The Manipulation of the Autophagic Pathway Sensitises Cervical Cancer Cells to Cisplatin Treatment.

by
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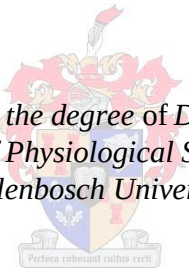

Dissertation presented for the degree of Doctor of Philosophy in the Faculty of Physiological Sciences at Stellenbosch University

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Declaration

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Date: March 2013

Abstract

Introduction

Cisplatin has been widely used to treat solid tumours and much success has come from the use of this drug in the treatment of head and neck, ovarian, testicular, cervical and small-cell lung cancers. However, the success of cisplatin treatment is limited due to its dose-limiting toxicity and its resulting side-effects, such as nephro- and ototoxicity. The devastating side-effects induced by cisplatin treatment provided the platform for this study whereby the aim was to lower the concentration of cisplatin while maintaining its cancer-specific cytotoxic action. Equally concerning is, cisplatin resistance which is becoming increasingly common, and this radically limits the clinical efficacy and utility of the drug. Adjuvant therapy has thus become necessary in an attempt to possibly curb or lessen the extent of cisplatin resistance. Due to the large body of evidence implicating the importance of autophagy in cancer, the prospect of targeting this mechanism has generally been accepted. Various chemotherapy agents induce autophagy in cancer cells; however the effect of cisplatin on autophagic induction has not been very well explored. We thus hypothesise that the manipulation of the autophagic pathway will sensitise cancer cells to a low concentration of cisplatin treatment. Furthermore, due to the functional interaction between Bcl-2 and Beclin-1 and its role in the regulation of autophagy, ratio analysis of Beclin-1 to Bcl-2 as means of detecting the role of autophagy within the cell under homeostatic and treatment/stress conditions has been conducted. Additionally, Bcl-2 has a prominent role in the malignant cell and it's over-expression has been found to confer resistance in a variety of cancerous cell lines. We therefore hypothesise that the silencing of Bcl-2 prior to cisplatin treatment will sensitise cervical cancer cells to apoptosis and increase the Beclin-1/Bcl-2 ratio in favour of apoptosis.

Materials and Methods

Three human cervical cell lines were used: a non-cancerous ectocervical epithelial cell line (Ect1/E6E7) and two cancerous cervical cell lines (HeLa and CaSki). In order to determine a concentration of cisplatin that was non-toxic to the non-cancerous Ect1/E6E7 cell line, a dose-response was performed.

With the use of an autophagy inhibitor (bafilomycin A1) and an autophagy inducer (rapamycin), autophagic flux capacities were assessed in each cell line through the Western blotting technique. In order to assess whether the chosen concentration of cisplatin induced autophagy, flow cytometry with the use of a Lysotracker™ dye was utilised, as well as analysis of autophagy protein levels (LC-3 II, Beclin-1 and p62). Autophagy modulation was achieved through two methods: pharmacological modulation with use of two recognised agents, namely bafilomycin A1 and rapamycin, and biological manipulation with the use of ATG5 and mTOR mRNA silencing. The effects of different treatment regimes on cell death was assessed with the use of PARP and caspase-3 cleavage through Western blotting, caspase-3/-7 activity (Caspase-Glo®), PI inclusion, LDH release and MTT reductive capacity. Additionally the effects of these treatment regimes on cell-cycle progression were also analysed.

Beclin-1 and Bcl-2 expression was determined through Western blotting and immunocytochemistry before and after treatment with cisplatin in HeLa and CaSki cells. To assess the reliance of the cervical cancer cells on Bcl-2 after cisplatin treatment, Bcl-2 knock-down was achieved through RNA interference, where after the Beclin-1/Bcl-2 ratio was assessed as well as apoptosis with the use of cleaved PARP analysis (Western blotting) and Caspase-Glo©.

For the *ex vivo* analysis, biopsies were collected from patients undergoing routine colposcopy screenings and hysterectomies at Tygerberg Hospital, Tygerberg, Western Cape. A total of 10 normal, 29 low-grade squamous intraepithelial lesions (LSIL), 33 high-grade squamous intraepithelial lesions (HSIL) and 13 carcinoma biopsies were collected for analysis, where after the expression profiles of two autophagy markers (mTOR and LC-3 II), as well as one anti-apoptotic marker (Bcl-2) were assessed. Protein levels were analysed through Western blot and confirmed through immunohistochemistry.

Results

Dose-response curves revealed that 15 µM of cisplatin did not induce cell death in the normal cervical epithelial cell line (Ect1/E6E7) and was therefore utilised through-out the remainder of the study. It was additionally determined that the CaSki cells were more resistant to cisplatin treatment when compared to the HeLa and Ect1/E6E7 cells.

Autophagic flux analysis revealed that, although all three cell lines were cervix derived, their autophagic flux capacities differed.

It was observed that the chosen concentration of cisplatin was able to induce autophagy in all three cell lines, with the HeLa cells demonstrating a particularly pronounced response. Autophagy modulation in conjunction with cisplatin treatment revealed the following: Autophagy inhibition with bafilomycin A1 lead to significant increases in caspase-3 and PARP cleavage and LDH release in both cervical cancer cell lines. The inhibition of autophagy through silencing of ATG5 induced caspase-3 cleavage and agrees with results obtained from pharmacological inhibition of autophagy with bafilomycin A1.

In addition to autophagic induction, a low concentration of cisplatin induced the up-regulation of Bcl-2, which when silenced significantly improved cisplatin-induced apoptosis in both cervical cancer cell lines.

Analysis of the expression profiles of mTOR and LC-3 in normal, pre-malignant (LSIL and HSIL) and cancerous cervical tissue revealed that autophagy is significantly up-regulated in HSILs and carcinoma of the cervix. Additionally, Bcl-2 expression is significantly increased in cervical carcinoma tissue, which agrees with results from other studies.

Conclusion

Autophagic flux capacities between the three cell lines investigated, derived from the same organ, differ significantly. This should be taken into consideration when autophagic modulation is being used as an adjuvant treatment. With regard to chemotherapy treatment in cervical cells, a low-concentration of cisplatin significantly induces autophagy in malignant and non-malignant cervix-derived cell lines where it serves a pro-survival mechanism. Inhibition of autophagy with bafilomycin A1 and ATG5 siRNA confirmed this survival effect in both cancerous cell lines where apoptosis was significantly increased. Interestingly, rapamycin pre-treatment together with cisplatin did not induce significant levels of apoptosis in HeLa cells where autophagy induction may have provided additional protection from the cytotoxic effects of cisplatin. Therefore the inhibition of autophagy through pharmacological and

biological inhibition improves the cytotoxicity of a low concentration of cisplatin and provides a promising new avenue for the future treatment of cervical cancer.

Bcl-2 up-regulation in response to cisplatin treatment also serves as a protective mechanism by which cervical cancer cells survive. The extent of apoptotic cell death observed after biological inhibition of Bcl-2 reiterates the fact that this response may be exploited in order to favour the use of lower concentrations of cisplatin. Analysis of clinical specimens emphasised the value of the *in vitro* work: Cervical cancer biopsies had increased expression of both LC-3 II and Bcl-2, indicating autophagy induction and apoptosis inhibition, respectively.

Thus two novel methods of improving cisplatin cytotoxicity have been demonstrated in the following study. Treatment regimens may administer more frequently and prolonged due to the minimal side-effects that accompanies low-dose cisplatin treatment.

Uittreksel

Inleiding

Sisplatien word algemeen gebruik vir die behandeling van soliede gewasse. Baie sukses is reeds deur die gebruik van díe middel behaal in die behandeling van kop en nek, ovariale, terstikulêre, servikale en klein-sel kankers. Die sukses van Sisplatien-behandeling word wel ingeperk deur die dosis-beperkende toksisiteit en die gevolglike newe-effekte soos nefrotoksisiteit. Hierdie verwoestende newe-effekte wat deur sisplatien behandelings geïnduseer word, het as die platform vir hierdie studie gedien. Die doel was om die sisplatien konsentrasies te verlaag, maar terselfdertyd die kankerspesifieke sitotoksisiteit te behou. Nog ’n punt van kommer is dat sisplatien-weerstandigheid aan die toeneem is, wat die kliniese effektiwiteit en gebruik van hierdie middel geweldig beperk. Byvoegmiddels het dus noodsaaklik geraak in die poging om die sisplatien-weerstandigheid te verhoed. As gevolg van verskeie bewyse wat die belangrikheid van outofagie in kanker impliseer, is die vooruitsig om hierdie meganisme te teiken, algemeen aanvaar. Verskeie chemoterapeutiese middels induseer outofagie in kanker selle, hoewel die effek van Sisplatien op outofagiese induksie nog nie goed ondersoek is nie. Ons hipotese is dus dat die manipulasie van die outofagiese pad die kankerselle sensitiseer tot ’n lae konsentrasie van sisplatien. Verder, as gevolg van die funksionele interaksie tussen Bcl-2 en Beclin-1, en hul rol in die regulering van outofagie, is verhouding-analises van Beclin-1 tot Bcl-2 uitgevoer met die doel om die rol van outofagie in die sel onder homeostatiese en behandeling/stres kondisies te bepaal. Verder is Bcl-2 bekend daarvoor om ’n prominente rol te speel in kwaadaardige selle, en die ooruitdrukking daarvan is gevind om weerstandigheid aan te help in ’n verskeidenheid van kankeragtige sellyne. Ons hipotetiseer dus dat geenonderdrukking van Bcl-2 voor die behandeling met sisplatien die servikale kanker selle sal sensitiseer tot apoptose en ’n verhoging in die verhouding van Beclin-1/Bcl-2 veroorsaak, wat in die guns van apoptose is.

Materiale en Metodes

Drie menslike servikale sellyne was gebruik: 'n nie-kankeragtige servikale epiteel sellyn (Ect/E6E7) en twee kankeragtige servikale sellyne (HeLa en CaSki). Om 'n konsentrasie van sisplatien te bepaal wat nie-toksies tot die nie-kankeragtige Ect1/E6E7 sellyn is, was 'n dosisrespons uitgevoer. Met die gebruik van 'n outofagiese inhibeerder (bafilomycin A1) en 'n outofagiese induseerder (rapamycin), is die outofagiese-fluks kapasiteite van elke sellyn deur die Western Blotting tegniek geassesseer. Om te bepaal of die gekose konsentrasie van sisplatien outofagie induseer, is vloeisitometrie met 'n Lysotracker™ kleurstof gebruik, sowel as analises op outofagie proteïenvlakke (LC-3 II, Beclin-1 en p62). Outofagie modulering is behaal deur twee metodes: farmakologiese modulering met twee erkende middels, naamlik bafilomycin A1 en rapamycin, en biologiese manipulasie met die gebruik van ATG5 en mTOR geenonderdrukking. Die effekte van die verskillende behandeling skedules op seldood was geassesseer deur gebruik te maak van PARP en kaspase-3 splitsing deur Western Blotting, kaspase-3/-7 aktiwiteit deur Caspase-Glo ®, PI-insluiting, LDH vrystelling en MTT reduserende kapasiteit. Verder is die effekte van hierdie behandeling skedules op selsiklus progressie ook geanaliseer.

Beclin-1 en Bcl-2 uitdrukking was ook bepaal deur Western Blotting en immunohistochemie voor en na behandeling met sisplatien in HeLa en CaSki selle. Om die afhanklikheid van die servikale kankerselle op Bcl-2 na sisplatien behandelings te toets, is Bcl-2 onderdruk deur RNA-inmenging, waarna Beclin-1/Bcl-2 verhouding geassesseer is, sowel as opoptose deur die gebruik van gesplitste PARP analises (Western Blotting) en Caspase-Glo©.

Vir die *ex vivo* analises is biopsies vanaf pasiënte wat roetine kolposkopie en histerektomies ondergaan, verkry (Tygerberg Hospitaal, Tygerberg, Westelike Provinsie). 'n Totaal van 10 normale, 29 lae-graad plaveisel intraepiteel letsels (LSIL), 33 hoe-graad plaveisel intraepiteel letsels (HSIL) en 13 karsinoom biopsies is verkry vir analises. Die uitdrukkingsprofiel van twee outofagiese merkers (mTOR en LC-3 II), asook een merker vir apoptose (Bcl-2), was geassesseer. Proteïen vlakke was ook deur Western Blotting geanaliseer en deur immunohistochemie bevestig.

Resultate

Dosisrespons kurwes het getoon dat 15 µM sisplatien nie seldood in die normale sellyn (Ect1/E6E7) geïnduseer het nie, en was daarom gebruik deur die res van hierdie studie. Verder is daar ook gevind dat CaSki selle meer weerstandig tot sisplatien behandelings is wanneer vergelyk word met die HeLa en Ect1/E6E7 selle. Outofagiese-fluks analises het getoon dat, alhoewel al drie sellyne vanaf die serviks afkomstig is, daar verskille is in hul outofagiese-fluks kapasiteit.

Daar is ook waargeneem dat die gekose konsentrasie van sisplatien in staat was om outofagie te induseer in al drie sellyne, met HeLa selle wat die mees merkbare respons getoon het. Modulering van outofagie in samewerking met sisplatien behandelings het die volgende onthul: inhibisie van outofagie deur bafilomycin A1 het gelei tot ’n beduidende verhoging in kaspase-3, PARP splitsing en LDH vrylating in beide servikale kankersellyne. Geenonderdrukking van ATG5 induseer kaspase-3 splitsing en stem ooreen met resultate wat verkry is deur farmakologiese inhibisie van outofagie met bafilomycin A1.

Bykomend tot outofagiese indusering, het ’n lae konsentrasie sisplatien die opregulering van Bcl-2 geïnduseer. Wanneer Bcl-2 geenonderdrukking in hierdie scenario toegepas was, het dit ’n beduidende verbetering in sisplatien-geïnduseerde apoptose in beide servikale kankersellyne getoon.

Analises van die uitdrukkingsprofiel van mTOR en LC-3 in normale, pre-maligne (LSIL en HSIL) en kankeragtige servikale weefsel, het getoon dat outofagie beduidend opgereguleer is in HSILs en servikale karsinome. Verder is Bcl-2 uitdrukking ook gevind om beduidend verhoog te wees in servikale karsinoomweefsel, wat ooreenstem met resultate verkry in ander studies.

Gevolgtrekking

Outofagiese-fluks kapasiteite tussen die drie sellyne, afkomstig van dieselfde orgaan, toon beduidende verskille. Hierdie bevinding moet in ag geneem word wanneer outofagiese-modulering as ’n bevorderingsbehandeling gebruik word. Met betrekking tot chemoterapie behandeling in servikale selle; ’n lae konsentrasie van sisplatien veroorsaak ’n beduidende indusering van outofagie in kwaadaardige en nie-kwaadaardige serviks-afkomstige sellyne, en dien as ’n oorlewingsmeganisme.

Inhibisie van outofagie met bafilomycin A1 en ATG5 siRNA het hierdie beskermings effek bevestig, aangesien apoptose beduidend verhoog was in beide kankersellyne. Interessant genoeg het rapamycin pre-behandeling tesame met sisplatien nie beduidende vlakke van apoptose in HeLa selle geïnduseer nie. Outofagie induksie mag dalk addisionele beskerming teen die sitotoksiese effekte van sisplatien gebied het. Daarom het die inhibisie van outofagie deur farmakologiese en biologiese inhibering die sitotoksisiteit van 'n lae konsentrasie sisplatien bevorder, wat 'n belowende bevinding is vir die toekomstige behandeling van servikale kanker.

Bcl-2 opregulering as gevolg van sisplatien behandelings dien ook as beskermings meganisme waarby servikale kankerselle oorleef. Die mate van apoptotiese seldood wat waargeneem word na biologiese inhibering van Bcl-2, wys weer op die feit dat hierdie respons uitgebuit kan word vir die gebruik van laer konsentrasies van sisplatien. Analises van die kliniese monsters het ook die waarde van die *in vitro* werk versterk: Servikale kanker biopsies het verhoogde uitdrukking van beide LC-3 II en Bcl-2 getoon, wat aandui dat outofagie geïnduseer en apoptose geïnhibeer word. Daar is dus twee nuwe metodes vir die verbetering van sisplatien-toksisiteit in hierdie studie gedemonstreer. Behandeling regimes kan meer gereeld en vir langer tydperke toegepas word, aangesien die newe-effekte van lae-dosis sisplatien behandelings minimaal is.

Research Outputs

1. Submitted Manuscripts:
    - **Leisching GR**, Loos B, Nell T, Engelbrecht A-M. *Sutherlandia* extract modulates the PI-3 kinase pathway and induces apoptosis in a colon cancer cell line.
2. In preparation:
    - **Leisching GR**, Loos B, Engelbrecht A-M. Cross-talk between autophagy and apoptosis: An investigation into the signalling mechanisms.
    - **Leisching GR**, Loos B, Engelbrecht A-M. Inhibition of autophagy sensitises cervical cells to undergo apoptosis in response to low-dose cisplatin treatment.
3. Conference proceedings:

Cancer & Metabolism: Pathways to the future, September 19-21, 2010, Edinburgh Scotland.

- **Leisching GR**, Loos B, Engelbrecht A-M. Cross-talk between autophagy and apoptosis: An investigation into the signalling mechanisms.

Physiological Society of Southern Africa, September 22-25 2010, Port Elizabeth, South Africa

- **Leisching GR**, Loos B, Engelbrecht A-M. Cross-talk between autophagy and apoptosis: An investigation into the signalling mechanisms.
    - Second prize in the Whyndam oral presentation.

Physiological Society of Southern Africa, September 10-13 2012, Stellenbosch, South Africa

- **Leisching GR**, Loos B, Engelbrecht A-M. Low-dose, high-impact: Circumventing cisplatin toxicity.
    - Winner in Wyndham oral presentation.

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List of Abbreviations

&: and

1º: Primary

2º: Secondary

ΔΨm: Change in mitochondrial membrane potential

A

ANOVA: Analysis of variance

Apaf-1: Apoptosis protease activation factor-1

ATG: Autophagy-related genes

ATP: Adenosine triphosphate

B

Bax: Bcl-associated partner containing six exons

Bcl2: B-cell lymphoma 2

Bid Bcl2 -interacting domain

C

C: Control

$CaCl_{2:}$ Calcium chloride

CARD: Caspase recruitment domains

Cisplatin: *cis*-diaminedichloroplatinum(II)

Caspase: Cysteine aspartate-specific protease

CO_2: Carbon dioxide

Cl.-PARP: cleaved-poly ADP ribose polymerase

D

DED: Death effector domains

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

E

EDTA: Ethylenediaminetetraacetic acid

ER: Endoplasmic reticulum

et al: Et alii

F

FADD: Fas-associated death domain

FAS-L: FAS ligand

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

G

GFP-LC3: Green fluorescent protein -microtubule-associated protein light chain 3

H

HPV: Human papilloma virus

hsc: heat shock cognate

HSIL: High-grade squamous intraepithelial lesion

I

IC: Inhibitory concentration

K

KSFM: Keratinocyte Serum-Free Medium

L

LC-3: Microtubule-associated protein light chain-3

LDH: Lactate dehydrogenase

LSIL: Low-grade squamous intraepithelial lesion

M

mPTP: Mitochondrial permeability transition pore

mRNA: messenger Ribonucleic acid

mTOR: Mammalian target of rapamycin

mTORC1: Mammalian target of rapamycin complex 1

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N

NaCl: Sodium chloride

NAD: Nicotinamide adenine dinucleotide

NADH: Nicotinamide adenine dinucleotide reduced

NADPH: Nicotinamide adenine dinucleotide phosphate

NaF: Sodium fluoride

NH_2: Amine group

O

O_2

OD: Optical density

P

P: phosphate

PARP: Poly ADP ribose polymerase

PBS: Phosphate buffered saline

PCD: Programmed cell death

Penstrep: Penicillin/Streptomycin

PI: Prodium Iodide

PI3-K: Phosphotidylinositol 3-kinase

PKB: Protein kinase B

PMSF: Phenylmethylsulphonyl fluoride

PTEN: Phosphatase and tensin homolog

PVDF: Polyvinylidene fluoride

R

RIPA: Radio immunoprecipitation assay

ROS: Reactive oxygen species

RT: Room temperature

S

Sc. seq.: Scrambled sequence

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM: Standard error of the mean

SiRNA: Small interfering ribonucleic acid

SQSTM1: Sequestome 1

T

tBid: Truncated Bid

TBS-T: TRIS-buffered saline-Tween

TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand

TRIS-HCl: Tri-(hydroxyl-methyl)-aminomethane-hydrogen chloride

U

UV Ultraviolet

V

vs: versus

1

Overview

1.1. Problem Statement and Motivation

Cervical cancer is the second most frequently diagnosed cancer in women worldwide despite effective screening programmes (Garcia et al., 2007, Jemal et al., 2008). Currently, cisplatin is used as cytotoxic agent for the management of this particular disease (Monk et al., 2005), however the severity of the side-effects of cisplatin treatment limits the use of this drug, particularly at high doses. The patient is often left with long-term damage which further decreases the quality of life (Redeker et al., 2000, Carlson et al., 2001).

Cancer cells are also becoming increasingly resistant to cisplatin treatment. A mechanism which is used by cancer cells to induce chemo-resistance is to increase autophagic activity in the cell (Chen et al., 2010, Notte et al., 2011). Autophagy, a process that functions as an intracellular quality control mechanism, has attracted much interest in the field of cancer research. Normally, basal autophagy is responsible for protein turnover and the elimination of damaged organelles and cytoplasmic components to maintain homeostasis in the cell (Maiuri et al., 2007). Under pathological conditions however, autophagy is considered to serve as a pro-survival mechanism, but inappropriate activation of autophagy can also result in cell death (Yang et al., 2008). Autophagy is up-regulated under conditions such as nutrient depletion, hypoxia and chemotherapy (Tsuchihara et al., 2008, Morselli et al., 2009, Brech et al., 2009).

Therefore the motivation for this study was to explore new avenues of chemotherapy and adjuvant treatment that would favour the use of lower concentrations of cisplatin, thereby limiting the side-effects that are commonly observed in normal tissue. Furthermore, mechanisms to reduce chemo-resistance through the manipulation of autophagy will also be investigated.

1.2 Hypothesis

It is thus hypothesised that modulation of the autophagic pathway will increase the cytotoxicity of cisplatin in cervical cancer cells, thus making the cancer cells more responsive to treatment and less chemo-resistant, thereby sensitising cervical cancer cells to cell death.

1.3 General Aims

An *in vitro* cervical cancer model made use of three cervix derived human cell lines: a normal non-cancerous ectocervical cell line (Ect1/E6E7), an adenocarcinoma cell line (HeLa) and a metastatic squamous cell carcinoma cell line (CaSki). Chapter 4 addresses four important pre-requisites/aims for this study: Firstly, to determine a concentration of cisplatin that is non-toxic to the normal, non-cancerous cervical cell line (Ect1/E6E7, Chapter 4.1), secondly to determine whether autophagic flux capacities could provide insight into the aggressive nature i.e. resistance, of the two cervical cancer cell lines (Chapter 4.2), thirdly to determine whether the three cell lines (from the same tissue) respond in a similar or different manner to autophagic stimulation, and lastly, to determine the value of autophagic flux as a pre-evaluation which will serve as a predictive tool in selecting a suitable autophagic modulator. Autophagic modulation may be achieved by the inhibition of autophagy with the use of bafilomycin A1, or its induction with the use of rapamycin before treatment with cisplatin.

There is very little information concerning cisplatin-induced autophagy as a focus point in the literature, particularly in malignant and non-malignant cervical cell lines. Therefore this is a relevant and worthwhile avenue to explore since it may shed light not only on the intracellular effects of cisplatin, but whether non-cytotoxic doses may be a future possibility in the treatment of cervical cancers. Therefore Chapter 5 aims to characterise the effects of the non-toxic concentration of cisplatin on autophagy, i.e. assess whether autophagy is up-regulated in response to this particular concentration of cisplatin and secondly to assess whether the cervical cell lines respond in a similar manner to treatment with cisplatin.

Literature suggests that cells utilise autophagy as a mechanism of surviving unfavourable conditions induced by anticancer drugs (Tiwari et al., 2008), therefore Chapter 6 aims to evaluate autophagic modulation as a pre-treatment to cisplatin with the use of two pharmacological agents (bafilomycin A1 and rapamycin) and two biological agents (ATG5 siRNA and mTOR siRNA). This will determine the reliance of both cancer cell lines on autophagy as a mechanism of resistance, and therefore the extent of cell death that is induced following autophagy induction or inhibition in combination with cisplatin treatment. This may then demonstrate the efficacy of autophagic flux pre-evaluation.

Cisplatin resistance is increasingly common, and has limited the efficacy and use of this drug in the clinic. Apart from autophagy, Bcl-2 up-regulation has been implicated in this resistance to cisplatin in a variety of non-cervical cancer cell lines (Miyashita and Reed, 1993, Dole et al., 1994, Miyake et al., 1998, Zangemeister-Wittke et al., 1998, Park et al., 2001, Oltersdorf et al., 2005, Cho et al., 2006, Michaud et al., 2009). Additionally, the functional interaction between Beclin-1 and Bcl-2 on autophagy and apoptosis respectively is highlighted as an important point of convergence between the two pathways (Pattingre et al., 2005, Maiuri et al., 2007), therefore the ratio of Beclin-1 to Bcl-2 is recommended as a means of assessing the role of autophagy under conditions of apoptosis (Pattingre and Levine, 2006a). Chapter 7 therefore aims to i) assess Bcl-2 expression under control and cisplatin treatment conditions, ii) assess the ratio of Beclin-1 to Bcl-2 under control and treatment conditions, iii) re-assess the Beclin-1/Bcl-2 ratio after silencing Bcl-2, and finally iv) to evaluate the extent of cell death (if any) with the use of Bcl-2 silencing as a pre-treatment to cisplatin therapy.

In order to determine the significance of the *in vitro* results, it was necessary to evaluate clinical specimens for autophagic (LC-3 II and mTOR) and apoptotic markers (Bcl-2). Due to the fact that cervical cancer development begins with a well-defined pre-malignant phase (Ambros and Kurman, 1990) that is characterised by low- and high-grade squamous intraepithelial lesion (LSIL and HSIL) development, Chapter 8 focused on evaluating the expression profile of two autophagy markers (mTOR and LC-3) and an apoptotic marker (Bcl-2) during each phase.

Before a study of this nature can be attempted, a thorough knowledge and insight into the aetiology and pathology of cervical cancer is required. In addition, autophagy

and apoptosis as well as the interaction between these two pathways need to be outlined for the purpose of this study. The current understanding and knowledge of these aspects will now be addressed in the literature discussion (Chapter 2).

2

Literature Review

2.1. Cervical Cancer

2.1.1. Incidence

The fight against cervical cancer thus far is a promising one; from the introduction of the Papanicolaou (Pap) test and liquid-based cytology, to the more recent development and release of the human papilloma virus (HPV) vaccine which is currently in use as a primary preventative measure for cancer of the cervix. Unfortunately, the disproportionate burden of cervical cancer in developing countries is due to the lack of screening, and as a result, 80% of all new cases in the world will occur in these under developed regions (Garcia et al., 2007). In South Africa alone, 16.48 million women are at risk of developing this disease (WHO/ICO, 2009). If introduction of a wide-spread immunization programme was introduced, it could lower the incidence of cervical cancer by up to 70% and greatly reduce the costs of cervical cancer screening (Woodman et al., 2007). It is however unlikely that secondary prevention through screening programmes would be scaled down or completely withdrawn for a number of reasons: older women that are not covered by the vaccination programme will still be at risk; protection by the vaccines are not absolute and the longevity is still uncertain up to this point with protection lasting a minimum 5 years (Villa et al., 2006, Harper et al., 2006). Equally important to note is that vaccines will only protect against HPV types that are targeted by the vaccine and the possibility of genotype replacement cannot be excluded.

Screening and vaccine programmes should ideally be integrated, as each alone will not reach all women. Effective strategies are needed to reach vulnerable populations where screening is needed most, and ensure that it is cost-effective (specifically for low-income districts) and that promotion of these programmes are community based and culturally sensitive. Thus the burden of cervical cancer is a long way from being resolved and the need for new and improved therapeutic strategies exists.

2.1.2. Epidemiology and Aetiology of Cervical Cancer

As early as 1842, it was determined by the Italian physician, Rigoni-Stern, that the development of cervical cancer was related to sexual contacts (Rigoni-Stern, 1842). Through the analysis of death certificates of women in Verona, Italy, during the period 1760-1839, he noted a high frequency of cervical cancer in prostitutes, widows and married women, but noticed its extreme rarity in virgins and nuns.

This notion rapidly set the wheels in motion to the discovery of the causal factor of cervical cancer, and by the 1970s, studies on the possible role of HPV in cancer induction was initiated. In 1982, a model was proposed whereby the high rates of cervical cancer in Latin American women could be attributed to a large number of sexual partners among males. This sexual behaviour pattern was paralleled by monogamy or few sexual partners among females (Skegg et al., 1982). Through this study, as well as others (Buckley et al., 1981, Brinton et al., 1989) it was determined that men operate as HPV vectors in the epidemiological chain and that HPV types related to cervical cancer are a wide-spread sexually transmitted disease. The massive growth of epidemiological and molecular data from then onward confirmed the carcinogenesis of HPV, and in 1991 it was officially concluded by the International Agency for Research on Cancer (IARC) that the association between HPV infection and cervical cancer is beyond reasonable doubt and that HPV is the aetiological factor in cervical cancer development (Bosch et al., 1992).

Over 100 HPV types have been identified, of these, 40 infect the genital tract (de Villiers et al., 2004). 15 high-risk or oncogenic types have been confirmed, of which HPV 16 followed by HPV 18, are the types found most commonly in squamous cell carcinomas (Clifford et al., 2003, Muñoz, 2003). HPV 18 is the type most strongly associated with adenocarcinomas of the cervix and is almost always integrated into the host genome in women with high-grade cervical intraepithelial neoplasia and invasive disease (Woodman et al., 2007). Although the incidence of squamous cell carcinoma has begun to decline in developed countries, adenocarcinoma of the cervix is now on the rise (Wang et al., 2004, Bray, 2005).

HPV types which infect the genital area can be divided into four groups depending on their oncogenic activity: high-risk types (HPV -16, -18, -31, -33, -35, -39, -45, -51, -52, 56, -58, and -59), probable high-risk types (HPV -26, -53, -66, -68, -73, 82), low-risk types (HPV -6, -11, -13, -40, -42, -43, -44, -54, -61, -70, -72, -81, and -89), and

types of undetermined risk (HPV -30, -32, -34, -62, -67, -69, -71, -74, -83, -84, -85, -86, -87, -90 and -91) (Muñoz et al., 2006). Because of the differing abilities of these HPV types to induce cellular oncogenic changes, it is becoming increasingly important in a clinical setting to implement HPV typing. This is a way of identifying patients who are at risk of developing cervical lesions and cancer, and as a result, patients can be managed and treated accordingly.

2.1.3. HPV Pathogenesis at the Cytological and Molecular Level

Genital HPV infections are transmitted by mucosa-to-mucosa or skin-to-skin contact (Burchell et al., 2006) where the virus enters presumably via microwounds in the epithelium. These openings allow the virus to reach the basal layer of cells (Fig. 2.1.), which consist of stem cells and undifferentiated cells that are continuously dividing (Longworth and Laimins, 2004). It is these basal undifferentiated cells that provide an ideal environment for copying viral DNA. The survival and thus successful reproduction of the HPV virus is highly dependent on the keratinocyte's differentiation program. Upon infection, a viral cascade is activated where the early genes are expressed (E1,E2 and E4-E7) (Table 1.). These are responsible for transcribing all

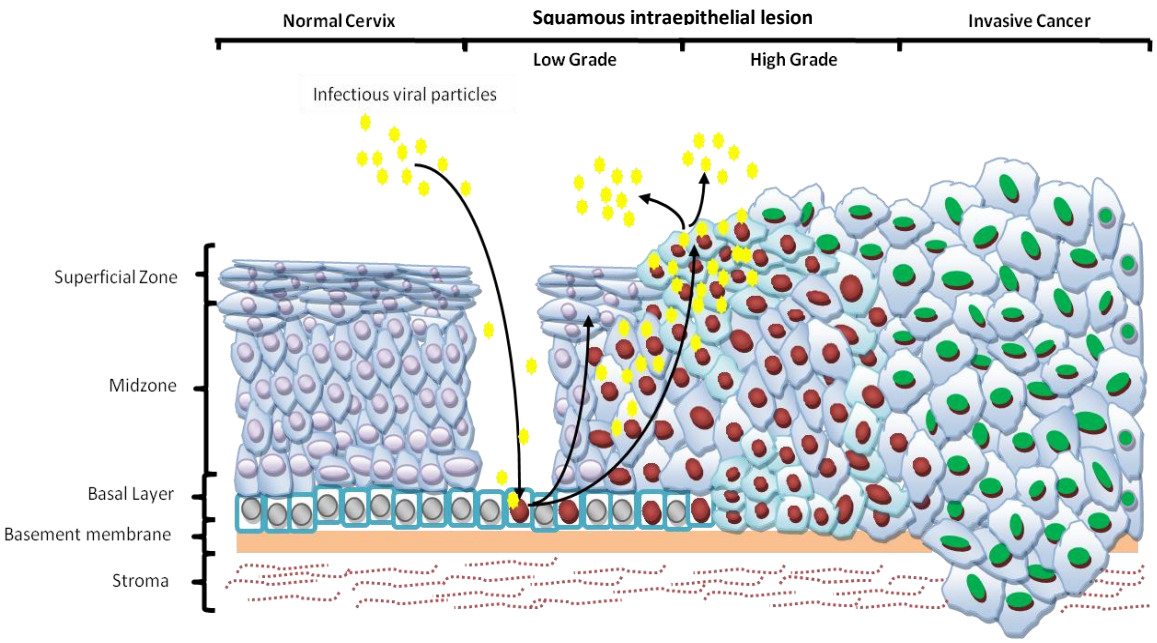


Figure 2.1. HPV-mediated progression to cervical cancer. The target of HPV is the basal cells where they are thought to enter via mico-abrasions in the cervix. Initial infection induces the expression of the early genes and the viral DNA replicates from episomal DNA (red nuclei). Progression into the upper layer causes the viral genome to replicate further, expressing the late genes which are responsible for encapsulating the viral genomes to form progeny virions in the nucleus. The shed virus then initiates new infections. Microinvasive and invasive carcinoma is associated with integration of the HPV genome into host chromosomes (green nuclei). Adapted and modified (Woodman et al., 2007)

viral proteins, except for those involved in capsid formation.

The HPV E6 protein has been the subject of great interest over the past few years with regards to its role in cervical cancer and its effect on the tumour suppressor protein p53. The E6 protein in high-risk HPVs are oncogenic, and this is mainly due to the fact that it binds with a higher affinity to p53 when compared to the E6 protein found in the low-risk types (Sotlar et al., 2004). p53, often referred to as the 'Guardian of the genome', plays a major role in DNA damage repair. Its expression is transiently increased, where it indirectly causes cell cycle arrest at the G_1 phase through its ability to control cell cycle progression. The E6 protein functions to either bind to and stimulate p53 degradation through a selective ubiquitin-dependant proteolytic pathway (Werness et al., 1990, Scheffner et al., 1990), or simply to bind to and inactivate p53. Ubiquitination and subsequent degradation of p53 decreases its half-life from several hours to less than 20 minutes in keratinocytes (Hubbert et al., 1992). The E6-mediated interference with p53 prevents the cell from undergoing apoptosis, thus resulting in genetic instability (chromosomal duplications and centrosomal abnormalities) and an increased risk of malignant conversion.

Table 1. Early genes expressed by HPV and their corresponding protein functions

Gene	Protein Function
E1	-Required for replication of extrachromosomal DNA and completion
	-Works in conjuction with E2 products
E2	-Full-length protein functions to transcrpitionally activate and bind to
	-DNA at the URR to enhance transcription of the early genes
	-Smaller protein inhibits transcription of early genes
	-Assists in extrachromosomal viral replication - works with E1
E4	-Required for maturation and development of the virus
	-Expressed during later stages of infection during viral assembly
E5	-Interacts with host cell membrane receptors EGF and PDGF
	-Thought to stimulate proliferation of infected cells
E6	-Crucial for viral replication, host cell immortalisation and transformation
	-Binds to p53 protein and causes its degradation through proteolytic mechanisms
E7	-Crucial for viral replication, host cell immortalisation and transformation
	-Binds to Rb protein causing its dissociation from E2F thus stimulating transcription of host cellular genes

The E7 viral product functions to bind to and interfere with the retinoblastoma (Rb) family of tumour suppressor proteins, as well as other proteins involved in cell cycle control. It reduces the ability of the Rb proteins to effectively manage the transition of the cycle through the G1/S phase (Steenbergen et al., 2005). As the HPV-infected basal cells divide, the copied viral genomes become separated into daughter cells, of which one begins to move toward the mid-zone where it nears differentiation. Uninfected cells will leave the cell cycle after progressing away from the basal layer

and lose their nuclei; this lack of nuclei at the suprabasal regions is characteristic of normal uninfected epithelia. In infected epithelia however, the keratinocytes remain active in the cell cycle and retain their nuclei (Longworth and Laimins, 2004). At this point, highly differentiated, infected cells re-enter the S-phase and activate the expression of viral components necessary for replication (Longworth and Laimins, 2004).

In the studies outlined above, it suggests that the functioning of E6 and E7 by the inactivation/degradation of p53 and Rb, respectively, is responsible for the oncogenic potential of HPV. However, malignant conversion is undesirable for the virus as well as the host because of the viral genome being integrated into the host genome, causing its disruption. Integrated HPV genomes almost always occur in women showing severe dysplasia or invasive disease where episomal forms are rarely detected (Woodman et al., 2007). This notion of integration being unfavourable to viral replication is established through the fact that cervical cancer cells do not produce viral particles. It therefore seems unlikely that the function of E6 and E7 in the viral life cycle is to contribute to oncogenic conversion (Vousden, 1993). A more probable hypothesis is that these proteins serve to generate a favourable cellular environment for replication. As mentioned previously, the viral life cycle is closely linked to the differentiation program of the keratinocyte and is highly dependent on the host replication machinery. It is thus important to continuously induce DNA replication in the host cell. The normal function of E6 and E7 may therefore be to prevent blocks in cell cycle progression and encourage production of DNA synthesis in keratinocytes.

The Bethesda system for reporting cervical or vaginal cytological diagnoses was introduced in order to establish uniform terminology and standardise diagnostic reports. An additional aspect was that it introduced a standardised approach for determining whether an individual specimen was adequate for evaluation in cytology smears stained by the Papanicolaou technique. For the forthcoming cytopathological discussion, the most recent 2001 Bethesda system (Apgar et al., 2003) will be used on a referral basis.

The cytological features of non-invasive HPV associated cervical lesions of the low-grade form are easily recognisable in cytological specimens. These features are indicative of productive viral infection and include: perinuclear halos, irregular nuclear

outlines, nuclear enlargement, nuclear hyperchromasia and multinucleation. Squamous epithelial cells exhibiting these characteristics are referred to as *koilocytes* and when these characteristics are evident in a cytological specimen, it is referred to as a low-grade squamous intraepithelial lesion (LSIL) (Apgar et al., 2003). Regression of HPV infection through the actions of the immune system occurs within 2 years of infection in approximately 90% of women (Wright, 2006), however these immune responses are poorly understood.

High-grade HPV-associated lesions exhibiting neoplasia are referred to as high-grade intraepithelial lesions (HSIL). These high-grade lesions may develop rather fast (within 2 years following normal cytology). HPV-induced cytological effects are present, but are usually less prominent than when compared to LSILs, most likely due to the fact that the viral genomes have been integrated and replication has come to a stop (mentioned previously). The immature basal cells have a minimal amount of cytoplasm and thus a high nucleus to cytoplasm ratio. Nuclear crowding, loss of normal cellular polarity and pleomorphisms are common features (Wright, 2006). Abnormal mitotic figures (aneuploidy) are characteristic of HSILs and their presence indicates that the lesion is neoplastic. If the viral infection has not been cleared by this stage cells begin to exhibit an expression pattern that is indicative of HPV-induced transformation. It is this step that sets the cell in motion toward gaining an immortal phenotype and subsequent invasive growth properties.

E6 and E7 expression play a major role in inducing transformation of the keratinocyte as a result of the deregulation of transcription seen in these oncogenes. Disruption of the viral genome as a result of integration into the host genome is the causal factor for the up-regulation of these genes seen in high-grade lesions and cervical cancer (Woodman et al., 2007, Steenbergen et al., 2005). Viral integration occurs downstream of the early genes, E6 and E7, frequently in the E1 or E2 region. This interference results in the loss of negative-feedback control by the viral regulatory E2 protein, which ultimately leads to complete silencing of p53 and Rb.

2.1.4. HPV Pathogenesis at the Histological Level

Cervical cancer is characterised by a well-defined pre-malignant phase which can be suspected upon cytological examination and confirmed through histological examination of cervical tissue. It was Richart (Richart, 1974) who introduced the concept that all types of precursor lesions to squamous cell carcinomas of the cervix represented a single disease process. The generic term used to describe this process was termed 'cervical intraepithelial neoplasia' (CIN).

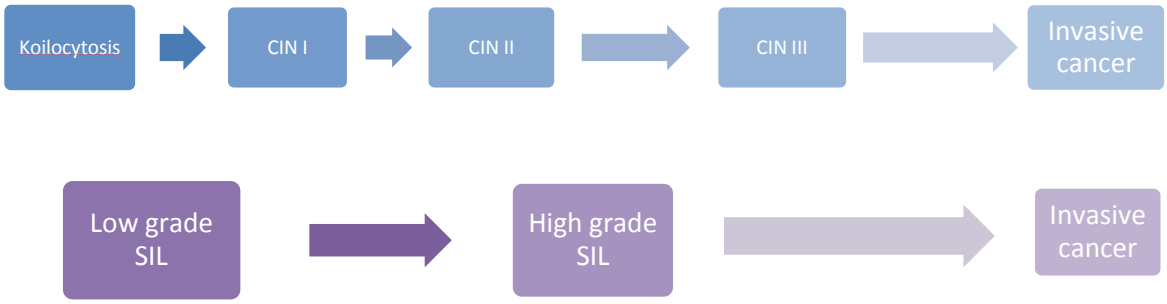


Figure 2.2. Terminology of precursor lesions of squamous cell carcinoma of the cervix. HPV infection induces cellular changes of the keratinocytes over time, consequently contributing to dysplasia and finally invasive cancer. CIN – Cervical intraepithelial lesions (terminology used to describe cytological changes), SIL – Squamous intraepithelial lesions (terminology used when describing histological changes of the cervix).

The pre-malignant changes are classified histologically on the basis of progressive atypia of epithelial cells: CIN1 (mild dysplasia) to CIN2 (moderate dysplasia) to CIN3 (severe dysplasia/carcinoma *in situ*) (Fig. 2.2.), and became widely adopted for the use in histopathology and cytopathology. The WHO classification system (Wells et al., 2003) is referred to from hereon in when discussing histopathological features of the pre-malignant/malignant cervix. Histological changes are seen in CIN1 where the architecture of the squamous epithelium becomes altered. A common feature is the thickening of the epithelium (acanthotic) as well as the formation of papillary projections with central fibrovascular cores. It is the proportion of the thickness of the epithelium showing mature and differentiated cells that is used for grading CIN and is dependent on the differentiation, maturation and stratification of the keratinocytes. In CIN1, undifferentiated cells are confined to the lower third of the epithelium where

good maturation of basaloid cells is seen. Mitotic figures are present, but are not very numerous. CIN2 is characterised by the appearance of mitotic figures in the lower half of the epithelium and dysplastic cellular changes which are more marked that those in CIN1. In CIN3, mitotic figures are seen through the full thickness of the epithelium, as well as the loss of cellular polarity. As a result, differentiation and stratification may be totally absent.

It is important to realise however, that certain lesions do not represent pre-cancer, and this is highly important for diagnostic specificity. For example, CIN1 is an insensitive histopathological sign of HPV infection, and is not pre-cancer. This was seen in studies done on histologically confirmed cases of CIN1 where it was shown that such lesions in fact represent a lower risk of progression to carcinoma than do the interpretations of LSILs (Cox et al., 2003). If a diagnosis indicates CIN1, this does not predict a significantly higher risk of CIN3 than does a negative biopsy (Cox et al., 2003). CIN3 on the other hand is suggestive of HPV-induced transformation and is a scientifically valid surrogate for long- term cancer risk as it can be estimated in clinical trials and prospective studies. In a comprehensive review on the natural history of CIN, it was found that only 32% of CIN3 lesions will regress when compared to 57% and 43% chance of regression of CIN1 and CIN2 lesions, respectively (Ostor, 1993). In the same study it was found that 56% of CIN3 lesions will persist if untreated, compared to 32% and 35% persistence of CIN1 and CIN2 lesions, respectively.

2.1.5. Invasive cancer: HPV involvement

The multi-step process toward the development of invasive cancer begins with persistence of high-risk HPV types in an individual where CIN lesions become progressively worse. The deregulation of E6 and E7 protein expression then occurs where the fine tuning of cell cycle control is altered, leading to genetic instability (mentioned previously). At this point, viral integration is often seen in conjunction with the evasion of the infection from the host immune system (Steenbergen et al., 2005). CIN2 is the result of these multi-factorial events and takes about 2-3 years to manifest.

It then takes a further 10-12 years for the lesion to gain an invasive phenotype (Koutsky et al., 1992). Within this time period, further cellular changes occur, such as genetic alterations involving oncogenes and tumour suppressor genes. The E6/E7

induced genetic instability causes tumour suppressor genes to become inactivated and oncogenes to become active. The inactivation of tumour suppressor genes causes immortalisation of the cell, as well as telomerase activation. Mortality stage barriers are bypassed with increased telomerase activation as it functions to maintain telomere length during cell division (Steenbergen et al., 2005). In a study conducted by Snijders *et al*, it was found that telomerase activity was found in 96% of squamous cell carcinomas and 40% of CIN3 lesions, with no detectable telomerase activity seen in normal cervices, CIN1 and CIN2 (Snijders et al., 1998). The final step is reached when the cell evolves from an immortal to an overt malignant and invasive phenotype.

Staging of cervical carcinomas is established using the FIGO (International Federation of Gynaecologists and Obstetricians) system. Invasive cancer cannot be visualised through gross inspection of the lesion, instead, diagnosis is based on histological examination of cervical tissue, preferably a cone, which must include the entire lesion. Invasion is characterised by the protrusion of malignant cells through the basement membrane, causing the margin of the invading nests to have a 'ragged' appearance. This irregularity in contour is the most reliable criterion in the diagnosis of invasive carcinoma. The extent to which the cells project into the stroma is a demonstration of the varying severities of invasion, where stage I is indicative of invasion confined strictly to the cervix, stage II is carcinoma that extends beyond the cervix and into the upper two-thirds of the vagina, but not as far as the pelvic wall. Stage III is characterised as carcinoma extending into the pelvic sidewall and lower third of the vagina, as well as hydronephrosis or a non-functioning kidney. Stage IV is carcinoma that has involved the mucosa of the bladder and/or rectum.

2.2. Cisplatin: The Platinum Drug

It has been almost 40 years since the clinical development of the neutral, square planar, coordination complex *cis*-diaminedichloroplatinum(II) (cisplatin). Cisplatin has been considered the most effective anticancer drug against cervical cancer in neoadjuvant and salvage treatment, with its success owing to the formation cytotoxic interstrand and intrastrand DNA cross-links. Cisplatin treatment is mainly administered to patients in the early stages of carcinoma (stages I to IIA), as well as patients with locally advanced or bulky cervical cancer. It can also be administered in

conjunction with radiation therapy followed by radical hysterectomy. It was previously unknown how the cellular uptake of platinum based complexes was regulated, but fairly recently, three copper transporters (CTR1, ATP7A and ATP7B) were found to be responsible (Lin et al., 2002, Kruh, 2003, Safaei et al., 2004a). Once cisplatin enters the cell, the drug undergoes strong hydration to form positively charged active species that interact with cellular nucleophiles (Andrews and Howell, 1990). There are many organelles and molecules with nucleophilic sites besides DNA, such as RNA, proteins, cytoskeletal microfilaments and membrane phospholipids that cisplatin also reacts with, therefore, despite the fact that it is generally accepted that genomic DNA is the critical pharmacological target of cisplatin, it is possible that it is not the causal factor of cisplatin-induced cytotoxicity.

It has been postulated that the cytotoxicity of the DNA adducts is translated into a programmed form of cell death, namely apoptosis (discussed in Section 2.4.) (Eastman, 1999). The advantage of apoptotic cell death, over necrotic cell death is the absence of an inflammatory response. Thus, apoptotic cell death is a favourable option when inducing cell death of cancer cells.

2.2.1. Cisplatin Resistance

Despite the broad clinical applications of cisplatin, resistance of cancer cells to the drug is becoming increasingly common and often culminates in chemotherapeutic failure. It has been suggested that this resistance may be attributed to a disruption in the normal apoptotic response. As a result of DNA damage incurred after cisplatin treatment, differential expression of various pro- and anti-apoptotic proteins has been observed. As mentioned earlier, p53 plays a major role in inducing apoptosis, the effects of which are seen by HPV-induced disruption of this protein resulting in major cellular and histological changes. In cisplatin resistant cells (HeLa/CDDP), it was demonstrated that even through the overexpression of p53 by transduction of a wild-type *p53* gene, apoptosis could not be induced (Minagawa et al., 1999), indicating that HeLa/CDDP cells were resistant to p53-mediated cisplatin-induced apoptosis. On the other hand, in normal cisplatin-sensitive cells, overexpression of the same wild-type gene increased the susceptibility to cisplatin-induced apoptosis. Thus, cisplatin-resistant cells have managed to suppress this pathway to an extent through the down-regulation of specific upstream initiator proteins and/or downstream effector proteins.

Resistance has also been attributed to a decreased influx of cisplatin into the cells. As mentioned earlier, CTR1, a membrane protein found to transport cisplatin into the cell, has altered expression levels in resistant cells. A study done on cisplatin resistant cervical cancer and ovarian cancer cells showed an almost 2-fold decrease in CTR1 expression when compared to the respective sensitive cell lines (Zisowsky et al., 2007). It is clear from the above discussion that these, as well as other factors are responsible for cisplatin resistance, whether they work in conjunction resulting in a cumulative effect, or work alone.

Another factor in rendering the cervical cancer cell resistant to cisplatin, is that which initiated the development of the cancer initially: HPV. Not surprisingly, the HPV oncogenic viruses have several mechanisms of suppressing apoptosis in order to survive. It was observed that HPV-positive cells were resistant to the anti-proliferative effects of cisplatin when compared to the viability of the HPV-negative cell line which was reduced by 51% over a 24h time period (Padilla et al., 2001).

2.2.2. Cisplatin: An *in vitro* perspective

Studies which focus on analysing the effects of chemotherapeutic drugs primarily use a concentration that inhibits 50% of cell growth (IC_{50}), a value that is obtained through analysing a dose response curve. Concentrations of cisplatin that have recently been used on various cell lines are specified in Table 2. When compared to the dose selected in this study (15 µM / 4.5 µg/ml for 24 h), the indicated doses are relatively high, and since the action of cisplatin is time-dependant, this too has significant effects on cytotoxicity. The strength of this approach is highlighted by the fact that this chosen concentration is up to 66 times less concentrated than the studies using cisplatin represented in Table 2. As mentioned previously, the efficacy of cisplatin treatment is underpinned by the toxic side effects which are often observed, thus there is a need for effective treatment options which utilise lower doses of cisplatin that are able to provide similar tumour-selective cell death with less cellular toxicity.

Table 2. *In vitro* cisplatin concentrations and treatment times on various cancer cell lines

Cisplatin dose	Treatment time	Cell type	Reference
20 µg/ml	24 h	PM1, MET1, MET4	Claerhout et al. 2010
64 µg/ml	24 h	A549/DDP	Ren et al. 2010
1500 µg	48 h	EC9706	Liu et al. 2011
15 - 60 µg/ml	48 h	CaSki	Sun et al. 2010
7.5 µg/ml	24 h	U251, C6, SHSY5Y, L929 HL-60, B16	Janjetovic et al. 2011
30 - 75 µg/ml	24 h	CHO, HeLa, CaSki, Jurkat	Keter et al. 2008
0.03 - 30 µg/ml	24h	HeLa, Siha, Caski, C33A	Ji and Zheng 2010
9 µg/ml	24 h	HeLa	Wang et al. 2000
0 - 300 µg/ml	4 - 18 h	HK2, Jurkat	Sancho-Martı´nez et al. 2011
10 µg/ml	24 h	SMMC-7721, HepG2	Chen et al. 2011
3 - 60 µg/ml	72 h	NRK-52E	Rovetta et al. 2012
5 µg/ml	48 h	SGC7901,BGC823	Feng et al. 2011
0 - 150 µg/ml	24 -48 h	SNU-1; 5; 16; 601; 638; 668	Huh et al. 2011
5 µg/ml	0 - 48 h	A2780, A2780/CP	Zhao et al. 2012

Furthermore, many studies fail to evaluate the effect of chemotherapy (particularly cisplatin) treatment *in vitro* on non-malignant cells, and this has created a gap in the literature.

2.3. Autophagy

Autophagy has been described as the first defence mechanism in response to a variety of cellular stressors (Moreau et al., 2010). Autophagy, a word derived from Greek meaning 'to eat one's self', embraces several different processes: microautophagy, macroautophagy and chaperone-mediated autophagy (Mizushima et al., 2008), all of which have a central function to degrade cellular proteins through the use of lysosomal machinery. Microautophagy involves the direct engulfment of cytoplasm at the surface of the degradative organelle by invagination, septation or protrusion resulting in direct sequestration of organelles by lysosomes. Chaperone-mediated autophagy is a process whereby unfolded proteins that contain a particular pentapeptide motif are chaperoned by the heat shock cognate (hsc) protein 70 to the lysosome for its subsequent degradation. Macroautophagy, hereafter referred to as autophagy, occurs at basal levels where it functions as a cytoplasmic 'quality control' process whereby long-lived/damaged organelles are eliminated (Mizushima et al., 2008). The role of autophagy in maintaining homeostasis extends further than sustaining a healthy cellular environment through organelle degradation; it is activated in response to nutrient depletion (Lum et al., 2005), it plays a role in cellular processes such as cellular development and differentiation (Levine and Kilonsky, 2004), and has been shown to play a protective role in the development of diseases such as cancer

(Levine and Kilonsky, 2004). Recently, it was proposed to serve as a mechanism by which tumour cells survive metabolically unfavourable conditions, such as hypoxia (Tsuchihara et al., 2008).

2.3.1 Autophagy: The Mechanism

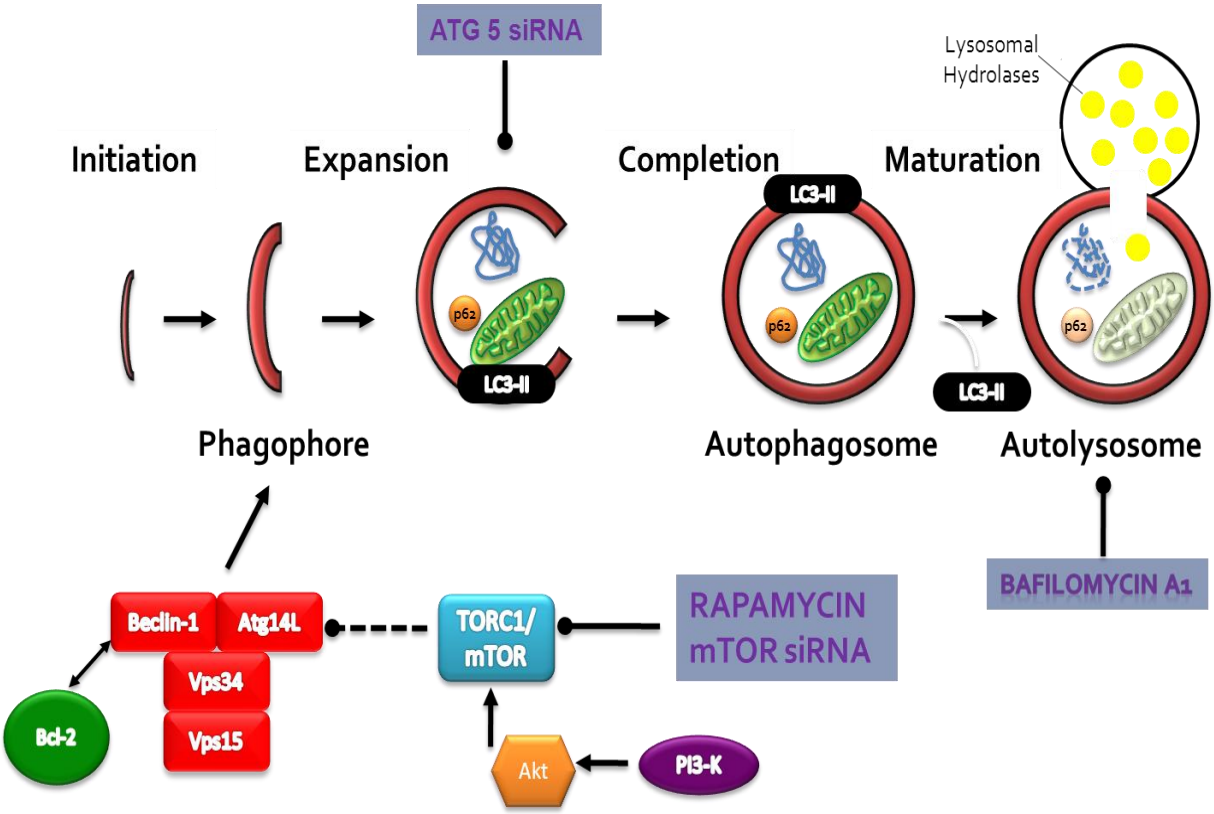


Figure 2.3. The process of autophagy. TORC1/mTOR, a rapamycin sensitive complex, is controlled upstream by PI3-K/Akt growth signalling complex where it negatively rates autophagy. Upon inhibition of TORC1/mTOR, Beclin-1 complexed with Vps34 and other essential proteins functions to sort vesicles and aid in membrane trafficking which initiates autophagosome formation (inhibited by silencing ATG5). Bcl-2, a Beclin-1 interacting protein, is able to bind to Beclin-1 and inhibit its pro-autophagic activity. Expansion of the phagophore and sequestration of organelles by p62 coincides with conversion of LC-3 I to LC-3 II, after which it is recruited to the forming the autophagosome and becomes membrane-bound. Completion and maturation of the autophagosome is signified by the fusion of the autophagosome with a lysosome to form an autophagolysosome. Lysosomal hydrolases then degrades the cellular components, including LC-3 II and p62. The macromolecular components are then made available to the cell's metabolism for other uses.

Autophagy is process that is controlled by the autophagy-related genes (ATG) originally found in yeast (Klionsky et al., 2003). An upstream component is responsible for the regulation and activation of these genes (Fig.2.3.). It

encompasses three main proteins: Phosphoinositide 3-kinase (PI3-k), Akt, and the mammalian target of rapamycin (mTOR) (Levine and Deretic, 2007). The class III PI3-k (Vps34) induce autophagy through binding with Atg 6 (Beclin-1) where it becomes localised in the trans-golgi network (the region where autophagosome formation is thought to take place). Here, Beclin-1 functions to sort specific autophagosomal components (Kihara et al., 2001). Vesical biogenesis is thought to occur *de novo* as the double-membraned vesicles do not appear to be budding off from pre-existing organelles. It is generally accepted that the source of the autophagosomal membrane is the endoplasmic reticulum (ER) and is supported by the fact that ER function and machinery are necessary for autophagy (Ishihara et al., 2001).

Shaping of the autophagosome is controlled by two pathways: the Atg12-Atg5 pathway and the microtubule-associated protein 1 light chain 3 (LC3/Atg8)-phosphatidylethanolamine (PE) pathway (Ohsumi, 2001)(Fig.2.4.). The Atg12 pathway begins with irreversible bond formation between Atg12 and Atg5. A conjugate is then formed with the additional binding of Atg7 and Atg10. The binding of Atg7 causes the activation of Atg12 which is then able to transfer and bind to Atg10 through a thioester bond. Finally, Atg12 binds covalently to Atg5 to form the final conjugate (Atg12-Atg5). This conjugate then binds non-covalently to Atg16 forming the Atg12-Atg5-Atg16 complex (Mizushima et al., 1999). It becomes membrane-bound and is a pre-requisite for the recruitment of LC3-II, the product of the second conjugated pathway.

The second pathway involved in autophagosome formation begins with the cleavage of LC3 to LC3-I by Atg4. Upon activation by Atg7, LC3-I is transferred to Atg3 where it eventually becomes conjugated to PE through an amide bond. The resulting LC3-II then becomes membrane-bound where it is able to function as a membrane protein (Yorimitsu and Kilonsky, 2005). It has thus been utilised as a marker for detecting autophagosomes. The pre-autophagosomal structure (PAS) is formed as a result. Expansion/elongation occurs through the supply of lipids; possibly in the form of lipid bilayers present in transport vesicles. At this point, the exact source of lipids is still unknown. It is thought that lipid delivery is as a result of the cycling of Atg9 from the mitochondria serving as the donor to the autophagosome serving as the acceptor membrane (Reggiori et al., 2005). Upon autophagosome completion, ATG proteins comprising of the vesicle-forming machinery dissociate through a process known as

uncoating. Only after uncoating, the autophagosome is able to fuse with a lysosome, indicating that coating may prevent premature fusion of the autophagosome and lysosome (this step may be inhibited by bafilomycin A1).

Fusion of the outer membrane of the autophagosome with the lysosomal membrane occurs with the assistance of specific fusion machinery. After the outer membrane has fused (forming the autophagolysosome), the inner membrane then enters the lysosome where it is now termed the autophagic body. Lytic enzymes then proceed to degrade the outer membrane, allowing for the cytoplasmic components to be released and subsequently degraded. The final stage of autophagy occurs when the degraded products are recycled and reused for synthesis of other proteins that are

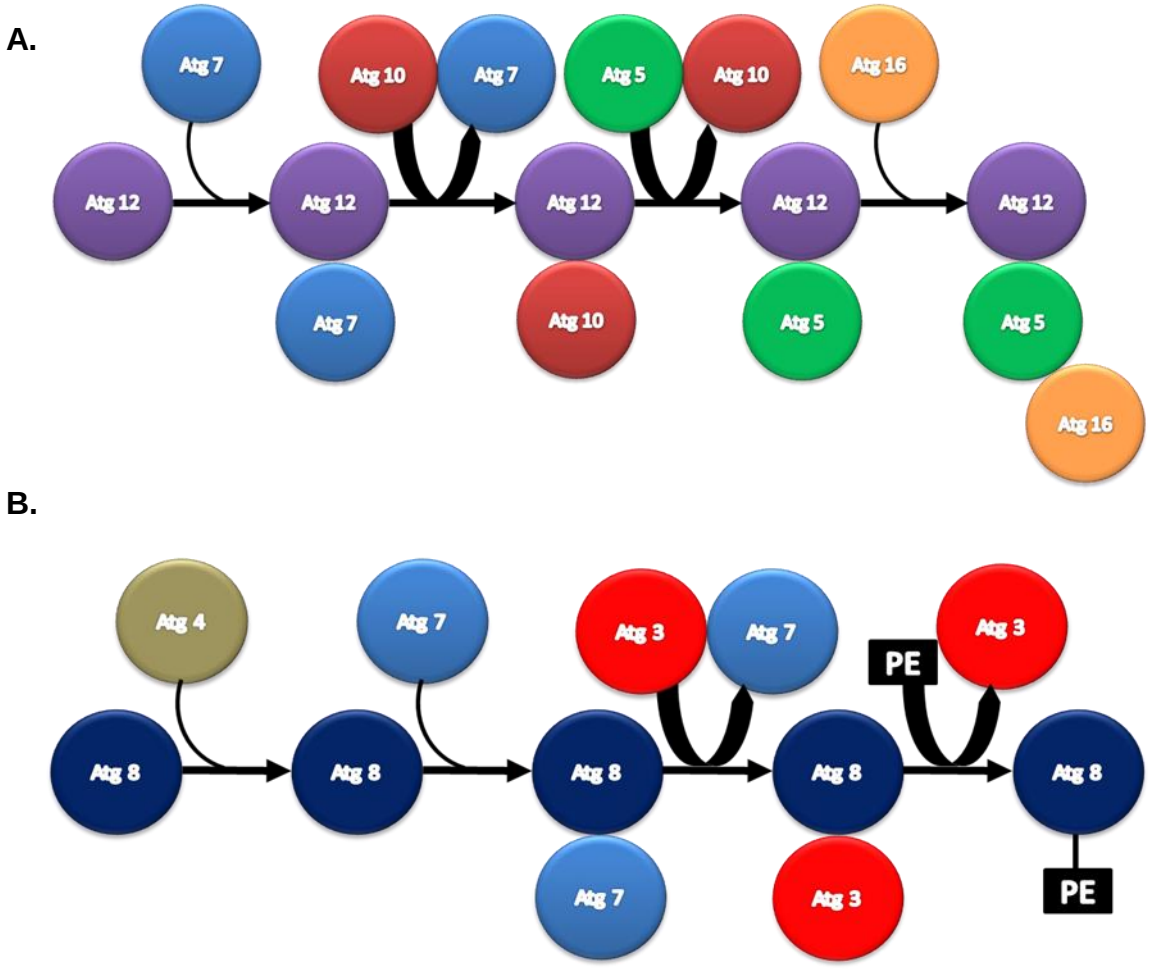


Figure 2.4. The molecular mechanism of autophagy. The process of autophagosome formation involves two ubiquitin-like conjugation pathways. **A.** The Atg5-Atg12 pathway which is responsible for driving the expansion of the membrane and dissociates just before, or immediately following completion. **B.** Atg8/LC-3 – PE pathway that results in covalent bond formation between ATG 8 and phosphatidylethanolamine (PE). This formation is essential for the sequestration and formation of vesicles in the autophagic pathway where it becomes degraded along with the cytoplasmic cargo. Adapted and modified (Yorimitsu and Klionsky, 2005).

needed or that are in short supply at that stage (Yorimitsu and Kilonsky, 2005).

