DIPLOMARBEIT

Titel der Diplomarbeit

Effects of Pyrrolidine Dithiocarbamate
on Apoptosis

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)

Verfasserin / Verfasser:	Markus Mandl
Matrikel-Nummer:	0201136
Studienrichtung (lt. Studienblatt):	Molekulare Biologie (A490)
Betreuerin / Betreuer:	Joachim Seipelt

Wien, am 14. Oktober 2007

Acknowledgements

First of all, I would like to thank my supervisor Joachim Seipelt for the opportunity to work on the interesting field of apoptosis.

I am very grateful to Barbara Holzer and Elisabeth Gaudernak for helpful ideas and discussions.

Special thanks to Andrea Triendl and Christina Heindl for taking care of my cells when I was absent.

Finally, I would like to thank my parents and my friends for supporting me during my study.

Summary

Pyrrolidine Dithiocarbamate (PDTC) is a metal chelating compound with antioxidant properties. PDTC is a copper and zinc ionophore and exerts many different effects in biological systems. It is able to inhibit the activation of NFκB, the replication of picornaviruses and proteasome-dependent proteolysis. PDTC exhibits pro- as well as anti-apoptotic effects depending on the model system used.

Apoptosis is a kind of programmed cell-death and is a very important mechanism in multicellular organisms to remove damaged or unwanted cells. The initiation of this cellular suicide program involves a lot of different signalling events which converge at the level of caspases. Pre-existing data of our laboratory suggest that PDTC act as an inhibitor of apoptosis.

The aim of this project was to find out which steps of the apoptotic pathway are affected by PDTC. It was assumed that the property of PDTC to act as zinc ionophore and subsequently increasing intracellular zinc levels, play an important role in inhibiting of apoptosis. This was supposed because zinc is known to inhibit caspases, which are proteases and activated during apoptosis. Further zinc is known to activate the PI3K/Akt pathway which provides survival-signals for the cell.

To answer the question how the anti-apoptotic effect of PDTC is achieved, HeLa cells, caspase-3 deficient MCF-7 as well as caspase-3 overexpressing MCF-7-cas3+ cells were used. Apoptosis was induced with Actinomycin D or Puromycin. These two substances were chosen due to the well known mode of action. Subsequently different hallmarks of apoptosis were analysed.

Herein it is shown that PDTC is able to inhibit cell death in HeLa, MCF-7 and MCF-7-cas3+ cells. This effect was abolished in the presence of the metal chelator EDTA in HeLa cells. This means that the anti-apoptotic property of PDTC depends on the presence of certain metal ions. The release of cytochrome c from mitochondria, which is characteristic for apoptosis, was delayed in the presence of PDTC.

The effects of PDTC on caspases were studied by using a caspase 3/7 assay, a cell-free system and the MCF-7 cell line. Herein PDTC seems not to act mainly on the level of caspases. Interestingly, the PI3K/Akt pathway is induced due to PDTC-treatment as demonstrated by western blot analysis. But inhibition of this pathway did not abolish the anti-apoptotic property of PDTC. In general the results suggest that PDTC acts mainly on pre- and/or mitochondrial stages of apoptosis.

Zusammenfassung

Pyrrolidine Dithiocarbamat (PDTC) ist eine Substanz die Komplexe mit Metallen bilden kann und anti-oxidative Eigenschaften besitzt. PDTC ist ein Kupfer- und Zink-Ionophor und bewirkt eine Vielzahl von Effekten in biologischen Systemen. Es inhibiert die Aktivierung von NFκB, die Replikation von Picornaviren und die Proteasome-abhängige Proteolyse. PDTC zeigt pro- als auch anti-apoptotische Wirkung, abhängig vom verwendeten Modellsystem.

Apoptose ist eine Form des programmierten Zelltods und ein wichtiger Mechanismus in multi-zellulären Organismen um beschädigte oder nicht mehr benötigte Zellen zu entfernen. Die Initiation dieses zellulären Selbstmordprogramms involviert eine Vielzahl von Signalwegen, die bei den Caspasen zusammenlaufen. Caspasen sind Proteasen, die im Laufe der Apoptose aktiviert werden. Bereits vorhandene Daten aus unserem Labor legten eine inhibitorische Wirkung von PDTC auf Apoptose nahe.

Das Ziel dieses Projekts war es herauszufinden, welche Stufen der Apoptose durch PDTC beeinflusst werden. Es wurde angenommen, dass die Eigenschaft von PDTC als Zink-Ionophor zu wirken und somit die intrazelluläre Konzentration von Zink zu erhöhen, eine wichtige Rolle bei der Inhibierung der Apoptose spielt. Der Grund dafür war, dass die Inhibierung von Caspasen durch Zink bereits beschrieben wurde. Weiters ist Zink in der Lage den PI3K/Akt Pathway zu aktivieren, der Überlebenssignale für die Zelle liefert.

Um die Frage zu beantworten, wie der anti-apoptoische Effekt von PDTC zu stande kommt, wurden HeLa Zellen sowie Caspase-3 defiziente MCF-7 und Caspase-3 überexpremierende MCF-7-cas3+ Zellen verwendet. Apoptose wurde in diesem Zellsystem induziert mit Actinomycin D oder Puromycin. Diese beiden Substanzen wurden aufgrund ihres bekannten Wirkungsmechanismus verwendet. Verschiedene Kennzeichen der Apoptose wurden anschließend untersucht.

In diesem Projekt wird gezeigt, dass PDTC eine anti-apoptotische Wirkung in HeLa, MCF-7 und MCF-7-cas3+ Zellen hat. Dieser Effekt wird durch EDTA, einem Komplexbildner, in HeLa Zellen aufgehoben. Dies bedeudet, dass der anti-apoptotische Effekt von PDTC von Metallionen abhängig ist.

Die Freisetzung von cytochrom c aus den Mitochondrien ist charakteristisch für Apoptose und wird durch PDTC verzögert. Die Effekte von PDTC auf Caspasen

wurden untersucht mittels Caspase 3/7 assays, einem zellfreien System sowie der Zelllinie MCF-7. In diesem Projekt wird gezeigt, dass PDTC nicht hauptsächlich auf Caspasen wirkt.

Interessanterweise wird der PI3K/Akt pathway durch PDTC aktiviert, wie mittels Western Blot Analysen gezeigt wird. Allerdings hebt die Inhibierung dieses Pathways nicht die anti-apoptotische Wirkung von PDTC auf. Die Ergebnisse dieses Projekts lassen auf einen pre- und/oder mitochondriellen Effekt von PDTC schließen.

Content

Introduction

I.1 The concept of cell death

In general there are many possibilities how a cell can die. The way how cell death is achieved depends on different factors and circumstances such as cell type and environmental conditions. In this case environment refers to the surrounding area of cells in a whole organism or in cell culture.

Basically at least three different kinds of cell death are distinguished, namely apoptosis, autophagy and necrosis (Fink and Cookson 2005; Galluzzi, Maiuri et al. 2007). All of them are described in detail later in this section.

Cell death was initially studied using the hermaphrodite *Caenorhabditis elegans*. It was found out that a significant fraction of cells generated during development die shortly after birth. Indeed, it is unlikely that most of them have time to perform any function. The resulting question is, why cells are generated and removed shortly afterwards. Cells die in a defined manner (programmed cell death) when they are produced in excess during development, already have served their purpose or if they are potential harmful for the organism. It is assumed that under certain circumstances it is simpler to use programmed cell death as a cellular suicide program than to generate a new cellular program that would avoid the formation of superfluous cells. Programmed cell death is used to refine stereotypical lineages and to produce sexually dimorphic structures in *Caenorhabditis elegans* (Donald L. Riddle 1997).

There is evidence that some kind of cell death is of ancient origin and evolutionary conserved. At first view, for unicellular organism it’s not necessary to have the ability to undergo programmed cell death. But if they live in close proximity of each other, it could be an advantage (Donald L. Riddle 1997). Recently it was shown that yeast cells can undergo apoptosis (Buttner, Eisenberg et al. 2006).

I.2 Classification of cell death

The process of cell death can classified according to the procedures taking place in a dying cell. There are three major types of cell death, namely apoptosis, autophagy and necrosis. Four criteria can be used to classify the type of cell death (Galluzzi, Maiuri et al. 2007):

1) **Morphological appearance**
2) **Enzymological criteria**
3) **Functional aspects: programmed vs. accidental**
4) **Immunological characteristics**

ad 1)

Apoptosis is the best studied kind of cell death and mainly defined by morphological alterations. These changes include chromatin condensation, nuclear fragmentation and the formation of apoptotic bodies. These vesicles are surrounded by a membrane and contain cytoplasmic organelles (Galluzzi, Maiuri et al. 2007). In whole organisms apoptotic bodies are engulfed by phagocytic cells (Fink and Cookson 2005; Jin and El-Deiry 2005; Galluzzi, Maiuri et al. 2007). Hallmarks of apoptosis are the activation of certain proteases called caspases and the fragmentation of DNA (Lawen 2003; Jin and El-Deiry 2005). The cell death of apoptosis is described in detail in section I.4. It is important to mention that apoptosis can occur without these two properties (Galluzzi, Maiuri et al. 2007).

During apoptosis all cellular structures and organelles are degraded rapidly. In contrast autophagy is a slow process. In autophagic cell death parts of the cytplasm are surrounded by double-membraned vacuoles and digested by lysosomal hydrolases (Galluzzi, Maiuri et al. 2007).

Morphological alterations during necrosis include an increase of cell volume (oncosis) thus leading to the rupture of the plasma membrane. In general necrosis lacks biochemical markers (Galluzzi, Maiuri et al. 2007). A summary of morphological features of cell death is shown in Fig. 1.

ad 2)

Different enzymatic processes are activated in different forms of cell death. The activation of caspases is associated with apoptosis. The permeabilization of the mitochondrial membrane plays a role in apoptosis as well as in necrosis (see I.4.2.). This process is considered as “point-of no return” thus committing the cell to undergoing cell death. It is reported that the metabolic status also can influence the kind of cell death. The presence of high glucose- and adenosintriphosphat (ATP)-levels favour the execution of apoptosis. This might be due to the fact that ATP is required for the activation of caspases. Depletion of ATP can switch cell death to necrosis (Galluzzi, Maiuri et al. 2007).

ad 3)

Very often the term “programmed cell death” is used as a quasi synonym for apoptosis. It is important to mention that necrosis can also be programmed (i.e. genetically predetermined). It is also suggested that a defined course of events take place in a necrotic cell (Galluzzi, Maiuri et al. 2007).

ad 4)

The widespread view that apoptosis is a physiological form of cell death whereas necrosis is the pathological form of cell death is oversimplified. In general it is true that apoptosis cause no inflammatory response in contrast to necrosis due to the release of cellular content. Some agents (for example: ionizing radiation) can induce a kind of cell death which is similar to apoptosis in the morphological pattern but accompanied by an immune response (Galluzzi, Maiuri et al. 2007).

I.3 Comparison of apoptosis, autophagy and necrosis

Three major types of cell death are described and further kinds are expected to exist (Fink and Cookson 2005): Apoptosis (type 1 cell death), autophagy (type 2) and necrosis (type 3) (Fink and Cookson 2005; Galluzzi, Maiuri et al. 2007). An overview of pathways leading to cell death is shown in Fig. 2.

The kind of death stimulus influence the way of cell death which is used (Galluzzi, Maiuri et al. 2007). For example DNA damage leads to caspase activation and to

apoptosis. Starvation of nutrients usually leads to autophagic cell death and osmotic lysis leads to necrosis (Galluzzi, Maiuri et al. 2007).

The morphological differences of apoptosis, autophagy and necrosis are depicted in Fig. 1.

Cell death mode	Characteristic morphological aspects	Notes
Apoptosis (Type 1)	● Rounding up of the cell ● Reduction of cellular and nuclear volume (pyknosis) ● Retraction of pseudopodes ● Nuclear fragmentation (karyorrhexis) ● Little modification of cytoplasmic organelles ● Plasma membrane blebbing	'Apoptosis' is the original term introduced by Kerr *et al.*[44] to define a cell death with specific morphological features.
Autophagy (Type 2)	● Lack of chromatin condensation ● Massive vacuolization of the cytoplasm (double-membraned autophagic vacuoles)	'Autophagic cell death' defines cell death occurring *with* autophagy, though it may misleadingly suggest a form of death occurring *through* autophagy.[45]
Necrosis (oncosis) (Type 3)	● Cytoplasmic swelling ● Rupture of plasma membrane ● Swelling of cytoplasmic organelles ● Moderate chromatin condensation	'Necrosis' identifies, in a negative fashion, cell death lacking the features of apoptosis or autophagy, and usually appears as oncosis.[46]

Fig. 1: Morphological aspects of different forms of cell death. Modified from (Galluzzi, Maiuri et al. 2007).

I.3.1 Apoptosis (type 1 cell death)

Apoptosis is the best studied type of cell death. It is described in detail in section I.4. Briefly, apoptosis can be divided into an initiation-, mediation- and execution phase. The initiation phase transmits information from a ligand to a receptor (Riedl and Salvesen 2007). There are two pathways, an extrinsic and an intrinsic one which can be triggered by various signals. For example DNA damage leads to the activation of the intrinsic pathway of apoptosis (Galluzzi, Maiuri et al. 2007). The extrinsic pathway of apoptosis is based on the interaction of extracellular ligands to their appropriate receptors on the cell surface. The receptor of the intrinsic pathway is Apaf-1, a multidomainprotein which is described on page 17. In the effector phase biochemical events are executed which result in the desired cellular response (Riedl and Salvesen 2007).The central effectors of apoptosis are specific proteases called caspases. Activator caspases cleave a lot of cellular substrates leading to typical morphological changes. This morphological pattern is described in Fig. 1. At the end cells are packaged into vesicles called apoptotic bodies which are cleared by phagocytosis (Fig. 2) (Jin and El-Deiry 2005). The regulation of this process is very complex and described in detail in section I.5.

I.3.2 Autophagy (type 2 cell death)

Autophagy is a cellular process which allows the degradation and recycling of macromolecules. Various kinds of stress can induce this mechanism such as deprivation of nutrients. Cells are able to provide nutrients for ATP synthesis by autophagy (Ferraro and Cecconi 2007).

It is important to mention that autophagy is a pro- survival as well as a pro-death mechanism (Ferraro and Cecconi 2007). In proper style the term “autophagy” refers to the survival function of this mechanism and the term “autophagic death” to the pro-death function. In this study as well as in other reviews regarding cell death “autophagy” means “autophagic cell death”. This is a kind of cell death which occur with (!) autophagy (Galluzzi, Maiuri et al. 2007).

The autophagic pathway starts with the engulfment of cytoplasmic material in membrane vesicles called autophagosomes. These vesicles finally fuse with lysosomes in a microtubule dependent manner and the content is degraded. *In vivo* cells undergoing autophagy are phagocytized (Fig. 2) (Fink and Cookson 2005). The morphological appearance of autophagic cells is mentioned in Fig. 1. It is suggested that autophagy can act under certain conditions as substitute of apoptosis. This is the case when apoptosis fails. Then cells have the option to die via autophagy (Ferraro and Cecconi 2007).

I.3.3 Necrosis (oncosis) (type 3 cell death)

Necrosis is characterised as a passive and accidental form of cell death (Galluzzi, Maiuri et al. 2007). But there is evidence that necrosis could also be programmed (Golstein and Kroemer 2007). Necrosis can occur during development; it can be triggered by plasma membrane receptors and is influenced by genetic factors. Further necrosis can be prevented by inhibition of certain enzymes and by the inhibition of caspases (Golstein and Kroemer 2007).

Oncosis is the prelethal pathway leading to cell death by cellular swelling (Fig. 2). This process leads to the depletion of energy stores and failure of ionic pumps in the plasma membrane (Fink and Cookson 2005). The gain of cell volume during oncosis leads to the rupture of the plasma membrane. Subsequently the cell content is released in an unorganized manner into the cellular environment. *In vivo* necrosis

causes an inflammatory response. Necrosis lacks specific biochemical markers thus it can be only detected by electron microscopy (Galluzzi, Maiuri et al. 2007).

I.3.4 Pyroptosis

This is a new form of cell death which is described in the literature (Fink and Cookson 2005). This form of cell death is induced by *Salmonella* and *Shigella* infections. Pyroptosis is mentioned here only for the sake of completeness because it is depicted in Fig. 2. Until now (Sept. 07) only nine papers are published in the PubMed concerning this kind of cell death.

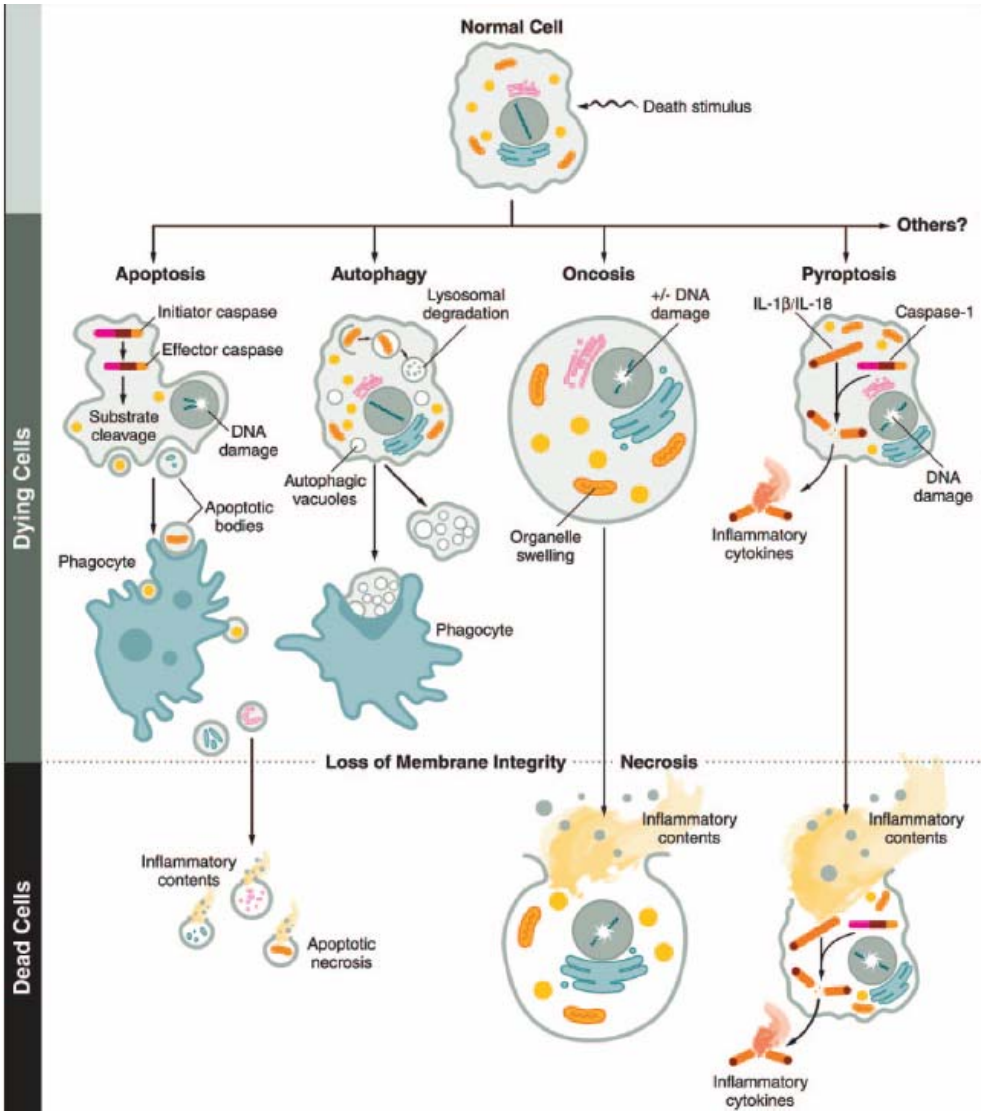


Fig. 2: Overview of different forms of cell death.
(Fink and Cookson 2005)

I.4 Apoptosis

The word apoptosis derives from the Greek language and denotes “falling of leaves from a tree in the autumn”. In cell biology the term apoptosis describes a specific morphology of cell death (Lawen 2003).

Apoptosis is important in development and tissue homeostasis of multicellular organisms. Throughout development more cells are produced than necessary. The excess of these cells might undergo apoptosis thereby contributing to the shape of tissues and organs. Apoptosis is a regulated process which is evolutionary conserved in different species. Malfunction of this regulation may lead to developmental defects, autoimmune diseases or cancer (Jin and El-Deiry 2005).

I.4.1 Morphological features of apoptosis

Different morphological changes can be observed during apoptosis (Fig. 1). In an early phase cells round up and loose their contact with their neighbours. They shrink, chromatin condenses and the DNA is fragmented. The nucleus and the plasma membrane become convoluted and form blebs. Finally the cell falls apart in membrane-encapsulated apoptotic bodies. These spheres are engulfed by macrophages of the surrounding tissue thus avoiding any inflammatory response (Lawen 2003; Jin and El-Deiry 2005).

I.4.2 Pathways of apoptosis

A variety of different signals can initiate the suicide program of a cell. There are two pathways responsible for apoptosis, an extrinsic- and an intrinsic- one. The extrinsic pathway signals due to the binding of external death inducing ligands to their appropriate receptors (for example: Fas:FasL). The intrinsic pathway is activated as consequence of DNA damage induced by radiation or chemicals as well as by growth factor withdrawal (Jin and El-Deiry 2005).

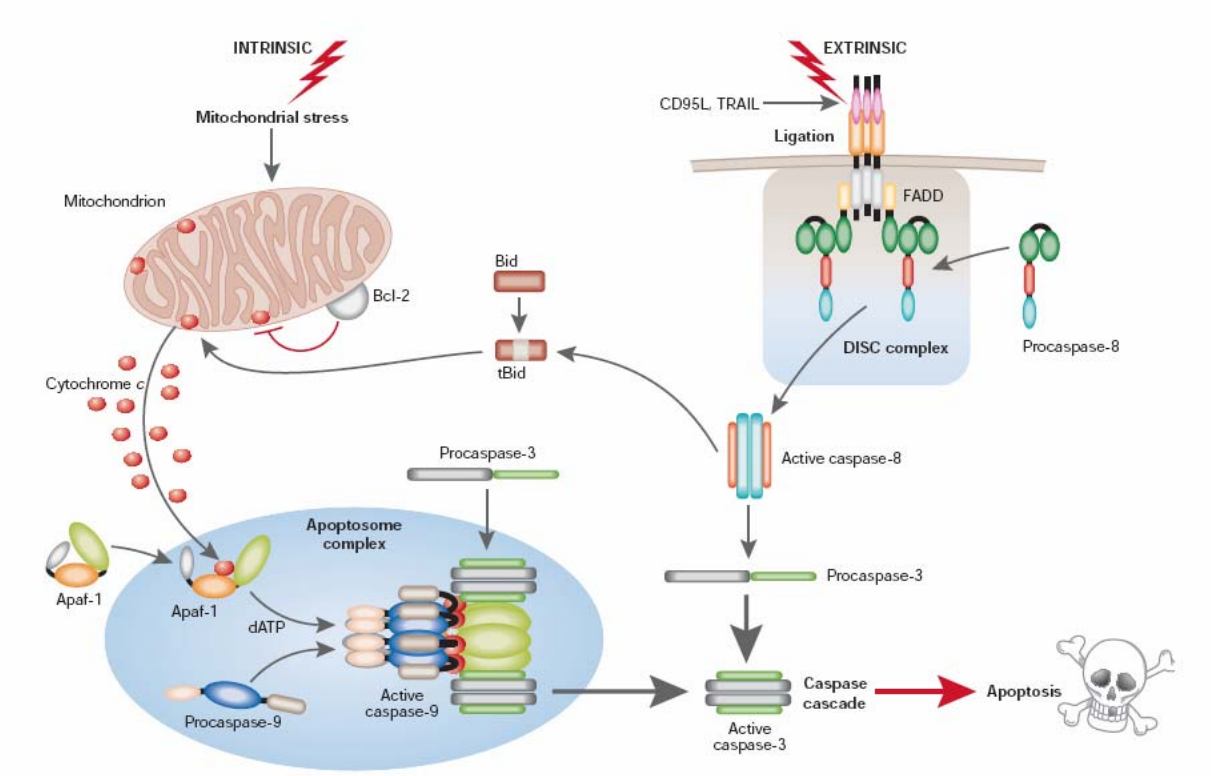


Fig. 3: Extrinsic- and intrinsic pathway of apoptosis.
(MacFarlane and Williams 2004)

a) Extrinsic Pathway

The extrinsic pathway is activated as a consequence of death-receptor/ligand interactions (Fig. 3). These are mainly TNF-TNFR1 and FasL-Fas. The tumor necrosis factor receptor (TNFR) superfamily consists of death receptors with different functions. The presence of cysteine rich domains in those receptors is responsible for ligand binding. Death receptors contain a cytosolic death domain. After ligand binding different intracellular adapter molecules (for example: FADD [fas associated death domain]) are recruited to the appropriate death receptor. This depends on the kind of receptor. The binding of adaptor proteins to the death receptor is mediated by the homophilic interaction of death domains. Further a signalling complex called DISC (death inducing signalling complex) is formed and caspase 8 or 10 is recruited depending on the receptor. The extrinsic as well as the intrinsic pathway converge at the level of caspases (Lawen 2003; Jin and El-Deiry 2005). The activation of caspases is described in section I.4.3.

b) Intrinsic Pathway

The intrinsic pathway is initiated inside the cell. Signals leading to the activation of this pathway are different forms of extra- and intracellular stress. For example, oxidative stress, radiation, withdrawal of growth factors or treatment with chemotherapeutic drugs (Jin and El-Deiry 2005). The Akt pathway which is activated by extracellular growth factors can regulate apoptosis (Alberts, Johnson et al. 2002; Jin and El-Deiry 2005) as depicted in Fig. 4. In addition there are many other signalling pathways in cells which regulate cell proliferation.

