INVESTIGATING THE EFFECT OF A NUCLEAR TRANSPORT INHIBITOR, INI-43, IN COMBINATION WITH CONVENTIONAL CHEMOTHERAPIES, CISPLATIN AND DOXORUBICIN, IN CERVICAL CANCER CELLS

Gabriella Mia Wilensky

Dissertation submitted in fulfillment of the requirements for the degree of

MSc (Med) in Medical Biochemistry

In the

Division of Medical Biochemistry and Structural Biology

Department of

Integrative Biomedical Sciences

UNIVERSITY OF CAPE TOWN

February 2023

DECLARATION

I, Gabriella Mia Wilensky, hereby declare that the work on which this dissertation/thesis is based is my original 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 empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate

Signature: ……

Date: ……11.02.23……

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following people:

My supervisor, Prof Virna Leaner, for her outstanding supervision, passion, encouragement, patience, and her open-door policy.

My co-supervisor, Dr Pauline van der Watt, for her endless encouragement and invaluable guidance and advice, and her considerable contribution of ideas.

Hajira Guzguy for assistance in the lab and tissue culture.

Graham Christians and Shaheida Johnson for running the lab and all their assistance

Students of lab 6.01 for creating the best working environment and for always being willing to help. Special thanks to Bianca Abrahams, Humaira Lambarey, Nonkululeko Mkwanazi, Dimakatso Senthebane and Shabita Rahman for their support and willingness to help.

National Research Foundation (NRF) for the Master's Innovation Scholarship.

My family and Rob, for their love and support.

TABLE OF CONTENTS

ABBREVIATIONS

~	Approximately
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
CEBPD	CCAAT/enhancer-binding protein delta
CDDP	Cisplatin
CI	Combination Index
cl. PARP	Cleaved Poly(ADP-ribose) Polymerase
CO_2	Carbon Dioxide
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
EGFR	Epidermal growth factor receptor
Fa	Fraction affected
g	Grams
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HDI	Human Development Index
HIFSC	Heat Inactivated Fetal Calf Serum
HPV	Human Papillomavirus
hr	Hour
IC_{50}	Half Inhibitory Concentration

IGF1-R	Insulin-like growth factor 1 receptor
INI-43	Inhibitor of Nuclear Import 43
KPNα	Karyopherin-α
KPNβ1	Karyopherin-β1
kDa	Kilodalton
M	Molar
ml	Millilitres
min	Minutes
mM	Millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NF-κB	Nuclear factor-κB
NLS	Nuclear Localisation Signal/Sequence
NPC	Nuclear Pore Complex
OD_{595}	Optical Density at 595nm
PARP	Poly (ADP-ribose) Polymerase
PBS	Phosphate Buffered Saline
RT	Radiation Therapy
RanGDP	Ran-Guanosine Diphosphate
RanGTP	Ran-Guanosine Triphosphate
RIPA	Radioimmunoprecipitation assay
Rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean

SINE	Selective Inhibitors of Nuclear Export
TBST	Tris Buffered Saline with Tween-20
V	Volts
XPO1	Exportin 1
γH2AX	139-serine phosphorylated histone H2A.X
µg	Microgram
µl	Microliter
µM	Micromolar

ABSTRACT

Background: Cervical cancer is the most common gynaecological cancer in South Africa, and the burden of disease remains high across the developing world. Like with many other cancers, treatment is limited due to drug resistance and adverse side effects. To overcome these limitations, it has become of interest to inhibit multiple pathways that cancer cells are reliant on, using combination therapies to induce maximal killing of cancer cells. Conventional chemotherapies used in the treatment of cancer include Cisplatin and Doxorubicin, both first-line drugs often used in combination with other treatments. Our laboratory has a focus on identifying novel therapeutic targets and found that Kpnβ1, a member of the nuclear transport protein family is overexpressed and necessary for the survival of cervical cancer cells. Inhibition of Kpnβ1 with a novel Inhibitor of Nuclear Import; INI-43 inhibits Kpnβ1-associated nuclear import pathways and results in cancer cell death. This study investigated the potential of Cisplatin and Doxorubicin when used in combination with INI-43 as an anti-cancer approach.

Methods: Cervical carcinoma cell lines, HeLa, ME-180 and SiHa, were treated with INI-43, Cisplatin and Doxorubicin individually or in combination with each other at fixed ratios. Cell viability was measured by the MTT assay and the Chou-Talalay method was adopted to investigate the nature of the drug interactions in the combination treatments. The Caspase-3/7 Glo Assay and Western blot analysis were performed to identify markers of apoptotic death, Caspase-3/7 activation and PARP1 cleavage, respectively, in single and combination treated cells. γH2AX was also investigated via western blot analysis to determine levels of DNA damage in single and combination treated cells.

Results: Combination index values were determined to be below 1 at all levels of cell death after treatment with INI-43 and Cisplatin and INI-43 and Doxorubicin combinations, in HeLa, ME-180 and SiHa cell lines, indicating a synergistic effect. Additionally, enhanced levels of PARP1 cleavage were detected in combination treated cells relative to those treated with individual drug doses, indicating that the observed synergism is attributed to both decreased cell viability and elevated levels of apoptotic cell death. Non-cancer ARPE-19 cells were unaffected by single agent treatments and, more importantly, remained unaffected after treatment with drug combinations which resulted in enhanced cell death in the cervical cancer cell lines tested.

Conclusion: INI-43, when combined with Cisplatin or Doxorubicin, results in synergistic cytotoxic effects in cervical cancer cells of different histological origins. These results suggest that combining conventional chemotherapies with targeted inhibitors of nuclear import pathways, has potential as an anti-cancer approach that warrants further investigation.

Key Words: Nuclear import inhibition, Combination therapy, Conventional chemotherapy, Cisplatin, Doxorubicin, Cervical cancer

CHAPTER 1

LITERATURE REVIEW

1.1 Cervical Cancer Incidence and Mortality

Globally, cancer is one of the leading causes of death and the incidence continues to rise despite significant efforts and progress on the research, prevention, and treatment fronts throughout the world[1]. In 2020 there were an estimated 19.3 million new cancer cases worldwide, whereas just a decade prior there were 14.1 million. Furthermore, there is an expected 47% rise in cancer incidence between now and 2040, meaning that a staggering 28.4 million cases are expected to challenge the global healthcare system that year[2].

A large majority of cancer cases are the result of long-term accumulation of genotoxic stress-induced mutations, with a multi-step and multi-gene model being generally accepted to describe tumorigenesis. However, in some cases, infectious agents can be the primary cause of cancer development, as is the case with Human Papillomavirus (HPV)-associated cervical cancer[3]. HPV is the most common sexually transmitted virus and persistent HPV infection is responsible for more than 95% of cervical cancer cases[4]. A better understanding of HPV, the different genotypes and their respective carcinogenic risks, has led to improved prevention of cervical cancer, most notably with the introduction of the first HPV vaccine in 2006.

Cancer of the cervix uteri (cervical cancer) is the fourth most common cancer among women worldwide, but the second most common among women in Africa[5]. It is currently the most

commonly diagnosed cancer in 23 countries and is the leading cause of cancer death in 36 countries[2]. The ten countries in the world with the highest incidence and death rates of cervical cancer (as of 2020) are all African countries, with Malawi ranking 1st in both categories[2]. Given the fact that 90% of new cervical cancer cases and deaths occur in low human development index (HDI) countries, it is obvious that the global disparity in cervical cancer incidence and mortality between low HDI and high HDI countries is immense[6]. In contrast to a cervical cancer diagnosis rate in the US of approximately 7.8 per 100,000 women per year, between 2015-2019, the rate was approximately 30 per 100,000 women per year in countries in Southern Africa and South America. However, this disparity is not only seen between low vs high HDI countries; it also presents within high HDI countries as those living in the poverty-stricken regions in high HDI countries suffer the same consequences as those living in low HDI countries. For example, the death rate of cervical cancer in the US (a high HDI country) is 2-fold higher for women living in high poverty areas[7]. The cervical cancer incidence and mortality rates worldwide are thus clearly influenced by socio-economic factors and poverty.

Due to effective preventative tools for cervical cancer, including the HPV vaccine as well as 'screen-and-treat'- initiatives, it is not surprising that high HDI countries have successfully reduced cervical cancer incidence and mortality over the last two decades, compared to low HDI countries where there has been no reduction in cervical cancer cases and deaths. The uneven distribution of incidence and mortality of this disease correlates with the levels of screening being performed in a country. Screening programs for cervical cancer aim to detect HPV-positive cells, pre-cancerous lesions, or cancer as early as possible, while it may still be curable. In the case of high income countries, where the burden of cervical cancer is relatively

low, there are screening rates of over 60%. This statistic is in grave contrast to results from a 2017 World Health Survey which reported the coverage of cervical cancer screening to be 10% in Sub-Saharan Africa. Furthermore, the report found that <1% of women in four West African countries had ever been screened for cervical cancer[8]. These statistics give further insight into the striking disparity of this disease and the strong correlation between low screening rates and high incidence and mortality rates of cervical cancer.

Since HPV vaccination (primary prevention) and screening and treatment of pre-cancerous lesions (secondary prevention) is lacking or inadequate in the majority of African countries, cervical cancer control mostly involves tertiary prevention which is defined as the diagnosis and treatment of invasive cervical cancer. Sadly, patients usually present at late stage disease at which the prognosis is poor and treatment options are limited[8,9]. Thus, there remains an urgent need for novel and/or improved therapeutic strategies for cervical cancer.

1.2 Common Therapeutic Strategies for Cervical Cancer: Surgery, Radiation and Chemotherapy

Surgery is most effective when performed on early-stage cancers as a means of 'curative' therapy, for example, if the tumour is localized and the entire tumour can be removed. The primary treatment for early stage cervical cancer patients is radical hysterectomy with pelvic lymphadenectomy[10]. When surgery is the primary treatment, adjuvant chemotherapy is often administered to give the patient the best chance of remission, as this decreases the risk of cancer recurrence by one third. In other instances, surgery is performed as a means of

'debulking' a tumour in order to make another treatment modality such as chemotherapy or radiation more effective.

Radiation therapy (RT) is the use of ionizing radiation to kill cancer cells by inducing irreparable DNA damage. A study by Barton et al. (2006) revealed that approximately 50 percent of all cancer patients can benefit from RT in the management of their disease, thus highlighting that RT is a critical element of an effective cancer treatment[11,12]. For most stages of cervical cancer, RT, a localized treatment modality, and chemotherapy, a systemic treatment modality, are given simultaneously (concurrent chemoradiation), because the chemotherapy enhances the effectiveness of the RT.

Chemotherapy drugs continue to be the backbone of cancer therapy. Chemotherapy involves the use of cytotoxic chemical agents, which are categorized as either conventional or targeted therapies[13,14]. Conventional chemotherapies exert their anti-cancer properties by interfering with fundamental cellular processes such as DNA synthesis and replication, thereby killing rapidly dividing cells. Conventional chemotherapies are relatively non-specific in targeting cancer cells because naturally proliferative non-cancer cells such as cells of the gastrointestinal tract (GIT) are also affected by their actions, hence they have their limitations. Common cancer chemotherapies used in the treatment of cervical cancer include Cisplatin and Doxorubicin.

1.3 Conventional chemotherapies: Cisplatin and Doxorubicin

1.3.1 Cisplatin

Over four decades ago, Cisplatin was approved by the FDA as the first platinum drug for the treatment of cancer[15,16]. Cisplatin continues to be a mainstay therapeutic to treat a wide range of sarcomas, carcinomas and germ cell cancers. Historically, Cisplatin has been the most effective first-line chemotherapy for the treatment of cervical cancer[17]. It is also often given intravenously before a cervical cancer patient's radiation session to sensitize the tumour to radiation. A study by Peters et al (2000) provides evidence to support this as patients in a randomized trial for locally advanced cervical cancer showed a survival advantage when treated with Cisplatin and radiotherapy rather than radiotherapy alone[18]. Therefore, although Cisplatin is highly effective as a single agent, in this capacity it acts as an effective radiosensitizer, which reiterates its broad, multi-faceted use in cancer therapy.

The mechanism of action of Cisplatin is well known, as it acts as an alkylating agent that induces irreparable DNA damage through crosslinking (mainly intrastrand), thereby activating the apoptotic pathway, leading to cell death[19] (Figure 1.1). More recent evidence shows that it may also exert its cytotoxic effect via a cytoplasmic component. The cytoplasmic cytotoxic molecular mechanisms of Cisplatin have not been elucidated thus far but are suspected to involve the accumulation of reactive oxygen species (ROS) and nitric oxide (NO)[20,21].

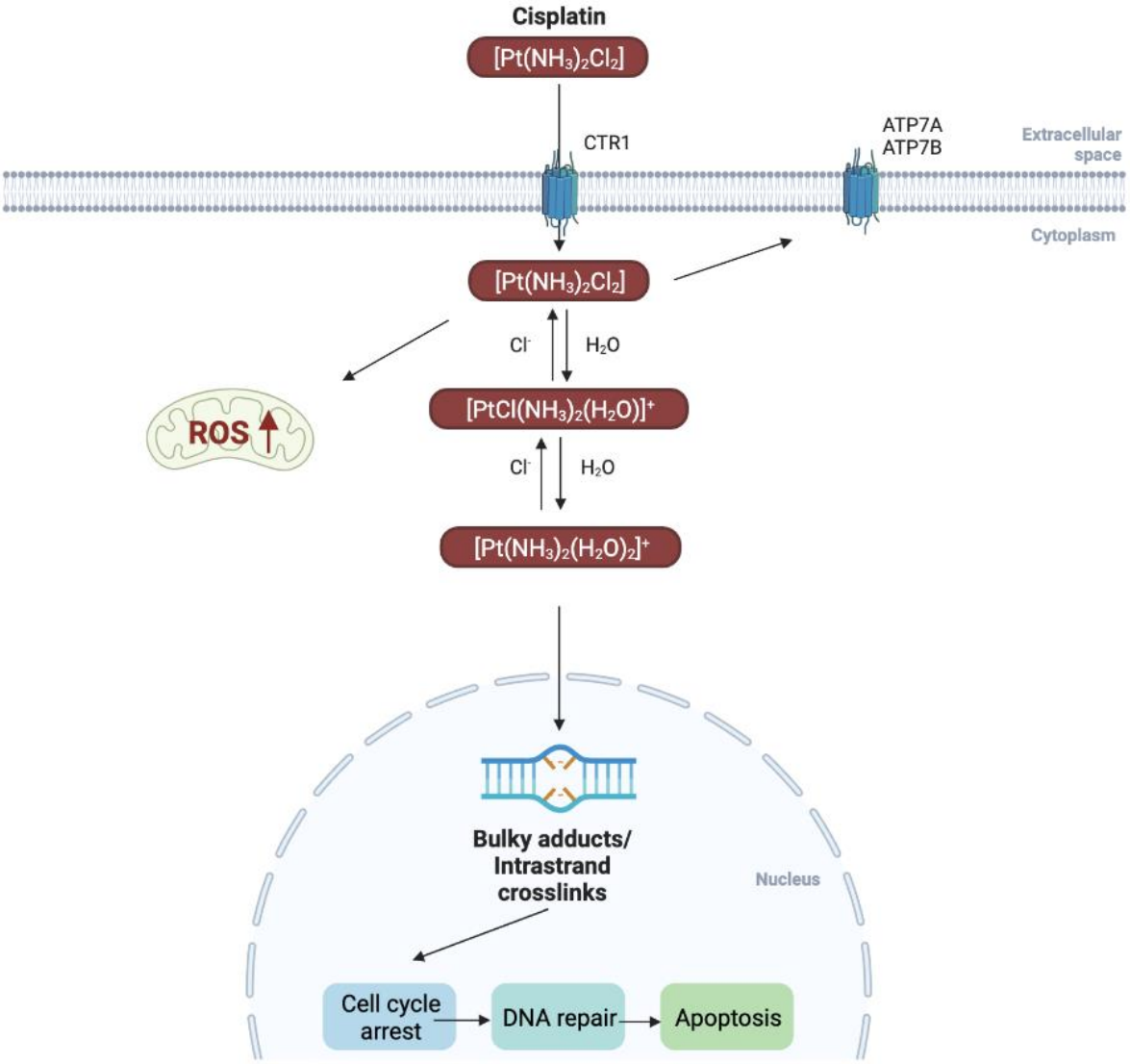


Figure 1.1. The Mechanism of Action of Cisplatin
Cisplatin is internalized into the cell via passive diffusion through the CTR1 copper transporter and aquated (bis-aqauated). The highly reactive hydrated Cisplatin covalently binds to the DNA and forms intrastrand adducts and interstrand crosslinks, inhibiting cell division as damaged DNA cannot repair itself. When Cisplatin-induced DNA damage exceeds the DNA repair capacity, Cisplatin exerts anti-cancer effects by inducing apoptosis. Copper efflux transporters ATP7A and ATP7B have been shown to be involved in efflux of Cisplatin. **[*Created with Biorender*]**

1.3.2 Doxorubicin

Doxorubicin is a type of anti-tumour antibiotic called an anthracycline. A meta-analysis of several randomized studies for breast cancer showed improved disease-free survival and overall survival rates achieved with anthracycline-based versus non-anthracycline chemotherapies. This supports Doxorubicin as the mainstay chemotherapy of current adjuvant treatments for early-stage breast cancer[22]. For over three decades Doxorubicin has also been a frontline conventional chemotherapy used to treat cancers such as cervical, endometrial, pancreatic, lung and stomach, amongst others[23].

Several mechanisms have been proposed for the cytostatic and cytotoxic actions of Doxorubicin. The two main ones being its intercalation into DNA and the generation of free radicals. Doxorubicin has been shown to interact with and inhibit topoisomerase II, which is a common target for many families of DNA intercalating anti-cancer drugs[24]. In this way, Doxorubicin distorts the DNA double helix, disrupting its uncoiling and inhibiting DNA and RNA synthesis, leading to inhibition of DNA replication and transcription, resulting in cell death (Figure 1.2). Additionally, the quinone structure of Doxorubicin lends itself to act as an electron acceptor in reactions mediated by oxo-reductive enzymes such as NADH dehydrogenase[25]. This free electron addition converts the quinones to semiquinone free radicals which are then able to exert damage to DNA, as well as to form superoxides, hydroxyl radicals and peroxides by interacting with molecular oxygen[26], causing lipid peroxidation and membrane damage (Figure 1.2). Other mechanisms of how Doxorubicin exerts its cytotoxicity have also been postulated, for example it has also been shown to disrupt iron metabolism by binding to iron in cells and forming a drug:iron complex which induces the formation of highly reactive hydroxyl radicals[27].

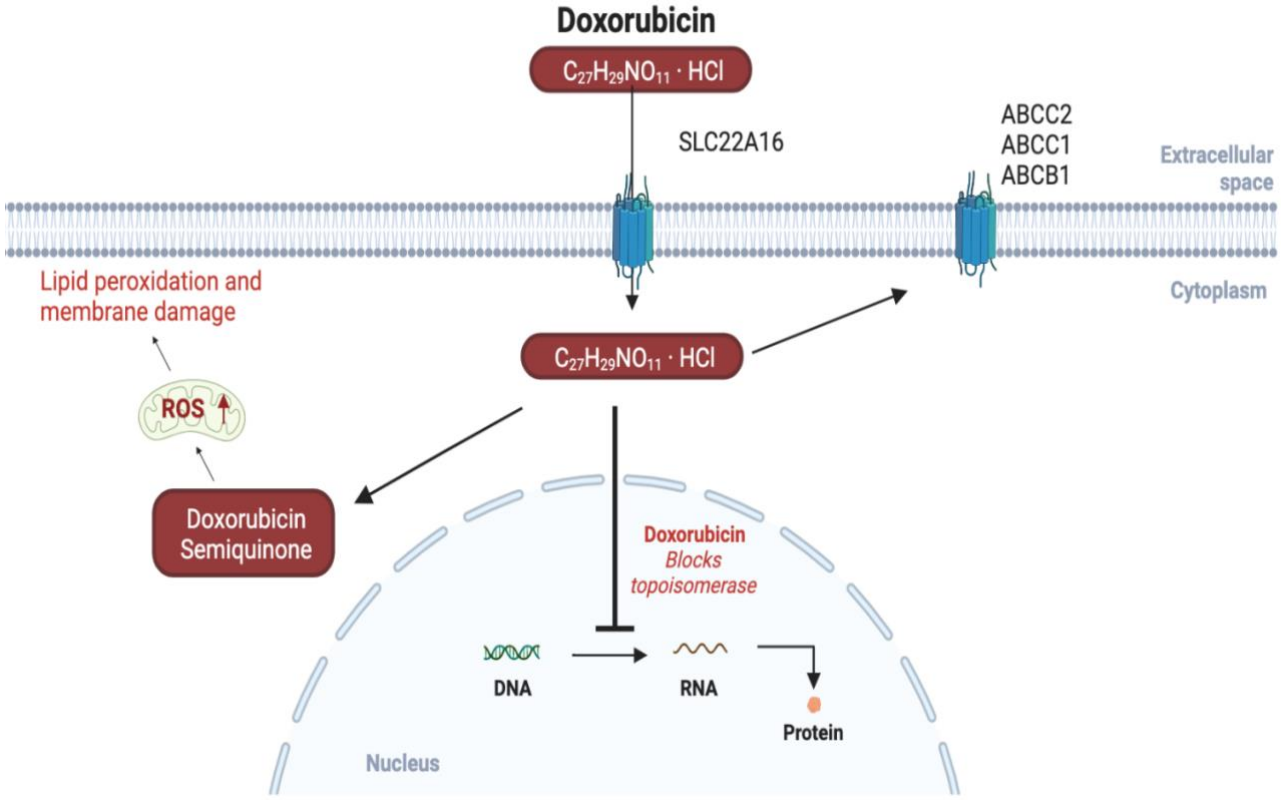


Figure 1.2. Doxorubicin disrupts the normal catalytic action of topoisomerase 2, causing DNA double strand breaks. Doxorubicin enters cell via the SLC22A16 transporter protein and intercalates into DNA, leading to inhibition of topoisomerase 2 (TOP2A). Doxorubicin also generates free radicals, leading to DNA and cell membrane damage. The ABC transporters (ABCC2, ABCC1 and ABCB1 are involved in Doxorubicin efflux. **[*Created with Biorender*]**

1.4 Major limitations of conventional chemotherapeutics, specifically Cisplatin and Doxorubicin

There are several shortfalls of traditional chemotherapies; one of the most notable being the adverse side effects that not only have a physical but also a psychological and economic impact on the patient's life. Another significant limitation is the chemoresistance that more often than not results from treatment with conventional chemotherapies such as Cisplatin and Doxorubicin.

Patients typically experience a favourable initial response to Cisplatin-based chemotherapy, but later relapse because the emergence of acquired Cisplatin resistance significantly reduces its clinical efficacy. Tissue culture studies investigating Cisplatin resistance suggest that the mechanisms of resistance include (1) Decreased intracellular accumulation or increased efflux, (2) drug inactivation, (3) alteration of drug target, (4) increased nucleotide excision-repair activity, (5) decreased mismatch-repair activity, and (6) evasion of apoptosis[28,29,30,31]. Many drugs, including Cisplatin, enter cells via cell receptors or transporters and decreased expression or mutation in these receptors or transporters results in impaired drug uptake. A study by DW Shen (2012) showed how impaired influx via genetic knockdown of CTR1 resulted in cellular resistance to Cisplatin *in vivo*[28]. Furthermore, ATP transporters are thought to contribute to Cisplatin and Doxorubicin resistance via drug sequestration and efflux which leads to reduced intracellular accumulation of the compounds[32,33].

A study by Christowitz et al (2019) suggests that the MAPK/ERK pathway is involved in Doxorubicin-induced drug resistance and tumour growth. The pathway is thought to achieve this through its adaptive role in protecting tumour cells from oxidative stress[34]. This is supported by other studies that have shown improved efficacy of Doxorubicin by inhibition of the ERK signalling pathway[35,36,37,38]. Another factor shown to contribute to Doxorubicin resistance is the alteration of the drug's target- topoisomerase 2. Various studies investigating the responsiveness of breast cancers to Doxorubicin treatment associate increased sensitivity with amplification of topoisomerase 2-alpha genes, whereas reduction in gene expression of topoisomerase 2-alpha contributes to acquired resistance to Doxorubicin[39,40]. Furthermore, Doxorubicin treatment has also been shown to induce autophagy. In certain cases autophagy has been shown to have a pro-survival role by preventing cellular damage and protecting

cancer cells against apoptosis and therefore contributing to acquired chemoresistance to Doxorubicin[41].

Despite Cisplatin being a first-line therapeutic treatment in various cancers, it has limited therapeutic response as a result of its serious side effects. The main dose-limiting toxicity is nephrotoxicity, but others include ototoxicity, gastrotoxicity, myelosuppression, and allergic reactions[42]. Carboplatin and Nedaplatin are analogues of Cisplatin and were developed as less nephrotoxic and neurotoxic treatment alternatives to Cisplatin. Patients treated with Nedaplatin or Carboplatin may have less toxicity and therefore better treatment compliance which would afford them to complete more chemotherapy cycles[43]. In the case of Doxorubicin, the most significant long-term toxicity is cardiac dysfunction, but other major toxicities have also been noted in the brain, kidney and liver – with over 40% of Doxorubicin-treated patients experiencing some form of liver injury[44,45]. Taken together, it is clear that the anti-cancer effectiveness of both drugs, like other conventional chemotherapies, is at the expense of other healthy tissues and organs of the body.

