Apoptotic Signaling beyond Caspase Activation in Hepatocytes

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Abbreviations

αCD95	**agonistic anti-CD95-antibody**
Ac-DEVD-AFC	**N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin**
ActD	**Actinomycin D**
AEBSF	**Pefabloc SC^{TM} / 4-(2-Aminoethyl)-benzenesulfonyl fluoride**
ATP	**Adenosintrisphosphate**
BIR	**Baculoviral inhibitory repeat**
BSA	**Bovine serum albumine**
Ca-074Me	**[L-3-trans-(Propoylcarbamoyl)-oxirane-2-carbonyl]-L-isoleucyl-proline methyl esther**
CARD	**Caspase recruitment domain**
CD	**Cluster of differentiation**
CD95	**Fas, Apo-1**
CD95L	**CD95-ligand**
CBHA	**m-Carboxycinnamic acid bis-hydroxamide**
CHAPS	**3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate**
CHX	**Cycloheximide**
Cyt c	**Cytochrome c**
E64-d	**(2S,3S)-trans-Epoxysuccinyl-Leucyl-amido-3-methylbutane ethyl ester**
DD	**Death domain**
DED	**Death effector domain**
DISC	**Death-inducing signaling complex**
DMSO	**Dimethyl sulfoxide**
DTT	**Dithiothreitol**
DNMT	**DNA methyltransferase**
ECL	**Enhanced chemiluminescence**
EDTA	**(Ethylenedinitrilo)tetraacetic acid**
EGFP	**Enhanced green fluorescence protein**
EGTA	**Ethylene-bis(oxyethylenenitrilo)tetraacetic acid**
FADD	**Fas-associated protein with death domain**
Fas	**FS-7-associated surface antigen**
FCS	**Fetal calf serum**
FLICE	**FADD-like IL-1beta-converting enzyme**
FLIP	**FADD-like IL-1beta-converting enzyme interacting protein**
HC	**Hepatocyte**
HCC	**Hepatocellular carcinoma**
HDAC	**Histone deacetylase**
HDI	**Histone deacetylase inhibitor**
HEPES	**4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid**
HSA	**Human serum albumin**
hu	**human**
i.v.	**intravenously**

Abbreviations

JNK	**Jun-terminal kinase**
KCl	**Potassium chloride**
LDH	**Lactate dehydrogenase**
mAb	**monoclonal antibody**
M344	**N-Hydroxy-7-(4-dimethylaminobenzol)aminoheptanamide**
MgCl2	**Magnesium chloride**
MOMP	**Mitochondrial outer membrane permeabilization**
MTI	**DNA methyltransferase inhibitor**
mu	**murine**
NaCl	**Sodium chloride**
NaH2PO4	**Sodium dihydrogenphosphate**
NF-κB	**Nuclear factor- κB**
NIW	**1-Naphthalenesulfonyl-Ile-Trp-CHO**
PAGE	**Polyacrylamide gel electrophoresis**
PARP	**Poly (ADP-ribose) polymerase**
PBS	**Phosphate buffered saline**
PCD	**Programmed cell death**
PS	**Phosphatidylserine**
RAIDD	**Rip-associated ICE-homologous protein with death domain**
RIP	**Receptor-interacting protein**
ROS	**Reactive oxygen species**
S.E.M.	**Standard error of means**
SD	**Standard deviation**
SDS	**Sodium dodecyl sulfate**
TLCK	**N,p-Tosyl-L-lysine-chloromethyl ketone**
TNF	**Tumor necrosis factor**
TNFR	**Tumor necrosis factor receptor**
TPCK	**N,p-Tosyl-L-phenylalanine-chloromethyl ketone**
TRADD	**TNF receptor-associated ptotein with death domain**
TRAF2	**TNF receptor-associated factor 2**
TRAIL	**TNF-related apoptosis inducine ligand**
Tris-HCl	**Tris(hydroxymethyl)aminomethane hydrochloride**
VPA	**Valproic acid**
vs.	**versus**
XIAP	**X-linked inhibitor of apoptosis protein**
zVAD-fmk	**N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone**

1 Introduction

1.1 The characteristics of programmed cell death

Balance between cell division and cell death is of utmost importance for the development and maintenance of multicellular organisms. Disorder of either process leads to severe patho-physiologic consequences like distorted embryogenesis; neurodegenerative disorders, autoimmune diseases and cancer [1]. Therefore, the equilibrium between life and death of an individual cell is tightly controlled and faulty elements can be effectively eliminated by a process called programmed cell death (PCD) [2]. The term "programmed" in PCD refers to the evolutionary conserved time- and position-programmed cell death during development of an organism, as firstly described by Sydney Brenner, Robert Horvitz and John Sulston. Their work on the contribution of PCD on the development of the model organism *Caenorhabditis elegans* was awarded with the 2002's Nobel price for Physiology or Medicine.

Programmed cell death is defined as a physiological mechanism, occurring in a temporal sequence, which is dependent on and executed by active cellular processes that can be intercepted by pharmacological interfering with intracellular signaling [1,3,4].

1.1.1 Apoptosis

Apoptosis was classified as a morphological entity in a landmark paper by Kerr and coworkers in 1972, who described the formation of apoptotic bodies from a cell [5]. The term apoptosis derived from the ancient Greek, defining the "falling off of petals from a flower". Carl Voigt, however, first described the phenomenon of apoptotic cells more than 100 years earlier in 1842 [6]. Apoptosis is a physiological form of PCD, however, it can also be induced by e.g. cancer drugs [6].

In the early 1990`s the study of cell death genes in the nematode worm *Caenorhabditis elegans* has led to the discovery of a family of cysteinyl-aspartate-cleaving proteases, shortly named caspases [7,8,9]. Caspases are regarded as the principal intracellular initiators and executors leading to apoptotic cell demise. The typical features of a cell dying apoptotically comprise nuclear condensation of chromatin, internucleosomal fragmentation of DNA, condensation of cytoplasm, rounding-up and detachment of cells and subsequently inclusion of cell contents into subcellular particles named apoptotic bodies [2]. Apoptosis is over 20 times faster than mitosis therefore sightings of dying cells *in vivo* are rare. Apoptotic cells are

removed by professional phagocytes or neighbouring cells without inflammatory reactions harming surrounding tissue.

Since the discovery of the utmost importance of apoptosis in many physiological and pathophysiological conditions, its regulation and activation by caspases has become a major research area. The term PCD is often used synonymously with apoptosis. However, PCD is the more general expression as it refers to both apoptotic and nonapoptotic cell death pathways. Not all forms of PCD share the characteristic morphology and sequences of apoptosis, but all types of PCD are highly regulated processes.

1.1.2 Programmed cell death without caspase activation

Contrary to earlier expectations, it shed to light that caspase activity is not the sole determinant of life and death decision in PCD [10,11]. Instead, it was demonstrated that inhibition of caspase activation revealed or even enhanced underlying caspase-independent death programmes. The existence of these back-up death pathways was uncovered in many cell death models including those triggered by death receptor agonists [12-14], cancer drugs [15], growth factor deprivation or expression of Bax-related proteins [16].

For example, as one of the first hints of caspase-independent PCD, it was shown by Xiang *et al*. in 1996, that inhibition of caspases did not protect Jurkat cells from cell death itself, but instead changed the morphology of the dying cells [16]. Additionally, the results obtained by Künstle *et al.* in 1999 that TNF-a mediated organ failure without caspase-3-like protease activation in mice liver and also the observations by Cauwel *et al*. in 2003 that TNF-a-mediated cell death in mice is enhanced under caspase inhibition, revealed that caspase-independent PCD is not restricted to *in vitro* models [17].

1.1.3 Necrosis

In contrast, necrosis or accidental cell death occurs particularly in pathological situations. This term refers to a passive, barely regulated form of cell demise, characterized by disintegration and lysis of the nucleus, edematous organelle- and cell-swelling [18]. The final rupture of the cell membrane spills cell contents into tissue causing leukocyte infiltration and a general inflammatory response [19].

1.2 Classification of programmed cell death

As a result of the investigation of caspase-independent forms of PCD, it became evident that the classical dichotomy of apoptosis and necrosis are only the extreme endpoints of a continuum of cell death modes. To categorize the different modes of cell death with their ample variance of morphological and biochemical features, several models were proposed.

1.2.1 Classification according to caspase involvement

Samali *et al.* proposed in 1999 to define apoptosis in biochemical terms as a strictly caspase-mediated cell death with the associated, characteristical morphology described in chapter 1.1.1. Samali *et al*. argue that caspase activation provides a common biochemical basis underlying the typical, apoptotic morphology despite the heterogeneity in the different cell types with regard to their activation and control of apoptosis. Other, caspase-independent forms should be simply termed as cell death until the distinguishing morphology and their underlying mechanism are clearly elucidated [20].

1.2.2 Classification according to morphology

Upon a lethal stimulus, a cell can die in different ways that can be classified according to their nuclear morphology and fate as proposed by Leist and Jäättelä in 2001, no matter if caspases are activated or not. Additional to the above described classical apoptotic and necrotic mode of cell death two additional subclasses are defined [3].

The term *apoptosis-like PCD* defines a sort of cell death characterized by a less compact chromatin condensation than in *classical apoptosis.* The type of condensation is geometrically rather complex with lumpy shapes as found by action of AIF, endonuclease G, cathepsins or other proteases. Like in classical apoptosis the recognition molecules like phosphatidylserine (PS) for macrophage phagocytosis are displayed on the cell's outer membrane. Most published forms of caspase-independent apoptosis fall into this category. This type of cell death is very rich in variances, as any degree and combination of apoptotic features can be found.

The term *necrosis-like PCD* is used to define a sort of cell death, which is characterized by the complete absence of chromatin condensation or at least chromatin clustered to loose speckles. Varying degrees of other apoptotic-like features, including PS exposure might occur.

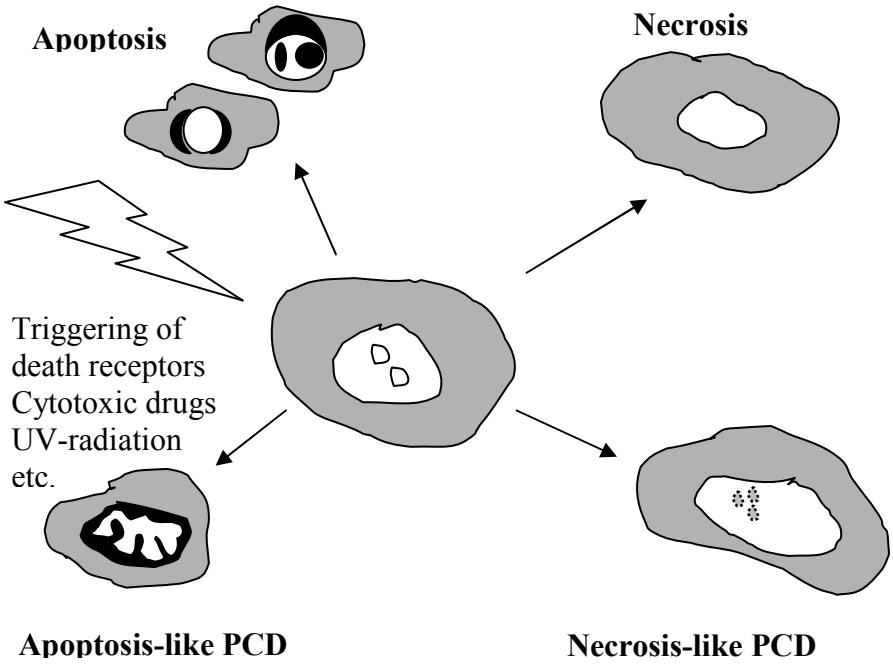


Fig 1.1: Classification of cell death according to the nuclear morphology of the dying cell according to the model proposed by Leist and Jäätellä [3].

1.2.3 Classification according to mechanistic features

In contrast to the descriptive classification model by Leist and Jäättelä, more specific definitions were proposed to categorize different kinds of PCD, which have a complete caspase independency in common.

Autophagy (derived from the Greek term for self-digestion): This process is characterized by sequestration and sub sequestration of cytoplasm and organelles into multimembrane autophagic vesicles. The subsequent delivery and degradation of these vesicles by the cell's own lysosomal system serves to eliminate long-lived proteins and organelle components. Within distinct degrees this process may function for cellular remodeling due to differentiation, stress or damage induced by cytokines. However, cells that undergo excessive autophagy are triggered to die without contribution of caspases [21].

Paraptosis: This process has only recently described as cytoplasmatic vacuolization beginning with progressive swelling of mitochondria and endoplasmatic reticulum. This process still remains widely obscure. It neither involves caspase activity nor does it display typical apoptotic hallmarks. It is mediated by mitogen-activated protein kinases [22] and can be

triggered by the TNF receptor family member TAJ/TROY [23] and insulin-like growth factor I receptor [22].

Mitotic catastrophe: This non-apoptotic pathway is triggered by mitotic failure caused by defective cell cycle checkpoints to hinder the development of aneuploid cells [24]. It can be induced by agents stabilizing/destabilizing microtubules and DNA damage. Whether mitotic catastrophe is indeed fully caspase-independent is still a matter of controversy [25].

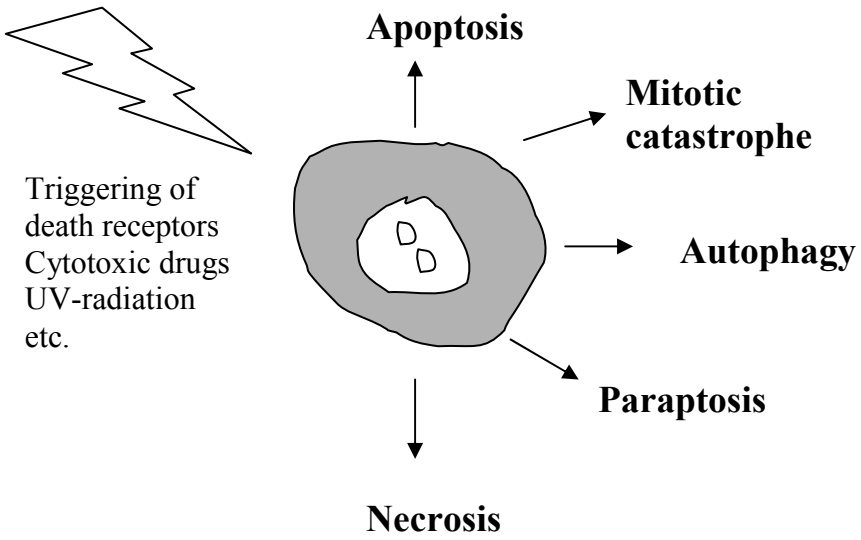


Fig 1.2: Classification of cell death according to mechanistic features (adapted from Broker [1]).

Death signaling in cells appears to be more complex than originally thought. The simple caspase activation model is not sufficient to explain the ample variance of morphological and biochemical features in PCD. Triggering cell death can result in multiple endpoints by action of signaling routes acting in concert with caspases or by utilizing non-caspase effectors. Exclusive definitions to categorize each type of cell death into distinct patterns are difficult to accomplish, since dying cells exhibit often markers and features of different types of PCD concomitantly, presumably due to shared pathways and mechanisms.

1.3 Apoptosis in the liver

The liver possesses a remarkable capacity for regeneration, unique among the tissues and organs of the human body. After injury or partial resection, as little as 25% of remaining liver tissue can regenerate into a whole organ again with fully reconstituted functions [26]. Quiescent cells are primed to enter cell cycle for proliferation under the influence of various proteins, growth factors and cell cycle-dependent kinases [27,28]. A key role for proliferation of the

regenerating liver has the cytokine TNF-α and its respective receptor TNF-R1 due to TNF-α mediated activation of c-Jun-terminal kinase (JNK) and the prosurvival transcription factor AP-1 and NF-κB, respectively [29,30]. After partial liver resection TNF-α levels in patients are rapidly increased [31]. Mice deficient for the NF-κB transducing gene RelA died already in an embryonic state due to massive liver apoptosis, which was dependent on TNF-R1. Mice deficient for both, TNF-R1 and RelA developed normally. A fact that impressively showed the pleiotropic functions of TNF-α and the overall necessity of the TNF-α/TNF-R1/NF-κB signaling system for the development and regeneration of the liver, as observed by Rosenberg *et al.* in 1998.

Therefore, TNF-α and also CD95L, another member of the TNF-superfamily play important roles in the induction of hepatic apoptosis. Enhanced TNF/ TNF-R1 expression was observed in alcoholic liver disease and fulminant hepatocytes apoptosis [32-34] and other diseases causing hepatic impairment like Wilson's disease, endotoxin-induced liver failure and ischemia/ reperfusion-induced liver damage [35-38]. In human patients suffering on hepatitis B an upregulation of CD95 expression with accompanied increase in liver apoptosis was observed [39-41]. Furthermore, an upregulation of CD95L was described for transformed, carcinogenic hepatocytes [42].

In summary, death receptors exert pleiotropic functions on the liver as they signal to both, cell proliferation and apoptosis depending on the circumstances. This tightly controlled regulation of death receptor-mediated apoptosis seems to play a significant role for the vitality of the liver. However, death receptor signaling pathways might also be a target for the prevention of exaggerated hepatocyte death in certain pathophysiologic conditions [43].

1.3.1 Death receptors and their ligands

The extrinsic pathway of apoptosis is induced by activation of so-called "death receptors". These death receptors are located on the cell surface and belong to the huge family of the tumor necrosis factor receptor (TNFR) gene superfamily, of which, up to now, 29 are known. They play important roles in a variety of biological events and control mechanisms. The most thoroughly studied are cell death induction and regulation of the inflammatory process [44]. The signaling of the apoptosis-inducing receptors TNFR-1, Fas/CD95 and TRAIL receptors DR-4 and DR-5 follows, in principal, an identical scheme of events [45]. The binding of the respective ligand results into trimerisation/clustering of the receptors on the cell surface. This signal is

transmitted through the membrane to the cytoplasmatic parts of the receptors, the so-called death domain (DD). The activated death domain constitutes an assembly point for the death-inducing signaling complex (DISC) leading subsequently to activation of the intracellular signaling cascade [2].

1.3.1.1 TNF-R1/TNF-a

The TNF receptor 1 (TNF-R1) is a 55 kDa transmembrane receptor, which is trimerized upon binding of its ligand TNF-a. The cytokine is mainly produced by monocytes and activated tissue macrophages (e.g. Kupffer cells in the liver) and in smaller amounts by several other cell types. TNF-a exerts pleiotropic effects on many different cell types and is implicated as an important mediator in various physiological and pathophysiological conditions [46]. Moreover, it has become clear that TNF-a is an important mediator of cell death in acute liver injury and has thus been studied extensively in detail [35-38].

TNF-R1 activation leads to the sequential formation of two signaling complexes. Complex one is located at the membrane and consists of the adaptor molecule TNF-R associated death domain (TRADD), the kinase receptor interacting protein (RIP) and TNF-R associated factor 2 (TRAF-2) beside the receptor itself. Complex one leads rapidly to activation of the prosurvival transcription factor NF-kB and JNK/AP1. Not until then, in a second step, the complex two is formed by TRADD and RIP1, which associate with Fas-associated protein with death domain (FADD) and caspase-8, responsible for induction of the caspase cascade. Therefore, only when the prosurvival checkpoint NF-kB failed to be activated, the cell is driven into apoptosis. Due to the pleiotropic signaling of TNF-a many cells can undergo apoptosis only after sensitization by repression of NF-kB activity by transcriptional/translational inhibitors.

1.3.1.2 CD95/CD95L

The CD95 receptor/CD95L system is a key signaling pathway involved in the regulation of homeostasis in several different cell types like the immune system, heart, kidney and the liver. The CD95 (APO-1, Fas) molecule, a 48 kDa transmembrane receptor, had originally been identified as a cell surface receptor that could mediate apoptotic cell death of transformed cells and cause regression of experimental tumors growing in nude mice [47,48].

The CD95 ligand (CD95L) in contrast is expressed predominantly on membranes of activated T-lymphocytes or natural killer cells, but can also be cleaved to act in soluble form. Beside its occurrence on immune cells, it is also constitutively expressed on immune-privileged sites

like eyes and testis in order to destroy immune and inflammatory cells. Furthermore, CD95L expression on cancer cells has been implicated in immune escape of tumors [49]. By constitutive expression of death receptor ligands like CD95L, tumors may adopt a killing mechanism from cytotoxic lymphocytes to delete the attacking antitumor T-cells through induction of apoptosis. Tumor cells also have developed passive strategies to escape CD95-mediated immune clearance by apoptosis, which include downregulation of their own CD95 receptors, secretion of decoy receptors or alterations in intracellular signaling pathways [49,50].

As a common theme, also CD95L leads to receptor trimerisation and DISC formation, which contrary to TNFR1 consists mainly of the receptor itself, FADD and caspase-8 resulting in caspase cascade activation. Additionally, it has been shown that death domain associated protein (DAXX), receptor interacting protein (RIP) kinase and RIP-associated ICH-1 homologous protein with a death domain (RAIDD) also associate with the cytoplasmatic part of CD95 receptor. The exact role of these proteins remains unclear, as they are dispensable for CD95L-induced apoptosis in lymphoid cells [51].

1.3.1.3 TRAIL

Two active TRAIL (TNF-related apoptosis-inducing ligand, APO-2L) receptors have been identified, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), which can initiate the apoptosis signaling cascade. TRAIL exerts selectively apoptosis-inducing activity on a wide variety of tumor cells. In contrast to CD95L action, healthy cells are believed to be protected by decoy receptors, which block competitively the binding upon active TRAIL receptors. Decoy receptor 1 does not possess a cytoplasmatic signaling domain while decoy receptor 2 has a truncated death domain. Another receptor, TRAIL-R5, also called osteoprotegerin is secreted into the extracellular fluid [52]. Also, analog to the CD95 system, resistance of tumor cells occasionally correlates with expression of decoy receptors or with alterations within the TRAIL-signaling pathways [53].

In general, TRAIL and CD95L signaling pathways and their effects are closely related, except the above mentioned exception. It was shown that the adaptor molecule FADD is required for both CD95L and TRAIL-mediated apoptosis [54,55]. The caspase cascade is triggered by binding and activation of caspase-8 and caspase-10 to the DISC of TRAIL and CD95L receptor, respectively [56]. In contrast to TNF-R1 signaling, the pathways induced by CD95 and the TRAIL receptors are regarded as less pleiotropic.

1.3.2 Caspase-dependent downstream signaling in apoptosis

1.3.2.1 Classification of caspases

To date 14 members of the caspase family are identified, which can be divided into three categories [57].

- Initiator caspases: This subclass is characterized by long prodomains of more than 90 amino acids. The prodomains consist of either a death effector domain (DED) or caspase recruitment domains (CARD), that are also present in adaptor molecules of the DISC such as FADD, RAIDD or also Apaf-1 serving as interaction motifs [58].
- Executioner or effector caspases: They are thought to be responsible for the actual demolition of the cell and tend to have short or absent pro-domains [59].
- Non-apoptotic caspases: Their main role lay rather in cytokine-processing than in apoptotic signaling [60].

Caspases are synthesized as inactive proenzymes, which undergo proteolysis and activation triggered usually by superior initiator caspases or by autocatalysis upon an apoptotic trigger [61]. The prodomains are cleaved off and the large and small subunits are separated to form an active heterotetramer of two small and two large subunits [60]. The regulated proteolysis of the caspases forms an irreversible and self-amplifying cascade leading eventually to apoptosis.

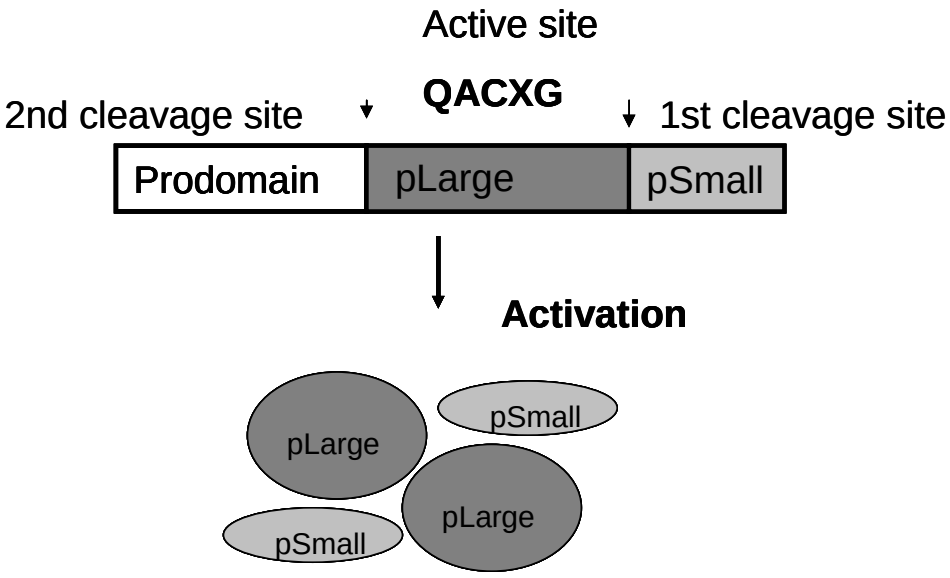


Fig. 1.3: The mechanism of caspase activation (adapted from Bleackley [100]).

1.3.2.2 The extrinsic pathway of apoptosis

Two major routes of apoptosis signaling are well defined. The *extrinsic pathway* involves recruitment of initiator caspase-8/-10 to the cytoplasmatic tails of the death receptor linked by adaptor proteins TRADD and FADD, which form the DISC leading to direct activation of the effector caspase cascade [62]. This pathway is mainly responsible for elimination of unwanted cells during development or immune system-mediated tumor removal.

The DISC-initiated apoptosis signal may be abrogated by the recruitment of the antiapoptotic mammalian Flice-inhibitory protein (cFLIP). It contains a DED domain and an inactive caspase domain, which prevents the recruitment of procaspase-8 (Flice) to the DISC and consequently its subsequent activation. DISC-linked cFLIP potently inhibits apoptosis induced by all known human death receptors. However, cFLIP does not inhibit the activation of procaspase-9, induced by irradiation or chemotherapeutic drugs *via* the mitochondria-dependent *intrinsic* pathway [52,63,64].

1.3.2.3 The intrinsic pathway of apoptosis

The second route, the *intrinsic or mitochondrial pathway* is initiated by a variety of apoptotic stimuli like ionizing radiation, heat shock, osmotic stress or chemotherapy which provokes the permeabilization of the mitochondrial outer membrane (MOMP). The released proapoptotic factors like cytochrome c induce the assembly of an effector caspase cascade-activating complex in the cytosol called apoptosome, which is comprised mainly of Apaf-1 and caspase-9 [65,66].

The inhibition of initiator and executioner caspases by antiapoptotic factors is an efficient cellular mechanism to suppress apoptosis signaling. Thus, members of the inhibitors of apoptosis-family (IAPs) were first identified in baculoviruses. The six human IAPs which have been identified up to now, share a common structural motif which is called baculoviral IAP repeat (BIR) [67]. The members of the IAP family have multiple biological functions that include binding of several caspases by their BIR domains. The X-linked inhibitor of apoptosis protein (XIAP), prevents or decelerates apoptosis by inhibition of caspase-3, -7 and -9 [68].

Nevertheless, if the extrinsic or the intrinsic pathway is activated, their signaling converges on the level of the executioner caspases -3/-6/-7. Caspase-3 is regarded as the most prominent executioner caspase, which cleaves an increasing number of substrates like structural proteins lamin A, vimentin and fodrin, the inhibitor of caspase-activated DNase (ICAD), the kinase RIP, its endogenous inhibitor XIAP or PARP [69]. Lamin A and PARP are also cleaved by the effector caspases-6 and -7, respectively.

