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Novel cGMP efflux inhibitors – Identified by virtual ligand screening (VLS) and confirmed by experimental studies

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Abstract

Elevated intracellular levels of cyclic guanosine monophosphate (cGMP) may induce apoptosis, and at least some cancer cells seem to escape this effect by increased efflux of cGMP, as clinical studies have shown that extracellular cGMP levels are elevated in various types of cancer. The human ATP binding cassette (ABC) transporter ABCC5 transports cGMP out of cells, and inhibition of ABCC5 may have cytotoxic effects. Sildenafil inhibits cGMP efflux by binding to ABCC5, and in order to search for potential novel ABCC5 inhibitors, we have identified sildenafil derivatives using structural and computational guidance and tested them for the cGMP efflux effect. Eleven compounds from virtual ligand screening (VLS) were tested *in vitro*, using inside-out vesicles (IOV), for inhibition of cGMP efflux. 7 of 11 compounds predicted by VLS to bind to ABCC5 were more potent than sildenafil, and the two most potent showed K_i-values of 50 #x02013;100 nM.

Introduction

Clinical studies have shown that extracellular cyclic guanosine monophosphate (cGMP) levels are elevated in various types of cancer.^{1–4} The human ATP binding cassette (ABC) transporter ABCC5 transports cGMP out of cells,⁵ and the increased cGMP efflux from cancer cells may be caused by an up regulation of ABCC5. cGMP regulates several apoptosis-associated genes, and intracellular elevation of cGMP concentration by inhibition of ABCC5 efflux may give cytotoxic effects.⁶

ABCC5 belong to the ABC transporters, which are structurally related membrane proteins featuring intracellular motifs that exhibit ATPase activity.⁷ This nucleotide binding domain (NBD), which contains the Walker A and Walker B motifs, cleaves ATP's terminal phosphate to energize the transport of substrate molecules against a concentration gradient. ABC genes are highly conserved within species, indicating that these genes may have been

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present since the beginning of eukaryotic evolution.^{7, 8} The overall topology of ABCC5 is divided into transmembrane domain (TMD) 1-NBD1-TMD2-NBD2.

No X-ray crystal structure of ABCC5 has been reported, but molecular models of ABCC5 may be constructed by homology using a known 3D crystal structure of an evolutionary related protein as a template. Docking, which is a method that suggests the preferred orientation of a drug molecule in the binding site of a drug target, can be used to predict the binding poses of substrates, known inhibitors and drug candidates to ABCC5, while the subsequent process of scoring can help rank-order them. In virtual ligand screening (VLS), drug candidates from compound databases can be selected using computer programs to theoretically predict whether they bind to ABCC5 using an ABCC5 model.

We have previously presented models of ABCB1,^{9, 10} ABCC4^{10, 11} and ABCC5^{10, 12} in outward-facing conformations and inward-facing conformations based on the *Staphylococcus aureus* ABC transporter Sav1866,¹³ which has been crystallized in an outward-facing ATP-bound state, and on the *Escherichia coli* MsbA, which has been crystallized in a wide open inward-facing conformation.¹⁴ Here we present a homology model of ABCC5 based on the X-ray crystal structure of the *Mus musculus* ABCB1 in a drug-bound conformation.¹⁵ ABCC5 efflux is inhibited by the phosphodiesterase 5 (PDE5) inhibitor sildenafil,⁵ and in order to search for potential ABCC5 inhibitors, the ABCC5 model was used for VLS of sildenafil derivatives. The template resolution was low (4.4 Å), and the amino acid sequence identity between *Mus musculus* ABCB1 and the human ABCC5 is 24%, clearly implying that the ABCC5 homology model has elements of uncertainty.

The aim of this study was to use structural and computational guidance to create chemical diversity around sildenafil and test it for the cGMP efflux effect. The methodologies of ligand-based drug design, which relies on knowledge of other molecules that bind to the biological target of interest, and structure-based drug design, which is based on knowledge about the three dimensional structure of the biological target, were combined, searching for sildenafil analogues in databases and using the ABCC5 model as an additional filter to select compounds to test for binding to ABCC5 *in vitro*. Eleven compounds from the VLS, that were selected based on score and drug likeness, were tested *in vitro* for a modulation of ABCC5 activity.