As a mechanism to abrogate the nonspecific cytotoxicity of conventional chemotherapies such as Cisplatin and Doxorubicin, different drug delivery/carrier systems have been developed to specifically direct the cytotoxic effects at the tumour. More notably, research into the discovery and development of conventional chemotherapies has plateaued whereas targeted therapies have been at the forefront of cancer drug discovery over recent years.

1.5 Targeted anti-cancer therapy

A deeper understanding of the complex and widely variable nature of cancer cells and the tumour microenvironment, as well as vast improvements in biotechnological advances, have brought about new anti-cancer strategies, one of them being targeted therapy. This new era of anti-cancer therapy has prompted the identification of previously undescribed cancer biomarkers and novel therapeutic targets. Newer treatment modalities such as targeted therapy and immunotherapy, as opposed to traditional systemic chemotherapy, have revolutionized cancer treatment in the past decade. Perhaps the most notable success story is that of the kinase inhibitor, Imatinib, for patients with BCR–ABL driven chronic myeloid leukaemia (CML) [46]. Imatinib therefore brought the concept of targeted therapy to the forefront of cancer research and became the 'poster child' for this new anti-cancer strategy.

In general, targeted therapies inhibit cancer-sustaining pathways that healthy cells are less reliant on for survival, and as a result of this increased specificity are expected to have an improved tolerability profile. Angiogenesis, for example, is a critical process in the development and progression of many cancers (including cervical cancer) and has therefore been one of the processes against which targeted therapies have been developed. Bevacizumab, an anti-angiogenesis agent, emerged as a promising targeted therapy and it is currently used as a first line treatment in combination with conventional chemotherapies such as Cisplatin and Paclitaxel to treat women with advanced cervical cancer[47]. The addition of Bevacizumab to combination chemotherapy in patients with recurrent, persistent, or metastatic cervical cancer was associated with an improvement of 3.7 months in median overall survival[48].

Identifying therapeutic targets critical for cervical cancer maintenance is crucial to improve the treatment response. In 2021, an antibody-drug conjugate directed against tissue factor, Tisotumab vedotin, was granted accelerated approval by the FDA for the treatment of recurrent or metastatic cervical cancer. Tisotumab vedotin works by binding to a novel clinical target, tissue factor, which is overexpressed on several solid tumours and has been linked to tumour promoting pathways[49,50]. Tisotumab vedotin is therefore a very recent example of a novel targeted therapy which is currently showing clinically significant and long-lasting remission with controllable and tolerable safety in a 'difficult-to-treat' group of cervical cancer patients[51].

In an effort to identify and develop other targeted therapies, research in our laboratory has a focus on identifying novel cancer biomarkers and therapeutic targets. As a result, multiple members of the nuclear transport system have been identified; their overexpression as well as their importance for cancer cell survival and function have been shown in several cancer types, including cervical cancer[52].

1.6 Targeting the nuclear transport system as a novel anti-cancer approach

A family of proteins showing great promise as targets for novel anti-cancer targeted therapies is the Karyopherin protein family of nuclear transport receptors. Nuclear transport machinery strictly regulates the trafficking of macromolecules across the nuclear membrane[53]. Karyopherins, which are further subdivided into the Karyopherin-α and Karyopherin-β subfamilies, facilitate the movement of cargo proteins/macromolecules and certain RNAs

across the nuclear pore complex (NPC) and are therefore crucial in orchestrating the nuclear transport process. Recent years have seen a rise in interest in the nucleo-cytoplasmic trafficking system as a novel anti-cancer target due to its association with cancer[54,55,56]. The dysfunction and dysregulation of this cellular process has been reported in a wide variety of tumours[57,58,59,60,61].

Overexpression of Karyopherin proteins in cancer cells has been linked to enhanced transport efficiency, thereby benefiting tumour cells by means of stimulating oncogenic signalling and supporting the high metabolic and proliferative demands of the cancer phenotype[62]. Exportins are the group of Karyopherin proteins responsible for transporting protein complexes out of the nucleus and they have been extensively studied for their potential role in cancer by transporting key mediators of oncogenesis across the nuclear membrane in cancer cells. Over-expression of the nuclear export protein XPO1 has been reported in several solid tumours and in haematological malignancies, including acute myeloid leukaemia (AML) and it is associated with advanced disease states and poor patient prognosis[63]. Small molecule inhibitors of XPO1 tested *in vitro*, *in vivo* and in preclinical models of AML exhibit potent antileukemic activity[64]. Furthermore, a study by Luo et al (2018) investigating XPO1 in diffuse large B-cell lymphoma revealed that higher XPO1 expression was correlated with a worse prognosis[65]. Although importins have not been as thoroughly investigated as exportins for their role in cancer, it was found that elevated expression of Importin-11 predicted poor survival of muscle-invasive bladder cancer patients[66]. A more recent study by Mis et al. (2019) revealed that Importin-11 levels are frequently overexpressed in human colorectal cancers. Furthermore, inhibiting the activity of Importin-11 suppressed the growth of tumours formed by APC mutant cancer cells isolated from patients[67]. Taken together, exportins and importins

both show promise as anti-cancer targets for a wide range of tumour types. The primary importin protein in the cells, Kpnβ1, is also being investigated in this regard.

1.7 Kpnβ1 as an anti-cancer therapeutic target

Karyopherin-β1 (Kpnβ1, also known as Importin β) is classified as an importin because it facilitates the transport of nuclear localisation signal (NLS)-containing proteins from the cytoplasm into the nucleus via the NPC[68]. Although Kpnβ1 has been shown to function independently, the more common "classical" import pathway also involves Karyopherin-α (Kpnα), which recognizes the NLS on the cargo protein, binds to it and thereafter binds to Kpnβ1. In this transport process Kpnα therefore acts as the link/adaptor protein between the cargo protein and Kpnβ1[56] (Figure 1.3).

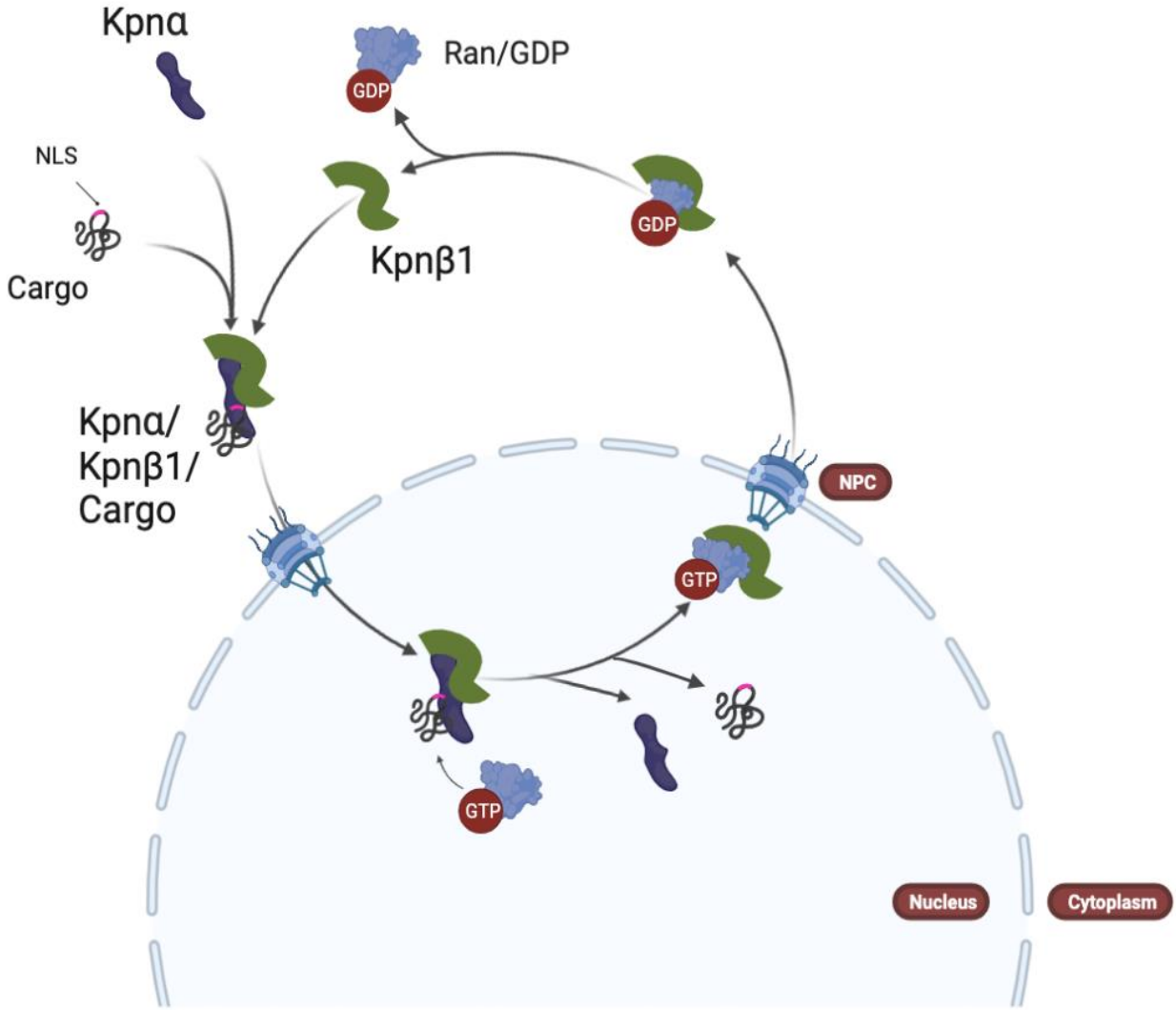


Figure 1.3. Simplified schematic showing the Karyopherin-β1/α mediated nuclear import cycle. Karyopherin-β1, Karyopherin-α and import cargo containing a nuclear localisation signal (NLS) form a trimeric complex in the cytoplasm. This complex transits through the nuclear pore complex (NPC) into the nucleus and binding of RanGTP dissociates the complex and releases the cargo. In the cytoplasm, hydrolysis of RanGTP releases Karyopherin-β1. **[*Created with Biorende*r]**

A study by van der Watt et al (2009) in our laboratory showed that Kpnβ1, is highly overexpressed in cervical cancer tumour biopsies and cell lines and is important for cancer cell survival and function[69]. As a result, potential inhibitors of Kpnβ1 were identified using a high-throughput *in silico* screen. The small molecule inhibitor of nuclear import-43 (INI-43) was investigated further as it interfered with the nuclear localization of Kpnβ1 and known

Kpnβ1 cargoes, NFAT, NFKB, AP-1 and NFY[56]. INI-43 treatment was also shown to inhibit oesophageal and cervical tumour growth in cancer xenograft models. Importantly, rescue experiments showed that Kpnβ1 over-expression reverted/rescued the repressed nuclear import of NFκB-p65 as a result of INI-43 treatment, as well as the cell death induced by INI-43, suggesting that the nuclear import inhibitory effect of INI-43 is exerted through Kpnβ1[70,56].

Kpnβ1 has also been shown to be important for the invasion and migration of cervical cancer cells, where its inhibition using siRNA or INI-43 resulted in significantly reduced cervical cancer cell migration and invasion. Its inhibition has also been shown to be associated with reduced expression of proinflammatory genes[62]. In small cell lung cancer, oncogenic drivers ASCL1 and NEUROD1 were found to be imported into the nucleus via Kpnβ1[71]. Kpnβ1 inhibition was shown to impair ASCL1 nuclear import, gene expression and small cell lung cancer growth. These studies broaden the support for targeting nuclear transport, specifically nuclear import via Kpnβ1, as an anti-cancer therapeutic strategy and a novel approach for targeted therapy.

1.8 The limitations of targeted therapy

While recent studies have identified key oncogenic players as targets for anti-cancer drugs (including nuclear transport proteins), concerns have also arisen over the effectiveness of targeting single proteins in cancer therapy. Since the early success of Imatinib, many targeted therapies which have been tried and tested as single agents, such as IGF-1R inhibitors, have been shown to fail to exhibit single agent activity and this has resulted in what some would argue as the 'premature' cessation of the development of these agents[72]. Ultimately, the idea

of a ‘one genetic abnormality - one drug’ drug development paradigm has proved disappointing, as a large number of targeted drugs (such as PI3K, AKT, MET and IGFR inhibitors) have not been successful in providing reproducible improvements in patient survival when used as single agents[73]. This is largely due to the development of chemoresistance.

While intrinsic and/or acquired chemoresistance is known to compromise the effectiveness of cancer chemotherapies, the issue of drug resistance is not unique to conventional chemotherapies. Despite many cancers starting out being susceptible to targeted and conventional chemotherapies, over the course of treatment they can develop resistance via numerous mechanisms, and this is referred to as acquired resistance.

It has become apparent that the full potential of targeted agents has not been realised due to the presence of *de novo* resistance, often resulting from compensatory signalling pathways or the development of acquired resistance. Mutations may occur in the target protein and therefore prevent drug-target interactions. In this manner, their characteristic specificity seems to also present as a weak spot to which cancer cells are able to adapt to and overcome by undergoing clonal evolution against the selective pressures of treatment. For example, overexpression of epidermal growth factor receptor (EGFR) has been shown in certain cancers such as colorectal and lung, making it an attractive anti-cancer target, however a study by Russo, Crisafulli and Sogari et al (2019) found that colorectal cancer cells treated with an EGFR inhibitor resulted in increased mutagenesis[74]. This drug driven mutagenesis is what helps the cancer cells gain resistance to the targeted therapy. Unfortunately, this has shown to be the case for various targeted therapies whereby the stress response translates to increased

mutagenesis in the cancer cells[75,76]. Another mechanism by which the efficacy of targeted therapy is compromised is via compensatory signalling pathways in response to treatment[77].

Overall, whether it be conventional or targeted therapies, resistance poses a major problem in oncology. Although various mechanisms by which cancer cells develop resistance to chemotherapies have been well elucidated, how to circumvent this resistance to improve anti-cancer efficacy remains to be defined.

1.9 Combination therapy approaches to treat cancer

The time consuming and expensive process of developing new anti-cancer drugs coupled with the ever-present burden of chemoresistance has resulted in the investigation of new treatment strategies such as combination therapy. Combination therapy can be defined as a treatment modality that involves combining two or more therapies in order to reduce treatment dose, which will limit drug resistance as well as toxicity against normal host cells[78].

Deploying a multi-therapeutic agent arsenal when fighting cancers is anticipated to hold an even greater chance of overcoming resistance when the drugs used have non-overlapping mechanisms of action, therefore inducing maximum cell death in the heterogenic tumour population[78]. On the other hand, when using monotherapies, the heterogenic nature of the tumour population increases the likelihood of subsequent resistant subpopulations, whereas this phenomenon may be circumvented by using drug combination regimens. Another advantage of combination therapy is that each drug can be used at its optimal dose, therefore

minimizing possible side effects. Not only is it beneficial to use a combination of drugs with different mechanisms of action, but because chemotherapy drugs often affect cancer cells at different points in the cell cycle, using a combination of drugs may further increase the chance that all of the cancer cells will be eliminated.

Both targeted and conventional chemotherapies are used in combination and have also shown to interact and collectively disrupt a number of cancer-related pathways. For example, recurrent or metastatic cervical cancer is often treated with a platinum-based chemotherapy in combination with the targeted agent Bevacizumab. A clinical trial with Bevacizumab used in combination with Cisplatin and Paclitaxel showed an improvement of 3.7 months in overall survival, compared to Cisplatin and Paclitaxel alone[48]. However, the biggest challenge of developing successful combination regimens is the narrow therapeutic index of each drug owing to overlapping toxicities[79]. Although, in the treatment of malignant melanoma with different combinations of targeted therapies, it was discovered that by combining Dabrafenib and Trametinib, and Vemurafenib and Cobimetinib it was possible to utilize the full dose of each targeted agent at the original schedule[80,81,82,]. Furthermore, in a study of four breast carcinoma cell lines, the addition of sulbactam (*β-lactamase inhibitor*) enhanced the cytotoxicity of Doxorubicin by blocking its efflux, thus triggering apoptosis in the breast cancer cells[83]. Results from a double blind, phase III trial also support the use of a targeted therapy in combination with conventional chemotherapy as patients with persistent, recurrent or metastatic cervical cancer who were treated with an immunotherapy in addition to platinum based chemotherapy (with or without Bevacizumab) had significantly longer progression-free and overall survival[84].

Nevertheless, combination therapy is not guaranteed to optimise therapy sensitivity and potentially enhanced toxic effects need to be taken into account. In 2010, a report of a phase III clinical trial showed no improved progression-free survival or overall survival in ovarian cancer patients treated with the addition of Gemcitabine (a targeted therapy) to Carboplatin-Paclitaxel, compared to those treated with only Carboplatin-Paclitaxel. The addition of Gemcitabine was also associated with more frequent Grades 3 to 4 hematologic toxicity and decreased quality of life[85]. Pre-clinical studies investigating inhibitors of oncogenes MEK and AKT in combination, revealed that sub-maximal inhibition of MEK and AKT pathways did not cause a significantly greater growth inhibition compared with growth inhibition caused by maximal MEK or AKT inhibition alone[86].

When chemotherapies are used in combination there are several possible outcomes of the interactions which may occur. There may be an additive, synergistic or antagonistic interaction. An additive interaction is when the effect of the combination is equal to the sum of the individual effects. A synergistic interaction is when the effect of the combination is greater than the sum of the individual effects and an antagonistic interaction is when the effect is less than the sum of the individual effects[87]. An ideal combination of anti-cancer drugs would result in a synergistic interaction and conversely an antagonistic interaction would be highly undesirable. These interactions are important considerations to take into account when designing drug or cancer treatment combinations.

In depth investigations need to be conducted prior to combination treatments of relevant cytotoxic agents. Prior knowledge of the mechanism of action for each drug is required as well as the nature of the drug interaction (additive, synergistic or antagonistic). Other factors

that need to be considered are the scheduling of administration and maintenance, as well as the balance between therapeutic benefit and harmful side effects. Each combination needs to be independently and objectively investigated and validated as these factors will vary from case to case. When the above specified factors are taken into consideration, combination therapy may be a powerful strategy when combining novel targeted therapies with conventional cytotoxic agents to improve treatment outcome.

1.10 Combination treatments involving nuclear transport inhibitors

The most extensively studied inhibitors of nuclear transport are the selective inhibitors of nuclear export (SINE), which been investigated in pre-clinical (animal) and clinical (human) studies. SINE compounds are currently being tested in combinations with various anti-cancer agents such as Doxorubicin and proteosome inhibitors (PI's). Treatment with SINE compounds leads to an accumulation of tumour suppressors in the nucleus of cancer cells and therefore reduces the amount of oncogene products which drive cell proliferation, ultimately triggering apoptosis [88]. Another important benefit to this type of treatment is that normal cells appear to be spared by SINE treatment. Selinexor is a first-in-class SINE compound and with the FDA's fast track was granted approval in 2020 for use in combination treatments with Dexamethasone with or without Bortezomib (PI) for relapsed or refractory multiple myeloma patients. The combination of Selinexor and a PI such as Bortezomib results in synergistic cytotoxic effects *in vitro* (sarcoma cells) and *in vivo* (mice xenograft), as revealed in various studies[89].

While nuclear export inhibitors are in preclinical and clinical development, and are being tested in combination with other therapeutics, more research is required to investigate the potential of nuclear import inhibitors in combination therapy. However, preliminary research indicates that concurrently targeting important nuclear import mechanisms can increase the effectiveness of available chemotherapies. CCAAT/enhancer-binding protein delta (CEBPD*)* is a well-known transcriptional factor that is activated by inflammatory cytokines and anti-cancer drug treatment; by inhibiting Importin 4-mediated nuclear import of CEBPD, chemosensitivity to Cisplatin was enhanced by DNA damage repair repression in cervical cancer cells and mice xenograft models[90]. Furthermore, cervical cancer cells that were pre-treated with the Kpnβ1 inhibitor, INI-43, were sensitized to Cisplatin[91]. These cells were treated sequentially, rather than concomitantly. In addition, Importazole, similar to INI-43, specifically blocks importin-β-mediated nuclear import, and was shown to sensitize resistant chronic myeloid leukaemia cells to the targeted therapy Imatinib by significantly inhibiting proliferation and enhancing apoptotic cell death. Thus, various studies have indicated the potential of nuclear import inhibitors to act synergistically with conventional chemotherapies or targeted therapies such as Imatinib.

1.11 Aim

This study aims to investigate whether the inhibition of nuclear import by INI-43 in combination with conventional chemotherapies, Cisplatin and Doxorubicin, has improved *in vitro* therapeutic efficacy relative to each agent as a monotherapy. The use of this type of combination strategy may alleviate some of the limitations of current cancer therapies such as chemoresistance and off target effects.

1.12 Objectives

(i) To determine the optimal cell killing concentrations of the nuclear import inhibitor, INI-43, in combination with Cisplatin and Doxorubicin.

(ii) To determine whether the effects of combination treatments are additive, synergistic or antagonistic using the Chou Talalay method.

(iii) To determine the effect of combination treatments on the viability of cancer and non-cancer cells.

(iv) To investigate the mechanism of cancer cell death upon treatment with inhibitors, by investigating PARP1 cleavage, Caspase-3/7 activity and γH2AX expression levels.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell lines

Three human cervical carcinoma cell lines, HeLa, SiHa and ME-180, and one non-cancer human retinal pigment epithelial cell line, ARPE-19, were used in this study. HeLa (HPV18) is derived from an adenocarcinoma of the uterine cervix and SiHa (HPV16) and ME-180 (HPV18) both originate from squamous epithelial carcinomas of the uterine cervix. The cervical cancer cell lines as well as the normal cell line were obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA). The use of non-cancer cervical epithelial cells is the ideal for comparison to cancer. Non-cancer primary cervical epithelial were not available at the time of this study. Such cell cultures have the added complication of growing in culture for a limited period of time and requiring special media and supplements to aid their growth. For this reason ARPE-19 cells which are able to grow in the laboratory were used for comparative purposes. Although of retinal origin, ARPE-19 cells are non-cancer epithelial cells and are therefore a satisfactory control for the cancer cells which are all of epithelial origin. Furthermore, they do not require additional supplements and use similar media to that used by the cancer cells.

2.1.2 Chemical Compounds

Two conventional chemotherapy drugs, Cisplatin and Doxorubicin, and one small molecule inhibitor, INI-43, were used in this study. INI-43, an inhibitor of nuclear transport, was purchased in powder form from Sigma Aldrich (St, Louis, MO, USA) and dissolved in DMSO to a stock concentration of 25 mM and stored at room temperature. Cisplatin (CDDP) was purchased from Sigma Aldrich (St, Louis, MO, USA) in powder form, dissolved to a stock concentration of 1 mg/ml (3.3 mM) in ddH2O, filtered using a 0.22 µM filter and stored at room temperature in the dark. The IC_{50} obtained using Cisplatin dissolved in water was compared to that obtained when Cisplatin was dissolved in 0.9 % NaCl and no significant difference in IC_{50} was observed. Doxorubicin was purchased from Sigma Aldrich (St, Louis, MO, USA) in powder form, dissolved to a stock concentration of 50 mg/ml in ddH_2O and kept refrigerated at 4°C.

Table 2.1. Small molecule inhibitor: INI-43

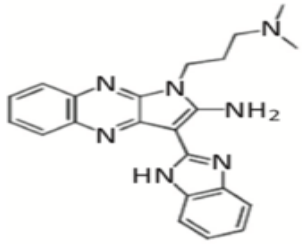
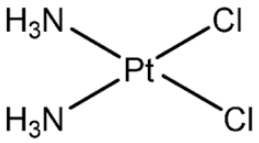
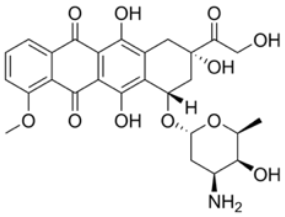
	Inhibitor of Nuclear Import (INI-43)
IUPAC name	(3-(1H-benzimidazol-2-yl)-1-(3-dimethylaminopropyl)pyrrolo[5,4-b]quinoxalin-2-amine)
Empirical Formula	$C_{22}H_{23}N_7$
Molar Mass (g/mol)	385.46
Structure	

Table 2.2. Conventional chemotherapies: Cisplatin and Doxorubicin

	Cisplatin	**Doxorubicin**
IUPAC name	cis-Diamminedichloroplatinum	(7*S*,9*S*)-7-[(2*R*,4*S*,5*S*,6*S*)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8,10-dihydro-7*H*-tetracene-5,12-dione
Empirical Formula	$[Pt(NH_3)_2Cl_2]$	$C_{27}H_{29}NO_{11}$
Molar Mass (g/mol)	301.1	543.5
Structure		

2.2 METHODS

2.2.1. Cell culture

The cervical cancer cell lines, HeLa, SiHa and ME-180, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, USA), the non-cancer cells were cultured in Dulbecco's Modified Eagle's Medium F-12 Nutrient Mixture (Invitrogen, USA), and both were supplemented with 10% heat inactivated fetal calf serum (Gibco, Paisley, Scotland) and penicillin streptomycin (100 µg/ml). All cell lines were grown in 100 mm cell culture dishes and incubated at 37°C at a constant 95% air and 5% CO_2 humidified atmosphere.

