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Investigation of the apoptosis signal transduction mediated by the marine pyridoacridine alkaloid Ascididemin in human leukemic Jurkat T cells

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Erklärung

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dedicated to my family

A. Contents

B. Abbreviations

ActD	Actinomycine D
AIF	Apoptosis-inducing factor
ANT	Adenin-nucleotide translocator
AP-1	Activator-protein 1
Apaf-1	Apoptotic protease activating factor 1
APS	Ammonium persulfate
ASC	Ascididemin
ATP /dATP	Adenosine-5'-triphosphate / 2'-Desoxyadenosine-5'-triphosphate
BH	Bcl-2 homology domain
BSA	Bovine serum albumine
C.	Caenorhabditis
CAD	Caspase-activated DNase
CARD	Caspase activation and recruitment domain
CD	Cluster of differentiation, nomenclature of antigenes
CD95	Designation of cell surface antigen/cell death receptor (APO-1/Fas)
CD95L	Ligand of the CD95 receptor
CHX	Cycloheximide
c-H_2DCFDA	Carboxy 2'7'-dichlorodihydrofluorescein diacetate
DD	Death domain
DED	Death effector domain
DIABLO	Direct IAP-binding protein with low pI
DISC	Death inducing signalling complex
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
DR	Death receptor
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
Erk	Extracellular-regulated kinase

FACS	Fluorescence-activated cell sorter
FADD	Fas-associated death domain protein
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FLICE	Fas-like interleukin-1β converting enzyme
FLIP	FLICE inhibiting protein; c - cellular, v - viral
Fmk	Fluormethylketone
FSC	Forward scatter
G	Relative centrifugal force (RCF or *g* force)
HBSS	Hank´s bufferd saline solution
Hepes	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HRP	Horseradish peroxidase
IAP	Inhibitors of apoptosis
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
JNK	c-Jun N-terminal kinase
kDA	Kilo Dalton
m	Milli
M	Molar
MAPK	Mitogen-activated protein kinases
MMP	Mitochondrial membrane permeabilisation; (OMP - outer, IMP - inner)
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n	Nano
NAC	N-Acetylcysteine
NADH	Nicotinamide adenine dinucleotid
NF-κB	Nuclear factor κB
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCD	Programmed cell death
PHA	Phythaemagglutinine
PI	Propidium iodide
PI3K	Phosphatidylinositide 3'-OH kinase

PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethanesulfonyl fluoride
PTPC	Permeability transition pore complex
PVDF	Polyvinylidene fluoride
rpm	Rounds per minute
ROS	Reactive oxygen species
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
Smac	Second mitochondria-derived activator of caspases
SSC	Side scatter
TBS-T	Phosphate buffered saline solution with Tween
TEMED	Tetramethylethylenediamine
TNF	Tumour necrosis factor
Tris	Tris-hydroxymethyl-aminomethan
VDAC	Voltage-dependent anion channel
$\Delta\psi_m$	Mitochondrial transmembrane potential
λ	Wavelenght
μ	Micro

C. Introduction

1 Background

The modern pharmacotherapy has its seeds in the application of natural drugs. Extracts of plants or animal products are used for medicinal treatment since thousands of years. The knowledge of natural drugs and their potential use in medicine continuously increased throughout the centuries. Advanced preparative and analytical methods in phytochemistry led to the isolation and structural characterisation of a huge number of natural compounds, usually defined as secondary metabolites. The screening with innovative biological assays allows the verification of their active principle. These improvements provided the basis for rational drug discovery and have fundamentally contributed to the development of important pharmaceuticals of natural origin.

Natural products are of great importance in the drug discovery process, particularly in the areas of infectious diseases and cancer. Recent analysis showed that for the period 1989 to 1995 over 60% of the approved drugs developed in these disease areas are of natural origin (Cragg *et al.*, 1997). Table 1 shows some plant- and microbal-derived anticancer drugs already in clinical use.

drug class	example	source organism	indication
plant-derived			
vinca alkaloids	vinblastine, vincristine	Catharanthus roseus	acut leukemia, lymphoma
lignans	etoposide*, teniposide*	Podophyllum species	acut leukemia, lymphoma
taxanes	paclitaxel, docetaxel*	Taxus species	breast cancer
camptothecines	topotecan *, irinotecan *	Camptotheca acuminata	ovarian cancer
microbal-derived			
anthracyclines	doxorubicin, daunomycin, idarubicin*	Streptomyces species	lymphoma, ovarian cancer
mitosanes	mitomycin	Streptomyces species	bladder cancer
glycopeptides	bleomycines A_2 and B_2	Streptomyces species	testicle tumour, lymphoma

Table 1: Representative plant-derived and microbal-derived anticancer drugs in clinical use.
** derived from a natural product source (e.g.semisynthetic)*
(modified adapted from Schwartsmann et al., 2002).

New strategies in drug discovery abandoned the exhausting, time- and capacity-consuming screening of natural products. Synthetic compound libraries and combinatorial chemistry libraries were employed for high throughput screening. These techniques allow the easy and convenient generation and testing of large numbers of pure products. However, the limited structural complexity and diversity of the mentioned libraries constricted the identification of interesting lead structures. Natural products, instead, exhibit a great variety of unusual chemical structures which underlines an important role of secondary metabolites as important source for new leads. Therefore, the synergistic use of natural product chemistry and combinatorial chemistry constitutes a promising approach in drug discovery and development yielding new effective therapeutics (Grabley and Thiericke, 1999).

Marine organisms, especially sponges, molluscs, bryozoans and ascidians represent a prolific source of structurally novel bioactive agents. During the past 30 years, a large number of new compounds with structures completely different from those isolated from terrestrial organisms, were successfully discovered from marine sources. The extreme conditions of the marine environment – light and pressure (deep sea), temperature and nutrients (deep-see hydrothermal vents), high salt content of the water – are decisive factors for the development of secondary metabolites of unprecedented structural diversity (Bracher F, 2002). Furthermore, due to the competition for light, space, and essential macro- and micronutrient within benthic communities, sessile marine organisms have adopted strategies that involve the use of chemical products, effective toxins against potential sessile competitors (Pawlik, 1993; Becerro *et al.*, 1997). Likewise, sessile marine organisms constitute potential substrates for fouling organism settlement, including bacterial colonisation. Thus, marine secondary metabolites additionally function as defence mechanism against fouling organisms (Wahl *et al.*, 1994). Considering these facts, it is not very surprising that isolated marine compounds predominantly exhibit antimicrobal and cytotoxic properties.

Cytarabine, which is used for the treatment of leukemia and lymphoma, was the first marine-derived anticancer agent to be developed for therapeutic application (Schwartsmann *et al.*, 2001). The first marine secondary metabolite which entered human clinical trials was the cyclopeptide didemnin B, isolated from the ascidian *Tridemnum solidum* (Vera and Joullie, 2002). Further marine products which undergo clinical trials at present are listed in table 2.

drug class	example	source organism (type)	development stage
cyclopeptide	Didemnin B	Tridemnum solidum	phase II
macrocyclic lactone	Bryostatin 1	Bugula neritina (bryozoan)	phase II
peptide	Dolastatin 10 (Cematodin)	Dolabella auricularia (mollusk)	phase II
polycyclic alkaloid	Ecteinascidin 743	Ecteinascidia turbitana (tunicate)	phase II, III
depsipeptide	Kahalahide F	Elysia rubefescens (mollusk)	phase I, II
cyclodepsipeptide	Aplidine	Aplidium albicans (tunicate)	phase I

Table 2: Current marine organism-derived anticancer drugs in development. (modified adapted from Schwartsmann et al., 2002).

These examples show the obvious importance of isolated marine compounds as potential anticancer drugs. However, the insufficient supply of material for isolation, structure elucidation and further biological and clinical testing represents one of the major problem in drug development of marine compounds. Furthermore, the ecological consequences resulting from excessive collection of the marine organisms must be considered. Therefore, besides chemical synthesis, efforts are being made for the *in vitro* cultivation of marine microorganisms which are discussed to be involved in the biosynthesis of marine secondary metabolites (Grabley and Thiericke, 1999; Bracher F, 2002).

2 Ascididemin

The class of pyridoacridine alkaloids and their biological activities

Polycyclic aromatic alkaloids, possessing a planar pyrido[4,3,2-mn]acridin skeleton, constitute a new, emerging class of marine compounds. In 1983, Schmitz *et al*. isolated amphidemin, the first example of a pyridoacridine alkaloid, from a Pacific sponge (*Amphimedon* species) (Schmitz *et al.*, 1983). Due to their significant biological activities there is currently great interest in marine pyridoacridine alkaloids. Most pyridoacridines exhibit *in vitro* cytotoxicity against a variety of tumour cell lines or antineoplastic activity in whole animal experiments (Molinski, 1993; Ding *et al.*, 1999; Burres *et al.*, 1989). The planar ring structure of the alkaloids promotes their intercalation into DNA (Burres *et al.*, 1989). As found for several DNA intercalators, like doxorubicin and etoposide, pyridoacridine alkaloids target topoisomerase II, an

enzyme of the replication machinery (McDonald *et al.*, 1994). The marine alkaloid dercitin serves as a lead compound for the development of new potential anti-HIV drugs (Taraporewala *et al.*, 1992). Some pyridoacridine alkaloids were found to strongly stimulate the release of Ca^{2+} from the sarcoplasmic reticulum (Kobayashi *et al.*, 1988b). A recent study reported insecticidal activity as well as specific binding to adenosine receptors and benzodiazepine binding sites of $GABA_A$ (γ-aminobutyrate) receptors of members of this structural class (Eder *et al.*, 1998).

Ascidians – the source of ascididemin

The pentacyclic aromatic alkaloid ascididemin (figure 1) was first isolated from the Okinawan tunicate *Didemnum* species in 1988 by Kobayashi *et al.* (Kobayashi *et al.*, 1988a). Later on, Bonnard *et al*. (Bonnard *et al.*, 1995) discovered ascididemin in an extract of the ascidian *Cystodytes dellechiajei*.

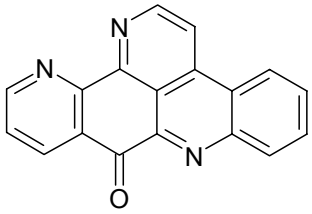


Figure 1: Chemical structure of ascididemin.

Tunicates are marine, filter-feeding animals classified in the phylum Chordata, which also includes the vertebrates, subphylum Urochordata. The most familiar tunicates are the ascidians (sea squirts; class: Ascidiacea).
The adult form of most tunicates shows no resemblance to vertebrate animals. The free-swimming tunicate larva (tadpole), instead, exhibits all the characteristic chordate features also found in the embryos of vertebrates: a notochord, a dorsal, hollow nerve cord, pharyngal gill slits and a muscular post-anal tail (figure 2). During metamorphosis the tail and the ability to move is lost and the nervous system largely disintegrates. The adult sea squirts are sedentary and cylindrical or globular animals. The soft body is surrounded by a thick tunic which is either transparent or brightly coloured due to pigmentary storage. Tunicates feed by drawing water through the incurrent siphon (buccal) at the top of the body. Food particles are filtered from the water by the pharynx and are passed into the digestive system. The water leaves through the excurrent siphon (atrial) at the side (figure 2).
Most solitary ascidians are hermaphrodites and reproduce by external fertilisation,

releasing eggs and sperm into the water and finally developing the larva. In addition, some species reproduce by budding, resulting in the formation of colonies of sea squirts. Within the colony the single zooids are either joined at their bases by slender stalks or embedded in one common tunic (figure 2).

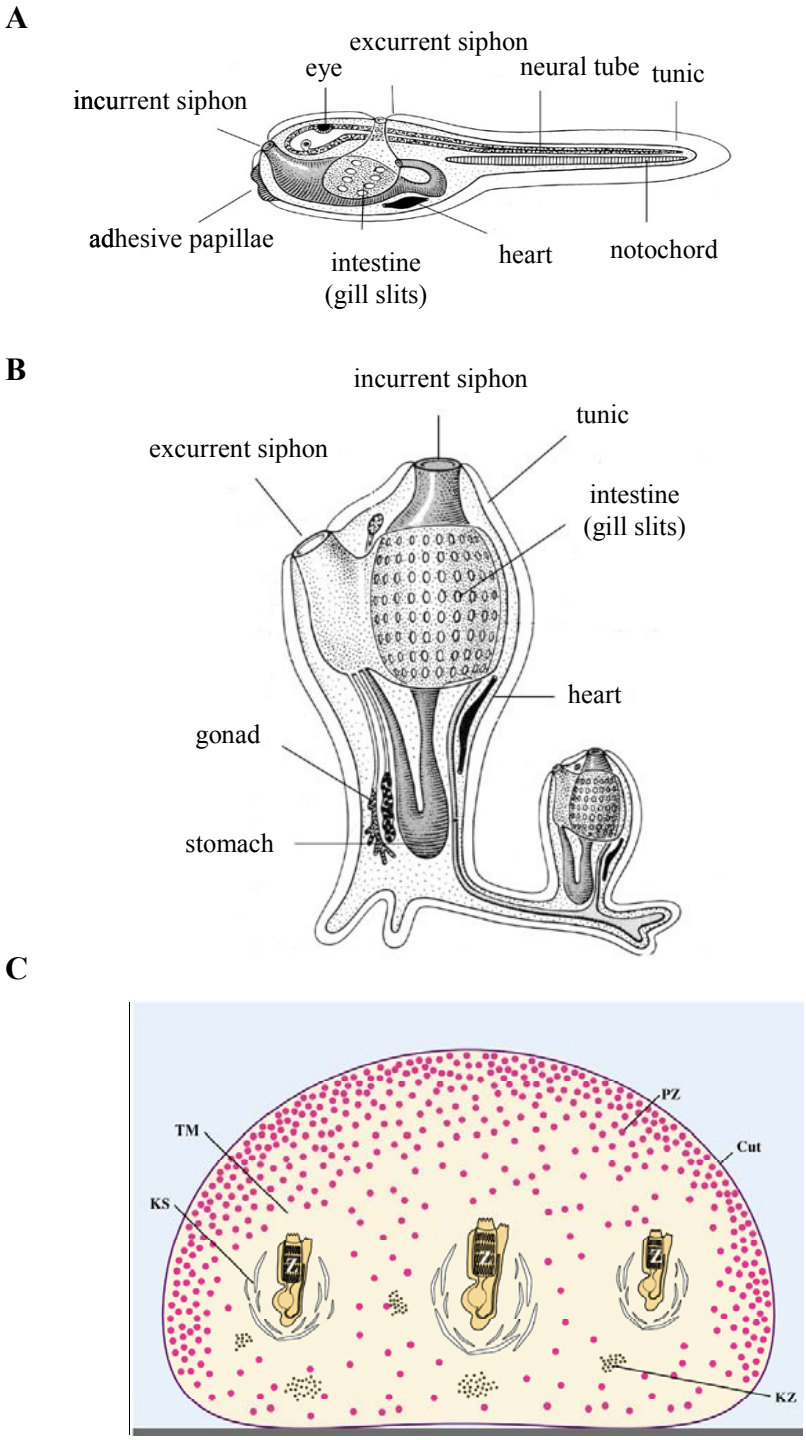


Figure 2: Schematic illustration of the free-swimming tunicat larva (A), an adult sea squirt (B) and a colony of ascidians, embedded in one common matrix (C). (Cut - cuticula, Z - zooid, PZ - pigmentary cells, TM - tunic matrix). (The pictures were kindly provided by Dr. E. Natzer and Prof. Dr. G. Wanner).

The species *Cystodytes dellechiajei* is a colony forming ascidian of either deep purple or green color (figure 3), producing pyridoacridine alkaloids like shermilamin B, kuanoniamine D and ascididemin. Steffan *et al.* have shown that amino acids are precursors of shermilamine B (Steffan *et al.*, 1993), the location of biosynthesis, however, yet remains unknown.

A

B

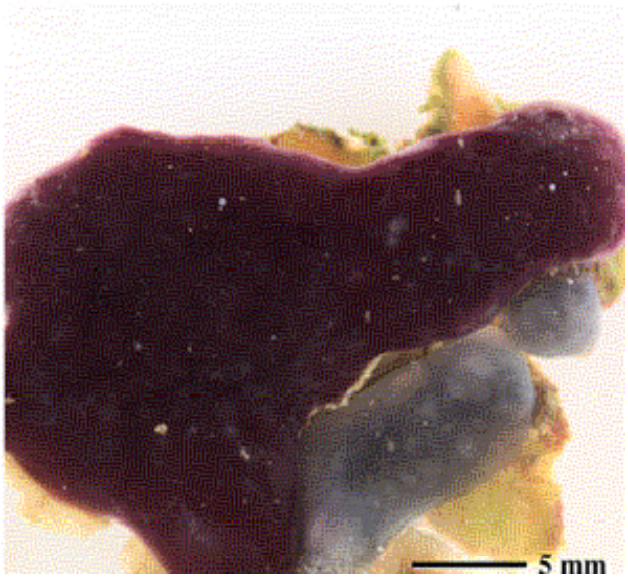


Figure 3: Habit of the colonies of Cystodytes dellechiajei.
A, Purple species of Cystodytes dellechiajei in its natural environment (The picture was kindly provided by Dr. B.Steffan);
B, Illustration of purple and green species of Cystodytes dellechiajei (The picture was kindly provided by Dr. E. Natzer and Prof. Dr. G. Wanner)

Dr. Eva-Maria (Rottmayer) Natzer, coworker of the group of Prof. Dr. G. Wanner (Botanical Institute, University of Munich; SFB 369, B4), recently discovered pigment cells in the tunic of purple colonies of *Cystodytes dellechiajei* containing red pigmentary grains (figure 4) which are supposed to consist of pyridoacridine alkaloids (Rottmayer, 2001). Pigment cells are distributed throughout the whole tunic matrix with accumulation in the external layer of the matrix. This observation corroborates the hypothesis that cytotoxic secondary metabolites function as defence mechanism against environmental insult.

Figure 4: Pigmenatry cells of the purple colonies of Cystodytes dellechiajei.
A, The pigmentary grains are found within vacuoles, visualised by light microscopy/differential interference contrast; B, A cluster of pigmentary grains, visualised by scanning electron microscopy (The pictures were kindly provided by Dr. E. Natzer and Prof. Dr. G. Wanner).

Biological properties of ascididemin

Reports exist of the potent Ca^{2+}-release activity of ascididemin (Kobayashi *et al.*, 1988a) as well as antibacterial and antifungal properties (Lindsay *et al.*, 1995). In particular, the cytotoxic potential against a variety of human solid tumour cell lines and human leukemia cell lines *in vitro* is well investigated (preclinical *in vitro* screening at the U.S. National Cancer Institute (NCI); Lindsay *et al.*, 1995; Bonnard *et al.*, 1995; Dassonneville *et al.*, 2000; Delfourne *et al.*, 2002). Most importantly, ascididemin was found to be equally toxic to drug-sensitive and multi-drug-resistant cell lines (Bonnard *et al.*, 1995; Dassonneville *et al.*, 2000). Spectroscopic measurements revealed that ascididemin intercalates into DNA, with preference to guanosine/cytidine (GC)-rich sequences (Bonnard *et al.*, 1995). Several *in vitro* studies with purified enzyme proved the inhibitory effect on topoisomerase II (Schmitz *et al.*, 1991; Dassonneville *et al.*, 2000). In contrast, whole cell experiments indicated that topoisomerase II might not be considered as a critical cellular target for ascididemin (Dassonneville *et al.*, 2000). Recently, it was reported that ascididemin is capable of damaging DNA via the production of reactive oxygen species (Matsumoto *et al.*, 2000). Additionally, ascididemin was recognised as a potent inducer of apoptosis in leukemia cell lines (Dassonneville *et al.*, 2000).

3 Aim of the work

Although the cytotoxic properties of pyridoalkaloids are well known, the molecular mechanisms that govern the cell death are yet poorly claryfied. Therefore, the aim of the present work was to investigate ascididemin-mediated signalling pathways leading to the apoptotic cell death in human leukemia Jurkat T cells focussing on

- the CD95 receptor system
- the activation of caspases
- the role of the mitochondria
- the involvement of mitogen-activated protein kinases (MAPK).

4 Mechanisms of cell death

Apoptosis and necrosis

There are two forms of cell death eukaryotic cells can undergo, apoptosis or necrosis. The term *apoptosis* (a combination of the greek words *apo* – off and *ptosis* – falling; in ancient greek used as description for the "falling off" or shedding of leaves and flowers from trees) first appeared in the biomedical literature in 1972, coined by Kerr *et al.* (Kerr *et al.*, 1972) to describe a structurally-distincitve form of cell death. The apoptotic process was recognised as a strongly regulated mechansim, expressed in the synonym of "programmed cell death" (PCD). Furthermore, studies on the nematode *Caenorhabditis elegans* provided evidence for the genetic basis of apoptosis which is highly conserved during evolution. (Note: Concerning these discoveries, S. Brenner, H.R. Horvitz and J.E. Sulston won the Nobel Prize in Physiology or Medicine in 2002.)

Apoptosis, often limited to a single cell or a small group of cells, is characterised by chromatin condensation and internucleosomal DNA degradation. Furthermore, cell shrinkage is accompanied by an extensive blebbing of the plasma membrane which results in the separation of the cell into a number of membrane-bound vesicels (apoptotic bodies). Structural alterations of the cell surface (e.g. translocation of phosphatidylserine to the outer cell membrane) ensure that apoptotic cells are recognised and phagocytised by either macrophages or adjacent epithelial cells (Duvall *et al.*, 1985). *In vitro*, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse. This terminal phase of *in vitro* cell death has been termed "secondary necrosis".

Apoptosis largely occurs under physiological conditions. Necrosis, in contrast, is a mode of cell death that happens in response to a variety of harmful conditions and toxic substances, such as hyperthermia, hypoxia, ischemia and metabolic poisons as well as direct cellular trauma, typically affecting groups of contiguous cells. The necrotic process is characterised by swelling of the cytoplasm and the organelles, especially the mitochondria, due to an influx of water and extracellular ions. Ultimately, the plasma membrane ruptures and cytoplasmatic contents including lysosomal enzymes are released into the extracellular space. Thus, necrotic cell death is often associated with extensive damage of surrounding cells and an intense inflammatory response in the corresponding tissue.

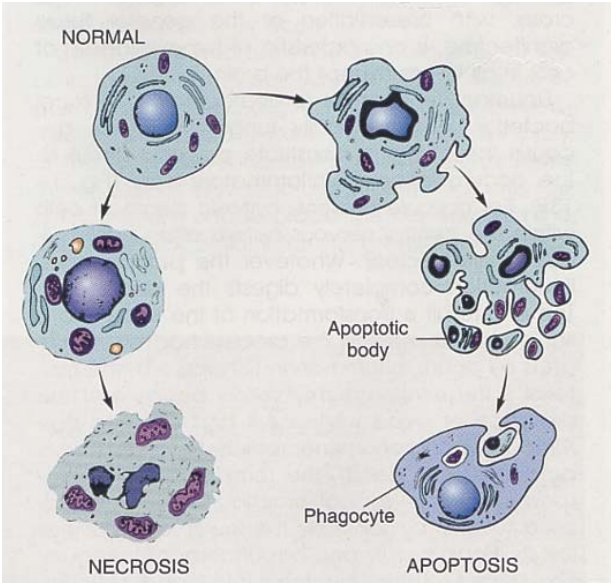


Figure 5: Schematic illustration of apoptotic and necrotic cell death. (adapted from Walker et al., 1988)

Physiological and pathophysiological relevance of apoptosis

Apoptosis has been recognised to be of major importance for development and tissue homeostasis. It is involved in the removal of surplus cells generated during mammalian development. During the ontogeny of many organs, for instance, an excess of cells is produced. At later stages of development cells are selectively removed by apoptosis to adjust the relative number of cells of different cell types to achieve proper organ function (Meier *et al.*, 2000).

Apoptosis in the immun system is a fundamental process in the regulation of lymphocyte maturation, in the assurance of self tolerance as well as in the downregulation of an immune response. Insufficient apoptosis leads to severe diseases like cancer and autoimmunity. Excessive apoptotic cell death, instead, is involved in the pathogenesis of AIDS, characterised by a depletion of $CD4^+$ T helper cells (Krammer, 2000).

Neuronal apoptosis is necessary for the development of the nervous system. However, massive apoptosis is implicated in neurodegenerative disorders like Alzheimer´s disease, Parkinson´s disease and Huntington´s disease. The formation of toxic abnormal protein structures and aggregates are supposed to contribute to the apoptotic cell death in specific neuronal subpopulations (Yuan and Yankner, 2000).

5 The signal transduction of apoptosis

Two convergent apoptotic signalling cascades have been described, referred to as extrinsic and intrinsic pathway. The extrinsic pathway is initiated at the cell surface by ligand-dependent triggering of receptors, called death receptors (DR). The intrinsic pathway emanates from alterations at the mitochondria level mediated by a variety of apoptotic stimuli including cellular stress, UV irradiation and chemotherapeutic agents.

5.1 Receptor-mediated apoptosis

DR are members of the tumour necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily and comprise a subfamily that is characterised by an intracellular domain, termed death domain (DD). Eight DRs have been described: TNF-R1, CD95 (Fas, APO-1), TRAMP (TNF-R-related apoptosis-mediating protein; DR3), TRAIL-R1 (TNF-related apoptosis-inducing ligand receptor 1; DR4), TRAIL-R2 (DR5), DR6, nerve growth factor (NGF-R) and ectodermal dysplasia receptor (EDA-R) (Schulze-Osthoff, 511). Among these, TNF-R1 and CD95 are the best characterised (Ashkenazi and Dixit, 1998).

CD95 receptor and CD95 ligand

The CD95 receptor (CD95) is a 45-kDa type I transmembrane protein which carries three cystein-rich repeats in its extracellular domain as recognition site for the corresponding receptor ligand (Itoh *et al.*, 1991; Orlinick *et al.*, 1997). CD95 is ubiquitously expressed on a variety of normal cells, including activated T- and B-cells and hepatocytes (Suda *et al.*, 1995). High levels of CD95 expression have also been detected on solid tumors of the breast, ovary, colon, liver and leukemia T cells (Nagata and Golstein, 1995).

The CD95 ligand (CD95L), a type II transmembrane protein of 40 kDa, is a member of the TNF-related cytokines (Suda *et al.*, 1993). CD95L expression is limited and has been detected on activated T-cells (Suda *et al.*, 1995), the corneal epithelium and the retina of the eye (Griffith *et al.*, 1995). Some tumours also express CD95L, that has been considered as a defence mechanism against immunological removal (Hahne *et al.*, 1996). Proteolytic cleavage of the membrane-bound CD95L by metalloproteinases generates the less active form of soluble CD95L (Mitsiades *et al.*, 2001).