1.3.2.4 Links between the pathways

The two pathways are linked at different levels. Activation of caspase-8 by receptor ligation may lead to cleavage of the proapoptotic protein Bid, whose truncated form tBid causes release of cytochrome c by the mitochondria, thereby linking death receptor signaling to mitochondria [70]. In addition, cleavage of the effector caspase-6 downstream of the mitochondria may feed back to the extrinsic pathway by activating caspase-8.

In type I cells, the quantity of activated caspase-8 is sufficient to directly trigger apoptosis *via* the extrinsic pathway for alone. In type II cell, like hepatocytes for instance, the amount of active caspase-8 generated at the DISC is insufficient to overrun intracellular inhibitory mechanisms and to trigger the caspase cascade. However, only minor caspase-8 cleavage activity is needed to generate tBid, which efficiently triggers MOMP to generate a mitochondrial amplification loop [65].

1.3.2.5 The role of mitochondria in apoptotic signaling

In recent years, it shed to light that the mitochondria constitutes a central integrating organelle for apoptosis regulation and execution. The members of the huge Bcl-2 family play a critical role in triggering MOMP. These proteins act to integrate upstream signals converging on the mitochondrial level. The actual pore-forming effector molecules are the proapoptotic Bcl-2 family members Bax and Bak. They reside within the cytosol and are activated transcriptionally or by conformational change induced by cleavage or binding to an activated proapoptotic Bcl-2 -like protein such as Bad, Bid, Bim, Noxa or PUMA, which functions as sensors for different forms of cellular stress induced by intrinsic stimuli. The antiapoptotic Bcl-2 family members Bcl-2 and Bcl-X_L oppose MOMP probably by heterodimerization with Bax-like proteins [4,71]. The proapoptotic and antiapoptotic family members constitute a rheostat that set the thresholds of susceptibility to apoptosis and translate signals into decisions of life and death *via* the intrinsic pathway which utilizes the mitochondria to amplify death signals [2].

1.3.3 Caspase-independent signaling in programmed cell death

The family of caspases has long been regarded as the central executors of apoptosis and PCD in general. However, recent findings of evolutionary conserved forms of PCD, acting caspase-independently, extended the knowledge about intracellular signaling pathways.

Many non-caspase proteases are able to cleave classical caspase substrates and thus might mimic their cellular effects. The best examined non-caspase proteases in caspase-independent PCD are calpains, cathepsins and granzymes. These proteases can act either with caspases in a cooperative way or also completely independent from caspase activity to trigger and execute PCD often characterized by a typically apoptotic morphology [72-76].

1.3.3.1 Calpain

Calpain is an ubiquitous cysteine protease with two major isoforms, m- and μ-calpain [77,78]. They reside inactive within the cytosol and are activated by intracellular elevation of Ca^{2+} concentration [73,75,79]. Activity may be further enhanced by proteolytic cleavage and association with membrane phospholipids, presumably by lowering the requirements of Ca^{2+}. Calpain activity is controlled by calpastatin, a natural inhibitor, which is subjected to cleavage by calpain or caspases. Calpains share some common substrates with caspases, e.g. Bid, Bax and even procaspases-3 and -9 themselves [80]. However, cleavage of the latter two neither activates nor inactivates these enzymes [2]. Several stimuli like irradiation, etoposide or neurotoxins, which are able to enhance intracellular Ca^{2+}-level induce calpain activity. Beside their cooperative activity within the classical caspase pathway several reports describe also caspase-independent apoptosis-like PCD induced by vitamin D analogues in breast cancer cells [81,82].

1.3.3.2 Cathepsins

Until recently, the large family of lysosomal proteases was believed to be responsible for disposal of faulty proteins und for degradation of extracellular matrix once secreted. However, more and more studies concede that they also have important roles in immunology and host defence as also in apoptosis, especially the cysteine proteases cathepsin B and L as well as the aspartate protease cathepsin D [83-86].

Under special conditions, they have been found to be translocated from the lysosomes into the cytosol and nucleus before the appearance of morphological changes indicative for PCD. Additionally, it was recognized that cathepsins are able to cleave caspases [87-91]. Cathepsins participate in caspase-dependent as well as caspase-independent PCD induced by TNF-a and by intrinsic stimuli like camptothecin, bile salts, oxidants and retinoids [92-95]. Interest in cathepsins was further boosted by the finding that the widely used caspase inhibitors zVAD-fmk, zDEVD-fmk and AcYVAD-cmk inhibited cathepsin B in an unspecific manner [96,97].

Therefore, it has to be clarified in detail whether distinct phenomenon are indeed driven by cathepsins rather than by caspase activity.

1.3.3.3 Serine proteases

There is growing evidence that serine proteases, in combination with or completely independent of caspases, take part in protein degradation during apoptosis. Most of the evidence is based on the observation that particular apoptotic events can be prevented by broad-range inhibitors of serine proteases such as TLCK or TPCK [98,99].

Granzmye A and B are the most prominent effectors of PCD within this class of proteases. They are stored in granules, which are utilized by cytotoxic T lymphocytes and natural killer cells for the elimination of pathogenic cells [2]. Upon activation, the contents of cytotoxic granules are released exocytotically to be delivered to target cells. The classical "lethal hit" model stated that following granule exocytosis, poly-perforin formed channels in the target cell membrane in a Ca^{2+}-dependent manner through which granzymes A and B gained entry to the target cell [100]. Studies employing mice lacking granzyme B have demonstrated that this protease is required for the granule-induced rapid caspase-mediated apoptosis [73]. Granzyme B, like caspases, exhibits the unique feature of cleaving after aspartic acid residues and can thus directly activate the caspase cascade. Other substrates comprise the inhibitor of caspase-activated DNase (ICAD), poly ADP-ribose polymerase (PARP) and the proapoptotic BH3-only protein Bid [101]. In the presence of caspase inhibitors, granzyme B triggers a slower, necrotic-like PCD [102]. Granzyme A has a different substrate specifity as it cleaves its substrates after lysine or arginine residues. This protease induces death by activation of a caspase-independent granzyme A activated DNase leading to DNA single strand breaks [103].

1.3.3.4 Mitochondrial effectors of caspase-independent PCD

Two caspase-independent proapoptotic factors are AIF and endonuclease G both translocating to the nucleus when released from mitochondria upon a cell death stimulus. They participate in DNA cleavage and chromatin condensation [104-106]. Whether these two proteins are released before, together or after cytochrom c release has been controversially discussed [107]. Endo G cleaves chromatin DNA into nucleosomal fragments independently of caspases. It is not exactly clear, how AIF contributes to large scale nuclear DNA fragmentation as it lacks intrinsic DNase activity, maybe it acts as a scaffolding protein to DNA for a yet unknown nuclease [104].

The serine protease Omi/HtrA2 is also released from mitochondrial intermembrane space into the cytosol and the nucleus. The mature 36 kDa form can both neutralize and inactivate XIAP by either binding in a manner similar to Smac/DIABLO or by cleavage, thus potentiating apoptosis [108-113]. However, Omi/HtrA2 can also act caspase-independently as effector molecule in a necrosis-like PCD, which exclusively relies on its protease function [114-116].

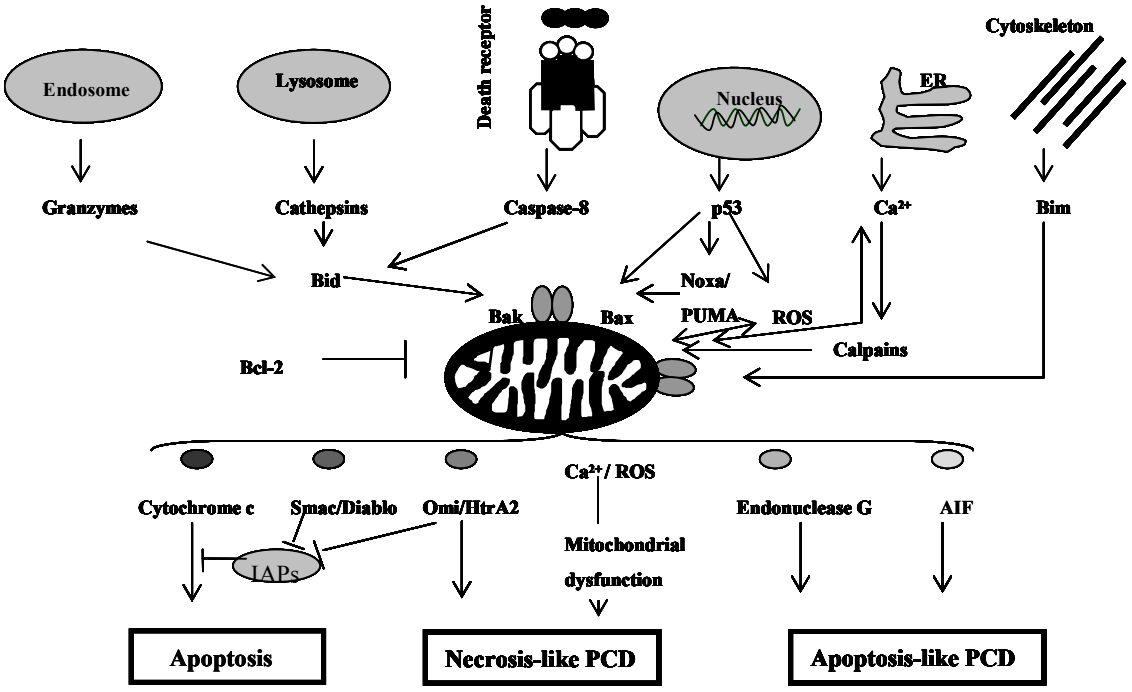


Fig.1.4: Control of mitochondrial outer membrane permeabilization (MOMP) and downstream signaling after MOMP (adapted from Jäättelä [4]).

In summary, it can be stated that the dependence of PCD in its diverse phenotypes on certain non-caspases, acting in parallel or cooperatively with caspase, might be extremely dependent on cell type and stimulus.

1.4 Epigenetic regulation of protein expression

The term epigenetic refers to mitotically and meiotically heritable alterations in the expression pattern of genes within a given cell by modifications of either DNA bases or of histones. In contrast to mutations, these modifications do not alter the primary DNA sequences and are thus potentially reversibel [117,118]. These processes are implicated in many aspects of cell biology like X-chromosomal inactivation, imprinting and cancer development and occur due to response to environmental or developmental factors or even spontaneously.

1.4.1 DNA methylation

DNA methylation seems to be unviersal in eukaryotes. In adult somatic tissues, DNA methylation typically occurs in a 5'-CpG-3' dinucleotide context called CpG motif. Between 60-70% of all CpG motifs are methylated, however, the methylation pattern is asymmetrically distributed in the genome as a whole and cell type-specific [119]. The post-synthetic covalent addition of a methyl group to cytosine is mediated by three known active DNA cytosine methyltransferase DNMT 1, 3a and 3b [120]. The enyzmes transfer the methyl moiety from the cellular methylgroup donor S-adenosylmethionine to the 5th position of the cytosine ring [121]. DNMT 1 is responsible for maintaining the methylation pattern during embryonal development and cell division [122,123]. It binds preferentially to hemimethylated DNA sequences. Especially this enzym seems to play an impotant role in cellular transformation. The other two known functional DNMTs 3a and 3b are regarded as *de novo* methylases and bind to both hemimethylated and naked DNA [124]. The CpG motifs are usually clustered in so-called CpG island, which are found in 5' regulatory regions and promoters of genes. DNA methylation may control pattern of gene expression in two ways. First, the methylation of DNA may itself physically impede the binding of transcription factors to the gene, thus blocking transcription. The methylgroup of 5-methylcytosine protrudes into the major groove of the DNA helix, where it can easily interfere with the binding of such proteins. Second, and likely more important, methylated DNA may be bound by proteins known as methyl-CpG-binding domain proteins (MBDs). MBD proteins then recruit additional proteins to the locus, such as histone deacetylases and other chromatin remodeling proteins [125,126]. DNA methylation is likely to be used for long-term silencing of gene expression as this modifications are normally established during development and maintained throughout the life. However, in older individuals, it is common to observe deviations of the expected patterns leading to abberant gene expression and increasing genomic instability [127]. Both processes, promoter hypermethylation leading to gene silencing and genome-wide hypomethylation resulting in genomic instability, have been observed [127].

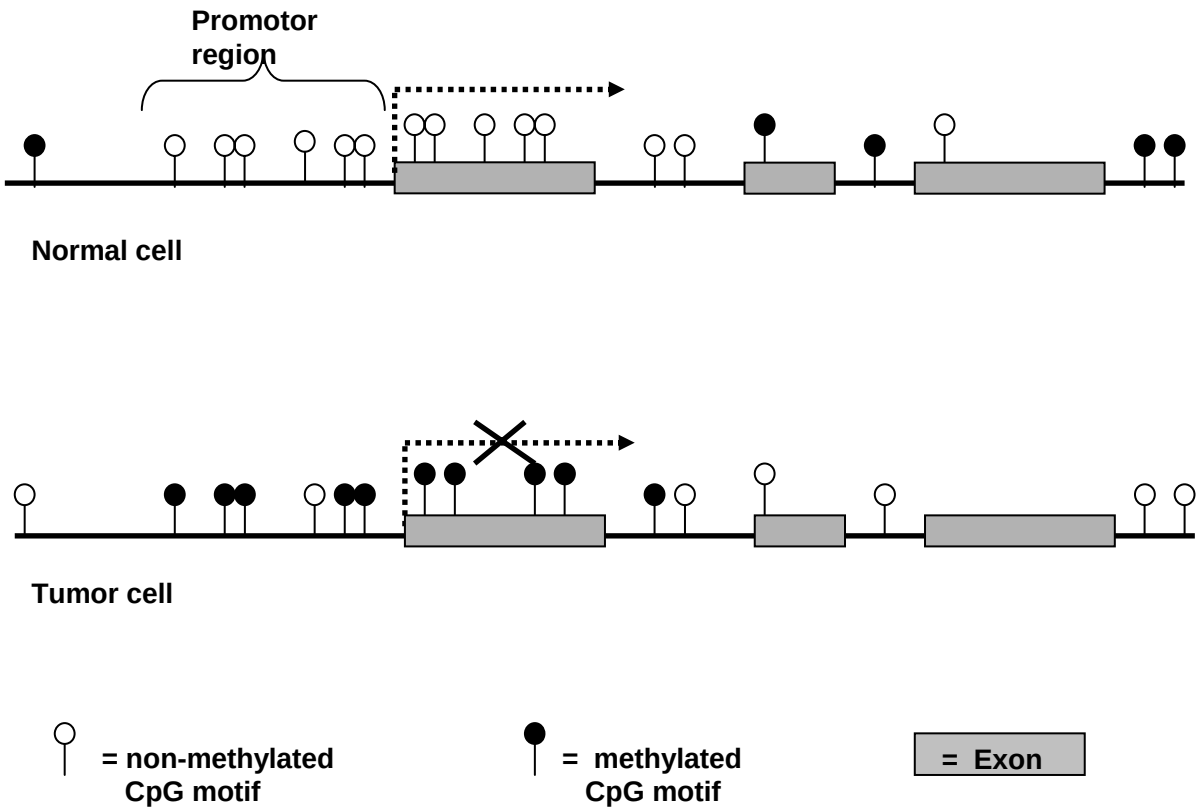


Fig.1.5: Distribution of CpG Dinucleotide in the human genome and differences in methylation patterns between normal cells and tumor resulting in gene silencing (adapted from Herman [288]).

1.4.2 Histone acetylation

Nucleosomes are the fundamental repeating subunits of all eukaryotic chromatin. They package DNA into chromosomes inside the nucleus and comprise the lowest level of compaction necessary for a proper storage but also for accessibility of DNA. Each individual nucleosome core particle consists of a complex of eight evolutionary highly conserved histone proteins, two molecules each of histones H2A, H2B, H3, and H4, and double-stranded DNA that is 146 nucleotide pairs long. The histone octamer forms a protein core around which the double-stranded DNA is wound [128,129]. Histones undergo posttranslational modifications which alter their interaction with DNA and nuclear proteins. The H3 and H4 histones have long tails protruding from the nucleosome, which can be covalently modified at several places. Modifications of the tail include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, citrullination, and ADP ribosylation [130].

Combinations of modifications are thought to constitute the so-called histone code. Histone modifications act in diverse biological processes such as gene regulation, DNA repair and

chromosome condensation. Loss of acetylation of lysine residues has been found to be most important in epigenetic gene silencing [131]. Acetylation/deacetylation is controlled by histone acetyl transferases (HATs) and histone deacetylases (HDACs). The dynamic equilibrium between the catalytic activities of this two enzyme families affects the cellular acetylation status and thereby influences how tightly or loosely the chromatin is packed [130]. The density of chromatin packaging decides whether it is accessible for the transcriptional machinery. Analog to DNA methylation, loss and gain of histone lysine acetylations are observed in cancer cells as this process eventually can lead to transcriptional repression of tumor suppressor genes or otherwise to enhancement of oncogene activity [132].

1.5 Cancer

Cancer is one of the principal causes of death in developed countries responsible for 7 million deaths every year. The term cancer generalised a total of approx. 200 distinct forms of diseases or disorders characterized by uncontrolled division of cells and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue through invasion or by implantation into distant sites by metastasis. The limitless capacity of proliferation is achieved not only by resistance to anti-growth signaling but also by self-sufficiency in growth signals. The thereby enhanced necessity of nutrient and oxygen supply is achieved by sustained angiogenesis. A typical feature of transformed cells is the non-responsiveness to apoptotic signals due to defects in apoptotic machinery thus evading immune clearance [133].

The characteristical features of transformed cells are the result of a multistage process, which has its origin in the alteration of genes or alteration of expression patterns of genes encoding proteins which modulate angiogenesis, metastatic or growth/apoptosis pathways [134-136]. The accumulation of mutated genes/imbalanced expression give rise to uncontrolled and often rapid proliferation of cells which lead to tumor formation. Benign tumors do not spread to other parts of the body or invade other tissues and are rarely a threat to life unless they extrinsically compress vital structures. Malignant tumors can invade other organs, spread to distant locations and disturb vital, physiological function of the affected tissues and organs [137]. The causative reason for tumorigenesis is usually unidentified, but it is known that the development of cancer involves an interaction between environment and heredity [138]. To principle mechanisms leading to an altered proteome within a cell were identified on the level of the DNA.

Mutations, that mean irreversible alteration of the DNA primary sequence, can cause cancer by abnormal pathophysiological enhancement of oncogene activity or by causing tumor suppressor genes to loose their function [133]. These alterations can be inherited through the germ line or more commonly arising in somatic tissue. Experimental animals with genetic deficiencies in DNA repair often show decreased lifespan and increased cancer incidence [139]. According to situation of organisms with long life spans risk tends to increase with age due to decreased DNA repair capacitiy [133]. A second cause of cancer development relying on DNA level was identified in epigenetic changes, which can, closely connected with mutational process, driving the development of tumors[140]. As described in detail in chapter 1.4, this change in gene expression involves either methylation of DNA in promotor regions of genes or post-translational modifications on the histones, which affects the packaging state and thus the accessibility of DNA for the transcriptional machinery [140,141]. If these processes are aberrantly regulated, it might cause also altered oncogene or tumor suppressor activity.

1.5.1 Hepatocellular carcinoma

Hepatocellular carcinoma (HCC, also called hepatoma) is a primary cancer of the liver. Most cases of HCC are secondary to either hepatitis infection (usually hepatitis B or C) or cirrhosis (alcoholism being the most common cause of hepatic cirrhosis), implying that alterations in growth control mechanisms during regeneration, which in part are mediated by cytokines, may be involved in hepatic carcinogenesis. In countries where hepatitis is not endemic, most malignant cancers in the liver are not primary HCC but due to metastasis of cancer from elsewhere in the body. Treatment options of HCC and prognosis are dependent on many factors but especially on tumor size and staging. In theory, cancers can be cured if entirely removed by surgery, but this is only possible when the tumor occurs as a clearly defined entity. This is the case in only a minority of patients. Often the tumors exist as poorly defined multiple spots with infiltrative growth patterns or are located in close contact with major blood vessels, especially when the cancer has metastasized. Culmination of metastases in the liver during progression of the disease is the case in more than 70% of all patients with colorectal cancer. Autopsy studies indicate that in at least half of these cases the liver is the sole metastatic site. These types of tumors are regarded to be non-resectable, thus the prognosis for survival is extremly poor for these patients. In the US, 140.000 new patients are diagnosed with colorectal cancer each year, approximately 20% to 30% will die of progressive metastatic disease confined to the liver. In the UK colorectal cancer is the second

most common cause of cancer death. Also in Germany colorectal cancer is widely distributed as it constitutes the second common cancerous disease.

1.5.2 Death receptor ligands in tumor therapy

In the 1980's clinical trials were performed to exploit the death-inducing capabilities of the cytokines TNF-a and CD95L against tumor cells. Both molecules exert remarkable results. The cytotoxicity of TNF-a, especially in combination with Interferon-g on transformed, malignant tumor cells, is so far unmatched by any other combination of cytokines[142,143]. CD95L proved to be effective since single injection of antibodies against human CD95 resulted in cure from xenotransplanted human tumors in immunodeficient nude and SCID mice [144,145]. Thus, stimulating the CD95 or the TNF-R1 receptor was evaluated as a novel approach for antitumor therapy and the hope of having an effective cure at hand rose.

However, both cytokines failed in trials as their systemic application lead to severe side reactions In case of TNF-a the patients suffered on fever, chills, anorexia, headaches, lethargy, fatigue and hypotension [142,143]. When overproduced, as e.g. in endotoxin (i.e. LPS = lipopolysaccharide)-induced shock or during experimental cerebral malaria, the cytokine may lead to fatal multiple organ failure or brain damage. The use of a species-specific monoclonal anti-murine CD95 antibody revealed also considerable systemic toxicity. Hepatocytes constitutively express CD95 on their surface and CD95 stimulation in mice has been shown to induce fulminant hepatic failure within hours [146,147].

More recently, TRAIL (Apo-2L) was shown to be selectively cytotoxic for tumor cells but exerted no or only minimal toxicity in normal tissues of mice and primates. Therefore, after the failed hopes with the other death-inducing cytokines, TRAIL was considered as the agent with the greatest potential for *in vivo* anticancer effects, either alone or in combination with chemotherapeutical drugs. It has been demonstrated that TRAIL-resistant tumor cells can be sensitized by subtoxic concentrations of drugs and cytokines towards TRAIL-mediated apoptosis significantly [148,149]. However, its safety has been challenged by a report that observed induction of apoptosis by TRAIL in cultured human hepatocytes, which were not obvious in trials with rodent and monkey hepatocytes [150,151].

To circumvent the obstacles of systemic treatment with death-inducing cytokines the isolated perfusion of the liver was developed (IHP =isolated hepatic perfusion). A promising treatment concept was the application of high, local doses of the alkylating agent melphalan either alone or in combination with TNF-a. The IHP proved to be technically feasible with negligible systemic toxicity due to only minor systemic drug exposure. The liver histology and

biochemistry showed only slight, transient disturbances. However, the lack of selectivity towards malignant cells turned out to be a major limitation of this attractive approach. Cell death was unspecifically initiated by chemotherapy not only in malignant cells but also in normal hepatocytes in absence or presence of TNF-a.

1.5.3 Chemotherapeutics in tumor therapy

The majority of chemotherapeutic drugs can be divided into alkylating agents, antimetabolites, anthracyclines, plant alkaloids and topoisomerase inhibitors. Most of them work by impairing mitosis, affecting effectively rapid dividing cancer cells. These agents cause cell death mostly by apoptosis which is triggered by induction of cytotoxic stress due to DNA damage, upregulation of death receptors or of proapoptotic factors and proteins and may be either dependent or independent of death receptor signaling. Death receptor-mediated and chemotherapy-induced apoptosis can converge at the level of the receptor, the DISC formation, the activation of the initiator caspase-8, at the level of the mitochondria or at the level of downstream effector caspase activation depending on the specific form of DNA damage, the cellular environment and the specific pathway(s) by which death receptor-mediated or drug-mediated apoptosis are induced. Although the major part of the tumors initially reacts to the therapy, drug resistance caused by a positive selection of clonal cells often arises due to a progressive alteration of the tumor cells. Also cell division effectively ceases near the center of a solid tumor, making them less sensitive to chemotherapeutical treatment. The acquired mechanisms of resistance by tumor cells include decreased uptake or increased efflux of chemotherapeutics, increased detoxification of chemotherapeutics, changes in expression level of drug targets and modulation of cell survival factors leading to deficiencies in apoptosis [152].

One target of drug discovery research is to find replacements or supplements for chemotherapeutics, which lost their effectiveness. A novel promising anticancer treatment in the context of replacement as well as supplementation is offered by epigenetic therapies.

Epigenetic drugs, whether demethylating agents or HDAC inhibitors target aberrantly heterochromatic, that means silenced regions of the DNA and leading to a reactivation of tumor suppressor genes and/or other genes, which exert important functions for normal cellular behaviour. Thus, the transformed state of cells can be potential reverted making them susceptible for chemo-, immuno- or radiotherapy.

2 Objectives of the study

Apoptosis is an inherent, tightly regulated form of cell death, essential for physiological development and tissue homeostasis. The protease family of caspases has long been regarded as the pivotal executioner of apoptosis. However, in recent years a variety of non-caspase proteases were identified, which contribute to apoptosis and other caspase-independent forms of cell death.

These caspase-independent pathways of programmed cell death constitute important alternatives for an organism against uncontrolled and potential harmful cell proliferation when caspase-mediated routes fail to be activated. Growing knowledge about alternative routes to programmed cell death can be exploited in cancer therapy to overcome chemoresistance of many tumors.

A new approach in cancer therapy is the pharmacological manipulation of epigenetic regulation of gene expression by a class of drugs named histone deacetylaseinhibitors (HDIs) and DNA methyltransferaseinhibitors (DMTIs). Especially the methyltransferase inhibitor 5-azacytidine represents a pioneering example as being the first DMTI approved for treatment of myelodysplastic syndromes.

In detail the following questions should be addressed, utilizing primary murine and human hepatocytes as well as the human hepatoma cell line HepG2:

- Characterization of the role of caspases in death receptor agonist-induced apoptosis in primary human hepatocytes.
- Characterization of the role of caspases and serine proteases on death receptor agonist–induced apoptosis on HDI/DMTI-sensitized hepatocytes.
- Characterization of the role of caspases and serine proteases in the intrinsic mode of apoptosis in hepatocytes.
- Investigation of the involvement of mitochondrial proapoptotic proteins as effectors within hepatocytes apoptosis under caspase arrest.
- Examination of DNA methyltransferase inhibitors in terms of sensitization of hepatocytes.
- Elaboration of the pharmacodynamic properties of 5-azacytidine as to its sensitizing effects on hepatocytes.

3 Materials and Methods

3.1 Materials and animals

3.1.1 Substances

CD95L was generously provided by Dr. Elisa Ferrando-May. Recombinant mouse TNF-a was obtained from Innogenetics (Ghent, Belgium). *Killer*Trail was purchased from Alexis Biochemicals (Gruenberg, Germany).

The DNA methyltransferase inhibitors 5-azacytidine; 5-aza-2'-deoxycytidine and S-(5'-adenosyl)-L-homocysteine were purchased from Sigma-Aldrich (Taufkirchen, Germany) except RG 108, which was purchased from Calbiochem (Schwalbach,Germany). Sodium valproate (Ergenyl®) was purchased from Sanofi-Synthelabo (Berlin, Germany), apicidin from Sigma-Aldrich (Taufkirchen, Germany), M344 and CBHA from Calbiochem (Schwalbach, Germany).