The most important events in the intrinsic pathway of apoptosis are taking place at the mitochondria. The key event is the mitochondrial outer membrane permeabilization (MOMP).

Responsible for this process are proteins of the Bcl-2 family. All members have at least one Bcl-2 homology domain in common. The Bcl-2 family is divided into pro- and anti-apoptotic members. The susceptibility of a cell to undergo apoptosis depends on the ratio of these two groups. The regulation of apoptosis is described in detail in section I.5. Bax and Bak are pro-apoptotic members of the Bcl-2 family. These two proteins form oligomers and insert into the outer mitochondrial membrane (OMM) which leads to the release of cytochrome c and other proteins (Lawen 2003; Jin and El-Deiry 2005).

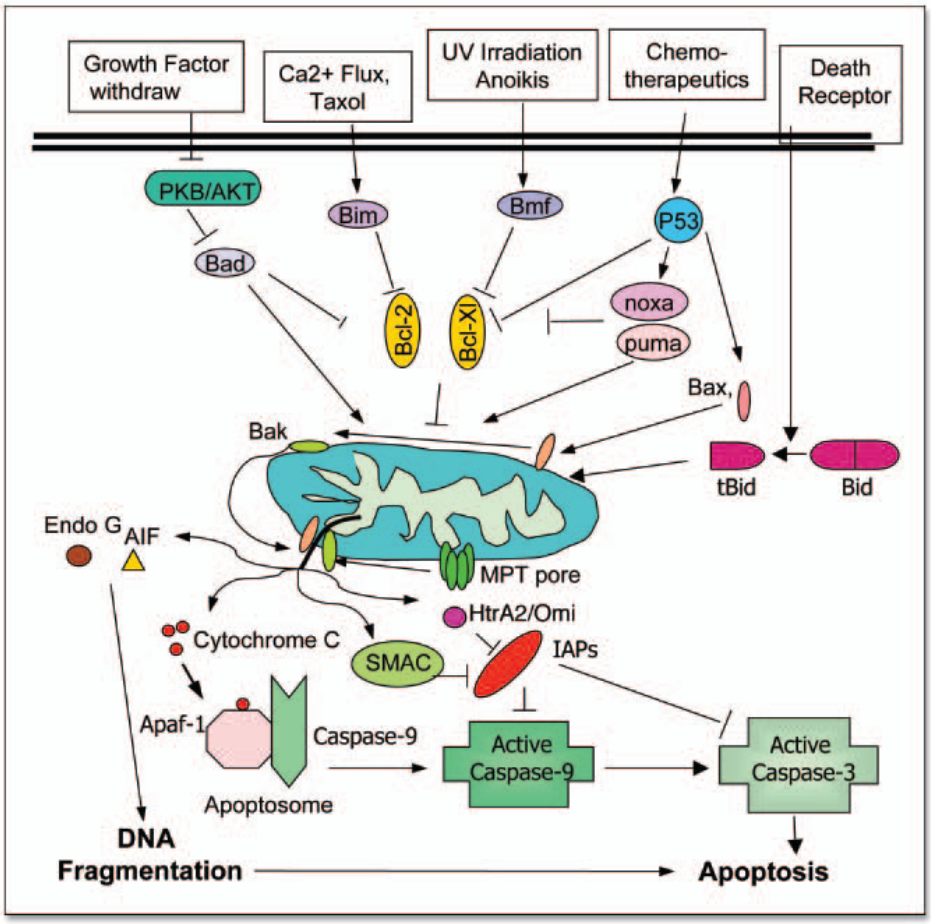


Fig. 4: Details of the intrinsic pathway of apoptosis.
(Jin and El-Deiry 2005)

Cytochrome c (cyt c) plays an important role in apoptosis as well as in the electron transport chain. When released into the cytosol cyt c binds Apaf-1, a scaffold protein, which leads in the presence of ATP/dATP to the formation of a complex called the apoptosome. Caspase-9 is recruited to the apoptosome and thus activated due to oligomerization (Jin and El-Deiry 2005). The apoptosome is an approx. 1 MDa complex and composed of seven Apaf-1, seven cyt c, seven (d)ATP and seven procaspase 9 molecules (Lawen 2003).

The apoptosome acts as a soluble receptor. It is thought that the formation of this signalling platform is a multistep process. Apaf-1 plays a key role in this process (Fig. 5). This multidomainprotein consists of an N-terminal caspase-recruitment domain (CARD), a nucleotide binding domain (NB-ARC) and a string of WD40 repeats in the C-terminus. The CARD domain is responsible for the recruitment of procaspase-9 by homotypic interaction. The function of the WD40 repeats is the binding of cytochrome c which is released from the mitochondria. The nucleotide binding domain contains an ATPase domain and is responsible for (d)ATP binding. Apaf-1

exists as a monomer in the absence of an apoptotic signal and contains a (d)ATP molecule in its centre. The crucial event of apoptosome formation is the binding of cytochrome c to the WD40 repeats. Afterwards the bound nucleotide is hydrolysed. Subsequently Apaf-1 exists in a semi-opened conformation. The NB-ARC region and the CARD domain of Apaf-1 remain inactive thus no recruitment of caspase-9 can occur. To enable the formation of the apoptosome Apaf-1 must undergo massive conformational changes (Riedl and Salvesen 2007).

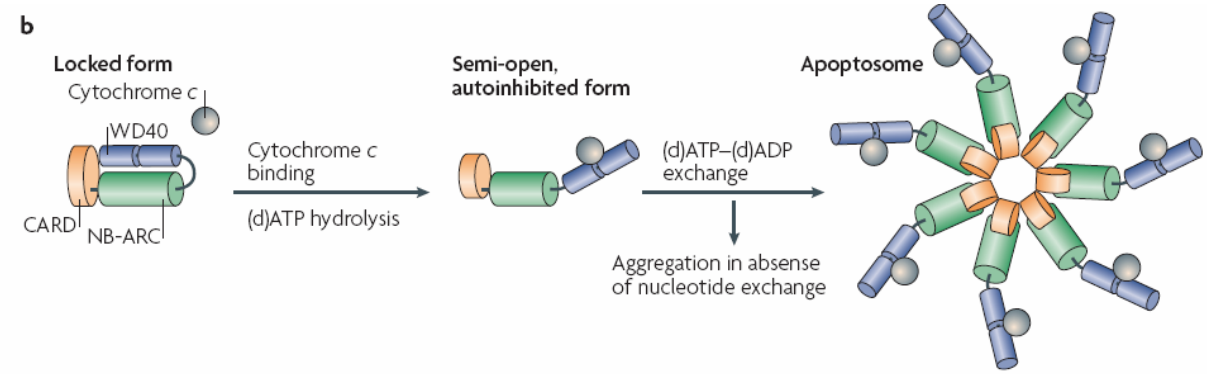


Fig. 5: Mechanism of apoptosome formation. Apaf-1 composed of CARD, WD40 and NB-ARC domains (left) binds cytochrome c when released from mitochondria. This leads to a semi-opened conformation of Apaf-1. (d)ATP exchange leads to formation of the apoptosome which in turn recruits procaspase-9. (Riedl and Salvesen 2007)

A second group of proteins released from the mitochondria are inhibitor of apoptosis (IAPs) antagonists. IAPs are cytosolic proteins and a kind of brake for apoptosis. IAPs are described in detail in section I.5.2. These proteins are able to inhibit active caspases. IAP antagonists function as competitive inhibitors of IAPs thus neutralizing their purpose. (Fan, Han et al. 2005)

I.4.3 Caspases

Caspases are aspartate-specific cysteine proteases. They belong to the interleukin-1ß-converting enzyme family. Fourteen different caspases are identified. All caspases have a pentapeptide active-site in common. The precursors of caspases are called procaspases and are zymogens. This means that they are activated by proteolytic cleavage. All caspases are able to autoactivate themselves as well as activating other caspases (Fan, Han et al. 2005).

Caspases are divided into three subfamilies: Apoptosis activators-, Apoptosis executioner- and inflammatory mediator caspases. Caspases 2, 8 and 10 belong to the first group. Apoptosis executioner caspases are caspase 3, 6 and 7. Caspase 1, 4, 5, and 11 belong to the third group of caspases mentioned. Caspases contain a protease domain which can be subdivided into a large catalytic subunit and a small catalytic subunit. A procaspase also contain a prodomain or an N-terminal peptide of variable length. Initiator- and inflammatory caspases have long prodomains. A prodomain contains the death effector domain (DED) and the caspase recruitment domain (CARD). DED and CARD mediate homophilic interactions and are important in procaspase activation (Rupinder, Gurpreet et al. 2007). The structural comparison of caspases is shown in Fig. 6.

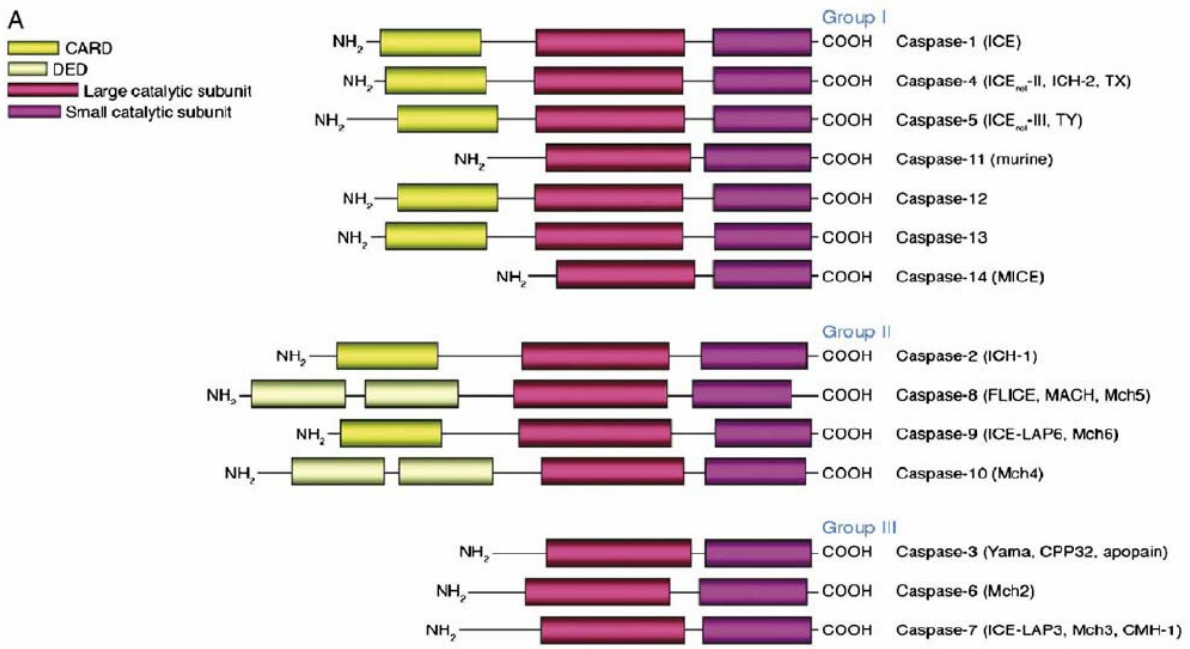


Fig. 6: Structural comparison of caspases. Group I: inflammatory caspases; Group II: apoptosis initiator caspases; Group III: apoptosis effector caspases.
(Rupinder, Gurpreet et al. 2007)

To activate an initiator caspase oligomerization or induced proximity is required. Thus adaptor molecules are recruited to a death complex. Subsequently, conformational changes of these molecules enable the recruitment of procaspases due to protein-protein interactions. The close proximity and this high local concentration of procaspases lead to autocatalytic activation. This mechanism is mediated by the N-terminal CARD and DED domains. There are three different death complexes: known as PIDDososme, DISC and apoptosome. They differ in the recruited adaptor molecules and caspases (Ho and Hawkins 2005).

The proteolytic cleavage of procaspases at specific Asp-X bonds, which is induced by close proximity of these molecules lead to the maturation of caspases. During proteolytic cleavage the prodomain is released. Mature caspases are heterotetramers. This heterotetramer is composed of two heterodimers which are aligned in a head-to-tail configuration. Two active sites are located at opposite ends thus a heterotetramer contains four active sites. The catalytic machinery involves a cysteine sulfhydrylgroup and a histidine imidazol ring (Rupinder, Gurpreet et al. 2007).

Fig. 7 shows a schematic representation of this process.

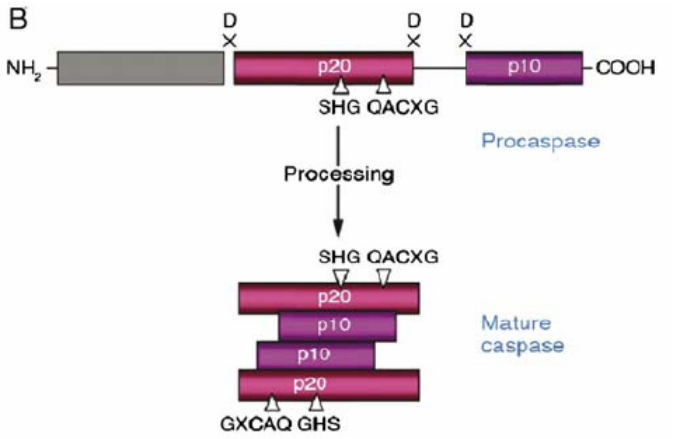


Fig. 7: Schematic representation of procaspase processing. Procaspase is cleaved at specific Asp-X bonds as indicated. p20: large catalytic subunit; p10: small catalytic subunit;
(Rupinder, Gurpreet et al. 2007)

Caspase-8 which plays an important role in the extrinsic pathway of apoptosis is able to induce cytochrome c release by truncation of Bid (Fig. 3 and Fig. 4). Bid is a pro-apoptotic member of the Bcl-2 family. The truncated form of Bid (tBid) subsequently translocates to the mitochondria and leads to the release of cytochrome c. Thus the mitochondrial pathway is activated as well. This mechanism acts as an amplification loop for the apoptosome formation. (Ho and Hawkins 2005) Activator caspases activate downstream executioner caspases by proteolytic processing.

I.4.4 Targets of caspases

Activated caspases initiate a death program by destroying key components of cellular infrastructure (Rupinder, Gurpreet et al. 2007). A key factor in apoptosis is caspase 3. Procaspase-3 is activated by caspase-3, -8 and -10. Substrates of caspase-3 include procaspase-3, -6, -9 and PARP [poly(ADP-ribose) polymerase] (Fan, Han et al. 2005).

Another substrate of caspase-3 is ROCK-1 (Rho-associated kinase). The cleavage of ROCK-1 during apoptosis generates a truncated molecule with increased kinase activity. This leads to cell contraction and membrane blebbing (Creagh and Martin 2001).

A lot of other cellular proteins are cleaved in apoptosis due to caspase activity. For example nuclear lamins which are cleaved by caspase-6 (Widlak 2000). This leads to the characteristic morphological changes described in section I.4.1. The executioner caspases-6 and -7 are homologous of caspase-3. Caspase-3 and -6 are able to activate each other thus creating a positive feedback loop.

PARP which is involved in DNA repair can be cleaved by all executioner caspases (Fan, Han et al. 2005). The function of PARP which is a nuclear enzyme is the addition of poly(ADP-ribose) to nuclear proteins. In the case of massive DNA damage the substrate of PARP, NAD (nicotinamide-dinucleotide) is depleted. Subsequently the ATP level decreases due to NAD synthesis. During apoptosis PARP is cleaved to preserve cellular ATP (Fink and Cookson 2005). The ATP level may influence the type of cell death. ATP is required for caspase activation. The cleavage of PARP is a hallmark of apoptosis and can be detected easily by western blot analysis.

During apoptosis DNA fragmentation can occur. This event is also triggered by caspases. Responsible for the generation of large-scale DNA fragments is an endonuclease called DNA Fragmentation factor 40 (DFF40) or caspase-activated DNase (CAD). This is an endonuclease specific for dsDNA but not for ssDNA or RNA. DFF40 generates blunt ends which can be detected by TUNEL staining. DFF40 attacks chromatin in the internucleosomal linker DNA and generates mono- as well as oligonucleosomal fragments. DFF40 requires the presence of Mg^{2+} but is inhibited by Zn^{2+}. Further it is assumed that DNA breakdown during apoptosis was developed to prevent the transfer of potentially harmful DNA (i.e. activated oncogenes or viral genes). It is important to mention that apoptosis can also occur without DNA fragmentation (Widlak 2000).

Chromatin condensation takes place at terminal stages of apoptosis. This can be triggered by DNA fragmentation per se in the case of DFF40. It was found out that this process does not require ATP (Widlak 2000).

DNA fragmentation can also be induced in a caspase-independent manner (Widlak 2000) by AIF (apoptosis inducing factor) as depicted in Fig. 4.

AIF is released from the mitochondria during apoptosis and translocates to the nucleus. AIF is a NADH oxidase and is important for optimal oxidative phosphorylation. In the nucleus it induces DNA fragmentation and chromatin condensation. The contribution of AIF to cell death depends on the cell type (Modjtahedi, Giordanetto et al. 2006).

I.4.5 Caspase inhibitors

The first caspase inhibitor discovered was a viral protein from cowpox virus designated as cytokine response modifier (CrmA). The advantage of a virus to inhibit caspases is an increasing of the viral replication time or preventing the generation of inflammatory cytokines. CrmA inhibits caspase-1 and -8 as well as caspase-10 in humans. CrmA acts as pseudosubstrate and binds to the active site of caspases.

A common used caspase inhibitor is N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone, abbreviated as zVAD-fmk. This is a general cell-permeable caspase inhibitor (Sigma-Aldrich). zVAD-fmk is an irreversible inhibitor due to its fluoromethylketone moiety. It serves as a pseudosubstrate for active caspases and blocks their catalytical center. zVAD-fmk inhibits all caspases except caspase-2 which is inhibited weakly (Callus and Vaux 2007). Furthermore, other inhibitors specific for different caspases exist.

The use of caspase inhibitors has revealed the existence of alternative cell death pathways, which may function as backup cell death program for apoptosis. zVAD-fmk modulates the three major types of cell death in different ways. It blocks apoptotic cell death while sensitizing cells to necrosis. zVAD-fmk may also trigger autophagic cell death (Vandenabeele, Vanden Berghe et al. 2006; Rupinder, Gurpreet et al. 2007).

I.5 Regulation of apoptosis

I.5.1 Upstream of mitochondria: Bcl-2 family proteins

The Bcl-2 family of proteins function as “life/death switch” by integrating inter- and intracellular signals. The Bcl-family is divided into three different subfamilies (Adams and Cory 2007):

pro-survival subfamily: Bcl-2, Bcl-X_L, Bcl-x, Mcl-1, A1, Bcl-B

Bax-like apoptotic subfamily: Bax, Bak, Bok

BH3-only proteins: Bik, Bad, Bid, Bim, Bmf, Hrk, Noxa, Puma

There are three Bcl-2 homology domains (BH1, BH2, BH3) which have the pro-survival subfamily and the Bax-like apoptotic subfamily in common. BH3-only proteins are largely unrelated to each other as well as to the pro-survival subfamily except the BH3 domain. The BH domains of each pro-survival member fold into a globular structure with a hydrophobic groove. On this groove a BH3 domain which is an amphipathic alpha helix can bind. This interaction neutralizes the pro-survival family member. BH3-only proteins seem to act as damage sensors and direct antagonists of Bcl-2 pro-survival members. BH3-only proteins are activated when apoptosis is induced (Adams and Cory 2007).

For example, Bim is associated with the cytoskeleton and released in response to apoptotic stimuli (Bouillet and Strasser 2002). Thus Bim is able to translocate to the mitochondria and the nucleus (Bouillet and Strasser 2002).

Bak is an integral membrane protein on the cytosolic face of mitochondria and ER. Bax is largely a cytosolic protein or loosely associated with mitochondria. During apoptosis Bax translocates to mitochondrial membranes. Subsequently Bax and Bak change their conformation and form membrane-associated homo-oligomers. Further the mitochondrial membrane is permeabilized leading to the release of cytochrome c (Adams and Cory 2007; Hacker and Weber 2007).

I.5.2 Inhibition of caspases by IAP (inhibitor of apoptosis) proteins

IAPs are regulatory proteins which contain several BIR (baculovirus inhibitor of apoptosis repeat) motifs and a RING domain. Some IAPs also contain a CARD (caspase recruitment domain) motif. The BIR domain enables IAPs to bind and thus to inhibit caspases. The RING domain allows the recruitment of ubiquitin-conjugating enzymes and to catalyse the transfer of ubiquitin from this enzyme to a substrate. Thus IAPs can target many proteins such as caspases or themselves to degradation by the proteasome. The best characterized IAP member is XIAP (X-linked inhibitor of apoptosis). XIAP binds active caspase-9 and -3 by its BIR domains. (Vaux and Silke 2005)

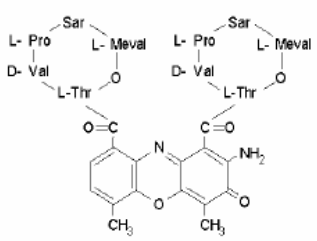
To enable the progression of apoptosis IAPs have to be inactivated. This is achieved by the release of IAP antagonists from mitochondria during apoptosis (Fan, Han et al. 2005). IAP antagonists can contain an IAP binding motif (IBM). IAP antagonists include Smac/DIABLO and HtrA2/Omi which share an IBM in common. They compete directly with caspase-9 for binding to XIAP as shown in Fig. 4. (Vaux and Silke 2005)

I.6 Induction of apoptosis

I.6.1 Induction of apoptosis with Actinomycin D and Puromycin

In this work apoptosis was induced by treating cells with 1 µM Actinomycin D or 50 µM Puromycin for different time points. These inducers were decided due to the well known mode of action.

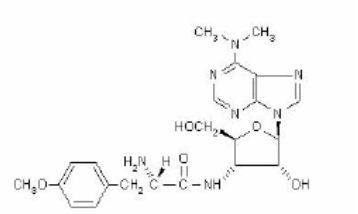
Actinomycin D is an antibiotic which forms a complex with dsDNA. Subsequently DNA-primed RNA synthesis is inhibited. Actinomycin D can also lead to DNA single-strand breaks (Sigma-Aldrich).



