The role of phosphodiesterase inhibitors in the management of pulmonary vascular diseases

Ghazwan Butrous¹,²,*

ABSTRACT
Phosphodiesterase inhibitors (PDE) can be used as therapeutic agents for various diseases such as dementia, depression, schizophrenia and erectile dysfunction in men, as well as congestive heart failure, chronic obstructive pulmonary disease, rheumatoid arthritis, other inflammatory diseases, diabetes and various other conditions. In this review we will concentrate on one type of PDE, mainly PDE5 and its role in pulmonary vascular diseases.

Keywords: pulmonary vascular disease, pulmonary phosphodiesterase inhibitors, pulmonary hypertension

http://dx.doi.org/10.5339/gcsp.2014.42
Submitted: 14 August 2014
Accepted: 11 September 2014
© 2014 Butrous, licensee Bloomsbury Qatar Foundation Journals. This is an open access article distributed under the terms of the Creative Commons Attribution license CC BY 4.0, which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

Cite this article as: Butrous G. The role of phosphodiesterase inhibitors in the management of pulmonary vascular diseases, Global Cardiology Science and Practice 2014:42
http://dx.doi.org/10.5339/gcsp.2014.42
INTRODUCTION

Phosphodiesterase inhibitors (PDE) can be used as therapeutic agents for various diseases such as dementia, depression, schizophrenia and erectile dysfunction in men, as well as congestive heart failure, chronic obstructive pulmonary disease, rheumatoid arthritis, other inflammatory diseases, diabetes and various other conditions. In this review we will concentrate on one type of PDE, mainly PDE5 and its role in pulmonary vascular diseases (PVDs). The PDE5 has been targeted by specific inhibitors (mostly cGMP analogs), such as sildenafil, vardenafil, and tadalafil (See Figure 1).

NO-cGMP SIGNALING PATHWAY AND ROLE cGMP AS A REGULATOR OF PULMONARY VASCULAR HOMEOSTASIS

Unlike the systemic circulation, the pulmonary circulation is a low pressure system, and therefore regulation may differ from the systemic circulation. The regulation and adaptation of the pulmonary circulation can be regulated by various remodelling mechanisms, including the process of angiogenesis (a physiological process through which new blood vessels are formed from pre-existing vessels) or vasculogenesis (the formation of new blood vessels when there are no pre-existing ones). The disturbance of these mechanisms can lead to the abnormal increase in blood pressure and to the development of pulmonary hypertension.

A major regulatory mechanism involved the discovery of the endothelium-derived relaxing factor (EDRF) by Furchgott and Zawadzki in 1980. EDRF contributes to the relaxation of arterial smooth muscle by acetylcholine, and was identified to be nitric oxide in 1987. Nitric oxide (NO) is a highly reactive molecule, which is synthesized from L-arginine by a nitric oxide synthase. There are usually a few forms of nitric oxide synthase, namely endothelial nitric oxide synthase (eNOS) in the vascular smooth muscle endothelial cells, and inducible nitric oxide synthase (iNOS) in the neuronal ends. When the nitric oxide is synthesized in the endothelial cells, it diffuses into the vascular smooth muscles, where it will stimulate another protein called soluble guanylyl cyclase. In turn, this protein helps produce cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). The other mechanism for stimulating production of cyclic GMP is through natriuretic peptide (the atrial natriuretic
peptide (ANP)) and brain natriuretic peptide (BNP). These ligands are linked to specific cell membrane receptors called natriuretic peptide receptors. In essence, these receptors are trans-membrane guanylyl cyclase which converts GTP to cGMP. cGMP will then stimulate cGMP-dependent protein kinase (PKG), which has many effects including smooth muscle relaxation. cGMP which plays a very important role in the physiology of the vascular system (see Figure 2 for more details. For full review see references).

The endothelial cells line the blood vessels, and play a crucial role in the physiology of blood vessels and the regulation of blood flow and pressure. They are similarly important in the remodeling process, which can be the result of pathological changes that take place during the pathogenesis of pulmonary hypertension.

Therefore cGMP plays a crucial part in the regulation and development of the pulmonary circulation, and in the regulation of pulmonary vascular tone, structure and survival. Thus its intracellular regulation is a very important step in the signaling mechanism. One of the major regulators of the cGMP is phosphodiesterase, and in particular PDE5.

CHEMISTRY AND ENZYMOLOGY OF PDE5
In chemistry, the hydroxyl groups in phosphoric acid react with hydroxyl groups on other molecules to form two ester bonds called phosphodiester bond. This reaction is catalyzed by ligases. This type of bond is widely seen in biology, for example in the backbone of DNA (DNA ligase during DNA synthesis).
replication) and RNA. It is also observed in some second messengers, such as cyclic nucleotide, and in particular in the case of cyclic nucleotide like cAMP (synthesized from ATP by adenylyl cyclase) or cGMP by guanylate cyclase catalyzes, which support the reaction of guanosine triphosphate (GTP) to $3',5'$-cyclic guanosine monophosphate (cGMP) and pyrophosphate, which act as secondary messenger signaling pathways (Figure 3).

A phosphodiesterase (PDE) is any enzyme that breaks a phosphodiester bond. There are many families of phosphodiesterase enzymes, mainly grouped on their target, the cyclic nucleotide. This family of enzymes degrades the phosphodiester bond in the second messenger molecules. Thus PDE plays a vital role in the regulation of cell cyclic nucleotide signaling within subcellular domains.19–23 Different PDEs of the same family are functionally related despite the fact that their amino acid sequences can show considerable divergence. Eleven PDEs have been identified in this family, each with different substrate specificities (hydrolysis of cAMP or cGMP) (Figure 4).

Each family is encoded by 1–4 genes, thus a total of 21 PDE Isoforms are recognized, and they are classified and named by a common convention. The most widely accepted nomenclature signifies the PDE family with an Arabic numeral,1–5,7–12 then a capital letter denotes the gene in that family, and a second and final Arabic numeral indicates the splice variant derived from a single gene (e.g., PDE1C3: family 1, gene C, splicing variant 3).24–26

THE MOLECULAR STRUCTURE OF THE PDE5

PDE5 enzyme in the cell is a homodimer, which means it is comprised of two identical units approximately equal in size. Each monomer contains a carboxyl-terminal catalytic domain, with a highly conserved zinc-binding motif, and regulatory domain with two allosteric binding pockets for cGMP, a phosphorylation site (Figure 5).6,25,27–29
Catalytic domains. All PDEs contain a conserved catalytic domain of approximately 270 amino acids at the carboxyl terminus. This domain contains the active pocket that accommodates ligands, such as cyclic nucleotides and PDE inhibitors, where catalysis hydrolysis of the phosphodiesterase bond takes place. The crystal structures of the catalytic domains suggest that this domain is composed of three subdomains. In PDE5 these domains are an N-terminal cyclin-fold domain (residues 537–678 about 141 amino acids), a linker helical domain (residues 679–725 about 46 amino acids), and a C-terminal helical bundle domain (residues 726–860 about 140 amino acids).

Functionally, the catalytic core can also be divided into sub-sites, these include metal-binding site (M site), which has some metal atoms (mainly zinc and magnesium), which bind to residues that are completely conserved in all PDE family members. Metal ions bound can stabilize binding of the ligands into the catalytic pocket and support conformational changes (see below). The other functional sites are the core pocket (Q pocket), hydrophobic pocket (H pocket) and Lid region (L region). In this catalytic pocket, ligands like cyclic nucleotide or PDE5 inhibitors have special bind with various amino acids that contribute to the affinity of the ligand to this site. Cyclic GMP will bind by hydrogen bond of various amino acids (Figure 6A). One of the most important amino acids is Gln817 (Glutamine at the site of 817 in the sequence of amino acid of the protein). This amino acid also contributes to the PDE5 selectivity toward cyclic nucleotide (cGMP vs cAMP). This is done by special orientation of the side chain of Gly817 that binds the purine moiety in cAMP or cGMP. The orientation can be done with the help of another amino (Gly775) in the Q core. This special mechanism has been dubbed “glutamine switch”. Furthermore, cGMP in particular is sandwiched between Gly817 and Phe820, described as “hydrophobic clamp”. Other amino acid are involved with other inhibitors which are highly conserved in all PDEs. The other import binding location within the catalytic pocket for cGMP is the H pocket. Although this pocket is of great importance to the cGMP binding, it is of less use to PDE5 inhibitors such as sildenafil.

The crystal structure of PDE5A in complex with sildenafil showed that the Q pocket accommodates the heterocyclic ring of the pyrazolopyrimidinone group of sildenafil. The amide moiety of the pyrazolopyrimidinone group forms a bidentate hydrogen bond with Gln817 similar to that of cGMP. In this region, Zn$^{2+}$ is also involved and can contribute to the site and stability of the binding, and thereby contribute to the PDE catalytic function. These is also some movement and conformational changes in this loop during activation of the enzyme, which plays an important part in increasing the affinity to the ligands. Furthermore, cGMP in particular is sandwiched between Gly817 and Phe820, described as “hydrophobic clamp”. Other amino acid are involved with other inhibitors which are highly conserved in all PDEs. The other import binding location within the catalytic pocket for cGMP is the H pocket. Although this pocket is of great importance to the cGMP binding, it is of less use to PDE5 inhibitors such as sildenafil.

