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Characterization of inhibition by vardenafil analogues of ATP-dependent transport of cGMP by the ABCC5 transporter.

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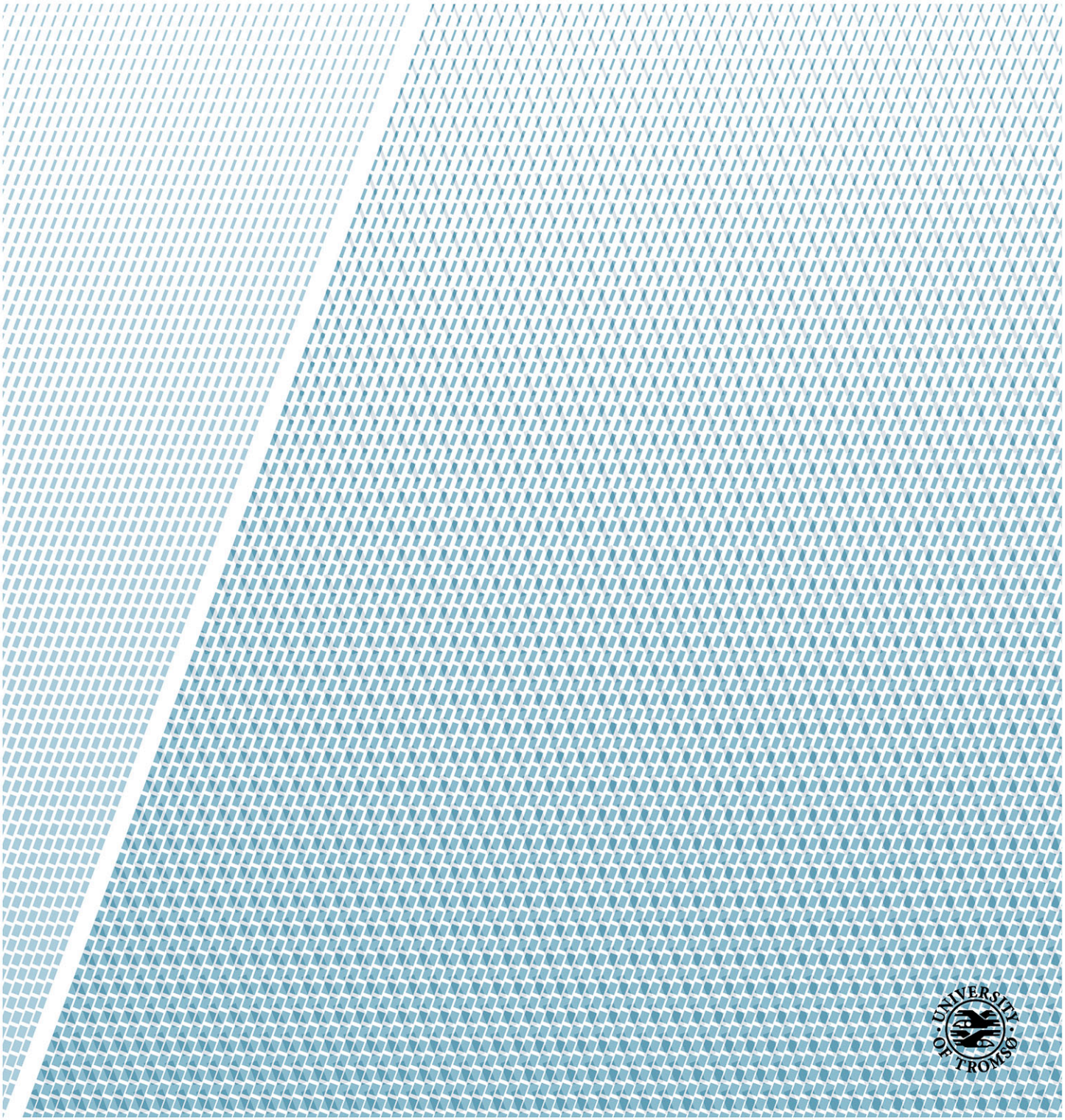

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Farzane Kuresh

ABBREVIATIONS

ABC	ATP-binding cassette
ABCC	ATP binding cassette, subfamily C
ABCC4	ATP binding cassette, subfamily C, member 4
ABCC5	ATP binding cassette, subfamily C, member 5
AchE	Acetylcholinesterase
BCRP	Breast cancer resistance protein (also known as ABCG2)
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
cN	Cyclic nucleotides
IOV	Inside out vesicles
MRD	Multi drug resistance
NBD	Nucleotide binding domain
NO	Nitric oxide
P-gp	P-glycoprotein (MDR1, ABCB1)
PDE	Phosphodiesterase
PDE5	Phosphodiesterase 5
PKA	Protein kinase A
PKG	Protein kinase G
RBC	Red blood cell
ROV	Right side vesicles
SUR	Sulfonylurea receptors
TMD	Transmembrane domain
VLS	Virtual ligand screening

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ABSTRACT

Background: Clinical studies have reported overexpression of PDE5 and elevation of intracellular cyclic GMP in various types of cancer cells.
ABCC5 transports intracellular cGMP out of the cells with high affinity, while PDE5 inhibitors prevent high affinity cGMP efflux by inhibiting ABCC5.
Increasing intracellular cGMP levels through inhibition of PDE5 and PDE5 export activity is hypothesized to promote apoptosis and growth restriction in tumor cells the tumor cells.
Vardenafil is a potent inhibitor of both PDE5 and ABCC5-mediated cGMP cellular efflux (Ki=3.4µl). Nineteen novel vardenafil analogs that have been predicted as potent inhibitors by VLS were chosen for tests of their ability to inhibit ATP- dependent transport of cGMP by measuring the accumulation of cyclic GMP in inside-out vesicles.

Aim: In this study, we investigated the ability of nineteen new compounds to inhibit ABCC5-mediated cGMP transport. We also determined the Ki values of the six most potent compounds.

Methods: Preparation of inside out vesicles and transport assay (12-well manifolds and 96-format assembly)

Results: Ki values for six of nineteen compounds that showed more than 50% inhibition of cGMP transport in the screening test were determined. Two of them were more potent than the positive control, sildenafil.

1 INTRODUCTION

1.1 Transport across cellular membranes

All cells depend on the movement of a broad array of molecules across their external (plasma) membranes for survival and normal physiological and developmental functions. Although some of this movement occurs through passive diffusion across the plasma membrane (e.g., water, steroid hormones), the movement of ions and most other polar or charged molecules depends on the presence of specialized membrane transport proteins, which can both facilitate and regulate the transport of specific molecules or classes of molecules across the plasma membrane, both in and out of the cell. Although eukaryotic cells also exchange molecules with their environment through endocytic and exocytic pathways, these pathways do not involve transport across the plasma membrane and largely serve different purposes than does membrane transport (1, 2).

Cell membranes are composed of lipid bilayers in which a large variety of proteins with different functions are associated or embedded, either as transmembrane proteins, which are in contact with both the extracellular intracellular (cytoplasmic) environments, or as membrane-associated proteins that are in contact with only one of the two. The lipid bilayer component of cell membranes is relatively impermeable to most biological molecules, a critical feature for the normal cellular function of cells, as it enables cells to carefully control their intracellular environments through the regulated influx and efflux of molecules. Lipid bilayers are permeable to varying degrees to small and nonpolar molecules (e.g., water and steroid hormones), but far less permeable to larger and, especially, more polar molecules. The lipid bilayer is especially impermeable to charged molecules, even small monoatomic ions, such as sodium and chloride ions (1, 2).

1.2 Membrane transporters

With the notable exception of some small, nonpolar molecules, such as oxygen, carbon dioxide, and steroid hormones, most molecules that traverse the plasma membrane – including nearly all polar and charged molecules – do so through specialized membrane transport proteins. Membrane transport proteins are multi-pass trans-membrane proteins that provide a passage through the hydrophobic interior of the membrane by creating a relatively hydrophilic channel or passageway for hydrophilic molecules. Transport of these molecules can be regulated, enabling cells to control the rate and direction of movement of molecules. Transport can be either passive, driven by a concentration or electrochemical gradient, or active, driven by coupling transport to ATP hydrolysis or another energy source, which allows transport to occur against concentration and electrical gradients. Membrane transport proteins enable cells to take up nutrients, such as sugars, and ions. They are also accountable for elimination of cellular waste, environmental toxins, and other xenobiotics, including drugs (3).

They are three major types of membrane transport protein: channels, passive transporters, and active transporters (4).

Membrane channels allow the passage of solutes through passive diffusion by providing a membrane-spanning channel. Movement through channels can be regulated through the opening and closing of the channel. Virtually all channels in eukaryotic plasma membranes serve to transport ions and may be referred to as ion channels. Ion channels are selective for the type of ion they transport, often allowing passage of only one type of ion or a very small set of closely-related ions. Ion channels function by opening transiently in response to a regulatory cue, which can be electrical or chemical.

Membrane transport proteins differ from channels in that they bind the solute to be transported and undergo a conformational change or changes in the process of transmitting the solute to the other side. Like ion channels, some membrane transport proteins allow passage of solutes without any requirement for energy input, and transport occurs passively from areas of high concentration to areas of low concentration. Passive transport is also sometimes referred to as facilitated transport (2).

Membrane transport that requires an energy input is termed active transport. Membrane transporters that mediate active transport physically couple the transport of the solute – along with the conformational changes in the transporter that must occur for this transport to occur – to one of two types of energy input – ATP hydrolysis or the co-transport of another solute along a concentration gradient which itself depends on ATP hydrolysis. Based on the nature of the coupled energy source, active transport can be divided into three main types: primary, secondary, and tertiary.

Primary active transport

In primary active transport, the membrane transporter is an ATPase, and the energy of ATP hydrolysis is coupled directly to the transport of solutes. One important and ubiquitous example of primary active transport is the pumping of sodium out of the cell, which is carried out by the sodium-potassium ATPase. The sodium-potassium ATPase couples ATP hydrolysis to the pumping of sodium ions out of the cell and potassium ions into the cell. In most cell types, the maintenance of high extracellular and low intracellular Na^+ is critical for numerous other energy- and voltage-dependent functions, including the active transport of solutes through the co-transport of Na^+ (see secondary active transport below).

Based on their structures, ATP-dependent transporters fall into three general classes as follows(5):

1) P-type transporters
2) F-type and V-type transporters
3) ATP binding cassette (ABC) transporters

Secondary and tertiary active transport

Secondary active transport differs from primary active transport in that transport of a solute in this case is physically coupled to the passive transport of another solute down a concentration or electrochemical gradient that itself was generated through ATP-dependent (primary) active transport. Transporters that mediate secondary active transport are not ATPases, but rather co-transporters. All known examples of secondary active transport are driven by ion gradients, and in the plasma membranes of animal cells, most secondary active transport is driven by the Na^+ gradient or the electrochemical gradient that is largely

determined by the Na^+ gradient. Thus, the Na-K ATPase indirectly drives numerous other active transport processes .

Some instances of active transport have been identified that depend on ion or other solute gradients that were generated through secondary active transport; these have been termed tertiary active transport, although mechanistically they are similar to secondary active transport.

1.3 ATP binding cassette (ABC) transporter

1.3.1 ABC transporters

ABC transporters are a large and functionally diverse class of ATP-dependent membrane transporters. They are among the largest and oldest superfamilies of related proteins, with representatives throughout the prokaryotic and eukaryotic world. They are found in all cells of all species, with multiple members of the family present in each cell type. Most members of this family that have been studied have been found mediate the active transport of solutes, but some members of the family act as regulated ion channels, not transporters, and some have non-transport functions.

As mentioned above, ABC transporters are primary active transporters. They are monomeric proteins structurally distinct from P-type transporters that represent the largest class of ATP-dependent transporters, mediating the active transport of a very large variety of molecules.

ABC transporters are responsible for the transport of a broad spectrum of molecules, including ions, lipids, sugars, polysaccharides, peptides, and proteins, etc. The transporters play a critical role in antibiotic and fungicide resistance. Mutation in human genes encoding ABC transporters causes diverse genetic disorders such as cystic fibroses, immune disorders, eye diseases, lipid disorders and skin diseases, etc. (6)

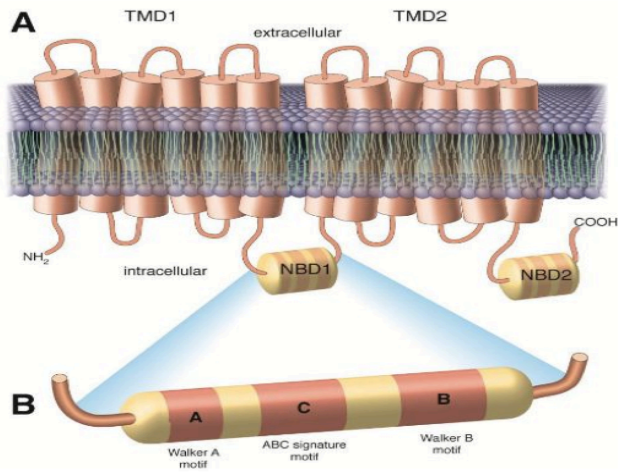
The physiological functions of ABC transporters in eukaryotes are still being examined. As the specificities of many of these molecules may be broad, their exact physiological significance may not be clear. However, it is clear that one of the general roles played by ABC transporters is in controlling the movement of molecules across important physiological barriers, such as the intestines, the blood-brain barrier, the placenta, and the active removal of foreign and toxic substances from cells and organs(7).

1.3.2 Structure, classification, and mechanism

To date, 49 ABC transporters have been identified in humans (8). These have been classified into seven families according to sequence homologies (ABCA through ABCG), with members of each family designated by a number (e.g., ABCA1, ABCA2, etc.).

The basic structure of ABC transporters includes four core domains: two trans-membrane domains (TMDs) and two nucleotide-binding domains (NBDs). These four domains can be present within one polypeptide chain, as so-called full transporters (e.g. ABCB1, ABCC4, ABCC5) or they can occur in two half transporters – each containing only two of the domains – that function as either homodimers or heterodimers (e.g.ABCG2/BCRP)(9) .

