Mechanisms of Apoptosis in Cancer:
Regulatory Role of Caspases

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Dedicated to my parents.

ABSTRACT

Die vorliegende Arbeit gewährt Einblick in biochemische Signalwege, die in verschiedenen Zellsystemen zu apoptotischem Zelltod nach Behandlung mit Zytostatika führen. Die Arbeit überspannt zwei Bereiche: die Identifizierung des initiierenden Signalweges bei Zytostatika-induzierter Apoptose in der humanen B-Zell Lymphom Zellinie BJAB und Einblick in Apoptose-bezogene Ereignisse, die durch Zytostatikabehandlung induziert werden. Die Verifizierung, daß Zytostatikabehandlung nicht nur das Zellwachstum hemmt sondern auch Apoptose induziert, ist der erste Schritt.

Der erste Bereich behandelt spezifische Ereignisse/Proteine die mit der Apoptose assoziiert sind: Um den initiierenden Signalweg bei Zytostatika-induzierter Apoptose zu bestimmen, wird die Kaskade der apoptotischen Ereignisse am Endpunkt der Apoptose beginnend aufgedeckt: Zytostatika-induzierter Zelltod wurde mittels Trypan-Blau Färbung untersucht und die Quantifizierung ergab, daß etwa 60% der behandelten BJAB-Zellen nach 72 Std. sterben. Weitere Charakteristika der Apoptose wurden, dem Signalweg aufwärts folgend, mittels immunchemischer, biochemischer und zellbiologischer Methoden untersucht. Die Fragmentierung der DNA wurde durch durchflußzytometrische Analyse bestimmt; Aktivierung der Caspasen wurde sowohl durch Western-Blot Analyse als auch in Caspase-spezifischen Aktivitäts-Assays detektiert. Hierdurch wird gezeigt, daß während der Apoptose die DNA degradiert wird, was in einer gesteigerten Zahl hypodiploider Zellen resultiert, die bis zu 60% nach 72 Std. erreichen. Eines der Caspase-Substrate, Poly-(ADP-Ribose)-Polymerase (PARP), wird spezifisch während der Zytostatika-induzierten Apoptose in BJAB-Zellen gespalten, was durch Western-Blot Analyse gezeigt wird. Desweiteren werden Caspase-3-, Caspase-9- und Caspase-8-Aktivierung mittels Western-Blot Analyse detektiert. Die Prozessierung der Caspase-8 warf die Frage auf, welches der Startpunkt der Caspase-Kaskade bei Zytostatika-induzierter Apoptose ist, da Caspase-8 im allgemeinen als die am Anfang der Protease-Kaskade stehende Initiator-Caspase bei der CD95/Fas induzierten Apoptose betrachtet wird. Der Zusammenbruch des mitochondrialen Membranpotentials, ein Ereignis, das oberhalb der Aktivierung von Caspase-9 gelegen ist, wurde mittels durchflußzytometrischer Analyse untersucht unter Gebrauch des Farbstoffes JC-1 (5,5´,6,6´-tetrachloro-1,1´,3,3´-tetraethyl-benzimidazolylcarbocyanin iodide), der sensitiv auf Veränderungen im mitochondrialen Membranpotential reagiert. Für BJAB-Zellen konnte ein signifikanter Verlust des mitochondrialen Membranpotentials gezeigt werden, der in 80% der mit Taxol-behandelten und 60% der Epirubizin-behandelten Zellen nach 72 Std. nachgewiesen wurde. Um im weiteren die Rolle der Caspase-8 Prozessierung zu untersuchen, wurden stabil transfizierte Zellen, die eine dominant-negative Mutante des Adaptermoleküls FADD (Fas assoziiertes Protein mit Todesdomäne) exprimieren, eingesetzt. FADD ist notwendig zur Formierung des Todes-indu-

zierenden Signalkomplexes (DISC) im CD95/Fas Signalweg; Überexpression der dominant-negativen Mutante des Moleküls FADD (FADDdn) unterbricht die Weiterleitung des CD95/Fas-Todessignals ins Zytoplasma und blockiert somit die Apoptose. Mittels Western-Blot erzielte Ergebnisse zeigen, daß die Transfektion mit FADDdn keinen signifikanten Einfluß auf die Caspase-8 Prozessierung hat. Die CD95/Fas unabhängige Prozessierung der Caspase-8 wurde in einem zellfreien Extrakt bestätigt, in dem die mitochondriale Induktion der Caspasen-Kaskade *in vitro* nachgestellt werden kann. Western-Blot Analyse und Substratassays mit Caspase-spezifischen Peptidsubstraten der *in vivo* und *in vitro* Experimente lokalisieren die Caspase-8-Prozessierung unterhalb der Procaspase-3 in den verschiedenen humanen B-Zell Lymphom Zellinien, die zum Einsatz kamen: BJAB/mock, BJAB/FADDdn, NALM6 und REH. Diese Tatsache wurde zudem durch den Einsatz spezifischer Caspase-Inhibitoren unterstrichen und durch die immunchemische Ausfällung der Procaspase-3 aus zellfreien Extrakten vor der Induktion der mitochondrialen Caspase-Kaskade. Die Prozessierung der Caspase-8 ist stark vermindert in Zytostatika-behandelten Zellen, die in Gegenwart von Z-DEVD-fmk, einem Caspase-3 spezifischen irreversiblen Inhibitor, inkubiert wurden. Immunchemische Depletion der Caspase-3 von zellfreien Extrakten blockiert die Prozessierung der Caspase-8 nach Initiierung der mitochondrialen Caspase-Kaskade.

Der zweite Bereich befaßt sich mit einem generelleren Ansatz: Ein hochauflösender Zugang zur Proteinanalyse wurde eingesetzt um einen Einblick in den Zytostatika-induzierten Zelltod in BJAB/mock Zellen zu erhalten. Die zweidimensionale Polyacrylamid-Gelelektrophorese (2DE PAGE) von Proben Zytostatika-behandelter Zellen und von Kontrollproben wurde durchgeführt und die Ergebnisse mittels subtraktiver Analyse ausgewertet. Diese Experimente führten zur Identifikation einiger Caspase-Substrate wie β-Aktin und Nucleolin. D4-GDI (rho GDP Dissoziationsinhibitor 2) wird ebenfalls als eines dieser Substrate gezeigt und extensiver experimenteller Aufwand wurde betrieben, um die *in vivo* verantwortliche Caspase festzumachen. Durch den Einsatz des Caspase-3 spezifischen Inhibitors Z-DEVD-fmk konnte gezeigt werden, daß D4-GDI in kultivierten, apoptotischen BJAB-Zellen von aktiver Caspase-3 gespalten wird. Dieses Ergebnis wurde in zellfreien Extrakten durch den Einsatz des Caspase-3 spezifischen Substrates Ac-DEVD-pNA als kompetitiver Inhibitor und immunchemischer Depletion von Procaspase-3 bestätigt. Beides führt zu einer verminderten Spaltung von D4-GDI. Da über D4-GDI bekannt ist, daß es ebenfalls als Substrat für Caspase-1 fungiert, wurde die Aktivierung von Procaspase-1 nach Zytostatikabehandlung in BJAB-Zellen untersucht. Die Western-Blot Analyse zeigte keinen Konzentrationsanstieg für die aktive Untereinheit p20 der Caspase-1. Im Aktivitätsassay, in dem das Caspase-1 spezifische Substrat Ac-WEHD-pNA eingesetzt wurde, konnte keine signifikante Spaltungsaktivität detektiert werden. Schließlich zeigte die massenspektrometrische Analyse des resultierenden D4-GDI Fragmentes, daß Caspase-3 und nicht Caspase-1 verant-

wortlich für die Spaltung von D4-GDI nach Zytostatikabehandlung von BJAB-Zellen ist. Das Peptid, welches die Caspase-1 Erkennungssequenz enthält, wurde in dem Apoptose-assoziierten D4-GDI Fragment detektiert. Darüberhinaus wurde demonstriert, daß der apoptotische Zelltod keinerlei Konsequenzen für Rho-GDI 1, ein Protein, das hohe Homologie zu D4-GDI aufweist, birgt. Hierdurch wird bekräftigt, daß die Spaltung zellulärer Proteine während der Apoptose hochspezifisch ist.

Die Ergebnisse der 2DE PAGE weisen darauf hin, daß die angewandte Methodologie nicht ausreicht, um Veränderungen regulatorischer Faktoren auf Proteinebene nach Zytostatikabehandlung von BJAB-Zellen zu detektieren. Signifikante Veränderungen der Proteinkonzentration konnten ausschließlich für Caspase-Substrate detektiert werden. Daher wurde versucht, dieses Problem zu lösen, indem die Probenpräparation spezifischer gestaltet wurde. Annexin-V gekoppelte magnetische Mikrokügelchen wurden eingesetzt, um apoptotische von nicht-apoptotischen Zellen zu trennen. Diese Methode reichert apoptotische Zellen durch Bindung an Phosphatidylserin (PS) an, welches aufgrund der Apoptose-Induktion auf der Außenmembran exponiert wird. Allerdings scheint die Exposition von PS ein spätes Ereignis bei Zytostatika-induzierter Apoptose in BJAB/mock-Zellen zu sein, da diese Methode nicht zur Identifikation regulatorischer Proteine während des apoptotischen Zelltodes führte. Dies wird aus der Tatsache geschlossen, daß die Spaltung von Caspase-Substraten in PS-negativen Zellen eindeutig detektiert werden konnte.

In einem weiteren Versuch wurde die Visualisierungstechnik durch metabolische Markierung der Proteine mit ^{35}S-Methionin und ^{35}S-Cystein sensitiviert. Durch diese Methode wird die Detektionsgrenze herabgesetzt und Veränderungen innerhalb eines spezifischen Zeitfensters können beobachtet werden. Die erhaltenen Ergebnisse zeigen deutlich, daß das metabolische Markieren nützlich für die Detektion regulatorischer Proteine ist, dafür jedoch andere Probleme mit sich bringt: In den meisten Fällen ist die Menge an regulatorischem Protein weit geringer als diejenige, welche für eine massenspektrometrische Analyse und Identifikation notwendig ist. In den bisherigen Experimenten konnte ein Spot detektiert werden, der signifikante Intensitätsunterschiede in Autoradiographien von 2DE PAGE Gelen aufweist. Die Identifizierung war bis jetzt allerdings nicht möglich.

ABSTRACT

This thesis will elucidate the biochemical pathways leading to apoptotic cell death after cytostatic drug-treatment of different cellular systems. The thesis will cover two areas: the identification of the initiating signalling pathway of drug-induced apoptosis in the human B-cell lymphoma cell line BJAB, and insights into apoptosis related events induced by cytostatic drug-treatment. Verification that cytostatic drug treatment not only inhibits cell growth but induces apoptosis is the first step.

The one area governs questions regarding specific events and/or proteins associated with apoptosis:

To elucidate the initiating pathway of drug-induced apoptosis the cascade of apoptotic events is unravelled starting at the most downstream event of apoptosis: drug-induced cell death was examined using the trypan blue exclusion assay and quantification revealed that about 60 % of treated BJAB cells died after 72 hs. Further hallmarks of apoptosis following the signalling pathways to far more upstream-located events were investigated using immunochemical, biochemical and cell biological methods. DNA fragmentation was determined using flow cytometric analysis; activation of caspases was detected by western blot analysis and specific caspase activity assays. Thereby it was shown that during apoptosis DNA is degraded resulting in an increased number of hypodiploid cells reaching up to 60 % after 72 hs. One of the most downstream substrates of caspases, PARP (poly-ADP ribose polymerase), was specifically cleaved during drug induced apoptosis in BJAB cells as shown by Western blot analysis. Moreover, caspase-3, caspase-9 and caspase-8 activation was detected by western blot analysis. Processing of caspase-8 rose the question for the initiating point of the caspase cascade during drug-induced apoptosis because caspase-8 is reported to be the most upstream-located initiator caspase in CD95/Fas-induced apoptosis. Disruption of mitochondrial membrane potential, an event that is located upstream of caspase-9 activation, was examined by flow cytometric analysis using the mitochondrial membrane potential sensitive dye JC-1 (5,5´,6,6´-tetrachloro-1,1´,3,3´-tetraethylbenzimidazolylcarbocyanin iodide) and BJAB/mock cells were shown to undergo significant loss of mitochondrial membrane potential reaching up to 80 % in taxol- and 60 % in epirubicin treated cells after 72 hs. To further investigate the role of caspase-8 processing stably transfected cells, harbouring a dominant-negative mutant of the adaptor molecule FADD (Fas associated protein with death domain), were used. FADD is necessary for formation of the death inducing signalling complex (DISC) in the CD95/Fas signalling pathway; overexpression of the dominant negative mutant of FADD (FADDdn) abrogates transmission of the CD95/Fas death signal into the cytoplasm and blocks apoptosis. Results derived from western blot analysis show that transfection with FADDdn had no significant influence on caspase-8 processing. CD95/Fas inde-

pendent processing of caspase-8 was confirmed using a cell free extract system which mimics the mitochondrial initiated caspase cascade. Western blot analysis and caspase substrate assays from the *in vivo* and *in vitro* experiments locate caspase-8 processing downstream of caspase-3 in different human B cell lymphoma cell lines used: BJAB/mock, BJAB/FADDdn, NALM6 and REH. This fact is strengthened by the usage of specific caspase inhibitors and by depleting caspase-3 from cell free extracts prior to induction of the mitochondrial caspase cascade. Caspase-8 processing was diminished in drug-treated cells incubated in the presence of Z-DEVD-fmk, a caspase-3 specific irreversible inhibitor. Immunochemical depletion of caspase-3 from cell free extracts blocked caspase-8 processing after initiation of the mitochondrial apoptotic cascade.

The second area governs a more general setup: A high resolution approach to protein analysis was used to gain insight into cytostatic drug-induced cell death of BJAB/mock cells. 2DE-PAGE analysis of samples derived from drug-treated cells and control samples was performed and evaluated by subtractive analysis. These experiments lead to the identification of some caspase substrates, such as β-actin and nucleolin. D4-GDI (rho GDP dissociation inhibitor 2) was also depicted as one of these caspase-substrates and extensive experimental effort was made to pinpoint the acting caspase *in vivo*. Using the caspase-3 specific inhibitor Z-DEVD-fmk it was shown that D4-GDI is being cleaved by active caspase-3 in cultured apoptotic BJAB cells. This result was confirmed in cell free extracts using the caspase-3 specific substrate Ac-DEVD-pNA as a competitive inhibitor and by immunochemical depletion of procaspase-3, both leading to decreases of D4-GDI cleavage. Because D4-GDI is also reported to serve as substrate for caspase-1, activation of caspase-1 upon drug treatment of BJAB/mock cells was investigated. Western blot analysis showed no increase in concentration of the mature subunit p20 of caspase-1. Additionally, in a caspase activity assay using the caspase-1 specific substrate Ac-WEHD-pNA no significant cleavage activity could be detected. Finally, mass spectrometric analysis of the resulting D4-GDI fragment clearly showed that caspase-3 and not caspase-1 is responsible for cleavage of D4-GDI after drug treatment of BJAB cells. The peptide containing the caspase-1 cleavage site was also detected in the apoptosis-associated fragment of D4-GDI. Moreover, it is demonstrated that apoptotic cell death bears no consequences for Rho-GDI 1, a protein which displays high homology to D4-GDI, thereby providing evidence that cleavage of cellular proteins during apoptosis is highly specific.

However, the results from 2DE PAGE analysis indicated that the applied methodology is not sufficient to detect changes of regulatory factors on protein level after drug-treatment of BJAB/mock cells. Significant changes in protein concentration could only be detected for caspase substrates. Therefore, some analytical efforts were made to overcome this problem. For this purpose, sample preparation was designed in a more spe-

cific way. Annexin V-coupled magnetic beads were used to separate apoptotic from non-apoptotic cells. This method enriches apoptotic cells by binding to posphatidylserine (PS) which is exposed to the outer membrane upon induction of apoptosis. However, exposure of PS appears to be a late event in drug-induced apoptosis of BJAB/mock cells. Thus, this method did not lead to the identification of regulatory proteins during apoptotic cell death. The fact that cleavage of caspase substrates was already detected in PS-negative cells further substantiates the finding that PS exposure is a late event in BJAB cells undergoing apoptosis.

In another attempt, the visualisation technique was sensitized by metabolic radioactive labeling (^{35}S-methionine and ^{35}S-cysteine) of proteins. By this method, detection limits are lowered and changes in a specific time frame can be monitored. Results obtained so far clearly indicate that metabolic labeling is suitable for detection of regulatory proteins but inherits other difficulties: in most cases the amount of regulatory proteins is far below the limit of mass spectrometric analysis and identification. In experiments performed so far, one spot displaying considerable variation in intensity was detected on images of 2DE PAGE gels derived from metabolic labeled samples but its identification was not yet successful.

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I INDICES

I-1 ABBREVIATIONS

°C	degree Celsius
µ	micro (x 10^{-6})
2DE PAGE	two-dimensional polyacrylamide gel electrophoresis
A	ampere(s)
aa	amino acid(s)
ACN	acetonitrile
AIF	apoptosis inducing factor
Apaf	apoptosis associated factor
APS	ammoniumperoxodisulfate
CAD	caspase activated DNase
CAPS	3-cyclohexylamino-1-propanesulfonic acid
CARD	caspase recruitment domain
caspase	cysteinyl aspartate-specific protease
CHAPS	3-(3-cholamidopropyl)dimethylammonio-1-propane sulfonate
cyt c	cytochrome c
Da	Dalton
dATP	desoxyadenosin triphosphate
DED	death effector domain
DD	death domain
DISC	death inducing signaling complex
DMSO	dimethylsulfoxide
DNA	desoxyribonuclic acid
DR	death receptor
DTT	dithiothreitol
DUT-N	desoxyuridine 5'-triphosphate nucleotidhydrolase
$\Delta\Psi_m$	mitochondrial membrane potential
ECL	enhanced chemoluminescence
EDTA	ethylendiamine tetraacetic acid
ER	endoplasmic reticulum
ESI	electrospray ionization
EST	expressed sequence tag
FACS	fluorescence activated cell sorting
FADD	Fas associated protein with death domain
FasR	Fas-receptor
g	gravitational constant (9.81 m s^{-2})
h	hour
HEPES	N-(2-hydroxyethyl)piperazine-N`-(2-ethanesulfonic acid)
HRP	horse radish peroxidase
IEF	isoelectric focusing

JC-1	5,5´,6,6´-tetrachloro-1,1´,3,3´-tetraethyl-benzimidazolylcarbocyanin iodide
k	kilo (x 10^3)
L	liter
M	mol / liter
m	milli (x 10^{-3})
min	minute(s)
mRNA	messenger RNA
MW	molecular weight
pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PARP	poly-ADP ribose polymerase
PBS	phosphate buffered saline
pH	potentio hydrogenii
pI	isoelectric point
PMSF	phenylmethylsulfonylfluoride
RNA	ribonucleic acid
s	second(s)
SDS	sodium dodecylsulfate
TEMED	N,N,N',N'-tetramethylethylendiamin
TFA	trifluoracetic acid
TNF	tumor necrosis factor
V	volt(s)

I-2 AMINOACID CODE

A	Ala	alanine
B	Asx	asparagine or aspartic acid
C	Cys	cysteine
D	Asp	aspartic acid
E	Glu	gluthamine
F	Phe	phenylalanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lysine
L	Leu	leucine
M	Met	methionine
N	Asn	asparagine
P	Pro	proline
Q	Gln	glutamine
R	Arg	arginine
S	Ser	serine
T	Thr	threonine
V	Val	valin
W	Trp	tryptophane
Y	Tyr	tyrosine
Z	Glx	glutamine or glutamic acid

I-3 FIGURE INDEX

I-4 TABLE INDEX

II FOREWORD

> Life
> the state of a material complex or individual characterized by the capacity to perform certain functional activities, including metabolism, growth, reproduction, and some form of responsiveness and adaptation. Life is further characterized by the presence of complex transformations of organic molecules and by the organization of such molecules into the successively larger units of protoplasm, cells, organs, and organisms.

This is the definition given in the Encyclopaedia Britannica. Just as there is a definition for life there is one for

> Death
> the total cessation of life processes that eventually occurs in all living organisms. The state of human death has always been obscured by mystery and superstition, and its precise definition remains controversial, differing according to culture and legal systems.

The present work neither deals with life nor with death in particular but it deals with the transition from life to death in certain circumstances. Although a vast amount of work has been devoted since the late 19th century to discover how cells multiply the study of cell death is of relatively recent concern. This theme first appeared in the Index Medicus - an index to medical literature - in 1979.

Definition: although the general meaning of *in vitro* is 'outside a living body' within this study the intention is slightly different and shall be understood as 'outside a living cell' (after destruction of the plasma membrane). Consequently experiments carried out with cell free extracts are called *in vitro* experiments. Experiments performed in cultured cells in contrast are referred to as '*in vivo*' experiments.

III INTRODUCTION

III-1 APOPTOSIS

III-1.1 EARLY DISCOVERIES

In 1890 William Councilman visualized cell death in humans for the first time (Councilman, 1890). He described vacuolated acidophilic bodies in liver tissue from yellow fever patients. But despite the observation of similar acidophilic bodies in other types of liver damage it took about 70 years and the use of electron microscopy to reveal that Councilmans acidophilic bodies were dying and/or dead cells (Kerr, 1969, Kerr, 1971). In parallel, it was shown that these cell death morphologies were not unique to liver damage but also occurred in the normal developing mouse during fusion of the palate (Farbman, 1968). Following these studies Kerr and colleagues published a detailed description of morphological changes associated with this form of cell death and called it apoptosis (Kerr, 1972). In the past several years it became clear that the observed morphological changes during cell death are determined by the activity of a group of cysteinyl proteases known as caspases (Alnemri et al., 1996).

III-1.2 ULTRASTRUCTURAL PHENOMENONS

Early ultrastructural features of apoptosis are the disintegration of cell junctions and condensation of the cytoplasm. The cells shrink and no swelling is observed as in the case of necrosis. At the surface of the nucleus lumps of chromatin aggregate, the nuclear membrane develops folds, and the nucleus splits into a number of membrane-bound, ultrastructurally well-preserved fragments. These fragments are shed and promptly taken up by specialized scavenger cells or even by ordinary, neighboring cells. The nuclear changes seem to be energy-dependent; they may reflect the fact that genes in the nucleus are beginning to express themselves in new ways, in response to unknown stimuli.

III-1.3 APOPTOSIS OVERVIEW

Apoptosis, also called programmed cell death, is a mechanism that allows cells to self-destruct when stimulated by an appropriate trigger. This program is activated for various reasons, e.g. when the cell is no longer needed within the body or when it becomes a threat to the health of the organism. Apoptosis is implicated in a variety of biological systems, such as embryonic development, differentiation, tissue homeostasis and defense against pathogens. Aberrant inhibition or initiation of apoptosis contributes to many diseases, including cancer. Basically, apoptosis is a normal physiological process that offets cell proliferation. Proteins involved in this process are evolutionarily conserved and homology is found ranging from the nematode *Caernorhabditis elegans*

(C. elegans) to man. The apoptotic machinery can be activated from inside (e.g. p53 signalling) or outside the cell (e.g. via death receptors) and further regulation is achieved by involvement of proteins of the BCL-2 family. The center of the apoptotic executive machinery is constituted by a family of cysteinyl proteases, termed caspases (cysteinyl aspartate-specific protease).

III-1.4 FROM NEMATODE TO MAN

Core components of the apoptotic machinery have been discovered in the *C. elegans* model system. Genetic studies identified three genes which are indispensable for removal of exactly 131 among 1090 somatic cells during development: the genes *CED-3*, *CED-4* and *CED-9* (Banda et al., 1992; Ellis et al., 1991; Laurent-Crawford et al., 1991). The protein encoded by *CED-3* (CED-3) is an effector of programmed cell death located most downstream in the cascade of the three proteins and the mammalian homologs are caspases. The *CED-9* protein (CED-9) represents an inhibitor of cell death in the nematode and is the prototypic member of the growing family of cell death regulators, the BCL-2 protein family. The *CED-4* gene product (CED-4) is located in between CED-3 and CED-9 and APAF-1 was identified as the mammalian counterpart (Zou, 1997, Chinnaiyan, 1997).

III-2 CASPASES: THE CENTRAL EXECUTIONERS OF APOPTOSIS

The first caspase was initially discovered as a cytokine-processing enzyme and designated interleukin-1β converting enzyme (Cerretti, 1992, Yuan, 1993). The presence of the conserved pentapeptide sequences QACR(N/Q)G at the caspase active site (Thornberry, 1998) and the rapid expansion of the expressed sequence tag (EST) database led to the identification of over 14 caspases in a short period of time (Fig. 1). Caspase-1 and -11 have been shown to function mainly in cytokine processing (Li, 1995, Kuida, 1995, Wang, 1998) whereas caspase-2, -3, -6, -7, -8, -9, -10 and -12 are involved in the regulation and execution of apoptosis (Kuida, 1996, Hakem, 1998, Varfolomeev, 1998, Begeron, 1998, Nakagawa, 2000). As the number of cloned caspases increases and since the human genome became sequenced, more proteins related to the caspases are being identified with divergence even at the highly conserved pentapeptide active site e.g.: caspase-homologue 1 (CSP-1) (Shaham, 1998) and DREDD caspase (Chen, 1998).

Caspases are synthesized as zymogens with the general organization shown in Fig. 2. (Cohen, 1997). An N-terminal prodomain is followed by sequences encoding a large and a small subunit. In some caspases the subunits are separated by a small spacer. This spacer is excised from the zymogen during maturation. All cleavages involved in the activation of procaspases occur at the carboxyl side of aspartate residues (Earnshaw, 1999) and the only known eukaryotic proteases with this specificity are the

caspases themselves and the cytotoxic T lymphocyte serine protease granzyme B (Greenberg, 1996, Pham, 1997, Thornberry, 1997). Caspase prodomains range in length from 23 amino acids (aa) for caspase-6 and -7 to 219 aa for caspase-10. Caspases with large prodomains are thought to be involved in the initiation of the apoptotic response (initiator caspases) and those harbouring a short prodomain are apparently activated in a first step by the initiator caspases followed by a second autoproteolytic step. These are therefore called effector caspases. On the basis of sequence alignments and functional studies two related motifs in the N-terminal region of initiator caspases have been discovered: the death effector domain (DED) (Chinnaiyan, 1995) and the caspase recruitment domain (CARD) (Hofmann, 1997). The DED is found in caspase-8 and 10 and is necessary for interaction of these caspases with adaptor proteins like FADD (Fas associated protein with death domain) (Chinnaiyan, 1995, Boldin et al., 1995) and TRADD (tumor necrosis factor associated protein with DD) (Hsu, 1995) in receptor mediated induction of apoptosis. The CARD is found in caspase-1, -2, -4 and -9 and was shown to be involved in promoting interactions of these caspases with one another and with other regulatory and adaptor proteins, e.g. APAF-1 (Brakebusch et al., 1992, Tartaglia, 1993, Takahashi et al., 1996, Itoh, 1993).