Figure 5. The schematic diagram of the PDE5 monomer, the PDE5 enzyme in the cell is a homodimer, which means it is comprised of two identical units approximately equal in size (see Figure 7). Each monomer contains a carboxyl-terminal catalytic domain (left) with a special pocket to occupy cGMP (or PDE5 inhibitor) for the catalytic role of enzyme (arrow) the detail of the pocket is shown in Figure 6. The other half (to the right is regulatory domain with two allosteric binding pockets for cGMP the regulatory domain GAF-A (yellow) and GAF –B (green), and, a phosphorylation site.

Regulatory domains: The N-terminal regulatory domain varies widely among the PDE classes and includes regions that auto-inhibit the catalytic domains, and may play part in controlling the subcellular localizations of this enzyme. In certain PDEs, phosphorylation of the enzyme can take place in this domain. The reader can consult various recent reviews on this topic. However, in this review we will concentrate on the Phosphodiesterase 5. The PDE5 regulatory domain has two domain tandems, GAF-A and GAF-B. The GAF acronym is derived from the names of the first three classes of proteins recognized to be contained in this domain: mammalian cGMP-binding PDEs, Anabaena adenyl cyclases, and Escherichia coli FhlA. These are a type of protein domain that is found in a wide range of proteins from all species.
questionable site for the binding of cGMP. In addition, it contains a single phosphorylation site (serine-102 in the human enzyme) that can be phosphorylated by Protein kinase G (PKG).

**PDE5 isoforms**: At present, only one gene for PDE5 has been discovered. Furthermore, the chromosomal location of the PDE5A gene was defined as chromosome 4q26. However, 3 variants (PDE5A1, 5A2, and 5A3) differ at their N-terminal regions. It is assumed, though it has not yet been clearly shown, that the different promoters for the PDE5 isoforms allow physiologically relevant differential control of PDE5 gene expression, thereby providing an additional mechanism for longer-term feedback regulation. In vitro tests have shown little differences among the

---

**Figure 6.** Schematic diagram of the surrounding of the Catalytic Pocket (arrow in Figure 5) See details in the text. (A) shows cGMP occupies the Catalytic Pocket and in (B) when PDE5 inhibitor (sildenafil) occupies the pocket. Note that the pyrazolopyrimidinone group (in red) is connected to the sildenafil. With the amino acid Gln 817 similar to that of cGMP (Glutamine switch with Gln775) with the support of Zn$^{2+}$ in the M site. The ethoxyphenyl group (blue) of sildenafil fits into the hydrophobic H pocket. The methylpiperazine group (green) of sildenafil is surrounded by other amino in particularly Tyr 664.
three isoforms in cGMP catalytic activities and in sensitivities to PDE5-specific inhibitors, but may have a tissue distribution pattern.\(^{41,42}\)

**LOCALIZATION OF THE PDE5 ENZYME**

Early identifications of PDE5 were reported in the 1970s and the early 1980s by various centers, and in particular by investigators from the Department of Physiology at Vanderbilt University in Nashville, Tennessee. Most of these are identified in many species and in various tissues with different concentration activity. There were high concentrations in the extracts of the lung, cerebellum, and Purkinje neurons, small intestine and platelets, and in certain tissue of the kidneys, particularly the proximal renal tubules and collecting duct. However, the concentration was low in extracts of the liver, adipose tissue, and skeletal muscle.\(^{43 - 50}\)

By 1990, most of the various forms of phosphodiesterases known today were recognized.\(^{51}\) However, there is also a differential quantity difference among the three isoforms. PDE5A1 and PDE5A2 are ubiquitous in many tissues, but PDE5A3 is specific to smooth muscle\(^{52}\) to maintain the contracted state of contractile organs such as the uterus and penis (penile corpus cavernosum). PDE5 is abundant in the lung,\(^{48,53}\) mainly in the pulmonary vessel smooth muscles as well as in pulmonary artery endothelial cells. However, the expression of PDE5 is greater in lung tissues from patients with pulmonary hypertension compared with controls, especially the expression of PDE5A1. In particular, the cells of intimal lesions and neomuscularised distal vessels see greater PDE5 expression, and this is true also in smooth muscle cells in the medial layer of the diseased pulmonary vasculature.\(^{54}\) In fact, PDE5 expression is 15 times higher in the lung than in the heart.

The subject of PDE5 extracts in the heart has long been controversial, as it may be present at very low levels in normal hearts, but PDE5 is normally expressed in the coronary vasculature and not in myocytes. Yet induction of PDE5 expression happens in the right and left ventricular hypertrophy. Similarly, heart failure of patients with pulmonary hypertension or other causes of left ventricle failure were reported,\(^{55 - 57}\) which suggests that right ventricle PDE5 expression could contribute to the pathogenesis of tight ventricular failure, probably via an increase in the myocardial oxidative stress which causes a rise of PDE5 expression in the failing heart.\(^{58}\) These findings suggest that right ventricle PDE5 expression could contribute to the pathogenesis of RV failure, and that PDE5 inhibitors increase RV inotropy and decrease RV afterload without significantly affecting systemic hemodynamics.

**Cellular distribution and subcellular localization:** PDE5A is generally considered to be a cytosolic protein in the smooth muscle of all vascular beds. There is evidence that PDE5A may be compartmentalized, and that at least a portion of PDE5 may be concentrated around various intracellular organelles. PDE5A has been found at the level of caveolin-rich lipid rafts, where it allows for a feedback loop between endothelial PDE5A and nitric oxide synthase (NOS3) via cGMP primary location of PDE5A at or near caveolae in vascular endothelial cells.\(^{59}\) Furthermore, PDE5A does not always maintain its sarcomeric localization but can take on a more diffuse distribution in the cytosol. In the hypertrophied LV myocytes, immunohistology has shown PDE5A normally localizes to the sarcomere z-disk.\(^ {60,61}\)

**THE DYNAMICITY OF THE PDE5 ENZYME**

In the last decade, much has been discovered about the dynamic of the PDE5 enzyme. The PDE5 holoenzyme can be present in inactive and active forms associated with conformational changes which are important in the PDE5 function.\(^ {34}\) These changes undergo both positive and negative regulatory feedback (Figure 7).

In general when cGMP is elevated, there will be a need to increases the activity of the PDE5 to lower the cGMP level, which then leads to a decrease of the activity of PDE5. Thus PDE5 obeys the Michaelis-Menten kinetics so that mass action applies only when the cGMP level is near or below the \(K_m\) of the enzyme for cGMP. cGMP occupy the allosteric binding sites in the regulatory domain, mainly in GAF-A whenever there is an increase in the intracellular cGMP. This allosterical activation is brought about by either a change in the dynamics of a protein or alteration in its mean structure, which is the beginning of the conformational changes of the PDE5 enzyme. It also exposes the enzyme for phosphorylation by protein kinase G (PKG). Phosphorylation itself will enhance the affinity of the allosteric binding in the GAF for more binding with cGMP.\(^ {37}\) The conformational changes and phosphorylation of the PDE5 will increase the exposure of the catalytic site, and enhance the affinity of the catalytic site to cGMP or PDE5 inhibitors such as sildenafil.\(^ {62 - 64}\)
Recent findings, however, showed that the conformational change is in fact multiform, and dynamically differs if the cGMP or PDE5 inhibitor occupy the catalytic site. For example, in the case of sildenafil occupation, the catalytic site will delay the reversal of these changes. However, it was also observed that PDE5 inhibitors would stimulate their own affinities for PDE5, partly by enhancing phosphorylation of PDE5, and further by enhancing conformational changes of the PDE5 holoenzyme. This action would also block the cGMP-lowering negative-feedback process initiated by cGMP elevation since they would occupy the catalytic site to prevent cGMP breakdown. This is important in consideration of the pharmacodynamics of these drugs, as it may be partly responsible for the prolongation of the PDE5 inhibitors action even beyond the pharmacological half-life of the inhibitors. These unique features of the PDE5 catalytic domain and the sildenafil configuration are key considerations for understanding the action of sildenafil and for development of PDE5 inhibitors.

Further details can be found in Francis et al. A reverse mechanism of deactivation of the enzyme can happen by dephosphorylating the enzyme with the help of the protein phosphatase type I (PP1 phosphatase) enzyme.