The NBD contains motifs; the Walker A and Walker B, the H loop and the Q loop, the ABC signature motif (also known as LSGGQ).
The TMDs contain polypeptide chains that span the membrane multiple times. Each trans-membrane domain typically forms six transmembrane alpha helics. Two TMDs creating a channel across membrane due to their pore like structure (6).



Figur 1. Structure of an ABC full transporter (Illustration by Roy Lysaa). (Skal prøve å tegne selv hvis jeg rekker)

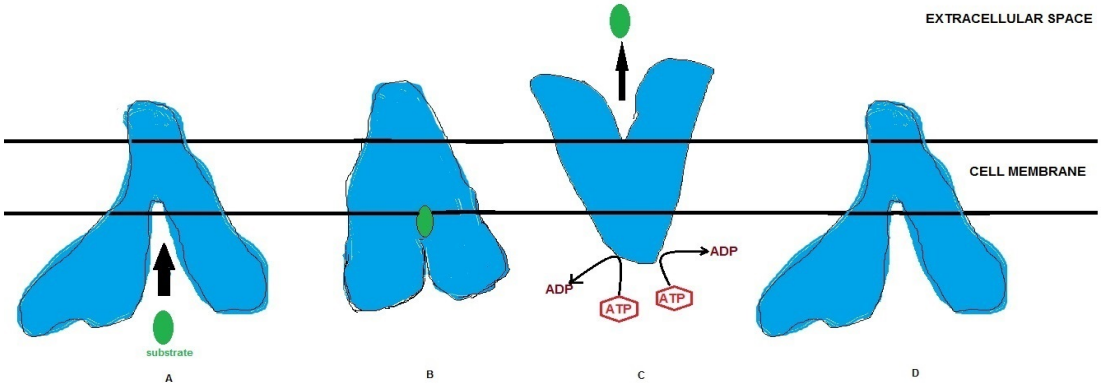
The pumping activity can be divided in several steps.

1) The ABC transporter recognizes the substrate. NMDs go through dimerization when substrate and ATP binds to the transporter.

2) The binding site changing direction from an outward to an inward facing conformation when ATP hydrolysis.

3) ATP hydrolysis dissociates the NBD dimmer and the binding site reoriented from an outward to an inward facing conformation and the substrate is transferred across the membrane to cytoplasm.

4) ADP and inorganic phosphate are released and ABC transporter returns to the initial state where TMDs back to the inward-facing conformation (10).



Figur 2. Illustration of a full transporter and ATPase activity.
A: Inward opening of the transporter domain. B: Conformational change caused by substrate binding. C: Separation of the membrane binding domains and release of substrate to the outward when binding of ATP. D: The transporter returns to the initial state when ATP hydrolysis.

1.3.3 ABC transporters in cancer multidrug resistance

The first identification of an ABC transporter occurred as a result of the appearance of multi-drug resistance among cancer patients receiving various types of cytostatic drugs. A protein originally referred to as P-glycoprotein (also known as MDR-1) was found to be expressed at higher levels in cells exhibiting multiple drug resistance. MDR-1 was eventually shown to be an ABC transporter capable of exporting multiple types of anti-cancer drugs out of the cytoplasm, and the phenomenon of multiple drug resistance in cancer was shown to be correlated with the selection of cells expressing higher levels of this protein. subsequently, another member of the ABC transporter superfamily – MRP – was also shown to mediate clinical cases of multiple drug resistance after cancer chemotherapy.

Since the identification of these two proteins as mediators of multiple drug resistance, there has been great interest in finding ways to overcome their effects and increase the effectiveness of anti-cancer drugs (11).

1.3.4 ATP binding cassette, subfamily C (ABCC)

The human ABCC subfamily can be referred to as the MRP (Multi resistance protein) transporters and all of the members are full transporters. ABCC subfamily consists of 13 members (see table1), which includes 10 MRPs (ABCC1-ABBC6 and ABCC10-ABCC7), ABC7 (also known as the cystic fibrosis trans-membrane conductance regulator, CFTR) and sulfonylurea receptors SUR1 (ABCC 8) and SUR2 (ABC 9). ABCC transporters play an important role in absorption, distribution and elimination of drugs due to their localization in intestinal and renal epithelial cells, blood tissue barriers and hepatocytes. They also protect tissue against the xenobiotic toxins (8).

Table 1. Human ATP binding cassette, subfamily C (ABCC), transporter genes and their functions, as listed in the HGNC database. (8)

Gene symbol	Protein	Chromosome location	Function
ABCC 1	MRP 1	16p13	Drug resistance
ABCC 2	MRP 2	10q24	Organic anion efflux
ABCC 3	MRP 3	17q22	Drug resistance
ABCC 4	MRP 4	13q31	Nucleoside transport
ABCC 5	MRP 5	3q27	Nucleoside transport
ABCC 6	MRP 6	16p13.11	Expressed primarily in liver and kidney
ABCC 7	CFTR	7q31.2	Chloride ion channel
ABCC 8	SUR1	11p15.1	Sulfonylurea receptor
ABCC 9	SUR2A	12p12.1	Encodes the regulatory SUR2A subunit of the cardiac K^+(ATP) channel
ABCC 10	MRP 10	6p12.3	Multidrug resistance
ABCC 11	MRP 11	16q12	Drug resistance in breast cancer
ABCC 12	MRP 12	16q12.1	Multidrug resistance
ABCC 13	MRP 13	21q11.2	Encodes a polypeptide of unknown function

1.4 Cyclic Nucleotides

Cyclic nucleotides (cNs) are found in all living organisms and play important roles in a wide range of biological functions, usually acting as second messengers in signal transduction pathways. Two cNs – cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) – are common in both prokaryotic and eukaryotic cells and have been extensively studied. Other, less common cNs may also function in some cell types, but these are less well understood ((12).

cAMP and cGMP are best known for their roles in mediating critical physiological processes. cAMP plays important roles in the regulation of metabolism, heart muscle contraction, among other things (13). Many of its important physiological functions are mediated through its regulation of protein kinase A (PKA). cGMP, on the other hand, is critically important in the regulation of vasodilation, affecting blood pressure, blood volume, and heart function, as well as in sensory transduction (e.g., vision), cell growth, neural plasticity, learning, and memory.

cAMP and cGMP levels in the cell or in particular subcellular locations are controlled by controlling the rate of synthesis, the rate of degradation, and the rate of export from the cell. cAMP and cGMP are synthesized from non-cyclic AMP and GMP through the action of specific nucleotide cyclases – adenylyl cyclases and guanylyl cyclases. In turn, they can be either degraded, through the actions of specific phosphodiesterases (PDEs), or exported, primarily through the actions of a subset of ABC transporters (14, 15).

Adenylyl cyclases (ACs) and guanylyl cyclases (GCs) can be either soluble (cytoplasmic) or membrane-associated (called "particulate" in older literature); membrane-associated and soluble nucleotide cyclases generally have different functions, responding to different upstream cues and generating cNs that function in different parts of the cell. Although there is specificity for one or the other mononucleotide by these cyclases, in some cases there is overlap in specificity, with a single cyclase being able to catalyze cyclization of either AMP or GMP, thereby potentially enabling crosstalk between the two systems at the level of cN synthesis (16).

GCs were the first enzymes to be shown to catalyze the production of a second messenger in response to ligand binding. They include seven membrane-associated receptors and two soluble, cytoplasmic forms. Membrane-associated GCs vary according to their tissue distribution and their ligand specificities. Among the ligands that can activate membrane-associated GCs are natriuretic peptides – which regulate the excretion of sodium in many cell types – cytokines, and small paracrine hormones, while the major ligand for soluble GCs is nitric oxide. Nitric oxide plays a critical role in the control of vasodilation; it stimulates cGMP production in response to various local physiological demands, such as the need for increased blood flow.

1.4.1 Cyclic guanosine monophosphate (cGMP)

cGMP was first identified in biological systems in rat urine, in 1963 (17). Early studies characterized the levels and biokinetics of cGMP in blood and urine, demonstrating that cGMP is commonly present in biological fluids and that exogenously administered cGMP is rapidly cleared by the kidneys (18). These were followed by discoveries that these extracellular levels of cGMP are altered in a number of pathological conditions, raising interest in the use of cGMP as a biomarker for disease and the potential role played by cGMP in disease. Studies on cGMP efflux in isolated organs and tissues first suggested the existence of a group of ATP-dependent transport molecules that could pump cGMP out of cells against a concentration gradient (14). The subsequent use of inside-out vesicles enabled detailed biochemical characterization of these efflux transporters and led to a more detailed understanding of their kinetics, mineral requirements, and specificities. These transporters were found to be members of the ATP-binding cassette family. One general finding of importance has been that specific inhibitors of phosphodiesterase 5 (PDE5) are also active against ATP-dependent efflux of cGMP. One of the major efflux transporters of cGMP is ABCC5, also known as multiple drug resistance protein 5 (MRP5). ABCC5 is upregulated in many types of cancer and mediates not only the efflux of cGMP, but also efflux of a wide variety of anticancer drugs. Inhibitors of PDE5, such as sildenafil, also show activity against ABCC5 transport activity and may be useful in ameliorating multiple drug resistance in cancer.

1.4.2 Cyclic adenosine monophosphate (cAMP)

cAMP was discovered in the 1950s as a novel compound produced in liver extracts in response to the addition of adrenalin or glucagon that was capable of stimulating glycogen breakdown into glucose (Corbin 2014 JBC). Sutherland showed that it was synthesized by an enzyme that came to be known as adenylyl cyclase and inactivated by the cleavage of its high energy phosphate bonds by enzymes that came to be called phosphodiesterases. This was the beginning of the biochemical understanding of cyclic nucleotide metabolism.

As described above and as for cGMP, cAMP levels are determined by the activities of nucleotide cyclases, phosphodiesterases, and efflux transporters. The major efflux transporter of cAMP is ABCC4, also known as MDR4.

1.4.3 Cyclic GMP in apoptosis and cancer

A potential relationship between cGMP levels and cancer has been suggested by many studies, although the nature of this relationship in many cases has remained unclear. However, there is more specific evidence to suggest an important therapeutic potential of targeting the enzymes and transport molecules that influence cellular cGMP levels. For one, cGMP is known to regulate a number of apoptosis-related genes, and cGMP levels in cells can influence the rate of apoptosis. Furthermore, the levels of both cGMP phosphodiesterases (PDE5) and cGMP exporters (ABCC5) have been found to increase in many cancer cell types, suggesting a selection for increased export and degradation of cGMP in cancer cells. Increasing intracellular cGMP levels through inhibition of phosphodiseterase and export activity is hypothesized to increase rates of apoptosis and thereby potentially serve to suppress tumor growth. Increased levels of cGMP may also have a more direct effect on proliferation of cancer cells (19).

1.5 Cyclic nucleotide phosphodiesterases

Cyclic nucleotide phosphodiesterase (PDE) activity was first described by Butcher and Sutherland in 1962 (20), shortly after the discovery of cAMP . Sutherland and his colleges determined that PDE activity is responsible for the hydrolysis of the 3' cyclic phosphodiester bond of cAMP to yield 5-AMP (21). Since then, a large number of cyclic nucleotide PDEs have been identified that primarily catalyze the hydrolysis of cAMP and cGMP.

Cyclic nucleotide PDEs fall into three broad classes based on structural similarities in their catalytic domains. Class I PDEs are the most studied and include the PDEs most commonly found to be critical in regulating specific cN levels in the cell in humans and other higher eukaryotes. Class II enzymes are less well studied and generally do not show substrate selectivity. Class III enzymes have only been found in bacteria and are structurally dissimilar to class I and class II enzymes (22) .

Class I PDEs that have been further categorized into 11 different PDE families (PDE1 to PDE11) based on their structural similarity, affinity for cAMP and cGMP, kinetic properties, intracellular localization, and sensitivity and response to inhibitors and effectors. Three of these families specifically hydrolyze cAMP (PDE4, PDE7, and PDE8), three selectively hydrolyze cGMP (PDE5, PDE6, and PDE9), and the remaining five can hydrolyze both cNs (23).

In most PDEs, including PDE5 family members, several protein domains occur N terminal to the catalytic domain that mediate various activities, including dimerization, cN binding, and recognition by kinases. One of these domains is the GAF domain; PDE gene families that have tandem GAF domains, recognized as the GAF-PDE subfamily, include PDE2, PDE5, PDE6, PDE10, and PDE 11. The known functions of GAF domains are to provide for dimerazation, to interact with regulatory proteins, and to interact with small ligands, such as cGMP and cAMP. Other PDEs (PDE1, PDE3, PDE4, PDE7, PDE8, PDE9) belong to the non-GAF-PDE subfamily that does not include GAF domains (24).

When cGMP binds to the allosteric site in the R domain, a conformational change

occurs, leading to phosphorylation of the enzyme. This causes an increase in the rate of cGMP hydrolysis by the catalytic domain. Hydrolysis of the 3' cyclic phosphodiester bond in cGMP leads to the production of 5 GMP, which has no second message activity. The allosteric cGMP binding sites and phosphorylation of a single serine by protein kinase G (PKG1) activates the PDE5 catalytic acitivity and result is a negative feedback regulation of cGMP/NO/PKG1 signaling (25).

A number of compounds with inhibitory activity toward PDEs were used for the treatment of specific medical conditions long before PDEs themselves had been identified. These included primarily three xanthines isolated from coffee beans: caffeine, theobromine and theophylline. In various combinations, these were used primarily for the treatment of asthma and cardiac insufficiency (26).

The first demonstration of inhibition of PDEs occurred in the late 1950s when caffeine (1, 3, 7-methylxanthine) was shown to specifically inhibit PDE activity toward cAMP in tissue extracts from liver, brain and heart (27). Later, Butcher and Sutherland discovered that theophylline and theobromine inhibit PDE activity and that theophylline was more potent than either caffeine or theobromine (20). However, these early PDE inhibitors were not selective for specific PDEs.

A chemist synthesized a collection of alkylxanthines to improve potency and selectivity of these compounds. As result, IBMX and IBMX analogs were produced that contributed as a valuable tool for studying cN signaling and defining characteristics of different PDEs (26).