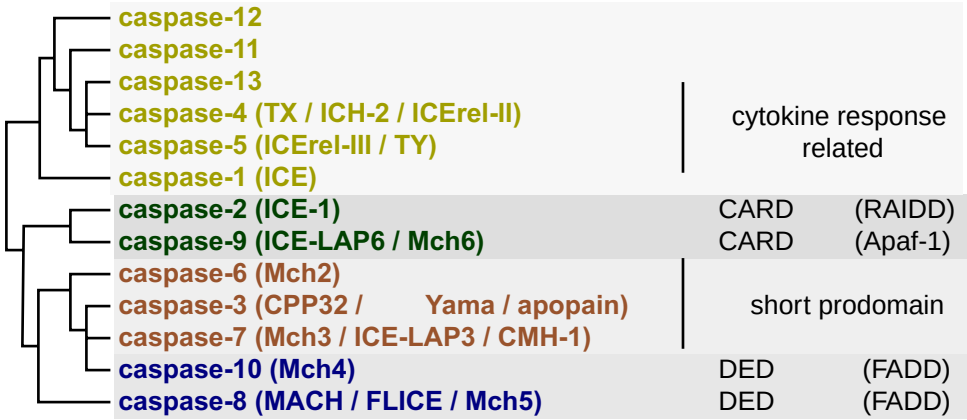

Fig. 1: Caspase dendrogram. The family of caspases can be divided into subgroups as depicted. Caspases 6, 4 and 1 are related to cytokine response and caspases 3, 6 and 7 are activated downstream of initiator caspase-8, -10, -9 and -2. Caspase-14 is not included and still under investigation.

Not only the length and structure of the prodomain defines the position of a caspase in the protease cascade, but the caspases display distinct intracellular localization (Zhivotovsky, 1999) which sometimes changes after activation. Zhivotovsky *et al.* found that in Jurkat cells procaspase-2 is distributed throughout the cytosol, microsomes and mitochondria, procaspase-3 and -9 are located in the cytosol and mitochondria whereas procaspase-7 and -8 were detected exclusively in the cytosol. After induction of apoptosis the localization of active caspases was different: caspase-2 was found only in the nuclei and caspase-3 was detected in cytosolic, mitochondrial and nuclear fractions. The authors further report the redistribution of caspase-7 (pro- and active form)

to the microsomal fraction, whereas active caspase-8 was predominantly present in the cytosol and to a minor extent in microsomal, mitochondrial and nuclear fractions. Active subunits of caspase-9 were cytosolic and probably associated with mitochondria.

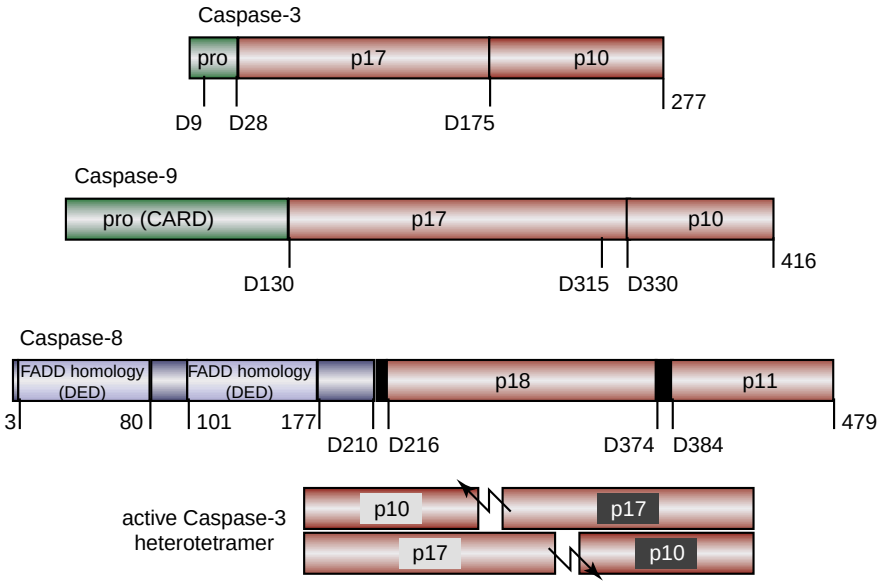

Fig. 2: Caspase structure. Although caspases belong to one family of enzymes they strongly differ in sequences of their prodomains. This reflects different signalling pathways of activation. Active caspases consist of two large and small subunits. The active site is formed by two different subunits from two different molecules as indicated by a flash.

III-3 Cell Death Pathways

Three main starting points of apoptosis are known; these are controled by death receptors, mitochondria and, as published recently, the endoplasmic reticulum (Nakagawa, 2000). Although much effort has been made concerning death receptor signalling as well as initiation of the apoptotic cascade by mitochondria, still tremendous investigation has to be made to elucidate the precise mode of action of these different pathways to death. Furthermore, there is an ongoing debate about potential cross-talk of different pathways. Nevertheless, parallel mechanisms of induction, execution and regulation of differentially triggered pathways are likely to exist (see Fig. 3)

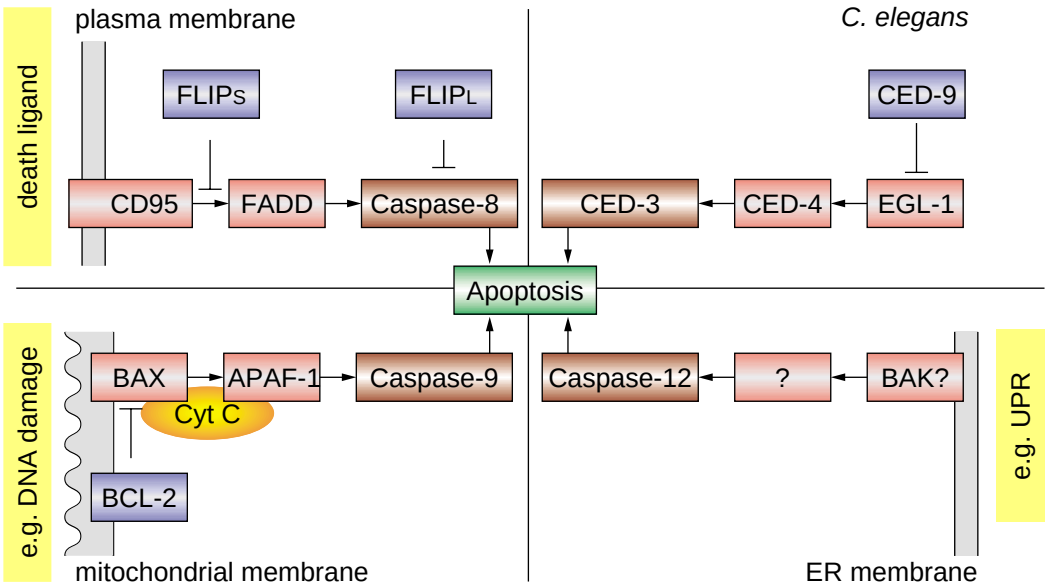

Fig. 3: Pathways to death. Distinct signals of induction of apoptosis always culminate in the same, stereo typic morphological and biochemical alterations of cells (see text for details).'?' indicate yet unknown steps. UPR = 'unfolded protein response'. ER = endoplasmic reticulum.

III-3.1 External Triggers: Death Receptors

The interaction of surface receptors with their cognate ligands is a fine-tuned mechanism to rapidly and selectively regulate life and death of a cell in higher organisms. Several receptors are capable of transmitting cytotoxic signals into the cytoplasm although many of these receptors display a wide range of apoptosis unrelated functions (e.g. cell activation, differentiation and proliferation). Moreover it is highly cell type specific whether the given signal leads to rapid cell death or proliferation. This is well documented for tumor necrosis factor (TNF) which exerts a costimulatory effect on naive lymphocytes and induces death signals in activated lymphocytes. The TNF-receptors (TNF-R) belong to an emerging family of receptors, the TNF receptor superfamily, with important function in differentiation, survival and cell death and for some members of this superfamily apoptosis inducing activity has been reported (Schulze-Osthoff, 1998).
One subgroup of TNF-R is characterized by the presence of a common death domain (DD. Itoh, 1993; Tartaglia, 1993) and therefore referred to as death receptors (DRs). Members of this subfamily are TNF-R1, CD95/Fas/APO-1, TRAMP/DR3/APO-3, TRAIL-R1/DR4/APO-2, TRAIL-R2/DR5, DcR1/TRAIL-R3 and DcR2/TRAIL-R4. DcR1 lacks the intracellular domian (Pan et al., 1997, MacFarlane et al., 1997, Sheridan et al., 1997) and DcR2 contains a truncated non-functional DD (Mongkolsapaya et al., 1998, Degli-Esposti et al., 1997, Degli-Esposti et al., 1997, Marsters et al., 1997). Receptor oligomerization upon ligand binding mediates transmission of the cytotoxic signal into the cytoplasm via the DDs. As an example, CD95-signalling is depicted in Fig.

4. The formation of the death inducing signalling complex (DISC) in CD95-induced apoptosis relies on FasL-(or other crosslinking agents) mediated receptor trimerisation and recruitment of adaptor molecules.

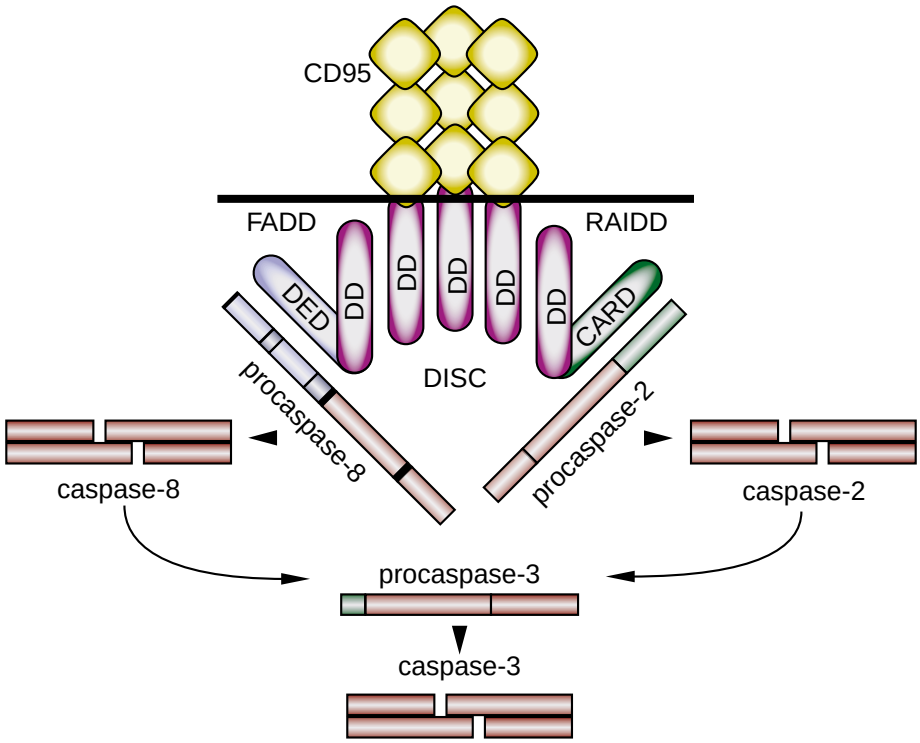

Fig. 4: CD95-signalling. After trimerisation of the receptor adaptor molecules are recruited (e.g. FADD, RAIDD) and mediate binding of procaspases. Close proximity induces autoactivation of the procaspases and initiates the downstream caspase cascade. Modified according to Schulze-Osthoff (1998). DISC = death inducing signalling complex, DD = death domain, DED = death effector domain.

These adaptor molecules consist of a DD, necessary for interaction with the cytoplasmatic receptor DD, and further a domain displaying the ability to associate with procaspases, the death effector domain (DED). The FADD DED was identified by transient expression of the N-terminal region of FADD (Chinnaiyan, 1995), which is sufficient to cause apoptosis. In contrast, overexpression of the C-terminal part, containing the DD, lacking the DED, protects the cell from receptor mediated apoptosis. This C-terminal deleted protein functions as a dominant negative mutant FADD (FADDdn). The finding that overexpression of inactive FADD or procaspase-8 (see Fig. 4) did not only block CD95 but also TNF-R1 signalling (Boldin et al., 1996, Chinnaiyan et al., 1996), although FADD does not bind to TNF-R1, led to the identification of TRADD (TNF-receptor associated protein with death domain). TRADD mediates binding of FADD after engagement of TNF-R1 thereby inducing procaspase-8 activation. Another pathway of CD95 may involve activation of procaspase-2 through RIP-interacting protein (RAIDD) (Duan and Dixit, 1997) reasoning that RAIDD contains a DD and a CARD domain, that is also present in caspase-2 (Schulze-Osthoff, 1998).
Besides apoptosis-mediating adaptor molecules harbouring a DED there are also neg-

ative regulatory elements containing this domain. These caspase-8 (former flice) inhibitory proteins (FLIPs) are known from several herpes viruses and molluscipox virus (Thome et al., 1997) and contain two DEDs. Two cellular FLIPs are known so far, $FLIP_L$ and $FLIP_S$, which both display significant antiapoptotic activity by incorporation into the DISC. Although $FLIP_L$ has sequence similarity to caspase-8, it blocks autoproteolytic activation of procaspase-8 when incorportated into the DISC due to replacement of the caspase-8 active site cysteine by a tyrosine in $FLIP_L$, rendering it proteolytically inactive (Irmler, 1997).

III-3.2 Internal Trigger: Mitochondria

Even cells without a mitochondrial genome (ρ^0 cells) can die by apoptosis (Jacobson, 1993). Consequently, for some time, mitochondria were thought not to play a role in the execution process of the apoptotic program. This assumption was underscored by the finding that the anti-apoptotic protein BCL-2, which resides in the outer mitochondrial membrane, still prevented death in these cells.

However, mitochondria are believed today to be a key component of the apoptotic machinery. It has been shown that in cell free extracts caspase activation by APAF-1 requires cytochrome c and dATP (Liu, 1996). APAF-1 contains an amino-terminal CED-3-like domain with a caspase recruitment domain (CARD), a central CED-4-like domain and a carboxyterminal stretch of 13 WD-40 repeats. WD-40 repeats mediate the interaction of proteins and are supposed to be the binding site for cytochrome c (Srinivasula, 1998). Multimerization of APAF-1, cytochrome c and dATP induces a conformational change in APAF-1 thereby exposing its CARD and mediating recruitement of procaspase-9. This multimolecular complex consisting of APAF-1, cytochrome c and dATP is called mitochondrial death inducing signalling complex (DISC). Procaspase-9 is recruited to the DISC by interaction of its prodomain with the APAF-1 CARD domain and activation is then achieved by autoprocessing induced by the proximity of the procaspase molecules (Kuida, 2000, Srinivasula, 1998). The stochiometry of the mitochondrial DISC is still under investigation. Interestingly, another CARD-protein, ARC (apoptosis repressor with CARD), has been identified in skeletal muscle and heart which ineracts with procaspase-8 and procaspase-2 but not procaspase-9 (Koseki et al., 1998). This indicates cross talk between CD95/Fas and mitochondrial death inducing signalling pathways. Two possible models that could explain the apotosis inhibitory function of ARC are discussed: repression of apoptosis by ARC might be caused by inhibition of caspase activation via direct binding to the death proteases or ARC could inhibit processed mature caspases.

Cytochrome c release from the intermembrane space is followed by mitochondrial permeability shift, ion flux and, in accordance with the acidic pH optimum of caspases, acidification of the cytoplasm (Matsuyama et al., 2000). Another protein liberated from

mitochondria is the apoptosis inducing factor (AIF). This molecule translocates to the nucleus and mediates caspase-independent DNA fragmentation (Susin et al., 1999, Daugas et al., 2000) into large fragments, a process which is different from the activity of caspase activated DNase (CAD). CAD is constitutively inhibited by the inhibitor of CAD (ICAD), which is cleaved by active caspase-3; the complex then dissolves and CAD executes its DNase activity (Enari et al., 1998). This is in agreement with the findings that in some cells apoptosis is changed to a more necrotic phenotype by addition of caspase inhibitors (McCarthy et al., 1997).

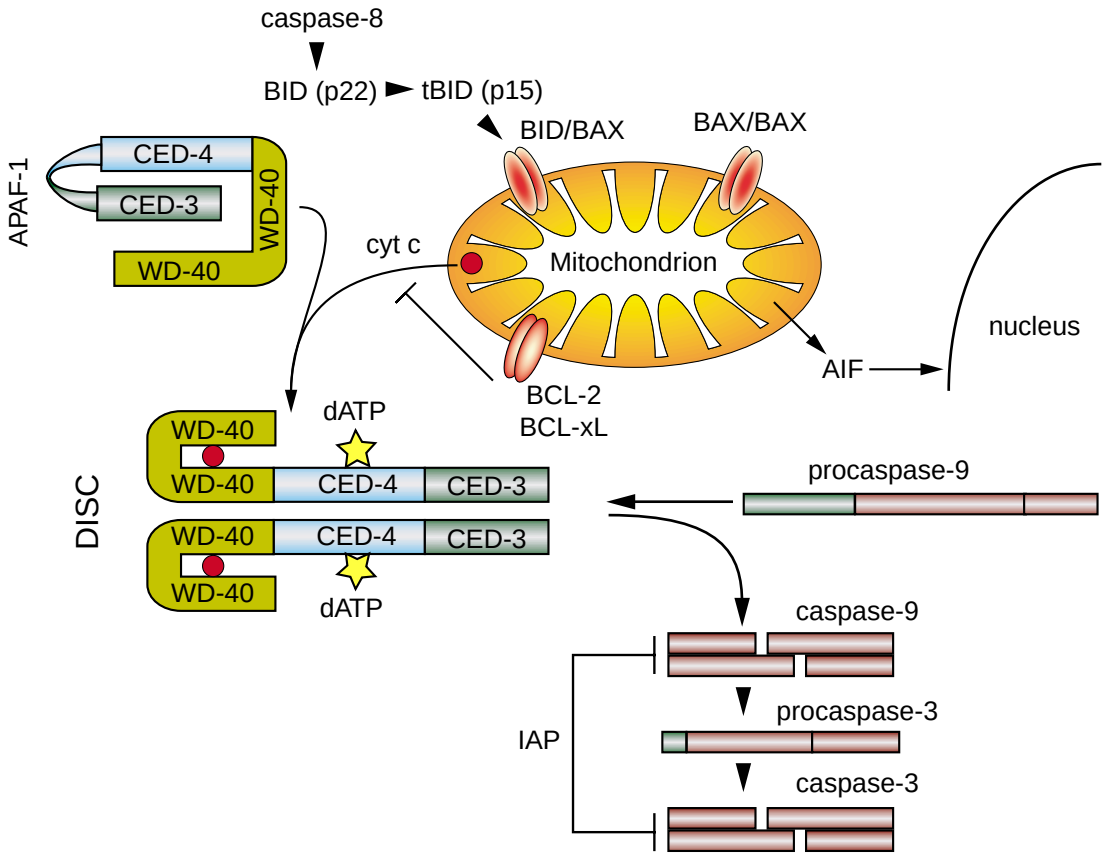

Fig. 5: Mitochondrial induction of caspase cascade. See text for details. Regulation by BCL-2 family proteins is described below (III-3.4), downstream regulators like IAPs are introduced in III-3.6. DISC = death inducing signalling complex, AIF = apoptosis inducing factor, IAP = inhibitor of apoptosis

In addition to these findings the opening of mitochondrial permeability transition (PT) pores is suggested to be the root mechanism for the release of apoptogenic proteins (Vander Heiden, 2000) from mitochondrial intermembrane space. The adenine nucleotide translocase (ANT), the voltage dependent anion channel (VDAC) and cyclophilin D (cyP D) are the basic units of the PT pore (Crompton, 1999) and the complex is located at contact sites between the mitochondrial inner and outermembrane. PT pore opening leads to matrix swelling and outermembrane rupture which in turn is reasoned to be the cause of cytochrome c and AIF release (Zamzami et al., 1995)

III-3.3 Internal Trigger: endoplasmic Reticulum

The contribution of the endoplasmic reticulum (ER) to apoptosis has only recently been discovered in mice. The initiator caspase in this pathway is caspase-12 which resides in the ER membrane directed to the cytosol and belongs to the caspase-1 subfamily. Activation of caspase-12 is triggered by ER stress in general which means inhibition of protein transport or inhibition of N-glycosylation or disruption of Ca^{2+} homeostasis in particular. Experiments in caspase-12 knock out mice indicate that activation of caspase-12 is independent from death receptor signalling and mitochondrial targeted apoptotic signals but essential for ER-stress induced apoptosis (Nakagawa, 2000).

III-3.4 Regulators of Apoptosis: BCL-2 Family Proteins

The proteins of the BCL-2 family play a pivotal role in the regulation of apoptosis. All members contain at least one of four conserved domains which are present in the prototypic BCL-2 protein and therefore are called BCL-2 homology (BH) domains (BH1, BH2, BH3, BH4). The family of BCL-2 proteins can be subdivided into pro- and antiapoptotic proteins. The latter, BCL-2, BL-xL and BCL-w, possess all four BH domains, and MCL-1 and A1 posses at least the BH1 and BH2 domain. Both, BH1 and BH2, are necessary to bind to and thereby inhibit the proapoptotic activity of BAX (Yin, 1994, Chittenden, 1995, Sedlack, 1997). In contrast, the proapoptotic proteins BAX, BAK and BOK lack the BH4 domain and the proteins BID, BAD, BIK, HRK and BIM are characterized by the presence of a BH3 domain only. Although the BH3 domain is necessary for bak and BID to bind to BCL-xL and promote cell death, the proapoptotic activity of this domain is not always dependent on its ability to interact with antiapoptotic proteins (Wang, 1996). Proapoptotic signalling of BCL-2 family proteins is regulated by heterodimerization of pro- and antiapoptotic proteins (e.g. BAX / BCL-2) and/or a conformational change in the complexed proapoptotic protein induced by another cell death promoting family member. Heterodimerization is not required for prosurvival signalling (Cheng, 1996, Kelekar, 1997).

The proapoptotic activity of BID is mediated by induction of a conformational change in BAX and translocation of BAX to the outer mitochondrial membrane followed by di- or oligomerization (Eskes, 1999, Desagher, 1999). This can also be the reason for release of cytochrome c and other apoptosis related proteins from the inter membrane space, because BAX, BCL-2 and BCL-xL bear the potential to form channels at least in lipid bilayers (Antonsson et al., 1997, Green, 1998, Minn, 1997). Emphasis to the pore forming capability of BCL-xL is given by the close spatial proximity of BH1, BH2 and BH3, creating an elongated hydrophobic cleft. This structure bears significant similarities to the pore forming domains of several bacterial toxins (colchicins A, E1 and diphteria toxin (Antonsson et al., 1997, Minn, 1997, Schendel et al., 1997, Schlesinger et al., 1997).

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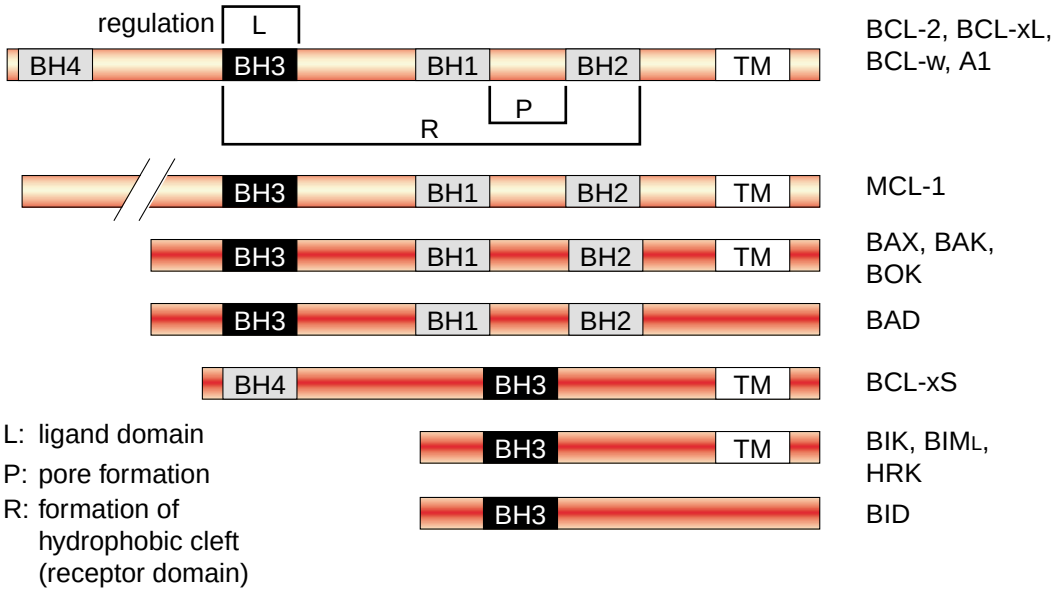

Fig. 6: BCL-2 family proteins. The gross protein domain structure (not in scale) for some BCL-2 related proteins. TM: membrane insertion domain according to Adams and Cory (1998) and Kroemer (1997).

Further prosurvival activity of BCL-2 and BCL-x is mediated by regulation of the VDAC, which resides in the outer mitochondrial membrane. Both proteins have been shown to interact with VDAC and thereby maintain exchange of complex anions between cytosol and inter-membrane space of mitochondria (Vander Heiden, 2000) and prevent release of cytochrome c (Shimizu, 2000). In contrast, the proapoptotic activity of BH3-only proteins is reported to induce cytochrome c release wthout permeability transition (Shimizu, 2000).

III-3.5 Caspase Substrates

Several protein substrates of caspases have been reported and some well characterized ones are summarised here.

The probably best characterized caspase substrate is PARP (poly-ADP ribose polymerase) which is cleaved early during apoptosis in many systems (Kaufmann, 1989; Kaufmann et al., 1993). PARP (116 kDa) is cleaved to a 24 kDa and a 89 kDa fragment representing the N-terminal DNA-binding domain and the C-terminal catalytic subunit, respectively. PARP is an ATP-dependent DNA repair enzyme, and specific cleavage during apoptosis abrogates both, its DNA repair (Casciola-Rosen et al., 1996) and ATPase activity. Caspase-7 and caspase-3 seem to be responsible for PARP degradation (Lazebnik et al., 1994, Cohen, 1997).

