EFFECT OF REACTIVE OXYGEN SPECIES IN CISPLATIN-INDUCED APOPTOTIC RESPONSE OF HCT 116 COLON CARCINOMA CELLS

by

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Submitted to Graduate School of Engineering and Natural Sciences

in partial fulfilment of

the requirements for the degree of

Master of Science

SABANCI UNIVERSITY

July 2009

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ABSTRACT

In this study primarily response to CDDP administration in terms of cell viability was examined. For demonstration of cell viability in response to CDDP treatment, MTT Assay was used in the absence or presence of the antioxidant, N-Acetyl-L-Cysteine (NAC) pre-treatment. It showed around 19% decrease in cell viability for wt and 21% for p53-/- in the absence of NAC at 24h and 30μM CDDP. It also revealed around 72% decrease for wt and 31% decrease for p53-/- in the absence of NAC at 48h and 30μM CDDP, yet in the presence of NAC the cell viability was found to be favoured by almost 20% for wt. To assess the extend of apoptotic response in the absence or presence of NAC pre-treatment Flow Cytometric Analyses by Annexin-V Labelling was applied. It revealed around 1.6 fold increase for wt and 2.4 fold increase for p53-/- in the absence of NAC at 24h and 30μM CDDP, yet in the presence of NAC the apoptotic response was found to be repressed by almost 20% for wt and 40% for p53-/- It also showed around 4.7 fold increase for wt and 2.6 fold increase for p53-/- in the absence of NAC at 48h and 30μM CDDP, yet in the presence of NAC the apoptotic response was found to be repressed by almost 57% for wt. The data showed that wt and p53-/- cells differed in cell viability depending on the dose of CDDP and antioxidant pre-treatment, indicating that some fraction of the apoptotic response was due to increased ROS levels.

In addition, DCFH-DA was exploited as the label for ROS in Flow Cytometric Analyses. Flow Cytometry showed around 4 fold increase for wt and 3.2 fold increase for p53-/- at 24h and 30μM CDDP yet in the presence of NAC the increase of ROS was found to be repressed by almost 50% for wt. It also revealed around 5.2 fold increase for wt and 5 fold increase for p53-/- in the absence of NAC at 48h and 30μM CDDP, yet in the presence of NAC the increase of ROS was found to be repressed by almost 66% for p53-/-. A second method, Thiobarbituric Acid Reactive Substances (TBARS) Assay was used to gain more insight, this time in terms of lipid peroxidation. TBARS Assay revealed 8.4 fold increase for wt and 5.9 fold increase for p53-/- in the absence of NAC at 24h and 30μM CDDP, yet in the presence of NAC the increase was found to be repressed by almost 75% for wt. It also showed around 1.5 fold increase for wt and 1.8 fold increase for p53-/- in the absence of NAC at 48h and 30μM CDDP, yet in the presence of NAC the increase was found to be repressed by around 13% for wt and 39% for p53-/-. Data acquired in this section indicated increased ROS and lipid peroxidation levels with CDDP treatment which could be overcome by NAC pre-treatment.

In further experiments, OxyBlot™ procedure was applied to total protein isolates and also to the pro-survival proteins of the Bcl-2 family proteins, Bcl-2, Mcl-1 and Bcl-x_L, fished out of the total protein suspensions via immunoprecipitation. The technique gave the picture of an increased protein carbonylation with increasing doses of CDDP (0, 30 and 60μM CDDP) and also exhibited that all three pro-survival proteins gave detectable carbonylation signal in the absence of the antioxidant and TP53 gene at 30μM CDDP, supporting the initial hypothesis of this work that loss of function of pro-survival proteins due to protein modifications contributes to apoptotic response in this experimental setup.

These results were discussed in the light of intracellular signalling cascades, especially those related to apoptosis and intracellular oxidative stress. As CDDP was found to be inducing apoptosis via affecting the overall redox status of the cell and the concept of sensitization to CDDP treatment could be an interesting approach for possible future applications of this work.

ÖZET

Bu çalışmada öncelikle, hücrelerin sisplatin uygulamasına canlılık açısından tepkisi incelendi. Hücre canlılığını göstermek için, MTT Analizi bir antioksidan olan asetilsisteinin (NAC) ön-uygulaması yapılarak ve yapılmadan kullanıldı. MTT Analizi 30μM sisplatin NAC olmadan 24h uygulandığında hücre canlılığında wt için yaklaşık %19 ve p53-/- için yaklaşık %21 azalma gösterdi. 30μM sisplatin NAC olmadan 48h uygulandığında ise, hücre canlılığında wt için yaklaşık %72 ve p53-/- için yaklaşık %31 azalma gösterdi ancak NAC verildiğinde wt için 20% artış gözlendi. Sisplatine bağlı apoptotik tepkinin boyutlarını NAC ön-uygulamasının yapıldığı ve yapılmadığı durumlarda görüntülemek için Annexin-V işaretlemesi kullanılarak Akış Sitometrisi kullanıldı. Akış Sitometrisi 30μM sisplatin NAC olmadan 24h uygulandığında apoptotik tepkide wt için yaklaşık 1.6 kat ve p53-/- için yaklaşık 2.4 kat artış gösterdi fakat NAC verildiğinde wt için 20% azalış gözlendi. 30μM sisplatin NAC olmadan 48h uygulandığında ise, apoptotik tepkide wt için yaklaşık 4.7 kat ve p53-/- için yaklaşık 2.6 kat artış gösterdi fakat NAC verildiğinde wt için 57% azalış gözlendi. Elde edilen data wt ve p53-/- hücrelerinin yaşayabilirlikte sisplatin dozu ve antioksidan ön-uygulamasına bağlı olarak değişiklik gösterdiğini bildirdi. Bu da apoptotik tepkinin bir kısmının yükselen ROT seviyeleriyle ilintili olduğunu işaret etti.

Ek olarak, Akış Sitometrisinde ROT için işaretleyici olarak DCFH-DA kullanıldı. Akış Sitometrisi 30μM sisplatin NAC olmadan 24h uygulandığında apoptotik tepkide wt için yaklaşık 4 kat ve p53-/- için yaklaşık 3.2 kat artış gösterdi fakat NAC verildiğinde wt için 50% azalış gözlendi. 30μM sisplatin NAC olmadan 48h uygulandığında ise, apoptotik tepkide wt için yaklaşık 5.2 kat ve p53-/- için yaklaşık 5 kat artış gösterdi fakat NAC verildiğinde p53-/- için 66% azalış gözlendi. İkinci bir yöntem, Tiyobarbitürik Asit Reaktif Maddeler (TBARS) Analizi, özellikle lipid peroksidasyonu hakkında daha fazla bilgi sağlamak üzere uygulandı. TBARS Analizi 30μM sisplatin NAC olmadan 24h uygulandığında wt için yaklaşık 8.4 kat ve p53-/- için yaklaşık 5.9 kat artış gösterdi fakat NAC verildiğinde wt için 75% azalış gözlendi. 30μM sisplatin NAC olmadan 48h uygulandığında ise, wt için yaklaşık 1.5 kat ve p53-/- için yaklaşık 1.8 kat artış gösterdi fakat NAC verildiğinde wt için %13 ve p53-/- için %39 azalış gözlendi. Bu bölümde elde edilen data ROT ve lipid peroksidasyonu seviyelerinin sisplatin uygulamasına bağlı olarak arttığını ve bunun aktioksidan verilerek geri çevrilebildiğini gösterdi.

Daha sonraki deneylerde, OxyBlot™ tekniği total protein izolasyonları ve immünçökeltme yöntemiyle total protein izolasyonlarından ayıklanmış Bcl-2 ailesi proteinlerinden kalım-yanlısı Bcl-2, Mcl-1 ve Bcl-x_L proteinleri için kullanıldı. Teknik artan sisplatin dozları (0, 20, 30, 60μM) ile protein karbonilasyonunun arttığını ve 30μM sisplatin ve antioksidan ile TP53 geninin yokluğu durumunda kalım-yanlısı proteinlerin üçünün de saptanabilir karbonilasyon sinyalleri verdiğini gösterdi. Bu da bu çalışmanın başlangıçtaki önsavı olan bu deney düzeneğinde kalım-yanlısı proteinlerin protein modifikasyonları nedeniyle işlev kaybına uğramalarının apoptotik cevaba katkıda bulunması fikrini destekledi.

Bu sonuçlar özellikle apoptoz ve hücre içi oksidatif stres bağlantılı sinyal kademeli dizilerinin ışığında tartışıldı. Sisplatinin hücrenin genel yükseltgenme-indirgenme durumunu etkilemek suretiyle apoptozu indüklediği bulunduğundan sisplatin uygulamasına hassaslaştırılma anlayışı bu çalışmanın gelecekteki olası uygulamaları için ilgi çekici bir yaklaşım olabilir.

ACKNOWLEDGEMENTS

The thesis you are holding in your hand right now is not exclusively my work but carries bits and pieces from all those wonderful people I have (met) in my life, particularly during my graduate studies. On the next two pages I would like to show my gratitude to these people for everything they have done for me.

First of all, I would like to thank my thesis advisor Hüveyda Başağa for this once-in-a-lifetime experience; it surely taught me a lot in every possible way and helped me to make up my mind what to do next with my life.

I also would like to express my gratitude to the jury members who spent some of their precious time for perfecting my thesis: Hikmet Budak who has been a comfortable haven to me whenever I was edgy by making things only easier for me and that exactly when I needed it the most; Batu Erman, the first person I have met at Sabancı University and probably the reason of my choice about where to conduct my graduate studies; Uğur Sezerman, an understanding and helpful educator and last but not least Alpay Taralp, an amazing person I have come to know only too late and am very grateful to his kind attitude even when he was finding snags in my work.

I couldn't have completed this work without the endless support of my family; especially of my brother Mehmet and my mother Şirin, to whom this work is solely dedicated to. I am most grateful to my father Turhan and my grandparents Mehmet and Yıldız Ateş for providing me a warm home, nurturing me with love and believing in me even in times when I was not believing in myself. I also am grateful to my aunt Aysel for putting up with me at my most irascible times and to my grandparents Hatice and Kazım Tülüce for letting me be their little spoiled granddaughter again despite my older age. Also, I am very thankful to my uncle Emin Tülüce who has supported me in hard times in every possible way although he's been thousands of miles away, to his wife Annalisa and the baby girls; Ilaria, Selin and Giada for bringing joy to my life. I love you all indescribably.

I am a very lucky person to have all these great friends I made from early childhood to graduate studies and without them I wouldn't be the person I am now.

Didar and the whole Talat family - Didem, Merih and Zübeyr – have presented me a second home where I always felt welcome and safe; with them I could be me as if I was part of the family organically. They have tolerated my ups and downs, showed me tough-love when necessary and shared my excitement, joy and worries cordially. Didar has worked equally hard with me on parts of this thesis where I did not know what and how to do anymore.

Also, the Çerçioğlu family especially Nilay Çerçioğlu Büyükkapancı and Eren Büyükkapancı have been there for me whenever I felt like giving up, I am grateful to them genially.
I would like to thank to all my other friends I grew up with and the lovely people they have brought to our lives for all the great time we had together and backing at times I have lost all strength to continue; especially to Kaya Tokmakçıoğlu I am most grateful for solving the single most crucial problem in my thesis in a very simple and clever way at a time when I was so confused and distracted and did not know any further.
Hagop Demir, Arman Kazancıoğlu, Alen Tahmizoğlu and Arda Tunç have brightened up my life on my cloudiest days and warmed my heart with their sincere affection; I am thankful.
I am very grateful to Meral Kence, my second mother, for guiding me in every aspect of life since we met in the first General Biology class at METU where I have made many of my close friends who have been great support: İnci Ayhan, Anıl Doğan, Soner Gündemir, Pınar Kafalı, Özlem Karalay and Ayça Mazman Whiting. Soner has supported me in many ways whether in shape of an earful, burst of laughters, tears of longing, an academic paper I did not have access to or just a song. I am certain and thankful that even more is yet to come.
I also would like to thank to my close friends İlkay Ayten Özel, Hande Çalışkan, Didem Tuğba Üstüner, Merve Yıldırım Budak and Servet Budak. Hande, although living thousands of miles away, has never left me alone and given me hope of a better future ahead of us. Merve and Servet have been like family; cousins, siblings or even parents. They have motivated me to do more and better and enriched my psyche.
Emre Aras, Selim Ayan, Erhat Nalbant and Övünç Üster have been patiently listening to me complain about minor things and alleviated them, I am cordially thankful.
I have met very nice people during my CCK experience at Karolinska Insitutet, Sweden; especially my supervisor Maria C. Shoshan, Joe Lawrence, lab-mates Emma Hernlund, Elvira Santic Ismail and Walid Fayyad have taught so many things and given confidence in my proficiencies; I am truthfully grateful.
Mert Akel, Aydın Albayrak, Belkıs Atasever, Bahar Soğutmaz Özdemir and Tuğsan Tezil have essentially been my life rings at SU. Mert, my fellow traveler, has put a roof over my head when I was practically homeless and has been pretty durable to my acts of temper. Tuğsan, my unofficial supervisor, has taken care of me and my cell cultures, helped me out with every issue in the lab and been a great friend to confide in. Thank you all heartily.
I hope with the closure of this chapter of my life I am headed to a better tomorrow with all these amazing people. Thank you.

Yıldız Özlem Ateş

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ABBREVIATIONS

APS	Ammonium persulfate
Bcl-2	B-cell lymphoma 2
Bcl-x_L	Basal cell lymphoma-extra large
BH	Bcl-2 Homology Domain
BSA	Bovine Serum Albumin
CDDP	cis-diamminedichloridoplatinum(II)
DCFH-DA	Dichlorofluorescein diacetate
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Endoplasmic Reticulum
EtOH	Ethanol
FBS	Fetal Bovine Serum
FM	Freezing Mixture
HCl	Hydrochloric Acid
HCT 116	human colon carcinoma cell line originated from a male
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic Acid
HRP	Horse Radish Peroxidase
KCl	Potassium Chloride
KH_2PO_4	Potassium Diphosphate
Mcl-1	Myeloid cell leukemia sequence 1
MetOH	Methanol
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular Weight
Na_2HPO_4	Disodium Phosphate
$Na_2HPO_4.12H_2O$	Disodium Phosphate Dodecahydrate
NAC	N- Acetyl-L-Cystein
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NP-40	Nonyl Phenoxylpolyethoxylethanol

p53	(tumour) protein 53
p53-/-	p53 knockout (lacking p53 expression)
PBS	Phosphate Buffered Saline
PMSF	Phenylmethanesulphonylfluoride
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulphate
TBA	Thiobarbituric Acid
TCA	Trichloroacetic Acid
TEMED	Tetramethylethylenediamine
Tiron	1,2-Dihydroxybenzene-3-5-disulfonate disodium salt
Tris	1,1,1-Tris(hydroxymethyl)-methanamide
UV	Ultraviolet
wt	wild type p53 (expressing p53)

1. INTRODUCTION

A carcinoma is any malignant cancer that arises from epithelial cells. Carcinomas invade surrounding tissues and organs and may metastasize to other sites such as lymph nodes. Although there are several different types of carcinoma, adenocarcinomas ("adeno" meaning "pertaining to a gland") which have glandular cell appearance in terms of histopathology, are very common and colorectal cancer (CRC) presents itself nearly 95% of all cases as an example to adenocarcinomas. This is because the colon has numerous glands within the tissue, which function in mucus secretion into the lumen of the colon and water absorption back into the blood, and colorectal adenocarcinomas are malignant epithelial tumours originating from glandular epithelium of the colorectal mucosa. The tissue forming these glands may undergo a number of changes at the genetic level such as loss of some genes including TP53 and move from benign (adenoma stage) to invasive, malignant CRC. [1] CRC is the third common form of cancer and the second leading cause of cancer related death in the Western World and there is a vast array of risk factors accompanying it such as exposure to some viruses, hereditary influences, smoking and diet, physical inactivity and exogenous hormones. CRC is seen in both sexes and although surgical intervention cures more than 50% of the CRC patients, recurrence is not uncommon that adjuvant (i.e. post-operation) as well as neoadjuvant (i.e. pre-operation) therapies are necessary. These include chemotherapeutics which in one way or another inhibit the growth of the neoplasm by cell cycle arrest via fixation of microtubules, targeting a specific enzyme or growth factor and/or induce programmed cell death as in apoptosis.

HCT 116, a cell line derived from the colon carcinoma of a male human patient is such an adenocarcinoma-like cell line. [2] It lacks the hMLH-1 protein, a part of the mismatch repair (MMR) system of the cell, and hence the cell is defective in DNA repair necessary for genome integrity. Deficiency in this MMR system is an advantage to tumourigenesis, as it happens with accumulation of several genomic alterations such as with enhanced proliferative capability and/or compromised programmed cell death and so increased metastatic potential. [3]

2. BACKGROUND

Cancer is thought to be caused by the interaction between genetic susceptibility and environmental toxins. Since it is not possible to eliminate all the risk factors, therapies have to be developed to treat and hopefully cure cancer. There are several different agents for cancer therapy which remove blockage to programmed cell death in a transformed cell and restore the natural autodestruction machinery by bringing the components such as regulatory Bcl-2 family proteins into action. [4]

2.1. Cancer

Cancer is the second most common cause of death in developed countries. About one third of the population contracts a cancer related disease during their lifetime and as such cancer therapy is a huge market that many investors are interested in. However, it is difficult to develop new treatment strategies, especially in the chemotherapy field mainly because cancer is a heterogeneous disease and many genetic and epigenetic factors play role in each neoplasm in the body of an affected individual. Additionally, chemicals used as antiproliferative agents are actually intended to cause cell death and this causes toxicity to the healthy tissues.

Cancer varies vastly depending on the cell type it originates from and the genetic alterations it carries. For one cell to acquire the necessary genetic changes within a reasonable time of disease progression the diseased tissue needs to be genetically unstable. Six common genetic alterations in cell physiology contributing to malignant growth have been suggested. [5]

- *Self-sufficiency in growth signals,* and/or
  Insensitivity to anti-growth signals
- *Evasion of cell death* - Apoptotic cell death is a suppressed mechanism for regulating the number of cells in an organism or cell population to keep healthy. The amplitude of proliferative or death signals decides whether or not the cell undergoes apoptosis. Tumour cells can acquire resistance to such death signals by inactivating pro-apoptotic signalling as in the case of loss of p53 expression, or increasing pro-survival Bcl-2 family protein amounts in the cell by

overexpression

- *Infinite replicative capacity*
- *Sustained angiogenesis*
- *Tissue invasion and metastasis*
- *Genome instability* - In order to gain the traits mentioned above within a reasonable time the tumour cells need to have a higher mutation rate than normal cells. This is mostly enabled by the inactivation of "genomic caretakers" such as p53 which is often found to be not-functional in cancer.

2.1.1. Genes Involved in Cancer

During carcinogenesis, the cell undergoes many genetic alterations as mentioned above. Some of these alterations are related to gain of function via specific point mutations, amplification or translocations as in the case of the oncogenes from proto-oncogene precursors. Oncogenes are dominant over the normal proto-oncogenes and change in the expression level or protein structure of these oncogenes promotes cell division independently of any external stimuli, a phenomenon critical for tumourigenesis. [6-7]

As there are oncogenes promoting the uncontrolled proliferation, there exist also tumour suppressor genes which as the name implies counteract and suppress a tumourigenic phenotype. [8] TSGs are important in many cellular functions such as apoptosis, signal transduction and DNA repair. They can be subdivided into “gatekeepers” which directly inhibit cell growth by suppressing proliferation or inducing apoptosis or differentiation, “caretakers” which ensure the fidelity of the genome through DNA repair or protection of genomic stability and the “landscapers” which are found in the mutated form in the cells surrounding the tumour and effect the microenvironment. [9] Rb and p53 are two of the best characterized TSGs in human cancers.

The TP53 gene encodes the p53 transcription factor (TF) sometimes referred to as the “guardian of the genome”. In response to stresses such as DNA damage, phosphorylation of p53 leads to its stabilization and increased activity as a transcription factor.

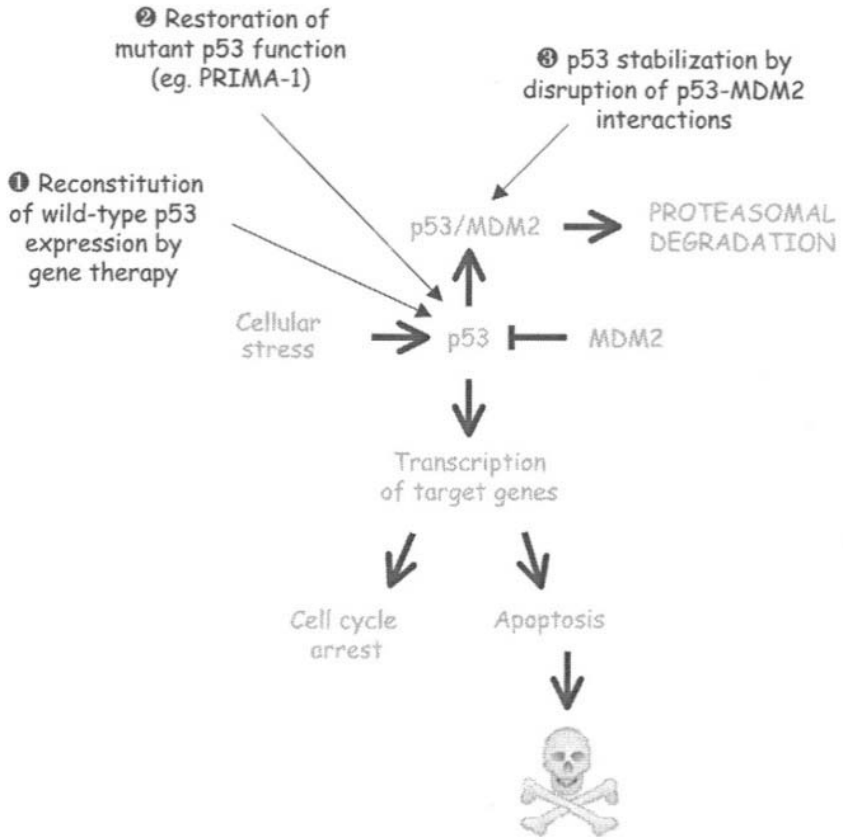


Fig. 2.a. Guardian of the genome. Cellular stress triggers accumulation of p53 and transactivation of target genes that induce cell cycle arrest or apoptosis. Almost 50% of all human cancers carry mutations in TP53. [10]

p53 also plays a central role in a cells decision to either induce cell cycle arrest or apoptosis. The p53-mediated apoptotic response involves induction of pro-apoptotic Bcl-2 family proteins including Bax, Noxa and Puma and repression of anti-apoptotic proteins such as Bcl-2. These effects together with p53-induced up-regulation of ROS generating enzymes all promote mitochondrial membrane permeabilization. [11] In addition, p53 can have transcription independent mechanisms of action activating pro-apoptotic proteins in mitochondria. [12-13] A role for caspase-2 during DNA damage induced apoptosis upstream of mitochondrial events has been established. [14] It promotes the expression of the p53-induced protein with a death domain (PIDD) which is part of a caspase-2 activating complex, the PIDDosome. [15] p53 has also been shown to induce oxidative phosphorylation by transactivating the SCO2 gene required for assembly of the cytochrome c oxidase complex, a key component of the respiratory chain. Loss of SCO2 by loss of p53, results in defective

oxidative phosphorylation, which in turn causes the metabolic shift towards glycolysis for ATP production. [16]

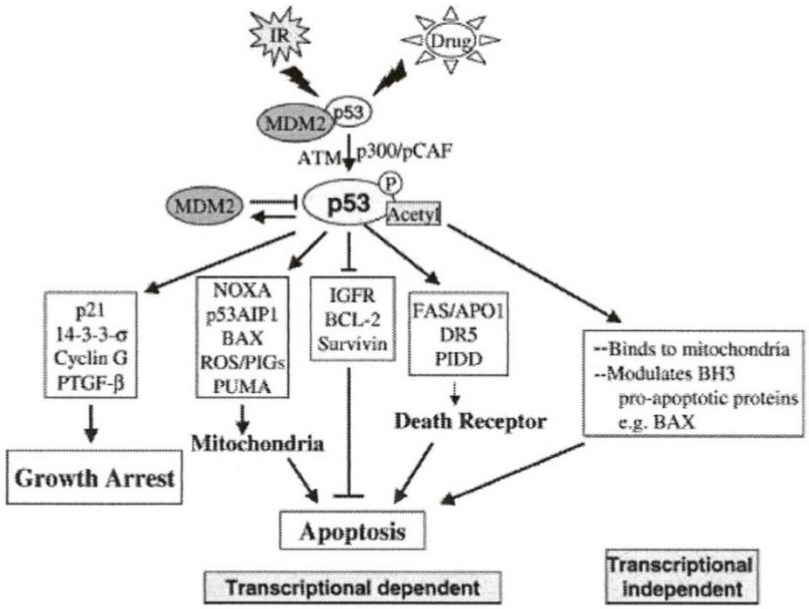


Fig. 2.b. p53 signalling pathways for growth arrest and apoptosis. Upon DNA damage, p53-MDM2 binding is dissociated as a result of p53 phosphorylation by ATM and acetylation by p300/pCAF leading to p53 activation. Activated p53 acts as a TF to transactive growth regulatory genes such as p21 to induce growth arrest. p53 regulates apoptosis in transcriptional-dependent and –independent manners, the former being with induction by transactivating the genes in both mitochondrial and death receptor pathways as well as transrepressing cellular survival genes such as Bcl-2 and the latter being with binding to mitochonia and modulates activity of BH3-containing pro-apoptotic proteins. [17]

Another gene controlled by p53 which has recently been identified, is p53-induced glycolysis and apoptosis regulator (TIGAR). TIGAR expression lowers the levels of glycolysis and induces up-regulation of the pentose phosphate pathway. This is involved in the synthesis of glutathione (GSH), which provides protection against increased ROS levels. Consequently, p53-induced expression of TIGAR lowers glycolysis and protects cells from ROS-mediated apoptosis. In tumour cells with mutated p53, TIGAR-mediated inhibition of glycolysis does not occur and this causes higher glycolytic rate of tumour tissues. [18] As a crucial "gatekeeper", p53 is the single most frequently mutated TSG in human cancer, inactivated in approximately 50% of all tumours. [19]

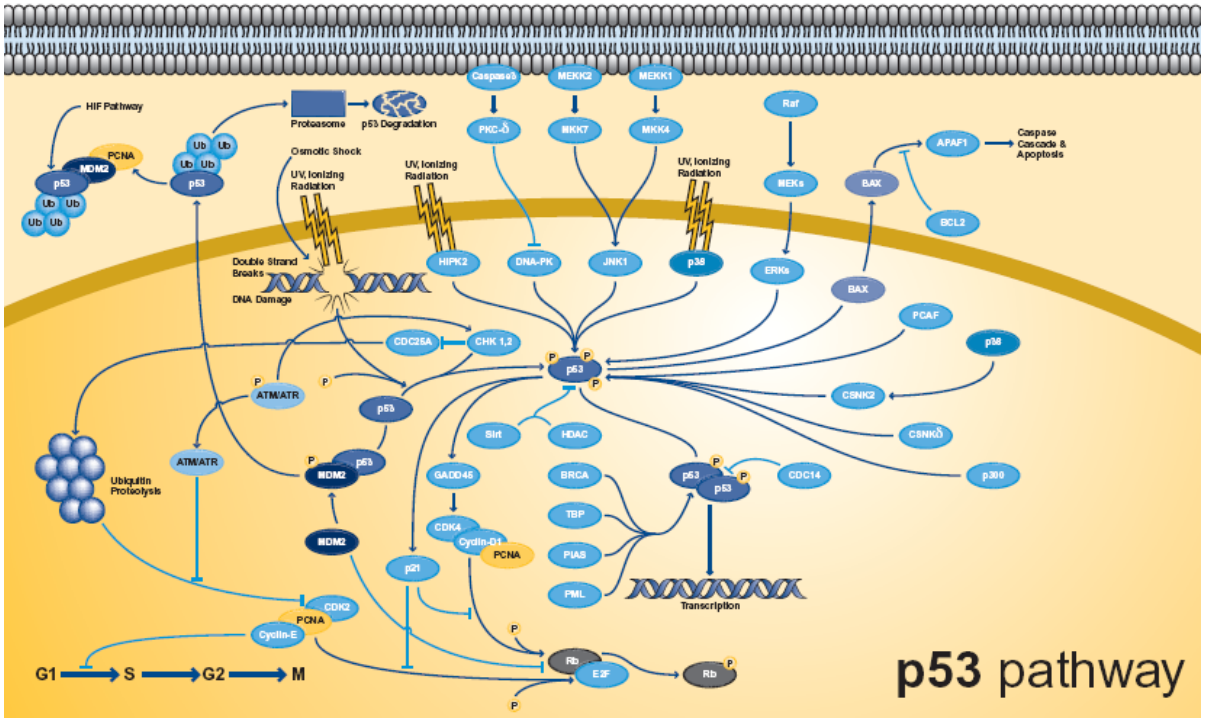


Fig 2.c. p53 pathway. [20]

2.1.2. Cancer Treatment

Because of the heterogeneous nature of cancer it is not possible to give a recipe on how to treat and hopefully cure a neoplasm. Nevertheless, the first approach, if applicable, is surgical excision. Removal of the tumour to any extend possible, minimizes the tumour burden and risk of subsequent metastases, as well. The second approach is radiation, where any local cancerous cell mass is the target due to its genomic instability and proliferative pressure it is under. These two approaches are not effective if the tumour has metastasized beyond lymph nodes. At that point, treatment needs to be systemic. Chemotherapeutics and secondary adjuvant therapies such as antibody and hormonal can be given systemically to either cure or at least slow down the progression of the disease and alleviate the symptoms associated. They need to be administered when the number of tumour cells is low enough to permit their destruction at doses that can be tolerated by the patient.

Chemotherapy acts by killing cells that divide rapidly - one of the main properties of cancer cells. This also involves harming cells that divide under normal circumstances resulting in side-effects. Most chemotherapeutic drugs work by impairing mitosis, effectively targeting

fast-dividing cells. Malignancies with slower growth rates tend to respond to chemotherapy much more modestly. Drugs affect "younger" tumours (i.e., less differentiated) more effectively, because mechanisms regulating cell growth are usually still preserved. With succeeding generations of tumour cells, differentiation is typically lost, growth becomes less regulated, and tumours become less responsive to most chemotherapeutic agents. Near the centre of some solid tumours, cell division has effectively ceased, making them insensitive to chemotherapy. Another problem with solid tumours is the fact that the chemotherapeutic agent often does not reach the core of the tumour. Also over time, cancer cells become more resistant to chemotherapy treatments. Recently, regulated pumps on the cell surface of cancer cells have been identified which actively move chemotherapeutics from inside the cell to the outside.

The majority of chemotherapeutic drugs can be divided in to alkylating agents, antimetabolites, anthracyclines, plant alkaloids, topoisomerase inhibitors, and other antitumour agents. All of these drugs affect cell division or DNA synthesis and function in some way.

2.1.2.1. Alkylating Agents and Platinum Compounds

Cancer chemotherapy evolved from the observed effects of sulphur mustard gas on bone marrow and lymphoid tissues after exposure during World War I which led to an ambitious search for other chemicals with such antitumour activities. The sulphur and nitrogen mustards are described to act as alkylating agents. They form strong electrophiles through the formation of carbonium ion intermediates. This results in the formation of covalent linkages by alkylation of various nucleophilic moieties. The chemotherapeutic and cytotoxic effects are not exclusively but directly related to the alkylation of DNA. The nucleophilic groups of proteins, RNA and many other molecules can also be the target of such attack. The alkylating agents are known to be more cytotoxic to rapidly dividing cells.

The first platinum antitumour compounds were discovered as a result of studying effects of electrical currents on bacterial growth. Growth inhibition was found to occur but was caused by a platinum complex of ammonia and chloride produced in the medium at the platinum

electrode. Several compounds were found to have antitumour effects and the most active platinum compound was found to be Cisplatin. [21]

Cisplatin, cis-diamminedichloroplatinum(II) (CDDP), has a central role in cancer chemotherapy. It is used to cure prostate cancer and also treat ovarian, cervical, head and neck and non-small cell lung cancers. The treatment is limited by side effects such as nephro- and neurotoxicity, of which neurotoxicity is dose-limiting and can result in peripheral neuropathy and hearing loss. [22] Resistance to the treatment is also common as some tumours have intrinsic mechanisms against treatment while others develop them during the course of treatment. A defective apoptotic program is one of the major contributors to CDDP resistance, together with increased drug efflux, decreased drug influx, increased cellular GSH (i.e. antioxidant defence of the cell) and metallothionein levels, increased DNA repair and oncogene expression. [23] In order to understand the cytotoxicity of CDDP and improve the therapeutic response, it is necessary to elucidate the molecular mechanisms of CDDP-induced cell death.