Results

In silico

The energy minimized ABCC5 model is shown in Figure 1. The model was in an inwardfacing conformation with the NBDs separated by approximately 18 Å. A large internal cavity open to the cytoplasm was formed by two transmembrane helix (TMH) bundles; TMHs 1, 2, 3, 6, 10, 11 and TMHs 4, 5, 7, 8, 9, 12. Key amino acid residues located in the large internal putative drug binding cavity included Gln190 (TMH1), Val411 (TMH5), Asn441, Thr444 and Lys448 (TMH6), Ser872 (TMH7), and Gln1138 (TMH12). The Walker A motifs consisted of a coiled loop and a short α -helix (P-loop), and the Walker B motifs were in β -sheet conformation and localized in the NBD's hydrophobic cores, which

were constituted of 5 parallel β -sheets and 1 anti-parallel β -sheet. The loop connecting NBD1 and TMD2 of the model featured 3 α -helices in the region between His767-Val817, while the region between Lys818-Val841 it was in an extended conformation. However, it should be kept in mind that this loop was not present in the template, and that modeling loops of this lengths is relatively inaccurate and consequently the modeled loop structures must be regarded as uncertain. The loop is approximately 40 Å from the binding pocket, and accordingly, the inclusion of this loop may not be necessary for the purpose of this study.

The Errat option of the Structural Analysis and Verification Server (SAVES) <http://nihserver.mbi.ucla.edu/SAVES/> reported that the overall quality factor of the ABCC5 model was 91.7, and a value above 90 indicates a good model. According to the Ramachandran plot provided by the Procheck option, 80.1% of the ABCC5 residues were in the most favored regions, 14.8% were in additional allowed regions, 2.5% were in generously allowed regions, and 2.6% were in disallowed regions. The summary of the Whatcheck option reported that the ABCC5 model was satisfactory.

The best docking score of the known binders was -29.5 kcal/mol, and this value was used as a threshold score for the VLS. Figure 2 shows sildenafil docked into the binding site of ABCC5. 30 compounds in the VLS had a better score than the threshold score of -29.5 kcal/mol, the best one being -37.9 kcal/mol. Table 1 shows the potential ABCC5 inhibitors from the VLS that were ordered from Ambinter. Docking revealed a tendency where the guanine-like moiety of the ligands interacted with Gln190 (TMH1) of ABCC5. Of the three binding site conformations included in the 4D docking procedure, the two conformations with the lowest energies were the conformations generally preferred by the ligands.

In vitro

A single concentration (32 μ M) of the compounds shown in Figure 3 were tested for their ability to inhibit [3 H]-cGMP uptake into inside-out vesicles (IOV). Sildenafil was introduced as a reference inhibitor, and the effect of the well-known PDE5 inhibitors (zaprinast and dipyridamole) were also determined. Figure 3 shows that sildenafil inhibited 85% of cGMP uptake. The corresponding values for zaprinast and dipyridamole were approximately 95%. However, several of the compounds brought forward by VLS showed even higher potency than sildenafil. Compounds **1** and **2** blocked the transport completely, whereas compounds **3**, **4**, **5**, **6** and **7** inhibited transport more effectively than the starting point molecule sildenafil.

K_i -values of the most potent inhibitors of cGMP transport were calculated, and while Sildenafil, which was used as reference inhibitor, showed a K_i -value of 1200 ± 170 nM, the corresponding values of compounds **1** and **2** were 75.3 ± 3.1 nM and 65.3 ± 6.4 nM, respectively.

