INVESTIGATING THE ROLE OF THE RENIN ANGIOTENSIN SYSTEM IN CANCER

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DECLARATION

I, Cherise Dunn, hereby declare that the contents of this thesis is my own unaided work, except where acknowledgements indicate otherwise; and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University. I am now presenting this thesis for academic examination towards the Degree of Doctor of Philosophy in Medical Biochemistry.

Signed: Signed by candidate Date: 12 May 2017

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"Education is the most powerful weapon which you can use to change the world."

-Nelson Mandela

TABLE OF CONTENTS

ABBREVIATIONS

°C	Degrees Celcius
%	Percentage
ACE-1	Angiotensin Converting Enzyme-1
ACE-2	Angiotensin Converting Enzyme-2
AKT	Protein Kinase B
AngII	Angiotensin II
Ang-(1-7)	Angiotensin-(1-7)
APS	Ammonium Persulphate
AT1R	Angiotensin Type-1 Receptor
AT2R	Angiotensin Type-2 Receptor
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
CAMKII	Calcium Calmodulin Kinase II
CDDP	Cisplatin, cis-diamminedichloridoplatinum (II)
CDK	Cyclin-dependent Kinase
CI	Combination Index
CO_2	Carbon Dioxide
C-PARP	Cleaved Poly(ADP-Ribose) Polymerase
Ctl	Control
DMEM	Dulbecco's Modified Eagle's Medium
dH_2O	Distilled Hydrogen Dioxide

DIZE	Diminazene aceturate
DNA	Deoxyribonucleotide
DOX	Doxorubicin
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ECL	Enhanced Chemiluminescence
EMT	Epithelial-Mesenchymal Transition
Fa	Fraction affected
FCS	Fetal Calf Serum
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HPV	Human Papillomavirus
Hr	Hour
HRP	Horse Radish Peroxidase
IC50	Half maximal inhibitory concentration
kDa	Kilodaltons
Log	Logarithm
Luc	Luciferase
MasR	MAS1 receptor
Mcl-1	Myeloid Cell Leukemia 1
MTT	3'-(4',5'-Dimethylthiazol-2'-yl)-2',5'-diphenyltetrazolium bromide
Na_3VO_4	Sodium orthovanadate
NaCl	Sodium chloride
NF-$_K$B	Nuclear Factor $_K$B
NSCLC	Non-small cell lung cancer

PAGE	Polyacrylamide Gel Electrophoresis
PARP	Poly(ADP-Ribose) Polymerase
PBS	Phosphate Buffered Saline
pCAMKII	phosphorylated calcium calmodulin kinase II
PRR	Pro(renin) receptor
p/s	Penicillin and Streptomycin
Rb	Retinoblastoma Protein
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic Acid
RNAi	RNA interference
RNase	Ribonuclease
RNAsin	Ribonuclease inhibitor
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS Polyacrylamide gel electrophoresis
SEM	Standard Error of the Mean
siRNA	Small interfering RNA
SV40	Simian Virus 40
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween
V	Volts
H2AX	139-serine phosphorylated histone H2A.X

Units

M	Molar
min	Minutes
µM	Micromolar
nM	Nanomolar
G	Gram
kg	Kilogram
mg	Milligram
µg	Microgram
ng	Nanogram
L	Litre
mL	Millilitre
µL	Microlitre
µm	Micrometre
nm	Nanometre
N	Normality

ABSTRACT

It has recently been discovered that cancer shares a link with metabolic diseases, including that of cardiovascular disease, diabetes, amongst others, where common sets of genes show similar gene expression. There is thus interest to investigate current therapies for metabolic diseases as possible anti-cancer agents. The renin-angiotensin system (RAS) regulates blood pressure and cardiovascular homeostasis through Angiotensin Converting Enzyme-1 (ACE-1) and its homolog ACE-2. RAS has also been implicated in the progression of various cancers due to the increased action of the vasoconstrictor, angiotensin II, which requires ACE-1 and specifically the Angiotensin Type 1 Receptor (AT1R) for its function. In this study, we investigated the potential association of the endogenous ACE-1 and ACE-2 enzymes in cervical cancer. Our results showed that ACE-1 and AT1R protein expression was elevated in cervical cancer cell lines compared to normal cells and that this correlated with elevated ACE-1 enzyme activity in cancer cells. Treatment with the ACE-1 inhibitors, Captopril and Lisinopril, reduced this activity. We showed that ACE-1 axis stimulation in cancer cells results in increased calcium signaling preferentially via the AT1R and this associates with cancer cell proliferation. Candesartan, an AT1R blocker significantly reduced these effects. ACE-2 expression and activity were decreased in cancer compared to normal cells. Our data shows that ACE2 activators, the natural peptide angiotensin 1-7 and small molecule Diminazene aceturate (DIZE) have anticancer effects with DIZE inducing a G2/M arrest in cancer cells.

We also investigated associations between drugs targeting RAS and current chemotherapeutic agents, Cisplatin (CDDP) and Doxorubicin (DOX). Our data shows that

ACE-1 axis inhibitors have an antagonistic effect on CDDP, while the ACE-2 activator DIZE associates synergistically with DOX. Taken together, these results suggest that elevated ACE-1 expression associates with cervical cancer and that the inhibitors of ACE-1 function or activators of ACE-2 function have potential as anticancer therapies as single agents or in combination treatments with current chemotherapeutics.

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer describes a large number of diseases characterised by the uncontrolled growth of abnormal cells with the ability to affect any part of the human body as they spread. Cancer is an enormous burden on society and a leading cause of death worldwide (Torre et al., 2015). Cancer is caused by a number of external factors, which include tobacco, an unhealthy diet, infectious agents as well as inherited genetic mutations (Torre et al., 2015). In 2012 GLOBOCAN estimated the number of cancer cases globally to be approximately 14.1 million with 8.2 million cancer deaths recorded (Torre et al., 2015). In developing countries cancer is responsible for 542 000 deaths with approximately 715 000 new cancer cases recorded in 2008. Cervical and breast cancers were diagnosed more frequently than other forms of cancer in women while cancers of the lung, bronchus and trachea, liver and stomach were the leading cancers in men (Torre et al., 2015) (Figure 1.1).

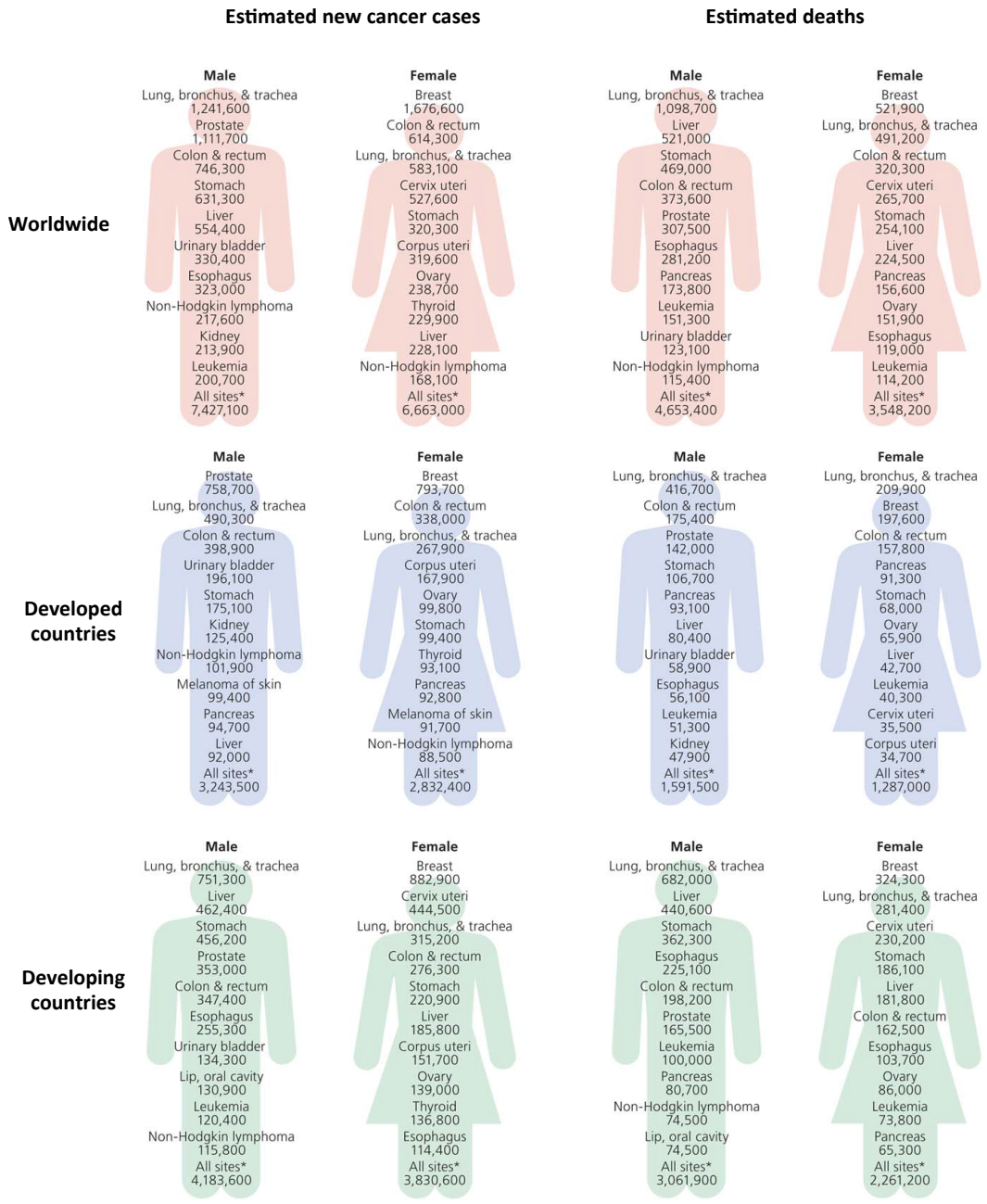


Figure 1.1: Estimated new cancer cases and deaths in 2012. From GLOBOCAN 2012 worldwide new cases of breast and cervical cancer are amongst the most frequently diagnosed and are two of the leading causes of cancer-related deaths globally and in developing countries. (Torre et al., 2015)

1.2 New and alternative cancer targets

Cancer has predominantly been known as a disease driven by rapid and uncontrolled proliferation. However, emerging evidence has shown that cancer can also be considered a metabolic disease, with associated metabolic reprogramming, disturbances in energy production and the disruption of normal cell signaling networks (Coller, 2014; Seyfried and Shelton, 2010; Seyfried et al., 2014).

1.2.1 Links between metabolism and cancer

Cancer cells re-engineer their metabolic pathways to drive proliferation and growth of tumours with emerging evidence indicating that oncogenic molecules such as c-MYC, hypoxia-inducible factor-1 (HIF-1) and AKT and tumour suppressors such as p53 enforce these metabolic shifts (Coller, 2014). In addition to reports that c-MYC has well defined roles in regulating proliferation, differentiation and apoptosis it has also been found to induce glucose consumption, lactate production which assist growing tumours in anaerobic microenvironments. Similarly, HIF-1 has also been found to facilitate the activation of oxygen independent modes of energy and is well documented as being stabilized during hypoxia leading to the transcription of target genes involved in angiogenesis. Under normal conditions the phosphatidyl inositol-3-kinase (PI3K) pathway is activated through growth signals. In many cancers, it is observed that this pathway is constitutively activated resulting in the activation of AKT kinase and the powerful activation of growth promoting program (Coller, 2014). In summary, these oncogenes contribute strongly to the metabolic reprogramming which takes place during carcinogenesis (Coller, 2014).

In response to DNA damage p53 is responsible for the regulation of many important cell processes such as cell cycle arrest and cell death but has also recently been investigated in the modulation of cellular metabolism (Coller, 2014).

Although metabolic changes in tumours have been documented in the past this shift was observed only as a secondary effect with uncontrolled proliferation being the major cause of cancer. Growing evidence suggests that metabolic changes are more fundamental than what was once thought. As a result of this shift metabolic diseases such as cardiovascular disease and diabetes have been linked to cancer (Hirsch et al., 2010; Reznik and Sander, 2015). By performing transcriptional profiling in two isogenic models of cellular transformation, Hirsch et al. (2010) identified a cancer gene signature associating with inflammatory and other metabolic diseases. Three groups of genes were identified which associate with cancer. These include genes associating with cancer cell functions such as enhanced proliferation, cell cycle control and cell death; genes associated with inflammation and immune system function as well as genes responsible for lipid metabolism, gastrointestinal disease, metabolic disease and cardiovascular disease.

Moreover, the study also found that the cancer gene signature was comprised of genes which were previously not associated with cancer, such as the lipid metabolism genes *OLR1* (oxidized LDL receptor 1)*, SREBP-1* (sterol element-binding protein 1)*, SNAP23* (SNARE protein synaptosomal-associated protein of 23 kDa) and *VAMP4* (Vesicle associated membrane protein-4). siRNA knockdown of the associated lipid metabolism proteins proved their importance in cellular transformation and growth of cancer cells.

As a result of emerging evidence there have been reports supporting findings that drugs used to treat metabolic diseases have anti-cancer properties (Figure 1.2 A). The anti-diabetic drug Metformin; anti-inflammatory drugs, including Celecoxib, Piroxicam and Sulindac; and lipid lowering drugs such as Simvastatin, Cerulenin and Mevastatin all successfully reduced or prevented cellular transformation (Figure 1.2 B). The ability of single cancer cells to form clonogenic colonies was also reduced after treatment with these drugs. In addition, Metformin, Cerulenin, Simvastatin and Sulindac were effective at reducing tumour growth in nude mice (Figure 1.2 C).

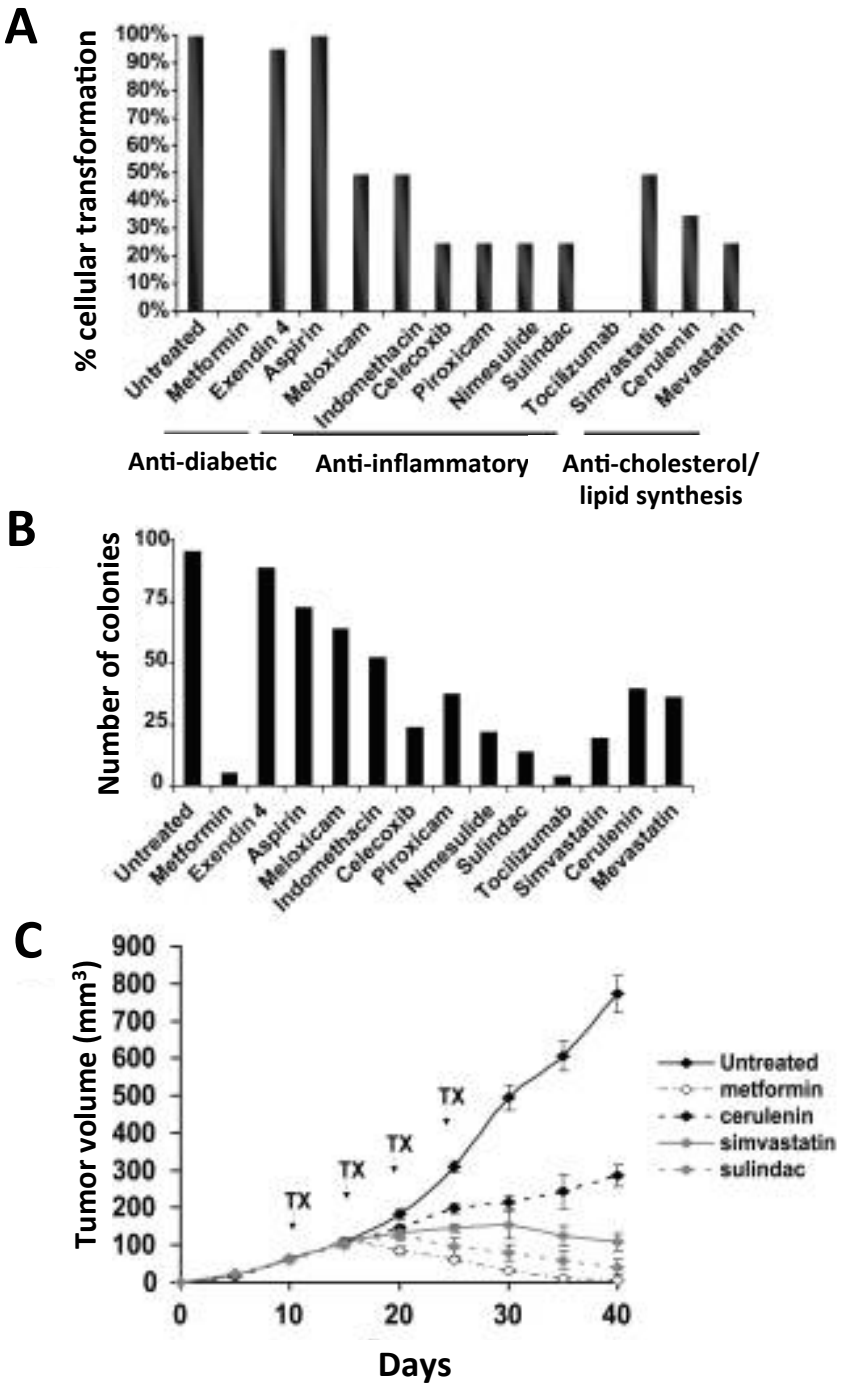


Figure 1.2: Many non-cancer drugs have anti-cancer effects. (A) Percentage of transformed cells (morphology assay) observed by treating TAM-induced MCF10 ER-Src cells with the indicated drugs. **(B)** Soft agar colony assay of the effect of the indicated drugs on transformation. **(C)** Tumour growth (mean ± SD) of MCF10 ER-Src cells after 4 cycles of i.p treatments with the indicated drugs (Hirsch et al., 2010).

Emerging evidence describes the multifaceted association between metabolism and cancer. (Reznik and Sander, 2015) examined the altered metabolism of breast and clear-cell kidney cancer, thereby discovering the altered co-expression patterns of four clusters of metabolic genes. As a result of this, mitochondrial dysfunction is exhibited in these cancer types promoting disease progression. Moreover, there is an association between atherosclerosis and cancer development through lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) (Jiang et al., 2011). LOX-1 is mainly expressed in endothelial cells and thus plays a role in angiogenesis, the creation of new blood vessels in healthy development but also a source of nutrition for a growing tumour. Jiang et al., (2011) found that LOX-1 is important for maintaining the transformed state of cells driving carcinogenesis and is associated with the risk factors for atherosclerosis, such as hypertension, hyperlipidemia and diabetes.

These studies suggest that metabolic reprogramming is associated with cancer. Consequently, genes associated with metabolic diseases provide attractive new targets for cancer therapies. Current drugs, in addition to their primary function of treating specific metabolic diseases, may be able to exert secondary functions with potent anti-cancer effects and thus provide a novel and alternative means of treating cancer.

1.3 The RAS as a potential cancer target

The renin-angiotensin system (RAS) was discovered in 1898 by the Finnish physiologist Robert Tigerstedt and his student Bergman through their investigations into pressor effects exerted by crude saline extract of rabbit kidney in rabbits (Kunikullaya et al., 2012; Marks and Maxwell, 1979; Phillips and Schmidt-Ott, 1999).

Today the RAS exists as a complex system responsible for regulation of arterial pressure and tissue perfusion. Fundamental to the pathway or cascade is angiotensin-converting enzyme-1 (ACE-1), which converts the peptide hormone angiotensin I to angiotensin II (Ang-II) (Deshayes and Nahmias, 2005; Peach, 1977). Ang-II mediates its biological effects through binding to two receptors which belong to the G-protein coupled receptor family, the angiotensin type I and type II receptors (AT1R and AT2R) (Figure 1.3). While both receptors are responsible for signal transduction, they result in opposing effects, with AT1R activation leading to vasoconstriction of blood vessels as well as increased cell proliferation, and AT2R activation resulting in vasodilation and reports of decreased cell proliferation (Ager et al., 2008).

As a result of the far reaching effects of ACE-1 and the RAS as a whole, numerous studies have shown the association between ACE-1 function and metabolic diseases such as diabetes as well as various cancer such as pancreatic and breast cancer (Anandanadesan et al., 2008; Du et al., 2012; Hsueh and Wyne, 2011). Thus, drugs used to target RAS might also prove effective against cancer. While some studies have shown an association between ACE-1 or AT1R hyperactivation with cancer (Escobar et al., 2004; Greco et al., 2002b; Vinson et al., 2012), others have been inconclusive (Cheng et al., 2015). Cohort studies, such as that

of (van der Knaap et al., 2008), assessed the effects of RAS inhibitors and ACE-1 insertion or deletion (I/D) polymorphism on risk of cancer. The data showed that persons with a DD genotype associate with high levels of ACE-1, and that RAS inhibitors protect against cancers in such individuals. Furthermore, a meta-analysis of the association of the ACE-1 gene polymorphism with cancer risk showed that the ACE-1 (I/D) polymorphism is associated with cancer in Caucasians (Zhang et al., 2014).

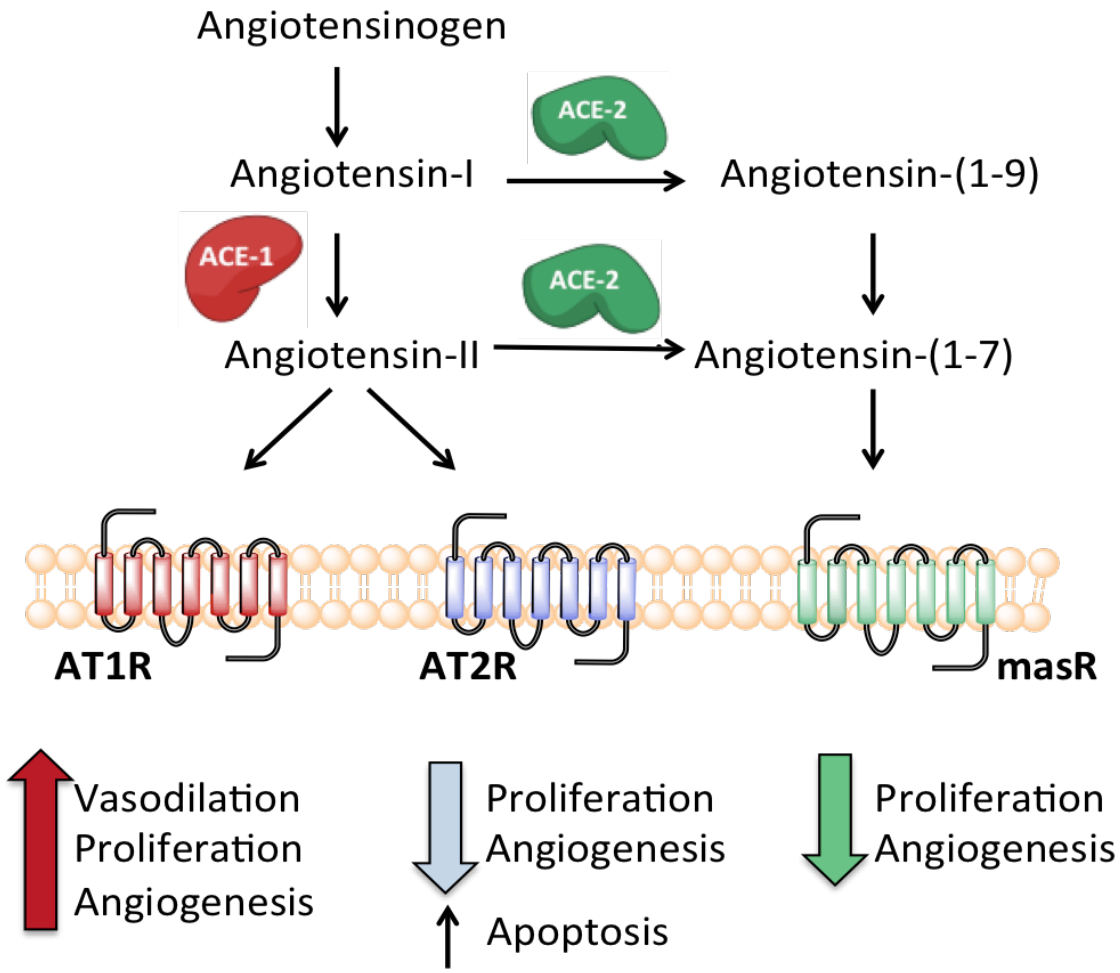


Figure 1.3: The extended renin angiotensin system. Angiotensin converting enzyme -1 (ACE-1) is responsible for cleavage of the inactive peptide angiotensin I, yielding angiotensin II which is the main effector peptide of the renin angiotensin system (RAS). Angiotensin-II causes a wide range of outcomes depending on the activation of either the angiotensin type I receptor (AT1R), or angiotensin type II receptor (AT2R). Apart from vasodilation, AT1R activation results in increased proliferation and angiogenesis in different cell types. Activation of the AT2R or the mas Receptor (masR) serves to counter such effects. MasR activation occurs through the binding of angiotensin- (1-7) as a result of cleavage of angiotensin-I by angiotensin converting enzyme-2 (ACE-2) and angiotensin- (1-9) by ACE-1.

1.3.1. Components of the RAS as potential anticancer targets

Cancer shares a link with various inflammatory and metabolic diseases, where common sets of genes show similar gene expression(Hirsch et al., 2010). As previously mentioned, it was shown that genes associating with cell cycle control, inflammation and lipid metabolism are also important for cancer function, thus showing increased expression in cancer tissues. The RAS has classically been known for its regulation of cardiovascular homeostasis and blood pressure control (Peach, 1977). However, there is increasing evidence that the major effector peptide of the RAS, Ang-II, as well as other components, are role players in pathophysiological conditions including the progression of many cancers (Ager et al., 2008; Atlas, 2007; Deshayes and Nahmias, 2005; Neo et al., 2010).

Apart from causing vasoconstriction, AT1R activation through binding of Ang-II has been observed to result in tissue remodelling and some of the hallmarks of cancer; cellular proliferation, angiogenesis, migration and invasion (Ager et al., 2008) (Figure 1.3). Ang-II exerts many of its effects through binding to the AT1R and interestingly it has been shown that in cancer the AT1R specifically is responsible for pathophysiological outcomes, i.e. pro-proliferative effects, whereas AT2R activation results in anti-proliferative effects (De Paepe et al., 2001 and Stoll et al., 1995).

Angiotensin-converting enzyme-2 (ACE-2) regulates the effects of ACE-1, through cleavage of both Ang-I and Ang-II to form Angiotensin 1-7 (Ang-(1-7)). Initial studies into the *Mas* oncogene, considered it an “orphan” G-protein receptor, without known functions (Santos et al., 2003). Studies by groups such as (Kostenis et al., 2005; Santos et al., 2007), later described the association between between AT1 Receptors, Mas and Ang-17. Since these

studies it is now well reported that binding of Ang-(1-7) to its specific receptor, the Mitochondrial Assembly Receptor (MasR), results in vasodilation and a reduction in proliferation and other pathophysiological actions of ACE-1 and the AT1R (Ager et al., 2008).

With growing evidence that Ang-II is synthesized not only systemically in circulation, but also at tissue sites and components of the RAS are expressed in tissues, the classic RAS paradigm has included the growing concept of tissue RAS.

The complexity of the RAS is a result of the wide spectrum of downstream effects induced through Ang-II activation of the AT1R. For this reason, crosstalk between signaling pathways exists facilitating an environment for carcinogenesis. For example, as well as being responsible for the proliferation of breast cells, Ang-II also stimulates the proliferation of endometrial, anterior pituitary and adrenocortical cells (Wegman-Ostrosky et al., 2013)

In the cancer setting Ang-II activation of the AT1R has been reported to stimulate the proliferation of breast, colorectal and prostate cancer cells (Ager et al., 2008; Lewandowska et al., 2011; Rodrigues-Ferreira et al., 2012). Since the discovery of the tissue RAS there has been much research targeting RAS components in various cancers (Table 1) (Neo et al., 2010; Wegman-Ostrosky et al., 2013). ACE-1 inhibitors such as Captopril have been shown to reduce growth and metastases *in vivo* of lewis lung carcinoma, renal carcinoma, colorectal, gastric cancer, and fibrosarcoma (Hii et al., 1998; Huang, 2007; Kosugi et al., 2006; Kowalski and Herman, 1996; Miyajima et al., 2002; Miyajima et al., 2015; Neo et al., 2007; Neo et al., 2010; Prontera et al., 1999; Suganuma et al., 2005; Volpert et al., 1996; Yoshiji et al., 2002).

AT1R inactivation with antagonists has also been seen to successfully reduce tumour growth of cancers including prostate, gastric, colorectal, breast, non-small cell lung and renal cancers (Alhusban et al., 2014; Araujo et al., 2015; Du et al., 2012; Kosaka et al., 2007; Kowalski and Herman, 1996; Nakagawa et al., 1995; Namazi et al., 2014; Okazaki et al., 2014). Studies have shown the blockade of the ACE-1 axis with ACE-1 inhbitors results in increased levels of Ang-I, due to a lack of feedback inhibition of renin secretion. Subsequent cleavage of Ang-I results in increased levels of Ang-(1-7), which is known to exert anti-cancer activity (Ritter, 2011; Santos et al., 2013). Any disruption within the complex cacades constituting the RAS has the potential to either promote, or reduce, the development of cancers, such as those mentioned above.

ACE-1 inhibitors or AT1R blockers could potentially be used as anticancer agents either as single treatments or in combination therapy as they are already being used in patients without any serious side effects, and as mentioned above, show signs of anticancer potential.

blockade in murine malignancy models. Adapted from (Ager et al., 2008)

ne/model	Agent	Tumour volume	Metastases	Refere
carcinoma 3LL	Captopril	Decreased	Decreased	Kowalski et a
rosarcoma	Captopril	Decreased	N/A	Volpert et a
noma SN 12K-1	Captopril	Decreased	N/A	Hii et al.,
carcinoma 3LL	Captopril (alone & combined with batimastat)	Decreased	Decreased	Prontera et a
cellular carcinoma	Captopril, perindopril and temocapril	Decreased	N/A	Yoshiji et al
stases of renal cinoma	Candesartan	Decreased	Decreased	Miyajima et a
cinoma SKOV-3	Candesartan	Decreased	N/A	Suganuma et
ncer KU-19-19	Candesartan	Decreased	N/A	Kosugi et al
ectal cancer liver tastases	Captopril	Decreased	Decreased	Neo et al.,
an gastric cancer xenograft	Candesartan	Decreased	N/A	Huang et al

essed. Several cancer cell lines have been used in xenograft or allograft animal models of metastases. These experiments nour volume and when assessed decreased metastasis

1.3.1.1 Inhibitors of the ACE-1 axis

Captopril, was the first synthetic ACE-1 inhibitor to be used in the treatment of hypertension and is a sulfydryl-containing alanine-proline analog (Figure 1.4). A short half-life and adverse side effects such as a metallic taste in the mouth, a rash, or a persistent cough, attributed to the sulfhydryl group unique to the compound, led to the development of improved ACE-1 inhibitors with carboxylate zinc binding groups, such as Lisinopril (Hanif et al., 2010).

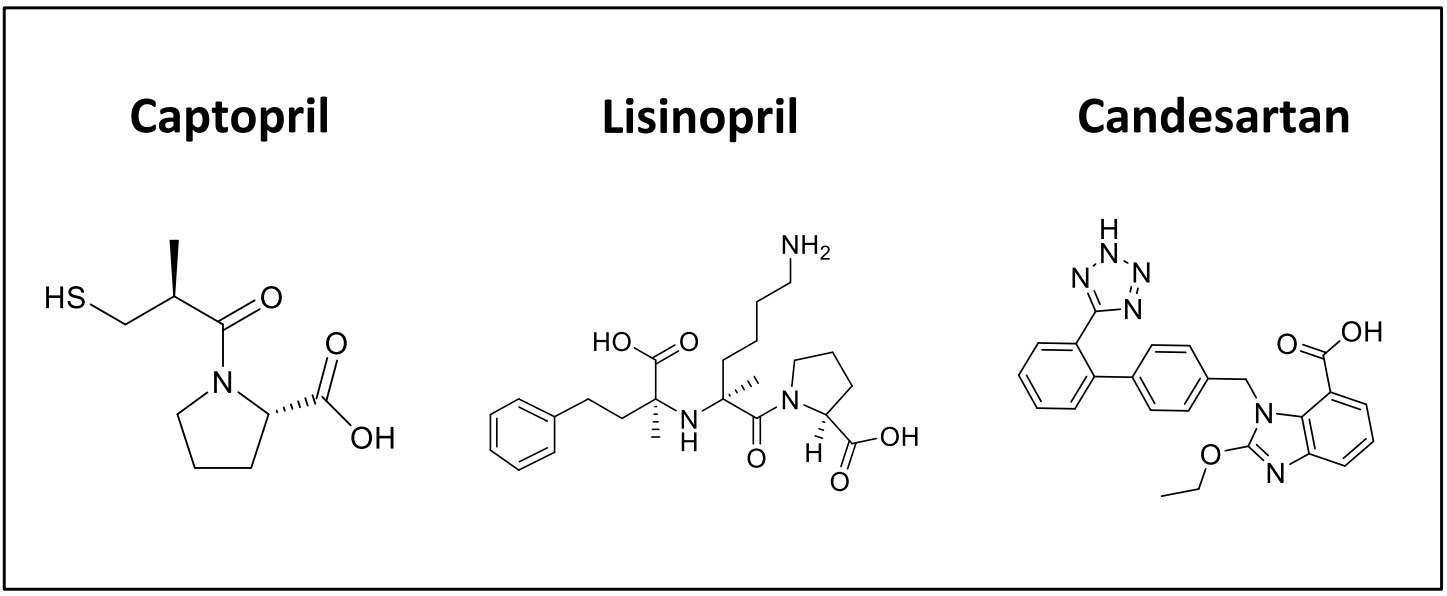
The chemical structures of the ACE-1 inhibitors are diverse leading to varying degrees of potency, bioavailability, plasma half-life, respective affinity for tissue bound ACE-1 and route of elimination from the body. In addition to this they also differ in terms of whether or not they are administered as prodrugs requiring subsequent activation (Table 2).

AT1R blockers were developed in an endeavour to improve the efficacy of the RAS blockade and improve the side effect profile of existing drugs. In 1971 Saralasin was introduced as the first AT1R blocker (Pals, D. T., Masucci, F. D., Denning, G.S.Jr., Sipos, F. and Fessler, D.C., 1971). Further research efforts were conducted to improve characteristics of this class as saralisin together with other antagonists such as sarmesin and sarilesin demonstrated poor bioavailabilty and short duration of action (Chung et al., 1999).

Current AT1R blockers have been further developed resulting in improved bioavailability compared to the first antagonists (Table 3). Similar to the ACE-1 inhibitors the chemical structures and pharmacological characteristics of AT1R blockers are variable although they

all show selective blockade of the AT1R while showing no off-target effects (Chung et al., 1999).

Candesartan is a benzimidazole-derived compound developed to further reduce ACE-1 inhibitor side effects, and block the activation of the AT1R preventing any downstream signaling (McClellan and Goa, 1998).



hemical structures of ACE-1 axis inhibitors. Captopril and Lisinopril target the inhibition of ACE-1 activity while Cand of the AT1R, activation through which results in a wide range of

rmacology of ACE-1 Inhibitors including Captopril and Lisinopril (Arora, 2013; Brown and Vaughan, 1998; Macdonalc

2002)

	Captopril	Enalapril	Lisinopril	Benazepril	Quinepril	Ra
Ligand	Sulfhydryl	Carboxyl	Carboxyl	Carboxyl	Carboxyl	Ca
drug	No	Yes	No	Yes	Yes	
C50	2×10^{-8} M	29.1 nM	4.7 nM	7 nM	8.3 nM	
ve drug, h	0.7 -0.9	2-8	6-8	1-2	2	
ve drug, h	1,7	11	12	10-11	1.9-2.5, 25 terminal	Tripha
elimination	kidney	kidney	kidney	kidney	kidney	k
range, mg	6.25 - 300	2.5 - 40	5 - 40	5 - 80	5 - 80	1.
lability, %	75 - 91	60	6 - 60	>37	>60	5

rmacology of clinically used AT1R antagonists

	Candesartan	Losartan	Olmesartan	Eprosartan	Irbesartan	Telmisartan	Valsart
Binding finity (K_d)	9.5 - 9.7	7.4 – 8.7	8.1	8.4 – 8.8	8.7 – 8.8	8.4	8.6
ıvailability	40%	33%	26%	13%	60-80%	67%	25%
oute of mination	kidney	kidney	kidney	kidney	kidney	kidney	kidney
eference	Chung et al., 1999 Vanderheyden et al., 1999	Diez et al., 2003, Timmermans et al., 1993	Kolke et al., 2001	Edwards et al., 1992	Vanderheyden et al., 1999	Wienen et al., 2000, Mclellan, K.J & Markham, A., 1998	De Gasparo Whitebrea 1995

1.3.1.2 Activating the ACE-2 axis of RAS

1.3.1.2.1 Activation of the ACE-2 axis using Ang-(1-7)

In addition to targeting the ACE-1 axis, there has also been an increase in the number of studies investigating the potential role that the ACE-2 axis may play as it is well documented as being the protective arm of the RAS (Ager et al., 2008; Ferrario, 1990b; Fleming et al., 2006; Wegman-Ostrosky et al., 2013). Activation of the ACE-2 axis using Ang-(1-7) in cancer studies has shown to have promising anti-cancer effects with disruption of growth-promoting signals as well as decreased angiogenesis, inflammation and metastasis of cancer cells (Feng et al., 2010a; Gallagher et al., 2014; Ni et al., 2012; Passos-Silva et al., 2015).

Furthermore, the overexpression of ACE-2 has been shown to produce anti-cancer effects through inhibition of angiogenesis and tumour cell invasion of non-small cell lung cancer cells (Feng et al., 2011a) as well as assisting with inhibiting acquired resistance to platinum-based chemotherapy in this cancer (Cheng et al., 2016).

1.3.1.2.2 Activation of the ACE-2 axis

Supplementing the fight against harmful effects of the ACE-1 axis are small molecule such as Diminazene aceturate (DIZE), a currently used ACE-2 enzyme activator (da Silva Oliveira and de Freitas, 2015; de Macedo et al., 2015; Kuriakose and Uzonna, 2014; Qi et al., 2013; Velkoska et al., 2015). Since its discovery, it has been utilised to increase ACE-2 activity in environments where ACE-2 activity was poor thus enabling the protective effects of ACE-2.

DIZE has been used since 1955 to treat animal trypanosomiasis, also referred to as sleeping sickness. It has been reported to possess trypanolytic properties, with the mechanism of action including the downregulation of phosphorylated mitogen activated protein kinases (MAPKs, including p38, ERK and JNK)(Peregrine and Mamman, 1993)Side effects associated with DIZE treatment in animals include tremors, sweating, convulsions and itching and thus has not yet been reported to be safe for testing in humans(Kuriakose et al., 2012)Considerations for future use in humans could involve structural modifications to the compound in order to maintain a high degree of potency with a reduction of side effects. Alternatively, studies could investigate the lowest possible dose for efficacy in animals and humans, as a single or combined treatment.

1.4 Signalling pathways associated with the tissue RAS and disease

1.4.1 Calcium Signalling

With the discovery of the local RAS in various tissues there is increasing interest to investigate how the system gets initiated, i.e the generation of angiotensin I. (Pro) renin is the inactive form of Renin and can be activated both proteolytically and non-proteolytically

(Sihn et al., 2010a). Very little is known regarding (pro) renin as well as the role of the (pro) renin receptor in cancer. It has been assumed that (pro) renin was recruited and locally activated within the tissue to aid in the generation of angiotensin II (Nguyen and Contrepas, 2008).

Binding of (pro) renin to the (pro) renin receptor has been shown to activate (pro) renin non-proteolytically with the result of local Angiotensin I generation (Sihn et al., 2010b). Studies using RAS inhibitors have shown that the activation of (pro) renin receptor can trigger intracellular signalling that is independent of Ang-II such as MAPK signalling (ERK 1/2) (Sihn et al., 2010a). Blockade of the AT1R can still result in signalling as activated (pro) renin can signal independently of AT1R activation.

Studies by (Cruciat et al., 2010) have identified an additional function of the (pro) renin receptor (PRR), acting as a V-ATPase which is an essential component of the Calcium-related Wnt signalling pathway (Figure 1.5). Through the phosphorylation of the Wnt co-receptor LRP6, they showed activated intracellular Wnt signalling was dependent on V-ATPase action. The (pro) renin receptor has been seen to trigger pro-proliferative and survival effects independent of Ang-II and the AT1R.

Cross talk between G protein-coupled receptors (like AT1R) and growth factor receptors (such as EGFR) can lead to the amplification of commonly used signalling pathways. This also encourages the overlap of other signalling mechanisms such as non-canonical Wnt/ Calcium signalling (Katoh, 2005). The Wnt/ Calcium pathway has been observed to activate Calmodulin-Dependent Kinase II (CAMKII) and protein kinase C (PKC) resulting in survival

and proliferation of cancer cells (Katoh, 2005). In addition to this, there have been reports that Ang-II via AT1R activation increases intracellular calcium in normal and breast cancer cells (Greco et al., 2002a).

Ang-II has been reported as being a mitogen promoting the growth of a variety of cell types through activation of protein tyrosine kinases. Activation of receptors to which tyrosine kinases are attached leads to growth stimulation. Further growth promoting effects occur via Ang-II stimulation of phosphatidylinositol (IP3) and diacylglycerol (DAG) resulting in an increase of cytosolic calcium and thus is not only responsible for proliferation of normal cells where it is synthesized but also neoplastic cells (Lewandowska et al., 2011; Wegman-Ostrosky et al., 2013). In a study by Greco et al (2002), phospholipase C was found to be activated triggering IP3 DAG. This then lead to the activation of PKC and calcium mobilization from the intracellular stores (Figure 1.5).

Calcium has been reported to be an important mediator of Ang-II effects and the expression of early cancer progression genes such as c-fos, c- jun and c-myc which are involved in the regulation of a number of target genes (Greco et al., 2003).

The nuclear factor of activated T cells (NFAT) protein family are transcription factors that have been implicated in cancer progression (Mancini and Toker, 2009). Overexpression of NFAT results in tumourigenic characteristics such as the promoted growth, migration, invasion, differentiation and survival of cells. NFAT is activated by the release of intracellular calcium stores as well as the addition of extracellular calcium to a cell. Since Ang-II has been well reported in the activation of calcium signalling in various cardiovascular diseases as well

as cancer (Kim and Iwao, 2000; Muscella et al., 2003a), we hypothesized that this signalling pathway might be altered in cervical cancer cells.