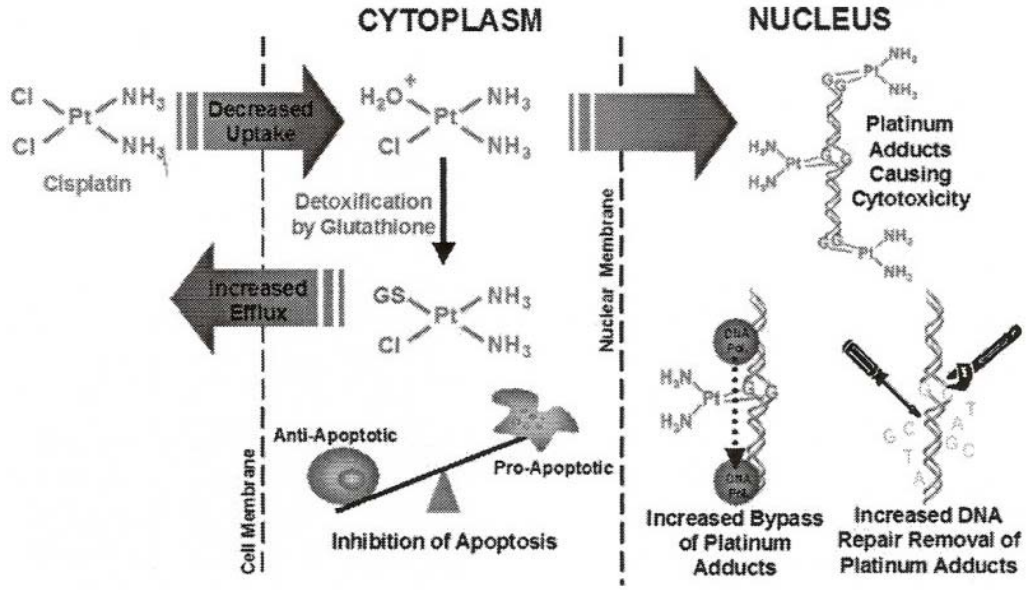


Fig. 2.d. CDDP resistance mechanisms. GS indicates glutathione and Pol polymerase. [24]

The cellular uptake of CDDP is not fully understood. It has been suggested that drug enters the cells partly by passive diffusion through transmembrane channels and partly by facilitated diffusion through an unidentified membrane transport system. [25]

CDDP is in its typical form a neutral inorganic complex that is activated upon entry into the cell, where the low chloride concentration facilitates replacement of the chloro-ligands of CDDP with water molecules. The aquated form is highly reactive and the resulting positively charged molecule can interact with nucleophilic sites of cellular proteins, membrane phospholipids, RNA and DNA. [26] It has been shown that CDDP induces apoptosis also in the absence of nucleus [27], indicating that, in addition to its DNA-damaging effects, CDDP causes cell death via the other cellular targets. [22] Approximately 1 % of the intracellular CDDP reacts with nuclear DNA to yield intra- and inter-strand DNA crosslinks and DNA-protein crosslinks. The most common adducts are intra-strand cross links between adjacent guanines and between neighbouring guanine and adenine, representing 65% and 25%, respectively, of the total number of adducts formed. [28]

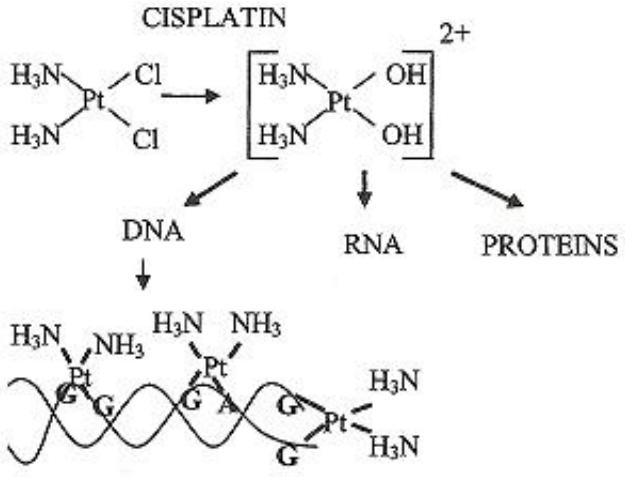


Fig. 2.e. Cisplatin and its DNA adduct. [29]

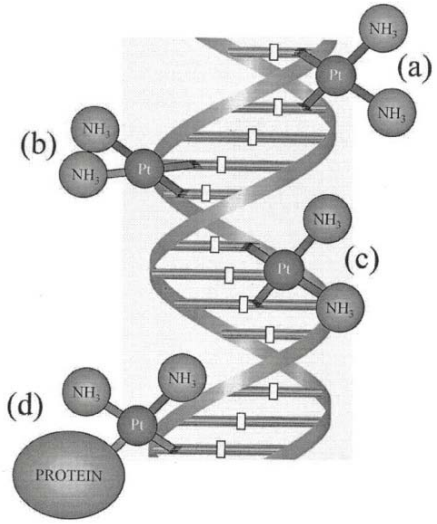


Fig. 2.f. Main adducts formed in the interaction of CDDP with DNA where (a) represents an interstrand crosslink, (b) a 1,2-intrastrand crosslink, (c) a 1,3-intrastrand crosslink and (d) protein-DNA crosslink. [30]

CDDP adducts are removed from DNA mainly by nucleotide excision repair (NER). The MMR system recognizes but does not remove the CDDP adducts since it always replaces the incorrect sequence in the daughter strand, leaving the CDDP adduct unrepaired. This initiates useless repair cycles which may generate DNA breaks and activate pro-apoptotic signals. [23]

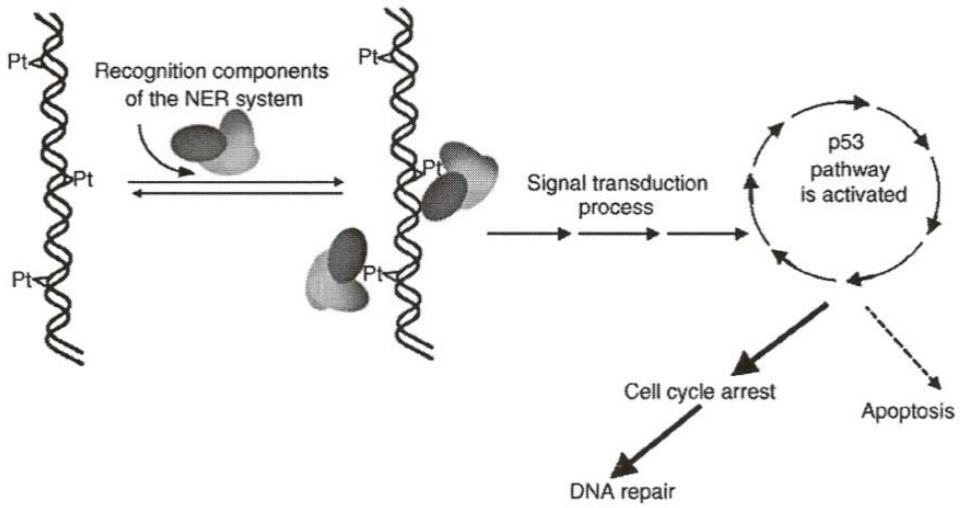


Fig. 2.g. Model for p53 involvement in the mechanism of resistance/sensitivity of tumours to CDDP. [31]

Binding of DNA-PK to damaged DNA results in phosphorylation and activation of two proteins involved in pro-apoptotic signalling, c-Abl and p53. Phosphorylation of p53 results in inhibition of its ubiquitination, leading to increased stabilization of the protein. p53 can initiate apoptosis as mentioned before by transcriptionally activating pro-apoptotic Bcl-2 family members such as Bax, Bak, Puma and Noxa, and repressing anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-x_L) and inhibitors of apoptotic proteins (lAPs). In addition, p53 can transactivate other genes that may contribute to apoptosis, including Apaf-1, PTEN, CD95 and TRAIL receptor 2. [32]

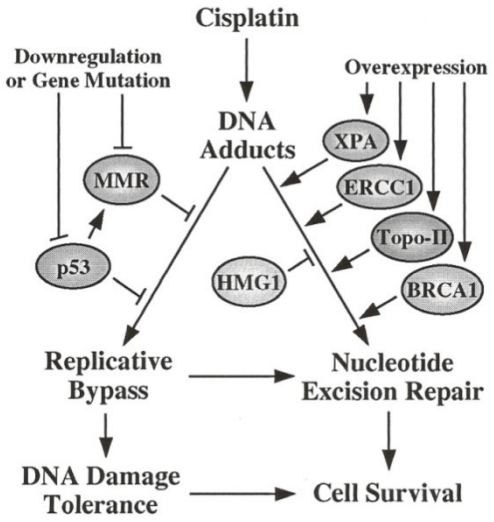


Fig. 2.h. Factors modulating repair of CDDP-induced DNA adducts and regulating replicative by-pass. [33]

The HMG proteins are a multifunctional family of small non-histone chromatin-associated proteins involved in gene regulation and maintenance of chromatin structure. Binding of HMG proteins to CDDP adducts may protect the adducts from recognition by DNA repair enzymes, resulting in higher cytotoxicity. Moreover, since these proteins have high affinity for CDDP-modified DNA, binding to DNA adducts may keep these proteins away from their normal binding sites, thus disturbing a diversity of cellular processes and potentially leading to cell death. [30]

2.1.3. Outcomes of Cancer Treatment

As more and more becomes known about the cellular response to damage inflicted by chemotherapy more insight is gained on the mode of action of drugs. Chemotherapeutics induce several responses affecting proliferation and survival of the cell, ranging from mitotic catastrophe, senescence and cell death modalities, i.e. autophagy, necrosis and apoptosis.

Mitotic catastrophe takes place with DNA damage and deficient cell cycle checkpoints which normally keep the cell cycle on hold until the damage is repaired. [34] Non-viable cells with smaller nuclei are formed with nuclear envelopes around clusters of missegregated chromosomes as a result of aberrant mitosis. [35] Mitotic catastrophe is induced by agents interfering with microtubule function as well as by DNA damage. [36-37] Mitotic catastrophe is also considered to be a starting point for cell death rather than a type of cell death by its own, and execution of cell death takes then place by apoptosis or necrosis. [38-39]

Senescence is defined as the condition of permanent growth arrest and was first described in cultured human fibroblasts that failed to divide after a limited number of cell divisions caused by the progressive shortening of telomeric ends by 50-100 base pairs per cell division. [40] The senescent cells are metabolically active and show distinctive modifications in their morphology such as enlarged and flattened cell shape and increased granularity. In addition to the telomere-dependent induction of replicative senescence, DNA damage and other types of stress responses also cause a senescence-like phenotype. [41-42] This response involves signalling which leads to stabilization of p53 and eventually growth arrest. [43] With tumour suppressors such as p53 acting as important regulators of senescence, senescence is considered as a tumour suppressor mechanism that cells need to overcome for tumour formation. [44] Tumour cells usually circumvent replicative senescence through up-regulation of telomerase [45] but can be strained into stress-induced premature senescence in response to chemotherapy and radiation. [41-42]

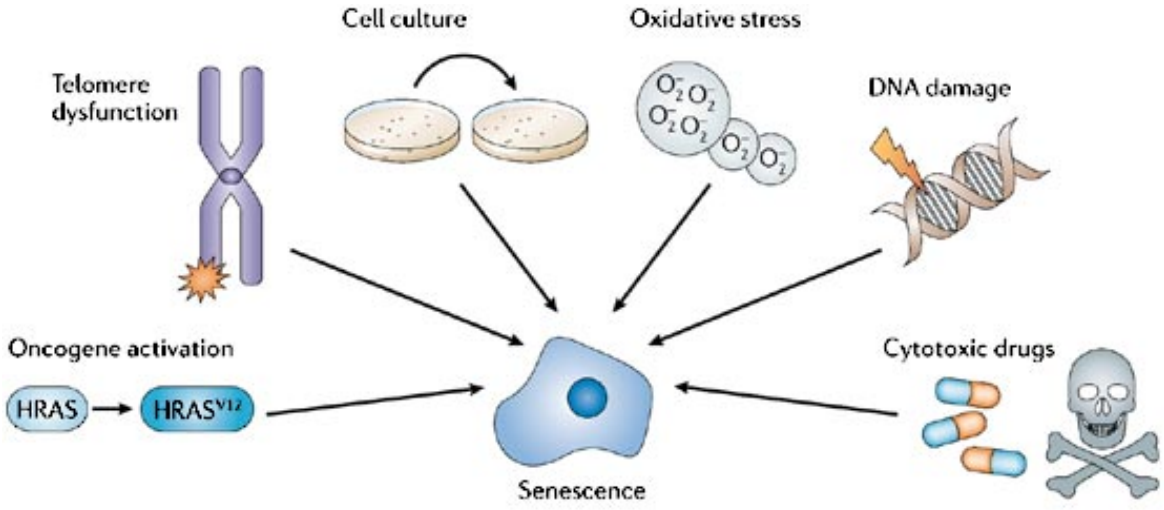


Fig. 2.i. The power and the promise of oncogene-induced senescence markers. [46]

Cell death is a fundamental and highly regulated process that is well conserved among diverse species. It is a vital process in development and homeostasis of normal organisms. All cells are equipped with a genetic program for self-destruction. Defects in cell death are observed in numerous physiological disorders including diabetes, neurodegenerative diseases and cancer.

The naturally occurring turnover of cells in the body is referred to as "programmed cell death". The term programmed cell death (PCD) was suggested in 1965 [47] and has been used to describe the phenomenon where cells follow a series of genetically controlled steps towards their own annihilation. It serves as a major mechanism for removal of redundant and potentially dangerous cells, such as virus-infected cells, self-reactive lymphocytes or tumour cells. [48]

Autophagy is an evolutionarily conserved process in which cytoplasmic contents of a cell are impounded within double membrane vacuoles called autophagosomes, which subsequently fuse with lysosomes where the material inside the autophagosomes are degraded. Autophagy functions as a protective response to various cellular stresses such as starvation, changes in cell volume, oxidative stress, accumulation of misfolded proteins, hormonal signals and irradition, in which the degradation of cytoplasmic material is used as nutrients and source of energy as an alternative form of programmed cell death. [49]

Necrosis has been referred to as the uncontrolled form of cell death which often occurs in response to a severe damage or physical insult. It is characterized by swelling of cytoplasm

and mitochondria, and loss of membrane integrity resulting in cell rupture and release of cellular contents into the surrounding tissue. [10] The resulting inflammatory response is allied with systemic toxicity and the activity of immune cells *in situ* may even support malignancy. [50] On the other hand, the stimulation of the immune response could potentiate the killing of tumour cells. [51] It is reported that necrosis may also be a regulated process triggered by various stimuli such as intracellular Ca^{2+} overload, excessive production of reactive oxygen species (ROS) and cellular energy depletion. [35] There also seems to be an overlap between apoptosis and necrosis in response to certain death inducing stimuli dependent on the dose.

The phenomenon of apoptosis was first described as a “shrinkage necrosis” where the cells turn into small, round, membrane encapsulated bodies with condensed chromatin and undamaged organelles. [52] It was then renamed as "apoptosis" with important roles in development, cellular turnover, tissue disorders and atrophy. Morphological changes defining apoptosis are condensation of nuclear chromatin, DNA fragmentation via activation of endonucleases along with cellular shrinkage and blebbing. Activation of caspase family proteases is one hallmark of apoptosis. The resulting apoptotic bodies are quickly recognized by their phosphotidylserine (PS) residues they expose on their cell membranes and engulfed by cells of the immune system or surrounding tissue so that they do not cause any tissue scarring or inflammation. [10] Apoptosis is an active, organized and intrinsic occurrence, in contrast to normal necrosis which is a rapid and "aggressive" process ensuing in interruption of cellular homeostasis. It occurs in tumours spontaneously as well as in response to some anticancer treatments. [53] Studies performed in the nematode *Caenorhabditis elegans* have provided important information on the strict genetic control of the apoptosis. [54] Several genes specifically required for induction and execution of apoptosis were identified and homologs of many of these genes have been found in mammals. These include both oncogenes such as Bcl-2 and tumour suppressor genes such as TP53. [55]

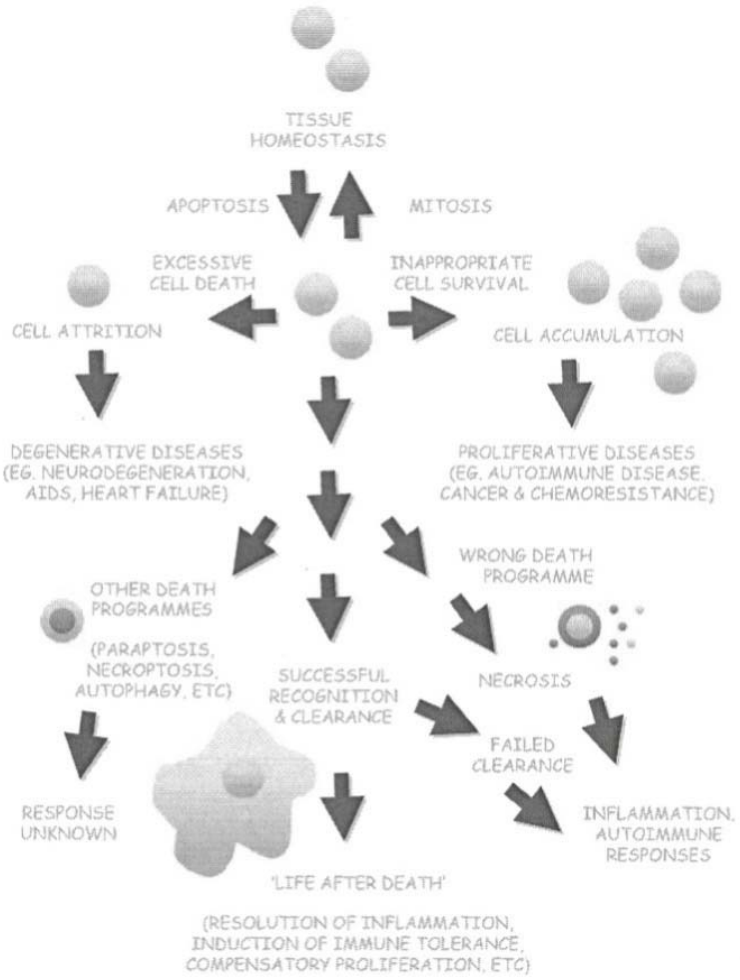


Fig. 2.j. Role of apoptosis in disease; it is required for the maintenance of tissue homeostasis. [10]

2.1.3.1. Biochemistry of Apoptosis

The apoptotic process can be divided into three phases:

1. Initiation phase - Many different intra- and extracellular signals such as signals from cell membrane, physical and chemical stresses, and oncogene expression have been shown to induce cell death. In this phase, the signals are detected and signalling pathways are induced in response to the stimuli.

2. Effector phase - In this phase the signals are transmitted and if necessary amplified inside the cell so that the execution can start.

3. Execution phase - The activated apoptotic machinery works on different cellular targets to cleave DNA and specific cellular proteins.

There are two initiator pathways which activate the execution of cell death: the extrinsic and the intrinsic pathways. Activation of either of these two pathways results in activation of caspases, the main executors of cell death.

Caspases are a family of cysteine proteinases conserved among a range of species which specifically cleave their substrates after aspartic acid residues. The distinctive substrate specificity is determined by the four residues amino-terminal to the cleavage site. Caspases exist in the cell as inactive proenzymes and need proteolytic cleavage of their prodomain for activation. Fully active caspases are tetramers consisting of two large (ca. 20kDa) and two small (ca. 10kDa) subunits. They are divided into two subfamilies; (1) proximal or initiator caspases, and (2) terminal or effector caspases.

Initiator caspases include caspase -1, -2, -4, -5, -8, -9, -10 and -12. Once activated, these initiator caspases process and activate the effector caspases.

Effector caspases are mainly activated by another proteinase, in most cases a caspase. Effector caspases possess short prodomains and include caspase-3, -6, -7, -11 and -13. Once activated, caspases, mainly caspase-3 and -7, cleave their specific substrates contributing to the morphological and functional changes associated with apoptosis, such as nuclear shrinking is caused by cleavage of nuclear lamin, and loss of cellular shape by cleavage of cytoskeletal proteins. Actually more than 100 substrates of the effector caspases have been identified so far. The proteins cleaved by caspases fall into four major categories: (1) apoptotic proteins such as Bid and Bcl-2 are activated or inactivated by caspase cleavage to promote cell death, (2) structural proteins are degraded, contributing to changes in cell shape and detachment from the matrix, (3) cellular DNA repair proteins involved in energy demanding processes are removed to save ATP for apoptotic mechanisms and (4) cleavage of cell cycle proteins is speculated to aid in cell death in response to improper cell cycle signalling by oncogenes. [51]

Due to there potential pro-apoptotic effects, caspases are strictly regulated both at transcriptional [56-57], and posttranslational level. Posttranslational modifications such as nitrosylation, oxidation, ubiquitination and phosphorylation have been shown to control caspase activity. [58]

The B-celllymphoma-2 (BcI-2) family proteins are regulatory proteins in the initiation of caspase activation and apoptosis. These proteins control the intrinsic apoptotic pathway by managing the release of caspase activating proteins from mitochondria via establishing mitochondrial membrane permeability. [59] In addition to this, Bcl-2 family proteins also

localize to other intracellular membranes such as the endoplasmic reticulum (ER) where they influence apoptotic signalling. In humans, twenty five members of this family have been discovered. The Bcl-2 family proteins can be divided in two groups: anti-apoptotic members, such as Bcl-2, Bcl-x_L Mcl-1, and pro-apoptotic member, such as Bak, Bax, Bim, Noxa. They are related through their conservation of helical sequences called Bcl-2 homology (BH) domains and each Bcl-2 family protein contains at least one of these four BH-domains, BH1,-2,-3, and -4. [60] The anti-apoptotic members display conservation in all four BH domains and a C-terminal hydrophobic tail allowing these proteins to be anchored to the membranes of mitochondria, ER and nucleus. All pro-apoptotic members lack BH4. [61] They are either multidomain proteins, possessing the BH1, -2 and -3 domains and intrinsic death-inducing activity as for Bax and Bak [4], or BH3-only proteins (BOPs) having homology only within the BH3 domain, also called the "minimal death domain" as for Bid, Bad, Bim. Pro-apoptotic family members are normally found in the cytosol or are loosely associated with membranes. After a death signal, these proteins translocate to the intracellular membranes, mostly the outer mitochondrial membrane, where they either insert into the membrane or interact with other proteins.

Bcl-2 family proteins can interact with each other, forming homodimers, heterodimers and oligodimers and can act as agonists or antagonists of their binding partners. Dimerization occurs through interaction between the amphiphatic BH3 α-helix of the pro-apoptotic proteins and the hydrophobic groove of the anti-apoptotic members, created by the α-helices in the BH1, -2 and -3 regions. [61] The ratio between pro- and anti-apoptotic proteins in the cell functions as a rheostat that sets the threshold for sensitivity to pro-apoptotic proteins. [62]

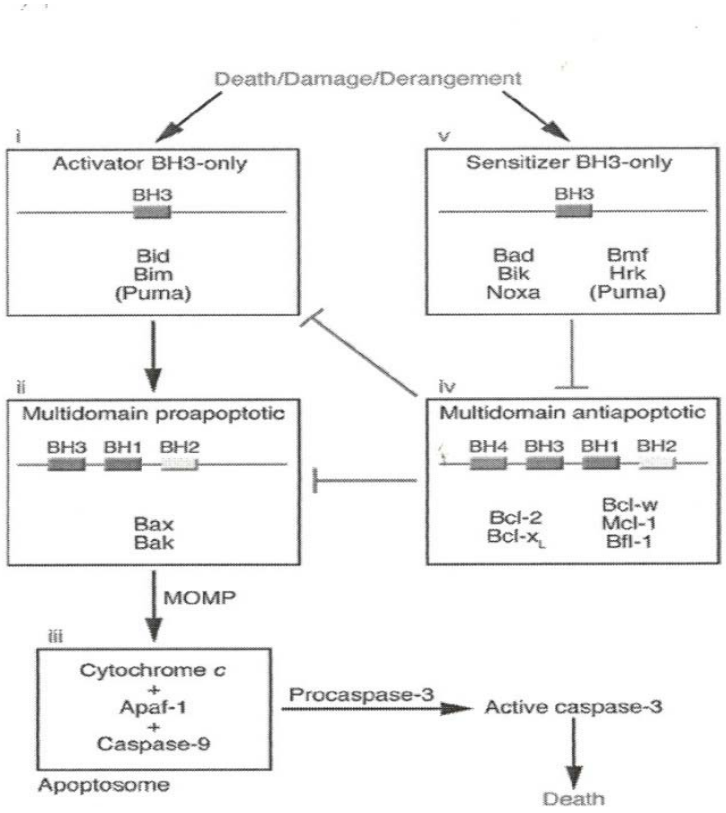


Fig. 2.k. A model of Bcl-2 family member control over PCD. In response to myriad cell death, damage or derangement signals BH3-only family members are activated (i). Activator BH3-only proteins interact with multidomain pro-apoptotic Bax and/or Bak inducing their oligomerization (ii) and thus resulting in MOMP, release of cyt c, apoptosome formation and caspase activion (iii). Bcl-2 and other multidomain anti-apoptotic proteins interrupt the death signal by binding and sequestering activator BH3-only proteins and perhaps also Bax/Bak (iv). Bcl-2 anti-apoptotic function may be antagonized by the competitive displacement of activator BH3-only molecules by sensitizer BH3-only proteins (v). [63]

The pro-apoptotic Bcl-2 proteins can be divided into two classes. The multi-domain protein Bax subclass (Bax, Bak and Bok) possess sequence homology for the BH1, -2, and -3 regions. [64] These proteins can promote apoptosis via their interaction with the mitochondrial membrane leading to release of cytochrome c and activation of caspases. The second subclass (Bik, Bim, Blk, Bid, Bad, Puma and Noxa) have strong homology only in the BH3 region. [65]

Bax and Bak have important roles in the intrinsic pathway of cell death at both the mitochondria and ER. Bak exists predominantly in mitochondria and ER membranes whereas

Bax is found mainly in the cytosol. The gene encoding Bax is a transcriptional target of the p53 protein in humans. [66] In response to death stimuli activated Bax and Bak undergo homo-oligomerization that results in the permeabilization of the outer mitochondrial membrane and the release of cytochrome c from the mitochondria. [67]

Bad, Bim, and Noxa can induce apoptosis via an interaction with either Bax or Bak and/or by generating stable complexes with anti-apoptotic Bcl-2/Bcl-x_L. [68-69] The genes encoding the BH3-only proteins Puma and Noxa are transcriptionally transactivated by p53. [70-71]

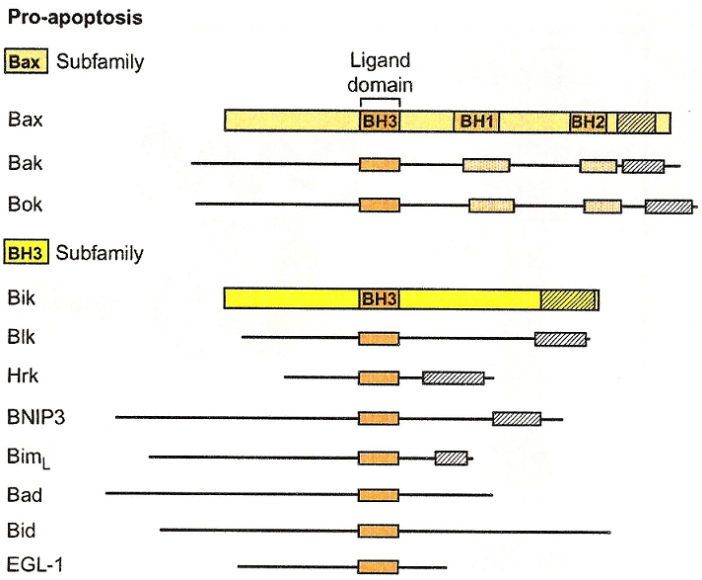


Fig. 2.l. Several Bcl-2 family proteins with similar structure and anti-apoptotic activity have been identified, including Mcl-1, Bcl-x_L, and Boo. [59] These Bcl-2 family proteins have a COOH-terminal hydrophobic transmembrane domain which directs them to the membranes of mitochondria, ER and the nucleus. [72]

Bcl-2 was first identified in the chromosomal breakpoint (t14:18) of chronic lymphocytic leukemia (CLL). [73] It was later described as an oncogene which protected against apoptosis. [74] Bcl-2 is now known to protect against most forms of apoptotic and sometimes necrotic cell death regardless of caspase involvement. [59] Association of Bcl-2 has been demonstrated in various processes, including regulation of calcium homeostasis [75-76], modulation of antioxidant pathways [77], promotion of gluthathione sequestration to the nucleus [78] and abrogation of cytochrome c release from mitochondria [79-80]. Bcl-2 resides in the outer mitochondrial membrane, ER and nuclear membranes. [81-82] It can also

bind non-homologous proteins such as Raf-l and calcineurin. Bcl-2 mediates translocation of Raf-l to the vicinity of the mitochondrial membrane. Once there, Raf-1 phosphorylates Bad, a pro-apoptotic BOP, the role of which is to heterodimerize with Bcl-2 and/or Bcl-x_L and abrogate their anti-apoptotic function. Phosphorylated Bad dissociates from Bcl-2 and Bcl-x_L and forms a cytosolic complex with a scaffold protein that inhibits interference of Bad with anti-apoptotic family members. [83-84] Moreover, by binding to calcineurin, a calcium-activated serine-threonine phosphatase, Bcl-2 may inhibit dephosphorylation of Bad. [85]

As Bcl-2 is constitutively membrane bound, Bcl-x_L associates with membranes only if stimulated. [86] The BH domains of Bcl-2 and Bcl-x_L have been shown to form a hydrophobic pocket in which Bak and Bax are bound and sequestered. [62] In addition, also BOPs including Bim, Bad and Noxa bind to Bcl-2 and Bcl-x_L, which indirectly inhibits Bak and Bax activation by oligomerization of the pro-apoptotic proteins and/or their insertion into the mitochondrial membrane. A model, where anti-apoptotic proteins inhibit activation of Bak and Bax by sequestering BOPs, has also been suggested. [69, 87] Bcl-x_L can bind and sequester the non-Bcl-2 protein Apaf-1 thus inhibiting formation of the apoptosome. [88-89] Bcl-x_L, together with Bcl-2, has been suggested to prevent mitochondrial membrane permeability either by physically or functionally interacting with voltage-dependent anion channel (VDAC), by neutralizing adenine nucleotide translocator (ANT) channel activity, or a combination of all of these [90] or by inhibition of pro-a pop to tic proteins such as Bax and Bak. [91]

Posttranslational modifications such as phosphorylation and cleavage regulate the activity of Bcl-2 and Bcl-x_L. Chemotherapeutic agents that cause microtubule disruption have been reported to induce phosphorylation of Bcl-2 and Bcl-x_L, abrogating their anti-apoptotic function. [92-93] It has been suggested that phosphorylation within the loop region of the Bcl-2 protein may determine the susceptibility to the cleavage by altering the conformational change and making the cleavage site more accessible to proteases. [94] Caspase-dependent cleavage of Bcl-2 and Bcl-x_L may occur in response to phenomena like Fas ligation, etoposide and growth factor withdrawal. Cleavage results in the exposure of the BH3 domains, converting these anti-apoptotic proteins into promoters of cell death. [95] Cleavage of Bcl-2 and Bcl-x_L can also be mediated by calpain, a calcium activated protease. [96]

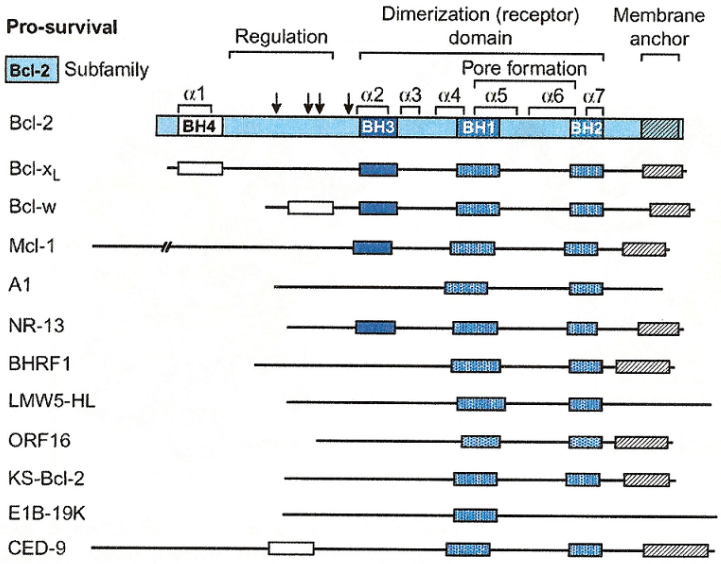


Fig. 2.m. Bcl-2 family proteins with similar structure and pro-apoptotic activity. [72]

2.1.3.2. Apoptotic Pathways

There are two apoptotic pathways which the cell can follow to commit apoptosis according to the origin of the death stimulus, extrinsic or intrinsic. These two pathways may overlap or take place at the same time, and some machinery they exploit may be common to both.

