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The roles of proteases in infection biology

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Content

Summary

The aim of this study was to elucidate the role of proteases in infection biology and their influence by PDTC. PDTC is a multifunctional metal chelating compound. Two main objectives could be achieved by using mainly two methods; FACS and Western Blot analysis.

First, it could be shown that PDTC is not able to inhibit the cleavage of eIF4GI by the viral $2A^{pro}$. For these experiments HeLa cells were used, which were stable transfected with the HRV2 $2A^{pro}$ construct under the control of a tetracycline inducible protein expression system. It could be shown that with and without addition of PDTC eIF4GI was partially cleaved by $2A^{pro}$. PDTC did not inhibit this partial cleavage.

Second, the antiapoptotic property of PDTC in HeLa cells was investigated. Based on morphological observations and inhibition of PARP cleavage and caspase activation it could be demonstrated that PDTC acts antiapoptotic in HeLa cells. Further, it could be shown that it acts also proteasome inhibitory in this cell line. To elucidate the underlying mechanism of the antiapoptotic property of PDTC, different approaches were defined. It may be possible that oxidative stress plays a considerable role during apoptosis and that the antioxidative property of PDTC may play an important role during its antiapoptotic effect. Further, it may be possible that the proteasome inhibitory function of PDTC plays an important role. Finally, the influence of members of the BCl-2 protein family by PDTC was a main objective. Previous data from our lab provided evidence that the inhibition of apoptosis is not simply based on inhibition of caspases, but that proteins of the BCl-2 protein family may a role. In addition, experiments with Pyrithione and Hinokitiol were performed to elucidate the role of these metal chelators during this process.

It could be shown that the antiapoptotic property of PDTC is neither linked to its antioxidative nor to its proteasome inhibitory property. As it was shown that induction of apoptosis by several inducers of apoptosis does not generally result in generation of oxidative stress, the antioxidative property of PDTC cannot be linked to its antiapoptotic effect. Experiments with MG132, a well known proteasome inhibitor, demonstrated that also this property of PDTC cannot be linked to its antiapoptotic effect in HeLa cells.

Further, it could be shown for the first time that PDTC acts antiapoptotic by preventing activation of BAX and BAK. Within this study it could be demonstrated that PDTC is an inhibitor of the conformational change of BAX and BAK that is triggered by several inducers of apoptosis. BAX and BAK, both members of the pro-apoptotic Bcl-2 protein family, play and essential role in the intrinsic apoptotic pathway by inducing cytochrome c release from mitochondria. This is due to a conformational change of these proteins. It was possible to demonstrate that this conformational change of BAX and BAK can be inhibited by PDTC and, thus, cell death prevented. It could not be shown whether PDTC acts directly or indirectly on these members of the BCl-2 protein family.

In this context it could also be shown that intrinsic as well as extrinsic apoptotic stimuli activate both, caspase 8 and 9. They are members of the cysteine protease family and known initiator caspases of the extrinsic, respectively intrinsic apoptotic pathway. This provided evidence for a strong crosstalk between the intrinsic and extrinsic apoptotic pathway. Further, it could be demonstrated that the antiapoptotic property of PDTC usually correlates with a strong reduction of caspase activation. Interestingly, PDTC was not able to inhibit caspase 8 activation by extrinsic apoptotic stimuli.

Finally, it could be shown that the metal chelators Pyrithione and Hinokitiol possess a similar biological activity as PDTC. Thus, metal ions may play a role during the antiapoptotic process.

Zusammenfassung

Ziel dieser Arbeit war es die Rolle von Proteasen im biologischen Infektionsprozess und die Einflussnahme von PDTC auf diesen Prozess zu untersuchen. PDTC ist ein multifunktioneller Metallchelator. Zwei hauptsächliche Fragestellungen konnten beantwortet werden. Dazu wurden vor allem zwei Methoden gewählt; FACS und Western Blot Analysen.

Zuerst konnte gezeigt werden, dass PDTC nicht im Stande ist die Spaltung von eIF4GI durch die virale Protease 2A zu inhibieren. Für diese Experimente wurde eine HeLa Zelllinie verwendet, welche mit dem HRV2 2A Protease Konstrukt stabil transfiziert war. Die Proteinexpression war unter Kontrolle eines Tetrazyklin indizierbaren Protein Expressionssystem. Es konnte gezeigt werden, dass mit oder ohne Zugabe von PDTC eIF4GI teilweise durch die Protease 2A gespalten wurde. PDTC war nicht im Stande diese Spaltung zu inhibieren.

Als zweites wurde die antiapoptotische Eigenschaft von PDTC in HeLa Zellen untersucht. Auf Basis morphologischer Beobachtungen und Inhibierung von PARP Spaltung und Kaspasen Aktivierung konnte gezeigt werden, dass PDTC eindeutig antiapoptotisch in HeLa Zellen wirkt. Weiters konnte gezeigt werden, dass PDTC auch inhibitorisch auf das Proteasom in dieser Zelllinie wirkt. Um den genauen Wirkungsmechanismus von PDTC zu untersuchen wurden verschiedene Fragestellungen definiert. Es könnte möglich sein, dass oxidativer Stress eine große Rolle während der Apoptose spielt und dass die antioxidative Eigenschaft von PDTC eine Rolle während dessen antiapoptotischen Wirkung spielt. Weiters könnte es möglich sein, dass die inhibitorische Wirkung von PDTC auf das Proteasom eine Rolle während dessen antiapoptotischen Wirkung spielt. Eine letzte große Fragestellung war die Einflussnahme von PDTC auf die Aktivität der BCl-2 Proteinfamilie. Vorhergehende Ergebnisse aus unserem Labor haben gezeigt, dass die Inhibierung der Apoptose durch PDTC nicht einfach durch die Inhibierung der Kaspasen erklärt werden kann. Vielmehr könnten Proteine der BCl-2 Familie eine entscheidende Rolle dabei spielen. Zusätzlich wurden noch Experimente mit Pyrithion und Hinokitiol durchgeführt, um deren Wirkung zu untersuchen.

Es konnte gezeigt werden, dass die antiapoptotische Wirkung von PDTC weder in Verbindung zu dessen antioxidativen noch zu dessen Proteasom inhibitorischen Wirkung steht. Da die Induktion der Apoptose durch diverse Apoptose induzierende Reagenzien zu keinem generellen oxidativen Stress führte, kann die antioxidative Wirkung von PDTC nicht ausschlaggebend für dessen antiapoptotische Wirkung sein. Experimente mit MG132, ein bekannter Proteasom Inhibitor, haben gezeigt, dass auch diese Eigenschaft von PDTC nicht mit dessen antiapoptotischen Wirkung in Zusammenhang gebracht werden kann.

Weiters konnte zum ersten Mal gezeigt werden, dass PDTC antiapoptotisch wirkt, indem es die Aktivierung der Proteine BAX und BAK inhibiert. In dieser Arbeit konnte gezeigt werden, dass PDTC ein Inhibitor der Konformationsänderung von BAX und BAK ist, die durch verschiedenste Apoptose induzierenden Reagenzien induziert wird. BAX und BAK, beide Mitglieder der proapoptotischen BCl-2 Proteinfamilie, spielen eine wichtige Rolle während der Apoptose,

indem sie Cytochrom C von den Mitochondrien freisetzen. Dieser Prozess wird durch die Konformationsänderung dieser Proteine induziert. In dieser Arbeit war es möglich zu zeigen, dass diese Konformationsänderung durch PDTC inhibiert werden kann und daher der Zelltod verhindert werden kann. Nicht gezeigt werden konnte, ob dies auf einem direkten oder indirekten Weg geschieht.

In diesem Zusammenhang konnte auch gezeigt werden, dass sowohl extrinsische als auch intrinsische Apoptose induzierende Reagenzien die Kaspasen 8 und 9 aktivieren. Beide sind Mitglieder der Familie der Cystein Proteasen und bekannte Initiator Kaspasen des extrinsischen, bzw. intrinsischen Apoptose Signalweges. Das gibt Grund zur Annahme für eine starke gegenseitige Einflussnahme beider Signalwege. Es konnte auch gezeigt werden, dass die antiapoptotische Wirkung von PDTC in der Regel mit einer starken Reduzierung der Kaspasen Aktivierung einhergeht. Bemerkenswert war, dass PDTC nicht im Stande war die Aktivierung der Kaspase 8 durch extrinsische Faktoren zu inhibieren.

Zuletzt konnte noch gezeigt werden, dass die Metallchelatoren Pyrithion und Hinokitiol eine ähnliche biologische Wirkung wie PDTC besitzen. Dies lässt vermuten, dass Metallionen eine Rolle während dieses Prozesses spielen.

Abbreviations

APAF1	Apoptotic protease activating factor 1
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BCl-2	B-cell lymphoma-2
BSA	Bovine serum albumin
CARD	Caspase recruitment domain
CD95	Fas ligand
CHX	Cycloheximide
DAPI	Diamidinphenylindoldihydrochlorid
DCF	Dichlorofluorescein
DED	Death effector domain
DISC	Death inducing signalling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxycycline hyclate
ECL	Enhanced chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
eIF4G	Eukaryotic initiation factor 4G
FACS	Fluorescent activated cell sorting
FasL	Fas ligand (also called CD95)
GFP	Green fluorescent protein
HBSS	Hank's buffered salt solution
HRP	Horseradish peroxidase
HRV	Human Rhinovirus

ICAM-1	Intercellular adhesion molecule 1
LDL	Low density lipoprotein
LGS	Lower gel solution (SDS-PAGE)
MEM	Modified Eagle's medium
NAC	N-Acetylcysteine
NF-κB	Nuclear factor κB
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffered saline
PDTC	Pyrrolidine dithiocarbamate
PFA	Paraformaldehyde
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediate
ROS	Reactive oxygen species
RT	Room temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ssRNA	Single stranded RNA
TBS	Tris buffered saline
TEA	Triethanolamine
TEMED	Tetramethylethylenediamine
TNF α	Tumor necrosis factor α
TNFR	Tumor necrosis factor receptor
UGS	Upper gel solution (SDS-PAGE)
WB	Western blot
WHO	World Health Organisation

1. Introduction

1.1 Apoptosis

Apoptosis, a mode of programmed cell death, is used by multicellular organisms to dispose unwanted cells. It is used in a diversity of settings and plays an essential role during embryogenesis as well as in adult organisms. In contrast to necrosis, apoptosis describes cell death that is carried out in a controlled manner to ensure that neighboring structures are unaffected. The importance of such a process can also be observed in several pathologies. For example, the incapacity to remove mutated cells can lead to severe cancer. The concept of apoptosis was discovered in the 1970s (Currie and Wylie, 1972). During last decades much progress was made in identifying the underlying mechanisms of apoptosis (reviewed in Taylor et al., 2008).

The great importance of apoptosis can best be outlined in discussing necrosis and its consequences for the organism. Necrosis, in contrast to apoptosis, describes rapid and uncontrolled cell death. It is accompanied by loss of membrane integrity and the release of cellular contents into the extracellular space. Therefore, usually it results in heavy damage of neighboring structures. This is the case in burns and cuts. In addition to the damage described, necrosis may also facilitate infections, an even more severe consequence. In biological terms there is one more severe outcome of necrosis. The uncontrolled release of cellular contents into the extracellular space alerts the immune system. Just because of their presence in the extracellular space, which is normally not the case, these cellular contents may be recognized as foreign entities and provoke an immune reaction. This immune reaction may result in further tissue damage or even escalation of the initial damage. Hence, it stands to reason that a process to remove unwanted cells in a safe manner, such as apoptosis, is essential for multicellular organisms.

The apoptotic process starts with death stimuli, which can have their origin outside (extrinsic pathway) or inside (intrinsic pathway) the cell. During the apoptotic signaling pathways caspases play an essential role as the main executors of apoptosis. Members of the BCl-2 protein family play a regulatory role during this process. Morphologically, apoptotic cell death can be observed in cell shrinkage and condensation of the nucleus and its fragmentation into smaller pieces, the so-called apoptotic bodies. In contrast to necrosis, apoptotic cells are readily recognized by phagocytes as being different from their viable counterparts and are rapidly engulfed and recycled by them. This ensures that neighboring structures remain unaffected.

Caspases, the executors of apoptosis

Caspases are the central components in the apoptotic process (reviewed in Riedl et al., 2004). They are so-named because they are cysteine proteases that cleave after an aspartate residue in their substrate. At least 14 mammalian caspases have been indentified so far; 11 of these were found in humans. There are two main categories of caspases that play a role in apoptosis, initiator and effector caspases.

At least 7 of the 14 known mammalian caspases have important roles in apoptosis. These include caspase 2, 8, 9 and 10 as initiator caspases and caspase 3, 6 and 7 as effector caspases. Typically, caspases are produced in cells as catalytically inactive zymogens and must undergo proteolytic activation during apoptosis.

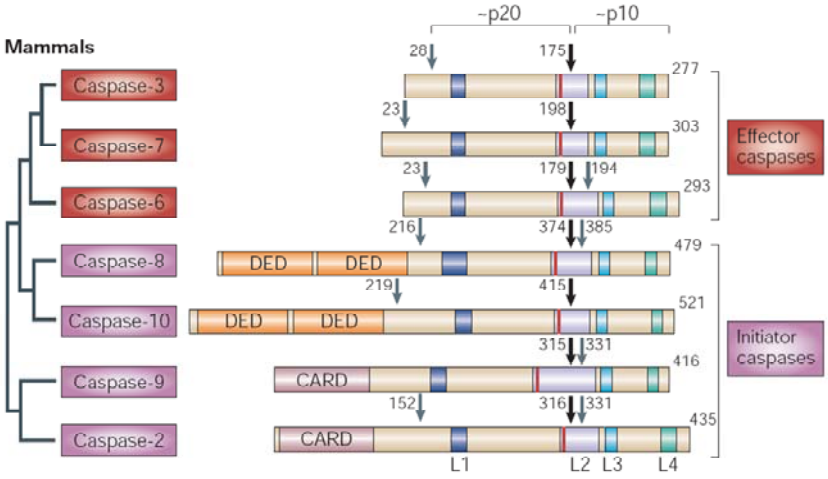

Fig. 1 Apoptotic caspases in mammals. Effector caspases, caspase 3-6-7, consist of small and large subunit, which are separated when activated through cleavage by initiator caspases. Initiator caspases, caspase 2-8-9-10, posses an extended N-terminal region, which contains a caspase recruitment domain (CARD) or death effector domain (DED). They are important for protein-protein interaction and auto-activation of initiator caspases.

(© Nature Publishing Group. Adapted from Riedl and Shi, 2004)

Initiator caspases are characterized by an extended N-terminal region. This region is used to recruit enzymes that are necessary for the activation of the apoptosis signaling pathway. The prodomains in initiator caspases contain two distinct, but structural related motifs; the caspase recruitment domain (CARD) and the death effector domain (DED). These motifs typically facilitate interactions with proteins that contain the same motif and play a crucial role in activation of initiator caspases as they are auto-activated. This process of activation requires protein-protein interactions and often also the assembly of a multi-component complex under apoptotic conditions. For example, the activation of pro-caspase 8 is facilitated by formation of the death inducing signaling complex (DISC) and the activation of pro-caspase 9 by apoptosome formation.

Effector caspases posses a small and large subunit similar to initiator caspases, but lack the extended N-terminal region. They are activated through cleavage by an initiator caspase at a specific internal Asp residue that separates the small and large subunit. Once activated, effector caspases are responsible for the proteolytic cleavage of a broad spectrum of cellular targets that lead to cell death.

Intrinsic and extrinsic pathways

The apoptotic response is mediated through the intrinsic or extrinsic apoptotic pathway, depending on the origin of the death stimuli (reviewed in Taylor et al., 2008). It is thought that there exists a crosstalk between both pathways.

The intrinsic apoptotic pathway is mediated by mitochondria. In response to apoptotic stimuli several proteins are released from the intermembrane space of mitochondria into the cytoplasm. One of the best characterized and most important proteins is cytochrome c. It is released into the cytoplasm in response to a wide range of apoptotic stimuli within the cell. These include DNA damage and oncogene activation. When released into the cytoplasm, cytochrome c binds to APAF1 and induces conformational changes that allow the formation of the apoptosome. Formation of the apoptosome activates caspase 9 and triggers the apoptotic signaling cascade that results in effector caspase activation and cell death.

