Sensitization by Histone Deacetylase Inhibitors of Receptor-Mediated Hepatic Apoptosis

DISSERTATION

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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 SCTM / 4-(2-Aminoethyl)-benzenesulfonyl fluoride**
ALT	**Alanin-aminotransferase**
BIR	**Baculoviral inhibitory repeat**
BSA	**Bovine serum albumine**
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**
DD	**Death domain**
DED	**Death effector domain**
DISC	**Death-inducing signaling complex**
DMSO	**Dimethyl sulfoxide**
DTT	**Dithiothreitol**
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**
KCl	**Potassium chloride**
LDH	**Lactate dehydrogenase**
mAb	**monoclonal antibody**
M344	**N-Hydroxy-7-(4-dimethylaminobenzol)aminoheptanamide**

Abbreviations

MgCl2	**Magnesium chloride**
mu	**murine**
NaCl	**Sodium chloride**
NaH2PO4	**Sodium dihydrogenphosphate**
NF-κB	**Nuclear factor- κB**
PAGE	**Polyacrylamide gel electrophoresis**
PBS	**Phosphate buffered saline**
RAIDD	**Rip-associated ICE-homologous protein with death domain**
RIP	**Receptor-interacting protein**
S.E.M.	**Standard error of means**
SD	**Standard derivation**
SDS	**Sodium dodecyl sulfate**
TNF	**Tumor necrosis factor**
TNFR	**Tumor necrosis factor receptor**
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 Cancer: General considerations

Cancer is a world wide health problem which causes 7 million deaths every year. The generic term cancer includes over 200 distinct diseases affecting over 60 human organs. However, there are six characteristic hallmarks, which acquire the most cancers [2]:

- Unlimited potential for cell division
- Invasion and metastasis
- Resistance to anti-growth signaling
- Self-sufficiency in growth signals
- Evasion of apoptosis
- Sustained angiogenesis

There are numerous indications, that these six characteristics are the result of a multistage process, which has its origin in the alteration of genes encoding proteins which modulate angiogenesis, metastatic, or growth/apoptosis pathways [3,4]. The accumulation of this six traits creats cells, which can proliferate unrestrictedly, form preneoplastic lesions, invade local tissues and establish distant metastasis [5]. The causative reason for tumorigenesis is usually unidentified, but it is known that the development of cancer involves an interaction between environment and heredity [6]. Initial somatic mutations can be induced either by faultiness of the DNA replication machinery or by environment factors such as radiation, chemicals and viruses [7]. Although cancer can be described as a genetic disease, it is exceptionally caused by a mutation in a single gene [6]. Three to seven mutations are necessary for carcinogenesis [7,8].

Most chemotherapeutical drugs target the limitless proliferation potential of transformed cells by exploiting their increased DNA replication, using alkylating agents (e.g. melphalan) and inhibitors of the DNA repair machinery (e.g.nitrosoureas). The specific engagement of microtubule modifiers, antimetabolites, steroids or hormonal therapies represents alternative strategies to arrest uncontrolled cell division.

Novel chemotherapeutics target directly specific signaling molecules which are involved in the cancer traits of growth signaling, invasion and angiogenesis. The increased specificity of these cancer treatments together with therapies, using immunotoxins or tumor vaccines decreased the toxicity to normal cells. The effectiveness of these therapies ultimately depends on the expression pattern of the targeted tumor.

Although the major part of the tumors initially reacts to the therapy, drug resistance caused by a positive selection of clonal cells often arises. The resistance to chemotherapeutic treatments includes reduced uptake or increased elimination of the drug, altered tumor microenvironments (e.g. acidity, hypoxia) and the disappearance of the target molecule [9]. 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. The term epigenetic is used to describe of changes in the pattern of gene expression that is mediated by mechanisms other than alterations in the primary nucleotide sequence. There are different molecular mechanisms that mediate epigenetic phenomenon:

- Covalent modification of core histones („Histone Code“)
- DNA methylation
- RNA interference or microRNAs
- Remodeling of Histones

Hepatocellular carcinoma

The liver represents a critical organ in human cancer. In the context of this thesis, hepatocellular carcinoma is one of the most frequent cancers worldwide with increasing incidence and the liver is the target of most other cancers due to metastasis. Hepatocellular carcinoma is related closely to chronic hepatitis and cirrhosis, implying that alterations in growth control mechanisms during regeneration, which in part are mediated by cytokines, may be involved in hepatic carcinogenesis.

Metastatic or primary non-resectable cancers confined to the liver represent an ongoing clinical problem: of 140.000 new patients diagnosed with colorectal cancer in the U.S. 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. More than 70% of all patients with colorectal cancer will develop liver metastasis during progression of the disease and autopsy studies indicate that in at least half of these cases the liver is the sole metastatic site. Unfortunately, the vast majority of colorectal metastases confined to the liver are considered to be non-resectable and the survival prognosis of these patients is extremely poor. Standard regimens using a variety of chemotherapeutics prolong the mean survival rate up to ten to fourteen months.

As an alternative to systemic treatment, the isolated perfusion of the liver (isolated hepatic perfusion, IHP) has been used. One of the historical examples of such treatment with high

local doses of chemotherapeutical agents is the alkylating agent melphalan either alone or in combination with tumor necrosis factor alpha (TNFα). It has been demonstrated that IHP is technically feasible with little systemic toxicity, low systemic drug exposure and minor transient disturbances of liver biochemistry and histology. However, the lack of selectivity towards malignant cells, i.e. the fact that cell death was initiated also in normal hepatocytes by chemotherapy in absence or presence of TNF has turned out to be a major limitation of this attractive approach.

1.2 Organization of DNA

Genomic DNA is the ultimate code of our heredity. The human genome contains estimated 35.000 genes located on about 2 meters total of DNA. Those 35.000 protein coding genes are transcribed into about 87.000 different mRNAs, using alternative gene splicing. Only 1% of the DNA or about 2.5 cm actually codes for functional genes.

The DNA in cells of eukaryotic species is sequestered in a nucleus, which occupies about 10% of total cell volume. The length of DNA-molecule would exceed the dimensions of this compartment. For that reason, the DNA must be compressed to fit into the space available. In the nuclei of all eukaryotic cells, genomic DNA is highly folded into a polymer called chromatin. This basic organization form of DNA is composed of multiple repeating units termed nucleosomes, which are comprised of 146 base pairs of DNA wrapped around a histone octamer that consists of two molecules each of the core histones H2A, H2B, H3 and H4. The Nucleosome arrays along the DNA are proposed to fold into a 30 nm fiber upon incorporation of a linker histone H1. Further compaction of the structure is achieved by folding this 30 nm fiber back and forth [10,11].

This structure is not static for packaging of the naked DNA molecule. In contrast, chromatin plays an active role in gene regulation by changing its structure. There are two chromatin structures, which differ in their biophysical conformation and in metabolic expression of their genes: Heterochromatin is highly condensed and remains transcriptionally inert in contrast to the decondensed, transcriptionally active structure which is called euchromatin. Given that histones and DNA in nucleosomes are held together by a large number of weak interactions, chromatin is generally repressive to extraneous access. The packing of DNA into a higher order and dynamic structure provides a central point of control for gene expression by regulating access of transcription factors to DNA.

Chromatin modification

The term “chromatin modification” describes post-translational modifications on the histones. Two of the most studied modifications are the acetylation and deacetylation of lysines in the tails of the core histones H3 and H4, which are controlled by histone acetyl transferases (HATs) and histone deacetylases (HDACs). The dynamic equilibrium between the catalytic activity of this two enzyme families affects the cellular acetylation status. [12]. The electrostatic interaction, between the phosphate backbone of nucleosomal DNA and the positive charge on the ε-amino groups of lysines cause the compact chromatin structure of heterochromatin maintaining chromatin in a transcriptionally silent state. At neutral pH lysine has a positively charged ammonium group, but addition of an acetyl group generates an uncharged amide, which reduces the affinity of histones tails to DNA. The transfer of acetyl-CoA to lysines by histone acetyl transferases neutralize the positive electric charge to form euchromatin. This results in enhancing access of transcriptional factors, transcriptional regulatory complexes and RNA polymerases to promoter regions of DNA. Consequently, highly acetylated (hyperacetylated) histones are associated with transcriptionally active sequences, while hypoacetylated histones are found in silenced chromatin.

Biochemical isolation of HATs revealed that they work in large multiprotein complexes that share some similarities regarding their subunit composition [13,14]. HATs are recruited to promoters by DNA-bound transcription factors. HATs function in association with protein complexes that may contain other HATs, transcription coactivator and corepressors [15]. HDAC complexes enzymatically remove the acetyl group from lysine residues of histone tails. In humans, 18 HDACs enzymes have been identified and ordered based on structural homologies with the three distinct yeast HDACs [16]. Both class I and class II are zinc-dependent enzymes for which a number of inhibitors have been developed. [17,18]. HDACs do not bind directly to DNA. They are recruited to transcription factors that can include corepressors, activators, chromatin remodelling proteins and HATs [19].

1.3 Apoptosis

The concept of programmed cell death was first described by biologists in the context of cell death during tissue remodeling for more than three decades. They proposed that cell death during development is characterized by specific events leading to defined self-destruction [20]. The term apoptosis is of Greek origin, having the original meaning of falling leaves from a tree. Kerr and coworkers used the term apoptosis to classify morphological changes leading to programmed cell death [21] a discovery which was finally awarded with the Nobel Prize in 2003.

The programmed or active cell death as a part of normal cell physiology is implicated in a variety of biological systems, such as tissue homeostasis, development in multicellular organisms, differentiation, proper function of immune system and removal of defect and therefore harmful cells. Dysregulation of apoptosis is involved in a large variety of human diseases, including AIDS (acquired immunodeficiency syndrome), viral infections, neurodegenerative disorders (Alzheimer's disease, Parkinson's disease), autoimmune diseases, ischemic disorders (e.g. myocardial infarction), some toxin-induced liver diseases and finally cancer, if the programme for active death is lost in a dedifferentiated cell [22].

The cellular interactions regulate cell death in two fundamentally different ways:

- Most cells require signals (trophic factors) to stay alive and will undergo programmed cell death in the absence of these signals.
- Some cells are triggered to undergo programmed cell death by specific signals (e.g. for a proper function of the immune system).

Additionally, apoptosis can be induced in response to various signals from inside the cell, e.g. by cellular stress triggered by oncogenes or drugs.

Apoptosis is characterized by specific, sequential morphological and biochemical changes. The events of a cell dying apoptotically comprise nuclear condensation of chromatin, condensation of cytoplasm, rounding up, blebbing of plasma membrane, intranucleosomal cleavage of the DNA and finally the fragmentation of the cell into compact membrane-enclosed structures, called apoptotic bodies which contain cytosol, condensed chromatin and organelles [23]. To avoid an inflammatory tissue reaction against apoptotic cells, enzymes responsible for the inflammatory response are inactivated and the apoptotic bodies are engulfed by phagocytes or neighbouring cells [24].

The classification of cell death as a classical apoptotic mode requires caspases a large enzyme family of intracellular cysteine proteases, that be activated in order to do their job in cleaving specific protein substrates [25]. Caspases, cysteine proteases homologous to C. elegans ced-3, are the dominant enzyme family in the apoptotic signaling network and are responsible for the most morphological features of apoptotically dying cells [26].

Initiation of Apoptosis

Inside the cell, caspases are generally synthesized as inactive proforms carrying a prodomain at their N-terminus. The prodomain is proteolytically removed during the activation process to form the catalytically active caspase. The proapoptotic caspases can be divided into the class of initiator caspases including procaspase-2, -8, -9 and -10 which carrying long prodomains and into the group of executioner caspases including procaspases-3, -6, and -7 which posses short prodomains. The prodomains of initiator caspases contain either death effector domains (DED) (e.g. Caspase-8) or caspase recruitment domains (CARD) (e.g. Caspase-9). These subdomains of procaspases decide about the recruitment of further accessory proteins to the interior part of the death receptors (e.g. CD95, extrinsic apoptosis pathway), or to large intracellular multi-protein complexes inside the cell (e.g. apoptosome, intrinsic apoptosis pathway).

Apoptotic signals coming from the inside of the cell often have the origin within the nucleus. DNA damage induced by irradiation or drugs initiate the activation and translocation of the p53 transcription factor into the nucleus, which in turn supports the expression of proapoptotic members of the Bcl-2 family and suppress antiapoptotic members of this protein family.

1.4 Death receptors and their ligands

The extrinsic apoptosis pathway is induced by activation of so called "death receptors" (DR). Death receptors are cell surface receptors and belong to the tumor necrosis factor receptor (TNFR) gene superfamily, including TNFR-1, Fas/CD95 and the Trail receptors DR-4 and DR-5 [27]. They transmit apoptosis signals initiated by specific ligands.