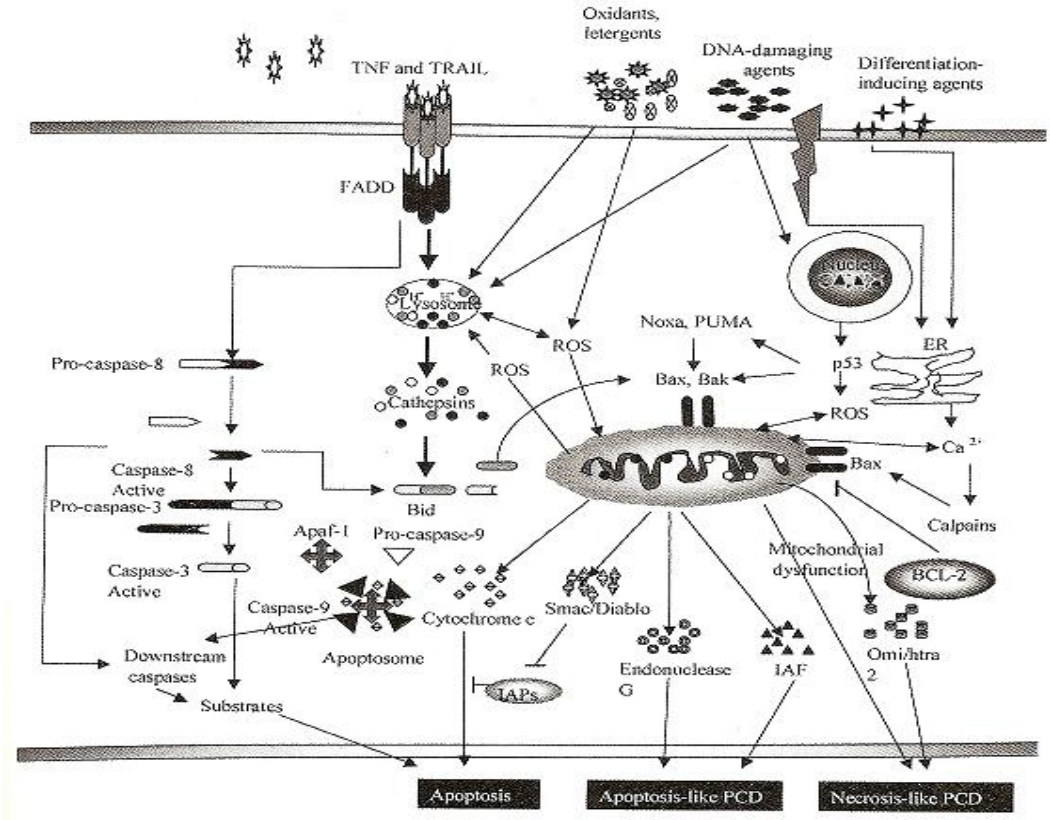


Fig. 2.n. Apoptotic pathways in overview. [97]

The extrinsic, or death receptor-mediated pathway, is triggered by ligand binding to cell surface death receptors. [62] The best characterized initiation of this pathway is through the tumour necrosis factor receptor (TNFR) family proteins; TNFR1, Fas (CD95 or Apo1) and DR4 (TRAIL-R1) along with -5 (TRAIL-R2). They contain structurally similar intracellular domains, death domains (DD) which is responsible for signalling initiation. [98] Upon ligand binding the receptor subunits trimerize and adaptor proteins TRADD (for TNF) and FADD (for FasL and TRAILs) are recruited to the receptor. Caspase-8 can cleave Bid to produce truncated Bid (tBid) which triggers the intrinsic apoptotic pathway by releasing cytochrome c from the mitochondria [99], activation of caspase-9 which cleaves caspase-3, which in turn further activates caspase-8 to amplify the signal. [100] Hence, caspase-8 is the key initiator caspase in the extrinsic pathway of apoptosis and Bid is a link between the extrinsic and intrinsic apoptotic pathways.

The intrinsic pathway utilizes organelles to amplify the death signals. [35] Mitochondria have a central role in intrinsic pathway of apoptosis. [90] Various signals induced by stress stimuli such as cytotoxic drugs, DNA damaging agents, hypoxia, heat shock, growth factor withdrawal, irradiation, ROS and death-receptor signalling converge on mitochondria. The mitochondrial events observed in response to cellular stress include permeabilization of the mitochondrial membranes. There are three models for this phenomenon:

- Model I

The permeability transition pore (PTP) is a polyprotein complex formed at the contact sites between the outer and the inner mitochondrial membrane. [101]

- Model II

Bcl-2 proteins may interact with proteins in the outer mitochondrial membrane, such as VDAC, and thereby regulate this channel's activity. [102] Since the pore size of VDAC is too small for passage of cytochrome c, it has been suggested that pro-apoptotic Bcl-2 members induce a conformational change leading to an increase in channel size. Anti-apoptotic Bcl-2 members would, according to this model, promote closure of the channel and thereby inhibit cytochrome c release. [103]

- Model III

Insertion of pro-apoptotic Bcl-2 family members into the outer mitochondrial membrane may be followed by formation of channels for passage of proteins localized in the mitochondrial intermembrane space. It has been shown that Bcl-2 family proteins can insert into synthetic lipid bilayers, oligomerize and form channels [104] but it remains unclear

whether these channels exist in cells and whether they would be large enough for passage of intermembrane proteins.

Once the mitochondrial membrane has become permeable some proteins are released from the mitochondrion during apoptosis. These are:

- *Cytochrome c*
- *Smac/DIABLO*
- *Omi/HtrA2*
- *Endonuclease G*
- *Apoptosis inducing factor (AIF)*

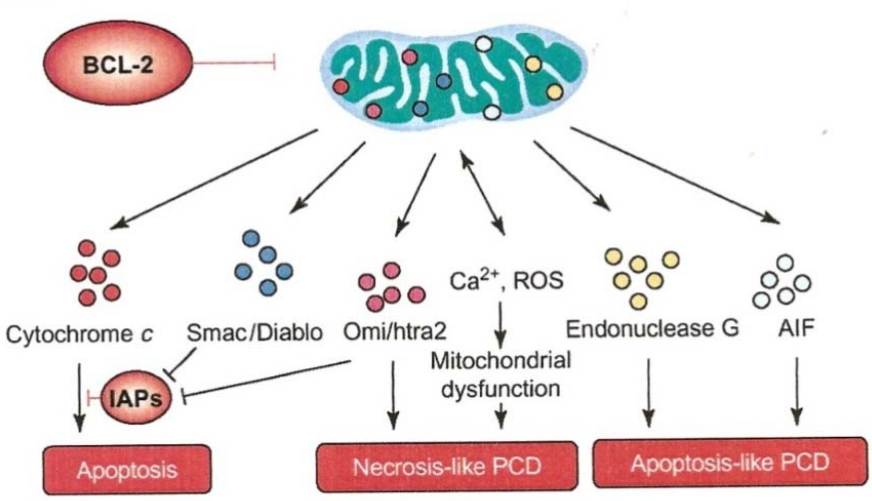


Fig. 2.o. Death pathways downstream of mitochondrial membrane permeabilization. cytochrome c via caspase activation, Smac/Diablo and Omi/HtrA2 via cytochrome c induced caspase activation by counteracting inhibitor of apoptosis proteins (IAPs), AIF via caspase independent death pathway culminating in DNA fragmentation and stage 1 chromatin condensation, EndoG via DNA cleavage and stage 1 chromatin condensation. Ca^{+2} and ROS via severe mitochondrial dysfunction. [105]

2.2. Oxidative Stress

Oxidative stress arises if there is an imbalance between the oxidative and reductive elements within a cell. The imbalance is mainly due to the loss of cell's ability to readily detoxify the reactive intermediates produced during cellular processes or easily repair the resulting damage on the components of the cell which is a reducing environment preserved by enzymes. These enzymes sustain the reduced status by continuous input of energy. Any disturbance of this

redox status results in changes in the cell due to the production of peroxides and free radicals (FRs) that damage proteins, lipids and nucleic acids within the cell.

Oxidative stress may work in two ways. It may stimulate the system for adaptation to the new circumstances in the environment the system has to cope with or it may cause damage to the components of the system. This solely depends on the concentration of prooxidant substances. Usually to overcome increased concentrations of prooxidants within the cells cells' response is to increase the expression of the genes encoding the components of the antioxidant defence of the cell. This is only helpful when the concentration of prooxidants is within certain limits.

2.2.1. Free Radicals, Oxygen and Reactive Oxygen Species

A (free) radical is a molecule with an unpaired (free) electron. The unpaired electron is a highly reactive "hot potato" that either "burns" (causes oxidative damage) or is passed from molecule to molecule so that the recipient becomes a FR and the donor is neutralized. Radicals usually carry zero net charge but can be positively (radical cation) and negatively (radical anion), too. The high reactivity of FRs comes from the fact that orbitals around the nucleus of an atom are more stable when they are occupied with a pair of electrons and not a single electron alone. FR damage can happen on lipids, proteins and nucleic acids in the cell.

The main site for FR production in the cell is the mitochondrion so most damage due to FRs is observed in mitochondrial membranes and mitochondrial DNA. [106] Some 1 to 5% fraction of the oxygen used in mitochondria to generate energy via aerobic respiration results in the formation of superoxide radicals.

There is a great range of FRs that can be formed in the human body such as H•, OH•, O_2^-, H_2O_2 (non radical oxygen derivative which acts as an oxidant in the cell), RO•, RO_2•, HO_2•. The reactivity of any FR varies according to its chemical properties.

Oxygen is a highly reactive nonmetallic period 2 element that readily forms compounds with almost all other elements. At standard temperature and pressure two atoms of the element bind to form dioxygen, a colorless, odorless, tasteless diatomic gas with the formula O_2. Oxygen is the third most abundant element in the universe by mass after hydrogen and helium and the most abundant element by mass in the Earth's crust. Diatomic oxygen gas constitutes 20.9% of the volume of air. It is vital for higher organisms but there is evidence that it can be

toxic as it inhibits cellular enzymes. Yet the rates of enzyme inhibition if any are too slow. So the damage attributed oxygen is due to the formation of oxygen FRs (OFR). [107]

Most FRs in biological systems are derivatives of oxygen (Reactive Oxygen Species, ROS) but there are also derivatives of nitrogen (Reactive Nitrogen Species, RNS). There are also Reactive Oxygen Intermediates, ROI which exist only for a short time so that they are relatively more reactive than other ROS.

Oxidant	Description
$\bullet O_2^-$, superoxide anion	One-electron reduction state of O_2, formed in many autoxidation reactions and by the electron transport chain. Rather unreactive but can release Fe^{2+} from iron-sulfur proteins and ferritin. Undergoes dismutation to form H_2O_2 spontaneously or by enzymatic catalysis and is a precursor for metal-catalyzed •OH formation.
H_2O_2, hydrogen peroxide	Two-electron reduction state, formed by dismutation of $\bullet O_2^-$ or by direct reduction of O_2. Lipid soluble and thus able to diffuse across membranes.
•OH, hydroxyl radical	Three-electron reduction state, formed by Fenton reaction and decomposition of peroxynitrite. Extremely reactive, will attack most cellular components
ROOH, organic hydroperoxide	Formed by radical reactions with cellular components such as lipids and nucleobases.
RO•, alkoxy and ROO•, peroxy radicals	Oxygen centred organic radicals. Lipid forms participate in lipid peroxidation reactions. Produced in the presence of oxygen by radical addition to double bonds or hydrogen abstraction.
HOCl, hypochlorous acid	Formed from H_2O_2 by myeloperoxidase. Lipid soluble and highly reactive. Will readily oxidize protein constituents, including thiol groups, amino groups and methionine.
ONOO-, peroxynitrite	Formed in a rapid reaction between $\bullet O_2^-$ and NO•. Lipid soluble and similar in reactivity to hypochlorous acid. Protonation forms peroxynitrous acid, which can undergo homolytic cleavage to form hydroxyl radical and nitrogen dioxide.

Table 2.a. Some common oxidants (adapted from [108-109])

A non-reactive molecule can be made into a FR in two ways: either it accepts an additional free electron and becomes a FR or it loses one of its electrons and is left with a single electron free to react with other molecules. A process called homolytic fission takes place when a covalent bond is broken and the shared electron pair is equally distributed among the parties of the covalent bond so that from a single covalently bonded molecule two radicals are formed. [110]

$$A : B \rightarrow A\bullet + B\bullet$$

An example to homolytic fission is a water molecule ending up as a hydrogen radical (H•) and a hydroxyl radical (OH•).

Another process called heterolytic fission takes place when a covalent bond is broken and the shared electron pair stays at one of the parties of the covalent bond so that from a single covalently bonded molecule one positively charged and one negatively charged ions are formed.

$$A : B \rightarrow A:^{-} + B^{+}$$

An example to heterolytic fission is a water molecule ending up as a hydrogen ion (H^{+}) and a hydroxide ion (OH^{-}).

The main source of FR generation is normal metabolic processes of the cell. Other extrinsic factors such as ultraviolet light (UV) also cause FR generation. During the process of respiration, cells are continuously subjected to oxidative stress as semi-reduced species of oxygen are also produced when molecular oxygen is reduced to water. Such semi-reduced species of oxygen (Reactive Oxygen Species, ROS) are highly reactive and initiate a series of oxidative reactions also referred to as oxidative stress.

As mentioned before, FRs are highly reactive towards cellular macromolecules such as lipids, proteins, and nucleic acids. They may cause modified enzyme function and increased cellular Ca^{+2} concentration by aggregating and cross linking, fragmenting and breaking down, modifying thiol groups of or nitrating phenolic compounds in proteins, decreased membrane fluidity by destroying of polyunsaturated fatty acids, forming reactive metabolites, altering activity of membrane bound receptor and transporters with lipids, inhibition of protein synthesis, activation of certain genes or translational errors by damaging bases, fragmenting or breaking deoxyribose rings in nucleic acids. On the other hand, FRs can also act as second messengers in the induction of molecular process.

2.2.2. Effects of ROS on Cellular Macromolecules

Protein oxidation is any covalent modification of a protein induced by ROI or by-products of oxidative stress. There is a vast array of agents that lead to protein oxidation. Some are chemical reagents such as H_2O_2, GSH, $^1O_2$; UV light, ozone; lipid peroxides such as HNE, MDA; mitochondria via electron transport chain leakage; P-450 enzymes and drugs and their metabolites. There are several different types of protein oxidative modifications such as sulfur oxidation as in cysteine disulfides; nitrosation, hydroxylation; hydro(pero)xy derivatives of aliphatic amino acids; amino acid interconversions such as histidine to asparagines or proline to OH-proline; lipid peroxidation adducts as with MDA, HNE; amino acid oxidation adducts such as p-hydroxyphenylacetaldehyde; peptide bond cleavages and protein carbonyls with side chain aldehydes and ketones. Amino acids have different tendencies to oxidation.

Cysteine	Disulfides, mixed disulfides (e.g., glutathiolation), HNE-Cys
Methionine	Methionine sulfoxide
Tyrosine	Dityrosine, nitrotyrosine, chlorotyrosines
Tryptophan	Hydroxy- and nitro-tryptophans, kynurenines
Phenylalanine	Hydroxyphenylalanines
Valine, Leucine	Hydro(pero)xides
Histidine	2-Oxohistidine, asparagine, aspartate, HNE-His
Glutamyl	Oxalic acid, pyruvic acid
Proline	Hydroxyproline, pyrrolidone, glutamic semialdehyde
Arginine	Glutamic semialdehyde, chloramines
Lysine	MDA-Lys, HNE-Lys

Table 2.b. The most susceptible amino acids and their main reaction products. [111-112]

The biochemical consequences of protein oxidative modification can be severe. It can cause loss or gain of enzyme activity, loss of protein function, loss of protease inhibitor activity, protein aggregation (e.g., IgG, LDL, prion protein), enhanced susceptibility to proteolysis , diminished susceptibility to proteolysis, abnormal cellular uptake (e.g., LDL), modified gene transcription (e.g., SoxR, IkB), increased immunogenicity (e.g., ovalbumin; HNE- or acrolein-LDL) etc. There are many diseases and conditions in which protein oxidation has been implicated such as atherosclerosis (LDL), rheumatoid arthritis (IgG, a-1-proteinase inhibitor), neurodegenerative diseases, Alzheimer's and Parkinson's disease and cancer.

Protein oxidation can be overcome by the use of antioxidants, scavengers such as methionine, antioxidant enzymes such as catalase and SOD, antioxidant enzyme mimics, chelators, depletion of O_2 and augmentation of cellular antioxidant systems as with acetylcysteine which increases the intracellular GSH.

There are advantages as well as disadvantages when using proteins as markers of oxidative stress. To start with, there is no single universal marker for protein oxidation. The types of modification depend highly on the nature of the oxidant. But the products are relatively stable to perform assays for the source of oxidant and there are very sensitive assays which can detect oxidized products in amounts less than 1 pmols. Different forms of oxidative modification have different functional consequences. For instance, methionine is highly susceptible but oxidation often does not affect protein function. But although carbonyls are often associated with dysfunction but these modifications may require more stringent oxidative conditions. Proteins, lipids, and DNA are modified by different oxidants to different degrees. DNA has more affinity to be modified in the presence of H_2O_2 than lipids which are far more reactive than proteins.

There are different methods for the detection of protein oxidation products depending on the oxidant and its target. In case of carbonyls, the first set of methods employ dinitrophenylhydrazine (DNPH) – coupled assays. These include techniques such as spectroscopy, high pressure liquid chromatography (HPLC), Western Blotting (OxyBlot™), ELISA and immunohistochemistry. [113-115]

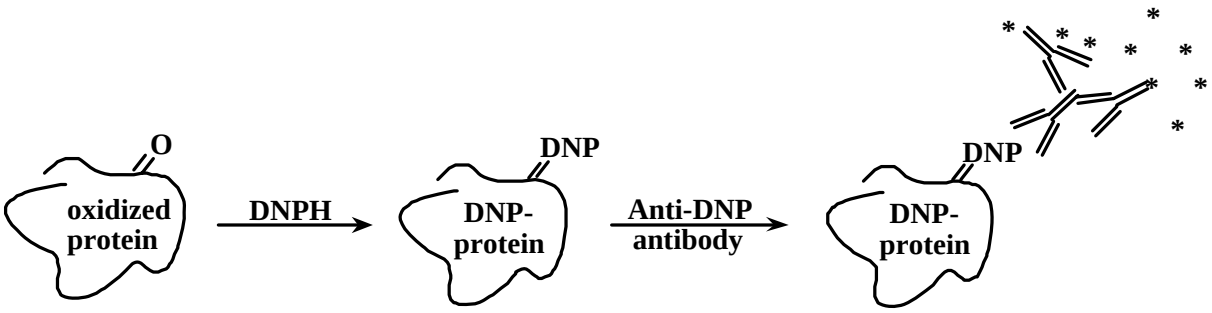


Fig. 2.p. Detection of oxidized proteins by use of DNPH. [113]

Carbonyl groups are relatively stable. They are normally present at low levels in most protein preparations (~1 nmol/mg protein ~ 0.05 mol/mol ~ 1/3000 amino acids). In vivo this background protein carbonyls can be elevated 2 to 8 fold under conditions of oxidative stress. They can be induced by almost all types of oxidants such as site-specific metal catalyzed oxidation, γ-irradiation, HOCl, ozone or lipid peroxide adducts. If the source of protein carbonyls is metal catalyzed oxidation the amino acids of interest are proline (g-glutamylsemialdehyde), arginine (g-glutamylsemialdehyde), lysine (amino-adipicsemialdehyde) and threonine (amino-ketobutyrate).

When detection of protein carbonyls will be done by western blotting there are some point which should be kept in mind. Firstly, carbohydrate groups of glycoproteins do not contribute to carbonyl levels [116] but free aldehyde groups from lipid peroxidation adducts (e.g., MDA) can react with DNPH. To overcome this phenomenon the adducts need to be stable. One other important point is that the western blot assay is only semi-quantitative but it can be used on cell and tissue extracts. [111]

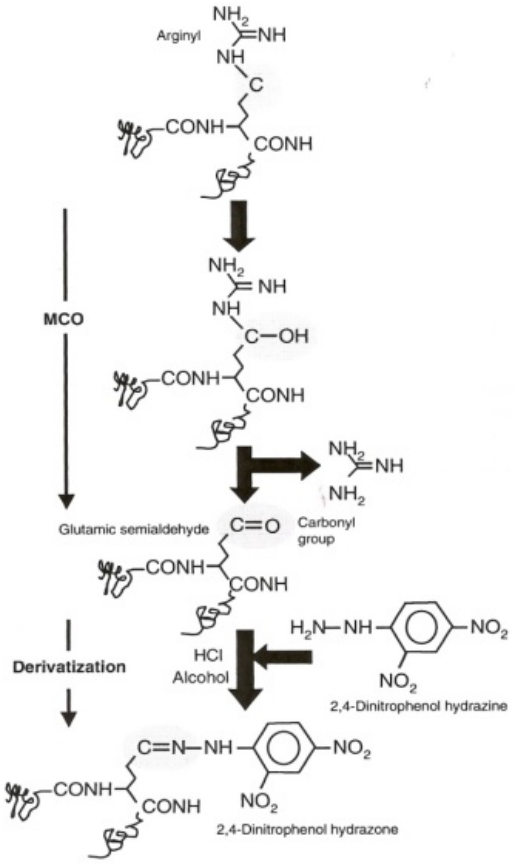


Fig. 2.r. Carbonylation and derivatization of a protein amino acid side chain. [117]

In case of protein sulfur group oxidations, cysteine and methionine are the most susceptible amino acids. This type of oxidation is distinguished from other oxidative protein modifications in that the cells have mechanisms to reverse the oxidation such as methonine sulfoxide reductase and GSH or thioredoxin redox systems and hence it may serve a regulatory function. Reversible oxidation/reduction of methionine may protect proteins from more damaging forms of oxidative modification as in carbonyl formation. [118]

Lipid peroxidation refers to the oxidative degradation of lipids. It is the process whereby FRs "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a FR chain reaction mechanism. It most often affects polyunsaturated fatty acids, because they contain multiple double bonds in between which lie methylene (i.e. -CH2-) groups that possess especially reactive hydrogens. As with any radical reaction the reaction consists of three major steps: initiation, propagation and termination.

Initiation is the step whereby a fatty acid radical is produced. The initiators in living cells are most notably reactive oxygen species (ROS), such as OH·, which combines with a hydrogen atom to make water and a fatty acid radical.

The fatty acid radical is not a very stable molecule, so it reacts readily with molecular oxygen, thereby creating a peroxyl-fatty acid radical. This too is an unstable species that reacts with another free fatty acid producing a different fatty acid radical and a lipid peroxide or a cyclic peroxide if it had reacted with itself. This cycle continues as the new fatty acid radical reacts in the same way.

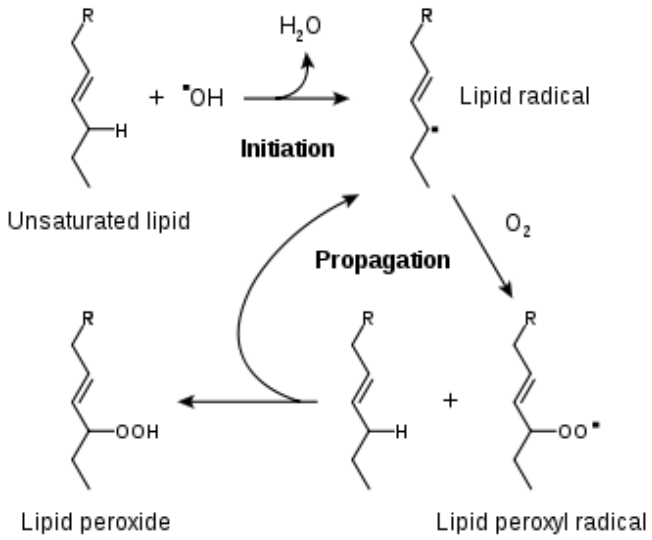


Fig. 2.s. Mechanisms of lipid peroxidation.

When a radical reacts it always produces another radical, which is why the process is called a "chain reaction mechanism." The radical reaction stops when two radicals react and produce a non-radical species. This happens only when the concentration of radical species is high enough for there to be a high probability of two radicals actually colliding. Living organisms have evolved different molecules that speed up termination by catching FRs and therefore protect the cell membrane. One important such antioxidant is vitamin E. Other anti-oxidants made within the body include the enzymes superoxide dismutase, catalase, and peroxidase.

If not terminated fast enough, there will be damage to the cell membrane, which consists mainly of lipids. [119]

In addition, end products of lipid peroxidation may be mutagenic and carcinogenic. For instance, the end product malondialdehyde reacts with deoxyadenosine and deoxyguanosine in DNA, forming DNA adducts to them. [120]

Malondialdehyde is the organic compound with the formula $CH_2(CHO)_2$. The structure of this species is more complex than this formula suggests. This reactive species occurs naturally and is a marker for oxidative stress. This compound is a reactive aldehyde and is one of the many reactive electrophile species that cause toxic stress in cells and form covalent protein adducts which are referred to as advanced lipoxidation end products (ALE), in analogy to advanced glycation end-products (AGE). The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism. TBARS Assay is a typical method using MDA as a marker of lipid peroxidation.

2.2.3. Antioxidant Defence Mechanism of the Cell

Cellular response to oxidative stress is a certainty in cells. [121] Various genes and gene proteins have been identified which act in the antioxidant defence mechanism of the cell. In addition to these, there are also other molecules called antioxidants acting chemically against the prooxidants in the cell. Antioxidants are defined as any substance that can delay or prevent the oxidation of a substrate when it is present in relatively smaller amounts than the oxidants. Antioxidants share the following assets:

- They have to cleanse the cells from FR species by protein catalysts (enzymes) or direct chemical reactions where they act as reactants
- They have to minimize the extend of FR formation
- They have to bind metal ions that are essential to convert poorly reactive species such as O_2^- and H_2O_2 into highly reactive species such as OH•
- They have to be able to repair the damage on the target
- or to destroy the damaged target and replace with newly synthesized molecules if damage cannot be repaired

The first line of defence mechanism against FRs in vivo is antioxidant enzymes or compounds with low molecular weight. GSH-dependent enzymes, i.e. glutathione peroxidase (GPX), glutathione reductase and glutathione transferase, catalase - which breaks down H_2O_2 to oxygen and water - and superoxide dismutase - which converts superoxide to H_2O_2 - account

for antioxidant enzymes. Low molecular weight compounds such as vitamin C, vitamin E, and carotenoids taken up with daily diet account for antioxidant molecules. There are also intrinsic molecules such as GSH, bilirubin and uric acid which act as antioxidant molecules in the human body.

Antioxidant enzymes provide an important defence against FRs in the human body. Glutathione peroxidase, glutathione reductase, catalase, thioredoxin reductase, superoxide dismutase, Heme oxygenase and biliverdin reductase, are the most important antioxidant enzymes.

Superoxide dismutase without glutathione peroxidase or catalase (CAT) to remove hydrogen peroxide is of little value. To eliminate hydrogen peroxide before a specific process by Fe^{+2} ion called Fenton Reaction can create the more reactive hydroxyl radical, cells utilize catalase and/or glutathione peroxidase.

The brain due to the high metabolic rate, high unsaturated fat content of the neurons and the fact that neurons are post-mitotic is more susceptible to FR damage than other tissues that its glutathione peroxidase activity is seven times more than its catalase activity. [122] Moreover, glutathione peroxidase is found throughout the cell as other antioxidant enzymes tend to be localized in different compartments in the cell.

Antioxidants are molecules that can neutralize FRs by accepting or donating an electron to overcome the unpaired electron condition. In most cases, the antioxidant molecule neutralizes a FR molecule as it becomes the FR itself but the FR it becomes is much less reactive than the FR it neutralized. It does so by diluting the unpaired electron in case it is a very large molecule or by being readily neutralized by another antioxidant and/or another mechanism for terminating its FR condition.

Molecules with loosely-held hydrogen atoms can use those hydrogen atoms like electrons to neutralize FRs. The hydrogen atoms are called reducing equivalents, and the molecules having such hydrogen atoms are said to be in a reduced state.

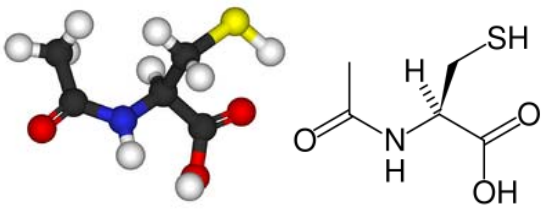


Fig. 2.t. N-acetyl-L-cysteine structure. .

N-Acetyl-L-Cysteine (NAC) is the amino acid L-Cysteine with an acetyl (-CO-CH_3) group attached to its amino (NH_2) group. The acetyl group increases the solubility and absorbability of the L-Cysteine. Amino acids which contain a sulphur group have antioxidant properties.

L-cysteine itself is not very water soluble and it is not well absorbed in the gastrointestinal tract. Dietary cysteine originates mainly from the breakdown ingested protein and peptides. Whey protein is a particularly rich food for cysteine.

Because cysteine is so unstable, the main extracellular source of intracellular cysteine is the dipeptide cystine (two conjugated cysteines). Cystine competes with glutamate for transport into cells such that conditions of elevated extracellular glutamate can lead to GSH depletion, worsened oxidative stress and cell death. [123]

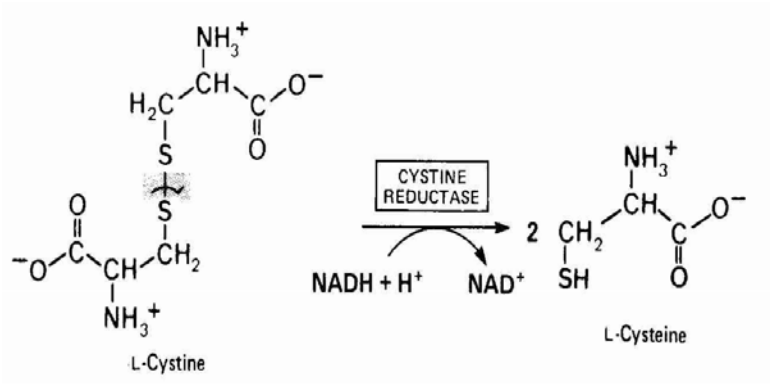


Fig. 2.u. Cystine to cysteine.

Oral supplementation of NAC provides an alternative means of extra intracellular GSH via elevated intracellular cysteine. NAC is rapidly absorbed after oral administration and reaches a maximum plasma level in 2-3 hours, with a half-life of about 6 hours. NAC readily enters cells.

Previous reports show evidence that NAC can rescue neurons from apoptotic death in the absence of growth factors by activation of the Ras-Extracellular signal Regulated Kinase

(ERK) pathway, an effect due to direct action on transcription factors by the thiol group, rather than by anti-oxidant effects. [124] In earlier studies it was shown that NAC has been used to regenerate oxidative phosphorylation complexes in mitochondria from age-related decline in function by sulphhydryl group action, rather than antioxidant effect [125] and that NAC protects against radiation damage by a direct radical scavenger action rather than by conversion to GSH [126]. By contrast it was also shown before that NAC added to cell culture causes increase in oxidative damage to DNA, an effect that can be inhibited by catalase and copper chelation. [127]

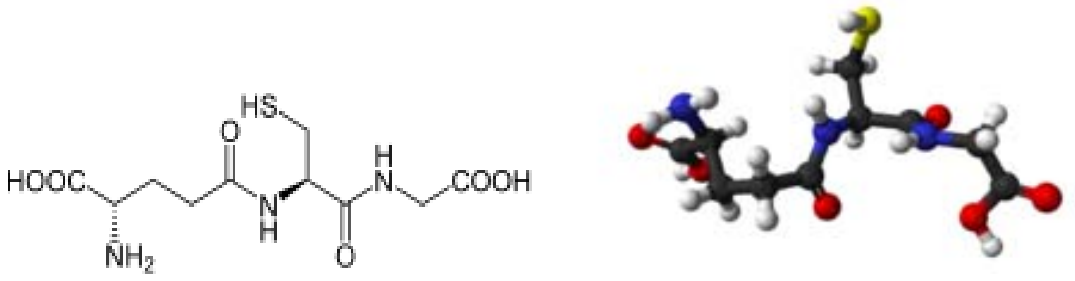


Fig. 2.v. L-gamma-glutamyl-L-cysteinylglycine structure.

L-gamma-glutamyl-L-cysteinylglycine, in short glutathione (GSH) is a tripeptide that contains an unusual peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side chains. GSH acts as an antioxidant and protects cells from damage by FRs. It is the predominant anti-oxidant in the aqueous cytoplasm of cells. Nearly all cells require GSH for viability and function although it is not an essential nutrient as it can be synthesized from three amino acids, L-cysteine, L-glutamic acid and glycine. NAC is processed in the cell to L-cysteine and used in the de novo synthesis of GSH. The synthesis takes place in two adenosine triphosphate-dependent steps: First, gamma-glutamylcysteine is synthesized from L-glutamate and cysteine via the enzyme gamma-glutamylcysteine synthetase (also called glutamate cysteine ligase, GCL). This reaction is the rate-limiting step in GSH synthesis as the availability of cysteine controls the reaction rate while glycine and glutamic acid are plentiful in cells. Second, glycine is added to the C-terminal of gamma-glutamylcysteine via the enzyme glutathione synthetase.

Previous studies showed that GSH can induce p53-dependent apoptosis in cancer cells. [128]

The hydrogen atom in the thiol (i. e. -SH) group of many sulphur-containing antioxidants as GSH can act as an electron for neutralizing free-radicals. The thiol groups are kept in a

reduced state at a concentration of approximately 5mM in animal cells. In healthy systems, more than 90% of the total GSH pool is in the reduced form (GSH) and less than 10% exists in the disulfide form (GSSG). An increased GSSG-to-GSH ratio is considered indicative of oxidative stress. GSH reduces any disulfide bond formed within cytoplasmic proteins to cysteine by acting as an electron donor where GSH is converted to its oxidized form GSH disulfide (GSSG). GSH is found almost exclusively in its reduced form since the enzyme glutathione reductase, which reverts GSH from its oxidized form, is constitutively active and inducible upon oxidative stress. The ratio of reduced GSH to oxidized GSSG within cells is can be employed as a measure of cellular toxicity.

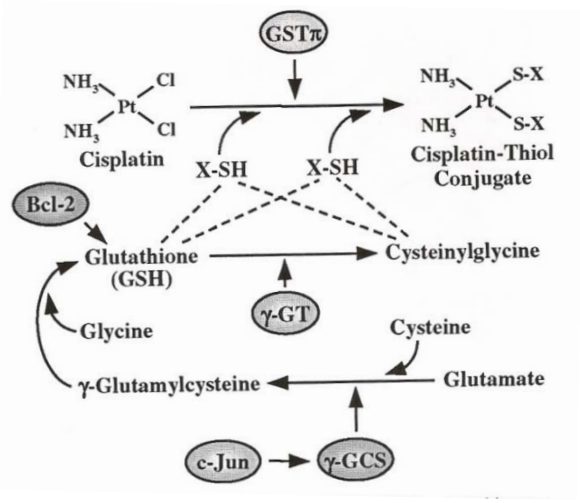


Fig. 2.w. Inactivation of CDDP by GSH. (X-SH indicates GSH or cysteinylglycine) [33]

GSH can scavenge peroxynitrite and hydroxyl radicals as well as convert hydrogen peroxide to water. In the process a glutathione radical GS• is formed but it is readily neutralized by another GS• to produce GSSG. GSSG can be converted back to GSH by NADPH-dependent glutathione reductase. Vitamin C can regenerate a Vitamin E radical and GSH can regenerate a Vitamin C radical to its antioxidant Vitamin C condition creating a chain of antioxidant molecule dependency.

