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Development of an assay for determining PDE5 enzyme activity and for screening of potential inhibitors of the PDE5 enzyme in cancer cells

MBI-3911 Masters' thesis in biomedicine

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Abstract

PDE5 is a member of the superfamily of phosphodiesterases, and it is identified as the main mechanism for breakdown of cGMP in mammals. Sildenafil is a well-known inhibitor of the PDE5 enzyme and it is also shown that sildenafil inhibits the ABCC5 transporter pump. ABCC5 is a member of the superfamily of ABC-transporters, and identified as an important transporter for mediating the cellular efflux of cGMP. Research group of Pharmacology and Toxicology at University in Tromsø recently showed that sildenafil analogs, IS-39213 and IS-60049, almost completely blocked the cGMP efflux in cancer cell lines C33A and C-4I by inhibiting the ABBC5 transporter pump. It is not known if these sildenafil analogs also inhibit the PDE5 enzyme. To further investigate this, an assay was developed for determining PDE5 activity and for screening of these potential inhibitors on the PDE5 enzyme in cancer cell lines C-4I and C-33A. Both IS-39123 and IS-60049 were shown to inhibit the PDE5 enzyme in the same degree as sildenafil, but the time available did not allow completion of the characterization of inhibitors and their mutual potency.

Abbreviations

5’-AMP = Adenosine 5’-monophosphate

5’-GMP = Guanosine 5’-monosphosphate

ABCC = ATP-binding-cassette transporter, subfamily C

ANP = Artrial natriuretic peptide

cGK = cGMP-dependent protein kinases

cAMP = Adenosine 3’,5’-cyclic monophosphate

cG-BPDE = cGMP-binding-specific phosphodiesterase

cGMP = Guanosine 3’,5’-cyclic monophosphate

CPM = Counts per minute

DEAE = Diethylaminoethyl

DPM = Disintegrations per minute

EDRF = Endothelial-derived relaxand factor

GC= Guanyl cyclase

IRAG = IP3 receptor-associcated cKG1B substrate

LSC = Liquid scintillation counter

MRP = Multidrug resistance protein

NO = Nitric oxide

PDE = Phosphodiesterase

PKG = Protein kinase G

SAX = Strong anion exchanger

SPE = Solid phase extraction

VSMC = Vascular smooth muscle cells

Contents

1 Introduction

1.1 Cyclic nucleotides

1.1.1 History

Cyclic nucleotides were discovered by Earl Sutherland and colleagues in the late fifties whilst working on glycogenolysis. This pioneering work led to Sutherland receiving the Nobel Prize in 1972, “for his discoveries concerning the mechanism of action of hormones through cAMP” [1]. Cyclic nucleotides, at first cyclic adenosine 5’,3’-monophosphate (cAMP), were described as a heat stable factor containing adenine, ribose and phosphate at a ratio of 1:1:1, and it was shown to be stable to acid and common phosphatases [2]. By 1958 the synthesis of cAMP by adenyl cyclase, and the degradation by phosphodiesterase (PDE) was described. Adenyl cyclase activity and hormone receptors were shown to be membrane confined [1]. At first the discovery of cyclic nucleotides, and their role as second messengers, was met with a lot of skepticism. It was not until Lipkin and colleagues determined the synthesis and structure of cAMP that the skepticism drifted towards acceptance. But little was still known about these second messengers, and at first Lipkin and colleagues gave it the name cyclic anhydrodiadenylic acid [3].

Another important landmark in the cyclic nucleotide history was the discovery of cyclic guanosine 3’,5’-monophosphate (cGMP) in rat urine by Ashman and Price, revealing that concentrations varied according to the hormonal state of the animal, confirming the importance of cGMP as a potential biological effector [4]. In the early seventies the research concerning cyclic nucleotides as second messengers exploded, and the researchers mostly focused on cAMP, whilst cGMP was dwelling in the background. In 1971, Rodbell and Birnbaumer, showed that hormone-sensitive cAMP production required GTP, thus postulating the theory of a transducer linking the cyclase to the receptor [5]. This intrigued researchers, and by the help of S49 lymphoma cells, thought to be lacking adenyl cyclase (named cyc^- cells), Ross and Gilman found evidence of a stimulatory G-protein [1, 6]. This stimulatory G-protein was sensitive to proteases and differed from adenyl cyclase, with ability to reconstitute hormone-sensitive adenyl cyclase activity in cyc^- membranes, showcasing that these cyc^- cells lacked the activating or coupling protein instead of lacking the adenyl cyclase [1, 6]. As the research on G-proteins expanded this initial postulate was confirmed.

1.1.2 Guanosine 3'-5'-cyclic monophosphate (cGMP)

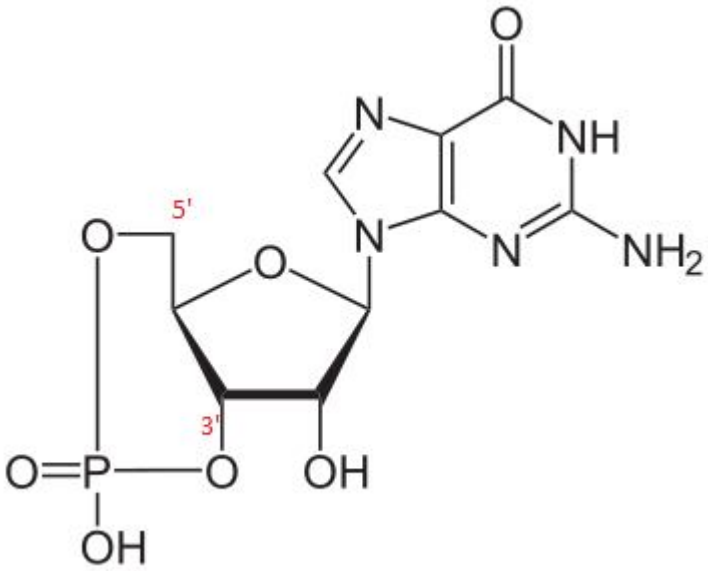

Figure 1: Chemical structure of guanosine 3',5'-cyclic monophosphate. The 3',5' highlights the cyclic structure and bonds formed (positions highlighted in red in this figure)

Even though the researchers determined the synthesis and breakdown of cGMP in the 1960s, it was not until the 1980s it was determined which hormones stimulated the synthesis, leading to an explosion of research concerning cGMP [1]. In the 1980s it was shown by Furchgott and colleagues that endothelial-derived relaxant factor (EDRF) stimulated smooth muscle cells to relax, and later on Ignarro and colleagues also showed that EDRF stimulated the production of cGMP [7, 8]. Ignarro further investigated the role of EDRF and concluded in 1987 that EDRF was the small reactive gas Nitric Oxide (NO) [8]. This baffled researchers and was met with skepticism in the beginning, since NO was a small reactive molecule many researchers did not see the potential of NO as a functional hormone. The skepticism faded away as more of the pieces in the puzzle of nitric oxide signaling were discovered by Ferid Murad amongst others. Murad showed that several hormones could stimulate a calcium-calmodulin-dependent NO synthase, producing NO and reciprocially stimulating soluble guanyl cyclase [9]. This pioneering work led to Furchgott, Ignarro and Murad receiving the Nobel Prize in 1998. Another important discovery was of a peptide produced in the heart, artrial natriuretic peptide (ANP), harboring the ability of increasing cGMP levels in smooth muscles, kidney and the adrenal gland [1]. ANP was later shown to act as a ligand for transmembrane guanyl cyclase (particular guanyl cyclase) [1]. This thesis will mainly focus on the nitric oxide pathway.

1.1.2.1 Synthesis

There are two distinct pathways regulating the synthesis of cGMP, both mentioned earlier, nitric oxide pathway and natriuretic pathway. Both pathways is coupled to a distinct guanyl cyclase isoform, either soluble guanyl cyclase (sGC) or particular guanyl cyclase (pGC), where the NO-mediated pathway is coupled to sGC and the natriuretic peptide pathway is coupled to the pGC [10]. Historically sGC is considered a cytosolic protein whilst pGC is considered as a membrane-bound protein. Recent studies shows a little more nuance concerning their localization, suggesting distinct pools of cGMP generation differing in downstream effects [11].

1.1.2.2 Nitric oxide pathway

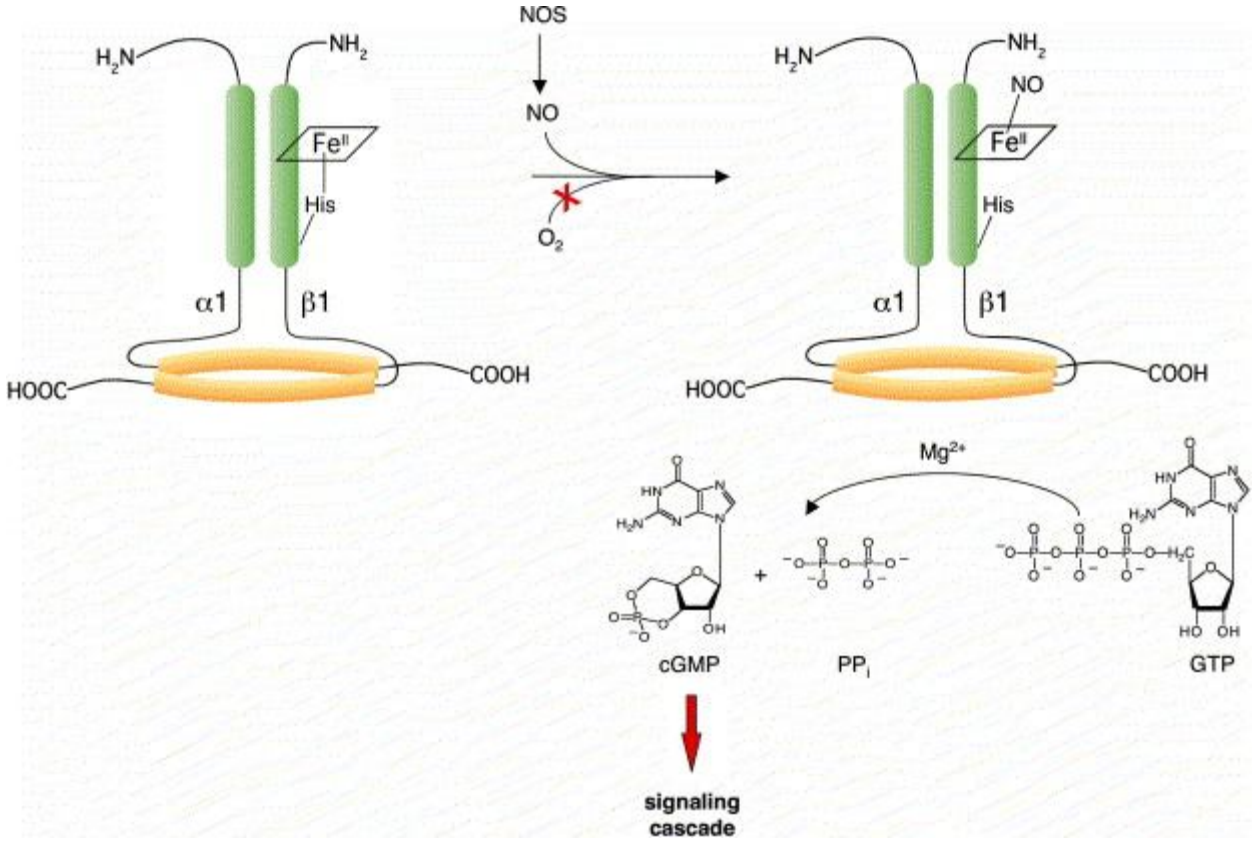

Figure 2: Schematic representation of the structure and function of the $\alpha_1\beta_1$ isoform of sGC. The heme moiety is important for binding of NO which further activates the cyclase and leads to conversion of GTP to cGMP. Figure taken from [12].

Soluble guanyl cyclase is a heterodimer consisting of an α subunit (82 kDa) and β subunit (70 kDa) and is shown to exist in four different isoforms, either one of the two α subunits (α_1 or α_2) and either one of the two β subunits (β_1 or β_2), where $\alpha_1\beta_1$ is the predominant isoform and most catalytically active [10, 13]. The $\alpha_1\beta_2$ and $\alpha_2\beta_2$ isoforms have not any reported cyclase activity, and the $\alpha_2\beta_1$ isoform has cyclase activity at a lesser extent than $\alpha_1\beta_1$ [10]. The heme moiety of the β_1 subunit is shown to be important for the distinct specificity for NO by activating sGC at nanomolar concentrations of NO [10, 14]. This conserved protoporphyrin-IX heme domain is ferrous and 5-coordinate, with a histidine residue at the protein derived axial ligand (H105), see figure 2 [10, 14]. The binding of NO to the heme moiety leads to formation of an inactive, but NO-responsive 6-coordinate nitrosyl intermediate that in the presence of magnesium, cGMP and pyrophosphate is further converted to a 5-coordinate nitroxyl complex which can be further activated by NO [10, 14]. The second NO-binding step leads to breakage of the iron-histidine bond between the heme-ring and the axial ligand of histidine, resulting in a conformational change in the catalytic site of the enzyme, thus accelerating the basal conversion of GTP to cGMP hundred folds, see figure 2 [10, 12, 14]. When lacking magnesium, cGMP or pyrophosphate, the 5-coordinate nitrosyl intermediate is unable to activate the enzyme, resulting in low or basal enzyme activity (low-activity state) for this NO-bound enzyme. When NO-levels and substrate/products-levels are high, these low-activity state sGC can be converted to a highly active state as a response to an acute increase of NO.
Alternatively the second NO-binding can be achieved by binding of NO to non-heme groups, also resulting in breakage of the iron-histidine bond [10]. The same case is observed for the non-heme binding of NO, at low levels the NO dissociates from the none-heme groups, leading to continual low levels of cGMP [10].

1.1.2.3 Function

cGMP plays an important role in various important physiologic processes in the cell, amongst others; ion channel conductance, cell growth, cardiovascular homeostasis, inflammation, apoptosis, cellular mobility and contractility [10]. This is achieved by functioning as a regulator of distinct intracellular molecular targets, and these effectors can mainly be grouped under three categories:

- cGMP-dependent protein kinases
- cyclic nucleotide gated cation-channels (CNG)
- hyperpolarization-activated and cyclic nucleotide-regulated cation-nonselective channels (HCN).