The signaling of apoptosis by members of the death receptor subfamily seems to follow basically the same sequential steps. The binding of a ligand results in a receptor trimerisation and clustering of intracellular parts of the death receptor, called death domain (DD). This allows binding of intracellular adapter molecules like FADD or TRADD, forming the so-called death inducing signaling complex (DISC). The DISC leads to the induction of apoptosis via recruitment and cleavage of procaspase-8. Activated Caspase-8 induces a caspase cascade by processing the effector caspase-3, -6 and -7 to initiate the degradation of the cell.

In some cellular systems, the direct activation of effector caspases is sufficient to induce apoptosis alone (type 1 signaling). In other cases, the signal coming from the DISC is not enough for execution of apoptosis (type 2 signaling) (Figure 1.1, left panel). The type 2 cells (e.g. hepatocytes or hepatoctes-derived cells as dealt with in this thesis) enhance the caspase signaling via a mitochondria-dependent, intrinsic pathway. Thus, activated caspase-8 also cleave Bcl-2 family members, i.e. postmitochondrial modulators, among them Bid to form tBid which acts together with the proapoptotic proteins Bax and Bak as a signal targeting mitochondria to facilitate the release of cytochrome c. In consequence, cytochrome c, dATP, APAF-1 and procaspase-9 form a complex called apoptosome. This complex enables the activation of caspase-9, which in turn activates the final executor, i.e. caspase-3 (Figure 1.1, right panel).

The inhibition of initiator and executioner caspases by antiapoptotic factors is an efficient cellular mechanism to suppress apoptosis signaling. Thus, members of the IAP-family (Inhibitor of Apoptosis Protein) 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) [28]. The members of the IAP family have multiple biological functions that include binding of several caspases by their BIR domains. XIAP, the X-linked Inhibitor of Apoptosis Protein, prevents or decelerates apoptosis by inhibition of caspase-3, -7 and -9 [29].

The DISC-initiated apoptosis signal may be abrogated by the recruitment of some proteins such as FLIP. The mammalian Flice-inhibitory protein (cFLIP), containing a DED domain and an inactive caspase domain prevents the recruitment of procaspase-8 (Flice) to the DISC and consequently its subsequent activation. 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 mitochondria-dependent pathway. [30-32]
Numerous pro- and antiapoptotic factors are part of a fine-tuned regulatory network. The apoptosis signaling pathways of viable cells are normally kept in an inactive state but the proteins of the apoptotic signaling pathway are genetically encoded and ready for the induction of programmed cell death by internal or external signals.´

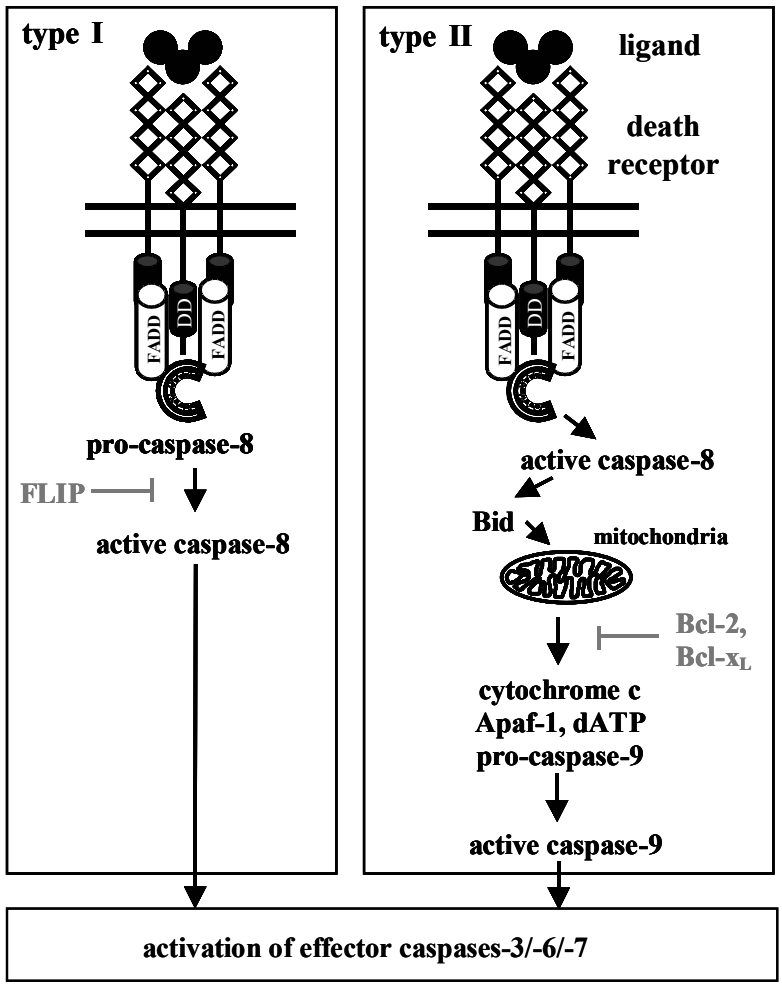

Figure 1.1. Signaling pathways of death receptor**.** For details, see text. DD, death domain; FADD, FLICE-associated death domain; FLIP, FLICE-inhibitory protein

1.4.1 CD95 / CD95L

The death receptor CD95 (Fas, Apo-1) controls the homeostasis of many tissues. CD95, abundantly expressed in the thymus, heart, kidney and liver, initiates apoptosis upon binding of Fas-ligand (FasL/CD95L). CD95L is synthesized in a membrane-resident form but can be converted in the soluble form by proteolytic cleavage. The physiological function of each form (membrane vs. soluble), which exist together *in vivo*, is still unclear. The CD95/CD95L arrangement plays a fundamental role in the immune system:

- Cytotoxic T cell-mediated killing of cells
- Deletion of activated T cells at the end of an immune response
- Destruction of inflammatory and immune cells in immune-privileged sites such as the eye, brain and testes by constitutively expressed CD95L.

The binding of CD95 by CD95L triggers a cascade of well characterized intracellular signaling events. The CD95L is a trimer and promotes the trimerisation of CD95 receptor after ligation. FADD associates with the intracellular death domain and recruits the initiator caspase-8 which is autoproteolytically cleaved. The activated caspase-8 then triggers the activation of the execution caspases such as caspase-3. [30]

Tumors possess a variety of mechanisms to escape the immune clearance by CD95-mediated apoptosis. One important mechanism used by tumor cells takes advantage of the manipulation of the CD95/CD95L system. Alterations of this system can be subdivided into passive and offensive strategies.

Thus, tumor cells can develop resistance to CD95-mediated apoptosis by

- downregulation of CD95 receptor expression
- secretion of decoy receptors to block CD95L on the surface of activated T lymphocytes
- alteration of intracellular mechanisms in CD95-mediated signaling pathway

The offensive immune escape mechanism has been coined “tumor counter attack”. Tumor cells of diverse origin have been reported to strongly express CD95L. Stable CD95L expression can only be observed in apoptosis-resistant tumors [33]. Analogous to the maintenance of the CD95L in the immune privilege, tumor cells use this system to suppress an antitumor immune response by elimination of the attacking antitumor lymphocytes. [34].

Chemotherapeutic drugs (e.g. bleomycin) can cause upregulation of CD95 by p53- and AP-1-dependent mechanisms and it is believed that this contributes to the elimination of tumor cells by inducing apoptosis [35-37]. However, many tumor cells are resistant to CD95-induced

apoptosis, especially after therapy [38]. Although some studies has been shown, that CD95 stimulation of cells under certain conditions can induce genes that have functions outside of apoptosis and that CD95 can promote proliferation of T cells and fibroblasts, specific tumor-promoting effects of CD95 stimulation for CD95 apoptosis-resistant cells have not been reported [39-42].

Additionally, upregulation of CD95L has also been demonstrated in alcohol-induced hepatitis, Wilson´s disease, Hepatitis B and graft versus host disease [43-45].

1.4.2 TRAIL-R1/2 / TRAIL

Five different Trail (TNF-related apoptosis-inducing ligand, APO-2L) receptors have been identified, but only two of them, TRAIL-R1 (DR4) and TRAIL-R2 (DR5) can initiate the apoptosis signaling cascade. The other three serve as decoy (Dc) receptors. DcR1 does not possess a cytoplasmatic signaling domain while DcR2 has a truncated death domain. However, in contrast to the other four receptors, Trail-R5, also called Osteoprotegerin is secreted into the extracellular fluid. [32]

Numerous of the signaling events of TRAIL-induced apoptosis are similar to CD95L. Binding of TRAIL to its death receptors triggers the recruitment of the adaptor molecule FADD to the cytoplasmatic death domain followed by procaspase-8 and its activation at the DISC [46].

TRAIL selectively causes apoptosis in a wide variety of tumor cells but not normal cells. It is believed, that the death receptors are mainly exist on transformed cells while its decoy receptors are expressed in normal cells. [47] However, the major disadvantage of TRAIL as a therapeutic drug is its toxicity to normal human hepatocytes, in contrast to monkey or mouse hepatocytes [48]. A method to avoid this apoptosis-inducing effect of TRAIL to normal human hepatocytes is the use of anti human TRAIL-R2 antibody which triggers exclusively cell death in transformed cells [49].

1.4.3 TNF-R1 / TNFα

Many data suggest that TNFα (Tumor necrosis factor α), a multifunctional cytokine mainly produced by monocytes and activated tissue macrophages (e.g. Kupffer cells) is involved in pathophysiological effects of inflammatation, cachexia and endotoxin shock [50,51]. However, the specific role of TNF in disease pathogenesis remains still poorly defined.

Binding of TNFα to the TNF-R1 leads to the activation of the transcription factors AP-1 and NF-kB followed by the induction of a number of proinflammatory, immunomodulatory and antiapoptotic genes. The ligation of TNFα on TNFR1 initiates receptor trimerisation and clustering of intracellular death domains followed by the binding of intracellular adapter

molecules called TNFR-associated death domain (TRADD) or RIP-associated ICH-1 / CED-3 homologous protein with a death domain (RAIDD) via interactions between death domains. TRADD has the ability to recruit different proteins to the activated receptor:

- Binding of TNF-associated factor 2 (TRAF2) leads to activation of NF-kB and the JNK/Ap-1 pathway.
- Association with FADD initiates the induction of apoptosis via the recruitment and cleavage of procaspase-8.

RAIDD recruits the initiator caspase-2 which also leads to the induction of apoptosis. [52,53].
Like TNFα itself, the receptor also exists in a membrane-associated and a soluble form. At high concentrations, soluble TNF receptors can neutralize most TNF activities and are cleaved from their membrane-associated form by the same enzyme which cleaves TNFα, i.e. TACE. TNFα is an extremely pleiotropic protein at the cellular level, with activities ranging from proliferation to cell death depending on the dose. Many tumor or cell types are resistant towards TNFα-induced apoptosis unless they have been sensitized to undergo cell death by inhibition of transcription or translation.

1.5 HDAC Inhibitors

The transcriptional blockade of hypoacetylated chromatin can be overcome by agents that inhibit HDACs. A number of structurally diverse natural and synthetic HDAC inhibitors (HDIs) have been identified. Compounds that are shown to inhibit class I and II HDAC activity by binding to the zinc-containing catalytic domains can be ordered into six structurally distinct classes: (1) small molecular weight carboxylates (e.g. valproic acid), (2) hydroxamic acids (e.g. CBHA), (3) benzamides (e.g. MS-275), (4) epoxyketones (e.g. trapoxin), (5) cyclic tetrapeptides (e.g. apicidin), (6) hybrid molecules (e.g. cyclic hydroxamic acid-containing peptide (CHAP 31)).
Insight into the inhibition of the HDAC activity by small molecule inhibitors came from the crystal structure of the histone deacetylase-like protein HDLP [54] and by crystallization of human HDAC8 [55]. The single catalytic domain of HDLP from the hyperthermophilic bacterium Aquifex aerolicus, also found in HDAC class I and II, consists of 375 amino acids arranged in a tubular deep pocket with an adjacent internal cavity and a wide bottom. Briefly, cocrystallization studies of HDLP and the hydroxamic acids trichostatin (TSA) or SAHA and HDAC8 with four structurally diverse hydroxamate inhibitors have shown that a Zn^{2+} ion-binding site positioned in the pocket is essential for a charge relay system which includes two histidine residues, two aspartic residues and a tyrosine residue. The chelation of zinc by

hydroxamic acid group is the main mechanism of HDAC enzyme inhibition. However, hydroxamic acids are subject of sulfatation, glucuronidation and enzymatic hydrolysis. Thus, some non-hydroxamate HDIs have been described, but all have reduced potency compared to hydroxamate inhibitors [56-58].

