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# Sildenafil negatively impacts tumor growth upon interfering with HSP90/PKD2 signaling axis

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**For my parents and beloved lover Mr.Shi**

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# Table of contents

**Table of contents……I**

**Abbreviations……III**

**1. Introduction……1**

**1.1 Drug repurposing in cancer……1**

**1.2 Phosphodiesterases（PDEs）……2**

1.2.1 General introduction……2

1.2.2 PDE5……3

1.2.3 PDE5 Inhibitors in Cancer……4

**1.3 Heat shock protein 90……6**

1.3.1 General introduction……6

1.3.2 Client proteins of HSP90……7

1.3.3 The role of HSP90 in cancer……7

1.3.4 HSP90 inhibitors……8

**1.4 Protein Kinases……9**

1.4.1 General introduction……9

1.4.2 The role of PKD2 in cancer……11

1.4.3 PKD2 is a client of HSP90……11

**1.5 Aims of the study……12**

**2. Materials and Methods……13**

**2.1. Materials……13**

2.1.1. Laboratory equipment……13

2.1.2. Chemicals and drugs……15

2.1.3. Cell lines……16

2.1.4. Cell culture items……17

2.1.5. Plasmids……17

2.1.6. Buffers……18

2.1.7. Antibodies……20

2.1.8. HSP90 inhibitor (PU-H71)……21

2.1.9. Materials for Immunohistochemistry……21

2.1.10. Materials for chicken CAM xenograft experiments……21

2.1.11. Software……22

**2.2. Methods……22**

2.2.1 Cultivation of cancer cell lines……22

2.2.2 Analysis of cancer cell proliferation……24

2.2.3 FACS analysis……25

2.2.4 Analysis of proteins……25

2.2.5 In vivo tumor xenografts using chicken chorioallantoic membrane (CAM) assay……26

2.2.6 Statistical analysis……27

3. Results ..... 28
3.1 Sildenafil treatment inhibits the growth of cancer cells in a concentration-dependent manner ..... 28
3.2 Sildenafil treatment inhibits the proliferation of cancer cells in a time-dependent manner ..... 29
3.3 Sildenafil treatment induces apoptosis in human cancer cells ..... 30
3.4 Sildenafil treatment suppresses tumor growth in vivo ..... 32
3.5 Sildenafil treatment is associated with altered HSP90 expression and PKD2 degradation ..... 34
3.6 Impaired proliferation of cancer cells following Sildenafil treatment can be partially restored by ectopic PKD2 ..... 35
3.7 PDE5 and HSP90 inhibitors synergistically suppress tumor growth *in vivo* 37
3.8 Sildenafil triggers tumor death through altered expression of HSP90 and degradation of PKD2 ..... 38
4. Discussion ..... 40
5. Summary ..... 45
6. References ..... 46
7. List of figures and tables ..... 67
7.1 Figures ..... 67
7.2 Tables ..... 67
8. Acknowledgements ..... 69
9. Curriculum vitae ..... 70

# Abbreviations

| | |
|---|---|
| °C | Degree Celsius |
| µg | Microgram |
| µl | Microlitre |
| µm | Micrometer |
| Akt | RAC-beta serine/threonine-protrein kinase |
| APS | Amonium Persulfate |
| AR | Androgen receptor |
| BCL-2 | B-cell lymphoma 2 |
| BCL-6 | B-cell lymphoma 6 |
| BCR-ABL | Breakpoint cluster region - Abelson tyrosine kinase oncorprotein |
| BRAF | B-Raf |
| BSA | Bovine serum albumin |
| CAM | Chorioallantoic membrane |
| cAMP | Cyclic adenosine monophosphate |
| CAMK | Calcium /calmodulin-dependent protein kinase |
| CDK4 | Cyclin-dependent kinase 4 |
| CDKs | Cyclin-dependent kinases |
| cGMP | Cyclic guanosine monophosphate |
| $CO_2$ | Carbon dioxide |
| CTD | Carboxy-terminal domain |
| ddH2O | Double-distilled water |
| $dH_2O$ | Distilled water |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl Sulfoxide |
| DPBS | Dulbecco's Phosphate Buffered Saline |
| ECL | Enhanced chemiluminescence |
| ED | Erectile dysfunction |
| EDTA | Ethylenediaminetetraacetic acid |
| EGFR | Epidermal growth factor receptor |
| EMT | Epithelial-mesenchymal transition |
| FACS | Fluorescence activated cell sorter |
| FDA | Food and Drug Administration |
| FBS | Fetal bovine serum |
| g | Gram |
| GRP94 | Glucose related protein 94 |
| Her2 | Human epidermal growth factor receptor 2 |
| $H_2O_2$ | Hydrogen peroxide |
| HIF | Hypoxia-inducing factor |
| HOP | Homedomain-only protein |
| HRP | Horseradish peroxidase |
| HSCC | Hypopharyngeal squamous cell carcinoma |

| | |
|---|---|
| HSP90 | Heat shock protein 90 |
| HSPs | Heat shock proteins |
| IHC | Immunohistochemistry |
| IARC | Internationa Agency for Research on Cancerl |
| JAK2 | Janus kinase 2 |
| $K_2HPO_4$ | Di-potassium hydrogen phosphate |
| KCl | Potassium chloride |
| KDa | Kilo Dalton |
| $KH_2PO_4$ | Potassium dihydrogen phosphate |
| LUTS | Lower urinary tract symptoms |
| M | Mole |
| mA | Milliampere |
| MAPK | Mitogen-activated protein kinase |
| MD | Middle domain |
| Mg | Milligram |
| $MgCl_2$ | Magnesium chloride |
| $MgSO_4$ | Magnesium sulfate |
| Min | Minute |
| ml | Milliliter |
| mm | Millimeter |
| mM | Millimole |
| MMP | Matrix metalloproteinase |
| n | Sample size |
| $Na_2HPO_4$ | Di-Sodium hydrogen phosphate |
| NaCl | Sodium chloride |
| NaF | Sodium fluoride |
| $NaH_2PO_4$ | Sodium dihydrogen phosphate |
| $NaN_3$ | Sodium azide |
| NaOH | Sodium hydroxide |
| NF-κB | Nuclear factor kappa B |
| NSAID | Non-steroidal anti-inflammatory drugs |
| nm | Nanometer |
| NTD | Amino-terminal domain |
| $O_2$ | Oxygen |
| Oct4 | Octamer-binding transcription factor 4 |
| PARP | Poly ADP-ribose polymerase |
| PAH | Pulmonary arterial hypertension |
| PBS | Phosphate buffered saline |
| PBST | 1× PBS containing 0.2% Tween 20 |
| PDEs | Phosphodiesterases |
| PDK-1 | Phosphoinositide-dependent kinase-1 |
| PEI | Polyethylenimine |
| pH | pH number |
| PI | Propidium Iodide |

| | |
|---|---|
| PKC | Protein kinase C |
| PKD2 | Protein kinase D2 |
| PKA | cAMP-dependent protein kinase |
| PKG | cGMP-dependent protein kinase |
| pMD2.G | Envelope plasmid |
| PP5 | Protein phosphatase 5 |
| psPAX2 | 2nd generation lentiviral packaging plasmid |
| PU-H71 | 6-Amino-8-(6-iodo-1,3-benzodioxol-5-yl) thio-N- (1-methyl-ethyl) - 9H-purine-9-propanamine |
| PVDF | Polyvinylidene difluoride |
| ROS | Reactive oxygen species |
| rpm | Revolutions per minutes |
| SDS | Sodium dodecyl sulfate |
| SEM | Standard error of the mean |
| SDS-PAGE | Sodium dodecyl sulfate - polyacrylamide gel electrophoresis |
| shRNA | Short hairpin RNA |
| STK33 | Serine/threonine kinase 33 |
| TBS | Tris Buffered Saline |
| TEMED | N,N,N‘,N‘-Tetramethylethylenediamine |
| Tris | Tris-aminomethane |
| TRAP-1 | Tumor necrosis factor receptor-associated protein 1 |
| USA | United States of America |
| UV | Ultraviolet |
| V | Volt |
| VEGF | Vascular endothelial growth factor |
| VEGF-A | Vascular endothelial growth factor A |
| VEGFR | Vascular endothelial growth factor receptor |
| WB | Western blot |

# 1. Introduction

## 1.1 Drug repurposing in cancer

Cancer is a global major public health issue, and a leading cause of death in the 21st century [1]. According to the global cancer statistics from The International Agency for Research on Cancer (IARC), there were estimated 18.1 million new cancer cases and 9.6 million cancer deaths in 2018 [1]. Lung was reported to be the organ with the highest incidence of cancer (18.4% of the total deaths), followed by female breast (11.6%), prostate (7.1%) and colorectal cancer (6.1%) [1]. Chemotherapy is the predominant therapy for advanced cancer patients [2]. Current chemotherapeutics include alkylating agents, antimetabolites, antitumor antibiotics, topoisomerase inhibitors and mitotic inhibitors [3, 4]. However, malignancies in advanced stages such as pancreatic, breast and prostate cancers are incurable with current treatments. Thus, there is an urgent need to develop more effective anti-cancer drugs. Innovations in cancer drug discovery appears to be a tough task, even though substantial amounts of money and time have been invested, only a limited number of new anti-cancer medications could be approved by the Food and Drug Administration（FDA）yearly [5]. In this case, more studies focusing on revealing alternative ways to shorten time, cut cost during cancer drug development are necessary [5]. Recently, a more attractive strategy is repurposing of well-established medications on the market. Drug repurposing or repositioning refer to converting existing drugs outside of their originally approved indications to treat other diseases [6]. For instance, aspirin is a non-steroidal anti-inflammatory drug (NSAID), originally developed for preventing pain and fever. Nevertheless, it was reported by Rothwell and colleagues that regular use of aspirin significantly decreased the long-term risk of several cancers and the risk of distant metastasis in observational studies [7]. The primary indication of

valproic acid is epilepsia, bipolar disorders and it is clinically used for schizophrenia. However, researchers also demonstrated that valproic acid could inhibit cancer cell proliferation, angiogenesis and trigger apoptosis by inhibiting histone deaceylase [8, 9, 10, 11]. The angiotensin II receptor blocker telmisartan is primarily used in the management of hypertension. Lately, telmisartan was shown to exhibit anti-proliferative effects in renal cell carcinoma, colon cancer, lung cancer, bladder cancer, prostate cancer and cervical cancer cell lines [12, 13, 14]. Moreover, metformin (Glucophage), originally described as one of the most commonly used diabetes drug, is now being considered as a cancer drug under trials of Phase II and Phase III [15]. Another example for drug repurposing is Sildenafil (Viagra), a phosphodiesterase inhibitor initially developed to treat angina, then achieves commercial success as treating erectile dysfunction (ED) [16, 17, 18]. Recent years, Sildenafil was demonstrated to have potential anti-oncogenic also pulmonary hypertension effects [19, 20，21], which will be further discussed in my thesis.

## 1.2 Phosphodiesterases（PDEs）

### 1.2.1 General introduction

The cyclic nucleotide phosphodiesterases (PDEs) are a large superfamily of enzymes that regulate cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) by hydrolysis [22]. The PDE family is composed of eleven members sharing the same conserved catalytic domain (i.e. the C domain), whereas the amino acid sequence toward the amino terminus varies significantly [23]. These PDE enyzmes are classified according to their substrate specificity. PDE5, PDE6, PDE9 are selective for cGMP; PDE4, PDE7, and PDE8 selectively hydrolyze cAMP while PDE1, PDE2, PDE3 and PDE10 represent dual substrate-degrading isozymes, hydrolyzing both cGMP and cAMP [23]. PDEs have been proved to play crucial roles in regulating intracellular cyclic nucleotide concentration, as well as acting as regulators of

biological processes in health and disease [24, 25, 26]. Therefore, PDE inhibitors are likely to activate cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG), as well as other cyclic nucleotide regulated processes by elevating a transient or sustained intracellular levels of cAMP and/or cGMP [27]. It is widely believed that selective PDE inhibitors can target specific functions and pathological conditions, which may render them therapeutic targets [28].

### 1.2.2 PDE5

#### 1.2.2.1 General review-characteristics, structure, distribution

PDE5 was originally discovered and isolated from rat lung [10] and platelets as cGMP binding protein [29]. The size of PDE5 varies between 90 kD to 100 kD, 90-kDa mainly depending on animal species and isoforms [30]. Nowadays, PDE5 is characterized by specific cGMP hydrolysis at low substrate levels and high affinity-binding sites for cGMP [22]. As showed in **Fig.1**, PDE5 has two GAF domains (GAF-A and GAF-B) and is activated by cGMP that specifically binds the GAF-A domain, a process modulated by phosphorylation [22, 31]. PDE5 enzymes are expressed in a variety of tissues/organs, such as corpus cavernosum, vessels, brain, lung, trachea, skeletal muscle, brain, kidney, pancreas, prostate, urethra, testis and bladder [30, 32].