**SELECTIVE PDE5 INHIBITORS: PHARMACOLOGY AND PHARMACOKINETICS**

The number of drugs that have been shown to inhibit the PDE5 selectively has been demonstrated since the early days of the discovery of this enzyme. The first drugs, including IBMX, dipyridamole and coffee, are all known to be potent but non-selective inhibitors. However, during the 1990s, a more specific drug, like Zaprinast, was described but could not find its way into clinical practice. Since then more specific enzymes have been developed, in particular sildenafil (Viagra, UK-92,480), tadalafil, vardenafil, Udenafil, Avanafil, and Mirodenafil. These drugs will inhibit the PDE5, and therefore increase cyclic GMP and the blood flow to the penile corpus cavernosum. Thus the last six drugs have been approved in the erectile dysfunction indication. It is very obvious that although they have very high specificity to PDE5, they still have effects on some other PDEs (Table 1), in particular PDE6 and PDE1, which has consequences for the side effects of these drugs particularly in the retina, wherein PDE6 is the predominant enzyme. There are also differential effects on the other PDEs, like PDE1C and PDE11, but these do not have clinical significance as far as we currently know. Most of the licensed drugs differ significantly in their chemical structure, but they all inhibit median inhibitory concentration (IC50) values in the low nM range (Table 1). Some of the basic pharmacology can also be seen in Table 1.
The drugs are mainly metabolized in the liver by cytochrome P450 (CYP) 3A4, and to a lesser extent by CYP2C9. Thus significant drug–drug interaction through CYP3A4 has been reported in many studies. This results in significant increases in the peak plasma concentration and area under curve of most PDE5 inhibitors with no effect on plasma half-life, in particular with co-administration of potent CYP3A4 inhibitors, such as erythromycin, ketoconazole and saquinavir. One important interaction pertinent to the topic of this review is the collaboration with another pulmonary hypertension drug, namely bosentan. Bosentan decreased the maximum plasma concentration of sildenafil by 55.4%, whilst sildenafil increased bosentan’s maximum plasma concentration by 42.0%. These combinations were very well tolerated in the clinical setting described below. The same can be applied to tadalafil where co-administration with bosentan decreased tadalafil exposure by 41.5% but incurred no significant changes in the bosentan level. However, these interactions are much smaller with ambrisentan, another once-a-day endothelial antagonist. HIV-infected patients taking protease inhibitors (saquinavir, indinavir and particularly ritonavir) significantly modify the pharmacokinetics of PDE5 inhibitors (sildenafil, tadalafil or vardenfil). This results in increased plasma concentration of both drug and metabolite, although a few recent papers do not report any significant adverse reactions to sildenafil during the follow-up period. Therefore, it is advisable to consider therapeutic drug monitoring during treatment in order to avoid possible over-dosage. On the basis of these interactions, phosphodiesterase inhibitors, when used for pulmonary arterial hypertension in HIV patients, should be used with caution and with considerable consideration, as it should be avoided in combination with many HIV drugs such as boceprevir and telaprevir.

### PDE5 INHIBITORS IN THE EXPERIMENTAL MODELS IN PULMONARY VASCULAR DISEASES

During the 1990s, there was some interest in the role of PDE5 inhibitors regulating or influencing the pulmonary pressures. In one of the early studies in awake lambs with U-46619-induced pulmonary hypertension, intravenous and aerosolized zaprinast or dipyridamole potentiated the vasodilating effects or prolonged the duration of action of intermittent NO inhalation.

It is well known that hypoxia-induced vasoconstriction and pulmonary hypertension occurs in response to low vascular oxygen tension. This represents the main physiological mechanism to match ventilation and perfusion in the lung. The hypoxic pulmonary vasoconstriction response diverts regional pulmonary blood flow away from poor ventilation to a better ventilated lung region, and thus preserves the ventilation perfusion mechanism and systemic oxygenation. Therefore, hypoxia-induced vasoconstriction and hypoxia-induced pulmonary hypertension is a very interesting model to study the effects of the selected PDE5 inhibitors. This model can be used as an acute model or chronic model. In the acute model, it has been shown that sildenafil significantly inhibits the increase in the pulmonary pressure produced by hypoxia, without affecting resting pulmonary pressure. One interpretation of this is that the hypoxia pulmonary vasoreaction normally involves retroactive inhibition of nitric oxide, therefore resulting in the subsequent decline of cGMP which normally exerts a calcium desensitization action. Exposure to chronic hypoxia induces a sustained pulmonary hypertension associated with structural and functional changes in the pulmonary arterial bed.

Zhao et al. showed in the isolated perfused lung of wild-type and endothelial nitric oxide synthase deficient mice that sildenafil markedly blunted acute hypoxic pulmonary vasoconstriction. Wild-type mice that dosed sildenafil orally throughout 3 weeks of exposure to hypoxia of 10% O2 exhibited a significant reduction in right ventricular systolic pressure, coupled with a small reduction in right ventricular hypertrophy and inhibition of pulmonary vascular remodeling. In endothelial nitric oxide synthase mutant mice, sildenafil attenuated the increase in right ventricular systolic pressure but without a significant effect on right ventricular hypertrophy or vascular remodeling, suggesting that sildenafil attenuates hypoxia-induced pulmonary hypertension in humans. This happens most likely
via the endothelial NOS-NO-cGMP pathway which contributes to the response to sildenafil, but other biochemical sources of cGMP also play a role. Sildenafil has beneficial pulmonary hemodynamic effects even when endothelial nitric oxide synthase activity is impaired. Later the same group of investigators noticed the identical phenomenon and documented that PDE5 is also found in newly masculinized distal pulmonary arteries exposed to hypoxia. PDE5 inhibition attenuates the rise in PAP and vascular remodeling when given before chronic exposure to hypoxia, and when administered as a treatment during ongoing hypoxia-induced pulmonary hypertension. NO-cGMP signaling is compensatory up-regulated in the hypoxic mouse model of PH, and sildenafil further augments this pathway to functionally alleviate pulmonary vasoconstriction.

There are many other signaling mechanisms that are involved with PDE5 inhibitors actions, indicating a major role of the NO/cyclic GMP pathway. Chronic hypoxia-induced alteration in RhoA signaling in the pulmonary artery leads to the abolition of RhoA/Rho kinase—mediated Ca$^{2+}$ sensitization of contraction, and is responsible for the decreased responses to contracting agonists in the pulmonary artery of rats. However, these alterations are prevented. Consequently, pharmacological inhibition of sildenafil-sensitive PDE5 down-regulates the Ca$^{2+}$ signaling pathway involved in this model of pulmonary hypertension.

Rats with monocrotaline-induced pulmonary hypertension were used extensively to understand the pathophysiological changes exerted by a PDE5 inhibitor. When sildenafil is chronically administered, the rats significantly increased plasma cGMP and inhibited the development of pulmonary hypertension and right heart hypertrophy, with preservation of gas exchange and systemic arterial pressure. It was also noticed that the death rate significantly decreased in those animals treated with sildenafil and prevented and reversed the development of pulmonary hypertension in monocrotaline-treated rats by improving the function of the endothelin system in pulmonary arteries. These models also demonstrated the number of lung arterioles was lowest, whereas the number of arterioles with muscularization of the medial layer was highest in the monocrotaline model that was not treated with sildenafil. Similarly, pulmonary mRNA expressions of tumor necrosis factor-α, caspase-3, plasminogen activator inhibitor-1, and transforming growth factor were higher, whereas bone morphogenetic protein type II (BMPR2) receptor, Bcl-2, and endothelial nitric oxide synthase were lower in the non-treated group, which also measured higher oxidative stress in the right ventricle.

In another study using this model, vardenafil significantly increased endothelial NO synthase expression and superoxide dismutase activity, and down-regulated nicotinamide adenine dinucleotide phosphate oxidase expression in rat lung tissue.

THE EARLY CLINICAL OBSERVATION WITH PDE5 INHIBITORS

Early studies supervised by the author and other workers to evaluate the effect of intravenous administration of sildenafil at various doses were conducted between 1998 and 2000. In particular Pfizer study 1024 was the first intravenous placebo control study. Various doses of intravenous sildenafil in the placebo-controlled study in the cardiac catheterization lab showed that sildenafil selectively reduced pulmonary pressure and pulmonary vascular resistance in more than 80 patients with pulmonary arterial hypertension. Additionally, pulmonary venous hypertension and pulmonary hypoxic hypertension placebos have shown (in unpublished data) that the intravenous administration of sildenafil can cause a reduction in the pulmonary pressure, which suggests more selectivity to the reduction of systemic blood pressure. It has been observed that there was no dose relationship noticed in early studies, although a decrease in the pulmonary pressure in congestive heart failure patients and patients with idiopathic pulmonary hypertension was noticed. Another observation stated that the effect reached a plateau at a plasma concentration of 100 ng per ml of sildenafil (Butrous et al. unpublished data) (Figure 8).

During this period, interest in the role of sildenafil in pulmonary hypertension gained significant momentum. These results have encouraged many early investigators to use sildenafil, and the first case report focused on a 21-year-old man who presented with a three year history of worsening dyspnea. He was diagnosed with primary pulmonary hypertension with severe heart failure and a four-month history of being unable to walk more than 90 meters. After declining treatment with continuous infusion of epoprostenol and lung transplantation, the patient instead complied with a maintenance dose of 100 mg five times per day. At follow-up at three months, the patient’s condition had improved dramatically, and he was able to perform one hour of regular aerobic exercise. As a result of these findings, many clinicians began to prescribe sildenafil on a compassionate basis, and it
showed success, which by word of mouth encouraged more people to consider this modality. One of the early studies on the use of sildenafil in the clinical setting was done by Wilkins et al.\textsuperscript{127} and Ghofrani et al.\textsuperscript{128} In both studies, sildenafil was used in combination with a short acting iloprost (prostanoids) to enhance its activity. Long-term treatment of patients with pulmonary arterial hypertension was investigated in a number of single-center studies. Kothari and Duggal\textsuperscript{129} were amongst the first, and treated 14 patients with severe pulmonary hypertension. They showed that oral sildenafil was well tolerated and led to an improved clinical condition and exercise performance. Their experiment was followed by several small studies that started to give credence to the idea that this sort of treatment could be used for the management of pulmonary hypertension.\textsuperscript{130 – 137}

**PHASE III CLINICAL TRIALS ON THE ROLE OF PDE5 INHIBITORS IN PULMONARY ARTERIAL HYPERTENSION**

The growing body of evidence from various small studies, the increase of compassionate use of sildenafil, and the proof of the mechanistic activities in the experimental setting between 1998 and\textsuperscript{2003} led to the decision to start the first Phase 3 study trial involving PDE5 inhibitor SUPER-1 (Sildenafil Use in Pulmonary HypERTension).\textsuperscript{138} The trial was set to prove that sildenafil is effective and safe for the management of pulmonary arterial hypertension. It was a randomized, placebo-controlled, multinational trial. It included 278 patients with symptomatic pulmonary arterial hypertension (idiopathic or associated with connective tissue disease or with repaired congenital systemic-to-pulmonary shunts). Patients were randomized to either placebo or sildenafil (20, 40 or 80 mg), to be taken orally three times daily for 12 weeks. Patients completing the 12-week randomized study could enter a long-term extension study.\textsuperscript{138} The study showed an increase in the distance walked in six minutes in patients receiving all doses of sildenafil with no significant difference between the three doses, compared to placebo. The mean placebo-corrected treatment effects were 45 to 50 meters (\(p < 0.001\)). (Figure 9 results of SUPER 1).