Discussion

In this study, molecular modeling techniques were used for construction of an ABCC5 model, and for identification of its interactions with sildenafil derivatives. Molecular modeling and docking may help to elucidate molecular interactions between drugs and drug targets,

aiding the search to understand the intermolecular forces involved in determining the potency and the specificity of the drug. However, homology modeling of transporters must be regarded with caution; there are few templates available, if any, and they often have low resolution, and homology between the target transporter and the template may also be low. The sequence identity between target and template in the present study was relatively low (24%), so even though a multiple sequence alignment, which highlights evolutionary relationships and increases the probability that corresponding sequence positions are correctly aligned, was used to guide the homology modeling procedure, the low homology gives elements of uncertainty to the resulting model. Homology modeling of membrane transporters often implies low homology; the sequence identity between the frequently used template *Aquifex aeolicus* leucine transporter LeuT_{Aa} and modeled monoamine transporters is ~20%,¹⁶ and the sequence identities in the transmembrane regions between the G-protein coupled receptors and Bacteriorhodopsin are 6–11%, even though their TMH arrangement is the same.¹⁷ The quality of the template must also be considered, both in regard to low resolution, and in regard to the fact that the amphiphilic nature of membrane proteins causes difficulties in experimental structure determination. Thus, even when crystallization is successful, the protein is no longer in its natural environment, and conformational disruption of the transporter due to the presence of detergent molecules during crystallization can not be excluded. Interestingly, recent cysteine cross-linking studies, linking residues in TMH3 and TMH9 of ABCB1,¹⁸ and between residues in the Cterminal ends of the two NBDs of ABCB1,¹⁹ have shown that ABCB1 still functions when covalently cross-linked. This indicates that the Sav1866 X-ray crystal structure,¹³ which is in an outward-facing conformation, also may serve as a template for modeling and running VLS on ABCC5, and also might yield interesting inhibitors.

Structural flexibility was taken into account when performing docking and VLS on the ABCC5 model. A crystal structure of a transporter may not be a realistic representation of the transporter in its native form, and transporters may undergo substantial conformational changes during the transport cycle. Large ranges of motion, changing the accessibility of the transporter from a cytoplasmic (inward) facing to an extracellular (outward) facing conformation, have been revealed from X-ray crystal structures of the bacterial ABC transporter lipid flippase, MsbA, trapped in different conformations.¹⁴ Induced-fit, which has been demonstrated in a cysteine-scanning mutagenesis and oxidative cross-linking study of substrate-induced changes in ABCB1,²⁰ and conformational changes due to transport, may be an important part of ligand recognition. The energy-based torsional sampling ("fumigation") generated additional conformations of the ligand binding area of ABCC5, with lower energies than the starting model. Of the three conformations used for docking and VLS, the two lowest energy conformations were preferred by the ligands, indicating that these conformations were more "druggable". The 4D docking, where the ligands are free to "choose" their preferred binding site conformation, may be viewed as a theoretical model of how the ligand is attracted to the drug target in a relatively more active conformation. Insight into structural changes of the drug target for yielding a lower energy drug - drug target complex may elucidate how the conformation of the binding site contributes to the adoption of an energetically favorable complex. Ideally, these observations can aid to predict how a designed drug will fit into the drug target.

In an interdisciplinary approach combining *in silico* studies (theoretical molecular modeling), and *in vitro* studies (experimental pharmacology), the ABC transporter model may be considered as a working tool for generating hypotheses and designing further experimental studies related to ABC transporter structure and function and their ligand interactions. *In vitro* studies and transporter modeling are complementary to each other in an iterating process towards a better understanding of the structure and function of these proteins. The VLS add-on to the Internal Coordinate Mechanics (ICM) program²¹ (ICM-VLS) has previously been applied to identify new leads for a number of targets.^{22, 23} In the present study, the VLS docking correctly predicted seven ligands as having a higher binding affinity to ABCC5 than sildenafil. The most potent compounds, compounds **1** and **2**, showed K_i -values of 50–100 nM. As far as we know, these are the most potent inhibitors described for ABCC5.

Both compounds **1** and **2** features a salicylic acid moiety, and the pKa of salicylic acid is 2.97. At physiological pH, the carboxylic acid moiety of salicylic acids tend to be negatively charged, and docking indicated that these compounds oriented the negatively charged COO-group towards Lys448 (TMH6). This ionic interaction may explain why these two compounds were the most potent ABCC5 inhibitors. The characteristic interaction of the guanine-like ring of the ligands with Gln190 (TMH1) of ABCC5, which was found in most predicted complexes, has also been observed in X-ray crystal structures of PDE5 complexed with either guanosine monophosphate (GMP) or sildenafil. In these complexes, the guanine ring of GMP, or the guanine-like ring of sildenafil, has hydrogen bond interactions with a glutamine (Gln817).²⁴