These proteins are mainly chimeric proteins containing an allosteric cGMP-binding site combined with other functional parts, like protein kinase domains, ion-transporting channel domains and GAF domains [15]. The structure and regulation of the cGMP-binding domain varies amongst the effectors and is usually categorized in three distinct groups:

- CAP-related family of binding sites, found in cyclic nucleotide dependent kinases and cyclic nucleotide gated channels
- catalytic sites of phosphodiesterase
- GAF domains, found in cGMP-dependent phosphodiesterase

1.1.2.4 cGMP-dependent protein kinases (cGKs)

The most studied of these effectors are the cGMP-dependent protein kinases (cGKs) and there are three types of cGKs known in mammals. Cytosolic cGK type I, residing in two isoforms (cGK1α and cGK1β) and membrane-bound cGK type II [16]. cGK type II mediates most of its effect on cGMP derived from particular guanyl cyclase (pGC), important for electrolyte transport and bone formation, whilst type I mediates the effect of the cGMP derived from soluble guanyl cyclase (sGC), an important transducer in cardiovascular homeostasis amongst other [16].

1.1.2.5 Roles of cGK type I

Mendelsohn and colleagues in Boston showed the importance of the cGK1α for the interaction with the regulatory subunit of myosin phosphatase of vascular smooth muscle cells (VSMC). This was achieved by generating a new mouse line with a mutation in the cGK1α isoform, disabling the binding of the isoform to the regulatory subunit of myosin phosphatase [16]. After 8 weeks these mutants already showed altered growth properties of VSMC, age-dependent hypertension and cardiac hypertrophy [16]. Their results showed that the interaction with the cGK1α isform is important for the dilation of VSMC, by activating the myosin phosphatase and further dephosphorylation of the myosin light chain [16].

Schlossman and colleagues in Munich showed the importance of the cGK1β isoform in the Inositol 1,4,5-trisphosphate (IP_3) signaling pathway, mediating changes in intracellular calcium concentrations. The cGK1β isoform was shown to interact with the IRAG protein (IP3 receptor-associated cKG1β substrate), forming a complex inhibiting the hormone induced calcium release regulated by IP_3 [17]. The calcium-release mediated by IP_3 is known to be important for regulating the relaxation of the aorta and colon amongst other. Schlossman created a mouse line expressing a mutated IRAG protein, unable to interact with the IP3-pathway. These mutants showed impaired relaxation of the aorta and colon, but no hypertension, supporting the role of the cGK1β-IRAG-Ca^{2+} pathway in smooth muscle relaxation and intestinal function, but not seen in regulating blood pressure by potassium channels [17].

1.2 Phosphodiesterases

1.2.1 History

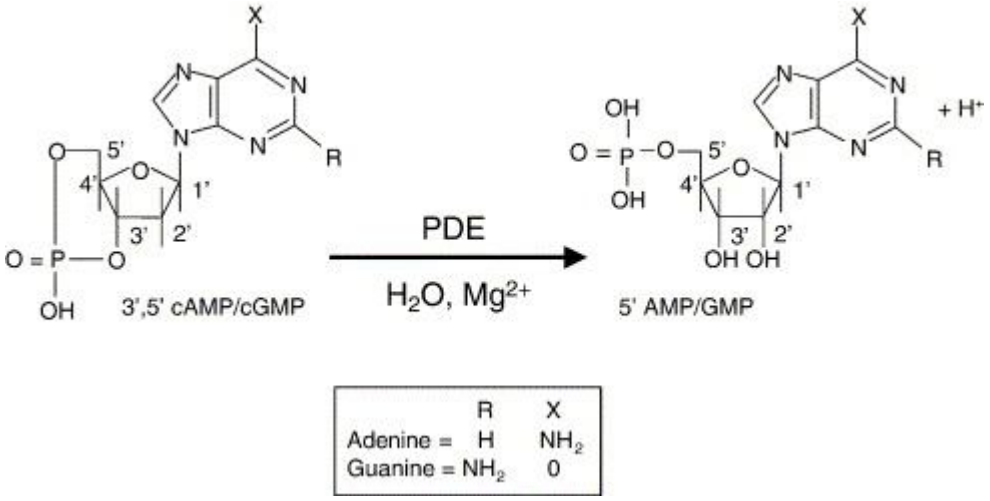

Figure 3: Hydrolysis of cyclic nucleotides to nucleotides by phosphodiesterases [18].

The discoveries of cyclic nucleotides in the sixties lead to the discovery of cyclic nucleotide phosphodiesterase (cnPDE), and in 1962 the first scientific paper concerning cnPDEs was published by Sutherland and Butcher. They observed an activity in fractions of homogenates of bovine heart where cAMP were inactivated, and described it as a significant physiological mechanism for inactivation of cAMP [19]. It was classified as the only mechanism for destruction of cAMP, the enzyme was found to be specific for the 3'-5'-bond and required magnesium to function properly, as observed in figure 3 [20]. In the beginning the biochemical characterization of PDE activity was achieved by studying cytosolic tissue fractions using anion exchange chromatography. These fractions dissociated differently and were differentiated by their respective substrate specificity and by their sensitivity to calcium-calmodulin (CaM), and they were given a name based on their elution order [21, 22].

The advancement in the field of molecular biology in the early 1980s, and in particular the advancement of sequencing and cloning, lead to a breakthrough in PDE research, the discovery of a relative large superfamily of PDEs consisting of at least five distinct PDE isoenzyme families [23]. The complete sequencing of various cnPDEs from different families lead to the understanding that most PDE isozymes were separate gene products with multivariant regulatory (e.g calmodulin or cGMP binding), and highly conserved catalytic domains [24]. These advancements led to confusing nomenclature and in the beginning of 1990s Joseph Beavo initiated an establishment of an official nomenclature for PDE isozymes. Where Roman numerals were given to family designations, and Arabic numerals were given to designate individual PDEs in a subfamily (thought to be products of alternative splicing) and Greek letters were used to categorize different subunits of a particular isozyme [24, 25]. For example, the PDE5A1 (Family: 5, gene: A, splicing variant: 1) The Roman numerals were arbitrary assigned, and not corresponding to orders of elution from fractioning processes (e.g DEAE-cellulose), as was the common nomenclature used before [25]. By time, roman numerals was exchanged with Arabic numerals as family designations, but both Roman and Arabic numerals are seen today.

By the turn of the millennium the research on phosphodiesterase exploded and attracted a lot of different pharmaceutical companies funding studies. This lead to a broader understanding of the premise that distinct phosphodiesterase families regulate specific cellular functions. These functions included regulation of insulin secretion, T-cell activation, fertility, growth amongst others [26]. Penile erectile function was a major target for pharmaceutical companies and promising trials with sildenafil showed that by inhibiting PDE5, enhancement of the penile rigidity was observed due to the increased concentration of cGMP in the corpus cavernosum, and was later marketed under the name Viagra® by Pfizer [26]. A lot of different isozymes were discovered over the years, and by 2003 it was shown that the PDE superfamily hailed from 21 genes coding for different PDEs, discovered from comparative, structural and functional studies [26-28]. These genes were further grouped into 11 enzyme families based on their primary amino acid sequence, overall domain structure, catalytic and regulatory considerations [29]. Combined with multiple promoters and alternative splicing, various PDE transcripts are expressed in cells and more than hundred open reading frames are recognized and continuously expanding [27]. Most of these PDE proteins residing from the transcripts, with a few exceptions, contain an identical catalytic domain, but differ at the C or N-terminal [27].

1.2.2 General structure

1.2.2.1 Catalytic domain

The catalytic domain of PDE4 was the first to be resolved at the atomic level, leading to an extensive research on other catalytic domains of phosphodiesterases [27]. The basic structure of the catalytic site of PDE4 includes 3 subdomains consisting of 16 alpha helices, defining the pocket where the substrate binds [27]. This pocket contains 11 of the 17 conserved residues in all PDEs, and many of the residues are implicated to be important for the catalytic function of PDE [27]. The importance of binding of Zinc was first observed in PDE V, achieved by conserved histidines and aspartates in the hydrolytic centre, but later revealed a similar importance for several other PDEs [27]. There is also observed the same effect of Magnesium binding, but with lower affinity [6]. Some phosphodiesterase selectively recognize cAMP, whilst other selectively binds cGMP, and many can bind both. It was proposed by Zhang et.al that this nucleotide specificity of the catalytic site is dependent of one particular invariant of glutamine (Q817), responsible for stabilizing the binding between the purine ring in the binding pocket and cGMP/cAMP, by forming hydrogen bonds [30]. Depending on the orientation of the glutamine residue, it can bind either cGMP, cAMP or both, yielding various possibilities of conformations [27, 30]. If the glutamine residue is not constricted by neighboring residues, and able to rotate freely, it can bind both nucleotides with relative high affinity by adopting different conformations [30]. For PDEs where the rotation is constricted by neighboring residues, selective binding is usually observed [27, 30]. It should be noted that this hypothesis is based largely on the structures for the catalytic domain solved with the 5'AMP or 5'GMP products complexed in the binding pocket, and it is not yet clear if the cyclic nucleotide substrates bind in the same manner as these products [27, 30].

1.2.2.2 Regulatory domains

Most of the regulatory functions of PDEs are related to the N-terminus of the protein. There are at least 3 domains present in multiple PDEs which are essential for ligand binding, oligomerization (dimerization) and kinase recognition/phosphorylation [27]. There is observed a remarkable homology in the arrangement of domains in different PDEs, and many propose that, though PDE is divergent, it has retained a common structural basis in the N-terminus, essential for oligomerization and regulating the catalytic activity [27]. The GAF-domain is a novel domain shown to be important for cGMP-specific phosphodiesterase (PDE2,5,6, 10 and 11), in both binding of cGMP and dimerization of the PDE, and still little is known about this domain [6]. The structure of GAF-

domain shows no resemblance to the structure of other cGMP-binding domains, differing in aa sequence, tertiary structure and other signature sequences [15]. Even though differing from other binding domains like CAP-related domains, it is remarkably specific to cGMP, and any given substitution of any residue of cGMP will drastically affect the binding of cGMP [15]. There are found other domains important for ligand binding in PDEs which do not have GAF-domains, but these are not found in multiple families, like PAS or UCR [27].

1.2.3 Function

PDE is a major regulator of the cyclic nucleotide biotransformation and have several other important functions in the cell. PDEs act as an effector of signal transduction by interacting with G-proteins and receptors, such is observed in the retina. In the retina, PDE6 is under control of the light receptor rhodepsin by the G-protein transducin, which binds to the catalytic site of PDE6, and a similar mechanism may also exist in the taste buds of the tongue [18, 31]. PDEs also serve a function in coordinating the cyclic nucleotide-dependent pathways with other signal transduction pathways, as PDEs are not solely regulated by cyclic nucleotides [31]. PDEs also function as homeostatic regulators via their role in feedback mechanisms, important for controlling the cyclic nucleotide concentration under stimulation by hormones et cetera, and there have been numerous reports implicating that PDEs also are involved in desensitization and termination of stimulation [31]. Studies of PDE4D knockout mice by Conti and colleagues have shown that PDE4D is important in both growth and fertility (only observed in females), since both genders were growth-retarded and nearly 50% of females shows signs of infertility, but the function and role of PDE4D in growth and fertility is not yet fully understood [18, 26, 32]. It was hypothesized that the cause stems from the decreased circulation of Insulin Growth Factor-1 (IGF-1), which is known to be under regulation by PDE3B, and thus mediating the insulin secretion from the B-cells in the pancreas [32].

1.3 Phosphodiesterase 5

The focus of this thesis is based on phosphodiesterase 5 (PDE5) and potential inhibitors for this particular enzyme. PDE5 was previously named cGMP-PDE, cGMP-binding-specific phosphodiesterase (cG-BPDE) or PDE V, but as mentioned earlier the proper nomenclature for this enzyme now is PDE5 [18]. The first discovery of PDE5 was in rat platelets by Hamet & Coquil in 1979, and in rat lung by Francis in 1980 [18]. It was purified and characterized and found to be a cytosolic isozyme hydrolyzing cGMP without activation by Ca^{2+}/calmodulin, and specifically inhibited by zaprinast and shown to be insensitive to rolipram [18, 33]. Zaprinast was the archetype of PDE5 inhibitors because of its specificity to PDE5, whilst rolipram was known to be a more unspecific inhibitor of various PDEs. Zaprinast was used in the characterization of the various functional properties of PDE5, and many of the functional characteristics of PDE5 are known by pharmalogical alleviation with zaprinast [18].

1.3.1 Structure

PDE5 is a homodimer of a 93 kDa subunit, shown to bind 0,93 cGMP/mol of subunit [18]. McAllister-Lucas was the first to deduce the structure of PDE5, based on cDNA clones of cG-BPDE from bovine lung [34]. They showed that the sequence enclosed a segment in the carobyxl-terminal (aa 578-812) which were homologous with the putative catalytic region conserved in all mammalian PDEs and contained another segment found in the amino-terminal end (aa 142-526) that was homologous to the putative cGMP binding region found in PDE2 and PDE6 and the photoreceptors PDEs [34]. Mcallister-Lucas also found that cG-BPDE contained the conserved serine residue (aa 92) phosphorylated by PKG [34]. Other researchers isolated and characterized human PDE5A, and mRNA was found to be expressed in aortic smooth muscle cells, placenta, skeletal muscle, pancreas, and was found in lesser extent in brain, liver and lung [18]. Two 5'-splice variants, PDE5A1 and PDE5A2 were identified by Kotera and Loughney which were differently distributed in tissue, but today it is known from studies in the corpus cavernosum, that only one PDE5A gene encodes three different isozymes from two alternate promoters [18]. The 3-dimensional structure of native PDE5A revealed that PDE5 is highly homologous with PDE6, and is a dimeric protein consisting of three distinct domains corresponding with the catalytic domain and 2 GAF-domains. Molecular modeling of the GAF A domain of PDE5A on the crystal structure of PDE2A shows that GAF A adopts structure similar to GAF B domain on PDEA2, providing the sole binding site for cGMP in PDE5 [18].

