the supernatant again before the centrifuge comes to a stop. With the previously very viscous solution this would be impossible to detect. Increasing the volume of buffer B confirmed the suspicion that large volumes of ghosts might have been lost previously at this step in the procedure.

#### 6.1.1.2 Centrifugation without brake

In order to achieve a complete sedimentation and full recovery of the fluffy ghosts, centrifugation hereafter was done completely without brake. This increased the time frame of the procedure largely, but the recovery of ghosts also increased from 1 ml to 4-5 ml. Increasing the volume of buffer B and performing centrifugation without the brakes gave a net result of 4 to 5 times more yield, and the diluted solution also made it easier to spot and avoid the relatively solid collection of proteases which appeared as a pellet in the bottom of the centrifuge tube.

### 6.1.2 Improvements to increase the inside-out yield of vesicles

#### 6.1.2.1 Adding the right volume of buffer C to the pelleted ghosts

In the next step in the procedure, where the vesicles are resuspended in the hypotonic buffer C (Solution for vesicles to form, 500 nM Tris•HCl, pH 8.2) in order to initiate vesiculation, it is important to make sure that all previously used buffer B is completely removed, as residues of this isotonic buffer is expected to interfere with and inhibit the vesicle formation which requires a strict hypotonic environment. But the fact that 4 ml packed RBCs can result in a volume of unsealed ghost that is between 4 and 5 ml, strongly indicates that the ghost fraction has some residue buffer B remnants within the fraction. This buffer residue cannot be removed unless much harder centrifugation is used that compresses the volume of the ghosts. To make sure that enough buffer C would be added at this stage in the procedure, the volume of ghosts was measured. According to the procedure, 39 volumes of buffer C were added to the volume of ghosts. Adding enough of buffer C is important to dilute buffer B remnants and outweigh the effect of the residue isotonic buffer B among the pelleted ghosts.

### 6.1.2.2 Avoiding divalent cations in the preparation of IOV

To achieve a high percentage of IOVs it is very important to incubate freshly made ghosts in an alkaline hypotonic buffer with low ionic strength in the absence of divalent cations (Steck and Kant 1974). The fact that divalent cations influences both the sidedness and the sealing of the vesicles was observed by Steck and Kant, but an explanation to this phenomena has not been presented so far. Previous studies (Steck and Kant 1974) have shown that vesiculation occured in medium with pH variations between 7.7 and 8.7, and that the yield of IOV increased in a more alkaline solution. However, certainly easily eluted polypeptides were lost from the membranes if the pH was at the highest level, and according to this fact the pH was kept between 8.0 and 8.2 in all solution used in this study. When vesicles are formed, they are stable in buffers with pH between 5.5 and 9.0 at 4°C for at least a week. If they are stored longer they may become unsealed again.

### 6.1.2.3 Avoiding sodium in all solutions used in preparation and transport assays

The modifications mentioned above made the yield of IOV higher than previously achieved, but when testing the vesicles in transport assays, a relatively high ATP independent transport into the IOV was still seen. It was decided to “switch off” any pump/transporter that gets it’s energy directly or indirectly from an existing sodium/potassium gradient. This was done by preparing vesicles and performing transport assays without sodium present. Sodium was omitted completely in every step in all procedures.

With these four modifications, the initial yield of ghosts increased, the percentage of IOV compared to ROV increased, typically between 60 to 80%, the IOV could be kept refrigerated for at least a week without decrease in transport activity as previously reported (Boadu et al. 2001), and the ATP-independent transport was not influenced by changes in inhibitor concentration or other solutes used in the transport assays.

### 6.1.3 Parameters influencing sidedness

High percentage of IOV cannot be explained by accidental wrapping of the cell membrane in one or the other direction, as the percentage of IOV then would be expected to be 50. The percentage of IOV compared to the percentage of ROV achieved in a vesicle preparation was significantly higher and was most likely ruled by different factors, and in this study observations indicated that individual parameters like drug use, diet, and phenotype could influence on the yield. But with the modifications mentioned above, the IOV sidedness was not seen below 60%.

## *6.2 Study of potential inhibitors for cyclic GMP transport*

Using IOV for transport studies has been proven useful as an experimental model system, and previous experiments by this model have shown that cGMP is transported by ABCC5 with high affinity (Schultz et al. 1998). It has been reported that phosphodiesterase5 inhibitors like sildenafil, zaprinast and dipyramidole are potent inhibitors to cGMP transport in IOV (Sundkvist et al. 2002).