2.3.2 Autophagic Flux

The capacity of a cell to induce autophagy is dependent on tissue type, which suggests that the reliance of cells on autophagy in different organ systems is dependent on their metabolic demand (Mizushima et al., 2004). This begs the question of whether all cancer cells have the same basal autophagic levels, or whether it differs among cell types. Also, do these cells have a similar or different capacity to induce autophagy, i.e. under certain conditions where autophagic flux is analysed, how much of the autophagic machinery is up-regulated when stimulated to its maximum? This may therefore represent an important indicator of aggressiveness and resistance. Autophagic flux can be described not only as the increased synthesis of LC-3, but completion of the process.

An accepted method for determining flux through the autophagic pathway is by analysing the turn-over of LC-3 II by Western blotting in the presence and absence of lysosomal degradation (Klionsky and Deter, 2008, Klionsky et al., 2012). If this pathway is blocked downstream where the fusion of autophagosomes with lysosomes is inhibited, thus preventing the recycling of the autophagosome, and induced upstream, autophagosomes (and therefore LC-3 II) will accumulate. bafilomycin A1 is a commonly used inhibitor of vacuolar formation and thus autophagy, and rapamycin, an inhibitor of mTOR, is a well- known autophagic inducer, both of which have been used in this study.

2.3.3. Dysregulation of Autophagy in Cancer

In the context of cancer, autophagy has been observed to exhibit both tumour-suppressive and tumour promoting functions under varying conditions. It may function as a temporary survival mechanism under various conditions of stress (Moreau et al., 2010), or cell death may ensue if this cellular stress leads to excessive or continuous autophagy (Liang and Jung, 2010). It is likely that it plays both roles, depending on the stage of cancer progression, the cell type, surrounding environment, amino acid levels, oxygen availability, viral invasion, etc. The engagement of autophagy by cancer cells is often observed during the late stages of tumour progression when it becomes up-regulated as a means of surviving unfavourable nutrient/oxygen conditions. Interestingly, literature suggests that both

the induction and the inhibition of autophagy in combination with chemotherapy treatment induces cell death.

a. Pro-Tumour Functions of Autophagy

It has been observed that autophagy is up-regulated under conditions such as nutrient depletion, hypoxia as well as chemotherapy (Tsuchihara et al., 2008, Morselli et al., 2009, Brech et al., 2009) and it has subsequently been proposed that autophagy promotes survival of cancerous cells under metabolic stress. Central to this metabolic stress is a lack of a steady supply of oxygen and substrates, a phenomenon commonly observed in primary, untreated tumours. These tumours do not have an established blood supply that is able to meet the high nutritional demands of aberrantly proliferating cells due to the requirement of amino acids and oxygen outweighing their respective availability supplied by the normal vasculature. Even after establishment of functionally and structurally adequate tumour vessels, the disorganised proliferation of these cells usually destroys the network of nutrient and oxygen supplying vessels, yet these cells are able to survive. During metabolic stress such as this, autophagy becomes up-regulated as a means of catabolising various unused/long-lived organelles as a source of metabolic substrates. By mitigating this metabolic stress, the integrity of the genome is preserved which will then confer resistance to anti-cancer therapy (Tsuchihara et al., 2008, Morselli et al., 2009).

The necessity for autophagy in cancer cells is observed in the following studies: In tamoxifen-resistant breast cancer cells, Beclin-1, ATG5 and ATG7 were eliminated through RNA interference, after which the cells were exposed to tamoxifen. This resulted in enhanced tamoxifen induced apoptosis (Qadir et al., 2008), which suggests that autophagy would normally assist in cell survival under these conditions. Likewise, the silencing of ATG proteins through siRNA sensitised cancer cells to radio- and chemotherapy (Tiwari et al., 2008). In cells that have detached from the extracellular matrix in order to metastasise, it was observed that autophagy was up-regulated (Lock and Debnath, 2008); presumably utilised as a way of surviving in the blood stream and in other organs where blood supply is limited. In cells defective in undergoing apoptosis (a common characteristic of cancer cells), autophagy is used to survive metabolic stress induced by radio- or chemotherapy (Kondo et al., 2005).

These examples substantiate the idea that cells utilise autophagy as a mechanism of surviving unfavourable conditions induced by anticancer drugs. Additional studies are required whereby autophagy is inhibited under conditions that induce its up-regulation, particularly with anti-cancer drugs, in order to elucidate at which point this inhibition becomes effective.

b. Anti-Tumour Functions of Autophagy

The first indication that autophagy may have a tumour suppressing effect emerged when it was observed that some cancer cells have a down-regulated autophagic response (Gunn et al., 1977, Kisen et al., 1993). A possible explanation for this may be that the up-regulation of the PI3-Kinase pathway is often observed in cancer cells (LoPiccolo et al., 2008, Jiang and Liu, 2008) which in turn results in the activation of the mammalian target of rapamycin (mTOR), a potent inhibitor of autophagy. This reveals an interesting point: autophagy- inducing molecules are under the control of tumour suppressor proteins such as phosphotensin-homologue deleted on chromosome ten (PTEN) and tuberous sclerosis (TSC) (Inoki et al., 2004).These are both tumour suppressors which function to suppress mTOR, a molecule which is a key regulator of cancer cell proliferation. Suppression of mTOR induces autophagy, whereas molecules which induce mTOR, such as the class I PI3-K and Akt, that are often up-regulated in various cancers (LoPiccolo et al., 2008, Jiang and Liu, 2008) , lead to the suppression of autophagy. Additional evidence to support these findings is the fact that autophagy genes themselves may operate as tumour suppressors (Liang and Jung, 2010). Although this suggests an anti-tumour function for autophagy, these molecules have roles in pathways that govern both life and death decisions, and thus reiterates the dynamics not only autophagy, but the cross-talk between this process and apoptosis.

Since autophagy has also been described as a ‘quality control’ process, it is not surprising that its down-regulation may prevent adequate maintenance of a healthy cellular environment, and may result in the accumulation of oncogenic events in the cell. In autophagy-defective cells, accelerated DNA damage was observed (Abedin et al., 2007, Katayama et al., 2007) as well as chromosomal instability followed by increased tumour susceptibility (Mathew et al., 2007). The exact mechanism for chromosomal instability is not known, however the accumulation of damaged

mitochondria and peroxisomes may play a role, as they are sources of excess reactive oxygen species (ROS), which induce genotoxic stress.

mTOR has been a central point of interest in terms of its regulation of autophagic function. Rapamycin, isolated from the microorganism *Streptomyces hygroscopicus*, functions to mechanistically inhibit mTOR (Vezina et al., 1975). Much success has come from the use of this inhibitor in cancer research (Vignot et al., 2005), and evidence suggests that the induction of autophagy has promising anti-tumour effects (Lefranc et al., 2007). For example, CaSki cells were observed to be increasingly sensitive to paclitaxel after the addition of rapamycin (Faried et al., 2006). Beclin-1 overexpression in the same cell line as well as various gastric cancer cell lines (MKN1, MKN28, MKN45, NUGC-3, AZ512 and TMK1) resulted in the sensitisation of these cells to undergo apoptotic cell death in the presence of cisplatin and other anti-cancer drugs (Furuya et al., 2005, Sun et al., 2010).

With regard to cisplatin treatment, overexpression of the Beclin-1 gene in CaSki and other cancer cells increases the chemosensitivity to cisplatin (Sun et al., 2010), and the combination of Beclin-1 over-expression together with anti-cancer drug administration is superior to that of anti-cancer treatment alone. Similarly, the induction of autophagy through the biological inhibition of mTOR using siRNAs restores cisplatin chemosensitivity in various lung cancer cell lines (Wangpaichitr et al., 2008).

Although these results contradict previous results related to cisplatin treatment, this differential response to the anti-cancer drug may be attributed to the following circumstances: firstly, the basal level of autophagy in the cell and its capacity to induce an autophagic response, secondly to what extent the cell relies/utilises autophagy in the presence of cisplatin, and lastly the dose and duration of cisplatin that is administered.

2.3.4. Cisplatin-Induced Autophagy

There is very little information concerning cisplatin-induced autophagy as a focus point in the literature, particularly in malignant and non-malignant cervical cell lines. Therefore this is a relevant and worthwhile avenue to explore since it may shed light not only on the intracellular effects of cisplatin, but whether non-cytotoxic doses may be a future possibility in the treatment of cervical cancers. More importantly, if

cisplatin does in fact induce autophagy, would it be possible to exploit this finding as a means of forcing the cell toward cell death?

Additionally, many studies fail to evaluate the effect of chemotherapy (particularly cisplatin) treatment *in vitro* on non-malignant cells and this should be addressed in the literature. There have been no studies to our knowledge that have assessed the effects of cisplatin and autophagy in the non-malignant Ect1/E6E7 cells, thus this will be assessed for the first time in this study. With that in mind however, a large amount of work has focused on the effects of cisplatin on cell death and autophagy in renal tubule epithelial cells, since cisplatin preferentially accumulates in this area, resulting in nephrotoxicity. It has been reported that cisplatin induces autophagy in renal epithelial cells *in vitro* and *in vivo* (Kaushal et al., 2008, Yang et al., 2008, Inoue et al., 2010) where it serves as a protective mechanism for the survival of these cells against apoptosis, which was determined through inhibiting autophagy with 3-MA and bafilomycin A1 (Periyasamy-Thandavan et al., 2008). Low-dose cisplatin (1 – 20 μM) was also observed to induce hepatic autophagy *in vitro* and *in vivo* where it plays a protective role against ischemia/reperfusion injury (Cardinal et al., 2009). The above results suggest that cisplatin may have an effect in activating the autophagic machinery in non-malignant cell types.

The effect of a low concentration of cisplatin in inducing autophagy is equally largely unknown, however a few studies have reported the following: Lui and colleagues reported an up-regulation of autophagy in oesophageal squamous carcinoma cells in response to cisplatin treatment (1.5 mg) through the analysis of LC-3 II and Beclin-1 (Liu et al., 2011). A similar response was observed in human U251 glioma, rat C6 glioma and mouse L929 fibrosarcoma cell lines in a dose and time-dependant fashion (Harhaji-Trajkovic et al., 2009), as well as in A549/DDP and A549 cells (Ren et al., 2010). This study by Ren and colleagues proposed that the cisplatin resistance observed in the lung carcinoma cells (A549/DDP) is attributed to the increased levels of autophagy when compared to the sensitive lung carcinoma cell line. These data suggest the potential importance of the role of autophagy in cisplatin treatment and therefore needs to be a point of focus in future studies.

2.4. Apoptosis

In instances of severe cellular stress, whether induced in the microenvironment or intracellularly, the organism is able to initiate cell death through apoptosis and avoid potentially life-threatening consequences. The term 'apoptosis' (Greek: apo- from, ptosis- falling) was coined in 1972 by Kerr, Wyllie and Currie to delineate the molecular events which lead to the distinct morphological cellular characteristics of cell death in living tissues (Kerr et al., 1972). Such characteristics include cellular shrinkage, membrane blebbing, chromatin condensation, and eventually fragmentation into apoptotic bodies. The fact that apoptosis is a gene-directed program has profound implications in our understanding of tissue homeostasis, as it implies that cell numbers can be regulated by factors that influence cell survival, proliferation, development (Zakeri and Lockshin, 2005, Zakeri and Lockshin, 2002) and aging (Lockshin and Zakeri, 1990). It is a multitude of interconnected pathways where the pattern of apoptotic death events manifest in a timely, ordered fashion. As a result, this process is often termed programmed cell death (PCD). PCD is essential in the removal of cells during development and degenerative disorders, as well as in cells that have been exposed to toxic compounds. Apoptosis occurs rapidly, leaving little trace, and as a consequence, the death of the cell is systemically unnoticed as no inflammatory response is initiated. In the last decade, the mechanisms of various signal transduction pathways that lead to PCD have been clarified, and with this information, apoptotic cascades have been described as intrinsic or extrinsic, caspase-dependent or -independent, p53-dependent or -independent and mitochondrial derived or death receptor governed.

a. Intrinsic Induction of Apoptosis

Intrinsically, apoptosis occurs as a result of a disturbance in the intracellular homeostasis (Fig.2.5.). Mitochondria play a critical role in the induction and execution of cell death in this pathway, and as a result it is often termed the 'mitochondrial death pathway'. It is initiated in response to intracellular death signals, such as DNA damage, growth factor withdrawal, oxidative stress or oncogene activation.

The decision of a cell's fate is dependent on the expression levels of the Bcl-2 family of intracellular proteins at any given time. The members of this family have been shown to both induce and inhibit the apoptotic process. They are characterised by the presence or absence of Bcl-2 homology (BH) domains, of which four have been

described: BH1, BH2, BH3, and BH4. The anti-apoptotic proteins, Bcl-2 and Bcl-X_L, both consist of all four BH domains, and function to antagonise pro-apoptotic protein function upstream of mitochondrially-mediated apoptosis. Two sub-families of pro-

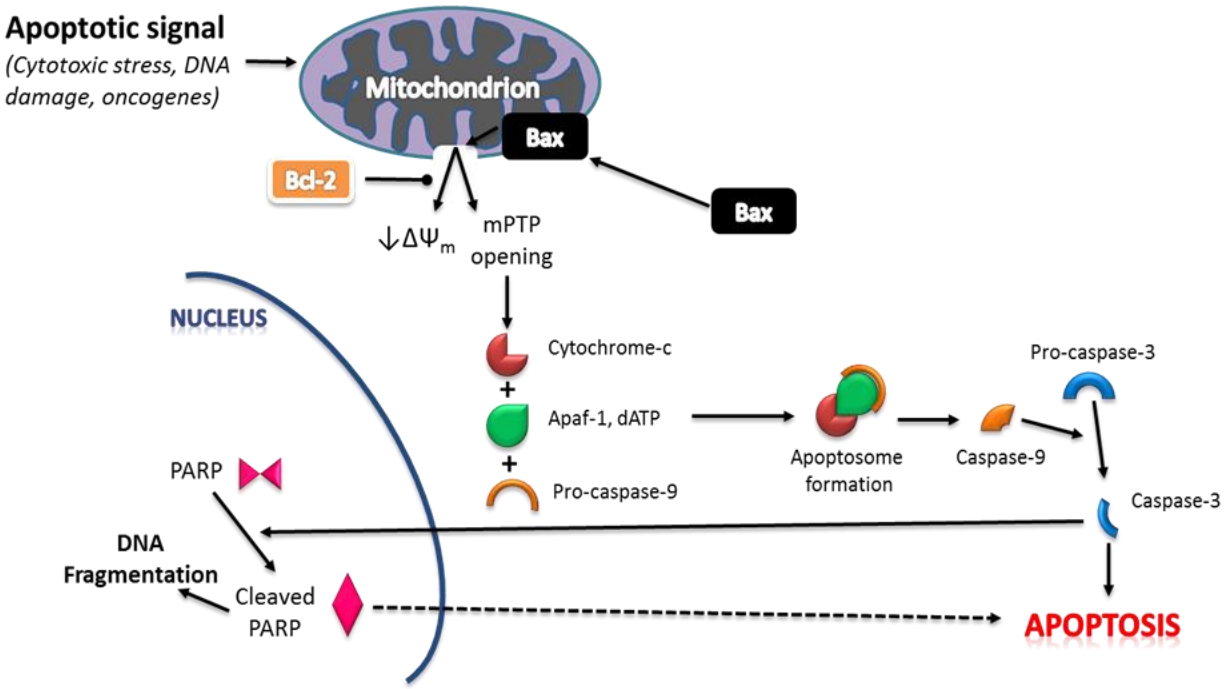


Figure 2.5. The Intrinsic Apoptotic Pathway. An apoptotic signal in the form of cytotoxic stress, DNA damage, etc. induces the translocation of Bax to the mitochondrial membrane which then facilitates the opening of the mitochondrial permeability transition pore (mPTP), leading to decrease in mitochondrial membrane potential ($\Delta\Psi_m$). Bcl-2, an anti-apoptotic protein is able to prevent this opening by interaction with Bax. Cytochrome-c is then released where it complexes with Apaf-1 and pro-caspase-9 to form an apoptosome. This facilitates cleavage of pro-caspase-9 into caspase-9 which then induces cleavage of the effector pro-caspase-3. Caspase-3 then cleaves PARP which prevents it from repairing DNA strand breaks and apoptosis is induced.

apoptotic proteins exist: the bax family (Bax, Bok, and Bak), which are comprised of the BH domains 1, 2, and 3, and the BH3 only family of proteins (Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, and PUMA). Both families of pro-apoptotic proteins are essential for the completion of apoptosis, and in some instances they work together in order to ensure it. A successful example of this is seen when Bid is cleaved by caspase-8 into the active tBid, which in turn induces Bax/Bak- dependent permeabilisation of the outer mitochondrial membrane and the subsequent release of cytochrome-c during Fas mediated apoptosis (Li et al., 1998). This relationship is established by the finding that apoptosis cannot be induced in the absence of Bak and Bax (Zong et al., 2001). There is still much to be learnt about the regulation of this family of proteins,

both between and within sub-families at the level of transcriptional control, protein translocation, and protein-protein interactions.

Apoptotic signals change the function of the Bcl-2 proteins leading to a change in mitochondrial integrity. One of these proteins is Bax, which is a pro-apoptotic protein whose expression is increased in response to death inducing stimuli. Bax moves from the cytosol and embeds itself into the mitochondrial membrane together with Bak, another pro-apoptotic protein. Here it is thought to cause the opening of the mitochondrial permeability transition pore (PTP), leading to a decrease in mitochondrial membrane potential and subsequent release of cytochrome-c. Bax and Bak are proven to be essential in mitochondrially mediated apoptosis since mitochondria deficient in either of them fail to release cytochrome-c (Wel et al., 2001). Cytochrome-c plays a crucial role in the electron transport chain and is found embedded in between the inner and outer membrane of the mitochondria. This supported the belief that the release of cytochrome-c is a crucial step in the execution of apoptosis. After its release, it binds to an adaptor protein known as Apaf-1 (apoptotic protease-activating factor-1), which, in the presence of ATP, induces self-oligomerisation. Once assembled, Apaf-1 recruits pro-caspase-9 and causes its auto-activation through a proximity-induced action. Procaspase-9 thus becomes the initiator caspase-9 that in turn activates the downstream effector caspase cascade.

Caspases. Cysteinyl aspartate-specific proteases (caspases) operate as a team to initiate and execute an orderly, irreversible and self-amplifying cascade that induces cell death (Cohen, 1997). Initially, caspases are synthesised as pro-caspases that are composed of a highly homologous protease domain, as well as an NH_2 terminal pro-domain. The critical protease domain contains two sub-units of approximately 10 and 20 kDa that form a heterodimer following its proteolytic processing. Two heterodimers then associate to form a tetramer which is the active form of the caspase (Walker et al., 1994). The NH_2 terminal pro-domain varies in length depending on the functional activity of the caspase. For example, initiator and inflammatory caspases possess long pro-domains that are more than 100 amino acids in length compared to the effector caspases which are characterised by pro-domains that are less than 30 amino acids long. Each long pro-domain host motifs that are essential for caspase activity, such as death effector domains (DEDs) in caspase-8 and -10, or caspase recruitment domains (CARD) in caspases -1, -2, -4, -

5, -9, -11, -12, -13, and -14. Both caspase-9 which serves as an initiator caspase during intrinsically induced apoptosis and caspase-8 which operates as an initiator caspase during extrinsically induced apoptosis, are able to cleave and activate effector caspases (-3, -6 and -7), thus rendering caspase-3, -6 and-7 the ultimate morphological and biochemical hallmarks of apoptosis, regardless of the apoptotic inducer.

PARP. Another hallmark of apoptotic cell death is the cleavage of poly (ADP- ribose) polymerase (PARP)(Berger and Petzold, 1985). Under normal circumstances, PARP catalyzes the poly (ADP- ribosyl)ation of many nuclear proteins, with NAD as a substrate (Hamid Boulares et al., 1999). It becomes activated when bound to DNA ends or strand breaks (DNA damage) and is needed during the repair of DNA damage. During cell death through apoptosis, caspase-3 is primarily responsible for the cleavage of PARP (Tewari et al., 1995, Nicholson et al., 1995), which inactivates the enzyme by destroying its ability to respond to DNA strand breaks within the cell, resulting in death. It has been suggested that the cleavage of PARP occurs as a means of preventing the depletion of energy (NAD and ATP) which is needed for late stage apoptosis (Berger and Petzold, 1985). As a result of PARP inactivation, DNA is fragmented into 200 base pair repeats that can be visualised on an agarose gel.

b. Apoptosis in Cancer

Early findings that indicate that apoptosis may have an effect on the development of a malignant phenotype goes back to the early 1970s where kinetic studies on tumour growth were conducted (Kerr et al., 1972). Tumour growth rates were less than 5% of that predicted by proliferation measurements alone, implying that cell loss from tumours may be substantial and that this ‘cell-loss factor’ may have a major impact on tumour growth or regression (Kerr et al., 1972, Wyllie et al., 1980). These observations, as well as subsequent studies on cytotoxic agents and tumour regression suggested not only that apoptosis contributed to cell loss in malignant tumours, but also that tumour progression may be promoted if apoptosis is disrupted.

Role in Tumourigenesis. One of the characteristics of tumour cells is their ability to evade apoptosis to allow them to survive and proliferate under stressful conditions, such as environments which lack nutrients and oxygen. In order to identify the role of apoptosis in tumour formation, it is important firstly, to identify apoptotic ‘triggers’ that a non-malignant cell encounters under various conditions, and secondly to determine

how these 'triggers' will provide insight into the driving forces of tumour evolution. In the skin, cells that are exposed excessively to UV rays undergo apoptosis presumably to remove cells that are extensively damaged (Ziegler et al., 1994). But loss of p53 in these damaged cells allows for their survival and subsequent transformation into malignant, tumourigenic cells (Ziegler et al., 1994). It is thus observed that a link between DNA damage, p53 signalling and apoptosis induction exists. Similarly, in patients treated with cisplatin, a common side-effect of nephrotoxicity is observed. Non-malignant renal tubular cells are especially sensitive to cisplatin treatment and it has been found that DNA damage (cross-links brought about by cisplatin treatment) in these cells induces apoptosis (Zhan et al., 1999) and that acute renal failure is associated with this treatment (Zhou et al., 1999).

Loss of p53 function in tumours is a common clinical phenomenon. Briefly, upon receiving the signal, cytoplasmic p53 undergoes (multi)mono-ubiquitination and rapidly translocates to the mitochondrial membrane where it interacts with members of the Bcl-2 family of proteins to cause permeabilisation of the outer membrane (Vaseva and Moll, 2008). This is accomplished through the interaction and subsequent neutralisation of the anti-apoptotic proteins Bcl-X_L and Bcl-2 which results in robust mitochondrial outer membrane permeabilisation and apoptosis (Mihara et al., 2003). If p53 becomes mutated (a common characteristic of many cancers) or degraded (as a result of infection with high risk-HPV), this down-stream cascade of events cannot occur, resulting in the malignant cell failing to die.

The Bcl-2 protein is another molecule that is altered in tumours. Its levels of expression have been used as a predictor of prognosis in ovarian cancer through the finding that late stage tumours had a higher expression of Bcl-2, which the authors suggest is the reason for the cells impaired ability to undergo apoptosis (Mano et al., 1999). Conversely, if Bcl-2 is found to be phosphorylated after treatment with a DNA damaging drug such as cisplatin, it is an indication that cell death through apoptosis is likely to follow, and that the drug is thus effective (Pratesi et al., 2000). Taken together, increased anti-apoptotic functioning of Bcl-2 plays a major role in tumour evolution (Adams and Cory, 2007).

2.5. The Beclin-1 Bcl-2 Interactome

The interaction between Beclin-1 and Bcl-2 proteins is an important point of convergence between the apoptotic and autophagic pathways. The initial discovery

of this complex was observed in a yeast two-hybrid screen where Beclin-1 was found to interact with Bcl-2 (Liang et al., 1999), and the significance of this was highlighted when Bcl-2 was down-regulated in HL60 cells resulting which resulted in cell death through autophagy (Saeki et al., 2000). At this point, little was known about the functional significance of this interaction, until work published by Pattingre and colleagues (Pattingre et al., 2005) suggested that Bcl-2 exerts an anti-autophagic function by binding to and inhibiting Beclin-1. Subsequently, the functional and spacial interaction between Beclin-1 and the anti-apoptotic proteins, Bcl-2 and Bcl-XL was elucidated (Maiuri et al., 2007).

Under conditions of homeostasis, the Beclin-1-Bcl-2 interaction ensures that autophagy is precisely regulated, and its activity is maintained particularly during nutrient depletion. Bcl-2/Bcl-XL firstly binds to and interferes with the pro-apoptotic proteins Bax and Bak at the mitochondrial membrane, thereby preventing apoptosis, and secondly it interacts with Beclin-1 to prevent excessive autophagy and possibly cell death (Pattingre et al., 2005). The importance of this interaction is underlined by the fact that Beclin-1 is a tumour suppressor protein and that inhibition of its function by Bcl-2 contributes to the oncogenic potential of Bcl-2 (Pattingre and Levine, 2006b). In an early study, the oncogenicity of Bcl-2 was observed in cervical carcinoma *in situ* samples as well as in invasive cervical carcinomas where it was determined that 81% and 62% of cases respectively were positive for Bcl-2 expression (Tjalma et al., 1998). In a study by Won and colleagues, tissue samples of invasive breast cancer were analysed for the presence of Bcl-2 and Beclin-1, and it was observed that Bcl-2 expression correlated with histological grade, distant metastasis and mitotic count, amongst other parameters (Won et al., 2010). Also, this expression was inversely correlated with the expression of Beclin-1, which the authors suggest is due to its inhibition by Bcl-2. In another study by Wang and colleagues (Wang et al., 2011), cervical squamous carcinoma tissue was analysed for the expression of Beclin-1. Although Bcl-2 expression was not analysed, a common trend was seen where Beclin-1 expression decreased significantly in malignant cervical cancer tissue when compared to those of normal or cervical intraepithelial neoplasia tissue.

The above evidence suggests that Bcl-2 not only has oncogenic potential, but also that its expression often occurs in the absence of Beclin-1 in a malignant cell. This association is likely to be more complex, nevertheless, it may bare the possibility that

potential for therapeutic treatment strategies exist. Bcl-2 antagonists have been applied clinically, and preliminary results seem promising (Leber et al., 2010).

3

Materials and Methods

3.1. Cell culture lines

HeLa and CaSki cells were purchased from Highveld Biological (South Africa) and grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (Gibco. Ltd) and 1% penicillin/streptomycin (P/S) (Sigma-Aldrich). Ect1/E6E7 cells (a kind gift from Dr. Donita Africander, Stellenbosch University, South Africa) were grown in Keratinocyte Serum-Free Medium (KSFM) supplemented with 4 mM $CaCl_2$, 0.1 ng/ml epidermal growth factor, 50 µg/ml bovine pituitary extract (Gibco. Ltd) and 1% P/S (Sigma-Aldrich). All cells were grown at 37°C and 5% CO_2 under humidified conditions. HeLa and CaSki cells were passaged upon reaching 70-80% confluency, and Ect1/E6E7 cells were passaged at a confluency of 60%.

3.2. Cisplatin preparation and treatment

Cisplatin (Sigma-Aldrich) was prepared before each treatment period by dissolving the powder in 0.9% NaCl solution to obtain a 0.001 M stock solution. It was then added to sub-confluent cells to reach final working concentrations of 5, 10, 15, 30, 60, 80,100 and 160 µM and incubated for a period of 24 h.

3.3. Autophagy modulators

Cells were treated at a confluency of 80 – 90% with 10 nM bafilomycin A1, 50 µM rapamycin or both (autophagic flux determination). In the case of dual treatment, cells were first treated with 10 nM bafilomycin A1 and left for a period of 24 h, where after 50 µM of rapamycin was added and left for a further 24 h. When acting as an adjuvant treatment, autophagic modulators were added to cells 24 h prior to cisplatin treatment.

3.4. MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is based on the principle of the reduction of MTT into blue formazan pigments by viable mitochondria in healthy cells and is therefore used to evaluate cytotoxicity

(Mosmann, 1983). HeLa and CaSki cells were both seeded in 24-well plates at a density of 3 x 10^4 cells/well and were allowed 24 h to adhere. Ect1/E6E7 cells were seeded in 24-well plates at a density of 5 x 10^4 cells/well and allowed 48 h to adhere. After the treatment period, 0.01 g/ml MTT was added to each well where after cells were incubated for 2 h at 37˚C in an atmosphere of 5% CO_2. HCl-isopropanol-Triton solution (1% HCl in isopropanol, 0.1% Triton X-100 in a 50:1 ration) was then added to each well and gently agitated for 5 min. Addition of this solution causes lysis of the cell membrane and the release of blue formazan pigments. The optical density (OD) was determined on a plate reader (EL-800, Micro-Tek instruments) at a wavelength of 540 nm and the values expressed as a percentage of the control.

3.5. Chromatin condensation analysis

Chromatin condensation (pyknosis) is a classical morphological characteristic of apoptosis (Kerr et al., 1972) and is one of the observable ultra-structural changes upon induction of this type of cell death (Kerr et al., 1972). The DNA intercalating flourochrome, Hoechst 33342 (1:200 dilution) was used to visualise this morphological nuclear change and was examined using a Nikon eclipse E 400 microscope. Cells were grown on coverslips in 6-well plates at a density of 2,5 x 10^5 cells/well for HeLa and CaSki cells, and 3,0 x 10^5 cells/well for the Ect1/E6E7 cells. They were then treated with varying concentrations of cisplatin and were incubated for a period of 24 h. Cells with condensed nuclear chromatin were counted and quantified as a percentage of the total number of cells.

3.6. Lysotracker™ (flow cytometry)

Cells were grown in T25 flasks at a seeding density of 700 000 cells/flask. After the treatment period, 2 ml Tryple-Xpress trypsin (Gibco) was added to each flask for 3-4 min until all cells had detached from the bottom of the flask. The cell suspension was then added to labelled 15 ml Falcon tubes (BD Biosciences) and centrifuged at 400 g for 3 min. The pellet was washed once with 0.1 M PBS where after Lysotracker™ (Invitrogen™, USA) was freshly prepared before use (1:10 000) and added to a final volume of 500 µl. Falcon tubes were then incubated at room temperature (RT) for 10 min and analysed. At least 10 000 cells were collected and analysed with a 488 nm laser at an emission of 610LP, 616/23BP.

3.7. Caspase Glo® Assay

The Caspase-Glo® Assay (Promega) is a luminescent assay that measures the activities of caspase-3 and -7, which are members of the cysteine aspartic acid-specific protease family that play vital roles in the execution of apoptosis. The principle of the assay is based on a caspase -3/7 luminogenic substrate which in the presence of cleaved caspase-3/7, enables a light reaction that is detectable at 490 nm $_{EX}$ and 510-570 nm $_{EM}$. Cells were seeded at a density of 10 000 cells/well in a 96-well plate. After the treatment period, the Caspase-Glo® buffer was added to the Caspase-Glo® substrate (as per manufacturer's instructions) and mixed to produce a working Caspase-Glo® reagent. This working solution was then added in equal volumes to cells containing medium (i.e. 100 µl medium and 100 µl Caspase-Glo® reagent). The liquid was mixed gently by pipetting and incubated at RT for 45 min where it was subsequently analysed using the GloMax luminometer (Glomax, Promega).

3.8. Lactate Dehydrogenase Assay

This Cytotoxicity Detection KitPLUS (Roche) is a colorimetric assay that measures lactate dehydrogenase (LDH) activity. LDH is a cytoplasmic enzyme that is readily released into the cell culture supernatant upon disruption of the plasma membrane. The principle of the assay is based on an enzymatic reaction: LDH catalyses the conversion of lactate to pyruvate with the simultaneous reduction of NAD^+ to NADH $^+$ H^+. A catalyst (diaphorase) then transfers the H and H^+ to the tetrazolium salt INT which is then reduced to formazan. Cells were grown in 96-well plates at a seeding density of 5000 cells/well in a final volume of 100 µl growth media and allowed 24 h to adhere. A background control was included to determine the LDH activity contained in the culture medium (blank) as well as a low control (LDH activity released from untreated cells) and a high control (maximum releasable LDH activity of the cells); these were performed on a separate plate in triplicate. After the treatment period, 5 µl 'Lysis Solution' was added to the high control wells and incubated for 10 min at 37 °C on a cell shaker. 100 µl "Reaction Mixture' was added to each well (including the high control, low control and background control) using a multi-pipette and then incubated at room temperature for 15 min and protected from light. Next, 50 µl 'Stop Solution' was added and the plates were allowed to shake for 10 s. An ELISA plate reader (EL-800, Micro-Tek instruments) was used to measure

the absorbance at 490 nm with a reference wavelength of 600 nm. The percentage of LDH release was determined as follows: LDH release (%) = (exp. value – low control) / (high control – low control) x 100.

3.9. Propidium Iodide (PI – Flow Cytometry)

Cells were grown in T25 flasks at a seeding density of 700 000 cells/flask. After the treatment period, 2 ml Tryple-Xpress trypsin (Gibco) was added to each flask for 3-4 min until all cells had detached. The cell suspension was then added to 15 ml Falcon tubes (BD Biosciences) and centrifuged at 6000 g for 3 min. The supernatant was removed and the pellet washed with 0.1 M PBS. The cells were centrifuged again at the same specifications and the supernatant was removed as before. PI (Sigma-Aldrich) was added to the unfixed cells to obtain a final concentration of 1 mg/ml, incubated for 10 min and analysed on the flow cytometer (BD FACSAria I). A minimum of 10 000 events were collected and analysed using a 488 nm laser and 610LP, 616/23BP emission filters. PI inclusion signified loss in membrane integrity and cell death. Values were represented as a percentage of the control.

3.10. Western Blotting

Cell were lysed with RIPA buffer (50 mM Tris-HCl pH 7.4, 10 ml of 10% NP-40, 2.5 ml of 10% Na-deoxycholate, 1 ml of 100 mM EDTA pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml leupeptin, 5 mg/ml SBT1, 1 M benzamidine, 0.1 mM sodium orthovandate, 50 mM sodium fluoride and 1 ml Triton X-100). The lysate protein content was determined using the Bradford technique (Bradford, 1976). Mini-PROTEAN® TGX™ (BIO RAD, USA) gradient gels were used (4-15%), which allowed for low as well as high molecular weight proteins to be probed for on the same membrane post-transfer (after stripping and re-probing).Cell lysates were diluted in Laemmli sample buffer and boiled for 5 min. 20 µg of protein was loaded and separated by electrophoresis. Proteins were then transferred to a PVDF membrane at 15 V and 0.05 A. Separated proteins were routinely stripped and re-probed with anti-actin antibody to ensure equal loading. Non-specific binding sites on the membranes were blocked with 5 g milk powder (fat-free) in 100 ml Tris-buffered saline-0.1% Tween 20 (TBS-T) and then incubated with primary antibodies (1:1000 dilution): Cleaved caspase-3, cleaved PARP, LC-3, p62, mTOR. ATG5, Bcl-2, Beclin-1 and β-actin (Beverly, MA, USA). Membranes were subsequently washed with large volumes of TBS-T (3 x 5 min) and the immobilized antibody was then

incubated with a horseradish peroxidase labelled secondary antibody (1:20 000 dilution) for 1 h. After washing again with TBS-T (3 x 5 min), membranes were covered with ECL™ detection reagents and quickly exposed to an autoradiography film (Hyperfilm ECL, RPN 2103) to detect light emission through a non-radioactive method. Films were densitometrically analysed (UN-SCAN-IT, Silkscience). All blots were scanned at a resolution of 150 dpi and the exact outline of each band was demarcated using this programme which takes into account all aspects of density and distribution. These analyses were performed under conditions where autoradiographic detection was in the linear response range.

3.11. RNA interference

mTOR and control/scrambled siRNA were purchased from Cell Signaling Technologies (Beverly, MA, USA) and supplied as a 10 µM stock solution. ATG5 siRNA was purchased from Invitrogen (USA) and supplied as a 20 pmol stock. The relevant proteins were silenced through reverse transfection. In brief, mTOR and control siRNAs were each diluted in 100 µl of serum-free, antibiotic-free DMEM/KSFM to a final concentration of 10 nM. ATG5 siRNA was diluted in 100 µl serum-free, antibiotic-free DMEM/KSFM to a final concentration of 20 nmol. This mixture was added to a well in a 24-well plate and mixed gently. 3 µl of FuGENE6 (Roche) was then added to this mixture and subsequently incubated for 20 min at room temperature (RT). During this time,cells were split from T75 flasks in antibiotic-free DMEM/KSFM and counted. 30 000 cells were then re-suspended in 300 µl antibiotic free media and added to the wells containing the siRNA-FuGENE6 mixture. The plate was gently rocked to allow adequate mixing of reagents and cells. Cells were then incubated for 48 h before continuing with treatments and analyses. Silencing was confirmed through Western blotting to detect changes in total amount of the targeted proteins.

3.12. Cell Cycle Analysis (flow cytometry)

Cell cycle analysis was performed by using the CycleTEST™ PLUS DNA Reagent kit (Becton Dickinson, CA, USA). CaSki and HeLa cells were seeded at a density of 250 000 cells per T25 flask and the Ect1/E6E7 cells at a density of 350 000 cells per T25 flask. Plating was performed 48 h before treatment. After the treatment period, cells were trypsinized and the cell suspension was centrifuged at 400 x g for 5 min at room temperature. The pellet was washed with 0.1 M PBS where after the cell

suspension was dentrifuged at the above specifications. 250 µl of trypsin buffer was added to each tube and allowed to incubate at room temperature for 10 min. Subsequently, 200 µl of RNase buffer and trypsin inhibitor was added to each tube and allowed to react for a further 10 min. Finally, 200 µl of ice cold PI stain was added to each tube and placed in the dark for an additional 10 min before analysis. At least 10 000 list-mode data events were acquired for each sample. A bandpass filter of 585/42 was utilised to detect light emitted between 564 and 606 nm. Results were obtained by using the CycleTEST™ PLUS DNA Reagent kit and ModFit LT software (Verity software house, Inc., ME, USA) on BD FACSAria I.

3.13. Immunocytochemistry

HeLa and CaSki cells were grown on coverslips in six-well plates (250 000 cells/well). After the treatment period, the medium was removed and cells were washed once with PBS. Cells were fixed with ice-cold methanol and acetone (1:1) and left to incubate at RT for 10 min. The fixative mixture was then removed and coverslips were allowed to air-dry for a further 10 min where after they were rinsed twice with PBS. Non-specific binding was prevented by incubating cells with 10% donkey serum for 1 h at RT. After this time period, the donkey serum was blotted off and Beclin-1 and Bcl-2 primary antibodies diluted in 1% bovine serum albumin (BSA, 1:50) was added to cells and allowed to incubate overnight at 4°C. Cells were then rinsed three times with PBS and allowed to incubate with the appropriate secondary antibody for 1 hr at RT. 10 min before the completion of incubation, Hoechst dye (1:200) was additionally added and kept in contact with cells for the remainder of the incubation period. Next, cells were rinsed and the coverslips were mounted on glass slides with DAKO fluorescent mounting medium (DAKO Inc., CA, USA). Slides were kept at -20°C until analysis.

3.14. Patients and specimen collection

Biopsies were collected from patients undergoing routine colposcopy screenings and hysterectomies at Tygerberg Hospital, Tygerberg, Western Cape (ethical reference number: N09/02/045). Samples were rinsed with PBS, placed in a cryovial and stored directly in liquid nitrogen until further use. A total of 10 normal, 29 LSIL, 33 HSIL and 13 carcinoma biopsies were collected for analysis.

3.15. Immunohistochemistry

Biopsies were embedded and sectioned at the pathology department at Tygerberg Hospital where 6 sections of each biopsy was prepared specifically for the purposes of this study. Prior to staining, tissue sections were deparaffinised in xylene and rehydrated in a graded series of ethanol. Epitope retrieval was acquired through trypsinisation for 1 h at 37°C. Blocking was done with donkey serum (1:50 dilution) for 1 h and blotted off. Primary antibodies recognising mTOR, Bcl-2 and LC-3B (Cell Signaling, USA) were diluted in 1% BSA (1:50 dilution) added to sections, and incubated overnight at 4 °C. Excess antibody was removed through rinsing 3 times with PBS where after a secondary antibody conjugated to a fluorophore was added (1:200 dilution) and incubated in the dark for 1 h at RT. Sections were washed 3 times with PBS and incubated with Hoechst 33342 dye (1:200 dilution) for 5 min at RT. After a final rinse with PBS, DAKO fluorescent mounting medium (DAKO Inc., CA, USA) was added and a coverslip placed carefully on each section for visualisation.

3.16. Statistical analysis

All values are represented as the mean ± standard error of the mean (SEM). A one-way or two-way analysis of variance (ANOVA) test was used where appropriate. A Bonferonni *post hoc* analysis was then employed to test for significance. The minimal level of significance was accepted as $p < 0.05$.

4

4.1. Dose-Response Curves and *In Vitro* Establishment of a Non-Toxic Dose of Cisplatin.

4.1.1. Introduction and Aims

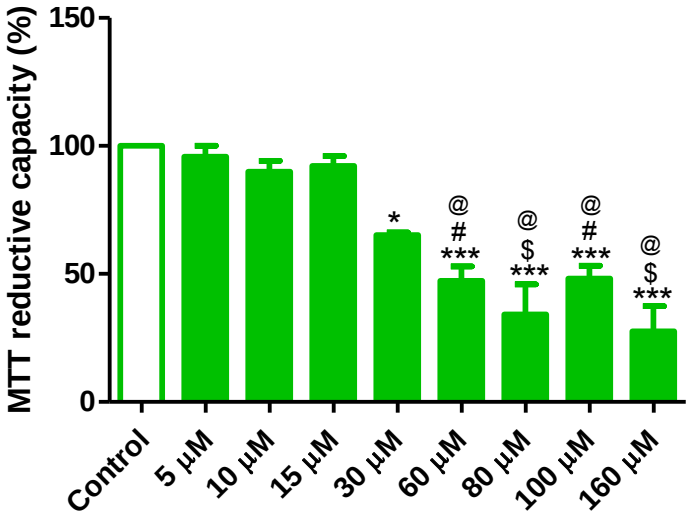
In South Africa, 16.48 million women are at risk of developing cervical cancer (WHO/ICO, 2009). Every year, 6742 women are diagnosed with cervical cancer, and of these, 3681 die from the disease (WHO/ICO, 2009). Women suffering from this disease have an overall decrease in quality of life (QOL), and on average, women who have cervical cancer have a decrease in life expectancy of 26 years (Herzog and Wright, 2007). Additionally, the administration of chemotherapy to cancer patients is more often than not a traumatic and painful experience and the side-effects that are commonly experienced during the treatment phase typically lead to a further decrease in QOL scores (Redeker et al., 2000). This is observed particularly in patients undergoing high-dose chemotherapeutic regimes (Carlson et al., 2001).

Here, an *in vitro* model was employed where a dose response using various concentrations of cisplatin was conducted, and its effects on cell death were analysed. The aims were to determine the concentration of cisplatin that did not significantly induce apoptosis in a non-cancerous Ect1/E6E7 cervical cell line. This dose response was conducted on two cervical cancer cell lines, namely CaSki, and HeLa cells, as a means of evaluating the response of these metabolically different cells and to compare their sensitivity to cisplatin treatment.

4.1.2. Results

All cell lines exhibit a dose-dependent decrease in cell viability, and increase in apoptosis in response to increasing concentrations of cisplatin.

A.



B.

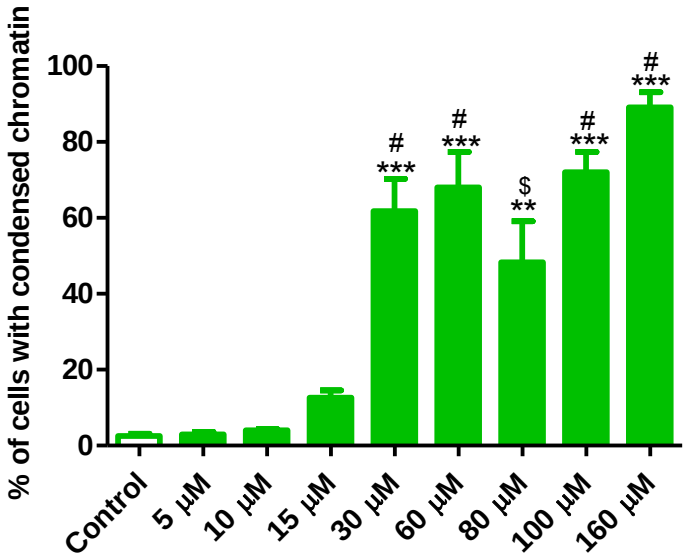


Figure 4.1. Cisplatin dose-response performed on Ect1/E6E7 cells for a period of 24 h. A. The MTT assay was employed in order to assess the effects of increasing doses of cisplatin on cell viability over a 24 hour time period. * $p < 0.05$, *** $p < 0.001$ vs. control, # $p < 0.05$ and $ $p < 0.001$ vs. 30 µM cisplatin, @ $p < 0.001$ vs. 15 µM cisplatin (n = 4). **B.** Chromatin condensation analysis using Hoechst 33342 dye was used to analyse the percentage of cells undergoing apoptosis in response to increasing concentrations of cisplatin over a 24 h time period. *** $p < 0.001$ vs. control, # $p < 0.001$ vs. 15 µM and $ $p < 0.01$ vs. 15 µM.

In order to establish a concentration of cisplatin that was not cytotoxic to non-cancerous, cervical epithelial cells (Ect1/E6E7), a dose-response was conducted where two methods were employed: one to assess overall cell viability (mitochondrial functioning) (Fig. 4.1.A) and one to assess apoptotic cell death (Fig.4.1.B). When compared to the control, Ect1/E6E7 cells did not show a significant reduction in their ability to reduce MTT to formazan when treated with 5, 10 or 15 μM of cisplatin (Fig.4.1.A). However, when treated with 30 μM of cisplatin, the reductive capacity decreased significantly (65.15 ± 0.98% vs. control, $p < 0.05$). Additionally, Ect1/E6E7 cells treated with 60, 80, 100 and 160 μM of cisplatin significantly decreased mitochondrial reductive capacity when compared to cells treated with 30 μM of cisplatin (60 μM: 47.35 ± 5.62% vs. 30 μM: 65.15 ± 0.98% $p < 0.05$;80 μM 34.13 ± 11.83% vs. 30 μM, $p < 0.001$; 100 μM: 48.20 ± 4.928% vs. 30 μM, $p < 0.05$; 160 μM: 27.55 ± 9.84% vs. 65.15 ± 0.98% $p < 0.001$). Furthermore, no significant differences between concentrations were observed in MTT reductive capacities of Ect1/E6E7 cells treated with 60, 80, 100 and 160 μM of cisplatin.

Condensed chromatin (pyknosis) was the next parameter assessed by means of Hoechst 33342 staining (Fig.4.1.B). Only cells treated with concentrations of 30 μM cisplatin and above exhibited a significant increase in cells with nuclear condensation, an indicator of apoptosis, when compared to the control (30 μM: 61.83 ± 8.50%; 60 μM: 68.00 ± 9.37%; 80 μM: 48.33 ± 10.83%; 100 μM: 72.03 ± 5.35%; 160 μM: 89.10 ± 4.00% vs. control $p < 0.001$).

15 μM of cisplatin was therefore chosen as the concentration to be utilised through-out the study as it did not induce significant changes in viability of the non-cancerous cells (Fig.4.1.A and B). All concentrations above 15 μM induced a significant decrease in mitochondrial MTT reductive capacity over a 24 h time period when compared to cells treated with 15 μM of cisplatin (30 μM: 65.15 ± 0.98%; 60 μM: 47.35 ± 5.62%; 80 μM: 34.13 ± 11.83%; 100 μM: 48.20 ± 4.93%; 160 μM: 27.55 ± 9.84% vs. 15 μM: 92.20 ± 3.84% $p < 0.001$). Similar results were observed in the fluorescent staining of DNA with Hoechst 33342: all concentrations of cisplatin induced significant increases in total percentage of nuclear condensation when compared to cells treated with 15 μM (30 μM: 61.83 ± 8.50%; 60 μM: 68.00 ± 9.37%; 100 μM: 72.03 ± 5.35%; 160 μM: 89.10 ± 4.00% vs. 15 μM: 12.63 ± 1.94% $p < 0.001$ and 80 μM: 48.33 ± 10.83% vs. 15 μM, $p < 0.01$).

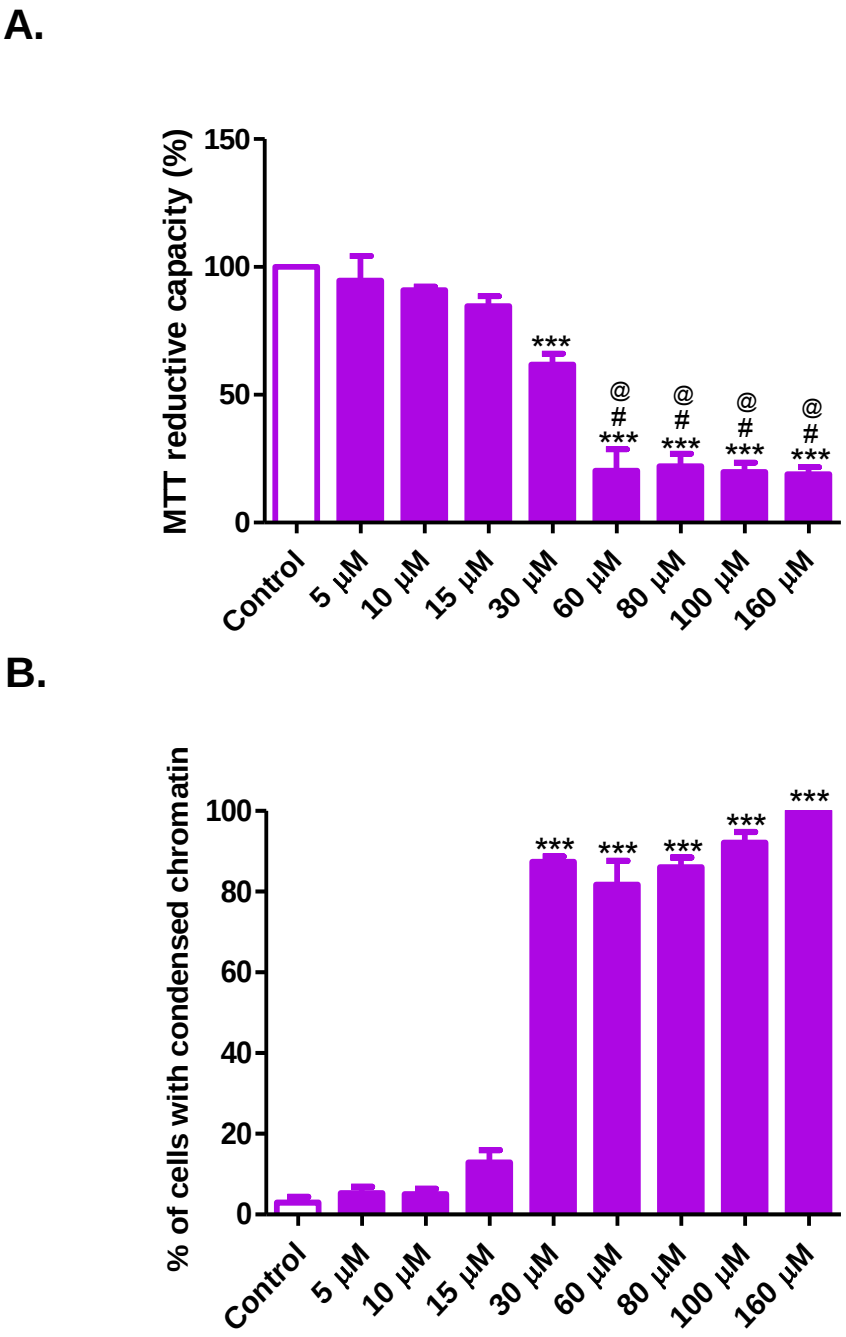


Figure 4.2. Cisplatin dose-response performed on HeLa cells for a period of 24 h. A. The MTT assay was employed in order to assess the effects of increasing doses of cisplatin on cell viability over a 24 hour time period. *** $p < 0.001$ vs. control, # $p < 0.001$ vs. 30 µM cisplatin, @ $p < 0.001$ vs. 15 µM (n = 4). **B.** Chromatin condensation using Hoechst 33342 dye was used to analyse the percentage of cells undergoing apoptosis in response to increasing concentrations of cisplatin over a 24 h time-point. *** $p < 0.001$ vs. control (n = 3).

When compared to the control, HeLa cells did not show a significant reduction in their ability to reduce MTT to formazan when treated with 5, 10 or 15 μM of cisplatin (Fig.4.2.A). However, when treated with 30 μM of cisplatin, the reductive capacity decreased significantly (61.8 ± 4.32% vs. control, $p < 0.001$). Additionally, HeLa cells treated with 60, 80, 100 and 160 μM of cisplatin further decreased the ability of the cells to reduce MTT to formazan when compared to cells treated with 30 μM of cisplatin (60 μM: 20.25 ± 8.49%; 80 μM: 22.03 ± 4.88%; 100 μM: 19.80 ± 3.58%; 160 μM: 18.90 ± 2.74% vs. 30 μM: 61.8 ± 4.32% $p < 0.001$). However, doses of 60 μM cisplatin and more did not induce any additional significant decreases in MTT reductive capacity.

Cisplatin treatment resulted in a dose-dependent increase in chromatin condensation in HeLa cells over a 24 h time period (Fig. 4.2.B). A significant increase pyknosis was observed when HeLa cells were treated with 30, 60, 80, 100 and 160 μM of cisplatin when compared to the control (30 μM: 87.33 ± 1.41%; 60 μM: 81.70 ± 5.93%; 80 μM: 86.07 ± 2.39%; 100 μM: 92.13 ± 2.66%; 160 μM: 100 ± 0.00% vs. control $p < 0.001$). No significant increase in pyknosis was observed when HeLa cells were treated with doses of 30 μM cisplatin and more.

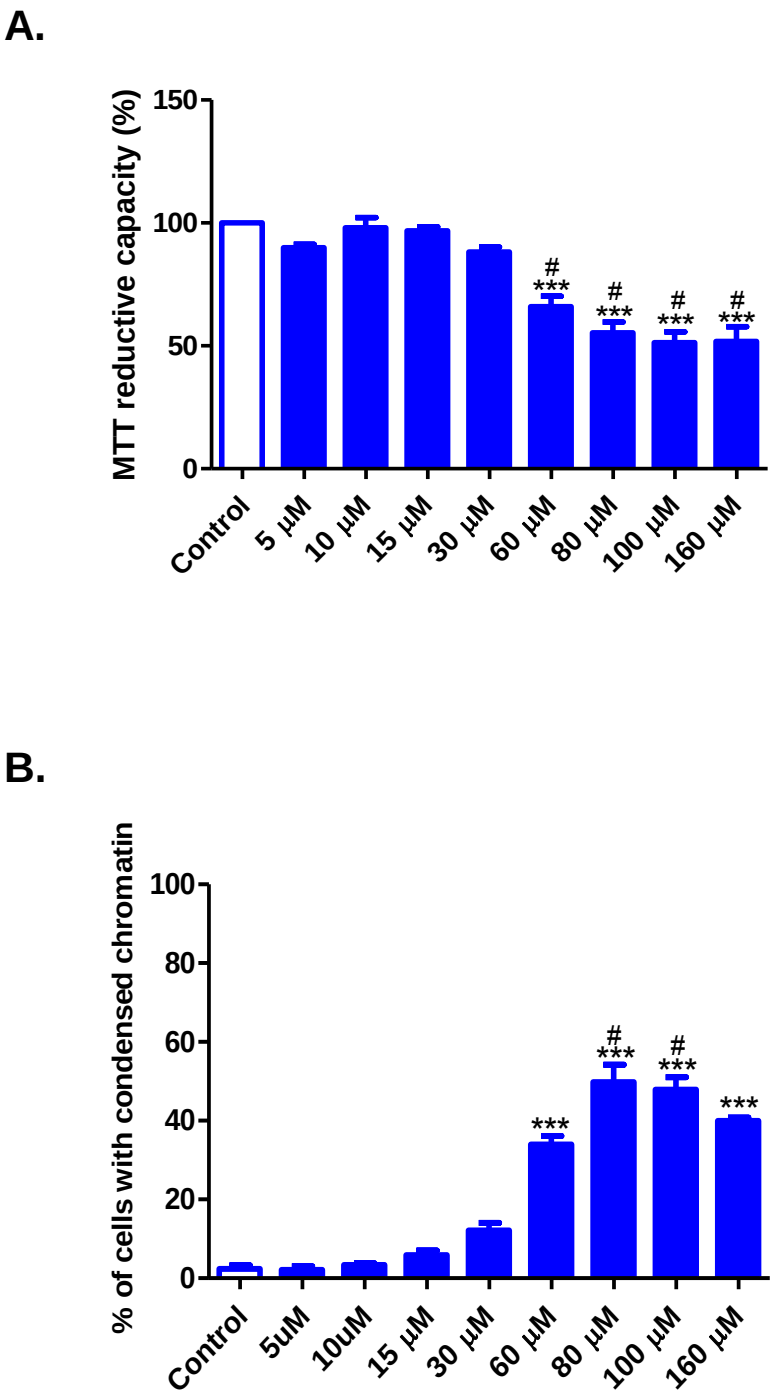


Figure 4.3. Cisplatin dose-response performed on CaSki cells for a period of 24 h. A. The MTT assay was employed in order to assess the effects of increasing doses of cisplatin on cell viability over a 24 hour time period. *** $p < 0.001$ vs. control, # $p < 0.001$ and vs. 15 µM cisplatin (n = 4). **B.** Chromatin condensation using Hoechst 33342 as an indication of apoptosis in response to increasing concentrations of cisplatin over a 24 h time period. *** $p < 0.001$ vs. control and # $p < 0.01$ vs. 60 µM (n = 3).

In response to an increasing concentration of cisplatin, the reductive capacity of CaSki cells was significantly reduced when treated with 60, 80, 100 and 160 µM of cisplatin (60 µM: 65.88 ± 4.33%; 80 µM: 55.35 ± 4.33%; 100 µM: 51.35 ± 4.30%; 160 µM: 51.75 ± 6.08% vs. control $p < 0.001$) in comparison to the control and no significant decrease in reductive capacity was observed when cells were treated with 5, 10, 15 or 30 µM of cisplatin over a 24 h time period (Fig.4.3.A). Concentrations of 60 µM cisplatin and higher did not induce any additional significant decrease in reductive capacity of CaSki cells.

Results obtained in the assessment of chromatin condensation (Fig. 4.3.B) were in agreement with the MTT analysis: Significant changes were observed only in CaSki cells treated with 60, 80, 100 and 160 µM of cisplatin for a 24 h time period (60 µM: 33.97 ± 2.19%; 80 µM: 49.77 ± 4.42%; 100 µM: 47.87 ± 3.20%; 160 µM: 39.95 ± 0.70% vs. control $p < 0.001$). Cells treated with 80 and 100 µM of cisplatin induced further significant increases in the percentage of apoptotic cells when compared to cells treated with 60 µM ($p<0.01$). No significant increases in chromatin condensation were observed when cells were treated with 5, 10, 15 or 30 µM of cisplatin over a 24 h time period.

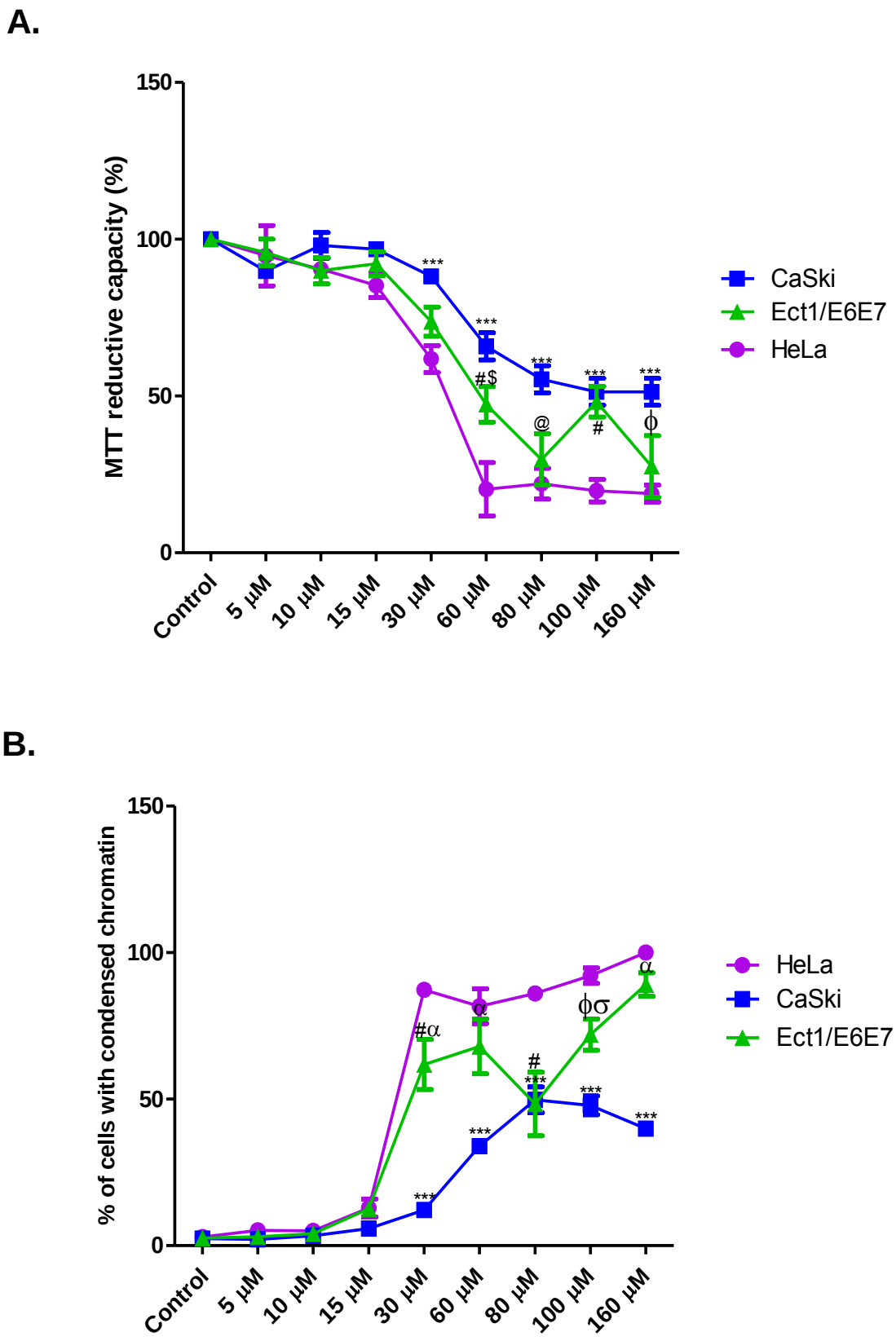


Figure 4.4. Cisplatin dose-response performed on HeLa, CaSki and Ect1/E6E7 cells. A. The MTT assay was employed in order to assess each cell line's ability to reduce MTT to formazan when treated with increasing doses of cisplatin over a 24 hour time period. *** $p < 0.001$ vs. HeLa # $p < 0.001$ vs. HeLa, $ $p < 0.05$, @ $p < 0.001$, ϕ $p < 0.01$ vs. CaSki. **B.** Chromatin condensation detected by Hoechst 33342 dye was used to analyse the percentage of cells undergoing apoptosis in response to increasing concentrations of cisplatin over a 24 h time period. *** $p < 0.001$, # $p < 0.001$, ϕ $p < 0.05$ vs. HeLa, α $p < 0.001$, σ $p < 0.01$ vs. CaSki.

Next, differences between the three cell lines with regards to their responses to cisplatin treatment, was assessed by measuring cell viability and apoptotic parameters.

In Fig.4.4.A, no significant differences were observed between the three cell line's capacity to reduce MTT when treated with 5, 10, or 15 µM of cisplatin. However, the reductive capacity of the CaSki cells was significantly higher than that of the HeLa cells when treated with 30, 60, 80, 100 and 160 µM of cisplatin (30 µM: 88.13 ± 2.10%; 60 µM: 65.88 ± 4.33%; 80 µM: 55.35 ± 4.33%; 100 µM: 51.35 ± 4.30%; 160 µM: 51.75 ± 6.08% vs. HeLa $p < 0.001$). CaSki cells were also more efficient in reducing MTT than when compared to the Ect1/E6E7 cells (60 µM: 47.35 ± 5.62% $p < 0.05$ vs. CaSki; 80 µM: 29.78 ± 8.26% $p < 0.001$ vs. CaSki; 160 µM: 27.55 ± 9.84%; $p < 0.01$ vs. CaSki). These results indicate that CaSki cells were least affected by cisplatin treatment at higher doses over a 24 h time period and were able to maintain close to 50% viability. On the other hand HeLa cells lost about 80% of their ability to reduce MTT and were greatly affected by cisplatin treatment.