These changes were observed as early as week 4, and were maintained at weeks 8 and 12. Sildenafil was also demonstrated to reduce mean pulmonary artery pressure and World Health Organization (WHO) functional class. Most adverse side-effects, such as flushing, dyspepsia, and diarrhea, were mild to moderate in intensity for all treatment groups.\textsuperscript{138} Based on these results, sildenafil was approved by the FDA and the EMEA in 2005 for the treatment of patients suffering from pulmonary arterial hypertension and was marketed as Revatio\textsuperscript{\textregistered} with 20 mg tablets based on a flat (non-significant) dose–effect relationship between 20–80 mg TID. It is worth noting that even the unpublished data from the Pfizer 1024 study, mentioned previously, showed a flat effect of mean pulmonary pressure for
all doses between 25–100 mg equivalents in plasma level (Figure 1iv data sildenafil). It is similarly worth mentioning that the three times per day dosing may not reflect the pharmacokinetics of sildenafil. The therapeutic window of sildenafil could be framed at approximately 30 minutes (median onset of action) and 4 hours (plasma half-life \([t\text{/}2]\)) post-dose. However, some data suggested that the duration of sildenafil activity might not be accurately reflected by plasma levels and the applied dosage, reflecting the discussion above that the conformational changes in the PDE5 enzyme can prolong the intracellular action of sildenafil despite the shorter half-life in the plasma. Moncada et al.\(^\text{139}\) noticed that in the majority of patients with erectile dysfunction sildenafil remains clinically active 12 hours after administration. Improvement of the erectile response persists beyond the time required for plasma clearance of the drugs (\([t\text{/}2]\) values of 4 and 18 h, respectively) to sub-therapeutic levels. These clinical observations may be partly explained by an unexpected persistence of the pharmacological effects of PDE5 inhibitors, which are likely caused by the conformational changes of the PDE5 which may remain as shown above. Additionally, sildenafil binding (and mostly likely other PDE5 inhibitors) to the catalytic site of PDE5 could occur at higher affinity intracellular than estimated previously, which might retard clearance of the inhibitor from the cells.\(^\text{125}\)

The 259 patients (out of 277) who finished the 12 weeks study of SUPER-1 were entered into an open-label uncontrolled extension study (SUPER-2)\(^\text{140}\) that continued until the last patient completed 3 years of sildenafil treatment. Patients titrated to sildenafil 80 mg TID. The reason for the higher dosage was the belief of the investigators that 80 mg would provide full PDE5 occupancy as shown by in-vitro enzymology of sildenafil. At 3 years from SUPER-1 baseline, 187 patients were alive. The overall Kaplan-Meier survival estimate was 79%. The most conservative figure for 3-year survival (i.e., assuming that all censored patients had died) is 68%. Patients with idiopathic pulmonary arterial hypertension had higher Kaplan-Meier 3-year survival rates than patients with pulmonary arterial hypertension associated with connective tissue disease (81% vs 72%), and patients walking ≥ 325 m at SUPER-1 baseline had better 3-year survival rates compared with those walking < 325 m at SUPER-1 baseline (84% and 70%, respectively).\(^\text{140}\) However, this open label extension study showed poor walkers (< 325 m) at SUPER-1 enrollment whose 6-minute walking distance failed to improve after the first 12 weeks of sildenafil treatment. Their reduced survival at 3 years may aid in the early identification of patients who have particularly poor prognoses, and therefore identify appropriate candidates for additional early therapy. Overall, most adverse events were of mild or moderate severity. At 3 years, 53 patients had died (censored, \(n = 37\)).\(^\text{140}\)

The second major phase 3 clinical trials was carried out with tadalafil based on the encouraging outcome of the SUPER-1 results and published case reports. Affuso et al. reported on a middle-aged woman affected by idiopathic pulmonary arterial hypertension whose quality of life and exercise tolerance improved remarkably after a six-month course of treatment with the long-acting phosphodiesterase-5 inhibitor tadalafil.\(^\text{141}\) PHIRST study (Pulmonary Arterial Hypertension and Response to Tadalafil)\(^\text{142}\) was a 16-week, double-blind, placebo-controlled study, involving 405 patients with pulmonary arterial hypertension either treatment-naïve or on background therapy.
with the endothelin receptor antagonist bosentan. Patients were randomized to placebo or tadalafil 2.5, 10, 20, or 40 mg orally once daily. Tadalafil increased the distance walked in 6 minutes in a dose-dependent manner; only the 40 mg dose met the pre-specified level of statistical significance ($p < 0.01$). In the bosentan-naive group, the treatment effect was 44 m (95% confidence interval, 20 to 69 m), compared with 23 m (95% confidence interval, −2 to 48 m) in patients on background bosentan therapy. Tadalafil 40 mg improved the time to clinical worsening ($p = 0.041$), incidence of clinical worsening (68% relative risk reduction; $p = 0.038$), and health-related quality of life. The changes in World Health Organization functional class were not statistically significant. The most common treatment-related adverse events reported with tadalafil were headache, myalgia, and flushing (Figure 10).

357 eligible patients entered into the PHIRST-1 received once-daily tadalafil at 20 mg or 40 mg in the double-blind, 52-week, uncontrolled extension study (PHIRST-2). 293 patients completed PHIRST-2 and showed significantly improved exercise capacity, clinical worsening, and quality of life. PHIRST-2 demonstrated that this dose was generally well tolerated up to 68 weeks. The addition of pulmonary arterial hypertension-specific therapy other than bosentan was not permitted in PHIRST-2. Despite this, the 161 patients on 20 mg and 40 mg in PHIRST-2 had an estimated survival of 95% and 97% respectively, and were associated with fewer clinical worsening events. The safety profile of tadalafil in PHIRST-2 was similar to that in PHIRST-1. Conversion from Sildenafil to Tadalafil in Pulmonary Arterial Hypertension (SITAR) Study has demonstrated that it is usually well tolerated, and may enhance clinical outcome.

In both SUPER-2 and PHIRST-2, the patients who started on placebo or a lower ineffective dose of tadalafil and were later treated with active medications during the extension study, showed less long-term effect than patients taking randomized active doses, consistent with the hypothesis that a delay in the initiation of an effective drug or dose may result in less long-term efficacy.

**MAJOR CLINICAL TRIALS ON PULMONARY ARTERIAL HYPERTENSION OF PDE5 INHIBITORS IN COMBINATION THERAPY**

The combination therapy has been advocated recently to add additive or synergistic effects, as there is currently no cure for the disease. Treatment with single agents remains far from satisfactory though it may maximize the therapeutic benefits such as symptom control and increased rate of survival. Most current clinical practice guidelines recommend combination therapy in functional classes III and IV if there is no improvement or if there is deterioration. The REVEAL registry demonstrated that nearly 50% of patients with pulmonary arterial hypertension received combination treatment. The majority of combination therapy involves PDE5 inhibitors. A few early clinical observations suggested that adding sildenafil to endothelin receptor antagonists bosentan, ambrisentan or prostacyclin caused additional clinical improvement in combination with sildenafil or tadalafil.
The first Phase 3 clinical trial to address the combination therapy by adding sildenafil to the prostacyclin derivatives\textsuperscript{162,163} was the PACE-1 study (Pulmonary Arterial Hypertension Combination Study of Epoprostenol and Sildenafil).\textsuperscript{164} Prostacyclin derivatives were always considered the main therapy for pulmonary arterial hypertension. PACE-1 was designed to investigate the safety and efficacy of adding oral sildenafil to long-term intravenous epoprostenol treatment. It involved 267 patients with pulmonary arterial hypertension who were receiving long-term intravenous epoprostenol therapy. The patients were randomized to receive placebo or sildenafil 20 mg three times daily, titrated to 40 mg and 80 mg three times daily as tolerated, at 4-week intervals. 97% of the patients completed the study. The results showed that a placebo-adjusted increase of 28.8 meters (95% CI, 13.9 to 43.8 meters) in the 6-minute walking distance occurred in patients in the sildenafil group (Figure 11). These improvements were most prominent amongst patients with baseline distances of 325 meters or more. By the end of week 16, fewer patients in the sildenafil group compared to the placebo group had clinical worsening events. Furthermore, sildenafil resulted in a greater change in mean pulmonary arterial pressure by 3.8 mm Hg relative to epoprostenol monotherapy. Overall, the combination was well tolerated.\textsuperscript{164}

The study was followed by an open extension study (PACES-2) for a duration of more than 3 years. Patients on placebo were given sildenafil. The study showed that the 6-minute walking distance improvement in PACE-1 was maintained in 59%, 44%, and 33% of patients at 1, 2, and 3 years respectively; the same was noticed with functional class. However, patients with PACES-1 baseline 6-minutes walking distance < 325 meters without 6-minutes walking distance improvement during the first 20 weeks of sildenafil treatment subsequently had poorer survival. Thus the PACE-2 provided some clinical assurance that the addition of sildenafil to patients on epoprostenol may carry some additional clinical benefits, as it appears generally well tolerated in pulmonary arterial hypertension patients.\textsuperscript{165,166}