The software ICM Pocket Finder had been used to find possible ligand binding cavities on the ABCC5 molecular model previously, and a cGMP model had been docked into two putative binding pockets in the ABCC5 model (Ravna et al., 2008). The ICM ligand docking procedure may also be used to predict whether certain small molecules may act as a ligand in a computer model. In this study 11 compounds structurally similar to sildenafil and predicted as possible inhibitors to cGMP transport using the ICM ligand docking procedure were chosen for IOV transport assay.

The different structures assayed are presented in Fig 8, and structural similarities to sildenafil is indicated with areas of different colors.

### 6.2.1 Limited water solubility and precipitation

The structure of most of these compounds lacking charged or polar groups may predict that the water solubility could be limited, but compounds like INI and INL contains an ionizible carboxyl group that is likely to make these compounds relatively water soluble (See Fig 8).

In this initial study of all compounds, 11 new compounds were tested in addition to the previously tested compounds sildenafil, zaprinast and dipyramidole that had already proved to be potent inhibitors of cGMP transport. To all compounds were added an initial volume of 50 μl DMSO that was necessary to dissolve some of the compounds to achieve a concentration of completely free solute. This made it possible to dissolve the solutes in

water to gain a supersaturated and unstable solution, but with 100% of the solute in its free form. This procedure was not necessary for all compounds, but in order to treat all compounds equally, they were all diluted the same way. The 50 μl of DMSO added resulted in a concentration of 68 mM DMSO in the final incubation, and at this low concentration DMSO did not incluence the cGMP uptake (Schultz et. al., 1997). Inhibitor dilutions in water were made to give a concentration of $10^{-4,5}$M in the final incubation, and hopefully the oversaturated solution would not precipitate until the incubation period was over.

### 6.2.2 Oversaturated solution and precipitation

For some compounds precipitation was seen shortly after addition of water, and these had to be dissolved again shortly before incubation started ( INB, INC, INE and INK). Some compounds precipitated during incubation at 37°C, some in room temperature. INI and INL did not precipitate even after days. The precipitation during incubation was only noticed by chance, as the procedure required swift operations from stop to the reaction followed by filtration, and as a result, every vial was not inspected for possible precipitation.

The relatively low inhibition potency achieved for some of the compounds were most likely due to precipitation leading to less solute inhibitor in free form. Therefore these compounds are poor candidates as potential ABCC5 inhibitors.

### 6.2.3 Comparing results for all inhibitors tested

INA (Sildenafil) contains a methyl group in its molecular structure, and the similarity to the structure of the switterionic Good buffer HEPES may predict a relatively good solubility in water. The compound used in this study was sildenafil citrate and solubility was in the literature stated as 3.5 mg/ml in water. This PDE5 inhibitor was included in this study as a positive control, as previous studies had shown that this compound had potent inhibiting effect on

ATP-dependent cGMP transport. The measurement of transport of cGMP with no inhibitor present but with 68 mM DMSO was considered as 100% effective transport into IOV, and compared to this the transport results for INA at 32 µM were 18% as effective transport with DMSO and 19% without DMSO present.

INB proved more difficult to dissolve in water compared to INA, and the molecular structure for this compound does not contain polar groups that would make the molecule more water soluble. For this compound precipitation was visible shortly after it was diluted in water to give a final incubation concentration of 32 µM, and thus the poor inhibiting effect seen may be due to the decrease in free drug following precipitation (52 % transport compared to sample with no inhibitor present).

INC also proved difficult to dissolve, and precipitation was seen during incubation for this compound. The inhibiting effect on ATP-dependent transport of cGMP proved somewhat better than INB, and probably due to a decrease in free solute concentration, this compound was not a potent inhibitor (38 % transport at a concentration of 32 µM).

For the test compound IND no precipitation was seen after it was dissolved and diluted to the different concentrations. This compound had a higher inhibiting effect than sildenafil in the test performed, and 5% of cGMP was transported into IOV at the concentration 32 µM.

In INE precipitation was noticed after incubation, and this precipitation may have occurred during the incubation period and thus influenced the concentration of free solute present in the reaction. Transport of cGMP into IOVs at a concentration of 32 µM was calculated to be 32% for this compound compared to non-inhibited transport.