The drug binding pocket of the ABCC5 model featured several polar amino acids (Gln190 (TMH1), Asn441 and Thr444 (TMH6), and Ser872 (TMH7)) and a positively charged amino acid (Lys448 (TMH6)), contributing to a molecular environment favorable for transport of organic anions. ABCC5 has been shown to transport cGMP with high affinity (K_m -value 2.1 μ M),⁵ and we have previously restored ATP-dependent cGMP transport into proteoliposomes by membrane proteins from human erythrocytes²⁵ and cGMP-induced ATPase activity.²⁵ At physiological intracellular cGMP concentration there are a number of leads that indicate ABCC5 as a high affinity and selective transporter for cGMP.²⁶ In the present study, the observed cGMP K_m -value of 2.2 μ M is in agreement with the K_m -value of 2.1 μ M reported earlier.⁵ The observation from the binding affinity studies of order of potency of the known ABCC5 inhibitors sildenafil, zaprinast and dipyridamole, with zaprinast and dipyridamole being equally potent, and both being more potent than sildenafil, were also in agreement with previous observations.²⁷

In the present VLS study, it was observed that each ligand had a tendency to prefer two main orientations, one towards Gln190 (in TMH1, corresponding to Leu65 (ABCB1)), and one towards Val253 (In TMH5, corresponding to Ile306 (ABCB1)). The differences in binding energy between different orientations of the same ligand did not differ significantly. This is in accordance with a study on ABCB1 where cysteine-scanning mutagenesis and reaction with an methanethiosulphonate (MTS) thiol-reactive analogue of verapamil (MTS-verapamil) showed that mutants Leu65Cys (TMH1) and Ile306Cys (TMH5) modified with MTS-verapamil have slightly different characteristics, indicating that the bound verapamil

molecules in these mutants have different orientations and that the protein can function quite well with the substrate bound in different orientations.²⁸ Theoretically, even though ten different conformations of each ligand were evaluated by ICM during VLS, focusing on only the ligand orientation with best score may lead to missing out better inhibitors oriented in a pose yielding a poorer score.

Despite more than 30 years of research and development of drugs for use in co-therapy to chemotherapeutic drugs to overcome multidrug resistance (MDR), these drugs have come no further than to late-phase clinical trials.²⁹ The possible roles of PDE5 inhibitors in overcoming MDR is currently gaining attention as emerging evidence indicates that sildenafil and sildenafil-like drugs may enhance the sensitivity of certain tumor cells to chemotherapeutic drugs. It has also been demonstrated that sildenafil also inhibits the activity of ABC transporters such as ABCB1 and ABCG2, reversing MDR in cancer cells mediated by these transporters.³⁰ In the present study, we chose to use a sildenafil substructure in order to achieve compounds for VLS, instead of using substructures of the slightly more potent zaprinast and dipyridamole. Zaprinast has been unsuccessful in clinical trials, and both zaprinast and dipyridamole are less selective than sildenafil.³¹ Very close analogs of sildenafil were considered in this study, and even though the search pattern used for finding sildenafil analogs was the ring system in Figure 5A, the 11 compounds that were selected for testing all have much more overlap with sildenafil than the ring system in figure 5A. Vendors presumably have generated libraries around the drug, and the additional drug-likeness filter could have biased the tested compounds even more towards close analogs of sildenafil. The ABCC5 model could also have been used to search for molecules that could not be trivially found from the starting sildenafil substructure, possibly discovering entirely new chemical scaffolds, but since sildenafil is a very interesting molecule in regard to MDR, the goal in this study was to create chemical diversity around sildenafil.

The IOV method is very time consuming, so using the ABCC5 model as an additional filter to select compounds to test for binding to ABCC5 in vitro, instead of testing all 105 compounds, may have saved time and lab resources. The structural and computational guidance used in the present study aided the identification of 7 compounds having higher effect on cGMP efflux effect than sildenafil. The sildenafil-like hits compounds from the VLS in the present study may represent candidates for lead optimization in the search for novel drugs that can be used to overcome MDR. Such drugs may also be used to inhibit cGMP efflux from cancer cells, possibly enhancing the body's own defense mechanism of increasing cGMP levels.

