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Silencing RBBP6 (Retinoblastoma Binding Protein 6) sensitizes breast cancer cells to staurosporine and camptothecin-induced cell death

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Silencing RBBP6 (Retinoblastoma Binding Protein 6) sensitizes breast cancer cells to *staurosporine* and *camptothecin*-induced cell death.

Pontsho Moela (318896)

A dissertation submitted to the Faculty of Science, University of Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Science.

Gauteng, Johannesburg, 2013.

Declaration

I declare that '**Silencing RBBP6 (Retinoblastoma Binding Protein 6) sensitizes breast cancer cells to *staurosporine* and *camptothecin*-induced cell death'** is my own unaided work. It is being submitted for the degree of Master of Science in the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

Pontsho Moela



_____

(Signed)

___January__day of____27th ___ 2014__

Research Outputs

Oral Presentation

Pontsho Moela, Mpho S. Choene and Lesetja R. Motadi. Silencing RBBP6 sensitizes breast cancer cells to *staurosporine*- and *camptothecin*-induced cell death. 5th Cross-Faculty Graduate Symposium, Wits University, Johannesburg, 01 August 2013

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Original Publication

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Abstract

Retinoblastoma Binding Protein 6 (RBBP6) is a multi-domain protein that uses its ring finger domain to interact with p53 and pRb tumour suppressor genes. The mechanism by which RBBP6 uses to degrade p53 is still unknown. Nonetheless, it is well known that RBBP6 promotes cell proliferation in several cancers by negatively regulating p53 via its E3 ubiquitin ligase activity (Ntwasa, 2008). Degradation of p53 by RBBP6 may compromise p53-mediated apoptosis in breast cancer.

This study is intended to investigate the potential applications of RNA interference (RNAi) to block RBBP6 expression as well as its subsequent effect on cell growth and apoptosis. To achieve these methodologies, the following techniques were used: RT-PCR, western blotting, xCELLigence system and flow cytometry. Our studies indicate that the knockdown of RBBP6 expression by siRNA modulates p53 gene involved in cell death pathways and apoptosis, showing statistically significant gene expression differences. RBBP6siRNA significantly reduced cell index (CI) compared to the control samples and we observed an inhibition of cellular proliferation in the interval of between 24 and 48 h, as shown in the data obtained by dynamic evaluation using the xCELLigence System. These results

were further confirmed by flow cytometry which showed some apoptotic activity. About 20.7% increase in apoptosis was observed in cells co-treated with RBBP6 siRNA and *camptothecin* when compared to *camptothecin*-only whereas in siRBBP6 and *Staurosporine* treated there was only 8.8% increase in apoptosis. These findings suggest that silencing RBBP6 may be a novel strategy to promote *staurosporine*- and *camptothecin*-induced apoptosis in breast cancer cells.

Keywords: Retinoblastoma Binding Protein 6, *staurosporine*, *camptothecin*

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I also deeply thank my mother Rose Moela and my grandmother Mahlake Moela for their endless support and patience throughout my academic years. To my sister Thabiso, thank you for always being so proud of my achievements. I admire your strong influence towards my academia. You instilled in me great words of encouragement when I was close to giving up.

Dedication

I dedicate this dissertation work to my late father and my late younger sister,

Reuben Sehlangu and Malebo Moela

Abbreviations

Apaf-1	Apoptosis protease-activating factor-1
Apo1	Apolipoprotein 1
APS	Ammonium persulfate
ATM	Ataxia telangiectasia
ATP	Adenine triphosphate
Bad	Bcl-x_L/Bcl-2-associated death protein
Bax	Bcl-2-associated death protein
BCA	Bicinchoninic acid
BCl-2	B cell leukaemia-2
Bid	B cell leukaemia lymphoma-2
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
BRCA1/2	Breast cancer 1/2 gene
BSA	Bovine serum albumin
Caspase	Cysteine aspartic-specific proteases
CCD	Charge-coupled device
cdk	Cyclin-D dependent kinase
cDNA	Complementary DNA

CI	Cell index
CPT	Camptothecin
DEPC	Diethyl pyrocarbonate
DISC	Death signalling complex
DMEM	Dulbecco's modified medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNTPs	Deoxyribonucleotides
dsRNA	Double-stranded RNA
EDTA	Ethylene diamine tetra acetic acid
ER	Estrogen receptor
FACS	Fluorescence activated cell sorter
FADD	Fas-associated death domain
Fas	Fibroblast-associated
FasL	Fas Ligand
FBS	Foetal bovine serum
FITC	Fluoresceine-isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HER2	Human Epidermal growth factor receptor 2
HRP	Horseradish peroxidase

Kb	Kilobase
kDa	Kilo Dalton
l	Litre
MDM2	Murine Double Minute 2
MOPS	4-Morpholine-propanesulfonic acid
mRNA	Messenger RNA
MW	Molecular Weight
NHL	Non-hodgkin lymphoma
P2P-R	Proliferation potential protein-related
P53/TP53	Protein 53/tumor protein 53
PACT	P53-associated cellular protein testis-derived
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium Iodide
PR	Progesterone receptor
pRB	Retinoblastoma protein
PS	Phosphatidyl serine
PTEN	Phosphate and tension gene
PTGS	Post transcriptional gene silencing

PVDF	Polyvinylidene difluoride
RBBP6	Retinoblastoma binding protein 6
RING	Really interesting new gene
RIPA	Radioimunoprecipitation assay
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RTCA	Real time cell analyser
RT-PCR	Reverse transcription PCR
SDEV	Standard deviation
SDS	Sodium dodecyl sulphate
siRNA	Short interfering RNA
ssRNA	Single-stranded RNA
STS	*Staurosporine*
TEMED	*N,N, N', N'*-Tetramethylethylenediamine
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
V	Volts
WHO	World health organisation

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Chapter 1: Introduction and Literature Review

1. Introduction

1.1 Breast Cancer

Breast cancer, characterised by tumorigenic growth in breast tissue, is the most prevalent type of cancer in women than in men (Ly et al. 2013). Breast cancer is classified into three subtypes according to the presence or absence of certain cancer cell receptors (Tinoco et al. 2013). These include the hormone receptor (HR) subtype (estrogen and/or progesterone receptors) which accounts for nearly 60% of all breast cancer incidences, the oncogene human epidermal growth factor receptor 2 (HER2/neu) subtype of which 20% is observed in all breast cancer cases and the triple negative breast cancer (negative for the expression of estrogen, progesterone and HER2/neu receptors) which also constitutes about 20% of all breast cancer cases (Tinoco et al. 2013).

The estrogen receptor positive breast cancers are the most common types of breast cancer because the estrogen and progesterone hormones are playing primary roles as transcription factors that mediate breast tumorigenesis, thus enabling the recurrence of the disease even after an effective targeting of the ER signalling (Lim, Metzger and Winer 2012).

In addition to the receptor-associated subtypes of breast cancer, researchers have documented intrinsic molecular subtypes: Luminal A and B, and HER2-like subsets (Tinoco et al. 2013); (Lim et al. 2012). These molecular subtypes correlate with the expression of ER, PR and HER2 in approximately 70% of breast cancer cases (Perou et al. 2000); (Sotiriou and Pusztai 2009).

1.1.1 Breast Cancer Epidemiology

Breast cancer is the most common cancer type amongst women, accounting for most cancer deaths second to cervical cancer worldwide (Jemal et al. 2012). Breast cancer incidence rate (number of people per 100 000 who develop breast cancer during a given time period) differs much across the world and this remarkable variance is attributed to substantial regional differences in the prevalence and distributions of socio-economic factors (Jemal et al. 2012). A highest incidence rate of 99.4 per 100 000 people in North America alone was observed (WHO, 2013). Moderately increasing incidence rates are observed in Europe, other parts of America as well as in the Southern Africa (WHO, 2008).

Mortality rates (number of people per 100 000 who die of breast cancer during a given time period) amongst women were estimated at 69%

worldwide (~519 000 deaths) in 2004 (WHO, 2004). Breast cancer survival rate (number of people per 100 000 who die of breast cancer during a given time period) is lower in black women (75%) than in white women (89%) as a result of poor prognosis in black women (Hortobagyi et al. 2005). This is because black women often present with late stage breast cancers with large and more aggressive tumours (Ghafoor et al. 2003).

The World Health Organisation statistics for the year 2010 indicate a steady increase of breast cancer cases worldwide where about 380 000 deaths are observed each year (WHO, 2010). In Africa, 92 600 breast cancer cases and 50 000 deaths were reported in 2008 statistics, with Southern African women showing highest breast cancer incidence rates when compared to women from all African countries (Jemal et al. 2012). The United Kingdom's incidence and mortality rate as a result of breast cancer, however, ranks the highest in the world with approximately 14000 deaths each year (McPherson, Steel and Dixon 2000).

1.1.2 Breast Cancer Risk Factors

In a study published by (Steiner, Klubert and Knutson 2008), breast cancer risk factors are divided into non-modifiable and modifiable factors. Advanced age and female gender are the major non-modifiable risk factors

associated with breast cancer and this is due to the fact that the patient's lifetime exposure to female hormones (progesterone and estrogen) associated with breast cancer would have increased significantly (Steiner et al. 2008). Other important non-modifiable breast cancer risk factors are menarche before the age of 12, menopause after the age of 45, genetic mutations and family history (Steiner et al. 2008; Nelson et al. 2012; Butt et al. 2012).

Women with first degree relatives demonstrate higher risk of developing breast cancer and the risk is much higher in women with first degree relatives who are diagnosed at a younger age compared to older first degree relatives (Nelson et al. 2012). Breast cancer cases arising from genetic mutations account for only about 5% and 60% of inherited breast cancers as a result of BRCA1 and BRCA2 gene mutations (Steiner et al. 2008).

Three 'hot spot' BRCA gene mutations (BRCA1-185delAG, BRCA1-5382insC and BRCA2-6174delT) have been identified as highly common in Ashkenazi Jewish population, making inherited breast cancer more prevalent in this descent group (Dumitrescu and Cotarla, 2005); (Steiner et al. 2008). Other genes known to contribute to breast cancer development are p53, PTEN (phosphate and tension gene) and ATM (ataxia-telangiectasia) (Dumitrescu and Cotarla, 2005).

Breast cancer risk factors that can be controlled (or modified) have also been documented (Steiner et al. 2008). Hormone therapy (hormone replacement therapy or oral contraceptives) use does not alleviate the risk of developing breast cancer. However, the risk becomes higher (30-50%) when usage exceeds five years (Hulka and Moorman 2008).

Pregnancy and breast feeding are also important risk factors of breast cancer that can be controlled (Steiner et al. 2008). Women (white and African-American) who give birth as early as 20 years of age or younger after a full term pregnancy and breast feed have a reduced lifetime risk of developing breast cancer when compared to nulliparous women (Ursin et al. 2004); (Lord et al. 2008); (Steiner et al. 2008).

Women with high breast density (amount of breast and connective tissue compared to fat) have been shown to have a greater risk of developing breast cancer (Lokate et al. 2011); (Schreer 2009); (Boyd et al. 2006). Behavioural and life style risk factors associated with the development of breast cancer include poor diet, i.e. high fat, low vegetable/fruit, low fibre and high in simple carbohydrates, overweight and obesity and decreasing physical activity (McTireman, 2003).

1.2 The Cell Cycle

The most important function of the cell cycle is to make sure that DNA is faithfully replicated in the S-phase and that identical chromosomes are equally distributed to two daughter cells during the mitotic (M) phase (Sherr, 1996). There are four coordinated processes involved in the eukaryotic cell cycle, namely cell growth, DNA replication process (or the interphase), the segregation of replicated chromosomes into two separate cells (or mitotic phase) and cell division (cytokinesis) (Vermeulen, Van Bockstaele and Berneman 2003) and (Cooper, 2000).

A typical human cell divides approximately every 24 hours and even though mitosis is the most dramatic stage of the cell cycle, it only lasts about an hour while the interphase takes up about 95% of the cell cycle duration during which cell growth and DNA replication occur in preparation for cell division (Cooper, 2000)

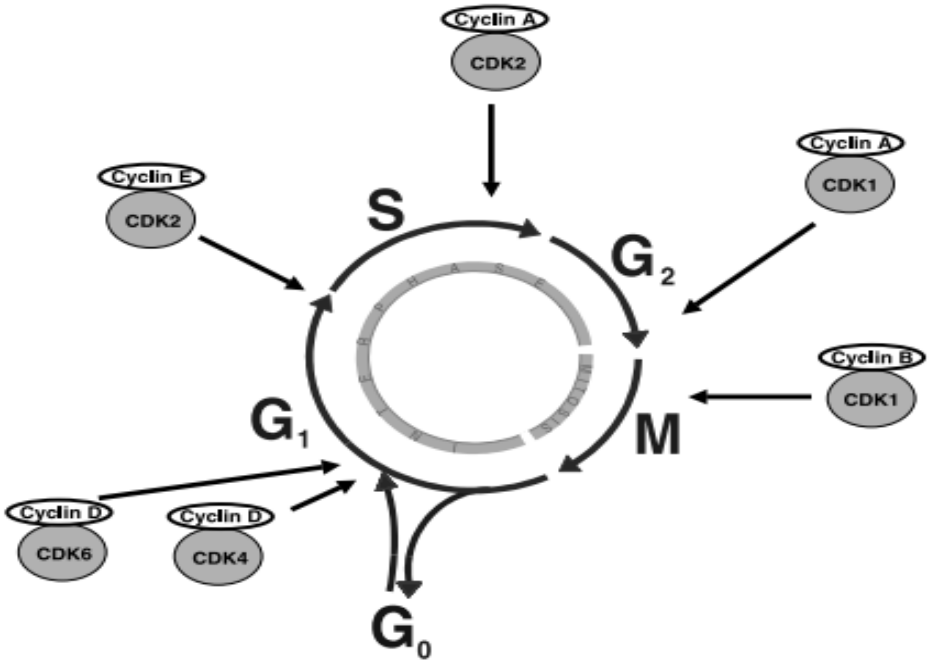


Figure 1': The stages of the cell cycle and the sites of regulatory cyclin/CDK complexes (Vermeulen et al. 2003).

The interphase is made up of G_1, S and the G_2 phase and DNA replication takes place in the S-phase which is preceded by the G_1 phase where preparation of DNA synthesis takes place and is followed by the G_2 where preparation of cell division or mitosis occurs (Vermeulen et al. 2003). Depending on the type of extracellular stimuli, cells in G_1 respond by either progressing to the S-phase or by entering a quiescent or resting phase called G_0 where they do not undergo any form of cellular growth though they are metabolically active (Sherr, 1996) and (Vermeulen et al. 2003).

Following the interphase is the M-phase where cell division occurs and it is divided into four stages, namely the prophase, metaphase, anaphase and telophase (Vermeulen et al. 2003).

1.2.1 Regulation of the cell cycle

Uncontrolled cell proliferation is the basic marker of cancer development and most cancers elicit damage in genes that are responsible for the regulation of the cell cycle (Sherr, 1996). Serine/threonine protein kinase family members known as the cyclin-dependent kinases (CDKs) are the key regulators of the transition from one cell cycle phase to another (Vermeulen et al. 2003).

Five CDKs have been identified to be activated during different stages of the cell cycle, i.e. CDK 4, 6 and 2 during G_1, CDK 2 during the S-phase and CDK 1 during G_2 and M-phase. Activated CDKs phosphorylate target proteins in order to induce downstream processes. CDKs are activated by cyclins which are required at different stages of the cell cycle. The type D cyclins (cyclin D1, D2, and D3) bind and activate CDK 4 and 6, and cyclin E and A bind to CDK 2. In addition to cyclin binding, CDKs are activated by phosphorylation at conserved serine/threonine regions and are counteracted by CDK inhibitors which bind to either CDKs alone or to CDK/cyclin complexes (Vermeulen et al. 2003).

1.3 Apoptosis

Chemotherapy has been a successful tool in the treatment of cancers such as testicular cancer and leukaemia. However, its cytotoxic effects have collateral damage to normal cells when used in the treatment of epithelial cancers such as breast, cervical, colon or lung (Johnstone, Ruefli and Lowe 2002). This, together with the fact that cancer cells have the ability to avoid apoptosis and continue proliferating, have led to current approaches in cancer therapy to focus on killing tumorigenic cells through the induction of apoptosis (Debatin 2004)

Apoptosis, as first described by Kerr *et al*. (1972), is a form of programmed cell death characterized by cell morphological changes that include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation and a subsequent cell death (Ouyang et al. 2012). Other forms of programmed cell death include autophagy and necrosis which are both distinguished from apoptosis by their morphological differences (Bialik et al. 2010).

1.3.1 Mechanisms of Apoptosis

Apoptosis is comprised of two signalling pathways, namely the death receptor pathway (extrinsic) and the mitochondrial pathway (intrinsic), (Wen et al. 2012). Activation of the extrinsic pathway is initiated by binding of the

Fas Ligand to the cell surface death receptor, Fas, a member of the TNF (tumour necrosis factor) receptor superfamily. Other members include the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor, Apo-2 and TNF-1 death receptors (Ghobrial, Witzig and Adjei 2005).

The Fas L-Fas complex forms a death inducing signalling complex (DISC) by aggregating with the Fas-associated death domain (FADD) adaptor protein and pro-caspase-8, thus leading to an active caspase-8 enzyme which subsequently activates pro-caspase-3, an enzyme required for execution of the apoptosis process (Ouyang et al. 2012). In other cells, caspase-8 interacts with death modulators (e.g. Bid) of the intrinsic pathway which lead to the release of cytochrome c (Ghobrial et al. 2005).

The intrinsic pathway is regulated by the Bcl-2 family proteins which become activated by post translational modifications followed by translocation to the mitochondria in response to either extracellular or intracellular stimuli. This triggers the release of cytochrome c from the outer mitochondrial membrane into the cytoplasm. The cytoplasmic cytochrome c interacts with pro-caspase-9 and Apaf-1 molecule to form an apoptosome body which leads to an active caspase-9 responsible for cleaving pro-caspase-3 into an active caspase-3 necessary for the induction of apoptosis (Ouyang et al. 2012) and (Ghobrial et al. 2005).

1.3.2 Bcl-2 Family Proteins

The Bcl-2 family proteins, first identified in follicular non-Hodgkin lymphoma (NHL), are known to control apoptosis at the intrinsic pathway level and are highly expressed in many other cancers. Therefore, numerous anti-tumour drugs exert their effects on the Bcl-2 proteins (Ghobrial et al. 2005). This family of proteins is made up of the proapoptotic Bcl-2 proteins which promotes apoptosis (Bax, Bak, Bad, Bcl-Xs, Bid and Bik) and the anti-apoptotic Bcl-2 proteins which suppress apoptosis (Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1) (Ghobrial et al. 2005).

The proapoptotic members promote apoptosis by mediating the release of cytochrome c from mitochrondria and the anti-apoptotic members block apoptosis by inactivating the pro-apoptotic members (Ouyang et al. 2012). Bax and Bak are termed BH3-only proteins and they exert their pro-apoptotic effects using the BH3 molecule (Ghobrial et al. 2005) and (Ouyang et al. 2012). In response to cellular stress, the proapoptotic proteins undergo activation processes such as dephosphorylation and cleavage followed by movement to the mitochondria where they initiate cytochrome c release (Ghobrial et al. 2005) and (Ouyang et al. 2012).

1.3.3 p53

The first human cDNA clones of the p53 tumour suppressor gene which codes for a protein of 393 amino acid were first isolated in the early 1980's and it was named so because it elicits a band size of 53kDa in agarose gel electrophoresis (Nag *et al*, 2013). The tumour suppressor gene is responsible for the regulation of cell cycle arrest, DNA repair and apoptosis and it is highly mutated in several cancers, thus leading to deregulation of the cell cycle and apoptosis impairment (Ghobrial et al. 2005).

Following nuclear stress such as radiation, p53 activates p21 gene which is responsible for the induction of cell cycle arrest during which damaged DNA is allowed to undergo repair. However, if damage is irreversible, p53 activates proapoptotic Bcl-2 protein members in order to induce apoptosis although the mechanism by which p53 induces apoptosis is not well understood (Ghobrial et al. 2005).

1.3.4 p53 and MDM2

Oncogenes and tumour suppressor genes maintain the balance between cell growth and cell death and p53 is one of the well-studied tumour suppressor genes that plays a role in the development and homeostasis of cells and tissues (Jiang et al, 2013). In addition to its downstream effectors such as cyclin dependent kinase inhibitor p21, pro-apoptotic bax and tumour necrosis

factor receptors (Fas and/or Apo1), p53 transcriptionally activates its negative regulator, MDM2, in an autoregulatory feedback loop (Jiang et al, 2013) and (de Rozieres *et al*, 2000).

MDM2 (Murine Double Minute 2) was established as an oncogene in a study which demonstrated that overexpression of the MDM2 gene rendered rodent fibroblasts tumorigenic in nude mice (Menedez et al, 2009). The MDM2 gene codes for a 491 amino acid protein and it contains two transcriptional promoter elements, one of which is p53-dependent (Menedez et al, 2009). Also known as a family member of the E3 ubiquitin ligases, MDM2 directly interacts with p53 to mediate its ubiquitination (Jiang et al, 2013). Following its expression, MDM2 translocates from the nucleus to the cytoplasm where it mediates the degradation of p53 and other targets (Menedez et al, 2009).

In most cancers, the mutated p53 or the function of wild-type p53 is inhibited by the MDM2 oncogene resulting in the impairment of tumour suppressive p53-pathways such as apoptosis and cell cycle arrest, thus interfering with the p53-MDM2 interaction as a cancer therapeutic strategy may be potential (Nag *et al*, 2013).

