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探討 Sildenafil 增強化療藥物 Doxorubicin 在人類荷爾蒙不依賴型前列腺癌細胞之抗癌作用機轉

Mechanism Study of Sildenafil as a Chemosensitizer of Doxorubicin against Hormone-Refractory Prostate Cancers

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本論文係張若凡君（R03423014）在國立臺灣大學藥學研究所完成之碩士學位論文，於民國 105 年 7 月 18 日承下列考試委員審查通過及口試及格，特此證明

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List of Abbreviations

ABC transporters	ATP-binding cassette transporters
ABCB1	ATP binding cassette subfamily B member 1
ABCC10	ATP binding cassette subfamily C member 10
ABCG2	ATP-binding cassette subfamily G member 2
ADT	Androgen deprivation therapy
AJCC	American Joint Committee on Cancer
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X
Bcl-2	B-cell lymphoma 2
Bid	BH3 interacting-domain death agonist
BSA	Bovine serum albumin
Caspase	Cysteine-aspartate-specific proteases
CD95	Cluster of differentiation 95
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
DAPI	4',6-diamidino-2-phenylindole
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DR3	Death receptor 3
DR4	Death receptor 4
DR5	Death receptor 5
DRE	Digital rectal examination
DSB	DNA double-strand break
ELISA	Enzyme-linked immunosorbent assay
FAP-1	Fas associated phosphatase-1
FBS	Fetal bovine serum
FDA	Food and Drug Administration

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FITC	Fluoresceine isothiocyanate
FLIP	Fas-associated death domain (FADD) interleukin-1-converting enzyme (FLICE)-like inhibitory protein
H2A.X	Histone H2A.X
HR	Homologous recombination
HRP	Horseradish peroxidase
HRPC	Hormone-refractory prostate cancer
LHRH	Luteinizing hormone-releasing hormone
NAC	N-acetylcysteine
NHEJ	Non-homologous end joining
PARP-1	Poly [ADP-ribose] polymerase 1
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.1% Tween 20
PDE5	Phosphodiesterase-5
PI	Propidium iodide
PI-3K	Phosphoinositide 3-kinase
PIKKs	Phosphatidylinositol 3-kinase-related kinases
PSA	Prostate-specific antigen
RNA	Ribonucleic acid
ROC	Reactive oxygen species
RPA14	Replication protein A 14 kDa subunit
RPA32	Replication protein A 32 kDa subunit
RPA70	Replication protein A 70 kDa subunit
SD	Standard deviation
siRNA	Small interfering RNA
Smac/DIABLO	Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI
TNFR1	Tumor necrosis factor receptor 1
Topo IIα	Topoisomerase IIα
TRUS	Transrectal ultrasound

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中文摘要

近年來，老藥新用在藥物開發上的發展潛力逐漸被受重視，像是威而鋼其學名 sildenafil (Phosphodiesterase-5 抑制劑)，為治療男性勃起功能障礙的藥物，但在最近研究發現，其在活體內外實驗中都具有加強 doxorubicin 毒殺賀爾蒙不依賴型前列腺癌細胞的能力，但其相關分子機制仍不清楚。在我們的研究結果中發現，sildenafil 本身並不會影響賀爾蒙不依賴型前列腺癌細胞 (PC-3 及 DU145) 的存活率，但是當其與 doxorubicin 同時作用下，sildenafil 能協同性地增強 doxorubicin 所誘導的前列腺癌細胞之細胞凋亡現象，包括增加核小體 DNA 斷裂、sub-G1 細胞數量、內因性與外因性細胞凋亡訊號，並減少抗凋亡 Bcl-2 蛋白質家族的表現量 (Mcl-1, Bcl-xL 及 Bcl-2)。此外，值得注意的是，雖然合併使用 sildenafil 與 doxorubicin 能增加 PC-3 細胞中的氧化壓力，但當我們利用抗氧化劑 (NAC 及 trolox) 來去除該氧化壓力後，卻無法阻止細胞凋亡的發生，意味著氧化壓力並非造成藥物合併後細胞凋亡增加之原因。有趣的是，我們也發現 sildenafil 可以加劇其他第二型拓譜異構酶 (etoposide 及 mitoxantrone) 所產生之細胞凋亡情況，然而 sildenafil 對於第一型拓譜異構酶 (camptothecin) 所導致的細胞凋亡則沒有影響。已知 doxorubicin 毒殺癌細胞的其一機制是增加 DNA 雙骨斷裂的產生，我們進一步探討 sildenafil 對於 doxorubicin 所誘導的 DNA 損傷及其修補機制之影響。實驗結果發現，doxorubicin 造成的 DNA 雙骨斷裂之兩種修復機制 (homologous recombination, HR; non-homologous DNA end joining, NHEJ) 都會被 sildenafil 所抑制，例如：合併使用 sildenafil 與 doxorubicin 會減少 HR 修補機制中 RPA32 的過量磷酸化、降低 Rad51 之表現量及其在細胞核內形成 foci 的能力;並在 NHEJ 修補機制中，減少 DNA-PKcs (Thr2609)的磷酸化、抑制 Ku80 鍵結到 DNA 斷裂尾端之能力。另外，已知 sildenafil 為 phosphodiesterase-5 (PDE5) 之抑制劑，我們也進一步探討抑制 PDE5 對於 sildenafil 增強 doxorubicin 毒殺效果的重要性。實驗結果顯示，不論是利用其他 PDE5 活性抑制劑 (vardenafil 或 tadalafil)，或是使用 siRNA 去抑制 PDE5 的表現，都可以加強 doxorubicin 之細胞毒殺效果，然而我們卻發現只有 PDE5 活性抑制劑才能有效減少 doxorubicin 所誘導的 DNA 雙骨斷裂 HR 修補機制。總結來說，sildenafil 可經由抑制 HR 與 NHEJ 途徑來減少 doxorubicin 所誘導的 DNA 雙骨斷裂之修補，導致核小體 DNA 片段化與內外因性細胞凋亡信號增強，藉此增加

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doxorubicin 毒殺賀爾蒙不依賴型前列腺癌細胞之能力。而目前我們不排除 PDE5 在此機制中可能扮演部分腳色，但這仍須要更進一步的研究去驗證。

關鍵字：Doxorubicin; Sildenafil; Hormone-refractory prostate cancer; HR; NHEJ; PDE5; Apoptosis

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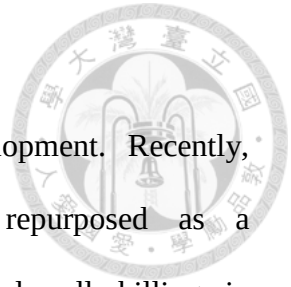

Abstract

Drug repositioning is a potential strategy for drug development. Recently, sildenafil, a phosphodiesterase-5 (PDE5) inhibitor, has been repurposed as a chemosensitizer to synergistically potentiate doxorubicin-induced cell killing in hormone-refractory prostate cancer (HRPC) both *in vitro* and *in vivo*. However the synergistic anticancer mechanism has not been well identified. In the present study, the data demonstrated that sildenafil by itself did not affect cell survival of PC-3 and DU145 (two HRPC cell lines), but significantly enhanced cell apoptosis induced by doxorubicin, as evidenced by the synergistic increase of nucleosomal DNA fragments and sub-G1 (apoptosis) population, and the activation of both intrinsic and extrinsic apoptotic pathways. Moreover, the anti-apoptotic Bcl-2 family proteins, Mcl-1, Bcl-xL and Bcl-2, were significantly downregulated by the combinatorial treatment with sildenafil and doxorubicin. It is noteworthy that an increase in cellular ROS at early combinatorial treatment was noted; however, both ROS scavengers, NAC and trolox, dramatically abolished the ROS production but failed to inhibit cell apoptosis, indicating the sensitization mechanism beyond the oxidative stress. Interestingly, sildenafil also enhanced cell apoptosis induced by other topoisomerase II inhibitors (e.g., etoposide and mitoxantrone) but not topoisomerase I inhibitor (e.g., camptothecin). Due to DNA-damaging properties of doxorubicin, the regulators and signaling of DNA double-strand break (DSB) and repair pathways were studied. As a result, the combinatorial treatment reduced the protein expression of hyperphosphorylated RPA32 and phosphorylated DNA-PKcs (Thr2609), which were involved in DSB repair pathways, homologous recombination (HR) and non-homologous DNA end joining (NHEJ), respectively. The defects in HR and NHEJ pathways were further substantiated by the reduced levels of nuclear Rad51 foci formation and DNA end-binding activity of

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nuclear Ku80. The role of PDE5 in the sensitization mechanism was examined as well. The data revealed that inhibition of PDE5 activity by two other inhibitors, vardenafil or tadalafil, or PDE5 knockdown by siRNA potentiated the cell-killing effect of doxorubicin. However, only PDE5 inhibitors but not PDE5 knockdown reduced the HR-mediated DSB repair in response to doxorubicin. In conclusion, the data suggest that sildenafil enhances doxorubicin-induced apoptosis in HRPC through the impairment of HR and NHEJ-mediated DSB repair systems, leading to synergistic increase of nucleosomal DNA fragments and activation of both intrinsic and extrinsic apoptotic pathways. Inhibition of PDE5 is, at least partly, responsible for the sensitization mechanism.

Key words: Doxorubicin; Sildenafil; Hormone-refractory prostate cancer; HR; NHEJ; PDE5; Apoptosis

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Aim of the study

According to the statistics from the World Health Organization in 2012, prostate cancer is the second most frequently diagnosed cancer and the fifth-leading cause of cancer death in men worldwide[1, 2]. Prevalence of prostate cancer is generally higher in developed countries than in developing countries. Based on the American Cancer Society's estimates for 2016, prostate cancer becomes the most common cancer among American men, and it is the second-leading cause of cancer death in men[3]. In Taiwan, cause of death statistics reported by the Ministry of Health and Welfare in 2014 show that prostate cancer is the seven-leading cause of cancer death in men[4]. Besides, the incidence and mortality rate of prostate cancer in Asian populations have gradually increased these years[5]. Moreover, since prostate cancer mainly occurs in men aged 65 or older, the incidence rate of prostate cancer in the aging society may be continuously rising.

Based on results of prostate biopsy graded with American Joint Committee on Cancer (AJCC) TNM system, prostate cancer can be categorized into four stages. For stage Ⅰ and Ⅱ, patients have no symptoms and cancer has not spread outside of the prostate. Treatment options usually include active surveillance, radical prostatectomy, and radiation therapy. 5-year survival rate of the early stages is almost 100%. For stage Ⅲ in which tumor has grown outside the prostate and may have spread to the seminal vesicles, the radiation plus hormone therapy is necessary. After treatment, the 5-year survival rate of this stage is still nearly 100%. However, patients with stage Ⅳ prostate cancer, which may has spread to nearby lymph nodes and bones, the 5-year survival rate decreases to 28%. In general, different types of hormone therapies such as luteinizing hormone-releasing hormone (LHRH) analog, LHRH antagonist, anti-androgen and

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estrogens are used initially to treat advanced prostate cancer. Unfortunately, nearly all metastatic prostate cancers treated with hormone therapies become resistant after a period of months or years. When prostate cancer no longer responses to hormone therapy, it is referred as hormone-refractory prostate cancer (HRPC). Chemotherapy is a key therapy for HRPC treatment. Among the chemotherapeutic agents, doxorubicin is one of the treatment options[6]. However, the major side effect of the drug is cumulative dose-dependent cardiotoxicity[7]. To minimize the side effect, combination of doxorubicin with other drugs may provide a good regimen by lowering the dosage while retaining the therapeutic function of doxorubicin[6]. Recently, sildenafil, an FDA-approved PDE5 inhibitor, has been repurposed to potentiate doxorubicin-induced killing in HRPC both *in vitro* and *in vivo*[8]. Nevertheless, the synergistic anticancer mechanism of doxorubicin combined with sildenafil has not yet been well identified. Therefore, the aim of this study is to delineate the sensitization mechanism of sildenafil in HRPC.

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Chapter 1: Introduction

1.1. Prostate

The prostate is a gland found only in male reproductive and urinary systems. It lies below the bladder and in front of the rectum. The function of the prostate gland is to produce some of weak alkaline fluid that is part of semen, which neutralize the acidity of the vaginal tract and prolong the lifespan of sperm. The growth and function of the prostate gland relies on androgens (male hormones) produced by the testes [9, 10].

1.2. Prostate cancer

Prostate cancer is a malignant tumor that starts when cells in prostate grow uncontrollably. Almost all prostate cancers (95%) belong to adenocarcinomas, which develop from the gland cells in the prostate. Although some of prostate cancers can grow and spread rapidly, most prostate cancers usually grow slowly [9, 10].

Incidence and mortality rates

According to the global cancer fact in 2012 estimated by the World Health Organization, prostate cancer is the second most frequently diagnosed cancer and the fifth-leading cause of cancer death in men worldwide[1, 2]. Based on the American Cancer Society's estimates for prostate cancer for 2016, prostate cancer is the most common cancer in American men, aside from skin cancer, and it is the second leading cause of cancer death[3]. In Taiwan, cause of death statistics reported by the Ministry of Health and Welfare in 2014 show that prostate cancer is the sixth-leading cause of cancer death in men[4].

Causes and risk factors

The exactly causes that lead to prostate cancer have not been well-identified. But some risk factors can be referred, such as age over 50, African American ethnicity,

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developed countries in the western world, inherited gene mutations (*RNASEL, BRCA1/2, MSH2 and MLH1 or HOXB13*), having higher levels of androgens, diet, obesity, etc[9].

Symptoms

Early stage of prostate cancer usually has no symptoms. More advanced prostate cancers may have problems urinating, blood in the urine or semen, erectile dysfunction, bone pain, leg pain and foot pain, etc[9, 10].

Diagnostic tests

1. Medical history and physical examination: Doctors diagnose prostate cancer through asking patients some questions about family history of prostate cancer and any changes in bladder habits, then using digital rectal examination (DRE) to check prostate for any lumps or changes in size and shape.
2. Prostate-specific antigen (PSA) test: PSA is a protein made by prostate gland. When the PSA level becomes abnormal in the blood, it may indicate the presence of prostate cancer. The higher the PSA level, the more possibly that prostate cancer occurs.
3. Transrectal ultrasound (TRUS): Image the prostate by using an ultrasound probe placed into the rectum. It is used to measure the size of prostate and check for abnormal areas.
4. Biopsy: During a biopsy, 6-12 biopsies were taken from different areas of prostate using a core needle biopsy. The collected tissues are tested in a laboratory to confirm whether cancer cells are present. If cancer cells exist, Gleason score is used to describe how aggressive cancer cells are.

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Stages and survival rates

Table 1. The stages and estimated 5-year survival rates for prostate cancers[9].

Stage by tumor location	Description	Stage grouping (TNM system)	Estimated 5-year survival rate
Local	Cancer is within the prostate	Ⅰ	~ 100%
		Ⅱ	
Regional	Cancer grows outside the prostate and spreads to the seminal vesicles	Ⅲ	~ 100%
	Cancer grows outside the prostate and spreads into tissues other than the seminal vesicles, such as the rectum, bladder, pelvis or nearby lymph nodes.	Ⅳ	
Distant	Cancer grows outside the prostate and spreads to distant lymph nodes, bones, or other organs.	Ⅳ	~ 28%

Prostate cancer treatments

Depends on the different stages of prostate cancer, ages and health states of patients, treatment options for men with prostate cancer may include[9-11]:

1. Watchful waiting or active surveillance: For men whose cancer is small and slow-growing, treatments such as surgery or radiation may not be suitable, because the side effects of these treatments may outweigh the benefits for the early stage prostate cancer. Therefore, active monitoring the prostate cancer using DRE and PSA blood test, or less intensive follow-up may be adopted.
2. Surgery: Radical prostatectomy is the main type of surgery to completely remove the localized prostate cancer plus some of the tissue nearby it, including the seminal vesicles.
3. Radiation therapy: Radiation therapy uses high-energy rays to kill cancer cells. The types of radiation therapy for prostate cancer include external beam radiation and

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brachytherapy (internal radiation therapy). They are usually combined with hormone therapy for treatment of more advanced prostate cancer.

4. Hormonal therapy: It is also known as androgen deprivation therapy (ADT), which slows the growth of prostate cancer by lowering the level of male hormones (androgens) in the body, or blocking the action of androgen from affecting prostate cancer cells. There are several types of hormone therapy can be used, including luteinizing hormone–releasing hormone (LHRH) agonists, LHRH antagonists, anti-androgens, CYP17 inhibitor or removal of the testicles (orchiectomy).
5. Chemotherapy: It is used to treat metastatic hormone-refractory prostate cancer. Some of chemotherapy drugs used to treat prostate cancer include:

   First-line chemotherapy combination: Docetaxel and a steroid drug prednisone

   Second-line chemotherapy combination: Cabazitaxel and prednisone

   Palliative chemotherapy: mitoxantrone and prednisone

   Others: Estramustine, epirubicin, paclitaxel, etc.

1.3. Human Prostate cancer cell lines

Table 2. The most commonly used human prostate cancer cell lines for research[12, 13]:

Cell lines	Source	Androgen sensitivity	*p53*	*PTEN*
PC-3	Lumbar metastasis	Insensitive	Deletion mutation	Deletion mutation
DU145	Brain metastasis	Insensitive	Missense mutation	$PTEN^{+/-}$
LNCaP	Lymph node metastasis	Sensitive	Silent mutation	Nonsense mutation

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1.4. Doxorubicin

Doxorubicin is a chemotherapy drug belonging to a class of compounds with similar structures, named anthracyclines. Doxorubicin was first isolated from Streptomyces peucetius var. caesius, a soil bacterium, in the 1970's[14], and has shown great efficacy to kill both solid and liquid tumors. It has been routinely used for the treatment of several cancers including breast, lung, gastric, ovarian, thyroid, non-Hodgkin's and Hodgkin's lymphoma, multiple myeloma, sarcoma and pediatric cancers[15]. Despite the common use of doxorubicin in the clinics, the mechanisms of doxorubicin are still not fully understood. So far, there are two proposed anticancer mechanisms of doxorubicin that are widely accepted. First, acting like almost all anthracycline drugs, doxorubicin intercalates into DNA and disrupts the topoisomerase-II (Topo II)-mediated DNA repair, leading to DNA double strand break. Second, redox-dependent metabolism of doxorubicin generates free radicals which damage the cellular membranes, DNA and proteins[16]. In addition to these two mechanisms, other models have also been proposed to explain the doxorubicin-mediated cell death, such as Topo-II-independent DNA adduct formation, ceramide overproduction and intercalation-induced DNA torsional stress and nucleosome destabilization[17]. Although doxorubicin exerts excellent killing effects in many cancers, its side effect of cumulative and dose-dependent cardiotoxicity has limited its usage[7].

1.5. Sildenafil

Sildenafil, also known as Viagra, is a cGMP-specific phosphodiesterase type 5 (PDE5) inhibitor. It has been approved by FDA for the indications of erectile dysfunction and pulmonary arterial hypertension since 1998 and 2005 respectively[18, 19].

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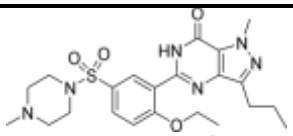
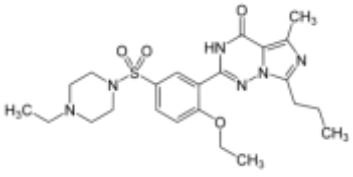
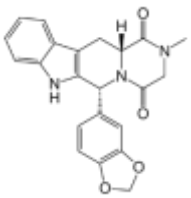
The selective vasodilatory mechanism of PDE5 inhibitors in penis and lung has been well identified. PDE5 is an enzyme that hydrolyzes second messenger molecule cGMP into biologically inactive 5'GMP. Therefore, sildenafil can increase levels of cGMP by inhibiting the degradation of cGMP via PDE5. PDE5 is found in particularly high concentrations in the corpus cavernosum and lung vasculature. The elevated cGMP in these tissues activates the downstream cGMP-dependent protein kinase (PKG) and cGMP-gated ion channels, finally resulting in the reduction in the intracellular calcium level. The decreased calcium level in cells leads to the relaxation of vascular smooth muscle, thereby increasing the blood flow in penis and lung. Of note, the activation of the NO/cGMP system by sexual stimulation or alveolar distension of lung is the prerequisite for sildenafil to exert its functions through cGMP/PKG signaling in penis or lung[20].

Other PDE5 inhibitors

Table 3. PDE5 inhibitors approved by FDA[21]

PDE5 inhibitor	IC50 for human recombinant PDE5A (nM)	Selectivity (nM)	Indication	Structure
Sildenafil	8.5	PDE1 (350) PDE6 (49)	ED, PAH	
Vardenafil	0.89	PDE1 (121) PDE6 (11)	ED	
Tadalafil	9.4	PDE11A (67)	ED, PAH, BPH	

* ED: Erectile dysfunction; PAH: Pulmonary arterial hypertension; BPH: Benign prostatic hyperplasia

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Drug repurposing of sildenafil as a chemosensitizer in cancers

Recently, sildenafil has been demonstrated to sensitize some types of cancer cells to several chemotherapy drugs. Some proposed mechanisms are shown as below.

Table 4. Proposed mechanisms for sildenafil as a chemosensitizer in cancers.

Drug combination	Cell lines	Proposed mechanisms	Ref.
Doxorubicin + Sildenafil	Prostate cancers (PC-3, DU145)	-Elevated ROS level -Enhanced intrinsic apoptosis (increased caspase-3 and -9; reduced Bcl-xL and p-Bad)	8
	Ovarian cancers (UCI 101, A2780, OSAC-1)	N/A	
Doxorubicin + Sildenafil	Prostate cancer (DU145)	-Enhanced extrinsic apoptosis (reduced FLIP-L and –S; decreased FAP-1)	22
Doxorubicin+ Sildenafil	Bladder cancers (HT-1376, J82, T24)	-Elevated ROS and NO -Enhanced extrinsic apoptosis, autophagy and necroptosis; -Increased DNA damage -PDE5 inhibition	23
Doxorubicin/ Gemcitabine + Sildenafil	Bladder cancers (HT-1376, J82, T24) and pancreatic cancer cells (PANC-1, Mia Paca2, AsPC-1)	N/A	
Etoposide + Sildenafil	Medulloblastoma cells	-Enhanced extrinsic apoptosis and autophagy -Increased DNA damage -Elevated NO -PDE5 inhibition	24
Colchicine/ Vinblastine/ Paclitaxel/ Docetaxel/ Cisplatin/ Mitoxantrone + Sildenafil	Multidrug-resistant cancer cells or ABC transporter-transfected cell lines	Inhibition the efflux function of ABCB1/P-glycoprotein, ABCG2 or ABCC10	25, 26

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1.6. Cell death

There are many types of cell death which can be classified according to its morphological appearance (which may be apoptotic, necrotic or autophagic), enzymological preference (the involvement of nucleases or of specific classes of proteases, such as caspases, calpains or cathepsins), functional aspects (programmed or accidental) or immunological characteristics (immunogenic or non-immunogenic)[27]. Among all types of cell death, programmed cell death has been regarded as a barrier to restrict cancer cells from keep surviving. Apoptosis, autophagy and necroptosis are the most common programmed cell death targeted by anticancer therapy nowadays[28].

Apoptosis

Apoptosis was first described by Kerr et al. in 1972, and is characterized with respect to the morphological changes in dying cells, including cell shrinkage, nuclear condensation and fragmentation, membrane blebbing, loss of adhesion to the neighboring cells and detachment from the extracellular matrix[29]. Biochemical changes include cleavage of high molecular weight of chromosomal DNA into internucleosomal fragments, phosphatidylserine externalization and numerous cleavages of intracellular substrate through specific proteolytic process. Of note, since apoptosis does not cause localized inflammatory response and damage to surrounding tissues, it has been considered as a useful target in cancer therapy[30].

Autophagy

Autophagy, is a catabolic process in which autophagosomes, double membrane-bound structures, encapsulate cytoplasmic macromolecules and organelles and destine these cellular components for degradation and renewal. Autophagy is an evolutionarily conserved strategy for cell to survive under stress conditions, such as nutrient deprivation, ROS, hypoxia, drug stimuli, endoplasmic reticulum stress, etc.