Studies of HDAC inhibitors (HDIs) demonstrated that only a subset of cellular genes is sensitive to histone acetylation in chromatin (< 2%). The reason for this selectivity is not clear. HDIs induce similar patterns of alterations of gene expression in different cell lines as well as definite differences by different agents [59,60]. As shown in Table 1., HDIs upregulate genes in transformed cells as well as repress the transcription of some other genes. The mechanisms of the transcriptional repression are unclear and may involve recruitment of corepressors or acetylation of nonhistone protein substrates [1].

HDIs, when administrated to different cancer cell types induce apoptosis and/or differentiation. Another consequence of HDAC inhibition in cultured cell lines includes the inhibition of cell proliferation by cell cycle arrest in G_1/S and/or G_2/M phase [61-64].

HDIs which are currently in clinical development show activity against malignant cell proliferation in culture and tumors growing in animal models. This antineoplastic activity may be characterized by the potency to induce apoptosis, cell cycle arrest or differentiation.

Regulated protein	Function	References
Downregulation by HDAC inhibitors		
TGF-β	Regulation of proliferation and differentiation	65,66
VRGF	Angiogenic factor	66,67
Cyclin A, B	Cell cycle regulator	68
HER2/neu	Growth factor receptor (EGFR class)	69
Upregulation by HDAC inhibitors		
Fas/Fas ligand	Proapoptotic	70
Bcl2	Proapoptotic	71
p53	Proapoptotic	66
Caspase 3	Cystein protease, proapoptotic	72
$p21^{WAF/Cip1}$	Cell cycle regulation	73,74

Table 1.1: Some of the Proteins whose transcriptional expression is altered in response to HDI treatment of cells [1].

Importantly, non-transformed, normal cells are resistant to HDAC inhibition. The absence of a G_2 checkpoint in tumor cells may account for the selective apoptotic response to HDIs [75].

HDIs increase the acetylation of histones in many different malignant and non-malignant tissue types, but some tissues show only little, if any metabolic responses upon treatment with HDIs (see also Figure 1.2). The global hyperacetylation of histone by HDIs cannot explain the selective changes of gene expression pattern. It may depend on the bioavailability and activity of HDIs or the acetylation of non histone proteins especially of tissue/development-specific (e.g. MyoD), oncogenetic (c-Myb), tumor-suppresssing (p53) or ubiquitous (e.g. HNF-4) transcription factors.

Generally, HDIs may induce apoptosis in human tumor cell lines by activating death receptor-dependent (e.g. upregulation of Fas/Fasl) and mitochondrial-mediated pathways (e.g. downregulation of antiapoptotic members of the Bcl-2 family). Thus, HDI- mediated modulation of the expression level of pro- and antiapoptotic proteins represent an efficient tool to amplify the cell death process [76-78] (see also Table 1).

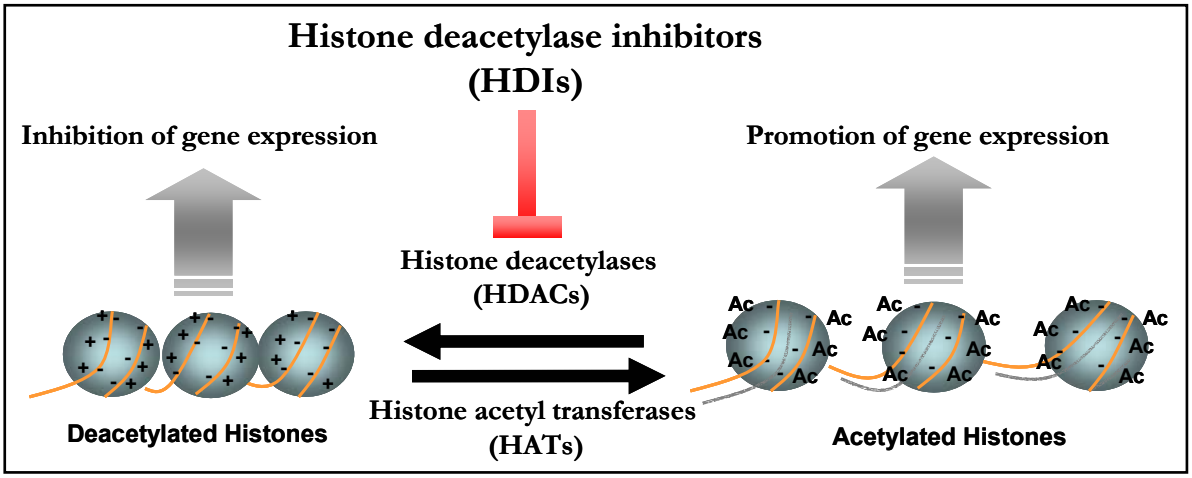

Figure 1.2: Post-translational modification of histones

2 Aims of the study

Preclinical and clinical studies suggest that inhibition of histone/protein deacetylation by targeting histone deacetylases (HDACs) represents a novel approach in cancer therapy. However, only recently it became evident that HDAC inhibitors by themselves do not only induce apoptosis but also render cells sensitive to death receptor agonists, i.e. TNF, TRAIL and CD95 ligand (CD95L), respectively. Consequently, the overall aim of this study was to elaborate a comprehensive understanding of the pharmacodynamic properties of HDAC inhibitors (HDIs) as to there sensitizing effects.

In detail the following questions should be addressed:

- In orientating cellular experiments the optimum conditions as to the activation of death receptors and the concentrations of HDIs should be defined such that a maximum sensitization window is made available.
- A sequential design has to be built up which starts from findings with transformed liver cells, compares them with primary mouse hepatocytes, extends them to whole mouse liver, and finally translate them to human primary liver cell cultures.

An investigation of the modification by HDIs of pro- and antiapoptotic protein factors needs to be carry out with the aim derive a plausible mechanism for the pharmacodynamic, i.e. apoptosis-sensitizing action of HDIs

3 Materials and Methods

3.1 Materials and animals

3.1.1 Substances

Activating anti-CD95 antibody (Jo2) and polyclonal IgG horseradish peroxidase-coupled secondary antibody (goat anti-mouse) were purchased from PharMingen (San Diego, CA, USA). CD95L was generously provided by Dr. Elisa Ferrando-May. Recombinant mouse TNF was obtained from Innogenetics (Ghent, Belgium) and human recombinant TNF was generously provided by Prof. Dr. D. Männel (University of Regensburg, Germany). *Killer*Trail was purchased from Alexis Biochemicals (Gruenberg, Germany). Mouse monoclonal anti-caspase-3, anti-Xiap and anti-Bax 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 and anti-Bid were obtained from New England Biolabs (Frankfurt am Main, Germany), rabbit polyclonal anti-c-Flip from Stressgen (Victoria, Canada). Mouse monoclonal anti-FADD was purchased from Biomol (Hamburg, Germany).

Sodium valproate (Ergenyl®) was purchased from Sanofi-Synthelabo (Berlin, Germany); apicidin from Sigma-Aldrich (Taufkirchen, Germany), M344 and CBHA from Calbiochem (Schwalbach, Germany). SAHA, LAQ-824 and MS-275 were generously provided by Altana Pharma AG.

Pefabloc® and the caspase substrate Ac-DEVD-afc were obtained from Biomol (Hamburg, Germany).

Primers for PCR were purchased from Thermo Electron Corporation (Dreieich, Germany), restriction and DNA modifying enzymes from New England Biolabs (Frankfurt am Main, 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.

3.2 Methods

3.2.1 Cell culture

HepG2 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in RPMI 1640 containing 10% FCS, 100 µg/ml penicillin and 100 U/ml streptomycin in a humidified incubator at 5% CO_2 / 95% air. FCS was inactivated by incubating in a wather bath at a temperature of 55°C for 30 minutes prior to addition to the medium. Cells were split twice a week in a ratio of 1:5, using Accutase® to detach adherent cells.

3.2.2 Isolation and culture of mouse hepatocytes

Hepatocytes were isolated from 8 weeks old mice by the two step collagenase perfusion method of Seglen [79] and cultured as described [80]. To separate the hepatocytes from 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 × g for 10 min at room temperature. Briefly, hepatocytes were plated in 200 µl RPMI 1640 medium containing 10% fetal calf serum (FCS), 100 µg/ml penicillin and 100 U/ml streptomycin in collagen-coated 24-well plates at a density of 8 x 10^4 hepatocytes per well. Cells were allowed to adhere to culture dishes for 3 h before the medium was exchanged to RPMI 1640 without FCS. Incubation of murine hepatocytes with indicated compounds started 30 min after medium exchange. Incubations were conducted in a humidified atmosphere at 37°C, 5% CO_2, 40% O_2 and 55% N_2.

3.2.3 Culture of primary human hepatocytes

Primary human hepatocytes were generously provided by Professor Dr. Nüssler (Charité, 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 5% CO_2, 37°C.

3.2.4 Treatment of cells

HepG2 cells, primary murine or human hepatocytes were preincubated with ActD, CHX or HDIs 3 hours before treatment with TNFα, *killer*Trail or αCD95/CD95L. If substances were dissolved in DMSO or Ethanol, the solvent alone was used in control incubations.

3.2.5 Isolated liver perfusion

Upon a lethal intravenous injection with 150 mg/kg pentobarbital-natrium and 0.8 mg/kg heparin, the vena portae and the vena cava inferior of the mouse liver were cannulated and ligated. After cannulation the organ was perfused blood-free before circulation has been closed, to guarantee a blood-free perfusion. Perfusion was carried out submarine (to avoid oedema formation) using a modified Krebs-Henseleit buffer (147 mM NaCl; 5.36 mM KCl, 0.34 mM Na2HPO4; 0.44 mM KH2PO4; 0.77 mM MgSO4; 10 mM D-Glucose; 9 mM HEPES; pH: 7.3; 325-330 mOsm) with a total volume of 25 ml buffer in a closed circulation mode under constant pressure conditions of 21.33 mm Hg. Osmolality of the perfusate fluid was set to 225-330 mOsm and checked before each individual experiment. The temperature of the perfusate was kept constant at 37°C, guaranteed by a separate thermo-circulation and oxygenation with pure oxygen at a pressure of 500 mbar was performed. During perfusion, the perfusate flow through the liver as well as the pressure were constantly measured and recorded. Samples for metabolite and enzyme measurements were taken from the perfusate at different time points, as indicated in the text. All technical equipment and tubes for performing isolated mouse liver perfusion was delivered by Hugo Sachs Electronik (March-Hugstetten, Germany).

3.2.6 Cytotoxicity assay

Cytotoxicity was measured by the reduction of tetrazolium dye Alamar Blue™ by viable cells. The assay was performed according to the manufacturer´s 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).

Also, release of cytosolic marker enzyme lactate dehydrogenase (LDH) was used as a parameter for cytotoxicity. 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).

The cytotoxicity after liver perfusion was quantified by the liver specific enzyme alanine aminotransferase (ALT) measurement out of perfusate samples taken at different time points during the experiment and stored at 4°C at the end of the experiment until measurement.

3.2.7 Caspase-3/-7 activity assay

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 PEFA-block 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) was followed 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 30 min at 37°C using a fluorometer plate reader 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 Preparation of S100 Fraction

HepG2 cells (6 x 10^6) were washed with ice-cold PBS and lysed with 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 incubation on ice for 15 min, cells were broken by passing 15 times through a G27 needle. Cell extracts were centrifuged at 900 x g, 10 min, 4°C. The supernatant was further centrifuged at 50,000 x g for 1 h. The supernatant (S100 fraction) was immediately frozen in liquid nitrogen 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), 5 µM cytochrome c and 1 mM dATP. The mixtures were incubated at 37°C for 30 min and were occasionally mixed.Ten µl of the reaction solution were used to determined the caspase-3,-7 activation.

3.2.9 Transient transfection

Transient Transfection was performed employing TransPass™ D2 from New England Biolabs (Frankfurt am Main, Germany) according to manufacter´s instructions. Briefly, HepG2 cells in 6-well plates were transfected with the human caspase-8 construct or the vector pCMV2 (Sigma-Aldrich, Taufkirchen, Germany) one day after plating. Four hours post-transfection the transfection complexes were removed and the incubation with Histonedeacetylases inhibitors was started directly after medium exchange for RPMI 1640

with FCS. Sixteen hours after removing the transfection complexes the caspase-3 -7 activation was determined as described above.

3.2.10 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 as described earlier. 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. Caspases-3, -8, -9 and XIAP were detected with the mouse monoclonal antibodies #610322 (clone 19), AM46T (clone 1-3), AM47T (clone 1-2) and #610716 (clone 28), respectively. Bid and c-FLIP was detected with a rabbit polyclonal antiserum (#2002; #AAP-440), for detection of BAX, FADD a mouse monoclonal antibody was used. 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 l H_2O_2 , pH 8.5).