As the PDE superfamily was characterized in detail and tests for inhibition of specific families or family members became possible, more specific PDE inhibitors were discovered. The most important of these for use in the clinic were the PDE5 inhibitors that were initially developed for use in the treatment of heart disease but which were first used clinically for the treatment of erectile dysfunction. These include sildenafil (brand name Viagra), vardenafil (brand name Levitra), and tadalafil (brand name Cialis). These were found to be highly specific for PDE5. These inhibitors act by virtue of their structural similarity to cGMP, blocking access of cGMP to the active site of PDE5 enzymes. Inhibition of PDE5 leads to increased cellular accumulation of cGMP and thereby biological effects that are cGMP dependent for a given cell type.

1.5.1 Therapeutic use of phosphodiesterase 5 inhibitors

Erectile dysfunction

PDE5A1 is highly abundant in the corpus cavernosum, where it hydrolyzes cGMP in smooth muscle cells. During sexual arousal, nitric oxide released from nerve terminals stimulates cGMP production, the accumulation of which leads to relaxation of smooth muscles and increased blood flow. Inhibition of PDE5 enables increased accumulation of cGMP and thereby increased blood flow into the corpus cavernosum (28).

Pulmonary arterial hypertension

Sildenafil is currently used for the treatment of pulmonary arterial hypertension. As in erectile dysfunction, the primary mechanism by which it improves the condition is through its effect on relaxation of smooth muscle cells in response to increased levels of cGMP(29).

Heart disease

The ability of PDE5 inhibition to increase local blood flow and affect other potential functions within the vasculature and the heart has been predicted to provide benefit to those suffering from both heart failure, in which expulsion of blood from the heart is insufficient, and survival of heart tissue after ischemic injury. In both cases, there is evidence that PDE5 inhibition can improve outcomes; in animal models it slows progression of heart failure and reduces the size and severity of heart damage, including necrosis, after heart attack. Some of the cardioprotective effects of PDE5 inhibition may depend on its known effects on smooth muscles, but some are also clearly related to other mechanisms that are not yet clearly identified (30).

Cancer

cGMP is known to promote apoptosis and slow cell growth, and PDE5 levels are elevated in many types of cancer (19, 30)This has led to the hypothesis that some cancer cells have selected for increased PDE5 levels as it confers a growth and survival advantage. Logically, then, it is postulated that inhibition of PDE5 in these cancer cells may have a therapeutic effect through promotion of apoptosis and suppression of growth. Several in vitro studies have demonstrated that PDE5 inhibitors can increase apoptosis and slow cell growth. Since PDE5 inhibitors are also able to inhibit the activity of the ABC transporter ABBC5, which is implicated in multiple drug resistance through its export of

many types of anticancer drug, PDE5 inhibitors could have secondary anticancer activity in the context of chemotherapy.

2 AIM OF THESIS

Nineteen new compounds were chosen as potential inhibitors of ABCC5 due to their structural similarity to vardenafil and their predicted high affinity for the ABCC5 transporter by molecular modeling and VLS. (Aina Ravna, Department of Medical Biology at UIT, personal communication).

We wish to test the ability of these nineteen compounds to inhibit ATP-dependent transport of cGMP was determined by measuring the accumulation of cyclic GMP in inside-out vesicles (IOVs). We also want to further investigate compound with the best inhibiting effect to determine their IC_{50} values and Ki values.

AIMS:

1) Test the ability to inhibit ATP dependent high affinity transport of cGMP of nineteen new compounds that are predicted as potential inhibitor by using VLS.

2) Determine whether a model with inside-out vesicles from human red blood cells could successfully be used to test potential inhibitors of cGMP transport via hRBC ABCC5.

3) Find the compounds that gave the best inhibiting effect (>50%) and characterize them with their IC50 and Ki values.

3 MATERIALS

3.1 Chemicals

Table 2. Overview of chemicals used in different methods

Chemical reagent	**Generic name**	**Manifacture/ Distributer**
ATCC	Acetylthiocholine chloride	Sigma
Triton-x100	polyethylene glycol p-(1,1,3,3tetramethylbutyl) phenyl ether	Sigma
KCl	Potassium chloride	Sigma Aldrich
Tris-HCl	Tris(hydroxymethyl) aminomethane hydrochloride	Merk
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid	Sigma Aldrich
Histodenz	**5**-(N-2,3-Dihydroxypropyl acetamido)-2,4,6-triiodo-N,N′-bis(2,3 dihydroxypropyl) isophthalamide	Sigma
ATP- Mg^{2+}	Adenosine 5′-triphosphate magnesium salt	Merk
[^{1}H]-cGMP	Guanosine 3',5'-cyclic phosphate	Sigma
[^{3}H]-cGMP		Sigma
K_2HPO_4-$3H_2O$	dipotassium hydrogen phosphate trihydrate	Merk

3.2 Overview of nineteen potential ABCC5 inhibitors (vardenafil analogs)

Table 3. Nineteen vardenafil analogs and sildenafil

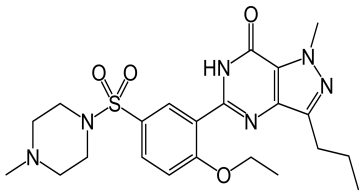
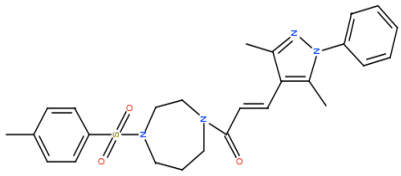
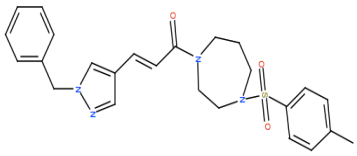
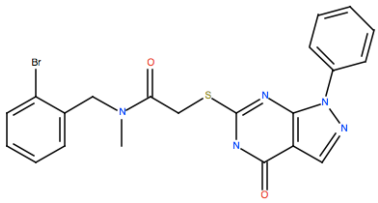
Compound	Formula and Molecular weight (g/mol)	Structure	Supplier and Compound ID
Sildenafil			
INH 1	**C26H30N4O3S** **478.60**		**Enamine** **Z131669058**
INH 2	**C25H28N4O3S** **464.57**		**Enamine** **Z131675110**
INH3	**C21H18BrN5O2S** **484.36**		**Enamine** **Z15383694**

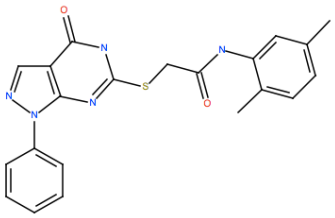
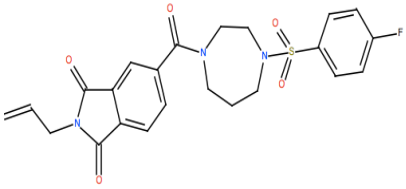
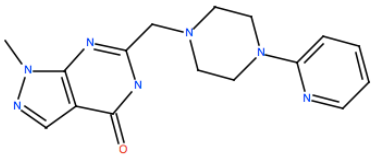
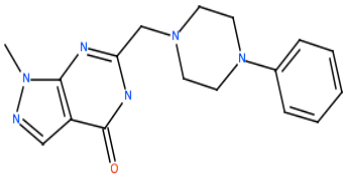
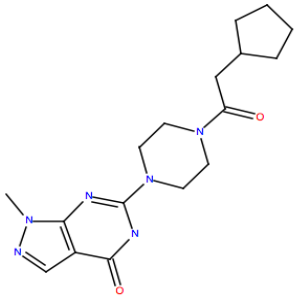
Table 3. *Continued*			
INH 4	**C21H19N5O2S** **405.47**		**Enamine** **Z15383727**
INH 5	**C23H22FN3O5S** **471.50**		**Enamine** **Z131699428**
INH 6	**C16H19N7O** **325.36**		**Enamine** **Z802694028**
INH 7	**C17H20N6O** **324.38**		**Enamine** **Z729878740**
INH 8	**C17H24N6O2** **346.41**		**Enamine** **Z1102995434**

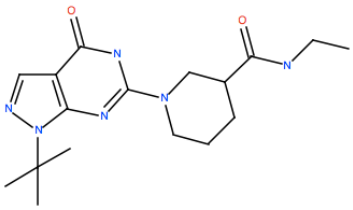
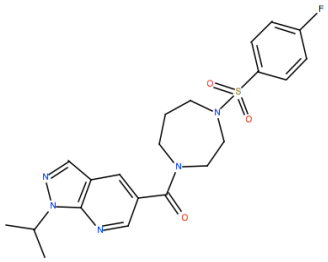
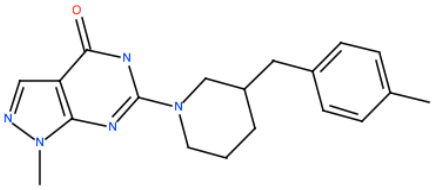
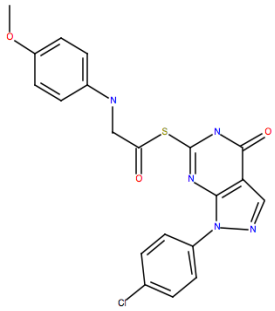
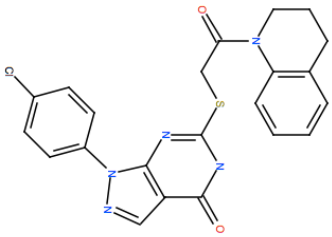
Table 3. *continued*			
INH 9	**C17H26N6O2** **346.42**		**Enamine** **Z1083966246**
INH 10	**C21H24FN5O3S** **445.51**		**Enamine** **Z218155582**
INH 11	**C19H23N5O** **337.41**		**Enamine** **Z1103000948**
INH 12	**C20H16CIN5O3S** **441.891**		**eMolecules** **C365-0139**
INH 13	**C22H18CIN5O2S** **451.929**		**eMolecules** **C365-0133**

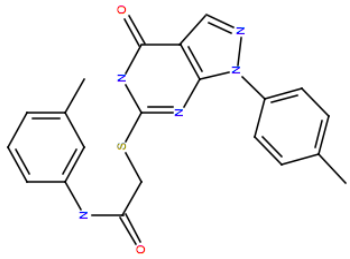
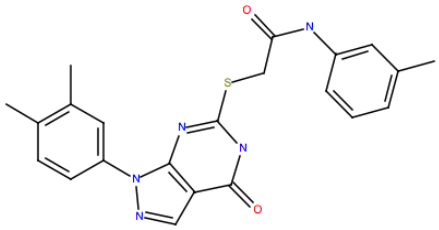
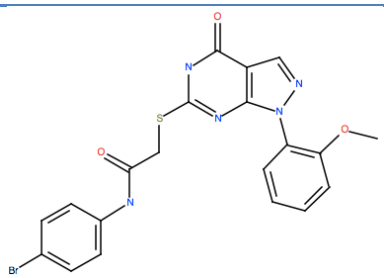
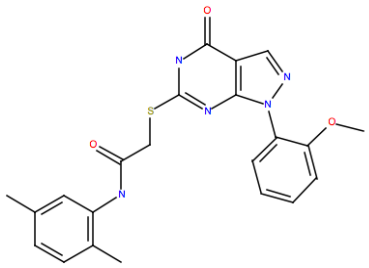
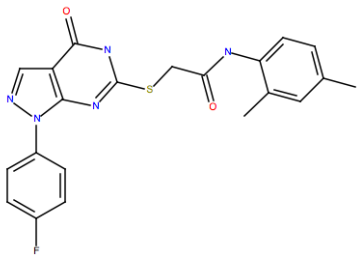
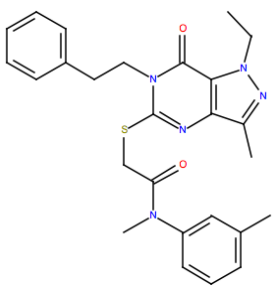
Table.3 *continued*			
INH 14	**C21H19N5O2S** **405.473**		**eMolecules** **C365-0215**
INH 15	**C22H21N5O2S** **419.499**		**eMolecules** **C365-0300**
INH 16	**C20H16BrN5O3S** **486.342**		**eMolecules** **G873-0200**
INH 17	**C22H21N5O3S** **435.499**		**eMolecules** **G873-0190**
INH 18	**C21H18FN5O2S** **423.463**		**eMolecules** **C099-0347**

Table.3 ***continued***

INH 19	**C26H29N5O2S** **475.606**		**eMolecules** **E960-0870**

3.3 Equipment

Table 4. List of equipment and instruments

Equitment	**Type/model**	**Manifacture/Distributer**
Analytic scale	PB3002	DeltaRange
Beth sonicator	Ultrasonic cleaner Model: USC100T	VWR
Centrifuge	Avanti J-26 XPI	Beckman Coulter
Centrifuge	5100	Kubota
Eppendorf – tube	Safe-lock tubes 1.5ml	Eppendorf AG,Germany
Filter	Nitrocellulose membrane 0.22 µm GSWP	Merk Millipore Ltd.
Liquid scintillation counter	Tri-Carb 1900 TR	Packard
Spectrophotometer	NanoDrop 2000c	Thermo Scientific
Spectrophotometer	Cary 60 UV-Vis	Agilent Technologies
Ultracentrifuge	Optima LE-80k	Beckman
Vaccum manifold		Millipore
Water purification system	Milli –Q plus	Millipore
TopCount Microplate		

3.4 Buffers, solutions and enzymes

Table 5. Buffer, solutions and enzymes

Method	Name	Composition
Preparation of inside-out vesicles from RBC	Buffer A (wash buffer)	5.0 mM Tris 113 mM K^+ 116 mM Cl^- Ad 1000 ml dH_2O pH 8.1
	Buffer B (hemolyzing buffer)	5.0 mM Tris 0.5 mM EGTA 4.0 mM K^+ 5.6 mM Cl^- Ad 1000 ml dH_2O pH 8.1
	Buffer C	0.5 mM Tris 0.2 mM Cl^- Ad 1000 ml dH_2O pH 8.2
	KPBS solution	9.57 mM Phosphate 157.7 mM K^+ 140 mM Cl^- Ad 1000 ml dH_2O