CD95-mediated death signals

Binding of CD95L or an agonistic antibody triggers the trimerisation of CD95, a process that in turn stimulates the generation of an death inducing signalling complex (DISC) (Dhein *et al.*, 1992; Kischkel *et al.*, 1995). Complex formation is initiated by the intracellular recruitment of the adapter protein FADD (Fas-associated death domain-containing protein) (Chinnaiyan *et al.*, 1996). FADD binds to the receptor *via*

homophilic interactions of the DD, present in both the intracellular part of CD95 and the C-terminus of FADD. FADD, in addition, contains a death effector domain (DED) at its N-terminus that couples to an analogous domain of procaspase-8/FLICE (Fas-like interleukin (IL)-1β converting enzyme) (see 1.4.2) (Muzio *et al.*, 1996). Following its activation within the DISC, caspase-8 initiates the proteolytic cascade by cleavage of downstream substrates.

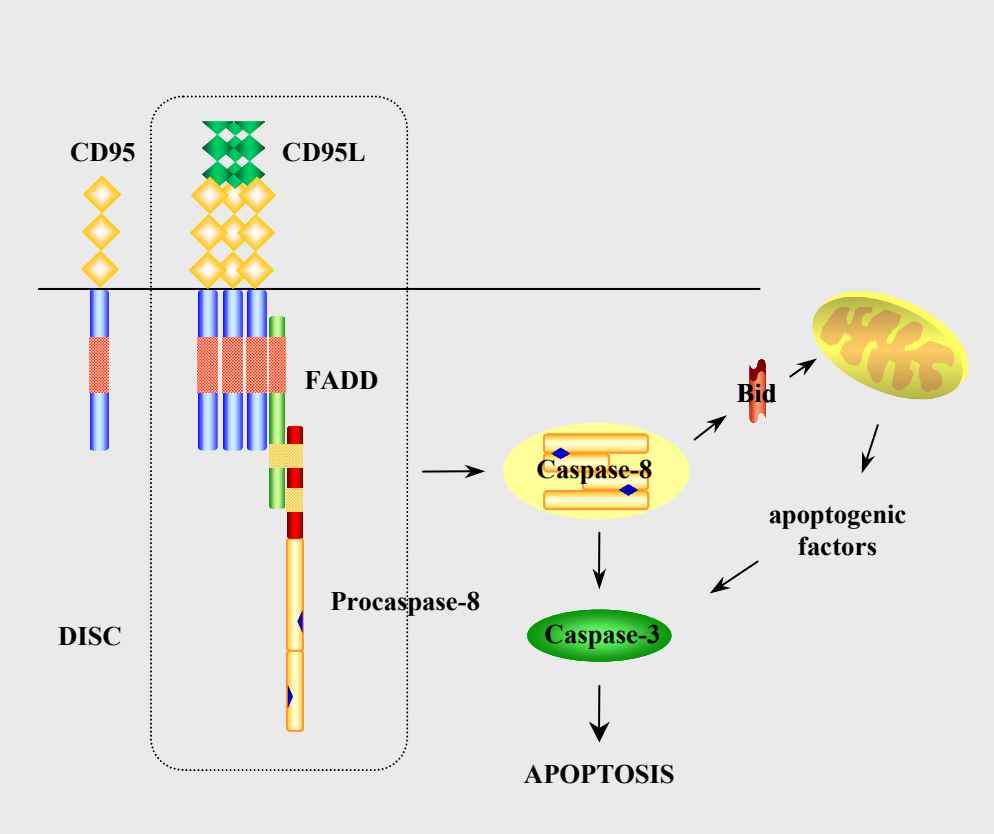


Figure 6: Schematic illustration of CD95-mediated apoptosis.

CD95-mediated apoptotic signals may also be transmitted by the recruitment of molecules other than FADD and caspase-8. RIP (receptor-interacting protein), RAIDD (RIP-associated ICH/Ced-3-homologous protein with DD), and procaspase-2 form part of a further signalling cascade (Duan and Dixit, 2002). Moreover, up-regulation of cytoplasmic DAXX (death domain-associated protein) leads to the induction of the SAPK/JNK (stress-activated protein kinase/c-Jun N-terminal kinase) pathway (see 1.4.5) (Yang *et al.*, 1997b).

Early signalling events of the death receptor can be blocked by cellular FLIP (FLICE/caspase-8 inhibitory protein) (Irmler *et al.*, 1997). Due to its structural homology with caspase-8, c-FLIP interferes with the activation of caspase-8 at the

DISC level. The DED-containing N-terminus of FLIP binds to the FADD adaptor, thus blocking the association of caspase-8 with FADD and preventing caspase activation (Irmler *et al.*, 1997). FLIP, first discovered in herpesviruses (v-FLIP), was supposed to attenuate apoptosis in order to evade the host´s immune response (Bertin *et al.*, 1997).

CD95-sensitive cells are classified concerning the signal transduction following receptor engagement. Apoptosis in typ I cells is initiated by activation of large amounts of caspase-8 at the DISC followed by the cleavage of caspase-3. In type II cells, instead, the mitochondria are involved in the signal transduction. Despite reduced DISC formation, caspase-8 is sufficiently activated to induce the mitochondrial apoptotic pathway *via* the cleavage of Bid (see 1.4.3) (Scaffidi *et al.*, 1998).

5.2 Caspases

Caspases, a family of cystein-dependent aspartate-specific proteases, play a fundamental role in the execution of apoptosis. These enzymes were found to share sequence homology with the cell death protease Ced-3 of the nematode *C. elegans* (Yuan *et al.*, 1993). To date, fourteen caspases have been identified in mammals, which are either involved in apoptosis or in inflammation.

Caspases are expressed constitutively as inactive proenzyms composed of a N-terminal prodomain, a large (~20 kDa) and a small (~10 kDa) subunit. Activation of caspases occurs through proteolytic processing of the zymogen. Cleavage after aspartate-residues of specific tetrapeptide recognition motifs results in the separation of the prodomain, the large and the small subunit. Formation of an heterodimer by the formers and further association to a heterotetramer generates the catalytic active enzyme (Wilson *et al.*, 1994) (figure 7).

Due to the high substrate specifity for aspartate-residues as cleavage sites activated caspases are able to process further procaspases which results in an amplifying caspase cascade. Gramzyme B, an aspartate-directed serin protease expressed in cytotoxic T lymphocytes, is the only known enzyme that also activates caspases (Thornberry *et al.*, 1997).

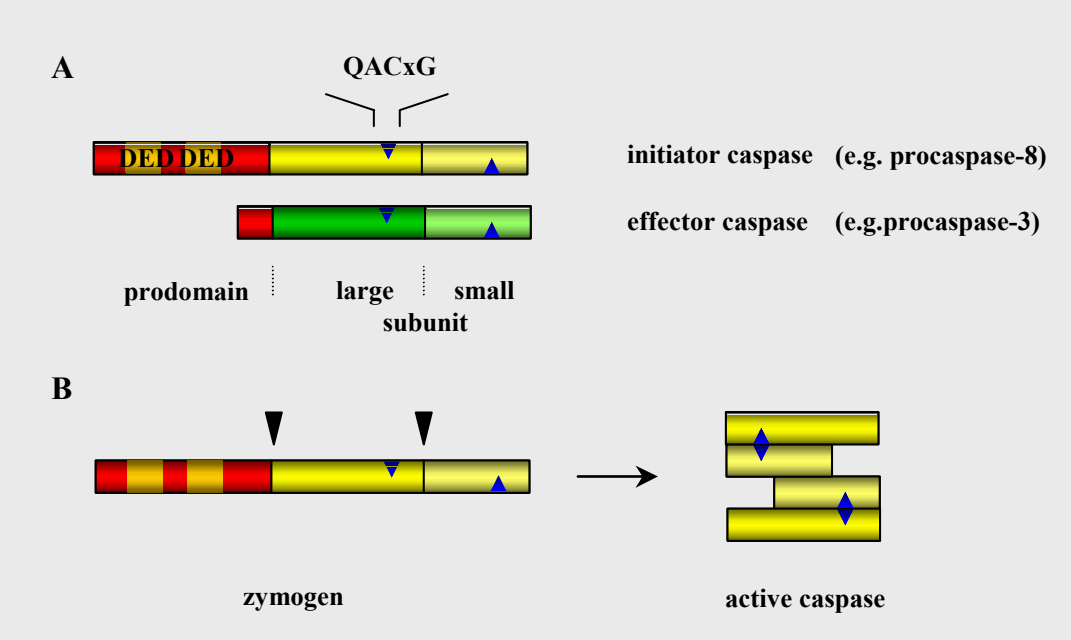


Figure 7: Schematic illustration showing the structures of procaspases and the activated form. A, The zymogen exists of a N-terminal prodomain (red), a large and a small subunit. The long prodomain of initiator caspases contains either a death effector domain (DED; two in the cases of procaspase-8) or a caspase recruitment domain (CARD; e.g. procaspase-9). Components of the catalytic site (blue triangle) are situated in both the large and the small subunit, whereby the absolutely conserved QACxG pentapeptide containing the catlaytic cystein is located in the former. B, Zymogens are cleaved after aspartate-residues of specific tetrapeptide recognition motifs (black arrows) to generate the heterodimeric active caspase.

The initial processing of caspases, however, requires binding to specific adapter molecules, like FADD (see 1.4.1) and Apaf-1 (apoptotic protease activating factor-1; see 1.4.3). The interaction occurs through distinct structural motifs, including DED and CARD (caspase recruitment domain), present in both the caspase prodomain and its corresponding adapter molecule. Within the complex, the caspase precursors are in close proximity allowing autocatalytic activation (Salvesen and Dixit, 1999; Earnshaw *et al.*, 1999). Based on the length of the prodomain, caspases involved in the apoptotic process can be classified as initiator caspases (caspase-2, caspase-8, caspase-9), characterised by long prodomains, and effector caspases (caspase-3, caspase-6, caspase-7) with short prodomains. Effector caspases are activated by the former and are involved in the cleavage of death substrates.

The morphological and biochemical changes that characterise apoptotic cell death result from the cleavage of death substrates. Caspases cleave essential structural components of the cytoskeleton such as actin which appears to be related to cell shrinkage and membrane blebbing (Cotter *et al.*, 1992; Mashima *et al.*, 1999). The externalisation of phosphatidylserine to the outer plasma membrane leaflet, a trigger for stimulating the phagocytosis of apoptotic cells by macrophages, also requires activated caspases

(Martin *et al.*, 1996). Proteolysis of lamins, the major nuclear structural proteins, may induce apoptotic nuclear condensation (Takahashi *et al.*, 1996). Likewise, the proteolytical processing of ICAD (inhibitor of caspase-activated DNase)/DFF (DNA fragmentation factor) results in the activation of CAD (caspase-activated DNase), an endonuclease responsible for the specific DNA fragmentation (Sakahira *et al.*, 1998). The inactivation of PARP (poly(ADP-ribose) polymerase), an energy-consuming DNA repair enzym, is supposed to conserve the cellular NAD^+ and ATP which, instead, could be utilised for the execution of apoptosis (Takahashi and Earnshaw, 1996).

To prevent accidental or spontaneous processing of caspases, the proteolytic system is both regulated at the level of zymogen activation (see 1.4.1) and by direct inhibition of enzymatically active caspases. Several viral gene products function as caspase inhibitors. Of these, the best known are CrmA (cytokine responsive modifier A) from cowpox virus and p35 from baculovirus (Zhou and Salvesen, 2000). Both are shown to block CD95-mediated apoptosis by stably binding to the active site of the proteases (Xue and Horvitz, 1995). IAP (inhibitors of apoptosis) are endogenous regulators of apoptosis, first identified in baculoviruses (Crook *et al.*, 1993; Duckett *et al.*, 1996). A potent inhibition of caspase-3, -7, and –9 was observed *in vitro* whereby distinct IAP domains either directly bind to the small subunit of caspase-9 or occlude the active site of caspase-3 and –7 (Srinivasula *et al.*, 2001; Huang *et al.*, 2001).

5.3 The mitochondrial apoptotic pathway

Mitochondrial dysfunction represents a key event in the apoptotic process. It is characterised by mitochondrial membrane permeabilisation (MMP), which can affect both the inner and outer mitochondrial membrane, loss of the transmembrane potential ($\Delta\psi_m$) and the release of apoptogenic factors from the intermembrane space to the cytosol.

The Bcl-2 family

Members of the Bcl-2 family are intimately involved in the regulation of the MMP. The bcl-2 proto-oncogene was first discovered in human B-cell lymphomas and was identified as a homologue of the *C. elegans* cell survival gene ced-9 (Tsujimoto *et al.*, 1984; Hengartner and Horvitz, 1994).The Bcl-2 family includes pro- as well as anti-apoptotic proteins which differ in the number of the conserved Bcl-2 homolog (BH) domains (figure 8). Most anti-apoptotic proteins, like Bcl-2 and Bcl-x_L, show strong sequence conservation in all four domains, whereas pro-apoptotic proteins, including Bax, Bad and Bid, frequently lack the BH4 domain. The latter are subdivided in the Bax subfamily, members of which contain BH1, BH2 and BH3, and the "BH3-domain-only" proteins, that show sequence homology only within the BH3 domain (Bid, Bad) (Reed, 1998). In viable cells anti-apoptotic proteins are localised in membranes such as the

outer mitochondrial membrane, the endoplasmic reticulum or the nuclear membrane, anchored through an additional carboxy-terminal hydrophobic domain. The pro-apoptotic proteins, instead, are mainly found in the cytosol (Krajewski *et al.*, 1993; Wolter *et al.*, 1997). The formation of Bcl-2/Bax heterodimers and the interaction of Bad with Bcl-2-Bcl-$x_L$within the outer mitochondrial membrane, respectively, suggests a neutralising competition of the proteins (Oltvai *et al.*, 1993; Ottilie *et al.*, 1997). Furthermore, Bad, Bcl-2 and Bcl-x_L can be inactivated by phosphorylation (see 1.4.5) (Datta *et al.*, 1997; Haldar *et al.*, 1995; Kharbanda *et al.*, 2000). In response to an apoptotic signal, Bax, for example, dimerises and translocates to the mitochondria where it becomes an integral pore forming membrane protein (Gross *et al.*, 1998). Additionally, following CD95 treatment, Bid is cleaved at its amino terminus by caspase-8. Truncated Bid (tBid) also translocates to the mitochondria, inserts into the membrane and interacts with Bax to cause mitochondrial permeability transition (Desagher *et al.*, 1999). In contrast, Bcl-2 antagonises the pore-forming activity of Bax, thus preventing the efflux of apoptosis-activating factors (Martinou and Green, 2001) (figure 9).

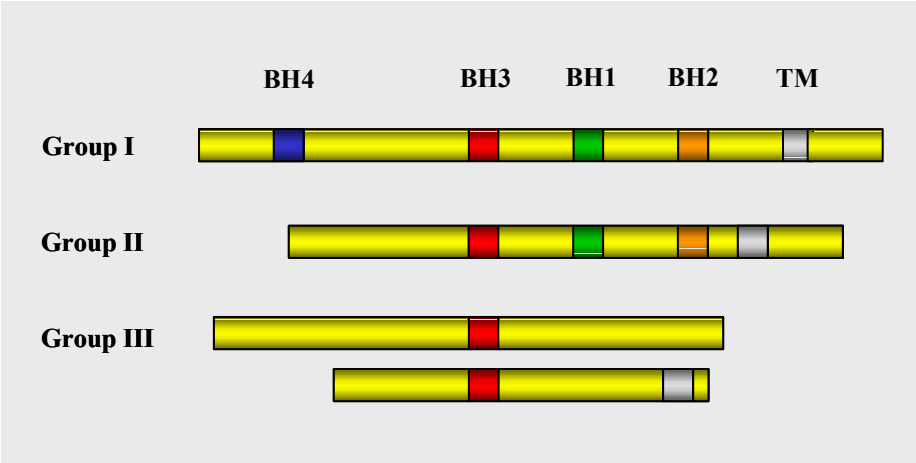


Figure 8: Schematic illustration of the Bcl-2 family.
Group I comprises anti-apoptotic proteins, such as Bcl-2 and Bcl-x_L, characterised by four short conserved Bcl-2 homology domains (BH1-BH4) and one C-terminal hydrophobic domain (TM) responsible for the localisation to membranes. Group II consists of pro-apoptotic proteins of the Bax subfamily which differ from group one in the lack of BH4. Group III represents the pro-apoptotic "BH-3-domain-only" proteins, either bearing the TM domain or not, like Bid and Bad. (adapted from Hengartner, 2000).

The permeability transition pore complex

The MMP also involves the permeability transition pore complex (PTPC), a multiprotein complex composed of the voltage-dependent anion channel (VDAC) of the outer membrane, the adenine nucleotide translocator (ANT) of the inner membrane and

various other proteins (Kroemer and Reed, 2000). Bcl-2 family members are supposed to interact with the PTPC and regulate its permeability. Several models are discussed for the MMP. Pore formation, for example, by opening of the ANT is accompanied by an decrease of $\Delta\psi_m$, or vice versa. Thereupon, the inner mitochondrial membrane becomes permeable for water and solutes leading to matrix swelling and, ultimately, to the rupture of the outer membrane (Crompton, 1999). Furthermore, permeabilisation of the outer membrane is supposed to result from a conversion of the VDAC in a Bcl-2/Bax-regulated fashion (Shimizu *et al.*, 1999) (figure 9).

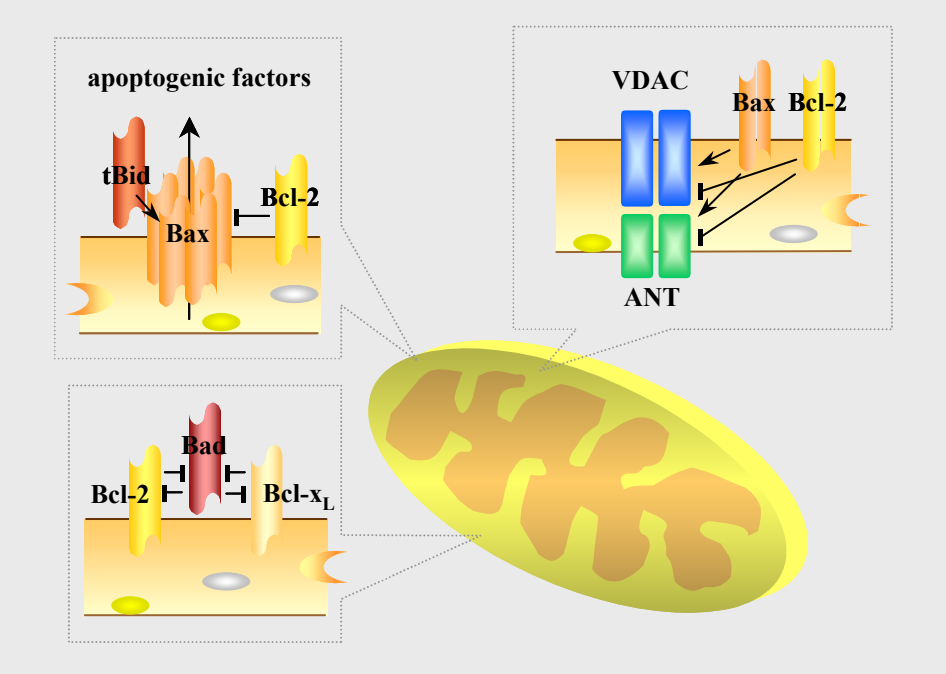


Figure 9: Schematic illustration of the interaction of Bcl-2 family members and their influence on the mitochondrial membrane permeabilisation.

Apotogenic factors

The release of apoptogenic factors from the mitochondrial intermembrane space into the cytosol, primarily the translocation of cytochrome *c*, constitutes a critical event in the mitochondrial signal transduction (figure 10). Once liberated from the mitochondria, cytochrome *c* binds to Apaf-1, the mammalian homolog of *C. elegans* Ced-4 (see 1.4.2) (Zou *et al.*, 1997). Apaf-1 contains binding sites for cytochrome *c* and dATP and oligomerises with other Apaf-1 molecules forming the apoptosome complex. The apoptosome recruits and binds the initiator caspase-9 (see 1.4.2). The processed caspase-9 is released from the multimeric complex and activates downstream effector caspases, thereby amplifying the caspase cascade (Saleh *et al.*, 1999). The mitochondrial protein AIF (apoptosis-inducing factor) translocates to the nucleus and is

found to induce caspase-independent peripheral chromatin condensation and DNA fragmentation (Lorenzo *et al.*, 1999). Smac/DIABLO (second mitochondria-derived activator of caspases; direct IAP-binding protein with low pI), an endogenous IAP inhibitor (see 1.4.2), sequesters IAP family members and neutralises their caspase-inhibiting activity (Du *et al.*, 2000).

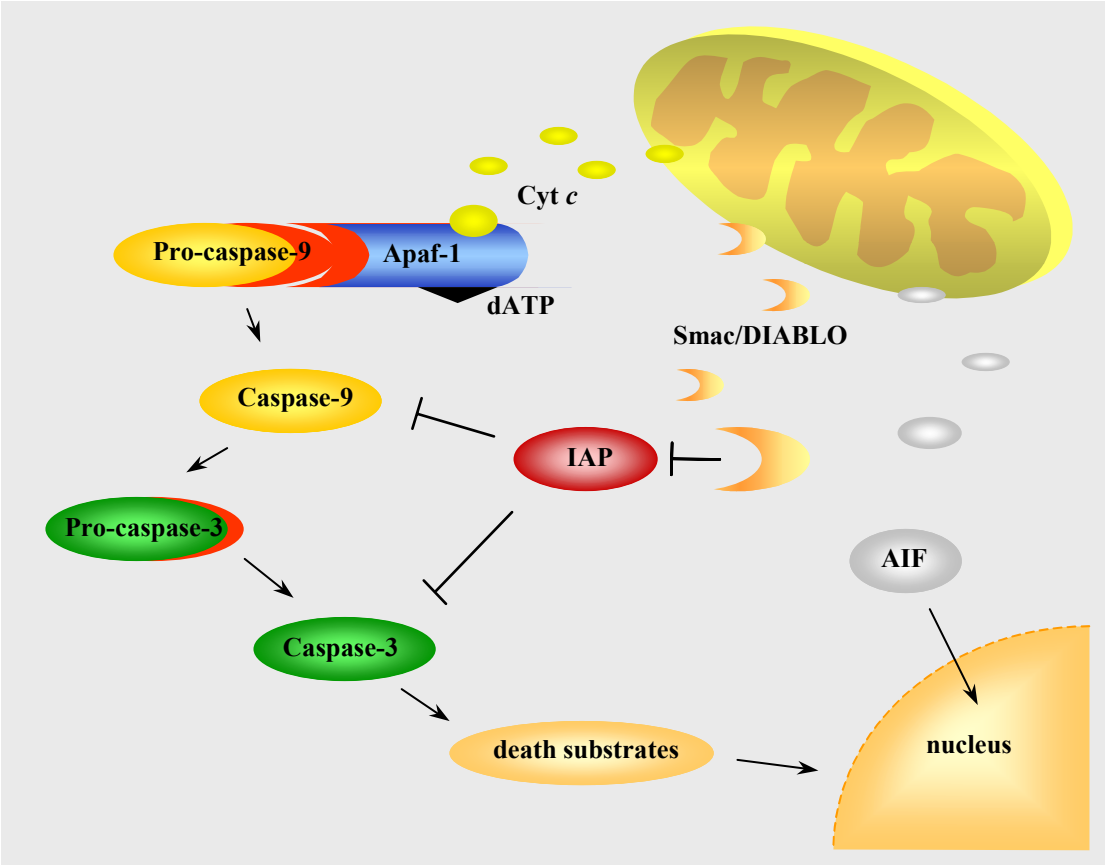


Figure 10: Schematic illustration of the effects of apoptogenic factors released from mitochondria.

5.4 Reactive oxygen species in apoptosis

Reactive oxygen species (ROS) are constantly generated during aerobic metabolism. ROS comprise free radicals like the superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$) and nitroxyl radical ($NO^{\cdot}$) as well as non-radicals including hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). Free radicals and their derivatives are involved in important physiological processes such as the regulation of the vascular tone and in receptor-mediated signalling pathways. Activated macrophages and neutrophiles produce large amounts of ROS involving the NADPH oxidase system as an antimicrobal defense

mechanism (respiratory burst) (Morel *et al.*, 1991). The presence of non-enzymatic (e.g. glutathione, vitamine A, C and E) as well as enzymatic (e.g. superoxid dismutase, catalase and glutathione peroxidase) antioxidants ensures the tight control of ROS generation and the maintenance of an intracellular redox balance. Cellular dysregulation in favour for an increase in ROS levels, e.g. resulting from excessive stimulation of NADPH oxidases, the uncoupling of the mitochondrial electron transport chain or severe changes in the thiol/disulfide redox state, cause oxidative cellular stress. Oxidative stress has been implicated in the pathogenesis of cancer, neurodegenerative diseases, atherosclerosis, AIDS and others (Droge, 2002).

Several studies provided evidence that ROS act as a mediator in apoptosis (Buttke and Sandstrom, 1994). Various chemotherapeutic drugs induce apoptosis accompanied by the intracellular production of ROS. Furthermore, the apoptotic response is inhibited by antioxidants or inducers of endogenous antioxidants (Verhaegen *et al.*, 1995). Likewise, a functional role for ROS was suggested for TNF- and CD95-induced apoptosis (Cossarizza *et al.*, 1995; Suzuki *et al.*, 1998). ROS, additionally, have been shown to modulate the expression of CD95 and CD95L (Delneste *et al.*, 1996; Bauer *et al.*, 1998). The transcription factors NF-κB (nuclear factor κB) and AP-1 (activator protein 1), implicated in the regulation of apoptosis, are both targtes of oxidative stimuli. NF-κB directly responds to oxidative stress in certain types of cells, whereas the activation of AP-1 is mainly attributed to the oxiative stress-induced MAPK signalling pathway (see 1.4.5) (Schreck *et al.*, 1991; Karin, 1995).

5.5 Mitogen-activated protein kinases (MAPK) and Akt

The mitogen-activated protein kinases (MAPK) are activated in response to a variety of extracellular stimuli and transduce signals from the cell surface to the nucleus. Currently, twelve MAPK have been identified wich can be divided into three subfamilies. The extracellular signal-regulated kinases (Erk) are mainly activated by growth factors and mitogenic stimuli and have been linked to cell survival. The c-Jun N-terminal kinases (JNK; also referred to as stress-activated protein kinases, SAPK) and the p38 kinases are activated by cellular stress including heat shock, UV irradiation, proinflammatory cytokines, DNA damaging agents and protein synthesis inhibitors and are related to the induction of apoptosis (Boulton *et al.*, 1991; Xia *et al.*, 1995). One common feature of the MAPK is their activation through phosphorylation within a protein kinase cascade (figure 11). MAPK are phosphorylated at both threonine and tyrosine residues by dual specificity MAPK kinases (MAPKK). The MAPKK are themselves phosphorylated and activated by upstream MAPKK kinases (MAPKKK) (Cobb and Goldsmith, 1995). Once activated, MAPK phosphorylate their substrates at specific serine/threonine residues. MAPK tragets include several transcription factors, thus MAPK are involved in the regulation of gene expression (Davis, 1993).