The caspase inhibitors z-Val-Ala-DL-Asp-(OMe)-fluoromethylketone (zVAD), z.-Val-Asp(OMe)-Val-Ala-Asp(OMe)-fluoromethylketone (zVDVAD), Ac-Asp-Glu-Val-Asp-chloromethylketone (Ac-DEVD), z-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone (zLEHD), z-Ile-Glu-Thr-Asp(OMe)-fluoromethylketone and Boc-Asp(OMe)-fluoromethylketone were purchased from Calbiochem (Schwalbach, Germany).

The serine protease inhibitors N-p-tosyl-L-lysine-chloromethylketone (TLCK), N-p-tosyl-L-phenylalanin-chloromethylketone (TPCK) and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) as well as the cytotoxic agents camptothecin and staurosporine were supplied by Sigma-Aldrich (Taufkirchen, Germany).

The caspase substrate Ac-DEVD-afc was obtained from Biomol (Hamburg, Germany).

AlamarBlue was obtained from BioSource (Solingen, Germany). Cytotoxicity detection kit (LDH) was used from Roche Diagnostics GmbH (Mannheim, Germany).

All standard chemicals were purchased from established suppliers, in most cases Sigma-Aldrich (Taufkirchen, Germany).

3.1.2 Cell culture materials

Cell culture plates (24 and 96 well), petri dishes and other plastic materials were purchased from Greiner (Frickenhausen, Germany). RPMI 1640, PBS, FCS as well as penicillin, streptomycin and Accutase® were obtained from PAA (Linz, Austria).

3.1.3 Animals

C57Bl6 wild type mice (25 g) were raised in the animal breeding facility of the University of Konstanz. Animals were held at 22°C and 55% humidity and given a constant day/night cycle of 12 h. All steps of animal handling were carried out according to the Guidelines of the National Institute of Health (NIH), the European Council (directive 86/609/EEC) and the national German authorities and followed the directives of the University of Konstanz Ethical Committee.

3.1.4 DNA vectors and constructs

The mammalian expression vector pCMV was purchased from Sigma-Aldrich (Taufkirchen, Germany). Stratagene (La Jolla, CA, USA) supplied the marker vector pEFGP-C1. The construct pCMV-C8, encoding the cDNA sequence of caspase-8, was kindly provided by Dr. Astrid Leja/Dr. Thomas Meergans (University of Konstanz, Germany).

3.1.5 Antibodies

Polyclonal IgG horseradish peroxidase-coupled secondary antibody (goat anti-mouse and goat anti-rabbit) were purchased from Pharmingen (San Diego, CA, USA). Mouse monoclonal anti-caspase-3 and anti-XIAP as well as anti-Apaf -1 were purchased from BD Biosciences Pharmingen (Heidelberg, Germany), mouse monoclonal anti-caspase-8 and -9 from Oncogene Research Products (Boston, MA, USA). Rabbit polyclonal anti-β-actin; anti-AIF; anti-Bid and anti-Bcl2 were obtained from New England Biolabs (Frankfurt am Main, Germany), rabbit polyclonal anti-cFlip from Stressgen (Victoria, Canada). Mouse polyclonal antibody anti-p53 was purchased from Santa Cruz (Santa Cruz, CA, USA). Mouse monoclonal anti-FADD was purchased from Biomol (Hamburg, Germany). All antibodies used were specific for human cells.

3.2 Methods

3.2.1 Cell culture

The cell line HepG2 was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and maintained in a humidified incubator at 5% CO_2 / 95% air.
HepG2 cells were cultured in RPMI 1640. The media was supplemented with heat-inactivated FCS. Cells were regularly passaged in a ratio of 1:3 to 1:5. The day before the experiment the cells were seeded in 96 well plates or 6 well plates at a density of 2.5 x 10^4 or 0.5 x 10^6 cells per well, respectively, depending on the experimental procedure.

3.2.2 Isolation and culture of mouse hepatocytes

Isolation of hepatocytes form 8 weeks old mice was performed by the two-step collagenase perfusion method of Seglen as modified by Leist [153,154]. To separate hepatocytes from remaining non-parenchymal cells, the pellet was resuspended in 32 ml Hanks' balanced salt solution (HBSS) and mixed with 20 ml of a Percoll solution followed by centrifugation at 800 x g for 10 min. at room temperature. To remove remaining Percoll, the pellet was additionally washed with HBSS by a subsequent centrifugation steps at 800 x g for 2.5 min at room temperature. The pellet was resuspended in RPMI 1640 medium containing 10% FCS. At least cells were plated with a density of 8 x 10^4 hepatocytes per well in a 24 well plate coated with collagen. Cells were allowed to settle down and adhere to culture dishes for minimum 3 h before the medium was exchanged RPMI 1640 excluding FCS. Incubation was conducted in a humidified atmosphere consisting of 55% N_2; 40% O_2 and 5% CO_2 at 37°C.

3.2.3 Culture of primary human hepatocytes

Primary human hepatocytes were generously provided by Professor Dr. Nüssler (Charite, Berlin). The hepatocytes were isolated from pathological inconspicuous specimens obtained from patients undergoing hepatic resections for the therapy of hepatic tumors. The cells were cultured in RPMI 1640 supplemented with 10% FCS and maintained in a humidified atmosphere with 5% CO_2 at 37°C.

3.2.4 Treatment of cells with inhibitors and cytokines

Before the experimental procedures the cells were brought into fresh medium after visual inspection for contamination or morphological variances.

In general (if not otherwise stated), the cells were sensitized with 75 µM 5-azacytidine 3h; with 100 µM CHX 2h or 1µg/ml ActD 30 min prior to treatment with cytokines, respectively. Inhibitors were e.g. dissolved in DMSO except TLCK (1mM HCl) and TPCK (Ethanol) and preincubated 30 min. prior to treatment with cytokines. Untreated control cells received the vehicle. Final concentration of DMSO never exceeded 1%.

3.2.5 Treatment with intrinsic inducers of apoptosis

Camptothecin and staurosporine were reconstituted in DMSO and diluted to working concentration in isotonic saline and applied 30 min. after addition of inhibitors. Control cells were treated with vehicle.

3.2.6 Treatment with UV radiation

Cells were treated with UV-B radiation with 312 nm of the desired intensity within a Fluo-link UV-crosslinking chamber (Fluo-Link; MWG biotech) without media and with covered control cells.

3.2.7 Cytotoxicity assays

Cytotoxicity was measured by the reduction of tetrazolium dye Alamar Blue™ by viable cells. The assay was performed according to the manufacturer instructions. Vehicle-treated cells were used to set basal level of cytotoxicity (i.e. 0% cytotoxicity), cells lysed with 0.1% Triton® X-100 were used to set maximum level (i.e. 100% cytotoxicity). This method, detecting the metabolic activity of cells does not distinguish between different modes of cell death, variances in proliferation and alterations of metabolic activity within cells due to mitostatic effects of drugs.

So in special cases, e.g. for cytotoxicity assay requiring periods of incubation exceeding 24h the more convenient LDH assay was used due to different growth rates of treated cells versus untreated control.

Thereby the release of cytosolic marker enzyme lactate dehydrogenase (LDH) was used as a parameter for cytotoxicity independently for each well independent of 0% cytotoxicity control. Lactate dehydrogenase was determined, using the Cytotoxicity Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) in culture supernatants (S), and in the remaining cell monolayer (C) after lysis with 0.1% Triton X-100. The percentage of lactate dehydrogenase release was calculated from the ratio of S/(S+C).

Before applying any cytotoxic assay the cells were examined with light microscopy to get a visual impression about morphological changes indicating the mode and extent of cell death.

3.2.8 Caspase-3/-7 activity assay

3.2.8.1 Experiments in 6 well culture plates

In cell culture experiments, cells were washed three times with phosphate-buffered saline (PBS) and lysed with hypotonic extraction buffer (25 mM HEPES, pH 7.5, 5 mM MgCl2, 1 mM EGTA, 1 mM Pefabloc plus 0.1% Triton X-100). After centrifugation (15 min, 13,000 x g, 4°C) of the lysates, supernatants were immediately frozen at -80°C. Generation of free 7-amino-4-trifluoromethylcoumarin (AFC) or 7-amino-4-methylcoumarin (AMC) was followed kinetically in assay buffer (50 mM HEPES, pH 7.4, 1% sucrose, 0.1% CHAPS, 10 mM DTT, 50 µM fluorogenic substrate DEVD-AFC (N-acetyl-Asp-Glu-Val-Asp-AFC, Biomol, Hamburg, Germany) for caspase -3/-7 activity for 30 min at intervals of 5 min. at 37°C using a fluorometer plate reader Wallac Victor² (Wallac Instruments, Turku, Finland) set at an excitation wavelength of 385 nm and an emission wavelength of 505 nm. Protein concentrations of corresponding samples were determined with the Pierce-Assay (Pierce, Rockford Illinois, USA), and the specific caspase-3/-7 like protease activity was calculated in pmol free AFC per min (µU) and mg protein using serially diluted standards (0-5 µM AFC).

3.2.8.2 Experiments in 96 well culture plates

Cells were washed three times with PBS and lysed within the cell culture plate with 30 µl hypotonic extraction buffer (25 mM HEPES, pH 7.5, 5 mM MgCl2, 1 mM EGTA, 1 mM AEBSF plus 0.1% Triton X-100). For complete lysis cells were incubated for 7 min. at 37°C and shaked for 2 min at full speed at RT. For determination of protein concentration 10 µl of lysate were spared. Release of free AFC was monitored in intervals of 5 min. over a period of 30 min at 37°C in a Wallac Victor² (Wallac Instruments, Turku, Finland).

3.2.9 Preparation of S-100 fraction

HepG2 cells (5-10 x 10^6) were detached from cell culture flask with the mild enzyme mix Accutase and lysed with cytosolic extraction buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1mM EGTA, 1 mM DTT) after washing two times in ice cold PBS. After incubation on ice for 20 min, cells were broken by passing 12 times through a G26 needle. Cell extracts were centrifuged at 900 x g, 10 min, 4°C to separate membranes and

debris. The supernatant was further centrifuged at 50,000 x g for 1 h within a Ti50 rotor at 4°C. Immediately after centrifugation the protein content of the samples was determined using Bradford assay (Bio-Rad Laboratories, Bio-Rad, Munich, Germany) and the supernatant (S-100 fraction) frozen in liquid nitrogen after supplement of a final concentration of 5% Glycerol and stored at -80°C for subsequent use in the cell-free caspase activation assay (see below).

Induction of apoptosis in a cell-free reconstitution system

The reaction mixture contained 25 µl of S100 fraction (~3 µg/µl), 10 µM cytochrome c; 1.5 mM DTT and 1mM dATP. The mixtures were incubated at 37°C 30 min before addition of caspase assay buffer (50 mM HEPES, pH 7.4, 1% sucrose, 0.1% CHAPS, 10 mM DTT, 50 µM fluorogenic substrate DEVD-afc (N-acetyl-Asp-Glu-Val-Asp-afc, Biomol, Hamburg, Germany). 10 µl of the reaction solution per incubation period were used to determine the caspase-3/-7 activation.

3.2.10 Transient transfection

For transfection experiments cells were seeded on a 6 well cell culture plate at a density of $0.5x10^6$ cells per well the day before

Transient transfection was performed employing Gene juice™ transfection reagent (Novagen, Nottingham, UK) according to manufacturer instructions. Briefly, HepG2 cells were transfected with 1µg of human caspase-8 construct, cloned into the mammalian expression vector pCMV2 (Sigma-Aldrich, Taufkirchen, Germany) in a DNA:transfection reagent ratio of 1:3. Immediately before the transfection procedure cells were brought into fresh RPMI 1640 media supplemented with the respective inhibitor. After an incubation period of 16 h without removing the transfection complexes the caspase-3/-7 activation was determined as described above.

For quantitative optical determination of apoptosis induction using a fluorescence microscope ratio of marker plasmid pEGFP-C1 (Stratagene, La Jolla, CA, USA) to other plasmid of 2.5:1 was used.

3.2.11 Microscopic determination of apoptotic markers

For qualitative determination of cells nuclei of apoptotic cells were stained with hoechst as indicated and phosphatidylserine exposure was detected by using the phospholipid binding dye Merocyanine 540 (Upstate, Lake Placid, New York, USA). Cells were incubated 10 min at 37°C with 10 µg/ml Merocyanine 540 in PBS, washed 2 x with PBS and examined by

fluorescence microscopy (rhodamine filter) immediately. Pictures were taken with a 20x LD PLAN-Neofluar TM N.A. 40 0.40 mounted on an Axiovert 100 (Zeiss, Oberkochen, Germany). The microscope was equipped with a Nikon Coolpix R 900.

3.2.12 SDS-PAGE/ Western Blot

Cultured cells were lysed with lysis buffer (250 mM sucrose, 50 mM Tris-HCl, 5 mM imidazole, 2.5 mM EDTA, 2.5 mM DTT, 0.1% Triton® X-100, pH 7.40) and protein concentration was measured using the Bradford protein assay (Biorad, Munich). Briefly, an aliquot of each sample equivalent to 30 µg protein was boiled after addition of the appropriate amount of 5x sample buffer (5 mM EDTA, 162 mM DTT, 5% SDS, 50% glycerol, 0.5% bromophenol blue, 188 mM Tris, pH 8.8). The samples were separated on 12% SDS-polyacrylamide gels (PAGE) and electrophoretically transferred onto nitrocellulose filters using the Bio-Rad electrotransfer system (Bio-Rad Laboratories, Munich, Germany). Equal transfer was verified by Ponceau-staining of the membranes. Antigen-antibody complexes were visualized with HRP-coupled secondary antibodies (goat anti-mouse and goat anti-rabbit, Dianova, Hamburg, Germany) and a custom-made ECL detection system (2.5 mM luminol, 0.4 mM para-coumaric acid, 10 mM Tris base, 0.15 % H_2O_2, pH8.5).

3.2.13 Detection of cytochrom c and AIF release

Treated cells were harvested at indicated time points with Accutase and washed two times with PBS. Cells were transferred into a 1.5 ml tube, spinned down and pellets were resuspended with cytosolic extraction buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1mM EGTA, 1 mM DTT). After an incubation period of 15 min. on ice, cell membranes were destroyed by shearing cells 12 times through a G26 needle. Lysates were centrifuged 15 min at 4°C with 3300 x g and obtained supernatants were additionally centrifuged at 13.000 rpm for 1 h at 4°C with an Eppendorf 5417 R centrifuge (Eppendorf; Hamburg, Germany). Supernatants were stored in -80°C.

3.2.14 Detection of DNA laddering

Per treatment, $5x10^6$ cells were seeded. Cells were harvested at indicated time points with Accutase and washed two times with PBS and transferred into a 1.5 ml tube. Pelleted cells

were resuspended in 0.5 ml of extraction buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5 % SDS, 0.5 mg/ml proteinase K) and incubated for 20 min. on ice.

Then the lysates were centrifuged at 13.000 rpm for 10 min. The DNA in the supernatant was purified and concentrated with Phenol/chloroform extraction twice and subsequent precipitation with 0.1 vol 3 M sodiumacetate and 2.5 vol ethanol overnight at -70°C. DNA was pelleted by centrifugation at 13.000 rpm, 30 min. at 4°C and resuspended in TE-buffer containing 20mg/ml RNase H with incubation at 37°C for 2h. DNA was runned on an agarose-gel 1% and visualized on an UV transilluminator.

3.2.15 Statistics

All data are given as means ± SD. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test of controls *vs.* treated groups. Statistical analysis that included all *vs.* all comparisons was done using Tukey Multiple Comparison Test. All statistics were calculated using the program GraphPad Prism® 4.01 (GraphPad Software Inc.) and a p value <0.05 was considered as being significant.

4 Results

4.1 The role of caspases in death receptor agonist-induced apoptosis of hepatocytes

It is well established that primary hepatocytes as well as cell lines derived from hepatomas, pre-treated with actinomycin D or cycloheximide, are highly sensitive to death receptor agonist-induced apoptosis elicited by the cytokines TNF-a, CD95L and TRAIL. So far caspases were regarded as the central enzymes responsible for characteristic morphological and biochemical changes in the context of apoptotic cell disassembly. However, data raised by Dünstl in 2004 provided evidence that protection of HepG2 cells by zVAD-fmk is unlikely to be due to global inhibition of caspases, but rather to a non-specific inhibition of a yet unidentified mechanism. Only the combined application of zVAD-fmk with the serine protease inhibitors TPCK/TLCK exhibited a protective effect suggesting a participation of serine proteases within death receptor agonist-induced apoptosis in HepG2 cells [155].

In order to evaluate these new findings and to verify the physiological meaning, the investigation was extended to primary human hepatocytes. Furthermore, a second approach of sensitizing towards death receptor-dependent apoptosis in HepG2 was chosen, by using histone deacetylase inhibitors and DNA methyltransferase inhibitors, drugs modulating gene expression.

4.1.1 Effect of caspase inhibition on death receptor agonist-induced apoptosis in primary human and murine hepatocytes

To assess whether caspase-independent mechanisms are operating under global caspase arrest comparable to HepG2 cells, primary human hepatocytes prepared from liver resection samples were incubated with sensitizing concentrations of actinomycin D and the cytokines TNF-a,CD95L or TRAIL, respectively.

In the presence of increasing concentrations of the pan-caspase inhibitor zVAD-fmk, cytotoxicity was not prevented in primary human hepatocytes except at concentration ranges beyond the respective IC_{50} value for half-maximal inhibition of caspase activity (Fig. 4.1a; b; c). These results were re-evaluated by using primary murine hepatocytes, (Fig. 4.1d), as these cells showed in previous findings no caspase-independent cell death [155]. Our findings received with ActD/CD95L- treated primary murine hepatocytes gave no clue of any cell death under

caspase arrest. The inhibition of caspases by zVAD-fmk was accompanied with complete protection against cell death.

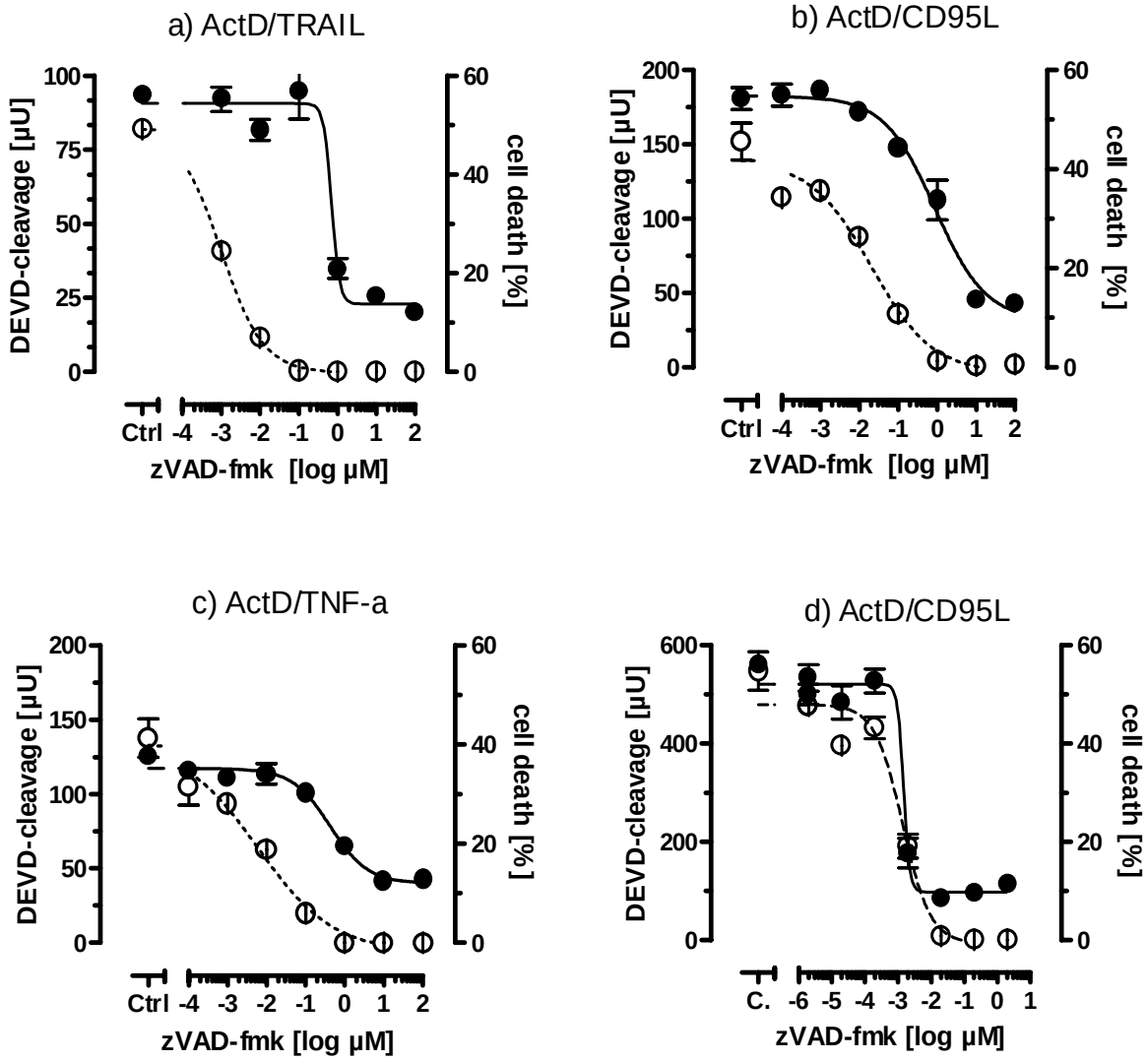


Fig 4.1: Concentration-dependent effect of zVAD-fmk on caspase activity (○) and cytotoxicity (●) in primary human hepatocytes (a; b; c) and primary murine hepatocytes (d) sensitized with 400 ng/ml ActD and treated with increasing concentrations of zVAD-fmk 30 min. prior to addition of::
a) 100 ng/ml TRAIL.
b) 10% v/v CD95L.
c) 100 ng/ml TNF-a.
d) 10% v/v CD95L.
Caspase activity and cytotoxicity was determined after 8h and 18h, respectively.
Data represent mean – SD.

Table 4.1 compiles the IC_{50} values of zVAD-fmk treatment for caspase activity and cytotoxicity obtained in primary human and murine hepatocytes. The ratio of these values highlights the clear differences at least for ActD/CD95L treatment between the two species. The concentrations of zVAD-fmk needed to prevent cell death was more than approximately

70 to 700 fold higher than the concentrations needed for completely abolishing caspase activity. In contrast to these findings, there was a coincident correlation of diminished caspase activity to resulting cytotoxicity by means of virtually identical IC_{50} of 13 nM and 14 nM, respectively, in primary murine hepatocytes.

Hepatocytes	Treatment	IC_{50} caspase activity [nM zVAD-fmk]	IC_{50} cytotoxicity [nM zVAD-fmk]	Ratio
human	ActD/TNF-a	6	418	1: 70
	ActD/CD95L	20	871	1: 44
	ActD/TRAIL	1	695	1: 695
murine	ActD/CD95L	13	14	1:1

Table 4.1: Comparison of IC_{50} values for inhibition of caspase activity and cytotoxicity by zVAD-fmk in primary human and primary murine hepatocytes treated with ActD and respective death receptor agonists.

4.1.2 Effect of caspase inhibition on death receptor agonist-induced apoptosis in HepG2 cells sensitized by inhibition of histone deacetylases and DNA methyltransferases

The influence of compounds orchestrating the epigenetic regulation of gene expression, like histone deacetylase inhibitors (HDIs) or DNA methyltransferase inhibitors (DMTIs) on apoptosis was subject of several recent studies. Beside their capacity to kill tumor cells *per se* as a promising feature for cancer therapy, HDIs are also able to sensitize cells towards death receptor agonist-induced apoptosis [156,157]. In course of these findings the DNA methyltransferase inhibitor 5-azacytidine was utilized in this thesis to examine its contribution to caspase-independent apoptosis. Furthermore, the sensitizing effect of 5-azacytidine was also subject of some mechanistical studies introduced in succeeding chapters.

The next series of experiments were dedicated to examine the influence of histone deacetylase or DNA methyltransferase inhibitors on the serine protease-dependent mechanism of apoptosis under caspase arrest. The procedure of treatment was similar as described before, except of using HDIs or DMTIs instead of ActD to sensitize the cells towards CD95L-induced apoptosis. The results received were essentially similar to those obtained with primary human hepatocytes and confirmed the results obtained with HepG2 by Dünstl [155] as seen in figure 4.2.

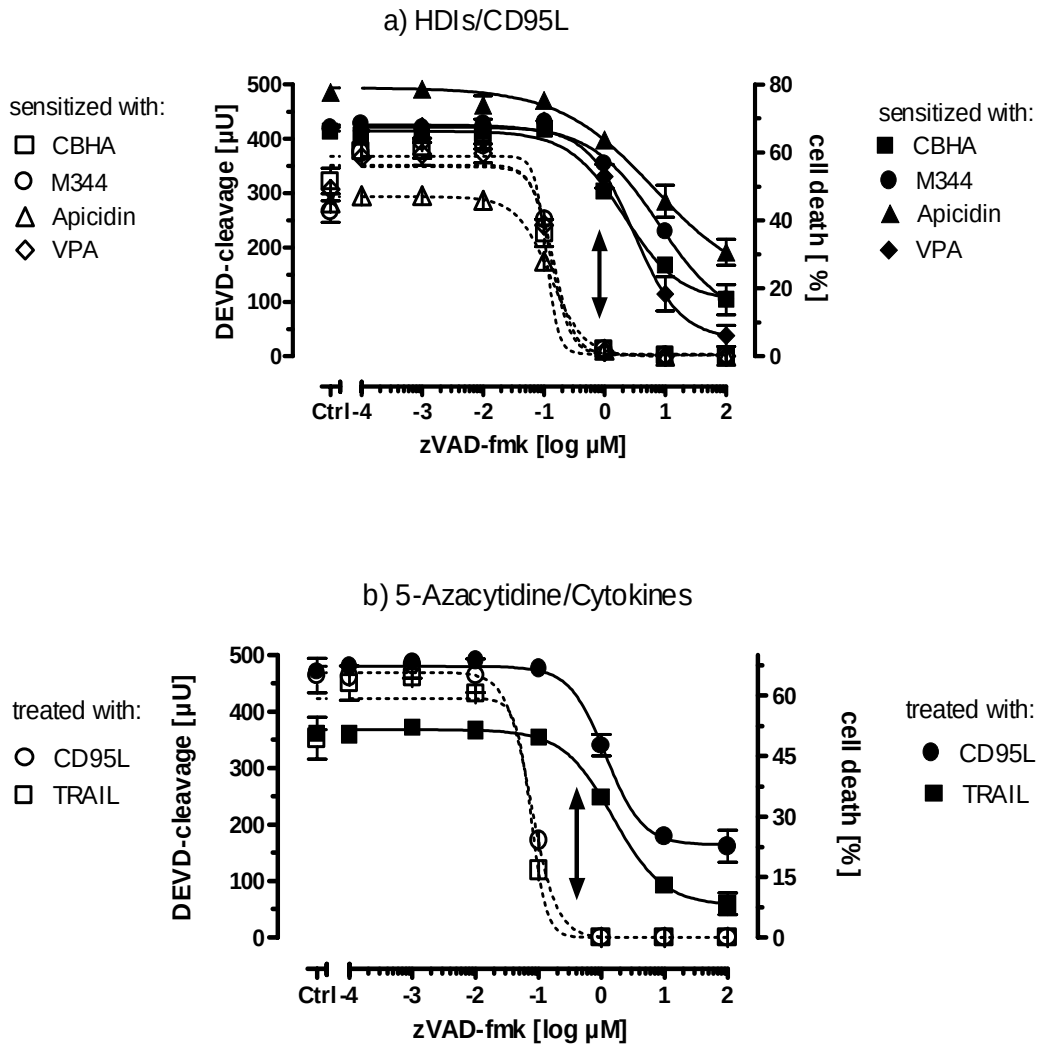


Fig. 4.2: Concentration-dependent effects of zVAD-fmk on caspase activity (open symbols) or cytotoxicity (filled symbol) on HepG2 treated with either CBHA (5 µM); M344 (1 µM); apicidin (0.5 µM) or VPA (12.5 mM) and 10% v/v CD95L (a) or with 75 µM 5-azacytidine and 10% v/v CD95L or 100 ng/ml TRAIL, respectively (b).
Caspase activity and cytotoxicity were determined after 8h and 18h, respectively.
Data represent mean – SD.