Buffers, solutions and enzymes *(continued)*

Separation gradients		5.0 mM Tris 3.6 mM K^+ 5.6 mM Cl^-
	Buffer D (27.6% Histodenz)	0.3 mM EGTA 0.3 336 mM Histodenz Ad 100 ml dH_2O pH 8.1 Density 1.146 g/ml
	Buffer E	5.0 mM Tris 173 mM K^+ 175 mM Cl^- 0.3 mM EGTA ad 100 ml dH_2O pH 8.1 Density 1.00 g/ml
	Gradient density #1	5.0 mM Tris 117 mM K^+ 119 mM Cl^- 0.3 mM EGTA 112 mM Histodenz pH 8.1 Density 1.048 g/ml
	Gradient density #2	5.0 mM Tris 60 mM K^+ 62 mM Cl^- 0.3 mM EGTA 224 mM Histodenz pH 8.1 Density 1.097 g/ml

Buffers, solutions and enzymes *(continued)*

Separation gradients *(continued)*	Gradient density #3	5.0 mM Tris 3.6 mM K^+ 5.6 mM Cl^- 0.3 mM EGTA 336 mM Histodenz pH 8.1 Density 1.146 g/ml
Transport assay	*Incubation solution 1* (with ATP) before mixing with vesicles and inhibitor solution	100.02 mM Tris 10.0 mM $MgCl_2$ 5.0 mM EGTA 9.02 µM [^{1}H]-cGMP 1.00 µM [^{3}H]-cGMP 504.2 mM K^+ 554.8 mM Cl^- 10.0 mM ATP
	Incubation solution 2 (without ATP) before mixing with vesicles and inhibitor solution	100.2 mM Tris 10.0 mM $MgCl_2$ 5.00 mM EGTA 9.02 µM [^{1}H]-cGMP 1.00 µM [^{3}H]-cGMP 504.1 mM K^+ 574.8 mM Cl^-

Buffers, solutions and enzymes *(continued)*

Transport assay *(continued)*	*Final incubation solution* (with ATP) after mixing with vesicles and inhibitor solution	20.00	mM Tris
		2.00	mM $MgCl_2$
		1.00	mM EGTA
		1.80	µM [^{1}H]-cGMP
		0.20	µM [^{3}H]-cGMP
		132.0	mM K^+
		139.0	mM Cl^-
		2.00	mM ATP
		X	mM Inhibitor solution
		(X is 1,0.1,0.01,0,001……0)	
		pH 8.00	
	Final incubation solution (without ATP) after mixing with vesicles and inhibitor solution	20.00	mM Tris
		2.00	mM MgCl2
		1.00	mM EGTA
		1.80	µM [1H]-cGMP
		0.20	µM [3H]-cGMP
		132.0	mM K+
		143.0	mM Cl^-
		X	mM inhibor solution
		(X is 1,0.1,0.01,0,001……0)	
		pH 8.00	

4 THEORY FOR METHODE

4.1 Preparation of inside-out vesicles (IOVs) from human erythrocytes

The two surfaces of cell membranes are functionally different due to their different ratios and types of amphipathic phospholipids, different membrane-associated proteins, and different orientations of proteins spanning the membrane. Closed membranous sacs – either sealed right side-out vesicles (ROV) or sealed impermeable IOVs can be prepared in order to chemically probe each face selectively (31).

ROVs are sealed membrane sacs that have the same sidedness as the original cell membranes (i.e., the extracellular face remains external). IOVs are sealed membrane sacs that have the opposite sidedness; the cytoplasmic side of the membrane faces outward and the original external surface faces the interior of the vesicles.

The method for preparation of IOVs used in this study was a modification of the original method by Steck et al., which was described in 1970 (32). Steck and his coworkers created of IOVs from erythrocytes by using low ionic strength and divalent cations. Linear density gradients were utilized to separate the inside-out vesicles from the right side out vesicles (32). IOVs have been used primarily to study efflux transporter activity, in particular that of ABC transporters (33).

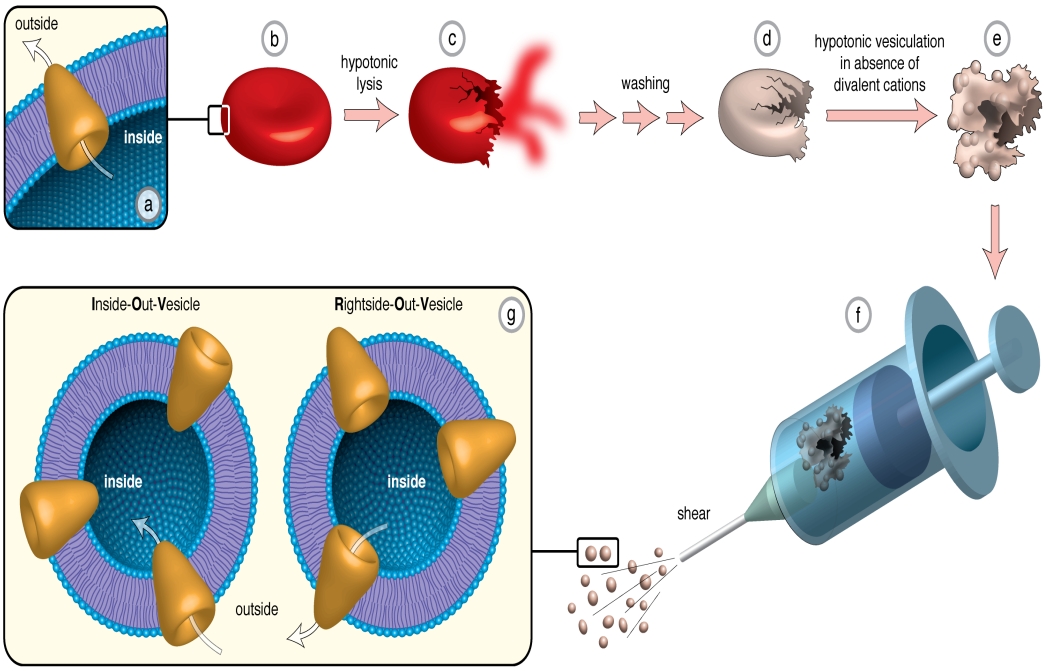


Figure 1: Illustration of the steps in IOV preparation from hRBC. (illustration by Roy Lysaa)
***A*: view of normal human erythrocytes membrane. *B*: normal shape of human erythrocyte. *C:* lysis of cell membrane by adding hypertonic solution for the removal of cell contents. *D:* Forming of erythrocytes ghosts when intracellular content and hemoglobin were removed after several hypotonic washing steps. *E*: Hypotonic vesiculation after incubation with very low ionic strength alkaline buffer. *F:* Shear applied by passing through thin cannula for homogenized sealed IOV. G: Enlarged view of an IOV and ROV.**

4.2 Sidedness test

The sidedness test method used was modified from the original method by Ellman et al., (1961)(34).This test can be utilized to detect the relative percentage of IOVs and ROVs in the prepared vesicle suspension by measuring acetylcholinesterase (AchE) accessibility.

Cell membrane proteins can be solubilized by detergents, which disrupt hydrophobic associations and destroy the bilayer. This causes membranes to become leaky. Detergents are small amphipathic molecules that contain hydrophobic tales and hydrophilic heads. The hydrophobic tales of these detergents displaces lipid molecules in the membrane since they binds to the hydrophobic regions of proteins. The hydrophilic polar heads of detergents can be either ionic or non-ionic. The detergent Triton X-100 has a nonionic, hydrophilic head and a hydrophilic polyethylene oxide tale (35).

The presence of large amounts of AchE in human red blood cells has been known since 1928 (36). AchE enzyme is anchored to the external side of cell membranes and is therefore present on the inner side of the IOVs and is not be available from the outside when IOVs are intact. The presence of detergent makes the IOV membranes leaky, and consequently gives substrates more access to the enzyme.

The relative percentage of ROVs and IOVs in the vesicle suspension was determined by measuring enzyme activity and comparing the levels in vesicles treated with detergent to those vesicles without detergent.

AchE catalyzes the conversion of acetylthiocholine into thiocholine and acetate. When thiocholine reacts with dithiobisnitrobenzoate ions, it produces a yellow color as an indicator. Enzyme activity can be determined by measuring absorbance at 412 nm. A UV-Vis spectrophotometer was used to measure absorbance.

The method is based on the following reactions:

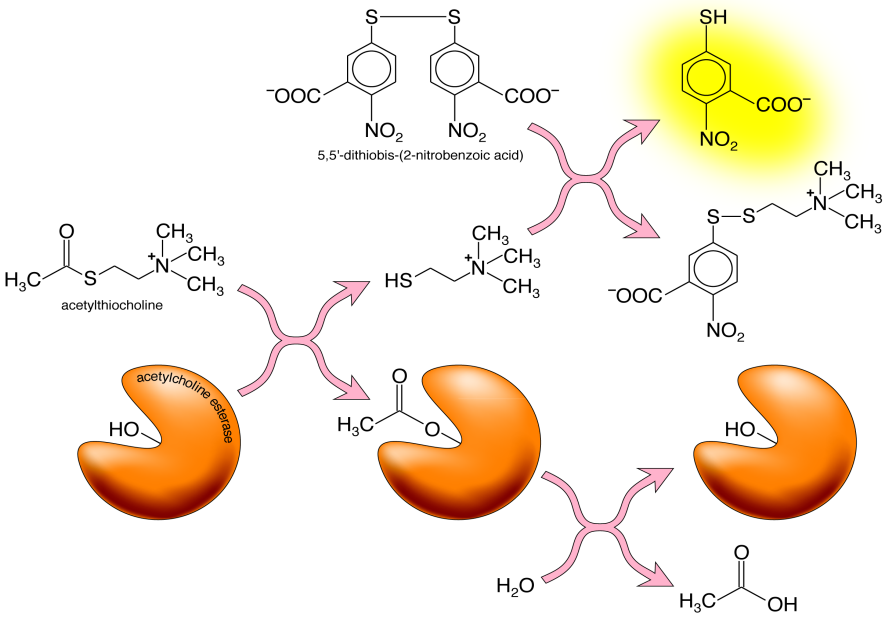


Figure 2: Diagram of acetylcholinesterase activity process. (Illustration by Roy Lysaa)
AchE dissociates acetylcholine into acetate and thiocholine. Thiocholine reacts non-enzymatically with 5,5'-dithiobis-(2-nitrobenzoic acid) and the strong yellow color, thionitrobenzoic acid, appears as a product.

4.3 Standardization of vesicle concentration

Determination of vesicle concentration by spectrophotometry

Protein concentration of vesicles can be measured by using a UV-Vis spectrophotometer at a wavelength of 420 nm. Protein concentration in the measured vesicle suspension is proportional with vesicle concentration. Purpose of measuring the protein concentration is to achieve the optimal concentration for vesicle to not clog the nitrocellulose filters with pore size 0.22 μm. The optimal concentration of vesicles, around 5mg/ml, can be achieved by dilution or concentration of the measured protein concentration.

Determination of vesicle concentration by hematocrit test

In this method, a hematocrit centrifuge was used to measure the fraction of true volumetric vesicle concentration. According to internal finding on the Medical Pharmacology and Toxicology research group, in the department of Medical Biology, 3% of total vesicle suspension volume is the preferred vesicle concentration in order to not clog the filter paper when using 96-well format assembly. To measure hematocrit % accurately, the suspension solution were concentrated 5 times more before measurement.

4.4 Transport Assay

4.4.1 General principles

Studies have shown that ABCC5 /MRP5 involved in extrusion of cGMP across the cell membrane against concentration gradients. (37)

Phosphodiesterases are enzymes that regulate the cellular level of cGMP. Inhibitors of phosphodiesterase 5 (PDE5) like sildenafil and vardenafil, have also been shown to be potent inhibitors of ABCC5-mediated cGMP cellular efflux (26).

Transport assays were utilized to test the above-mentioned nineteen vardenafil analogs for the cGMP efflux effect by measuring the cGMP activity in the IOVs. IOVs are suitable for biochemical and pharmacological characterization of cGMP transport since compounds will accumulate within the vesicles.

Radiolabeled cGMP uptake into IOVs can be determined by quantifying the radioactivity-retained filter.

Studies have shown that:

- The ideal pH for cGMP uptake into IOVs is pH 8,0 (38)
- The ideal concentration of Mg^{2+} is 10mM (39).
- The ideal concentration of ATP is 2mM (39).
- The ideal temperature for starting cGMP transport into IOVs is 37° and stopping transport is 0-4° (38).
- Ca^{2+} inhibits uptake of cGMP into IOVs (38). By adding 1mM of chelating agent EGTA, divalent cations are chelated. EGTA has more affinity for Ca^{2+} than Mg^{2+}. Therefore, the remained Ca^{2+} will be chelated while free Mg^{2+} will still exist during incubation.
- Na^{+} theoretically can have a positive effect on non ATP- dependent transport and consequently affect the result of the transport assay. Na+ was not included in any procedures in the present study.

In this study, the ICM program from Molsoft for VLS and Pharmacophore modeling were used to find potential inhibitors. Nineteen potential inhibitors were identified using ABCC5 as a target receptor. All of the compounds identified were vardenafil analogs (Aina Ravna, Department of Medical Biology at UIT, personal communication).

4.4.2 Screening test of potential inhibitors for cGMP transport

A screening test was used to get a coarse overview of the ability of compounds to inhibit ABBC5-mediated cGMP transport into IOVs. The screening test was performed on nineteen new compounds with the same concentration (10μM). Transport activity can be calculated by testing the compounds for cGMP transport including in the presence and absence of ATP. The transport of cGMP with no inhibitor present was considered as maximum transport (100%) into IOVs.

All the compounds were dissolved in purified water and diluted to achieve a concentration of 10μM (10-5M) before testing. The results of this test were used to calculate the amount of translocation of cGMP into IOVs as a percentage relative to the baseline. Sildenafil was used as positive control.

In this procedure, millipore water vacuum filtration sampling manifolds (12 well manifolds) and a liquid scintillation counter were used to perform the test.

4.4.3 Transport assay to find IC_{50} value

The six compounds that showed the most promising result from the screening test were chosen for find their IC50 values. Each of those compounds was diluted further to achieve the concentration range from 10^{-3} to 10^{-9}.