Molecular formula: $C_{62}H_{86}N_{12}O_{16}$
Molecular weight: 1255.42

Fig. 8: Structure of Actinomycin D (Sigma-Aldrich)

Puromycin is an antiobiotic of the aminonucleoside family. It is isolated from *Streptomyces alboniger*. Puromycin inhibits protein synthesis by mimicking the 3´end of an aminoacyl-tRNA, subsequently leading to premature chain termination. (Sigma-Aldrich)



Molecular formula: $C_{22}H_{29}N_7O_5 \cdot 2HCl$
Molecular weight: 544.43

Fig. 9: Structure of Puromycin (Sigma-Aldrich)

I.6.2 Induction of apoptosis in a cell-free system

In 1996 a cell-free system was reported where apoptosis could be induced by the addition of dATP and cytochrome c (Liu, Kim et al. 1996). The authors used cytosolic extracts from HeLa S3 cells and isolated nuclei. This system showed characteristic apoptotic properties as observed in cells like caspase-3 activity and DNA fragmentation. These *in vitro* studies enabled them to identify cytochrome c as an important factor in apoptosis.

The advantage of a cell-free system is the possibility to study events downstream of mitochondria in the apoptotic pathway regardless of pre-mitochondrial events.

I.7 The MCF-7 cell line as model system for apoptosis

The MCF-7 cell line is a human breast cancer cell line and derived from a patient in 1970. MCF-7 cells are widely used as model system for apoptosis and chemoresistance (Simstein, Burow et al. 2003). There are distinct lineages of this cell line with different properties in regard of apoptosis. MCF-7 cells are relatively insensitive to apoptotic inducers. It was concluded that MCF-7 variants reflect the apoptotic-resistant phenotypes of breast cancer *in vivo* (Simstein, Burow et al. 2003).
Important to mention is that the MCF-7 cell line does not express caspase-3. This is because of a deletion in the *casp-3* gene. But it was demonstrated that MCF-7 cells are able to activate other caspases. It is described that apoptosis can occur in MCF-7 cells by sequential activation of caspase-9, -7 and -6. Further it was shown that caspase-3 is not required for TNF-induced apoptosis. Characteristic features of apoptosis, like morphological changes or DNA fragmentation are not always observed in MCF-7 cells. If caspase-3 is restored, cells are more sensitive to apoptosis induction by etoposide (Simstein, Burow et al. 2003).
MCF-7 cells are used as model for chemoresistance. It was shown that long time exposure to TNF result in resistance to this drug. Interestingly, chemoresistance seems to be determined by the PI3K/Akt pathway (Simstein, Burow et al. 2003).

I.8 Assays to determine cell death

Several different techniques to analyse cell death exist. Here selected methods which are commonly used are discussed:

I.8.1 Exclusion of dyes

This method is based on the exclusion of vital dyes from cells due to plasma membrane integrity. Such dyes are membrane-impermeable and can only enter the cell when the plasma membrane is disrupted. (Fink and Cookson 2005; Galluzzi, Maiuri et al. 2007)
Examples are propidium iodide (PI) and trypan blue. PI is often used in flow cytometry whereas trypan blue is used in microscopy.
This method does not imply the mechanism of cell death because membrane destruction is a unique feature of dying cells. (Fink and Cookson 2005; Galluzzi, Maiuri et al. 2007)

I.8.2 Annexin V staining

Phosphatidylserine (PS) is usually located on the inner leaflet of the plasma membrane. The externalization of PS is a recognition signal for phagocytosis and takes place early during apoptosis when the plasma membrane is still intact. PS exposure can be detected by labelled Annexin V which binds specifically to this phospholipid. This interaction can be measured subsequently by flow cytometry (Fink and Cookson 2005; Galluzzi, Maiuri et al. 2007). It is reported that externalization of PS was also observed in oncotic cells (Fink and Cookson 2005). Further it has been shown that PS exposure also occur in T-cell activation without cell death (Galluzzi, Maiuri et al. 2007).

I.8.3 Immunoblotting

The activity of caspases can be determined by western blotting. These proteases are described in detail in section I.4.3 and cleave a lot of cellular substrates when active. Antibodies against such substrates can be used to detect caspase activity.

I.8.4 Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

The TUNEL staining is based on the activity of the terminal deoxynucleotidyl transferase which is used to label 3´ends of DNA breaks with biotinylated nucleotides. DNA fragmentation can occur during cell death but it is not exclusively seen in apoptosis (Galluzzi, Maiuri et al. 2007). The TUNEL staining is evaluated by microscopy. Detection of DNA laddering is one frequent used technique to analyse cell death. It can also be analysed by gel electrophoresis (Fink and Cookson 2005).

I.8.5 Cell based assays

A lot of different assays are available to measure viability or caspase activity of cells. In this study among other things a proliferation assay and a caspase assay purchased from Promega was used. Details can be found in the section materials and methods.

I.9 The PI3K/Akt pathway

Akt (also called protein kinase B, PKB) is a serine/threonin kinase. This kinase is activated in response to extracellular growth factors and survival stimuli. The Akt pathway is now thought to play an important role in signal transduction. It controls cell proliferation and survival (Song, Ouyang et al. 2005).

Three isoforms of Akt exist. All of them have a conserved structure. This includes an amino-terminal pleckstrin homolgy (PH) domain which enables binding to 3-phosphoinositides. Further Akt has a central kinase domain as well as a carboxy-terminal regulatory domain. Akt contains two phosphorylation sites Thr308 and Ser473. For full enzymatic activity phosphorylation of both sites is required. Akt is partially active if Thr308 is phosphorylated alone. But phosphorylation of Ser473 alone has only a little effect on its activity (Song, Ouyang et al. 2005).

I.9.1 Activation of Akt

Phosphoinisitide 3-kinase (PI3K) is activated by tyrosine kinase or G-protein coupled receptors. Subsequently PI3K phosphorylates phosphoinistol (4,5)-diphosphate (PIP2) generating phosphoinistol (3,4,5)-triphosphate (PIP3) which serves as second messenger (Fig. 10). This enables the recruitment of Akt to the plasma membrane. Akt binds PIP3 due to the PH domain. This alters the conformation of Akt and is a prerequisite for its activation. Akt consists of two phosphorylation sites. Thr308 is phosphorylated by phosphoinositide-dependent kinase-1 (PDK1) after Akt is recruited to the plasma membrane. The phosphorylation of Ser473 is required for full activation of Akt. However, this phosphorylation step is not understood completely. It was assumed that PDK1 also phosphorylates Ser473. But there is evidence that PDK1 contributes to this phosphorylation only in an indirect manner. It was reported further that Akt could autophosphorylate itself at this residue under certain conditions (Song, Ouyang et al. 2005).

Akt can be also activated in a PI3K independent manner. In this case Akt can be activated by PKA (protein kinsase A). For this kind of activation the PH domain of Akt1 is not necessary. The mechanism how Akt is activated in a PI3K independent manner is not clear (Song, Ouyang et al. 2005).

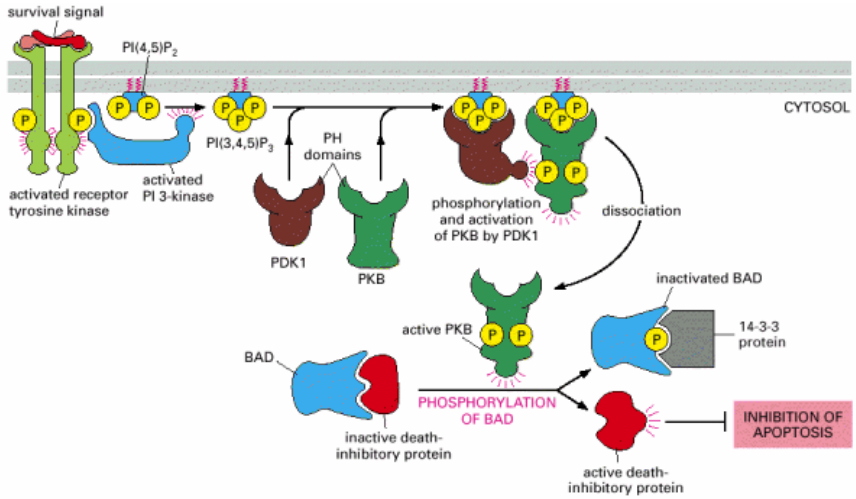


Fig. 10: PI3K signaling. One way in which PI3K promotes cell survival. (PKB=Akt) (Alberts, Johnson et al. 2002)

I.9.2 Consequences of activation of Akt

In general active Akt, which is a serine/threonin kinase provides a strong survival signal for the cell. Further Akt influences the apoptotic pathway directly by acting on the pro-apoptotic BAD protein, which is a member of the Bcl-2 family.

Human caspase-9 is also phosphorylated by Akt directly. This phosphorylation leads to an attenuation of the activity of caspase-9. This regulatory step is not conserved in lower mammalian species.

The Akt pathway also controls cell survival on the level of transcription. The family of Forkhead (FoxO) transcription factors are phosphorylated directly by Akt. The target genes of FoxO transcription factors include the gene of FasL as well as genes of intracellular components for apoptosis. Pro-survival genes are also expressed due to activation of nuclear factor-κB (NFκB) by Akt. These genes include Bcl-XL and caspase inhibitors.

I.10 Pyrrolidine dithiocarbamate (PDTC)

Pyrrolidine dithiocarbamate (PDTC) is a metal chelating compound and is studied for many years. Quite a lot of properties of this substance are described in the literature. The reported effects are controversial and depend on the model system used. This section summarizes current knowledge about PDTC.

PDTC is a copper and zinc ionophore with anti-oxidant properties. It is a thiol (R-SH) and thus PDTC can react with sulfhydrylgroups. PDTC is a white solid compound and stable when dissolved in water. Fig. 11 shows the structure of PDTC.

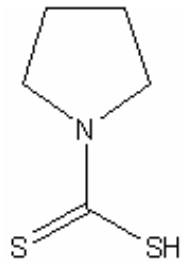


Fig. 11: Structure of PDTC

I.10.1 Reported effects of PDTC on apoptosis

PDTC was described as a pro-apoptotic substance by Erl et al. (Erl, Weber et al. 2000). In this study it was shown that PDTC can induce apoptosis in different primary cells such as human umblical vein endothelial cells (HUVEC) and human fibroblasts. It was found out that not only the cell type but also the cell density are crucial for induction of apoptosis. The authors demonstrated further that the presence of Cu^{2+} and Zn^{2+} are required for PDTC activity (Erl, Weber et al. 2000).
Another study demonstrates pro-apoptotic properties of PDTC at low concentrations (Della Ragione, Cucciolla et al. 2000). Herein the promyelocitic cell line HL-60 was used. It is shown that PDTC cause cytochrome c release after five hours. Additional isolated mitochondria were incubated with PDTC and release of cytochrome c was observed *in vitro*. Thus it was concluded that the mitochondria are the main target of PDTC (Della Ragione, Cucciolla et al. 2000).

Controversial to the previous cited study Cheng et al. demonstrated anti-apoptotic effects of PDTC in HL-60 cells (Cheng, Huang et al. 2006). In this study apoptosis was induced by luteolin. It was found out that PDTC decreases apoptosis in a dose dependent manner but it was unable to prevent luteolin-induced cytochrome c release. It was also demonstrated that Akt was phosphorylated in the presence of

luteolin and PDTC. But PDTC alone failed to cause the phosphorylation of Akt. The authors concluded that the inhibitory effects of PDTC might be due to activation of Akt and subsequently phosphorylation of caspase-9 (Cheng, Huang et al. 2006).

Another study reporting inhibitory effects of PDTC on apoptosis was published in 1997 by Nobel et al. (Nobel, Burgess et al. 1997). Herein the general mechanisms of ditihiocarbamates to inhibit apoptosis were analysed. The behaviour of these substances was investigated in etoposide-induced apoptosis in thymocytes. It was found out that dithiocarbamates are more potent to inhibit apoptosis in thymocytes when they are oxidized to their respective disulfides. This oxidation step is achieved in cell culture experiments by Cu^{2+} in the serum. The authors report an inhibitory effect of dithiocarbamates on the activation of caspase-3 in a cell-free system. Interestingly PDTC showed no such activity. However, it is proposed that dithiocarbamate disulfides themselves are anti-apoptotic by interaction with caspases. But they loose their inhibitory activity when they are metabolized (Nobel, Burgess et al. 1997).

I.10.2 Other effects of PDTC

Also other effects of PDTC are reported. One study reported that PDTC and zinc can inhibit proteasome-dependent proteolysis (Kim, Kim et al. 2004). It was found out that treatment of HeLa cells with PDTC resulted in the accumulation of several proteasome substrates. It is also shown that zinc and copper ions can influence ubiquitin-proteasome activity (Kim, Kim et al. 2004).

PDTC is used as an inhibitor of nuclear factor-κB (NF-κB). One paper reporting this fact is published by Kim et al. (Kim, Kim et al. 1999). In this study PDTC was not able to inhibit NF-κB in the absence of serum. Interestingly zinc and copper restored its inhibitory activity. A low zinc concentration was sufficient to observe this effect. The authors concluded that the interaction of PDTC with zinc is the likely mechanism of influencing NF-κB activity (Kim, Kim et al. 1999).

Further PDTC exhibits antiviral properties. It was shown that PDTC is able to inhibit the processing of viral polyprotein of picornaviruses (Krenn, Holzer et al. 2005; Lanke, Krenn et al. 2007). The authors demonstrated that PDTC interferes with viral RNA synthesis thus inhibiting viral replication. These properties of PDTC are mediated by the import of zinc ions into cells (Lanke, Krenn et al. 2007).

Effects of PDTC were also observed *in vivo*. It is reported that PDTC improves cognitive function in a transgenic animal model of Alzheimer´s disease (Malm, Iivonen et al. 2007). In this study less phosphorylation of the tau protein was found due to PDTC treatment. Further it was observed that PDTC increases Akt phosphorylation as well as copper concentration in the brain. Therefore it was hypothesized that PDTC provides this beneficial effect in the animal model due to copper-mediated activation of Akt (Malm, Iivonen et al. 2007).

I.11 Cellular processes affected by zinc

Zinc is a ubiquitous trace element which is important as structural constituent of many proteins and enzymes. In multicellular organisms 40 % of intracellular zinc is located in the nucleus and 50 % in the cytosol. Zinc is required for the biological function of 300 enzymes. The deprivation of this trace element for example by chelation leads to cell death (Stefanidou, Maravelias et al. 2006).

I.11.1 The PI3K/Akt pathway can be stimulated by zinc

Zinc is known to cause insulin-like effects in various cell types. This includes the activation of the PI3K/Akt pathway. It was shown that the activity of PI3K can be increased in response to zinc exposure. A primary cellular target of zinc upstream of PI3K was not found (Barthel, Ostrakhovitch et al. 2007).

Only certain ions are able to activate the PI3K/Akt pathway. These ions are Zn^{2+}, Cu^{2+} and Cd^{2+}. All of them show similarities regarding activation of Akt. Zinc, copper and cadmium are known as thiol-reactive ions and they cause phosphorylation of Akt in a PI3K dependent manner. This was concluded due to the fact that this phosphorylation can be abolished by the presence of PI3K-inhibitors such as wortmannin (Barthel, Ostrakhovitch et al. 2007).

Protein tyrosine phosphatases (PTPases) are targets of such thiol-reactive compounds. One of them is PTEN which dephosphorylates PIP3. In airway epithelial cells it was shown that PTEN protein levels are lost due to Zn^{2+} treatment subsequnetly leading to activation of Akt. By contrast zinc was shown to stimulate cell death via Akt signaling in neurons. This means that the outcome of zinc treatment depends on the cell type (Barthel, Ostrakhovitch et al. 2007).

I.11.2 Zinc as regulator of apoptosis

Zinc is a biometal and shows cytoprotective effects (Truong-Tran, Carter et al. 2001). If cells are supplemented with zinc *in vitro* as well as *in vivo* the susceptibility to undergo toxin-induced apoptosis decreases. For this effect the labile intracellular zinc-pools are important. It is reported that a deprivation of zinc *in vivo* leads to an increase of apoptosis (Truong-Tran, Carter et al. 2001). Also *in vitro* studies revealed that apoptosis is induced by treatment of cells with zinc-depleted medium or TPEN, a zinc chelator. This zinc deficiency- induced apoptosis depends on caspase-3 activity. Further it was shown that zinc was able to block apoptosis by all apoptosis-inducing treatments tested. This finding leads to the idea that zinc suppresses a common event (Truong-Tran, Carter et al. 2001).

In vivo studies support the idea that intracellular zinc levels can be changed easily and thus protect cells and tissues from apoptosis. But two major problems arose from *in vitro* studies. These studies were often done with supraphysiological zinc concentrations. The reason for this is the poor uptake of zinc into cells. But the problem is that high concentrations of intracellular zinc can cross-link proteins non-specifically (Truong-Tran, Carter et al. 2001). The second difficulty is the possibility that zinc depletion and supplementation studies measured different effects of zinc. This might be the case if zinc has more than one target in the cell. However, cytoprotective agents seems to act at pre-mitochondrial or mitochondrial stages, rather than on the level of caspases (Truong-Tran, Carter et al. 2001).

As cytosolic target of zinc, caspase-3 was identified (Truong-Tran, Carter et al. 2001). This finding is controversal because other studies disagree and find caspase-3 relatively zinc-insensitive (Truong-Tran, Carter et al. 2001). Interestingly, in airway epithelial cells zinc was found to be co-localized with procaspase-3. There is evidence that zinc blocks the activation of caspase-3 rather than the enzyme itself. However, in cell-free systems it was shown that the presence of zinc inhibits PARP-cleavage by caspase-3 (Truong-Tran, Carter et al. 2001).

This and other studies lead to the conclusion that zinc may interact with a sulfhydryl group in caspase-3 (Fig. 12) (Truong-Tran, Carter et al. 2001).

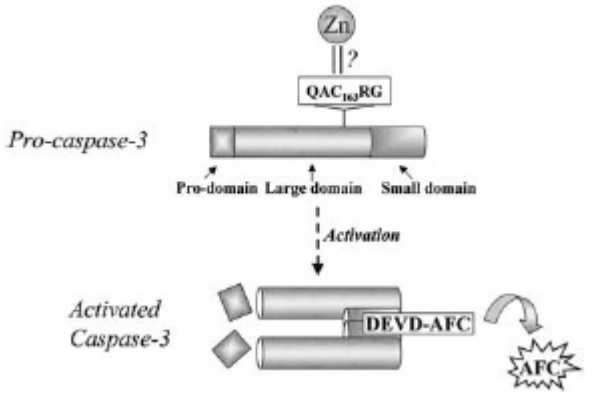


Fig. 12: Model of caspase-3 inhibition by zinc. It is proposed that zinc binds to Cys163 in the active centre and inhibiting this enzyme reversibly. DEVD-AFC represents a fluorogenic substrate which is cleaved due to caspase-3 activity.
(Truong-Tran, Carter et al. 2001)

Aim

Pyrrolidine Dithiocarbamate (PDTC) is a substance which exhibits quite a lot of different effects in biological systems. PDTC is described as inducer as well as inhibitor of apoptosis (Nobel, Burgess et al. 1997; Della Ragione, Cucciolla et al. 2000; Erl, Weber et al. 2000; Cheng, Huang et al. 2006). This property depends on the model system used. In our laboratory pre-exisiting data suggests an anti-apoptotic effect of PDTC.

Further it is described that PDTC is able to inhibit cellular processes like proteasome-dependent proteolysis, the activation of NFκB as well as the replication of picornaviruses (Krenn, Holzer et al. 2005; Lanke, Krenn et al. 2007). In all these cases the presence of certain metal ions (i.e. zinc, copper) is a prerequisite for the function of PDTC. It was also shown that PDTC increases the concentration of intracellular zinc (Lanke, Krenn et al. 2007).

The aim of this project was to find out how PDTC is able to inhibit apoptosis and which steps of the apoptotic pathway are affected.

It was assumed that the increase of intracellular $[Zn]^{2+}$ by PDTC is important in preventing apoptosis. Zinc is known to inhibit caspase-3 and to activate the PI3K/Akt pathway, which provides survival signals for the cell (Truong-Tran, Carter et al. 2001; Barthel, Ostrakhovitch et al. 2007).

To analyse the anti-apoptotic effect of PDTC HeLa cells, caspase-3 deficient MCF-7 and caspase-3 overexpressing MCF-7cas3+ cells were used. Apoptosis was induced in these cell lines with Actinomycin D or Puromycin. These two substances were used to induce cell death due to the well known mode of action.

First, to determine the impact of PDTC on apoptosis in the model system used viability- and/or caspase-3/7 assays were done. The influence of PDTC on the release of cytochrome c from mitochondria was analysed by subcellular fractionation.

Second, to analyse the effect of PDTC on caspase-9 processing and subsequently on caspase-3 a cell-free system composed of cytosolic extracts and nuclei of HeLa S3 cells was used. Apoptosis was induced in this system by addition of cytochrome c and (d)ATP. Effects of PDTC on caspase-3 were also analysed in caspase-3 overexpressing MCF-7-cas3+ cells as well as in caspase-3 deficient MCF-7 cells by using flow cytometry.

Third, the ability of PDTC to activate the PI3K/Akt pathway, which provides a survival signal for cells (Alberts, Johnson et al. 2002), was determined by western blotting. Further it was checked if the inhibition of this pathway by wortmannin could abolish the anti-apoptotic effect of PDTC.

III Materials and Methods

III.1 Tissue Culture

III.1.1 Cell lines

Cell line	Description	Culture Conditions	Source
HeLa	cervix carcinoma cells	RPMI 1640, 10 % Fetal calf serum, 2 mM L-Glutamine, 100 U/ml Penicillin, 100µg/ml Streptomycin	ATCC CCL-2
HeLa S3	cervix carcinoma cells	DMEM, 10 % Fetal calf serum, 2 mM L-Glutamine, 100 U/ml Penicillin, 100µg/ml Streptomycin	ATCC CCL-2.2
MCF-7	breast cancer cells	RPMI 1640, 10 % Fetal calf serum, 2 mM L-Glutamine, 100 U/ml Penicillin, 100µg/ml Streptomycin	Rainer, Jäniche, Düsseldorf
MCF-7-Cas3+	breast cancer cells overexpressing caspase-3	RPMI 1640; 10 % Fetal calf serum, 2 mM L-Glutamine, 100 U/ml Penicillin, 100µg/ml Streptomycin	Rainer, Jäniche, Düsseldorf

III.1.2 Media and Chemicals

- RPMI growth medium
  RPMI 1640 -L-Glutamine, Gibco
  10 % heat inactivated FCS
  1 % L-Glutamine stock solution
  1 % Penicillin-Streptomycin stock solution

- DMEM growth medium
  DMEM, Gibco
  10 % heat inactivated FCS
  1 % Penicillin-Streptomycin stock solution

- 1x Trypsin-EDTA, PAA

- Fetal calf serum (FCS), Gibco
  heat inactivation by incubation at 56°C for 30 min

- 100x L-Glutamine, Gibco
  200 mM stock solution

- Penicillin-Streptomycin, stock solution, Gibco
  10.000 U/ml Penicillin,
  10.000 µg/ml Streptomycin

- 10x PBS
  11,5 g $Na_2HPO_4*2H_2O$
  80 g NaCl
  2 g KCl
  2 g KH_2PO_4
  ad 1l dH_2O; pH 7,4

- Dimethylsulfoxid (DMSO),
  Sigma Aldrich

- Pyrrolidine Dithiocarbamate (PDTC),
  ALEXIS Biochemicals
  500 mM stock solution in dH_2O,
  stored at -20°C;
  10 mM working solution is
  prepared by dilution 1:50 in PBS

- Actinomycin D, Sigma Aldrich
  1 mM; dissolved in DMSO;
  stored at -20°C

- Puromycin Dihydrochloride,
  Sigma Aldrich
  10 mM; dissolved in dH_2O;
  stored at -20°C

- Wortmannin, Sigma Aldrich
  2,3 mM; dissolved in DMSO;
  stored at -20°C

- EDTA, 50 mM, sterile

III.1.3 Cell culture

Cells are grown in tissue culture flasks (Nunc) at 37°C, 5 % CO_2 in humidified atmosphere. To split cells, the medium is discarded and the monolayer is rinsed with 1xPBS. One ml Trypsin-EDTA is added to a 75 cm^2 flask. The cells are incubated for 5-10 min until the cells detach from the surface. 10 ml fresh growth medium are added to neutralize the Trypsin and a homogenous cell suspension is created by pipetting up/down. In the case of HeLa cells, the suspension is ready to use for passaging into a new flask/dish. All other cells are centrifuged after trypsinisation to remove the trypsin completely. After resuspending in fresh growth medium the cells are transferred into desired flasks/dishes.