1.3.2 Functional roles

1.3.2.1 Role in erectile dysfunction

PDE5, as earlier mentioned, was first indicated as a potential target in treatment of vasorelaxation, as specific inhibition of PDE5 by zaprinast induced an increase of cGMP associated with the vasorelaxing effect achieved by the NO/cGMP-pathway [18]. At first specific PDE5 inhibitors were derived from zaprinast as potential anti-hypertensive agents or as coronary vasodilators, but surprisingly during clinical studies, sildenafil proved to be a potent effector for erectile dysfunction by increasing the penile blood flow. This baffled many researchers, and the research drifted towards the potential of treating erectile dysfunction as it was shown that these inhibitors worked specifically on penile blood flow and the enhancement of intracellular cGMP in corpus cavernosum did not generate major unwanted effects in other tissues [35]. This is a question which is still largely unanswered, but there a couple of possibilities directing the answer to the unevenness distribution of PDE5 in tissues and some claim it is due to the sexual stimulation, which is needed for sildenafil to function properly, leading to a specific release of NO in the penis [35]. This is further backed by the presence of a 5'-flanking androgen receptor found in the PDE5 promoter suggesting that PDE5 is under regulation by androgens, important effectors in sexual stimulation [35]. Morelli and colleagues showed in 2004 that there was a physiological predominance of PDE5 in the corpus cavernosum, and this predominance is androgen-dependent. Morelli further showed that PDE5 expression were 10-fold more abundant in the corpus cavernosum than in other male reproductive tissues, and even 10-to 100-fold higher than in non-reproductive tissues [35].

1.3.2.2 Other functional roles

PDE5A transcripts are found in many tissues, and high levels of PDE5 are observed in lung, which could be explained by the observed role of PDE5 activation during pulmonary hypertension, but yet the mechanism is poorly understood, but the research concerning PDE5 inhibitors as potential agent for treating pulmonary hypertension or respiratory distress expands [18]. There is also found evidence of PDE5 in the brain, and Prickaerts and colleagues showed that inhibition of PDE5 in the brain improved the memory consolidation of object information [18].

1.4 ATP-Binding-Cassette-transporters, subfamily C (ABCC-transporters)

Even though the cell membrane is close to impermeable for cyclic nucleotides, the presence of cyclic nucleotides in both urine and blood has been described since the early sixties [4]. The primary mechanism for metabolizing cyclic nucleotides is the phosphodiesterases, but as there were observed cyclic nucleotides in both blood and urine, there clearly existed another mechanism for excreting excess cyclic nucleotides, and this mechanism baffled researchers for ages. Many observed that cyclic nucleotide transport was unidirectional, extruded against a concentration gradient, and was inhibited by the non-specific organic transport inhibitor, probenecid [36, 37]. It was observed in many different cells that this excretion was concurring with the synthesis of cyclic nucleotides, as for cGMP, it was observed in endothelial cells amongst others, that upon guanyl cyclase stimulation by nitric oxides/natriuretic proteins, there was an export mechanism present [37].

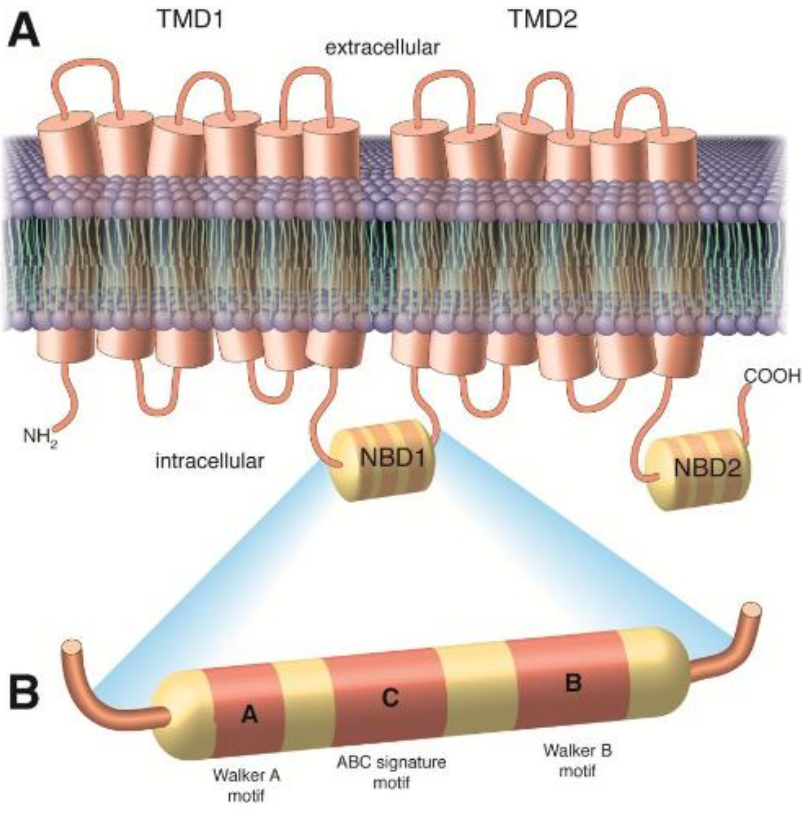

Figure 4: A. General structure of ABCC5 embedded in the lipid bilayer, consisting of two transmembrane domains with 6 alpha helices and two ATP binding domains, NBD1 and NBD2. **B.** Highlighting the nucleotide binding domain, NBD1, containing the Walker A and B motifs and another signature ABC motif. Figure created by Roy Lysså.

Members of the family of multidrug resistance protein (MRP), more precisely known as ABCC (ATP-Binding-Cassette transporter, subfamily C), are known for transporting amphilic anions, and since the first characterization of the MRP1 protein in 1994, these transporters have been suggested as potential transporters of cyclic nucleotides, thus responsible for the presence of cyclic nucleotides in blood and urine [37]. As more ABCC transporters were characterized it became clear that many of these were responsible for the excretion of cyclic nucleotides in cells working as efflux pumps. Studies showed that ABCC4, ABCC5 and ABCC11 have a P-glyco-protein-like core comprising of two transmembrane domains, and two nucleotide-binding domains responsible for cleaving ATP's terminal phosphate-group to energize the transport of substrates, cyclic nucleotide amongst others, against a concentration gradient [36, 38]. These domains differentiates these transporters from others transporters in the subfamily, as they usually consist of a more amino-terminal transmembrane domain (TMD_0) [36, 37]. It is known that ABCC4 is a selective, moderate high affinity transporter for cAMP, ABCC5 is a selective high affinity transporter for cGMP, whilst ABCC11 is a non-selective low affinity transporter for both cAMP and cGMP, and it is also shown that there is an additional site present in both ABCC4 and ABCC5 with high K_m for both nucleotides [36, 37]. Jedlitschky and colleagues showed that the ABCC5 pump transports cGMP with high affinity, K_m-value was determined to 2,1 µM [37].

1.4.1 Innhibitors

Research group of Medical Pharmacology and Toxicology at University of Tromsø has investigated the role of a series of compounds which is shown to inhibit the ABCC5 transporters. These inhibitors are based on the chemical diversity of sildenafil, which is a well-known inhibitor of the PDE5 enzyme. They were designed by ligand-based drug design, applying the three dimensional structure of the ABCC5-transporter and searching databases for additional sildenafil analogs [38]. Potential inhibitors were confirmed by virtual ligand screening (VLS) and experimental studies of cGMP efflux on erythrocyte inside-out vesicles [38]. These findings showed that two compounds were of particular interest as they were shown to almost completely block the ABCC5 pump, and these two compounds were IS-39213 and IS-60049 [38].

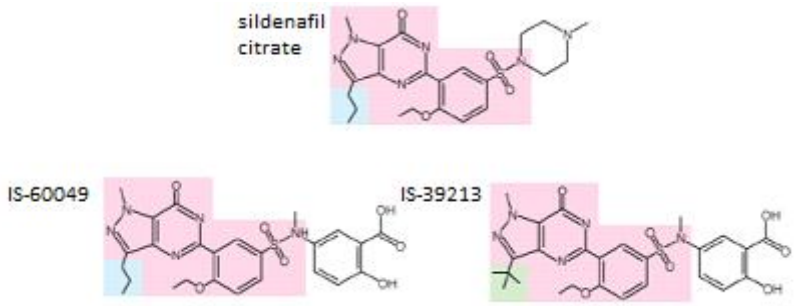

Figure 5: Chemical structures of sildenafil and sildenafil analogs, IS-60049 and IS-39213. The shaded pink area represents common backbone for all compounds. The blue color represents propyl groups whilst the green color represents an isobutylgroup. Figure modified from [38], with permission.

Both IS-39313 and IS-60049 were shown to be very potent inhibitors of the ABCC5 pump as the efflux of cGMP was shown to be 0 %, compared to sildenafil where there were observed a cGMP-efflux of 16,2 %, at equivalent inhibitors concentrations present [38]. The K_i-value for IS-39313 was calculated to 75,3 ± 3,1 nm, and for IS-60049 65,3 ± 6,3 nm, compared to sildenafil where the K_i was measured to 1200 ± 170 nm, proving these are more potent inhibitors of the ABCC5 pump [38]. Currently there are no experimental data from research investigating the potential role of these compounds as inhibitors of the PDE5 enzyme, and this thesis will further investigate their role as potential inhibitors of the PDE5 enzyme.

2 Aim of study

The aim of this study was to develop a robust assay for determining the PDE5 activity in cancer cells. This assay should be further used to determine both the activity of PDE5 in cancer cells and for screening of potential inhibitors of the PDE5 enzyme. Our research group, Medical Pharmacology and Toxicolgy at University of Tromsø, has investigated the role of sildenafil analogs as inhibitors of the ABCC5 transporter pump, but currently none of these inhibitors have been tested as inhibitors of the PDE5 enzyme. Therefore this became a task for me to look further into by screening these inhibitors on cancer cell lines, C-4I and C-33A, for testing their potential of inhibiting the PDE5 enzyme as well.

3 Materials

Table 1: Composition of lysisbuffer, pH 7,5

Reagents	Concentrations	Manufacturer	Catalog number from manufacturer
TRIS (hydroxymethyl aminomethane)	20,0 mM	Merck	1.08219.1000
Propionic acid	16,0 mM	Sigma-Aldrich	P1386
EGTA (Ethyleneglycol-bis(2-aminoethylether)-N,N,N,N'. N'-tetraacetic acid)	1,0 mM	Sigma-Aldrich	D2158
Benzamidine	1,0 mM	Sigma-Aldrich	B6506
DTT (dithiothreitol)	1,0 mM	Fluka	43819
Sodium orthovanadate	1,0 mM	Sigma-Aldrich	S6508
Protease cocktail inhibitor	Protease Inhibitors (between 0,8 and 1004 µM)	Sigma-Aldrich	P8340

Table 2: Composition of PDE5 assay buffer, pH 7,5

Reagents	Final concentrations	Manufacturer	Catalog number from manufacturer
TRIS (hydroxymethyl aminomethane)	20,0 mM	Merck	1.08219.1000
Propionic acid	16,0 mM	Sigma-Aldrich	P1386
EGTA (Ethyleneglycol-bis(2-aminoethylether)-N,N,N,N'. N'-tetraacetic acid)	1,0 mM	Sigma-Aldrich	D2158
Magnesium acetate tetrahydrate	6,9 mM Mg^{2+} 13,8 mM acetate	Sigma-Aldrich	M5661
Guanosine 3',5'-cyclic monophosphate (cGMP)	9,0 µM	Sigma-Aldrich	G6129
Guanosine 3',5'-cylic phosphate. ammonium salt, [8-^{3}H]-	1mCi/ml	Perkin-Elmer	NET337001MC
Bovine serum albumin (BSA)	0,5 mg/ml	Sigma-Aldrich	A7511

4 Theory for methods

4.1 Cancer cell lines

Cancer cell lines C-4I and C-33A were chosen as the complex biological material for this assay. These cell lines were chosen because they have been used for studies concerning PDE5 by our research group. Both cell lines are robust and easy to work with and the doubling time is fast, approximately 24 hours, making them not so time-consuming to work with. A study done by Eggen and colleagues at University of Tromsø, has determined the mRNA expression of PDE5A in cancer cell lines, showing satisfying expression of PDE5A expressed in both C-33A and C-4I [39]. A percentage change of 1,6 observed for C-4I, and 1,2 for C-33A compared to GAPDH. Cell line WI-38 had the highest observed relative PDE5A mRNA expression, 38,1, but this is a not a cancer cell line, and prior studies show that they grow very slowly [39]. These were not chosen even though they had far more superior PDE5 mRNA expression, partly because they were time-consuming to work with, but mostly because they stems from a normal cell line, and we wanted to investigate the role in cancer cell lines.

4.1.1 C-33A

C-33A is a cancer cell derived from a series of cervical cancer of biopsies performed by one of the pioneers in gynecological cancer research, Dr. N. Auersperg. It stems from a female of cacausian heritage aged 66, and the initial disease was retinoblastoma [40]. The C-33A cell line initially expressed an hypodiploid karyotype with an epithelial morphology, but karylogical instability was observed with continued passage [41]. Expression of p53 is elevated and the retinoblastoma protein (pRB) is present, but in abnormal size [42].