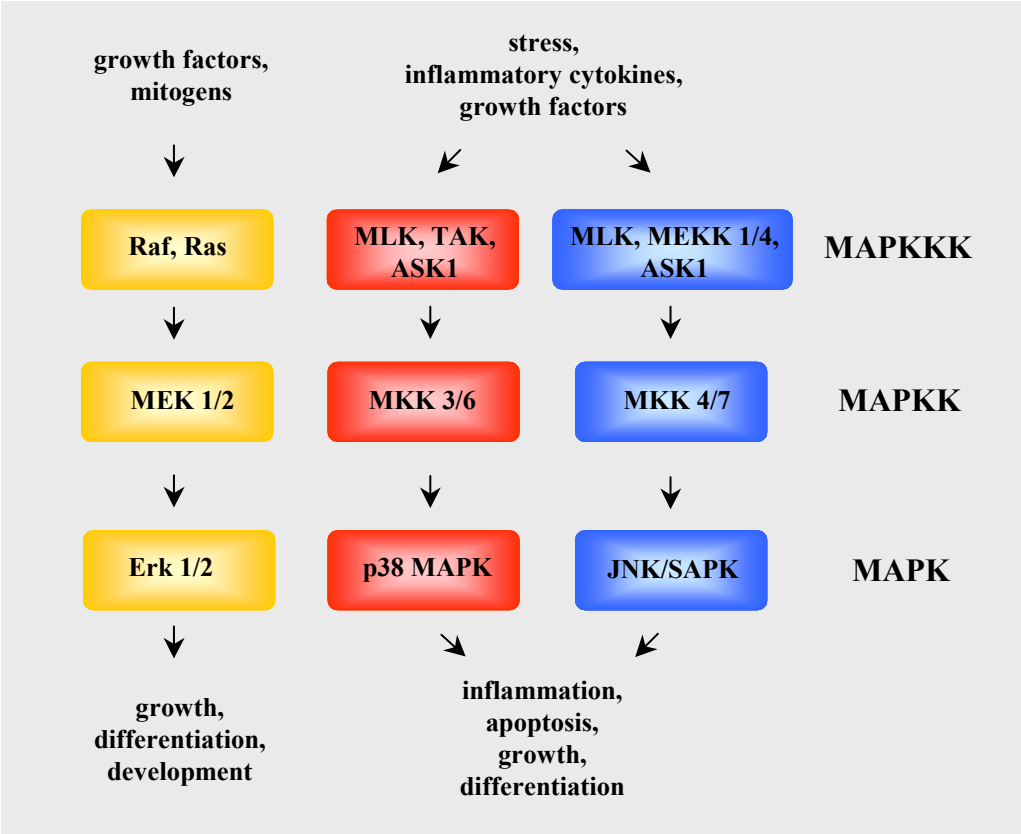


Figure 11: Schematic illustration of the MAPK signalling cascade.

Signalling pathways involving the serine/threonine kinase Akt, also termed protein kinase B (PKB), are implicated in cell survival. A large number of studies have demonstrated that activated Akt blocks apoptosis induced by diverse stimuli, including growth factor withdrawal, UV irradiation, DNA damage and anti-CD95 antibody (Kennedy *et al.*, 1997; Mildner *et al.*, 2002; Karpinich *et al.*, 2002; Hausler *et al.*, 1998). After binding to phospholipids at the inner surface of the plasma membrane, that are generated by phosphatidylinositide 3´-OH kinase (PI3K), Akt is activated through phosphorylation both at a threonine and a serine residue (Alessi *et al.*, 1997; Toker and Newton, 2000). Recent studies have proven that Akt directly targets proteins involved in the apoptotic signalling. Akt inactivates caspase-9 through phosphorylation, thereby blocking the transduction of death signals arising from mitochondrial pertubation (see 1.4.3) (Cardone *et al.*, 1998). Likewise, the pro-apototic Bad is inactivated by Akt-mediated phosphorylation (Datta *et al.*, 1997). Furthermore, Akt promotes cell survival by regulation of FLIP expression (see 1.4.1) (Panka *et al.*, 2001).

5.6 Approaches in cancer therapy

The apoptotic process has been recognised as an important target for therapeutic intervention and rational drug discovery (Nicholson, 2000; Schmitt and Lowe, 1999). Promising strategies in cancer therapy tend to reconstitute the ability of tumour cells to undergo apoptosis, in many instances lost due to genetic changes.

For instance, the discovery that TRAIL (TNF-related apoptosis-inducing ligand) almost selectively induces apoptosis in tumor cells, while sparing healthy cells, revealed the therapeutic potential of recombinant TRAIL for cancer treatment (Ashkenazi *et al.*, 1999). In pre-clinical *in vivo* studies promising results have been obtained, however, a recent study showed that TRAIL exerts *in vitro* cytotoxicity against human hepatocytes (Gliniak and Le, 1999; Jo *et al.*, 2000).

The tumour suppressor gene p53 is a predestinated target for gene therapy since it is mutated in at least 50% of all human tumors. The transfer of p53-expressing adenoviruses has been shown to promote tumour regression in preclinical models of solid tumors. Greater efficacy was shown for adenovirus-mediated p53 gene therapy when combined with the application of DNA damaging drugs (Fujiwara *et al.*, 1994). Currently, clinical phase II and III trials are underway (Ehlert and Kubbutat, 2001).

The Bcl-2 antisense G-3139, also termed Genasense, has reached phase III of clinical trials for leukemia and melanoma. Co-administration studies of Bcl-2 antisense and conventional anticancer drugs for solid tumour as well as relapsed acute leukemia have proceeded into clinical phase II (Ehlert and Kubbutat, 2001).

In 2001, the first rationally developed anticancer drug which targets a specfic oncoprotein was approved for chronic myelogenous leukemia (CML). Glivec® (STI571, Imatinib) selectively inhibits the tyrosin kinase Bcr-Abl, a fusion protein originated from a reciprocal translocation between chromosomes 9 and 22. The shortened version of chromosome 22, which is termed Philadelphia chromosome, is characteristic for CML and, therefore, represents an unique target for therapeutical intervention (Rowley, 1973; Capdeville *et al.*, 2002).

These few examples should demonstrate that an increased understanding of the molecular mechanisms that govern the apoptotic process or resistance to apoptosis in healthy and malignant cells provides an indispensable basis for effective and selective cancer therapies.

D. Materials and Methods

1 Materials

1.1 Ascididemin

Ascididemin, isolated and synthesised according to the following protocols, was kindly provided by Michael Estermeier from the group of Dr. Bert Steffan (Department of Chemistry, University of Munich; SFB 369, A4).

Isolation of ascididemin

Green morphs of *Cystodytes dellechiajei*, collected in Spain, were homogenised in a blender and extracted first with methanol, followed by repeated extraction with methanol / chloroform 1:1 (v/v) containing 1% of a 30% ammonium hydroxide solution. The organic layers were combined, filtered, and evaporated in vacuo yielding a green residue. The crude extract was suspended in water and partitioned successively against toluene and 2-butanol. After evaporation, the toluene-soluble material was subjected to chromatography on a Sephadex LH-20 column (120.0 cm × 3.5 cm) using methanol/chloroform 1:1 (v/v) as eluent to give a fraction containing primarily ascididemin. Pure, brightly yellow coloured ascididemin (10.8 mg, 0.01% wet weight) was obtained after further purification by HPLC on a Bischoff Prontosil Eurobond C-18 reversed-phase column (5 μm, 8 mm × 250 mm) employing a linear gradient from 80% aqueous trifluoroacetic acid (0.1%) / 20% acetonitril up to 80% acetonitril in 30 minutes.

Synthesis of ascididemin

Additional ascididemin (99% purity) was obtained by total synthesis according to the method of Bracher (Bracher F, 1989). The purity of ascididemin was evaluated by HPLC on an analytical Bischoff Prontosil Eurobond C-18 column (5 μm, 4 mm × 250 mm) using the conditions described above.

Derivatives of ascididemin were obtained by total synthesis according to Bracher (Bracher F, 1992) with some modifications (Lindsay *et al.*, 1997).

Isolated and sythesised ascididemin did not diverge in their cytotoxicity and their property to induce apoptosis in leukemia Jurkat T cells as determined by morphological apoptotic features, MTT assay and DNA fragmentation (data not shown).

1.2 Biochemicals, Reagents, Dyes and Solutions

carboxy-H_2DCFDA	Molecular Probes, Eugene, OR, USA
CD95 Ab (ZB4)	Medical & Biological Laboratories Co., Nagoya, Japan
CD95L, soluble	Alexis Biochemicals, Grünberg, Germany
Complete™	Roche, Mannheim, Germany
Cycloheximide	Sigma, Taufkirchen, Germany
Etoposide	Calbiochem, Bad Soden, Germany
FCS	PAA Laboratories, Cölbe, Germany
Geniticin sulfate (G418)	PAA Laboratories, Cölbe, Germany
Hoechst 33342	Sigma, Taufkirchen, Germany
JC-1	Molecular Probes, Eugene, OR, USA
L-glutamine	PAN Biotech, Aidenbach, Germany
MTT	Sigma, Taufkirchen, Germany
NAC	Sigma, Taufkirchen, Germany
PHA	Sigma, Taufkirchen, Germany
PMA	Sigma, Taufkirchen, Germany
Propidium iodide	Sigma, Taufkirchen, Germany
RPMI 1640	PAN Biotech, Aidenbach, Germany
SB203580 (p38 inhibitor)	Calbiochem, Bad Soden, Germany
SP 600125 (JNK inhibitor)	Calbiochem, Bad Soden, Germany
Staurosporine	Calbiochem, Bad Soden, Germany
Wortmannin (PI3K inhibitor)	Alexis Biochemicals, Grünberg, Germany
zIETD-fmk (caspase-8 inhibitor)	Calbiochem, Bad Soden, Germany
zVAD-fmk (broadspectrum caspase inhibitor)	Bachem, Heidelberg, Germany
zVDVAD-fmk (caspase-2 inhibitor)	Calbiochem, Bad Soden, Germany

PBS

Na_2HPO_4	1.48 g
KH_2PO_4	0.43 g
NaCl	7.20 g
H_20	ad 1000 ml

Hanks´ balanced salt solution (HBSS)

$CaCl_2$ x 2 H_2O	0.95 mM
KCl	5.3 mM
KH_2PO_4	0.44 mM
$MgCl_2$ x 6 H_2O	0.49 mM
$MgSO_4$ x 7 H_2O	0.41 mM
NaCl	136.75 mM
Na_2HPO_4 x 2 H_2O	0.34 mM
Hepes	20 mM
adjusting to pH 7.35	

2 Cell culture

2.1 Cell lines

The human leukemia T cell lines Jurkat (clone J16) as well as Jurkat^R, which are deficient of CD95 (Peter *et al.*, 1995) (kindly provided by Drs P.H. Krammer and H. Walczak, Heidelberg, Germany) were cultured in RPMI 1640 medium, containing 2 mM L-glutamine (PAN Biotech, Aidenbach, Germany) and supplemented with 10% fetal calf serum (FCS) (PAA Laboratories, Cölbe, Germany).

Jurkat T cells stably transfected with control plasmid (*neo*) or plasmid with *bcl-x$_L$* or b*cl-2* inserts (Walczak *et al.*, 2000) (kindly provided by Drs P.H. Krammer and H. Walczak, Heidelberg, Germany) were cultured as described above. Every fifth passage, the medium was additionally supplemented with 1 mg/ml of the antibiotic geniticin sulphate (G418) (PAA Laboratories, Cölbe, Germany) for selection of the transfected cells.

2.2 Culture techniques

Cell cultivation

Cells were cultured in culture medium in a 75 cm^2 tissue culture flask (Peske, Aindling-Pichl, Germany). Cell density was always kept under 1 x 10^6/ml to avoid mutations of the cells. Passaging of the cells was done three times a week. Experiments were performed with cells cultivated not longer than passage number twenty. Further passaging of immortalised cell lines is possible but the risk of genotypic changes increases.

Cultivation and experimental incubation were performed in a cell incubator (Heracell, Heraeus, Hanau, Germany) at 37°C, 90% air humidity and 5% CO_2.

Seeding of cells

For experiments cell concentration must be standarised by counting. Cell number was determined using a Fuchs-Rosenthal counting chamber (hemacytometer). The required cell concentration was adjusted by diluting the cell suspension with fresh culture medium. Cells were seeded in appropriate density in 96- or 24- well tissue culture plate and kept in the incubator for 12 h – 16 h before treatment.

Cell freezing

Microbal contamination or genotypic changes may appear in long-term cell cultures, leading to the loss of well-characterised cell lines. To prevent cell loss, cells can be

frozen and stored almost indefinitely at a very low temperature in liquid nitrogen (-196°C).

Freezing medium

RPMI 1640	70 ml
FCS	20 ml
DMSO	10 ml

For cryoprotection dimethylsulfoxide (DMSO) is added to the freezing medium. After preparation the freezing medium is sterile filtered.

5 x 10^5 cells/ml were pelleted by centrifugation (200x*g*, 10 min, 4°C) and resuspended in freezing medium. The cell suspension was equally transferred to cryovials (1.5 ml). Gradual decrease in temperature ensures gentle freezing: the cryovials were first put into a polystyrene box and placed in a –86°C freezer. After one day the vials were transferred to a liquid nitrogen container.

Cell thawing

The cryovial was carefully taken out of the liquid nitrogen and immediately thawed in a water-bath (37°C). Cells were transferred to a tissue culture flask (75 cm^2) containing 20 ml prewarmed culture medium (37°C). After 24 h medium was changed to remove cell debris, leaked toxic cell content and the DMSO, derived from the freezing medium.

3 Measurement of cell viability

MTT assay

This assay is a quantitative colorimetric method for determination of cell survival and proliferation. The assessed parameter is the metabolic activity of viable cells. Metabolically active cells reduce pale yellow tetrazolium salt (MTT) to a dark blue water-insoluble formazan which can be, after solubilisation with DMSO, directly quantified. The absorbance of the formazan directly correlates with the number of viable cells (Mosmann, 1983).

9 x 10^5 cells/ml (100 µl/well) were seeded in a 96-well tissue culture plate. Cells were either left untreated or stimulated with ascididemin in different concentrations (0.3 µM – 50 µM) for 24 h. Actinomycin D (1 µg/ml) was used as positive control, DMSO (1%)

as vehicel control. 10 µl of MTT solution (dissolved in PBS at a stock concentration of 5 mg/ml; sterile filtered and protected from light) was added to each well and incubated for 2 h at 37°C in a CO_2 incubator. Accumulated cristal formazan was solubilised by adding 190 µl DMSO to each well and gently shaking the culture plate in the dark at room temperautre for 1 h. Absorbance of the coloured solution was measured at $\lambda = 550$ nm with an ELISA plate reader (SLT Spectra, SLT Labinstruments, Crailsheim). The absorbance in the DMSO wells was used to determine the zero inhibition growth level for each microtiter plate in an experiment. Statistical analysis of three independend experiments performed in triplicate and calculation of IC_{50} were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, California, USA).

4 Flow cytometry

In a flow cytometric system, large numbers of cells or particles flow within a laminar fluid stream in a single file passing a laser beam where they are individually evaluated. As the focused laser beam interacts with a cell, scattered light and, in the case of using fluorescent antibodies or dyes, fluorescence signals are created at the same time. The electronic signals are converted into digital values and are illustrated in dot plots or histogram plots.

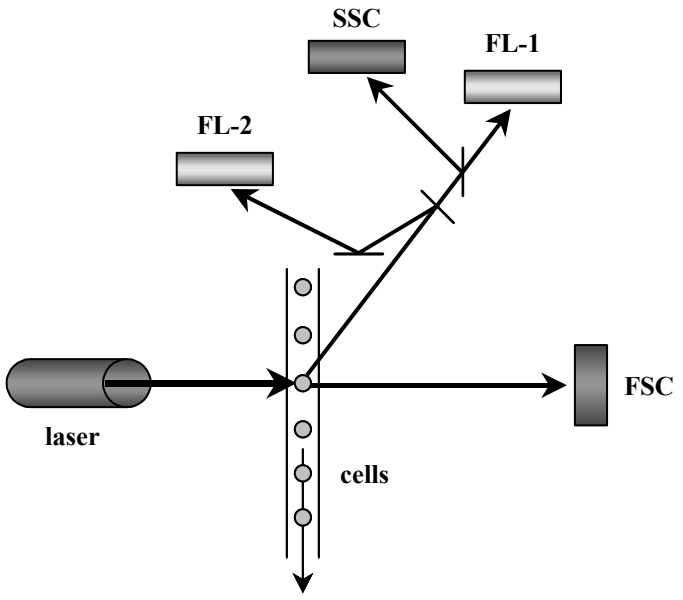


Figure 12: Schematic illustration of a flow cytometer.

All measurements were performed on a FACScalibur (Becton Dickinson, Heidelberg, Germany), equipped with an 488 nm argon-ion laser, using CellQuest™ software.

FACS buffer

NaCl	8,12 g
KH_2PO_4	0,26 g
Na_2HPO_4	2,35 g
KCl	0,28 g
Na_2EDTA	0,36 g
LiCl	0,43 g
Na-azide	0,20 g
H_2O	ad 1000 ml, pH 7,37

4.1 Determination of cell size and granularity

The intensity of light scattered in the forward direction (FSC) generally correlates with cell size, the intensity of scattered light measured at 90° to the laser beam (side scatter; SSC) correlates with cell granularity. Changes of morphological features of apoptotic cells affect their light scattering properties: cell shrinkage and fragmentation reduces the FSC signal, increase of granularity enhances SSC. The detected signals are illustrated in dot plots (FSC versus SSC). Discrimination between normal and apoptotic cells can be made due to the distribution of the dots within the plot (Gorman *et al.*, 1997b).

7 x 10^5 cells/ml (1 ml/well) were seeded in a 24-well tissue culture plate. Cells were either left untreated or treated with ascididemin (5 µM) for different peroids of time. Cells were harvested in polypropylene tubes by centrifugation (600x*g*, 10 min, 4°C), washed with cold PBS and resuspended in a volume of 500 µl of cold PBS for flow cytometric analysis. Adjustments of the cytometer settings (FSC, SSC) were carried out with untreated control cells.

4.2 Detection of apoptosis by Annexin V-FITC

During apoptosis a number of changes in cell surface markers occur. An early event is the loss of asymmetry in cell membrane phospholipids, altering both the hydrophobicity and charge of the membrane surface. In living cells, the distribution of phospholipids is asymmetric, with the inner membrane containing anionic phospholipids (e.g. phosphatidylserine, PS) and the outer membrane having mostly neutral phospholipids. Upon induction of apoptosis, however, the amount of PS on the outer surface of the membrane increases (Fadok *et al.*, 1992). Recognition of phospholipids by phagocytes *in vivo* results in the removal of apoptotic cells. Annexin V, a calcium-dependent phospholipid-binding protein, has a high affinity for PS (Vermes *et al.*, 1995). Hence, FITC-labeled Annexin V can be used to identify apoptotic cells by flow cytometry.

Additional incubation with propidium iodide (PI) (see 2.4.3) is used to distinguish between viable, early apoptotic, necrotic or late apoptotic cells. Necrotic or late apoptotic cells will bind Annexin V-FITC and stain with PI because of membrane rupture while PI will be excluded from viable (FITC negative) and early apoptotic (FITC positive) cells.

Detection of apoptosis after ascididemin treatment by Annexin V-FITC was performed using a Annexin V-FITC Apoptosis Detection Kit from Calbiochem (Schwalbach, Germany) according to the manufacturer´s instruction.

Cells (7 x 10^5 cells/ml; 1 ml; 24-well tissue culture plate) were either left untreated or stimulated with ascididemin (5 µM) for different periods of time. Cells were harvested by centrifugation (1,000x*g*, 5 min, RT), washed with cold PBS, resuspended in a volume of 500 µl cold 1X Binding Buffer and incubated with 1.25 µl Annexin V-FITC solution for 15 min at room temperature. After centrifugation (1,000x*g*, 5 min, RT) the obtained pellet was resuspended in 500 µl 1X Binding Buffer and 10 µl PI solution was added. The samples were placed on ice in the dark and immediately analysed by flow cytometry. To set up the flow cytometer apoptosis-induced cells stained with FITC only (FL-1; λ_{em}: 530 nm) and labeled with PI only (FL-2; λ_{em}: 585 nm), respectively, were used.

4.3 Quantification of apoptosis

DNA fragmentation constitutes one biochemical hallmark of apoptosis. Thus, measurement of DNA content makes it possible to identify apoptotic cells, to recognise the cell cycle phase specificity and to quantitate apoptosis. For flow cytometry analysis of the relative nuclear DNA content the fluorescent dye propidium iodide (PI), which becomes highly fluorescent after binding to DNA, is most commonly used. After permeabilisation PI binds to DNA in cells at all stages of the cell cycle, and the intensity with which a cell nucleus emits fluorescent light is directly proportional to its DNA content. The results of the measurement are illustrated in a histogram, where the number of cells (counts) is plotted against the relative fluorescence intensity of PI (FL-2; λ_{em}: 585 nm; red fluorescence). The histogram reflects the cell cycle distribution of the cell population. Staining normal untreated cells with PI, most of the cells are in G_0/G_1 phase (DNA content: 2n) and emit light at a uniform frequency, depicited in the prominent G_0/G_1 peak of the histogram. Cells in G_2/M phase (DNA content: 2 x 2n) emit light with double intensity of the G_0/G_1 cells and therefore appear at higher values. Cells passing through the S phase (DNA content between 2n and 2 x 2n) emit light of an intensity range that falls between the G_0/G_1 peak and G_2/M peak. Staining procedure using a hypotonic buffer (see below) results in extraction of the fragmented low molecular weight DNA of apoptotic nuclei and leads to their decreased stainability with PI. Apoptotic nuclei appear as a broad hypodiploid DNA peak at lower fluorescence

intensity compared to nuclei in G_0/G_1 phase ("sub-G_1"). For more exact evaluation of the "sub-G_1" peak the logarithmic mode of PI fluorescence acquisition was used for experiments.

PI staining solution

Propidium iodide	50 µg	
RNAse	500 µg	
Sodium citrate	0,1% (w/v)	
Triton X-100	0,1% (v/v)	
PBS	ad 1 ml	(light protection)

7 x 10^5 cells/ml (1 ml/well), seeded in a 24-well tissue culture plate, were either left untreated or stimulated with ascididemin. The used stimulation time and concentration of ascididemin as well as the preincubation with inhibitors was dependent on the experiments performed. Cells were harvested in polypropylene tubes by centrifugation (600x*g*, 10 min, 4°C), washed with cold PBS and resuspended in 500 µl hypotonic PI staining solution. The tubes were placed at 4°C in the dark over night. Cell fluorescence (FL-2) as well as scatter characteristics (FSC/SSC) were aquired. For the adjustment of instrument settings untreated control cells were used. Marked regions of "sub-G_1" were evaluated for percentage of apoptotic cells.

4.4 Measurement of mitochondrial transmembrane potential ($\Delta\psi_m$)

Mitochondrial dysfunction within the apoptotic process is often associated with loss of the mitochondrial inner transmembrane potential. One possibility to visualise alterations in the mitochondrial membrane potential is staining with potentiometric fluorescent dyes. The cationic carbocyanine dye JC-1 exhibits potential-dependent fluorescence emission: JC-1 accumulates in the mitochondrial matrix under the influence of $\Delta\psi_m$ and forms red fluorescent JC-1 aggregates (λ_{ex}: 585 nm; λ_{em}: 590 nm); mitochondrial depolarisation leads to a loss of JC-1 aggregates and an increase in green fluorescent JC-1 monomers (λ_{ex}: 510 nm; λ_{em}: 527 nm) (Cossarizza *et al.*, 1996).

To determine the reduction of $\Delta\psi_m$, ascididemin (5 µM)-treated or untreated cells (7 x 10^5 cells/ml; 1 ml; 24-well tissue culture plate) were incubated with 1.25 µg/ml JC-1 (dissolved in DMSO at a stock concentration of 5 mg/ml; light protection), first for 10 min in a CO_2 incubator and, after transferring the samples to polypropylene tubes, for

further 20 min at room temperature. Alteration of membrane potential was assayed detecting FL-1 (green fluorescence; λ_{em}: 530 nm).

The fluorescence emission shift was also observed using fluorescence light microscopy (see 2.5.3).

4.5 Measurement of ROS generation

Apoptosis can be initiated by oxidative stress mediated by the generation of reactive oxygen species (ROS) (Buttke and Sandstrom, 1994). For the investigation of ROS levels carboxylated 2'7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) is commonly used (Burow and Valet, 1987). Carboxy-H_2DCFDA is a cell-permeant indicator for reactive oxygen species, particularly peroxide, that is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell yielding a green fluorescent product. The dye has two negative charges at physiological pH, allowing for better retention by cells.

For equal loading the entire volume of all samples (7 x 10^5 cells/ml; 1 ml/sample) is pelleted by centrifugation (400x*g,* 10 min, 20°C). The obtained pellet is resuspended with 195 µl prewarmed HBSS, loaded with 5 µl of a 200 µM carboxy-H_2DCFDA solution (dissolved in water-free DMSO at a stock concentration of 20 mM; dilution made with HBSS; light protection) and incubated for 30 min in a CO_2 incubator. The dye was removed by centrifugation and washing with HBSS. The pellet was resuspended in adequate volume HBSS (1 ml/sample), the cell suspension was equally transferred to polypropylene tubes and placed into a water-bath (37°C). Carboxy-H_2DCFDA-containing cells were left untreated, stimulated with either ascididemin (5 µM) or staurosporine (0.5 µM) as positive control and measured at different points of time. To estimate intracellular peroxide production, fluorescence intensity (FL-1; λ_{em}: 530 nm) was recorded. Cells incubated with carboxy-H_2DCFDA only were employed to monitor basal peroxide synthesis. Fluorescence intensity was obtained as histogram statistics.

5 Microscopy

5.1 Morphology of apoptotic cells

Cell shrinkage, membrane blebbing and the formation of apoptotic bodies are characteristic events during apoptosis which can be easily detected by light microscopy.

Cells (8 x 10^5 cells/ml; 1 ml; 24-well tissue culture plate) were either left untreated or stimulated with ascididemin (5 µM) for different periods of time. Cells were viewed at a 200-fold magnification with a light microscop (Axiovert 25, Zeiss, Munich, Germany).

5.2 Hoechst staining

Cells undergoing apoptosis display an increase in chromatin condensation. Morphologically, the nuclei of apoptotic cells become smaller than those of normal cells and become hyperfluorescent when labeled with some nuclear stains. The bisbenzimide dye Hoechst 33342 is a cell-permeant, adenosine/thymidine (AT) selective, minor groove-binding DNA dye that brightly stains the condensed chromatin of apoptotic cells while staining the looser chromatin of viable cells only moderately.