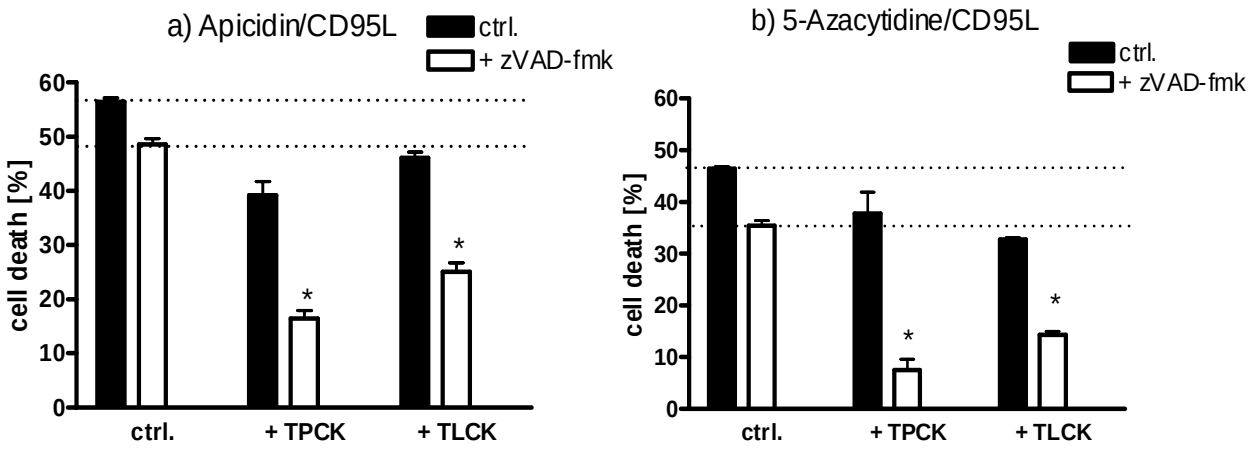
The zVAD-fmk effect condensed in half-maximal inhibition of either caspase activity or cytotoxicity is similar to the values shown in table 4.1. These results exhibit likewise caspase-independent mechanism of apoptosis acting in this model.

Treatment	IC_{50} caspase activity [nM zVAD-fmk]	IC_{50} cytotoxicity [nM zVAD-fmk]	Ratio
Apicidin/ CD95L	128	6418	1:50
CBHA/ CD95L	110	2117	1:19
M344/ CD95L	144	7846	1:54
VPA/ CD95L	133	3050	1:23
5-Azacytidine/ CD95L	77	1117	1:15
5-Azacytidine/ TRAIL	59	1604	1:27

Table 4.2: Comparison of IC_{50} values for caspase activity and cytotoxicity by zVAD-fmk in HepG2 cells treated with death receptor agonists and various drugs inhibiting histone deacetylases and DNA methyltransferases.

4.1.3 Protective effects of serine protease inhibitors on cytotoxicity with HepG2 sensitized by inhibitors of histone deacetylase and DNA methyltransferases

The protective effects of serine protease inhibitors were tested under the condition of sensitization by HDIs or 5-azacytidine. In this setting, the combined protective effect of the unspecific inhibitors TPCK and TLCK for chymotrypsin-like and trypsin-like serine proteases, respectively, as well as the broadband serine protease inhibitor AEBSF, applied in combination with zVAD-fmk, was clearly shown. Neither one of the substances alone was able to confer protection as shown in figure 4.3.



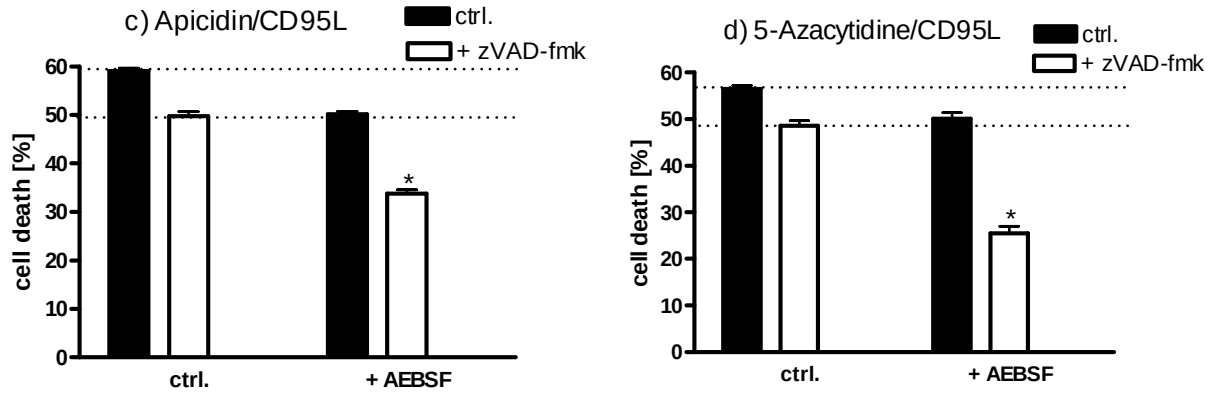


Fig.4.3: Effect of serine protease inhibitors on HepG2 cells treated with 10% CD95L after sensitization with either 0,5 µM Apicidin (a; c) or 75 µM 5-azacytidine (b;c). Serine protease inhibitors were applied as indicated in concentrations of 25 µM TPCK; 100 µM TLCK or 400 µM AEBSF in combination with 1,5 µM zVAD-fmk or with saline (ctrl.).
Data represents mean – SD.
*: $p < 0,01$ for saline *vs.* zVAD-fmk. One-way ANOVA, Dunnet's Multiple Comparison Test.

In all model systems assayed, only concentrations of zVAD–fmk, which are distinctly higher than the concentrations needed for complete caspase -3/-7 inhibition conferred protection to hepatocytes by yet unknown mechanisms. Additionally, the combined use of low concentrations of zVAD-fmk and the unspecific serine protease inhibitors TPCK, TLCK and AEBSF resulted in a significant decrease of cytotoxicity. These findings indicate a general involvement of a serine protease-dependent mechanism active in primary human hepatocytes and hepatoma cell line HepG2 at least supporting the caspase-based signaling pathways of apoptosis.

4.1.4 Efficacy of small peptide caspase inhibitors

4.1.4.1 Effect of zVAD-fmk on various caspases in HepG2 cells

To exclude the possibility that the pan-caspase inhibitor zVAD-fmk has not the same efficacy in inhibiting relevant caspases, the caspase -2;-3;-6;-7;-8;-9;-10 activity was determined using specific substrates. HepG2 cells were treated with increasing concentrations of zVAD-fmk prior to induction of apoptosis with TNF-a, CD95L or TRAIL to quantify the specific caspase activities (Fig.4.4). As summarized in table 4.3, all IC_{50} values were below 200 nM. At a concentration of 1.5 mM zVAD-fmk all caspases were completely inhibited.

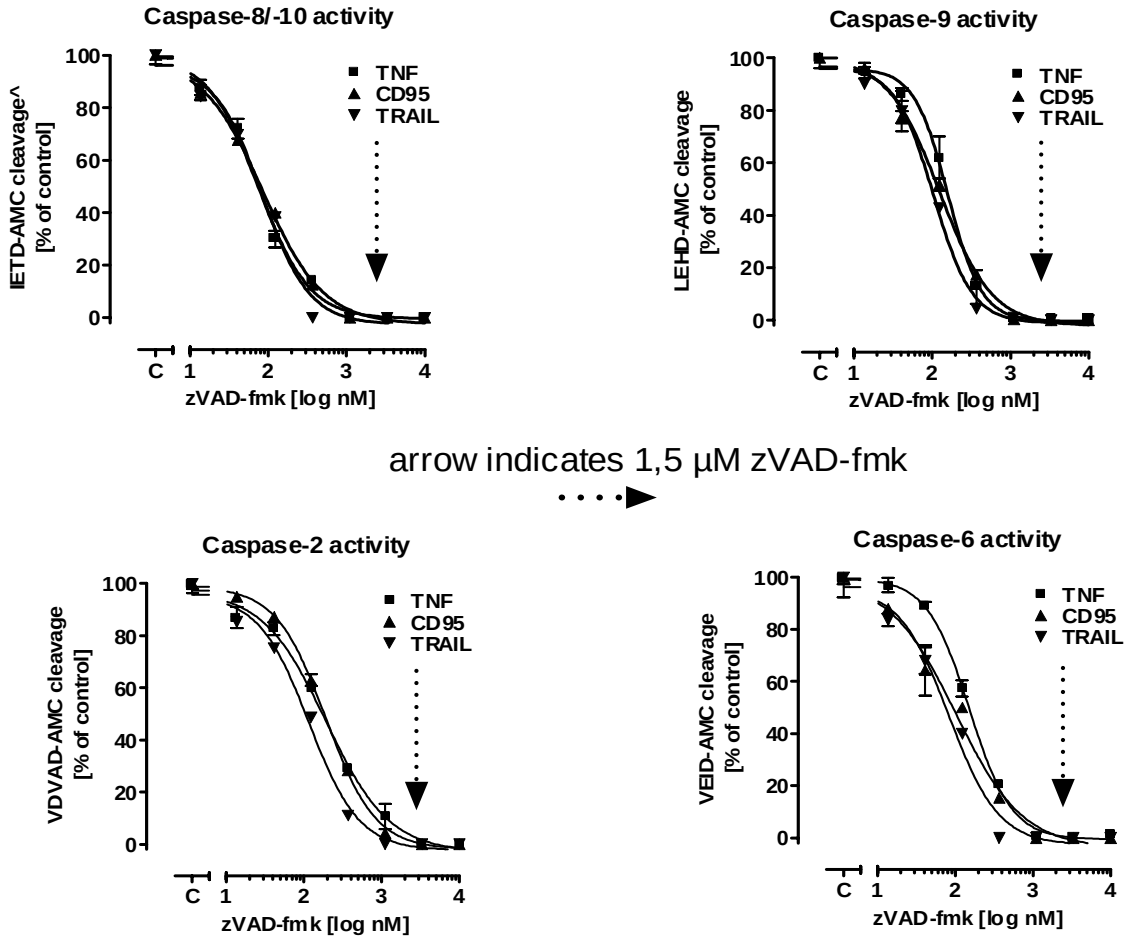


Fig. 4.4: Concentration-dependent effect of zVAD-fmk on activity of various apoptosis relevant caspases in HepG2 treated with either 1µg/ml Act and 100 ng/ml TNFa; 100 µM CHX and 10% v/v CD95L or 100 µM CHX and 100 ng/ml TRAIL. Caspase activity was measured after 8h with respective fluorochrome coupled substrate.
Data represent mean – SD. Cleavage rate means percent of control without zVAD-fmk treatment.

		IC_{50} caspase activity [nM]		
Substrate	Caspase	ActD/TNF-a	CHX/CD95L	CHX/TRAIL
IETD-AMC	Casp -8/-10	75	85	84
LEHD-AMC	Casp -9	159	125	104
VDVAD-AMC	Casp -2	193	191	116
VEID-AMC	Casp -6	152	103	84

Table 4.3: Comparison of IC_{50} values for inhibition of activity of different caspases by zVAD-fmk in HepG2 cells treated with death receptor agonists.

4.1.4.2 Effect of various caspase inhibitors on different apoptotic models

Various caspase inhibitors were tested for their influence on CD95L-induced apoptosis and on apoptosis induced by staurosporine, a potent inducer of the intrinsic mode of cell death, in HepG2 cells.

The inhibitors used, pan-caspase inhibitors (i.e. zVAD-fmk and Boc-D-fmk) just as the specific inhibitors zDEVD-fmk, zIETD-fmk and zLEHD-fmk for caspase-3, caspase-8/-10 or caspase-9, respectively, conferred no protection of HepG2 cells in concentration ranges where caspase activity was evidentially abolished. Notably, whereas all small peptide caspase inhibitors used were able to prevent cells in high concentration from death receptor agonist-induced apoptosis (Fig. 4.5a), no protection was observed using the mitochondrial inducer staurosporine (Fig. 4.5b). Also worth mentioning is the fact that the specific inhibitors are able to decrease effector caspase -3/-7 DEVD cleavage activity to zero within concentration ranges, which are not associated with any alterations of cytotoxicity as seen in fig. 4.5 a and b.

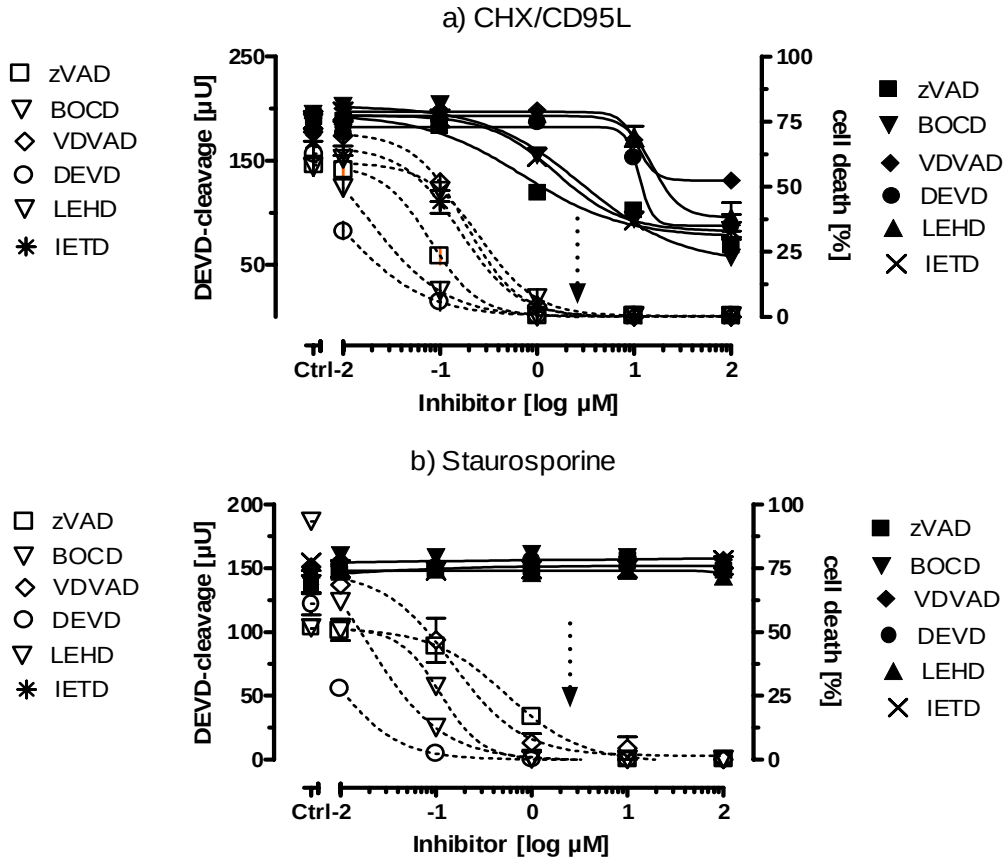


Fig. 4.5: Concentration-dependent effect of various caspase inhibitors on cytotoxicity and caspase -3/-7 activity in HepG2 cells treated with either 100 µM CHX prior addition of 10% v/v CD95L ligand or with 10 µM Staurosporine**.** Caspase activity and cytotoxicity was determined after 8h and 18h, respectively. Data represents mean ± SD.

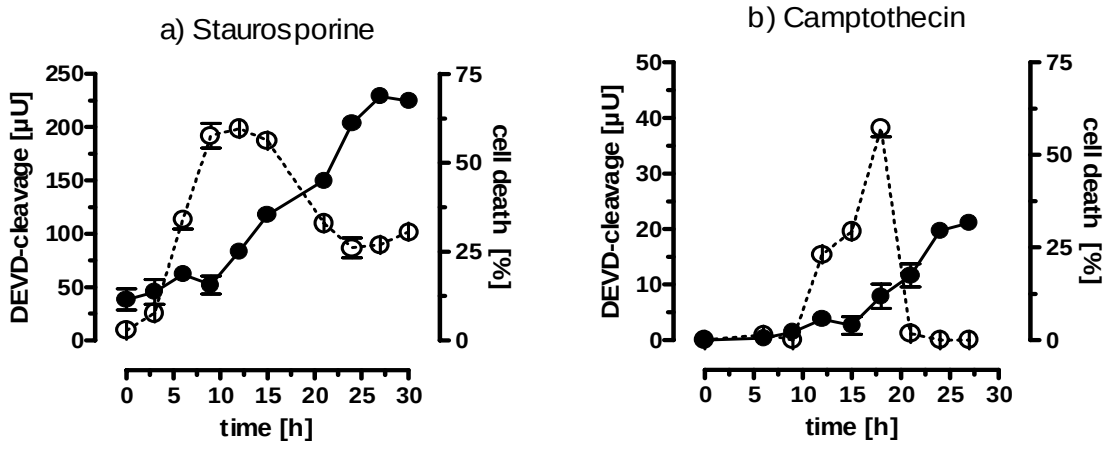
4.2 The role of caspases in the intrinsic mode of cell death

These results led us to review the data obtained with death receptor agonist-induced apoptosis in the context of the role of caspase activity within the intrinsic pathway of apoptosis which is triggered by *e.g.* chemotherapeutics or UV-radiation causing cellular stress response, whose signaling converges on the mitochondrial level to induce mitochondrial membrane permeabilization (MOMP). In recent years, it turned out that MOMP leads to cell death either by cytochrome c release followed by caspase activation [158] or by the release of caspase-independent death effectors like endonuclease G; Omi/HtrA2 [104] and apoptosis inducing factor (AIF) [159].

The aim of the following experiments was to elucidate the role of caspases in the intrinsic mode of cell death and to explore to which extent serine proteases might play a role for apoptotic signaling in HepG2 cells. For this purpose, the potent intrinsic stimuli camptothecin, staurosporine and UV-radiation were utilized.

4.2.1 Kinetics of caspase activity and cytotoxicity

Figure 4.6 shows the time dependence of caspase activity and cytotoxicity of HepG2 cells treated with staurosporine, camptothecin or UV-radiation. The time course of events differed between the several stimuli. Treatment with staurosporine and camptothecin resulted in a clear definable peak of caspase activity before cell death occurred. However, the activation of the caspase cascade was more rapid and pronounced in case of staurosporine. The induction of caspase activity after UV radiation was more protracted and coincided with the beginning of cell demise. The data presumably reflects the different modes of action and cellular targets of these compounds.



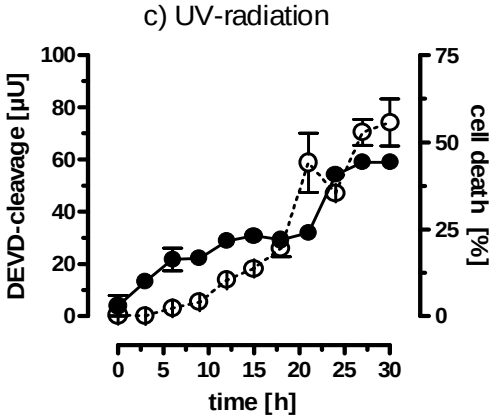
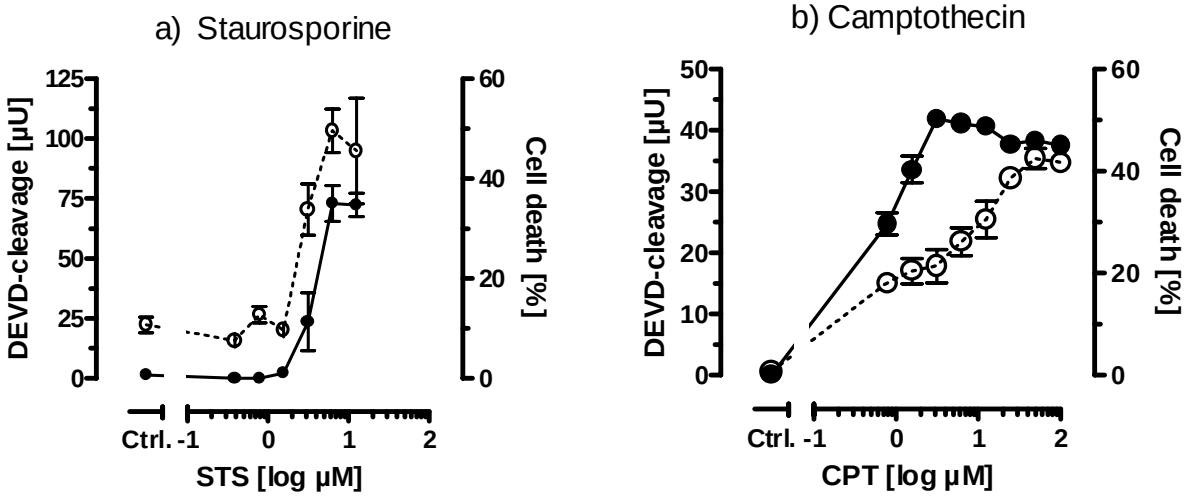


Fig. 4.6: Time course of caspase activity (o) and cytotoxicity (•) in HepG2 after treatment with either 10 µM Staurosporine (a), 10 µM Camptothecin (b) or UV-radiation (c) with an itensity of 0.5 J/cm². Data represents mean – SD.

4.2.2 Correlation of caspase activity and cytotoxicity

To point out whether a possible correlation exists between the degree of caspase activity and the subsequent cytotoxicity, HepG2 cells were treated with increasing concentrations of the respective stimuli. The concentration-dependent induction of effector caspase activity correlated directly with the enhancement of cell death as seen in Fig. 4.7.



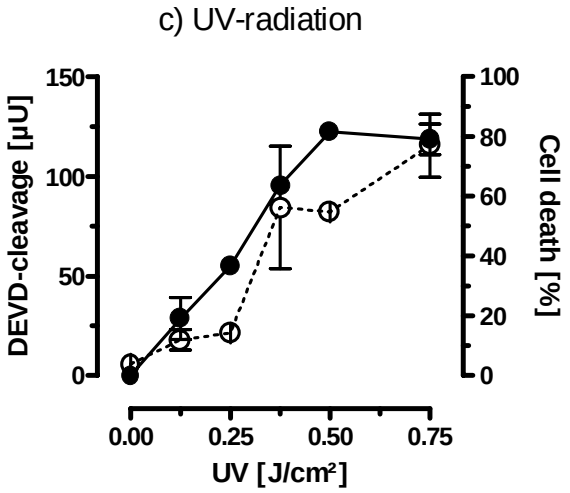


Fig. 4.7: Concentration curve for caspase activation (o) and cytotoxicity (•) after treatment of HepG2 cells with increasing concentrations of following stimuli:
a) 0-15 µM Staurosporine
b) 0-100 µM Camptothecin
c) 0-0,75 J/cm² UV B-radiation
Cytotoxicity was detected after 18h in case of staurosporine and after 30h in case of camptothecin and UV.
Data represents mean – SD.

Altogether the time and concentration dependence of cytotoxicity induced by the respective stimuli reflected the degree of the prefixed activation of caspases, which might be indicative for a causal relationship between both events.

4.2.3 Effect of caspase inhibition on the intrinsic pathway of apoptosis in HepG2 cells

The next experiment was performed to verify the previous results obtained with caspase-independent cell death induced by staurosporine as a general mechanism valid for miscellaneous intrinsic inducers of cell death in this model. To investigate whether or not caspase inhibition is sufficient to confer protection to HepG2 cells, increasing concentrations of the broadband caspase inhibitor zVAD-fmk were applied prior to treatment with either UV-radiation, camptothecin or staurosporine.

Also with these stimuli acting *via* different activation modes of cell death, in the state of a virtually abolished caspase activity, no protection against cell death was observed. Notably, also high concentrated zVAD-fmk failed to protect cells, except in case of camptothecin, where a protective effect was achieved with 100 µM zVAD-fmk as shown in figure 4.8c. To verify the results with regard to death receptor ligand-induced apoptosis, HepG2 cells were treated with CHX/CD95L and increasing concentrations of zVAD-fmk in a parallel experiment. The results verify the non-protective effects of zVAD-fmk even in high concentrations applied within the staurosporine and UV-triggered apoptosis (Fig. 4.8 d).

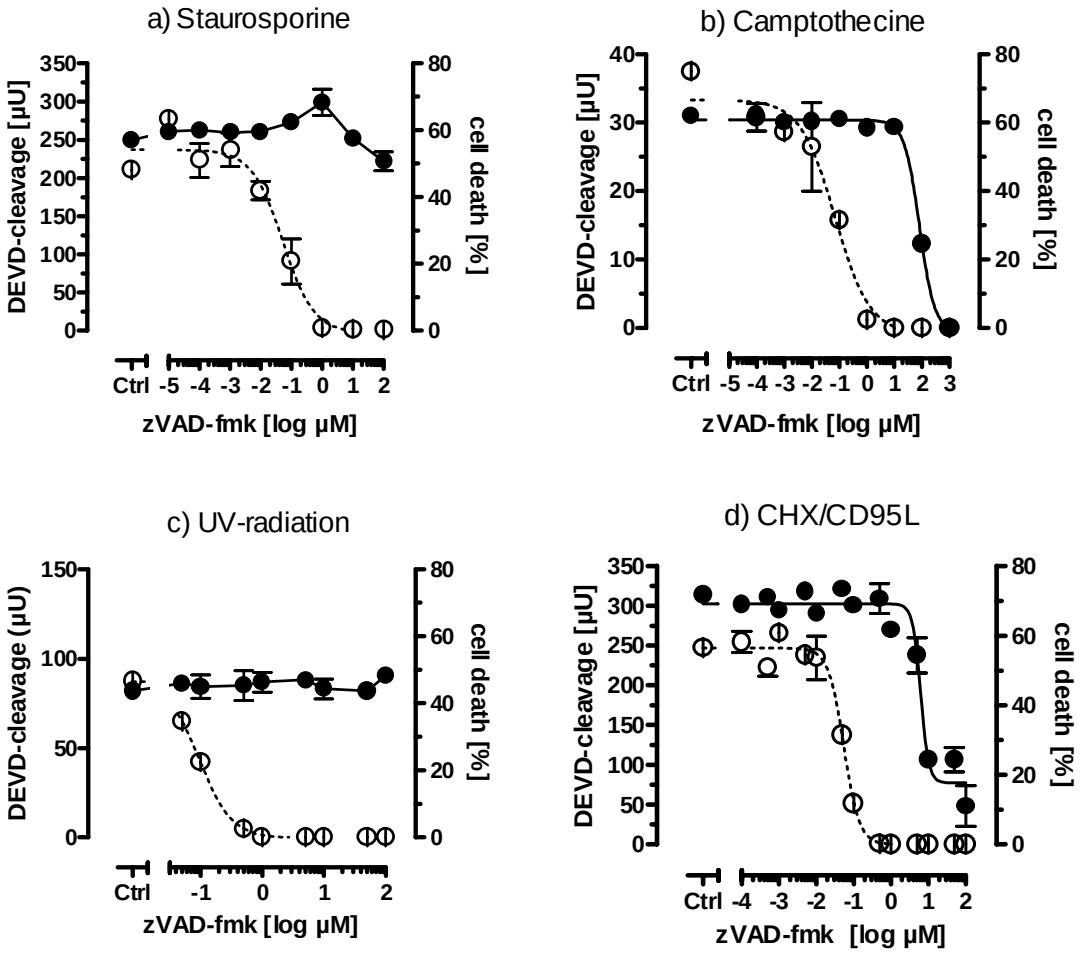


Fig. 4.8: Concentration-dependent effect of zVAD-fmk on caspase activity (o) and cytotoxicity (•) in HepG2 cells treated with 10 µM staurosporine (a); 10 µM camptothecin (b); 0.5 J/cm² UVB -radiation (c) and 100 µM CHX; 10% v/v CD95L (d). Data represents mean – SD.