4.1.2 C-4I

C-4I is also a cancer cell line derived from biopsies performed by Dr. N. Auersperg. It stems from a 41 year old female of Caucasian heritage with an exophytic stage II, grade IV invasive squamous carcinoma of the cervix [40]. Cell form is stratified epithelial colonies and show properties of squamous cell differentiation in long term culture [40]. It is tumorigenic with an epithelial morphology and contains human papillomavirus type 18 DNA sequences and expresses HPV-18 RNA

4.2 Anion exchange chromatography

4.2.1 Solid Phase Extraction

Solid phase extraction (SPE) is a step-wise form of chromatography designed to extract, partition or/and adsorb chosen analytes from a liquid phase onto a stationary phase (solid phase) [43]. The stationary phase varies, it could be a sorbent, resin or silica gel, depending on the analyte wanted for extraction. SPE is one of the most powerful techniques available for rapid and selective sample preparation prior to analytical chromatography or to simply extract certain analytes from a sample matrix [43]. There are various types of SPE methods available, depending on the analyte wanted for extraction in the sample matrix. There are 3 common variants of SPE, depending on the analyte of choice and the sample matrix of the liquid phase, and these three methods are reversed-phase, ion-exchange and normal-phase [43]. Reversed phase is based on hydrophobic interactions between the functional groups of the sorbent in the stationary phase and the hydrophobic character of analyte in sample matrix [43]. Reversed phase SPE retains most molecules with any hydrophobic character, making it the least specific of the different retention methods [43]. Ion-exchange chromatography is based on electrostatic interactions between the sorbent in the stationary phase and the analyte in the sample matrix, therefore both the analyte and the sorbent must be in its ionized form [43]. Normal-phase extraction is based on hydrophilic interactions between the sorbent in the stationary phase and the analyte in the sample matrix. In order to achieve these hydrophilic interactions, the analyte must be introduced to the sorbent in a non-polar sample, and typical sample matrices used for normal-phase include hydrocarbons and fatty acids diluted in an organic solvent [43].

For aqueous solutions (i.e. biological fluids, water, aqueous extracts of tissue etc.) reversed phase or ion-exchange are the methods available, depending on the polarity of the compounds. If it is moderately polar or non-polar, reversed phase is the best choice, and if the analyte in the sample matrix is weak cations/anions, ion-exchange is the better choice [43]. If the sample matrix is an organic solution (i.e. organic extracts of tissues, hexane, dichloromethane etc.), both normal phase and ion-exchange SPE can be applied, depending on the polarity of the analyte, for moderately polar or polar analytes, normal phase are the best choice, and if it the analyte in the sample matrix is strong cations/anions, ion-exchange can be applied [43].

4.2.1 Basic steps of SPE ion-exchange

1. Sample Pre-treatment: Samples are diluted with buffer of appropriate pH to ensure that the functional groups of the analyte are ionized [43].
2. Condition/equilibration: Conditioning of the SPE device is important for activating the sorbent, ensuring the sorbent will stay in its ionized form, and open the silica pores. For aqueous solutions methanol is often the choice. The equilibration step is important for ensuring that the solvent is in its ionized form, thus ensuring proper retention. For equilibration washing buffer with appropriate pH is often applied [43]
3. Sample loading: Sample should be loaded in a consistently manner, and the flow rate should not increase 1-2 drops/second to allow proper retention of analyte. [43]
4. Washing: Washing is applied to remove unwanted/unretained materials from the stationary phase. When choosing washing buffer one should pay attention to the pH of the buffer. The ionic strength of the buffer should not be so strong that it would neutralize the charge of either the sorbent or the analytes functional groups, leading to pre-maturely elution of analyte. But still strong enough to remove unwanted/unretained material from the sorbent. Often the washing buffer of choice is the same buffer as the sample matrix is dissolved/equilibrated in. [43]
5. Elution: For ion-exchange sorbents it is important to choose solutions which will neutralize either the analytes or the sorbents functional groups, reversing the retention caused by electrostatic interactions. Alternatively solvents with high ionic strength, displacing the adsorbed analyte, leading to elution of the analyte. [43]

4.2.3 Discovery® strong anion exchangers (DSC-SAX)

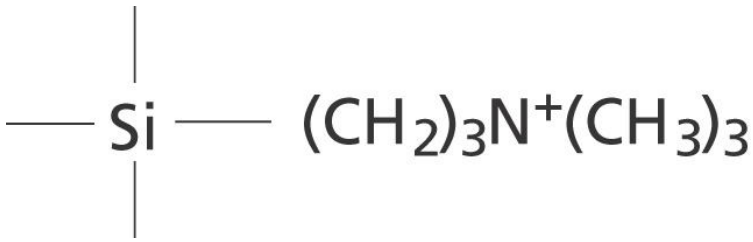

Figure 6: Chemical formula of the quaternary amine polymerically bound to the silica gel found in DSC-SAX columns. This quaternary amine will attract negatively charged ions via electrostatic interactions, leading to binding of negatively charged ions

Discovery® strong anion-exchange setup (DSC-SAX) by Supelco is ideal for adsorbing strong anions and other weak binding anions. The stationary phase (sorbent) is a quaternary amine group polymerically bounded to a silica surface [44]. The quaternary amine group is a strong base existing as a positively-charged cation (see figure 6) attracting anionic compounds in the sample matrix, giving it the name a strong anion exchanger (SAX) [44]. For conditioning of the DSC-SAX column, methanol is recommended for activation (ionization) of the polymerically bound quaternary amine in the silica gel. For washing an appropriate buffer should be applied, 2 pH levels above/below the pH of the sorbent, to ensure that unwanted retention and occupying of potential binding sites for samples do not occur. The superior choice for elution is benzenesulfonate, but others elution solvents work well, like citrate, chloride etc. [43]. Sodium benzenesulfonate is a strong acidic solvent, leading to disruption of the electrostatic interactions between the sorbent and ionic analytes due to the change of pH.

4.3 Radioactivity

A nuclide is defined as an atom with a particular number of protons and neutrons residing in the nucleus. A radionuclide is defined as a nuclide with the property of spontaneously converting mass into energy, and this energy is emitted as energetic particles and electromagnetic radiation [45]. This process of emitting radiation is more precisely defined as radioactive decay, but also disintegration or transformation is used. Radionuclides eventually decay into stable nuclides, and some even undergo a series of decays before reaching a stable nuclide. Radioactive decay happens spontaneously due to the instability in the nucleus of the unstable atom and without any interaction with other particles outside of the atom [45]. The process of radioactive decay is entirely random, thus making it impossible to predict, but when a large number of identical radionuclides are present, the fraction of unstable atoms that will decay over time can be specified [45]. Half-life is often used as a quantity to describe the radioactive decay. Half-life is defined as the time required for one half of the unstable atoms present to decay. The half-life of radionuclides is unaffected by chemical and physical environment of the atom, and the half-life of radionuclides differs from another, making it particular useful for measurement of radiation [45]. The quantity of radioactive material present is expressed in terms of the rate of decay at that given time. The most common unit for expressing radioactivity is Becquerel (Bq), given as one disintegration (decaying radionuclide) per second, but often Curie (Ci) is used, 1 Ci equals $3{,}7 * 10^{10}$ Bq [45].

There are four major types of radiation emitted by radionuclides, defined on the basis of type, energy and intensity of the radiation emitted.

- Alpha particles: Particles with a massive charge, identical with the ^{4}He nuclei. Emitted from the nucleus with discrete energies. For example, ^{234}U. [45]
- Beta particles: Particles with light charge, found in both positive (positron) and negative (negatron) forms. They have the same mass as an electron and emitted from the nucleus in a continuous range until maximum energy is reached. For example, ^{3}H, ^{14}C. [45]
- Gamma rays: Electromagnetic radiation emitted from the nucleus with discrete energies. For example, ^{137}Cs. [45]
- X-rays: Electromagnetic radiation emitted from the electron shells of an atom with discrete energies. For example: ^{131}I. [45]

Currently there resides three different sources for radioactive material, naturally-occurring, reactor-produced (by-product) or accelerator-produced. Naturally-occurring radioactive material is any radioactive material occurring naturally on earth, accelerator-produced material is produced by charged particle accelerators, and reactor produced (by-products) is produced in nuclear reactors, either as a by-product of the fission process or by the neutrons emitted [45]. Many of the radiochemicals used in modern biology are by-products material, where the nonradioactive atoms are substituted with radioactive counterparts, yielding a radioactive compound which is chemically identical to the initial nonradioactive compound [45]. A common radioactive applicant in modern biology is [^{3}H], also known as tritiated chemicals. The hydrogen isotope ^{3}H will decay into the daughter particle ^{3}He, emitting a beta-particle and one undetectable antineutrino.

$$ {}^{\beta}_{1}H \rightarrow {}^{3}_{2}He^{+} + e^{-} + \bar{\nu}_{e} $$

Figure 7: Formula for the decay of the hydrogen isotope ^{3}H

For measurement of the radioactive decay one can apply a beta-counter, based on the liquid scintillation counter (LSC), measuring the beta particles emitted.

4.3.1 Liquid scintillation counting

In the early stages of detection of radiation it was common to apply a ionization detection, using an insulating gas in a counting chamber [46]. The pulse of conductivity between two electrodes were measured, as emitted radiation leads to ionizing of atoms in the insulating gas, thus increasing the conductivity of the gas [46]. This had severe limitations as only gas phase isotopes could be detected. This was solved by applying a solid scintillation counting procedure. Many of the irradiated atoms are not fully ionized by collision with emitted radiation particles, but are promoted to an excited state [46]. Excited atoms have the ability of returning to ground state by emitting energy in form of a photon of light. By applying a crystal of organic or inorganic material which could be irradiated by the radioactive sample, the light emitted from the crystal could be used to determine the amount of radioactivity in the sample [46]. This is an excellent method for measuring gamma radiation, but has limitations when applied to alpha- and beta-radiation, as the crystal must be protected from contamination by the sample, and therefore the crystal must protected by a barrier, often this barrier lead to alpha- and beta-radiation not reaching the scintillation crystal due to the short path length of the radiation [46].

This was solved by applying the radioactive sample in a liquid scintillator cocktail rather than a solid crystal. A liquid scintillation cocktail were designed to absorb the energy emitted from radioisotopes, and re-emit the energy in form of light photons which could be measured by the use of a photomultiplicator system [46]. The liquid scintillation cocktail contains both a solvent and a phosphor. The solvent is used for the energy absorption whilst the phosphor converts the absorbed energy to light [46]. Scintillation cocktails must therefore act as an efficient collector of energy and must transfer the energy to phosphor molecules rather than dissipating the energy in other processes [46]. Phosphor must be dissolved in the cocktail and the cocktail should not quench the scintillation process occurring in phosphor. Phosphors are divided into two classes, primary and secondary scintillators. Primary scintillators are responsible for the conversion of energy to light, whilst the secondary scintillators (wavelength shifters) are responsible for capturing the emitted light from the excited primary scintillator, and re-emitting it as a longer wavelength signal [46]. This is due to the fact that most primary scintillators emit light at 408 nm, not ideal for measuring the response by a photomultiplier [46].

4.3.2 Beta-counters

Each beta-particle emitted in the scintillation cocktail leaves a trail of energized solvent molecules, and these molecules transfer their energy to scintillators molecules, emitting light with a characteristic wavelength [46]. Since each scintillation molecule emits only one photon when activated, the number of photons generated will be directly proportional to the path length of the beta-particle, which is based on the energy of the emission [46]. Thus will the intensity of each light pulse correspond with the energy emission and the number of pulses per second will correspond with number of radioactive emissions. Pulses are collected into channels and the counts per minute (cpm) is measured for each channel is recorded, where each channel corresponds to specific beta-energies, like [^{3}H] emissions or [^{32}P] emissions [46]. The cpm will thus be proportional to the amount of isotope in the sample. In a theoretical ideal cocktail, all of the emitted beta-energy is converted to light, but this is not the case. Many beta-counter accounts for this by giving the results as disintegrations per minute (dpm), correcting for the efficiency and background, giving the actual amount of decay per minute [46].

4.4 PDE5 assay

Currently there are many different commercial kits available for measuring PDE5 activity, but the disadvantage of many of these are that they do not work well with complex biological material, thus requiring purified PDE5. The purification of PDE5 is a difficult task, and not very often performed, and very time-consuming. The sum of these factors makes these commercial kits not ideal for determining the PDE5 activity in cancer cells. Many of these methods deliver purified PDE5 which can be used for screening potential inhibitors of PDE5. A widely used kit for this task is the PDE5A assay kit by BPS Bioscience, applying fluorescently labeled cGMP, generating fluorescent 5'-GMP, and PDE5 activity can be calculated by measuring the fluorescence of the product [47].

Currently there are a couple of different methods available for measuring PDE 5 activity in biological material like cancer cells. The most common methods apply the use of radioactive labeled cGMP, snake venom and mostly use anion exchange chromatography for separation [48-50], but some researchers also apply a fluorescent labeled silica-gel for separation [51]. This anion-exchange method stems from the early seventies, developed by the pioneers of cyclic nucleotide research, Earl Sutherland and Joseph Beavo. This method, though with slight modifications, is still used to this date, showcasing the robustness of the method. Therefore we modeled an assay for measuring PDE5 activity in cancer cells and for screening of potential inhibitors after the principle in this method. Many modifications have been applied and these modifications, and the process and consideration of these modifications, will be described in detail in the methods section.

4.4.1 History of PDE 5 assay designed by Beavo

In the beginning of the sixties when phosphodiesterase was discovered, there was observed a need for measuring the activity of PDEs, as it became clear that PDEs were responsible for the breakdown of cyclic nucleotides. Sutherland and Butcher designed a method for measuring PDE activity based on the 5' nucleotidase activity found in *Crotalus atrox* venom, harboring the ability of hydrolyzing 5'AMP/GMP (breakdown product of PDE) to adenosine/guanosine and inorganic phosphate [20]. For kinetic studies concerning PDE activity they applied the phosphate method, designed by Buell et. al, thus correlating the inorganic phosphate formed in the hydrolyzing reaction of 5'-AMP/GMP to the activity of PDEs [20]. As the field of molecular biology expanded and the use of radio-labeled substrates were introduced, Beavo and Sutherland started the progress of developing an assay for determining the PDE activity by amount of cyclic nucleotides hydrolyzed, instead of measuring a bi-product, thus making it a more robust method, discarding the

measurement of a bi-product formed in the dephosphorylating of 5'-AMP/GMP [20, 48]. Hence Beavo established a method for measuring the PDE activity, largely based on the original method described by Sutherland and Butcher, except this method applied the use of radioactive labeled substrate ([^{3}H]-cyclic nucleotides) [48]. The principle of the method was simple, but effective. The radiolabeled cGMP (see below in figure 8) has tritium (^{3}H) as substitution for hydrogen at position 8' of the guanine, making the compound chemically identical to cGMP, but harbors radiation. Therefor the breakdown of 5'-GMP to guanosine and inorganic phosphate will not cleave the radioactive group, thus is [^{3}H]-guanosine also radioactive. This is taken advantage of in the assay and a flow-chart of the procedure is given in figure 9.