Proteolysis of lamins during apoptosis is supposed to be executed by caspase-6 as caspase-6 cleaves lamin A after the specific sequence VEID (Orth et al., 1996, Takahashi et al., 1996). This sequence is located in a well conserved α-helical rod domain and cleavage may result in disruption of lamin-lamin interaction followed by some of

the characteristic nuclear changes during apoptosis (Ucker et al., 1992, Oberhammer et al., 1994, Lazebnik et al., 1995, Greidinger et al., 1996).
U1-70kDa is cleaved specifically during apoptosis, and the RNA-binding domain is separated from the arginine-rich region (Casciola-Rosen et al., 1996, Tewari, 1995). This event is most likely mediated by caspase-3 and separating the domains of the enzyme from one another may have a dominant-negative effect on U1-70kDa activity resulting in blockade of splicing and probably influencing cellular repair pathways which rely on new mRNA synthesis (Cohen, 1997).
Protein kinase C δ (PKCδ) is cleaved by caspase-3 but not caspase-1, -4 and -6 (Casciola-Rosen et al., 1996, Song et al., 1996) to form an catalytically active fragment. Overexpression of an active PKCδ fragment is associated with chromatine condensation indicating that proteolytic activation of PKCδ contributes to some features of the apoptotic phenotype (Emoto et al., 1995, Ghayur et al., 1996).
An important mediator of cell cycle progression, the retino blastoma protein (Rb), is also proteolytically cleaved during apoptosis (Janicke et al., 1996, Browne et al., 1994, An and Dou, 1996). Rb is relevant for cell cycle progression after phosphorylation by cyclin-dependent kinases and an anti-apoptotic function has been reported (Kouzarides, 1995). Different sized cleavage products can be observed probably displaying cleavage activity of different caspases. The cleavage product produced after proteolysis at an DEAD motif fails to bind the regulatory protein MDM2 (Janicke et al., 1996).
In addition, numerous downstream substrates of caspases have been identified, many of these being involved in DNA repair, RNA maturation or maintainance of structural integrity.

III-3.6 CASPASE INHIBITORS

Viral and Cellular Caspase Inhibitors
For an infected cell the far most effective way to prevent viral replication is to kill itself. Since apoptosis is used to defend against viruses, some viruses carry cell death inhibitors to block this response of their host cells. Several viral cell death inhibitors act by binding to activated caspases.
The cowpox virus encodes the cytokine response modifier A (crmA) which inhibits caspase-1, thereby reducing the defensive inflammatory response triggered by IL-1β (McFadden, 1995). Moreover, it was found that crmA prevents defensive suicide of infected cells thereby providing more time for viral replication (Ray, 1996). This also stressed the close proximity of inflammatory response and apoptosis. With respect to the inhibitory constants it can be stated that crmA inhibits active caspase-1 and caspase-8 (Los, 1995, Tewari, 1995, Varfolomeev, 1998) and is unlikely to inhibit caspase-3, -6 and -7 *in vivo* (Zhou et al., 1997, Garcia-Calvo, 1998). Besides the fact

that it inhibits caspase-9 with a K_i of 2 nM (compared to 0.3 nM for caspase-8) it is stated that it cannot inhibit caspase-9 *in vivo*.(Kuida, 1998, Smith, 1996).CrmA belongs to the family of serine proteases, the pseudosubstrate residues are LVAD and after cleavage polypeptides are released.

The baculoviral p35 not only blocks apoptotic response to infections in insect cells but is in addition capable of inhibiting the *C. elegans CED-3* protein and caspase-1, -3, -6, -7, -8 and -10 (K_i < 10 nM; Bump et al., 1995, Zhou, 1998). P35 neither inhibits non-caspase cysteinyl proteases nor serine proteases. In contrast to crmA the cleaved subunits of p35 remain in an inhibitory complex that can be dissolved by SDS (Zhou, 1998).

Inhibitor of apoptosis proteins (IAPs) were first identified in baculoviruses (Clem, 1991, Clem, 1994). Up to now, six human IAP relatives have been identified: NAIP, cIAP1, cIAP2, XIAP, survivin and apollon (Deveraux, 1998, Chen, 1999) and at least cIAP1, cIAP2 and XIAP have been shown to directly inhibit caspase activity. IAPs share a common structural motif which is called the baculoviral IAP repeat (BIR). The BIR-domain is a cysteine- and histidine-rich sequence motif of 70 aa length and has been shown to bind to and inhibit caspases (Deveraux et al., 1997, Roy et al., 1997, Deveraux, 1998, Takahashi et al., 1998). XIAP inhibits caspase-8-induced protease acticity at the level of caspase-3 and caspase-7 whereas cytochrome c induced activation is additionaly prevented upstream of the effector caspases by direct inhibition of caspase-9 processing.

Synthetic Caspase Inhibitors

A number of specific caspase inhibitors have been developed based on the substrate cleavage sites of prefered substrates or a combinatorial approach (see table 1) to find optimum peptide sequences of the different caspases. These inhibitors act as pseudosubstrates for active caspases and are therefore competitive inhibitors. Most widely used are specific tetrapeptide inhibitors but also trimers, especially with the sequence VAD, are applied for certain purposes. Specific inhibitors resemble the structure Z-P^4-P^3-P^2-D-end (Z = benzyloxycarbonyl-). The end-group varies according to the purpose of the tetrapeptide. As end-groups for caspase substrates mostly chromophors are used, especially pNA (para-nitroanilid) and AMC (aminomethylcoumarin) as colorimetric and fluorometric substrates, respectively. Caspase inhibitors are capped with fluoromethylketone (fmk) or chloromethylketone (cmk). Those groups produce irreversible inhibitors because a thiomethylketone is formed with the active site cysteine of the caspase resulting in inactivation of the enzyme. In experiments with cultured cells the fmk group should be prefered because of superior membrane permeability (Ekert, 1999)

.

Table 1: Preferred sequence specificity of caspases. .

Caspase	Optimal Sequence ($P^4P^3P^2P^1$)
caspase-1	WEHD
caspase-4	(W/L)EHD
caspase-5	(W/L)EHD
C. elegans CED-3	DETD
caspase-3	DEVD
caspase-7	DEVD
caspase-2	DEHD
caspase-6	VEHD
caspas-8	LETD
caspase-9	LEHD

Different substrate specificity allows to subdivide caspases into three groups. Despite the absolute requirement for D in the P^1 position caspase-1, -4 and -5 display a preference for hydrophobic amino acids at the P^4-position. The second subgroup consists of caspase-2, -3, -7 and CED-3, these enzymes show specificity for aspartate in the P^4 position and the general motif is represented by DEXD. At last, for caspase-6, -8 and -9 comprise the third subgroup and for their proteolytic activity the common cleavage sequence is (L/V)EXD.

III-4 CHEMOTHERAPHEUTHIC DRUGS

III-4.1 TAXOL

The cytotoxicity of taxol is based on its ability to alter the formation of microtubuli. In contrast to vincaalcaloids, which inhibit tubulin polymerisation, taxol enhances formation of microtubuli (Horwitz, 1992, Nicolaou, 1994, Pazdur, 1993). Microtubuli play an important role during mitosis: the cytoskeleton breaks down and the mitotic spindle apparatus is build of the tubulin pool. Upon taxol treatment, microtubuli differ from physiologically constituted polymers: they show a decreased diameter, consist of 12 instead of 13 profilaments and are shorter in length. Furthermore, they are more resistant to depolymerisation by decreased temperature or increased Ca^{2+} concentration. Taxol operates in all phases of the cell cycle. During mitosis star-shaped microtubuli formation is observed and in other cell cycle phases microtubuli bundles are formed. Isolation of tubulin in stable structures consequently blocks formation of the mitotic spindle-apparatus (Schiff and Horwitz, 1979, Horwitz et al., 1994).

III-4.2 EPIRUBICIN

Epirubicin most effectively influences exponentially growing cells in the S- and G_2-phase of the cell cycle. The mechanism of epirubicin treatment is not yet fully understood but includs intercalation into DNA and non-covalent binding to DNA, inhibition of topoisomerases (topo) I and II and other helicases and production of reactive oxygen species (ROS) (Chan et al., 1996). Intercalation seems to play a minor role in cancer therapy because high concentrations of epirubicin are required for this effect and are not achieved under therapeutic treatment conditions. Topo II mediates alteration of the three dimensional structure in condensed DNA and inhibition of this enzyme results in persisting strand-breaks. Topo II-inhibitors bind to the DNA-topo II-complex and inhibit re-ligation of the single strands by formation of an irreversible, ternary complex (Ross, 1978, Ross, 1981, Tewey, 1984).

IV AIM OF THIS STUDY

Two major cell-intrinsic pathways have been demonstrated over the past five years to induce apoptosis. One pathway starts with death receptor ligation and the other involves cytochrome c release from mitochondria. Cytostatic drug-induced apoptosis has been reported to be mediated by induction of death receptor ligand expression (Friesen et al., 1996, Friesen et al., 1997, Müller et al., 1997, Fulda et al., 1997, Fulda et al., 1998). However, other groups announced that chemotherapeutic drugs induce apoptosis independent of CD95/Fas signalling, exclusively via the mitochondrial apoptotic pathway (McGahon et al., 1998, Adjei et al., 1998, Eischen et al., 1997, Micheau et al., 1999). Therefore, it is still a matter of debate how cytostatic drugs induce apoptosis, and whether cytostatic drug-induced apoptosis is mediated via the CD95/Fas pathway or is independent of death receptor signalling.

Thus, the aim of this study was the analysis of cytostatic drug-induced apoptosis in the human B-lymphoid cell line BJAB. Two different cytostatic drugs, taxol and epirubicin, were chosen for examination of their effects on cultured BJAB cells. These drugs target different cellular molecules and therefore provide a more general answer to the mode of action of cytostatic drugs. The goal was to investigate which apoptotic cascade(s) is (are) initiated, and especially the order in which caspases are activated in this experimental setup had to be determined. Furthermore, the question whether the results obtained in BJAB cells as a model system represent a general principle of cytostatic drug-induced apoptosis was addressed. For this NALM6 and REH B-lymphoid cell lines were examined to provide evidence for a general statement on the mechanisms involved in cytostatic drug-induced apoptosis in B cells.

Anitbodies against various apoptosis-related proteins can be used to answer most of these questions. However, this approach is not suitable to reveal yet unknown proteins involved in cytostatic drug-induced apoptosis. In order to investigate general effects of cytostatic drug-treatment on protein level, two-dimensional polyacrylamide gelelectrophoresis (2DE PAGE) had to be performed to detect apoptosis-associated proteins. Identification of specific caspase substrates could give clues to downstream effects of caspase activation and elucidate consequences of protease activation during apoptosis in more detail. Furthermore, regulatory proteins acting upstream of caspase activation can eventually be detected by 2DE PAGE.

V MATERIAL

V-1 CHEMICALS AND REAGENTS

Table 2: Chemicals and Reagents. All reagents used were p.a. grade.

Substance	Supplier
3-(cyclohexylamino)-propane-1-sulphonic acid (CAPS)	SIGMA, München, Germany
^{35}S-Methionine/cysteine labeling mix	Amersham Buchler, Braunschweig, Germany
40% acrylamide/bisacrylamide 29:1	Roth GmbH & Co, Karlsruhe, Germany
Acetonitril (ACN), HPLC grade	MERCK, Berlin, Germany
Acrylamide	Carl Roth GmbH & Co, Karlsruhe, Germany
Agarose	BioRad, München, Germany
Ammoniumperoxodisulfate (APS)	Carl Roth GmbH & Co, Karlsruhe, Germany
3-[(3-Cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS)	SIGMA, München, Germany
Coomassie Brilliant Blue R-250	Carl Roth GmbH & Co, Karlsruhe, Germany
dATP	SIGMA, München, Germany
Dithiothreitol (DTT)	SIGMA, München, Germany
epirubicin	Pharmacia Upjohn, Erlangen, Germany
Ethanol	Carl Roth GmbH & Co, Karlsruhe, Germany
Ethylendiamine tetra acetic acid (EDTA)	Carl Roth GmbH & Co, Karlsruhe, Germany
Fetal Calf Serum	Life Technologies GmbH, Karlsruhe, Germany
HCOOH, 37% stock	Carl Roth GmbH & Co, Karlsruhe, Germany
N-[2-hydroxyethyl]piperazine-N`-[2-ethane-sulfonic acid] (HEPES)	Carl Roth GmbH & Co, Karlsruhe, Germany
IEF gel solution	WITA, Teltow, Germany
Leupeptin	SIGMA, München, Germany
L-Glutamine	Life Technologies GmbH, Karlsruhe, Germany
Methanol	Carl Roth GmbH & Co, Karlsruhe, Germany
$MgCl_2$	Carl Roth GmbH & Co, Karlsruhe, Germany
Nitrocellulose membrane	Schleicher & Schüll, Dassel, Germany
Non fat dry milk powder	Glücksklee
Penicillin/Streptomycin	Life Technologies GmbH, Karlsruhe, Germany
Phenyl-methyl-sulfonyl-fluoride (PMSF)	SIGMA, München, Germany
Ponceau-S	SIGMA, München, Germany

Table 2: Chemicals and Reagents. All reagents used were p.a. grade.

Substance	Supplier
Potassium chloride (KCl)	Carl Roth GmbH & Co, Karlsruhe, Germany
Propidium iodide (PI)	Carl Roth GmbH & Co, Karlsruhe, Germany
Protein-A Sepharose	SIGMA, München, Germany
RPMI 1640	Life Technologies GmbH, Karlsruhe, Germany
SDS-PAGE gel solution	WITA, Teltow, Germany
Sodium chloride (NaCl)	Carl Roth GmbH & Co, Karlsruhe, Germany
Sodium dodecylsulfate (SDS)	Carl Roth GmbH & Co, Karlsruhe, Germany
Sucrose	Carl Roth GmbH & Co, Karlsruhe, Germany
taxol (paclitaxel)	Bristol Arzneimittel, München, Germany
Tris-(hydroxymethyl)-amino-methane (TRIS)	Carl Roth GmbH & Co, Karlsruhe, Germany
Triton X-100	Carl Roth GmbH & Co, Karlsruhe, Germany
Tween 20	Carl Roth GmbH & Co, Karlsruhe, Germany

V-2 CELL LINES

Table 3: Cell lines.

cell line	description
BJAB	Burkitt like lymphoma cell line transfectants BJAB/mock, BJAB/crmA and BJAB/FADDdn were kindly provided by Dr. Klaus Schulze-Osthoff, University of Tübingen, Tübingen, Germany
NALM6	human B-cell lymphoma cell line
REH	human B-cell lymphoma cell line

V-3 ENZYMES AND PROTEINS

Cytochrome c: SIGMA, München, Germany

RNase A: Carl Roth GmbH & Co, Karlsruhe, Germany

Trypsin: Promega, Mannheim, Germany

Bovine Serum Albumin (BSA):SIGMA, München, Germany,

V-4 SPECIAL REAGENTS

Z-DEVD-fmk, Z-WEHD-fmk: irreversible, cell permeable specific caspase inhibitors were from Kamyia Biomedical company, Seattle, WA, USA.

Ac-DEVD-pA, Ac-IETD-pNA:specific colorimetric caspase substrates were purchased from Calbiochem-Novabiochem GmbH, Bad Soden, Germany.

5,5´,6,6´-tetrachloro-1,1´,3,3´-tetraethyl-benzimidazolylcarbocyanin iodide (JC-1):was purchased from Molecular Probes, Leiden, The Netherlands.

V-5 ANTIBODIES

Secondary antibodies, anti-mouse and anti-rabbit, coupled to horse radish peroxidase were purchased from Promega, Mannheim, Germany. Anti-goat antibody coupled to horse-radish peroxidase was purchased from Santa Cruz, California, USA .

Table 4: Primary antibodies.

antibody directed against	description
caspase-1	polyclonal rabbit; # sc-622; Santa Cruz, California, USA
caspase-1 p20 subunit	polyclonal goat; # sc-1780; Santa Cruz, California, USA
caspase-3	polyclonal rabbit; # 65906E; Pharmingen, Hamburg, Germany
caspase-8	monoclonal mouse; kindly provided by Dr. Klaus Schulze-Osthoff, University of Tübingen, Tübingen, Germany
caspase-9	polyclonal rabbit; # 68086E; Pharmingen, Hamburg, Germany
rho-GDI/D4-GDI	polyclonal rabbit; # 66586E; Pharmingen, Hamburg, Germany
PARP	polyclonal rabbit; # 65196E; Pharmingen, Hamburg, Germany

V-6 KITS

Bicinchonic assay	(Pierce) KMF Laborchemie Handels GmbH, St. Augustin, Germany
Enhanced chemoluminescence (ECL)	Amersham Buchler, Braunschweig, Germany

VI METHODS

VI-1 CENTRIFUGATION (GENERAL COMMENT)

If not otherwise mentioned cells are harvested by centrifugation at 300 x g at 4°C for 5 min. This corresponds to 2500 rpm in an Eppendorf centrifuge and 1200 rpm in a Varifuge 3.0R (Heraeus). Cells were handled on ice if no further treatment or growing was intended.

VI-2 CELL CULTURE

Human Burkitt Like Lymphoma cells, BJAB, stably transfected with crmA, FADDdn or vector control (pcDNA 3) were kindly provided by Klaus Schulze Osthoff (University of Tübingen, Tübingen, Germany). Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.56 g/L L-glutamine and incubated at 37°C in 95% air and 5% CO_2. Cells were subcultured every 3-4 days by dilution of the cells to a concentration of 1 x 10^5 cells / mL. NALM6 and REH cells were cultured under the same conditions as BJAB cells.

VI-3 INDUCTION OF APOPTOSIS

VI-3.1 CELL CULTURE

Cells were grown in RPMI 1640 supplemented with 10% (v/v) fetal calf serum (FCS), 0.56 g/L L-glutamine, 100 000 i.u. penicillin and 0.1 g/L streptomycin. Cells were subcultured every 4 days by dilution of the cells to a concentration of 1x10^5 /mL.

VI-3.2 CYTOSTATIC DRUG TREATMENT

Cells were seeded at a density of 4x10^5 /mL 24 hs prior to starting an experiment. For induction of apoptosis cells were then harvested and resuspended at a density of 1x10^5 cells/mL with the desired concentrations of the respective cytostatic drugs. In standard experiments taxol and epirubicin were used at a concentration of 0.12 µM and 1.85 µM, respectively.

VI-3.3 INHIBITOR EXPERIMENTS

For inhibition of caspase activity, cells were incubated with different irreversible, cell permeable peptide inhibitors at various concentrations.

1. Z-DEVD-fmk (inhibitor of caspase-3-like activity)
2. Z-WEHD-fmk (inhibitor of caspase-1-like activity)

Stock solutions of the inhibitors were prepared in DMSO or EtOH as indicated and stored at 4°C. Inhibitors were added to the cell culture 2 hs prior to treatment with cytostatic drugs, and control cultures containing the same concentration of DMSO or EtOH, respectively were grown in parallel.

VI-4 Quantification of Apoptotic Cells

VI-4.1 Trypan Blue Exclusion

Cells were harvested at 400 x g for 5 min and incubated with Trypan blue solution for 5 min. Blue cells were assumed as dead. The number of living and dead cells was assessed by counting in a Neubauer counting chamber. At least 100 cells were counted.

VI-4.2 Modified Cell Cycle Analysis

After treatment, cells were spun down at 300 x g for 5 min. and resuspended in cold PBS. Cells were then fixed in PBS/2% H_2CO (v/v) on ice for 30 min. and spun down again. After fixation, cells were incubated with 66 % EtOH in PBS for 15 min. on ice or at -20°C over night. RNA was digested after further centrifugation by addition of 50 µL of PBS containing 40 µg/mL RNase and incubation at 37°C for 30 min. Then, cells were pelleted again and DNA was stained by addition of 200 µL of propidium iodide (50 µg/mL) in PBS.
Percentage of hypodiploid cells was assessed by flow cytometric analysis (Nicoletti et al., 1991) using a FACScan (Becton Dickinson, Heidelberg, Germany) equipped with CELLQuest software. Apoptotic cells were determined by quantitating the sub-G_1 peak using the analysis software mentioned above.

VI-4.3 Depolarisation of Mitochondria (JC-1 Assay)

Cells were harvested, washed in PBS and then collected by centrifugation at 300 x g. 1 x 10^5 cells were resuspended in RPMI 1640 without phenol red and supplements (glutamine, penicillin, streptomycin) with or without 2.5 µg/mL 5,5´,6,6´-tetrachloro-1,1´,3,3´-tetraethyl-benzimidazolylcarbocyanin iodide (JC-1). Samples were incubated for 30 min. at 37°C with shaking. After centrifugation at 300 x g, 4°C for 5 min. cells were first washed, resuspended in 200 µL ice-cold PBS and analysed immediately by flow cytometry. Cells with decreased fluorescence compared with untreated control cells were assumed to display lower mitochondrial membrane potential. Data were collected on a FACScan and quantification was performed using the CELLQuest software.

VI-5 *IN VITRO* INDUCTION OF MITOCHONDRIAL APOPTOTIC CASCADE

Buffer H

20	mM	HEPES, pH 7.0
10	mM	KCl
2	mM	$MgCl_2$
1	mM	EDTA

Cell extracts were prepared as decribed (Deveraux et al., 1997). After harvesting, cells were washed twice in PBS and once in buffer H, spun down again and cells were incubated in buffer H (containing 100 µM PMSF) on ice for 15 min.. Rupture of membranes was achieved by passing the cells through a 0.80 mm x 0.40 mm needle 15 times. The suspension was cleared by centrifugation at 16000 x g twice for 15 min. at 4°C. Clear supernatants were used for *in vitro* activation of mitochondrial apoptotic cascade or to determine inherent caspase activity. Mimicry of mitochondrial induction of apoptosis (*in vitro* caspase activation) was achieved by addition of 1 mM DTT, 1 µM horse heart cytochrome c and 1 mM dATP and incubation at 30°C for 5 min. followed by incubation at 37°C for different periods of time. Control extracts were likewise incubated in the absence of cytochrome c and dATP.

VI-6 CLEAVAGE OF CASPASE SPECIFIC COLORIMETRIC SUBSTRATES

Buffer B

50	mM	HEPES, pH 7.4
100	mM	NaCl
1	mM	EDTA
0.1	%	CHAPS
10	%	saccharose
5	mM	DTT

Cell extracts were prepared as described above, and protein concentration was determined using the BCA assay kit. 10 µL of extract were mixed with 90 µL of buffer B. Just prior to measurement of absorption at 405 nm 2 µL of colorimetric substrate were added. Samples were incubated at 37°C and absorption values were determined after appropriate periods of time according to the speed of absorption increase. Specific caspase activity was calculated from the extinction values and the protein concentration of the extract.

VI-7 SAMPLE PREPARATION FOR GEL ELECTROPHORESIS

The sample preparation was different according to the purpose of the analysis. In general, cell pellets and protein solutions were handled at 4°C to avoid protein degradation. Nevertheless, buffers always contained a protease inhibitor mix, either PMSF/pepstatin/leupeptin or the protease inhibitor cocktail from Boehringer.

VI-8 One Dimensional Polyacrylamide Gel Electrophoresis

VI-8.1 Preparation of SDS-PAGE gels

According to the apparent molecular weight of the proteins in question, gels containing different concentrations of acrylamide were used. The composition of different gels is given in table 5. Separation was performed at 200 V until the methylene blue front had left the gel's end.

Table 5: Composition of polyacrylamide gels. 10% aqueous APS was prepared freshly and the acrylamide stock solution contained acrylamide/bisacrylamide in a ratio of 29:1.

stacking	acrylamide (40%) / mL	Tris/Cl (0.5 M) pH 6.8 / mL	H_2O / mL	SDS (10%) / µL	TEMED / µL	APS (10%) / µL
4 %	0.375	0.380	2.185	30	3	3
running	Acrylamide (40%) / mL	Tris/Cl (1.5 M) pH 8.5 / mL	H_2O / mL	SDS (10%) / µL	TEMED / µL	APS (10%) / µL
8 %	2.0	2.5	5.3	100	8	100
10 %	2.5	2.5	4.7	100	4	100
12 %	3.0	2.5	4.3	100	4	100
16 %	4.0	2.5	3.3	100	4	100

VI-8.2 Sample Preparation for One Dimensional Gel Electrophoresis

Buffer L

10	mM	Tris/Cl, pH 7.5
300	mM	NaCl
1	%	Tritin-X 100
2	mM	$MgCl_2$
5	mM	EDTA
1	µM	pepstatin
1	µM	leupeptin
100	µM	PMSF

Cells were treated with the respective cytostatic drugs or left untreated. After incubation, cells were harvested at 300 x g for 5 min. and washed twice in an appropriate volume of PBS. After washing, lysis was achieved by addition of buffer L and using a Vortex for 30 s. Debris was spun down at 16000 x g for 15 min and the supernatant was collected an used as sample.

VI-8.3 Determination of Protein Concentration

Protein concentration was determined using the BCA assay kit from Pierce according to the manufacturer's protocol. 2 - 5 µL of sample were diluted in 200 µL of BCA solution in an ELISA plate (flat bottom) and incubated at 37°C for 30 min. in the dark. Absorption was measured using an ELISA reader at 590 nm. BSA was used as standard and the protein concentration was calculatied on the basis of the derived standard curve.

VI-9 High Resolution Two Dimensional PAGE Gel Electrophoresis

VI-9.1 Preparation of First Dimension PAGE Gels (Isolelctric focusing gels)

Separating gel solution			Cap-gel solution		
3.5	%	acrylamide	12	%	acrylamide
0.3	%	piperazine diacrylamide	0.13	%	piperazine diacryl-amide
9	M	urea	9	M	urea
4	%	WITAlyte	4	%	WITAlyte
5	%	glycerol	5	%	glycerol
0.06	%	TEMED	0.06	%	TEMED

For preparation of IEF rod gels, premixed acrylamide solutions from WITA GmbH (Teltow, Germany) were used. Solutions were thawed and degassed for 15 min. using a water-jet pump. 25 µL of 0.8 % APS were added to 975 µL acrylamide solution, mixed gently and the liquid was pulled in either 0.9 mm diameter or 1.5 mm diameter glass capillaries of 23 cm length fastened in a gel casting apparatus. The designated volume in the capillaries was left empty to allow sample application and addition of cap-gel solution respectively. After polymerisation (30 min.) capillaries were turned upside down, cap-gel solution (390 µL) was also degassed and 10 µL of 0.8 % APS were added. The solution was mixed gently and filled into the upper end of the capillary using a tuberculine syringe.
After 15 min. of incubation, water resulting from polymerisation was removed. Capping ends of the capillaries were overlayed with water. The openings at the sample end of the capillaries were covered with a water droplet and both ends were closed with parafilm. Gels were used after 3 days of polymerisation in the dark at room temperature.