In this procedure, 96-format assembly was used for perform the transport assay. 96-format assembly was designed by Roy Andre Lysaa employs principles similar to those of a cell harvester. Sildenafil has been used as reference inhibitor.

A TopCount micro-plate scintillation counter was used to measure transport activity.

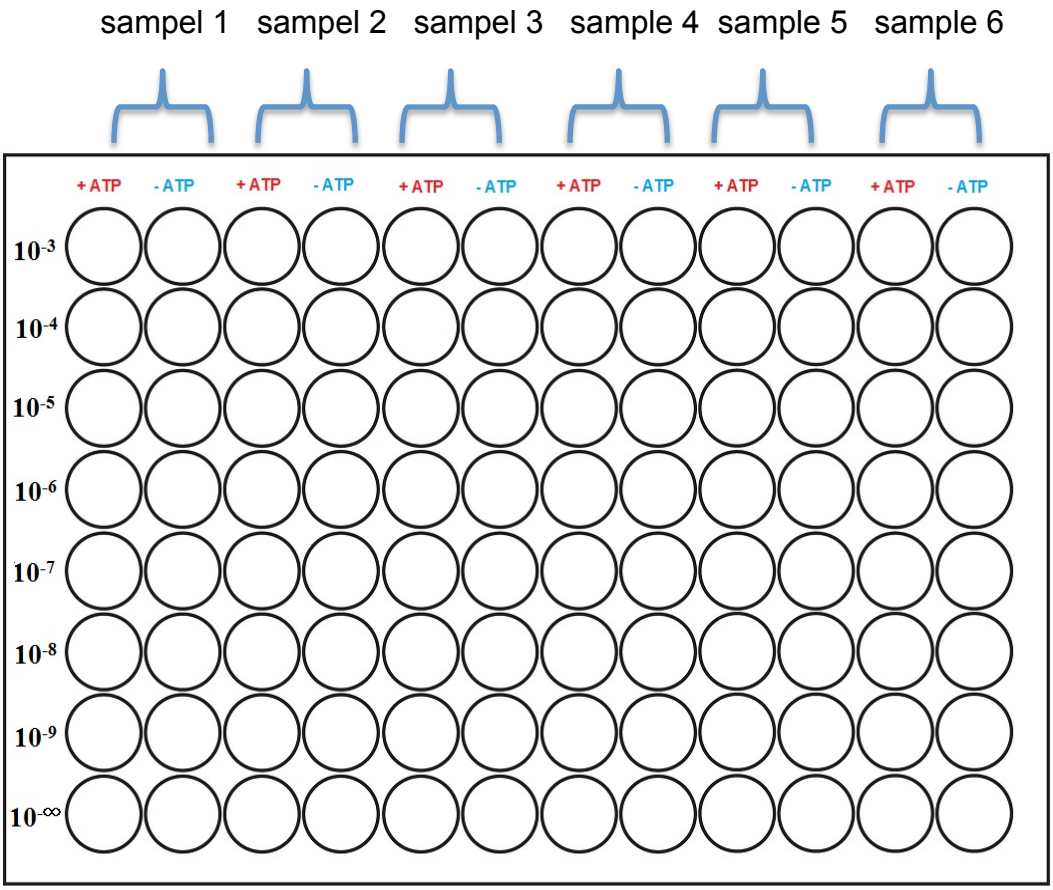


Figure 3. 96-deep-well plate. Each sample include 8 different concentrations(10-3 to 10-∞) with ATP and without ATP. 6 samples can be prepared in one plate at the same time.

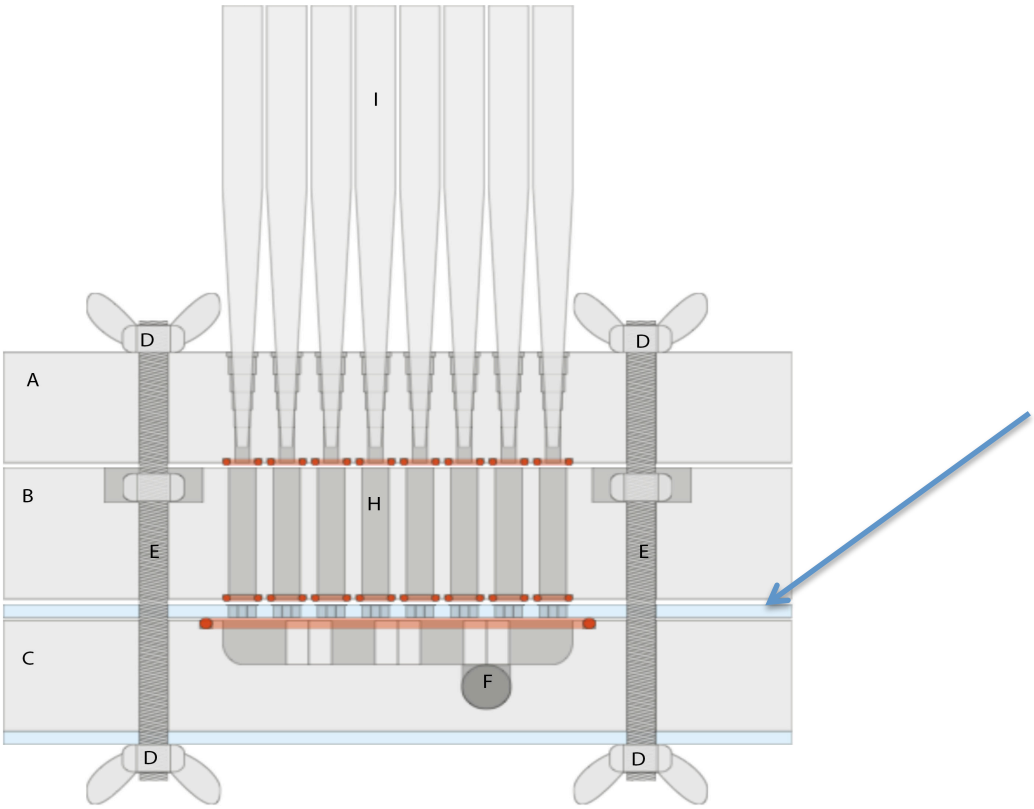


Figure 4: Illustration of 96-format assembly (illustration by Roy Lysaa) A: Top plate with pipette tips attached. B: Middle plate with 96 wells. C: Vacuum manifold that have metal plate on the top. D: wing screws for tighten the plates, one wing screw on each corner of the plate. 4 on the top and 4 on the bottom. E: Set screws, one for each corner of plates. Total 4 set screws. F: suction tube for the vacuum manifold pointing up on the paper sheet. H: 96 wells on the middle plate B that filled with samples. I: 1000 µl pipette tips attached to 96 holes on top plate A. Red colors represent rubber orrings that are placed on top of each hole. Arrow pointing at nitrocellulose filter 0.2 µm that is placed between the metal bottom C plate and middle plate B.

4.4.4 Calculation of half maximal inhibitory concentration (IC50) value

The IC50 value is the needed concentration of substrate or drug that inhibits a specific biological or biochemical activity by fifty percent

The IC50 value is estimated by measuring the cGMP translocation activity of ABCC5 transporters into IOVs against a range of dilutions of inhibitors

Linear regression can be used to calculate the IC50.The response-curve fits better to a straight line if the x-axis is logarithm-transformed when working with dose response data. In this study, the percent activity plotted as a function of the log inhibitor concentration.

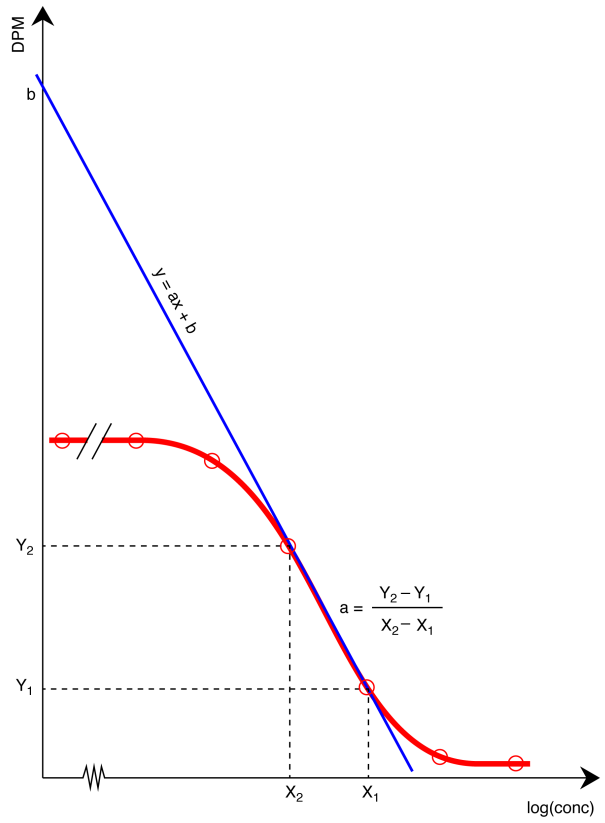


Figure 5: Sketch of the principles for determining the IC50 value.

b = intersection between line and second axis
a = slope ,
The equation for a straight line is : y = ax + b,
By inserting the right values in this equation IC50 value can be calculated.
IC50 = x-value that corresponds to the y-value that is half of the y-value at 100% activity.
Calculated X value when Y is 50% = IC50 value.

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

5 METHODS

5.1 Preparation of IOVs

5.1.1 Preparation of unsealed ghosts from Red Blood Cells (RBCs)

The fresh blood sampled in this method was collected in anticoagulant EDTA tubes from healthy human volunteers. Blood was maintained 0-4°C during all remaining steps.
Plasma and buffy coat were aspirated from vials after sedimentation in a swing bucket-rotor at 1000x g (2200 rpm, Kubota) for 15 minutes.
Cells were washed 3 times with 5 volumes of buffer *A* by centrifuging 10 minutes at 1000 x g (2601 rpm, Beckman Coulter, rotor JA-18) in slow brake mode. Each time, the supernatants and buffy coats were carefully aspirated.

40 volumes of buffer *B* were added to the cells, and cells were re-suspended well to initiate hemolysis of the RBCs. The suspension were then distributed to centrifuge vials. After centrifugation at 20000 x g (12859 rpm, Beckman Coulter, rotor JA-18) for 20 minutes without brake, the supernatants were removed by using an electrical pipette for large volumes and a plastic Pasteur pipette for small volumes. Care was taken to avoid disturbing the sediment, which included the fluffy membrane ghost pellet and a small hard protease button. Fluffy membranes were transferred to a new vial while taking care not to interfere with the hard protease button, which is rich in contaminating proteases. The fluffy ghost membranes were washed with buffer B until they turned milkey-white and supernatant was colorless.

5.1.2 Preparation of sealed vesicles

The milky-white ghost membranes were re-suspended in 39 volumes of *buffer C* to initiate vesiculation. The suspension were incubated in a refrigerated room of 0-4°C for 2-18 hours before membranes were pelleted by centrifugation at 47900 x g (Beckman Counter, rotor JA-18) for 20 minutes in weak brake mode. Supernatants were carefully removed and the sedimented pellets were transferred into a new vial after they were re-suspended in an appropriate volume of buffer C. The volumes depended on the amount of collected pellets and were usually around 300 µl of buffer C per vial.

Collected ghost pellets were homogenized by passing them 3 times through a No.27 G ¾, 0,4 x 19 mm cannula attached a 10 ml syringe.
By performing this process, vesicles were separated from unsealed ghosts.

Linear density gradients were used to separate IOVs from the ghosts and ROVs. Three different densities were prepared using buffer D diluted with buffer E:

	Density	Dilution	mM Histodenz
→	1,048 g/ml	1:2 (E:D)	112
→	1,097 g/ml	2:1 (E:D)	224
→	1,146 g/ml	Buffer E(undiluted)	336

Suitable amount of vesicles were layered on the top of gradients. Gradients containing vesicles were centrifuged in Beckman ultracentrifugation at 100 000 x g (rotor SW40 TI, 237000 rpm) for 2-18 hours at 0-4 °C.

The uppermost band was collected through the vial wall by using a thin needle and syringe. 40 volumes of KPBS were added the collected band, and the vesicles were washed by centrifuging at 28 000 x g for 30 minutes in order for remove the Histodenz. Supernatants were carefully removed and pellets were re-suspended in suitable volume of KPBS, around 1000µl.

5.2 Sidedness test

Sample solutions with the detergent Triton X-100 (SSX) and sample solution without detergent (SSO) were prepared in 1ml cuvettes/cells by mixing the following:

SSX	SSO
- 10 µl of vesicles	- 10 µl of vesicles
- 100 µl of FX	- 10 µl of FO
- 920 µl of incubation solution A	- 920 of incubation solution A
- 63 µl of ATCC	- 63 of ATCC

The absorbance of these solutions at 412 nm was set as zero.

SSX and SSO solutions were mixed well before starting measurements.

Absorbance at 1 and 3 minutes after the start of the reaction was measured for each sample solution with a spectrophotometer. Three samples were prepared in parallel for each condition.

Calculation % of IOVs

$$\%ROV = \frac{\Delta abs\ X3 - \Delta abs\ X1}{\Delta abs\ O3 - \Delta abs\ O1}\ x\ 100\%$$

% IOV = 100% - %ROV

Where;

$\Delta abs\ X3$ = *Absorbance with Triton measured 3 minutes after start*

$\Delta abs\ X1$ = *Absorbance with Triton measured 1 minute after start*

$\Delta abs\ O3$ = *Absorbance without Triton measured 3 minutes after start*

$\Delta abs\ O1$ = *Absorbance without Triton measured 1 minute after start*

5.3 Standardization of vesicle concentration

Determination of vesicle concentration by using spectrophotometry

NanoDrop 2000c wavelength of 420nm was used to determinate the protein concentrations of vesicle suspension.
All readings were taken using 2µl of solution placed on the microvolume pedestal of the spectrophotometer. KPBS, the dilution buffer, was used to define the zero concentration, and each sample was read three times.
The microvolume pedestal was cleaned with soft tissue paper before adding each new sample. After protein concentrations were determined, the vesicle suspensions were diluted with KPBS to a final protein concentration of 5-6 mg/ml.