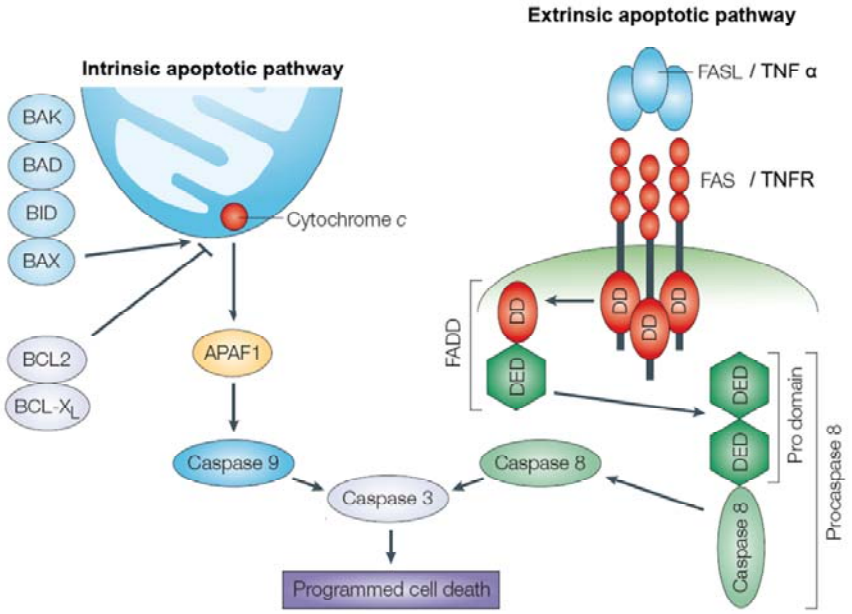

Fig. 2 Simplified representation of the intrinsic and extrinsic apoptotic pathway. Activation of caspase 9 after cytochrome c release from mitochondria leads to activation of caspase 3 during the intrinsic apoptotic pathway. Members of the BCl-2 protein family play a regulatory role during this process. Activation of caspase 8 after binding of a death ligand to its receptor and subsequent activation of caspase 3 results in cell death during the extrinsic apoptotic pathway.

The extrinsic apoptotic pathway is initiated by binding of an extracellular death ligand to its cell surface death receptor. These include FasL and Fas as well as TNF α and TNFR. The binding of the ligand to its receptor leads to the recruitment of further cytosolic factors and result in the formation a death inducing signaling complex (DISC). Formation of DISC leads to activation of caspase 8, which then results in effector caspase activation and cell death.

Emerging evidence for diverse non-apoptotic mechanisms of cell death

Emerging evidence suggest that apoptosis is not the only mechanism of controlled cell death (reviewed in Degterev et al., 2008). Evidence for three pathways of cell death has recently emerged. They are called type II cell death, necroptosis and PARP1-mediated cell death. Type II cell death is characterized by the accumulation of double membrane enclosed vesicles. As these vesicles are characteristic of autophagy, it is thought that autophagy may play an essential role during this type of cell death. However, the role of autophagy is still under debate. Necroptosis represents a special type of programmed necrosis. Necroptosis can be induced with classical apoptotic stimuli, such as death ligands, when apoptosis is inhibited by specific apoptosis inhibitors. In addition, it shows some morphological features of necrosis, which explains the name. PARP1 is a nuclear enzyme that is involved in maintaining genome stability. Over-activation of PARP1 can lead to caspase independent cell death in several ways for which details remain unclear so far. Initial DNA damage that results in energy collapse or mitochondrial dysfunction may play an essential role during this type of cell death.

Which role these pathways of non-apoptotic cell death may play has to be shown. The question whether non-apoptotic cell death can also occur under normal circumstances when apoptosis is possible remains to be discussed. It may be that they represent rudimentary back-up forms of cell death.

1.2 The BCl-2 protein family

Members of the BCl-2 (B-cell lymphoma-2) protein family play an essential role during apoptosis (reviewed in Youle et al. and Chipuk et al., 2008). They are key regulators of cytochrome c release from mitochondria and it is thought that they play a role in crosstalk between the extrinsic and intrinsic apoptotic pathway. They can be divided into two major groups of proteins; proapoptotic and antiapoptotic BCl-2 proteins.

In mammals there are at least 12 core BCl-2 proteins. These include BCl-2 itself as well as proteins that have a secondary or tertiary structure that is similar to BCl-2. They posses either proapoptotic or antiapoptotic properties. The structure of antiapoptotic BCL-2 proteins may include BH1-4 motifs, whereas proapoptotic members posses only BH1-3 motifs. In addition, several BH3-only proteins exist, which share only the BH3 motif with the core BCl-2 proteins. They seem to lack a close evolutionary relationship to core BCl-2 proteins. However, they are important regulators of BCl-2 proteins, with which they interact through the BH3 motif. The

BH1, BH2 and BH3 motif fold to line a hydrophobic pocket that can bind BH3-only proteins. Most prominent members of the proapoptotic BCl-2 proteins are BAX and BAK, the most prominent member of antiapoptotic BCl-2 proteins is BCl-2 itself. BAX and BAK induce cytochrome c release from mitochondria through a conformational change that is triggered by apoptotic stimuli. BCL-2 is thought to inhibit this conformational change and, therefore, inhibit apoptosis. The exact mechanism of this interaction is not yet known.

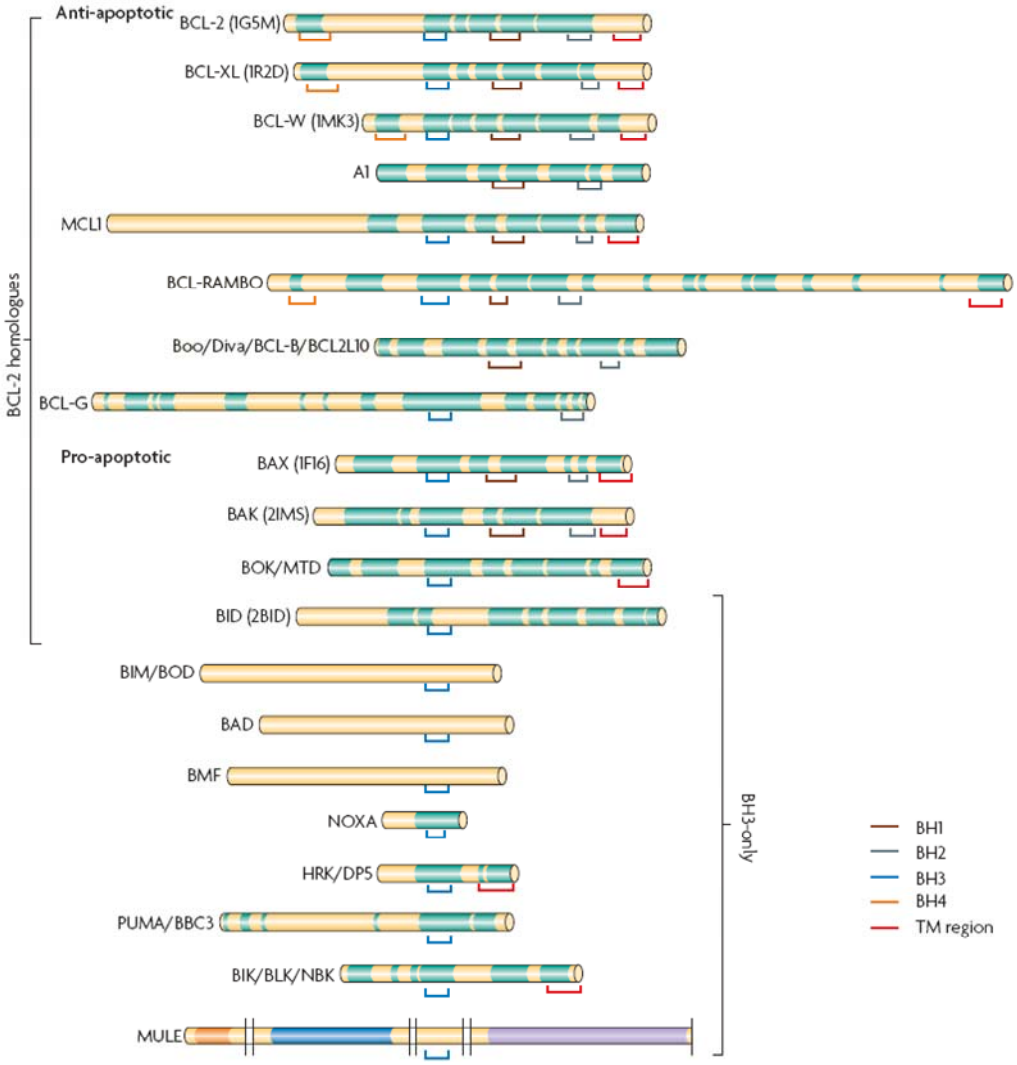

Fig. 3 Members of the core BCl-2 protein family and BH3-only proteins. They can be divided into antiapoptotic and proapoptotic proteins. Structural motifs, BH1-4, are shown. Members of antiapoptotic BCl-2 proteins posses BH1-4 motifs, whereas proapoptotic proteins have BH1-3. BH3-only proteins are key regulators of core BCl-2 proteins.

Both pathways of apoptosis, the intrinsic and extrinsic pathway, require the activation and action of several caspases to successfully kill a cell. The intrinsic apoptotic pathway requires in addition the regulatory action of members of the BCl-2 protein family. There is also raising evidence that there may exist a strong crosstalk between both pathways that is mediated by

core BCl-2 proteins or BH3-only proteins. Thus, BCl-2 proteins may also play a considerable role during the extrinsic apoptotic pathway via a crosstalk to the intrinsic pathway.

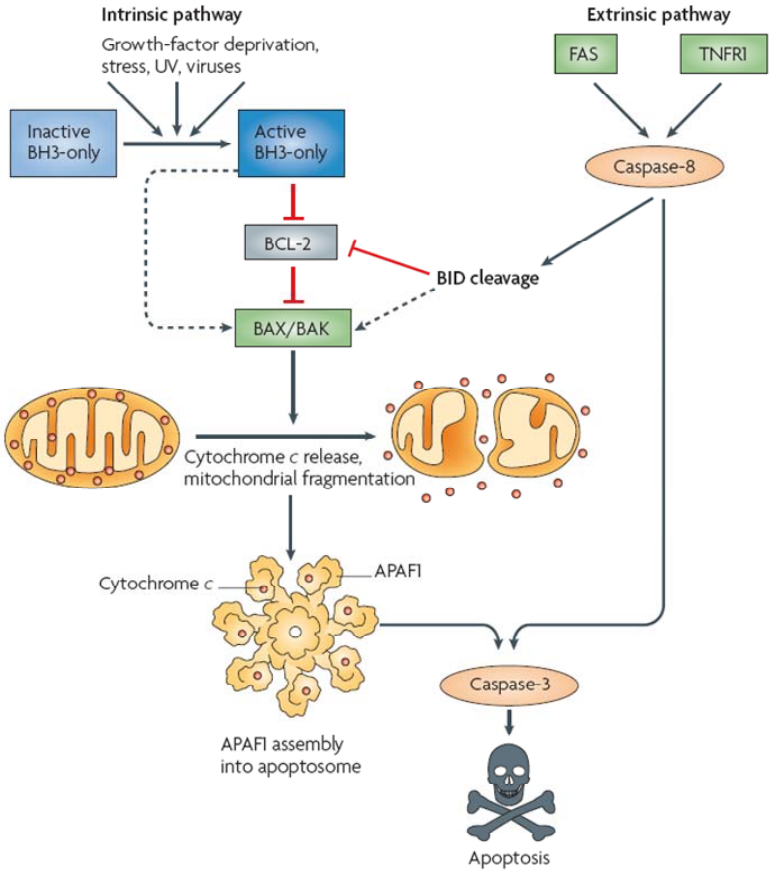

Fig. 4 Scheme depicting intrinsic and extrinsic pathways of apoptosis. BH3-only proteins may act proapoptotic by inhibiting antiapoptotic BCl-2 proteins or by direct activation of BAX/BAK. The extrinsic pathway may intersect with the intrinsic pathway through BID, a member of BH3-only proteins.

(© Nature Publishing Group. From Youle and Strasser, 2008)

During the intrinsic apoptotic pathway cytochrome c release from mitochondria plays an essential role. It binds to APAF1 and triggers the formation of the apoptosome and, thus, cell death. Cytochrome c release from mitochondria is regulated by BCl-2 proteins. It is known that BAX and BAK induce cytochrome c release from mitochondria through a conformational change that is induces by apoptotic stimuli. Two steps in the activation of BAX could be observed: First, the translocation of BAX to mitochondria and, second, the N-terminal conformational change and the insertion into the mitochondrial membrane. This activation step from soluble, cytosolic BAX to membrane inserted BAX in apoptotic cells seems to be essential for cytochrome c release. If the release of proteins from the space between the outer and inner mitochondrial membrane is triggered by proapoptotic BCl-2 proteins in a direct or indirect way, has to be shown.

The antiapoptotic BCl-2 protein members inhibit BAX and BAK. How this inhibition is overcome is not yet clear. There are two main hypotheses: It is thought that either BH3-only proteins

bind direct to antiapoptotic BCl-2 proteins and, thus, inhibit their function or that BAX and BAK may be directly activated by some BH3-only proteins. However, it seems that intrinsic apoptotic stimuli disrupt the balance between antiapoptotic and proapoptotic BCl-2 proteins and, therefore, induce the apoptotic process.

A direct contribution of BCl-2 proteins to the extrinsic apoptotic pathway is not yet known. However, it is known that the extrinsic pathway can intersect with the intrinsic pathway through caspase 9 activation mediated by BID. In this context, the C-terminal truncated form of BID, a member of the proapoptotic BH3-only proteins, translocates to mitochondria and promotes caspase activation. Which importance this intersection plays during cell death is not yet clear. One could think that this feedback mechanism could be necessary for cell death and retain cells before overhasty cell death decisions.

1.3 Oxidative stress

Early in evolution oxygen and its reactive metabolites were a major threat to eukaryotic cells. The reactive nature of oxygen made it necessary to develop defense mechanisms to protect molecules from damage. With the dangerous side of oxygen controlled, it was possible to use oxygen for the aerobic metabolism.

Oxidative stress is defined as an imbalance that favors the production of reactive oxygen species (ROS) over antioxidant defense (reviewed in Ott et al., 2007). This can become dangerous for mitochondrial function and cell viability. The mitochondrial respiratory chain is the major source of ROS production. In the case of oxidative stress, high amounts of ROS are produced in the mitochondrial respiratory chain. At the same time, mitochondria are an important target for the damaging effects of ROS. Thus, mitochondrial function may fail and cells cannot be provided anymore with sufficient ATP for their survival. This can lead in cellular damage and apoptosis if the repair and defense mechanisms fail.

A small amount of oxygen consumed during respiration is converted into superoxide radicals. This produces a relatively stable intermediate, the superoxide anion, which serves as the precursor of most ROS. Superoxide dismutase is able to convert the superoxide anion to hydrogen peroxide. Subsequent interaction of hydrogen peroxide and superoxide anion can generate the highly reactive hydroxyl radical. Two major defense mechanisms where established within the mitochondrial matrix to further metabolize hydrogen peroxide. Glutathione peroxidase and peroxiredoxin are able to convert hydrogen peroxide to water.

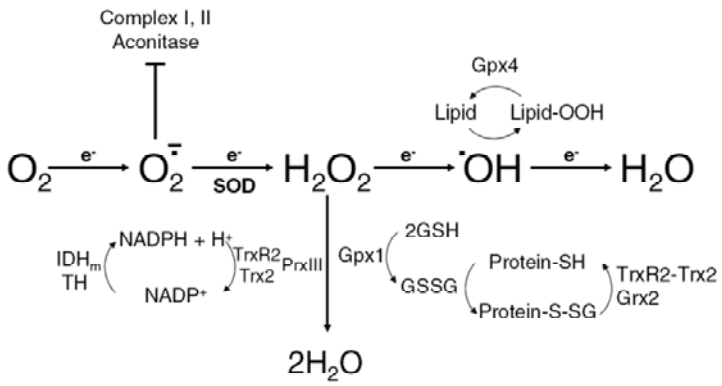

Fig. 5 Formation, effects and inactivation of reactive oxygen species in mitochondria. GSH reduced glutathione, GSSG glutathione disulfide, Gpx glutathione peroxidase, Grx glutaredoxin, IDH_m mitochondrial isocitrate dehydrogenase, NADP nicotinamide adenine dinucleotide phosphate, Prx peroxiredoxin, SOD superoxide dismutase, TH transhydrogenase, Trx thioredoxin, TrxR thioredoxin reductase.

1.4 Proteasome dependent proteolysis: Ubiquitinylation

One of the most important apparatus that destroys aberrant proteins is the proteasome (reviewed in Nandi et al., 2006). The proteasome, an ATP dependent proteolytic system, is present in many copies throughout the cytosol and the nucleus. It is essential for a cell to possess an apparatus that is able to degrade unwanted proteins. These may be misfolded proteins or proteins that are only needed for a short time and have to be removed afterwards. The proteasome recognizes unwanted proteins by their ubiquitin tag. The ubiquitin conjugating system marks proteins for destruction. They may be either monoubiquitinylated or polyubiquitinylated. It is thought that it is this polyubiquitin chain on a target protein that is recognized by a specific receptor in the proteasome. The exact role of mono- and polyubiquitinylation in the cellular context is not yet known. Ubiquitin has been reported to play a role also in intracellular trafficking.