Table 4.4 highlights the concentrations for half-maximal inhibition of caspase activity of HepG2 cells stimulated with either death receptor agonist CD95L or intrinsic stimuli which are common in either set up, in contrast to complete lack of protection with high concentrations of zVAD-fmk within the mitochondrial mode of HepG2 cell death.

	IC_{50} caspase activity [nM zVAD-fmk]	**IC_{50} cytotoxicity** [nM zVAD-fmk]
Staurosporine	52	-
Camptothecin	69	approx. 80.000
UV-radiation	95	-
CHX/CD95L	54	6924

Table 4.2: Comparison of IC_{50} values for inhibition of caspase activity and cytotoxicity by zVAD-fmk in HepG2 cells treated with stimuli inducing the mitochondrial mode of cell death and control experiment done with CHX/CD95L.

4.2.4 Morphology of mitochondrial mode of cell death under caspase arrest

To check for morphological features of apoptosis with assays performable by light- and fluorescence microscopy, HepG2 cell were stimulated with camptothecin, staurosporine or UV-radiation after treatment with either 1.5 µM zVAD-fmk or with vehicle.

After an appropriate incubation time the cells were examined for membrane blebbing, chromatin condensation and for exposure of phosphatidylserine on the outer membrane as typical markers of apoptosis, which were clearly observable for all treatments applied. Irrespective of any caspase arrest, the visible parameters of apoptosis were qualitatively indistinguishable within a given stimulus. The pictures taken with staurosporine treated HepG2 cells are shown as a representative example for all three applied stimulus.

Thus, a switch from apoptosis to *e.g.* necrotic-like cell death under caspase arrest could be excluded. The control cells showed the inconspicuous morphology of healthy cells.

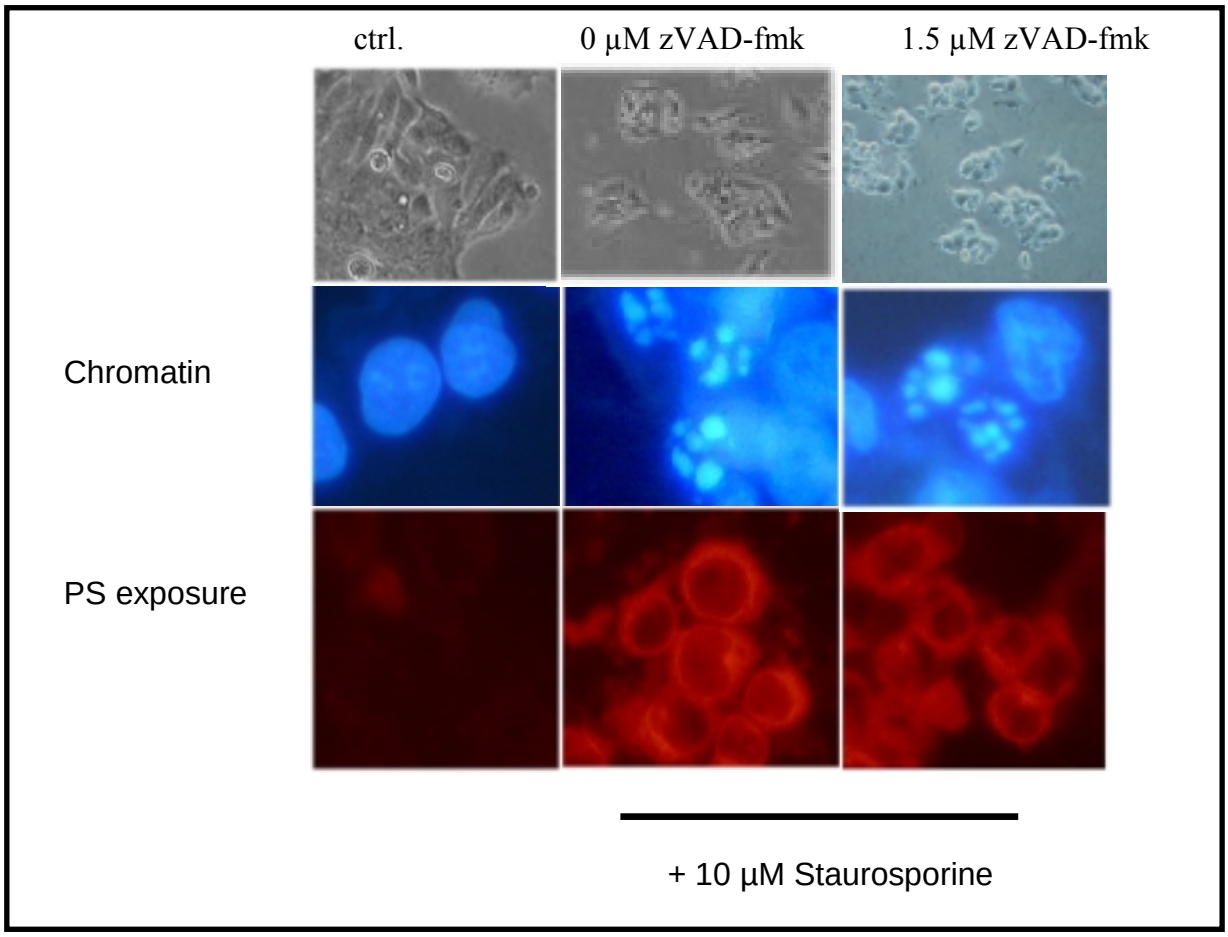


Fig.4.9: Morphology (upper row); chromatin condensation (middle row) and exposure of phophatidylserine (lower row) on the outer membrane of HepG2 cells treated with 10 µM staurosporine. Phosphatidylserine exposure was detected after 6h, chromatin condensation was detected after 8h, and morphology was pictured after 18h.

4.2.5 Protective effects of serine protease inhibitors on cytotoxicity in HepG2 cells induced by stimuli of the mitochondrial mode of cell death?

In order to further characterize the caspase-independent cell death of HepG2 cells induced by stimuli of the intrinsic mode of cell death, a variety of caspase inhibitors were tested. The main focus was laid on serine protease inhibitors, which showed protective effects in experiments performed with death receptor agonists.

Thus, HepG2 cells were treated with suitable amounts of either UV-radiation, camptothecin or staurosporine in various combinations of zVAD-fmk and serine protease inhibitors like TPCK, TLCK and AEBSF pictured in fig. 4.10. In contrary to the cytoprotective effects mediated by the combinatorial use of TPCK/TLCK and zVAD-fmk in death receptor ligand – induced cell death no protection was observed under any condition investigated. Even though

each inhibitor was applied over a wide concentration range, no protective effects were observed.

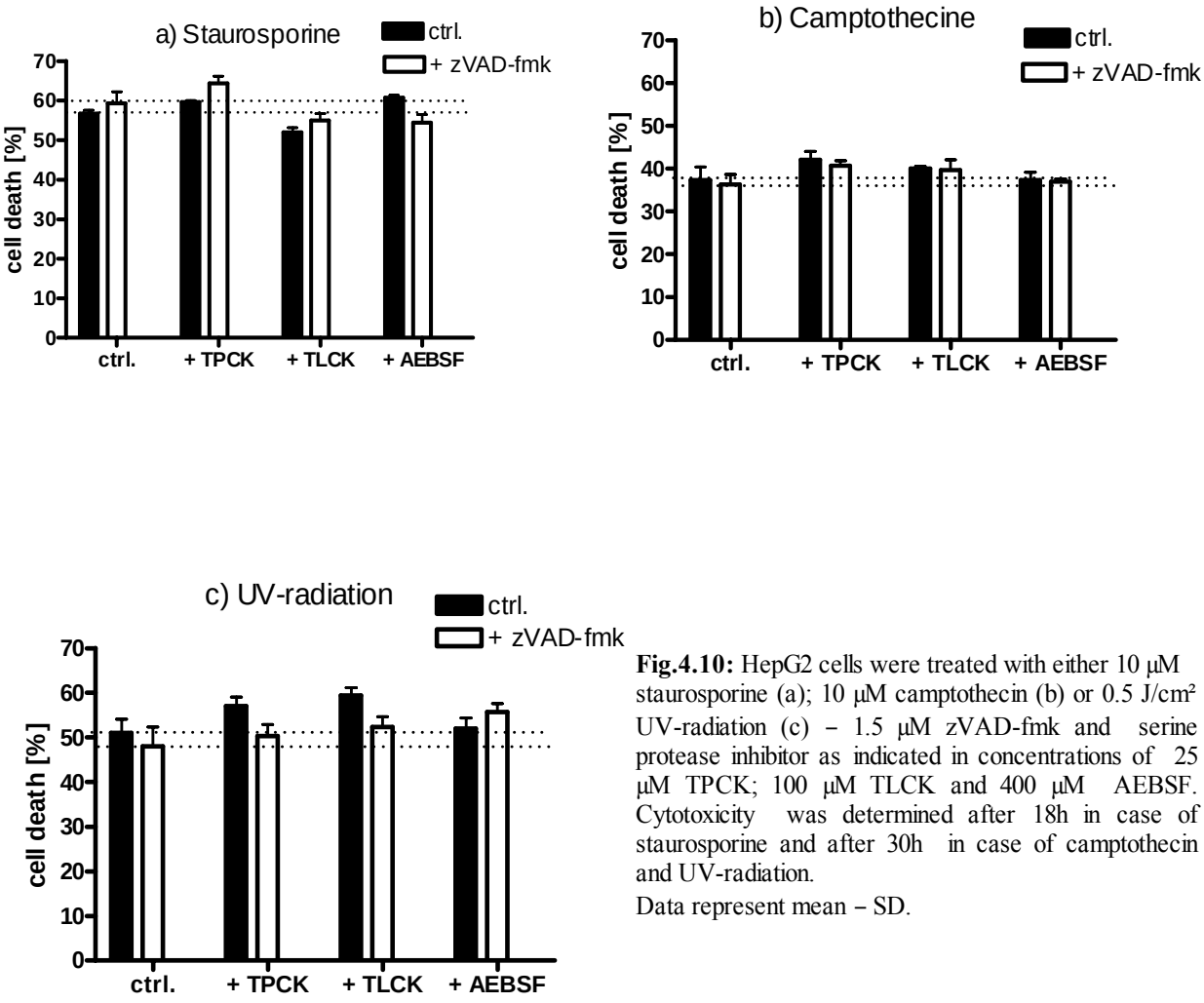


Fig.4.10: HepG2 cells were treated with either 10 µM staurosporine (a); 10 µM camptothecin (b) or 0.5 J/cm² UV-radiation (c) – 1.5 µM zVAD-fmk and serine protease inhibitor as indicated in concentrations of 25 µM TPCK; 100 µM TLCK and 400 µM AEBSF. Cytotoxicity was determined after 18h in case of staurosporine and after 30h in case of camptothecin and UV-radiation.
Data represent mean – SD.

We triggered apoptosis via the extrinsic or the intrinsic pathway to display the role of caspase activation in HepG2 cells. Our results indicate that caspase activity seems to be dispensable for execution of apoptotic cell death. However, the characteristics appear to be different, depending on the used stimuli and their respective signaling pathways. The intrinsic inducers avoid the hypothetical serine protease activation under global caspase arrest. Nevertheless both caspase-independent pathways seem to converge at a common executing mechanism leading to cell demise, which exhibits apoptosis-like morphology.

4.3 Role of apoptosis inducing factor (AIF) in apoptosis under caspase arrest in HepG2 cells

The next question to be answered addresses the factors promoting and executing the cell disassembly, such as condensation and fragmentation of DNA. Since the phenomenon of caspase-independent apoptosis could be seen in the extrinsic and the intrinsic mode of cell death, which are connected via Bid cleavage, the main focus was set onto the mitochondria harbouring a set of proteins, which are capable of executing apoptosis independent of caspases. The protein apoptosis inducing factor (AIF) was under extensive examination in recent years and is known to act in a caspase-independent manner. In healthy cells AIF is sequestered into the mitochondrial intermembrane space but once released during a cellular insult, it is cleaved and translocated into the nucleus. There is evidence that AIF induces chromatin condensation and cleavage of DNA into high molecular weight fragments [160].

To investigate the relevance of AIF within apoptosis under caspase arrest we tried to determine the mode of DNA fragmentation and the kinetic of AIF release during an apoptotic insult. A second attempt was to inhibit AIF activity. Since it is known that AIF translocation can indirectly prevented by inhibiting PARP activity [160], we utilized 3-aminobenzamide (3-AB), a potent PARP inhibitor, to assay its effect on AIF translocation and cell death in HepG2 cells.

4.3.1 Kinetics of AIF release during death receptor agonist induced apoptosis

The time course of AIF and cytochrom c release into the cytosol was determined after 1.5h and 3h after addition of CD95L in absence and presence of 1.5 µM of zVAD-fmk. Figure 4.11 demonstrates that AIF release is a fast event occurring concomitant with cytochrome c release unaffected by any caspase arrest.

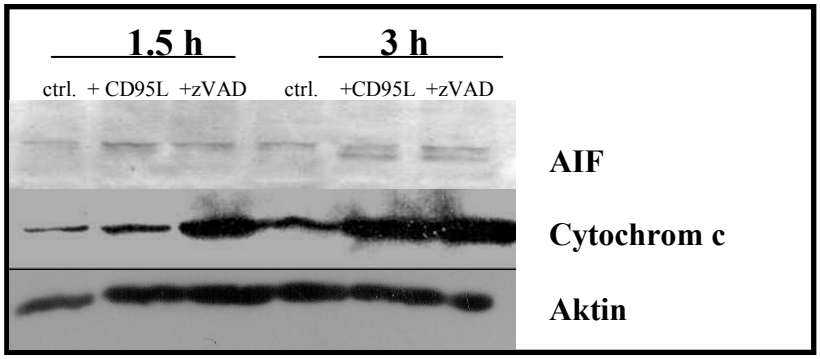


Fig. 4.11: Time course of cytochrom c - and AIF release from mitochondria into cytosol in HepG2 cells treated with 100 µM CHX and ±1.5 µM zVAD-fmk prior to addition of 10% v/v CD95L. Each lane was loaded with 30 µg protein.

The double bands appear due to 67 kDa premature, nonapoptogenic form of AIF with a mitochondrial localization signal, which is removed within the mitochondria to form the 57 kDa mature form of AIF. The 57 kDa form is released after apoptosis induction.

4.3.2 Pattern of DNA fragmentation of apoptosis under caspase arrest

It is well established that AIF induces a characteristic high molecular weight DNA fragmentation pattern of 50-200 kilobase pairs [159]. While the classical laddering of DNA performed by the caspase dependent CAD/ICAD system is composed of 126 bp and multiples, we assayed the pattern evolving from fragmentation under caspase arrest. The DNA in CD95L stimulated cells appeared as a typical "ladder" of fragmented DNA, independent from pre-treatment with zVAD-fmk.

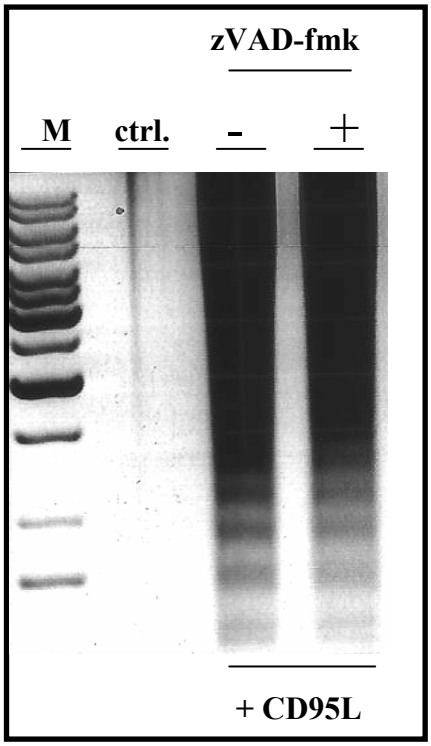


Fig. 4.12: DNA fragmentation pattern of HepG2 cells after treatment with 10 % v/v CD95L ±1.5 µM zVAD-fmk. Cells were lysed 8h after apoptosis induction and purified DNA was loaded on a 1% agarose gel and visualized by ethidiumbromid.

4.3.3 Prevention of AIF release under PARP inhibition

The AIF translocation was actually abolished under PARP inhibitory conditions in HepG2 cells. The release from AIF as part of the apoptotic response after stimulation with CD95L was clearly visible. AIF was also released under caspase inhibitory conditions in case of CD95L, however, to a smaller extent, which is reflected by the decrease in cytotoxicity under zVAD-fmk (compared to Fig 4.14). But it was clearly visible that AIF remained into the mitochondria in cells treated with 3-AB. Cytochrome c release was not impaired in any condition (Fig 4.13).

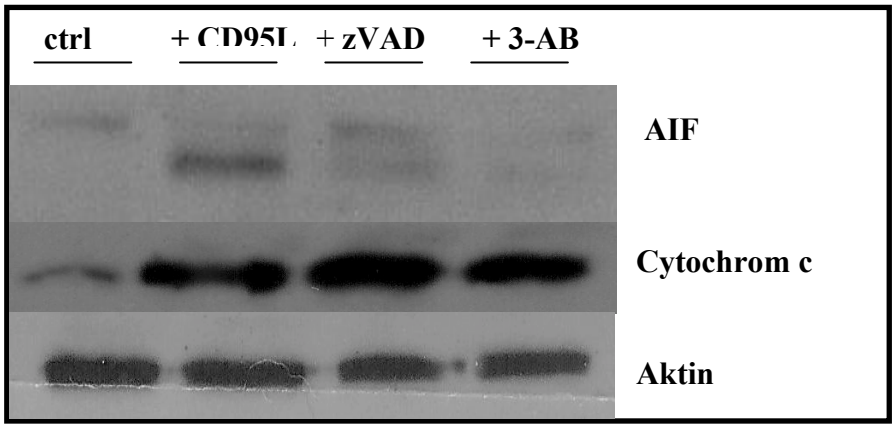


Fig. 4.13: Release of cytochrome c and AIF from mitochondria into cytosol of HepG2 cells treated with either 10 % v/v CD95L and ± 1.5 µM zVAD-fmk. Cells were lysed 3h after treatment. Each lane was loaded with 30 µg protein.

To investigate the influence of the PARP inhibitor 3-AB on cytotoxicity under caspase promoting compared to caspase inhibitory conditions HepG2 cells, pre-treated with or without zVAD-fmk, were stimulated with either CD95L or staurosporine. In spite of using increasing concentrations of 3-AB, no protective effect was observed, also in conditions were AIF translocation was prevented by 3-AB (Fig.4.14).

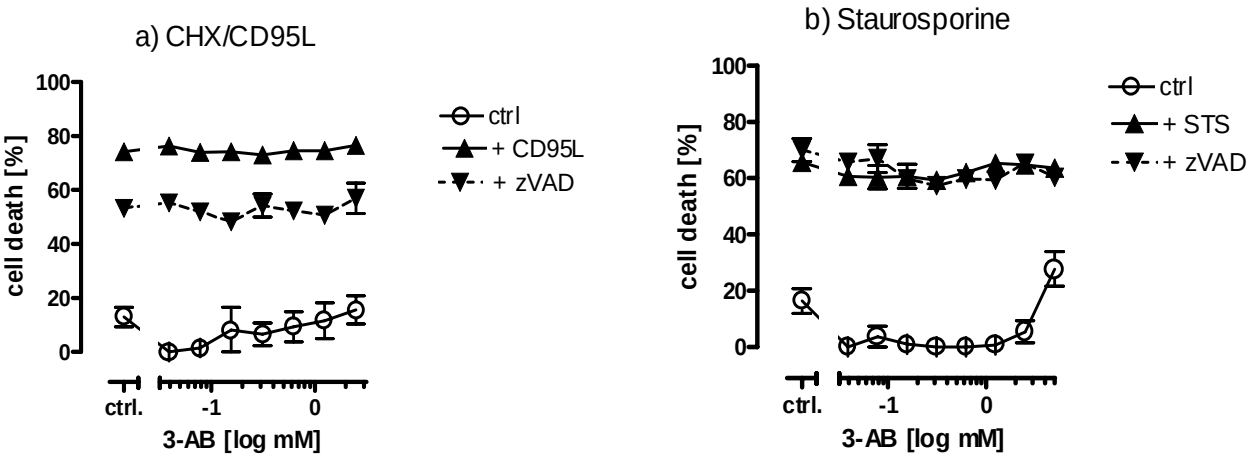


Fig. 4.14: Effect of increasing concentrations of 3-AB on CD95L (a) and staurosporine (b) mediated cytotoxicity in HepG2 cells under caspase promoting and caspase inhibitory conditions by 1.5 µM zVAD-fmk. Cell were treated with either 10% v/v CD95L or 10 µM staurosporine. Cytotoxicity was determined after 18h.
Data represent mean – SD.

4.4 5-Azacytidine: new properties of an old molecule

Experimental studies conducted in this study addressing the mechanism of caspase-independent apoptosis revealed that the DNA methyltransferase inhibitor 5-azacytidine was able to sensitize hepatocytes towards death receptor agonist-induced apoptosis. As 5-azacytidine represents a clinically relevant drug, the potential sensitizing effects on hepatocytes were examined in detail to elucidate the major pharmacodynamic properties of this compound.

4.4.1 Effect of 5-azacytidine on death receptor agonist-induced cytotoxicity

4.4.1.1 5-Azacytidine mediated sensitization in primary human hepatocytes

The influence of 5-azacytidine on death receptor agonist-induced apoptosis was examined in primary human hepatocyte cultures. The hepatocytes were isolated from pathological inconspicuous specimens obtained from patients undergoing hepatic resections for the therapy of liver tumors. As seen in figure 4.15, 5-azacytidine rendered hepatocytes sensitive to the action of the death receptor agonists TNF-a;CD95L and TRAIL in a concentration-dependent manner.

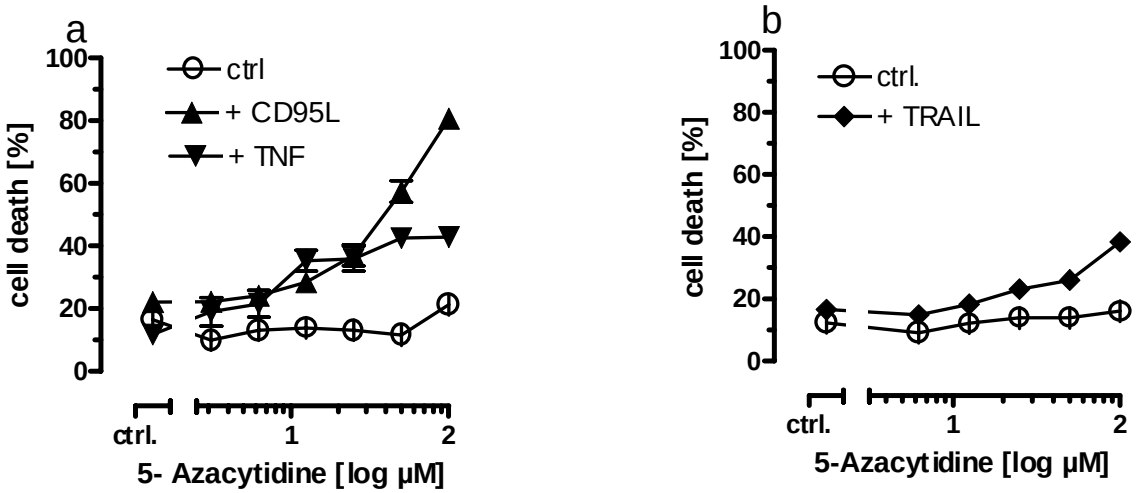


Fig. 4.15: Effect of increasing concentrations of 5-azacytidine on primary human hepatocytes treated with either 100 ng/ml TNF-a; 10 % v/v CD95L (a) or 100 ng/ml TRAIL (b). Cytotoxicity was determined after 18h.
Data represent mean – SD.

4.4.1.2 5-Azacytidine mediated sensitization in primary murine hepatocytes

The results shown above were confirmed in analogous experiments utilizing hepatocytes isolated from murine liver. Since murine liver cells were insensitive against TRAIL-induced apoptosis, the assays were conducted with TNF-a and CD95L.

The first set of experiments was done to define the optimal concentration for sensitization. Increasing concentrations of 5-Azacytidine were added on primary murine hepatocytes alone or in combination with invariable concentrations of the cytokines TNF-a and CD95L (data not shown). From these data 75 µM 5-azacytidine was derived as the most efficient concentration for sensitization. In counter-check experiments the sensitizing effects were confirmed by treatment of primary murine hepatocytes with increasing concentrations of the cytokines at a fixed concentration of 75 µM 5-azacytidine. As seen in figure 4.16, increasing concentrations of TNF-a and CD95L resulted in enhanced cytotoxicity and effector caspase-3/-7 activity.

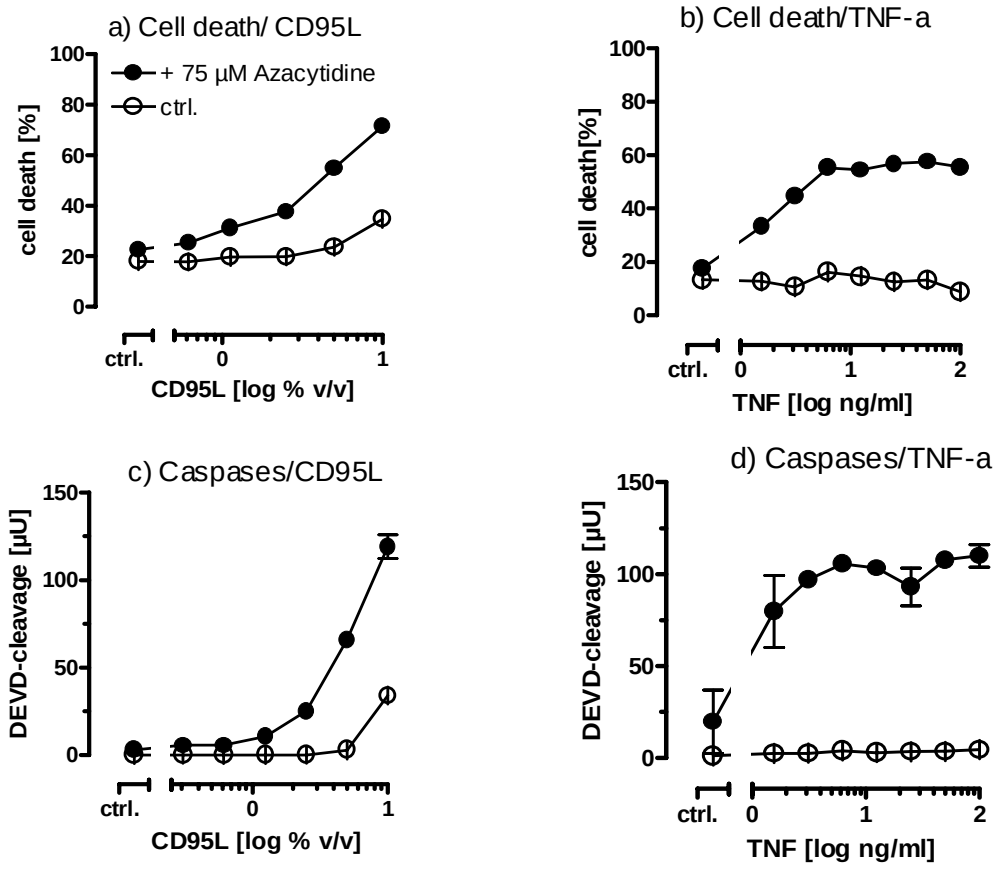


Fig. 4.16: Concentration-response curves of death receptor ligands with determination of cytotoxicity (a,b) and their respective effector caspase activity (c,d). Primary murine hepatocytes were treated with 75 µM 5-azacytidine prior addition of increasing concentrations of CD95L and TNF-a. Cytotoxicity was determined after 18h.
Data represent mean − SD.