Figure 4.4.B depicts a comparison between all three cell lines and the percent of pyknosis when treated with increasing concentrations of cisplatin for a period of 24 h. These results mirror those observed in Figure 4.4.A: no significant differences were observed between the three cell lines with regard to the percentage of pyknosis present when treated with 5, 10, or 15 µM of cisplatin. Similarly, CaSki cells exhibited the least amount of pyknosis and thus apoptotic cell death when compared to HeLa cells (30 µM: 12.13 ± 1.93%; 60 µM: 33.97 ± 2.19%; 80 µM: 49.77 ± 4.42%; 100 µM: 47.87 ± 3.20%; 160 µM: 39.95 ± 0.70% vs. HeLa $p < 0.001$), and Ect1/E6E7 cells (30 µM: 61.83 ± 8.50%; 60 µM: 68.00 ± 9.37%; 160 µM: 89.10 ± 4.00% vs. CaSki $p < 0.001$; 100 µM: 72.03 ± 5.35% $p < 0.01$). These results strengthen those observed in Fig.4.4.B suggesting that CaSki cells are indeed more resistant to undergoing cell death than both HeLa and Ect1/E6E7 cells when treated with increasing doses of cisplatin for 24 h.

4.1.3. Discussion

Although cisplatin is considered the most effective anti-cancer drug for treatment in both neoadjuvant and salvage treatment of cervical cancer (Benedetti Panici et al., 2001, 2003), its success is hindered by the dose-limiting factors commonly observed during and after treatment, such as nephrotoxicity, ototoxicity, neurotoxicity and gastrointestinal toxicity (Vermorken et al., 1983, Safirstein et al., 1986, Blakley et al., 1994, Cardinaal et al., 2000, Sergi et al., 2003). Furthermore, patients with cervical cancer experience a decrease in QOL (Herzog and Wright, 2007) which is exacerbated further by chemotherapy treatment (Monk et al., 2005).

The necessity for a lower dose of cisplatin exhibiting similar anti-cancer effects to that of the higher doses would therefore be ideal. For this purpose, it was necessary first and foremost to conduct a dose-response as a means of determining a non-toxic concentration of cisplatin, as well as to evaluate each cell's response to cisplatin treatment. The aims were firstly to determine a dose of cisplatin that is non-toxic to non-cancerous ectocervical cells (Ect1/E6E7); secondly to conduct this analysis on two cervical cancer cell lines (HeLa and CaSki) in order to assess their propensity to undergo cell death, and finally to compare the dose-responses to one another as a means of determining their sensitivity/resistance to cisplatin in comparison to one another.

The result of the MTT assay indicates that MTT reductive capacity, and therefore cell viability, decreases as the concentration of cisplatin increases, which infers that the effects of cisplatin treatment is dose dependent (Fig.4.1.A). This agrees with other *in vitro* studies (Choi et al., 2004, Kuwahara et al., 2000, Cheng et al., 2010, Funaoka et al., 1996) conducted on HeLa, CaSki and other cell lines. Moreover, the results suggest that concentrations of 60 µM and more do not result in any additional significant decreases in MTT reductive capacity, suggesting no added benefit as the dose increases further. This observation agrees with Stewart and colleagues who proposed that dose-response curves flatten at higher doses (Stewart et al., 2007). The results examining chromatin condensation agree with those observed in the MTT assay: a dose-dependent effect whereby increasing concentrations of cisplatin induce an increase in pyknosis (Fig. 4.1.B).

Based on the results, it was concluded that 15 µM of cisplatin would be selected as the working concentration for all subsequent experimental studies, as both the MTT

and chromatin condensation assays revealed no significant changes when compared to the control (Fig.4.1.A and B).

The results obtained in the HeLa cells show a similar response to the Ect1/E6E7 cells (Fig. 4.2. A. and B.), however HeLa cells display a more pronounced decrease in MTT reductive capacity and increase in apoptosis induction, indicating an increased sensitivity to concentrations of 30 μM and higher. The fact that the non-cancerous cells exhibit this 'tolerance' to cisplatin treatment may be due to the differences in cell cycle activity and rate of proliferation.

When dose-response analysis was performed on the CaSki cells, tolerance to cisplatin treatment was observed (Fig. 4.3.A. and B.). The previously described response to increasing concentrations of cisplatin was observed (as seen with the other two cell lines), however to a much lesser extent. Significant effects on MTT reductive capacity and apoptosis induction were only observed following treatment with 60 μM and more, with a loss in MTT reductive capacity and increase in apoptotic induction by about 44% and 35% respectively (Fig. 4.3.A. and B.). In other words, CaSki cells were able to tolerate double the concentration that the Ect1/E6E7 and HeLa cells were able to with not even a 50% loss in their overall viability. As with the previous two dose-response graphs, a plateau was reached in MTT reductive capacity at higher doses, indicating that concentrations of 60 μM and more offered no significant added benefit in increasing cell death. However, this was not the case when analysing the extent of apoptosis through chromatin condensation, a slightly different picture emerged: significant increases in the percentage of total cells with condensed chromatin were evident in CaSki cells treated with cisplatin up until 80 μM, where after a gradual trend toward a decrease in apoptotic cells was observed. Undetected necrosis may be ascribed to the observed result. The CaSki cells appear to tolerate increasing concentrations of cisplatin to better extent than the other two cell lines.

Figures 4.4.A. and B. are presented such that HeLa, CaSki and Ect1/E6E7 dose-responses are plotted on the same axis. When both parameters were analysed, the CaSki cell's ability to reduce MTT was significantly improved when compared to the HeLa and Ect1/E6E7 cells. This was mirrored in Figure 4.4.B where CaSki cells displayed a lesser propensity to undergo apoptosis than both HeLa and Ect1/E6E7 cells. This data suggests that CaSki cells are more resistant to the intracellular

effects of cisplatin treatment than the HeLa cells, which agrees with a study conducted by Funaoka and colleagues (Funaoka et al., 1996).

It has been hypothesised that the shape of the dose-response curve will provide insight into possible resistance mechanisms both *in vitro* and *in vivo* (Stewart et al., 1996). It has been postulated that if resistance is seen at lower doses, this is due to excess of a resistance factor (i.e. active resistance) that may be overwhelmed by the administration of high concentrations of chemotherapy drugs. Active resistance factors have been described as being those that high-dose chemotherapy and resistance modulating agents interfere with that would under normal circumstances improve therapeutic efficacy, i.e. DNA repair mechanisms, drug efflux, glutathione, etc. It has therefore been proposed that the presence of an active resistance factor is characterised by a shoulder on the log response vs. dose curve with the eventual saturation of the resistance factor.

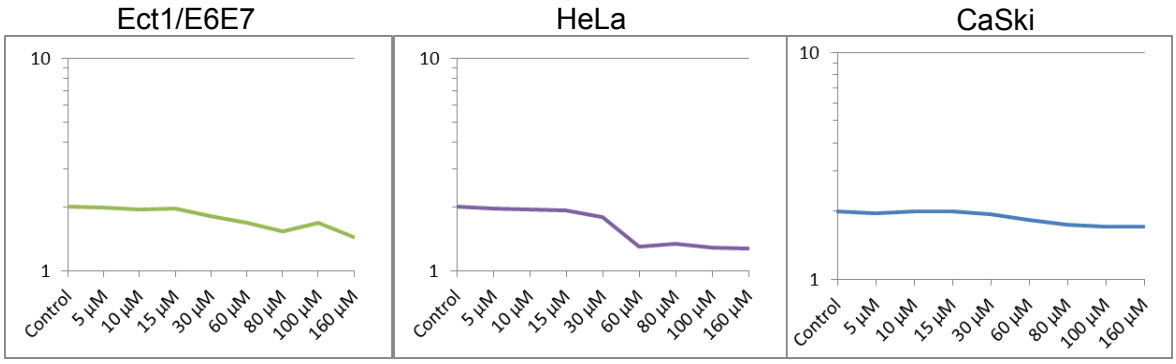


Figure 4.5. Log response vs. dose curve of Ect1/E6E7, HeLa and CaSki cells. Both Ect1/E6E7 and CaSki cells display similar composite curves which indicate a passive resistance mechanism against cisplatin treatment according to Stewart and colleagues (Stewart et al., 1996, Thompson, 2008) . The HeLa cells composite curve is characterised by a shoulder at low doses of cisplatin, indicating induction of an active resistance factor, and a terminal plateau indicating a form of passive resistance (Stewart et al., 1996)

Conversely, passive resistance in this setting has been described as the ineffectiveness of high doses and resistance modulating agents in inducing additional increases in cell death due to defective apoptosis for example, and generally arises from gene deletion/down-regulation/mutation or decreased drug affinity for a target. This may be characterised by a relatively flat log response vs. dose curve (Stewart et al., 1996, Stewart et al., 2007, Stewart, 2007).

The log response vs. dose curve of the Ect1/E6E7, HeLa and CaSki cell lines were plotted and analysed, with each plot representing the log of each mean. Our results indicate that the CaSki cell's response to cisplatin is governed by a passive resistance mechanism as the log response vs. dose curve is relatively flat. Given the fact that CaSki cells are positive for HPV DNA, a virus with oncoproteins which are known to degrade and inactivate proteins essential for cell cycle control, it may indicate such passive resistance as described by Stewart and colleagues (Stewart et al., 1996).

The composite curve of the HeLa cells on the other hand takes on a sigmoid shape, which might be an indication of active resistance toward cisplatin treatment at low doses and passive resistance at higher doses which is depicted by the terminal plateau. The active resistance factor that the HeLa cells are engaging in order to tolerate the low doses of cisplatin could be the up-regulation of autophagy. Literature suggests that in response to chemotherapeutic agents autophagy is in fact increased as a protective mechanism (deBruin and Medema, 2008) and may contribute to resistance (Chen et al., 2010). Since there is very little information supported by the literature that suggests that cisplatin prompts autophagic induction, this will be addressed in subsequent chapters.

The log response versus dose curve of the Ect1/E6E7 cells is relatively flat and is similar to the CaSki composite curve which may suggest a passive resistance mechanism against cisplatin treatment. However one might anticipate a slight shoulder on the composite curve. Forthcoming chapters will provide insight into possible factors that may confer this passive resistance.

A number of conclusions may be drawn from the above study: Firstly, 15 μM of cisplatin was selected as the final dose due to its non-toxic effects on a non-cancerous cervical cell line. This low dose of cisplatin also did not induce cytotoxic effects in the cancer cells, therefore, a major aim of this study will be to increase the cytotoxic effect of cisplatin on cancer cells with the aid of adjuvant therapy. Secondly, we confirmed that cisplatin has a dose-dependent effect on all three cell lines and that it induces apoptosis and decreases viability. Thirdly we have demonstrated that CaSki cells have an increased tolerance to cisplatin treatment and that this response is likely to be cell type dependent. Lastly through plotting the log response versus

dose we provided data of value for resistance mechanisms engaged by the cell in response to treatment.

4.2. Characterisation of Autophagic Flux in Three Different Cervical Cell Lines.

4.2.1. Introduction and Aims

The mechanism by which autophagy may be up-regulated could partly be due to metabolic reprogramming from oxidative phosphorylation to aerobic glycolysis, commonly termed the 'Warburg effect' (Warburg, 1925). This provides the cancer cells with the unique ability to increase the efficiency of its energy production to compensate for increased growth rates that favour anabolic metabolism. Metabolic reprogramming is therefore imperative in order for cancer cell survival under anaerobic conditions where autophagy plays a major role in tumour survival (Lozy and Karantza, 2012). With the above in mind, determination of autophagic flux in the different cell lines is thus imperative.

Autophagic flux determination may then possibly allow us to answer questions that arose from Chapter 4.1., such as the reasons behind the differential tolerance to cisplatin treatment observed between the three cell lines. Additionally, we may be able to predict, depending on how reliant the cell is on autophagy, whether the inhibition or induction of autophagy may drive the cell toward death. The aim of this chapter is therefore i) to establish the capacity of each cell line to induce an autophagic response and determine whether the three cell lines respond in a similar or different manner, ii) to determine the value of autophagic flux as a pre-evaluation which will serve as a predictive tool in selecting a suitable autophagic modulator.

4.2.2. Results

All Three Cell Lines Exhibit Differential Flux through the Autophagic Pathway.

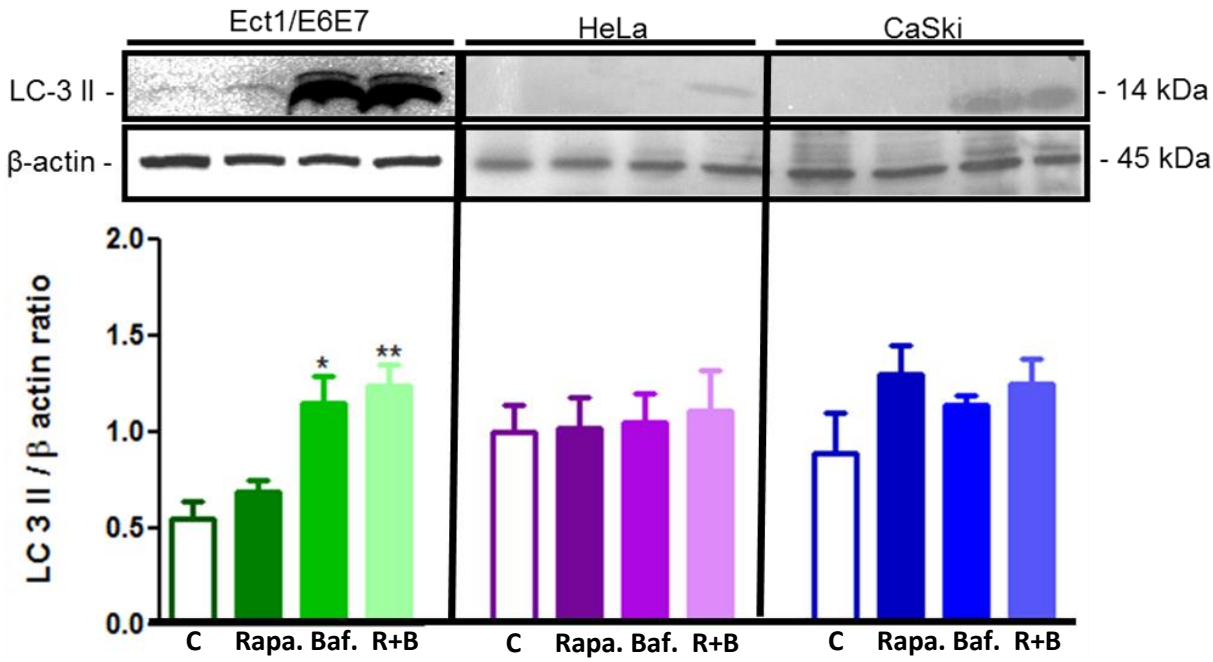


Figure 4.6. Representative blot and corresponding densitometry of autophagic pathway flux in Ect1/E6E7, HeLa and CaSki cells. All three cell lines were either treated with: rapamycin (Rapa.), bafilomycin A1 (Baf.), or both (R + B) and analysed for the expression of LC-3 II. * $p < 0.05$ and ** $p < 0.01$ vs. control Ect1/E6E7, n = 3.

Autophagic flux was analysed in Ect1/E6E7, HeLa and CaSki cells whereby each cell line was either not treated, treated with rapamycin alone, bafilomycin A1 alone or Rapamycin and bafilomycin A1 together (Fig. 4.6.). Flux capacity of each cell line is indicated by the group treated with both autophagic modulators (Rapa. + Baf.). Densitometry indicates that LC-3 II expression is significantly increased in Ect1/E6E7 cells when compared to the control (1.23 ± 0.11 vs. control, * $p < 0.05$ and ** $p < 0.01$). No significant differences in LC 3 II expression (flux) was observed in both HeLa and CaSki cells.

4.2.3. Discussion

The importance of autophagy in whole body metabolism is an interesting concept: under normal homeostatic conditions, it functions as an anti-tumour mechanism whereby it prevents genomic instability and other oncogenic events from taking place (Abedin et al., 2007, Katayama et al., 2007), essentially maintaining the integrity of many organs. On the other hand, under conditions of starvation, autophagy is induced in order to supply substrates for energy metabolism. The capacity of a cell to induce autophagy is dependent on tissue type, which suggests that the reliance of cells on autophagy in different organ systems is dependent on their metabolic demands (Mizushima et al., 2004). An example of this is observed in the liver where protein turn-over rates are especially high; consequently, the demand on autophagy is similarly great, particularly under starvation conditions. Conversely, under starvation conditions in the brain, autophagy is not induced, even after 48 hours of food with-drawl in mice (Mizushima et al., 2004). It is possible that this could be due to fuels such as glucose and ketone bodies being supplied by other organs for utilization by the brain. The diversity of autophagic induction between tissues is further emphasised by the fact that it is constitutively activated in thymic epithelial cells under nutrient-rich conditions and functions to degrade cytosolic antigens to be expressed on major histocompatibility (MHC II) class II proteins for recognition and selection by lymphocytes (Mizushima et al., 2004). Therefore, the basal levels of autophagy and the propensity of the cell to up-regulate autophagy does indeed appear to be dependent on tissue type and whether the particular organ itself has a high metabolic demand.

On the subject of metabolic demand, it is now clear that once cells within a particular tissue become malignant, their metabolism alters and allows them not only to favour anabolic reactions to support cell growth, but also to induce autophagy as a means of surviving harsh, unfavourable conditions. This fact gave rise to the notion that it functions as mechanism of resistance against the death-inducing effects of various anti-cancer drugs (Chen et al., 2010, Notte et al., 2011). The reliance of cancer cells on autophagy may also differ between cancer types and may contribute to the aggressiveness of the particular cancer. With the above in mind, the aim of this chapter was to assess each of the cell line's capacity to induce autophagy through analysing the presence of LC3 II by Western blot in the presence of rapamycin and bafilomycin A1. HeLa, CaSki and Ect1/E6E7 cells are isolated from the cervix: CaSki

and Ect1/E6E7 cells are both squamous cells originating from the ectocervix, with former having a malignant phenotype, and HeLa cells (also malignant) originate from a glandular cell type in the endocervix. Thus, an additional aim was to assess whether cells from the same organ have differential flux through the autophagic pathway, and in this way it is hoped that it may provide information on the particular organ/tissue in question with regards to the reliance of the malignant cell on autophagy and/or metabolic demands of the of tissue.

Studies have identified CaSki cells to possess a more resistant phenotype than most cervical cancer cell lines, including HeLa cells (Funaoka, Shindoh et al. 1996; Padilla 2006, Padilla 2002). Although speculative, it may be the ability of these cells to induce autophagy that aids them in developing this resistant phenotype. We thus examined the autophagic flux capacities of each cell line to possibly move toward proving this hypothesis. In Figure 4.6., LC-3 II expression was assessed using Western blotting under various conditions (corresponding densitometry below blots). Autophagic flux was determined by inducing autophagy upstream by using rapamycin, and inhibiting the fusion of autophagosomes with lysosomes downstream with bafilomycin A1, thus preventing the recycling of LC-3 II (as measured by Western blot). This results in the accumulation of autophagosomes in the cytoplasm which is indicative of the flux through the pathway. Only the non-cancerous Ect1/E6E7 cells induce significant increases in LC-3 II. Although not statistically significant, visual observation of the Western blot suggests that flux through the autophagic pathway is somewhat less in the CaSki cells than when compared to its non-carcinogenic counterpart, which agrees with other studies (Gunn et al., 1977, Kisen et al., 1993, Kirkegaard et al., 2004). A similar observation (although more pronounced), was seen in HeLa cells which originate from glandular cells in the endocervix: a weak capacity to induce autophagy upon stimulation with the autophagic modulators was exhibited by these cells. This data strengthens previous findings that autophagic induction is cell type dependent. We have further demonstrated that there are also differences in the same cell type between normal and transformed cells.

In the previous chapter it was demonstrated that CaSki cells had the highest tolerance to treatment with cisplatin, and the HeLa cells had the lowest tolerance. From the data obtained in this chapter it could be speculated that in response to cisplatin treatment, HeLa cells could not increase flux through the pathway at a

sufficient enough rate to act as a protective mechanism, and at higher doses underwent subsequent cell death. On the other hand, the CaSki cells displayed the highest tolerance to cisplatin treatment, and combined with the above data it is suggested that its ability to induce increased flux through the autophagic pathway may have functioned as a protective mechanism to prevent cell death induction. However, no literature (to our knowledge) which assessed autophagic flux in these cell lines is available to compare our results with. Therefore in conclusion, each cell line displays differential flux through the autophagic pathway and this may provide insight to the aggressiveness of the particular cell type and that this differential flux is in fact cell type dependent.

It can thus be concluded that these three cell lines have a differential ability to induce autophagy and that they are differentially resistant to cisplatin treatment. The major aim of this study is therefore, with the aid of adjuvant therapy through the manipulation of autophagy, to sensitise the cervical cancer cells to cisplatin treatment. This aim will be addressed in the following two chapters.

5

The Effect of a Non-Toxic Concentration of Cispatin in Inducing Autophagy in Three Cervical Cell Lines.

5.1. Introduction and Aims

Cisplatin-induced autophagy is not sufficiently addressed within the literature, particularly in cervical cancer cell lines. This is therefore a relevant and worthwhile avenue to explore since it may not only provide information on the intracellular effects of cisplatin, but whether non-cytotoxic doses may be a future possibility in the treatment of cervical cancers. Additionally, it is possible that the autophagic response that is induced by cisplatin treatment may be exploited in order to improve cisplatin-induced apoptosis.

With the above in mind, the aims of this chapter are to determine whether cisplatin induces autophagy at the chosen dose of 15 μM in Ect1/E6E7, HeLa and CaSki cells, and whether this response differs between these three cervical cell lines.

5.2. Results

5.2.1. Comparison of three cervical cell lines to cisplatin induced autophagy with the use of Lysotracker™.

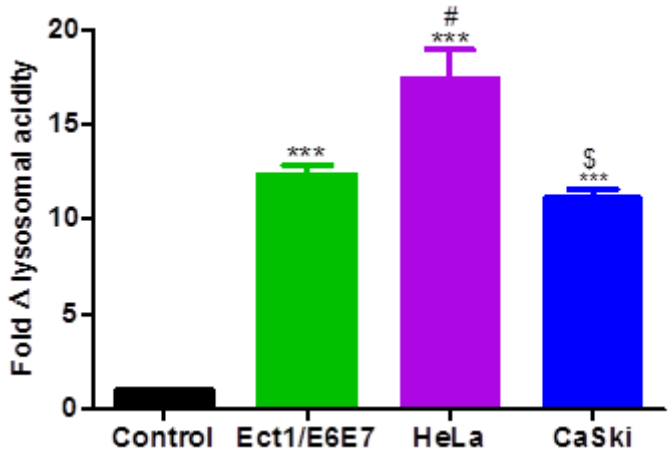


Figure 5.1. Comparison of changes in mean lysosomal compartmentalisation (flouresence) between Ect1/E6E7, HeLa and CaSki cells (flow cytometry). *** $p < 0.001$ vs. control, # $p < 0.05$ vs. Ect1/E6E7 and $ $p < 0.001$ vs. HeLa, n = 3-7.

Methods for monitoring autophagy include the use of dyes such as Lysotracker™, and although it's a reliable approach, results should be strengthened with the use of other acceptable techniques. The responses of all three cell lines to cisplatin treatment were compared to one another to assess whether lysosomal biosynthesis differed significantly. All cell lines exhibit increased lysosomal compartmentalisation in comparison to the control (Ect1/E6E7: 12.36 ± 0.45, HeLa: 17.41 ± 1.52, CaSki: 11.12 ± 0.46 vs. control, *** $p < 0.001$). HeLa cells exhibit significantly higher mean lysosomal acidity in response to cisplatin treatment when compared to the Ect1/E6E7 and CaSki cells (12.36 ± 0.45 vs. HeLa,# $p < 0.05$ and 11.12 ± 0.462 vs. HeLa, $ $p < 0.001$).

5.2.2. The effect of cisplatin treatment on p62, Beclin-1 and LC-3 II in three cervical cell lines.

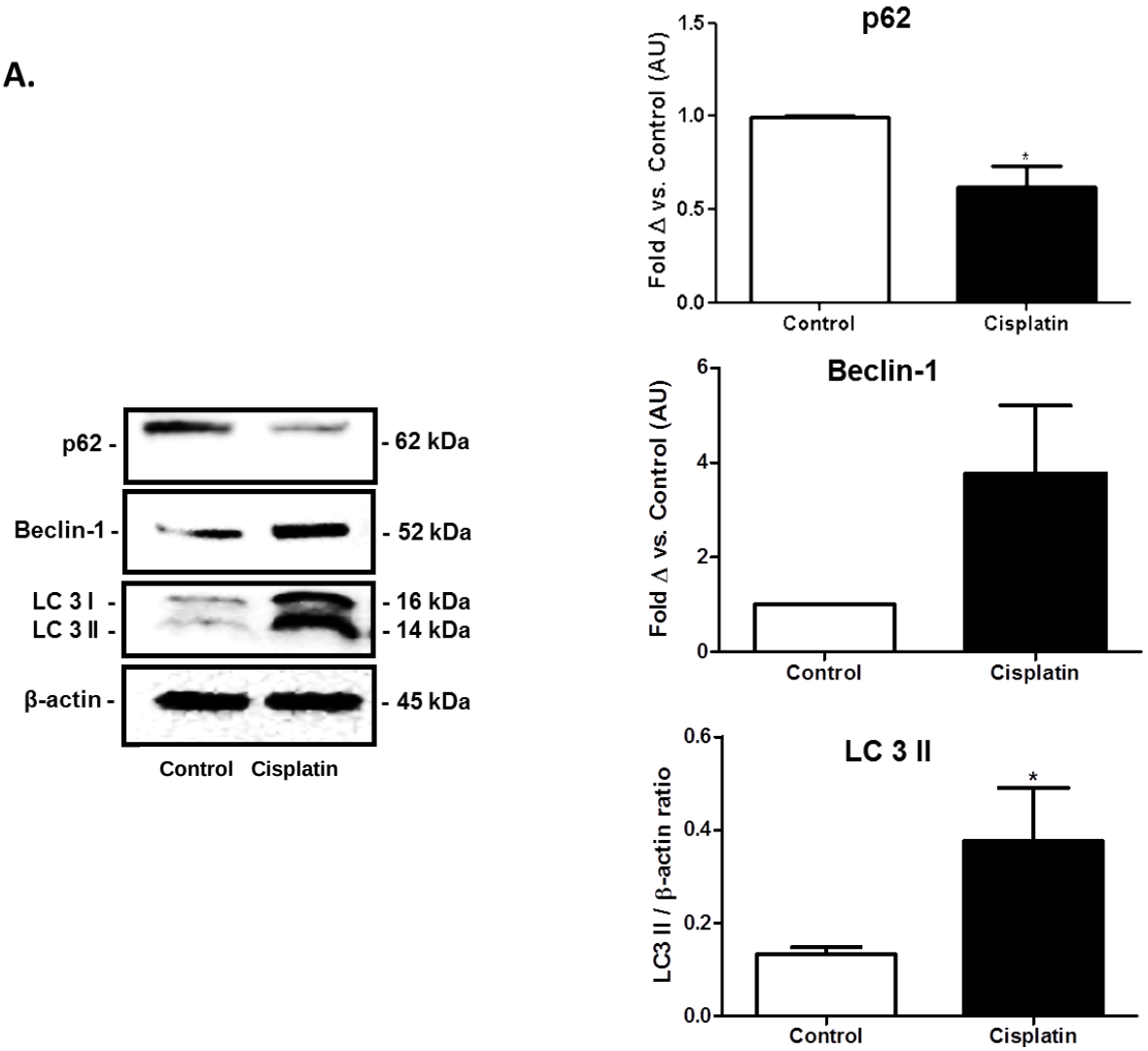


Figure 5.2.A. Cisplatin (15 µM, 24 h) induces autophagy in Ect1/E6E7 cells. Western blots (left) and corresponding densitometry data (right) depict changes in p62, Beclin-1 and LC-3 II protein expression in response to treatment with 15 µM cisplatin for a 24 h time period (AU – arbitrary units) . * $p < 0.01$ vs. control, n > 4.

Changes in endogenous p62, Beclin-1 and LC 3 II protein levels were detected by using the Western blotting technique. Significant changes in protein levels were observed with LC-3 II and p62 (0.38 ± 0.11 and 0.62 ± 0.115 vs. control, *$p < 0.05$).

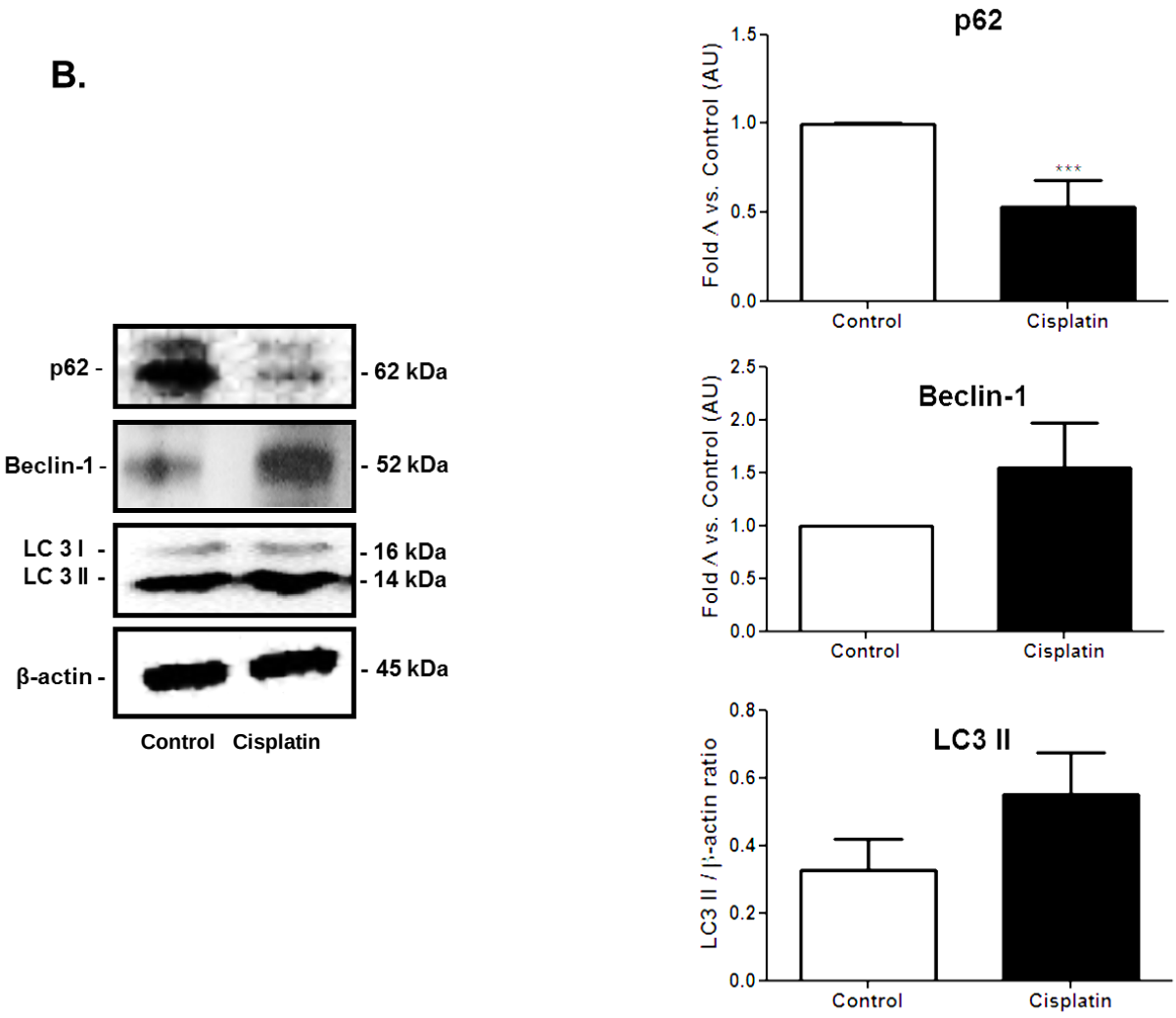
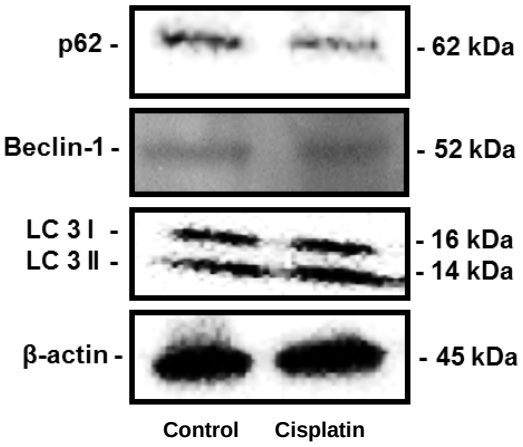
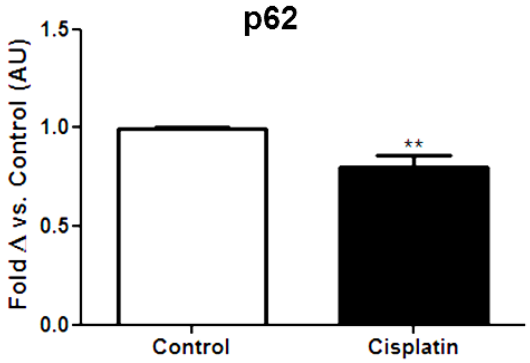


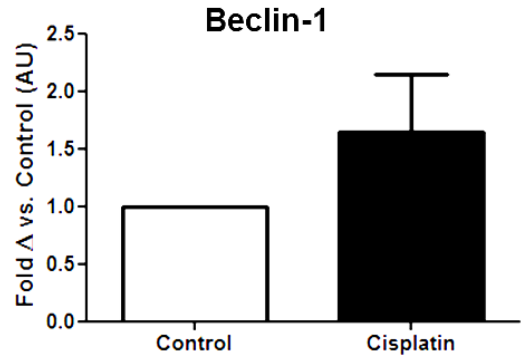
Figure 5.2.B. Cisplatin (15 μM, 24 h) induces autophagy in HeLa cells. Western blots (left) and corresponding densitometry data (right) depict changes in p62, Beclin-1 and LC-3 II protein expression in response to treatment with 15 μM cisplatin for a 24 h time period (AU – arbitrary units) . *** $p < 0.01$ vs. control, n > 4.

Treatment of HeLa cells with 15 μM cisplatin for a 24 h time period induced a significant decrease in p62 protein levels when compared to the control (0.53 ± 0.15 vs. control, *** $p < 0.001$).

C.







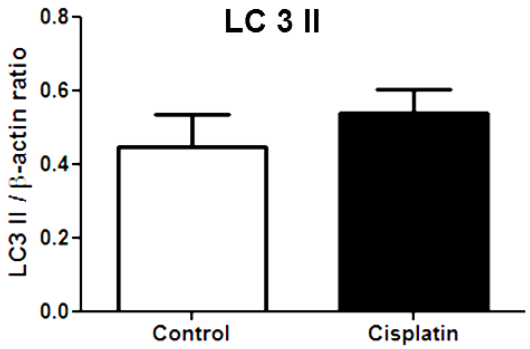


Figure 5.2.C. Cisplatin (15 µM, 24 h) induces autophagy in CaSki cells. Western blots (left) and corresponding densitometry data (right) depict changes in p62, Beclin-1 and LC 3 II protein expression in response to treatment with 15 µM cisplatin for a 24 h time period (AU – arbitrary units) . ** $p < 0.01$ vs. control, n > 4.

CaSki cells treated with 15 µM cisplatin for a 24 h time period induced a significant decrease in p62 protein levels when compared to the control(0.80 ± 0.06 vs. control, ** $p < 0.01$).

5.3. Discussion

The side-effects and long-term damage induced by cisplatin chemotherapy limits its clinical efficacy, therefore the challenge lies in achieving a similar level of cancer cell death with the use of a lower, non-toxic dose of cisplatin with the aid of adjuvant therapy .It was first necessary to characterise the effects of this lower dose (15 μM) in the three cell types with regards to autophagy in order to determine whether this response may be exploited.

It was observed that the Ect1/E6E7, HeLa and CaSki cells did not exhibit any significant deviations in cell viability or apoptotic cell death when treated with concentrations of cisplatin up to 15 μM (Chapter 4.1.). It was hypothesised that a mechanism of resistance was initiated by the cervical cells that permitted a large degree of tolerance to cisplatin exposure for the 24 h time period. The possibility that this was indeed resistance being observed was strengthened by work conducted by Stewart and colleagues who suggest that the various shapes displayed by the log vs. dose response curve may be indicative of the presence of active or passive resistance factors (Stewart et al., 1996). It was previously mentioned in Chapter 4.1. that the HeLa cells are relying predominantly on an active mechanism of resistance, of which autophagy was selected as a possible candidate. The CaSki and Ect1/E6E7 cell's log vs. dose response curve suggest more of a passive resistance mechanism at play. This chapter therefore aimed firstly to determine whether cisplatin at the chosen concentration induces autophagy, and secondly whether this response was similar or differs between cell lines by comparing them to one another.

Toward the completion of the autophagic process, it is required that the autophagosome fuses with a lysosome in order to degrade the endocytosed and autphagocytosed organelles/proteins (Kroemer and Jaattela, 2005), therefore, an increase in lysosomal production is synchronised with the induction of autophagy, and it is this characteristic that can be utilised to assess the extent of autophagy that is induced by the cell.

5.3.1. Differential induction of autophagy in three cervical cell lines.

Since the responses to cisplatin treatment and autophagic flux capacities differed between all 3 cell lines (Chapter 4.1. and 4.2.), it was necessary to determine whether the levels of cisplatin-induced autophagy were similar or different between

cell lines i.e. did the cells respond in a similar fashion with regard to autophagy induction when exposed to 15 µM cisplatin for 24 h.

Lysotracker™ is a weakly basic dye that concentrates in acidic cellular organelles such as lysosomes and has been commonly used to evaluate their biosynthesis in the context of autophagy (Hu et al., 2012, Viola et al., 2012, Kaminskyy et al., 2012). It was utilised in this study to assess the effects of low-dose cisplatin in inducing autophagy in three cervical cell lines (Fig. 5.1). The Ect1/E6E7, HeLa and CaSki cells all exhibited significant increases in intracellular acidity as measured by the flow cytometer when treated with 15 µM cisplatin for 24 h. Additionally, it is clear that both the Ect1/E6E7 and CaSki cells induce a similar level of autophagy when exposed to the chosen dose of cisplatin. On the other hand, the results additionally indicate that the HeLa cells had significantly higher mean lysosomal acidity/flourescence in the presence of cisplatin when compared to both the Ect1/E6E7 and CaSki cells (Fig. 5.1.) which suggests autophagy up-regulation. This implies that the HeLa cells have differential sensitivity to cisplatin and the ability to up-regulate autophagy. It may be likely as no cell death was observed when the HeLa cells were treated with 15 µM cisplatin for 24 h. If it is recalled from the log vs. dose response curve, it was deduced (according to the hypothesis put forward by Stewart and colleagues (Stewart et al., 1996), that an active resistance mechanism is utilised by the cells at these lower doses of cisplatin. We can now partially confirm this hypothesis; however the expression of various autophagic proteins would need to be evaluated to confirm this finding. Also, manipulation of this pathway with inhibitors/inducers of autophagy with cisplatin treatment will reveal how necessary this mechanism is in order for HeLa cell survival.

This suggests that autophagy is most likely induced in response to cisplatin treatment, nevertheless, confirmation is required through the detection of the up-regulation of LC-3 II protein through Western blotting. Lui and colleagues used a similar lysosomal stain, known as MDC as a method for the detection of autophagy in response to cisplatin treatment in oesophageal cancer cells, which they later confirmed through immunoblotting with LC-3 II (Liu et al., 2011). Lysosomal staining with the use of acridine orange was utilised as a method of autophagy detection in a wide variety of cancer cell types in response to cisplatin treatment (Harhaji-Trajkovic et al., 2009). For the purposes of this study, it is noteworthy to mention, that the

above studies use high doses of cisplatin treatment. Nevertheless, the commonality of this particular method for the detection of autophagy verifies its reliability.

Interestingly the function of these lysosomes may not be exclusively for the use of autophagy. Lysosomes can also function as intermediary compartments to either store metals from influx pathways or distribute them to efflux systems (Eaton and Qian, 2002, Larsen et al., 2003). With the use of x-ray microanalysis and ion microscopy, it was observed that platinum was detectable in lysosomes of the kidney in guinea pigs treated with cisplatin (Saito and Aran, 1994). Similarly, fluorescein-tagged cisplatin was detected not only in the nucleus, but in cytoplasmic vesicles and lysosomes of cisplatin resistant- and ovarian cancer cells (Molenaar et al., 2000, Safaei et al., 2005a). Studies then confirmed that cisplatin resistance is attributed to abnormal protein trafficking and secretion (Chauhan et al., 2003, Safaei et al., 2005a) and that cisplatin-resistant cell types are associated with less lysosomes when compared to their sensitive counterparts (Safaei et al., 2004b). If Chapter 4.1 is recalled, it was established that the HeLa cells are more sensitive to cisplatin treatment in comparison to the CaSki cells (Fig. 4.4.), and taking into account this observation by Safaei and colleagues (Safaei et al., 2004), this may also be an explanation for the differential lysosomal induction induced by the three cervical cell lines. The question of whether the increased production of lysosomes is due to autophagy induction or a method of trafficking cisplatin toward efflux pathways will be addressed in the next chapter.

The use of Lysotracker™ as a measure of autophagic activity is generally reliable; however protein expression analysis of the various markers of autophagy is required in order to confirm these findings. Three proteins were evaluated for the up-regulation of autophagy: p62, Beclin-1 and LC 3 II.

5.3.2. Cisplatin stimulates an increase in the molecules responsible for autophagic induction and execution.

The results demonstrated in this study show significant decreases in p62 protein levels were observed in all three cervical cell lines (Figure 5.2. A, B and C) in response to cisplatin treatment. P62 is a functionally diverse protein that is implicated in obesity (Rodriguez et al., 2006) and cancer (Mathew et al., 2009). It is also present in cytoplasmic inclusions of protein aggregation diseases (Zatloukal et al., 2002). Its

functional significance in autophagy has not gone unnoticed: not only does it recruit LC3 to the autophagosomal membrane, but Pankiv and colleagues also proposed that it binds directly to LC-3 to facilitate degradation of proteins under various conditions where itself becomes degraded (Pankiv et al., 2007). Therefore total cellular levels of p62 correlate inversely with autophagic activity, as well as with other parameters commonly utilised for the analysis of autophagy (Mizushima et al., 2010). The role of p62 in cancer cell survival was emphasised when it was linked to tumour transformation and progression *in vivo* (Duran et al., 2008), where after its importance for cancer cell survival under hypoxic conditions was also established (Jaakkola and Pursiheimo, 2009). A recent study revealed that cisplatin resistant ovarian carcinoma cells express higher levels of p62 than their sensitive counterparts, and this is attributed to the fact that endoplasmic reticulum stress is minimised by the binding of p62 to ubiquitinated proteins for degradation by autophagy (Yu et al., 2011a). With regards to cisplatin treatment on endogenous p62 levels, it was observed in one study that p62 protein expression decreased gradually over time as autophagy is induced in hepatoma cells (Xu et al., 2012a).

An equally important marker of autophagy is Beclin-1; it forms part of an upstream complex that is responsible for the initiation of autophagy, and is negatively regulated by mTOR. It plays a tumour-suppressive role in cancer and its expression promotes autophagic cell death in tumours (Liang et al., 1999, Cao and Klionsky, 2007). No significant increase in Beclin-1 protein expression was observed in response to cisplatin treatment in all three cervical cell lines. This is in contrast to other studies: It was determined that autophagy activation in response to cisplatin treatment is regulated by Beclin-1 and an up-regulation of this protein is observed *in vitro* in breast cancer cells, cervical cancer cells (Hou et al., 2010b, Xu et al., 2012c) as well as other cells (Harhaji-Trajkovic et al., 2009). Beclin-1 up-regulation was also observed *in vivo* in liver tissue of mice treated with cisplatin (Cardinal et al., 2009). Additionally, Beclin-1 over expression enhances cisplatin-induced apoptosis in gastric cancer cells. It may be argued here that the concentration of cisplatin utilised in the above mentioned studies are higher (> 20 μM) than the concentration used in this study (15 μM), and may therefore stimulate autophagy to a much greater extent, leading to a significant increase in Beclin-1 expression.

Finally, LC-3 II protein expression was assessed and quantified in all three cell lines in response to cisplatin treatment (Fig. 5.2.A, B and C). Significant increases in LC-3

II expression was only observed in the non-cancerous Ect1/E6E7 cells. The lack of a significant increase in both cancer cell lines may be due to the continuous recycling of LC-3 II (Kabeya et al., 2000). Upon stimulation of autophagy, LC-3 is cleaved to form LC 3 I, which then becomes conjugated to phosphatidylinositol (PE) resulting in a membrane-bound LC-3 II protein (Yorimitsu and Kilonsky, 2005). During the later stages of autophagy, LC-3 II may become degraded or recycled back to cytosolic LC-3 I (Kabeya et al., 2000), therefore even though autophagy may be induced, protein levels of LC-3 II may appear lower than expected. Therefore although LC-3 II is a promising marker of autophagosomal presence, and thus autophagy, it is necessary to evaluate other markers for confirmation.

After the assessment of lysosomal biosynthesis as well as proteins involved in autophagy initiation and completion in all three cervical cell lines, it may be concluded that 15 μM of cisplatin treatment for a 24 hour time period sufficiently induces autophagy. Additionally, lysosomal biosynthesis between the cell lines appear to differ, i.e. HeLa cells induce synthesis of a significantly larger amount of lysosomes in response to cisplatin than when compared with the other two cell lines, indicating that they may be more dependent on autophagy to tolerate cisplatin treatment. In order to assess whether autophagy may be imperative for the survival of the cervical cancer cells at this low-dose treatment, the autophagic pathway will have to be induced and inhibited in conjunction with cisplatin treatment.

6

The Effect of Autophagic Modulation in Sensitising Cervical Cancer Cells to Cisplatin Treatment.

6.1. Introduction and Aims

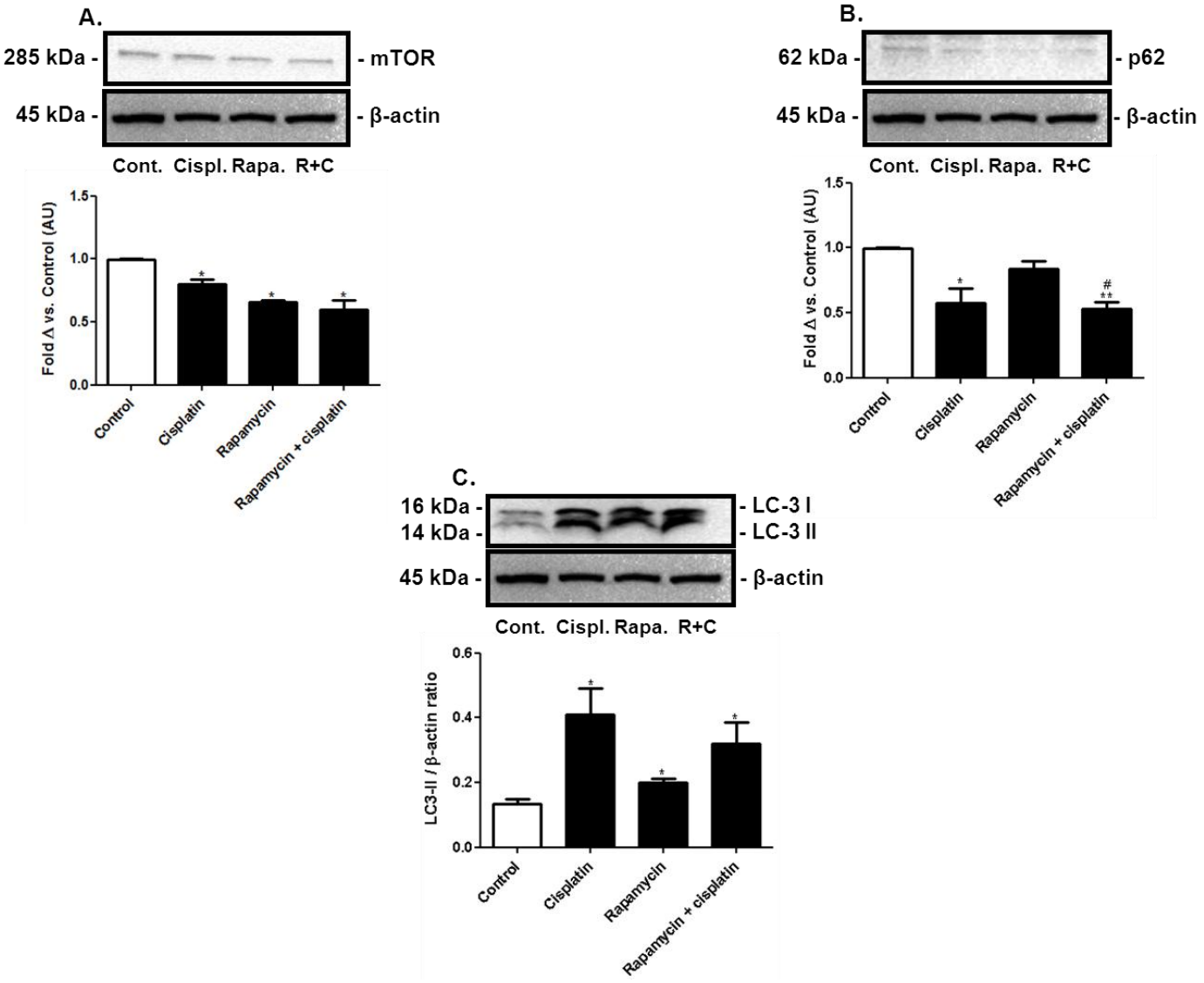
Studies have shown that the modulation of autophagic activity can sensitise cancer cells to chemotherapeutic agents (Zhuang et al., 2009) as well as augment chemotherapy-induced apoptosis (Notte et al., 2011). However, the effect of autophagic modulation as a pre-treatment strategy in conjunction with a low-concentration of cisplatin on cell death induction remains to be elucidated. The importance of the cell cycle and its role as an indication for sensitivity in combination chemotherapy has been realised (Shah and Schwartz, 2001). Cell-cycle mediated drug resistance occurs when treatment with one agent impacts the cell-cycle such that the subsequent chemotherapy agent administered immediately afterward is less effective (Shah and Schwartz, 2001).

The aims of this chapter are therefore to i) determine whether the induction or inhibition of autophagy proves to be more efficient in increasing cisplatin-induced apoptosis in two cancerous cell lines (HeLa and CaSki)., ii) conduct cell cycle analysis to determine whether the chemotherapy agents of choice act on cell cycle progression.

6.2. Results

6.2.1. Pharmacological induction of autophagy in combination with cisplatin treatment: Effects on autophagy and apoptosis.

a. The effect of rapamycin (induction of autophagy) and cisplatin on autophagy in Ect1/E6E7 cells.



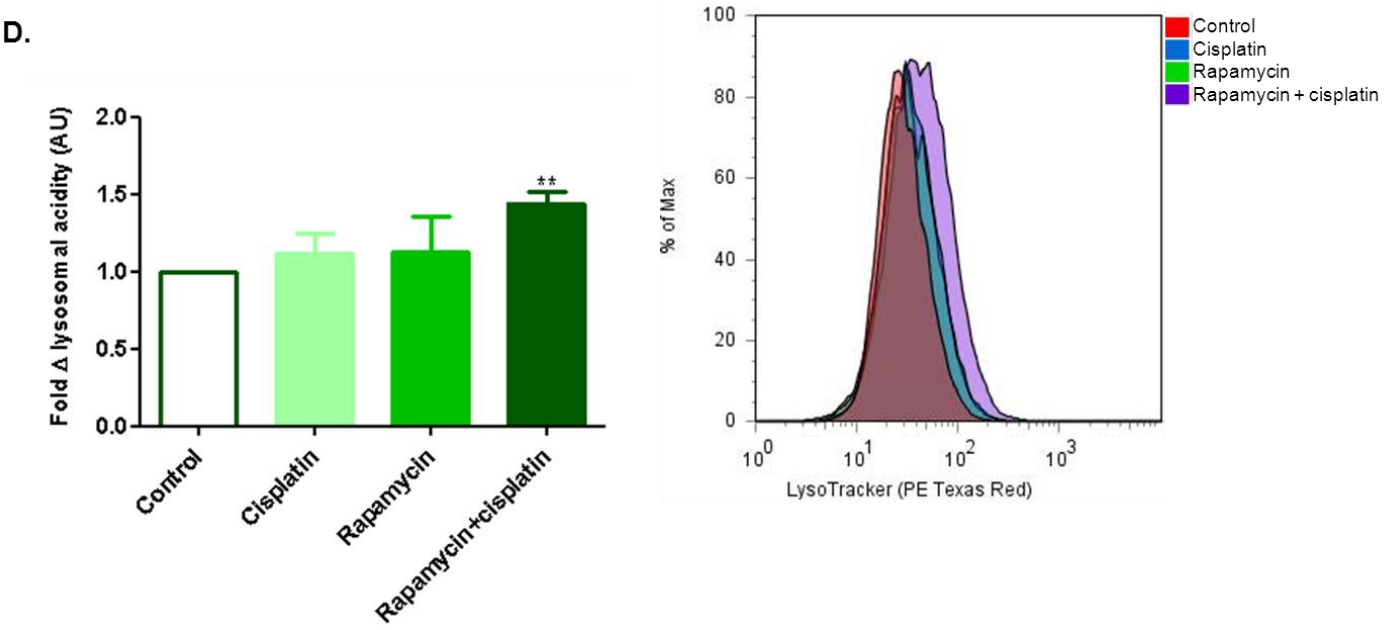
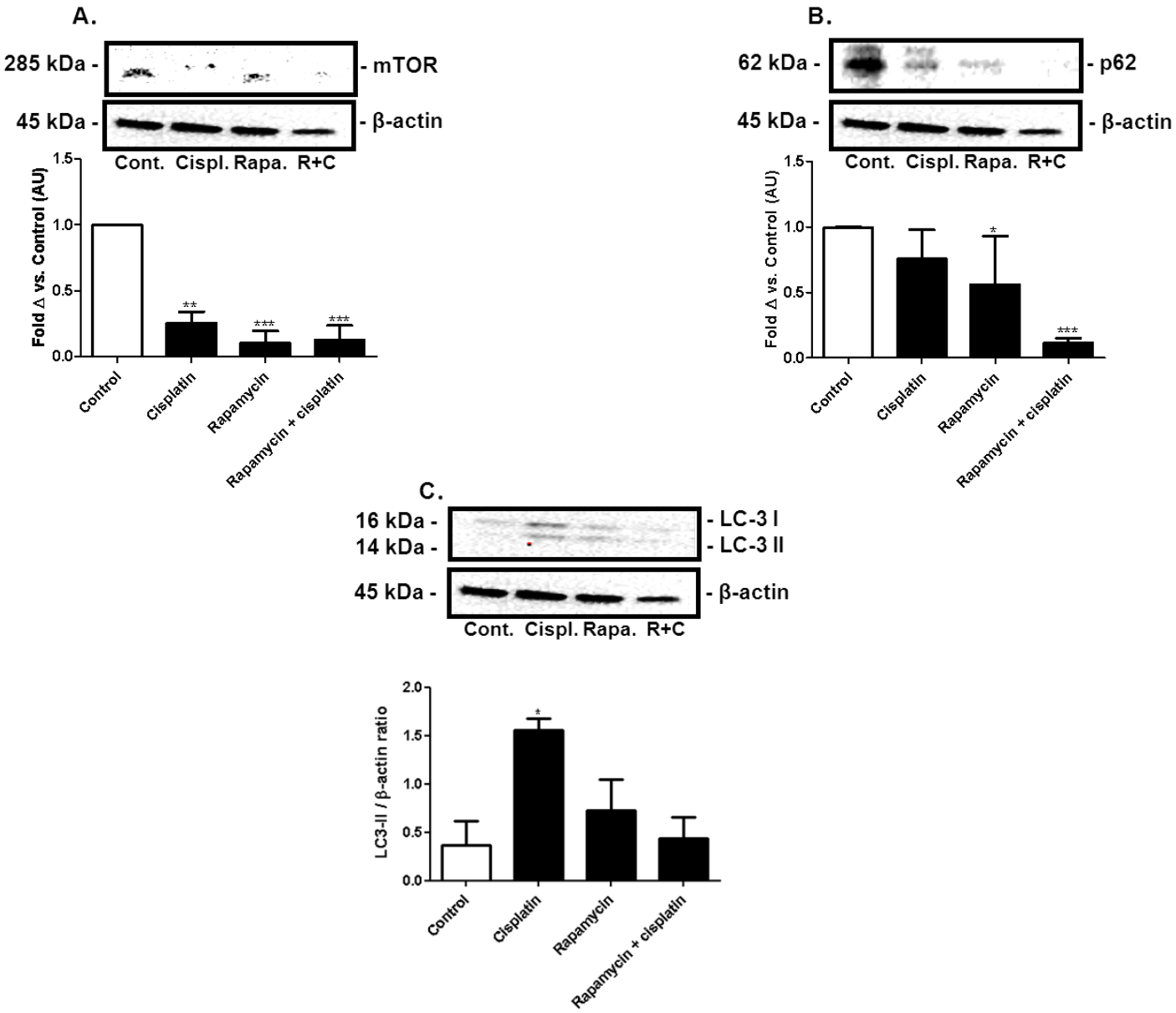


Figure 6.2.1. The effect of rapamycin and cisplatin on autophagic induction in Ect1/E6E7 cells. **A.** mTOR activation, * p< 0.05 vs. Control, n = 3. **B.** p62 degradation, * p < 0.05, ** p < 0.01 vs. Control and # p< 0.05 vs. rapamycin, n = 3. **C.** LC-3 II protein levels, * p < 0.05 vs. Control, n = 3. Corresponding densitometry below each representative Western blot. **D. Fluorescence intensity:** Lysosomal acidic compartment**,** ** p < 0.01 vs. Control, n = 4. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Cont.-control, Cispl.-cisplatin, Rapa.- rapamycin, R+C.- Rapamycin + cisplatin, AU- Arbitrary units.

The effect of rapamycin and cisplatin treatment on mTOR, p62 and LC-3 II protein expression in Ect1/E6E7 cells was analysed using the Western blotting technique and flow cytometry. Cisplatin, rapamycin as well as rapamycin + cisplatin (R+C) all significantly reduced mTOR protein expression in Ect1/E6E7 cells in comparison to the control (cisplatin: 0.80 ± 0.04; rapamycin: 0.66 ± 0.02; R+C: 0.60 ± 0.07 vs. control, * $p < 0.05$, Fig. 6.2.1. A). A significant decrease in p62 protein expression levels in Ect1/E6E7 cells was observed after treatment with cisplatin (0.58 ± 0.11 vs. control, * $p < 0.05$) as well as with the combination of rapamycin and cisplatin (0.53 ± 0.05 vs. control, ** $p < 0.01$ vs. rapamycin, # $p< 0.05$, Fig. 6.2.1. B). The analysis of LC-3 II protein levels in Ect1/E6E7 cells revealed a significant increase in the cisplatin, rapamycin and combination (R+C) treatment groups (cisplatin: 0.41 ± 0.08, rapamycin: 0.20 ± 0.01 and R+C: 0.32 ± 0.07 vs. control, * $p < 0.05$, Fig. 6.2.1.C). The lysosomal acidic compartment was analysed with the use of Lysotracker™, and fluorescence intensity subsequently quantified on the flow cytometer (Fig. 6.2.1. D). A significant increase in mean fluorescence intensity was observed in the combination group (1.44 ± 0.07 vs. control, * $p <0.05$).

b. The effect of rapamycin and cisplatin on autophagy and in HeLa cells.



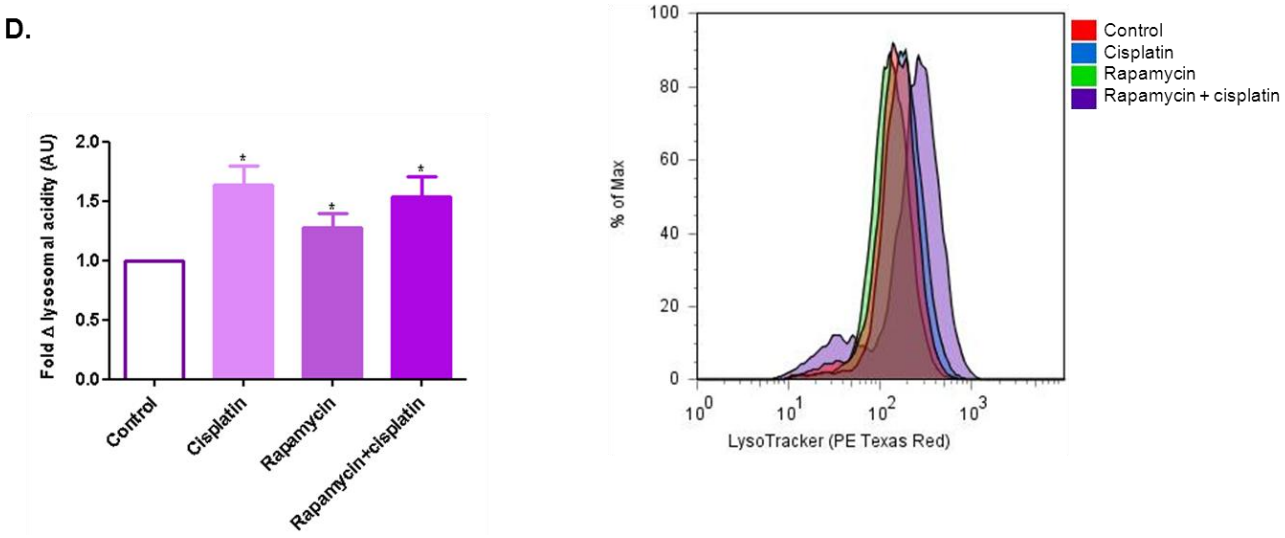
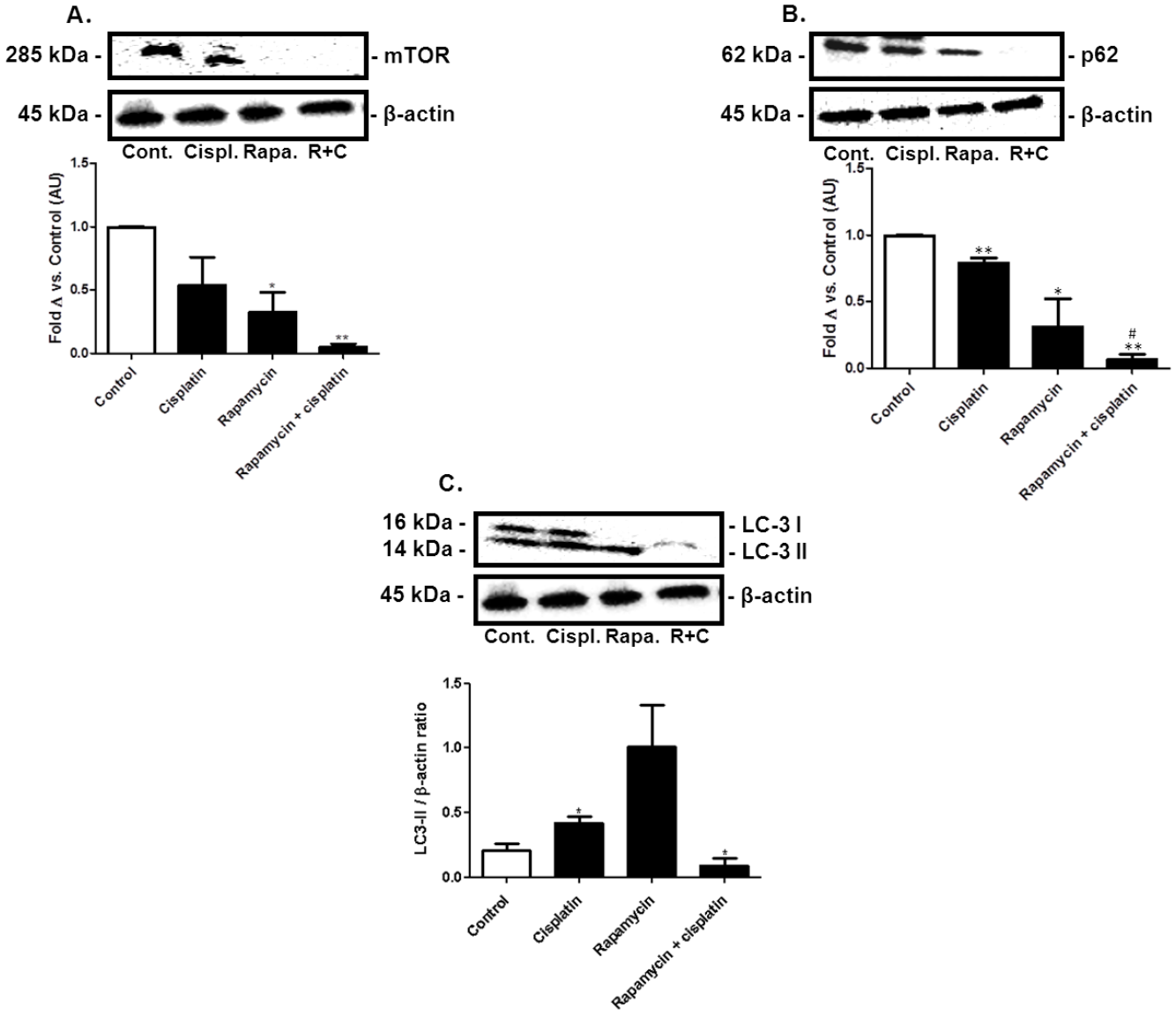


Figure 6.2.2. The effect of rapamycin and cisplatin on autophagic induction in HeLa cells. A. mTOR activation, *** $p < 0.001$, ** $p < 0.01$ vs. Control, n = 3. **B.** p62 degradation, *** $p < 0.001$; * $p < 0.05$ vs. Control, n = 3. **C.** LC-3 II protein levels, n = 3. Corresponding densitometry below each representative Western blot. **D.** Lysosomal compartmentalisation, * $p< 0.05$ vs. control, n = 7. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Cont.-control, Cispl.-cisplatin, Rapa.-rapamycin, R+C.- Rapamycin + cisplatin, AU- Arbitrary units.