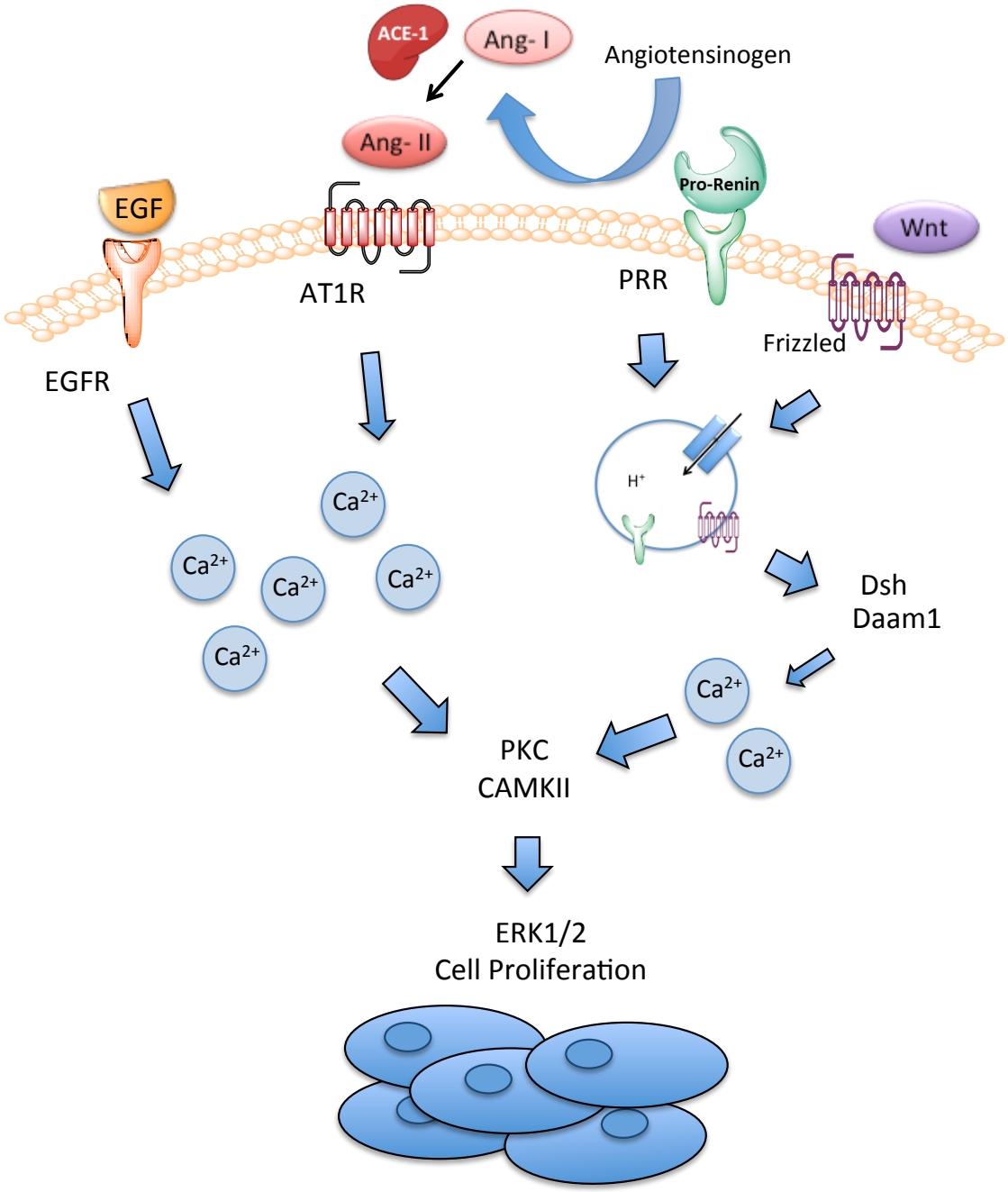


Figure 1.5: Tissue RAS signaling pathways. Via possible crosstalk between activated GPCRS, AT1R and EGFR or via Wnt signaling pathways, Intracellular calcium signaling is disrupted in cancer cells leading to increased cytosolic calcium activating downstream proliferation signals.

1.5 Drug repurposing as anticancer treatments

As traditional anticancer drug discoveries and research takes years and a large budget before a new drug can reach patients an alternative approach is much needed. “Drug repurposing”, also known as “drug repositioning”, provides the opportunity for the identification of alternative therapeutic indications for existing approved drugs (Oprea and Mestres, 2012). This nonconventional approach to traditional drug discovery methods is useful in that existing drugs have already undergone extensive screening and received approval for safety in patients. Thus, discovery of suitable anticancer drug candidates and subsequent research on them could result in the drug reaching the cancer patient sooner than new, or traditionally discovered drugs (Jin et al., 2012).

As drug repurposing is becoming increasingly attractive due to the many benefits, several strategies to effectively identify and analyzing potential cancer targets and thus anticancer drugs are being utilised. One such strategy is based on the fact that many currently used drugs possess, in addition to their primary targets, some form of secondary activity. Another strategy derived from numerous studies, such as those previously discussed, makes use of the fact that different diseases share common molecular pathways resulting in a drug exerting therapeutic effects in diseases that share core pathways (Gupta et al., 2013; Hirsch et al., 2010).

Over the years non-cancer drugs have been selected for study for potential anticancer activity as their biological activity has been well described. Examples of a few such drugs include Aspirin, Metformin, Thalidomide, Methotrexate and Noscapin (Table 4), which had

initial therapeutic indications toward pain relief, nausea, immunosuppressant therapy or diseases including diabetes and malaria.

Table 4: Non-cancer drugs and their original and new anticancer indications.

Drug	Original therapeutic indication (mechanism)	New anticancer indication (mechanism)
Aspirin	Analgesic, antipyretic (COX-1 ↓ , COX-2↓)	Colorectal cancer (COX-2↓ , NF-$_{\kappa}$B ↓, AP-1 ↓)
Metformin	Diabetes (AMPK↑^{a})	Breast, adenocarcinoma, prostate, colorectal (AMPK↑^{a}, NF-$_{\kappa}$B↓ , TNF↓, MCP-1↓)
Thalidomide	Antiemetic in pregnancy (TNF-α↓)	Multiple myeloma (NF-$_{\kappa}$B↓, STAT3↓)
Methotrexate	Acute leukemia (DHFR↓)	Osteosarcoma, breast cancer (NF-$_{\kappa}$B ↓, TNF -α↓)
Noscapin	Antitussive, antimalarial, analgesic (bradykinin↓)	Multiple cancer types (NF-$_{\kappa}$B↓, HIF-1 -α↓, Bcl-2↓, p21↑, p53↑)
Statins	Myocardial infarction (HMG-CoA reductase ↓)	Prostate cancer, leukemia (NF-$_{\kappa}$B↓, HMG-CoA reductase↓)
Nitroxaline	Antibiotic	Bladder, breast cancer (MetAP-2↓)
Rapamycin	Immunosuppressant (mTOR↓)	Lymphoma, leukemia, colorectal cancer (NF-$_{\kappa}$B↓, IL-6↓, IKK↓)

Abbreviations: DHFR, dihydrofolate reductase; HIF-1α, hypoxia-inducible factor-1 α; MetAP, methionine aminopeptidase; MCP-1, monocyte chemoattractant protein-1; ↑^{a}, activation; ↑, upregulation;↓, downregulation. Adapted from Gupta et al., (2013).

The abandoned antihypertensive agent, Mibefradil, is another example of successful drug repurposing. Short administration of the drug followed by conventional anticancer agents has been proven to enhance the therapeutic potential of the conventional anticancer regimen (Krouse et al., 2015). While Mibefradil was removed from the market due to drug-drug interactions, it could be rescued as the short administration of the drug prior to chemotherapy remedied this effect and improved the therapeutic potential of chemotherapy. Evolving cancer chemotherapy regimens to include a repurposed drug may have the potential to reduce the dosage, as well as potential side effects, of well known chemotherapeutic agents, such as Cisplatin (CDDP) or Doxorubicin (DOX).

1.6 Explorations in combination treatment

1.6.1 Current anticancer treatments

The chemotherapy landscape has changed and improved over the years. Chemotherapy is aggressive on the body as surrounding healthy cells are often sacrificed in order to kill nearby cancer cells. Good patient management includes analyzing the best choice of treatment methods and frequent monitoring of toxic side effects. Changes in practice have seen improvement in the tolerability of many drugs including CDDP (Fennell et al., 2016).

CDDP and DOX are two of the most widely used chemotherapeutic agents on the market today for many cancers some of which include lung, neck, bladder, cervical, ovarian and testicular (Figure 1.6). CDDP is a platinum based compound effective against carcinomas, germ cell tumours, sarcomas and lymphomas (Dasari and Tchounwou, 2014). Furthermore, it is an alkylating agent specifically intercalating with DNA destabilizing the double helix into

adducts in the *cis* configuration. This DNA damage and subsequent interference with DNA repair mechanisms induces apoptosis in cancer cells (Dasari and Tchounwou, 2014). In cervical cancer CDDP is one of the most effective frontline chemotherapeutic drugs (Fennell et al., 2016; Long, 2007; Thigpen et al., 1981)

DOX is an anthracycline antibiotic used most importantly for the treatment of solid tumours (Hortobagyi, 1997; Lenglet and David-Cordonnier, 2010). DOX is highly toxic inducing DNA damage via inhibition of topoisomerase II as well as free radical formation leading to apoptosis of cancer cells (Patel and Kaufmann, 2012). While DOX is extremely effective in killing cancer cells, one of the dominant side effects of this harsh treatment is cardiomyopathy (Zhang et al., 2016).

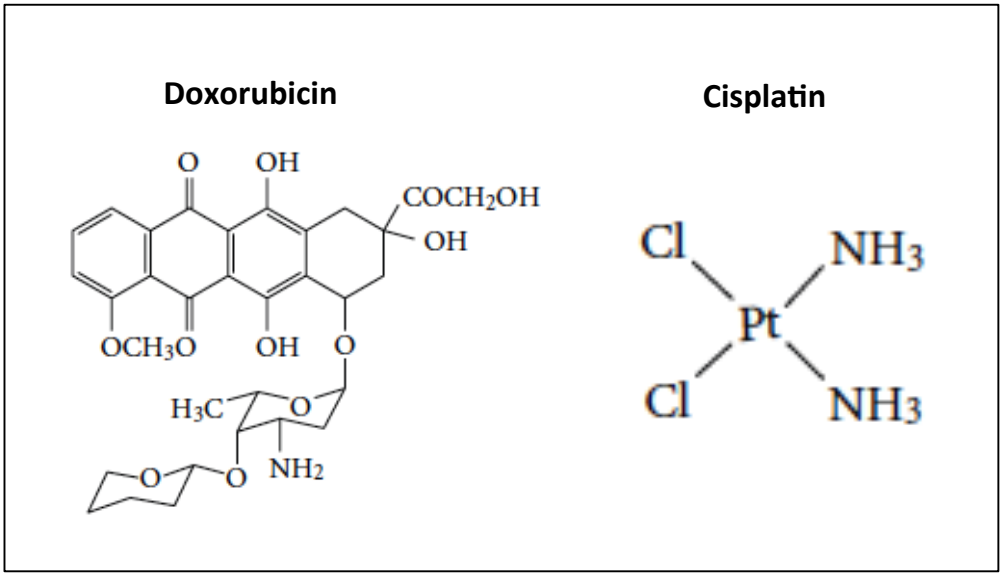


Figure 1.6: Chemical structures of Doxorubicin and Cisplatin. Adapted from (Lenglet and David-Cordonnier, 2010)**.**

1.6.2 Combination treatments in cancer

Combination treatments in cancer have become standard practice in order to increase drug response and improve tolerability. In an effort to combat chemoresistance, research investigating effective combination treatments has been conducted with promising results (Jia et al., 2009; Pritchard et al., 2012; Pritchard et al., 2013; Ricevuto et al., 2010). Some of the potential benefits of using combination treatments include the possible amplification of the effective agent when treating cancer cells. Combination therapies are tested in the hope that effects of the combined usage may be greater than either of the individual treatments. The ultimate aim of combination treatments is it maximize efficacy of treatment while minimizing toxicity.

In cervical cancer, a number of effective alternative therapies to CDDP have been investigated some of which include paclitaxel, topotecan, ifosfamide, gemcitabine, irinotecan (Coleman et al., 1986; Fushiki et al., 1997; Kim et al., 1999; Kumar and Gupta, 2016; Li et al., 2015; Muderspach et al., 2001; Urruticoechea et al., 2010; Valle et al., 2010; Verschraegen et al., 1997). CDDP combination treatments in cervical as well as other cancers using drugs such as paclitaxel have also been investigated with great success (Crino et al., 1997; Esteban et al., 2006; Koizumi et al., 2008; Larasati et al., 2014; Minami et al., 2013; Takahashi et al., 2012). It was shown by (Larasati et al., 2014) that the combined treatment of cinnamon essential oil with CDDP induces HeLa cell cycle arrest thereby inhibiting proliferation. Furthermore, in PC-9 and H1650 non-small cell lung cancer cells as well as in xenograft models, (Minami et al., 2013) showed that the combined treatment of the PARP inhibitor, olaparib, with CDDP was more effective at killing cancer cells than each

drug individually. CDDP combination therapy has proven effective in improving prognosis of patients in phase I to phase III studies. The prognosis of gastric cancer patients in a phase I study was improved after triple combination treatments of paclitaxel, CDDP and S-1 (Takahashi et al., 2012). In a phase II study investigating the effects of CDDP combination treatment with the EGFR inhibitor, erlotinib, it was found that combination treatment exerted significant antitumour activity in cervical cancer patients (Nogueira-Rodrigues et al., 2014). A phase III study by (Goto et al., 2016) showed that a second-line chemotherapy of CDDP, etoposide and irinotecan improved overall survival of small-cell lung cancer patients when compared to topotecan monotherapy.

DOX combination treatments have also seen much success in cancer. The use of docetaxel (DOC) with DOX in PC3 and DU145 prostate cancer cells resulted in a synergistic effect (Tsakalozou et al., 2012). Similarly to CDDP, in addition to *in vivo* studies, the use of DOX in combination treatments has proven successful in phases I to III trials. A phase I trial by (Thomas et al., 2014) showed that the combined treatment of DOX with a histone deactylase inhibitor, belinostat, together with CDDP and cyclophosphamide was effective against thymic epithelial tumours with improved progression-free survival. The phase II study by (Pautier et al., 2015) showed that in combination with Trabectedin, DOX was an effective first-line treatment of metastatic leiomyosarcomas, which typically show limited sensitivity to chemotherapy. Furthermore, a phase III study by (Judson et al., 2014a) showed that the combined treatment of DOX with ifosfamide for first-line treatment of metastatic soft tissue sarcoma was effective in the shrinkage of tumours. These studies provide evidence that combination treatments, with the use of DOX and CDDP in such treatments, lay the foundation for effective chemotherapy strategies.

Drug interaction effects in combination therapy

Drug combinations can result in one of three outcomes with regard to their interaction: synergism, antagonism or additive effects (Chou and Talalay, 1984). Such evaluations are best determined using mathematical software, which carefully interrogates the combination of two or more drugs. Chou and Talalay are considered the drug combination pioneers as their combined input to understand combination therapies has assisted studies worldwide to accurately define the effects resulting from multiple drug interactions. “Synergism, antagonism and additivity can be defined as the interaction between two or more components such that the combined effect is superior, inferior or equal, respectively, to the expected sum of the individual drug effects”(Chou and Talalay, 1984; Chou, 2006; Mayer and Janoff, 2007; Merlin, 1994). The benefit of combination treatment to cancer patients has been promising with researchers showing synergistic drug combination treatments improve patient outcomes compared to single treatments. In a phase II trial by (Finn et al., 2015), it was shown that a small molecule inhibitor of cyclin dependent kinases 4/6, palbociclib, maintained its synergism with the anti-oestrogen treatment, letrozole. In both cohorts studied it was found that median progression-free survival of patients was significantly greater in the group receiving the combination treatment. As a result a phase III trial is being pursued. Furthermore, a review of some notable combined immunotherapy to cancer patients by (Melero et al., 2015) highlights the benefits of effective combination treatments. One such study conducted by (Reck et al., 2013) was a phase II trial investigating first-line therapies in extensive disease-small-cell lung cancer. It was observed that the sequential administration of an anti-CTLA4 monoclonal antibody, ipilimumab,

together with carboplatin and paclitaxel, improved immune related progression-free survival of patients when compared to control groups.

The use of Chou and Talalay's method of fixed ratio treatments has advanced the systematic screening of multiple drug combinations. This form of analysis was previously unrecognized and much research has provided insight as to how dependent synergistic effects can be on drug ratios (Chou, 2006; Mayer and Janoff, 2007).

1.6.2.1 RAS inhibitors as combination treatments

As RAS inhibitors are already approved for clinical use and have shown to have anticancer effects (see section 1.3.1), it would be greatly beneficial to make use of their anticancer function to augment current anticancer regimens. While the use of antihypertensive agents has been shown to provide protection against harmful side effects of DOX or CDDP treatment such as cardio or nephrotoxicity (Akolkar et al., 2015; Saleh et al., 2009), repurposing the RAS inhibitors to target cancer in combination treatments has not been widely explored. The *in vivo* study by (Akolkar et al., 2015)showed that the use of the ACE-1 inhibitor, perindopril, and the AT1R antagonist, valsartan, had a prophylactic effect against cardiotoxicity induced through the treatment of DOX and trastuzumab, the monoclonal antibody used to treat HER2 positive breast cancer. It was shown that RAS inhibition in the combination treatment improved the survival of mice compared to the control group. (Saleh et al., 2009) also showed the therapeutic benefit of RAS inhibition on CDDP-induced nephrotoxicity, where the ATR antagonist, Losartan, showed protective effects against kidney injury in rats.

1.7 Project Aims

This study was based on the hypothesis that altered expression and activity of the RAS may contribute to cancer development. Thus, the aim was to determine the role of the RAS in different cancer cell lines. The objectives were:

1. To investigate the effects of ACE-1 axis of the RAS, i.e. Ang-II, ACE-1 and the AT1R, expression and ACE-1 activity in cancer cells, with respect activation of intracellular signalling
2. To investigate the effect of ACE-2 axis activation on cancer cell biology using Ang-(1-7), and the small molecule ACE-2 activator DIZE
3. To investigate the effects of combined treatment with RAS inhibitors/antagonists and chemotherapeutic agents on cancer cell biology

CHAPTER 2

ACE-1 AXIS PROTEIN EXPRESSION AND ACTIVITY IN CANCER CELLS

2.1 INTRODUCTION

Since the discovery that cancer shares a link with metabolic diseases, more reports have been recorded implicating the Renin Angiotensin System (RAS) in the development of various cancers(Carl-McGrath et al., 2007; Dinh et al., 2001; Ino et al., 2011; Wegman-Ostrosky et al., 2015). The direct and indirect effects of RAS signaling on cancer cells are increasingly being documented. Potential therapeutic strategies such as AT1 receptor (AT1R) blockers (ARBs) have demonstrated anticancer effects and are thus being further researched. Literature studies have reported the up-regulation of AT1R protein expression within breast cancer tissue and the blockade of AT1R signaling via ARB's such as Irbesartan, reduced cancer cell proliferation(Anandanadesan et al., 2008; Du et al., 2012; Escobar et al., 2004; Hunyady and Catt, 2006; Muscella et al., 2003b; Puddefoot et al., 2006; Vinson et al., 2012; Wegman-Ostrosky et al., 2013; Zhao et al., 2010a). Cancer cells within the developing tumour can be directly affected via RAS signals, whereas the growth of vascular cells can promote angiogenesis and thus indirectly influence tumour growth and metastasis. RAS signaling stimulates hematopoiesis (Park and Zambidis, 2009) and the proliferation of various cell types including endothelial cells, squamous cells lining either blood or lymph vessels (Wegman-Ostrosky et al., 2013), vascular smooth muscle cells, glomerular mesangial cells, and hepatic stellate cells, (Bataller et al., 2000; Griendling et al., 1997; Ray et al., 1991).

It has been reported that the RAS plays numerous roles in cancer progression, such as proliferation, angiogenesis, migration and invasion (Dorsam and Gutkind, 2007; Escobar et al., 2004; Greco et al., 2002b; Hunyady and Catt, 2006; Puddefoot et al., 2006; Wegman-Ostrosky et al., 2013). ACE-1 axis signalling, meaning the activation of the AT1R by Angiotensin II (Ang-II), has been shown to control the proliferation of endometrial, colorectal, breast, anterior pituitary and adrenocortical cells (Ager et al., 2008; Ino et al., 2011; Kuniyasu, 2012; McEwan et al., 1996; Muscella et al., 2002; Pawlikowski et al., 1999). While there is literature on RAS downstream signalling in endothelial cells, calcium signalling in particular, little is known regarding signalling surrounding ACE-1 axis signalling in cancer cells, particularly cervical cancer.

In this chapter we investigated the expression and activity of ACE-1 axis components of the RAS. The potential cell killing effect of the ACE-1 inhibitors, Captopril, Lisinopril and the AT1 receptor antagonist, Candesartan, was explored. We also investigated the ability of ACE-1 axis inhibition to disrupt the cell cycle and associated cell killing events.

2.2. RESULTS

2.2.1. Expression and activity of ACE-1 axis components, ACE-1 and AT1 Receptor in transformed, cancer and non-cancer cell lines

Using western blot analysis protein expression levels of components of the ACE-1 axis, namely the ACE-1 enzyme and the AT1 Receptor, were investigated in a panel of cancer and transformed cell lines of different tissue origin, including breast cancer (MDA-MB-231), cervical cancer (HeLa and SiHa) and hepatocellular carcinoma (HepG2) and a transformed cell line, SVWI38. Expression levels were compared to that observed in non-cancer WI38 cells. Results showed elevated ACE-1 expression in the cancer and transformed cells compared to the non-cancer WI38 cells (Figure 2.1A). Using a selected cell lines, we next investigated whether elevated ACE-1 expression correlated with ACE-1 activity. ACE-1 protein expression had good correlation with ACE-1 enzyme activity, as measured with a fluorimetric assay using the ACE-1 specific substrate, benzyloxycarbonyl-Phe-His-Leu (ZFHL), with the cancer cell lines showing elevated activity (Figure 2.1B).

After establishing that ACE-1 protein expression was elevated in cancer cells and that there was an increase ACE-1 enzyme activity in cancer cells compared to control, we next investigated protein expression of the AT1R (Figure 2.2 A), as there is evidence linking the activation of the AT1R with the pathophysiological effects of Ang-II (Abd El-All et al., 2015; Blaszczak-Swiatkiewicz and Mikiciuk-Olasik, 2015; Chu et al., 2015; El Rashedy and Aboul-Enein, 2013; Koronkiewicz et al., 2015; Yadav et al., 2015; Yurttas et al., 2015).

Our results suggest elevated AT1R expression in the majority of cancer cell lines investigated compared to that in the non-cancer WI38 cell line. Together these results show that two key components of the RAS, ACE-1 and AT1R, have elevated expression in cancer cells.

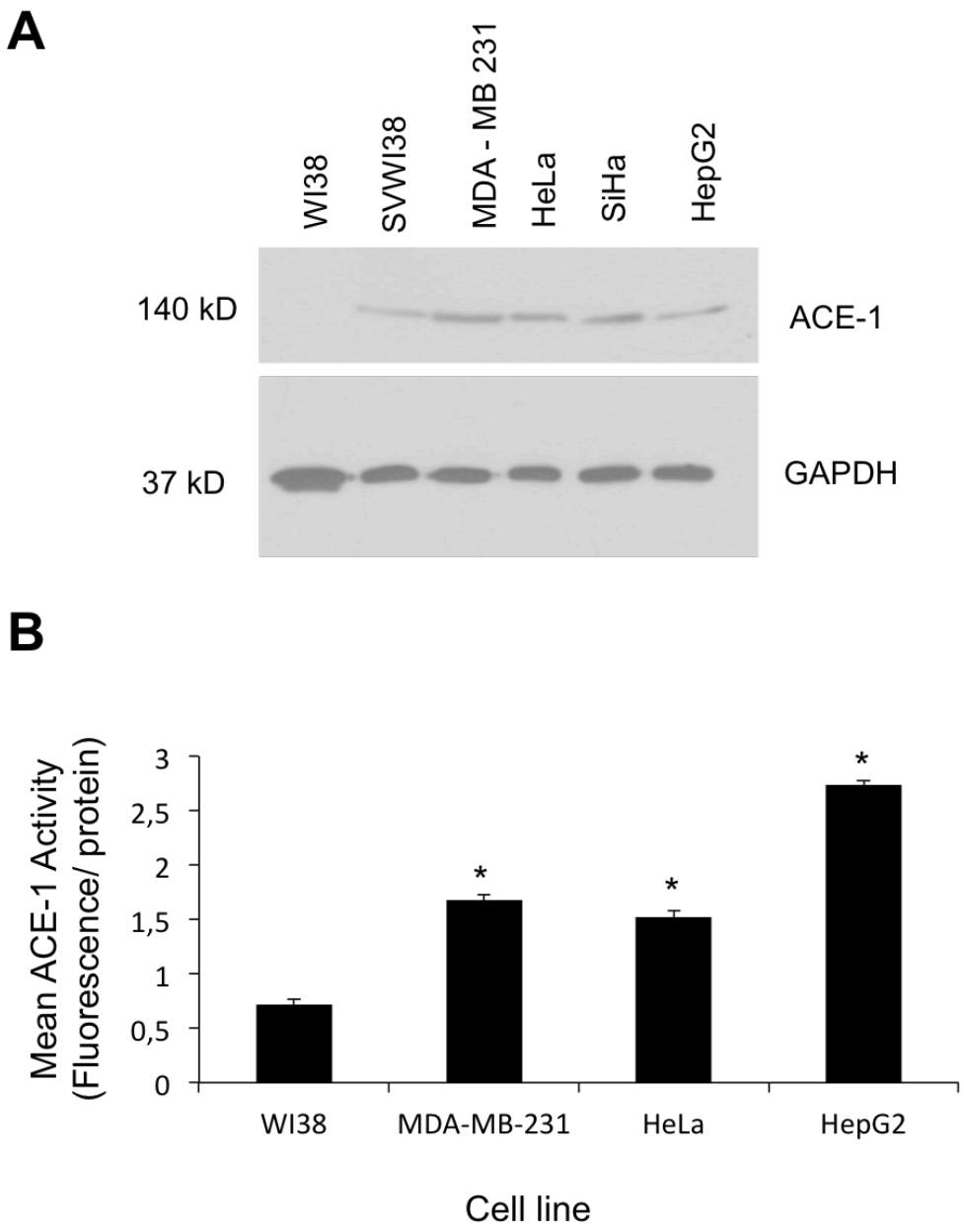


Figure 2.1: Endogenous ACE-1 expression and activity is elevated in cancer cells compared to normal cells. A: Western blot analysis showing ACE-1 expression in WI38 (normal fibroblasts), SVWI38 (transformed fibroblasts) and breast cancer, MDA-MB-231, cervical cancer, HeLa and SiHa, and hepatocellular carcinoma, HepG2, cell lines. GAPDH was used as a control for protein loading. **B:** ACE-1 activity measured in normal and cancer cells using the ACE-1 enzyme assay showing cleavage of the ZFHL substrate. Results shown are the mean +/- SEM of experiments performed in triplicate and repeated at least two independent times. *p-value <0.05.

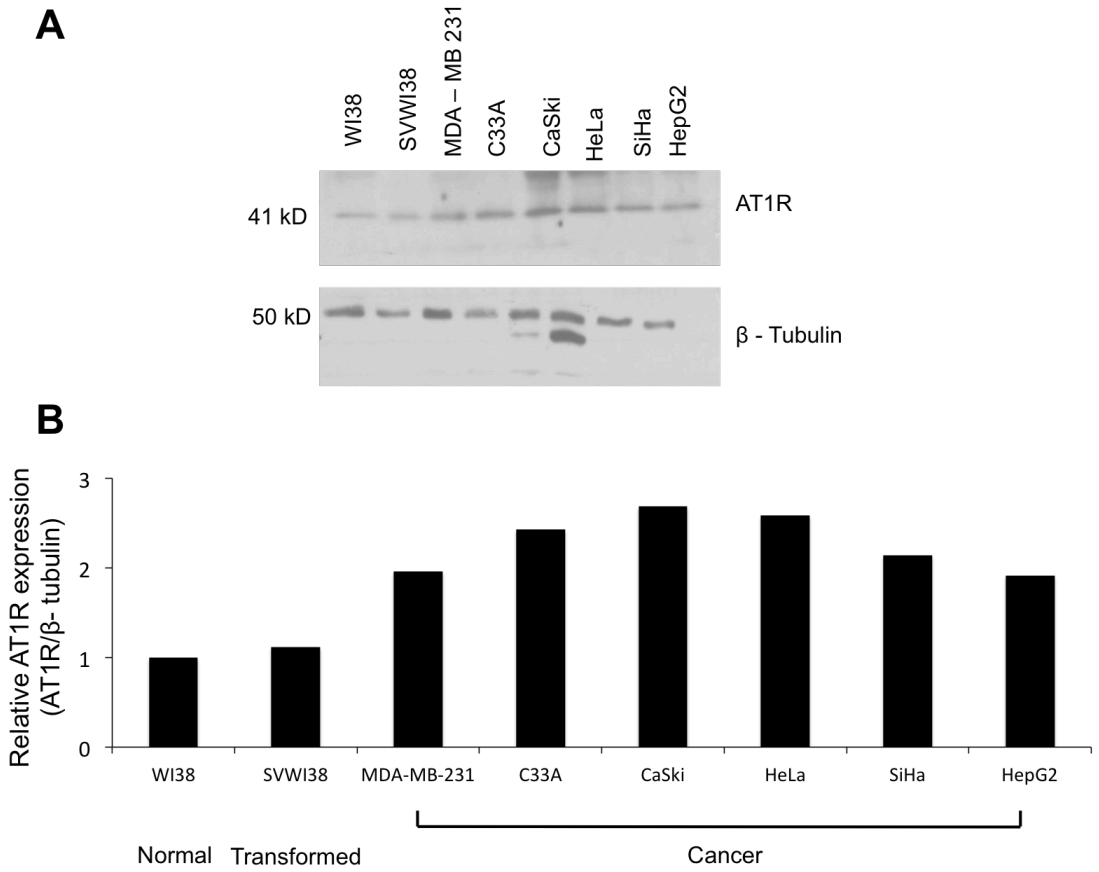


Figure 2.2: Endogenous AT1R protein expression is elevated in cancer compared to normal cells. A: Western blot analysis showing AT1R expression in normal fibroblasts, WI38, transformed, SVWI38, breast cancer, MDA-MB-231, cervical cancer C33A, CaSki, HeLa and SiHa, and hepatocellular carcinoma, HepG2, cell lines. β-tubulin was used as a control for protein loading. **B:** Quantification of band intensity relative to β-tubulin showing AT1R expression in cancer cells. Results shown represent experiments performed two independent times.

2.2.2 ACE-1 axis inhibition reduces proliferation of cancer cells

Having shown that ACE-1 and AT1R expression was elevated in cancer cells we next explored the functional relevance of this by inhibiting their activities. ACE-1 inhibitors, Captopril and Lisinopril, and the AT1R antagonist, Candesartan, were tested for effects in a panel of cancer cell lines. The cervical cancer cell lines tested included HeLa, CaSki and SiHa. The MDA-MB-231 breast cancer cell line was also included.

The 50% inhibitory concentrations (IC_{50}) for Captopril, Lisinopril and Candesartan were determined in non-cancer (WI38 and FG0) and cancer cell lines (HeLa, CaSki, SiHa, MDA-MB-231 and HepG2). Non-cancer cell lines, WI38 and FG0, appeared insensitive to treatment with the ACE-1 inhibitors and AT1R antagonist and thus an IC_{50} concentration could not be determined. The cancer cell lines (HeLa, CaSki, SiHa and HepG2) all on the other hand showed IC_{50} concentrations for Captopril, Lisinopril and Candesartan of 310 to 367 µM, 400 to 478 µM, 150 to 182 µM respectively.

The results showed that a lower dose of the AT1R antagonist, Candesartan, was required to give 50% maximal inhibition compared to the ACE-1 inhibitors, Captopril and Lisinopril.

Table 1: IC_{50} values of ACE-1 inhibitors, Captopril and Lisinopril and AT1R antagonist, Candesartan in normal and cancer cells. IC_{50} values were generated for non-cancer cells (WI38 and FG0) and cancer cell lines (HeLa, CaSki, SiHa and MDA-MB-231). Experiments were repeated three independent times.

Cell line	Captopril		Lisinopril		Candesartan	
	IC_{50} (µM)	95% CI	IC_{50} (µM)	95% CI	IC_{50} (µM)	95% CI
WI38	CNBD	CNBD	CNBD	CNBD	CNBD	CNBD
FGO	CNBD	CNBD	CNBD	CNBD	CNBD	CNBD
HeLa	310	229 – 412	400	277 – 577	150	119 – 182
CaSki	325	247 – 427	412	395 – 430	151	36 – 640
SiHa	325	211 – 497	450	396 – 505	150	127 – 179
MDA-MB-231	367	237 – 568	478	354 – 647	182	91 – 364

CNBD - Could not be determined

Since ACE-1 and AT1R expression were elevated in cancer cells, their requirements for cancer cell proliferation were investigated by inhibiting their activity using two approaches, an ACE-1-specific siRNA, and with the ACE-1 inhibitors, Captopril and Lisinopril. The effect of inhibiting activation of the AT1R on cell proliferation was also monitored (Figure 2.3).

The proliferation assays showed a significant reduction in HeLa cell proliferation with the ACE-1 inhibitor, Lisinopril, and the AT1R antagonist, Candesartan (Figure 2.3 A). For Captopril, a small but non-significant, decrease in cell proliferation was only observed after 3 days treatment. All 3 agents had a significant inhibitory effect on the proliferation of CaSki cells (Figure 2.3 B). The requirement of ACE-1 activity was further investigated by silencing of ACE-1 using siRNA, which resulted in a significant reduction in HeLa cell proliferation (Figure 2.3 C) similar to that seen for Lisinopril. Western blot results showing ACE-1 knockdown can be found in Appendix

These results show that inhibiting ACE-1 expression with siRNA and its activity with small molecules interferes with the proliferation of cancer cells.

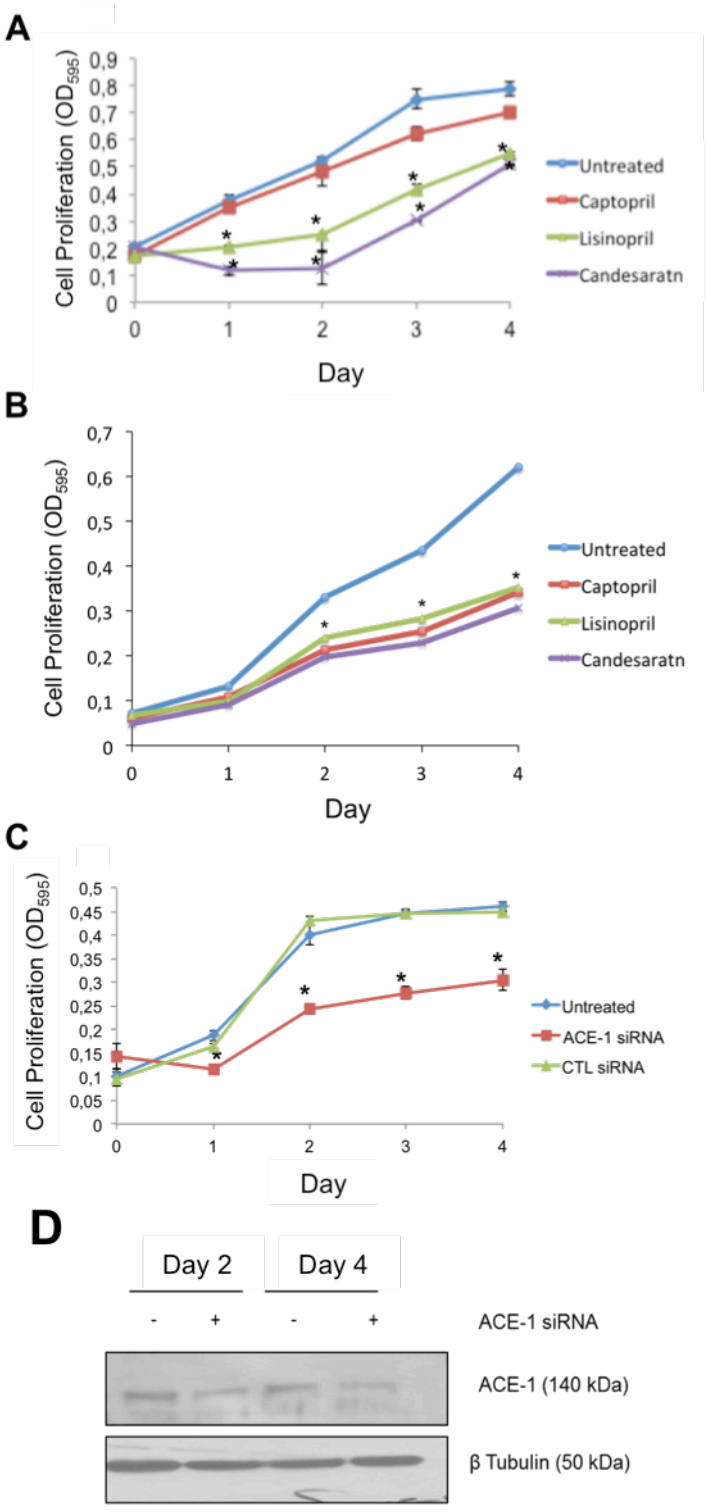


Figure 2.3: Effect of ACE-1 axis treatments on HeLa and CaSki cell proliferation. A: HeLa and **B:** CaSki cells were treated with Captopril, Lisinopril and Candesartan at IC_{50} concentration and the effect on cell proliferation monitored using the MTT assay. **C:** Transient knockdown of ACE-1 using siRNA in HeLa cells significantly reduces proliferation of HeLa cells. Control siRNA was used to control for the effect of DNA transfection Results are the mean +/- SEM of experiments performed in triplicate and repeated three times. *p-value <0.05.

To investigate whether inhibition of ACE-1 axis proteins associates with cell death, we used the Annexin V assay to determine the percentage of live, early and late apoptotic as well as necrotic cells after treatment with Captopril, Lisinopril or Candesartan.

Our results show that Captopril had a small but significant inhibitory effect on the percentage of live cells on all three cell lines tested, HeLa, CaSki and MDA-MB-231 (Figure 2.4). Accompanying this was a small but significant increase in cells in early, late apoptosis and necrosis. Lisinopril treatment similarly resulted in an increase in cells undergoing apoptosis and necrosis (Figure 2.5). Candesartan treatment resulted in a significant increase in necrosis in HeLa and CaSki cells and an increase in both apoptosis and necrosis in MDAMB-231 cells (Figure 2.6).

This data suggests that inhibitors of ACE-1 axis proteins influence cell death having signs of both apoptosis and necrosis.

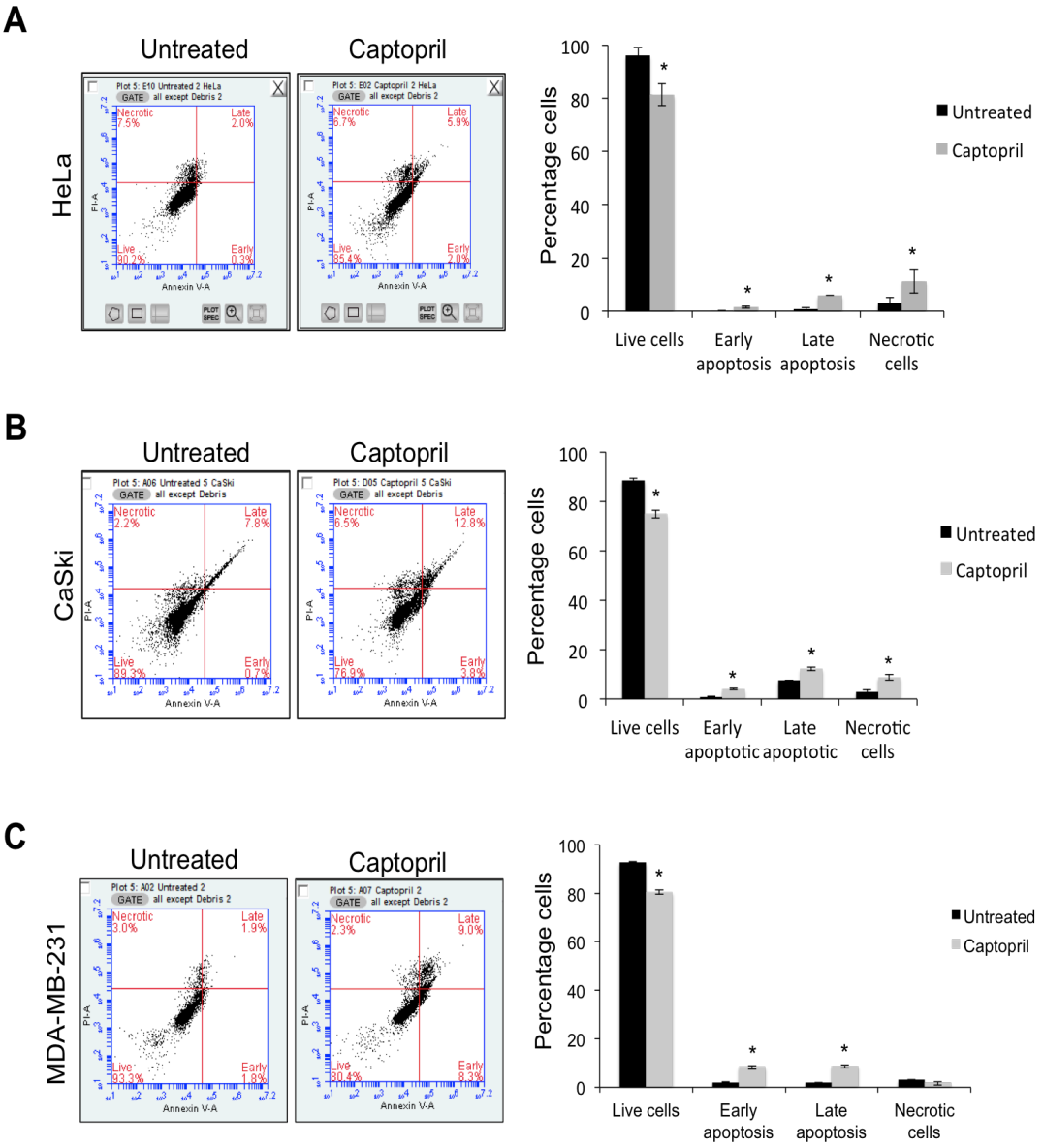


Figure 2.4: Captopril induces apoptosis and necrosis in cancer cells. Annexin V profiles indicate that a 48 hr treatment with the IC_{50} concentration of Captopril reduces the number of live cells via apoptosis in **A:** HeLa, **B:** CaSki and **C:** MDA-MB-231 cells. Annexin V profiles for one representing experiment is shown for each cell line and bar chart results are the mean +/- SEM of experiments performed in triplicate and repeated two independent times (*p-value<0.05).