3.2.11 Statistics

All data are given as means ± SEM. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni'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 HDAC inhibition in death receptor-induced apoptosis of HepG2 cells

Various classes of HDIs have been shown to enhance or even enable at all the cytotoxicity mediated by chemotherapeutic drugs or cytokines in a variety of carcinoma cell lines. HepG2 cells, isolated from tumor tissue of a 15-year old Argentine boy with hepatocellular carcinoma (HCC) in 1975 [81], are one of the most widely used HCC lines, because they express a variety of liver functions (e.g. fibrinogen, bile acids) [82] and represent a useful biological system to investigate hepatic apoptosis [83-85]. It is well-known that the ligands of the TNF superfamily alone are not able to induce apoptosis in HepG2 cells. In order to overcome this resistance, the control experiments were carried out in the presence of actinomycin D (ActD), i.e. a transcription inhibitor with the rationale to suppress the synthesis and the hence action of anti-apoptotic proteins.

4.1.1 Sensitization of HepG2 cells by HDIs

To ascertain the optimal sensitizing concentration towards TRAIL-, TNF- or CD95L- initiated cytotoxicity, HepG2 cells were incubated with increasing concentrations of ActD and the HDIs valproic acid, M344, CBHA and apicidin alone or together with the cytokines as indicated. Although structural dissimilar, all four HDIs were capable of promoting the TRAIL- and CD95L-, but not of the TNFα- induced toxicity in HepG2 cells. Unlike in this setting, the hepatoma cell line was sensitized toward all three cytokines tested in the presence of ActD (Figure 4.1).

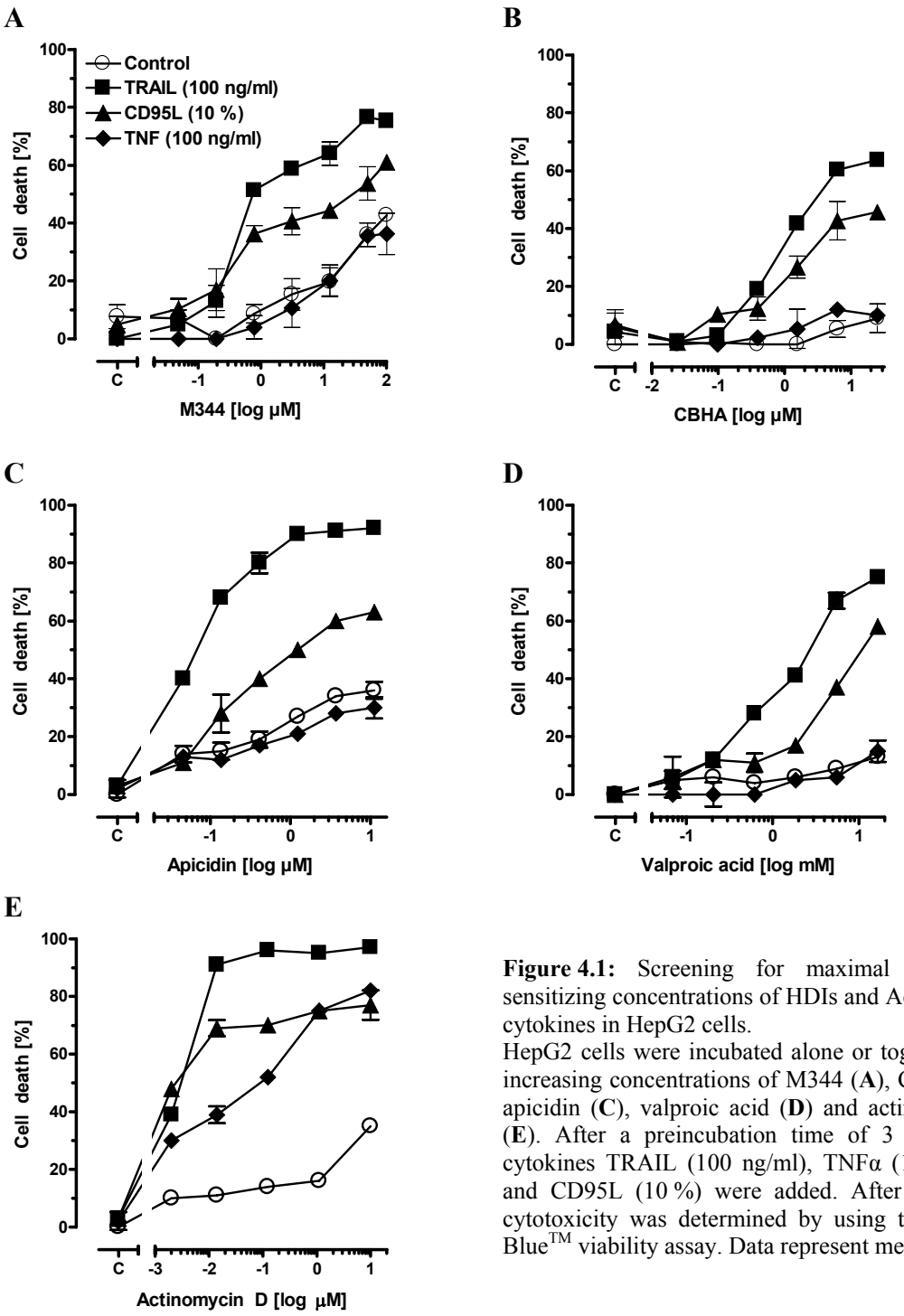

Figure 4.1: Screening for maximal non-toxic, sensitizing concentrations of HDIs and ActD toward cytokines in HepG2 cells.
HepG2 cells were incubated alone or together with increasing concentrations of M344 (**A**), CBHA **(B)**, apicidin (**C**), valproic acid (**D**) and actinomycin D (**E**). After a preincubation time of 3 hours, the cytokines TRAIL (100 ng/ml), TNFα (100 ng/ml) and CD95L (10 %) were added. After 18 hours, cytotoxicity was determined by using the Alamar Blue™ viability assay. Data represent mean ± SD.

4.1.2 Concentration finding studies with death receptor agonists

In a next set of experiments, the sensitizing effects were examined with regard to the concentration dependencies of TRAIL-, TNFα- or CD95L-induced cytotoxicity. To this end, constant concentrations of the HDIs were used which were defined by the ratio of the basic cytotoxicity *versus* the enhancement of toxicity by coincubation with the ligand. This experimental setup confirmed the previous results: M344, CBHA, apicidin and valproic acid caused a concentration-dependent toxicity of CD95L and TRAIL, but not of TNFα in HepG2 cells, while ActD enhanced the toxicity of all death receptor ligands (Figure 4.2).

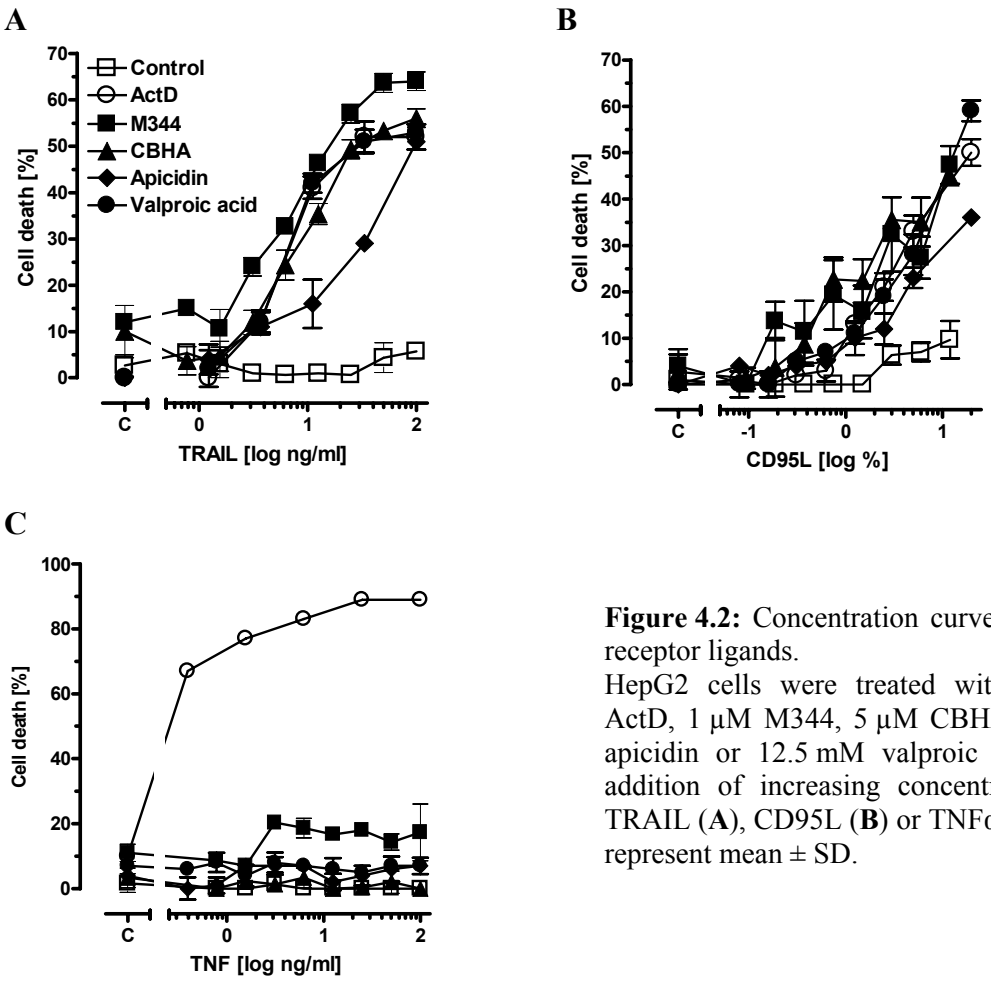

Figure 4.2: Concentration curves of death receptor ligands.
HepG2 cells were treated with 1 µg/ml ActD, 1 µM M344, 5 µM CBHA, 0.5 µM apicidin or 12.5 mM valproic acid prior addition of increasing concentrations of, TRAIL (**A**), CD95L (**B**) or TNFα (**C**). Data represent mean ± SD.

4.1.3 Kinetics of cytotoxicity and caspase activity

After engagement of a death receptor by its ligand, the activation of executioner caspases like caspase-3 represent a key event in apoptosis. Consequently, the mode of cell death induced by TRAIL, CD95L and TNFα was investigated by recording the kinetics of caspase-3-like activity and cytotoxicity in sensitized hepatocytes for 12 and 24 hours, respectively. In fact, HepG2 cells treated with HDIs alone exhibited no significant cytotoxicity than those treated with HDIs *plus* CD95L or TRAIL. In other words, it is the presence of HDIs which allows the induction of apoptotic cell death to occur at all with these two DR ligands. In contrast, neither caspase activation nor toxicity could be observed when HepG2 cells were treated with TNFα in the presence of CBHA, M344, valproic acid or apicidin (data not shown). No caspase-3-like activity and cytotoxicity was detected in HepG2 cells treated with HDIs alone, using the defined sensitizing concentrations described in the previous experiments. When CD95 or TRAIL were activated in the presence of HDIs (Figure 4.3 C, D) or ActD (data not shown), however, a peak of caspase activity was reached after approximately 10 hours in all