Cells (7 x 10^5 cells/ml; 1 ml; 24-well tissue culture plate) were either left untreated or treated with ascididemin (5 µM) for different periods of time. Cells were harvested by centrifugation (110x*g*; 10 min; 4°C), the obtained pellet was resuspended in 1 ml PBS. Each sample was stained with 10 µl Hoechst solution (dissolved in double destilled water at a stock concentration of 0.1 mg/ml) for 5 min at 37°C in the dark. The dye was removed by centrifugation (110xg; 10 min; 4°C) and washing with PBS. Condensed chromatin was visualised with a 200-fold magnification using a Zeiss Axiovert 25 microscope (Zeiss, Munich, Germany), filter 02 (λ_{ex}: 365 nm; λ_{em}: 420 nm).

5.3 JC-1

(see 2.4.4)

For fluorescence light microscopy sample were prepared as described under 2.4.4 with some modifications. Incubation with 1.25 µg/ml JC-1 was carried out for 30 min in a CO_2 incubator. The fluorescence emission shift of JC-1 resulting from the mitochondrial depolarisation was visualised with a 200-fold magnification using a Zeiss Axiovert 25 microscope (Zeiss, Munich, Germany), filter 09 (λ_{ex}: 450-490 nm; λ_{em}: 520 nm).

6 Fluorimetric caspase activity assay

Most of the proteolytic cleavages during apoptosis results from the activation of caspases, a family of cysteine-dependent proteases. These enzymes recognise specific tetra- or pentapeptide motifs in their substrates and cleave exclusively on the carboxyl side of aspartate residues. Caspase activation can be measured by applying a synthetic peptide substrate which is coupled to a fluorophor. Cleavage of the fluorogenic substrate by the activated enzyme leads to increased fluorescence (Gurtu *et al.*, 1997). The generated fluorophore is proportional to the concentration of activated caspase. 7-amino-4-trifluoromethyl coumarin (AFC) is a commonly used fluorophore. The liberation of AFC shows a blue to green shift in fluorescence at an excitation wavelength of 390 nm and an emission wavelength of 505 nm.

Activity of caspase-2 and –9 was determined using a Caspase-2 and a Caspase-9 Activity Assay Kit (fluorometric), respectively, from Calbiochem (Schwalbach, Germany) according to the manufacturer's instruction.

Cells ($1x10^6$ cells/ml; 1 ml; 24-well tissue culture plate), either untreated or treated with ascididemin (5 µM) for different periods of time were collected by centrifugation (400x*g*, 10 min, 4°C), washed with cold PBS, transferred to reaction tubes and frozen at –20°C over night. The pellet was resuspended in 50 µl lysis buffer and incubated on ice for 10 min. The samples were centrifuged (500x*g*, 5 min, 4°C), 50 µl of the cleared lysat was transferred to a round-bottom 96-well microtiter plate (Greiner, Frickenhausen, Germany) and 50 µl of reaction buffer (containing 10 mM DTT) was added to each sample. 5 µl of VDVAD-AFC in case of measuring caspase-2 activity and 10 µl of LEHD-AFC in case of measuring caspase-9 activity were added to each well. Generation of free AFC at 37°C was kinetically determined by fluorescence measurement (λ_{ex}: 385 nm; λ_{em}: 505 nm) using a microplate reader (Fluostar, BMG GmbH, Offenburg, Germany).

7 Western blot analysis

Apoptosis related proteins were investigated by Western blot analysis.

7.1 Preparation of samples of whole cell lysates

Lysis buffer

Tris-HCl pH 7.5	30 mM	
NaCl	150 mM	
EDTA	2 mM	
Triton X-100	1% (v/v)	
Complete™ (25x)		freshly added before use

Lysis buffer (phosphorylated proteins)

Hepes	50 mM	
NaCl	50 mM	
EDTA	5 mM	
$Na_4P_2O_7$ x 10 H_2O	10 mM	
NaF	50 mM	
Na_3VO_4	1 mM	
PMSF	1 mM	freshly added before use
Triton X-100	1%	
Complete™ (25x)		

SDS Sample buffer (5x)

Tris-HCl (pH 6.8) 3.125 M	100 µl
Glycerol	500 µl
SDS 20%	250 µl
DTT 16%	125 µl
Pyronin Y 5%	5 µl
H_2O	ad 1000 µl

Cells (8 x10^6 cells/ml; 1 ml; 24-well tissue culture plate) were either left untreated, stimulated with ascididemin (5 µM) or preincubated with inhibtor as indicated. Cells were harvested by pooling triplicates and subsequent centrifugation (400x*g*, 10 min,

4°C). The obtained pellet was washed with cold PBS and stored at –86°C over night. Cells were lysed in 100 µl of appropriate lysis buffer for 30 min at 4°C and centrifugated (10,000x*g*, 10 min, 4°C). After determination of protein concentration by the method of Bradford (Bradford M., 1976), an appropiate volume of sample buffer was added to the probes. After boiling at 95°C for 5 min, the samples were either stored at –20°C or immediately used for protein electrophoresis.

7.2 Preparation of samples of cytosolic and mitochondrial fraction

Involvement of mitochondria in apoptosis can lead to the release of various factors into the cytoplasm from mitochondria, e.g. cytochrome *c*. To detect the occurrence of certain proteins in the cytoplasm the separation of cytosolic and mitochondrial fraction is necessary.

Permeabilisation buffer

Mannitol	210 mM	
Hepes	10 mM	
EGTA	0.2 mM	
Succinate	5 mM	
Sucrose	70 mM	freshly added before use
BSA	0.15%	
Digitonin	80 µg/ml	
adjusting to pH 7.2		

Release of cytochrome *c* from mitochondria was analysed according to Leist *et al.* (Leist *et al*., 1998). Cells (8 x 10^5 cells/ml; 1 ml; 24-well tissue culture plate) were either left untreated, stimulated with ascididemin (5 µM) or preincubated with inhibitor as indicated. Cells were harvested by pooling triplicates and subsequent centrifugation (1500 rpm, 10 min, 4°C). Cell pellets were resuspended in permeabilisation buffer and incubated for 20 min at 4°C. Permeabilised cells were centrifuged (170x*g*, 10 min, 4°C). The supernatant was removed and centrifuged again (13,000x*g*, 10 min, 4°C). The obtained cytosolic fraction was analysed for protein concentration, diluted with the appropriate volume of sample buffer and stored at –20°C for Western blot analysis. The remaining pellet from the first centrifugation step after permeabilisation was lysed in 0.1% Triton / PBS (15 min, 4°C), centrifuged (13,000x*g*, 10 min, 4°C) and the supernatant containing the mitochondrial fraction was further prepared as described for the cytosolic fraction.

7.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Separation of proteins was performed by denaturating SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). This technique allows the electrophoretic separation of denaturated proteins according to their size. Sodium dodecyl sulfate (SDS), a highly negative charged detergent, solubilises proteins and leads to a constant net charge per mass unit. Hence, SDS-polypeptide complexes migrate toward the anode through the polyacrylamide gel according to their molecular weight. In addition, the differences in molecular shape are compensated by the loss of the tertiary and secondary structures because of the disruption of the hydrogen bonds and unfolding of the molecules. By adding of a reducing agent like dithiothreitol (DTT) disulfid bonds are cleaved and proteins are totally unfolded. The molecular weight of the investigated proteins is estimated by applying molecular weight standards (Rainbow marker RPN 720, Amersham, Buckinghamshire, England). Discontinuous gel electrophoresis is commonly used. A stacking gel that allows protein concentration in the gel, is overlaid on to a separation gel which allows protein separation. Percentage of acrylamide/ bisacrylamide gels should be chosen according to the expected molecular weight of the protein of interest.

Electrophoresis was carried out in an vertical apparatus Mini Protean II (BioRad, Hercules, CA, USA). Two gel runs were performed in parallel.

For gel preparation an acrylamide 30% / bis-acrylamide 0.8% 37.5:1 (v/v) stock solution (Rotiphorese™ Gel 30) from Roth (Karlsruhe, Germany) was applied.

Separation gel 10%		**Stacking gel**	
PAA solution 30%	6.25 ml	PAA solution 30%	2.125 ml
1.5 M Tris, pH 8.8	4.69 ml	1.25 M Tris, pH 6.8	1.25 ml
SDS 10%	0.188 ml	SDS 10%	0.125 ml
H_2O	7.625 ml	H_2O	8.75 ml
10 min degassing		10 min degassing	
TEMED	18.75 µl	TEMED	25 µl
APS	93.75 µl	APS	125 µl

Electrophoresis buffer	
Tris base	3 g
Glycine	14.4 g
SDS	1.0 g
H_2O	ad 1000 ml

Samples with equal amount of protein and the molecular weight marker were loaded into the slots of the prepared polyacrylamid gels. Electrophoresis was run at 100 V for 21 min for stacking and at 200 V for 45 min for resolving of the proteins. (power supply: Biometra, Göttingen, Germany).

7.4 Western blotting and detection of proteins

The specific detection of proteins can be carried out after their electrophoretic transfer from the gel to an immobilising membrane (blotting membrane), incubation with specific antibodies and subsequent chemiluminescence visualisation.

Anode solution I pH 10.4		**Anode solution II pH 10.4**	
Tris base	15 g	Tris base	1.5 g
Methanol	100 ml	Methanol	100 ml
H2O	400 ml	H_2O	400 ml

Cathode solution pH 7.6	
ε-amino-n-caproic acid	2.6 g
Methanol	100 ml
H_2O	400 ml

TBS-T pH 8.0	
Tris base	3 g
NaCl	11.1 g
Tween 20	2 ml
H_2O	ad 1000 ml

Transfer of proteins onto a polyvinylidene fluoride (PVDF) microporous membrane (Immobilon-P, Millipore, Bedford, MA, USA) was performed by semi-dry blotting between two horizontal graphite electrodes (Fastblot B 43, Biometra, Göttingen, Germany). The discontinuous buffer system leads to an equal and effective protein transfer with sharp signals.

The blotting membrane was cut to the size of the resolving gel and soaked for 5 min in methanol, H_2O and anode solution II, respectively. Forming an air bubble free stack, 6 sheets of blotting paper (BioRad, Hercules, CA, USA) impregnated with anode solution I, followed by 3 sheets impregnated with anode solution II, were placed on the graphite plate (anode). Onto this stack first the membrane, then the gel was laid and subsequently covered with 9 sheets of blotting paper moisturised with cathode solution.

Blotting was performed at a current of 0.8 mA per cm^2 of blotting surface and 115 V for 45 min.

After transfer, the membrane was blocked with 5% (m/v) nonfat dry milk (Blotto, BioRad, Hercules, CA, USA) in TBS-T pH 8.0 for 1 h at room temperature to mask unspecific binding sites. The blocked membrane was incubated with a diluted antibody solution of the adequate primary antibody (see below) at 4°C overnight on a shaking platform. Three washing steps for 10 min in TBS-T pH 8.0 preceded the incubation for 1 h at room temperature with the secondary horseradish peroxidase (HRPO)-labeled antibody. After repetition of the three washing steps, chemiluminescence detection was performed using Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products, Zaventem, Belgium) and imaged by a Kodak Digital Science™ Image station 440CF (NEN Life Science, Zaventem, Belgium).

antibody	isotype	dilution	company
Bid	rabbit IgG	1:2000, BSA	kindly provided by X. Wang (Luo *et al.*, 1998)
Bcl-2	goat IgG	1:1000, Blotto	Santa Caruz, Heidelberg, Germany
phospho Bcl-2 (Ser70)	rabbit IgG	1:1000, BSA	Cell Signaling, Frankfurt/M, Germany
Bcl-x_L	rabbit IgG	1:1000; Blotto	Santa Cruz, Heidelberg, Germany
Caspase-2	mouse IgG1	1,5 µg/ml, Blotto	Becton Dickinson/PharMingen Heidelberg, Germany
Caspase-3	mouse IgG 2a	1:1000, Blotto	Becton Dickinson/Transduction Lab., Heidelberg, Germany
Caspase-8	mouse IgG 2b	1:2000, Blotto	Upstate Biotechnology, Lake Placid, USA
Caspase-9	mouse IgG1	0,5 µg/ml, Blotto	Calbiochem, Bad Soden, Germany
PARP	mouse IgG1	1 µg/ml, Blotto	Calbiochem, Bad Soden, Germany
Cytochrome *c*	mouse IgG 2b	1 µg/ml, Blotto	Becton Dickinson/PharMingen Heidelberg, Germany
phospho JNK (Thr183/Tyr185)	mouse IgG1	1:2000, Blotto	Cell Signaling, Frankfurt/M, Germany
phospho p38 (Thr180/Tyr182)	rabbit IgG	1:1000, BSA	Cell Signaling, Frankfurt/M, Germany
phospho p44/42 (Erk) (Thr202/Tyr204)	mouse IgG	1:2000, Blotto	Cell Signaling, Frankfurt/M, Germany
phospho Akt (Ser473)	rabbit IgG	1:1000, BSA	Cell Signaling, Frankfurt/M, Germany

Table 3: List of antibodies

7.5 Coomassie blue staining

Nonspecific binding of dyes to protein structures allows visualisation of proteins separated by gel electrophoresis. This method includes fixation of proteins in ethanol-acetic acid and a staining step. Coomasie blue staining is useful for control of equal protein loading and effective blotting.

Staining solution		**Destaining solution**	
Coomassie blue	0.3%	Glacial acetic acid	10% (v/v)
Glacial acetic acid	10% (v/v)	Ethanol 96%	30% (v/v)
Ethanol 96%	45% (v/v)	H_2O	
H_2O			
filtration			

After Western blotting, gels were shaken with staining solution for 45 min and subsequently destained with destaining solution three times for 15 min. Proteins remained in the gel became visible as blue bands.

8 Statistics

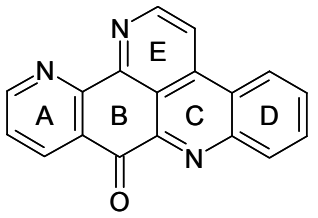
Statistical analysis were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, California , USA). Student´s t-test (unpaired, two-tailed) and ANOVA (one-way) combined with post test Dunnett were applied as indicated. Analysing two groups with significantly different variances, student´s t test was combined with Welch´s correction.

E. Results

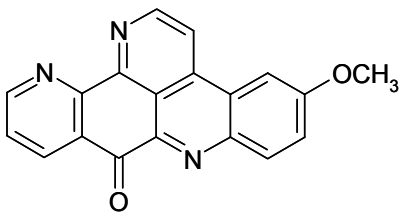
1 Ascididemin induces apoptosis in leukemia Jurkat T cells

1.1 Ascididemin exhibits cytotoxicity against leukemia Jurkat T cells – structure-activity relationship

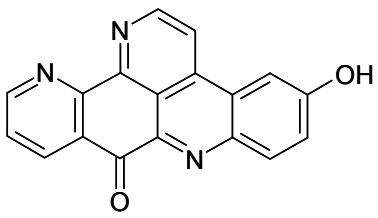
Ascididemin, whose chemical structure is shown in figure 13 A, showed dose-dependent cytotoxic potency against the acute leukemia T cell line Jurkat determined by using the colorimetric MTT assay. Cell viability was significantly decreased upon stimulation with ascididemin at concentrations in the range of 0.4 µM up to 25 µM (figure 14). The IC_{50} value, i.e., the drug concentration inhibiting the mean growth value by 50%, was calculated as 0.6 µM.



A



B



C

Figure 13: Ascididemin (A) and its analogues 5-methoxyascididemin (B) and 5-hydroxyascididemin (C)

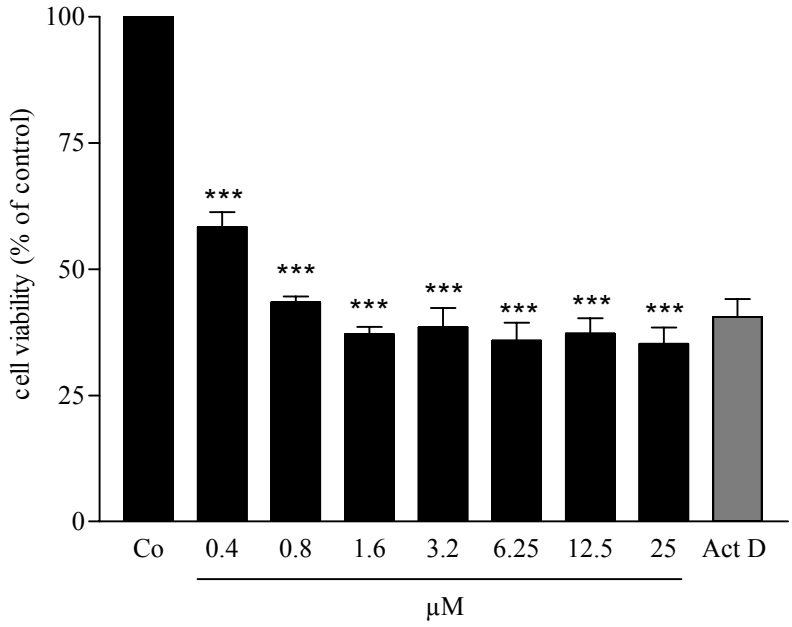


*Figure 14: Dose-dependent in vitro cytotoxicity of ascididemin against leukemia Jurkat T cells. Cells were incubated with increasing concentrations (0.4 µM-25 µM) of ascididemin for 24 h. Cell viability was quantified by applying the MTT assay as described under "Materials and Methods". Bars represent the mean ± SEM of three independent experiments performed in triplicate; *** $p<0.001$ represents significant differences compared to controle values, whereby control was set as 100% (ANOVA/Dunnett).*

Testing the two ring D analogues, 5-methoxyascididemin (figure 13 B) and 5-hydroxyascididemin (figure 13 C), revealed lower cytotoxic properties for 5-methoxyascididemin (IC_{50} = 6.5 µM) compared to ascididemin. 5-hydroxyascididemin exhibited no remarkable cytotoxic effect towards Jurkat T cells in this test system (figure 15).

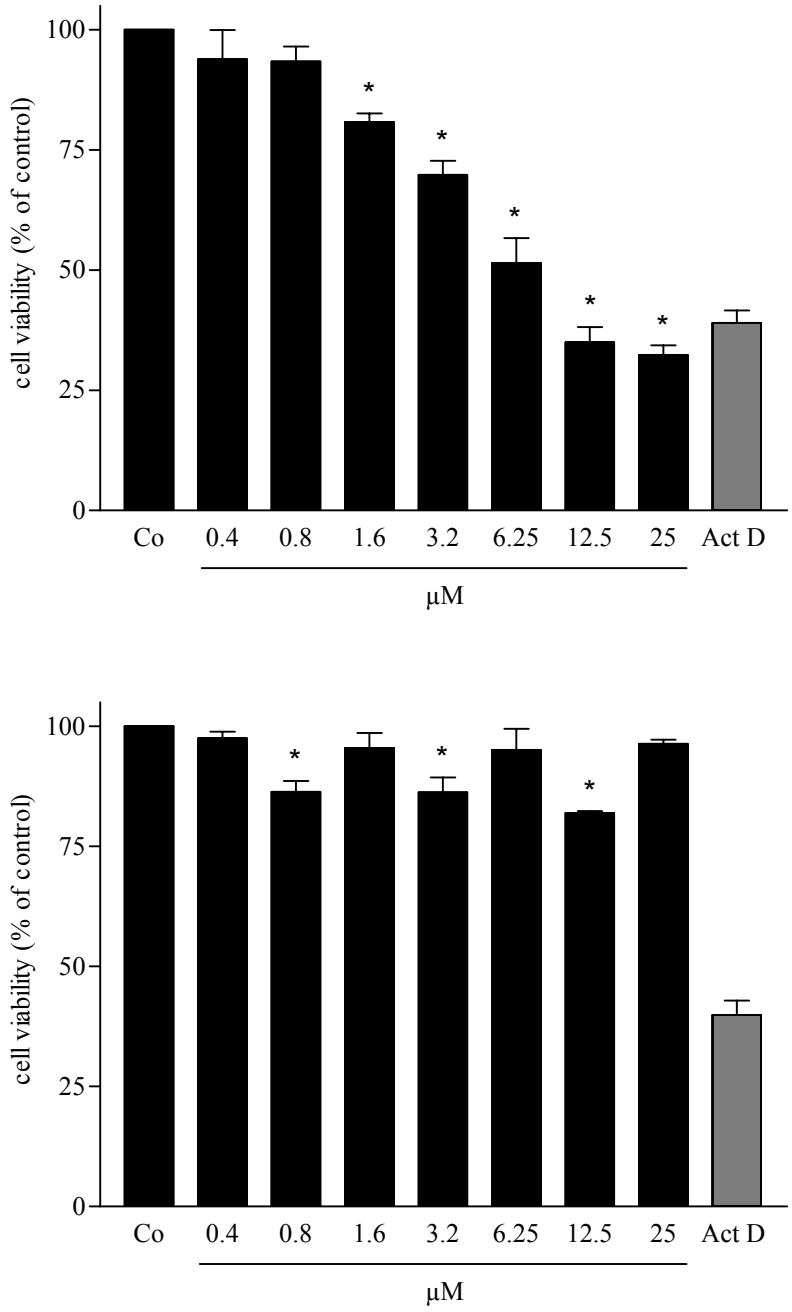


Figure 15: Structure-activity relationship of the ascididemin analogues 5-methoxyascididemin and 5-hydroxyascididemin.
*Jurkat T cells were incubated with increasing concentrations (0.4 µM-25 µM) of 5-methoxyascididemin (upper panel) and 5-hydroxyascididemin (lower panel) for 24 h. Cell viability was quantified by applying the MTT assay as described under "Materials and Methods". Bars represent the mean ± SEM of three independent experiments performed in triplicate; * $p<0.05$ (ANOVA/Dunnett) represents significant differences compared to controle values, whereby control was set as 100%.*

1.2 Ascididemin induces apoptotic cell death

For discrimination between apoptotic and necrotic cell death, morphological criteria combined with commonly accepted biochemical methods were used.

Ascididemin treated cells showed the characteristic morphological and biochemical features of apoptosis like membrane blebbing, cell shrinkage, chromatin condensation, DNA fragmentation and formation of apoptotic bodies.

Light microscopy revealed the occurrence of cell shrinkage and apoptotic bodies after stimulation with ascididemin (5 µM) for 8h (figure 16).

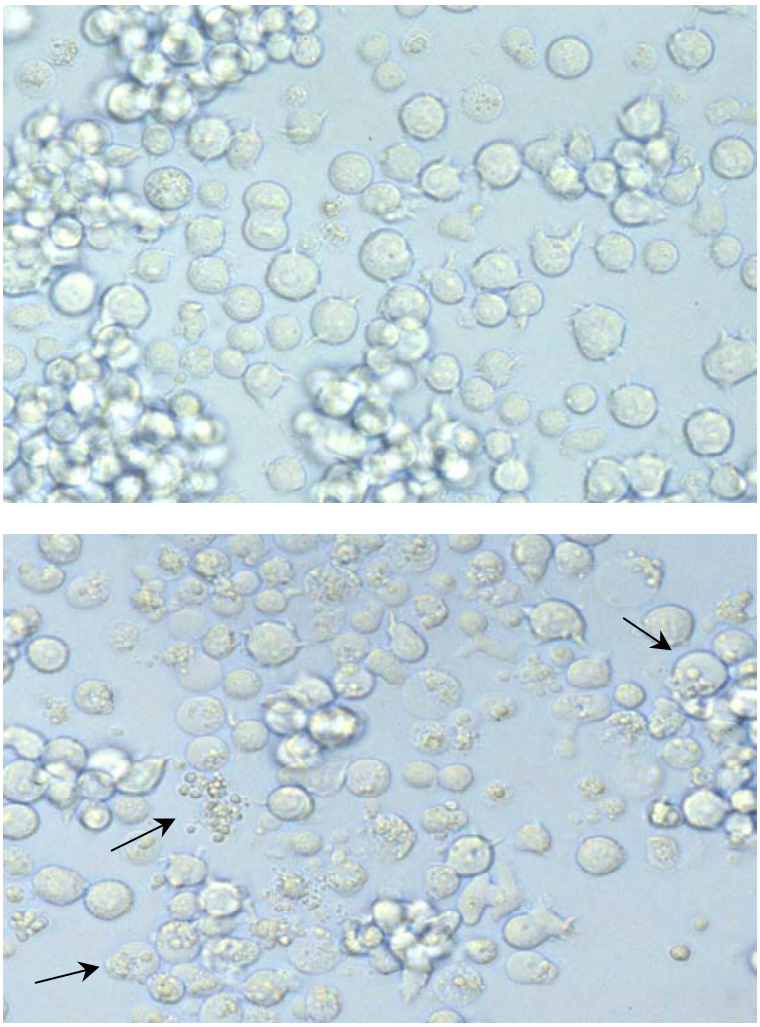


Figure 16: Ascididemin-treated Jurkat T cells exhibit characteristic morphological apoptotic features: Light microscopy
The upper pannel shows untreated control cells. Ascididemin-treated (5 µM, 8 h) cells showing characteristic membrane blebbing, cell shrinkage and formation of apoptotic bodies are depicted in the lower panel (original magnification 200-fold).

Changes in cell size and granularity

A further method for detection of alteration in cell morphology was flow cytometry analysis of cell size and granularity. Changes in light scatter, i.e. a decrease in forward scatter (FSC) as a sign of lower cell size and an increase in side scatter (SSC) as a sign of granularity compared to control cells, are characteristic for cells dying of apoptosis. Two representative dot plots of untreated and ascididemin-treated Jurkat T cells (5 µM, 24 h) are depicted in figure 17.

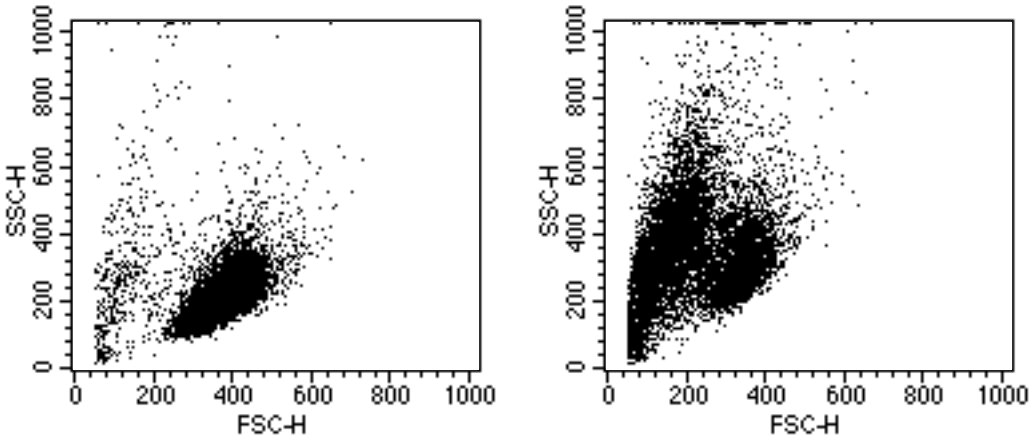


Figure 17: Ascididemin-treated Jurkat T cells exhibit characteristic morphological apoptotic features: FACS analysis
Dot plot of untreated Jurkat T cells exhibiting almost uniform cell size and granularity (left panel); characteristic dot plot of apototic cells after ascididemin treatment (5 µM, 24 h; right panel): occurrence of a second population of apoptotic cells in the range of lower FSC and similar or higher SSC compared to control cells. Measurement was carried out by flow cytometry as described under "Materials and Methods".