2.2.2. Mycoplasma testing

In order to confirm the cells were free of mycoplasma contamination, mycoplasma testing was routinely performed by growing the cells in penicillin-free and streptomycin-free Dulbecco's Modified Eagle's Medium (DMEM) for three or four days. The cells were then plated onto coverslips and fixed and stained with Hoechst fluorescent DNA-binding stain before mounting and visualization using the EVOS M5000 Imaging System (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.3. Cell Viability Assays

2.2.3.1 MTT assay to determine IC_{50} values

Cells were seeded in triplicate into 96-well plates at a density of 5000 cells per well in 90 µl culture medium and incubated overnight. All MTT experiments included wells with only

culture medium as the cell-free control (media blanks). The cells were then treated with 10 µL of a range of concentrations of the drug (INI-43, Cisplatin, Doxorubicin) for 24 hours, after which 10 µL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Aldrich, USA) solution was added to the cells. The formazan crystals were then solubilized four hours later using Solubilisation Solution (10% SLS in 0.01 mol/L HCL). The following day a BioTek microplate spectrophotometer was used to determine absorbance at 595nm. The absorbance reading is directionally proportional to the number of live cells in culture. IC_{50} curves were generated using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA) as follows: OD_{595nm} readings were base-line corrected against media blanks and plotted against Log(x) transformed drug concentrations to produce sigmoidal dose-response curves, from which IC_{50} values and respective 95% confidence intervals were calculated.

2.2.3.2 Combination Index determination

To assess whether the cytotoxicity was enhanced when INI-43 was used in combination with either Cisplatin or Doxorubicin at fixed ratios, the Chou-Talalay method was adopted. Cell viability was measured by MTT assay and OD_{595nm} readings (mean SEM, n=3) were converted to fraction affected (Fa) values, as indicated below, which represent the fraction of cells killed at a specific drug concentration. Fa values and corresponding drug doses (for single and combination treatments) were entered into CompuSyn software (CompuSyn, Inc., Paramus, NJ) to generate a report consisting of combination index values (CI). CI < 1 indicates synergism, CI = 0 indicates additive effects and CI > 1 indicates antagonism.

$$Fa=1-\frac{\text{mean OD595nm treated}}{\text{mean OD595nm control (untreated)}}$$

2.2.3.3 Cell proliferation assays

To investigate the proliferation of cells after individual drug treatment or combination drug treatment, cervical cancer cells were plated in triplicate at 1500 cells per well and ARPE-19 cells were plated in triplicate at 3000 cells per well (due to their slower proliferation rate) in 96-well plates and left to adhere overnight. The cells were then treated with DMSO (control) or individual treatments, or individual treatments and their respective combinations, after which cell proliferation was analysed every 24 hours for four days, using the MTT method. OD_{595nm} readings were converted to relative cell proliferation values and plotted against time in days on Excel to produce cell proliferation curves.

2.2.3.4 Colony formation assay

1000 cells were plated in 6-well plates at single cell density and left to adhere overnight. The following day, cells were treated with single agent treatments of INI-43, CDDP or DOX for 24 hours and cells treated with DMSO served as control. After 24 hr drug treatment, the drug containing media was removed and replaced with fresh media. Every two days (48 hours) the media was replaced and after eight days the cells were rinsed with cold PBS and fixed with fixation solution for five minutes at room temperature and then stained with 0.5% crystal violet solution (Sigma-Aldrich) and incubated at room temperature for 2 hours. After a 2 hr incubation, the crystal violet solution was removed and plates were rinsed in tap water. Plates were left to air-dry overnight and the next day images were captured with a standard camera. Crystal violet stain was then solubilized using 33% glacial acetic acid, transferred to a 96-well plate and absorbance was measured at OD_{595nm} using a Biotek microplate spectrophotometer (Winooski, VT, USA).

2.2.4 Apoptosis assays

2.2.4.1 Caspase 3/7 assay

Cells were plated in 96-well plates at a density of 5000 cells per well in 90 µl cell culture medium and left to adhere overnight. The next day the cells were treated with 10 µl of INI-43, Cisplatin and Doxorubicin, individually or in combination. 24 hr post treatment, 50 µl of media was removed from each well and 50 µl of Caspase-Glo reagent (Promega, Madison, WI, USA) was added to each well. The plate was then covered with foil and left at room temperature for 30 minutes. After 30 minutes, 90 µl from each well was transferred to a white plate. Luminescence was measured using the Veritas microplate luminometer (Promega) and normalized to OD_{595nm} readings of MTT experiments performed in parallel.

2.2.4.2 Western blot analysis

2.2.4.2.1 Protein extraction

Cancer cells were plated at 300 000 and normal cells were plated at 500 000 cells per 60 mm cell culture dish and the following day the cells were treated using the specified drug concentrations for 24 hours. The following day the cell floaters were collected by collecting the media from each dish into corresponding 14 ml cell culture tubes. Cell floaters were collected because PARP cleavage occurs as a late event in apoptosis, hence apoptotic cells might be starting to detach from the dish. In addition, INI-43 induces mitotic arrest (van der Watt et al., 2015), hence INI-43-treated cells round up and are less firmly attached to the cell culture dish and can easily detach. Two successive washes of 1 ml cold 1 X PBS were performed and then also added to the corresponding 14 ml cell culture tubes and then centrifuged. The supernatant was then suctioned off and 60 µL RIPA was added to the cell

pellet. 60 µl cold RIPA containing 1 X protease and phosphatase inhibitors was added to each dish to lyse the cells and the cells were scraped using a cell scraper and then pipetted into the corresponding Eppendorf tube with the resuspended cell floater pellet. While cells were being scraped from one dish the other dishes were kept on ice.

2.2.4.2.2 Sample Processing

Eppendorf tubes were sonicated for 10 seconds, followed by a 10 000 rpm centrifugation for 10 minutes at 4°C. The supernatant was then transferred to a new Eppendorf tube (on ice) and was used as the protein sample (as the pellet contains the cell debris).

2.2.4.2.3 Protein Quantification using the BCA assay

Protein quantification of the harvested protein samples was performed in a 96-well plate using the BCA (Bicinchoninic Acid) Assay (Pierce® BCA Protein Assay kit, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Protein concentration was measured using a standard curve from a serial dilution of bovine serum albumin (BSA) standards, with concentrations ranging from 0 to 2000 µg/ml. After 30 minute incubation at 37°C, absorbance was read at OD_{595nm} using a Biotek microplate spectrophotometer (Winooski, VT, USA).

2.2.4.2.4 Preparation and electrophoresis of gels

The 10% and 15% resolving and the 4% stacking gel were prepared as outlined in the tables below. The resolving gel was poured into the gel casting plates and 1 ml of isopropanol was added on top in order to create a straight gel. Once the gel had set the isopropanol was

removed and the gel was gently rinsed and then the stacking gel was poured on top and the comb was inserted to create the wells.

Tables 2.3 A and B. Preparation of 15% and 10% resolving gels and 4% stacking gel

A)

Resolving gels	**15%**	**10%**
dH_2O	3.3 ml	2.75 ml
1M Tris pH 8.8	3.9 ml	3.75 ml
10% SDS	150 µl	100 µl
30% Acrylamide	7.5 ml	3.35 ml
10% APS	150 µl	200 µl
TEMED	15 µl	20 µl

B)

Stacking gel	**4%**
dH_2O	3.65 ml
1M Tris pH 6.8	625 µl
10% SDS	50 µl
30% Acrylamide	650 µl
10% APS	60 µl
TEMED	6 µl

After the stacking gel had set, 4 X loading dye was added to the protein samples and then they were denatured by heating at 90°C for 5 minutes using a heating block. Equal amounts of protein (25 µg) were loaded onto the SDS-polyacrylamide gels and run in 1 X running buffer at 150 V through the stacking gel, then 180 V for approximately 1 hr. 5 µl of broad range protein ladder was loaded in the first lane to be used as a marker to determine the size of the protein bands. After electrophoresis, the proteins were transferred to a nitrocellulose membrane in cold 1 X transfer buffer at 100 V for 70 minutes. After transfer, the membrane was blocked in 5% skimmed milk powder/TBST for 1 hr at room temperature. For PARP1 analysis: the membrane was cut just below the 72 kDa mark (red marker) to probe for PARP1

(116 kDa) and cleaved PARP1 (89 kDa) above this mark and loading control GAPDH (35 kDa) below this mark. For γH2AX analysis: the membrane was cut at the 20 kDa mark (blue marker) to probe for GAPDH above this mark and γH2AX below this mark (15 kDa). GAPDH was used in this study because it is a constitutively expressed protein, meaning it is typically present at relatively constant levels across various cell types and experimental conditions, ensuring that differences in signal intensity are primarily attributed to changes in the protein of interest, not loading variations.

2.2.4.2.5 Immunoblotting

Primary antibody was added (as indicated in Table 2.4 below) and the membranes were left overnight at 4°C on a gentle horizontal shaker. The following day the membranes were washed in TBST and placed on the shaker three times for 5 minutes at room temperature. After washing, the membranes were incubated in secondary antibody on a gentle shaker for 1hr at room temperature. The membranes were then washed again three times for 5 min in TBST at room temperature.

Table 2.4. Antibodies and incubation conditions for detection of proteins of interest

Protein	Primary Antibody	Condition	Secondary Antibody	Condition
PARP (116 kDa)	Rabbit αPARP1/2 (Cell Signaling Technology #9542)	1:1000 in TBST	Goat αRabbit (BioRad)	1:5000 in TBST
GAPDH (37 kDa)	Rabbit αGAPDH (Sigma #G9545)	1:10000 in TBST	Goat αRabbit (BioRad)	1:5000 in TBST
γH2AX (15 kDa)	Rabbit αP-Histone H2A.X (Cell Signalling #2577)	1:500 in TBST	Goat αRabbit (BioRad)	1:5000 in TBST

2.2.4.2.6 Visualisation and densitometry

The protein bands of interest were detected using chemiluminescent LumiGLO Substrate (KPL, USA) by mixing the required amount of substrate in the ratio 1:1 reagent A to reagent B. The substrate was applied to the membrane upon visualization and the digital image was obtained using the iBright FL1500 Imaging System and the band intensities were quantified relative to the loading control using ImageJ software.

2.2.5 Observation of morphological changes and cell photography

Images of HeLa, ME-180 and SiHa cells treated with monotherapies and combination therapies of INI-43, Cisplatin and Doxorubicin for 24 hours were captured using the EVOS M5000 Imaging System (Thermo Fisher Scientific, USA) at 10X magnification and 70% confluency. Cell morphology changes observed under the microscope following 24 hr treatments were noted.

2.2.6 Statistical analyses

The Student's t test was used to compare mean values when comparing 2 groups and the ANOVA technique was used when comparing mean values between 3 groups. P value <0.05 was considered statistically significant.

CHAPTER 3

INVESTIGATING THE EFFECT OF INI-43, CISPLATIN AND DOXORUBICIN AS SINGLE AGENT TREATMENTS

3.1 INTRODUCTION

Extreme biological heterogeneity between different cancers as well as within a specific type of cancer has made it exceptionally challenging to understand and treat this complex disease. Although conventional chemotherapies have had some success in treating cancer, they seem to reach a plateau of efficacy and this, coupled with a better understanding of cancer biology, has led to the development of targeted anti-cancer drugs. Hence, over the past 30 years, the focus of new drug development has been on targeting known oncogenic drivers[46].

However, conventional chemotherapies such as Cisplatin and Doxorubicin are still the first line therapeutics for many cancers, including cervical cancer and breast cancer, amongst others. Conventional chemotherapies like these lack specificity in targeting cancer cells and are restricted in their efficacy by dose-limiting toxicities. On the other hand, molecularly targeted agents exploit the oncogene addictions and specific hallmarks of cancer cells. In this way, the hallmarks and oncogene addictions of cancer cells become the vulnerabilities to which targeted agents are developed.

Previous work in our lab involved a structure-based virtual screen in which 12 million chemical compounds were screened to identify small molecules with potential to bind and inhibit

Kpnβ1, a nuclear import receptor that has been shown in our lab to be overexpressed at both mRNA and protein levels, and is essential for cervical cancer cell survival and proliferation[52]. Through this computational screen, and subsequent in vitro testing, compound 43 (INI-43) was identified. In contrast to the general cytotoxicity of Cisplatin and Doxorubicin, rescue experiments in our laboratory have shown that INI-43 exerts its effects primarily via inhibiting Kpnβ1, thus classifying it as a targeted therapy. Due to the success of combining targeted and conventional chemotherapies in combination therapy, it is of interest to determine whether INI-43 and Cisplatin/Doxorubicin could be useful combinations. To this end, the effects of the drugs as single agents needed to first be determined. In this chapter the effects of INI-43, Cisplatin and Doxorubicin as monotherapies were investigated on cell sensitivity, cell proliferation, reproductive (colony-forming) ability and apoptosis, using cervical cancer cell lines, HeLa, SiHa and ME-180, and the non-cancer epithelial cell line ARPE-19.

3.2 RESULTS

3.2.1 IC_{50} determination for INI-43, Cisplatin and Doxorubicin as single agent treatments in cervical cancer cells

Cell line sensitivities of HeLa, SiHa and ME-180 to INI-43, Cisplatin (CDDP) and Doxorubicin (DOX) as single agent treatments were assessed by determining the IC_{50} values, which is the effective concentration at which 50% cell death is observed. Cells were treated with a range of concentrations of either INI-43, CDDP or DOX for 24 hours and cell viability was measured using the MTT assay. Absorbance readings (OD_{595}) were plotted against Log(x) transformed drug concentrations to generate sigmoidal dose-response curves, from which IC_{50} values were determined (Fig. 3.1). The average IC_{50} results with 95% confidence intervals are tabulated in Table 3.1.

As shown, the three cervical cancer cell lines HeLa, ME-180 and SiHa had IC_{50} values of approximately 10.6, 11.3 and 14.9 μM respectively for INI-43 (Table 3.1), indicating that at these concentrations there was half maximal cell kill. Interestingly, it was observed that INI-43 exerts its cytotoxicity rapidly as cell death was observed under the microscope within a few hours post treatment. In addition, there appears to be a very narrow concentration window in which INI-43 kills the cells, as seen by the steep hillslopes of the representative curves in Figure 3.1 (A, D, G) (a very small difference in INI-43 concentration is needed for less than 50% cell kill versus near or complete cell death).

The IC_{50} values for the two conventional chemotherapies CDDP and DOX were also similar across the three cancer cell lines. The IC_{50} values for CDDP in HeLa, ME-180 and SiHa were 34.4, 36.5 and 41.7 µM respectively. For DOX, the IC_{50} values in HeLa, ME-180 and SiHa cell lines, were 1.1, 1.2 and 1.8 µM respectively. Interestingly, the respective IC_{50} for INI-43, CDDP and DOX was higher in SiHa cells compared to the other cervical cancer cell lines, in line with its classification as a more chemo-resistant cell line.

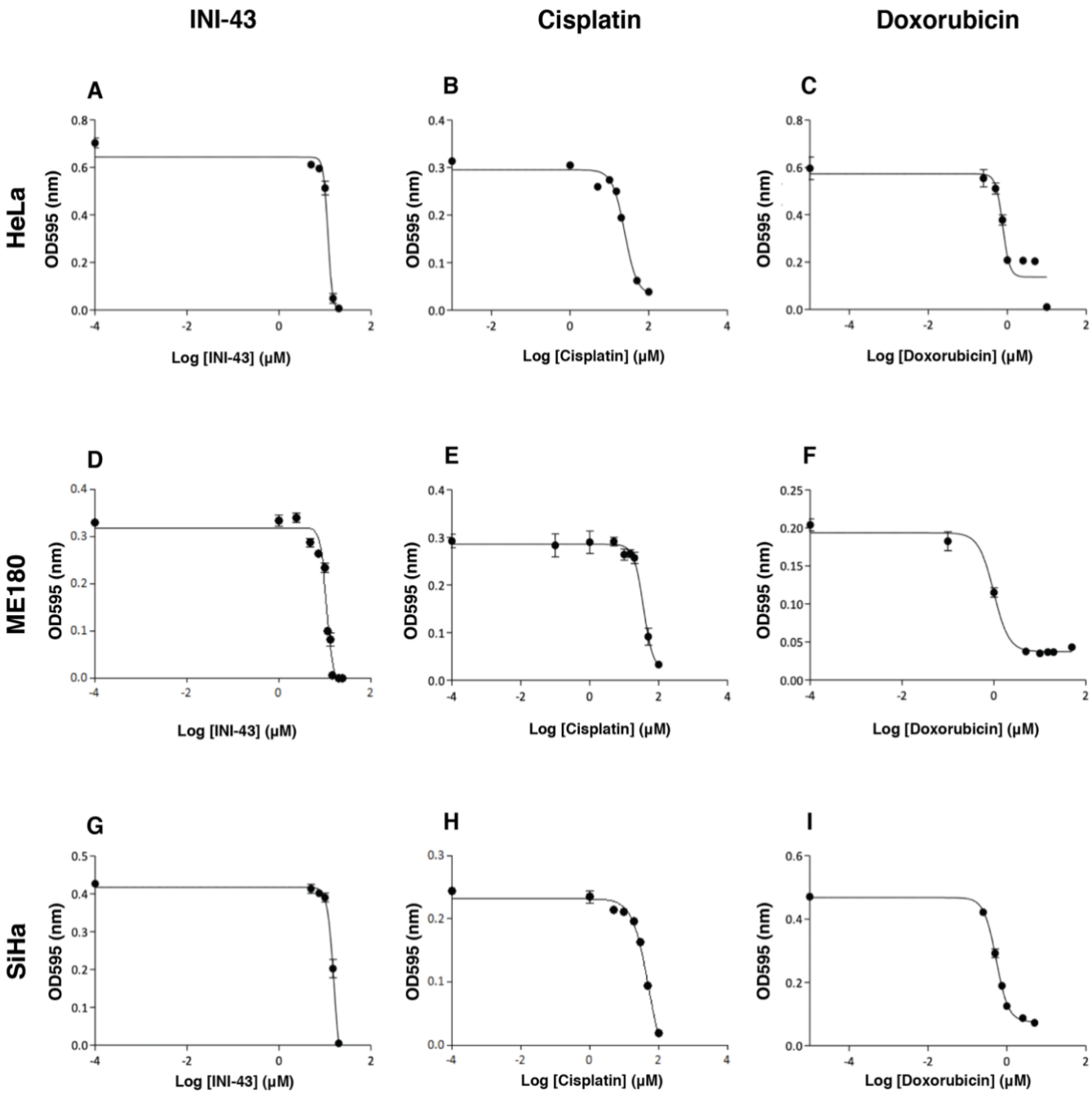


Figure 3.1. Representative survival curves demonstrating sensitivities of cervical cancer cell lines to INI-43, Cisplatin and Doxorubicin as monotherapies. Cervical cancer cell lines**:** HeLa, ME-180 and SiHa were treated with a range of INI-43 (A,D,G), Cisplatin (B,E,H) or Doxorubicin (C,F,I) concentrations for 24 hours and cell viability was quantified using the MTT assay. Dose-response curves were generated and IC_{50} values were calculated using Graph Pad Prism software. IC_{50} determination experiments were performed at least 3 independent times in triplicate and individual data points of representative curves are depicted as the mean OD_{595nm} reading ± SEM.

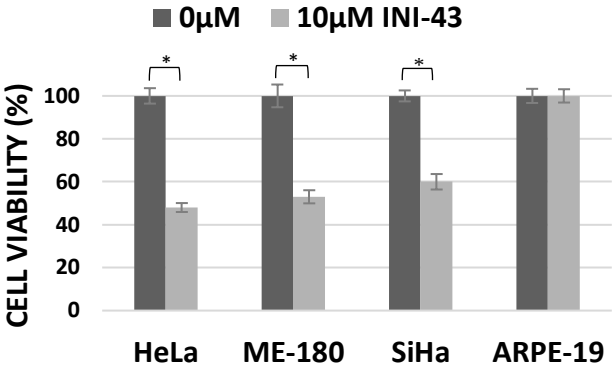
Table 3.1. Average IC_{50} values of INI-43, Cisplatin and Doxorubicin in cervical cancer cell lines.

	INI-43 (µM)	95% CI	CDDP (µM)	95% CI	DOX (µM)	95% CI
HeLa	10.6	8.2-14.4	34.4	28.5-38.4	1.1	0.5-2.2
ME-180	11.3	9.1-16.6	36.5	29.9-42.3	1.2	0.8-2.6
SiHa	14.9	12.8-19.8	41.7	30.1-48.9	1.8	0.3-3.0

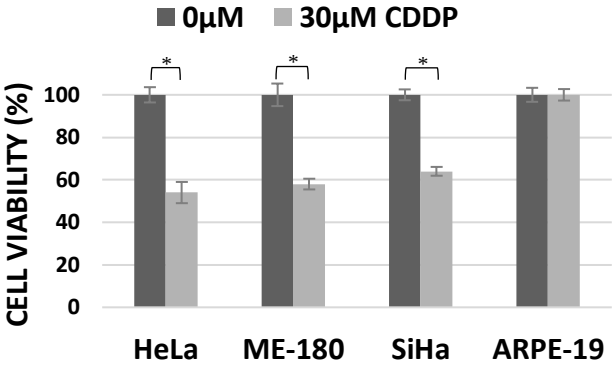
3.2.2 Effect of approximate IC_{50} concentrations of INI-43, Cisplatin and Doxorubicin on non-cancer cells

The non-cancer cell line, ARPE-19, was next treated with INI-43, CDDP and DOX, at the approximate IC_{50} concentrations obtained for the cervical cancer cell lines and cell viability was determined using the MTT assay. Treatment of the cancer cell lines with an approximate IC_{50} of 10 µM for INI-43 had significant cell killing effects in HeLa, ME-180 and SiHa cells, while the non-cancer cell line was insensitive to this concentration (Fig. 3.2 A). The non-cancer cell line was also insensitive to the approximate IC_{50} concentrations of 30 µM CDDP (Fig. 3.2 B) and 1 µM DOX (Fig. 3.2 C), in contrast to the significant cell killing effect seen in the cancer cell lines. For INI-43, CDDP and DOX treatment in the non-cancer cells, no signs of cell death were visible and 100% cell viability was determined (Fig. 3.2 A, B, C), thus ARPE-19 cells exhibited a significantly reduced sensitivity to the three drugs compared to the cancer cell lines.

A



B



C

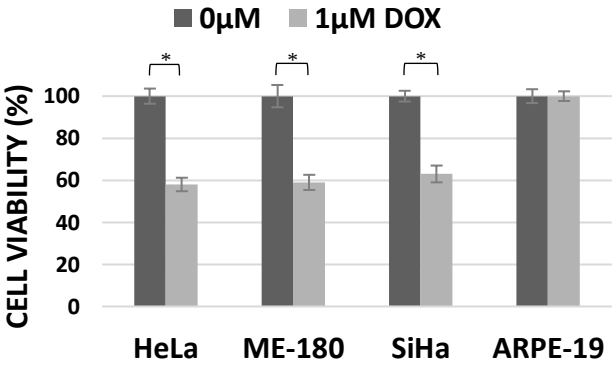


Figure 3.2. The effect of INI-43, CDDP and DOX on the viability of cervical cancer and non-cancer epithelial cells. Cells were plated at 5000 per well in a 96-well dish and allowed to settle overnight, followed by incubation with 0 μM, 10 μM INI-43, 15 μM CDDP or 1 μM DOX for 24 hours. Viable cells were determined using the MTT reagent and represented as % of total cell viability (100%). Results shown are mean ± SEM of experiments performed in 3 replicates and repeated at least two independent times, *$p < 0.05$.

3.2.2 Effect of single agent treatments on proliferation of cervical cancer cells and non-cancer epithelial cells

Having shown that INI-43, CDDP and DOX selectively reduced cervical cancer cell viability, we next investigated their individual effects on anchorage-dependent cell proliferation. HeLa, SiHa, ME-180 and ARPE-19 cells were treated with the vehicle control (DMSO), ½ IC_{50} values and IC_{50} values of INI-43, CDDP and DOX and cell proliferation was monitored for four days using the MTT assay.

5 µM (½ IC_{50}) INI-43 had little effect on the proliferation of the three cancer cell lines as well as non-cancer ARPE-19 cells. HeLa, ME-180 and SiHa cells treated with 10 µM (IC_{50}) INI-43 treatment showed significant inhibition of proliferation and this effect was sustained for the four-day period with no recovery observed. Proliferation of the non-cancer ARPE19 cells, on the other hand, was not affected by 10 µM INI-43 (Fig. 3.3 A).

15 µM (½ IC_{50}) and 30 µM (IC_{50}) CDDP-treated cancer cells showed significant inhibition of cell proliferation from day 1 until day 4. For DOX, ½ IC_{50} (0.5 µM)-treated cells showed significantly reduced proliferative abilities and IC_{50} (1 µM)-treated cells showed complete cell death from day 2 to day 4 with no recovery observed for all three cancer cell lines. Non-cancer ARPE-19 cells remained unaffected by all single agent treatments tested. This supports earlier findings that cancer cells are more sensitive to INI-43, CDDP and DOX treatment compared to non-cancer cells.