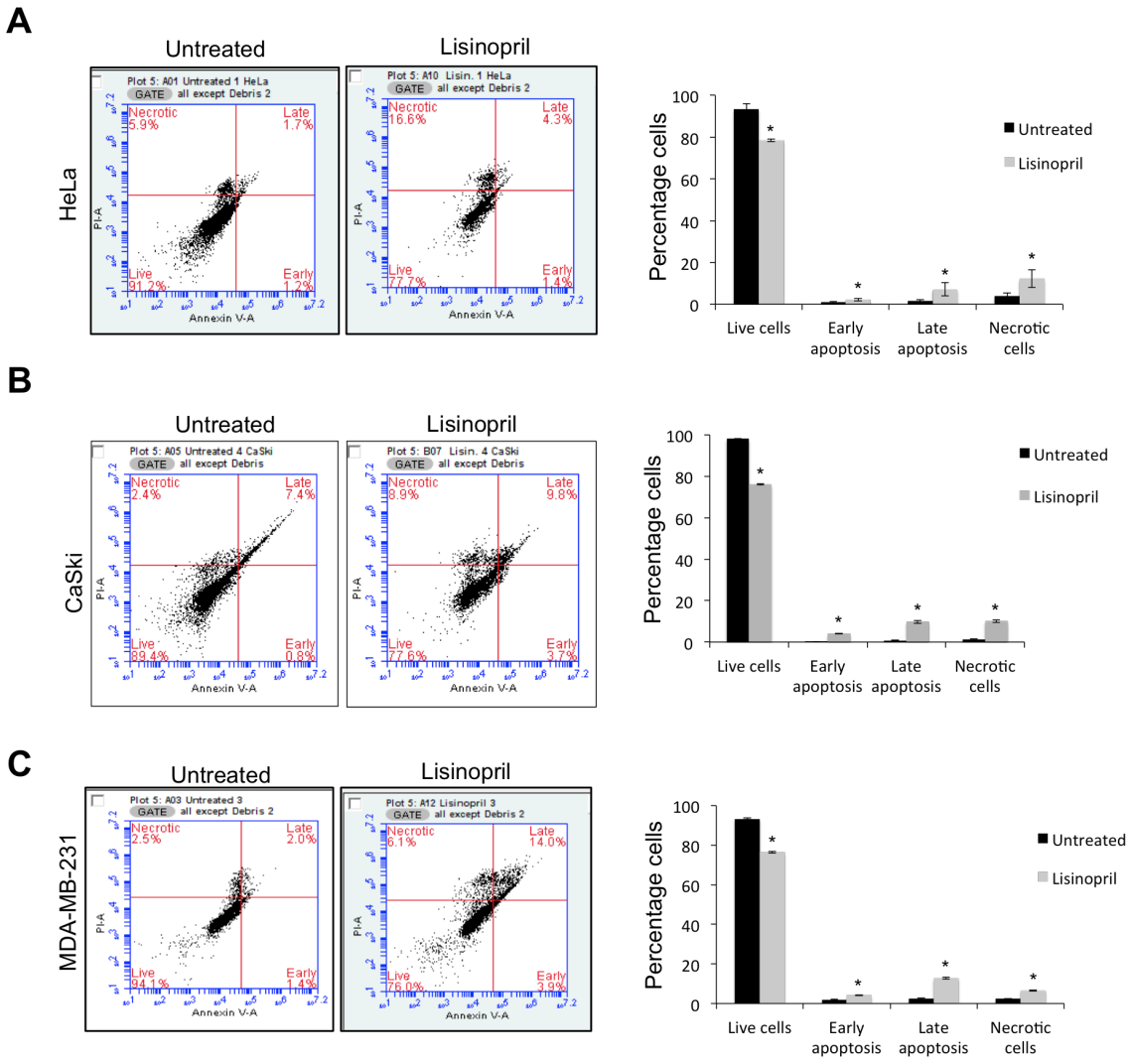


Figure 2.5: Lisinopril induces apoptosis and necrosis in cancer cells. Annexin V profiles show that a 48 hr treatment with the IC_{50} concentration of Lisinopril reduces the number of live cells via apoptosis and necrosis in **A:** HeLa, **B:** CaSki and **C:** MDA-MB-231 cells. Annexin V profiles for one representing experiment is shown for each cell line and bar chart results are the mean +/- SEM of experiments performed in triplicate and repeated two independent times (*p-value<0.05).

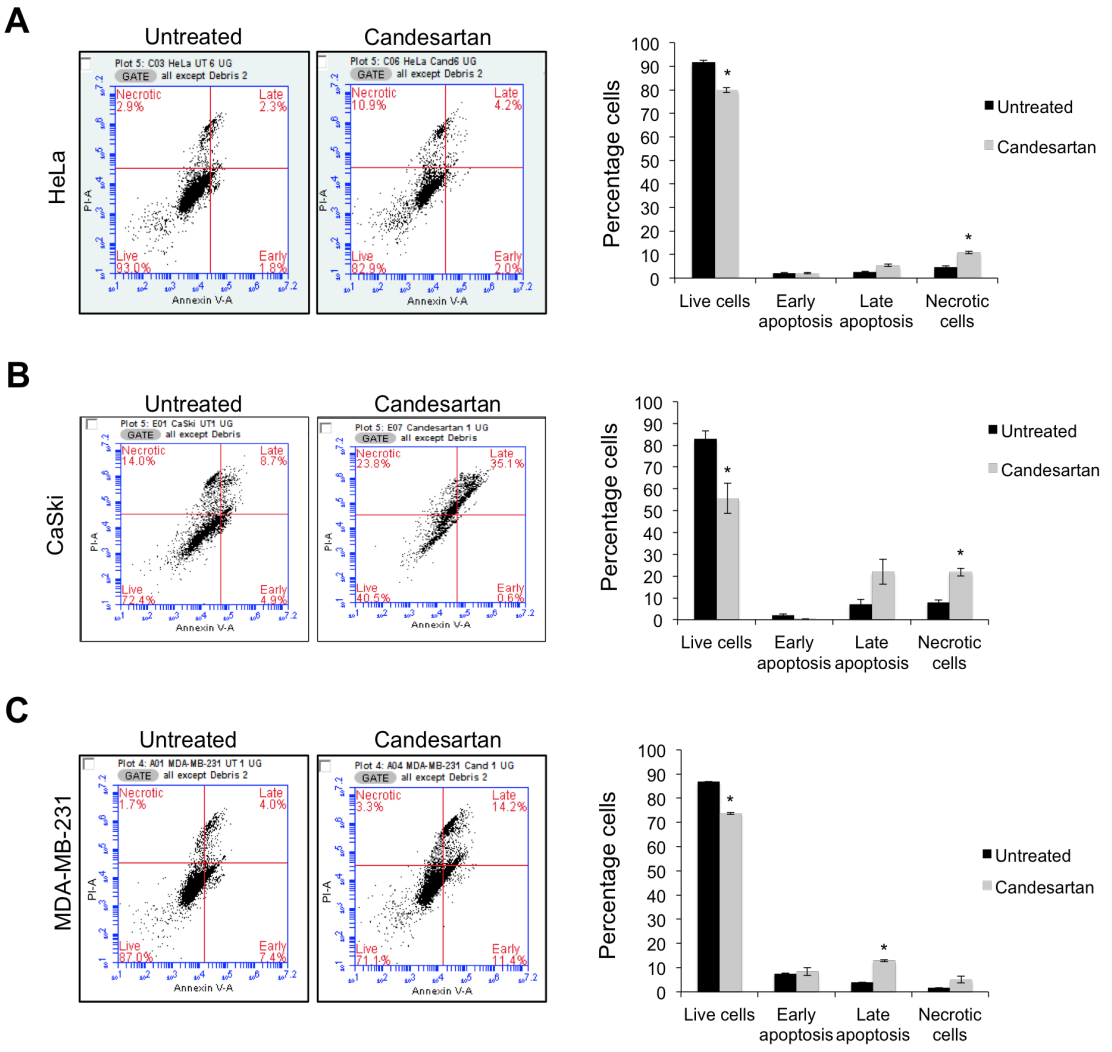


Figure 2.6: Candesartan reduces live cells via necrosis in cancer cells. Annexin V profiles show that a 48 hr treatment with the IC_{50} concentration of Candesartan reduces the number of live cells via necrosis in **A:** HeLa, **B:** CaSki and late apoptosis in **C:** MDA-MB-231 cells. Annexin V profiles for one representing experiment is shown for each cell line and bar chart results are the mean +/- SEM of experiments performed in triplicate and repeated three independent times (*p-value<0.05).

2.2.3 Ang-II stimulates cancer cell growth

As we observed that ACE-1 and AT1R protein expression was elevated in cancer cells and that ACE-1 enzyme activity was also increased in cancer cells we investigated whether cancer cells would respond to Ang-II treatment. Ang-II signaling is predominantly via the AT1R(Ager et al., 2008) thus the following investigations would confirm our earlier observations that the ACE-1 axis is present and active in cancer cells. HeLa and SiHa cell proliferation was monitored over 48 hours in the presence of increasing concentrations of Ang-II. Our results show a dose dependent increase in the proliferation of both HeLa and SiHa cells with increasing Ang-II, with 400 nM showing an approximate two-fold increase in cell proliferation (Figure 2.7 A and B).

To confirm that Ang-II increase in proliferation was as a result of activation of the AT1R, we inhibited its activity using Candesartan. The antagonist for the AT2R, a synthetic small molecule called PD123319 was used to monitor possible effects via the AT2R. We found in both HeLa and SiHa cells, that the stimulatory effect of Ang-II on their proliferation was significantly inhibited by Candesartan (Figure 2.8 A and C). The AT2R antagonist PD123319 had no effect (Figure 2.8 B and D).

These results suggest that Ang-II stimulation of HeLa and SiHa cancer cell proliferation occurs preferentially via the AT1R rather than the AT2R.

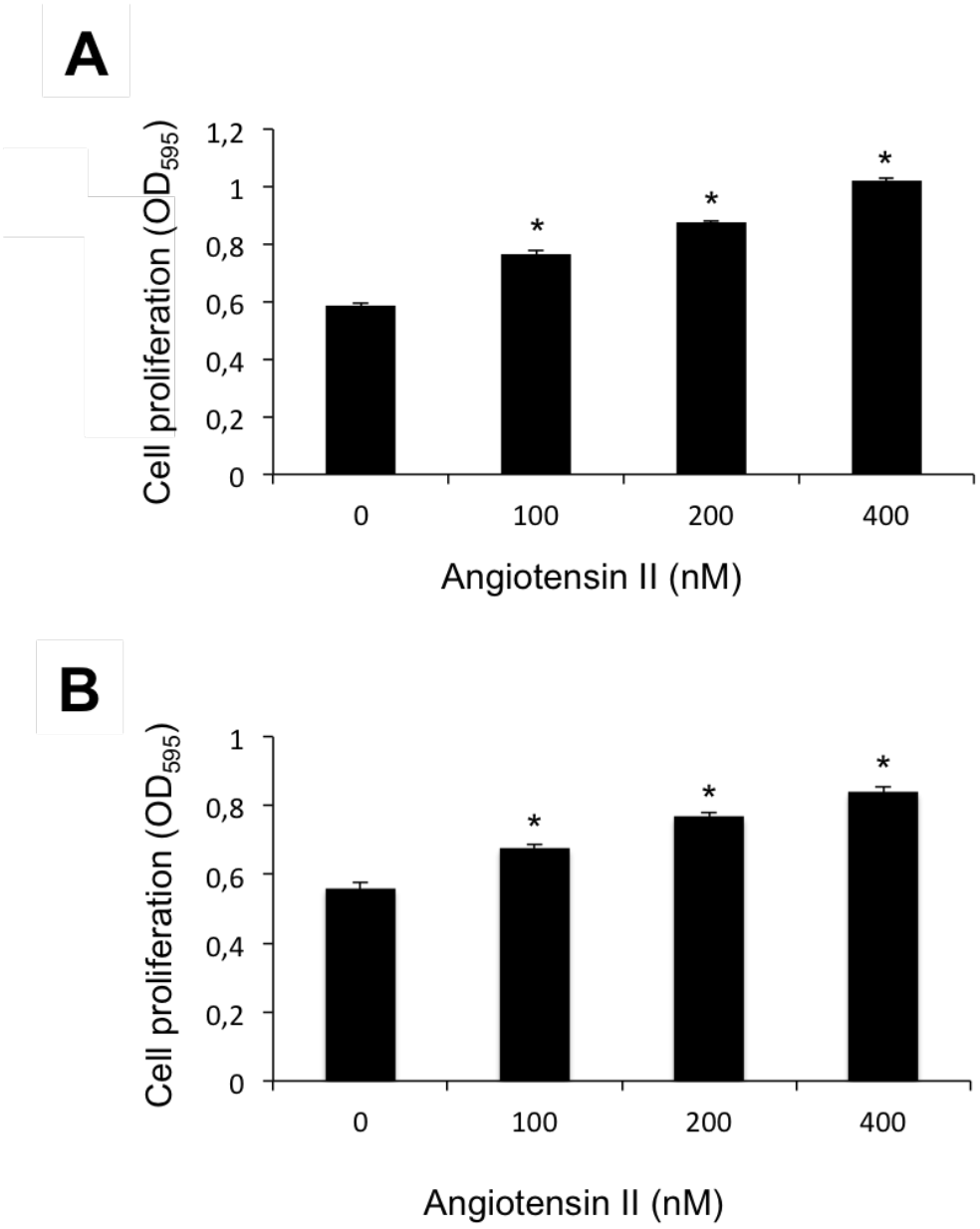


Figure 2.7: Effect of Ang-II on cancer cell proliferation. Ang-II treatment significantly increases HeLa (**A**) and SiHa (**B**) cell proliferation in a dose dependent manner. Results shown are the mean +/- SEM of experiments performed in triplicate and repeated at least two independent times. *p-value<0.05.

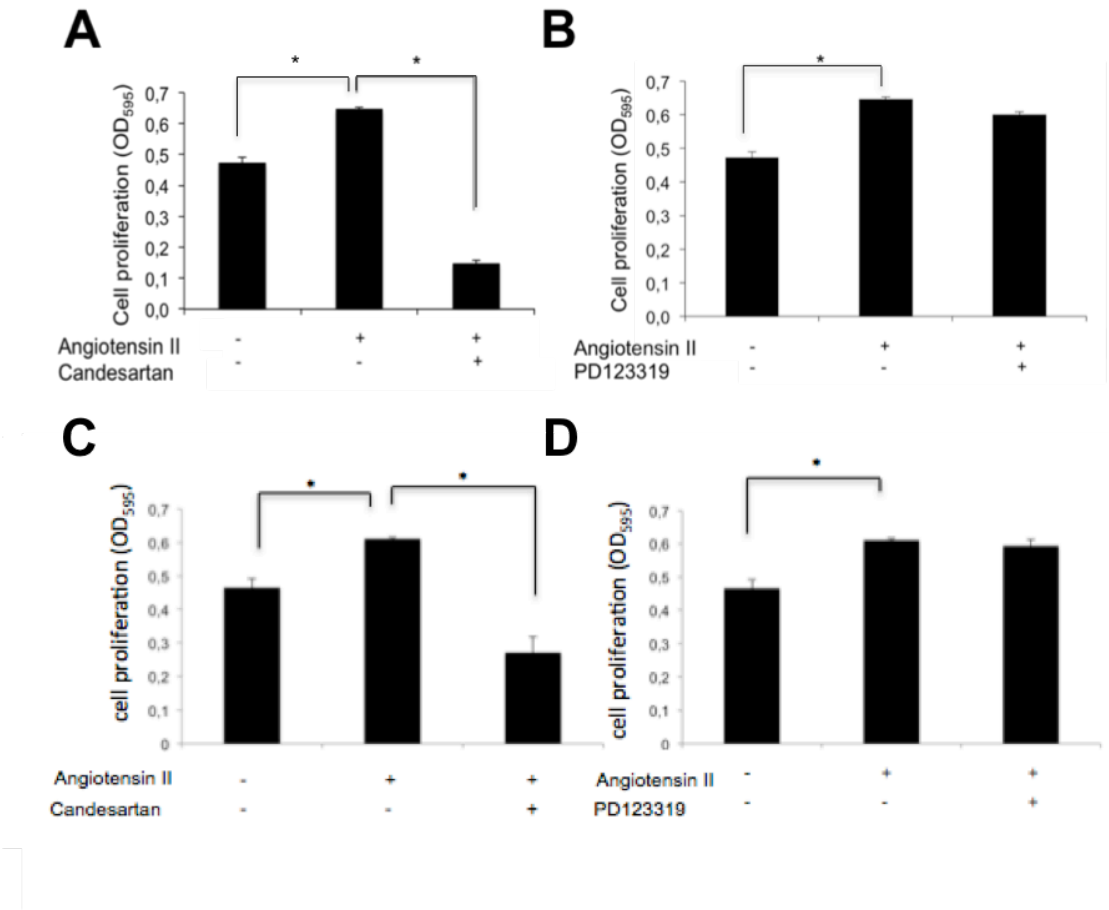


Figure 2.8: AT1R inhibition reduces cancer cell proliferation.

A and B: Stimulated proliferation of HeLa cells with Ang-II is reduced after treatment with Candesartan and not PD123319**. C. and D:** Similar results are observed with SiHa cells. Results shown are the mean +/- SEM of experiments performed in triplicate and repeated two independent times. *p-value<0.05.

2.2.4 Ang-II treatment associates with the release of intracellular calcium stores

Ang-II alters calcium signalling in a variety of cells types including endothelial and vascular smooth muscle cells (Haller et al., 1999; Helou and Marchetti, 1997; Montiel et al., 2003; Nitschke et al., 2000; Pueyo et al., 1998; Touyz and Schiffrin, 1997; Tran et al., 2000). We were therefore interested in determining whether Ang-II had effects on calcium signaling in cancer cells.

We first investigated the effect of Ang-II on calcium release from intracellular calcium stores in cancer cells. Calcium release was measured using a calcium fluorescent indicator, Fluo-4 AM. After incubation with 0.75mM Fluo-4 AM for 30 minutes, the release of calcium stores within the live cancer cells was monitored using the BD Accuri flow cytometer. Baseline calcium levels were monitored for two minutes to ensure there were no fluctuations before Ang-II treatment.

Our results show that Ang-II treatment releases intracellular calcium stores in HeLa and SiHa cells as observed by a significant increase in calcium fluorescence in both the cancer cell lines used (Figure 2.9 A and C). Pre-treatment with the AT1R inhibitor, Candesartan, significantly decreased the Ang-II induced- release of intracellular calcium in both cell lines (Figure 2.9 B and D). It was observed that Candesartan treatment had no effect on the release of calcium in HeLa or SiHa cells (Figure 2.9 E and F).

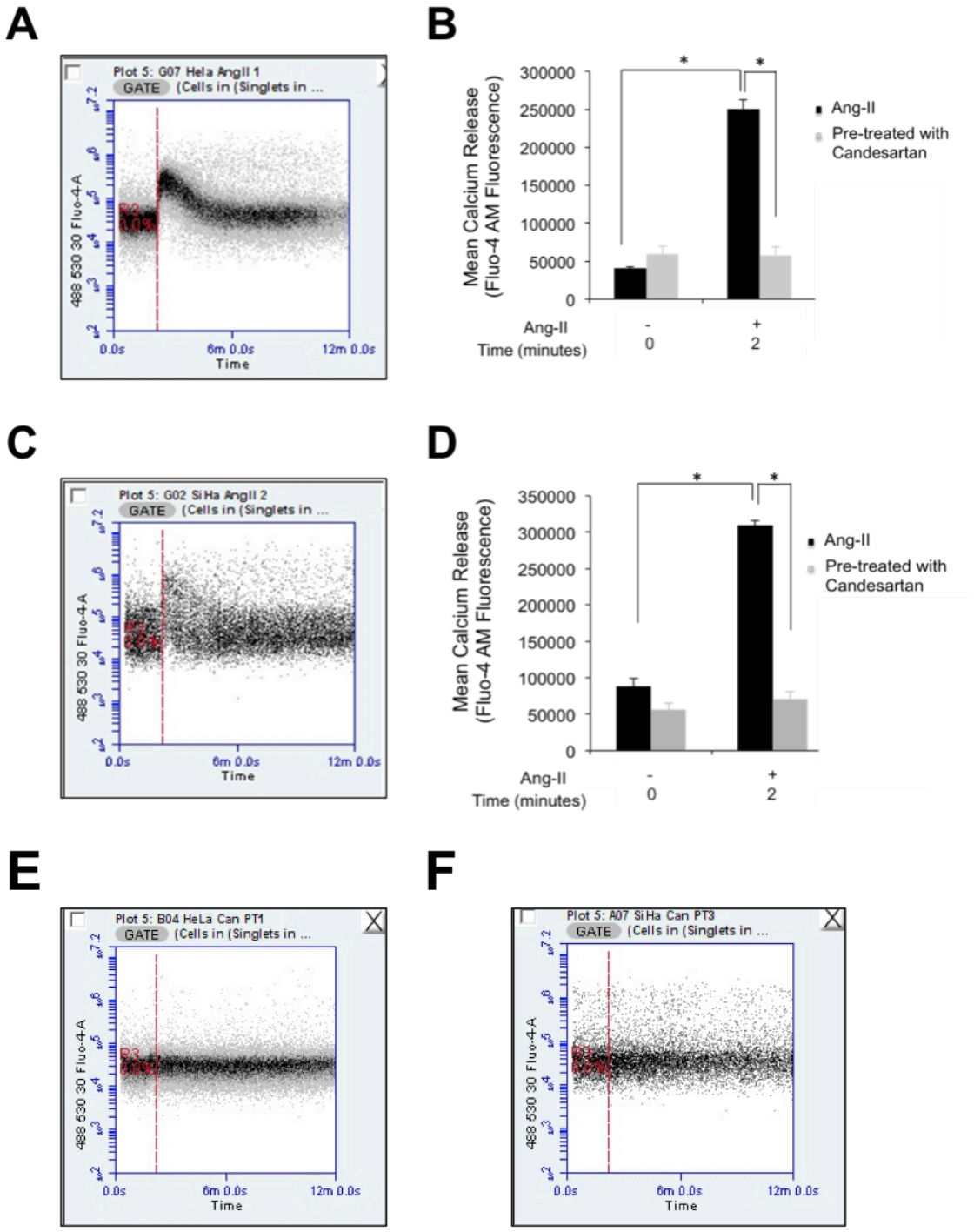


Figure 2.9: Effect of stimulation and inhibition of the ACE-1 axis on release of intracellular calcium in cancer cells. **A. and C:** One representative Calcium release profile of HeLa and SiHa cells after treatment with 400 nM Ang-II using flow cytometry. **B. and D:** Bar chart quantification showing that Ang-II significantly stimulates the release of intracellular calcium in cancer cells which Candesartan pretreatment can prevent. **E and F:** Representative calcium release profile of HeLa and SiHa cells after treatment with IC_{50} Candesartan. Results shown are the mean +/- SEM of experiments performed in triplicate and repeated three independent times, *p-value<0.05.

Having observed the release of intracellular calcium we next investigated the activation of calcium/calmodulin-dependent kinase II (CAMKII), a key enzyme associated with calcium signalling.

Ang-II treatment resulted in a substantial increase in phosphorylated CAMKII in HeLa cells within 1 minute of treatment that tapered off with increasing time (Figure 2.10 A). Phosphorylated CAMKII was observed after 5 minutes of Ang-II treatment in CaSki cells (Figure 2.10 B). Pre-treatment of both HeLa and CaSki cells for 24 h with Candesartan prevented the phosphorylation of CAMKII (Figure 2.10 C and D). Densitometric quantification of the protein bands, indicating relative pCAMKII expression in the two cell lines can be found in Appendix I (Figure A.1).

These results suggest that calcium signalling pathways are activated in cancer cells in response to Ang-II via the AT1 receptor resulting in activation of calcium responsive kinases such as CAMKII.

Having shown that upstream signaling pathways are activated by Ang-II in cancer cells we next investigated whether this had an effect on transcription factors known to be responsive to calcium signaling. NFAT, is a transcription factor that is known to translocate to the nucleus after the release of calcium stores where it activates its target genes. We used the NFAT luciferase assay to monitor effects of Ang-II and its inhibitor, Candesartan. Our results show that Ang-II treatment had a dramatic stimulatory effect on NFAT

transcriptional activity and that this was inhibited by the AT1R antagonist, Candesartan (Figure 2.11).

Together, our results presented in this chapter provides evidence that ACE-1 and the AT1R associates with the proliferation of cancer cells. Ang-II stimulates cancer cells via activation of the AT1R, which results in the release of intracellular calcium stores and activation of CAMKII and transcription factors such as NFAT.

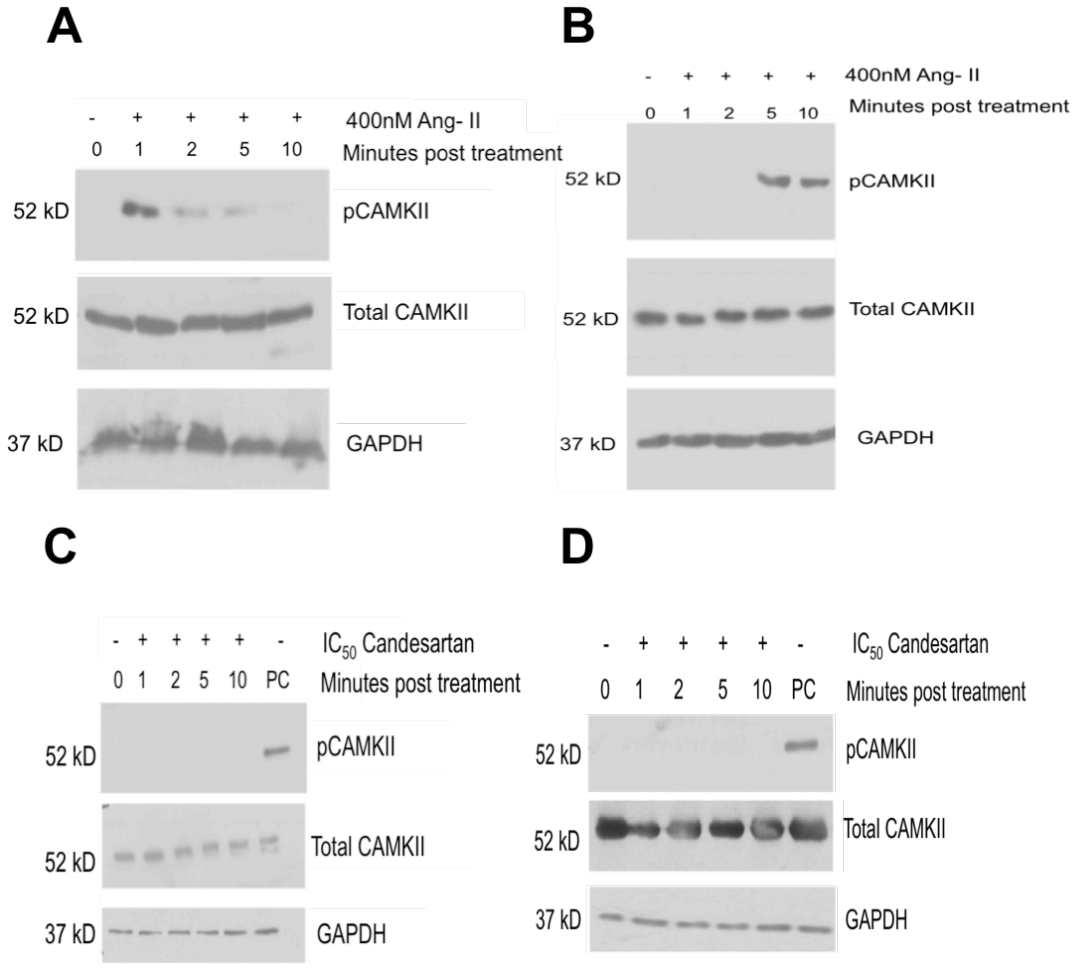


Figure 2.10: Effect of Ang-II treatment on CAMKII phosphorylation in HeLa and CaSki cells. **A & B:** Phosphorylation of CAMKII can be seen after minutes of exposure of 400 nM Ang-II in HeLa and Caski cells **C:** HeLa and CaSki **(D)** cells were pre-treated with Candesartan for 24 h before being subjected to Ang-II treatment for the indicated amounts of time. Candesartan prevented the Ang-II-induced phosphorylation of pCAMKII. Positive controls (PC), where HeLa and CaSki cells showed pCAMKII expression (after AngII treatment for 1 and 5 minutes respectively) were included to highlight this. Results shown are representative of two independent experiments.

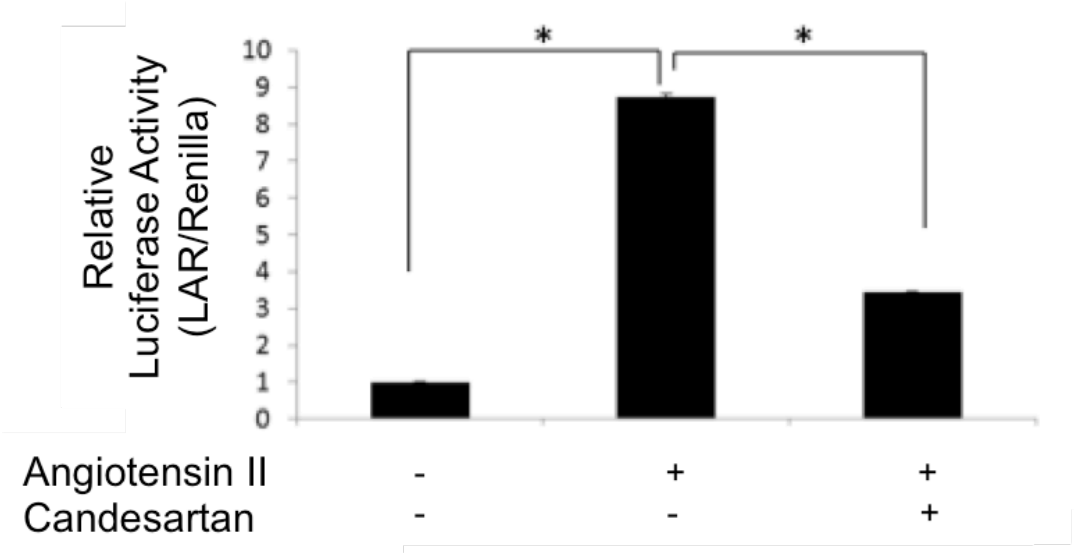


Figure 2.11: Candesartan prevents Ang-II stimulated NFAT transcriptional activity.
Ang-II treatment significantly stimulates NFAT activity in HeLa cells, which can be reduced with Candesartan. Results shown are the mean +/- SEM of experiments performed in triplicate and repeated three independent times, *p-value<0.05.

2.3 DISCUSSION

There is growing evidence that chronic conditions such as diabetes and cardiovascular disease share common differential gene expression patterns with cancer incidence (Chochieva et al., 2008; Hirsch et al., 2010; Reddy et al., 1995; Small et al., 1997; Suganuma et al., 2005; Yoshiji et al., 2002). The aim of this study was to investigate whether components of the Renin Angiotensin System, in particular ACE-1 and the AT1R, associate with the cancer phenotype using a panel of cancer cells of different tissue origin. We report that our results suggest elevated ACE-1 expression and activity in cancer cell lines compared to normal cells. AT1R expression levels were similarly found to be greater in cancer cells than normal cells.

Ang-II has been shown to associate with the AT1R with pathophysiological consequences (Chochieva et al., 2008; Zhao et al., 2010b). It is known to regulate expression of growth factors and promote the proliferation of various cell types as well as other cancer hallmarks such as cell migration and invasion (Kikkawa et al., 2004; Muscella et al., 2002; Rodrigues-Ferreira et al., 2012; Suganuma et al., 2005). In line with these studies we observed that Ang-II stimulates cervical cancer cell proliferation. Our work supports findings associating Ang-II primarily with the AT1R with diseased states. As Ang-II can bind both AT1 and AT2 receptors, thus we were interested to determine whether its effects on cervical cancer cell proliferation occurred via AT1R or AT2R activation. We found that blocking the AT2R with PD123319 had no effect on Ang-II stimulated proliferation suggesting that the AT1R is the preferred receptor. This was confirmed by Candesartan treatment, which prevented Ang-II stimulated proliferation.

Inhibition of ACE-1 or AT1R has been reported to cause varying effects depending on the cell type. A study by Carl-Mcgrath et al., (2007) showed that inhibition of ACE-1, or either of the receptors, AT1 or AT2, resulted in increased proliferation and reduction in invasive ability of gastric cancer cell lines. In pancreatic cancer, using PK9 and HS766T cell lines, ACE-1 axis inhibition using Captopril or Candesartan reduced cell proliferation (Arafat et al., 2007) as well as inhibiting tumour growth and angiogenesis in vivo (Miyajima et al., 2002). In our study, we show that ACE-1 inhibition using siRNA or the inhibitors, Captopril and Lisinopril, as well as the AT1R antagonist, Candesartan, reduce cervical cancer cell proliferation, suggesting that cancer cells are dependent on the ACE-1 axis for their proliferation and that ACE-1 and AT1R inhibitors have potential anti-cancer effects. We also showed that these inhibitors did not adversely affect the non-cancer cells investigated in our study.

Out of the three antihypertensive agents used in our study, Candesartan, the AT1R antagonist, was the only small molecule, which is a benzimidazole-derived compound. Benzimidazole derivatives have been shown to exert diverse biological activities including those of fungicides, bacteriosides and bacteriostatics. More recently, there has been growing evidence suggesting benzimidazole derivatives have anticancer properties (Abd El-All et al., 2015; El Rashedy and Aboul-Enein, 2013; Yadav et al., 2015; Yurttas et al., 2015). In vitro studies investigating the anti-tumour effect of benzimidazole derivatives have been carried out using colon, liver, cervix, uterine sarcoma, osteosarcoma, lung and breast cancer cells(Abd El-All et al., 2015; Blaszczak-Swiatkiewicz et al., 2012; El Rashedy and Aboul-Enein, 2013; Nofal et al., 2011; Reddy et al., 2015; Yadav et al., 2015; Yurttas et al., 2015). Results presented in our study suggest that Candesartan treatment of cervical cancer cells results in

cell death due to necrosis. Breast cancer cells (MDA-MB-231) showed a mix of apoptosis and necrosis. These data support findings that the ACE-1 axis of RAS is active in breast cancer cells and that ACE-1 axis inhibitors can reduce Ang-II induced proliferative effects (Du et al., 2012; Okazaki et al., 2014; Puddefoot et al., 2006; Rodrigues-Ferreira et al., 2012).

There is evidence in the literature that Ang-II and its receptor, AT1, have a role in increasing intracellular calcium levels. Intracellular calcium is an important regulator of various signalling mechanisms and the transcription of genes involved in cell processes like growth and proliferation (Monteith et al, 2007; Parkash and Asotra, 2010). Release of calcium from mitochondrial or endoplasmic reticulum stores as well as calcium influx can disrupt calcium homeostasis and potentially induce a variety of effects, one of which being cell death, if the threshold of calcium release within a cell is surpassed (Monteith et al, 2007; Parkash and Asotra, 2010). We thus investigated calcium signalling pathways in cancer cells with high ACE-1 expression. We monitored the release of intracellular calcium in live cells directly with a flow cytometry approach using the calcium fluorophore Fluo-4 AM. This is a novel approach that uses live cells whilst monitoring effects on calcium signaling. Calcium signalling events, especially those involving calcium, typically occur within a very short space of time. We observed that Ang-II treatment resulted in the release of intracellular calcium stores in HeLa, CaSki and SiHa cells and that Candesartan prevented this. We hypothesize that inhibition of calcium signalling pathways with Candesartan may interfere with downstream signalling events in cancer cells

To test this we investigated the expression levels of a key calcium signalling protein, calcium/calmodulin-dependent kinase II (CAMKII). Ang-II treatment resulted in the rapid

phosphorylation of CAMKII, which could be observed within minutes in HeLa and CaSki cells. Ang-II also resulted in the activation of the transcription factor NFAT, which is responsive to calcium release. Phosphorylation of CAMKII and NFAT activation was significantly inhibited by Candesartan treatment. Muthalif et al. (1998), describes a link between Ang-II and CAMKII signalling in vascular smooth muscle cells. However, our study is the first to report Ang-II activation of calcium signaling pathways in cervical cancer cells.

In conclusion, we have established that the ACE-1 axis (i.e ACE-1 and AT1R) of the RAS is highly expressed in cervical cancer compared to normal cells and promotes the proliferation of cancer cells. We have observed that Ang-II can act as a growth promoter and the inhibitors of the ACE-1 axis, can reduce Ang-II-induced proliferation. Our results show an interesting association between the ACE-1 axis in cancer cells and calcium signalling proteins like CAMKII. We propose that elevated ACE-1 and AT1R in cancer cells results in increased signalling via the AT1 receptor, which when bound by Ang-II results in the release of intracellular calcium stores. This triggers the phosphorylation of calcium responsive proteins including CAMKII and NFAT (Figure 2.12). The activation of the signalling cascade is necessary for cervical cancer proliferation as treatment with ACE-1 and AT1R inhibitors blocks the release of calcium stores, phosphorylation of CAMKII and activation of NFAT.

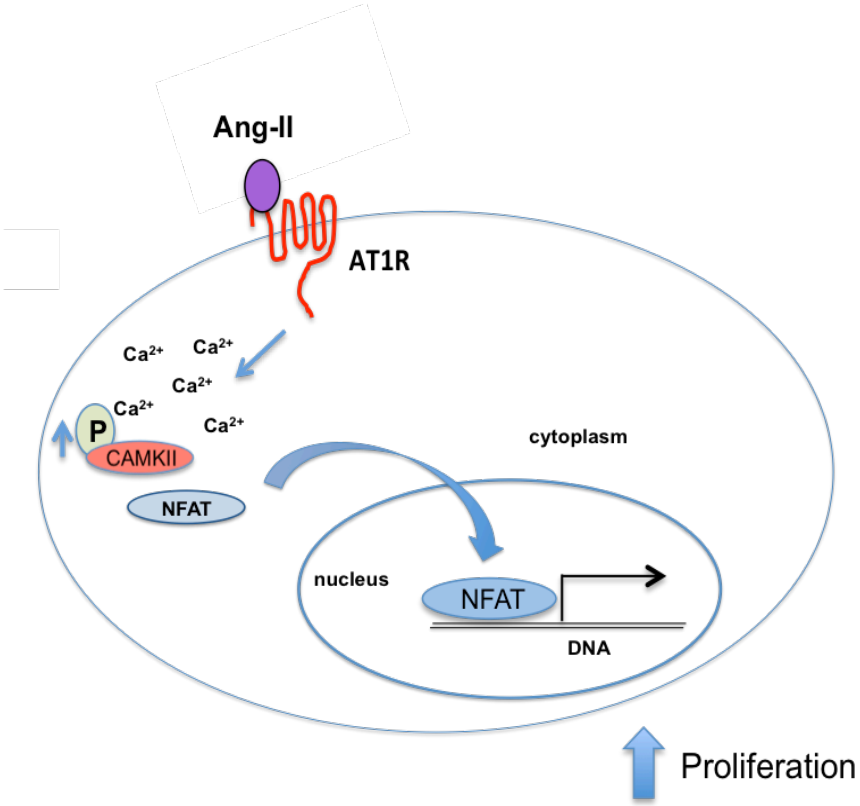


Figure 2.12: Schematic representation showing ACE-1 axis association with activation of intracellular signalling in cancer cells. ACE-1 axis proteins, ACE-1 and AT1R have elevated expression and activity in cancer cells. Activation of the AT1 receptor by Ang-II results in the release of intracellular calcium stores which triggers the activation of signaling molecules including CAMKII and NFAT. We propose that the events are in part required for the proliferation of cervical cancer cells.

CHAPTER 3

INVESTIGATING THE ROLE OF THE ACE-2 AXIS IN CANCER CELLS

3.1 INTRODUCTION

The ACE-2 enzyme within the RAS serves to counterbalance the effects of the ACE-1 axis (Ager et al., 2008; Duenas-Gonzalez et al., 2008a; Guang et al., 2012; Mizuiri and Ohashi, 2015; Verma et al., 2012). Many studies have begun to demonstrate that these enzymes function beyond that of blood pressure regulation (Ager et al., 2008; Deshayes and Nahmias, 2005; Guang et al., 2012; Ino et al., 2011; Juillerat-Jeanneret, 2007; Mizuiri and Ohashi, 2015). As ACE-1 and ACE-2 typically exist in a balanced environment, it is conceivable that dysregulation of these proteins may be associated with diseased states within various tissues and organs. In some of these instances where ACE-2 itself is down-regulated, ACE-1 effects overwhelm that particular environment (Mizuiri and Ohashi, 2015). In the study carried out by Mizuiri and Ohashi (2015), the activation of the AT1R was associated with the aetiology of renal disease, whereas activation of the ACE-2 axis via Ang-(1-7), resulted in renoprotective effects in addition to other beneficial effects such as reduced vasoconstriction, cell proliferation and water retention. Examples where the activation of the ACE-2 axis has been successful in protecting against disease include studies focused on promoting antithrombotic activity, cardiovascular disease as well as diabetic retinopathy which eventually leads to blindness (Fraga-Silva et al., 2010).

Recent research has begun to address whether the stimulation of ACE-2 function, or overexpression of ACE-2 in diseased states is linked to positive effects(Feng et al., 2010a;

Feng et al., 2011b; Ingelfinger, 2006; Lovren et al., 2008; Rentzsch et al., 2008; Verma et al., 2012; Zhong et al., 2011). To this end, there are encouraging results where ACE-2 overexpression has resulted in the reduction of diabetes-induced retinopathy (Verma et al., 2012) as well as potential anti-angiogenic effects together with the suppression of ACE-1 and AT1R expression *in vivo* (Feng et al., 2011b). ACE-2 overexpression has also been reported to inhibit cell proliferation in the pancreatic cancer cell lines, BxPC3 and SW1990 (Zhou et al., 2011) and reduce epithelial-mesenchymal transition (EMT) in A549 non-small cell lung cancer cells (Qian et al., 2013).

Activation of the ACE-2 axis using Ang-(1-7) in cancer studies has shown to have promising anti-cancer effects with disruption of growth-promoting signals as well as decreased angiogenesis, inflammation and metastasis of breast, prostate and hepatocellular carcinoma cells(Feng et al., 2010a; Gallagher et al., 2014; Ni et al., 2012; Passos-Silva et al., 2015). In 2009, a pharmacokinetic and Phase I study of Ang-(1-7) was carried out to establish the recommended phase II dose for treating patients with advanced cancers as well as to assess pharmacokinetics of the peptide hormone(Petty et al., 2009)

In addition to natural activators of the ACE-2 axis, synthetic small molecules such as Diminazene aceturate (DIZE) have been shown to enhance ACE-2 activity in hypertension(de Macedo et al., 2015; Kuriakose and Uzonna, 2014; Qi et al., 2013; Velkoska et al., 2016)Thus far, literature reports of the ACE-2 activator DIZE, also referred to as its trade name Berenil, have primarily involved studies making use of it as an anti-trypanosomal drug in livestock (Burudi et al., 1994; Diack et al., 1997; Kaminsky et al., 1993; Peregrine and Mamman, 1993; Poot et al., 1990). However DIZE has also been used for assisting with the regulation of

blood pressure, reducing inflammation and producing gastroprotective effects in mice (Souza et al., 2016; Tao et al., 2016; Velkoska et al., 2016). Furthermore, it has been reported that DIZE inhibits chromatin condensation(Haaf and Schmid, 2000; Poot et al., 1990) and could potentially be utilized for growth inhibition purposes in cancer.

In this chapter we investigated ACE-2 expression and activity in cancer cells using a natural or synthetic small molecule.

3.2 RESULTS

3.2.1 Endogenous expression and activity of ACE-2 is lower in cancer cell lines compared to normal cells

Our earlier results showed that ACE-1 expression was elevated in cancer compared to normal cell lines under tissue culture conditions. Here we aimed to determine the expression of ACE-2 as well as the potential role the ACE-2 axis in cancer cells.