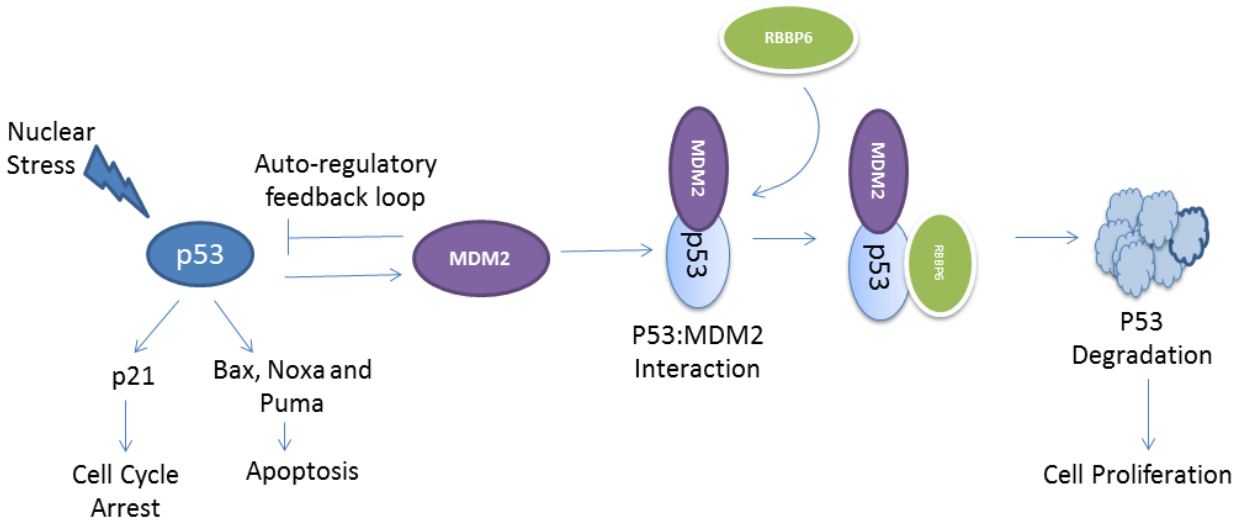


Figure 2': A simplified diagrammatic representation of interactions among p53, MDM2 and RBBP6.

Under normal cellular conditions, p53 tumour suppressor gene is kept under tight regulation by the MDM2-P53 auto-regulatory feedback loop. In response to stress stimuli such as DNA damage or radiation, activated p53 interacts with genes such as p21, Bax, Noxa and Puma which are responsible for the induction of cell cycle arrest and apoptosis as illustrated in figure 2. During cancer development, the interaction between p53 and MDM2 mediates p53 interaction with the ring finger domain of ubiquitin ligases, in this case the RBBP6 ubiquitin ligase, for degradation of the p53 tumour suppressor protein. This compromises the occurrence of p53-mediated apoptosis and facilitates cell proliferation.

1.4 RBBP6

Screening tests performed over a decade ago revealed Retinoblastoma Binding Protein 6 (RBBP6) as a multi-domain protein that uses its ring-finger domain to interact with the p53 and pRb tumour suppressor genes (Pugh et al. 2006); Pretorius *et al*, 2013). Also known as RBQ-1, PACT or P2P-R, the RBBP6 mRNA occurs in the form of three major transcripts, transcript 6.1, 6.0 and 1.1kb as a result of alternative splicing which code for protein isoforms 1, 2 and 3, respectively (Pugh et al. 2006).

RBBP6 is highly expressed in cancer of the oesophagus as well as other cancers and this makes it a potential target in the treatment of cancers with intact p53 (Ntwasa, 2008); (Pugh et al. 2006). In addition to its several biological functions such as transcription, translation and ubiquitination, it is associated with the execution of p53 degradation following inhibition of the tumour suppressor gene by MDM2, thus facilitating cell proliferation (Ntwasa, 2008); (Pretorius *et al*, 2013). However, little is known about the mechanism by which RBBP6 uses to degrade the p53 tumour suppressor protein.

A study performed by Li et al., (2007) indicates that knockdown of RBBP6 in mice leads to enhanced p53 accumulation which favours apoptosis and a slow growth in the mice. RBBP6 is responsible for negatively regulating p53

by interacting with MDM2, thus leading to p53 depletion. Silencing RBBP6 in mice led to an up-regulation of p53 (Li et al., 2007).

Although monotherapy archives a significant response rate in cancer patients, drug resistance is still a major hiccup to be addressed in cancer therapy. Recently, most of the efficient regimes for anticancer activity include multidrug combinations, thus investigating optimum combinations may be useful for cancer treatment (Arakawa *et al*, 2009). RNA interference (RNAi) is a regulatory mechanism that uses small interfering RNA (siRNA) molecules to degrade specific mRNA and silence gene activity (Aagaard and Rossi, 2007).

Camptothecin causes DNA strand breaks during the S-phase of the cell cycle by stabilizing DNA-topoisomerase I complexes in order to induce apoptosis in cancer cells (Arakawa *et al*, 2009) and *staurosporine* induces apoptosis by inhibiting protein kinases (Xue *et al*, 2003). In this study, we report on additive cytotoxic effects between gene therapy (RNA interference) and apoptosis-inducing agents (*camptothecin* and *staurosporine*) in human breast cancer cells.

Chapter 2: Aim and Objectives

The aim of this study was to promote the induction of apoptosis in breast cancer cells following gene silencing in combination with *staurosporine* and *camptothecin* treatments. Since RBBP6 is involved in the degradation of p53 thus reducing p53-mediated apoptosis to facilitate cell proliferation, the effect of co-treatment with RBBP6 gene silencing and apoptosis-inducing agents on p53 expression was evaluated at both mRNA and protein level.

Objective 1: To silence RBBP6, MDM2 and p53 by transfecting human breast cancer cell lines with siRBBP6, siMDM2 and sip53

Objective 2: To check whether RBBP6, MDM2 and p53 genes were silenced by measuring gene expression levels using Real Time RT-PCR technique

Objective 3: To check the effects of RBBP6 and MDM2 gene silencing on the expression of p53 gene using Real Time PCR technique

Objective 4: To co-treat human breast cancer cell lines with siRNAs (RBBP6, MDM2 and p53) and apoptosis-inducing agents (*staurosporine* and *camptothecin*)

Objective 5: To confirm gene silencing by measuring protein expression levels of RBBP6, MDM2 and p53 using western blot analysis following co-treatment

Objective 6: To measure expression levels of Bax and BCl-2 following co-treatment using real time RT-PCR and western blot analysis

Objective 7: To measure cell growth using *eXCELLigence* system and apoptosis using flow cytometry analysis following co-treatment

Chapter 3: Materials and Methods

3.1 Materials

3.1.1 Cell lines

Different breast cancer cell lines and a non-tumorigenic cell line were used in this study as both a primary source of mRNA and protein and for studying growth and morphology after co-treatment. The cell lines include non-invasive MCF-7 breast cancer cells, invasive CAMA-1 breast cancer cells and MRC-5 lung fibroblasts. All cell lines were obtained from Japan Health Resource Centre.

3.1.2 Primers

The following primer sequences which were designed using Primer3Plus software were used to amplify the corresponding genes:

RBBP6 Primers

Forward primer: CAGCGACGACTAAAAGAAGAG

Reverse primer: GAGCGGCTGAATGATCGAGA

p53 Primers

Forward primer: TGACACGCTTCCCTGGATTG

Reverse primer: ACCATCGCTATCTGAGCAGC

MDM2 Primers:

Forward Primer: ATCAGGCAGGGGAGAGTGAT

Reverse Primer: TCTACATACTGGGCAGGGCT

Bax Primers

Forward Primer: CCGCCGTGGACACAGAC

Reverse Primer: CAGAAAACATGTCAGCTGCCA

BCl-2 Primers

Forward Primer: TCCGATCAGGAAGGCTAGAGTT

Reverse Primer: TCGGTCTCCTAAAAGCAGGC

GAPDH Primers

Forward Primer: CAGCCGCATCTTCTTTTGCG

Reverse Primer: TGGAATTTGCCATGGGTGGA

3.1.3 RNAi Oligos

Silencing was archived by the use of Ambion's *Silencer*® Select Pre-designed siRNAs supplied by Life Technologies™ which target the RBBP6, MDM2 and p53 genes. The siRNA molecules are chemically modified 21-mer double stranded nucleotides that are recognised by the cytoplasmic RNA-induced

silencing complex (RISC) of mammalian cells to mediate inactivation of target genes (Aagaard and Rossi, 2007). Delivery of the Ambion's *Silencer*® Select Pre-designed short interfering RNAs into the mammalian adherent cells was achieved by the use of the lipid-based siPORT™ *NeoFX*™ transfection agent also manufactured by Ambion®. Oligonucleotide targeting GADPH served as a positive control and a non-targeting oligonucleotide served as a negative control.

3.1.4 *Staurosporine*

Chosen as one of the apoptosis-inducing agents in this study, *staurosporine* (Calbiochem®) is known for its protein kinase inhibiting property and as a strong inducer of apoptotic cell death (Xue *et al*, 2003). The mechanism by which *staurosporine* uses to induce apoptosis is still controversial. Several studies report that the mitochondrial apoptotic pathway plays a central role in the *staurosporine*-induced cell death while other reports show that there is a requirement for the activation of caspases in *staurosporine*-induced apoptosis. The protein kinase inhibitor is largely thought to mediate translocation of bax to the outer mitochondrial membrane where it permeabilises the membrane, thus leading to the release of cytochrome c from mitochondria into the cytoplasm (Zhang *et al*, 2004 and Xue *et al*, 2003).

3.1.5 *Camptothecin*

Camptothecin (Calbiochem®) is a plant extract isolated from *Camptotheca acuminata* tree which is originally cultivated in most regions of China where it is used for anticancer treatment in Chinese traditional medicine. It is classified as a quinoline alkaloid that exerts its cytotoxic effects by the induction of apoptosis. *Camptothecin* binds to DNA topoisomerase I complex to form a ternary structure that stabilizes the complex to block religation of DNA, thus causing DNA strand breaks that result in apoptosis (Nieves-Neira and Pommier 1999).

Clinical trials on this apoptosis-inducing agent revealed low solubility and adverse drug reactions which led to the synthesis of more soluble and beneficial *camptothecin* derivatives which demonstrate good activity against tumours of lung, breast, colorectal and ovarian (Nieves-Neira and Pommier 1999). Examples are *irinotecan hydrochloride* and *topotecan* which have been clinically approved by the FDA and are clinically used in anticancer therapy (Arakawa *et al*, 2009).

3.2 Methods

3.2.1 Cell culture routine

Human monolayer normal and cancer cell lines were grown in HyClone® DMEM/High Glucose growth medium supplemented with 10% FBS (fetal bovine serum) and 1% antibiotic (penicillin/streptomycin) and routinely maintained at 37°C in a 5% CO_2 incubator. The cells were fed every second day of the week by replacing old growth medium with equal amount of fresh growth medium after rinsing twice with 2ml PBS. The cells were sub-cultured when at near-confluency at least once per week and excess sub-cultures were preserved at ≥ -80°C in DMEM/High Glucose growth medium supplemented with 10% FBS and 20% DMSO (dimethyl sulfoxide).

3.2.2 RNA interference (RNAi)

Principle

RNA interference (RNAi), also called post transcriptional gene silencing (PTGS), is a eukaryotic regulatory mechanism that plays an important role in directing development and suppressing parasitic gene expression. It uses small interfering RNA (siRNA) molecules to degrade specific mRNA and silence gene activity (Aagaard and Rossi, 2007). The RNAi pathway is initiated by cleavage of long double stranded RNA by DICER enzyme into short interfering double stranded RNA molecules of about 20 nucleotides in length.

These short interfering RNA molecules have a characteristic 5'phosphate and a two nucleotide 3'overhang which allows them to be recognised by the RNAi machinery that eventually leads to degradation of target mRNA (Meister and Tuschl, 2004). The short double stranded siRNA separates into two single stranded RNA (ssRNA) named the guide strand and the passenger strand. The passenger strand becomes degraded and the guide strand associates with the RNA-induced silencing complex (RISC) where it specifically base-pairs with complementary target mRNA. This induces catalytic cleavage of the target gene by a component of the RISC complex known as Argonaute enzyme (Aagaard and Rossi, 2007 and Meister and Tuschl, 2004).

Introduction of artificial siRNAs has been adopted as a tool to target and inactivate gene expression both *in-vitro* and *in-vivo*. Most human diseases such as cancer, viral infections, autoimmune diseases and genetic disorders are associated with alterations in one or more genes and therefore this makes the RNAi intervension an attractive strategy for gene-related therapeutics (Aagaard and Rossi, 2007). In this study, we used RNAi to silence RBBP6, MDM2 and p53 in breast cancer cell lines.

Technique

Seventy to eighty percent (70-80%) confluent cells were transfected with 100nM siRNAs targeting RBBP6, MDM2 and p53 over a 24 hour period. Briefly, the adherent cells were trypsinized and resuspended in an antibiotic-free media. The cell suspension was then mixed with siRNA/transfection agent complex in a 24-well plate and incubated at 37°C for 24 hours. Post transfection cells were exposed to 0.25μM *staurosporine* and 0.25μM *camptothecin* for additional 24 and 48 hours and then harvested for subsequent analysis.

3.2.3 RNA Extraction

Principle

RNA is used as a starting point for downstream processes such as reverse transcription real-time PCR, conventional PCR, array analysis and cDNA library constructions. However this single stranded ribonucleic acid is highly unstable with a very short half-life once extracted from cells or tissues. It is the ubiquitous presence of RNAses on all tissues (especially on skin, in blood, and in most bacteria and fungi in the environment) that make RNA easily degradable. High quality total RNA is therefore an absolute prerequisite in the performance of molecular techniques (Tan and Yiap, 2009). Most RNA isolation procedures make use of RNAse inhibitory agents such as chaotropic ions (e.g. guanidine salt), sodium dodecylsulfate (SDS)

denaturant or phenol-based compounds. The commercial RNA isolation kit used in this study relies on guanidine chaotropic salt.

Technique

The general purification steps which were used in this study following a 24, 48 and 72 hour co-treatment of nearly confluent cultured cells with Nucleospin® RNA II total RNA isolation kit include: effective disruption of cells → denaturation of nucleoprotein complexes → inactivation of DNAses → washing steps for the removal of salts, metabolites and macromolecular cellular components → elution of pure RNA. The resulting RNA was quantified using the nanodrop technique and RNA purity was confirmed by ensuring that A260/A280 ratio was >1.7 and the RNA integrity was confirmed by examination of the 18S and 28S RNA bands on agarose gel stained with ethidium bromide.

3.2.4 Reverse Transcription

Principle

In order to perform RT-PCR, the obtained RNA template must first be converted into a complementary DNA (cDNA) by the use of reverse transcriptase enzyme. In this study we performed reverse transcription using

the ImProm-II™ Reverse Transcription System commercial kit manufactured by Promega.

Technique

Cells were co-treated with siRNAs and apoptosis-inducing agents for 24, 48 and 72 hours before RNA isolation. Sequence specific primers (RBBP6, MDM2 and p53) designed using the Primer3Plus bioinformatics tool together with the obtained total RNA template, formed major components of the PCR cocktail used in the synthesis of cDNA. The RNA reverse transcription was performed according to the manufacturer's detailed protocol using the Multigene Gradient Thermal Cycler.

3.2.5 Real Time RT-PCR

Principle

To check whether the transfected cells were successfully silenced and to study gene expression changes following co-treatment of cells, real time RT-PCR was performed. The total mRNA transcribed into complementary cDNA was amplified and quantified by the use of real time polymerase chain reaction. Real time RT-PCR differs from conventional PCR in that instead of detecting and quantifying amplified DNA at the end of the reaction, the amount of PCR product is measured at each cycle in real time. Measurement

of the amount of DNA at each cycle is accomplished by the use of fluorescent dyes capable of intercalating each of the newly synthesized double stranded DNA to produce a signal that's directly proportional to the amount of PCR product.

SYBR Green is the commonly used fluorescence dye due to its advantages, i.e. it is inexpensive, easy to use and highly sensitive. The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products which results in an over estimation of the target concentration. Other commonly used probes include TaqMan, Molecular Beacons and Scorpions.

Technique

In this study, the SYBR® Green JumpStart Taq ReadyMix (SIGMA®) dye was used to quantify the amount of gene expression from cDNA synthesized from total RNA that was isolated from cultured cells exposed to 24, 48 and 72 hour treatment with 100nM siRNAs and 0.25µM apoptosis inducing agents. The real time RT-PCR was performed in a 20µl reaction mixture containing 2100ng/ul cDNA, SYBR Green, forward and reverse primers. (GADPH: Forward – 5'-GAG TCA ACG GAT TTG GTC GT- 3', Reverse – 5'-TTG ATT TTG GAG GGA TCT CG- 3'; RBBP6: Forward – 5' –CAG CGA CGA CTA AAA GAA GAG TCT- 3', Reverse – 5' – GGT AAT TGC

GGC TCT TGC CT- 3'; p53: Forward – 5' -GTT CCG AGA GCT GAA TGA GG- 3', Reverse – 5' -TGA GTC AGG CCC TTC TGT CT -3', MDM2: forward– 5' ATCAGGCAGGGGAGAGTGAT_3' and Reverse– 5' TCTACATACTGGGCAGGGC-3' under the following conditions: 36 cycles of 94°C for 35s, 59°C for 45s, and 72°C for 45s.

3.2.6 Western Blot

Principle

Western blot analysis continues to be a routine technique in most laboratories to determine the level of expression of proteins. This protein analysis technique was introduced by Towbin *et al*. in 1979. The term blotting refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. The first step in a western blotting procedure is to separate the macromolecules using gel electrophoresis. Following electrophoresis, the separated molecules are transferred or blotted onto a second matrix, generally a nitrocellulose or polyvinylidene fluoride (PVDF) membrane. Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane. The transferred protein is complexed with an enzyme-labelled antibody as a probe. An appropriate substrate is then added to the enzyme and together they produce a detectable product such as a chromogenic or

fluorogenic precipitate on the membrane for colorimetric or fluorometric detection, respectively.

The most sensitive detection methods use a chemiluminescent substrate that, when combined with the enzyme, produces light as a by-product. The light output can be captured using film, a CCD camera or a phosphorimager that is designed for chemiluminescent detection. Whatever substrate is used, the intensity of the signal should correlate with the abundance of the antigen on the blotting membrane.

Detailed procedures of a western blot vary widely. One common variation involves direct vs. indirect detection. With the direct detection method, the primary antibody that is used to detect an antigen on the blot is also labelled with an enzyme or a fluorescence dye. This detection method is not widely used as most researchers prefer the indirect detection method. In the indirect method, a primary antibody is added first to bind to the antigen. This is followed by a labeled secondary antibody that is directed against the primary antibody. Labels include biotin, fluorescence probes such as fluorescein or rhodamine and enzyme conjugates such as horseradish peroxidase or alkaline phosphatase. In this study, the indirect method was used since it offers more advantages over the direct method, i.e. it has increased sensitivity, a wide

variety of labelled secondary antibodies are available commercially and immunoreactivity of the primary antibody is not affected by labeling.

Technique

Whole cell protein was extracted using RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 10% sodium dodecylsulfate (SDS), and 3 µl/ml aprotinin and 5µg/ml leupeptin in PBS, pH 7.4). Seventy two (72) hours post transfection and co-treatment with either *staurosporine* or *camptothecin*, cells were washed twice with cold PBS, then resuspended in 500µl RIPA buffer and collected by scraping. The total protein was then separated from cell debri by centrifugation at 14000 rpm for 15 minutes followed by quantification with Pierce® BCA Protein Assay Kit. The protein was heated at 95°C for 5 minutes and 30µg was loaded per well for electrophoretic separation in 40% acrylamide gel preparation at 100V for 1hour. The protein was transferred onto a nitrocellulose membrane using wet electro-transfer method overnight at 30V followed by incubation with primary antibody after 1 hour of blocking with 5% non-fat milk buffer. The weak light signal produced by HRP-linked secondary antibody was detected and enhanced using the Pierce® ECL Western Blotting Chemiluminescence Substrate and the blots were imaged by the CCD-based ChemiDoc™ MP system.

3.2.7 xCELLigence System

Principle

To study the effect of the co-treatment on cellular growth of the studied cell lines, the xCELLigence system for real time analysis of mammalian cell was used. This label-free assay allows the possibility for real time measurement of cellular responses and this eliminates some of the shortcomings of end-point assays. The xCELLigence system uses specially designed microtiter plates containing interdigitated gold microelectrodes to non-invasively monitor the viability of cultured cells using electrical impedance as a read out in the form of cell index (CI).

Technique

Before cells were seeded, 16-well E-plates containing antibiotic-free medium were imposed to current flow on the xCELLigence instrument to record background readings. 1 x 10^5 cells were then seeded in each well of the E-plates simultaneously with 100nM siRNA targeting RBBP6, MDM2 and p53 genes in the same antibiotic-free medium. The E-plates were connected back on current flow supplied by the xCELLigence instrument that was placed in a 37°C incubator and the experiment was allowed to run for 24 hours. Twenty four hours (24) hours post transfection cell were further treated with 0.25μM apoptosis-inducing agents (*staurosporine* and *camptothecin*). The experiment was continued for an additional 24 hours. Cell Index values were recorded at

15 minute interval sweeps until the end of the experiment under the following xCELLigence parameters: [1st step: 1 sweep, 1 minute interval, 00:00:39 total time; 2nd step: 100 sweeps, 15 minute interval, 24:45:39 total time; 3rd step: 100 sweeps, 15 minute interval, 49:30:39 total time].

3.2.7 Flow Cytometry

Principle

To check whether the observed cell death caused by co-treating cultured cells with siRNA + apoptosis-inducing agents was as a result of apoptosis and not necrosis, flow cytometry analysis technique was used. Loss of plasma membrane asymmetry and attachment are the earliest features of apoptosis. In early apoptotic cells, the membrane phospholipid phosphatidyl serine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment.

Annexin V, a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS, binds to the exposed PS. Annexin V may be conjugated to fluorochromes including FITC. This format retains its high affinity for PS and thus acts as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis. Since externalization of PS occurs in the earlier stages of apoptosis, FITC Annexin V staining can identify

apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation.

FITC Annexin V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore staining with FITC Annexin V is typically used in conjugation with vital dyes such as propidium iodide (PI) to allow identification of early apoptotic cells (PI negative, FITC Annexin V positive). Viable cells with intact membrane exclude PI whereas the membranes of the dead and damaged cells are permeable to PI. Viable cells are considered FITC Annexin V and PI negative. Cells that are in early apoptosis are considered FITC Annexin V positive and PI negative, and cells that are in late apoptosis are considered both FITC Annexin V and PI positive.

Technique

Cultured cells were seeded in 24-well plates and simultaneously transfected with siRNAs targeting RBBP6, MDM2 and p53 for 24hours and treated for an additional 24 hours with apoptosis-inducing agents (0.25 μM *staurosporine* and *camptothecin*). The treated cells were then trypsinized, transferred to 15 ml tubes, pelleted for 2 minutes at 1500 rpm and resuspended in 100μl 1X binding buffer (annexin V-FITC Apoptosis Detection Kit, abcam®) at a concentration of $1x10^4$ cells/ml. The cell solution

was then transferred into 1ml tubes and 5µl of FITC Annexin V and 5µl of PI were added. This was followed by gentle vortexing and incubation for 15 minutes at room temperature in the dark. 400µl 1X binding buffer was then added to each tube and the cell solutions were analysed by flow cytometry within 1 hour.