Determination of vesicle concentration by using hematocrit

The fraction of the packed vesicles measured according to the following steps:

- 500µl of vesicle suspension was centrifuged at 13000 G for 1 minute in a microfuge tube.
- 400µl of supernatant was removed from the microfuge tube.
- The remaining 100µl vesicle pellet was re-suspended.
- The suspension was placed in a hematocrit tube and centrifuged for 5 minutes in a hematocrit centrifuge.
- The fraction of packed vesicles was measured.

The measured fraction of packed vesicles was multiplied by 5 to find out the concentration of the vesicle suspension. The vesicle suspension was diluted with KPBS to achieve the preferred vesicle concentration, which is 3%.

5.4 Transport Assay

5.4.1 Screening test of potential inhibitors for cGMP transport

Weighing and dissolution procedure

Sildenafil and nineteen test compounds were weighed using a milligram scale and transferred to TT vials. Purified water (Milli-Q) was added to each to a final concentration of 10µM. Particles were dissolved in water by shaking the vials. Ultrasound (ultrasonic clear) was used to crush remaining visible particles when necessary.

Preparation and incubation procedure

The frozen vesicle suspensions were thawed quickly in a 37°C water bath with gentle shaking and then placed on ice for 30 minutes prior to filtration. Vesicle suspensions were filtered and kept on ice.

Assay procedure

All samples were kept on ice except during incubation in a warm bath.

Incubation solution 1 (with ATP) and incubation solution 2 (without ATP) were prepared.

Total volumes used per sample (in one vial) were as follows:

- 50µl incubation solution 1 or incubation solution 2.
- 150µl inhibitor solution (10^{-5} M) or water.
- 50µl vesicles.

Transport started when vesicles were added after pre-incubation for 2.5 minutes in a water bath at 37°C. Vesicles were mixed well each time before adding to vials containing the incubation solution plus inhibitor solution.

Filter manifolds were prepared by wetting nitrocellulose filters, pore size 0.22 µm, with KPBS and placing in wells. After preparation, plates containing filter manifolds were stored in at 4°C.

Filtration procedure

This procedure was performed in a refrigerated room at 4°C.

After incubation, the transport reaction was stopped by adding 3ml of ice-cold KPBS to each incubation vial, and the suspensions were added to the filter wells on the vacuum manifolds. Any possible remaining incubation solution in the vial was flushed out with additional 3ml cold KPBS and applied to the same filter, making sure that filters did not dry before this addition. Filters were then rinsed 3 times with 2 ml cold KPBS

Scintillation procedure

This procedure was performed at room temperature for practical reasons.

After filtration, filters were removed from the manifolds, and each filter was transferred to one scintillation vial.

2ml of ethyl acetate was added to each vial to dissolve the filter.

10ml of scintillation liquid was added to each vial and mixed well by shaking before being placed in the scintillation machine for counting. Each experiment included 4 parallels with ATP and 3 parallells without ATP.

5.4.2 Transport assay to find IC_{50} value

The frozen vesicle suspensions were thawed quickly in a 37 °C water bath with shaking and then placed on ice for 30 minutes prior to filtration. Vesicle suspensions were filtered and standardized by using hematocrit before use.

Assay procedure

Two incubation solutions, solution 1 with ATP and solution 2 without ATP were prepared. Solution 1 was added to 96 well plate in column one and solution 2 was added in column 2.

Inhibitor solution in concentration range from 10^{-3} to 10^{-9} and $10^{-\infty}$ was added in both of the columns (See figure 3). At last, same amounts of the vesicle suspension were added in all the wells in both columns. For each experiment, there were four parallels for each concentration of inhibitor with ATP and 3 parallels for each concentration of

inhibitor without ATP. There were applied three experiments in different times for each inhibitor solution.

Same procedure has been applied for all of the six compounds and for sildenafil.

The final incubation concentrations were 2.00 mM Tris 2.0 mM MgCl, 1.0 mM EGTA, 1.80 μM [^{1}H]-cGMP, 0.20 μM [^{3}H]-cGMP, 132.0 mM K^{+}, 139.0 mM Cl and 2.0 ATP or no ATP, pH 8.0.

Filtration procedure

Filter paper was moistened by dipping into the KPBS solution before being placed on the metal plate.

The manifold (top plate with holes) was placed on top of the metal plate and screwed tight with a screw nut from the bottom of the metal plate.

KPBS was added to the wells of the manifold before samples were added.

400μl KPBS was added to the wells, followed by 200μl of sample.

600μl KPBS was added to the unused wells where no sample was added.

The total volume in each well in the filter manifold was 600μl.

Samples were added from the 96-well microplates to the wells in the filter manifold.

Each tube (2ml pipette) in the tube rack was filled with a small amount (~ 250μl) of KPBS using an 8-channel dispenser. The tube rack was then shaken gently once in a downward motion to get rid of air trapped in the bottom of the tubes. The tube rack was placed on top of the filter manifold.

Each tube was filled to the rim with KPBS by using an 8-channel dispenser, while avoiding air bubbles.

The vent to the vacuum manifold space was closed to start suction.

The tube rack was removed when all tubes were empty. The filter manifold was removed when all the wells were empty.

The filter was carefully removed from the metal plate and placed on the counting plate.

Counting procedure

The counting plate with filter was placed in the drying cabinet in 30 minutes.15ml of counting cocktail was added after counting plate was in the drying cabinet for 30 minutes at 37 °. Plastic was placed on top of the counting plate before counting with the counting machine.

6 RESULTS

6.1 Screening test of potential inhibitors for cGMP transport

Nineteen new compounds were chosen as potential inhibitors due to their structural similarity to vardenafil and their potential affinity to the ABCC5 transporter by molecular modeling and VLS.

In this test, a single concentration (10 µM) of the compounds,was tested for their ability to inhibit ATP dependent high affinity transport of cGMP uptake into IOV. Water without inhibitor was added to incubation solution to obtain the control transport level (100%) for cGMP. All compounds were tested included 3 parallels with ATP and without ATP. All results presented activity of cGMP transport in percentage, which correspond differences between cGMP accumulation in IOVs with ATP and without ATP (results with ATP – without ATP). Sildenafil was used as reference inhibitor. See fig.5.

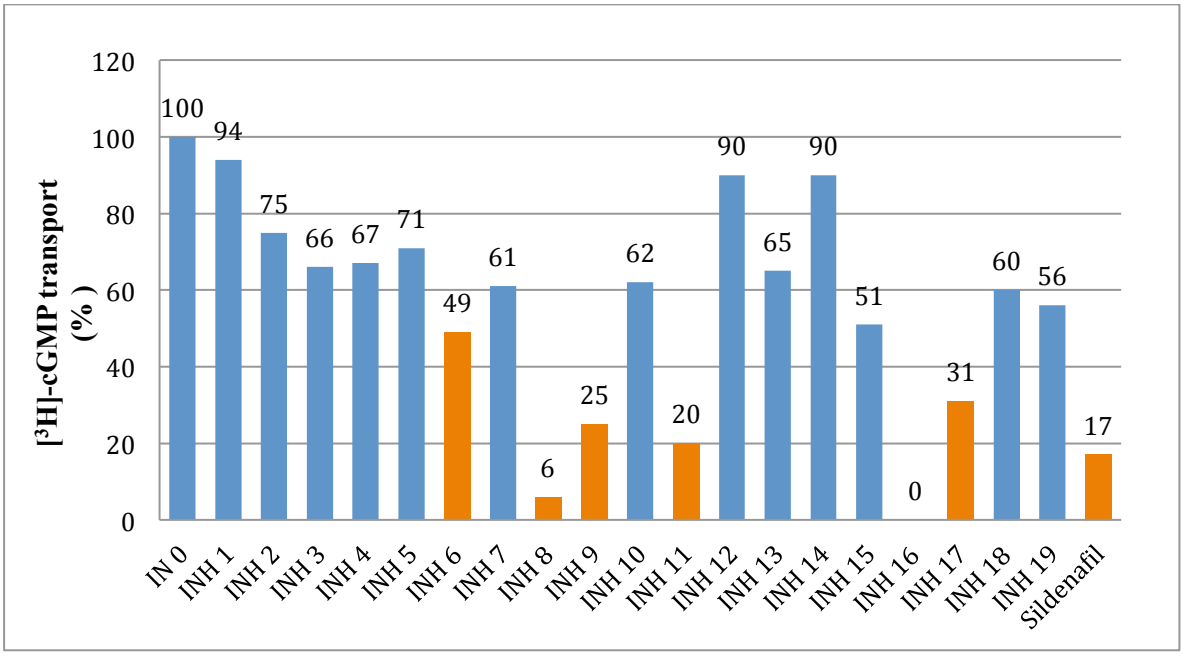


Figure 6. Transport activity of cGMP of nineteen vardenafil analogs and sildenafil into IOVs presented in percentage. Only differences between cGMP accumulation with and without ATP shown.

Six of those nineteen compounds inhibited more that 50%, which are INH 6 (51%) , INH 8 (94%), IN 9 (75%), INH 11 (80%), INH 16 (100%) , INH 17(69%). Sildenafil inhibited 83% (17% activity) of cGMP uptake. Two (INH 8 and INH 16) of nineteen compounds showed higher inhibition (%) then sildenafil in this test. INH 16 blocked the transporter completely.

Table 6. Six compounds showed more then 50% inhibition of cGMP uptake into IOVs.

Compound	Activity (%)	Inhibition (%)
INH 6	**49 %**	**51%**
INH 8	**6%**	**94%**
INH 9	**25%**	**75%**
INH 11	**20%**	**80%**
INH 16	**0%**	**100%**
INH 17	**31%**	**69%**
Sildenafil	**17%**	**83%**

6.2 Transport assay to find IC_{50} value

Six (see table 6) of nineteen compounds showed more than 50% inhibiting effect on the screening test were chosen to test further to calculate IC_{50} value. Their ability to inhibit ATP dependent [^{3}H]-cyclic nucleotide accumulation in IOVs was determined in concentration range from 10^{-3} to 10^{-9} M. 10-∞ M with no inhibitor added was used as maximum transport (100 % activity). The IC_{50} values were determined according to Chou (40) and transformed to Ki values according to Cheng and Prusoff (41). Substrate concentration was 2 µM for cGMP, and Km-value used was 2.6 µM that was based on earlier reported values by Orvoll et al., 2013 (42). Sildenafil was used as positive control. The results are presented as mean value ± SEM. IC50 value and Ki value for each compounds presented in table 7.

6.2.1 IC_{50}-curve for INH 6

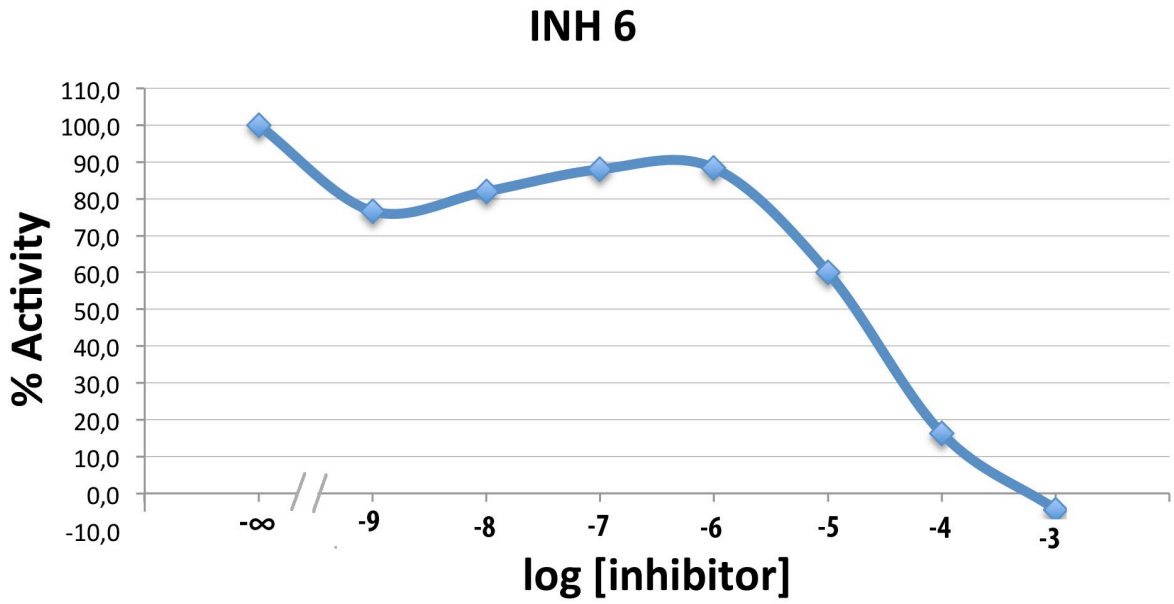


Figure 7. IC_{50}-curve for INH 6. Inhibition of ATP-dependent [^{3}H]-cyclic nucleotide accumulation in IOVs in the present of INH 6. Inhibitor concentrations are in the range between 10^{-3} and 10^{-9} M, and $10^{-\infty}$ M with no inhibitor added represent the maximum transport (100% activity). The activity presented is disintegration per minute (dpm) calculated to percentage. The results are presented as mean values ± SEM from three separate experiments.