VI-9.2 Preparation of Second Dimension PAGE gels (SDS-PAGE Gels)

Buffer P		
285	mM	Tris base
90	mM	Tris/Cl
3.5	mM	SDS

For this purpose, WITA gel solutions were used which are delivered in 73.5 mL aliquots. After thawing the desired amount of acrylamide solution (1 aliquot per gel of 0.9 mm ∅ , 3 aliquots per 2 gels of 1.5 mm ∅) 1.28 % APS (5 mL per aliquot) was added and the solution was mixed gently. Glas plates were fixed in the appropriate gel casting apparatus and 40 % glycerol (aqueous solution) was filled between the glass plates at the bottom, acrylamide solution was poured into from the top. Filled plates were covered with parafilm. After 30 min. of polymerisation, casted gels were turned upside down, the glycerol was removed and the top was covered with buffer P and parafilm. Gels were stored at 4°C overnight and ready to use the following day.

VI-9.3 Sample Preparation for Two Dimensional Gel Electrophoresis

For subtractive analysis of 2DE PAGE gels, high reproducibility is essential and sample preparation is the most crucial step in 2DE PAGE. When subtractive analysis was desired sample preparation was performed in parallel.

In general, cells were harvested in the same manner as for one dimensional gel electrophoresis. After washing in PBS the weight of the pellet was determined (=W / mg) and lysis was achieved by addition of 1.08 x W mg urea. Resulting volume (=V) was assumed to be 2 x W and sulfide bonds were reduced by addition of 0.05 x V DTT (1.4 M stock solution, 70 mM final concentration). Sample was supplemented with 2 % Servalyte (pH 2-4) and protease activity abolished with PMSF (1 mM final concentration, 200 mM stock in ethanol) and pepstatin (1 µg/mL final concentration, 100 µg/mL stock). The resulting suspension was mixed using a Vortex for about 15 min. at room temperature. DNA was separated by centrifugation in special tube at 100,000 x g for 20 min at 4°C and the supernatant was collected as protein sample and stored at -80°C.

VI-9.4 Isoelectric Focusing (First Dimension)

Buffer K (degassed)

9	M	urea
5	%	ethylendiamine
5	%	gylcerol

Buffer A (degassed)

3	M	urea
		ortho-phosphoric acid

Sephadex solution

100	µL	sephadex solution
108	mg	urea
10	µL	ampholytes pH 2-11

Overlay solution

5.3	µL	urea
5	mg	Servalyte pH 2-4
0.7	µL	glycine

Buffer I (no pH adjustment!)

285	mM	Tris base
90	mM	Tris/Cl
3.5	mM	SDS

Water was removed from both ends of the IEF gels, the bottom end filled with buffer K and gels were set up in the IEF apparatus. Sample ends were covered with 5 mm sephadex solution and the sample applied on top of it (10 µL for 0.9 mm diameter rod gels, 20-30 µL for 1.5 mm diameter rod gels). Sample was covered carefully with 5 µL overlay solution and buffer A filled on top of it. Buffer K was filled onto the bottom of the apparatus and buffer A into the top reservoir so that the gel tops were covered. Electric current program is given in table 6.

After finishing isoelectric focusing, gels were pressed onto a special tray using a syringe equipped with a 200 µL pipet tip filled with buffer I. Gels were equilibrated in buffer I two times 5 min. and stored at -80°C until use.

Table 6: Electric current program for IEF. Gels were run at room temperature for 21 hs 15 min using the indicated program on a BioRad Power Pack 3000.

U/Volt	time
100	1 h
200	1 h
400	17.5 hs
650	1 h
1000	30 min.
1500	10 min.
2000	5 min

VI-9.5 SDS-PAGE (SECOND DIMENSION)

Buffer AG

1	%	agarose
0.1	%	SDS
125	mM	Tris pH 6.8 (adjusted with H_3PO_4)

SDS PAGE Buffer

25	mM	Tris base
129	mM	glycine
0.1	%	SDS

The covering solution was removed from the gels' top slits and IEF gels were placed on top. Rod gels were fixed with 1 % buffer AG. Second dimension gels were placed into the running apparatus filled with SDS-PAGE buffer. Bromphenol blue solution was added to the top reservoir (kathodic reservoir) and separation was performed at 11°C for 5-7 hs. Running conditions were as given in table 7.

Table 7: Electric current programs for second dimension SDS-PAGE gels. Protein separation was performed at 11°C for 5-7 hs and stopped at the time when bromphenol blue front reached the indicated gel end (1 cm prior to the end of glass plates).

I/mA	time
65	15 min.
85	5 - 7 hs

0.9 mm thickness

I/mA	time
120	15 min.
160	5 - 7 hs

1.5 mm thickness

VI-9.6 SDS-PAGE Gel Staining

Fixation solution		
5	%	acetic acid
50	%	ethanol

After two dimensional gel electrophoresis, gels were stained with respect to the purpose of the gels. Analytical gels (in general 0.9 mm gels) were stained using the protocol of Heuckeshofen and Dernik (Heuckeshofen and Dernick, 1985), preparative gels (1.5 mm) were either stained with Coomassie Brilliant Blue or silver stained according to the protocol of Blum et al. (Blum et al., 1987). All gels were fixed in fixing solution for at least 2 hs or over night prior to staining. If blotting was desired, gels were left unfixed and proteins were transferred to the membrane immediately after finishing second dimension protein separation.

VI-9.7 Silver Stain (Analytical, Heuckeshofen and Dernick, 1985)

Incubation solution		
0.5	M	NaAc
0.1	M	$Na_2S_2O_3$
1.25	%	glutaraldehyde
30	%	ethanol

Staining solution		
0.2	%	$AgNO_3$
0.01	%	CH_2O (37% stock)

Developer solution		
240	mM	Na_2CO_3
0.01	%	CH_2O (37% stock)

Stop solution		
100	mM	EDTA

Gels were fixed over night at 4°C or room temperature with shaking in fixation solution, incubated in incubation solution for 1 h with shaking and equilibrated in water (3 times 20 min.). Proteins were stained by application of staining solution for 30 min. and gels were rinsed in water (30 s). Subsequently, the staining pattern was visualised using developer solution (3 - 7 min.). After removal of the developer solution, reaction was stopped with stop solution.

VI-9.8 Coomassie Brilliant Blue Staining (CBB Staining)

CBB-staining solution		
0.1	%	CBB R-250
10	%	acetic acid
50	%	ethanol

Destaining solution		
5	%	acetic acid
50	%	ethanol

After fixation, gels were incubated in CBB staining solution over night and then destained by several changes in destaining solution until a satisfactory staining pattern was observed. Gels were stored at 4°C in a plastic bag.

VI-9.9 SILVER STAIN (PREPARATIVE, BLUM ET AL., 1987)

This method was applied to gels desired for spot identification since the method applied to analytical 2DE PAGE gels wasn't adequate (proteins are permanently fixed in the acrylamide matrix due to the reaction with glutaraldehyde).
The gels were fixed and subsequently the acid was removed by stepwise washing with 30% ethanol (20 min.) and water (20 min.). Sensitation was accomplished by applying 0.02 % $Na_2S_2O_3$ solution for about 1 min and sodium thiosulfate solution was discarded and gels rinsed two times with water. Gels were equilibrated in staining solution for 45 min., rinsed two times in water and proteins were visualized by application of developer solution until a satisfying spot intensity was reached. Reaction was stopped with stop solution and the gels were stored in a plastic bag at 4°C.

VI-10 BLOTTING

10xCAPS

100	mM	CAPS; pH 11

Blotting buffer

10	mM	CAPS
10	%	MeOH

Blotting was performed using a BioRad semi dry blotting apparatus (Transblot SD cell). Membranes and filter paper were swollen in CAPS buffer for several minutes and the blotting sandwich consisted of (anode) 3 x filter paper, NC membrane, SDS-PAGE gel, 3 x filter paper (kathode). The size of the filter papers and the membrane fitted exactly the gel's size. Blotting was performed at 1 mA / cm^2 for 45 min. - 75 min. in regard to percentage of the acrylamide in the gel and the molecular mass of the protein(s) in question. Membranes were stained with Ponceau Red to verify homogenous protein transfer and destained in H_2O. Membranes were dryed and stored at room temperature in a plastic bag.

VI-11 IMMUNOCHEMICAL DEPLETION OF CASPASE-3

Caspase-3 was immunoprecipitated from cellular extracts of BJAB cells as described (Geilen et al., 1997). Briefly, 100 µl of cellular extract were incubated with 0.5 mg of protein A-Sepharose and 20 µl (4 µg) of polyclonal rabbit anti-human caspase-3 antibody at 4°C for 4 hs with moderate shaking. The protein A-Sepharose immune complex was sedimented by centrifugation at 300 g, 4°C for 5 min. The cleared supernatant was used for *in vitro* activation by addition of dATP and cytochrome c as described above. Control extracts were prepared in parallel in the presence of protein A sepharose but in the absence of antibody.

VI-12 IDENTIFICATION OF PROTEINS

VI-12.1 IMMUNOCHEMICAL IDENTIFICATION

10xPBST

10	x	PBS
0.5	%	Tween 20

Blocking Buffer

1	x	PBST
10	%	blocking reagent
	or	
3	%	non-fat dry milk powder

Immunodetection of protein was performed as described (Wieder et al., 1994). Membranes were blocked for one hour in blocking buffer using either non-fat dry milk powder or casein as blocking reagent. The first antibody was diluted in blocking buffer and applied for one hour at room temperature with moderate shaking in a 15 cm diameter petri dish. Then, membranes were briefly washed in PBST and incubated in PBST twice for 10 min.. Secondary antibody was diluted in PBST and applied for one hour at room temperature with shaking. Excess antibody was washed away with PBST and membranes were again shaken in PBST twice for 10 min.. Freshly prepared enhanced chemoluminescence (ECL) solution was applied for 2 min. and membranes were dried between filter paper. After positioning the membranes in the cassette (aligned to one edge) films (also aligned to the same edge) were exposed to the membranes for different periods of time to obtain optimal results. Processed images were identically positioned in the film box and marker bands were marked on the films.

VI-12.2 MASS SPECTROMETRIC IDENTIFICATION

Equilibration buffer

200	mM	Tris/Cl, pH 8.5

Digestion buffer

100	mM	Tris/Cl, pH 8.2
1	mM	Ca_2Cl

Tryptic In-Gel Digest of Coomassie Stained Gel Pieces

Coomassie destaining buffer

Equilibration buffer : ACN 1:1

Spots were cut from two dimensional PAGE gels and equilibrated in an appropriate volume of coomassie destaining buffer for 30 min. at 30°C with shaking. Buffer was discarded and when necessary the wash was repeated. Gel pieces were then nearly dried

in a SpeedVac (5-10 min.) and digestion was achieved by addition of 0.1-0.2 µg trypsin in an appropriate volume of digestion buffer. Digest was performed at 37°C with shaking over night. Reaction was stopped by addition of 10 % trifluoroacetic acid to give a final concentration of 2 % - 3 % and shaking at 60 °C for 20 min.

Tryptic In-Gel Digest of Silver Stained Gel Pieces

Reducing buffer (prepared freshly)		
15	mM	ferricyanide (30 mM stock)
50	mM	$Na_2S_2O_3$ (50 mM stock)

Destaining of spots was achieved by incubation in reducing buffer until brownish color was removed completely. Gel pieces were equilibrated in plenty of water. Water was changed several times to assure complete removal of the chemical agents.

Preparation of Samples for Mass Spectrometry

Elution buffer		
60	%	acetonitril (ACN)
0.1	%	TFA

After tryptic digest supernatant was collected and peptides were further eluted from gel pieces by shaking in Tris/Cl, pH 8.5 : ACN 1:1 for 20 min at 37°C. Supernatant was collected, ACN evaporated by centrifugation in a SpeedVac for 20 min. and peptides resuspended in 0.1 % TFA aqueous solution. Then peptides were collected on a Zip Tip by pipetting the solution several times through the Tip. Peptides were eluted from the Tip with max. 5 µL elution buffer and ACN again was evaporated in a SpeedVac. Peptides were resuspended in 60 % MeOH, 1 % HCOOH. Alternatively after binding to the Zip Tip, peptides were eluted directly with 60 % MeOH, 1 % HCOOH.

VI-13 ANNEXIN-V BASED CELL FRACTIONATION

BJAB/mock cells were treated with 0.12 µM taxol or 1.85 µM epirubicin for 48 hs and fractionation was performed according to the manufacturers' protocol. Briefly, approximately 1 x 10^7 cells were resuspended in 80 µL and 20 µL of a suspension of Annexin-V coupled microbeads were added. After incubation at 4°C, cells were washed in 1 mL of binding buffer and applied to a positive selection column in the presence of a magnetic field, equilibrated in 3 mL Annexin-V binding buffer. PS-negative cells which passed through the column were collected and the column was washed with 3 x 500 µL of binding buffer. The column was removed from the magnetic field and PS-positive cells were eluted in 1000 µL Annexin-V binding buffer using the supplied plunger to flush the cells out. Collected cells were spun down at 300 x g and the resulting pellet was used for further investigation.

VI-14 METABOLIC ^{35}S-LABELING

Cells were starved in methionine/cysteine free RPMI 1640 medium for 2 hs at a density of 4 x 10^5 cells/mL. Then, ^{35}S-labeled methionine/cysteine labeling mix and cytostatic drugs or labeling mix alone were added (50 µCi/mL). Cells were cultured for 6 hs - 12 hs. Then, the cells were harvested by centrifugation at 300 x g, washed in 1 x PBS and the resulting pellet was used as sample.

VII RESULTS

VII-1 CYTOSTATIC DRUG TREATMENT OF BJAB CELLS

In a first set of experiments, BJAB/mock cells were incubated with different concentrations of taxol or epirubicin for different periods of time. Mock transfected BJAB cells (BJAB/mock) were used as control. The range of tested concentrations was 0.012 µM to 1.2 µM for taxol and 0.19 µM to 18.5 µM for epirubicin and time of incubation was 24 hs, 48 hs, 72 hs and 96 hs. It turned out that 0.12 µM taxol and 1.85 µM epirubicin efficiently induced apoptosis after 72 hs of treatment (60 - 70 %) and these drug concentrations were therefore used as standard concentrations.

VII-1.1 CELL DEATH UPON TREATMENT WITH CYTOSTATIC DRUGS

To examine cytostatic drug-induced cell death and growth inhibition, cells were incubated in the presence or absence of 0.12 µM taxol and 1.85 µM epirubicin. Induction of cell death was confirmed by trypan blue exclusion. As shown in Fig. 7, populations of treated cells and cells left untreated diverged from one another. While cell death increased upon treatment with taxol or epirubicin and reached 70 % it was below 10 % in control populations.

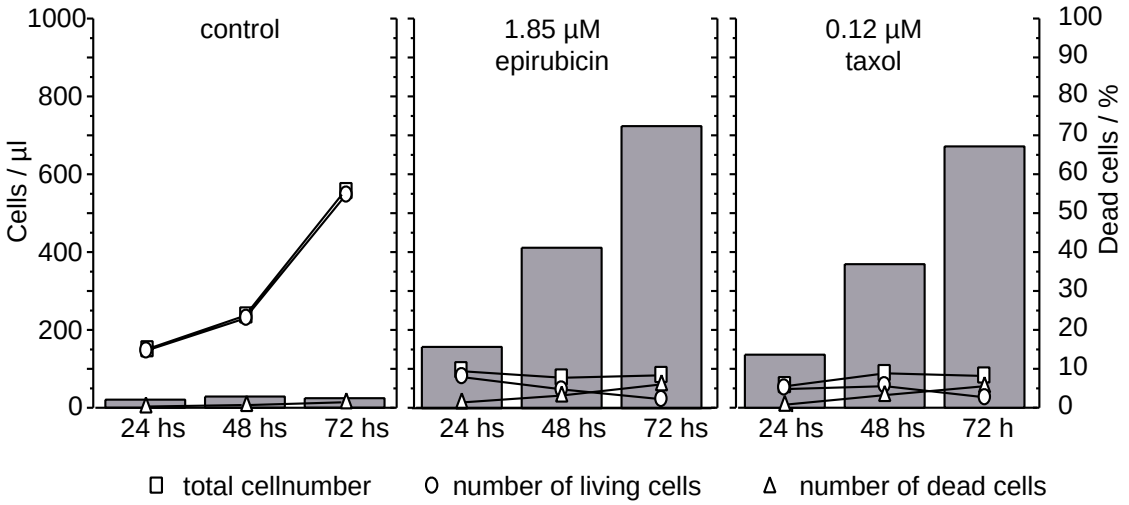

Fig. 7: Drug-induced cell death. The number of dead cells was determined using trypan blue exclusion. Blue cells were assumed to be dead and unstained cells scored as living. Bars display percentage of dead cells (right axis). Cell numbers are indicated at the left axis. Total cell number = dead (stained) cells + living (unstained) cells.

Epirubicin treatment stopped cell growth within 24 hs, number of total cells decreased and number of dead cells increased as the number of living cells decreased. This is reflected by the increasing percentage of dead cells finally reaching about 70 % after 72 hs of incubation. Taxol treament caused comparable effects but inhibition of cell growth was somewhat delayed. Percentage of dead cells was about 60 % after 72 hs of treatment with taxol (Fig. 7).

VII-1.2 DNA FRAGMENTATION

To distinguish between necrotic and apoptotic cell death, a modified cell cycle analysis was performed. This method is suitable to detect DNA content of cells by staining fixed cells with propidium iodide (PI) and thereby discriminating between cells in different phases of cell cycle: $G_{0/1}$, $G_{2/M}$ and hypodiploid cells. Because apoptosis results in DNA strand breaks (Earnshaw, 1999) upon activation of DNA digesting enzymes, e.g. CAD (Enari et al., 1998), this method can be used to determine the number and percentage of apoptotic cells in a cell population. The results of a representative time-course experiment are shown in Fig. 8. The number of apoptotic cells increased with time of incubation and reached about 60 % after 72 hs in both taxol- and epirubicin-treated cell populations thereby confirming the data obtained by trypan blue exclusion.

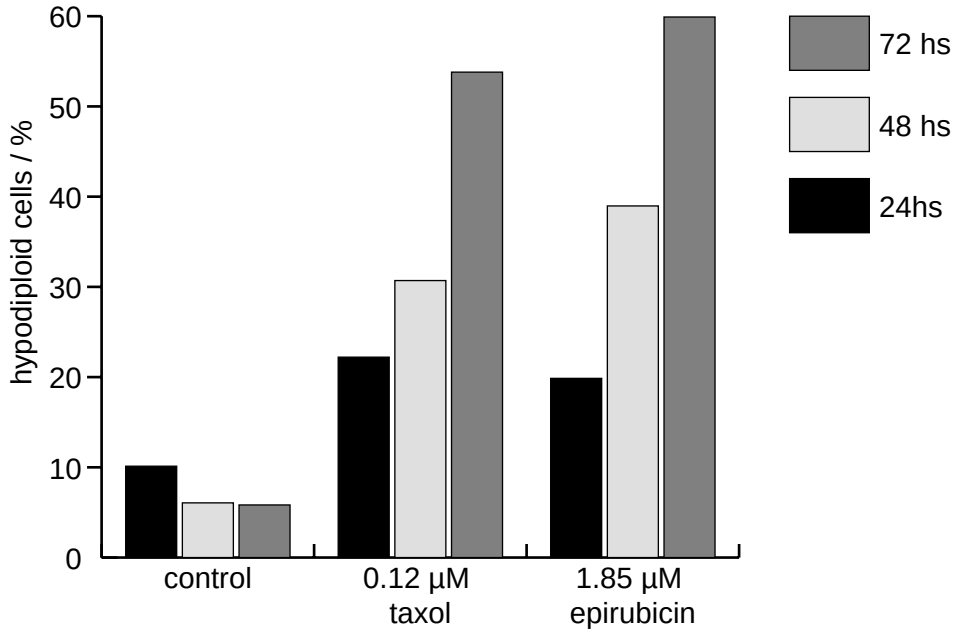

Fig. 8: Modified cell cycle analysis of drug-treated BJAB/mock cells. BJAB/mock cells were incubated with taxol and epirubicin for 24 hs, 48 hs and 72 hsas depicted. Subsequently modified cell cycle analysis was performed. The number of hypodiploid cells is given as percent of 10000 events counted.

VII-1.3 CLEAVAGE OF PARP

Poly-(ADP-ribose)-polymerase, a 116 kDa protein located in the nucleus, is a typical substrate of caspase- 3 and cleaved during apoptosis resulting in the formation of a 84 kDa and a 32 kDa fragment (Wieder et al., 1997). As the detection of hypodiploid cells by FACS analysis indicated cell death via apoptosis rather than necrosis this protein was chosen as a marker protein for apoptosis. SDS PAGE was performed using a 10 % polyacrylamide gel and Western blot analysis with an anti-PARP antibody (Ab) revealed specific, time-dependent cleavage of PARP upon cytostatic drug treatment (Fig. 9)

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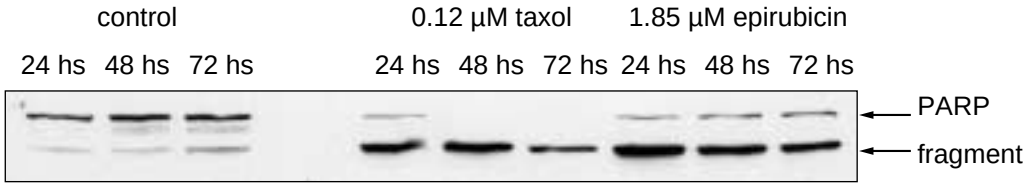

Fig. 9: Cytostatic drugs induce cleavage of PARP. BJAB/mock cells were incubated with the indicated concentrations of cytostatic drugs for 24 hs, 48 hs and 72 hs. Western blot analysis revealed the specific cleavage of PARP. The full length PARP and the corresponding 84 kDa fragment are indicated at the right margin.

These experiments confirmed apoptotic cell death and further indicated activation of caspase- 3 during drug induced apoptosis. Control samples did not show significant cleavage of PARP.

VII-2 Unraveling the Apoptotic Signalling Pathway

VII-2.1 Caspase-3

It is well known that a family of cysteinyl proteases, the so called caspases, is involved in apoptotic cell death. Caspases are expressed as zymogens in many cells and activated by limited proteolysis to form active heterotetramers (Earnshaw, 1999). Thus, immunodetection of caspase subunits can be used to show processing and activation of the zymogen.

Detection of caspase-3 subunit (p17) was performed by Western blot analysis using a polyclonal Ab specific for procaspase-3 and the mature p17 subunit. Fig. 10 shows that the 17 kDa subunit, p17, of the active caspase-3 heterotetramer is formed upon treatment of BJAB/mock cells with cytostatic drugs. Epirubicin-induced processing of caspase-3 preceded taxol-induced effects and already reached its maximum after 24 hs. Taxol-induced formation of the p17 subunit reached its maximum after 48 hs. These results clearly show that the cytostatic drugs taxol and epirubicin at the indicated concentration (0.12 µM and 1.85 µM; respectively) induce apoptosis rather than necrosis.

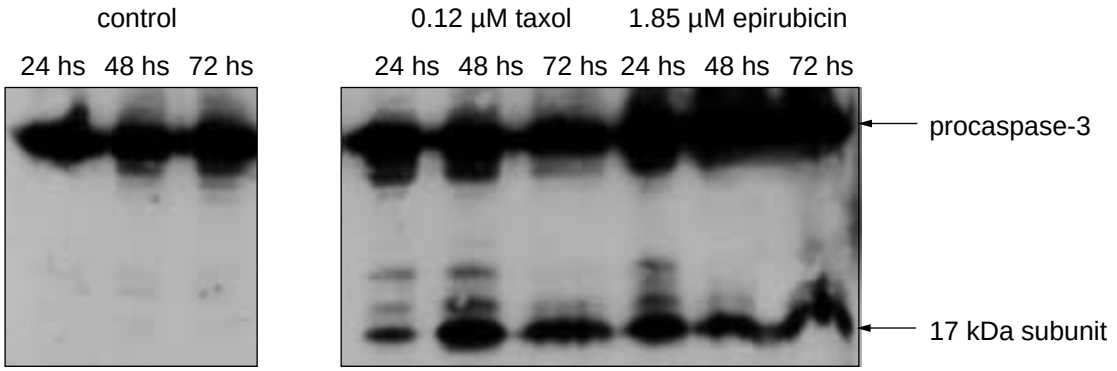

Fig. 10: Cytostatic drugs induce processing of caspase-3. BJAB/mock cells were incubated with the indicated concentrations of cytostatic drugs for 24 hs, 48 hs and 72 hs. Western blot analysis shows the specific processing of caspase-3 resulting in time-dependent appearance of the p17 subunit. The positions of procaspase-3 and the 17 kDa subunit (p17) are indicated at the right margin.

VII-2.2 CASPASE-9

As caspase-3 is the most prominent downstream effector caspase, drug induced caspase-3 processing could happen via two differing signalling pathways: (I) death receptor mediated signalling via TNF-R superfamily or (II) activation of mitochondria. To investigate these two possibilities, a pAb specific for caspase-9 (proenzyme and subunit) was used. As caspase-9 is activated by formation of a cytosolic complex consisting of APAF-1, cytochrome c, dATP and caspase-9 (Li et al., 1997) after mitochondrial permeability shift and release of cytochrome c (Bossy-Wetzel et al., 1998), detection of caspase-9 subunits would indicate processing of caspase-3 via the mitochondrial signalling pathway. Results are shown in Fig. 11.