The ubiquitin conjugating system

Ubiquitin is prepared for conjugation to other proteins by the ATP dependent ubiquitin activating enzyme E1. This creates an activated, E1-bound ubiquitin that is subsequently transferred to one of a set of ubiquitin conjugating enzymes (E2). The E2 enzymes act in conjunction with accessory proteins (E3). The E2-E3 complex is called ubiquitin ligase, in which E3 binds to a degradation signal in proteins. This helps E2 to form a polyubiquitin chain linked to a lysine of the substrate protein. It is this polyubiquitin chain on a target protein that is recognized by a specific receptor in the proteasome. The ubiquitin-proteasome system consists of many distinct but similarly organized proteolytic pathways, which have in common the E1 enzyme and the proteasome. They differ by the composition of the E2-E3 ubiquitin

ligase and accessory factors. In mammals there exist at least 30 different E2 and hundreds of different E3 enzymes that may form this complex.

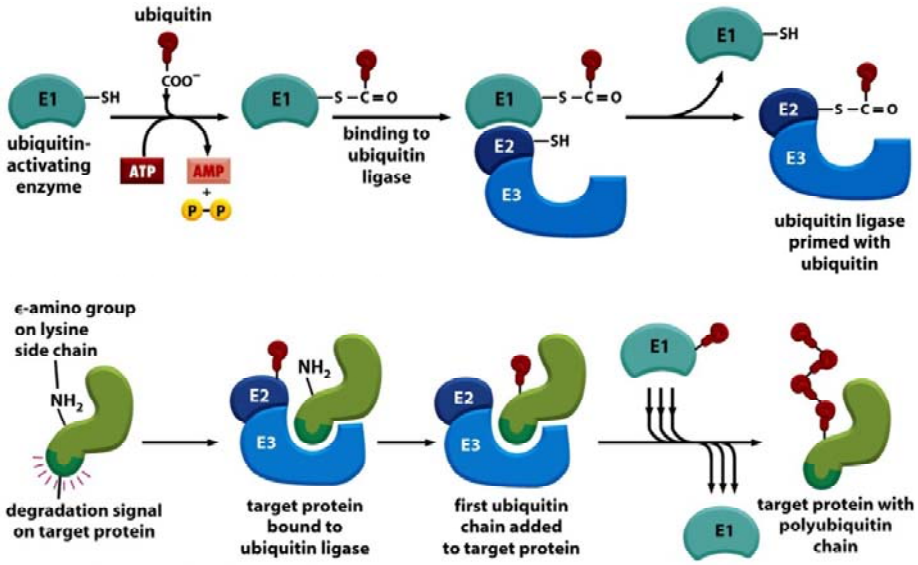

Fig. 6 Ubiquitin and the marking of proteins with polyubiquitin chains. The process of polyubiquitinylation marks proteins for degradation by the proteasome.

1.5 PDTC, Pyrithione and Hinokitiol

PDTC

Pyrrolidine dithiocarbamate (PDTC, see Fig. 7) is a multifunctional, metal chelating compound. Several properties of PDTC are described in the literature. These include pro- and antiapoptotic properties, pro- and antioxidative properties, antiviral properties, antimicrobial properties and inhibitory functions on proteasomal activity and NF-κB activation. The biological functions of PDTC are controversial discussed in the literature. In our lab we could show that PDTC acts antiapoptotic, antiviral (HRV) and that it inhibits proteasome depend proteolysis in HeLa cells.

PDTC is described in the literature as a proapoptotic as well as an antiapoptotic compound. Erl and colleagues showed that PDTC induced apoptosis depends on cell type, density, and the presence of Cu^{2+} and Zn^{2+} (Erl et al., 2000). They showed that PDTC induces apoptosis in a dose depend manner in rat smooth muscle cells, human fibroblasts and endothelial cells at low but not at high cell density. Further, they showed that Cu^{2+} and Zn^{2+} are necessary for the apoptotic effect of PDTC. In addition, it was shown that PDTC induces apoptosis by a cytochrome c dependent mechanism in HL-60 cells (Della Ragione et al., 2000) and that it exerts anti-proliferative and proapoptotic effects in renal cell carcinoma cell lines (Morais et al., 2006). Recently, Daniel and colleagues showed that PDTC zinc and copper complexes

induce apoptosis in human breast cancer cells by inhibiting the proteasomal activity (Daniel et al., 2008).

In contrast, Verhaegen and colleagues demonstrated the inhibition of apoptosis by antioxidants in HL-60 cells (Verhaegen et al., 1995). They reported that PDTC was able to inhibit UV-induced apoptosis as well as apoptosis induced by a range of cytotoxic drugs, such as Actinomycin D. In another study, it was demonstrated that dithiocarbamates inhibit apoptosis by directly preventing caspase 3 activation (Nobel et al., 1997). Further, it was shown that PDTC was able to inhibit luteolin induced apoptosis in a dose-dependent manner in HL-60 cells (Cheng et al., 2006).

PDTC is generally characterized as an antioxidant. In their study, Moellering and colleagues showed that PDTC exerts antioxidative effects in bovine aortic endothelial cells (Moellering et al., 1999). However, during last year evidence for its prooxidative effect has been reported. Evidence for this was shown by Tapia and colleagues in a study on proteinuria. In their study they showed that PDTC enhances oxidative stress in proteinuria (Tapia et al., 2008). In addition, it was shown that oxygen radicals may play a central role in the induction of apoptosis and, thus, PDTC may also act antiapoptotic through its antioxidative property (Verhaegen et al., 1995).

PDTC and zinc inhibit proteasome dependent proteolysis in HeLa cells (Kim et al., 2004). They could show that treatment with PDTC resulted in the accumulation of several proteasome substrates including p53 and p21 in HeLa cells. In addition, it was shown that PDTC complexes with copper and can act as proteasome inhibitor and apoptosis inducer in human breast cancer cells (Daniel et al., 2005). Further, they could show in more detail that PDTC zinc and copper complexes induce apoptosis in tumor cells by inhibiting the proteasomal activity (Milacic et al., 2008). It has also been shown that PDTC reduces coxsackievirus B3 replication through inhibition of the ubiquitin proteasome pathway (Si et al., 2005).

PDTC acts also antiviral and antimicrobial. The antiviral property could be shown with human rhinoviruses by Gaudernak and colleagues. They discovered that PDTC is an extremely potent compound against HRV and poliovirus infection in cell culture using HeLa cells (Gaudernak et al., 2002). It could further be shown that this antiviral activity involves metal ions (Krenn et al., 2005) and that PDTC inhibits picornavirus polyprotein processing and RNA replication by transporting zinc ions into cells (Lanke et al., 2007; Krenn et al., in press). The antibacterial activity of PDTC was shown by Kang and colleagues using diverse bacterial strains (Kang et al., 2008).

PDTC is a potent inhibitor of NF-κB activation in intact cells (Schreck et al., 1992); this inhibition requires the presence zinc (Kim et al., 1999).

Pyrithione and Hinokitiol

Pyrithione and Hinokitiol are structural diverse but functional similar metal chelating compounds compared to PDTC. It was shown that Pyrithione transports zinc ions into cells and that it inhibits NF-κB activation in a ten time more potent manner as PDTC (Kim et al., 1999). Hinokitiol is a cell permeable tropolone analog that exhibits similar properties compared to PDTC. It is a known effective chelator of iron, zinc and magnesium.

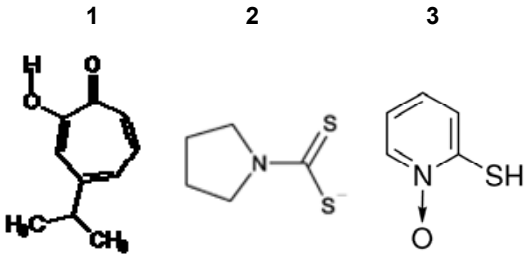

Fig. 7 Chemical structure of Hinokitiol (1), PDTC (2) and Pyrithione (3).

1.6 Rhinovirus and its 2A protease

Rhinoviruses belong to the family of *Picornaviridae*. Currently, more than 100 serotypes of rhinoviruses are known. They are nonenveloped viruses with a single stranded RNA (ssRNA) genome of positive polarity. This virus family contains many important human and animal pathogens. These include poliovirus, hepatitis A virus, foot and mouth disease virus and also rhinoviruses. Much progress was made during last years in the fight against poliovirus. It may be possible to eradicate poliovirus in the near future as it was already achieved for smallpox by the WHO.

Rhinoviruses are the viruses most commonly isolated from persons experiencing mild upper respiratory illnesses (common colds). They are spread from person to person by means of virus contaminated respiratory secretions. Symptoms of an infection with rhinovirus include all known symptoms of a common cold. Usually, these symptoms decay after a few days and do not require a medical intervention. However, in young children, elderly or immunodeficient people rhinovirus may cause more severe symptoms that require medical interventions. Since today, no effective causative treatment or vaccine is available.

Rhinoviruses can be classified into two groups. Viruses of the so called major group, which consist of about 90% of known rhinoviruses, are using ICAM-1 as receptor. The so called minor group viruses, which consist of about 10% of known rhinoviruses, are using members of the LDL receptor for internalization. A different classification into groups A and B is done by sequence homology of VP4/VP2.

The viral replication cycle of rhinovirus starts with its binding to a cellular receptor. The virion is then internalized in a process called receptor mediated endocytosis. Subsequently, the viral RNA is released into the cytoplasm of the host cell in a process involving conformational changes of the capsid. The multiplication of rhinoviruses occurs entirely in the cytoplasm. First, the viral RNA is translated directly into a single polyprotein, which is further processed by $2A^{pro}$ and $3C^{pro}$. Replication takes place in membrane vesicles derived from endoplasmatic reticulum and the Golgi complex. During this process negative stranded RNAs are synthesized that serve as templates for the generation of positive sense genomes. The newly synthesized positive sense RNA can then be used as template for further replication cycles or packaged into newly synthesized virions that become infectious after a maturation cleavage. Finally, infectious virus is released by lysis of the cell.

The genome of rhinoviruses codes for a total of 11 proteins. These include the four viral capsid proteins, called VP1-4. The other seven proteins include viral proteins 2B and 2C that induce changes in the intracellular membrane network, 3A that inhibits intracellular transport, 3B that serves as primer for RNA synthesis, 3D that is a RNA dependent RNA polymerase and the $2A^{pro}$ and $3C^{pro}$, which play an essential role in cleavage of the polyprotein. In addition, $2A^{pro}$ plays an essential role in host cell translational inhibition.

Viral protease 2A and its effect on host cell translation

Virus infection of most cell types by human rhinovirus induces a rapid and nearly complete inhibition of host cell protein synthesis (reviewed in Lloyd, 2006). The main target of the $2A^{pro}$ during the infection process is the translation initiation factor eIF4G. There are two major forms of eIF4G, termed eIF4GI and eIF4GII. They share only about 46% of homology, but are highly conserved in key regions that bind to other translation factor. In HeLa cells eIF4GI is the dominant form, comprising approximately 90% of total eIF4G. eIF4G can simultaneously bind several initiation factors and, thus, is involved in translation initiation. This process is interrupted through cleavage by $2A^{pro}$.

2. Materials and methods

2.1 Solutions and reagents

Acrylamide 30%	30% Acrylamide + 0,8% Methylenbisacrylamide in dH_2O, filtrate
Actinomycin D	SIGMA-ALDRICH Stock solution: 1 mM in DMSO, -20°C
Anode 1 buffer	300 mM Tris-Base 20% MeOH in dH_2O, do not adjust pH
Anode 2 buffer	25 mM Tris-Base 20% MeOH in dH_2O, do not adjust pH
Antibody dilutions, primary	5 ml 1 x TBS + a few grains of BSA, approximately 20 mg + 0,05% NaN_3 in a 50 ml tube
APS 10%	10% APS in dH_2O, 4°C
Cathode buffer	40 mM Amino-n-caproic acid 25 mM Tris-Base 20% MeOH in dH_2O, do not adjust pH
Cycloheximide	SIGMA-ALDRICH Stock solution: 100 mM in dH_2O, -20°C
DCF	SIGMA-ALDRICH Stock solution: 10 mM in DMSO, -20°C
Doxycycline hyclate	SIGMA-ALDRICH Stock solution: 1 mg/ml in dH_2O, 4°C
E.coli	*E.coli* XL-1 blue
ECL Super Signal West Pico	PIERCE
Embedding medium	DakoCytomation Fluorescent Mounting Medium

FasL/CD95 antibody	EXBIO antibodies, Lot FP10503
G418	PAA, Potency: 735 mg/g Stock solution: 50 mg/ml in dH_2O, 4°C
HBSS 1 x	GIBCO 400 mg/l KCl 60 mg/l KH_2PO_4 350 mg/l $NaHCO_3$ 8 g/l NaCl 48 mg/l Na_2HPO_4 1 g/l D-Glucose in dH_2O
Hinokitiol	Calbiochem Stock solution: 100 mM in DMSO, -20°C
Hygromycine B in PBS	Invitrogen
LB medium	1% trypton 0,5% yeast extract 1% NaCl in dH_2O, autoclave add 100 µg/ml antibiotic if desired
LGS	1,5 M Tris-Base 0,4% SDS in dH_2O, pH=8,8 (HCl)
Lipofectamine 2000	Invitrogen
Marker prestained	Precision Plus Protein Standards from BIO-RAD
MG132	Calbiochem Stock solution: 1 mM in DMSO, -20°C
Milk powder solution	5% milk powder in 1 x TBS
OptiMEM	GIBCO
Orange G 10 x	1 mM EDTA 10% Ficoll 0,1% Orange G in 0,5 x TEA buffer

PBS 10 x	65 mM $Na_2HPO_4 \cdot 2H_2O$ 1,4 M NaCl 27 mM KCl 15 mM KH_2PO_4 in dH_2O
PBS/Triton X100	1 x PBS + 0,1% Triton X100
PDTC	Alexis Biochemical Stock solution: 10 mM in PBS, 4°C
Perm-Wash 10 x	BD 1 x Perm-Wash in dH_2O
Perm-Wash/BSA	1 x Perm-Wash + 1% BSA
PFA 4%	4 g in 10 ml dH_2O stir at 60°C for several hours until it is dissolved add few drops of conc. NaOH until clearance of solution fill up to 100 ml with 1 x PBS store aliquots at -20°C
Plasmid purification kit	MACHEREY-NAGEL Nucleo Bond PC100
Puromycin	SIGMA-ALDRICH Stock solution: 10 mM in dH_2O, -20°C
Pyrithione	SIGMA-ALDRICH Stock solution: 100 mM in dH_2O, -20°C
RPMI 1640 medium	GIBCO + 2 mM L-Glutamine + 1% Pen/Strep + 10% FCS Stock solutions: 200 mM L-Glutamine from GIBCO 10000 units/ml Penicillin from GIBCO 10 mg/ml Streptomycin from GIBCO
Running buffer 10 x for SDS-PAGE	1,9 M glycine 250 mM Tris-Base 1% SDS in dH_2O

Sample buffer for protein extracts	50 mM Tris/HCl 6 M urea 30% glycerine 2% SDS a few grains of Bromophenol blue in dH_2O
Stack solution for SDS-PAGE	12,5 ml UGS 6,5 ml 30% Acrylamide 31 ml dH_2O
Stripping buffer	130 mM Tris/HCl 2% SDS in dH_2O, pH=6,7 add 360 µl β-mercaptoethanol to 50 ml buffer before use
TBS 10 x	200 mM Tris-Base 1,4 M NaCl in dH_2O
TEA buffer 10 x	0,5 M Tris-BASE 0,83 M NaAc 50 mM EDTA in dH_2O, pH 8,2 (HAc)
TEMED	Merck
Tissue culture flaks and plates	Corning Incorporated
TNF α	SIGMA-ALDRICH Stock solution: 10 µg/ml in dH_2O, -20°C
Transfection medium	RPMI 1640 medium + 2 mM L-Glutamine + 1% Pen/Strep + 2% FCS
Trypsin/EDTA 1 x	PAA
UGS	0,5 M Tris-Base 0,4% SDS in dH_2O, pH=6,8 (HCl)
X Posure Film	CL-X Posure Film from Thermo scientific

2.2 Biological function of selected compounds

Puromycin is a protein synthesis inhibitor that causes premature chain termination.

Actinomycin D is a transcription inhibitor that forms stable complexes with double stranded DNA and that causes single strand breaks in DNA.

TNF α is mainly secreted by macrophages and one of the most important cytokines in immune response. By binding to its receptors it activates the caspase signalling pathway.

FasL/CD95 antibody stimulation results in aggregation of its intracellular death domains and, therefore, activation of the caspase signalling pathway.

CHX is a translation inhibitor that is used to improve the apoptotic effect of TNF α and FasL/CD95 antibody.