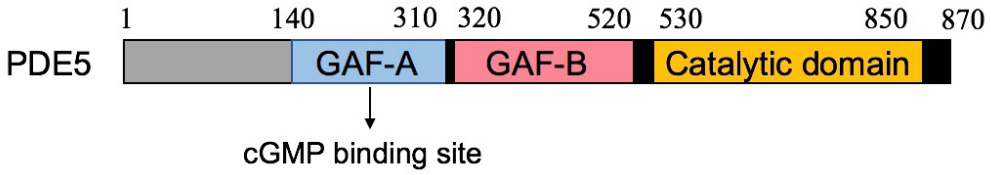


**Figure 1: The structural model of cGMP-specific PDE5.**

PDE5 has two GAF domains (GAF-A and GAF-B). PDE5 can be activated by high-affinity cGMP binding to the GAF-A domain. Binding affinity is modulated by phosphorylation of a nearby site.

#### 1.2.2.2 PDE5 inhibitors - Sildenafil

PDE5 inhibitors decrease intracellular accumulation of cGMP, resulting in the activation of cGMP-dependent protein kinase G (PKG) with subsequent phosphorylation of specific substrate proteins [33]. cGMP is a second messenger that activates the serine/threonine protein kinase PKG, shown to play a vital role in a variety of biological processes, including calcium homeostasis, platelet activation and adhesion, smooth muscle relaxation, cardiac functions, vasodilation, cell differentiation, proliferation and apoptosis [34].

Sildenafil is a selective PDE5 inhibitor, which was originally developed to treat heart disease. Sildenafil was marketed by Pfizer in 1998 with the trade name of Viagra as an effective treatment for erectile dysfunction in male patients [35]. Sildenafil accelerates intracellular cGMP levels by binding PDE5 and restrains the biological transformation of 3′,5′-cGMP to 5′-GMP, thus increasing blood flow to the corpus cavernosum [36]. Furthermore, there are more new proved applications of Sildenafil, such as treatment of lower urinary tract symptoms (LUTS) and pulmonary arterial hypertension (PAH) [37]. The successful application of Sildenafil in treating ED has stimulated investigations in several other clinical fields, including cardiovascular [38, 39, 40], cutaneous [41, 42], reproductive [43, 44, 45], pulmonary [46, 47, 48], neurologic [49, 50] as well as gastrointestinal disorders [51, 52, 53]. Interestingly, an emerging role for Sildenafil is potential effect on suppressing tumor growth in various cancer types.

### 1.2.3 PDE5 Inhibitors in Cancer

Numerous studies have shown that PDE5 expression is elevated in many human carcinomas, for instance metastatic breast, leukemia, bladder, lung, cervical, colorectal, prostate, pancreatic and lung tumors as compared to normal or surrounding non-neoplastic tissues [54, 55, 56, 57, 58, 59, 60, 61,

62], suggesting an important role for PDE5 during tumor progression. Mei XL and colleagues reported that Sildenafil intervention was associated with growth inhibition of human colorectal cancer cells by elevating intracellular reactive oxidative species (ROS) levels both in *vitro* and in *vivo* [62]. Furthermore, Sildenafil and Vardenafil induced caspase-dependent apoptosis in B-cell chronic lymphatic leukemia in *vitro* [63]. In another study, Sildenafil was involved in therapy regimens in a colon tumorigenesis model (i.e. Balb/c mice). In this model, Sildenafil was demonstrated to suppress tumorigenesis in inflammation-dependent colon cancer and to regulate the inflammatory microenvironment by blocking leukocyte infiltration [64]. Another PDE5 inhibitor, Exisulind, triggered growth inhibition and apoptosis in human prostate cancer by blocking the androgen receptor (AR) signaling pathway [65]. Moreover, Sulindac, selectively inhibited colon tumor cell growth by activating the cGMP/PKG pathway and suppressing Wnt/β-catenin signaling [66].

Many studies proved that PDE5 inhibitors had synergistic effects with various chemotherapeutics [67, 68, 69, 70, 71]. For example, PDE5 inhibitors enhanced the lethality of pemetrexed through inhibiting multiple chaperone proteins and via the actions of cyclic GMP and nitric oxide [67]. A recent study suggested that Sildenafil potentiated the antitumor activity of cisplatin by inducing apoptosis and inhibiting proliferation and angiogenesis [68]. Booth and colleagues discovered that Sildenafil could interact with the approved NSAID celecoxib to kill a variety of tumor cell types at physiologic concentrations [69]. It was also demonstrated by Li et al. that oral administration of the PDE5 inhibitor vardenafil potentiated the anti-tumor efficacy of anti-HER2 monoclonal antibody trastuzumab treatment in a xenograft mouse model of lung cancer [70]. In a study using medulloblastoma cell lines, Sildenafil was shown to significantly increase cell death when combined with vincristine, etoposide, as well as cisplatin [71].

Plenty of evidence illustrates that Sildenafil leads to tumor death and may be an adjuvant chemotherapy for cancers [72]. The underlying molecular

mechanisms of these effects are not fully understood and currently still being under investigation. Interestingly, elevation of PKG activity following PDE5 inhibition was corroborated with decreased expression of heat shock protein 90 (HSP90) [73, 74].

## 1.3 Heat shock protein 90

### 1.3.1 General introduction

Heat shock proteins (HSPs) are highly conserved stress response chaperone proteins, which are induced by environmental and cellular stressful conditions such as temperature change [75, 76]. HSPs participate in protein quality control by folding and stabilizing proteins to maintain cellular integrity and to protect cells [77, 78]. The family members of HSPs are named according to their molecular weight. Heat shock proteins 90 (HSP90) forms a family of chaperones with the molecular weight around 90 kD [79]. HSP90 is highly conserved and ubiquitously expressed aiding in the folding, stabilizing and remodeling of a plethora of proteins [77]. HSP90 can be found in all living species expect archaea [80]. Human HSP90 proteins members can be classified according to their cellular distribution. For instance, HSP90$\alpha$ and HSP90$\beta$ isoforms exist in the cytoplasm, tumor necrosis factor receptor-associated protein 1 (TRAP-1) is distributed in the mitochondria [81], and glucose related protein 94 (GRP94) is expressed in the ER [82]. As shown in **Fig.2**, HSP90 is an ATP-dependent molecular chaperone that exists as a dimeric protein composed of an N-terminal ATP-binding domain (NTD) [83], a C-terminal dimerization domain (CTD) [84] and a middle co-chaperone and client-binding domain (MD) [85].

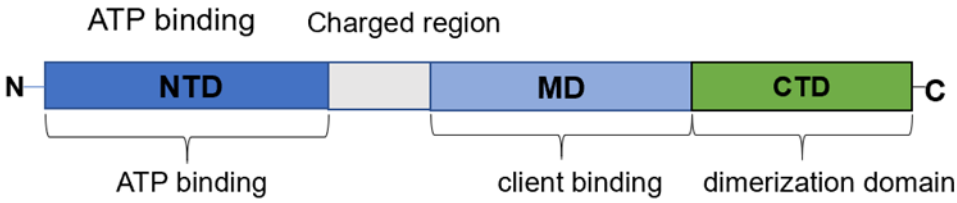


**Fig 2: The structural model of Hsp90.**

Hsp90 is composed of an N-terminal ATP-binding domain (N); a middle domain (M); and a C-terminal dimerization domain (C). A charged region is between the N and M domains. All three domains interact with different substrates.

### 1.3.2 Client proteins of HSP90

HSP90 is a biological catalyst which functions in non-covalent binding interacting partners, that are regarded as “clients” [86, 87]. HSP90 interacts with approximate 400 client proteins, many of which play essential role in the disease progression, including cancer, Alzheimer’s disease, as well as inflammatory diseases [88]. Client proteins of HSP90 include kinases like Akt2, CDKs, PKC as well as steroid receptors such as BCL-6, CAR, p53, Oct4 [88, 89, 90]. The interaction of HSP90 with its clients is aided by the so-called co-chaperones such as AHA1, HOP, p23 and CDC37 [86, 87]. Among these co-chaperones, CDC37 emerges to be a vital signal transduction molecule as a necessary adjoint for stable folding of protein kinases [86, 87]. CDC37 is involved in the inhibition of the HSP90 ATP-ase activity, thereby prolonging the interaction between the chaperone and its clients [91].

### 1.3.3 The role of HSP90 in cancer

HSP90 is overexpressed in multiple cancers, such as lung [92], gastric [93], breast cancers [94, 95] and glioblastoma [96], and is associated with tumor growth, differentiation. High levels of HSP90 are correlated with poor prognosis [97]. Many studies demonstrated that HSP90 promotes tumorigenesis through protein clients, many of which are oncogenic proteins, including tyrosine kinase

receptors (such as EGFR, Her2, and VEGFR), signal transduction proteins (such as Bcr-Abl, Alk, Braf, and Akt), transcription factors (such as androgen receptors, and HIF-1α), cell cycle regulatory proteins (such as Cdk4, Rb, and cyclin D) and antiapoptotic proteins (such as Bcl2) [98]. Furthermore, HSP90 was shown to regulate PKM2 via Thr-328 phosphorylation, thus promoting cell glycolysis, proliferation and inhibiting apoptosis in hepatocellular carcinoma [99]. In addition, HSP90 activates HIF‐1α and NF‐kB, leading to the enhanced epithelial-to mesenchymal transition (EMT), invasion, and metastasis in cancer cells [100]. Furthermore, HSP90 interacts with extracellular matrix metalloproteinase MMP-2 and MMP-9, which breaks extracellular matrix, leading to invasion and metastasis of tumors, such as breast cancer [101]. High HSP90 levels are corroborated with elevated expression of vascular endothelial growth factor receptors (VEGFRs) involved in the endothelial cell - dependent tumor angiogenesis [102]. Altogether, HSP90 overexpression translates in increased proliferation, migration, invasion and angiogenesis. Since the chaperone also plays an important role in tumor growth by maintaining the stability of various proteins upregulated in tumor cells, such as protein kinases C and D, SRC22, AKT23, PDK-1 and STK33, inhibitors for HSP90 are potential targets when considering future cancer therapy cocktails [103].

### 1.3.4 HSP90 inhibitors

HSP90 inhibitors have attracted special attention from researchers. Although several HSP90 inhibitors have been developed and entered clinical trials for diverse cancers, none of them has been approved for clinical use due to lack of efficacy and severe side effects. One pharmacological mechanism of HSP90 inhibitors is to suppress its ATP-ase activity upon binding to the ATP pocket in the N-terminus [104]. This in turn prevents the N-terminal closure of HSP90 dimer, disrupting the HSP90 chaperone cycle during the maturation of the client protein [104]. For example, PU-H71, a water-soluble purine-based analog

selective for HSP90, is currently tested in two clinical trials [105, 106]. PU-H71 inhibits the activity of the chaperone by blocking ATP binding of complexed HSP90. Consequently, PU-H71 triggers the destabilization and subsequent proteasome-mediated degradation of HSP90 client proteins [107, 108, 109]. There are also other designed inhibitors with diverse mechanisms such as disrupting the function of the chaperone [110]. Overall, HSP90 inhibitors can be a potentially effective therapeutics in cancers.

## 1.4 Protein Kinases

### 1.4.1 General introduction

Protein phosphorylation catalyzed by numerous protein kinases is a fundamental process of regulating cellular functions [111, 112, 113]. Since the first characterization of the phosphorylation reaction in glycogen metabolism and the description of glycogen phosphorylase in the 1960s, kinases have attracted the interest of many scientists [114]. Protein kinases are enzymes that activate other proteins by chemically adding phosphate groups to them. The chemical activity of kinases involves the transfer of a terminal γ-phosphate group of adenosine triphosphate (ATP) to serine, threonine or tyrosine residues, under the well-known process of phosphorylation [114]. Human genome sequencing revealed that around 2% of the human genome encodes for protein kinases [115, 116]. Kinases are classified in groups, families and subfamilies and are implicated in the signal transduction in eukaryotic cells by modifying substrate activity [114]. Besides, protein kinases have been proven to participate in several cellular processes and pathways, including metabolism, transcription, cell cycle progression, cytoskeletal rearrangement, cell movement, apoptosis and differentiation [115]. The protein kinase D (PKD) family belongs to the calcium/calmodulin dependent protein kinase (CaMK) superfamily [116]. The PKD family consists of three isoforms in mammals: PKD1/PKCμ, PKD2, PKD3/PKCν [117]. PKD1 is the first identified family

member in 1994, originally recognized as a member of the PKC family and was named as PKCμ [118]. Two additional PKD family members PKD2 and PKD3, were discovered in 2001 and 1999, respectively [119, 120]. As shown in **Fig. 3**, PKD2 includes an N-terminal cysteine-rich DAG/phorbol ester binding domain, a central protein binding pleckstrin homology (PH) domain, and a C-terminal catalytic domain that shares homology with the calmodulin-dependent kinases [118, 119, 120, 121]. Although all three members of PKD family exhibit high sequence homology, they have distinct structural and isoform-specific functions [119, 122, 123, 124]. PKDs can be triggered by a multitude of stimuli such as G protein-coupled receptors, tumor-promoting phorbol esters, reactive oxygen species and receptor tyrosine kinases [117, 125, 126]. PKDs are also directly activated by PKCs through phosphorylation at serine residues within the activation loop of the catalytic domain [127, 128]. The phosphorylation of the PKD isoforms results in activation of various signaling pathways including NF-κB, MAPK and CREB [129, 130]. PKDs have many cellular functions involved in various cellular and physiological processes, including cell migration, proliferation, survival, angiogenesis, protein transport, EMT, and transcriptional regulation [131].