As the methyltransferase inhibitor 5-azacytidine proved to sensitize hepatocytes towards apoptosis, we tried to work out following topics:

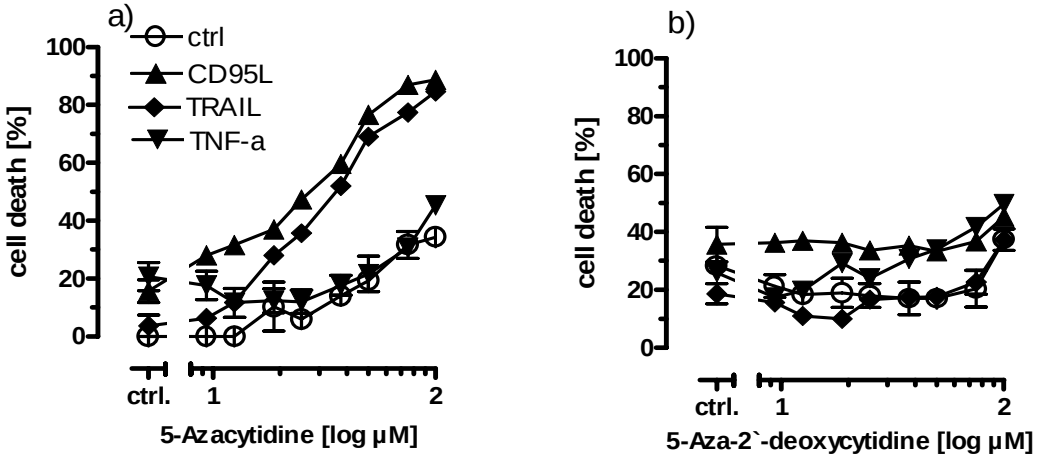
- Examination of DNA methyltransferase inhibitors in terms of sensitization of hepatocytes
- Mechanistic studies for clarification of the action point(s) of 5-azacytidine.

For this purpose the human hepatoma cell line HepG2 was chosen.

4.4.1.3 Capability of various DNA methyltransferase inhibitors to sensitize the human hepatoma cell line HepG2 cells against death receptor agonist-induced apoptosis

To elucidate potential augmentation of death receptor-triggered cytotoxicity with different kinds of DNA methyltransferase inhibitors, HepG2 cells were incubated with increasing concentrations of 5-azacytidine, the analogue 5-aza-2'-deoxycytidine, S-(5'-adenosyl)-L-homocysteine and RG 108. To define the optimal concentration for sensitization *versus* a minimal basal toxicity, the indicated substances were incubated with HepG2 cell in increasing concentration alone or in combination with the cytokines TNF-a, CD95L or TRAIL (Figure 4.17).

Besides the positive controls, in terms of ActD and CHX, only the combination of 5-azacytidine combined with TRAIL or CD95L showed a significant concentration-dependent increase of cytotoxicity. Unlike in primary hepatocytes, 5-azacytidine was not capable of promoting TNF-a induced cytotoxicity. The no-effect data for the analogue 5-aza-2'-deoxycytidine and for positive controls see figure 4.16, data for RG 108 and S-(5'-adenosyl)-L-homocysteine are not shown.



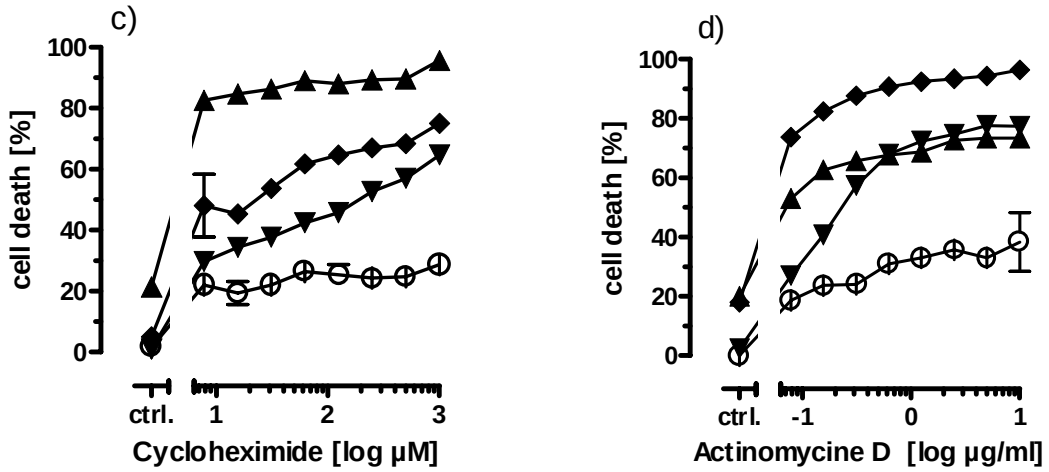


Fig. 4.17: Screening for maximal non-toxic, sensitizing concentrations of MTI and ActD/CHX toward cytokines in HepG2 cells. HepG2 cells were incubated with increasing concentrations of 5-azacytidine (a), 5-aza-2'-deoxycytidine (b), cycloheximide (c) and actinomycine D (d) 3h prior to addition TRAIL (100 ng/ml), TNFa (100 ng/ml) and CD95L (10 % v/v). Cytotoxicity was determined after 18h.
Data represent mean ± SD.

4.4.1.4 Kinetics of caspase activity and cytotoxicity in HepG2 cells after sensitization with 5-azacytidine

After death receptor trimerisation and assembly of the death inducing signaling complex (DISC) upon binding of a death ligand, a hierarchical sequence of death signaling leads to subsequent activation of the effector caspases -3/-7, detectable by DEVD cleavage, which in turn activate other enzymes responsible for cellular degradation.

In the used cellular system an increase in cytotoxicity occurred over time after a peak of maximal caspase activity. Data in figure 4.18 demonstrated that the overall series of events was similar with CD95L and TRAIL, even though the induction was more rapid in case of TRAIL. The peak of caspase activity occurred around 8-10h, while the toxicity reached the plateau phase after 18h to 21h. It is concluded from these kinetic observations that the emerging cytotoxicity is likely to be the consequence of the activation of caspases.

Neither caspase -3/-7 like activity nor any cytotoxicity was detected in HepG2 cells treated with 5-azacytidine alone or in combination with TNF-a.

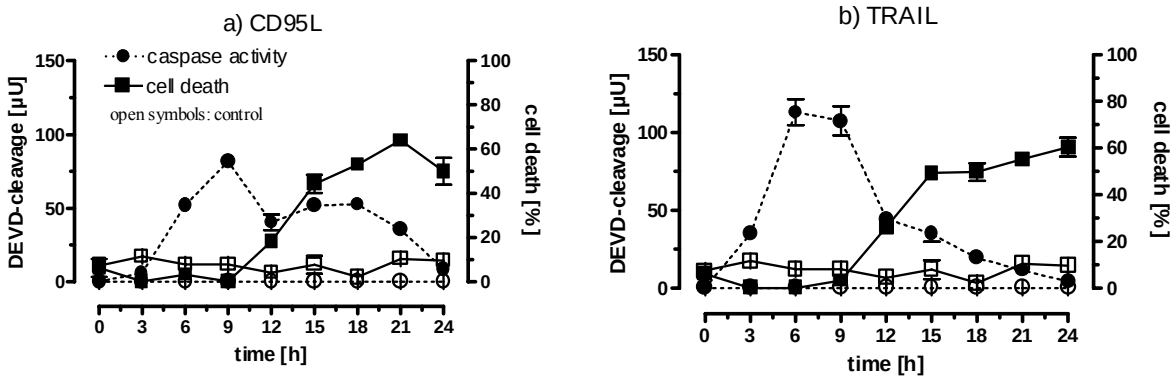
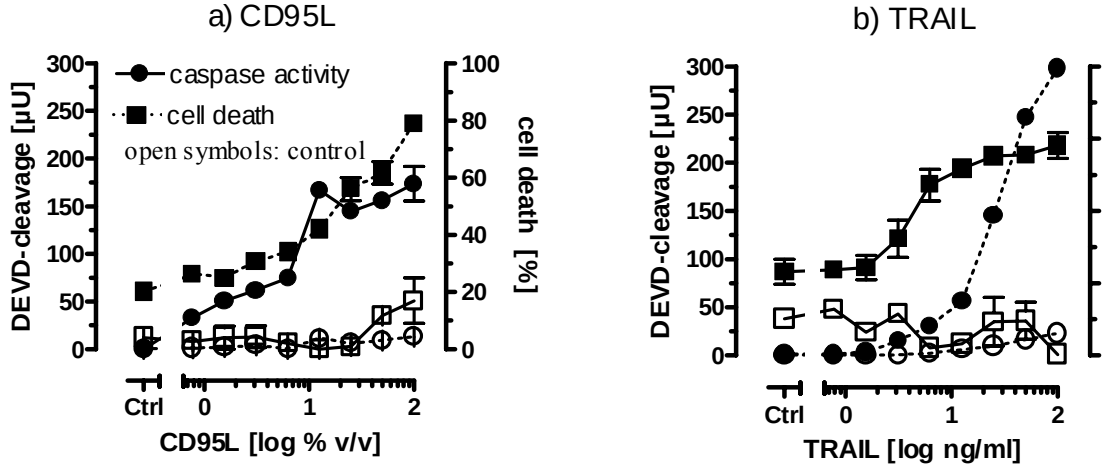


Fig. 4.18: Time course of caspase activity (•) and resulting cytotoxicity (■) after treatment of HepG2 cells with either 75 µM 5-azacytidine alone (open symbols) or in combination with 10 % v/v CD95L (a) and TRAIL 100 ng/ml (b). Cytotoxicity and caspase activity was determined at the indicated time points. Data represent mean ± SD.

4.4.1.5 Correlation of caspase activity and cytotoxicity HepG2 cells after sensitization with 5-azacytidine

In the next series of experiments a reversed set-up was chosen in order to correlate the death receptor-agonist concentration with cytotoxicity and caspase activity. HepG2 cells, pre-treated with a constant concentration of 75 µM 5-azacytidine were incubated with increasing concentrations of either CD95L or TRAIL. As seen in fig. 4.19 increasing concentrations of death receptor agonist caused increasing caspase activity and cytotoxicity.

Also in this set of experiments the non-sensitization effect of 5-aza-2'deoxycytidine, S-(5'-adenosyl)-L-homocysteine and RG 108 were affirmed. The positive control ActD enhanced the cytotoxicity as well as caspase activity with either cytokine utilized (data not shown).



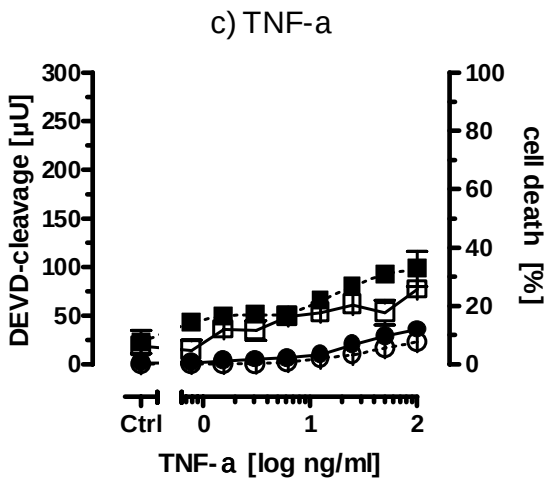


Fig. 4.19: Concentration curve for caspase activation (●) and cytotoxicity (■) after treatment of HepG2 cells with increasing concentrations of following stimuli after pretreatment with 75 µM 5-azacytidine:
a) 0-10% v/v CD95L.
b) 0-100 ng/ml TRAIL.
c) 0-100 ng/ml TNF-a.
Caspase activity was determined after 8h, cytotoxicity was determined after 18h.
Data represents mean – SD.
Open symbols represent control without 5-azacytidine pretreatment.

In conclusion, the results described in the two previous chapters showed a time- and concentration-dependent activation of caspases and a subsequent cytotoxicity.

4.4.1.6 Morphology of cell death in HepG2 cells

In order to verify the nature of the 5-azacytidine-mediated sensitization towards death receptor agonist-induced apoptosis the morphology of the dying cells was assessed by light- and fluorescence microscopy. Unlike untreated controls, the cells treated with CD95L and 5-azacytidine showed a rounding up and membrane blebbing (zeiosis), the typical nuclear morphology of condensed chromatin stained with Hoechst and exposure of phosphatidylserine on the outer membrane. The cells treated with 5-azacytidine alone showed no morphological changes in all parameters analyzed. In the case of CD95L, the cells showed only slight signs of apoptotic morphology. This could be tentatively explained with the fact that CD95L without sensitization is able to induce apoptosis to a certain degree. Similar results are observed in the center of morphological examinations of primary murine hepatocytes treated with 5-azacytidine and CD95L alone or in combination (data not shown).

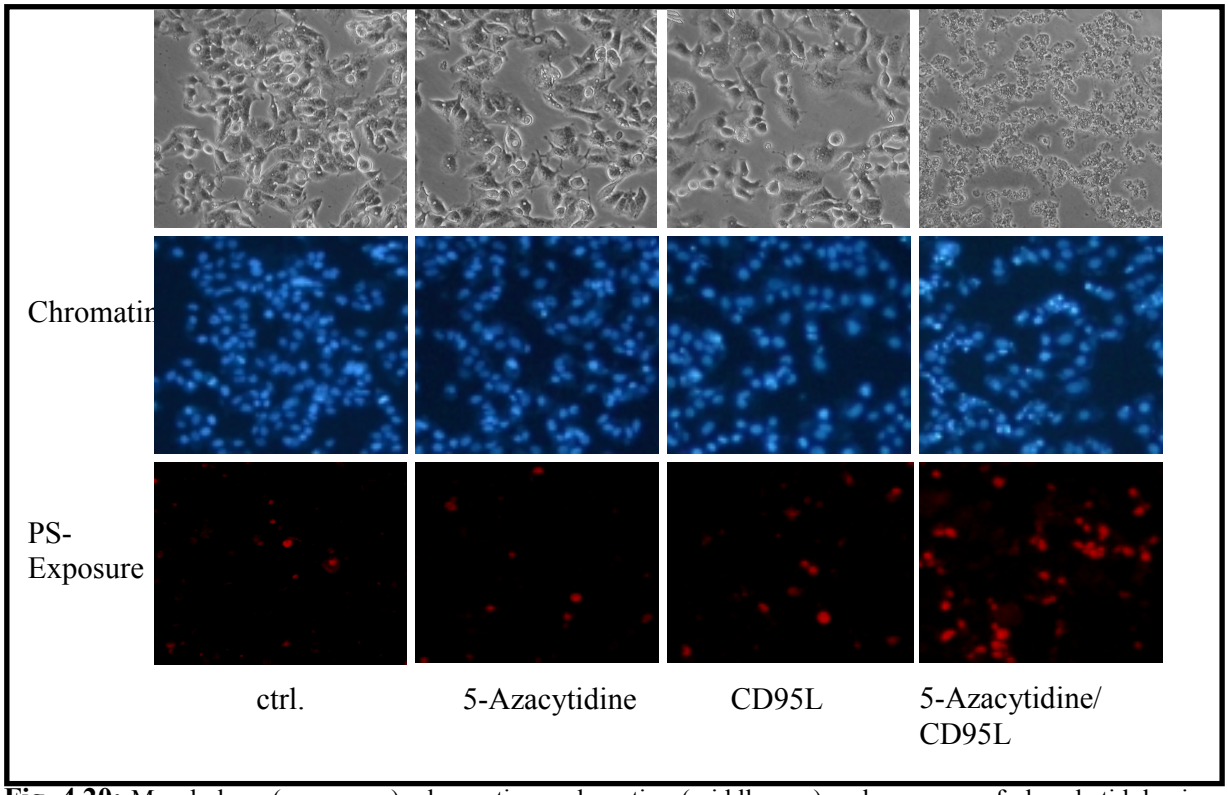


Fig. 4.20: Morphology (upper row); chromatin condensation (middle row) and exposure of phosphatidylserine on the outer membrane of HepG2 cells treated with either 75 µM 5-azacytidine; 10% v/v CD95L or in combination with both compounds. Pictures were taken 6h for PS exposure, 8h for chromatin condensation and 18h for morphology after stimuli.

4.4.2 Mechanistic rational for sensitizational effects of 5-azacytidine

4.4.2.1 Kinetics of altered protein expression pattern

The characteristics of apoptosis are thought to be the result of an irreversible activation of proteases, such as caspases, which are stored preformed as zymogens within the cytosol. This network of enzymes is tightly controlled by regulatory proteins with pro- or antiapoptotic features, which ensures that the cell death programs works properly in case of a physiological trigger or to secure the cell from accidentally triggered caspase cascades.

Cells can become hypersensitive or *vice versa* resistant towards apoptotic stimuli by modulating protein expression levels. Thus, alterations in the levels of various central regulator proteins, which could have profound effects on apoptotic execution under the influence of 5-azacytidine were examined. Cells were treated with 75 µM 5-azacytidine for up to 15h; samples for SDS-PAGE and subsequent western blotting were taken every 3 hours. Three groups of proteins were examined for changes: initiator and effector caspases; antiapoptotic proteins and proapoptotic proteins.

During the time course the expression levels of the main players in the network, the executioner caspase-8 and-9 as well the hierarchical subsequent main effector caspase- remains largely unaffected.

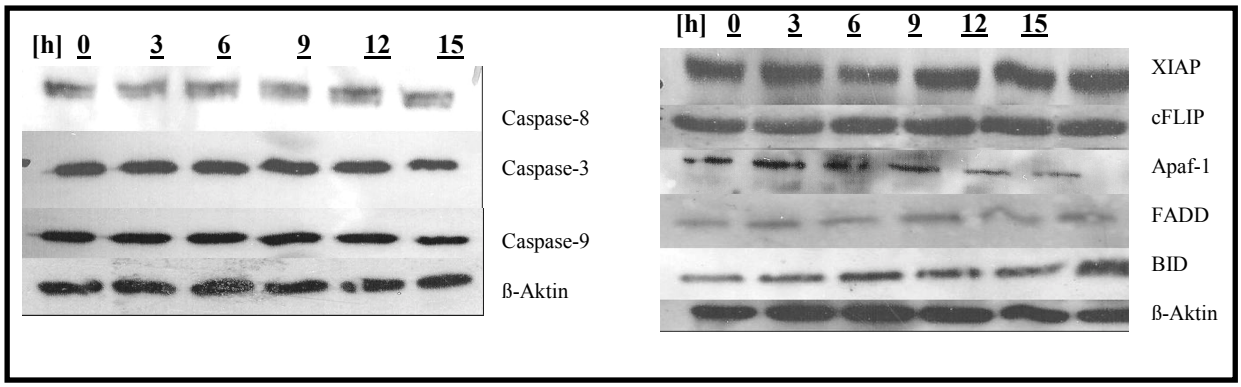


Fig. 4.21: Time course of expression of proteins was detected using cell lysates from HepG2 cells treated with 75 µM 5-azacytidine for up to 15 h. Each lane was loaded with 30 µg protein.

The proapoptotic proteins FADD and Bid showed no significant alterations over time. The expression level of Apaf-1 seemed to be slight down regulated after 9h. Furthermore, the group of well known antiapoptotic proteins, like the caspase-8 competing protein cFLIP as well as the endogenous caspase inhibitor XIAP showed no detectable change in their cellular level.

However, a change in p53 and BAX expression level was observed, most dramatic during the first 6h after 5-azacytidine treatment. In detail, during the first 2h an increase in cytosolic level of p53 was observable and after 3h the level of BAX was clearly enhanced (fig. 4.22a,b)

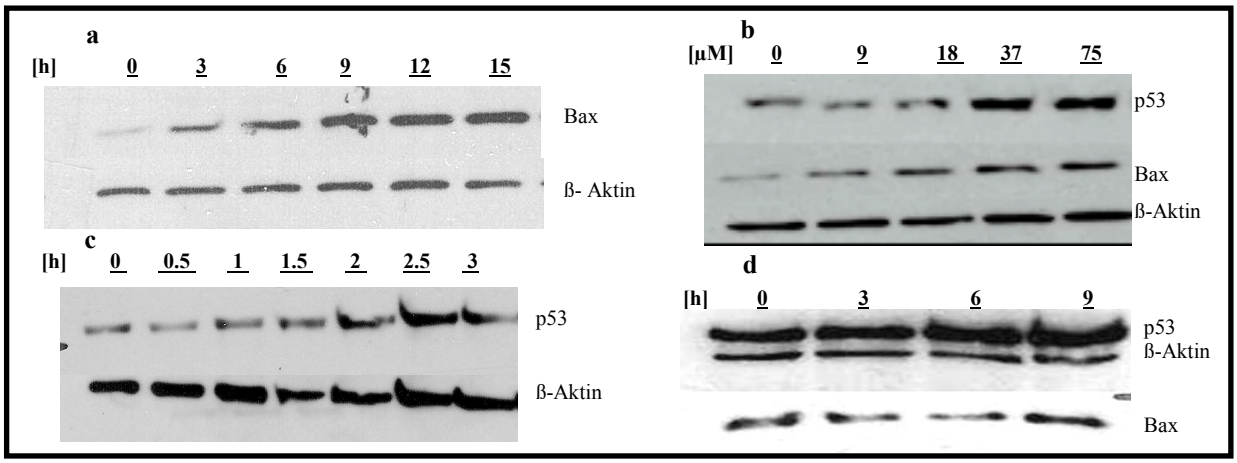


Fig. 4.22: Time course of expression of the proapoptotic proteins Bax and p53 was detected using cell lysates from HepG2 cells treated with 75 µM 5-azacytidine for up to either 15h or 3h in case of p53. Each lane was loaded with 30µg protein (**a, b**). Concentration-dependent modulation of expression of the proapoptotic proteins Bax and p53 was detected using cell lysates from HepG2 cells treated with 0-75 µM 5-azacytidine harvested after 3h. Each lane was loaded with 30µg protein (**c**). Time course of expression of the proapoptotic proteins Bax and p53 was detected using cell lysates from HepG2 cells treated with 75µM 5-aza-2'-deoxycytidine for up to 9h. Each lane was loaded with 30µg protein (**d**).

To check the concentration dependence of this effect, the lysates of cells treated with increasing concentrations of 5-azacytidine incubated over a time range of 3h were analyzed by Western blot. Also in this set-up an increase of both proteins was clearly observable (Fig. 4.22 c.) From this point of view, it can be concluded that sensitization of HepG2 cells against death receptor agonist-induced apoptosis might be due to an enhanced influence of proapoptotic proteins. Supporting such a view, the analogue 5-aza-2'-deoxycytidine, which is not able to sensitize cells showed no influence of BAX and p53 expression in HepG2 cells (Fig. 4.22 d).

4.4.2.2 Effect of 5-azacytidine on inducers of the intrinsic pathway of apoptosis

To strengthen the notion that upregulation of p53/Bax expression is responsible for sensitizing effect of 5-azacytidine, a model based on the intrinsic pathway of apoptosis was utilized. The signaling pathways induced by these stimuli converge on the level of BAX/BAK leading to release of proapoptotic factors from mitochondria. HepG2 cells were treated with either UV-radiation or camptothecin after preincubation with either saline or 75 µM 5-azacytidine. As indicated in figure 4.23 the treated cells showed a significant higher rate of caspase activity as well as a resulting higher cytotoxicity compared to untreated control cells.

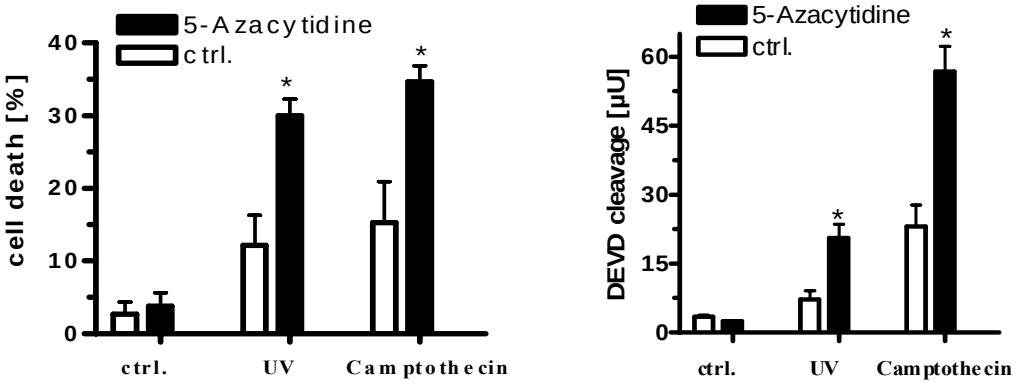


Fig. 4.24: HepG2 cells were preincubated with 75 µM 5-azacytidine 3h prior to addition of 0,3 µM Camptothecin or radiation with 30 mJ/cm² UV B-radiation. Caspase activity (a) was determined after 22h and cytotoxicity (b) was determined after 30h.
Data represent mean ± SD.
*: $p < 0.01$ for control *vs.* 5-azacytidine. Dunnett's Multiple Comparison Test.

4.4.2.3 Characterization of modifications of 5-azacytidine downstream of the death receptor

In previous experiments the whole cell was investigated. Here distinct, separated components of the cellular apoptosis signaling pathways were examined to check separately different parts of the network in terms of involvement of 5-azacytidine.

A DISC independent model of triggering apoptosis was used, exploiting the self-catalyzing/ activating properties of caspase-8. This phenomenon is explained by the *induced proximity model,* which forced HepG2 cells, transient transfected with a vector bearing the cDNA sequence encoding for caspase-8 to go into apoptosis efficiently. The resulting caspase-3/ -7 activity was measurable in a time-dependent manner corresponding with Western blot derived data visualizing expression levels of the heterologous expressed caspase-8 (data not shown).

With regard to these data, all following experiments were measured 18h after transfection. The success of the transfection procedure was monitored by parallel use of a vector bearing the green fluorescent protein EGFP. Compared to control cells, a concentration-dependent increase of caspase-3/-7 activity was observed in cell treated with 5-azacytidine. These data showed that 5-azacytidine was able to enhance caspase-8 mediated effector caspase activity. This effect culminated in cell treated with 75 µM 5-azacytidine more than 2-fold compared with non-treated cells and more than 5-fold compared to cells receiving the same 5-azacytidine concentration but transfected with an empty "mock" vector (Fig. 4.24).