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However, when coping with excessive stress or being dysregulated, autophagy may lead to non-apoptotic cell death. In cancers, autophagy plays dual roles, either tumor suppressor or protector[31]. The regulation of autophagy in cancers is complicated and depends on different cell conditions[28].

Necroptosis

Necroptosis, different from necrosis, is a form of programmed necrosis, which can be executed by regulated mechanisms like apoptosis, but in a caspase-independent manner. Necroptosis can be induced by the activation of the TNF receptor superfamily, Toll-like receptors (TLRs), cellular metabolic and genotoxic stresses or various anticancer agents. One of the critical characteristics of necroptosis is to form necrosome by receptor-interacting protein kinase 1 (RIP1) and RIP3. The formation of the necrosome can be inhibited by chemical compounds such as necrostatin-1, which is the most widely used agent to detect necroptosis in cells. The advantage of the necroptosis is that it can target apoptosis-resistant forms of cancers[28, 32].

1.7. Apoptosis

Apoptosis is the major type of programmed cell death and can be divided into two core pathways, the extrinsic death receptor-mediated apoptosis and intrinsic mitochondria-dependent apoptosis (Appendix 1).

Extrinsic death receptor-mediated apoptosis

Type 1 TNF receptor (TNFR1) and Fas (CD95) are two most well-known death receptors (DR), and their corresponding ligands are TNF and Fas ligand (FasL). The intracellular portion of death receptor is known as the death domain (DD). Once three or more DR-ligands bind to DR, they initiate trimerization of DR in which DD were brought together to form a binding site for the adaptor protein. The specific adaptor

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proteins respectively for TNFR1 and Fas are called TNF receptor-associated DD (TRADD) and Fas-associated DD (FADD). The function of TRADD or FADD is to recruits pro-caspase-8 to form a complex of ligand-receptor-adaptor protein-pro-caspase-8, named the death-inducing signaling complex (DISC), which is responsible for the cleavage of pro-caspase-8 to caspase-8. Activated caspase-8 can then stimulate apoptosis through activation of caspase-3, or promote crosstalk signaling to enhance intrinsic apoptosis by truncated Bid (tBid)[33].

Intrinsic mitochondria-dependent apoptosis

The intrinsic pathway is initiated by the cellular stimuli such as DNA damage, oxidative stress, growth-factor deprivation, hypoxia, etc. Regardless of the stimuli, this pathway eventually increases mitochondrial permeability and releases several pro-apoptotic molecules such as cytochrome-c, Smac/DIABLO, apoptosis-inducing factor (AIF) and endonuclease-G into the cytoplasm. Among these released molecules, cytochrome c bind to cytosolic apoptotic protease activating factor 1 (Apaf-1), procaspase-9 and ATP to form apoptosome, which subsequently activates downstream caspases to induce apoptosis. The intrinsic apoptosis induced by this pathway is closely regulated by the Bcl-2 family[33, 34].

Bcl-2 family

Proteins in Bcl-2 family are subdivided into three groups based on structural and functional similarities. Group I is multi-domain (BH1-BH4) anti-apoptotic proteins including Bcl-2, Bcl-xL, Mcl-1, Bcl-w, Bcl-b and A1. Groups Ⅱ is multi-domain (BH1-BH4) pro-apoptotic proteins, including Bax, Bak and Bok. Groups Ⅲ is BH-3 only proteins, containing Bim, Bad, tBid, PUMA, NOXA, Bik, Bmf and Hrk[35] (Appendix 2).

In general, anti-apoptotic Bcl-2 family members can promote ADP/ATP exchange

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and prevent the opening of permeability transition pores, thus maintaining the mitochondrial membrane permeability. In contrast, both pro-apoptotic Bak and Bax can oligomerize to form a pore on the outer mitochondrial membranes, leading to release of pro-apoptotic substances from the mitochondria. Several models have been proposed to understand the consequence of the interactions between these three subgroups of Bcl-2 protein family. In the "displacement" or called "indirect activation" model, anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-xL and Mcl-1 directly bind to pro-apoptotic Bak or Bax to prevent them from forming oligomeric pore on the outer membrane of mitochondria. In another model called "direct activation", BH3-only proteins with high affinity to Bax and Bak are termed as "activators," while those that only bind the anti-apoptotic proteins are named "sensitizers." The activator BH3 such as Bim and puma directly interact with and activate Bax, Bak and tBid to promote mitochondrial outer membrane permeabilization (MOMP). While the anti-apoptotic proteins such as Bcl-2 and Bcl-xL indirectly inhibit MOMP by binding to BH3 activators. However, the BH3 sensitizer proteins such as Bmf and Bad can further interact with anti-apoptotic proteins, thus releasing the BH3 activator for promoting MOMP by activation and oligomerization of Bax and Bak[36, 37].

Caspase

Caspases, cysteine-aspartate-specific proteases, are a family of protease enzymes playing important roles in apoptosis. Caspase has a cysteine in its active site that hydrolyzes target protein at the C-terminal of an aspartic acid amino acid. Caspases have been generally classified by their known roles in apoptosis (caspase-2, -3, -6, -7, -8, -9 and -10), and in inflammation (caspase-1, -4, -5, -12). Caspases involved in apoptosis can be further subclassified by their mechanism of actions as either initiator caspases (caspase-8 and -9) or effector caspases (caspase-3, -6, and -7). Caspases are initially

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produced as inactive monomeric pro-caspases, which can be cleaved and activated by either exposure to another activated caspase, autocatalysis or association with an activator protein complex, for instance apoptosome[38].

PARP cleavage

PARP-1, also known as NAD^+ ADP-ribosyltransferase 1, is a nuclear enzyme that catalyzes the transfer of poly(ADP-ribose) from its substrate β-NAD^+ onto itself and other nuclear proteins in response to DNA single-strand breaks in the base excision repair pathway. During apoptosis, activated caspases-3 and -7 have been shown to cleave PARP-1 into fragments of 89 and 24 kDa. The cleaved PARP-1 becomes incapable of responding to DNA damage. Therefore, PARP cleavage with fragments of 89 or 24 kDa has become a useful hallmark of apoptosis[39, 40].

1.8. Oxidative stress

Oxidative stress occurs in the biological systems when the levels of oxidants or reactive oxygen species (ROS) overwhelm the antioxidants or radical scavenging mechanisms, leading to damages of cellular components including proteins, lipids and DNA. ROS are molecules derived from oxygen that have accepted extra electrons and can oxidize other molecules. ROS includes hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), etc. Mitochondrial respiratory chain and various intracellular enzymes such as NADPH oxidase, xanthine oxidase and lipooxygenases are all endogenous ROS generators. In normal cellular metabolism, the optimal amount of ROS plays an important role in signal transduction. On the other hand, ROS can also be produced from exogenous sources such as radiation and chemotherapies, which has been one kind of mechanism to kill cancer cells. To reduce excessive ROS, cells are equipped with different enzymatic antioxidant defenses such as

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superoxide dismutases and catalase, and nonenzymatic antioxidants including glutathione and thioredoxin[41].

1.9. DNA double-strand break signaling and repair

Formation of DNA double-strand breaks (DSBs)[42]

DSBs can be generated in various routes. In addition to "programmed" DSB formation during V(D)J recombination, class switch recombination and meiosis, DSB can also be formed by the "accidental" events, such as ionizing radiation, treatment of radiomimetic drugs like topoisomerase II poisons, or topoisomerase I poisons/crosslinking agents-mediated replication fork collapse (one-ended DSB).

DSB-induced DNA damage response[43-45]

The DNA damage response (DDR) is a network of cellular pathways that sense DNA lesions, activate cell cycle checkpoints and allow DNA repair during cell cycle arrest to avoid the generation of deleterious mutations. When the amount of DNA damage exceeds the repair capacity, DDR signaling will eliminate damaged cells by triggering apoptosis or senescence. In general, three main roles are involved in the DDR—DNA damage sensors, signal transducers and effectors.

When DSBs occur, the DSB sensor, MRE11/RAD50/NBS1 (MRN) complex, will bind to and recruit partially autoactivated ATM, one of phosphoinositide 3-kinase (PI3K)-like protein kinases, to the DSB site, then inducing fully activation of ATM[46]. Activated ATM phosphorylates numerous local substrates including histone variant H2A.X (γ-H2A.X) around DSB. γ-H2A.X can extend megabase pair distances from the DSB and trigger histone modifications around the DSB to increase DNA accessibility for downstream protein assembly.

DNA damage sensors transmit signals to transducers, which then amplify and

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transduce signals to downstream effectors to result in cell cycle arrest. Chk2 and Chk1 are important transducers of ATM and ATR in response to DSB and DNA single-strand break (SSB) respectively. ATM/Chk2 and ATR/Chk1 pathways were historically thought to act in parallel with overlapping functions. However, more recently studies found these two pathways can be an upstream-downstream relationship, which explains why both ATM/Chk2 and ATR/Chk1 are activated and responsible for DSB-induced cell cycle arrest[47-50]. The proposed model[45] (Appendix 3) suggests that upon DSBs, MRN complex recognizes the DSB and leads to recruitment and full activation of ATM. The activated ATM then can phosphorylate several effector kinases including Chk2, which results in G1 arrest through ATM-Chk2-p53-p21 pathway. Activated ATM can also promote the enrollment of CtIP to the site of DSB, where CtIP interacts with and stimulates the nuclease activity of MRE11 of MRN complex to start the end resection of DSB and generate short tracts of ssDNA. Other nucleases and helicases, such as Exo1 and BLM, further resect the ssDNA to form the more extensive regions for RPA to bind and initiate the homologous recombination-mediated DSB repair. Importantly, it has been noted that the exposed ssDNA regions act like SSBs to activate ATR/Chk1 pathway. Activated Chk1 can induce p53-independent S phase arrest via phosphorylation of Cdc25A for degradation[51], or it can turn on G2/M checkpoint by phosphorylating and promoting Cdc25C for association with 14-3-3 proteins, preventing Cdc25C from activating mitotic Cdk1/cyclin B complex[52, 53]. Cell cycle arrest modulated by these transducer and effector kinases is important for the additional replication checkpoint responses (fork stabilization, inhibition of origin firing and S/M checkpoint) and the following DSB repair.

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DNA double-strand break repair (Appendix 4)

Except for one-ended DSB which can only be repaired by homologous recombination (HR), two-ended DSB, such as IR or topoisomerase II-mediated DNA damage, can be repaired by either HR or non-homologous DNA end joining (NHEJ)[54].

Homologous recombination (HR)

HR is an error-free DSB repair pathway. It is restricted to the late S and G2 phases of the cell cycle, where the homologous sequence located on the sister chromatid is available to serve as a donor template for repair of the damaged strand. The repair process of HR can be divided into three phases[55, 56].

Phase Ⅰ: Presynapsis

The ends of DSB are initially resected by MRN complex (Mre11-Rad50-Nbs1) and endonuclease CtIP complexed with BRCA1 in a 5' to 3' direction to generate short 3'-overhangs of single-strand DNAs (ssDNA). Further end resection is subsequently extended by Exo1, Dna2 and BLM to ensure the maintained resection. The resected ssDNA-ends are then coated by replication protein A (RPA) filaments, which keep ssDNAs unwound. Later, Rad51 together with other mediator proteins, such as BRCA2, Rad52 and Rad51 paralogs, replace RPA to form helical nucleoprotein filament on DNA.

Phase Ⅱ: Synapsis

Rad51 nucleofilaments promote the searching for homologous DNA sequences (sister chromatid in mitosis) similar to that of the 3'-overhangs, and catalyze strand invasion with the formation of displacement loop (D-loop).

Phase Ⅲ: Postsynapsis

After the successful strand invasion, DNA synthesis of the invading strand is carried out

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by DNA polymerase using the donor sequence serving as a template. Depending on the different types of HR, D-loop can be resolved by dissociation of one of the invading strands (synthesis-dependent strand annealing pathway, SDSA), or through migrating double Holliday junction intermediate that is dissolved by BLM–RMI–TOP3 or cleaved by resolvases.

Non-homologous end joining (NHEJ)

NHEJ is an error-prone DNA double-strand break (DSB) repair pathway that is active throughout all cell cycle phases. Compared to HR pathway, NHEJ is a much faster repair process because it simply joins the DSB ends without ensuring the restoration of the original DNA sequence around the DSB site. It has been known that NHEJ is the predominant pathway to repair IR or topoisomerase Ⅱ inhibitors-induced DSBs in mammalian cells.

The repair process of NHEJ [55, 56]*:*

The initiation of NHEJ begins from the binding of the Ku70/Ku80 heterodimer (Ku) to the exposed ends of DSB. Upon binding to DNA, Ku-DNA complex recruits and activates DNA-PKcs to the site of DSB. Activated DNA-PKcs has two important functions. It first thethers two opposing ends of DSB closly, and then recruits end-processing factors (for example Artemis, polynucleotide kinase/phosphatase (PNKP), AP endonuclease 1 (APE1) and tyrosyl–DNA phosphodiesterase 1 (TDP1)) to process the ends of DSB, which allows for religation by the XRCC4- XLF- LIG4 complex together with the polymerases λ and μ.

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Chapter 2: Materials and Methods

2.1. Materials

Human prostate adenocarcinoma cell lines, PC-3 and DU-145, were purchased from American Type Culture Collection (Rockville, MD, USA). RPMI 1640 medium, fetal bovine serum (FBS), penicillin, streptomycin and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from GIBCO/BRL Life Technologies (Grand Island, NY). Control siRNA, antibodies of PARP-1, Bax (6A7), Bcl-2 (C-2), Bcl-xL, Bak (G-23), Mcl-1 (22), Rad51, α-tubulin (B-7), DNA-PKcs (H-163), anti-mouse and anti-rabbit IgGs were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies of γ-H2A.X (Ser139), Bid, caspase-8, cleaved caspase-9 and Ku80 were from Cell Signaling Technologies (Boston, MA). Caspase-3 was from Imgenex, Corp. (San Diego, CA). Antibodies of p-Chk2 (Thr68) and p-DNA-PKcs (Thr2609) (10B1) were from Abcam PLC, Inc. (Massachusetts, US). Antibody of RPA32 (12F3.3) was from GeneTex Inc. Antibody of PDE5 was from OriGene Technologies, Inc. (Rockville, MD, USA). PDE5 siRNA was from GE Healthcare Dharmacon Inc. (Chicago, USA). Doxorubicin, camptothecin, etoposide, sildenafil, vardenafil, tadalafil, propidium iodide (PI) and all other chemical compounds were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Methods

2.2.1. Cell culture

HRPC cell lines, PC-3 and DU-145, were cultured in RPMI 1640 medium with 5% FBS (v/v), penicillin (100 units/ml) and streptomycin (100 μg/ml). Cultures were maintained in a 37℃ incubator with 5% CO_2. When cells were 90%-100% confluent, cells were detached by using 0.05 % trypsin-EDTA for passaging.

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2.2.2. PI staining and flow cytometric analysis

Cells with the indicated treatments were harvested by trypsinization, fixed with 70% (v/v) alcohol at -20℃ for at least 30 minutes and washed with PBS. After centrifugation, cells were resuspended with 0.5 ml PI solution containing Triton X-100 (0.1% v/v), RNase (100 μg/ml) and PI (80 μg/ml). DNA content was analyzed with FACScan and CellQuest software (Becton Dickinson, Mountain View, CA).

2.2.3. Western blotting

Sample preparation

After the indicated treatment, the cells were trypsinized and lysed with 60 μl ice-cold lysis buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin, 1mM Na_3VO_4, 1mM NaF and 1mM dithiothreitol). The lysate was incubated on ice for 10 minutes and then clarified by centrifugation at 14,000 rpm (13,000 ×g) at 4℃ for 20 minutes. After the centrifugation, supernatant (cell extract) was collected to determine protein concentration using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). To prepare sample for loading into gels, 5× sample buffer (0.3 M Tris pH 6.8, 10 % SDS, 50 % glycerol, 10 % β -mercaptoethanol, 0.02 % bromophenol blue) was added to the cell extract in a ratio of 1:4, and samples were mixed thoroughly then being heated at 95℃ for 5 minutes. Samples can be stored at -20℃ for future use.

Protein separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The polyacrylamide gel used in a single electrophoresis run can be divided into stacking gel and separating gel. The acrylamide percentage (6~14%, pH 8.8) of the

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separating gel depends on the sizes of the target proteins in the sample. After the gelation of the separating gel in the casting frame, stacking gel (acrylamide 5%, pH 6.8) was added on top of the separating gel and a gel comb was inserted into the stacking gel. The fully gelated polyacrylamide gels were then set up into the vertical gel electrophoresis tank, and the running buffer (25 mM Tris-base, 192 mM glycine, 3.5 mM SDS, pH 8.3) was poured into the inner and outer chamber of tank. The protein marker and the equal amount of proteins (30 μg) from each prepared sample were loaded into wells, and the gel was ready to be run at 60 V (for protein stacking) ~ 90 V (for protein separation) for about 2.5 hours.

Transferring the protein from the gel to the membrane

When performing a wet transfer, the separating gel was put into the a "transfer sandwich" (from cathode to anode pad: sponge-filter paper-gel-PVDF membrane-filter paper-sponge), which was placed in a tray with transfer buffer (25 mM Tris-base, 192 mM glycine, 20 % methanol, pH 8.3). Transferring the protein from the gel to the membrane at 65~75 V (about 200~250 mA) for 2~2.5 hours.

Immunoblotting

After an overnight incubation in PBST/5% nonfat milk at 4 ℃, the membrane was washed with PBS/0.1% Tween 20 (PBST) for three times and immuno-reacted with the indicated first antibody (1:1000~1:3000 dilutions) for 2 h at room temperature. After four washings with PBST, the anti-mouse or anti-rabbit IgG-HRP secondary antibody (1:5000 dilution) was applied to the membranes for 1 h at room temperature. The membranes were washed with PBST for 1 h and the detection of signal was performed with an enhanced chemiluminescence detection kit (Amersham Biosciences). The light signal was captured by X-ray films. The relative protein levels were quantified using the Bio-Rad Quantity One software (Hercules, CA, USA).

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2.2.4. Measurement of reactive oxygen species (ROS)

Cells pretreated with or without the antioxidant NAC (1 mM) or trolox (0.3 mM) were then incubated with 0.1 % DMSO (control), doxorubicin or/and sildenafil for the indicated times. Thirty minutes before the termination of the incubation period, DCFH-DA (final concentration of 10 mM) was added to the cells and incubated for the last 30 min at 37℃. Cells with different drug treatments were then harvested respectively for the detection of ROS production (%) by measuring the percentage of DCF fluorescence-positive cells in the collected total cells (10000 events) using FACScan flow cytometric analysis.

2.2.5. Comet assay

After treatment, the cells were pelleted and resuspended in ice-cold PBS. The resuspended cells were mixed with 1.5% low melting point agarose. This mixture was loaded onto a fully frosted slide that had been pre-coated with 0.7% agarose and a coverslip was then applied to the slide. After the gelation of the cell mixture, the coverslip was removed. The slides were then submerged in pre-chilled lysis solution (1% Triton X-100, 2.5 M NaCl, and 10 mM EDTA, pH 10.5) for 30 minutes at 4℃. After soaking with pre-chilled unwinding and electrophoresis buffer (0.3 N NaOH and 1 mM EDTA) for 30 minutes, the slides were subjected to electrophoresis for 15 minutes at 0.5 V/cm (25 mA). After electrophoresis, slides were stained with 1X SYBR Gold (Molecular Probes) and nuclei images were visualized and captured at 200X magnifications with an Zeiss AxioImager A1 fluorescent microscope (Zeiss, Germany) equipped with a CCD camera (Optronics, Goleta, CA). Over one hundred of cells in each sample were scored to calculate the average of comet tail moment (Tail moment = $\%DNA_{tail} \times Length_{tail}$) using TriTek CometScore^{TM} software.

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2.2.6. Nuclear extraction

Nuclear extracts were prepared by sequential cell lysis and nuclear lysis. Cell pellet was suspended in 200 μl buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol and 0.2 mM PMSF. The cells were subjected to vigorous vortex for 20 seconds, ice-cold incubation for 10 min to disrupt the cell membrane and centrifuged at 2000 rpm for 2 min. The pelleted nuclei were washed twice with 100 μl buffer containing 10 mM HEPES, 50 mM NaCl, 25% glycerol and 0.1 mM EDTA without resuspension the pellet. After removal of the washing buffer by centrifugation at 2000 rpm for 5 min, the pelleted nuclei were lysed with 30 μl buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 M EDTA, 0.5 mM dithiothreitol and 0.2 mM PMSF. After 20 minutes on ice, the lysates were centrifuged at 14,000 rpm for 5 min. The supernatants containing the solubilized nuclear proteins were used for Western blotting.

2.2.7. DNA fragmentation assay

The DNA fragmentation was determined using the Cell Death Detection ELISA^{PLUS} kit (Roche, Mannheim, Germany). The assay was based on the quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono- and oligo-nucleosomes) in cells after the induction of cell death. After treated with the indicated agents, cells were lysed and centrifuged, and the supernatant was used for the detection of nucleosomal DNA fragments according to the manufacturer's protocol.

2.2.8. Small interfering RNA (siRNA) transfection

PC-3 cells were seeded into a 6-well plate with 30% confluence for each well and grown for 24 hours to 50% confluence. Each well was washed twice with PBS and 1

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mL of serum-free Opti-MEM (Life Technologies, Ground Island, NY) was added. Aliquots containing control or PDE5 siRNA in serum-free Opti-MEM were transfected into cells using Lipofectamine 2000 according to the instructions. The sequences of PDE5 siRNA are GAAGACAGCUCCAAUGACA, GAAAUCAGGUGCUGCUUGA, GAUGACAGCUUGUGAUCUU and GGAAACGGUGGGACAUUUA. After transfection for 5 hours, cells were washed twice with PBS and incubated in 10% FBS-containing RPMI-1640 medium for 48 hours. The cells were then treated with or without doxorubicin for 48 hours, and the level of protein of interest was detected using Western bot analysis.

2.2.9. Immunofluorescence staining of nuclear Rad51 foci

PC-3 cells were grown on coverslips placed in a 6-well plate (1.8×10^5 cells/well). All procedures for immunofluorescence staining were conducted at room temperature. Following treatment with 1 μM doxorubicin or/and 10 μM sildenafil for 24 hours and 8 hours of cell recovery in drug-free medium, PC-3 cells were washed twice with PBS and fixed with 4 % paraformaldehyde in PBS for 20~30 min. After fixation, cells were washed three times with PBS, permeabilized with 0.1% Triton X-100 for 10 minutes followed by three times of wash with PBS and then blocked with 5% BSA/PBS for 1 hour. To examine the nuclear Rad51 foci, cells were subsequently stained with the anti-Rad51 antibody (1:200 dilution in 2.5% BSA/PBS) for 1 hour with gentle agitation and washed three times with PBS. Cells were next incubated with the FITC-conjugated secondary antibody for 1 hour (1:100 dilution in 2.5% BSA/PBS) with gentle agitation. After washing cells three times with PBS, nuclear staining was performed using 0.15 μg/ml DAPI for 5~10 minutes. Cells on coverslip were finally washed three times with PBS. The air-dried coverslips were next mounted onto glass slides using prolong®

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diamond antifade mountant (Thermo Fisher Scientific inc., Waltham, MA, USA). The slides were then kept in the dark at 4℃ for at least one day to dry the antifade mountant. The immunofluorescent images of nuclear Rad51 foci and nuclei were captured at 630X magnifications (63x/1.4 NA oil immersion objective lens) using Zeiss AxioImager A1 fluorescent microscope (Zeiss, Germany) equipped with a CCD camera (Optronics, Goleta, CA). At least 100 cells were examined in each sample, and the percentage of cells containing over five Rad51 foci in each sample was estimated.