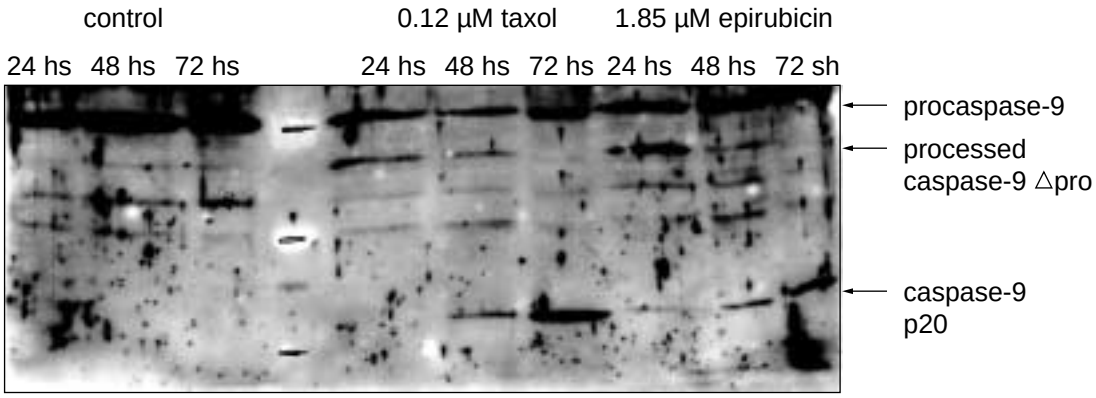

Fig. 11: Cytostatic drugs induce processing of caspase-9. BJAB/mock cells were incubated with the indicated concentrations of cytostatic drugs for 24 hs, 48 hs and 72 hs. Western blot analysis shows the specific processing of caspase-9 resulting in time dependent appearance of the p20 subunit. The positions of procaspase-9 and the 20 kDa subunit (p20) are indicated at the right margin.

As shown in Fig. 11, drug treatment of BJAB/mock cells led to processing of procaspase-9 indicating that induction of the apoptotic cascade occurs via the mitochondrial signalling pathway. To confirm this by another methodology, flow cytometric analysis of mitochondrial membrane potential was performed using the dye JC-1.

VII-2.3 Depolarisation of Mitochondria

The cationic carbocyanine dye JC-1 is a fluorescent marker for mitochondrial depolarization during apoptosis. The most distinctive feature of JC-1 is its potential sensitive emission color shift resulting in a decrease of the red/green fluorescence intensity ratio in response to mitochondrial depolarisation. BJAB/mock cells were incubated either with taxol or epirubicin or in control medium for different periods of time and FACS analysis was performed after staining mitochondria as described in the Methods section (VI-4.3).

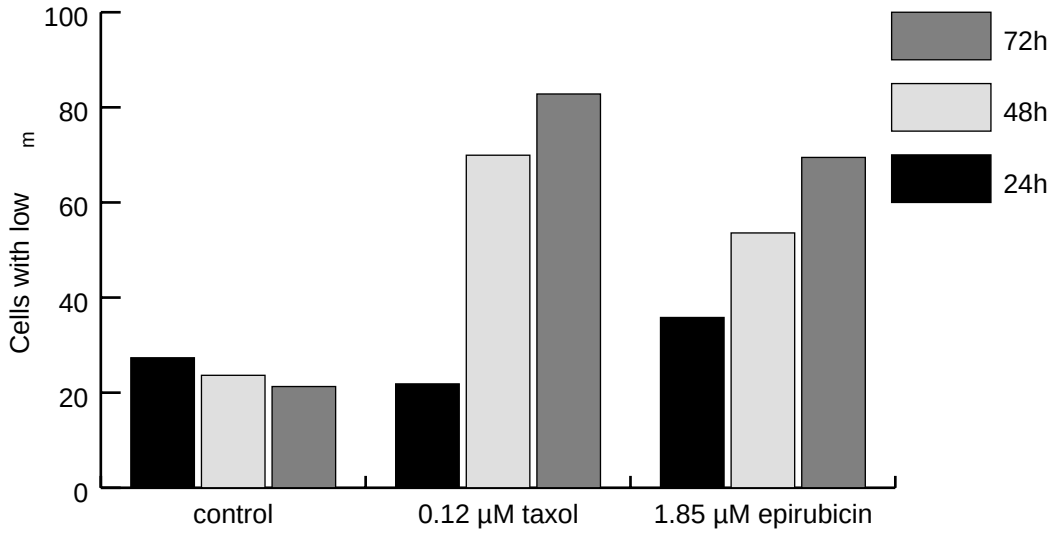

Fig. 12: Loss of mitochondrial membrane potential. BJAB/mock cells were incubated with the indicated concentrations of cytostatic drugs for 24 hs, 48 hs and 72 hs. Then mitochondrial membrane potential was analyzed by the JC-1 assay. Cells displaying decreased mitochondrial membrane potential were scored as apoptotic. Results are given in % total of 10 000 events counted in FACS analysis.

As shown in Fig. 12, depolarisation of mitochondrial membrane potential again increased with time as did the above mentioned markers of apoptosis (PARP cleavage, processing of caspase-3) after incubation with taxol or epirubicin. In contrast, control samples didn't show significant increase in mitochondrial depolarisation. Thus, activation of the mitochondrial apoptotic pathway seems to be an integrated part of the apoptotic machinery after drug exposure of BJAB cells.

VII-2.4 CASPASE-8

Detection of a drug-induced permeability shift of mitochondria and activation of caspase-9 does not exclude the possibility of a parallel induction of the CD95/Fas signalling pathway. As caspase-8 is the initiator caspase in CD95/Fas signalling a mAb detecting procaspase-8 and the processed forms of this enzyme (Bantel et al., 1999) was used in Western blot analysis (Fig. 13).

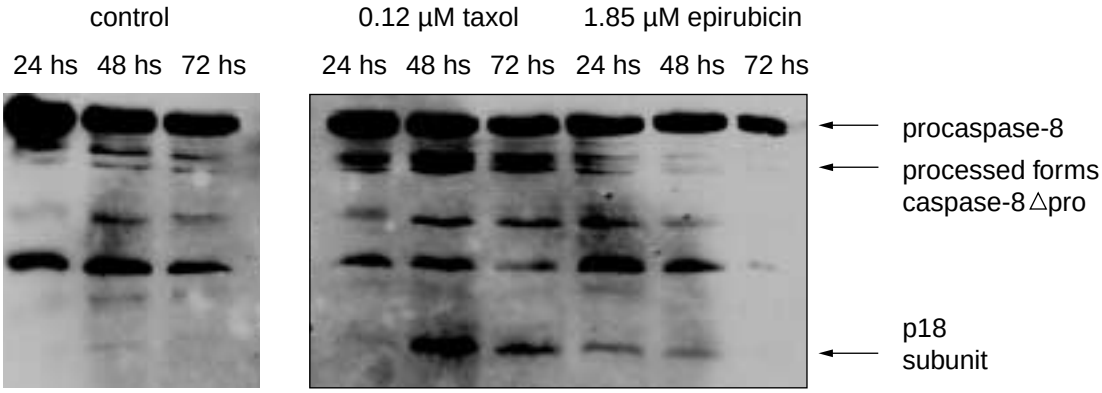

Fig. 13: Caspase-8 processing after drug treatment. After treatment of BJAB/mock cells with the indicated concentrations of taxol or epirubicin, protein samples (20 µg per lane) were separated by SDS PAGE and subsequent Western blot analysis was performed using an Ab specific for procaspase 8 and processed forms of caspase 8 (forms lacking the prodomain: Δpro; active 18 kDa subunit: p18)

Surprisingly, not only the procaspase-8 content decreased but the concentration of the 18 kDa subunit, p18, of the active heterotetramer increased in a time dependent manner after induction of apoptosis by taxol and epirubicin (see Fig. 13). However, the appearance of p18 was delayed in relation to caspase-3 activation rising the question whether this cleavage of procaspase-8 was a consequence of caspase-3 activation.

VII-2.5 CASPASE-3 ACTIVITY ASSAY

As detection of the caspase-3 p17 subunit only indicates cleavage of the zymogen it was also tested if this cleavage was accompanied by caspase-3 activation. In this experiment the substrate Ac-DEVD-pNA was used to assay caspase-3-like cleavage activity in extracts from BJAB/mock cells incubated with the respective cytostatic drugs for 24 hs and 48 hs. The result is shown in Fig. 14: caspase-3 activity was clearly detected and activation of the caspase was confirmed.

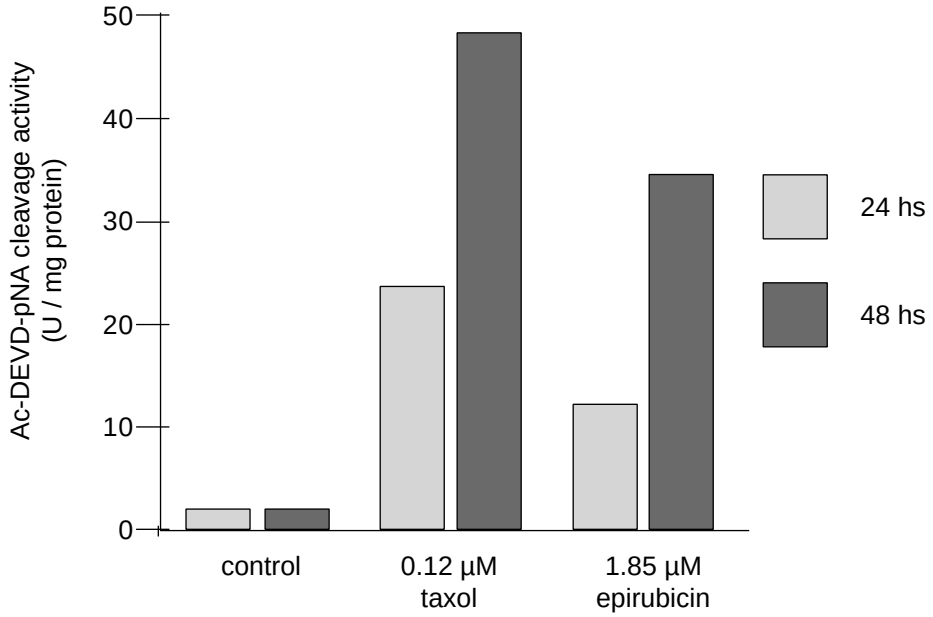

Fig. 14: Ac-DEVD-pNA cleavage activity. BJAB/mock cells were incubated with or without cytostatic drugs as indicated for 24 hs or 48 hs. Cellular extracts were prepared and the colorimetric substrate assay performed as described. Ac-DEVD-pNA cleavage activity was calculated on the basis of the absorption coefficient of pNA and normalized with respect to protein concentration.

VII-3 CASPASE-8 AS INITIATOR OR SUBSTRATE?

The observation that the appearance of caspase-8 p18 subunit was delayed during induction of apoptosis led to the assumption that caspase-8 may be activated downstream of caspase-3 to induce some kind of amplification of the apoptotic cascade (Slee et al., 1999). This theory was reasonable because BJAB cells lack FasL mRNA and FasL mRNA is not increased after cytostatic drug treatment (Daniel et al., 1997). Thus, activation of caspase-8 via CD95/Fas signalling was unlikely to occur.

VII-3.1 INHIBITOR EXPERIMENTS

The usage of the irreversible caspase-3 inhibitor Z-DEVD-fmk promised to answer the question if caspase-8 is activated downstream of caspase-3. First BJAB/mock cells were cultured in the presence of 0.12 µM taxol by addition of different concentrations of Z-DEVD-fmk for 48 hs. The percentage of apoptotic cells was determined by flow cytometric analysis (Fig. 15). Although it was not possible to completely block drug induced apoptosis, Fig. 15 shows that addition of Z-DEVD-fmk clearly reduced the rate of DNA fragmentation even at low concentrations <10 µM. Addition of the peptide inhibitor to cultured cells during incubation with cytostatic drugs led to a clear shift of the 17 kDa subunit of caspase-3 to a higher apparent molecular weight of about 18 and 20 kDa, indicating that the inhibitor had irreversibly bound to the enzyme.

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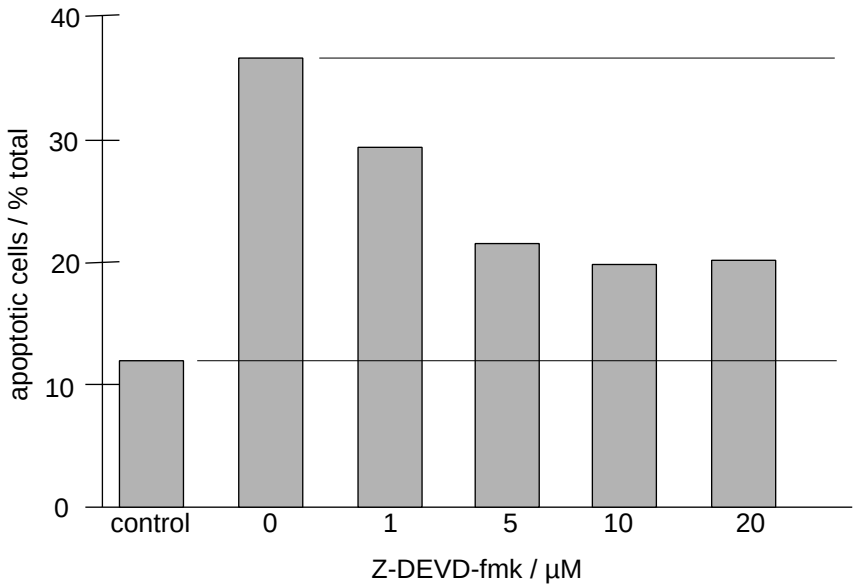

Fig. 15: Z-DEVD-fmk reduces the rate of taxol-induced apoptosis. BJAB/mock cells were incubated with 0.12 µM taxol for 48 hs in the presence of the indicated concentrations of Z-DEVD-fmk. Subsequently, cell cycle analysis was performed as described. Number of hypodiploid cells (apoptotic) is given as percent of 10000 events counted in flow cytometric analysis.

Inhibition of caspase-3-like activity in this experimental setting was confirmed by Western blot analysis of the cleavage of the caspase-3 substrate PARP, which was completely inhibited when drug-treated cells were incubated in the presence of the peptide inhibitor (data not shown). In contrast to caspase-3, treatment of BJAB/mock cells with Z-DEVD-fmk did not lead to a molecular weight upshift of the active subunit of caspase-8, but completely blocked taxol- and epirubicin-induced processing of its proform (Fig. 16).

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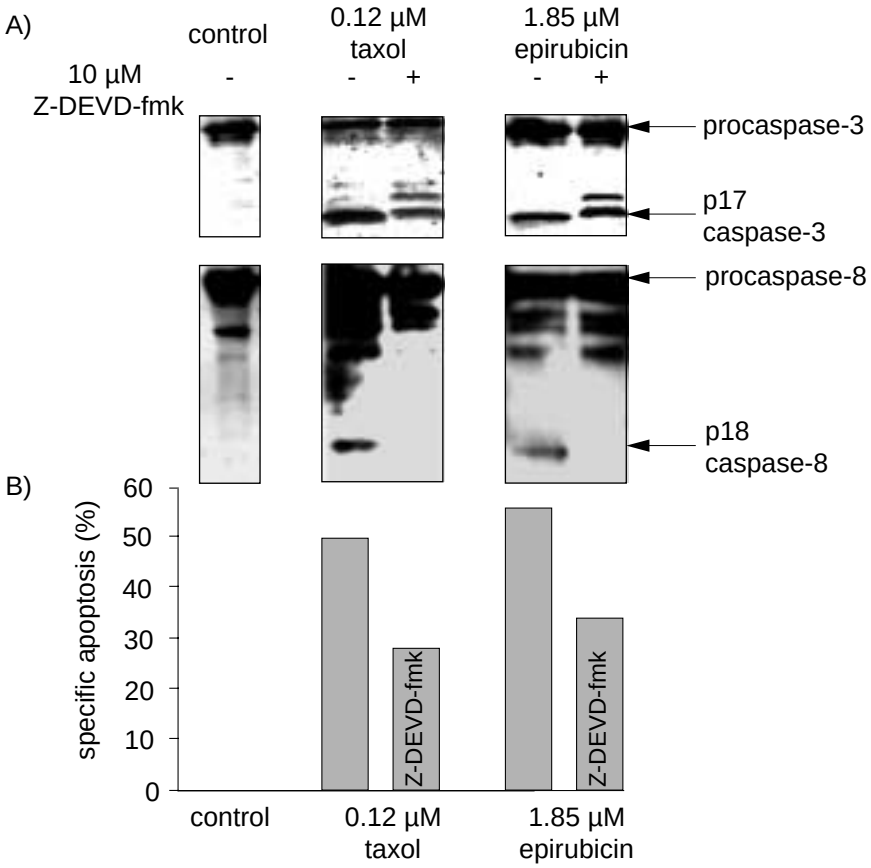

Fig. 16: Z-DEVD-fmk blocks drug-induced processing of caspase-8. Cells were cultured for 48 hs with 0.12 µM taxol or 1.85 µM epirubicin in the presence or absence of 10 µM Z-DEVD-fmk. Caspase-3 and caspase-8 processing was examined by Western blot analysis (A) and specific apoptosis was determined by assaying DNA fragmentation (B).

VII-3.2 TIME-DEPENDENT CASPASE-8 ACTIVITY INCREASE *IN VITRO*

To evaluate processing of caspase-8 upon induction of the mitochondrial apoptotic cascade *in vitro* („*in vitro* activation"), cell extracts were prepared and the effects of cytochrome c and dATP were studied. A time course experiment was performed in which samples were incubated at 30°C in the presence of cytochrome c and dATP or kept at room temperature. Caspase activities were measured in corresponding samples using the peptide substrates Ac-DEVD-pNA and Ac-IETD-pNA (Fig. 17). Samples were also analysed by Western blot analysis (Fig. 18) to correlate appearance of immunoreactive bands to the caspase activities.

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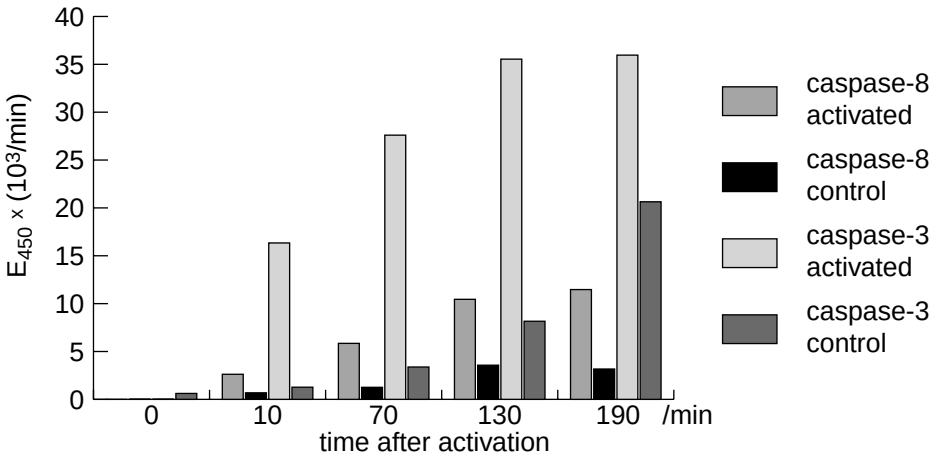


Fig. 17: Caspase-3 and caspase-8-like cleavage activity after *in vitro* activation. As depicted mitochondrial caspase cascade was initiated by addition of cytochrome c and dATP and incubation at 30°C for the indicated periods of time. Control samples were equally incubated without addition of cytochrome c and dATP. Absorption resulting from specific cleavage of Ac-DEVD-pNA and Ac-IETD-pNA was determined and calculated as ΔE / min.

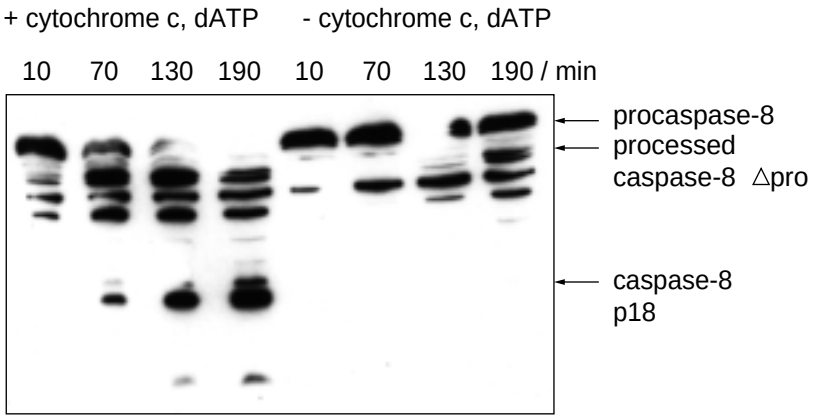


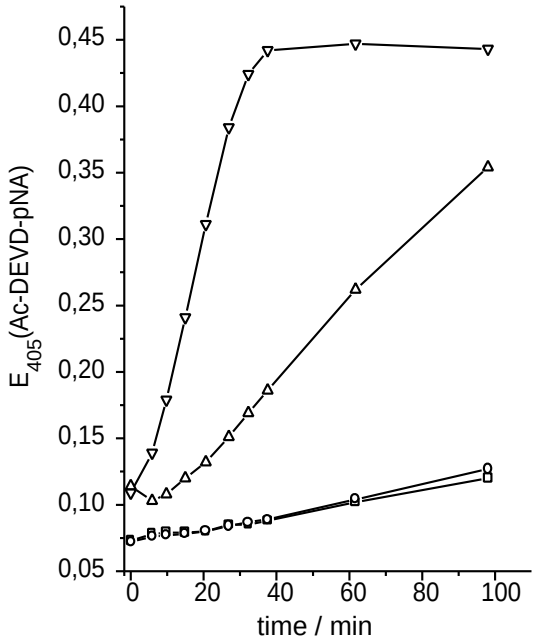
Fig. 18: Caspase-8 processing after different periods of *in vitro* activation. Identical samples used for determination of peptide substrate cleavage activity were examined by Western blot analysis detecting caspase 8 pro- and processed forms.

VII-3.3 Immunoprecipitation of Caspase-3 *In Vitro*

Caspase-3 and Caspase-8 Activity Assay

To further confirm caspase-8 activation downstream of caspase-3, cell extracts from control BJAB/mock cells were prepared as described. The sample was splitted. Caspase-3 was immunoprecipitated by addition of caspase-3 Ab and protein A-sepharose and incubation at 4°C with moderate rocking for 1 h. Control samples were incubated in the presence of protein A-sepharose. Samples were splitted again and positive controls were activated by addition of cytochrome c and dATP and incubation at 30°C.

Following this, caspase-3-like activity was measured using the specific colorimetric substrate, Ac-DEVD-pNA. As shown in Fig. 19, caspase-3 activity was diminished in the caspase-3-depleted sample and reached only about 20 % of the activity measured in the undepleted control sample. Samples were removed after different periods of *in vitro* activation and Western blot analysis was performed to further examine caspase-8 processing.



	caspase-3 immunoprecipitation	cytochrome C/ dATP
□	+	-
○	-	-
△	+	+
▽	-	+

Fig. 19: Time course of Ac-DEVD-pNA cleavage. Samples which had been treated as indicated were examined on their ability to process Ac-DEVD-pNA by measuring absorption at 405 nm.

Western Blot Analysis of Caspase-8 Processing

The same samples used to assay Z-DEVD-pNA cleavage activity after depletion of caspase-3 were used for Western blot analysis. 15 µL of sample were separated by SDS-PAGE and transfered to nitro-cellulose membrane. Caspase-8 detection was performed as described using the suitable Ab. Fig. 20 shows that processing of pro-caspase-8 was blocked in caspase-3-depleted extracts and can therefore be considered as caspase-3-dependent in this experimental setup.

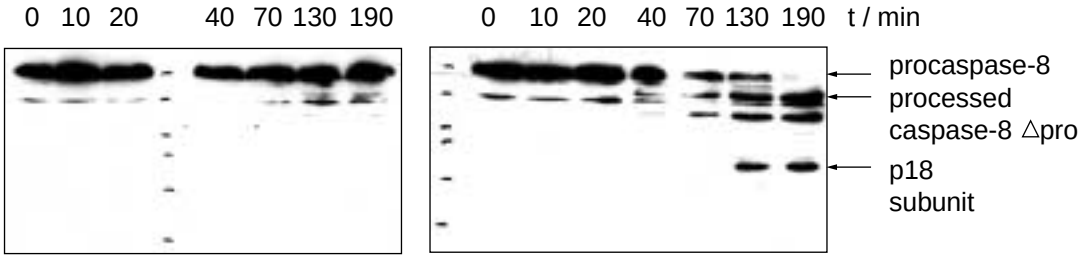


Fig. 20: Caspase-8 activation in caspase-3-depleted cell extracts. Extracts were prepared and caspase-3 was immunoprecipitated as described. After different periods of *in vitro* activation, samples were examined by Western blot analysis.

Colorimetric Substrate of Caspase-3 as Inhibitor In Vitro

Since the idea of caspase-8 activation downstream of the effector caspase-3 is controversally discussed additional experimental evidence for this phenomenon was necessary. Therefore control extracts from BJAB/mock cells were prepared and splitted. To investigate the role of active caspase-3 on caspase-8 processing in more detail, Z-DEVD-pNA was added to the extracts just before and directly after activation with cytochrome c and dATP. The colorimetric substrate was used instead of the irreversible inhibitor Z-DEVD-fmk because it was possible to simultaneously monitor activation of caspase-3 by measuring absorption. In this experimental setting, an excess of the peptide substrate was used to investigate the effect of caspase-3 inhibition on cleavage of caspase-8. As shown in Fig. 21, caspase-3 processing is inhibited in samples taken from this experiment after *in vitro* activation for up to 20 min. Interestingly, the appearance of mature p17 is completely blocked in the presence of Ac-DEVD-pNA.

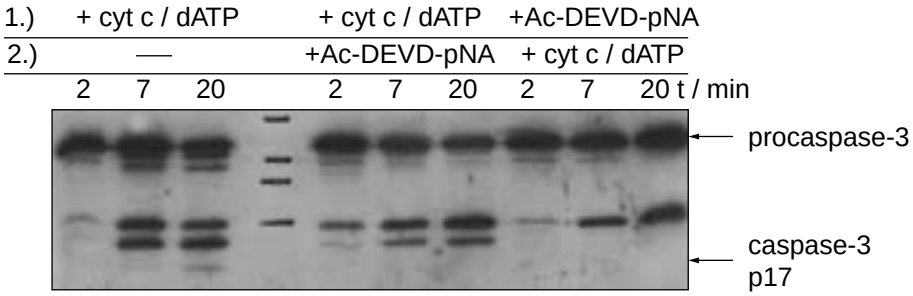


Fig. 21: Influence of Ac-DEVD-pNA on caspase-3 processing *in vitro*. The addition of Ac-DEVD-pNA clearly delayed caspase-3 processing. This effect was stronger in samples which had been supplemented with Ac-DEVD-pNA prior to activation than in those which had been supplemented after activation.