3.2.8 Statistical analysis

The results of each series of experiments (performed in duplicates) are expressed as the mean values ± standard deviation of the mean (SD). Levels of the statistical significance were calculated using the paired student t-test when comparing two groups or by analysis of variance (ANOVA). P-values of ≤ 0.05 were considered significant.

Chapter 4: Results

4.1 Introduction

Retinoblastoma binding protein 6 (RBBP6) negatively regulates p53 with its E3 ubiquitin ligase function and thus promotes cell proliferation. This event may in turn compromise the occurrence of p53-mediated apoptosis in breast cancer cells. Therefore, in this study we hypothesized that silencing the expression of RBBP6 may enhance apoptosis induced by either *staurosporine* or *camptothecin*. To address this question, RNA interference, real time RT-PCR, western blotting, xCELLigence system and flow cytometry were used.

4.1. PCR Amplification of Target Genes

Before cDNA synthesis by reverse transcription could be performed, the integrity of total mRNA isolated from MRC-5 fibroblast, MCF-7 and CAMA-1 breast carcinomas was evaluated using ethidium bromide-stained agarose gel electrophoresis. The synthesized cDNA served as a template in the amplification of RBBP6, MDM2, p53, bax, bcl-2 and GAPDH genes as shown by agarose gel electrophoresis, amplification of each gene was successful at different annealing temperatures (**figure 3)**.

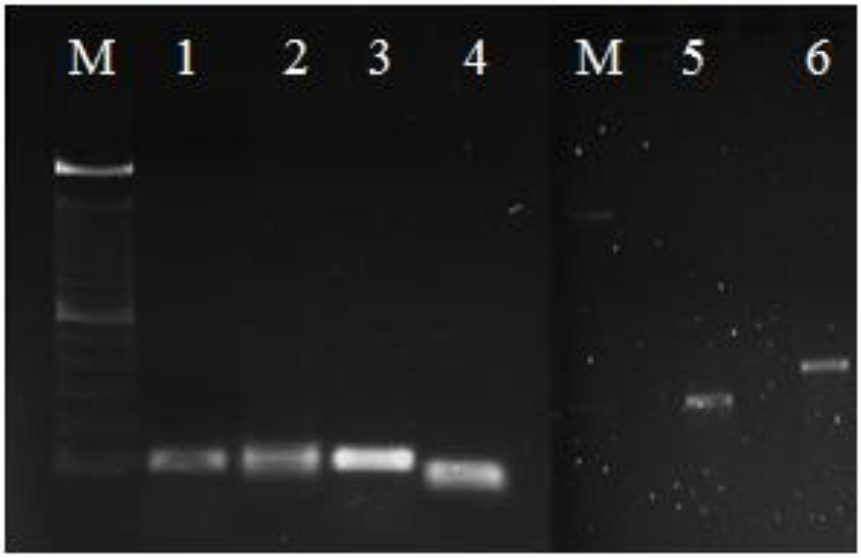


Figure 3': Agarose gel electrophoresis showing amplification of target genes in MRC-5 fibroblasts. Lane M represents DNA molecular weight marker and lanes 1, 2, 3, 4 and 5 represent amplification of the following genes: RBBP6, MDM2, BCl-2, bax, p53, and RBBP6, respectively.

4.2 Gene Silencing and mRNA Expressional Analysis

Gene silencing by RNAi leads to a highly potent repression of gene expression mainly because it employs cellular machinery that effectively allows targeting of specific mRNA transcripts (Aagaard and Rossi, 2007). In this study the RNAi technique was employed to down-regulate RBBP6 and its associated genes, namely p53 and MDM2. To check whether or not gene silencing was successful, real time RT-PCR which relies on reverse transcription of total RNA to synthesize complimentary DNA that serves as a template during real time PCR amplification of the genes of interest was used.

Quantitative PCR analysis was used to evaluate the transcript on the silenced RBBP6, p53, MDM2, and the effect they have on Bax and Bcl2 genes as shown in **figure 1**. As expected we have observed 37% decrease in RBBP6 expression following silencing of MCF-7 cells with 100nM siRNA while in combination with *camptothecin* the expression was further reduced by about 49%. In MCF-7 cells that where treated with *Staurosporine and si*RBBP6, expression was reduced by only 22%. In siMDM2, we observed a much higher silencing following co-treatment with both *staurosporine* and *camptothecin* at 50% and 51%, respectively (**figure1.1a: A&B**). In CAMA-1 cells, about 87% and 90% gene silencing of RBBP6 and MDM2, respectively, were observed after siRNA co-treatment with *staurosporine* whereas combination of *camptothecin* with siRBBP6 and siMDM2 led to about 53% and 21% gene silencing, respectively **(figure1.1b: A&B)**

The present study emphasizes the important role of p53 and Bax pro-apoptotic genes in activating apoptosis and reducing cell proliferation. The effect of RBBP6 and MDM2 silencing on p53 expression in both MCF-7 and CAMA-1 cells is shown in **figure 1.1a: C&D** and **figure1.1b: C&D,** respectively. As observed in MCF-7, siRBBP6 in combination with *camptothecin* treatment led to a 13% increase in the expression of p53 whereas siMDM2 in combination with *camptothecin* resulted in 31% increase

expression (**figure1.1a: C& D**). However, co-treatment with *staurosporine* showed little to no-effect in the expression of p53 after treatment with siRBBP6 or siMDM2 (**figure1.1a: C& D**). In CAMA-1 cells, there were about 17% and 33% up-regulation of p53 following co-treatment with siMDM2-*staurosporine* and siMDM2-*camptothecin*, respectively **(figure1.1b: C&D)**.

One important factor in the apoptosis induction is the Bax/Bcl2 ratio which determines whether the cells survive or die. We have observed up to double increase in the Bax/Bcl2 ratio following MDM2 silencing (**figure1.2: B&D**) and RBBP6 silencing (**figure1.2: D**) in MCF-7 and CAMA-1 cells. Bax was significantly increased following MDM2 and RBBP6 silencing that was followed by treatment with Staurosporine (**figure1.2: A&C**). Silencing both RBBP6 and MDM2 seem to have favoured Bax/Bcl2 ratio that is for apoptosis in both MCF-7 and CAMA-1. In MRC5 fibroblast cells that we considered normal cells, p53 was only slightly increased when RBBP6 and MDM2 silenced even when treated with both agents (**figure1.2**).

These findings suggest that RBBP6 and MDM2 silencing were successful. The findings also suggest that combination of siRNAs (RBBP6 and MDM2) and *camptothecin* further reduces expression of RBBP6 and MDM2. Co-treatment of siMDM2 and *staurosporine* showed further significant reduction

in MDM2 expression whereas for RBBP6 there was little effect on its expression after co-treatment with siRBBP6 and *staurosporine*. The findings also suggest that both RBBP6 and MDM2 silencing does inducep53 expression.

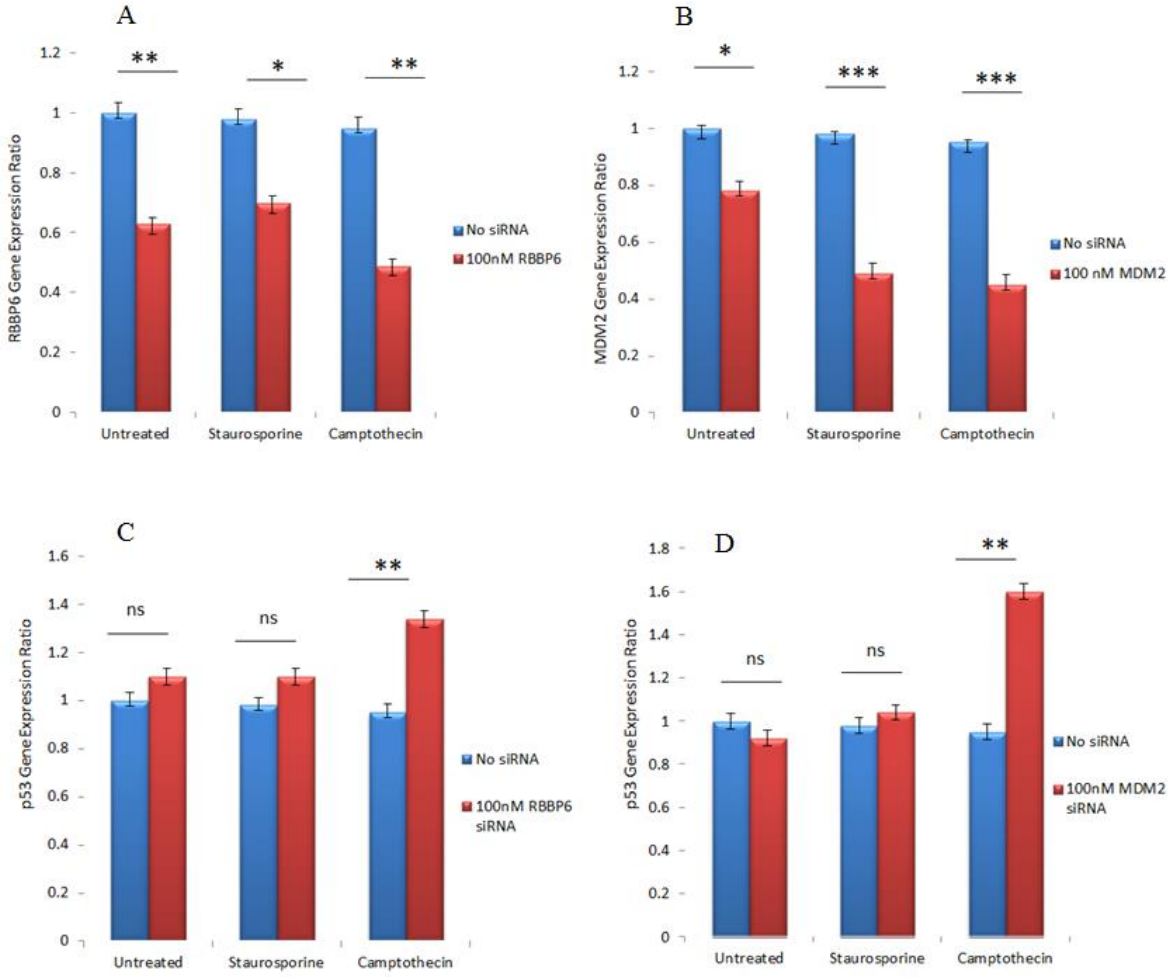


Figure 1.1a: Relative quantification of gene expression in MCF-7 cells was performed using real time RT-PCR. **A** and **B**, gene expression of RBBP6 and MDM2 in cells that were not transfected and either not treated, treated with *staurosporine* or *camptothecin*, silenced with either MDM2 or RBBP6 siRNAs and treated with either *staurosporine* or *camptothecin*. **C** and **D**, p53 gene expression after co-treatment of MCF-7 cells with siRNAs targeting RBBP6 and MDM2, respectively, and the apoptosis-inducing agents

(*staurosporine* and *camptothecin*). The experiments were done in duplicates and the data is statistically significant, p-value <0.05.

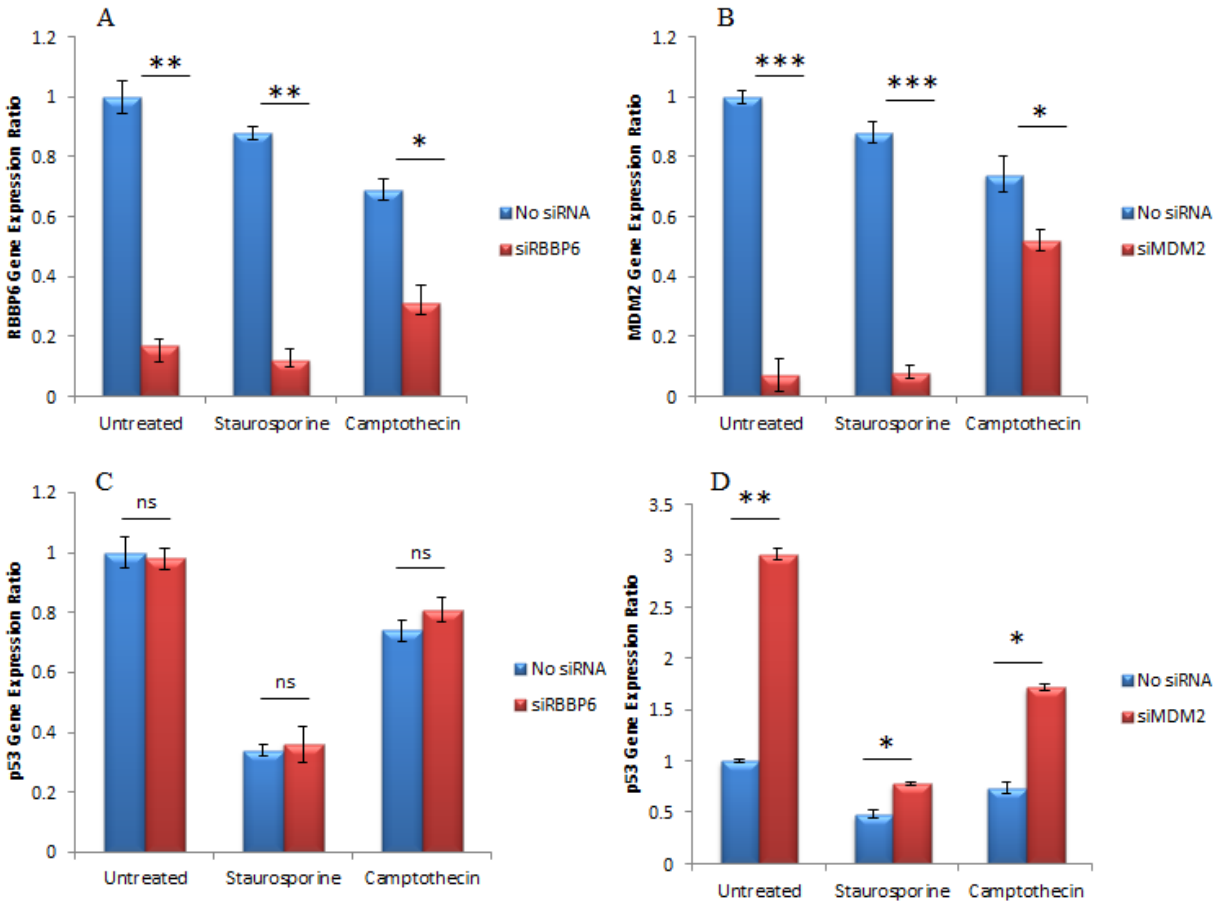


Figure 1.1b: Relative quantification of gene expression in CAMA-1 cells was performed using real time RT-PCR. **A** and **B** gene expression of RBBP6 and MDM2 in cells that were not transfected and either not treated, treated with *staurosporine* or *camptothecin*, silenced with either MDM2 or RBBP6 siRNAs and treated with either *staurosporine* or *camptothecin*. **C** and **D**, p53 gene expression after co-treatment of CAMA-1 cells with siRNAs targeting RBBP6 and MDM2, respectively, and the apoptosis-inducing agents (*staurosporine* and *camptothecin*). The experiments were done in duplicates and the data is statistically significant, p-value <0.05.

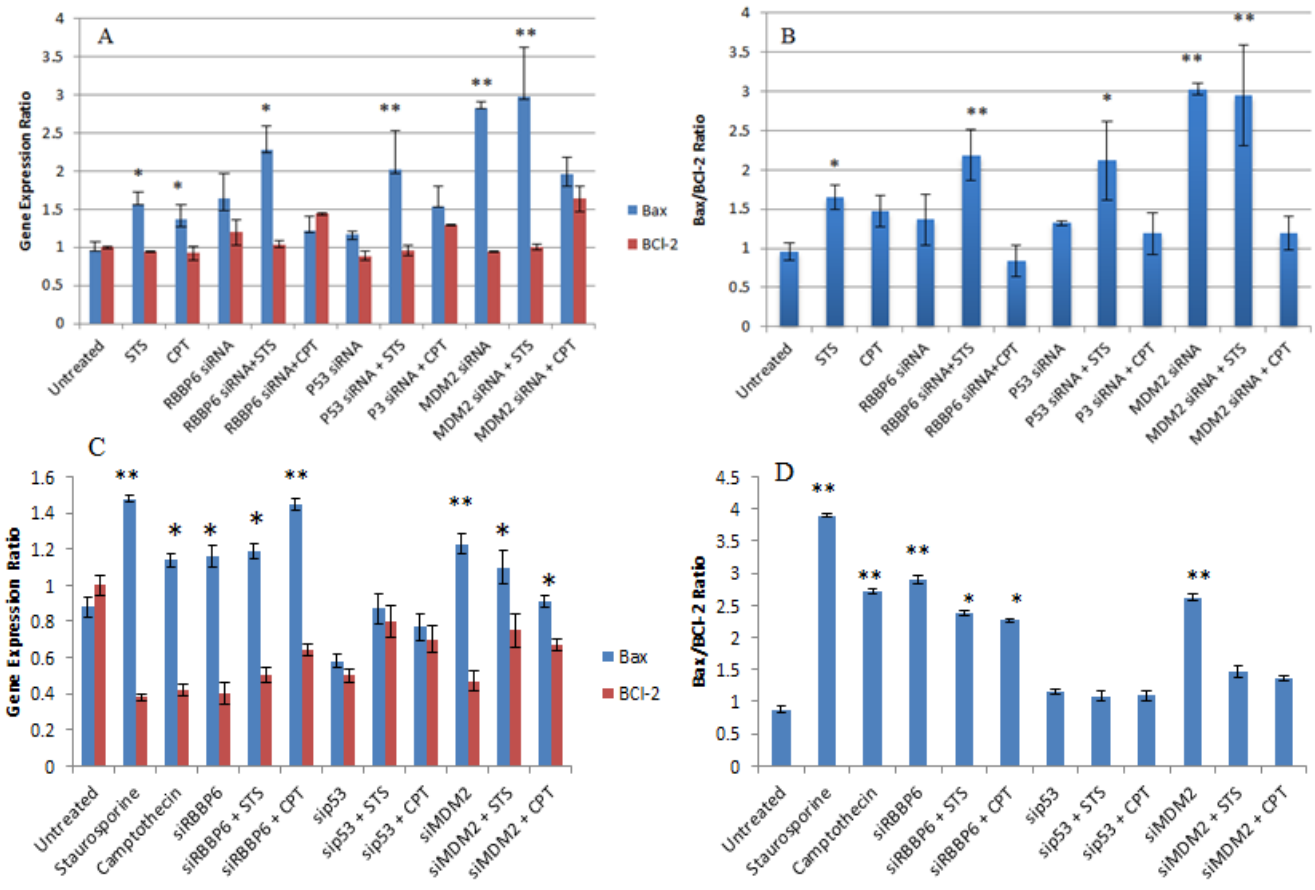


Figure 1.2: Relative quantification of gene expression in MCF-7 and CAMA-1 cells was performed using real time RT-PCR. **A** and **C**, gene expression of Bax and BCl-2 in MCF-7 and CAMA-1, respectively, that were not transfected and either not treated, treated with *staurosporine* or *camptothecin*, silenced with either RBBP6-, p53- and MDM2-targeting siRNAs then treated with either *staurosporine* or *camptothecin*. **B** and **D**, Bax/BCl-2 ratios after co-treatment of MCF-7 and CAMA-1 cells, respectively, with siRNAs targeting RBBP6, p53 and MDM2, and the apoptosis-inducing agents (*staurosporine* and *camptothecin*).

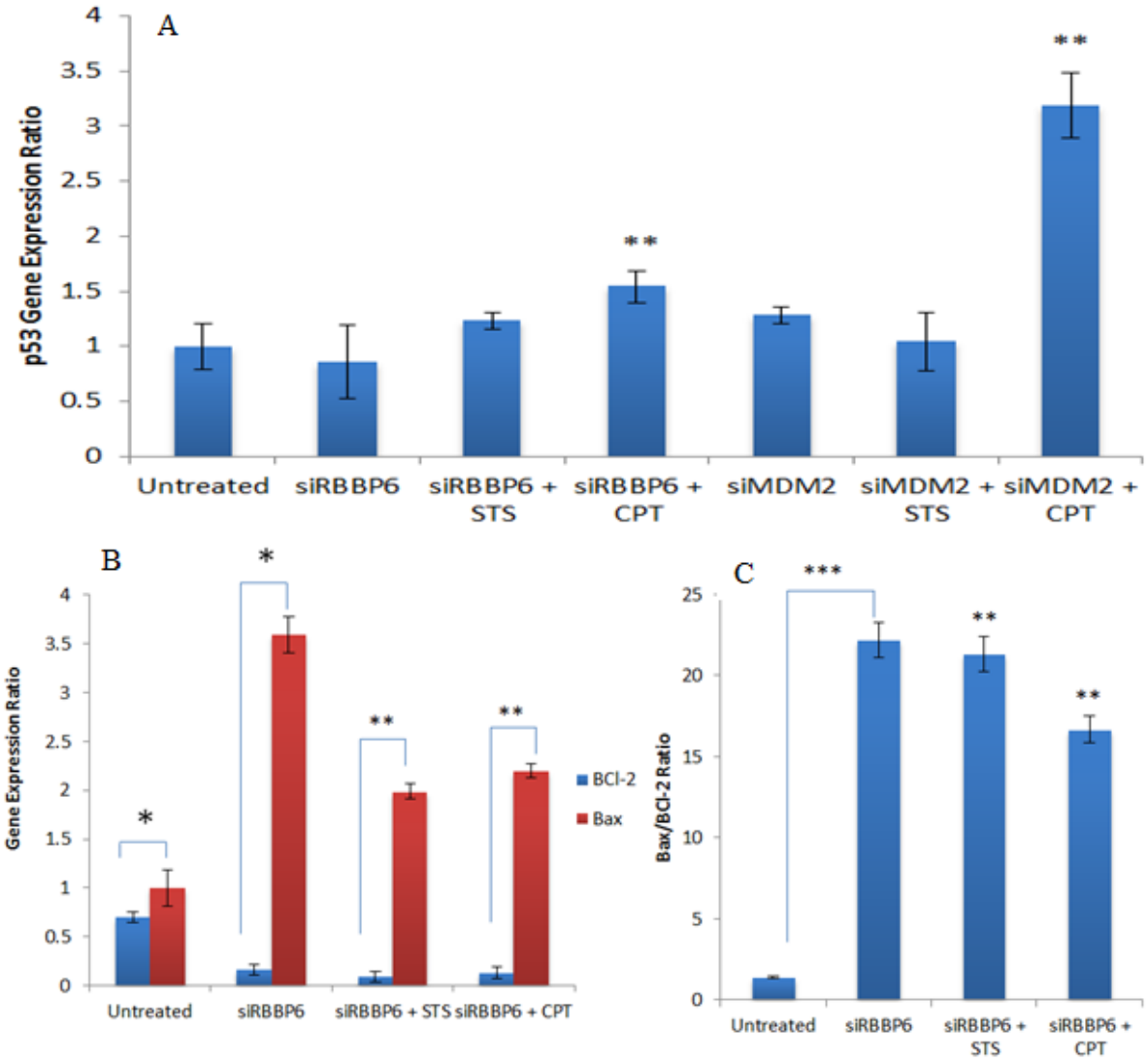


Figure 1.3: Relative quantification of gene expression in MRC-5 cells was performed using real time RT-PCR. **(A)** shows gene expression of p53 in cells that were not treated, silenced with either MDM2 or RBBP6 siRNAs and then treated with either *staurosporine* or *camptothecin*. **B and C**, Bax, BCl-2 relative expression and Bax/BCl-2 ratios after co-treatment of MRC-5 cells, respectively, with siRBBP6 and the apoptosis-inducing agents (*staurosporine* and *camptothecin*).