2.2.10. DNA end-binding activity of Ku80 protein

Assessment of DNA end-binding activity of Ku80 was carried out by using a Ku70/Ku80 DNA Repair kit (Active Motif). Briefly, equivalent amounts of nuclear proteins (4 μg) were loaded into an oligonucleotide coated 96-well plate. Then, Ku80 proteins contained in nuclear extract specifically bound to the oligonucleotide. Anti-Ku80 antibody provided by this kit detected DNA bound-Ku80. Addition of the secondary HRP-conjugated antibodies and developing solution provided a colorimetric readout (λ = 450 nm) quantified by spectrophotometry.

2.2.11. Data Analysis

Data are presented as mean±standard deviation (SD) for the indicated number of separate experiments. Statistical analysis of data was performed with one-way analysis of variance followed by Bonferroni t-test and *p*-values < 0.05 were considered significant.

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Chapter 3: Results

3.1. Effect of doxorubicin and sildenafil on cell cycle progression in PC-3 and DU145 cells

The cell population of the cell cycle in both PC-3 and DU145 cells treated with doxorubicin and sildenafil was determined by PI staining and analyzed using FACScan flow cytometry. Results showed that sildenafil alone did not affect the cell cycle distribution (Fig. 1). However, it significantly potentiated doxorubicin-induced increase of sub-G1 phase population, while decreased both S and G2/M cell population in PC-3 cells (Fig. 1). Similar effects in increasing sub-G1 population were observed in DU145 cells (Fig. 2). Since sub-G1 cell population was considered as apoptotic cells with DNA fragmentation followed by loss of DNA content, the validation of apoptotic cell death was conducted.

3.2. Validation of sildenafil-mediated sensitization of doxorubicin-induced apoptosis

Several assays were performed to confirm whether sildenafil could synergistically enhance apoptotic cell death induced by doxorubicin. The results of microscopic examination showed that, in contrast to doxorubicin alone, the combinatory treatment of doxorubicin and sildenafil for 24 h caused a profound increase of cell shrinkage and apoptotic bodies in PC-3 cells (Fig. 3A). It was worth noting that although sildenafil was able to sensitize PC-3 cells to doxorubicin, sildenafil itself did not have any cytotoxic effect on PC-3 cells. Besides, cell death detection ELISA^{PLUS} kit which detects nucleosomal DNA fragments in the cytoplasm of apoptotic cells was employed to validate cell apoptosis. As a consequence, sildenafil alone did not induce an increase of

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nucleosomal DNA fragments in PC-3 cells, but significantly increased doxorubicin-induced effects by 1.67 times (doxorubicin, 1.68-fold; combination, 2.80-fold) (Fig. 3B). Moreover, the expression of apoptotic markers in intrinsic and extrinsic pathway was monitored by Western blot in PC-3 cells. The apoptotic markers including cleaved caspase-9 and -8 which served as initiator caspases in intrinsic and extrinsic pathways, respectively, and their common downstream substrates, effector caspase-3 and PARP-1, were examined. The results demonstrated that the combinatorial treatment for 24 hours significantly increased the expression of cleaved caspase-3, 8, 9 and PARP-1 as compared to doxorubicin alone (Fig. 3C). These results suggested that sildenafil was able to potentiate doxorubicin-induced cell apoptosis by activation of both intrinsic and extrinsic apoptotic pathways.

3.3. Effect of doxorubicin or/and sildenafil on the expression of Bcl-2 family proteins

Bcl-2 family proteins, consisting of anti-apoptotic and pro-apoptotic members, are gatekeepers of mitochondria and crucial regulators of apoptosis particularly in the intrinsic pathway to govern the mitochondrial outer membrane permeability. Since the combinatorial treatment enhanced the activation of caspase-9, a key initiator caspase in the mitochondria-involved intrinsic apoptotic pathway, the effect of the combinatorial treatment on the expression of Bcl-2 family proteins was further examined by Western blot. Consequently, the combinatorial treatment for 24 h could further reduce doxorubicin-mediated decease of anti-apoptotic Bcl-2 family proteins, including Mcl-1, Bcl-xL and Bcl-2, but not those of pro-apoptotic members, such as Bax and Bak, in PC-3 cells (Fig. 4A and 4B). Moreover, combinatorial treatment further downregulated the protein level of Bid pro-form (Fig. 4A and 4B). The decreased Bid pro-form was

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indicative of the caspase-8 activation since Bid served as a downstream substrate of caspase-8. Taken together, the data suggest that sildenafil may augment the mitochondria-dependent apoptosis induced by doxorubicin through modification of specific members of Bcl-2 family.

3.4. Effect of doxorubicin or/and sildenafil on ROS production

Since mitochondrial dysfunction is often associated with an increase in ROS production, the intracellular ROS levels in PC-3 cells after combinatorial treatment were determined by DCFH-DA assay. The data revealed that the short-term (e.g., 3 h) exposure to doxorubicin or sildenafil alone or combinatorial treatment significantly elevated cellular ROS levels. All the increased levels of ROS production were dramatically abolished in the presence of ROS scavengers, NAC and trolox (Fig. 5). To further determine whether the oxidative stress contributed to the apoptotic sensitization, the flow cytometric analysis of PI staining was performed to determine apoptotic sub-G1 population. As a result, neither NAC nor trolox significantly blunted the synergistic cell apoptosis caused by the combinatorial treatment (Fig. 6). The data suggest that the increase of oxidative stress is not responsible for the apoptotic sensitization mechanism.

3.5. Effect of sildenafil on doxorubicin-induced DNA double-strand break signaling and repair system

Doxorubicin is a DNA damaging drug known to cause DNA double-strand breaks (DSB). The effects of sildenafil on doxorubicin-mediated DSB signaling and repair system were examined. We first employed the alkaline comet assay to monitor the chromosomal DNA integrity. Comet tail moment was utilized as a scoring parameter to

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assess the DNA damage level in individual cells. The data revealed that although doxorubicin alone induced a rapid and profound increase of comet tail moment, sildenafil did not augment the DNA damage levels caused by doxorubicin (Fig. 7). The results of Western blot also showed that sildenafil did not increase doxorubicin-induced phosphorylation of histone H2A.X at Ser139 (γ-H2A.X) and Chk2 phosphorylation at Thr68 (the hallmarker and transducer kinase of DSB, respectively) at an early exposure time (e.g., 3 hours) (Fig. 8A, B). Of note, sildenafil significantly increased the levels of doxorubicin-induced γ-H2A.X formation at a longer exposure time (e.g., 24 hours) (Fig. 8A). The results indicate that sildenafil is unable to potentiate direct DNA damage induced by doxorubicin, but can ultimately sensitize the DNA damaging effect and apoptosis through certain programmed mechanism, such as impairment of DNA repair systems. Accordingly, several markers involved in DNA repair were examined. The data demonstrated that sildenafil blunted the initial RPA32 hyperphosphorylation induced by doxorubicin (Fig. 8C and 8D) and lowered doxorubicin-elicited DNA-PKcs phosphorylation (Thr2609) (Fig. 8F and 8G). In addition, sildenafil further decreased doxorucibin-induced down-regulation of Rad51 (Fig. 8C and 8E). Similar effects were observed in DU145 cells with combinatorial treatment (Fig. 9). It has been well recognized that RPA32 binds to ssDNA during the initial phase of homologous recombination (HR) pathway and its hyperphosphorylation plays a critical role in promoting DSB repair to maintain genome stability in response to DNA damage[57, 58]. Rad51 plays a major role in homologous recombination (HR) for repairing DSB[59]. Differently, DNA-PKcs is required for non-homologous end joining (NHEJ) pathway of DNA repair, which rejoins DSBs[60]. Altogether, the data suggest that sildenafil may impair both HR and NHEJ pathways of DNA repair during doxorubicin-induced DNA damage effect.

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3.6. Effect of combinatorial treatment on DNA end-binding capacity and protein expression of Ku80

Ku heterodimers (Ku70/Ku80) are crucial DSB sensors in NHEJ pathway. They can bind directly to the ends of DSB, and serve as a molecular scaffold to recruit the core NHEJ machinery including DNA-PKcs to repair DSB. To monitor the activity of NHEJ, the DNA end-binding capacity of Ku80 was determined. The data demonstrated that the combinatorial treatment of PC-3 cells with doxorubicin and sildenafil significantly, although moderately, decreased the DNA end-binding capacity of nuclear Ku80 (Fig. 10A). Total and nuclear Ku80 levels were detected by Western blot analysis, and the Ku80 expression was not modified in the presence of doxorubicin and sildenafil (Fig. 10B). The results indicate that the combinatorial treatment can reduce the NHEJ activity by decreasing the DNA end-binding capacity of nuclear Ku80 without changing its protein expression.

3.7. Effect of combinatorial treatment on nuclear foci formation and expression of Rad51

Rad51, a recombinase, has been well recognized to play a central role in HR pathway to accurately repair DSB by catalyzing homology searching and strand exchange reactions. Successful HR repairing requires the formation of Rad51 nucleofilaments on single-strand DNA, which can be seen as Rad51 foci in nucleus using immunefluorescence imaging. Therefore, we further examined the nuclear Rad51 foci formation in PC-3 cells. The data showed that doxorubicin dramatically induced the formation of nuclear Rad51 foci; however, the effect was significantly inhibited by sildenafil (Fig. 11A-11C). Besides, Western blot of nuclear extract also showed the diminished expression of Rad51 after the combinatorial treatment for 24 hours (Fig.

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11D). The results indicate that sildenafil may impair doxorubicin-elicited HR-mediated DSB repair by lowering the total protein level of Rad51, thereby decreasing the formation of nuclear Rad51 foci.

3.8. Effect of other PDE5 inhibitors on doxorubicin-induced cell apoptosis

To realize whether inhibition of PDE5 activity was indispensable for the sensitization mechanism, two other FDA-approved PDE5 inhibitors, vardenafil and tadalafil, were examined in PC-3 cells in this study. The data showed that tadalafil alone but not vardenafil alone induced a small but significant production of nucleosomal DNA fragments (apoptosis). Furthermore, both PDE5 inhibitors sensitized doxorubicin-induced effects although vardenafil exhibited a much higher sensitization activity than that of tadalafil (Fig. 12A). The data of Western blot analysis also showed that both PDE5 inhibitors potentiated doxorubicin-induced caspase-3 activation and the cleavage of its downstream substrate PARP-1 (Fig. 12B and 12C).

The synergism of doxorubicin and PDE5 inhibitors (vardenafil or tadalafil) on apoptosis was further substantiated in DU145 using PI staining and FACScan flow cytometry for the detection of sub-G1 population (apoptotic cells). The results revealed that both vardenafil (Fig. 13A) and tadalafil (Fig. 13B) could significantly potentiate sub-G1 population induced by doxorubicin in a dose-dependent manner.

Taken together, the data suggest that the inhibition of PDE5 activity may play a crucial role in the sensitization mechanism when combined with doxorubicin.

3.9. Effect of other PDE5 inhibitors on HR-mediated repair of doxorubicin-induced DSB

To investigate whether two other PDE5 inhibitors, vardenafil and tadalafil, could

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also reduce the expression of HR-related proteins in response to doxorubicin, the Western blot analysis was performed to detect protein levels of hyperphosphorylated RPA32 (p-RPA32) and Rad51 in PC-3 cells. The results revealed that only vardenafil but not tadalafil could reduce the doxorubicin-induced hyperphosphorylation of RPA32 (Fig. 14 A and B). However, both vardenafil and tadalafil could lead to a decrease in Rad51 protein level when combined with doxorubicin although vardenafil was more effective than tadalafil (Fig. 14 A and B). Notably, there was a strong negative correlation (R^2 = 0.9304) between the levels of Rad51 protein expression and nucleosomal DNA fragments in the presence of doxorubicin and different PDE5 inhibitors (sildenafil, vardenafil or tadalafil) (Fig. 15), supporting that the down-regulation of Rad51 protein and the impaired DNA repair might contribute to the sensitization effect on cell apoptosis.

3.10. Effect of PDE5 knockdown on doxorubicin-induced cell death and DSB signaling and repair

Although sildenafil, vardenafil and tadalafil are all potent and selective PDE5 inhibitors approved by FDA, they have also been reported to have inhibitory effects on other PDE isoenzymes, such as PDE1, 6 or 11. Thus, to determine the role of PDE5 but not the other subtypes in the sensitization mechanism is critical. To this end, we knocked down PDE5 using small interfering RNA (siRNA) to further check doxorubicin-mediated effect. The data showed that PDE5 knockdown did not change the cell cycle distribution of the control group in PC-3 cells but decreased both S and G2/M cell population, while significantly increased the sub-G1 population induced by doxorubicin (Fig. 16A and B). However, PDE5 knockdown unexpectedly reduced the doxorubicin-induced caspase-3 cleavage but had no effect on PARP-1 cleavage (Fig.

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16C). The PDE5 knockdown also showed different regulation from that of PDE5 inhibitors on several proteins markers of DNA repair (Fig. 17). Altogether, the data indicate that the mechanism of the sensitization effect caused by PDE5 knockdown was not similar to that of PDE5 inhibitors.

3.11. Effect of sildenafil on the sensitization of apoptosis induced by other topoisomerase inhibitors

In addition to doxorubicin, several other topoisomerase inhibitors were also examined for the effect of sildenafil-mediated chemosensitization on cell apoptosis. Cell apoptosis was determined by PI staining and flow cytometric analysis for the detection of sub-G1 population. The data in Figure 18 showed that sildenafil could significantly potentiate cell apoptosis induced by two other topoisomerase Ⅱ inhibitors, etoposide and mitoxantrone, but not by topoisomerase I inhibitor, camptothecin (Fig. 18).

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Chapter 4: Discussions

Sildenafil is an FDA-approved PDE5 inhibitor for the treatment of erectile dysfunction and pulmonary arterial hypertension, but it has been recently repurposed as a potential chemosensitizer for anticancer applications in both *in vitro* and *in vivo* studies. Several *in vitro* studies have shown that sildenafil could potentiate cell death caused by different chemotherapeutic agents such as doxorubicin, mitomycin C, gemcitabine, cisplatin, etoposide, paclitaxel or vincristine in prostate, bladder, ovarian, pancreatic or brain cancer cell lines in either an additive or synergistic manner[8, 23, 24]. Among these drug combinations, sildenafil combined with doxorubicin have been further shown to significantly inhibit PC-3 prostate tumor xenograft growth in nude mice when compared to doxorubicin alone[8]. However, the more detailed anticancer mechanisms and the upstream cellular regulation of the synergism between doxorubicin and sildenafil in hormone-refractory prostate cancer (HRPC) have not yet been well identified. Therefore, the sensitization mechanism has been elucidated in the present study.

4.1. Effect of sildenafil on apoptosis induced by doxorubicin in PC-3 cells

Previous study reported that sildenafil enhanced cell-killing effect of doxorubicin in PC-3 and DU145 cells partly by increasing intrinsic apoptosis through up-regulation of caspase-3, 9 activities, reduced expression of Bcl-xL and phosphorylation of Bad[8]. Based on our results, we found that doxorubicin combined with sildenafil potentiate the intrinsic cell apoptosis in PC-3 cells through the activation of caspase-9 and -3, and decreased level of anti-apoptotic proteins, Mcl-1, Bcl-xL and Bcl-2. In addition to mitochondria-dependent apoptotic pathway, we suggested the death receptor-mediated extrinsic apoptosis was also augmented by the drug combination due to the observation

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of increased cleaved caspase-8 and reduced pro-form of Bid, one of the pro-apoptotic members in Bcl-2 family. Several cell death receptors have been shown to active caspase-8, including CD95, DR3, DR4, DR5 and TNFR1[61]. In this study, we did not determine what death receptor was crucial for synergistic activation of caspase-8 in response to the combinatorial treatment. However, two recent studies have demonstrated that knockdown of CD95 or Fas-associated death domain protein, or overexpression of short forms of FLIP could suppress synergistic killing effect caused by the drug combination of doxorubicin and sildenafil in bladder cancer cells and prostate cancer DU145 cells[22, 23]. Therefore, it is possible that the combinatorial treatment might induce CD95-mediated apoptosis, thereby activating caspase-8. Besides, the reduced pro-form of Bid might imply the existence of the elevated level of truncated Bid (tBid), which was known to be cleaved by activated caspase-8 and was responsible for the crosstalk between intrinsic and extrinsic apoptotic pathways[61]. Nevertheless, more evidence should be provided to support the increase of tBid. Altogether, the data suggest that the combination of sildenafil and doxorubicin is able to induce cell apoptosis in HRPC through both intrinsic and extrinsic apoptotic pathways.

4.2. The role of ROS production in the sensitization mechanism

Das et al. suggested that sildenafil by itself could not elevate the cellular ROS level, but it significantly boosted doxorubicin-induced ROS production, thus contributing to the enhanced apoptosis in HRPC cells[8]. However, we noted that they measured the ROS level after a long-term drug treatment (24, 48 or 72 hours). It has been reported that the event of late increase in intracellular ROS induced by genotoxic stress may be mediated by caspase-induced feedback amplification, and could be reversed by caspase inhibitors, overexpression of Bcl-2 or inhibition of mitochondrial permeability transition pore

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opening[62]. According to our measurement of nucleosomal DNA fragments in apoptotic cells, we found that cell apoptosis caused by the drug combination could be detected after 15 hours of drug treatment. We thus considered the late increase in ROS production (24, 48 or 72 hours) may be a consequence of the potentiated apoptosis caused by the drug combination but not the initial mechanism that led to synergistic cell death. Therefore, we monitored the production of ROS after a short-term drug treatment (3 hours) and found that sildenafil by itself even generated a higher level of intracellular ROS than doxorubicin did. When sildenafil combined with doxorubicin, an additive increase in ROS level could be observed. We further confirmed the role of ROS in the synergistic apoptotic effect of drug combination using ROS scavengers and revealed that although both NAC and trolox could reverse the additive increase of cellular ROS, they did not abolish the rise of sub-G1 population (apoptosis) and rescue the apoptotic morphology (data not shown) caused by the combinatorial treatment. These results indicated that mechanism other than ROS production may contribute to the synergy of cell apoptosis.

4.3. Effect of sildenafil on doxorubicin-induced DNA double-strand break signaling and repair

In addition to generation of ROS, another proposed anticancer mechanism of doxorubicin is to inhibit the topoisomerase IIα (Topo IIα)-mediated DSB re-ligation, resulting in Topo IIα-DNA cleavage complexes and later transforming into DNA double-strand breaks (DSBs) by proteasome pathway[63, 64]. Based on the known action of doxorubicin, we then studied the effect of sildenafil on doxorubicin-induced DSB signaling and repair.

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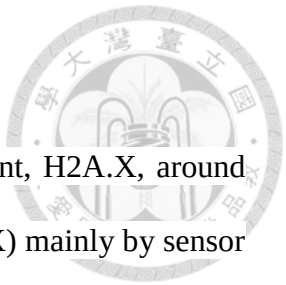

4.3.1. DNA double-strand break signaling

It has been reported that once DSBs occur, histone H2A variant, H2A.X, around the site of DSB are subsequently phosphorylated at Ser139 (γ-H2A.X) mainly by sensor kinase ATM, one of PIKKs members. Therefore, γ-H2A.X is regarded as a marker of DSBs[65]. Our data revealed that sildenafil did not apparently affect the early phosphorylated level of H2A.X (Ser139) in response to DNA damage induced by doxorubicin. Although the enhanced expression of γ-H2A.X could be detected after a long-term combinatorial treatment (24 h), it may be a result of apoptotic DNA fragmentation[66]. This implied that sildenafil may not potentiate the doxorubicin-induced DSBs, which was further supported by the result of comet assay. In addition to γ-H2A.X, Chk2 (a DNA damage checkpoint protein) is also phosphorylated by ATM at the position of Thr68 in response to DSB formation. Phosphorylation of Chk2 at Thr68 initiates its full activation, which enables it to act as a signal transducer to phosphorylate several substrates responsible for halting the cell cycle, initiation of DNA repair, and the induction of apoptosis after DNA damage[65]. Our data demonstrated that sildenafil did not change the doxorubicin-induced phosphorylation of Chk2 (Thr68) at any time point. This may imply that the synergistic apoptosis resulted from the drug combination was Chk2-independent. Of note, it has been reported that p-Chk1 has partially redundant functions with p-Chk2[67]. One prior report has demonstrated that doxorubicin induces the phosphorylation of both Chk1 and Chk2, and inhibition of Chk1 but not Chk2 function bypassed the DNA damage checkpoint, revealing that Chk1, but not Chk2, is important for doxorubicin-induced cell cycle arrest[68]. To determine whether p-Chk1 plays the role instead of p-Chk2 in the synergy of drug combination, more studies about p-Chk1 should be conducted[69].

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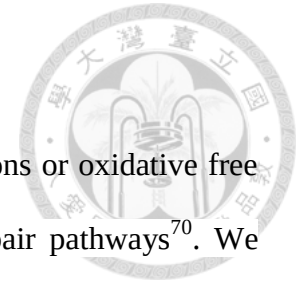

4.3.2. DNA double-strand break repair

It has been indicated that DSB caused by topoisomerase poisons or oxidative free radicals could be repaired through both HR and NHEJ DNA repair pathways[70]. We found that although sildenafil could not enhance the doxorubicin-induced DNA damage, combination of these two drugs significantly downregulated the levels of three crucial DSB repair proteins including p-DNA-PKcs (Thr2609) in NHEJ, and RPA32 and Rad51 in HR.

Ku70/Ku80 heterodimers are crucial DSB sensors to recognize and bind to the ends of DSB, which is required for directing DNA-PKcs to DSB and triggering its kinase activity for activation of NHEJ repair machinery[70]. Our results demonstrated that doxorubicin combined with sildenafil not only lowered the level of p-DNA-PKcs (Thr2609), but also significantly decreased the DNA-end binding capacity of Ku80 without changing its protein level, confirming the decrease in NHEJ activity. Even though NHEJ is an error-prone DSB repair pathway compared to HR, it has been proved as a fast, efficient and indispensable way for repair of DSB induced by doxorubicin[71, 72]. Therefore, the declined NHEJ activity may sensitize cancer cells to doxorubicin. For instance, one study has reported that KU-0060648, a potent dual inhibitor of DNA-PK and PI-3K, enhances etoposide and doxorubicin-induced *in vitro* and *in vivo* cytotoxicity in human breast and colon cancer cells[73]. Hence, we suggested the impaired NHEJ activity caused by the drug combination may partly confer the improved efficacy of doxorubicin in PC-3 cells.