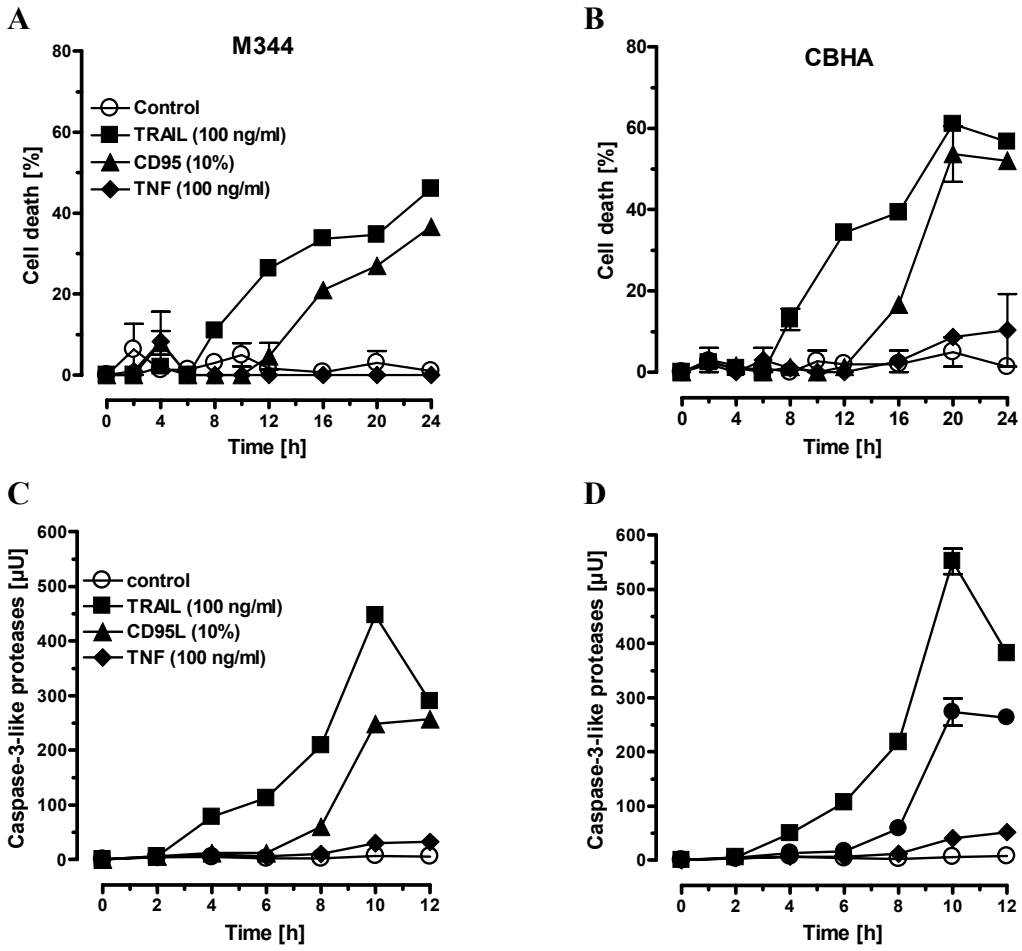

Figure 4.3: Time course of cytotoxicity (**A, B**) and caspase activity (**C, D**).
HepG2 were treated with 1 µM M344 (**A, C**) or 5 µM CBHA (**B, D**) alone or together with the ligands TRAIL (100 ng/ml), CD95L (10%) or TNFα (100 ng/ml). Cytotoxicity and caspase-3-like activity was determined at the indicated time points. Data represent mean ± SD.

experimental set-ups. It is concluded from these kinetic observations that the emerging cytotoxicity is likely to be the consequence of the activation of caspases.

4.1.4 Kinetics of protein expression pattern

The transmission of apoptosis signals and their execution is regulated by a network of proteins. The primary effect of HDIs is to modulate gene expression. Consequently, the upregulation of proapoptotic and/or downregulation of antiapoptotic proteins are likely mechanisms of sensitization towards apoptosis signals. Various cells enhance the apoptotic signal coming from the DISC *via* the intrinsic pathway. It was therefore of interest to examine the influence of HDIs on the expression of some proapoptotic proteins.

The approach was taken by Western blot analysis using cell lysates from HepG2 cells treated with 5 µM CBHA for up to 21 hours. As shown in Figure 4.4 the levels of the executioner caspase-3 as well as both initiator caspases-8 and -9 remained largely unaffected in the presence of CBHA. Similarly, the expression levels of Bid and Bax, which initiate the intrinsic pathway, were unchanged. The expression of FADD, which triggers the recruitment of caspase-8 into the DISC, is slightly enhanced in the present of CBHA in a period of 21 hours.

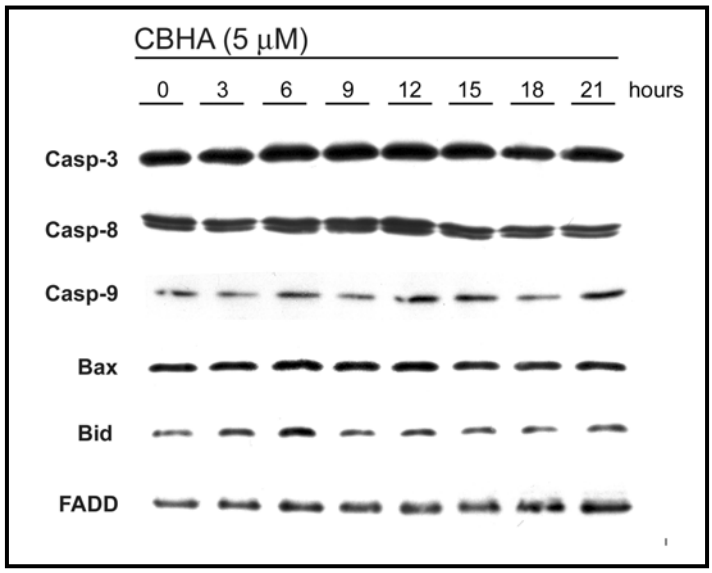

Figure 4.4: Time course of various proapoptotic proteins expression. Expression of caspase-3, -8 and -9 and the proapoptotic proteins Bax, Bid and FADD was detected using cell lysates from HepG2 cells treated with 5 µM CBHA for up to 21 hours. Each lane was loaded with 30 µg protein.

The inhibition of caspase activation by endogenous proteins is one of the essential cellular mechanisms to prevent the induction and execution of apoptosis signaling. As described, the FLICE-inhibitory protein cFLIP and XIAP belong to antiapoptotic proteins which directly inhibit the activation or activity of caspases. Both proteins are involved in the regulatory networks of TRAIL- as well as of CD95L-triggered apoptosis. Downregulation of cFLIP or XIAP was reported in various cell lines in response to HDIs [86]. Therefore, the expression level of these two antiapoptotic proteins was analyzed using lysates from HepG2 cells, treated with CBHA, M344, or valproic acid (VPA). The protein level of cFlip – decreased at 3 to 6 hours – was barely detectable in response to all three HDIs used at 18 hours. At 21 hours, cFlip was essentially undetectable. Similarly, the expression of XIAP is slightly decreased over time. It is concluded from these findings that the sensitization of HDIs towards TRAIL- and CD95L-triggered apoptosis is likely to be due to the downregulation of antiapoptotic proteins.

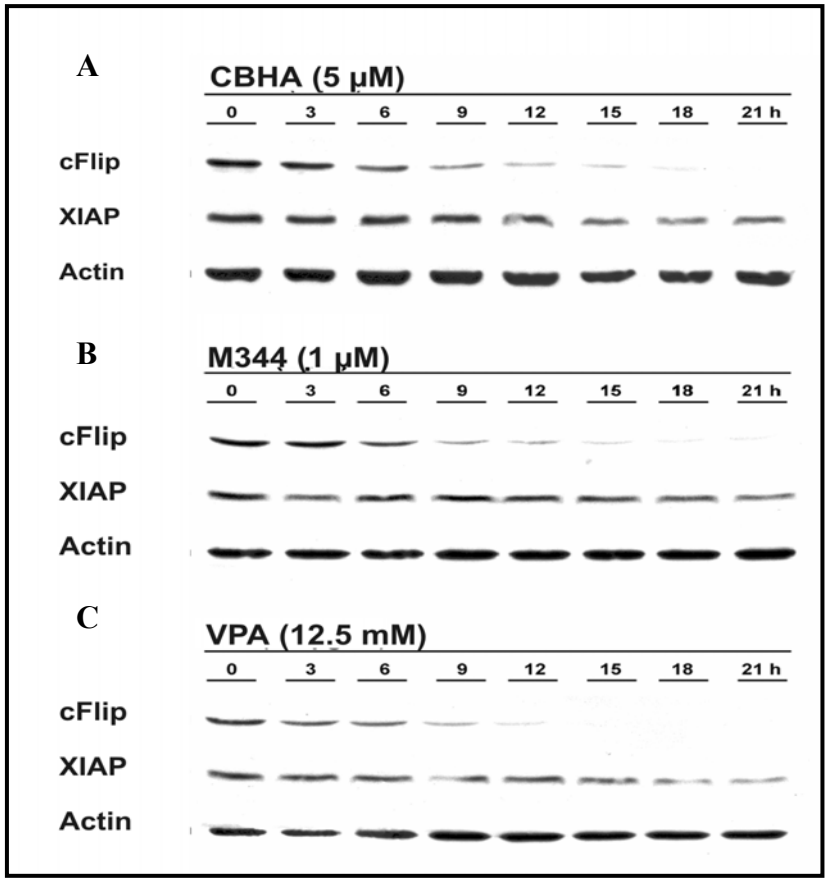

Figure 4.5: Time course of cFlip and XIAP expression. Expression of cFlip and XIAP was detected by Western blot analysis using cell lysates from HepG2 cells treated with CBHA (**A**), M344 (**B**) or VPA (**C**) for up to 21 h. Each lane was loaded with 30 µg protein.

4.1.5 Characterization of modifications by HDIs downstream of the death receptor

The following experiments were dedicated to the events downstream of the death receptors in order to collect more information on the mechanism of sensitization by the HDIs. In order to address the extrinsic pathway first, HepG2 cells were transfected with caspase-8 via pCMV2 vector. Cells transfected either with the empty vector or with the vector encoding for caspase-8 were incubated with various HDIs, or with ActD. Caspase-3/-7 was assessed after 16 h as a readout for the activation of the extrinsic pathway. The results in Figure 4.6 A demonstrate that the transfection of caspase-8 increased the caspase-3-like activity in the cells about two-fold (Figure 4.6 A, controls). As compared to untreated caspase-8-transfected cells, treatment with HDIs increased executioner caspase-3/-7 activity by a factor of 2 to 3. These data show that, HDIs are able to enhance the caspase-8 mediated caspase-3/-7 activation. In a second

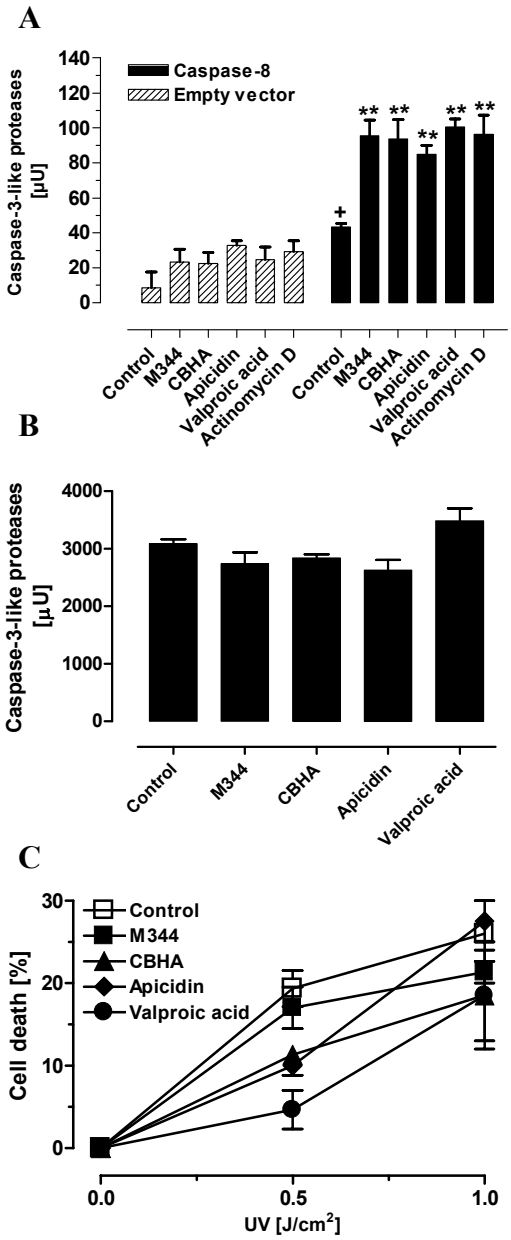

Figure 4.6: Events downstream of the death receptor.
(**A**) HepG2 cells were transfected with either caspase-8 or with empty vector. Cells were treated with M344 (1 µM), CBHA (5 µM), apicidin (0.5 µM) or VPA (12.5 mM). After 16 hours the caspase-3 like activity was measured by quantification of the DEVD-afc cleavage.
(**B**) S100 extracts derivated from HepG2 cells treated with M344 (1 µM), CBHA (5 µM), apicidin (0.5 µM) or VPA (12.5 mM) for 21 hours were incubated with 5 µM Cytochrome C and 1 mM dATP at 37°C. After 30 min, caspase-3-like activity was determined.
(**C**) HepG2 cells were preincubated with M344 (1 µM), CBHA (5 µM), apicidin (0.5 µM) or VPA (12.5 mM). After 3 h, the cells were treated using UV-radiation and cytotoxicity was assessed 24 h later.
Data represent mean ± SD.
+: $p < 0.05$ for empty vector control *vs* caspase-8 control.
**: $p < 0.01$ for caspase-8 control *vs* caspase-8 plus HDIs. Dunnett's Multiple Comparison Test

approach, characteristics of the intrinsic pathway were addressed in a cell-free systems using dATP/cytochrome c in cytosolic extracts of HepG2 cells pretreated with HDIs. Figure 4.6B shows that caspase-3/-7 activity was not significantly altered by any HDI tested. In a third series of experiments, a death receptor-independent but mitochondria-dependent model of apoptosis was chosen, which consisted in irradiation of HepG2 cells with various doses of UV light. The data in Figure 4.6 C show that the HDIs tested had essentially no influence on this type of cell death. These findings show mitochondria are most likely not involved in a HDI-mediated sensitization. The whole set of experiments in Figure 4.6 rather suggests that the site of action of HDIs is the activation step from the initiator caspase-8 to the executioner caspase-3/-7, be it directly or indirectly via suppression of anti-apoptotic factor such as XIAP.