Area [cm^2]	Trypsin [ml]	Medium [ml]
75	1	12
225	2-2,5	30

III.1.4 Counting of cells

Cells are counted by using a hemato-cytometer (Neubauer improved). Therefore a 50 µl aliquot of the desired cell suspension is taken and diluted 1:5 or 1:10 with medium. Cells are counted in all four chambers of the hemato-cytometer and the average is calculated. Cell concentration is determined according to the following equation:

$$[\text{Average}] \times [\text{Dilution}] \times 10^4 = \text{cells/ml}$$

III.1.5 Freezing/Thawing of cells

A Cryo-freezing container (Nalgene) is pre-cooled at 4°C. Cells are harvested by trypsinisation. Desired cell number ($1*10^6$-$3*10^6$ cells) in a total volume of 900 µl is transferred into a cryo-tube (Nunc) and 100 µl DMSO added. The cryo-tube is inverted immediately several times and placed into the pre-cooled freezing chamber. Afterwards the freezing-chamber is put at -80°C for at least 24 h. Finally the cryo-tube is transferred into liquid nitrogen.

To thaw cells the desired cryo-tube is immediately thawed at 37°C in the water bath. The cell suspension is transferred into a 50 ml Falcon where the cells are rinsed slowly with 10 ml growth medium. The cells are centrifuged (300 g, 4 min) and the residual supernatant is removed. Finally the cells are resuspended in the desired volume and seeded into a culture-flask. On the next day the medium is changed to remove the last traces of DMSO.

III.2 Protein detection methods

III.2.1 Subcellular fractionation

Material:

- SEM buffer

250 mM	Sucrose
10 mM	4-Morpholine-propanesulfonic acid (MOPS)
1 mM	Ethylenediamine-tetraacedic acid (EDTA)
100 mM	KCl
1,5 mM	$MgCl_2$
1 mM	Ethylen-glycol-bis(2-aminoethylether)-N,N,N´,N´-tetraacetic acid (EGTA)
pH 7,2; KOH	

- 25x Protease Inhibitor Cocktail
  Dissolve one tablette of EDTA-free Complete Protease Inhibitor (Roche) in 2 ml dH_2O. Aliquot and store at -20°C.
- 1xPBS
- Douncer, 7 ml, Wheaton

Method:

To analyse the release of cytochrome c from mitochondria into the cytosol which is a hallmark of apoptosis, subcellular fractionation was done. This method results in three different fractions designated as “total cell extract”, “Mitochondria” and “Cytosol”.

$5*10^6$ cells are seed on one 15cm dish and incubated for two days at 37°C in humidified atmosphere. Medium is removed and cells are treated with desired drug. After different time points the supernatant which contains floating cells is transferred to a 50 ml Falcon tube. Attached cells on the dish are washed with cold 1xPBS. After PBS is removed completely 0,75 ml SEM buffer containing protease inhibitors is added to each dish. Cells are harvested by using a cell scraper and transferred to a Eppendorf tube.

The floating cells in the Falcon tube are centrifuged (300 g, 4°C, 4 min), supernatant is removed by decantation. To wash cells, approximately 10 ml of ice cold 1xPBS are added. The cell pellet is carefully resuspended by shaking the tube slightly and

centrifuged again (300 g, 4°C, 4 min). Afterwards PBS is removed completely. To merge the cell pellet with the already harvested cells, a small amount of the cell-suspension in the Eppendorf tube is added to the cell pellet. Cells are resuspended by pipetting and transferred to the Eppendorf tube again. This is necessary to minimize the volume.

Finally cells are transferred to a pre-cooled 7 ml Douncer (Wheaton) on ice. Cells are disrupted with 100 strokes using a type B pestill. Successful disruption is checked by trypan blue staining. An aliquot of 50 µl is taken and designated as “total cell extract” containing nuclei. Disrupted cells are transferred into an Eppendorf tube and centrifuged in a micro-centrifuge (600 g, 4°C, 5 min). The supernatant is transferred to a new tube.

The pellet is resuspended in 100 µl SEM buffer containing protease inhibitors followed by centrifugation (600 g, 4°C, 5 min). This step is necessary to increase the yield of mitochondria. Both supernatants are merged and centrifuged (13600 g, 4°C, 15 min). 100 µl of the supernatant are taken and designated as “cytosolic fraction”. The mitochondrial pellet is washed with 100 µl SEM buffer containing protease inhibitors and centrifuged (13600 g, 4°C, 15 min). Finally the mitochondrial pellet is resuspended in 50 µl SEM buffer containing protease inhibitors. All fractions are stored at -20°C until further analysis.

III.2.2 Cell-free System for apoptosis

Material:

- Buffer N (hypotonic)
  10 mM Hepes
  2 mM $MgCl_2$
  25 mM KCl
  pH 7,5; KOH
- Buffer N+
  10 mM Hepes
  2 mM $MgCl_2$
  25 mM KCl
  250 mM Sucrose
  pH 7,5; KOH
- Buffer A
  20 mM Hepes
  10 mM KCl
  1,5 mM $MgCl_2$
  1 mM EDTA
  1 mM EGTA
  pH 7,4; KOH
- Aprotinin, 1 mg/ml
- Pepstatin, 1 mg/ml
- Leupeptin, 10 mg/ml
- 100 mM Phenylmethylsulfonyl-fluoride (PMSF) stock solution in methanol
- 214 mM Dithiothreitol (DTT)
- 40 mM ATP
- 100 mM dATP
- 800 µM Cytochrome c,
  from bovine heart, >95%,
  Sigma Aldrich
  dissolved in PBS and stored at -20°C
- 0,4 % w/v Trypan blue
- 2 M Sucrose

Method:

To analyse the influence of PDTC on the apoptotic events downstream of cytochrome c release a cell-free system was used similar to those published (Liu, Kim et al. 1996).

$7{,}5*10^6$ HeLa S3 are seeded on 15 cm dishes and incubated for two days at 37°C in humidified atmosphere. The supernatant is discarded and the cells are immediately washed with ice-cold PBS. All further steps are carried out on ice. PBS is removed completely and 0,75 ml Buffer A supplemented with Pepstatin (1:1000), Leupeptin (1:1000), Aprotinin (1:1000), PMSF (1:1000) and DTT (1:214) are added. Cells are harvested by using a cell-scraper, transferred into an Eppendorf tube, incubated 20 min on ice and finally pushed through a 26G needle. The success of the disruption is checked by trypan blue staining. Usually approximately 40 strokes are

required to reach 90 % blue cells. The homogenate is centrifuged in a micro-centrifuge (13.600 g, 15 min, 4°C) and the supernatant transferred into a pre-cooled 1,5 ml Beckman-ultra-centrifuge tube and the nuclear-pellet is kept on ice. Now the supernatant is re-centrifuged using a Beckman ultra-centrifuge (Optima TLX Ultracentrifuge, rotor TLA 100.3, 100.000 g, 1 h, 4°C). The resulting supernatant is designated as S100 cytosolic-fraction.

The nuclear pellet is resuspended by pipetting in 1 ml Buffer N (hypotonic) supplemented with protease-inhibitors as described above, 125 µl 2 M sucrose are added and mixed well by inversion. The nuclei are centrifuged (300 g, 4 min, 4 °C) using a swinging-bucket rotor (Heraeus Sepatech #2704).

The supernatant is discarded and the pellet is resuspended in 1 ml BufferN+ supplemented with protease-inhibitors as described followed by a second centrifugation (300 g, 4 min, 4°C, swinging-bucket rotor Heraeus Sepatech #2704). The supernatant is removed and the nuclei are resuspended in 100 µl Buffer N+ containing the same protease inhibitors as described above.

Nuclei and cytosolic fraction are mixed in a ratio 1:10. To induce apoptosis 1 mM dATP, 1 mM ATP and 10 µM cytochrome c are added. As negative control one reaction is prepared without the addition of cytochrome c. Reactions are incubated at 37°C, 600 rpm and aliquots of each are taken after desired time points. Immediately 4x sample buffer is added to the aliquots and heated for approximately 30 min at 95°C to stop the reaction. Samples are stored at -20°C and are analysed by western blotting.

III.2.3 Preparation of cell extracts

Material:

- 5x Sample buffer
  - 10 ml Glycerol
  - 5 ml ß-Mercaptoethanol
  - 3 g SDS
  - 3,8 g Tris-(hydroxymethyl)-aminomethane (Tris)
  - 3 mg Bromphenolblue
  - pH 6,8; HCl
- 1x sample buffer
  - dilute 5x sample buffer 1:5 with dH_2O

Method:

Cells are seeded in 12 well plates and treated as desired. To collect floating cells, the medium is transferred into an Eppendorf-tube and centrifuged (15.800 g, 3 min, RT). The supernatant is discarded. Attached cells are lysed by adding 50 µl 1x sample buffer. Proteins are harvested by scraping with the pipette-tip and added to the cell-pellet. Samples are heated at 95°C for approximately 30 min prior western blot analysis.

III.2.4 Tris/Tricine PAGE

Material:

- Acryl-/Bisacrylamide stock solution
  300 g Acrylamide
  8 g Methylenbisacrylamide
  Dissolve in 1l dH_2O. Filter and store at 4°C.

- Anode buffer
  0,2 M Tris
  pH 8,9; HCl

- APS
  10 % w/v
  Ammoniumperoxodisulfat in dH_2O

- Cathode buffer
  0,1 M Tris
  0,1 M Tricine
  0,1 % w/v
  Sodiumdodecylsulfate (SDS)
  no pH

- 87% v/v Glycerol

- 5x Sample buffer
  10 ml Glycerol
  5 ml ß-Mercaptoethanol
  3 g SDS
  3,8 g Tris
  3 mg Bromphenolblue
  pH 6,8; HCl

- Stack Solution
  12,5 ml 4xUGS
  6,5 ml 30% Acrylamide
  31 ml dH_2O

- TEMED
  N,N,N´,N´-Tetramethylethylethylene-diamine

- 3x Tris/HCl SDS buffer
  1,5 M Tris
  0,15 % w/v SDS
  pH 8,45; HCl

- 4x UGS (upper gel solution)
  0,5 M Tris
  0,4 % w/v SDS
  pH 6,8; HCl

Method:

Tris/Tricine PAGE is used for good separation of small proteins. Gel apparatus is assembled according to the manufacturer's instructions. For preparing one seperating gel the following solutions are mixed.

	1 x
Acrylamide 30 %	2 ml
3x Tris/HCl/SDS pH 8,45	2 ml
dH_2O	1,4 ml
Glycerol	0,6 ml
APS	36 µl
TEMED	3,6 µl

After the solution is filled into the gel apparatus 1 ml of dH_2O is added carefully to ensure a smooth surface. After 30 min the seperating gel is polymerised, the stack gel can be prepared. The water-layer of the seperating gel is removed prior adding the stack gel. 2 ml stack solution, 12 µl APS and 4 µl TEMED are mixed. Immediately afterwards the comb is put in the polymerising stack gel.

After the stack gel is polymerised the comb is removed and the gel put into the electrophoresis unit. Cathode buffer is added to the upper chamber and anode buffer to the lower one.

To prepare the samples an appropriate volume of sample buffer is added. Afterwards all samples are heated at 95°C for approximately 30 min, chilled on ice and centrifuged shortly. All samples are loaded at room temperature to avoid any precipitation of proteins. To run a gel a current of 20 mA/gel is applied. The loading dye reaches the end of the gel after approximately 1,5 h. Finally the gel is subjected to western blot analysis.

III.2.5 SDS PAGE

Material:

- Acryl-/Bisacrylamide stock solution
  300 g Acrylamide
  8 g Methylenbisacrylamide
  Dissolve in 1l dH_2O. Filter and store at 4°C.
- APS
  10 % w/v
  Ammoniumperoxodisulfat in dH_2O
- 4x LGS
  1,5 M Tris
  0,4 % w/v SDS
  pH 8,8; HCl
- TEMED
  N,N,N´,N´-Tetramethylethylethylene-diamine
- 10 x Running buffer
  3,8 M Glycin
  0,5 M Tris
  1 % w/v SDS

Method:

A 7,5 % acrylamide gel is used to separate large proteins. In principle the method is the same as described above. For one separating gel the following solutions are mixed together:

	1 x
Acrylamide 30 %	1,5 ml
4x LGS	1,5 ml
dH_2O	3 ml
APS	36 µl
TEMED	3,6 µl

The rest of the procedure is the same as described for the Tricine PAGE except that there is only one running buffer.

III.2.6 Western Blot

Material:

- Anode Buffer 1
  300 mM Tris
  20 % v/v Methanol
- Anode Buffer 2
  25 mM Tris
  20 % v/v Methanol
- Albumin Bovine Serum (BSA),
  Fraction V, >96%, Sigma Aldrich
- Cathode Buffer
  40 mM 6-Amino-n-hexanoic acid
  25 mM Tris
  20 % v/v MeOH
- ECL-Detection Kit, Pierce
- Whatman Paper
- PROTRAN(R), Nitrocellulose
  Transfer Membrane, 0,2 µm,
  Schleicher & Schuell
- 1x PBS
- PBST
  1xPBS
  0,1 % v/v Tween 20
- Ponceau S, Sodium salt,
  Practical grade, Sigma Aldrich
  0,1 % w/v in 1% v/v acetic acid
- Non-fat dry milk
- 10% w/v NaN_3
- Blocking solution
  5 % w/v non-fat dry milk in
  PBST

Antibodies:

Primary Antibodies:

	Company	Dilution used for Western Blot	Source
α Actin	Sigma, A2066	1:8.000	Rabbit
α pAkt(Ser473)	Cell Signaling, 4058S	1:1.000	Rabbit
α Akt	Cell Signaling, 9272	1:1.000	Rabbit
α Cleaved Caspase 9	Cell Signaling, 9501S	1:500	Rabbit
α COX IV	abcam, ab14744	1:2.000	Mouse
α Cyt c	Invitrogen, 33-8500	1:500	Mouse
α PARP	Santa Cruz, sc7150	1:2.500	Rabbit

Secondary Antibodies:

	Company	Dilution used for Western Blot
α Mouse-HRP	Jackson	1:40.000
α Rabbit-HRP	Jackson	1:20.000

Method:

Whatman paper is cut into 7 x 10 cm sheets. For one gel 10 sheets are required. A piece of nitrocellulose membrane with the dimensions 6 x 8 cm is cut out. To prepare a western blot the semy dry blotting machine is opened, three Whatman sheets are soaked with Anode buffer 1 and placed on the anode. Two sheets are soaked with Anode buffer 2 and placed above. The membrane is pre-rinsed with dH_2O and put next.

The gel is removed from the electrophoresis unit and one glass plate is removed. A dry sheet of Whatman paper is placed on the gel. Now the gel is carefully removed from the second glass plate and incubated in Cathode buffer. Air bubbles between gel and Whatman paper are removed. Finally the gel is put onto the nitrocellulose membrane. At the end four Cathode buffer-soaked sheets of Whatman paper are put on the sandwich. To ensure that no air bubbles can disturb the blotting process the surface of the sandwich is smoothed by using a Falcon tube.

A constant voltage of 15 V is applied for at least one hour. The success of the blotting process is checked by Ponceau staining. Therefore the membrane is incubated in the Ponceau solution for approximately five min under slightly shaking. If necessary the membrane is cut in two pieces to probe with different antibodies simultaneously. The membrane is then de-stained with dH_2O. To block unspecific binding sites the nitrocellulose membrane is put into a 50 ml Falcon tube and approximately 5 ml blocking solution are added. The Falcon is put on a roller in the cold room (4°C) for one hour. Afterwards the blocking solution is discarded and the membrane is washed twice with PBST (5 min, 4°C, roller). The membrane is probed with the desired primary antibody. Therefore the antibody is diluted according to the table above in 4 ml PBST containing a small amount of BSA and 0,1 % w/v NaN_3 to re-use the antibody.

The membrane is incubated with the primary antibody over night at 4°C on a roller. Antibody dilutions can be stored at 4°C and re-used. Afterwards the blot is washed

three times with PBST. The secondary antibody is diluted in PBST and applied to the blot for two hours. Finally the blot is washed twice with PBST at room temperature under slightly shaking and washed once with PBS.
For detection an equal amount of both ECL-solutions are mixed and applied to the blot immediately. Usually 1 ml of each solution was used for one blot. After an incubation period of five min at room temperature the membrane is placed into the film cassette and exposed to an x-ray film (CL-X Posure Film, Pierce). Exposure time ranges from 30 sec – 15 min. The film is developed by using an Agfa Curix60 developing machine.

III.2.7 Stripping of nitrocellulose-membranes

Material:

- 0,2 M NaOH
- Blocking solution
  5 % w/v non-fat dry milk in PBST

Method:

The membrane is washed with dH_2O for five min at room temperature under slightly shaking. 0,2 M NaOH is applied for five min. The blot is washed once with dH_2O for five min. Finally the membrane is blocked for one hour at 4°C and washed twice with PBST. Now the blot can be re-probed with a new primary antibody.

III.3 Flow Cytometry

Material:

- 438 µM Rhodamine 123 (R123) in Methanol
- Propidiumiodide (PI), 10 mg/ml in dH_2O

Method:

The R123/PI staining allows discrimination between early- and late phase of apoptosis. Early apoptotic cells loose their mitochondrial membrane potential which

can be seen in R123 staining. Late apoptotic and necrotic cells loose their plasma membrane integrity thus PI can accumulate in these cells.

$1,5*10^6$ cells are seeded on 60 mm dishes and incubated over night at 37°C. Cells are treated with desired drugs for certain time points.

The supernatant is transferred into a 15 ml Falcon tube, the attached cells washed once with sterile 1xPBS and 0,5 ml trypsin is added per dish. Cells are incubated for approximately 5 min at 37°C until detachment.

Four ml medium are added to each dish to neutralize the trypsin and the cell suspension is transferred into the 15 ml Falcon tube. Cells are centrifuged (300 g, 4 min, RT) and resuspended in 3 ml medium. To one ml of cell suspension containing $1*10^6$ cells, 0,5 µl Rhodamine are added (200 nM final). Cells are incubated 20 min at 37°C in the dark. A control of un-stained cells is also required. Cells are centrifuged (300 g, 4 min, RT, swinging bucket rotor: Heraeus Sepatech #2704) and resuspended in one ml sterile PBS. One µl PI is added and cells are analysed by flow cytometry (BD LSR flow cytometer). Fluorescence is excited by a 488 nm laser and green (R123) and red fluorescence (PI) is collected.

The instrument settings of the BD LSR are given in the following table:

Detector	Voltage	AmpGain
FSC-H	---	1
SSC-H	680	4
FL1-H	400	Log
FL2-H	700	Log
FL4-H	719	2
FL5-H	500	4
FL3-H	530	Log
SSC-W	730	Log

III.4 Caspase 3/7 Assay

Material:

- Caspase-Glo(R) 3/7 Assay Kit, Promega, G8091

Method:

This assay is based on the cleavage of an artificial caspase substrate by active caspase 3/7 subsequently generating a substrate for luciferase. The emitted light is therfore proportional to the caspase 3/7 activity.
1,5 *10^6 cells are seeded on one 96-well plate and incubated over night at 37°C. On the next day cells are treated with 50 µl/well 50 µM Puromycin, 1 µM Actinomycin D +/- 125 µM PDTC. For control desired wells are untreated or vehicle-treated (DMSO). The Caspase-Glo(R) 3/7 Reagent is prepared according to the manufacturers instructions.
To start the assay the 96-well plate and the assay reagent are equilibrated to room temperature for a few minutes. 50 µl assay reagent are added to each well and mixed gently by shaking the plate. After incubation for 30 min at room temperature the reaction mixture is transferred into a 96-well white-walled plate. Finally the luminescence is read by a plate-reading luminometer (Berthold Technologies, Centro LB 960) for 10 s/well.

III.5 Crystal Violet staining

Material:

- 0,2 % w/v crystal violet in 2 % v/v Ethanol
- 1 % w/v SDS
- 1xPBS

Method:

The principle of the assay is the retention of a dye by fixed cells on a plate. When the dye is solubilized the absorbance is proportional to the amount of attached cells.
Cells are grown in a 96 well plate and treated as desired. Afterwards the medium is discarded and cells are washed twice with 100 µl/well 1xPBS. 50 µl/well crystal violet solution is added followed by incubation for 20 min at room temperature. The plate is

washed carefully with tap water to remove the excess of crystal violet and dried over night at room temperature. The dye is solubilized with 50 µl/well 1 % w/v SDS and by shaking the plate slightly. Absorbance is measured by a plate reader (Labsystems Multiskan RC) at 560 nm.

III.6 Proliferation Assay

Material:

- CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay, G109C, Promega

Method:

This assay is based on the reduction of the tetrazolium salt MTS by dehydrogenase enzymes of viable cells. The generated formazan product is colored, soluble and can be measured at 490 nm.

Cells are seeded in 96 well plates and treated as desired with a total volume of 100 µl/well. MTS and PMS solution are thawed at room temperature or at 37°C. Two ml MTS solution and 100 µl PMS solution are mixed immediately before use. 20 µl of this solution are added to each well of the 96 well plate. Cells are incubated at 37°C for 1-4 h. Absorbance is read at 490 nm using a plate reader (Labsystems Multiskan RC).

IV Results

IV.1 PDTC inhibits apoptosis in HeLa cells

In the literature pro- as well as anti-apoptotic effects of PDTC are described (Nobel, Burgess et al. 1997; Della Ragione, Cucciolla et al. 2000; Erl, Weber et al. 2000; Cheng, Huang et al. 2006). Pre-existing data of our laboratory indicates an anti-apoptotic effect of PDTC. To induce apoptosis HeLa cells were treated with Actinomycin D or Puromycin. The decision to use these inducers is based on their well known mode of action. Actinomycin D inhibits transcription whereas Puromycin inhibts translation (see introduction for details). HeLa cells were treated with 1 µM Actinomycin D or 50 µM Puromycin for different time points. These concentrations were decided, due to the appearance of cell death after 8-10 h. Cells were co-treated with or without 125 µM PDTC. This concentration of PDTC was not toxic for HeLa cells in the desired time frame. Subsequently different hallmarks of apoptosis were analysed. First of all the possibility of PDTC to counteract Actinomycin D or Puromycin-induced apoptosis was checked by microscopic observation, crystal violet staining, proliferation- and caspase 3/7 assay.

Fig. 13 shows typical microscopic observations of HeLa cells treated with Actinomycin D with or without PDTC for different time points. DMSO was used as vehicle control. When apoptosis was induced with Actinomycin D no morphological intact cells are visible after 12 h. In contrast, if PDTC is present approximately fifty percent of the cells remain morphological intact after 12 h.