Besides the reduced NHEJ activity, we found that eight hours after the drug treatment, sildenafil downregulated doxorubicin-induced hyperphosphorylation of RPA32. RPA32 is a regulatory subunit of RPA, which is a heterotrimeric protein complex with two other subunits RPA70 and RPA14. RPA bind to single-strand DNA

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during DNA replication or upon DNA damage to facilitate nascent strand synthesis, or activate cell-cycle checkpoint mediators and DNA repair machineries involved in HR or/and nucleotide excision repair. RPA undergoes both stress-dependent and -independent phosphorylation on the N-terminus of the RPA32 subunit. Under un-stressed cell cycle, RPA32 is phosphorylated by cyclin-cdk complexes at Ser-23 and Ser-29 during DNA replication and mitosis. Upon DNA damage, PIKKs hyperphosphorylate RPA at five or more additional sites out of possible nine sites, including Ser4, Ser8, Ser11, Ser12, Ser13, Thr21, Ser23, Ser29, and Ser33 on the N-terminus of RPA32[74, 75]. The hyperphosphorylated form of RPA32 has been shown to have a significantly reduced mobility on SDS–PAGE and can be recognized by Western blot analysis[76, 77]. It has been suggested that RPA32 hyperphosphorylation leads to a change in RPA conformation, which reduces the interaction between RPA and proteins involved in DNA replication but without changing the interaction with proteins involved in DNA repair. This indicates that RPA32 hyperphosphorylation promotes the DNA repair[58, 75]. Therefore, the initial reduction in hyperphosphorylation of RPA32 caused by doxorubicin and sildenafil in HRPC cells may imply a decrease in HR-mediated repair of DSBs. The impairment of HR was further substantiated by a dramatic reduction of another protein, Rad51, which has been regarded playing a central role in HR pathway to accurately repair DSBs by catalyzing homology searching and strand exchange reactions. The data revealed that doxorubicin in combination with sildenafil apparently lowered the total protein expression and nuclear foci formation of Rad51 in HRPC cells, confirming the diminished HR-mediated DSB repair. This decrease in total and nuclear expression of Rad51 can be further discussed in three aspects including the down-regulation of *Rad51* transcription, increased degradation and reduced nuclear localization of Rad51. According to the previous studies, the decreased

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transcription of *Rad51* in response to the specific stimulation in cancer cells has been associated with the regulation of the E2 factor (E2F) family of transcription factors. Bindra and Glazer have demonstrated that the hypoxia-induced downregulation of Rad51 in MCF7 breast cancer cell line is mediated by the increased occupancy of transcriptional repressor E2F4/p130 complexes at the E2F site in *RAD51* promoter[78]. Besides, Bonavida and Yakovlev revealed that NO/RNS stimulation caused by inflammation-relevant concentrations (50–200μM) of NO/RNS donor, SNAP, would not directly cause DNA damage in MCF-10A and A549 cancer cells, but inhibit the transcription of DNA repair genes including *Rad51* and *BRCA1* by changing their promoter occupancy from complexes containing activator E2F1 to complexes containing repressor E2F4[79, 80]. Similar mechanism is also found in the pan-histone deacetylase (HDAC) inhibitor-induced reduction of *Rad51* transcription in both gastric and prostate cancer cells[81, 82]. On the other hand, gefitinib, a tyrosine kinase inhibitor, has been reported to induce cytotoxicity in drug sensitive human non-small cell lung cancer cells through not only lowering the mRNA level of Rad51 but also enhancing the 26S proteasome-mediated degradation of Rad51[83]. In the present study, the decreased level of Rad51 in nucleus in HRPC cells co-treated with doxorubicin and sildenafil might be a consequence of either the total reduction of cellular Rad51 or the decreased nuclear localization of Rad51. So far, two different mechanisms have been proposed to explain how Rad51 localizes in the nucleus. Since Rad51 does not have a nuclear localization signal (NLS), Rad51C, one of Rad51 paralogs, containing a functional C-terminal NLS has been shown to directly interact with Rad51 to assist its nuclear entry[84]. After Rad51 enters the nucleus, CRM1/exportin 1 may bind to the nuclear export signal (NES) within Rad51 for nuclear export. Another mechanism indicates that the BRC4 domain of BRCA2 is able to mask the Rad51 NES, leading to nuclear retention of RAD51[85]. Based

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on these prior studies, the effect of doxorubicin or/and sildenafil on E2F transcription factors, Rad51 ubiquitination and Rad51C and BRCA2-mediated nuclear localization of Rad51 may be intriguing issues waited to be investigated.

4.4. Effect of PDE5 inhibitors or PDE5 knockdown on doxorubicin-induced cell death and DSB signaling and repair

Two other PDE5 inhibitors have been utilized to further identify the role of PDE5 in the sensitization mechanism. We found that inhibition of PDE5 activity using two other PDE5 inhibitors, vardenafil and tadalafil, could also potentiate the cell-killing effect of doxorubicin in both PC-3 and DU145 cells. We found that although sildenafil, vardenafil and tadalafil were all selective and potent PDE5 inhibitors approved by FDA, they exerted different abilities to increase doxorubicin-induced cell apoptosis (vardenafil>sildenafil>tadalafil), which closely correlated with their abilities to lower the protein expression of Rad51. This result strongly supported our proposed mechanism that impaired HR repair played a major part to contribute to the synergistic apoptosis. However, it has not been well identified whether the reduction of Rad51 level caused by the drug combination is an on-target or off-target effect of PDE5 inhibitors. Therefore, we further knocked down PDE5 using siRNA to examine its synergy with doxorubicin and the effect on DNA damage response. The results revealed that knockdown of PDE5 could also sensitize PC-3 cells to doxorubicin with the evidence of an increased sub-G1 population using PI staining and flow cytometric analysis. However, unexpected results of Western blot showed that knockdown of PDE5 followed by the treatment of doxorubicin for 48 hours reduced the level of cleaved caspase-3, and decreased the expression of DNA damage response and repair proteins including γ-H2A.X, p-Chk2 (Thr68), p-RPA32 and Rad51. Of note, although PARP-1

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has been known as a substrate of caspase-3, an apoptotic marker, the level of cleaved PARP-1 was not decreased. It has been suggested that the caspase-independent PARP-1 cleavage may be related to other types of cell death but not apoptosis, such as cathepsin D/AIF (apoptosis-inducing factor)-mediated apoptosis-like programmed cell death or cathepsin B/D-mediated necrosis [86, 87]. However, it needs further investigation to know the complicated regulation for PARP-1. Based on these data, we concluded that knockdown of PDE5 changes the cell fate of PC-3 cells. For example, unlike the usage of PDE5 inhibitors, knockdown of PDE5 sensitize PC-3 cells to doxorubicin in a caspase-independent mechanism. Besides the possible changes of cell fate, we considered knockdown of PDE5 followed by the treatment of doxorubicin may resemble the sequential treatment with PDE5 inhibitor followed by doxorubicin, which could not completely mimic the co-treatment of these two drugs. Therefore, take these observations into consideration, PDE5 knockdown is not similar to the presence of PDE5 inhibitors and can not explain the relationship between DNA damage response proteins and the inhibition of PDE5 caused by the PDE5 inhibitors.

4.5. Effect of other topoisomerase inhibitors or/and sildenafil on cell apoptosis

Previous studies have indicated that PDE5 inhibitors could sensitize multidrug-resistant cancer cells to chemotherapy drugs by inhibiting drug efflux of the ATP-binding cassette transporters (ABC transporters) including ABCB1 (p-glycoprotein), ABCG2 and ABCC10[25, 88, 89]. However, this transporter-relevant mechanism can only be used to explain the synergism observed in multidrug-resistant cancer cells overexpressing specific ABC transporters. The HRPC cell lines PC-3 and DU145 do not express p-glycoprotein, ABCG2 and ABCC10[90-94]. Besides, p-glycoprotein and ABCG2 have been shown to pump both topoisomerase Ⅰ and Ⅱ

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inhibitors out of cells [95]. According to our results, we found that sildenafil could only sensitize PC-3 cells to topoisomerase Ⅱ inhibitors including doxorubicin, etoposide and mitoxantrone, but not topoisomerase Ⅰ inhibitor camptothecin. The finding strongly supports that sildenafil may not improve the efficacy of doxorubicin in HRPC cells by interfering with drug efflux function of p-glycoprotein and ABCG2.

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Chapter 5: Conclusion

Sildenafil does not increase doxorubicin-induced DNA double-strand breaks (DSBs) in hormone-refractory prostate cancer cells (HRPC), but it significantly impairs the doxorubicin-elicited DSB repair systems including homologous recombination (HR) and non-homologous end joining (NHEJ) pathways, thus leading to the synergistic increase in nucleosomal DNA fragments and enhanced activation of intrinsic and extrinsic cell apoptosis. Besides, both inhibition of PDE5 activity and knockdown of PDE5 can sensitize HRPC to doxorubicin, indicating PDE5 may partly contribute to the sensitization mechanism. However, the role of PDE5 in DSB repair still remains unclear (Fig. 19).

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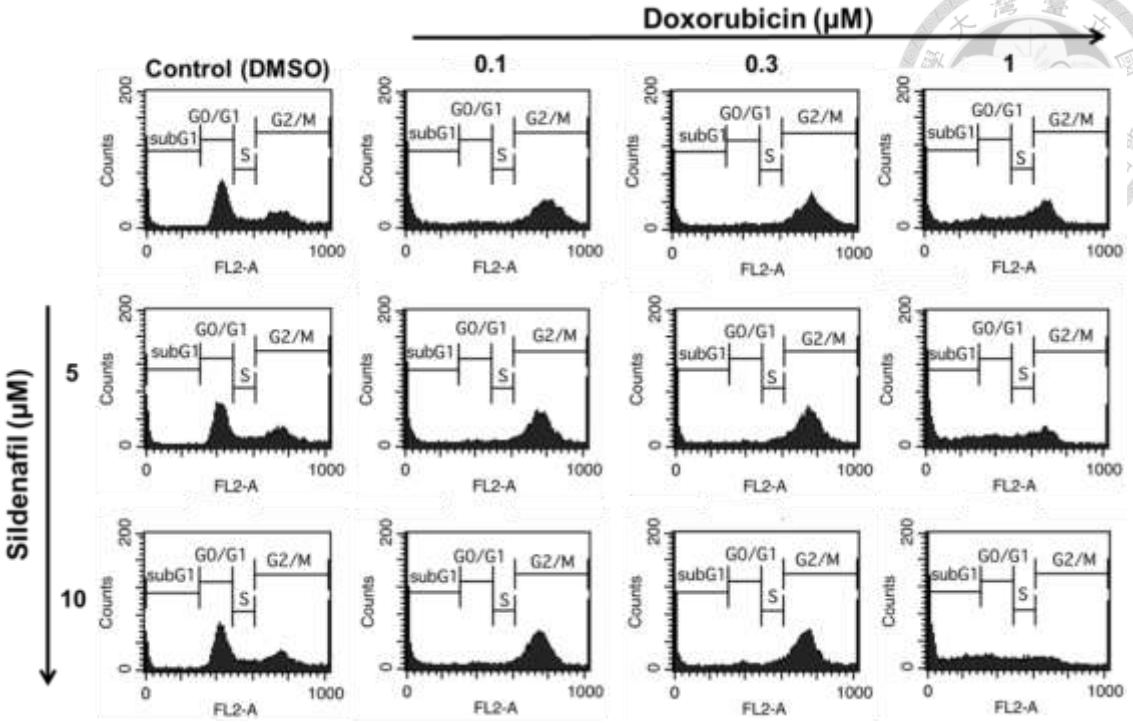

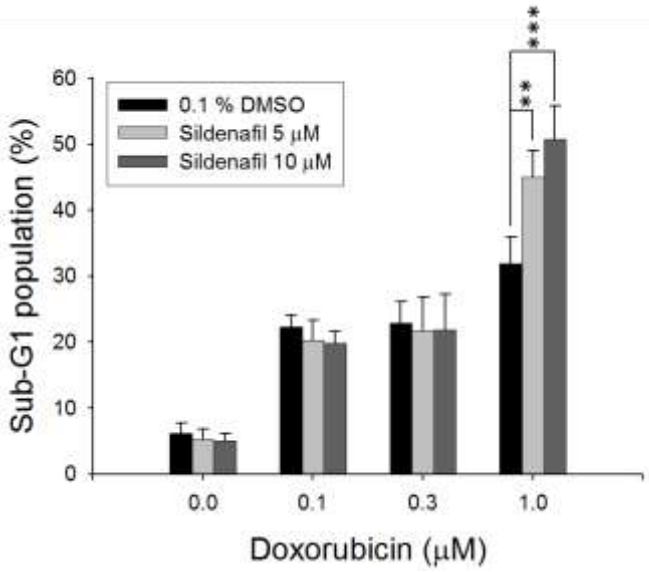

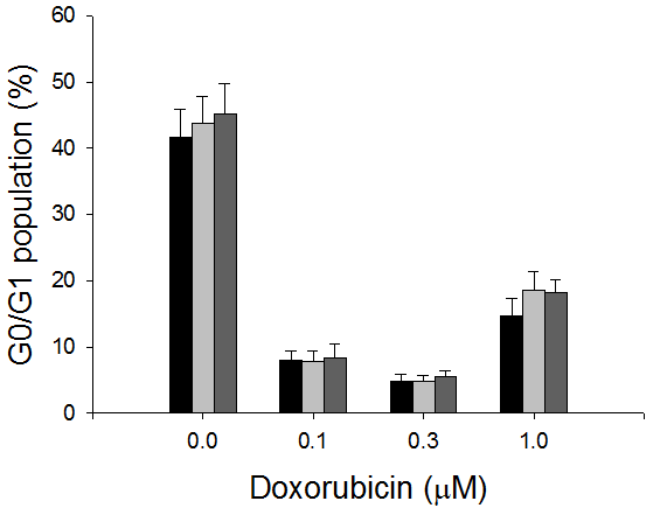

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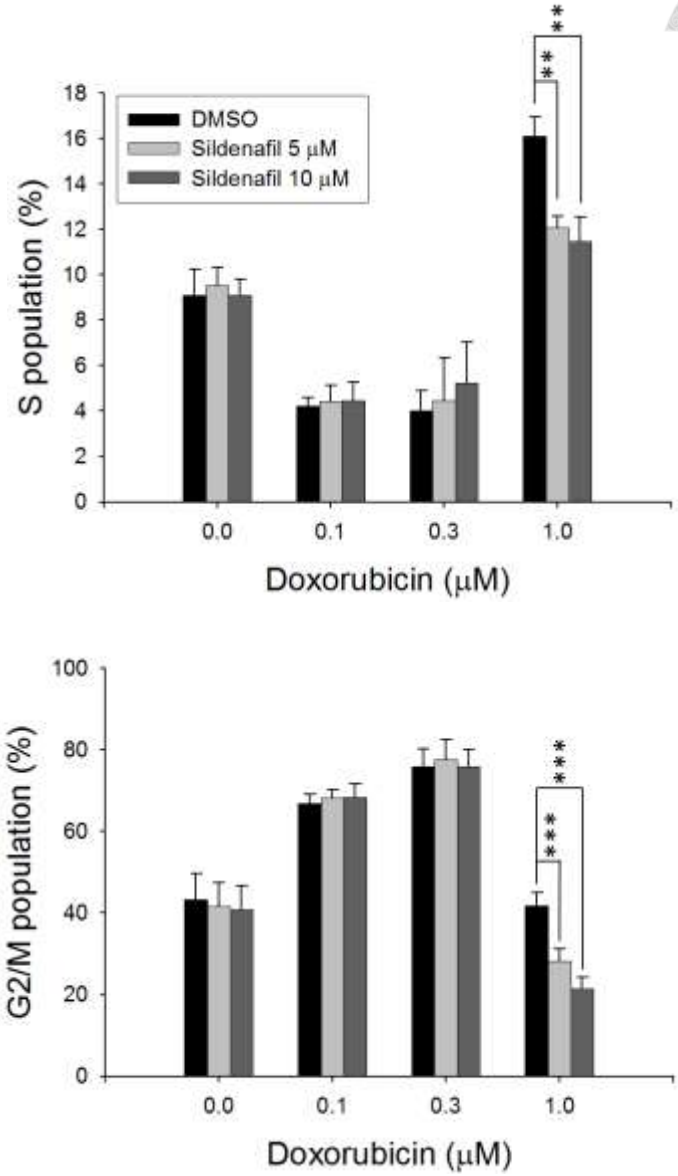

Figure 1. Effect of doxorubicin and/or sildenafil on cell cycle distribution in PC-3 cells.

PC-3 cells were treated with 0.1% DMSO, graded concentrations of doxorubicin (0.1, 0.3 or 1 μM) or/and sildenafil (5 or 10 μM) for 48 hours. The cells were fixed with 70% ethanol and stained with propidium iodide to analyze the distribution of cell populations in cell cycle phases (sub-G1, G0/G1, S and G2/M phase) by FACScan flow cytometric analysis. The data are presented as mean±SD of three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$.

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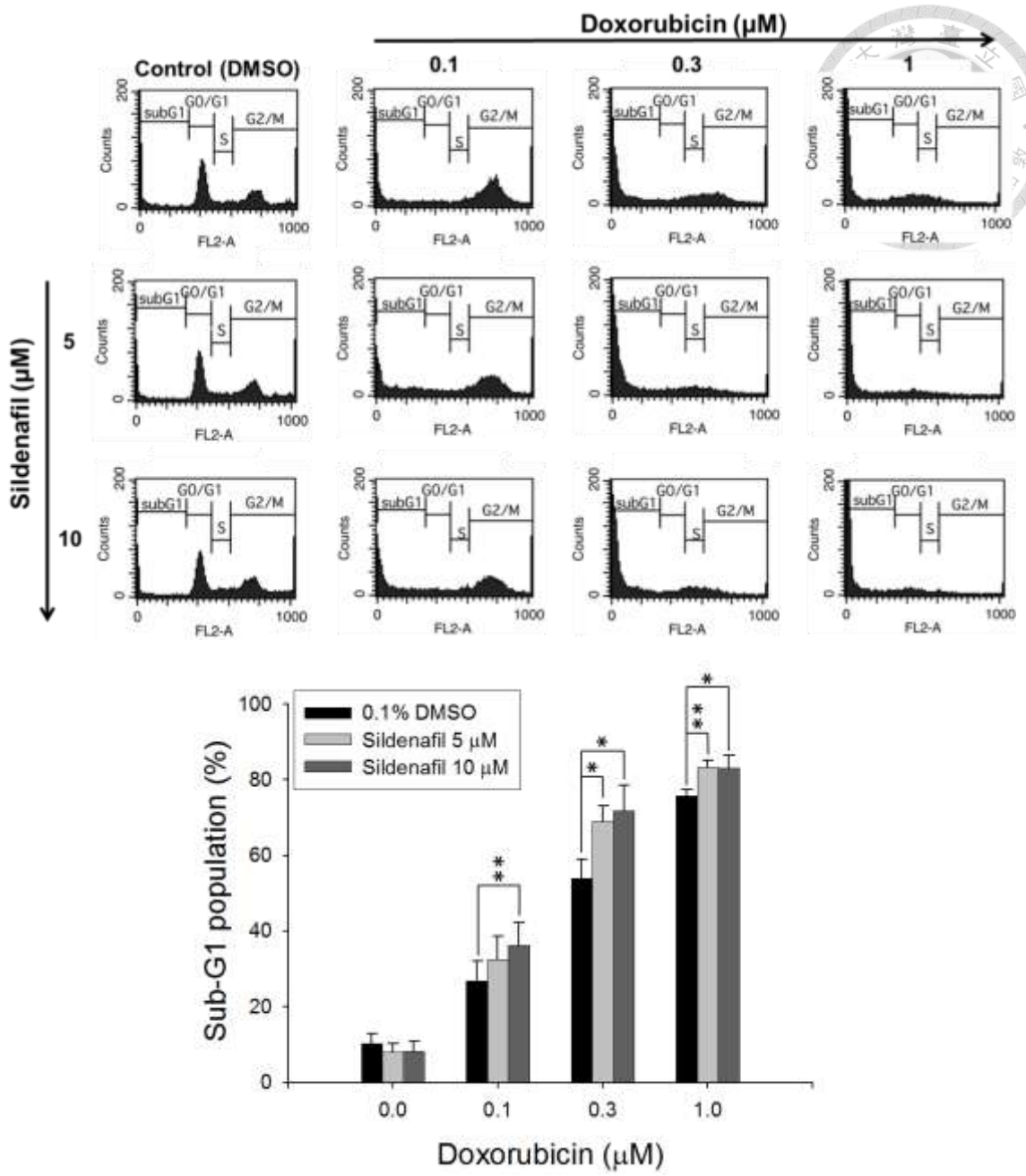

Figure 2. Effect of doxorubicin and/or sildenafil on cell cycle distribution in DU145 cells.

DU145 cells were treated with 0.1% DMSO, graded concentrations of doxorubicin (0.1, 0.3 or 1 μM) or/and sildenafil (5 or 10 μM) for 48 hours. The cells were fixed with 70% ethanol and stained with propidium iodide to analyze the distribution of cell populations in cell cycle phases (sub-G1, G0/G1, Sand G2/M phase) by FACScan flow cytometric analysis. The data are presented as mean±SD of three independent experiments. *p < 0.05 and **p < 0.01.

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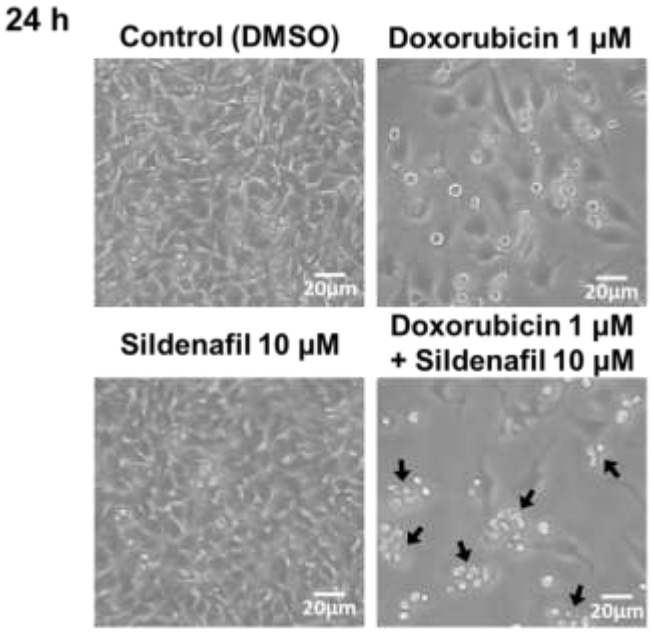

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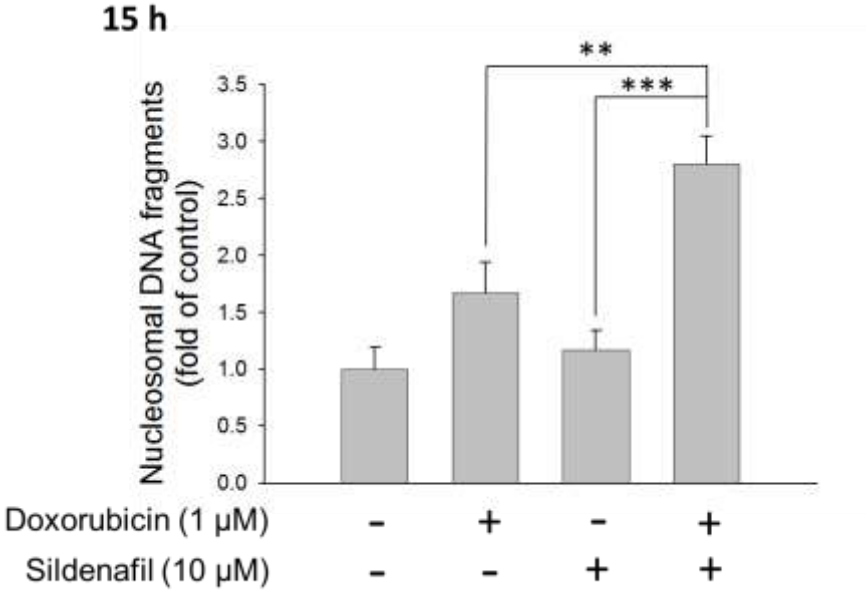

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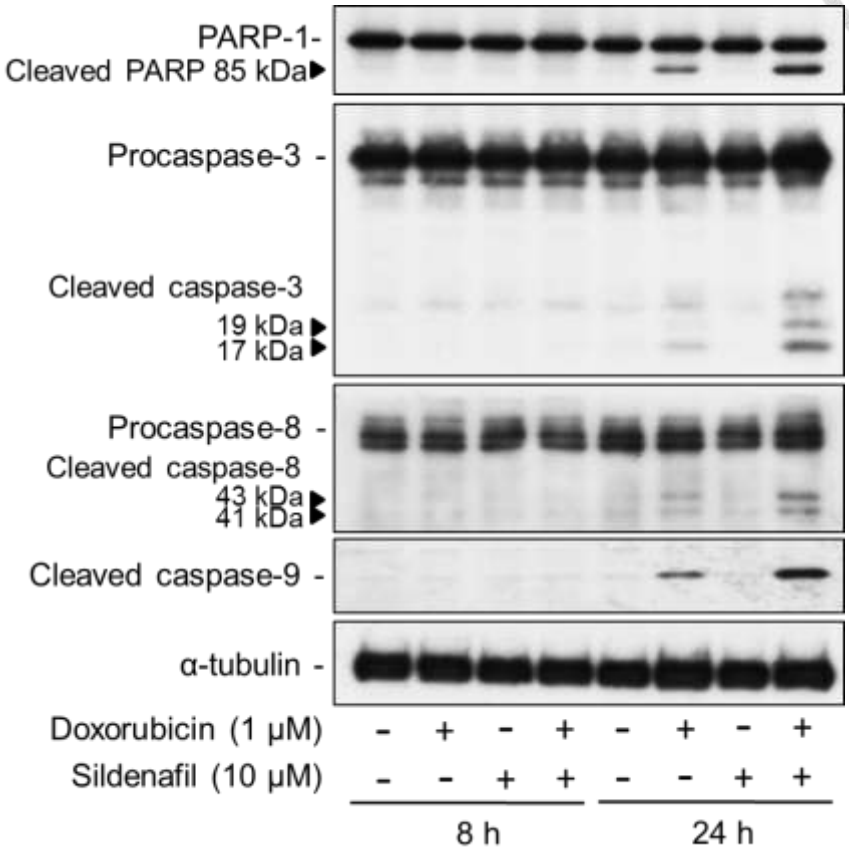

Figure 3. Effect of sildenafil on doxorubicin-induced apoptosis in PC-3 cells.