Detection of phosphatidylserine translocated to the cell surface

Ascididemin treatment led to the exposure of phosphatidylserine (PS) on the outside of the plasma membrane, a characteristic event in early stages of apoptosis, detected by Annexin V-FITC stainig and FACS analysis (figure 18). Costaining with propidium iodide (PI) allowed discrimination between apoptotic and necrotic cells.

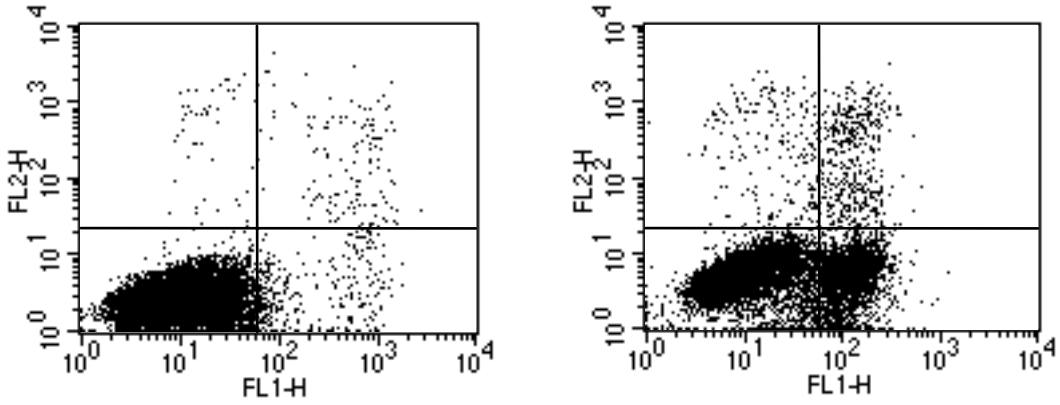


Figure 18: Ascididemin induces translocation of phosphatidylserine to the outer cell membrane.
FACS analysis of either untreated (left panel) or ascididemin-stimulated cells (5 µM, 8 h; right panel) after staining with Annexin V-FITC (FL-1) and PI (FL-2): cells appearing in the lower rigth quadrant show positive Annexin V-FITC staining, which indicates phosphatidylserine translocation to the cell surface, and no DNA stainig with PI, demonstrating intact cell membranes, both features of early apoptosis. Necrotic or late apoptotic cells would appear in the upper right quadrant: Annexin V-FITC- and PI-positive. The figure shows representative histograms out of three independent experiments.

Hoechst staining of condensed chromatin

Morphological changes during apoptotis also affect the nucleus. Staining apoptotic cells with the fluorescent dye Hoechst 33342 visualised condensed chromatin and fragmented nuclei in response to ascididemin treatment by fluorescence microscopy (figure 19).

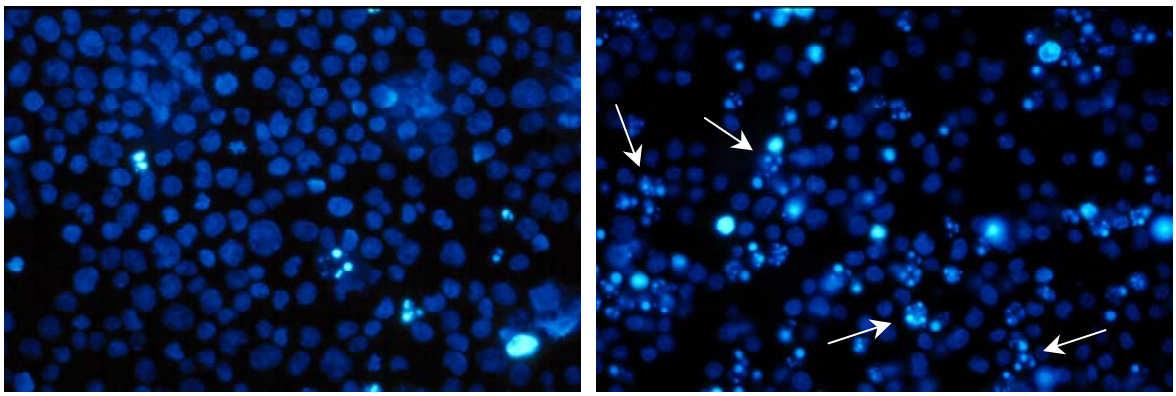


Figure 19: Ascididemin treatment of Jurkat T cells leads to chromatin condensation and fragmented nuclei.
After treatment with ascididemin (5 µM, 8 h) cells were stained with Hoechst 33342 as described under "Materials and Methods": nuclei of untreated cells show diffuse blue fluorescence (left panel); apoptotic nuclei exhibit bright blue fluorescence due to condensed chromatin (right panel). The pictures show representative photographs out of three independent experiments (original magnification 200-fold).

FACS analysis of DNA fragmentation

Apoptotic signalling leads to activation of the nuclear endonuclease CAD which results in fragmentation of the genomic DNA. Analysis of propidium iodide (PI) stained nuclei by flow cytometry showed that ascididemin-treated cells displayed a population of nuclei with hypodiploid DNA content (figure 20). As depicted in the histogram of ascididemin-treated cells, ascididemin predominantly targeted cells in S phase.

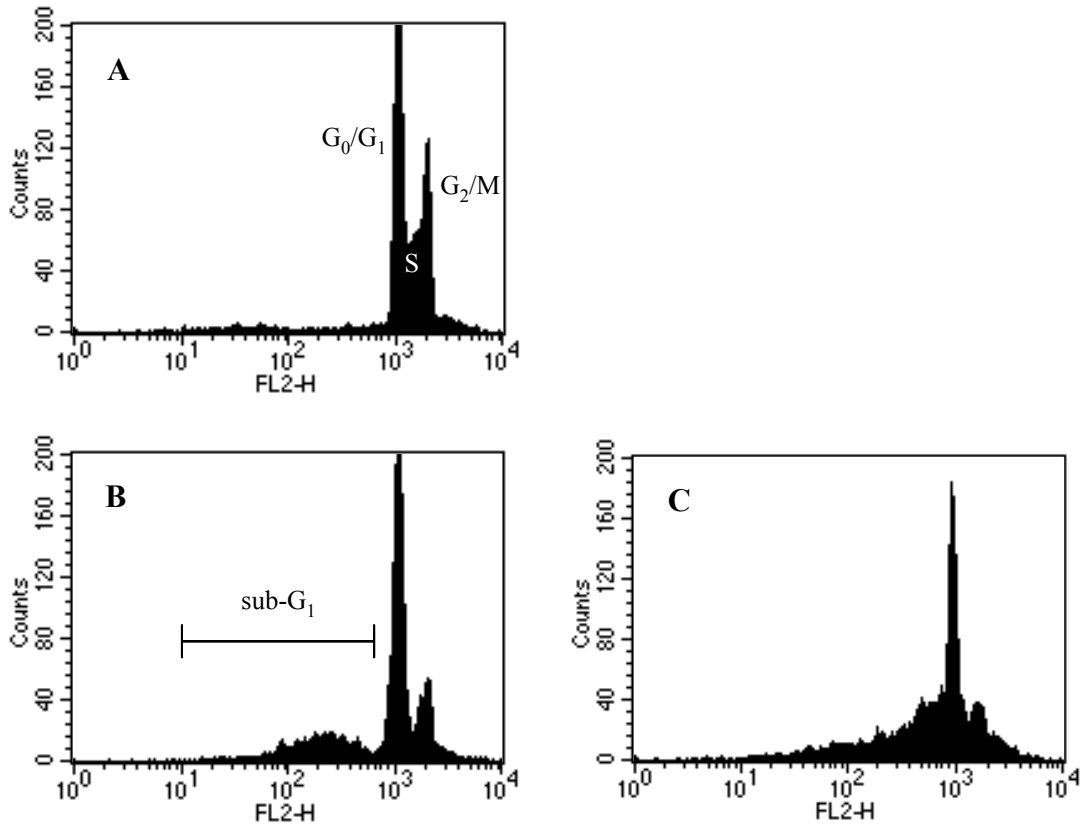


Figure 20: Jurkat T cells exhibit DNA fragmentation after ascididemin treatment.
FACS analysis of PI-stained nuclei of either untreated cells (A) or cells treated with ascididemin (5 µM) for 8 h (B) and 24 h (C) as described under "Materials and Methods": the histograms show the distribution of nuclei with different DNA content; counts left of the "G_0/G_1-peak" ("sub-G_1") demonstrate the appearance of nuclei with a hypodiploid DNA content.

Statistical analysis of the "sub-G_1" region revealed a significant, dose- and time-dependent induction of apoptosis in response to ascididemin (figure 21). A concentration of 0.5 µM ascididemin (16 h) already showed a significant increase in the percentage of apoptotic cells. Treatment with 5 µM ascididemin led to a significant augmentation of apoptotic cells after 8 h.

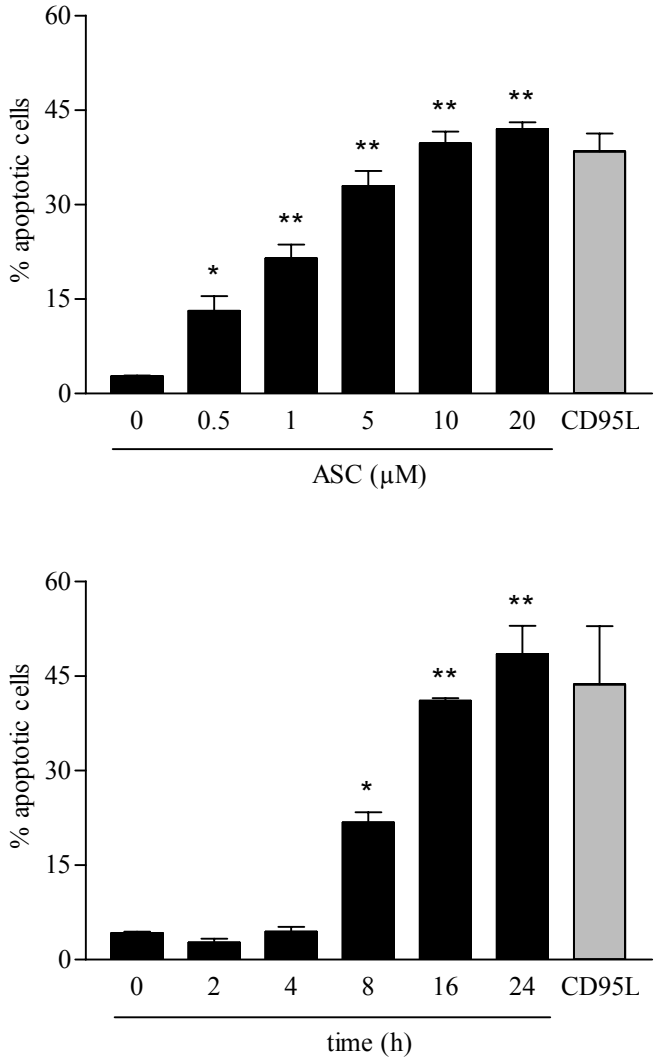


*Figure 21: Ascididemin induces apoptosis dose- and time-dependently in Jurkat leukemia T cells. Dose-dependent induction of apoptosis by ascididemin (upper panel): cells were incubated with increasing concentrations (0.5-20 µM) of ascididemin for 16 h; time-dependency of ascididemin-induced apoptosis (lower pannel): cells were incubated with ascididemin (5 µM) for the indicated time periods (0-24 h). Soluble CD95 ligand (CD95L) (100 ng/ml, 24h) was used as positive control. % Apoptotic cells, percentage of cells with hypodiploid DNA content as described under "Materials and Methods". Bars represent the mean ± SEM of three independent experiments performed in duplicate; * $p<0.05$, ** $p<0.01$ significantly different compared to untreated control (0 h) (ANOVA/Dunnett).*

2 The CD95 receptor/CD95 ligand system in ascididemin-induced apoptosis

Several DNA damaging agents, such as doxorubicin and etoposide, have been reported to induce the expression of CD95 ligand (CD95L) and mediate cell death by subsequent interaction with the CD95 receptor (CD95) (Mo and Beck, 1999; Friesen *et al.*, 1996).

First, to investigate whether protein synthesis is required for ascididemin-induced apoptosis, Jurkat T cells were preincubated with the protein synthesis inhibitor cycloheximide (CHX) (Chlichlia *et al.*, 1998). Acquisition of fragmented DNA after treatment with ascididemin (5 µM, 8 h) by flow cytometry showed no significant reduction of ascididemin-mediated DNA fragmentation in addition with CHX treatment (1 µg/ml, preincubation 1 h) (figure 22). In contrast, inhibition of protein synthesis by CHX affected DNA fragmentation after etoposide treatment (Siitonen *et al.*, 2000). These data suggested that synthesis of new protein is not required for ascididemin mediated cell death.

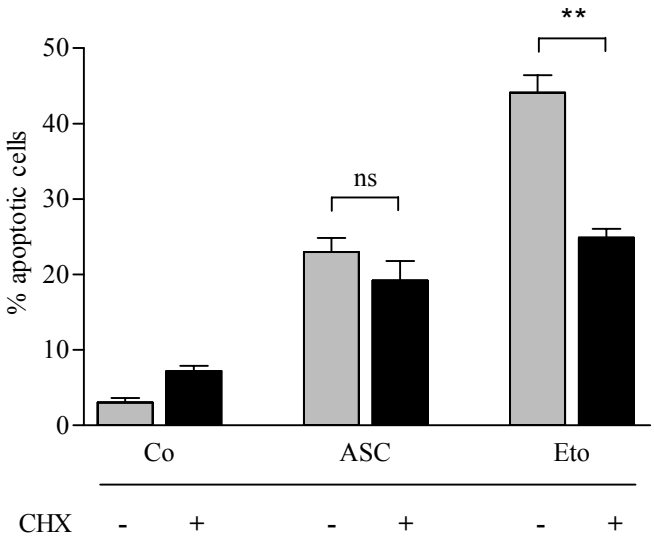


Figure 22: Protein synthesis is not required for ascididemin induced apoptosis.
*Cells were either left untreated (Co) or stimulated with ascididemin (5 µM) for 8 h after preincubation with cycloheximide (CHX) (1 µg/ml) for 1 h. Etoposide (25 µg/ml) was used as positive control. % Apoptotic cells was determined by FACS analysis of DNA fragmentation as described under "Materials and Methods". Bars show mean ± SEM of three independent experiments performed in duplicate. Ns, no significance compared to values seen in ascididemin-treated cells, ** $p<0.01$ significantly different compared to values seen in etoposide-treated cells (Student´s t-test).*

Inhibition of CD95 receptor ligation

Blocking the CD95 receptor of CD95-sensitive Jurkat T cells with a neutralising anti-CD95 antibody (ZB4) was applied to determine whether the engagement of CD95 is necessary for ascididemin-induced apoptosis. Jurkat T cells preincubated with ZB4 (1 µg/ml) for 1 h showed a significantly reduced apoptosis rate in response to soluble CD95L (100 ng/ml) compared to cells treated with CD95L only. In contrast, ZB4 was unable to diminish ascididemin (5 µM)-triggered apoptosis (figure 23). In fact, apoptosis was significantly increased in ZB4-pretreated and ascididemin-treated cells compared to cells treated with ascididemin only. This was most likely due to the cytotoxic potential of ZB4 itself observed in this cell system. These results indicate that ascididemin-induced apoptosis occurs independently of CD95L binding to the CD95 receptor.

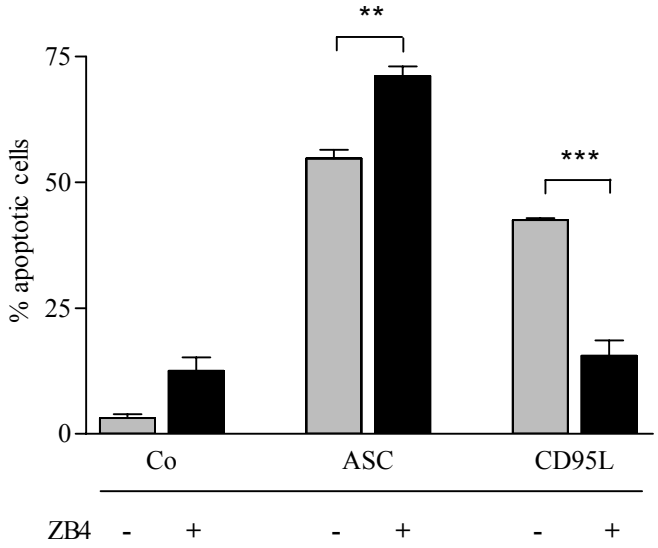


*Figure 23: Ascididemin-induced apoptosis occurs independently of CD95L binding to the CD95 receptor. FACS analysis of DNA fragmentation of Jurkat T cells with either blocked (preincubation with anti-CD95 antibody ZB4, 1 µg/ml, 1 h) or unblocked CD95 receptor and subsequent addition of ascididemin (ASC, 5 µM) or soluble CD95L (100 ng/ml) as positive control for 24 h. Bars indicate mean ± SEM of three independent experiments performed in triplicate. ** $p<0.01$ represents significant difference compared to values seen in ascididemin treated cells, *** $p<0.001$ represents significant difference compared to values seen in CD95L treated cells (Student´s t-test).*

Induction of apoptosis despite lack of CD95 receptor

Trimerisation, i.e. activation of the CD95 receptor does not only occur after binding of the CD95L but also in response to different stimuli that have been reported to directly induce clustering of the CD95 receptor, independently of CD95L (Aragane *et al.*, 1998; Gajate *et al.*, 2000). To examine whether ascididemin might activate the CD95 receptor system also by mechanisms independent of CD95L, ascididemin was applied to the subclone Jurkat^R which lacks the CD95 receptor. CD95-resistant Jurkat^R T cells underwent apoptosis in a concentration-dependent manner after stimulation with ascididemin for 24 h. At higher concentrations (5-20 µM) the effect was less pronounced than in CD95-sensitive Jurkat T cells (figure 24).

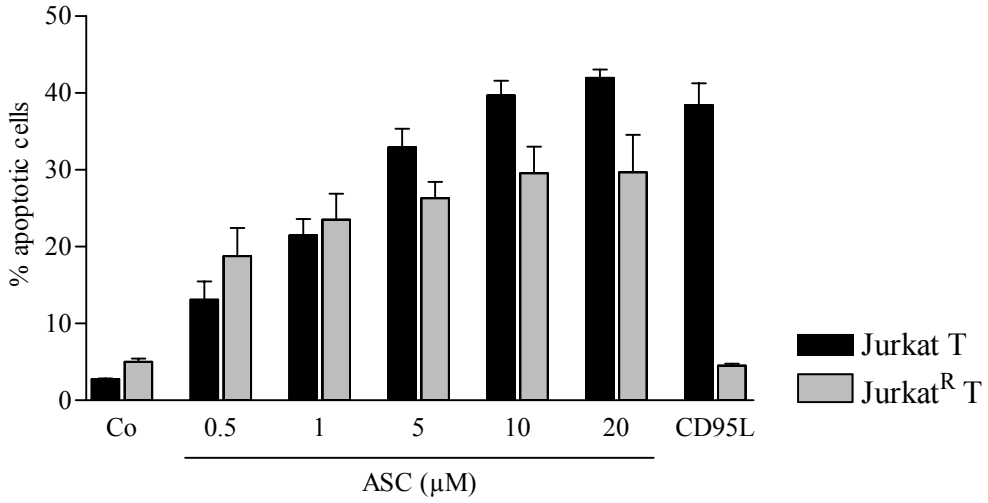


Figure 24: Ascididemin induces apoptosis in CD95-resistant Jurkat^R T cells.
*Jurkat T cells (black bars) as well as Jurkat^R T cells (grey bars) were either left untreated (Co) or incubated with ascididemin at the indicated concentrations for 16 h. Soluble CD95L was used as positive control. % Apoptotic cells was quantified by flow cytometry as described under "Materials and Methods". Bars represent mean ± SEM of three independent experiments performed in duplicate. ** $p<0.01$ represents significant difference compared to values seen in Jurkat T cells, *** $p<0.001$ represents significant difference compared to values seen in Jurkat T cells (Student´s t-test).*

3 The role of mitochondria in ascididemin-triggered apoptosis

Besides triggering the "extrinsic " pathway due to receptor activation, apoptotic signals can be mediated by a mitochondria-dependent "intrinsic" pathway. Mitochondrial membrane permeabilisation is a critical event in the process leading to apoptosis. Apoptogenic factors released from mitochondria participate in the transduction and amplification of apoptotic signals (Bernardi *et al.*, 1999; Kroemer *et al.*, 1998).

3.1 Ascididemin affects mitochondrial membrane permeabilisation

Release of cytochrome c

To address the question whether ascididemin targets mitochondria, the release of cytochrome *c* from mitochondria into the cytosol was investigated by western blot analysis. Time-dependence studies revealed the appearance of cytochrome *c* in the cytosolic fraction, indicating outer mitochondrial membrane permeabilisation (figure 25). A stepwise translocation of cytochrome *c* from the mitochondria into the cytosol was observed with significant amounts of cytochrome *c* detectable in the cytosol as early as 4 h after cell treatment with ascididemin (5 µM).

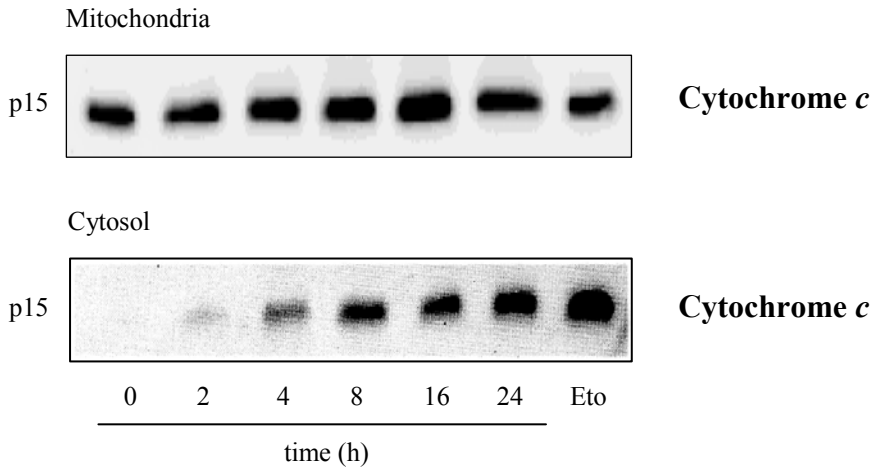


Figure 25: Ascididemin induces time-dependent cytochrome c release from mitochondria. Jurkat T cells were either left untreated (0 h) or stimulated with ascididemin (5 µM) for different time points as indicated. Cytosolic and mitochondrial fraction were analysed for cytochrome c by western blot as described under "Materials and Methods". The figure shows one representative western blot out of three independent experiments.

Loss of mitochondrial transmembrane potential ($\Delta\psi_m$)

Dissipation of the mitochondrial transmembrane potential ($\Delta\psi_m$) is related to increased inner mitochondrial membrane permeability (Kroemer and Reed, 2000). Ascididemin led to mitochondrial depolarisation as detected by flow cytometry as well as fluorescence microscopy applying the potential-sensitive dye JC-1. First signs of a loss of $\Delta\psi_m$ were already apparent 2 h after the addition of ascididemin as seen by an increase in FL-1 intensity (figure 26) and the occurrence of a bright green fluorescence, respectively (figure 27).

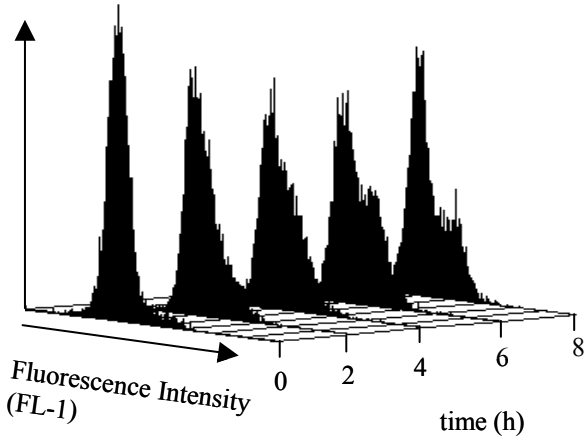


Figure 26: Ascididemin treatement leads to the dissipation of the mitochondrial membrane potential. FACS analysis of ascididemin-induced time-dependent $\Delta\psi_m$ dissipation in response to ascididemin (5 µM). Jurkat T cells were treated with ascididemin for the indicated time periods and subsequently loaded with the fluorochrome JC-1 (1.25 µg/ml) as described in "Materials and Methods". An increase in green fluorescence (FL-1) is indicative of a loss of $\Delta\psi_m$. Depicted histogram shows one representative out of three independent experiments.

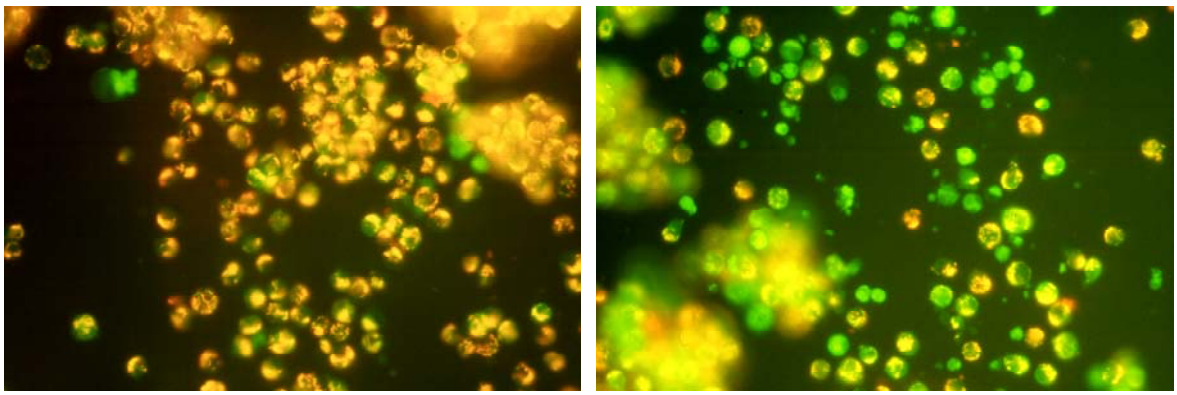


Figure 27: Fluorescence emission shift of JC-1 due to the dissipation of the mitochondrial membrane potential.
Fluorescence microscopy photographs visualising the fluorescence emmision shift of JC-1:

untreated control cells exhibit bright red fluorescence (left panel), ascididemin-treated cells (2 h) show green fluorescence indicating mitochondrial depolarisation (right panel). Samples were prepared as described under "Materials and Methods". The pictures show representative photographs out of three independent experiments (original magnification 200-fold).