Hinokitiol, Pyrithione and PDTC are structural diverse but functional similar metal chelating compounds.

2.3 Antibodies

Primary antibodies	Company	Dilution (used for)
BAK	Calbiochem (AM03)	1:500 (FACS)
BAX	BD (556467)	1:1000 (FACS)
Caspase 8	Cell Signaling (9746)	1:1000 (WB)
Caspase 9	Alexis Biochemicals (ALX210838)	1:1500 (WB)
	and Cell Signaling (9502)	1:1500 (WB)
PARP	Santa Cruz (sc7150)	1:2500 (WB)
eIF4GI	Kindly provided by R. Rhoads. Rhoads R. et al, 1992	1:1000 (WB)
Ubiquitin	DakoCytomation (Z0458)	1:2000 (WB)

Secondary antibodies	Company	Dilution (used for)
Alexa 488	Molecular Probes	1:1000 (FACS)
Mouse HRP	Jackson	1:15000 (WB)
Rabbit HRP	Jackson	1:15000 (WB)
Actin	SIGMA	1:1000 (WB)

2.4 Cell lines

HeLa cells (strain Ohio), a human cervix carcinoma cell line, were used mainly to elucidate the antiapoptotic property of PDTC in FACS and Western Blot experiments. They were obtained from ECACC, Salisbury, UK.

In addition, HeLa cells stable transfected with a HRV2 $2A^{pro}$ construct were used to elucidate the role of the protease. Protein expression in these cells is under the control of a tetracycline inducible protein expression system (tet on), which was induced by addition of 1µg/ml doxycycline. HeLa cells stable transfected with HRV2 $2A^{pro}$ (HeLa $2A^{pro}$) and HRV2 $2A^{pro}$ C106A (HeLa $2A^{proi}$) were kindly provided by Dr. Barbara Holzer from our lab. In the latter case, the biological function of the protease was disrupted by replacing the active site nucleophil cysteine by alanine (C106A). In both stable transfected constructs, $2A^{pro}$ is expressed as a fusion protein to EGFP.

For transient transfections with the described constructs a HeLa cell line (HeLa tet on) for protein expression under the control of the tet on system was kindly provided by Prof. Roland Foisner from the Max. F. Perutz Laboratories. Protein expression was induced by addition of 1µg/ml doxycycline.

2.5 Handling of cells

All cell lines were cultured in RPMI 1640 medium. HeLa $2A^{pro}$, HeLa $2A^{proi}$ and HeLa tet on cells were cultured in RMPI 1640 medium without Penicillin and Streptomycin. 100 µg/ml G418 and Hygromycine B were added as selective antibiotics to these cell lines. Hygromycine B was used as selective antibiotic for the transfected constructs containing the gene of interest; G418 for the construct containing the transactivator of the tet on system. Both together form the tet on protein expression system. All cell lines were split in the range of 1:3 to 1:20, depending on the grade of confluence.

Splitting cells

Growth medium was removed and cells washed once with PBS. Then, cells were treated with Trypsin/EDTA for a few minutes at 37°C, 5% CO_2 until complete detachment. This reaction was stopped by adding fresh medium and, subsequently, cells were diluted into fresh medium and incubated at 37°C, 5% CO_2.

For a T75 tissue culture flask 5 ml PBS were used to wash cells, treatment with Trypsin/EDTA was done with 1 ml and this reaction stopped by adding 9 ml of fresh medium. Finally, cells were cultivated with 15 ml medium.

Freezing cells

First of all, an appropriate amount of RPMI 1640 medium containing 10% DMSO was prepared and sterilized by filtration. Then, cells were washed and trypsinized as described above. Subsequently, cells were centrifuged for 4 min at 300 g and resuspended in the prepared medium. Cryo tubes, containing 1 ml of cells, were frozen at -80°C using a Cryo 1°C Freezing Container over night and the following day transferred into liquid nitrogen for long term storage. To obtain vials with 10^6-10^7 cells, a confluent T162 tissue culture flask was used and cells split into three Cryo tubes.

Thawing cells

First, a 50 ml tube and a T75 tissue culture flask with 10 ml prewarmed medium were prepared. Then, one vial of frozen cells, containing 10^6-10^7 cells, was thawed quickly at 37°C and cells were transferred into the prepared 50 ml tube. To remove DMSO, cells were centrifuged for 4 min at 300 g, resuspended in 5 ml fresh medium and added to the tissue culture flask. Cells were incubated at 37°C, 5% CO_2. The day after, medium was changed to remove dead cells and remaining DMSO. Subsequently, cells were incubated until complete confluence and split once before use for experiments.

Seeding cells for experiments

Experiments were done in 6- or 12-well plates. To obtain the desired grade of confluence for experiments cells were counted after trypsination using a counting chamber. Then, an appropriate amount of cells was diluted into fresh medium. Finally, 2 ml per well of cells were incubated over night at 37°C, 5% CO_2. The next day, 50-80% confluent cells were used for experiments.

Treatment of cells with reagents

Medium was changed before any treatment of cells for a given time period with reagents. 1 ml medium was used in the case of 12-well plates, 2 ml medium in 6-well plates.

2.6 Transfection using Lipofectamine 2000

Transfections were done with 2 µg DNA per well of a 12-well plate using the following protocol:

2 µg DNA as well as 2,5 µg Lipofectamine 2000 were added to 125 µl OptiMEM each. The rate of 1:1,25 for DNA:Lipofectamine 2000 was determined experimentally. After a 5 min incubation period equal amounts of DNA/OptiMEM and Lipofectamine 2000/OptiMEM were mixed and incubated for 20 min at RT. Aliquots with a total volume not exceeding 375 µl were mixed for optimal complex generation. Meanwhile, cells were washed with PBS and 1 ml transfection medium was added to each well. After the incubation period, 250 µl of the DNA/Lipofectamine 2000/OptiMEM mix were added drop wise and equally distributed to each well. After 4 hours incubation at 37°C, 5% CO_2 cells were washed with PBS and incubation continued with standard medium. Cells were split to reduce the cell number if necessary. The next day experiments were performed with the transfected cells.

2.7 Protein extracts

Cells were washed once with PBS. Then, 50 µl (12-well plate) sample buffer were added to each well and cell extracts transferred into Eppendorf tubes. Cell extracts were obtained by smearing the sample buffer on the surface with the tip of the pipette.

If cells were already detached from the surface during the experiment, floating cells were collected by centrifugation. In this way, both adherent and detached cells are combined in the cell extract. To collect cells, medium was transferred into Eppendorf tubes and centrifuged for 4 min at 300 g. Then, the pellets were washed with PBS using the PBS with witch the 12-well plate was washed in the meantime. After centrifugation the supernatant was removed. Again 50 µl sample buffer were added to each well of the 12-well plate and cell extracts transferred to the corresponding Eppendorf tube. Cell extracts were frozen at -20°C until further use.

2.8 Sample preparation for fluorescence microscopy

Samples were prepared for fluorescence microscopy as protein expression control for with doxycycline induced HeLa $2A^{pro}$ or HeLa $2A^{proi}$ cells. In both transfected constructs, $2A^{pro}$ is expressed as a fusion protein to EGFP. Therefore, sterile cover slides were placed into 12-well plates before seeding cells. Sterilization was obtained by flaming the cover slides with absolute ethanol.

For final sample preparation cells were washed once with PBS and treated with 500 µl 4% PFA for 10 min. Subsequently, fixed cells were washed twice with PBS and treated with 500 µl PBS/Triton X100 for 5 min to permeabilize cells for the following DAPI staining. After another

two washing steps with PBS cells were treated with 500 µl DAPI (0,04 mg/ml) for 10 min. Cell nuclei were stained to obtain a reference for GFP positive cells. Finally, cells were washed three times with PBS and the cover slides placed on object plates containing a drop of DAKO embedding medium. Samples were dried and stored in dark until microscopy.

2.9 FACS

ROI detection by FACS

Cells were seeded in 6-well plates and treated with desired reagents at 50-70% confluence. Until final washing steps cells were incubated at 37°C, 5% CO_2. 45 min before the end point of the treatment cells were loaded by addition of 5 µM DCF. As a negative control unloaded cells were used, as a positive control cells treated with 10 mM H_2O_2 for 1-2 hours. After 45 min cells were prepared for the measurement.

From now on all work was done in a dark environment. Washing steps were done in the biosafety cabinet without light and all FACS tubes were covered with aluminium foil. To obtain cells in solution medium was removed, cells were washed once with PBS, trypsinized with 200 µl Trypsin/EDTA and the reaction was stopped by adding 800 µl fresh medium. Then, cells were transferred into clear FACS tubes and centrifuged for 4 min at 300 g. The supernatant was removed and cells were resuspended in 1 ml HBSS and centrifuged again. Finally, cells were resuspended in 1 ml HBSS and kept on ice until immediate measurement. A FACSCalibur from BD (Becton Dickinson) was used for measurement using the cell quest pro software and measuring 10.000 cells per sample. The excitation was 480 nm, the emission 525nm.

Detection of BAK/BAX conformational change by FACS

Cells were seeded in 6-well plates and treated with desired reagents at 50-70% confluence. Until final washing steps cells were incubated at 37°C, 5% CO_2.

The first day, medium was removed, cells were washed with PBS, trypsinized with 200 µl Trypsin/EDTA and the reaction stopped by adding 800 µl fresh medium. To include cells that were detached during the experiment, medium and PBS from washing step were collected in a 50 ml tube, together with the trypsinized cells. Subsequently, cells were centrifuged in 50 ml tubes for 4 min at 300 g and 4°C. Cells were resuspended in 1 ml PBS and transferred into Eppendorf tubes. After centrifugation cells were resuspended in 800 µl 2% PFA and incubated at 4°C for 10 min before centrifugation. In the next step, cells were resuspended in 1ml 100 mM NH_4Cl in PBS and incubated at 4°C for 5 min before centrifugation. Then, cells were resuspended in 1 ml PBS and kept at 4°C over night or work proceeded.

To permeabilize cells, cells were washed twice with 500 µl Perm-Wash. All washing steps were again done with centrifugation for 4 min at 300 g and 4°C. Then, cells were incubated in 500 µl

Perm-Wash for 15 min at 4°C before being centrifuged. Cells were resuspended in 500 µl Perm-Wash/BSA and incubated for 10 min at RT. After centrifugation cells were resuspended in the primary antibody dilution and incubated for one hour. All three antibodies, BAK, BAX and Alexa 488 were diluted in Perm-Wash/BSA and incubation was done with 100 µl at 4°C with constant rotation in a 50 ml tube. Afterwards, 450 µl Perm-Wash/BSA were added and cells incubated at RT for 5 min before centrifugation. Then, cells were incubated with the secondary antibody, Alexa 488, for 30 min. Subsequently, 450 µl Perm-Wash/BSA were added and cells again incubated at RT for 5 min before centrifugation. Finally, cells were washed twice with 500 µl Perm-Wash and once with 500 µl PBS. Cells were resuspended in 500 µl PBS and transferred into clear FACS tubes for the measurement. If the measurement was not done immediately, cells were kept at 4°C in the meantime. A FACSCalibur from BD (Becton Dickinson) was used for measurement using the cell quest pro software and measuring 10.000 cells per sample. The excitation/emission maxima of Alexa488 are 488/519 nm.

2.10 SDS-PAGE and Western Blot

SDS-PAGE

For SDS-PAGE 7,5% to 12,5% acrylamide gels were used, depending on protein size. First the separating gel was prepared and covered with dH_2O until it was polymerized. Then, dH_2O was removed and the stacking gel prepared. Finally, the gel was loaded using usually 10 µl of protein samples and 5 µl Precision Plus Protein Standards marker from BIO-RAD. Protein samples were heated before at 95°C for 5-20 min. Electrophoresis was done at 15 mA/gel. Gels were prepared as followed:

Separating gel	7,5%	10%	12,5%	**Stacking gel**	
30% Acrylamide	1,5 ml	2 ml	2,5 ml	Stack solution	2 ml
4 x LGS	1,5 ml	1,5 ml	1,5 ml	10% APS	12 µl
H_2O	3 ml	2,5 ml	2 ml	TEMED	4 µl
10% APS	36 µl	36 µl	36 µl		
TEMED	3,6 µl	3,6 µl	3,6 µl		

Western Blot

Western blotting was done using a semi dry method. Whatman papers soaked in Anode 1, Anode 2 and Cathode buffer and nitrocellulose membrane soaked in dH_2O were used for the blotting sandwich. They were arranged as shown in Fig. 8. A Whatman paper soaked in Cathode buffer was used to detach the acrylamide gel from the glass plate. Blotting was done with 15 V for 90 min. Afterwards, membranes were blocked with 5% milk powder solution in TBS for at least 30 min. Subsequently, membranes were incubated with the primary antibody over night at 4°C in a 50 ml tube with constant rotation. Antibodies were diluted in 5 ml TBS + BSA + 0,05% NaN_3 in a 50 ml tube. Membranes were washed three times for 5 min with TBS before incubation with the secondary antibody for one hour at RT. Secondary antibodies conjugated to HRP were used. All steps were done with gentle shaking. After another three washing steps detection was performed using ECL Super Signal West Pico. Both ECL reagents were mixed 1:1 and membranes incubated for 1-2 min. Then, membranes were covered in cling film and films exposed initially for 1 min.

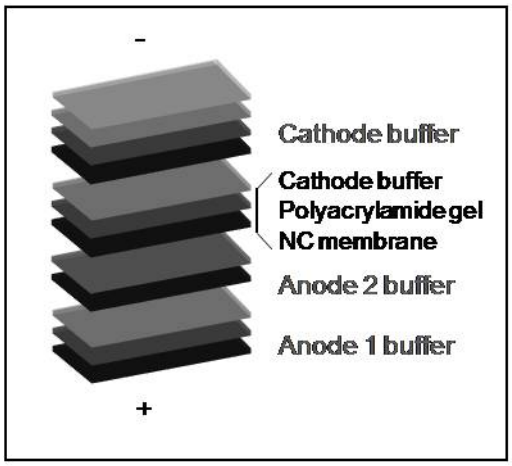

Fig. 8 Blotting sandwich for Western Blot

If membranes were reused for another antibody, they were stripped for 20 min at 50°C in stripping buffer. β-mercaptoethanol was freshly added each time to the stripping buffer.

2.11 Transformation and Midi prep

Competent *E.coli*

A 50 ml over night culture of *E.coli* XL-1 blue was prepared. The following day, 2 ml were added to 100 ml LB medium without antibiotics. Bacteria were grown with vigorous shaking until an OD_{600} of 0,4. Then, bacteria were centrifuged for 5 min at 1400 g and supernatant removed. 50 ml (half volume) sterile $CaCl_2$ were added and the solution kept on ice for 20 min. Bacteria were again centrifuged and supernatant removed. Subsequently, the pellet was

resuspended on ice in 1/15 volume sterile $CaCl_2$ + 15% glycerol. Aliquots of 200 µl were shock frozen in liquid nitrogen and stored at -80°C. Aliquots can be used for a few months.

Transformation

Competent *E.coli* XL-1 blue were used. One aliquot of competent *E.coli* was thawed by leaving it 10 min on ice. Then, 1-3 µg DNA of a midi prep were added to 100 µl competent *E.coli* and the mix left on ice for 15 min. Subsequently, the mix was heat shocked for 45 sec at 42°C and immediately transferred back to ice and 200 µl prewarmed LB medium was added. Incubation was done at 37°C for 30 min before plating on LB agar plates with antibiotic. 50 µl of the incubated bacteria as well as 100 µl of the higher concentrated bacteria solution were plated. The higher concentration was obtained by spinning down for 30 sec with up to 2900 g and subsequent resuspending of bacteria in 100 µl LB medium. Agar plates were incubated over night at 37°C. A negative control was done to exclude contaminations.

Glycerol stocks of transformed bacteria were prepared by mixing 700 µl of an over night culture with 300 µl autoclaved glycerol and freezing it with liquid nitrogen before storage at minus 80°C.

Midi prep

Midi preps were done according to manufactures instructions using the Macherey Nagel Nucleo Bond PC100 kit. 100 ml LB medium with 100 µg/ml antibiotic was inoculated and grown over night with shaking at 37°C. The day after, midi prep was performed. Finally, a control agarose gel electrophoresis was done and DNA concentration measured. DNA was stored at -20°C.

A 1% agarose gel was prepared and electrophoresis done with 120 V using a Horizon 11-14 from GibcoBRL. To prepare the gel agarose was solved in 0,5 x TEA buffer and heated in the microwave to melt it. Samples were loaded using 1 x Orange G.

DNA concentration was measured using a NanoDrop spectrophotometer from Thermo scientific. 1:10 or 1:20 dilutions were prepared if necessary.