Coordinates of the ABCC5 model are available upon request.

Experimental section

Software

The ICM program²¹ version 3.6-1e was used for homology modeling, compound docking and sildenafil substructure search. The ICM program package included the ICM VLS add-on and access to Molcart, a database of chemical structures for ~4M of commercially available compounds.

Chemicals

cGMP (Sigma Aldrich, St. Louis, MO, USA), 8[³H]-cGMP (Perkin Elmer, Boston, MA, USA), Sildenafil (a kind gift from Pfizer Inc., NY, USA), 11 compounds based on VLS were purchased from Ambinter (c/o Greenpharma SAS, 3, allée du titane, 45100 Orléans, France). Other chemicals of analytical grade.

Homology modeling

A multiple sequence alignment of mouse ABCB1 (SWISS-PROT accession number P06795), human ABCB1 (SWISS-PROT accession number P08183), human ABCC4 (SWISS-PROT accession number O15439), human ABCC5 (SWISS-PROT accession number O15440), human ABCC11 (SWISS-PROT accession number Q9BX80), *Escherichia coli* MsbA (SWISS-PROT accession number P60752) and *Vibrio cholerae* MsbA (Q9KQW9) was generated using T-COFFEE³² version 4.71 (<http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee.cgi/index.cgi>) and used to guide the input alignment (Figure 4) for the homology modeling.

The ABCC5 model was constructed using the X-ray crystal structure of the *Mus musculus* ABCB1¹⁵ (PDB code 3G60), complexed with the ligand cyclic-tris-(R)-valineselenazole (QZ59-RRR), as a template. The ICM homology modeling module constructs the molecular model by homology from core sections defined by the average C-alpha atom positions in conserved regions. Loops were automatically constructed in the ICM homology module by selecting the best-fitting loop based on calculating maps around loops that were retrieved from the PDB databank³³ and matched in regard to sequence similarity and sterical interactions with the surroundings of the model, and scoring of their relative energies. The loop connecting NBD1 and TMD2 in the template, which is missing in the template, was included in the loop search procedure.

The ABCC5 model was refined by globally optimizing side-chain positions and annealing of the backbone using the RefineModel macro of ICM. The RefineModel macro includes (1) a side-chain conformational sampling using 'Montecarlo fast',³⁴ (2) 5 iterative annealings of the backbone with tethers, and (3) a second side-chain conformational sampling using 'Montecarlo fast'. In 'Montecarlo fast', the conformational space of a molecule is sampled with the ICM global optimization procedure. An iteration of the procedure, which consists of a random move followed by a local energy minimization, and calculation of the complete energy, is accepted or rejected based on energy and temperature. Tethers in the iterative annealings of the backbone are harmonic restraints pulling an atom in the model to a static point in space represented by a corresponding atom in the template. The RefineModel macro was followed by the "Regul" option of ICM. "Regul", which denotes "Regularization", is a procedure for fitting a protein model with the ideal covalent geometry of the residues.

The refined ABCC5 model was energy minimized using the leaprc.ff03 force field of the AMBER 9.0 program package.³⁵ Two energy minimizations were performed; (1) with restrained backbone by 500 cycles of the steepest descent minimization followed by 500 steps of conjugate gradient minimization, and (2) with no restraints by 1000 cycles of the steepest descent minimization followed by 1500 steps of conjugate gradient minimization. A

10 Å cutoff radius for non-bonded interactions and a dielectric multiplicative constant of 1.0 for the electrostatic interactions were used in the molecular mechanics calculations.