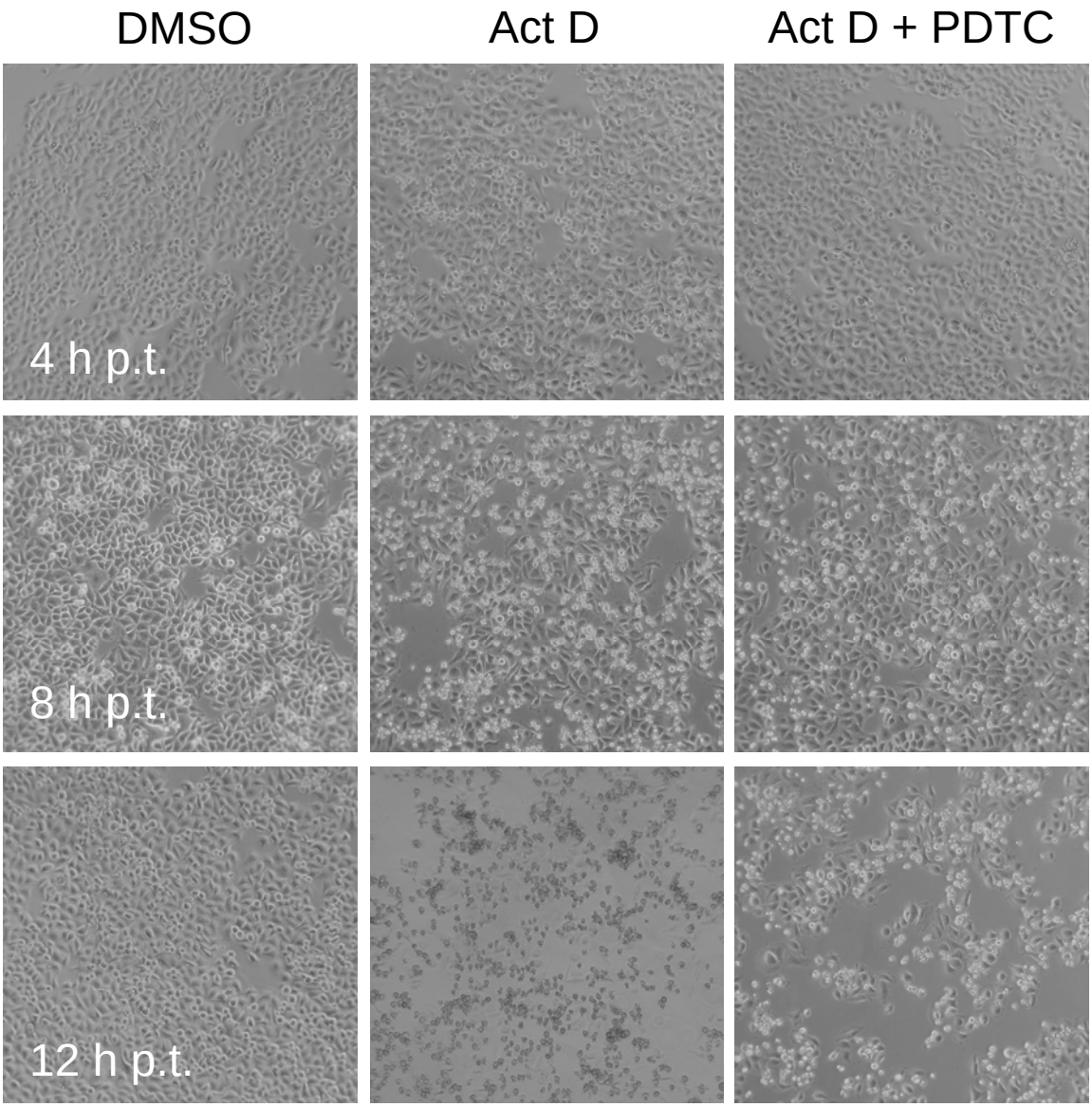


Fig. 13: HeLa cells were treated with 1 µM Actinomycin D +/- 125 µM PDTC for 4, 8 and 12 h respectively. More viable cells are observed when PDTC is present.

Fig. 14 shows HeLa cells treated with 50 µM Puromycin +/- PDTC for 4, 8 and 12 hours. The same situation as shown in Fig. 13 can be observed. When HeLa cells were treated with Puromycin alone, cell death appeared after 8 h. At this time point about 10 percent of Puromycin treated HeLa cells are morphologically intact. In contrast in the presence of PDTC, approximately 60 percent of the cells remain intact after 8 h.

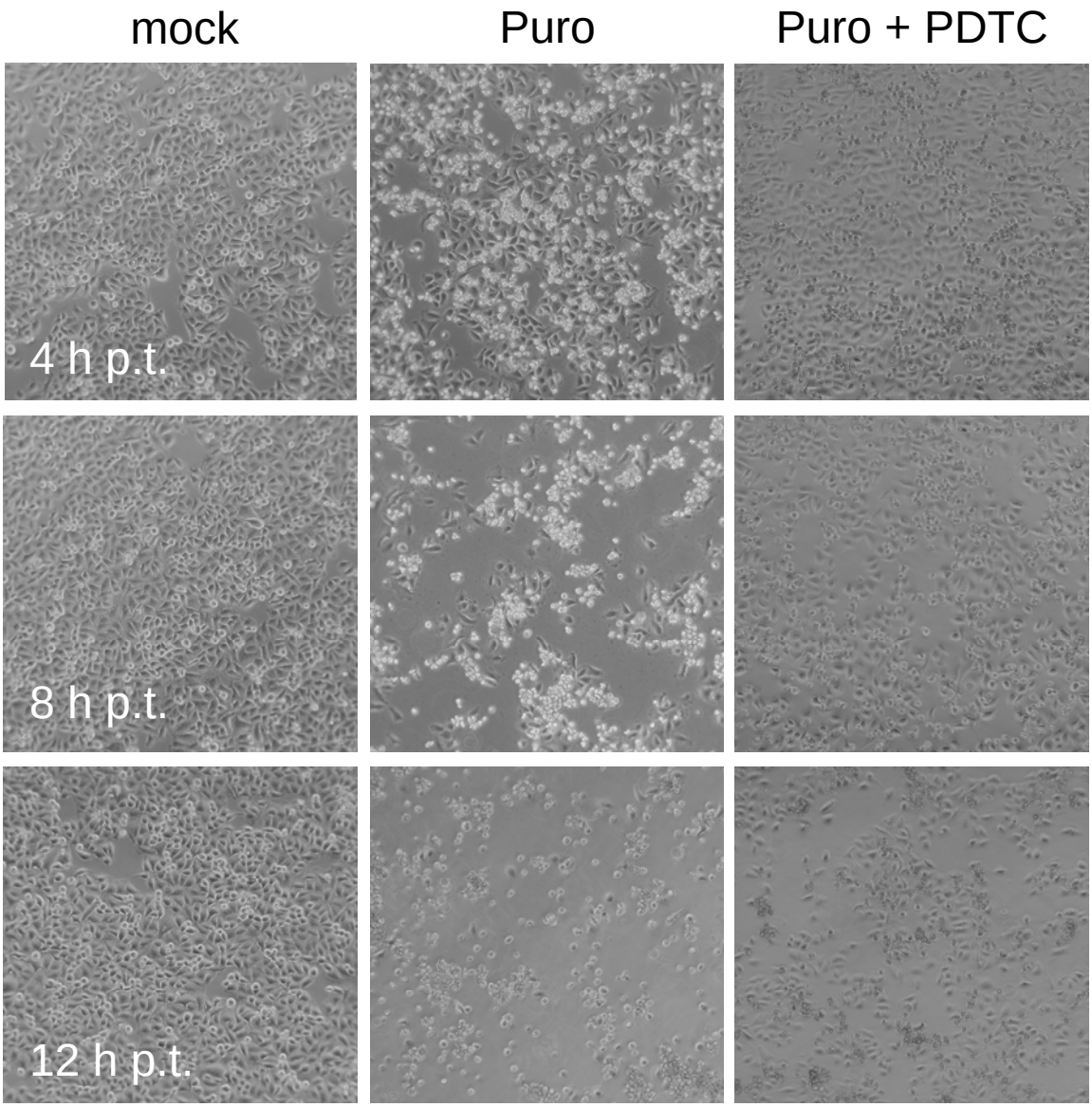


Fig. 14: HeLa cells were treated with 50 µM Puromycin with or without PDTC for indicated time points.

To quantify the observed protective effect of PDTC a crystal violet assay was done as described in material and methods. HeLa cells were treated with Actinomycin D or Puromycin +/- PDTC. After certain time points cells were stained with crystal violet. Finally the dye was solubilized and the absorbance measured. This assay is used to determine the amount of attached cells on the culture dish.

Fig. 15 shows such an experiment. If apoptosis was induced by Actinomycin D or Puromycin, the amount of attached cells decreases in a time dependent manner (cyan and blue bars). This reflects the amount of apoptotic cells which are washed away during the staining procedure. PDTC, DMSO and untreated cells (mock) serve as controls. No significant differences between the controls can be seen indicating

that neither PDTC nor DMSO alone induce cell death in HeLa cells under the conditions used. If PDTC was present during induction of apoptosis this decrease was slower. This finding reflects the protective effect of PDTC (cyan- and blue-striated bars). This experiment was done twice revealing similar results.

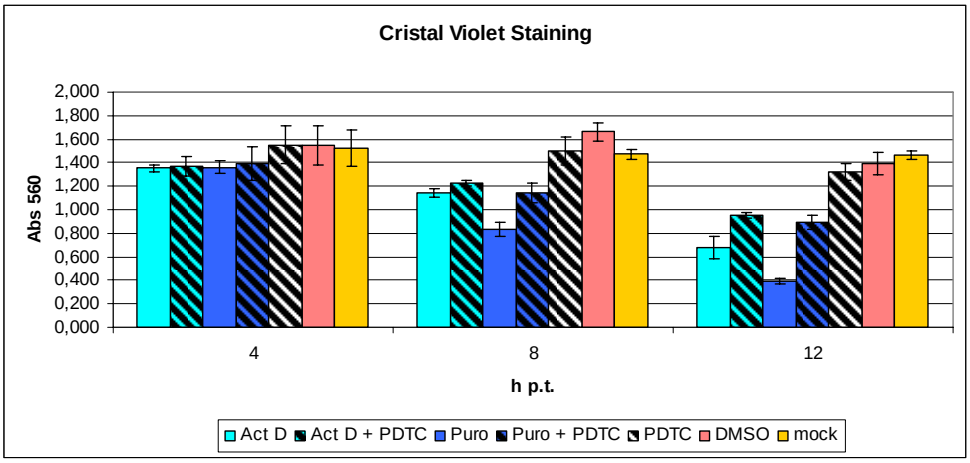


Fig. 15: Crystal violet staining of HeLa cells. Apoptosis was induced with 1 µM Actinomycin D or 50 µM Puromycin +/- 125 µM PDTC.

To measure the viability of HeLa cells after induction of apoptosis by Actinomycin D or Puromycin a proliferation assay was performed. This method was chosen because the activity of dehydrogenase enzymes in cells is a parameter for viability. Cells were treated with the inducer with or without PDTC for 4,8 and 12 h respectively.

The result of a proliferation assay is shown in
Fig. 16. Herein apoptosis was induced in HeLa cells with 1 µM Actinomycin D or 50 µM Puromycin for indicated time points. The experiments shown in Fig. 15 and Fig. 16 were done at the same time on parallel plates. Thus a similar decline in viability should be observed (cyan and blue bars). But this is not the case in Fig. 16 in Actinomycin D-induced apoptosis (cyan bars). Here the signal remains nearly on the same level. In Puromycin-induced apoptosis a decrease of the signal could be observed (blue bars). This might reflect different mechanisms of apoptosis induction. For control cells were treated with PDTC, DMSO or left untreated (mock). Here no decline of viability was seen.
A protective effect of PDTC could only be observed in Puromycin-induced apoptosis after 12 h. In this case the viability of Puromycin-PDTC co-treated cells was as high as those of untreated cells.

Further it is important to mention that PDTC reacts with the assay reagent. It was observed that blanks (= medium + treatment) containing PDTC change the colour from yellow to light brown during assay development (not shown). To eliminate this background, blanks were subtracted from each sample.

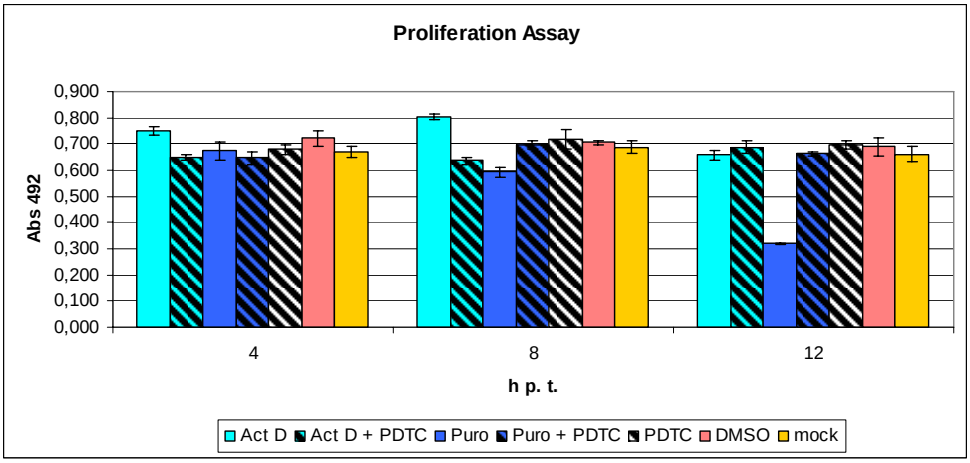


Fig. 16: Proliferation assay of HeLa cells treated as described in Fig. 15.

A hallmark of apoptosis is the activation of caspases. Thus caspase activity should increase during apoptosis induction whereas viability decreases. To confirm previous results the caspase-3/7 activity was measured by using Caspase-Glo(R) 3/7 Assay (Promega). This assay is based on the cleavage of an artificial substrate by caspase-3 and -7 subsequently providing a substrate for the luciferase. The measured luminescence is proportional to caspase activity. Caspase-3 and -7 are executioner caspases and activated at the end of the cascade leading to the characteristic pattern of apoptotic cells.

Caspase 3/7 activity in Actinomycin D- and Puromycin-induced cell death is shown in Fig. 17. HeLa cells were treated again with 1 µM Actinomycin D or 50 µM Puromycin +/- 125 µM PDTC for indicated time points. For control cells were treated with PDTC, DMSO or left untreated (mock). In these cases no significant differences in caspase-3/7 activity were observed. The activity of the executioner caspases-3 and -7 increases in a time dependent manner when apoptosis is induced with Actinomycin D or Puromycin (cyan and blue bars). PDTC co-treated cells show less caspase-3/7 activity (cyan- and blue-striated bars).

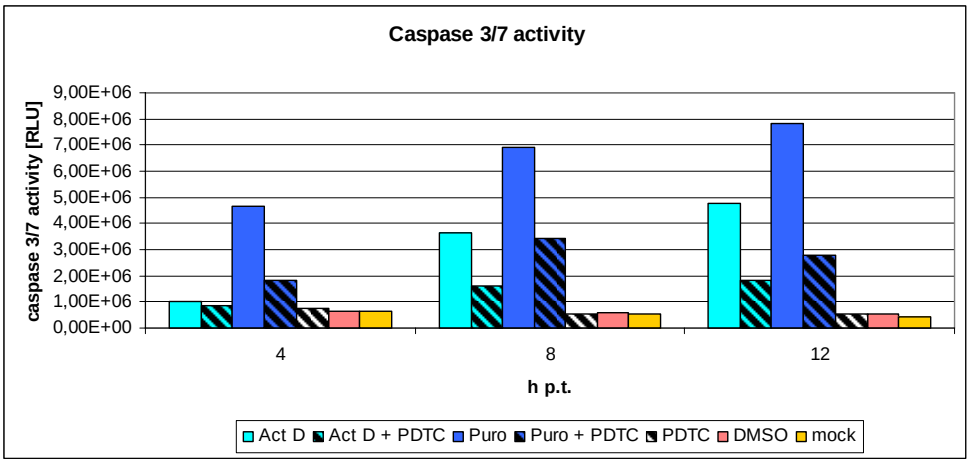


Fig. 17: Caspase 3/7 activity of Actinomycin D- and Puromycin-induced apoptosis in HeLa cells. Cells were treated as in Fig. 15.

The results shown in the previous figures demonstrate that PDTC has a clear protective effect against apoptosis in HeLa cells. This is shown by microscopic observations (Fig. 13 and Fig. 14), a crystal violet assay which measures the amount of attached cells on the culture dish (Fig. 15) as well as by the activation of caspase-3/7 (Fig. 17).

If PDTC is present during induction of apoptosis more cells are attached on the surface and seem to be morphological intact (Fig. 13; Fig. 14; Fig. 15). Further the activity of executioner caspases-3 and -7 are reduced in the presence of PDTC (Fig. 17). This effect is independent of the induction of apoptosis by Actinomycin D or Puromycin.

Interestingly the protective effect of PDTC could only be observed in a proliferation assay in Puromycin-induced apoptosis after 12 h (Fig. 16). This assay is based on the activity of dehydrogenase enzymes of living cells. As shown in Fig. 16 the viability did not decrease in a time dependent manner after induction of apoptosis. The second problem by using this assay is that PDTC seems to react with the assay reagent. To eliminate the background blanks (=medium+treatment) were subtracted.

IV.2 The anti-apoptotic effect of PDTC depends on the presence of metal ions

The presence of metal ions especially copper and zinc are described to be fundamental for PDTC to develop its function. For example it is described that EDTA is able to counteract PDTC thus abolishing its anti-viral activity against picornaviruses (Lanke, Krenn et al. 2007).

To investigate if EDTA is also able to eliminate the anti-apoptotic effect of PDTC a crystal violet staining was done. HeLa cells were seeded on 12 well dishes and apoptosis induced with 50 µM Puromycin or 1 µM Actinomycin D +/- PDTC. In the case of Actinomycin D-induced apoptosis DMSO was used as vehicle control. Different concentrations of EDTA were added to desired wells as indicated in Fig. 18. After 10 h the cells were washed once with PBS and stained with crystal violet. This time point was chosen to see significant differences between apoptotic cells and cells which are protected by PDTC.

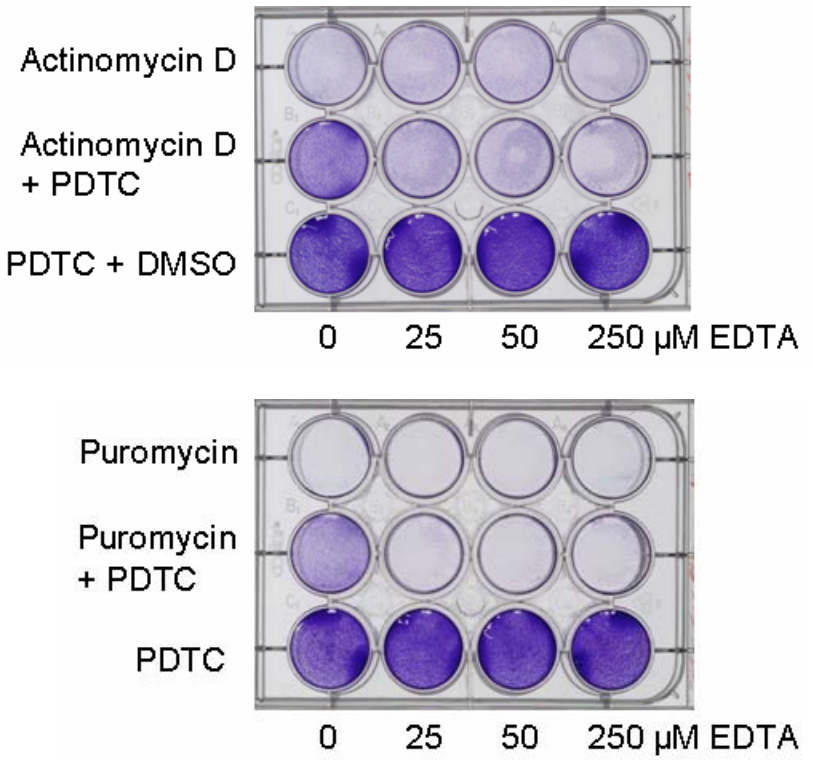


Fig. 18: Crystal violet staining of HeLa cells. Cells were treated with 1 µM Actinomycin D or 50 µM Puromycin +/- 125 µM PDTC as well as indicated concentrations of EDTA for 10 h.

Fig. 18 shows that EDTA can abolish the anti-apoptotic effect of PDTC in HeLa cells. In the upper part of Fig. 18 HeLa cells were treated with Actinomycin D +/- 125 µM PDTC and indicated concentrations of EDTA for 10 h. For control HeLa cells were treated with PDTC, DMSO as vehicle control as well as varied concentrations of EDTA. In the absence of EDTA the protective effect of PDTC is clearly visible. The presence of 25 µM EDTA abolished the majority of the anti-apoptotic effect. At higher concentrations of EDTA no protective effect of PDTC was observed anymore.
The same experiment with Puromycin as apoptotic inducer is shown in the lower part of Fig. 18. Here apoptosis was induced with 50 µM Puromycin +/- 125 µM PDTC. Desired concentrations of EDTA were added and cells were incubated for 10 h. PDTC/EDTA treated cells serve as negative control. Again the protective effect of PDTC in Puromycin-induced is clearly visible if EDTA is absent. Addition of 25 µM EDTA abolished this effect completely after 10 h.

The result shown in Fig. 18 clearly demonstrates that the presence of metal ions in the medium is a prerequisite for the anti-apoptotic effect of PDTC.

IV.3 Effects of PDTC on the intrinsic pathway of apoptosis

To analyse the pathway in more detail subcellular fractionation was done. This method was chosen to analyse the release of cytochrome c from mitochondria and to investigate other apoptotic marker as described below. Therefore HeLa cells were seeded in 15 cm dishes, treated with 1 µM Actinomycin D or 50 µM Puromycin +/- 125 µM PDTC as described in materials and methods. After different time points cells were disrupted and separated by centrifugation into three fractions ("mitochondria", "cytosol", "nucleus"). Hallmarks of apoptosis of each fraction were analysed by western blotting:

Cytochrome c release:

During apoptosis cyt c disappears from the mitochondria and appears in the cytosol. COX IV (cytochrome c oxidase IV) was used as mitochondrial loading control in western blot analysis. The purity of the cytosolic fraction i.e. the absence of mitochondria was also determined by COX IV as mitochondrial marker (not shown).

Cleaved caspase 9:

Cyt c which is released into the cytosol forms together with Apaf 1 and caspase 9 a complex called the Apoptosome. Subsequently caspase 9 is cleaved and activated. The appearance of a 37 kDa cleavage product of caspase-9 in the cytosol was analysed.

PARP cleavage:

PARP is located in the nucleus and cleaved as a consequence of caspase 3 activity. Subsequently an 87 kDa cleavage product of PARP can be detected.

To analyse small proteins like cyt c (15 kDa), COX IV (17 kDa) or cleaved caspase 9 (37 kDa) a tricine PAGE was used. To analyse PARP cleavage protein samples were subjected to a 7,5 % acrylamide SDS PAGE as described in material and methods. Apoptotic markers were analysed by western blotting.

Fig. 19 shows the result of the fractionation of Actinomycin D-induced HeLa cells co-treated +/- PDTC. The concentrations used are the same as described in Fig. 15. Cells were analysed after 6, 8 and 10 h. The amount of cytochrome c in mitochondria did not decrease in a significant way when apoptosis is induced with Actinomycin D. For control cells were treated with PDTC or DMSO as vehicle control. Neither PDTC nor DMSO leads to the release of cytochrome c. When PDTC is present during induction of apoptosis no big differences regarding the amount of cytochrome c in the mitochondria were observed.
On the other hand cytochrome c appears in the cytosolic fraction when it is released. After 6 h p.t. cytochrome c can be detected in the cytosol of Actinomycin D-induced HeLa cells (Fig. 19, “Cytosol”). The amount of released cytochrome c seems to be constant at later time points.
If HeLa cells were treated with Actinomycin D and PDTC, after 6 h no cytochrome c was detected in the cytosolic fraction. Two hours later (8 h p.t.) a slight band appeared when cells are co-treated with Actinomycin D + PDTC. After 10 h p.t. nearly the same amount of cytochrome c is released into the cytosol independent of the presence of PDTC during induction of apoptosis.