4.2 HDAC inhibition in CD95-induced apoptosis in primary murine and human hepatocytes

With the known sensitization of HDIs towards transformed, highly proliferating cells, it was of interest to study these pharmacodynamic properties in primary, healthy liver cells. Therefore, orientating experiments were made with M344, CBHA, apicidin and VPA in primary mouse hepatocyte cultures. These cells, insensitive towards TRAIL-induced apoptosis, were therefore driven into apoptosis via using the death receptor antagonistic αCD95. Similar results were obtained as with the HepG2 cells, except that higher concentrations of HDIs were needed for the sensitization (data not shown). All further experiments with HDIs concentrate on apoptosis initiated *via* the CD95 system, because the engagement of the TNF-R had no effect in the presence of HDIs (data not shown). Figure 4.7A shows, that the coincubation with HDIs or ActD concentration-dependently enhanced αCD95-mediated cytotoxicity. The results clearly demonstrate that with any of the HDIs studied, a sensitization of receptor-mediated, apoptotic cell death was also observed in primary cells. As expected, the increase in apoptotic cells death was paralleled by a correspondingly increased activation of caspase-3/-7 (Figure 4.7 B,C).

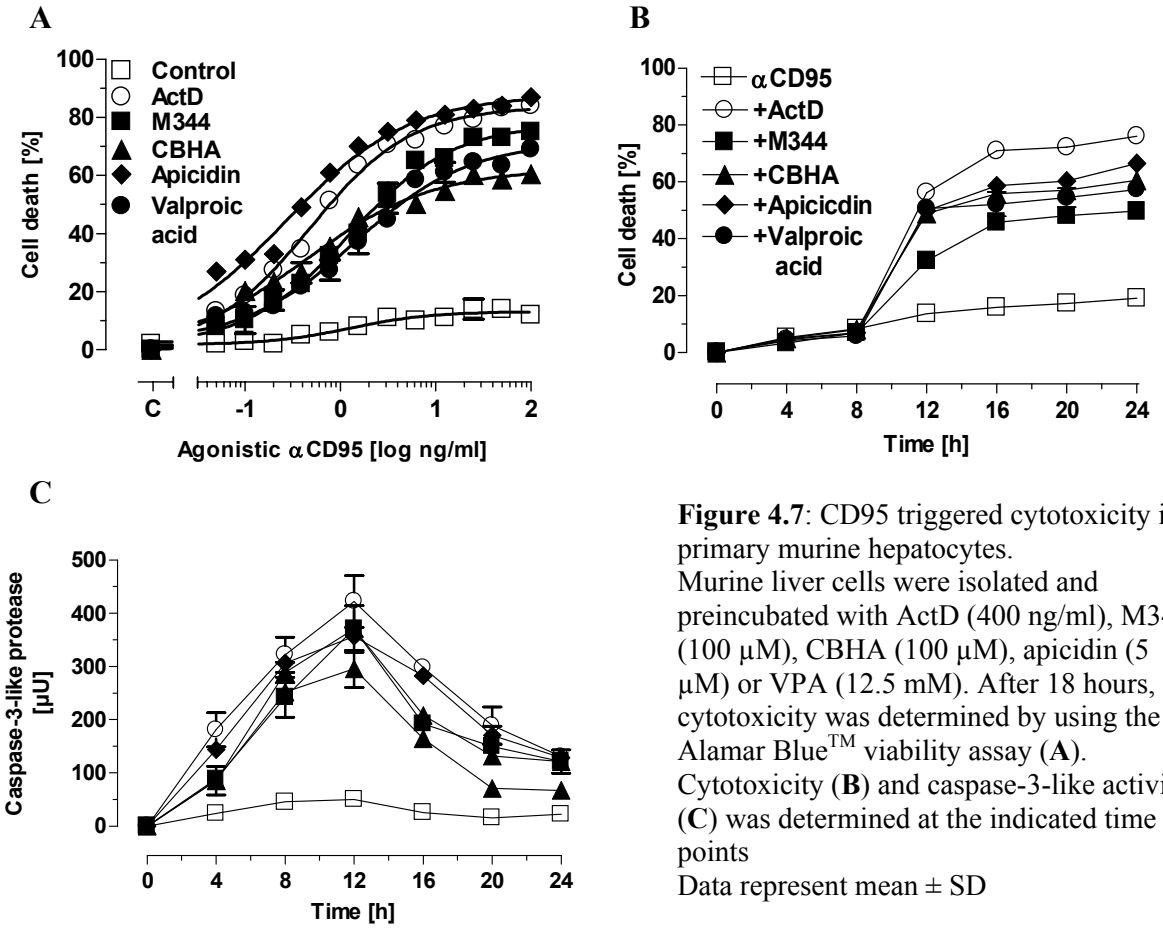

Figure 4.7: CD95 triggered cytotoxicity in primary murine hepatocytes.
Murine liver cells were isolated and preincubated with ActD (400 ng/ml), M344 (100 µM), CBHA (100 µM), apicidin (5 µM) or VPA (12.5 mM). After 18 hours, cytotoxicity was determined by using the Alamar Blue™ viability assay (**A**). Cytotoxicity (**B**) and caspase-3-like activity (**C**) was determined at the indicated time points
Data represent mean ± SD

4.2.1 HDI mediated sensitization to CD95-induced apoptosis *in situ*

Further experiments were then carried out applying the isolated, recirculating mouse liver perfusion as a hepatic in situ model. This system involves also the non-parenchymal cells in the physiological composition and architecture. In this model, control organs did not undergo a significant hepatotoxicity for up to 480 min as indicated by the absence of ALT. In contrast to a perfusion with 0.5 µg αCD95, the treatments with 10 µg or 1 µg of this agonistic antibody alone induce a significant hepatotoxicity. It was also shown that an onset of ALT release was dependent on the concentration of the apoptotic stimulus. In presence of the HDI apicidin however, the hepatotoxicity of CD95 stimulation was significantly enhanced in all three concentration settings (Figure 4.8). This finding demonstrates the sensitization of apicidin to the whole organ, i.e. healthy cells in their natural surrounding, towards a CD95-dependent apoptosis.

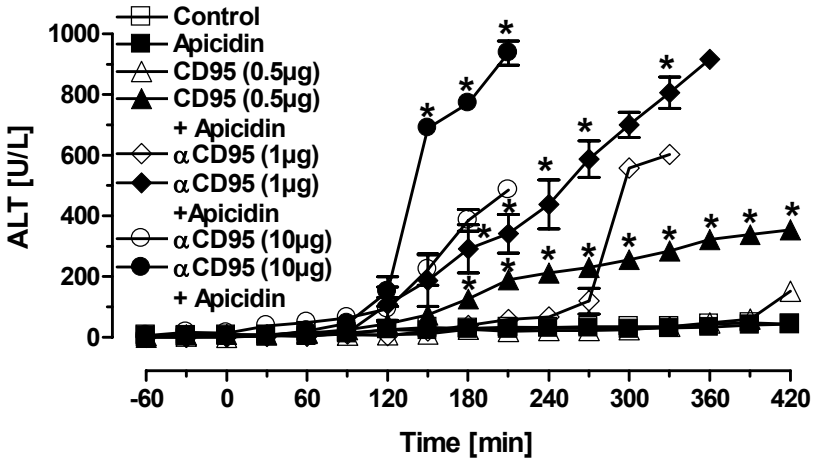

Figure 4.8: αCD95-mediated hepatoxicity in isolate perfused mouse liver.
Livers from C57Bl6 mice were isolated perfused in situ with 5 µM apicidin and different concentrations of αCD95 as indicated up to 8 h. At various time points liver injury was quantified by measurement of the liver-specific enzyme alanine aminotransferase (ALT).
Data represent mean ± SD *: $p < 0.05$ for αCD95 *vs* αCD95 + apicidin. Bonferroni's posttest

4.2.2 HDI mediated sensitization in primary human hepatocytes

In a final proof-of-principle experiment, the influence of HDIs on CD95-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 hepatic tumors. Three further HDIs, which are under clinical development on top of those model compounds were used in the experiments. As shown in Figure 4.9, any of the six HDIs involved into the experiment caused a significant sensitization towards a death receptor-induced apoptosis in human liver cells. This finding demonstrates that the conditions observed in animal tissue – *in vitro* as well as *in situ* – seems to be apply for the human situation.

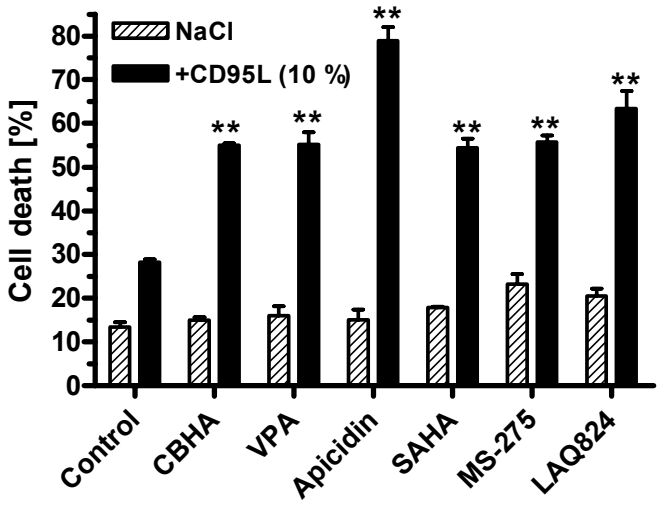

Figure 4.9: CD95L-triggered cytotoxicity in the presence of HDIs in primary human hepatocytes.
Primary human liver cells were preincubated with CBHA (100 µM), VPA (12.5 mM), apicidin (5 µM), SAHA (100 µM), MS-275 (100 µM) or LAQ824 (100 µM) 3 hours before treatment with CD95L. After 18 h cytotoxicity was assessed by determination of LDH release.
Data represent mean ± SD. **: $p < 0.01$ for CD95L *vs* CD95L plus HDI. Dunnett's Multiple Comparison Test

Taken together, all results presented in this thesis converge to a uniform picture, i.e. the sensitization by HDIs towards a death receptor-mediated apoptosis in a HCC line as well as in primary murine and human hepatocytes. The site of action was identified as being located upstream of the activation of executioner caspases and downstream of the receptor assembly events, which allows to derive a potential tentative mechanism.

5 Discussion

5.1 Methodological aspects of this study

5.1.1 Choice of HDACs as a molecular target

This study was undertaken to get insight into the molecular events that occur in receptor-dependent apoptosis after inhibition of HDACs. It is focussed on the liver, because this organ is the metabolic center of the body with service function for non-hepatic organs, and also performs other functions crucial to the mammalian organism, such as elimination of tumor cells, acute phase reaction, and xenobiotic metabolism.

Death receptors and their ligands exert important regulatory functions in the maintenance of tissue homeostasis and the physiological regulation of programmed cell death. Apoptosis involves a genetic reprogramming of the cell to promote an evolutionary conserved intracellular pathway leading to specific, sequential morphological and biochemical changes within the cell. The human HCC line HepG2 is normally resistant to a death receptor-induced apoptosis, which can be overcome by a transcriptional (e.g. ActD) or translational arrest, presumably by blocking the synthesis of anti-apoptotic protein factors.

HDIs, a group of heterogeneous structural diverse compounds are being studied with respect to their chemotherapeutic properties in a number of neoplasias, [87]. This philosophy is based on their hitherto assumed efficacy for inducing differentiation and apoptosis in tumor cells, with little or no toxicity to normal cells [17,88]. It has been suggested that the basis for the tumor specificity of HDIs consists in their interference with death receptor-dependent pathways and/or a defective G2 checkpoint in the cell cycle [75,89,90]. Yet, a universal molecular mechanism that transduces HDI-dependent cell death has not been identified. Several hints exist towards caspase activation which is a general event of HDI-dependent apoptosis. In different studies caspase-2, -3, -7, -8 and -9 processing and activation was observed in HDI-dependent apoptosis [78,91,92].