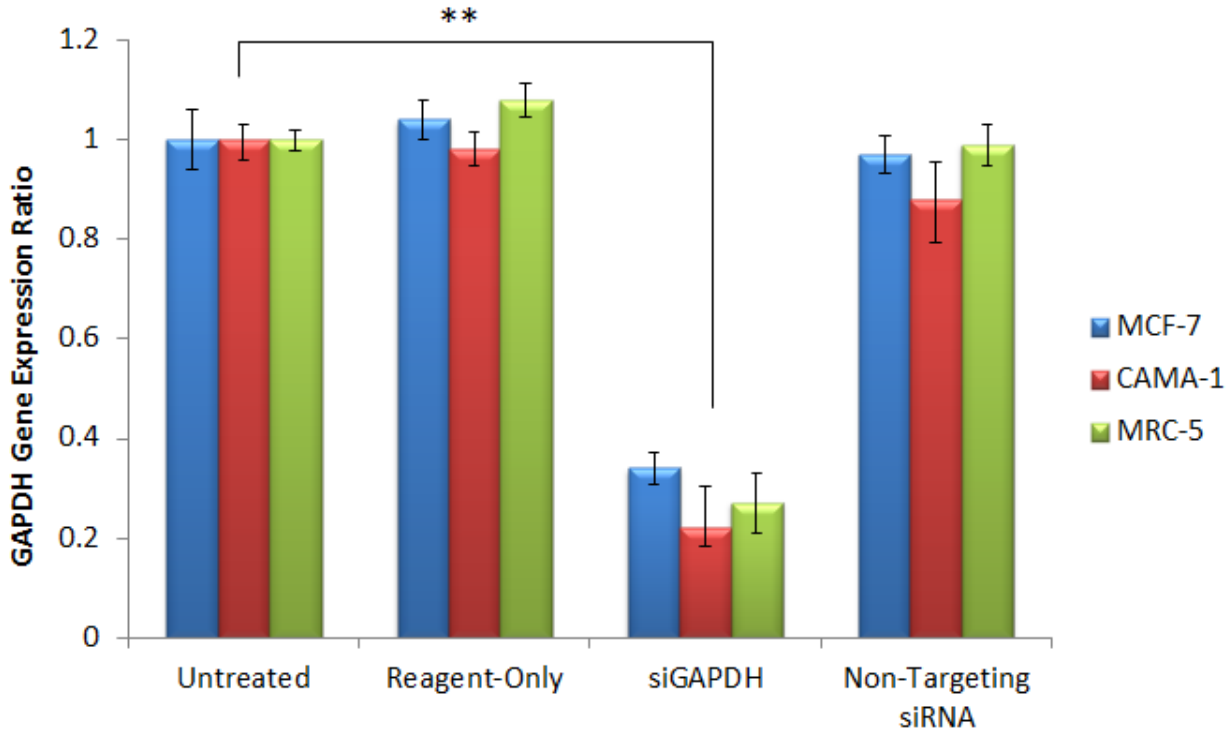


Figure 1.4: Relative quantification of GAPDH gene expression in MCF-7 (blue), CAMA-1(red) and MRC-5 (green) cells was performed as a control using real time RT-PCR before transfection and after transfection with either i) reagent-only, ii) siGAPDH (positive control) and iii) non-targeting siRNA (negative control).

4.3 Protein Expression by Western Blot Analysis

Having shown that using quantitative PCR silencing RBBP6 and MDM2 was successful in mRNA, it was necessary to further confirm the success at a protein level because in most cases mRNA is later translated by the cell machinery into functional protein. In this study, western blotting was employed to analyse protein expression following RBBP6 and MDM2 silencing. Briefly, cells were transfected for 48 hours with siRNAs (siRBBP6, sip53 and siMDM2) and then treated with either *staurosporine* or *camptothecin* for a further 24 hours, followed by whole cell protein extraction and analysis.

The expression of RBBP6, MDM2 and p53 proteins was significantly reduced following silencing in MCF-7, CAMA-1 and MRC-5 cells with their respective siRNAs in comparison to protein expression in cells that were untreated and those that were treated with *staurosporine* or *camptothecin* alone (**figure 2**). In MCF-7, Combination of siRNAs with either *staurosporine* or *camptothecin* almost led to a complete repression of RBBP6 and MDM2 proteins whereas reduction in p53 protein expression seems to be constant in siRNA-only and siRNA + *staurosporine*/*camptothecin*-treated cells (**figure2.1: A-C**). Silencing both RBBP6 and MDM2 followed by treatment with *Staurosporine* in MCF-7 cells resulted in an increase in bax

protein expression (**figure2.1: D**) whereas that of Bcl2 seemed to be reduced or remained unchanged (**figure2.1: E**).

Expression of RBBP6, p53 and MDM2 proteins after silencing RBBP6 gene were also measured in CAMA-1 and MRC-5 cell lines (**figure2.2: A- B)**. Expression of RBBP6 seems to decrease significantly in CAMA-1 cell lines following treatment with *camptothecin.* However, treatment with staurosporine slightly lowers RBBP6 expression in CAMA-1 cells (**figure2.2: A)**. siRBBP6 lowers RBBP6 expression and when combined with *camptothecin* the RBBP6 protein is almost completely silenced (**figure2.2: A)**. MDM2 expression in CAMA-1 cells is not affected much by *camptothecin*, nor RBBP6 silencing and siRBBP6-*camptothecin* treatment. However, treatment with staurosporine or staurosporine + siRBBP6 lowers the expression of MDM2 (**figure2.2: A).**

As expected, RBBP6 silencing up-regulates the expression of p53. However, combination of siRBBP6 and either staurosporine or *camptothecin* does not seem to alleviate p53 expression **(figure2.2: A)**. It was also noted that staurosporine treatment lowers the expression of p53 as well (**figure2.2: A)**. In MRC-5 cells MDM2 expression remains almost the same throughout treatment (**figure2.2: B).** RBBP6 expression decreases steadily and p53

remains constantly up-regulated after siRBBP6 treatment, siRBBP6+STS and siRBBP6+CPT (**figure2.2: B).**

SiRBBP6 in MCF-7, CAMA-1 and MRC-5 cells and siMDM2 in MCF-7 led to an up-regulation of p53 protein (**figure2.1: A-C** and **figure2.2 A-B**) whereas sip53 caused MDM2 down-regulation (**figure2: B**). These findings suggest that there might be a relationship between RBBP6, MDM2 and p53 at both mRNA and protein level.

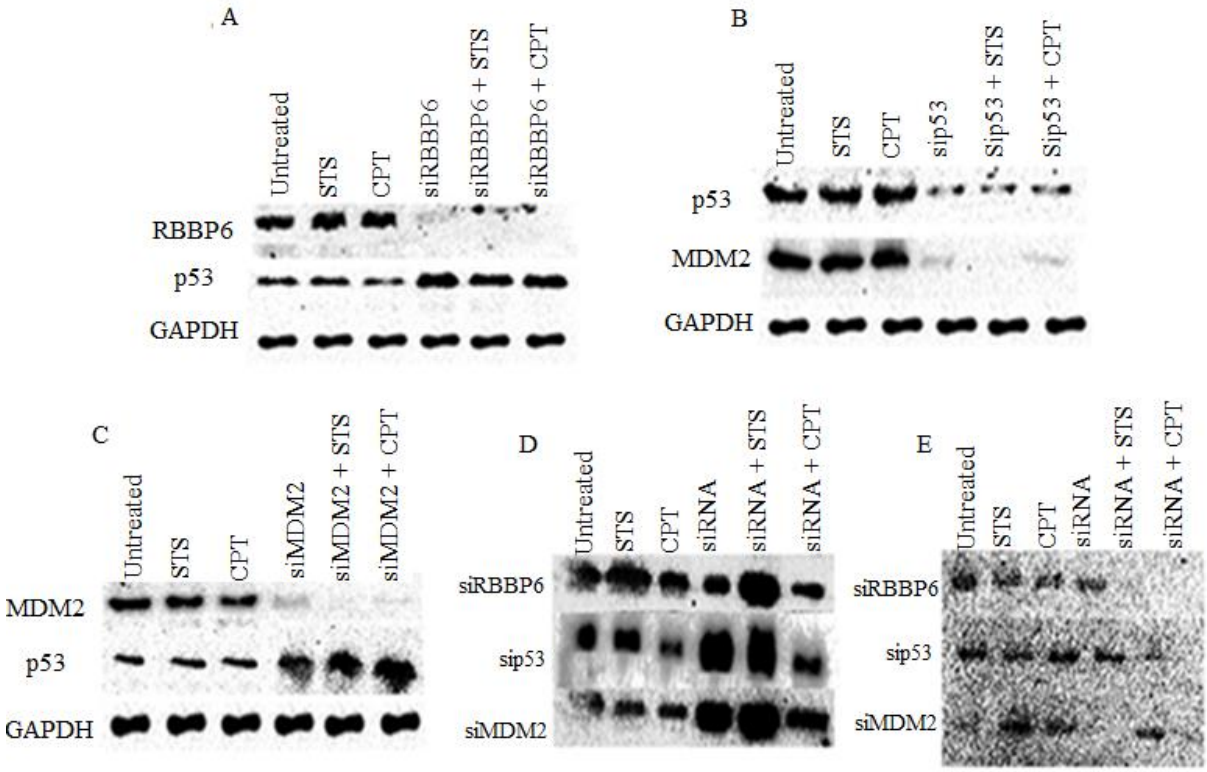


Figure 2.1: Protein expression in MCF-7 cells was performed using western blot technique. GAPDH was used to confirm that an equivalent amount of protein was loaded into each well. **A,** protein expression of RBBP6 and p53 in cells that were either not treated or co-treated with RBBP6 siRNAs and *staurosporine* (STS) or *camptothecin* (CPT). **B**, p53 and MDM2 protein expression after co-treatment of MCF-7 cells with

siRNA targeting p53 and the apoptosis-inducing agents (*staurosporine* and *camptothecin*). **C**, MDM2 and p53 protein expression after silencing with siRNAs targeting MDM2 and the apoptosis-inducing agents (*staurosporine* and *camptothecin*). **D** and **E**, protein expression of bax and BCl-2, respectively, in cells that were either not treated or co-treated with RBBP6-, p53- and MDM2-targeting siRNAs and *staurosporine* (STS) or *camptothecin* (CPT).

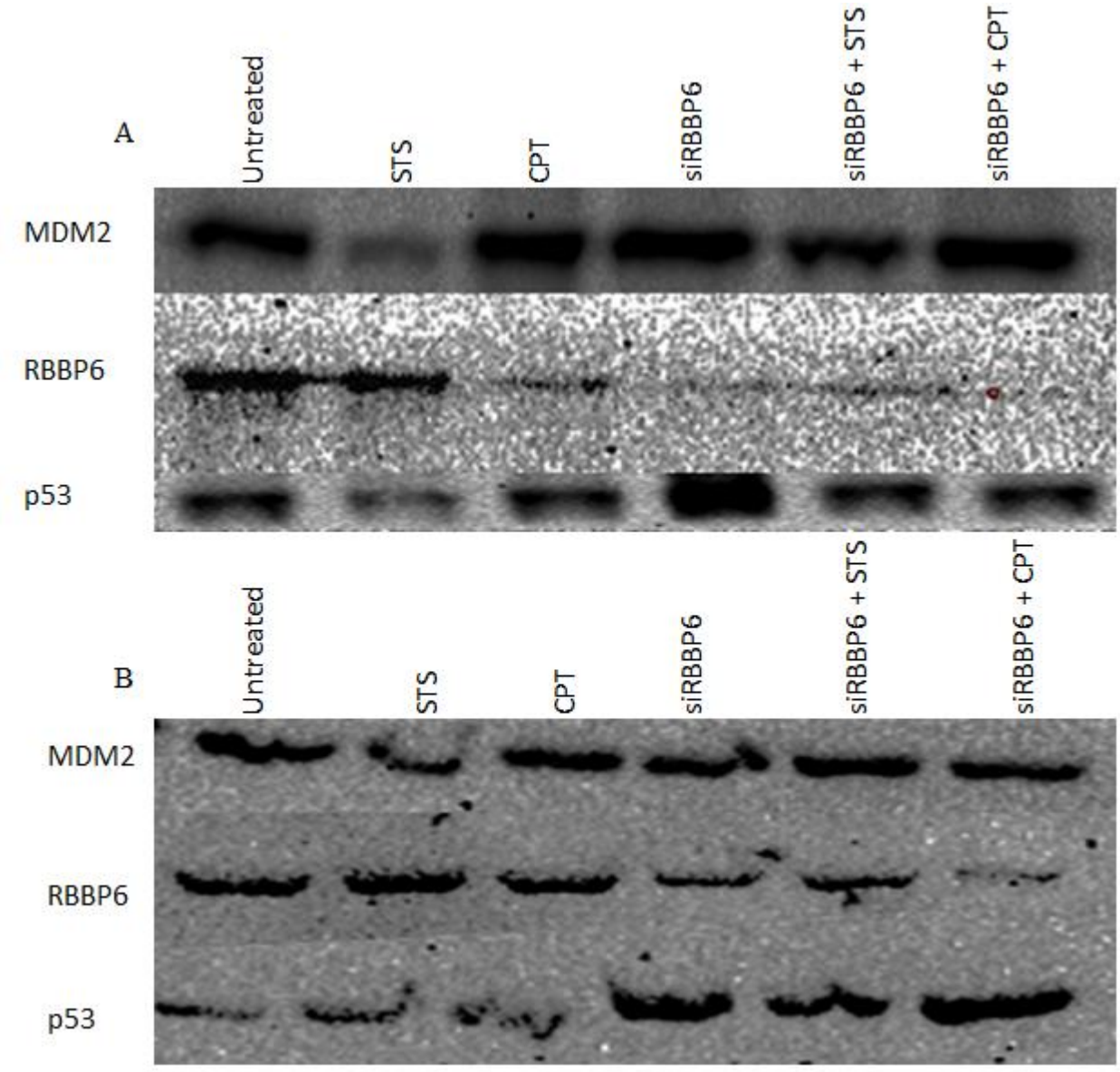


Figure 2.2: Protein expression in CAMA-1 **(A)** and MRC-5 **(B)** cells was performed using western blot technique. MDM2, RBBP6 and p53 protein expression was analysed in CAMA-1 and MRC-5 cells after co-treatment with RBBP6 siRNA and *staurosporine* (STS) or *camptothecin* (CPT).

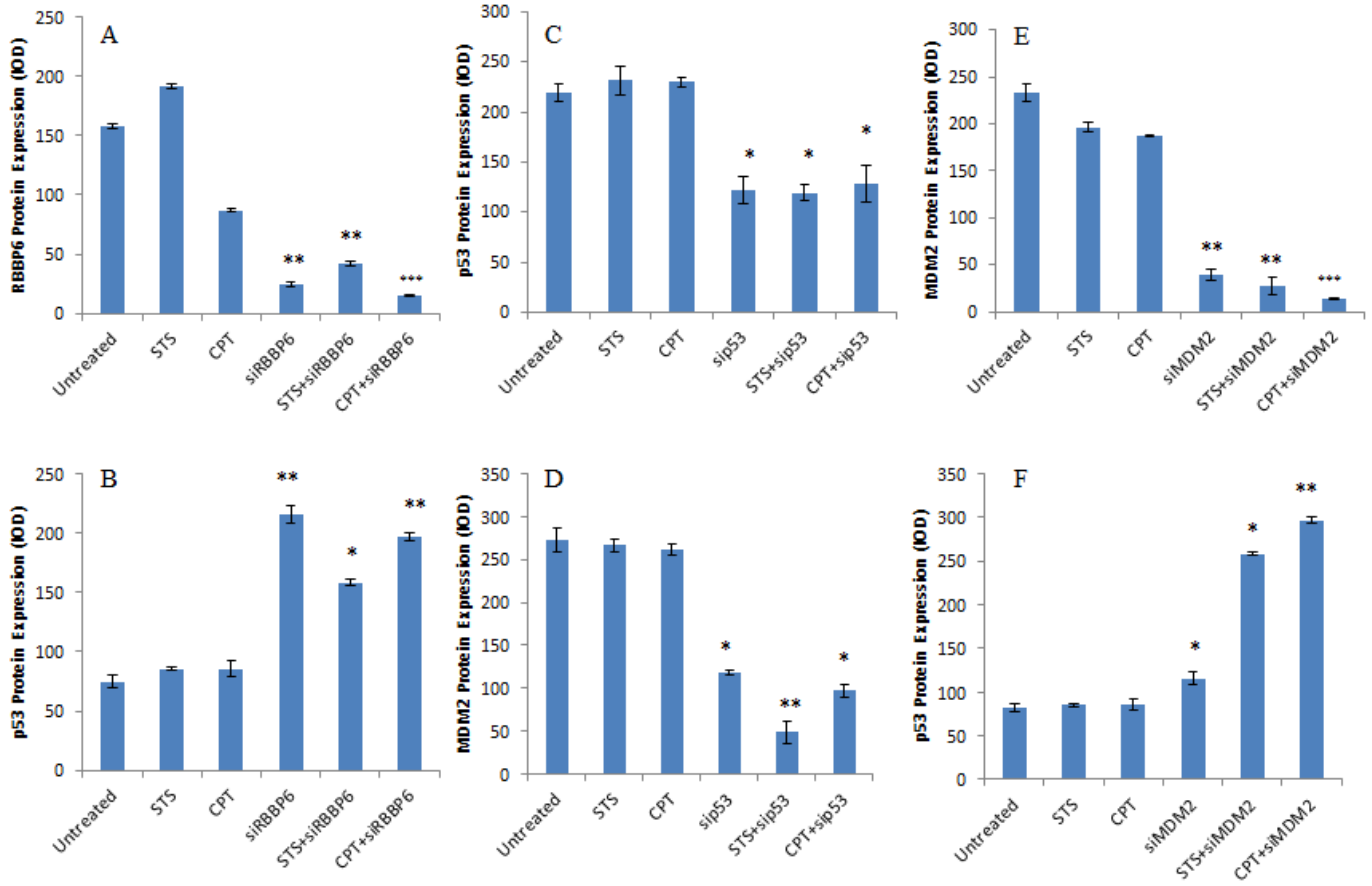


Figure 2.3: Statistical analysis of western blot protein expression analysis in MCF-7 cell line. T-test was used to generate p-values in order to compute the difference between treated and untreated scores. **A, B, C, D, E** and **F**, statistical analysis between untreated and silenced and between treated/silenced + treated (as described on y and x-axes). * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$ and ^{ns} indicates $p \geq 0.05$.

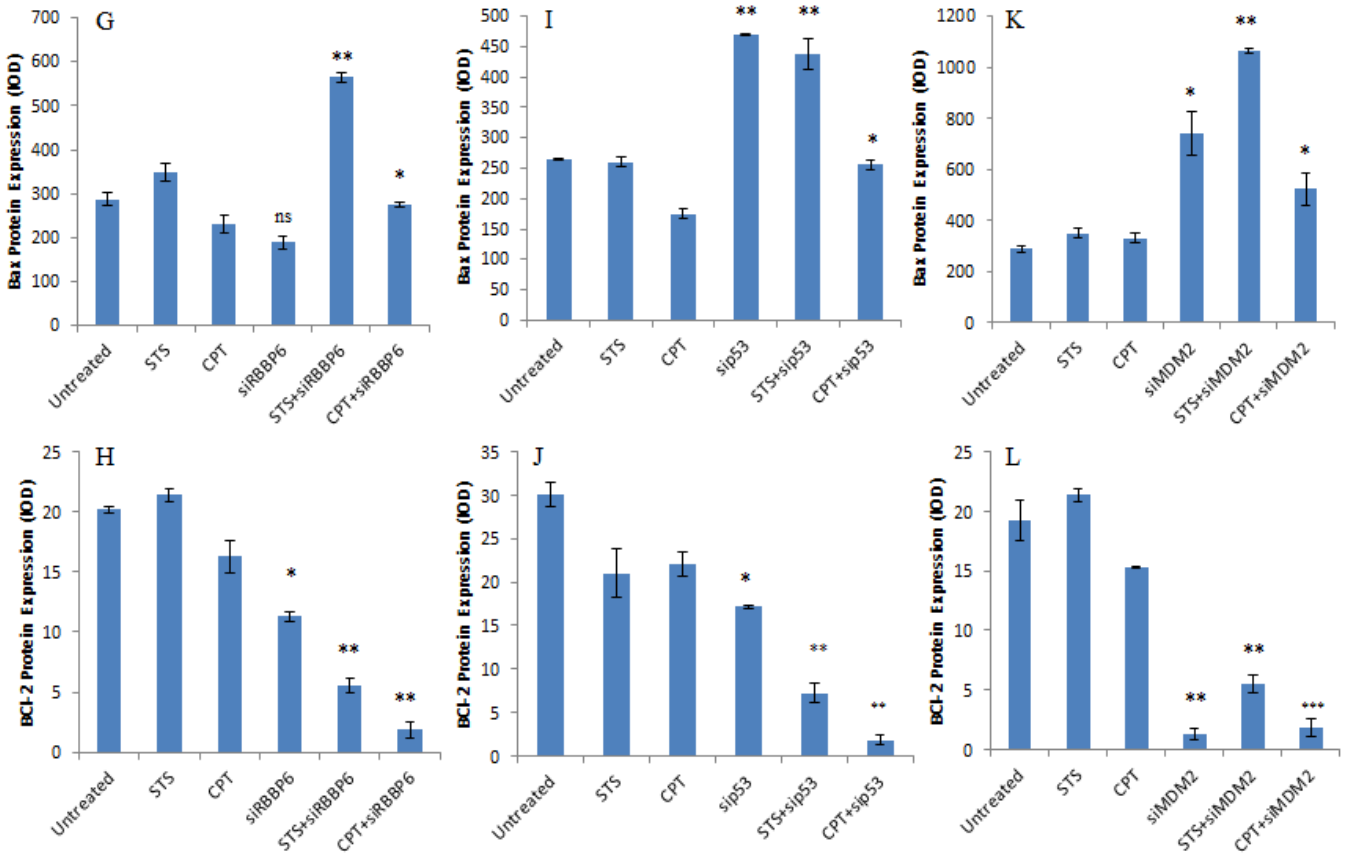


Figure 2.4: Statistical analysis of western blot protein expression (bax and bcl-2) analysis in CAMA-1 cell line. T-test was used to generate p-values in order to compute the difference between treated and untreated scores. **G, H, I, J, K** and **L**, statistical analysis between untreated and silenced and between treated/silenced + treated (as described on y and x-axes). * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$ and ^{ns} indicates $p \geq 0.05$.