Western blot analysis showed that, in contrast to ACE-1 expression, endogenous ACE-2 protein levels were reduced in cancer cell lines compared to non-cancer WI-38 cells and transformed SVWI-38 cells (Figure 3.1). To determine whether low ACE-2 expression correlated with ACE-2 activity levels in these cancer cells, we determined ACE-2 enzymatic activity using a flurogenic assay. This assay measures the cleavage of an ACE-2-specific fluorogenic substrate, (7-methoxycoumarin-4-yl) acetyl-Ala-Pro-Lys-(2,4-dinitrophenyl). Results were represented as relative fluorescence to microgram (μg) protein. Our results showed that the cancer cell lines HeLa, CaSki and MDA-MB-231, showed significantly lower levels of ACE-2 activity compared to a non-cancer cell line, FG0 (Figure 3.1 B). SVWI38 cells which had high ACE-2 expression, showed surprisingly low ACE-2 activity. The reason for this is not clear at present, but could be that SVWI38 cells contain a different isozyme of ACE-2, possibly the ACE-2 enzyme expressed by SVWI38 possesses different post transcriptional modifications, or perhaps while ACE-2 is expressed in SVWI38 cells, the enzyme may require activation by a stimulus to increase activity. A further possibility is that the antibody may have shown signs of non-specificity as ACE-2 activity in SVWI38 cells was reduced compared to ACE-2 expression in these cells. Our results for the cancer cells nonetheless show a correlation of decreased ACE-2 expression with ACE-2 activity.

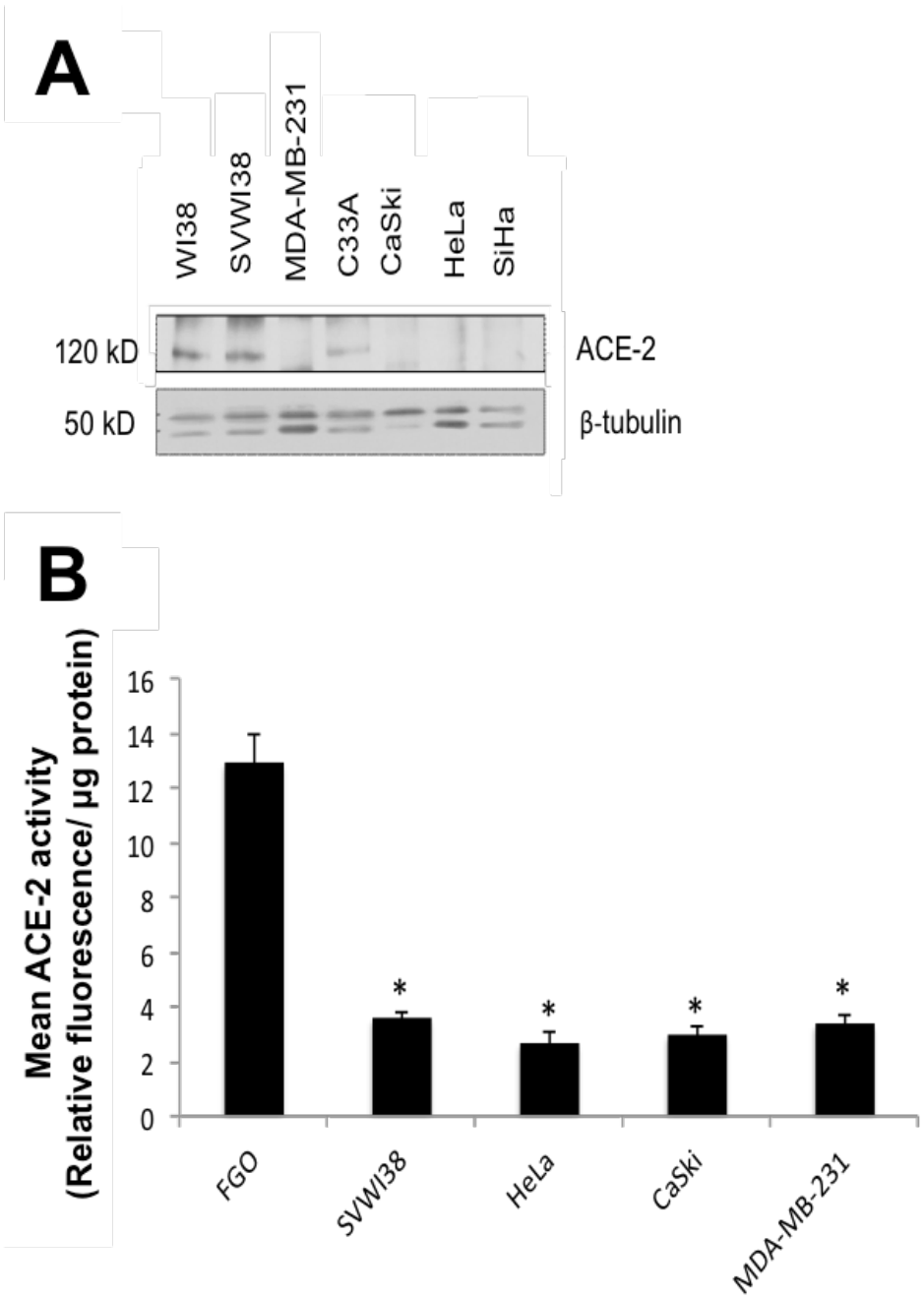


Figure 3.1: Endogenous ACE-2 expression and activity is lower in cancer cells compared to normal cells. (A.) Western blot analysis showing ACE-2 expression in WI38 (normal fibroblasts), SVWI38 (transformed fibroblasts) and cancer cell lines. B-tubulin was used as a control for protein loading. **(B.)** ACE-2 activity measured in normal and cancer cells using the ACE-2 enzyme assay measuring cleavage of the fluorogenic peptide, (7-methoxycoumarin-4-yl) acetyl-Ala-Pro-Lys-(2,4-dinitrophenyl). Results shown are the mean +- SE of experiments performed in triplicate and repeated at least two independent times. *$p<0.05$.

3.2.2 Activation of the ACE-2 axis in cancer cells disrupts cancer cell proliferation

Endogenous Ang-(1-7) can increase the beneficial effects associated with the ACE-2 axis through the stimulation of the Mas receptor(Ager et al., 2008; Chappell et al., 2014; Ferrario, 2011; Santos et al., 2013; Simoes e Silva et al., 2013; Xu et al., 2011)

Thus, we investigated whether the ACE-2 cleavage product, Ang-(1-7), could exert an anti-proliferative effect in the cervical cancer cell lines, HeLa and CaSki, as well as the breast cancer cell line MDA-MB-231. Cells treated with an increasing concentration of Ang-(1-7) showed a significant decrease in proliferation compared to control cells (Figure 3.2). This suggested that activation of ACE-2 regulated pathways has an inhibitory effect on cervical cancer cells.

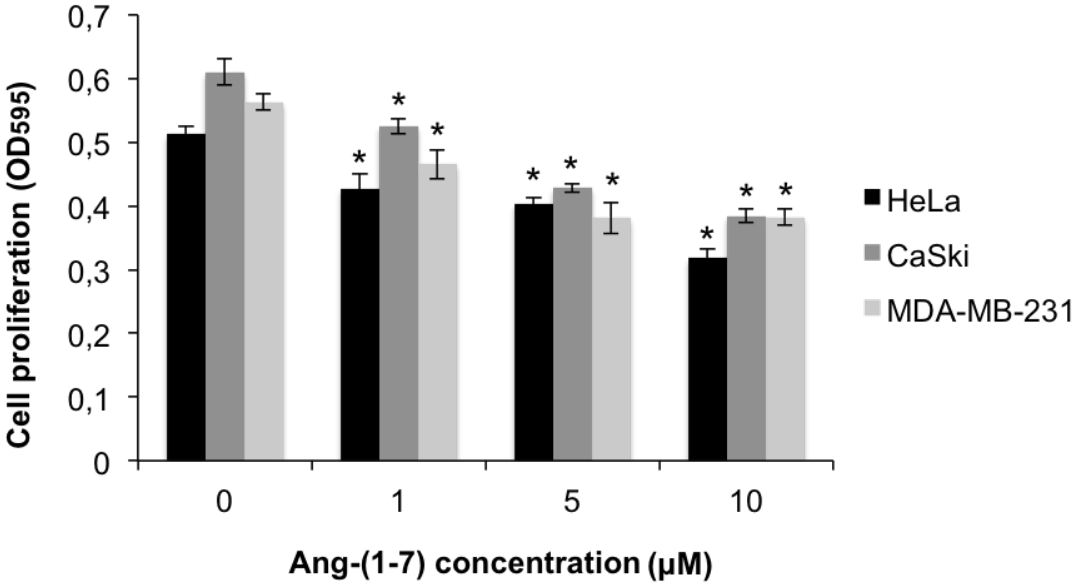


Figure 3.2 Natural peptide, Ang-(1-7) reduces cancer cell proliferation. A 48 hour treatment of Ang-(1-7) significantly reduced cell proliferation of HeLa and CaSki cervical cancer cells as well as MDA-MB-231, breast cancer cells (*$p<0.05$). Results shown represent the mean +/-SD of experiments performed in triplicate and repeated at least two independent times.

3.2.3 The small molecule, DIZE, significantly increases ACE-2 enzyme activity in normal and cancer cell lines

Since we observed that the Ang-(1-7) could exert anti-proliferative effects in cancer cells, we were interested in determining whether DIZE would behave in a similar manner as more recent studies have suggested it exerts a stimulatory effect on the ACE-2 enzyme (da Silva Oliveira and de Freitas, 2015; Kuriakose and Uzonna, 2014; Qi et al., 2013; Souza et al., 2016; Tao et al., 2016)

We used a sensitive flurogenic assay to measure ACE-2 activity in the absence and presence of DIZE treatment. Our results show that DIZE had a significant stimulatory effect on ACE-2 activity in all of the cell lines tested (Figure 3.3). DIZE-stimulated ACE-2 activity in the cancer cell lines was still below that of the untreated non-cancer cell line FGO.

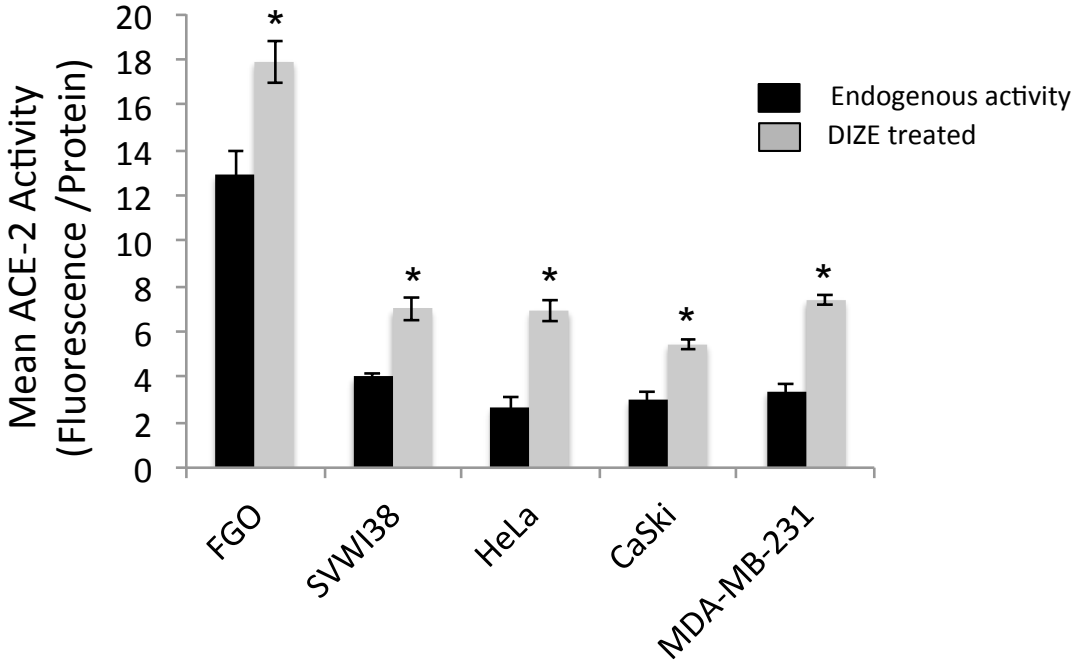


Figure 3.3: DIZE increases ACE-2 enzyme activity in normal and cancer cell lines. Cells were pre-treated with 1µM DIZE for 1 hour prior to being incubated with the fluorogenic ACE-2 specific substrate, (7-Methoxycoumarin-4-yl) acetyl dinitrophenyl for 1 hour. The amount of substrate cleavage was determined for each sample. Fluorescence readings were normalised to µg protein. DIZE significantly increases ACE-2 enzyme activity in the normal and cancer cell lines (*$p<0.05$). Results shown represent the mean +/- SEM of experiments performed in triplicate and repeated at least two independent times.

3.2.4 The ACE-2 axis activators, Ang-(1-7) and DIZE, have inhibitory effects on cancer cell proliferation and clonogenic potential

Having observed that DIZE could stimulate ACE-2 activity in cancer cells, we next investigated its effects on cancer cell survival by measuring its IC_{50} in treated cells. DIZE had little to no effect on the non-cancer cells, WI38 and FG0, and IC_{50} values could not be determined. In contrast, IC_{50} concentrations of 3 µM for HeLa cells, and 10 µM for CaSki and MDA-MB-231 were calculated (Table 3.1).

To monitor the effects of activating the ACE-2 axis on cancer cell proliferation, MTT assays were performed on HeLa and MDA-MB-231 cells treated with either DIZE or Ang-(1-7). Our results show that from day two onwards, treatment with DIZE resulted in a significant reduction in the proliferation of HeLa and MDA-MB231 cells at IC_{50} and 2 x IC_{50} concentrations (Figure 3.4 A and B). Similarly treatment with either 1 or 2 µM Ang-(1-7) showed reduced cancer cell proliferation at 48 hours and subsequent time points (Figure 3.4 C and D).

Table 3.1. IC_{50} values of DIZE in non-cancer and cancer cell lines. MTT assays were performed to determine DIZE IC_{50} values for WI38, FG0 non-cancer cell lines and HeLa, CaSki and MDA-MB-231 cancer cell lines. Experiments were performed in triplicate and repeated at least two independent times for non-cancer and cancer cell lines.

Cell Line	IC_{50} (µM)	95% Confidence Interval
FG0	CNBD	CNBD
WI38	CNBD	CNBD
HeLa	3	2.1 - 5
CaSki	10	6 - 17
MDA-MB-231	10	7.6 – 11.9

CNBD- Could not be determined

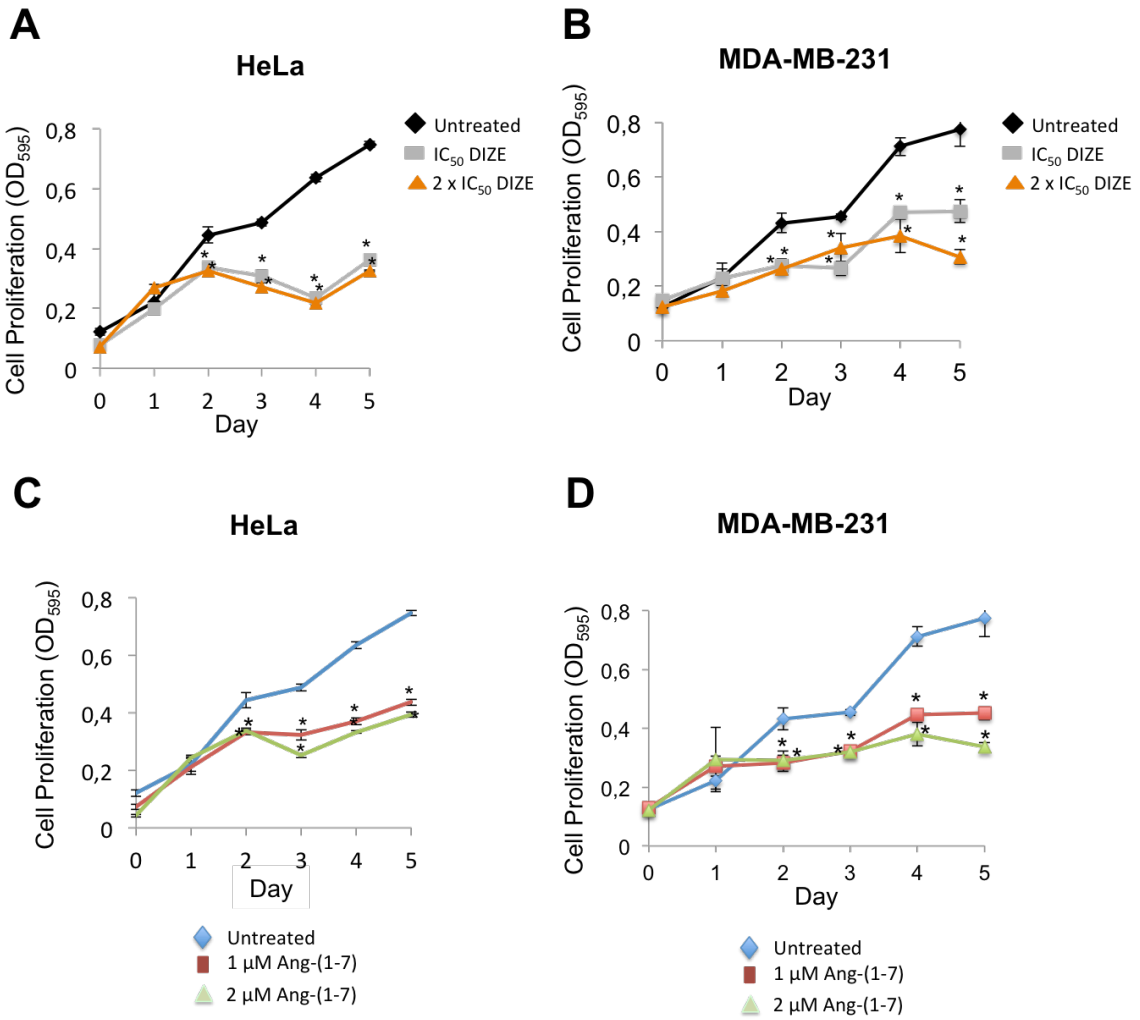


Figure 3.4: DIZE and Ang-(1-7) reduce cancer cell proliferation. HeLa **(A.)**, and MDA-MB-231 **(B.)** cells were treated with the indicated concentrations of DIZE or Ang-(1-7) **(C. and D.)** once off and proliferation monitored for 5 days. Results shown are representative of experiments performed in triplicate and repeated at least two independent times, (*p<0.05).

Having observed an anti-proliferative response in the cancer cell lines to Ang-(1-7) and DIZE treatment, we next investigated their effect on clonogenic colony formation. Briefly, HeLa, CaSki and MDA-MB-231 cells were seeded into a 6-well tissue culture plate at a low enough number so that single cells could establish clonogenic colonies. The treated groups received either 1 µM Ang-(1-7), half IC_{50} or IC_{50} concentrations of DIZE for 24 hours followed y incubation for 10 days upon which visible colonies could be observed using phase microscopy. Colonies were stained with crystal violet and colonies were counted using ImageJ. Results showed that Ang-(1-7) reduced colony formation and the survival fraction of clonogenic colonies for HeLa (Figure 3.5 A), CaSki (Figure 3.5 B) and MDA-MB-231 (Figure 3.5 C) cells. Similarly, DIZE treatment at half IC_{50} and IC_{50} concentrations significantly reduced the number of colonies in all of the cancer cell lines tested (Figure 3.6 A, B and C). These results provide unequivocal evidence that stimulation of the ACE-2 axis inhibited cancer cell proliferation.

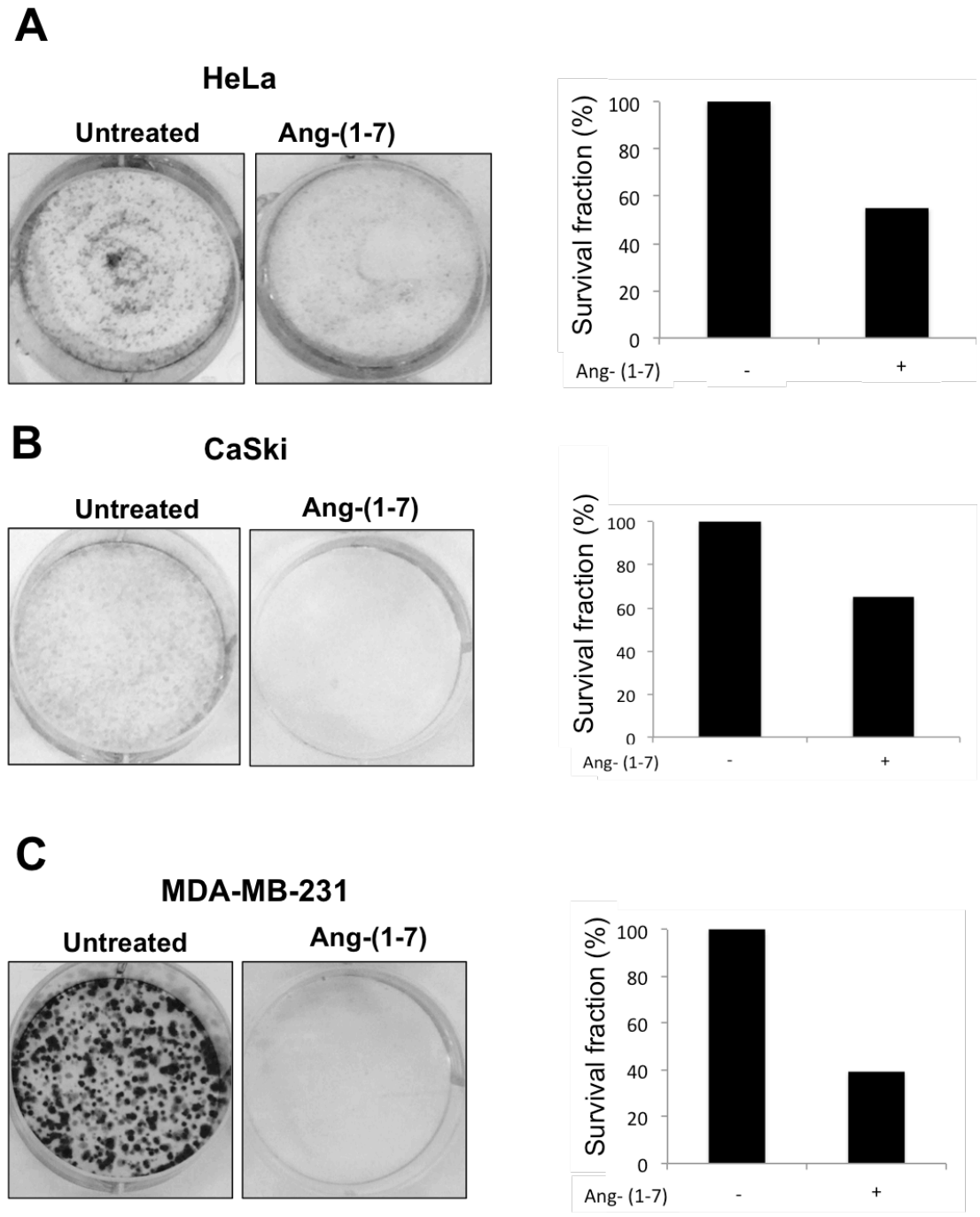


Figure 3.5: Ang (1-7) reduces clonogenic colony formation of cancer cells. HeLa **(A.),** CaSki **(B.)** and MDA-MB-231 **(C.)** cells were treated with 1µM Ang-(1-7) once off and clonogenic colony formation monitored for 10 days. Colonies were stained with crystal violet and allowed to dry before colonies were counted using ImageJ. For each cell line the % survival fraction indicates Ang-(1-7) treatment reduced the number of clonogenic colonies able to form after 10 days. Results shown is representative of experiments performed in triplicate and repeated two independent times.

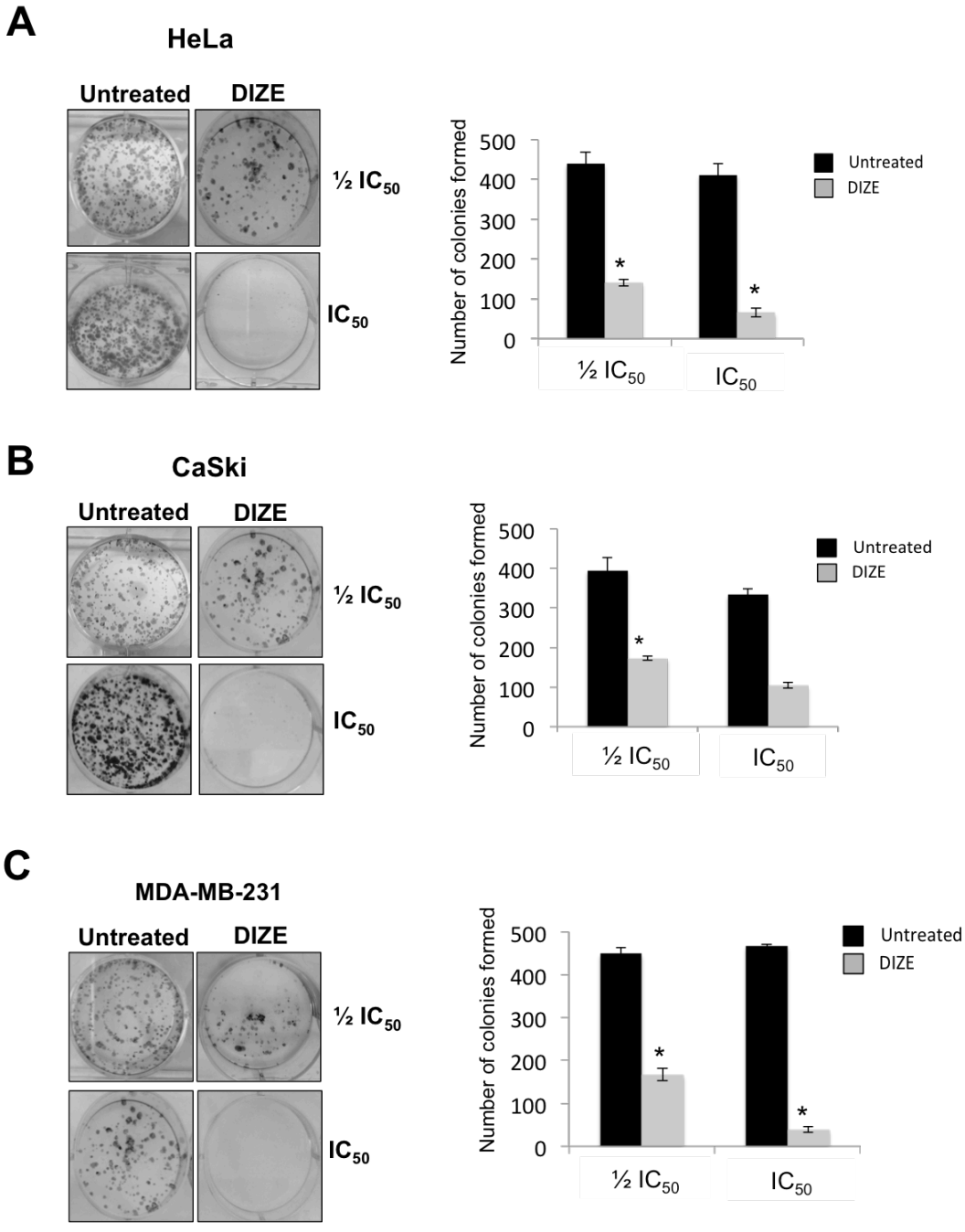


Figure 3.6: DIZE reduces colony formation of cancer cells. HeLa **(A.),** CaSki **(B.)** and MDA-MB-231 **(C.)** cells were treated with IC_{50} DIZE once off and clonogenic colony formation monitored for 10 days. Colonies were stained with crystal violet and allowed to dry before colonies were counted using ImageJ. DIZE treatment significantly reduced the number of clonogenic colonies able to form after 10 days, (*$p<0.05$). Results shown are the mean +/- SEM of experiments performed in triplicate and repeated three independent times.

3.2.6 ACE-2 activation by DIZE results in altered cell cycle progression

Using phase microscopy we observed that DIZE treatment caused a substantial increase in the appearance of rounded cells, some of which were still adherent on the cell culture dish while others were floating in the culture medium (Figure 3.7 A). We postulated that the rounded cells may be indicative of cells undergoing mitotic arrest. A significant increase in rounded cells compared to healthy adherent cells was quantified using ImageJ (Figure 3.7 B). In the DIZE treated samples, the degree to which healthy adherent cells were beginning to round and lift from the culture dish varied between the replicates, however, a greater number of rounded and non-adherent cells were observed (Figure 3.7 B). Cell cycle analysis was performed on control and DIZE treated cells. Our results showed a clear and significant increase in the G2/M population of DIZE treated HeLa, CaSki and MDA-MB-231 cells (Figure 3.8 A, B and C). A notable increase of cells in the subG1 population was observed for CaSki and MDA-MB-231 cells. While DIZE treatment significantly increased the subG1 population in HeLa cells, the increase was not as robust as for CaSki and MDA-MB-231 cells. However, after DIZE treatment, HeLa cells did show a greater increase in the percentage of cells in the G2/M phase of the cell cycle compared to the other cell lines.

We monitored the expression levels of two proteins, phosphorylated Histone H3 (pHisH3) and Mcl-1, that have been reported to correlate with the G2/M phase of the cell cycle following ACE-2 activation with DIZE. Histone H3 phosphorylation takes place during chromosome condensation during mitosis, specifically during the G2 phase of the cell cycle and is completed and maintained during late prophase and metaphase. As cells exit mitosis dephosphorylation of Histone H3 occurs(Hans and Dimitrov, 2001; Hendzel et al., 1997; Van

Hooser et al., 1998). Mcl-1 is a member of the Bcl-2 family of proteins and is known to regulate protein function by having a role in cancer cell survival as an apoptotic protein and through its involvement in cell cycle progression. Mcl-1 degradation is typically observed with the onset of apoptosis (Akgul, 2009; Bednarek et al., 2007; Michels et al., 2005; Perciavalle and Opferman, 2013)

Our results show that DIZE treatment caused an increase in pHisH3 levels (Figure 3.9 A). Mcl1 levels conversely were progressively decreased over 48 hours in response to DIZE treatment (Figure 3.9 B). These results support the notion that ACE-2 activation results in cells being delayed in the G2/M phase of the cell cycle.

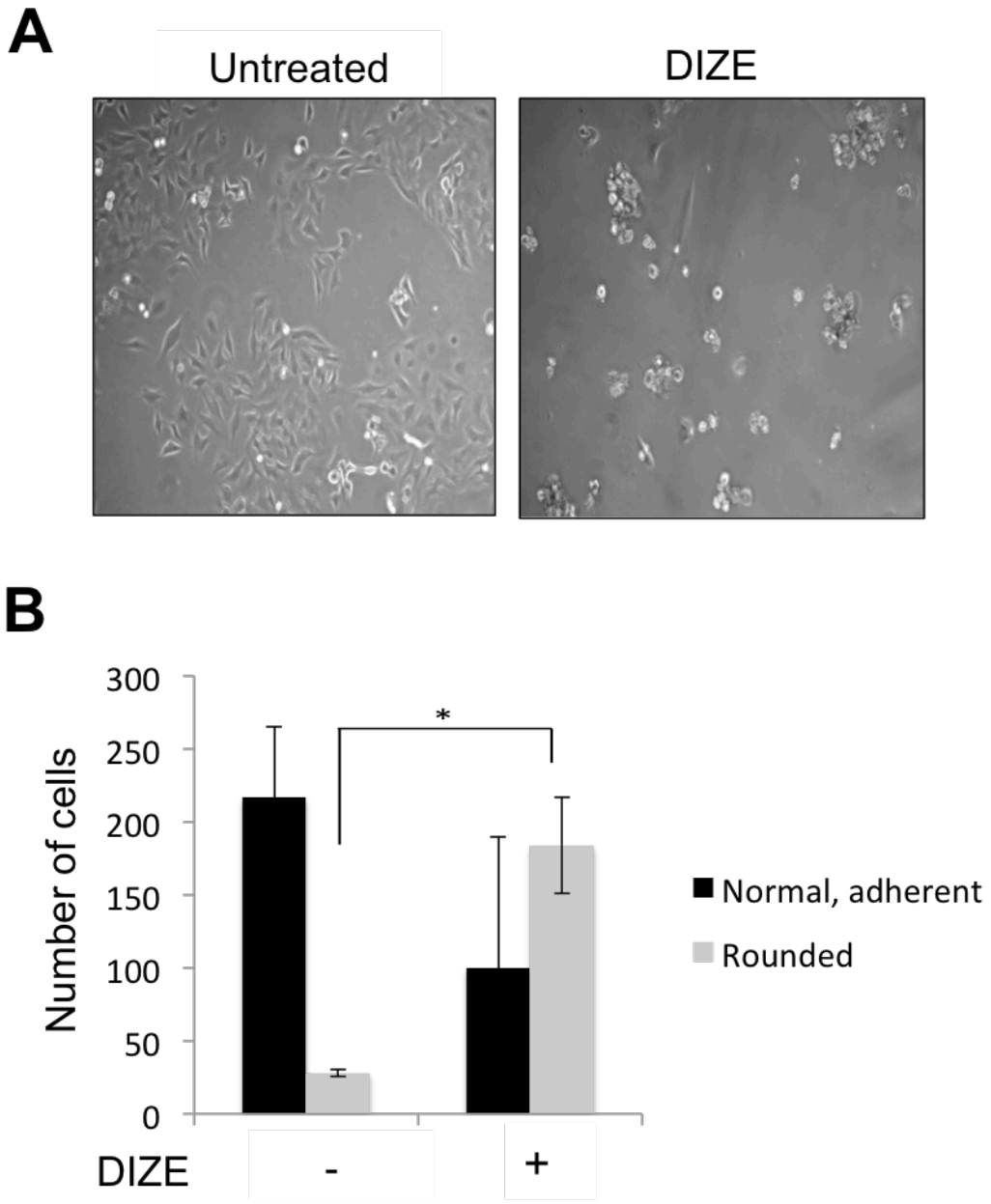


Figure 3.7: DIZE affects HeLa cell morphology by increasing the number of rounded cells as determined by phase microscopy. A. HeLa cells were treated with the IC_{50} concentration of DIZE for 48 hours and images captured using the phase microscope. B: Image J quantitation of rounded cells compared to healthy, adherent cells. Results shown are the mean +/- SEM of experiments performed in triplicate where rounded or normal, adherent cells were counted in >100 cells per condition (*$p<0.05$). Experiments were repeated at least two independent times.

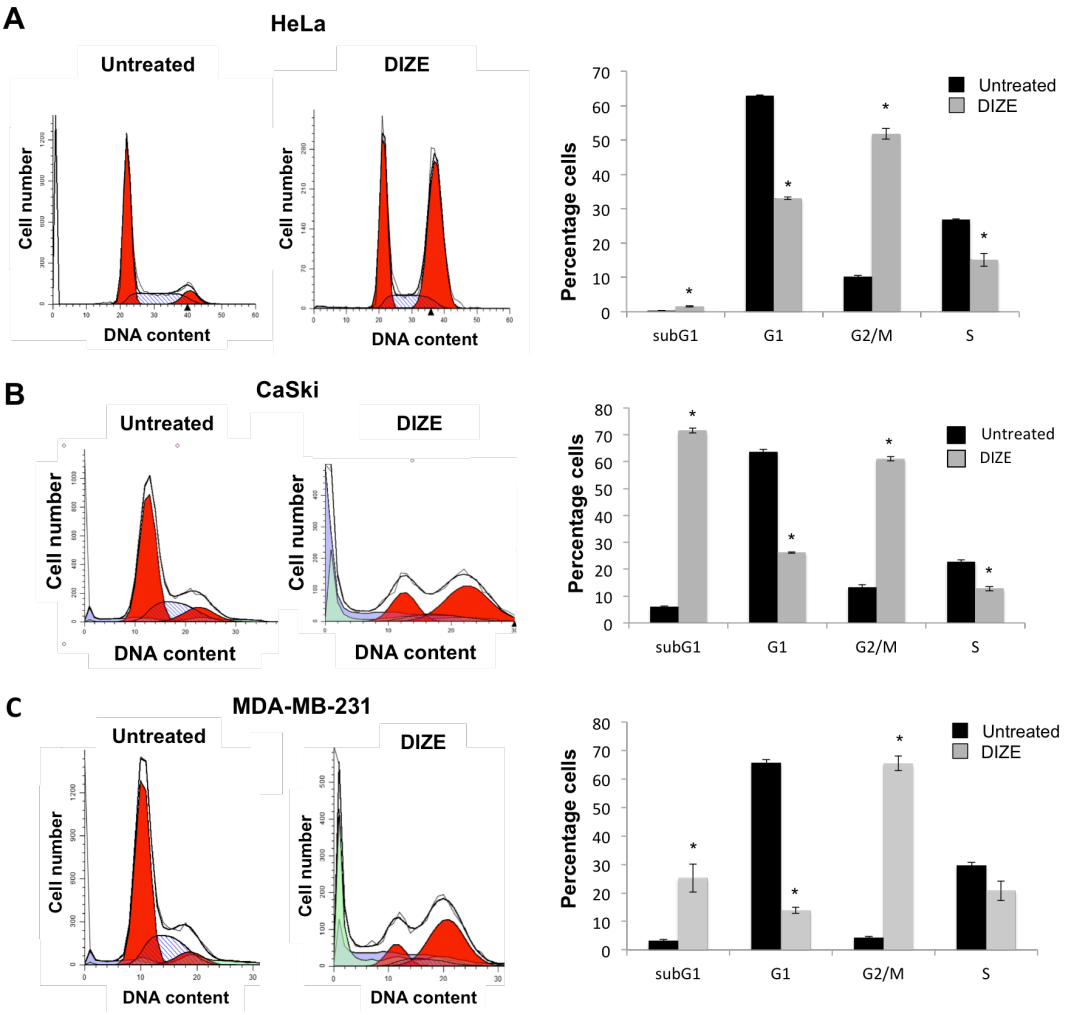


Figure 3.8: DIZE treatment induces a G2/M arrest in cancer cells using flow cytometry. HeLa **(A.)**, CaSki **(B.)** and MDA-MB-231 **(C.)** cell cycle profiles showing the effects of IC_{50} DIZE treatment for 48 hours. Quantitation of cell cycle data showing significant changes in all phases of the cell cycle (*$p<0.05$). Results shown are the mean +/- SEM of experiments performed in triplicate and repeated at least two independent times.

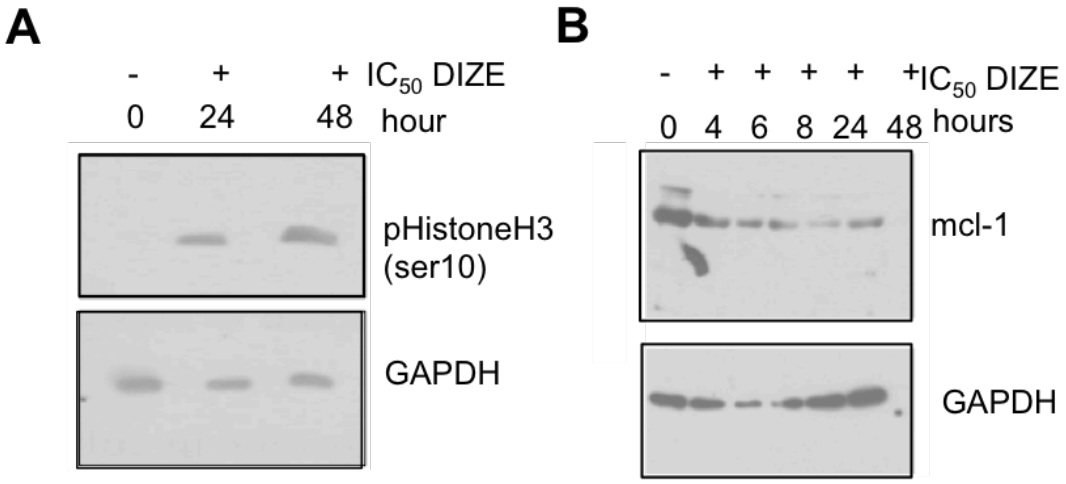


Figure 3.9: Expression of G2/M cell cycle proteins induced by DIZE treatment in HeLa cells. Western blot analysis showing fluctuation of the expression levels of the G2/M cell cycle proteins. **(A.)** pHistone H3 protein expression is elevated after IC_{50} DIZE treatment. **(B.)** Mcl-1 protein degradation, indicative of G2/M arrest, was observed from 4 hours to 48 hour DIZE treatments. Experiments shown were repeated two independent times.

3.2.6 Activation of ACE-2 by DIZE results in cell death via apoptosis

As ACE-2 activation of cancer cells resulted in a significant increase in the subG1 cell population we next investigated whether this correlates with cell death via apoptosis. The degree of cell damage and death caused by DIZE was determined using the Annexin V assay. During apoptosis, the membrane phospholipid phosphatidylserine (PS) translocates from the inner to the outer cell membrane while the plasma membrane remains intact. Fluorochrome-labelled Annexin V has a high affinity for PS thus binding would indicate the onset of apoptosis. Propidium iodide (PI) is also used to distinguish viable cells from damaged or dying cells as those with damaged membranes will readily take up PI whereas healthy cells exclude it. Thus the combination of Annexin V and PI allows the differentiation of healthy cell populations from those undergoing early apoptosis, late apoptosis or necrosis(Riccardi and Nicoletti, 2006; van Engeland et al., 1996; Vermes et al., 1995)

Our results show that DIZE treatment at its IC_{50} concentration in HeLa, CaSki and MDA-MB-231 cells, resulted in a significant reduction in the percentage of live cells and a significant increase in the percentage of late apoptotic and necrotic cells (Figure 3.10 A, B and C).

We independently confirmed that DIZE treatment resulted in cell death via apoptosis by monitoring the PARP cleavage. PARP functions to repair DNA damage and when cells undergo apoptosis, PARP is cleaved by caspase-3 preventing DNA repair and ultimately leading to cell death(Boulares et al., 1999; Duriez and Shah, 1997; Kaufmann et al., 1993; Soldani et al., 2001)Western blot analysis of PARP identified PARP cleavage in DIZE-treated HeLa cell lysates confirming cell death was mediated by apoptosis (Figure 3.11 A). We also

observed an increase in p53 expression after treatment (Figure 3.11 B). The p53 tumor suppressor protein regulates cell death through the detection of stress signals. Apoptosis can be initiated by p53 via the activation of pro-apoptotic proteins such as Bax, Puma and Noxa, or the repression of anti-apoptotic proteins such as Bcl-2 and Bcl-$_{XL}$ and Mcl-1 (Amaral et al., 2010; Elmore, 2007; Vaux and Strasser, 1996)

Together, our results suggest that activation of ACE-2 by small molecules such as DIZE associates with G2/M cell cycle arrest and accompanying cancer cell death via apoptosis and necrosis.