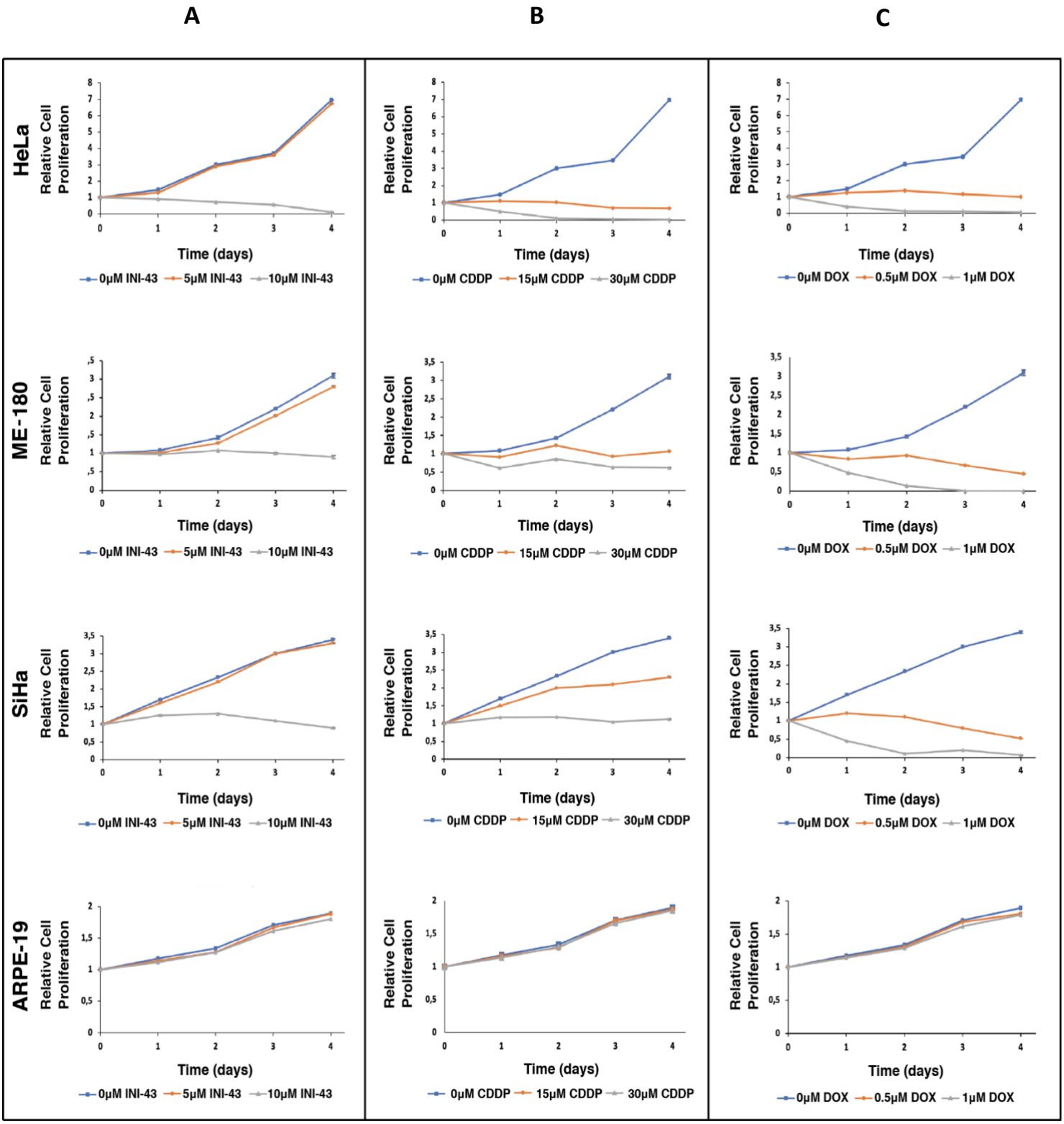


Figure 3.3. Proliferation of cervical cancer and non-cancer cell lines treated with INI-43, CDDP and DOX over 4 days. Cells were seeded at 1500 cells per well in 96-well plates and treated with 0 μM, 5 μM INI-43, 10 μM INI-43, 15 μM CDDP, 30 μM CDDP, 0.5 μM DOX or 1 μM DOX the following day. Proliferation was examined every 24 hours using the MTT reagent for 4 days and normalized to the viable cells on day 0. Results shown are mean ± SEM of 3 replicates, and each experiment was repeated at least two independent times.

3.2.2 Effect of single agent treatments on colony formation of cervical cancer cells

We next tested the effect of INI-43, CDDP and DOX on cervical cancer cell colony formation, by performing the anchorage-dependent colony formation assay. This assay is also an *in vitro* cell survival assay but different from the proliferation assay because it assesses the ability of the cells to form colonies after the drug-containing media has been removed. This assay is based on the ability of a single cell to grow into a colony, where a colony is defined as 50 cells or more[92].

Cells were sparsely seeded and treated with the concentrations previously used for the proliferation assay; that is, the approximate ½ IC_{50} and IC_{50} values for INI-43, CDDP and DOX, and treatment was for a 24 hr period. The drug-containing growth media was then removed and replaced with fresh media. After 8 days incubation, with growth media replaced every 48 hours, viable cells were fixed and stained with crystal violet and macroscopic colonies were visually examined.

In all three cancer cell lines, there was no observable difference in colony formation between untreated cells and 5 µM INI-43-treated cells (Fig. 3.4 A). For 10 µM INI-43 treated cells however, there were no visible colonies (Fig. 3.4 A). There were also no visible colonies for 0.5 µM and 1 µM DOX treated cells (Fig. 3.4 B), as well as 15 µM and 30 µM CDDP treated cells (Fig. 3.4 C). Colonies were solubilised and absorbance was measured to quantify cancer cell colonies. These findings, when coupled with the proliferation assay results, demonstrated that 10 µM INI-43, 1 µM DOX and 30 µM CDDP effectively inhibited cancer cell survival and growth.

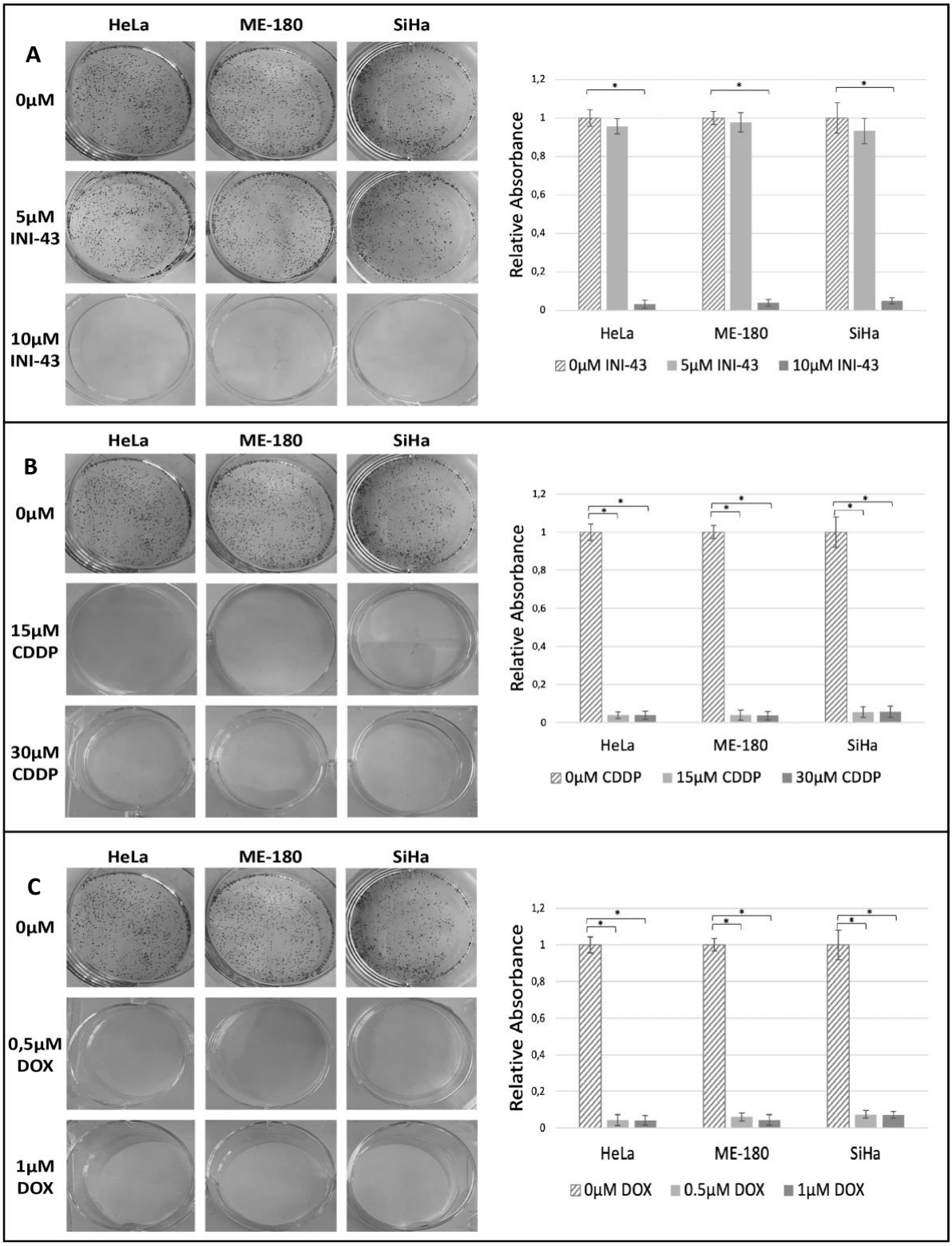


Figure 3.4. Colonies of HeLa, ME-180 and SiHa cells formed after INI-43, CDDP and DOX exposure. Cells were plated sparsely at 1000 per dish followed by treatment at approximate ½ IC_{50} or IC_{50} of each drug. After 24 hours, drug containing media was removed and media was replenished every second day for 8 days. Viable colonies were fixed and stained with crystal violet, images were captured and absorbance was measured after solubilisation with acetic acid. Images are representatives of experiments performed in triplicate and repeated two independent times. Quantified results are mean ± SEM experiment performed in triplicate, *$p < 0.05$.

3.2.3 Single agent treatments of INI-43, Cisplatin and Doxorubicin result in activation of the apoptotic cell death pathway

Next, a defining characteristic of the apoptotic pathway was investigated, namely PARP1 cleavage. The PARP1 antibody detects endogenous levels of full length PARP1 (116 kDa), as well as the large fragment (89 kDa) of PARP1 resulting from Caspase cleavage. HeLa, ME-180 and SiHa cells were treated with ½ IC_{50} or IC_{50} concentrations of each agent individually for 24 hours. Proteins were harvested and Western blot analysis was performed to detect PARP1 cleavage and GAPDH was used as a loading control. The ratio of cleaved PARP1 (89 kDa) to uncleaved PARP1 (116 kDa) normalised against GAPDH (the loading control) was quantified using Image J analysis and represented in Figure 3.5.

A dose-dependent increase in levels of cleaved PARP1 after treatment with drugs was observed for all three cancer cell lines. HeLa cells appeared to be the most sensitive to INI-43 as 5 µM INI-43 induced some PARP1 cleavage (Fig. 3.5 A) but this concentration of INI-43 had no effect on PARP1 cleavage in ME-180 (Fig. 3.5 B) and SiHa cells (Fig. 3.5 C). However, after treatment with 10 µM INI-43, similar levels of cleaved PARP were detected in HeLa and ME-180 cells and there was some cleaved PARP1 induced in the SiHa cells as well. In HeLa and ME-180 cells, PARP1 cleavage was also induced to a similar level after DOX and CDDP treatments (Fig. 3.5 A and B), while in SiHa cells PARP1 cleavage was slightly less pronounced but still evident at the higher concentrations of DOX and CDDP (Fig. 3.5 C). This confirms that the cells are undergoing apoptosis.

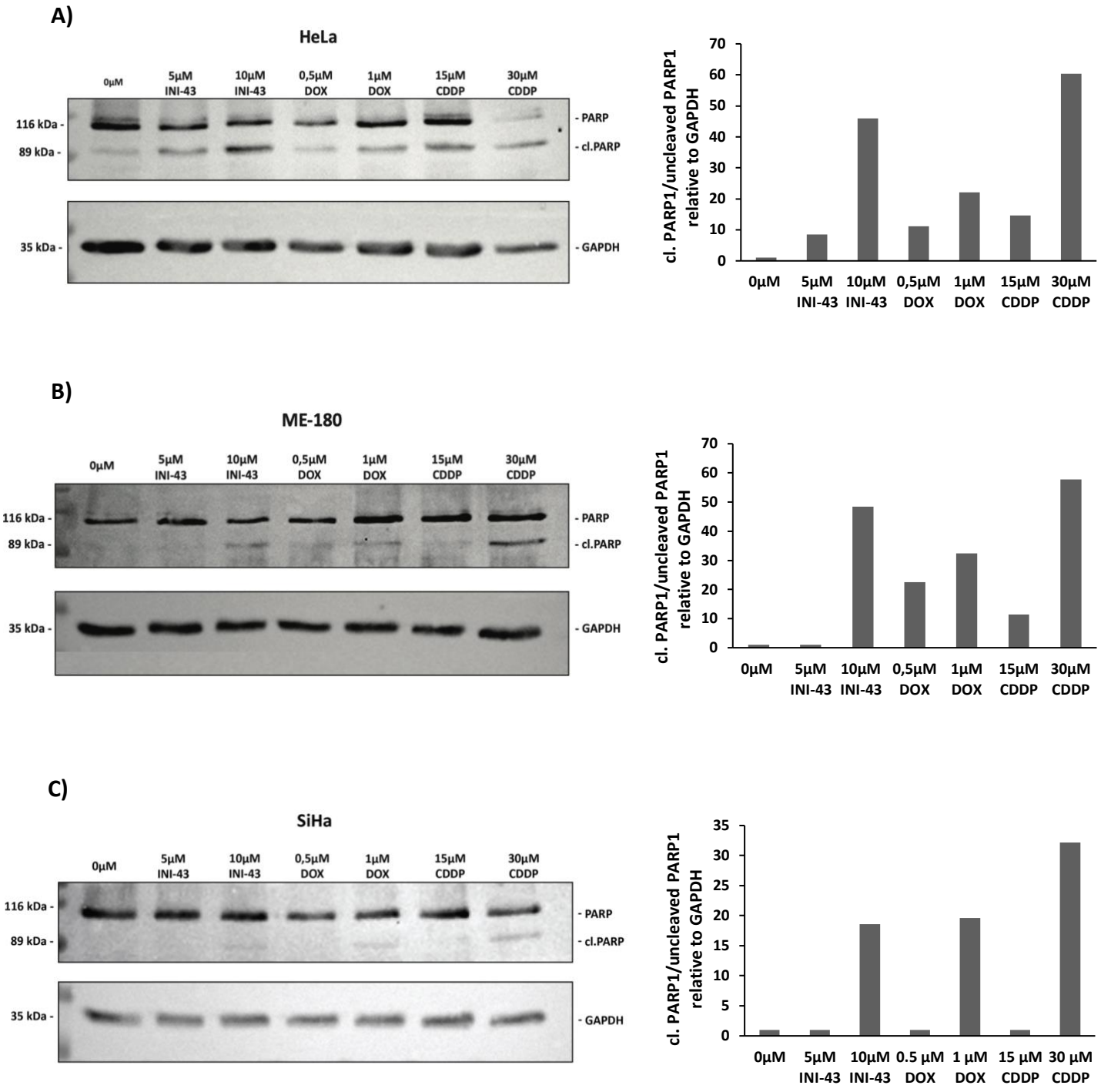


Figure 3.5. PARP1 cleavage after individual treatment of cervical cancer cells with INI-43, Cisplatin and Doxorubicin. (A) HeLa, **(B)** ME-180 and **(C)** SiHa cells were treated, for 24 hours, with either no drug, 5 μM or 10 μM INI-43, 0.5 μM or 1 μM DOX, 15 μM or 30 μM CDDP (½ IC_{50} and IC_{50} of each drug). 24 hours later, proteins were harvested and Western blot analysis was performed to identify uncleaved PARP (116 kDa) and cleaved PARP (cl. PARP, 89 kDa) using an αPARP1 antibody. GAPDH was used as the loading control. Band intensity was measured using the ImageJ software and the ratio of cl. PARP to PARP (normalized against GAPDH) was plotted for each treatment group. The experiment was repeated twice in each cell line.

3.3 DISCUSSION

In this chapter the effects of individual treatments of INI-43, CDDP and DOX on cervical cancer and non-cancer ARPE-19 cells were investigated using multiple techniques. This chapter focused on identifying the IC_{50} value of each drug in each cell line and exploring cell proliferation and reproductive (colony-forming) ability after 24 hr monotherapy treatments. This chapter also investigated the effect the single agent treatments had on PARP1 cleavage as a measure of apoptosis and this was achieved by performing the analytical technique, Western blotting.

IC_{50} values were determined for INI-43, CDDP and DOX by performing the MTT assay. The IC_{50} values for each single agent were similar across the three cervical cancer cell lines, with SiHa cells being slightly more resistant to INI-43 (~15 µM compared to ~10 µM), CDDP (~40 µM compared to ~30 µM) and DOX treatment (~1,8 µM compared to ~1 µM). This is in agreement with previous studies of cell morphology, MTT and colony formation assays which revealed SiHa cells to be less sensitive to CDDP compared to CasKi and HeLa cells[93,94,95]. Furthermore, HeLa cells were the most sensitive to CDDP compared to Caski and SiHa cells, which is in line with results from this study which found HeLa cells to be the most sensitive and SiHa cells to be slightly less affected by CDDP, demonstrated by SiHa cells having a higher IC_{50} value than HeLa and ME-180 cell lines. A study by Hafiza and Latifah (2014) found the CDDP IC_{50} value in SiHa cells to be approximately 40 µM[96], which supports the IC_{50} values determined in this study. In addition, a study by Funaoka et al. (2006) investigated the resistance of SiHa cells to CDDP and proposed that the levels of p21 play a role, whereas a study by Venkatraman et al. (2005) attributed the CDDP resistance to NFκB activity in SiHa cells. Hence, SiHa cells are

widely used in the literature as a chemo-resistant cell line and thus it is interesting that results from this study identify them as being more resistant to INI-43 than the other cervical cancer cell lines used in this study, namely HeLa and ME-180. The longer doubling time of SiHa cells (±2.6 days compared to 1/1.5 days for HeLa and ME-180 respectively[97]) may partially explain why they are slightly less sensitive to INI-43 than HeLa and ME-180 cells. SiHa cells replicate at a slower rate and therefore fewer cells will be affected by its anti-mitotic activity in a given 24 hr treatment period.

DOX treatment resulted in the lowest IC_{50} concentrations, exhibiting cytotoxicity in concentrations between 1 µM and 2 µM for all three cancer cell lines, which is in line with what previous studies have found in cervical cancer cells (IC_{50} 1.66 ± 0.30)[98,99]. The IC_{50} values determined for the three cervical cancer cell lines used in this study are also similar to IC_{50} results in breast cancer cells treated with DOX (Mcf-7 DOX IC_{50} 2.2±4.3)[100]. The 'Genomics of Drug Sensitivity' database describes SiHa cells to be more resistant to DOX than HeLa and ME-180 cells[101]. A study by Filippova et al. (2014) supports the DOX IC_{50} results in this study as it showed SiHa cells to be more resistant to DOX than CaSki cells, and linked the chemoresistance of SiHa cells to a higher level of anti-oxidant acting proteins[102]. This is in line with the higher IC_{50} values determined in this study for SiHa cells compared to HeLa and ME-180 cell lines.

Most importantly, this study identified all three chemotherapies to be significantly more cytotoxic to cancer cells in comparison to a non-cancer epithelial cell line. In contrast to the three cancer cell lines, cell viability was unaffected for ARPE-19 cells treated at 10 µM INI-43, 30 µM CDDP and 1 µM DOX; doses that killed all three cancer cell lines used in this study.

Both *in vitro* cell survival assays; cell proliferation and colony formation, showed similar trends after 24 hr single agent treatments. For both assays, results after 5 µM INI-43 treatment were not significantly different to untreated cells and 10 µM INI-43 had significant inhibitory effects in the HeLa, ME-180 and SiHa cell lines. This is in line with a recent study by Kelenis et al. (2022) which showed that Kpnβ1 inhibition obliterated the proliferation and colony formation of lung cancer cells[71]. A study by Zhu et al. (2018) showed similar results in a glioblastoma cell line where after Kpnβ1 knockdown, cell proliferation and colony formation were inhibited[103]. Treatment with 0.5 µM DOX and 15 µM CDDP also showed complete inhibition of colony formation. The DOX result is supported by a recent study by Mehraj et al. (2022) which showed significant reduction of colonies formed in triple negative breast cancer cells after treatment with DOX at less than a tenth of the concentration that was used in this study[104]. The CDDP result is in line with results from a study by Wu et al. (2016) which showed the proliferation and colony formation of SiHa cells after CDDP treatment to be significantly reduced.

Western blot analysis supported the results obtained in the cell proliferation and colony formation assays. PARP is a DNA repair protein cleaved in mid/late stage apoptosis, thus cleaved PARP1 was used as a measure of Caspase cascade activation and execution of the apoptotic process. Cleaved PARP1 was observed after single agent treatment using IC_{50} concentrations of the drugs (10 µM INI-43, 1 µM DOX and 30 µM CDDP). For HeLa cells, unexpectedly, a faint cleaved PARP1 band is seen in the untreated group, however, when the cleaved PARP1 band is quantified relative to the uncleaved PARP1 band, the amount of cleaved PARP1 is not significant. Also, when compared in relation to the bands for treatment groups, it is not significant. An underlying level of apoptosis is expected to be found in a HeLa

cell culture, due to the fast-growing nature of the cells. Although HeLa cells exhibited an increase in the level of cleaved PARP1 at a concentration of 5 µM INI-43, this effect was not nearly as apparent as at 10 µM INI-43 treatment. As expected, treatment with the approximate IC_{50} concentrations, 10 µM INI-43, 1 µM DOX and 30 µM CDDP, showed higher levels of cleaved PARP1 compared to their respective lower concentrations that were tested. HeLa cells showed the most prominent cleaved PARP1 bands compared to ME-180 and SiHa cells, supporting the findings from 3.2.1 and 3.2.2 which show HeLa to be more sensitive than ME-180 and SiHa cells and SiHa cells to be most resistant to all three single agent treatments. Although INI-43 was not used specifically, the study by Zhu et al. (2018) showed that Kpnβ1 knockdown induced significant, dose-dependent PARP cleavage in glioblastoma cells[103]. Additionally, a study by Suh et al. (2010) showed PARP cleavage following DOX treatment in CaSki and C33A cervical cancer cell lines[105].

This study explored the biological effects of single agent treatments of INI-43, CDDP and DOX in cervical cancer cell lines. Since 24 hr treatment with INI-43 at concentrations below 10 µM, specifically 5 µM INI-43, had minimal or no effect on cervical cancer cell viability, this sublethal concentration was next used to investigate whether INI-43 could enhance the efficacy of the conventional chemotherapies CDDP and DOX, including their apoptotic effects and DNA-damaging abilities. Chemoresistance that develops after single agent chemotherapy is a critical factor as to why it is necessary to find a combination therapy whereby one drug can sensitise the cancer cells to CDDP/DOX – this is what we aim to determine using INI-43.

CHAPTER 4

INVESTIGATING THE EFFECT OF INI-43, CISPLATIN AND DOXORUBICIN IN COMBINATION TREATMENTS

4.1 INTRODUCTION

Adverse chemotherapy side effects and the complexity of acquired drug resistance suggests that targeted and combination therapies may offer improved efficacy in treating cancers in the clinic[77]. Novel therapeutic agents that can act synergistically with existing, conventional therapies are anticipated to improve the likelihood of successful therapy response and survival rates[106]. The addition of molecularly targeted treatments to conventional therapies, such as platinum-based regimens, is a relatively recent anti-cancer subject of study[84]. For example, a recent clinical trial found the addition of the angiogenic inhibitor, Bevacizumab, to Carboplatin and Paclitaxel combination chemotherapy yielded higher response rates and increased overall survival[107]. Thus, although it is a relatively new anti-cancer strategy, there are already successful cases, whereby combining a targeted therapy with conventional chemotherapies results in an improved treatment response.

In both combinatorial and single agent research there have been more studies of exportins and their inhibitors compared to importins and their inhibitors[108]. Specifically, targeting Exportin 1 (CRM1/XPO1) has been extensively studied as a single agent therapy as well as part of combination treatments in preclinical and clinical trials[109,110,111]. Although significant preclinical and clinical work is currently being carried out for inhibitors of Exportin 1, such as

the recently FDA-approved Selinexor, studies of nuclear import inhibitors are still in the early stages and investigations into their anti-cancer effects are limited to preclinical models. However, preliminary research indicates that concurrently targeting nuclear import pathways can increase the effectiveness of available chemotherapy drugs[112].

This chapter investigates the combined effects of the targeted nuclear import inhibitor, INI-43, with conventional chemotherapies, either Cisplatin or Doxorubicin, in cervical cancer cell lines representative of squamous cell cervical carcinoma (ME-180 and SiHa) and adenocarcinoma of the cervix (HeLa), and ARPE-19 non-cancer cells.