5.1.2 Choice of conditions and agents (HDIs)

In this thesis, the first set of experiments with HepG2 cells was carried out to find a suitable working window for HDI concentrations vs. effects (Figure 4.1) and preincubation times (data not shown) which allow to quantify the sensitization towards death receptor-induced apoptosis. We used four chemically different HDIs, because it is well known that these agents may cause different effects in the same biological system [59,60]. The hydroxamic acids CBHA

and M344, as well as the cyclic peptide apicidin were utilized in a micromolar concentration range. Valproic acid (VPA) was used at a final concentration of 12.5 mM in all experiments. VPA is among the structural class of carboxylates, which have much weaker HDAC inhibition constants (K_is), commonly in a millimolar range [93]. The relative cytotoxicity was determined, using the tetrazolium dye Alamar Blue™, a growth indicator which based on the detection of metabolic activity [94]. In contrast to the LDH release assay, this method identifies the relative changes of a biological system without to distinguish between cell death, variances in proliferation and alterations of the metabolic activity within the cell. Thus, to get an impression of possible cellular morphological changes a visual control has been done directly before starting the assay.

5.2 Differential sensitization by HDIs of HepG2 cells to CD95L and TRAIL, but not to TNFα

It was demonstrated, that CD95L and TRAIL in combination with the HDIs strongly induce caspase activity and cytotoxicity in HepG2 cells (Figures 4.1, 4.2 and 4.3). These findings are in accordance with previously reported works done in other cell lines [95,96]. However, the cytokine TNFα exerts no cytotoxicity in the presence of HDIs in this experimental set up (Figure 4.1). This new finding contrasts with data published for breast cancer cell lines MCF-7, T47-D and BT-20 [97]. Chopin *et al.* showed that treatment with sodium butyrate, which belongs to the HDI class of carboxylates just as valproic acid, enhances CD95L-, TRAIL- and TNFα-induced apoptosis in these cancer cell lines. In contrast to HepG2 cells, these cells are not resistant towards death receptor-induced apoptosis. The data from Chopin *et al*. suggest a synergistic enhancement of a TNFα-, CD95L- or TRAIL-triggered apoptosis, but not blunting the inhibition by the HDI sodium butyrate. Furthermore, the alterations in the expression pattern, induced by the treatment with HDIs, are cell type-specific [1]. The data presented here clearly show that a liver tumor-derived cell line behaves differently compared to a breast tumor-derived one.

5.3 From alterations of the expression pattern in HepG2 cells by HDIs towards a molecular mechanism

HDIs cause an opened chromatin conformation associated with the promotion of the gene expression due to enhanced access of transcription complexes to the DNA [98]. Moreover, HDIs are capable of up- and downregulation of genes. The mechanism of the transcriptional repression is discussed in terms of the recruitment of corepressors or acetylation of nonhistone proteins [1]. Based on these general interrelationships, one possible explanation for the sensitization of the HDIs is the stimulation of the CD95L- and TRAIL-induced pathway. Here, this hypothesis was confirmed and a more detailed explanation is proposed: it was demonstrated that the HDIs valproic acid, CBHA and M344 caused a downregulation of the antiapoptotic proteins cFLIP and XIAP (Figure 4.5) as well as a slight upregulation of the proapoptotic protein FADD, observed in the presence of CBHA. The protein level of the proapoptotic proteins Bax, Bid and the caspases-3, -8 and -9 was unaffected (Figure 4.4).

The DISC is thought to act as adaptor protein complex by linking members of the tumor necrosis factor receptor (TNFR) superfamily to downstream signaling pathways. The

transmission of the signal into the cell, induced by the engagement of a death receptor is inhibited by FLICE-inhibitory protein (cFLIP) blocking the recruitment of the procaspase-8 into the DISC and subsequently its autoproteolytical maturation. In this study here, the level of the DISC inhibitor cFLIP was rapidly decreased at 3 to 6 hours in the presence of the HDIs. Even though, cFLIP is involved in CD95L, TRAIL and TNFα-induced apoptosis [99,100], downregulation of this antiapoptotic protein appears to have no effect on the initiation of TNFα-induced cytotoxicity in HepG2 cells. Yeh *et al.* demonstrated that cFLIP$^{-/-}$ embryonic mouse fibroblasts are highly sensitive to CD95L- but also TNF-induced apoptosis, showing rapid induction of caspase activities. NF-kappaB activation is intact in TNF-stimulated cFLIP$^{-/-}$ cells [101]. In contrast, stable overexpression of cFLIP results in resistance to receptor-mediated apoptosis [102]. The adaptor molecule FADD is recruited by the CD95-, TRAIL- and TNF-receptor upon binding of a ligand and interacts with the initiator caspases-8 [100,103]. Upregulation of FADD assists the transmission of the proapoptotic signal into the cell triggered by the binding of CD95L and TRAIL (Figure 4.4). The endogenous caspase inhibitor XIAP, which prevents apoptosis by inhibition of caspases, was slightly decreased over time (Figure 4.5). Thus, downregulation of XIAP affects apoptotic events downstream of the death receptors. This protein influences the activity of the executioner caspases-3 and -7 directly or indirectly through inhibition of the initiator caspase-9. Hence, it is reasonable to assume that downregulation of XIAP allows the enhancement of caspase activitation. Moreover, the rapid downregulation of the DISC inhibitor cFLIP in the presence of HDIs is identified as a central element of the sensitization towards CD95L- and TRAIL- triggered apoptosis by these compounds in HepG2 cells.

Under the experimental conditions applied here, the HDIs studied failed to overcome the resistance towards TNFα-triggered apoptosis in HepG2 cells. TNF-R1 induced apoptosis is mediated *via* FADD and consequently also under the control of cFLIP. However, TNF-R1 also leads to activation of the transcription factor NF-κB, a repressor of apoptosis [104]. Published evidence is unclear as to this issue here: it has been reported that HDIs can suppress [105], or activate the gene expression of antiapoptotic proteins by NF-κB [106,107]. Independent activation of NF-κB by HDIs would also negatively affect the apoptosis induced by UV-radiation or even eliminate a sensitization towards CD95L- or TRAIL-induced apoptosis. It is assumed, that the HDIs appear to have no influence on a TNF-R1-mediated activation of NF-κB and NF-κB-regulated genes. This could be an explanation for the selective sensitization of the HDIs in HepG2 cells.

5.4 The site of modulation by HDIs: the extrinsic apoptosis pathway

The presence of HDIs increased the caspase-3/-7 activity in the context of overexpressed caspase-8 in HepG2 cells (Figure 4.6A). A cellular system was generated that mimics the cascade of caspase activation downstream of the DISC, i.e. a model of the extrinsic pathway. To evaluate the functionality of this system, the enhanced caspase-8 activity was measured and the apoptotic morphology of transfected cells was assessed by cotransfection of EGFP and caspase-8 [45]. The increased level of caspase-8 is known to cause the autoproteolytical maturation of this initiator caspase, which in turn directly activates the executioner caspases-3 and -7 within the cell [23]. This means that overexpression of caspase-8 in HepG2 cells is sufficient to trigger the apoptotic cascade. This experiment shows that the alteration in expression pattern caused by HDIs also affects proteins of the apoptosis signaling pathway which act downstream of the death receptor. Since the expression of XIAP, which binds and inactivates the executioner caspases was clearly decreased after 16 hours, it is likely that downregulation of XIAP is indeed the event, which causes the sensitization in this experimental setup (see also Figure 4.5). Thus, downregulation of cFLIP appears not to be the exclusive event for sensitization.

The executioner caspases-3 and -7 can also be activated by caspase-9 *via* the intrinsic pathway. For instance, the benzamide MS-275 induces apoptosis *via* downregulation of cFLIP but also *via* modulation of the intrinsic pathway in the myeloid leukaemia cell line U937 [108,109]. The mitochondrial apoptotic pathway activated by addition of dATP and cytochrome c to cell extracts of treated HepG2 cells to initiate the Apaf-1/procaspase 9/cyt-c apoptosome cascade [110,111] was not affected by HDIs (Figure 4.6B). These data show that the HDIs are unlikely to be responsible for the control of apoptosome complex formation. Bratton *et al*. demonstrate that XIAP, is normally present in high molecular weight complexes in inactivated cell lysates, but directly interacts with the apoptosome in cytochrome c/dATP-activated lysates. XIAP associates with oligomerized Apaf-1 and/or processed caspase-9 and influences the activation of caspase-3, but also binds activated caspase-3 produced within the apoptosome and sequesters it within the complex [112]. However, Datta *et al*. shows that only overexpression of XIAP caused a significant reduction of caspases-9, -3 and -7 activation in activated S100 extracts [113]. This can be interpreted such that the physiological XIAP concentration has no influence on this massive caspase activity in this experimental model.

According to the previous experiment, we detected no increased cytotoxicity after irradiation with UV light in the presence of HDIs in HepG2 cells (Figure 4.6C). The apoptotic parameters (e.g. caspase activity) of this model have been investigated (data not shown). UV

radiation is a DNA-damaging stimulus that activates a p53-dependent apoptotic response [114]. DNA-damaging stimuli, such as UV, activate the intrinsic death effector pathways that perturb mitochondrial structure and function, leading to the release of cytochrome c [115]. Cytosolic cytochrome c forms the apoptosome complex together with APAF-1, dATP, and the initiator procaspase-9 to cause activation of caspase-9 and to trigger subsequent effector caspase activation. Additionally, the HDIs studied don't appear to exert activation of p53 in HepG2. Studies have argued that HDIs could induce caspase activation dependently as well as independently of p53 [63,66,116] In the meantime, it has been suggested that the basis for tumor specificity of HDIs is p53-independent [89]. Taken together, these results favour the notion that that the HDIs used here preferentially modulate the extrinsic pathway.

5.5 The situation in normal primary murine and human hepatocytes

A common goal of new drug discovery programs is the anticancer treatment that destroys tumors while having minimal toxicity on normal body tissue. In vivo and in vitro tests have shown, that HDIs are relatively non-toxic to normal cells and tissues but are selectively cytotoxic for a range of cancer cell lines [75,117,118]. In accordance with the preceding results, a possible sensitizing effect of the HDIs towards αCD95- and TNFα-triggered apoptosis was investigated in healthy primary murine (*in vitro, in situ*) and human hepatocytes (*in vitro*). In contrast to HepG2 cells, primary murine hepatocytes turned out not to be resistant and to react- even though slightly- towards αCD95 exposure. As with transformed malignant cells, the presence of HDIs strongly increased αCD95-induced cytotoxicity in primary murine as well as human hepatocytes. In further analogy to the findings in HepG2 cells, the HDIs were not able to sensitize the hepatocytes towards TNFα-triggered apoptosis (data not shown). Interestingly, concentrations of CBHA, M344 and apicidin needed for sensitizing primary hepatocytes were 10 - 100 -fold higher as compared to HepG2 cells. However, this was not the case for VPA.

The expression level of cFLIP, XIAP and FADD was investigated in the presence of apicidin, CBHA, M344 and VPA in primary murine hepatocytes, too. Amazingly, no alterations were detected in response of these HDIs (data not shown), which rules out quite a number of possible explanations for the sensitization towards CD95-induced apoptosis. Still the possibility remains open that these drugs upregulate and activate the transcription factor p53 which in turn renders the cells sensitive to CD95-mediated apoptosis [103,119].

The liver composed of hepatocytes as well as of non-parenchymal cells (e.g. endothelial cells, Kupffer cells) is the metabolic center of the body. The fact that in isolated perfused mouse

livers, displayed increased αCD95-induced hepatotoxicity in the presence of 5 μM apicidin demonstrates that the key results of this study apply also to the whole intact organ. This is insofar of prime interest, as Phase I and Phase II clinical trails with VPA (Phase I), SAHA (Phase II), Ms-275 (Phase II) and LAQ824 (Phase I) are ongoing. It has to be noted, that the hepatocytes were isolated from patients undergoing hepatic resection for the therapy of hepatic tumors. Although, crude contaminations with tumorgenic cells were ruled out by pathologists it can not be entirely excluded, that some less neoplastic cells also existed in this experimental setup. However, the results clearly show the potential risk of HDIs which partially are under clinical development towards healthy human hepatocytes.