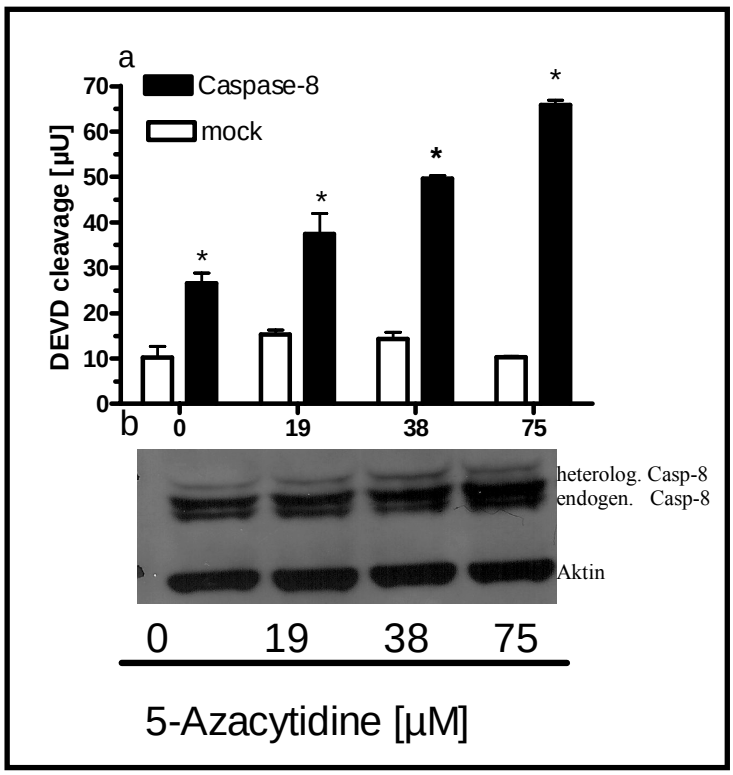


Fig. 4.24: HepG2 cells were transfected with either caspase-8 or with mock vector. Cells were treated with 5-azacytidine as indicated. After 18 hours the caspase activity was measured by quantification of the DEVD-afc cleavage (a).
*: $p < 0.01$ for caspase-8 control vs caspase-8 plus 5-azacytidine. Dunnett's Multiple Comparison Test. Data represent mean ± SD.

In parallel a second batch of treated cells was lysed in order to visualize the heterologous caspase-8 expression with western blot. Each lane was loaded with 30 µg protein (b).

4.4.2.4 Characterization of modifications of 5-azacytidine downstream of the mitochondria

The following experiments were dedicated to further pinpoint the site of 5-azacytidine action. Therefore, the cytosol of cells with the preformed components of the apoptosome, caspase-9 and Apaf-1 was separated from the mitochondria and other organelles.

This technique offers the possibility to reconstruct the self assembly of the apoptosome by addition of cytochrom c/dATP within cytosolic lysates of 5-azacytidine treated cells. The data obtained from this system showed that 5-azacytidine had no influence on DEVD cleavage activity and therefore had no modulatory effect on components localized downstream of the mitochondria.

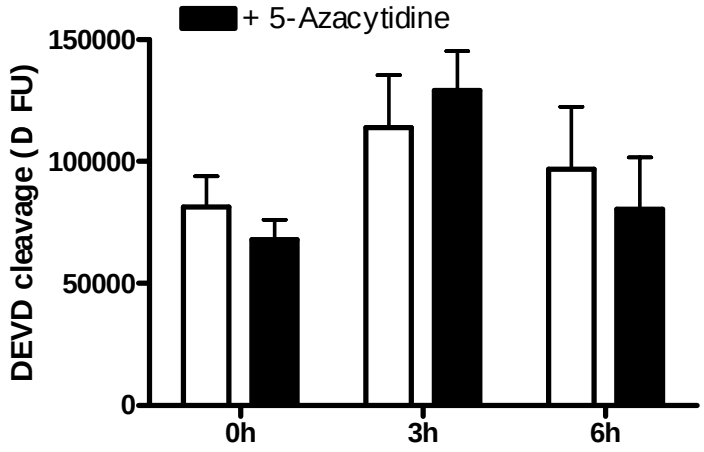


Fig. 4.25: HepG2 cells preincubated with either 75 µM 5-azacytidine or vehicle for the indicated time points before fractionation in S-100 lysates. After pretreatment for 30 min. with 0,3 mM dATP and 10 µM cytochrom c , the caspase -3/-7 activity was measured .
Data represents mean – SD.

Altogether, the data imply the ability of 5-azacytidine to sensitize primary as well as transformed hepatocytes to apoptosis due to a mechanism located between the DISC and the mitochondrial level, most likely by modulating the expression levels of p53 and BAX. As far as other DMTIs does not show any signs of sensitization, these ability is not due to methyltransferase inhibitory activity of 5-azacytidine.

5 Discussion

5.1 Liver-specific aspects of the study

The liver is one of the major metabolic organs in the body with several functions like glycogen storage, acute phase reaction and drug detoxification. Development and maintenance of tissue homeostasis is achieved by physiological regulation of programmed cell death. Growing evidence suggets that apoptosis can contribute to inflammatory disorders and to hepatic impairment induced by acute or chronic exposure to xenobiotics and toxins. The presence of several differentially regulated apoptosis-mediating receptors and their respective ligands on hepatocytes may explain the liver's susceptibility to autoimmune reactions, toxins and viruses [161]. Long lasting exposure to such insults causes chronic liver diseases, which can culminate in severe hepatic impairment and cancer. Hepatocellular carcinomas are still a significant clinical problem as standard treatments achieve only limited success.

One of the major recent intentions of developing anti-cancer drugs is to selectively trigger apoptotic cell death in tumor cells without harming surrounding healthy tissue. One predominant characteristic of transformed cells is their limitless proliferation capacity. Traditional cancer drugs target on this feature to achieve specific cytotoxic effects. However, therapeutic success is limited by two major obstacles, namely *adverse drug reactions* of healthy tissues and organs and *chemoresistance* of tumor cells, e.g. when key components of the apoptotic machinery become dysfunctional.

5.2 Caspase-independent apoptosis in the liver

Induction of apoptosis in its classic form, i.e. by activating caspases, is major mechanism of action contributing to tumor cell death by chemo-; radiation- or immunotherapy. Although many tumors initially respond to therapy, transformed cells can subsequently gain adaptations to escape and survive the treatment. Modulation of cell survival factors or defects in death receptor pathways lead to disorders in apoptosis cascade [162,163] and thus to chemoresistance [164,165]. However, caspase-independent apoptosis and other forms of cell death may also contribute to the response to cancer therapy [166,167]. Today's efforts concentrate on developing agents aiming to kill tumor cells apoptotically by by-passing their survival adaptations or by restoring the defects in key components of their cell death machinery. Therefore, the search

for suitable strategies to counteract chemoresistant phenotypes can benefit from the elucidation of novel signaling pathways leading to apoptosis [168].

Recent investigations in various cell lines resulted in a modified view of apoptotic signaling after death receptor ligation relies not only on the activation of caspases but also on non-caspase proteases that contribute to apoptosis either in concert with caspases or completely independent [1,59].

Studies conducted in hepatocytes revealed the existence of a serine protease–dependent mechanism of death receptor agonist-induced apoptosis under caspase arrest [155].

These data give rise to the question, addressed in the present study, whether the observed caspase independency is a general feature of hepatoma cells in contrast to non-transformed hepatocytes. This topic could be of therapeutic interest, since low doses of zVAD-fmk might protect normal tissue from cytotoxic effects of death receptor agonists, while transformed cells could be selectively targeted by zVAD-fmk *in vivo.* Otherwise, it could be possible that human primary hepatocytes, unlike their murine counterparts, exhibit a similar caspase independent mechanism, which could be due to differences between human and murine species.

5.2.1 Methodological aspects of the study I

5.2.1.1 Cellular model

In the present study, primary human hepatocytes, isolated from pathological inconspicuous specimens obtained from patients undergoing hepatic resections for the therapy of hepatic tumors, were utilized to compare their susceptibility to apoptosis under caspase arrest with that of primary murine hepatocytes and the human hepatoma cell line HepG2. The cell line HepG2, established from tumor tissue of a male Caucasian, still displays a variety of liver cell-specific functions (e.g. fibrinogen and bile acid production, low density lipoprotein uptake [169-172]. It proved to be a convenient model of liver cell apoptosis induced by a variety of drugs or death receptor agonists [20,173-175].

5.2.1.2 Choice of agents and conditions

Hepatocytes are normally resistant to death receptor agonist-induced apoptosis, which can be overcome by the use of sensitizing agents. In this thesis the unspecific transcriptional inhibitor Actinomycin D was utilized to sensitize hepatocytes towards TNF-a, CD95L and TRAIL induced apoptosis. Additionally, histone deacetylase inhibitors (HDI) and the DNA

methyltransferase inhibitor 5-azacytidine were used to sensitize hepatocytes. A set of experiments with primary human and murine hepatocytes as well as with HepG2 cells was carried out to find a suitable concentrations of the respective agent and preincubation times (data not shown), which allow the quantification of the sensitization towards death receptor-induced apoptosis.

5.2.1.3 Drugs and activators of the intrinsic pathway of apoptosis

Three potent inducer of the intrinsic pathway of apoptosis in hepatocytes, exhibiting different modes of action, were chosen.

- Camptothecin: The clinical relevant DNA topoisomerase I inhibitor leads to single and double DNA strand breaks during replication [176]. Afterwards, p53 is activated resulting in transcription of proapoptotic genes [177] and caspase-8, -9 and -3 activation [178].

- UV-B radiation: UV radiation is a DNA-damaging stimulus that activates a p53-dependent response leading to induction of the mitochondrial way of apoptosis [179]

- Staurosporine: The protein tyrosine kinase inhibitor staurosporine is a strong inducer of apoptosis in different cell types [180,181] which acts in general via the mitochondrial way of apoptosis [182].

The time- and concentration-dependency of caspase activity and accompanying cytotoxicity was determined with all three compounds to define suitable working concentrations. The strict dependence of caspase activation and cytotoxicity is taken by analogy as evidence for an interdependence of the two processes. Staurosporine triggered apoptosis regardless of the cell cycle phase, thus a sharp peak of caspase activation and a high degree of cytotoxicity were observed comparable to death receptor agonist-induced apoptosis. In contrast, the mode of action of camptothecin and UV-radiation were cell cycle-dependent, thus their effects are less pronounced.

The relative cytotoxicity was determined with the tetrazolium dye Alamar Blue™. Since the viability assays do not distinguish between the different forms of cell death, the dying cells were examined for morphological apoptotic markers such as chromatin fragmentation and exposure of phosphatidylserine on the outer membrane. To get an impression of possible

cellular morphological changes, a visual control was done in every experiment directly before starting the assay.

5.3 Functional aspects on the role of caspases in death receptor agonist-induced apoptosis in hepatocytes

5.3.1 Caspase-independent apoptosis sensitized by ActD towards death receptor agonists in primary hepatocytes

In contrast to primary murine hepatocytes, inhibition of caspases by zVAD-fmk failed to abrogate cytotoxicity in primary human hepatocytes (Fig.4.1). The strong discrepancy between the IC_{50} values of caspase activity and prevention of cell death in primary human hepatocytes suggests that protection was due to unspecific inhibition of a non-caspase target. Based on these data, it is concluded that caspases are activated in but are not obligatory for death receptor agonist-induced apoptosis in these cells. Thus, the direct dependency of cytotoxicity on caspase activity seems to be an exclusive feature of primary murine hepatocytes for still unknown reasons. In general, this means that the occurrence of caspase-independent apoptosis is not solely restricted to hepatoma cell lines, as this feature is also inherent in primary human hepatocytes.

5.3.2 Caspase-independent apoptosis sensitized by HDIs/5-azacytidine towards death receptor agonists in HepG2 cells

Unlike the “classical” sensitizers ActD/CHX, histone deacetylase inhibitors are capable of sensitizing hepatocytes towards death receptor agonist-induced apoptosis without affecting general translation/transcription. HDIs can overcome chemoresistance by direct modulation of expression levels of apoptosis-related proteins [183,184]. HDI-mediated apoptosis is regarded as caspase-dependent process [185, 186]. This more specific approach of sensitization was applied to corroborate the obtained data regarding caspase-independent apoptosis. These substances represent efficient drugs to amplify cell death processes and could prove to be useful tools to study caspase-independent apoptosis.

Although HDIs interfere with both positive and negative regulators of apoptotic signaling, only a minor reduction or delay in cell death was observed under complete caspase arrest. A similar pattern was observed in HepG2 cells sensitized with the DNA methyltransferase inhibitor 5-azacytidine.

The findings that inhibition of caspases by zVAD-fmk failed to abrogate cytotoxicity in human primary hepatocytes and in the human hepatoma cell line HepG2 strongly suggests that caspases are activated in but are dispensable for death receptor agonist-induced apoptosis in these cells.

5.3.3 Role of p53 in caspase-independent apoptosis

A recent study published by Stambolsky *et al.* in 2006 describes the regulation of AIF expression by basal levels of p53. Cells harbouring functional p53 will constitutively contain higher levels of mitochondrial AIF, which is believed to induce cell death without caspase contribution [159,187]. Stambolsky *et al.* conclude that caspase-independent apoptosis is compromised in cells lacking functional p53 [188].

In this thesis, several cell lines were tested for death receptor agonist induced apoptosis under caspase arrest. All cell lines tested, which exhibit caspase-independent apoptosis, harbour also a functional p53 [189]. This includes also the murine hepatoma cell line Hepa 1-6 and the non-transformed cell line AML-12, derived from mice transgenic for transforming growth factor TGF-a [190]. Notably, in the human hepatoma cell line HuH7, harbouring a dysfunctional p53 mutant, caspase inhibition was strictly correlated with reduction in cytotoxicity (data not shown). These results do not explain the lack of caspase-independent apoptosis in cytotoxicity in primary murine hepatocytes, which also contain wild type p53. However, with regard to the results obtained with murine Hepa 1-6 and AML-12 cells, it can be concluded that species differences alone are obviously not the reason for the apparent lack of caspase-independent cell death in primary murine hepatocytes.

5.3.4 Role of apoptosis inducing factor (AIF) in caspase-independent apoptosis

In CD95L-triggered apoptosis in HepG2 cells, the release of cytochrome c from mitochondria was not impaired in the presence of zVAD-fmk (fig. 4.11). It is clear that once released from mitochondria AIF acts independently of caspases, but it is still a matter of debate if the release itself needs caspase activity [105,187,191,289]. There are studies, which present evidence for both possibilities depending on cell type and stimulus. In our cell model, the use of zVAD-fmk impaired the release of AIF partly, concomitant with a slight reduction of cell death as seen in figure 4.13 and 4.14. These results indicate that caspase activity has indeed some influence on AIF release in apoptotic signaling and that cell death in this model depends in part on presence of AIF. Seth *et al.* presented in 2005 evidences for caspase-2-driven release of AIF

from mitochondria in renal tubular epithelial cells, which could be impaired by caspase-2 inhibitor. Figure 4.13 showed that AIF-release is only partly inhibited by zVAD-fmk and could therefore also contribute to caspase-independent apoptosis.

However, two lines of evidence suggest that the presence of AIF in the cytosol is, at least, not solely responsible for CD95L-triggered DNA fragmentation and apoptosis under caspase arrest in HepG2 cells.

First, Yu *et al*. showed in 2002 that PARP-1 activation is required for translocation of AIF out of the mitochondria to the nucleus to induce cell death, which can be prevented by PARP inhibitors and in PARP knock out mice. PAR-polymer was revealed as an AIF-releasing factor that plays important roles in PARP-1-dependent cell death [187,192]. In our model, neither CD95L-triggered nor staurosporine-induced cell death was prevented by the PARP-1 inhibitor 3-aminobenzamide in presence or absence of zVAD-fmk. However, at least in case of CD95L-triggered apoptosis, this inhibitor prevented the release of AIF from the mitochondria as seen on the Western blot in figure 4.13.

Second, in caspase-driven apoptosis oligonucleosomal stretches of DNA of about 200 base pairs (or multiples thereof) are generated by caspase-activated DNase (CAD) [57,159].The pattern of DNA fragmentation should alter under caspase arrest with AIF as the sole major effector. AIF induces caspase-independent formation of large 50 kb chromatin fragments [159], clearly distinct from the patterns of DNA fragmentation by CAD [193]. However, in our experiments also in the presence of zVAD-fmk, the pattern consisted of CAD-typical oligonucleosomal laddering (fig. 4.12).

So AIF seems to be not essential in this model, however, it is possible that AIF works in concert with other apoptotic factors. The endonuclease G could contribute to caspase-independent apoptosis in HepG2 cells or hepatocytes in general. After being released from mitochondria it leads to an oligonucleosomal pattern of DNA fragmentation [194,195]. Nevertheless, it is speculated if p53 status of a cell could also contribute to endonuclease G level and activity in analogy to AIF to explain the conspicuous coincidence of functional p53 and caspase-independent apoptosis within the tested cell lines[188].

Also, it cannot be excluded that a functional p53 is necessary for the action of another, yet unknown mediator of caspase-independent apoptosis. In future approaches, the quantification of AIF and endonuclease G levels in cell lines exhibiting caspase-independent apoptosis in contrast to cell lines, which do not, could help to reveal the responsible factors.

5.3.5 Protective effects of serine protease inhibitors in HepG2 cells

The unspecific serine protease inhibitors TPCK and TLCK mediated a significant cytoprotective effect in HepG2 cells sensitized either by the HDI apicidin or 5-azacytidine. However, this protection was only observable in the presence of caspase-inhibitory concentrations of zVAD-fmk. These results confirm the data initially rendered by Dünstl in HepG2 cells, sensitized with ActD/CHX [155]. Additionally, it was found that in HepG2 cells sensitized with apicidin and 5-azacytidine also the serine protease inhibitor AEBSF had protective effects in combination with low, but caspase-inhibitory concentration of zVAD-fmk.

These data fit well into the emerging notion that inhibition of caspase activation does not necessarily protect cells against any apoptosis inducing stimulus [1]. The view that the uncovering of back-up death pathways taking over after inhibition of caspase activity by pan-caspase inhibitors may lead to a general review of the role of caspases in apoptosis. Selective, tightly controlled proteolysis is a defining characteristic of apoptosis resulting in the ordered disassembly of the cell [5]. Cellular demise with typical apoptotic morphology is induced by caspase-mediated cleavage of specific substrates like lamins, cytoskeletal proteins, inhibitor of caspase activated DNase or p21 kinase 2 [196,197]. It was recognized that many non-caspase proteases can cleave at least some of the typical caspase substrates, thus mimicking or taking over the role of caspases [73,198-200], followed by apoptosis or at least a regulated apoptosis-like cell death. Over recent years a number of publications described the contribution of serine proteases to apoptotic signaling and execution of apoptosis [59,99,100,108,201,202]. However, the relationship between serine proteases and apoptotic signaling remains unclear, because signaling pathways may vary strongly between different cell types and stimuli used. The site of action of serine proteases may be allocated upstream or downstream of caspases or even attributed to completely caspase-independent signaling pathways [101].

IN our experiments, the unspecific serine protease inhibitors conferred protection only in combination with caspase inhibitory concentrations of zVAD-fmk in either set up utilized in this thesis. Neither serine protease inhibitor alone was sufficient to protect cells from apoptosis. Also exclusive caspase arrest showed only minor protective effects in this model. These data confirm the notion that HepG2 cells can either switch to a serine protease dependent mechanism under caspase arrest, or, at least, possess a parallel serine protease-based apoptotic pathway.

5.3.6 Inhibition profile of caspase inhibitors

The small molecule inhibitor zVAD-fmk is regarded as competitive and irreversible inhibitor of caspases. In HepG2 cells treated with either TNF-a,CD95L or TRAIL all major caspases contributing to death receptor agonist-induced apoptosis were inhibited completely at the applied concentration of 1.5 µM zVAD-fmk, inclusive caspase-2, which is less sensitive to zVAD-fmk as detected by cleavage assays utilizing the respective caspase substrates (fig. 4.4) [61]. Additionally, a set of pan-caspase inhibitors and inhibitors regarded to be specific for distinct caspases were tested for their effect on apoptosis induced by either CD95L or staurosporine. At concentrations higher than approx. 1 µM, all inhibitors used abolished caspase activity. The commonly used caspase inhibitors are based on tetrapeptide recognition motifs of caspase substrates, which exhibit relatively broad specificity [203]. Accordingly, it is clear that although peptide-based caspase inhibitors may be used to incriminate caspases in whole cell models of apoptosis and in animal models of disease in general, none of these compounds can be used to implicate a particular caspase [61]. The prevention of apoptosis in CD95L stimulated cells at high concentrations of inhibitors can be explained by unspecific effects on other cellular targets. It is described, for example, that zVAD-fmk and DEVD-fmk can act on several cathepsins [96] and granzyme B [203]. So these inhibitors exert action not only restricted to caspases but also to other, yet unknown cellular targets.

Notably, neither caspase inhibitor used was able to prevent cytotoxicity in staurosporine treated cells, not even in high concentrations of up to 100 µM.

5.3.7 Caspase independent apoptosis induced by intrinsic stimuli in HepG2 cells

Most drugs currently used in cancer therapy exert general cytotoxicity due to their action as antimetabolites, cytostatics or due to their DNA damaging properties [168]. However, regardless of classification, induction of apoptosis has been identified as the major mechanism of action by chemotherapeutics in tumor cells since the resulting signaling converges to induce outer membrane permeabilization on mitochondria [204,205]. Caspase activation is closely linked to permeabilization of the mitochondrial outer membrane leading subsequently to cell death [206,207].Therefore, in analogy to the experiments conducted with death receptor agonists the role of caspases in hepatocyte apoptosis induced by intrinsic stimuli was determined.

5.3.7.1 Caspase independent apoptosis induced by staurosporine, camptothecin and UV-radiation in HepG2 cells

In this thesis, it was shown that cell death induced by intrinsic stimuli staurosporine, camptothecin and UV-radiation in HepG2 cells was not affected by increasing concentrations of zVAD-fmk, despite caspase activity was abrogated. The results pictured in figure 4.8 lead to the conclusion that caspases are not essential in the mitochondrial pathway of apoptosis since caspase inhibition by zVAD-fmk had no effect on cytotoxicity.

For a better understanding, the events which take place during cell death, can be categorized into three phases. *The proximal phase*, which covers upstream signals and events setting the thresholds for cell death. The *intermediate phase*, comprising the events around mitochondria leading to MOMP and, the *terminal phase*, which covers the signaling pathways downstream of mitochondrial outer membrane permeabilization (MOMP) leading subsequently to cellular degradation. The *intermediat*e and the *terminal phase* are much better investigated than the p*roximal phase*, which often remains obscure [208]. Recent data indicate that MOMP prompts several caspase-dependent as well as caspase-independent pathways. The most investigated pathway is induced by cytochrome c/apoptosome/caspase-9 with contribution of SMAC/DIABLO and Omi/HtrA2 [57,196]. Due to its protease activity Omi/HtrA2 can also have caspase-independent functions to cell death [108,109]. Other mitochondrial death effectors are the above mentioned AIF and endonuclease G, responsible for caspase-independent DNA degradation.

In many models of cell death the master controllers of apoptosis operate at the level of the mitochondria. Numerous pathways, induced by a plethora of stimuli, converge in the proximal phase on this central organelle leading to MOMP. Over the past years, it has become increasingly clear that the point of no return to cell death lies upstream of MOMP [209-213]. The use of caspase inhibitors downstream of MOMP induction cannot protect from cell death, it might only delay the kinetics or change the phenotype [209,210]. Several studies described caspase-independent cell death induced by either staurosporine, which depends on signaling pathways active in parallel to and independently of the caspase cascade in several model systems with either cathepsin D, AIF or both molecules initiated as effectors [214-217]. The results, presented in this study, confirm this notion as neither stimuli assayed, showed any reduction of cell death despite the inhibition of caspases.

In HepG2 cells, caspase-independent apoptosis triggered by death receptor agonists most likely involves on one or several serine proteases according to protective effects of serine protease inhibitors. Results by Dünstl in 2004 showed that combined application zVAD-fmk and TLCK prevented release of cytochrom c out of the mitochondria [155]. Thus, the serine

protease(s) is (are) most likely located upstream of the mitochondria within the *proximal phase.*

The data obtained with HepG2 treated with staurosporine and UV-radiation confirms these data, hence serine protease inhibitors had no protective effect. Presumably, these stimuli trigger signaling pathways bypassing the hypothetical, proximal located, serine proteases and leading directly to MOMP. In contrast, cell death induced by camptothecin is also inhibited by high concentrations of zVAD-fmk. This result leads to the question, whether this could be due to inhibiting a still unknown target of zVAD-fmk in analogy to cytotoxicity pattern obtained in death receptor agonist-induced apoptosis under zVAD-fmk. It is known that cathepsin B, a lysosomal protease is released from mitochondria by camptothecin [95,155]. Cathepsin B triggers cleavage of classical caspase substrates like Bid, leading to MOMP, which could be inhibited by higher concentrations of zVAD-fmk [96]. However, the unspecific calpain/cathepsin inhibitor E-64d, as well a variety of other inhibitors of cathepsins B, L and D (CA-074Me, NIW and pepstatin A, respectively) did not have any protective effect in either setting. In any case, serine protease inhibitors failed also to protect cells from camptothecin-induced cell death leading to the conclusion that different signaling pathways *proximal* of the mitochondria are utilized in the intrinsic and extrinsic mode of cell death, which must still be clarified in detail.

5.3.7.2 Morphological aspects

A necrotic type of cell death under caspase arrest can be excluded based on morphological observation of the typical signs of apoptosis. Neither exposure of phosphatidylserine on the outer membrane nor chromatin condensation was abrogated by inhibition of caspases. Also, the membrane integrity of the dying cells was intact as determined by Sytox-staining. The DNA-binding fluorescent dye did not pass the cell membrane (pictures not shown). Many studies described that phosphatidylserine exposure can be uncoupled from caspase activation in many model systems [218,219] as observed in HepG2 cells treated with zVAD-fmk.

The shape of chromatin condensation in cells treated either with UV-radiation, camptothecin or staurosporine was geometrically complex. The presence of zVAD-fmk did not alter the pattern of chromatin condensation within cells receiving a given stimulus. So, caspase inhibition does neither influence the respective phenotype of apoptotic chromatin condensation nor the exposure of the phagocytotic recognition signal phosphatidylserine.

The more or less compacted chromatin condensation of the HepG2 cells in combination with phosphatidylserine exposure qualifies this examined kind of cell death in HepG2 as at least

apoptosis-like according to the classification of PCD proposed by Leist and Jäättelä in 2001, no matter if caspases are active or not [3].

The situation in primary mouse hepatocytes is different, where radiation with UV led to apoptosis, which was strictly dependent on caspases, since inhibition of caspases conferred protection [220]. Camptothecin alone induced only a slight increase in cell death of primary hepatocytes, since these cells are mitotically inactive and thus, are not affected by CPT-induced DNA damage. But apoptosis induction in primary murine hepatocytes by combinatorial treatment with camptothecin and TNF-a also showed a strict dependency on caspase activity [221]. These results lead to the conclusion that caspase independent apoptosis, either induced by death receptor agonists or intrinsic stimuli, depends on cell type specific mechanisms.

5.4 Sensitization effects of 5-azacytidine

5.4.1 Methodological aspects of the study II

5.4.1.1 Choice of the molecular target

A new concept to overcome chemoresistance is the pharmacological intervention to aberrant epigenetic silencing of e.g. tumor suppressor genes by application of DNA methyltransferase inhibitors (DMTI) as these changes are potentially reversible in contrast to neoplastic transformations induced by DNA mutations [222]. The DMTIs 5-azacytidine and its analogue 5-aza-2'-deoxycytidine have been shown to be effective in the reversal of transformation-mediated hypermethylation *in vivo* [223] and suppression of cancer-specific cellular phenotypes [224,225]. Yet they may also exhibit adverse drug reactions such as inherent cytotoxicity [226].

In terms of pharmacology, adverse drug reaction (ADR) designates an unwanted effect caused by drug administration, which contributes to patient morbidity and mortality as defined by the World Health Organisation (WHO) in 1994: *'An adverse drug reaction is any response to a drug that is noxious and unintended and that occurs at doses used in man for prophylaxis, diagnosis, or therapy.'* These ADRs can affect every organ system in the body, in particular the liver [227]. Thus, drug-induced liver injury is the most common reason for drug withdrawal and causes more than 50% of acute liver failure in the USA [228]. The specified complications range from mild, asymptotic changes to severe hepatic disorders. The high susceptibility of the liver is founded on its function to metabolize xenobiotics. Therefore, the potential hepatotoxicity of newly applied compounds has to be assessed carefully against the background of destroying tumors while having minimal toxicity on normal body tissue.