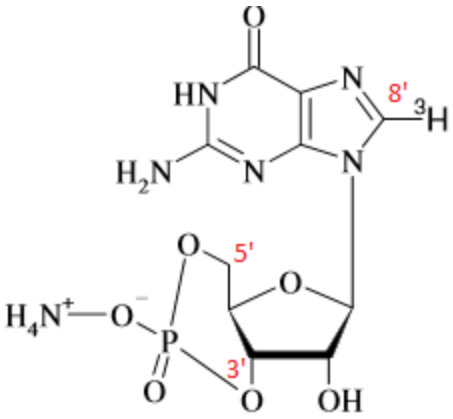

Figure 8: Chemical structure of [8-^{3}H]-guanosine 3',5'-cyclic monophosphate. The radionuclide is found at position 8 in the guanosine.

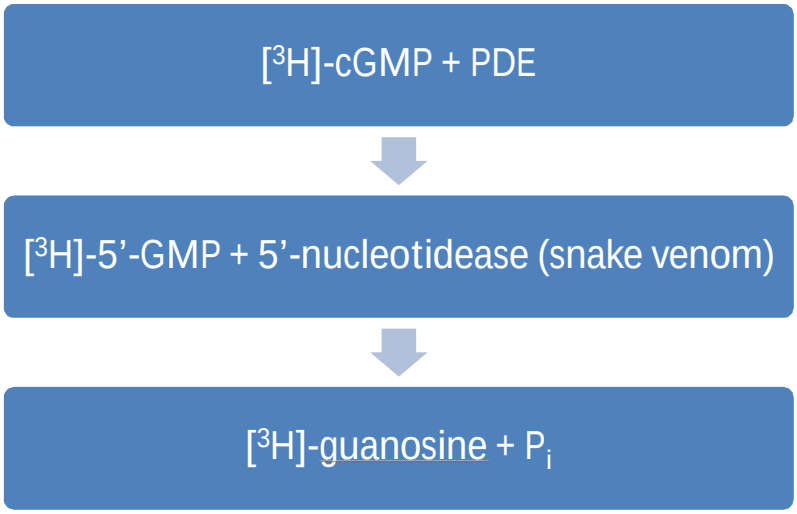

Figure 9: Flow-chart of breakdown of [^{3}H]-cGMP

Remaining cyclic nucleotide could then be separated from dephosphorylated tritiated products by anion exchange chromatography. For separation Beavo and colleagues applied a Dowex-2 resin exchange column [48]. Since radiolabeled cyclic nucleotides and nucleotides are negatively charged they will be attracted to the Dowex-2 resin column, which is positively charged. [^{3}H]-nucleoside have no charge, lacking the phosphate group responsible for the negatively charge of both cyclic nucleotides and nucleotides, and will be subsequently washed out in the washing progress, as only negatively charged compounds will be attracted to the column. Therefore one can measure the activity of PDE5 indirectly, by comparing the initial amount of radioactivity for [^{3}H]-cGMP with the amount bound to the column. This can be done as only tritiated cGMP and 5'-GMP, which is not hydrolyzed by snake venom, will bind to the column. The ratio between initial amount of radioactivity and measured radioactivity for the bound radionuclides thus correlates with the activity of PDE5. Subsequently one can measure the amount of radioactivity of the washing water, containing tritiated nucleosides not bound to the column. Measuring of the radioactivity in the eluate gives more precise results as the washing water contains certain amounts of tritiated substrate not bound to column initially, as results show that approximately 5 % of tritiated substrate bind to the column [48]. Often there are large amounts of washing water and there are limitations concerning the solubility of large aqueous solutions in the scintillation cocktail used for LSC.

4.4.2 Original set-up of the PDE5-assay as performed by Beavo

Cyclic nucleotides, containing either cGMP or cAMP at 500,000 dpm/ml were incubated at 30 °C with a phosphodiesterase preparation for 15 minutes in a medium consisting of 10 µM of Tris buffer, 0,5 µM $MgCl_2$, pH 7,5 in final volume of 250 µL[48]. Reaction were stopped by boiling in a water bath at 95-98 °C for 75 seconds, samples stores on ice for 1-2 min, and afterwards excess *Crotalus atrox* venom (0,05 to 0,10 mg) was added and incubated for 10 min at 30°C [48]. After incubation with venom, the reaction was applied to a Dowex-2 resin column (4,5 x 0,5 cm), pre-conditioned with 0,04 M Tris-Cl buffer to appropriate pH 7,5 [48]. Column was washed three times with 26 mL Tris-Cl buffer and eluted with 35 mL of 0,3 M HCl.[48] Aliquots of both washing-fractions and eluate-fractions were counted in a liquid scintillation spectrometer [48].

5 Methods

5.1 Cell-culturing

Cell-lines C-33A and C-4I were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in flasks from Nunc containing polystyrene for adhesion. Cells were cultured in RPMI-1640 w/glutamine (Cat. No. R0833, Sigma-Aldrich), with 10 % (v/v) fetal bovine serum (Cat. No. R7524, Sigma-Aldrich), and 10000 units penicillin and 10 mg streptomycin (Cat. No P0781, Sigma-Aldrich). Cell density was monitored daily by observation in a microscope. Cells were split by trypsination with 0,25 % Trypsin-EDTA (Cat. No. T4049, Sigma-Aldrich) when the cell density were satisfying. This was performed to achieve satisfying amount of cells. When satisfying amount of cells were present and cell density was satisfying in each flask, 7-8 T-175 (Nunc) flasks were harvested for each cell line. Before harvesting of the cells, cells were washed 3X in ice-cold PBS (Cat. No. D8573, Sigma-Aldrich). Cells were covered in a minimal amount of ice-cold lysis buffer (for composition see table 1) and harvested by scraping. Harvest by trypsination was not the chosen method as there was uncertainty about how trypsin would affect the PDE5 enzyme, as there always would be some small amount of trypsin left after centrifugation which potentially could degrade the PDE5 enzyme. Harvesting of cells using lysisbuffer had previously shown good results from studies by Sonnenburg and Beavo, so the lysisbuffer was modeled after the lysisbuffer used by Sonnenburg[49]. Lysisbuffer thus contained different materials needed for lysing cells and protecting enzymes found in the cytosol, like various protease inhibitors (benzamidine, protease inhibitors found in protease cocktail inhibitor), inhibitors of endogenous phosphatases (orthovanadate), reducing agent (DTT) and a calcium chelator (EGTA). 3mL of ice-cold lysisbuffer was used for 2 T-175 flasks, scraping cells first, and scraped cells were transferred to the next flask. Steps were repeated, yielding a total volume of approximately 12 mL scraped cell lysate for 7-8 T-175 flasks. Cell lysate was homogenized using 25 strokes by the pestle of a Dounce homogenizer in a chilled room. Homogenized cell lysate were centrifuged at 100,000g for 1h at 4°C, and supernatant collected. Ultracentrifugation leads to sedimentation of the lysate and since PDE5 is a cytosolic protein it will be found in the supernatant, as the pellet mainly contains cell walls, broken cells etc. Lysate was frozen at -80 °C until use.

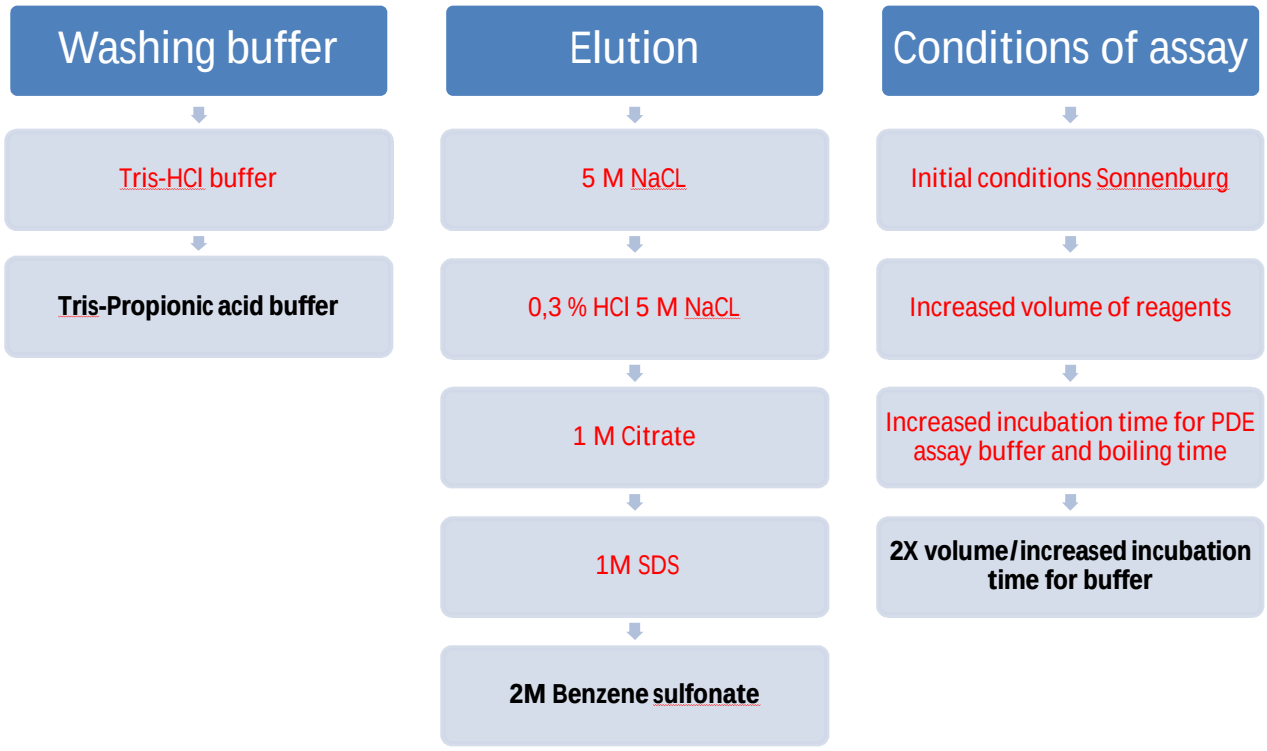

Figure 10: Flow-chart for the process leading to the final conditions of the assay. Boxes marked with red indicate steps tried out and not yielding promising results, and boxes marked with black indicate the final choice.

5.2 Development of PDE5 assay

The assay developed is very similar to the assay developed by Beavo in 1970, thus with some modifications. A brief overview of the modifications are given in the flow chart sketched in figure 10. The assay developed is based on the assay performed by Sonnenburg et.al which is largely based on the initial method described by Beavo, but Sonnenburgs assay gave more details concerning concentrations of solutions used and gave a proper description of preparation of cell lysates [49].

5.2.1 Choice of column for anion-exchange

The first problem encountered was choosing the best suited column for anion exchange. The initial assay used a Dowex-2 resin column, but today better choices for this assay are available. A widely used column for anion ion-exchange chromatography is a column based on Sephadex, a cross-linked dextran-gel. The composition of the gel varies, but the stationary phase usually consists of bead-formed gel, cross-linking dextran with an ion exchange group, like diethylaminoethyl (DEAE) [52]. DEAE-Sephadex works well as an anion-exchange column and has shown good results for weak anions of low molecular weight, like cGMP. A major disadvantage of DEAE-Sephadex columns is the preparation and packaging of columns, making it very time-consuming. Another widely used column for weak anion exchange chromatography is the SAX-column, which can be bought pre-packaged and ready to go, like the DSC-SAX columns described earlier. But it was uncertain how well this column could bind [^{3}H]-cGMP, as no prior research concerning [^{3}H]-cGMP binding was found.

5.2.2 Pilot assays of columns

To look further into this a pilot study was constructed, investigating the ability of binding of [^{3}H]-cGMP, to both DEAE-Sephadex columns and to DSC-SAX columns. The sephadex column of choice were DEAE-Sephadex A-25 (Cat.no 17-0170-01, GE Healthcare), packed according to protocol in empty columns [52], and the pre-packaged DSC-SAX 100mg/1ml columns from Supelco (Cat. No 52662-U, Sigma-Aldrich). The set-up of the anion ion-exchange pilot assay were largely based on the set-up from the assay of Sonnenburg and Beavo, and recommendations from manufacturer, but with certain modifications described in detail later [48, 49, 52]. These results showed that both the DSC-SAX columns and DEAE-Sephadex columns adsorbed [^{3}H]-cGMP well (results not shown). It became clear that the DSC-SAX columns were the better choice because packing of the DEAE-Sephadex columns were time-consuming, and DEAE-Sephadex is a very fluffy material, making it more laborious to handle than the pre-packaged, ready-to-go DSC-SAX columns. The sedimentation process of the DEAE-Sephadex material is difficult to reproduce for each column, thus it could lead to more variations in between the columns. DEAE-Sephadex columns were discarded and the process of optimization of the conditions of the DSC-SAX 100mg/1ml column from Supelco was started.

5.2.3 Optimization of DSC-SAX 100mg/1ml columns

During the process of optimization various problems were encountered, and many modifications were made to achieve a robust assay with reproducible results. The first problem encountered was the elution process. The first choice of eluent was 5M NaCl, as this was used by Sonnenburg and colleagues [49]. It was shown that 5M NaCl was not a proper eluent for this DSC-SAX column as a second elution step showed that a large portion of [^{3}H]-cGMP was still retained in the column after the first step of elution (results not shown). These findings were not in consensus with results achieved by others [49], and what was to be expected. As the original assay described by Beavo used 0,3 % HCl, a combination of 5 M NaCl and 0,3 % HCl solution were prepared and tested as an elution solvent [48, 49]. This showed better results, but still a large fraction of [^{3}H]-cGMP was still retained in the column (results not shown). As both of these assays applied another column than the DSC-SAX column used, we had to dig further into literature concerning the DSC-SAX column. The hand-book from Supelco states that sodium benzenesulfonate is the far superior eluent for the DSC-SAX column applied in this assay [43], but as benezenesulfonate had to be ordered other substances were tried as elution solvents in the meantime. Citrate was the second strongest elution solvent, and sodium dodecyl sulphate (SDS), a solvent similar in structure to sodium benzensulfonate were applied as elution solvent. These elution solvents did not show satisfying results. Citrate was not completely dissolved in the scintillation cocktail mix making the elution results vary. SDS dissolved in the scintillation cocktail, but there was not observed proper elution. Pilot studies using sodium benzenesulfonate showed very satisfying results from elution as it completely dissolved in the scintillation cocktail, and the second elution step showed that little to none [^{3}H]-cGMP was retained in the column (see table 3) after the first elution step. For washing of the column a washing buffer consisting of 20 mM Tris and 16,4 mM propionic acid, pH 7,5, was used. This washing buffer was based on the set-up of Sonnenburg with a Tris-HCl washing buffer, but HCl was exchanged with propionic acid due to the role of chloride as a potential eluent of the column[49]. It was shown that this washing buffer worked very well, and as little as 1,2 % of the [^{3}H]-cGMP were washed away, see results in table 3.