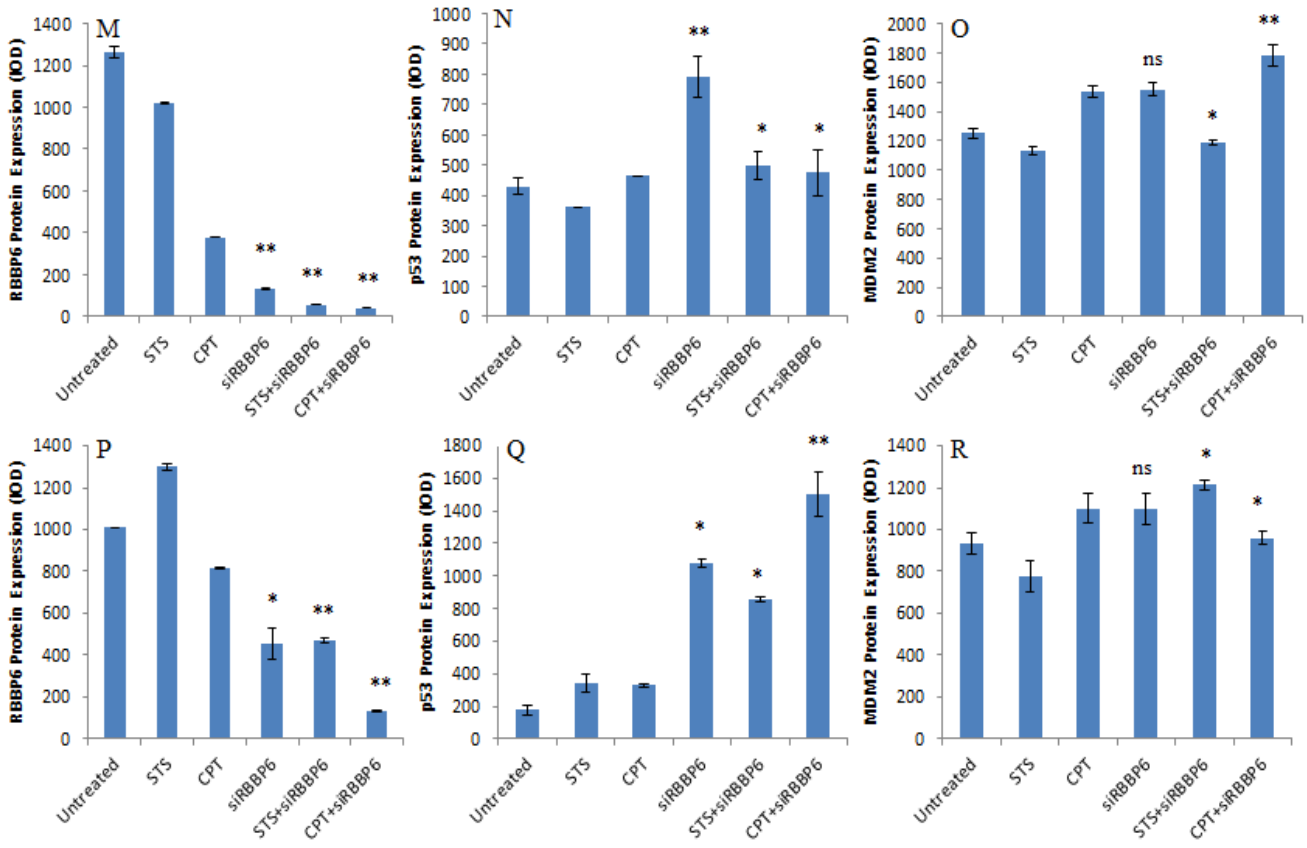


Figure 2.5: Statistical analysis of western blot protein expression analysis in CAMA-1 cell line **(M, N and O)** and in MRC-5 cell line **(P, Q and R).** T-test was used to generate p-values in order to compute the difference between treated and untreated scores, untreated and silenced and between treated/silenced + treated (as described on y and x-axes). * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$ and ^{ns} indicates $p \geq 0.05$.

4.4 Analysis of Cell Growth after Co-treatment

The objective of this section was to monitor the effect of RBBP6 silencing and its related genes on cell viability of cultured adherent cells. To accomplish this, the xCELLigence real time cell analyser was used which is a non-invasive label-free technique that is based on an increase in electrode impedance in response to cell adhesion and spreading on the surface of a biocompatible gold-coated 16-well E-plate. The cells were monitored over a period of about 2 days with 24 hours of silencing-only followed by another 24 hours of co-treatment with either *staurosporine* or *camptothecin*.

In response to gene silencing and induction of apoptosis with either *staurosporine* or *camptothecin*, we verified the growth inhibitory effect of siRBBP6, siMDM2 and sip53 (100nM) in MCF-7and CAMA-1 breast cancer cells (**figure 3.1 and 3.2**). Growth curves were normalised to the cell index (CI) at the last measured time point before compound addition for each well. MCF7 growth curve, which is presented in **figure 3.1** showed a significant reduction in cellular growth after silencing with siRBBP6 and siMDM2 whereas sip53 reduces cell growth at a slower rate. Combination of siRNAs and the apoptosis-inducing agents (*staurosporine* or *camptothecin*) further reduces cell growth as shown in **figure 3.1: B and C**. *Staurosporine* reduced

cell growth at a much faster rate as compared to *camptothecin* (**figure 3.1: B and C**).

In CAMA-1 breast cancer cells, RBBP6-knock down population shows a higher rate of growth inhibition following treatment with *camptothecin* as compared to the p53- and MDM2-knock down populations (**figure 3.2: E**). When comparing results obtained from breast cancer cells (MCF-7 and CAMA-1) to those obtained from MRC-5 fibroblasts, almost similar pattern of growth inhibition was observed however at a minimal level (**figure3.3: G-I**). Staurosporine and siRBBP6 did not have as much effect as in MCF7 cells (**figure 3.3: I**).

These findings suggest that both co-treatments (siRNAs + *staurosporine* and siRNAs + *camptothecin*) induce cell death. However, the siRNAs + *staurosporine* combination might be more potent in the cells as seen by the rapid fall in cellular growth.

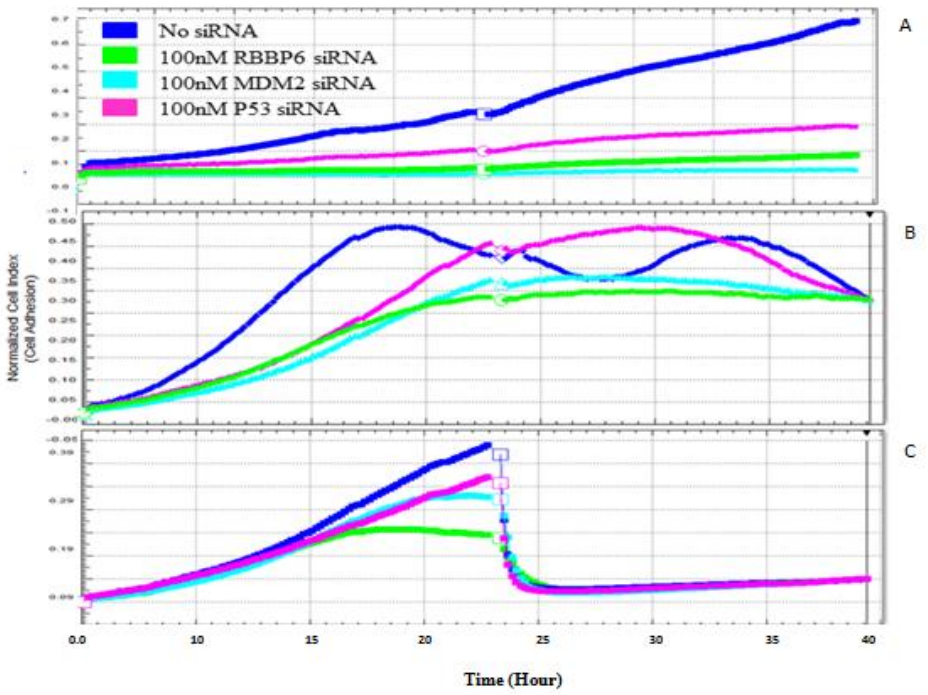


Figure 3.1: Cell growth of MCF-7 cells was analysed using the xCELLigence system which relies on the generation of electrical impedance as cell growth by 16-well plates coated with a gold microelectron covering at the base of each well. The y-axis shows normalised cell index or cell adhesion over a period of about 48 hours (x-axis). The growth patterns highlighted in blue show cells that are not treated; those highlighted in green, light blue and pink show cells that were transfected with siRBBP6, siMDM2 and sip53, respectively. **A** shows growth curves of cell populations that were silenced only and not treated and treatment with *staurosporine* or *camptothecin* (**B** and **C**, respectively) was introduced 24 hours post transfection

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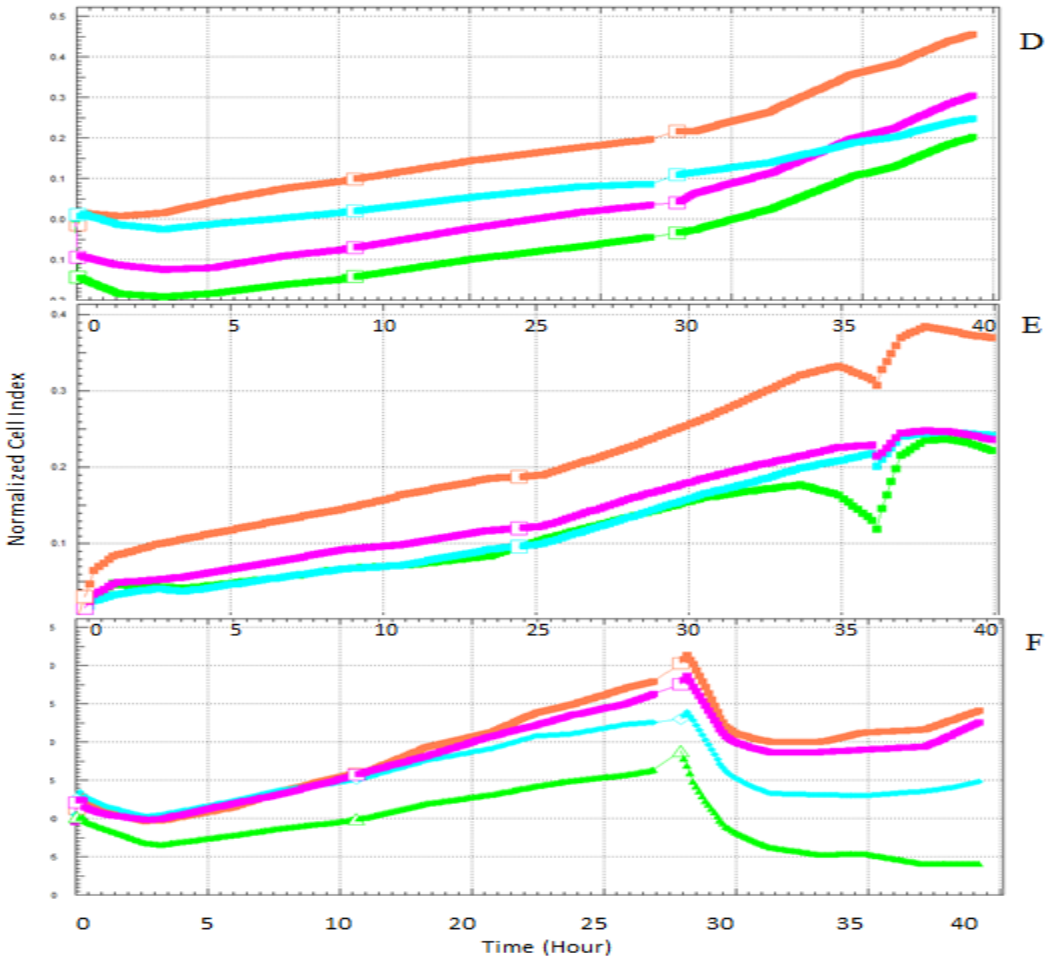


Figure 3.2: Cell growth of CAMA-1 cells was analysed using the xCELLigence system which relies on the generation of electrical impedance as cell growth by 16-well plates coated with a gold microelectron covering at the base of each well. The y-axis shows normalised cell index or cell adhesion over a period of about 48 hours (x-axis). The growth patterns highlighted in orange show cells that were exposed to transfection reagent only and those highlighted in green, light blue and pink show cells that were transfected with siRBBP6, siMDM2 and sip53, respectively. **D** shows growth curves of cell populations that were silenced only and not treated and treatment with *staurosporine* or *camptothecin* (**E** and **F**, respectively) was introduced 24 hours post transfection.

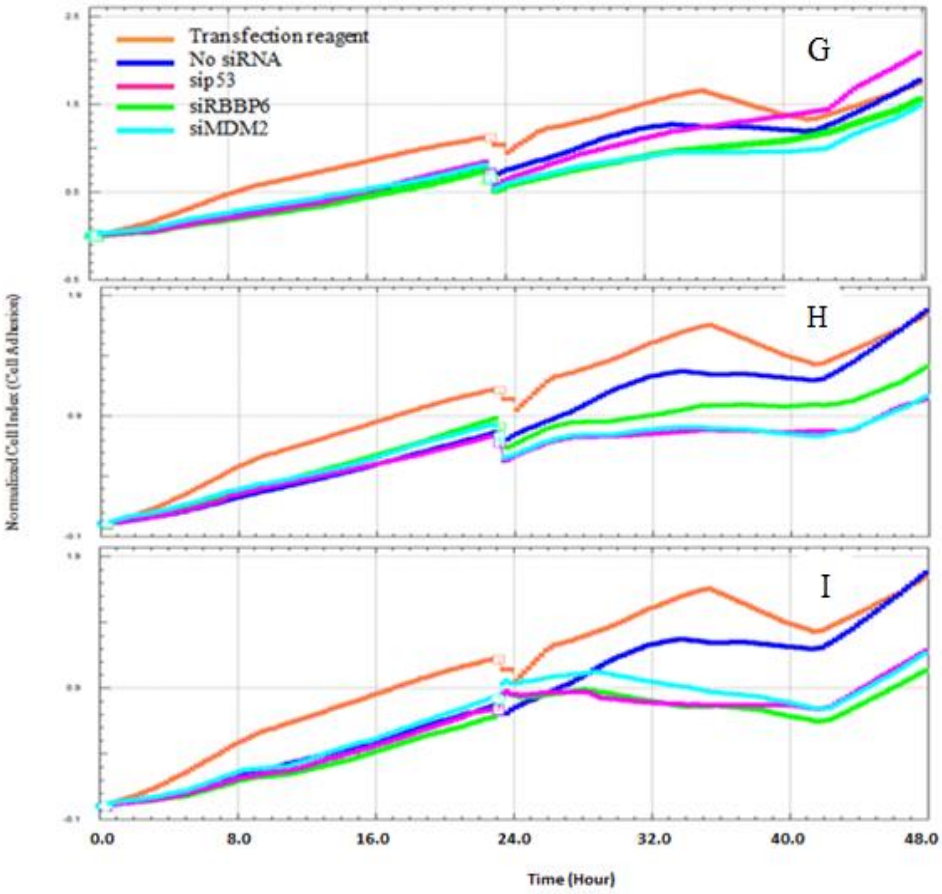


Figure 3.3: Cell growth of MRC-5 cells was analysed using the xCELLigence system which relies on the generation of electrical impedance as cell growth by 16-well plates coated with a gold microelectron covering at the base of each well. The y-axis shows normalised cell index or cell adhesion over a period of about 48 hours (x-axis). The growth patterns highlighted in orange show cells that were exposed to transfection reagent only and those highlighted in green, light blue and pink show cells that were transfected with siRBBP6, siMDM2 and sip53, respectively. **G** shows growth curves of cell populations that were silenced only and not treated and treatment with *staurosporine* or *camptothecin* (**H** and **I**, respectively) was introduced 24 hours post transfection

4.5 Apoptosis Detection

As shown in the previous section, gene silencing coupled with either of the apoptosis inducers (*staurosporine* or *camptothecin*) led to reduced cell viability in breast cancer cells MCF-7 and CAMA-1. To identify whether the

observed cell death was as a result of apoptosis or necrosis, flow cytometry analysis was used. Annexin V and PI staining make flow cytometry an effective technique to distinguish between necrosis, early and late apoptosis in cultured cells. In this section, we measured apoptosis following co-treatment with siRNAs (siRBBP6, sip53, and siMDM2) and apoptosis inducers.

Analysis by flow cytometry revealed that the percentage of apoptotic cells following treatment with *camptothecin* and *staurosporine* was significantly increased in MCF-7 cells to 53.2% and 55.9% respectively ($p<0.05$) (**figure4: c and d**) in comparison to untreated cells at only 0.8% **(figure4: b)**. siRBBP6 significantly increased apoptosis in MCF-7 cells treated with *camptothecin* (20.7% increment). However, only 8.8% increase in apoptosis was observed in MCF-7 cells that were treated with siRBBP6 and *staurosporine* (**figure5: e and f**). MDM2 silencing in MCF-7 significantly induced apoptosis in *camptothecin*-treated cells by 26.6% as compared to that of s*taurosporine* where only 0.5% increase in apoptosis was observed (**figure5: g and h**). sip53 reduces *camptothecin*-induced and *staurosporine*-induced apoptosis by 26% and 30.7%, respectively in MCF-7 (**figure6: I and j**) which was far less than those of MDM2 and RBBP6.

In CAMA-1 cells, silencing RBBP6 in combination with *staurosporine* decreased the level of *staurosporine*-induced apoptosis from 59.1% to 50.7%

whereas combination of siRBBP6 with *camptothecin* shifted *camptothecin*-induced apoptosis from 56% to 61% **(figure8: B-F)**. Similarly, MDM2 silencing led to a negative shift of apoptosis when combined with *staurosporine* (from 59.1% to 54.2) and a positive shift when combined with *camptothecin* (56.1% to 67.3%) **(figure8: J-L)**. Untreated CAMA-1 cells showed only 0.3% apoptosis. We further observed that apoptosis induction in CAMA-1 cells that were p53-silenced, sip53+STS- and sip53+CPT-treated was minimal (46.9%, 39.1% and 28.6%, respectively) in comparison to apoptosis induced in CAMA-1 cells that were treated with only *staurosporine* or *camptothecin* (59.1% and 56.1, respectively) **(figure8: G-I)**.

Apoptosis induction in MRC-5 fibroblast was fairly low following treatment with *staurosporine* or *camptothecin* (15.3% and 18.7%, respectively) as compared to the one observed in MCF-7 and CMA-1 cells **(figure9: B and C)**. Only 2.5% apoptosis was observed in untreated MRC-5 cells. Silencing MRC-5 with siRBBP6, sip53 and siMDM2 induced 15.8%, 12.3% and 20.5% apoptosis, respectively **(figure9 and 10)**, whereas combination with *staurosporine* led to 13.5%, 13.3% and 12.6% apoptosis induction, respectively **(figure 9 and 10)**. Combination of siRBBP6, sip53 and siMDM2 with *camptothecin* in MRC-5 led to 12%, 14.8% and 15.4% apoptosis, respectively **(figure 9 and 10)**.

Double silencing with siRBBP6 and sip53 in both CAMA-1 and MCF-7 cells showed a slightly lower apoptosis in comparison to siRBBP6 only **(figure 11).** siRBBP6/sip53 double silencing led to 36.7% apoptosis in MCF-7 and 39.4% apoptosis in CAMA-1 cells. Co-treatment with *staurosporine* led to 50.7% apoptosis in MCF-7 and 50.8% in CAMA-1 cells whereas co-treatment with *camptothecin* led to 50.9% and 41.8% apoptosis in MCF-7 and CAMA-1 cells, respectively **(figure 11)**.