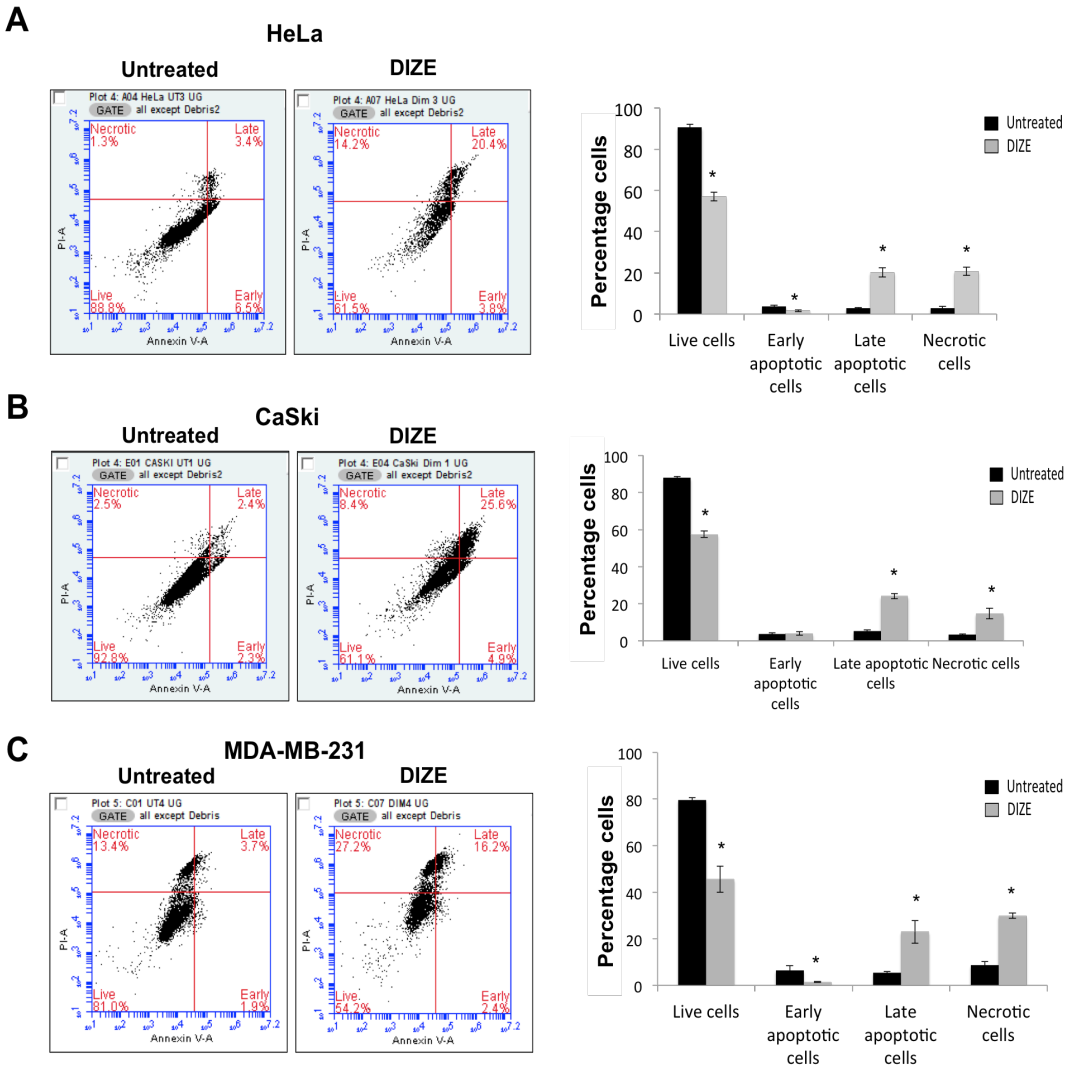


Figure 3.10: Effect of DIZE on cancer cell death using the Annexin V assay. HeLa, CaSki and MDA-MB-231 cells were treated with the IC_{50} concentration of DIZE for 48 hours and effects on cell death investigated using the Annexin V assay. Annexin V profiles of HeLa **(A.)**, CaSki **(B.)** and MDA-MB-231 **(C.)** cells showing cells falling into four quadrants indicative of live cells, early apoptotic, late apoptotic and necrotic cells. Quantitation of Annexin V profiles showing a significant reduction in the percentage of live and early apoptotic cells and a significant increase in the percentage of late apoptotic and necrotic cells (*$p<0.05$). Results shown are the mean +/- SEM of experiments performed in triplicate and repeated two independent times.

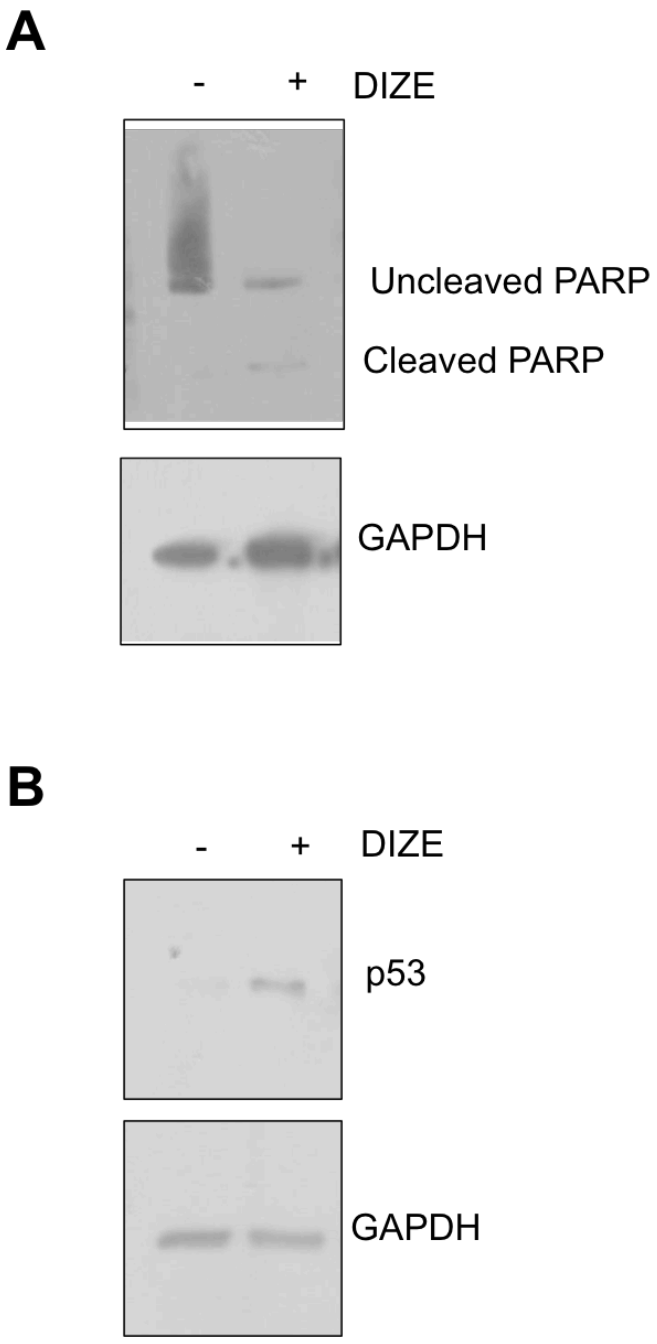


Figure 3.11: Effects of DIZE on PARP cleavage and p53 expression in HeLa cells (A.) Western blot analysis of PARP cleavage and (B.) p53 expression after IC_{50} DIZE treatment for 48 hours. PARP cleavage and p53 were used to confirm the induction of apoptosis in HeLa cells. GAPDH was used as a loading control. Experiments were repeated three independent times.

3.2.7 ACE-2 activation by Ang-(1-7) associates with cell death via apoptosis

We investigated the effects of Ang-(1-7) treatment on cell cycle progression in HeLa cells revealed no change in the percentage of cells in the G1, G2/M and S phases of the cell cycle. However, a 7-fold increase in cells in the subG1 population was observed (Figure 3.12). Annexin V staining was performed to monitor the modes of cell death associated with Ang-(1-7) treatment. In HeLa (Figure 3.13 A), CaSki (Figure 3.13 B) and MDA-MB-231 cells (Figure 3.13 C) Ang-(1-7) treatment caused an increase in cell death via both apoptosis and necrosis.

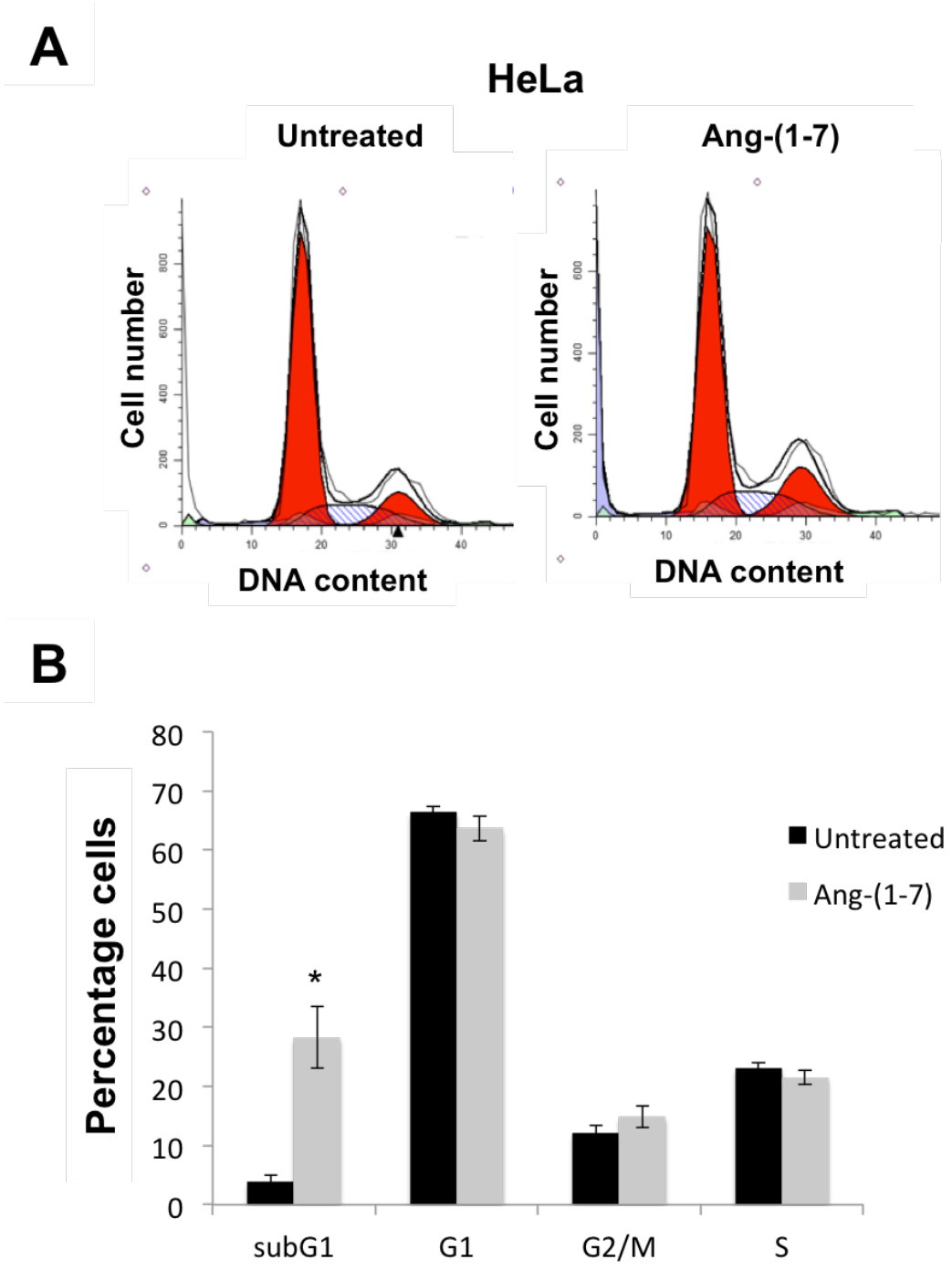


Figure 3.12: Effect of Ang-(1-7) on HeLa cell cycle profile using flow cytometric analysis. (A) Representative picture of HeLa cell cycle profile showing the effects of Ang-(1-7) on cancer cell cycle profile after 1 µM Ang-(1-7) treatment for 48 hours. **(B).** Quantitation of cell cycle data showing a significant increase in the subG1 population (*p<0.05). Results shown are the mean +/-SEM of experiments performed in triplicate and repeated twice.

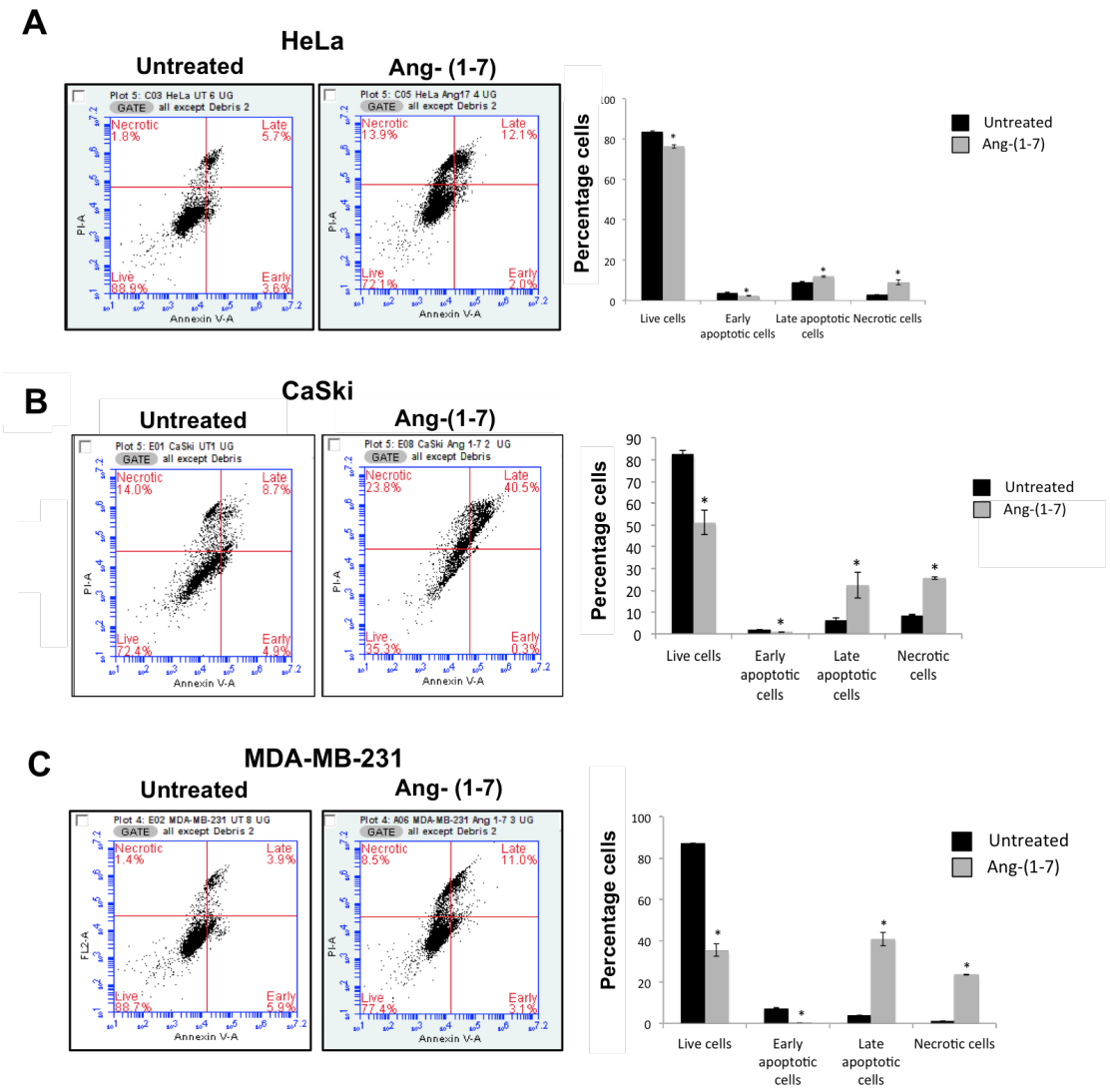


Figure 3.13: Effect of Ang-(1-7) on cancer cell death.

HeLa, CaSki and MDA-MB-231 cells were treated with 1 µM Ang-(1-7) for 48 hours and effects on cell death investigated using the Annexin V assay. Annexin V profiles of HeLa **(A.)**, CaSki **(B.)** and MDA-MB-231 cells **(C.)** with cells falling into four quadrants indicative of live cells, early apoptotic, late apoptotic and necrotic cells as shown. Quantitation of Annexin V profiles shows a significant reduction in the percentage of live cells and early apoptotic cells as well as a significant increase in the percentage of late apoptotic and necrotic cells (*p<0.05). Results shown are the mean +/-SEM of experiments performed in triplicate and repeated twice.

3.3 DISCUSSION

There are numerous reports describing the involvement of the ACE-1 axis in diseases such as hypertension and cardiovascular disease. Our earlier results suggest that the ACE-1 axis is functional in certain cancer cell lines. There is a large body of evidence supporting the role of ACE-2 axis in disease and cancer(Ager et al., 2008; Babacan et al., 2015; Cutler, 1999; Dinh et al., 2001; Fazeli et al., 2012; Ino et al., 2011; Kuniyasu, 2012; Mizuiri and Ohashi, 2015; Verma et al., 2012). Thus, the role of the ACE-2 axis and the effect of ACE-2 activation on specific cancer cell lines were investigated.

The hypothesis that the ACE-2 axis may be able to counter ACE-1 effects in cancer cells was tested using Ang-(1-7), the ACE-2 cleavage product. Treatment using Ang-(1-7) significantly reduced cervical and breast cancer cell proliferation and induced cancer cell death via apoptosis (Figure 3.13). These findings support the notion that Ang-(1-7) confers protection against ACE-1 cancer-promoting effects, and inhibits the migration and invasion of A549 human lung adenocarcinoma cells(Ni et al., 2012) The anti-cancer activity of Ang-(1-7) has also been described using *in vitro* and *in vivo* model systems(Feng et al., 2010b; Gallagher et al., 2014; Ni et al., 2012; Simoes E Silva and Teixeira, 2016)

Our study suggests that endogenous ACE-2 levels and enzyme activity is low in cancer cells and that DIZE treatment significantly enhances ACE-2 activity in cervical and breast cancer cell lines. We further showed that cancer cell proliferation was significantly inhibited by the ACE-2 activators DIZE and Ang-(1-7). This suggests that the ACE-2 even though expressed at

low levels in cancer cells, can be activated using small molecules such as DIZE and Ang-(1-7) leading to cancer cell death.

Since the discovery of RAS components in cancer it has been suggested *in vitro* as well as in animal models that ACE-1 via Ang-II and AT1R activation promotes tumour growth through cell proliferation, vascularization and metastatic progression (Du et al., 2012; Duenas-Gonzalez et al., 2008b; Kuniyasu, 2012; Miyajima et al., 2015; Stevens et al., 2015). The well-documented approach in cell lines, animal models and patient studies, has been to target AT1R activation, and this has been effective in cancer of the prostate, breast and colon (Alhusban et al., 2014; Babacan et al., 2015; Kuniyasu, 2012; Namazi et al., 2015). An alternate approach is aimed at exploiting ACE-2/Ang-(1-7). A recent study by (Yu et al., 2016) reported that down-regulation of the ACE-2 axis promotes breast cancer cell metastasis via increased calcium signaling. For the first time, we have shown that ACE-2 activation in cervical and breast cancer cell lines using DIZE, resulted in decreased cell proliferation that associated with a G2/M arrest and cancer cell death via apoptosis.

There is great interest in repurposing compounds or investigating further potential applications for drugs currently on the market. For this reason, more studies investigating the anti-cancer effects and downstream signaling of DIZE is required. Despite potential off target effects our data suggests that DIZE has promise as an anti-cancer agent. No pharmacokinetic studies have yet been reported for DIZE thus more investigation is required into DIZE and structurally similar compounds.

Taken together, the data presented in this chapter suggest that activators of the ACE-2 axis might have therapeutic potential against cervical and breast cancer as new agents or in combination treatments with current chemotherapeutics. Moreover ACE-2 axis activation through DIZE treatment results in selective killing effects in cancer cells and not normal cells.

CHAPTER 4

INVESTIGATING THE EFFECT OF RAS TREATMENT IN COMBINATION WITH DOX OR CDDP IN CANCER CELLS

4.1 INTRODUCTION

Considerable efforts have been made in pre-clinical laboratory research methodology to identify efficacious treatment options for cancer patients. Drug combination therapies, new drug delivery techniques, and novel approaches are aimed at making cancer treatments more effective by decreasing side effects and drug resistance (Nastiuk and Krolewski, 2016). While solid tumour malignancies can be effectively treated with surgery and radiotherapy, the need for effective treatment of metastatic cancers makes researching chemotherapy, and in particular novel combination treatments, crucial for emerging therapeutic options.

Cisplatin (CDDP) and Doxorubicin (DOX) are effective anticancer drugs and are used against a wide variety of human cancers including oesophageal, lung, bladder, ovarian, cervical, testicular, prostate, and breast cancer (Dasari and Tchounwou, 2014; Dhar et al., 2011; Goodsell, 2006; Khan et al., 1982; Morris et al., 1992; Patel and Kaufmann, 2012). CDDP destabilizes DNA through intercalation (Dasari and Tchounwou, 2014) while DOX induces DNA damage through topoisomerase II inhibition and by the formation of free radicals (Eom et al., 2005). While these drugs are known to induce apoptosis in cancer cells, drug resistant tumours and severe side effects are common (Akiyama et al., 1999; Dasari and Tchounwou, 2014; Keizer et al., 1990; Patel and Kaufmann, 2012; Rajeswaran et al., 2008). Due to the cytotoxic nature of such drugs they are often administered as a combination treatment, where the combination of different drugs can achieve a synergistic therapeutic effect. This

allows for the dose to be lowered, and therefore toxicity minimized (Akiyama et al., 1999; Apostolou et al., 2013; Ardizzoni et al., 1991; Crino et al., 1997; Judson et al., 2014b; Lee et al., 2014).

Although CDDP is a compelling chemotherapeutic drug, it has been reported that patients treated with this drug may develop resistance or may experience relapse (Akiyama et al., 1999; Dancey and Chen, 2006). In order to overcome CDDP resistance other platinum compounds, such as carboplatin, have been developed, showing strong chemotherapeutic properties (Dasari and Tchounwou, 2014; Natarajan et al., 1999). Furthermore, combination therapies have previously been shown to be effective against drug resistance while reducing the harmful side effects associated with CDDP (Dasari and Tchounwou, 2014; Lee et al., 2014; Morris et al., 1992).

As with CDPP, the use of the chemotherapeutic agent DOX is limited by drug resistance (Housman et al., 2014). When DOX is used in conjunction with other drugs, such as CDDP and the Hsp90 inhibitor, Gamitrinib, enhanced anticancer effects have been demonstrated. Gamitrinib, together with DOX, reduced the growth of tumours in breast and prostate cancer xenograft models without worsening the cardiotoxic side effects of DOX (Dombernowsky et al., 1996; Park et al., 2014). Combined treatment of DOX with natural compounds such as grape seed extract and curcumin analogs have been successful in the treatment of breast cancer (Dayton et al., 2011; Notarbartolo et al., 2005; Sharma et al., 2004). Paclitaxel, a mitotic inhibitor isolated from the bark of *Taxus brevifolia, (*the northwest Pacific Yew tree) has successfully been used with both DOX and CDDP to treat

breast, oesophageal and cervical cancer (Cragg, 1998; Dombernowsky et al., 1996; Lee et al., 2014; Li et al., 2015).

In addition to standard chemotherapeutic agents, repurposed drugs such as ACE-1 inhibitors, are proving to have potential valuable in combatting cancer. It has been reported that ACE-1 inhibition results in the reduction of side effects caused by chronic DOX and CDDP treatment in rats. One such study by (Hiona et al., 2011) showed that pre-treatment of the ACE-1 inhibitor, Enalapril, reduced DOX-induced cardiomyopathy in rats through the prevention of free radical formation and preservation of mitochondrial function. Research published by Sacco et al. (2001; 2009) showed that the ACE-1 inhibitor, Zofenopril prevented cardiac injury without interfering with the anti-tumour activity of DOX. Combined treatments of ACE-1 inhibitors with CDDP have also been reported to be beneficial. Moreover, a xenograft study by (El-Sayed et al., 2008) demonstrated how the ACE-1 inhibitor, Captopril, provided a protective role against CDDP-induced-kidney damage through antioxidant effects provided by the drug's sulfhydryl group.

Earlier data in our study has shown that inhibition of the ACE-1 axis of the Renin Angiotensin System (RAS) as well as activation of the ACE-2 axis results in promising anticancer effects. The effect of combination treatments using CDDP or DOX together with inhibitors of the ACE-1 axis, Captopril or Lisinopril, or activators of the ACE-2 axis, such as Diminazene aceturate (DIZE) are investigated in this chapter for potential anticancer effects in cervical and breast cancer cells.

4.2 RESULTS

4.2.1 Effect of combination treatments of DOX and ACE-1 inhibitors on cancer cells

DOX was used in combination with the ACE-1 inhibitors, Captopril and Lisinopril, to investigate the combined effect of these drugs. HeLa and MDA-MB-231 cells were treated with the half maximal inhibitory concentration (IC_{50}) of either of the ACE-1 inhibitors, Captopril or Lisinopril, together with a range of concentrations of DOX to investigate the effect of such combinations on the IC_{50} of DOX. Results showed that the addition of the IC_{50} concentration of Captopril, in HeLa or MDA-MB-231 cells, did not result in a significant change in the IC_{50} of DOX (Figure 4.1 A). Similarly, in both cell lines, treatment with the IC_{50} concentration of Lisinopril together with a range of DOX, resulted in no significant change in the IC_{50} concentration of DOX (Figure 4.1 B).

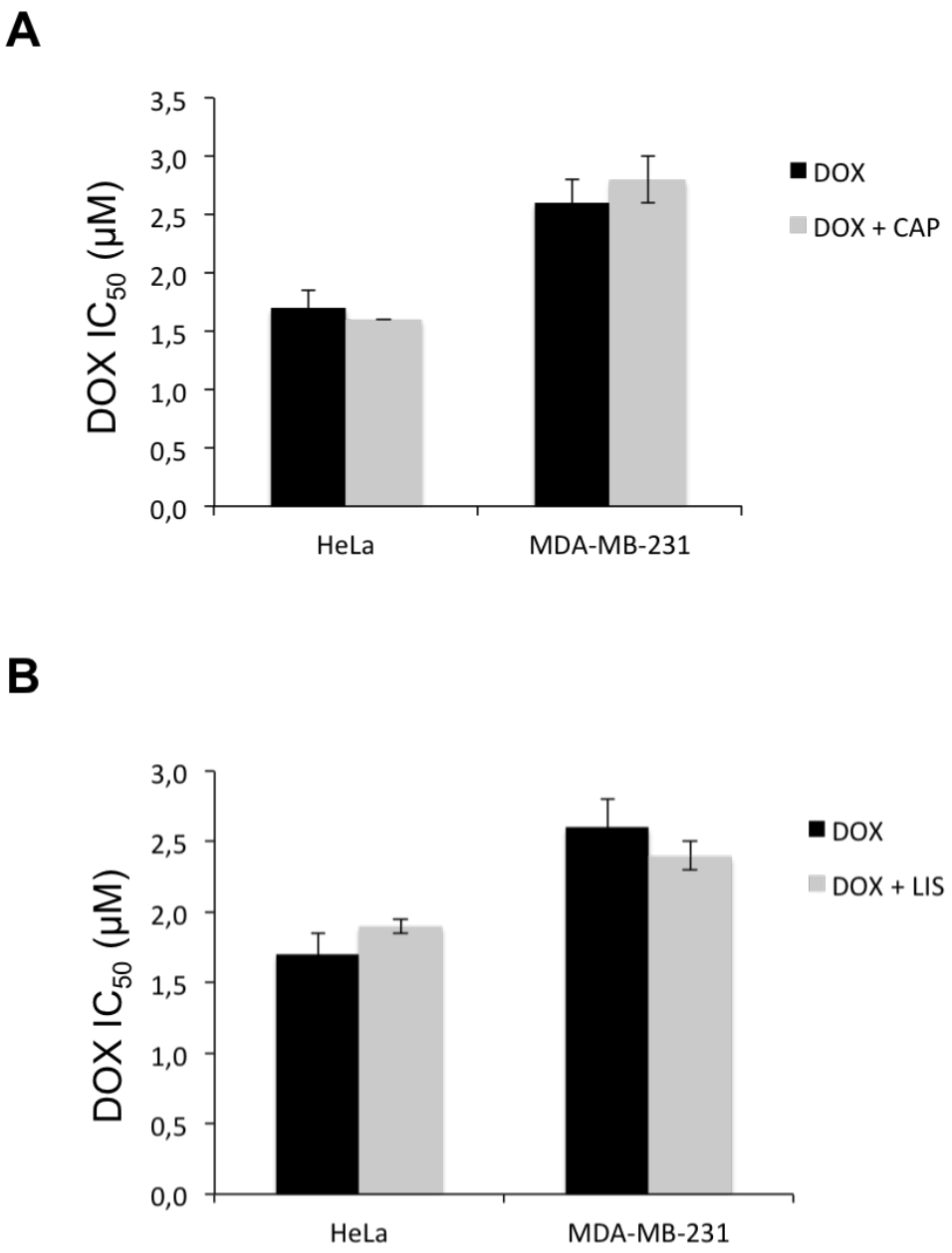


Figure 4.1: Effect of ACE-1 inhibitor treatment on DOX IC_{50} concentration. (A.) HeLa and MDA-MB-231 cells were treated with either Captopril or Lisinopril (B.) and the effect of co-treatment on DOX IC_{50} determined with the MTT assay. No significant change in IC_{50} was observed. Results shown are the mean +/- SEM of experiments performed in triplicate and repeated two independent times.

4.2.2 Effect of combination treatments of CDDP and ACE-1 inhibitors on cancer cells

CDDP was used with either of the ACE-1 inhibitors and the effect on HeLa, CaSki and MDA-MB-231 cell viability determined using the MTT assay (Figures 4.2 and 4.3). Results showed that co-treatment with the IC_{50} concentration of either of the ACE-1 inhibitors, Captopril or Lisinopril reduced the cell killing effect of CDDP. The IC_{50} concentration of CDDP was significantly increased from 19 µM to 37 µM in HeLa cells and from 24 µM to 32 µM in MDA-MB-231 cells when combined with Captopril (Figure 4.2 A). A similar trend was observed in Caski cells, even though the change in CDDP IC_{50} concentration was marginal, increasing from 13 µM to 19 µM (Figure 4.2 A). This data suggests that Captopril reduces the sensitivity of cancer cells to CDDP.

In all cell lines tested, Lisinopril significantly increased the IC_{50} of CDDP. Combined treatment of Lisinopril increased the IC_{50} of CDDP from 18 µM to 97 µM in HeLa cells, 13 µM to 79 µM in CaSki cells and 26 µM to 214 µM in MDA-MB-231 cells (Figure 4.2B).

The Chou and Talalay method was then used to determine the potential association of the ACE-1 inhibitors with CDDP (Chou and Talalay, 1984; Chou, 2006). This method involves a combination treatment of the drugs in question, using a constant ratio of drug A with drug B. Together with Compusyn's mathematical analysis, the Chou-Talalay combination index (CI) method allows for the quantitative determination of synergism (where CI < 1), additivity (where CI = 1), and antagonism (where CI > 1) in drug combinations. Where the Log (CI) is shown then Log (CI) < 0 indicates synergism, Log (CI) = 0 indicates additivity, and Log (CI) > 0 indicates antagonism. Where Log CI profiles are shown this is indicative of the degree of antagonism determined between the two drugs. Results show that when HeLa, CaSki or

MDA-MB-231 cells were treated with CDDP and Captopril in a 1:1 ratio, i.e. where the IC_{50} of CDDP was used with the IC_{50} of Captopril in a constant ratio, a reduced cell killing effect was observed in each of the cancer cell lines tested (Figure 4.3 A). Our results suggest that CDDP is more effective as a single treatment. Determination of the CI of the combined treatment of CDDP with Captopril showed that there was an antagonistic association, where the Log CI of the two drugs was greater than 0, thus reducing the cell killing effect of the combined treatment. This trend was observed in each of the cell lines tested (Figures 4.3 B).

A similar result was observed in these cell lines when a combined treatment of CDDP with Lisinopril in a 1:1 ratio was applied (Figure 4.4). The combined treatment of CDDP with Lisinopril resulted in a marginal effect on cancer cell viability when compared to CDDP single treatment (Figure 4.4 A). Determination of the CI values for the combined treatment in HeLa, CaSki and MDA-MB-231 cells all indicated antagonistic associations between the drugs (Figure 4.4 B).

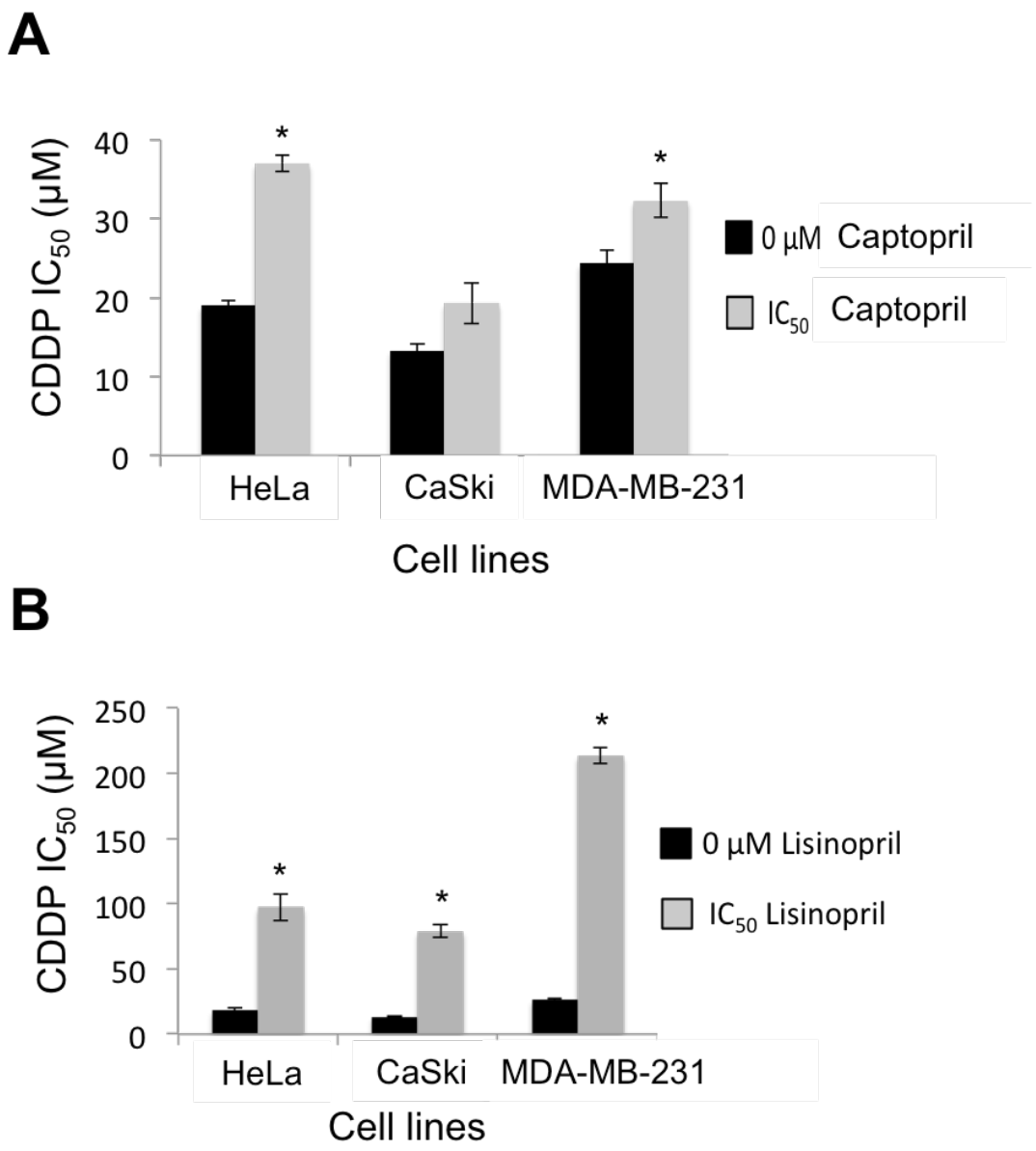


Figure 4.2: ACE-1 inhibitors decrease sensitivity of cancer cells to CDDP treatment. HeLa, CaSki and MDA-MB-231 cancer cells were treated with varying concentrations of CDDP, with or without the IC_{50} of Captopril (A) or Lisinopril (B) for 48 hours. A significant increase in the IC_{50} of CDDP was observed In HeLa and MDA-MB-231 cell lines (*$p<0.05$). Results shown are the mean +/- SEM of experiments performed in triplicate and repeated at least two independent times.

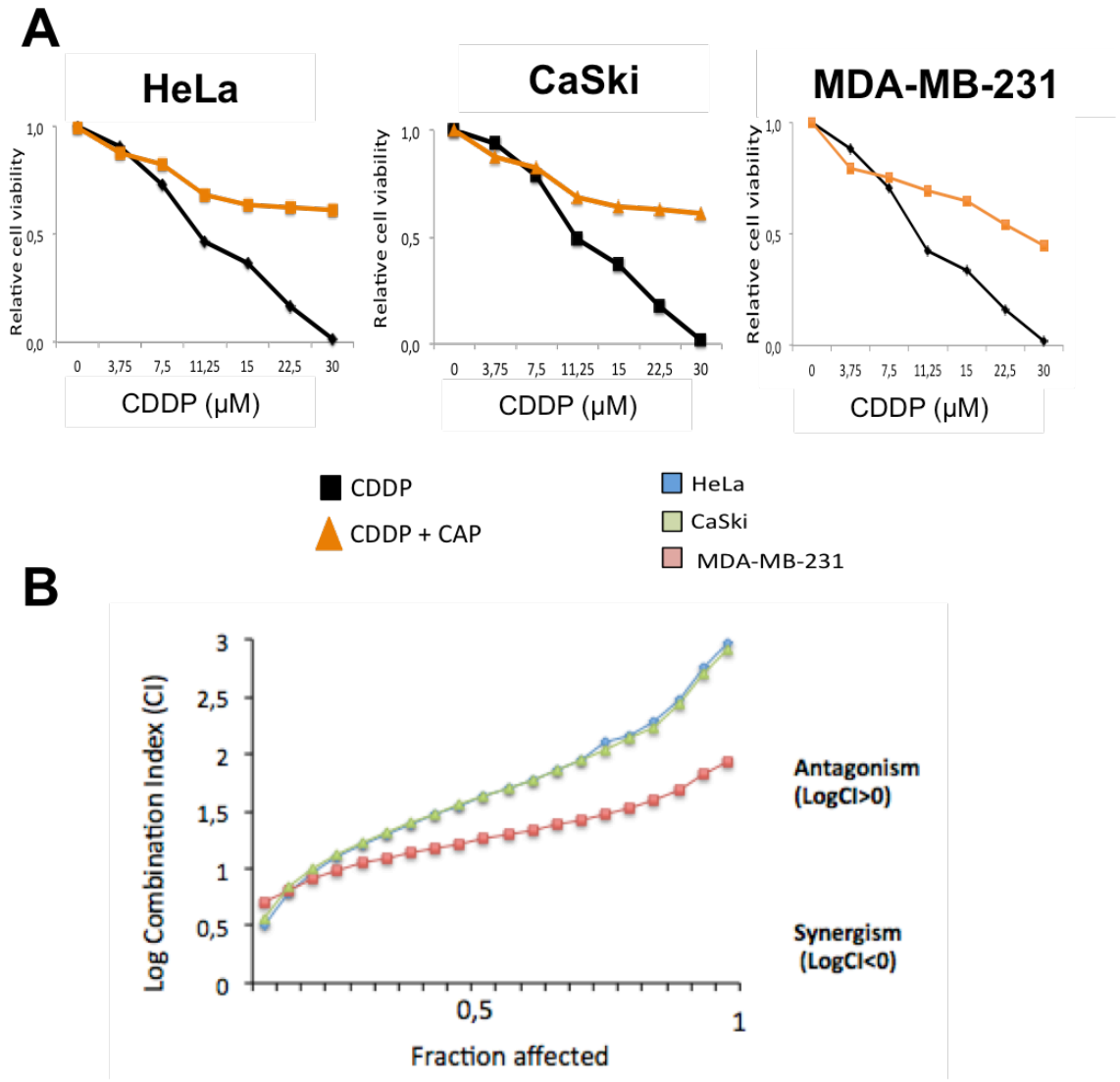


Figure 4.3: CDDP and CAP combination treatment produces antagonistic effects in cancer cells. (A.) HeLa, CaSki and MDA-MB-231 cells were treated with a 1:1 ratio of the IC_{50} concentrations of CDDP and Captopril and cell viability measured after 48 hours using the MTT assay. CDDP as a single treatment effectively kills HeLa, CaSki and MDA-MB-231 cancer cells whereas the 1:1 combined treatment of CDDP with Captopril results in a poorer reduction in the cell viability of treated cells **(B.)** The CI for 1CDDP:1Captopril was determined and suggests an antagonistic effect (LogCI >0) in these cell lines. Results shown are the mean +/- SEM of experiments performed in triplicate and repeated at least two independent times.

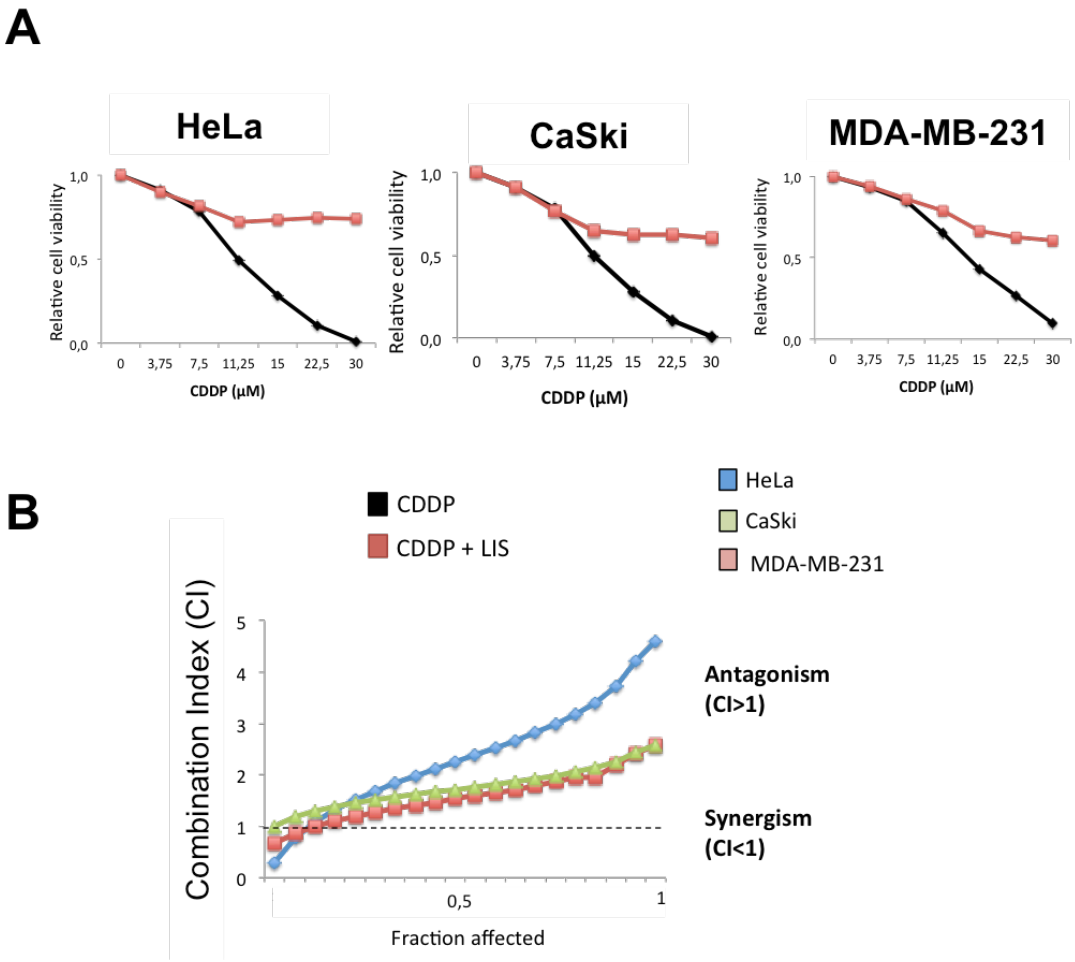


Figure 4.4: CDDP and Lisinopril combination treatment produces antagonistic effects in cancer cells. (A.) HeLa, CaSki and MDA-MB-231 cells were treated with a 1:1 ratio of the IC_{50} concentrations of CDDP and Lisinopril and cell viability measured after 48 hours using the MTT assay. The 1:1 combined treatment results in a marginal reduction in cell viability when compared to CDDP single treatment **(B.)** The CI for CDDP:Lisinopril was determined and shows a predominantly antagonistic effect (CI>1) in these cell lines. Results shown are the mean +/- SEM of experiments performed in triplicate and repeated at least two independent times.