3.2 Ascididemin-induced apoptosis is a mitochondria-dependent process

Antiapoptotic members of the Bcl-2 family, like Bcl-x_L and Bcl-2, have been shown to block mitochondrial cytochrome *c* release and subsequent apoptosis in response to a variety of stimuli (Yang *et al.*, 1997a; Vander Heiden *et al.*, 1997; Scaffidi *et al.*, 1998). To clarify the role of mitochondria in ascididemin-induced apoptosis, Jurkat T cells stably transfected with either vector alone (Jurkat/*neo*), vector containing *bcl-x_L* inserts (Jurkat/*bcl-x_L*), or vector containing *bcl-2* inserts (Jurkat/*bcl-2*) were investigated for DNA fragmentation in response to ascididemin (figure 28). Dose-response studies revealed prevention of apoptosis in both cell lines overexpressing the antiapoptotic proteins (figure 29). These data suggest a mitochondria-dependent apoptotic signalling mediated by ascididemin.

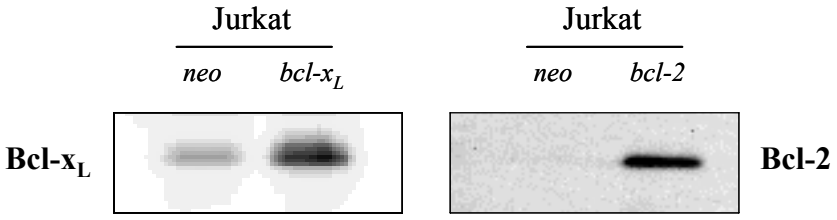


Figure 28: Western blots demonstrating the expression level of Bcl-x_L and Bcl-2 protein in Jurkat T cells transfected with vectors containing either bcl-x_L or bcl-2 inserts or control vector (neo).

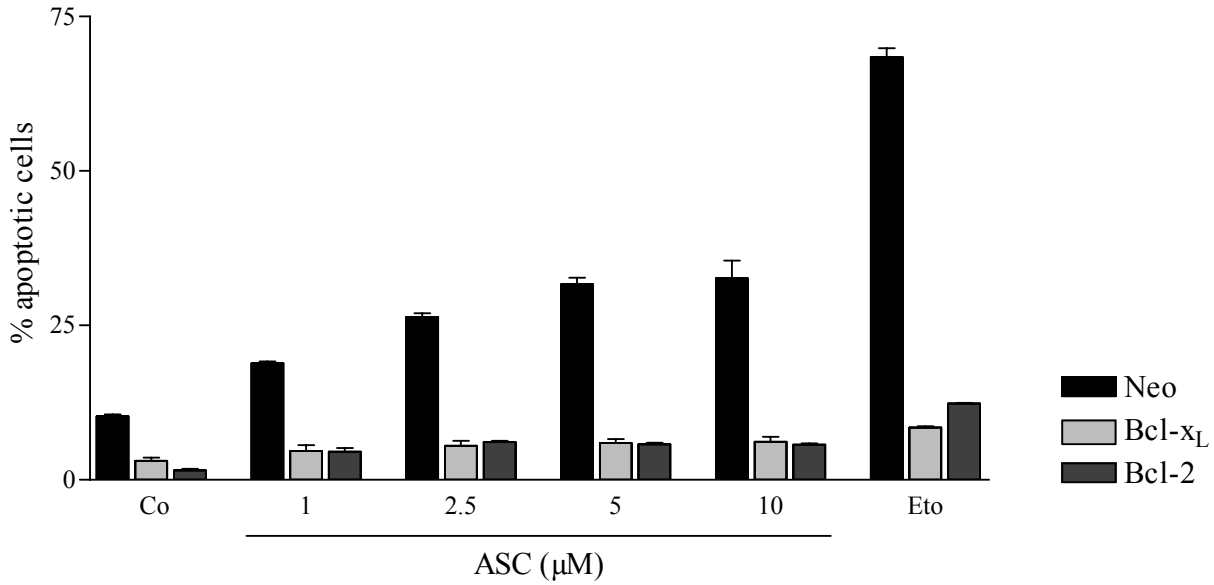


Figure 29: Ascididemin induces a mitochondria-dependent apoptotic pathway.
Overexpression of Bcl-x_L or Bcl-2 inhibits ascididemin-induced apoptosis. Control cells (Jurkat/neo), cells overexpressing Bcl-x_L (Jurkat/bcl-x_L) or Bcl-2 (Jurkat/bcl-2) were incubated with the indicated concentrations of ascididemin for 16 h. % Apoptotic cells were quantified by FACS analysis of DNA fragmentation as described under "Materials and Methods". Bars indicate the mean ± SD of two independent experiments performed in duplicate.

4 The role of caspases in the ascididemin-induced apoptotic pathway

The central component of apoptotic signalling is the proteolytic system of caspases, a family of cystein-dependent aspartate-directed proteases. These enzymes participate in a cascade that is triggered in response to proapoptotic signals and ends in the cleavage of a variety of proteins, resulting in disassembly of the cell.

4.1 Ascididemin induces activation of caspase-9, -8 and –3

To elucidate the involvement of caspases in ascididemin-mediated apoptosis the activation of certain caspases were determined by western blot analysis.

Treatment of Jurkat T cells led to the activation of caspase-3, -8, and –9. Time-dependence studies revealed the processing of procaspase-9 after 4 h of incubation with ascididemin (5 μM), cleavage of caspase-8 and –3, respectively occurred after 8 h

(figure 30). Cleavage of the caspase-3 substrate PARP showed a similiar activation pattern as caspase-3 (figure 31).

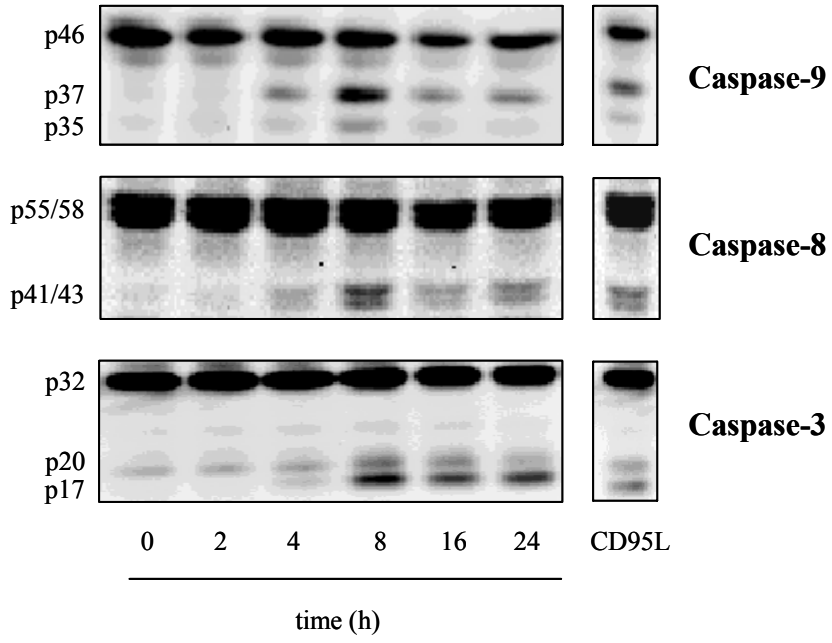


Figure 30: Caspase activation in response to ascididemin treatment.
Representative western blots showing the time-dependent (0-24h) processing of procaspases (caspase-9, -8 and –3) to their active intermediates after treatment with ascididemin (5 µM) for different time periods. Soluble CD95L (200 ng/ml, 8 h) was used as positive control. All experiments were performed three times with similar results.

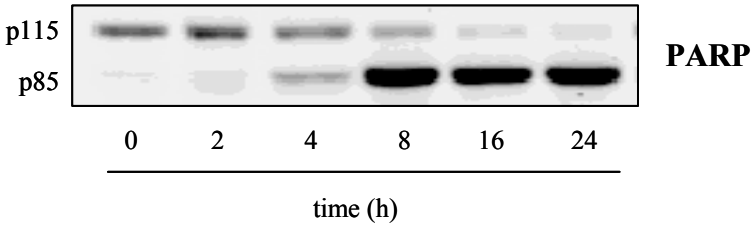


Figure 31: Ascididemin induces cleavage of PARP.
Cells were either left untreated (0 h) or incubated with 5 µM ascididemin for different time periods and analysed for cleavage of PARP as described under "Materials and Methods". The figure shows one representative western blot out of two independent experiments.

In regard to the kinetics of caspase activation, the processing of caspase-8, commonly classified as initiator caspase, occurred after cleavage of the initiator caspase-9. In contrast, activation of caspase-8 correlated well with the activation of the executioner caspase-3. Thus, these data suggest that caspase-8 rather acts as an executioner caspase in the apoptotic signalling mediated by ascididemin.

4.2 Caspase activation is necessary for ascididemin-induced apoptosis

To evalute the contribution of caspase activation to apoptosis induced by ascididemin the broadspectrum caspase inhibitor zVAD-fmk was applied. Using flow cytometry analysis of DNA fragmentation a complete abrogation of ascididemin-induced (5 µM) apoptosis after pretreatment with 25 µM zVAD-fmk for 1 h was observed (figure 32).

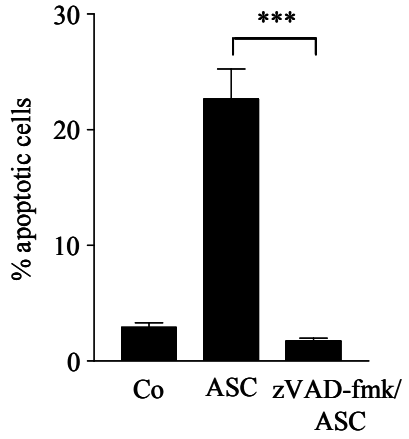


*Figure 32: Inhibition of caspases by zVAD-fmk protects against ascididemin-induced apoptosis. Cells were left untreated (Co), treated with ascididemin (ASC, 5 µM, 8 h), or pretreated with zVAD-fmk (25 µM, 1 h) and then incubated with ascididemin (ASC, 5 µM). Apoptotic cells were quantified by FACS as described under "Materials and Methods". Bars represent the mean ± SE of three independent experiments performed in duplicate; ***p< 0.001 implicates significant difference compared to values seen in ascididemin-treated cells (Student´s t test).*

4.3 Caspase-2

Caspase-2 is activated in response to various apoptotic stimuli, including DNA damaging agents, TNF-α, and CD95 ligation (Harvey *et al.*, 1997; Duan and Dixit, 2002; Droin *et al.*, 2001). As caspase-2 was demonstrated to be a nuclear resident protein (Colussi *et al.*, 1998; Paroni *et al.*, 2002) and ascididemin is reported to affect the nucleus, the impact of caspase-2 on ascididemin-induced apoptosis was investigated.

4.3.1 Activated caspase-2 is necessary for ascididemin-induced apoptosis

Time-response studies indicated that ascididemin (5 µM) stimulated the processing of procaspase-2 as early as 4h after stimulation (figure 33). Etoposide was used as positive control (Robertson *et al.*, 2000).

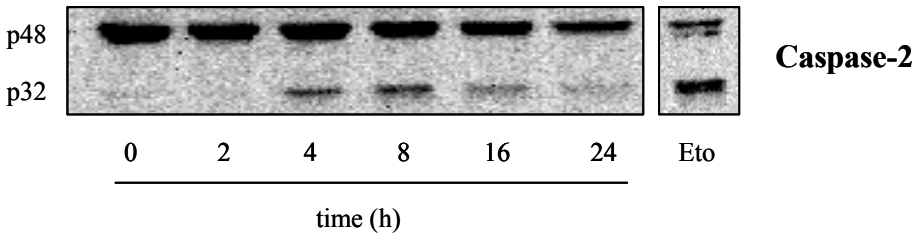


Figure 33: Activation of caspase-2 in response of ascididemin treatment.
Cells were either left untreated (0 h) or incubated with ascididemin (5 µM) for different time periods and processed caspase-2 was detected as described under "Materials and Methods". Etoposide (25 µg/ml, 8 h) was used as positive control. The figure shows one representative western blot out of three independent expriments.

The crucial involvement of caspase-2 in the apoptotic signalling of ascididemin was proven by prevention of DNA fragmentation in the presence of the specific caspase-2 inhibitor zVDVAD-fmk (25 µM, preincubation 1 h) (figure 34).

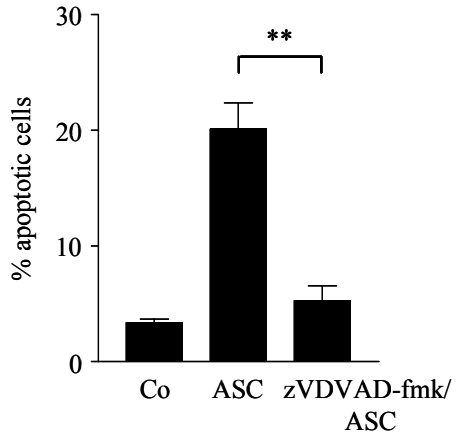


Figure 34: Caspase-2 activation is required for ascididemin-induced apoptosis.
*FACS analysis as described under "Materials and Methods" of Jurkat T cells either left untreated (Co), treated with ascididemin (ASC, 5 µM, 8 h) or pretreated with the specific caspase-2 inhibitor zVDVAD-fmk (25 µM, 1 h) and subsequently stimulated with ascididemin (5 µM). Bars indicate the mean ± SEM of three independent experiments performed in duplicate; ** $p < 0.01$ implicates significant difference compared to values seen in ascididemin-treated cells (Student´s t test).*

4.3.2 Caspase-2 acts as an initiator caspase

The hierarchical collocation of caspase-2 within the caspase cascade is discussed controversially. On the one hand, caspase-2 activation is reported as an initial and very early event, respectively, (Harvey *et al.*, 1997; Droin *et al.*, 2001; Robertson *et al.*, 2002). On the other hand, caspase-2 has been shown to be activated downstream of caspase-9 and caspase-3 (Slee *et al.*, 1999; Li *et al.*, 1997; O'Reilly *et al.*, 2002).

Caspase-2 activation occurs upstream of mitochondria

The fact that the processing of procaspase-2 kinetically paralleled the cleavage of procaspase–9 (figure 30) raises the question of whether caspase-2 acts as initiator caspase within the ascididemin-induced caspase cascade. To investigate at what stage in the apoptotic pathway caspase-2 activation occurs, Jurkat/*bcl-x*$_L$ cells, which are protected against ascididemin-induced mitochondrial apoptosis (figure 29), were examined for the processing of caspase-2 after treatment with ascididemin (5 µM). In fact, the cleavage of caspase-2 was found in both cell types in response to ascididemin indicating that caspase-2 activation occurs upstream of mitochondria (figure 35). However, in Jurkat/*bcl-x*$_L$ caspase-2 was activated to a lower extent than in Jurkat/*neo*. Etoposide (25 µg/ml) was used as positive control (Robertson *et al.*, 2000).

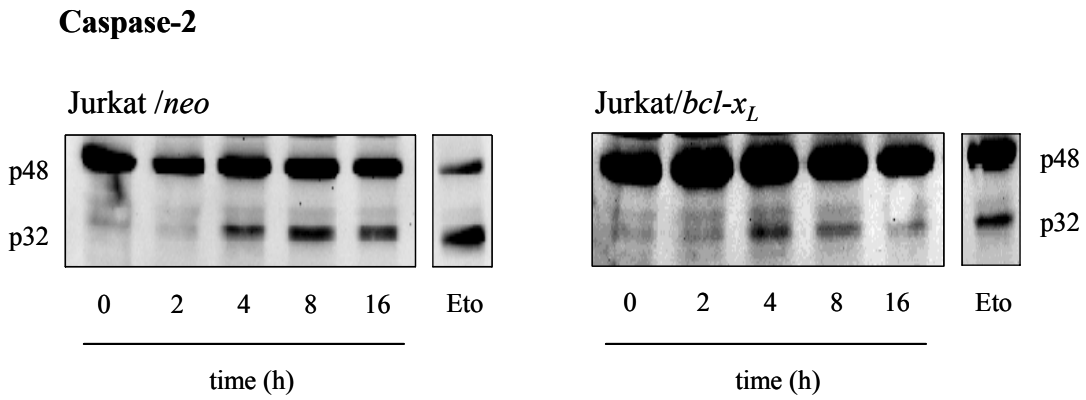


Figure 35: Caspase-2 is activated upstream of mitochondria.
Representative western blot showing the time-dependent cleavage of procaspase-2 to its active subunit in Jurkat/neo and Jurkat/bcl-x$_L$ cells. Control cells (Jurkat/neo) and cells overexpressing Bcl-x$_L$ (Jurkat/bcl-x$_L$) were incubated with ascididemin (5 µM) for the indicated time periods. Etoposide (Eto, 25 µg/ml, 8 h) was used as positive control. Experiments were performed three times with similar results.

To confirm that caspase-8 functions as an effector caspase in the apoptotic pathway of ascididemin, activation of caspase-8 was investigated in Jurkat/*neo* and Jurkat/*bcl-x*$_L$. As depicted in figure 36, cleavage of caspase-8, in fact, only occurred in Jurkat/*neo*,

whereas overexpression of Bcl-x_L prevented the activation of caspase-8. These results corroborate again that caspase-8 acts as an effector caspase downstream of mitochondria.

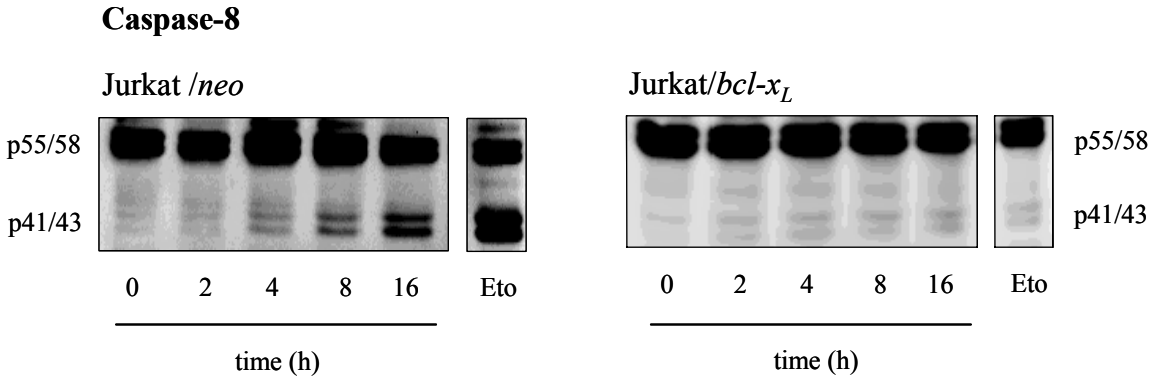


Figure 36: Processing of procaspase-8 occurs downstream of mitochondria.
Representative western blots indicating activation of caspase-8 as a mitochondria-dependent event. Control cells (Jurkat/neo) and cells overexpressing Bcl-x_L (Jurkat/bcl-x_L) were incubated with ascididemin (5 µM) for the indicated time periods. Etoposide (Eto, 25 µg/ml, 8 h) was used as positive control. Experiments were performed three times with similar results.

Caspases contribute to the cleavage of caspase-2

Since caspase-2 was activated to a lower extent upstream of mitochondria, an amplification of caspase-2 activation within the caspase cascade was supposed. Indeed, inhibition of active caspases by the broadspectrum caspase inhibitor zVAD-fmk (25 µM; preincubation 1 h) led to an attenuation of processing of caspase-2 in response to ascididemin (5 µM, 8 h) (figure 37).

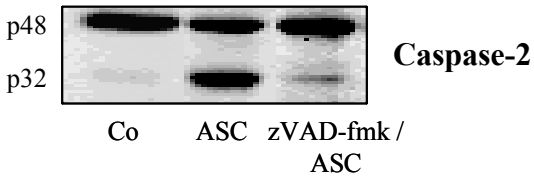


Figure 37: zVAD-fmk reduces activation of caspase-2.
Jurkat T cells were either left untreated (Co) or stimulated with ascididemin (5 µM, 8 h) in the absence (ASC) or presence of the broadspectrum caspase inhibitor zVAD-fmk (25 µM, preincubation 1 h) and probed for cleavage of caspase-2 as described under "Materials and Methods". The figure shows one representative western blot out of three independent experiments.

Activated caspase-2 affects cytochrome c release

Due to the observation that caspase-2 is activated upstream of mitochondria a possible influence on cytochrome *c* release came into question. To determine whether caspase-2 was required for ascididemin-induced cytochrome *c* release, Jurkat T cells were treated with 5 µM ascididemin for 8 h in the absence or presence of the specific caspase-2 inhibitor zVDVAD-fmk (25 µM). The results indicated that pretreatment with zVDVAD-fmk for 1 h diminished translocation of cytochrome *c* from the mitochondria into the cytoplasm (figure 38). Thus, caspase-2 is considered as an important inductor of cytochrome *c* release upstream of mitochondria.

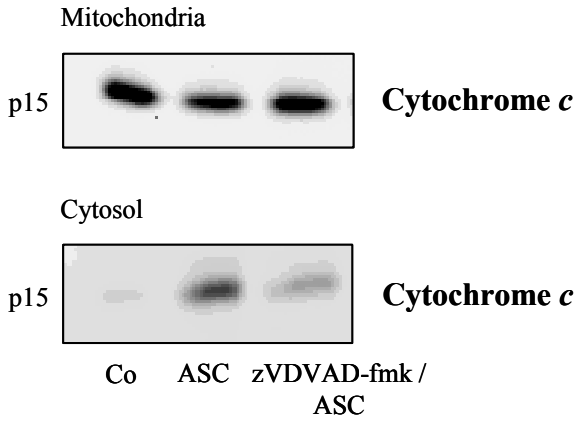


Figure 38: Caspase-2 inhibition affects ascididemin-triggered cytochrome c release.
Jurkat T cells, either left untreated (Co) or treated with 5 µM ascididemin for 8 h in the absence (ASC) or presence of zVDVAD-fmk (pretreatment 1 h) were analysed for cytochrome c release by western blot analysis as described under "Materials and Methods". The figure shows one representative western blot out of two independent experiments.

Caspase-2 contributes to the cleavage of caspase-9

Since caspase-2 inhibition attenuated ascididemin-mediated cytochrome *c* release the involvement of caspase-2 in the activation of caspase-9 was assumed. To test this hypothesis, Jurkat T cells were investigated for caspase-9 cleavage by western blot analysis. Cells were stimulated with ascididemin (5 µM) for 8 h in the absence or presence of the specific caspase-2 inhibitor zVDVAD-fmk (25 µM, pretreatment 1 h). The obtained data demonstrated that inhibition of active caspase-2 resulted in reduced cleavage of caspase-9 (figure 39). Hence, caspase-2 activation is supposed as an initial event in ascididemin-mediated apoptosis.

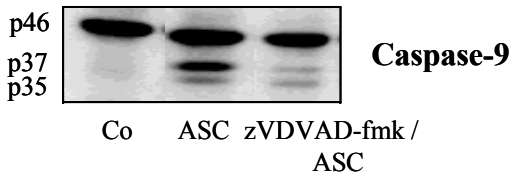


Figure 39: Processing of caspase-9 is influenced by inhibition of caspase-2.
Representative western blot showing reduced processing of procaspase-9 into its intermediates in response to ascididemin (5 µM) stimulation of Jurkat T cells for 8 h with additional pretreatment for 1 h with the specific caspase-2 inhibitor zVDVAD-fmk (25 µM). The figure shows one out of three independent experiments.

Caspase-2 is activated prior to the processing of caspase-9

To confirm the finding of an initial activation of caspase-2, measurement of caspase-2 activity in relation to caspase-9 activity was performed by applying fluorometric caspase activity assays. Following treatment of Jurkat T cells with 5 µM ascididemin for up to 8 h a significant increase in caspase-2 activity was already detected after 3 h of incubation, whereas caspase-9 is still inactive (figure 40). These data offer additional support that caspase-2 acts as an initiator caspase in the ascididemin-triggered apoptotic signalling.

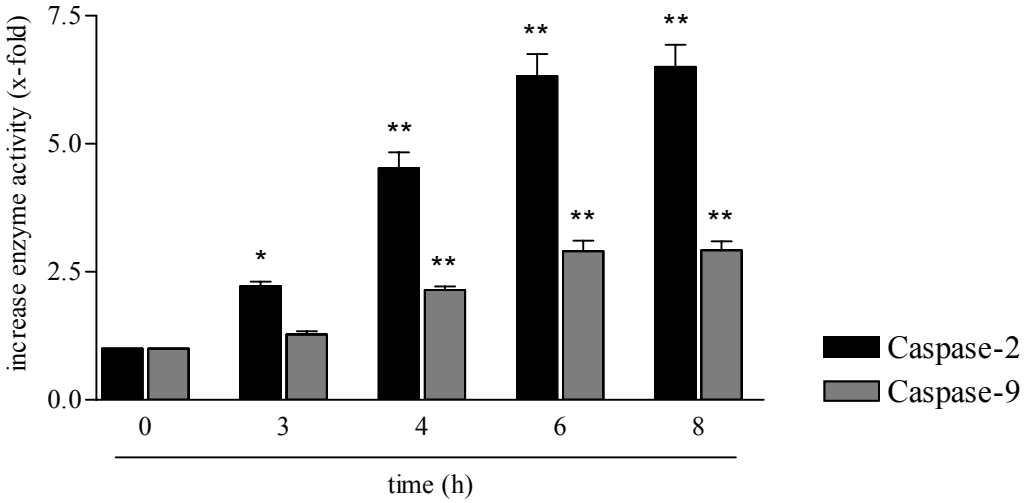


Figure 40: Caspase-2 activity preceds caspase-9 activity.
*Comparison of caspase-2 and caspase-9 enzyme activity in Jurkat T cells treated with ascididemin (5 µM) for the indicated time periods. Measurement was performed by the fluorometric VDVAD-AFC cleavage assay and LEHD-AFC cleavage assay, respectively, as described under "Materials and Methods". Bars represent the mean ± SD of three independent experiments performed in duplicate. * $p<0.05$, ** $p<0.01$ significantly different compared to untreated control (0 h) (ANOVA/Dunnett).*

Activated caspase-2 cleaves Bid

The proapoptotic protein Bid transduces the apoptotic signal from activated caspase-8 to mitochondria in the CD95 receptor mediated signalling cascade. *In vitro* studies demonstrated that also recombinant active caspase-2 is able to cleave Bid (Paroni *et al.*, 2001). To investigate whether caspase-2 transduces the apoptotic signal *via* cleavage of Bid in this system, Jurkat T cells were stimulated with ascididemin (5 µM, 8 h) in the presence or absence of the specific caspase-2 inhibitor zVDVAD-fmk (25 µM, pretreatment 1 h). The results revealed Bid as a target of activated caspase-2 in the response of ascididemin treatment (figure 41).