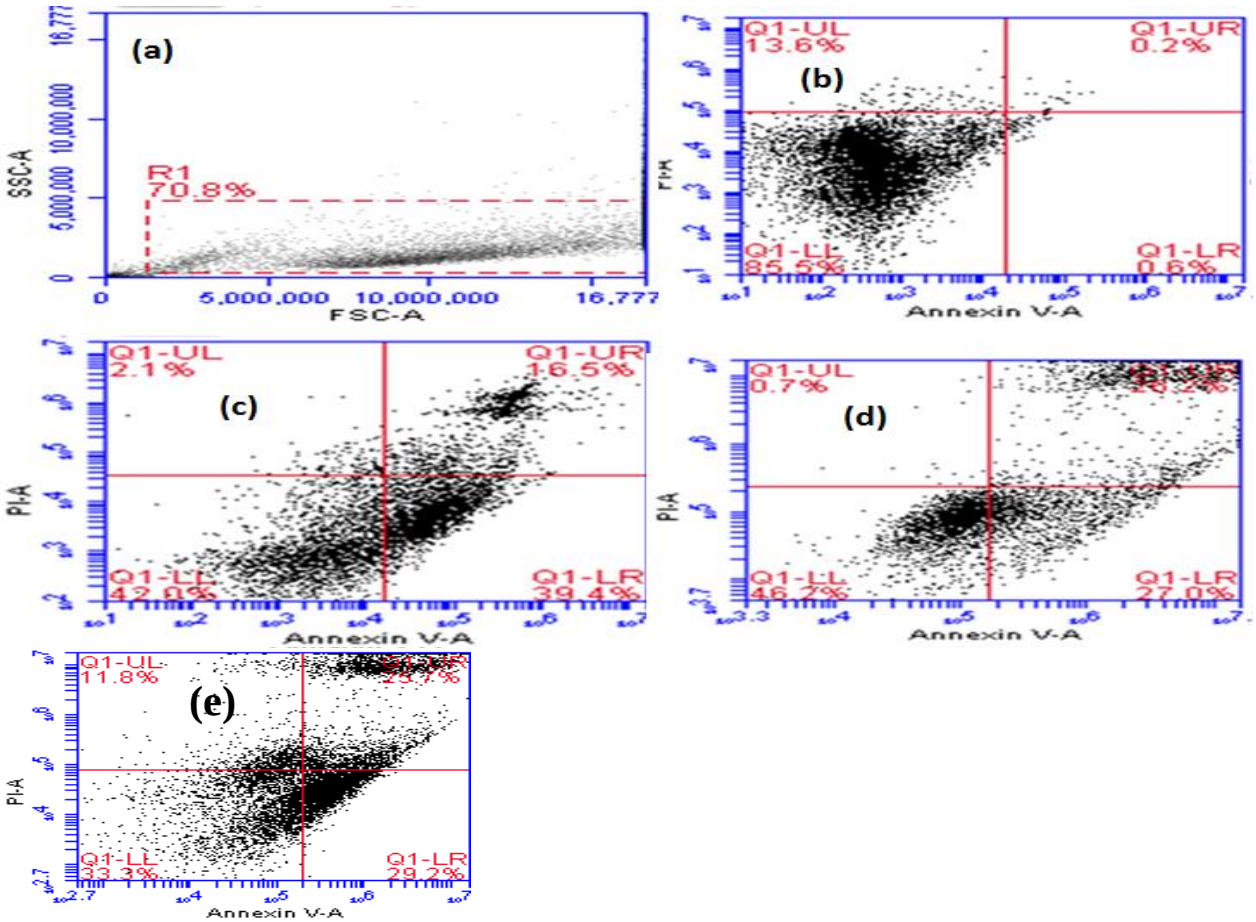


Figure 4: Apoptosis of MCF-7 cells was analysed using flow cytometer with annexin V and PI. Upper left quadrants show necrotic cells which stain positive for PI and negative for annexin V, upper right quadrants show cells undergoing late apoptosis with positive staining for both PI and annexin V. Lower right quadrants show cells undergoing early apoptosis and are staining positive for annexin V and negative for PI; and lastly the lower left quadrants show viable cells. **(a)** Gating of cells showing the side and

forward scatter. **(b)** Shows cells before treatment, **(c) and (d)** show cells after treatment with 0.25µM *staurosporine* and 0.25µM *camptothecin*, respectively. **(e)** Shows cells after transfection with siRBBP6-only.

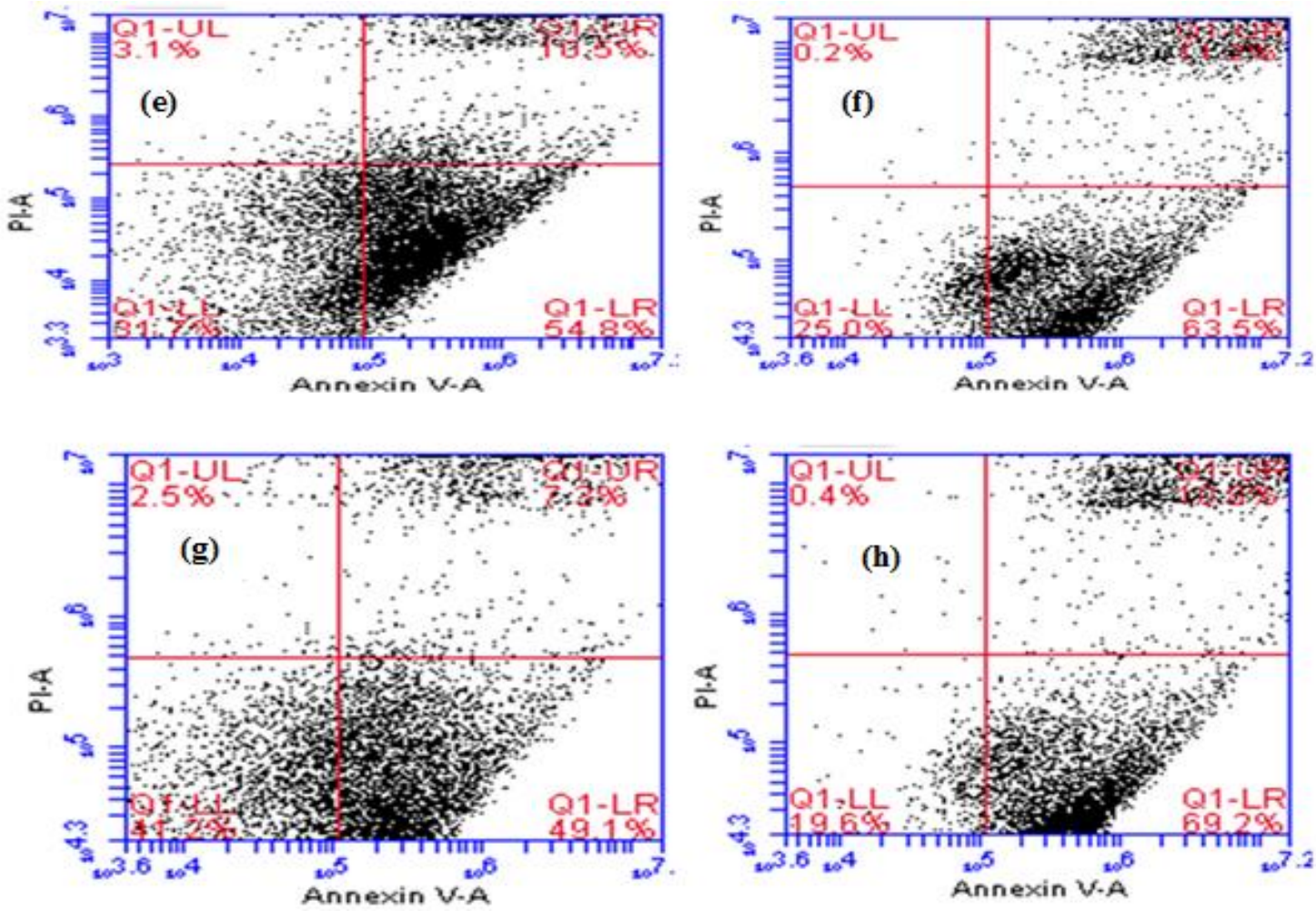


Figure 5: Apoptosis of MCF-7 cells was analysed using flow cytometer with annexin V and PI. **(e)** MCF-7 cells after co-treatment with 100nM RBBP6 siRNA and 0.25µM *staurosporine* and **(f)** MCF-7 cells after co-treatment with 100nM RBBP6 siRNA and 0.25µM *camptothecin*. **(g)** MCF-7 cells after co-treatment with 100nM MDM2 siRNA and 0.25µM *staurosporine* and **(h)** MCF-7 cells after co-treatment with 100nM MDM2 siRNA and 0.25µM *camptothecin*.

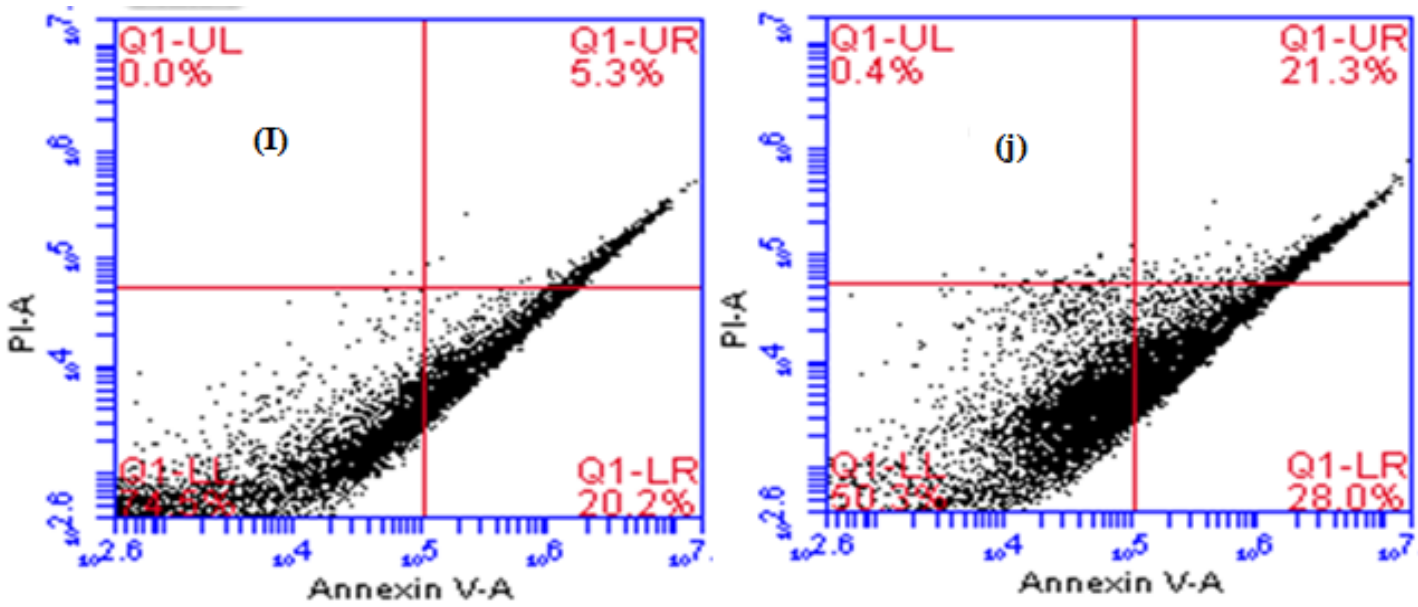


Figure 6: Apoptosis of MCF-7 cells was analysed using flow cytometer with annexin V and PI. **(h)** MCF-7 cells after co-treatment with 100nM p53 siRNA and 0.25µM *staurosporine* and **(i)** MCF-7 cells after co-treatment with 100nM p53 siRNA and 0.25µM *camptothecin*.

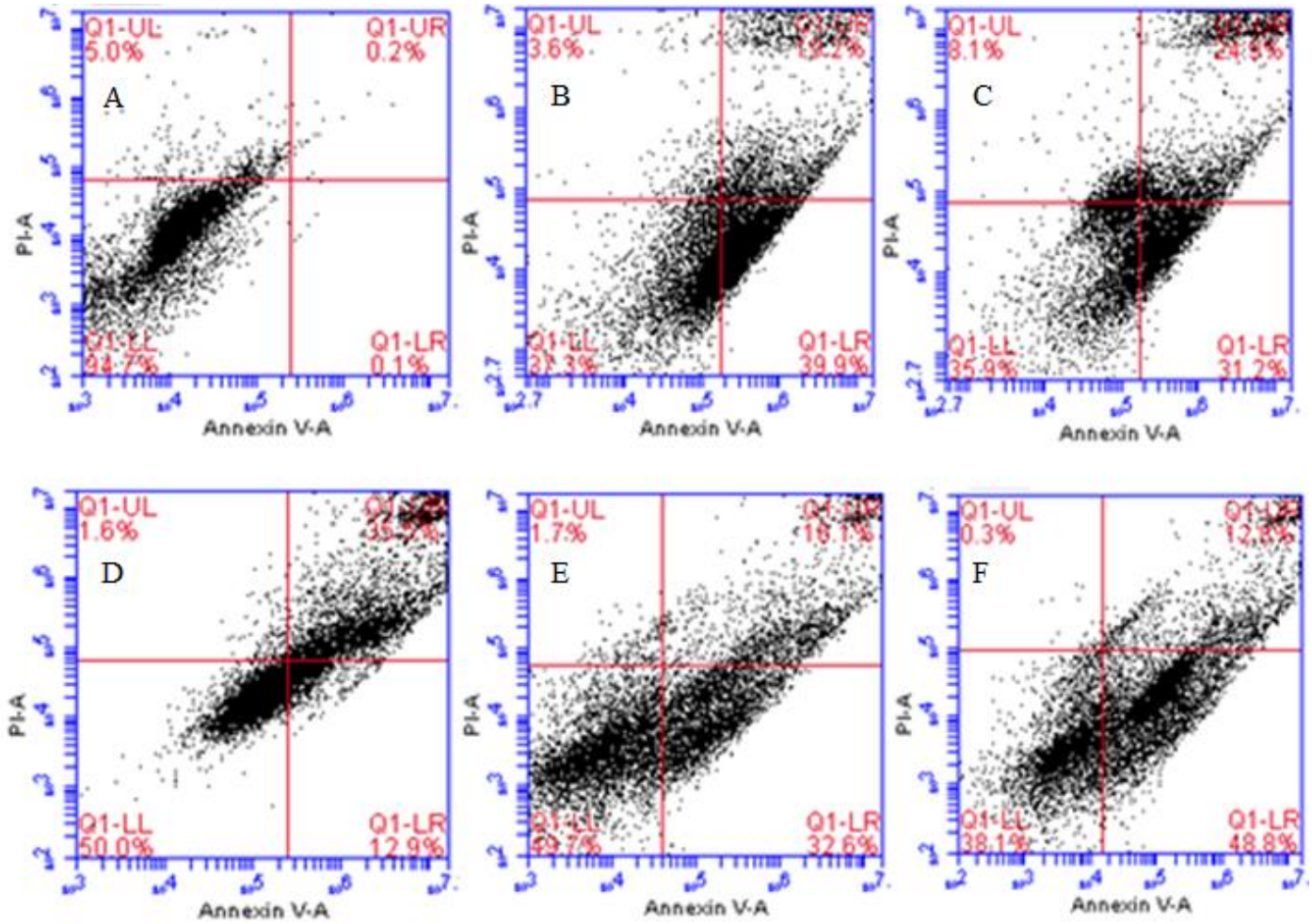


Figure 7: Apoptosis of CAMA-1 cells was analysed using flow cytometer with annexin V and PI. **(A)** apoptosis in untreated cells, **B** and **C** cells after treatment with *staurosporine* and *camptothecin*, respectively. **D**, **E**, and **F**, apoptosis in cells co-treated with 100nM siRBBP6, 100nM siRBBP6 + 0.25µM *staurosporine* and 100nM siRBBP6 + 0.25µM *camptothecin*, respectively.

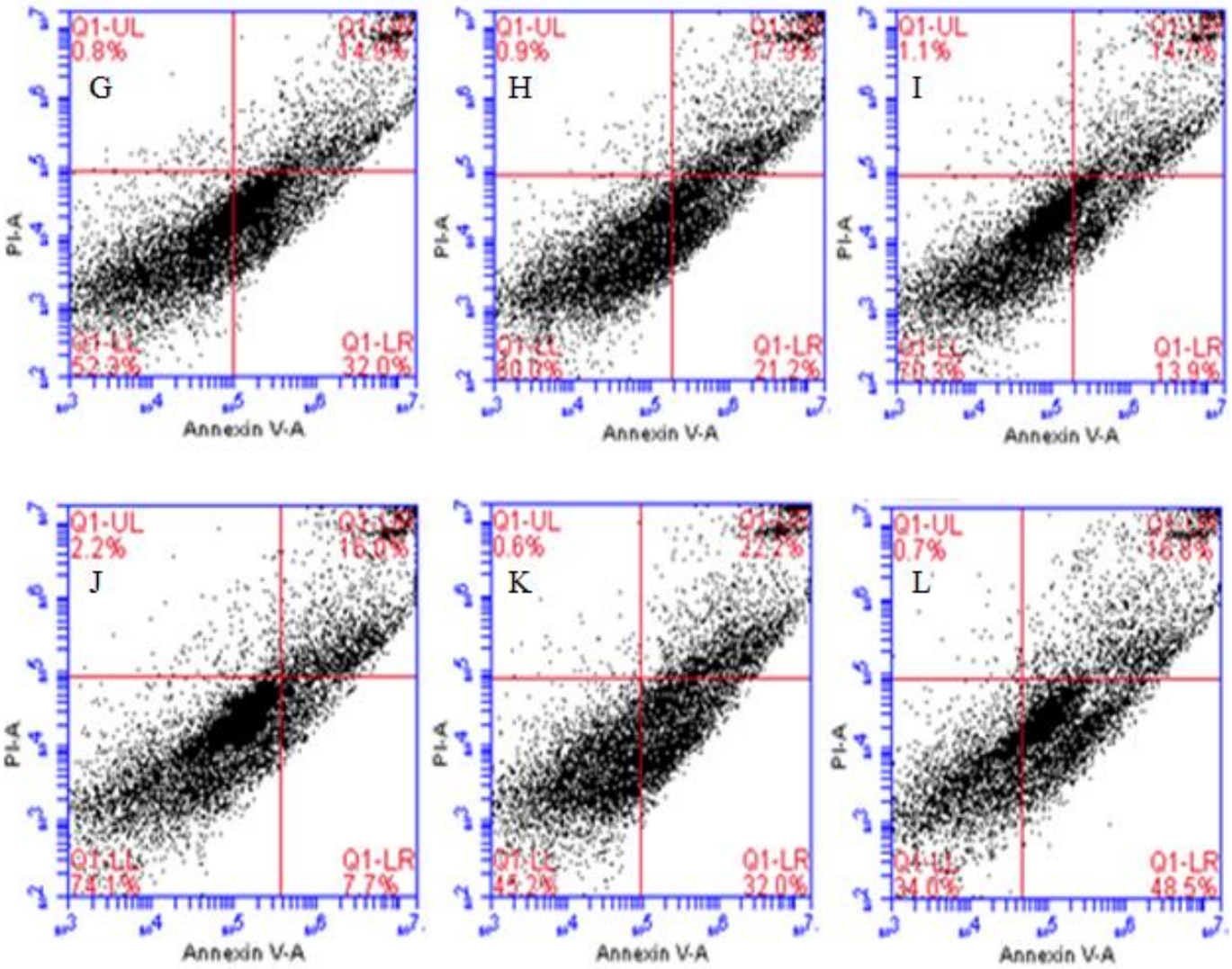


Figure 8: Apoptosis of CAMA-1 cells was analysed using flow cytometer with annexin V and PI. **G**, **H** and **I**, apoptosis in cells co-treated with 100nM sip53, 100nM sip53 + 0.25µM *staurosporine,* and 100nM sip53 + 0.25µM *camptothecin,* respectively, and **J, K** and **L**, apoptosis in cells co-treated with 100nM siMDM2, 100nM siMDM2 + 0.25µM *staurosporine* and 100nM siMDM2 + 0.25µM *camptothecin,* respectively.

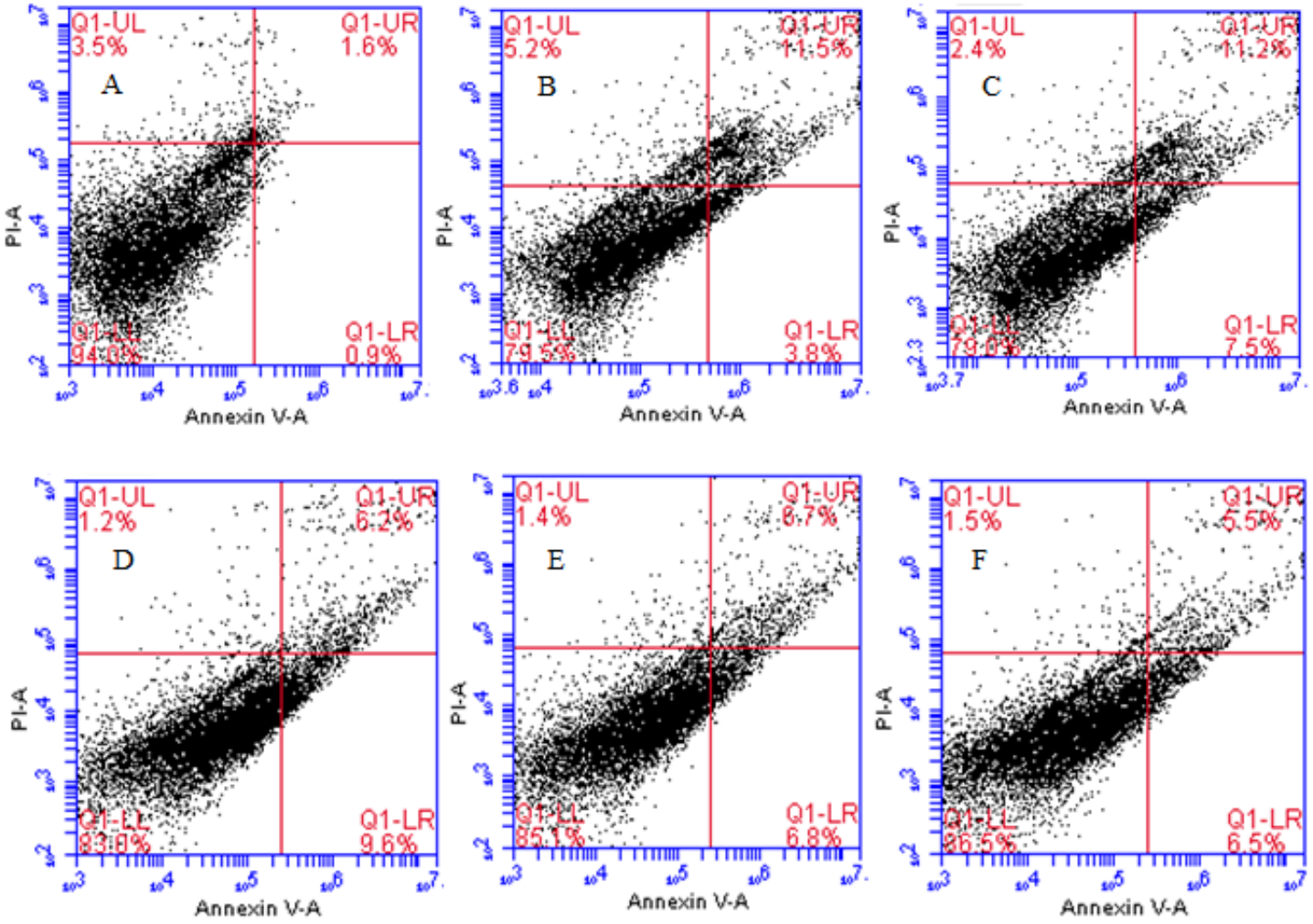


Figure 9: Apoptosis of MRC-5 cells was analysed using flow cytometer with annexin V and PI. **A** apoptosis in untreated cells, **B** and **C** cells after treatment with *staurosporine* and *camptothecin*, respectively. **D**, **E**, and **F**, apoptosis in cells co-treated with 100nM siRBBP6, 100nM siRBBP6 + 0.25µM *staurosporine* and 100nM siRBBP6 + 0.25µM *camptothecin*, respectively.