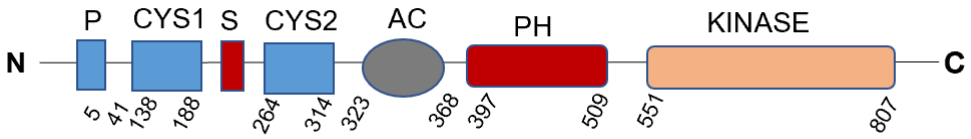


**Fig 3: The structural model of PKD2.**

P: proline rich domain; CYS: cysteine-rich Zn finger domain; AC: acidic domain; PH: pleckstrin homology domain; CAT: catalytic domain. Numbers indicate amino acid number. PKDs are composed of an N-terminal cysteine-rich DAG/phorbol ester binding domain, a central protein binding pleckstrin homology (PH) domain, and a C-terminal catalytic domain that shares homology with the calmodulin-dependent kinases.

### 1.4.2 The role of PKD2 in cancer

Many studies have demonstrated elevated PKD2 expression in various tumors, including prostate, breast, pancreatic, prostate, gastric, colorectal carcinoma, and myeloid leukemia [132, 133, 134]. PKD2 was reported as an important mediator of tumor cell - endothelial cell communication in gastrointestinal tumors by regulating VEGF-A expression and stimulating blood vessel formation [132]. PKD2 accelerates pancreatic cancer cell invasion and angiogenesis by stimulating expression of matrix metalloproteinases 7 and 9 (MMP7/9) [133]. Investigation of the role of PKD2 in chronic myelogenous leukemia (CML) demonstrated the kinase as an effector of GA binding protein (GABP), a tetrameric transcription factor involved in cell cycling [134]. PKD2 promotes angiogenesis in endothelial cells through the modulation of vascular endothelial growth factor receptor-2 and fibroblast growth factor receptor-1 expression [135]. In addition, tumor angiogenesis is enhanced by PKD2 also through mast cell recruitment upon the activation and interaction with ERK1/2 and NF-kB in prostate tumors [136]. PKD2 promotes invasion in prostate cancer upon activation of NF-kB and HDAC1-mediated expression of urokinase-type plasminogen activator (uPA) [137]. Furthermore, PKD2 mediates drug resistance, affecting the proliferation and migration of cancer cells. PKD2 has a positive regulatory effect on the migration of the drug-resistant breast cancer cells, while it has a negative regulatory effect on the migration of parental MCF-7 cells under the same conditions [138]. In triple-negative breast cancer cell lines such as HCC1806, PKD2 inhibition leads to decreased cell proliferation [139]. Furthermore, PKD2 also has been reported as a client of HSP90 participating at the chaperone's tumor angiogenesis program [140, 141].

### 1.4.3 PKD2 is a client of HSP90

Moulick and colleagues involved a proteomic approach combined with bioinformatic pathway analysis, to identify signaling networks which are

coordinated by heat shock protein 90 (HSP90), and identified PKD2 as a potential HSP90 “client” in chronic myeloid leukemia [140]. Our laboratory group also reported that the chaperone binds to and stabilizes PKD2 in various human cancer cell lines [141]. Interestingly, HIF-1a, an oxygen sensor protein also reported as a client of HSP90 [142] was shown to be modulated by PKD2 [143]. Stabilization of PKD2 by HSP90 also leads to the activation of NF-kB and its target VEGF-A, thus promoting cancer cell proliferation and stimulating blood vessel formation in hypoxic tumors [141].

## 1.5 Aims of the study

As described above, the repurposing of Sildenafil maybe an innovative therapy to target many cancers. However, the molecular mechanisms by which Sildenafil inhibits tumor progression remain unclear.

The main objective of this study was to investigate the effect of Sildenafil on tumor lethality and the molecular mechanism governing this event. In my thesis, the following investigations were addressed:

1. Investigation of the effect of low doses of Sildenafil on tumor cell proliferation and viability in vitro and in vivo.
2. Characterization of HSP90/PKD2 signaling following Sildenafil treatment of various cancer cell lines.
3. Delineation of the effect of combined therapy of PDE5 and HSP90 inhibitors in tumor growth.

# 2. Materials and Methods

## 2.1. Materials

### 2.1.1. Laboratory equipment

**Table 1:** Overview with employed equipment.

| Equipment | Company / Producer |
|---|---|
| Accu-jet® pro pipette controller | BRANDTECH, Germany |
| Autoclave VX-150 | Systec, Wettenberg, HE, Germany |
| Beckman Coulter Gallios 3/10 AT01005 | Beckman Coulter, USA |
| Block Heater/SBH130D | Stuart, UK |
| Camera Alpha 77, CAM macro images | Sony, Japan |
| Cell Culture Dishes (100×20 mm, sterile) | #664-160, CELLSTAR, Germany |
| Cell Culture Plates 6-Well | #657-160, CELLSTAR, Germany |
| Cell Culture Plates12-well | #665-180, CELLSTAR, Germany |
| Cell scraper | #83.1831, Sarstedt, Nuembrecht, NRW, Germany |
| Cell scraper, 16cm | #83.1832, Sarstedt, Nuembrecht, NRW, Germany |
| Centrifuge / Heraeus Megafuge 1.0R | Heraeus, ThermoFisher Scientific, USA |
| Centrifuge 5417C, 4℃ | Eppendorf, Hamburg, HH, Germany |
| Centrifuge Heraeus Labofuge 400 | Thermo Fisher Scientific, Waltham, MA, USA |
| Centrifuge tubes 15ml, sterile | #62-554-502, SARSTEDT, Germany |
| Centrifuge tubes 50ml, sterile | #62-547-254, SARSTEDT, Germany |
| Cold storage room 4°C / D 95030 | Viessmann HOF, Allendorf (Eder), HE, Germany |
| Counting chamber Neubauer | Laboroptik, Lancing, UK |
| Cover slips (22×22 mm) | Menzel-GLASER, Germany |
| Disposable cuvettes | #7590-15, Brand, Germany |
| Filter pipette tips 10µl | #70-1130-410, SARSTEDT, Germany |
| Filter pipette tips 1000µl | #70-762-411, SARSTEDT, Germany |
| Filter pipette tips 200µl | #70-760-411, SARSTEDT, Germany |
| Freezer -20°C / UIG1323 | Liebherr, Bulle, FR, Switzerland |
| Filter Paper | #3030-917, Whatman, GE healthcare, USA |
| Freezer -80°C | Heraeus, ThermoFisher Scientific, USA |
| Freezing tubes, 1.5ml, sterile | Roth, Karlsruhe, BW, Germany |
| Fridge 4°C | Liebherr, Bulle, FR, Switzerland |
| Fume cupboard | #D28857 Syke, Wesemann, Germany |
| Gel electrophoresis apparatus | Bio-Rad, Hercules, CA, USA |
| Glass tube (5ml) | #55.1579, Sarstedt, Germany |
| Gloves (M 7-8,powder-free) | Sempermed, Wimpassing, Austria |
| Gloves (Medium, powder-free) | #700113, Ansell, Belgium |

| | |
|---|---|
| HERAcell 240i CO2 Incubator | Heraeus, ThermoFisher Scientific, USA |
| Ice machine AF 80 | Scotsman, Vernon Hills, IL, USA |
| Imaging unit Fusion SL | Vilber lourmat, Peqlab, Germany |
| Inolab pH meter Level 2 | WTW Inc., Weilheim, Germany |
| Inverted microscope | # AE31, Motic, Germany |
| Inverted Microscope CKX41 | Olympus, Tokyo, Japan |
| Lamino airflow hood / HERA Safe | Heraeus, ThermoFisher Scientific, USA |
| Liquid nitrogen tank | Cryoson, Kleinkahl, BY, Germany |
| Liquid nitrogen tank (Cryoson/ UN1977) | CROSPEED®MED, Lindle, Germany |
| Luminometer | #Lumat LB 9501, Berthold, Germany |
| Magnetic stirrer / MR3000 | Heidolph, Schwabach, BY, Germany |
| Magnetic stirrer / MR3001 | Heidolph, Schwabach, BY, Germany |
| Microliter syringe 50µl/1705 RN | HAMILTON, Reno, Nevada, USA |
| Microtube (1.5 ml) | SARSTEDT, Germany |
| Microtube (1.5 ml) | SARSTEDT, Germany |
| Mini-Protean Tetra System | Bio-Rad, Hercules, CA, USA |
| Multipipette / Transferpette -8, 20-200µl | Eppendorf, Merk |
| Parafilm | #PM-996, BEMIS flexible packaging, USA |
| Pipette tips 10µl | #S1110-3000, STARLAB, Germany |
| Pipette tips 1000µl | #S111-6001, STARLAB, Germany |
| Pipette tips 200µl | #S111-0006, STARLAB, Germany |
| Pipettes 2, 10, 20, 100, 200 & 1000µl | Eppendorf, Germany |
| Power Pac Basic | Bio-Rad, Hercules, CA, USA |
| Power Pac HC | Bio-Rad, Hercules, CA, USA |
| Power Pac200 | Bio-Rad, Hercules, CA, USA |
| Scale / HG Tunning-Fork | ViBRA, shinko Denshi, Japan |
| Scale / HGTuning-Fork Scale / 2000-0.01g | Shinko Denshi, Germany |
| Scale / Sartorius BP-210-S | Sartorius, Germany |
| Scale / Sartorius TE313S | Sartorius, Germany |
| SDS-PAGE gel cassettes | Bio-Rad, Hercules, CA, USA |
| Serological Pipet 10ml | #357530, FALCON, USA |
| Serological Pipet 10ml, sterile | #4488, Costar, USA |
| Serological Pipet 1ml, sterile | #433402, Costar, USA |
| Serological Pipet 25ml, sterile | #4489, Costar, USA |
| Serological Pipet 2ml, sterile | #356507, FALCON, USA |
| Serological Pipet 5ml | #357529, FALCON, USA |
| Serological Pipet 5ml, sterile | #4487, Costar, USA |
| Shaker / Polymax 1040 | Heidolph, Schwabach, BY, Germany |
| Shaker / PROMAX 1020 | Heidolph, Schwabach, BY, Germany |
| Spectrophotometer / Ultrospec 3000 UV/Visiable | Pharmacia Biotech, Germany |
| Syringes Luer-Lok (1ml, sterile) | #300013, Becton Dickinson Plastipak™, USA |
| Test-tube-rotator | # SRT9, Stuart, UK |

| | |
|---|---|
| Thermomixer 5320 | Eppendorf, Germany |
| Thermomixer 5436 | Eppendorf, Germany |
| Trans-Blot Cell system | Bio Rad, Hercules, CA, USA |
| Water warming bath | # SWB25, ThermoScientific, USA |
| Water warming bath | # GFL-Type 1003, Eurolux, Germany |

### 2.1.2. Chemicals and drugs

**Table 2:** Overview with employed chemicals and drugs.

| Chemicals and Drugs | Cat. No. / Company |
|---|---|
| Acrylamide solution 30% | #3029.1, Roth, Carl Roth, Germany |
| Albumin bovine Fraction V pH7.0 (BSA) | #11930.04, Serva, Germany |
| 10x Annexin V Binding Buffer | ab14084, Abcam, UK |
| Amonium Persulfate (APS) | #A3678, Sigma Aldrich, USA |
| Annexin V Apoptosis detection reagent (500x) | ab14082, Abcam, UK |
| Blasticidin S | #A3784.0010, AppliChem, Germany |
| Bromophenol blue sodium salt | #114405-5G, Sigma Aldrich, USA |
| Calcium chloride | #CN93.2, Carl Roth, Germany |
| Citric acid | #C0759-5KG, Sigma Aldrich, USA |
| Complete Mini EDTA-free, Protease inhibitor | #11836170001, Roche, Switzerland |
| Dimethyl Sulphoxide (DMSO) | #D2650, Sigma Aldrich, USA |
| di-potassium hydrogen phosphate (K2HPO4) | #26931.263-500g, VWR, ThermoFisher Scientific, USA |
| di-Sodium hydrogen phosphate (Na2HPO4), anhydrous | #P030.1, Carl Roth, Germany |
| EDTA, anhydrous | #E6758-100G, Sigma,Germany |
| EGTA | #3054.3, Carl Roth, Germany |
| Ethanol, absolute | #32205-2.5L, Honeywell, Sigma Aldrich, USA |
| Ethanol, absolute | #32205-2.5L, Honeywell, Sigma Aldrich, USA |
| Glycerin | #7530.1, Carl Roth, Germany |
| Glycerol | #15523-1L-R-D, Sigma Aldrich, USA |
| Glycine | #A1377-1000, AppliChem, Germany |
| Hydrochloric acid 37% | #30721-1L, Honeywell, Sigma Aldrich, USA |
| Hydrogen peroxide （H2O2）30% | # 27500, Fischer, Germany |
| Isopropanol | #20842.330-2.5L, VWR Chemicals, USA |
| Luminol | #123072-25G, Sigma Aldrich, USA |
| Magnesium chloride (MgCl2) | #2189.1-500G, Sigma Aldrich, USA |
| Magnesium sulffate (MgSO4) | #0261.1, Carl Roth, Germany |