5.2.3 Pilot study of the DSC-SAX 100mg/1ml ability of adsorbing [^{3}H]-cGMP

For conditioning of the DSC-SAX 100mg/1mL column (Cat. No. 52662-U, Sigma-Aldrich) 500 µL of methanol was added, and for equilibration 500 µLTris-propionic acid (washing buffer), pH 7,5 was added. Sample used were 1mCi/ml [^{3}H]-cGMP (Cat. No. NET337001MC, Perkin-Almer, Waltmar, MA, USA) diluted in lysisbuffer, yielding final concentration of approximately 800000 dpm/ml of [^{3}H]-cGMP in a total volume of 500 µL. Samples were applied to a preconditioned/equilibrated DSC-SAX column in triplets. Sample was drained with gravity, thus not exceeding 1-2 drops per minute, as the sample had to be drained slowly and dropwise for allowing hydrostatic interactions to occur. After the sample was drained, the column was washed 5 times with 100 µL washing buffer. Washing water was collected, and 400µL of washing water was transferred to a scintillation vial containing 10 mL scintillation cocktail, Ultima Gold XR LSC cocktail (Cat. No. L8411, Sigma-Aldrich), and radioactivity was measured using a beta-counter, Packard tri-carb 1900TR liquid scintillation analyzer. For elution 500 µL of 2M sodium benzenesulfonate (Cat. No. 147281-256, Sigma-Aldrich) was chosen and applied dropwise to the column, and eluate was collected. 400 µL of eluate was transferred to a scintillation vial containing 10 mL scintillation cocktail, Ultima Gold XR, and radioactivity was measured by the same beta-counter. This elution step was repeated, to assure proper elution and thus assuring that there was no [^{3}H]-cGMP retained in the column.

5.2.4 Pilot studies of PDE5 assay

At first the conditions of the assay were modeled after the Beavo assay, as described in section 4.4.2, using 50 µL cell lysate diluted in 100 µL water, which was pre-incubated at 30°C for 1 min. For starting the reaction, 150 µL PDE assay buffer (for composition see material section) was added, containing approximately 4230000 dpm/ml [^{3}H]-cGMP (Cat. No. NET337001MC, Perkin-Almer, Waltmar, MA, USA), 6,9 mM Mg^{2+}(key activator of PDE5 enzyme), 3,4 mM EGTA (chelating calcium) and 0,5 mg/ml BSA needed for PDE5 to function properly in a total volume of 250 µL. This reaction mix was then incubated for 10 min at 30 °C [49]. Reaction was stopped by immersion in a boiling water bath at 100 °C for 1 min, and then cooled on ice for 1-2 minutes. For breakdown of hydrolyzed 5'-GMP, 2,5 µL 10 mg/ml *Crotalus atrox* snake venom (Cat. No. N5880, Sigma-Aldrich) was added and incubated for 10 min at 30 °C, and then diluted in 250 µL washing buffer (Tris-propionic acid, pH 7,5) to achieve proper pH, yielding a final concentration of 12670000 dpm/ml of radionuclides. Lysis buffer (containing no lysate) was used as blank to mimic conditions found in cell lysate, and treated exactly the same as the samples. Samples were then

applied to a pre-condition/equilibrated DSC-SAX 100mg/1ml column, as described. For washing, 100 µL washing buffer (Tris-propionic acid, pH 7,5) were applied 5 times. For elution 500 µL of 2M benzenesulfonate was applied. 400 µL of eluate was transferred to a scintillation vial containg 10 mL scintillation cocktail (Ultima Gold XR), and radioactivity was measured using a beta-counter as described.

The results (not shown) from these pilot studies showed little to no difference between the blank and the samples. These results were not in consensus with expectations. The expression of PDE5A mRNA was shown to be relatively low in these cancer cell lines [39], so we assumed that if the amount of lysate and the incubation period was increased, there would be observed more promising results, as shown in the flow-chart in figure 10. These tweaks showed promising results and was further investigated by performing a linearity test for a series of dilution of cell lysate, and a linearity test where the incubation time for the PDE 5 assay buffer varied. Both of these linearity tests showed a satisfying linearity (see results section), suggesting that this was a robust assay and we could proceed with testing inhibitors. There was also observed that increasing the boiling time to 5 minutes lead to decomposition of [^{3}H]-cGMP and poor binding to the column, and varying retention of [^{3}H]-cGMP in the column (results not shown).

5.3 Final PDE 5 assay set-up

100 µL of ice-cold fresh lysate was diluted in 200 µL water in an eppendorf tube, 5 parallels, and pre-incubated for 1 min at 30 °C. When inhibitors were tested, they were diluted in water yielding a final inhibitor concentration of 0,5 µM. Inhibitors used were sildenafil citrate (Cat. No PZ0003, Sigma-Aldrich), IS-39212 (Ambinter, Greenpharma SAS, Orlèans, France) and IS-60049 (Ambinter, Greenpharma SAS, Orlèans, France). The PDE assay was started by adding 200 µL ice-cold PDE assay buffer (for composition see table 3 in materials section), containing approximately 6335000 dpm/ml [^{3}H]-cGMP (Cat. No. NET337001MC, Perkin-Almer, Waltmar, MA, USA), yielding a final volume of 500 µL. Reaction mix was incubated at 30 °C for 25 min. Reaction was stopped by immersion in a boiling water bath at 100 °C for 1 min, and reaction mix was cooled on ice for 1-2 min, and then 5 µL 10 mg/ml *Crotalus atrox* snake venom (Cat.No N5880, Sigma-Aldrich) was added and reaction mix was incubated for 10 min at 30 °C. After incubation with snake venom, the reaction mix was diluted in 500 µL washing buffer (Tris-propionic acid, pH 7,5) to achieve appropriate pH of the sample matrix, yielding a final concentration of approximately

1267000 dpm/ml of [^{3}H]-radionuclides. Sample matrix was then applied to a pre-conditioned/equilibrated DSC-SAX 100mg/1mL column (Cat. No. 52662-U, Sigma-Aldrich) with 500 µL methanol used for conditioning and 500 µL washing buffer (Tris-propionic acid, pH 7,5) for equilibrating. Sample was drained by gravity for achieving a slow and proper adsorption, not exceeding 1-2 drops per second. After the sample was drained through the column, the column was subsequently washed five times with 100 µL washing buffer, dropwise and by the use of gravity. The column was then eluted with 500 µL 2M sodium benzenesulfonate (Cat. No. 147281-256, Sigma-Aldrich) dropwise and by the use of gravity. 400 µL of eluate was transferred to a scintillation vial, containing 10 mL scintillation cocktail, Ultima Gold XR LSC cocktail (Cat. No L8411, Sigma-Aldrich) and radioactivity of eluates was measured using a beta-counter, Packard tri-carb 1900TR liquid scintillation analyzer.

6 Results

6.1 Adsorption of [^{3}H]-cGMP to DSC-SAX columns

The results presented in table 3 show that the adsorption of [^{3}H]-cGMP to the DSC-SAX column is very satisfying. The percentage of [^{3}H]-cGMP not adsorbed in the column is as little as 1,2 %. In the initial assay designed by Beavo there was observed as high as 5 % unretained [^{3}H]-cGMP, so these results indicate that the DSC-SAX columns are superior for binding of [^{3}H]-cGMP. The second elution step showed that 2M sodium benzenesulfonate was a superior choice for elution, as there was a vanishing amount of [^{3}H]-cGMP retained on the column after the first elution. Relying on these results the testing of PDE5 activity in cell lysates could precede as this column was shown as a very good choice for this assay.

Table 3: Results from pilot assay testing adsorption of [3^H]-cGMP to DSC-SAX columns. Samples were run in triplicates in conditions described in section 5.2, results given as corrected mean value of dpm measured. Correction factor is 1,25 due to only 400 µL of initial 500µL eluate was added in scintillation cocktail

Washing solution, dpm	**1. elution, dpm**	**2. elution,dpm**
8995±189	772366 ± 35246	5386 ± 560
Unretained [^{3}H]-cGMP		1,2 %

6.2 Linearity assays

Results from the lysate dilution series show a satisfying linearity between the measured mean radioactivity of eluates as a function of percentage of lysate, as seen in figure 11. This shows that there is observed a linear correlation between the activity of PDE5 and the measured radioactivity for each eluate. This is an important step in the process of validating a method, as there has to be a linear correlation between the amount of sample and the activity measured for the method to be trustworthy and gain reproducible results. This shows that the method and the final conditions used for the assay are robust and ready for further testing.

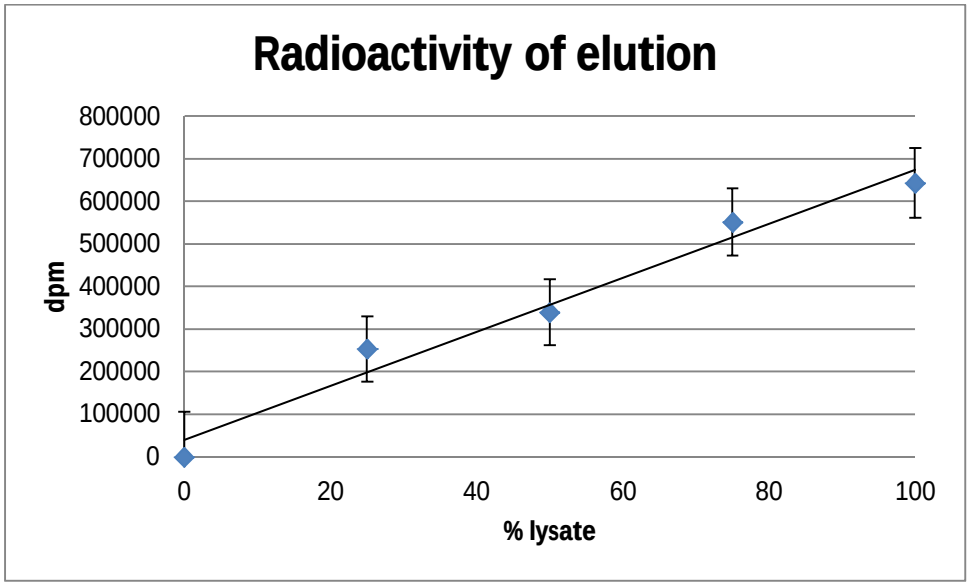

Figure 11: Results from the dilution series of the lysate. Cancer cell line used was C-4I. A series of dilutions of the lysate were analyzed (100 %, 75 %, 50 % 25 % and 0%) on the fixed conditions for the assay, as described earlier in section 5.3. Samples were run in triplicates. Results presented as mean percentage of the difference between samples and blank. Lysisbuffer was used as blank to mimic the conditions of the lysate.

The results from the linearity assay where the incubation time were varied at fixed conditions show a satisfying amount of linearity between mean measured radioactivity for eluates and incubation time, as seen in figure 12. There should be observed linearity between the incubation time and the measured mean radioactivity as the activity of PDE5 should increase at a constant level when the incubation increases, assuming the enzyme is saturated. Even though the standard deviation is high for each incubation time, there is observed a fair linearity. This is another important step for validating a method, as there should be correlation between the activity and the incubation time for producing trustworthy and reproducible results. These results show that the conditions of the assay are satisfying, making this an even more robust assay ready for screening of inhibitors.

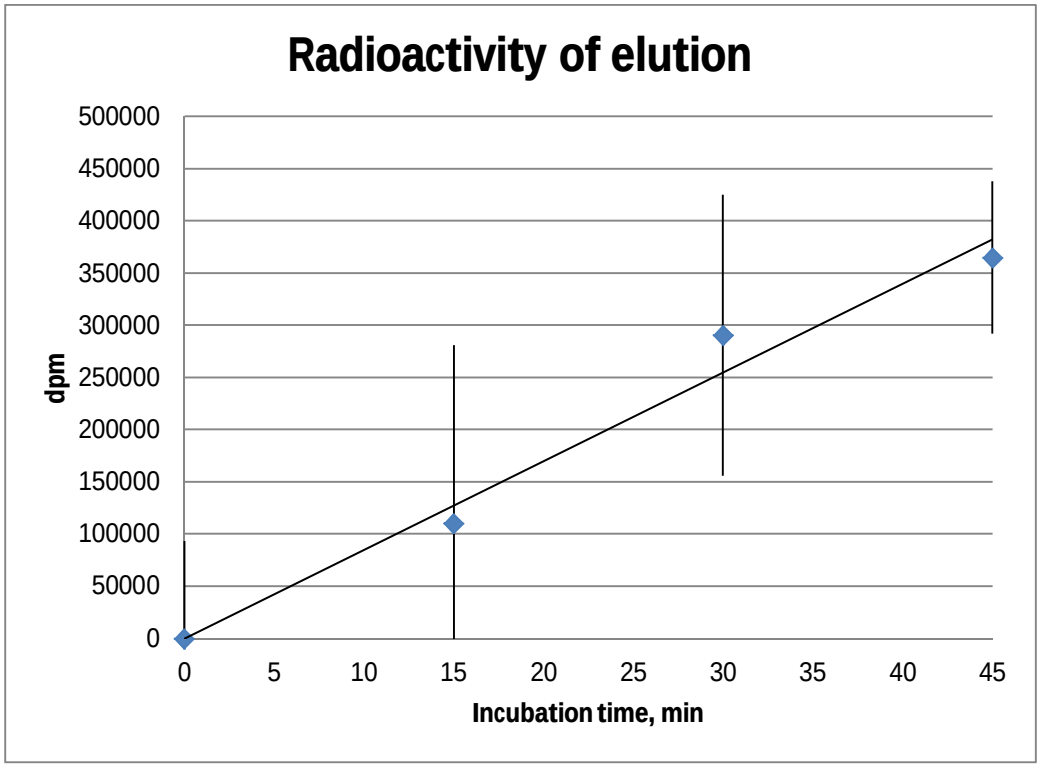

Figure 12: Results from the linearity assay where the incubation time was varied. Cancer cell line used was C-4I. Fixed conditions were applied, as described in section 5.3, but the incubation time with PDE5 assay buffer of each reaction varied (0 min, 10 min, 20, min, 30min and 40 min). Samples were run in triplicates. Results presented as the mean time difference between samples and blank