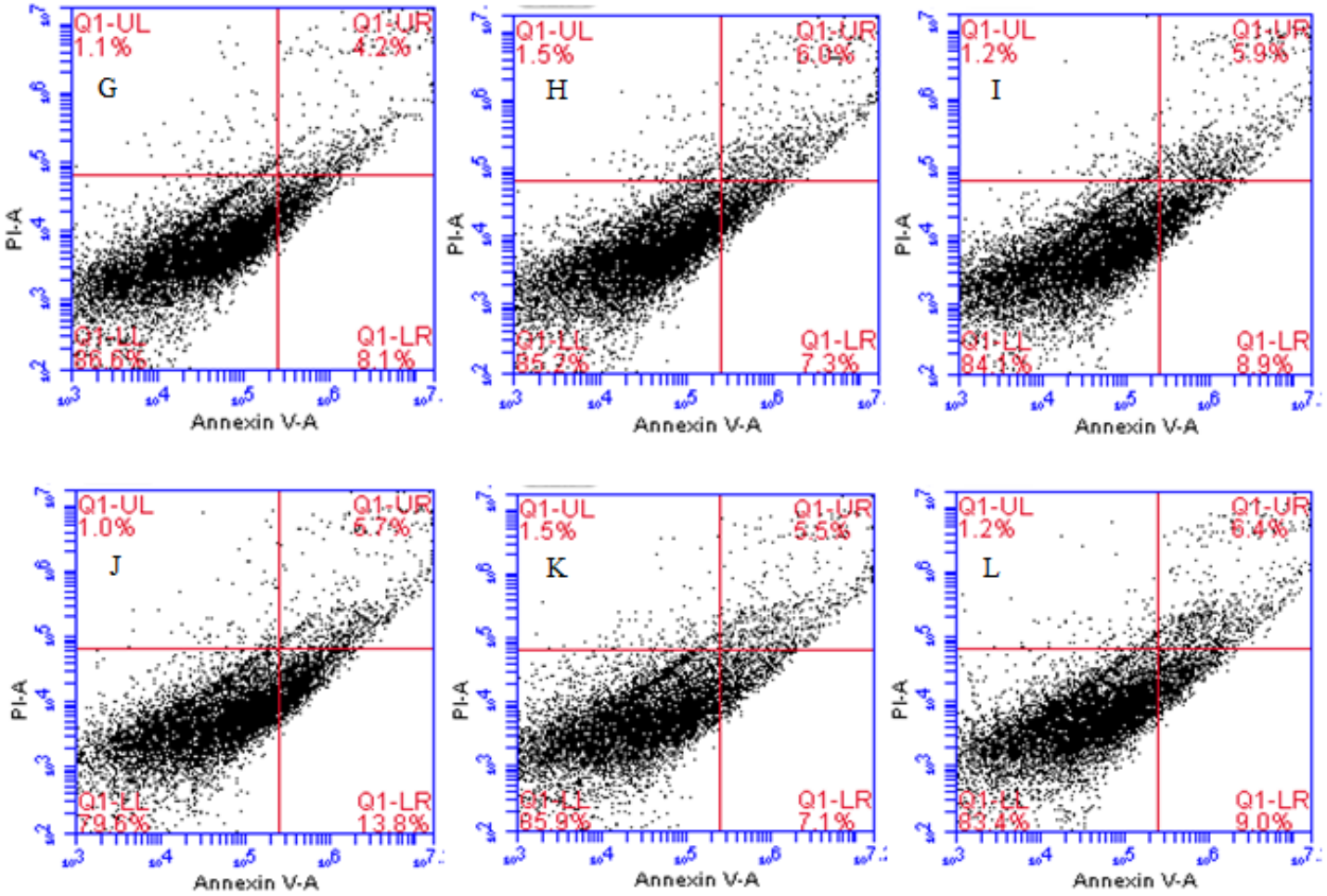


Figure 10: Apoptosis of MRC-5 cells was analysed using flow cytometer with annexin V and PI. **G**, **H** and **I**, apoptosis in cells co-treated with 100nM sip53, 100nM sip53 + 0.25µM *staurosporine*, and 100nM sip53 + 0.25µM *camptothecin*, respectively, and **J, K** and **L**, apoptosis in cells co-treated with 100nM siMDM2, 100nM siMDM2 + 0.25µM *staurosporine* and 100nM siMDM2 + 0.25µM *camptothecin*, respectively.

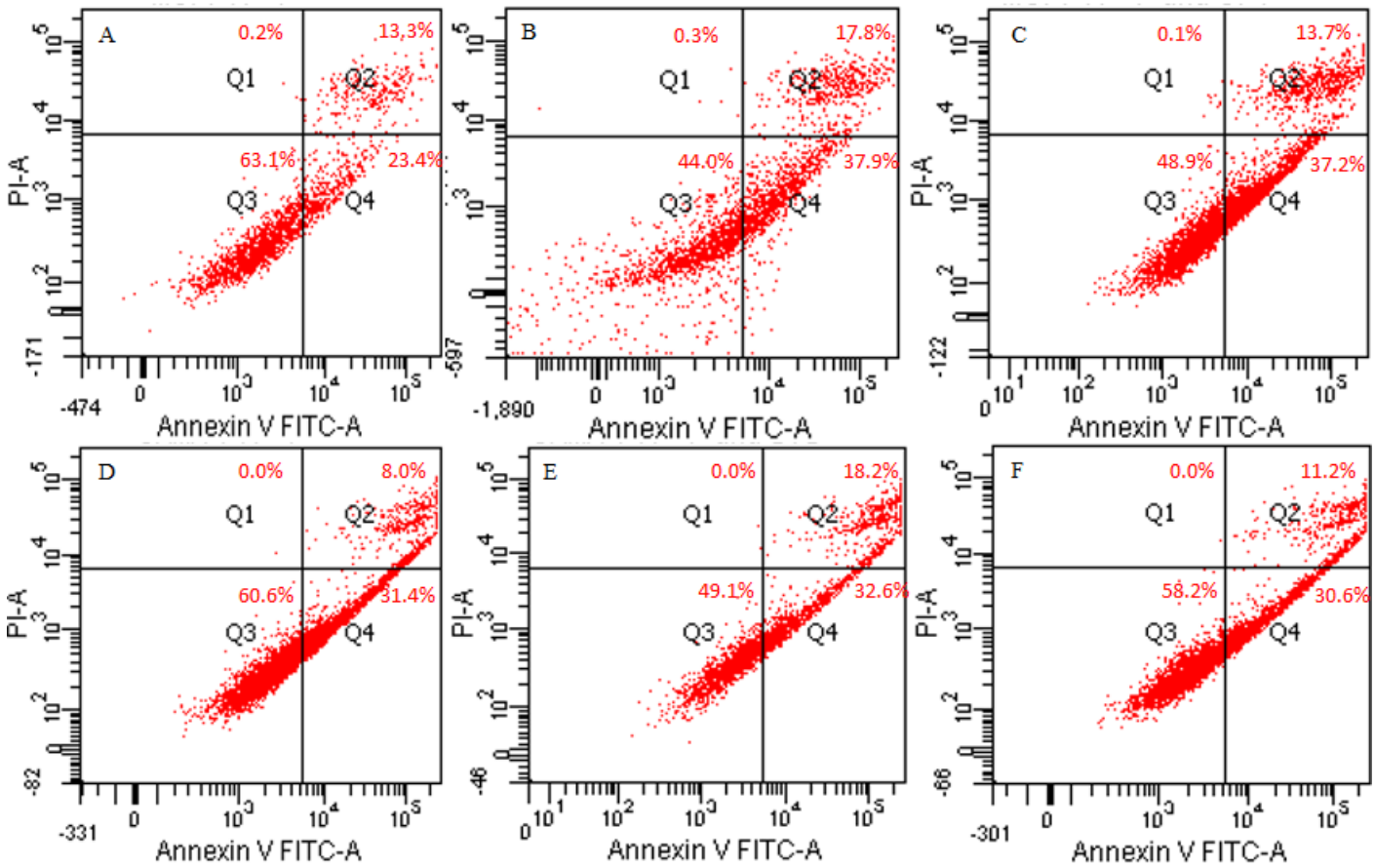


Figure 11: Apoptosis of MCF-7 **(A, B, and C)** and CAMA-1 **(D, E, and F)** cells double-silenced with siRBBP6 and sip53 and co-treated with either 0.25µM *staurosporine* or 0.25µM *camptothecin* was analysed using flow cytometer (annexin V and PI). **A** and **D**, shows cells double-silenced with siRBBP6/sip53, **B** and **E** shows cell co-treated with siRBBP6/sip53 + STS, and **C** and **F** shows cells co-treated with siRBBP6/sip53 + CPT.

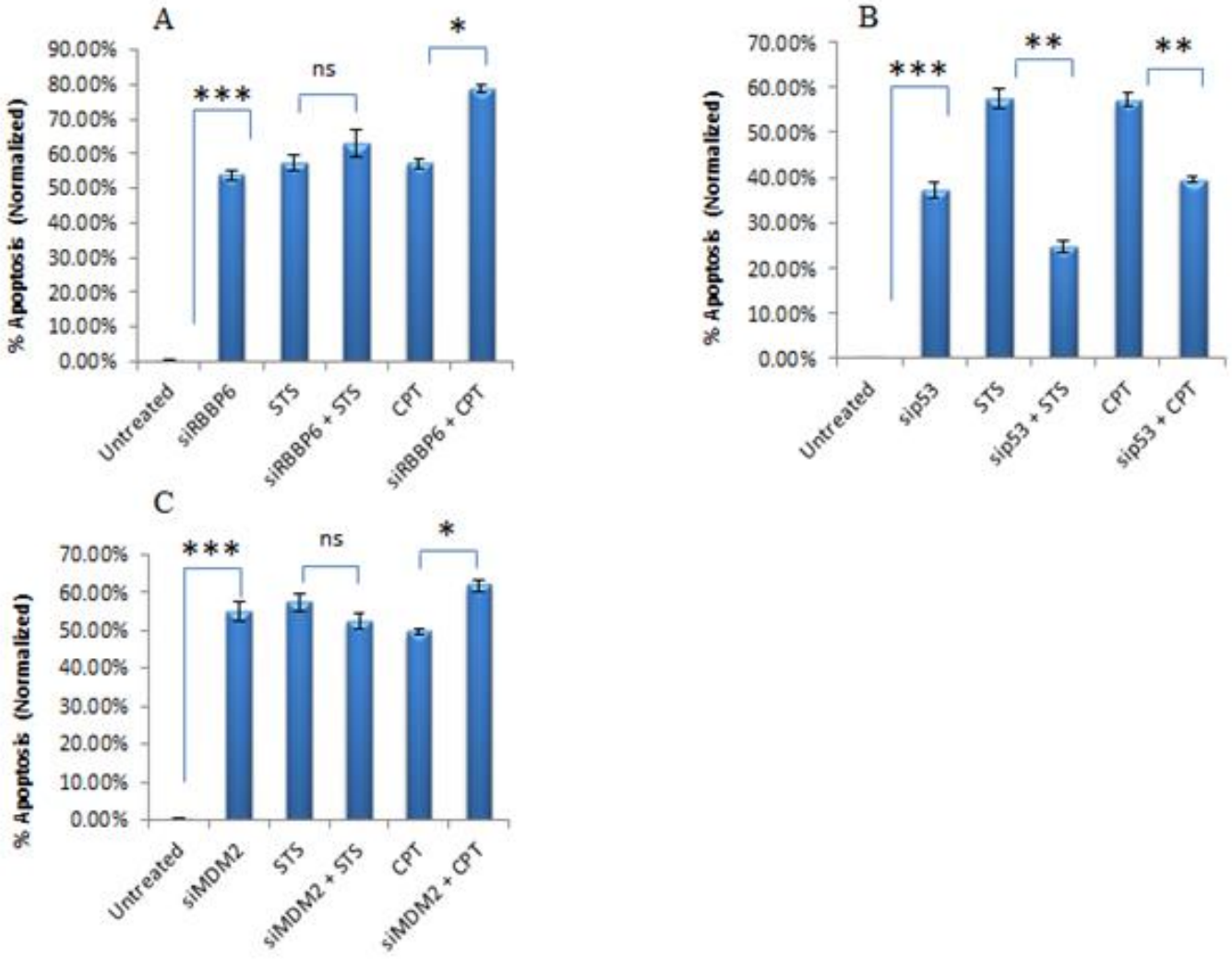


Figure12: Statistical analysis of flow cytometry-obtained apoptosis (%) in MCF-7 cell line. T-test was used to generate p-values in order to compute the difference between treated and untreated scores. **A, B** and **C**, statistical analysis between untreated and silenced and between treated and silenced + treated in MCF-7 cells. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$ and ^{ns} indicates $p \geq 0.05$.

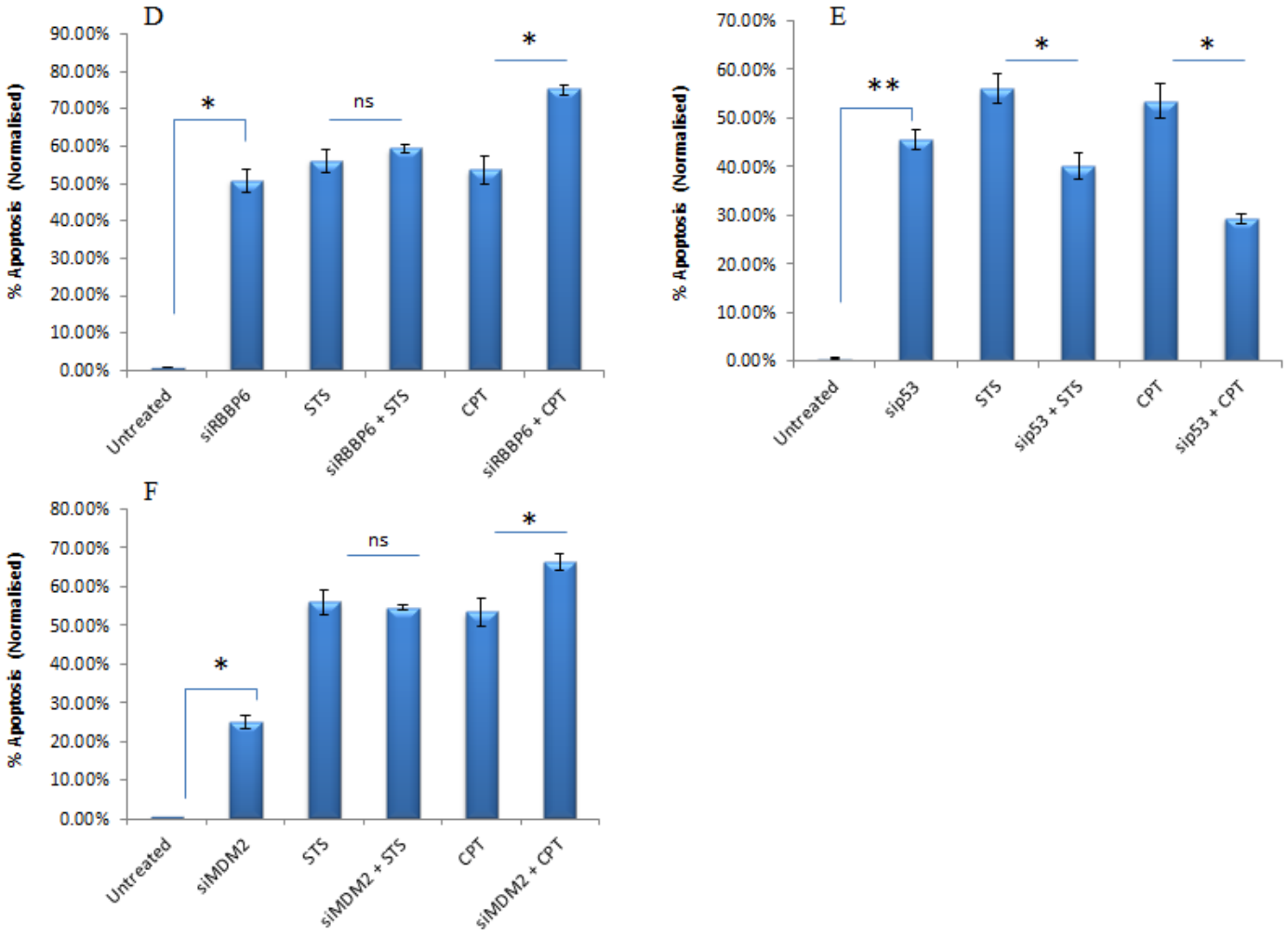


Figure 13: Statistical analysis of flow cytometry-obtained apoptosis (%) in CAMA-1 cell line. T-test was used to generate p-values in order to compute the difference between treated and untreated scores. **D, E** and **F**, statistical analysis between untreated and silenced and between treated and silenced + treated in MCF-7 cells. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$ and ^{ns} indicates $p \geq 0.05$.

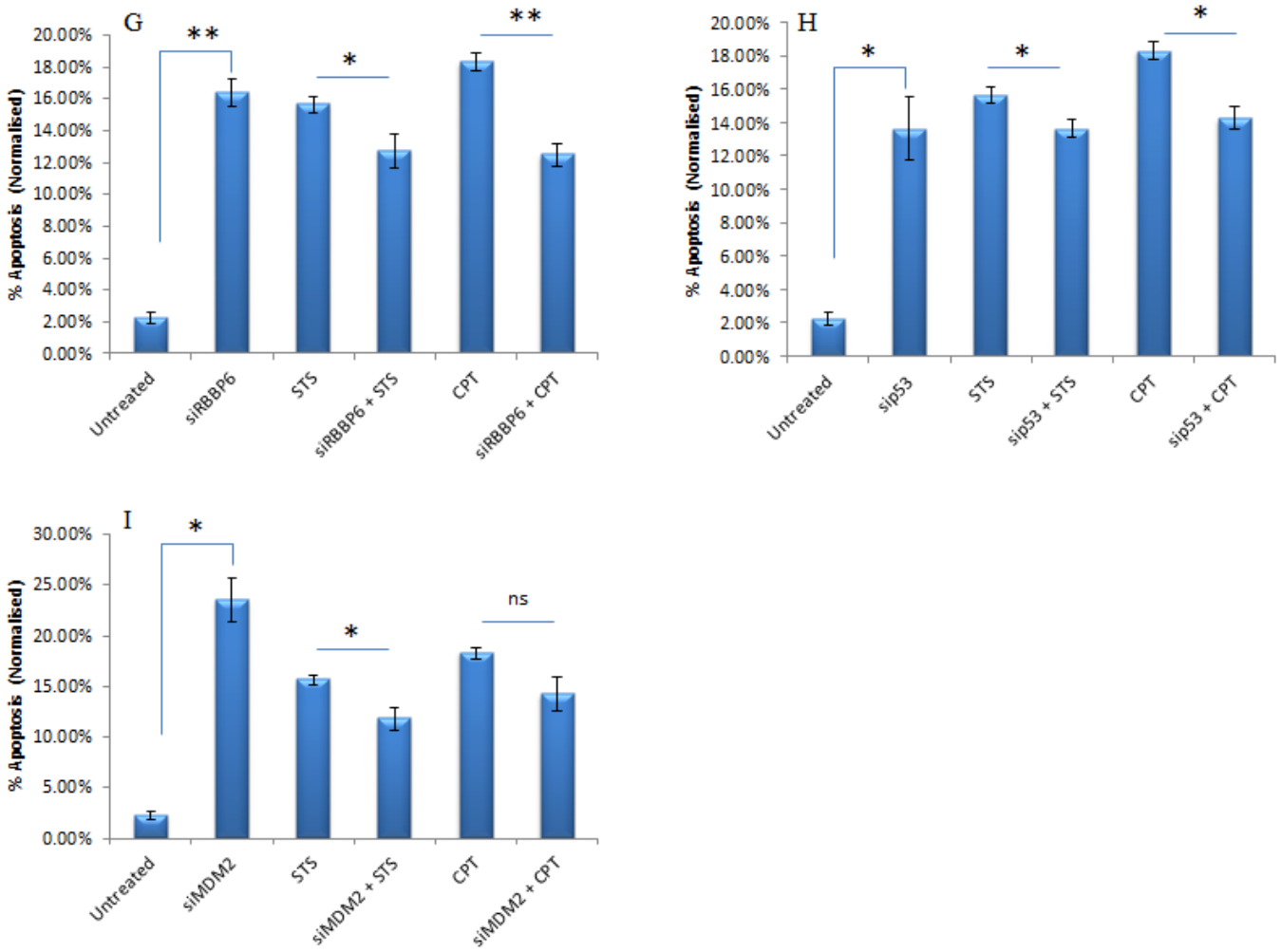


Figure 14: Statistical analysis of flow cytometry-obtained apoptosis (%) in MRC-5 cell line. T-test was used to generate p-values in order to compute the difference between treated and untreated scores. **G, H** and **I**, statistical analysis between untreated and silenced and between treated and silenced + treated in MCF-7 cells. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$ and ^{ns} indicates $p \geq 0.05$.

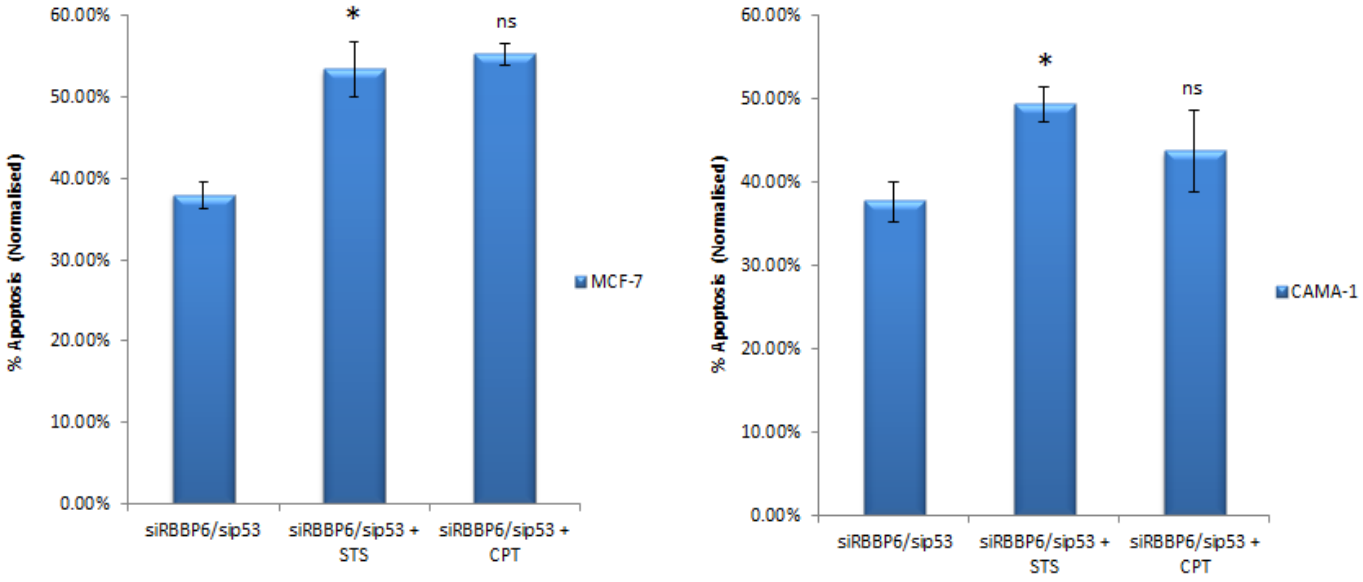


Figure 15: Statistical analysis of flow cytometry-obtained apoptosis (%) in MCF-7 and CAMA-1 cell lines. T-test was used to generate p-values in order to compute the difference between treated and untreated scores. The **s**tatistical analysis was computed between double-silenced and co-treatment with either *staurosporine* or *camptothecin*. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$ and ^{ns} indicates $p \geq 0.05$.