| | |
|---|---|
| Methanol | #32213-2.5L, HoneyWell, Sigma Aldrich, USA |
| N,N,N‘,N‘-Tetramethylethylenediamine (TEMED) | #T22500-100ML, Sigma Aldrich, USA |
| PageRuler Plus Prestained Protein Ladder | #26617, Thermo Fisher Scientific, USA |
| p-Coumaric acid | #C9008-25G, Sigma, Germany |
| Penicillin | Sigma, Germany |
| Polybrene | #TR-1003-G, Merck, Germany |
| Polyethylenimine (PEI) | #23966-2, Polyscience, USA |
| Potassium chloride (KCl) | #1.04936.1000, Merck, Germany |
| Potassium dihydrogen phosphate (KH2PO4) | #1.04873.1000, Merck, Germany |
| Protein Assay Dye Reagent Concentrate / BradFord Assay | #500-0006, BIO-RAD, USA |
| Propidium Iodide | ab14085, Abcam, UK |
| Puromycin dihydrochloride | #P8833-10MG, Sigma Aldrich, USA |
| PVDF membrane | #IPVH00010, Merck Millipore, USA |
| Skim milk powder | #,71066-500G, Fluka anylytical |
| Sildenafil | #PZ0003, Sigma, USA |
| Sodium azide | #S8032-25G, Sigma Aldrich, USA |
| Sodium chloride (NaCl) | #31434-1KG-R, Sigma Aldrich, USA |
| Sodium dihydrogen phosphate （NaH2PO4）, dihydrate | #T879-1, Carl Roth, Germany |
| Sodium dihydrogen phosphate （NaH2PO4）, monohydrate | #1.06346.0500, Merck, Germany |
| Sodium dodecyl sulphate (SDS) | #20765.03-1Kg, Serva, Germany |
| Sodium fluoride (NaF) | #S7920-100G, Sigma Aldrich, USA |
| Sodium hydroxide, pellets (NaOH) | #A1551.1000, AppliChem, Germany |
| Sodium hydroxide, solution (NaOH), 0.2mmol/L | #1.09140.1000, Merck, Germany |
| Sodium hydroxide, solution (NaOH), 2mmol/L | #1.09136.1000, Merck, Germany |
| Streptomycin | Sigma, Germany |
| Tris | #A2264-1000, Applichem, Germany |
| Trypanblue | #A0668.0025, AppliChem, Germany |
| Tween® 20 | #P7949-500ML, Sigma Aldrich, USA |

### 2.1.3. Cell lines

Employed cells were used in the early passages and kept in culture no longer than 6 to 8 weeks.

**Table 3:** Overview with employed cell lines.

| Cell line | Description |
|---|---|
| MIA PaCa2* | Human pancreatic epithelial carcinoma |
| Panc1* | Human pancreatic epithelial carcinoma |
| HEK293T§ – virus packaging cells | Human embryonic kidney |
| MDA-MB-231& | Human breast epithelial adenocarcinoma (derived from metastatic site: pleural effusion) |
| HCT-116# | Human colon epithelial carcinoma |
| SW-480$ | Human colon adenocarcinoma |

#Authenticated at Multiplexion, Heidelberg, Germany.

*Purchased from Leibnitz-Institute DSMZ- German Collection for Microorganisms and Cell Culture GmbH, Germany

§Purchased from Invitrogen, Germany

$Kindly offered by the Department of Surgery, Medical University of Ulm

&Kind gift of the Institute of Pharmacology for Natural Products and Clinical Pharmacology, University of Ulm, Germany

### 2.1.4. Cell culture items

**Table 4**: Overview with employed cell culture items.

| Media | Cat. No / Producer |
|---|---|
| Dulbecco's Modified Eagle Medium (DMEM) | #41965-039, Gibco, ThermoFisher Scientific,UK |
| McCoy's 5A Medium | #M8403-500ML, Sigma Aldrich, USA |
| RPMI Medium 1640 1× | #31870-025, Gibco, ThermoFisher Scientific, UK |
| Fetal bovine serum ( FBS ) | #S0615, Biochrom GmbH, Merck, Germany |
| Penicillin/Streptomycin (10000Units/mL penicillin and 10000ug/mL streptomycin) | #15140-122, Gibco, ThermoFisher Scientific, USA |
| 0.5% Typsin/EDTA 10× (no phenol red) | #15400-054, Gibco, ThermoFisher Scientific, UK |
| Dulbecco's Phosphate Buffered Saline (DPBS) 1× | #14190-094, Gibco, ThermoFisher Scientific, UK |

### 2.1.5. Plasmids

**Table 5:** Overview with the plasmids employed in this study.

| Vector | Type | Company |
|---|---|---|
| pLenti6.2-V5-DEST | Gateway cloning vector | #23490, Addgene, MA, USA |
| pLenti6.2-V5-DEST-PKD2 | PKD2 overexpression | Generated in the lab |
| pLenti6.2-GW/EmGFP | Empty vector | Invitrogen, Germany |
| pMD2.G | Packaging plasmid | #12259, Addgene, MA, USA |
| psPAX2 | Packaging plasmid | #12260, Addgene, MA, USA |

### 2.1.6. Buffers

Overview with employed buffers used in this study.

**Table 6:** 4× Laemmli-sample buffer.

| Component | Concentration |
|---|---|
| Tris-HCl pH 6.8 | 0.25 M |
| Glycerin | 26% |
| SDS | 20% |
| β-mercaptoethanol | 10% |
| Bromphenol blue | 0.004% |

**Table 7:** 1× SDS-running buffer (1× SDS-PAGE buffer).

| Component | Concentration |
|---|---|
| Tris | 25 mM |
| Glycin | 192 mM |
| SDS | 0.1% |

**Table 8:** 10×SDS-running buffer (10× SDS-PAGE buffer).

| Component | Weight |
|---|---|
| Tris | 151.1g |
| Glycine | 720.6g |
| SDS | 50g |
| Up to volume of 1500ml with $dH_2O$ | |

**Table 9:** Wet transfer buffer.

| Component | Weight |
|---|---|
| Tris | 12g |
| Glycin | 56.7g |
| Methanol | 800 ml |
| Up to volume of 4000ml with dH2O | |

**Table 10:** 10× PBS buffer.

| Component | Concentration |
|---|---|
| NaCl | 1.5 M |
| KCl | 26 mM |
| Na2HPO4 | 80 mM |
| NaH2PO4 | 17 mM |

**Table 11:** Washing buffer / 1×PBST (1× PBS containing 0.2% Tween 20).

| Component | Volume |
|---|---|
| PBS buffer 10× (see above) | 100 ml |
| dH2O | 900 ml |
| Tween20 | 2 ml |

**Table 12:** 5% non-fat milk in 1×PBST.

| Component | Weight |
|---|---|
| non-fat milk | 10 g |
| Up to 200ml with fresh washing buffer | |

**Table 13:** IP- lysis buffer.

| Component | Concentration |
|---|---|
| Tris/HCl pH=7.6 | 10 mM |
| EDTA pH=8.0 | 5 mM |
| NaCl | 50 mM |
| NaF | 50 mM |
| Triton X-100 | 1% |

Note: IP lysis buffer could be used for protein extraction for WB. 1 tablet of Protease inhibitor (Complete-Mini, EDTA free) was dissolved in 10 ml of IP lysis buffer.

**Table 14:** Gels used for WB were prepared with 1.0 mm comb.

| Component | Separating gel 10 % | Collecting gel 4 % |
|---|---|---|
| $dH_2O$ | 8.16 ml | 6 ml |
| 30 % Polyacrylamide | 6.66 ml | 1.35 ml |
| Tris 1.5 M pH=8.8 | 5 ml | - |
| Tris 0.5 M pH=6.8 | - | 2.5 ml |
| 10 % SDS | 200 µl | 100 µl |
| 10 % APS | 400 µl | 200 µl |
| TEMED* | 30 µl | 20 µl |

Note: APS and TEMED were only added just before casting the gel between the glass plates in order to avoid premature polymerization.
Abbreviations: TEMED, N,N,N‘,N‘-Tetramethylethylenediamine; APS, ammonium persulfate.

**Table 15:** Composition of ECL solution.

| Component | Solution 1 | Solution 2 |
|---|---|---|
| Water | 18 ml | 18 ml |
| Tris-HCl 1M pH=8.5 | 2 ml | 2 ml |
| 30% $H_2O_2$ | 12 µl | - |
| 90 mM p-Coumaric acid | - | 88µl |
| 250 mM Luminol | - | 200µl |
| Mix solution 1 and solution 2 before use. | | |

Note: Preparation of 90 mM p-Coumaric acid: 0.15g p-coumaric acid were reconstituted in 10ml DMSO. Preparation of 250 mM Luminol: 0.44 g 3-Aminophthalhydrazide were reconstituted in 10 ml DMSO.

### 2.1.7. Antibodies

#### 2.1.7.1. Primary antibodies

All primary antibodies were diluted in 5% dry fat milk in 1×PBS-Tween20. The PVDF membranes were incubated with primary antibody overnight.

**Table 16:** List with primary antibodies used in our study.

| Antigen | Isotype | Dilution | Storage | Manufacturer | Cat. No |
|---|---|---|---|---|---|
| β-actin (Clone AC-15) | Mouse, monoclonal | 1:2000 | -20 °C | Sigma-Aldrich | #A5441 |
| HSP90β (Clone D-19) | Goat, polycolonal | 1:500 | 4 °C | Santa Cruz Biotechnology | #SC-1057 |
| Ki 67(Clone MIB-1) | Mouse, monoclonal | 1:100 | -20 °C | Agilent | GA62661-2 |
| PARP (Clone 46D11) | Rabbit, polyclonal | 1:1000 | -20 °C | Cell Signaling Technology | #9542S |
| PKD2 | Rabbit, polyclonal | 1:1000 | 4 °C | Calbiochem | #ST1042 |

Abbreviations: HSP90β, heat shock protein 90 beta; PKD2, protein kinase D2.

### 2.1.7.2. Secondary antibodies

All Secondary antibodies were diluted in 5% dry fat milk in 1×PBS-Tween20. The PVDF membranes were incubated with secondary antibody for 2 hours.

**Table 17:** List with secondary antibodies used in our study.

| Type | Isotype | Dilution | Storage | Manufacturer | Cat. No |
|---|---|---|---|---|---|
| Horseradish peroxidase (HRP)-conjugated | Rabbit anti-goat IgG | 1:10000 | -20 °C | GE Healthcare UK Limited | #A5420 |
| Horseradish peroxidase (HRP)-conjugated | Sheep anti-mouse IgG | 1:2500 | 4 °C | GE Healthcare UK Limited | #NA931 |
| Horseradish peroxidase (HRP)-conjugated | Donkey anti-rabbit IgG | 1:2500 | 4 °C | GE Healthcare UK Limited | #NA934 |

Abbreviations: IgG, immunoglobulin G.

### 2.1.8. HSP90 inhibitor (PU-H71)

PH-H71 inhibitor was kindly provided by Dr. Gabriela Chiosis at the Memorial Sloan-Kettering, New York, USA, which holds the intellectual rights. Samus Therapeutics, of which Dr. Chiosis has partial ownership, has licensed PU-H71.

### 2.1.9. Materials for Immunohistochemistry

**Table 18:** List with materials for Immunohistochemistry used in our study.

| Type | Isotype | Dilution | Storage | Manufacturer | Cat. No |
|---|---|---|---|---|---|
| Ki67, clone MIB-1 | Mouse | 1:100 | 4 °C | DAKO | |

### 2.1.10. Materials for chicken CAM xenograft experiments

The fertilized eggs used for tumor xenografts were purchased from LSL Rhein-Main, Dieburg Germany.

### 2.1.11. Software

**Table 19:** Software used in the current study.

| Name | Company |
|---|---|
| Microsoft Office | Microsoft, USA |
| Adobe Phososhop CS5.1 | Adobe, USA. |
| GraphPad Prism 7.0 software | GraphPad Software, Inc, La Jolla, CA, USA |
| Image J software | National Institute of Health, Bethesda, MD, USA |
| FlowJo software | Becton Dickinson Biosciences, USA |

## 2.2. Methods

### 2.2.1 Cultivation of cancer cell lines

#### 2.2.1.1 General cell culture techniques (centrifugation and quantification of cells number)

Centrifugation of trypsinised cells was performed in Heraeus centrifuges for 5 minutes at 900 rpm at room temperature. Thereafter, pelleted cells were resuspended in fresh growing media. For quantification of cells number a Neubauer chamber was used. 10 µl cell suspension was placed between chamber and coverslip and cells quantified on 4 marked quadrates. Discrimination between living and dead cells was given by staining with Trypan blue, a dye which enters into the cells upon membrane disruption. Final number of cells is given by the formula:

Living cells / ml media = quantified white cells / 16 quadrates x $10^4$ x Dilution

#### 2.2.1.2 Cultivation of adherent cancer cells

The early passages of human pancreatic MIA PaCa-2 and Panc1, human breast MDA-MB-231 cancer cells as well as HEK293T human embryonic kidney cell line were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2% glutamine, and 1%

penicillin/streptomycin. Human colorectal HCT-116 cancer cells were cultured in McCoy'5A medium supplemented with 10% FBS and 1% penicillin/streptomycin. Human colorectal SW480 adenocarcinoma cell lines were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin. Cancer cell lines were obtained from DSMZ-German collection of Microorganisms and Culture Cells (Braunschweig, Germany) or authenticated at the Multiplexion (Heidelberg, Germany). HEK293T cell line was used to produce lentivirus - containing supernatants. Cells were washed with pre-warmed sterile Dulbecco's Phosphate Buffered Saline (DPBS 1×). After removing FBS residues, cells were incubated with 1 ml pre-warmed trypsin/EDTA solution at 37℃ for 2 minutes, cells were observed under microscopy for detachment. After cell detachment, FCS-containing media was added to inactive the trypsin. The cell suspension was collected and centrifuged at 900 rpm for 4 minutes. Then, the supernatant was removed and cell pellet was resuspended in a minimal volume of pre-warmed complete growth medium. Split cells were used either for various experiments or further propagated. The medium exchange was performed every 2 – 3 days. Cells were split at a confluence of 70 – 90%.