In the case of Actinomycin D treated cells, the release of cytochrome c correlates well with the appearance of the 37 kDa cleavage product of cleaved caspase 9. It can be detected after 6 and 8 h p.t. and disappears at 10 h. PDTC and DMSO treated cells which serve as negative control did not show caspase-9 processing. Interestingly no caspase-9 processing could be detected in Actinomycin D + PDTC treated cells, despite the release of cytochrome c (Fig. 19).

PARP cleavage was analysed to monitor the progress of apoptosis. PARP is cleaved as a consequence of caspase-3 activity late in the apoptotic program. PDTC or vehicle treated cells (DMSO) show no PARP cleavage. The cleavage product is observed after 6 h p.t. if cells are induced with Actinomycin D alone and increases in a time dependent manner. PDTC co-treatment reduces the the level of cleaved PARP after 8 and 10 h. This experiment was done twice revealing similar results.

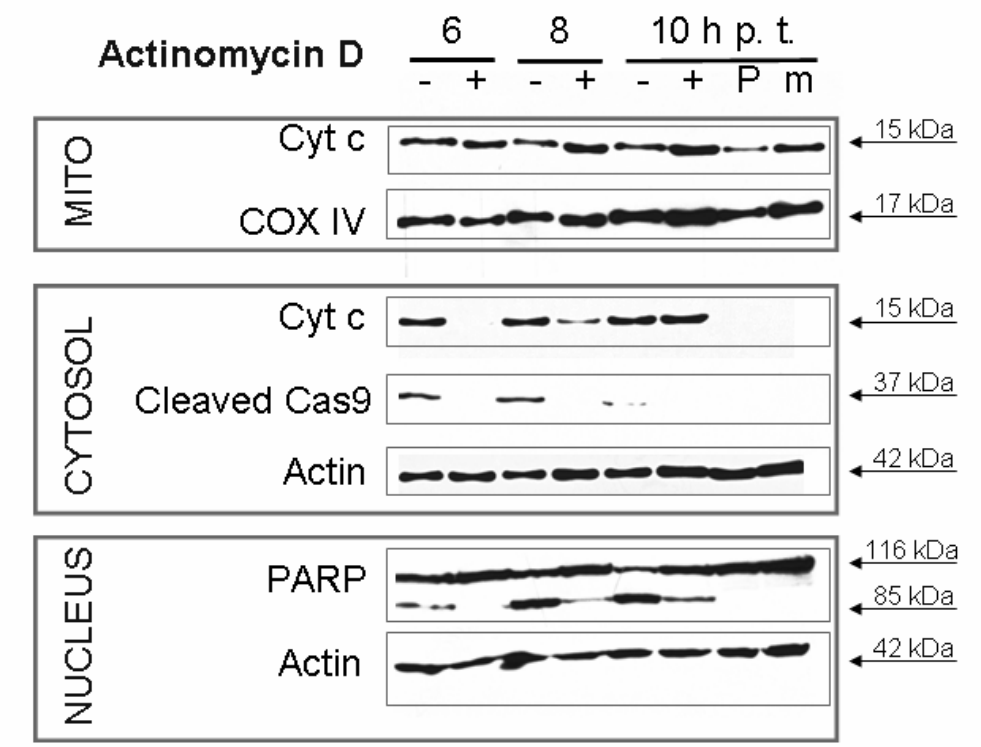


Fig. 19: Subcellular fractionation of Actinomycin D-induced HeLa cells. Treatment as described in Fig. 15. Abbreviations: +/- with/without PDTC, P PDTC only, m mock(DMSO treated)

The experiment shown in Fig. 19 demonstrates that PDTC delays cytochrome c release in Actinomycin D-induced apoptosis in HeLa cells. The absence of cleaved caspase 9 in PDTC co-treated cells but the appearance of cytochrome c in the cytosol could indicate a separate effect on the apoptosome-formation.

The same experiment as shown in Fig. 19 was done with Puromycin to confirm the previous result. Again, apoptosis was induced in HeLa cells with Puromycin in the absence or presence of PDTC (Fig. 20). After indicated time points, subcellular fractionation was done as described in materials and methods.

When apoptosis is induced the amount of cytochrome c in the mitochondrial fraction decreases in a time dependent manner and cannot be detected anymore at 7 h p.t (Fig. 20). In contrast, a decrease in the mitochondrial cytochrome c level was not observed in Actinomycin D-induced apoptosis (Fig. 19).

PDTC or untreated cells (mock) which serve as negative control show an equal level of cytochrome c in the mitochondrial fraction (Fig. 20). This means that PDTC did not trigger cytochrome c release in our model system.

In the presence of PDTC during Puromycin-induced apoptosis more cytochrome c is found in the mitochondria.

In the cytosolic fraction cytochrome c appears after 4-5 h p.t. Again PDTC and untreated cells show no cytochrome c release. If PDTC is present during Puromycin-induced apoptosis the release of cytochrome c is delayed and can be detected at 5 h p.t.. In general less cytochrome c is released when cells are co-treated with PDTC. These findings correlate well with the situation in the mitochondrial fraction.
Cleaved caspase-9 is detected in Puromycin-induced HeLa cells after 3 h. If cells are co-treated with Puromycin and PDTC the cleavage product appears at 4 h p.t..
Cleaved PARP is detected after 4 h in cells treated with Puromycin alone. In the presence of PDTC the cleavage product is absent at this time point. At further time points only a weak band of the cleavage product is obtained when cells are co-treated with PDTC.

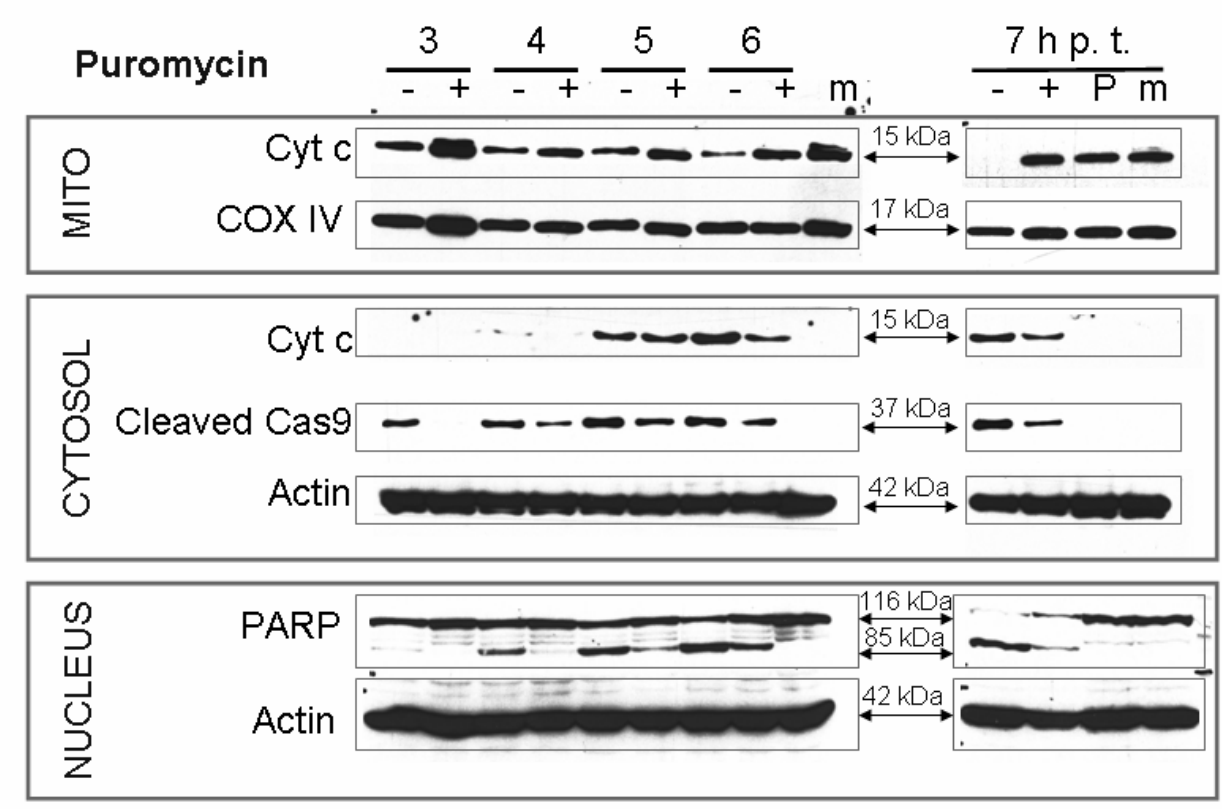


Fig. 20: Subcellular fractionation of Puromycin-induced HeLa cells +/- PDTC. Treatment as described in Fig. 15. Abbreviations: +/- with/without PDTC, P PDTC only, m mock

The western blots shown in Fig. 20 demonstrate that PDTC affects cytochrome c release in Puromycin-induced apoptosis in HeLa cells. Less cytochrome c is released when cells are co-treated with PDTC. Subsequently less caspase 9 and PARP is cleaved.

IV.4 Effects of PDTC on caspase-9 processing and caspase-3 activity in a cell-free system

In Fig. 19 it is shown that in Actinomycin D-induced apoptosis in HeLa cells the release of cytochrome c is delayed in the presence of PDTC. But no cleaved caspase 9 was detected. A similar situation is observed in Puromycin-induced apoptosis shown in Fig. 20.

To analyse if the effects of PDTC on caspase 9 processing and PARP cleavage are consequences of previous effects on cytochrome c release or separate ones, a cell-free system was used similar to those published (Liu, Kim et al. 1996). Apoptosis was induced in this system by addition of cytochrome c and (d)ATP. The principle of the assay is depicted in Fig. 21.

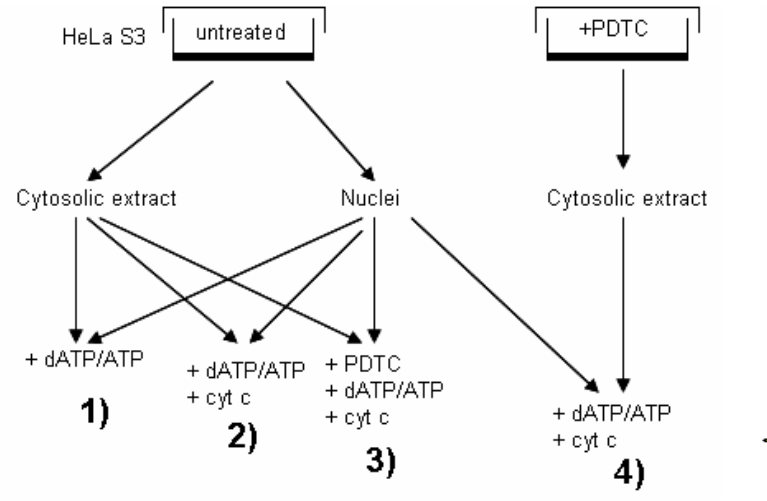


Fig. 21: Shematic diagram of the cell-free system. Cytosolic extract and nuclei were prepared from untreated HeLa S3 cells. Apoptosis was induced by addition of cytochrome c (cyt c) and (d)ATP in the absence 2) or presence of PDTC 3). In 1) apoptosis was not induced (control). A cytosolic extract of PDTC-treated HeLa S3 was prepared and apoptosis induced 4).

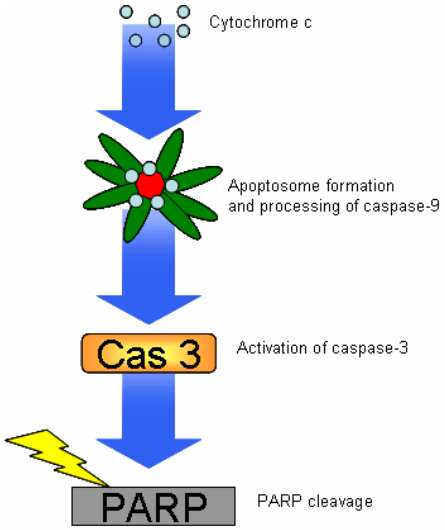


Fig. 22: Apoptotic events taking place in the presence of cytochrome c. Apaf-1, cytochrome c and (d)ATP form the apoptosome which leads to the recruitment of procaspase-9. Active (processed) caspase-9 activates caspase-3. Further different cellular substrates are cleaved (e.g. PARP).

Two 15 cm dishes of HeLa S3 were used. One dish was pre-treated with 125 µM PDTC for 1h. The reason to do so was the question if a metabolic processing of PDTC could influence apoptosis. The second dish was incubated with fresh growth medium for the same time. Cells were harvested and nuclei and cytosolic fractions prepared as described in materials and methods (Fig. 21).

Nuclei from untreated cells were mixed either with the cytosolic fraction of PDTC-treated cells (Fig. 21 4)) or with the cytosolic fraction of untreated cells (Fig. 21 1-3)). To one reaction containing the cytosol of untreated cells 125 µM PDTC was added (Fig. 21 3)).

After induction of apoptosis by addition of cytochrome c and dATP/ATP reactions were incubated at 37°C. The events which are taking place in the cell-free system are depicted in Fig. 22. The addition of cytochrome c and dATP/ATP leads to the formation of the apoptosome. Subsequently caspase-9 is processed leading to activation of caspase-3. Finally PARP is cleaved as a consequence of caspase-3 activity. At different time points an aliquot was taken and 4x sample buffer added to stop the reaction. Processing of caspase-9 and PARP cleavage was determined by western blotting.

Fig. 23 shows the result of such an experiment. The negative control (i.e. no apoptosis induced; Fig. 21 1)) is shown in lanes A1, A5, B1 and B5. In this reaction cytochrome c is missing thus no apoptotic event should occur at any time point. This control is necessary to ensure that no unspecific degradation of caspase-9 or PARP occurs. Only a very small amount of caspase-9 and PARP was cleaved without induction of apoptosis after 180 min (lane B5).

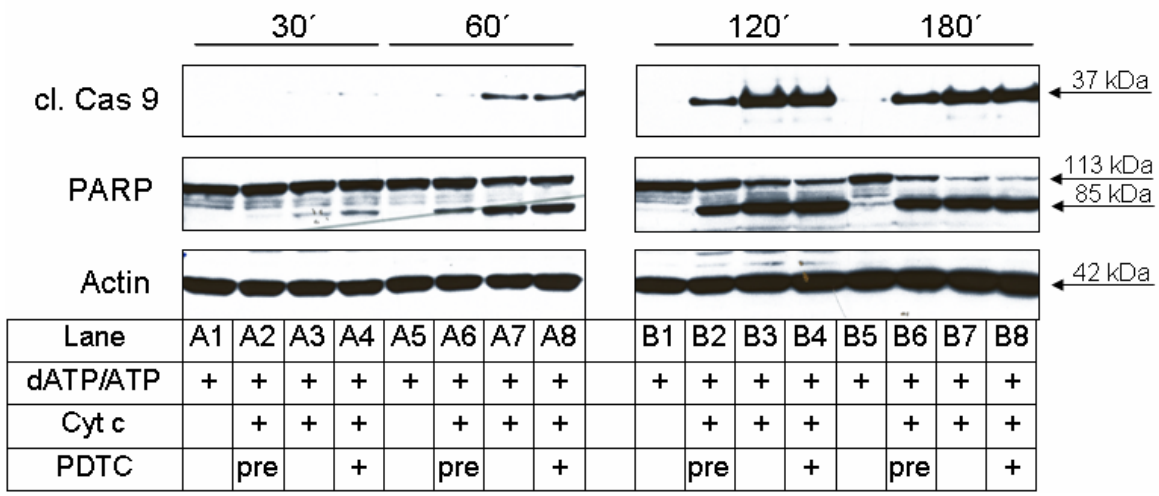
The positive control (i.e. induction of apoptosis) is shown in lanes A3, A7, B3 and B7. In this reaction all apoptotic events as depicted in Fig. 22 should take place. When apoptosis was induced by addition of cytochrome c and dATP/ATP, cleaved caspase-9 was detected after 60 min (lane A7). The level of cleaved caspase-9 increases in a time dependent manner (lane B3 and B7).

PARP cleavage correlates well with caspase 9 processing (lanes A3, A7, B3 and B7). The cleavage product of PARP appears after 30 min of incubation and increases in a time dependent manner. After 180 min of incubation approximately 90 percent of PARP are cleaved (lane B7).

If PDTC was added to the cell-free system (Fig. 23 lanes A4, A8, B4 and B8; Fig. 21 3)) no anti-apoptotic effect was observed at any time point. Caspase-9 processing and PARP cleavage occurred with same rate as in the positive control (lanes A3, A7, B3 and B7).

If the cytosolic fraction of PDTC-treated cells was used and apoptosis induced by addition of cytochrome c + dATP/ATP (Fig. 23 lanes A2, A6, B2 and B6; Fig. 21 4)),

an anti-apoptotic effect was observed. After 60 min nearly no caspase-9 was processed (lane A6) compared to the positive control in lane A7. This effect was continued at later time points. Caspase-9 processing was inhibited at 120 and 180 min after induction of apoptosis (lanes B2 and B6). These findings correlate well with the cleavage of PARP. Less PARP was cleaved at all time points when the cytosolic fraction of PDTC-treated cells was used (lanes A2, A6, B2 and B6) compared to the positive control (lanes A3, A7, B3 and B7).
Actin was used here as loading control. This experiment was done twice revealing similar results.



Lane	A1	A2	A3	A4	A5	A6	A7	A8		B1	B2	B3	B4	B5	B6	B7	B8
dATP/ATP	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+
Cyt c		+	+	+		+	+	+			+	+	+		+	+	+
PDTC		pre		+		pre		+			pre		+		pre		+

Fig. 23: Apoptosis in HeLa S3 cell free extracts. Apoptosis was induced by the addition of dATP/ATP and cytochrome c. Cell extracts of PDTC pre-incubated or untreated cells were used. PDTC was added as indicated. Caspase 9 processing and PARP cleavage were analysed at different time points.
Abbreviations: cl. Cas9: cleaved caspase 9; cp: cleavage product; pre: cytosolic extract of PDTC pre-incubated cells;

The result shown in Fig. 23 demonstrates that PDTC has no anti-apoptotic effect when apoptosis was induced in the cell-free system. In contrast, if apoptosis was induced using a cytosolic extract of PDTC-treated HeLa S3 cells, anti-apoptotic effects were observed. This means that an interaction between PDTC and living cells is the prerequisite revealing its anti-apoptotic activity.

IV.5 Effects of PDTC on caspase-3

Previously it was described that caspases could be inhibited by zinc ions (Truong-Tran, Carter et al. 2001). It was also demonstrated that PDTC increases intracellular zinc (Lanke, Krenn et al. 2007). This could be the reason for the anti-apoptotic effect of PDTC.

To check if PDTC acts on caspase 3 directly, the caspase 3-overexpressing cell line MCF-7-cas3+ was used. As control MCF-7 cells were used which lack a functional *caspase-3* gene. It was analysed if PDTC is able to inhibit apoptosis in these cell lines.

IV.5.1 Effects of PDTC on Puromycin-induced cell death in MCF-7 and MCF-7-cas3+

To induce apoptosis MCF-7 cells and caspase 3-overexpressing MCF-7-cas3+ cells were treated with 50 µM Puromycin +/- 125 µM PDTC. Puromycin was chosen because of its well known mode of action. Viability of both cell lines under these conditions was checked visually as well by proliferation- and crystal violet assay.

The pictures showed in Fig. 24 are typical microscopic observation of MCF-7 cells undergoing Puromycin-induced apoptosis. After 4 h of induction approximately 30 percent of cells showed typical morphological properties of apoptosis. The fraction of apoptotic cells increases dramatically in a time dependent manner. 8 h p.t. approximately 70 percent of Puromycin-treated cells are rounded up. After 12 h nearly all cells are dead and floating.

In contrast, if PDTC is present during Puromycin-induced apoptosis the cells are protected. About 20 percent of MCF-7cells are rounded up at any time point when co-treated with Puromycin and PDTC.

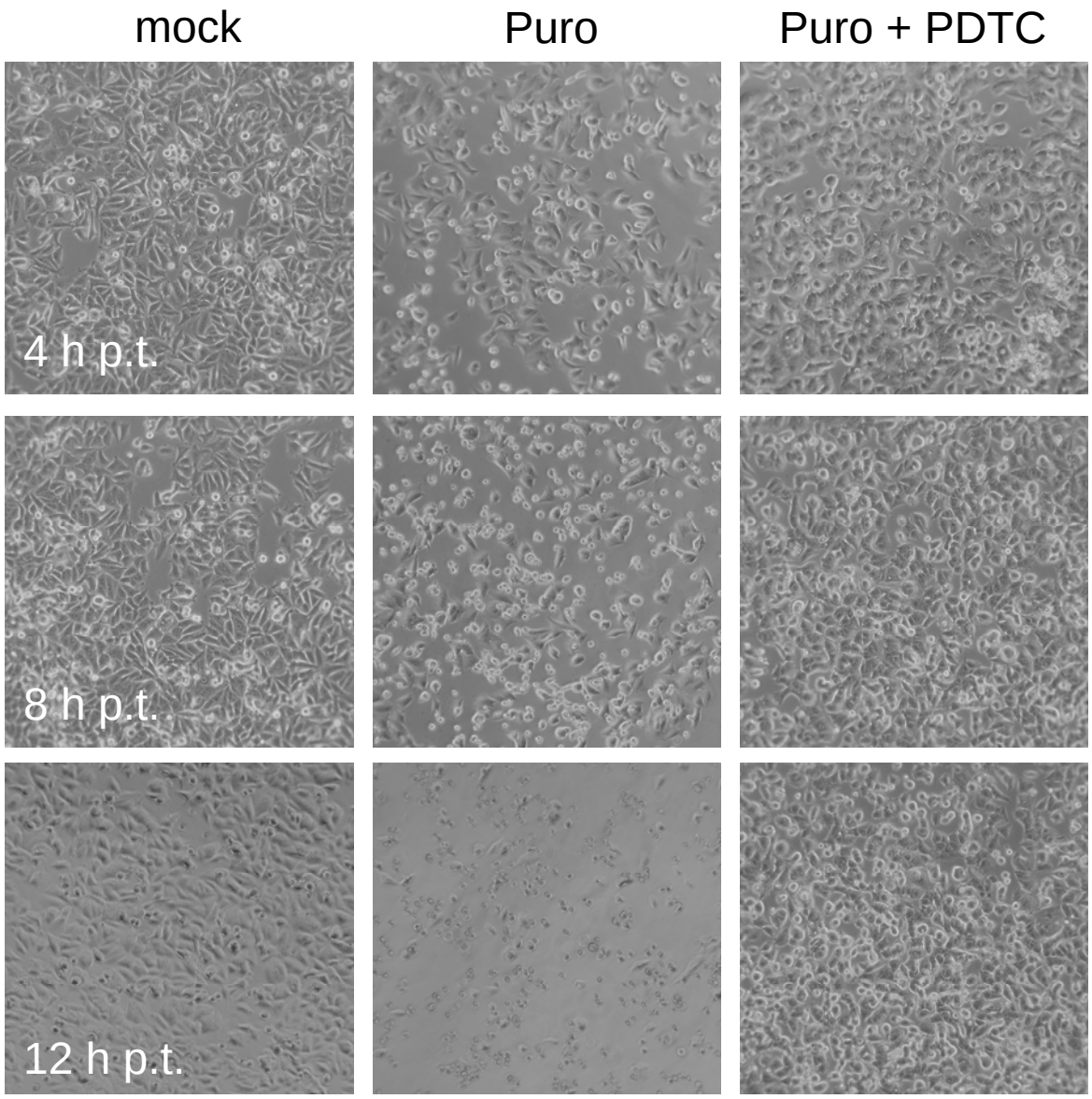


Fig. 24: MCF-7 cells treated with 50 µM Puromycin +/- 125 µM PDTC

In Fig. 25 MCF-7-cas3+ cells undergoing Puromycin-induced apoptosis are shown. After 4 h of induction of apoptosis about 30 percent of the cells are rounded up. This value increases to approximately 70 percent after 8 h. Finally after 12 h near all cells show an apoptotic morphology. In the presence of PDTC after 4 and 8 h about 10-20 percent of the cells are dead. Approximately 30 percent of round cells are found after 12 h when PDTC is present during Puromycin-induced apoptosis. This finding is similar to those obtained from caspase-3 deficient MCF-7 cells (Fig. 23).