Further works were done with patients treated with subcutaneous treprostinil (which is another prostacyclin derivative) by adding sildenafil as an add-on therapy. Patients were evaluated in a 12-week open-label study. The combination was well tolerated with improvement in the mean treadmill time from 465 ± 167 seconds to 656 ± 205 seconds after 12 weeks of treatment with statistical improvement in the dyspnea–fatigue score.\textsuperscript{167}

The combination of oral bosentan and sildenafil proved to be one of the most common combinations used in clinical practice as shown by the REVEAL database.\textsuperscript{162} Clearly, randomized, double-blind, placebo-controlled studies are warranted to define the role and type of combination therapies in pulmonary arterial hypertension.\textsuperscript{168,169} Two clinical studies in particular are worth mentioning here, namely COMPASS-1 and COMPASS-3. The former was an open-label study which investigated the acute pharmacodynamic effects of a single oral dose of sildenafil (25 mg) in 45 patients with PAH on the background of bosentan during right heart catheterization to evaluate the acute hemodynamic effects.
Further reduction was observed in the PVR following sildenafil, and the amount of reduction was comparable to that of nitric oxide (NO). The totality of the findings suggests that the addition of sildenafil to bosentan treatment can have additive effects.\(^{157}\) Additionally, COMPASS-3 assessed the benefits of a bosentan-based stepped approach using a 6-minute walking distance of 380 meters as the functional threshold. By study’s end, 100 patients were enrolled in COMPASS-3 and 31 patients (31%) reached the 380 meter threshold, either at week 16 (monotherapy, \(n = 16\)) or week 28 (combination, \(n = 15\)).\(^ {170}\)

Combination of Bosentan and Sildenafil vs Sildenafil Monotherapy on Morbidity and Mortality in Symptomatic Patients with Pulmonary Arterial Hypertension (COMPASS-2) was a phase IV multicenter, double-blinded, randomized, placebo-controlled, event-driven study enrolling 180 patients on sildenafil by adding bosentan to assess morbidity and mortality. However, in March 2014, the sponsoring companies announced COMPASS-2 did not meet the primary endpoint of time to first morbidity or mortality event, despite the fact that adding bosentan on top of sildenafil showed an improvement of 21.8 meters in 6 minutes walking distance at week 16 (\(p = 0.01\)). Furthermore, a placebo-corrected incidence of 15.4% in liver enzyme elevations was reported, which remains greater than three times the upper limit of normal over a median exposure to double-blind treatment of 23 months.\(^ {171}\)

Due to the potential combination use, the pharmacokinetic interactions between these two drugs were assessed in a crossover study in 26 healthy adults. Single-dose pharmacokinetics of ambrisentan (10 mg) and its metabolite, 4-hydroxymethyl ambrisentan, were determined in the absence and presence of multiple doses of tadalafil (40 mg QD). Similarly, single-dose pharmacokinetics of tadalafil (40 mg) was evaluated in the absence and presence of multiple doses of ambrisentan (10 mg QD). In the presence of tadalafil, ambrisentan maximum plasma concentration (\(C_{\text{max}}\)) was similar (105.0% [90% CI: 95.9–115.0%]) and systemic exposure (\(AUC_{0–1}\)) was slightly decreased (87.5% [84.0–91.2%]), compared with ambrisentan alone. The safety profile of the drugs combined was similar to that of either drug alone. No dose adjustments should be necessary when these drugs are co-administered. These results are in contrast to previous reports that the sulfonamide-based ERA bosentan can cause marked decreases in the exposure of tadalafil.\(^ {172}\)

The ATHENA-1 study is an open-label, multicenter study evaluating the effect of ambrisentan in patients with pulmonary arterial hypertension and a suboptimal response to monotherapy with a PDE-5 inhibitor. The primary end point will examine reductions in pulmonary vascular resistance at 24 weeks, with change in functional capacity as a secondary end point.\(^ {173}\) ATHENA-1 has recently completed enrollment. Initial results suggest that 33 patients who received ambrisentan in addition to PDE5 inhibitors showed some numerical improvement as well as high survival rates over 48 weeks.\(^ {174}\)

One large, ongoing, randomized clinical trial, AMBITION (A Study of First-Line Ambrisentan and Tadalafil Combination Therapy in Subjects With Pulmonary Arterial Hypertension),\(^ {175}\) is designed to address whether up-front combination therapy with ambrisentan and tadalafil is superior to monotherapy with either of these agents in terms of the primary end point of time to clinical worsening and secondary end points of 6 minutes walking distance and WHO functional class up front combination strategy. The results of this study are expected in 2015 and should further help in evaluating the benefit of up front combination strategy.

A retrospective analysis of medical journals from pulmonary arterial hypertension patients from 2000–2011 suggests that first-line combination therapy may more potently improve haemodynamics and may represent a new prognostic marker in pulmonary arterial hypertension.\(^ {176}\)

### PDE5 INHIBITORS IN SICKLE CELL ANEMIA PATIENTS

Sickle cell anemia has been attributed as a risk factor for the development of pulmonary hypertension.\(^ {177,178}\) It has been estimated that pulmonary hypertension can be found in 10% of patients during right heart catheterizations,\(^ {179,180}\) and about 32–40% patients when assessed by the elevation of tricuspid regurgitant jet velocity echocardiography.\(^ {181–183}\) It is a global disease, but the burden of sickle cell disease is highest in sub-Saharan Africa.\(^ {184}\)

It was hypothesized that PDE5 inhibitors, by increased nitric oxide availability, may be beneficial in patients with sickle cell disease and/or other hemolytic disorders, characterized by a state of relative NO deficiency. This NO deficiency may be due to the decreased NO synthesis by haemoglobin released during the process of haemolysis.\(^ {185–188}\) In the first open label uncontrolled pilot trial, sildenafil was evaluated in 12 patients with sickle cell disease and pulmonary hypertension and was found to decrease the estimated pulmonary artery systolic pressure and increase the 6-min walking distance
by an average of 78 m, \( p = 0.0012. \) Sildenafil has also been tried in a number of patients with other haemoglobinopathies (for example, thalassemia) who were suffering from severe pulmonary hypertension. In these cases, investigators noticed a significant decrease in pulmonary pressure and improvement in exercise capacity and functional class, and reported no significant adverse events. These findings were confirmed by others in other countries, such as Mehari et al. from USA, and Fonseca et al. from Brazil. The latter studied more than 160 patients with sickle cell disease and clinical suspicion of PH and found that 10% of the total cohort had pulmonary hypertension. PH was the major independent risk factor for death and risk in both studies, and the risk of pulmonary hypertension was associated with the severity of hemolytic anemia, suggesting that patients with all forms of chronic hemolytic anemia should be evaluated for possible pulmonary hypertension.

The scientific background and these early observations led to the design of a multi-center, placebo-controlled, double-blind, 16-week trial evaluating sildenafil therapy, sponsored by the National Institute of the United States of America. This study was called PHaSST (treatment of Pulmonary Hypertension and Sickle cell disease with Sildenafil Therapy) which originally planned to screen approximately 1000 patients with sickle cell disease, ages 12 and above, with an enrollment goal of 132 patients. The pulmonary hypertension in this trial was Doppler-defined (TRV \( \geq 2.7 \text{ m/s} \)), based on the premise that patients within this criteria would have a higher risk of mortality. However, the NHLBI study was prematurely stopped due to a significant increase in serious adverse events, specifically vaso-occlusive crises in the sildenafil arm (46% vs. 22% in placebo arm; \( p = 0.048 \)) after 29 subjects had completed the 16 week assessments and 74 subjects had been randomized. All 33 subjects with TRV \( \geq 3.0 \text{ m/s} \) underwent right heart catheterization (RHC) and had acute testing with sildenafil 60 mg; although mean pulmonary arterial pressure decreased from baseline (\( p = 0.01 \)), change in PVR was not significant. After 16 weeks of sildenafil, there was no difference in the TRV change and no difference was detected in 6 minutes walking distance for those experiencing a vaso-occlusive crisis versus those who were crisis-free.

### PDE5 INHIBITORS IN SCLERODERMA PATIENTS

Although scleroderma is part of WHO class I, the effect of PDE5 inhibitors is less compared to its effect in idiopathic pulmonary arterial hypertension, suggesting that scleroderma patients may respond differently to pulmonary arterial hypertension therapy than idiopathic pulmonary arterial hypertension patients. Differences in therapy response found in scleroderma-associated pulmonary hypertension patients may involve the unique pathophysiology and disease progression associated with scleroderma, as opposed to idiopathic pulmonary arterial hypertension. This could suggest that the cohort in this study represented a population less likely to respond to additional therapy because of a more rapid disease progression and clinical deterioration. Mathai et al. studied a small cohort of patients (both idiopathic and scleroderma pulmonary hypertension) who had failed bosentan monotherapy, and were subsequently treated with bosentan – sildenafil combination therapy. Patients with idiopathic pulmonary arterial hypertension who failed bosentan monotherapy experienced a significant increase in the mean 6 minutes walking distance after 3 months of receiving additional sildenafil therapy, whereas no significant difference in the mean 6minute walking distance was observed in the scleroderma associated pulmonary arterial hypertension subjects. The results of this study show that SSD-associated pulmonary arterial hypertension was not responsive to additional sildenafil therapy, although beneficial effects were found in the idiopathic pulmonary arterial hypertension group after failing bosentan monotherapy.