#### 2.2.1.3 Freezing and thawing of cell lines

Cancer cells in exponentially growing phase were trypsinised and centrifuged for 5 minutes at 900 rpm. After resuspension of cells in freezing media containing 90% FBS and 10% DMSO, 1 ml was distributed in each liquid-N2 dedicated vials. Cells were placed for 1 hour in ice and then transferred to the -80°C freezer. Frozen cells were transferred to the liquid nitrogen after one week. Cells were thawed by quickly bringing them from the liquid nitrogen and immediately placed in 37°C water bath. Then, transfer 7 ml pre-warmed complete growth medium drop-wise into the centrifuge tube containing the thawed cells. Cells were centrifuged at 900 rpm for 5 minutes. After the

centrifugation, cancer cells were gently resuspended in fresh growing medium and plated in culture dishes.

#### 2.2.1.4 Transfection of virus-producing HEK 293FT cells and harvesting of virus-containing supernatants

HEK 293FT cells were plated at a density of $1x10^6$ per 10 cm plate 24 hours prior the transfection procedure. In an Eppendorf tube 3 µg of DNA together with 300 ng pMD2 and 1.8 µg psPAX2 were resuspended in up to 900 µl FBS and penicillin/streptomycin-free DMEM media. 30 µl polyethyleneimine (PEI) was added to the mix which was then incubated for 20 minutes at ambiental temperature. Supernatants were harvested after 24 and 48 hours. Virus-containing supernatants were filtered through a 0.2 µm filter. This procedure was performed in the security level 2 (S2) laboratory at the Medical University of Ulm.

#### 2.2.1.5 Lentiviral-mediated transduction of cancer cells

Target cancer cells were plated so that a cell confluence of about 40-50% is reached in the day of the viral transduction. Growing media was changed with 3 ml fresh media and 1ml lentivirus-containing supernatant. To the 4 ml final volume 6 µl polybrene (15µg/ml) was added before incubation at 37°C. Transduced cells were washed 24 hours later when the corresponding antibiotic (puromycin, 2 µg/ml or blasticidin 3µg/ml) was added. Selection was conducted for the next 3 days (puromycin) or 5 days (blasticidin). Assays were performed few days after selection to avoid eventual effects of the antibiotic.

### 2.2.2 Analysis of cancer cell proliferation

Cancer cell lines were seeded in triplicate in 12 well dishes ($3×10^4$ cells/well). Cells were incubated with various concentration of Sildenafil and proliferation was monitored up to 144 hours. Every day for a period of seven days, cells from

a set of three wells were collected and cell number was determined as described at 2.2.1.1.

### 2.2.3 FACS analysis

Cancer cells were incubated with 25 µM Sildenafil for three days. After washing with PBS, cells were subjected to Annexin V / Propidium Iodide staining according to the manufacturer recommendations. Acquisition was conducted with a Beckman Coulter Gallios 3/10 AT01005 machine; analysis of data was performed with Kaluza Analysis 2.1. software.

### 2.2.4 Analysis of proteins

#### 2.2.4.1 Total protein extraction from cultured cells

Cancer cells were harvested from culture dishes and transferred into 15 ml conical tubes. After centrifugation (4 minutes, 1200 rpm), pellet was washed with 600 µl PBS and finally lysed in IP-lysis buffer containing 10 mM Tris-HCl, 5 mM EDTA, 50 mMNaCl, 50 mM NaF and 1% Triton X100 supplemented with Complete Protease Inhibitor Cocktail and PhosStop tablets. After centrifugation of probes (10 min, 14,000 rpm at 4°C) cleared lysate was retrieved.

#### 2.2.4.2 Measurement of protein concentration

In order to measure the protein concentration a 1:5 dilution of Protein Assay Dye Reagent was used. In brief, 1 µl cell lysate was suspended in 1000 µl Protein Assay Reagent before incubation for 5 minutes at RT. measuring The optical density (OD) at 595 nm wavelength was measured with a spectrophotometer.

#### 2.2.4.3 Western blot analysis

In order to separate and detect a particular protein, cell lysate was subjected to

SDS-PAGE gel electrophoresis. The composition of the lower/separation gel and upper/collecting gel was prepared as presented in table 14. After casting, the separating gel was allowed to polymerize for about 1 hour. Upon casting the collecting gel, a “comb” was placed at the loading end, and polymerization was allowed for an additional hour. The electrophoresis occurred in a BioRad Electrophoresis chamber filled with running buffer. 50 to 100 µg whole cell extract were mixed with Laemmli buffer freshly supplied with 5% β-mercaptoethanol. Denaturation took place for 10 minutes at 96°C in a heating device. Spun down protein samples were loaded into gel slots previously rinsed with running buffer. The separation of proteins occurred about 1 hour at 160 V at the room temperature. Transfer of proteins from gel to a methanol-activated polyvinylidene difluoride membrane (PVDF) was performed under a constant current of ~400 mA for two hours at 4°C using as so called “wet blotting” method. Membranes were blocked in 5 % non-fat dry milk in PBS containing 0.2% Tween-20 for 1 hour at room temperature in order to minimize unspecific binding. 0.2% Tween-20 in PBS was used for all subsequent washes. PVDF membranes were then incubated at 4°C on a rocking shaker, overnight with the first specific antibody for desired protein. Incubation with the secondary antibody covalently conjugated with horse radish peroxidase which binds to already bound first antibody and catalyses a chemiluminescent reaction occurred for 1 hour at room temperature. Finally, after three consecutive washes each of 10 minutes at room temperature, ECL substrate was added to the membrane and the specific bands for desired proteins could be visualized by using a ChemiSmart machine.

### 2.2.5 In vivo tumor xenografts using chicken chorioallantoic membrane (CAM) assay

Tumor xenografts experiments were used to substantiate the *in vitro* data with respect to tumor growth. 1.5 million MIA PaCa2 pancreatic or HCT-116 colon

cancer cells were used for CAM tumor xenografts. Genetically modified cancer cells were implanted on day 8 after fertilization inside of a silicon ring with the diameter of 5 mm. Tumors were allowed to form and grow for the next 4 days. Treatment with HSP90 and/or PDE5 inhibitor occurred 24 and 48 hours after tumor cell implantation respectively. On day 12 after fertilization tumors were retrieved, photographed and fixed in formalin. Formalin-fixed tumors were then embedded in paraffin using standard protocols. Immunohistochemistry was conducted with Ki67.

### 2.2.6 Statistical analysis

Statistical analysis as well as graphing was accomplished using Prism 7.03 (GraphPad Software). Mean and statistical significance were calculated and analysed using t-test. All data are representative of at least three experiments.

# 3. Results

## 3.1 Sildenafil treatment inhibits the growth of cancer cells in a concentration-dependent manner

Sildenafil, the selective phosphodiesterase 5 (PDE5) inhibitor was reported to impair the growth of colon cancer cells at concentrations varying from 190 to 271 μM. As these concentrations of Sildenafil are far from being physiologically relevant in clinical interventions, we interrogated whether lower concentrations could have a negative impact on cancer cell proliferation. The effect of Sildenafil was investigated in our group in several cancer cells including breast, lung and pancreatic cancer. In the first experiments, I substantiated the previous data in the above mentioned cancer cells by taking under investigation two colon cancer cell lines (i.e. HCT-116 and SW480). These were treated for three days with a concentration of Sildenafil ranging from 0 - 50 μM. The cell number was quantified in a triplicate setting after 72 hours. Our results revealed that the two human colon cancer cell lines displayed a decreased proliferation to Sildenafil treatment (**Fig. 4A** and **B**). These impaired proliferation rates were similar to breast, lung or pancreatic cancer cell lines from experiments previously conducted in the lab. Furthermore, we observed that HCT-116 cells are more sensitive to Sildenafil than SW480 cells. While proliferation of HCT-116 is negatively impacted already at a concentration of around 10 μM, SW480 cells appear to be more resistant at this concentration. However, both of them displayed adequately impaired proliferation at the concentration of 25 μM. Therefore, this concentration was chosen for subsequent experiments.

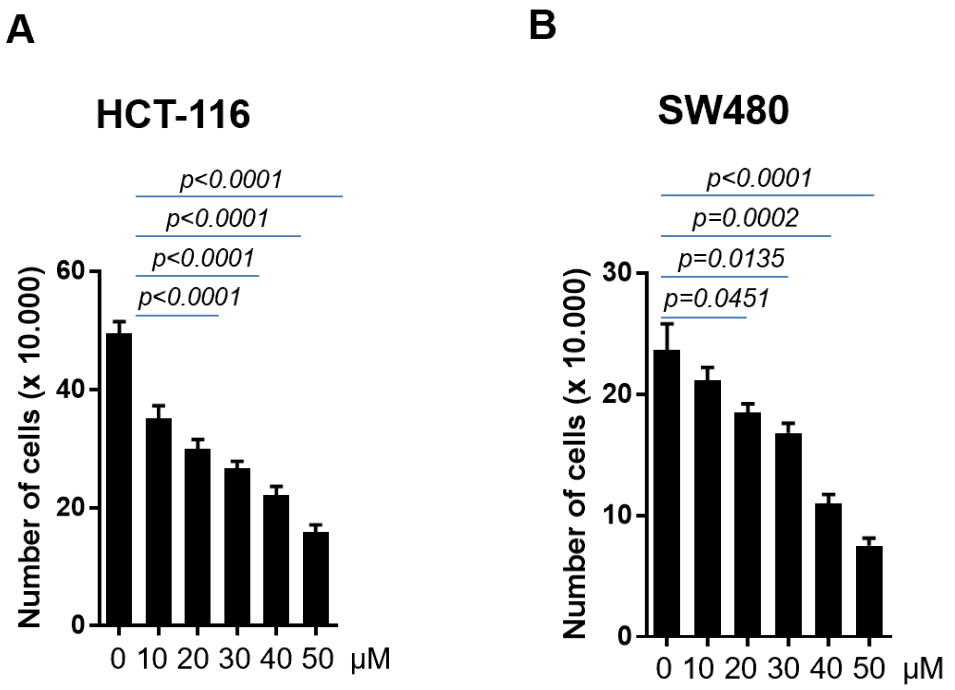


**Figure 4: Sildenafil treatment is associated with decreased growth of colon cancer cells in a concentration dependent manner.**

**A, B,** HCT-116 and SW480 colon cancer cells ($2x10^4$) were seeded in 12-well dishes and incubated with various concentration of Sildenafil as indicated. The cell number was determined after 72 hours. One of three independent experiments performed in triplicate is shown. Error bars represent the standard errors of mean of three independent experiments. These results are in line with experiments obtained with MIA Paca2, Panc-1, A549, MDA-MB-231 cancer cells conducted by Yang Liu in our lab.

Taken from Lu et al., 2020 (ref. 161).

## 3.2 Sildenafil treatment inhibits the proliferation of cancer cells in a time-dependent manner

Next, we sought to explore how the proliferation is impacted over the time upon treatment with Sildenafil. HCT-116, SW480 colon cancer cells were left untreated or treated with 25 µM Sildenafil for six days. The proliferation was assessed every 24 hours. As depicted in the **Fig. 5A** and **B**, the proliferation of cancer cells was not only impaired in a dose-dependent, but also in a time-dependent manner. Incubation of colon cancer cells for 72h, 96h, 120h and 144h resulted in a markedly decreased in proliferation (**Fig. 5A** and **B**). Our observation is consistent with the results from the treatment of breast, lung and

pancreatic cancer cell lines. Altogether, these results indicate the suppressive effect of Sildenafil on cancer cell proliferation.