In Fig. 6.2.2. A, mTOR protein expression levels were significantly decreased in all treatment groups in HeLa cells (cisplatin: 0.26 ± 0.09 vs. control, ** p< 0.01; Rapa: 0.10 ± 0.09 and R+C: 0.13 ± 0.11 vs. control, *** p < 0.001). Similarly, p62 protein levels decreased significantly (Fig. 6.2.2. B, however only in the rapamycin and combination groups (0.56 ± 0.37 vs. control, * p < 0.05 and 0.11 ± 0.04 vs. control,*** p< 0.001). LC-3 II levels increased significantly in the cisplatin treated group (* p < 0.05 vs. control, n = 4). Assessment of the lysosomal acidic compartment (mean fluorescence) revealed a significant increase in all treatment groups (cisplatin: 1.64 ± 0.16; Rapa.: 1.28 ± 0.12 and R+C: 1.54 ± 0.17 vs. control, * p < 0.05, Fig. 6.2.2. D)

c. The effect of rapamycin and cisplatin on autophagy and in CaSki cells.



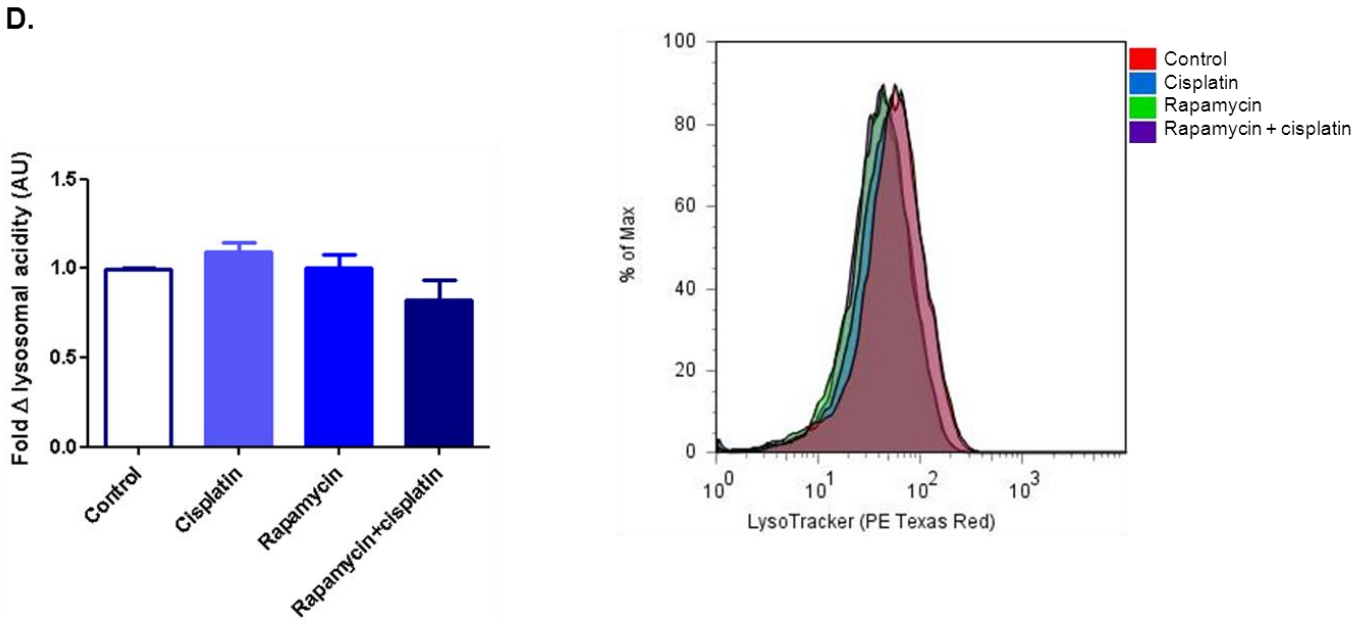
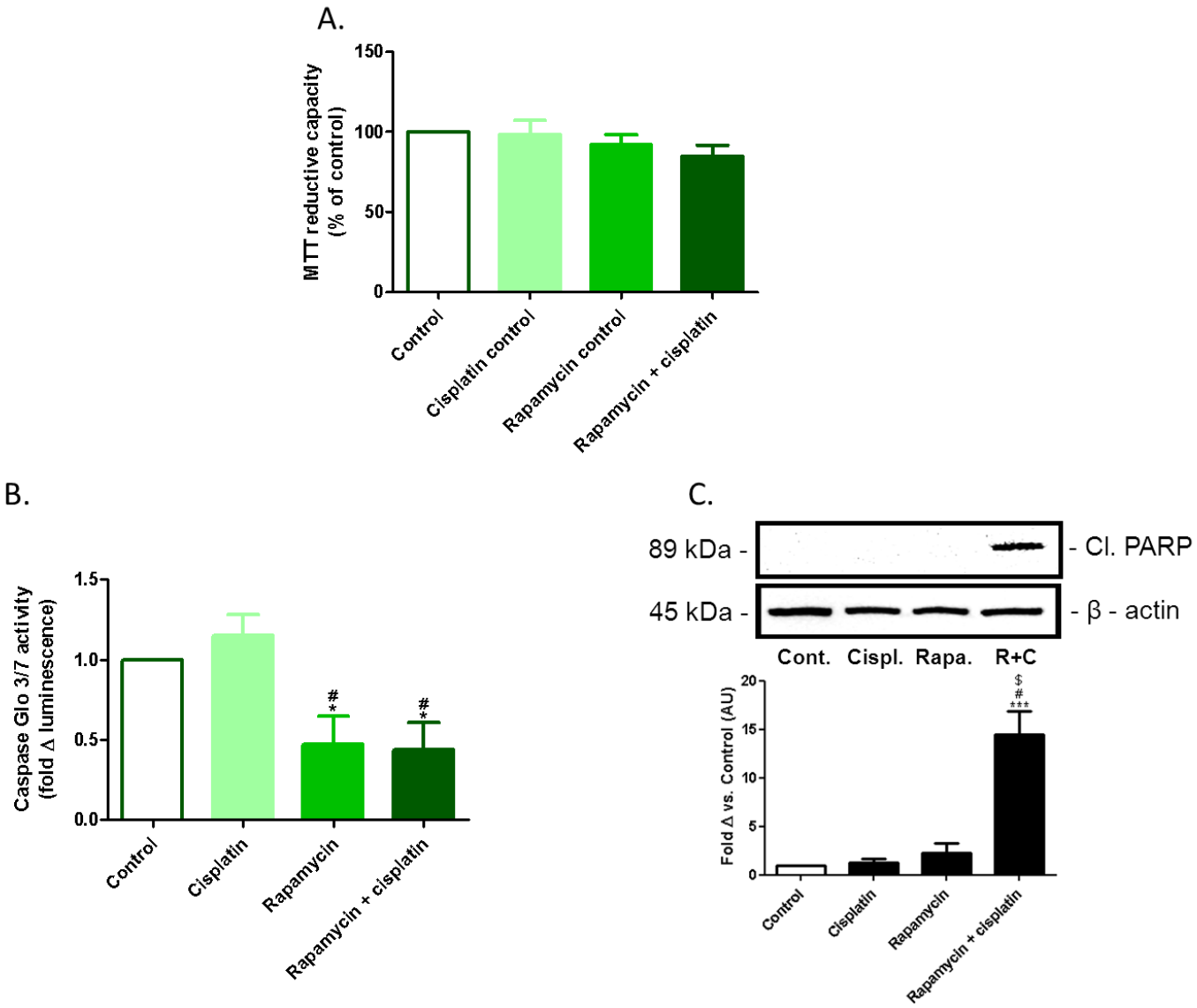


Figure 6.2.3. The effect of rapamycin and cisplatin on autophagic induction in CaSki cells. **A.** mTOR activation, * $p < 0.05$, ** $p < 0.01$ vs. Control, n = 3. **B.** p62 degradation, * $p < 0.05$; ** $p < 0.01$ vs. Control, # $p < 0.01$ vs. cisplatin, n = 3. **C.** The combination group had significantly lower levels of LC-3 II protein when compared to CaSki cells treated with rapamycin alone, * $p< 0.05$ (n = 3, Representative blot). Corresponding densitometry below each representative Western blot. **D.** Lysosomal acidic compartmentalisation, n = 7. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM Cont.-control, Cispl.-cisplatin, Rapa.- rapamycin, R+C.- Rapamycin + cisplatin, AU- Arbitrary units.

Intracellular mTOR protein levels were significantly lower in the rapamycin and combination groups in comparison to the control (0.32 ± 0.16 * $p < 0.05$ and 0.05 ± 0.03 ** $p < 0.01$ vs. control, Fig. 6.2.3.A). CaSki cells treated with cisplatin, rapamycin and the combination of the two exhibited significantly lowered p62 protein levels (cisplatin: 0.79 ± 0.04** $p < 0.01$; rapa.: 0.31 ± 0.21 * $p< 0.05$; R+C: 0.07 ± 0.04 **$p<$ 0.01 vs. control and 0.07 ± 0.04 # $p < 0.01$ vs. cisplatin, Fig. 6.2.3.B). Analysis of LC-3 II protein levels revealed that cisplatin induced a significant increase in LC-3 II signal (0.42 ± 0.45 vs. control, * $p < 0.05$) and the combination group significantly decreased levels of LC-3 II when compared to CaSki cells treated with rapamycin alone (0.09 ± 0.06 vs. rapamycin, * $p < 0.05$, Fig. 6.2.3. C). Assessment of the lysosomal acidic compartment by fluorescence intensity analysis did not reveal any significant changes in any of the treatment groups in the CaSki cells.

d. The effect of rapamycin and cisplatin on apoptosis in Ect1/E6E7 cells.



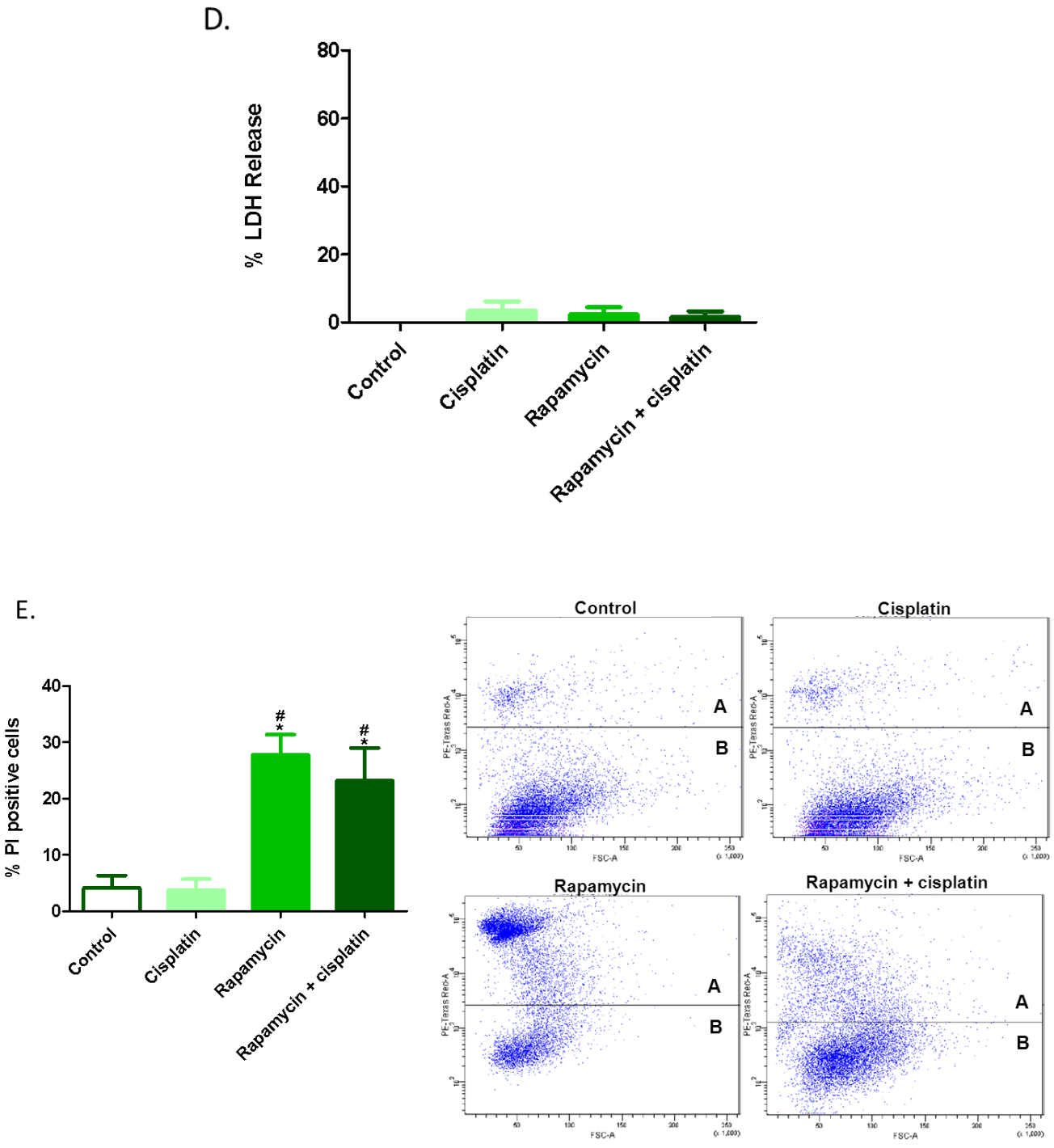
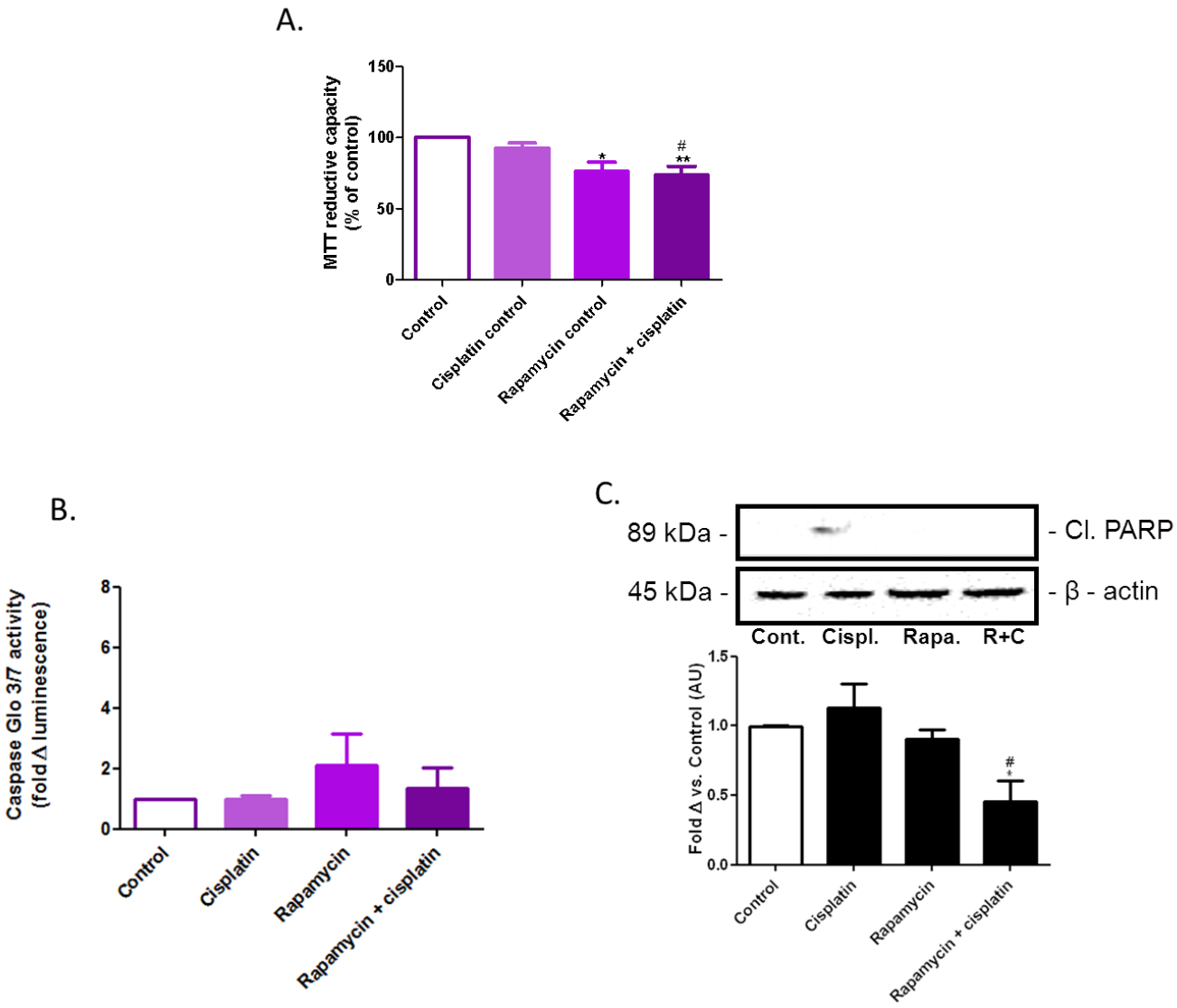


Figure 6.2.4. Effect on the induction of autophagy on apoptosis in Ect1/E6E7 cells. A. Cell viability (MTT reductive capacity), n = 5. **B.** Active caspase -3 and -7 activity * $p< 0.05$ vs. control and # $p< 0.05$ vs. cisplatin, n = 3. **C.** PARP cleavage, *** $p< 0.001$ vs. control, # $p< 0.001$ vs. cisplatin, and $ $p< 0.001$ vs. rapamycin, n = 3. **D.** LDH release, n = 5. **E.** Propidium iodide inclusion (flow cytometry), PI positive: quadrant A, PI negative: quadrant B, * $p < 0.05$ vs. control and # $p < 0.05$ vs. cisplatin, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM Cont.-control, Cispl.-cisplatin, Rapa.- rapamycin, R+C.- Rapamycin + cisplatin, AU- Arbitrary units.

Ect1/E6E7 cells did not display any significant differences in viability upon cisplatin treatment (Fig. 6.2.4. A.), based on the MTT reductive capacity. Caspase -3 and -7 activity was significantly reduced in the rapamycin and combination group when compared to the control and cisplatin treated cells (rapa.: 0.47 ± 0.17% and R+C: 0.44 ± 0.17% vs. control * $p < 0.05$; Rapa. and R+C vs. cisplatin, # $p < 0.05$, Fig. 6.2.4.B). Cleaved PARP protein levels in Ect1/E6E7 cells were observed to significantly increase in cells treated with the combination of rapamycin and cisplatin (14.49 ± 2.42 vs. control, *** $p < 0.001$, 14.49 ± 2.42 vs. cisplatin, # $p< 0.001$ and 14.49 ± 2.42 vs. rapamycin, $ $p< 0.001$, Fig. 6.2.4. C.). No significant changes in LDH release was observed in any of the treatment groups (Fig. 6.2.4. D). The quantification of PI positive Ect1/E6E7 cells was achieved through flow cytometry. A significant increase in PI positive cells was observed in the rapamycin and combination groups (27.75 ± 3.60% and 23.18 ± 5.79% vs. control, * $p < 0.05$; 27.75 ± 3.60% and 23.18 ± 5.79% vs. cisplatin, # $p < 0.05$, Fig. 6.2.4. E).

e. The effect of rapamycin and cisplatin on apoptosis in HeLa cells.



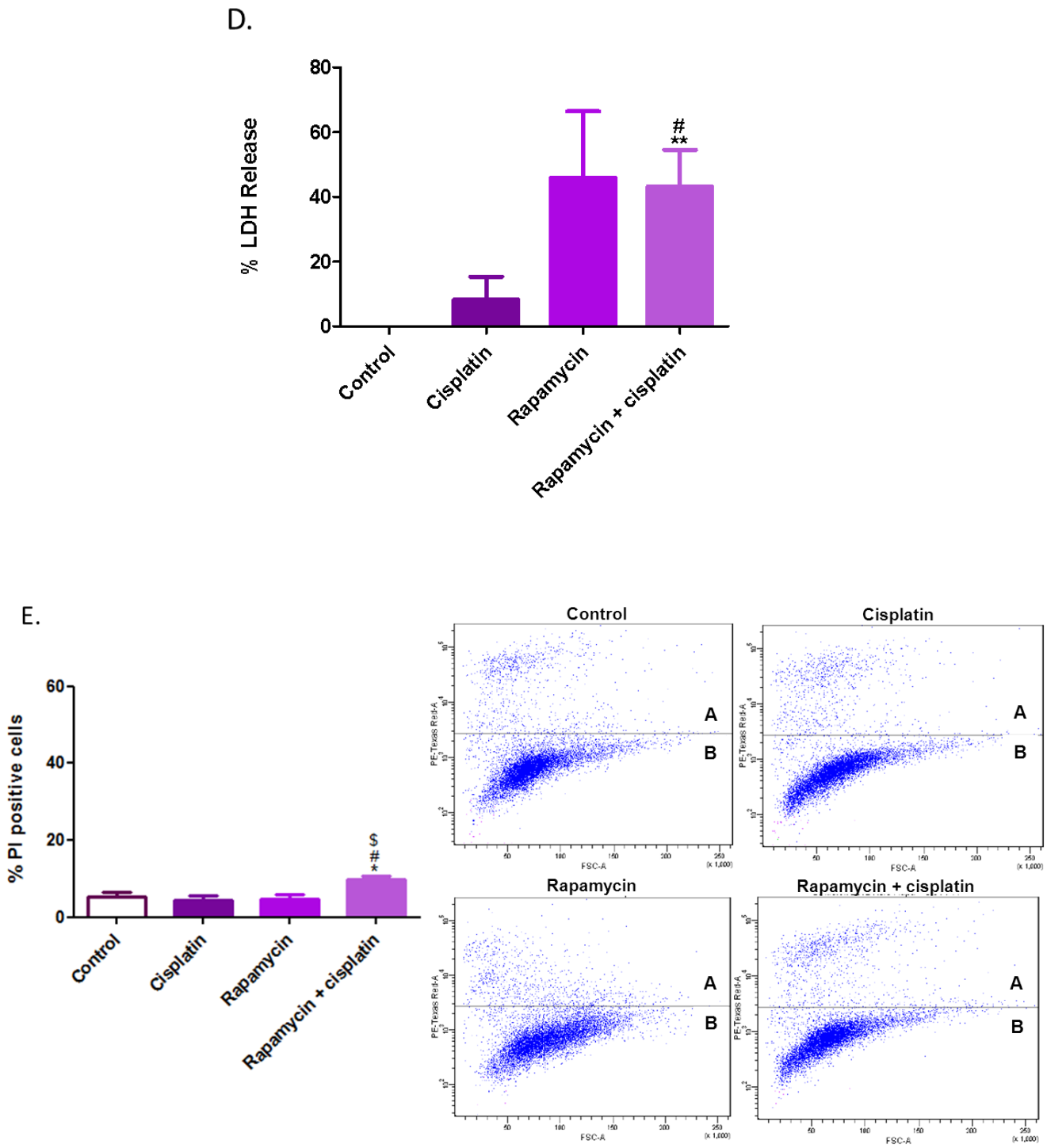
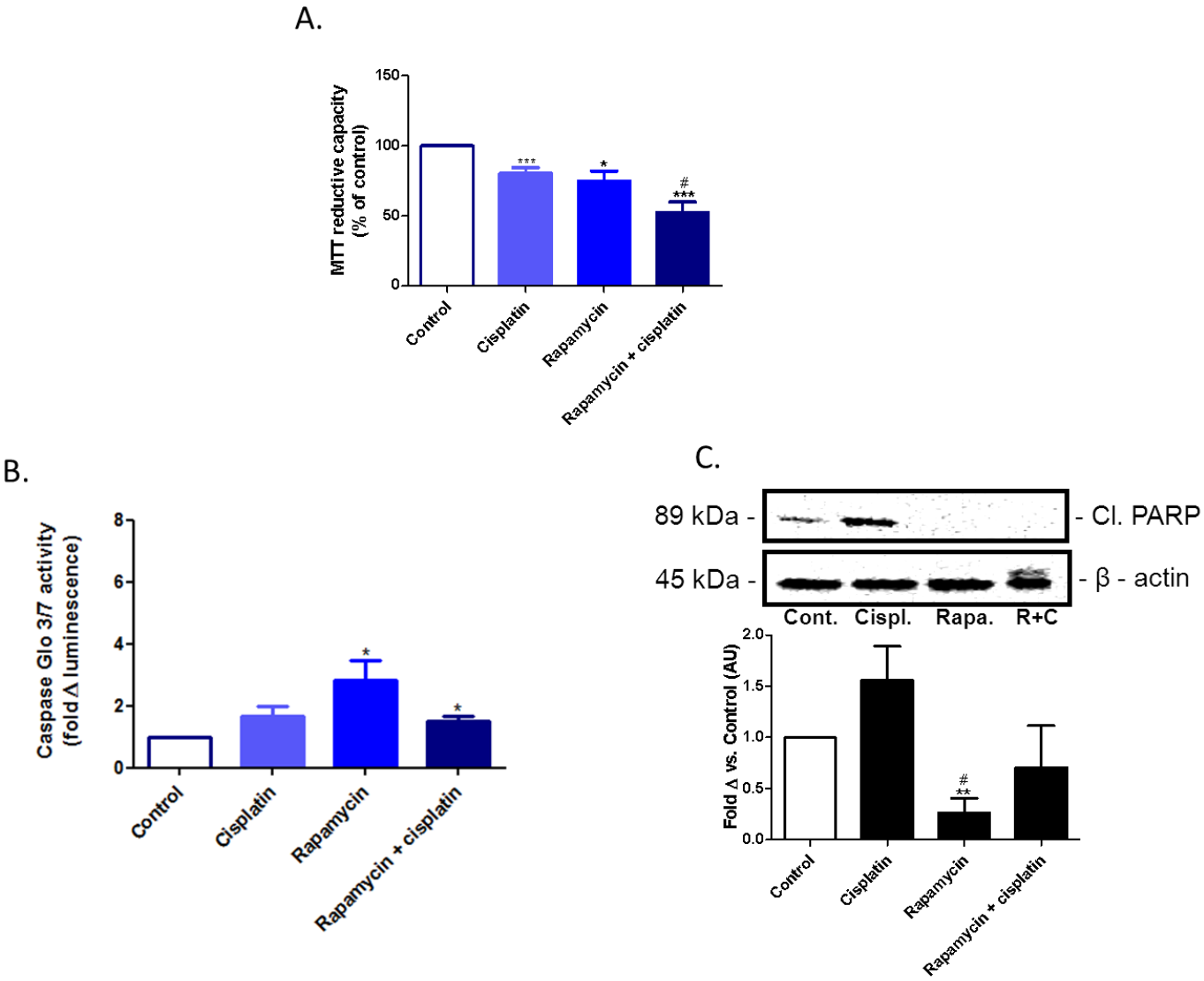


Figure 6.2.5. Effect on the induction of autophagy on apoptosis in HeLa cells. **A.** Cell viability (MTT), * p< 0.05 and ** p< 0.01 vs. control, # p < 0.05 vs. cisplatin, n = 7. **B.** Active caspase -3 and -7 activity, n = 3. **C.** PARP cleavage, representative blot, * p < 0.05 vs. control and # p < 0.05 vs. cisplatin, n = 4. **D.** LDH release, ** p < 0.01 vs. control and # p < 0.05 vs. cisplatin, n = 5. **E.** Propidium iodide inclusion (flow cytometry), PI positive: quadrant A, PI negative: quadrant B * p < 0.05 vs. control, # p < 0.05 vs. cisplatin and $ p < 0.05 vs. rapamycin, n = 4. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Cont.- control, Cispl.-cisplatin, Rapa.- rapamycin, R+C.- Rapamycin + cisplatin, AU- Arbitrary units.

HeLa cell viability was significantly reduced in the rapamycin and combination treatment groups (76.17 ± 6.45% * $p < 0.05$ and 73.42 ± 6.40% ** $p < 0.01$ vs. control, 73.42 ± 6.38% # $p< 0.05$ vs. cisplatin, Fig. 6.2.5. A.). Significant cleavage of PARP was observed in the combination treated group (0.45 ± 0.15 * $p < 0.05$ vs. control and # $p < 0.05$ vs. cisplatin, Fig.6.2.5.C). HeLa cells treated with the combination of rapamycin and cisplatin exhibited a significant increase in the percentage of LDH released (43.33 ± 11.18% ** $p < 0.01$ vs. control and 43.33 ± 11.18% # $p < 0.05$ vs. cisplatin, Fig. 6.2.5. D). A significant increase in the percentage of PI positive cells was observed in the combination group (9.83 ± 0.90% vs. control, * $p < 0.05$; 9.83 ± 0.90% vs. cisplatin, # $p < 0.05$ and 9.83 ± 0.90% vs. rapamycin $ $p < 0.05$).

f. The effect of rapamycin and cisplatin on apoptosis in CaSki cells.



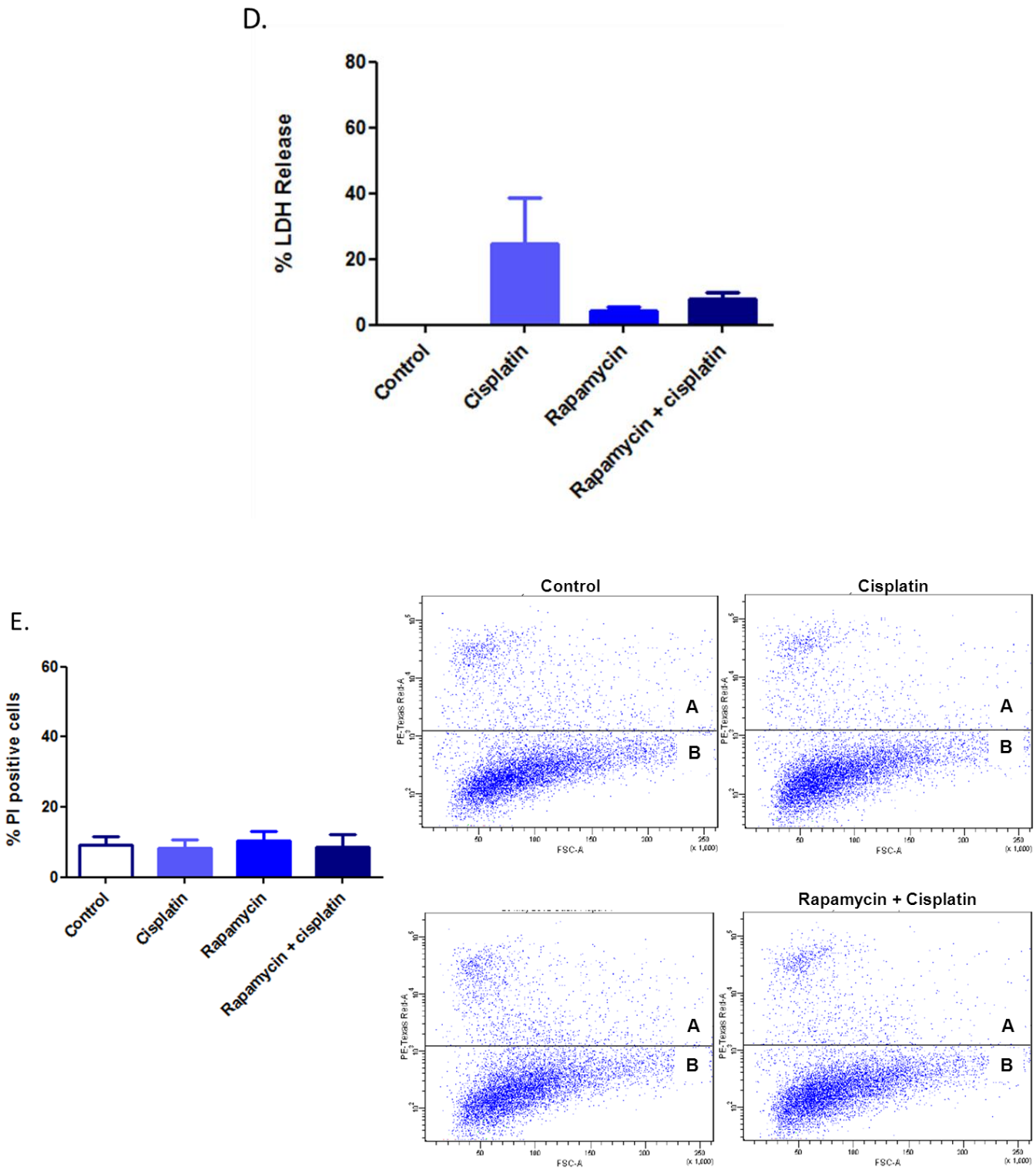


Figure 6.2.6. Effect on the induction of autophagy on apoptosis in CaSki cells. **A.** Cell viability (MTT), * $p < 0.05$ and *** $p < 0.001$ vs. control, # $p< 0.05$ vs. cisplatin, n = 7. **B.** Caspase -3 and -7 activity, * $p < 0.05$ vs. control, n = 3. **C.** PARP cleavage (representative blot), ** $p< 0.01$ vs. control and # $p< 0.05$ vs. cisplatin, n = 4. **D.** LDH, n = 4. **E**. Propidium iodide inclusion (flow cytometry), PI positive: quadrant A, PI negative: quadrant B, n = 4. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Cont.- control, Cispl.-cisplatin, Rapa.- rapamycin, R+C.- Rapamycin + cisplatin, AU- Arbitrary units.

Cell viability (MTT) of CaSki cells treated with cisplatin, rapamycin and the combination of the two revealed a significant decrease in cell viability (80.37 ± 4.04%; 74.76 ± 7.36% and 52.33 ± 7.65% vs. control * $p < 0.05$, *** $p < 0.001$, 52.33 ± 7.65% vs. cisplatin, # $p < 0.05$, Fig. 6.2.6.A). Caspase -3/-7 activity of CaSki cells treated with rapamycin and rapamycin and cisplatin increased significantly in both groups when compared to the control (2.8 ± 0.65 and 1.52 ± 0.13 vs. control, * $p < 0.05$, Fig. 6.2.6.B). PARP cleavage was significantly decreased in CaSki cells treated with rapamycin alone when compared to the control (0.26 ± 0.15 vs. control, ** $p < 0.01$) and the cisplatin group (0.26 ± 0.15 vs. cisplatin, # $p < 0.05$, Fig. 6.2.6. C.

g. Analysis of cell cycle progression modulated by rapamycin and cisplatin treatment in Ect1/E6E7 cells.

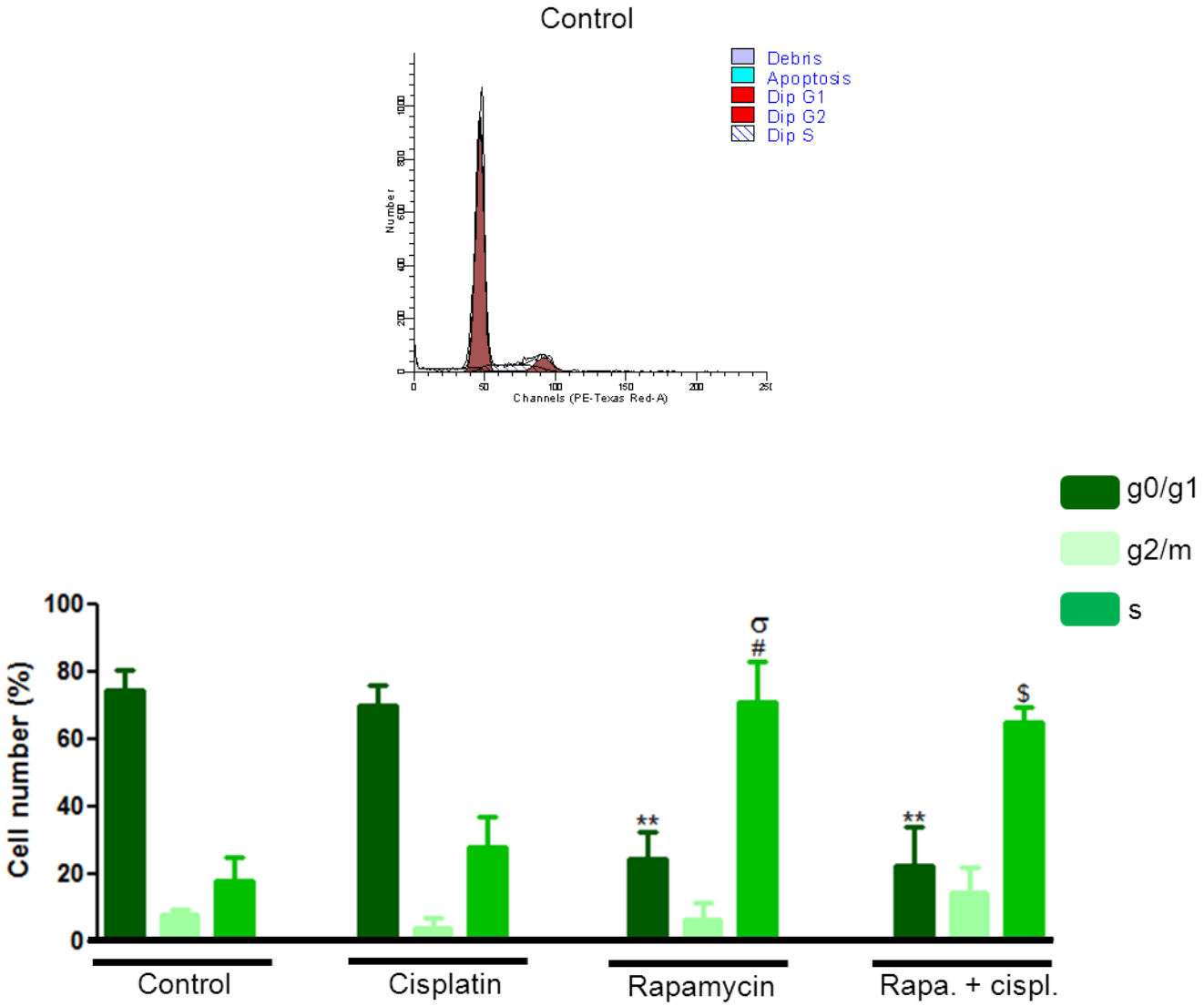


Figure 6.2.7. Modulation of cell cycle progression by rapamycin and cisplatin in Ect1/E6E7 cells. Cells were either treated with rapamycin (50 μM), cisplatin (15 μM) or the combination of the two for 24 h. Cell cycle distribution pattern was assessed by flow cytometry, ** $p < 0.01$ vs. control G0/G1, # $p < 0.01$ vs. control S and $ $p < 0.05$ vs. control S; σ $p < 0.05$ vs. cisplatin S, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Rapa. + cispl. = rapamycin + cisplatin.

The distribution of cells in different phases of the cell cycle phase after treatment with rapamycin, cisplatin, or the combination of the two is illustrated in Fig. 6.2.7. Induction of autophagy with rapamycin resulted in a significant decrease in the percentage of cells in the G1 phase in comparison to the control (23.70 ± 7.90% and 21.91 ± 11.20% vs. control G1 ** $p < 0.01$), with a concomitant increase in the percentage of cells in the *S* phase when treated with rapamycin and the combination of rapamycin and cisplatin (70.42 ± 12.00% vs. control *S*, # $p < 0.01$ and 64.29 ± 4.58% vs. control *S*, $ $p < 0.05$) and cisplatin treated Ect1/E6E7 cells (70.42 ± 12.00% vs. cisplatin, σ $p < 0.05$).

h. Analysis of cell cycle progression modulated by rapamycin and cisplatin treatment in HeLa cells.

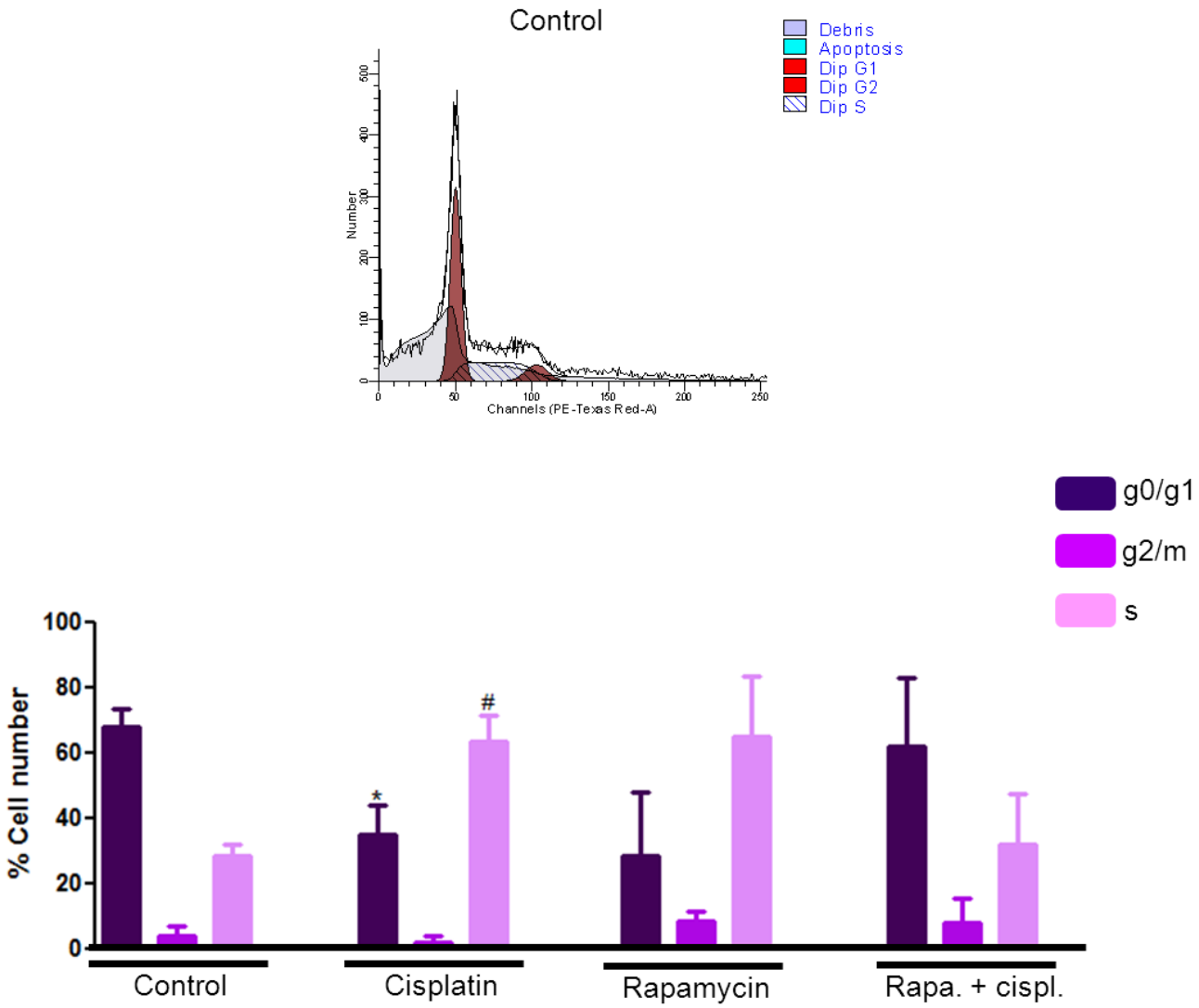


Figure 6.2.8. Modulation of cell cycle progression by rapamycin and cisplatin in HeLa cells. Cells were either treated with rapamycin (50 µM) or cisplatin (15 µM) or the combination of the two for 24 h. Cell cycle distribution assessed by flow cytometry,* $p < 0.01$ vs. control G0/G1, # $p < 0.01$ vs. control, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Rapa. + cispl. = rapamycin + cisplatin.

Cell cycle analysis of HeLa cells reveals a significant decrease in the G1-phase after treatment with cisplatin (35.03 ± 8.76% vs. control G1, * $p < 0.05$) and S phase (63.14 ± 8.23% vs. control S, # $p < 0.05$, Fig. 6.2.8).

i. Analysis of cell cycle progression modulated by rapamycin and cisplatin treatment in CaSki cells.

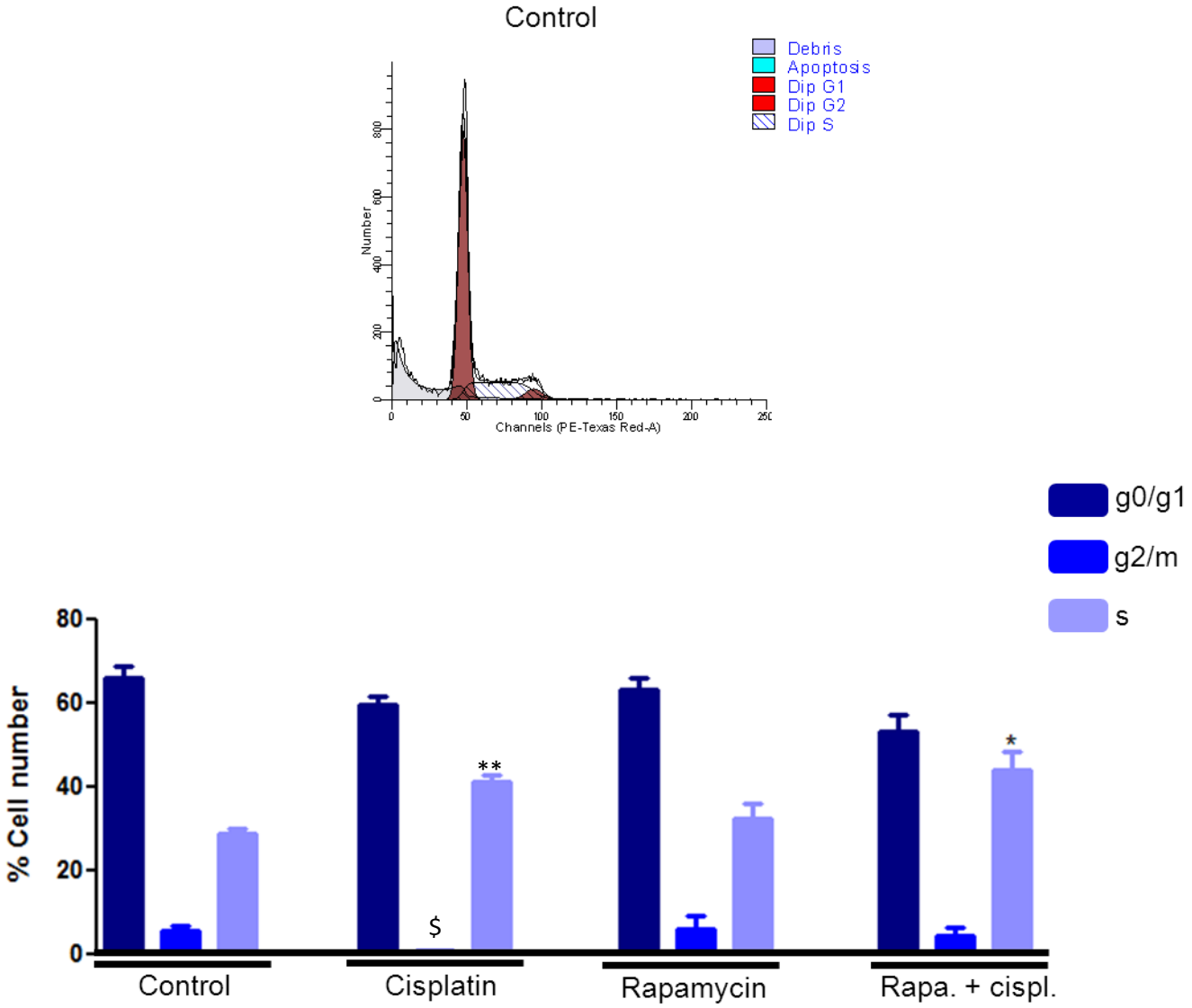


Figure 6.2.9. Modulation of cell cycle progression by rapamycin and cisplatin in CaSki. Cells were either treated with rapamycin (50 μM), cisplatin (15 μM) or the combination of the two for 24 h. * $p < 0.05$ and ** $p < 0.01$ vs. control S, $ $p < 0.01$ vs. control G2/M, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Rapa. + cispl. = rapamycin + cisplatin.

CaSki cells did not undergo any prominent shifts in the various cell cycle phases, however treatment with cisplatin induced a significant decrease in the G2/M phase with a concomitant increase in the S phase in comparison to the control (0.16 ± 0.16% and 40.66 ± 1.70% ** $p < 0.01$ vs. control S and $ $p < 0.01$ vs. control G2/M). The combination of rapamycin and cisplatin led to a significant increase in the S phase (43.29 ± 4.55% vs. control S, * $p < 0.05$ and, Fig. 6.2.9).

6.2.2. Biological induction of autophagy in combination with cisplatin treatment: Effects on autophagy and apoptosis.

a. The response of mTOR silencing on autophagy in Ect1/E6E7 cells.

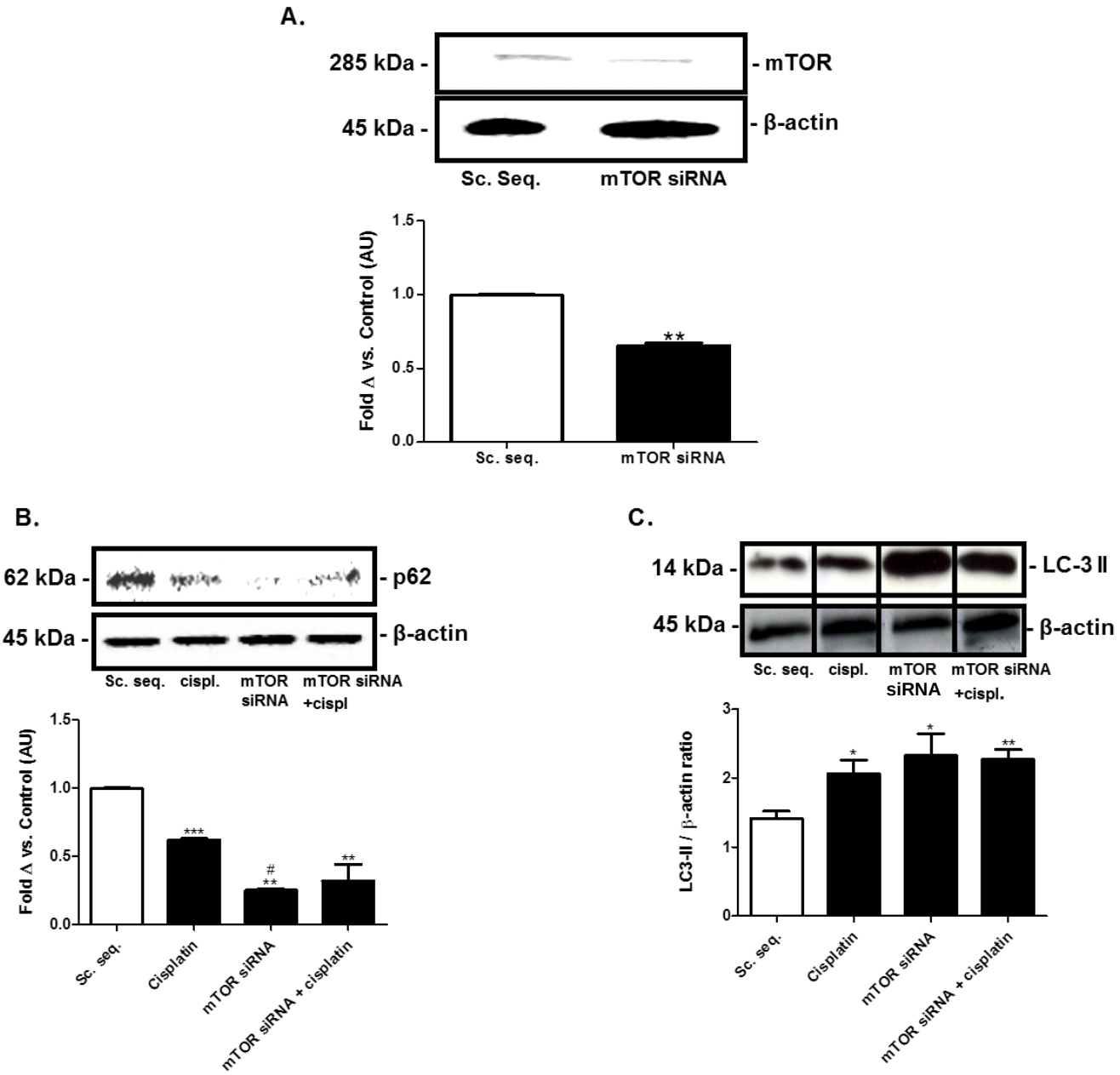


Figure 6.2.10. mTOR silencing in Ect1/E6E7 cells and the effect on autophagy. A. Silencing of mTOR ** $p < 0.05$ vs. sc. seq., n = 3. **B.** Degradation of p62, ** $p < 0.01$, *** $p < 0.001$ vs. sc. seq., # $p < 0.01$ vs. cisplatin, n = 3. **C.** LC-3 II protein levels, * $p < 0.05$ and ** $p < 0.01$, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM, representative blots. Sc. seq. = scrambled siRNA sequence/control, AU = arbitrary units.

Silencing of mTOR in Ect cells was confirmed through Western blotting (0.66 ± 0.02 vs. sc. seq., $p < 0.01$, Fig. 6.2.10.A). p62 significantly decreased in Ect1/E6E7 cells treated with cisplatin, silenced for the mTOR protein, as well as in the combination group in comparison to the control/scrambled sequence (0.62 ± 0.01, 0.25 ± 0.01 and 0.32 ± 0.12 vs. sc. seq., ** $p < 0.01$ and *** $p < 0.001$). Additionally, Ect1/E6E7 cells in which mTOR was silenced had a significantly lower intracellular p62 level than those cells treated with cisplatin (0.25 ± 0.01 vs. cisplatin, # $p < 0.01$, Fig. 6.2.10. B). LC-3 II signal significantly increased in the cisplatin, silenced and combination groups in comparison to the control/scrambled sequence (2.07 ± 0.19, 2.33 ± 0.31 and 2.27 ± 0.14 vs. sc. seq., * $p < 0.05$, ** $p < 0.01$, Fig 6.2.10.C).

b. The effect of mTOR silencing on autophagy in HeLa cells.

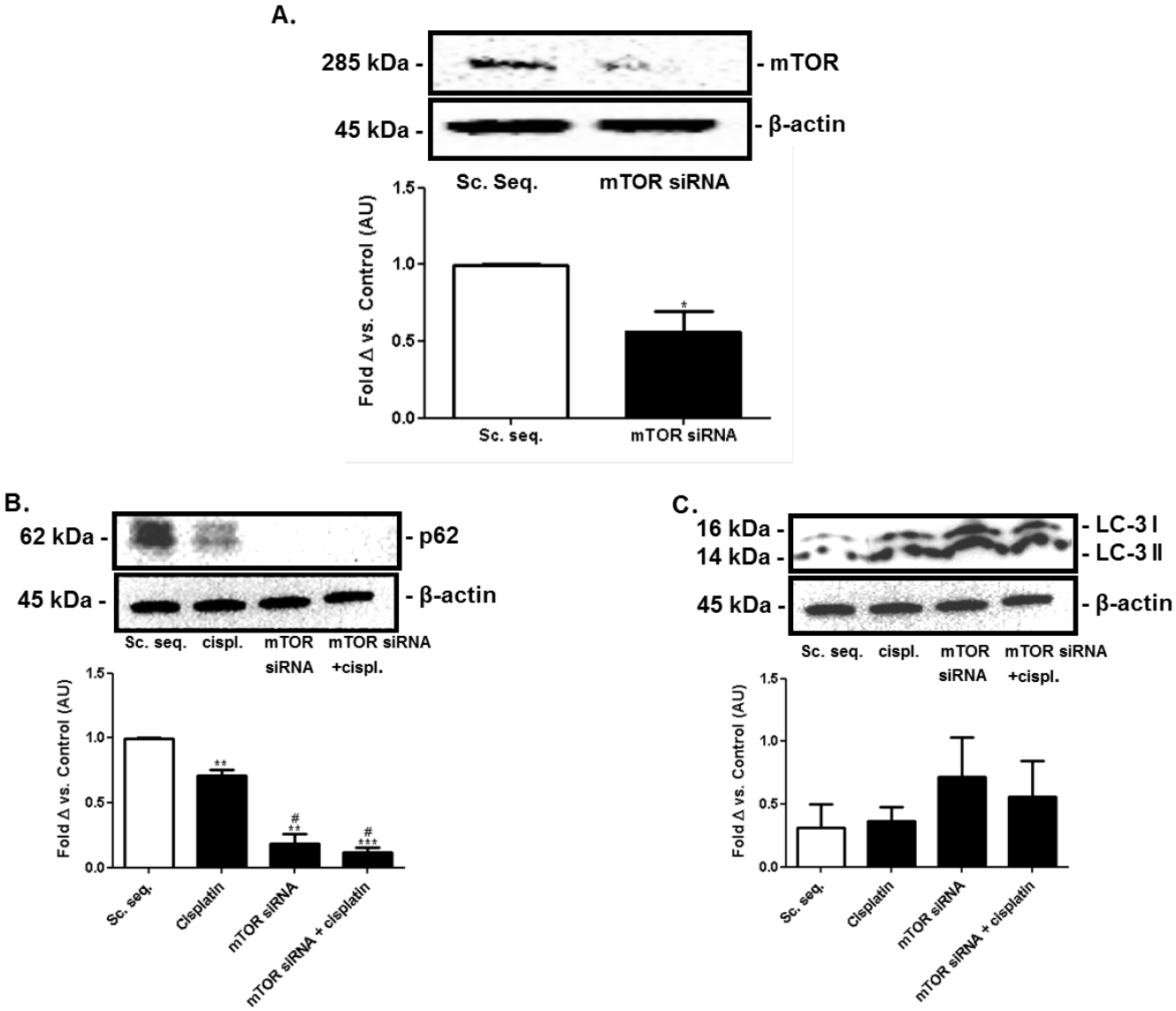


Figure 6.2.11. mTOR silencing in HeLa cells and the effect on autophagy. A. Silencing of mTOR * p < 0.05 vs. sc. seq., n = 3. **B.** p62 degradation, ** p < 0.01, ***p < 0.001 vs. sc. seq., # p< 0.01 vs. cisplatin, n = 3. **C.** LC-3 II protein levels, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM, representative blots Sc. seq. = scrambled siRNA sequence/control, AU = arbitrary units.

mTOR silencing in HeLa cells was confirmed through Western blotting (0.56 ± 0.13 vs. sc. seq.* $p < 0.05$, Fig. 6.2.11. A). p62 protein levels were significantly decreased in the cisplatin group, the mTOR siRNA group, as well as in the combination group when compared to the control (cisplatin: 0.71 ± 0.05, mTOR: 0.18 ± 0.08 ** $p < 0.01$ and 0.12 ± 0.04 *** $p < 0.001$ vs. sc. seq.; mTOR and mTOR siRNA + cisplatin: # $p < 0.001$ vs. cisplatin, Fig. 6.2.11. B.). LC-3 II expression levels did not increase significantly in any of the treatment groups (Fig. 6.2.11. C).

c. The effect of mTOR silencing on autophagy in CaSki cells.

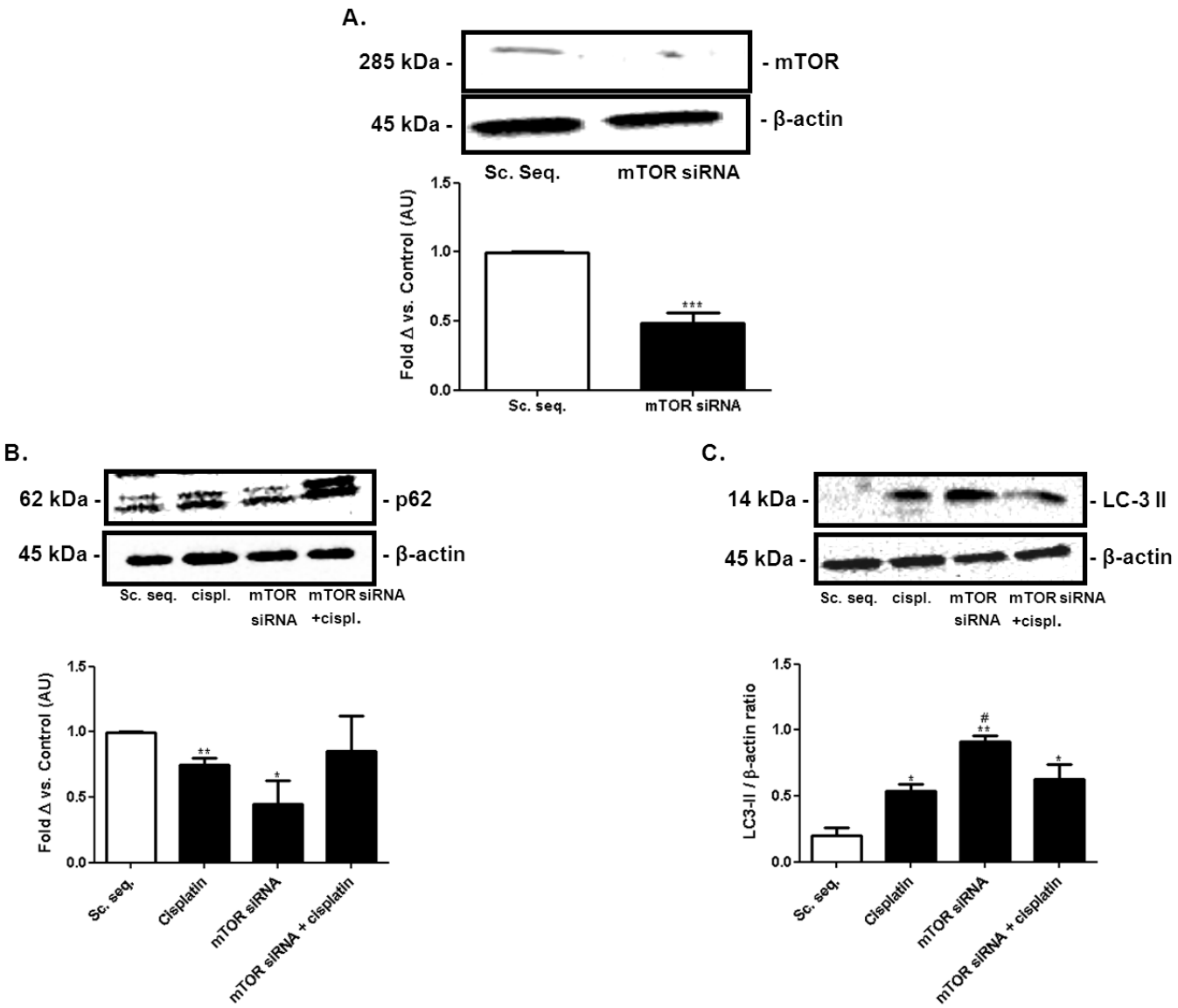


Figure 6.2.12. mTOR silencing in CaSki cells and the effect on autophagy. A. Silencing of mTOR *** $p <$ 0.001 vs. sc. seq., n = 3. **B.** p62 degradation, * $p <$ 0.05, ** $p <$ 0.01 vs. sc. seq., n = 3. **C.** LC-3 II protein levels * $p <$ 0.01 vs. sc. seq. and # $p <$ 0.01 vs. cisplatin, n = 3 (Representative blot). Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM, representative blots. Sc. seq. = scrambled siRNA sequence/control, AU = arbitrary units.