The GSH system (GSH, glutathione peroxidase and glutathione reductase) is a key defense against hydrogen peroxide and other peroxides. There are four forms of glutathione peroxidase (GPx) enzymes: (1) cystolic Glutathione Peroxidase (cGPx, ubiquitously distributed), (2) Phospholipid Hydroperoxidase Glutathione Peroxidase (PHGPx, in plasma membranes to reduce hydroperoxides of complex lipids), (3) plasma Glutathione Peroxidase (pGPx, in blood plasma) and (4) Gastro-Intestinal Glutathione Peroxidase (GIGPx, in the liver

and GI tract only). The kidney manufactures most pGPx. Selenium is an essential component of GPx, so the relative preservation of enzyme levels during selenium deficiency may provide a guide to their relative importance. [129]

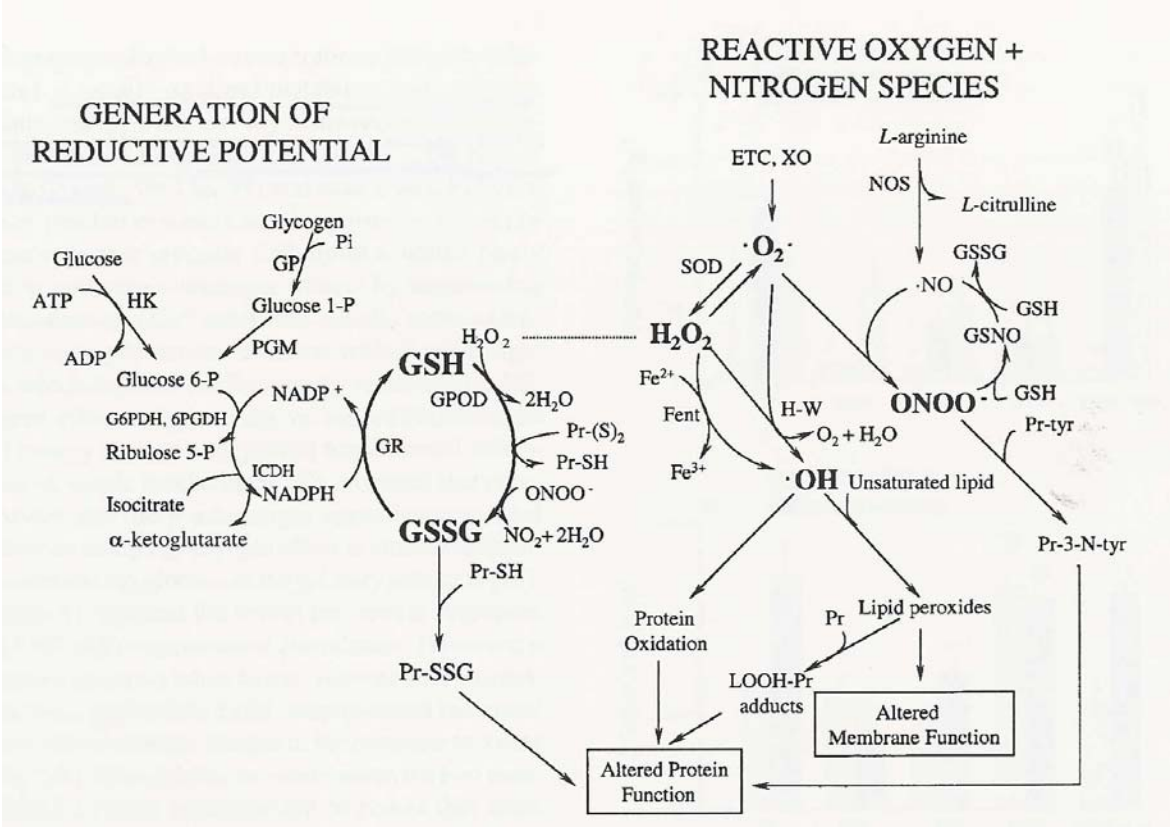


Fig. 2.y. GSH/GSSG Redox Pair Anti-Oxidant Defence.

2.2.4. Oxidative Stress and Cellular Signalling

Usually extracellular environment is more oxidized than intracellular environment causing formation of disulfide bridges between cysteine amino acid residues exposed to the exterior so that complementary surfaces have the ability to maintain a covalent bond that stabilizes the structure. This is important for extracellular proteins constantly exposed to a variety of proteases capable of degrading proteins with loose structures easily. On the other hand, inside the cell the environment is reduced. Here, cysteine residues are not involved in the formation of disulfide bonds, unless intracellular redox balance is disturbed via oxidative stress. The formation of disulfide bonds alters both conformation and activity of a number of enzymes, especially phosphatases. These enzymes usually control the activity of protein kinases, i. e. protein phosphorylases. Inactivation of a specific phosphatase by oxidative stress results in

sustained activity of kinases which in a cell means increasing activation of certain intracellular signal cascades. Such alterations in the intracellular signal cascades proceeding through subsequent phosphorylations of certain kinases that operate in a pathway end in phosphorylation of proteins in many cell compartments, such as mitochondria or nucleus. This modification of specific regulatory proteins can result in a number of changes, ranging from ionic signals to wide alterations in patterns of gene expression resulting in protein products such as antioxidant enzyme, growth arrest, DNA repair, mitochondrial electron transport, cell adhesion, cytokine, and glucose-regulated proteins. As a consequence, a cell may change its rate of proliferation, or die, depending on the signal networks. An intracellular oscillation of oxidant levels has been previously reported to be associated with the maintenance of the rate of cell proliferation. [130]

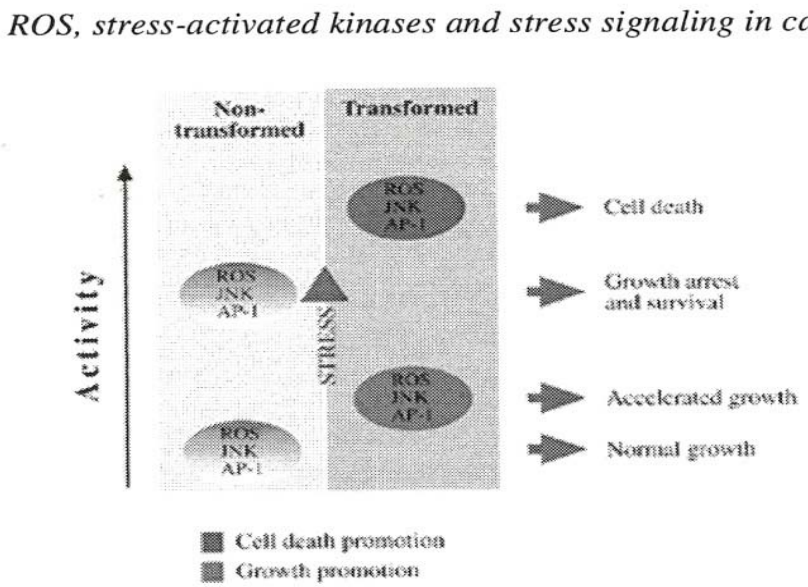


Fig. 2.z. Model for dysregulation of ROS and SAPKs in transformed cells setting the cellular response to anticancer agents. [131]

Hydrogen peroxide is one of the best-studied oxidising agents, which is used as endogenous messenger within the cell.

It is still not fully elucidated how changes in the intracellular ROS levels are recognized and translated into control of expression of certain genes including antioxidant enzymes, repair proteins and transcription factors which are inducible transcriptional activator proteins that enable activation of a certain gene as a response to physiological and ROS triggered signals. These activator proteins respond to diverse stimuli by binding to specific DNA sequences and

either stimulating or inhibiting the transcription of the nearby gene. Oxidative stress response is related in the activation of various functionally unrelated genes of signal transduction and cell proliferation processes. The mammalian oxidative stress has a protective function. Activation of the genes depends not only on intracellular redox status but also on other physiological signals, whether extrinsic or intrinsic. In both cases ROS act as intracellular signalling, i. e. secondary messenger molecules. Hence, it can be deduced that ROS induced gene expression is not only restricted to unfavourable environmental conditions but has a broader and elemental role in cellular processes.

Intracellular ROS levels and the induction of apoptosis are shown to be closely associated with each other. ROS usually act as mediator of apoptotic response. First evidence to this is that the increased ROS or oxidative damage marker levels are observed in cells committing apoptosis. Second evidence shows that antioxidants protect cells from committing apoptosis which was induced by different stimuli. It has been shown before that apoptosis proceeds as intracellular ROS levels increase or intracellular antioxidant concentrations decrease. If a cell commits apoptosis, there are many potential sources of ROS, the most important being the mitochondria. With the release of cytochrome c from mitochondria, electron transport chain gets blocked and consequently superoxide and other ROIs are generated.

In this study, the aim was to elucidate further on the mechanisms behind CDDP-induced cell death, particularly in terms of increased ROS levels and their possible effect on protein targets of CDDP. The proteins under examination were pro-survival proteins of the apoptotic machinery, specifically Bcl-2, Mcl-1 and Bcl-x_L. An additional parameter, the presence (wt) or absence (-/-) of TP53 gene in the HCT 116 human colon carcinoma cell line employed throughout the studies, was put into the equation as the protein product of this gene is known to be a transcription factor to several other genes including those expressing proteins which provide protection against increased ROS levels.

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Buffers and Solutions

The list of buffers and solutions with respective data on them is given in the appendix A.

3.1.2. Chemicals and Antibodies

The list of chemicals and antibodies exploited is given in the appendix B.

3.1.3. Molecular Biology Kits

The list of molecular biology kits exploited is given in the appendix C.

3.1.4. Laboratory Equipment

The list of laboratory equipment exploited is given in the appendix D.

3.1.5. Other Materials Needed

The list of other materials needed is given in the appendix E.

3.2. METHODS

3.2.1. Cell Culture and Treatments

HCT 116 wt and p53-/- cell lines were kindly provided by Bert Vogelstein at Johns Hopkins Oncology Centre, Baltimore, USA.

Both cell lines were cultured in modified McCoy's 5A medium and maintained in a humidified incubator steadily at 37˚C and 5% CO_2.

Instructions on basic cell culture techniques, how to employ a hematocytometer for determination of cell density and administration of drug and/or supplement are given in appendix F.

3.2.2. Assessment of Cell Viability

MTT Assay procedure was performed according to manufacturer's instructions. Detailed information is given in appendix F.

3.2.3. Assessment of Apoptotic Response

Flow Cytometric Analyses by Annexin-V Labelling was performed according to manufacturer's instructions. Detailed information is given in appendix F.

3.2.4. Assessment of ROS Production

Flow Cytometric Analyses by DCFH-DA Labelling was performed according to manufacturer's instructions. Detailed information is given in appendix F.

3.2.5. Assessment of Lipid Peroxidation

TBARS Assay was performed according to the previously optimized procedure. Detailed information is given in appendix F.

3.2.6. Assessment of Protein Carbonylation via OxyBlot™

Detailed information on protein isolation, immunoprecipitation, Bradford's protein content assay, SDS-PAGE gel preparation and electrophoresis, Western blotting, OxyBlot™ procedure and respective detection techniques are given in appendix F.

3.2.7. Statistical Analyses

Data were interpreted as means ± standard deviation (SD). For statistical comparison t-test was applied. p-values smaller than 0.05 (*) and 0.01 (**) were considered to be statistically significant.

4. RESULTS

4.1. Effect of CDDP on Cell Viability

To investigate the effect of CDDP administration on HCT 116 wt and p53-/- cells in terms of cell viability MTT Assay was employed. In MTT Assay 96-well plates were seeded with the two cell lines at a density of $2.0x10^4$ cells/well for 24h and $1.2x10^4$ cells for 48h treatments. Data obtained from seven different sets were given as average percentage with respect to controls ± SD.

To demonstrate the relation between dose and cell viability different concentrations of CDDP (0, 12, 30 and 60μM) were administered to HCT 116 wt and p53-/- cells for a pre-set duration of treatment (24 and 48h).

For 24h treatment cell viability was found to be following a decreasing pattern similar in both cell lines with increasing doses of CDDP. Here, for the wt cells the cell viability decreased down to 94.4, 80.8 and 72.3% for 12, 30 and 60 μM CDDP, respectively when the untreated control was taken as 100%. For p53-/- cells the cell viability decreased down to 95.8, 76.4 and 55% for 12, 30 and 60 μM CDDP, respectively when the untreated control was taken as 100%. The p53-/- cells proved to be less proliferative than the wt cells. (Fig. 4.1.a.)

For 48h treatment cell viability was found to be following a declining pattern in both cell lines with increasing doses of CDDP. Here, for the wt cells the cell viability decreased down to 54.3, 30.51 and 26.96% for 12, 30 and 60 μM CDDP, respectively when the untreated control was taken as 100%. For p53-/- cells the cell viability decreased down to 78.03, 66.84 and 38.81% for 12, 30 and 60μM CDDP, respectively when the untreated control was taken as 100%. The wt cells showed intermediately less proliferative than the p53-/- cells. (Fig. 4.1.b.)

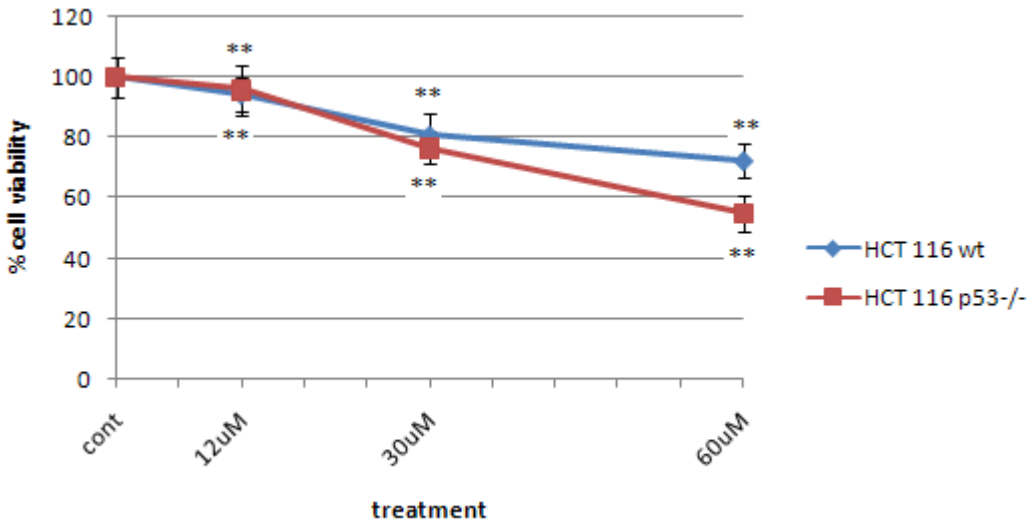


Fig. 4.1.a. MTT Assay for assessment of cell viability. Both cell lines were seeded in 96-well plates at a density of $2.0x10^4$ cells/well and treated 24h with different concentrations of CDDP. Data shown are normalized % mean ± %SD from seven different data sets.(*: $p < 0.05$ and **: $p < 0.01$)

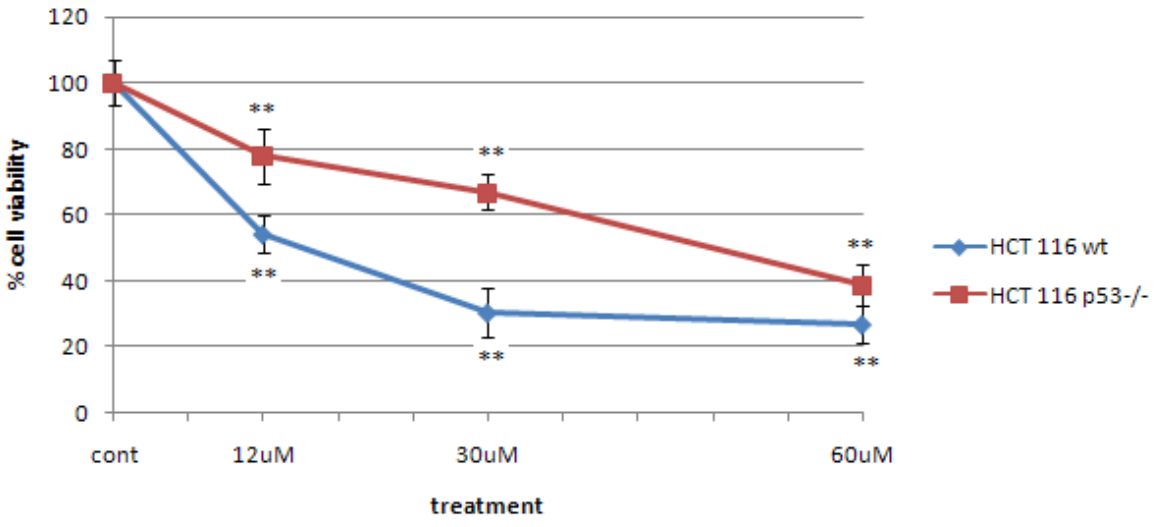


Fig. 4.1.b. MTT Assay for assessment of cell viability. Both cell lines were seeded in 96-well plates at a density of $1.2x10^4$ cells/well and treated 48h with different concentrations of CDDP. Data shown are normalized % mean ± %SD from seven different data sets.(*: $p < 0.05$ and **: $p < 0.01$)

To demonstrate the relation between duration of treatment and cell viability pre-set concentrations of CDDP (0 and 30µM) were administered to HCT 116 wt and p53-/- cells for different durations of treatment (24h and 48h).

Cell viability was found to be lessening in both cell lines in time. In 24h, for the wt cells the cell viability decreased down to 80.8% as for p53-/- cells 76.4% for 30µM CDDP when the untreated control was taken as 100%. In 48h, for the wt cells the cell viability decreased down to 30.5% as for p53-/- cells 66.8% for 30µM CDDP when the untreated control was taken as 100%. The wt cells showed a steeper loss of cell viability in 48h than the p53-/- as for 24h the decrease in both cell lines was proximate. (Fig. 4.1.c.)

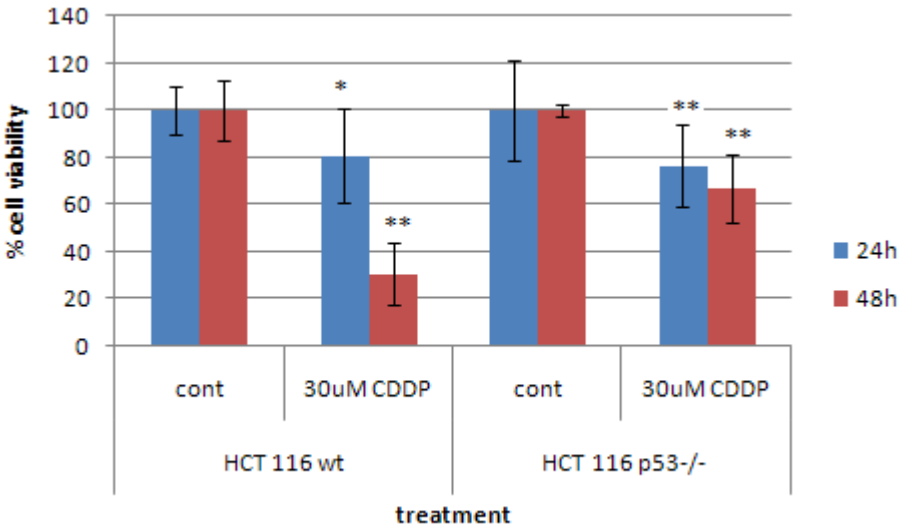


Fig. 4.1.c. MTT Assay for assessment of cell viability. Both cell lines were seeded in 96-well plates at a density of $2.0x10^4$ cells/well for 24h and $1.2x10^4$ cells/well for 48h and treated with different concentrations of CDDP. Data shown are normalized % mean ± %SD from seven different data sets.(*: $p < 0.05$ and **: $p < 0.01$)

Administration of NAC was performed an hour before the CDDP treatment at a final concentration of 0.5mM. CDDP was then added into the same medium either at increasing concentrations (0, 12, 30 and 60µM) for pre-set duration of treatment (24h or 48h) or at pre-set concentrations (0 and 30 µM) for increasing duration of treatment (24h and 48h) to clarify the dose- and time-dependencies.

For 24h treatment cell viability was found to be restored to a certain extend as responsive to NAC pre-treatment in both cell lines. Here, for the wt cells the change in cell viability was found to be 2.12, 4.84 and 2.1% for 12, 30 and 60 µM CDDP, respectively when compared to CDDP only with the untreated control taken as 100%. For p53-/- cells the change was again positive with 2.74, 5.24 and 8.43% for 12, 30 and 60 µM CDDP, respectively when compared to CDDP only with the untreated control taken as 100%. The p53-/- cells showed susceptibility to loss of cell viability in the presence of NAC as in the absence, too than the wt cells and they proved to be slightly more responsive to NAC pre-treatment than the wt cells for 24h treatment. (Fig. 4.1.d)

For 48h treatment cell viability was found to be restored to a certain extend as responsive to NAC pre-treatment in both cell lines. Here, for the wt cells the observed change was 14.4, 17.2, 5.42% for 12, 30 and 60 µM CDDP, respectively when compared to CDDP only with the untreated control taken as 100%. For p53-/- cells the incline was observed as 4.82, 13.2, 4.24% for 12, 30 and 60 µM CDDP, respectively when compared to CDDP only with the untreated control taken as 100%. The wt cells proved to be more prone to loss of cell viability in the presence of NAC as in the absence, too than the p53-/- cells yet the p53-/- cells proved to be slightly more responsive to NAC pre-treatment than the wt cells. (Fig. 4.1.e.)

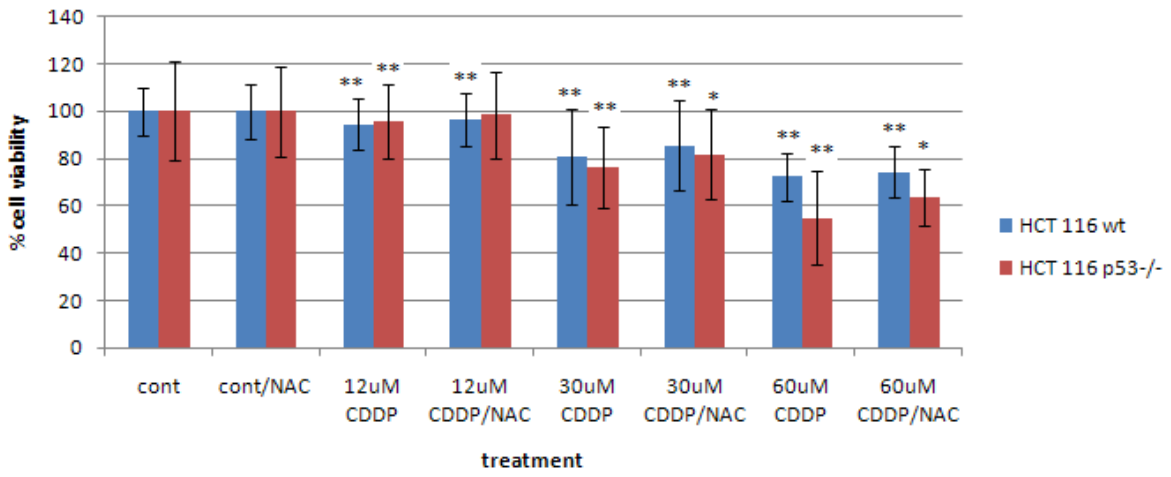


Fig. 4.1.d. MTT Assay for assessment of cell viability. Both cell lines were seeded in 96-well plates at a density of $2.0x10^4$ cells/well and treated 24h with different concentrations of CDDP in the presence of 0.5mM NAC. Data shown are mean ± SD from seven different data sets.(*: $p < 0.05$ and **: $p < 0.01$)

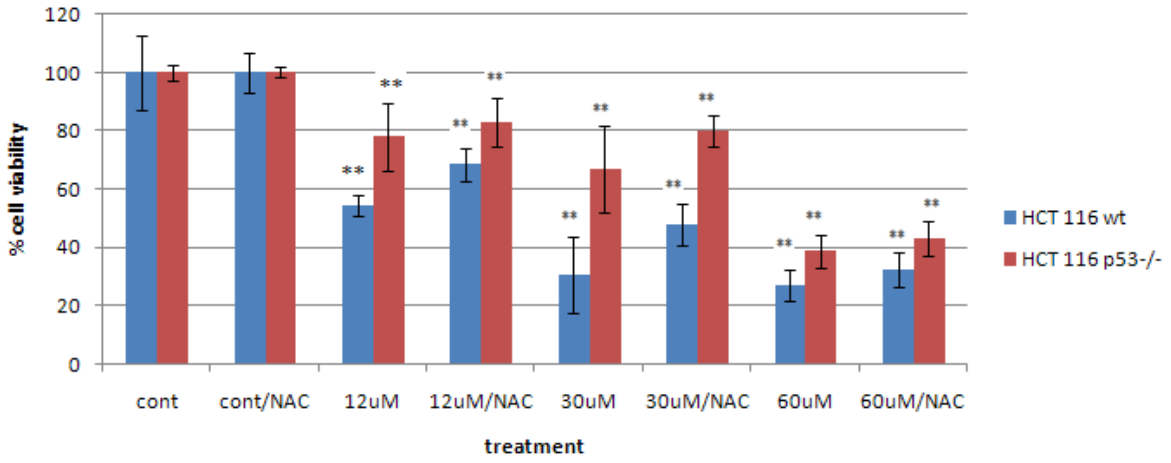


Fig. 4.1.e. MTT Assay for assessment of cell viability. Both cell lines were seeded in 96-well plates at a density of 1.2x104 cells/well and treated 48h with different concentrations of CDDP in the presence of 0.5mM NAC. Data shown are mean ± SD from seven different data sets.(*: $p < 0.05$ and **: $p < 0.01$)

Cell viability was found to be restored to a certain extend as responsive to NAC pre-treatment in both cell lines with time. In 24h, for the wt cells there was increase by 4.84% as for p53-/- cells by 5.24% for 30µM CDDP when compared to CDDP only with the untreated control taken as 100%. In 48h, for the wt cells there was increase by 17.2% as for p53-/- cells by 13.2% for 30µM CDDP when compared to CDDP only with the untreated control taken as 100%.The p53-/- cells proved to be more responsive to NAC pre-treatment for 24h treatment with CDDP as it was the wt responded better to NAC pre-treatment in terms of restoration of cell viability for 48h treatment with CDDP. (Fig. 4.1.f.)

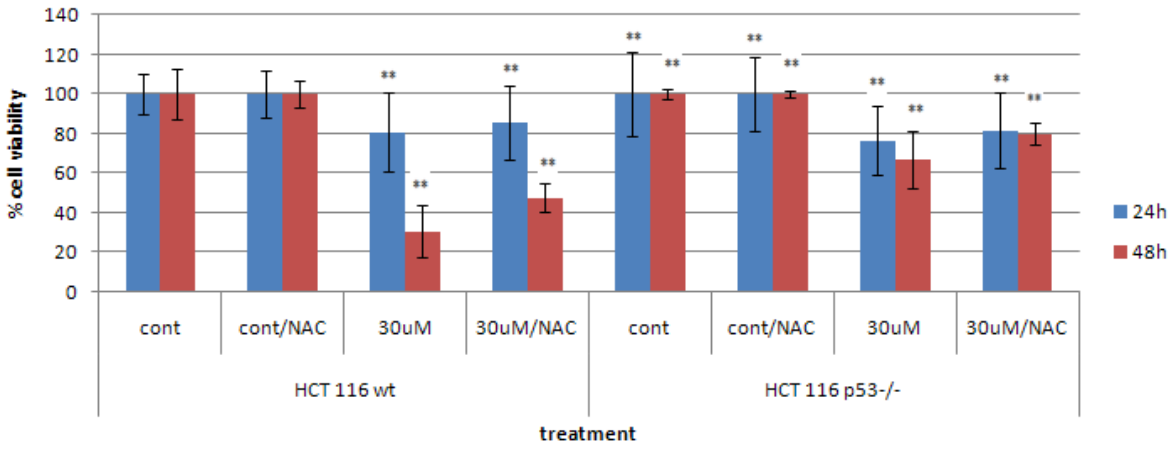


Fig. 4.1.f. MTT Assay for assessment of cell viability. Both cell lines were seeded in 96-well plates at a density of 2.0x104 cells/well for 24h and 1.2x104 cells/well for 48h and treated with different concentrations of CDDP in the presence of 0.5mM NAC. Data shown are mean ± SD from seven different data sets.(*: $p < 0.05$ and **: $p < 0.01$)

4.2. Effect of CDDP on Apoptotic Response

To investigate the effect of CDDP administration on HCT 116 wt and p53-/- cells in terms of apoptotic response Flow Cytometric Analyses by Annexin-V Labelling was employed. In Flow Cytometric Analyses by Annexin-V Labelling 12-well plates were seeded with the two cell lines at a density of $2.5x10^5$ cells/well for 24h and $1.5x10^5$ cells for 48h treatments. Data obtained were given as folds normalized according to the controls.

To demonstrate the relation between dose and apoptotic response different concentrations of CDDP (0, 12, 30 and 60µM) were administered to HCT 116 wt and p53-/- cells for a pre-set duration of treatment (24 and 48h).

For 24h treatment apoptotic response was found to be following an escalating pattern similar in both cell lines with increasing doses of CDDP. Here, for the wt cells the increase continued by 2.86, 3.83 and 4.9 fold for 12, 30 and 60 µM CDDP, respectively when compared to the untreated control in a normalized manner. For p53-/- cells sensitivity to apoptosis continued by 2.01, 2.43 and 3.58 fold for 12, 30 and 60µM CDDP, respectively when compared to the untreated control in a normalized manner. The wt cells showed stronger apoptotic response than the p53-/- cells. (Fig. 4.2.a.)

For 48h treatment apoptotic response was found to be following an inclining pattern in both cell lines with increasing doses of CDDP. Here, for the wt cells the increase continued by 3.46, 4.71 and 9.58 fold for 12, 30 and 60 µM CDDP, respectively when compared to the untreated control in a normalized manner. For p53-/- cells apoptotic response grew more solid by 2.33, 2.58 and 4.19 fold for 12, 30 and 60µM CDDP, respectively when compared to the untreated control in a normalized manner. The wt cells were proven to be more apoptotic in comparison to the p53-/- cells, especially at 60 µM CDDP. (Fig. 4.2.b.)

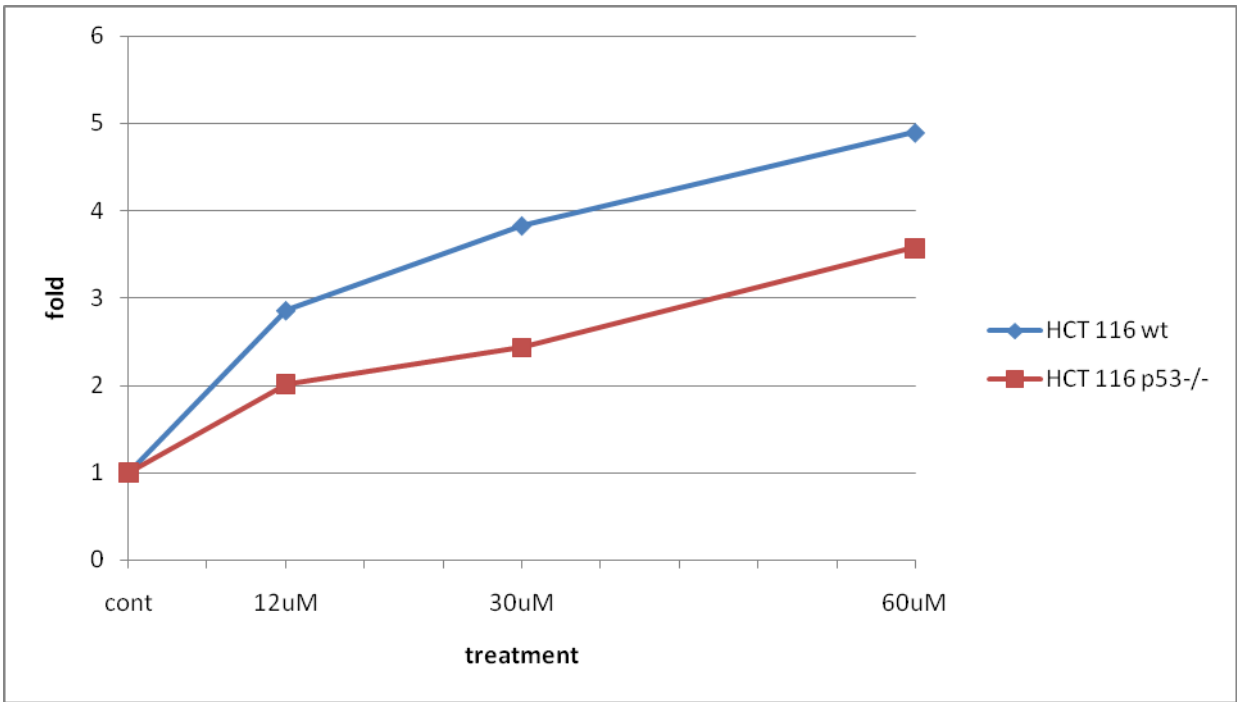


Fig.4.2.a. Annexin-V Labelling for apoptotic response in Flow Cytometry. Both cell lines were seeded in 12-well plates at a cell density of 2.5x105 cells/well and treated 24h with different concentrations of CDDP. Data shown are normalized according to the controls of one experiment.