3. Results

3.1 PDTC does not inhibit eIF4GI cleavage by HRV2 2A protease

To elucidate the antiviral role of PDTC during the HRV infection process, the influence of PDTC on the activity of the viral $2A^{pro}$ was investigated. Therefore, HeLa cell lines stable transfected with an active (HeLa $2A^{pro}$) or inactive (HeLa $2A^{proi}$) HRV2 2A protease construct under the control of a tetracycline inducible protein expression system were used. To obtain these cell lines cell clones from a single integration event were chosen by using a single cell culture method and selecting for the co-expressed marker. To investigate the influence of PDTC, protein expression in these cell lines was initiated by addition of doxycycline and eIF4GI cleavage by the $2A^{pro}$ was determined in the presence and absence of PDTC. As negative control HeLa $2A^{proi}$ cells were used. The active site nucleophil of the protease in this cell line was replaced and, thus, its biological activity disrupted. As positive control for the cleavage products, HeLa cells were infected with HRV14. From the literature it is known that eIF4GI is cleaved by HRV $2A^{pro}$ during the infection process. This cleavage results in host cell translational shutoff (reviewed in Lloyd, 2005).

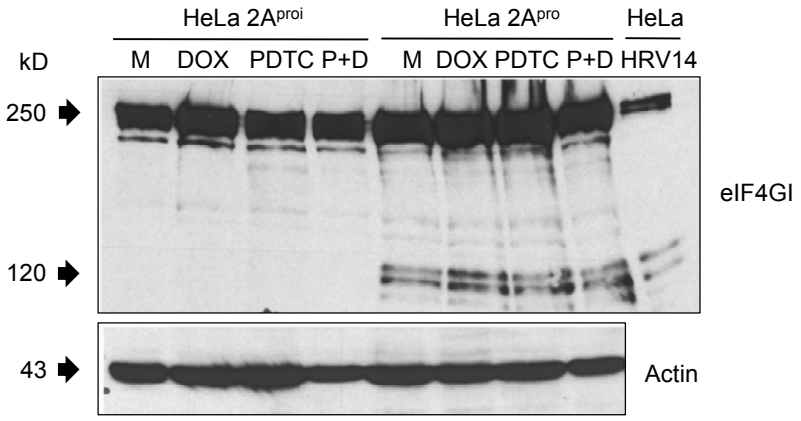

Fig. 9 PDTC does not inhibit eIF4GI cleavage by HRV2 2A protease. Protein expression in HeLa $2A^{pro}$ and HeLa $2A^{proi}$ cells was induced by addition of 1µg/ml doxycyclin (DOX). In addition, cells were treated with 125 µM PDTC (PDTC) or 125 µM PDTC and 1 µg/ml doxycycline (P+D). HeLa cells infected with HRV14 were used as positive control. M represents untreated cells. Protein extracts were prepared after 20 h and Western Blot analysis for eIF4GI and actin as loading control performed.

Fig. 9 shows that PDTC does not inhibit eIF4GI cleavage by HRV2 $2A^{pro}$. It can be seen that no eIF4GI cleavage occurs with HeLa $2A^{proi}$ cells as it was expected. To verify the cleavage products that can be seen in the case of HeLa $2A^{pro}$ cells, eIF4GI cleavage by HRV14 is shown. Further, this figure shows that eIF4GI cleavage occurs already without the inducer doxycycline in HeLa $2A^{pro}$ cells. Moreover, no increase of eIF4GI cleavage can be seen after addition of doxycycline. Thus, these facts outline that the protein expression system is leaky and not inducible in this cell line. However, it was possible to demonstrate that PDTC is not able to

inhibit the partial eIF4GI cleavage by the viral protease as it can be seen with PDTC treated HeLa $2A^{pro}$ cells.

In addition, fluorescence microscopy was performed as control of protein expression. In both inserted constructs $2A^{pro}$ is expressed as a fusion protein to EGFP. Up to 20% green fluorescent cells could be seen after 24h of doxycycline addition to HeLa $2A^{proi}$ cells. No or only very few green fluorescent cells could be observed in non treated HeLa $2A^{proi}$ cells. In the case of HeLa $2A^{pro}$ cells no green fluorescent cells were visible. In addition, no GFP could be detected in Western blot analysis as it was possible with HeLa $2A^{proi}$ cells. Thus, protein expression in HeLa $2A^{proi}$ cells seems to be inducible, whereas protein expression in HeLa$2A^{pro}$ cells is not inducible. The reason for it is unknown.

As these experiments demonstrated that the function of viral proteases is not influenced by PDTC we decided to further investigate its influence on human caspases, members of the cysteine protease family. Previous data from our lab showed that PDTC acts antiapoptotic in HeLa cells.

3.2 PDTC shows antiapoptotic properties in HeLa cells

PDTC is a multifunctional, metal chelating compound. Several properties of PDTC are described in the literature, including antiapoptotic and proapoptotic effects that apparently depend on the cellular system used (Erl et al., 2000). To demonstrate the antiapoptotic effect of PDTC in HeLa cells, cells were treated with inducers of apoptosis and, in addition, with PDTC. Apoptosis was induced with Puromycin, Actinomycin D, TNF α/CHX or FasL antibody/CHX. Puromycin and Actinomycin D were selected as intrinsic apoptosis inducers. TNF α/CHX and FasL antibody/CHX were chosen as extrinsic inducers of apoptosis. CHX was used to improve the apoptotic effect of the extrinsic apoptosis inducers. As a marker of apoptosis PARP cleavage was analyzed by Western Blot. It is well known that PARP is cleaved during apoptosis at a late step. In addition, caspase 8 and 9 activation was investigated in these experiments to determine how they are influence by PDTC during its antiapoptotic action.

Morphologically, HeLa cells treated with one of the apoptotic stimuli should significant progress of cell death after 8 hours. In contrast to healthy cells, they were rounded and detached from the surface. This apoptotic phenotype could be abolished if PDTC was added in addition.

Fig. 10 shows the antiapoptotic effect of PDTC. It can be seen that induction of apoptosis by the different inducers of apoptosis results in PARP cleavage as well as caspase activation. Untreated cells or cells only treated with PDTC do not show any PARP cleavage or caspase activation. A significant reduction of PARP cleavage and caspase 9 activation can be observed when PDTC is added. Interestingly, caspase 8 activation by extrinsic apoptotic stimuli could not be inhibited by PDTC. Caspase 8 activation by intrinsic apoptotic stimuli could be inhibited with

PDTC. Although caspase 8 activation by extrinsic stimuli is not inhibited, PARP cleavage was inhibited and cells were viable. Further, it can be seen that PARP cleavage induced by Actinomycin D and FasL antibody/CHX could be inhibited completely, whereas PARP cleavage induced by Puromycin and TNF α/CHX could not be inhibited completely. Morphologically, in all cases the antiapoptotic effect of PDTC could be clearly seen.

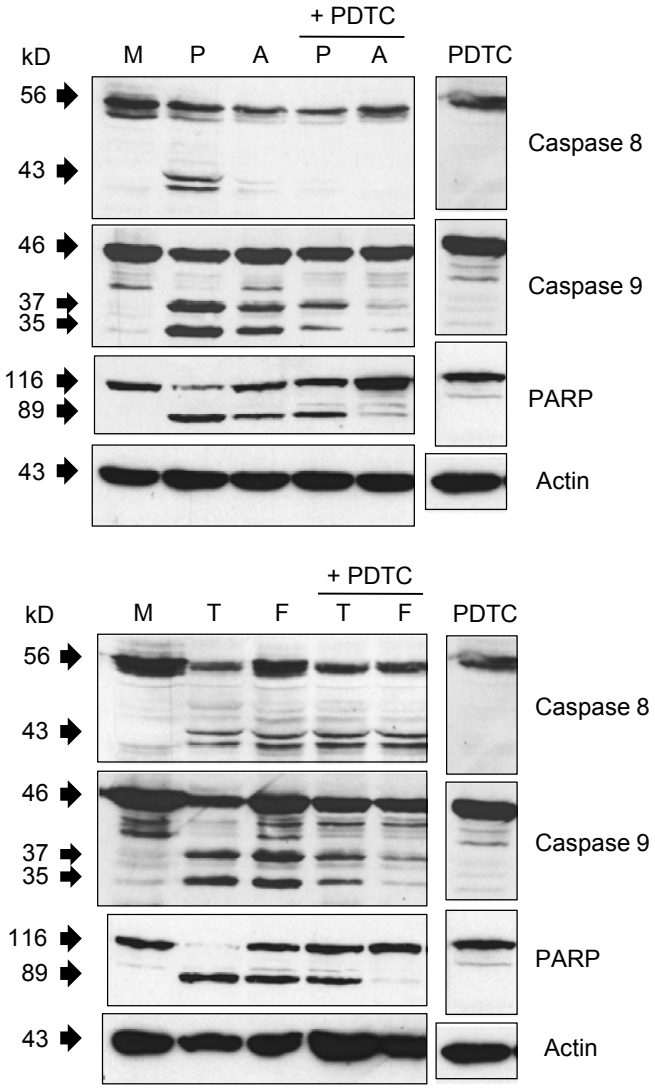

Fig. 10 PDTC acts antiapoptotic in HeLa cells. Apoptosis was induced with 50 µM Puromycin (P), 1 µM Actinomycin D (A), 50 ng/ml TNF α and 10 µM CHX (T) or 0,3 µg/ml FasL antibody and 10 µM CHX (F). In addition, cells were treated with 125 µM PDTC. M represents untreated HeLa cells. Protein extracts were prepared after 8 h and Western Blot analysis for caspase 8, caspase 9, PARP and actin as loading control performed.

In addition, Fig. 10 shows that both, caspase 8 and 9, are activated by intrinsic as well as extrinsic apoptotic stimuli. This suggests a strong feedback mechanism between both pathways of apoptosis. In this context, it is known that proteins from the BCl-2 protein family may play an essential role during apoptosis. It is thought that they possess a key regulatory role during these signalling processes (reviewed in Youle and Strasser, 2008).

To further elucidate the underlying mechanism of the antiapoptotic property of PDTC, diverse strategies were planed. Previous data from our lab using a cell free system showed that the antiapoptotic effect of PDTC is not simply based on inhibition of caspases. Thus, other regulators of apoptosis may be influenced by PDTC. The influence of PDTC on members of the BCl-2 protein family was of particular interest as these proteins are thought to be important regulators of apoptosis. In addition, also the role of oxidative stress during apoptosis and the potential antioxidative property of PDTC were of interest. It is known from literature that oxidative stress may play a considerable role during apoptosis (reviewed in Ott et al., 2007). Further, it was shown that PDTC inhibits the proteasome dependent proteolysis in HeLa cells (Kim et al., 2004). Thus, also this property of PDTC was of interest in context to its antiapoptotic effect. Moreover, Pyrithione and Hinokitiol were used to further elucidate the potential role of metal ions during PDTC activity. They are structural diverse but functional similar metal chelating compounds. This could be shown in experiments of our lab (Krenn et al., in press).

3.3 The antiapoptotic property of PDTC is not linked to proteasomal degradation

To elucidate the potential role of the proteasome inhibitory function of PDTC during its antiapoptotic effect, HeLa cells were treated with inducers of apoptosis and, in addition, with PDTC or MG132. MG132 is a well known proteasome inhibitor and was used to compare its effect with that of PDTC. PARP cleavage was analyzed by Western Blot as a marker of apoptosis. Apoptosis was induced with Puromycin, Actinomycin D or TNF α/CHX in these experiments.

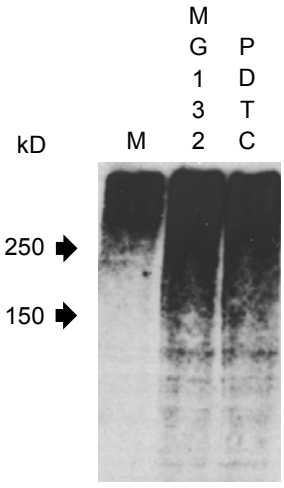

Fig. 11 PDTC inhibits the proteasome dependent proteolysis in HeLa cells. HeLa cells were treated with 1 µM MG132 or 125 µM PDTC for 8 h. M represents untreated cells. Western Blot analysis for Ubiquitin was performed.

First of all, it is shown in Fig. 11 that PDTC inhibits proteasome dependent proteolysis in HeLa cells. It can be seen that cells treated with both compounds, MG132 and PDTC, accumulate ubiquitinylated proteins and, thus, act proteasome inhibitory.

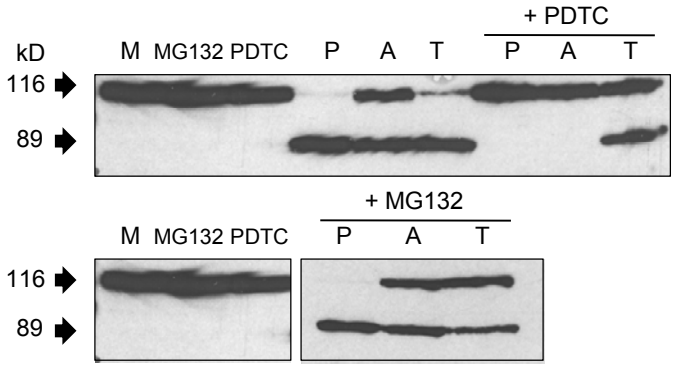

Fig. 12 The antiapoptotic effect of PDTC is not linked to proteasome inhibition. Apoptosis was induced with 50 µM Puromycin (P), 1 µM Actinomycin D (A) or 50 ng/ml TNF α and 10 µM CHX (T). In addition, cells were treated with 125 µM PDTC or 1 µM MG132. M represents untreated cells. Protein extracts were prepared after 8 h and Western blot analysis for PARP performed.

Fig. 12 shows that the antiapoptotic property of PDTC is not linked to proteasomal degradation. Induction of apoptosis by the different inducers of apoptosis results in PARP cleavage which can be reduced significantly by addition of PDTC. By addition of MG132 no significant reduction of PARP cleavage could be obtained. It could further be seen morphologically that MG132 does not possess a similar antiapoptotic effect compared to PDTC. Whereas cells treated with PDTC and the apoptotic inducers did not show an apoptotic phenotype, cells treated with MG132 showed an apoptotic phenotype. Rounded and detached cells could be observed as it was the case for cells only treated with the apoptotic inducers. Therefore, it can be concluded that the proteasome inhibitory property of PDTC is not responsible for its antiapoptotic effect.

3.4 The antiapoptotic property of PDTC is not linked to oxidative stress

To elucidate the role of oxidative stress during the antiapoptotic effect of PDTC reactive oxygen intermediates were measured by FACS. In addition, PARP cleavage was analyzed by Western Blot analyses.

For the measurement of reactive oxygen intermediates HeLa cells were treated with the apoptosis inducers and, in addition, with PDTC. As negative control untreated cells were used and as positive control cells treated with H_2O_2. NAC, a well known antioxidant, was used to compare its effect to that of PDTC in Western Blot analyses. Therefore, HeLa cells were treated with the apoptosis inducers and, in addition, with PDTC or NAC. In all experiments apoptosis was induced with Puromycin, Actinomycin D or TNF α/CHX.

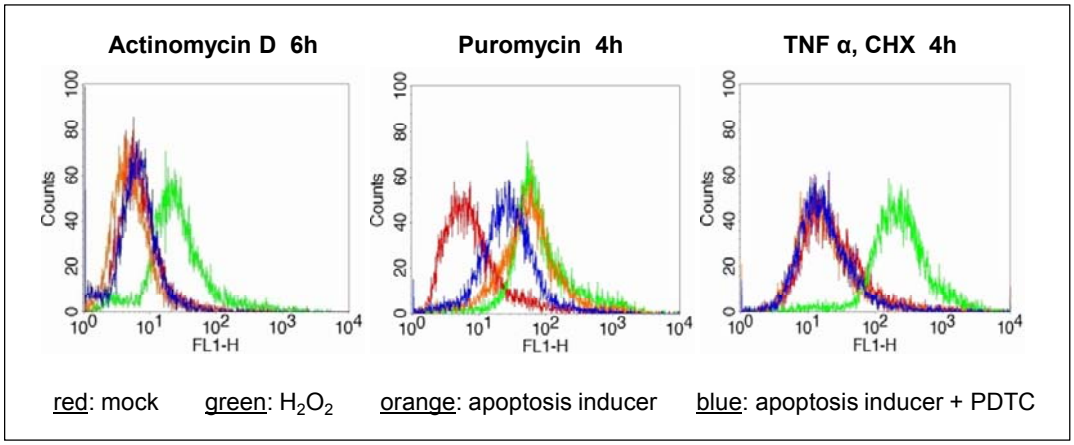

Fig. 13 Oxidative stress is not linked to the antiapoptotic effect of PDTC. HeLa cells were treated with 1 µM Actinomycin D, 50 µM Puromycin or 50 ng/ml TNF α and 10 µM CHX for the indicated time points as apoptosis inducers. In addition, cells were treated with 125 µM PDTC. As positive control cells treated with 5 mM H_2O_2 for 2 h were used. ROI detection by FACS was performed.