6.2.2 IC_{50} curve for INH 8

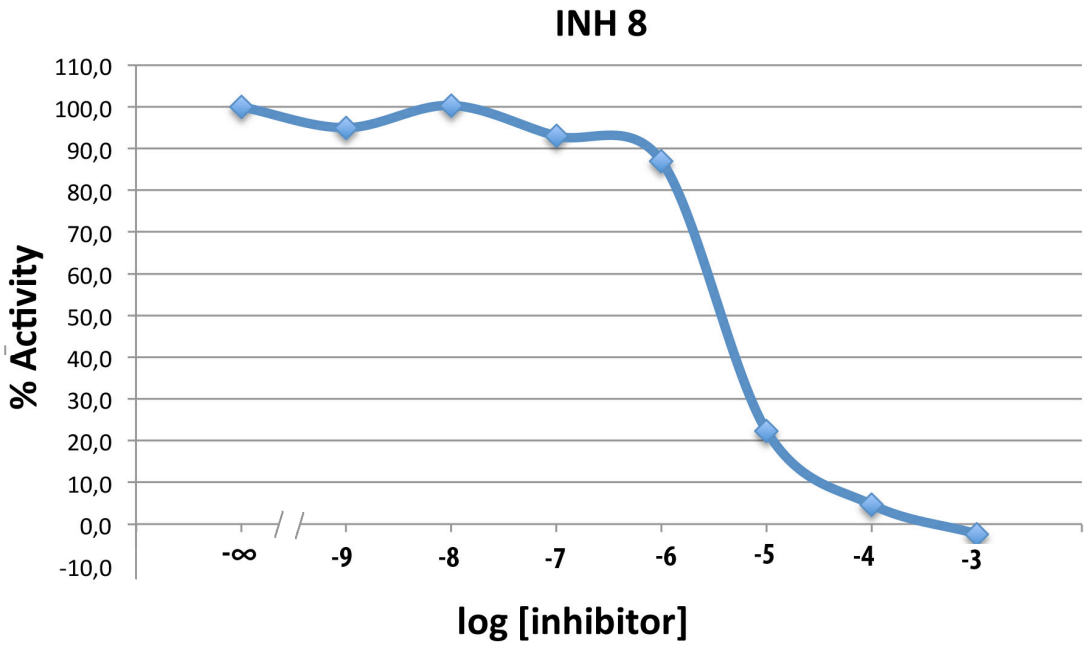


Figure 8. IC_{50}-curve for INH 8. Inhibition of ATP-dependent [^{3}H]-cyclic nucleotide accumulation in IOVs in the present INH 8. Inhibitor concentrations are in the range between 10^{-3} and 10^{-9} M , and $10^{-\infty}$ M with no inhibitor added represent the maximum transport (100% activity). The activity presented is disintegration per minute (dpm) calculated to percentage. The results are presented as mean values ± SEM from three separate experiments.

6.2.3 IC_{50}-curve for INH 9

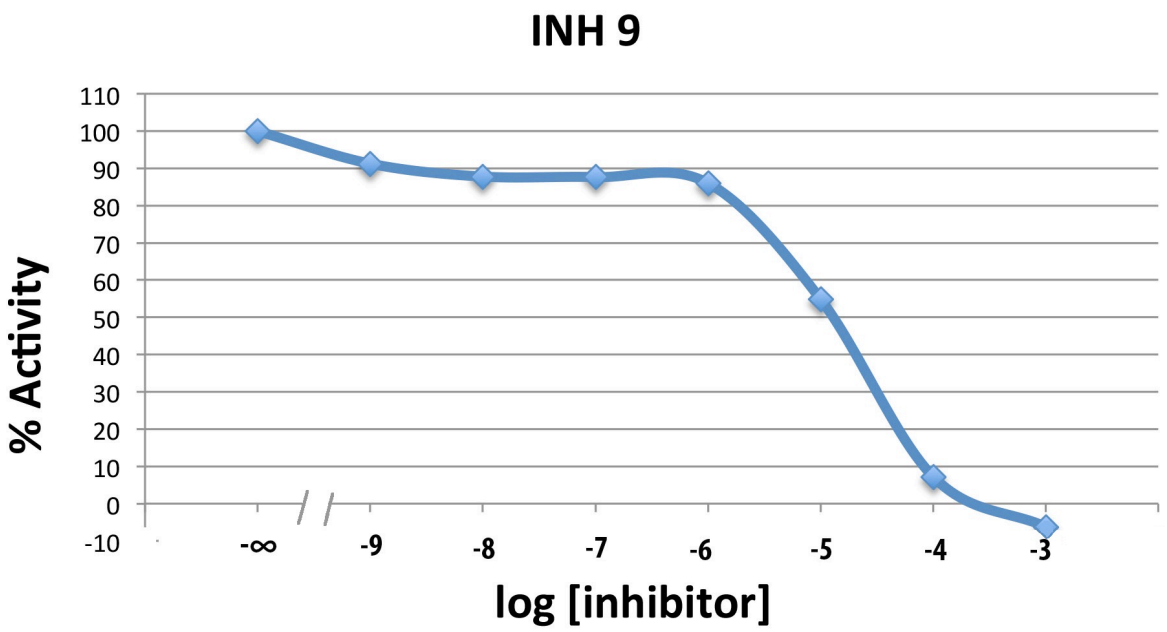


Figure 9. IC_{50}-curve for INH 9. Inhibition of ATP-dependent [^{3}H]-cyclic nucleotide accumulation in IOVs in the present INH 9. Inhibitor concentrations are in the range between 10^{-3} and 10^{-9} M , and $10^{-\infty}$ M with no inhibitor added represent the maximum transport (100% activity). The activity presented is disintegration per minute (dpm) calculated to percentage. The results are presented as mean values ± SEM from three separate experiments.

6.2.4 IC_{50}-curve for INH 11

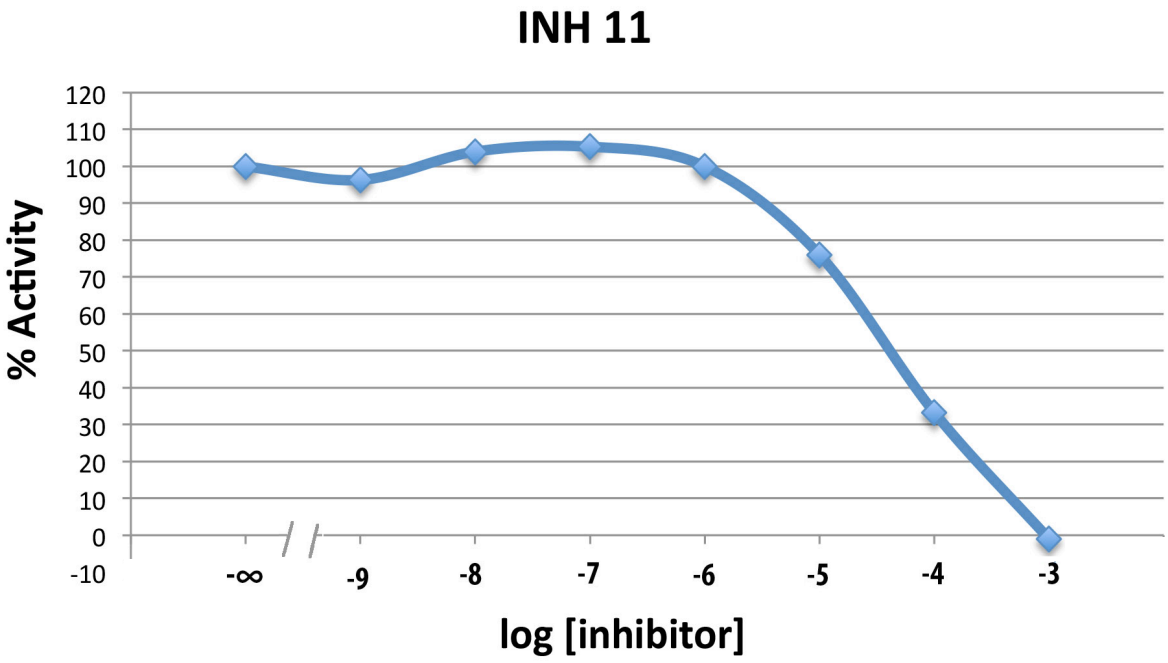


Figure 10. IC_{50}-curve for INH 11. Inhibition of ATP-dependent [^{3}H]-cyclic nucleotide accumulation in IOVs in the present INH 11. Inhibitor concentrations are in the range between 10^{-3} and 10^{-9} M , and $10^{-\infty}$ M with no inhibitor added represent the maximum transport (100% activity). The activity presented is disintegration per minute (dpm) calculated to percentage. The results are presented as mean values ± SEM from three separate experiments.

6.2.5 IC_{50}-curve for INH 16

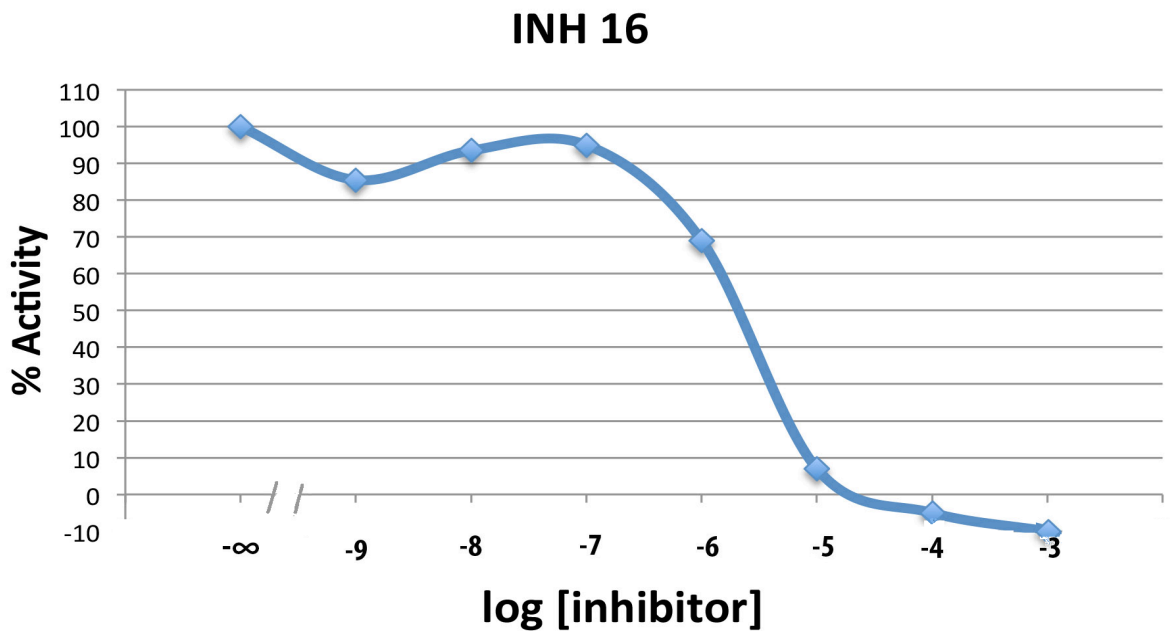


Figure 11. IC_{50}-curve for INH 16. Inhibition of ATP-dependent [^{3}H]-cyclic nucleotide accumulation in IOVs in the present INH 16. Inhibitor concentrations are in the range between 10^{-3} and 10^{-9} M, and $10^{-\infty}$ M with no inhibitor added represent the maximum transport (100% activity). The activity presented is disintegration per minute (dpm) calculated to percentage. The results are presented as mean values ± SEM from three separate experiments.

6.2.6 IC_{50}-curve for INH 17

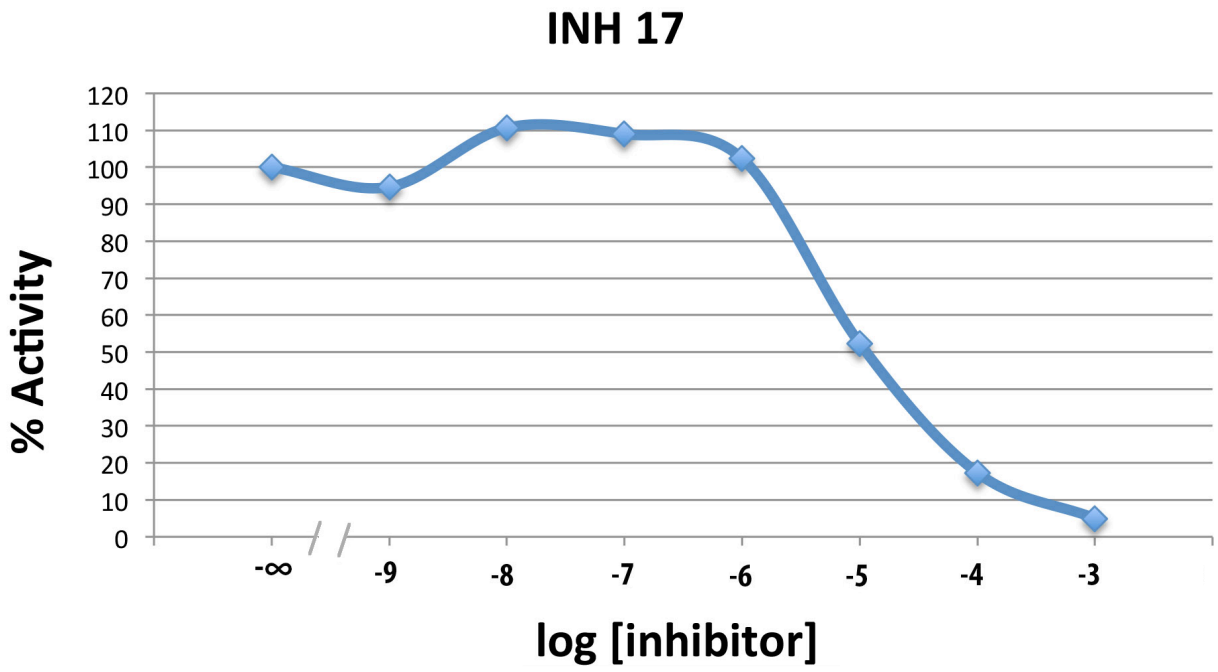


Figure 12. IC_{50}-curve for INH 17. Inhibition of ATP-dependent [^{3}H]-cyclic nucleotide accumulation in IOVs in the present INH 17. Inhibitor concentrations are in the range between 10^{-3} and 10^{-9} M, and $10^{-\infty}$ M with no inhibitor added represent the maximum transport (100% activity). The activity presented is disintegration per minute (dpm) calculated to percentage. The results are presented as mean values ± SEM from three separate experiments.