6.3 Relative PDE5 activity for each cell line

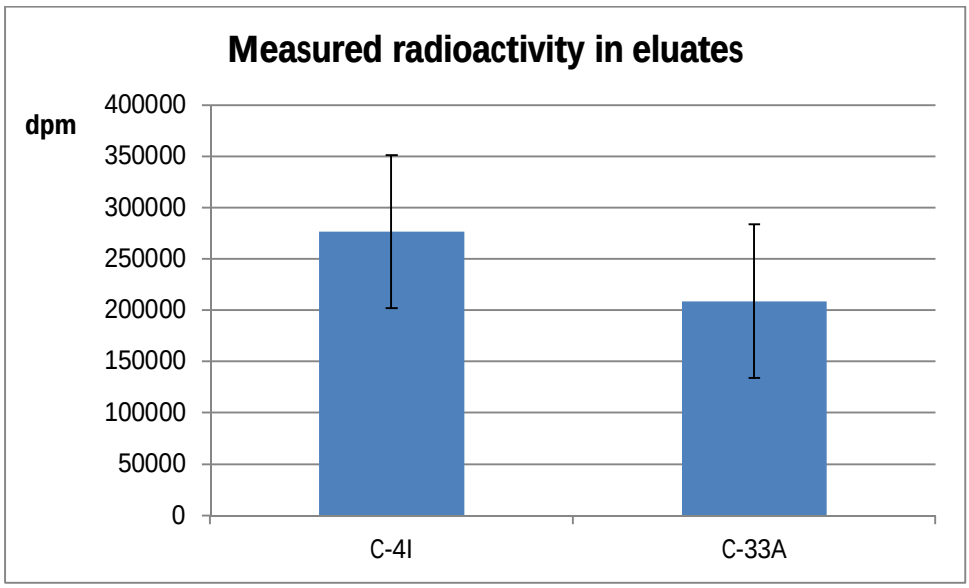

Figure 13: Results from determining the activity of PDE5 for both C-4I cells and C-33A cells, conditions described in section 5.3. Samples were run in 5 parallels, and radioactivity was measured for each eluate, and presented in these columns as mean difference between blank and samples. Blank consisting of lysisbuffer, mimicking the conditions of the lysate, but not containing any actual cell lysate. Values for eluates was corrected as only 400 µL of total 500 µL eluate was transferred, correction factor was 1,25.

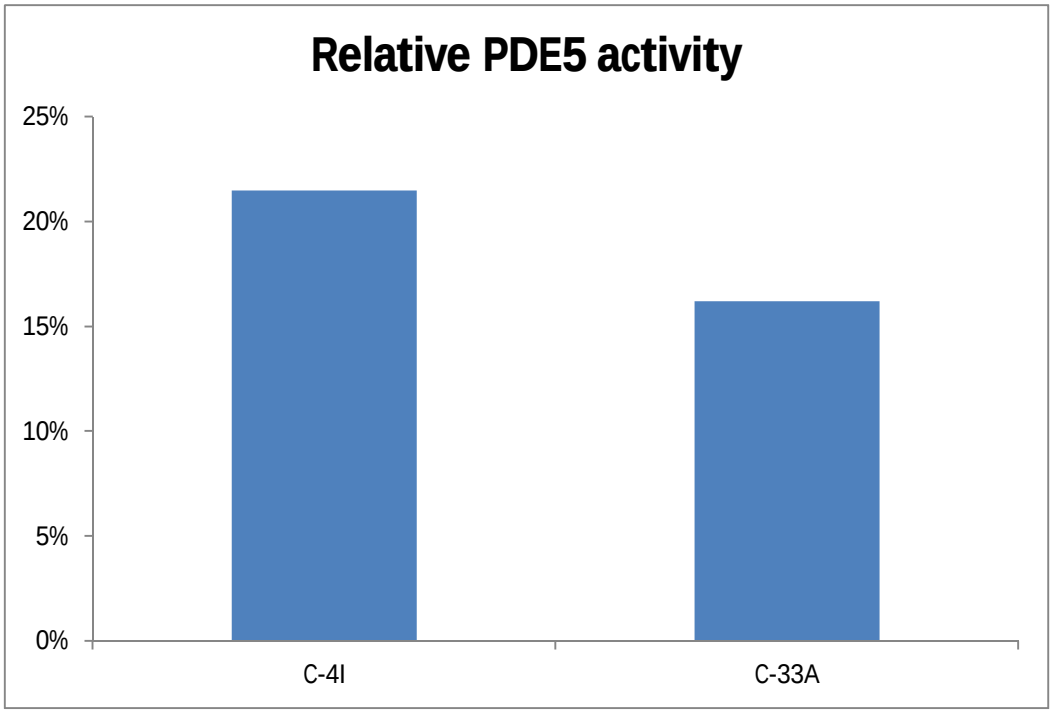

Figure 14: Relative PDE5 activity for cancer cell lines, C-4I and C-33A. The relative activity is given as the ratio between the measured radioactivity for blank and samples in comparison with the initial amount of radioactivity. Calculated as: $\frac{DPM\ blank - DPM\ eluate}{DPM\ total} * 100\ \%$

Washing water was not collected as the eluate contains [^{3}H]-cGMP and [^{3}H]-5'-GMP, and washing water contains [^{3}H]-guanosine which is not negatively charged, thus not binding to the column. Therefore the decrease in measured radioactivity in the eluate for each cell line compared to blank (containing lysisbuffer without cell lysate) is consistent with the PDE5 activity found in the lysate. This is due to the simple fact that when lysate is present, containing PDE5, [^{3}H]-cGMP will be hydrolyzed to [^{3}H]-5'-GMP, and this is the substrate for the 5'-nucleotidase found in snake venom from *Crotalus Atrox*. The 5'-nucleotidase hydrolyzes [^{3}H]-5'-GMP further to [^{3}H]-guanosine which does not bind to the column, and is subsequently washed away in the washing process. When there is no PDE5 activity present, as is the case for the blank (lysisbuffer containing no lysate), [^{3}H]-cGMP is not hydrolyzed, hence no [^{3}H]-guanosine will be formed, leading to almost complete binding of initial amount of radioactive [^{3}H]-cGMP. Therefore one can give relative PDE5 activity as the difference between the measured radioactivity for the blank and the measured radioactivity for each eluate, compared to the initial amount of radioactivity for the eluate. It is therefore

important that the PDE5 enzyme is saturated, so it works with constant velocity during the incubation time. Depletion of the substrate during the incubation period leads to reduced velocity since the velocity is correlated to the concentration of the substrate. Backing on the linearity assays shown in figures 11 and 12 we concluded that in the applied conditions for the assay, as described in section 5.3, the enzyme is saturated. If it is not saturated in the assay, the results will vary, as more [^{3}H]-cGMP are not hydrolyzed, yielding false results which are not trustworthy. Since both the samples for lysates were treated exactly the same and fresh lysate was used, the day-to-day variation was eliminated.

6.4 Screening of potential inhibitors on cancer cell lines C-33A and C-4I

Table 4: Raw-data for figure 15. DPM values corrected with conversion factor 1,25 as described. The relative PDE5 inhibition is explained later in section 6.5

	Blank (uninhibited)	Sildenafil	IS-60049	IS-39213
Corrected DPM values ± SD	329490 ± 33341	386136 ± 32088	387544 ±13067	417292 ±27813
Mean DPM - Blank	0	150193 ± 46275	140662 ±13070	171341 ± 43420
Relative PDE5 inhibition, %	0 %	16 %	15 %	18 %

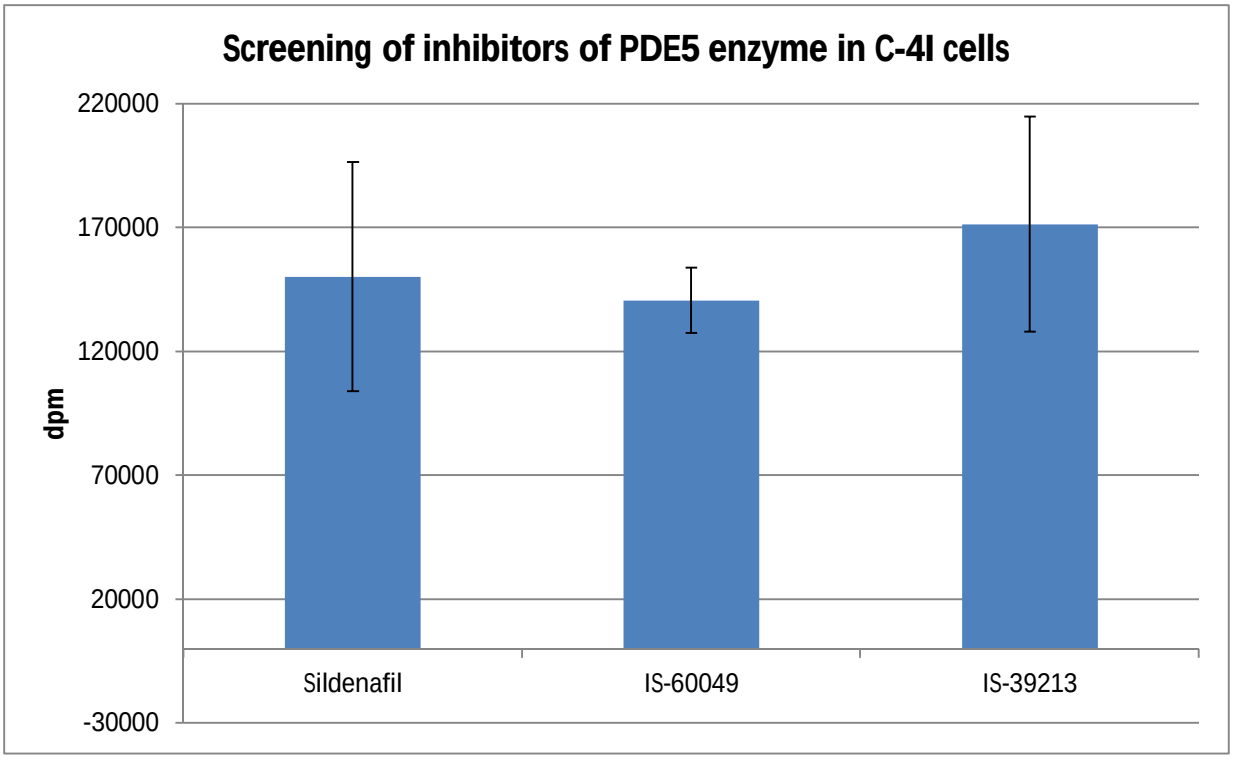

Figure 15: Results from screening of inhibitors on C-33A cells. Samples were run in 5 parallels. Conditions of assay were as described in section 5.3, final concentration of each inhibitor was 0,5 µM. Results presented as the mean difference between the measured radioactivity for the blank and samples containing inhibitor. Blank consisting of uninhibited C-33A-cells

Table 5: Raw-data for figure 16. DPM values corrected with conversion factor 1,25 as described and relative PDE5 inhibition is explained later in section 6.5

	Blank (uninhibited)	**Sildenafil**	**IS-60049**	**IS-39213**
Corrected DPM values	364679 ± 17205	482671 ± 13931	484430 ± 13520	521615 ± 28176
Mean DPM – blank		117993 ± 22139	119752 ± 21883	156936 ± 33014
Relative PDE5 inhibition		13 %	13 %	17 %

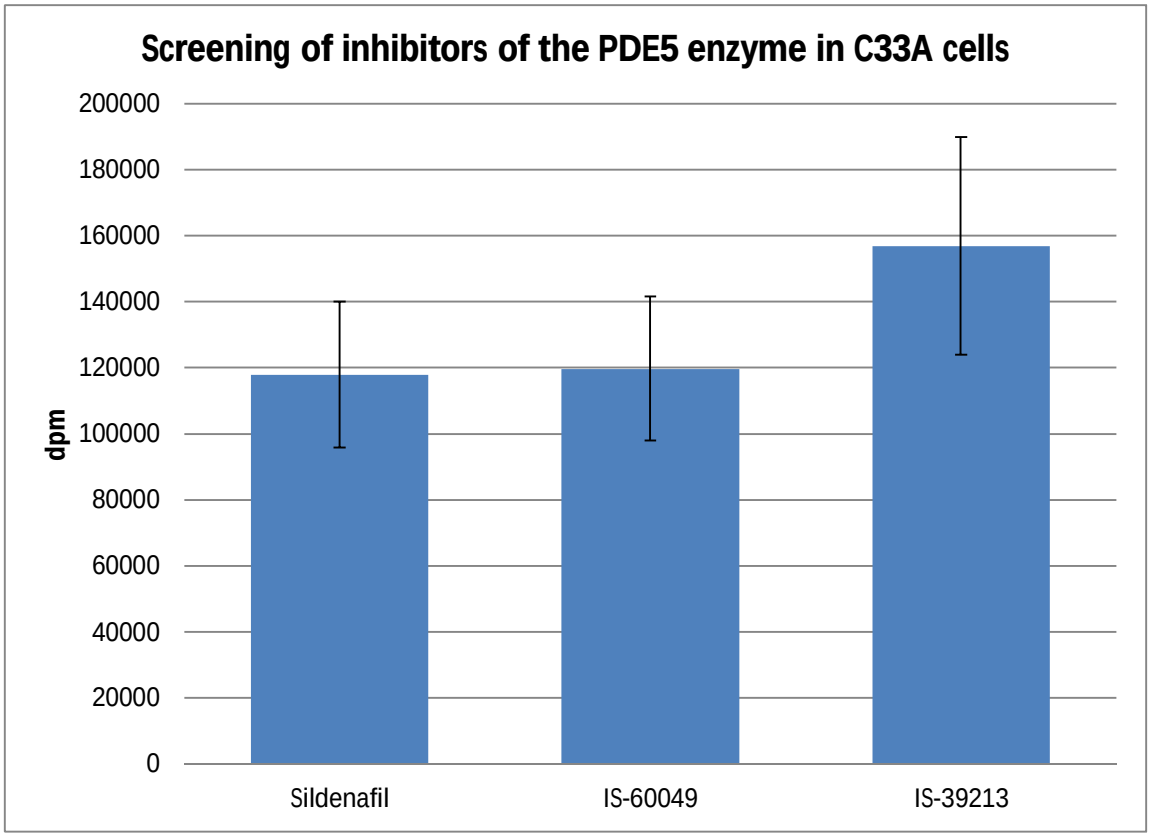

Figure 16: Results from screening of potential inhibitors of the PDE5 enzyme in C-4I cells. Samples were run in 5 parallels. Conditions of assay were as described in section 5.3, final concentration of each inhibitor was 0,5 µM. Results presented as the mean difference of measured radioactivity for blank and samples containing inhibitors. Blank consisting of uninhibited C-4I cells.