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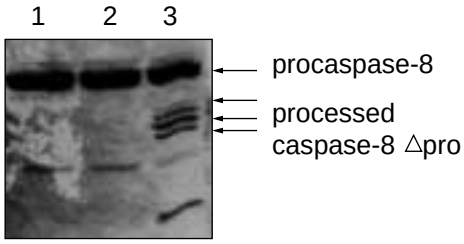

Fig. 22: Influence of Ac-DEVD-pNA on caspase-8 processing *in vitro*. Following 2 hs of activation, samples were examined using Western blot analysis. (1) Ac-DEVD-pNA was added prior to addition of cytochrome c and dATP, (2) Ac-DEVD-pNA was added after addition of cytochrome c and dATP; (3) no colorimetric peptide substrate was added.

Due to addition of the colorimetric substrate Ac-DEVD-pNA processing of caspase-8 was delayed (Fig. 22). This confirmed that caspase-8 processing occurs downstream of mitochondria and even downstream of caspase-3, at least *in vitro*.

VII-4 CYTOSTATIC DRUG TREATMENT OF BJAB/FADDDN CELLS

To further exclude an involvement of the CD95/Fas signalling pathway, BJAB cells stably transfected with a dominant-negative FADD mutant (FADDdn) were used. As the adaptor molecule FADD recruits caspase-8 to the CD95/Fas signalling complex (death inducing signalling complex, DISC (Chinnaiyan et al., 1996) after receptor trimerisation and thereby induces caspase-8 processing and activation this signalling pathway is blocked in FADDdn transfectants. CD95/Fas-induced apoptosis was indeed shown to be completely inhibited in BJAB/FADDdn cells (Bantel et al., 1999) and transfection with FADDdn completely prevents caspase-3 activation upon triggering the cells with agonistic anti-CD95 antibodies (Chinnaiyan et al., 1996). Activation of caspase-8 and / or caspase-3 in this cell type does therefore not occur upon induction of apoptosis via CD95/Fas receptor trimerisation.

VII-4.1 DNA FRAGMENTATION IN CYTOSTATIC DRUG-TREATED BJAB/FADDDN CELLS

In a first set of experiments, drug-induced DNA fragmentation in BJAB/FADDdn cells was assessed as compared with vector transfected BJAB/mock cells. Specific apoptosis was calculated by subtracting values obtained in control samples (spontaneous apoptosis) from values obtained for drug-treated cells. Equal percentage of specific DNA fragmentation was detected after 48 hs of incubation with 0.12 µM taxol and 1.85 µM epirubicin, respectively, in BJAB/mock and BJAB/FADDdn. As shown in Fig. 23, taxol- as well as epirubicin-induced apoptosis was not significantly influenced by overexpression of FADDdn. Thus, drug-induced apoptosis in BJAB cells seems to be independent of CD95/Fas signalling.

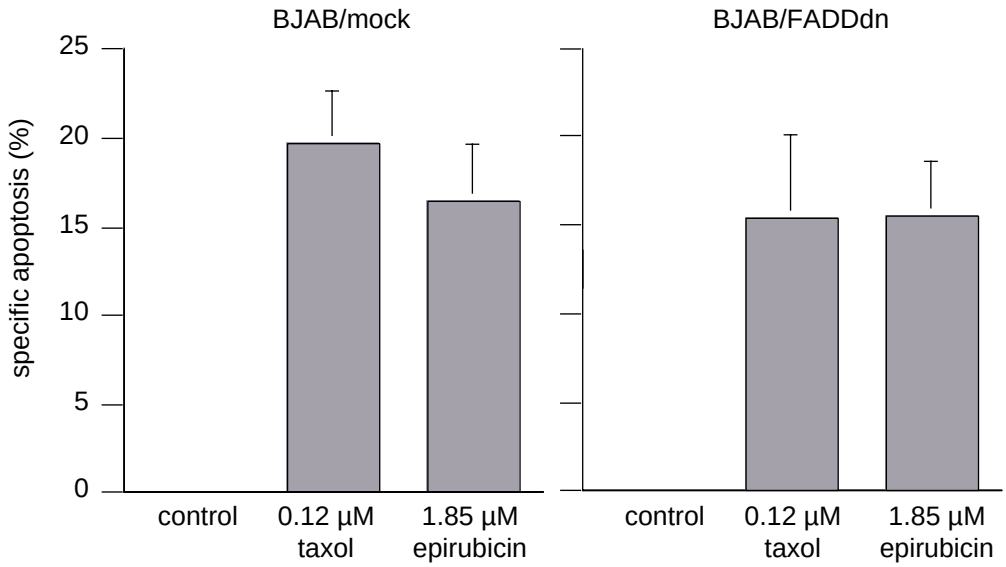

Fig. 23: Drug-induced apoptosis in BJAB/FADDdn and BJAB/mock cells. Cells were incubated with the indicated concentrations of cytostatic drugs for 48 hs and modified cell cycle analysis was performed as described. Values are given as specific apoptosis ± S.D. (n = 3).

VII-4.2 Mitochondrial Depolarisation

In recent publications, the critical role of mitochondrial damage for drug-induced apoptosis (Fulda et al., 1998, Melendez-Zajgla et al., 1999) and CD95-dependent signalling to mitochondria via caspase-8-mediated cleavage of BID has been demonstrated (Li et al., 1998). This BID cleavage product (tBID) is claimed to induce release of BAX from the BAX/BCL-2 complex and thereby induce formation of large pores releasing cytochrome c from the intermembrane space. Additionally, BID itself is reported to induce this effect without cleavage to tBID by caspase-8 (Eskes, 1999). We thus measured mitochondrial activation after treatment with taxol and epirubicin in our experimental system. Treatment of BJAB/mock cells with both agents led to a significant mitochondrial permeability shift and to a significant increase of cells with low $\Delta\Psi_m$ in a time dependent manner. This drug-induced mitochondrial permeability transition was not influenced by overexpression of FADDdn (Fig. 24), thereby excluding the possibility that the mitochondrial activation in BJAB cells was mediated by a CD95/Fas-dependent mechanism. Time course experiments in BJAB/mock, BJAB/FADDdn cells revealed that the mitochondrial permeability transition already occurred 24 hs after addition of epirubicin and taxol to the culture medium and thus preceded or at least coincided with the processing of procaspase-8.

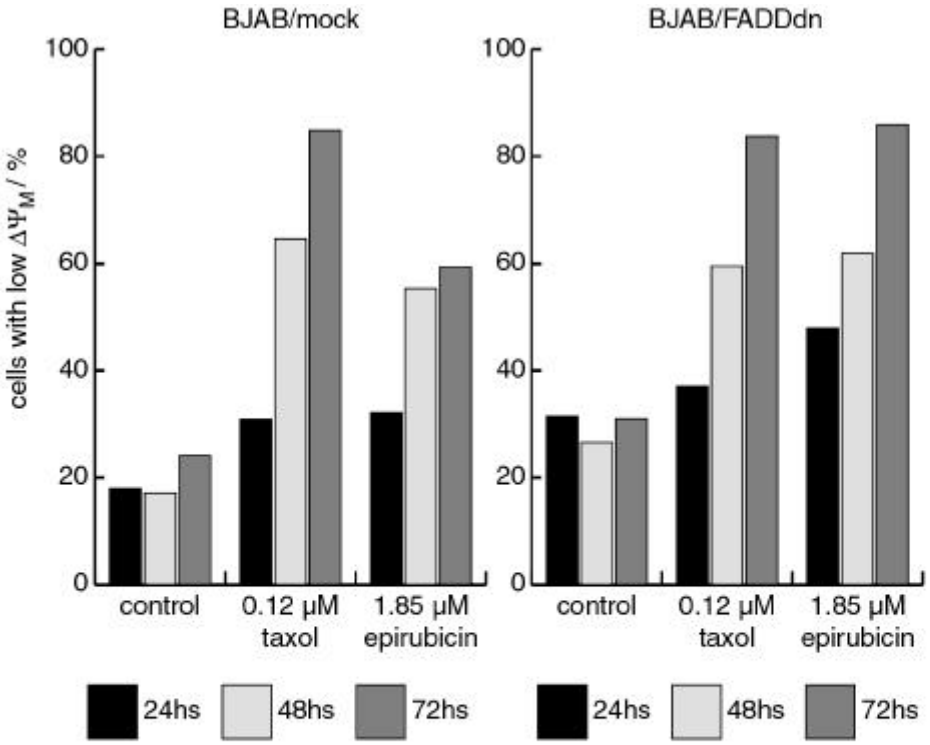

Fig. 24: Drug-induced loss of $\Delta\Psi_m$ in BJAB/mock versus BJAB/FADDdn cells. BJAB/mock and BJAB/FADDdn cells were incubated with the indicated concentrations of cytostatic drugs for different periods of time. Then mitochondrial membrane potential was analyzed by the JC-1 assay. Cells displaying decreased mitochondrial membrane potential were scored as apoptotic. Results are given in percent of 10 000 events counted in FACS analysis.

VII-4.3 Caspase-3 and Caspase-8 Western Blot Analysis

To further confirm that caspase-8 processing upon drug treatment in BJAB cells is independent of CD95/Fas signalling, Western blot analysis was performed. After challenge with taxol and epirubicin caspase-3 Western blot analysis was performed (Fig. 25, Fig. 26) and caspase-8 p18 was detected. The cleavage of caspase-8 in these cells coincided with the appearance of the 17 kDa active subunit of caspase-3 and was not delayed in comparison to drug-treated BJAB/mock cells.

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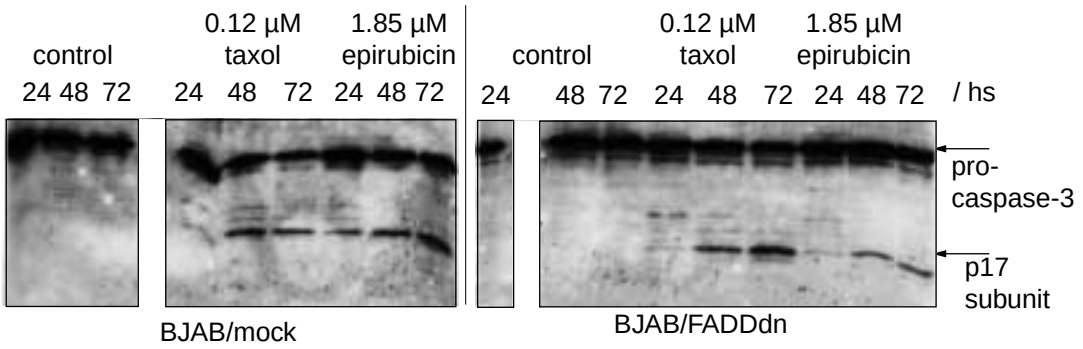

Fig. 25: Caspase-3 activation in BJAB/mock and BJAB/FADDdn cells. Cells were incubated with or without cytostatic drugs for different periods of time as depicted. Caspase-3 activation was detected using Western blot analysis. Positions of procaspase-3 and the 17 kDa subunit (p17) of the active heterotetramer are indicated at the right margin.

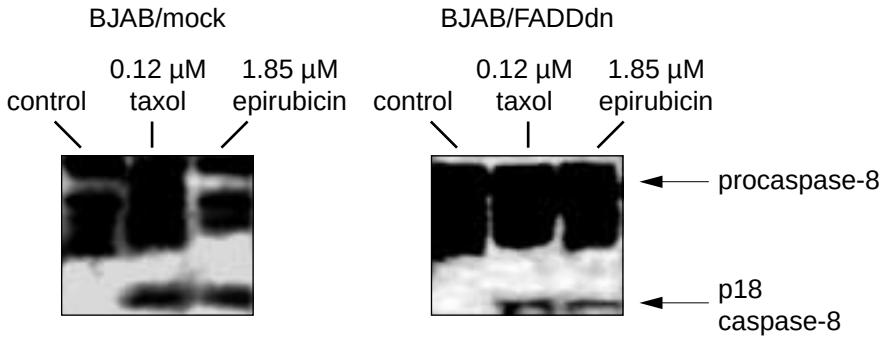

Fig. 26: Caspase-8 activation in BJAB/mock and BJAB/FADDdn cells. BJAB/mock or dominant negative FADD (FADDdn)-transfected BJAB cells were either left untreated or incubated with 0.12 µM taxol or 1.85 µM epirubicin for 48 hs. Then, Western blot analyses were performed with anti-human caspase-8 antibody. The positions of procaspase-8 and the 18 kDa active subunit of caspase-8 are indicated at the right margin.

VII-5 Cytostatic Drug Treatment of NALM6 and REH Cells

As the results described above strongly indicated that processing and activation of caspase-8 occurs separate from CD95/Fas signalling in BJAB/mock and BJAB/FADDdn cells, the question arose whether this is a general feature of CD95/Fas resistent cells. To answer this question, two other B-lymphoid cell types, namely NALM6 and REH, were used and DNA fragmentation and depolarisation of mitochondria was investigated using the appropriate assay systems. Furthermore, Western blot analysis was performed to visualize caspase-8 processing.

VII-5.1 DNA Fragmentation in NALM6 and REH Cells

To verify drug induced apoptosis in these two B-lymphoid cell types, modified cell cycle analysis was performed. Cells were incubated with 0.12 µM taxol or 1.85 µM epirubicin for up to 72 hs and the percentage of apoptotic cells was determined by flow cytometric analysis. Results are shown in Fig. 27.

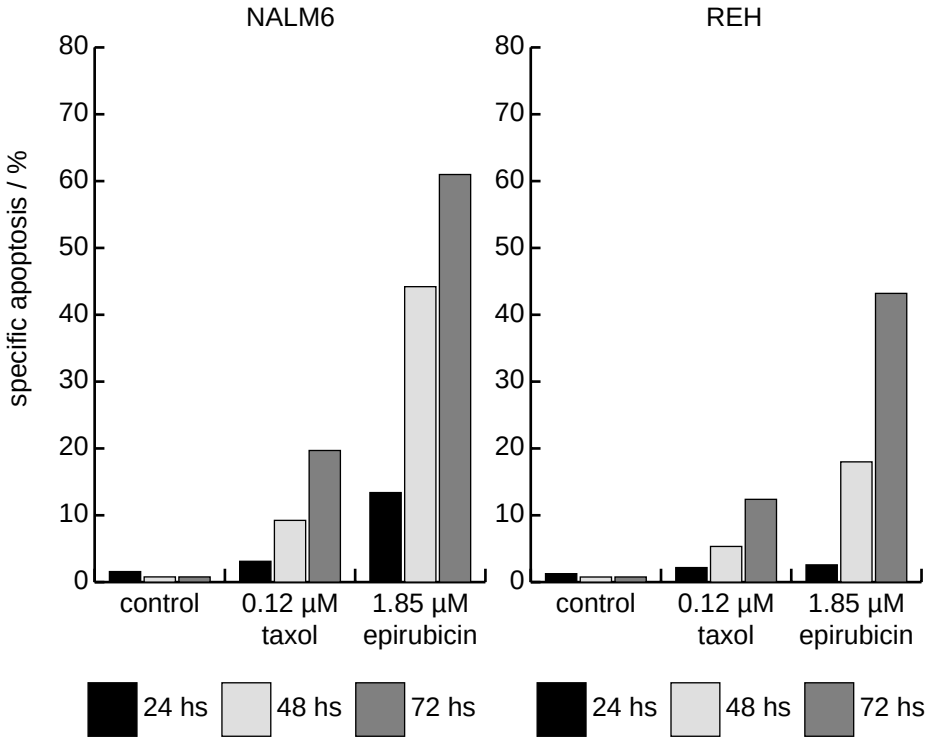

Fig. 27: DNA fragmentation in NALM6 and REH cells. Cells were treated with 0.12 µM taxol or 1.85 µM epirubicin for the indicated periods of time and subsequently modified cell cycle analysis was performed as described. Number of hypodiploid cells is given as % specific apoptosis.

VII-5.2 Mitochondrial Depolarisation in NALM6 and REH Cells

NALM6 and REH cells were challenged with taxol or epirubicin for 24 hs and 48 hs and depolarisation of mitochondria was detected by staining cells with the JC-1 dye and subsequent flow cytometric analysis (Fig. 28). Loss of $\Delta\Psi_M$ was already detected after 24 hs of treatment with taxol and epirubicin and reached up to 80 % - 90 % in REH cells. NALM6 cells displayed about 40 % of drug-induced depolarisation of mitochondria after 48 hs of treatment with the respective cytostatic drugs.

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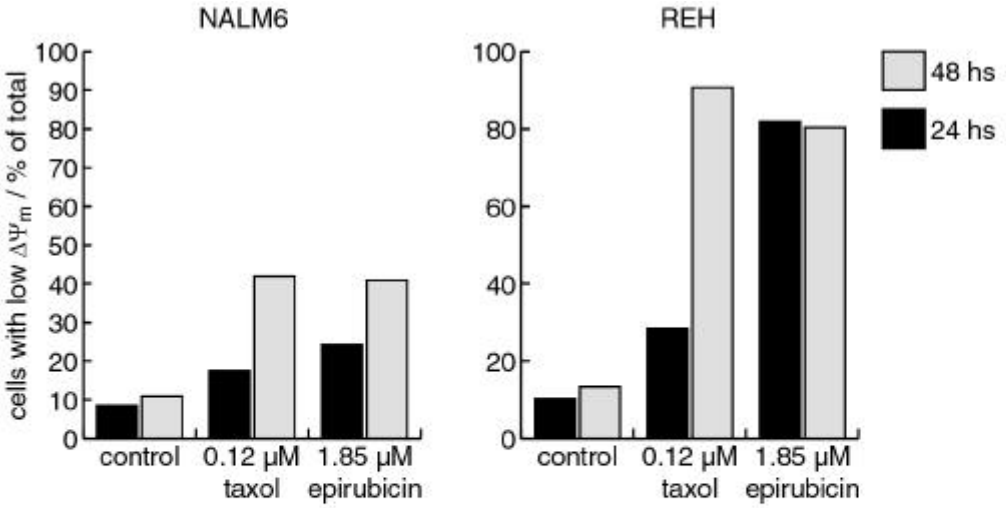

Fig. 28: Drug-induced loss $\Delta\Psi_m$ in NALM6 and REH cells. Nalm6 and REH cells were incubated with the indicated concentrations of cytostatic drugs for 24 hs and 48 hs. Then mitochondrial membrane potential was analyzed by the JC-1 assay. Cells displaying decreased mitochondrial membrane potential were scored as apoptotic. Results are given in percent of 10 000 events counted in FACS analysis.

VII-5.3 Activation of Caspase-3 in NALM6 and REH Cells

To render caspase-3 responsible for processing of caspase-8 in NALM6 and/or REH cells first caspase-3 processing was assessed by caspase activity assay using the colorimetric peptide substrate Ac-DEVD-pNA. Cells were cultured in the presence or absence of taxol for 48 hs or epirubicin for 24 hs.

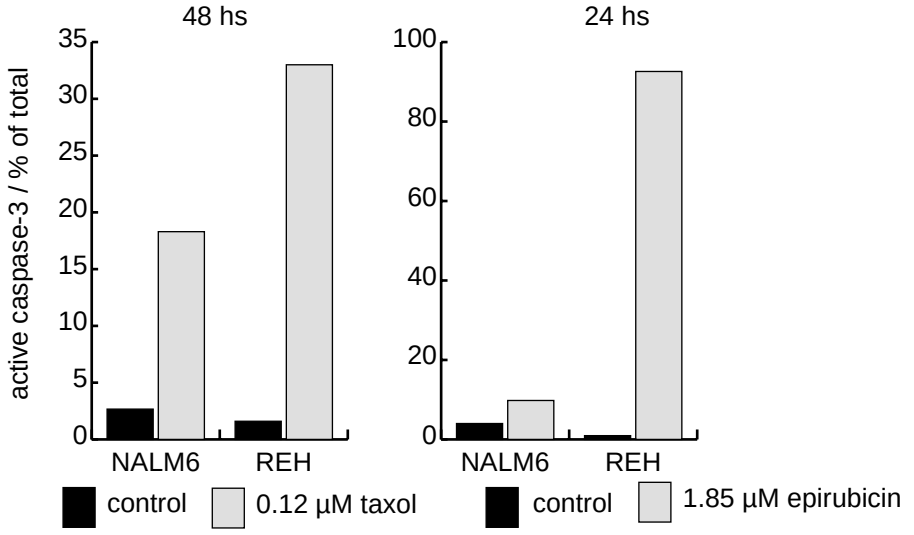

Fig. 29: Induction of caspase-3 activity after drug treatment. NALM6 and REH cells were incubated with 0.12 µM taxol for 24 hs and 1.85 µM epirubicin for 48 hs. Cell extracts were prepared as described and Ac-DEVD-pNA cleavage was determined by measurement of E_{405} in an ELISA reader. *In vitro* activation of identical extracts was performed. Caspase-3-like activity of *in vitro* activated extracts was defined as 100 %.

VII-5.4 Caspase-8 Activity in NALM6 and REH Cells

Ac-IETD-pNA cleavage was likewise examined. However, determination of the total activity (i.e. 100 % caspase-8 activation after addition of cytochrome c and dATP) was not possible. Thus, specific caspase-8-like activity was calculated on the basis of protein concentration and the absorption coefficient of the cleaved substrate. As shown in Fig. 30, only in REH cells significant amounts of caspase-8-like activity were measured after challenge with taxol and epirubicin, whereas in NALM6 cells induction of caspase-8-like activity was not observed..

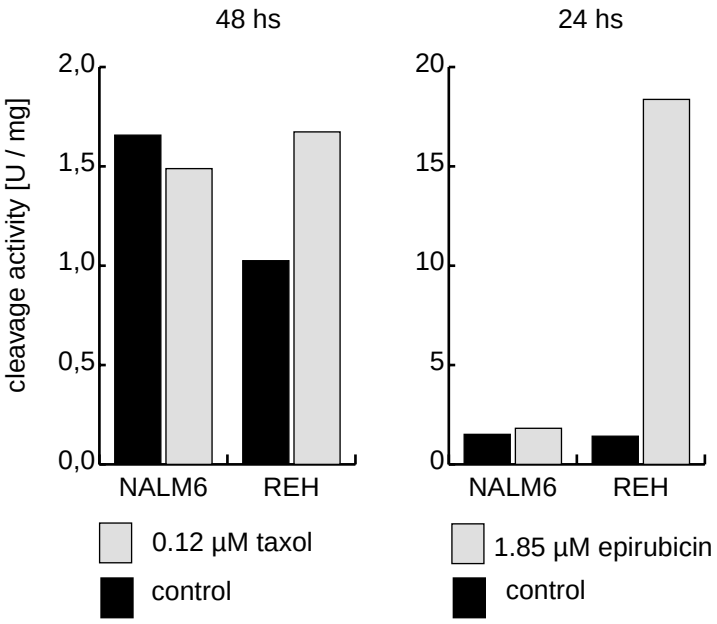

Fig. 30: Induction of caspase-8-like cleavage activity after drug treatment. NALM6 and REH cells were incubated with 0.12 µM taxol for 48 hs and 1.85 µM epirubicin for 24 hs. Cell extracts were prepared as described and Ac-IETD-pNA cleavage was determined by measurement of absorption increase. Protein concentration was measured using the BCA assay kit and specific caspase-8-like activity was calculated in U/mg protein.

VII-5.5 Processing of Caspase-8 in NALM6 and REH Cells

In these experiments, effects of cytostatic drug treatment on caspase-8 processing were examined in different B-lymphoid cell types by Western blot analysis. The mature subunit p18 of caspase-8 was detected after 24 hs of treatment with the cytostatic drugs (Fig. 31). These data confirmed caspase-8 processing in REH cells after induction of apoptosis by taxol and epirubicin. In accordance with the caspase-8 activity assay processing of caspase-8 was less pronounced in epirubicin-treated NALM6 cells.

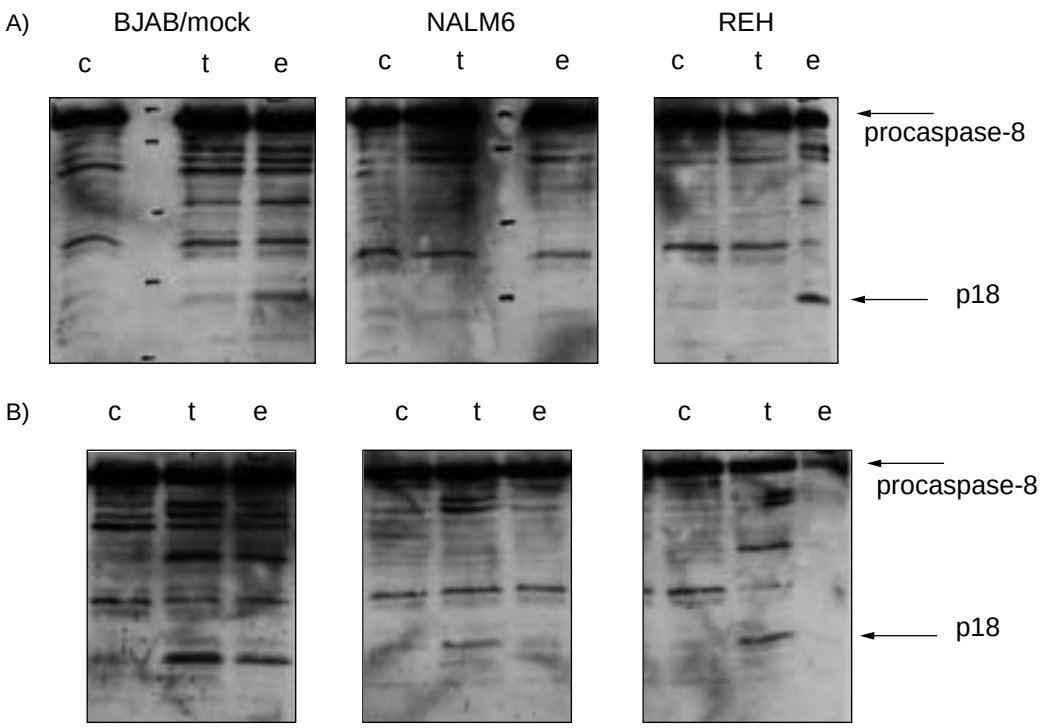

Fig. 31: Drug-induced caspase-8 processing in BJAB/mock, NALM6 and REH cells. Cells were incubated with 0.12 µM taxol (t), 1.85 µM epirubicin (e) or control medium (c) for 24 hs (A) and 48 hs (B). Caspase-8 processing was detected using Western blot analysis. The positions of procaspase-8 and the mature 18 kDa subunit (p18) are indicated at the right margin.