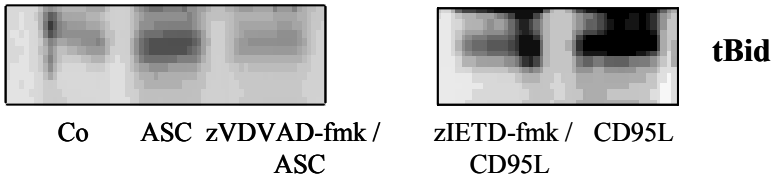


Figure 41: Activated caspase-2 cleaves Bid.
Representative western blot showing abrogation of Bid cleavage in response to pretreatment with 25 µM of the specific caspase-2 inhibitor zVDVAD-fmk (1 h) and subsequent stimulation with ascididemin (5 µM, 8 h) in comparison of treatment with ascididemin alone. Soluble CD95L (100 ng/ml, 8 h) alone as well as preincubation with zIETD-fmk, the specific caspase-8 inhibitor, (10 µM, 1 h) followed by stimulation with soluble CD95L were used as positive control. The figure shows one out of three independent experiments.

5 Ascididemin treatment leads to generation of reactive oxygen species

Oxidative stress has been proposed as a potential mediator of apoptosis (Buttke and Sandstrom, 1994). Anticancer drugs like camptothecin and actinomycin D have been shown to rapidly produce reactive oxygen species (ROS) during the induction of leukemic cell apoptosis (Creagh and Cotter, 1999; McGowan *et al.*, 1996; Gorman *et al.*, 1997a). Matsumoto and coworkers showed that ascididemin is able to cleave isolated plasmid DNA mediated by ROS *in vitro* (Matsumoto *et al.*, 2000). To assess initial intracellular peroxide production in Jurkat T cells FACS analysis was performed empolying the redox-sensitive fluorescent dye carboxy-H_2DCFDA. Ascididemin treatment (5 µM) of Jurkat T cells caused a significant peroxide production as early as 30 min after stimulation (figure 42). Staurosporine (0.5 µM) was used as positive control (Cai and Jones, 1998).

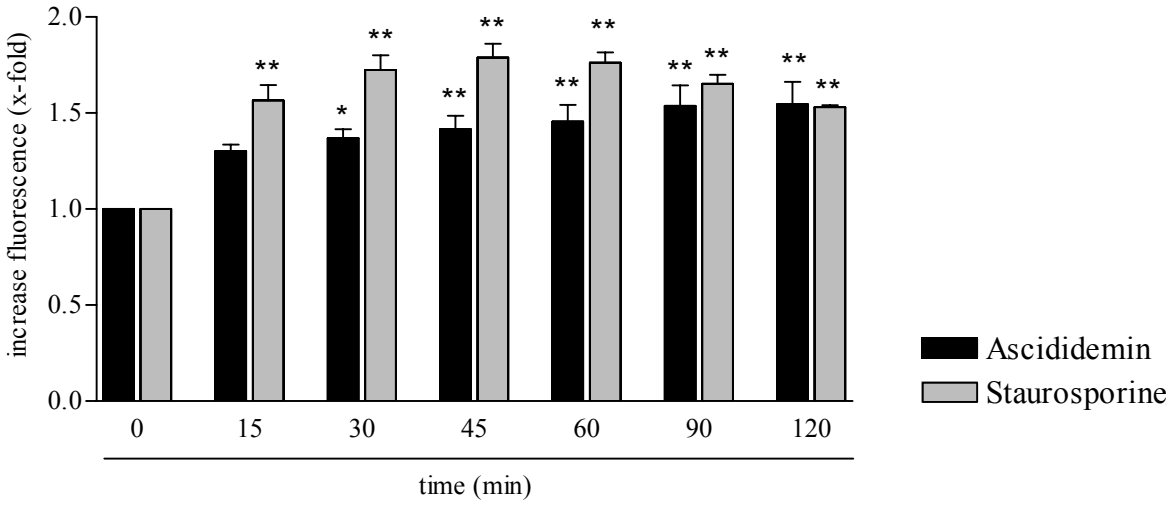


Figure 42: Ascididemin induces generation of reactive oxygen species in Jurkat T cells.
*Cells were preloaded with 5 µM carboxy-H_2DCFDA and exposed to 5 µM ascididemin. At indicated time points, the intracellular fluorescence was quantified by flow cytometry as described in "Materials and Methods". Bars indicate the mean ± SE of three independent experiments performed in duplicate. * $p<0.05$, ** $p<0.01$ significantly different compared to values seen in untreated control cells (0 min) (ANOVA/Dunnett).*

To elucidate if ROS play a critical role in the apoptotic process mediated by ascididemin, DNA fragmentation of ascididemin treated Jurkat T cells (5 µM; 8 h) was assessed in the presence or absence of 20 mM N-acetylcysteine (NAC), a well established thiol antioxidant. Pretreatment of NAC did not alter the rate of apoptotic cells in comparison to the rate obtained for ascididemin alone (figure 43). These data suggest that ROS do not affect late apoptotic events in ascididemin-induced apoptosis.

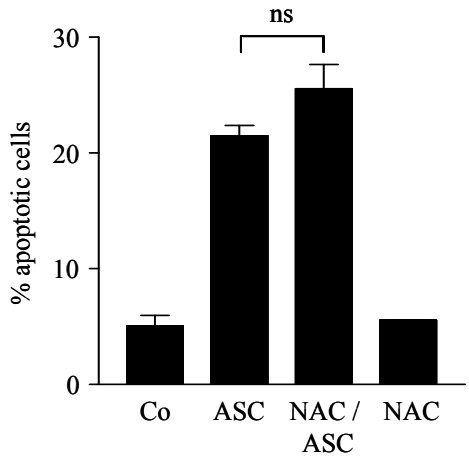


Figure 43: N-acetylcysteine (NAC) does not reduce ascididemin-triggered DNA fragmentation. Cells were left untreated (Co), treated with ascididemin (ASC, 5 µM, 8 h), or pretreated with NAC (20 mM, 1 h) and then incubated with ascididemin (ASC, 5 µM). Apoptotic cells were quantified by FACS as described under "Materials and Methods". Bars represent the mean ± SEM of three independent experiments performed in duplicate; ns p> 0.05 implicates no significance compared to values seen in ascididemin-treated cells (Student´s t test).

6 The role of MAPK and Akt in ascididemin-triggered apoptosis

6.1 The activation of MAPK

In response to oxidative stress the activation of mitogen-activated protein kinases (MAPK) have been reported (Shiah *et al.*, 1999; Banno Y. *et al.*, 2001). The activation of c-Jun N-terminal kinases (JNK) and p38 kinases is mainly correlated with cell death or apoptosis. In contrast, activated extracellular signal-regulated kinases (Erk) are related to cell survival and proliferation. Given the generation of ROS by ascididemin, activation of JNK, p38 and Erk, three subfamilies of the MAPK, could be considered. To address this question, western blot analysis of phosphorylated, i.e. activated, MAPK was performed. Time-course studies clearly detected phosphorylated p38 and JNK after treatment with ascididemin (5 µM) for 2 h and 4 h, respectively (figure 44).

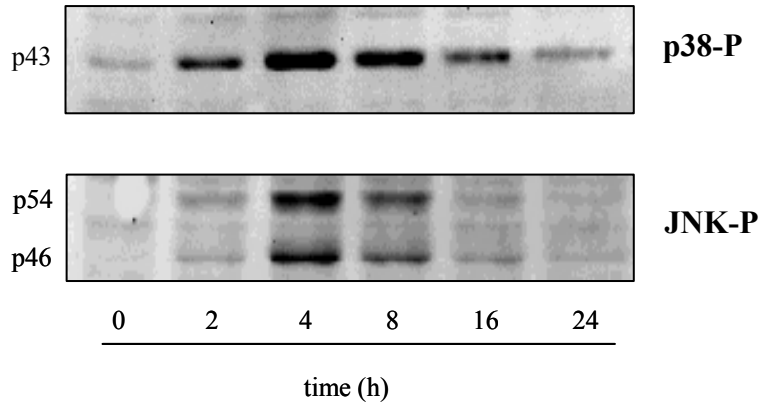


Figure 44: Phosphorylation of p38 and JNK following ascididemin treatment in Jurkat T cells. Cells were either left untreated or treated with ascididemin (5 µM) for the indicated time periods and probed for phosphorylation of p38 (upper panel) and JNK (lower panel), respectively, by western blot analysis as described under "Materials and Methods". The figure shows one representative western blot out of three independent experiments for each kinase.

In contrast, the level of phosphorylated Erk was unaltered, rather slightly decreased, in response to ascididemin treatment (figure 45). Phytohaemagglutinine (PHA) in combination with phorbol 12-myristate 13-acetate (PMA), a known activator of the Erk cascade, was used as positive control (Li *et al.*, 1999).

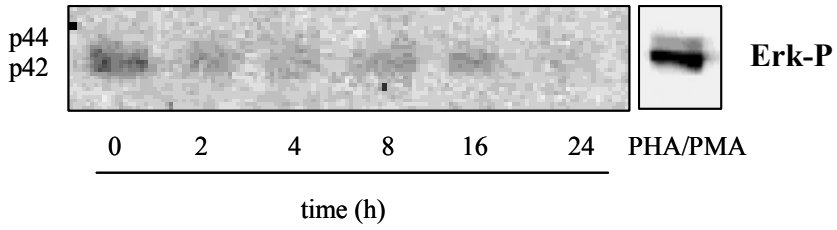


Figure 45: Erk is not phosphorylated in response to ascididemin treatment of Jurkat T cells. Cells were either left untreated or treated with ascididemin (5 µM) for the indicated time periods and probed for phosphorylation of Erk by western blot analysis as described under "Materials and Methods". PHA (1 µg/ml)/PMA (100 nM) treatment (5 min) was used as positive control. The figure shows one representative western blot out of three independent experiments.

6.2 Influence of activated JNK and p38 on ascididemin-mediated apoptosis

The impact of activated MAPK in apoptosis is highly divergent. Apoptotic as well as anti-apoptotic influences during the response to different stimuli are reported (Ortiz *et al.*, 2001; Xia *et al.*, 1995; MacFarlane *et al.*, 2000; Lenczowski *et al.*, 1997). The precise role of these activated kinases appears to be largely dependent on cell typ and stimulus. To examine the influence of activated p38 and JNK on ascididemin-induced apoptosis in Jurkat T cells, DNA fragmentation was measured by FACS analysis employing the specific kinase inhibitors SB203580 (Young *et al.*, 1997) and SP600125 (Bennett *et al.*, 2001), respectively. Both inhibit the catalytic activity of the specific kinase. Preincubation with the p38 inhibitor SB203580 (10 µM; 45 min) did not alter the rate of ascididemin-mediated apoptosis (5 µM; 8 h), indicating that activation of p38 occurs without any relevance to the apoptotic signalling (figure 46, left panel). In contrast, application of the JNK inhibitor SP600126 (10 µM; 15 min) caused a significant reduction of DNA fragmentation (figure 46, right panel). Due to the fact that DNA fragmentation could be blocked only partially by SP600125, the role for active JNK to the apoptotic pathway of ascididemin is rather contributory.

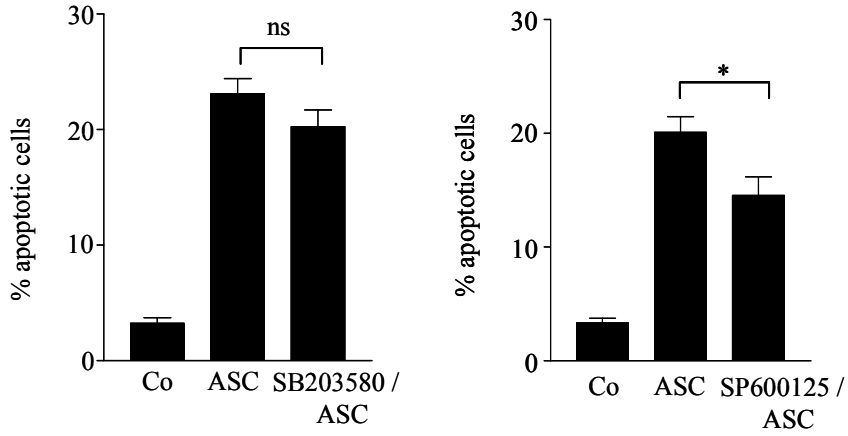


*Figure 46: Causal involvement of active p38 and active JNK in ascididemin-mediated apoptosis. FACS analysis of DNA fragmentation in Jurkat T cells was performed as described in "Materials and Methods". Cells were either preincubated with the specific inhibitor of p38 (SB203580, 10 µM, 45 min) (left panel) and the specific inhibitor of JNK (SP600125, 10 µM, 15 min) (right panel), respectively, and subsequently stimulated with ascididemin (ASC, 5 µM, 8 h) or treated with ascididemin alone, as indicated. Control cells (Co) were left untreated. Bars represent the mean ± SEM of three independent experiments performed in duplicate. Ns $p>0.05$ no significance compared to values seen in ascididemin-treated cells, * $p<0.05$ significantly different compared to values seen in ascididemin-treated cells (Student´s t-test).*

JNK activation occurs upstream of mitochondria

Some reports demonstrated that Bcl-x_L and Bcl-2 are able to inhibit the induction of JNK (Srivastava *et al.*, 1999; Park *et al.*, 1997). Therefore, a possible relationship between mitochondrial pertubation and phosphorylation of JNK was examined. Overexpression of Bcl-2 or Bcl-x_L in Jurkat T cells abolished apoptosis in response to ascididemin (figure 29). However, no difference in ascididemin-induced activation of JNK was detected in these cell lines compared to control cells (Jurkat/*neo*) (figure 47). These results indicate that JNK is activated independently of mitochondrial events.

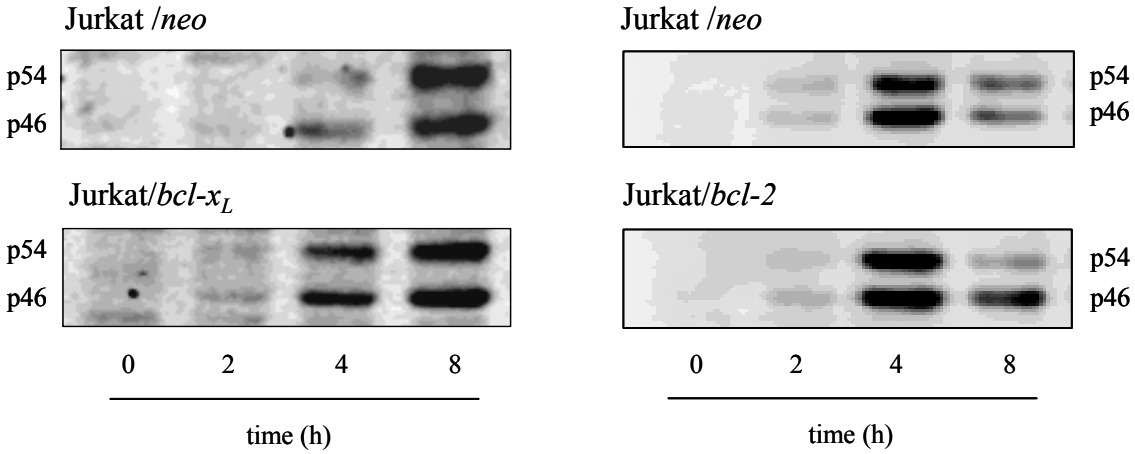


Figure 47: JNK activation occurs upstream of mitochondria.
Control cells (Jurkat/neo) and cells overexpressing Bcl-x_L (Jurkat/bcl-x_L) and Bcl-2 (Jurkat/bcl-2) were incubated with ascididemin (5 µM) for the indicated time periods. Western blot analysis of phosphorylated JNK was carried out as described under "Materials and Methods". The figure shows one representative out of three independent experiments.

Activation of JNK is necessary for cytochrome c release

Previous studies reported that JNK is able to translocate to and directly activate mitochondria (Kharbanda *et al.*, 2000; Aoki *et al.*, 2002), and that JNK is required for cytochrome *c* release (Tournier *et al.*, 2000). Employing the specific JNK inhibitor SP600125 (10 µM; preincubation 45 min) a putative impact of activated JNK on the mitochondrial release of cytochrome *c* into the cytoplasm in response to ascididemin treatment (5 µM; 6 h) was investigated. Western blot analysis confirmed a strong impact of active JNK on cytochrome *c* release in Jurkat T cells (figure 48).

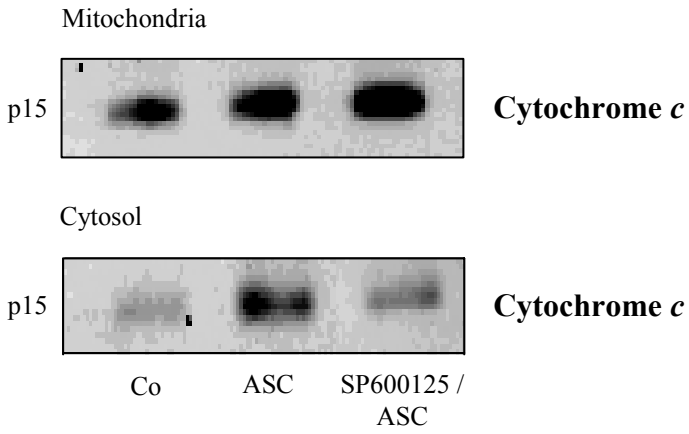


Figure 48: Activated JNK is required for cytochrome c release.
Jurkat T cells were either left untreated (Co), treated with ascididemin (ASC, 5 µM, 6 h) alone or pretreated with the JNK inhibitor SP600125 (10 µM, 45 min) and then incubated with ascididemin (5 µM). Sample preparation and western blot analysis was performed as described under "Materials and Methods". Experiments were performed three times with similar results.

Bcl-2 as possible target of JNK

Several studies demonstrated that phosphorylation and therefore inactivation of the anti-apoptotic protein Bcl-2 is associated with cell death (Haldar *et al.*, 1995). JNK was found to mediate the phosphorylation of Bcl-2 (Maundrell *et al.*, 1997; Yamamoto *et al.*, 1999). To test whether Bcl-2 is phosphorylated in response to ascididemin treatment, western blot analysis was performed. In contrast to taxol (1 µM), used as positive control (Yamamoto *et al.*, 1999), ascididemin did not induce phosphorylation of Bcl-2 (figure 49).

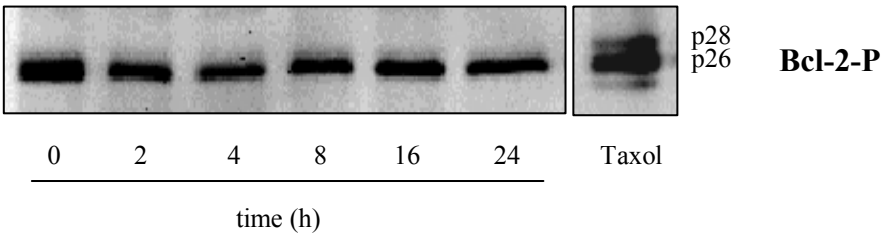


Figure 49: Ascididemin does not induce phosphorylation of Bcl-2.
Jurkat T cells were treated with ascididemin (5 µM) for the indicated time periods and probed for phosphorylated Bcl-2 as described under "Materials and Methods". Taxol (1 µM, 24 h) was used as positive control. Experiments were performed three times with similar results.

6.3 The activation of Akt

Akt is activated by ascididemin

The PI3K-regulated protein kinase Akt plays, like Erk, a critical role in cell survival. Western blot analysis exhibited that ascididemin (5 µM) activated Akt in Jurkat T cells in a time-dependent manner up to 8 h (figure 50).

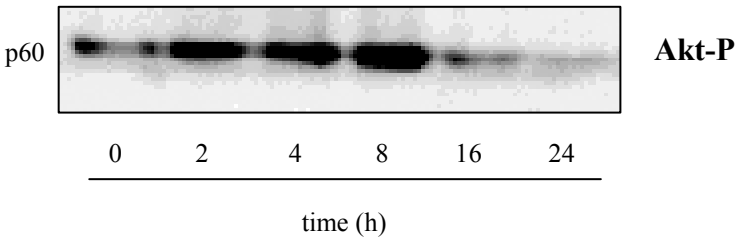


Figure 50: Ascididemin activates Akt in Jurkat T cells.
Cells were either left untreated or treated with ascididemin (5 µM) for the indicated time periods and analysed for phosphorylation of Akt by western blot analysis as described under "Materials and Methods". Figure shows one representative western blot out of three independent experiments.

Activated Akt does not affect ascididemin-induced apoptosis

Tang *et al.* (Tang *et al.*, 2001) could show that activated Akt delays etoposide-induced apoptosis. To investigate a potential significance of the active survival kinase Akt in the apoptotic pathway of ascididemin, Jurkat T cells were treated with ascididemin (5 µM) in the presence or absence of wortmannin (50 nM; 45 min preincubation), a PI3K inhibitor, and monitored for DNA fragmentation by flow cytometry. Indeed, inhibition of PI3-kinase by wortmannin abolished the ascididemin-induced phosphorylation of its downstream mediator Akt (figure 51, left panel). However, inhibition of Akt activation did not change the rate of apoptosis compared to ascididemin treatment alone (figure 51, right panel). Together, these data show that activation of the survival kinase Akt is without any relevance to the apoptotic process triggered by ascididemin.

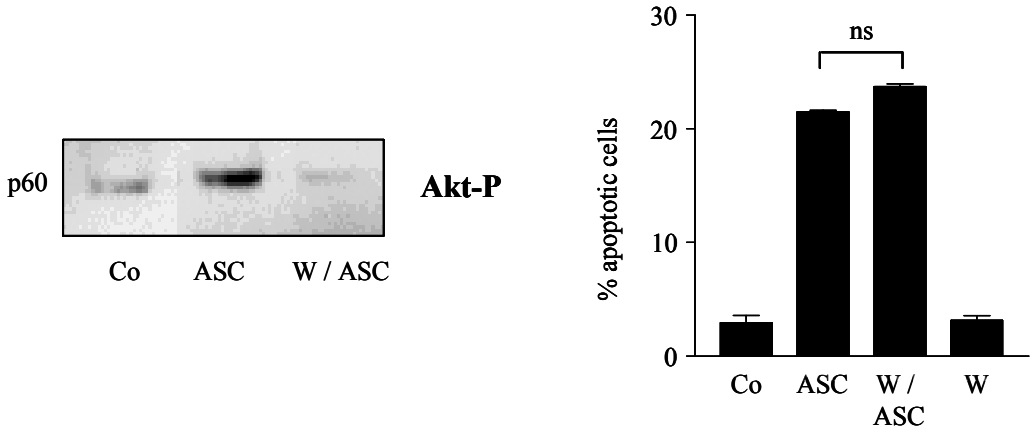


Figure 51: Activated Akt does not affect ascididemin-induced apoptosis.
One representative western blot out of three showing the inhibitory effect of wortmannin (50 nM, preincubation 45 min) on Akt phosphorylation in response to ascididemin treatment (5 µM, 2 h) (left panel). Jurkat T cells were either treated with ascididemin (5 µM, 8 h) alone or pretreated with the PI3K inhibitor wortmannin (50 nM, preincubation 45 min) and subsequently stimulated with ascididemin. Control cells (Co) were left untreated (right panel). FACS analysis of DNA fragmentation was performed as described under "Materials and Methods". Bars indicate the mean ± SEM of three independent experiments performed in duplicate. Ns implicates no significance compared to values seen in ascididemin-treated cells (Student´s t-test).

7 The signalling upstream of mitochondria

Since it was shown that caspase-2 (see 3.4.3.2) as well as JNK (see 3.6.1.3) act upstream of mitochondria, it should be clarified whether a cross-talk exists between the caspase signalling and the MAPK pathway.

7.1 Role of ROS in the activation of JNK and caspases

Cellular oxidative stress is a key mediator for activating members of the MAPK pathways, e.g. the JNK pathway. In order to determine if phosphorylation of JNK is induced by ROS, Jurkat T cells were treated with ascididemin (5 µM, 4 h / 8 h) in the presence or absence of N-acetylcysteine (NAC). JNK phosphorylation was almost completely blocked by NAC only at the early time point (4 h), indicating that ROS is essential for the initial activation of the JNK pathway (figure 52).

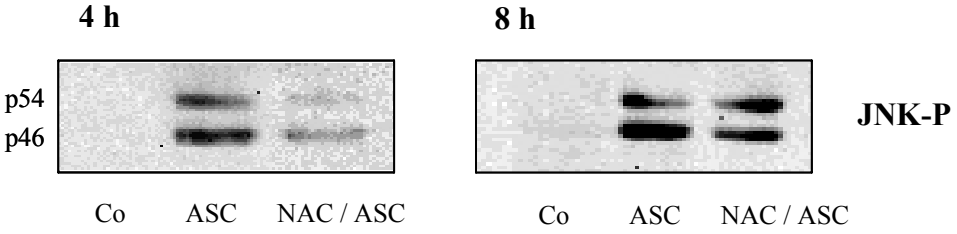


Figure 52: N-acetylcysteine (NAC) blocks phosphorylation of JNK at early time points.
Jurakt T cells were either left untreated (Co), treated with ascididemin (5 µM) for 4 h (left panel) and 8 h (right panel), respectively, or pretreated for 1 h with NAC (20 mM) and subsequently stimulated with ascididemin (5 µM). Phosphorylated JNK was detected by western blot analysis as described under "Materials and Methods". The figure shows one representative western blot out of three independent experiments.

To test a possible impact of ROS on the processing of caspases, cells were treated as in the experiment described above. Western blot analysis detecting caspase-2 and caspase-9 revealed a selective abrogation of caspase cleavage in response to NAC pretreatment followed by ascididemin incubation. Processing of caspase-2 was unaffected by NAC, whereas cleavage of caspase-9 was abolished (figure 53). These data indicate that activation of caspase-2 occurs independently of ROS. However, ROS are involved in the activation of caspase-9.

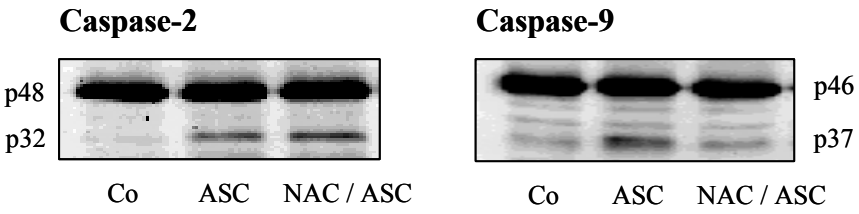


Figure 53: NAC selectively inhibits caspase cleavage.
Jurakt T cells were either left untreated (Co), treated with ascididemin (5 µM, 4 h) or pretreated for 1 h with NAC (20 mM) and subsequently stimulated with ascididemin (5 µM). Cleavage of caspase-2 (left panel) and caspase-9 (right panel), respectively, was detected by western blot analysis as described under "Materials and Methods". The figure shows one representative western blot out of three independent experiments.