Efficient silencing of mTOR was confirmed through Western blotting (0.48 ± 0.08 vs. sc. seq., *** $p < 0.001$, Fig. 6.2.12. A). Levels of p62 decreased significantly in comparison to the control in CaSki cells treated with cisplatin, and in the silenced group (0.75 ± 0.05 * $p < 0.01$ and 0.45 ± 0.18 * $p < 0.01$ vs. sc. seq., Fig. 6.2.12. B). In Lastly, a significant increase in LC-3 II protein expression was observed in CaSki cells treated with cisplatin, silenced for mTOR and the combination of the two (cisplatin: 0.54 ± 0.05, mTOR: 0.91 ± 0.04 vs. sc. seq. 0.63 ± 0.12, * $p < 0.05$, ** $p < 0.01$ vs. sc. seq. and mTOR siRNA vs. cisplatin # $p < 0.01$, Fig. 6.2.12. C.).

d. The effect of mTOR silencing with cisplatin treatment on apoptosis in Ect1/E6E7 cells.

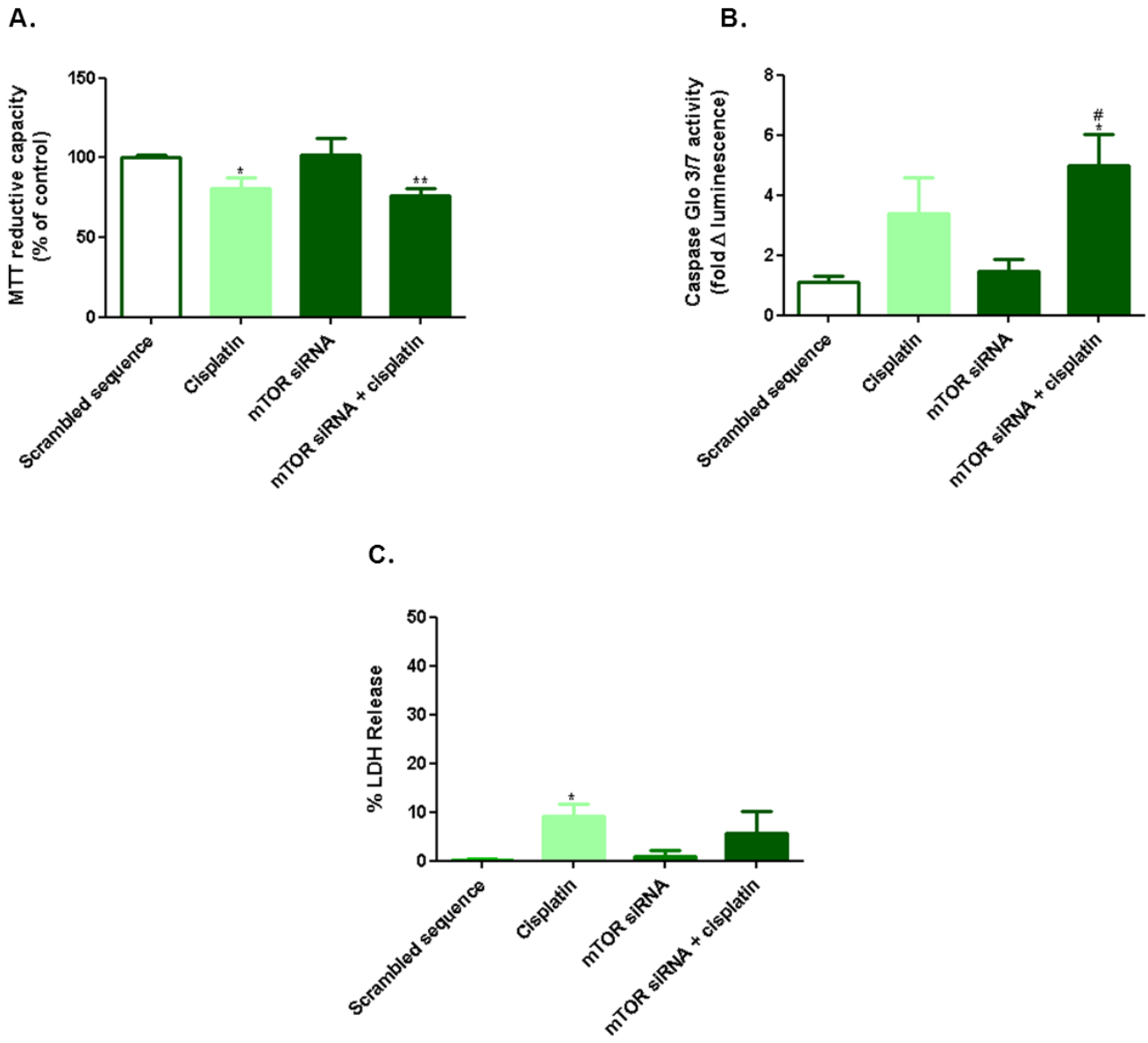


Figure 6.2.13. Effect of the silencing of mTOR on apoptosis in Ect1/E6E7 cells. **A.** Cell viability (MTT), * $p < 0.05$ and ** $p < 0.01$ vs. control, n = 3 **B.** Caspase -3/-7 activity * $p < 0.05$ vs. sc. seq., # $p < 0.05$ vs. mTOR, n = 3. **C.** LDH release * $p < 0.05$ vs. sc.seq., n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Sc. seq. = scrambled siRNA sequence/control, AU = arbitrary units.

Cell viability (MTT) results reveal significant decreases in the cisplatin and combination groups (80.87 ± 6.4%, *p < 0.05 and 76.37 ± 4.35%, ** p < 0.01 vs. sc. seq. Fig. 6.2.13. A). Silencing of mTOR and combination treatment with cisplatin induced a significant up-regulation in caspase-3/-7 activity in CaSki cells (4.97 ± 1.05 vs. sc. seq. * p < 0.05 and 4.97 ± 1.05 vs. mTOR siRNA # p < 0.05, Fig. 6.2.13. B). LDH release significantly increased in cells treated with cisplatin alone (9.20 ± 2.58% vs. sc. seq. * p < 0.05, Fig. 6.2.13. C).

e. The effect of mTOR silencing with cisplatin treatment on apoptosis in HeLa cells.

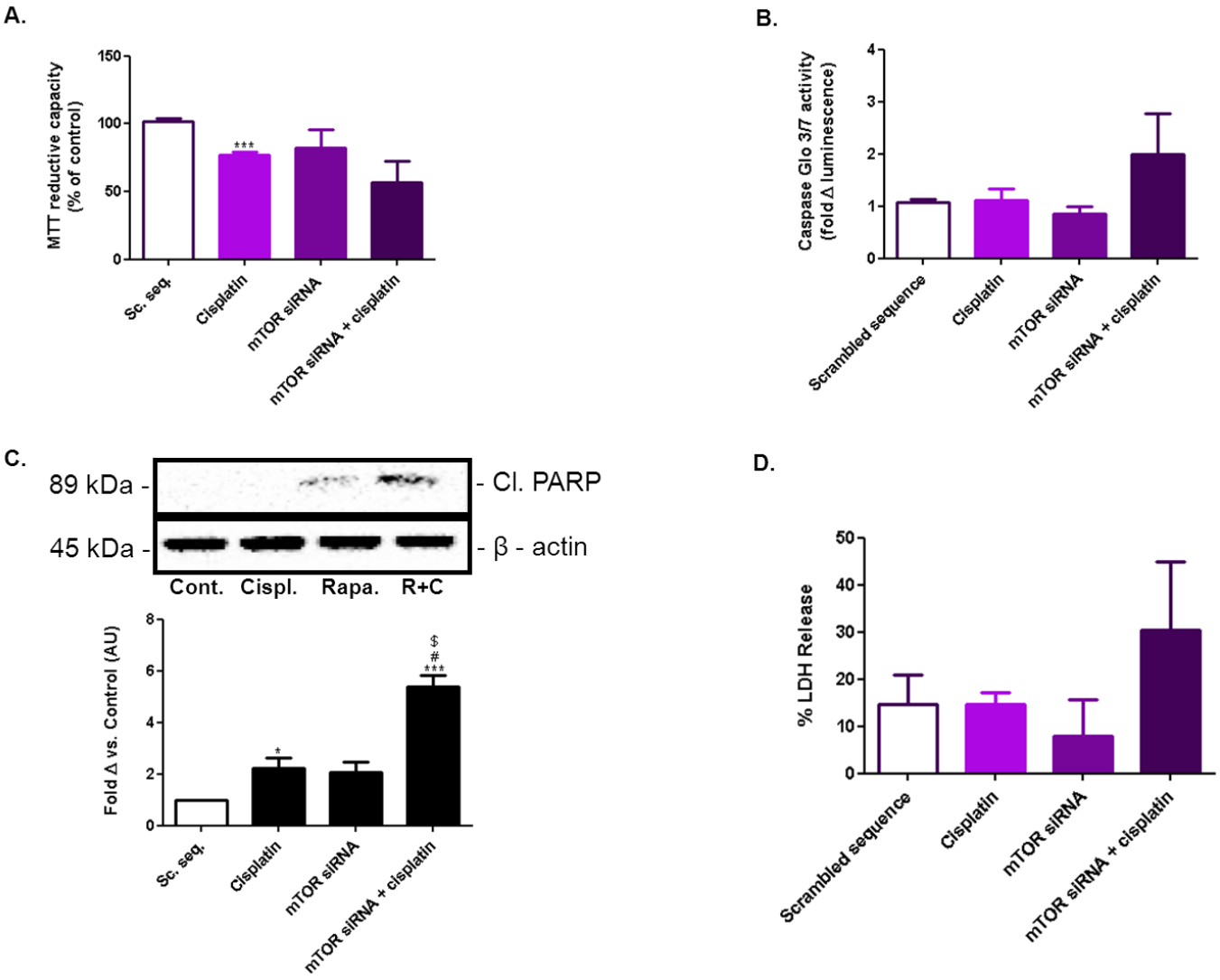


Figure 6.2.14. **Effect of the silencing of mTOR with cisplatin treatment on apoptosis in HeLa cells**. **A.** Cell viability (MTT), *** $p < 0.001$ and vs. sc. seq., n = 6 **B.** Caspase -3/-7 activity, n = 4. **C.** PARP cleavage, * $p < 0.05$ and *** $p < 0.001$ vs. sc. seq., # $p < 0.01$ vs. cisplatin and $ $p< 0.01$ vs. mTOR, n = 3 (corresponding representative blot above). **D.** LDH release, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Sc. seq. = scrambled siRNA sequence/control, AU = arbitrary units.

Cell viability (MTT) was significantly reduced in HeLa cells treated with cisplatin (76.78 ± 2.53% vs. sc. seq. * $p < 0.001$, Fig. 6.2.14. A). No significant changes were observed in caspase-3/-7 activity in any of the groups (Fig. 6.2.14. B). Cleaved PARP protein levels increased in HeLa cells treated with cisplatin and cisplatin treatment with silencing (2.21 ± 0.42 * $p< 0.05$ and 5.39 ± 0.43 *** $p < 0.001$ vs. sc. seq; 5.39 ± 0.43 # $p < 0.01$ vs. cisplatin; 5.39 ± 0.43 $ $p < 0.001$ vs. mTOR, Fig. 6.2.14. C). Analysis of LDH release did not reveal any significant changes in any of the groups (Fig.6.2.14. D).

f. The effect of mTOR silencing with cisplatin treatment on apoptosis in CaSki cells.

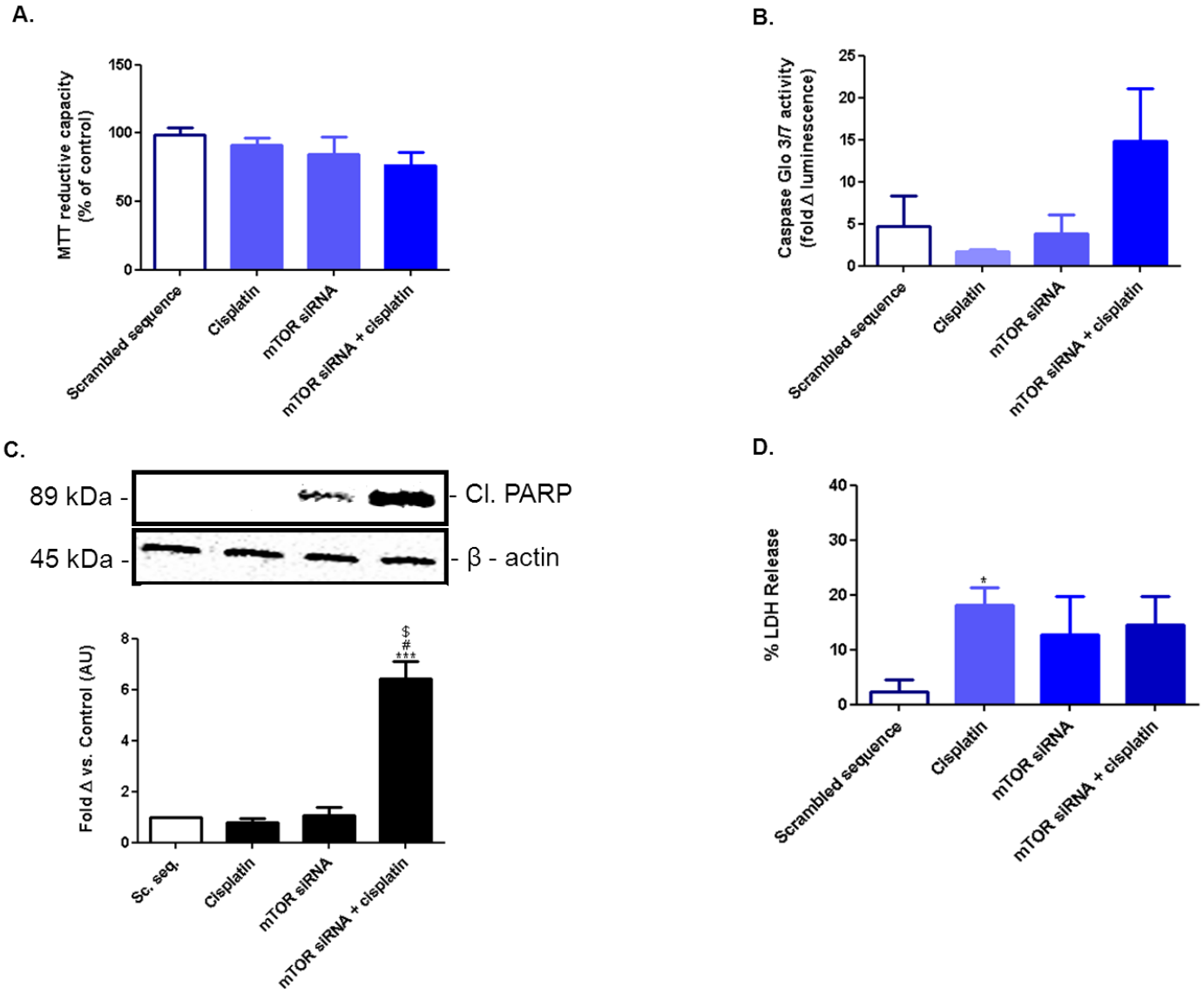
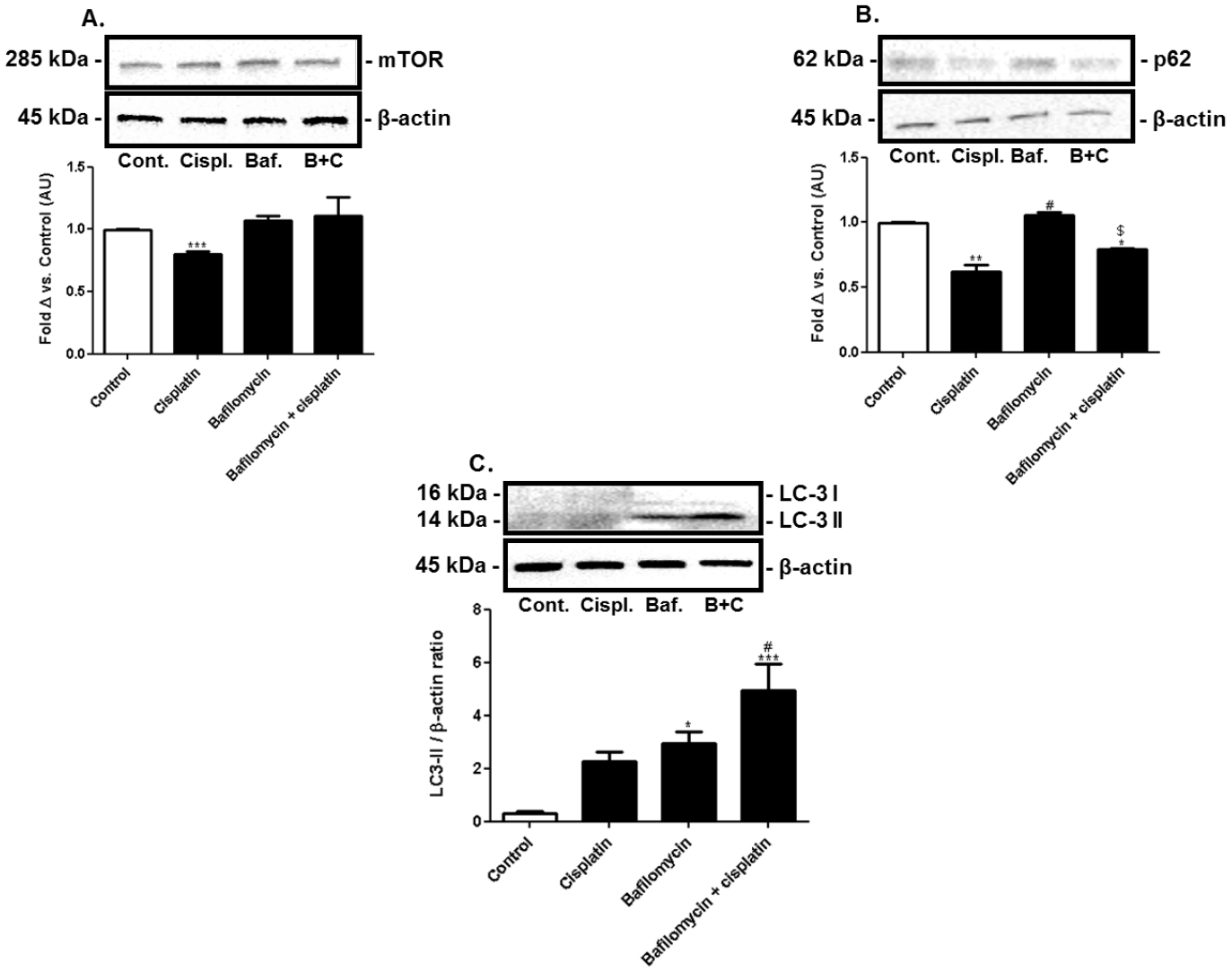


Figure 6.2.15. Effect of the silencing of mTOR on apoptosis in CaSki cells.**A.** Cell viability (MTT), n = 3 **B.** Caspase -3/-7 activity, n = 4. **C.** PARP cleavage *** $p < 0.001$ vs. sc. seq., # $p < 0.001$ vs. cisplatin and $ $p < 0.001$ vs. mTOR, n = 3 (corresponding representative blot above). **D.** LDH release * $p < 0.05$, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Sc. seq. = scrambled siRNA sequence/control, AU = arbitrary units.

Cell viability (MTT) of CaSki cells was not altered significantly in any of the treatment groups (Fig. 6.2.15. A), similarly, caspase -3/-7 activity did not change significantly (Fig. 6.2.15. B). Intracellular levels of cleaved PARP significantly increased in cells that were silenced for mTOR and subsequently treated with cisplatin (6.42 ± 0.69 vs. sc. seq. *** $p < 0.001$; 6.42 ± 0.69 vs. cisplatin # $p < 0.001$; 6.42 ± 0.69 vs. mTOR $ $p < 0.001$, Fig. 6.2.15. C). CaSki cells treated with cisplatin exhibited a significant increase in LDH release when compared to the control (18.12 ± 3.12% vs. sc.seq. * $p < 0.05$, Fig. 6.2.15 D).

6.2.3. Pharmacological inhibition of autophagy in combination with cisplatin treatment: Effects on autophagy and apoptosis.

a. The effect of bafilomycin (inhibition of autophagy) and cisplatin on autophagy in Ect1/E6E7 cells.



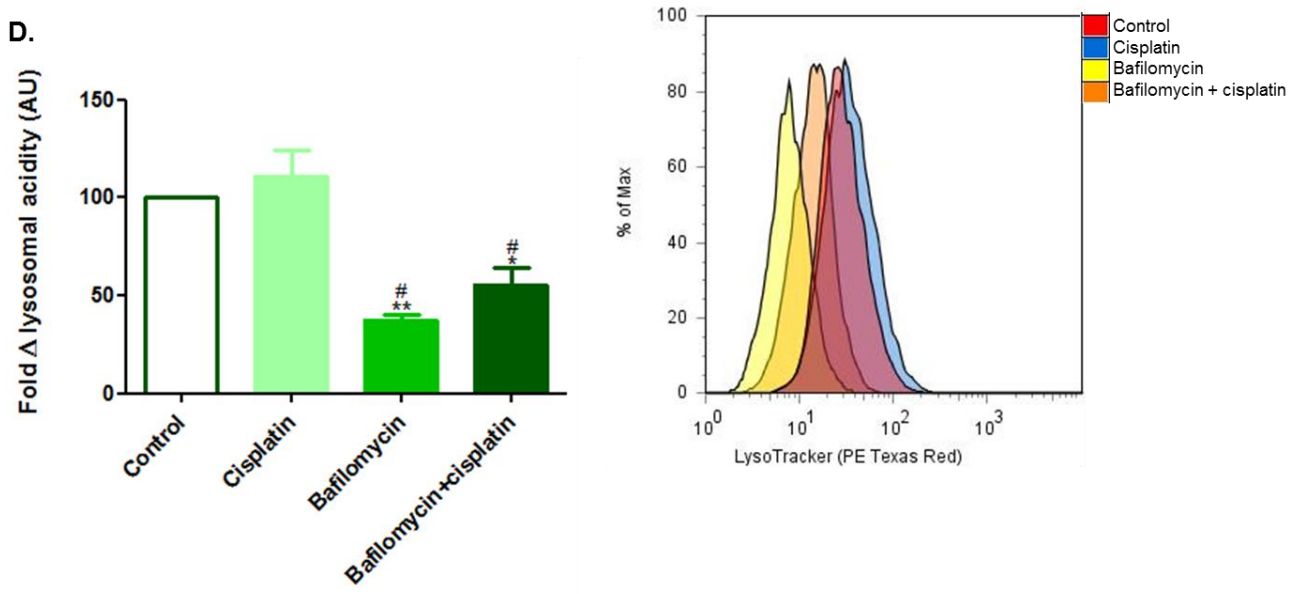
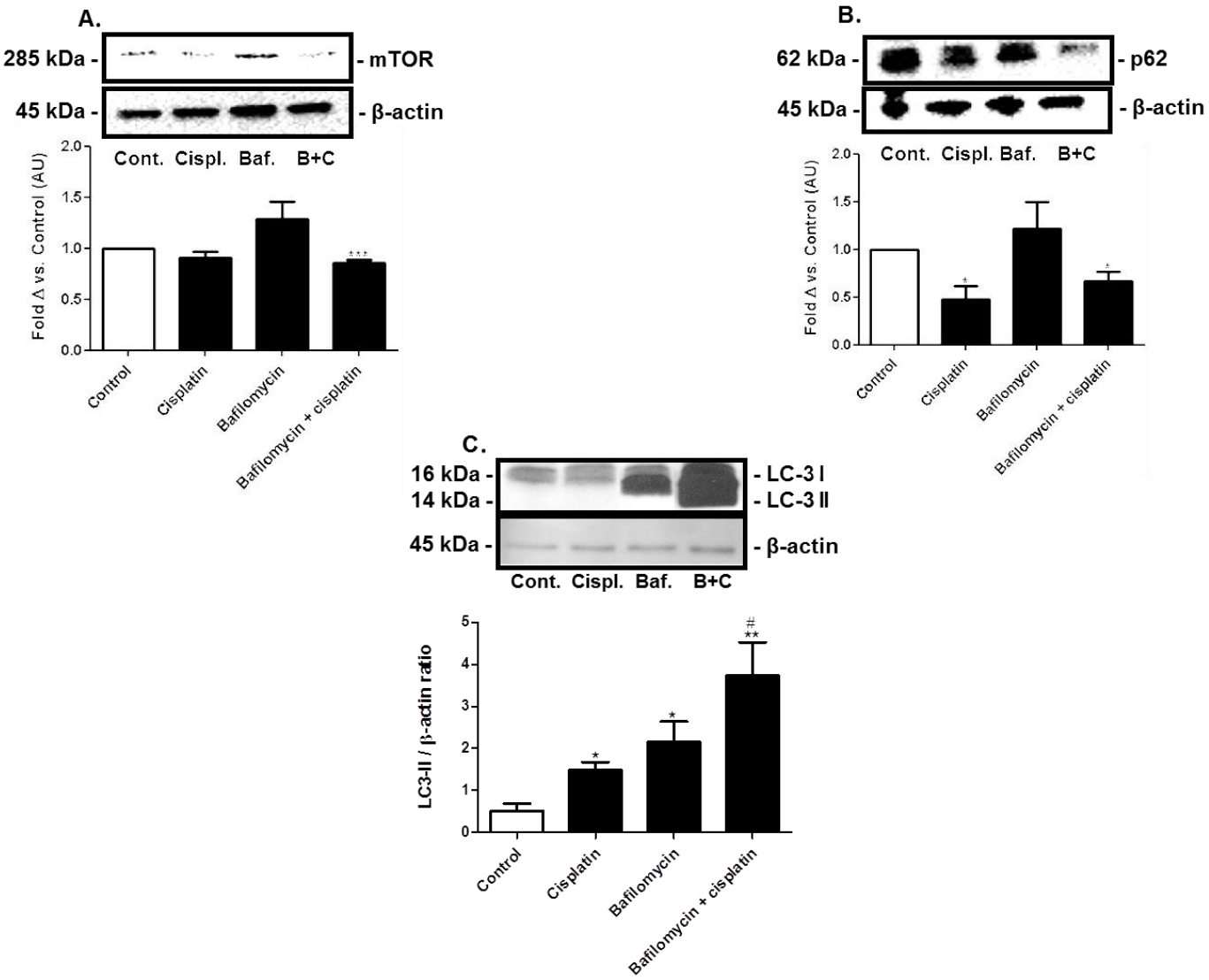


Figure 6.2.16. The effect of bafilomycin and cisplatin on autophagy in Ect1/E6E7 cells. A. mTOR protein expression, *** $p < 0.001$ vs. Control, n = 3. **B.** p62 degradation, * $p < 0.05$, ** $p < 0.01$ vs. Control, # $p < 0.01$ vs. cisplatin and $ $p< 0.05$ vs. bafilomycin, n = 3 (Representative blot). **C.** LC-3 II protein levels, * $p < 0.05$, *** $p < 0.001$ vs. Control and # $p< 0.05$ vs. cisplatin, n = 4. Corresponding densitometry below each representative Western blot. **E.** Lysosomal acidic compartment determined through flow cytometry, * $p < 0.05$, ** $p < 0.01$ vs. Control and cisplatin # $p < 0.01$ vs. cisplatin, n = 4. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Cont.-control, Cispl.-cisplatin, Baf.-Bafilomycin A1, B+C.- Bafilomycin A1 + cisplatin, AU- Arbitrary units.

Intracellular levels of mTOR decreased significantly in response to cisplatin treatment in Ect1/E6E7 cells when compared to the control (0.80 ± 0.02 vs. control, *** p < 0.001, Fig. 6.2.16. A). p62 protein levels in Ect1/E6E7 cells decreased significantly when treated with cisplatin and the combination of cisplatin and bafilomycin when compared to the control (0.62 ± 0.05, ** p < 0.01 and 0.79 ± 0.01, * p < 0.05 vs. control, Fig. 6.2.16 B). Additionally, p62 was significantly increased in cells treated with bafilomycin when compared to the cisplatin group (1.01± 0.03 vs. cisplatin, # p < 0.01), as well as between the bafilomycin and combination group (0.79 ± 0.05 vs. bafilomycin, $ p < 0.05, Fig. 6.2.16. B). LC-3 II protein levels increased significantly in the bafilomycin and combination groups (2.93 ± 0.47, * p < 0.05 and 4.93 ± 1.00 *** p < 0.001 vs. control and 4.93 ± 1.00 vs. cisplatin # p < 0.05, Fig. 6.2.16. C.). Ect1/E6E7 cells treated with bafilomycin and the combination of bafilomycin + cisplatin exhibited significant decreases in mean lysosomal acidic compartmentalisation when compared to the control (55.35 ± 8.48, * p < 0.05, 37.5 ± 2.89, ** p < 0.01 vs. control and 55.35 ± 8.48, * p< 0.05, 37.5 ± 2.89 vs. cisplatin # p< 0.01, n = 4, Fig. 6.2.16. D.).

b. The effect of bafilomycin and cisplatin on autophagy in HeLa cells.



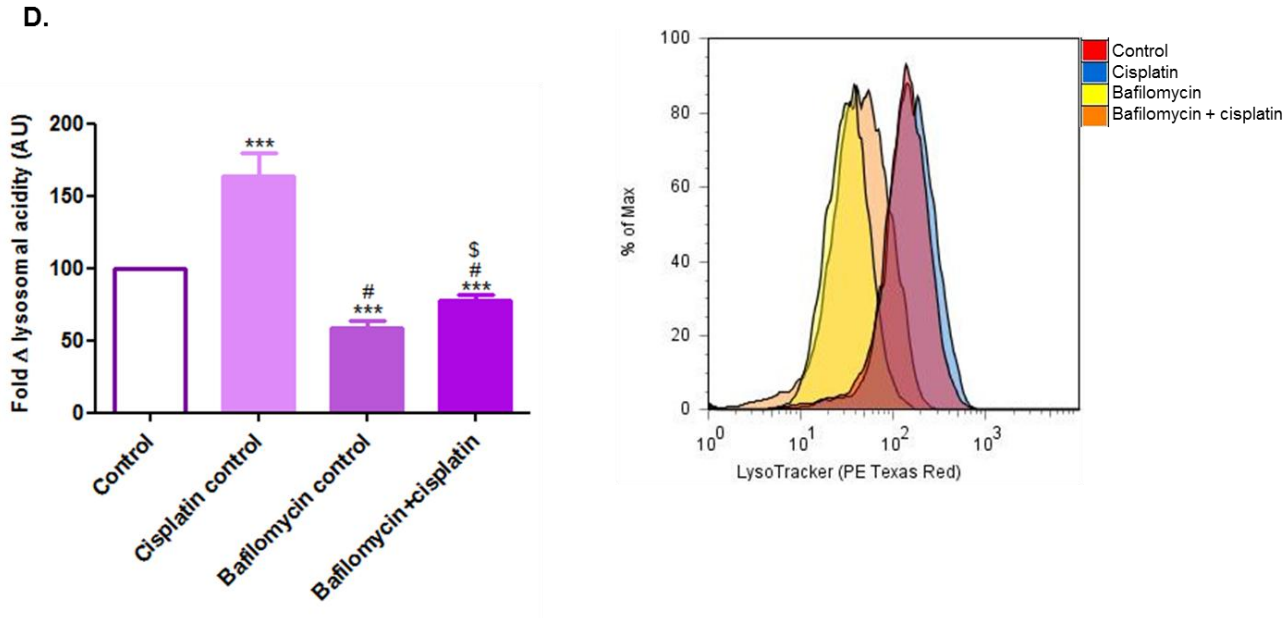
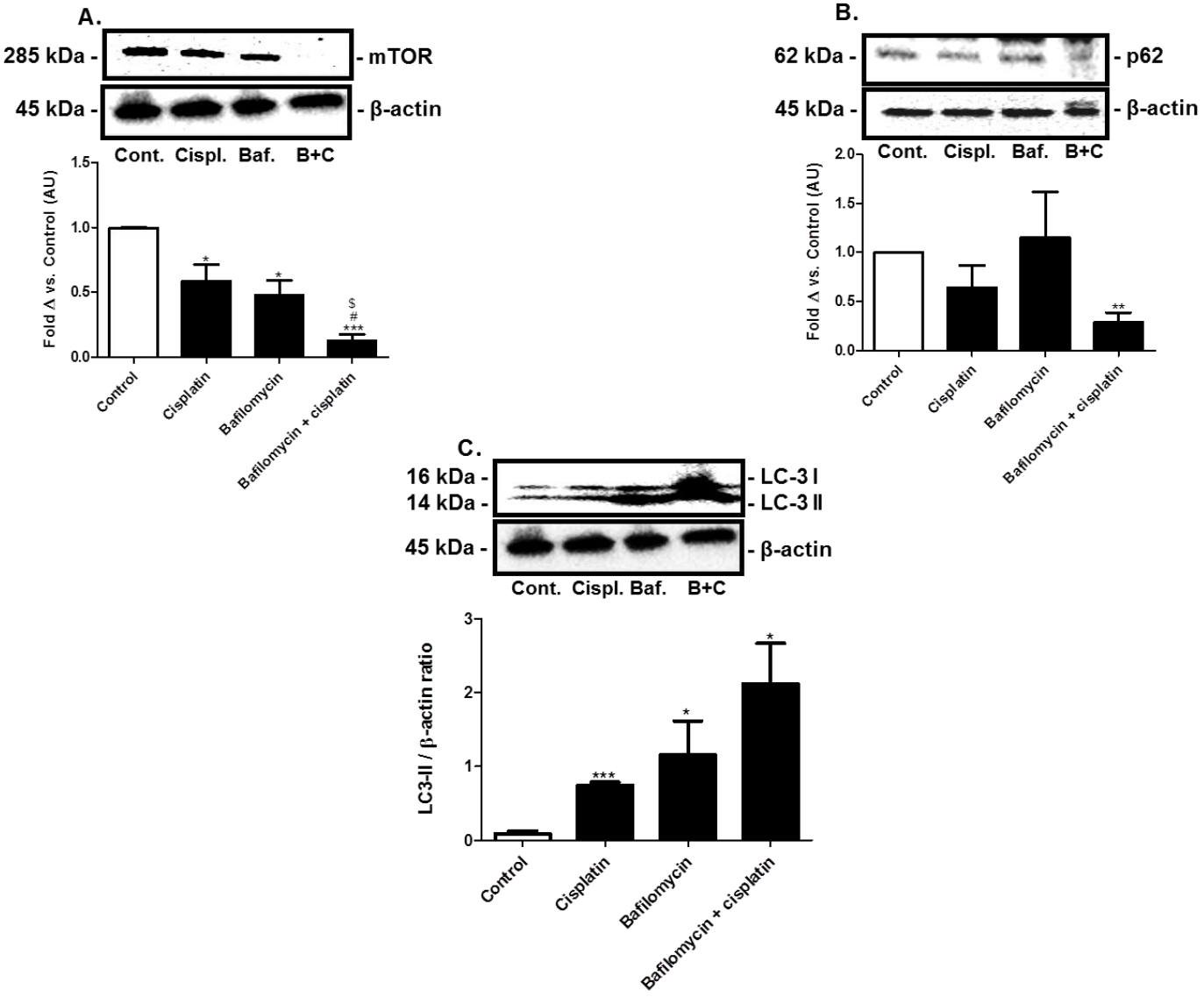


Figure 6.2.17. The effect of bafilomycin and cisplatin on autophagy in HeLa cells. **A.** mTOR protein expression, *** $p < 0.001$ vs. Control, n = 3. **B.** p62 degradation, * $p < 0.05$ vs. Control, n = 3. **C.** LC-3 II expression levels, * $p < 0.05$, *** $p < 0.001$ vs. Control and # $p < 0.01$ vs. cisplatin, n = 5. Corresponding densitometry below each representative Western blot. **D.** Lysosomal acidic compartmentalisation determined through flow cytometry, *** $p < 0.01$ vs. Control and cisplatin # $p < 0.001$ vs. cisplatin, $ $p < 0.05$ vs. bafilomycin. n = 5. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Cont.-control, Cispl.-cisplatin, Baf.- Bafilomycin A1, B+C.- Bafilomycin A1 + cisplatin, AU- Arbitrary units.

Intracellular protein levels of mTOR exhibited a significant decrease in HeLa cells treated with bafilomycin and cisplatin (0.86 ± 0.03 vs. control, *** $p < 0.001$, Fig. 6.2.17. A). p62 protein levels decreased in a similar fashion, however HeLa cells treated with cisplatin also exhibited a significant decrease in p62 when compared to the control (cisplatin: 0.47 ± 0.14 vs. control, * $p < 0.05$ and bafilomycin + cisplatin: 0.67 ± 0.1 vs. control * $p < 0.05$, Fig. 6.2.17. B). LC-3 II signal increased significantly in response to bafilomycin as well as the combination of bafilomycin + cisplatin (2.15 ± 0.49 vs. control * $p < 0.05$, 3.73 ± 0.80 vs. control ** $p < 0.01$ and 3.73 ± 0.80 vs. cisplatin # $p < 0.01$, Fig. 6.2.17. C). HeLa cells treated with cisplatin exhibited a significant increase mean fluorescence intensity of the lysosomal acidic compartment in comparison to the control (163.8 ± 15.67%, *** $p < 0.001$ vs. cisplatin) and cells treated with bafilomycin and the combination of bafilomycin + cisplatin exhibited a significant decreasein mean fluorescence intensity (58.82 ± 5.30% and 77.70 ± 4.20%, *** $p < 0.001$ vs. control; 58.82 ± 5.30% and 77.70 ± 4.20% vs. cisplatin # $p < 0.001$ and 77.70 ± 4.20%, $ $p < 0.05$, n = 4, Fig. 6.2.17. D).

c. The effect of bafilomycin and cisplatin on autophagy in CaSki cells.



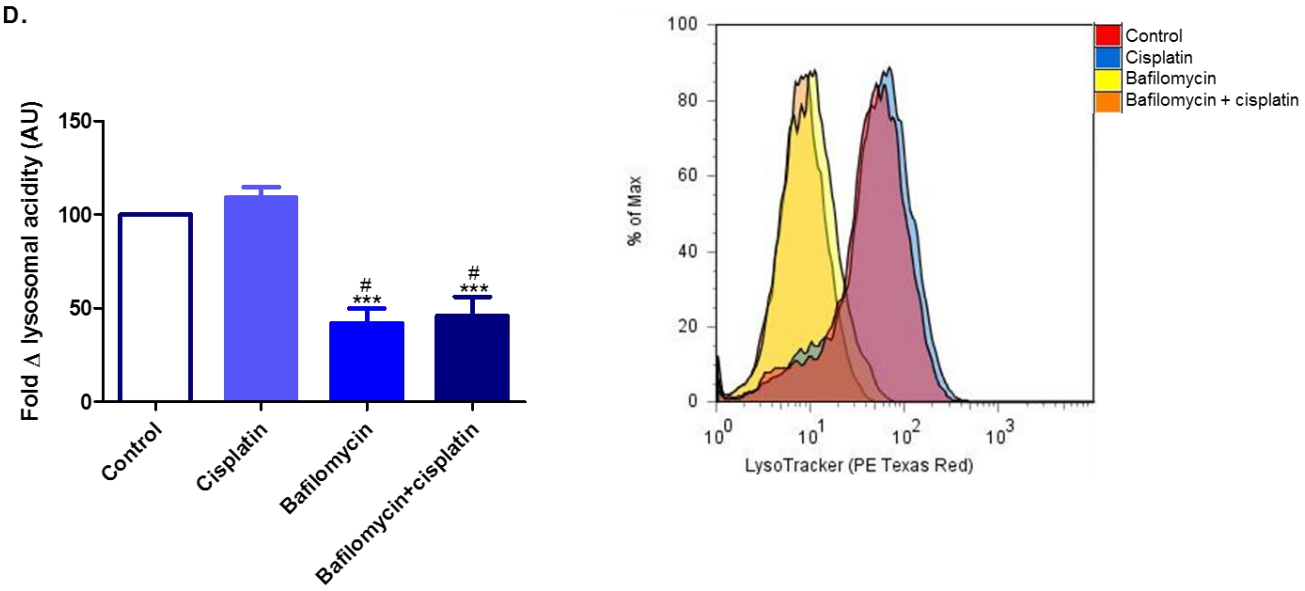
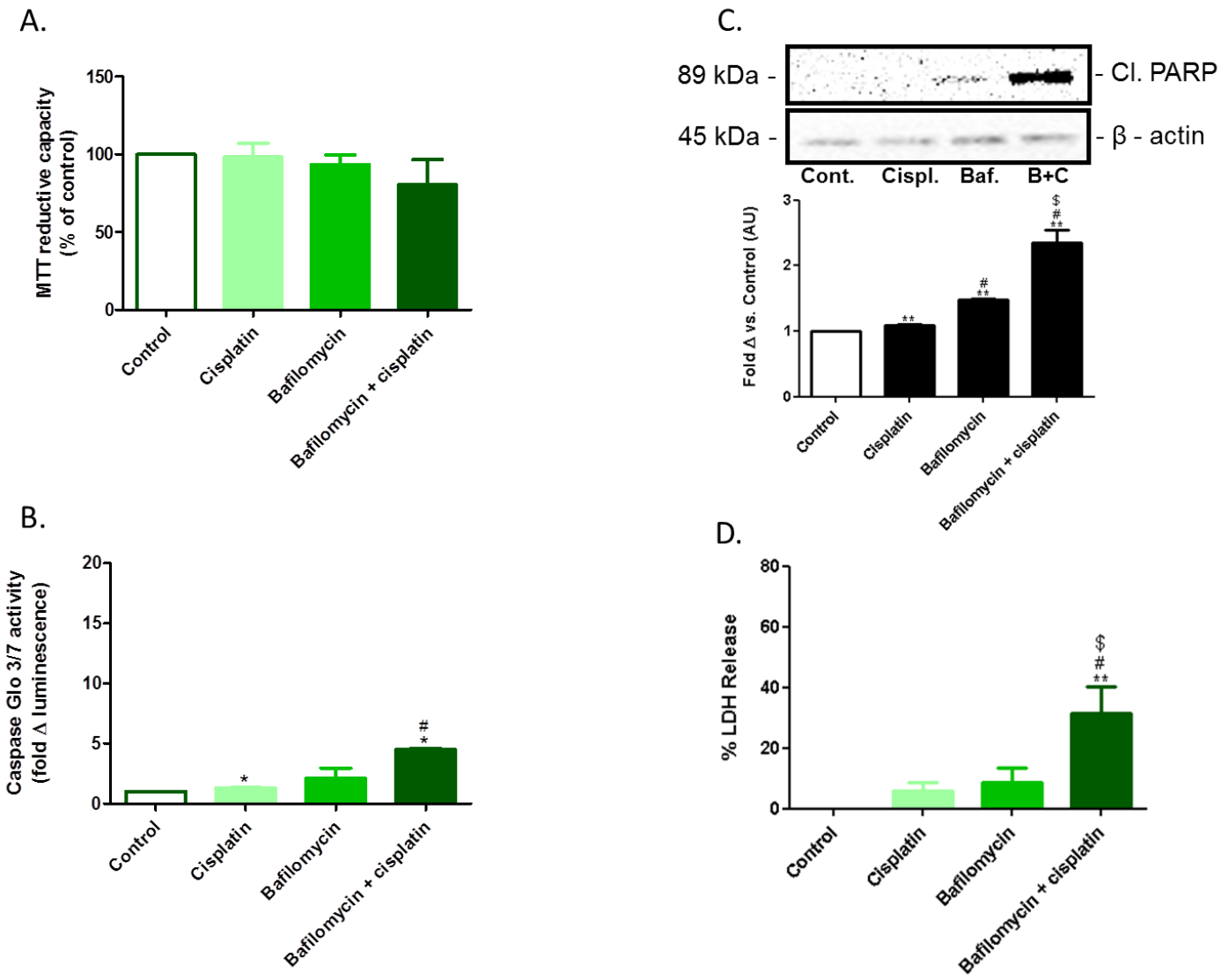


Figure 6.2.18. The effect of bafilomycin and cisplatin on autophagy in CaSki cells. **A.** mTOR protein expression, * $p < 0.05$ and *** $p < 0.001$ vs. control, # $p < 0.05$ vs. cisplatin and $ $p < 0.05$ vs. bafilomycin, n = 3. **B.** p62 degradation, ** $p < 0.01$ vs. control, n = 3. **C.** LC-3 II expression levels * $p < 0.05$, *** $p < 0.001$ vs. control, n = 4. Corresponding densitometry below each representative Western blot. **D.** Lysosomal acidic compartmentalisation determined through flow cytometry, *** $p < 0.001$ vs. Control and cisplatin # $p < 0.001$ vs. cisplatin, n = 6. . Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Cont.-control, Cispl.-cisplatin, Bafi.- Bafilomycin A1, B+C.- Bafilomycin A1 + cisplatin, AU- Arbitrary units.

Intracellular levels of mTOR decreased significantly in response to cisplatin, bafilomycin and the combination treatment in CaSki cells when compared to the control (0.58 ± 0.13, 0.48 ± 0.11 and 0.13 ± 0.05 vs. control, * $p < 0.05$, *** $p < 0.001$, Fig. 6.2.18. A.). The combination group also exhibited a significant decrease in mTOR protein expression when compared to the cisplatin (0.13 ± 0.05 vs. cisplatin, # $p < 0.05$) and bafilomycin groups (0.13 ± 0.05 vs. bafilomycin, $ $p < 0.05$.). p62 protein levels in CaSki cells decreased significantly only when treated with the combination of cisplatin and bafilomycin in comparison to the control (0.29 ± 0.1, ** $p < 0.01$ vs. control, Fig. 6.2.18. B.). LC-3 II protein levels increased significantly in the cisplatin, bafilomycin and combination groups (0.74 ± 0.05, 1.16 ± 0.46 and 2.12 ± 0.55 * $p < 0.05$ vs. control, Fig. 6.2.18. C.). CaSki cells treated with bafilomycin and the combination of bafilomycin + cisplatin exhibited a significant decrease in mean fluorescence intensity indicating the lysosomal acidic compartment when compared to the control (41.88 ± 8.00% and 45.92 ± 10.19%, *** $p < 0.001$ vs. control and 41.88 ± 8.00% and 45.92 ± 10.19% vs. cisplatin # $p < 0.001$, n = 6 (Fig. 6.2.18. D).

d. The effects of bafilomycin and cisplatin on apoptosis in Ect1/E6E7 cells.



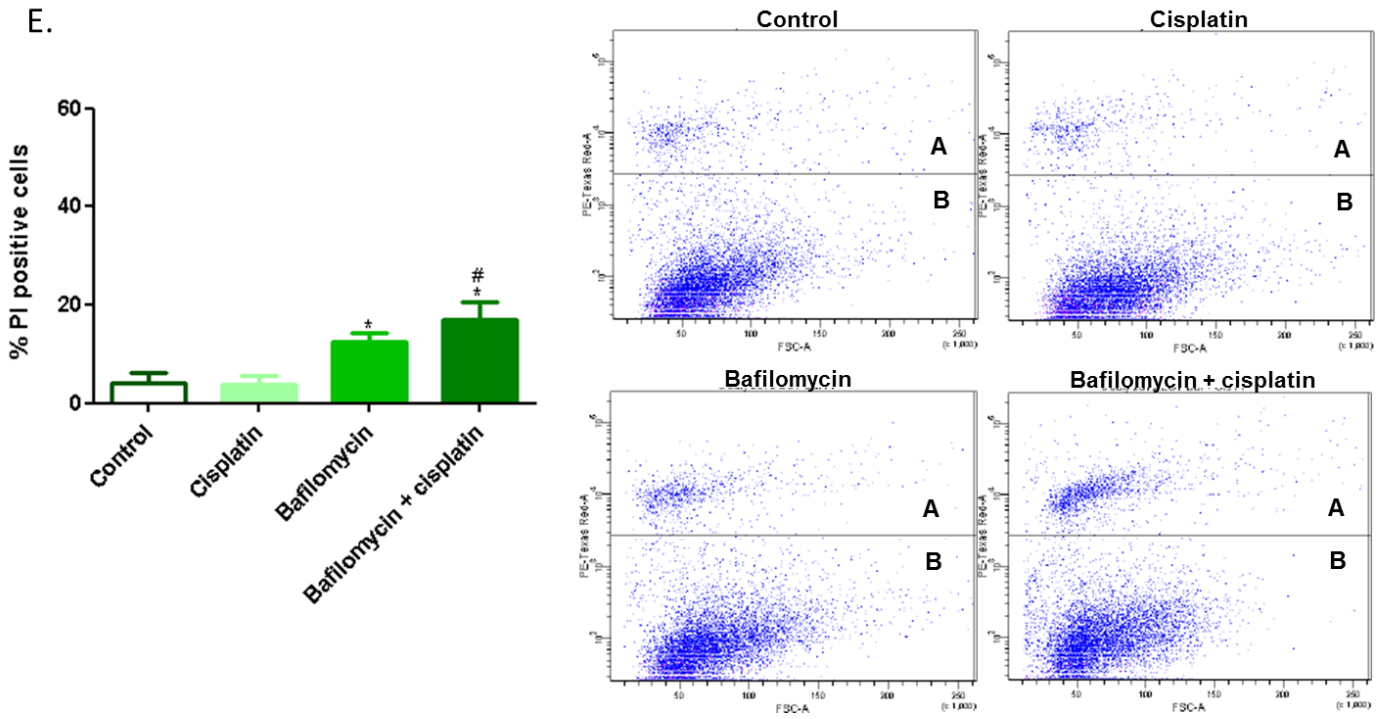
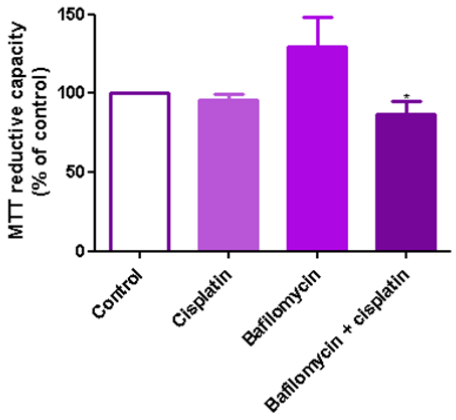


Figure 6.2.19. **Effect of the inhibition of autophagy on apoptosis in Ect1/E6E7 cells**. **A.** Cell viability (MTT), n = 5. **B.** Caspase -3 and -7 activity, * $p < 0.05$ vs. control and # $p < 0.05$ vs. cisplatin, n = 3. **C.** PARP cleavage, *** $p < 0.001$ vs. control, # $p < 0.001$ vs. cisplatin, and $ $p < 0.001$ vs. rapamycin, n = 3. **D.** LDH release, n = 5. **E.** Propidium iodide inclusion (flow cytometry), PI positive: quadrant A, PI negative: quadrant B, * $p < 0.05$ vs. control and # $p < 0.05$ vs. cisplatin, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Cont.-control.Cont.-control, Cispl.-cisplatin, Baf..- bafilomycin A1, R+C.- Bafilomycin A1 + cisplatin, AU- Arbitrary units.

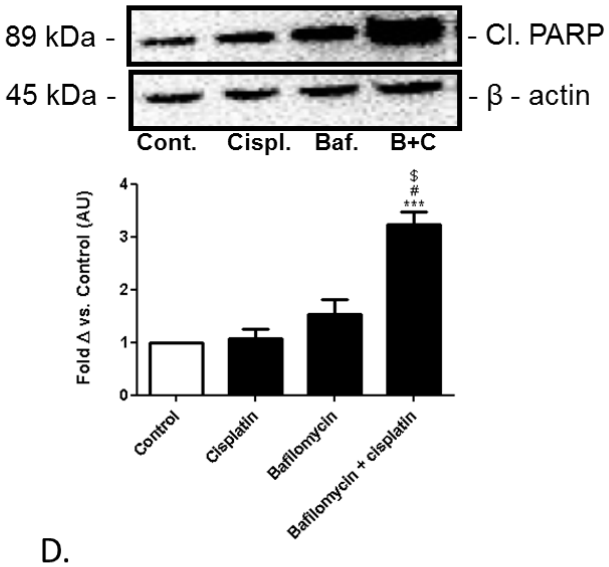
Ect1/E6E7 cells did not display any significant differences in the MTT reductive capacity in any of the treatment groups (Fig. 6.2.19.A.). Caspase -3 and -7 activity was significantly increased in the cisplatin and combination groups (1.28 ± 0.04 and 4.49 ± 0.09 vs. control * $p < 0.05$; 4.49 ± 0.09 vs. cisplatin, # $p < 0.05$, Fig. 6.2.19.B). Cleaved PARP protein levels in Ect1/E6E7 cells increased in cells treated with cisplatin, bafilomycin and the combination of bafilomycin and cisplatin (1.10 ± 0.01, 1.48 ± 0.02 and 2.35 ± 0.21 vs. control, ** $p < 0.01$, 1.48 ± 0.02 and 2.35 ± 0.21 vs. cisplatin, # $p < 0.01$ and 2.35 ± 0.21 vs. bafilomycin, $ $p < 0.05$, Fig. 6.2.19.C). A significant increase in LDH release was observed in Ect1/E6E7 cells treated with bafilomycin + cisplatin (31.40 ± 9.05% vs. control, ** $p < 0.01$, 31.40 ± 9.05% vs. cisplatin # $p < 0.05$ and 31.40 ± 9.05% vs. bafilomycin, $ $p < 0.05$, Fig. 6.2.19.D). A significant increase in PI positive cells was observed in the bafilomycin and combination groups (12.55 ± 1.75% and 16.92 ± 3.76% vs. control, * $p < 0.05$; 16.92 ± 3.76% vs. cisplatin, # $p < 0.05$, Fig. 6.2.19.E).

e. The effects of bafilomycin and cisplatin on apoptosis in HeLa cells.

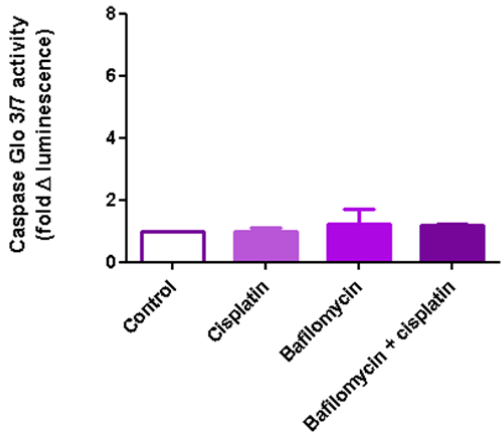
A.



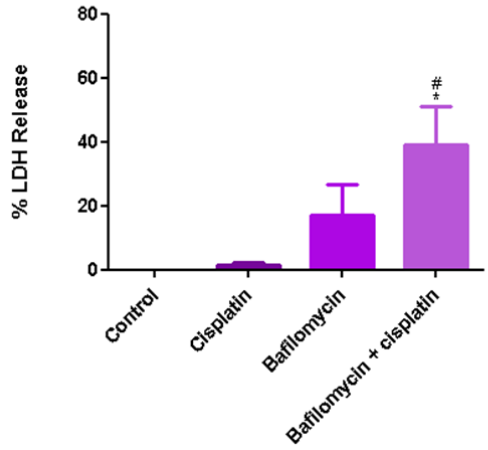
C.



B.



D.



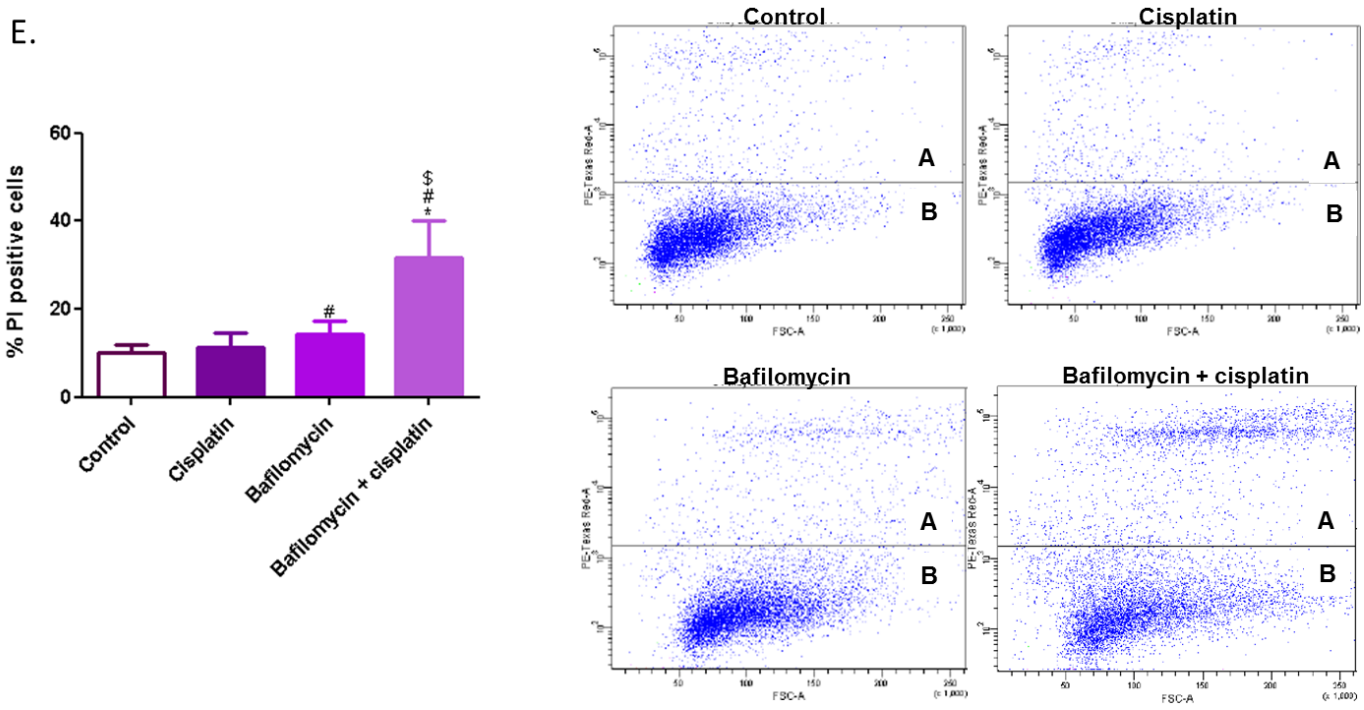
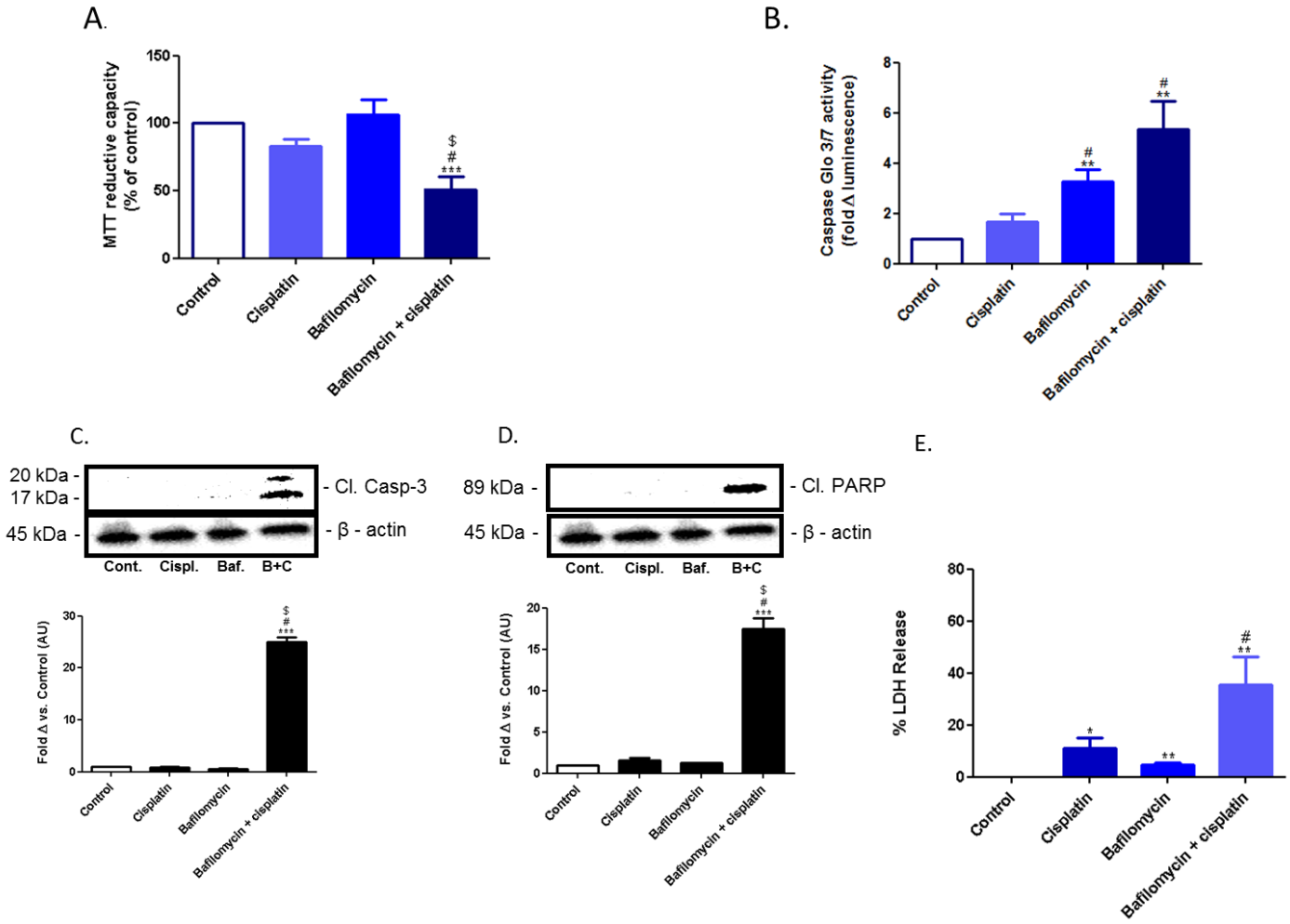


Figure 6.2.20. Effect of the inhibition of autophagy on apoptosis in HeLa cells. **A.** Cell viability (MTT) **p*<0.05 vs. bafilomycin, n = 8. **B.** Caspase -3 and -7 activity, n = 3. **C.** PARP cleavage, *** $p < 0.001$ vs. control, #$p < 0.001$ vs. cisplatin and \$ $p< 0.01$ vs. bafilomycin, n = 4. **D.** LDH release, * $p < 0.05$ vs. control and # $p < 0.05$ vs. cisplatin, n = 5. **E. .** Propidium iodide inclusion (flow cytometry), PI positive: quadrant A, PI negative: quadrant B, * $p< 0.05$ vs. control, # $p< 0.05$ vs. cisplatin and \$ $p < 0.05$ vs. bafilomycin, n = 4. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Cont.-control, Cispl.-cisplatin, Rapa.- Bafilomycin A1, B+C.- Bafilomycin A1 + cisplatin, AU- Arbitrary units.

Cell viability (MTT) was significantly decreased in the combination treatment group when compared to the bafilomycin group (86.54 ± 8.15% vs. bafilomycin, * $p < 0.05$, Fig. 6.2.20 A). The analysis of the presence of active caspase -3 and -7 did not reveal any significant changes in their activity in any of the treatment groups (Fig. 6.2.20. B). Cleaved PARP levels increased significantly in HeLa cells treated with the combination of bafilomycin and cisplatin (3.23 ± 0.23 vs. control, ***$p<0.001$, 3.23 ± 0.23 # $p < 0.001$ vs. cisplatin and 3.23 ± 0.23, $ $p < 0.01$ vs. bafilomycin, Fig.6.2.20.D). HeLa cells treated with bafilomycin + cisplatin exhibited a significant increase in LDH release (39.20 ± 11.78%, vs. control, ** $p < 0.05$ and 39.20% ± 11.78 vs. cisplatin, # $p < 0.05$, Fig. 6.2.20.E). A significant increase in the percentage of PI positive cells was observed in the combination treatment group (20.00 ± 3.81% vs. control, * $p < 0.05$; 20.00 ± 3.81% vs. cisplatin, # $p < 0.05$ and 20.00 ± 3.81% vs. bafilomycin, $ $p < 0.05$).

f. The effects of bafilomycin and cisplatin on apoptosis in CaSki cells.