PC-3 cells were treated with 0.1% DMSO, doxorubicin (1 μM) or/and sildenafil (10 μM) for indicated hours. Changes in cell morphology were observed by microscopic examination (A). *Scale bar*, 20 μm. Arrows, apoptotic cells. (B) Cell Death Detection ELISA^{PLUS} kit was employed to detect apoptotic cells through measuring the level of nucleosomal DNA fragments. The data are presented as mean±SD of three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$. (C) The expressions of apoptotic markers including caspases-3, -8 and-9 and PARP-1 were detected by Western blot.

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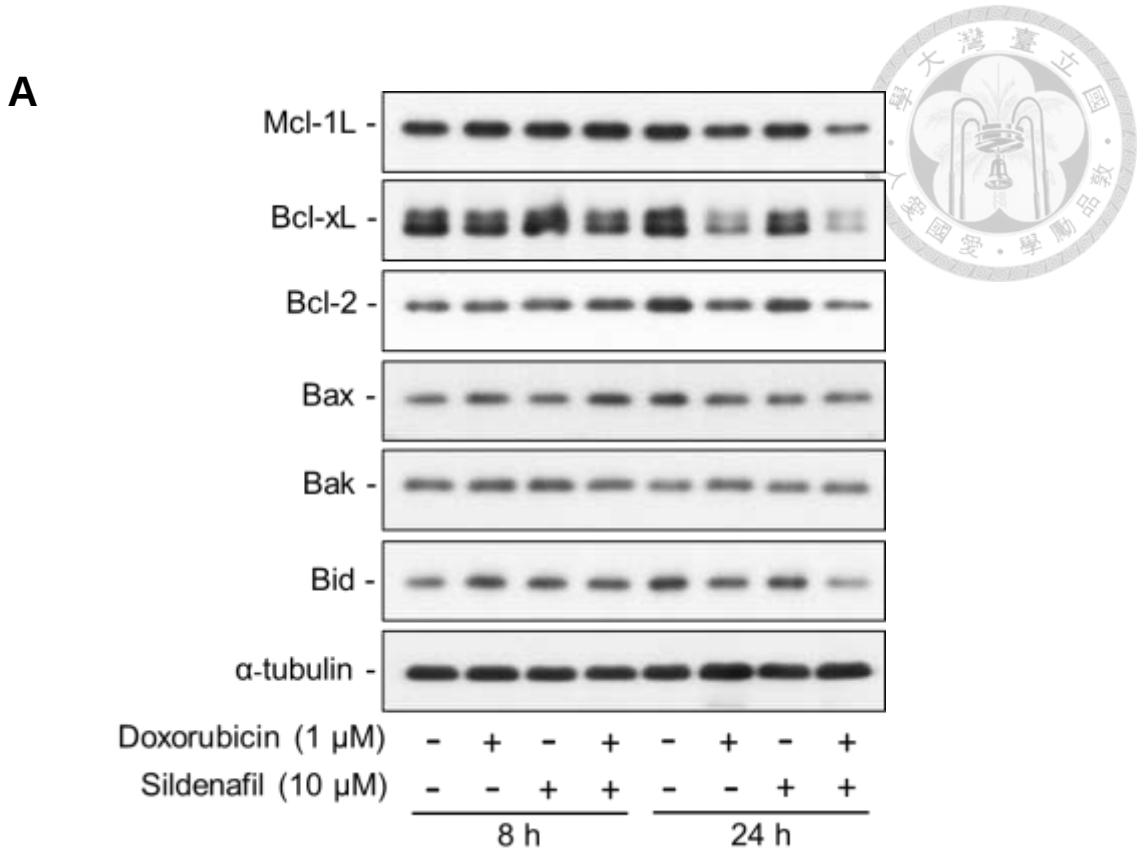

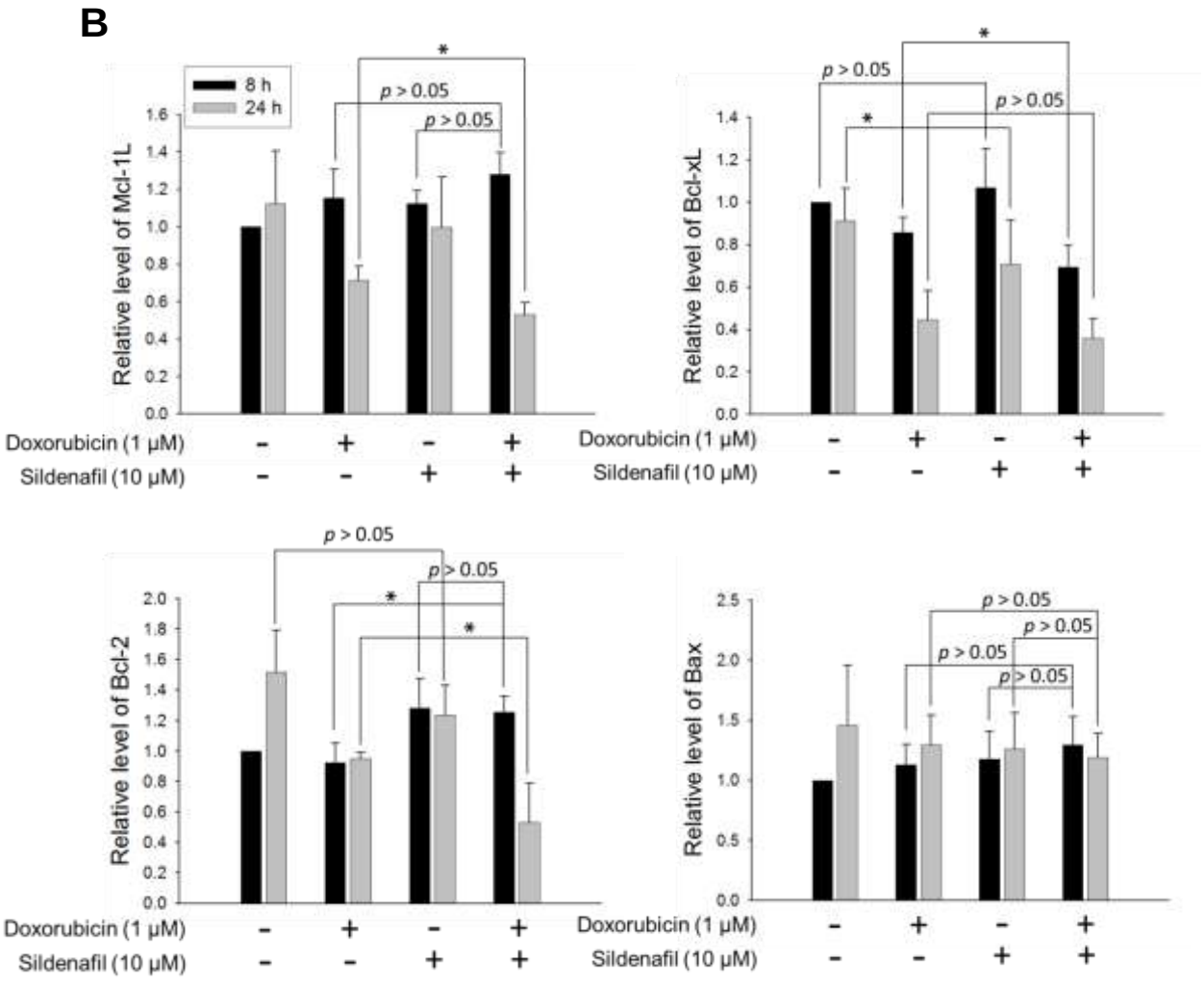

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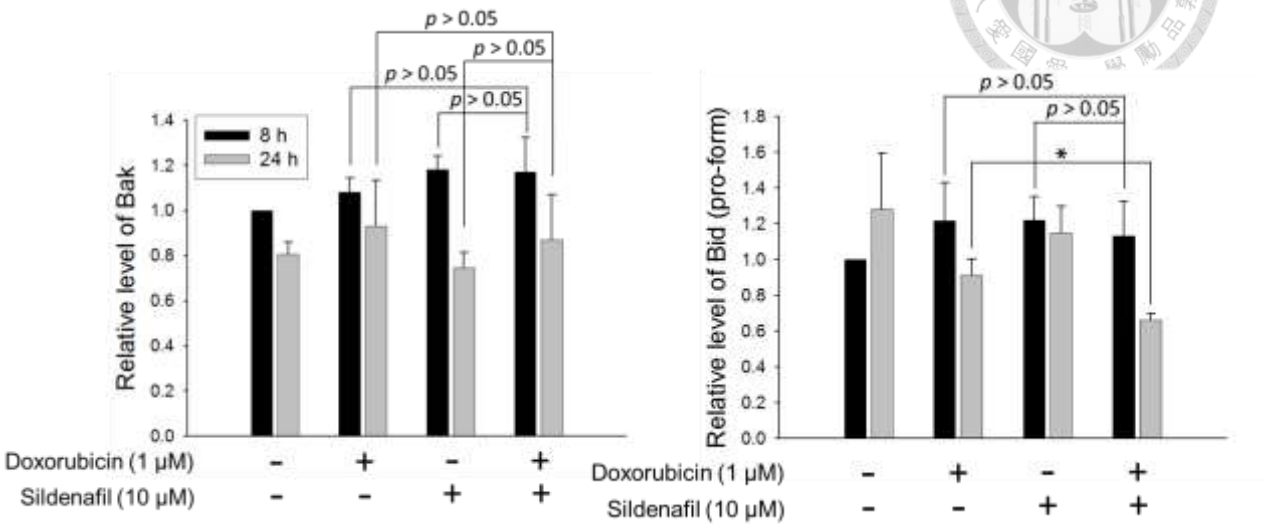

Figure 4. Effect of doxorubicin and/or sildenafil on the expression of Bcl-2 family in PC-3 cells.

PC-3 cells were treated with 0.1% DMSO, doxorubicin (1 μM) or/and sildenafil (10 μM) for 8 or 24 hours. (A) The expression of several members in Bcl-2 family was detected by Western blot. (B) The relative protein levels were quantified using the Bio-Rad Quantity One software with α-tubulin as the internal control. The data are presented as mean±SD of three to four independent experiments and normalized to the protein level of the control group (8 hours). *$p < 0.05$.

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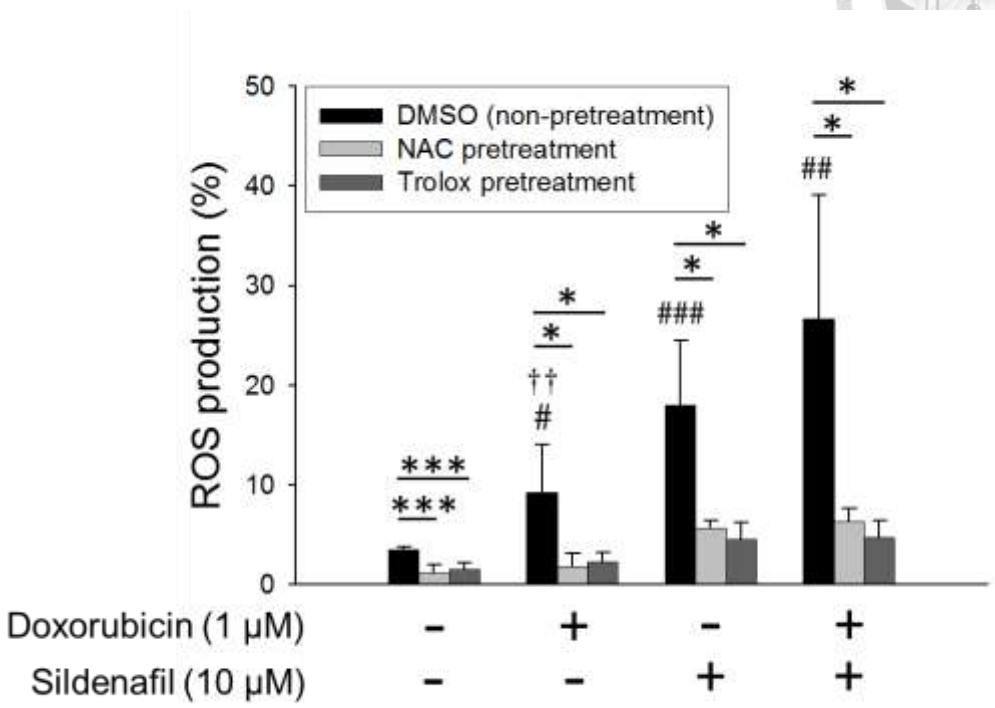

Figure 5. Effect of doxorubicin and/or sildenafil on ROS production in PC-3 cells.

PC-3 cells were pretreated with 0.1% DMSO (non-pretreatment group), NAC (1 mM) or trolox (0.3 mM) for 30 min, and then co-incubated with doxorubicin (1 μM) or/and sildenafil (10 μM) for 3 hours. Before the termination of incubation, cells were incubated with 10 μM DCFH-DA for 30 min to probe the intracellular ROS. ROS production (%) was detected using FACScan flow cytometry by measuring the percentage of DCF fluorescence-positive cells in total cells. The data are presented as mean±SD of three independent experiments. $^{*}p < 0.05$ and $^{***}p < 0.001$ *vs.* respective non-pretreatment group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ *vs.* control group without pre-treatment. $^{\dagger\dagger}p < 0.01$ *vs.* group of drug combination without pre-treatment.

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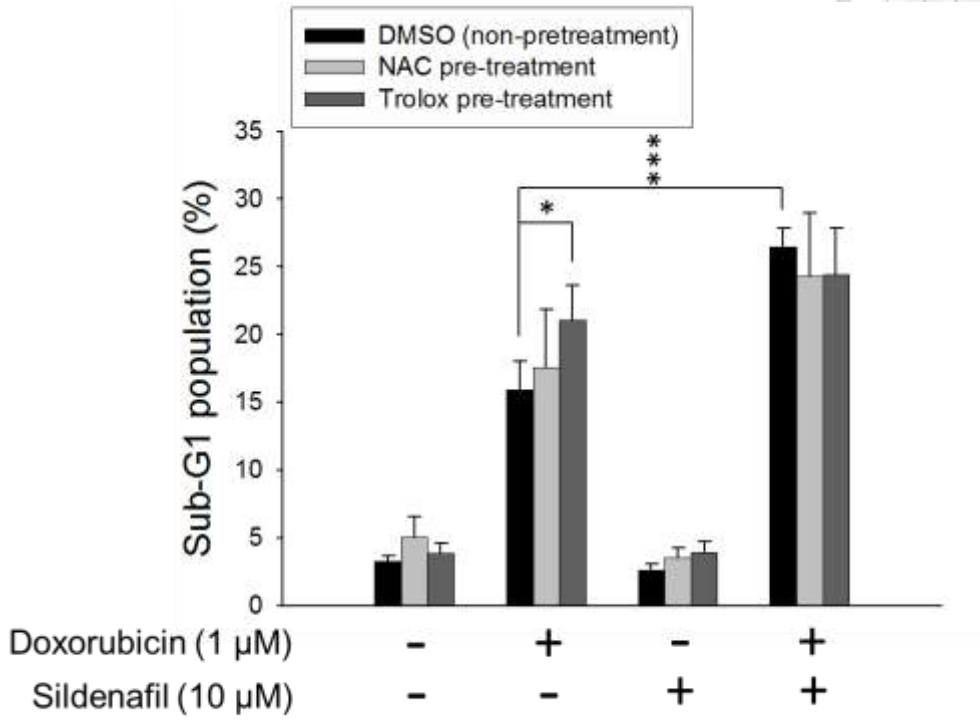

Figure 6. Effect of ROS scavengers NAC and trolox on cell apoptosis induced by doxorubicin and sildenafil.

PC-3 cells were pretreated with 0.1% DMSO (non-pretreatment group), NAC (1 mM) or trolox (0.3 mM) for 30 min, and then co-incubated with doxorubicin (1 μM) or/and sildenafil (10 μM) for 48 h. After the indicated treatment, cells were fixed with 70% ethanol and stained with propidium iodide to analyze the sub-G1 population (apoptotic cells) by FACScan flow cytometric analysis. The data are presented as mean±SD of three independent experiments.*$P < 0.05$ and ***$P < 0.001$.

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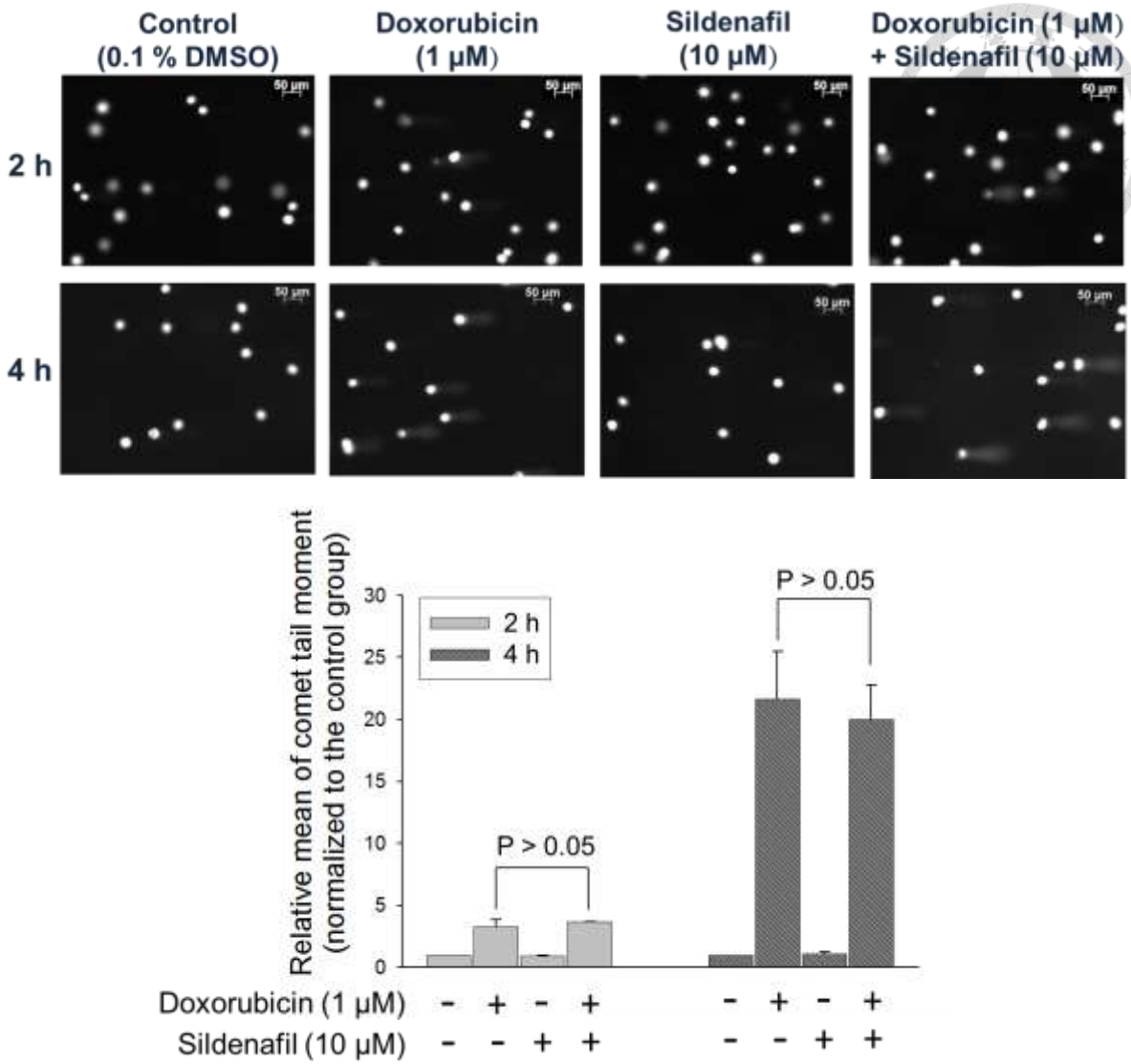

Figure 7. Effect of doxorubicin and/or sildenafil on the integrity of chromosomal DNA in PC-3 cells.

PC-3 cells treated with doxorubicin (1 μM) or/and sildenafil (10 μM) for 2 or 4 hours were examined for the integrity of chromosome DNA by measuring the amount of DNA single- and double-strand breaks using alkaline comet assay. The DNA integrity in individual cells was scored by the parameter of comet tail moment (Tail moment = $\%DNA_{tail} \times Length_{tail}$) using TriTek CometScore^{TM} software, and at least 100 cells were randomly scored per sample for calculating the mean value of comet tail moment. The quantitative data are presented as the relative mean of comet tail moment (normalized to that of control group) ± SD of two (2 h) or three independent (4 h) experiments.