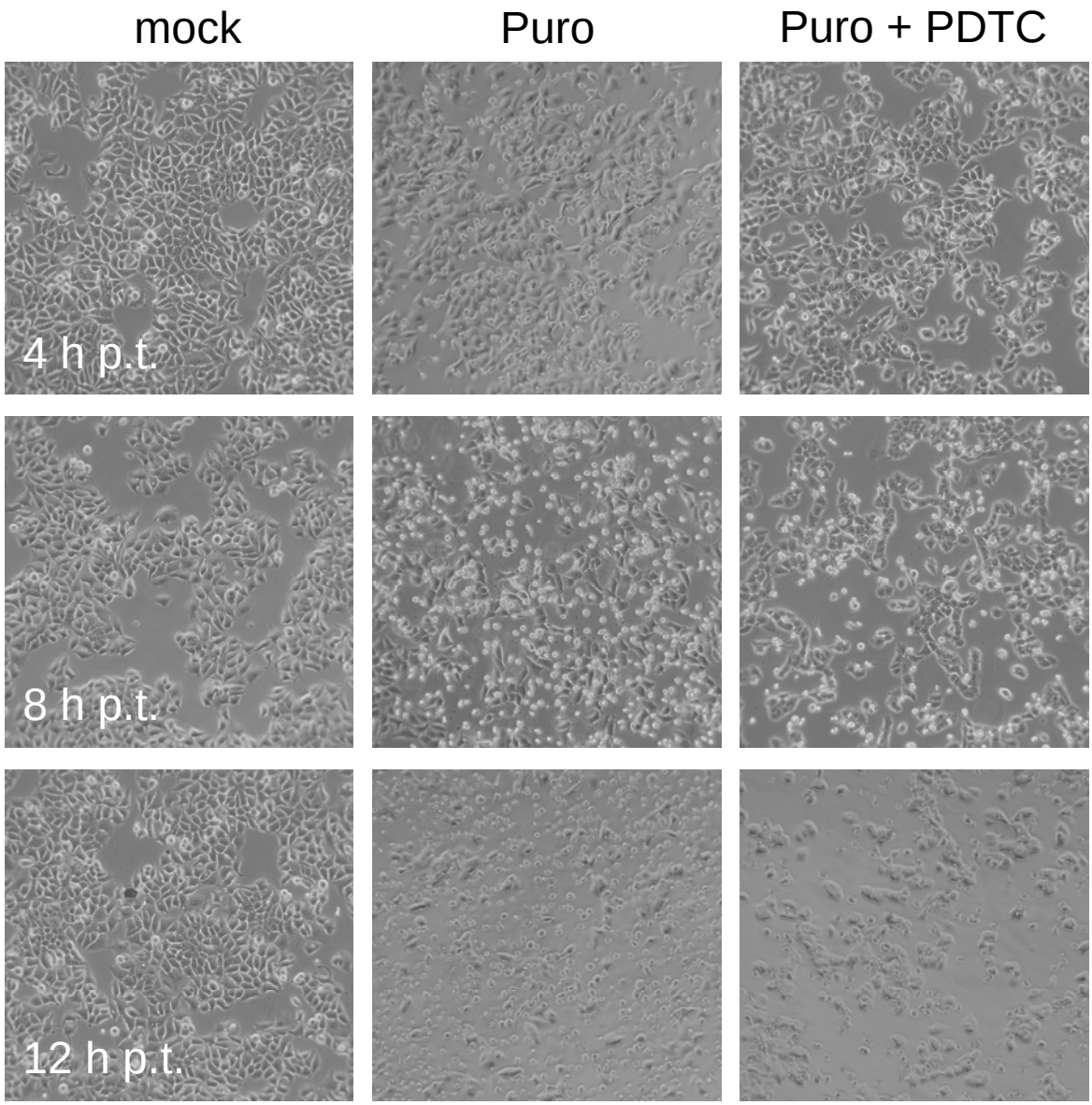


Fig. 25: MCF-7-cas3+ cells treated with 50 µM Puromycin +/- 125 µM PDTC

The pictures shown in Fig. 24 and Fig. 25 demonstrate that PDTC has anti-apoptotic properties in MCF-7 and MCF-7-cas3+ cells. In both cell lines PDTC prevents the development of characteristic morphological features of Puromycin-induced apoptosis.

To quantify the observed effect of PDTC in MCF-7 cells a proliferation- and crystal-violet assay was done. Fig. 26 shows the results of these assays. Both cell lines MCF-7 and caspase-3 overexpressing MCF-7-cas3+ were treated with 50 µm Puromycin +/- 125 µM PDTC for indicated time points. Proliferation assay and crystal violet staining were done on parallel plates.

In both cell lines Puromycin reduces viability slightly when measured by proliferation assay (blue bars). For control cells were left untreated (mock). When PDTC was present (striated bars) the signal increases strongly and reaches a value above of untreated cells. To ensure that this effect is not due to the interference between PDTC and the assay reagent, blanks (= medium + treatment) were subtracted.

The crystal violet assays shown in the lower panel in Fig. 26 correlate well with the observed morphological changes of both cell lines due to Puromycin-treatment (see Fig. 24 and Fig. 25 for comparison). The signal which reflects the amount of attached cells decreases in both cell lines in a time dependent manner. This represents apoptotic cells which are washed away during the staining procedure. Again cells were treated with PDTC alone or left untreated as control. PDTC alone did not cause significant reduction of the signal in MCF-7 cells (Fig. 26 lower left panel) and showed no effect in MCF-7-cas3+ cells (Fig. 26 lower right panel).

As depicted in Fig. 24 and Fig. 25, PDTC shows a protective effect on Puromycin-induced cell death in MCF-7 and MCF-7-cas3+ cells (Fig. 26 lower panel, blue-black striated bars).

This experiment was done twice and similar results were obtained.

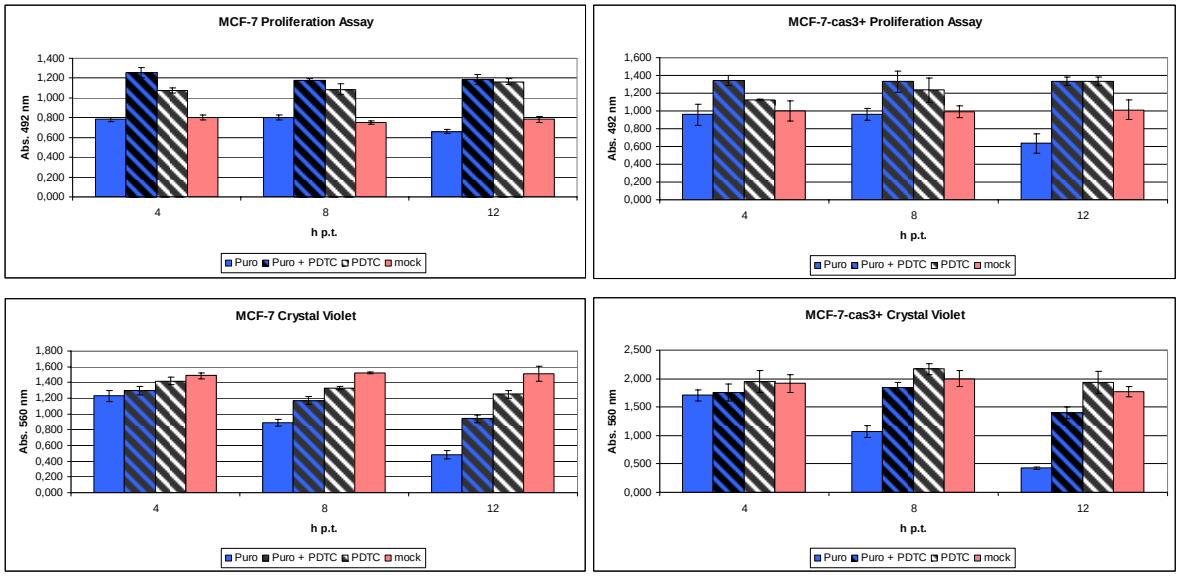


Fig. 26: Proliferation assay and crystal violet staining of MCF-7 and MCF-7-cas3+ cells. Cells were treated with 50 µM Puromycin +/- 125 µM PDTC. Experiment done on parallel plates.

The results shown in Figures 24-26 demonstrate a protective effect of PDTC on Puromycin-induced cell death in MCF-7 and MCF-7-cas3+ cells. This can be seen clearly in microscopic observations and crystal violet staining. In contrast, the proliferation assay which is based on the activity of dehydrogenase enzymes of living cells did not reveal a distinct result.

IV.5.2 Effects of PDTC on early- and late phase of apoptosis

One aim of this project was to investigate if PDTC acts on caspase-3 directly. Thus caspase-3 overexpressing MCF-7-cas3+ cells and caspase-3 deficient MCF-7 cells were used. PDTC exhibits a protective effect against Puromycin-induced apoptosis in both cell lines as shown in Fig. 24-26. This means that caspase-3 can not be the only target of PDTC.

However, it is described that caspase inhibitors can change the kind of cell death (Vandenabeele, Vanden Berghe et al. 2006). Thus a rhodamine/propidium iodide staining was done which allows the discrimination between an early phase of apoptosis and a late phase of apoptosis / necrosis.

Rhodamine (R123) is a lipophilic cationic fluorochrome which accumulates in the mitochondria of living cells. In early apoptotic cells this accumulation does not occur due to loss of mitochondrial transmembrane potential (Fig. 27, gate R1). Late apoptotic/necrotic cells stain only with propidium iodide (PI) due to the loss of plasma membrane integrity (Fig. 27, gate R2).

To analyse if PDTC acts on caspase-3 directly, apoptosis was induced in MCF-7 and MCF-7-cas3+ cells with 50 µM Puromycin +/- 125 µM PDTC for 8 h. This time point was chosen due to low differences between Puromycin and Puromycin+PDTC treated cells at previous time points. After treatment MCF-7 and MCF-7-cas3+ cells were harvested by trypsinization and subjected to R123/PI staining. For control cells were also treated with PDTC alone or left untreated. Cell fluorescence was analyzed by flow cytometry after 8 h p.t. as described in materials and methods. The results of such an experiment are shown in Fig. 27 and Fig. 28.

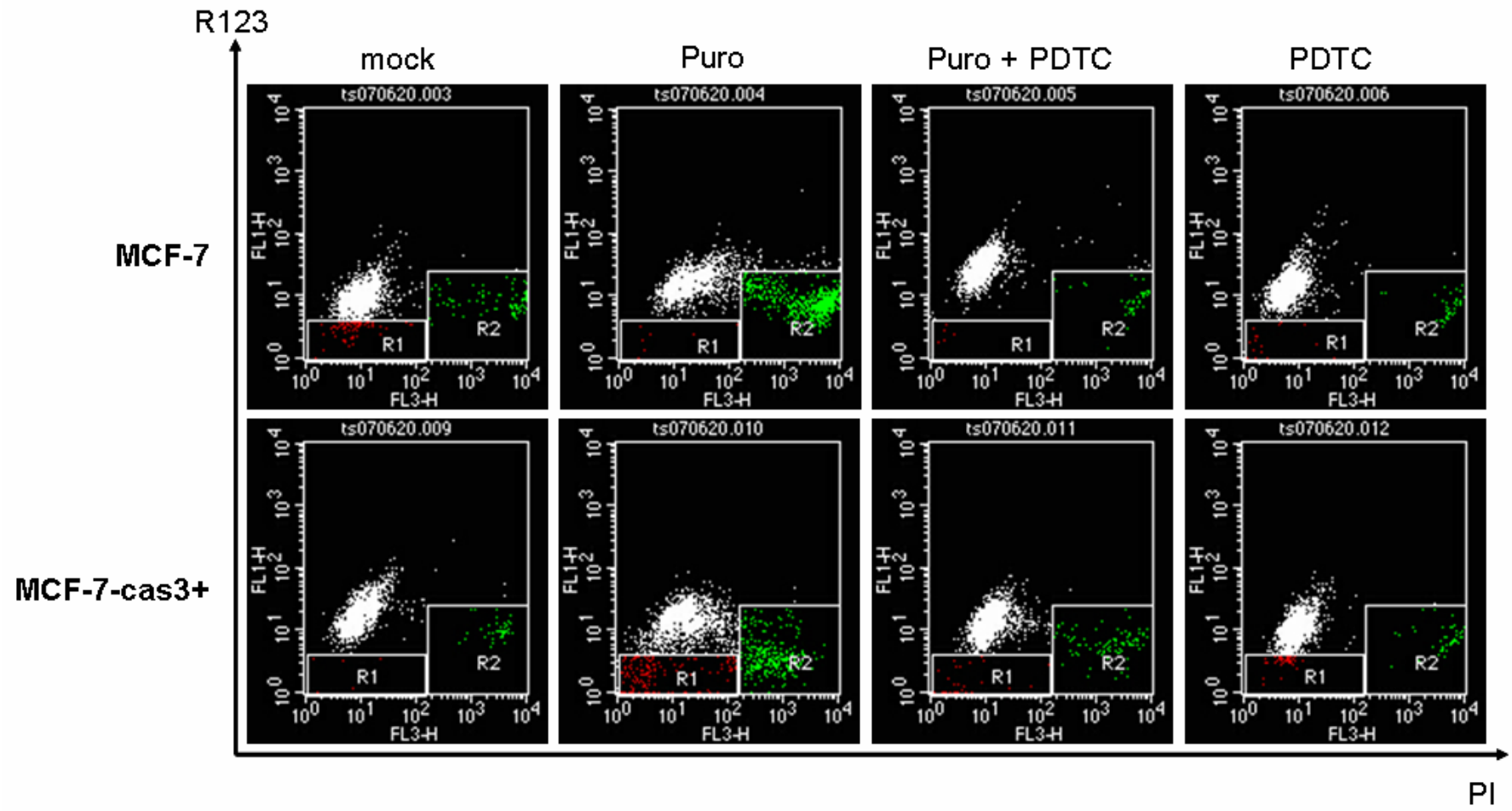


Fig. 27: Rhodamin (R123) / Prpoidium iodide (PI) staining of MCF-7 and MCF-7-cas3+ cells. Cell fluorescence was determined by flow cytometry allowing discrimination of early- (R1) and late-phase (R2) of apoptosis. Apoptosis was induced in these cell lines with 50 µM Puromycin +/- 125 µM PDTC for 8 h.

When cell death is induced with Puromycin, both cell lines show a different pattern of apoptosis. Eight hours after induction of apoptosis approximately 4 % of MCF-7 cells are found in the early phase and 30 % are found in the late phase of apoptosis (Fig. 28). In contrast, in caspase 3-overexpressing MCF-7-cas3+ cells approximately 7 % are found in the early phase of apoptosis and approximately 13 % in the late phase (Fig. 28).

But when both cell lines were treated with Puromycin + PDTC the pattern looks nearly the same after 8 h (Fig. 27). If the anti-apoptotic effect of PDTC depends mainly on the inhibition of caspase-3, the distribution of caspase-3 deficient MCF-7 cells between early- and late phase of apoptosis should be unaffected when treated with Puromycin + PDTC. Further, if caspase-3 is the main target of PDTC, the distribution of MCF-7-cas3+ cells between early- and late phase of apoptosis should look similar to the pattern of MCF-7 cells, when PDTC is present during induction of apoptosis.

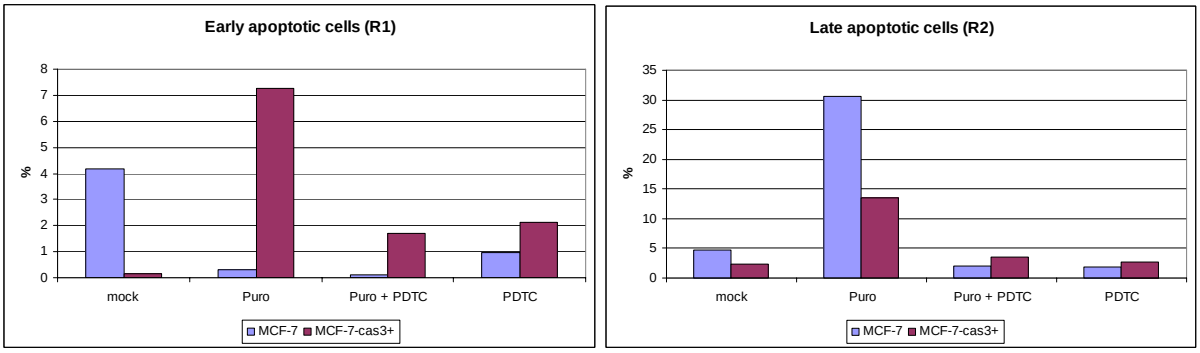


Fig. 28: Quantification of R123/PI staining. Percentage of cells found in gate R1 (early phase) or gate R2 (late phase) in Fig. 27.

The results shown in Fig. 27 and Fig. 28 demonstrate that PDTC does not act mainly on caspase-3. Thus caspase-3 is not the major target of PDTC.

IV.6 Effects of PDTC on the PI3K/Akt pathway

In the literature it is described that PDTC can increase the intracellular concentration of zinc (Lanke, Krenn et al. 2007). Further it is described that the PI3K/Akt pathway can be activated by Zn^{2+}, Cu^{2+} and Cd^{2+} ions. In this case the phosphorylation of Akt occurred in a PI3K-dependent manner. This was concluded by the fact that the phosphorylation of Akt was abolished by the presence of wortmannin, a PI3K inhibitor (Barthel, Ostrakhovitch et al. 2007). Not surprisingly there are two reports concerning the phosphorylation of Akt due to PDTC treatment. In one study it is shown that PDTC leads to phosphorylation of Akt only when apoptosis is induced (Cheng, Huang et al. 2006). In contrast PDTC alone was not able to activate Akt (Cheng, Huang et al. 2006).

However, the idea emerged that the activation of the PI3K/Akt pathway could be the major reason for the anti-apoptotic effect of PDTC. First of all it was investigated if PDTC was able to induce the phosphorylation of Akt and if this event is based on the activity of PI3K.

Therefore HeLa cells were pre-incubated with 1 µM wortmannin, a PI3K inhibitor, or an appropriate volume of DMSO as vehicle control for one hour. Afterwards cells were treated with 125 µM PDTC in the presence or absence of 1 µM wortmannin for different time points. DMSO was added to all samples which did not contain wortmannin. Activation of Akt was determined by western blotting using a specific antibody against pAkt (Ser473). The membrane was stripped afterwards and probed with an antibody against total Akt as loading control.

The result of such an experiment is shown in Fig. 29. In lanes 1,4 and 7 HeLa cells were incubated with growth medium and DMSO which serves as solvent control. Only after 60 min a weak phosphorylation of Akt could be detected under these conditions (lane 7). If PDTC (and DMSO) was present a phosphorylation could be detected after 20, 40 and 60 min of incubation (lanes 2,5,8). If PI3K was inhibited by wortmannin no Akt phosphorylation could be observed in the presence of PDTC (lanes 3,6,9). This experiment was done three times obtaining similar results.

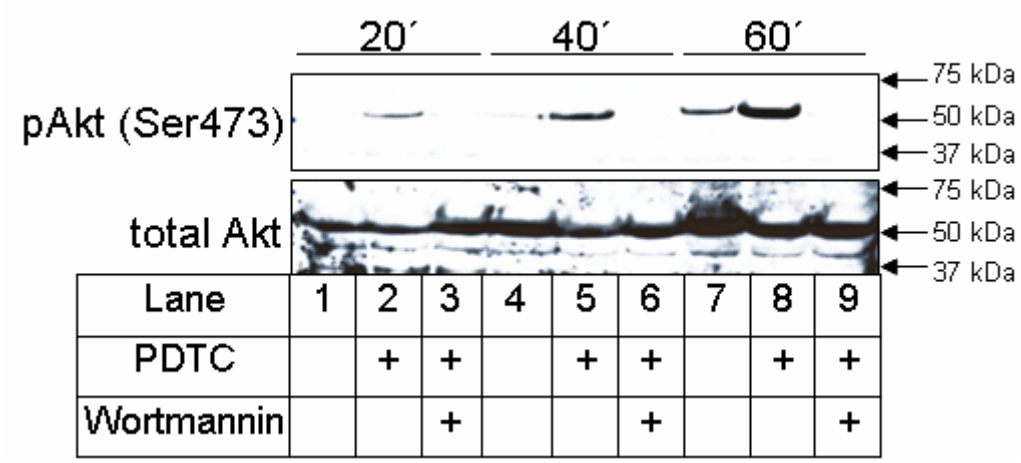


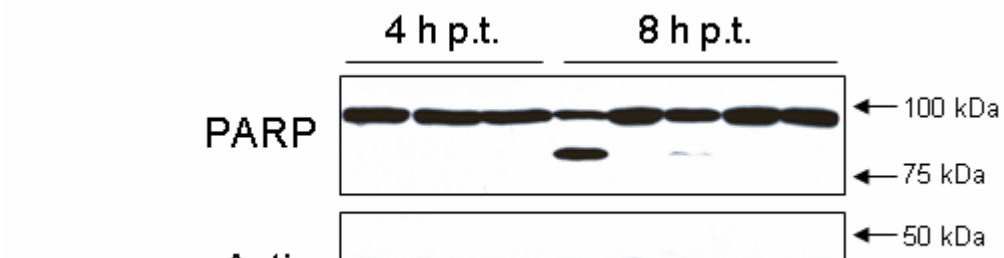
Fig. 29: PDTC-induced phosphorylation of Akt (Ser473)

The result in Fig. 29 demonstrates that PDTC is able to induce the phosphorylation of Akt. Further it is shown that PI3K activity is a prerequisite for PDTC-induced Akt phosphorylation.

The Akt pathway provides survival signals for the cell and is able to inhibit apoptosis. These signalling events can be prevented by the inhibition of PI3K by wortmannin. If the activation of Akt by PDTC is the reason for its anti-apoptotic activity, wortmannin should abolish the anti-apoptotic effect of PDTC.

To answer this question HeLa cells were pre-incubated with 1 µM wortmannin or an appropriate volume of DMSO as vehicle control for one hour. Apoptosis was induced with 1 µM Actinomycin D or 50 µM Puromycin. Cells were incubated with the inducer in presence or absence of PDTC +/- 1 µM wortmannin. DMSO as vehicle control was added to all samples which did not contain wortmannin. After 4 and 8 h p.t. protein extracts were prepared. PARP cleavage was determined by western blotting to determine apoptosis. Actin was detected on the same blot as loading control. The result of such an experiment is shown in Fig. 30 and Fig. 31.
In Fig. 30 apoptosis was induced with Actinomycin D. After 4 h p.t. no effect could be observed (Fig. 30, lanes 1-3). After 8 h p.t. PARP cleavage was detected when apoptosis was induced (lane 4). If cells were co-treated with PDTC no PARP cleavage was seen (lane 5). As control cells were treated with wortmannin alone (lane 7) or only with DMSO (lane 8). No PARP cleavage was detected in these cases.

When apoptosis was induced with Actinomycin D in the presence of PDTC and wortmannin, only a small amount of cleaved PARP was observed (lane 6). This means that the activation of the PI3K/Akt pathway seems not to be the major reason for the anti-apoptotic activity of PDTC.



Lane	1	2	3	4	5	6	7	8
Actinomycin D	+	+	+	+	+	+		
PDTC		+	+		+	+		
Wortmannin			+			+	+	

Fig. 30: Wortmannin vs. PDTC in Actinomycin D-induced apoptosis in HeLa cells.