5.6 Pharmacokinetic aspects of HDIs

The relative rank order of potency for HDIs towards the sensitization of CD95 induced hepatotoxicity was identical in HepG2 and primary cells:

$$\text{Apicidin} > \text{M344} \geq \text{CBHA} >> \text{VPA}$$

In contrast to VPA, the concentrations of the other HDIs, which were necessary to sensitize towards CD95-induced apoptosis, are significant higher in primary murine hepatocytes (apicidin: 10x; M344: 100x; CBHA: 20x). The experimental setup in the context of this study allowed to detected only acute effects of HDIs towards death receptor-induced apoptosis, rather than long-term effects. Although generally undesirable, the inhibition of intracellular HDAC activity commonly requires continuous systemic circulation and drug exposure to achieve maximal clinical response. This could causes a transient accumulation of the HDIs in the liver followed by hepatoxic side effects. Rapid clearance, a high degree of protein binding, rapid metabolism, or rapid inactivation of reactive functional groups are factors that can adversely affect HDI bioviability and antitumor activity. Additionally, the requirement of constant systemic exposure to gain an active antitumor drug concentration will most likely limit the clinical development of any HDIs [120]. In conclusion, this study emphasizes that the potential clinical applications of HDIs may be limited by pathophysiological conditions involving CD95L (e.g. alcohol-induced hepatitis, Wilson's disease, Hepatitis B) where the presence of HDIs can potentiate CD95L-induced hepatoxicity.

5.7 Pharmacodynamic evaluation of the HDIs potential

It was established, that the HDIs, used in this study are able to sensitize HepG2 cells as well as primary murine and human hepatocytes towards a CD95 death receptor-induced apoptosis. In accordance with the literature, TRAIL selectively caused apoptosis of HepG2 but not of primary murine cells [47]. It is discussed, that the greatest potential of HDIs lies in their ability

to modulate the activity of other therapeutic agents [121]. Thus, pretreatment with the HDIs sodium butyrate, SAHA and LAQ824 has been shown to enhance TRAIL-triggered apoptosis in the context of preclinical studies [122,123]. Because of its toxicity to normal human hepatocytes, further investigations are necessary to evaluate a possible therapeutic cotreatment [48]. On the other hand, TRAIL appears to be also involved in fatty liver disease [124]. Thus, it is possible that HDIs also enhance TRAIL-mediated effects in the liver. In the case of TNFα, no hepatotoxicity was observed in all experimental setups. A HDIs-mediated sensitization towards the pleiotropic cytokine TNFα performing physiological and pathophysiological functions could associate with fatal systemic side effects. Thus, the systemic application of TNFα showed a very low maximal tolerated dose in human [125].

VPA needs an additional comment in this discussion because it is a FDA (Food and Drug Administration)-approved drug for treatment of epilepsy for many years. Recent discovery of its HDI property has evoked much research interest in the epigenetics field. VPA was shown to reduce lung tumor growth of breast cancer cells [126] and induce apoptosis in human melanoma cell lines [127]. Although VPA is not potent, it is well tolerated in humans at near-millimolar serum levels [126,128]. However, some severe side effects of VPA are well known. Its pharmacological resurrection as HDI is probably due to recognition of VPA-mediated neural tube defects in human, mouse and other vertebrate embryos [129-131]. Among further adverse effects, VPA also affect liver function, leading to an elevation of the liver transaminases and to a reduction of liver biosynthesis including coagulation factors [132]. A recent survey study of valproate use in the United States in nearly 400.000 patients between 1978 and 1984 has shown that children under 2 years of age who received the drug as part of multiple anticonvulsant therapy were at risk (20 fold increase) of developing fatal hepatotoxicity. The exact pathophysiological mechanisms which elicited these side effects are not established. However, it is well-known, that VPA exerts the anticonvulsant effect in micromolar concentrations, but inhibits HDACs in millimolar concentrations. In contrast to the other HDIs investigated, no potency differences were detected for the various cell types. This means that VPA has indeed the pharmacodynamic property of enhancing CD95L-mediated hepatotoxicity, be it in healthy, diseased or malignant liver tissue.

5.8 Conclusions

The lines of evidence presented in this study originate from findings obtained in malignantly transformed liver cells, primary mouse hepatocytes, whole mouse liver, and finally, from translating representative experiments to human primary liver cell cultures. This experimental

approach allowed a consistent insight into the differential sensitization by HDIs of the death receptor-dependent pathway in the liver (see also Figure 5.1). A generally valid overall mechanism could not be derived. Instead, the following plausible mechanisms of this HDI-dependent sensitization of hepatic cell death can be proposed:

- rapid downregulation of the DISC inhibitor cFLIP
- downregulation of the cytosolic caspase inhibitor XIAP
- the notion that the HDIs used here preferentially modulate the extrinsic pathway.

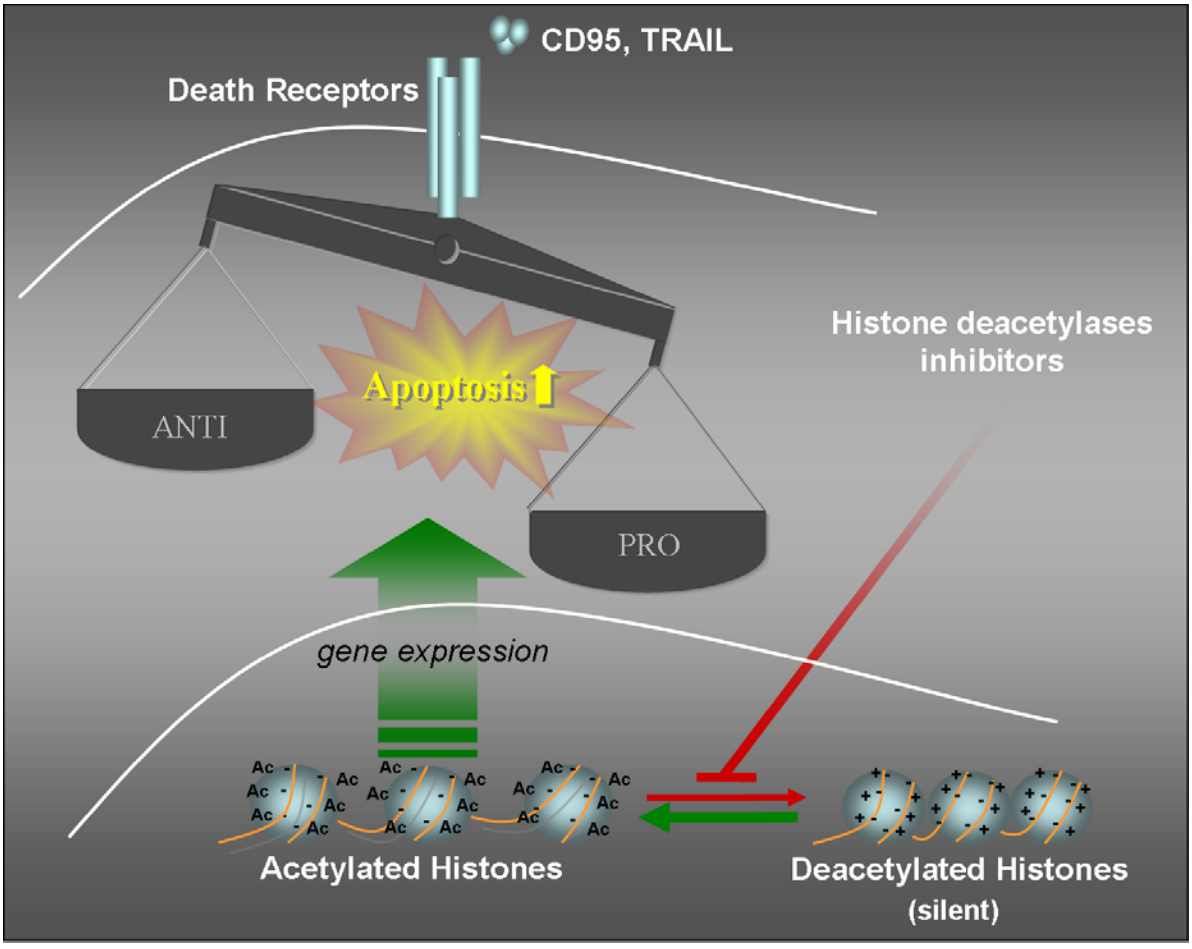

Figure 5.1: Proposed mechanism of HDIs-mediated sensitization.

6 Summary

The apoptosis receptors agonists TNFα, CD95 Ligand and TRAIL are not only important regulators of programmed cell death, but also of tissue homeostasis. Numerous pro- and antiapoptotic proteins control the balance between tissue proliferation and removal.

Histone deacetylase inhibitors (HDIs) are a chemically heterogeneous group of compounds which lead globally to hyperacetylation of histones. The resulting alterations of gene expression patterns inhibit cell proliferation and tissue growth. This property makes HDIs a new class of chemotherapeutics with potential against neoplasia.

The influence of the HDIs apicidin, CBHA, M344 and VPA on the extent of death receptor-dependent apoptosis was investigated with the following results:

- In the human liver cancer cell line HepG2 and in primary mouse liver cell cultures, treatment with these various HDIs selectively caused a sensitization towards CD95L- and TRAIL-triggered apoptosis, but not towards the one initiated by TNFα.
- After exposure of HepG2 cells to the various HDIs, a strong downregulation of the distal receptor protein cFLIP, which inhibits the assembly of the death-inducing signaling complex of apoptosis (DISC), was found. In addition, the expression of the anti-apoptotic protein XIAP was decreased, while pro-apoptotic adapter protein FADD was increased. From these findings, a plausible mechanism for the pharmacodynamic, i.e. apoptosis-sensitizing action of HDIs is proposed.
- HDIs preferentially modulated the extrinsic death receptor signaling pathway in HepG2 cells.
- The relative rank order of the sensitizing potency of the various HDIs examined was similar in all cell types: Apicidin > M344 ≥ CBHA >> VPA
- As a representative example, VPA was examined in primary human hepatocytes. In fact, a similar sensitization towards CD95 was found as in HepG2 cells.

The design of this study comprised malignantly transformed liver cells, primary mouse hepatocytes, whole mouse liver, and human primary liver cell cultures. Its results extend the biochemical and pharmacological basis for the development of this class of chemotherapeutics. It provides new evidence for the limitations in clinical use under pathophysiological conditions where the CD95 or TRAIL system is preactivated.

7 Zusammenfassung

Die Zytokine TNFα, CD95 Ligand und TRAIL spielen in der Regulation des programmierten Zelltodes und der Homöostase von Geweben eine fundamentale Rolle. Zahlreiche pro- und antiapoptotische Proteine kontrollieren ein Fließgleichgewicht zwischen Auf- und Abbau von Geweben.

Histondeacetylase-Iinhibitoren (HDIs) sind eine Gruppe chemisch heterogener Substanzen, deren Anwendung zu einer umfassenden Hyperacetylierung von Histonproteinen führt. Die daraus resultierende Veränderung des Expressionsmusters führt zu einer zellulären Proliferationshemmung und Wachstumsinhibition von Geweben. Diese Eigenschaften verleihen den HDIs ein chemotherapeutisches Potenzial zur Behandlung von Neoplasien.

Im Rahmen dieser Studie wurde der Einfluss der HDIs CBHA, Apicidin, M344 und Valproinsäure auf die Todesrezeptor-induzierte Apoptose untersucht. Folgende Ergebnisse wurden erzielt:

- Die HDIs sensitivierten sowohl die humane Tumorzelllinie HepG2 als auch isolierte primäre, humane und murine Leberzellen gegenüber einer CD95L und TRAIL, jedoch nicht gegenüber einer TNFα induzierten Apoptose.
- Die Expression des Proteins cFLIP, das eine vollständige Assemblierung des DISCs (death-inducing signalling complex of apoptosis) hemmt, war in den sensitivierten Zellen deutlich herunterreguliert. Außerdem war die Expression des antiapoptotischen Proteins XIAP vermindert, während das proapoptotische Adapterprotein FADD verstärkt exprimiert wurde. Daraus wird ein plausibler Mechanismus vorgeschlagen.
- Die HDIs modulierten in erster Linie die extrinsische Signalkaskade des Todesrezeptors.
- Das Sensitivierungspotenzials der verschiedenen HDIs folgt in den untersuchten Zelltypen der Reihenfolge: Apicidin > M344 ≥ CBHA >> VPA
- Valproinsäure sensitivierte humane Leberzellen im gleichen Konzentrationsbereich wie HepG2 Zellen gegenüber CD95.

Die sequenziell angelegte Studienplanung baut auf Experimenten auf mit transformierten Leberzellen, primären Mausleberzellen, der intakten Mausleber und primären humanen Hepatozyten. Die Ergebnisse erweitern die biochemische und pharmakologische Basis zur Entwicklung dieser Chemotherapeutika. Es werden Limitationen der klinischen Anwendung diskutiert, die auftreten können unter pathophysiologischen Bedingungen, bei denen die Mediatoren CD95L und TRAIL eine Rolle spielen.

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8 References

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