7.2 Activated JNK contributes to caspase activation

To identify putative interactions between the signalling pathway of caspases and the regulation of phosphorylation of JNK, the broadspectrum caspase inhibitor zVAD-fmk was used. Pretreatment for 1 h with zVAD-fmk (25 µM) and subsequent incubation with ascididemin (5 µM, 8 h) did not interfere with JNK phosphorylation, demonstrating that the activation of JNK occurs independently of active caspases (figure 54).

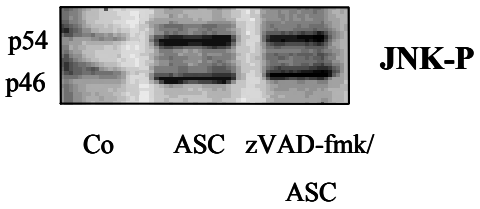


Figure 54: Caspase inhibition by zVAD-fmk does not interfere with JNK phosphorylation. Jurkat T cells were either left untreated (Co), treated with ascididemin (5 µM, 8 h) or pretreated for 1 h with the broadspectrum caspase inhibitor zVAD-fmk (25 µM) and subsequently stimulated with ascididemin (5 µM). JNK phosphorylation was detected by western blot analysis as described under "Materials and Methods". The figure shows one representative western blot out of three independent experiments.

On the other hand, inhibition of caspase-2 and caspase-9 activation by the specific JNK inhibitor SP600125 suggested a contributory role of active JNK to the activation of caspase-2 and caspase-9 at least at early time points (4h) (figure 55).

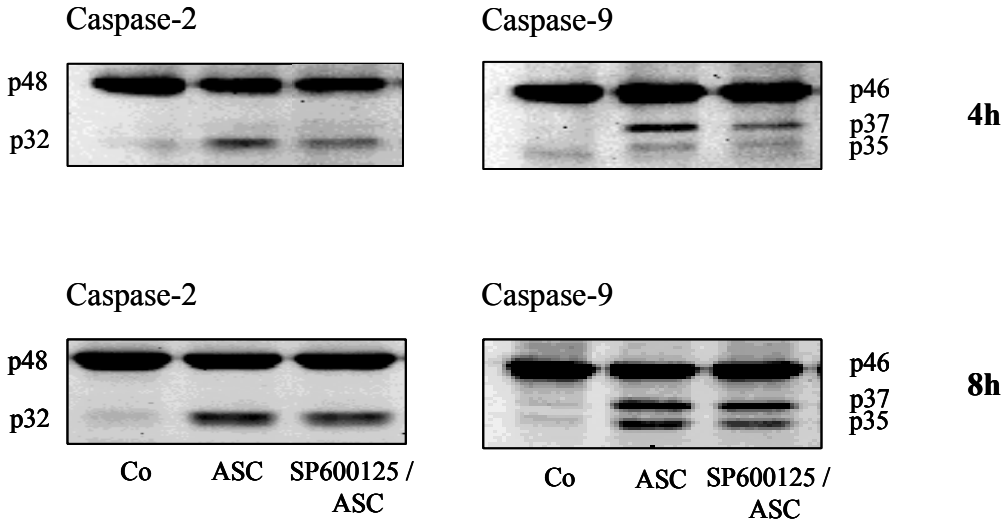


Figure 55: Cleavage of caspase-2 and caspase-9 is reduced by the specific JNK inhibitor SP600125 at early time points.
Jurkat T cells were either left untreated (Co), treated with ascididemin (ASC, 5 µM, 6 h) alone or pretreated with the specific JNK inhibitor SP600125 (10 µM, 45 min) and then incubated with ascididemin (5 µM). Sample preparation and western blot analysis was performed as described under "Materials and Methods". Experiments were performed three times with similar results.

F. Discussion

The marine pyridoacridine alkaloid ascididemin has previously been shown to exhibit antitumour properties against different cancer cell lines (Delfourne *et al.*, 2002; Bonnard *et al.*, 1995). Aim of the present studies was to clarify its cytotoxic effect towards the human acute leukemia T cell line Jurkat with emphasis on the apoptotic signalling pathway triggered by ascididemin.

1 Ascididemin-induced apoptosis in Jurkat T cells

1.1 Cytotoxicity of ascididemin and two analogues

The results obtained by applying the MTT assay demonstrate that ascididemin exhibits marked dose-dependent cytotoxic potency against Jurkat T cells *in vitro* with an IC_{50} value of 0.6 µM.

Concerning the structure-activity relationship of ascididemin, Lindsay *et al.* showed that the nitrogen in ring A and a completed ring E are prerequisites for the biological activity of ascididemin (Lindsay *et al.*, 1995). Furthermore, a previous study reported that the ring D-analogue 5-methoxyascididemin showed improved cytotoxic activity against solid tumours. The effects, however, were strongly dependent on the cancer cell line (Delfourne *et al.*, 2002). Compared to the parent alkaloid, 5-methoxyascididemin showed a lower level of cytotoxicity towards Jurkat T cells, demonstrated by a 10-fold higher IC_{50} value (IC_{50} = 6.5 µM). Moreover, 5-hydroxyascididemin failed to show any relevant cytotoxic properties. In conclusion, the substitution pattern of 5-methoxyascididemin and 5-hydroxyascididemin negatively affects the cytotoxic activity of the natural compound ascididemin against the human leukemia cell line Jurkat.

1.2 Ascididemin induces apoptotic cell death in Jurkat T cells

The induction of apoptosis in response to the exposure of cytotoxic drugs is subject of intense investigation. Apoptosis is charactarised by a variety of distinct morphological and biochemical features that are used to discriminate the apoptotic cell death against necrosis. Commonly accepted methods were used to evaluate ascididemin-induced cell death in Jurkat T cells.

Light microscopy combined with flow cytometry visualised the occurrence of the characteristic cell shrinkage and formation of apoptotic bodies in response to ascididemin treatment. Alterations of the cell membrane, namely translocation of phosphatidylserine to the outer cell membrane, was detectable by flow cytometry using Annexin V-FITC. Costaining with propidium iodide (PI) allowed to clearly exclude necrotic cell death.

Apoptotic morphological changes concerning the nucleus were detected by staining of condensed chromatin with Hoechst 33342 and subsequent fluorescence microscopy. Flow cytometry of PI stained nuclei revealed DNA fragmentation in a dose- and time-dependent manner. Ascididemin, a DNA damaging agent due to its intercalating properties, predominantely targets cells in the S phase.

2 Ascididemin-induced apoptosis occurs independently of a functional CD95 receptor/CD95 L interaction in Jurkat T cells

The CD95 receptor is expressed on most T-acute lymphatic leukemia (T-ALL) cell lines such as Jurkat and on leukemic cells from patients. However, the contribution of the CD95 pathway to the cytotoxic activity of anticancer drugs remains controversial. Anticancer drugs, like the DNA damaging topoisomerase II enzyme inhibitors etoposide and doxorubicin, have been shown, with some exceptions (McGahon *et al.*, 1998), to enhance both CD95 receptor and CD95 ligand (CD95L) expression on tumor cells (Micheau *et al.*, 1997; Mo and Beck, 1999; Siitonen *et al.*, 2000). However, the concept of involvement of the CD95 receptor/CD95L system in drug-induced apoptosis (Fulda *et al.*, 2000; Friesen *et al.*, 1996) has been challenged by several recent studies (Villunger *et al.*, 1997; Micheau *et al.*, 1999; Siitonen *et al.*, 2000; Eischen *et al.*, 1997): though CD95L is upregulated, the apoptotic cell death occurs independently of CD95 signalling. Direct activation of the CD95 receptor independent of death receptor ligand ligation has also been proposed (Gajate *et al.*, 2000; Aragane *et al.*, 1998).

The present data show that functional CD95 receptor/CD95L interaction is not a prerequisite for ascididemin-triggered apoptosis in Jurkat T cells. Inhibition of protein synthesis with cycloheximide (CHX) provided evidence that ascididemin-triggered apoptosis did not require synthesis of new protein. Thus, the requirement of new CD95L expression can be ruled out. Inhibition of CD95 receptor engagement with a neutralising anti-CD95 antibody (ZB4) had no effect on the rate of apoptotic cells compared to unblocked cells. This observation supports the assumption of an apoptotic signalling independent of a functional CD95 receptor/CD95L interaction. Jurkat^{R} T cells, a CD95-resistant Jurkat subline characterised by the lack of expression of receptor

protein, were also sensitive for ascididemin-induced apoptosis in a dose-dependent manner.

However, at higher concentrations of ascididemin, the rate of apoptotic cells was slightly decreased in comparison to CD95-sensitive Jurkat T cells. This observation might point to the requirement of intracellular events at the receptor level for the apoptotic signalling mediated by high concentrations of ascididemin. Micheau *et al*. were able to show that anticancer drugs can induce trimerisation of the CD95 receptor independently of CD95L as well as the recruitment of the adapter molecule FADD to the receptor in the cytotoxic process leading to apoptosis (Micheau *et al.*, 1999). However, the recruitment of the CD95 receptor might rather contribute to than playing a major role in the apoptotic signal transduction of ascididemin.

3 Mitochondria play a critical role in ascididemin-triggered apoptosis

Besides the death receptors, mitochondria play a central role in the transduction of apoptotic signals triggered by many stimuli. Mitochondrial membrane permeabilisation and the release of apoptogenic factors, e.g. cytochrome *c*, are key events in the process leading to apoptosis (Kroemer and Reed, 2000). Anti-apoptotic proteins of the Bcl-2 family, like Bcl-x_L and Bcl-2, exert their protective effect at the mitochondrial level (Yang *et al.*, 1997a; Decaudin *et al.*, 1997).

The results of the present work show that, indeed, redistribution of cytochrome *c* into the cytoplasm occurred in response to ascididemin treatment. Translocation of cytochrome *c* from the mitochondrial intermembrane space to the cytosol occurs in consequence of outer mitochondrial membrane permeabilisation (OMP). Inner mitochondrial membrane permeabilisation (IMP) is manifested as a dissipation of the transmembrane potential ($\Delta\psi_m$). In most cases mitochondrial dysfunction implicates both, OMP and IMP. However, OMP and IMP are not necessarily coupled as shown for staurosporine (Bossy-Wetzel *et al.*, 1998). Determination of a reduction in $\Delta\psi_m$ by using the fluorochrome JC-1 indirectly evidenced IMP in Jurkat T cells treated with ascididemin. The decrease of $\Delta\psi_m$ preceded the release of cytochrome *c*. Together these data clearly indicate that ascididemin induces mitochondrial dysfunction in Jurkat T cells.

The present data further demonstrate a causal involvement of mitochondria in ascididemin-mediated apoptosis, as overexpression of the anti-apoptotic proteins Bcl-x_L or Bcl-2 protected cells from cell death.

4 Activated caspases are essential for the ascididemin-induced pathway

Caspases, intracellular cystein proteases, play an essential role in the initiation and execution phases of both receptor- and chemical-induced apoptotic cell death. However, the number of studies, however, proposing an additional caspase-independent cell death is growing (Mathiasen *et al.*, 1999; Roberts *et al.*, 1999; Nylandsted *et al.*, 2000).

As it is demonstrated in the present work, ascididemin triggers the activation of the initiator caspase-8, and –9, respectively, and the executioner caspase-3. Cleavage of its substrate poly(ADP-ribose) polymerase (PARP) confirmes active caspase-3. The fact that DNA fragmentation was entirely abrogated by the broadspectrum caspase inhibitor zVAD-fmk provides evidence for the essential role of activated caspases in the signal transduction. Interestingly, the early processing of procaspase-9, the apical caspase in the mitochondrial pathway, was followed by the simultaneous cleavage of procaspase-8 and procaspase-3. In regard to the kinetics, caspase-8 can be considered to act as an executioner caspase in the apoptotic signalling triggered by ascididemin, concomitant the findings of a CD95 receptor-independent apoptotic signalling (see 3.2) (Engels *et al.*, 2000; Dirsch *et al.*, 2002). The failure of cleavage of caspase-8 in Bcl-x_L overexpressing Jurkat T cells underlines the classification as a downstream caspase.

5 Caspase-2 acts as initiator caspase in ascididemin-mediated apoptosis

Caspase-2 is activated during apoptosis by a variety of apoptotic stimuli, including CD95L, TNFα, growth factor withdrawal and DNA damaging agents (Harvey *et al.*, 1997; Droin *et al.*, 2001; Robertson *et al.*, 2002; Stefanis *et al.*, 1998). Although caspase-2 is classified as initiator caspase due to its long prodomain, the hierachical collocation within the caspase cascade is discussed controversially. For instance, caspase-2 is related to TNF receptor 1 (TNFR1) as well as CD95 death signalling due to its recruitment to these receptors mediated by the adaptor molecule RAIDD (Duan and Dixit, 2002). Involvement in the CD95-mediated pathway is additionally implicated by the contribution of caspase-2 to caspase-8 activation (Droin *et al*., 2001). In contrast, some studies query that caspase-2 plays a major role as initiator caspase by showing that Apaf-1 together with caspase-9 (O'Reilly *et al.*, 2002) or the effector caspase-3 (Paroni *et al.*, 2001; Li *et al.*, 1997; Slee *et al.*, 1999) are required for the activation of caspase-2. These different observations may mainly reflect a cell-type and stimulus dependent manner of caspase-2 activation. Subcellular fractionation studies revealed

that procaspase-2 is present in several intracellular compartments, including cytosol, Golgi, mitochondria and the nucleus (Susin *et al.*, 1999; Mancini *et al.*, 2000; Paroni *et al.*, 2002; Zhivotovsky *et al.*, 1999). Colussi and coworker (Colussi *et al.*, 1998) were the first to show the presence of nuclear localisation sequences in certain regions of the prodomain of caspase-2 that regulate its nuclear import. In the following, Paroni and colleagues (Paroni *et al.*, 2002) additionally demonstrated that caspase-2 can trigger mitochondrial dysfunction without relocalising into the cytoplasm. Furthermore, nuclear localisation of the caspase-2 adapter molecule RAIDD was observed (Shearwin-Whyatt *et al.*, 2000). Interestingly, a very recent report showed that the cell cycle nuclear protein cyclin D3 interacts with and activates caspase-2 (Mendelsohn *et al.*, 2002). Thus, it might be conceivable that drugs affecting the nucleus may lead to the activation of caspase-2 and subsequent mitochondrial cell death.

Indeed, the present data show early activation of caspase-2 in ascididemin treated Jurkat T cells. The findings that the specific caspase-2 inhibitor zVDVAD-fmk was able to abolish DNA fragmentation clearly indicate the significant role of caspase-2 in the apoptotic signal transduction triggered by ascididemin. Studies with Bcl-x_L overexpressing Jurkat T cells provide evidence that, in fact, processing of procaspase-2 occurs upstream of mitochondria. Further activation of caspase-2 results from an amplification within the caspase cascade, which is a common event during the apoptotic process, as processing of procaspase-2 was reduced by the broadspectrum caspase inhibitor zVAD-fmk. Most importantly, caspase activity studies revealed that caspase-2 is the most apical caspase in the ascididemin-induced caspase cascade.

Very recently, Robertson *et al.* reported that activated caspase-2 acts upstream of mitochondria to regulate cytochorme *c* release following etoposide treatment (Robertson *et al.*, 2002). The present results demonstrate that activated caspase-2, indeed, interferes with the mitochondrial pathway. Activated caspase-2 is required for mitochondrial cytochrome *c* release and subsequent processing of caspase-9 in response to ascididemin treatment.

In vitro studies showed that recombinant caspase-2 can cleave Bid. Cleaved Bid in turn translocates to the mitochondria causing mitochondrial dysfunction (Paroni *et al.*, 2001). So far nothing is known about the signal transduction of activated caspase-2 to mitochondria *via* truncated Bid mediated by anticancer drugs. Concerning etoposide, the results of Robertson *et al.* suggest that active caspase-2 targets mitochondria and stimulates the release of cytochrome *c* without the requirement of Bid. In addition, purified caspase-2 can directly induce the release of apoptogenic factors from mitochondria (Guo *et al.*, 2002). Surprisingly, Bid cleavage in Jurakt T cells exposed to ascididemin could be attenuated by the specific caspase-2 inhibitor zVDVAD-fmk. These data indicate that activated caspase-2 is able to cleave Bid in response to ascididemin. Thus, it is suggested that Bid links activated caspase-2 and mitochondrial dysfunction in the apoptotic process of ascididemin.

6 ROS and the apoptotic pathway mediated by ascididemin

A critical role for oxidativ stress in the induction of apoptosis is provided, on the one hand, by studies which show that low levels of reactive oxygen species (ROS) induce apoptosis (Dumont *et al.*, 1999) and, on the other hand, by the observation that various antioxidants, such as N-acetylcysteine (NAC) are able to inhibit apoptotic cell death (Dirsch *et al.*, 1998; Friesen *et al.*, 1999; Verhaegen *et al.*, 1995). Additionally, ROS generation has been reported to occur in CD95-mediated apoptosis (Suzuki *et al.*, 1998) as well as following exposure to UV irradiation (Gorman *et al.*, 1997a) and chemotherapeutic drugs (Creagh and Cotter, 1999). In contrast, some reports demonstrate a rather contributory role of oxidative stress for the induction of apoptosis (Gorman *et al.*, 1997a). Sentürker *et al.* even showed that chemotherapeutic drugs are able to trigger apoptosis without generation of ROS (Senturker *et al.*, 2002). The results of the present work show that treatment of Jurkat T cells with ascididemin leads to a very early generation of ROS. However, oxidative stress does not play a major role in the apoptotic process as NAC did not affect DNA fragmentation. Nevertheless, ROS might be considered as a contributory factor to the initiation of ascididemin-induced apoptosis. Taking into account that DNA fragmentation appears at late stages of apoptosis the inhibitory effect of NAC may be overcome by parallel signalling pathways. Furthermore, in respect of the JNK pathway, the present results point to some impact of ROS in ascididemin-induced signalling (see 4.7).

7 The role of MAPK and Akt in ascididemin-triggered apoptosis

Signal transduction pathways involving the mitogen-activated protein kinases (MAPK) including the c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK), the p38 MAPK and the extracellular signal-regulated kinases (Erk), have been shown to differentially contribute to pro- and antiapoptotic pathways. Erk are mainly activated by growth factors and mitogenic stimuli and pivotally linked to cell survival and mitogenesis (Chang and Karin, 2001). JNK and p38, on the other hand, are activated by a diverse array of cellular stresses, including ionising irradiation, ROS and cytotoxic agents, and either have been associated with apoptosis (Ortiz *et al.*, 2001; Xia *et al.*, 1995; Zanke *et al.*, 1996; Deschesnes *et al.*, 2001).

The results of the present work demonstrate that ascididemin induces the activation of JNK and p38 but not the activation of Erk.

Although the activation of p38 is a very early event in the signalling triggered by ascididemin, the inhibition of the catalytic activity by the specific p38 inhibitor

SB203580 did not attenuate apoptosis. These results negate a significant impact of p38 on the apoptotic process mediated by ascididemin.

Inhibitory studies with the specific JNK inhibitor SP600125 demonstrated the causal involvement of activated JNK in the programmed cell death signalling induced by ascididemin. The fact that SP600125 only partially reduced the rate of apoptotic cells points out that the apoptotic response of Jurkat T cells to ascididemin does not solely derive from the JNK cascade. The present data show that JNK is activated independently of active caspases upstream of mitochondria as neither the broadspectrum caspase inhibitor zVAD-fmk nor overexpression of Bcl-x_L and Bcl-2, respectively, could abrogate the phosphorylation of JNK (MacFarlane *et al.*, 2000; Cahill *et al.*, 1996; Srivastava *et al.*, 1999). ROS could be identified as initial mediator of JNK activation as the antioxidant NAC prevented this process at early time points (Shiah *et al.*, 1999). In accordance with reports of mitochondria as targets of JNK as well as requirement of JNK for cytochrome *c* release (Kharbanda *et al.*, 2000; Aoki *et al.*, 2002; Tournier *et al.*, 2000), it is proven that active JNK contributes to ascididemin-induced mitochondrial dysfunction: the translocation of cytochrome *c* from the mitochondria into the cytoplasm and the subsequent activation of caspase-9 is diminished in the presence of SP600125. However, Bcl-2 as direct target of JNK can be excluded (Maundrell *et al.*, 1997). Taking into account that inhibition of active JNK only partially reduces the processing of the initiator caspase-2, a parallel activation of the caspase cascade is hypothesised. In conclusion, the results support the view that the redox sensitive activation of JNK contributes to the induction of the apoptotic process. Upon further progression, parallel signalling pathways, such as the caspase cascade followed by mitochondrial amplification, exceed the outcome of the JNK cascade.

Several studies clearly demonstrated the survivial functions of Akt in the apoptotic process (Datta *et al.*, 1999; del Peso *et al.*, 1997; Cardone *et al.*, 1998). Inhibition of Akt significantly increased the susceptibility of cells to chemotherapeutic- and CD95-induced apoptosis (Tang *et al.*, 2001; O'Gorman *et al.*, 2001). However, the rate of ascididemin-triggered apoptosis was not altered in response to the inhibition of Akt activation by the PI3-kinase inhibitor wortmannin. This observation indicates that cell death signals predominate in the cellular response to ascididemin.

G. Summary

The present work characterises the apoptotic signal transduction mediated by the marine pyridoacridine alkaloid ascididemin (5 μM) in human leukemia Jurkat T cells (fig. 56).

Ascididemin was shown to induce apoptosis *via* a signalling cascade strongly dependent on the activation of caspases and mitochondrial dysfunction, but independent of CD95 receptor signalling.

In particular, caspase-2 was identified not only to be the most apical caspase of the caspase cascade but also to be essential for the apoptotic process triggered by ascididemin. Furthermore, it could be shown that caspase-2-mediated cleavage of Bid represents a possible link between active caspase-2 and mitochondrial perturbation in response to ascididemin. These results demonstrate a central role of caspase-2 in mitochondria-mediated signal transduction. This represents a new aspect of a mitochondria-dependent pathway.

Ascididemin-induced mitochondrial dysfunction was demonstrated to be associated with the release of cytochrome *c* from the intermembrane space to the cytosol and the loss of the mitochondrial transmembrane potential.

It was further shown that ascididemin triggers the redox-sensitive activation of JNK. A contributory role of active JNK to the induction of mitochondrial dysfunction and subsequent apoptosis in response to ascididemin could be verified.

In summary, this thesis contributes to the understanding of the molecular mechanism regulating ascididemin-mediated apoptotic cell death in human leukemia Jurkat T cells. The anti-leukemic potential of ascididemin might be useful especially in the treatment of cells with low chemosensitivity caused by resistance in CD95 signalling.

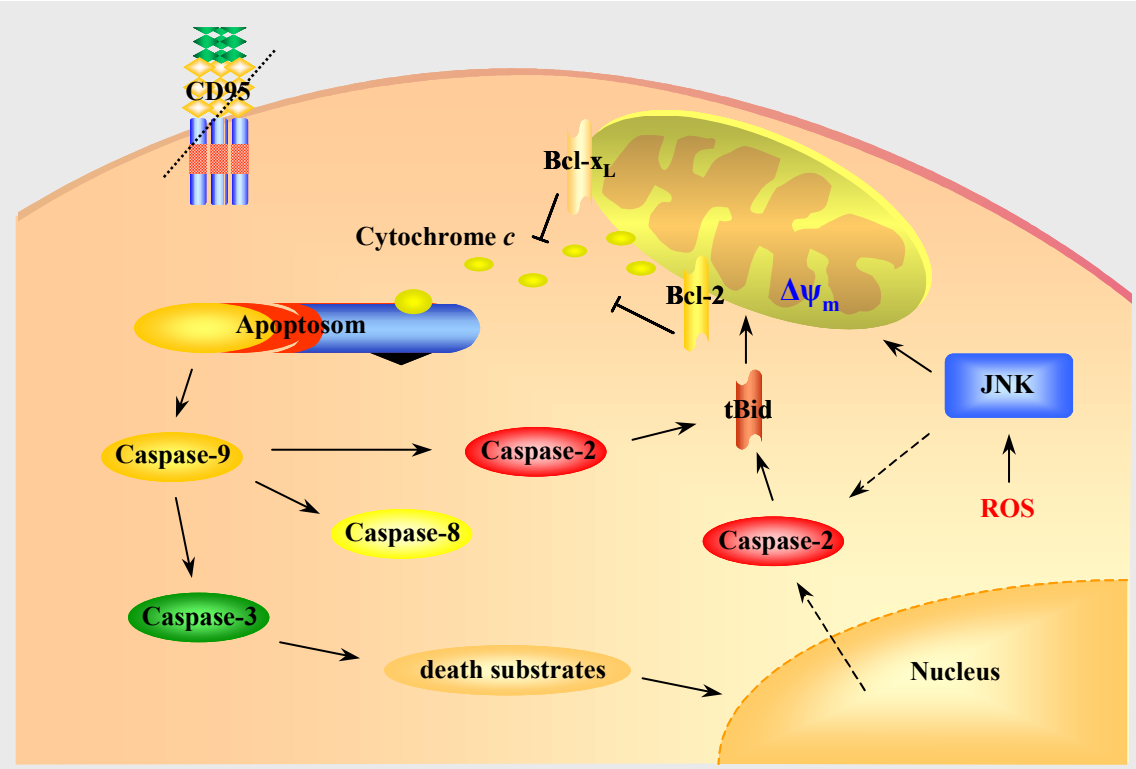


Figure 56: Schematic illustration of the postulated signalling pathway induced by ascididemin. Since ascididemin is characterised as a DNA intercalating agent, it is proposed that the apoptotic signal arises from the nucleus. The signalling pathway is transduced by activation of caspase-2 and JNK, routed via mitochondria, amplified by the caspase cascade and finally targets a variety of death substrates. Signal transduction via the CD95 receptor is not involved.

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I. Appendix

1 Publications

1.1 Abstracts

Kirschke SO, Dirsch VM, Estermeier M, Steffan B, Vollmar AM. The marine alkaloid ascididemin triggers apoptosis by a mechanism that does not involve caspase activation upstream of mitochondria. Free Radical Biology and Medicine 2001 Nov, 31 (Supp 1):316. Poster presentation at the 8th Annual Meeting of the Oxygen Society, Durham, North Carolina, USA (2001).

Kirschke SO, Dirsch VM, Estermeier M, Steffan B, Vollmar AM. Apoptotic signaling of ascididemin, a marine pyridoacridine alkaloid. Naunyn-Schmiedeberg's Archieves of Pharmacology 2002 Mar, 365 (Supp 1):40. Poster presentation at the 42. Tagung der deutschen Gesellschaft für klinische Pharmakologie und Toxikologie, Mainz, Germany (2002).

1.2 Original publications

Dirsch VM, Kirschke SO, Estermeier M, Steffan B, Vollmar AM. Apoptosis signaling triggered by the marine alkaloid ascididemin is routed via caspase-2 and c-Jun NH_2-terminal kinases to mitochondria.

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Feling N, Kirschke SO, Steglich W. Isolation and structural characterisation of Flettichromen, a cytotoxic secondary metabolite of Albatrellus flettii.

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3 Curriculum vitae

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