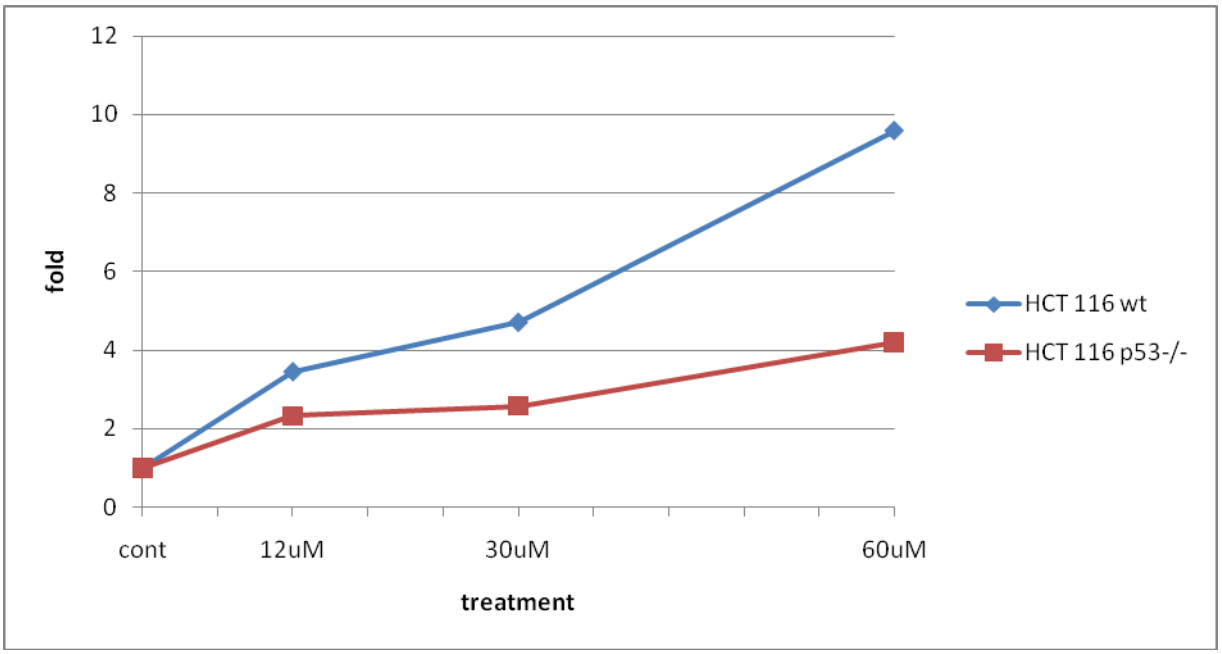


Fig. 4.2.b. Annexin-V Labelling for apoptotic response in Flow Cytometry. Both cell lines were seeded in 12-well plates at a cell density of 1.5x105 cells/well and treated 48h with different concentrations of CDDP. Data shown are normalized according to the controls of one experiment

To demonstrate the relation between duration of treatment and apoptotic response pre-set concentrations of CDDP (0 and 30μM) were administered to HCT 116 wt and p53-/- cells for different durations of treatment (24h and 48h).

Apoptotic response was found to be intensifying in both cell lines with time. In 24h, for the wt cells the strength amplified by 3.83 fold and for p53-/- cells by 2.43 fold for 30μM CDDP when compared to CDDP only in a normalized manner according to untreated controls. In 48h, for the wt cells the apoptotic response was more powerful by 4.71 fold and for p53-/- cells by 2.58 fold for 30μM CDDP when compared to CDDP only in a normalized manner according to untreated controls. The wt cells showed more resilient apoptotic response in 48h than in 24h as the p53-/- cells showed an only slight one in both 24h and 48h and also when compared to wt cells. (Fig. 4.2.c.)

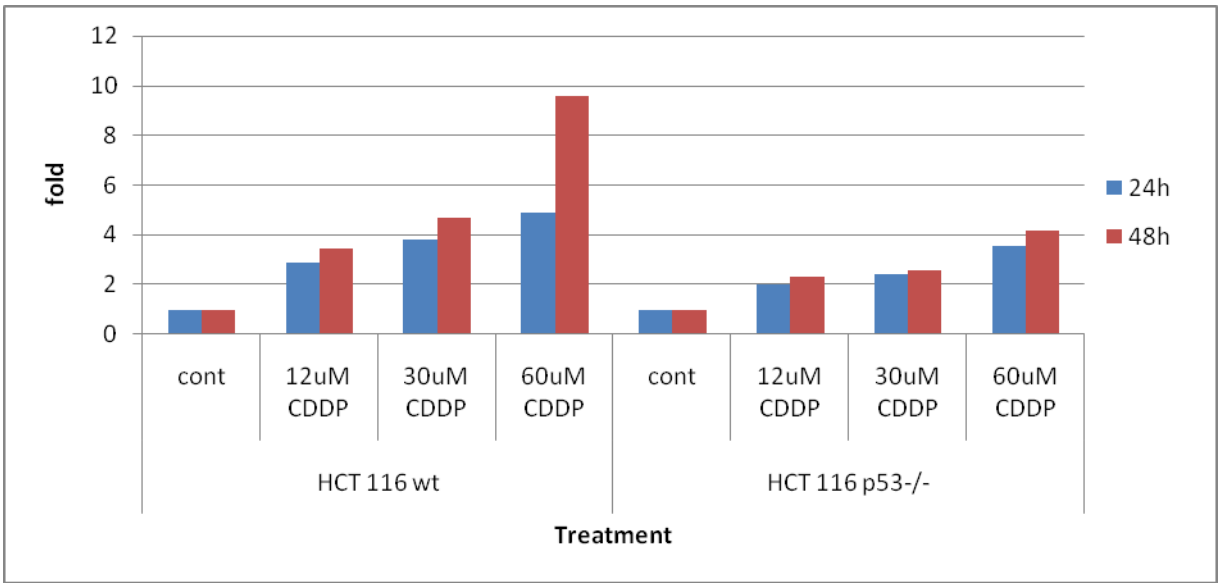


Fig. 4.2.c. Annexin-V Labelling for apoptotic response in Flow Cytometry. Both cell lines were seeded in 12-well plates at a cell density of 2.5x105 cells/well for 24h and 1.5x105 cells for 48h and treated with pre-set concentrations of CDDP (0 and 30µM). Data shown are normalized according to the controls of one experiment.

Administration of NAC was performed an hour before the CDDP treatment at a final concentration of 0.5mM. CDDP was then added into the same medium either at increasing concentrations (0, 12, 30 and 60µM) for pre-set duration of treatment (24h or 48h) or at pre-set concentrations (0 and 30 µM) for increasing duration of treatment (24h and 48h) to clarify the dose- and time-dependencies.

For 24h treatment apoptotic response was found to be declining as responsive to NAC pre-treatment in both cell lines. Here, for the wt cells the intensity of the response lessened by 1.49, 0.23 and 0,91 fold for 12, 30 and 60µM CDDP, respectively when compared to CDDP only in a normalized manner according to untreated controls. For p53-/- cells the apoptotic response went down by 0.74, 1 and1.44 fold for 12, 30 and 60µM CDDP, respectively when compared to CDDP only in a normalized manner according to untreated controls. The wt cells underwent more apoptosis in the presence of NAC as in the absence, too than the p53-/- cells yet it is the p53-/- cells which proved to be more responsive to NAC pre-treatment than the wt cells. (Fig. 4.2.d.)

For 48h treatment apoptotic response was found to be falling as responsive to NAC pre-treatment in both cell lines. Here, for the wt cells there was a decrease in apoptotic response by 1.21, 1.97 and 6.86 fold for 12, 30 and 60µM CDDP, respectively when compared to CDDP only in a normalized manner according to untreated controls. For p53-/- cells the drop was by 0.8, 0.17 and 1.29 fold for 12, 30 and 60µM CDDP, respectively when compared to CDDP only in a normalized manner according to untreated controls. The p53-/- cells proved to be intermediately more prone to apoptosis in the presence of NAC as in the absence, too than the wt cells yet it is the wt cells which proved to be more responsive to NAC pre-treatment than the p53-/- cells. (Fig. 4.2.e.)

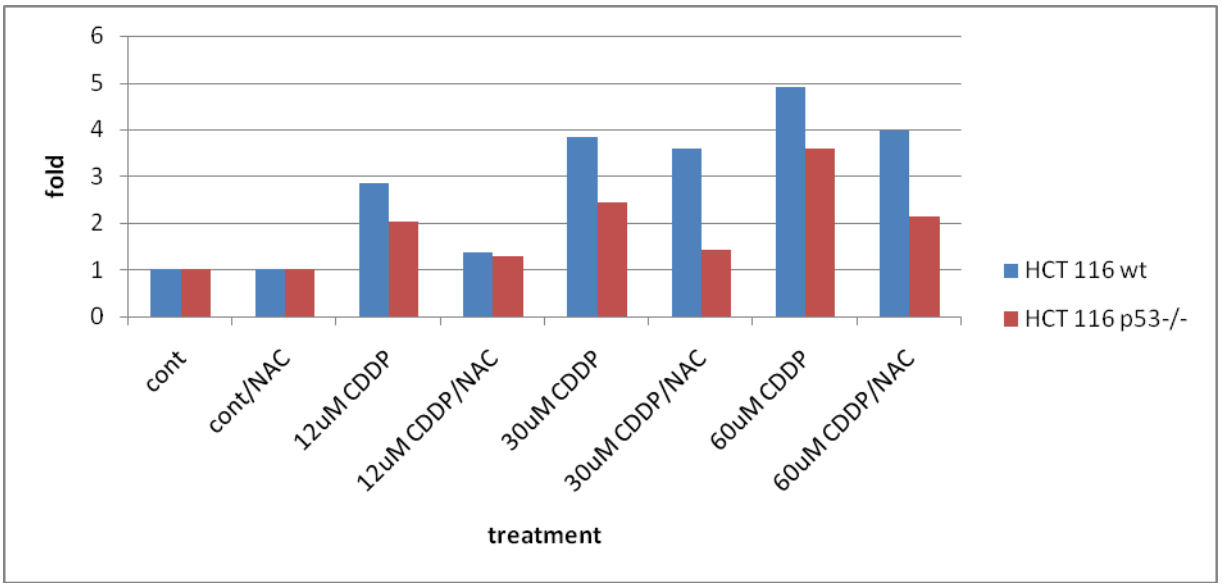


Fig. 4.2.d. Annexin-V Labelling for apoptotic response in Flow Cytometry. Both cell lines were seeded in 12-well plates at a cell density of $2.5x10^5$ cells/well and treated 24h with different concentrations of CDDP in the presence of 0.5mM NAC. Data shown are normalized according to the controls of one experiment.

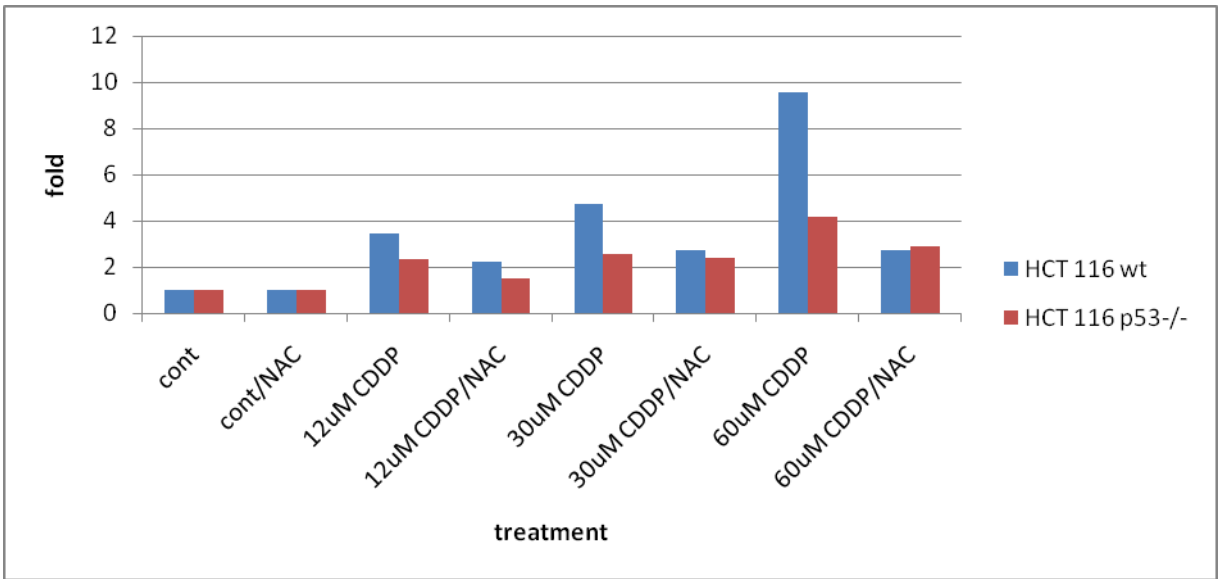


Fig. 4.2.e. Annexin-V Labelling for apoptotic response in Flow Cytometry. Both cell lines were seeded in 12-well plates at a cell density of $1.5x10^5$ cells/well and treated 48h with different concentrations of CDDP in the presence of 0.5mM NAC. Data shown are normalized according to the controls of one experiment.

Apoptotic response was found to be diminishing as responsive to NAC pre-treatment in both cell lines with time. In 24h, for the wt cells the loss in intensity of the apoptotic response by 0.23 fold and for p53-/- cells by 1 fold for 30µM CDDP when compared to CDDP only in a normalized manner according to untreated controls. In 48h, for the wt cells the drop was by 1.97 fold and for p53-/- cells by 0.17 fold for 30µM CDDP when compared to CDDP only in a normalized manner according to untreated controls. The p53-/- cells proved to be more responsive to NAC pre-treatment than the wt cells in 24h yet the late effect of NAC pre-treatment on wt was observed for 48h. (Fig. 4.2.f.)

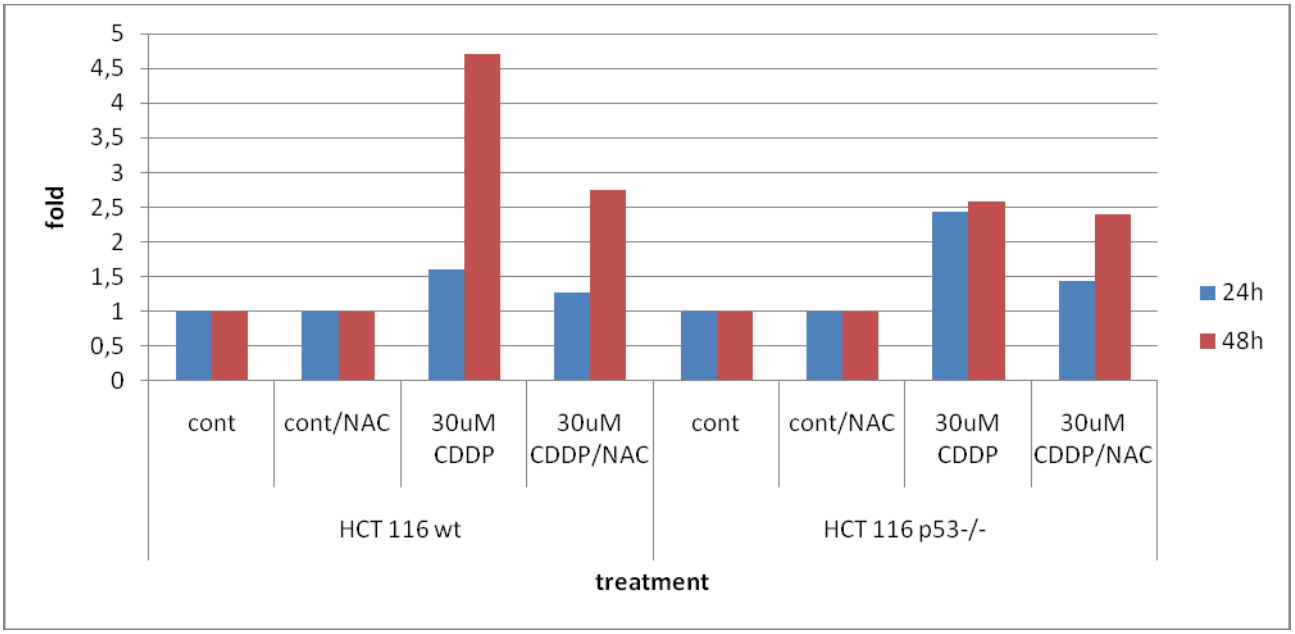


Fig. 4.2.f. Annexin-V Labelling for apoptotic response in Flow Cytometry. Both cell lines were seeded in 12-well plates at a cell density of 2.5x105 cells/well for 24h and 1.5x105 cells for 48h and treated with pre-set concentrations of CDDP (0 and 30μM) in the presence of 0.5mM NAC. Data shown are normalized according to the controls of one experiment

4.3. Effect of CDDP on ROS Production

To investigate the effect of CDDP administration on HCT 116 wt and p53-/- cells in terms of intracellular ROS levels Flow Cytometric Analyses by DCFH-DA Labelling of ROS was employed. In Flow Cytometric Analyses by DCFH-DA Labelling of ROS 12-well plates were seeded with the two cell lines at a density of $2.5x10^5$ cells/well for 24h and $1.5x10^5$ cells for 48h treatments. Data obtained were given as folds normalized according to the controls.

To demonstrate the relation between dose and intracellular ROS levels different concentrations of CDDP (0, 12, 30 and 60μM) were administered to HCT 116 wt and p53-/- cells for a pre-set duration of treatment (24 and 48h).

For 24h treatment intracellular ROS levels were found to be following an increasing pattern in both cell lines with increasing doses of CDDP. Here, for the wt cells the intensification in ROS production continued by 3.32, 3.97 and 4.53 fold for 12, 30 and 60 μM CDDP, respectively when compared to the untreated control in a normalized manner. For p53-/- cells the increase in ROS production continued by 3.04 and 3.24 fold for 12 and 30μM CDDP,

respectively when compared to the untreated control in a normalized manner, yet there was a sudden decrease in ROS levels in this cell line for treatment with 60µM CDDP by 1.2 fold compared to the 30µM CDDP treatment. When the datum of the sudden decrease omitted, p53-/- cells showed an intermediate dominance in intracellular ROS levels than the wt cells. (Fig. 4.3.a.)

For 48h treatment intracellular ROS levels were found to be following an escalating pattern very similar in both cell lines with increasing doses of CDDP. Here, for the wt cells the amplification in intracellular ROS levels continued by 4.53, 5.18 and 5.79 fold for 12, 30 and 60 µM CDDP, respectively when compared to the untreated control in a normalized manner. For p53-/- cells the increase was sustained by 3.67, 5 and 5.75 fold for 12, 30 and 60µM CDDP, respectively when compared to the untreated control in a normalized manner. The wt cells showed a slightly sharper increase in ROS levels at the 12µM CDDP concentration than the p53-/- cells which caught up with the wt at 60 µM CDDP that at the end the p53-/- cells were proven to be producing more ROS than the wt cells to a slight extend. (Fig. 4.3.b.)

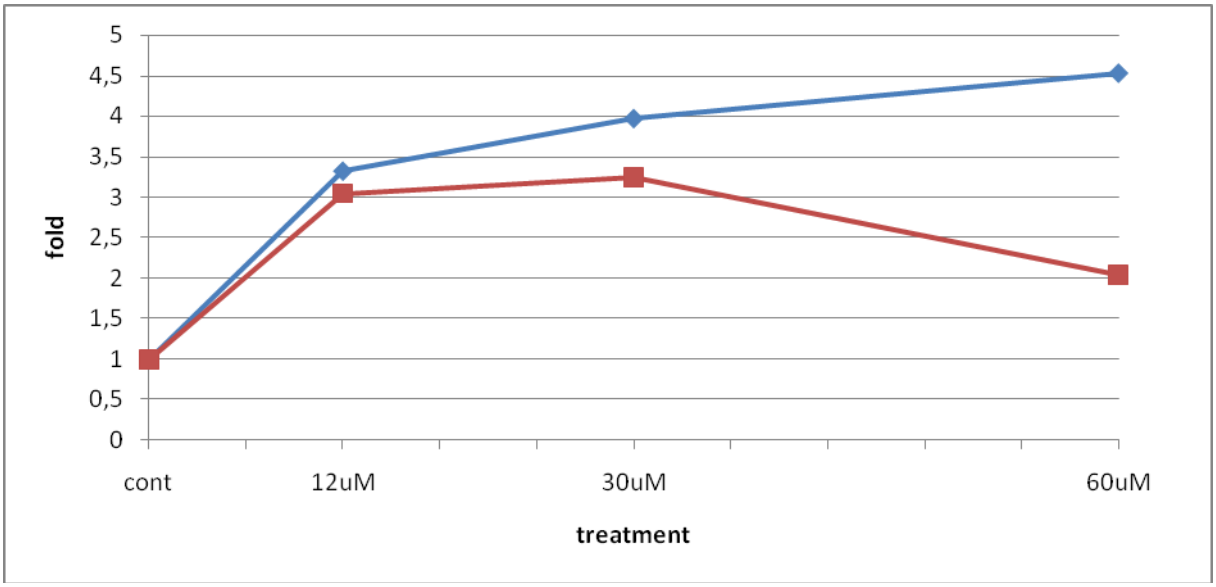


Fig. 4.3.a. DCFH-DA Labelling of ROS in Flow Cytometry. Both cell lines were seeded in 12-well plates at a cell density of 2.5x105 cells/well and treated 24h with different concentrations of CDDP. Data shown are normalized according to the controls of one experiment.

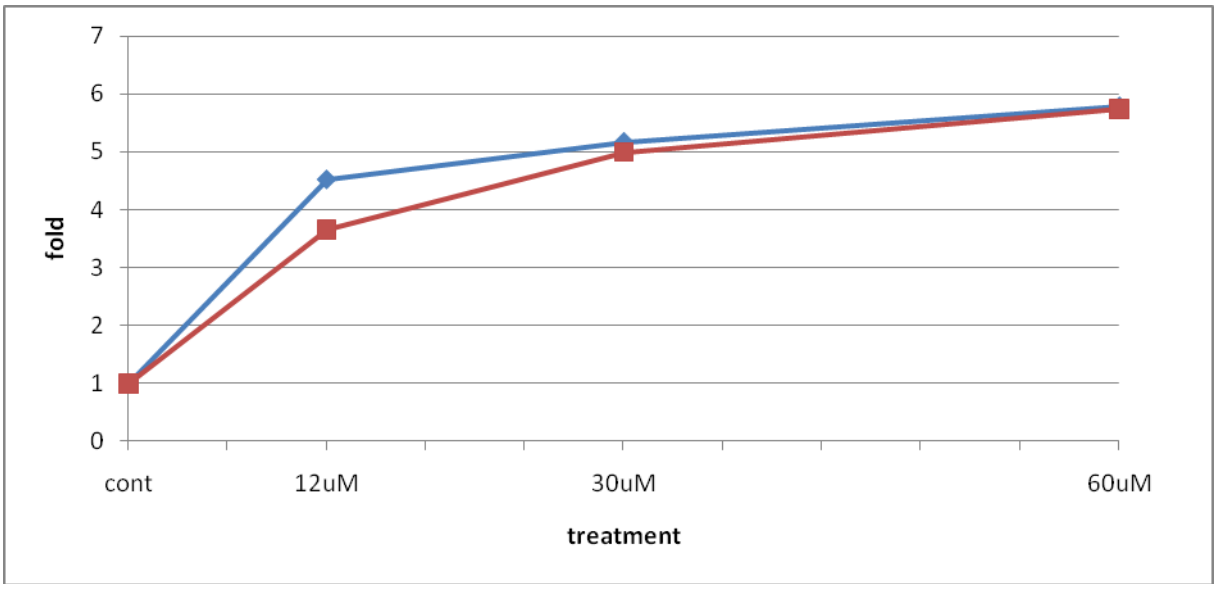


Fig. 4.3.b. DCFH-DA Labelling of ROS in Flow Cytometry. Both cell lines were seeded in 12-well plate at a cell density of 1.5x105 cells/well and treated 48h with different concentrations of CDDP. Data shown are normalized according to the controls of one experiment.

To demonstrate the relation between duration of treatment and intracellular ROS levels pre-set concentrations of CDDP (0 and 30µM) were administered to HCT 116 wt and p53-/- cells for different durations of treatment (24 and 48h).

Intracellular ROS levels were found to be rising in both cell lines with time. In 24h, for the wt cells the extension of ROS levels was by 3.97 fold and for p53-/- cells by 3.24 fold for 30µM CDDP when compared to the untreated control in a normalized manner. In 48h, for the wt cells the raise in ROS levels was by 5.18 fold and for p53-/- cells by 5 fold for 30µM CDDP when compared to the untreated control in a normalized manner. Although wt cells showed more steep increase of ROS levels in 24h than the p53-/- cells, both cell lines revealed proximate ROS levels at 48h. (Fig. 4.3.c.)

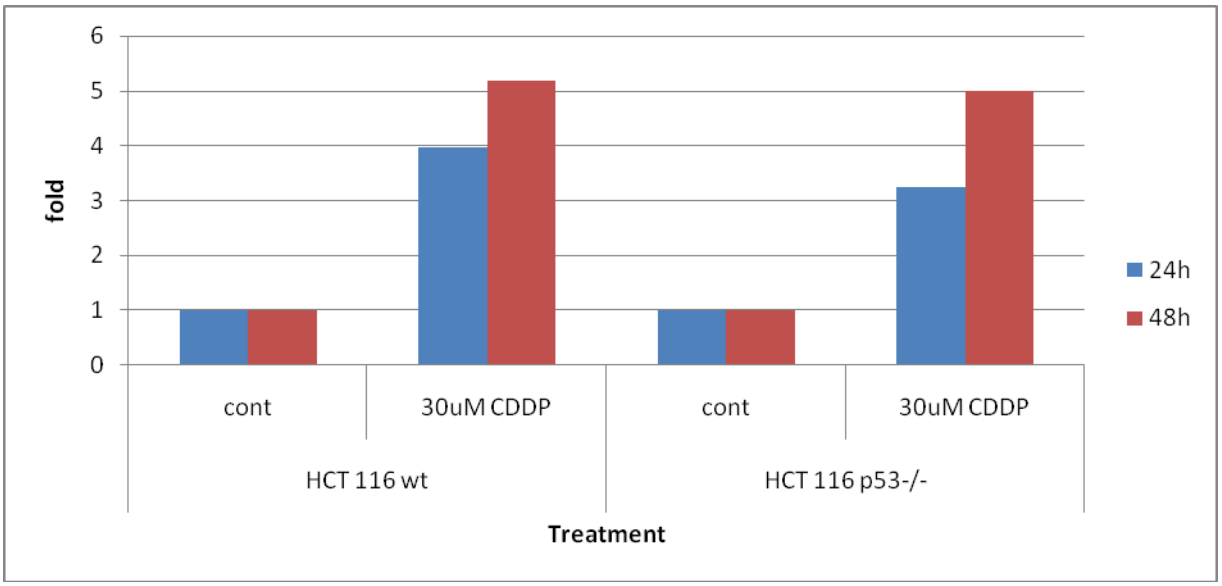


Fig. 4.3.c. DCFH-DA Labelling of ROS in Flow Cytometry. Both cell lines were seeded in 12-well plates at a cell density of 2.5x105 cells/well for 24h and 1.5x105 cells for 48h and treated with pre-set concentrations of CDDP (0 and 30µM). Data shown are normalized according to the controls of one experiment

Administration of NAC was performed an hour before the CDDP treatment at a final concentration of 0.5mM. CDDP was then added into the same medium either at increasing concentrations (0, 12, 30 and 60µM) for pre-set duration of treatment (24h or 48h) or at pre-set concentrations (0 and 30 µM) for increasing duration of treatment (24h and 48h) to clarify the dose- and time-dependencies.

For 24h treatment intracellular ROS levels were found to be decreasing as responsive to NAC pre-treatment in both cell lines. Here, for the wt cells there was a decline in ROS levels by 1.32, 1.93 and 1.69 fold for 12, 30 and 60µM CDDP, respectively when compared to CDDP only in a normalized manner according to untreated controls. For p53-/- cells the fall was 0.06, 0.12 and 0.58 fold for 12, 30 and 60µM CDDP, respectively when compared to CDDP only in a normalized manner according to untreated controls. Here again the sudden lessening in ROS levels in p53-/- cell line for treatment with 60µM CDDP by 1.2 fold compared to the 30µM CDDP treatment should be kept in mind yet the datum was omitted when comparison between the two cell lines was performed. Besides, the drop-off due to NAC pre-treatment was still observed. The p53-/- cells showed more ROS production in the presence of NAC as

in the absence, too than the wt cells but the wt cells proved to be more responsive to NAC pre-treatment than the p53-/- cells as well. (Fig. 4.3.d.)

For 48h treatment intracellular ROS levels were found to be lessening as responsive to NAC pre-treatment in both cell lines. Here, for the wt cells the ROS levels diminished by 0.5, 0.08 and 0.59 fold for 12, 30 and 60µM CDDP, respectively when compared to CDDP only in a normalized manner according to untreated controls. For p53-/- cells the drop was by 0.74, 1.7 and 0.5 fold for 12, 30 and 60µM CDDP, respectively when compared to CDDP only in a normalized manner according to untreated controls. The p53-/- cells showed slightly sharper increase in intracellular ROS levels in the presence of NAC as in the absence, too than the wt cells but the wt cells proved to be more responsive to NAC pre-treatment than the p53-/- cells. (Fig. 4.3.e.)

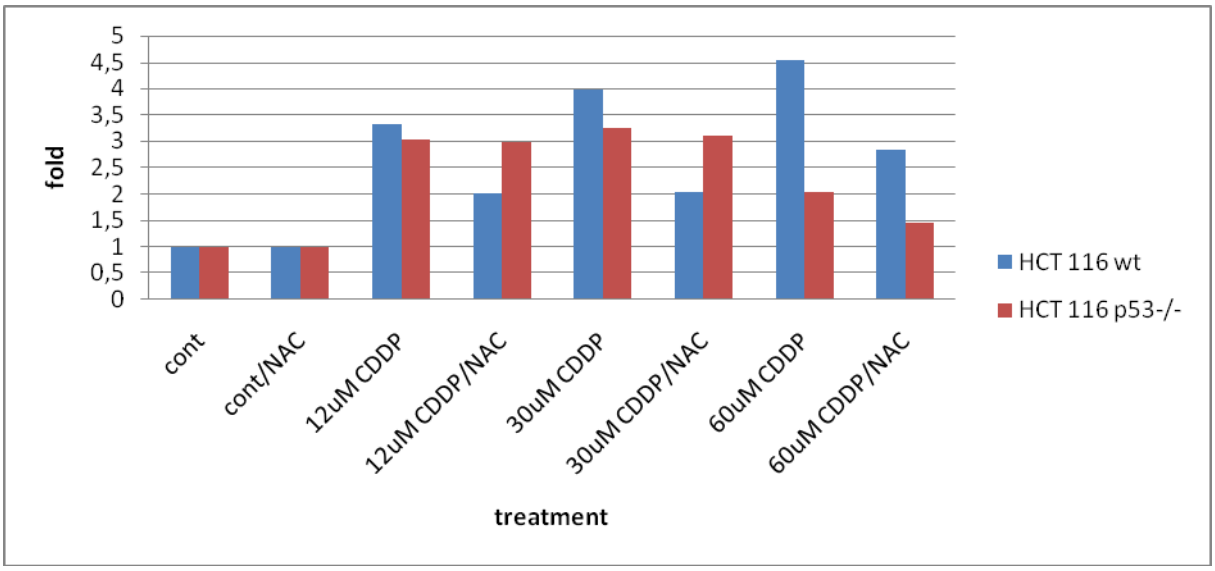


Fig. 4.3.d. DCFH-DA Labelling of ROS in Flow Cytometry. Both cell lines were seeded in 12-well plates at a cell density of $2.5x10^5$ cells/well and treated 24h with different concentrations of CDDP in the presence or absence of 0.5mM NAC. Data shown are normalized according to the controls of one experiment.