To support the findings that the combined treatments of CDDP with Captopril or Lisinopril reduced sensitivity of cancer cells to CDDP, the effect of these combined treatments on cancer cell death was investigated using the Annexin V assay. Annexin V profiles of a representative experiment show that in HeLa cells the combination of CDDP together with Captopril reduced the ability of CDDP to kill cancer cells, making the combination less effective than a single treatment of CDDP alone (Figure A.2, Appendix I). The percentage live cells increased from 60.2% to 97.9 % when Captopril was combined with CDDP (Figure 4.5 A). The Annexin V profile of Captopril single treatment (Figure A.2, Appendix I) showed that there was a small, but significant effect on cancer cell death as 12.8% of cells were either apoptotic or necrotic after treatment (Figure A.2, Appendix I).

Similarly in CaSki cells, Annexin V profiles of the single treatments of CDDP or Captopril, compared to the combined treatment of the two drugs, show that a greater percentage of cells were undergoing apoptosis or necrosis (Figure A.3, Appendix I). The percentage of live cells after treatment with both drugs was observed as being 98.3% whereas only 76.2% live cells remained after Captopril single treatment and 58 % after CDDP treatment (Figure 4.5 B). A similar result can be seen in MDA-MB-231 breast cancer cells (Figure A.4, Appendix I; Figure 4.5 C).

Similar results were observed after combined treatments of CDDP with Lisinopril in the cancer cell lines. Annexin V profiles show that combined treatment in HeLa cells did not result in a cell killing effect (Figure 4.6 A). The Annexin V profile of cells treated with both CDDP and Lisinopril appear similar to that of the control cells, whereas profiles of cells treated with either of the single agents show that there was an increase in the percentage

of apoptotic and necrotic cells and thus a decrease in the percentage of live cells (Figure A.5, Appendix I).

In CaSki cells treatment of both CDDP and Lisinopril showed similar results to that of HeLa cells and Annexin V profile of both drugs showed no significant difference to that of the untreated sample (Figure A.6, Appendix I). Quantitation of each of the single treatments however, showed a significant reduction in the percentage live cells and a significant increase in cells undergoing cell death (Figure 4.6 B). A similar result can be seen in MDA-MB-231 breast cancer cells (Figure A.7, Appendix I; Figure 4.6 C).

While the preliminary data presented here requires further validation, results suggest that combination treatments of CDDP with the ACE-1 inhibitors, Captopril and Lisinopril, may not have beneficial effects in the treatment of cancer. Such results may have future implications for example, patients on ACE-1 inhibitors who develop cancer. Results presented in this chapter suggest that it may not be a good approach to treat cancer patients with CDDP while they are on ACE-1 inhibitors.

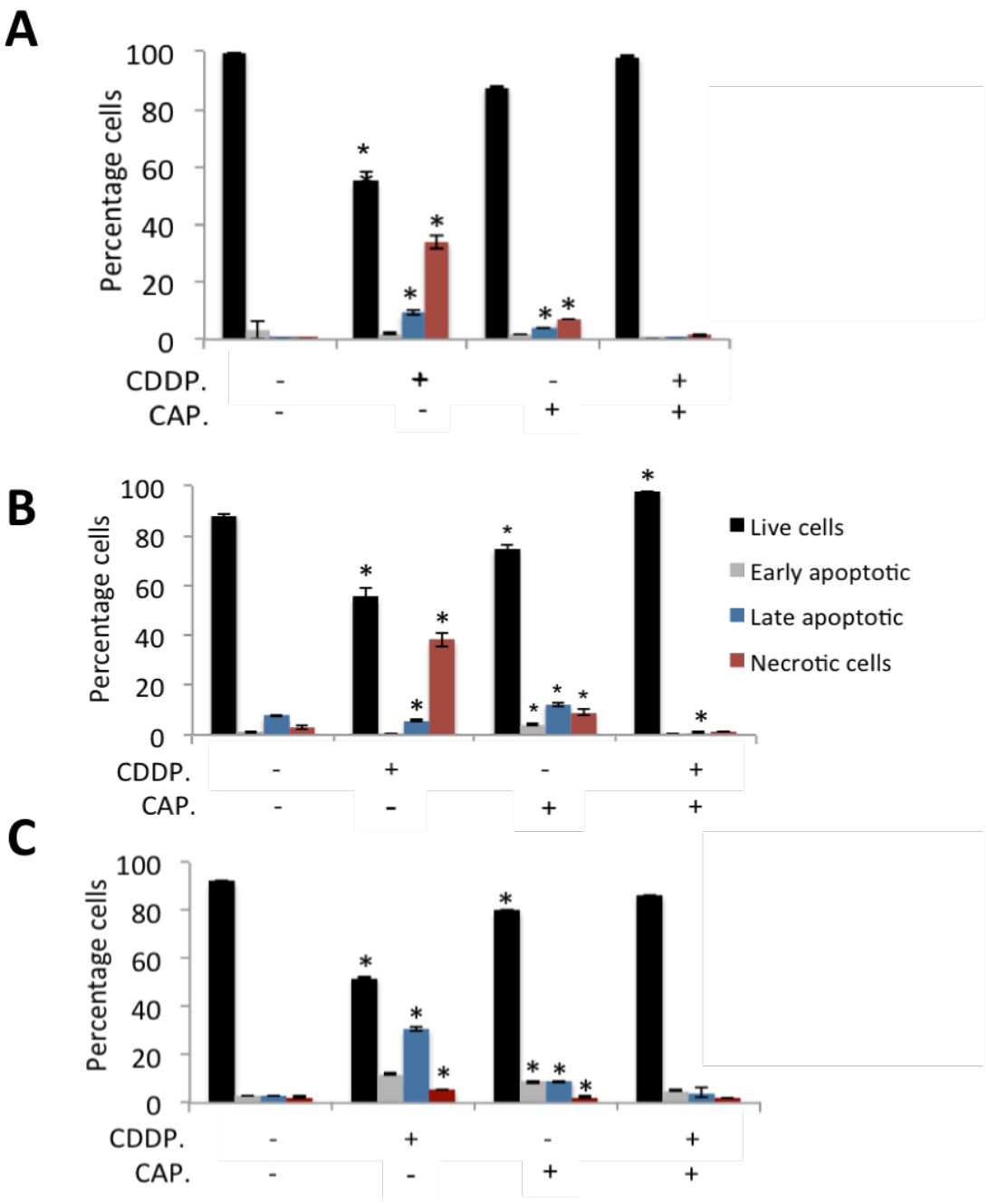


Figure 4.5. The effect of CDDP and Captopril combined treatment on cancer cell death using the Annexin V assay. HeLa **(A.)**, CaSki **(B.)** and MDA-MB-231 **(C.)** cells were treated with the IC_{50} concentrations of CDDP and Captopril for 48 hours and effects on cell death investigated using the Annexin V assay. Quantitation of Annexin V profiles showing that the combination treatment results in no significant effect on cancer cell death. CDDP and Captopril single treatments showed a significant increase in the percentage of late apoptotic and necrotic cells (*$p<0.05$). Results shown are the mean +/-SEM of experiments performed in triplicate and repeated twice.

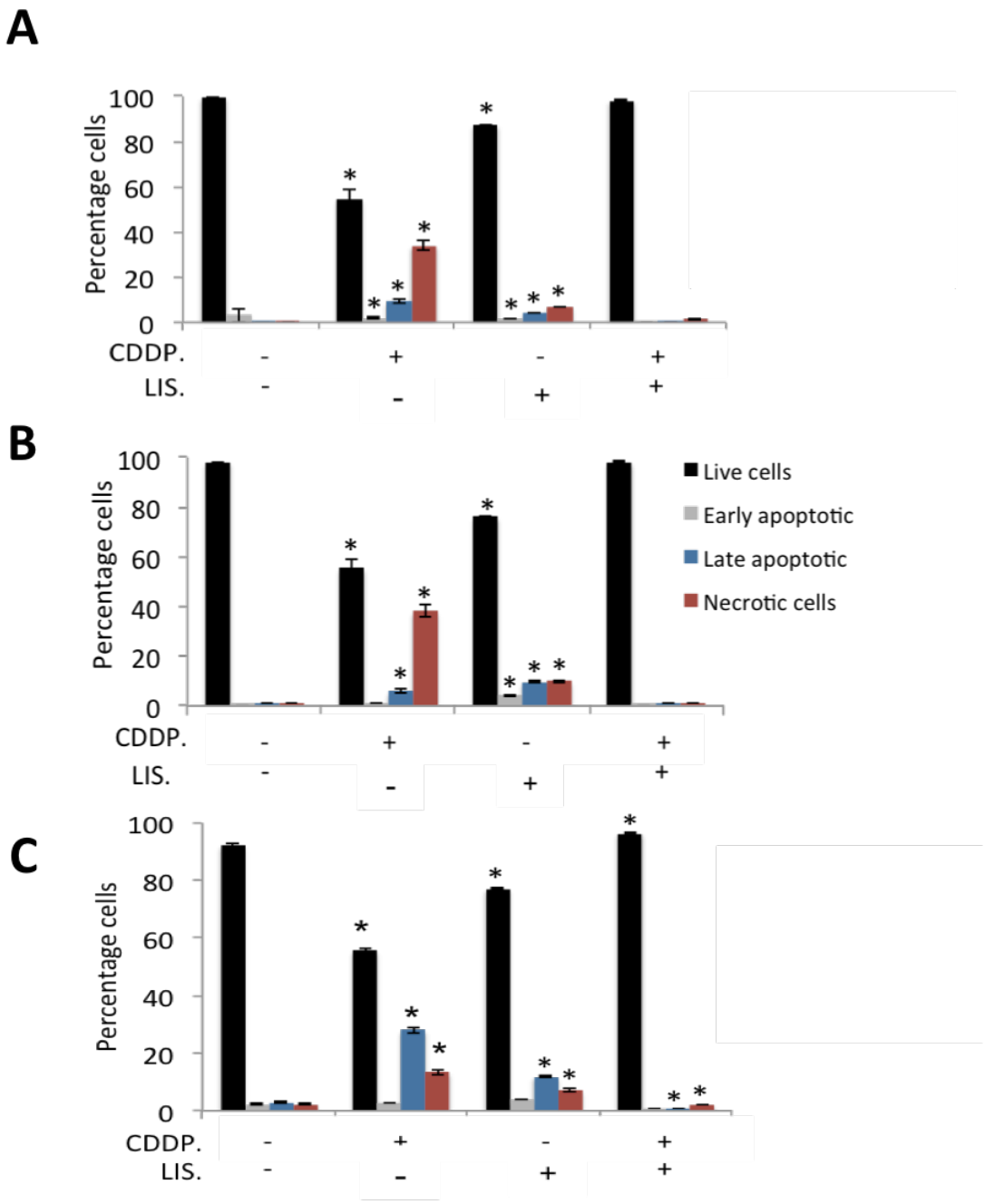


Figure 4.6. The effect of CDDP and Lisinopril combined treatment on cancer cell death using the Annexin V assay. HeLa **(A.)**, CaSki **(B.)** and MDA-MB-231 **(C.)** cells were treated with the IC_{50} concentrations of CDDP and Lisinopril for 48 hours and effects on cell death investigated using the Annexin V assay. Quantitation of Annexin V profiles showing no significant effect on cancer cell death. CDDP and Lisinopril single treatments showed a significant reduction in live cells and a significant increase in the percentage of late apoptotic and necrotic cells (*$p<0.05$). Results shown are the mean +/-SEM of experiments performed in triplicate and repeated twice.

4.2.3 Combined treatments of chemotherapeutic drugs, CDDP and DOX, with ACE-2 activator, DIZE

4.2.3.1 Investigating the effects of CDDP and DIZE combined treatment in cancer cells

The previous chapter provided evidence showing that DIZE has anticancer properties, significantly reducing cervical and breast cancer cell proliferation via a G2/M block in the cell cycle. Here we investigate the effects of combination treatment of DIZE with CDDP and DOX on cancer cells.

HeLa, CaSki and MDA-MB-231 cells were treated with CDDP and the IC_{50} concentration of DIZE for 48 hours in order to determine if the activation of the ACE-2 axis in conjunction with a chemotherapeutic agent such as CDDP could produce an enhanced cell killing effect. Activation of the ACE-2 axis with DIZE treatment in all three cell lines did not alter CDDP cytotoxicity, as no significant change, positive or negative, in the IC_{50} concentration of CDDP was observed (Figure 4.7).

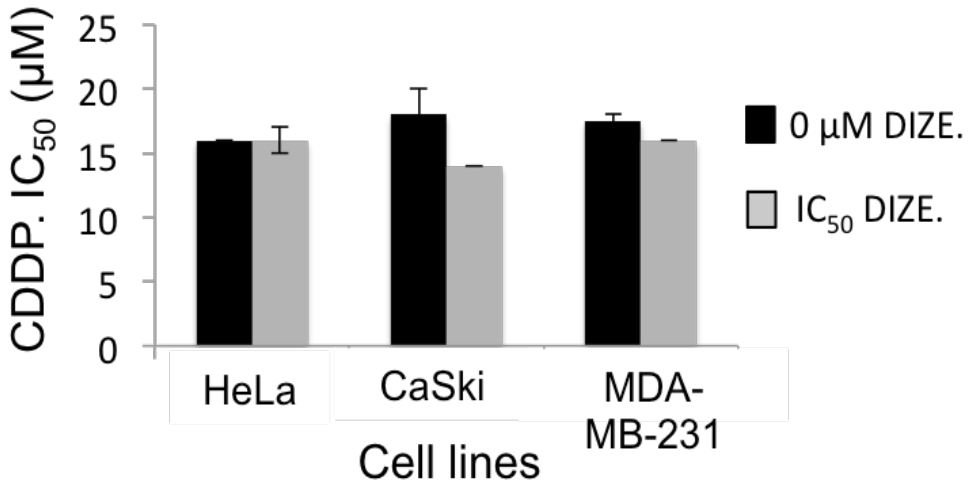


Figure 4.7: Effect of DIZE treatment on CDDP IC_{50} concentrations in cancer cell lines. (A.) HeLa, CaSki and MDA-MB-231 cancer cells were treated with varying concentrations of CDDP, with or without the IC_{50} concentration of DIZE, for 48 hours. The IC_{50} concentration of CDDP as a single treatment as well as with DIZE was determined using the MTT assay. No change in the IC_{50} concentration of CDDP was observed in any of the cell lines (*$p<0.05$). Results shown are the mean +/- SEM of experiments performed in triplicate and repeated twice.

4.2.3.2 Investigating the effects of DOX and DIZE combined treatment in cancer cells

The combined treatment of DOX with DIZE was next investigated in HeLa, CaSki and MDA-MB-231 cells. Results show that DIZE had a dramatic effect on the IC_{50} concentration of DOX, significantly reducing the required concentration of DOX to kill 50 % of cells, from the micromolar to nanomolar range (Figure 4.8). The IC_{50} concentration of DOX changed from 1.8 µM, 2.6 µM and 2.7 µM to 24 nM, 39 nM and 44 nM for HeLa, CaSki and MDA-MB-231 cells, respectively (Table 4.1). This significant decrease in the IC_{50} of DOX suggests that DOX and DIZE may use similar mechanisms of action, or alternatively, synergistic mechanisms resulting in enhanced cell death.

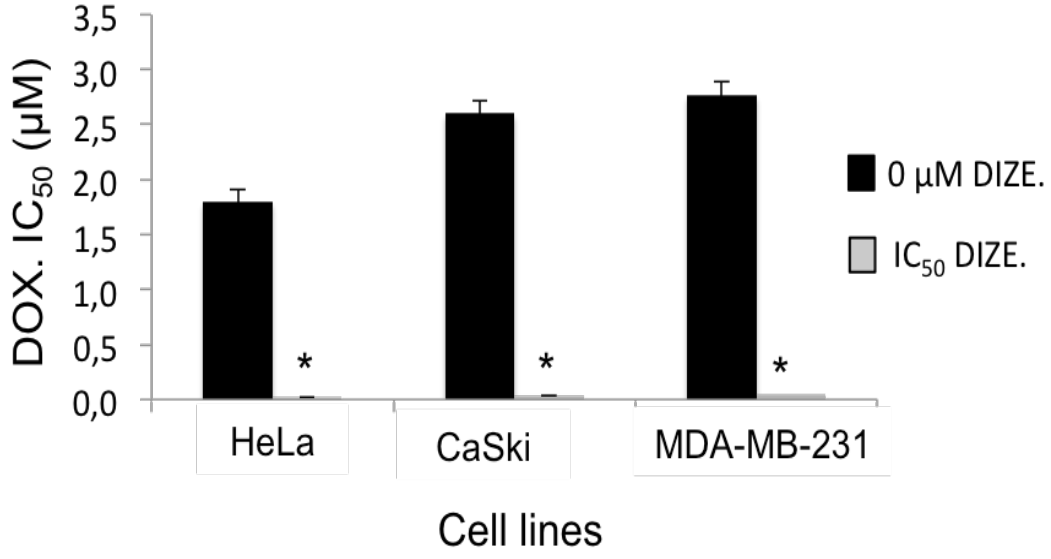


Figure 4.8: Effect of DIZE treatment on DOX IC_{50} concentrations in cancer cell lines HeLa, CaSki and MDA-MB-231 cancer cells were treated with varying concentrations of DOX, with or without the IC_{50} concentration of DIZE, for 48 hours. The IC_{50} concentration of DOX as a single treatment as well as with DIZE was determined using the MTT assay. A significant reduction in the IC_{50} concentration of DOX was observed across all of the cell lines (*$p<0.05$). Results shown are the mean +/- SEM of experiments performed in triplicate and repeated three independent times.

Table 4.1. IC_{50} concentrations determined for DOX and the combined treatment of DOX and the ACE-2 activator, DIZE

Cell Line	DOX. (μM)	DOX. + DIZE. (nM)
HeLa	1.8 (+- 0,115)	24 (+- 0,003)
CaSki	2.6 (+- 0,066)	39 (+- 0,001)
MDA-MB-231	2,7 (+- 0,120)	44 (+- 0,001)

A strong reduction in DOX IC_{50} concentration was observed in HeLa and CaSki, cervical cancer cells and MDA-MB-231, breast cancer cells. Results shown are the mean +/- SEM of experiments performed in triplicate and repeated three independent times.

To quantitatively analyse the dose-effect relationships which may exist when cancer cells are treated with DOX and DIZE in both the 1DOX:1DIZE (Table A.1, Appendix II) and 1DOX:3DIZE (Table A.2, Appendix II) treatment ratios, the Chou-Talalay constant ratio method and Compusyn software were used. The Log combination index (LogCI) values of the fractions of cells affected by DOX and DIZE treatment suggest whether potential associations exist between the two drugs where (LogCI >0 is indicative of antagonism, LogCI <0 of synergism, and LogCI= 0 an additive association between the drugs in question). Results show that 75% of HeLa cells, 80% of MDA-MB-231 cells and 97% of CaSki cells were affected through a synergistic association between DOX and DIZE when treated in a 1DOX:1DIZE ratio of the respective IC_{50} concentrations (Figure 4.9A).

When cells were treated with a 1DOX:3DIZE treatment results showed a more even spread of data with a similar trend moving toward synergism. Results showed that 65% of MDA-MB-231 cells, 75% of CaSki cells and 90% of HeLa cells were sensitive to the combined treatment due to a synergistic association between the drugs (Figure 4.9 B). This suggests that HeLa cells are more sensitive to a higher dose of both DOX and DIZE whereas slightly lower concentrations of DIZE are required to have a more substantial overall effect on CaSki and MDA-MB-231 cells.

While DOX is a potent anticancer drug on its own, the combined use with the ACE-2 activator DIZE provides a greater cell killing effect. Preliminary results presented here show that further investigations into the overall effects on cancer cells, such as CaSki or MDA-MB-231 cells, require lower concentrations of DIZE to be used in co-treatments with DOX.

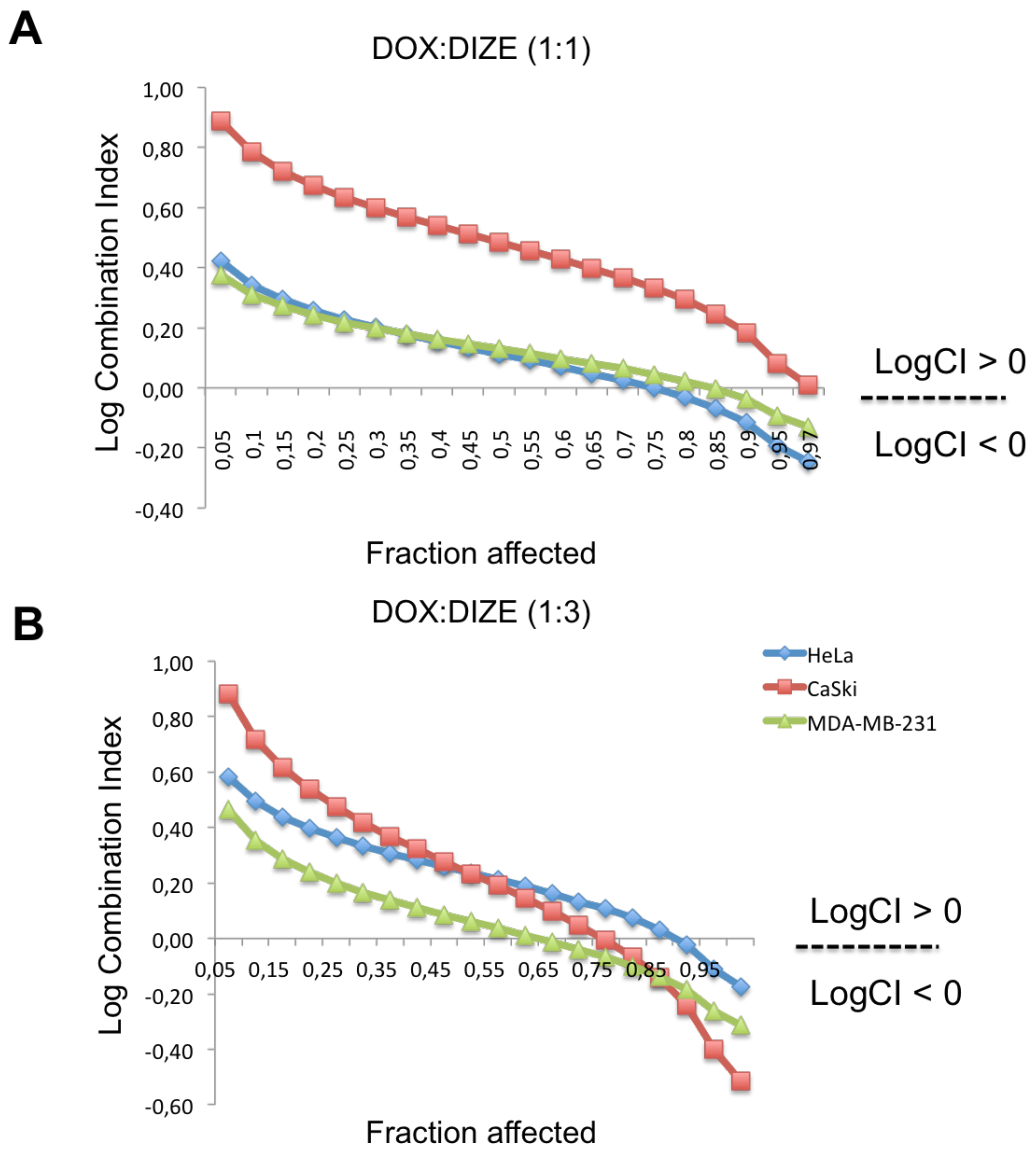


Figure 4.9: Combination index for combined treatments of DOX and DIZE in cancer cell lines. HeLa, CaSki and MDA-MB-231 cells were treated with a constant ratio of 1DOX:1DIZE or 1DOX:3DIZE ratio for 48 hours. Thereafter combination indices were calculated using Compusyn analysis. Results shown are the mean +/- SEM of experiments performed in triplicate and repeated two independent times.

4.2.3.3 Effect of combined DOX and DIZE treatment on cancer cell morphology and cell cycle progression

Whist monitoring the DOX with DIZE co-treatments using phase microscopy, we observed changes in HeLa and CaSki cell morphology. Cells treated with the DOX single treatment were adherent and appeared to be flatter and larger in size compared to control cells (Figure 4.10 A). Many of the DIZE treated cells were still adherent, and those which were, appeared rounded and smaller in size compared to control cells. In addition, many floating clumps of cells could be seen suggestive of cell death. Observations of cells treated with DOX and DIZE combined treatment showed very few adherent cells and clumps of rounded cells and floating cells were observed (Figure 4.10 A). Similar results could be seen for CaSki cells (Figure 4.10 B). Cells, which lose their ability to be adherent are often cells undergoing cell death processes and while those that have a more rounded or flattened morphology suggests changes in cell cycle progression.

Possible explanations for rounded cells are that cells are entering G2/M arrest or becoming apoptotic, while a flattened cytoplasm with a large nucleus usually appearing like a “fried egg” could be indicative of senescence or a G1/S arrest. These observations of change in morphology support our earlier evidence that DIZE treatment results in a G2/M block in cancer cells as well as causes death. Furthermore, it is well known that treatments using low concentrations of DOX result in the induction of senescence in cells (Altieri et al., 2012; Chang et al., 1999; Eom et al., 2005; Ewald et al., 2010; Roninson, 2003).

These observations suggest that both HeLa and CaSki cells exposed to combined treatment of IC_{50} concentrations of DOX and DIZE become increasingly sensitive to DOX treatment as fewer cells were adherent and showed signs of healthy morphology. Increased cell death was observed with both cell lines.

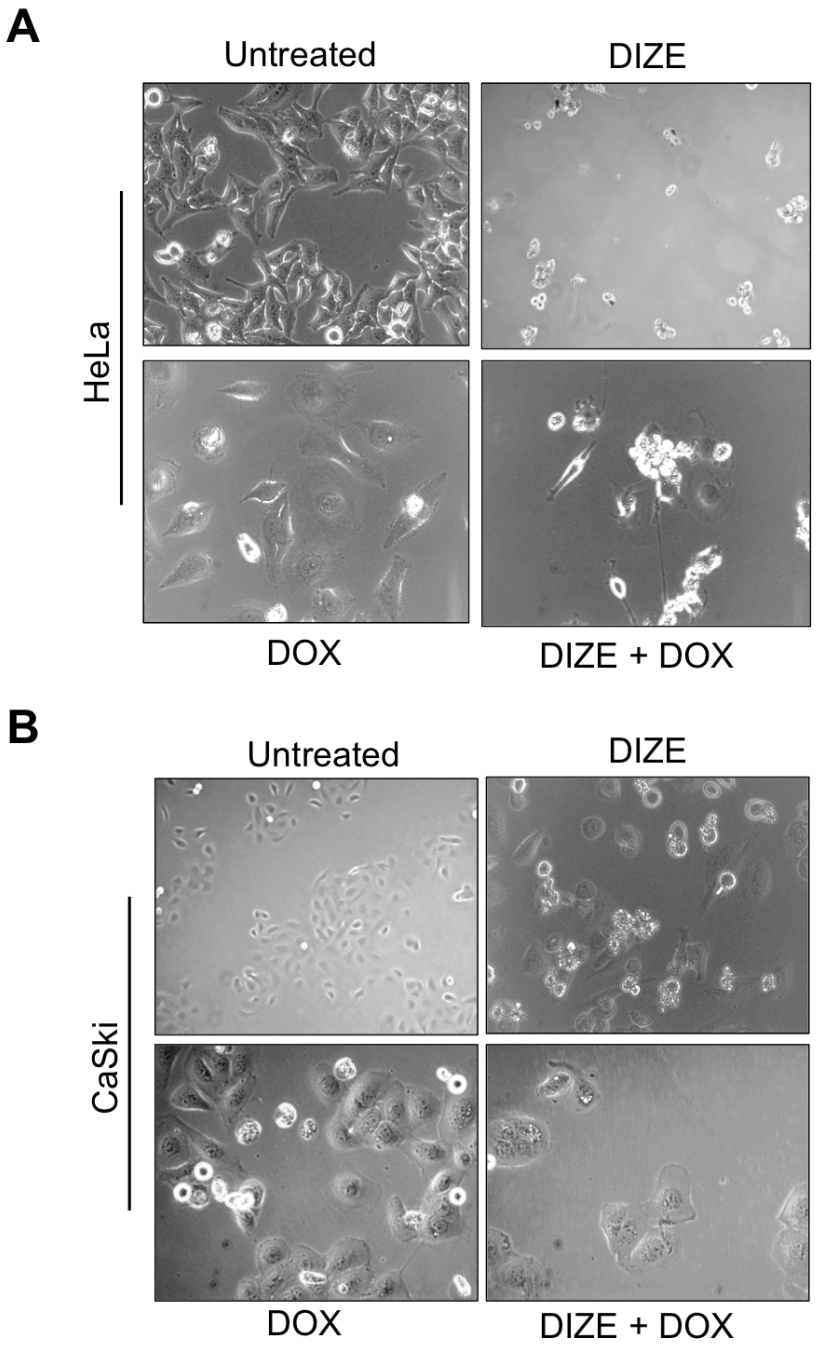


Figure 4.10: Morphology of cancer cells is affected by combined treatment of DOX and DIZE. (A.) Combined DOX with DIZE treatment in HeLa and **(B)** CaSki cells affects cell morphology. An increase in rounded and apoptotic cells as well as senescent appearing cells were observed after 48 hour co-treatment. Results shown are representative of experiments performed in triplicate and repeated two independent times.

Since the combination treatment was observed to significantly inhibit cancer cell proliferation and affect morphology, the effect of the co-treatment on cell cycle progression as well as the ability of HeLa, CaSki and MDA-MB-231 cancer cells to form clonogenic colonies were evaluated next.

For cell cycle analysis, HeLa and CaSki cells were treated with the IC_{50} of DIZE and the reduced dose of DOX, which showed efficacy in combination (Figure 4.11). The DOX dose in HeLa and CaSki cells was 24nM and 39nM, respectively. Cells were co-treated for 48 hours and cell cycle analysis performed on the BD Accuri C6 flow cytometer. DIZE single treated HeLa cells had an increase in the G2/M phase of the cell cycle (Figure 4.11 A), supporting the rounded morphology observed under the microscope. In DOX single treated cells, an increase in the subG1 and G1 phases of the cell cycle were observed compared to control cells. Results using the combination treatment conditions show an increase in the subG1 and G1 phases of the cell cycle.

In CaSki cells a similar trend was seen after the treatments. Compared to control cells DOX treated cells showed an increase in subG1 and G1 phases of the cell cycle while DIZE treated cells showed an increase in G2/M and subG1 cell cycle phases (Figure 4.11 B). Cells treated with the combined treatment of DOX and DIZE showed an increase in subG1 and G1 phases. Taken together these results show that the combined treatment of DOX and DIZE alter cancer cell morphology, which associates with a delay in G1/S transtion.

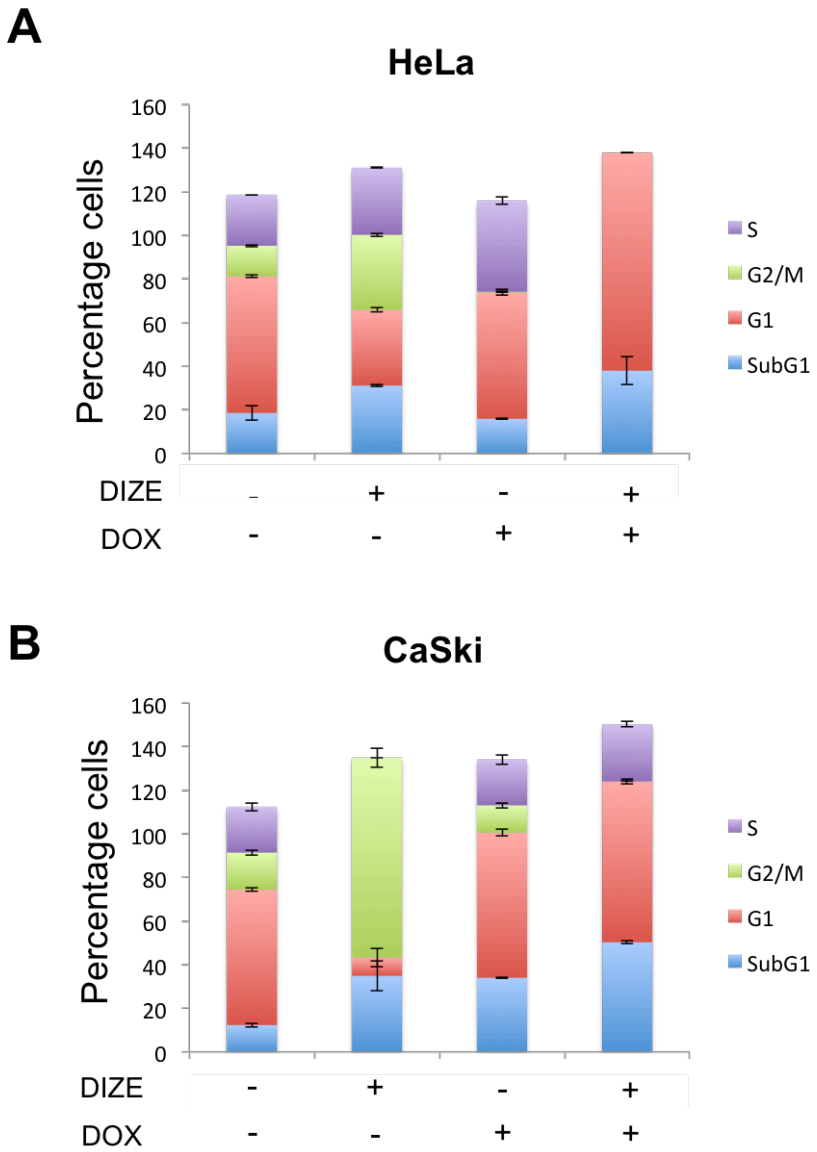


Figure 4.11: Cell cycle analysis of HeLa and CaSki cells after co-treatment of DOX and DIZE. (A.) HeLa cells were treated for 48 hours with IC_{50} of DOX and 24nM DIZE. (B.) CaSki cells were treated with the IC_{50} of DOX and 39 nM DIZE. Cell cycle analysis was performed the following day. Co-treatment of DOX and DIZE results in an increase in the subG1 and G1 populations of cells in both HeLa and CaSki cells. Results shown are the mean +/- SEM of experiments performed in triplicate and repeated two independent times.

4.2.3.4 Effect of combined DOX and DIZE treatment on cancer cell clonogenic ability

To determine the clonogenic ability of cancer cells after co-treatment the clonogenic colony forming assay was performed. Cells treated with the IC_{50} concentration of DIZE were unable to form colonies. Hence a lower concentration (½ the IC_{50} concentration) of DIZE was used in this experiment. Cells were treated with either ½ the IC_{50} concentration of DIZE, the IC_{50} concentration of DOX, or a combination of both, and clonogenic colony formation monitored for 10 days. Results showed that in all of the cell lines DOX and DIZE co-treatment reduced the number of clonogenic colonies able to survive by nearly 100-fold, compared to either treatment alone for HeLa (Figure 4.12), CaSki (Figure 4.13) and MDA-MB-231 cells (Figure 4.14).

DOX and DIZE in combination (1/2 IC_{50} DIZE with DOX) was effective in reducing the number of clonogenic HeLa (Figure 4.12 A and B), CaSki (Figure 4.13 A and B) or MDA-MB-231 (Figure 4.14 A and B) colonies able to survive after 10 days, even more so than the single treatment of either drug. This data shows that DIZE enhances DOX cytotoxicity reducing the ability of cancer cells to form clonogenic colonies.

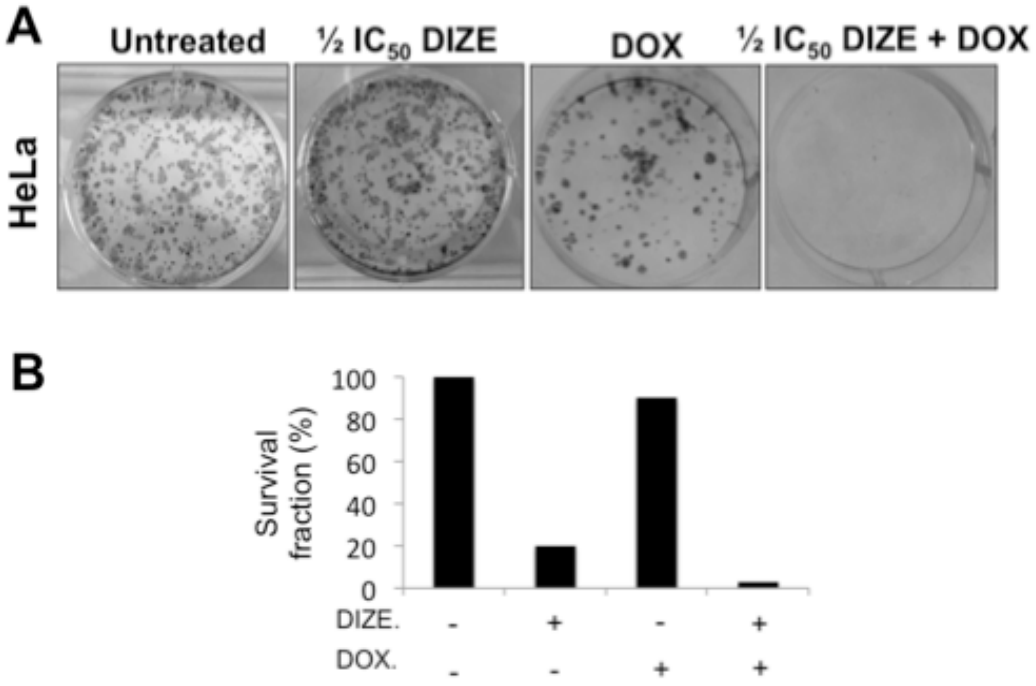


Figure 4.12: DOX and DIZE co-treatment reduces the clonogenic ability of HeLa cells. (A.) HeLa cells were treated once off with ½ IC_{50} DIZE and clonogenic ability monitored for 10 days. Thereafter colonies were stained with crystal violet and colonies quantified with ImageJ **(B.)**. The percentage survival fraction is indicated in the bar graphs for the treatment conditions. Results shown are representative of experiments performed in triplicate and repeated two independent times.

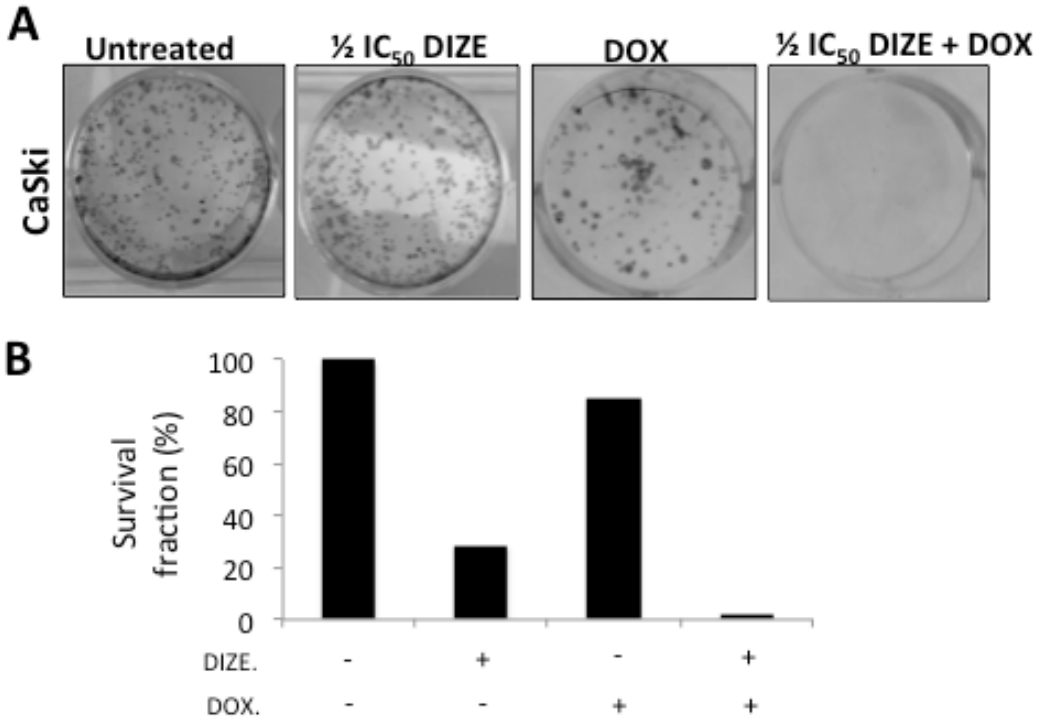


Figure 4.13: DOX and DIZE co-treatment reduces the clonogenic ability of CaSki cells. (A.) CaSki cells were treated once off with the IC_{50} DOX and ½ IC_{50} DIZE and clonogenic ability monitored for 10 days. Thereafter colonies were stained with crystal violet and colonies quantified with ImageJ **(B.)** The percentage survival fraction is indicated in the bar graphs for the treatment conditions. Results shown are representative of experiments performed in triplicate and repeated two independent times.