The stereochemical quality of the ABCC5 model was checked using the SAVES Metaserver for analyzing and validating protein structures <http://nihserver.mbi.ucla.edu/SAVES/>. Programs run were Procheck,³⁶ What_check,³⁷ and Errat.³⁸

4D VLS docking

In order to investigate putative ligand binding modes in the highly flexible transporter protein, energy-based torsional sampling was used to generate additional conformations of the ligand binding area of ABCC5. This computational technique called “fumigation”³⁹ is aimed at generating more “druggable” conformations of ligand binding pockets. Fumigation is based on torsional sampling of the binding pocket side chains in the presence of a repulsive density representing a generic ligand, using the ICM biased probability Monte Carlo sampling procedure. The pocket used for torsional sampling was defined using the ligand skin mesh of QZ59-RRR from the template.¹⁵

In order to get a threshold score for the VLS, known binders to ABCC5 were docked using 4D docking. Thus, the VLS threshold was based scores calculated by docking sildenafil, MK571, glibenclamide, sulfapyrazone, trequinsin, benzbromarone, verapamil, zaprinast, dipyridamole, probenecid and cGMP. Ligands were prepared in the ICM ligand editor and converted to 3D when setting up the ligand during the docking session. Charges were also assigned in this step. A 4D docking procedure was used employing the binding pocket conformational ensembles. In this approach, the pocket ensemble conformations is used as an extra, fourth dimension of the ligand sampling space, allowing ligand docking to the multiple binding pocket conformations in a single docking simulation.⁴⁰ The 3 binding pocket conformations with lowest energy were used in the 4D docking procedure.

The Molcart chemical management system, featuring a number of compound databases (including Ambinter, Chembridge, Lifechemicals, etc.) that can be analyzed and searched using ICM cheminformatic tools, was used to retrieve compounds with a common substructure as in sildenafil (Figure 5). This substructure is a guanine-like moiety, resembling the guanine part of cGMP. A database of 105 sildenafil-like compounds was obtained and used for a 4D VLS docking into the ABCC5 transporter. ICM-VLS, which is a combination of internal coordinate docking methodology and global optimization scheme, provides empirically adjusted scoring functions and accurate and fast potentials, yielding efficient virtual screening methodology in which ligands are fully and continuously flexible. 11 hits with scores above the score threshold were selected based on the drug-likeness score and ordered from Ambinter (<http://www.ambinter.com/>) for in vitro testing. The ICM drug-likeness score is predicted based on 5000 marketed drugs from the World Drug Index (WDI) (positives) and 10000 non-drug compounds (negatives).

Preparation of IOV

In the present study, a modified version of the Steck IOV preparation⁴¹ was used. Fresh human EDTA blood was used to produce IOVs from human erythrocytes. All steps after collecting the blood were performed at 0–4°C. The cells were sedimented by centrifugation

2300g for 15 minutes. Plasma and buffycoat were discarded, and the red blood cells were washed 3 times by centrifugation at 1000g 5 mM Tris•HCl, 113 mM KCl, pH=8.1. Cells were lysed in 10 volumes of 5 mM TrisHCl, 0.5 mM EGTA, 4 mM KCl, pH=8.1 and washed by repeated centrifugation at 20000g for 20 minutes and resuspension in the same buffer until ghosts were milky white. Vesiculation was initiated by adding 39 volumes of 500 nM TrisHCl, pH=8.2 to one volume of cell suspension. The vesiculation was completed by homogenization of vesicles and unsealed ghosts by passing the suspension five times through a 27G cannula. IOVs, right-side out vesicles and unsealed vesicles and ghosts were separated by ultracentrifugation ($100.000 \times g$) over night using a density gradient from 1,048 g/ml to 1,146 g/ml Histodenz (Sigma-Aldrich, St. Louis, MO, USA) in 5 mM Tris, 3 mM KCl, 0.3 mM EGTA. The uppermost band was collected, washed and resuspended in 1.47 mM KH_2PO_4 , 81 mM K_2HPO_4 and 140 mM KCl, pH 7.6. Sidedness was verified using acetylcholinesterase accessibility.