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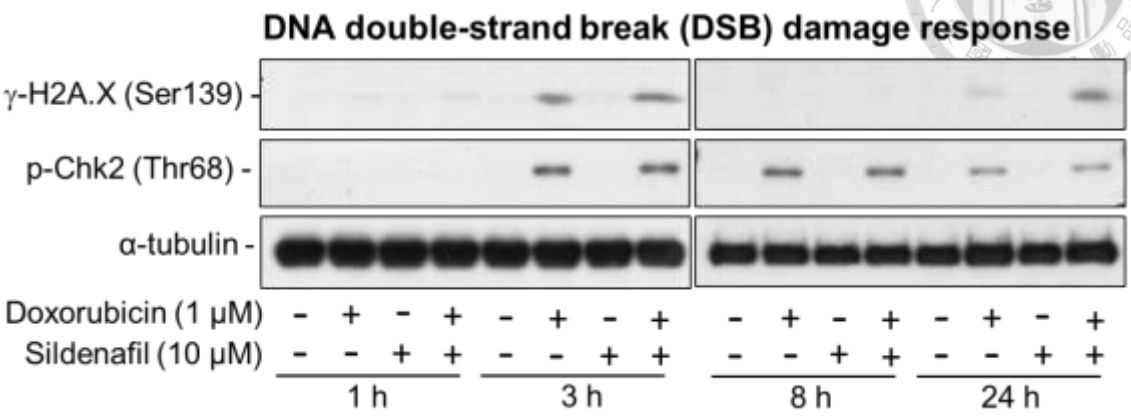

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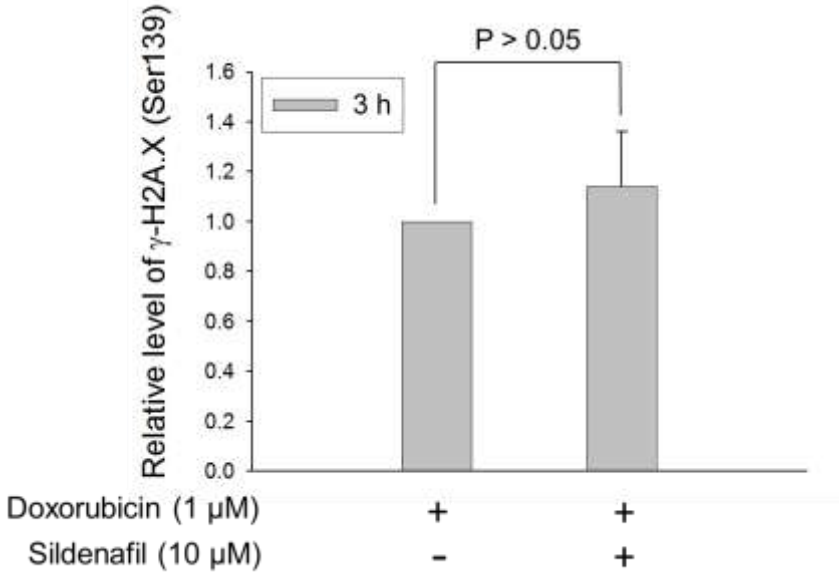

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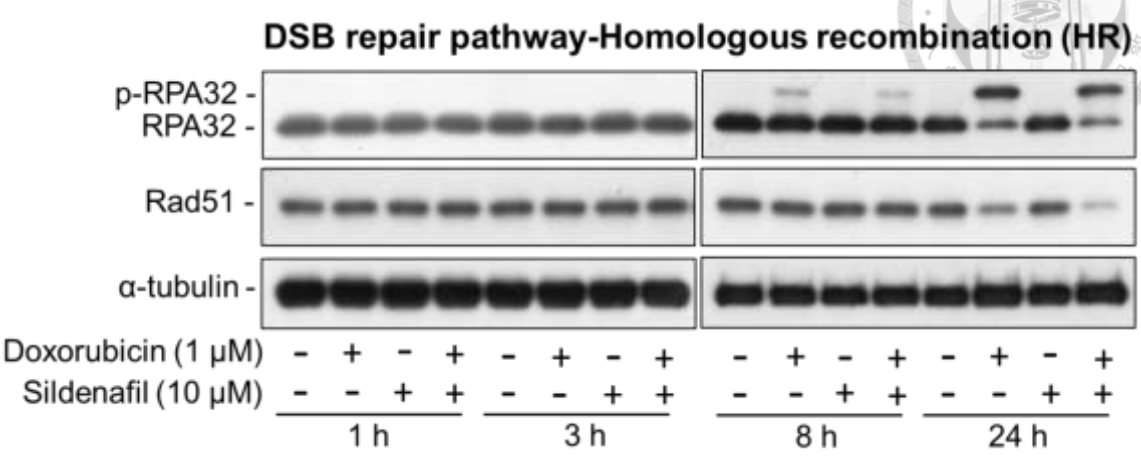

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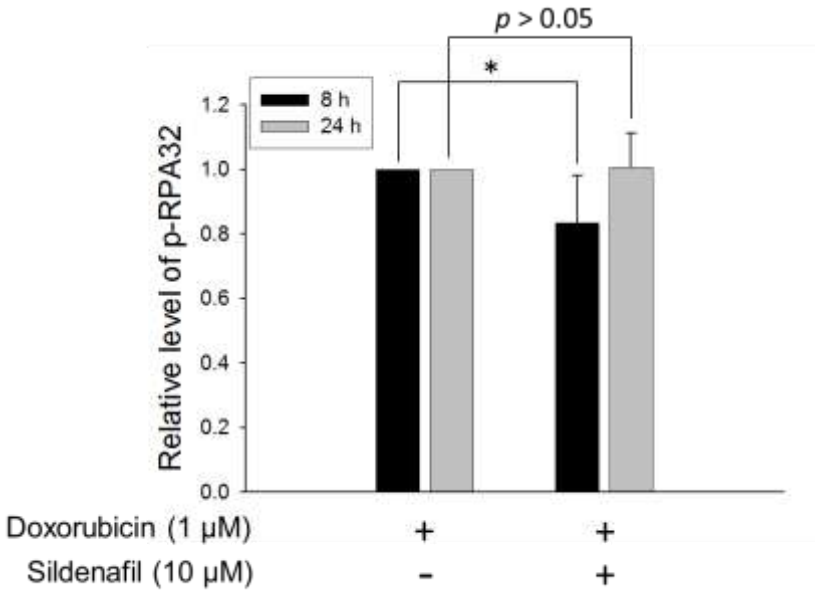

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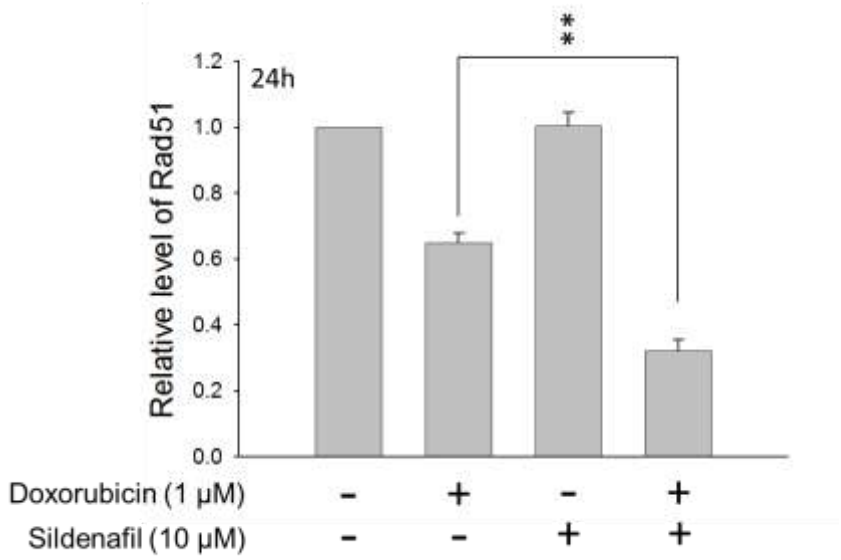

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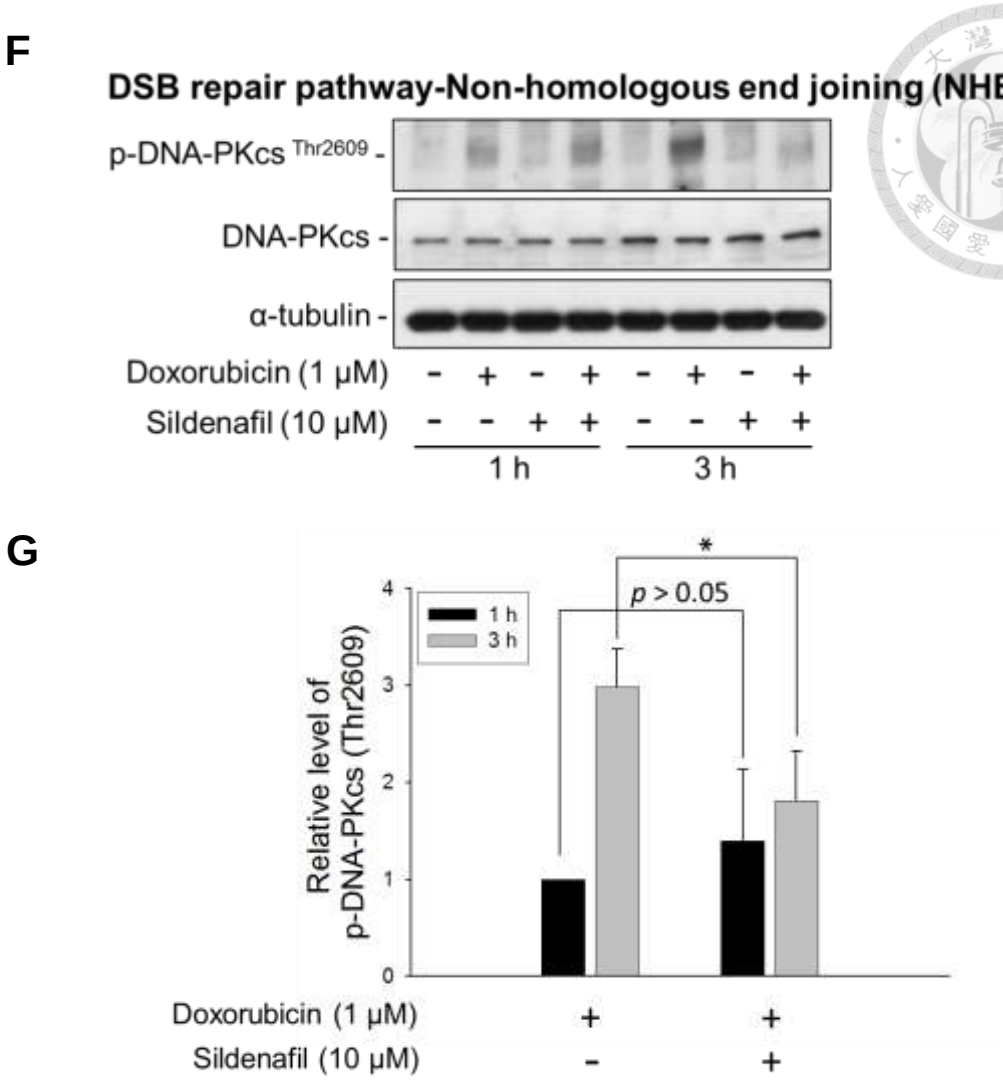

Figure 8. Effect of sildenafil on doxorubicin-induced DNA double-strand break (DSB) signaling and repair in PC-3 cells.

PC-3 cells were treated with 0.1% DMSO, doxorubicin (1 μM) or/and sildenafil (10 μM) for indicated hours. The expression of several proteins involved in DSB signaling (A) and repair pathways including homologous recombination (HR) (C), and non-homologous end joining (NHEJ) (F), was monitored by Western blot. (B, D, E and G) The relative protein levels were quantified using the Bio-Rad Quantity One software. The data are presented as mean±SD of two (for Rad51) or three independent experiments. *$p < 0.05$ and **$p < 0.01$.

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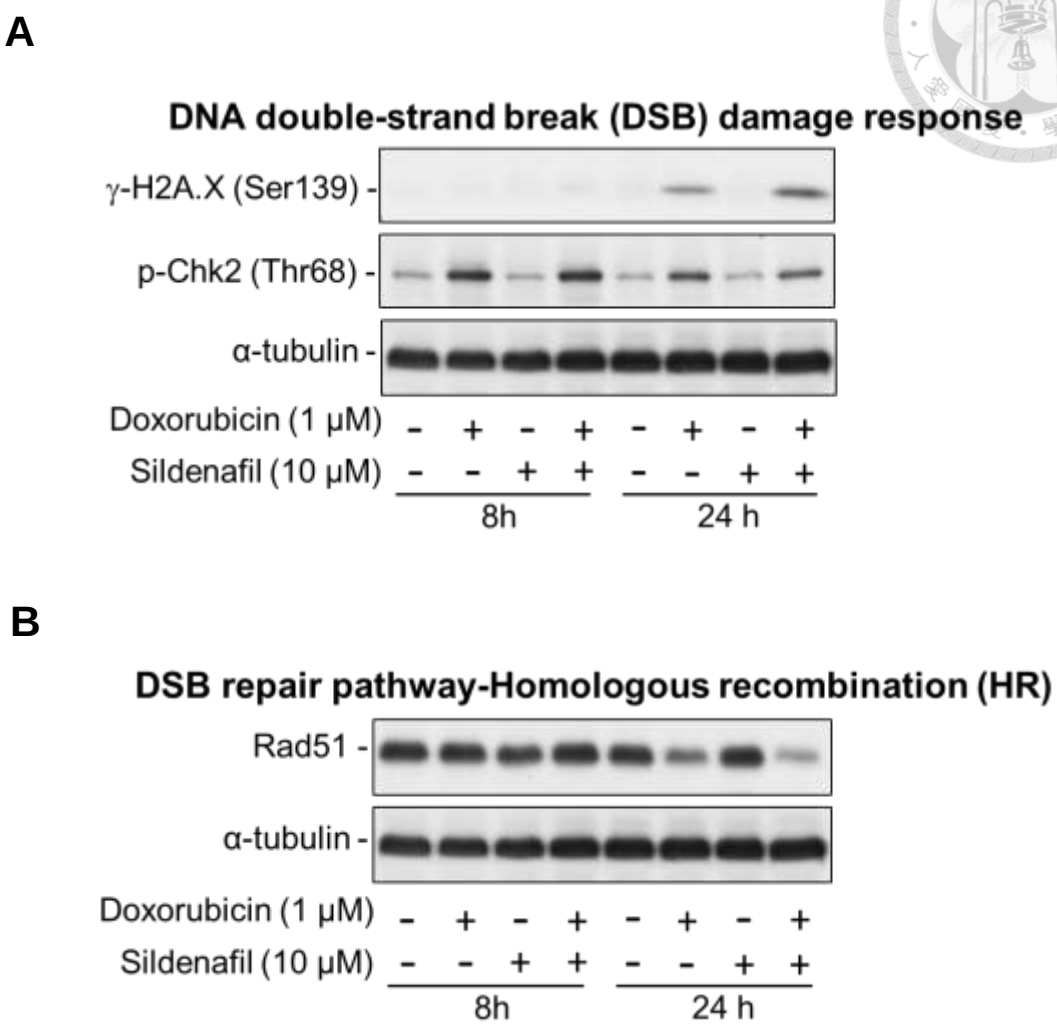

Figure 9. Effect of sildenafil on doxorubicin-induced DNA double-strand break (DSB) signaling and repair in DU145 cells.

DU145 cells were treated with 0.1% DMSO, doxorubicin (1 μM) or/and sildenafil (10 μM) for indicated hours. The expression of several proteins involved in DSB signaling (A) and repair (B) was monitored by Western blot.

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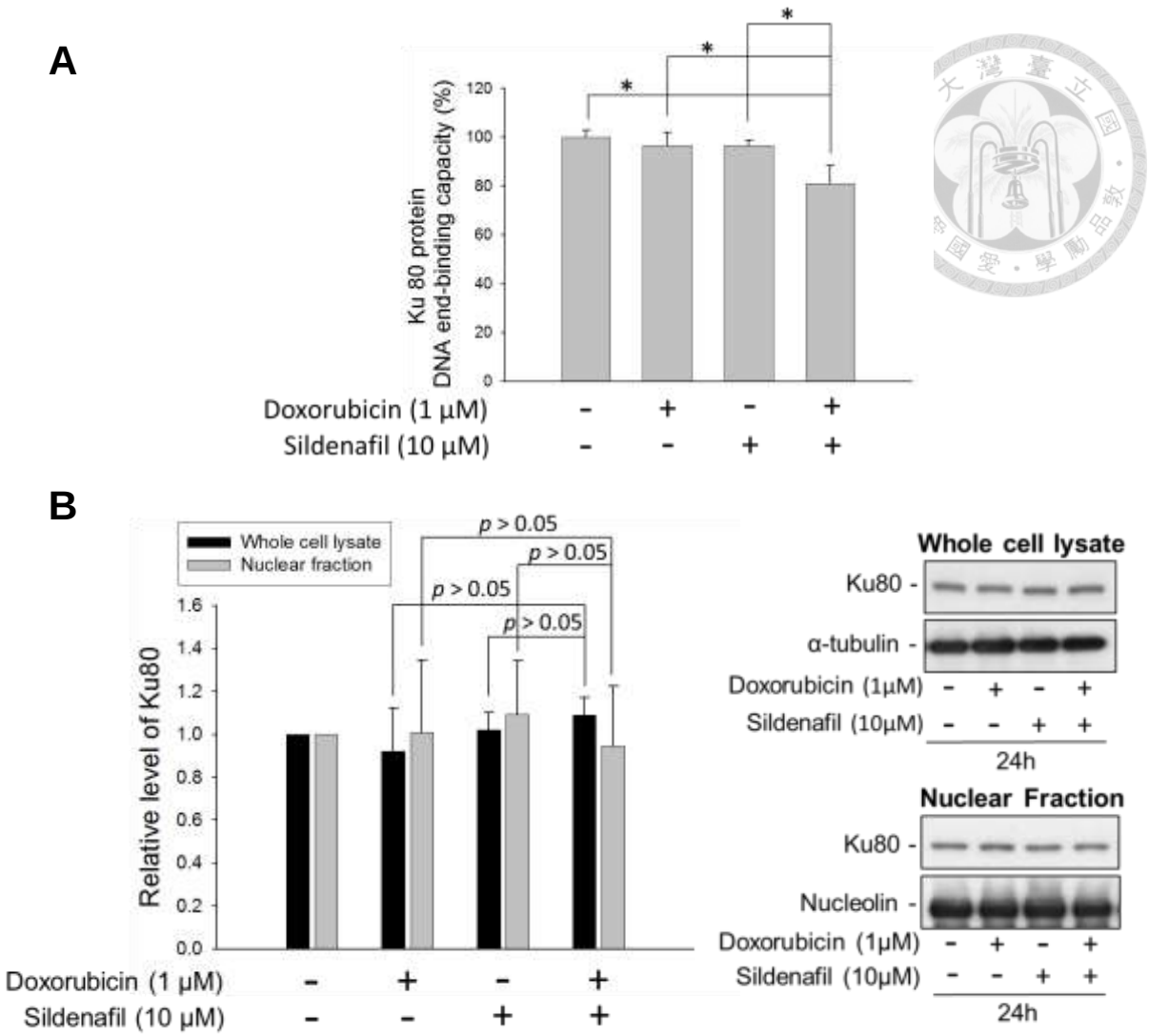

Figure 10. Effect of doxorubicin and/or sildenafil on DNA end-binding capacity, total and nuclear expression of Ku80 in PC-3 cells.

PC-3 cells treated with doxorubicin (1 μM) or/and sildenafil (10 μM) for 24 h were harvested, and whole cell lysates and nuclear fractions were then collected separately. (A) The DNA end-binding activity of Ku80 contained in nuclear fractions was assayed using a Ku70/ Ku86 DNA Repair kit. The data are presented as mean±SD of three independent experiments. *$p < 0.05$. (B) The expression of Ku80 in whole cell lysates and nuclear fractions was respectively detected by Western blot and quantified by Bio-Rad Quantity One software. Nucleolin was used as an internal control of nuclear proteins. The quantitative data are presented as mean±SD of two or three independent experiments and normalized to the protein level of the control group.

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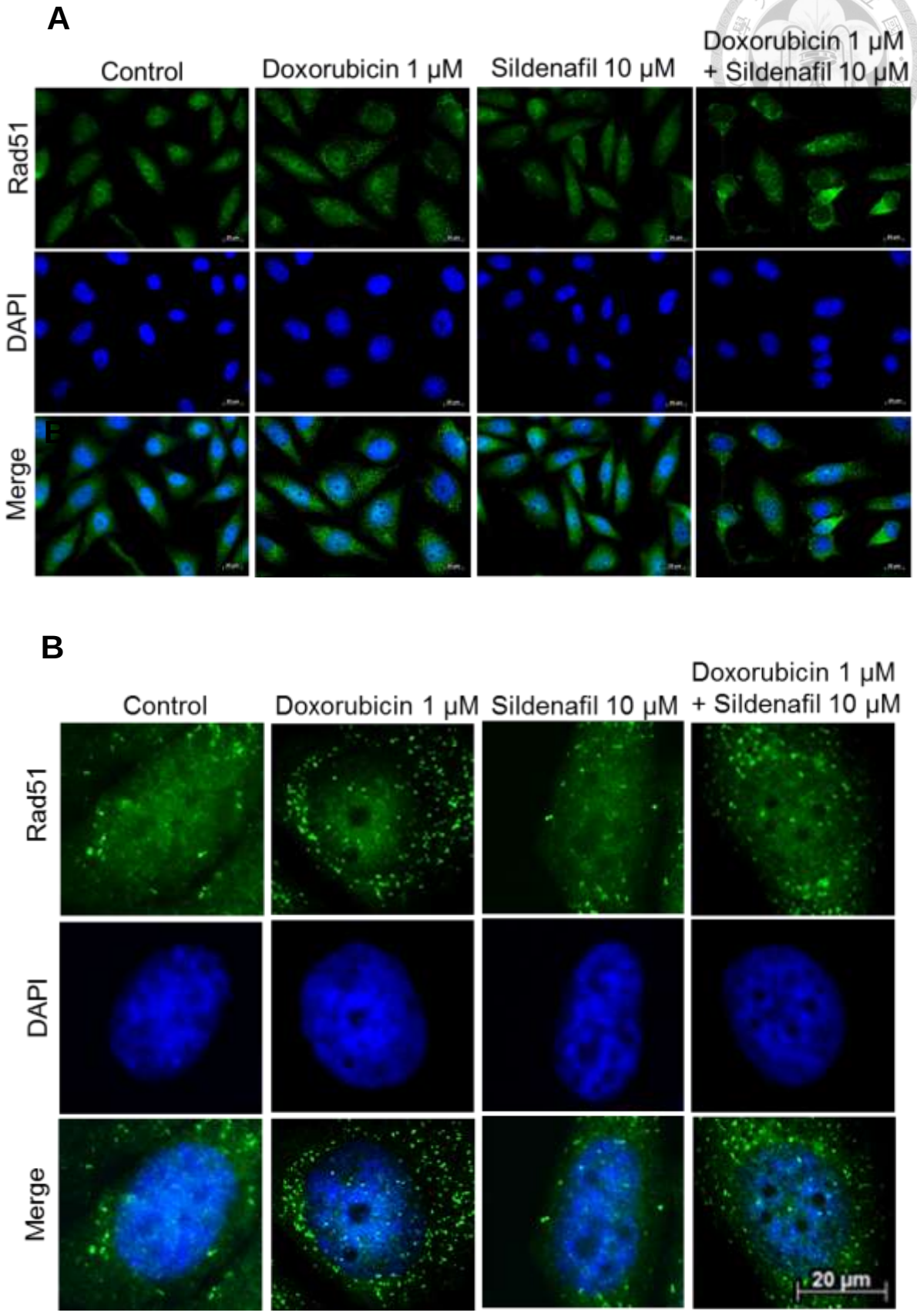

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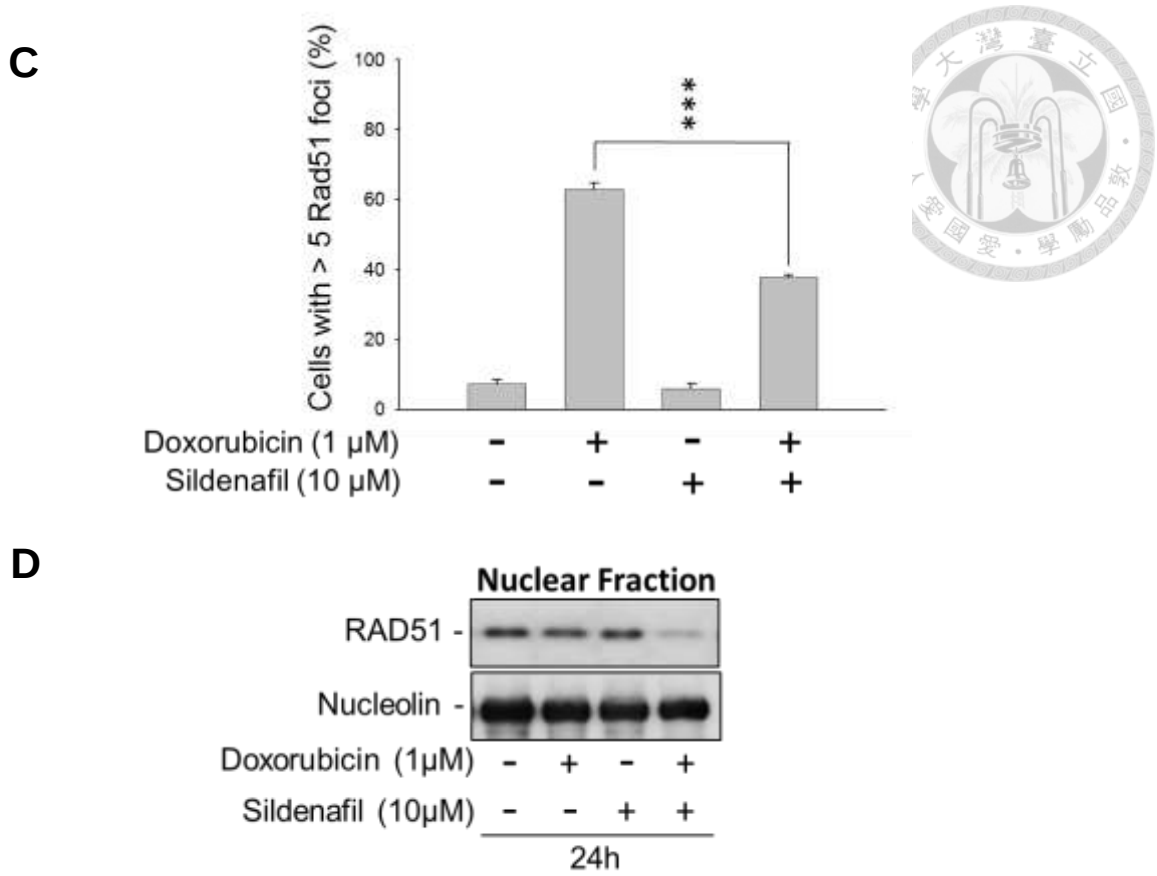

Figure 11. Effect of doxorubicin and/or sildenafil on the nuclear foci formation and expression of Rad51 in PC-3 cells.