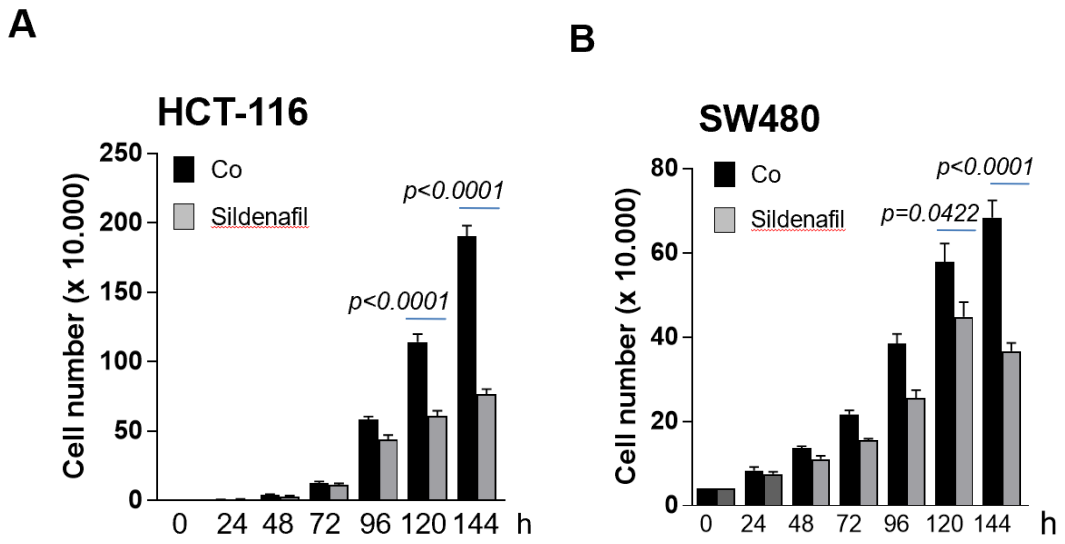


**Figure 5: Sildenafil treatment inhibits the growth of cancer cells in a time dependent manner.**

**A, B**, HCT-116 and SW480 colon cancer cells ($2x10^4$) were seeded in 12-well plates and incubated with 25 µM Sildenafil. The cell number was quantified every 24h in the next six days. One of three independent experiments performed in triplicate is shown. Error bars show the standard errors of mean of three independent experiments. These results are in line with experiments obtained with MIA Paca2, Panc-1, A549, MDA-MB-231 cancer cells conducted by Yang Liu in our lab.

Taken from Lu et al., 2020 (ref. 161).

## 3.3 Sildenafil treatment induces apoptosis in human cancer cells

Having analyzed the effect of Sildenafil on cancer cell proliferation, we determined next whether the drug may impact the cell viability as well. Therefore, colon and pancreatic cancer cells were treated with 25 µM Sildenafil for 72 hours before being stained with Annexin V/PI and subsequently examined by FACS analysis. Our data demonstrates that Sildenafil intervention significantly triggered apoptosis in both MIA PaCa-2 and HCT-116 compared to control group (**Fig. 6A** and **B**). Both fractions of early and late stage apoptotic

cells were significantly elevated with Sildenafil incubation (**Fig. 6A** and **B**).

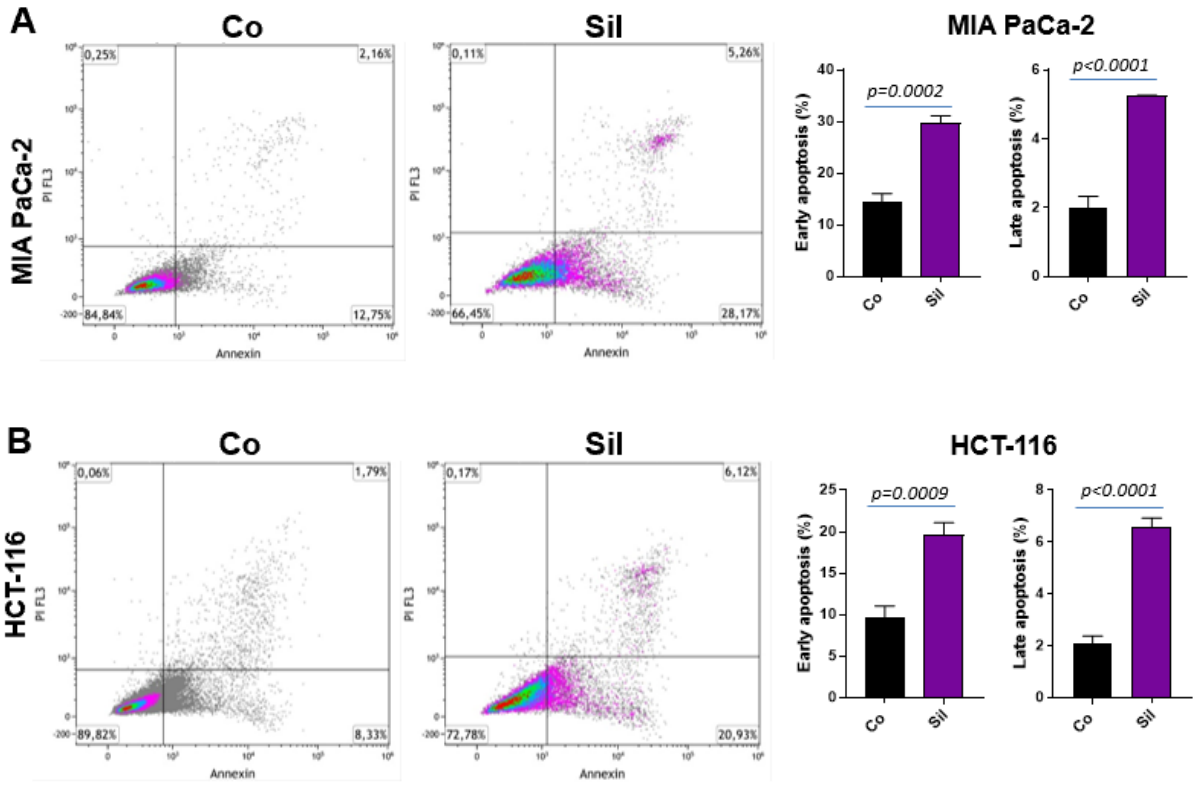


**Figure 6: Sildenafil treatment is corroborated with impaired cancer cell viability.**

**A**, MIA PaCa2 pancreatic and **B**, HCT-116 colon cancer cells were treated with 25 µM Sildenafil (Sil) for three days. The apoptosis was detected by flow cytometry with Annexin V / Propidium Iodide (PI) staining. The FACS analysis was conducted in triplicate. The data is shown as mean +/- SEM of three independent experiments.

Taken from Lu et al., 2020 (ref. 161).

In order to substantiate the FACS results, we investigated the apoptosis marker, cleaved PARP by Western blotting. MIA PaCa-2 and HCT-116 cancer cells were incubated with Sildenafil. Cell were harvested after three days and used for preparation of whole cell lysates before western blot analysis. Cleaved PARP levels were substantially augmented upon incubation with the drug as presented in **Fig. 7.**

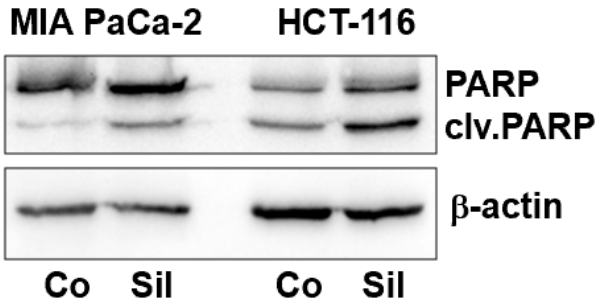


**Figure 7: Sildenafil treatment is associated with elevated cell death.**

Protein lysates of MIA PaCa2 and HCT-116 cells were subjected to western blotting using PARP specific antibody.β-actin was used as loading control. A representative western blot is shown.

Taken from Lu et al., 2020 (ref. 161).

## 3.4 Sildenafil treatment suppresses tumor growth in vivo

Our data showing that Sildenafil is able to inhibit proliferation and impair cancer cell viability *in vitro* prompted us to determine whether PDE5 inhibitor is reflected by decreased tumor progression *in vivo*. In this experiment, the chicken chorioallantoic membrane (CAM), a well-established model of *in vivo* tumor formation was involved. MIA PaCa-2 pancreatic cancer and HCT-116 colon cancer cells were transplanted onto the surface of CAM of fertilized chicken eggs at day 8 of embryonic development. Xenografted tumors were treated with Sildenafil after 24 and 48 hours respectively. Four days after implantation, formed solid tumors were resected and fixed in formalin. The microtumors, which can be observed with the naked eye, were photographed. Using ImageJ software, I calculated the area of the tumors xenografted inside of a 5 mm diameter silicon ring. The quantifications show that Sildenafil treatment was associated with significantly decreased tumor area (**Fig. 8A** and **B**).

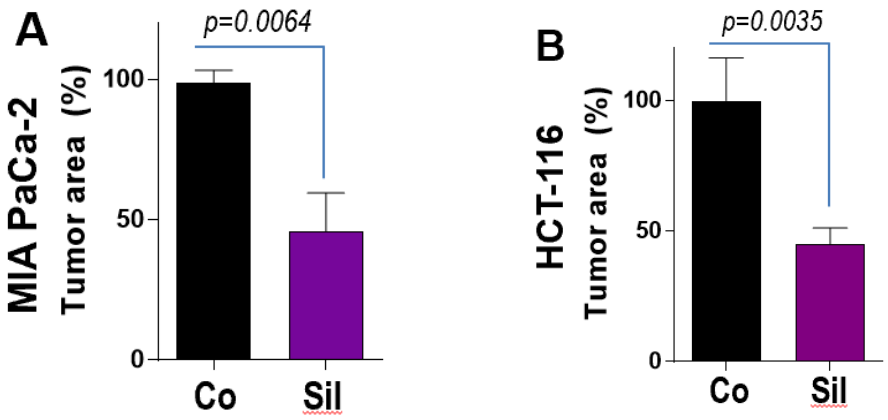


**Figure 8: The tumor area is decreased after sildenafil intervention.**

**A, B,** HCT-116 colon or MIA PaCa2 pancreatic cancer cells ($1.5x10^6$) were transplanted at day 8 of embryonic development. Tumors were treated with 25 µM Sildenafil (Sil) after 24 and 48 hours after the xenograft procedure. Four days after implantation, tumors were retrieved and their area was calculated. The charts presenting the tumor area show the mean +/- SEM of four to seven tumors.

Taken from Lu et al., 2020 (ref. 161).

Tumors fixed in formalin were subjected for immunohistological staining. Ki67 marker was used in order to detect proliferating cells. Digital photographs were used for quantification of the Ki67 proliferation index. My data shows that Sildenafil application resulted in significantly decreased tumor growth (**Fig. 9A** and **B**).

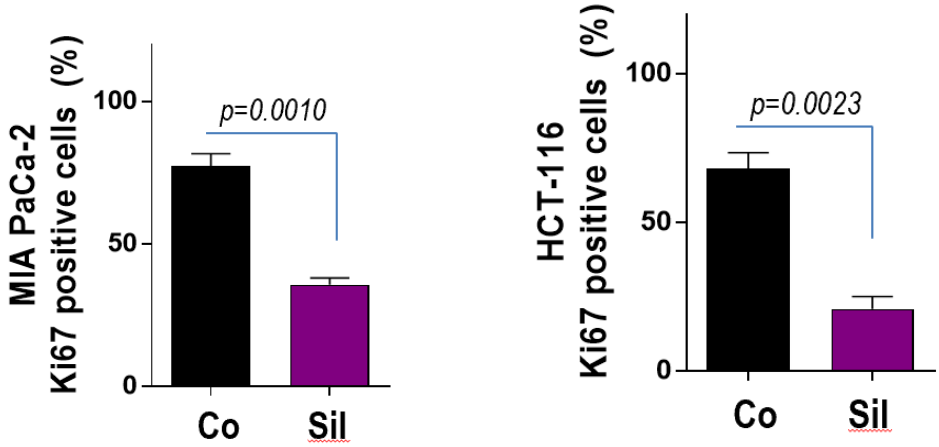


**Figure 9: Sildenafil treatment suppresses tumor growth in vivo.**

Ki67 proliferation index for HCT-116 **(A)** and MIA PaCa2 **(B)** cells growing on CAM is presented. Error bars represent mean +/- SEM of at least six microscopic fields, each containing at least

200 cells.

Taken from Lu et al., 2020 (ref. 161).

## 3.5 Sildenafil treatment is associated with altered HSP90 expression and PKD2 degradation

The data above demonstrated that Sildenafil negatively impacted cell proliferation and viability. In the next set of experiments we investigated potential molecules and signaling pathways that might be involved. Since earlier reports linked combinatorial intervention of Sildenafil/pemetrexed to killing tumor cells by altering the expression of HSP90, HSP70 or GRP78, we sought to investigate whether Sildenafil treatment affects the levels of the HSP90 chaperone. Therefore, Panc1 and MIA PaCa-2 pancreatic cancer cells were treated with Sildenafil at a concentration of 25 μM and 50 μM respectively. Lysates were subjected to western blot analysis in order to check HSP90 protein levels. Our data show that HSP90 expression is reduced following Sildenafil treatment in both cell lines (**Fig. 10A**). The 2D densitometry analysis demonstrates that the significant reduction of HSP90 occurred in a dose-dependent manner (**Fig. 10A**). Our laboratory reported that protein kinase D2 (PKD2) participates to the growth, migration, metastasis and invasion of various tumor types including pancreatic tumors. Interestingly, PKD2 stabilization was demonstrated to be orchestrated by the HSP90/CDC37 chaperone complex. Thus, we examined whether Sildenafil treatment also had an impact on PKD2 expression. Our data demonstrate an association between altered HSP90 expression and increased degradation of PKD2 (**Fig. 10B**). Altogether, our findings indicate that the decreased proliferation and viability after incubation of cancer cells with Sildenafil could be partially funneled through the HSP90/PKD2 signaling pathway.