4.2 RESULTS

4.2.1 Determining effective ratios to be used in combination experiments

Since IC_{50} values for INI-43, CDDP and DOX in each cell line had been determined (Fig. 3.1), we next investigated the effects of INI-43 in combination with either CDDP or DOX. In order for each drug to induce a similar level of cell kill when used in combination, ratios were constructed based on the approximate single agent IC_{50} values. The combinations were designed using the equipotent constant-ratio design (or diagonal technique) as proposed by Chou and Talalay, (1984)[113].

For experimental conditions, 5000 cells per well were plated in a 96-well plate and the next day the cells were treated with single agent and combination treatments in constant ratios. For the INI-43:CDDP combination, a ratio of 1:3 (INI-43:CDDP) was chosen as it aligned with the IC_{50} values previously determined, which were approximately 10µM for INI-43 and 30 µM for CDDP. For the DOX combination, a ratio of 5:1 (INI-43:DOX) was chosen, based on approximate, previously determined IC_{50} values. The fixed ratios of nuclear import inhibitor: chemotherapeutic agent and the range of concentrations that was used to treat the cells is outlined below in Table 4.1 and 4.2.

Table 4.1. INI-43 and Cisplatin concentrations used for Combination Index determination experiments. Cells were treated with INI-43 and Cisplatin in combination at a fixed 1:3 ratio using the concentrations indicated below.

INI-43 (μM)	Cisplatin (μM)
Ratio 1:3	
1	3
2.5	7.5
5	15
7.5	22.5
10	30
12	36
13.5	40.5

Table 4.2. INI-43 and Doxorubicin concentrations used for Combination Index determination experiments. Cells were treated with INI-43 and Doxorubicin in combination at a fixed 5:1 ratio using the concentrations indicated below.

INI-43 (μM)	Doxorubicin (μM)
Ratio 5:1	
0.5	0.1
2.5	0.5
5	1
7.5	1.5
10	2
15	3

4.2.2 Investigating the effect of combination treatments with fixed ratios of INI-43:Cisplatin and INI-43: Doxorubicin.

HeLa, ME-180 and SiHa cells were treated with INI-43 and CDDP/DOX at the tabulated concentrations (Table 4.1 and 4.2) and cell viability was measured 24 hours after treatment using the MTT assay. Absorbance values were entered into CompuSyn software, a programme designed by Chou and Martin (2005) for quantitation of synergism and antagonism.

Combination index (CI) values were generated to determine the degree of drug interaction, based on the Chou-Talalay method. Combination index values generated by Compusyn were plotted against the fraction of affected cells (Fig. 4.1 and 4.2). As described by Chou et al. (2006), a CI value of less than 1 is defined as synergism, with values closer to 0 being described as strongly synergistic. A CI value greater than 1.10 is defined as antagonistic and CI values that fall between 1 and 1.10 are indicative of an additive interaction[87].

4.2.2.1 Effect of combining INI-43 and Cisplatin on cervical cancer cell viability

After 24 hr INI-43 and CDDP combination treatment, HeLa, ME-180 and SiHa cells showed a similar trend of interactions when CI values were plotted against fraction affected (Fa). For HeLa and SiHa cells, synergistic drug interactions were observed for all combinations of INI-43 and CDDP tested (Fig. 4.1 A and C). The most prominent effects of synergism were observed when the Fa>0.5, which is a promising finding as stronger synergism at a higher Fa value (>0.5) is most desirable in terms of clinical translation. At Fa=0.5 ME-180 cells showed an additive relationship but at higher Fa values a synergistic relationship prevailed (Fig. 4.1B). Since higher Fa values are more clinically relevant, it is important to acknowledge the improved drug interactions as Fa values increased. The combination index values obtained were maintained well below 1 at all levels of cell death in HeLa and SiHa cell lines, indicating a strong synergistic effect when the targeted agent (INI-43) was used in combination with conventional chemotherapy CDDP in these cell lines. The effects of INI-43:CDDP combinations in comparison to single treatments on cell viability for all three cell lines are shown in Fig. 4.1 A, B and C. These results show that the combination of INI-43 and CDDP caused a much greater inhibition on HeLa, ME-180 and SiHa cervical cancer cell viability compared to treatment with either single agent.

4.2.2.2 Effect of combining INI-43 and Doxorubicin on cervical cancer cell viability

INI-43 in combination with DOX at a fixed 5:1 ratio displayed moderate synergistic effects in the HeLa, ME-180 and SiHa cell lines (Fig. 4.2). The CI values were below 1 for HeLa and ME-180 cell lines at all concentration points tested, indicating a synergistic interaction with the combination of INI-43 and DOX (Fig. 4.2 A and B). SiHa cells, however, showed increasing CI values as Fa values increased (Fig. 4.2 C). The combination treatment therefore resulted in synergistic interactions but at Fa=0.5, this changed to an additive interaction (CI=1). The effect of INI-43:DOX combinations in comparison to single treatments on cell viability for all three cell lines are shown in Fig. 4.2 A, B and C. These results show that the combination of INI-43 and DOX caused a more pronounced inhibition on HeLa, ME-180 and SiHa cervical cancer cell viability compared to treatment with either single agent.

The Compusyn software is also able to predict dose reduction index (DRI) values for experimental and non-experimental data points. The DRI is a measure of the extent to which the dose of one or more agents in the combination can be lowered while still achieving the same cell kill effect comparable with those achieved with single agents. A DRI greater than 1 indicates a favourable dose reduction and the DRI was another way in which the combinations of the drugs were assessed. The software calculated that for HeLa cells treated with the INI-43 and CDDP combination, ~8 fold less CDDP was required, when combined with INI-43, to achieve 50% inhibition. A dose-reduction was similarly achieved when INI-43 was combined with DOX, however, the effect was not as pronounced as with CDDP. For HeLa cells treated with the INI-43 and DOX combination, ~2 fold less DOX was required, when combined with INI-43, to achieve 50% inhibition. Similar dose reduction results were found in the ME-180 and SiHa cell lines.

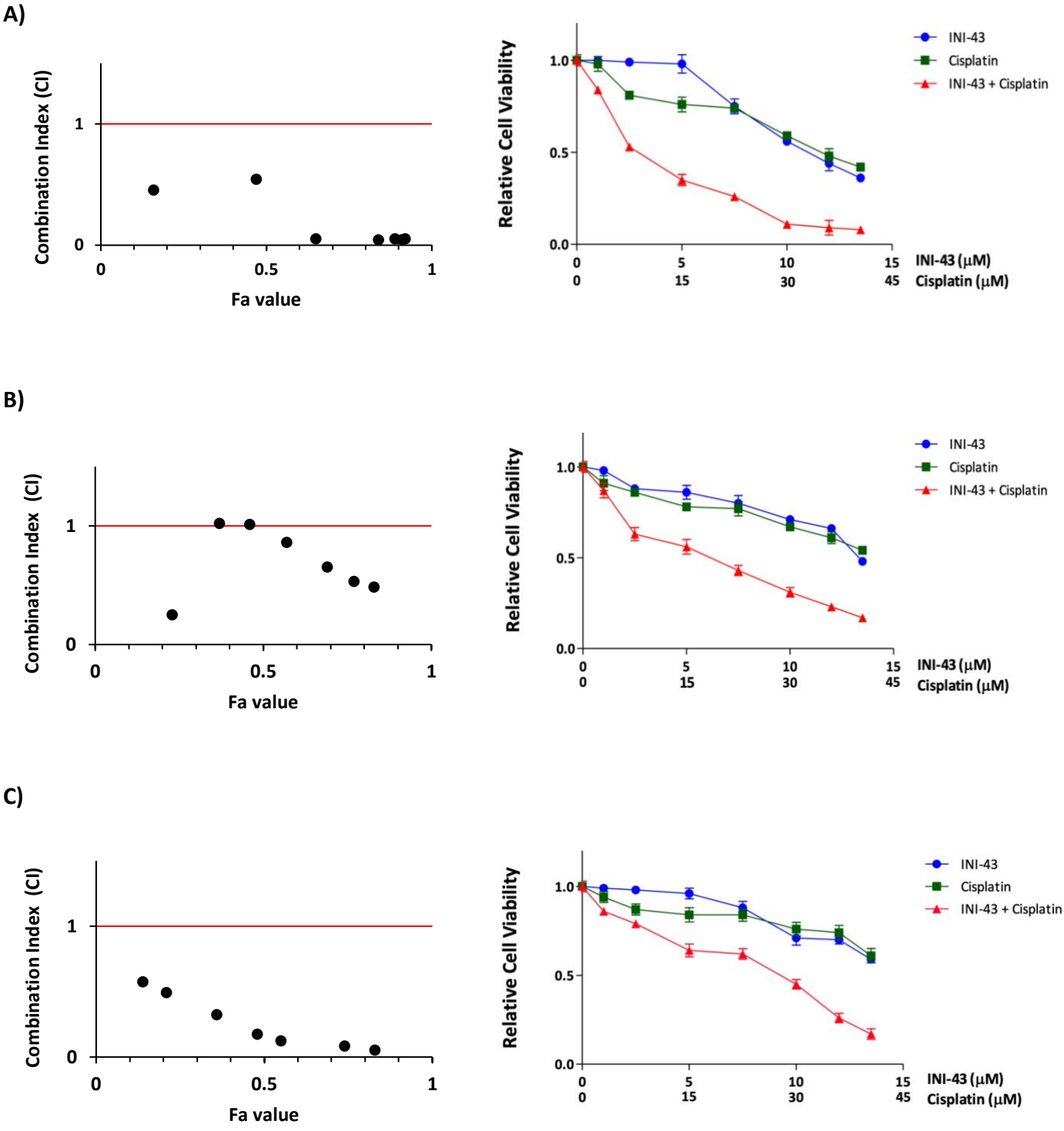


Figure 4.1. Effect of combination treatments of INI-43 and Cisplatin in HeLa, ME-180 and SiHa cell lines. (A) HeLa, **(B)** ME-180 and **(C)** SiHa cells were treated, for 24 hours, with a range of INI-43 and CDDP concentrations as single agents or in combination with each other at a fixed ratio of 1:3 (INI-43: CDDP). CI plots for (**A**) HeLa and (**B**) ME-180 and (**C**) SiHa cell lines were constructed by plotting CI values against Fa. Fa = 1 indicates complete cell death and Fa = 0 indicates no cell death. CI < 1 indicates synergism, CI = 1 indicates additive effects and CI>1 indicates antagonism. Data points plotted represent actual experimental results generated by the CompuSyn software. Relative Cell Viability graphs were plotted on GraphPad Prism 8. Results shown are of experiments performed in triplicate and repeated at least two independent times.

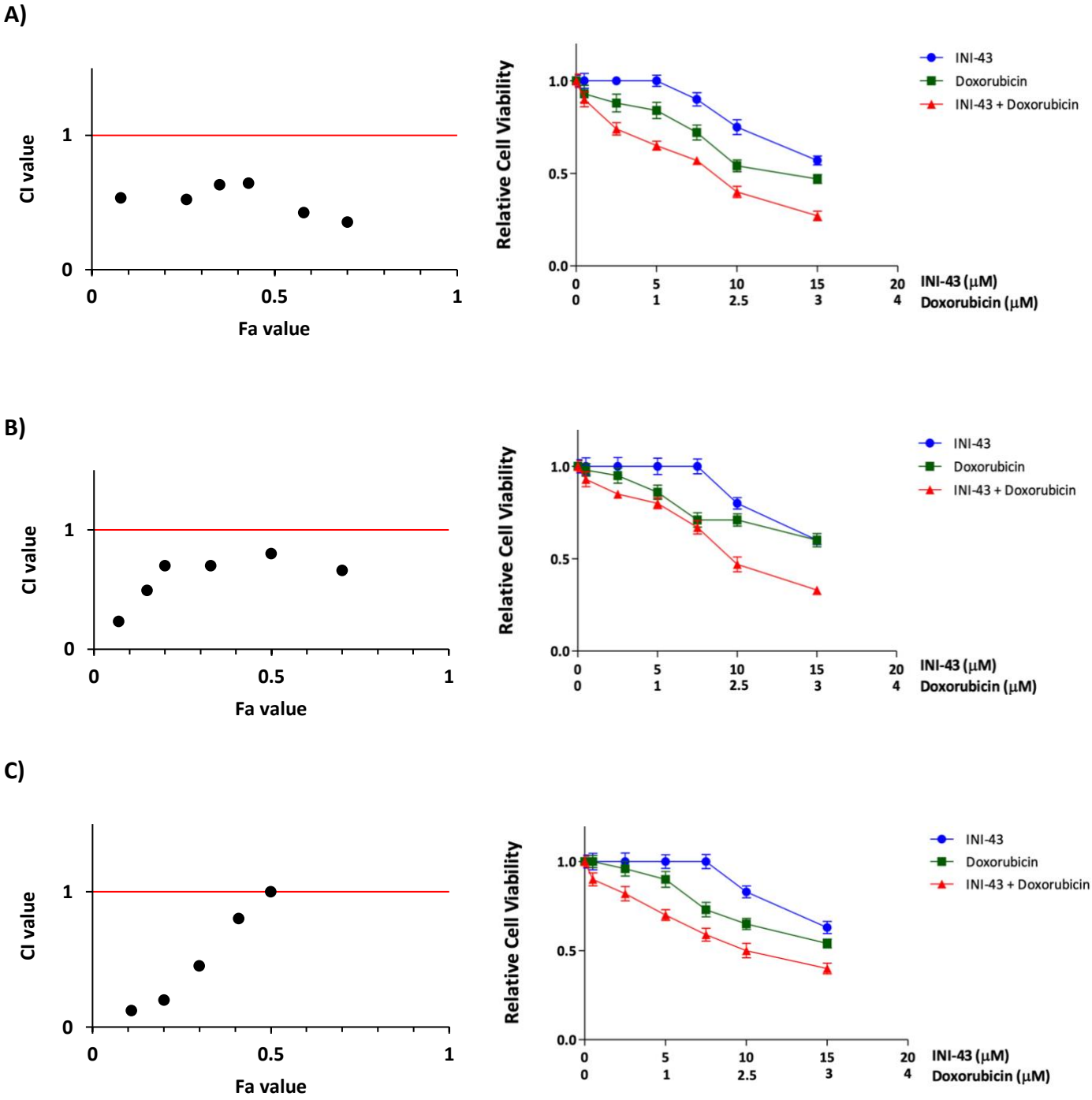
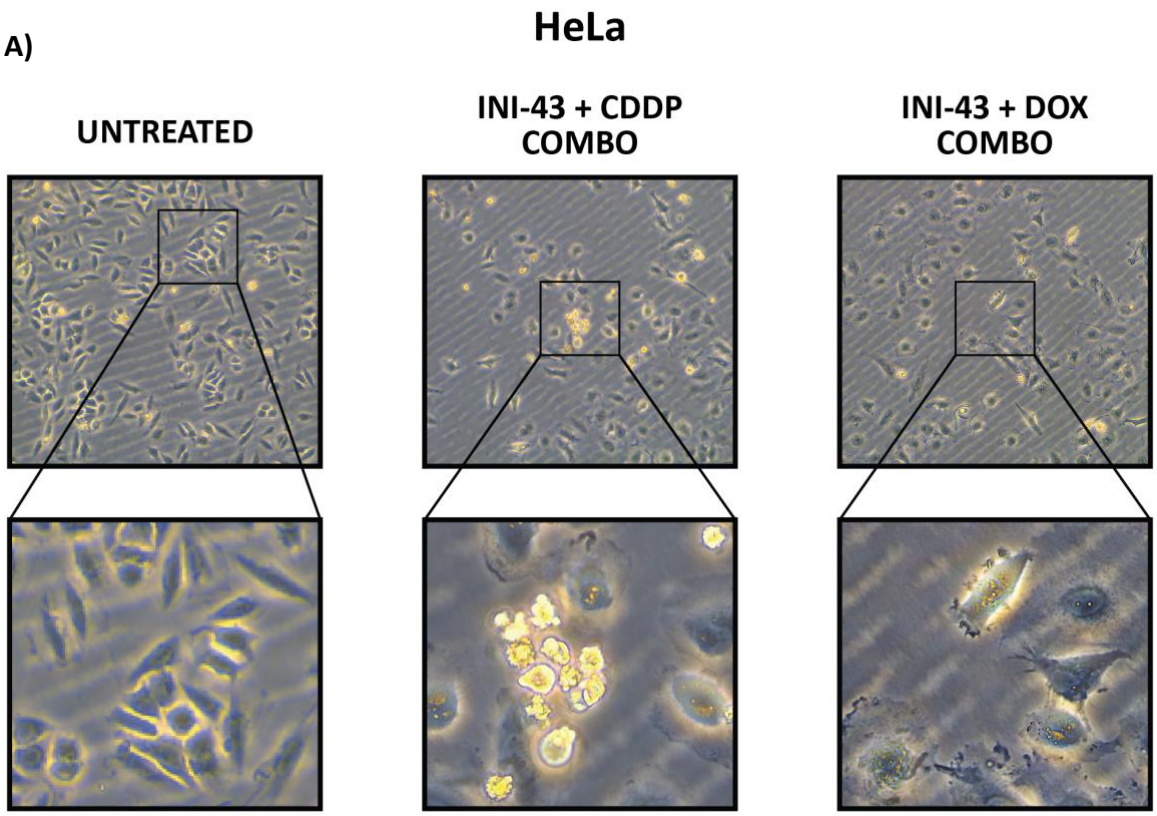


Figure 4.2. Effect of combination treatments of INI-43 and Doxorubicin in HeLa, ME-180 cells and in SiHa cell lines. (A) HeLa, **(B)** ME-180 and **(C)** SiHa cells were treated, for 24 hours, with a range of INI-43 and DOX concentrations as single agents or in combination with each other at a fixed ratio of 5:1 (INI-43: DOX). CI plots for (**A**) HeLa and (**B**) ME-180 and (**C**) SiHa cell lines were constructed by plotting CI values against Fa. Results shown are of experiments performed in triplicate and repeated at least two independent times.

4.2.3. Effect of combination treatments on cell morphology

To provide visual evidence of the combined effects determined in the Compusyn/cell viability experiments, images of cervical cancer cells 24 hr post treatment were captured using a digital inverted microscope. Changes in cell number and morphology after treatment were noted. In both combinations (INI-43 with both CDDP and DOX), a notable decrease in cell number was observed for all three cell lines; HeLa, ME-180 and SiHa (Fig. 4.3 A, B and C). In addition, morphological changes in the size and shape of the treated cells were observed. Following treatment, the cells appeared to have shrunk and adopted a more rounded shape. The rounded up cells also had more extracellular vesicles (a sign of apoptotic blebbing), suggesting that cell death in the combination treatment was as a result of apoptosis.



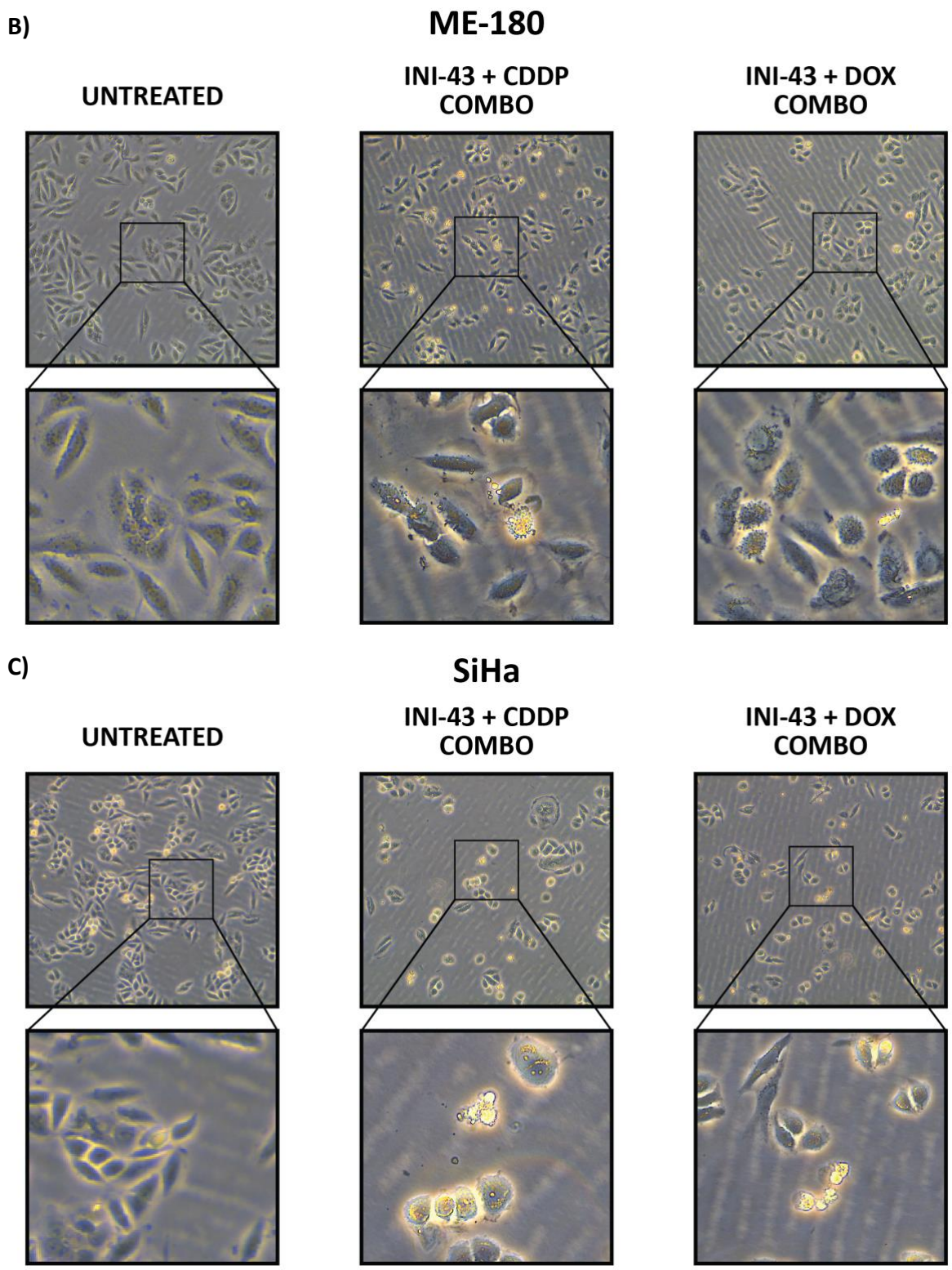


Figure 4.3. Effect of combination treatment on cervical cancer cell morphology. **(A)** HeLa, **(B)** ME-180 and **(C)** SiHa cells were treated with control (DMSO) or combination therapies of INI-43 and Cisplatin and INI-43 and Doxorubicin for 24 hours. Images were captured using the EVOS M5000 Imaging System (Thermo Fisher Scientific, USA) at 10X magnification and 60/70% confluency. The zoomed in area in combination treatments is representative of cells with rounded up appearance and showing signs of membrane blebbing Results are representative of experiments performed in triplicate and repeated at least two independent times.

4.2.4 Determining the levels of PARP1 cleavage and Caspase 3/7 activation in cervical cancer cells treated with INI-43 and Cisplatin in combination and INI-43 and Doxorubicin in combination

Since significant inhibition of cell viability and visible signs of cell death was observed upon combination treatment, we next investigated whether the enhanced cell death in the combination treated cells was as a result of increased apoptosis. Markers of apoptosis, such as PARP1 cleavage and Caspase 3/7 activation, were assayed as they represent two different stages of the apoptotic pathway. HeLa and ME-180 cells were treated with ½ IC_{50} concentrations individually (5 µM INI-43, 15 µM CDDP, 0.5 µM DOX) or their combinations (5 µM INI-43 + 15 µM CDDP, 5 µM INI-43 + 0.5 µM DOX) for 24 hours, after which apoptosis assays were conducted. PARP1 cleavage was evaluated by Western blot analysis and the amount of cleaved PARP1 present under each experimental condition was analysed and expressed relative to uncleaved PARP1.

4.2.4.1 Effect of combination treatments on PARP1 cleavage

During apoptosis, PARP1 is cleaved into two fragments of 89 kDa and 24 kDa. The levels of the 89 kDa cleaved PARP1 fragment were measured as an indication of apoptosis and expressed relative to total levels of uncleaved PARP1 which decreases upon cleavage. Densitometrical analysis of PARP1 Western blots was used to quantify cleaved PARP1 (89 kDa) relative to uncleaved PARP1 and normalised to GAPDH. Results from Western blot experiments for the cervical cancer cell lines; HeLa and ME-180 showed enhanced relative cleaved PARP1/uncleaved PARP1 after combination treatment compared to the respective individual treatments (Fig. 4.4 A and 4.5 A). Quantification of the cleaved PARP1 relative to

uncleaved PARP1 and normalised to the loading control GAPDH revealed an increase in cleaved PARP1 in the INI-43 and CDDP combination treatments to be greater than the sum of the effects observed after individual treatments (Fig. 4.4 B and 4.5 B). Similar results were observed for the INI-43 and DOX combination (Fig. 4.4 C and 4.5 C).