### PDE5 INHIBITORS IN HIV PATIENTS

HIV infection increases the likelihood of developing pulmonary hypertension in both adult and pediatric age groups. The French national study reports that two-thirds of their patients with HIV-associated pulmonary hypertension used pulmonary arterial hypertension-specific therapy, yet none of these drugs have been specifically studied in a randomized clinical trial that warrants high confidence. Moreover, concomitant medication with current HAART may cause some interaction. It is now recognized that most of these patients need specific treatment in the form of established current therapies of drugs that interfere with endothelial receptors, nitric oxide or prostacyclin pathways.

PDE5 inhibitors, in particular sildenafil, have been amongst the first oral therapies implemented in the treatment of pulmonary hypertension secondary to HIV, but there are very few case reports and no...
proper clinical trials. Nonetheless, it was observed that HIV-infected patients taking protease inhibitors (saquinavir, indinavir and particularly ritonavir) significantly modify the pharmacokinetics of sildenafil, presumably through inhibition of CYP3A4. This results in increased plasma concentration of both drug and metabolite, although a few recent papers do not report any significant adverse reactions to sildenafil during the follow-up period. Therefore, it is advisable to consider therapeutic drug monitoring of sildenafil during treatment in order to avoid possible over-dosage.

**PDE5 INHIBITORS IN ADULT CONGENITAL HEART DISEASES PATIENTS**

Unfortunately, most of the landmark trials for pulmonary arterial hypertension included only a small number of congenital heart disease (CHD) patients. However, due to the similarities in pathophysiology between PAH and PAH-CHD, all of the targeted therapies are used and approved for the treatment of pulmonary arterial hypertension-congenital heart disease. Randomized trials with sildenafil (SUPER-1) and tadalafil (PHIRST) included 7% and 12% congenital heart diseases patients, respectively. They both found improvements in exercise capacity in the treatment arms. Eisenmenger syndrome is characterized by elevated pulmonary vascular resistance and right-to-left shunting of blood through a systemic to pulmonary circulation connection. Some favorable results have also been reported in patients with Eisenmenger syndrome treated with the sildenafil or tadalafil. Furthermore, sildenafil may help in improving exercise performance in children and young adults with single-ventricle physiology after the Fontan operation.

Recent observations confirm that patients with Eisenmenger syndrome had the highest baseline pulmonary vascular resistance and the lowest exercise capacity. Upon treatment with pulmonary arterial hypertension-specific drugs, either monotherapy or in combination, the survival of the patients with idiopathic pulmonary arterial hypertension appeared to be worse when compared with the pulmonary arterial hypertension-congenital heart diseases subgroups.

In addition, PDE5 inhibitors were also considered in combination in adult congenital heart disease, in particular in Eisenmenger syndrome. A report on the case of a 68-year-old woman with Eisenmenger syndrome related to congenital heart disease shows that she was treated with inhaled iloprost and oral sildenafil for 2 years. A crossover study in Eisenmenger syndrome patients included 21 patients on open-label bosentan. After 3 months of therapy, the addition of sildenafil did not add significant improvement in the 6 minutes walking distance, but did show some improvement in oxygenation by 5%.

**PDE5 INHIBITORS IN CHRONIC THROMBOEMBOLIC PULMONARY HYPERTENSION PATIENTS**

Chronic thromboembolic pulmonary hypertension (CTEPH) affects up to 3.8% of patients with acute major pulmonary embolism. If left untreated, CTEPH leads to progressive pulmonary hypertension with additional pulmonary vascular remodeling. Pulmonary endarterectomy is considered the first choice of treatment for selected CTEPH patients, yet only a small percentage of these patients are eligible for pulmonary thrombendarterectomy. Patients deemed non-operable have to be treated with targeted therapies. PDE5 inhibitors, in particular sildenafil, have been tested in several small trials. The first uncontrolled clinical trial had 12 non-operable chronic thromboembolic pulmonary hypertension patients who were treated by sildenafil. Ghofrani et al. found that after approximately 6 months of sildenafil treatment, pulmonary hemodynamics and exercise capacity improved significantly. This was followed by a larger open-label uncontrolled clinical trial. 104 patients from the same center with inoperable CTEPH were treated with 50 mg sildenafil three times a day, which resulted in significant long-term functional improvement. Yet the acute effect of sildenafil may not predict the long-term outcome of therapy. In a small, double-blind, placebo-controlled pilot study, 19 subjects with inoperable CTEPH were randomly assigned to sildenafil or placebo for 12 weeks. Overall, investigators found no significant differences between the two groups with respect to change in exercise capacity, but significant improvements were seen in WHO class and pulmonary vascular resistance (PVR). This suggests partial beneficial effect and may necessitate further larger-scale trials of sildenafil in inoperable CTEPH. None of the PDE5 inhibitors available now are licensed for CTEPH patients, although recently Riociguat, a soluble stimulator which shares the same NO-cGMP pathway by stimulating guanylate cyclase enzyme, has been approved for this indication with chronic thromboembolic pulmonary hypertension.
PDE5 INHIBITORS IN HIGH ALTITUDE PULMONARY VASCULAR DISEASES

There is interest in the use of PDE5 inhibitors for the treatment of pulmonary hypertension associated with hypoxia. Several studies have been conducted in subjects during hypoxia and at high altitude. Acute mountain sickness and high altitude pulmonary hypertension are two syndromes of high altitude illness. Recent clinical studies showed the beneficial effects of phosphodiesterase type 5 (PDE-5) inhibitors on the treatment of pulmonary hypertension. Meta-analysis to evaluate the clinical efficacy of PDE-5 inhibitors on high altitude hypoxia and its complications suggested that exaggerated hypoxic pulmonary vasoconstriction is a key factor in the development of high altitude pulmonary edema (HAPE). Sildenafil improves cardiac output and exercise performance during acute hypoxia, but not normoxia.223

Richalet et al. studied the effects of oral sildenafil in 12 normal subjects who were exposed for 6 days at 4,350 meter in a randomized, double-blind, placebo-controlled study, and found that sildenafil protects against the development of altitude-induced pulmonary hypertension and improves gas exchange, limiting the altitude-induced hypoxemia and decrease in exercise performance.224

Another study included 188 natives from the Naryn region in Kyrgyzstan, which is situated 2500 meters above sea level. 44 natives underwent cardiac catheterization whilst 29 (66%) had a resting mean pulmonary artery pressure (PAP) above 25 mm Hg. 22 patients with a raised mean PAP were randomized to receive sildenafil (25 or 100 mg) or matching placebo taken 8 hourly for 12 weeks. Patients on sildenafil lowered their mean PAP compared to those on placebo (n = 8) (95% CI – 12.4 to – 1.3). Both doses improved the 6-minute walking distance by about 40 meters overall.225 During a planned trip, 14 healthy volunteers (12 men and 2 women) were enrolled to evaluate the effect of sildenafil’s exercise capacity at Mount Everest Base Camp at an altitude of 5400 meters. Half of the participants were experienced mountaineers the other half were well-trained, experienced trekkers who had repeatedly traveled to altitudes above 3500 meters. Oral sildenafil reduced pulmonary hypertension both at rest and during exercise while maintaining gas exchange and systemic arterial pressure.226

In further analysis, the 14 members of a Mount Everest expedition were monitored during an acute hypoxic challenge at sea level, and further environmental hypoxia exposure at altitudes of 3440 m and 5245 m and 2 weeks after return to sea level. They received either placebo or 50 mg sildenafil in a double-blind randomized cross-over design. Reportedly, sildenafil significantly decreased systolic pulmonary arterial pressure and improved right ventricular function.227

However, another study used 62 healthy lowland volunteers (36 male; median age 21 years, range 18 to 31) on the Apex 2 research expedition, who were flown to La Paz, Bolivia (3650 m), and after 4 – 5 days acclimatization ascended over 90 min to 5200 m. Chronic sildenafil administration showed no significant difference in PASP at 5200 m between sildenafil and placebo groups. Median AMS score on day 2 at 5200 m was significantly higher in the sildenafil group (placebo 4.0, sildenafil 6.5; p = 0.004) but there was no difference in prevalence of acute mountain sickness between groups on pulmonary artery systolic pressure and symptoms of acute mountain sickness.228

A further 16 healthy subjects were included in a randomized, double-blind, placebo-controlled, cross-over study on the effects of 50 mg sildenafil on echocardiographic indexes of the pulmonary circulation and on cardiopulmonary cycle exercise in normoxia, in acute normobaric hypoxia (fraction of 10% inspired O₂), and then again after 2 weeks of acclimatization at 5000 m on Mount Chimborazo (Ecuador). In normoxia, sildenafil had no effect on maximum VO₂ or O₂ saturation but in acute hypoxia, sildenafil increased maximum VO₂. In chronic hypoxia, sildenafil did not affect maximum VO₂ or O₂ saturation, but did decrease pulmonary vascular resistance by 30% to 50% in different conditions. It was concluded that sildenafil increases exercise capacity in acute normobaric hypoxia and that this is explained by improved arterial oxygenation, rather than by a decrease in afterload of the right ventricle.229

However, in a single randomized placebo-controlled study, 10 high-altitude pulmonary edema-susceptible subjects received tadalafil 10 mg twice daily during a rapid ascent to 4559 m.230 Only 1 of the remaining 8 subjects who received tadalafil developed high-altitude pulmonary edema, but 2 of the subjects receiving tadalafil withdrew from the study due to severe acute mountain sickness on arrival at 4559 m. In the placebo group, 7 of 9 subjects developed high-altitude pulmonary edema. The occurrence of acute mountain sickness with tadalafil may have discouraged further trials for the prevention of high-altitude pulmonary edema.
In a recently published paper on Meta-Analysis of Clinical Efficacy of Sildenafil, on High Altitude Hypoxia and Its Complications, Yu et al.235 reviewed five clinical trials.224,226–229 A total of 60 subjects received sildenafil, and 72 subjects were given placebo. In accordance with previous reports, short-term treatment with sildenafil (1–2 days) significantly reduced pulmonary artery systolic pressure at rest (MD -4.53; 95% CI -6.72, -2.34; p < 0.0001). However, treatment with sildenafil (1–2 days) did not improve oxygen saturation after exposure to high altitude (MD 0.07; 95% CI -1.26, 1.41; p = 0.91). Moreover, no significant difference was observed in heart rate between the sildenafil and placebo-treated groups (MD 6.95; 95% CI -3.53, 17.43; p = 0.19). AMS score did not improve after treatment at different time points. In this case, the investigators concluded that short-term treatment with sildenafil can attenuate the altitude-induced high pulmonary systolic arterial pressure, but has no significant beneficial effects on arterial oxygen saturation, heart rate, and acute mountain sickness.