(A) PC-3 cells were treated with doxorubicin (1 μM) or/and sildenafil (10 μM) for 24 hours followed by 8 hours of cell recovery in drug-free medium, and then fixed with 4 % paraformaldehyde. The formation of nuclear Rad51 foci in PC-3 cells was determined by immunofluorescence staining. Images were captured using a Zeiss AxioImager A1 fluorescent microscope with a 63x oil immersion objective equipped with a CCD camera.Green: Rad51, FITC; Blue: Nuclei, DAPI. (B) The enlarged images of typical cells selected from (A) were shown. (C) The percentage of cells containing over five Rad51 foci in each condition was estimated with minimum of 100 cells counted. The data are presented as mean±SD of three independent experiments. ***$p <$ 0.001. (D) The expression of nuclear Rad51 in PC-3 cells treated with indicated drug for 24 h was evaluated by Western blot. Nucleolin was used as an internal control of nuclear proteins.

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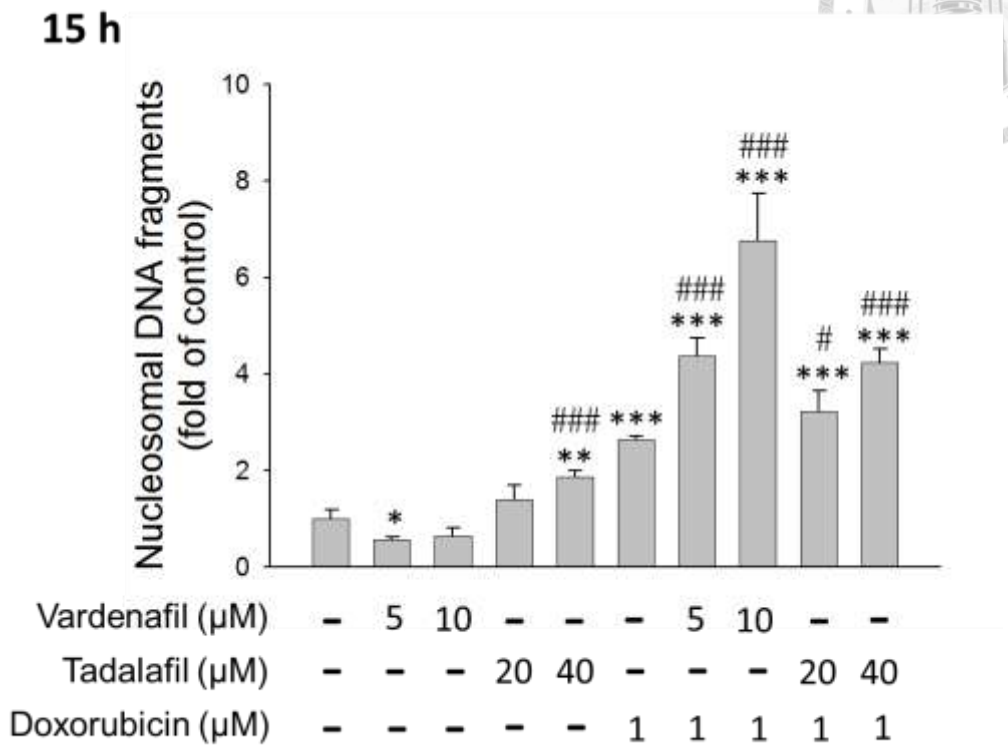

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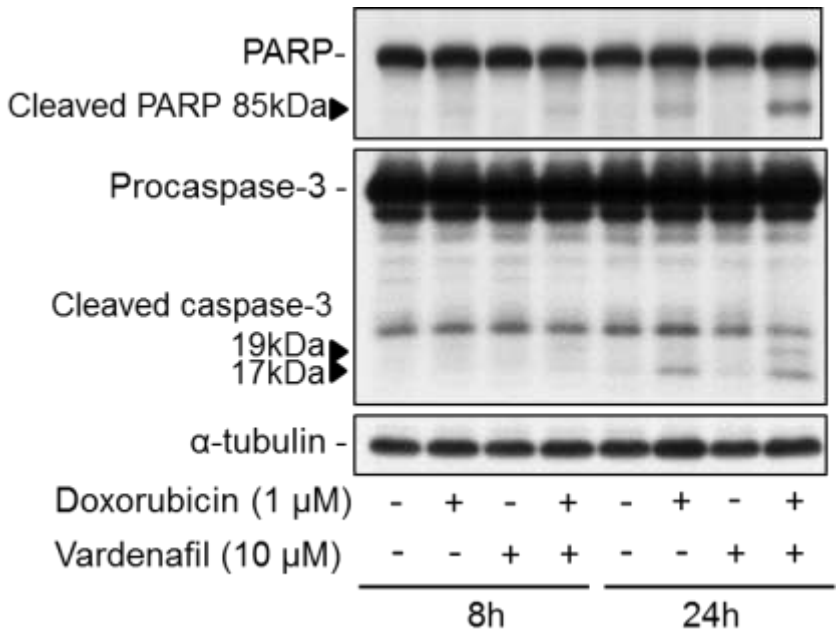

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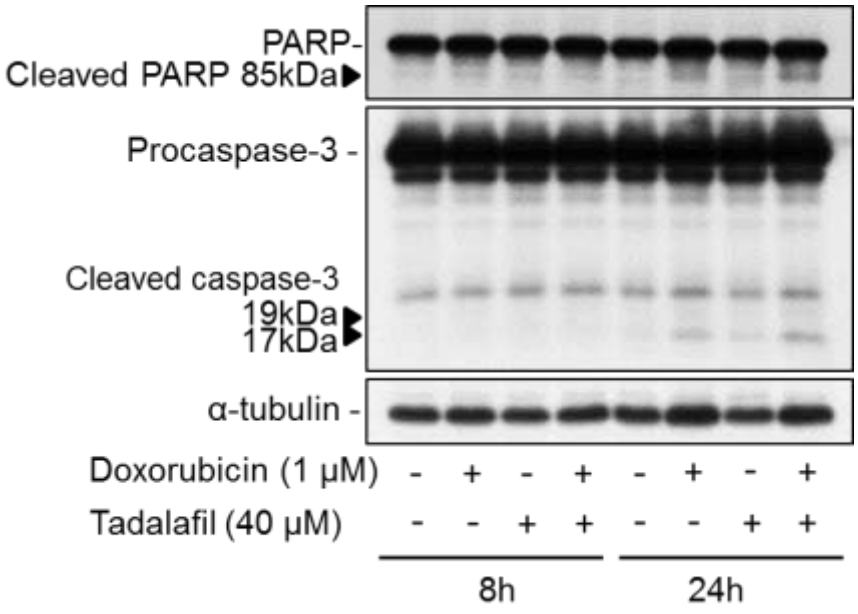

Figure 12. Effect of other PDE5 inhibitors, vardenafil and tadalafil, on doxorubicin-induced cell apoptosis in PC-3cells.

PC-3 cells were treated with 0.1% DMSO, doxorubicin (1 μM) or/and other PDE5 inhibitors, vardenafil (5 or 10 μM) or tadalafil (20 or 40 μM), for indicated hours. (A) Cells were harvested and apoptotic cells were determined using Cell Death Detection ELISA^{PLUS} kit by measuring cellular level of nucleosomal DNA fragments. The data are presented as mean±SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ *vs.* control group. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ *vs.* doxorubicin group. (B, C) The expression of apoptotic markers including caspases-3 and PARP-1 was detected by Western blot.

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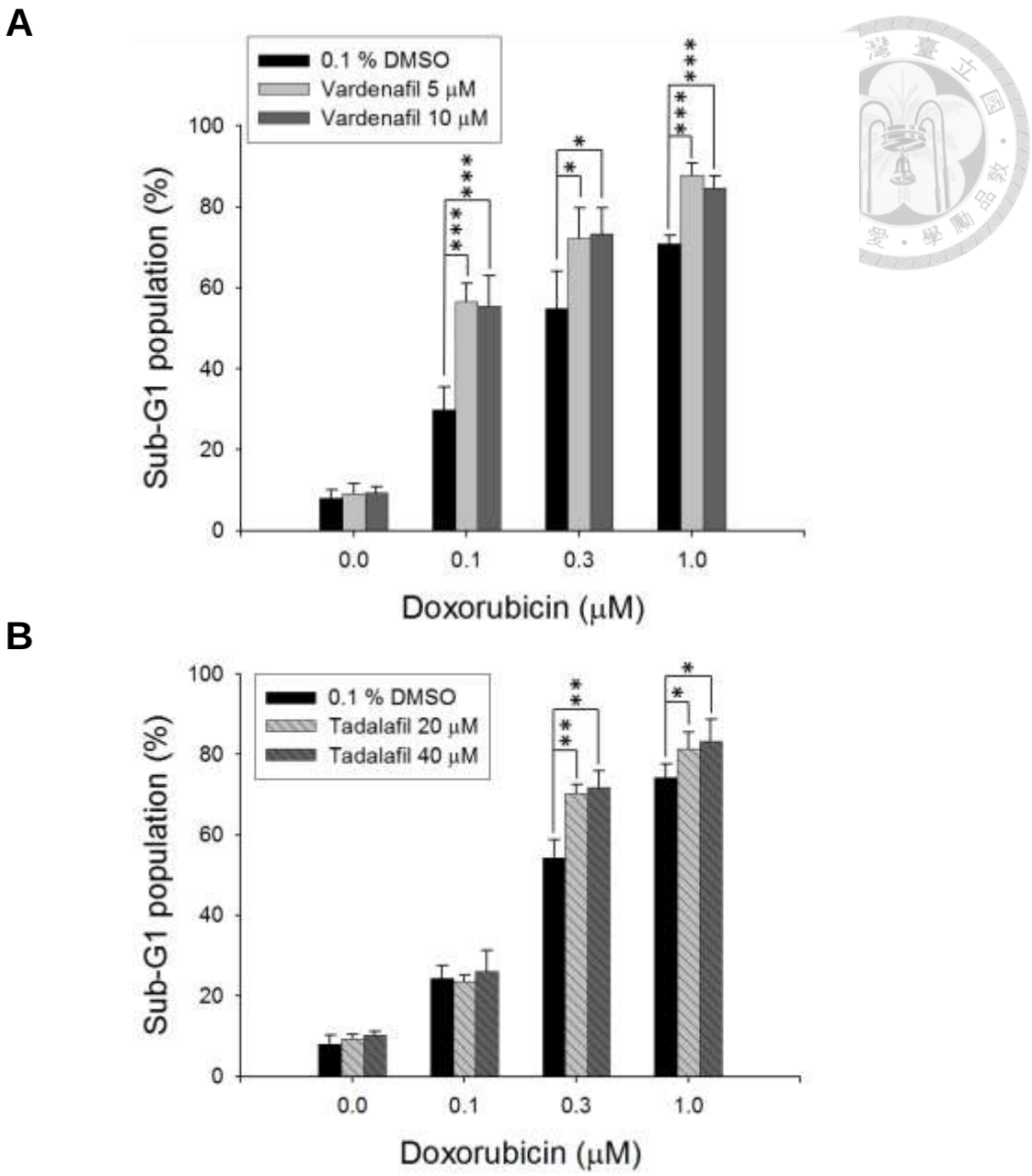

Figure 13. Effect of other PDE5 inhibitors, vardenafil and tadalafil, on doxorubicin-induced cell apoptosis in DU145 cells.

DU145 cells were treated with 0.1% DMSO, doxorubicin (1 μM) or/and other PDE5 inhibitors, vardenafil (5 or 10 μM, A) or tadalafil (20 or 40 μM, B), for 48 hours. The cells were fixed with 70% ethanol and stained with propidium iodide to determine the percentage of apoptotic cell population (sub-G1 population) using FACScan flow cytometric analysis. The data are presented as mean±SD of three independent experiments.*$p < 0.05$, **$p < 0.05$ and ***$p < 0.001$.

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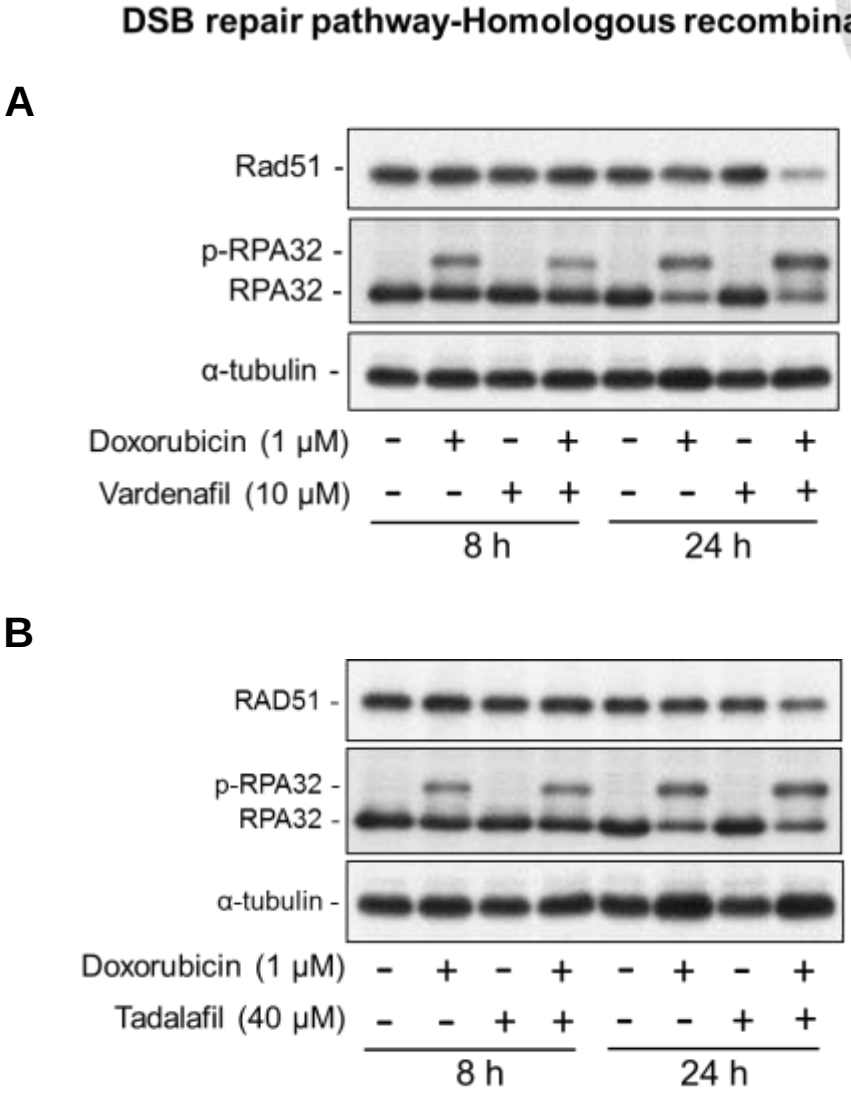

Figure 14. Effect of PDE5 inhibitors, vardenafil and tadalafil, on HR-mediated repair of DSB induced by doxorubicin in PC-3 cells.

PC-3 cells were treated with 0.1% DMSO, doxorubicin (1 μM) or/and other PDE5 inhibitors, 10 μM of vardenafil (A) or 40 μM of tadalafil (B), for indicated hours. The expression of HR-related proteins was monitored by Western blot.

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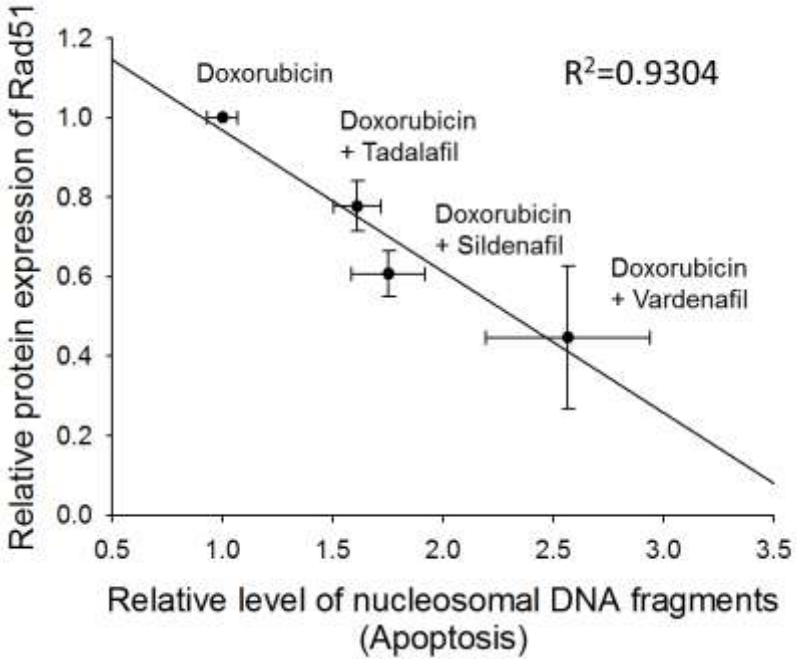

Figure 15. Correlation between Rad51 expression and the level of nucleosomal DNA fragments (apoptosis) in PC-3 cells treated with doxorubicin or in combination with different PDE5 inhibitors.

Data points are shown for 4 treatment groups in PC-3 cells, including doxorubicin (1μM) alone and its combination with three different PDE5 inhibitors (10 μM sildenafil, 10 Mm vardenafil, or 40 μM tadalafil). The x axis is the relative level of nucleosomal DNA fragments (apoptosis) detected by Cell Death Detection ELISA^{PLUS} kit between 4 treatment groups. The y axis is the relative protein expression of Rad51 monitored by Western blot between 4 treatment groups. Values of both x and y axis parameters are normalized to that in the doxorubicin alone group. The data are presented as mean±SD of two or three independent experiments. The line is a linear regression fit, with R^2 = 0.93.

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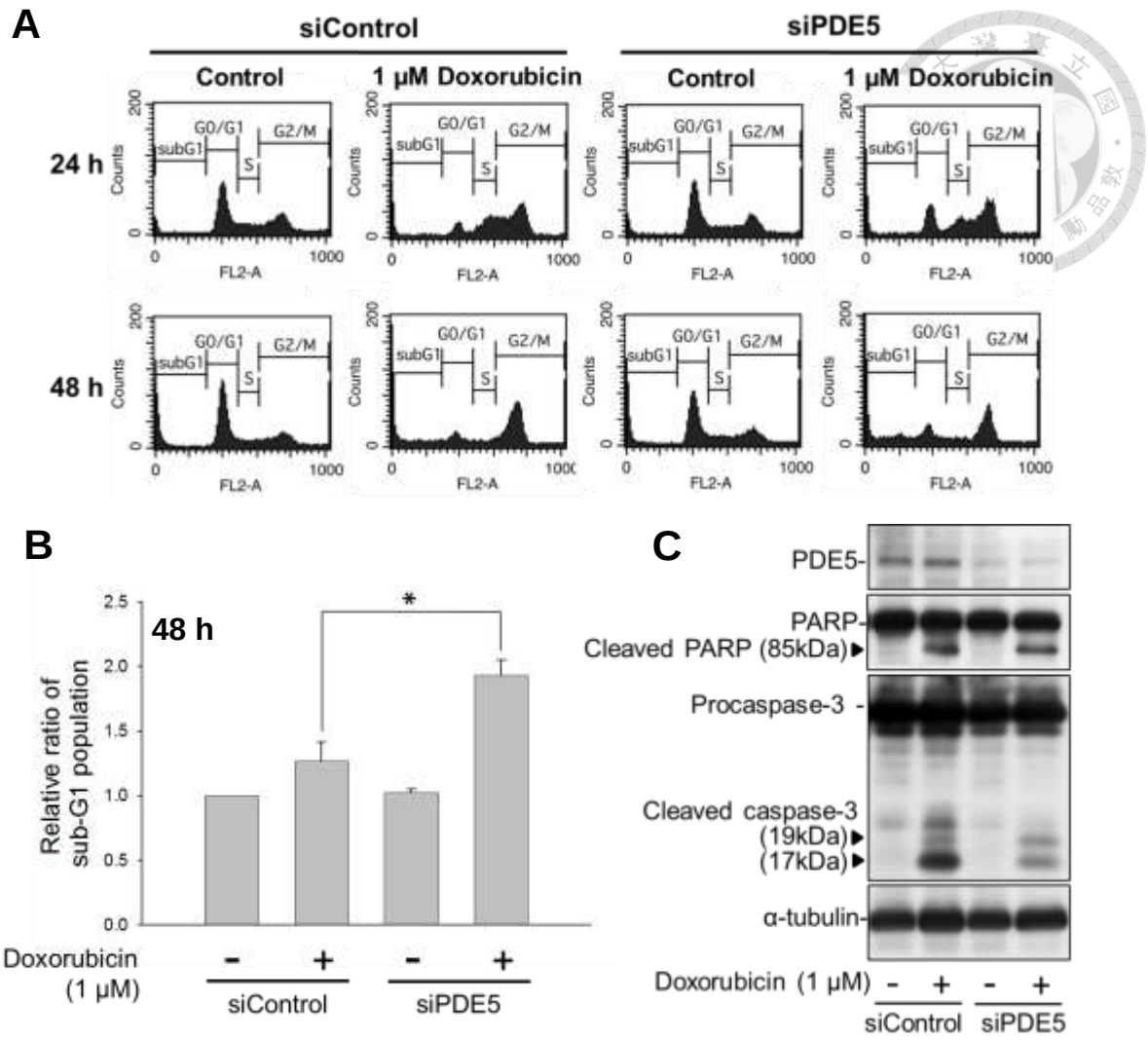

Figure 16. Effect of PDE5 knockdown on doxorubicin-induced cell death in PC-3cells.

PC-3 cells were transfected with 10 nmole control siRNA (siControl) or 25 nmole PDE5 siRNA (siPDE5) for 5 hours. After 5 hours of siRNA transfection followed by 48 hours of cell recovery in serum-containing medium, cells were treated with or without 1 μM doxorubicin for 24 or 48 hours. PC-3 cells were harvested to analyze cell cycle by PI staining and flow cytometry (A). (B) Quantitative bar-graph showed the relative ratio of sub-G1 population, normalized to that in siControl group without adding doxorubicin, between different treatment groups. The data are presented as mean±SD of two independent experiments. *$p < 0.05$. (C) Western blot was used to exam the knockdown efficiency of PDE5 and the level of cleaved caspase-3 and PARP-1.