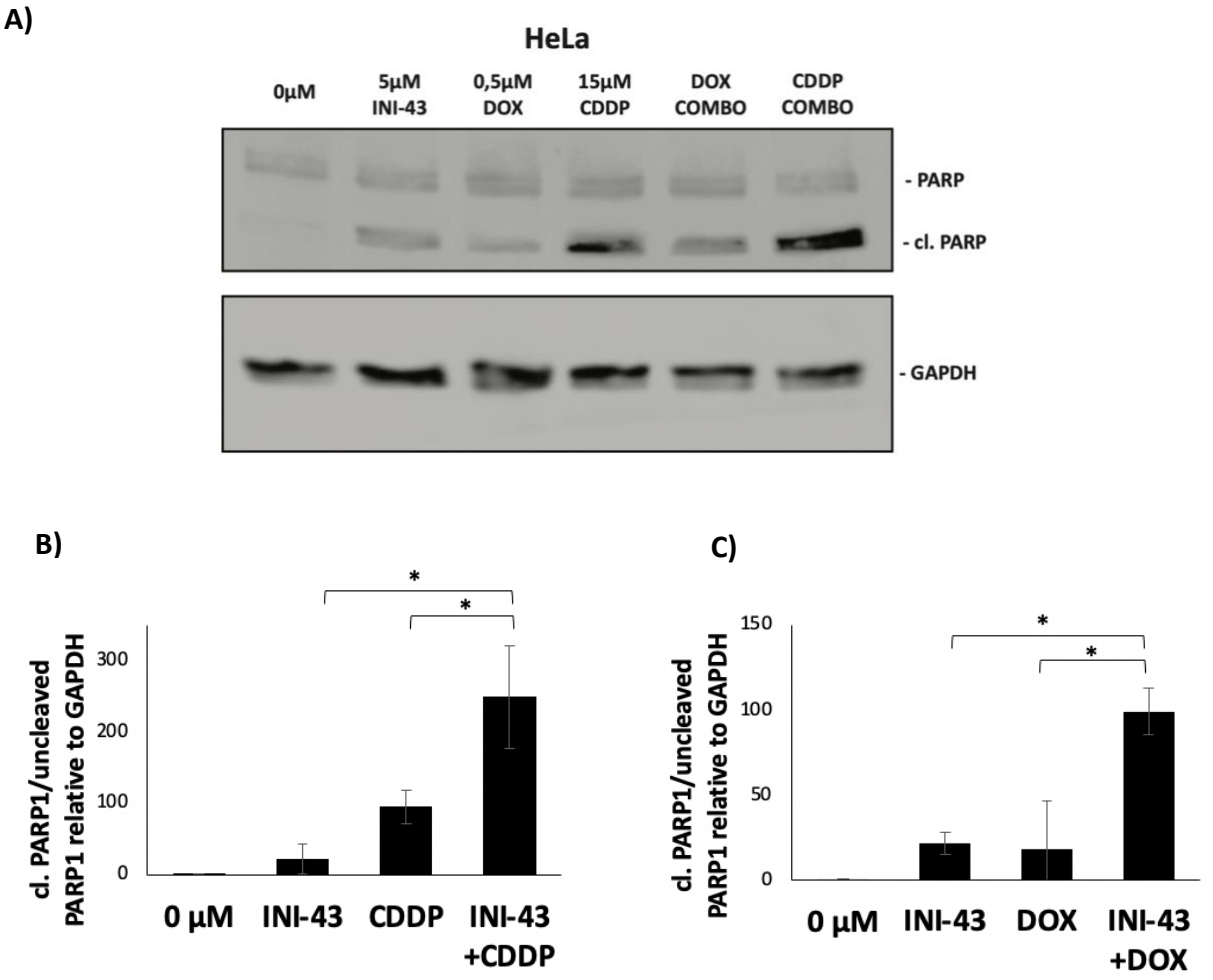


Figure 4.4. Effect of combination treatments of INI-43 and Cisplatin and INI-43 and Doxorubicin on apoptotic cell death in cervical cancer cells. HeLa cells were treated, for 24 hours, with no drug, 5 μM INI-43, 0.5 μM DOX, 15 μM CDDP or 5 μM INI-43 + 0.5 μM DOX or 5 μM INI-43 + 15 μM CDDP. Protein was harvested and Western blot analysis was performed to determine relative levels of uncleaved PARP1 (116 kDa) and cleaved (cl) PARP1 (89 kDa) using an αPARP1 antibody. GAPDH was used as the loading control. Band intensity was determined using ImageJ analysis software and the ratio of cl PARP1 to uncleaved PARP1 (normalized against GAPDH) was plotted for each treatment group. **(A)** Western blots shown are representative results of experiments repeated 3 independent times in each cell line. **(B, C)** Bar charts represent the mean ± SEM of experiments performed three independent times. *=$p<0.05$

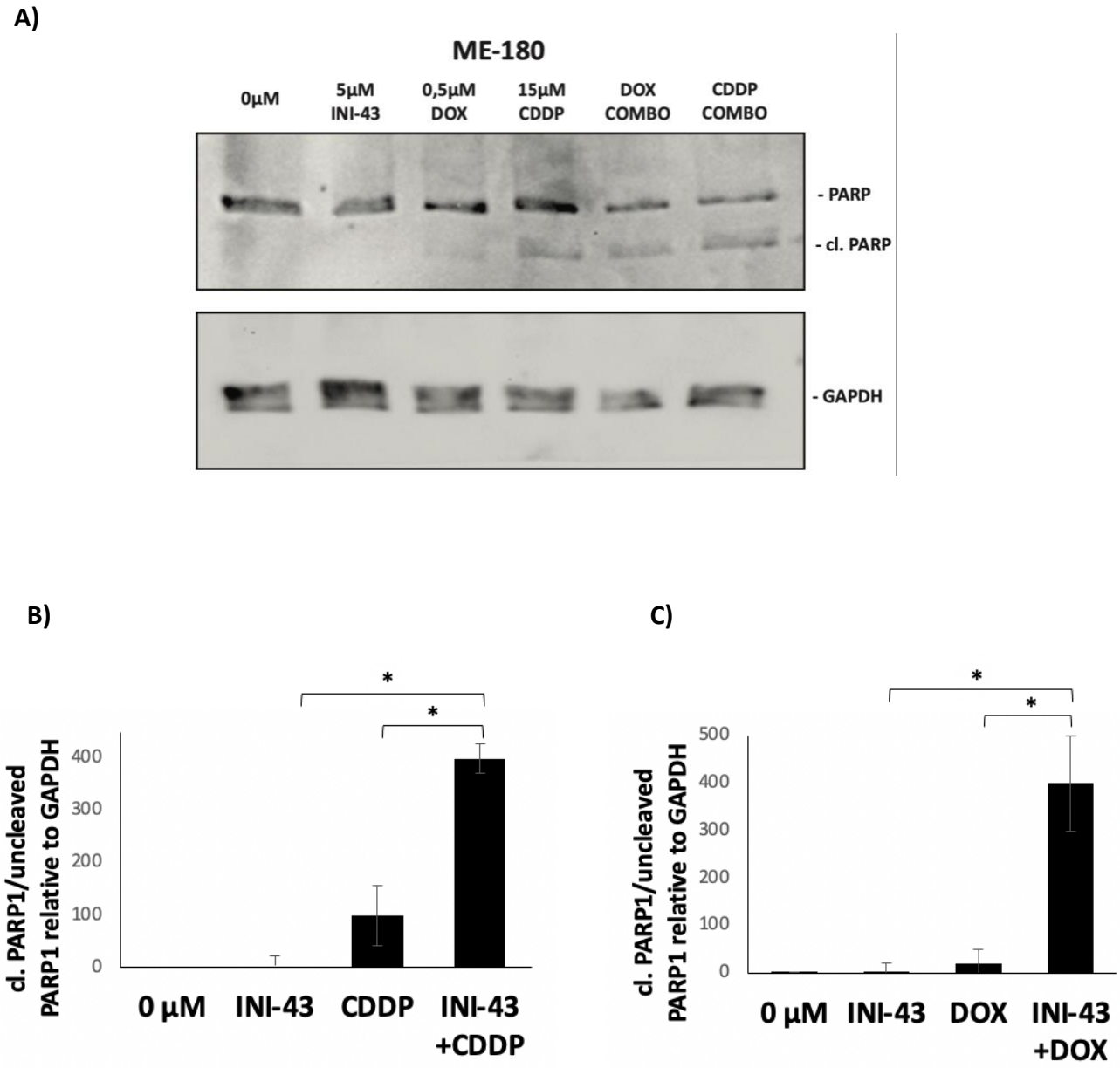


Figure 4.5. Effect of combination treatments of INI-43 and Cisplatin and INI-43 and Doxorubicin on apoptotic cell death in cervical cancer cells. ME-180 cells were treated, for 24 hours, with no drug, 5 μM INI-43, 0.5 μM DOX, 15 μM CDDP or 5 μM INI-43 + 0.5 μM DOX or 5 μM INI-43 + 15 μM CDDP. Protein was harvested and Western blot analysis was performed to determine relative expression levels of uncleaved PARP1 (116 kDa) and cleaved (cl) PARP1 (89 kDa) using an αPARP1 antibody. GAPDH was used as the loading control. Band intensity was determined using ImageJ analysis software and the ratio of cl PARP1 to uncleaved PARP1 (normalized against GAPDH) was plotted for each treatment group. **(A)** Western blots shown are representative results of experiments repeated 3 independent times in each cell line. **(B, C)** Bar charts represents the mean +/_ SEM of experiments performed three independent times. *=$p<0.05$

4.2.4.1 Effect of combination treatments on Caspase 3/7 activity

To independently determine the effects of the combination treatments, Caspase 3/7 assays were used as a proxy for apoptosis. For the Caspase-Glo 3/7 Assay, a luminogenic Caspase 3/7 substrate which is cleaved by active Caspases to produce luminescence serves as an indicator of apoptosis-related Caspase 3/7 activity. MTT assays for viable cells were set up concurrently and Caspase 3/7 results were normalized to the MTT results to control for possible differences in cell number between conditions. The Caspase-Glo 3/7 assay results show that individual INI-43 treatments resulted in negligible Caspase 3/7 activation while individual treatments with CDDP and DOX significantly increased Caspase 3/7 activation (Fig. 4.7 A, B and C). The combination treatments resulted in the greatest Caspase 3/7 activity, which was significantly greater than that observed after individual treatments.

Together, the data suggest that combined treatment of cervical cancer cells with INI-43 and Cisplatin/Doxorubicin results in enhanced apoptosis, compared to treatment with drugs on their own.

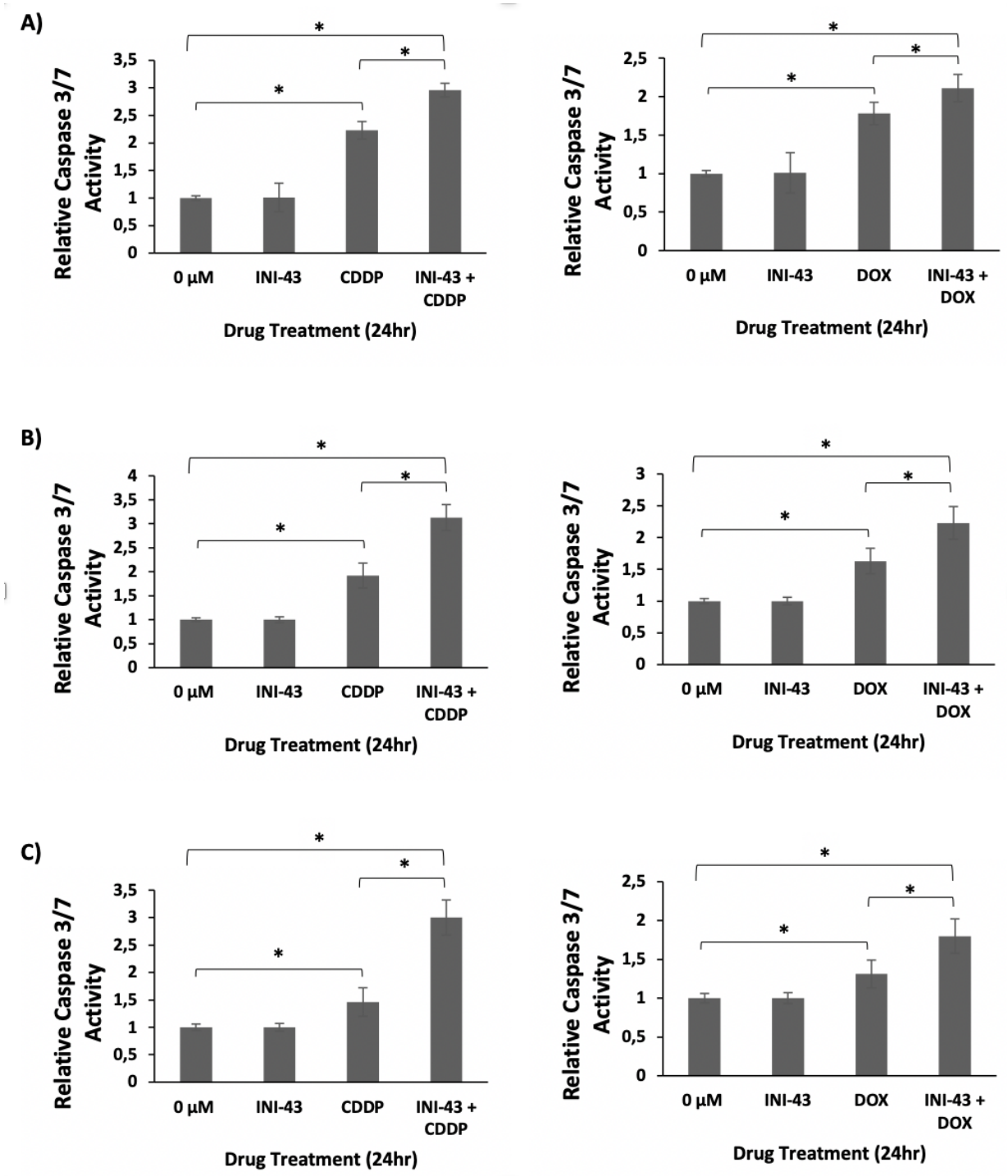


Figure 4.7. Caspase 3/7 activity following individual and combination treatments. Caspase 3/7 activity was determined and normalized to viable cells as measured using the MTT assay for **(A)** HeLa, **(B)** ME-180 and **(C)** SiHa cells. Results shown are the mean ±SEM of the experiments performed in triplicate, *=p<0.05

4.2.5 Determining if combination treatments associate with DNA damage

Next, we wanted to investigate whether the increased levels of apoptosis, as characterized by increased levels of cleaved PARP1 and enhanced Caspase 3/7 activity, associated with enhanced DNA damage upon drug treatment. Phosphorylation of histone H2AX, forming γH2AX, is well-described in the literature as a sensitive molecular marker for DNA damage (specifically, double strand breaks)[114]. Therefore, γH2AX levels following single and combination treatment were analysed by Western blot analysis.

Results showed that γH2AX levels were enhanced upon CDDP treatment in all three cell lines; HeLa, ME180 and SiHa cells (Fig. 4.8 A, B, C), and upon DOX treatment in ME180 and SiHa cells (Fig. 4.8 B, C), indicating that these conventional chemotherapies induce a DNA damage response in the cells upon treatment, albeit to different extents in the different cell lines. Interestingly, however, INI-43 was not able to further enhance the phosphorylation of H2AX, in HeLa and SiHa cells, while in ME180 cells, the addition of INI-43 was found to enhance γH2AX levels induced by DOX (Fig. 4.8 B). Since γH2AX as a marker of DNA damage is expected to be detected before PARP1 cleavage in the apoptotic pathway, it was proposed that γH2AX might be enhanced at a shorter time point, therefore an 8 hr timepoint was chosen to investigate this in the INI-43:CDDP combination. However, at this timepoint as well, γH2AX levels in HeLa, ME-180 and SiHa cells after the INI-43:CDDP combination treatment were not enhanced compared to 15 μM CDDP on its own (Appendix B 1). While the experiment may still need further optimisation, in terms of drug concentration and time, these results suggest that the synergistically enhanced cell death upon combination treatment with INI-43 and CDDP, and INI-43 and DOX, is likely not due to enhanced levels of DNA damage upon co-

treatment with drugs, but rather via another mechanism. While the results show that there is no induction of H2AX phosphorylation upon combination treatment, additional experimentation is required to definitively rule out DNA damage as a contributing factor to the cell death observed.

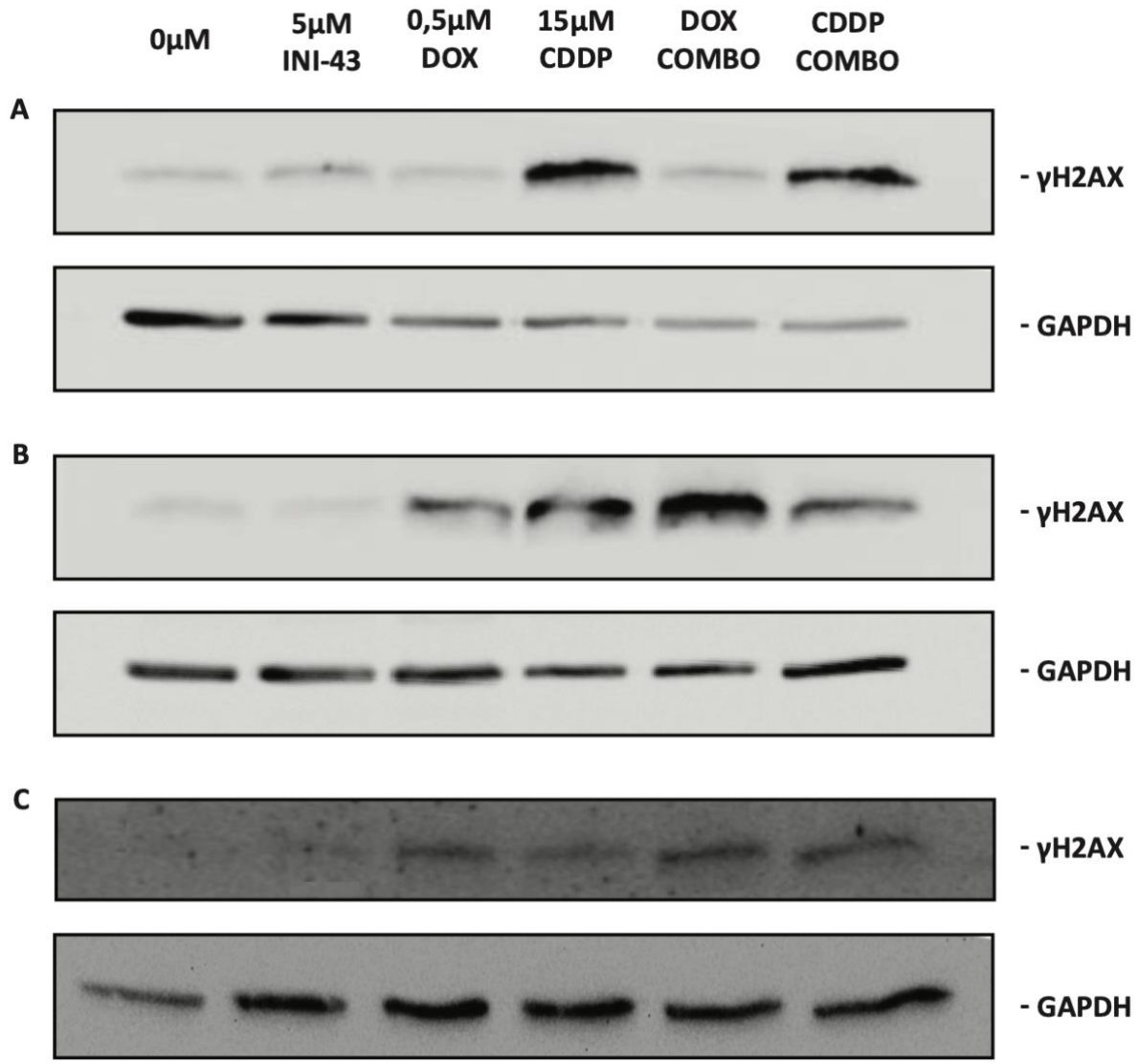


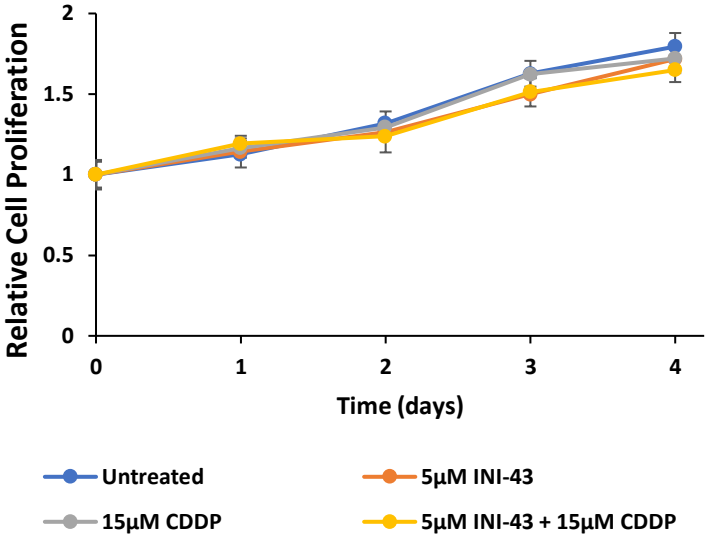
Figure 4.8. Levels of γH2AX after INI-43, DOX and CDDP treatment individually and in combination in cervical cancer cells. (A) HeLa, (B) ME-180 and (C) SiHa cells were treated, for 24 hours, with no drug, 5 µM INI-43, 0.5 µM DOX, 15 µM CDDP or DOX combo (5 µM INI-43 + 0.5 µM DOX) or CDDP combo (5 µM INI-43 + 15 µM CDDP). Proteins were harvested and Western blot analysis was performed to determine relative levels of γH2AX (15kDa). GAPDH was used as the loading control.

4.2.6 Effect of combination treatment on non-cancer cells

Since our results showed that INI-43 treated in combination with CDDP or DOX exhibited synergistic interactions in the cervical cancer cell lines HeLa, ME-180 and SiHa, we next wanted to determine if the combinations that had inhibitory effects on the proliferation of cancer cells would have any effect on a non-cancer cell line, ARPE-19. Previously we showed that single agent treatments, at concentrations that killed the cancer cell lines, had no effect on ARPE-19 cell proliferation/viability, hence it was of interest to determine if the ARPE-19 cells were sensitive to combination treatments at drug concentrations that resulted in synergistically enhanced cervical cancer cell death.

For the proliferation assay, ARPE-19 cells were plated at ~3000 cells per well and treated with either the vehicle control (DMSO), ½ IC_{50} values (taken from cancer cell results) of INI-43 (5 µM) or CDDP (15 µM) or DOX (0.5 µM) or their respective combinations, whereafter cell proliferation was monitored daily for four days using the MTT assay. The results show that individual treatments of 5 µM INI-43 and 15 µM CDDP as well as the combination treatment had no effect on cell proliferation (Fig. 4.9 A). Similar results were obtained for DOX, where both individual and the INI-43 and DOX combination had no effect on the proliferation of ARPE-19 cells (Fig. 4.9 B). These results show that non-cancer ARPE-19 cells remained unaffected by single drug treatments, but importantly also remain unaffected by combination drug treatments that have significant inhibitory effects on HeLa, ME-180 and SiHa cervical cancer cell lines.

A)



B)

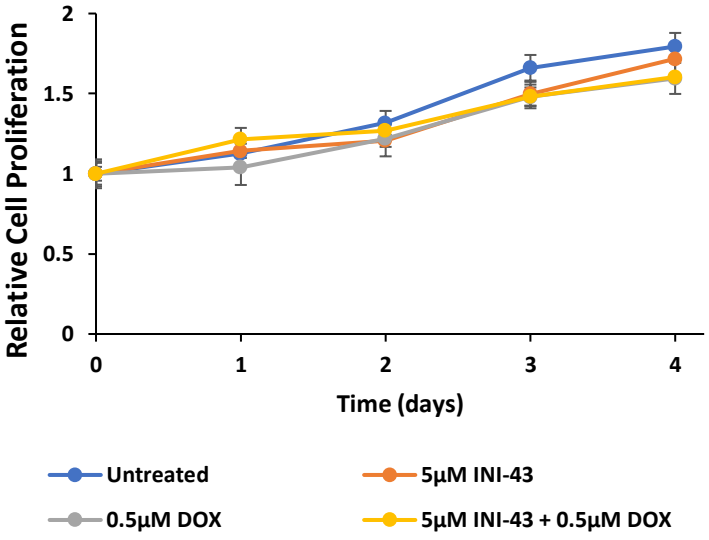


Figure 4.9. Cell proliferation of ARPE-19 cells following combination treatment. Non-cancer ARPE-19 cells were seeded at 3000 cells per well in 96-well plates and treated with **(A)** ½ IC_{50} INI-43 or ½ IC_{50} CDDP or their combination, or **(B)** ½ IC_{50} INI-43 or ½ IC_{50} DOX or their combination. Proliferation was examined every 24 hours using the MTT reagent for 4 days after treatment and normalized to the viable cells on day 0. Results shown are mean ± SEM of experiments performed in triplicate and repeated three independent times.