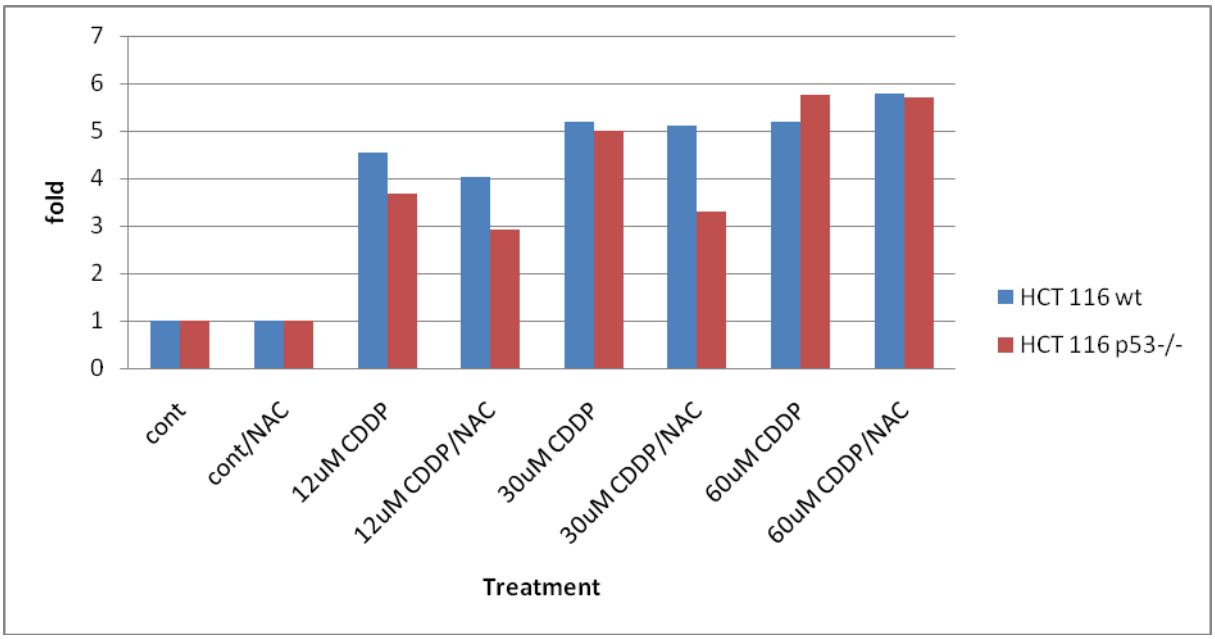


Fig. 4.3.e. DCFH-DA Labelling of ROS in Flow Cytometry. Both cell lines were seeded in 12-well plates at a cell density of $1.5x10^5$ cells/well and treated 48h with different concentrations of CDDP in the presence or absence of 0.5mM NAC. Data shown are normalized according to the controls of one experiment.

Intracellular ROS levels were found to be decreasing as responsive to NAC pre-treatment in both cell lines with time. In 24h, for the wt cells the intracellular ROS levels dropped by 1.93 fold and for p53-/- cells by 0.12 fold for 30µM CDDP when compared to CDDP only in a normalized manner according to untreated controls. In 48h, for the wt cells the decline in ROS levels was 0.08 fold and for p53-/- cells by 1.7 fold for 30µM CDDP when compared to CDDP only in a normalized manner according to untreated controls. The wt cells proved to be more responsive to NAC pre-treatment than the p53-/- cells in 24h yet the late effect of NAC pre-treatment on p53-/- was observed for 48h. (Fig. 4.3.f.)

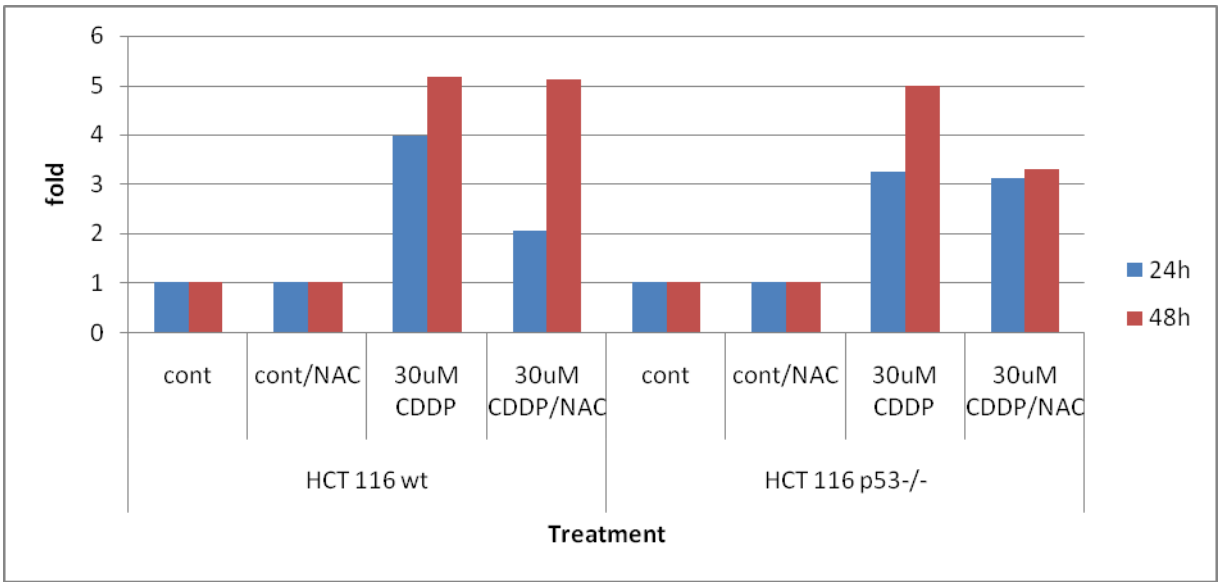


Fig. 4.3.f. DCFH-DA Labelling of ROS in Flow Cytometry. Both cell lines were seeded in 12-well plates at a cell density of 2.5x105 cells/well for 24h and 1.5x105 cells for 48h and treated with pre-set concentrations of CDDP (0 and 30µM) in the presence or absence of 0.5mM NAC. Data shown are normalized according to the controls of one experiment.

4.4. Effect of CDDP on Lipid Peroxidation

To investigate the effect of CDDP administration on HCT 116 wt and p53-/- cells in terms of intracellular lipid peroxidation levels TBARS Assay was employed. In TBARS Assay 12-well plates were seeded with the two cell lines at a density of $2.5x10^5$ cells/well for 24h and $1.5x10^5$ cells for 48h treatments. Data obtained from three different sets were given as average absorbance ± SD.

To demonstrate the relation between dose and intracellular lipid peroxidation levels different concentrations of CDDP (0, 12, 30 and 60µM) were administered to HCT 116 wt and p53-/- cells for a pre-set duration of treatment (24 and 48h).

For 24h treatment intracellular lipid peroxidation levels were found to be following an increasing pattern very similar in both cell lines with increasing doses of CDDP. Here, for the wt cells the amplification in intracellular lipid peroxidation levels continued given as average absorbances from 0.64 in untreated control to 0.7, 0.74 and 0.99 for 12, 30 and 60 µM CDDP, respectively. For p53-/- cells the magnification in intracellular lipid peroxidation levels persisted given as average absorbances from 0.31 in untreated control to 0.33, 0.48 and 0.71

for 12, 30 and 60µM CDDP, respectively. The wt cells showed more lipid peroxidation in untreated control which can be considered as a background level that was significantly smaller for the p53-/- cell line. The increase from 12 to 30µM CDDP was sharper in p53-/- than wt yet this was evened out at 60µM CDDP so that overall change in lipid peroxidation levels was intermediately higher in p53-/- cells in comparison with wt cells. (Fig. 4.4.a.)

For 48h treatment intracellular lipid peroxidation levels were found to be following an escalating pattern in both cell lines with increasing doses of CDDP. Here, for the wt cells the increase continued given as average absorbances from 0.47 in untreated control to 0.56, 0.66 and 0.72 for 12, 30 and 60 µM CDDP, respectively. For p53-/- cells the rise in intracellular lipid peroxidation levels persisted given as average absorbances from 0.5 in untreated control to 0.56 and 0.77 for 30 and 60µM CDDP, respectively whereas for 12µM CDDP there was almost no increase and the average absorbances remained proximate. (Fig 4.4.b.)

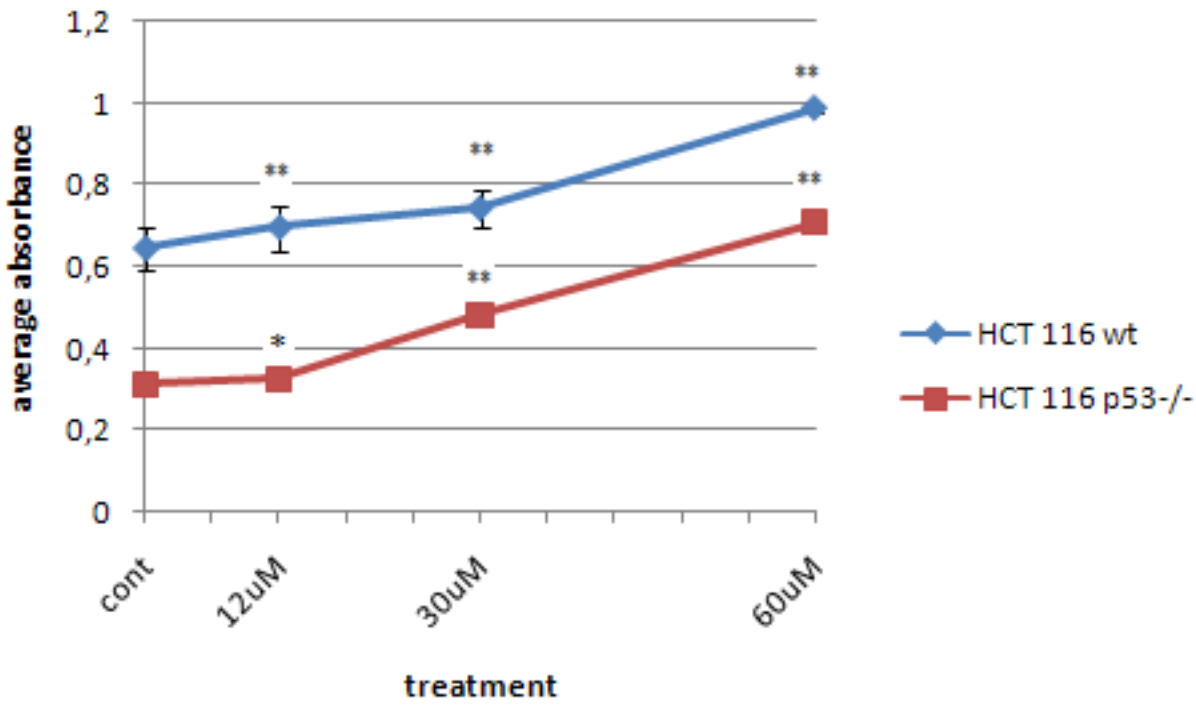


Fig. 4.4.a. TBARS Assay for assessment of ROSlipid peroxidation. Both cell lines were seeded in 12-well plate at a cell density of 2.5x105 cells/well and treated 24h with different concentrations of CDDP. Data shown are mean ±SD from three different data sets.(*: $p < 0.05$ and **: $p < 0.01$)

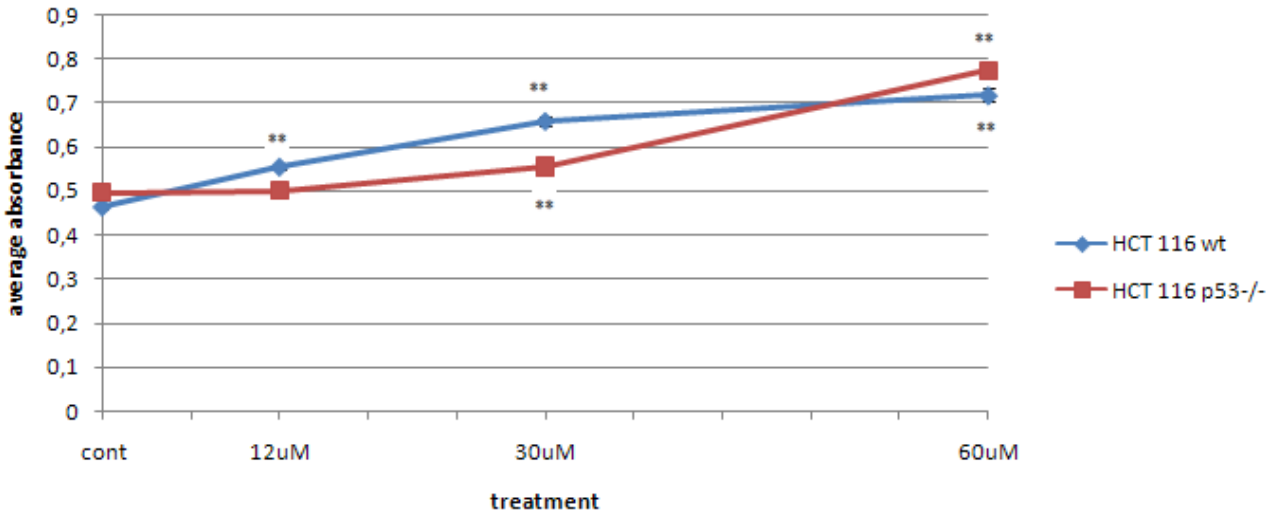


Fig. 4.4.b. TBARS Assay for assessment of ROSlipid peroxidation. Both cell lines were seeded in 12-well plate at a cell density of 1.5x105 cells/well and treated 48h with different concentrations of CDDP. Data shown are the mean ±SD from three different data sets.(*: $p < 0.05$ and **: $p < 0.01$)

To demonstrate the relation between duration of treatment and intracellular lipid peroxidation levels pre-set concentrations of CDDP (0 and 30µM) were administered to HCT 116 wt and p53-/- cells for different durations of treatment (0', 15', 30', 1h, 2h, 4h and 8h as well as 24 and 48h).

Intracellular lipid peroxidation levels were found to be following a certain pattern in both cell lines within short intervals of time. When treated with 30µM CDDP, for the wt cells the change in time given as average absorbances was from 0.03 in t=0 to 0.44 in t=30min, 0.18 in t=1h, 0.11 in t=2h, 0.41 in t=4h and 0.15 in t=8h and for p53-/- cells from 0.03 in t=0 to 0.62 in t=15min, 0.54 in t=30min, 0.36 in t=1h, 0.2 in t=2h, 0.33 in t=4h and 0.3 in t=8h. There was almost no change in wt cells at the end of 15min after CDDP administration, other than that both cell lines seemed to experience a sudden increase in lipid peroxidation levels shortly after CDDP administration which was compensated more and more in time having a minimum point at 2h, yet this trend only continued up until a certain duration of treatment, specifically at around 4^{th} hour when lipid peroxidation levels had a peak that again was pulled down at 8h. (Fig. 4.4.c.)

Intracellular lipid peroxidation levels were found to be increasing in both cell lines with time. In 24h, for the wt cells the intensification in intracellular lipid peroxidation levels given as average absorbances was from 0.64 in untreated control to 0.74 and for p53-/- cells from 0.31 in untreated control to 0.48 for 30µM CDDP. In 48h, for the wt cells the increase in lipid peroxidation levels given as average absorbances was from 0.47 in untreated control to 0.66 and for p53-/- cells from 0.5 in untreated control to 0.56 for 30µM CDDP. Although wt cells showed sharper rise of lipid peroxidation levels in 24h than the p53-/- cells, the slope slowed down in 48h so that the outcomes were closer. (Fig. 4.4.d.)

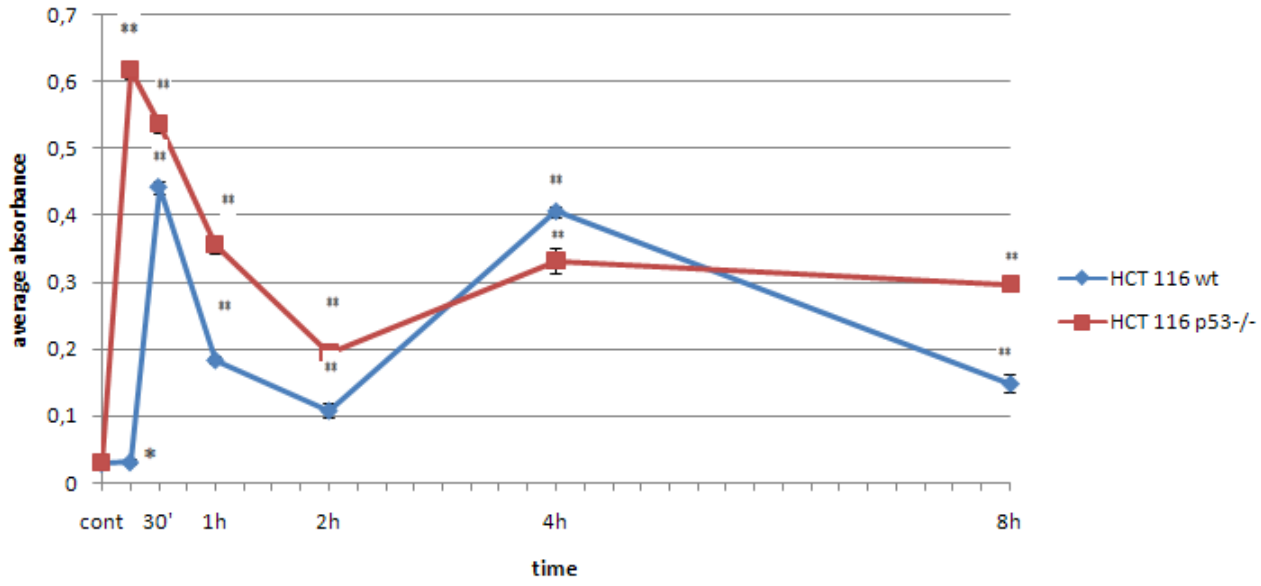


Fig. 4.4.c. TBARS Assay for assessment of lipid peroxidation. Both cell lines were seeded in 12-well plate at a cell density of $2.0x10^5$ cells/well and treated with pre-set concentrations of CDDP (0 and 30 µM) for different durations of treatment (0, 15', 30', 1h, 2h, 4h and 8h). Data shown are mean ± SD from three different data sets. (*: $p < 0.05$ and **: $p < 0.01$)

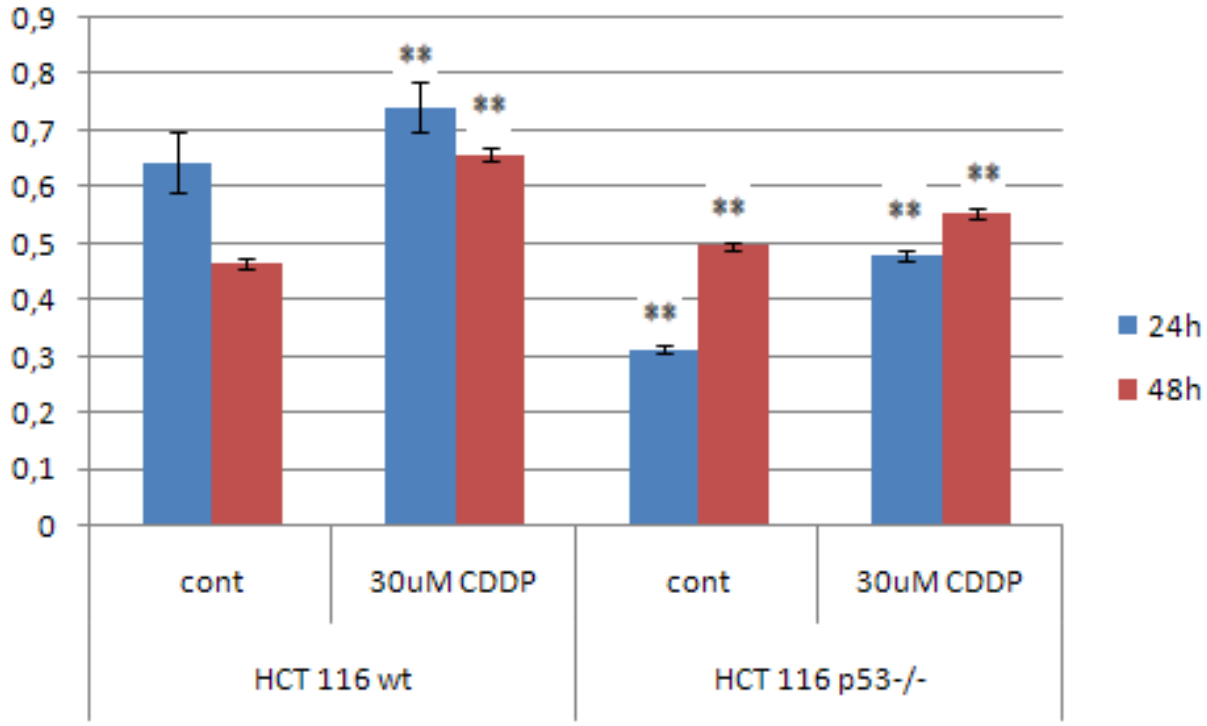


Fig. 4.4.d. TBARS Assay for assessment of lipid peroxidation. Both cell lines were seeded in 12-well plates at a cell density of 2.5x105 cells/well for 24h and 1.5x105 cells for 48h and treated with pre-set concentrations of CDDP (0 and 30μM). Data shown are mean ± SD from three different data sets. (*: $p < 0.05$ and **: $p < 0.01$)

Administration of NAC was performed an hour before the CDDP treatment at a final concentration of 0.5mM. CDDP was then added into the same medium either at increasing concentrations (0, 12, 30 and 60μM) for pre-set duration of treatment (24h or 48h) or at pre-set concentrations (0 and 30 μM) for increasing duration of treatment (24h and 48h) to clarify the dose- and time-dependencies.

For 24h treatment intracellular lipid peroxidation levels were found to be declining as responsive to NAC pre-treatment in both cell lines. Here, for the wt cells the decrease given as difference in average absorbances was 0.13 in untreated control, 0.11 and 0.041 for 12 and 60μM CDDP, respectively. There was however an increase in average absorbance (0.045) for 30μM CDDP. For p53-/- cells the decrease given as difference in average absorbances was 0.034 in untreated control, 0.018, 0.99 and 0.001 for 12, 30 and 60μM CDDP, respectively. The wt cells showed slightly sharper increase in the presence of NAC as in the absence, too

and they proved generally to be more responsive to NAC pre-treatment than p53-/- cells in 24h. (Fig. 4.4.e)

For 48h treatment intracellular lipid peroxidation levels were found to be lessening as responsive to NAC pre-treatment in both cell lines. Here, for the wt cells the decrease given as difference in average absorbances was 0.04 in untreated control and 0.055 for 30µM CDDP. There was however an increase in average absorbance (0.258) for 60 and no change for 12 µM CDDP. For p53-/- cells the decrease given as difference in average absorbances was 0.087 in untreated control, 0.082, 0.65 and 0.244 for 12, 30 and 60µM CDDP, respectively. The wt cells showed slightly more significant amplification in lipid peroxidation levels in the presence of NAC as in the absence, too yet it is the p53-/- cells which proved to be more responsive to NAC pre-treatment than wt cells in 48h. (Fig. 4.4.f.)

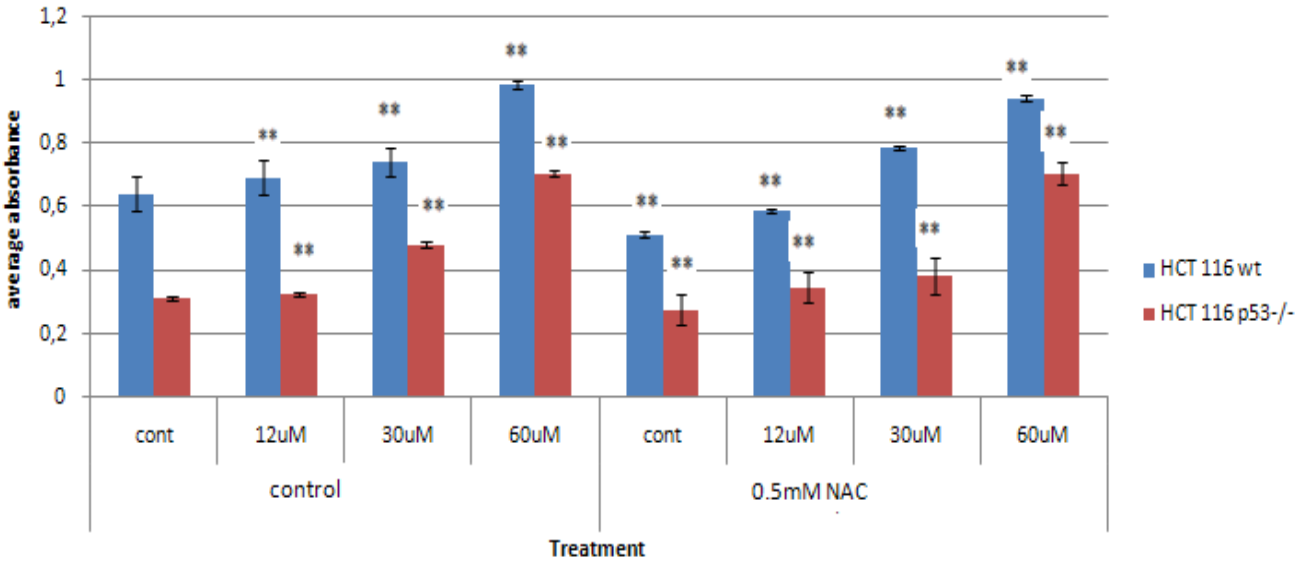


Fig. 4.4.e TBARS Assay for assessment of lipid peroxidation. Both cell lines were seeded in 12-well plate at a cell density of $2.5x10^5$ cells/well and treated 24h with different concentrations of CDDP in the presence or absence of 0.5mM NAC. Data shown are mean ± SD from three different data sets.(*: $p < 0.05$ and **: $p < 0.01$)

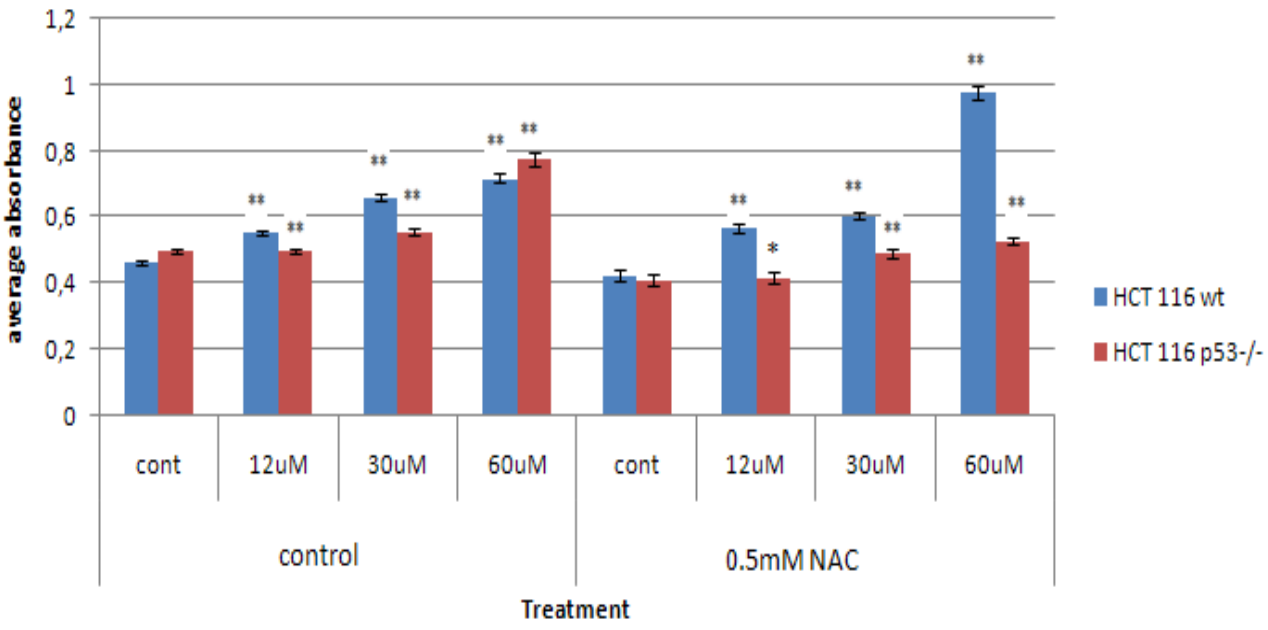


Fig. 4.4.f. TBARS Assay for assessment of lipid peroxidation. Both cell lines were seeded in 12-well plate at a cell density of $1.5x10^5$ cells/well and treated 48h with different concentrations of CDDP in the presence or absence of 0.5mM NAC. Data shown are mean ± SD from three different data sets.(*: $p < 0.05$ and **: $p < 0.01$)

Intracellular lipid peroxidation levels were found to be falling as responsive to NAC pre-treatment in both cell lines with time. In 24h, for the wt cells the decrease given as difference in average absorbances was 0.13 in untreated control as for p53-/- cells 0.034 in untreated control and 0.99 for 30µM CDDP. There was however a rise in average absorbance (0.045) for 30µM CDDP. In 48h, for the wt cells the decrease given as difference in average absorbances was 0.039 in untreated control and 0.055 for 30µM CDDP as for p53-/- cells 0.087 in untreated control and 0.065 for 30µM CDDP. The p53-/- cells proved to be more responsive than wt cells in both 24h and 48h treatments. (Fig. 4.4.g.)

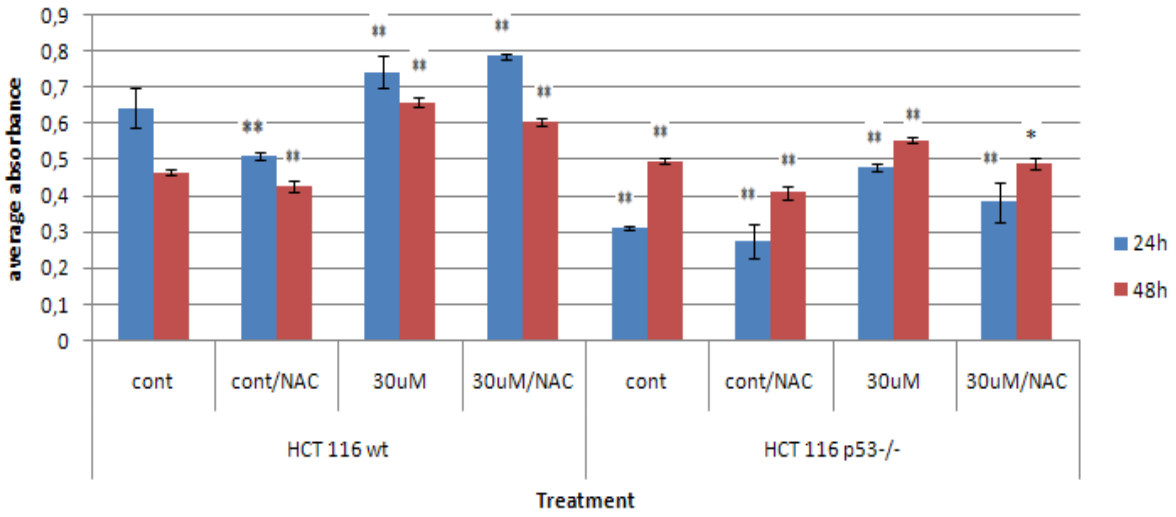


Fig. 4.4.g. TBARS Assay for assessment of lipid peroxidation. Both cell lines were seeded in 12-well plate at a cell density of $2.5x10^5$ cells/well for 24h and $1.5x10^5$ cells for 48h and treated with pre-set concentrations of CDDP (0 and 30µM) in the presence or absence of 0.5mM NAC. Data shown are mean ± SD from three different data sets. (*: $p < 0.05$ and **: $p < 0.01$)

Results in Overview

Comparison of the results has been performed according to the slopes of trendlines for each set of data specific for one cell type and absence or presence of NAC pre-treatment at either various doses of CDDP. The sharper slope being A and that from the other cell line being B, from the direct proportion for A corresponding to 100%, B corresponds to x% and (100-x)% is the difference between the intensities in their response to treatment whether reaction to CDDP, reaction to CDDP + NAC or response strength to NAC pre-treatment. Significance levels have been established for this obtained difference:

- > 10% slight difference
- >25% intermediate difference, and
- <25% significant difference in this concept.

time	*24h*						*48h*					
	CDDP		**CDDP +NAC**		**response to NAC**		**CDDP**		**CDDP +NAC**		**response to NAC**	
MTT	*p*	***	*p*	***	*p*	*	*w*	**	*w*	**	*p*	*
Annexin-V	*w*	***	*w*	***	*p*	***	*w*	***	*p*	**	*w*	***
DCFH-DA	*p*	**	*p*	***	*w*	***	*p*	*	*p*	**	*w*	*
TBARS	*p*	**	*w*	*	*w*	**	*p*	2	*w*	***	*p*	*

Table 4. Summary of the outcomes of the experiments regarding cell type, NAC pre-treatment and duration of treatment with various concentrations of CDDP. * indicates slight (up to 10%), ** indicates intermediate (up to 25%) and *** indicates significant (more than 25%) difference and "*w*" is short for wt and "*p*" for p53-/- cell type.

4.5. Effect of CDDP on Protein Carbonylation

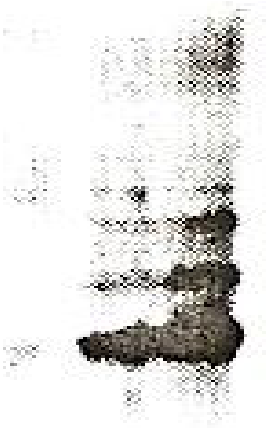
For obtaining protein isolates four 100mm dishes were seeded with HCT 116 wt cells at a density of $4.0x10^6$ cells/dish and the dishes were kept in the incubator until attachment of cells. Upon removal of the initial medium, CDDP was administered at increasing concentrations (0, 20, 30 and 90μM) for pre-set duration of treatment (6h and16h) to clarify the dose- and time-dependencies of protein carbonylation.

Total protein isolates were harvested after 6h and 16h of CDDP administration and used as samples in the OxyBlot™ procedure.