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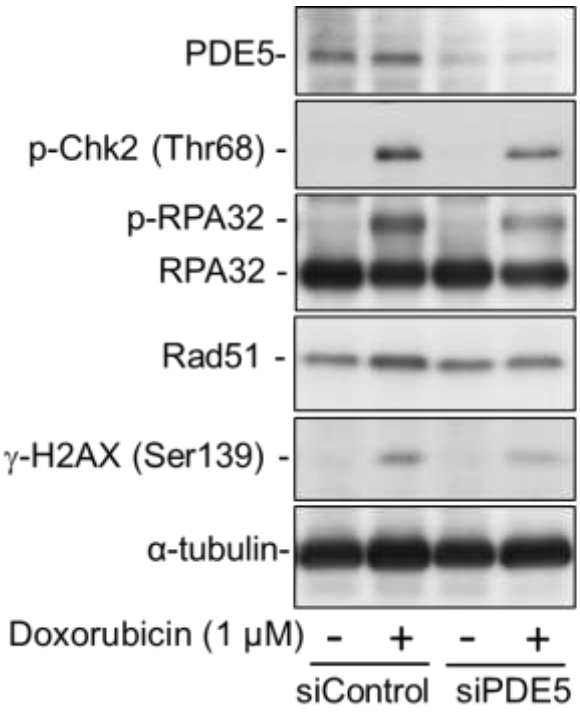

Figure 17. Effect of PDE5 knockdown on doxorubicin-induced DSB signalling and repair in PC-3 cells.

PC-3 cells transfected with 10 nmole control siRNA (siControl) or 25 nmole PDE5 siRNA (siPDE5) were treated with or without 1 μM doxorubicin for 48 hours. After treatment, cells were harvested to detect the expression of proteins involved in DSB signaling and repair using Western blot.

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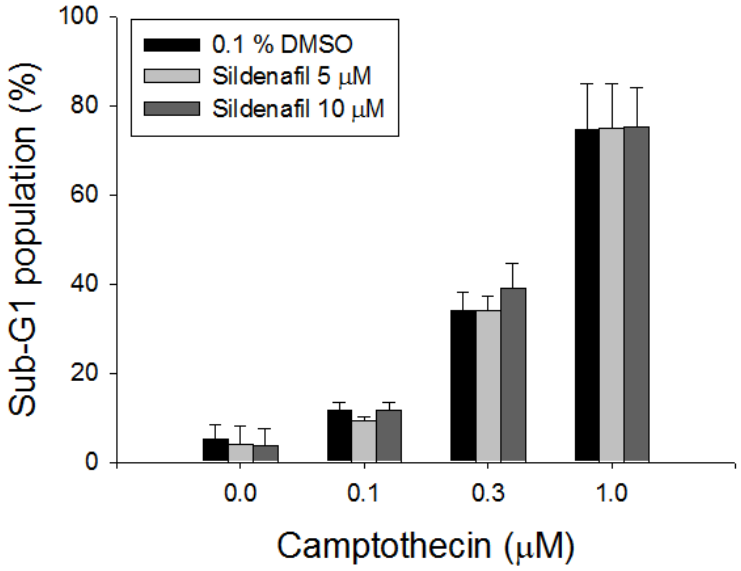
A



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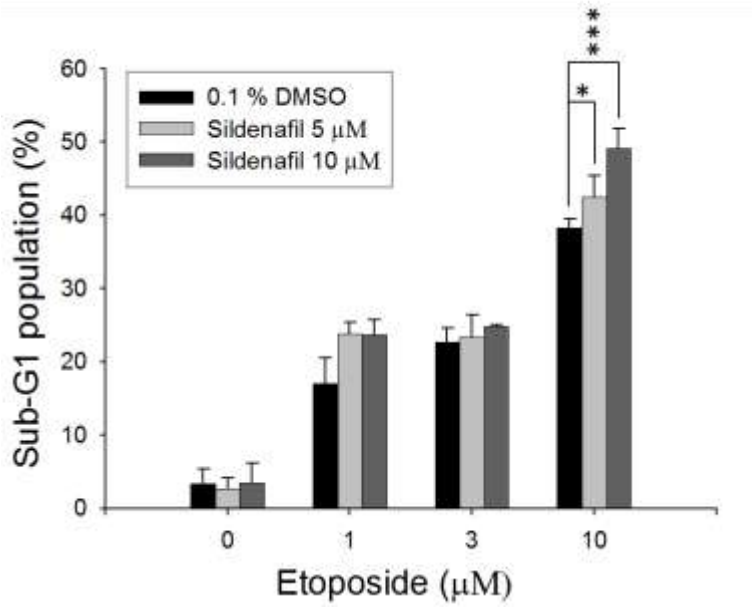

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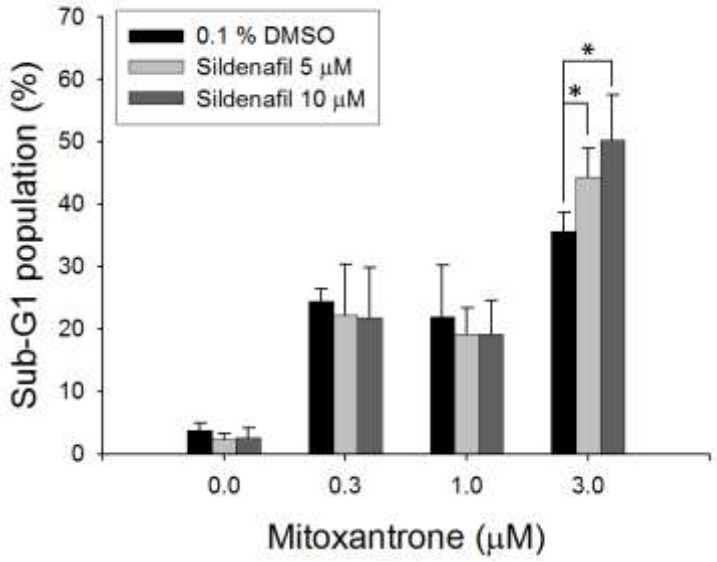

Figure 18. Effect of other topoisomerase inhibitors and/or sildenafil on cell death in PC-3 cells.

PC-3 cells were treated with 0.1% DMSO, graded concentrations of topoisomerase Ⅰ/Ⅱ inhibitor, camptothecin (A), etoposide (B) or mitoxantrone (C), or/and sildenafil (5 or 10 μM) for 48 hours. The cells were fixed with 70% ethanol and stained with propidium iodide to determine the amount of apoptotic cells by measuring sub-G1 population through FACScan flow cytometric analysis. The data are presented as mean±SD of three independent experiments. *$p < 0.05$ and ***$p < 0.001$.

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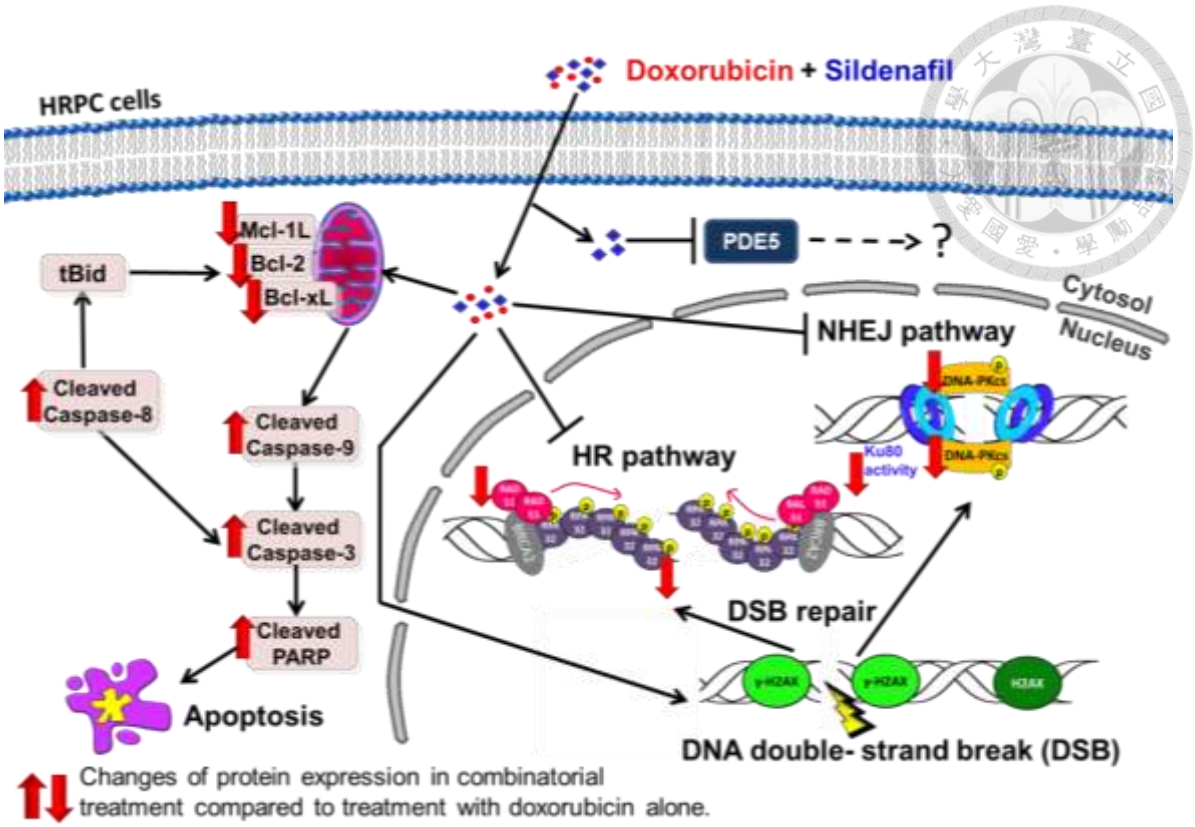

Figure 19. A schematic of how sildenafil sensitizes HRPC cells to chemotherapy drug doxorubicin.

Sildenafil does not increase DNA double-strand breaks (DSB) induced by doxorubicin, but it significantly impairs DSB repairing pathways including homologous recombination (HR) and non-homologous end joining (NHEJ). In addition, inhibition of PDE5 activity or knockdown of PDE5 is suggested to potentiate doxorubicin-induced killing of HRPC cells. However, the role of PDE5 in DSB repair still remains unclear.

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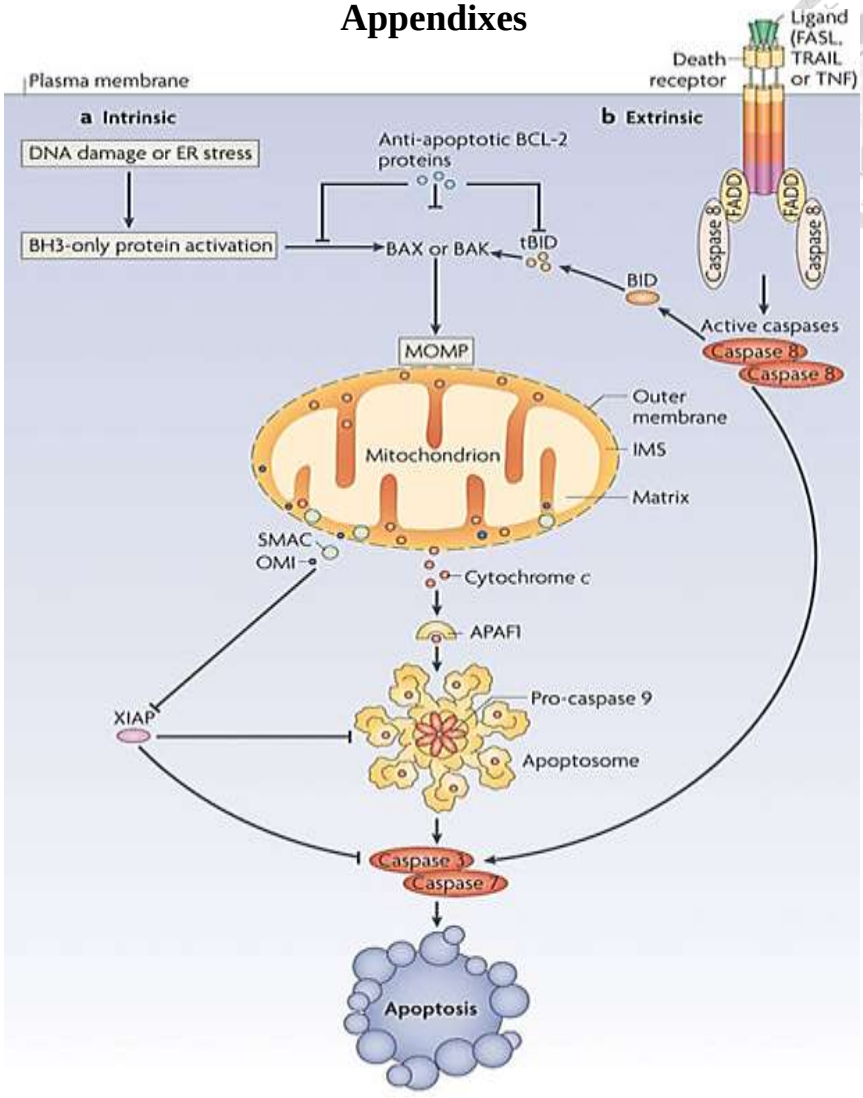
Appendixes



Appendix 1. Intrinsic and extrinsic apoptotic pathways.

The extrinsic pathway is initiated by the trimerization of death receptors upon binding with their ligands, which is followed by death-inducing signaling complex (DISC) formation with the activation of caspase-8. The activated caspase-8 can directly cleave procaspase-3 to result in apoptosis, or it can cleave Bid to truncated Bid for crosstalk between intrinsic apoptotic pathway. For the intrinsic apoptotic pathway, after the apoptotic stimuli, the mitochondrial outer membrane permeabilization occurs due to the dysregulation of Bcl-2 protein family, leading to cytochrome c release, apoptosome formation and then caspase-9 activation.

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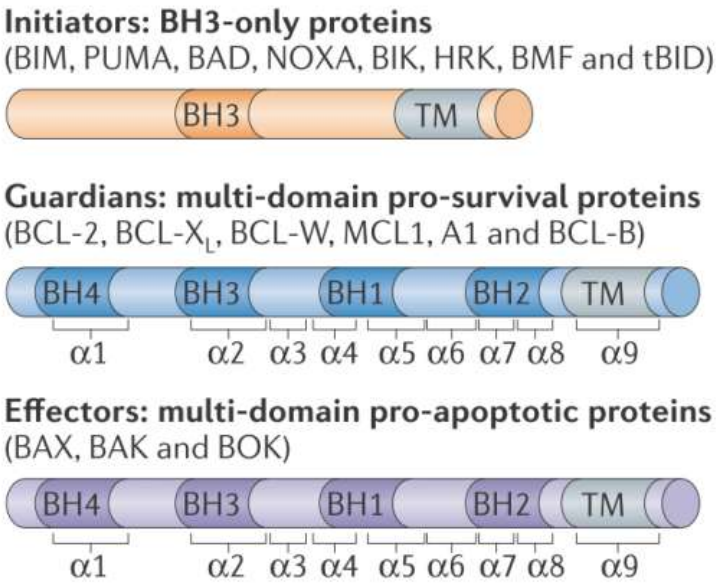

Appendix 2. Classification of Bcl-2 family

Bcl-2 family proteins are subdivided into three groups based their structural and functional similarities. Group I is multi-domain (BH1-BH4) anti-apoptotic proteins including Bcl-2, Bcl-xL, Mcl-1, Bcl-w, Bcl-b and A1. Groups Ⅱ is multi-domain (BH1-BH4) pro-apoptotic proteins, including Bax, Bak and Bok. Groups Ⅲ is BH-3 only proteins, containing Bim, Bad, tBid, PUMA, NOXA, Bik, Bmf and Hrk.

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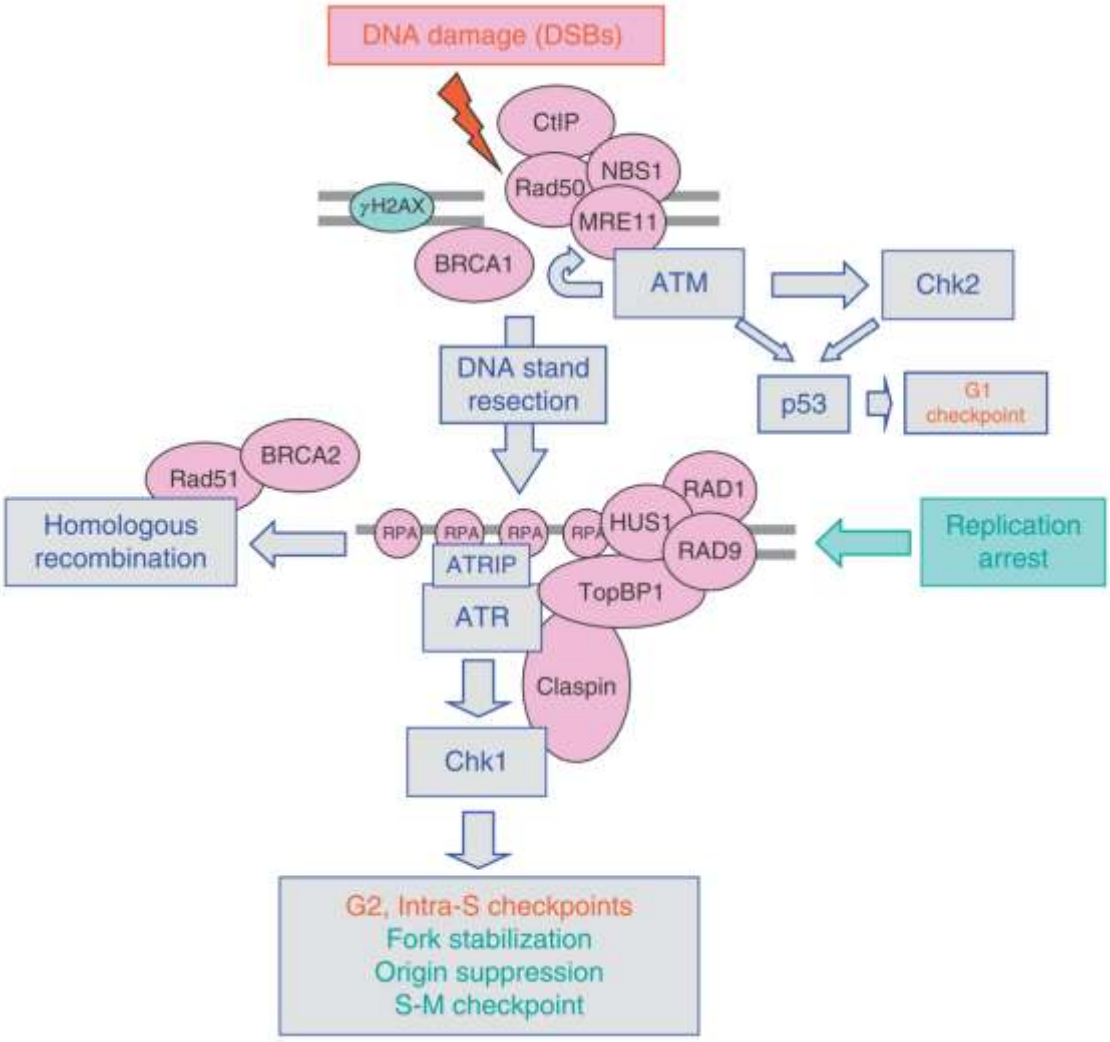

Appendix 3. DNA damage signaling in response to DNA double-strand breaks (DSBs).

In response to DSBs, ATM is activated by autophosphorylation and direct interaction with MRN complexe at the site of DSB. The activated ATM can turn on G1 checkpoint by ATM/Chk2/p53/p21 pathway. After CtIP, BRCA1 and other nucleases and helicases recruited by ATM conduct the DNA strand resection to form tracts of single-strand DNA (ssDNA) at DNA damage site, RPA then can bind to ssDNA and induce ATR/Chk1 activation. ATR/Chk1 is responsible for p53-independent S and G2/M phase arrest. *Advances in cancer research* **2010,** *108*, 73-112[45]

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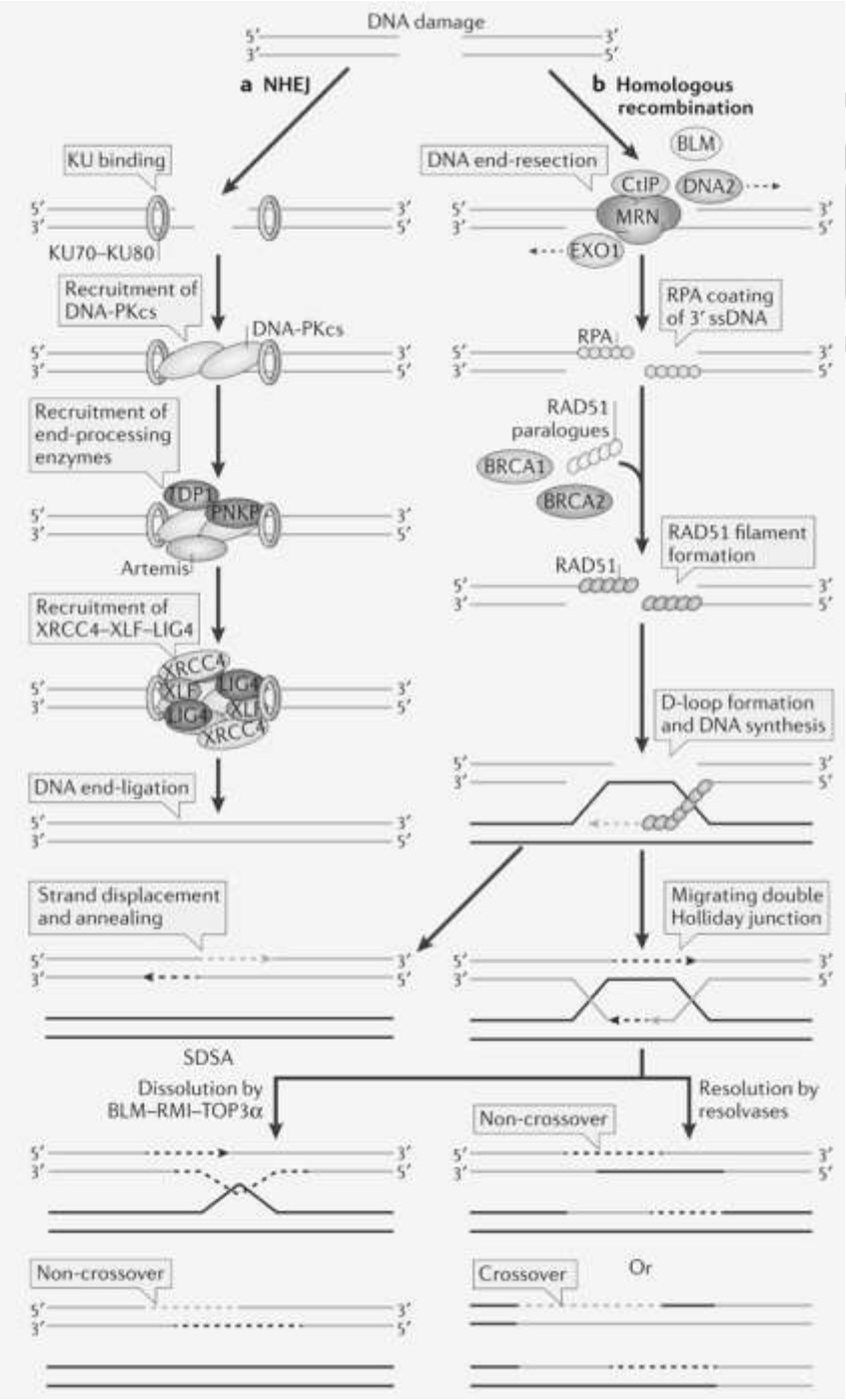

Appendix 4. Homologous recombination (HR) and non-homologous end joining (NHEJ) pathways for repairing DNA double-strand breaks (DSBs).

HR and NHEJ are two pathways to repair the DSBs in eukaryotic cells. HR repairs the DSBs with the core machinery, MRN/RPA/Rad51/resolvase, in an error-free manner during the late S and G2 phases of cell cycle, while, NHEJ repairs the DSBs with the core machinery, Ku/DNA-PKcs/XRCC4, in an error-prone manner during the whole cell cycle. *Nature reviews. Molecular cell biology* **2014,** *15* (1), 7-18[55]

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