INF – no precipitation was observed for this compound, and the calculated transport of cGMP into IOVs in the presence of this molecule was 12% at a

concentration of 32 µM. The inhibiting potency for this compound was better than the one observed for sildenafil in this test.

ING was among the compounds where precipitation was seen, but the precipitation may have occurred after the main period of incubation, as the transport of cGMP with this compound present was only 5% at a concentration of 32 µM.

INH seemed to be a reasonably potent inhibitor of the active transport of cGMP into IOV, and no precipitation was observed for this compound. Transport of cGMP was calculated to be 9%, which is better than the result for sildenafil at a concentration of 32 µM.

INI has a carboxyl group as part of its molecule structure, and this ionized group may contribute to the relatively high solubility in water. This compound had a very high potency as cGMP transport inhibitor in this initial test. The transport calculated with this compound present in a concentration of 32 µM was -2 %.

INJ also showed a relatively high potency as a possible inhibitor to cGMP transport in the concentration tested, with a transport of cGMP with this compound present calculated to be 8% compared to total transport at a concentration of 32 µM.

The compound INK showed precipitation during incubation, and thus the decrease in free solute from the calculated 32 µM may have influenced the relative low inhibiting effect observed for this compound (37% transport).

INL is very similar to INI, they both have an ionizing carboxyl group in the molecule structure, see Fig 8 for comparing the two compound. Like INI this compound was easily dissolved after pH adjustment to 8.3 with KOH, and it showed a high potency inhibiting ATP-dependent transport of cGMP in this

study. Transport for the concentration tested was 0% compared to total export at a concentration of 32 µM.

INM – zaprinast has an acceptable solubility and had in previous tests shown inhibiting effect of high potency in cGMP transported into IOV. Value found in this test at a concentration of 32 µM showed 6% transport compared to total transport.

INN – dipyramidole as well as INM was included as a positive controls in the test, and this compound was relatively easy to dissolve and had a high potency inhibiting effect at the concentration of 32 µM as was tested in this study (6% transport).

All the different compounds chosen had an inhibiting effect on cGMP transport into IOVs, and only four of the 11 tested compounds showed less potency than sildenafil in this study. For these compounds precipitation was observed, which may account for some of the relatively low potency as inhibitors for the ATP-dependent cGMP transport.

## *6.3 $IC_{50}$ STUDY*

The compounds that were chosen for further investigation were the compounds that revealed the best inhibiting effects on the cGMP transport into IOV and for which no precipitation was seen during or shortly after incubation in the previous transport assays. In addition to INA, which had been investigated earlier and in this study was used as a positive control, transport studies with various concentrations of inhibitor were made to find $IC_{50}$ values for the different compounds and thereby gain more knowledge about their properties as cGMP transport inhibitors. Zaprinast and dipyramidole had previously proved to be high affinity competitors to the ATP-dependent transport of cGMP (Sundkvist et al. 2002) and they were not investigated further in this study.

### 6.3.1 INA

INA was tested in a concentration range between 1 mM to 1 nM, and also with no inhibitor added as a control. The values obtained for the samples of the ATP dependent transport (active transport) for this sample subtracted the values obtained for the ATP independent transport (unspecific dissociation/energy independent transport) was regarded the net 100% transport. Values obtained for the samples containing inhibitor were related to the 100% value. DMSO was not added to initially dissolve the inhibitor in this test as sildenafil is relatively soluble in water.

The $IC_{50}$-curve for INA showed higher transport into IOV at inhibitor concentration 10 nM than without inhibitor present, but as similar values were obtained for 100nM, 1 nM and for control (absence of inhibitor), the lowest concentration of inhibitors presented a higher plateau.

### 6.3.2 IND

This test compound was initially dissolved in DMSO. Due to this and limited amount of test compound available made it necessary to use 32µM as the

highest concentration. Dilution factor was 10, ranging from 32µM to 0,32nM and also with 0 inhibitor present. Precipitation was observed neither during nor shortly after incubation for this compound. According to results seen in the initial test, the potency of this inhibitor can be considered in the high range, with an $IC_{50}$ value of 1.2 µM. This test suggests that this compound is a more potent inhibitor than sildenafil.