Transport assay

cGMP is transported out of cells via ABCC5 with a K_m value of 2.1 μM .⁵ In the present study cGMP uptake into IOVs was determined for an inhibitor concentration range of 0 – 320 μM , and the [^3H]-cGMP transport was characterized with a K_m value of $2.2 \pm 0.35 \mu\text{M}$. IOVs were incubated for 60 minutes with or without 2.0 mM ATP in a mixture containing 20 mM Tris•HCl, 10 mM MgCl_2 , 1 mM EGTA, 2 μM [^3H]-labeled cGMP, 121 mM KCl, pH=8.0 at 37°, and inhibitor in a concentration range from 0 μM to 316 μM . The transport process was stopped with addition of ice-cold 1.47 mM KH_2PO_4 , 8,1 mM K_2HPO_4 and 140 mM KCl, pH 7.6. The IOVs were separated from the incubation medium by filtration (nitrocellulose membrane, 0.22 μm GSWP, Millipore, Billerica, MA, USA). The radioactivity on the filters was quantified by liquid scintillation (Ultima Gold XR, Packard, Groningen, The Netherlands) in a Packard 1900 TR Liquid Scintillation analyzer. DMSO was needed to dissolve some of the inhibitors and was added to a similar concentration in the control samples (without inhibitors).

Determination of K_i -values

The IC_{50} -values were determined and transformed to K_i -values according to Cheng and Prusoff.⁴²

Statistics

The results are presented as mean value $\pm$ SEM. Unpaired two-tailed t test with Welch correction was performed using GraphPad InStat version 3.06, GraphPad Software, San Diego, California, USA.

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Nonstandard abbreviations

ABC	ATP binding cassette
cGMP	cyclic guanosine monophosphate
GMP	guanosine monophosphate
ICM	Internal Coordinate Mechanics
ICM-VLS	The virtual ligand screening (VLS) add-on to the Internal Coordinate Mechanics (ICM) program
IOV	inside-out vesicles
MDR	multidrug resistance
MTS	methanethiosulphonate
NBD	nucleotide binding domain
PDE5	phosphodiesterase 5
SAVES	Structural Analysis and Verification Server
TMD	transmembrane domain
TMH	transmembrane helix
VLS	virtual ligand screening
WDI	World Drug Index

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Figure 1. Superimposition of the energy minimized ABCC5 model (purple) and the template X-ray crystal structure of the *Mus musculus* ABCB1¹⁵ (green). The green dotted line of template indicates the missing loop connecting NBD1 and TMD2. The ligand QZ59-RRR co-crystallized with *Mus musculus* ABCB1¹⁵ is shown in space-filling solid style.

Figure 2. Sildenafil docked into the binding site of ABCC5 viewed from the extracellular side. Amino acids with hydrogen bond interactions with sildenafil are displayed as sticks colored according to atom type (C = light yellow; H = grey; O = red; N = blue; sulfur = yellow); Gln190 (TMH1), Asn441 (TMH6), and Gln1138 (TMH12).

Figure 3.

The sildenafil-like substances were selected by criteria described in Materials and Methods. They were tested for their ability to inhibit ATP-dependent high affinity transport of [³H]-cGMP (3.3 μM). A single inhibitor concentration (32 μM) approximately 10 times above the concentration of [³H]-cGMP was employed to unveil the most potent inhibitors. In addition, sildenafil, zaprinast and dipyridamole were included as positive controls. Unpaired t test with Welch correction was performed and showed that all substances were statistically significant different ($p < 0.05$) either more potent or less potent than sildenafil, with exception of compounds **8** and **9**. The results are presented as mean value + SEM.

Figure 4. Alignment of *Mouse* ABCB1 and human ABCC5 used as input alignment for the ICM homology modeling module. TMHs are indicated as boxes. Secondary structure of template is indicated: Alpha helix = red cylinder; 3_{10} helix = purple cylinder; beta sheet = green arrow.

Figure 5.

A. The sildenafil substructure used to retrieve sildenafil-like compounds from Molcart. B. Molecular structure of sildenafil. C. Molecular structure of cGMP.

Table 1

Potential ABCC5 inhibitors from the VLS that were ordered from Ambinter, with compound code, IUPAC name, Pubchem compound ID, ICM drug likeness score (predicted based on 5000 marketed drugs from WDI (positives) and 10000 non-drug compounds (negatives)), ICM docking score, % of cGMP efflux, and K_i values.