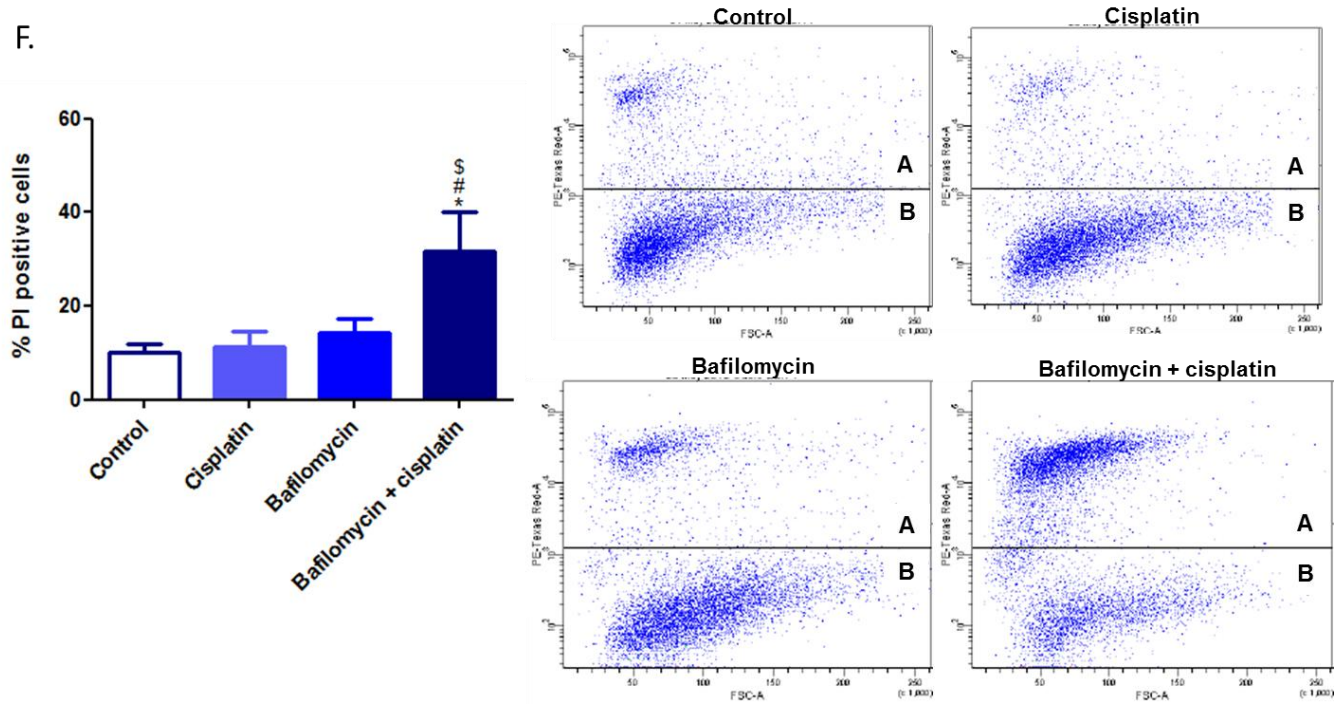


Figure 6.2.21. Effect of the inhibition of autophagy on apoptosis in CaSki cells. **A.** Cell viability (MTT), ****p*<0.05 vs. control, * *p* < 0.05 vs. cisplatin and $ *p* < 0.001 vs. bafilomycin, n = 8. **B.** Caspase -3 and -7 activity, ** *p* < 0.01 vs. control and # *p* < 0.05 vs. cisplatin, n = 3. **C.** Cleaved caspase-3, *** *p* < 0.001 vs. control, #*p*<0.001 vs. cisplatin and $ *p* < 0.001 vs. bafilomycin, n = 4. **D.** Cleaved PARP, *** *p* < 0.001 vs. control, #*p*<0.001 vs. cisplatin and $ *p* < 0.001 vs. bafilomycin, n = 4. **E.** LDH release, * *p* < 0.05, ** *p* < 0.01 vs. control and # *p* <0.05 vs. bafilomycin, n = 5. **F**. Propidium iodide inclusion (flow cytometry), PI positive: quadrant A, PI negative: quadrant B, * *p* < 0.05 vs. control, # *p* < 0.05 vs. cisplatin and $ *p* < 0.05 vs. bafilomycin, n = 4. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Cont.-control, Cispl.-cisplatin, Baf.- bafilomycin A1, B+C.- Bafilomycin A1 + cisplatin, AU- Arbitrary units.

Cell viabilty (MTT) significantly decreased in the combination treatment group in comparison to the control group (51.07 ± 9.36% vs. control, ***p < 0.001), cisplatin group (51.07 ± 9.36% vs. cisplatin, # p < 0.05) and the bafilomycin group (51.04 ± 9.36% vs. bafilomycin, *** p < 0.001, Fig. 6.2.21.A). Active caspase -3 and -7 revealed a significant increase in activity in the bafilomycin (3.27 ± 0.46 vs. control, ** p < 0.01 and 3.27 ± 0.46 vs. cisplatin, # p < 0.05) and combination group (5.36 ± 1.12 vs. control, ** p < 0.01 and 5.36 ± 0.46 vs. cisplatin, # p < 0.05, Fig. 6.2.21.B). Western blot analysis of cleaved caspase-3 indicated significantly increased levels in CaSki cells treated with combination of bafilomycin and cisplatin when compared to the control (24.97 ± 0.99 vs. control, *** p < 0.001), cisplatin (24.97 ± 0.99 vs. cisplatin, # p < 0.001) and bafilomycin groups (24.97 ± 0.99 vs. bafilomycin, $ p < 0.001, Fig. 6.2.21.C). Cleaved PARP levels increased significantly in HeLa cells treated with the combination of bafilomycin and cisplatin (17.42 ± 1.31 vs. control, *** p < 0.001, 17.42 ± 1.31 # p < 0.001 vs. cisplatin and 17.42 ± 1.31, $ p < 0.001 vs. bafilomycin, Fig. 6.2.21.D.). LDH release in CaSki cells significantly increased in comparison to the control (11.08 ± 4.18% * p < 0.05 and 4.53 ± 0.76% p < 0.01 vs. control), additionally, cells treated with bafilomycin + cisplatinalso exhibited a significant increase in LDH released in comparison to the control (35.58 ± 10.87%, ** p < 0.001 vs. control) and bafilomycin treatment groups (35.58 ± 10.87% vs. bafilomycin, # p < 0.05, Fig. 6.2.21.E). A significant increase in the percentage of PI positive cells was only observed in the combination treatment group (31.83 ± 8.40 vs. control, * p < 0.05; 31.83 ± 8.40% vs. cisplatin, # p < 0.05 and 31.83 ± 8.40% vs. bafilomycin, $ p < 0.05).

g. Analysis of cell cycle progression modulated by bafilomycin and cisplatin treatment in Ect1/E6E7 cells.

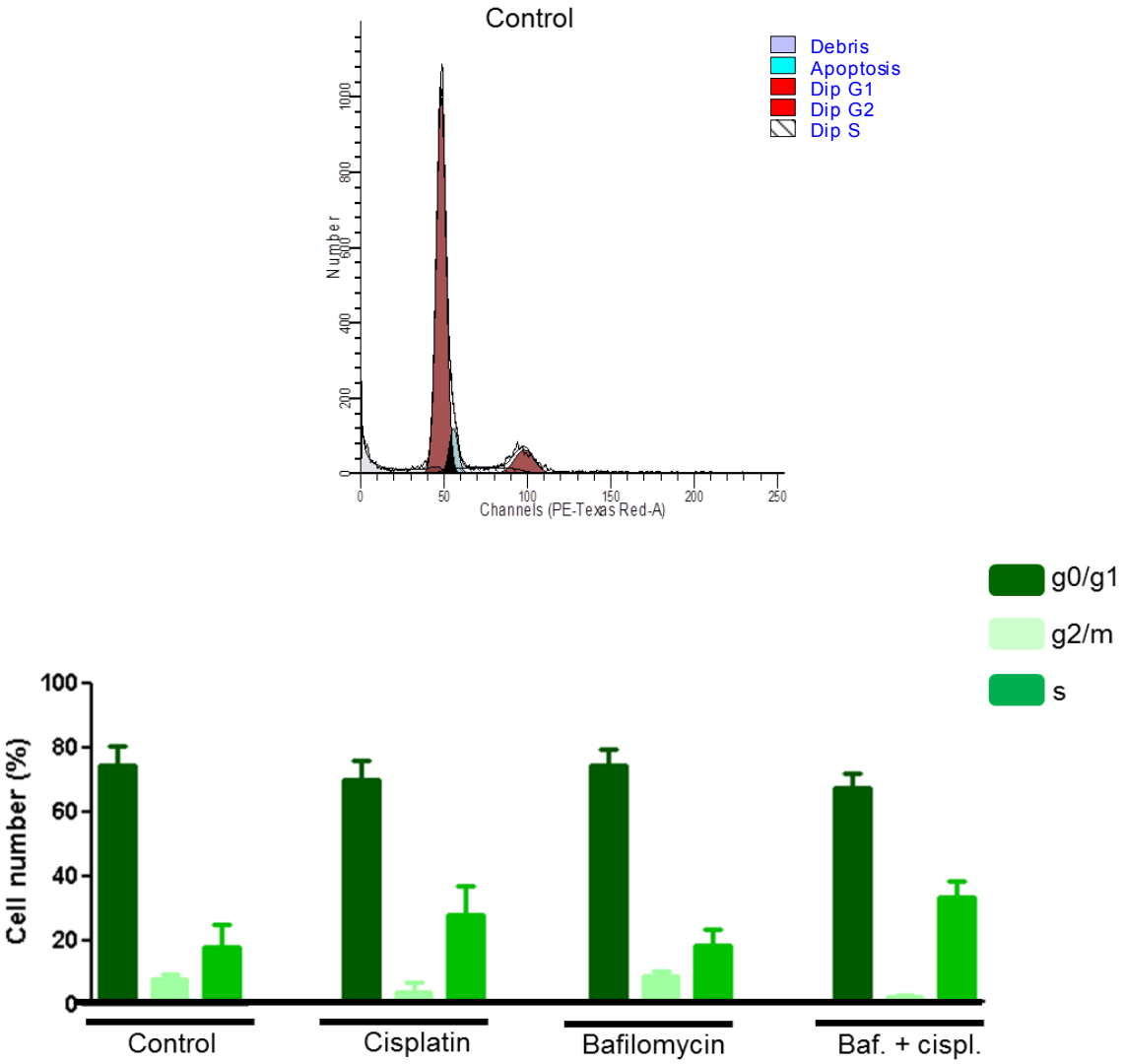


Figure 6.2.22. Modulation of cell cycle progression by bafilomycin and cisplatin in Ect1/E6E7 cells. Cells were either treated with bafilomycin (10 nM) or cisplatin (15 µM) or the combination of the two for 24 h, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Baf.. + cispl. = bafilomycin + cisplatin.

Cell cycle analysis of Ect1/E6E7 cells did not reveal any significant changes in the percentage of cells in the various stages of the cell cycle.

h. Analysis of cell cycle progression modulated by bafilomycin and cisplatin treatment in HeLa cells.

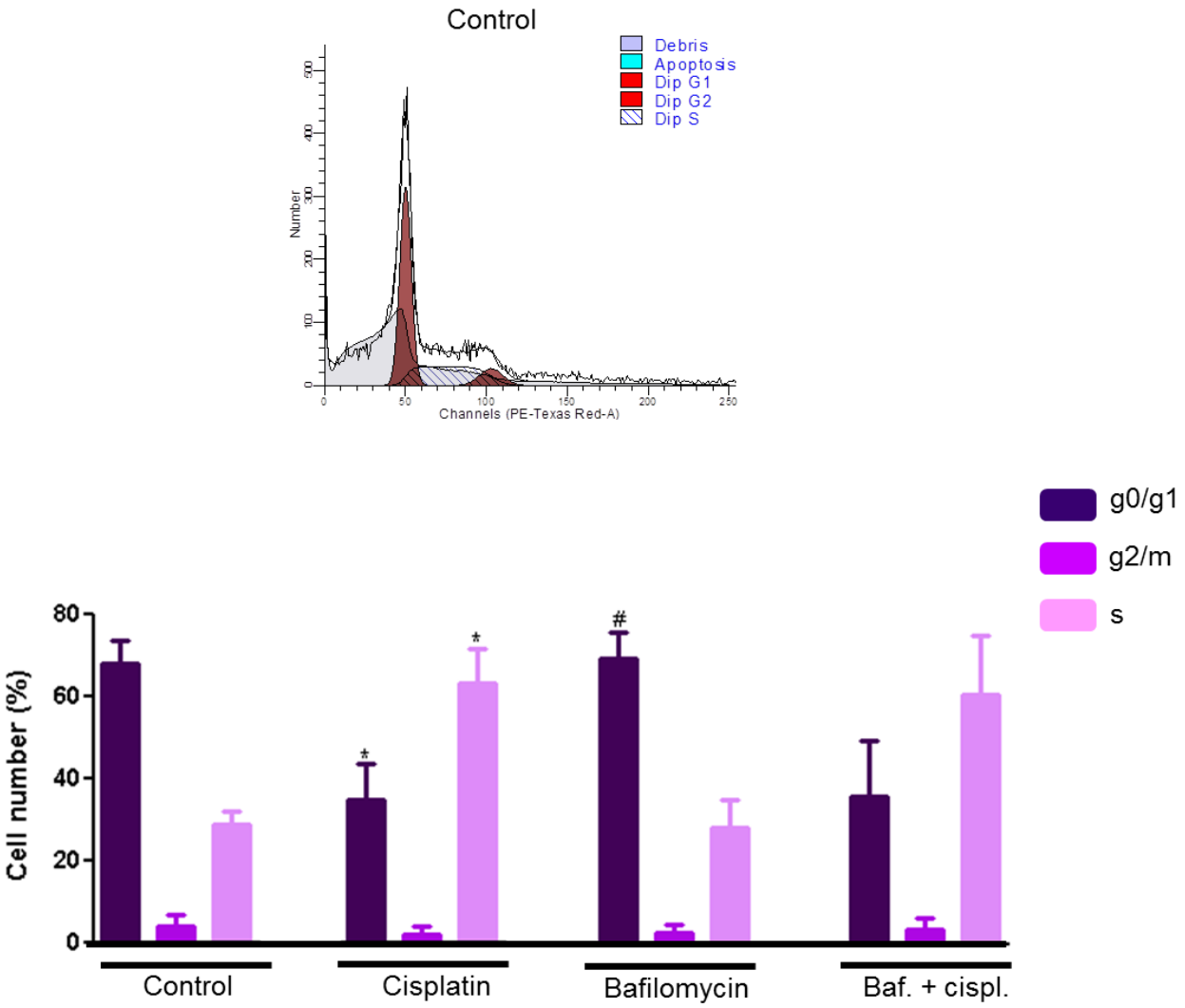


Figure 6.2.23. **Modulation of cell cycle progression by bafilomycin and cisplatin in HeLa cells**. Cells were either treated with bafilomycin (10 nM) or cisplatin (15 µM) or the combination of the two for 24 h, * $p < 0.05$ vs. control G1/G0 and S, # $p < 0.05$ vs. cisplatin G1, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM baf. + cispl. = bafilomycin + cisplatin.

Significant alterations in cell cycle phases were observed with cisplatin treatment in comparison to the control (34.79 ± 8.80% vs. control G1, * $p < 0.05$) and cisplatin treatment in comparison to HeLa cells treated with bafilomycin (69.31 ± 6.50% vs. cisplatin G1, # $p < 0.05$). Cisplatin treated cells also exhibited a shift in the percentage of cells into the S-phase (63.14 ± 8.25% vs. control S, * $p < 0.05$, Fig. 6.2.23.).

i. Analysis of cell cycle progression modulated by bafilomycin and cisplatin treatment in CaSki cells.

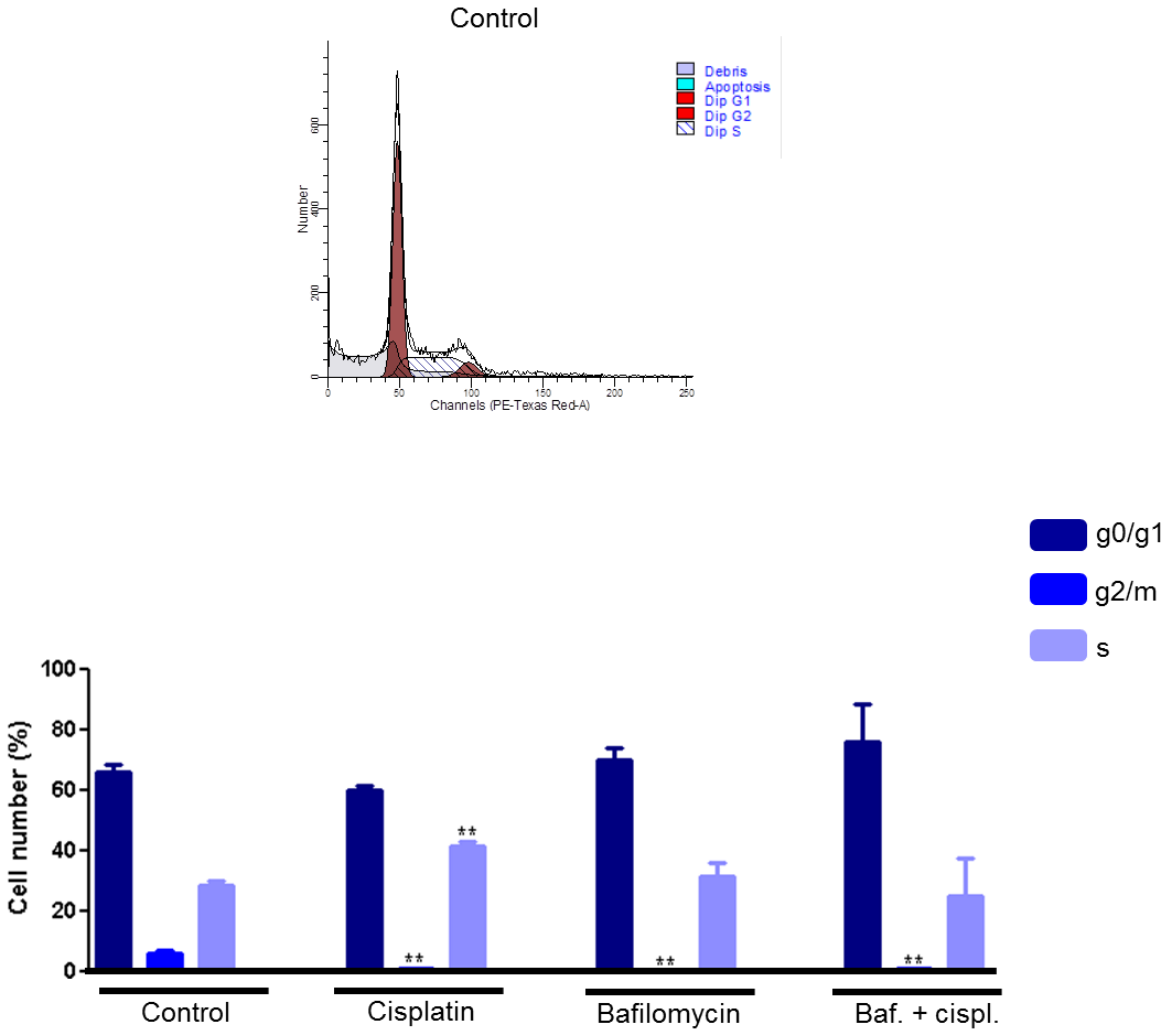


Figure 6.2.24. Modulation of cell cycle progression by bafilomycin and cisplatin in CaSki cells. Cells were either treated with bafilomycin (10 nM) or cisplatin (15 µM) or the combination of the two for 24 h, ** p < 0.01 vs. control S, ** p < 0.01 vs. control G2/M, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM, baf. + cispl. = bafilomycin + cisplatin.

CaSki cells treated with cisplatin exhibited a significant increase in the S-phase (40.66 ± 1.70% vs. control S, ** $p < 0.01$) with a concomitant decrease in the G2/M phase (0.16 ± 0.16% vs. control, $p < 0.01$). Similarly, CaSki cells treated with bafilomycin as well as the combination of bafilomycin and cisplatin both exhibited a significant decrease in the percentage of cells in the G2/M phase (0.00 ± 0.00% and 0.11 ± 0.11% vs. control G2/M, ** $p < 0.01$, Fig. 6.2.24.).

6.2.4. Biological inhibition of autophagy in combination with cisplatin treatment: Effects on autophagy and apoptosis.

a. The effect of ATG5 silencing on autophagy in Ect1/E6E7 cells.

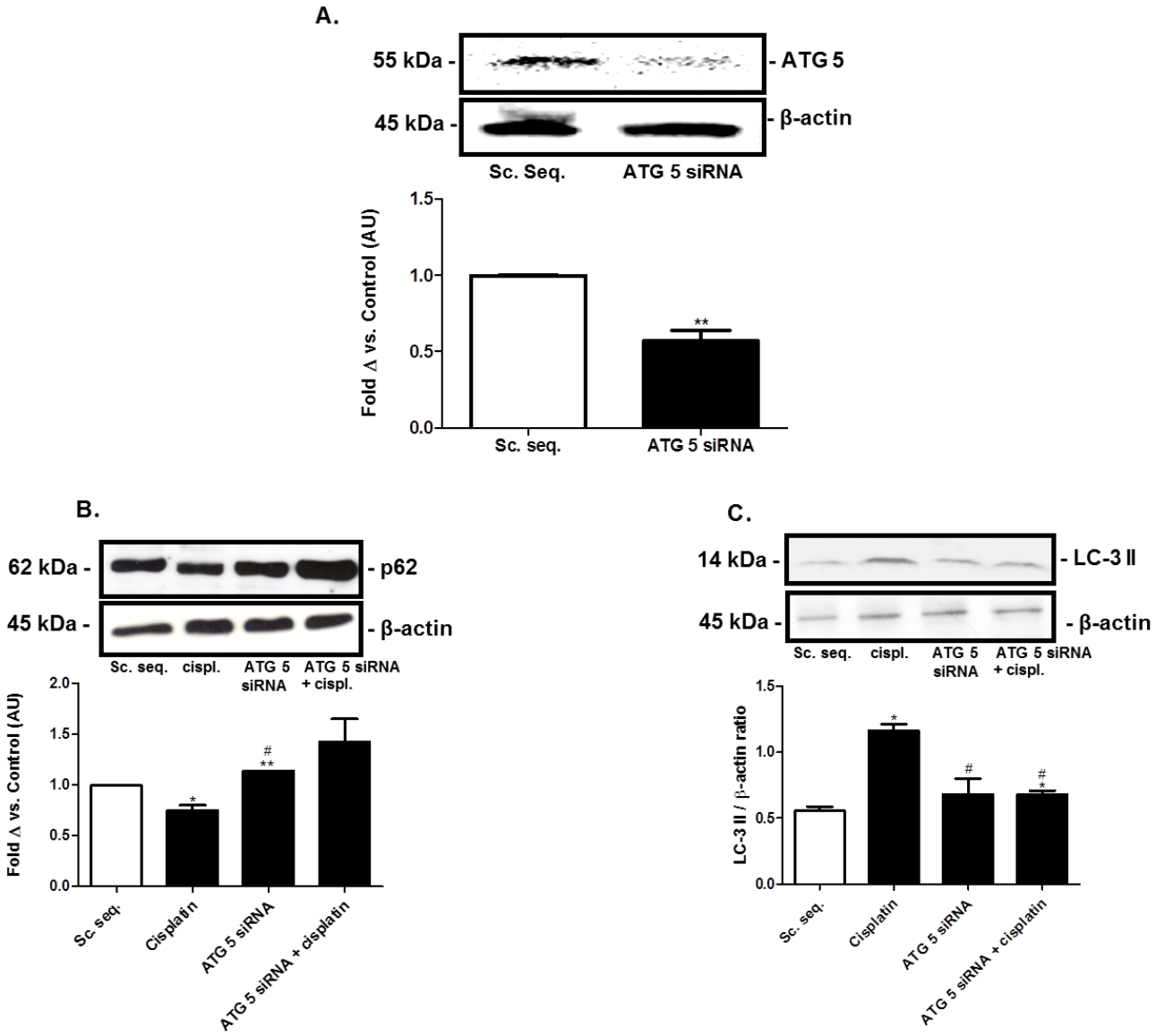


Figure 6.2.25. **Atg5 silencing and the effect on autophagy in Ect1/E6E7 cells**. **A.** Silencing of ATG 5 confirmed through Western blotting ** $p < 0.01$ vs. sc. seq., n = 3. **B.** p62 degradation, * $p < 0.05$ vs. sc. seq., ** $p < 0.01$ vs. sc. seq. and # $p < 0.05$ vs. cisplatin, n = 3. **C.** LC-3 II protein levels, * $p < 0.05$ vs. sc. seq., # $p < 0.05$ vs. cisplatin, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM Sc. seq. = scrambled siRNA sequence/control, cispl. = cisplatin, AU = arbitrary units.

Efficient silencing of ATG was confirmed through Western blotting (0.57 ± 0.07 vs. sc. seq., ** $p < 0.01$, Fig. 6.2.25.A.). Intracellular protein levels of p62 were significantly decreased in cells treated with cisplatin when compared to the control (0.75 ± 0.05 vs. control, * $p < 0.05$) and increased in cells where ATG 5 was silenced (1.14 ± 0.01 vs. control, ** $p < 0.01$ and # $p< 0.05$ vs. cisplatin, Fig. 6.2.25.B.). LC-3 II protein levels increased significantly in cells treated with cisplatin (1.17 ± 0.05 vs. control, * $p < 0.05$) and the combination of silencing with cisplatin treatment (0.68 ± 0.03 vs. control, * $p < 0.05$). Additionally, in both groups where ATG 5 was silenced, LC-3 II protein expression was significantly lower when compared to the cisplatin treated group (0.68 ± 0.12 and 0.68 ± 0.03 vs. cisplatin, # $p < 0.05$, Fig. 6.2.25.C.).

b. The effect of ATG5 silencing on autophagy in HeLa cells.

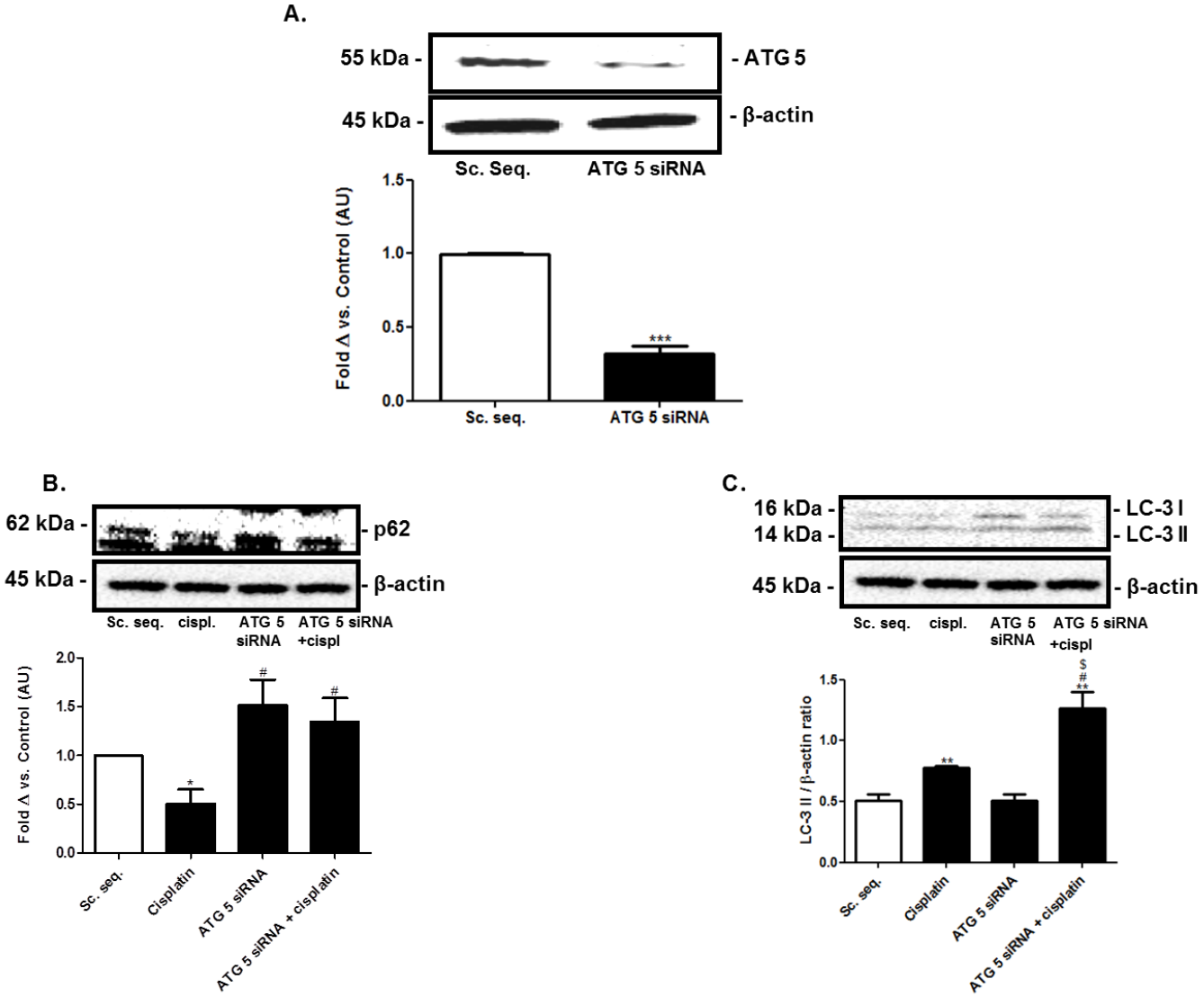


Figure 6.2.26. Atg5 silencing and the effect on autophagy in HeLa cells. **A.** Silencing of ATG 5 confirmed through Western blotting *** $p < 0.001$ vs. sc. seq., n = 3. **B.** p62 degradation, * $p < 0.05$ vs. sc. seq., # $p < 0.05$ vs. cisplatin, n = 3. **C.** LC-3 II protein levels, ** $p < 0.01$ vs. control, # $p < 0.05$ vs. cisplatin and $ $p < 0.05$ vs. ATG 5 siRNA, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM Sc. seq. = scrambled siRNA sequence/control, cispl. = cisplatin, AU = arbitrary units.

Efficient silencing of the ATG 5 protein was confirmed through the Western blotting procedure (0.32 ± 0.06 vs. sc. seq. *** $p < 0.001$, Fig. 6.2.26.A). Assessment of markers of autophagy revealed a significant decrease in p62 intracelluar protein levels after treatment with cisplatin (0.51 ± 0.14 vs. sc. seq., * $p < 0.05$) in comparison to the control cells. Additionally, in comparison to the cisplatin treated HeLa cells, a significant increase in p62 was observed in silenced cells (1.52 ± 0.26 vs. cisplatin, # $p< 0.05$) and within the combination group (1.35 ± 0.24 vs. cisplatin, # $p< 0.05$, Fig. 6.2.26.B). Cisplatin treatment in CaSki cells induced a significant increase in LC-3 II protein expression when compared to the control (0.78 ± 0.02 vs. control, * $p< 0.01$, Fig. 6.2.26.C). Additionally, silencing of ATG5 combined with cisplatin treatment induced a significant increase in LC-3 II protein levels when compared to the control (1.27 ± 0.13 vs. control, ** $p < 0.01$), cisplatin (1.27 ± 0.13 vs. cisplatin, # $p < 0.05$) and ATG 5 (1.27 ± 0.13 vs. ATG 5 siRNA, $ $p < 0.05$, Fig. 6.2.26.C).

c. The effect of ATG5 silencing on autophagy in CaSki cells.

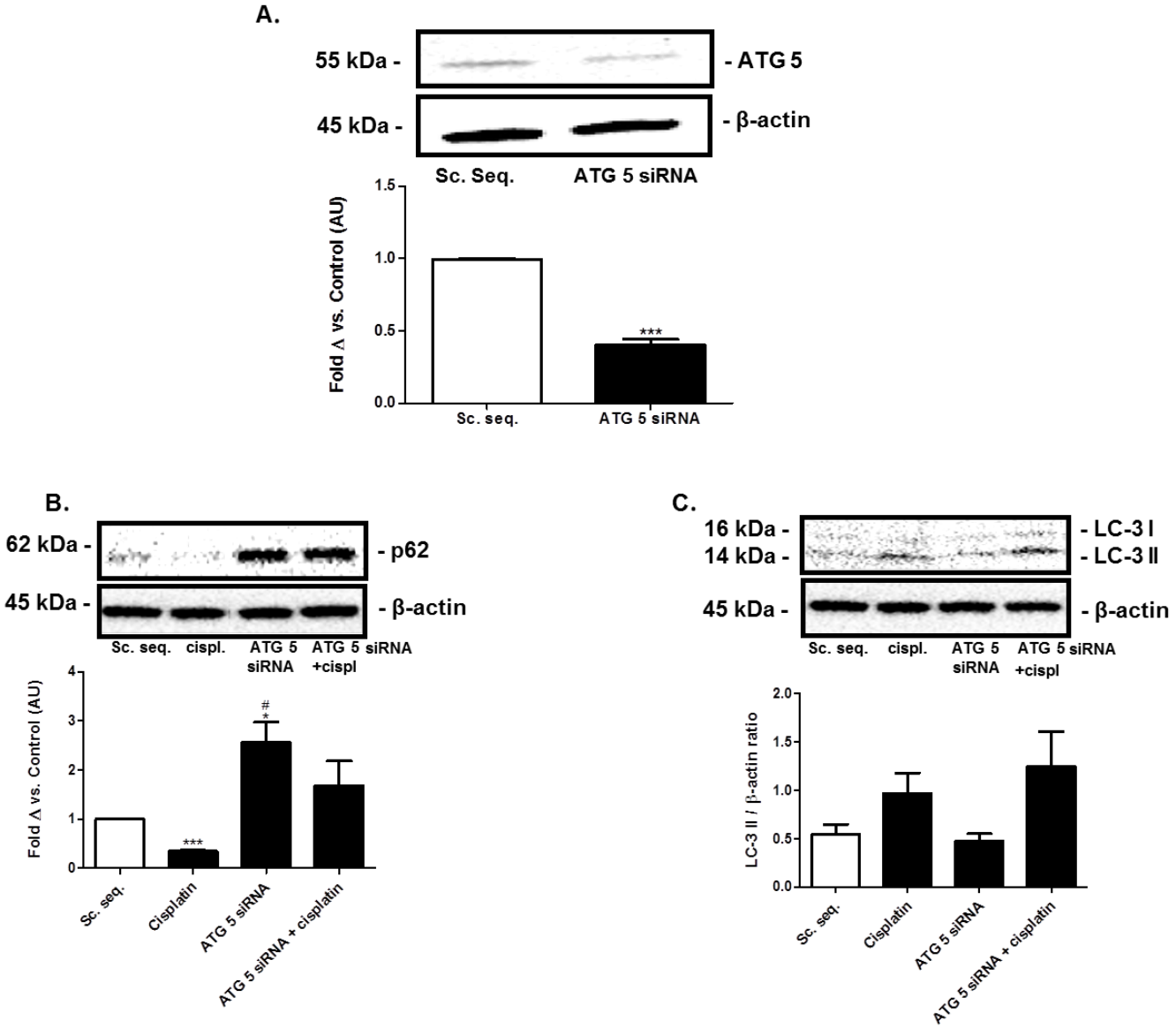


Figure 6.2.27. Atg5 silencing and the effect on autophagy in CaSki cells. **A.** Silencing of ATG 5 confirmed through Western blotting *** $p < 0.001$ vs. sc. seq., n = 3**.** p62 degradation, *** $p < 0.001$ vs. sc. seq., * $p < 0.05$ vs. control and # $p < 0.05$ vs. cisplatin, n = 3. **C.** LC-3 II protein levels, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Sc. seq. = scrambled siRNA sequence/control, cispl. = cisplatin, AU = arbitrary units.

Efficient silencing of the ATG 5 protein was confirmed through Western blotting (0.40 ± 0.04 vs. sc. seq. $p < 0.001$, Fig. 6.2.27.A). Assessment of markers of autophagy revealed a significant decrease in p62 intracelluar protein levels after treatment with cisplatin (0.35 ± 0.02 vs. sc. seq., *** $p < 0.001$) and a significant increase in p62 after silencing of ATG 5 (2.56 ± 0.42 vs. control, * $p < 0.05$), in comparison to the control cells (Fig. 6.2.27.B). Additionally, in comparison to the cisplatin treated CaSki cells, a significant increase in p62 was observed where ATG 5 was silenced (2.56 ± 0.42 vs. cisplatin, # $p < 0.01$, Fig. 6.2.27.B.). No statistically significant changes in LC-3 II protein expression were observed in any of the treatment groups (Fig. 6.2.27.C.).

d. The effect of ATG5 silencing on apoptosis in Ect1/E6E7 cells.

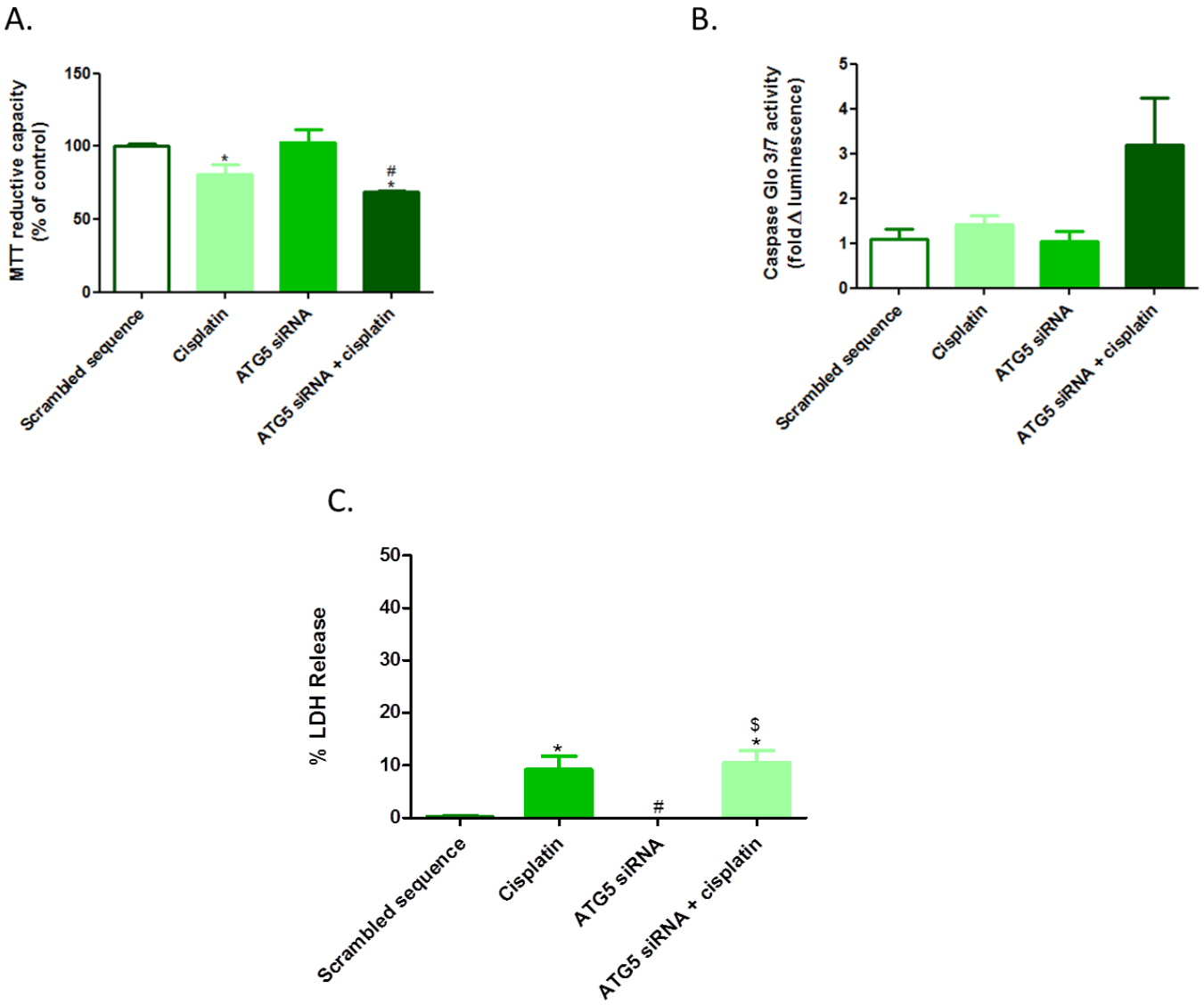


Figure 6.2.28.. **Effect of the silencing of ATG 5 on apoptosis in Ect1/E6E7 cells. A.** Cell viability (MTT), * $p < 0.05$ vs. sc. seq. and # $p < 0.05$ vs. cisplatin, n = 4 **B.** Caspase-3 and -7 activity, n = 4. **C.** LDH release, * $p < 0.05$ vs. sc.seq., # $p < 0.05$ vs. cisplatin and $ $p < 0.05$ vs. ATG5 siRNA, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Sc. seq. = scrambled siRNA sequence/control, AU = arbitrary units.

Analysis of apoptotic markers after silencing of ATG 5 in combination with cisplatin revealed a significant decrease in MTT reductive capacity of Ect1/E6E7 in the cisplatin and combination groups (80.87 ± 6.40%, 68.40 ± 1.34% vs. sc.seq., * $p < 0.05$ and 68.40 ± 1.39% vs. ATG 5 siRNA, # $p < 0.05$, Fig. 6.2.28.A.). Caspase -3 and -7 activity did not change significantly in any of the treatment groups (Fig. 6.2.28.B.). LDH release increased significantly in the cisplatin and combination groups in comparison to the control/scrambled sequence (9.20 ± 2.58% and 10.47 ± 2.34% vs. sc. seq., * $p < 0.05$). Additionally, silencing resulted in a significant decrease in LDH release in comparison to cells treated with cisplatin (0.01 ± 0.00% vs. cisplatin, # $p < 0.05$, Fig. 6.2.28.C).

e. The effect of ATG5 silencing on apoptosis in HeLa cells .

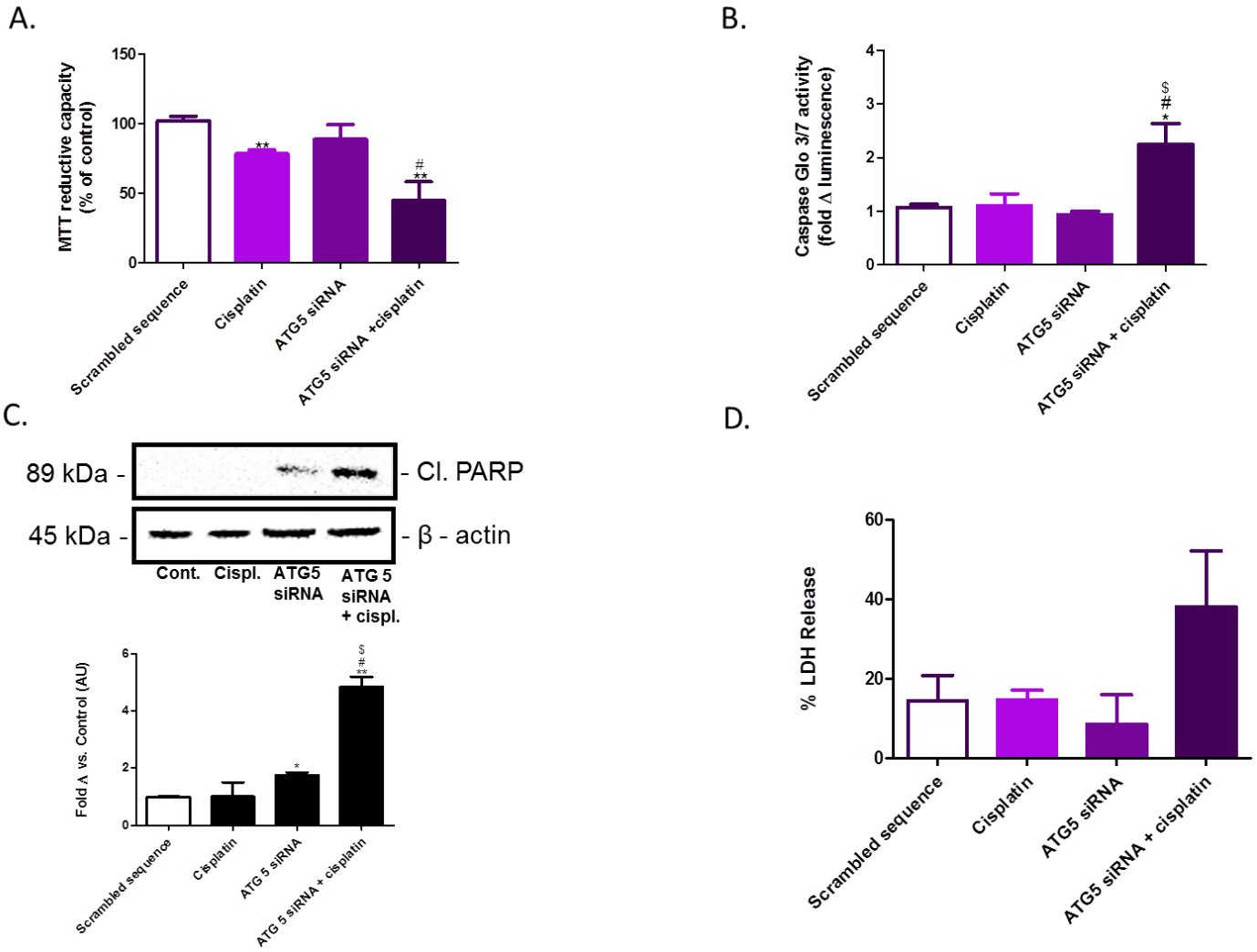
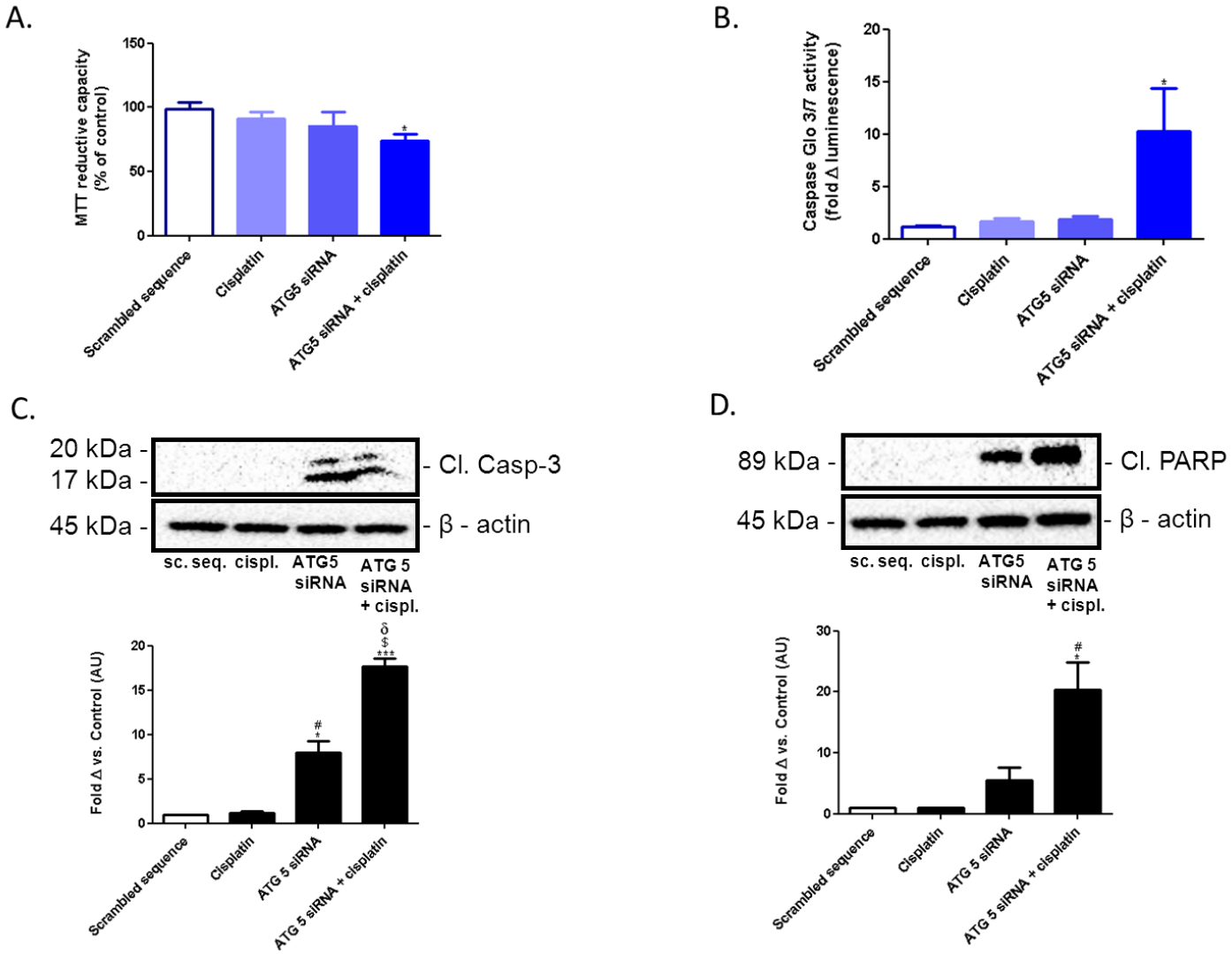


Figure 6.2.29. Effect of ATG5 silencing on apoptosis in HeLa cells. **A.** Cell viability (MTT), ** $p < 0.01$ vs. sc. seq. and # $p < 0.05$ vs. cisplatin, n = 4 **B.** Caspase-3 and -7 activity, * $p < 0.05$ vs. control, # $p < 0.05$ vs. cisplatin and $ $p < 0.05$ vs. ATG5 siRNA, n = 4. **C.** PARP cleavage, * $p < 0.05$ and ** $p < 0.01$ vs. sc. seq., # $p < 0.01$ vs. cisplatin and $ $p < 0.05$ vs. ATG 5 siRNA, n = 3 (corresponding representative blot above). **D.** LDH release, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Sc. seq. = scrambled siRNA sequence/control, AU = arbitrary units.

Silencing of ATG 5 was utilised in order to confirm previous findings which indicated that the inhibition of autophagy combined with cisplatin treatment induces cell death. A significant decrease in cell viability of HeLa cells treated with cisplatin was observed (78.18 ± 2.73% vs. scr. seq, ** $p < 0.01$, Fig. 6.2.29.A). Additionally, HeLa cells that were silenced for ATG 5 and treated with cisplatin thereafter displayed a significant reduction in viability when compared to the control cells (44.53 ± 13.54% vs. scr. seq., ** $p < 0.01$) and cells silenced for ATG 5 alone (44.53 ± 13.54% vs. ATG 5 siRNA, # $p < 0.05$, Fig. 6.2.29.A.). Caspase activity remained unchanged in all groups except in the combination group where ATG 5 was silenced with subsequent treatment with cisplatin (2.26 ± 0.38 vs. control, * $p < 0.05$, 2.26 ± 0.38 vs. cisplatin, # $p < 0.05$, and 2.26 ± 0.38 vs. ATG 5 siRNA, $ $p< 0.05$, Fig. 6.2.29.B.). Cleavage of PARP, which is indicative of apoptotic induction, was significantly increased in the silenced and combination group in comparison to the control (1.75 ± 0.1 and 4.85 ± 0.36 vs. sc.seq., * $p < 0.05$, ** $p < 0.01$). Furthermore, PARP cleavage in the combination group was significantly increased in comparison to the cisplatin (4.85 ± 0.36 vs. cisplatin, # $p< 0.01$) and the ATG5 siRNA group (4.85 ± 0.36 vs. ATG 5 siRNA., $ $p< 0.05$, Fig. 6.2.29.C.). No significant changes in LDH release were observed in either of the treatment groups (Fig. 6.2.29.D.).

f. The effect of ATG5 silencing on apoptosis in CaSki cells



D.

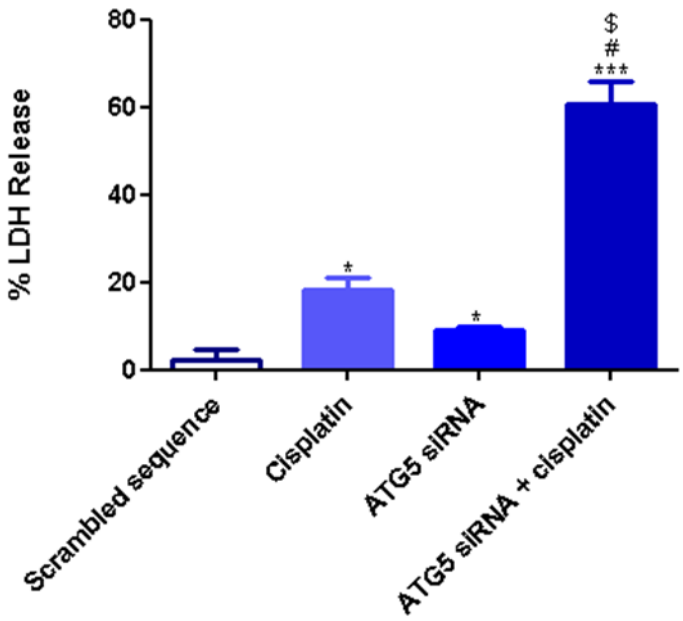


Figure 6.2.30. **Effect of the silencing of ATG 5 on apoptosis in CaSki cells**. **A.** Cell viability (MTT), * $p < 0.05$ vs. sc. seq., n = 3. **B.** Caspase-3 and -7 activity, * $p < 0.05$ vs. sc. seq., n = 4. **C.** Caspase-3 cleavage, * $p < 0.05$ and *** $p < 0.001$ vs. sc. seq., # $p < 0.05$ and $ $p < 0.001$ vs. cisplatin and δ $p < 0.01$ vs. ATG 5 siRNA, n = 3. **D.** PARP cleavage, * $p < 0.05$ vs. sc. seq. and # $p < 0.05$ vs. cisplatin, n = 3. **E.** LDH release, * $p < 0.05$, *** $p < 0.001$ vs. sc. seq., # $p < 0.001$ vs. cisplatin and $ $p < 0.001$ vs. ATG 5 siRNA, n = 3. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM. Sc. seq. = scrambled siRNA sequence/control, AU = arbitrary units.

Cell viability (MTT) decreased significantly in the combination group in comparison to the control (74.23 ± 5.07% vs. sc. seq., * $p < 0.05$, Fig. 6.2.30.A). Caspase activity was observed to increase significantly only in the combination group (10.28 ± 4.07 vs. sc. seq. * $p < 0.05$, Fig. 6.2.30.B). Furthermore, Western blot analysis of caspase-3 cleavage indicated significantly higher intracellular levels in the silenced and combination treatment groups in comparison to the control (7.99 ± 1.28 and 17.63 ± 0.97 vs. sc. seq. * $p < 0.05$, *** $p < 0.001$) and cisplatin (7.99 ± 1.28 and 17.63 ± 0.97 vs. cisplatin, # $p < 0.05$, $ $p < 0.001$, Fig. 6.2.30.C). A significant difference in caspase-3 cleavage was observed between the silenced and combination group (17.63 ± 0.97 vs. ATG 5 siRNA, # $p < 0.01$, Fig. 6.2.30.C.). Similarly, cleaved PARP levels were increased, however only in the combination group in comparison to the control (20.43 ± 4.44 vs. sc. seq., * $p < 0.05$) and the cisplatin group (20.43 ± 4.44 vs. cisplatin, # $p < 0.05$). LDH release increased significantly in CaSki cells treated with cisplatin (18.12 ± 3.12% vs. sc. seq., * $p < 0.05$), silenced for ATG 5 (9.23 ± 0.66% vs. sc. seq., * $p < 0.05$) as well as silenced for ATG 5 with cisplatin treatment (60.77 ± 5.28% vs. sc. seq., *** $p < 0.001$; 60.77 ± 5.28% vs. cisplatin, # $p < 0.001$ and 60.77 ± 5.28% vs. ATG 5 siRNA, $ $p < 0.001$, Fig. 6.2.30.D).

g. Summary of results.

Table 3.a. Summary of results: The effect of rapamycin and cisplatin on autophagy and apoptosis in three cervical cell lines.

	RAPAMYCIN								
	Ect1/E6E7			**HeLa**			**CaSki**		
	Cisplatin	**Rapamycin**	**R+C**	**Cisplatin**	**Rapamycin**	**R+C**	**Cisplatin**	**Rapamycin**	**R+C**
AUTOPHAGY									
mTOR	↓	↓	↓	↓	↓	↓	–	↓	↓
p62	↓	–	↓	–	↓	↓	↓	↓	↓
LC-3 II	↑	↓	↓	–	–	–	↑	–	↓
Lysosomes	–	–	↑	↑	↑	↑	–	–	–
MTT	–	–	–	–	↓	↓	↓	↓	↓
CELL DEATH									
Caspase-Glo	–	↓	↓	–	–	–	–	↑	↑
PARP cleavage	–	–	↑	–	–	↓	–	↓	–
PI inclusion	–	↑	↑	–	–	↑	–	–	–
LDH Release	–	–	–	–	–	↑	–	–	–
CELL CYCLE	–	↓ G1,↑S	↓ G1,↑S	↓ G1,↑S	–	–	↑S	–	–

↑ - significant increase, ↓ significant decrease, – not significant

Table 3b. Summary of results: The effect of mTOR silencing and cisplatin on autophagy and apoptosis in three cervical cell lines.

	mTOR siRNA								
	Ect1/E6E7			**HeLa**			**CaSki**		
	Cisplatin	**mTOR siRNA**	**mTOR siRNA + cispl.**	**Cisplatin**	**mTOR siRNA**	**mTOR siRNA + cispl.**	**Cisplatin**	**mTOR siRNA**	**mTOR siRNA + cispl.**
AUTOPHAGY									
p62	↓	↓	↓	↓	↓	↓	↓	↓	–
LC-3 II	↑	↑	↑	–	↑	↑	↑	↑	↑
MTT	↓	–	↓	↓	–	–	–	–	–
CELL DEATH									
Caspase-Glo	–	–	↑	–	–	–	–	–	–
PARP cleavage	–	–	–	↑	–	↑	–	–	↑
LDH Release	↑	–	–	–	–	–	↑	–	–

↑ - significant increase, ↓ significant decrease, – not significant

Table 4 a. Summary of results: The effect of bafilomycin and cisplatin on autophagy and apoptosis in three cervical cell lines.

	BAFILOMYCIN A1								
	Ect1/E6E7			**HeLa**			**CaSki**		
	Cisplatin	**Bafilomycin A1**	**B+C**	**Cisplatin**	**Bafilomycin A1**	**B+C**	**Cisplatin**	**Bafilomycin A1**	**B+C**
AUTOPHAGY									
mTOR	↓	–	–	↓	–	↓	↓	↓	↓
p62	↓	–	↓	↓	–	↓	–	–	↓
LC-3 II	↑	↑	↑	–	↑	↑	↑	↑	↑
Lysosomes	–	↓	↓	↑	↓	↓	–	↓	↓
MTT	–	–	–	–	–	–	–	–	↓
CELL DEATH									
Caspase-Glo	↑	–	↑	–	–	–	–	↑	↑
PARP cleavage	↑	↑	↑	–	–	↑	–	–	↑
Caspase-3 cleavage	x	x	x	x	x	x	x	x	↑
PI inclusion	–	↑	↑	–	–	↑	–	–	↑
LDH Release	–	–	↑	–	–	↑	–	–	↑
CELL CYCLE	–	–	–	↓ G2/M,↑S	–	–	↑S	↓ G2/M	↓ G2/M

↑ - significant increase, ↓ significant decrease, – not significant, x not detected.

Table 4 b. Summary of results: The effect of ATG5 silencing and cisplatin on autophagy and apoptosis in three cervical cell lines.

	ATG5 siRNA								
	Ect1/E6E7			**HeLa**			**CaSki**		
	Cisplatin	**ATG5 siRNA**	**ATG5 siRNA + cispl.**	**Cisplatin**	**ATG5 siRNA**	**ATG5 siRNA + cispl.**	**Cisplatin**	**ATG5 siRNA**	**ATG5 siRNA + cispl.**
AUTOPHAGY									
p62	↓	↑	–	↓	↑	↑	↓	↑	–
LC-3 II	↑	–	↑	↑	–	↑	–	–	–
MTT	↓	–	↓	↓	–	↓	–	–	↓
CELL DEATH									
Caspase-Glo	–	–	–	–	–	↑	–	–	↑
PARP cleavage	–	–	–	↑	–	↑	–	–	↑
Caspase-3 cleavage	x	x	x	x	x	x	x	↑	↑
LDH Release	↑	–	–	–	–	–	↑	↑	↑

↑ - significant increase, ↓ significant decrease, – not significant, x not detected.

6.3. Discussion

The previous observation that a low, non-toxic concentration of cisplatin induces autophagy brought forth the possibility that this response may be exploited in order to improve cisplatin-induced apoptosis with the hope of eliminating the need for the high concentrations that are currently in clinical use. If such a lower cisplatin concentration with autophagy induction/inhibition enhances cell death, this could decrease the severity of the side-effects considerably, and as a result of this, chemotherapy treatment regimens may be prolonged. It has been shown in literature that both the induction and inhibition of autophagy can improve the sensitivity of various cancer cell types to chemotherapy treatment (Levy and Thorburn, 2011, Notte et al., 2011). This part of the study therefore aimed to determine whether the induction or inhibition of autophagy may contribute to the improvement of cisplatin-induced apoptosis in two cancerous cell lines (HeLa and CaSki).

6.3.1. The effect of the combination of rapamycin and cisplatin treatment on autophagy in three cervical cell lines.

Firstly, autophagy was induced with the use of an established pharmacological agent known as rapamycin. It specifically inhibits mTOR, an upstream autophagic modulator, thereby inducing autophagy. Since cisplatin induces autophagy on its own, two things should be considered: Firstly, will the induction of autophagy drive the cells into metabolic failure? And secondly, will induction of autophagy above the cisplatin-induced levels function as an additional resistance mechanism against cisplatin treatment? The effect of the combination treatment (rapamycin and cisplatin) on autophagy was initially assessed, and the effects on apoptosis induction was evaluated.

Ect1/E6E7

Treatment with rapamycin alone and in combination with cisplatin induced a significant decrease in the intracellular levels of mTOR when compared to the control cells (Fig. 6.2.1. A). Since cisplatin treatment alone induced a decrease in mTOR protein levels, it might have been expected that mTOR protein levels would be lower in the combination group than in the other two treatment groups, however this was not the case. A possible explanation for this could be that since autophagy has been

up-regulated by pre-treatment with rapamycin, a negative feedback mechanism could be responsible for preventing further up-regulation upon treatment with cisplatin. This phenomenon has been observed in oncogene-induced senescence where autophagy was activated. This correlated with negative feedback in the mTOR pathway in human diploid fibroblasts (Young et al., 2009).

An equally important marker of autophagy, p62, was analysed and although rapamycin treatment alone did not induce significant degradation through autophagy induction, the combination of cisplatin and rapamycin did (Fig.6.2.1.B.). Intracellular levels of p62 in this group were similar to those seen in cells treated with cisplatin alone, thus the combination treatment did not prove to be more effective in inducing autophagy and this result may in effect be attributed to cisplatin treatment alone. Although p62 plays a role in a myriad of other important cellular processes, during the dynamics of autophagy its degradation is indicative of autophagy up-regulation (Mizushima et al., 2010). In the context of non-cancerous cells, rapamycin was observed to decrease protein levels of p62 in human embryonic kidney cells by a minimal 32% (Tung et al., 2010), which agrees with the observations obtained in this study where rapamycin did not decrease p62 protein levels in the non-cancerous Ect1/E6E7 cells. This may therefore suggest that essentially, the effect of rapamycin on p62 protein levels may be negligible in these cells since its effects were minor in comparison to the other untreated (control) and treated groups.

Lastly, the presence of LC-3 II protein (and therefore autophagosomes) was assessed (Fig. 6.2.1.C.). Both rapamycin treated groups had significantly higher levels of LC-3 II protein in comparison to the control group. The combination treatment of rapamycin and cisplatin induced a significant decrease in p62 protein levels in comparison to the control and rapamycin groups and exhibited a concomitant increase in LC-3 II protein levels. Interestingly, since p62 and LC-3 II function at the same level of the autophagic signalling cascade i.e. the autophagosome, the relative protein levels of each should in theory correlate with one another such that a decrease in p62 is indicative of an increase in autophagy (LC-3 II) (Mizushima et al., 2010) . This increase in LC-3 II in a non-cancerous cell line in response to rapamycin treatment is supported by a study in which a primary culture of rodent pancreatic islets was observed to initiate a similar response (Tanemura et al., 2009). The Western blotting data suggest that even though the upstream autophagy molecules do not exhibit pronounced changes in their

expression in response to the combination of rapamycin and cisplatin, downstream at the level of the autophagosome, the effect is more pronounced (although an additive effect is not necessarily observed).

In order to build upon the Western blotting data obtained (Fig. 6.2.1. A, B and C), analysis of the lysosomal acidic compartment as an indication for autophagic activity was assessed through the use of flow cytometry (Fig. 6.2.1. D.). The results suggest that Ect1/E6E7 cells treated with a combination of rapamycin and cisplatin were the only group that had significantly increased levels of the acidic lysosomal compartment. The completion of the autophagic process requires that autophagosomes fuse with lysosomes to form autophagolysosomes with the eventual purpose of degrading the intracellular components so that they may be utilised by the cell's metabolism (Klionsky and Deter, 2008). Therefore their presence may be used as a marker for the up-regulation of autophagy. Since lysosomal biogenesis is in effect one of the final stages of the autophagic pathway, an increase in the presence of lysosomes is likely to correlate with a decrease in p62 and increase in LC-3 II protein levels. This was indeed the case since the combination group exhibited significant changes in both of these markers. An additive effect with rapamycin and cisplatin treatment in Ect1/E6E7 cells was not observed upon analysis of lysosomal biogenesis.

The capacity of the Ect1/E6E7 cells to induce autophagy was determined to be relatively high upon stimulation with the combination of rapamycin and bafilomycin (Fig. 4.6.). The positive results observed here i.e. significant molecular and lysosomal changes in response to rapamycin and cisplatin treatment, may be as a result of this increased autophagic capacity. The true significance of this will be determined after the analysis of the responses of the two cancerous cell lines to this treatment regime.