PARP1 and γH2AX levels were also evaluated after individual and combination treatment of ARPE-19 cells. ARPE-19 cells were treated with the same concentrations of drugs used in the proliferation assay and after 24 hours protein was harvested, and Western blot analysis was performed to determine relative cleaved PARP1 and γH2AX (15 kDa). Protein lysates obtained from combination treated HeLa cells were included concurrently to draw comparison between expression levels in cancer and non-cancer cell lines, essentially serving as a positive control. Results showed that there was no visible cleaved PARP1 after treatment of ARPE19 cells, and only very faint bands were visible for γH2AX in the ARPE-19 cells. (Fig. 4.10 A and B). These results support the fact that the drug combinations that induce apoptosis in cervical cancer cells, have no observable effect on the non-cancer cells.

4.3 DISCUSSION

In order to address acquired drug resistance and the side-effects of anti-cancer agents, recent research has focused on finding novel combinations of anti-cancer agents with non-overlapping mechanisms of action. In line with this, this study focused on the use of a novel anti-cancer small molecule, INI-43, in combination with front line chemotherapeutic agents, CDDP and DOX. This chapter focused on investigating whether cytotoxicity could be synergistically enhanced when treating cervical cancer cells with the combination of drugs compared to the respective monotherapies.

Firstly, combination index (CI) values were determined using Compusyn software. Compusyn generates a combination index value by comparing the effect resulting from the combination of two drugs directly to the effect of its individual components. The CI value is thus a quantitative measure of whether the drug combination is synergistic, antagonistic or additive. Since the three cancer cell lines (HeLa, ME-180 and SiHa) were relatively similar in their sensitivities to INI-43, CDDP and DOX (as determined by IC_{50} experiments in Chapter 3), the same ratios were employed for the three cell lines for subsequent combination index (CI) determination experiments. An effective combination was defined as a synergistic one, whereby there was an increase in effectiveness following drug combination therapy as compared to the drug treatments alone. Synergism thus demonstrates the combination's superiority to each of its individual components. As shown in the CI vs Fa plots, synergistic combination index values were determined after combining INI-43 with both CDDP and DOX. Several combination studies involving either CDDP or DOX with other anti-cancer compounds, including targeted therapies, have outlined synergistic interactions similar to those seen in

the combination index plots of this study. Recently a study by Mehraj et al. (2022) showed that DOX worked synergistically with Adapalene, a retinoid found to have anti-cancer potential, in triple negative breast cancer cells[104]. Another combination study published in 2022 showed that pancreatic cancer cells treated with DOX in combination with a potent targeted therapy, Sildenafil, resulted in CI values below 1 which represent a synergistic interaction[115]. Interestingly, this same compound was also found to act synergistically with CDDP to enhance cell toxicity in human breast adenocarcinoma cells[116]. Additionally, a recent study by Sarwar et al. (2022) in HeLa cells showed CDDP and a phytochemical used in combination resulted in synergistic effects[117]. Thus, both conventional chemotherapies determined in this study to have synergistic effects with INI-43, have been found in multiple previous studies to act synergistically with different types of compounds including anti-cancer drugs and natural compounds.

Many mainstay chemotherapies mediate their cytotoxicity via enhanced induction of apoptotic cell death; thus, in this study we investigated whether there were increased levels of well-known apoptotic markers, namely cleaved PARP1 and activated Caspase 3/7, following combination treatments. Our study showed enhanced levels of cleaved PARP1 in all three cervical cancer cell lines after combination treatments which indicates an increased level of apoptosis occurring in combination treated cells. The Western blot results were supported by the Caspase 3/7 results which show significantly increased activity of Caspase 3/7 in combination treatments compared to the monotherapies. Importantly, the sum of the increase in Caspase activity for the monotherapies (INI-43 and either CDDP or DOX) was less than the increase in Caspase activity in the respective combination treated cells, thus indicating a synergistic effect which supports the synergistic interaction. In line with this, a

study by Gamen et al. (1997) showed that Caspases 3 and 7 were both activated in Jurkat (leukemia) cells following treatment with DOX. Importantly, they showed that the apoptotic process induced after DOX treatment was mediated by these Caspases[118]. In this study, DOX induced the activity of these Caspases at a concentration previously identified in the literature (0.5 μM). A study by Gamen et al. (2000) showed that DOX treatment resulted in the disruption of the mitochondrial membrane potential, along with Caspase activation to promote the apoptotic pathway[119]. CDDP has also been shown to synergize with a novel anti-cancer agent, caffeic acid, which increased Caspase 3 and 7 activity in two cervical cancer cell lines (HeLa and CaSki) to a greater degree than using either agent alone[120]. Furthermore, a study targeting Exportin 1 using the inhibitor LMB in combination with CDDP revealed increased levels of apoptosis in SiHa cells. The IC_{50} values determined for CDDP in that study were reduced by more than 10-fold with the addition of the Exportin 1 inhibitor[95]. Taken together, the enhanced apoptosis shown via increased levels cleaved PARP1 as well as increased activity of Caspase 3/7 following combination treatments in all three cervical cancer cell lines, suggest that the synergistic interaction of the drugs occurs via interplay during the apoptotic pathway.

We next wanted to investigate a potential mechanism from which the synergistic cell death occurs. A study by Chi et al. (2021) demonstrated enhanced γH2AX levels when SiHa cells that had been pre-treated with INI-43 were treated with CDDP, however the experimental design of our study differed in that treatment with INI-43 and the conventional chemotherapies was concurrent rather than sequential. Results from our study differ in that γH2AX levels assayed 24 hours after treatment, were not different between combination treated and single agent treated, suggesting that the enhanced levels of apoptosis observed were not due to increased

DNA damage in these conditions. While we also investigated γH2AX levels at an earlier time point (8 hours post-treatment), it is possible that the induction of γH2AX foci could occur even earlier than that, and that enhanced levels of H2AX phosphorylation might be observed after combination treatment at earlier time points. Sears et al. (2016) showed that treatment of non-small cell lung cancer H460 cells with CDDP resulted in nuclear H2AX phosphorylation 30 minutes after treatment[121]. It is possible that investigating γH2AX levels at an earlier time point might result in different findings, and thus further optimisation of the experiment is required before we can rule out DNA damage as a player in the INI-43-induced sensitisation of cells to CDDP and DOX.

Since conventional chemotherapies have very limited therapeutic indices, it was necessary to test whether the combinations which led to enhanced cell death in cervical cancer cells also had an effect on non-cancer cells. Non-cancer ARPE-19 cells were therefore treated with the INI-43:CDDP or INI-43:DOX combinations and there was no effect on the rate of cell proliferation when ARPE-19 cells were treated with the combination therapies, compared to untreated cells. Furthermore, no increase in cleaved PARP1 supported the fact that the combination did not induce apoptosis in the non-cancer cell line (Appendix B, Figure B.2). There was also no visible DNA damage, as shown with no γH2AX expression, in the combination treated cells. It is therefore promising that INI-43:CDDP and INI-43:DOX combinations do not appear to affect non-cancer cell proliferation, apoptosis, or degree of DNA damage, while the same combinations induce cervical cancer cell death.

Both CDDP and DOX have narrow therapeutic indices, owing to their severe toxicities as well as the emergence of drug resistance. As a result, there is a need to find innovative drugs that

enhance their effectiveness while minimizing their toxicity to normal cells. Combination therapies allow simultaneous targeting of crucial molecular mechanisms, which is suspected to reduce therapeutic resistance as cancers rely on a limited number of essential molecular mechanisms for their survival and growth. In this chapter, we describe that INI-43, at a sub-lethal dose, significantly increased CDDP and DOX-induced apoptosis. Similar levels of synergism were observed when INI-43 was combined with both CDDP and DOX. Further work is required to investigate the mechanisms driving the synergistic response, and to determine whether similar effects are observed in different cancer types. It will also be of interest to determine whether other nuclear import inhibitors, such as Importazole, are similarly able to sensitise cancer cells to chemotherapy drugs; CDDP and DOX.

CHAPTER 5

CONCLUSION

5.1 Main conclusions

There is a drive in the field of cancer research to combat drug toxicity and limit intrinsic and/or acquired chemoresistance. Novel targeted anti-cancer agents, such as INI-43 used in this study, are of growing interest because of their enhanced specificity and lower toxicity. Furthermore, combination therapies have been shown to combat drug resistance by inhibiting multiple pathways that cancer cells are reliant on and thus induce maximal cancer cell kill effect. In fact, the model of independent drug action suggests that due to patient-to-patient variability, combining drugs with different mechanisms of action can increase the number of positive responses simply by providing more than one opportunity for the patient to benefit from monotherapies[122]. Importantly, identifying a drug combination that exhibits synergy has the added benefit of allowing the same level of efficacy to be achieved at lower drug doses. Targeted therapies have been found to be particularly effective in the context of combination treatments, therefore this study set out to investigate the combined effects of the targeted agent INI-43 with conventional chemotherapies, Cisplatin or Doxorubicin, and to determine whether synergistic interactions exist between the drugs, and whether, ultimately, these are good drug combinations to pursue in further studies.

The anti-cancer effects of INI-43, Cisplatin and Doxorubicin as monotherapies were first investigated. Importantly, results from this study showed the three cervical cancer cells to be

significantly more sensitive to INI-43, Cisplatin and Doxorubicin treatments compared to the non-cancer ARPE-19 cells. In contrast to the three cervical cancer cell lines, cell viability was unaffected for ARPE-19 cells treated with the approximate IC_{50} doses of the cancer cell lines used in this study.

The focus of the study was to determine whether cancer cell death could be enhanced when combining INI-43 with either Cisplatin or Doxorubicin. To assess this, combination index (CI) values were determined using the Chou Talalay method. Combination index values were determined to be near or below 1 at all levels of cell death after treatment with INI-43 and Cisplatin and INI-43 and Doxorubicin combinations, in HeLa, ME-180 and SiHa cell lines, indicating a synergistic effect.

In investigating the effect of individual and combination treatments on the apoptotic process, a dose-dependent increase in the levels of cleaved PARP1 was observed in all three cancer cell lines after treatment with INI-43 or Cisplatin or Doxorubicin as monotherapies. A more noteworthy finding was that PARP1 cleavage was significantly enhanced after combination treatments compared to the individual treatments in all three cancer cell lines. Apoptosis was also investigated via the Caspase-Glo 3/7 assay which showed significantly increased levels of Caspase 3/7 after combination treatments compared to single treatments. Interestingly, the sum of the increase in Caspase 3/7 activity in single treatments was less than the increase in Caspase 3/7 activity in the combination treated cells, therefore indicating synergism. Thus, both the Western blot and Caspase-Glo 3/7 assay results suggest that INI-43 treatment enhances the anti-cancer effect of Cisplatin and Doxorubicin via enhancing apoptosis, a

desired pathway for cell death in anti-cancer therapy. Importantly, the enhanced apoptotic effect was observed even though sub-lethal concentrations of INI-43 were used.

The effect of the combined treatment on non-cancer cells of epithelial origin was investigated and there was no significant difference in the proliferation of untreated and combination treated ARPE-19 cells. Additionally, there was no PARP1 cleavage after any of the treatments. Thus, the non-cancer cell line tested in this study was insensitive to the combination treatments that resulted in significant cell death in the three cancer cell lines. This is promising in that the sparing of non-cancer cells suggests that drug toxicity might be minimal, however, findings were limited to one cell line, and non-cancer cells of various origins (including fast-dividing non-cancer cells) need to be tested before accurate conclusions can be drawn.

In conclusion, our study demonstrates that concomitant treatment of cervical cancer cells with conventional chemotherapies, Cisplatin or Doxorubicin, together with a small molecule targeted against nuclear import, INI-43, results in synergistic anti-cancer effects. To our knowledge, this study using cervical cancer cells grown in tissue culture conditions is a first to show synergistic interactions between INI-43 and conventionally used chemotherapies.

Synergism cannot be determined in humans, hence preclinical studies like this, using cancer cell lines, are critical in determining which combinations to pursue in further studies. If *in vivo* work were to validate these results, it would suggest that using the combination treatments in the clinic could allow for doses to be lowered as well as reducing the possibility of drug resistance developing.

5.2 Limitations and future work

Results of this study warrant further investigation into the mechanism of cell death and the impact of synergistic drug concentrations on additional cancer and non-cancer cells as a novel anti-cancer approach. While three cervical cancer cell lines (HeLa, ME-180 and SiHa) were used in this study to circumvent cell line specific effects, we only had access to one non-cancer cell line, a retinal epithelial cell line (ARPE-19). A non-cancer epithelial line of cervical tissue origin would have been ideal but was not available during the course of this study. Future work will include a panel of cancer (not only cervical cancer cell lines) to determine if the synergistic effects observed in cervical cancer cells can be extended to other cancer types. In addition, a panel of normal cell lines, preferably including non-cancer cervical cells, to rule out that the effects seen are not cell line specific can be included in a larger study.

Further research should also include investigations into the proteins that Kpnβ1 imports and their expression levels in the nucleus before and after single and combination treatments as this will provide insights into the mechanisms underlying the synergistic relationship between INI-43 and Cisplatin/Doxorubicin, and as such point to which cancers/patients are likely to benefit from these combinations. This is important in the worldwide drive towards precision medicine. Understanding the molecular mechanisms involved could also allow for the determination of which other drugs might similarly work well with INI-43.

This study was limited to *in vitro* work, and it is therefore imperative that future studies include *in vivo* testing of the drug combinations in an animal model. If *in vivo* work were to validate the results obtained in this study, it would provide supportive evidence that the

combination treatments could have value in a clinical setting allowing for doses to be lowered as well as reducing the possibility of drug resistance developing.

Finally, it will be interesting to determine whether the addition of another drug to the combinations investigated in this study might further enhance the anti-cancer effects. Ultimately, tackling tumour heterogeneity using multiple agents and minimising drug resistance by inhibiting multiple pathways is key to providing the greatest chance of therapeutic success.

REFERENCES

1. Bray, F., Laversanne, M., Weiderpass, E. & Soerjomataram, I. The ever-increasing importance of cancer as a leading cause of premature death worldwide. *Cancer* **127**, 3029–3030 (2021).

2. Sung, H. *et al.* Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA. Cancer J. Clin.* **71**, 209–249 (2021).

3. Schiffman, M., Castle, P. E., Jeronimo, J., Rodriguez, A. C. & Wacholder, S. Human papillomavirus and cervical cancer. *Lancet* **370**, 890–907 (2007).

4. Walboomers, J. M. M. *et al.* Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* **189**, 12–19 (1999).

5. Jedy-Agba, E. *et al.* Trends in cervical cancer incidence in sub-Saharan Africa. *Br. J. Cancer* **123**, 148–154 (2020).

6. Singh, G. Global Inequalities in Cervical Cancer Incidence and Mortality are Linked to Deprivation, Low Socioeconomic Status, and Human Development. *Int. J. MCH AIDS* **1**, (2012).

7. Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2019. *CA. Cancer J. Clin.* **69**, 7–34 (2019).

8. Forum, P. Coverage of Cervical Cancer Screening in 57 Countries : Low Average Levels and Large Inequalities. **5**, 863–868 (2008).

9. Olson, B. *et al.* Cervical cancer screening programs and guidelines in low- and middle-income countries. *Int. J. Gynecol. Obstet.* **134**, 239–246 (2016).

10. Guimarães, Y. M., Godoy, L. R., Longatto-Filho, A. & Reis, R. dos. Management of Early-Stage Cervical Cancer: A Literature Review. *Cancers (Basel).* **14**, 575 (2022).

11. Barton, M. B., Frommer, M. & Shafiq, J. Role of radiotherapy in cancer control in low-income and middle-income countries. *Lancet Oncol.* **7**, 584–595 (2006).

12. Jaffray, D. A. & Gospodarowicz, M. K. Radiation Therapy for Cancer. in *Disease Control Priorities, Third Edition (Volume 3): Cancer* (2015). doi:10.1596/978-1-4648-0349-9_ch14.

13. Padma, V. V. An overview of targeted cancer therapy. *BioMedicine* **5**, 19 (2015).

14. Baudino, T. Targeted Cancer Therapy: The Next Generation of Cancer Treatment. *Curr. Drug Discov. Technol.* **12**, 3–20 (2015).

15. Kelland, L. The resurgence of platinum-based cancer chemotherapy. *Nat. Rev. Cancer* **7**, 573–584 (2007).

16. Lebwohl, D. & Canetta, R. Clinical development of platinum complexes in cancer therapy: an historical perspective and an update. *Eur. J. Cancer* **34**, 1522–1534 (1998).

17. Lorusso, D., Petrelli, F., Coinu, A., Raspagliesi, F. & Barni, S. A systematic review comparing cisplatin and carboplatin plus paclitaxel-based chemotherapy for recurrent or metastatic cervical cancer. *Gynecol. Oncol.* **133**, 117–123 (2014).

18. Todo, Y. & Watari, H. Concurrent chemoradiotherapy for cervical cancer: background including evidence-based data, pitfalls of the data, limitation of treatment in certain groups. *Chinese J. Cancer Res.* **28**, 221–227 (2016).

19. Siddik, Z. H. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* **22**, 7265–7279 (2003).

20. Pabla, N. & Dong, Z. Cisplatin nephrotoxicity: Mechanisms and renoprotective strategies. *Kidney Int.* **73**, 994–1007 (2008).

21. Fuertes, M., Castilla, J., Alonso, C. & Pérez, J. Cisplatin Biochemical Mechanism of Action: From Cytotoxicity to Induction of Cell Death Through Interconnections Between Apoptotic and Necrotic Pathways. *Curr. Med. Chem.* **10**, 257–266 (2003).

22. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* **365**, 1687–1717 (2005).

23. Lipshultz, S. E. *et al.* Assessment of dexrazoxane as a cardioprotectant in doxorubicin-treated children with high-risk acute lymphoblastic leukaemia: long-term follow-up of a prospective, randomised, multicentre trial. *Lancet Oncol.* **11**, 950–961 (2010).

24. Cummings, J., Anderson, L., Willmott, N. & Smyth, J. F. The molecular pharmacology of doxorubicin in vivo. *Eur. J. Cancer Clin. Oncol.* **27**, 532–535 (1991).

25. Bachur, N. R., Gordon, S. L. & Gee, M. V. A general mechanism for microsomal activation of quinone anticancer agents to free radicals. *Cancer Res.* **38**, 1745–50 (1978).

26. Bachur, N. R., Gordon, S. L. & Gee, M. V. Anthracycline antibiotic augmentation of microsomal electron transport and free radical formation. *Mol. Pharmacol.* **13**, 901–10 (1977).

27. Eliot, H., Gianni, L. & Myers, C. Oxidative destruction of DNA by the adriamycin-iron complex. *Biochemistry* **23**, 928–936 (1984).

28. Shen, D.-W., Pouliot, L. M., Hall, M. D. & Gottesman, M. M. Cisplatin Resistance: A Cellular Self-Defense Mechanism Resulting from Multiple Epigenetic and Genetic Changes. *Pharmacol. Rev.* **64**, 706–721 (2012).

29. Kartalou, M. & Essigmann, J. M. Mechanisms of resistance to cisplatin. *Mutat. Res. Mol. Mech. Mutagen.* **478**, 23–43 (2001).

30. Sedletska, Y., Giraud-Panis, M.-J. & Malinge, J.-M. Cisplatin Is a DNA-Damaging Antitumour Compound Triggering Multifactorial Biochemical Responses in Cancer Cells: Importance of Apoptotic Pathways. *Curr. Med. Chem. Agents* **5**, 251–265 (2005).

31. Brabec, V. & Kasparkova, J. Modifications of DNA by platinum complexes. *Drug Resist. Updat.* **8**, 131–146 (2005).

32. Kuo, M. T., Chen, H. H. W., Song, I.-S., Savaraj, N. & Ishikawa, T. The roles of copper transporters in cisplatin resistance. *Cancer Metastasis Rev.* **26**, 71–83 (2007).

33. Christowitz, C. *et al.* Mechanisms of doxorubicin-induced drug resistance

and drug resistant tumour growth in a murine breast tumour model. *BMC Cancer* **19**, 757 (2019).

34. Christowitz, C. *et al.* Mechanisms of doxorubicin-induced drug resistance and drug resistant tumour growth in a murine breast tumour model. *BMC Cancer* **19**, 1–10 (2019).

35. Shukla, A. *et al.* Blocking of ERK1 and ERK2 sensitizes human mesothelioma cells to doxorubicin. *Mol. Cancer* **9**, 314 (2010).

36. McCubrey, J. A. *et al.* Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR Cascade Inhibitors: How Mutations Can Result in Therapy Resistance and How to Overcome Resistance. *Oncotarget* **3**, 1068–1111 (2012).

37. Sheng, Y., You, Y. & Chen, Y. Dual-targeting hybrid peptide-conjugated doxorubicin for drug resistance reversal in breast cancer. *Int. J. Pharm.* **512**, 1–13 (2016).

38. Chen, T. *et al.* Dasatinib reverses the multidrug resistance of breast cancer MCF-7 cells to doxorubicin by downregulating P-gp expression via inhibiting the activation of ERK signaling pathway. *Cancer Biol. Ther.* **16**, 106–14 (2015).

39. Järvinen, T. A. H. *et al.* Amplification and Deletion of Topoisomerase IIα Associate with ErbB-2 Amplification and Affect Sensitivity to Topoisomerase II Inhibitor Doxorubicin in Breast Cancer. *Am. J. Pathol.* **156**, 839–847 (2000).

40. Press, M. F. *et al.* Alteration of Topoisomerase II–Alpha Gene in Human Breast Cancer: Association With Responsiveness to Anthracycline-Based Chemotherapy. *J. Clin. Oncol.* **29**, 859–867 (2011).

41. Xiao, B. *et al.* The true colors of autophagy in doxorubicin-induced cardiotoxicity. *Oncol. Lett.* **18**, 2165–2172 (2019).

42. Florea, A.-M. & Büsselberg, D. Cisplatin as an Anti-Tumor Drug: Cellular Mechanisms of Activity, Drug Resistance and Induced Side Effects. *Cancers (Basel).* **3**, 1351–1371 (2011).

43. SHAN, J. *et al.* Nedaplatin- versus cisplatin-based chemotherapy in the

survival time of patients with non-small cell lung cancer. *Mol. Clin. Oncol.* **3**, 543–549 (2015).

44. Tacar, O., Sriamornsak, P. & Dass, C. R. Doxorubicin: An update on anticancer molecular action, toxicity and novel drug delivery systems. *J. Pharm. Pharmacol.* **65**, 157–170 (2013).

45. Meredith, A.-M. & Dass, C. R. Increasing role of the cancer chemotherapeutic doxorubicin in cellular metabolism. *J. Pharm. Pharmacol.* **68**, 729–741 (2016).

46. Westin, J. R. & Kurzrock, R. It's About Time: Lessons for Solid Tumors from Chronic Myelogenous Leukemia Therapy. *Mol. Cancer Ther.* **11**, 2549–2555 (2012).

47. Tewari, K. S. *et al.* Bevacizumab for advanced cervical cancer: final overall survival and adverse event analysis of a randomised, controlled, open-label, phase 3 trial (Gynecologic Oncology Group 240). *Lancet* **390**, 1654–1663 (2017).

48. Tewari, K. S. *et al.* Improved Survival with Bevacizumab in Advanced Cervical Cancer. *N. Engl. J. Med.* **370**, 734–743 (2014).

49. Hong, D. S. *et al.* Tisotumab Vedotin in Previously Treated Recurrent or Metastatic Cervical Cancer. *Clin. Cancer Res.* **26**, 1220–1228 (2020).

50. Ruf, W. Tissue factor and cancer. *Thromb. Res.* **130**, S84–S87 (2012).

51. Song, X. *et al.* Tisotumab vedotin for the treatment of cervical carcinoma. *Drugs of Today* **58**, 213 (2022).

52. Van Der Watt, P. J. *et al.* The karyopherin proteins, Crm1 and Karyopherin β1, are overexpressed in cervical cancer and are critical for cancer cell survival and proliferation. *Int. J. Cancer* (2009) doi:10.1002/ijc.24146.

53. Stewart, M. Function of the Nuclear Transport Machinery in Maintaining the Distinctive Compositions of the Nucleus and Cytoplasm. *Int. J. Mol. Sci.* **23**, 2578 (2022).

54. Kosyna, F. K. & Depping, R. Controlling the gatekeeper: Therapeutic

targeting of nuclear transport. *Cells* **7**, (2018).

55. Hill, R., Cautain, B., De Pedro, N. & Link, W. Targeting nucleocytoplasmic transport in cancer therapy. *Oncotarget* **5**, 11–28 (2014).

56. Stelma, T. *et al.* Targeting nuclear transporters in cancer: Diagnostic, prognostic and therapeutic potential. *IUBMB Life* **68**, 268–280 (2016).

57. van der Watt, P. J., Ngarande, E. & Leaner, V. D. Overexpression of Kpnβ1 and Kpnα2 importin proteins in cancer derives from deregulated E2F activity. *PLoS One* (2011) doi:10.1371/journal.pone.0027723.

58. Alshareeda, A. T. *et al.* KPNA2 is a nuclear export protein that contributes to aberrant localisation of key proteins and poor prognosis of breast cancer. *Br. J. Cancer* **112**, 1929–37 (2015).

59. Gousias, K., Niehusmann, P., Gielen, G. H. & Simon, M. Karyopherin a2 and chromosome region maintenance protein 1 expression in meningiomas: novel biomarkers for recurrence and malignant progression. *J. Neurooncol.* **118**, 289–296 (2014).