These protein isolates revealed carbonylation signals increasing in intensity (i.e. darker bands) as with increasing doses of CDDP as well as increasing duration of treatment. (Fig. 4.5.a. and Fig. 4.5.b.)

lane	1	2	3	4
CDDP in µM	0	20	30	90

Fig. 4.5.a. OxyBlot™ picture of samples harvested at 6h after administration of CDDP



lane	1	2	3	4
CDDP in µM	0	20	30	90

Fig. 4.5.b. OxyBlot™ picture of samples harvested at 16h after administration of CDDP

OxyBlot™ procedure was also applied to Bcl-2, Bcl-x_L, and Mcl-1 protein samples purified by immunoprecipitation from total protein isolates of cell cultures treated with 0 or 30μM of CDDP in the absence and presence of NAC pre-treatment for 24h. (Fig. 4.5.c, Fig. 4.5.d. and Fig. 4.5.d, respectively)

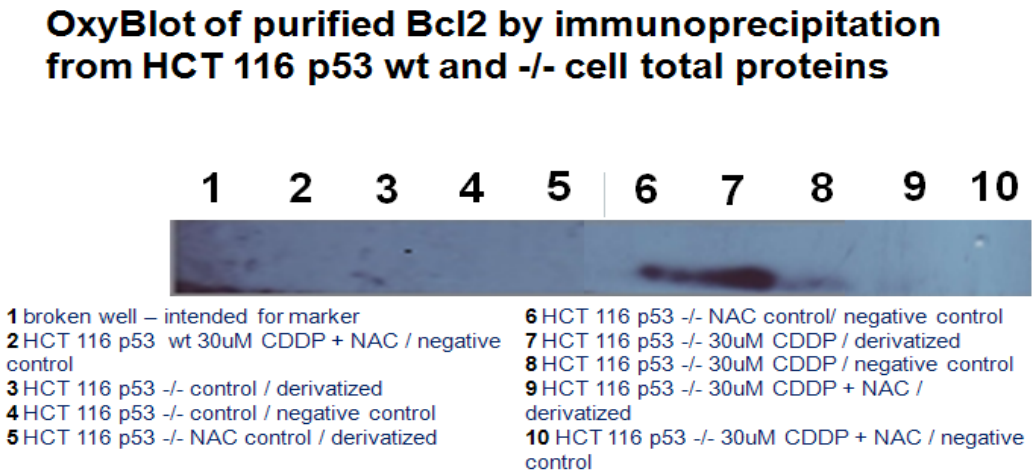


Fig. 4.5.c. OxyBlot™ picture of purified Bcl-2 samples from cell cultures harvested 24h after administration of CDDP

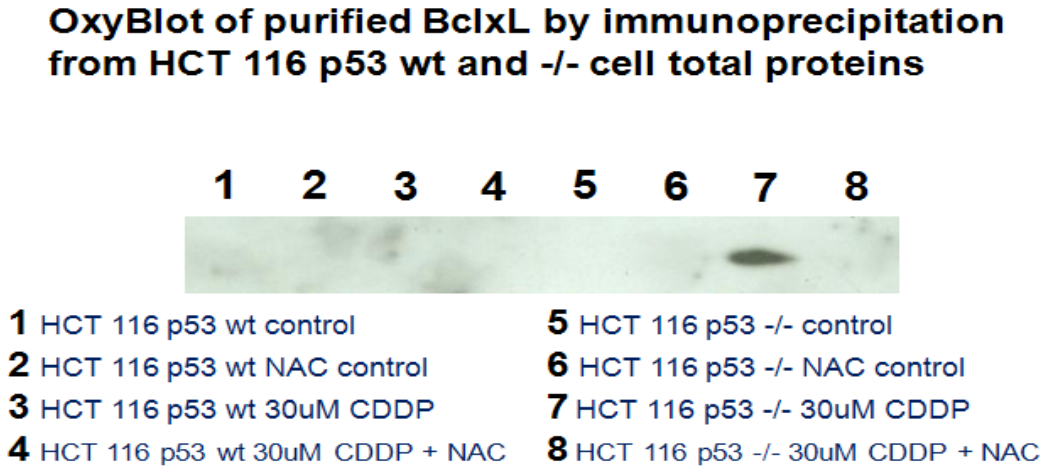


Fig. 4.5.d. OxyBlot™ picture of purified Bcl-x_L samples from cell cultures harvested 24h after administration of CDDP

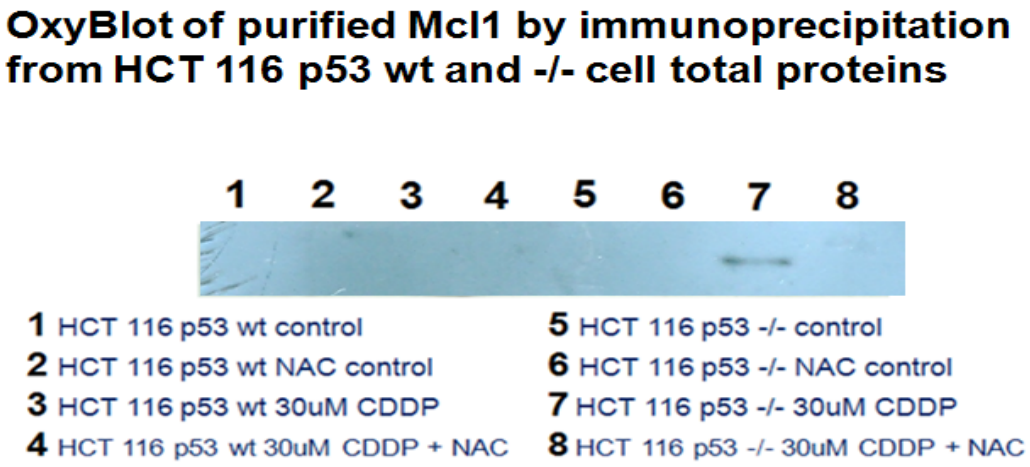


Fig. 4.5.e. OxyBlot™ picture of purified Mcl-1 samples from cell cultures harvested 24h after administration of CDDP

4.6. Effect of CDDP on Cell Morphology

HCT 116 wt and p53-/- cells were treated with various concentrations of CDDP in the presence or absence of NAC. Pictures of each culture were taken after 24h with the light microscope at a magnification of 40X.

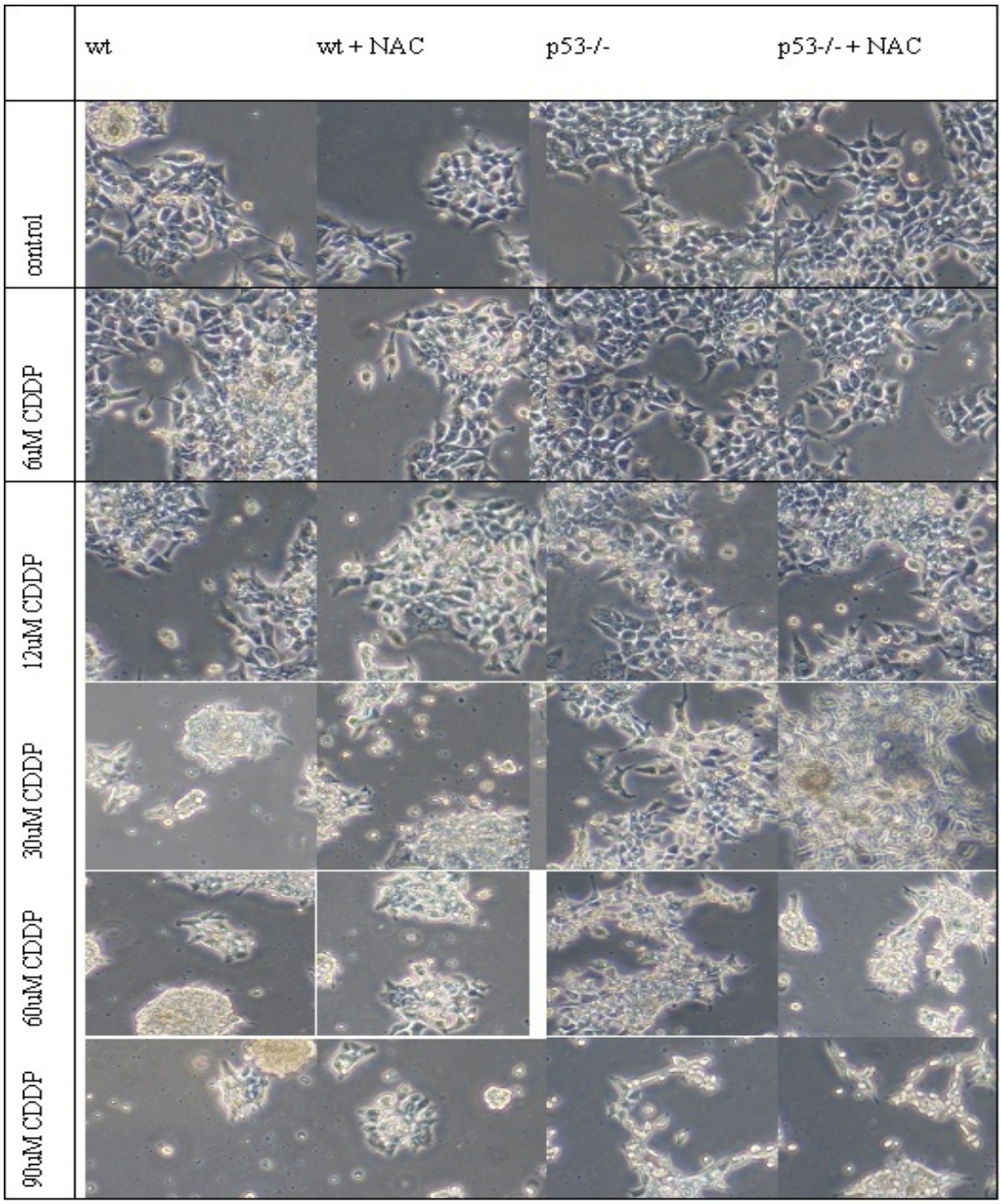


Table 4.6. Pictures of wt and p53-/- cells taken under light microscope 40X after 24h treatment with various concentrations of CDDP

5. DISCUSSION

Results in the first two sections regarding the cell viability and apoptotic response to CDDP treatment indicated that CDDP decreased the cell viability and increased apoptotic cell death and these effects are directly proportional to concentration and duration of treatment.
Here, for determination of the effect of treatment on the cell viability MTT Assay, a standard colorimetric assay for measuring the activity of enzymes in mitochondria that reduce MTT to formazan, giving a purple color as a measure of mitochondrial activity and also cytotoxicity caused by mitochondrial dysfunction, was employed. One disadvantage of this assay has been reported as it may give false positives with reactive oxygen metabolites. It was previously used in other studies as an assay for determining the enzymatic activity in mitochondria only without deducing assumptions about changes in cell viability. [132] Yet, the objective of the assay in this study was assessment of cell viability and the obtained data indicated that cell viability tended to lessen with increasing doses of CDDP and with time, an effect that was reversed to some extend by the NAC pre-treatment and this proving that some fraction of loss of cell viability is ROS-dependent.

For 24h treatment cell viability was found to be following a decreasing pattern similar in both cell lines with increasing doses of CDDP. The p53-/- cells proved to be less proliferative than the wt cells by ca. 39% indicating the positive role of p53 on the mechanisms of cell survival and also of the intracellular redox equilibrium. (Fig. 4.1.a.) Cell viability was found to be restored to a certain extend as responsive to NAC pre-treatment in both cell lines. The p53-/- cells showed steeper decline in cell viability in the presence of NAC as in the absence than the wt cells pointing out to the importance of p53 as a guardian of the genome and hence cell proliferation. [10] They also proved to be more responsive to CDDP administration in the presence of NAC pre-treatment than the wt cells by ca. 32% confirming the prior connections of cell viability and protective role of p53. [9] (Fig. 4.1.d) Overall for 24h p53-/- cells were ca. 10% more responsive to NAC pre-treatment than the wt cells.

For 48h treatment cell viability was found to be following a decreasing pattern in both cell lines with increasing doses of CDDP. The wt cells lost their viability more by ca. 11% than the p53-/- cells representative of the fact that the unrepairable damage exerted by ROS given enough time can end up with loss of viability, a phenomenon augmented by the p53 related induction of cell death machinery. [10] (Fig. 4.1.b.) With NAC pre-treatment cell viability was found to be restored to a certain extend in both cell lines. The wt cells proved to be

relatively more proliferative in the presence of NAC as in the absence by ca. 15% than the p53-/- cells this being a sign of p53-related cell death machinery working properly when damage is extensive. [12] Here, Yet the p53-/- cells proved to be ca. 4% more responsive to NAC pre-treatment than the wt cells - a value which could be neglected yet pointing out towards an affirmation of the same behaviour for 24h treatments. (Fig. 4.1.e.)

Cell viability was found to be decreasing in both cell lines in time. The wt cells proved to be less viable in 48h than the p53-/- as for 24h the diminishing in cell viability in both cell lines was proximate. (Fig. 4.1.c.) Cell viability was found to be restored to a certain extend as responsive to NAC pre-treatment in both cell lines with time. The p53-/- cells were more responsive to NAC pre-treatment for 24h treatment with CDDP as it was the wt cells more responsive to NAC pre-treatment for 48h treatment with CDDP. (Fig. 4.1.f.)

To sum up, the p53-/- cells were more sensitive than the wt cells in 24h in terms of loss of cell viability but as duration of treatment was longer wt cells lost viability more than p53-/- regardless of NAC pre-treatment. This proves the p53 protein being an activator of mechanisms to repair damage done especially on the DNA, that its absence results in loss of viability even in shorter terms of time, but also being a promoter of cell death machinery when the damage is beyond repair as with longer exposures to CDDP. [14]

In this study, for determining the apoptotic cell death Flow Cytometric Analyses by Annexin-V Staining, a technique based on counting, examining, and separating of cells suspended in a stream of fluid regarding their affinity to fluorescently labelled Annexin A5 probe which binds to the negatively charged phospholipids (phosphatidylserine) exposed on the cell surface of apoptotic cells, was employed. Data showed that apoptotic response tends to increase with increasing doses of CDDP and time, an effect that was reversed by the NAC pre-treatment to some extend.

For 24h treatment apoptotic response was found to be following an increasing pattern similar in both cell lines with increasing doses of CDDP. The wt cells showed stronger apoptotic response by ca. 33% than the p53-/- cells certainly due to the role of p53 in induction of apoptosis upon the protein's stabilization via phosphorylation in stress conditions. [15] (Fig. 4.2.a.) For 24h treatment apoptotic response was found to be repressed as responsive to NAC pre-treatment in both cell lines. The wt cells showed more apoptosis in the presence of NAC as in the absence than the p53-/- cells by ca. 65% again signifying the importance of p53 in regulation of apoptosis [17] yet it is the p53-/- cells which proved to be more responsive to NAC pre-treatment by ca. 44% than the wt cells as they lack the p53 protein. (Fig. 4.2.d.)

For 48h treatment apoptotic response was found to be following an increasing pattern in both cell lines with increasing doses of CDDP. The wt cells underwent more apoptosis by ca. 64% than the p53-/- cells, especially at 60 μM CDDP where the damage is broader with 48h in comparison to 24h and thus the p53-related portion of the apoptotic response becomes relatively more dominant. (Fig. 4.2.b.) For 48h treatment apoptotic response was found to be restrained as responsive to NAC pre-treatment in both cell lines. The p53-/- cells showed sharper promotion of apoptotic response in the presence of NAC as in the absence than the wt cells by ca. 22% presumably indicative of p53-independent induction of apoptosis due to stress such as lysosome-related activation of cathepsins or down-regulation of TIGAR due to loss of p53 [18] yet it is the wt cells which proved to be more responsive to NAC pre-treatment by ca. 47% than the p53-/- cells indicative of the weight of p53 on apoptotic machinery. [12-13] (Fig. 4.2.e.)

Apoptotic response was found to be increasing in both cell lines with time. The wt cells showed sharper amplification in apoptotic response in 48h than in 24h as the p53-/- cells showed slight increase in both 24h and 48h and also when compared to wt cells. (Fig. 4.2.c.) Apoptotic response was found to be declining as responsive to NAC pre-treatment in both cell lines with time. The p53-/- cells proved to be more responsive to NAC pre-treatment than the wt cells in 24h yet the late effect of NAC pre-treatment on wt was observed for 48h. (Fig. 4.2.f.)

In short, the wt cells showed stronger reaction than the p53-/- cells in both 24 and 48h treatments in the absence of NAC but when the cells were treated with CDDP in combination with the antioxidant for 24h the wt and for 48h the p53-/- cells underwent more apoptosis. So the increased ROS levels do induce apoptosis significantly in both durations of treatment in the presence of p53 and absence of NAC but it is the p53 molecule which is central for the apoptotic signalling in shorter treatment when the ROS levels drop. [16, 18]

Results in next two sections regarding the ROS and lipid peroxidation levels revealed that CDDP indeed amplifies the intracellular ROS levels and this effect is directly proportional to concentration and duration of treatment. Here, the two methods utilized revealed different patterns for CDDP administration in the absence or presence of NAC pre-treatment where p53-/- cells are more prone to amplification in intracellular ROS levels than the wt cells and they also show inclined lipid peroxidation levels in the absence of NAC pre-treatment as it is the wt cells which have higher lipid peroxidation levels in the presence of NAC pre-treatment

regardless of duration of treatment. This suggests that the p53 protein causes a metabolic shift due to loss of TIGAR and SCO2 resulting in high intracellular ROS.

For the assessment of changes in the intracellular ROS levels Flow Cytometric Analyses by DCFH-DA Labelling of ROS, a technique based on counting, examining, and separating of cells suspended in a stream of fluid regarding their luminescence which results from the intracellular ROS converting the 2',7'-dichlorofluorescin diacetate (DCFH-DA) probe to the highly fluorescent compound 2',7'-dichlorofluorescein (DCF) which then via the deacetylation by esterases and subsequent oxidation by ROS in the cell turns to non-fluorescent 2',7'-dichlorofluorescin, was employed. Data showed that intracellular ROS levels tended to increase with increasing doses of CDDP and time, an effect that was reversed by the NAC pre-treatment.

For 24h treatment intracellular ROS levels were found to be following an increasing pattern in both cell lines with increasing doses of CDDP. The p53-/- cells showed more ROS by ca. 17% than the wt cells demonstrating the direct proportion of p53 with metabolism related genes such as SCO2 and TIGAR which when down-regulated contribute to increased rates of glycolysis that in turn causes increased ROS levels and/or decreased rates of pentose phosphate pathway (PPP) that is very important in the cellular GSH synthesis. [16, 18] The unexpected datum at 60μM CDDP administered to p53-/- cells of this experimental setup was omitted when calculating the percentages for comparison of the behaviour of the two cell lines. The decrease in intracellular ROS levels indicates most certainly an experimental error and the fact that there is only one set of experiments in ROS level investigation makes it impossible to formulate a better judgement. (Fig. 4.3.a.) For 24h treatment intracellular ROS levels were found to be decreasing as responsive to NAC pre-treatment in both cell lines. Here again the sudden drop-off in ROS levels in p53-/- cell line for treatment with 60μM CDDP by 1.2 fold compared to the 30μM CDDP treatment should be kept in mind yet the decrease due to NAC pre-treatment was still observed. The p53-/- cells showed more ROS in the presence of NAC as in the absence by ca. 59% signifying the regulatory duty of p53 on metabolic characteristics of the cancer cell. [16, 18] than the wt cells but the wt cells proved to be more responsive to NAC pre-treatment by ca. 41% than the p53-/- cells. (Fig. 4.3.d.)

For 48h treatment intracellular ROS levels were found to be following an increasing pattern very similar in both cell lines with increasing doses of CDDP The wt cells showed a slightly stronger enhancement in ROS levels at the 12μM CDDP concentration than the p53-/- cells

which caught up with the wt at 60 μM CDDP that at the end the p53-/- cells showed only slightly sharper increase by ca. 6% than wt cells, a value which could be neglected. (Fig. 4.3.b.) For 48h treatment intracellular ROS levels were found to be lessening as responsive to NAC pre-treatment in both cell lines. The p53-/- cells showed slightly steeper rise in ROS levels in the presence of NAC as in the absence, too by ca. 16% than the wt cells but again the wt cells proved to be more responsive to NAC pre-treatment by ca. 9% than the p53-/- cells again a value which could be neglected yet maybe due to mechanisms involving the p53 and its regulatory role as a transcription factor. (Fig. 4.3.e.)

Intracellular ROS levels were found to be increasing in both cell lines with time. Although wt cells showed sharper increase of ROS levels in 24h than the p53-/- cells, both cell lines revealed proximate ROS levels at 48h. (Fig. 4.3.c.) Intracellular ROS levels were found to be diminishing as responsive to NAC pre-treatment in both cell lines with time. The wt cells proved to be more responsive to NAC pre-treatment than the p53-/- cells in 24h yet the late effect of NAC pre-treatment on p53-/- was observed for 48h. (Fig. 4.3.f.)

Likewise, for assessment of intracellular lipid peroxidation levels Thiobarbituric Acid Reactive Substances (TBARS) Assay, a method based on screening and monitoring of lipid hydroperoxides and aldehydes, such as malondialdehyde (MDA), in the cell culture media and cell lysate as indicators of oxidative stress, was used. Here, MDA combines with thiobarbituric acid (TBA) to form a fluorescent adduct, that is measured spectroscopically and TBARS are expressed as MDA equivalents. This assay although considered as significantly error prone gives valuable information about the effect of increased ROS levels within the cell on lipids, one of the main macromolecules. Data showed that intracellular lipid peroxidation levels tended to increase with increasing doses of CDDP and time, an effect that was reversed to some extend by the NAC pre-treatment.

For 24h treatment intracellular lipid peroxidation levels were found to be following an increasing pattern very similar in both cell lines with increasing doses of CDDP although starting from the control samples the two cell lines showed a certain, consistent difference in absorbance readings throughout the experimental setup. The wt cells showed higher lipid peroxidation levels in untreated control which can be considered as a background or experimental noise that was significantly smaller for the p53-/- cell line. The rise in intracellular lipid peroxidation levels from 12 to 30μM CDDP was sharper in p53-/- than wt yet this was evened out at 60μM CDDP so that overall change in lipid peroxidation levels was

intermediately higher in p53-/- cells by ca. 17% in comparison with wt cells suggesting p53 to act as a transcription factor to some antioxidant enzymes yet not fully uncovered. [11] (Fig. 4.4.a.) For 24h treatment intracellular lipid peroxidation levels were found to be decreasing as responsive to NAC pre-treatment in both cell lines. There was however an increase in average absorbance (0.045) for 30μM CDDP which can only be considered as an experimental error bearing in mind that the whole experimental setup was reasonably consistent in data and expected outcomes throughout. The wt cells proved to have slightly higher lipid peroxidation levels in the presence of NAC by ca. 4% - a value which could be neglected yet they proved generally to be more responsive to NAC pre-treatment by ca. 20% than p53-/- cells in 24h. (Fig. 4.4.e)

For 48h treatment intracellular lipid peroxidation levels were found to be following an increasing pattern in both cell lines with increasing doses of CDDP. The amplification from 30 to 60μM CDDP was sharper in p53-/- than wt and the p53-/- cells showed higher lipid peroxidation levels by ca. 14%. (Fig 4.4.b.) For 48h treatment intracellular lipid peroxidation levels were found to be lessening as responsive to NAC pre-treatment in both cell lines. There was however an enhancement in average absorbance (0.258) for 60μM and no change for 12μM CDDP. As the latter can be ignored, the former indicating the increase designates an experimental error. The wt cells showed sharper increase in the presence of NAC by ca. 77% yet it is the p53-/- cells which proved to be more responsive to NAC pre-treatment by ca. 4% - a value which could be neglected than wt cells in 48h if relevant. (Fig. 4.4.f.)

Intracellular lipid peroxidation levels were found to be following a certain pattern in both cell lines within short intervals of time. There was almost no change in wt cells at the end of 15min after CDDP administration, other than that both cell lines seemed to experience a sudden increase in lipid peroxidation levels shortly after CDDP administration which was compensated more and more in time having a minimum point at 2h, yet this trend only continued up until a certain duration of treatment, specifically at around 4th hour when lipid peroxidation levels had a peak that again was pulled down at 8h. (Fig. 4.4.c.) This pattern is pointing towards to a certain cascade of intracellular events regarding the antioxidant defence mechanism of the cell. Intracellular lipid peroxidation levels were found to be increasing in both cell lines with time. Although wt cells showed sharper rise of lipid peroxidation levels in 24h than the p53-/- cells, the slope slowed down in 48h so that the outcomes were closer. (Fig. 4.4.d.) Intracellular lipid peroxidation levels were found to be diminishing as responsive to NAC pre-treatment in both cell lines with time. There was however an amplification in

average absorbance (0.045) for 30μM CDDP. The p53-/- cells proved to be more responsive to NAC pre-treatment than wt cells in both 24h and 48h treatments. (Fig. 4.4.g.)

From results obtained in the section regarding protein oxidation it can be deduced that p53 is an effective protector against protein carbonylation due to oxidants in the cell. [9] It does not only keep the genome but also the proteome of the cell intact whether directly or indirectly. Absence of p53 protein makes the cell more prone to non-functional proteins; this may affect the apoptotic response in a positive way if it happens in pro-survival proteins, and in a negative way if in anti-survival proteins. Data obtained in this work only reveal that pro-survival proteins are modified and hence might have lost function and thus be contributing to the apoptotic response. Yet again the apoptotic machinery is very complex and further studies are obligatory to reveal to which extend non-functional proteins enhance the escape from programmed cell death.

For assessment of the extend of carbonylation as an indicator of oxidative modification on proteins OxyBlot™, a protocol for immunoblot detection of carbonyl groups (aldehydes and ketones) introduced into proteins at lysine, arginine, proline or threonine residues in a site-specific manner by oxidative reactions with strong oxidants, was employed.

In a previous study performed in the laboratory of Maria C. Shoshan at Cancer Centrum of Karolinska Institute, Sweden carbonylation signals were shown to be intensifying with increasing doses of CDDP and time for total protein isolates of wt cells. (Fig. 4.5.a. and Fig. 4.5.b.) This was confirmative of the relation between CDDP and intracellular ROS levels increasing with increasing doses of CDDP and duration of treatment along with the damaging effects of ROS on cellular macromolecules, in this case proteins.

To determine the extend of carbonylation specifically of pro-survival proteins, Bcl-2, Bcl-x_L and Mcl-1 OxyBlot™ procedure was applied to purified samples from total protein isolates by immunoprecipitation, a technique of precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein to isolate and concentrate a particular protein from a sample containing many thousands of different proteins by coupling the protein and the antibody to a solid substrate, was used. The amount of purified proteins in each experiment was evidently very small that detectable amounts unquestionably account for carbonylation in those protein samples.

Coupling the two procedures (IP and OxyBlot™) has been quite tricky but that it actually worked was proven by the results as well as the markers and underivatized/negative controls run occasionally along with the derivatized samples. Each time negative controls (samples which have not undergone derivatization process) were run in the same or separate gels and never gave any signal signifying the coherence of the procedure.

All three blots for Bcl-2 (Fig. 4.5.c), Bcl-x_L (Fig. 4.5.d) and Mcl-1 (Fig. 4.5.e) showed detectable carbonylation signals for samples obtained from total protein isolates of p53-/- cell cultures treated with 30μM CDDP in the absence of NAC whereas there was no signal in the wt samples treated with 30μM CDDP in the absence of NAC and all other samples whether controls or CDDP along with NAC even with longer exposure times (up to 2h). This ensures the protective role of p53 in case of an oxidative insult on the cell. [11, 16, 18]

In Fig. 4.5.c. showing the OxyBlot™ of Bcl-2 IP the lanes adjacent to the well holding the IP from the p53-/- cell cultures treated with 30μM CDDP in the absence of NAC reveal bands but the reason of these false positives is a simple spillage which took place when samples were loaded onto the gel.

Effect of CDDP administration on cell morphologies of the two cell lines in the absence and presence of NAC has also been investigated for a pre-set duration of treatment (24h)with increasing doses of CDDP. As can be seen on Table 4.6. increasing doses of CDDP has overwhelming effects on cells which can be reversed by the NAC pre-treatment to a certain extend. Here, wt cells seem to be more responsive to NAC pre-treatment when it comes to morphological changes but as the data from the here performed assays indicate morphological changes certainly do not represent all that can be encountered inside the cell upon an induced disturbance, such as in this case, oxidative stress.

One point worth mentioning is that the highly transformed cells such as the p53-/- cell line employed in this work show a certain resistance to apoptosis especially when the intracellular ROS levels decrease. This phenomenon, called the threshold concept, regarding cells' proliferative vs. apoptotic behaviour is shown in figures below.

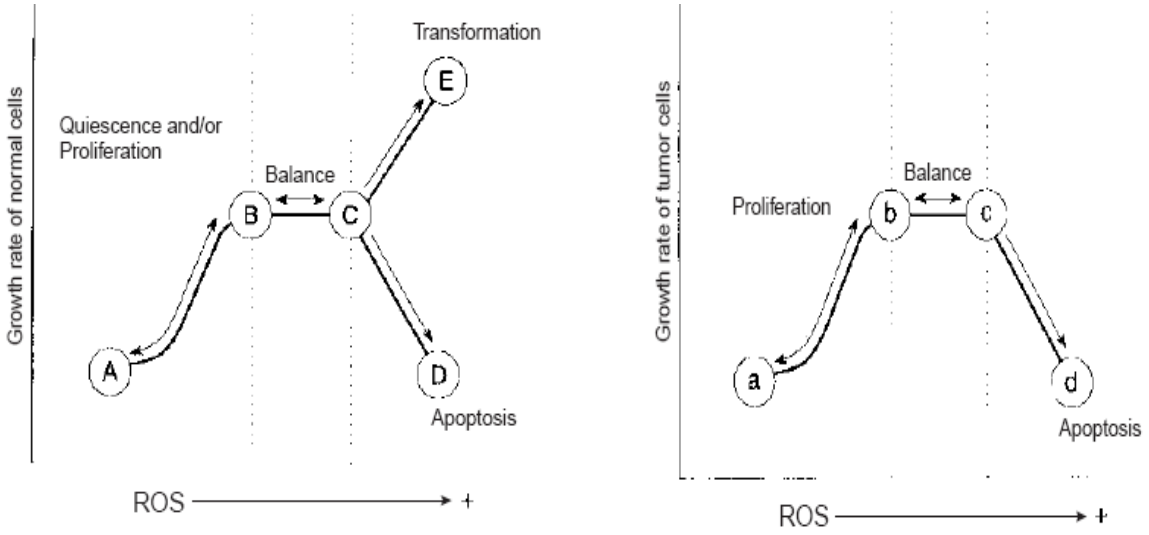


Fig. 5.a. and 5.b. On the left, the graph represents the hypothetical response of normal cells to ROS. Under normal conditions cell growth rate varies between A and B, between B and C the destructive capacity of ROS is balanced by the protective effects of the cellular antioxidants, and beyond a threshold C malignant transformation may occur (E) or cell death is observed (D). On the right the graph represents the hypothetical response of transformed cells to ROS. At lower ROS levels cell growth might be stimulated followed by an adaptive production of antioxidants between a and b which may contribute to resistance of tumour cells to subsequent treatments, between b and c destructive activity of ROS is compensated by the antioxidants and at a threshold point c where ROS levels are further increased or antioxidants reduced the antioxidant mediated self defence becomes exhausted leading to cell death. [133]

6. CONCLUSION and FUTURE DIRECTIONS

Data obtained in this study confirmed the direct proportion between the dose and/or duration of treatment with the initial hypothesis of this work – i.e. a direct proportion between intracellular ROS levels and dose and/or time of CDDP administered which can be reversed by NAC pre-treatment to some extend. Schematic representation is given below.

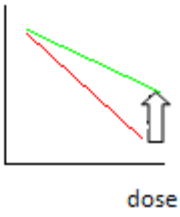
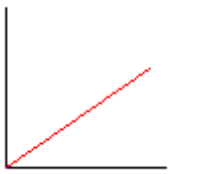
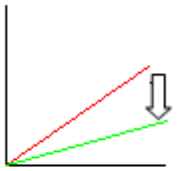
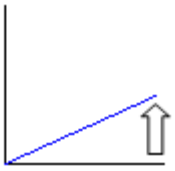
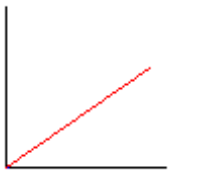
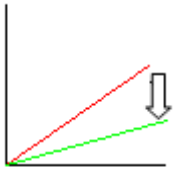
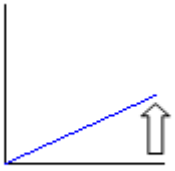
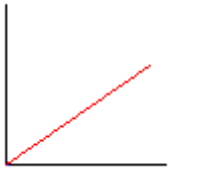
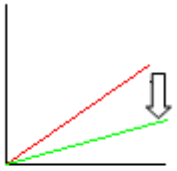
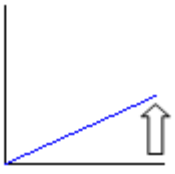
	CDDP	**CDDP + NAC**	**response to NAC**
Cell Viability			
Apoptotic Response			
ROS Production			
Lipid Peroxidation			

Table 6. Schematic representation of the data obtained from various assays performed in this study, all generalized according to the intensity of reaction to increasing doses of treatment in the absence (red) and presence (green) of antioxidant pre-treatment and the difference they caused (blue).