Fig. 13 shows a representative FACS graph for each of the used apoptosis inducers. It shows that the potential antioxidative property of PDTC cannot be linked to its antiapoptotic property. Untreated cells shown in red represent the negative control; cells treated with H_2O_2 shown in green were used as positive control. H_2O_2 treatment led to the generation of reactive oxygen intermediates. Treatment with Puromycin for 4 hours resulted in ROI generation, which could be slightly reduced by addition of PDTC. Treatment with Actinomycin D for 6 hours or TNF α/CHX for 4 h did not result in considerable ROI generation. It has to be mentioned that also treatment with Actinomycin D or TNF α/CHX in some cases resulted in slight ROI generation. However, as this is not generally the case as it is with Puromycin, ROI generation cannot be linked to the antiapoptotic effect of PDTC as PDTC always acts antiapoptotic. The induction of apoptosis is not accompanied by ROI generation and, thus, the antiapoptotic effect of PDTC cannot depend on reduction of ROI.

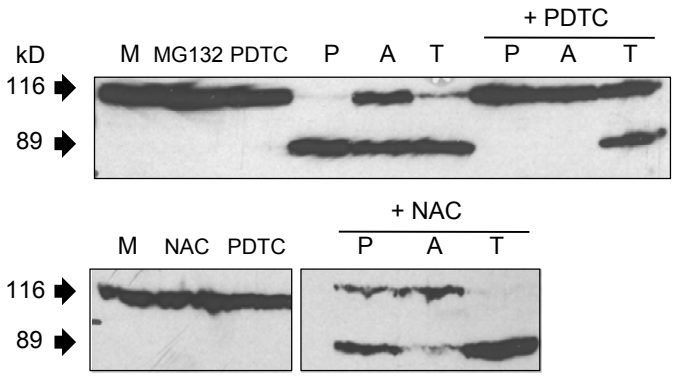

Fig. 14 The antiapoptotic effect of PDTC is not linked to oxidative stress. Apoptosis was induced with 50 µM Puromycin (P), 1 µM Actinomycin D (A) or 50 ng/ml TNF α and 10 µM CHX (T). In addition, cells were treated with 125 µM PDTC or 10 mM NAC. M represents untreated cells. Protein extracts were prepared after 8 h and Western blot analysis for PARP performed.

To further substantiate these data, Fig. 14 shows that NAC, a well known antioxidant, and PDTC do not possess a comparatively antiapoptotic effect. It can be seen that PARP cleavage induced by the apoptosis inducers can be significantly reduced by addition of PDTC, but not NAC. Moreover, this could be seen morphologically where PDTC but not NAC was able to abolish the apoptotic phenotype. Thus, it can be concluded that the potential oxidative property of PDTC is not linked to its antioxidative property.

3.5 The antiapoptotic effect of PDTC may involve metal ions

Former data from our lab demonstrated that the biological function of PDTC involves metal ions (Krenn et al., 2005). It was shown that the antiviral effect of PDTC was abolished by metal ion chelating agents such as EDTA. To further elucidate the potential role of metal ions, the antiapoptotic effect of PDTC was compared with those of Hinokitiol and Pyrithione. They are structural diverse but functional similar metal chelating compounds compared to PDTC. This could be shown in former experiments of our lab (Krenn et al., in press).

To compare the antiapoptotic effect of PDTC, Pyrithione and Hinokitiol, PARP cleavage was analyzed by Western blot. Therefore, HeLa cells were treated with the inducers of apoptosis and, in addition, with PDTC, Pyrithione or Hinokitiol. $MgCl_2$ was added in addition to Hinokitiol due to its toxic effect on HeLa cells without it. In addition, caspase 8 and 9 activation was investigated to elucidate the influence of all three chemical compounds on these caspases. For these experiments Puromycin, Actinomycin D, TNF α/CHX or FasL antibody/CHX were used as apoptosis inducers.

Fig. 15 shows the antiapoptotic effect of PDTC, Pyrithione and Hinokitiol/$MgCl_2$ in HeLa cells. The antiapoptotic effect of PDTC and the implications of caspase activation have already been discussed with Fig. 10, which is a reduced presentation of this figure. Fig. 15 shows in addition that Pyrithione and Hinokitiol/$MgCl_2$ possess a similar antiapoptotic property as PDTC. A significant reduction of PARP cleavage can also be observed by addition of Pyrithione and Hinokitiol/$MgCl_2$. In addition, inhibition of caspase 9 activation can be seen as with PDTC treated cells. Further, it could be shown that also Pyrithione and Hinokitiol are not able to inhibit caspase 8 activation by extrinsic apoptotic stimuli as discussed for PDTC. The antiapoptotic effect of all three compounds could be seen morphologically.

Concluding, it was shown that Pyrithione, Hinokitiol/$MgCl_2$ and PDTC act similar. This suggests that the antiapoptotic effect of these chemical compounds may involve metal ions.

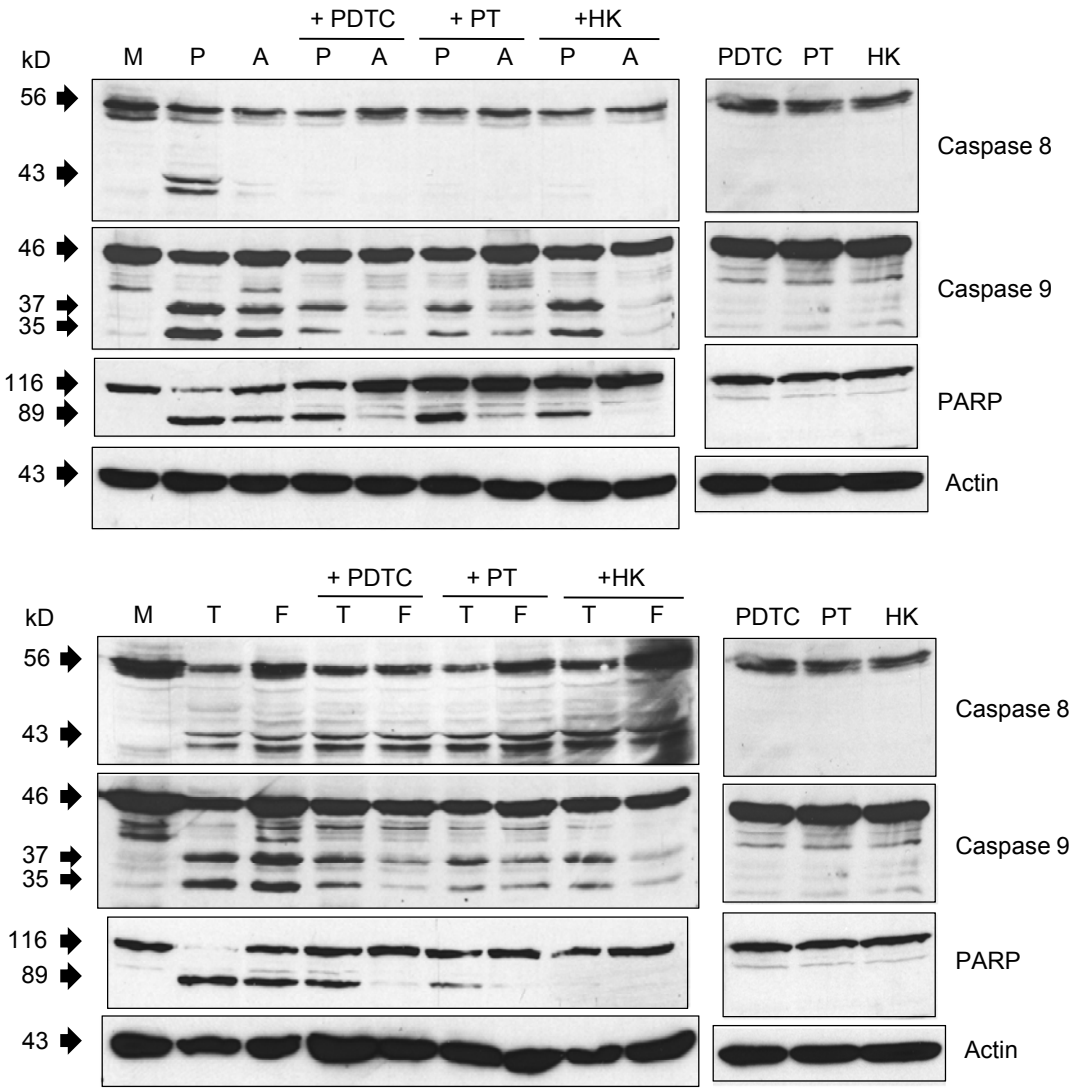

Fig. 15 PDTC, Pyrithione and Hinokitiol/$MgCl_2$ act similar in HeLa cells. Apoptosis was induced with 50 µM Puromycin (P), 1 µM Actinomycin D (A), 50 ng/ml TNF α and 10 µM CHX (T) or 0,3 µg/ml FasL antibody and 10 µM CHX (F). In addition, cells were treated with 125 µM PDTC, 12,5 µM Pyrithione (PT) or 125 µM Hinokitiol and 30 mM $MgCl_2$ (HK). M represents untreated cells. Protein extracts were prepared after 8 h and Western Blot analysis for caspase 8, caspase 9, PARP and actin as loading control performed.

3.6 PDTC inhibits apoptosis by preventing activation of BAX/BAK

To elucidate the potential influence of PDTC on members of the BCl-2 protein family, two proapoptotic BCl-2 protein members were chosen, BAX and BAK. BAX and BAK play a role in the intrinsic apoptotic pathway by inducing cytochrome c release from mitochondria. This action is due to a conformational change, which leads to cytochrome c release and, thus, progression of the apoptotic pathway. The potential role of BAX and BAK is well described in the literature and became an interesting target for this work as these proteins are thought to be responsible directly for cytochrome c release through their integration into the outer mitochondrial membrane (reviewed in Youle and Strasser, 2008).

To investigate the influence of PDTC on BAX and BAK, their conformational status was analyzed by FACS. Therefore, BAX and BAK antibodies were used, which specifically recognise their conformational change during the apoptotic process. Apoptosis was induced in HeLa cells with Puromycin, Actinomycin D, TNF α/CHX or FasL antibody/CHX in these experiments. In addition, cells were treated with PDTC to investigate the antiapoptotic action of it. Different time points were chosen to analyze the time depended effect of the apoptosis inducers and the effect of PDTC. As negative control, untreated cells as well as cells only treated with PDTC were used.

Fig. 16 shows the inhibitory effect of PDTC on BAX and BAK in graphs that were directly obtained from the FACS measurement. It shows that PDTC inhibits the conformational change of BAX and BAK during apoptosis induced by Puromycin. The peak shift along the x-axis that can be observed represents the conformational change of BAX and BAK that occurs during apoptosis. In Puromycin treated cells it can be seen that the conformational change of BAX and BAK progresses with time. By addition of PDTC this conformational change and, thus, the peak shift along the x-axis, can be inhibited. These FACS results correlate with morphological observations in which the antiapoptotic effect of PDTC could be clearly seen.

For an easier presentation of these results gates were set as they can be seen in Fig. 16. Fig. 17 to 19 show the conformational change of BAX and BAK in percentage based on these gate settings. Original data obtained from FACS measurement with the other inducers of apoptosis can be found in Appendix 1-3.

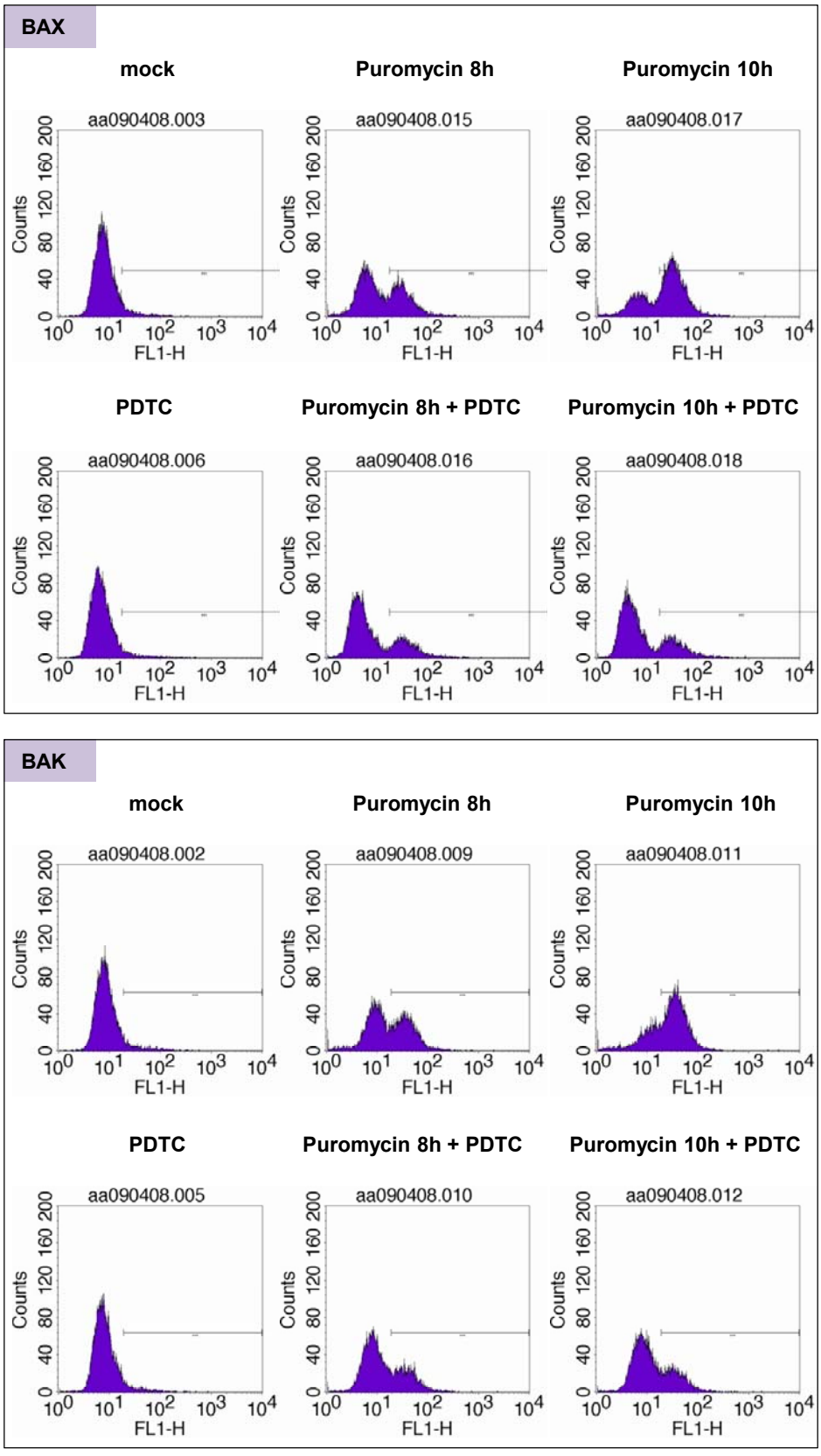

Fig. 16 PDTC is an inhibitor of the conformational change of BAX and BAK during apoptosis. Apoptosis in HeLa cells was induced with 50 µM Puromycin for indicated time points. In addition, cells were treated with 125 µM PDTC. Mock represent untreated cells. FACS analysis was performed using BAX and BAK antibodies, which specifically recognise the conformational change of BAX and BAK. This results in a peak shift along the x-axis.

Fig. 17 shows the inhibition of the conformational change of BAX and BAK by PDTC in percentage based on gate settings during apoptosis induced with Actinomycin D or Puromycin. Mock represents untreated cells or cells treated only with PDTC. For both apoptosis inducers 6, 8 and 10 hours time points were chosen to show the progress of the apoptotic process and, thus, the increasing percentages of BAX/BAK conformational change. It can be seen that there is a constant increase of proteins undergoing this conformational change with time. Further, it could be shown that by addition of PDTC this conformational change can be inhibited. Moreover, this inhibitory function of PDTC seems to form a plateau at about 20-30% in late time points. Although PDTC did act clearly antiapoptotic as it could be seen morphologically and also in previous experiments discussed in this report, the basal level of untreated or only with PDTC treated cells was not obtained after addition of PDTC. This indicates that a higher amount of BAX and BAK undergoing a conformational change is necessary for cytochrome c release.