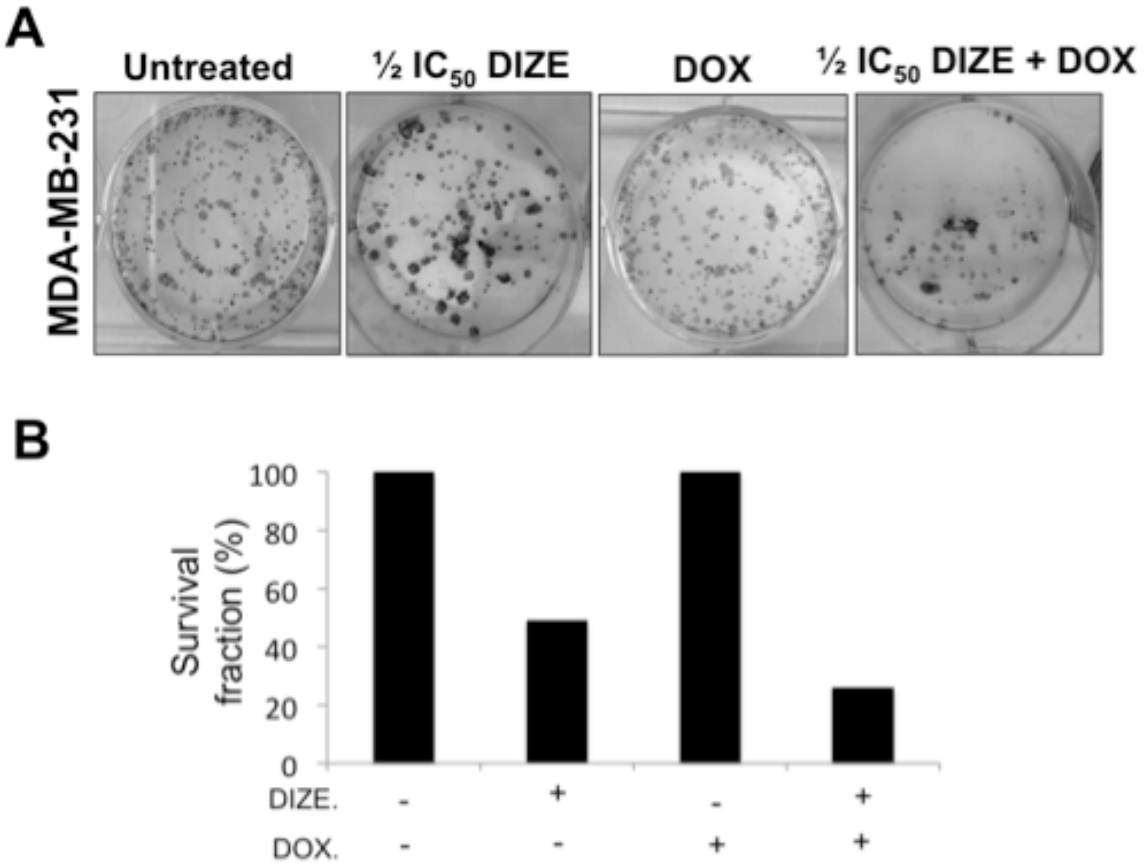


Figure 4.14: DOX and DIZE co-treatment reduces the clonogenic ability of MDA-MB-231 cells. (A.) MDA-MB-231 cells were treated once off with the IC_{50} of DOX and ½ IC_{50} of DIZE and clonogenic ability monitored for 10 days. Thereafter colonies were stained with crystal violet and colonies quantified with ImageJ (B.). Percentage survival fraction is indicated in the bar graphs for the treatment conditions. Results shown are representative of experiments performed in triplicate and repeated two independent times.

4.2.3.5 DOX and DIZE combined treatment results in increased cell death via apoptosis and necrosis

To investigate the effect of the combined treatment of DOX and DIZE on cancer cell death, the Annexin V assay was then used. HeLa (Figure 4.15 A), CaSki (Figure 4.15 B) and MDA-MB-231 cells (Figure 4.15 C) were treated with either the IC_{50} concentration of DIZE, the lower, nanomolar concentration of DOX (being 24 nM, 39 nM and 44nM for HeLa, CaSki and MDA-MB-231 cells, respectively), or the combination of DIZE and DOX. Annexin V results of DIZE treated HeLa cells show that the percentage live cells decreased from 88.8% in control cells to 61.5%, and to 57.8 % in combined DOX and DIZE treated cells (Figure 4.15 A). Since the DOX concentration was now lower, the percentage live cells in the DOX treated sample, was now comparable to that in the control. Quantifying the percentage cells in each quadrant of the Annexin V profile results show after DIZE treatment there was a significant decrease in percentage live cells, with a significant increase in the percentage necrotic cells. No significant change in cells was seen using a DOX single treatment at the lowered concentration. The Annexin V profiles of HeLa treated cells show a significant decrease in the percentage live cells was observed in combination treatment of DOX and DIZE accompanied by a significant increase in late apoptotic and necrotic cells (Figure 4.15 A, Figure A.8, Appendix III).

The Annexin V profiles of CaSki cells showed that DIZE treatment reduced the percentage of live cells and a greater scatter of cells in the quadrants representative of apoptotic and necrotic processes were observed (Figure 4.15 B, Figure A.9, Appendix III). Annexin V profiles of DOX and DIZE treated cells showed fewer cells in the lower left quadrant

representative of live cells with a significant increase in the percentage of apoptotic and necrotic cells (Figure A.9, Appendix III).

A similar observation was found in MDA-MB-231 cells where DIZE treatment significantly reduced the percentage live cells compared to the untreated (Figure 4.15 C). The Annexin V profiles of DOX and DIZE treated cells showed fewer cells in the lower left quadrant representative of live cells with a significant increase in the percentage of late apoptotic and necrotic cells (Figure A.10, Appendix III).

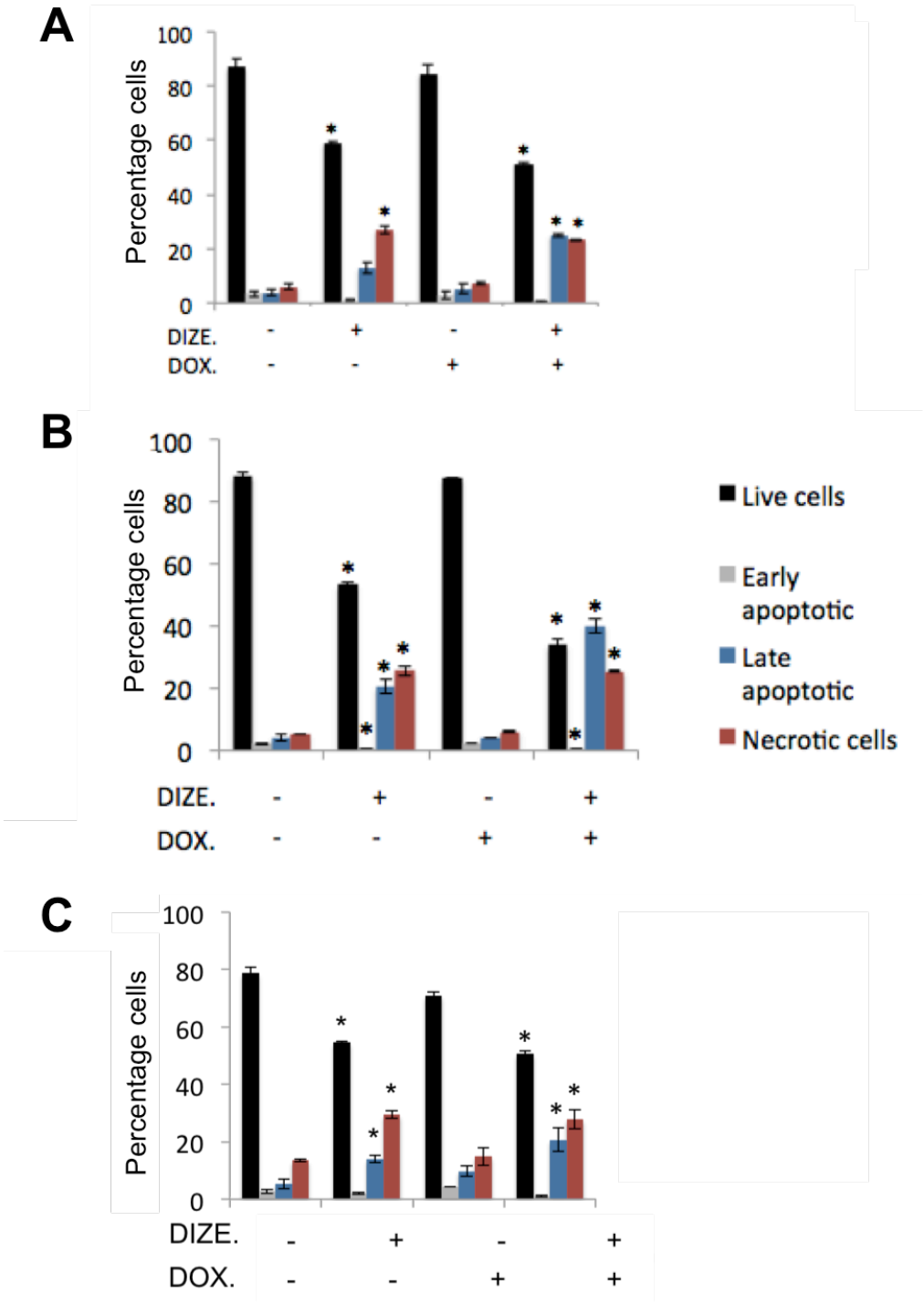


Figure 4.15: The effect of DOX and DIZE combined treatment on cancer cell death using the Annexin V assay. HeLa **(A.)**, CaSki **(B.)** and MDA-MB-231 **(C.)** cells were treated with the IC_{50} concentrations of DOX and DIZE for 48 hours and effects on cell death investigated using the Annexin V assay. Quantitation of the respective Annexin V profiles shows a significant reduction in the percentage of live cells and a significant increase in late apoptotic and necrotic cells compared to untreated cells (*$p<0.05$). Results shown are the mean +/- SEM of experiments performed in triplicate and repeated twice.

To further investigate the effect of DIZE and DOX combined treatment on HeLa cell death, western blot analysis was performed to determine PARP cleavage as an indicator of cell death via apoptosis. Results show an induction of PARP cleavage after the combination treatment, which is more pronounced when compared to either DIZE or DOX single treatments (Figure 4.16). This confirms the elevated level of apoptosis after DIZE and DOX combined treatment.

Taken together, results from this chapter suggest that ACE-1 inhibitors reduce the sensitivity of cancer cells to CDDP while having no significant effect on DOX potency. However, the ACE-2 activator, DIZE significantly increases the sensitivity of cancer cells to DOX treatment altering cancer cell morphology and inducing cell death via apoptosis and necrosis. The DOX and DIZE combination is associated with an increase in cells delayed in the G1 phase of the cell cycle and cell death via apoptosis.

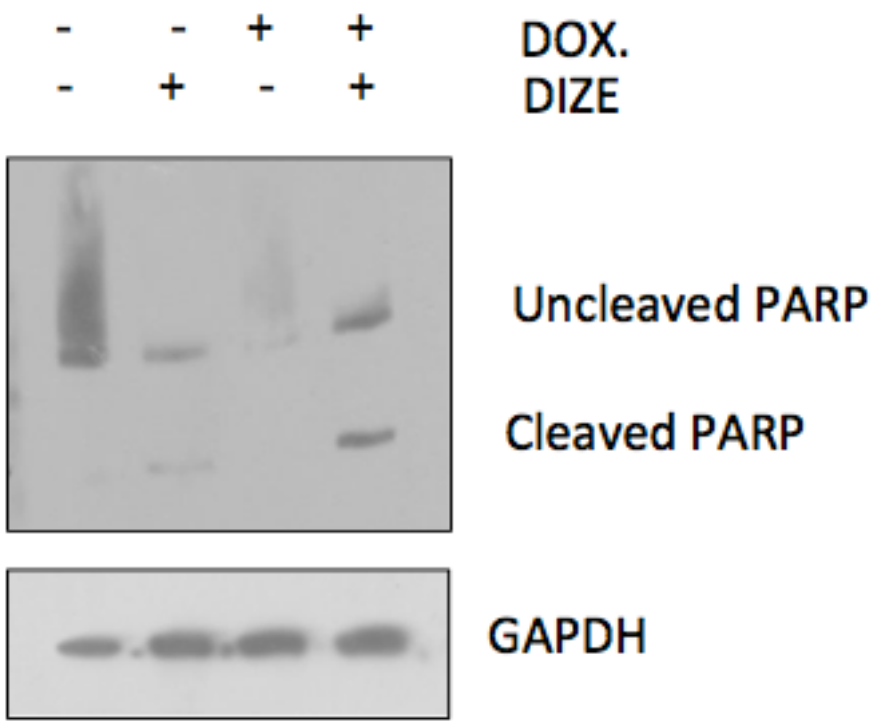


Figure 4.16: Effects of DOX and DIZE treatment on HeLa cell death. Western blot analysis showing increased PARP cleavage after a combined treatment of 24nM DOX with the IC_{50} concentration of DIZE in HeLa cells. Results shown were repeated two independent times.

4.3 DISCUSSION

In this chapter we investigated possible effects in the combined use of ACE-1 inhibitors, as well as the ACE-2 axis activator DIZE, and currently used chemotherapeutic agents, CDDP and DOX, on cancer cell viability. Current chemotherapeutic agents, while being highly effective in killing cancer cells, are known for their harsh side effects and it is hopeful that investigations into the possible combined treatments will result in the improvement of patient outcomes. Due to the cytotoxic nature of such drugs they are often administered as a combined treatment (Budman et al., 2002; Esteban et al., 2006; Fernandez and Sessel, 2009). In light of this, it is important to note that not all combination treatments may show a preferred outcome or provide evidence of tolerability in patients, as was observed by Giacconne et al., (2004) where the efficacy of a combined treatment of Gefitinib with Gemcitabine and CDDP was compared to the single treatments in advanced non-small cell lung cancer (NSCLC) patients. For this reason it is important to investigate combination treatments in model systems like cultured cells and animal studies before advancing them to clinical trials.

Since literature has shown that RAS inhibitors (i.e ACE-1 inhibitors) possess chemoprevention and anticancer effects, which our earlier data supported, we investigated whether the anticancer effects of DOX and CDDP could be enhanced through the use of ACE-1 inhibitors, Captopril and Lisinopril, as well as the ACE-2 activator, DIZE. It has been reported that ACE-1 inhibitors may be used with DOX to act as cardioprotective agents reducing the main side effect of DOX, cardiac damage. An *in vivo* study by(Sacco et al., 2009)showed that ACE-1 inhibitors, Captopril, Lisinopril and Zofenopril provide protection

against DOX-induced cardiac damage. Furthermore,(Akolkar et al., 2015)reported from their *in vivo* study that Captopril and Lisinopril prevent cardiotoxicity induced by combined treatments of DOX with Trastuzumab, a monoclonal antibody used to treat breast cancer. Our findings however, indicate that ACE-1 inhibitors do not significantly affect DOX potency in HeLa and MDA-MB-231 cells, as the concentration of DOX required to kill 50% cancer cells remained unchanged. The data does not indicate whether the ACE-1 inhibitors used could provide potential protective effects to a surrounding tumour microenvironment. Future co-culture experiments of cancer with healthy cells could perhaps provide insight on such a matter.

Our study shows that ACE-1 inhibitors negatively affect CDDP potency in an antagonistic manner. CompuSyn analysis identified that a 1:1 ratio of the IC_{50} concentrations of either Captopril or Lisinopril was antagonistic if used in combination with CDDP. Our results, using cells in culture, suggest that a combined use of CDDP with Captopril or Lisinopril may not be beneficial. Literature reports however, support the use of cardioprotective drugs like ACE-1 inhibitors in patients undergoing chemotherapy to prevent chemotherapy-induced cardiotoxicity (Cardinale et al., 2006). While this might be the case for some chemotherapies, further research is warranted to investigate the potential benefits and adverse effects of these combinations as the mechanisms behind ACE-1 inhibitor-induced prevention of cardiotoxicity is unclear.

The cause for the observed antagonism could perhaps be due to the structure of ACE-1 inhibitors and thus conflicting mechanisms of action. In a study conducted by (Lesan et al., 2014) the anti-diabetic drug, Metformin, was shown to antagonize CDDP function. It was

proposed that this could be due to the reduction of oxidative stress caused by Metformin. As Captopril contains a sulfhydryl group, which is a known scavenger of reactive oxygen species (ROS) (Chopra et al., 1989; Napoli et al., 2004), the antioxidant capabilities of Captopril, may similarly to Metformin, be a possible reason why Captopril antagonizes CDDP function in our study. Further investigation into the reasons behind the antagonistic effect Lisinopril has with CDDP need to be conducted.

The data presented here suggest that ACE-1 inhibitors induce cancer cell death on their own and this supports literature findings describing ACE-1 inhibition as potential anticancer drugs (Araujo et al., 2015; Babacan et al., 2015; Kowalski and Herman, 1996; Wang et al., 2008). In combination however, the cell killing effect of the ACE-1 inhibitors together with CDDP was reduced. This could possibly be as a result of the promotion of the c-Jun N terminal kinase (JNK) survival pathway that ACE-1 inhibitors are known to activate in endothelial cells (Kohlstedt et al., 2006; Ryan and Sigmund, 2004). Depending on the cell type as well as the nature and duration of death stimuli, the JNK pathway can either be seen as pro- or anti-apoptotic (Liu and Lin, 2005).

Since the CDDP combination treatment with the ACE-1 inhibitors Captopril or Lisinopril proved to be antagonistic we investigated the effect of the ACE-2 axis activator DIZE on CDDP outcome, based on the natural counteractive role that the ACE-2 axis plays. Our data show that DIZE did not enhance the cytotoxicity of CDDP. In a study using MCF-7 and MDA-MB-231 breast cancer cells, Bielawski et al. (2008) showed that platinum-containing DIZE derivatives, possessed strong DNA-binding activity. A possible reason for the lack of effect

on CDDP outcome on cancer cells in our study could perhaps be that DIZE out competes CDDP in binding DNA.

We did however find that combination treatments of DIZE with DOX allowed the IC_{50} concentration of DOX to be reduced substantially from the micromolar to the nanomolar range. In addition both a 1:1 and 1:3 DOX and DIZE combination treatment showed synergistic associations at the higher spectrum of the combined treatments. There are reports in the literature of DOX combination treatments that show, in addition to cytotoxic effects, synergistic effects, such as the combined treatment of curcumin with DOX (Judson et al., 2014b; Notarbartolo et al., 2005; Park et al., 2014) showed that the addition of the Hsp90 inhibitor, Gamitrinib, to DOX therapy in breast and prostate xenograft models, sensitized tumour growth in a synergistic manner.

The chemical structure of DIZE contains phenylhydrazine, a potent DNA damaging agent (Ferrali et al., 1997; Yamamoto and Kawanishi, 1992). A possible reason for the synergistic associations between the ACE-2 activator, DIZE and DOX may simply be due to the enhanced DNA damage capabilities of the combined treatment. As the mechanism of action of DOX is topoisomerase II inhibition (Eom et al., 2005; Lenglet and David-Cordonnier, 2010; Patel and Kaufmann, 2012), the added stress induced by DIZE could be a factor leading to the potential drug synergism observed in this study. The observation of a partly antagonistic association between DOX and DIZE correlating with lower concentrations of the combined treatment may be of value as Fernandez et al., (2009) provide evidence of their hypothesis that antagonism between drug A, (referred to by the authors as the “editor”) and drug B

(the primary drug), could play an effective therapeutic role if drug A suppresses the downstream signaling responsible for side effects induced by drug B.

We also show morphological evidence and results from the Annexin V assay which suggest that DOX and DIZE co-treatment results in an increase in apoptotic, necrotic and senescent cells which is supported by data showing an increase in cells in the G1 and subG1 population of the cell cycle after treatment. This suggests that DOX and DIZE co-treatment induces apoptosis, necrosis and possibly senescence or quiescence. The β–galactosidase assay together with western blot evidence of senescence protein markers such as the cyclin-dependent kinase inhibitors, p16 and p21, could provide further support for our findings. P16 and p21 regulate cell cycle at the G1 to S phase transition and are up-regulated during cellular senescence. Alternatively the combined treatment of DOX and DIZE could be increasing the subG1 population of cells by mediating quiescence. (Yumoto et al., 2014) suggest cells are pushed to dormancy through high levels of high levels of TGFβ1 as well as disruptions in the ERK MAPK and p38 MAPK pathways. Future investigations into the effect of DOX and DIZE treatment on markers such as these could provide further insight into this drug combination.

While current chemotherapies are effective at killing cancer cells they are toxic to healthy cells in patients. Our results have shown that not all combination treatments with currently available chemotherapeutic agents will be synergistic and have an improved end result. The association analysis of the drugs from combined DOX and DIZE treatment suggests that small molecules with the potential to activate the ACE-2 enzyme may have further positive

effects on other chemotherapeutic agents and such combinations may be promising in the future.

CHAPTER 5

CONCLUSIONS

While there are studies indicating the existence of the RAS not only systemically but also within several organs at tissue level, there is contention over the impact that the RAS and/or inhibitors thereof impact cancer. Recent evidence suggests that RAS components influence carcinogenesis with examples including AT1R expression being differentially up-regulated in cancers such as breast, ovarian and colorectal (Ager et al., 2011; Chen et al., 2013; Ino et al., 2006). Ang-II, the ligand for AT1R, has also been shown to be associated with an increase in proliferation of various cancer cell types including ovarian and breast; the activation of invasiveness of cervical cancer cells, as well as angiogenesis and metastasis of other gynecologic cancers such as endometrial, ovarian and gestational choriocarcinoma (Chen et al., 2013; Du et al., 2012; Ino et al., 2011; Kikkawa et al., 2004; Puddefoot et al., 2006; Suganuma et al., 2005; Uemura et al., 2003).

This study provides evidence showing high ACE-1 as well as AT1R expression in cancer cell lines. Ang-II treatment resulted in a significant increase of cervical cancer cell proliferation. Our data also shows that ACE-1 protein expression associates with increased ACE-1 enzyme activity in breast and cervical cancer cell lines. These findings are in agreement with previous studies reporting differential expression of RAS components, e.g. AT1R, observed in brain, lung, pancreatic, prostate, ovarian and breast cancer when compared to normal tissue counterparts (Ager et al., 2008; Deshayes and Nahmias, 2005; Suganuma et al., 2005).

We hypothesized that increased RAS components may have significance to cancer cell biology. To explore this, the effects of RAS inhibitors, specifically ACE-1 axis inhibitors, as well as activators of ACE-2 function were investigated. Our initial experiments focused on the ACE-1 axis of the RAS and we observed that exposure of cancer cells to Captopril, Lisinopril or Candesartan treatment inhibited cancer cell proliferation. Using the Annexin V assay it was determined that the mode of cell death was via apoptosis. Further investigations into the effects of ACE-1 inhibition in cancer are required as existing data is conflicting. As our results were obtained from *in vitro* studies, they may be considered preliminary, however, do support existing bodies of work such as that of (Miao et al., 2016; Wilop et al., 2009), which showed that ACE-1 inhibition together with chemotherapy, resulted in a positive impact on progression-free survival, regardless of whether the non-small cell lung cancer patients were in early or advanced stages of disease progression.

Our data also shows that Ang-II treatment of cancer cells results in the release of calcium from intracellular stores and the activation of calcium signaling pathways via phosphorylation of CAMKII and the transcription factor, NFAT. Inhibiting the Ang-II receptor, AT1R, blocked these effects. Typically in healthy cells, growth factors set off spikes of calcium release to act as signals carefully regulating cell cycle progression (Berridge, 1995; Berridge et al., 2000; Santella, 1998). Calcium release signals at the various phases activate cell cycle events including: early genes responsible for inducing resting cells to re-enter the cell cycle, DNA synthesis at the G_1/S transition phase and mitotic events. It has been reported that in cancer cells, the increasing rate of continuous calcium signals associates with increased cell proliferation(Berridge, 1995; Berridge et al., 2000; Capiod et al., 2007;

Roderick and Cook, 2008). In our study we showed that inhibition of AT1R function, using Candesartan, significantly reduced these effects.

This study also reports that activation of the ACE-2 axis, through natural or synthetic small molecules, reduced cancer cell proliferation, with DIZE in particular inducing a G2/M arrest in cancer cells, suggesting a mechanism of action different to that of Ang-(1-7). It has been documented that in the cardiovascular context, the ACE-2 axis serves to counter ACE-1 axis effects and restore balance where AT1R activation has led to deleterious consequences (Ferrario, 1990a; Ferrario, 1990c). Similar observations of hyper-activated ACE-1 axis have also been reported in other diseases including atherosclerosis, dementia and metabolic syndrome, which could lead to cardiovascular disease, a stroke or diabetes (Engeli et al., 2000; Engeli and Sharma, 2002; Engeli, 2006; Mogi and Horiuchi, 2009; Sata and Fukuda, 2010). This leads us to believe that the natural, or synthetic activation of the ACE-2 axis using treatments with Ang-(1-7), or the small molecule DIZE, in cervical and breast cancer serves to protect against harmful ACE-1 axis effects. We found that both treatments significantly reduced the clonogenic ability of single cancer cells, with DIZE effective at half the IC_{50} concentration and through the Annexin V assay, we found that both Ang-(1-7) and DIZE induced cell death via apoptosis or necrosis.

The use of RAS inhibitors and ACE-2 axis activation in combination drug treatment to fight cancer is another promising route, which could be explored in xenograft models. Further studies using xenograft mouse models as opposed to transgenic mice, could offer a more feasible solution as research could be implemented sooner and at lower cost. Our *in vitro* study found that combination treatments using ACE-1 axis inhibitors and CDDP were

antagonistic to the effects of CDDP only. On the other hand we found that combination treatments of DOX and the ACE-2 activator, DIZE, were synergistic allowing for a significant lowering of the DOX concentration associated with cancer killing effects. Whether this will correlate with a reduction in side effects associated with DOX treatments will require further investigation using *in vivo* models.

While there are no reports of direct combination treatments using Captopril or Lisinopril in conjunction with CDDP to treat cancers, there is evidence for the impact of ACE-1 inhibitors and AT1R blockers (note the names of these drugs were not included in the study) on the survival of non-small-cell lung cancer patients receiving platinum-based chemotherapy. Patients who had received long term (minimum of three months) treatments of antihypertensive agents showed improved survival than patients who had not received antihypertensive treatment before chemotherapy (Wilop et al., 2009). Our method of combination treatment using Captopril or Lisinopril with CDDP did not involve a pre-treatment, so an improved outcome may possibly result if this method were to be used in future investigations. Combination treatments using CDDP have had mixed outcomes showing both antagonistic as well as synergistic effects. The combination treatment of the EGFR antagonist, Gefitinib, together with CDDP has been reported to have an antagonistic response in a number of non-small-cell lung cancer cell lines (Tsai et al., 2011). In cervical cancer however, CDDP combination treatments with naturally derived compounds such as cinnamon essential oil, myricetin and methyl eugenol, showed synergistic effects in HeLa cells inhibiting proliferation via apoptosis (Larasati et al., 2014; Yi et al., 2015). The synergistic effects induced through these combination treatments could possibly be due to the antioxidant properties associated with the natural compounds and knowledge of these

possible associations could empower the growing body of evidence supporting the use of combined treatments.

Similarly to CDDP combined treatments, those involving DOX and antihypertensive agents suggest that pre-treatment of ACE-1 inhibitors offer protection against the side effects of DOX (Hiona et al., 2011; Sacco et al., 2009). Our study shows that ACE-2 activation with DIZE reduces the effective concentration of DOX needed to kill 50% of the cells treated. Such a dose reduction could be further investigated to determine whether protective effects are indeed possible with the use of DIZE. There are numerous reports stating the synergistic effects of combined treatments using DOX (Alberts et al., 1981; Ardizzoni et al., 1991; Judson et al., 2014b; Lee et al., 2014; Morris et al., 1992; Park et al., 2014; Wu et al., 2013). Our data is novel in that, to our knowledge, this is the first study to report synergistic effects as a combined treatment consisting of an ACE-2 axis activator and DOX. Such combinations using small molecules capable of activating the ACE-2 axis in cancer warrant further investigations. Our study provides insight into future possible therapeutic strategies to treat cervical cancer. Moreover, our results suggest further investigation into the treatment regimens for cervical cancer patients receiving ACE-1 inhibitors as our preliminary data shows that antihypertensive treatment reduces the effect of the potent chemotherapeutic drug, CDDP.

Future work could investigate the role of the RAS in the cervical cancer context within animal models to get a clearer understanding of the functioning of RAS actions using a model, which better mimics the cancer environment. Further study of this complex system is required to better understand the roles played by the various components in the cervical

and breast cancer environment. In addition, possible experiments into alternative combined treatments using ACE-1 inhibitors or ACE-2 activators should be performed, as well as investigations into the transcriptional regulation of poorly expressed ACE-2 protein in cancer cells. While we were not without limitations in our study our results provide a more holistic view of the role played by integral RAS components in cervical and breast cancer.

The key findings from our study can be summarized as follows: We observed elevated ACE-1 expression and enzyme activity *in vitro,* with Ang-II promoting cancer cell proliferation, via the AT1R, in a dose dependent manner. AT1R function activates calcium signaling pathways resulting in increased cancer cell proliferation, which ACE-1 inhibition can reduce, through the induction of apoptosis or necrosis. We observed lower ACE-2 expression and activity in cancer cells, which DIZE treatment could rescue. Treatment with DIZE or Ang-(1-7) reduced cancer cell proliferation, with DIZE inducing a G2/M arrest and cell death via apoptosis or necrosis. Both treatments prevented the clonogenic ability of single cancer cells with DIZE being effective at half IC_{50} concentration. We found while ACE-1 inhibitors induced cell death on their own, they reduced the sensitivity of cancer cells to chemotherapeutic drugs, DOX and CDDP, with associations between ACE-1 inhibitors and CDDP showing antagonistic effects. We show that DIZE does not affect the potency of CDDP whereas combined treatment with DOX, results in the reduced ability of cells to produce clonogenic colonies as well as an enhanced cell killing effect. The data suggests that inhibitors of the ACE-1 axis, or activators of ACE-2 function, could serve as potential anti-cancer agents with improved effects if used in conjunction with currently used chemotherapies such as DOX.

CHAPTER 6

MATERIALS AND METHODS

6.1 MATERIALS

6.1.1 Cell lines

The following cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA):

- cervical cancer cell lines C33A (HPV-negative), CaSki, HeLa and SiHa (all HPV-positive), and breast cancer cell line, MDA-MB-231
- Non-cancer, lung fibroblast cell line, WI38 and the SV40 transformed lung fibroblasts (SVWI38)

FG0 skin fibroblasts were a gift from Dr. A.D Marais (Groote Schuur Hospital, Cape Town). HepG2, liver carcinoma cells were obtained from the UCT Heart Foundation

6.1.2 Cell culture media

Most cell lines were cultured under adherent conditions in complete medium consisting of Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% heat inactivated fetal calf serum (FCS) (Gibco, Life Technologies, Carlsbad, CA, USA) with an antibiotic cocktail of 100 U/ml Penicillin and 100 µg/ml Streptomycin. Alternatively, HepG2 cells were cultured adherently in antibiotic free, DMEM and 10% (v/v) non-heat-inactivated FCS. All cell lines were maintained in a humidified incubator at 37°C and 5% CO_2 and routinely passaged every 2-3 days. Cells were grown to 60-80% confluency before being trypsinized with 0.05% trypsin and EDTA.

When freezing stocks of cells, cells were resuspended in cell freezing media (100U/ml penicillin and 100 µg/ml Streptomycin, 20% FCS and 10% DMSO) and 1 ml was aliquoted per cryotube. Cells were placed at -80 °C for up to a week before being placed in liquid nitrogen for long term storage. Sensitive cell lines such as WI38 and HepG2 were thawed into media containing 20% FCS for the first 24 hours while less sensitive cell lines were thawed into their normal growth media.

6.1.3 Transfections with siRNA

Transient knockdown of ACE-1 protein expression was achieved using short-interfering RNA (siRNA) technology. ACE-1 siRNA (sc-270350, Santa Cruz Biotechnology, Dallas, TX, USA) mediated knockdown was performed using 20 nM siRNA with a 3:1 ratio of transfection reagent (HiPerfect, Qiagen, MD, USA) to siRNA. The transfection mix was added to the medium and left for 5 minutes after which either control or ACE-1 specific siRNA (Santa Cruz Biotechnology) was added. The mixture was left for 15 minutes and added dropwise to the cells, which thereafter were left in the incubator for 6 hours. This medium was then replaced with normal complete medium (DMEM, 10% FCS with Penicillin and Streptomycin antibiotics). Control siRNA (sc-37007, Santa Cruz Biotechnology) was used to control for the effect of DNA transfection. siRNAs were acquired as lyophilized powders and were suspended in RNAse-free water to give a stock concentration of 10 µM.

6.1.4 Small molecules and inhibitors

Captopril and Lisinopril were a gift from Prof. E.D Sturrock at the Zinc Metalloprotease group, IBMS, UCT and were resuspended in sterile water before being stored at 4°C. Candesartan cilexitil (Sigma, Aldrich, Germany) was resuspended in sterile water and stored at 4°C. The AT2R antagonist, PD123319 (Sigma, Aldrich, Germany) was resuspended in sterile water and stored at 4°C. Ang-II and Ang-(1-7) were purchased from Sigma and stored at -20 °C. Diminazene aceturate (Sigma) was dissolved in water and stored at room temperature. Doxorubicin and Cisplatin were resuspended in sterile water and and stored at -80°C. 12-o-tetradecanoylphorbol-13- acetate (TPA), was purchased from Sigma Aldrich® Co and dissolved in tissue grade DMSO.

6.2 METHODS

6.2.1 Transfections with siRNA

HeLa cells were plated in 60 mm dishes at a concentration of 400 000 cells per dish. The following day the transfection mix was prepared using DMEM (Gibco) media free of antibiotics and fetal calf serum (FCS), HiPerfect lipid reagent ACE-1 siRNA (sc-270350, Santa Cruz Biotechnology, Dallas, TX, USA) mediated knockdown was performed using 20 nM siRNA with a 3:1 ratio of transfection reagent (HiPerfect) to siRNA. The transfection mix was added to the medium and left for 5 minutes after which either control or ACE-1 specific siRNA (Santa Cruz Biotechnology) was added. The mixture was left for 15 minutes and added dropwise to the cells, which thereafter were left in the incubator for 6 hours. This medium was then replaced with normal complete medium (DMEM, 10% FCS with Penicillin

and Streptomycin antibiotics). Control siRNA (sc-37007, Santa Cruz Biotechnology) was used to control for the effect of DNA transfection. siRNAs were acquired as lyophilized powders and were suspended in RNAse-free water to give a stock concentration of 10 µM.

6.2.2 Western blot analysis

Protein lysates from cells were extracted using protein lysis buffer containing RIPA and a fresh mixture of sodium orthovanadate (Na_3VO4) and protease inhibitors. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Thermo Scientific, Rockford, IL, USA). Equal amounts of protein were loaded into SDS Polyacrylamide gel and ran at 150V for approximately 1 hour 30 minutes or until proteins were sufficiently separated according to the molecular size of the protein of interest. The protein molecular weight marker, Spectra-BR (Thermo Fisher Scientific Fermentas, Illinois, USA) for the determination of protein size. 20-30 µg of total protein was loaded onto SDS Polyacrylamide gels prepared according to (Table 6.1) Proteins were then transferred to a Hybond-ECL nitrocellulose membrane (ACE-Amersham, Buckinghamshire, UK) for 1 hour at 100 V. Membranes were blocked for an hour at room temperature in 5% milk and thereafter the appropriate dilution of primary antibody specific for the protein of interest (Table 6.2) was added and incubated overnight at 4°C. β-tubulin or GAPDH were used as the loading controls in these experiments. Chemiluminescence, was used to detect bands and depending on the strength of the signal either Lumiglo or Lumiglo Reserve (KPL 112, Inc., Gaithersburg, MD, USA), were used. When different antibodies were used on the same blot, the membranes were stripped in 1 M Glycine, pH 2.5 for 5 minutes then neutralised with 1/10 volume 1 M Tris-Cl, pH 7.5. The membranes were then washed four times with

TBST after which blocking and re-probing with the primary antibody of interest could proceed.

Table 6.1 Preparation of resolving and stacking gels for thickness and pore sizes (%)

Resolving Gel Solution	**7.5 % (1.5 mm gel)**	**10% (1.5 mm gel)**	**15% (1.5 mm gel)**
Resolving gel buffer	3 ml	3 ml	3 ml
Distilled water	3.75 ml	3 ml	1.5 ml
30% Acrylamide	2.25 ml	3 ml	4.5 ml
10 % Ammonium Persulphate (APS)	180 µl	180 µl	180 µl
TEMED	18 µl	18 µl	18 µl
Stacking Gel Solution	**1.5 mm**	**1 mm**	
Stacking gel buffer	1.5 ml	750 µl	
Distilled water	3.5 ml	1.75 ml	
30% Acrylamide	1 ml	500 µl	
10% APS	60 µl	30 µl	
TEMED	6 µl	3 µl	

Table 6.2 Antibody concentrations and incubation conditions

Primary Antibody	Primary Antibody Conditions	Secondary Antibody	Secondary Antibody Conditions	Detection Substrate
Rabbit anti-ACE-1	1:1000 in 5% milk (in TBST)	Goat anti-rabbit [Bio-Rad]	1:5000 in TBST	Lumiglo
Rabbit anti-AT1R (306) [sc-579, Santa Cruz Biotechnology]	1:1000 in 5% milk (in TBST)	Goat anti-rabbit [Bio-Rad]	1:5000 in TBST	Lumiglo
Goat anti-ACE-2	1:1000 in 5% milk (in TBST)	Donkey anti-goat [Pierce]	1:2000 in TBST	Lumiglo
Rabbit anti-β-tubulin (H-235) [sc-9104, Santa Cruz Biotechnology]	1:1000 in TBST	Goat anti-rabbit [Bio-Rad]	1:5000 in TBST	Lumiglo
Mouse anti-GAPDH [sc-47724, Santa Cruz Biotechnology]	1:1000 in TBST	Goat anti-mouse [Bio-Rad]	1:5000 in TBST	Lumiglo
Mouse anti-pCAMKII (22B1) [sc-32289, Santa Cruz Biotechnology]	1:1000 in 5% milk (in TBST)	Goat anti-mouse [Bio-Rad]	1:5000 in TBST	Lumiglo
Rabbit anti-Total CAMKII (M-176) [sc-9035, Santa Cruz Biotechnology]	1:1000 in 5% milk (in TBST)	Goat anti-rabbit [Bio-Rad]	1:5000 in TBST	Lumiglo
Rabbit anti- mcl-1 (H-260) [sc-20679, Santa Cruz Biotechnology]	1:500 in 5% milk (in TBST)	Goat anti-rabbit [Bio-Rad]	1:2500 in TBST	Lumiglo Reserve
Rabbit anti-pHistone H3 (ser10) [sc-8656, Santa Cruz Biotechnology]	1:1000 in 5% milk (in TBST)	Goat anti-rabbit [Bio-Rad]	1:5000 in TBST	Lumiglo
Rabbit anti- PARP [sc-7150, Santa Cruz Biotechnology]	1:1000 in 5% BSA	Goat anti-rabbit [Bio-Rad]	1:5000 in 5% milk (in TBST)	Lumiglo
Mouse anti- p53 [M7001, Dako]	1:500 in TBST	Goat anti-mouse [Pierce]	1:2000 in TBST	Lumiglo Reserve

6.2.3 IC_{50} Determination and Cell Proliferation (MTT) assay

3000 cells in 90 µL complete medium per well were seeded in triplicate in a 96 well plate and allowed to settle overnight in a conditioned incubator. The next day cells were treated with a dose range of Captopril, Lisinopril or Candesartan. In order to factor in background absorbance readings, media blanks were treated with the same dose range of drugs. Cells were incubated for 48 hours with the respective drugs when 10 µL MTT reagent, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) was added to assess metabolically active cells. Four hours later 100 µL SDS containing solubilisation reagent was added and the absorbance read the following morning at 595 nm using a Biotek microplate spectrophotometer (Winooski,VT,USA).

6.2.4 Transfections with ACE-1 siRNA

250 000 cells were plated in 35 mm dishes and allowed to settle overnight in a humidified chamber at 37°C and 5% CO_2. The following day a transfection mixture was prepared in DMEM (Gibco) medium free of fetal calf serum (FCS) and antibiotics. A transfection reagent (HiPerfect, Qiagen, MD, USA) was added to the medium and left for 5 minutes after which either control or ACE-1 specific siRNA (Santa Cruz Biotechnology) was added. The mixture was left for 15 minutes and added dropwise to the cells which were thereafter left in the incubator for 6 hours. This medium was then replaced with normal complete medium (DMEM, 10% FCS with Penicillin and Streptomycin antibiotics). The effect of ACE-1 knockdown was investigated accordingly using western blot analysis.

6.2.5 Combination Treatments

To test the effects of the combined treatments on cancer cells, i.e. whether ACE-1 inhibitors Captopril and Lisinopril, had an effect on the efficacy of CDDP or DOX, or if DIZE could enhance the effect of DOX in cancer cells, we investigated the effects of the combined treatments using the fixed ratio method of (Chou and Talalay, 1984)MTT assay. CDDP and the ACE-1 inhibitors, Captopril and Lisinopril, were treated in a 1:1 ratio of the respective IC_{50} concentrations of each drug appropriate to the cell lines tested. Cells were treated with DOX and DIZE in a fixed 1:1 or 1:3 ratio compared to single treatments (see Tables A2 and A3 in Appendix II). 5000 cells/well in a 96-well plate were left to adhere overnight under incubated conditions. Cells were treated with the drugs of interest for 48 hours giving the total volume per well of 100 µL. The next day MTT reagent was added with the addition of Solubilization solution four hours later. The absorbance was measured the following morning at OD_{595}nm using a Biotek microplate spectrophotometer.

6.2.6 ACE-1 functional assay

To determine the endogenous ACE-1 activity in normal and cancer cells, a modification of the spectrofluorimetric ACE-1 enzyme assay was used where cleavage of the substrate benzyloxycarbonyl-Phe-His-Leu (ZFHL, Sigma, USA) is monitored. After a 48 hour treatment with Captopril or Lisinopril, cell lysates were incubated for two hours (instead of 30 minutes) at 37°C with the ACE-1 specific substrate ZFHL. The reaction was stopped with 0.28M NaOH solution and the sensitive fluorescent agent, o-phthalaldehyde (Sigma, USA) added to derivatise the HL released from the substrate. This reaction was stopped with 3N HCl and after 10 minutes read in a Cary Eclipse fluorescent spectrophotometer (Varian Inc., USA)

with an excitation wavelength of 360nm and an emission wavelength of 485nM. A sample of purified testes ACE-1 was used as the positive control.

6.2.7 ACE-2 functional assay

To determine endogenous ACE-2 enzyme activity in normal and cancer cells the ACE-2 specific substrate, Mca-Y-V-A-D-A-P-K(Dnp)-OH (fluorogenic peptide substrate; R&D Systems Inc, USA) was used. Cells were incubated with the substrate for 1 hour at 37°C with a total reaction volume of 100 uL. The reaction mixture contained final concentrations of 100mM Tris, pH 7, and 300 mM NaCl. Reaction product was measured by the increase in fluorescence detected at excitation and emission wavelengths of 320nm and 430 nm respectively using a Cary Eclipse fluorescence spectrophotometer. Fluorescent readings were normalised to 100 µg protein.

6.2.8 Calcium release assay

Cells were trypsinized, washed, placed in eppendorf tubes at 1 x 10^6 cells/ml, and incubated in the dark with 1.5 uM Fluo-4 AM (Molecular Probes, Eugene, OR, USA) in complete DMEM at 37°C for 20 minutes. Cells were then washed 3 times with Ca^{2+}/Mg^{2+} PBS buffer using centrifugation (1 min at 1700rpm) and resuspended in Ca^{2+}/Mg^{2+} PBS. Propidium iodide was added for 5 minutes to stain DNA content of live cells. Fluorescence data was thereafter analysed on the C6 Accuri flow cytometer. Baseline calcium levels were monitored for 1 minute to ensure stable readings before 400nm Ang-II was added. Thereafter calcium levels were monitored for a further 11 min. Fluorescence was measured before and after treatment with Ang-II. To determine whether the IC_{50} of Candesartan could prevent the

release of intracellular calcium, an overnight pretreatment was applied to cells before the fluorescence data was collected.