HeLa

As with the Ect1/E6E7 cells, the combination of rapamycin and cisplatin treatment on autophagy was assessed through the analysis of three autophagy markers: mTOR, p62 and LC-3 II. Analysis of mTOR revealed significantly lowered expression in all groups (Fig. 6.2.2. A), additionally, no additive effect in further decreasing mTOR expression was observed in cells treated with the combination of rapamycin and cisplatin. Since rapamycin is an mTOR inhibitor, a decrease in its expression was expected. Interestingly, the response of the HeLa cells to rapamycin treatment

suggests a certain degree of sensitivity when compared to results obtained with Ect1/E6E7 cells, i.e. the same concentration of rapamycin had a more pronounced effect in inhibiting mTOR (Fig.6.2.1. A.). Furthermore, cisplatin treatment alone was able to significantly decrease mTOR expression, which agrees with a study conducted by Faried and colleagues who observed that a concentration of cisplatin that inhibited 20% of HeLa and CaSki cell proliferation (i.e. a low concentration, 6.28 μM) was able to effectively down-regulate mTOR (Faried et al., 2008b). The analysis of mTOR under these treatment conditions suggests that both rapamycin and cisplatin are able to effectively reduce expression of mTOR in HeLa cells and that the combination of rapamycin and cisplatin treatment does not prompt additional inhibition of mTOR.

The analysis of p62, a late stage autophagic marker, revealed significant decreases in intracellular protein levels in the rapamycin and combination treatment groups. Treatment with rapamycin induced p62 degradation in the HeLa cells which is in agreement with a study performed by Bjorkoy and colleagues (Bjorkoy et al., 2005). The presence of p62 in the combination group is markedly lower than both the rapamycin and cisplatin control groups, which points towards a possible additive effect induced by the two pharmacological agents.

Since p62 protein levels in the combination treatment group (Fig. 6.2.2. B) were particularly reduced; LC-3 II protein levels should be consequently high. This was not the case for both rapamycin treated groups (Fig. 6.2.2.C). A study conducted by Chang and colleagues did not observe an enhancement of LC-3 II after rapamycin treatment in hepatomblastoma cell lines (Chang et al., 2011). Similarly, treatment with rapamycin did not induce pronounced up-regulation of LC-3 II in lung cancer cells (Kim et al., 2009). It is noteworthy to mention that although LC-3 II protein levels did not appear to be up-regulated, this does not infer that autophagy is not activated. This is established by the fact that during the late stages of autophagy, LC3-II is also degraded along with other cytoplasmic constituents, therefore generating a ‘negative result’ as such (Kabeya et al., 2000, Tanida et al., 2005, Mizushima et al., 2010). Therefore the data obtained from Western blotting suggests an up-regulation of autophagy in HeLa cells treated with rapamycin and cisplatin in combination, however cisplatin treatment had no effect on rapamycin-induced autophagy.

Analysis of lysosomal acidity revealed significant increases in all treatment groups, which combined with the Western blotting data suggest that firstly, rapamycin does induce autophagy in HeLa cells, secondly that these cells are in the late stages of autophagy and finally that cisplatin has no effect on rapamycin-induced autophagy. If it is recalled from chapter 3, HeLa cells demonstrated insignificant flux through the autophagic pathway, but were able to respond efficiently to the combination treatment in inducing autophagy, therefore the significance of this lowered flux may be observed after analysis of apoptosis.

CaSki

Lastly, analysis of the aggressive CaSki cells on rapamycin and cisplatin induced autophagy revealed the following: mTOR expression was significantly reduced in cells treated with rapamycin and the combination of rapamycin and cisplatin in comparison to the control (Fig.6.2.3. A.).

Intracellular p62 protein levels decreased significantly in the combination group and this result implies that autophagy has been activated (Mizushima et al., 2010). Here it is evident that the addition of cisplatin after pre-treatment with rapamycin has an additive effect in reducing p62 protein expression when compared to cells treated with cisplatin alone (Fig. 6.2.3.B.). Thus far, these results are indicative of an up-regulation in autophagy in response to the combination treatment with rapamycin and cisplatin.

LC-3 II protein levels in the cisplatin group were significantly increased (Fig. 6.2.3. C), and the addition of cisplatin after pre-treatment with rapamycin significantly decreased LC-3 II protein levels in comparison to the rapamycin treated group. As with the HeLa cells, CaSki cells treated with a combination of rapamycin and cisplatin may have very low/undetectable levels of LC-3 II due to its high rate of fusion of autophagosomes with lysosomes (Scheffner et al., 1991). From the analysis of these three autophagic markers, it may be concluded that autophagy is likely to be occurring in CaSki cells after treatment with rapamycin and cisplatin in combination.

In order to support the above results, changes in the lysosomal acidic compartment with the use of Lysotracker™ were assessed (Fig. 6.2.3. D). Surprisingly, the results obtained were contradictory to the Western blot data (Fig. 6.2.3. A-C); no significant changes were observed in the rapamycin and even the combination treated group. A

study conducted by Safaei and colleagues may provide a possible explanation for this finding: the presence of lysosomes in cisplatin-sensitive and cisplatin-resistant ovarian carcinoma cell lines were detected with the use of Lysotracker™. It was observed that the cisplatin-resistant cell type had markedly fewer lysosomes than the cisplatin-sensitive counterparts (Safaei et al., 2005b). Since it was concluded that the CaSki cells had the greatest tolerance and therefore resistance to cisplatin treatment (Chapter 2), this may be a possible explanation for the results described (Figure 6.2.3. C).

From this section, the following conclusions may be drawn: The non-cancerous Ect1/E6E7 cells significantly induced autophagy after exposure to the combination treatment (Fig. 5.3.1). The HeLa and CaSki cells exhibited decreased LC-3 II at the end of the treatment period which indicates late-stage autophagy due to LC-3 II degradation. Autophagic flux measurements in the CaSki cell line (Chapter 4.2.) indicate that they have sufficient capacity to induce autophagy and thus we can attribute this to the results obtained here. To determine whether the combination treatment sensitised cervical cancer cells to cisplatin treatment, apoptosis was hence analysed.

6.3.2. The effect of the combination of rapamycin and cisplatin treatment on apoptotic induction in three cervical cell lines.

Analysis of apoptosis and membrane integrity was assessed by the analysis of cell viability (MTT), caspase-3/-7 activity, PARP cleavage, LDH release and PI inclusion.

Ect1/E6E7

Firstly, cell viability of Ect1/E6E7 cells was assessed through the MTT assay which revealed no significant changes in the combination treatment group (Fig. 6.2.4. A). This result is desirable considering that the goal of this study is to maintain the integrity of the non-cancerous cells. Analysis of caspase-3 and -7 activity revealed significant decreases in their activity in the combination group in comparison to the control and cisplatin treated group (Fig. 6.2.4. B). A explanation for this may be that once caspase-3 is activated, XIAP specifically reduces the level of active caspase-3 through binding with the caspase and degrading it via its E3 ubiquitin protein ligase (Suzuki et al., 2001). Therefore caspase-3 and -7 were both likely to be activated at

an earlier stage, and at the end of the treatment period had been degraded by this ubiquitin system, creating the above mentioned result (Fig. 6.2.4. B).

A more direct biochemical marker of apoptosis is the cleavage of PARP (Berger and Petzold, 1985) which revealed that only treatment with the combination of rapamycin and cisplatin induced significant cleavage (Fig. 6.2.4. C). Furthermore, the combination treatment group was positive for propidium iodide (PI) inclusion, as observed in figure 6.2.4. E. A lack of LDH release with an increase in PI inclusion seems conflicting since a positive result for both tests is dependent on the permeabilisation of the plasma membrane. Keilhoff and Wolf observed that LDH release occurred only in response to cellular stress and as cell death progressed, LDH activity in the medium decreased significantly (Keilhoff and Wolf, 1993). This supports the results obtained in Figures 6.2.4. D. and E, which suggest that these cells are in the late stages of apoptosis. It is noteworthy to mention that only about 25% of the total Ect1/E6E7 cell population exhibited apoptotic characteristics in response to rapamycin and cisplatin treatment (Fig. 6.2.4. E) which is favourable since damage to non-cancerous cells is central to inducing the side-effects commonly observed by chemotherapy treatment.

HeLa

Cell viability (MTT) decreased significantly, although only by about 25% in cells treated with the combination of rapamycin and cisplatin. Additionally, the addition of cisplatin after pre-treatment with rapamycin has no effect on rapamycin-induced MTT reductive capacity. What can be inferred however is that treatment with rapamycin may affect mitochondrial functioning since this assay is based on the reductive function of these metabolic organelles. Data to support this was demonstrated by Scheike and colleagues who observed that treatment with rapamycin lowered mitochondrial oxygen consumption and decreased oxidative capacity in Jurkat cells (Schieke et al., 2006).

Analysis of activity of the effector caspsae -3 and -7 did not reveal significant increases in any of the treatment groups (Fig. 6.2.5. B.), and this correlated with a lack of PARP cleavage (Fig. 6.2.5. C). The HeLa cells may not be undergoing apoptosis at this point in the treatment protocol, however an increase in the incubation time with cisplatin may elicit an apoptotic response since its effects are known to be time-dependent (Choi et al., 2004, Cheng et al., 2010).

Possibly the most striking result observed is that the combination of rapamycin and cisplatin treatment decreased PARP cleavage in HeLa cells (Fig. 6.2.5. C). Although not significant, the level of PARP cleavage in cells treated with rapamycin and cisplatin appear to be considerably lower (P = 0.053), this may point to the ability of rapamycin to assist in HeLa cell survival in the face of cisplatin treatment. It has previously been established that cisplatin increases autophagy (Chapter 5) and this is a likely mechanism of resistance/survival, thus pre-treatment with an autophagy inducer, such as rapamycin, may be protective against the damaging effects of cisplatin. Equally important is the finding that rapamycin inhibits the protective functioning of PARP, thereby preventing the poly(ADP-ribosyl)ation of DNA and preventing DNA repair (Fahrer et al., 2009).

Evaluation of LDH release in HeLa cells revealed an increase after treatment with the combination of rapamycin and cisplatin (Fig.6.2.5. D) which may be indicative of a stress response (Keilhoff and Wolf, 1993) due to the metabolic/mitochondrial disruptions induced by treatment with rapamycin (Schieke et al., 2006).The percentage of PI positive cells in the rapamycin and cisplatin treated group were significantly higher than the other treatment groups (Fig. 6.2.5. E), but only by about 4% (mean = 9.83%). It can therefore be concluded that the combination treatment possibly induces minor mitochondrial dysfunction and membrane permeabilisation in a small percentage of HeLa cells after the treatment period, however the results do not suggest that this is an effective method in inducing apoptosis in adenocarcinoma cells.

The rationale behind inducing autophagy with a pharmacological agent such as rapamycin, and subsequently attempting to increase autophagic levels with a substance also known to induce autophagy, such as cislpatin, is that massive autophagic up-regulation (above a defined threshold) could result in large parts of the cytosol being degraded, leading to irreversible cellular atrophy (Gozuacik and Kimchi, 2007). Referring back to the autophagic flux analysis of the HeLa cells, it was clear that their capacity to induce autophagy upon stimulation was partial, therefore it may have been predicted that this method of attempting to induce cell death *with* autophagy (Kroemer and Levine, 2008) may not have been successful.

CaSki

The MTT results revealed that the combination of rapamycin and cisplatin treatment decreased MTT reductive capacity by roughly 48% (Fig. 6.2.6. A) and when compared to the rapamycin treated group, the addition of cisplatin caused a further decrease of 22.43%. This result infers that under these conditions, mitochondrial activity is likely to be compromised (Slater et al., 1963) and that cisplatin worsens this rapamycin-induced state. Under these conditions one may expect an increase in cleavage of caspases -3 and -7 (due to cytochrome-c release from the mitochondrial membrane). This was indeed the case and both groups treated with rapamycin exhibited increases in caspase activity (Fig. 6.2.6. B.).

PARP cleavage, one of the biochemical characteristics of apoptosis was absent in CaSki cells treated with the combination of rapamycin and cisplatin (Fig. 6.2.6. C). This is similar to the results observed in the HeLa cells (Fig. 6.2.5. C), however the absence of PARP in the rapamycin group appears to be more pronounced and may once again be attributed to the rapamycin itself (Fahrer et al., 2009). Even though there is an absence of cleaved PARP, apoptosis is not dependant its cleavage and is able to occur independently (Wang et al., 1997).

Analysis of the membrane integrity did not reveal any significant changes in any of the treatment groups (Fig. 6.2.6. D. and E.). Since the early markers of apoptosis were suggestive of apoptotic induction, it is likely that an increase in incubation time with cisplatin will produce positive results.

Referring back to the characterisation of autophagic flux (Fig. 4.6.), these cells had an increased capacity to induce autophagy upon stimulation, as well as a high basal level of autophagic activity, therefore a method such as this whereby autophagy was induced with rapamycin and then stimulated again with cisplatin, in addition to the already elevated basal autophagic levels is promising. The importance of autophagic flux evaluation before treatment intervention such as this is necessary, since it proved to be ineffective in inducing apoptosis in the HeLa cells with a low autophagic flux, but was able to induce apoptosis in the CaSki cell lines with relatively higher autophagic flux.

Collectively, the results suggest apoptosis induction. Sensitisation of CaSki cells to cisplatin treatment through mTOR inhibition with the use of rapamycin has proven to be successful (Faried et al., 2008b), however the dose of cisplatin utilised in that study was 116.6 μM (in comparison to 15 μM used in this study). It has been

demonstrated here that with the use of a concentration almost eight-fold lower, that apoptotic cell death was achieved in the aggressive CaSki cell line.

6.3.3. The effect of rapamycin and cisplatin treatment on the modulation of cell cycle progression.

The importance of the cell cycle and its role as an indication for sensitivity in combination chemotherapy has been realised (Shah and Schwartz, 2001). Cell-cycle mediated drug resistance occurs when treatment with one agent impacts the cell-cycle such that the subsequent chemotherapy agent administered immediately afterward is less effective (Shah and Schwartz, 2001). It is therefore necessary to conduct cell cycle analysis to determine whether the chemotherapy agents of choice act on cell cycle progression.

Ect1/E6E6

Treatment with cisplatin did not induce any significant alterations in the distribution of cells in the various stages of the cell cycle, however treatment with rapamycin significantly decreased the percentage of cells in the G1/G0 phase with a concomitant increase in the percentage of cells in the S phase (Fig. 6.2.7.). This is contrary to other studies which observed that rapamycin induces G1 phase cell cycle arrest in yeast and non-cancerous mammalian cells (Heitman et al., 1991, Morris, 1991, Dumont et al., 1990, Powell et al., 1999). Literature suggests that a shift into the S phase generally occurs in response to DNA strand breaks (Kastan et al., 2000), and since there have been no particular roles for p53 and p21 in controlling the progression of the S phase, the delay may be beneficial for the cells (in order to repair strand breaks) without inducing permanent arrest (Bartek and Lukas, 2001). It was established that Ect1/E6E7 cells exhibit inclusion of PI (Fig.6.2.4.E), which includes DNA degradation, in response to both rapamycin and the combination treatment. As previously mentioned, rapamycin inhibits PARP activity (Fahrer et al., 2009), thus resulting in its reduced activity to aid in the DNA repair process (Hamid Boulares et al., 1999). Due to the inactivity of PARP, a shift into the S phase may be the result. Cisplatin does not appear to play a role in prompting this S phase shift.

HeLa

A significant increase in the percentage of cells that entered the S phase after cisplatin treatment was observed (Fig. 6.2.8). In cancer cells, this is a typical observation (Qin and Ng, 2002, Sorenson and Eastman, 1988a, Sorenson and Eastman, 1988b, Eastman, 2009, Shah and Schwartz, 2001). Surprisingly, entry into the S phase resulted in a 2-fold decrease in sensitivity of cancer cells to cisplatin and may confer protection to cisplatin induced apoptosis (Mueller et al., 2006), thus a shift into this phase does not signify cell death in this model. This can be supported by the results obtained in Chapter 4.1 where it was demonstrated that 15 μM cisplatin does not induce apoptosis. Treatment with rapamycin indicated a similar profile to that of the cisplatin treated groups. Interestingly, the addition of cisplatin seemed to revert the cell cycle profile back to a state that resembles the control group.

CaSki

As observed with the HeLa cells, the more aggressive CaSki cells exhibit an increase in the percentage of cells in S phase, however a decrease in the percentage of cells in the G1 phase was not observed (Fig. 6.2.9). The combination treatment did induce an increase in the percentage of cells in S phase, but only by 14.81%. The fact that these cells do exhibit any obvious shifts or arrests may prove to be promising due to the fact that cells that do undergo growth arrest may be protected from apoptosis and be resistant to the particular drug (Shah and Schwartz, 2001).

6.3.4. The effect of mTOR silencing combined with cisplatin treatment on autophagy in three cervical cell lines.

In order to control for any off target effects that rapamycin may have, it was necessary to biologically inhibit mTOR through RNA interference. This technique has been widely used and accepted to assess gene function (Sharp, 2001).

Ect1/E6E7

The silencing of mTOR efficiently induced the degradation of p62 with and without the addition of cisplatin (Fig. 6.2.10. B). Additionally, the presence of cisplatin had no additive effect on improving p62 degradation through the silencing of mTOR. Analysis of LC-3 II confirmed that silencing of mTOR in combination with cisplatin treatment increases autophagy (Fig. 6.2.10. C), which agrees with results obtained using rapamycin and cisplatin and the effect of this combination on autophagy (Fig. 6.2.1.C.). Cisplatin does not exhibit any additive effect on LC-3 II expression levels

when added to culture after the silencing period. The results obtained above which suggest that the silencing of mTOR induces autophagy in non-cancerous cell lines agree with other studies (Bohensky et al., 2010, Sakiyama et al., 2009). Furthermore, rapamycin proves to be an acceptable pharmacological inducer of autophagy in a non-cancerous cervical cell line.

HeLa

Efficient inhibition of mTOR was observed after silencing HeLa cells (Fig. 6.2.11. A). The results obtained with regards to autophagic induction mirror those obtained with rapamycin treatment (Fig. 6.2.2.), however the effects of mTOR silencing on p62 were more pronounced (Fig. 6.2.11. B).

Intracellular levels of LC-3 II did not exhibit any significant increases in response to the silencing of mTOR (Fig. 6.2.11. C). Corresponding p62 protein levels suggest its degradation (6.2.11. B.), and its lowered expression correlates with autophagic induction (Mizushima et al., 2010). Furthermore, during the late stages of autophagy, LC-3 II itself becomes degraded along with other cytoplasmic constituents, thus rendering the protein levels as insignificant (Kabeya, Mizushima et al. 2000; Tanida, Minematsu-Ikeguchi et al. 2005; Mizushima, Yoshimori et al. 2010). In conclusion, the inhibition of mTOR through pharmacological and biological methods prove to be sufficient in inducing autophagy in HeLa cells, but no additive effect was observed with cisplatin treatment in silenced cells.

CaSki

The silencing of mTOR in CaSki cells produced similar results to those obtained with the use of rapamycin treatment (Fig. 6.2.3.), however p62 protein levels were significantly reduced after treatment with rapamycin and cisplatin in combination, whereas biological inhibition of mTOR in combination with cisplatin did not produce such an effect (Fig. 6.2.12. B). Silencing of mTOR without cisplatin treatment decreased p62 protein levels, which is indicative of autophagy induction, however, this was lost after cisplatin treatment. P62 has been implicated in cisplatin resistance in ovarian cancer cells by clearing ubiquinated proteins and preventing endoplasmic reticulum stress (Yu et al., 2011b). Additionally, it was observed that p62 protein levels were increased in cisplatin treated cells that were still viable (Moretti et al.,

2009). Taken together, it may be speculated that the addition of cisplatin returns p62 levels to that of the viable control cells thus conferring survival.

Silencing of mTOR alone increased LC-3 II protein expression, more so than the control and the cisplatin treated group, however, this effect was lost after cisplatin treatment. It could be due to a down-regulation of autophagy, or simply a loss in LC-3 II due to its degradation with other cytoplasmic constituents during the autophagic process. p62 protein levels point to the former however, unless autophagy is able to proceed without p62 degradation.

Taken together, these results suggest that the inhibition of mTOR in CaSki cells through silencing induces autophagy, however the addition of cisplatin leads to a decrease in this response, as indicated by the p62 and LC-3 II protein levels. The return of p62 levels back to a control state may be indicative for cell survival.

6.3.5. The effect of the combination of mTOR silencing and cisplatin treatment on apoptotic induction in three cervical cell lines.

It was necessary to determine whether the apoptotic effects induced through treatment with rapamycin were a result of the inhibition of mTOR, and not as a result of potential off-target molecular effects. Thus silencing was employed to inhibit the translation of mTOR mRNA into the protein. Some markers and methods of apoptosis measurement (that were used to assess the effects of rapamycin and cisplatin) were not used in this section, as silencing in this context is utilised purely as a proof of concept.

Ect1/E6E7

Treatment with rapamycin in combination with cisplatin at the end of the treatment period displayed characteristics of membrane permeabilization, indicating late-stage apoptosis (Fig.6.2.4. D and E). Conversely, the silencing of mTOR combined with cisplatin did not exhibit such characteristics, instead, a significant decrease in cell viability was observed (Fig. 6.2.13. A) and caspase-3 and -7 activity was increased (Fig. 6.2.13. B). Cisplatin alone did not increase caspase-3 and -7 activity significantly; this points to the fact that the addition of cisplatin *after* silencing induced caspase -3 and -7 cleavage effectively, however, protein levels of cleaved caspase -

3 were not detectable by Western blot, indicating that the percentage of cells undergoing apoptosis was likely to be minor. Similarly, PARP cleavage was also undetectable through Western blotting.

In conclusion, above data suggest that treatment with the combination of rapamycin and cisplatin induced apoptosis at an earlier time point, thus causing the Ect1/E6E7 cells to undergo plasma membrane permeabilisation at the end of the treatment period. Conversely, the silencing of mTOR with cisplatin treatment induced a slower onset of apoptosis. This may be attributed to the fact that rapamycin, apart from inhibiting mTOR, also induces derangements in mitochondrial function (Schieke et al., 2006), therefore this may have contributed to an earlier onset of apoptosis in the Ect1/E6E7 cells. Nevertheless, the effect of mTOR inhibition combined with low-dose cisplatin treatment is mildly cytotoxic to these non-cancerous cells, thus this avenue of treatment proves to be promising, provided that it is successful in inducing cell death in the cancerous cell lines.

HeLa

Analysis of apoptotic markers in HeLa cells treated with the combination of rapamycin and cisplatin revealed a slight decrease in cell viability (MTT) and LDH release, however no evidence of apoptosis was observed (Fig. 6.2.5.). Inhibition of mTOR through silencing yielded somewhat different results (Fig. 6.2.14.): Cleaved PARP protein levels were higher in comparison to the other two treatment groups (and control), which is contrast to the levels of cleaved PARP observed after treatment with rapamycin and cisplatin (Fig. 6.2.5. D.). Rapamycin was observed to inhibit PARP (Fahrer et al., 2009), which accounted for the lack of cleavage observed. It was proposed that since no definite signs of autophagy were present, rapamycin treatment may play a protective role and aid in HeLa cell survival. This notion has now partially been discarded since PARP cleavage was detected.

Above results are in contrast to other studies which found that the inhibition of mTOR in conjunction with cisplatin treatment induces apoptosis in leukemia cells (Shi et al., 1995), ovarian cancer cells (Bae-Jump et al., 2009, Peng et al., 2010), basal-like breast cancer cells (Wong et al., 2011), laryngeal cancer cells (Lei et al., 2009) and in oesophageal squamous cell carcinoma in nude mice (Hou et al., 2010a). Interestingly, very few studies address this method of inducing apoptosis in cervical cancer cells, as well as the effect it may have on non-cancerous cells. In a study

conducted by Faried and colleagues, mTOR inhibition with rapamycin and subsequent treatment with cisplatin induced apoptosis in both HeLa and CaSki cells (Faried et al., 2008b), however, the reason for this may be attributed to the fact that the concentrations of cisplatin used were based on the IC_{50} values, i.e. HeLa = 15.7 µM and CaSki = 116.6 µM. Another study conducted by Ji and Zheng found that the inhibition of mTOR with rapamycin and siRNAs sensitised HeLa cells to cisplatin treatment at concentrations ranging from 0.1 – 100 µM (Ji and Zheng, 2010). Sensitising cervical cancer cells to cisplatin treatment will likely induce apoptosis when the concentration of cisplatin is already high. The challenge therefore lies in being able to induce a similar magnitude of apoptosis with a lower concentration of cisplatin, thus eliminating the need for high concentrations and the debilitating side-effects which accompany it. Due to this reason alone, the combination of rapamycin and cisplatin treatment is not effective in inducing apoptosis in HeLa cells, but the possibility that an increase in exposure time to cisplatin, thereby improving the chance for apoptotic induction should not be excluded.

CaSki

Silencing of mTOR in combination with cisplatin resulted PARP cleavage but no other markers of apoptosis were affected (Fig. 6.2.15). This finding confirms the fact that PARP cleavage should not be utilized as the only marker of apoptosis, as rapamycin has clearly inhibited its expression in both the HeLa and CaSki cells (Fig. 6.2.5.D. and 6.2.6. D.). Therefore other biochemical markers such as cleaved caspase-3 and -7 should be used in addition. The more pronounced effect on apoptosis in CaSki cells treated with rapamycin could be attributed to the fact that silencing offers only partial inhibition of mTOR (48% in this case), whereas rapamycin was able to reduce mTOR expression by 68%.

The use of rapamycin as a means of sensitisation to chemotherapy treatment in CaSki cells was demonstrated by (Faried et al, 2006), where it was observed that rapamycin treatment sensitises CaSki cells to treatment with paclitaxel (Faried et al., 2006). Thus this is a treatment option; however it seems to be dependent on high doses of chemotherapy treatment. Nevertheless, rapamycin, and not silencing as means of inhibiting mTOR is more efficient in inducing apoptosis in CaSki cells, and this result may be improved by increasing the exposure time to cisplatin treatment.

It can be concluded that the use of rapamycin as a means of sensitising CaSki cells to cisplatin treatment reveals promising results. This is supported by the fact that only minor induction of autophagy in non-cancerous cells is observed. Therefore, rapamycin as a pre-treatment followed by low-dose cisplatin is an encouraging future prospect for the treatment of squamous cell carcinoma of the cervix.

6.3.6. The effect of autophagy inhibition with bafilomycin in combination with cisplatin treatment on autophagy in three cervical cell lines.

Bafilomycin A1 is a macrolide antibiotic isolated from *Streptomyces griseus,* which is able to inhibit V-type H^+ -ATPases (vacuolar proton pump) at concentrations as low as 10 nM (Bowman et al., 1988) and is able to inhibit cell growth at high concentrations (> 10 µM) (Ohkuma et al., 1993). Since this study is based on inhibiting autophagy through the impedance of autophagosome-lysosome fusion, a non-toxic dose of 10 nM was chosen.

Ect1/E6E7

Treatment with bafilomycin did not appear to alter mTOR expression levels in Ect1/E6E7 cells (Fig. 6.2.16. A). It may be speculated that the lack of degradation of damaged cytoplasmic constituents would result in a positive feedback signal as means of further activating mTOR in an attempt to clear these constituents. A study conducted by Wang and Miller observed this in fibroblasts after treatment with bafilomycin (Wang and Miller, 2012). In the present study, mTOR levels remained similar to that of the control. A possible explanation for this is explained in study conducted by Ohsaki and colleagues who demonstrated that inactivated mTOR is present in the lysosomes, and are subsequently recruited to the autophagolysosomes to be degraded, a process that is inhibited by bafilomycin A1 (Ohsaki et al., 2010). This may explain the reason for the unchanging intracellular protein level: lysosomal production is lowered due to bafilomcin A1 treatment, thus mTOR cannot be recruited to lysosomes to eventually be degraded through the process of autophagy, and therefore accumulates in the cytosol.

Further downstream, the analysis of p62 protein levels in the bafilomycin A1 treated group were similar to that of the control, however in the combination group, p62 protein levels were decreased in comparison to both the control and bafilomycin

group. Since bafilomycin prevents lysosomal biogenesis (Bowman et al., 1988), cytoplasmic constituents sequestered by autophagosomes may not be degraded. p62 is one such protein that is recruited to the autophagosome (Bjorkoy et al., 2005, Pankiv et al., 2007, Bjorkoy et al., 2009), therefore increasing its presence in the cytosolic fraction. It is important to keep in mind that bafilomycin A1 does not afford complete inhibition of lysosomal production (Fig.6.2.16. D.), therefore the addition of cisplatin may promote the utilisation of the available lysosomes, thereby minimally degrading p62.

LC-3 II levels increased significantly in response to treatment with bafilomycin with and without cisplatin treatment (Fig. 6.2.16.C). The accumulation of LC-3 II proteins was expected after treatment with bafilomycin (Klionsky et al., 2008) and has been observed in non-cancerous cells elsewhere (Kawakami et al., 2009, Ding et al., 2010, Kimura et al., 2012). Finally, the analysis of lysosomal presence was conducted with the use of a Lysotracker ™ through flow cytometry. Both bafilomycin A1 treated groups had significantly decreased lysosomal acidity which confirmed vacuolar ATPase disruption.

HeLa

A significant decrease in mTOR protein expression was observed in the combination treatment group (Fig. 6.2.17. A). This may have had a substantial effect on autophagic induction since p62 protein levels in the combination group were also significantly lower when compared to that of the control (Fig. 6.2.17. B). The addition of bafilomycin under control conditions is indicative of the basal autophagic flux and indicative for the requirement of autophagy in quality control purposes. But once the cell encounters a stressor (i.e. cisplatin), the specific up-regulation in autophagy and lysosome production is required. Bafilomycin A1 does not completely inhibit the vacuolar ATPase pump (Fig.5.3.17. E.), therefore the degradation of p62 is still able to occur.

If the focus is shifted toward LC-3 II expression (Fig. 5.3.17. D.), it is clear that the addition of cisplatin markedly increases both LC-3 I and II expression in the combination treatment group. A large and possible lethal accumulation of autophagosomes with ‘yet- to- be- degraded’ components are taking up some much needed space in the cytosol. Whether this accumulation proves to be lethal or not will have to be addressed.

Analysis of lysosomal acidity in HeLa cells treated with bafilomycin revealed significant decrease in lysosome acidification (Fig. 6.2.17. D). Furthermore, the up-regulation of lysosomal biogenesis in the presence of cisplatin in the combination treatment group increased 0.7 fold. This is contrast to another study which determined that the addition of cisplatin to bafilomycin pre-treated renal tubular epithelial cells prevented formation of cisplatin induced autophagic lysosomes (Yang et al., 2008). Nevertheless, this correlates with p62 data and suggests that even though lysosomal production is partially inhibited, HeLa cells are able to attempt to discard any cytoplasmic constituents damaged by cisplatin treatment. It was briefly mentioned in chapter 4 that lysosomes are utilised by cancer cells as intermediary compartments for the storage of cisplatin (Molenaar et al., 2000, Safaei et al., 2005a) in order to distribute it to efflux systems, which include vesicles (Eaton, 2002 Safaei, 2005). The increase in lysosomal production may be for this reason, or for their use in the formation of autophagolysosomes.

In conclusion, bafilomycin treatment alone significantly and successfully inhibits autophagy in HeLa cells.

CaSki

mTOR expression in both bafilomycin treated groups decreased significantly (Fig. 6.2.18. A). Interestingly, a synergistic effect was observed in the combination group which exhibited a further decrease in intracellular mTOR levels, therefore the presence of cisplatin was likely to play a role in the activation (inhibition) of mTOR. Even though this activation is further upstream, its effects are observed in p62 protein levels downstream in the combination group (Fig. 6.2.18. B), which is indicative of p62 degradation and autophagy. Bafilomycin alone had no effect on p62 protein levels; however treatment with cisplatin seemed to partially disrupt its anti-autophagic activity and is suggested by a significant decrease in intracellular protein levels.

LC-3 II levels were significantly increased in both bafilomycin treated groups, however a synergistic effect due to the addition of cisplatin was not observed (Fig. 6.2.18. C). A study conducted by Lui and colleagues observed that the addition of 3-MA prior to cisplatin treatment decreased the conversion of LC-3 I to LC-3 II in oesophageal squamous cell carcinoma (Liu et al., 2011), which is in contrast to this study. The concentration of cisplatin utilised in the afore mentioned study was 1.5

mg, which is especially high, therefore the decrease in the autophagic response may be due to apoptosis induction that bypasses autophagy. The results obtained as mentioned above which analyse the effect of bafilomycin with cisplatin treatment on mTOR protein levels has not been addressed in the literature, thus there is limited information to compare these results with.

The intracellular lysosomal acidic compartment, as mentioned with the use of flow cytometry indicates the effectiveness of bafilomycin in inhibiting lysosomal biogenesis in CaSki cells, and this effectiveness is maintained after treatment with cisplatin (Fig. 6.2.18. D). Due to this, it cannot be deduced that the increase in p62 degradation is due to the increased presence of lysosomes.

It may be concluded that bafilomycin as a single treatment agent inhibits mTOR to the same extent as cisplatin, but is able to prevent p62 and LC-3 II degradation efficiently in CaSki cells. The addition of cisplatin after pre-treatment with bafilomycin appears to abolish its anti-autophagy effects. Its subsequent effects on cell death induction will be addressed in the following section.

6.3.7. The effect of autophagy inhibition with bafilomycin in combination with cisplatin treatment on cell death induction in three cervical cell lines.

Ect1/E6E7

The inhibition of autophagy with and without cisplatin had no effect on the MTT reductive capacity of Ect1/E6E7 cells (Fig. 6.2.19. A.), thus bafilomycin A1 as a single treatment agent does not disturb mitochondrial functioning, and the presence of cisplatin equally has no effect. It has been speculated that bafilomycin A1 may have an alternative function besides that of inhibiting vacuolar ATPase. In neuronal cells it was observed to maintain mitochondrial integrity under autophagic stress (Shacka et al., 2006). This may explain the efficient MTT reductive capacity (cell viability) observed in these cells in both bafilomycin A1 treated groups.

Caspase-3 and -7 activity increased 4.49 fold in comparison to the control which is indicative of their cleavage and apoptosis induction (Fig. 6.2.19.B). Here, bafilomycin A1 pre-treatment enhanced cisplatin-induced caspase activity. This is the first indication that Ect1/E6E7 cells induce autophagy as a potential survival mechanism

in the face of cisplatin treatment. These results are in agreement with recent studies conducted on renal proximal tubule cells (Periyasamy-Thandavan et al., 2008, Kaushal et al., 2008, Inoue et al., 2010, Pallet and Anglicheau, 2009, Takahashi et al., 2012).

PARP cleavage levels correspond to caspase activity observed in Figure 6.2.16. B. due to the fact that caspase-3 was found to be responsible for the cleavage of PARP (Nicholson, Tewari et al., 1995). Bafilomycin and cisplatin act synergistically to produce a 2.35 fold increase in PARP cleavage when compared to the control (Fig. 6.2.19. C). LDH release increased significantly to about 31% (Fig. 6.2.19. D.), which indicates a significant stress response (Keilhoff and Wolf, 1993) in these cells. Permeabilisation of the plasma membrane, thus allowing for the inclusion of PI was occuring in only 16.92% of the non-cancerous Ect1/E6E7 cells treated with the combination of cisplatin and bafilomycin (Fig. 6.2.19.E).

Minor levels of cell death was observed in these non-cancerous cells which approves this method of inducing cell death as appropriate, especially for the purposes of this study.

HeLa

At the end of the treatment period, HeLa cells were observed to have a slight but significant decrease in MTT reductive capacity when treated with the combination of bafilomycin A1 and cisplatin, suggesting minor mitochondrial dysfunction (Fig. 6.2.20. A). A study conducted by Shingu and colleagues observed that bafilomycin treatment alone decreased mitochondrial membrane potential by an insignificant 3.43% (Shingu et al., 2009), which suggests that its effects on mitochondria are negligible. This result is supported by a study which observed that bafilomycin alone did not affect the MTT reductive capacity in glioma and fibrosarcoma cell lines, however the combination of bafilomycin and cisplatin reduced this capacity to below 40% of the control (Harhaji-Trajkovic et al., 2009). The concentration of cisplatin that was used was 50 µM, which as a single agent, reduced the MTT reductive capacity to 50% of the control.

The first indication that HeLa cells require autophagy in order to survive cisplatin treatment was observed in Figure 6.2.20.C. Here pre-treatment with bafilomycin and subsequent treatment with cisplatin increased PARP cleavage significantly in

comparison to the control and the other treatment groups. Bafilomycin improved the cytotoxicity of low-dose cisplatin in HeLa cells. There is very little, to no information regarding the effects of bafilomycin A1 treatment in particular, and cisplatin treatment in inducing PARP cleavage in HeLa cells, thus there these results cannot be contextualized with others. However, this is observed with other autophagy inhibtors such as 3-MA and chloroquine (discussed below).

The percentage of LDH release increased by almost 40% in the combination treatment group (Fig. 6.2.20.D). In a study conducted by Harhaji-Trajkovic and colleagues, bafilomycin treatment alone increased LDH release in mouse fibrocarcoma, rat glioma and human glioma cell lines; however the concentration used was 10 times higher than the concentration used in this study (Harhaji-Trajkovic et al., 2009). This provides further evidence that bafilomycin A1 treatment in this study induces low levels of cytotoxicity. More importantly is the percentage of cells positive for PI inclusion where bafilomycin A1 and cisplatin acted in a synergistic manner to induce cell death (Fig. 6.2.20.E).

It can be concluded that HeLa cells likely require autophagy in order to survive cisplatin treatment and that the up-regulation of autophagy functions as a resistance mechanism in these cells. Pre-treatment with bafilomycin, and therefore the inhibition of autophagy, sensitises cervical adenocarcinoma cells (HeLa) efficiently to cisplatin treatment. The literature agrees with this finding; however the use of bafilomycin does not feature prominently in a setting such as this. Instead, autophagy inhibition through the use of 3-MA and chloriquine is better documented. A recent study observed that the combination of cisplatin (6 µg/ml) with 3-MA or chloriquine enhanced cisplatin cytotoxicity in HeLa cells (Xu et al., 2012b). Similarly, efficient cell death was observed in metastatic skin carcinoma when autophagy was inhibited with 3-MA and subsequently treated with 20 µg/ml cisplatin (Claerhout et al., 2010). Hepatocarcinoma cells underwent efficient cell death in response to combination treatment with 3-MA and 8 µg/ml cisplatin (Guo et al., 2012). Indeed, these studies have found success in inducing cell death through the inhibition of autophagy, however the concentrations of cisplatin used in each study are based on the IC_{50} and are therefore already toxic to the cancer cells. In contrast, it has been demonstrated in the present study that the inhibition of autophagy is sufficient to enhance the cytotoxicity of a low, non-toxic dose of cisplatin in HeLa cells.

CaSki

The effect of the combination of bafilomycin and cisplatin treatment was effective in decreasing CaSki cell viability (Fig. 6.2.21. A). An additive effect was observed which decreased cell viability by almost 50%. The success of this result is highlighted by a study where bafilomycin pre-treatment with subsequent addition of 50 μM cisplatin (IC_{50}) reduced MTT to just below 40% of the control (Harhaji-Trajkovic et al., 2009). This concentration is over 3 times that of the concentration used in this study and as a single agent induces a decrease of 50% in the MTT reductive capacity. This in turn was associated with an increase in caspase -3 and -7 activity and cleavage was observed (Fig. 6.2.21. B and C). As with the HeLa cells, this result indicates a reliance of the CaSki cells on autophagy as a mechanism of survival in the face of cisplatin treatment.

LDH release increased by 35.58% and the percentage of cells positive for PI increased by 31.83%.(Fig. 6.2.21. E. and F). Taken together, the above results suggest strongly that pre-treatment with an autophagy inhibitor effectively sensitises the aggressive CaSki cells to a low concentration of cisplatin treatment.

6.3.8. The effect of bafilomycin and cisplatin treatment on modulation of the cell cycle.

Ect1/E6E7

Ect1/E6E7 cells do not appear to be affected by the combination treatment and continue to grow normally (Fig. 6.2.22).

HeLa

The response of HeLa cells to cisplatin exhibit the characteristic shift from G1/G0 into the S phase that is commonly observed after cisplatin treatment (Qin and Ng, 2002, Sorenson and Eastman, 1988b, Shah and Schwartz, 2001). Interestingly, even though apoptosis was observed when HeLa cells were treated with the combination of bafilomycin and cisplatin, no significant shifts between the cell cycle phases was observed (Fig.6.2.23). The distribution of cells between the phases in the combination group resemble those in the cisplatin treatment group, thus this is likely due to cisplatin treatment alone. This lack of arrest is not beneficial to cancer cells

due to the fact that they utilise this mechanism to their advantage in order to repair any damage that may have been incurred during treatment (Shah and Schwartz, 2001).

CaSki

Cell cycle analysis of the aggressive CaSki cells did not exhibit any change of the cell cycle, even though apoptosis was actively occurring (6.2.24.). This is a promising result as arrests, particularly in the S phase result in a 2-fold decrease in sensitivity to cisplatin (Donaldson et al., 2006) and may induce resistance against apoptosis (Mueller et al., 2006).

6.3.9. The effect of the combination of ATG5 silencing and cisplatin treatment on autophagic induction in three cervical cell lines.

Bafilomycin is known as an inhibitor of autophagy (Yamamoto et al., 1998, Klionsky et al., 2008), however it blocks autophagy far downstream and therefore still allows for the initial molecular aspects to occur. Biological inhibition of autophagy using ATG5 siRNA may therefore be better suited as a method of inhibition as it has a specific target and prevents the maturation of autophagosome (Mizushima et al., 1998). Hence, ATG5 silencing will be adressed in the following chapter.

Ect1/E6E7

Silencing of ATG 5 inhibited autophagy in Ect1/E6E7 cells as demonstrated in Figure 6.2.25. The lack of p62 degradation is observed in both groups where ATG5 was silenced, indicating that autophagasome maturation was (partially) disrupted. Similar results were observed in primary human keratinocytes, a cell line very similar to Ect1/E6E7 cells; however shRNA ATG5 were used instead (Lee et al., 2011). ATG 5 silencing was also observed to increase p62 protein levels in baby mouse kidney cells (Mathew et al., 2009). These results support the afore mentioned findings in the Ect1/E6E7 cells and confirm the autophagy inhibitory effect of ATG5 siRNA in non-cancerous cell lines. LC-3 II expression was as expected: silencing alone did not alter LC-3 II expression when compared to that of the control; however, the addition of cisplatin induced a slight increase in its expression (Fig. 6.2.25. C). Collectively these results confirm the inhibition of autophagy this *in vitro* model of non-cancerous Ect1/E6E7 cells with the use of ATG5 siRNA.

HeLa

The effect of the silencing of ATG 5 on HeLa cells prevented the degradation of p62, as observed by the increase in the intracellular protein levels in both silencing groups (Fig. 6.2.26.. B). LC-3 II protein levels remained low in the silenced group that had not been treated with cisplatin (Fig. 6.2.26.. C). Unexpectedly, the addition of cisplatin induced an increase in autophagosome production (Fig. 6.2.26.. C). It is important to mention that silencing only partially inhibits the translation of mRNA into protein, therefore a small amount of translation is still able to occur. This may explain the increase in autophagosomal production in the combination group.

CaSki

The inhibition of ATG5 prevented the degradation of p62 significantly and is indicative of decreased autophagosome production and function (Fig. 6.2.27.. B).

6.3.10. The effect of ATG5 silencing combined with cisplatin treatment on apoptotic induction in three cervical cell lines.

Ect1/E6E7

Cell viability (MTT) was reduced in the Ect1/E6E7 cells in the combination group (Fig. 6.2.28.A). A study on the effect of ATG 5 silencing and cisplatin treatment demonstrated a significant increase in caspase -3 activity in renal tubule epithelial cells (Yang et al., 2008). This may be comparable to this result on the one hand, but on the other hand, the concentration of cisplatin utilised was 50 µM, more than three times the concentration that was used in the present study. LDH release was significantly elevated in the combination group and it is clear here that the addition of cisplatin played a role in this stress induced response (Fig. 6.2.28.C).

Overall, the results obtained with biological inhibition of autophagy compared to those obtained with pharmacological inhibition are relatively similar. More importantly however, the effect on cell death in this non-cancerous cell line is very limited. It may be argued that the low concentration of cisplatin chosen (15 µM) may have contributed to this positive result. A gap in the literature has now been addressed since it has been demonstrated here for the first time that this particular treatment protocol has very little effect on the integrity of a non-cancerous cell type.

HeLa

The effects of autophagy inhibition with the use of ATG 5 siRNA plus cisplatin decreased MTT reductive capacity in HeLa cells by almost 56% (Fig. 6.2.29. A) compared to a 14.5% decrease with the use of bafilomycin and cisplatin (Fig. 6.2.5.A). Interestingly, caspase -3 and -7 activity increased significantly in response to biological inhibition of autophagy with cisplatin treatment (Fig. 6.2.29. B). If this result is compared to the effect of the pharmacological agent bafilomycin, effector caspase activity was comparable to that of the control (Fig. 6.2.5. B).

The biological inhibition of autophagy with the use of ATG5 markedly improved cisplatin cytotoxicity in HeLa cells and underlines the importance of the caution one should be reminded of when using chemical agents. The effect observed with the use of these agents should be verified with the appropriate siRNA or shRNA.

CaSki

The converse was observed in CaSki cells after the analysis of cell viability (MTT): only a 26% decrease in reductive capacity was observed with the use of biological autophagy inhibition in conjunction with cisplatin treatment, compared to 49% decrease in MTT reductive capacity with the use of bafilomycin as an autophagy inhibitor (Fig. 6.2.30. A).

Caspase activity increased and was detectable through Western blotting, which indicates the effectiveness of this method in inducing apoptosis. Additionally, silencing of ATG5 alone induced a significant increase in caspase-3 cleavage. PARP cleavage was equally high (Fig. 6.2.30. D) in the combination group LDH release increased to 61% in comparison to 35% after treatment with bafilomycin and cisplatin.

It may therefore be concluded that autophagy is utilised by these cervical cancer cells as a survival mechanism after treatment with a low concentration of cisplatin and efficient apoptotic cell death is induced in both cancerous cell lines whether autophagy is inhibited pharmacologicaly or biologically. Furthermore, the effects of cell death on the non-cancerous cell line is minimal, and therefore proves to be an effective method for the treatment of cervical cancer

7

The Role of Bcl-2 in Cisplatin Treatment in Cervical Cancer Cells.

7.1. Introduction and Aims

The interaction between Beclin-1 and Bcl-2 proteins is an important point of convergence between the autophagic and apoptotic pathways respectively. Due to the functional interaction between Bcl-2 and Beclin-1 and its role in the regulation autophagy, ratio analysis of Beclin-1 to Bcl-2 as means of detecting the role of autophagy within the cell under homeostatic and treatment/stress conditions is recommended (Pattingre and Levine, 2006a). Additionally, Bcl-2 has a prominent role in the malignant cell and has been found to confer resistance in a variety of cancerous cell lines (Campos et al., 1993, Cho et al., 2006, Michaud et al., 2009, Miyashita and Reed, 1993). The response of Bcl-2 to a low concentration of cisplatin and its effect on preventing apoptosis in cervical cancer cells has not yet been assessed.

Therefore, the aims of this chapter are i) to evaluate the basal levels of Bcl-2 and Beclin-1 in HeLa and CaSki cell lines, ii) to determine the relationship (ratio) between Beclin-1 and Bcl-2 under control conditions and in response to cisplatin treatment to determine whether their interaction may indicate cellular resistance, and finally iii) to silence Bcl-2 to further define its role during cisplatin treatment.

7.2. Results

7.2.1. The Beclin-1 and Bcl-2 expression levels under basal (NT) as well as cisplatin treated (T) conditions in HeLa and CaSki cells.

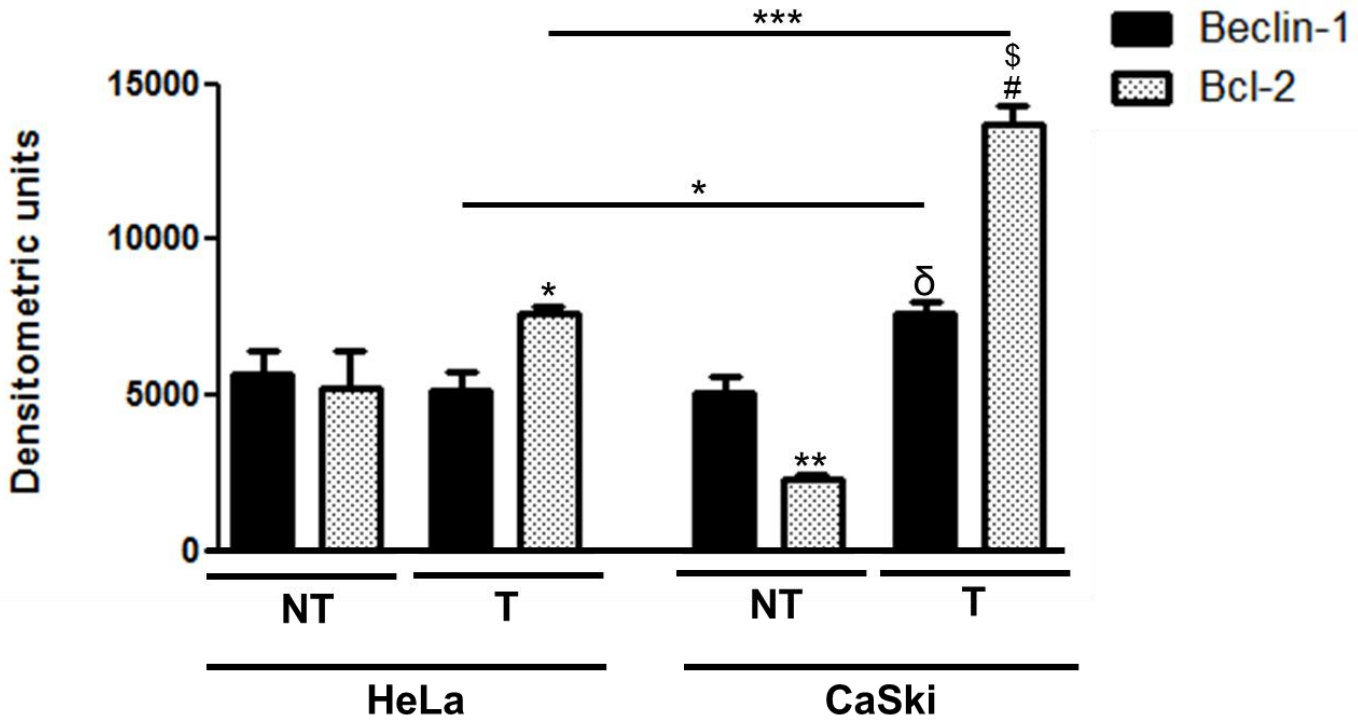
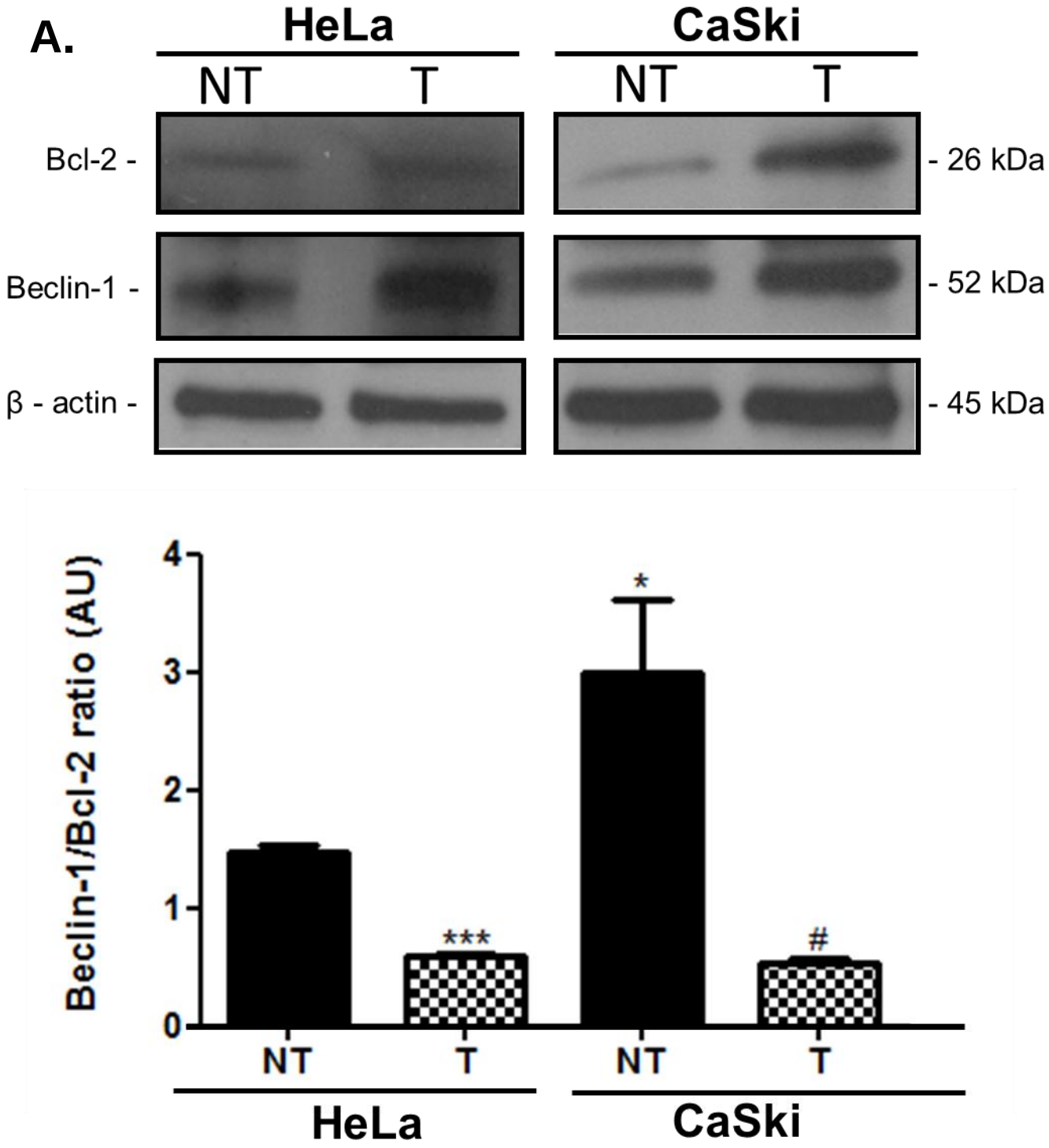


Figure 7.2.1. Densitometric analysis of Beclin-1 and Bcl-2 protein levels in non- treated (NT) and treated (T) HeLa and CaSki cells as obtained through densitometric analysis of Western blots. Bcl-2 and Beclin-1 protein levels in HeLa and CaSki cells were analysed for changes in expression after the addition of 15 µM cisplatin for a period of 24 h. Analysis within each cell line: *p<0.05 vs. Beclin-1 T (HeLa), ** p < 0.01 vs. Beclin-1 NT (CaSki), # p < 0.001 vs. Beclin-1 T (CaSki), $ p < 0.001 vs. Bcl-2 NT (CaSki) and δ p < 0.05 vs. Beclin-1 NT (CaSki), analysis between cell lines: * p < 0.05, *** p < 0.001, n = 3.

Bcl-2 and Beclin-1 protein expression levels were analysed before (NT) and after (T) treatment with cisplatin for a period of 24 h (Fig. 7.2.1. A). After treatment with cisplatin, Bcl-2 protein levels increased significantly in HeLa cells (7648 ± 183.40 * $p < 0.05$ vs. Beclin-1 T (HeLa). At basal levels, Bcl-2 protein expression was significantly lower than Beclin-1 protein levels in CaSki cells (2314 ± 122.80 ** $p < 0.01$ vs. Beclin-1 NT (CaSki). The addition of cisplatin (T) induced a significant increase in Beclin-1 and Bcl-2 protein levels in CaSki cells (7656 ± 353.80 vs. Beclin-1 NT (CaSki), δ $p < 0.05$, 13673 ± 590.1, # $p < 0.001$ vs.Bcl-2 NT (CaSki) and $ $p < 0.001$ vs. Beclin-1 T (CaSki).

Comparisons *between* the two cervical cancer cell lines revealed that Bcl-2 and Beclin-1 protein expression in CaSki cells significantly increased in response to cisplatin treatment when compared to the treated HeLa cells (7656 ± 353.80 vs. Beclin-1 T (HeLa), * $p < 0.05$ and 13673 ± 590.10 vs. Bcl-2 (T) HeLa, *** $p < 0.001$).

7.2.2. The Beclin-1/Bcl-2 ratio in the response to cisplatin treatment in HeLa and CaSki cells.



B.

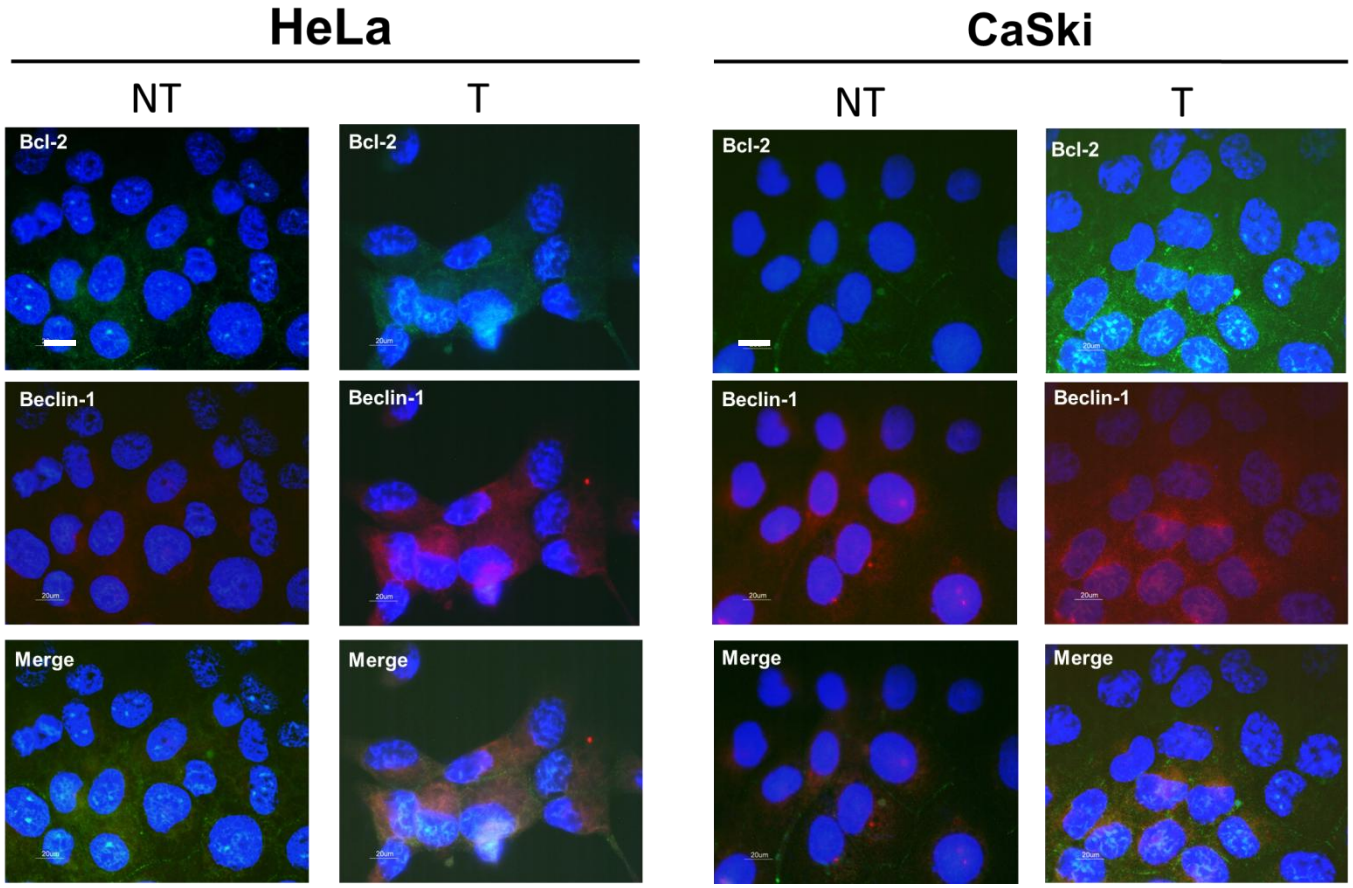


Figure 7.2.2. Analysis of Beclin-1 and Bcl-2 protein levels under basal conditions as well as in response to cisplatin treatment (15 µM; 24 h). A. Analysis of the Beclin-1/Bcl-2 ratio in HeLa and CaSki cells not treated (NT) or treated (T) with 15 µM cisplatin for 24 h, * $p < 0.05$ and *** $p < 0.001$ vs. HeLa NT, # $p < 0.01$ vs. NT (CaSki), n = 4. **B.** Representative immunofluorescent images of HeLa and CaSki cells depicting expression of Bcl-2 and Beclin-1 under basal conditions (NT) and in response to cisplatin treatment (T). Blue- Hoechst 33342, green- Bcl-2 and red- Beclin-1. Scale bar = 20 µm, n = 3.

Analysis of the Beclin-1/Bcl-2 ratio reveals that HeLa cells treated with cisplatin display a significant decrease in the Beclin-1/Bcl-2 ratio in comparison to the HeLa control cells (NT) (0.59 ± 0.05 vs. HeLa (NT), *** $p < 0.001$, Fig. 7.2.2. A.). CaSki cells exhibit a significantly higher ratio at basal levels in comparison to the HeLa cells (3.4 ± 0.80 vs. HeLa NT, * $p < 0.05$) After treatment, the ratio decreases significantly in comparison to the CaSki control cells (NT) 0.53 ± 0.05 vs. CaSki NT. # $p < 0.01$). The presence of Beclin-1 and Bcl-2 proteins was analysed with the use of immunofluorescence with and without the addition of cisplatin (Fig. 7.2.2. B.). Images represent Beclin-1 and Bcl-2 expression determined through Western blotting in Figure 7.2.2. A. Both figures indicate an increase in Beclin-1 and Bcl-2 protein expression increases in response to cisplatin treatment.

7.2.3. The effect of Bcl-2 silencing in HeLa and CaSki cells on intracellular Beclin-1 protein levels.

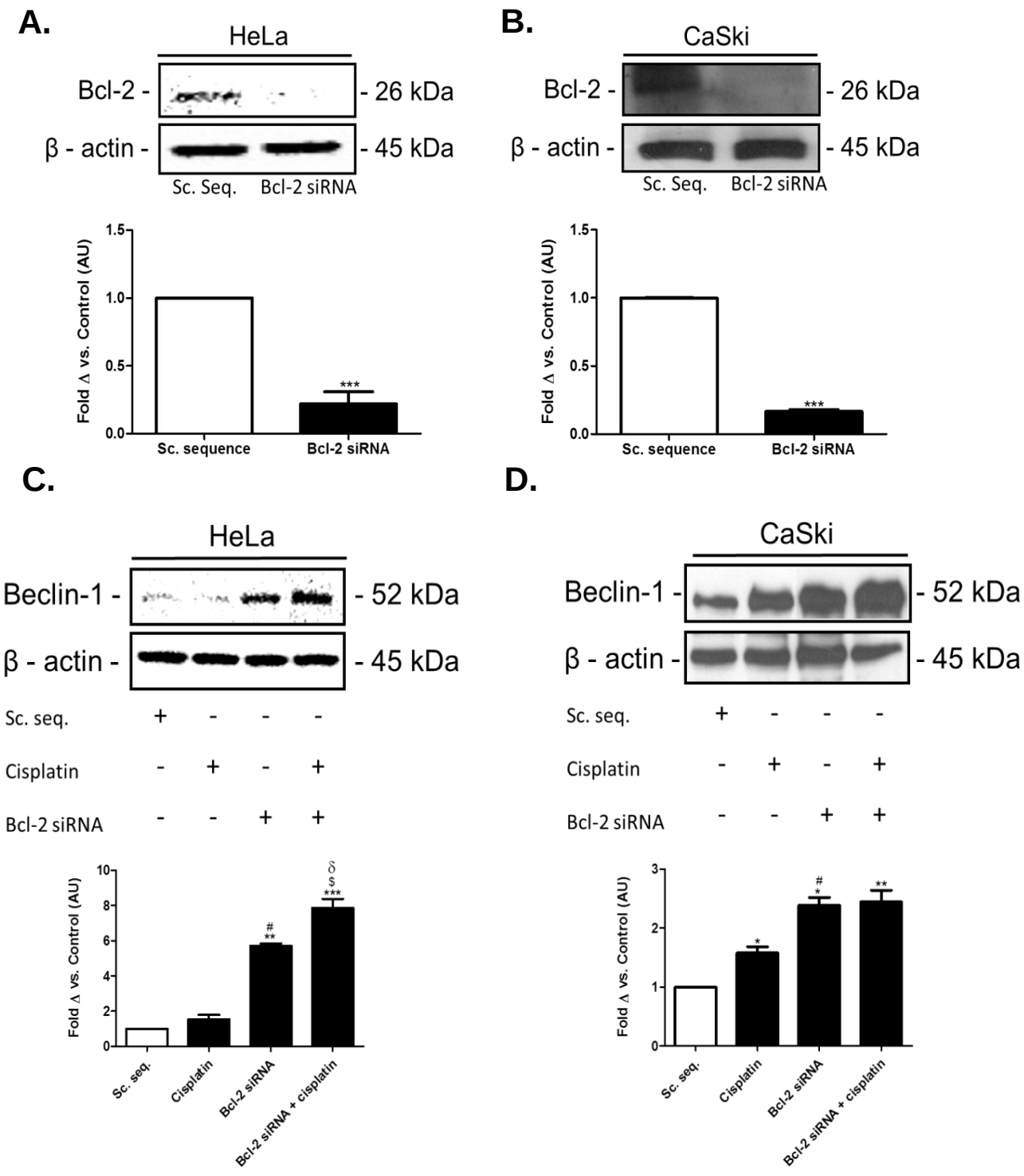


Figure 7.2.3. Silencing of Bcl-2 in HeLa and CaSki cells and resulting expression levels of Beclin-1. A. and **B.** Silencing of Bcl-2 confirmed through Western blotting, *** $p < 0.001$, n = 3. **C.** Beclin-1 protein expression in HeLa cells, ** $p < 0.01$, *** $p < 0.001$ vs. Sc. seq. (control), # $p < 0.01$ and $ $p < 0.001$ vs. cisplatin and δ $p < 0.05$ vs. Bcl-2 siRNA, n=3. **D.** Beclin-1 protein expression in CaSki cells, * $p < 0.05$, ** $p < 0.01$ vs. Sc. seq. (control) and # $p < 0.05$ vs. cisplatin, n=3.