Chapter 5: Discussion and Conclusions

Discussion

Several E3 enzymes are associated with cancer development and are therefore highly expressed in a number of tumours (Chen *et al*, 2006). RBBP6 has been identified as an E3 ubiquitin ligase that is markedly up-regulated in tumours of the lung (Motadi *et al*, 2011). Because RBBP6 possesses a p53 binding domain, the p53 tumour suppressor is prone to ubiquitination by the ring finger domain of RBBP6, thus leading to cancer progression (Malloy *et.al*, 2012; Vassilev *et al*, 2004). RBBP6 therefore serves as a hallmark target in the development of anticancer therapeutics and findings in this study reveal how a synergistic effect of two biological compounds could become a potential treatment against breast cancer by targeting RBBP6.

We demonstrated at both the mRNA and protein level that it is possible to markedly silence the expression of RBBP6 in human breast cancer cells (MCF-7 and CAMA-1) using RNA interference. We further demonstrated that combinational therapy of siRBBP6 and either *camptothecin* or *staurosporine* further reduces expression of RBBP6 and lead to reduction in cell proliferation **(figure 1)**. However, siRBBP6 in combination with *staurosporine* does not significantly reduce RBBP6 gene expression. These differences in the effects of

these two combinational therapies (RBBP6 siRNA/*staurosporine* and RBBP6 siRNA/*camptothecin*) on RBBP6 expression may be attributable to the fact that the two apoptosis-inducing agents possess different mechanisms of apoptosis induction.

Camptothecin acts as a topoisomerase I inhibitor thus causing DNA strand breaks during the S-phase of the cell cycle which leads to apoptosis in cancer (Arakawa *et al.*, 2009). The mechanism by which *staurosporine* uses to induce apoptosis is not well understood. However, it is suspected that it uses its protein kinase inhibiting function to mediate translocation of bax from the cytosol to mitochondria where it causes the release of cytochrome c from the outer mitochondrial membrane (Xue *et al*., 2003; Motadi *et al* 2007). Silencing of RBBP6 in these cells markedly enhanced the level of apoptosis induced by *camptothecin.* However, apoptosis induced by *staurosporine* was not significantly enhanced by RBBP6 silencing. The results also strongly suggest that the enhanced apoptosis is as a result of the synergistic effect between RBBP6 siRNA and *camptothecin* as treatment with *camptothecin* alone (or siRNA alone) inducing less apoptosis.

p53 induces apoptosis by transcriptionally activating bax and causing its translocation to the mitochondria where it prorates the outer mitochondrial membrane (Shen and White, 2001). P53-silenced cells were not undergoing as

much apoptotic cell death as those that were RBBP6-silenced and from this it is arguable that some of the observed apoptosis might be attributed to some p53-mediated pathway. These findings suggest that by blocking the gene expression of both MDM2 and RBBP6 the activation of other proapoptotic genes is promoted, leading to the activation and upregulation of different intracellular signaling pathways, including BCl-2 gene family, inflammatory caspases and death receptors (Porichi *et al* 2009; Morissette *et al* 2007). These signalling pathways promote p53 mediated cell death.

Under normal physiological conditions, wild type TP53 tumour suppressor has a short half-life due to its targeted degradation by MDM2 E3 ubiquitin ligase (Bai and Zhu, 2006). This auto-regulatory feedback loop keeps TP53 under tight regulation to maintain normal cell growth and prevent tumorigenesis (Malloy *et.al*, 2012; Vassilev *et al*, 2004). In response to stress signals such as DNA damage, activated TP53 stimulates genes such as p21 and Bax which are responsible for the induction of cell cycle arrest and apoptosis, respectively (Bai and Zhu, 2006). Inhibition of RBBP6 led to overexpression of the p53 tumour suppressor at both the mRNA and protein level. This suggests that by silencing RBBP6 we were able to block the interaction between RBBP6 and p53 and thus prevented p53 degradation.

During their auto-regulatory feedback loop, p53 activates MDM2 which in turn has the ability to block the transactivation activity of p53 by tightly binding the p53 protein (Malloy *et.al*, 2012). MDM2 is also involved in the nuclear transport of p53 and also serves as an E3 ubiquitin ligase that promotes degradation of p53 (Vassilev *et al.*, 2012). We demonstrated in this study that by tempering with the p53:MDM2 auto-regulatory feedback loop, i.e. silencing MDM2, also frees p53 as evidenced by the up-regulation of both p53 mRNA and p53 protein **(figure 1)** and the fact that p53 silencing leads to MDM2 down-regulation suggests that the p53:MDM2 interaction does exist and it compromises the function of p53 during cancer development (Shangary S and Wang S 2008). These findings suggest that in the absence of RBBP6 and the p53:MDM2 interaction p53 degradation by the E3 ubiquitin ligases is abrogated, thus leading to p53-mediated apoptosis. Normal skin fibroblasts were less responsive to the co-treatment and this somehow suggests that silencing RBBP6 does not exhibit any cytotoxicity to noncancerous cells.

We showed a good correlation between cellular growth reduction and apoptosis induction in MCF-7 cells, for example siRBBP6 combined with *camptothecin* led to a significant cellular growth rate reduction and an increase in apoptosis induction in comparison to cells that were untreated or treated with *camptothecin* only. However combination of siRBBP6 with *staurosporine* led to

a much faster reduction in growth rate of both MCF-7 and CAMA-1 cells **(figure 3.1 and 3.2)**. The observed difference in the potency of *camptothecin* and *staurosporine* on cell growth may be attributable to the fact that the two agents induce apoptosis using different mechanisms. We have shown that *camptothecin* induces a much higher apoptosis than *staurosporine* when combined with siRBBP6 and this may be due to the fact that *camptothecin* interacts directly with proapoptotic genes in order to induce apoptosis (Zeng et al, 2012). Wild type p53 has been shown to enhance *camptothecin*-induced apoptosis (Zhang et al, 2000 and Li et al, 2000) and this may also explain the observed enhanced apoptosis in cells co-treated with siRBBP6+*camptothecin*.

Interestingly, we also showed that sip53 combined with either *camptothecin* or *staurosporine* leads to a slight increase in cell growth in comparison to co-treatment with siRBBP6 or siMDM2 and either of the agents **(figure 3.1)**. This observation may suggest that p53 is required in the induction of cell death and to explore this further, apoptosis detection was carried out in p53-knockdown MCF-7 cells. We showed that there is minimal apoptosis induction in p53-silenced cell population in comparison to cells co-treated with either siMDM2 or siRBBP6. P53 is involved in the induction of apoptosis via the intrinsic apoptotic pathway by promoting transcription; translocation and oligomerization of bax on the outer mitochondrial membrane in order to

facilitate cytochrome c release (Bai and Zhu, 2006) and these findings suggest that silencing p53 reduces p53-mediated apoptosis.

Cellular growth studies have shown that CAMA-1 cells deteriorate in response to siMDM2+ *camptothecin* and siRBBP6 + *camptothecin* co-treatments. However, in comparison to MCF-7, growth reduction lasted for a short period of time before the cells would recover and this may be due to the fact that CAMA-1 cells are metastatic which makes them more aggressive and less responsive to the co-treatment (Kenny et al, 2007). The fact that there was no increase in cell growth following sip53 co-treatment may suggest that CAMA-1 cells do not depend mainly on p53 to undergo cell death. Due to their aggressiveness, CAMA-1 generally underwent less apoptosis in comparison with that observed in MCF-7. The co-treatment may be less cytotoxic to normal cells because according to our findings there was little to no effect in terms of apoptosis induction or cell growth reduction in MRC-5 fibroblasts **(figure 3.3, 9 and 10).** We have also shown that p53-knockdown in MCF-7 and CAMA-1 cells that were also silenced with siRBBP6 induced a slightly lower apoptosis following co-treatment with either *staurosporine* or *camptothecin*. p53 might be responsible for some of the observed apoptosis induction in RBBP6-silenced cells and this may be due to the fact that siRBBP6 has led to p53 up-regulation in both cell lines.

Conclusion

p53 could promote the convergence of the extrinsic and intrinsic apoptotic pathways, including in MCF7 breast cancer cells. The present study provides early insight into the mechanisms of RBBP6 pathways in mediating p53 in breast cancer cells as the mRNA and protein expression data show. Manipulating the expression of the RBBP6 and MDM2 genes is required for the induction of apoptosis by different Bcl-2 gene family members. Therefore, these results provide a promising path to understanding further the role of RBBP6 in cancer development and treatment.

Chapter 6: References

Aagaard L., Rossi J. J. (2007) RNAi Therapeutics: Principles, prospects and challenges. *Advanced Drug Delivery Reviews* 59:75-86

Arakawa Y., Saito S., Yamada H., Aiba H (2009) Simultaneous treatment with *camptothecin* and valproic acid suppresses induction of Bcl-XL and promotes apoptosis of MCF-7 breast cancer cells. *Apoptosis* 14:1076-85

Bai L., Zhu W. G. (2006) p53 structure, function and therapeutic applications. *Journal ossf Cancer Molecules* 2(4):141-53

Bialik, S., E. Zalckvar, Y. Ber, A. D. Rubinstein & A. Kimchi (2010) Systems biology analysis of programmed cell death. *Trends in Biochemical Sciences,* 35**,** 556-564.

Boyd, N. F., L. J. Martin, L. Sun, H. Guo, A. Chiarelli, G. Hislop, M. Yaffe & S. Minkin (2006) Body size, mammographic density, and breast cancer risk. *Cancer Epidemiol Biomarkers Prev,* 15**,** 2086-92.

Butt, Z., S. F. Haider, S. Arif, M. R. Khan, U. Ashfaq, U. Shahbaz & M. H. Bukhari (2012) Breast cancer risk factors: A comparison between pre-menopausal and post-menopausal women. *Journal of the Pakistan Medical Association,* 62**,** 120-124.

Chen C., Seth A. K., and Aplin A. E. (2006) Genetic and expression abberations of E3 ubiquitin ligases in human breast cancer. *Mol Cancer Res*, 4(10):695-707

Cooper G. M. (2000) The cell: a molecular approach, 2nd Ed. Sunderland (MA): *Sinauer Associates*

De Roziere S., Maya R., Oren M., and Lozano G. (2000) The loss of MDM2 induces p53-mediated apoptosis. *Oncogene*, 19:1691-1697

Debatin K. M. (2004) Apoptosis pathways in cancer and cancer therapy. *Cancer Immunology Immunotherapy,* 53**,** 153-159.

Dunitrescu R. G. and Cotarla I. (2005) Understanding breast cancer risk – where do we stand in 2005? *J Cell Mol Med* 9(1):208-21

Ghafoor, A., A. Jemal, E. Ward, V. Cokkinides, R. Smith & M. Thun (2003) Trends in breast cancer by race and ethnicity. *CA Cancer J Clin,* 53**,** 342-55.

Ghobrial, I. M., T. E. Witzig & A. A. Adjei (2005) Targeting apoptosis pathways in cancer therapy. *Ca-a Cancer Journal for Clinicians,* 55**,** 178-194.

Hortobagyi, G. N., J. de la Garza Salazar, K. Pritchard, D. Amadori, R. Haidinger, C. A. Hudis, H. Khaled, M. C. Liu, M. Martin, M. Namer, J. A. O'Shaughnessy, Z. Z. Shen & K. S. Albain (2005) The global breast cancer burden: variations in epidemiology and survival. *Clin Breast Cancer,* 6**,** 391-401.

Hulka, B. S. & P. G. Moorman (2008) Breast cancer: hormones and other risk factors. *Maturitas,* 61**,** 203-13; discussion 213.

Jemal, A., F. Bray, D. Forman, M. O'Brien, J. Ferlay, M. Center & D. M. Parkin (2012) Cancer burden in Africa and opportunities for prevention. *Cancer,* 118**,** 4372-4384.

Jiang T., Zhoub C., Gud J., Liud Y., Zhaod L., Lia W., Wanga G., Lid Y. (2013) Enhanced therapeutic effects of cisplatin on prostate cancer in tumour-bearing mice by transfecting the attenuated Salmonella carrying a plasmid co-expressing p53 gene and MDM2 siRNA. *Cancer Letters* 337(1):133-42

Johnstone, R. W., A. A. Ruefli & S. W. Lowe (2002) Apoptosis: A link between cancer genetics and chemotherapy. *Cell,* 108**,** 153-164.

Kenny P. A., Lee G. Y., Myer C. A., Nevel R. M., Semeik J. R., Spellman P. T., Lorenzl K., Lee E. H., Barcellos-Hoffl M. H., Peterson O. W., Gray J. W., and Bissell M. J. (2007) The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Mol Oncol* 1(1):84-96

Kerr J. F., Wyllie A. H., and Currie A. R. (1972) Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br J Cancer*, 26(4):239-257

Li G., Bush J. A. and Ho V. C. (2000) p53-dependent apoptosis in melanoma cells after treatment with camptothecin. *J Invest Dermatol* 115:514-519

Lim, E., O. Metzger & E. P. Winer (2012) The Natural History of Hormone Receptor-Positive Breast Cancer. *Oncology-New York,* 26**,** 688-+.

Lokate, M., P. H. M. Peeters, L. M. Peelen, G. Haars, W. B. Veldhuis & C. H. van Gils (2011) Mammographic density and breast cancer risk: the role of the fat surrounding the fibroglandular tissue. *Breast Cancer Research,* 13.

Lord, S. J., L. Bernstein, K. A. Johnson, K. E. Malone, J. A. McDonald, P. A. Marchbanks, M. S. Simon, B. L. Strom, M. F. Press, S. G. Folger, R. T. Burkman, D. Deapen, R. Spirtas & G. Ursin (2008) Breast cancer risk and hormone receptor status in older women by parity, age of first birth, and breastfeeding: a case-control study. *Cancer Epidemiol Biomarkers Prev,* 17**,** 1723-30.

Ly, D., D. Forman, J. Ferlay, L. A. Brinton & M. B. Cook (2013) An international comparison of male and female breast cancer incidence rates. *International Journal of Cancer,* 132**,** 1918-1926.

Malloy K. L, Choi H., Fiorilla C., Matainaho V. T., Gerwick W. H. (2012) Hoiamide D, a marine cyanobacteria-derived inhibitor of p53/MDM2 interaction. *Bioorganic and Medicinal Chemistry Letters* 22:683-88

McPherson, K., C. M. Steel & J. M. Dixon (2000) ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics. *BMJ,* 321**,** 624-8.

McTireman A. (2003) Behavioural risk factors in breast cancer: can risk be modified? *Oncologist* 8:326-34

Meister G., Tuschl T. (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature* 431:343-49

Mendez D., Inga A., Resnick MA (2009) The expanding universe of p53 targets. *Nat Rev Cancer* 9:742-37

Motadi L. R, Bhoola K. D, Dlamini Z. (2011) Expression and function of retinoblastoma binding protein 6 (RBBP6) in human lung cancer. *Immunobiology* 216:1065-73

Motadi L. R., Misso N. L., Dlamini Z. and Bhoola K. D. (2007). Molecular genetics and mechanisms of apoptosis in carcinomas of the lung and pleura: therapeutic targets. International Immunopharmacology *7*: 1934-1947.

Nag S., Qin J., Srivenugopal K. S., Wang M., and Zang R. (2013) The MDM2-p53 pathway revisited. *The Journal of Biomedical Research*, 27(4):254-271

Nelson, H. D., B. Zakher, A. Cantor, R. Fu, J. Griffin, E. S. O'Meara, D. S. Buist, K. Kerlikowske, N. T. van Ravesteyn, A. Trentham-Dietz, J. S. Mandelblatt & D. L. Miglioretti (2012) Risk factors for breast cancer for women aged 40 to 49 years: a systematic review and meta-analysis. *Ann Intern Med,* 156**,** 635-48.

Nieves-Neira, W. & Y. Pommier (1999) Apoptotic response to camptothecin and 7-hydroxystaurosporine (UCN-01) in the 8 human breast cancer cell lines of the NCI Anticancer Drug Screen: multifactorial relationships with topoisomerase I, protein kinase C, Bcl-2, p53, MDM-2 and caspase pathways. *International Journal of Cancer,* 82**,** 396-404.

Ntwasa M. (2008) Retinoblastoma binding protein 6 is a potential target for therapeutic drugs. Biotechnology and Molecular Biology 3(2):024-031

Ouyang, L., Z. Shi, S. Zhao, F. T. Wang, T. T. Zhou, B. Liu & J. K. Bao (2012) Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell Prolif,* 45**,** 487-98.

Perou, C. M., T. Sorlie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, C. A. Rees, J. R. Pollack, D. T. Ross, H. Johnsen, L. A. Akslen, O. Fluge, A. Pergamenschikov, C. Williams, S. X. Zhu, P. E. Lonning, A. L. Borresen-Dale, P. O. Brown & D. Botstein (2000) Molecular portraits of human breast tumours. *Nature,* 406**,** 747-52.

Porichi O, Nikolaidou ME, Apostolaki A, Tserkezoglou A, Arnogiannaki N, Kassanos D, Margaritis L, Panotopoulou E. 2009. BCL-2, BAX and P53 expression profiles in endometrial carcinoma as studied by real-time PCR and immunohistochemistry. Anticancer Res. 29(10):3977-82.

Pretorius A, Kaur M, Wamalwa M, February MF (2013) Functional analysis and characterization of the human RBBP6 promoters based on a combination of molecular biology and in silico approaches provide additional evidence for RBBP6 role in apoptosis. *JBio* 1(1):2251-3159

Pugh, D. J., E. Ab, A. Faro, P. T. Lutya, E. Hoffmann & D. J. Rees (2006) DWNN, a novel ubiquitin-like domain, implicates RBBP6 in mRNA processing and ubiquitin-like pathways. *BMC Struct Biol,* 6**,** 1.

Schreer, I. (2009) Dense Breast Tissue as an Important Risk Factor for Breast Cancer and Implications for Early Detection. *Breast Care,* 4**,** 89-92.

Shangary S., Wang S. (2008). Targeting the MDM2-p53 interaction for cancer therapy. Clin Cancer Res. 14(17): 5318–5324.

Shen Y. and White E. (2001) P53-dependent apoptosis pathways. *Adv Cancer Res*, 82:58-84

Sherr C. J. (1996) Cancer cell cycles. *Science* 274:1672-77

Sotiriou, C. & L. Pusztai (2009) MOLECULAR ORIGINS OF CANCER Gene-Expression Signatures in Breast Cancer. *New England Journal of Medicine,* 360**,** 790-800.

Steiner, E., D. Klubert & D. Knutson (2008) Assessing Breast Cancer Risk in Women. *American Family Physician,* 78**,** 1361-1366.

Tan S. C., and Yiap B. C. (2009) DNA, RNA and protein extraction: The past and the present. *J Biomed Biotechnol* 1-10

Tinoco, G., S. Warsch, S. Gluck, K. Avancha & A. J. Montero (2013) Treating Breast Cancer in the 21st Century: Emerging Biological Therapies. *Journal of Cancer,* 4**,** 117-132.

Towbin H., Staehelin T., Gordon J. (1979) Electrophoresis transfer of protin from polyacrylamide gel to nitrocellulosesheets: procedure and some applications. *Proc Natl Acad Sci*, 76(9):4350-4354

Ursin, G., L. Bernstein, Y. P. Wang, S. J. Lord, D. Deapen, J. M. Liff, S. A. Norman, L. K. Weiss, J. R. Daling, P. A. Marchbanks, K. E. Malone, S.

G. Folger, J. A. McDonald, R. T. Burkman, M. S. Simon, B. L. Strom & R. Spirtas (2004) Reproductive factors and risk of breast carcinoma in a study of white and African-American women. *Cancer,* 101**,** 353-362.

Vassilev L. T., Vu B. T., Graves B., *et al.* (2003) In Vivo activation of the p53 pathway by small molecule antagonists of MDM2. *Science* 343:844-48

Vermeulen, K., D. R. Van Bockstaele & Z. N. Berneman (2003) The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif,* 36**,** 131-49.

Wen, X., Z. Q. Lin, B. Liu & Y. Q. Wei (2012) Caspase-mediated programmed cell death pathways as potential therapeutic targets in cancer. *Cell Prolif,* 45**,** 217-224.

World Health Organisation [WHO] (2010) World health report. Executive summary. Retrieved on 27 April 2011 from http://www.who.int/whr/2010/en/index.html

World Health Organisation [WHO] (2008) Breast cancer: prevention and control. Retrieved on 15 February 2013 from http://www.who.int/cancer/detection/breastcancer/en/

World Health Organisation [WHO] (2004) The Global burden of disease. Retrieved on 15 February 2013 from http://www.who.int/healthinfo/global_burden_disease/2004_report_update/en/index.html

Xue L., Chiu S., Oleinick N. L. (2003) *Staurosporine*-induced death of MCF-7 human breast cancer cells: a distinction between caspase-3-dependent steps of apoptosis and the critical lethal lessions. *Experimental Cell Research* 283:135-45

Zeng C. W., Zhang X. J., Lin K. Y., Ye H., Feng S. Y., Zhang H., and Chen Y. Q. (2012) Camptothecin induces apoptosis in cancer cells via miR-125b mediated mitochondrial pathways. *Molecular Pharmacology*

Zhang X. D., Gillespie S. K., Hersey P. (2004) Staurosporine induces apoptosis of melanoma by both caspases-dependent and caspases-independent apoptotic pathways. *Mol Cancer Ther* 3:187-197

Zhang Z. W., Patchett S. E. and Farthing M. J. G. (2000) Topoisomerase I inhibitor (camptothecin)-induced apoptosis in human gastric cancer cells and the role of wild-type p53 in the enhancementof its cytotoxicity. *Anti-cancer Drugs* 11(9):757-764