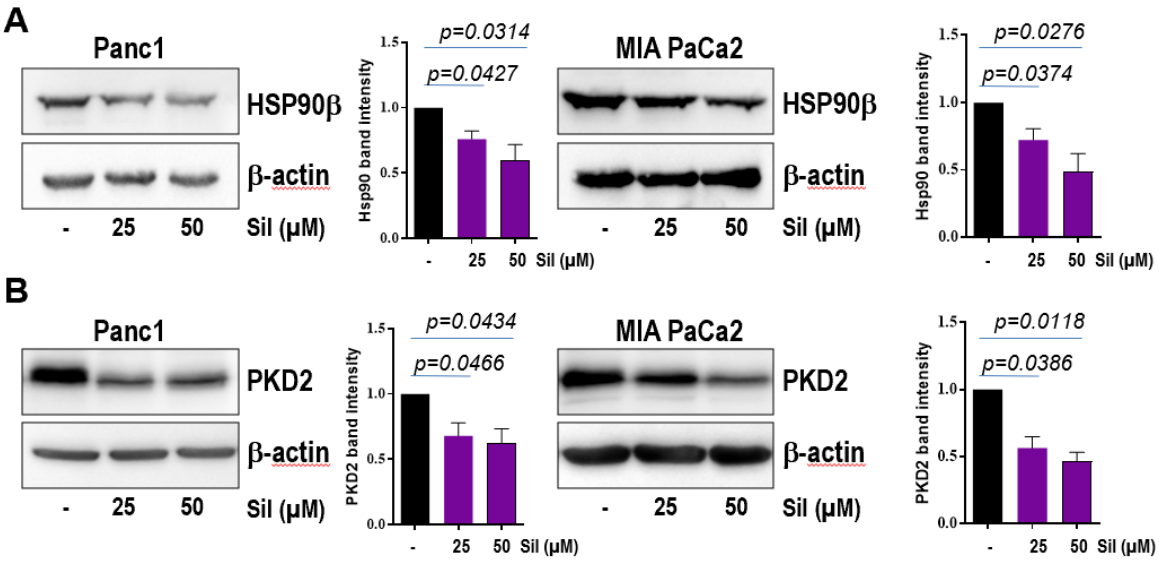


**Figure 10: Cancer cells proliferation impaired by Sildenafil treatment is associated with altered HSP90 expression and PKD2 degradation.**

**A**, **B**, Panc1 and MIA PaCa-2 pancreatic cancer cells were incubated with 25 and 50 μM Sildenafil for 3 days. Protein lysates of cancer cells were subjected to western blotting using with HSP90β- and PKD2-specific antibodies. β -actin served as loading control. The representative western blots of three independent experiments are shown. Densitometry analysis of three to four blots is presented.

Taken from Lu et al., 2020 (ref. 161).

## 3.6 Impaired proliferation of cancer cells following Sildenafil treatment can be partially restored by ectopic PKD2

In order to further delineate the contribution of kinase to the proliferation, we generated cancer cells stably transduced with a PDK2 expression vector or empty vector. Successful ectopic expression of PKD2 in various cancer cell lines is shown in **Fig. 11A**. Transduced cancer cell lines were treated with 25 μM Sildenafil every 24h for 3 days. Our results show that the mere expression of PKD2 is associated with augmented proliferation (**Fig. 11B – E**). These data are in line with previous data in our laboratory. Furthermore, we could reconfirm the impaired proliferation of SW480 after Sildenafil treatment (**Fig. 11B**) similar to the data presented in **Fig. 4B**. Most importantly, overexpression of PKD2 was corroborated with a partial rescue of proliferation after Sildenafil treatment (**Fig.**

**11B – E**). Taken together, our findings infer the alteration of HSP90 and PKD2 degradation as possible molecular mechanism leading to cell death after Sildenafil treatment.

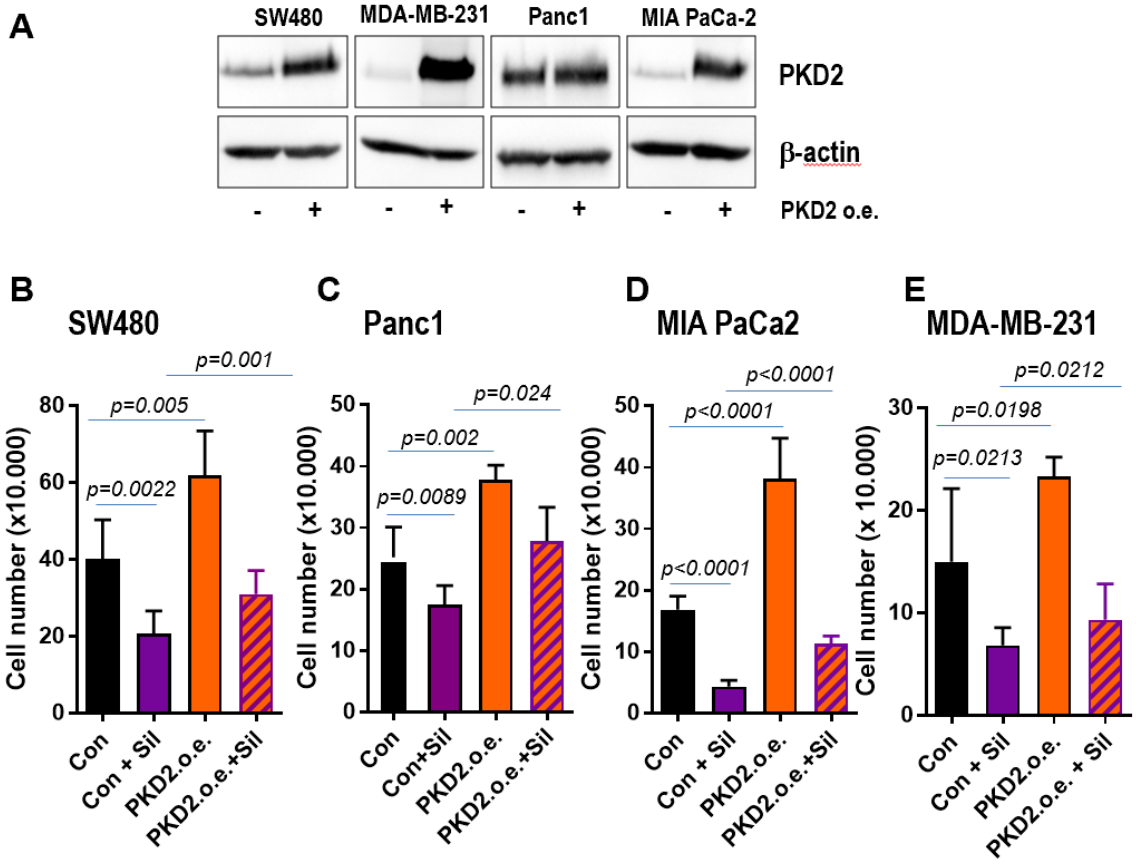


**Figure 11: Impaired proliferation of cancer cells following Sildenafil treatment can be partially restored by ectopic PKD2.**

**A**, PKD2 over-expression in various cancer cells was achieved via lentiviral-mediated transduction. Protein lysates of cancer cells were subjected to western blotting using with PKD2 specific antibody. β-actin served as a loading control. The representative western blots results of three independent experiments were shown. **B, C, D, E, F,** transduced cancer cells were seeded in 12 well dishes and left untreated or treated with 25 μM Sildenafil at 0h, 24h, 48h time points. The cell number was quantified at 72h. One of three independent experiments performed in triplicate is shown. Error bars represent the standard errors of the mean of three independent experiments.

Taken from Lu et al., 2020 (ref. 161).

## 3.7 PDE5 and HSP90 inhibitors synergistically suppress tumor growth *in vivo*

In this set of experiments we sought to lower even further the concentration of Sildenafil (i.e. down to 15 μM). In addition, we involved in our study the PU-H71, an ATP-competitive HSP90 inhibitor. Previous studies showed that 1 μM PU-H71 is associated with substantial cell death. Indeed, we could reconfirm these results in our western blot analysis (**Fig. 12**). However, the western blot analysis shows the 50 nM PU-H71 had no effect on PARP cleaved (**Fig. 12**). Therefore, we selected this concentration of PU-H71 for further experiments where this inhibitor was combined with Sildenafil. Consequently, a combination of 15 μM Sildenafil and 50 nM PU-H71 were used in our *in vivo* experiments.

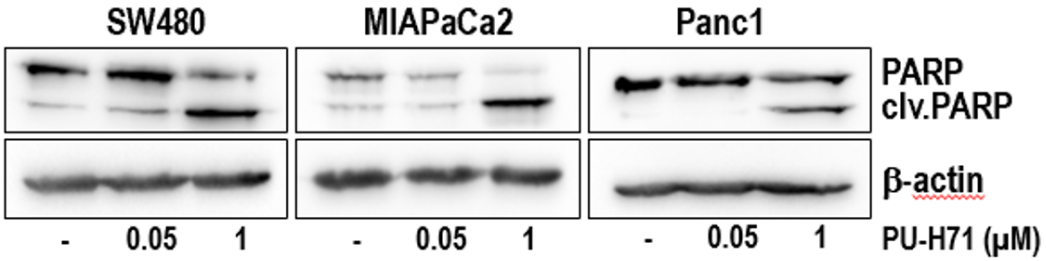


**Figure 12: PDE5 and HSP90 inhibitors have synergistic effect on inhibiting tumor growth in vivo.**

Various cancer cell lines were incubated with different concentrations of PU-H71 for 24 hours as indicated. Lysates were subjected to western blot analysis with PARP-specific antibody. β-actin served as a loading control.

Taken from Lu et al., 2020 (ref. 161).

The CAM model was involved in this set of experiments. SW480 cells were seeded inside the silicon ring and the growing tumors were treated either with 50 nM PU-H71 or 15 μM Sildenafil or a combination of both compounds 24 and 48 hours after the xenograft procedure. Four days after implantation tumor size was measured. No difference in tumor size was observed with Sildenafil or PU-H71 treatment alone (**Fig. 13**). However, combination of both drugs resulted in a significant decrease of tumor size (**Fig. 13**). These results suggest that use

of these inhibitors could be of relevance when designing future cancer therapy cocktails.

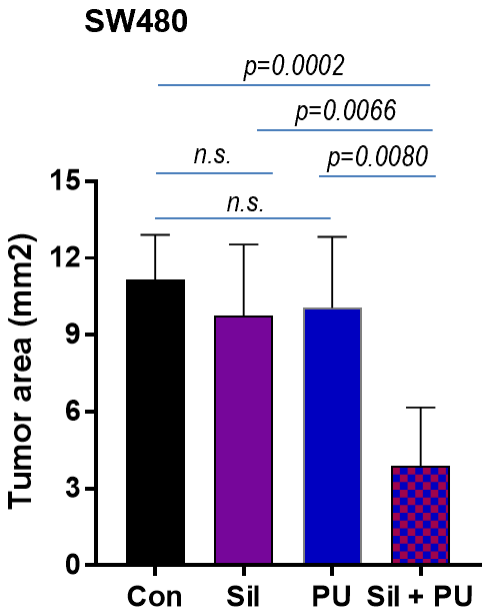


**Figure 13: PDE5 and HSP90 inhibitors have synergistic effect on inhibiting tumor growth *in vivo*.**

SW480 colon cancer cells ($1x10^6$) were implanted on the surface inside of chicken chorioallantoic membrane 8 days after fertilization. Tumors were treated either with PU-H71 or Sildenafil or combination of both compounds as indicated. Quantification of tumor area is presented. Error bars represent mean +/- SEM of five to seven tumors.

Taken from Lu et al., 2020 (ref. 161).

## 3.8 Sildenafil triggers tumor death through altered expression of HSP90 and degradation of PKD2

It is known that PDE5 inhibitors Sildenafil may hamper tumor progression by decreasing intracellular accumulation of cGMP, thus resulting in the activation of PKG, reported to be associated with apoptosis, growth suppression and inhibition of angiogenesis. In our study, we demonstrated that tumor lethality induced by Sildenafil treatment is associated with altered expression of HSP90 chaperone and the degradation of one of its client proteins, PKD2.