In Puromycin-induced apoptosis a similar situation was observed as shown in Fig. 31. Again HeLa cells were treated with the inducer in presence or absence of PDTC +/- wortmannin. After 4 h no significant effect was observed. After eight hours PARP was cleaved nearly completely when apoptosis was induced by Puromycin (Fig. 31, lane 4). In the presence of PDTC approximately fifty percent of PARP were cleaved (lane 5). If the cells were co-treated with Puromycin, PDTC and wortmannin, PARP cleavage was about fifty percent after 8 h (Fig. 31, lane 6). For control cells were treated with wortmannin alone or with DMSO as vehicle control. Herein no PARP cleavage could be detected (lanes 7 and 8).

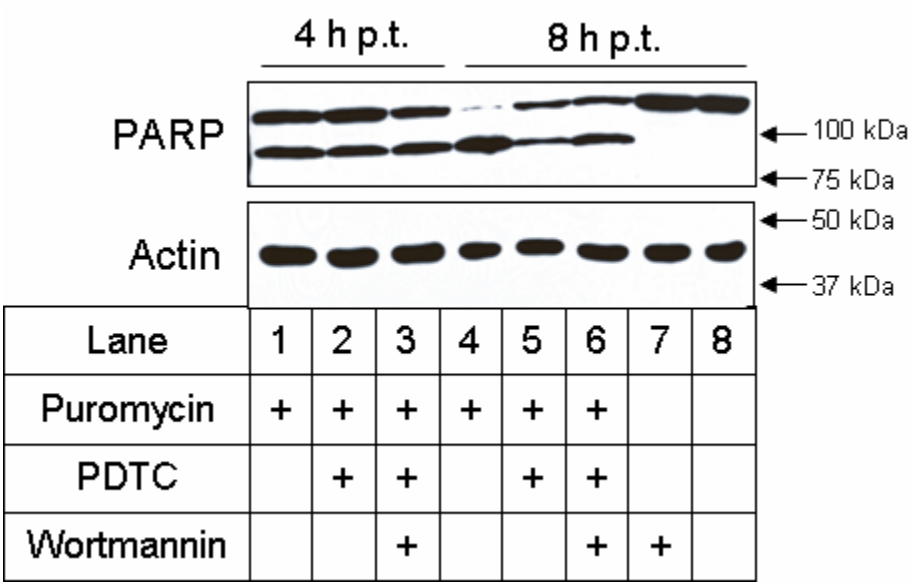


Fig. 31: Wortmannin vs. PDTC in Puromycin-induced apoptosis in HeLa cells.

The results shown in Fig. 30 and Fig. 31 demonstrate that the PI3K inhibitor wortmannin can not counteract PDTC in regard to Actinomycin D and Puromycin-induced apoptosis in HeLa cells. This means that PDTC-induced activation of the PI3K/Akt pathway (Fig. 29) is not the main reason for the anti-apoptotic activity of PDTC.

V Discussion

The effects of PDTC (Pyrrolidine dithiocarbamate) described in the literature range from pro-apoptotic, anti-apoptotic, inhibition of picornaviruses, inhibition of the proteasome as well as inhibition of NFκB (Kim, Kim et al. 1999; Della Ragione, Cucciolla et al. 2000; Erl, Weber et al. 2000; Kim, Kim et al. 2004; Cheng, Huang et al. 2006; Lanke, Krenn et al. 2007). In most cases the activity of PDTC described depends on the presence of metal ions especially zinc and copper.
The aim of this project was to investigate the mode of action of PDTC regarding apoptosis. Due to the fact that PDTC is able to increase the intracellular concentration of zinc (Lanke, Krenn et al. 2007), and that zinc ions may affect apoptosis (Truong-Tran, Carter et al. 2001), two scenarios were assumed:

First the increase of intracellular zinc could inhibit caspase-3 activity by interfering with the active center as described (Truong-Tran, Carter et al. 2001).

Secondly the increase of intracellular zinc could trigger a survival signal thus inhibiting apoptosis. One possibility is the activation of the PI3K/Akt pathway. It was described that zinc ions are able to activate this pathway and there are also two papers concerning the phosphorylation of Akt due to PDTC treatment (Cheng, Huang et al. 2006; Barthel, Ostrakhovitch et al. 2007; Malm, Iivonen et al. 2007).

In this project it is shown that PDTC exhibit an anti-apoptotic effect in Actinomycin D- and Puromycin-induced apoptosis in HeLa cells. Further an inhibitory effect was also observed in Puromycin-induced apoptosis in caspase-3 deficient MCF-7 and caspase-3 overexpressing MCF-7-cas3+ cells.
It is shown that the anti-apoptotic effect of PDTC depends on metal ions. This is because EDTA, which chelates divalent cations, can counteract PDTC.
The release of cytochrome c, a hallmark of apoptosis is delayed in the presence of PDTC in Actinomycin D- as well as in Puromycin-induced apoptosis. Processing of caspase-9 was not observed in Actinomycin D-induced apoptosis in HeLa cells even if cytochrome c was released.
In a cell-free system the effects of PDTC on caspase-9 and caspase-3 were investigated. PDTC exhibit no anti-apoptotic effect in this system when apoptosis was

induced by the addition of cytochrome c and (d)ATP. In contrast, if apoptosis was induced using cytosolic extracts of PDTC-treated cells, a weak inhibitory effect of PDTC was observed.

PDTC was also able to inhibit Puromycin-induced apoptosis in caspase-3 deficient MCF-7 as well as in caspase-3 overexpressing MCF-7-cas3+ cells.

Further it is demonstrated that in our system PDTC leads to rapid activation of the PI3K/Akt pathway, which can influence apoptosis. Surprisingly inhibition of this pathway did not abolish the anti-apoptotic effect of PDTC.

V.1 The anti-apoptotic effect of PDTC

It is reported in the literature that apoptosis can be inhibited as well as induced by PDTC (Nobel, Burgess et al. 1997; Della Ragione, Cucciolla et al. 2000; Erl, Weber et al. 2000; Cheng, Huang et al. 2006). The latter effect of PDTC depends on cell type, cell density and on the presence of zinc and copper (Erl, Weber et al. 2000).

In this study PDTC exhibit a protective effect against Actinomycin D- and Puromycin-induced apoptosis in HeLa cells. Such an effect was also observed in caspase-3 deficient MCF-7 and caspase-3 overexpressing MCF-7-cas3+ cells when apoptosis was induced with Puromycin. These findings would suggest a common mechanism of PDTC. Regarding effects of PDTC on apoptosis described in the literature and compared with the results of this project, the question raises why PDTC can act as apoptotic inducer as well as inhibitor. In other words: which criteria must be fullfilled to enable PDTC to act as an inhibitor of apoptosis? Answering this question seems to be important to reveal the mode of action of PDTC.

In the literature it is described that zinc or copper ions are a prerequisite for PDTC to exhibit its biological activity (Kim, Kim et al. 1999; Erl, Weber et al. 2000; Kim, Kim et al. 2004; Lanke, Krenn et al. 2007).

To check if the presence of metal ions is necessary for the anti-apoptotic effect of PDTC, EDTA should be able to counteract PDTC. In this study EDTA was able to abolish the anti-apoptotic effect in Actinomycin D- as well as in Puromycin-induced apoptosis in HeLa cells. This demonstrates that the presence of certain metal ions in the medium is necessary for PDTC to develop its function. It is likely that these ions are Zn^{2+} and Cu^{2+}, because it is reported in several publications that zinc and copper ions are associated with the effect of PDTC (Kim, Kim et al. 1999; Erl, Weber et al.

2000; Kim, Kim et al. 2004; Krenn, Holzer et al. 2005). In the medium itself there are of course inorganic salts but no zinc or copper (Invitrogen). Thus the serum which is added to the medium seems to be the source of such ions. The concentration of zinc or copper ions in the serum used can not be indicated because this is not mentioned in the certificate of analysis or in any other product description. The concentration of zinc in calf serum is about 76 µg/dl (Nisbet, Yarim et al. 2006).
However, EDTA is a metal chelating compound which forms complexes mainly with divalent cations. The resulting question is if zinc or copper ions are necessary for the anti-apoptotic effect of PDTC because EDTA does not only form complexes with these two ions.

V.2 Effects of PDTC on cytochrome c release

The release of cytochrome c from the mitochondria into the cytosol is a hallmark of apoptosis. In the literature it is mentioned that PDTC cause the release of cytochrome c from mitochondria in HL-60 cells (Della Ragione, Cucciolla et al. 2000; Cheng, Huang et al. 2006). Interestingly Ragione et al. describes PDTC as apoptotic inducer whereas Cheng et al. demonstrate an anti-apoptotic effect of PDTC in Luteolin-induced apoptosis, despite the release of cytochrome c due to PDTC-treatment (Della Ragione, Cucciolla et al. 2000; Cheng, Huang et al. 2006). In Ragione et al. it is shown that incubation of purified mitochondria with PDTC alone lead to the release of cytochrome c. Thus it was concluded that the mitochondria are the primary targets of PDTC (Della Ragione, Cucciolla et al. 2000).
Here in this project it is shown that PDTC delayed the release of cytochrome c in Actinomycin D- and Puromycin-induced apoptosis in HeLa cells. PDTC alone was unable to cause the release of cytochrome c in HeLa cells. This indicates a pre- and/or mitochondrial effect of PDTC.

V.3 Effects of PDTC on caspases

It is described in the literature that PDTC increases intracellular zinc levels (Krenn, Holzer et al. 2005; Lanke, Krenn et al. 2007). Truong-Tran et al. describe the effects of zinc on the activation of caspases especially on caspase-3 (Truong-Tran, Carter et

al. 2001). Thus it was assumed that the import of zinc by PDTC leads to the inhibition of caspases and therby contributing to the anti-apoptotic effect observed.
In this project it is shown that the release of cytochrome c is delayed in the presence of PDTC in Actinomycin D- and Puromycin-induced apoptosis in HeLa cells. Further, in Actinomycin D-induced apoptosis caspase-9 is not processed (i.e. cleaved) even if cytochrome c is released (Fig. 19). This might be a consequence of delayed cytochrome c release or indicate an effect on caspase-9 processing.

To investigate the effects of PDTC on caspase-9 processing in more detail, a cell-free system was performed similar to those published (Liu, Kim et al. 1996). The advantage of such a system is that pre- and mitochondrial events of apoptosis are removed. This is because apoptosis is induced in the cell-free system by the addition of cytochrome c and (d)ATP. The cleavage of PARP was also analysed in this system and reflects the activity of caspase-3. In the presence of PDTC no anti-apoptotic effect was observed regarding caspase-9 and PARP cleavage at any time point (Fig. 23). This finding suggests that neither caspase-9 nor caspase-3 is affected by PDTC directly.
In Truong-Tran et al. it is mentioned that in cell-free systems PARP cleavage was inhibited in the presence of zinc (Truong-Tran, Carter et al. 2001). Thus the same experiment was done using a cytosolic extract of PDTC treated HeLa S3 cells. In this case a weak anti-apoptotic effect of PDTC on the processing of caspase-9 was observed. This result obtained from the cell-free system is similar to those determined in Actinomycin D-induced apoptosis in HeLa cells. This might be due to import of ions into the cells or due to metabolic processing of PDTC.
The effects of PDTC on caspase-3 were also analysed by using the caspase-3 deficient cell line MCF-7 as well as caspase-3 overexpressing cells (MCF-7-cas3+). It is described that apoptosis can occur in MCF-7 cells by sequential activation of caspase-9, -7 and -6 (Simstein, Burow et al. 2003). As seen in Fig. 24, Fig. 25, Fig. 26 and Fig. 27 an anti-apoptotic effect of PDTC in Puromycin-induced apoptosis was observed in MCF-7 and MCF-7-cas3+ cells. These findings exclude caspase-3 as main target of PDTC.

V.4 PDTC-induced Akt phosphorylation has no effect on apoptosis in HeLa cells

In the literature it is described that PDTC leads to the phosphorylation of Akt in Luteolin-induced apoptosis in HL-60 cells (Cheng, Huang et al. 2006). But PDTC alone failed to activate the PI3K/Akt pathway in this study. Further it is described that Akt is phosphorylated due to treatment of zinc in HeLa cells in a PI3K-dependent manner (Barthel, Ostrakhovitch et al. 2007). Hence the idea evolved that PDTC activates the PI3K/Akt pathway leading to survival signals. Fig. 29 demonstrates that PDTC cause the phosphorylation of Akt at Ser473 shortly after treatment in HeLa cells. This phosphorylation is abolished by the presence of wortmannin, which is a PI3K inhibitor. This finding means that the activity of PI3K is a prerequisite for PDTC-induced Akt phosphorylation. A similar situation was observed for zinc-induced Akt phosphorylation in HeLa cells (Barthel, Ostrakhovitch et al. 2007).

The wortmannin-sensitivity of PDTC-induced Akt phosphorylation was the requirement for the next experiment. If the activation of the PI3K/Akt pathway is the reason for the anti-apoptotic effect of PDTC, it should be abolished by wortmannin. Surprisingly this is not the case as shown in Fig. 30 and Fig. 31. Wortmannin failed to counteract PDTC after 8 h.

V.5 Conclusions

Taking together, these findings suggest that PDTC acts mainly at pre- and/or mitochondrial stages of apoptosis. It is demonstrated that PDTC delays the release of cytochrome c from the mitochondria which is a hallmark of apoptosis. An inhibitory effect of PDTC on caspase-9 processing is observed in Actinomycin D-induced apoptosis in HeLa cells and in the cell-free system. Further it is demonstrated that PDTC does not act mainly on caspase-3 as assumed at the beginning.

Surprisingly the activation of Akt by PDTC which is known to inhibit apoptosis is not the reason for its anti-apoptotic activity.

V.6 Open questions and future experiments

PDTC is able to inhibit the activation of NFκB (Kim, Kim et al. 1999). This ubiquitously expressed transcription factor is known to exhibit anti-apoptotic as well as pro-apoptotic effects (Shishodia and Aggarwal 2002). The function of NFκB regarding cell death depends on cell type and apoptotic stimuli. NFκB promotes cell survival due to inducing the transcription of anti-apoptotic genes such as members of the IAP or Bcl-2 family. Anti-apoptotic effects of NFκB are also mediated by down-regulation of apoptotic genes. There are also findings which suggest that the anti-apoptotic effects of this transcription factor are based on protein-protein interaction rather than *de novo* protein synthesis (Shishodia and Aggarwal 2002).
In the case of Puromycin-induced apoptosis *de novo* protein synthesis can not occur due to inhibition by this inducer. If PDTC acts on NFκB, the anti-apoptotic effect can be mediated only by protein-protein interactions of NFκB. In this case NFκB must exhibit pro-apoptotic properties in Puromycin-induced apoptosis in HeLa and MCF-7 cells.
An important question is why PDTC exhibit pro- as well as anti-apoptotic properties. Certainly the different roles of NFκB as well as the fact that PDTC acts on this transcription factor could explain this observation. Provided the inhibition of NFκB by PDTC is the reason for the anti-apoptotic effect.

The results of this project suggest a pre- and/or mitochondrial effect of PDTC. Ragione et al. also concluded that mitochondria are the primary targets of PDTC (Della Ragione, Cucciolla et al. 2000). In contrast to the results in this diploma thesis Ragione et al. describe PDTC as inducer of apoptosis in HL-60 cells (Della Ragione, Cucciolla et al. 2000). Different cellular models can also be the reason for the different effects of PDTC observed (Erl, Weber et al. 2000).
To clearify if PDTC acts on the level of mitochondria, it should be investigated if PDTC is able to inhibit or to promote cytochrome c release of isolated mitochondria *in vitro*. Therefore cytochrome c release can be triggered by recombinant Bax protein or by the addition of calcium.

VI References

Adams, J. M. and S. Cory (2007). "The Bcl-2 apoptotic switch in cancer development and therapy." Oncogene **26**(9): 1324-37.

Alberts, B., A. Johnson, et al. (2002). Molecular Biology of the Cell, Garlandscience.

Barthel, A., E. A. Ostrakhovitch, et al. (2007). "Stimulation of phosphoinositide 3-kinase/Akt signaling by copper and zinc ions: mechanisms and consequences." Arch Biochem Biophys **463**(2): 175-82.

Bouillet, P. and A. Strasser (2002). "BH3-only proteins - evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death." J Cell Sci **115**(Pt 8): 1567-74.

Buttner, S., T. Eisenberg, et al. (2006). "Why yeast cells can undergo apoptosis: death in times of peace, love, and war." J Cell Biol **175**(4): 521-5.

Callus, B. A. and D. L. Vaux (2007). "Caspase inhibitors: viral, cellular and chemical." Cell Death Differ **14**(1): 73-8.

Cheng, A. C., T. C. Huang, et al. (2006). "Pyrrolidine dithiocarbamate inhibition of luteolin-induced apoptosis through up-regulated phosphorylation of Akt and caspase-9 in human leukemia HL-60 cells." J Agric Food Chem **54**(12): 4215-21.

Creagh, E. M. and S. J. Martin (2001). "Caspases: cellular demolition experts." Biochem Soc Trans **29**(Pt 6): 696-702.

Della Ragione, F., V. Cucciolla, et al. (2000). "Pyrrolidine dithiocarbamate induces apoptosis by a cytochrome c-dependent mechanism." Biochem Biophys Res Commun **268**(3): 942-6.

Donald L. Riddle, T. B., Barbara J. Meyer, James R. Priess (1997). C. Elegans II, COLD SPRING HARBOR LABORATORY PRESS 1997.

Erl, W., C. Weber, et al. (2000). "Pyrrolidine dithiocarbamate-induced apoptosis depends on cell type, density, and the presence of Cu(2+) and Zn(2+)." Am J Physiol Cell Physiol **278**(6): C1116-25.

Fan, T. J., L. H. Han, et al. (2005). "Caspase family proteases and apoptosis." Acta Biochim Biophys Sin (Shanghai) **37**(11): 719-27.

Ferraro, E. and F. Cecconi (2007). "Autophagic and apoptotic response to stress signals in mammalian cells." Arch Biochem Biophys **462**(2): 210-9.

Fink, S. L. and B. T. Cookson (2005). "Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells." Infect Immun **73**(4): 1907-16.

Galluzzi, L., M. C. Maiuri, et al. (2007). "Cell death modalities: classification and pathophysiological implications." Cell Death Differ **14**(7): 1237-43.

Golstein, P. and G. Kroemer (2007). "Cell death by necrosis: towards a molecular definition." Trends Biochem Sci **32**(1): 37-43.

Hacker, G. and A. Weber (2007). "BH3-only proteins trigger cytochrome c release, but how?" Arch Biochem Biophys **462**(2): 150-5.

Ho, P. K. and C. J. Hawkins (2005). "Mammalian initiator apoptotic caspases." Febs J **272**(21): 5436-53.

Invitrogen. "Media formulations RPMI1640, Cat. No. 31870." from http://www.invitrogen.com/content.cfm?pageId=95&fuseaction=MediaForm.dsp_mediaForm&productId=191.

Jin, Z. and W. S. El-Deiry (2005). "Overview of cell death signaling pathways." Cancer Biol Ther **4**(2): 139-63.

Kim, C. H., J. H. Kim, et al. (1999). "Zinc is required in pyrrolidine dithiocarbamate inhibition of NF-kappaB activation." FEBS Lett **449**(1): 28-32.

Kim, I., C. H. Kim, et al. (2004). "Pyrrolidine dithiocarbamate and zinc inhibit proteasome-dependent proteolysis." Exp Cell Res **298**(1): 229-38.

Krenn, B. M., B. Holzer, et al. (2005). "Inhibition of polyprotein processing and RNA replication of human rhinovirus by pyrrolidine dithiocarbamate involves metal ions." J Virol **79**(22): 13892-9.

Lanke, K., B. M. Krenn, et al. (2007). "PDTC inhibits picornavirus polyprotein processing and RNA replication by transporting zinc ions into cells." J Gen Virol **88**(Pt 4): 1206-17.

Lawen, A. (2003). "Apoptosis-an introduction." Bioessays **25**(9): 888-96.

Liu, X., C. N. Kim, et al. (1996). "Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c." Cell **86**(1): 147-57.

MacFarlane, M. and A. C. Williams (2004). "Apoptosis and disease: a life or death decision." EMBO Rep **5**(7): 674-8.

Malm, T. M., H. Iivonen, et al. (2007). "Pyrrolidine dithiocarbamate activates Akt and improves spatial learning in APP/PS1 mice without affecting beta-amyloid burden." J Neurosci **27**(14): 3712-21.

Modjtahedi, N., F. Giordanetto, et al. (2006). "Apoptosis-inducing factor: vital and lethal." Trends Cell Biol **16**(5): 264-72.

Nisbet, C., G. F. Yarim, et al. (2006). "Effects of trichophytosis on serum zinc levels in calves." Biol Trace Elem Res **113**(3): 273-80.

Nobel, C. S., D. H. Burgess, et al. (1997). "Mechanism of dithiocarbamate inhibition of apoptosis: thiol oxidation by dithiocarbamate disulfides directly inhibits processing of the caspase-3 proenzyme." Chem Res Toxicol **10**(6): 636-43.

Riedl, S. J. and G. S. Salvesen (2007). "The apoptosome: signalling platform of cell death." Nat Rev Mol Cell Biol **8**(5): 405-13.

Rupinder, S. K., A. K. Gurpreet, et al. (2007). "Cell suicide and caspases." Vascul Pharmacol **46**(6): 383-93.

Shishodia, S. and B. B. Aggarwal (2002). "Nuclear factor-kappaB activation: a question of life or death." J Biochem Mol Biol **35**(1): 28-40.

Sigma-Aldrich. "Actinomycin D datasheet." from http://www.sigmaaldrich.com/sigma/datasheet/a9415dat.pdf.

Sigma-Aldrich. "Puromycin datasheet." from http://www.sigmaaldrich.com/sigma/datasheet/p9620dat.pdf.

Sigma-Aldrich. "z-VAD-fmk datasheet." from http://www.sigmaaldrich.com/sigma/datasheet/v116dat.pdf.

Simstein, R., M. Burow, et al. (2003). "Apoptosis, chemoresistance, and breast cancer: insights from the MCF-7 cell model system." Exp Biol Med (Maywood) **228**(9): 995-1003.

Song, G., G. Ouyang, et al. (2005). "The activation of Akt/PKB signaling pathway and cell survival." J Cell Mol Med **9**(1): 59-71.

Stefanidou, M., C. Maravelias, et al. (2006). "Zinc: a multipurpose trace element." Arch Toxicol **80**(1): 1-9.

Truong-Tran, A. Q., J. Carter, et al. (2001). "The role of zinc in caspase activation and apoptotic cell death." Biometals **14**(3-4): 315-30.

Vandenabeele, P., T. Vanden Berghe, et al. (2006). "Caspase inhibitors promote alternative cell death pathways." Sci STKE **2006**(358): pe44.

Vaux, D. L. and J. Silke (2005). "IAPs, RINGs and ubiquitylation." Nat Rev Mol Cell Biol **6**(4): 287-97.

Widlak, P. (2000). "The DFF40/CAD endonuclease and its role in apoptosis." Acta Biochim Pol **47**(4): 1037-44.

Curriculum Vitae

Personal Details:

Name:	Markus Mandl
Date of birth:	22/03/1983, Bruck/Mur, Styria
Citizenship:	Austrian

Languages:

German, English

Secondary education:

September 1997 – June 2002	Secondary College for Chemical Technology (HBLVA für chemische Industrie), Specialisation: Biochemistry, Bio- and Gene-technology, Vienna, Austria

University studies:

October 2002 – autumn 2007	Molecular Biology (A490) at the University of Vienna, Austria
since October 2005	Political Science (A300) at the University of Vienna, Austria

Research experience:

March 2004 – June 2005	Free contract of employment at the Institute of Biomedical Research, Medical University of Vienna, Austria (Cell culture routine)

Congresses:

July 7-12th 2007	FEBS Congress “Molecular Machines”, Vienna, Austria (Participant with poster)