6.2.7 IC_{50}-curve for Sildenafil

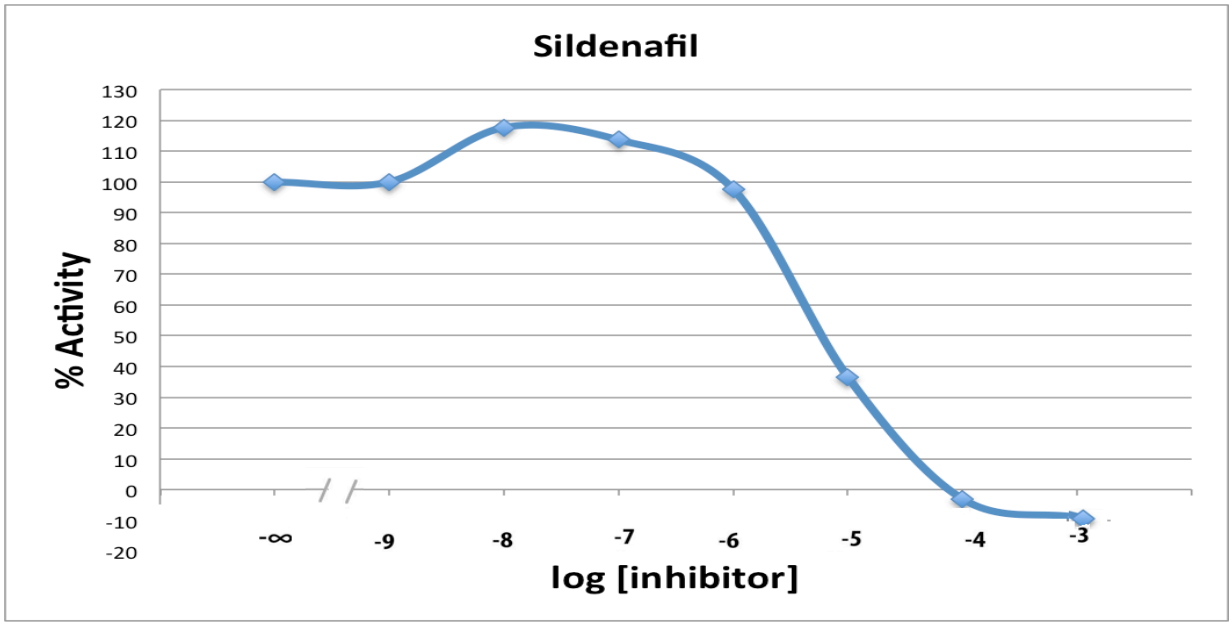


Figure 13. IC_{50}-curve for sildenafil. Inhibition of ATP-dependent [^{3}H]-cyclic nucleotide accumulation in IOVs in the present Sildenafil. Inhibitor concentrations are in the range between 10^{-3} and 10^{-9} M , and $10^{-\infty}$ M with no inhibitor added represent the maximum transport (100% activity). The activity presented is disintegration per minute (dpm) calculated to percentage. The results are presented as mean values ± SEM from three separate experiments.

Table 7. IC_{50}-values, Ki-values and SEM of six most potent inhibitors and sildenafil. Order of potency: INH 16 > INH 8 > Sildenafil > INH 9 > INH 17 >INH 6 > INH 11

Compound	IC50 value (µM)	SEM	Ki value
INH 6	18.0	1.9	10.1
INH 8	3.7	1.9	2.1
INH 9	13.2	1.5	7.5
INH 11	40.9	1.6	23.1
INH 16	2.0	1.3	1.1
INH 17	13.5	1.8	7.7
Sildenafil	6.4	1.9	3.6

7 DISCUSSION

Nineteen new compounds were chosen as potential inhibitors of ABCC5 due to their structural similarity to vardenafil and their predicted high affinity for the ABCC5 transporter by molecular modeling and VLS. (Aina Ravna, Department of Medical Biology at UIT, personal communication).

Even though computer programs are very helpful to theoretically predict potential ABCC5 inhibitors, the crystal structure may not be a realistic representation of the transporter. The protein is not in its natural environment during crystal structure determination, and yet it can undergo substantial conformational changes during the transport activity in vivo. By combining transporter modeling and in vitro studies, we can achieve a better understanding of the structure and function of these proteins.

In the present study, the ability of the nineteen compounds to inhibit ATP- and temperature-dependent transport of cGMP was determined by measuring the accumulation of cyclic GMP in inside-out vesicles (IOVs).

7.1 Preparation of inside-out vesicles and sidedness test

Human erythrocytes express ABCC4 and ABBC5 (37), and ABCC5 is a selective high affinity transporter for cGMP (43). The membrane is asymmetrical and has different features on its two surfaces that make it possible to study each face selectively. IOVs prepared from human red blood cells were suitable for our study of cGMP efflux and made it possible to control substrate and inhibitor concentrations.

The preparation of IOVs used in this study was a modification of the original method by Steck et al., 1970 (32). Some improvements have been made by the Medical Pharmacology and Toxicology research group, in the department of Medical Biology, UIT. The following improvements were used in this study in order to stabilize and increase the yield of the IOVs.

- Adding the right volume of hypertonic buffer C for vesicle formation, which requires an absolute hypotonic environment. It is important to completely remove the previously used isotonic buffer, buffer B, which can inhibit the formation of vesicles if any remains. To make sure that enough buffer C was added at this stage, 39 volumes or more of buffer C were added to one volume of ghosts.

- Avoiding adding divalent cations in the preparation of IOVs. Steck and Kant observed that divalent cations influenced the sidedness and sealing of the vesicles. It was important to incubate the ghost membranes in a hypertonic buffer with low ionic strength in the absence of divalent cations (31). They also showed that vesiculation occurred when the pH was between 7.7 and 8.7 (31). Given the fact that polypeptides are lost from the membrane when the pH is at its highest level, pH levels were kept between 8.0 and 8.2 in all solutions in this study, even though a previous study stated that the yield of IOVs increased in a more alkaline solution.

However, these improvements increased the yield of the IOVs. The amount of the yield and sidedness of IOVs varied widely from one batch to another. This variation could have several explanations.

- Homogenized sealed IOVs achieved by passing them through a thin cannula. The time in passage through the cannula varied from one batch to another; it was either 2 or 3 times. Homogenizing the sealed IOVs by passing them though a cannula 3 times gave a slightly higher yield of IOVs then 2 times.
- Gradients containing vesicles were centrifuged in an ultracentrifuge at 100 000 x g for 2-18 hours at 0-4 °C. The uppermost band was collected after centrifugation. It has been observed that 2 -3 hours of centrifugation gave more clear and visible bands in gradients; therefore, a greater amount of IOVs was collected from these runs. It was observed that the bands were diffuse and difficult to collect precisely after 16 - 18 hours of centrifugation and that made difficult to collect precisely only the uppermost band. The reason for this is not clear, but it might be explained by the fact that longer centrifugation times cause sedimentation that disturbs the two layers. Another possible explanation is that the gradients have disintegrated and made the density in the gradient vial homogeneous.
- Any possible contamination can change the pH level in buffers and can disturb IOV formation.

7.2 Transport assay

The ATP binding cassette family members ABCC4 and ABCC5 are anion transporters that have been identified as transport proteins for cyclic nucleotides (37, 44). Both ABCC4 and ABCC5 coexist in human erythrocytes (43).
We support the idea that ABCC5 has high affinity for cGMP and ABCC4 has high affinity for cAMP, but both ABCC4 and ABCC5 also transport the other cyclic nucleotide, albeit with low affinity. We believe that high affinity efflux of cGMP and cAMP is specific since they are unable to interact at physiologic concentrations (16).

The biokinetics of cGMP is can be described by three processes:
1. Synthesis; synthesis by guanylate cyclases
2. Degradation; conversion of cGMP to GMP by nucleotide phosphodiesterases (PDEs)
3. Efflux; excretion of unchanged cGMP by transport proteins in cell membranes (14).

cGMP is eliminated by PDE5 and ABCC5, and both have similar affinities for cGMP . Specific inhibitors of PDE5, such as sildenafil, have also been identified as potent inhibitors of ABCC5 (37). This inhibition prevents high affinity cGMP efflux (45).
Our aim is to find compounds that have a dual and balanced action. If the inhibition of PDE5 and ABCC5 is too high their physiologic function might be compromised.

7.2.1 Specificity

The term specificity is used to differentiate between members of the same family, including between members of the PDE family and members of the ABC transporter subfamily C.
Our initial goal was to test cGMP and cAMP transport inhibition by vardenafil analogs in order to compare their affinities and specificities for ABCC5. The ratio between Ki-values for inhibition of [^{3}H]-cGMP and [^{3}H]-cAMP transport explains the specificity of ABCC5 (46). Previous studies have reported that vardenafil (Ki-ratio = 0.18) had higher ABCC5 specificity then sildenafil (Ki-ratio = 0.45) (46).

In the present study, nineteen compounds were tested for their ability to inhibit ABCC5-mediated cGMP transport, and the Ki values for the six most potent

compounds among the nineteen were determined. Some of the compounds have also been tested for their ability to inhibit cAMP transport. These results are not included in this study since the available time for experiments was not sufficient to complete experiments. We could clearly see that these compounds had a tendency to inhibit cAMP transport, but fluctuations occurred. Possible reasons for the fluctuations are methodological problems and/or biological effects. However, the time available made it impossible to clarify this.

7.2.2 Solubility of compounds

All of the nineteen vardenafil analogs and sildenafil were dissolved in water in our study. According to the literature, vardenafil hydrochloride is very poorly dissolved in water and has a solubility of 0.11 mg/mL. For this reason, 0.1 M HCl was added to some of the compounds that contained amine groups in order to improve the solubility by increasing the polarity of the molecule. Howevere, the solubility of those compounds was not improved. Some of the compounds dissolved easily in water, while the non-dissolved visible particles for some of the compounds had to be crushed by using ultrasound. Even though, those suspensions were shaken well before use, variation in samples has to be taken in consideration. Sildenafil citrate dissolved well in water and it has solubility of 3.5 mg/mL.

7.2.3 Comparing the results from transport assay to find IC_{50} value

Six of the nineteen compounds exhibited more than 50% inhibition in the screening test. They were chosen to be further tested to determine their IC_{50} values. The potent inhibitor sildenafil was used as a positive control.

Previous studies had shown that vardenafil is a strong inhibitor of ABC-mediated cGMP transport, with a Ki value 0.62 µM (46), while different Ki values have been reported for sildenafil in different publications, including 0.24 µM, 3.6 µM, and 1.2 µM (37, 45, 47).

Two of the six compounds tested were more potent than sildenafil. Among all the tested compounds, INH 16, with a Ki-value of 1.1 ±1.3, was the most potent inhibitor of ATP-dependent cGMP transport. INH 8 was the second most potent inhibitor, with a Ki value of 2.1 ±1.9, while sildenafil was the next most potent inhibitor, with a Ki value of 3.6 ±1.9.

The order of potency in the [^{3}H]-cGMP transport assay for the six compounds that were tested is as follows (with Ki values and SEM) :

INH 16 (1.1 ± 1.3) > INH 8 (2.1 ± 1.9) > Sildenafil (3.6 ± 1.9) > INH 9 (5.7 ± 1.5) > INH 17 (7.7 ± 1.8) >INH 6 (10.1 ± 1.9) > INH 11 (23.1 ±1.6)
The calculated standard errors of the mean (SEM) for all of these inhibitors were very similar to each other, which made our results trustworthy.

The IC^{50} curves for these six inhibitors and sildenafil are described in figure 7-13. We could clearly see that they all had an initial plateau. Even though there are ups and downs on the plateau, it is possible to draw a straight line. Thereafter, the line declined steeply and then flattened out. The curves for all of the inhibitors, except INH 17, crossed the y-axis at the highest concentrations, which means under 0% transport. This theoretically impossible incident may be caused by methodological problems and/or biological effects.

7.2.4 96-format assembly versus 12-wells manifold

Water vacuum filtration sampling manifolds with 12-wells have long been used to separate IOVs from the incubation medium in transport assays. In this study, a 12-well manifold was used in the transport assay to test potential inhibitors (screening test). These manifolds gave reliable and good results and allowed us to filter up to twelve samples simultaneously.

A new filtration technology was developed in the Medical Pharmacology and Toxicology group, Department of Medical Biology, UIT, was utilized in this study for transport assays to find IC50 values.

Advantages of 96-format assembly compared to 12 well manifold

- The total volume of required solutions (incubation solution, inhibitor solution, vesicles) in transport assays are almost 5 fold less in the 96 -format assembly than in the 12 well manifolds. Less use of expensive solutions like radioactive solutions makes 96-format assembly more economical to use.
- 12 well manifolds can filter up to 12 samples, while the 96-assembly can filter up to 96 samples at once. Therefore, the 96-format assembly is a more time-saving technique.
- The 96-format assembly is a relatively simple and reproducible technique

Disadvantages of the 96-format assembly

- Due to manipulation of small amounts of solutions and vesicles, use of this technique can affect accuracy. Better accuracy can be achieved through training and experience.

8 CONCLUTION

In this study, the first aim was to test the ability to inhibit ATP dependent high affinity transport of cGMP of nineteen new compounds predicted to be inhibitors by VLS. Their ability to inhibit cGMP transport was determined by measuring the accumulation of cyclic GMP in inside-out vesicles (IOVs).
The percentage cGMP inhibitory activity for all the compounds varied widely. Only six of them showed more then 50% inhibition under the conditions of the assay.

The second aim was to determine whether an experimental model using inside-out vesicles from human red blood cells could successfully be used to test potential inhibitors of cGMP transport via ABCC5. The method for preparation of IOVs used in this study was a modification of the original method, which was described in 1970.
Even better results were achieved after some improvements were applied, including stabilization and increased yield of IOVs. Human erythrocytes express ABBC5, and due to membrane asymmetry, it is possible to study each face selectively. IOVs from human red blood cells (hRBC) were successful as tools to test potential inhibitors of cGMP transport via ABCC5, for the reason that it was possible to control substrate and inhibitor concentrations.

The third aim was to find the compounds that gave the best inhibiting effect (>50%) and to determine their IC50 and Ki values. As mentioned earlier, six of the compounds exhibited a high degree of inhibition in the screening test. Their IC_{50} values and Ki values were calculated. The order of potency in the [^{3}H]-cGMP transport assay for the six compounds that were tested is as follows (with Ki values and SEM) :
INH 16 (1.1 ± 1.3) > INH 8 (2.1 ± 1.9) > Sildenafil (3.6 ± 1.9) > INH 9 (5.7 ± 1.5) > INH 17 (7.7 ± 1.8) >INH 6 (10.1 ± 1.9) > INH 11 (23.1 ±1.6)

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