Thus, this part of the study was dedicated to elucidate the yet unknown effects of commonly used DNA methyltransferase inhibitors in particular with regard to their influence on death receptor agonist-induced apoptosis in liver cells.

5.4.1.2 Choice of agents

This part of the thesis was conducted to elucidate the newfound sensitization effect of 5-azacytidine towards death receptor agonist-induced apoptosis in hepatocytes. Further investigations were done to answer the question whether this effect is a general mechanism of DNA methyltransferase inhibition or is limited to 5-azacytidine.

A set of four commonly used DNA methyltransferases were assayed

- 5-Azacytidine: An analogue of the naturally occurring nucleoside cytosine, which incorporates into DNA/RNA and thereby depletes DNA methyltransferase activity

- 5-Aza-2'-deoxycytidine: An analogue of 5-azacytidine, which has also been tested in clinical trials with promising results in treatment of myeloid leukemia [225,229]. It is currently also subject of trials to overcome chemoresistance in tumor cell lines [168,230,231].

- RG 108: A newly designed lead structure capable of specific inhibition of DNA methyltransferase activity by binding directly to the active centre [232].

- S-(5′-Adenosyl)-L-homocysteine: An inhibitor of the methyl group donor S-adenosyl-methione, which inhibits a broad spectrum of cellular methyltransferases [234].

The DNA methyltransferase inhibitor 5-azacytidine is of particular interest because it has proven to be effective in phase III clinical trials and was recently approved for clinical use by the Food and Drug Administration (FDA) for the treatment of myelodysplastic syndrome. It is a pioneering example of an agent that targets 'epigenetic' gene silencing, a mechanism that is exploited by cancer cells to inhibit the expression of genes that counteract the malignant phenotype [235]. Additionally, it is believed that 5-azacytidine directly mediates cytotoxicity on rapidly dividing cancer cells, which escapes the normal growth control for yet unknown reasons [236,237]. However, some studies described the induction of apoptosis in HL 60 cells and in neural progenitor cells due to DNA damaging by 5-azacytidine [238-240].

5.4.2 5-Azacytidine mediated sensitization in hepatocytes

5.4.2.1 Basic conditions and characteristics of sensitization effects of 5-azacytidine on hepatocytes

To study acute cytotoxicity of drug exposure in hepatocytes, 5-azacytidine was applied 3h prior to addition of stimulus. The reduction of the experimentally determined preincubation time led to a decrease in cytotoxicity in this model (data not shown). To define an effective, sensitizing concentration towards death receptor agonist-induced apoptosis, experiments were carried out with increasing concentrations of 5-azacytidine (Fig. 4.15; 4.17). In the presence of increasing concentrations of death receptor agonists enhanced caspase activity and cytotoxicity was observed in 5-azacytidine treated cells (Fig. 4.16; 4.19).

The sequential time course of caspase activity and cytotoxicity, determined in HepG2 (Fig.4.18) fits well into the concept of caspase-8 triggered effector caspase activation. Moreover, this effect is expected to be concentration-dependent under non-saturating conditions. The morphological studies show a clear apoptotic morphology of HepG2 cells and also of primary murine hepatocytes (data not shown) sensitized by 5-azacytidine towards death receptor agonist-induced cell death as the typical hallmarks like chromatin condensation and phosphatidylserine exposure are clearly visible. In summary, these data affirm the notion of an apoptotic cell death with caspase involvement induced by death receptor agonists in hepatocytes sensitized with 5-azacytidine.

5.4.2.2 The sensitization effects of 5-azacytidine in hepatocytes

Many studies in recent years showed that inhibition of DNA methyltransferase activity can lead to antineoplastic effects. Especially 5-aza-2'-deoxycytidine exerts remarkable effects in overcoming chemoresistance against TRAIL-induced apoptosis in various tumor cell lines like glioblastoma, neuroblastoma and non small lung cancer cell lines [168,230,231]. However, no analogous sensitizing effect was observable with any DMTI other than 5-azacytidine in HepG2 cells in this study.

Upon uptake into cells, 5-azacytidine and 5-aza-2'-deoxycytidine are phosphorylated in a multistep process to pyrimidinetriphosphates, which are incorporated into DNA by DNA polymerase a [241,242]. After incorporation, the compounds form covalent adducts with DNA-methyltransferase followed by depleting enzyme activity [243]. Thus, DMTIs revert the transformed phenotypes as the hypermethylation-associated epimutations need to be actively maintained after each cell division [244.245]. Primary human and murine hepatocytes in cell culture, as used in this study, should not been targeted by DMTIs, since post-mitotic, i.e. non-

replicating cells would preserve their methylation pattern. The sensitization effects of 5-azacytidine on hepatocytes seem to be an exclusive feature of this compound and are not based on the DNA methyltransferase inhibitory activity. The other DMTIs, used in this study, showed no sensitization effects in the chosen set-up arranged to study acute cytotoxic effects by coincubation with cytokines in either hepatocyte type used.

5.4.2.3 Selective sensitization by 5-azacytidine of HepG2 cells to CD95L and TRAIL, but not to TNF-α

In contrast to observations in primary hepatocytes, 5-azacytidine sensitizes HepG2 cells selectively towards CD95L and TRAIL but not to TNF-a. This phenomenon was also observed in the mouse hepatocyte cell line AML-12, assayed in this study, which was also solely sensitized towards CD95L (data not shown). Similar observations were described in HepG2 cells sensitized by JNK- and histone deacetylase inhibitors [155,246]. This phenomenon needs further clarification and cannot be explained by the data raised of this study. One of the possible explanations might be that there are major differences in the activation of the transcription factor NFkB between primary hepatocytes and neoplastically transformed liver cells. The stimulation of TNF-R1 by binding of TNF-a leads to pleiotropic effects within the cells, which allows for the induction of apoptosis when NFkB, a repressor of apoptosis, fails to be activated [247,248]. In contrast to primary hepatocytes 5-azacytidine might either stimulate NFkB or fail to block its action in HepG2 cells. However, a stimulated, proliferative pathway activated by 5-azacytidine would also negatively affect apoptosis triggered by CD95L and TRAIL. Thus, the direct activation of NFkB by 5-azacytidine seems to be little likely to explain our findings.

Another possible explanation for selective sensitization might come from studies by Friesen *et al.* and Müller *et al*. in 1997 and 1998, respectively, who have shown that apoptosis induced in tumor cells involves the CD95 system [249-251]. Treatment of hepatoma cell lines with chemotherapeutic agents causes upregulation of the CD95 ligand and receptor, leading to autocrine and paracrine cell death. Upregulation of the CD95 system in hepatocellular carcinomas was controlled by activation of p53 after DNA damage and therefore sensitized cells to apoptosis [252]. The TRAIL receptors DR4 and DR5 have also been demonstrated to be induced by DNA-damaging agents in a p53-dependent fashion [253-256]. Therefore, the activation of p53 by 5-azacytidine, as described in chapter 4.4.2.1 could contribute to selective sensitization of HepG2 cells towards CD95L and TRAIL triggered apoptosis.

Furthermore, it has to be considered that 5-azacytidine and 5-aza-2'-deoxycytidine could lead to an unspecific inhibition of transcription and translation. Due to its ribonucleoside structure, 5-azacytidine is incorporated into DNA as RNA and thus might affect protein translation [257]. However, 5-azacytidine exhibits enhanced cytotoxicity during the S-phase, supporting the greater impact of it effects on DNA rather than on RNA [258]. In contrast to treatment with 5-azacytidine, ActD and CHX are capable of sensitizing HepG2 towards all three cytokines. The non-sensitization effect of 5-azacytidine towards TNF-a therefore exclude the possibility that the underlying mechanisms depend on global inhibition of transcription or translation.

5.4.2.4 Pharmacological aspects

The concentrations of 5-azacytidine needed for sensitization of primary hepatocytes and HepG2 cells towards death receptor agonist-induced apoptosis were identical. Due to its long term mechanism of action and its short half-live of 22 minutes within the organism, 5-azacytidine needs to be administered daily over a period of 4 weeks to achieve a maximal clinical effect. After application, the drug goes into systemic circulation and is distributed over the tissues, reaching also the liver.

The low micromolar plasma concentrations determined in this thesis, for sensitization of healthy and transformed hepatocytes in culture, are also found *in vivo* in serum levels of patients [227,235]. Additionally, xenobiotics may undergo concentration in the liver by various processes, including active transport systems [259].

The data according the sensitization effects of 5-azacytidine were critical in respect to the notion that non-transformed hepatocytes are believed to be relatively insensitive to cytotoxic effects of 5-azacytidine. However, their sensitizing action could lead to severe hepatic implications in patients. The concomitant presence of endogenous immune modulating cytokines like TNF could cause indirect hepatotoxicity. Such a situation can occur in patients with clinical infectious diseases. Such incidences are rarely observed [235,260]. The cytokine TRAIL is of clinical interest as it exerts specific cytotoxicity on transformed cells, while healthy cells seem to be resistant [54,62]. This is not the case in healthy human hepatocytes, which could be a potential drawback for the systemic application of TRAIL in cancer therapy [150-151]. Also, in combination with TRAIL 5-azacytidine might exert potential harmful effects.

In conclusion, the hepatotoxic side effects observed in clinical studies could be explained by sensitization against cytokines which might be released from endogenous sources during hepatic diseases (e.g. Wilson's disease, alcohol induced hepatitis, hepatitis b, hepatic tumors)

and thus potentiate their pathophysiological effects. This acute sensitizing effect applies to 5-azacytidine as such and seems not to be a general feature of DNA methyltransferase inhibitors. This notion is supported by the observation that 5-aza-2'-deoxycytidine and RG 108, a direct inhibitor of DNA methyltransferases, have no acute sensitization effect in HepG2 in the used set-up.

5.5 Mechanistic rationale for sensitizing effects of 5-azacytidine on HepG2 cells

5.5.1 Modulation of protein expression levels

Treatment with 5-azacytidine increased p53 and Bax levels in a time- and concentration-dependent manner, as detectable on Western blot. Furthermore, there seemed to be a defined progression of events as the p53 level preceded the alterations in Bax expression. The modulation of p53 occurred already after approx. 1h, followed by increased Bax expression after 3 to 9 hours. Notably, the most sensitizing effect regarding the cytotoxic outcome was achieved, when the cells had been pre-treated 3h prior to addition of the stimuli.

Based on these findings, the following general scenario could be discussed for the sensitization of cell death in hepatocytes by 5-azacytidine according to "*switched rheostat model*" [208]. Application of 5-azacytidine induces a rapid change in p53 levels, resulting in a transcriptional induction of Bax in treated cells by a yet unknown mechanism, presumably DNA damage. The ratio of Bax to Bcl2 constitutes a *rheostat* setting the threshold of susceptibility to apoptosis via the mitochondria pathway, which amplifies death signals. Since Bax is not active *per se,* it needs a direct activator. *The switch*, in this case BH3-only molecules like tBid (as generated by activation of caspase-8 upon receptor trimerization), Bim, Bad, PUMA, Noxa and stabilized p53 depending on the stimulus or cause of cellular stress [2]. In summary, these events are sufficient to trigger cell death in hepatocytes treated with death receptor agonist.

Several studies described changes in p53 level in several transformed cell lines treated with 5-azacytidine and 5-aza-2'deoxycytidine [240,261]. The formation of DNA methyltransferase-DNA adducts covalently crosslinked by 5-azacytidine/5-aza-2'-deoxycytidine could be one possible mechanism leading to genotoxic stress. [262,263]. In healthy cells, the p53 protein level is kept at low concentrations by its short half life of 20 minutes due to proteosomal degradation. Additionally, it is discussed that p53 exists in a latent state. Under these conditions p53 must receive an activation signal [264,265]. Within minutes, levels of p53 rise dramatically in case of

sustained DNA damage, because of lengthening of the proteins half-life [266]. This phenomenon occurred indeed in 5-azacytidine treated HepG2 cells, in which increased p53 levels were detectable within half an hour. The transcription of the proapoptotic Bcl2-family member Bax is increased by p53. Additionally, it is discussed that p53, stabilized by cellular stress can lead also directly to Bax activation [267].

Bax is localized in an inactive form within the cytoplasm in non-stressed cells [268]. After receiving an activation signal, Bax and its accompanied proapoptotic protein Bak function as a gateway to mitochondria, which leads to release of cytochrome c and other proapoptotic molecules [269,270.] Hepatocytes are believed to be type II cells, which require the mitochondrial amplification loop for proper conduction of apoptosis [271,272]. A direct link between Bax activation and DR-agonist-induced signaling was described by Werner *et al*. who showed that TRAIL and CD95L signal to mitochondria through nearly identical pathway involving the DISC-accessory protein FADD, caspase-8/-10 and truncated Bid [273-276]. The emergence of active tBid after treatment with CD95L was detectable in HepG2 cells (data not shown).

In this study, the protein levels of caspases as well as of the investigated proapoptotic proteins were found to be unaffected in 5-azacytidine treated cells. In other studies, the caspase-inhibiting proteins cFLIP and XIAP were often found to be downregulated after chemotherapeutic treatments with e.g. cisplatin or histone deacetylase inhibitors [277-278]. These effects were sufficient to sensitize cells to death receptor agonist-induced apoptosis [184]. Also the unspecific action of inhibitors of transcription and translation, ActD and CHX caused a sensitization by downregulation of cFLIP [279-281]. However, the expression level of these antiapoptotic proteins remained stable in our model. It seems that 5-azacytidine acts by upregulation of proapoptotic factors rather than by downregulation of proapoptotic proteins due to translational/transcriptional inhibition. Notably, after treatment of HepG2 cells with 5-aza-2'deoxycytidine, which exerts no sensitizing effects, a slight alteration in p53 level was observable, but there were no signs of Bax modulation.

5.5.2 Possible site of action

In order to establish a DISC- independent model of apoptosis, caspase-8 was transiently over-expressed in HepG2 cells to activate the components of the CD95L/ TRAIL signaling pathway. The subsequent, significant increase in caspase-3/-7 activity was measurable 16h after transfection when enhanced caspase-8 expression was detectable directly by Western blot. Cells co-transfected with an EGFP expression vector displayed an apoptotic morphology of the chromatin (data not shown). In summary, the results are compatible with the events

according to the "*induced proximity model*" [2], since the generation of high local concentrations of caspase-8 leads to autoactivation, which is sufficient to trigger the caspase cascade in HepG2 cells.

Also, in this set-up, the sensitizing effects of 5-azacytidine were clearly detectable by significantly higher caspase-3/-7 activity compared to untreated cells. Therefore, the site of action seems to be located downstream of the DISC. Lowering the thresholds for apoptosis is also detectable in this model, since caspase-8 activates the extrinsic branch, directly triggering effector caspase cascade as well as the intrinsic branch by cleavage of Bid leading to MOMP.

These data are in accordance with Western blot analysis regarding the expression levels of DISC related proteins FADD, cFLIP and endogenous caspase-8. However, the possibility that an upregulation of CD95 and TRAIL receptor on the cell surface contributes to sensitization effects of 5-azacytidine cannot be completely excluded. Such mechanisms are described for CD95 receptor after treatment with chemotherapeutic agents including cisplatin, mitomycin, methotrexate, mitoxantrone, doxorubicin and bleomycin in a variety of different cell lines dependent on the presence of a functional p53 gene [174,282]. Also TRAIL DR-5 seems to be a *bona fide* target gene for p53 [254,283].

It is well established that cellular stress like DNA damage induced either by UV-radiation or by camptothecin [177-179,284] is mediated by p53-dependent mechanisms, leading directly or indirectly to Bax/Bak activation and subsequent apoptosis execution through perturbation of mitochondrial structure and to caspase-9 activation *via* apoptosome formation. Thus, a 5-azacytidine triggered increase of p53/Bax should sensitize cells to apoptosis. This was indeed the case in our study, as 5-azacytidine enhanced the effector caspase activity and the resulting toxicity of HepG2 cells treated with camptothecin and UV-B radiation, which strengthen the notion of a vital role of the p53/ Bax complex for sensitizing effects of 5-azacytidine.

Processes downstream of the mitochondria, leading to apoptosome formation constitute another mechanism to sensitize cells to apoptosis. XIAP is an inhibitor of both proteolytically processed caspase-9 and -3. Its downregulation or inhibition by SMAC/DIABLO and Omi/HtrA2 can lower the thresholds to apoptotic signaling [2]. The apoptosome formation can be artificially activated by addition of dATP and cytochrome c to S-100 cell extracts, depleted of mitochondria. This protein complex joins caspase precursor via the linking molecule Apaf-1 to generate an active caspase -9 [158,285,286]. The principal suitability of the system was tested with treatment of PETCM, an inhibitor of prothymosin-a, which prevents APAF-1 from apoptosome formation in a competitive manner to dATP and cytochrome c [287], resulting in enhanced caspase activity (data not shown).

Neither the apoptosome formation nor the proteins involved in signaling pathways and regulation mechanisms downstream of the mitochondria seemed to be affected by 5-azacytidine treatment at any time point. Also the expression levels of caspase-9 and Apaf -1 are nearly unaffected, as demonstrated by Western blot analysis.

From these data it is concluded, that a major component of 5-azacytidine derived sensitization of HepG2 cells towards death receptor agonist-induced apoptosis is localized between the DISC complex and the mitochondrial level in the proximal phase of cell death signaling and relies on the action of the enhanced levels of the proapoptotic proteins p53 and Bax. It is most likely the upregulation of these proteins which sensitizes the cells in lowering the threshold for triggering MOMP. The terminal phase of cell death signaling downstream of mitochondria seems not to be directly affected by such a protein modulatory action of 5-azacytidine. An experiment to further test this hypothesis could be the overexpression of the antiapoptotic protein Bcl-2, which should protect the mitochondria from the sensitizing effect of 5-azacytidine.

6 Summary

The present study investigated the role of caspases and serine proteases in hepatocyte apoptosis triggered by death receptor agonists and by stimuli inducing the intrinsic pathway of apoptosis. In addition, the potential of various epigenetic drugs to sensitize towards a serine protease-dependent mechanism of apoptosis under caspase arrest was examined within the HepG2 cell model.

1. In contrast to primary murine hepatocytes, inhibition of caspases by zVAD-fmk was not sufficient to protect primary human hepatocytes and the human hepatoma cell line HepG2 from death receptor agonist-induced apoptosis.
2. HepG2 cells, sensitized towards CD95L- and TRAIL-triggered apoptosis by the histone deacetylase inhibitors apicidin, CBHA, M344 and VPA as well as by the DNA methyltransferase inhibitor 5-azacytidine, were able to undergo apoptosis under caspase arrest, which could only be prevented by additional, simultaneous application of the serine protease inhibitors TPCK, TLCK and AEBSF.
3. The inducers of the intrinsic pathway of apoptosis camptothecin, staurosporine and UV-radiation led to a time- and concentration-dependent activation of caspases resulting in cell death with typical apoptotic morphology in HepG2 cells. However, use of caspase inhibitor zVAD-fmk and/or serine protease inhibitors neither prevented nor changed the morphology of apoptosis.
4. The pan-caspase inhibitor zVAD-fmk is able to inhibit a set of initiator and effector caspases. Furthermore, the application of specific caspase-inhibitors abolished DEVD cleavage-activity but failed to protect HepG2 cells from CD95L and staurosporine induced apoptosis.
5. The release of the caspase-independent proapoptotic molecule AIF from the mitochondria was prevented by application of PARP inhibitor 3–aminobenzamid without protecting HepG2 cells from apoptosis. Additionally, under caspase arrest the pattern of oligonucleosomal DNA fragmentation is not altered as commonly observed under sole influence of AIF.

The results of this thesis demonstrate that a general caspase inhibition does not protect human primary hepatocytes and HepG2 cells from apoptosis. As mechanistic rationale a serine protease-dependent pathway acting in parallel to caspase signaling can be provided, at least for death receptor agonist-induced apoptosis.

6. Summary

The antineoplastic agent 5-azacytidine is a pioneering example of DNA methyltransferase inhibitors, which was recently approved for treatment of myelodysplastic syndromes. The pharmacodynamic properties of 5-azacytidine were elucidated to profile the newfound sensitizing effect on hepatocyte apoptosis**.** In summary, following results were obtained:

1. 5-Azacytidine sensitized hepatocytes towards death receptor agonist-induced apoptosis.
2. Treatment with 5-azacytidine selectively caused a time- and concentration-dependent sensitization towards CD95L- and TRAIL-triggered caspase-activation and apoptosis, but exerts no effect with TNF-α in HepG2 cells.
3. Sensitization capability of 5-azacytidine is not primarily based on its inhibitory effect on DNA methyltransferases as other common used inhibitors like the analogue 5-aza-2'-deoxycytidine exert no sensitization effects on hepatocytes.
4. After exposure to HepG2 cells, 5-azacytidine induced a time- and concentration- dependent upregulation of the proapoptotic proteins p53 and Bax.
5. 5-Azacytidine enhanced effector caspase activity when the extrinsic pathway of apoptosis was activated without DISC contribution by transient overexpression of caspase-8.
6. Caspase activity and cytotoxicity of p53/Bax-dependent intrinsic stimuli was enhanced by 5-azacytidine
7. In contrast, artificial activation of the intrinsic pathway within S-100 cytosolic fractions of HepG2 via dATP/cytochrom c failed to induce upregulation of caspase activity after 5-azacytidine treatment.

The design of this study comprised malignantly transformed liver cells, primary mouse and human primary hepatocytes to detect acute effects of 5-azacytidine on apoptosis. The results obtained reveal that 5-azacytidine sensitizes hepatocytes against death receptor agonists. As a mechanistic rationale, evidence for a p53/Bax-dependent sensitizing mechanism acting in-between the death inducing signaling complex (DISC) and above the mitochondrial level were presented. In summary, the findings offer a mechanistic explanation for the adverse hepatotoxic properties of 5-azacytidine observed in patients with pre-existing liver disorders.

7 Zusammenfassung

Die vorliegende Arbeit hatte zum Ziel, die Beteiligung von Caspasen und Serinproteasen an der Apoptose von Leberzellen zu untersuchen, welche zum einen durch Todesrezeptorliganden und zum anderen durch Stimulantien des intrinsischen Weges ausgelöst wurde. Zusätzlich wurden verschiedene pharmakologische Wirkstoffen, welche epigenetische Regulationsmechanismen beeinflussen, auf ihre Fähigkeit hin untersucht, das HepG2-Zellmodell gegenüber Todesrezeptor-stimulierte Apoptose zu sensitiveren.

1. Im Gegensatz zu primären murinen Hepatozyten war die Hemmung von Caspasen in primären humanen Hepatozyten und HepG2-Zellen durch zVAD-fmk nicht ausreichend, um einen Schutz gegenüber Todesrezeptor-vermittelter Apoptose zu erwirken.
2. HepG2-Zellen, welche durch die Histondeacetylasehemmer Apicidin, CBHA, M344 und VPA sowie durch den Methyltransferasehemmer 5-Azacytidine sensitiviert wurden, gingen trotz Caspasehemmung in die Apoptose, welche nur durch gleichzeitige Gabe der Serinproteasehemmer TPCK, TLCK und AEBSF verhindert werden konnte.
3. Die Stimulantien des intrinsischen Signalweges der Apoptose Camptothecin, Staurosporin und UV-Strahlung führten zu einer zeit- und konzentrationsabhängigen Aktivierung von Caspasen, welche zu einem Zelltod mit typisch apoptotischer Morphologie führte. Der Einsatz des Caspasehemmers zVAD-fmk und/oder von Serinproteasehemmern verhinderte weder die Apoptose noch führte er zu einer veränderten Ausprägung.
4. Der allgemeine Caspasehemmer zVAD-fmk hemmte sowohl Initiator- als auch Effektorcaspasen. Weiterhin führte der Einsatz von spezifischen Caspasehemmern zu einer vollständigen Aufhebung der DEVD-Spaltung durch Effektorcaspaseaktivität ohne allerdings die Zellen vor CD95L- und Staurosporin-vermittelter Apoptose zu schützen.
5. Die Freisetzung des Caspase-unabhängigen agierenden proapoptotischen Moleküls AIF aus dem Mitochondrium wurde durch den PARP-Hemmer 3-Aminobenzamid verhindert ohne allerdings die Zellen vor CD95L- und Staurosporin-vermittelter Apoptose zu schützen. Das Muster der oligonucleosomalen DNA-Spaltung ist unter Einfluss von zVAD nicht verändert, wie es üblicherweise in einen AIF-abhängigen Zelltod der Fall wäre.

Die Ergebnisse der Arbeit zeigen, das primäre humane Hepatozyten und HepG2-Zellen nicht durch eine generelle Caspasehemmung vor Apoptose geschützt werden. Es scheint, dass zumindest in der Todesrezeptor-vermittelten Apoptose Serinprotease-abhängige Signalwege existieren, welche parallel zu den Caspase-abhängigen Signalwegen agieren.

7. Zusammenfassung

Die gegen Neoplasien wirksame Substanz 5-Azacytidin, welche seit kurzem zur Behandlung myelodysplastischer Syndrome zugelassen wurde, ist ein bahnbrechendes Beispiel für Methyltransferasehemmer. Deren pharmakodynamischen Eigenschaften wurden untersucht, um die sensitivierende Wirkung auf Hepatozyten zu charakterisieren.

1. 5-Azacytidin sensitivierte Hepatozyten gegenüber Todesrezeptor-stimulierter Apoptose.
2. In HepG2-Zellen sensitivierte 5-Azacytidin zeit- und konzentrationsabhängig selektiv gegenüber CD95L-und TRAIL-vermittelter Caspaseaktivierung und Apoptose, zeigte aber keine Effekte gegenüber TNF-a.
3. Die sensitivierende Wirkung beruhte nicht auf der Hemmung von Methyltransferasen, da andere bekannte Methyltransferasehemmer wie z.B. das Analogon 5-Aza-2'-deoxycytidin keine sensitivierenden Eigenschaften aufwiesen.
4. In 5-Azacytidin behandelten Zellen wurden die proapoptotischen Proteine p53 und Bax zeit- und konzentrationsabhängig hochreguliert.
5. Nach Rezeptor/DISC unabhängiger Aktivierung des extrinsischen Signalweges durch Überexpression der aktiven Caspase-8, war die Effektorcaspaseaktivität in 5-Azacytidin vorbehandelten Zellen gegenüber Kontrollzellen erhöht.
6. Die Caspaseaktivierung und zytotoxische Wirkung von p53/Bax-abhängigen intrinsischen Stimulantien war nach Sensitivierung mit 5-Azacytidin erhöht.
7. Im Gegensatz dazu war nach künstlicher Aktivierung des intrinsischen Signalweges durch Zugabe von dATP/Cyctochrom C zu einer cytosolischen S-100 Fraktion von 5-Azacytidin vorbehandelten HepG2-Zellen keine Erhöhung der Effektorcaspaseaktivität messbar.

Der Entwurf der Studie beinhaltete primäre humane und murine Hepatozyten wie HepG2-Zellen, um akute Wirkungen von 5-Azacytidin auf die Apoptose zu bestimmen. Die Ergebnisse zeigen, dass 5-Azacytidin Hepatozyten gegenüber Todesrezeptorvermittelte-Apoptose sensitiviert. Als Ursache, werden Beweise für eine p53/Bax-abhängigen Mechanismus vorgestellt, welcher, angesiedelt zwischen DISC und Mitochondrium, zu einer verstärkten Empfindlichkeit gegenüber Apoptose führt. Zusammengefasst geben die Ergebnisse eine ursächliche Begründung für die leberschädigenden Nebenwirkungen von 5-azacytidin, welche in Patienten mit Lebervorerkrankungen beobachtet wurden.

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