60. Altan, B. *et al.* Nuclear karyopherin-α2 expression in primary lesions and metastatic lymph nodes was associated with poor prognosis and progression in gastric cancer. *Carcinogenesis* **34**, 2314–2321 (2013).

61. Zhu, J. *et al.* Upregulation of KPNβ1 in gastric cancer cell promotes tumor cell proliferation and predicts poor prognosis. *Tumor Biol.* (2016) doi:10.1007/s13277-015-3839-7.

62. Stelma, T. & Leaner, V. D. KPNB1-mediated nuclear import is required for motility and inflammatory transcription factor activity in cervical cancer cells. *Oncotarget* (2017) doi:10.18632/oncotarget.15834.

63. Shao, W. Y. *et al.* Phenethyl isothiocyanate suppresses the metastasis of ovarian cancer associated with the inhibition of CRM1-mediated nuclear export and mTOR-STAT3 pathway. *Cancer Biol. Ther.* **18**, 26–35 (2017).

64. Ranganathan, P. *et al.* XPO1 Inhibition using Selinexor Synergizes with Chemotherapy in Acute Myeloid Leukemia by Targeting DNA Repair and Restoring Topoisomerase IIα to the Nucleus. *Clin. Cancer Res.* **22**, 6142–

6152 (2016).

65. Luo, B. *et al.* Expression of exportin-1 in diffuse large B-cell lymphoma: immunohistochemistry and TCGA analyses. *Int. J. Clin. Exp. Pathol.* **11**, 5547–5560 (2018).

66. Zhao, J. *et al.* Whole-exome sequencing of muscle-invasive bladder cancer identifies recurrent copy number variation in IPO11 and prognostic significance of importin-11 overexpression on poor survival. *Oncotarget* **7**, 75648–75658 (2016).

67. Mis, M. *et al.* IPO11 mediates βcatenin nuclear import in a subset of colorectal cancers. *J. Cell Biol.* **219**, (2020).

68. van der Watt, P. J., Stowell, C. L. & Leaner, V. D. The Nuclear Import Receptor Kpnβ1 and Its Potential as an AntiCancer Therapeutic Target. *Crit. Rev. Eukaryot. Gene Expr.* **23**, 1–10 (2013).

69. Van Der Watt, P. J. *et al.* The karyopherin proteins, Crm1 and Karyopherin β1, are overexpressed in cervical cancer and are critical for cancer cell survival and proliferation. *Int. J. Cancer* **124**, 1829–1840 (2009).

70. Van Der Watt, P. J. *et al.* Targeting the nuclear import receptor Kpnβ1 as an anticancer therapeutic. *Mol. Cancer Ther.* **15**, 560–573 (2016).

71. Kelenis, D. P. *et al.* Inhibition of Karyopherin β1-Mediated Nuclear Import Disrupts Oncogenic Lineage-Defining Transcription Factor Activity in Small Cell Lung Cancer. *Cancer Res.* **82**, 3058–3073 (2022).

72. Basu, B., Olmos, D. & de Bono, J. S. Targeting IGF-1R: throwing out the baby with the bathwater? *Br. J. Cancer* **104**, 1–3 (2011).

73. Yap, T. A., Bjerke, L., Clarke, P. A. & Workman, P. Drugging PI3K in cancer: refining targets and therapeutic strategies. *Curr. Opin. Pharmacol.* **23**, 98–107 (2015).

74. Russo, M. *et al.* Adaptive mutability of colorectal cancers in response to targeted therapies. *Science (80-.).* **366**, 1473–1480 (2019).

75. Cipponi, A. *et al.* MTOR signaling orchestrates stress-induced mutagenesis,

facilitating adaptive evolution in cancer. *Science (80-.).* **368**, 1127–1131 (2020).

76. Venkatesan, S., Swanton, C., Taylor, B. S. & Costello, J. F. Treatment-Induced Mutagenesis and Selective Pressures Sculpt Cancer Evolution. *Cold Spring Harb. Perspect. Med.* **7**, (2017).

77. Lopez, J. S. & Banerji, U. Combine and conquer: Challenges for targeted therapy combinations in early phase trials. *Nat. Rev. Clin. Oncol.* **14**, 57–66 (2017).

78. Mokhtari, R. B. *et al.* Combination therapy in combating cancer. *Oncotarget* (2017) doi:10.18632/oncotarget.16723.

79. Park, S. R., Davis, M., Doroshow, J. H. & Kummar, S. Safety and feasibility of targeted agent combinations in solid tumours. *Nat. Rev. Clin. Oncol.* **10**, 154–168 (2013).

80. Long, G. V *et al.* Dabrafenib and trametinib versus dabrafenib and placebo for Val600 BRAF-mutant melanoma: a multicentre, double-blind, phase 3 randomised controlled trial. *Lancet* **386**, 444–451 (2015).

81. Grob, J. J. *et al.* Comparison of dabrafenib and trametinib combination therapy with vemurafenib monotherapy on health-related quality of life in patients with unresectable or metastatic cutaneous BRAF Val600-mutation-positive melanoma (COMBI-v): results of a phase 3, open-l. *Lancet Oncol.* **16**, 1389–1398 (2015).

82. Ribas, A. *et al.* Combination of vemurafenib and cobimetinib in patients with advanced BRAFV600-mutated melanoma: a phase 1b study. *Lancet Oncol.* **15**, 954–965 (2014).

83. Wen, S., Su, S., Liou, B., Lin, C. & Lee, K. Sulbactam-enhanced cytotoxicity of doxorubicin in breast cancer cells. *Cancer Cell Int.* **18**, 128 (2018).

84. Sun, J.-M. *et al.* Pembrolizumab plus chemotherapy versus chemotherapy alone for first-line treatment of advanced oesophageal cancer (KEYNOTE-590): a randomised, placebo-controlled, phase 3 study. *Lancet* **398**, 759–771 (2021).

85. du Bois, A. *et al.* Phase III Trial of Carboplatin Plus Paclitaxel With or Without Gemcitabine in First-Line Treatment of Epithelial Ovarian Cancer. *J. Clin. Oncol.* **28**, 4162–4169 (2010).

86. Stewart, A., Thavasu, P., de Bono, J. S. & Banerji, U. Titration of signalling output: insights into clinical combinations of MEK and AKT inhibitors. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol.* **26**, 1504–10 (2015).

87. Chou, T.-C. Theoretical Basis, Experimental Design, and Computerized Simulation of Synergism and Antagonism in Drug Combination Studies. *Pharmacol. Rev.* **58**, 621–681 (2006).

88. Gandhi, U. H. *et al.* Clinical Implications of Targeting XPO1-mediated Nuclear Export in Multiple Myeloma. *Clin. Lymphoma Myeloma Leuk.* **18**, 335–345 (2018).

89. Kashyap, T. *et al.* Selinexor, a Selective Inhibitor of Nuclear Export (SINE) compound, acts through NF-κB deactivation and combines with proteasome inhibitors to synergistically induce tumor cell death. *Oncotarget* **7**, 78883–78895 (2016).

90. Zhou, Y. *et al.* Inhibiting Importin 4-mediated nuclear import of CEBPD enhances chemosensitivity by repression of PRKDC-driven DNA damage repair in cervical cancer. *Oncogene* **39**, 5633–5648 (2020).

91. Chi, R. A., van der Watt, P., Wei, W., Birrer, M. J. & Leaner, V. D. Inhibition of Kpnβ1 mediated nuclear import enhances cisplatin chemosensitivity in cervical cancer. *BMC Cancer* **21**, 106 (2021).

92. Franken, N. A. P., Rodermond, H. M., Stap, J., Haveman, J. & van Bree, C. Clonogenic assay of cells in vitro. *Nat. Protoc.* **1**, 2315–2319 (2006).

93. Funaoka, K. *et al.* High-risk HPV-positive human cancer cell lines show different sensitivity to cisplatin-induced apoptosis correlated with the p21Waf1/Cip1 level. *Cancer Lett.* **108**, 15–23 (1996).

94. Venkatraman, M., Anto, R. J., Nair, A., Varghese, M. & Karunagaran, D. Biological and chemical inhibitors of NF-κB sensitize SiHa cells to cisplatin-induced apoptosis. *Mol. Carcinog.* **44**, 51–59 (2005).

95. Yi, S. A., Kim, G. W., Yoo, J., Han, J.-W. & Kwon, S. H. HP1γ Sensitizes Cervical Cancer Cells to Cisplatin through the Suppression of UBE2L3. *Int. J. Mol. Sci.* **21**, 5976 (2020).

96. Saiful Yazan, L. & Wan Nor Hafiza. Potential implications of GRP58 expression and susceptibility of cervical cancer to cisplatin and thymoquinone-based therapy. *Onco. Targets. Ther.* 1375 (2014) doi:10.2147/OTT.S62928.

97. Kalu, N. N. *et al.* Genomic characterization of human papillomavirus-positive and -negative human squamous cell cancer cell lines. *Oncotarget* **8**, 86369–86383 (2017).

98. Sobierajska, E. *et al.* Unusual enhancement of doxorubicin activity on co-delivery with Polyhedral oligomeric silsesquioxane (POSS). *Materials (Basel).* **10**, (2017).

99. Nurcahyanti, A. D. R. & Wink, M. L-Canavanine potentiates the cytotoxicity of doxorubicin and cisplatin in arginine deprived human cancer cells. *PeerJ* **2016**, (2016).

100. Kapadia, G. J. *et al.* Synergistic cytotoxicity of red beetroot (Beta vulgaris L.) extract with doxorubicin in human pancreatic, breast and prostate cancer cell lines. *J. Complement. Integr. Med.* **10**, (2013).

101. Yang, W. *et al.* Genomics of Drug Sensitivity in Cancer (GDSC): a resource for therapeutic biomarker discovery in cancer cells. *Nucleic Acids Res.* **41**, D955–D961 (2012).

102. Filippova, M. *et al.* Cellular Levels of Oxidative Stress Affect the Response of Cervical Cancer Cells to Chemotherapeutic Agents. *Biomed Res. Int.* **2014**, 1–14 (2014).

103. Zhu, Z. C., Liu, J. W., Li, K., Zheng, J. & Xiong, Z. Q. KPNB1 inhibition disrupts proteostasis and triggers unfolded protein response-mediated apoptosis in glioblastoma cells. *Oncogene* (2018) doi:10.1038/s41388-018-0180-9.

104. Mehraj, U. *et al.* Adapalene and Doxorubicin Synergistically Promote Apoptosis of TNBC Cells by Hyperactivation of the ERK1/2 Pathway Through ROS Induction. *Front. Oncol.* **12**, (2022).

105. Suh, D. S. *et al.* Differential apoptotic response in HPV-infected cancer cells of the uterine cervix after doxorubicin treatment. *Oncol. Rep.* **23**, 751–6 (2010).

106. Ayoub, N. M. Editorial: Novel Combination Therapies for the Treatment of Solid Cancers. *Front. Oncol.* **11**, (2021).

107. Tewari, K. S. *et al.* Final Overall Survival of a Randomized Trial of Bevacizumab for Primary Treatment of Ovarian Cancer. *J. Clin. Oncol.* **37**, 2317–2328 (2019).

108. Mahipal, A. & Malafa, M. Importins and exportins as therapeutic targets in cancer. *Pharmacol. Ther.* **164**, 135–143 (2016).

109. Wang, A. Y. & Liu, H. The past, present, and future of CRM1/XPO1 inhibitors. *Stem cell Investig.* **6**, 6 (2019).

110. Azizian, N. G. & Li, Y. XPO1-dependent nuclear export as a target for cancer therapy. *J. Hematol. Oncol.* **13**, 61 (2020).

111. Galinski, B. *et al.* Therapeutic Targeting of Exportin-1 in Childhood Cancer. *Cancers (Basel).* **13**, 6161 (2021).

112. Nachmias, B. & Schimmer, A. D. Targeting nuclear import and export in hematological malignancies. *Leukemia* **34**, 2875–2886 (2020).

113. Chou, T.-C. & Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* **22**, 27–55 (1984).

114. Kawashima, S. *et al.* γ-H2AX as a potential indicator of radiosensitivity in colorectal cancer cells. *Oncol. Lett.* **20**, 2331–2337 (2020).

115. Das, A. *et al.* Sildenafil (Viagra) sensitizes prostate cancer cells to doxorubicin-mediated apoptosis through CD95. *Oncotarget* **7**, 4399–4413 (2016).

116. Hassanvand, F. *et al.* Sildenafil enhances cisplatin-induced apoptosis in human breast adenocarcinoma cells. *J. Cancer Res. Ther.* (2020) doi:10.4103/jcrt.JCRT_675_19.

117. Sarwar, S., Yu, J. Q., Nadeem, H. & Huq, F. Synergistic Cytotoxic Effect from Combination of Wedelolactone and Cisplatin in HeLa Cell Line: A Novel Finding. *Drug Des. Devel. Ther.* **Volume 14**, 3841–3852 (2020).

118. Gamen, S. *et al.* Doxorubicin-induced apoptosis in human T-cell leukemia is mediated by caspase-3 activation in a Fas-independent way. *FEBS Lett.* **417**, 360–364 (1997).

119. Gamen, S. *et al.* Doxorubicin Treatment Activates a Z-VAD-Sensitive Caspase, Which Causes ΔΨm Loss, Caspase-9 Activity, and Apoptosis in Jurkat Cells. *Exp. Cell Res.* **258**, 223–235 (2000).

120. Koraneekit, A. *et al.* Synergistic effects of cisplatin-caffeic acid induces apoptosis in human cervical cancer cells via the mitochondrial pathways. *Oncol. Lett.* (2018) doi:10.3892/ol.2018.8256.

121. Sears, C. R., Cooney, S. A., Chin-Sinex, H., Mendonca, M. S. & Turchi, J. J. DNA damage response (DDR) pathway engagement in cisplatin radiosensitization of non-small cell lung cancer. *DNA Repair (Amst).* **40**, 35–46 (2016).

122. Plana, D., Palmer, A. C. & Sorger, P. K. Independent Drug Action in Combination Therapy: Implications for Precision Oncology. *Cancer Discov.* **12**, 606–624 (2022).

APPENDIX A

A.1.Tissue culture solutions

Cell freezing media
70% DMEM
20% Fetal Calf Serum
10% DMSO

10 X PBS
40g NaCl
1 g KCl
5.75 g $Na_2HPO_4.7H_2O$
1 g KH_2PO_4
Up to 500 ml with dH_2O

MTT (5 mg/ml)
100 mg MTT
20 ml 1 X PBS
Vortex and incubate at 37°C for 15min
Filter through a 0.2 μm filter
Wrap in foil and store at 4°C for up to 1 month

Solubilisation Reagent
25 g SLS
Up to 250 ml with dH_2O
Add 76.6 μl conc HCl

Fixation Solution
12.5 ml glacial acetic acid
87.5 ml methanol

Crystal Violet Solution
500 mg crystal violet
25 ml methanol
75ml dH_2O

A.2. Protein solutions

RIPA Buffer
150 mM Sodium Chloride
1% Trition X-100
1% Sodium Deoxycholate
0.1 % SDS
10 mM Tris-Cl, pH 7.4

4% Stacking gel
3.65 ml dH_2O
625 µl 1M Tris pH 6.8
50 µl 10% SDS
650 µl 30% Acrylamide
60 µl 10% APS
6 µl TEMED

10% Resolving gel
2.75 ml dH_2O
3.75 ml 1M Tris pH 8.8
100 µl 10% SDS
3.35 ml 30% Acrylamide
2000 µl 10% APS
20 µl TEMED

15% Resolving gel
3.3 ml dH_2O
3.9 ml 1M Tris pH 8.8
150 µl 10% SDS
7.5 ml 30% Acrylamide
150 µl 10% APS
15 µl TEMED

10 X Running buffer
20 g Glycine
31.6 g Tris
50 ml 10% SDS
Up to 500 ml with dH_2O

10 X Transfer buffer
72 g Glycine
19 g Tris
Up to 500 ml with dH_2O

1X Transfer buffer
100 ml 10 X Transfer buffer
200 ml Isopropoanol/Methanol
700 ml dH_2O

Stripping buffer
15 g Glycine
1 g SDS
10 ml Tween-20
Adjust pH to 2.2
Up to 1L with dH_2O

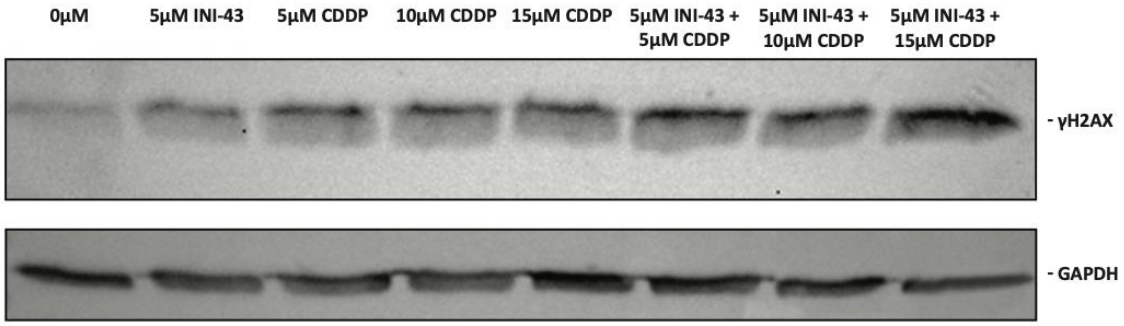
TBST
50 mM Tris-Cl, pH7.5
150 mM NaCl
0.05% Tween-20

A.3. PROTEIN MARKER

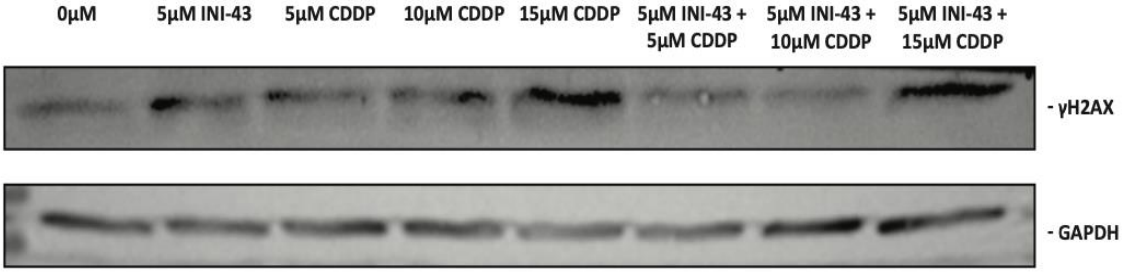
Figure A.1. **Protein molecular weight marker: Color Prestained Protein Standard (10-250 kDa).** Ladder used to determine the molecular weight of proteins subjected to polyacrylamide gel electrophoresis (10 and 15% SDS-PAGE gels). (New England Biolabs, US)

APPENDIX B

HeLa



ME-180



SiHa

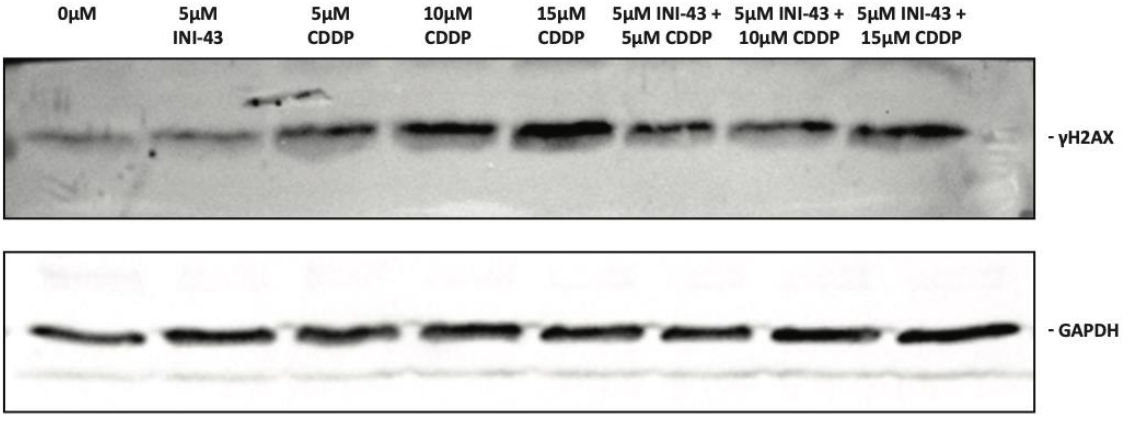
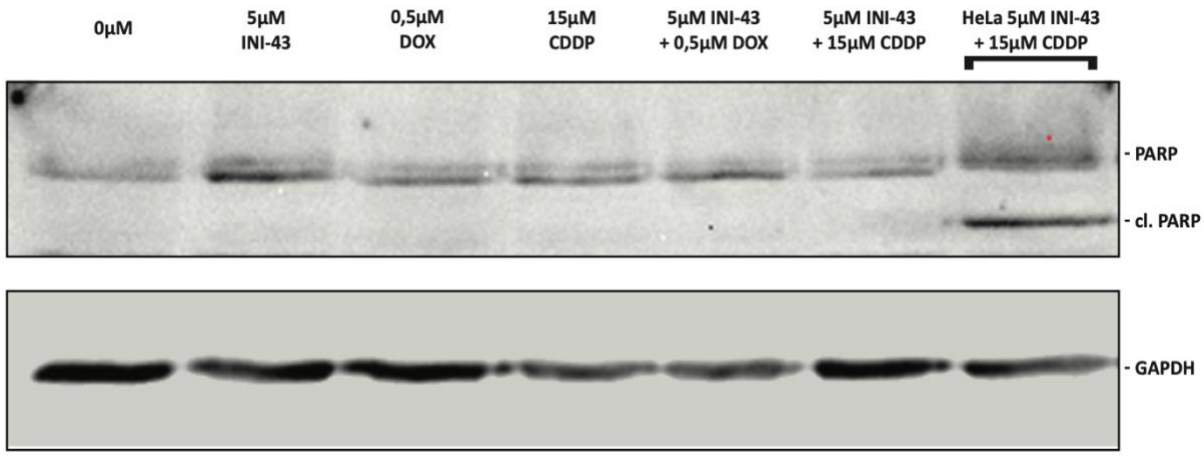


Figure B.1. Levels of γH2AX after INI-43 and CDDP treatment individually and in combination. **(A)** HeLa **(B)** ME-180 and **(C)** SiHa cells were treated, for 8 hours, with no drug, 5 μM INI-43, 5 μM CDDP, 10 μM CDDP, 15 μM CDDP and 5 μM INI-43 + 5 μM CDDP, 5 μM INI-43 + 10 μM CDDP, 5 μM INI-43 + 15 μM CDDP. Proteins were harvested and Western blot analysis was performed to determine relative levels of γH2AX (15 kDa). GAPDH was used as the loading control.

A)



B)

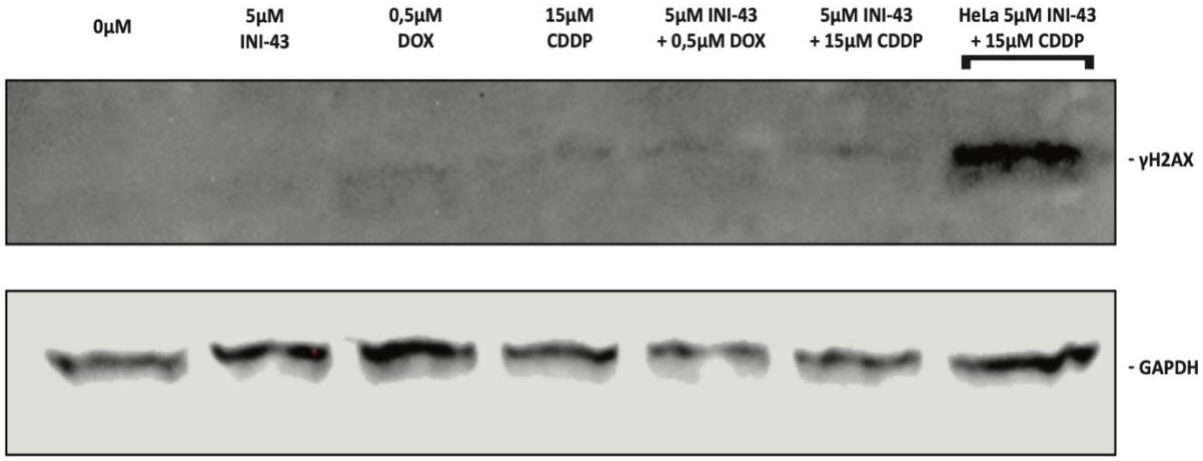


Figure B.2. PARP1 cleavage and γH2AX levels following combination treatment in ARPE-19 cells. Non-cancer ARPE-19 cells were treated, for 24 hours, with no drug, 5 μM INI-43, 0.5 μM DOX, 15 μM CDDP or 5 μM INI-43 + 0.5 μM DOX or 5 μM INI-43 + 15 μM CDDP. Protein was harvested and Western blot analysis was performed to determine relative levels of **(A)** uncleaved PARP1 (116 kDa) and cleaved PARP1 (89 kDa) using an αPARP1 antibody, as well as **(B)** γH2AX using an αPhospho-Histone H2A.X antibody. GAPDH was used as the loading control for both experiments. HeLa extracts from cells treated with 5 μM INI-43 + 15 μM CDDP were used as a positive control for PARP1 cleavage and induction of γH2AX. Western blots shown are representative results of experiments repeated at least 2 independent times.