VII-6 SUMMARY OF FUNCTIONAL INVESTIGATIONS

The following list summarizes the functional characterization of caspase signalling in B-lymphoid cell types

1. Cytostatic drug treatment of human B-lymphoid cells induces apoptosis (cleavage of PARP, processing and activation of caspase-3).
2. Caspase-9 is activated and mitochondrial membrane potential is disrupted after challenge with cytostatic drugs.
3. Caspase-8 is also activated, but this activation is inhibited by the specific caspase-3 inhibitor Z-DEVD-fmk.
4. Caspase-3 depletion of cell free extracts blocks caspase-8 processing after addition of cytochrome c and dATP.
5. Time-dependent caspase-8 activation in cell free extracts shows a slower kinetic as compared with caspase-3 activation.
6. Caspase-8 activation is similar in BJAB/mock and BJAB/FADDdn transfected cells.

VII-7 Detection of Apoptosis Related Proteins

High resolution 2DE-PAGE is the state of the art method to identify yet unknown (apoptosis related) proteins. Therefore, this method was used to examine effects of cytostatic drugs in the experimental system, i.e. in BJAB cells. To obtain further information about apoptosis related proteins, BJAB cells transfected with crmA were used. CrmA (cytokine response modifier A) is an antiapoptotic protein derived from the cow pox virus mainly inhibiting caspase-8 (K_i = 0.3 - 0.95 nM)but also caspase-3 (K_i = 1600 nM) (Zhou et al., 1997). BJAB/crmA cells should show the same regulatory response to cytostatic drug treatment as compared to mock-transfected controls. On the other hand, the response downstream of caspase activation should be inhibited. In first experiments, standard markers of apoptosis were examined, e.g. DNA fragmentation, depolarisation of mitochondria, PARP-cleavage and activation of caspases. These experiments are shown in detail below. Taken together, apoptosis was significantly delayed in BJAB/crmA cells in comparison with vector control transfected BJAB/mock cells but not abolished.

VII-7.1 DNA Fragmentation is Delayed in CrmA Transfected BJAB Cells

To assess downstream events in apoptotic cell death DNA fragmentation upon treatment with cytostatic drugs was examined. BJAB/crmA cells were incubated with 0.12 µM taxol and 1.85 µM epirubicin for 24 hs, 48 hs and 72 hs and hypodiploid cells were quantified by flow cytometric analysis (Fig. 32). This experiment revealed that DNA fragmentation is clearly delayed in crmA transfected cells. However, apoptosis was not completely blocked. Apoptosis in these cells reached about 20% after 72 hs of drug-treatment.

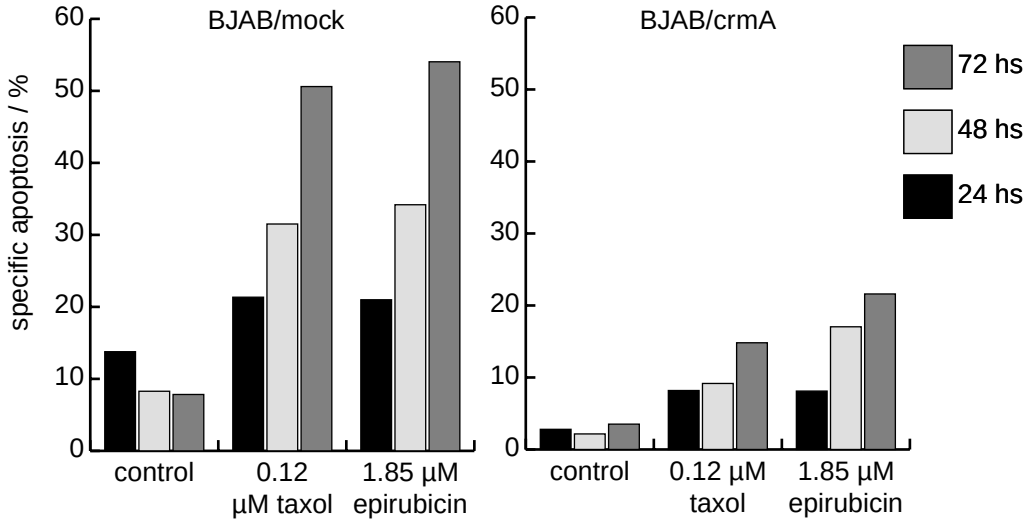

Fig. 32: DNA fragmentation is reduced in crmA-transfected BJAB cells. BJAB/mock and BJAB/crmA cells were incubated with cytostatic drugs for different periods of time as indicated. Modified cell cycle analysis (10000 events counted) showed that crmA transfection had significant impact on percentage of hypodiploid cells.

VII-7.2 Caspase-3 Activation is Delayed in CrmA Transfected BJAB cells

Caspase-3 Processing

Caspase-3 processing after treatment of BJAB/crmA cells was examined by Western blot analysis. The intensity of the immunoreactive band representing the mature subunit of caspase-3, p17, was significantly reduced after 24 hs and 48 hs of cytostatic drug treatment in BJAB/crmA cells as compared to BJAB/mock cells but increased until 72 hs of treatment (Fig. 33). This indicated that caspase activation is strongly inhibited but not blocked in crmA-transfected BJAB cells.

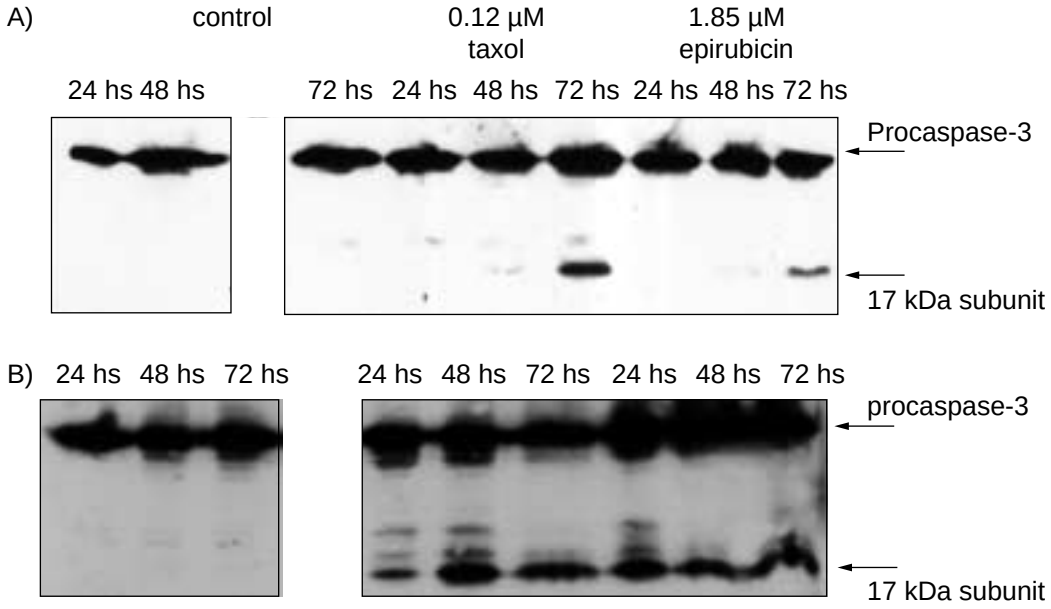

Fig. 33: Caspase-3 processing is delayed in BJAB/crmA. A) BJAB/crmA cells were incubated with cytostatic drugs for different periods of time as indicated and Western blot analysis was performed as described. The positions of procaspase-3 and the active 17 kDa subunit, p17, are indicated at the right margin. B) As a control, BJAB/mock cells were likewise incubated with cytostatic drugs and caspase-3 processing was analyzed.

VII-7.3 HIGH RESOLUTION 2DE-PAGE

After characterization of the crmA-transfected cell line samples were prepared for 2DE PAGE. Analytical two dimensional gel electrophoresis was performed. The gels were stained according to the protocol of Heuckeshofen et al. (1985). Subtractive analysis revealed several spots varying in intensity, especially in the low molecular weight range. Further information was deduced from comparison of samples derived from the two different cell lines (BJAB/mock and BJAB/crmA) as well as treatment with the respective cytostatic drugs. On basis of this overall correlation, it is a plausible consideration that most of the spots displaying decreased intensity in samples of drug-treated cells represented caspase substrates. Thus, taxol and epirubicin resulted in decrease of the same spots which therefore are assumed to be related to apoptosis in general and not to regulatory effects of these cytostatic drugs which head for different cellular agents. Moreover, the experiments showed that spot variations were the same in BJAB/mock and BJAB/crmA cells although drug-induced effects were in BJAB/crmA cells were delayed in general. Further experiments were therefore performed exclusively with BJAB/mock.

Altered spots were cut from preparative gels, digested with trypsin and mass spectrometric analysis was performed. Moreover, not only spots displaying changes in intensity but also spots representing proteins with obviously constant protein concentration were identified in order to calibrate the 2DE gels with respect to the apparent molecular weight and pH gradient. Results of data bank searches are given in table 8 and positions of corresponding spots on 2DE PAGE gels are shown in Fig. 34. Enlargements of certain areas are shown in Fig. 35 - Fig. 39. Mass spectrometric analysis was performed by Eva-Christina Müller.

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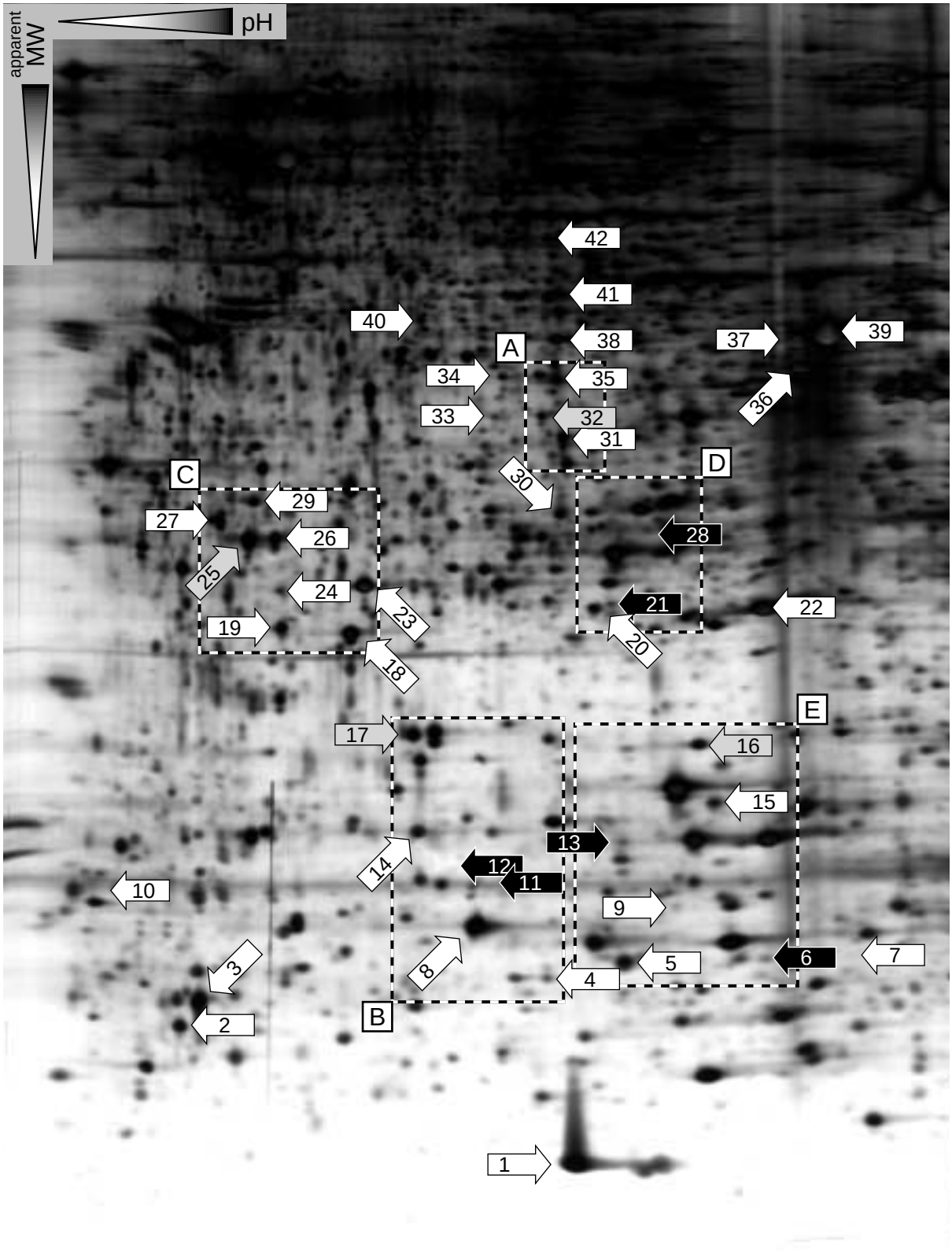

Fig. 34: Representative 2DE PAGE gel. Two dimensional separation of proteins from a control sample is shown. Spots within the indicated areas A - E were identified by mass spectrometric analysis. Results were correlated with those obtained from cytostatic drug-treated cells and cell extracts with or without *in vitro* activation as depicted on the following pages. Colors indicate change in spot intensity: white: no significant change; grey: decrease; black: increase. The identity of the spots is given in table 8.

Table 8: Spots identified by mass spectrometric analysis. The corresponding positions are indicated in Fig. 34.

Spot Number	Protein	(SwissProt) Accesion Number	comments
1		P00248	Ubiquitin
2	THIO	P10599	Thioredoxin
3	TBB1	P07437	Tubulin β-1 chain
4		Q15336	Li-Cadherin
5	YHIT	P49774	Hypothetical HIT-likeprotein U296A
6	ACTB	P02570	β-Actin, **increase**, C-terminal fragment
7	AR16	O15511	ARP2/3 complex 16 KDA subunit
8	FABE	Q01469	Fatty acid binding protein
9	G3P2	P04406	Glyceraldehyd 3-phosphate dehydrogenase
10	RLA2 /	P05387 /	60S acidic ribosomal protein P2/
	MLEN	P16475	Myosin light chain alkali, non-muscle isoform
11	ACTB	P02570	β-Actin, **increase**, N-terminal fragment
12	NUCL	P19338	Nucleolin, **increase**, fragment
13	CYPH	P05092	Cyclophilin A, **increase**
14	STHM	P16949	Stathmin
15	DEST	P18282	Destrin / actin depolymerizing factor
16	BTF3	P20290	RNA polymerase B transcription factor 3, **decrease**
17	DUT	P33316	Deoxiuridine 5'-triphosphate nucleotidohydro-lase, **decrease**
18	TDX1	P32119	Thioredoxin peroxidase 1
19		G3641298	FIFO type ATPase
20	TDX2	Q06830	Thioredoxin peroxidase 2
21	GDIS	P52566	Rho GDP-dissociation inhibitor 2, **increase**
22	TDX2	Q06830	Thioredoxin peroxidase 2
23	GTP	P09211	Glutathione S-transferase P
24	GTP	P09211	Glutathione S-transferase P
25	GDIS	P52566	Rho GDP-dissociation inhibitor 2, **decrease**
26	RANG	P43487	RAN-specific GTPase-activating protein
27	GDIR	P52565	Rho GDP-dissociation inhibitor 1

Table 8: Spots identified by mass spectrometric analysis. The corresponding positions are indicated in Fig. 34.

Spot Number	Protein	(SwissProt) Accesion Number	comments
28	TCPD	P50991	T-complex protein 1, δ subunit, **increase**
29	PRC8	P25788	Proteasome component C8
30	IF4H	Q15056	eukaryotic translation initiation factor 4H
31	PNPH	P00491	Purine nucleoside phosphorylase
32	ICE3	P42574	Caspase-3 (proform), **decrease**
33	ANX4	P09525	Annexin IV
34		HSU86782	Pad1
35	ENOA	P06733	Alpha Enolase
36	ROA2	P22626	Heterogeneous nuclear ribonucleoprotein A2/B1
37		EST: G1303338	
38		PIR:JW0079	hnRNP like protein
39	G3P2	P04406	
40	IDHA	P50213	Socitrate dehydrogenase (NAD) subunit α, mitochondrial
41	CABA	Q99020	CARG-binding factor-A (mouse)
42	LA	P05455	Lupus LA protein

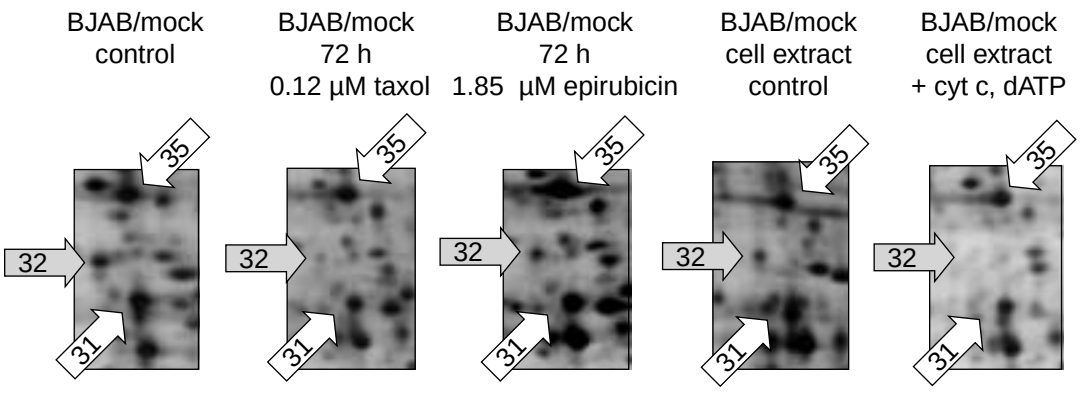

Fig. 35: (AREA A) Decrease of procaspase-3. Cells or cellular extracts were treated as indicated in the figure. Then, 2DE PAGE analysis was performed. 32 = procaspase-3; 35 = alpha enolase; 31 = purine nucleotide phosphorylase.

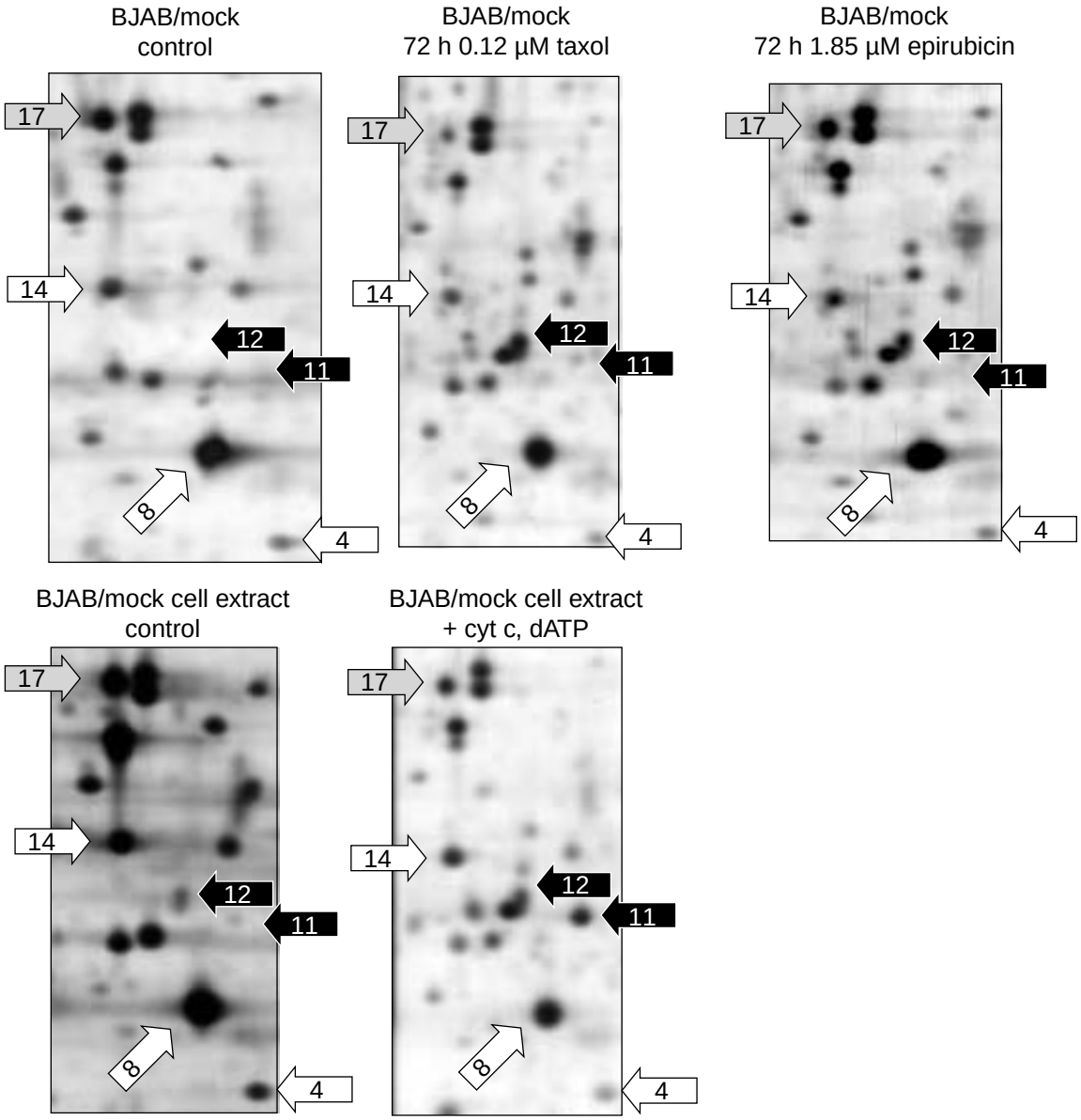

Fig. 36: (AREA B) Detection of a nucleolin and β-actin fragment. 2DE PAGE analysis was performed after cytostatic drug-treatment or after *in vitro* activation as indicated in the figure. 17 = DUT; 14 = STHM; 12 = nucleolin (fragment); 11 = β-actin (fragment); 8 = FABE; 4 = poly-c.

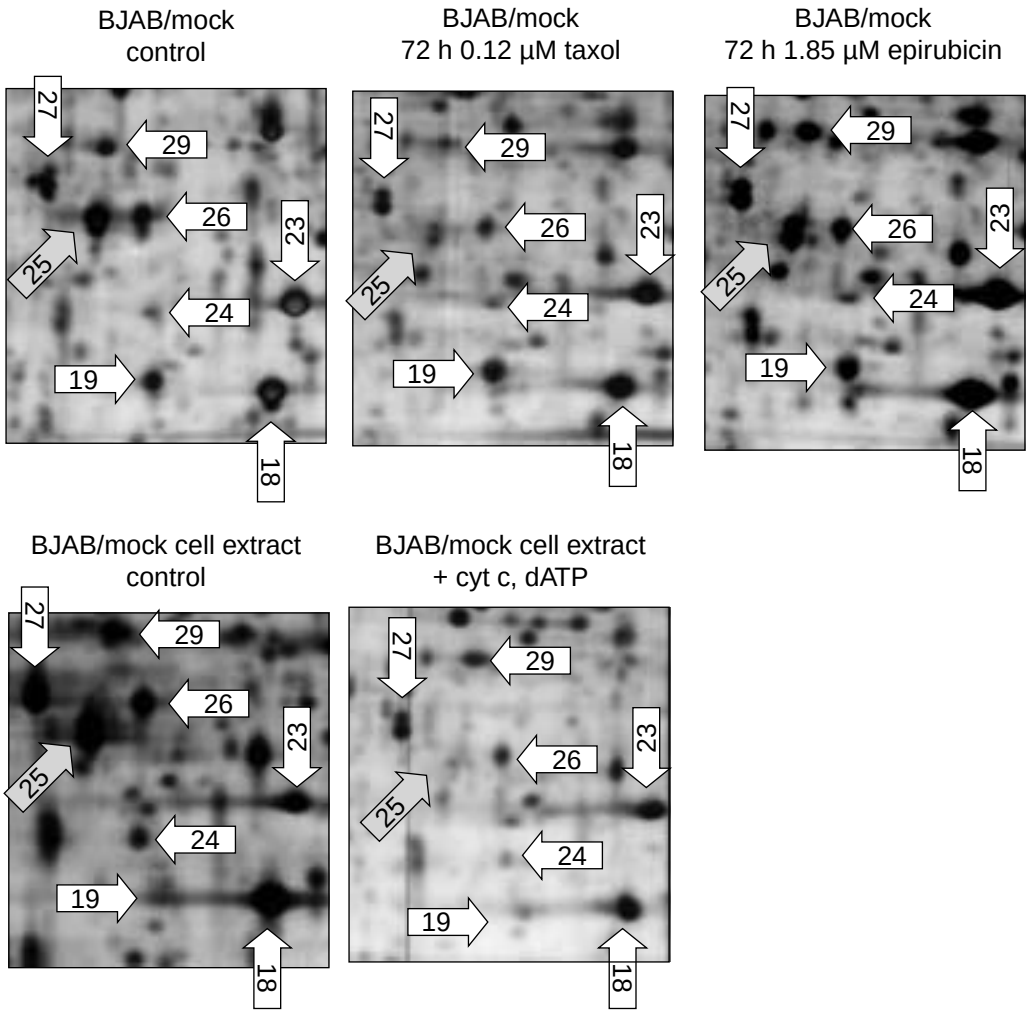

Fig. 37: (AREA C) Decrease of D4-GDI concentration. After treatment of cultured cells or cellular extracts as indicated in the figure, 2DE PAGE analysis was performed. 29 = PRCδ; 27 = rho-GDI; 25 = D4-GDI; 26 = RANG; 23 and 24 = GTP; 19 = FIFO type ATPase; 18 = TDX1.

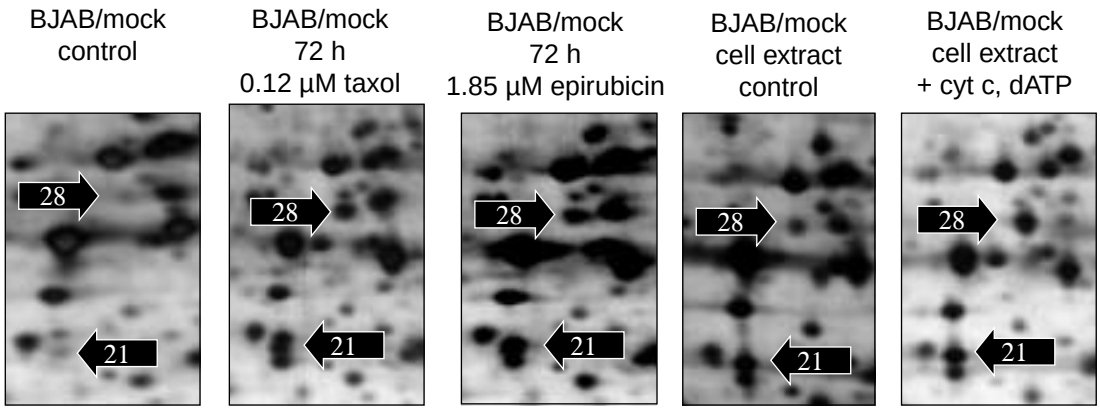

Fig. 38: (AREA D) D4-GDI-fragment and tcpd-fragment. Increase in intensity of the spots identified as D4-GDI (fragment, spot #21) and t-complex binding protein (δ-subunit, fragment, spot #28) was readily detected in lysates of drug-treated BJAB/mock cells and cell extracts after induction of the mitochondrial apoptotic cascade after performing 2DE PAGE.