PDE5 INHIBITORS IN PEDIATRIC POPULATION

Animal and clinical data suggested that PDE5 is involved in the regulation of pulmonary vascular reactivity during the perinatal period and may potentiate birth-related pulmonary vasodilator stimuli.16,232–234 In late-gestation fetal lambs with chronic pulmonary hypertension, the prophylactic use of sildenafil prevents the rise in pulmonary vascular tone and altered vasoreactivity caused by ductus arteriosus compression. These results support the hypothesis that elevated PDE5 activity is involved in the consequences of chronic pulmonary hypertension in the perinatal lung.235 Additionally, it was noticed that sildenafil induces a transient pulmonary vasodilation and mediates a sustained change in vascular reactivity, especially to birth-related stimuli in the ovine fetal lung.234 The first observation by Atz and his colleagues276 suggested that sildenafil may potentiate pulmonary vasodilation with NO or ameliorate the deleterious effects of abrupt discontinuation of NO (pulmonary hypertension crisis) by increasing intracellular and circulating cGMP, preventing rapid depletion of cGMP when the gas is withdrawn.236–237 This was confirmed during the treatment of pulmonary hypertension crises after withdrawal of NO in study with 103 eligible patients.238 In turn, this led to interest in using sildenafil in a pediatric age group for patients with primary pulmonary hypertension.239 These studies have generally suggested favorable effects and outcomes in infants and young children with pulmonary arterial hypertension, but these reports are mainly uncontrolled observations except for one single-center, placebo-controlled trial for PPHN.240

The report of sildenafil treatment in critically ill infants with persistent pulmonary hypertension in the neonate ward in five centers involved a total of 36 neonates. Intravenous sildenafil was well tolerated with short-term improvements in oxygenation. Acute and sustained improvements in oxygenation were noted in those neonates who received the higher infusion doses.241,242

A separate study on patients with congenital heart disease compared the effects of inhaled NO before and after the specific inhibition of intravenous sildenafil. Investigators noticed that intravenous sildenafil is as effective as inhaled NO as a pulmonary vasodilator in children with congenital heart disease.243 In another study it was noticed that intravenous sildenafil reduced pulmonary artery pressure and shortened time to extubation and intensive care unit stay in children with postoperative PH with congenital heart disease (Figure 12).244 After Fontan surgery, sildenafil infusion acutely improves cardiopulmonary hemodynamics, increasing cardiac index. For the range of doses studied, exposure was within the acute safety range reported in adult subjects.245 Other smaller series suggested that sildenafil can be beneficial for children as an add-on to a failure or lesser response to bosentan monotherapy.246

Many treating physicians have extrapolated from adult trials that the disease and its response to treatment can differ between children and adults.247–252 Sildenafil has been used extensively off-label for the treatment of neonates, infants, and children with pulmonary arterial hypertension associated with diverse heart and lung diseases. In a small open label study with sildenafil at 0.25 to 1 mg/kg 4 times daily to 14 children, group diagnoses were primary (n = 4) and secondary (n = 10).

Investigators found that sildenafil therapy was well tolerated and resulted in a significant increase of the distance walked in the 6-minute walking test.253 Therefore, a 16-week, placebo-controlled, dose-ranging study evaluated the effects in a trial of oral Sildenafil in Treatment-Naive Children, Aged 1 to 17 Years, With Pulmonary Arterial Hypertension (STARTS-1).254 Children (n = 235; weight ≥ 8 kg) were randomized to low-, medium-, or high-dose sildenafil or placebo orally 3 times daily for 16 weeks. The primary comparison was a percent change from baseline in peak oxygen consumption (PVO2) for the 3 sildenafil doses combined versus placebo. Exercise testing was performed in 115 children able to
exercise reliably; the study was powered for this population. Secondary end points (assessed in all patients) included hemodynamics and functional class. The results showed that low-dose sildenafil was ineffective but P\text{VO}_2, functional class, and hemodynamics improved with medium and high doses versus placebo, whilst most adverse events were mild to moderate in severity. STARTS-1 completers could enter the STARTS-2 extension study, wherein patients who received sildenafil in STARTS-1 continued the same dose, whilst placebo-treated patients were randomized to low-, medium-, or high-dose sildenafil. Because STARTS-2 did not include a placebo arm, the impact of sildenafil monotherapy on long-term survival is difficult to discern.

In STARTS-2, however, the statistical analysis suggested increased mortality with higher doses and appeared to occur after 2 years of treatment and in children with idiopathic pulmonary arterial
hypertension/ with a baseline values above median values for PVRI (15.1 Wood units/m²), mean pulmonary arterial pressure of 62 mm Hg, and right atrial pressure of 7 mm Hg. With all patients having the potential to complete 3 years of treatment (including some receiving 7 years of treatment), 35 deaths have been reported. Deaths were reported on treatment (n = 26) or during follow-up (n = 9). The incidence of deaths is currently 9% (5 of 55), 14% (10 of 74), and 20% (20 of 100) for patients randomized in STARTS-1 or -2 to low-, medium-, and high-dose sildenafil, respectively. This led to some regulatory concern with the FDA warning that “children taking a high dose of Revatio had a higher risk of death than children taking a low dose”. For this reason, sildenafil has not been approved for the treatment of pulmonary arterial hypertension in children.

Nevertheless, despite these concerns the European Medicines Agency (EMA) approved sildenafil therapy for use in children with pulmonary arterial hypertension, but added caveats regarding dosage. The Pediatric Pulmonary Hypertension Network (PPHNet), which includes leaders of pediatric pulmonary hypertension programs throughout North America, has heavily discussed this issue and suggested in recent communications that the Drug Safety Communication was overly conservative in the FDA conclusions about both effectiveness and mortality risk. Furthermore, on deeper examination of the mortality data from STARTS-2 and acknowledging the many limitations of the STARTS trial, PPHNet offered a series of recommendations regarding the use of sildenafil and concluded STARTS-2 to be a failed trial.

On careful re-analysis the group stated that “although children randomized to higher compared with lower sildenafil doses had an unexplained increased mortality, all sildenafil dose groups displayed favorable survival for children with pulmonary arterial hypertension”. Combined with STARTS-1 efficacy results, the long-term survival rates favor use of lower sildenafil doses. After interim STARTS-2 survival data were reviewed, a recommendation was issued to down-titrate all patients remaining in the study to lower sildenafil doses. Therefore this subject is still controversial and need careful re-evaluation.

THE EFFECT OF PDE5 INHIBITORS ON THE RIGHT VENTRICULAR FUNCTION IN PULMONARY HYPERTENSION

PDE5 has been demonstrated to be upregulated in rat right ventricular hypertrophy myocardium, whilst inhibition of PDE5 (with either MY-5445, DA-8159 or sildenafil) significantly increases contractility in the perfused heart (modified Langendorff preparation) and isolated cardiomyocytes. In right ventricular hypertrophy myocardium, but not normal right ventricle, PDE5 inhibition leads to increases in both cGMP and cAMP in right ventricular hypertrophy. However, the abnormal right ventricle attenuated the compensatory development of right ventricular hypertrophy, and pulmonary artery medial wall thickening prevented myocardial fibrosis induced by MCT. These finding were confirmed in surgical specimens from 9 patients, which showed that PDE5 is not expressed in the myocardium of the normal human right ventricle but markedly upregulated in hypertrophied right ventricle myocardium. Further, tissue extracts from the right ventricle of 20 patients also confirmed severity-dependent upregulation of myocytes PDE5 expression in the RV and the impact of this upregulation on myocardial contractility. Thus the ability of PDE5 inhibitors to increase right ventricle inotropy and to decrease RV afterload without significantly affecting systemic hemodynamics makes them ideal for the treatment of diseases affecting the right ventricle, including pulmonary arterial hypertension.

PDE5 INHIBITORS IN LEFT HEART FAILURE

Myocardial PDE5 expression and cellular distribution were determined in left ventricular samples from patients with end-stage CHF and normal donors and from mice after transverse aortic constriction induced CHF. Compared with donor human hearts, myocardial PDE5 protein was increased 4.5-fold in CHF samples, and the increase of myocardial PDE5 expression was significantly correlated with myocardial oxidative stress markers 3'-nitrotyrosine or 4-hydroxynonenal expression. Reducing oxidative stress by treatment with M40401 attenuated cardiomyocyte PDE5 expression. This, with selective inhibition of PDE5, protected the heart against pressure overload-induced left ventricular hypertrophy and congestive heart failure.