Silencing of Bcl-2 was confirmed through the Western blotting technique in both HeLa (0.22 ± 0.09 vs. sc. seq., *** $p < 0.001$) and CaSki cells (0.17 ± 0.02 vs. sc. seq., $p < 0.001$, Fig. 7.2.3. A. and B.). Due to the important relationship between Beclin-1 and Bcl-2, it was necessary to assess Beclin-1 protein levels in response to Bcl-2 silencing with and without cisplatin treatment (Fig. 7.2.3. C. and D.). Beclin-1 protein levels increased significantly in both treatment groups where Bcl-2 was silenced in HeLa cells (5.72 ± 0.12 vs. control, * $p < 0.01$ and cisplatin # $p < 0.01$; 7.86 ± 0.52 vs. control, *** $p < 0.001$, # $p < 0.01$ vs. cisplatin and δ $p < 0.05$ vs. Bcl-2 siRNA). A similar observation was made with the CaSki cells, however cisplatin treatment alone increased Beclin-1 expression significantly (1.58 ± 0.11 vs. control, * $p < 0.05$). A significant increase was also observed in both groups where Bcl-2 was silenced (2.39 ± 0.14 vs. control, * $p < 0.05$ and # $p < 0.05$ vs. cisplatin; 2.44 ± 0.20 vs. control, $p < 0.01$).

7.2.4. The Beclin-1/Bcl-2 ratio in HeLa and CaSki cell lines after Bcl-2 silencing.

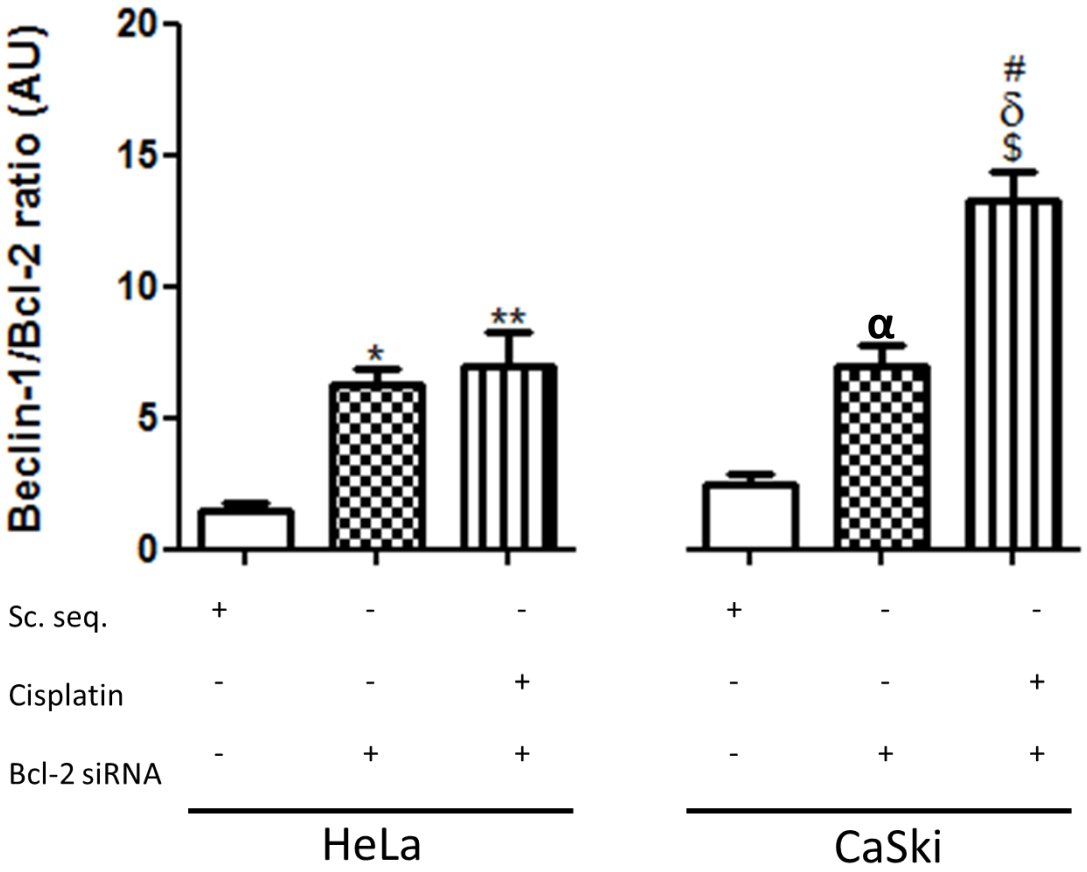


Figure 7.2.4. The Bcl-2/Beclin-1 ratio increases in HeLa and CaSki cells after the silencing of Bcl-2. Ratios were determined according to densitometric analysis of Beclin-1 and Bcl-2. * $p < 0.05$ and ** $p < 0.01$ vs. sc. Seq. HeLa; α $p < 0.05$ and $ $p < 0.001$ vs. sc. Seq. CaSki; δ $p < 0.05$ vs. Bcl-2 siRNA CaSki; # $p < 0.01$ vs. Bcl-2 + cisplatin HeLa, n = 3. Sc. Seq. = scrambled sequence (control), AU = arbitrary units.

The Beclin-1/Bcl-2 ratio was assessed after silencing of Bcl-2 in HeLa and CaSki cells (Fig.7.2.4.). The ratio increased significantly in both silencing groups in HeLa cells (6.23 ± 0.64 and 6.97 ± 1.27 vs. sc. seq. HeLa, * $p < 0.05$ and ** $p < 0.01$). The silencing of Bcl-2 in CaSki cells increased the Beclin-1/Bcl-2 ratio significantly in comparison the scrambled sequence (7.00 ± 0.80 vs. sc. seq. CaSki, α $p < 0.05$). The addition of cisplatin in this group increased the ratio significantly in comparison to the scrambled sequence (13.25 ± 1.15 vs. sc. seq. CaSki, $ $p < 0.001$), Bcl-2 siRNA (13.25 ± 1.15 vs. Bcl-2 siRNA CaSki, δ $p < 0.05$) and in comparison to Bcl-2 siRNA + cisplatin ratio in HeLa cells (13.25 ± 1.15 vs. Bcl-2 siRNA + cisplatin HeLa, # $p < 0.01$).

7.2.5. The effect of Bcl-2 silencing on apoptosis during cisplatin treatment in cervical cancer cells.

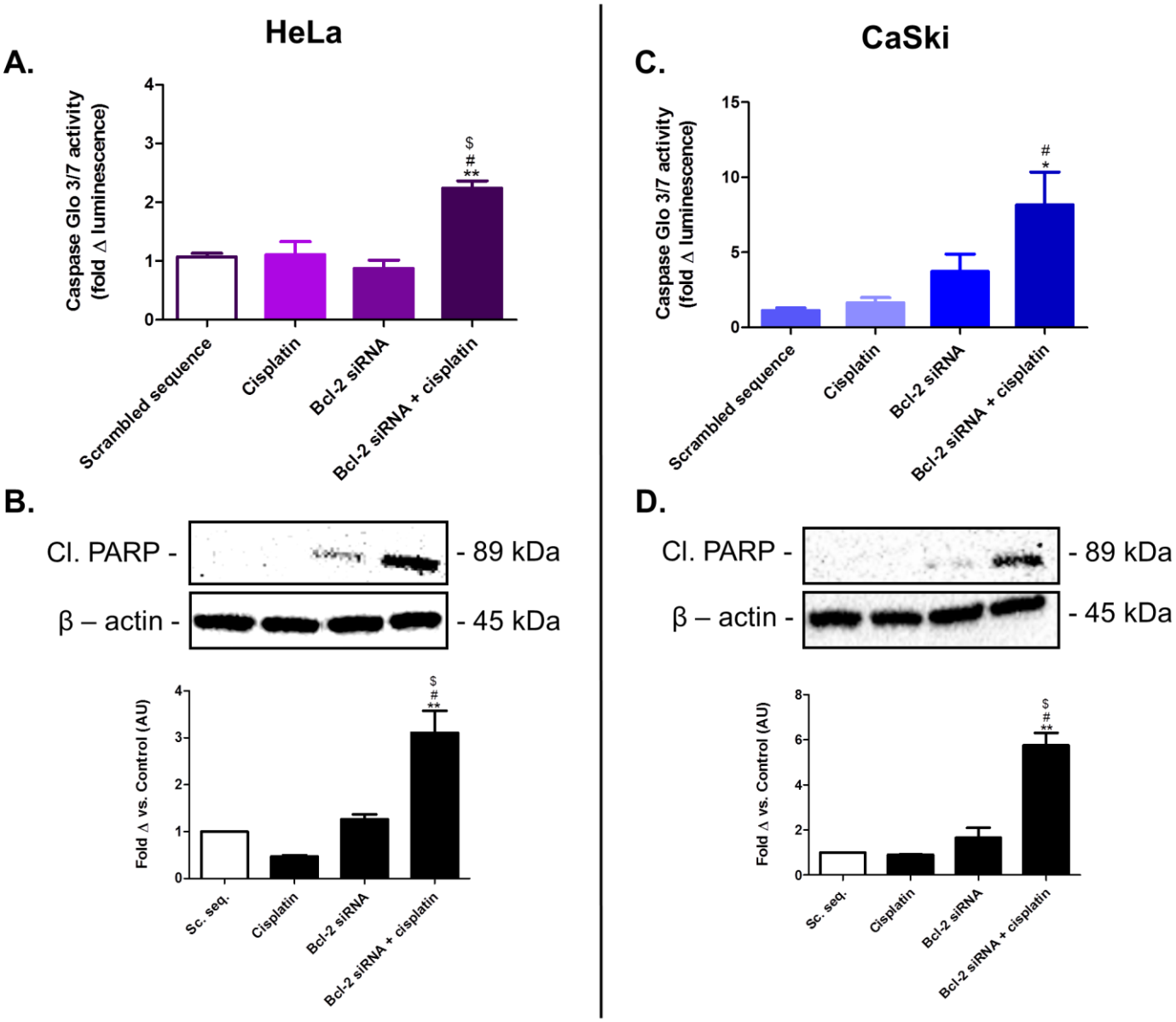


Figure 7.2.5. The effect of Bcl-2 silencing on apoptosis during cisplatin treatment in cervical cancer cells. A. Caspase -3/-7 activity, ** $p < 0.01$ vs. sc. seq. # $p < 0.01$ vs. cisplatin and $ $p < 0.01$ vs. Bcl-2 siRNA, n = 4. **B.** PARP cleavage levels, ** $p < 0.01$ vs. sc. seq., # $p < 0.001$ vs. cisplatin and $ $p < 0.01$ vs. Bcl-2 siRNA, n = 3. **C.** Caspase -3/-7, * $p < 0.05$ vs. sc.seq. and # $p < 0.05$ vs. cisplatin, n = 3. **D.** PARP cleavage, ** $p < 0.01$ vs. sc.seq., # $p < 0.01$ vs. cisplatin and $ $p < 0.01$ vs. Bcl-2 siRNA, n = 3. Sc. seq. – scrambled sequence (control), AU – arbitrary units.

Effector caspase activity was analysed as a marker of apoptosis which revealed that the silencing of Bcl-2 significantly increased cisplatin-induced caspase activation in HeLa cells (2.24 ± 0.13 vs. sc. seq., ** $p < 0.01$, vs. cisplatin, # $p < 0.01$, vs. Bcl-2 siRNA, $ $p < 0.01$, Fig. 7.2.5. A). Equally, the silencing of Bcl-2 significantly increased cisplatin-induced PARP cleavage in HeLa cells (3.11 ± 0.17 vs. sc. seq., ** $p < 0.01$, vs. cisplatin # $p < 0.001$, vs. Bcl-2 siRNA, $ $p < 0.01$, Fig. 7.2.5. B).

CaSki cells exhibited a similar response to Bcl-2 silencing with cisplatin treatment: Effector caspase activity increased significantly after Bcl-2 was silenced and cells were treated with cisplatin (8.17 ± 2.17 vs. sc. seq., * $p < 0.05$, vs. cisplatin, # $p < 0.05$, Fig, 7.2.5. C.). PARP cleavage increased concomitantly in this groups in addition to caspase activation (5.75 ± 0.55 vs. sc. seq., ** $p < 0.01$ vs. sc.seq., vs. cisplatin, # $p < 0.01$, vs. Bcl-2 siRNA, $ $p < 0.01$.

7.2.6. Comparison of caspase-3/-7 activity and PARP cleavage between HeLa and CaSki cells in response to Bcl-2 silencing with cisplatin treatment.

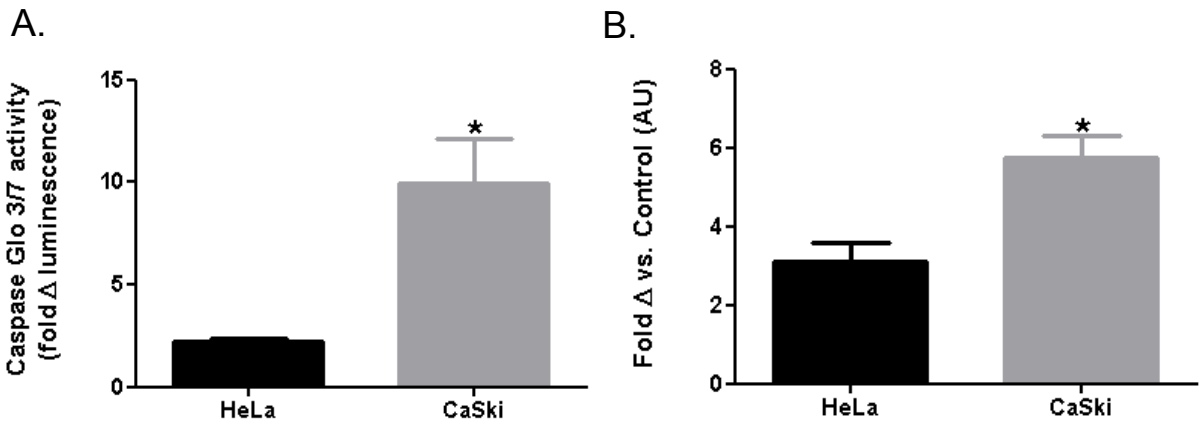


Figure 7.2.6. Comparison of caspase-3/-7 activity and PARP cleavage between HeLa and CaSki cells after Bcl-2 silencing with cisplatin treatment (15 µM, 24 h). A. Caspase activity, **p*<0.05 vs. HeLa. **B.** PARP cleavage, * *p* < 0.05 vs. HeLa. AU- arbitrary units.

CaSki cells exhibit significantly higher effector caspase activity in comparison to HeLa cells after the combination of Bcl-2 silencing and cisplatin treatment (9.95 ± 2.15, * *p* < 0.05 vs. HeLa). The comparison of PARP cleavage between the two cell lines reveal significantly increased protein levels in CaSki cells (5.75 ± 0.55, * *p* < 0.05 vs. HeLa).

7.3. Discussion

The role of Bcl-2 in response to a low concentration of cisplatin treatment in cervical cancer cells remains poorly understood, and since its effects on inducing cisplatin resistance may be involved, it is necessary to dissect the role of the anti-apoptotic protein, Bcl-2 in cisplatin resistance in HeLa and CaSki cells. It was demonstrated in previous chapters that at this particular dose and time point (15 μM; 24 h), no significant cell death occurred (Chapter 4.1), however, an autophagic response was induced (Chapter 4.2). Furthermore it was established that this autophagic response promoted the survival of cervical cancer cells during cisplatin treatment (Chapter 5). Even though the response to cisplatin-induced autophagy is Beclin-1 dependant (I.e. an increase is Beclin-1 was observed), intracellular protein levels of Bcl-2 were also observed to increase. This seems paradoxical considering the current understanding of the interaction between Beclin-1 and Bcl-2 (Pattingre et al., 2005). This suggests that an increased expression of Bcl-2, along with increased autophagy may play a role in conferring these cervical cancer cells resistant to cisplatin treatment.

Bcl-2 and Beclin-1 protein expression levels were analysed under control conditions, as well as after a 24 h treatment period with 15 μM cisplatin (Fig. 7.1.). In HeLa cells the protein expression levels of Bcl-2 and Beclin-1 were found to be similar at basal levels, however after cisplatin treatment, Bcl-2 protein levels increased significantly, and Beclin-1 levels remained unchanged. In CaSki cells a significant decrease in Bcl-2 protein levels was observed before treatment, which increased significantly after treatment with cisplatin. Basal levels of Bcl-2 have previously been demonstrated to serve as an indicator of resistance to chemotherapy: higher basal Bcl-2 levels in colon cancer cells have been shown to be indicative of increased resistance to 5-flouracil (Violette et al., 2002). This is not the case between HeLa and CaSki cell lines since the basal Bcl-2 levels tend toward a decrease in CaSki cells at the homeostatic state in comparison to the HeLa cells ($P = 0.07$), however they are more resistant to cisplatin treatment. The increased response of Bcl-2 to cisplatin treatment in both cell lines is in contrast to a study which demonstrated that over time, Bcl-2 protein levels decrease in HeLa cells in response to 40 μM of cisplatin (Maldonado et al., 1997). A possible explanation for decreased Bcl-2 protein levels in their study may be attributed to the fact that such a high concentration of cisplatin might directly induce apoptosis and counteract any form a resistance which is normally employed to attempt cellular survival. The use of a concentration similar to the one used in the

present study (4.5 µg/ml or 15 µM) revealed that after 24 h of treatment with 10 µM cisplatin, Bcl-2 expression levels did not change, however its over-expression with a vector containing full-length Bcl-2 was able to suppress cisplatin-induced apoptosis in rat neuroblastoma cells (Park et al., 2001). In bladder cancer cells however, a slight increase in Bcl-2 was observed after treatment with 10 µg/ml of cisplatin for a 24 h time period (Cho et al., 2006) which agrees with our results (Figure 7.1.). Moreover, a study demonstrated that intracellular glutathione levels are regulated partly by Bcl-2 and confer resistance of MCF-7 breast cancer cells to cisplatin treatment. Additionally it was observed that at low concentrations, Bcl-2 was able to induce resistance even in the presence of BSO which is an inhibitor of the rate-limiting enzyme responsible for glutathione synthesis (Rudin et al., 2003). These studies point to a probable role for Bcl-2 as a mechanism of resistance to cisplatin in HeLa and CaSki cells at low-concentrations of cisplatin. To our knowledge, the increased expression of Bcl-2 in CaSki cells after treatment with a low-concentration of cisplatin has not been documented before. Our data strongly suggest that at this concentration, cervical cancer cells up-regulate Bcl-2 as a mechanism by which the cells survive cisplatin treatment. Referring back to chapter 4 where both HeLa and CaSki cells induced autophagy in response to cisplatin treatment, and considering the relationship between Beclin-1 and Bcl-2 on autophagy (Pattingre et al., 2005), it may indicate that Beclin-1 and Bcl-2 are acting independently of another: Beclin-1 as a pro-autophagy protein, and Bcl-2 as an anti-apoptotic protein. It is likely that when forming a complex, the above mentioned functions are still maintained. In support of this notion, it was observed that Bcl-2 was still able to exert its anti-apoptotic function even while being bound to Beclin-1 (Ciechomska et al., 2009). Co-immunoprecipitation is required for future studies in order to validate this claim.

Due to the importance of the functional interaction between Bcl-2 and Beclin-1 and its role in the regulation autophagy, ratio analysis of Beclin-1 to Bcl-2 as means of detecting the role of autophagy within the cell under homeostatic and treatment/stress conditions is recommended (Pattingre and Levine, 2006a). Our data indicate that the Beclin-1/Bcl-2 ratio is significantly higher in the CaSki cells when compared to the HeLa cells at basal levels (Fig. 7.2.2. A.). This is indicative of an increased basal autophagic activity (Pattingre et al., 2005), which agrees with results obtained in Chapter 4.2., where basal levels of CaSki cells exhibited increased LC-3 II protein levels (Fig. 4.6.). After cisplatin treatment, this ratio decreased significantly

in both cervical cancer cell lines, which may indicate a decrease in autophagic activity. This is contrast to other autophagic markers that were analysed which demonstrated that cisplatin treatment alone is sufficient to induce autophagy (chapter 4). Both Beclin-1 and Bcl-2 protein levels increased (Fig.7.2.1.). Bcl-2 may therefore play an alternative role at this particular cisplatin concentration in cervical cancer cells, one which is independent of Beclin-1 interaction, and therefore autophagic control.

Qualitative images depicting Bcl-2 and Beclin-1 expression levels in non-treated (NT) and treated (T) cells confirm data generated through western blot analyses of the expression levels of the individual proteins, however, treatment with cisplatin was observed to induce the accumulation of Bcl-2 in the nuclei of both HeLa and CaSki cells (Fig.7.2.2. B). This may be indicative of apoptosis, since its nuclear translocation may prevent it from exerting its anti-apoptotic effects, i.e. complexing with bax at the mitochondrial membrane. A similar response was observed in neuronal nuclei of cells that were damaged through hypoxia-ischaemia (Zhu et al., 2010), which suggests that nuclear translocation occurs in cells which are undergoing cell death. This reiterates the notion that in this *in vitro* model, Bcl-2 and Beclin-1 work independently.

In order to further elucidate this potential independent role of Bcl-2, silencing was performed. Bcl-2 was efficiently silenced in both HeLa and CaSki cells (Fig. 7.2.3. A. and B.), after which Beclin-1 expression was assessed. In HeLa cells, Beclin-1 expression was significantly elevated in both silencing groups, and the addition of cisplatin synergistically elevated this expression when compared to the silencing of Bcl-2 alone (Fig. 7.2.3. C.). A similar result was observed in the CaSki cells where silencing of Bcl-2 induced an up-regulation in Beclin-1 expression, however in this case, the addition of cisplatin after Bcl-2 silencing had no additional effect on increasing Beclin-1 protein levels (Fig. 7.2.3. D.). These results infer that Bcl-2 does control Beclin-1 expression to a certain extent (i.e. bound to Beclin-1), particularly in response to cisplatin treatment in HeLa cells, i.e. cisplatin treatment alone did not substantially increase Beclin-1 as observed when Bcl-2 was silenced. Nevertheless, silencing of Bcl-2 up-regulated Beclin-1 expression in both cervical cancer cell lines which agrees with a study that Bcl-2 silenced MCF-7 breast cancer cells increased Beclin-1 expression, and after 72 h resulted in autophagic cell death (Akar et al., 2008).

The above results prompted the re-analysis of the Beclin-1/Bcl-2 ratio in the context of Bcl-2 silencing (Fig. 7.2.4.). The ratio increased significantly in both cell lines with and without the treatment of cisplatin. However in CaSki cells, the addition of cisplatin exhibited a synergistic effect in increasing this ratio. In order to interpret the above results to make a distinction between autophagy acting as a survival mechanism and autophagy as a mechanism of cell death, Beclin-1 expression is required to be elevated for the latter to occur (Shimizu et al., 2004). This observation is similar to our findings where a significant increase in Beclin-1 protein levels, as well as Beclin-1/Bcl-2 ratios were observed in HeLa and CaSki cells after silencing of Bcl-2 with and without additional treatment with cisplatin.

Analysis of apoptotic death markers suggests a role of Bcl-2 as a mechanism which delays the onset of apoptosis, thereby conferring resistance to cisplatin treatment. Both HeLa and CaSki cells displayed increased caspase -3/-7 activity in response to the combination of Bcl-2 silencing and cisplatin treatment (Fig. 7.2.3. A and C.). Equally indicative of cell death induction was a significant increase in PARP cleavage in both cell lines (Fig. 7.2.3. B. and D.). Since the up-regulation of Bcl-2 in response to cisplatin was significantly higher than that of the HeLa cells (Fig. 7.1.), it may be argued that their ability to resist the intracellular effects of cisplatin is particularly dependent on the up-regulation of Bcl-2. Figure 7.2.6. demonstrates that the silencing of Bcl-2 sensitises CaSki cells to cisplatin-induced apoptosis to a much greater extent than in the HeLa cells.

Taken together, the results obtained on the Beclin-1/Bcl-2 ratio after silencing, as well as PARP cleavage and caspase activity, cell death *with* autophagy (Galluzzi et al., 2009, Levine and Yuan, 2005) may be occurring in this *in vitro* model, as the presence of apoptosis is evident. Silencing of Bcl-2 improved sensitivity to cisplatin treatment in bladder cancer cells (Cho et al., 2006), melanoma cells (Wacheck et al., 2003), ovarian cancer cells (Wang et al., 2009), non-small lung cancer cells (Losert et al., 2007) and lung adenocarcinoma cells (Huang et al., 2007), however its effect on cervical cancer cells using a low concentration of cisplatin has not been addressed in the literature.

In summary, we demonstrate that in cervical cancer cells, Bcl-2 is up-regulated as a potential means of providing partial resistance against a low concentration of cisplatin treatment. Silencing of Bcl-2 is able to greatly improve cisplatin sensitivity in both cell

lines, particularly in the aggressive CaSki cells. Furthermore, we have demonstrated that the exaggerated up-regulation of Bcl-2 by CaSki cells in response to cisplatin treatment is likely to contribute to (in combination with autophagy) the aggressive, metastatic nature of this cell type. Therefore, our results confirm another 'side-effect' of cisplatin treatment, i.e. dependence on Bcl-2 expression for cellular survival. This method, in combination with autophagy inhibition as pre-treatments is a promising method to greatly improve the chemotherapeutic value of a low concentration of cisplatin in a cervical cancer setting *in vitro*, and is recommended as a future study.

8

The Expression Profile of Bcl-2, mTOR and LC-3 in the Various Stages of Cervical Cancer Development.

8.1. Introduction and Aims

Due to the fact that cervical cancer is characterised by a well-defined pre-malignant phase (Ambros and Kurman, 1990) without an immediate onset that commonly occurs with other cancers, it offers an opportunity for the assessment of molecular changes that may occur during the transformation phase.

Due to the role of autophagy and apoptosis in cancer, the aim of this chapter is to assess the expression profiles of mTOR and LC-3 and one anti-apoptotic marker (Bcl-2) during the various stages of tissue transformation as this has not been addressed in the literature.

8.2. Results

8.2.1. The mTOR expression profile in normal, low-grade and high-grade squamous intraepithelial lesion tissue and cancerous cervical tissue.

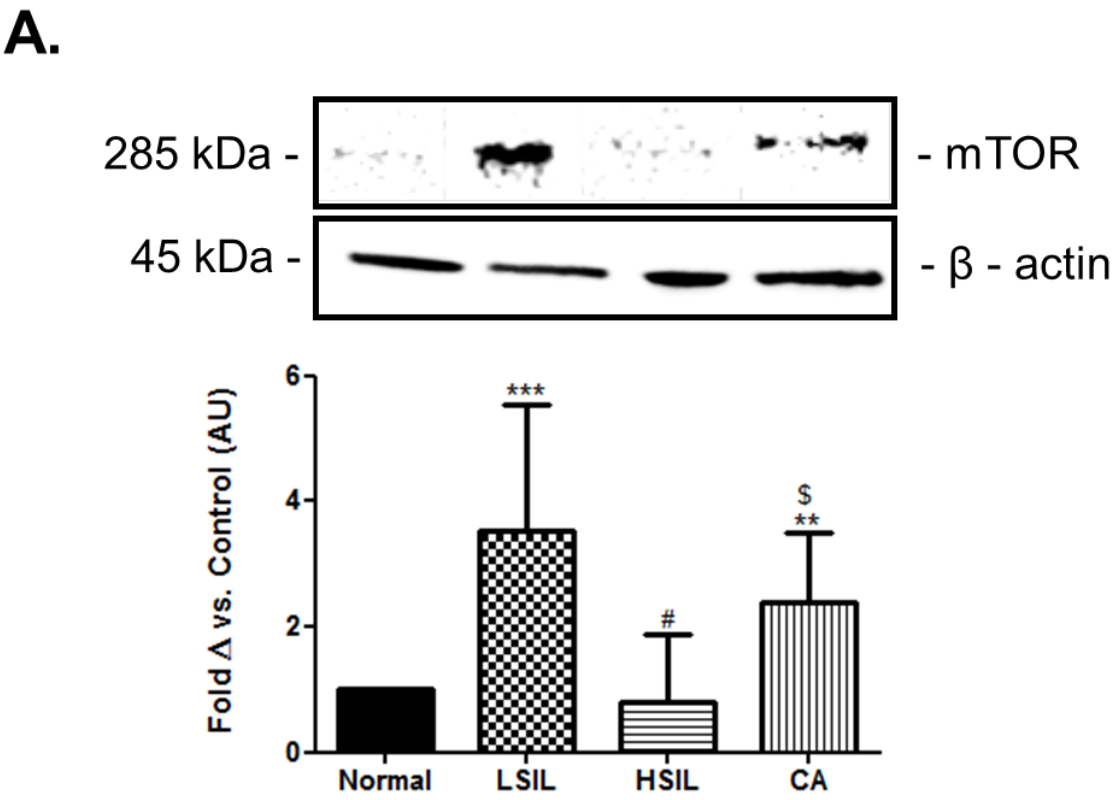


Figure 8.2.1.A. The mTOR expression profile in normal, low-grade and high-grade squamous intraepithelial lesions and cervical carcinoma tissue in cervix. Representative Western blot, ***$p<0.001$, ** $p < 0.01$ vs. normal, # $p < 0.001$ vs. LSIL, $ $p < 0.001$ vs. HSIL. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM, Normal: n=10, LSIL: n=29, HSIL: n=33, CA: n=13. LSIL: low-grade squamous intraepithelial lesion, HSIL: high-grade squamous intraepithelial lesion, CA: carcinoma.

B.

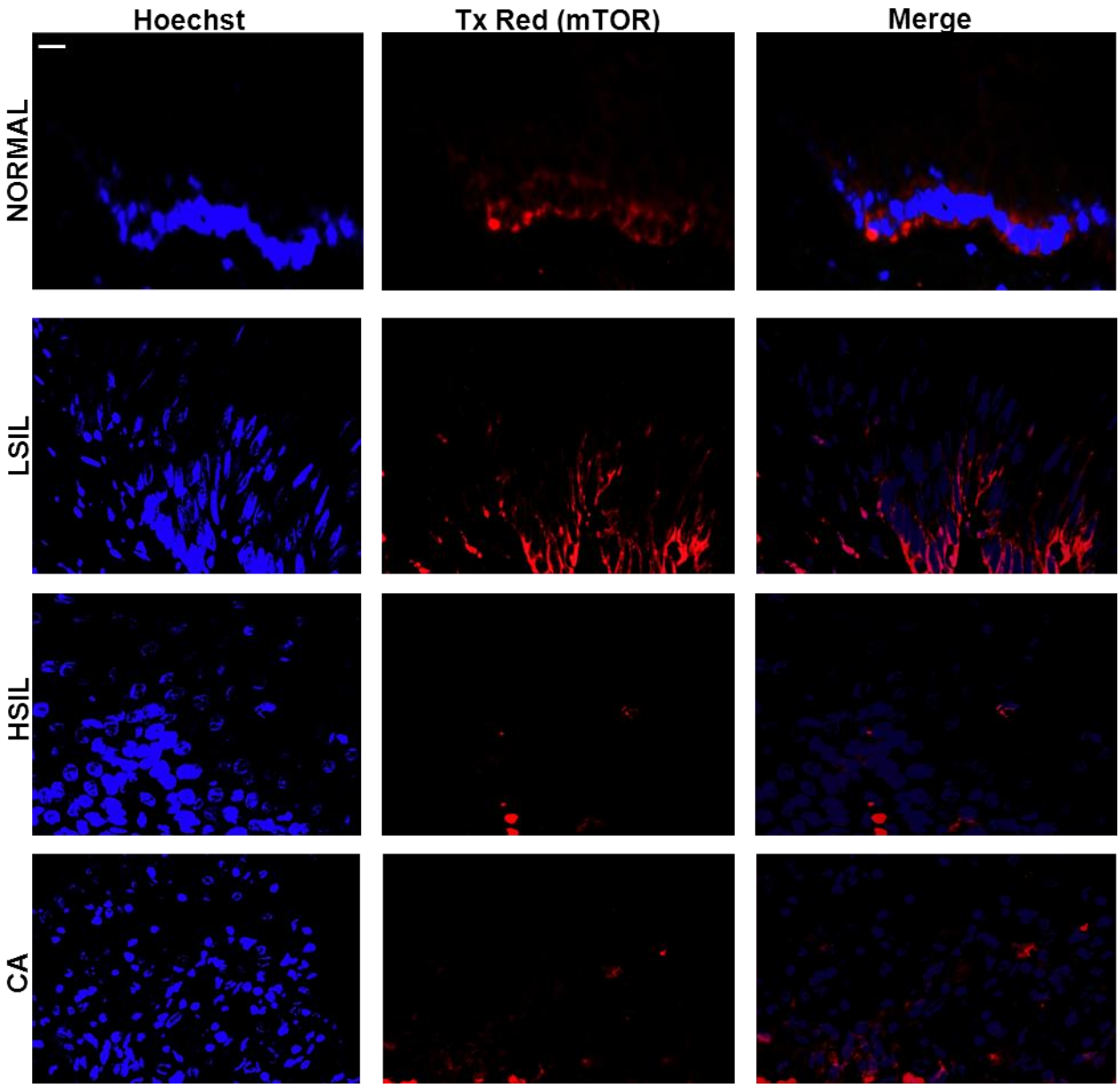


Figure 8.2.1.B. Immunohistochemistry of mTOR (red) in normal, LSIL, HSIL and CA. Representative images of mTOR expression profiles in normal (n=4), LSIL (n=6), HSIL (n=8) and cervical carcinogenesis (n=5), scale bar: 20 µm. LSIL – low-grade squamous cervical carcinoma, HSIL – high-grade squamous cervical carcinoma, CA- carcinoma.

mTOR protein expression was significantly increased in low-grade squamous intraepithelial lesions (LSIL) in comparison to the normal cervical tissue (3.52 ± 0.71 vs. normal, *** $p < 0.001$). In cervical carcinoma tissue. mTOR expression was significantly increased in comparison to the normal cervical tissue (2.39 ± 0.29 vs. normal, ** $p < 0.01$ vs. normal) and high-grade squamous intraepithelial lesion (HSIL) tissue (2.39 ± 0.29 vs. HSIL, $ $p < 0.001$). HSIL cervical tissue had significantly decreased expression levels of mTOR in comparison to the LSIL cervical tissue (0.81 ± 0.24 vs. LSIL, # $p < 0.001$, Fig. 8.2.1. A).

The expression of mTOR was assessed with the use of immunohistochemistry on tissue sections that are representative of each stage the progression towards cervical cancer development. Fluorescent images suggest an increase in mTOR expression in LSIL (Fig. 8.2.1. B).

8.2.2. The Bcl-2 expression profile in normal, low-grade and high-grade squamous intraepithelial lesion tissue and cancerous cervical tissue.

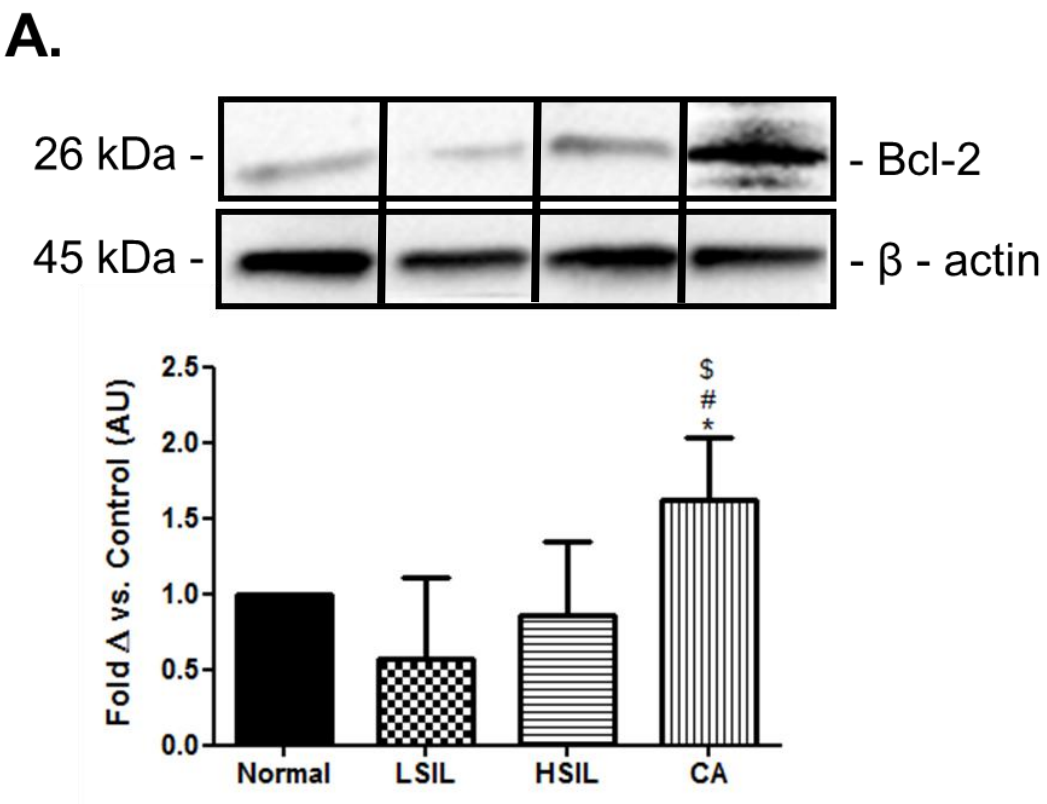


Figure 8.2.2.A. The Bcl-2 expression profile in normal, low-grade and high-grade squamous intraepithelial lesions and cervical carcinoma tissue in cervix. Representative Western blot, * p < 0.05 vs. normal, # p < 0.001 vs. LSIL, $ p < 0.05 vs. HSIL. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM, normal (n=4), LSIL (n=6), HSIL (n=8) and cervical carcinogenesis (n=5). LSIL: low-grade squamous intraepithelial lesion, HSIL: high-grade squamous intraepithelial lesion, CA: carcinoma.

B.

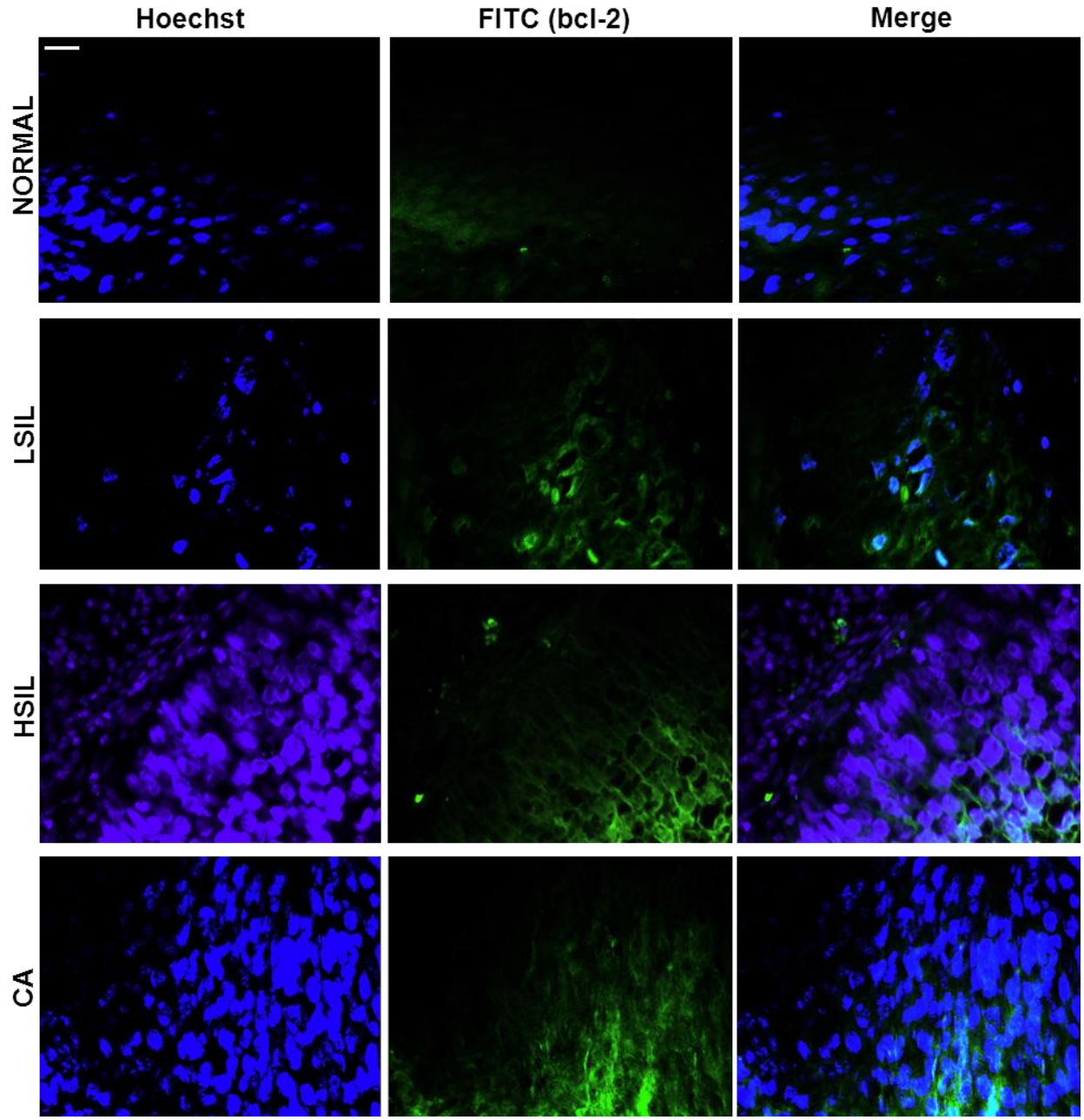


Figure 8.2.2. B. Immunohistochemistry of Bcl-2 (green) in normal, LSIL, HSIL and CA. Representative images of Bcl-2 expression profiles in normal, LSIL, HSIL and cervical carcinogenesis, scale bar: 20 µm. LSIL – low-grade squamous cervical carcinoma, HSIL – high-grade squamous cervical carcinoma, CA- carcinoma.

Bcl-2 expression was significantly increased in cervical carcinoma tissue in comparison to normal tissue (1.62 ± 0.16 vs. normal, * $p < 0.05$), LSIL tissue (1.62 ± 0.16 vs. LSIL, # $p < 0.001$) and HSIL tissue (1.62 ± 0.16 vs. HSIL, $ $p < 0.01$, Fig. 8.2.2. A).

Immunohistochemistry revealed that cervical carcinoma tissue had increased Bcl-2 protein expression (Fig. 8.2.2. B).

8.2.3. The LC-3 expression profile in normal, low-grade and high-grade squamous intraepithelial lesion tissue and cancerous cervical tissue.

A.

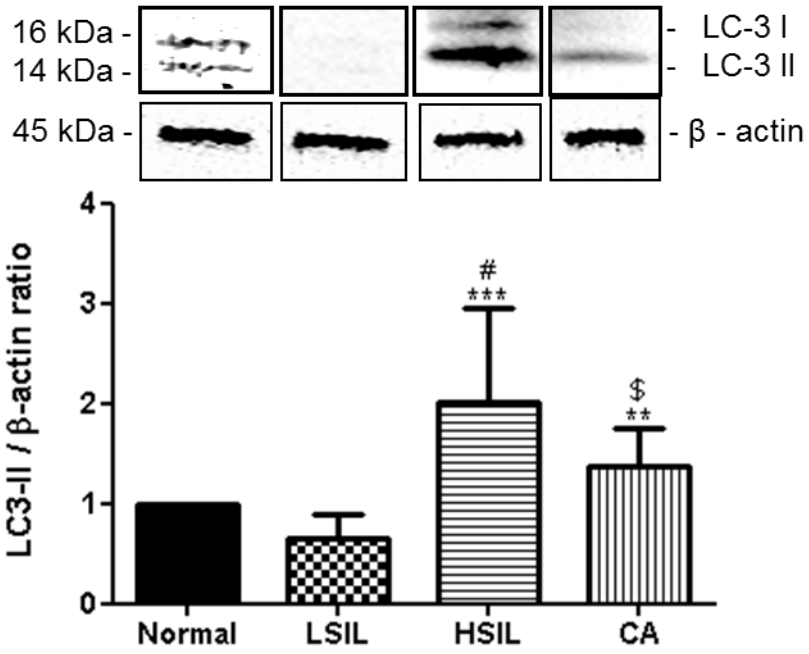


Figure 8.2.3.A. The LC-3 II expression profile in normal, low-grade and high-grade squamous intraepithelial lesions and cervical carcinoma tissue in cervix. Representative Western blot, *** $p < 0.001$, ** $p < 0.01$ vs. normal, # $p < 0.001$ vs. LSIL, $ $p < 0.05$ vs. LSIL. Statistical analysis: ANOVA and Bonferroni correction. All results expressed as mean ± SEM, Normal: n=10, LSIL: n=29, HSIL: n=33, CA: n=13. LSIL: low-grade squamous intraepithelial lesion, HSIL: high-grade squamous intraepithelial lesion, CA: carcinoma.

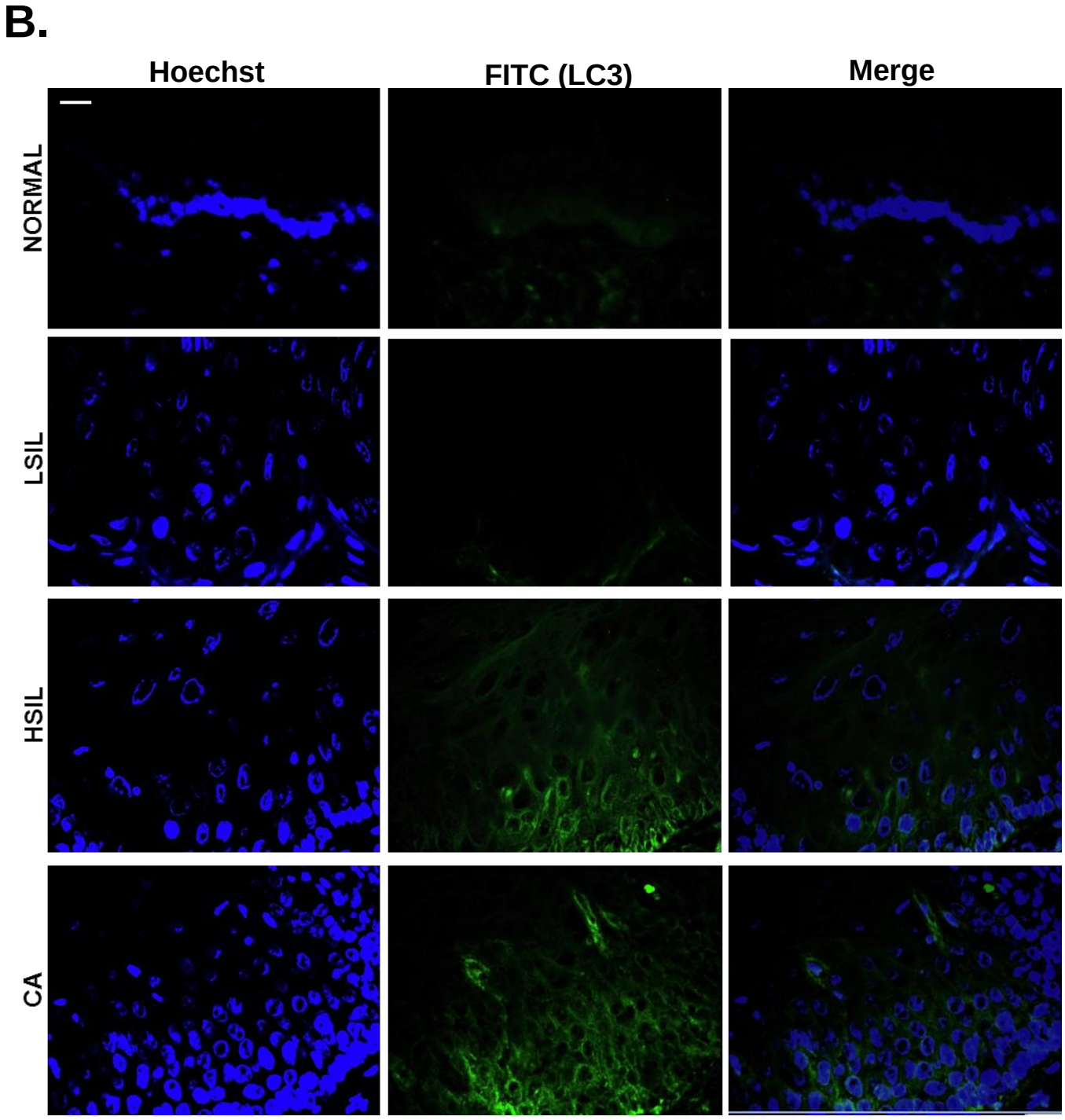


Figure 8.2.3.B. Immunohistochemistry of LC-3 (green) in normal, LSIL, HSIL and CA. Representative images of LC-3 expression profiles in normal (n=4), LSIL (n=6), HSIL (n=8) and cervical carcinogenesis (n=5), scale bar: 20 µm. LSIL – low-grade squamous cervical carcinoma, HSIL – high-grade squamous cervical carcinoma, CA- carcinoma.

Western blot analysis revealed an increase in LC-3 II protein levels in HSIL tissue in comparison to normal cervical tissue (2.01 ± 0.31 vs. normal, ***$p < 0.001$) and LSIL tissue (2.01 ± 0.31 vs. LSIL, # $p < 0.001$). Cervical carcinoma tissue also has significantly increased LC-3 II protein levels in comparison to normal cervical tissue (1.37 ± 0.14 vs. normal, ** $p < 0.01$) and LSIL tissue (1.37 ± 0.14 vs. normal, $ $p < 0.05$, Fig. 8.2.3. A).

Immunohistochemistry of reveal that LC-3 expression is increased in HSIL and cervical carcinoma tissue sections (Fig. 8.2.3. B).

8.3. Discussion

Cervical cancer is characterised by a well-defined pre-malignant phase (Ambros and Kurman, 1990) which is attributed to infection with the human papilloma virus (HPV). These pre-malignant phases are characterised by low- and high-grade squamous intraepithelial lesion (LSIL and HSIL) development after infection with the virus (Nobbenhuis et al., 1999). The lesions that develop become a platform for the pathological expansion of the cancer and compromise the control of proliferation and differentiation of the tumour cells (Green and Evan, 2002). Central to the proliferation of the tumour cells is the aberrant alteration of survival pathways and changes in the apoptotic threshold of these cells.

The importance of autophagy in cervical cancer cells has been demonstrated in previous chapters. Additionally, basal autophagic levels as well as flux capacities proved to differ considerably between normal and cancerous cervical cell types, which enabled to point out each cancer cell type as likely being either resistant or sensitive to undergo cell death upon autophagic modulation. It is therefore of interest to examine clinical specimens for the presence of autophagy at various pathological stages and possibly utilise the results obtained from the *in vitro* data as a means of determining whether adjuvant treatment in combination with a low-dose of cisplatin would be effective in a clinical setting of cervical cancer treatment.

Little is known regarding the role of autophagy and apoptosis in the development of cervical cancer through the analysis of clinical specimens, therefore the aim of this chapter was to assess the role of autophagy through mTOR and LC-3 II protein expression as well as apoptosis through Bcl-2 expression in the progression from normal to cancerous cervical tissue.

8.3.1. mTOR

The molecular link between mTOR and cancer was discovered when it was observed that the TSC complex (mutations of which cause the hamartomatous syndrome) upstream of mTOR negatively regulates its activity (Inoki et al., 2002). Additionally, it had been observed that the mTOR pathway is constitutively activated in a variety of cancers (West et al., 2002), which subsequently results in the inhibition of mTOR and the induction of a variety of processes, including autophagy (Loewith et al., 2002). The analysis of mTOR protein expression in normal, LSIL, HSIL and cancerous

cervical tissue revealed that both the LSIL and cancerous cervical tissue had increased mTOR protein expression levels in comparison to the normal cervical tissue (Fig. 8.2.1.A) whereas HSIL cervical tissue had significantly lower mTOR expression levels in comparison to both LSIL and cancerous cervical tissue, and was comparable to the control. To support this observation, activated mTOR (lowered total mTOR) was observed to be present in HSILs (Feng et al., 2009), however, its activation was also observed in squamous cell carcinoma (Feng et al., 2009), advanced squamous cell carcinoma (Faried et al., 2008a) and adenocarcinoma of the cervix (Faried et al., 2008a). Since mTOR inhibition results in the activation of autophagy, it may be argued that this process is activated in normal and HSIL tissue, more so than in LSIL and cancerous cervical tissue. The observation that mTOR levels are significantly increased in the cancerous tissue when compared to the normal tissue may suggest that autophagy has an important role in normal cellular homeostasis. This notion is supported by the fact that under normal conditions, autophagy is activated in order to prevent genomic instability and other oncogenic events from taking place (Abedin et al., 2007, Katayama et al., 2007). In cancer, protein synthesis is required to outweigh protein degradation in order for tumour growth (Otsuka and Moskowitz, 1978), and the expression of mTOR is therefore required to increase cell size and mass (Fingar et al., 2002). Additionally, during the early stages of tumourigenesis, autophagic degradation and proteolysis were observed to be lower when compared to their non-cancerous counterparts (Gunn et al., 1977, Kisen et al., 1993, Kirkegaard et al., 2004). In order to support this statement however, a more direct marker of autophagy, such as LC-3 II is required to be assessed.

8.2.2. Bcl-2

Analysis of cervical pre-malignant and malignant tissue revealed that Bcl-2 protein expression was significantly increased in cervical carcinoma tissue samples (Fig. 8.2.2. A) and suggests that the malignant state requires such an alteration in order to maintain the malignant phenotype. The importance of proliferation in cancer, and therefore a decreased ability to undergo apoptosis underlines the characteristic nature of cancer. Bcl-2, an anti-apoptotic protein, is thus a crucial regulator of apoptosis inhibition in this diseased state.

A systematic survey and meta-analysis of the transcriptional profiles of a variety of cancers indicated that the dysregulation of Bcl-2 is a key distinguishing factor between normal and cancer cells (Slavov and Dawson, 2009), moreover, its increased expression has been correlated with increased resistance of a variety of cancers to chemotherapy drugs, including cisplatin (Carson and Ribeiro, 1993, Campos et al., 1993). As mentioned previously, evidence documenting the expression of Bcl-2 in cervical cancers is confounding. An increase in Bcl-2 protein expression demonstrated in this study agrees with a study conducted by Dimitrakakis and colleagues which demonstrated that Bcl-2 protein expression is directly related to the grade of cervical intraepithelial neoplasia (Dimitrakakis et al., 2000), however it is in contrast to another study which demonstrated that Bcl-2 protein expression in neoplastic cervical tissue in significantly decreased in comparison to the normal cervical tissue (Tjalma et al., 1998). A possible approach by which to define this role more clearly would be to evaluate the corresponding beclin-1 protein levels, since the interaction between these two molecules acts as a rheostat in determining the decision between autophagy and apoptosis initiation (Pattingre et al., 2005). A different, more recent study demonstrated that beclin-1 protein expression levels in cervical carcinoma was significantly lower than when compared to the normal squamous epithelial tissue (Zhu et al., 2012). This finding supports this study, as well as the study conducted by Dimitrakakis and colleagues which both suggest increased Bcl-2 protein expression levels in cervical carcinoma.

Previous *in vitro* work (Chapter 7) revealed that basal levels of Bcl-2 differ significantly between the cancerous HeLa and CaSki cells (Fig. 7.2.1.), however whether this expression is elevated compared to the normal Ect1/E6E7 cells, as observed in the tissue, remains to be determined. Following treatment with cisplatin however, Bcl-2 expression increased, and after silencing apoptosis was observed, which indicates that cervical cancer cells are dependent on Bcl-2 expression as a means of preventing apoptosis. Therefore, the *in vitro*, as well as the *ex vivo* data strongly suggest that Bcl-2 is a promising therapeutic target for the treatment of cervical cancer. Future studies may focus on silencing of Bcl-2 prior to a low-concentration of cisplatin treatment to further improve cell death *in vitro,* which may serve as an additional method for the treatment of cervical cancer.

8.2.3. LC-3 II

Western blot analysis suggests that both HSIL and cervical carcinoma tissue had increased LC-3 II protein levels in comparison to the control and LSIL tissue samples (Fig. 8.2.3. A). Due to the fact that LC-3 II is a reliable, direct marker of autophagy (Mizushima, 2004), these results suggest the up-regulation of autophagy under both these pre-malignant and malignant circumstances. These results are supported by the down-regulation of mTOR protein expression HSIL tissue (Fig. 8.2.1.) as previously discussed; however this result is not as conclusive when mTOR expression in the cancerous cervical tissue is analysed.

The result obtained in Figure 8.2.3. is in contrast to a study which observed that cancerous cervical tissue had significantly lowered LC-3 protein levels in comparison to normal cervical tissue (Zhu et al., 2012), its expression in pre-malignant tissue was not assessed, and to our knowledge has not been evaluated before. In order to provide a possible explanation as to why autophagy may be up-regulated in pre-malignant HSILs could be due to the presence of the HPV virus: In addition to the role of autophagy as a survival mechanism, it has also been implicated as an anti-viral mechanism by which a cell rids itself of intracellular pathogens through sequestration of the viral counterparts within autophagosomes for degradation through fusion with lysosomes (Kirkegaard et al., 2004, Lee and Iwasaki, 2008, Hussey et al., 2009). Conversely, it can also be utilized and exploited by the invading virus as a method of replication or as an exit strategy, and in this case functions as a pro-viral mechanism (de Bruin and Medema, 2008). This has not been proven directly in HPV, however a study revealed that the transfection of cervical cells with HPV E6 and E7 proteins induced an ‘autophagic-like response’, and thus this may serve as a possible explanation for the increased autophagy levels. An additional reason may be that high-grade lesions develop rapidly and corresponds to morphological features of the basal cells, including a minimal amount of cytoplasm and thus a high nucleus to cytoplasm ratio (Wright, 2006). Therefore autophagy may be required to sustain this rapid growth by continuously supplying substrates for anabolic reactions.

Generally, once the cervix presents with HSILs, surgical methods are put in place for its removal, which leads to post-operative effects and discomfort (Zivadinovic et al., 2012). The fact that HSILs have increased levels of autophagy in comparison to the

control tissue (Fig. 8.2.3.) suggests that its progression toward malignancy may be controlled through the pharmacological inhibition of autophagy. Hydroxychloriquine, an anti-malarial agent and autophagy inhibitor (Rubinsztein et al., 2007) may be a possible candidate. This is however dependent on acquiring conclusive evidence that autophagy is indeed increased in HSILs. Future studies are required to determine the role of autophagy in HPV infection, as this may provide an additional avenue for the treatment of pre-malignant lesions induced by HPV infection.

The *In vitro* work conducted in Chapter 6 suggests that in cervical cancer, autophagy inhibition in conjunction with a low-concentration of cisplatin treatment is effective in inducing efficient cell death. Due to the fact that autophagy is significantly increased in cancerous cervical tissue (Fig. 8.2.3.), this method indeed indicates a promising approach for treatment of patients with cervical cancer.

In conclusion, HSIL cervical tissue exhibited increased autophagic activity when compared to the normal and LSIL cervical tissue counterparts which, to our knowledge, has been demonstrated for the first time. Additionally, cervical carcinoma tissue expresses increased Bcl-2 protein levels, as well as LC-3 II protein levels, which is indicative of suppressed apoptosis and increased autophagy respectively.

9

Summary and Conclusion

Due to the severity of the side-effects which often accompany cisplatin-based chemotherapeutic regimes, alternative treatment strategies are required. These should make use of a lower dose of cisplatin which, with the use of an adjuvant treatment, improves the chemotherapeutic value of such a dose, with minimal cytotoxic effects on non-cancerous cells. An *in vitro* model of cervical cancer was employed which made use of three human cervical cell lines: a non-cancerous ectocervical cell line (Ect1/E6E7), an adenocarcinoma cell line (HeLa) and a metastatic squamous epithelial cell line (CaSki).

Cisplatin dose-response curves revealed that 15 µM (4.5 µg/ml cisplatin) had no significant effects on cell death induction in the non-cancerous cells, and was therefore selected to be used throughout the remainder of the study. This concentration had no significant effects on cell death induction in both cancerous cell lines.

In organs that have a high-metabolic demand, the level of autophagic activity is equally high (Mizushima et al., 2004). In cancer cells, metabolism is altered in order to favour anabolic reactions as a mechanism of sustaining increased growth rates, thus basal autophagic levels and flux capacities may reveal valuable information as to the aggressiveness of various cancers, as well as their reliance on autophagy. Our data demonstrate that each cervical cell line had a differential capacity to induce autophagy upon stimulation, with the HeLa cells having the lowest capacity. This data strengthens previous findings that autophagic induction is cell type dependent. Moreover, we have demonstrated that there are also differences in the same cell type between normal and transformed cells.

It was then required that the role of autophagy be evaluated in response to the chosen concentration of cisplatin, and it was observed that autophagy was induced in all three cell lines in response to 15 µM cisplatin for a 24 h treatment period. We then determined the significance of this response through inhibiting as well as inducing autophagy prior to cisplatin treatment, and subsequently analysing a variety of cell

death markers. We have demonstrated here for the first time that the inhibition of autophagy with bafilomycin A1 or ATG5 siRNA in combination with a non-toxic concentration of cisplatin induced efficient apoptotic cell death in both cervical cancer cell lines, with minimal cell death induced in the normal, non-cancerous cells. We therefore confirm that autophagy inhibition prior to a low-concentration of cisplatin treatment significanlty improves the cytotoxicity of cisplatin in cervical cancer cells, and that cisplatin-induced apoptosis may be exploited as a means of improving the clinical efficacy of the drug.

The prominent role of Bcl-2 in cisplatin resistance in other cancer cell lines prompted the analysis of the response of this anti-apoptotic protein to a low-concentration of cisplatin treatment. Bcl-2 was up-regulated significantly in both cancerous cell lines 24 h after cisplatin treatment. Here we demonstrate the requirement of Bcl-2 up-regulation in cervical cancer cells through silencing of Bcl-2 prior to cisplatin treatment. Significant increases in cisplatin-induced apoptosis in both cell lines were observed, however the CaSki cells were affected to a larger extent. Additionally, the Beclin-1 Bcl-2 ratio increased significantly in both cell lines which indicates that cell death is occurring *with* autophagy. Our data strongly suggest that both cervical cell lines, particularly the aggressive CaSki cells are dependent on the up-regulation of Bcl-2 in response to cisplatin treatment, and that this is a distinct intracellular characteristic which may be exploited in order to improve cisplatin-induced apoptosis. Future studies are required to assess whether the effects on cell death are improved if Bcl-2 silencing together with autophagy inhibition is used prior to a low-concentration of cisplatin treatment.

In order to determine the value of the above findings, clinical specimens were collected to assess the expression profile of autophagic (mTOR and LC-3) and anti-apoptotic (Bcl-2) markers in normal, pre-malignant (LSIL and HSIL) and cancerous cervical tissue. mTOR and LC-3 II expression in the various stages of cervical cancer development suggest that autophagy is significantly up-regulated in HSILs and carcinoma of the cervix and that Bcl-2 expression, which is in agreement with other studies, is increased exclusively in cancerous cervical tissue. These data underline the relevance of the results obtained in the *in vitro* study: basal autophagic levels are increased in cervical carcinoma, which is likely to respond well to pre-treatment with an autophagic inhibitor with a low-dose of cisplatin treatment. This response may be further improved with the inhibition of Bcl-2 as an additional pre-treatment.

The value of this treatment method, if moved into the clinic, is that a low-dose of cisplatin could be used with a pre-treatment such as autophagy and/or Bcl-2 inhibition, which may then allow prolonged exposure to cisplatin chemotherapy and therefore longer treatment regimes, with minimal amount of side-effects.

10

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