### 6.3.3 INI

This compound was easier to dissolve than IND after pH adjustment with KOH, most probably due to its ionizable carboxyl group and polar hydroxyl croup. The carboxyl group is probably ionized above pH 8, and probably contribute to the compound being more water soluble than some of the other sildenafil derivatives tested. In figure Q a plateau was seen in the concentration range between 100nM and 1nM for this compound at approximately 85% of the maximum transport for samples without inhibitor. As the points showed in the graphs are mean values from 12 samples (3x test x 4 parallels) with ATP present and 9 samples (3x test x 3 parallels), this observed phenomenon is probably not a coincidence due to measurement variance, but so far no explanation has been found.

As expected from the initial test for all 11 new compounds, this inhibitor seems to be a more potent inhibitor to cGMP transport than sildenafil. The $IC_{50}$ value found for this compounds was 0,75 µM.

### 6.3.4 INL

This possible inhibitor has a molecular structure that is very similar to INI with a carboxyl group and a hydroxyl group in corresponding sites. INL proved to be relatively soluble in water, and no precipitation was seen in a solution of this compound even after days refrigerated. The $IC_{50}$ curve in figure R showed that the concentration needed to inhibit half of the total cGMP transport by this compound was calculated to be 0,29 µM. The $IC_{50}$ value for this inhibitor was

the lowest among the compounds selected in this study. Thus this compound was predicted the most potent inhibitor of ATP-dependent cGMP transport among all the compounds tested.

### 6.3.5 General considerations regarding high affinity binding to ABCC5

By molecular modeling (Ravna et al. 2008) two binding pockets were predicted in the transmembrane area of ABCC5. Each of the predicted binding pockets had a positively charged amino acid in a position suggesting it could be a possible recognition site for organic anions. When docking of cGMP were performed by the ICM program, the program suggested a salt bridge between the positively charged amino acid Lysine and the negatively charged phosphate group of cGMP. A salt bridge was also suggested in the other binding pocket between the negatively charged phosphate group on cGMP and the positively charged amino acid arginine. It is expected that this account for the high affinity binding that expected to take place at the intracellular site of the ABCC5 transporter, before the translocation caused by conformational changes in the binding site changes the binding affinity and the substrate is released into the extracellular side of the membrane.

A strong electrostatic attraction may be formed between the two positively charged amino acids in the putative binding pockets of ABCC5 and the carboxyl groups found in the molecular structure of INI and INL. These carboxyl groups are most likely negatively charged at pH 8, which was used in the transport assays. Ionizible carboxyl groups were not found in the structure of the other compounds tested, and this may be the main reason for the difference seen between all compounds tested in addition to decreased water solubility and precipitation problems for some of the compounds. The very high affinity found between ABCC5 and the two inhibitors INI and INL both in preliminary tests of all 11 compounds and regarding $IC_{50}$-values for each of the two compounds is very likely due to strong electrostatic attraction between the positive charged amino acids in the transporter's suggested

binding pockets and the negatively charged carboxyl groups in the molecular structures of these two compounds.

## *6.4 Saturation study*

According to the mass action law (Michaelis-Menten kinetics), with increased substrate concentration, the transport would reach a maximum and thereby reduce the fraction of [$^3$H]-cGMP measured. The fraction of [$^3$H]-cGMP was kept constant at a concentration of 0,4524 μM and the concentrations used for [$^1$H]-cGMP ranged from 1,55 to 143 μM. The difference between the two cGMP compounds regarding affinity to the transporter could be neglected and thus the same transport system was used for both compounds.

This result shows a two component saturation curve with high and low affinity, as presented earlier by Boadu et al. in 2001 (Boadu et al. 2001).

**Table 2**: Calculated $K_m$-values for a two component saturation curve with fixed concentration of [$^3$H]-cGMP and increasing concentrations of [$^1$H]-cGMP

| | Low $K_m$* (μM) | High $K_m$** (μM) |
|---|---|---|
| Mean | 5.2 | 971 |
| SD | 1.4 | 45 |

*) High affinity,

**) Low affinity.

Previous studies have shown that ABCC5 is a high affinity efflux transporter of cGMP, and thus the high affinity transport shown in this study is executed by ABCC5. The low affinity transport may be explained by at least 3different possibilities:

1. There may be ABCC5 transporters with low affinity to cGMP, these transporters may be triggered by a high concentration of cGMP. One explanation may be that the omission of $Na^+$ throughout all procedures, may have influenced the low affinity state of the transporter (releasing position) in a way that decreased the releasing of the substrate, and thus both [$^3$H]-cGMP and [$^1$H]-cGMP will be attached to the IOV longer than expected. Higher levels of [$^3$H]-cGMP will be measured as a result.