6.5 Relative PDE5A inhibition of cancer cell lines C-33A and C-4I

Since inhibiting of the PDE5 enzyme leads to an increase in measured radioactivity, the relative PDE5 inhibition of each inhibitor can be calculated. This can be done by applying a simple formula. Assuming the PDE5 enzyme is saturated, the full activity of the uninhibited enzyme could be given by comparing the measured radioactivity in the eluate with the initial amount of radioactivity, and this is set as 100 % PDE activity. When an inhibitor is present the activity of PDE5 decreases, leading to an increase in measured radioactivity for the eluate for the inhibited cells. By comparing the ratio of measured radioactivity in inhibited cells with the activity found in uninhibited cells in comparison to the initial amount of radioactivity, the relative PDE5 inhibition can be given as:

$$(1- \frac{DPM\ total - DPM\ sample\ eluate}{DPM\ total - DPM\ blank\ eluate}) * 100\ \%$$

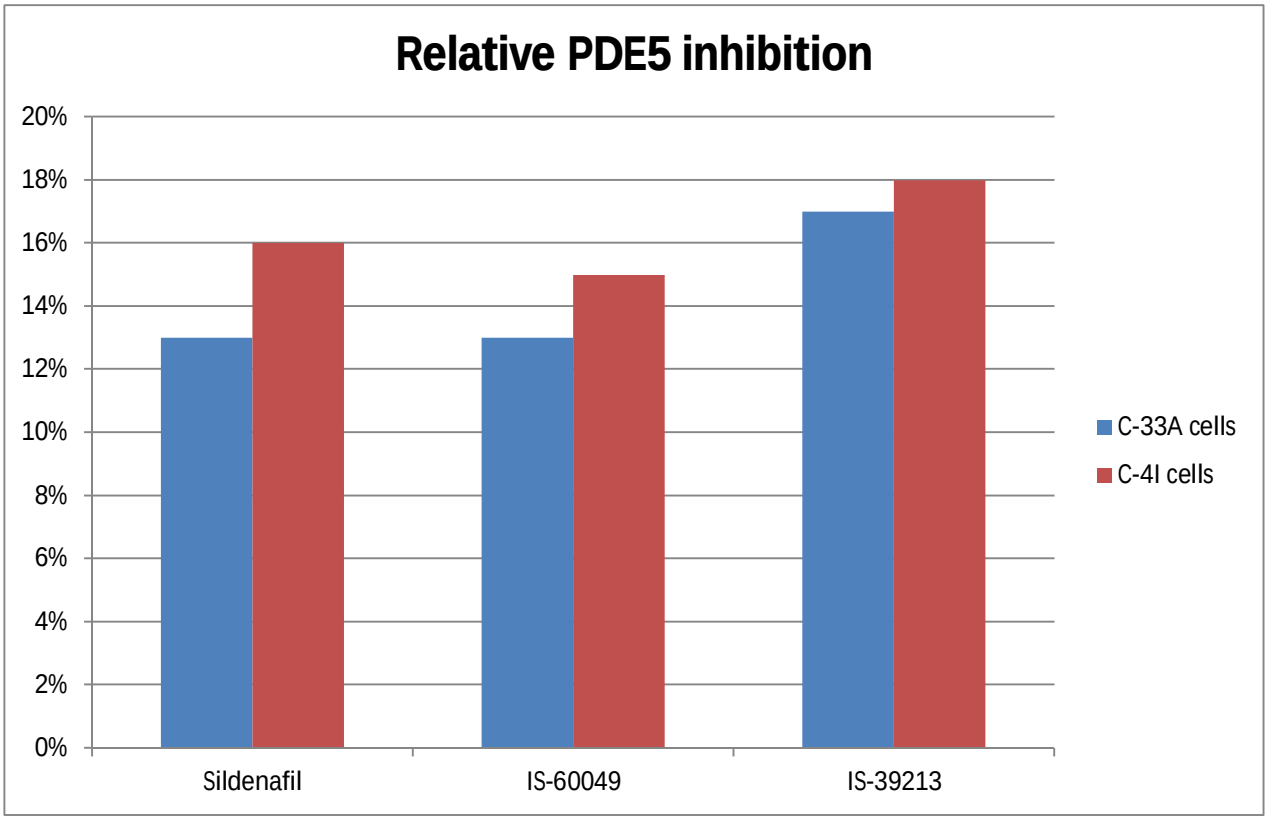

Figure 17: Relative PDE5 inhibition for all inhibitors on both cancer cell lines, raw data taken respectively from table 4 and 5.

7 Discussion

Many clinical studies have shown that cyclic nucleotides are found in varying levels in urine, and it have been suggested by many as a prognostical marker for various types of cancer, like uterine cervix cancer and ovarian cancer [53, 54]. The role of cGMP in regulation of apoptosis is well-documented, and it is shown to work as a regulator of many pro-apoptotic, and anti-apoptotic pathways like protein kinase G I, JNK-pathway, beta-catenin amongst others [55]. Following these findings and the knowledge of ABCC-transporters, there has been a large interest in investigating the role of inhibitors of the ABCC5-transporter as a tool in cancer research. Suggesting that the high cGMP-efflux observed in cancer cells is due to the upregulation of the ABCC5 transporter [38].

Many studies have confirmed that in various cancers and cancer cell lines there is observed an upregulation of ABCC5 expression, and this may be responsible for the observed levels of cyclic nucleotides found in urine. Mourskaia and colleagues recently showed that ABCC5 transporters were overexpressed in breast cancer osseus metastases derived from primary breast tumors, suggesting ABCC5 transporters as a potential therapeutic target for breast cancer [56]. Eggen and colleagues at University of Tromsø also showed that there was an observed increase of ABCC5 regulation during growth phase of human cervical cancer cell lines, whilst the expression of PDE5 virtually remained unchanged [39]. This was further backed as the normal W-38I cells showed a moderate upregulation of ABCC5 transporters during growth, whilst the expression of PDE5 dramatically increased [39]. These findings supports that the upregulation of ABCC5 is one of the mechanisms observed in cancer cells for escaping apoptosis by extruding a potential pro-apoptotic initiator like cGMP.

Various research groups, including our research group, Medical Pharmacology and Toxicology at University of Tromsø, are investigating this further by looking into potential inhibitors blocking the ABCC5 transporter pump. It is shown by studies that sildenafil is a potent inhibitor of the ABCC5 transporter pump [37], and it has been shown by Shi and colleagues that sildenafil inhibits the ABCB1 and ABCG2 transporters, thus implicating that sildenafil and other PDE5 inhibitors may enhance the sensitivity of certain cancer cells to chemotherapy, by reversing the multi drug resistance observed in many cancer cells [57]. Based on these findings our group has investigated potential inhibitors based on sildenafil, as described earlier [38]. These studies showed there were two inhibitors which were of particular interest, IS-60040 and IS-39213, almost completely blocking the ABCC5 pump as measured efflux of cGMP out of the cell was 0%, compared to sildenafil which had an observed efflux of 16,2 % [38]. These are promising results and may give rise to a potential new cancer treatment by strengthening the apoptotic machinery in the cell by blocking the export of potential pro-apoptotic effectors, like cGMP in cancer cells, and by reversing the multi drug resistance observed in many cancer cells, thus allowing chemotherapeutic substances to have longer effect on the cell.[37, 38] Further investigations are needed to explore the potential role of these inhibitors as a cancer tool, but it is clear that there is a potential in cancer medicine for these compounds. Prior to my research it has not been confirmed by experimental studies that these thought-to-be inhibitors of PDE5 actually inhibit the PDE5 enzyme.

7.1 Results from determining relative PDE5 activity for each cell line

The results observed when determining the relative PDE5 activity show that there clearly is a PDE5 activity present in these cells. These findings are in accordance with the results from Eggen and colleagues where they showed that the mRNA expression of PDE5A in these cancer cell was low, as described earlier. The data observed is not strong enough to conclude there is a difference between the relative PDE5 activity in the C-4I cells compared to C-33A, as the standard deviation overlaps. These findings serve merely as an implication of a higher relative PDE 5 activity in C-4I cells than in C33A cells, further supported by the findings of Eggen and colleagues. Further studies are needed to conclude if these observations are a result of the varying expression of PDE5A mRNA in these cancer cell lines. These data should have been supported by a relative comparison to concentration of proteins in these cell lysates, but there was not time enough to determine the concentration of proteins in each lysate.

7.2 Results from screening of potential inhibitors

The observed increase in radioactivity in eluates from uninhibited cells to inhibited cells, is consistent with the inhibition of the PDE5 enzyme in these cancer cell lines, as described earlier. Sildenafil is a well-known inhibitor of the PDE5 enzyme and thus serving as a control for inhibition of PDE5 activity in both cancer cell lines, and compounds used for screening are sildenafil analogs. The inhibitor concentration chosen, 0,5 µM was chosen based on the IC_{50}-value for sildenafil, 0,0039µM [58]. We chose 0,5 µM as inhibitor concentration to ensure there was inhibition present, as there was not time enough to conclude more studies concerning different inhibitor concentration. Results shown in figure 15 and 16, show that there is an observed difference between the uninhibited cells and cell lysates exposed to potential inhibitors for both cancer cell lines. The observed increase in radioactivity between the different compounds for both cancer cell lines is insufficient to be conclusive about the potency of inhibition. The standard deviation for measured radioactivity compared to uninhibited cells overlaps when comparing each inhibitor with each other. Therefore the data of observed difference in between inhibitors are not strong enough to conclude anything about the potency of inhibition of PDE5 activity on both cancer cell lines. Still there is strong evidence implicating that these compounds are inhibitors of the PDE5 enzyme in the same degree as sildenafil.

7.3 Relative PDE5 inhibition

These results give an overview of the relative inhibition of each inhibitor on each cancer cell line when inhibitor concentration was 0,5 µM. This concentration was chosen based on the IC_{50}-value for sildenafil, which was stated to be 0,0039 µM [58], as mentioned earlier. There is observed a higher inhibition for all inhibitors on the C-4I cells compared to the C-33A cells. The reason for this could be explained by the findings of Eggen and colleagues (described earlier), as they observed that PDE5A mRNA expression was slightly higher for C-4I cells than for C-33A cells. It was also implicated by the results from the relative PDE5 activity that C-4I cells may have a higher activity than the C33A cells, but these data are not strong enough. These results gives a good indication that there is an inhibition of PDE5 present in both cancer cell lines for both of the tested compounds, even though the relative inhibition observed is small, but findings are in coherence with the relative small expression of PDE5 mRNA observed for these cancer cells. It gives a clear implication that the inhibition is in the same degree as for sildenafil, as there is little difference in between the different inhibitors. These results can be trusted as an indication, but could not be used for concluding anything. Even though they give the same results when two different cell lines are applied, but more extensive testing and follow-up experiments are needed for concluding anything.

8. Conclusion

The assay developed is a robust assay producing satisfying results and reproducible results, so the main goal of this thesis was achieved. This assay is shown to work well for measuring the relative PDE5A activity in complex biological materials, like cancer cells. It was also shown that it works well for screening of potential inhibitors of PDE5 enzyme found in cancer cells. The results from the screening of inhibitors on cancer cells serve merely as an implication that there is inhibition present, but could not be used to conclude anything. Therefore the second goal was also achieved, as these are just screening results, and further studies are needed to conclude anything. The basic principle of a screening process is screening potential inhibitors for a potential effect and these results from the screening has to be further validated by follow up studies and more precise methods. Even though the follow-up studies needed for validating these results are not performed it strongly implies that these inhibitors inhibit the PDE5 enzyme, but the potency of the inhibition is not known. These data show that IS-60049 may be a more potent inhibitor of the PDE5 enzyme than sildenafil, but the data collected is not strong enough to conclude this. The role of inhibiting the PDE5 enzyme has to be accounted for when these inhibitors are evaluated as a potential tool in cancer research, as they are shown to inhibit the PDE5 enzyme as well, and thus the effects of inhibiting the PDE5 enzyme has to be investigated further. These effects may also give rise to many of the unwanted effects observed with inhibition of the PDE5 enzyme.

9. Future aspects

Further studies are needed to conclude these findings. The obvious choice of follow-up research is to determine both $IC\text{-}_{50}$ and K_i values of the inhibitors. The IC-50 value could easily be determined by varying the inhibitor concentration at fixed assay conditions and plotting the data observed in a dose-response curve. These results could be further plotted in a Lineweaver-Burke plot and K_i-value of the inhibitors could easily be determined. This was initially planned, but there was unfortunately not enough time to collect the data needed to do this as the progress of growing cancer cells and developing the assay took more time than initially planned.

These results should be further investigated by comparison with a different independent method to see if results coincide. This would give more proof, and could be used for drawing a conclusion. This was also planned by comparing with the commercially available kit by BPS Bioscience, as described earlier, but unfortunately there was not enough time to conclude this assay either.

10. References

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