Works in failing hearts (tachypacing model), showing PDE5 inhibition, could blunt β-adrenergic–stimulated inotropy. Takimoto et al. reported that sildenafil could both block and reverse maladaptive cardiac remodeling induced by sustained pressure overload using a mouse model...
(aortic banding). These data suggested that PDE5 may be unregulated in the failing and hypertrophied left ventricle and may down-regulate itself in normal myocardium. Other investigators demonstrated benefits of PDE5 inhibition on myocardial post ischemic injury and cell survival signaling and showed that PDE5 inhibition enhances ischemia-induced angiogenesis with mobilization of endothelial progenitor cells through a protein kinase G–dependent HIF-1/vascular endothelial growth factor pathway. These data suggest that PDE5 inhibition may be a new treatment strategy for cardiac hypertrophy and remodeling and may have a therapeutic potential to treat ischemic cardiovascular diseases.

Lewis et al. showed that in patients with systolic heart failure, sildenafil acts as a selective pulmonary vasodilator during rest and exercise in patients with heart failure and pulmonary hypertension, and improves exercise capacity and quality of life in patients with systolic heart failure and with secondary pulmonary hypertension. A small double-blind study with 45 heart failure patients (New York Heart Association class II-III) who were randomly assigned to placebo or sildenafil (50 mg three times per day) for 1 year found that sildenafil improves functional capacity and clinical status and that long-term use of sildenafil was well tolerated. This study also showed sustained and significant improvement in the left ventricle (diastolic function properties and cardiac geometry), which may suggest that PDE5 inhibitors can be a target therapy for heart failure with preserved ejection fraction or diastolic heart failure. The above finding led to the design of the RELAX trial (Phosphodiesterase-5 Inhibition to Improve CLinical Status And EXercise Capacity in Diastolic Heart Failure), which was a multicenter, randomized double-blind, placebo-controlled treatment study designed clinical trial conducted within the National Heart Lung and Blood Institute-sponsored heart failure clinical research network. Its aim was to test the hypothesis that chronic PDE5i (sildenafil 20 mg TID for 12 weeks followed by 60 mg TID for 12 weeks) improves exercise capacity and clinical status in patients with heart failure with preserved ejection fraction (HfPEF).

In this study the investigators adopted the change in VO2 from baseline to 24 weeks as the primary endpoint with change in 6-minute walking distance at 12 and 24 weeks, and the change in a composite clinical score at 24 weeks as secondary endpoints. The trial recruited 216 patients (median age 69 years) and 48% were women. However, RELAX was neutral, reporting no benefit of sildenafil compared with placebo in the primary end point or in any secondary functional and structural end points including markers of clinical status. The initial results showed that sildenafil treatment in HfPEF was associated with modest impairment on resting LV contractility despite beneficial effect on the right ventricle function and LV afterload. These offsetting effects on the ventricular vascular function may partially explain the lack of clinical benefit from PDE5i observed in the RELAX trial. However, the RELAX study provided many useful clinical observations on the natural history of HfPEF. Clinicians and researchers may argue about the selection of endpoints and interpretation of the data, but this is beyond the scope of this review and more information can be obtained elsewhere.

### PDE5 INHIBITORS IN CHRONIC LUNG DISEASES

Hypertension associated with chronic obstructive pulmonary diseases (COPD) and interstitial lung disease has also been considered with regards to PDE5 inhibitors. Chronic obstructive lung disease affects the entire lung including the pulmonary vasculature, rather than just the airways. Pulmonary hypertension has long been recognized in a subset of patients with obstructive lung diseases. The etiology can be due to hypoxia vasoconstriction leading to permanent medial hypertrophy and remodeling, but other pathological factors may play a part leading to complex interactions including in situ thrombosis, decreased expression of vascular endothelial growth factor and other contributory mechanisms includes inflammation. Therefore, there may be a case for pharmacologic treatment of pulmonary hypertension in selected patients with advanced COPD and right heart failure. It was thought that PDE5 inhibitors might have a therapeutic potential for chronic airway diseases, in particular due to their effect on the remodeling process. Acute sildenafil use resulted in transient airways dilatation, reduced gas trapping and vasodilation. However, the main concern with these agents, like with other vasodilators, is perturbation of ventilation–perfusion (V/Q) matching. There are some suggestions that sildenafil (and potentially other PDE5 inhibitors) may cause preferential pulmonary vasodilation and improve gas exchange in patients with severe lung fibrosis and secondary pulmonary hypertension, and thus preserve V/Q matching. The hypothesis proposes that poorly ventilated areas of the lung would be expected to have lower NO and cGMP levels and so the local effect of PDE5 inhibition would be less than in a well-ventilated lung.
Initial small observations of six patients suffering from severe COPD suggested some benefit in the use of PDE5 inhibitors, as they demonstrated significantly improved hemodynamic parameters following administration of oral sildenafil. Additionally, the walking distance in the 6-minute walking distances increased significantly in the five patients who accepted a second right heart catheterization. Nevertheless it will be a challenge for randomized controlled trials to overcome the difficulties of the diagnosis of right ventricular failure and the definition of a relevant primary endpoint in pulmonary hypertensive patients with COPD. Larger placebo control studies with sildenafil in patients with severe COPD and moderately increased pulmonary arterial pressure did not improve the results of pulmonary rehabilitation in exercise tolerance.

While these small trials are encouraging, recent larger studies indicate that sildenafil does not have a beneficial effect on exercise capacity in patients with COPD and emphysema without pulmonary hypertension or on the outcomes of respiratory rehabilitation in COPD patients with moderate pulmonary hypertension. In addition, some observed that sildenafil significantly worsened gas exchange at rest as well as quality of life. There is now an ongoing study (TADA-PHiLD trial) which is a multicenter, prospective, randomized, placebo-controlled, double-blind clinical trial that aims to characterize the efficacy and safety profile of PDE5 inhibitors (specifically tadalafil) in patients with chronic obstructive lung disease and moderate to severe pulmonary hypertension. Moreover, circulatory exercise impairment remains unstudied, and the low prevalence of this condition might explain why these studies have not yet been performed to date.

The presence of pulmonary hypertension in idiopathic pulmonary fibrosis patients is associated with poor survival. Based on prior studies, it can be estimated that about 60% of patients with idiopathic pulmonary fibrosis have pulmonary hypertension. Due to the paucity of drugs for this condition, many investigators tried to argue that improving the pulmonary circulation may benefit some of these patients. In a small trial, sildenafil improved the exercise tolerance as measured by 6-minute walking distance in 57% of the patients, which prompted a large, well-controlled trial. In fact, a larger study was funded by the National Heart, Lung, and Blood Institute and others and named STEP-IPF (Sildenafil Trial of Exercise Performance in Idiopathic Pulmonary Fibrosis). This study was a double-blind, randomized, placebo-controlled trial and included patients with advanced idiopathic pulmonary fibrosis (IPF), and selected for patients with complicating pulmonary hypertension by virtue of the inclusion criterion of a single breath diffusing capacity (DLCO) of < 35% of predicted. The first period consisted of 12 weeks of a double-blind comparison between sildenafil and placebo control. The primary outcome was the proportion of patients with an increase in the 6-minute walking distance of 20% or more. A total of 180 patients were enrolled in the study. No benefit was shown for sildenafil in the primary outcome, but the presence of some positive secondary outcomes created clinical equipoise for further research. There were small but significant differences in arterial oxygenation, carbon monoxide diffusion capacity, the degree of dyspnea, and quality of life favoring the sildenafil group. Serious adverse events were similar in the two study groups. There is good biological and physiological rationale as well as outcome data to support studies addressing PH complicating IPF. There are many elements that are integral to a successful study: a drug that works, administration of the drug at an appropriate dose, a well-targeted patient cohort that is followed long enough to see an effect, and a carefully constructed end point. If all such elements are aligned, a positive study of a pulmonary vasoactive agent might indeed demonstrate an important aspect of IPF to be targeted.

CONCLUSION
PDE5 inhibitors have contributed to our knowledge of the pathophysiology of pulmonary vascular disease, and have become the leading oral therapy for pulmonary hypertension worldwide and in particular in the developing world. That is mainly due to the availability and more reasonable cost than other classes of pulmonary hypertension-specific drugs. However, PDE5 inhibitors are not universal and its effects depend on the etiology, as they are most effective in idiopathic and primary pulmonary hypertension. PDE5 inhibitors are proven less effective in other forms of the disease, such as for patients with pulmonary arterial hypertension, even though they have been approved by many authorities for this indication. Similarly, it can be useful in some selected patients with left heart failure (both systolic and diastolic), in selected patients with hypoxia pulmonary hypertension, and in interstitial lung diseases, though these are not indicated yet. Finally, they cannot be universal drugs, even with the same indication and etiology. There is wide clinical variation in patients’ responses;
this is partly because these drugs can act differently at certain stages of the disease progression. Furthermore, PDE5 inhibitors, like all other currently available specific pulmonary hypertension drugs, have been useful in improving clinical presentation and may delay clinical worsening, but will not cure the condition.

Though most of these drugs are now generic and no longer inspire big clinical trials, their appeal is still high in identification of the right pathology and patient phenotype and genotype that can benefit from this class of drugs. This needs further clinical and basic research for years to come.

REFERENCES


LEVITRA [Internet]. Available from: http://www.levitra.com/.