In light of all the results from the experiments of this study, the concept of sensitization seems to be relevant. As known, in preventive medicine ROS have long been associated with neoplastic transformation and antioxidants which scavenge FRs have been favoured as prophylactic agents protecting from ROS-induced carcinogenesis. But in clinical practice the generation of ROS is regarded as a useful process common to all non-surgical anticancer therapies. So presence of antioxidants in neoplastic tissue can decrease the efficacy of treatment regarding the fraction of cell death dependent on ROS generation for its action and hence favouring tumour cell growth and development of multidrug resistance, the latter being a serious problem in treating cancer, by DNA strand breaks, chromosomal abnormalities, oxidative base modifications and cellular transformation aiding oncogenic activation and promotion of growth factors and cytokines by ROS acting as secondary messengers. [133] So cancer cells can be sensitized to treatments such as CDDP administration which causes increased intracellular ROS levels and this being a certain fraction of its efficacy by not using antioxidants alongside the treatment if not even increase intracellular ROS levels more by use of oxidants or inhibitors of antioxidants. If normal cells suffer from severe damage due to increased systemic ROS, antioxidants can only be used to reduce side effects allowing higher doses of chemotherapeutic in the prospect to obtain more antiproliferative effect due to other modes of action of the drug than increased ROS levels. The whole thing can be visualized as a balance and the determination of an optimum treatment with respect to the system's capability to deal with increased ROS levels in both healthy and cancerous tissues is of critical importance. One other important point is with techniques which have difficulty in effectively delivering therapy to the tumour may induce not only tumour growth but also the potential resistance of the tumour to subsequent treatments. Local or targeted drug delivery techniques would here be of great benefit. Overproduction of ROS may not always initiate the apoptotic response to an expected extend as with the p53-/- cells and unlike low ROS levels that induce expression of certain antioxidant enzymes such as SOD in the cell may in turn exhaust the protective effectiveness of antioxidants resulting in cell death. This is only favourable if the cell that dies is a tumour cell and with a normal cell this means severe side effects of the drug. CDDP is known to have serious side effects such as nephrotoxicity and hearing loss.

This study showed that CDDP treatment increases intracellular ROS levels which contribute to apoptotic cell death in response to treatment. The mechanisms behind contain the transcription factor/effector p53 and carbonylation of pro-survival Bcl-2 family proteins. In case for p53 cells which carry constitutively active TP53 can be used throughout the same

experimental setup. Data obtained when compared to the results presented in this work would further enlighten the involvement of p53 to the modes of action of this drug. Also, use of a positive control and/or inhibitor of antioxidants would enable better interpretation of the outcomes of this study. For carbonylation of proteins, the first thing which needs to be done is to prove that carbonylation on these proteins indeed cause loss of function. For this, the primary step is to predict sites for carbonylation on individual proteins with respect to their biochemistry, i.e. amino acid sequence and structure in space. Experimentally this can be achieved by on-blot tryptic digestion followed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS). Data obtained from MALDI MS can be used for protein identification via database search and differences can be interpreted as modifications. In this work, immunoprecipitation was used to isolate a certain protein from a total protein mixture the technique can be applied to the latter to achieve less diverse sample. Although carbonylation is an irreversible modification getting rid of the sepharose beads after immunoprecipitation is difficult and interferes with the later OxyBlot™ procedure. At that point, a more efficient and less damaging method needs to be established. Candidates for this are salting out to interfere with the solubility and heat treatment to break the covalent bonds holding the sepharose bead antibody and protein together.

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APPENDIX A: Buffers and Solutions

A.1. Modified McCoy's 5A Medium

solvent	McCoy's 5A Medium
ingredient	*final concentration*
L-glutamine	2mM
Penicillin/Streptomycin (P/S)Solution (100U/l)	1U/l≡1%(v/v)
Fetal Bovine Serum (FBS)	10%(v/v)
storage	at 4˚C until use

A.2. 10X Phosphate Buffered Saline (PBS)

solvent	ddH_20
ingredient	*final concentration*
NaCl	1.3688M
KCl	30.18mM
$Na_2HPO_4.12H_2O$	64.97mM
KH_2PO_4	15.06mM
final pH adjusted to	7.4
storage	at room temperature

A.3. 1X PBS

Dilution with ddH_20 in the ratio 9:1, ddH_20:10X PBS

A.4. Freezing Mixture (FM) for Cell Stocks

solvent	FBS
ingredient	*final concentration*
DMSO	10%(v/v)
storage	at -20˚C until use

A.5. 1000X N- Acetyl-L-Cystein (NAC) Solution

solvent	ddH_2O
ingredient	*final concentration*
NAC	0.5M
storage	at 4˚C until use

A.6. MTT Solution

solvent	PBS
ingredient	*final concentration*
MTT Labelling Reagent	5mg/ml
storage	at 4˚C for 4 weeks in dark

A.7. Annexin-V Incubation Buffer

solvent	ddH_2O
ingredient	*final concentration*
Hepes	10mM
NaCl	140mM
$CaCl_2$	2.5mM
storage	at 4˚C until use

A.8. Dichlorofluorescein diacetate (DCFH-DA) Stock Solution

solvent	DMSO
ingredient	*final concentration*
DCFH-DA	20.6mM
storage	at 4˚C until use

A.9. DCFH-DA Incubation Solution

solvent	PBS
ingredient	*final concentration*
DCFH-DA	5μM
storage	in dark; use immediately

A.10. Thiobarbituric Acid (TBA) Solution

solvent	DMSO
ingredient	*final concentration*
DCFH-DA	67%(w/v)
storage	at room temperature

Subsequent dilution with ddH_2O in the ratio 9:1, ddH_20:67%(w/v) TBA yielding the 6.7%(w/v) TBA Solution

A.11. Trichloroacetic Acid (TCA) Solution

solvent	ddH_2O
ingredient	*final concentration*
TCA	10%(w/v)
storage	at room temperature

A.12. Thiobarbituric Acid Reactive Substances (TBARS) Assay Standards

solvent	EtOH
ingredient	*final concentration*
1,1,3,3-Tetramethoxypropane	0.4167%(v/v)
storage	at room temperature

Subsequent dilution with ddH_2O in the ratio 49:1, ddH_2O:0.4167%(v/v) 1,1,3,3-Tetramethoxypropane yielding the 500uM stock.

tube number	*final concentration in µM*	*ddH_2O in µl*	*1,1,3,3-Tetramethoxypropane in µl*
1	0	500	0
2	0.625	500	500 from tube 3
3	1.25	500	500 from tube 4
4	2.5	500	500 from tube 5
5	5	500	500 from tube 6
6	10	800	200 from tube 7
7	50	500	500 from tube 8
8	100	800	200 om 500µM stock

A.13. 10% NP-40

Dilution with ddH20 in the ratio 9:1, ddH20:100% NP-40

A.14. Cell Lysis Buffer (CLB)

solvent	ddH_2O
ingredient	*final concentration*
NaCl	150mM
NP-40	1%
Tris-HCl	50mM
final pH adjusted to	8
storage	at -20˚C until use
additions prior to use	*final concentration*
PMSF	0.1mM
protease cocktail inhibitor	10%(v/v)
either 2-Mercaptoethanol	1.5%(v/v)
or Dithiothreitol	50mM

A.15. Bradford Reagent for Protein Content Assay

Dilution with ddH_20 in the ratio 4:1, ddH_20:Bradford Stock Solution

A.16. Bovine Serum Albumin (BSA) Stock Solution

solvent	ddH_2O
ingredient	*final concentration*
BSA	1mg/ml
storage	at -20˚C until use

Subsequent dilutions with ddH_20 in the ratios 1:1, 3:1, 7:1, etc. ddH20:BSA Stock Solution

A.17. 1X DNPH Solution

Dilution with ddH_20 in the ratio 9:1, ddH_20:10X DNPH Solution

A.18. 1X Derivatization Control (DC) Solution

Dilution with ddH_20 in the ratio 9:1, ddH_20:10X DC Solution

A.19. 1X Gel Loading Buffer for OxyBlot™

solvent	ddH_2O
ingredient	*final concentration*
Tris-HCl at pH 6.8	62.5mM
Glycerol	10%(v/v)
2-Mercaptoethanol	0.17875M
SDS	2%(w/v)
Bromophenol Blue	0.002%(v/v)
storage	at -20˚C until use

A.19. 4X Tris-Cl Buffer for Separating Gel Solution

solvent	ddH_2O
ingredient	*final concentration*
Tris	1.5M
SDS	0.4%(w/v)
final pH adjusted to	8.8

storage	at 4˚C

A.20. 15% Separating Gel Solution

solvent	ddH_2O
ingredient	*final concenntration*
4X Tris-Cl Buffer at pH 8.8	25%(v/v)
30% Acrylamide / 0.8% Bisacrylamide Solution	50%(v/v)
storage	at 4˚C until use
additions prior to use	*volume*
TEMED	0.067%(v/v)
10%(w/v) APS	0.33%(v/v)

A.21. 4X Tris-Cl Buffer for Stacking Gel Solution

solvent	ddH_2O
ingredient	*final concentration*
Tris	0.5M
SDS	0.4%(w/v)
final pH adjusted to	6.8
storage	at 4˚C until use

A.22. Stacking Gel Solution

solvent	ddH_2O
ingredient	*volume*
4X Tris-Cl Buffer at pH 6.8	25%
30%Acrylamide/8%Bisacrylamide Solution	13.05%(v/v)
storage	at 4˚C until use
additions prior to use	*volume*
TEMED	0.1%(v/v)
10%(w/v)APS	0.5%(v/v)

A.23. 10X Running Buffer (RB)

solvent	ddH_20
ingredient	*final concentration*
Tris base	0.25M
Glycine	1.92M
SDS	1%(w/v)
final pH adjusted to	8.5
storage	at room temperature until use

A.24. 1X RB

Dilution with ddH_20 in the ratio 9:1, ddH_20:10X RB

A.25. 10X Transfer Buffer (TB)

solvent	ddH_20
ingredient	*final concentration*
Tris base	0.125M
Glycine	0.96M
final pH adjusted to	8.3
storage	at room temperature until use

A.26. 1X TB

Dilution with ddH_20 in the ratio 9:1, ddH_20:10X TB

additions prior to use	*final concentration*
MetOH	20%(v/v)

A.27. 1X PBS-T

Dilution with ddH_20 in the ratio 9:1, ddH_20:10X PBS

additions prior to use	*final concentration*
Tween®20	0.05%

A.28. Blocking/Dilution Buffer for OxyBlot™

solvent	1X PBS-T
ingredient	*final concentration*
BSA	1%(w/v)
storage	at 4˚C until use

A.29. 1˚ Antibody (Ab) Solution for OxyBlot™

Dilution with Blocking/Dilution Buffer for OxyBlot™ in the ratio 149:1, Blocking/Dilution Buffer for OxyBlot™:1˚ Ab

A.30. 2˚ Ab Solution for OxyBlot™

Dilution with Blocking/Dilution Buffer for OxyBlot™ in the ratio 299:1, Blocking/Dilution Buffer for OxyBlot™:2˚ Ab

A.31. Chemiluminescent Reagent

Mix Reagent A and Reagent B in equal amounts to end up with an adequate volume covering the blot(s)

A.32. Developer Solution

Dilution with ddH_2O in the ratio 4:1, ddH_2O:Developer Stock Solution

A.33. Fixer Solution

Dilution with ddH_2O in the ratio 4:1, ddH_2O:Fixer Stock Solution

APPENDIX B: Chemicals and Antibodies

Name	*Supplier*	*Catalog Number*
1,1,1-Tris(hydroxymethyl)-methanamide	Fluka, Switzerland	93349
1,1,3,3-Tetramethoxypropane		
1X Trypsin-EDTA	Biological Industries, Israel	03-050-1
2-Mercaptoethanol	Sigma, Germany	M370-1
Acrylamide/Bisacrylamide	Sigma, Germany	A3699
Ammonium persulfate	Sigma, Germany	A3678
Annexin-V-Fluorescein	Alexis Biochemicals, USA	ALX-209-250-T100
Anti-Bcl-2 Antibody	Cell Signal Tech, USA	2872
Anti-Bcl-x_L Antibody	Cell Signal Tech, USA	2762
Anti-Mcl-1 Antibody	Cell Signal Tech, USA	4572
Bovine Serum Albumin		
Bradford Stock Solution		
Bromophenol Blue		
cis-diamminedichloridoplatinum(II)		
Dichlorofluorescein diacetate		
Dimethyl sulfoxide	Sigma, Germany	D2650
Dithiothreitol		
Ethanol	Riedel-de-Haén, Germany	32221
Fetal Bovine Serum	Sigma, Germany	F2442
Glycerol	Riedel-de-Haén, Germany	15523
Glycine	Amnesa, USA	0167
Hydrochloric Acid	Merck, Germany	100314
Isopropanol	Riedel-de-Haén, Germany	24137
Liquid Nitrogen	Karbogaz, Turkey	-
McCoy's 5A Medium	Biological Industries, Israel	01-075-1
Methanol	Riedel-de-Haén, Germany	24229
N- Acetyl-L-Cystein	Sigma	
Nonyl phenoxylpolyethoxylethanol	Sigma, Germany	13021
Penicillin/Streptomycin Solution	Biological Industries, Israel	-

Phenylmethanesulphonylfluoride	Sigma, Germany	P7626
Phosphate Buffered Saline	Sigma, Germany	P4417
Potassium Chloride	Fluka, Switzerland	60129
Protease Inhibitor Cocktail tablets	Roche, Germany	11697498001
Protein G Sepharose		
Sodium Chloride	Riedel-de-Haén, Germany	13423
Sodium Dodecyl Sulphate	Sigma, Germany	L4390
Sodium Hydroxide	Merck, Germany	106462
Tetramethylethylenediamine	Sigma, Germany	T7029
Thiobarbituric Acid		
Trichloroacetic Acid		
Tween®20	Merck, Germany	822184
Developer		
Fixer		
Calcium Chloride		
Potassium Diphosphate		
Disodium Phosphate Dodecahydrate		
Disodium Phosphate		

APPENDIX C: Molecular Biology Kits

Name	*Supplier*	*Catalog Number*
MTT Cell Proliferation Kit	Roche, Germany	1465007-001
Annexin-V-Fluos Stain Kit	Roche, Germany	11988548001
M30-Apoptosense® ELISA	Peviva, Sweden	PE0024
CytoTox96® Non-Radioactive Cytotoxicity Assay	Promega, USA	G1780
OxyBlot™ Protein Oxidation Detection Kit	Chemicon Int., USA	S7150

APPENDIX D: Laboratory Equipment

Autoclave: Hirayama, Hiclave HV-110, JAPAN

Certoclav, Table Top Autoclave CV-EL-12L, AUSTRIA

Balance: Sartorius, BP211D, GERMANY

Sartorius, BP221S, GERMANY

Sartorius, BP610, GERMANY

Schimadzu, Libror EB-3200 HU, JAPAN

Centrifuge: Eppendorf, 5415C, GERMANY

Eppendorf, 5415D, GERMANY

Eppendorf, 5415R, GERMANY

Kendro Lab. Prod., Heraeus Multifuge 3L, GERMANY

Hitachi, Sorvall RC5C Plus, USA

Hitachi, Sorvall Discovery 100 SE, USA

Deepfreeze: -70^{o} C, Kendro Lab. Prod., Heraeus Hfu486 Basic, GERMANY 132

-20^{o} C, Bosch, TURKIYE

Distilled Water: Millipore, Elix-S, FRANCE

Millipore, MilliQ Academic, FRANCE

Electrophoresis: Biogen Inc., USA

Ice Machine: Scotsman Inc., AF20, USA

Incubator: Memmert, Modell 300, GERMANY

Memmert, Modell 600, GERMANY

Laminar Flow: Kendro Lab. Prod., Heraeus, HeraSafe HS12, GERMANY

Magnetic Stirrer: VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY

VELP Scientifica, Microstirrer, ITALY

Microliter Pipette: Gilson, Pipetman, FRANCE

Mettler Toledo, Volumate, USA

pH meter: WTW, pH540 GLP MultiCal®, GERMANY

Power Supply: Biorad, PowerPac 300, USA

Refrigerator: 4˚C, Bosch, TÜRKiYE

Shaker: Forma Scientific, Orbital Shaker 4520, USA

GFL, Shaker 3011, USA

New Brunswick Sci., Innova™ 4330, USA

Spectrophotometer: Schimadzu, UV-1208, JAPAN

Water bath: Huber, Polystat cc1, GERMANY

BD Biosciences FACS Canto Flow Cytometer

APPENDIX E: Materials otherwise needed

PVDF membrane		
Whatman paper		
Culture plates		

APPENDIX F: Methods

F.1. There have been four parameters in this study, enabling a broader view of the outcome of CDDP treatment according to presence or absence of TP53 gene thus presence (wt) or absence (p53-/-) of p53 protein, concentration of CDDP, absence or presence of NAC pre-treatment and duration of CDDP treatment. The scheme below shows these parameters in detail.

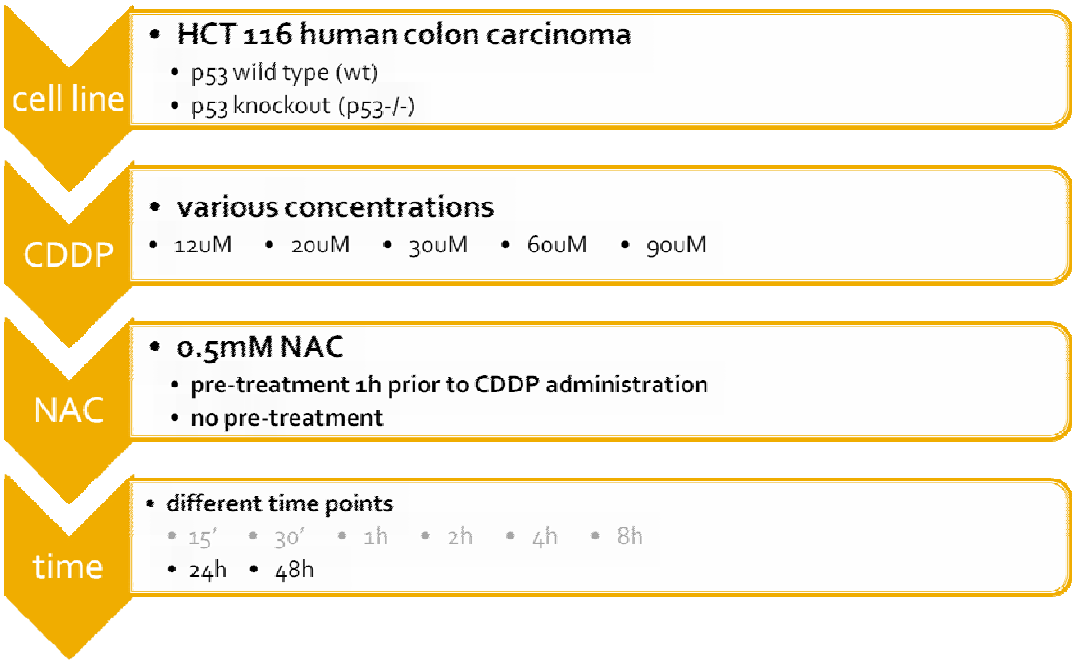

Fig F.1. Parameters of this work. Below are given the various values they were addressed to throughout the study.

F.2. Cell Culture and Treatments

After growth at >90% confluency of cells the medium was removed and the monolayer of cells attached on the culture dish/flask was washed with 1X sterile PBS. Following removal of the PBS, 1X sterile Trypsin solution was applied and the dish/flask was kept in the incubator for 5 to 10min until the cells were detached. The trypsinization process was ended by the addition of complete medium atop. The resulting cell suspension was examined for its cell density by the use of a hematocytometer under light microscope.

There are two counting chambers in a hematocytometer. Each contains nine fields and five of these nine are to be examined for the number of cells on them. The counting is performed by taking the cells on the top and left grids which touch the middle line and all in the central

area. So 2 x 5 = 10 fields give the number of cells in 1µl of cell suspension. For the cell density per ml of cell suspension the former needs to be multiplied by 10^3.

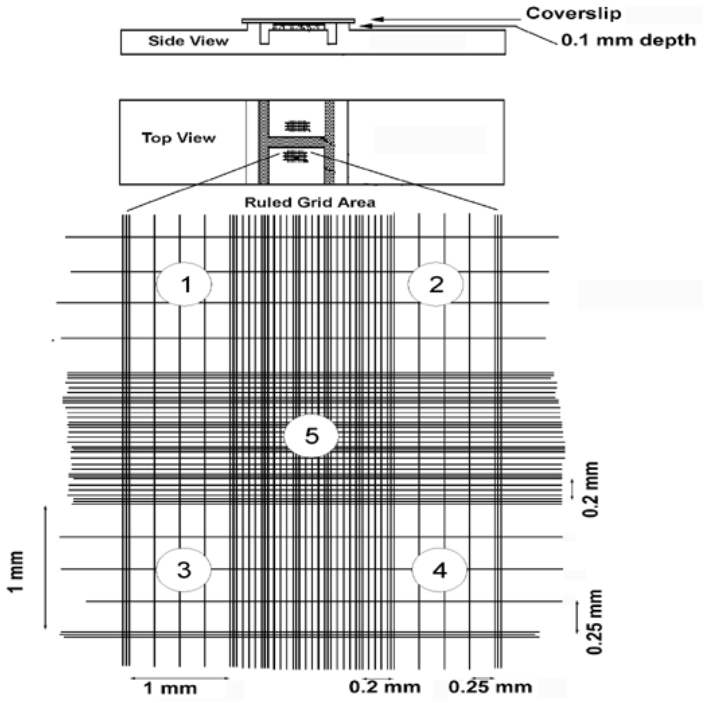

Fig. F.2. Schematic representation of a counting chamber on a hematocytometer. The area is divided into nine equal squares, five of which as depicted above are employed for counting.

	100mm dish	***60mm dish***	***35mm dish***	***T_{25} flask***	***T_{75} flask***	***96 well plate***	***12 well plate***	***6 well plate***
Number of cells seeded	$2.2x10^6$ to $4.4x10^6$	$0.9x10^6$ to $1.8x10^6$	$3.5x10^5$ to $7x10^5$	$1x10^6$ to $2x10^6$	$3x10^6$ to $6x10^6$	$1.2x10^4$ to $2.4x10^4$ / well	$1.5x10^5$ to $3x10^5$ / well	$4x10^5$ to $8x10^5$ / well
Final volume of medium	20ml	10ml	4ml	10ml	15ml	200µl / well	2ml / well	4ml / well
Volume of 1X PBS used	10 ml	5ml	3ml	5ml	8ml	200µl / well	1ml / well	2ml / well
Volume of 1X Trypsin-EDTA used	8ml	3ml	2ml	3ml	5ml	100µl / well	500µl / well	750µl / well

Table F.2: Basic numbers in cell culturing for various culture equipment of different shape and surface area. The number of cells to be seeded depends on the nature and size of the cells and requirements of the experiment. Volume of medium, 1X PBS and 1X Trypsin-EDTA may vary according to current cell density, requirements of the experiment and practice.

Stock cell suspensions were prepared whenever sufficient number of cells was excess. Optimally, 10^6 cells in a cell suspension of corresponding volume were centrifuged for 5min at 300g and at the end the supernatant was removed. The pellet was resuspended in 1ml FM and transferred into cryovials which are kept at -80˚C in a beaker containing isopropanol for a steady temperature decrease of 1˚C / min and then stored in liquid nitrogen until use. One such cryovial can be thawed and the content be suspended in medium at 37˚C where after enough time for the cells to attach the medium has to be replaced with fresh medium to get rid of the toxic DMSO from the freezing mixture.

After growth at >60% confluency of cells, the medium was removed and CDDP was administered at pre-set final concentrations of 0 (control), 6, 12, 20, 30, 60 and 90µM in fresh

medium. Administration of NAC was performed an hour before the CDDP treatment at a final concentration of 0.5mM. The medium containing the drug and/or supplement was not removed until the end of the pre-set duration of the treatment.

F.3. Assessment of Cell Viability via MTT Assay

Both cell lines were seeded into flat bottom 96-well plates at a cell density per well given in the table above and the plates were kept in the incubator until attachment of cells. Upon removal of the initial medium, treatment was performed in a volume of 100µl of fresh medium per well for the pre-set duration. Afterwards, 10µl of MTT Labelling Reagent were added atop in each well to achieve a final concentration of 0.5mg/ml. The plate is then incubated at 37˚C and 5% CO_2 for 4h in the incubator. Latterly, 100µl of 1X Solubilization Solution per well were added and the plates were again incubated, this time overnight. After this second incubation period, the wells were measured as samples for their absorbance at the wavelength of 550nm where a reference wavelength of 655nm is set prior to spectrophotometrical analyses. Data were interpreted as percent of relative cell viability by calculating the ratio of absorbance from treated samples times 100 to absorbance of control samples.

F.4. Assessment of Apoptotic Response via Flow Cytometric Analyses by Annexin-V Labelling

Both cell lines were seeded into 12-well plates at a cell density per well given in the table above and the plates were kept in the incubator until attachment of cells. Upon removal of the initial medium, treatment was performed in fresh medium for the pre-set duration. Afterwards, the medium from each well was discarded and after washing with adequate volume of 1X PBS 0.5ml of 1X Trypsin solution was added to detach the cells. The contents were collected into FACS tubes and some volume of complete medium was introduced to stop the trypsinization process. The tubes were then centrifuged for 5min at 300g and the supernatants were discarded. The pellets were resuspended in 0.5ml of PBS and the tubes were again centrifuged for 5min at 300g. The supernatants were again discarded and the pellets were resuspended in 100 µl of Annexin-V Incubation buffer to which 2 µl of Annexin-V-Fluorescein which labels the exposed phosphotidylserines on cell surfaces as a marker of early apoptosis were added. The tubes were then kept in dark for 15min at room temperature. After a third turn of centrifugation for 5min at 300g the supernatants were discarded and the pellets were resuspended in 0.5ml of PBS. The suspensions were then analyzed by BD FACS

Diva. Data were interpreted as percentages of a certain population of a pre-set size which then were converted into folds with respect to the control values.

F.5. Assessment of ROS Production via Flow Cytometric Analyses by DCFH-DA Labelling

Both cell lines were seeded into 12-well plates at a cell density per well given in the table above and the plates were kept in the incubator until attachment of cells. Upon removal of the initial medium, treatment was performed in fresh medium for the pre-set duration. Afterwards, the medium from each well was discarded and after washing with adequate volume of 1X PBS 0.5ml of 1X Trypsin solution was added to detach the cells. The contents were collected into FACS tubes and some volume of complete medium was introduced to stop the trypsinization process. The tubes were then centrifuged for 5min at 300g and the supernatants were discarded. The pellets were resuspended in 0.5ml of PBS and the tubes were again centrifuged for 5min at 300g. The supernatants were again discarded and the pellets were resuspended in 100 µl of DCFH-DA Incubation Solution. The tubes were then kept in dark for 25min at room temperature. After a third turn of centrifugation for 5min at 300g the supernatants were discarded and the pellets were resuspended in 0.5ml of PBS. The suspensions were then analyzed by BD FACS Diva. Data were interpreted as percentages of a certain population of a pre-set size which then were converted into folds with respect to the control values.

F.6. Assessment of Lipid Peroxidation via TBARS Assay

Both cell lines were seeded into 12-well plates at a cell density per well given in the table above and the plates were kept in the incubator until attachment of cells. Upon removal of the initial medium, treatment was performed in fresh medium for the pre-set duration. Afterwards, the medium from each well was discarded and 0.5ml of PBS was added to aid in cell harvesting by means of a scraper. The contents of each well were transferred into Eppendorf tubes. The wells were washed again with 0.5ml of PBS and the secondary contents were also collected in corresponding tubes. The tubes were then centrifuged for 5min at 300g. The supernatants were discarded and the pellets resuspended in 0.5ml of PBS. After a second turn of centrifugation for 5min at 300g the supernatants were discarded and 110 µl of 10% SDS were added atop the pellets and the tubes were frozen at -20˚C. After thawing, a third round of centrifugation took place at 4˚C for 10 min at 3000g. 10 µl of each supernatant was taken onto an ELISA plate for protein content assay. To the rest 200 µl of ice-cold TCA

Solution were added and the tubes were kept for 15min on ice. A fourth centrifugation was then performed at 4˚C for 15min at 2200g. In the meantime, standards were prepared as instructed. Latterly, into a second set of Eppendorf tubes 200µl of each standard and sample are transferred. Into each tube 200µl of TBA Solution were added and the tubes were heated on a heating block for 10min at 100˚C. After cooling the content of the tubes were measured as standards and samples for their absorbance at the wavelength of 550nm. Data were interpreted with respect to the data from the standards.

F.7. Assessment of Protein Carbonylation via OxyBlot™

F.7.1. on Total Protein

Both cell lines were seeded into dishes at a cell density per well given in the table above and the dishes were kept in the incubator until attachment of cells. Upon removal of the initial medium, treatment was performed in fresh medium for the pre-set duration. Afterwards, the medium from each dish was discarded and adequate volume of ice-cold 1X PBS was added to aid in cell harvesting by means of a scraper. The contents of each dish were transferred into tubes of adequate volume. The dishes were washed again with adequate volume of ice-cold PBS again and the secondary contents were also collected in corresponding tubes. The tubes were then centrifuged for 30Sec at 13200rpm. The supernatants were discarded and the pellets resuspended in adequate volumes of CLB. The tubes were then kept on ice for 30min. Subsequently, a second round of centrifugation was performed for 10min at 13200rpm. The supernatants were kept at -20˚C for shorter and at -80˚C for longer periods of time.

Protein content assay procedure was performed according to manufacturer's instructions. Into the wells of an ELISA plate, triplets or quadruplets of each sample of same or different dilutions with ddH2O were transferred in aliquots of 5µl. Triplets or quadruplets of BSA standards of concentration 0, 10, 5, 2.5, 1.25, 0.625 etc. are also transferred into other wells of the plate and atop of all wells 95µl of diluted Bradford Solution were added. The wells were measured as samples and standards for their absorbance at the wavelength of 595nm by the presence of a blank. Data were interpreted by using a BSA Standard Curve where its equation needs to have a R^2 value larger than 0.9 and smaller than 1.0.

OxyBlot™ procedure was performed according to manufacturer's instructions. After performing a series of dilutions to bring each protein sample to the same concentration of the smallest value in the current pool of samples, corresponding volumes of each sample for 15 to

20 µq of total protein were taken into two sets of Eppendorf tubes and the volumes are completed to 5 µl with ddH2O. At this point, if fishing out of a certain protein from a total protein solution was formerly performed, regardless of the concentration of the protein in the immunoprecipitation product 5 µl aliquots of each sample were taken. To both sets of tubes 5 µl of 12%SDS are added. Then, to one of the sets 10 µl of 1X DNPH Solution and to the other set 1X DC Solution were added. After incubation of the tubes for 15min at room temperature, in both sets 7.5 µl of Neutralization Solution were added. Addition of certain amount of 1X Gel Loading Buffer for OxyBlot™ does not affect the electrophoresis in any way.

SDS-PAGE Gels were prepared according to the instructions of the equipment's manufacturer. Fresh separating gel solution was poured in between the two glass panes which prove a vacancy of 1mm thickness and overlaid by a layer of isopropanol to stop contact with air. Once the separating gel was cast and set, the isopropanol layer was removed and the contact areas washed with ddH2O. The stacking gel solution was then poured atop. This time instead of the isopropanol layer a comb of desired well number was placed sealing the air contact areas. Once the stacking gel was set, the comb was removed. Treated samples, negative controls and if empty wells available mixture of standard proteins attached DNP residues with or without addition of gel loading buffer were transferred into the wells in all cases without heating prior to loading. The gels were run in 1X RB for about 2h at room temperature with a constant voltage of 70V.

Once the gels were run, the proteins were transferred onto MetOH activated PVDF membranes, by means of electricity. The assemblies consisting of a sponge, Whatman paper, gel, PVDF membrane, a second layer of Whatman paper and a second sponge were immersed in 1X TB prior to alignment with respect to the direction of electricity. A constant current of 110mA was applied for 2h at room temperature by the presence of an ice-block to overcome the increasing temperature in time. After the transfer the membranes were blocked in 1X Blocking/Dilution Buffer for 2h at room temperature. The blots were then incubated with the 1˚Ab Solution specific for derivatized sites on the proteins overnight in cold room with gentle shaking. Subsequently, the blots were rinsed twice, washed once for 15min and then twice for 5min with 1X PBS-T with gentle shaking at room temperature. They were then incubated with 2˚Ab Solution specific for 1˚Ab overnight in cold room with gentle shaking. Afterwards, they again were rinsed twice, washed once for 15min and then twice for 5min with 1X PBS-T with gentle shaking at room temperature and kept in cold 1X PBS-T until detection.

The blots were drained from excess PBS-T and placed on to a layer of plastic film in a cassette with their protein-carrying sides up. Atop the freshly prepared mixture of chemiluminescent reagents was applied and a second layer of plastic film was overlaid so that the more sensitive X-Ray film does not come into contact with the liquids. The light emitted changes the film in a way that after immersing the film into the Developer Solution, subsequent rinsing in water, immersing into the Fixer Solution and a final rinsing in water the areas exposed to light yield darker bands than the background. In this sense, the detection of carbonylated proteins in the samples was performed.

F.7.2. on Immunoprecipitates

Firstly, from the least concentrated in the current pool of protein isolates the highest possible amount of protein for a final volume of 600µl was calculated. This enabled highest protein yields after fishing out a certain protein. Usually, the amount of protein was around 800µg. The more concentrated samples, on the other hand, were diluted with CLB to give the final volume of 600µl in each Eppendorf tube. Into all tubes 1µl (≡1µg) of capture Ab was added and they were mixed gently at room temperature. In the meantime, 30µl per tube of 50% slurry Protein G Sepharose suspension were taken in an Eppendorf tube and centrifuged for 30sec at 13200rpm at room temperature. The supernatant was discarded and the pellet resuspended in starting volume of cold CLB. This process was repeated five times and at last the pellet was resuspended in adequate volume of CLB. From this suspension 30µl were transferred into each tube and kept overnight in cold room with gentle shaking. Afterwards, the tubes were centrifuged for 30sec at 13200rpm at 4˚C and the supernatants were discarded. The pellets were resuspended either with 5µl of CLB or 1X Gel Loading Buffer for OxyBlot™ and pipetted up and down for about 5min to separate protein complexes from the beads mechanically as heating of samples prior to loading is not allowed in the OxyBlot™ procedure. The tubes were then centrifuged for 1min at 13200rpm at room temperature and supernatants were treated as protein samples in the OxyBlot™ procedure.