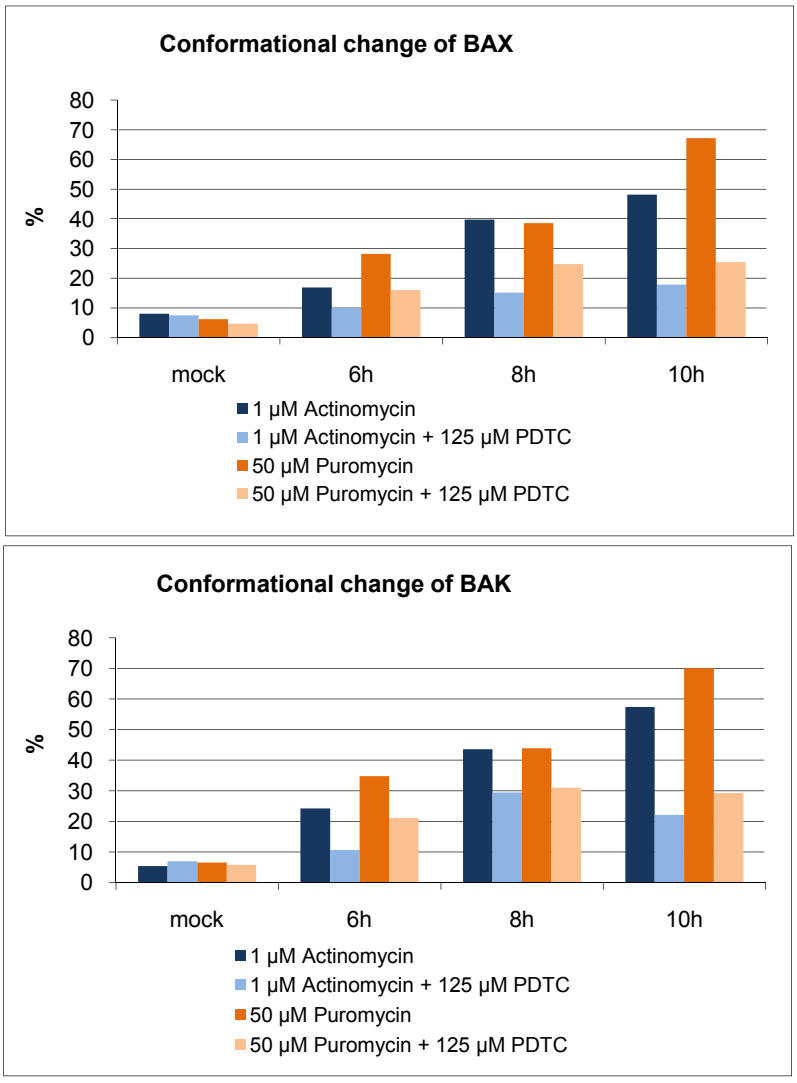

Fig. 17 Conformational change of BAX/BAK shown in percentage based on gate settings. Apoptosis was induced with 50 µM Puromycin or 1µM Actinomycin D. In addition, cells were treated with 125 µM PDTC. Mock represent untreated or PDTC treated cells.

Fig. 18 shows the inhibition of the conformational change during apoptosis induced with TNF α/CHX. For these experiments a 4 and 6 hours time point was chosen as TNF α/CHX showed to be a faster inducer of apoptosis in HeLa cells. Similar results as shown in Fig. 17 can be seen. PDTC showed to be an inhibitor of BAX and BAK conformational change forming a plateau at again about 20%.

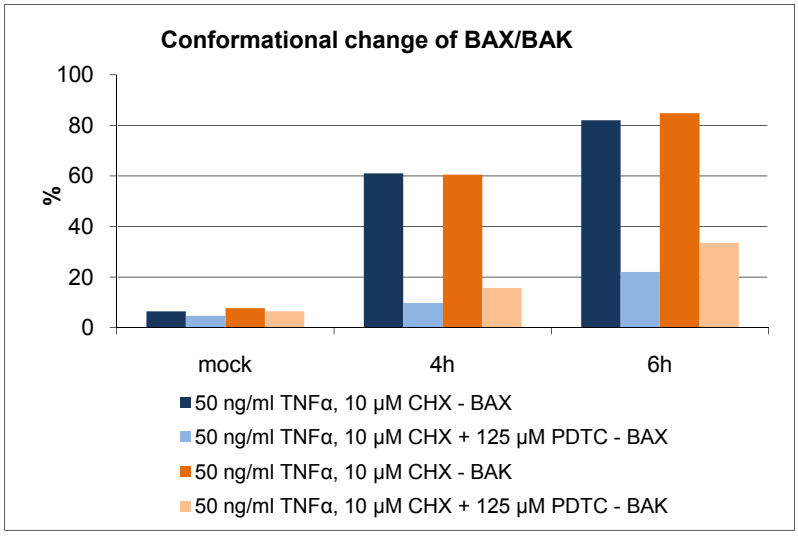

Fig. 18 Conformational change of BAX/BAK shown in percentage based on gate settings. Apoptosis was induced with 50 ng/ml TNF α and 10 µM CHX. In addition, cells were treated with 125 µM PDTC. Mock represent untreated or PDTC treated cells.

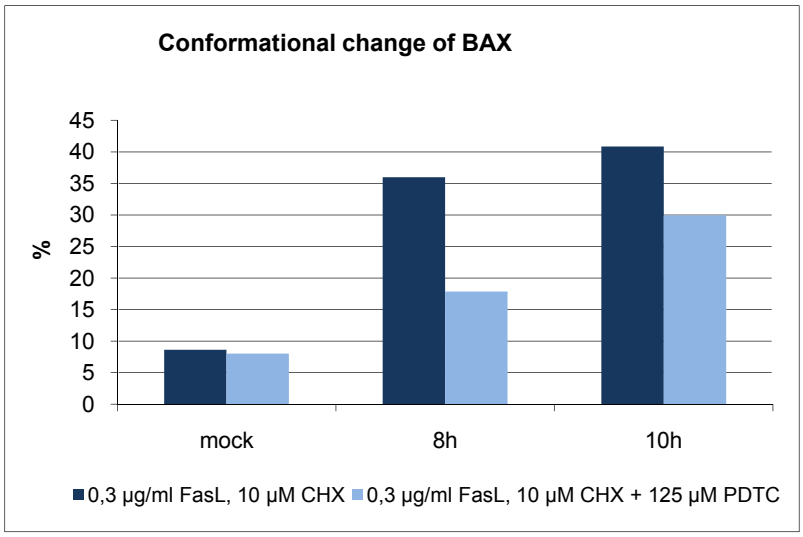

Fig. 19 Conformational change of BAX shown in percentage based on gate settings. Apoptosis was induced with 0,3 µg/ml FasL antibody and 10 µM CHX. In addition, cells were treated with 125 µM PDTC. Mock represent untreated or PDTC treated cells.

Fig. 19 shows this inhibition by PDTC after apoptosis was induced with FasL antibody/CHX only for BAX. For reasons that could not be overcome experiments with the BAK antibody did not anymore result in a peak shift along the x-axis after treatment with an inducer of apoptosis as it was the case in former experiments. As this represents the positive control of this

experimental setup, obtained results are not shown. Choosing an 8 and 10 hour time point similar results were obtained as for the other apoptosis inducer.

In addition, in these figures it can be seen that the antiapoptotic activity of PDTC by inhibiting the conformational change of BAX and BAK is obtained with intrinsic as well as extrinsic apoptotic stimuli. Thus, a strong feedback mechanism between both pathways is likely. Evidence for this could also be seen in Fig. 15. Both, caspase 8 and 9 are activated by intrinsic as well as extrinsic apoptotic stimuli.

Concluding, these experiments demonstrated that PDTC is able to inhibit the conformational change of BAX and BAK during apoptosis. It could be shown that PDTC acts antiapoptotic during this step of the apoptotic process by influencing BAX and BAK. It could not be shown whether PDTC acts directly on BAX and BAK.

3.7 Pyrithione inhibits the conformational change of BAX during apoptosis

To further elucidate the mode of action of PDTC the same experimental setup for FACS measurement was used with Pyrithione as with PDTC in previous experiments. As it could be shown that the function of PDTC may involve metal ions, Pyrithione may have a similar effect on BAX and BAK as PDTC.

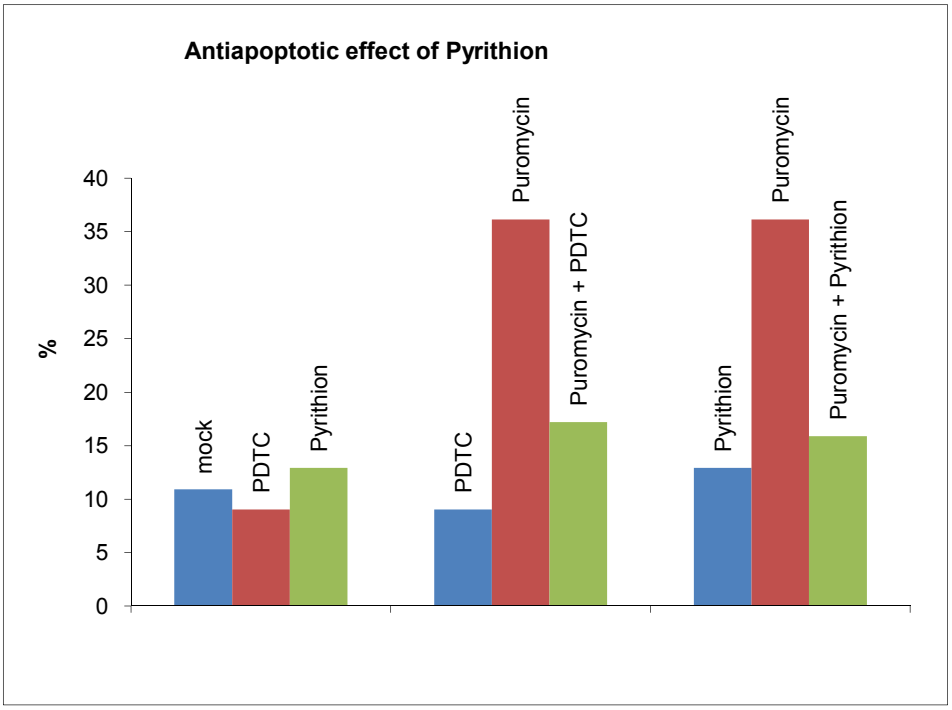

Fig. 20 Conformational change of BAX is shown in percentage based on gate settings. Apoptosis was induced with 50 µM Puromycin for 8 h. In addition, cells were treated with 125 µM PDTC and 12,5 µM Pyrithion. Mock represent untreated cells.

Fig. 20 shows that Pyrithione and PDTC act similar. The figure shows the inhibition of the conformational change of BAX during apoptosis induced with Puromycin. Mock represents untreated cells or cells that were treated only with PDTC or Pyrithione. It can be seen that both, PDTC and Pyrithione, inhibit the conformational change of BAX induced with Puromycin. This suggests that the mode of action of metal chelators may play a considerable role during this inhibition. To further investigate the role of metal ions during this process should be one aim of future work. Original data obtained from FACS measurement of these experiments can be found in Appendix 4.

4. Discussion

4.1 PDTC and its action on viral 2A protease

HRV $2A^{pro}$ and $3C^{pro}$ play an essential role during cleavage of the polyprotein, which is an essential step during the viral replication cycle. In addition, the viral $2A^{pro}$ plays a role in host cell translational shutoff. In this context, eIF4G is the main cleavage target of the viral protease. There are two major forms of the eukaryotic initiation factor 4G, termed eIF4GI and eIF4GII. They play an important role in host cell translation initiation. It was already shown in the literature that eIF4 subunits are cleaved by the 2A protease from human coxsackie- and rhinovirus (Lamphear et al., 1993). Further, in their study they showed that infection of cells with picornaviruses of the rhino- and enterovirus groups causes a shutoff in translation of cellular mRNAs but permits viral RNA translation to proceed. In addition, it was shown that cleavage of eIF4GI by picornaviral $2A^{pro}$ may directly occur (Sommergruber et al., 1994).

In this study it was shown for the first time that PDTC has no influence on the biological activity of the HRV2 $2A^{pro}$ in cell culture. Partial cleavage of eIF4GI by the $2A^{pro}$ could be observed with and without addition of PDTC. No difference of eIF4GI cleavage was observed in HeLa cells. This means that PDTC does not act directly on the $2A^{pro}$ during its antiviral activity. The antiviral property of PDTC was shown in previous studies (Gaudernak et al., 2002; Lanke et al., 2007).

4.2 The antiapoptotic property of PDTC

PDTC is a chemical compound that is described in the literature to possess diverse properties. This includes pro- and antiapoptotic properties, pro- and antioxidative properties as well as a proteasome inhibitory property.

The antiapoptotic property of PDTC is discussed very controversial in the literature. It was shown by Erl and colleagues that PDTC induced apoptosis depends on cell type and density (Erl et al., 2000). There is evidence for the proapoptotic effect of PDTC (Della Ragione et al., 2000; Morais et al., 2006; Daniel et al., 2008) as well as for the antiapoptotic effect of PDTC (Verhaegen et al., 1995; Nobel et al., 1997; Cheng et al., 2006) as described in the introduction. In this study it could be clearly shown that PDTC acts antiapoptotic in HeLa cells.

It was shown that the antiapoptotic property of PDTC is not linked to its potential antioxidative property. As it was shown that induction of apoptosis by several inducers of apoptosis does not generally result in generation of oxidative stress, the potential antioxidative property of PDTC cannot be linked to its antiapoptotic effect. ROI production induced by Puromycin, the only apoptosis inducer used that induced oxidative stress, could only slightly be reduced by

addition of PDTC. Moreover, in some cases during our experiments with PDTC it acted slightly prooxidative. Thus, albeit PDTC is generally described as an antioxidant, there is also evidence for its prooxidative property. This could further be shown by Tapia and colleagues (Tapia et al., 2008). Concluding, in this study it could be shown that PDTC may not be a strong antioxidative chemical compound. However, further experiments would be necessary to elucidate this property of PDTC.

Further, in this study it could be shown that PDTC is a potent proteasome inhibitor in HeLa cells. This was already shown by Kim and colleagues. They showed that PDTC and zinc inhibit proteasome dependent proteolysis in HeLa cells (Kim et al., 2004). Interestingly, during this study it could also be shown that PDTC does not inhibit the proteasome dependent proteolysis in HeLa $2A^{proi}$ cells (data not shown). This may suggest that the proteasome inhibitory effect of PDTC is dependent on several, not yet defined, intracellular factors. It was shown within this study that the antiapoptotic effect of PDTC cannot be linked to its proteasome inhibitory effect.

Most interesting in this study it was shown for the first time that PDTC acts antiapoptotic by preventing the activation of BAX and BAK. Weather PDTC acts directly or indirectly on BAX and BAK could not be shown. It is well described in the literature that BAX and BAK, both members of the proapoptotic BCl-2 protein family, play an essential role in cytochrome c release from mitochondria and, thus, apoptosis (reviewed in Youle et al. and Chipuk et al., 2008). Thus, this mechanism could be of great importance during the apoptotic process.

It could be demonstrated that Pyrithione and Hinokitiol possess a similar biological activity as PDTC. This may suggest that the antiapoptotic effect of PDTC may involve metal ions as they are all known metal chelators. However, further experiments are necessary to confirm it. From the literature it is already known that PDTC acts antiviral by transporting zinc ions into cells (Lanke et al., 2007). Further, Erl and colleagues reported in their study that the biological activity of PDTC depends on the presence of copper and zinc ions (Erl et al., 2000). In addition, the antiviral activity of Pyrithione and Hinokitiol against picornaviral infections was recently discovered by our group (Krenn et al., in press). These findings suggest that the antiapoptotic effect of PDTC may also involve metal ions.

Finally, it could be demonstrated that both, intrinsic as well extrinsic apoptotic stimuli activate caspase 8 and 9. This suggests a cross talk between both pathways of apoptosis. It was already shown that the extrinsic pathway may intersect with the intrinsic pathway through BID cleavage (reviewed in Youle and Strasser, 2008). Further work is necessary to elucidate the role of BCl-2 proteins during this crosstalk.