6.2.9 NFAT Luciferase assay

To investigate if Ang-II effects within cancer cells are a result of NFAT related-Calcium signalling the NFAT luciferase assay will be used. 30 000 HeLa cells were seeded in a volume of 500 µL per well in a 24 well plate and left to settle overnight in a humidified incubator at 37°C and 5% CO2. The next day cells were transfected with 100 ng GFP-NFAT, NFAT-luciferase and the Renilla plasmid. To normalize for transfection efficiency cells were co-transfected with this serving as a control along with promoter constructs. After 8 hours, medium is replaced with complete medium. The cells were treated with 400 nM Ang-II or 190 µM Candesartan for 24 hours. Control samples were stimulated with 100 nM TPA and 1.3 µM Ionomycin to perforate the cell membrane allowing calcium influx thereby activating NFAT. 5 hours after TPA and Ionomycin treatment the control sample were ready for assaying. All samples were prepared using replacing media with 2 1xPBS washes and 100 µL 1xPassive Lysis Buffer (Promega). Firefly luciferase activity was determined with the Dual Luciferase Kit (Promega) and luminescence measured using the Glomax 96 well microtitre luminometer (Promega).

6.2.10 Cell cycle analysis

For analysis of the effects of the ACE-1 axis inhibitors, Ang-(1-7) and DIZE on the cell cycle, 150 000 cancer cells were plated in 60 mm dishes, treated with the appropriate drug, and then harvested at the appropriate time point. Once floating cells were collected cells were

harvested using trypsin and cells resuspended in 2 ml media and fixed in 8 ml ice-cold 100 % ethanol. Cells in ethanol were then incubated at -20°C for up to two weeks. Prior to sample analysis ethanol was subsequently removed by centrifugation and 1 x 10^6 cells transferred to an eppendorf, where 50 µg/ml RNase was added to remove RNA. 2 µl from a 100 µg/ml solution of Propidium iodide was then added to stain the DNA content of the cells and allowed to incubate for 20 minutes in the dark at room temperature. Cell cycle profiles were analysed using the BD Accuri C6 Flow Cytometer (BD BioSciences, NJ, USA). The percentage of cells at different stages of the cell cycle was performed using Modfit 3.2 (Verity Software House, USA).

6.2.11 Annexin V/Propidium Iodide Assay

The Annexin V/Propidium Iodide detection assay (Thermo Fisher Scientific, USA) was performed to determine the mode of cell death of cancer cells after treatment with either the ACE-1 axis inhibitors, ACE-2 axis activators or combination drug treatments used in this study. Cells were harvested and washed twice with cold sterile 1x PBS before being resuspended in 1ml of 1 x Annexin V solution containing 1x Annexin binding buffer with 1 µl PI and 2 µl FITC Annexin V (supplied in the kit). Samples were gently mixed and incubated at room temperature in the dark for 15 minutes before anaylsis on the BD Accuri C6 flow cytometer. Fluorescence of the stained cells was measured using the FL1 and FL3 channels and the distinction of cell populations undergoing apoptosis or necrosis observed.

6.2.12 Clonogenic Colony Formation Assay

This *in vitro* assay monitors the ability of a single cell to form colonies. Briefly, 1000 HeLa, CaSki or MDA-MB-231 cells were seeded into each well of a 6 well plate. Treatments were plated in triplicate and cells were allowed to settle overnight. The next day cells were treated for 24 hours with ½ IC_{50} or IC_{50} of DIZE, or 1μM Ang-(1-7). Fresh media was then applied to the cells and colony formation was subsequently monitored over a 10 day period. Visible colonies were then stained overnight with 0.5% crystal violet staining solution (see solutions), carefully rinsed with water and allowed to dry before being photographed with a high definition (HD) resolution camera and colonies quantified using ImageJ software. Plating efficiency (PE%) and survival fraction (SF%) was calculated using the following equations: (PE%)= number of colonies/ number of cells plated x 100; (SF%) = PE of treated sample/PE of control x 100. Cell survival was calculated as the number of counted colonies/[number of seeded cells x PE control/100].

6.2.14 Statistical Analyses

Experiments were performed in triplicate and represented as the mean +/ standard error of the mean (SEM) and repeated at least twice. For comparisons, the Student's two-tailed paired t-test was used with a p value of <0.05 considered statistically significant.

6.3 SOLUTIONS

6.3.1 Tissue culture solutions

Cell-freezing media

70 % DMEM

20 % FCS

10 % DMSO

10 X PBS

40 g NaCl

1 g KCl

5.75 g Na2HPO4.7H2O

6.3.2 MTT (5 mg/ml)

100 mg MTT

20 ml 1 X PBS

Vortex and incubate at 37°C for 15 min, filter through a 0.2 µm filter. Wrap in foil and store at 4°C for up to one month.

Solubilisation Reagent

25 g SLS

Make up to 250 ml with dH2O then adding 76.6 µl conc. HCl

6.3.3 Western Blot solutions

Resolving gel buffer

36.2 g Tris, 0.8 g SLS

Dissolve in 150 ml dH2O, pH to 8.9 with 1 N HCL or 1 N NaOH and make up to 200 ml with dH2O. Store at 4°C.

Stacking Gel Buffer

5.9 g Tris, 0.4 g SLS

Dissolve in 70 ml dH2O, pH to 6.8 with 1 N HCL or 1 N NaOH and make up to 100 ml with dH2O. Stored at 4°C.

10x Running buffer

30.2 g Tris, 144 g Glycine, 10 g SDS

Make up to 1 L with dH2O

1x Running buffer

100 ml of 10x running buffer 900 ml of dH2O

10x Transfer Buffer

144 g Glycine 38 g Tris, make up to 1 L with dH2O

1x Transfer Buffer

100 ml 10x transfer buffer (in this order) 700 ml dH2O, 200 ml methanol/isopropanol

10x Tris Buffered Saline (TBS)

60.5 g Tris, 87.6 g NaCl

Dissolve in 700 ml dH2O, pH to 7.5 with 1 N HCL or 1 N NaOH and make up to 1 L with dH2O.

1x TBST

100 ml 10x TBS, 900 ml dH2O and 1 ml Tween 20

4 X Laemmli Loading Dye

250 mM Tris-Cl, pH 6.8 6 % SDS, 0.005 % Bromophenol Blue, 40 % Glycerol and 10 % β-mercaptoethanol

Blocking solution

0.5 g fat free milk powder (Elite)

10ml 1 x TBST

RIPA Buffer

150 mM Sodium Chloride, 1 % Triton X-100, 1 % Sodium Deoxycholate, 0.1 % SDS and 10 mM Tris-Cl, pH 7.4

6.3.4 ACE-1 functional assay solutions

0.28M NaOH

24 mg/ml (in methanol) o-phthaldialdehyde

3N HCl

6.3.5 ACE-2 functional assay solutions

100mM Tris, pH 7

300 mM NaCl

6.3.6 Calcium release solutions

Ca^{2+}/Mg^{2+}/PBS

0.1 mM $CaCl_2$

0.1 mM $MgCl_2$

1x PBS

6.3.7 Clonogenic colony formation solutions

0.5% crystal violet staining solution

0.5g crystal violet powder

80ml dH_20

20ml methanol

Dissolve crystal violet powder in H_20 thereafter add methanol. Store solution at room temperature in the dark and use within 2 months.

APPENDIX I

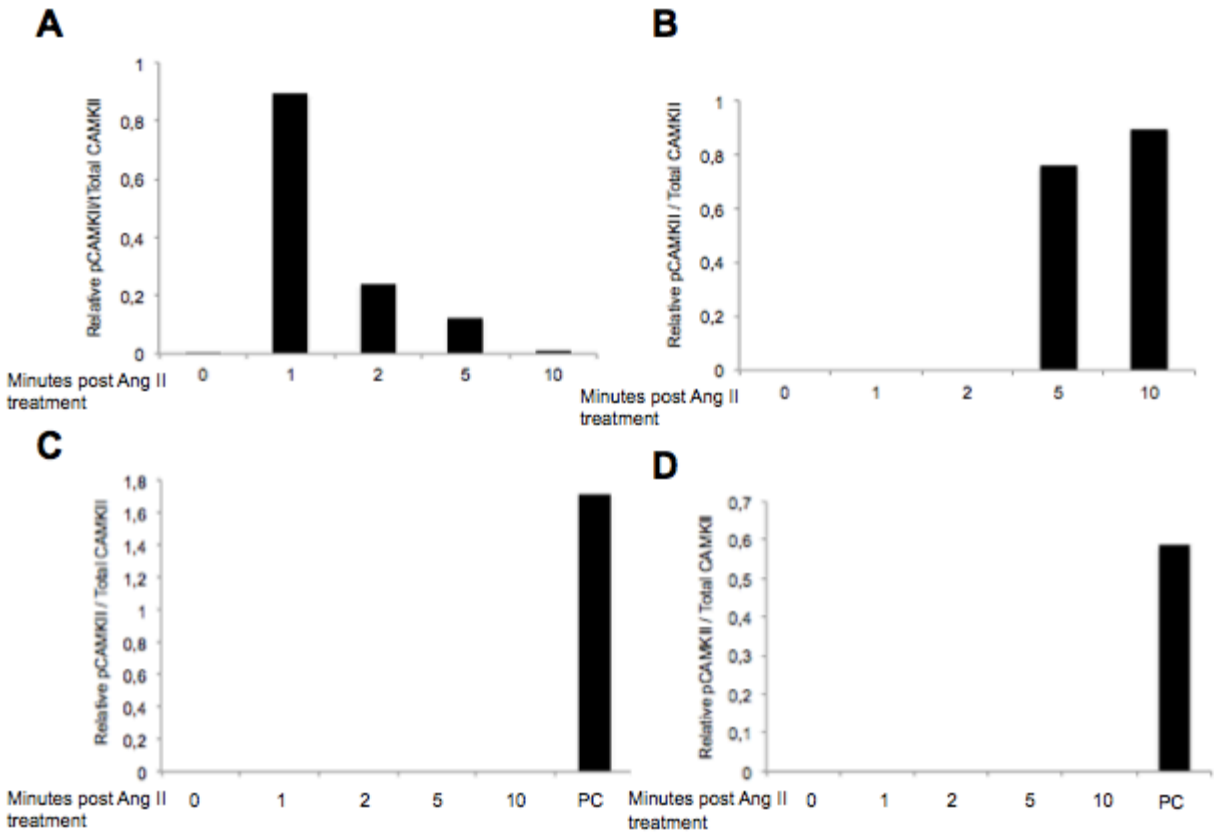


Figure A.1: Densitometric quantification of pCAMKII protein bands relative to total CAMKII in HeLa and CaSki cells. Densitometry of HeLa **(A)** and CaSki **(B)** protein bands from western blots detecting relative pCAMKII expression after minutes of Ang II treatment. Candesartan treatment prevented phosphorylation in HeLa **(C)** and CaSki **(D)** cells. Relative phosphorylation of positive controls for each cell line were shown. Representative experiments of two independent experiments were shown.

Annexin V profiles of the combined treatment of CDDP and Captopril in HeLa, cervical cancer cell line.

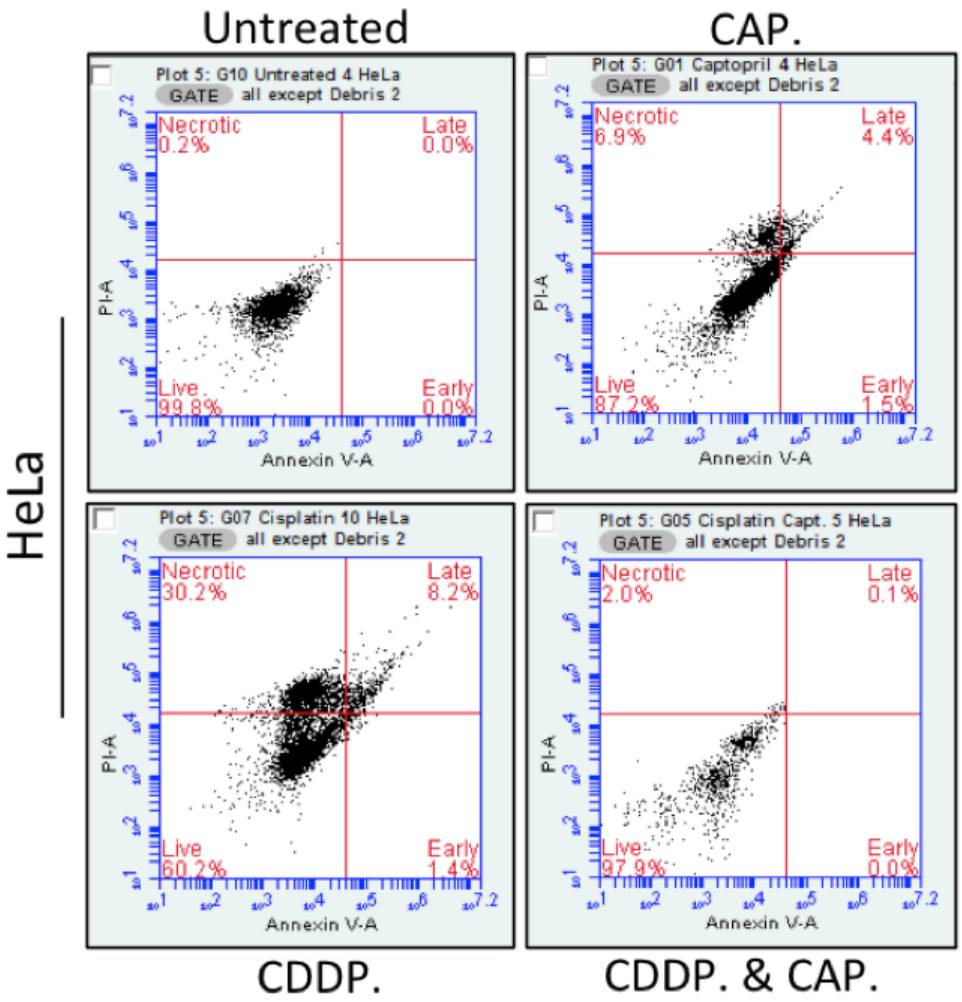


Figure A.2: The effect of CDDP and Captopril combination treatment on HeLa cervical cancer cell death using the Annexin V assay. HeLa cells were treated with the IC_{50} concentrations of CDDP and Captopril for 48 hours and effects on cell death investigated using the Annexin V assay. Profiles of HeLa cells with cells falling into four quadrants as previously described Combined treatment of CDDP with Captopril showed no significant effect on HeLa cell death, however, CDDP and Captopril single treatments showed a significant decrease in the percentage of live cells and a significant increase in the percentage of late apoptotic and necrotic cells (*$p<0.05$). Experiments were performed in triplicate and repeated twice.

Annexin V profiles of the combined treatment of CDDP and Captopril in CaSki, cervical cancer cell line.

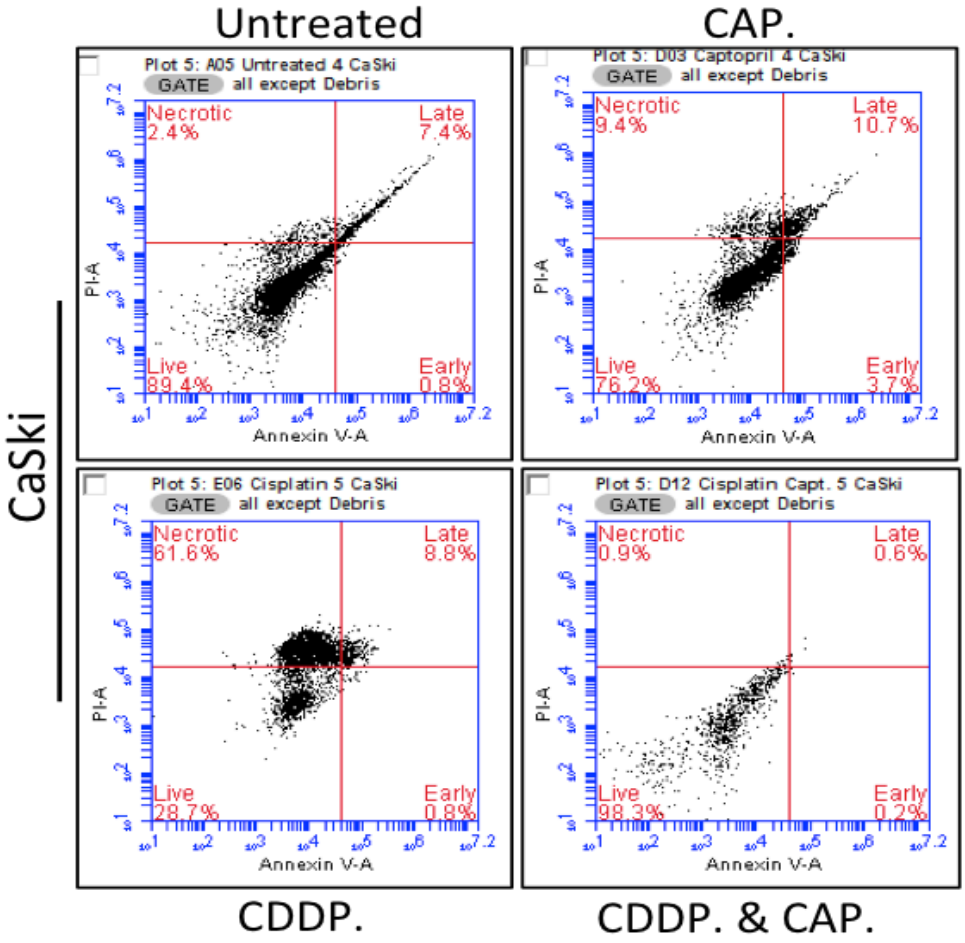


Figure A.3: The effect of CDDP and Captopril combination treatment on CaSki cervical cancer cell death using the Annexin V assay. CaSki cells were treated with the IC_{50} concentrations of CDDP and Captopril for 48 hours and effects on cell death investigated using the Annexin V assay. Profiles of CaSki cells with cells falling into four quadrants as previously described. Combined treatment of CDDP with Captopril showed no significant effect on CaSki cell death, however, CDDP and Captopril single treatments showed a significant decrease in the percentage of live cells and a significant increase in the percentage of late apoptotic and necrotic cells (*$p<0.05$). Experiments were performed in triplicate and repeated twice.

Annexin V profiles of the combined treatment of CDDP and Captopril in MDA-MB-231, breast cancer cell line.

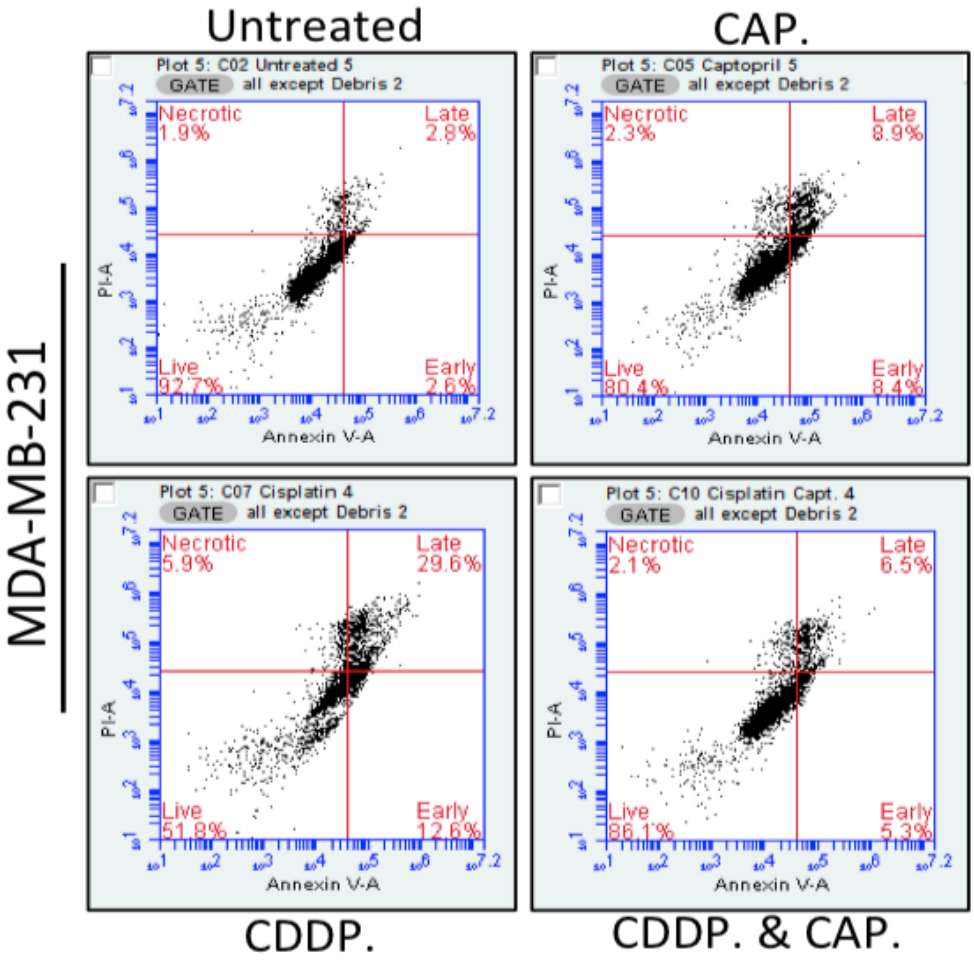


Figure A.4: The effect of CDDP and Captopril combination treatment on MDA-MB-231 breast cancer cell death using the Annexin V assay. MDA-MB-231 cells were treated with the IC_{50} concentrations of CDDP and Captopril for 48 hours and effects on cell death investigated using the Annexin V assay. Profiles of MDA-MB-231 cells with cells falling into four quadrants as previously described. Combined treatment of CDDP with Captopril showed no significant effect on MDA-MB-231 cell death, however, CDDP and Captopril single treatments showed a significant decrease in the percentage of live cells and a significant increase in the percentage of late apoptotic and necrotic cells (*$p<0.05$). Experiments were performed in triplicate and repeated twice.

Annexin V profiles of the combined treatment of CDDP and Lisinopril in HeLa cervical cancer cell line.

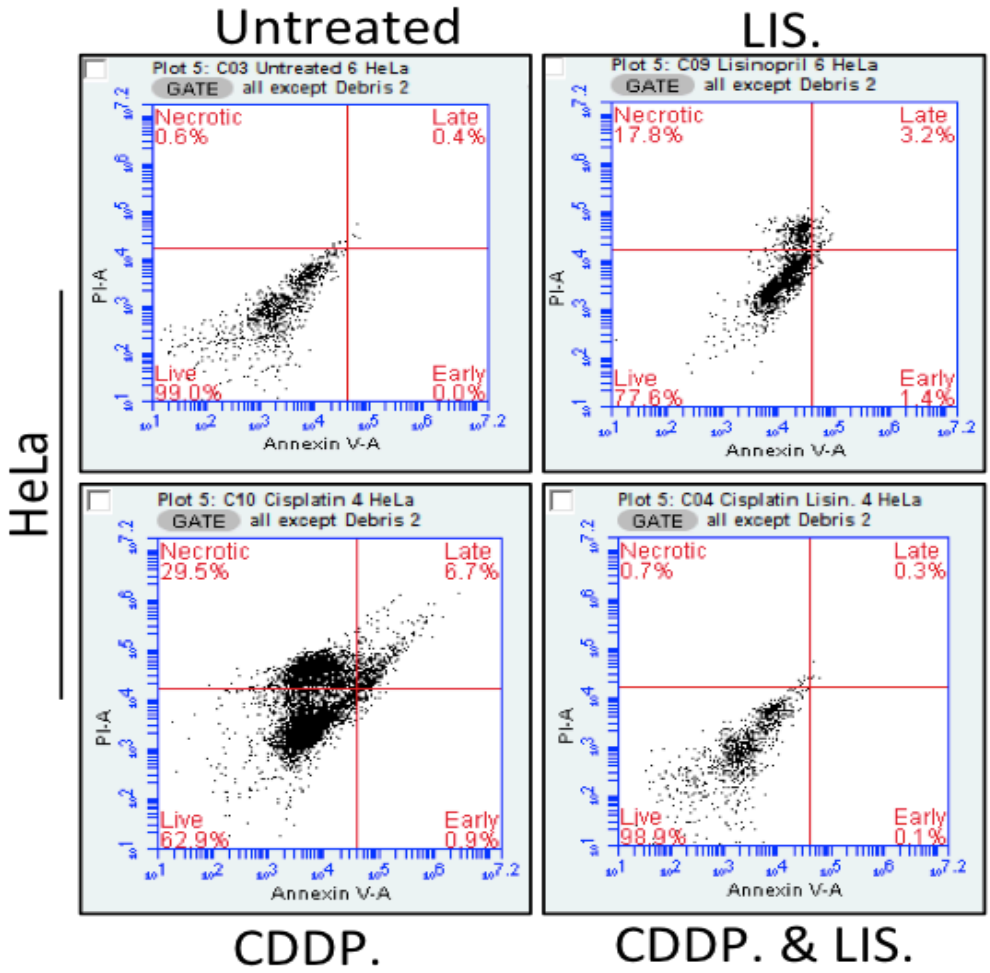


Figure A.5: The effect of CDDP and Lisinopril combination treatment on HeLa cervical cancer cell death using the Annexin V assay. HeLa cells were treated with the IC_{50} concentrations of CDDP and Lisinopril for 48 hours and effects on cell death investigated using the Annexin V assay. Profiles of HeLa cells with cells falling into four quadrants as previously described Annexin V profile of the combined treatment shows no significant effect on cell death. CDDP showed a significant decrease in the percentage of live cells and a significant increase in the percentage of late apoptotic and necrotic cells. The Lisinopril profile showed a significant decrease in live cells as well as a significant increase in necrotic cells (*$p<0.05$). Experiments were performed in triplicate and repeated twice.

Annexin V profiles of the combined treatment of CDDP and Lisinopril in CaSki cervical cancer cell line.

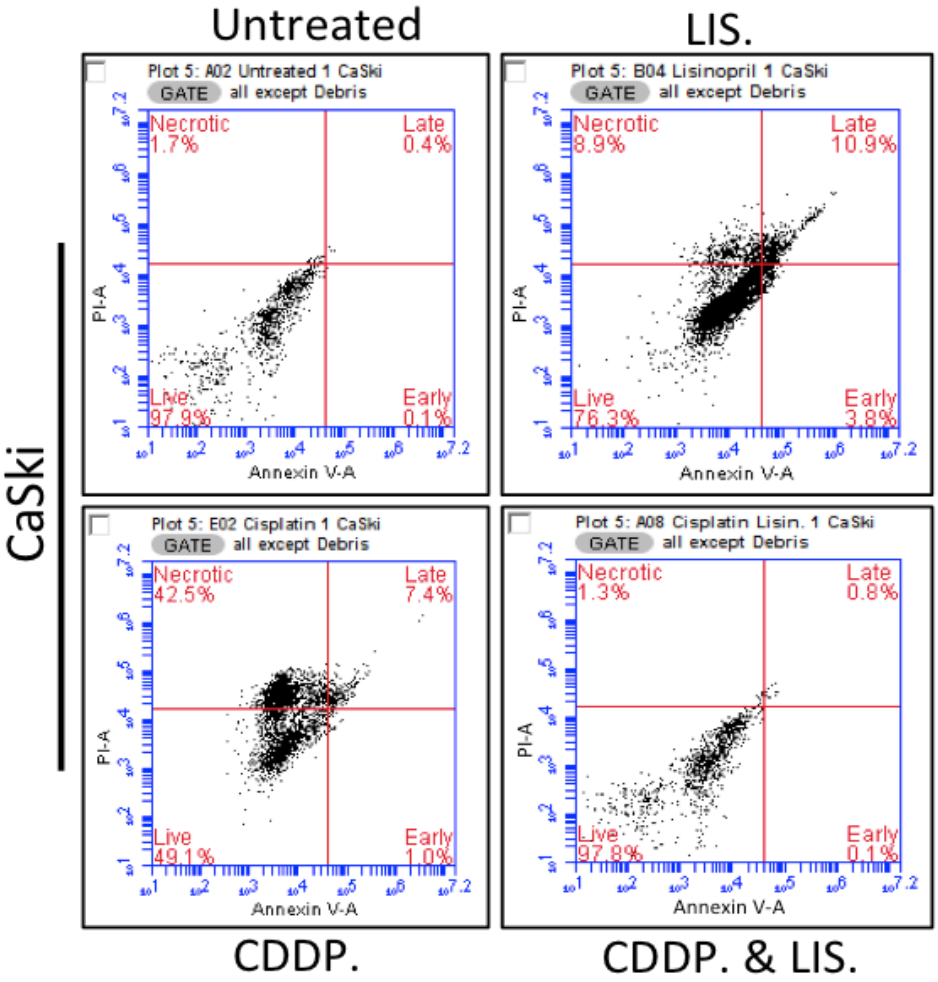


Figure A.6: The effect of CDDP and Lisinopril combination treatment on CaSki cervical cancer cell death using the Annexin V assay. CaSki cells were treated with the IC_{50} concentrations of CDDP and Lisinopril for 48 hours and effects on cell death investigated using the Annexin V assay. Profiles of CaSki cells with cells falling into four quadrants as previously described. Profile of the combined treatment shows no significant effect on cell death. CDDP showed a significant decrease in the percentage of live cells and a significant increase in the percentage of late apoptotic and necrotic cells. The profile of Lisinopril showed a significant decrease in live cells as well as a significant increase in apoptotic and necrotic cells (*$p<0.05$). Experiments were performed in triplicate and repeated twice.

Annexin V profiles of the combined treatment of CDDP and Lisinopril in MDA-MB-231, breast cancer cell line.

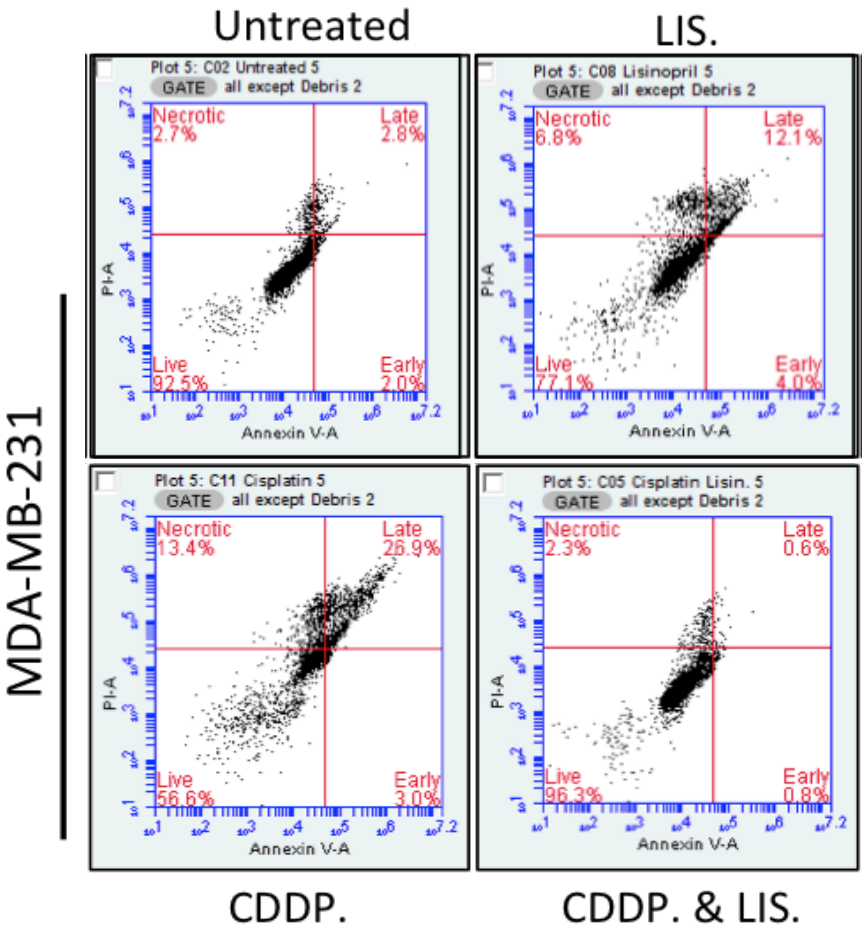


Figure A.7: The effect of CDDP and Lisinopril combination treatment on MDA-MB-21 breast cancer cell death using the Annexin V assay. MDA-MB-231 cells were treated with the IC_{50} concentrations of CDDP and Lisinopril for 48 hours and effects on cell death investigated using the Annexin V assay. Profiles of MDA-MB-231 cells with cells falling into four quadrants as previously described. Profile of the combined treatment shows no significant effect on cell death. CDDP showed a significant decrease in the percentage of live cells and a significant increase in the percentage of late apoptotic and necrotic cells. The profile of Lisinopril showed a significant decrease in live cells as well as a significant increase in apoptotic and necrotic cells (*$p<0.05$). Experiments were performed in triplicate and repeated twice.

APPENDIX II

Table A.1. 1DOX:1DIZE concentrations used in Combination Index determination

HeLa		CaSki		MDA-MB-231	
DOX (μM)	DIZE (μM)	DOX (μM)	DIZE (μM)	DOX (μM)	DIZE (μM)
0.45	0.75	0.65	2.5	0.68	2.5
0.9	1.5	1.3	5	1.35	5
1.35	2.25	1.95	7.5	2	7.5
1.8	3	2.6	10	2.7	10
2.7	4.5	3.9	15	4.1	15
3.6	6	5.2	20	5.4	20

Table A.1. DOX and DIZE concentrations used in determination of the association between the drugs. Cells were treated with the combined DOX and DIZE treatment or single treatments of each drug. In each experiment the combination treatment maintained a fixed ratio of 1:1.

Table A.2. 1DOX:3DIZE concentrations used in Combination Index determination

HeLa		Caski		MDA-MB-231	
DOX (μM)	DIZE (μM)	DOX (μM)	DIZE (μM)	DOX (μM)	DIZE (μM)
0.45	2.25	0.65	7.5	0.68	7.5
0.9	4.5	1.3	15	1.35	15
1.35	6.75	1.95	22.5	2	22.5
1.8	9	2.6	30	2.7	30
2.7	13.5	3.9	45	4.1	45
3.6	18	5.2	60	5.4	60

Table A.2. DOX and DIZE concentrations used in determination of the association between the drugs. Cells were treated with the combined DOX and DIZE treatment or single treatments of each drug. In each experiment the combination treatment maintained a fixed ratio of 1:3.

APPENDIX III

Annexin V profiles of the combined treatment of DOX and DIZE in HeLa, cervical cancer cell line.

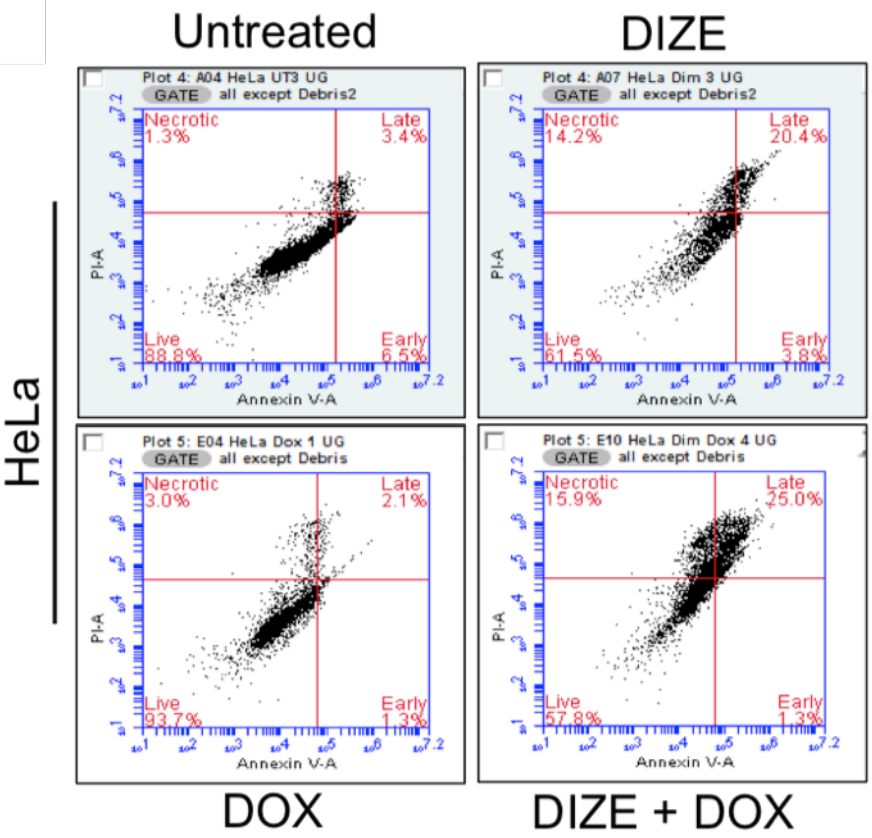


Figure A.8: The effect of DOX and DIZE combination treatment on HeLa cervical cancer cell death using the Annexin V assay. HeLa cells were treated with the IC_{50} concentrations of DOX and DIZE for 48 hours and effects on cell death investigated using the Annexin V assay. The above shows Annexin V profiles of HeLa cells with cells falling into four quadrants as previously described. Experiments were performed in triplicate and repeated twice.

Annexin V profiles of the combined treatment of DOX and DIZE in CaSki, cervical cancer cell line.

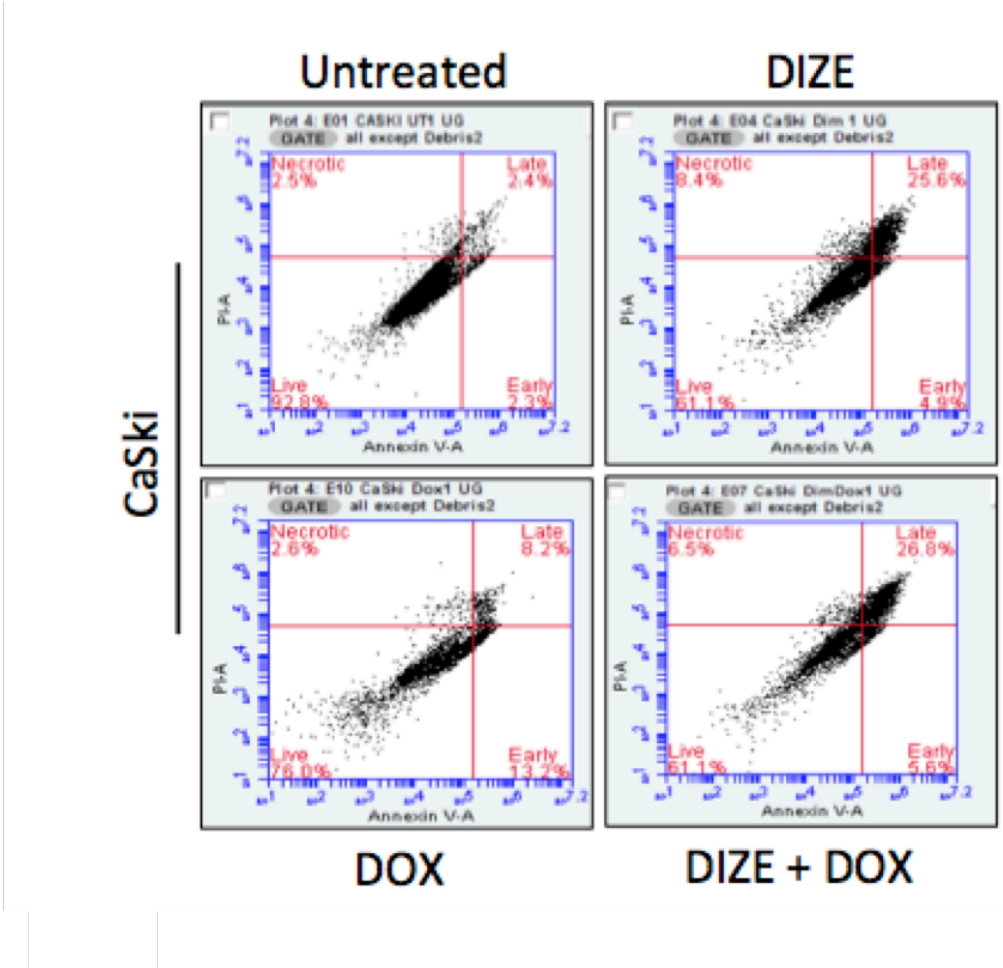


Figure A.9: The effect of DOX and DIZE combination treatment on CaSki cervical cancer cell death using the Annexin V assay. CaSki cells were treated with the IC_{50} concentrations of DOX and DIZE for 48 hours and effects on cell death investigated using the Annexin V assay. The above shows Annexin V profiles of CaSki cells with cells falling into four quadrants as previously described. Experiments were performed in triplicate and repeated twice.

Annexin V profiles of the combined treatment of DOX and DIZE in MDA-MB-231, breast cancer cell line.

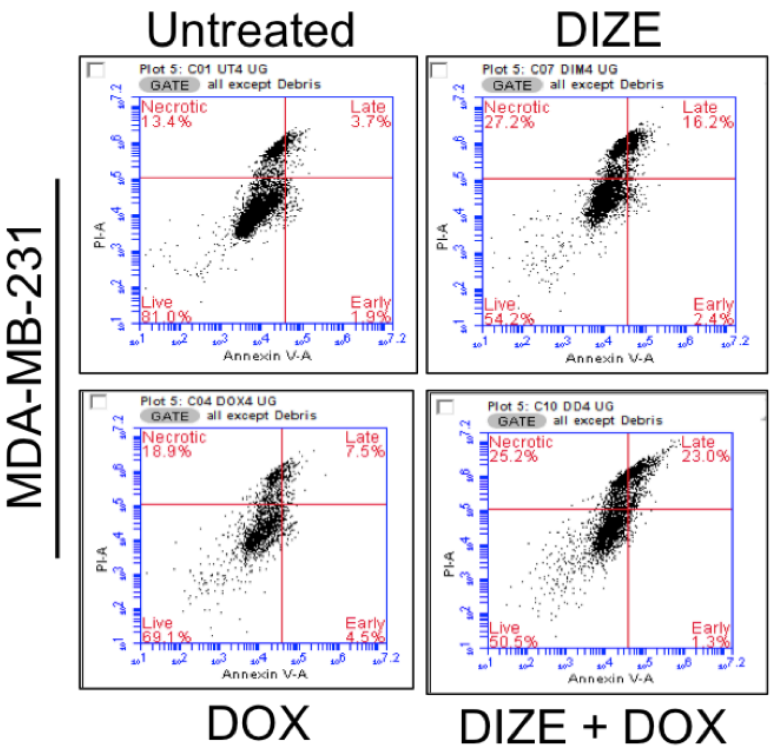


Figure A.10: The effect of DOX and DIZE combination treatment on MDA-MB-231 breast cancer cell death using the Annexin V assay. MDA-MB-231 cells were treated with the IC_{50} concentrations of DOX and DIZE for 48 hours and effects on cell death investigated using the Annexin V assay. The above shows Annexin V profiles of MDA-MB-231 cells with cells falling into four quadrants as previously described. Experiments were performed in triplicate and repeated twice.

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