2. Another explanation could be that as the concentration of cGMP in this test is much higher than the physiological concentration, the cells (here represented by IOV) may have additional low affinity ATP-dependent transporters with the ability to transport solutes at extremely high concentration. We know that the ABC transporter ABCC4 which is a high affinity transporter of cyclic adenosine monophosphate (cAMP) also has the ability to transport cGMP with low affinity, and this transporter is also present in RBCs (Wu et al. 2005).

3. Organic anion transporter 2 (OAT2) which has been documented as a cGMP transporter (Cropp et al. 2008) in other tissues may also be present in RBC, but so far this is not established. However, the last mentioned transporter has the ability to transport organic anions in both directions, and functions as both an efflux and influx pump. It was noted though that when a high outward directed concentration gradient of cGMP was generated, the net effect of cGMP transport was in the efflux direction.

The extended extrusion mechanism that is revealed in this displacement study, could be the result of the activity of more than one transporter, and even though the number of transporters and carriers already characterized is huge, there may still be others that have not been discovered yet.

## *6.5 The study of the influence of magnesium concentration on cyclic GMP transport*

In previous studies, it was found that magnesium affected the activity of various transporters, and high concentration of magnesium had an inhibiting effect of the transporter P-glycoprotein (Doige et al., 1992). Testing the transport of cGMP by ABCC5 in IOV in combination with an inhibitor to investigate possible effects of magnesium seemed interesting. The compound INI was chosen in a concentration range between 1µM and 1 nM and with no inhibitor added as lower transport than expected was seen in concentrations between 100 nM and 10 nM for this inhibitor, and a possible explanation could be a complex formation between the methyl group on the INI molecule and the Mg+ present in the incubation solution.

The result revealed that a magnesium concentration of 10 mM reduces the ATP dependent transport of cGMP into IOV in inhibitor concentrations lower than 100 nM and that a plateau in transport is reached from this concentration and in lower concentration of inhibitors. The same inhibitor concentrations tested with 1 mM showed increasing transport until the inhibitor concentration was as low as 10 nM and the transport at this point was 100%.

# 7 CONCLUSION

In this study, the first aim was to establish a refined IOV method in order to test the effect of possible inhibitors on the cGMP transported by ABCC5. With the 4 improvements done to the procedure (see results or discussion), the fraction of IOV was not seen below 60%. The omission of sodium in all solutions used throughout all procedures, reduced the ATP-independent association/transport seen previously to a low basic level that was not influenced by inhibitor concentrations or other solutes in the incubation vials. It may be concluded that refining the method concerning yield and IOV sidedness was fulfilled.

The second aim was to confirm the predicted inhibiting effect of 11 selected compound postulated as potential inhibitors by the use of VLS. All compounds tested were predicted by VLS to be more potent inhibitors than sildenafil. Due to limited water solubility and the fact that precipitation was observed during the incubation period for some compounds, the results were not as predicted by VLS for all potential inhibitors. It was observed that four of the compounds were prone to precipitate, an thus the inhibiting effect for these were poorer than for sildenafil . All the other potential inhibitors showed a higher inhibition on cGMP transport than sildenafil.

# 8 FUTURE ASPECTS

The aims of this study were achieved, but there are still a lot of questions to be answered. First, it will be necessary to find out whether the compounds tested also prove to be inhibitors of PDE5, like sildenafil. The aim is to find inhibitors with selective effect on the ABCC5 transporter. Second, by the use of antibodies and Western Blot, try to find out more about which transporters the human erythrocyte contains. This may answer some questions about whether another transporter than ABCC5 is responsible for the low affinity transport of cGMP (see saturation study). Third, site directed mutagenesis studies with mutations in amino acids predicted from the molecular model to take part in ligand binding, and subsequent testing of vesicles prepared from transfected cells, may provide more knowledge about the predicted position of

the binding site(s) in the ABCC5 transporter. Fourth, testing the same inhibiting compounds on IOV prepared from other cell types from the perspective that they may be used in combination with chemotherapy may also be necessary.

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