4.3 Proposed interaction and outlook

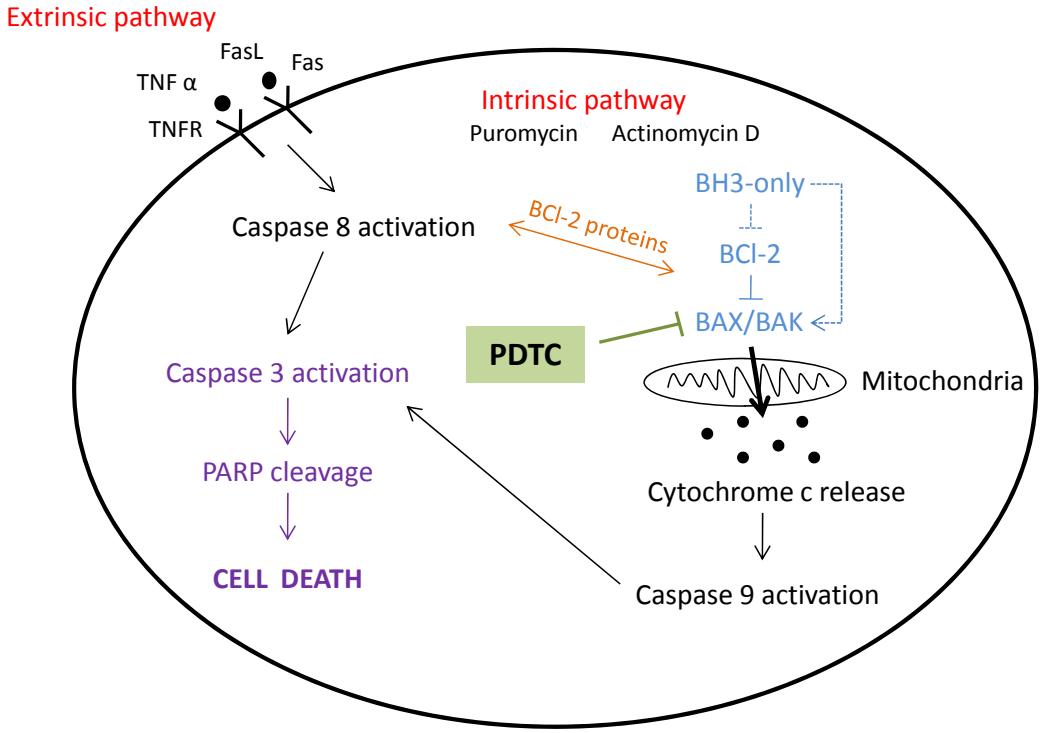

Fig. 21 Schematic view of apoptosis, the action of BCl-2 family proteins and the mode of action of PDTC.

In the first part of this study it was possible to show that PDTC cannot inhibit the cleavage of eIF4GI by the HRV2 $2A^{pro}$ although the used experimental system was leaky. Next to that, the role of the viral $3C^{pro}$ and its influence by PDTC should be investigated. Therefore, a tighter regulated protein expression system should be selected. In addition, in vitro experiments with $3C^{pro}$ could be undertaken. Reasons for its importance can also be found in the literature where it is described that both viral proteases may be necessary for efficient and complete host cell translation shutoff (reviewed in Lloyd, 2005).

Further, during this study it was possible to demonstrate that PDTC acts antiapoptotic by preventing the conformational change of BAX and BAK. It could not be shown if PDTC acts directly or indirectly on these members of the BCl-2 protein family. To further elucidate the effect of PDTC on members of the BCl-2 protein family, the influence of PDTC on antiapoptotic members of the BCl-2 protein family and on BH3-only proteins should be investigated. From literature it is known that the antiapoptotic BCl-2 protein members inhibit BAX and BAK, both members of the proapoptotic BCl-2 proteins. How this inhibition is overcome is not yet clear. There are two main hypotheses. It is thought that either BH3-only proteins bind directly to antiapoptotic BCl-2 proteins and, thus, inhibit their function or that BAX and BAK may be directly activated by some BH3-only proteins (reviewed in Youle et al. and Chipuk et al., 2008). The investigation of the influence of PDTC on members of the BCl-2 protein family could further give insights into the assumed strong crosstalk between the extrinsic and intrinsic apoptotic pathway.

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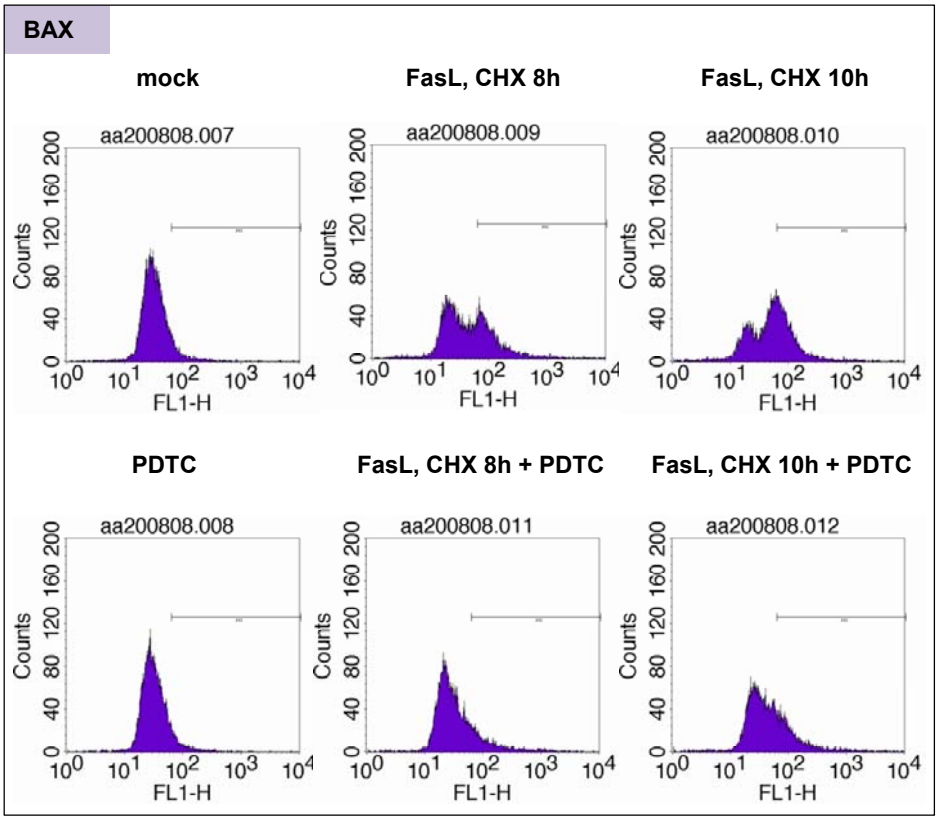
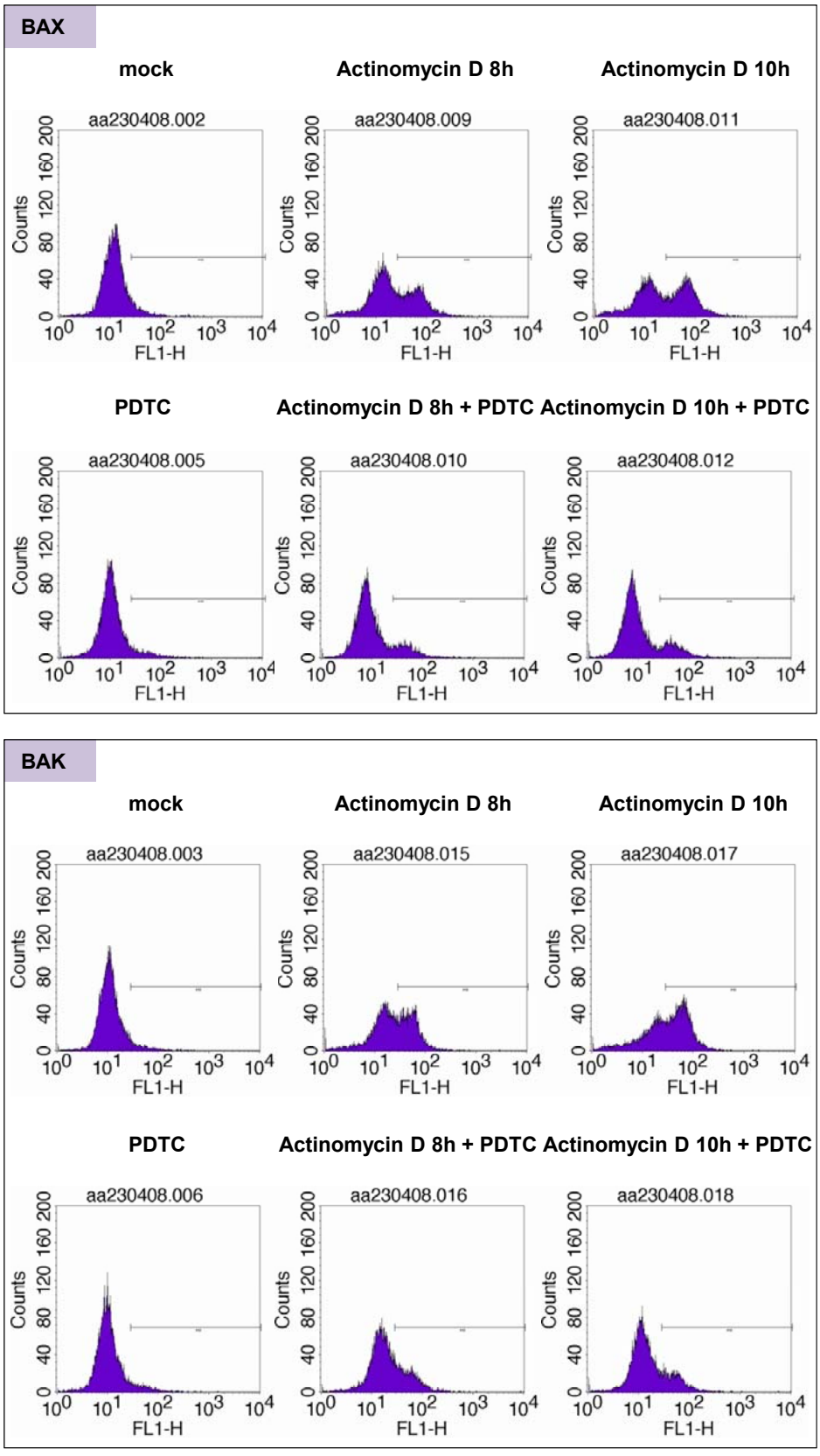
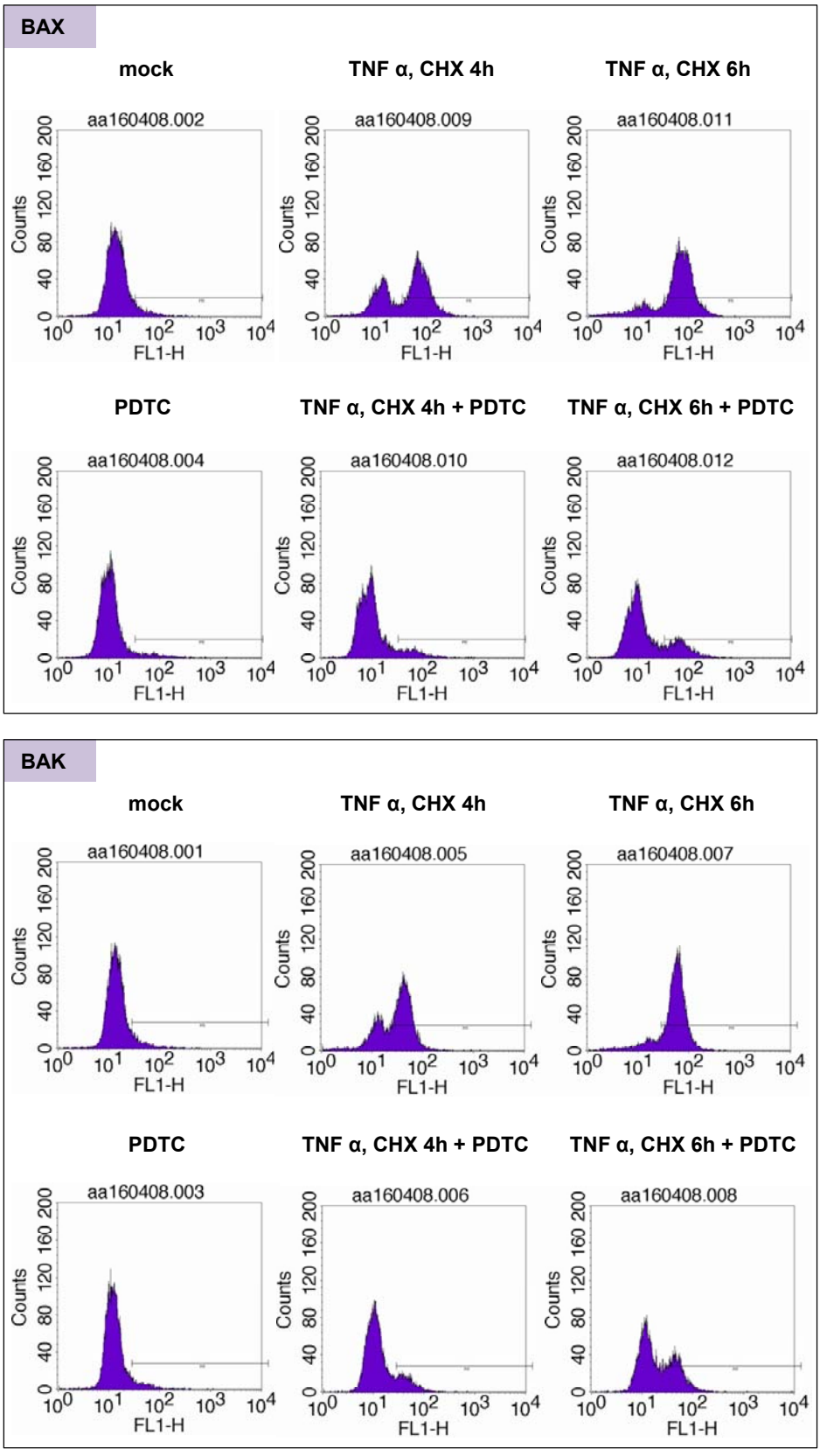
Appendix



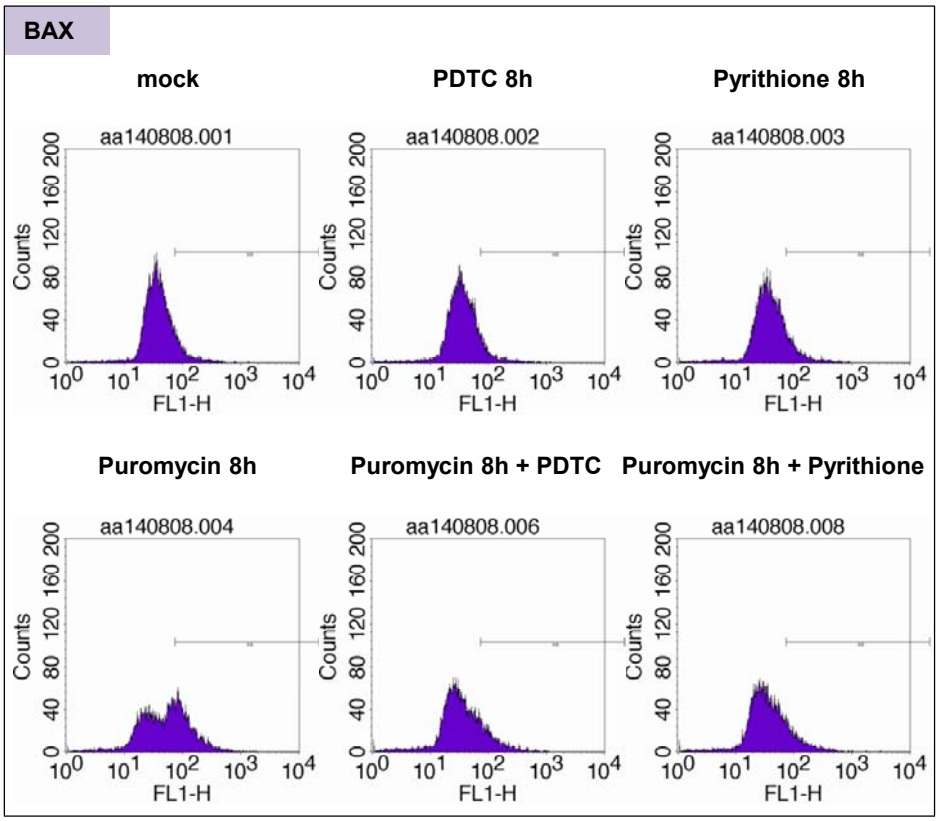
Appendix 3 PDTC is an inhibitor of the conformational change of BAX during apoptosis. Apoptosis in HeLa cells was induced with 0,3 µg/ml FasL antibody and 10 µM CHX for indicated time points. In addition, cells were treated with 125 µM PDTC. Mock represent untreated cells. FACS analysis was performed using BAX antibody.



Appendix 1 PDTC is an inhibitor of the conformational change of BAX and BAK during apoptosis. Apoptosis in HeLa cells was induced with 1 μM Actinomycin D for indicated time points. In addition, cells were treated with 125 μM PDTC. Mock represent untreated cells. FACS analysis was performed using BAX and BAK antibodies.



Appendix 2 PDTC is an inhibitor of the conformational change of BAX and BAK during apoptosis. Apoptosis in HeLa cells was induced with 50 ng/ml TNF α and 10 μM CHX for indicated time points. In addition, cells were treated with 125 μM PDTC. Mock represent untreated cells. FACS analysis was performed using BAX and BAK antibodies.



Appendix 4 Pyrithione and PDTC act similar. Apoptosis in HeLa cells was induced with 50 µM Puromycin for indicated time points. In addition, cells were treated with 125 µM PDTC or 12,5 µM Pyrithione. Mock represent untreated cells. FACS analysis was performed using BAX antibody.

Curriculum vitae

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