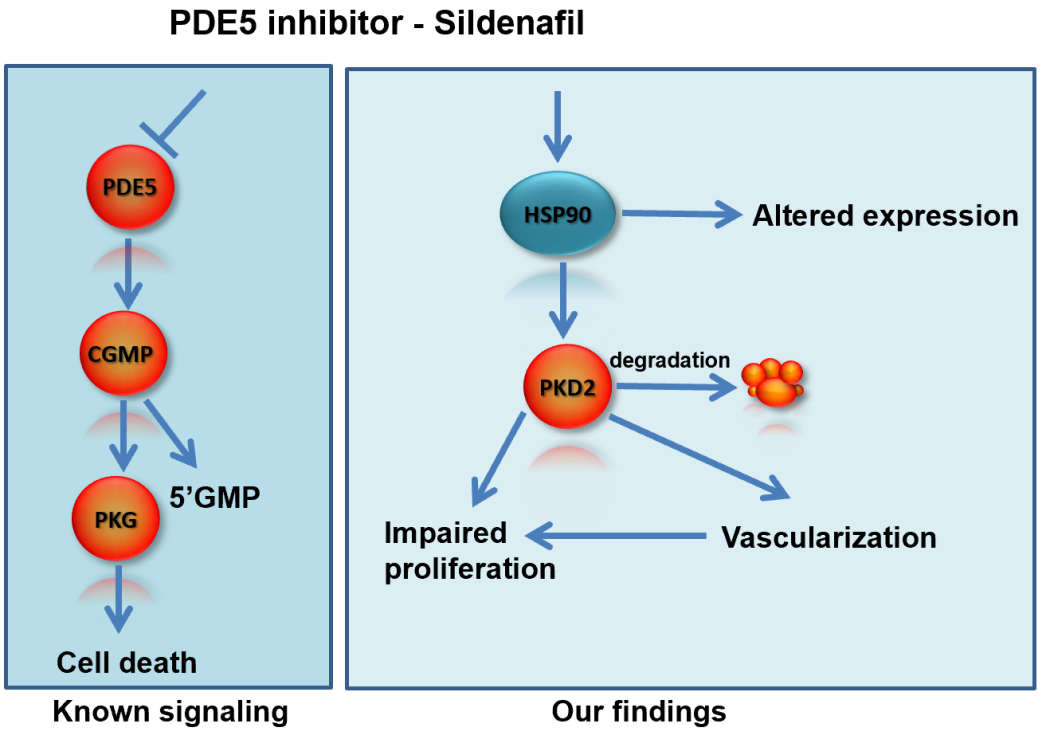


**Figure 14: Sildenafil triggers tumor death through altered expression of HSP90 and degradation of PKD2.**

Impaired cancer cell growth is induced by alteration of HSP90 expression and subsequent PKD2 degradation following PDE5 inhibition.

# 4. Discussion

The development of cancer represents an intricate process mirrored by a myriad of epigenetic and genetic events that promote abnormal cell division and growth that ultimately translate to augmented invasion and spreading to remote parts of the body. At the beginning of this century, Hanahan and Weinberg delineated several, so called hallmarks of cancer. These depict how tumors acquire distinctive and/or complementary capabilities [144], by which a robust proliferative signaling paralleled by circumvention of the control by tumor suppressors and augmented angiogenesis favours growth, invasion and metastatic dissemination [144]. Nowadays, noticeable improvements have been achieved in the field of cancer treatment. However, the problems regarding prolonging overall survival and improving life quality of cancer patients are still bothering researchers. In recent years, repurposing mature medications on the market opened new anticancer avenues with the advantage of higher efficiency, decreased side effects and development costs. Sildenafil, a selective inhibitor of PDE5, originally developed to treat pulmonary arterial hypertension, then approved to tackle the erectile dysfunction [145, 146], was lately demonstrated to have a negative effect on cancer cell growth. Although the suppressive effect of Sildenafil has been confirmed, the molecular mechanism of how Sildenafil inhibits tumor cell proliferation and viability is not fully understood.

In this thesis, we have demonstrated that Sildenafil treatment is associated with impaired cell proliferation, elevated apoptosis and suppressive tumor growth effects. Moreover, we delineated that decreased proliferation and viability after Sildenafil incubation is paralleled by altered HSP90 expression, which impacts on the degradation of PKD2, a client protein of HSP90. Finally, our study demonstrated that combination of PDE5 and HSP90 inhibitors have synergetic effect on inhibiting tumor growth.

Sildenafil was used for treating erectile dysfunction by enhancing the cGMP

levels, which results in the activation of protein kinase G [147]. Considering cGMP is a second messenger involved in many biological processes such as cell proliferation and apoptosis, scientists attempted to explore the role of Sildenafil in regulating tumor development and progression. Numerous studies reported that PDE5 expression is elevated in various human tumors including bladder, lung, colorectal, prostate and pancreatic cancers [148]. For example, Sarfati and colleagues found that Sildenafil elevated apoptosis of B-chronic lymphocytic leukemia cells, and apoptosis was dependent on the activation of intracellular caspase 3 [149]. These findings inspired us to interrogate whether low concentrations of Sildenafil could inhibit growth of other human cancer cells. Our lab previously substantiated the effect of Sildenafil in several cancer cells such as breast, lung and pancreatic cancer. We extended these findings to colon (HCT-116, SW480) cancer cell lines with various concentrations of Sildenafil. Our findings confirmed that 25 μM Sildenafil treatment not only impaired proliferation but also accelerated cell death in colon cancer cell lines. In accordance with our results, Mei and colleagues previously demonstrated that Sildenafil suppressed the growth and induce cycle arrest of colorectal cancer in vitro and in subcutaneous xenografts by augmenting intracellular reactive oxidative specie (ROS) levels [150]. However, in their study, the growth of colorectal cancer cells was inhibited at a concentration ranging from 190 to 271 μM [150].

Moreover, multiple studies demonstrated the mechanisms by which Sildenafil triggers tumor cell death. Sildenafil treatment could potentiate doxorubicin-induced apoptosis of prostate cancer cells by enhancing the generation of reactive oxygen species, elevated activity of caspase-3 and caspase-9 asscoiated with decreased expression of Bcl-xL [151]. In line with this, Sildenafil was reported to augment the lethality of pemetrexed by inhibiting various chaperone proteins regulating the activity of cyclic GMP and nitric oxid [152]. In this study, the combination of pemetrexed and Sildenafil resulted in altered HSP90, HSP70 or / and GRP78 expression [152]. Notably, HSP90 participates

in folding and stabilization of around 400 client proteins. Many of these client proteins belong to oncoproteins and play an essential role in angiogenesis, cell death, proliferation and metastasis. In line with these reports, our findings demonstrate that Sildenafil treatment leads to reduced expression of HSP90.

Our lab previously demonstrated the role of PKD2 in regulating tumor cell-endothelial cell communication in gastrointestinal tumors [143]. Furthermore, PKD2 acts as a client protein of HSP90 and activates the NF-κB/VEGF-A signal cascade to boost tumor growth [141]. Therefore, we attempted to examine whether decreased HSP90 expression following Sildenafil incubation was accompanied by PKD2 alteration. Our findings demonstrated that Sildenafil treatment-induced HSP90 alteration was associated with degradation of PKD2 in pancreatic and breast cancer cell lines. Rescue experiments involving ectopic expression of PKD2 showed restored viability of cancer cells after incubation with Sildenafil. These findings indicate that the suppressed proliferation and viability following Sildenafil treatment is partially funneled through HSP90/PKD2 signaling pathway. However, we are aware that other potential molecular mechanisms may contribute to the process of Sildenafil-related tumor cell death. For instance, PDE5 inhibitors were demonstrated to negatively impact colon tumor cell growth upon activation of cGMP/PKG pathway, which involves the inhibiton of Wnt/β-catenin signaling pathway and is correlated with decreased cyclin D1 and survivin [27]. The apoptosis induced by PDE5 inhibitor exisulind in colon tumor cell lines was associated the cGMP/PKG pathway activation and augmented phosphorylation of β-catenine [153, 154]. Moreover, PDE5 inhibitors selectively induced cell death in breast tumor cells, followed by increased β-catenin phosphorylation, decreased β-catenin mRNA and protein levels, and decreased expression of Wnt/β-catenin–regulated proteins [155]. These findings suggest Sildenafil to be implicated in Wnt/β-catenin-regulated transcription. In conclusion, our study confirmed that the HSP90/PKD2 signaling pathway partly contributes to the Sildenafil-induced

cell death. Therefore, HSP90 could be regarded as an essential target for future therapeutic strategies.

Interestingly, combination of PDE5 inhibitors with other therapeutic regimens potentiated the efficacy in suppressing the growth of various cancer cells. The combination of Sildenafil and celecoxib was associated with decreased breast tumor growth in vivo. At the same time the anti-cancer effect could be potentiated by multiple sclerosis medication FTY720, upon blocking sphingosine-1-phosphate (S1P) and elevation of ceramide levels [156]. Another study reported that the capacity of doxorubicin in killing castration-resistant prostate cancer cells was increased once combined with sildenafil or vardenafil, and enhanced cell death was achieved triggered by suppressing the expression of the anti-apoptotic Bcl-2 family members [157]. Indeed, Booth and colleagues reported that Sildenafil used at a concentration of 2 μM potentiated the lethality of pemetrexed through inhibition of multiple chaperone proteins [152]. To note, Sildenafil alone at 2 μM concentration had no impact on cell viability. Furthermore, another study showed that Sildenafil at 2 μM potentiated pemetrexed and sorafenib by effectively inactivating NF-κB, mTOR and STAT transcription factors [158]. Thus, it appears that combination of PDE5 inhibitors with various drugs is very effective in achieving anti-cancer effects. Our lab previously reported that 1 μM PU-H71 (an ATP-competitive HSP90 inhibitor) induced apoptosis in cancer cells, and was associated with the degradation of HSP90 client proteins [159, 160]. In our study, we involved 20x less PU-H71 as used in the previous studies i.e.50 nM PU-H71. At this concentration, PU-H71 does not have any negative effect on cell viability. Then, we sought to combine minimal doses of PU-H71 with Sildenafil in order to investigate whether they had synergic effect toward cancer cell death. In combined therapy, 50 nM PU-H71 was able to potentiate the killing effect of 15 μM Sildenafil in tumor cells. Therefore, our findings are consistent with previous studies that the killing effect of Sildenafil could enhance the killing effect of other drugs.

In summary, our study proposes a new potential anti-cancer therapy upon

repurposing existing mature medications. However, these findings should be further substantiated by involvement of *in vivo* animal models and the underlying mechanisms should be further investigated to provide more evidence about the relevance in biological systems.

# 5. Summary

The repurposing of existing mature medications has become an attractive avenue in the fight against cancer. Phosphodiesterase 5 (PDE5) inhibitors have been demonstrated to suppress the growth of various cancers. However, data on treating tumors with comparably low concentrations of PDE5 inhibitors and its underlying mechanisms are not fully understood.

Here we report that low doses of Sildenafil incubation of tumor cells led to impaired cell proliferation and augmented apoptosis in vitro, as well as suppressed tumor growth in vivo. Incubation of cancer cells with sildenafil was associated with alteration of heat shock protein 90 (HSP90) chaperone expression, and subsequent degradation of protein kinase D2 (PKD2), Sildenafil was able to potentiate the cell killing effect of PU-H71 at low doses.

Altogether, our study demonstrates that repurposing of drugs, either alone or in combination with anti-tumor medications emerges as a new potential anti-cancer strategy.

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# 7. List of figures and tables

## 7.1 Figures

**Figure 1**: The structural model of cGMP-specific PDE5.

**Figure 2**: The structural model of Hsp90.

**Figure 3**: The structural model of PKD2.

**Figure 4**: Sildenafil treatment is associated with decreased growth of colon cancer cells in a concentration dependent manner.

**Figure 5**: Sildenafil treatment inhibits the growth of cancer cells in a time dependent manner.

**Figure 6**: Sildenafil treatment is corroborated with impaired cancer cell viability.

**Figure 7**: Sildenafil treatment is associated with elevated cell death.

**Figure 8**: The tumor size is decreased after sildenafil intervention.

**Figure 9**: Sildenafil treatment suppresses tumor growth in vivo

**Figure 10**: Cancer cells proliferation impaired by Sildenafil treatment is associated with altered HSP90 expression and PKD2 degradation.

**Figure 11**: Impaired proliferation of cancer cells following Sildenafil treatment can be partially restored by ectopic PKD2.

**Figure 12**: PDE5 and HSP90 inhibitors have synergistic effect on inhibiting tumor growth in vivo.

**Figure 13**: PDE5 and HSP90 inhibitors have synergistic effect on inhibiting tumor growth in vivo.

**Figure 14**: Sildenafil triggers tumor death through altered expression of HSP90 and degradation of PKD2.

## 7.2 Tables

**Table 1**: Overview with employed equipment.

**Table 2**: Overview with employed chemicals and drugs.

**Table 3**: Overview with employed cell lines.

**Table 4**: Overview with employed cell culture items.

**Table 5**: Overview with the plasmids employed in this study.

**Table 6**: 4× Laemmli-sample buffer.

**Table 7**: 1× SDS-running buffer (1× SDS-PAGE buffer).

**Table 8**: 10×SDS-running buffer (10× SDS-PAGE buffer).

**Table 9**: Wet transfer buffer.

**Table 10**: 10× PBS buffer.

**Table 11**: Washing buffer / 1×PBST (1× PBS containing 0.2% Tween 20).

**Table 12**: 5% non-fat milk in 1×PBST.

**Table 13**: IP- lysis buffer.

**Table 14**: Gels used for WB were prepared with 1.0 mm comb.

**Table 15**: Composition of ECL solution.

**Table 16**: List with primary antibodies used in our study.

**Table 17**: List with secondary antibodies used in our study.

**Table 18**: List with materials for Immunohistochemistry used in our study.

**Table 19**: Software used in the current study.

# 8. Acknowledgements

Acknowlegements have been deleted for reasons of data protection.

# 9. Curriculum vitae

Curriculum vitae has been deleted for reasons of data protection.