Compound	Ambinter compound code	IUPAC name	Pubchem compound ID	Molecular structure	Drug likeness	ICM Score	% cGMP efflux	K_i (nM)
Sildenafil		5-[2-ethoxy-5-(4-methylpiperazin-1-yl)sulfonylphenyl]-1-methyl-3-propyl-4H-pyrazolo[4,3-d]pyrimidin-7-one	CID 5212		0.9335	-23.55	16.2	1200 ± 170
Zaprinast		5-(2-propoxyphenyl)-2,3-dihydrotriazolo[4,5-d]pyrimidin-7-one	CID 5722		0.4367	19.61	5.7	
Dipyridamole		2-[[2-[bis(2-hydroxyethyl)amino]-4,8-di(piperidin-1-yl)pyrimido[5,4-d]pyrimidin-6-yl]-(2-hydroxyethyl)amino]ethanol	CID 3108		0.7904	19.08	5.9	
1	STOCK3S-39213	5-[[3-(3-tert-butyl-1-methyl-7-oxo-4H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxyphenyl]sulfonyl]amino]-2-hydroxybenzoic acid	CID 1598490		0.7573	30.93	0	75.3 ± 3.1
2	STOCK1S-60049	5-[[4-ethoxy-3-(1-methyl-7-oxo-3-propyl-4H-pyrazolo[4,3-d]pyrimidin-5-yl)phenyl]sulfonyl]amino]-2-hydroxybenzoic acid	CID 1899750		1.064	29.06	0	65.3 ± 6.4

Compound	Ambinter compound code	IUPAC name	Pubchem compound ID	Molecular structure	Drug likeness	ICM Score	% cGMP efflux	K _i (nM)
3	PHAR099048	4-ethoxy-3-(1-methyl-7-oxo-3-propyl-4H-pyrazolo[4,3-d]pyrimidin-5-yl)-N-[3-(1-methylpyrrolidin-2-yl)benzenesulfonamide	CID 4921527		1.246	32.33	5.5	
4	STOCK1S-59261	4-ethoxy-N,N-diethyl-3-(1-methyl-7-oxo-3-propyl-4H-pyrazolo[4,3-d]pyrimidin-5-yl)benzenesulfonamide	CID 1899174		0.3768	31.02	6.1	
5	STOCK1S-52373	4-ethoxy-N-methyl-3-(1-methyl-7-oxo-3-propyl-4H-pyrazolo[4,3-d]pyrimidin-5-yl)benzenesulfonamide	CID 1896380		0.2554	-31.24	5.5	
6	STOCK1S-62278	4-ethoxy-N-(2-hydroxyethyl)-3-(1-methyl-7-oxo-3-propyl-4H-pyrazolo[4,3-d]pyrimidin-5-yl)benzenesulfonamide	CID 1900265		0.3706	-30	8.1	
7	STOCK1S-53050	4-ethoxy-N-(2-hydroxyethyl)-N-methyl-3-(1-methyl-7-oxo-3-propyl-4H-pyrazolo[4,3-d]pyrimidin-5-yl)benzenesulfonamide	CID 1896597		0.5718	31.47 12.0	12.0	
8	STOCK1S-57138	5-[5-(azepan-1-ylsulfonyl)-2-ethoxyphenyl]-1-methyl-3-propyl-4H-pyrazolo[4,3-d]pyrimidin-7-one	CID 1897952		0.7381	31.98	22.0	
9	STOCK1S-53798	5-(2-ethoxy-5-piperidin-1-ylsulfonylphenyl)-1-methyl-3-propyl-4H-pyrazolo[4,3-d]pyrimidin-7-one	CID 1896867		0.9196	29.64	37.1	

Compound	Ambinter compound code	IUPAC name	Pubchem compound ID	Molecular structure	Drug likeness	ICM Score	% cGMP efflux	Ki (nM)
10	STOCK1S-68740	4-ethoxy-N,N-dimethyl-3-(1-methyl-7-oxo-3-propyl-4H-pyrazolo[4,3-d]pyrimidin-5-yl)benzenesulfonamide	CID 1902581		0.3803	34.62	37.7	
11	STOCK1S-53657	N-benzyl-4-ethoxy-3-(1-methyl-7-oxo-3-propyl-4H-pyrazolo[4,3-d]pyrimidin-5-yl)benzenesulfonamide	CID 1896826		0.5251	34.76	49.6	