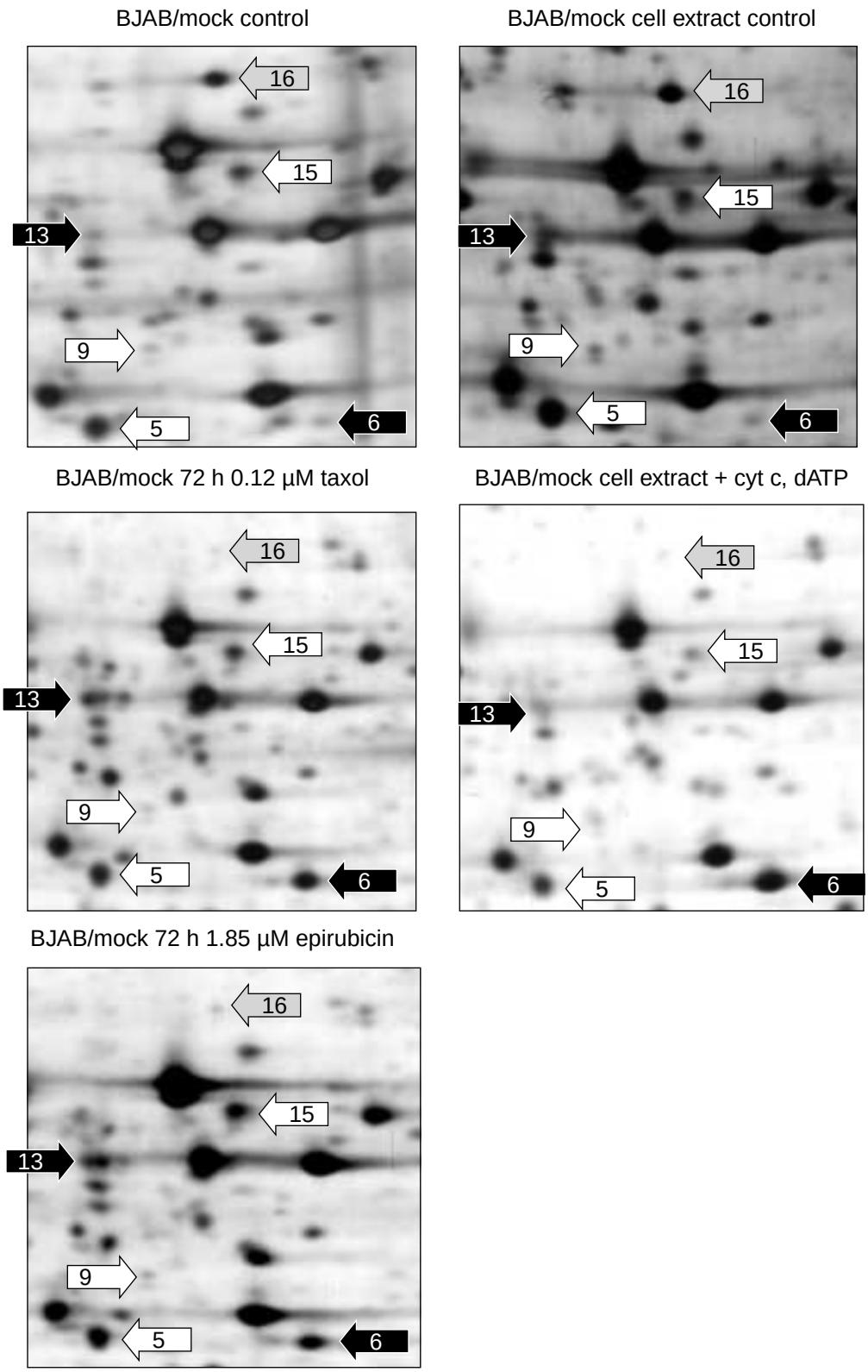

Fig. 39: (AREA E) BTF3 and β-actin fragment. Protein samples of drug-treated cells or *in vitro* activated extract and control samples were separated by 2DE PAGE. Results are shown above. Change in intensity of the spots representing the transcription factor BTF3 (spot #16) and an β-actin fragment (spot #6) was, as shown above, significant. 16 = BTF3; 15 = destin; 13 = cyclophilin A; 9 = G3P2; 6 = β-actin (fragment); 5 = hint protein.

Areas containing spots displaying significant variability in intensity are enlarged in Fig. 35 - Fig. 39. Significant decrease of intensity was detected for procaspase-3, D4-GDI, DUT whereas spot intensity was obviously increased for fragments of β-actin, nucleolin, D4-GDI, tcpd and a spot representing cyclophilin A. One of the most variable spots was identified as D4-GDI. In the following investigations, the significance and mechanism of this phenomenon was studied in detail.

VII-8 D4-GDI IN DRUG INDUCED APOPTOSIS

D4-GDI (rho-GDI 2) is one of three known small GTP dissociation inhibitors and exclusively expressed in haematopoietic tissues. Rho-GDI (rho-GDI 1) is expressed ubiquitously and rho-GDI 3 in brain, lung kidney, testis and pancreas (Sasaki and Takai, 1998). Since the intensity of the identified spot representing D4-GDI significantly decreased upon treatment of BJAB cells with cytostatic drugs further investigation intended to clarify several questions, e.g. if D4-GDI is specifically cleaved during apoptosis and if so, which caspase or protease is responsible for cleavage of D4-GDI (Brockstedt et al., 1998).

VII-8.1 D4-GDI / RHO-GDI IS SPECIFICALLY CLEAVED DURING APOPTOSIS

BJAB/mock cells and BJAB/crmA cells were cultured in the presence or absence of 0.12 µM taxol or 1.85 µM epirubicin for 24 hs, 48 hs and 72 hs. After determination of protein concentration, 15 µg of protein were separated by SDS-PAGE and blotted onto NC membrane followed by Western blot analysis using a pAb specific for rho-GDI and D4-GDI (Fig. 40).

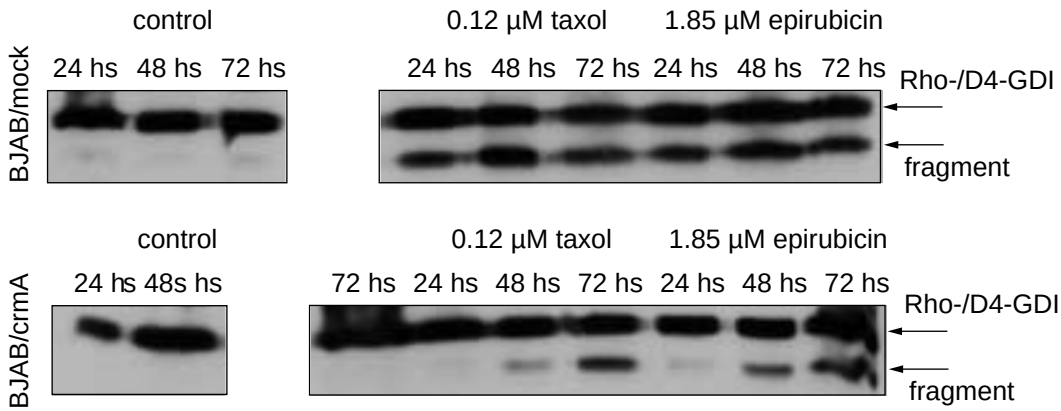

Fig. 40: Cleavage of rho-GDI / D4-GDI after drug treatment of BJAB cells. BJAB/mock and BJAB/crmA cells were incubated with cytostatic drugs for the indicated periods of time. Western blot analysis revealed the specific cleavage of either rho-GDI or D4-GDI. The positions of the bands representing rho-/D4-GDI and the corresponding fragment are indicated at the right margin.

In response to cytostatic drug treatment one additional band was detected. As the decrease of D4-GDI concentration during apoptosis had already been shown by 2DE

PAGE it was reasonable that this band represented a D4-GDI fragment resulting from specific cleavage of the full length protein during apoptosis. Nonetheless, there was yet no information about the effects of drug treatment on rho-GDI in BJAB cells and additional experiments were necessary to obtain further information about the origin of the appearing band.

VII-8.2 2DE PAGE AND WESTERN BLOT ANALYSIS

To determine the identity of the immunoreactive bands proteins were separated by analytical 2DE PAGE and blotted onto NC membranes. Proteins were visualized by Ponceau Red staining and subsequently membranes were scanned on a Linotype Hell flat bed scanner. Western blot analysis was performed using the above mentioned pAb specific for rho-GDI and D4-GDI, and immunoreactive spots were visualized using the ECL detection system (Fig. 42). Three protein spots were detected which were assumed to represent rho-GDI 1, D4-GDI (rho-GDI 2) and a fragment of one of these proteins. The position of detected spots was correlated with Ponceau staining of the membranes using computer aided image processing. As the spot representing D4-GDI had already been identified (spot #2, Fig. 42) the immunoreactive spot located at a higher apparent molecular weight and lower isoelectric point (spot #1, Fig. 42) was considered to be rho-GDI and the third spot, located at lower apparent molecular weight (about 23 kDa) and higher isoelectric point, was considered to represent the fragment formed upon induction of apoptosis (spot #3, Fig. 42).

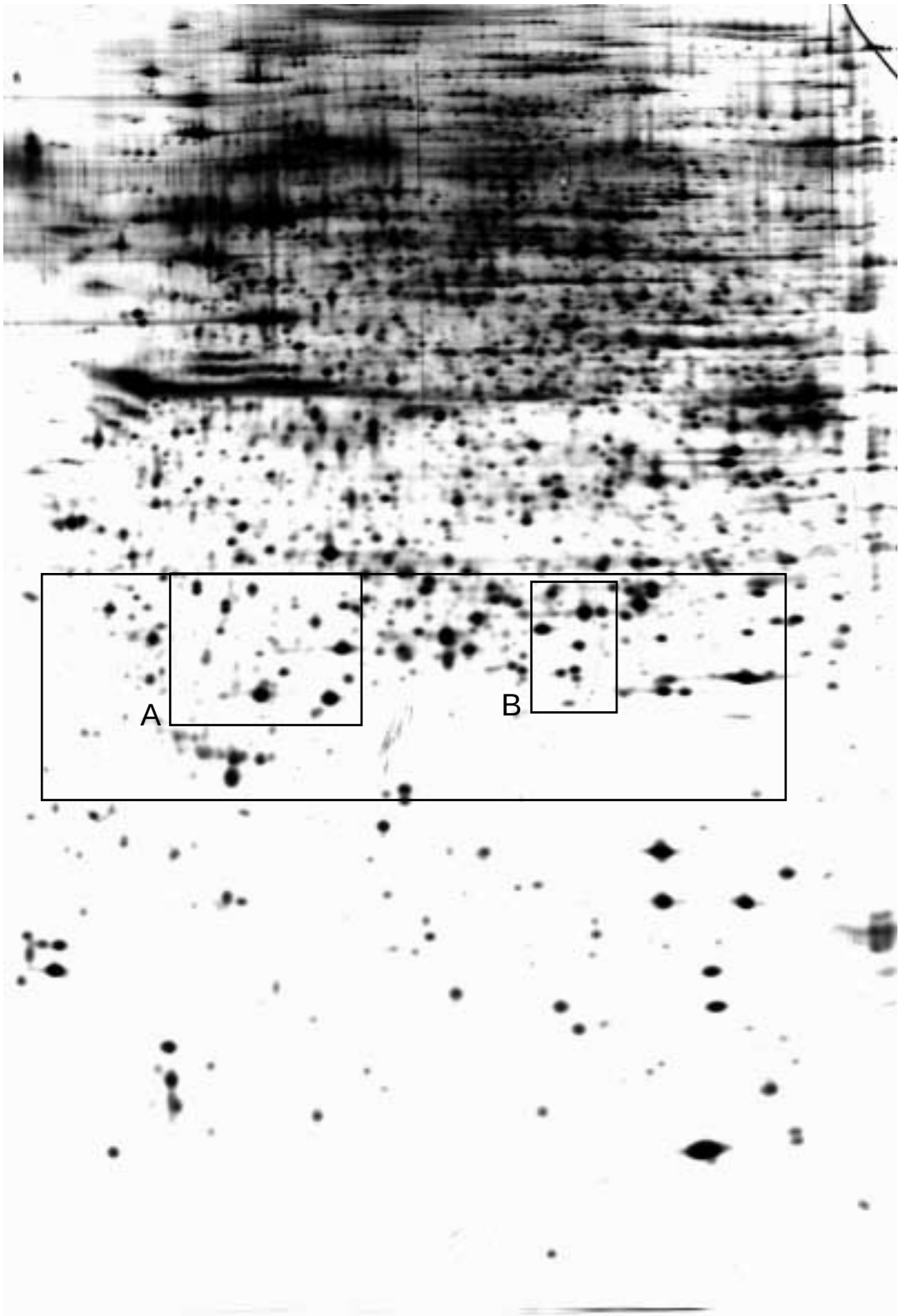

Fig. 41: Example of a 2DE PAGE gel. The indicated region of the 2DE PAGE gel and results obtained by Western blot analysis of the corresponding area are correlated in detail in Fig. 42.

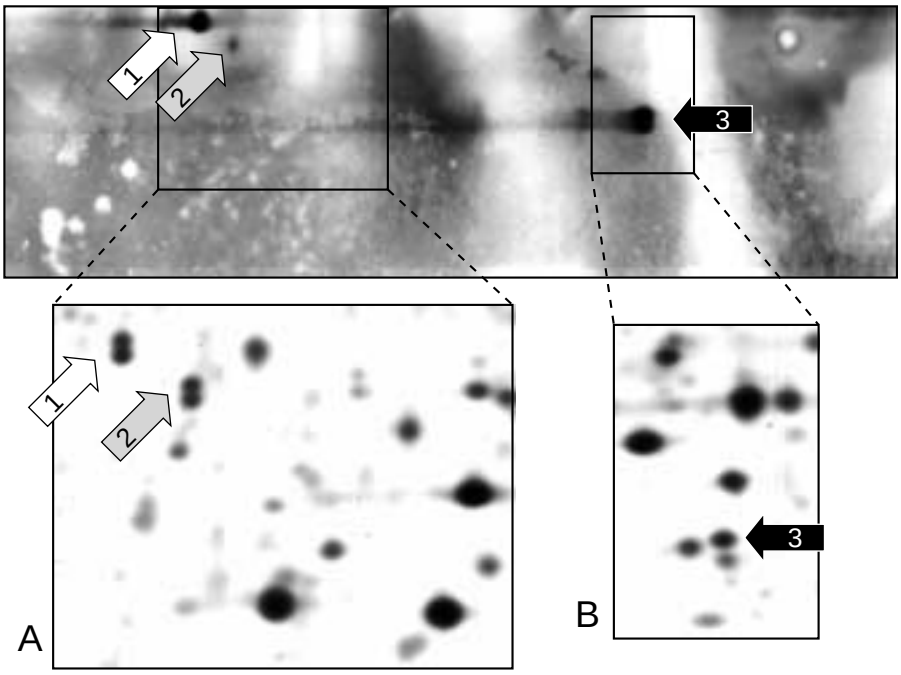

Fig. 42: Correlation of Western blot analysis and silver stain of 2DE PAGE gels. 2DE PAGE gels of drug-treated (0.12 µM taxol, 72 hs) BJAB/mock cells were prepared in parallel and either silver stained (A and B) or blotted and analyzed using a rho-GDI / D4-GDI specific antibody. The positions of the immunoreactive spots are indicated in A and B, the overall location of the area taken for Western blot analysis is depicted in Fig. 41. Spot 2 was already identified as D4-GDI, spot #1 was assumed to represent rho-GDI and spot #3 represents a fragment of either of this proteins.

VII-8.3 Identification of the Immunoreactive Spots

As the identity of the spots still had to be confirmed again 2DE PAGE was performed (analytical scale) and proteins were visualized by staining according to Blum et al. followed by spot excision, tryptic digest and mass spectrometric examination of the resulting peptide mixtures. Data bank search on the basis of peptide masses and internal sequences led to confirmation of the results obtained by Western blot analysis of the 2DE gels (Fig. 42). This indicates that D4-GDI is specifically cleaved by one of the caspases activated during drug-induced apoptosis. Cleavage of rho-GDI 1 does not occur as the concentration of rho-GDI 1 did not change in samples derived from apoptotic cells. Sequence analysis of rho-GDI and D4-GDI revealed that rho-GDI lacks a specific caspase-3 cleavage site which is only present in D4-GDI. Thus, it was likely that the fragment represents a cleavage product of D4-GDI resulting from caspase-3 activity. Mimicing mitochondrial activation of the apoptotic cascade which primarily activates caspase-9 and caspase-3, was performed to obtain further information about the sequential caspase cascade responsible for D4-GDI cleavage.

VII-8.4 *IN VITRO* ACTIVATION OF THE MITOCHONDRIAL APOPTOTIC CASCADE INDUCES D4-GDI CLEAVAGE.

In order to obtain larger amounts of the cleavage product it was tested whether rho-GDI 1 / D4-GDI was cleaved after *in vitro* activation of cellular extracts derived from BJAB/ mock cells. Mitochondrial initiation of the caspase cascade was achieved by addition of 10 µM cytochrome c and 1 mM dATP followed by incubation at 30°C for 5 min. The control sample was kept on ice without addition of cytochrome c and dATP. After activation, a time course was obtained by taking samples of equal volume from both extracts after different periods of time and proteins were immediately denatured by addition of excess sample buffer (5xPP) and boiling at 95°C for 5 min. Subsequent Western blot analysis revealed that rho-GDI 1 / D4-GDI was specifically cleaved only in activated extracts thereby confirming that a caspase which had been activated downstream of the mitochondrial initiation of the caspase cascade was necessary to cleave rho-GDI 1 / D4-GDI producing the observed fragment (Fig. 43).

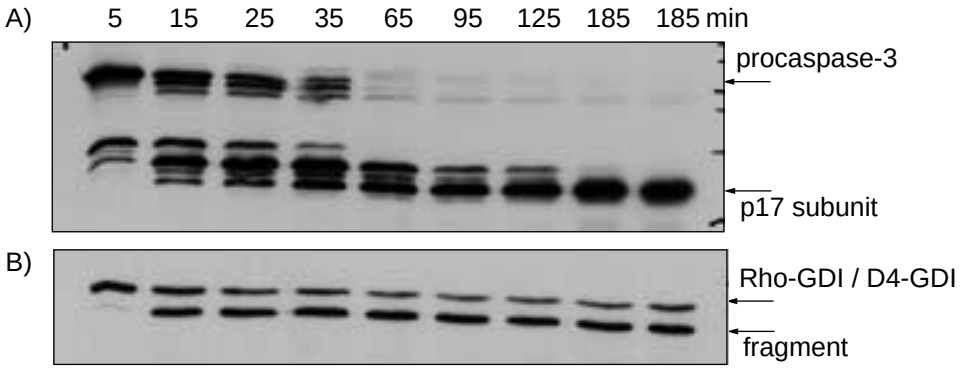

Fig. 43: Formation of the rho-GDI 1 / D4-GDI fragment after *in vitro* activation. Cell extracts were prepared and the mitochondrial caspase cascade was initiated by addition of cytochrome c and dATP. Samples were analyzed by Western blot analysis using anti-caspase-3 Ab (A) or anti-rho-GDI 1 / D4-GDI Ab (B). The positions of the respective protein bands are indicated at the right margin.

Thus, induction of the mitochondrial caspase cascade lead to formation of the band representing the D4-GDI fragment and allowed production of larger amounts of the fragment by *in vitro* activation of extracts. The resulting protein mixture was then used for further analysis by 2DE PAGE. Specific cleavage of D4-GDI (decrease in spot intensity) and formation of the spot in question (increase in intensity) was also confirmed detected in analytical 2DE gels (Fig. 44).

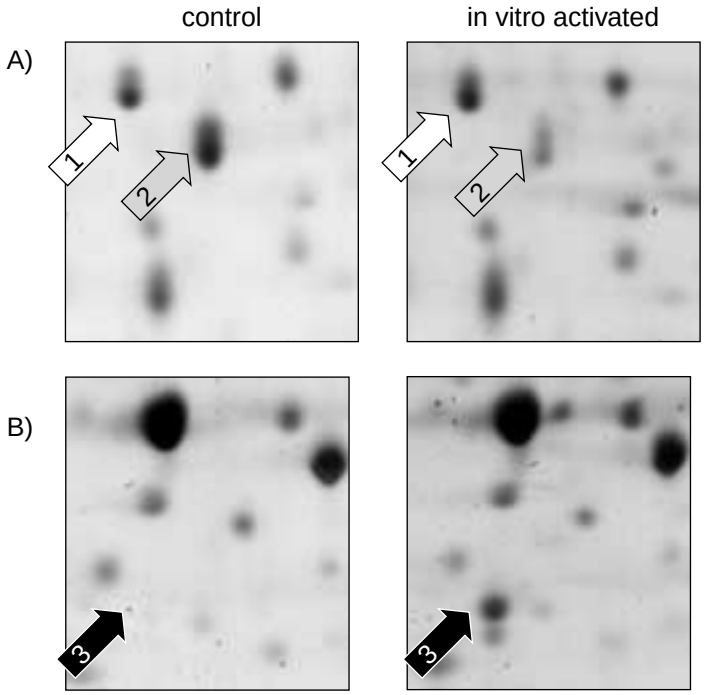

Fig. 44: Rho-GDI 1, D4-GDI and D4-GDI fragment in 2DE PAGE gels of cell free extracts. Extracts were prepared as described and protein samples were separated using semipreparatve 2DE PAGE followed by silverstaining according to Blum et al. (1987). Positions of the identified spots are indicated and enlarged areas correspond to those depicted in Fig. 41. 1 = rho-GDI 1; 2 = D4-GDI; 3 frag-

VII-8.5 Role of Caspase-1 in Drug-induced Apoptosis

D4-GDI has been reported to be a substrate for caspase-3 (Na et al., 1996) and caspase-1 (Danley et al., 1996). In previous experiments, caspase-3 activation of caspase-3 has already been shown. However, effects of cytostatic drug treatment on caspase-1 processing and activation still had to be examined. This was necessary because experimental evidence had been provided that caspase-1 is activated during apoptosis, at least after taxol treatment (Ibrado et al., 1998, Kottke et al., 1999).

Western Blot Analysis

Protein samples derived from BJAB/mock cells incubated with 0.12 µM taxol or 1.85 µM epribucin were used for SDS-PAGE, blotted onto NC membrane and a pAb was used to detect procaspase-1 (see Fig. 45 A). A time-dependent decrease in procaspase-1 content was observed. On the other hand, using an Ab specific for the mature caspase-1 p20 subunit, an increase of p20 was not detected (Fig. 45 B) indicating that caspase-1 is degraded rather than processed to form the active enzyme.

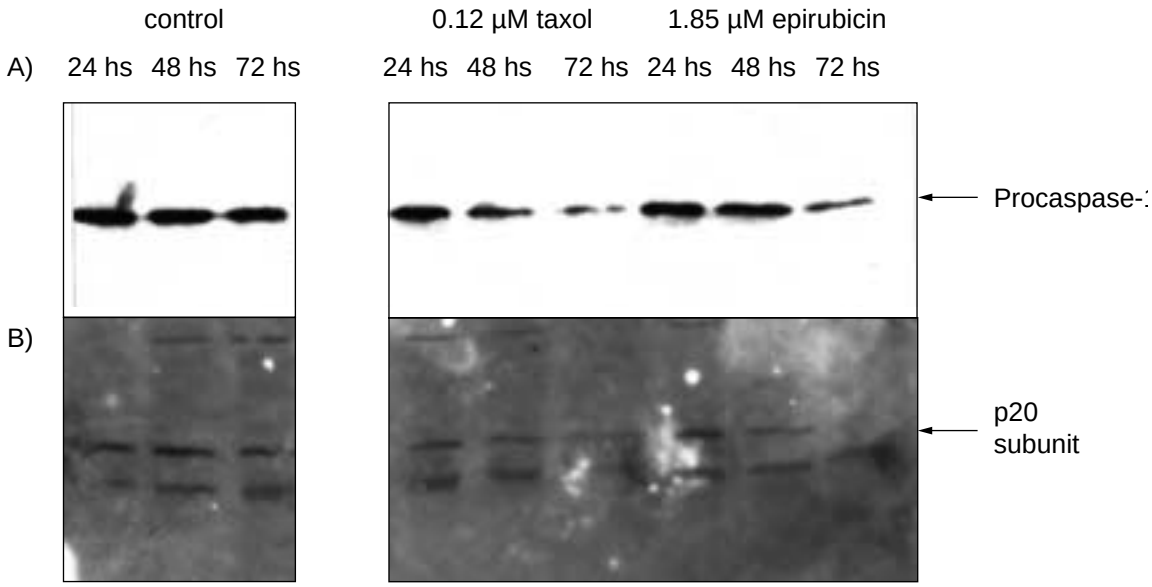

Fig. 45: Cleavage of procaspase-1 in drug-treated BJAB cells. Western blot analysis of identical samples used for detection of caspase-3 processing and PARP cleavage. A time-dependent decrease in procaspase-1 content is clearly visible (A). However, using a caspase-1 p20 specific Ab, an increase in p20 content was not detected (B).

Nonetheless, the data obtained so far did not exclude a role of caspase-1 in drug induced apoptosis, and the possibility of caspase-1 activation was further investigated using the caspase-1 specific colorimetric substrate Ac-WEHD-pNA.

Caspase-1 Activity Assay

BJAB/mock cells were either treated with control medium, incubated with 0.12 µM taxol or 1.85 µM epirubicin for 48 hs and cell extracts were prepared. Extracts were either activated by addition of cytochrome c and dATP and incubation at 30°C for 30 min or

kept on ice without addition of supplements. For detection of caspase-1-like activity Ac-WEHD-pNA was used. This substrate reflects the preferred caspase-1 substrate sequence (Rano et al., 1997) and cleavage after Asp_1 releases pNA resulting in an increase of absorption at 405 nm. Absorption was measured after 3 hs of incubation at 37°C and no significant increase in absorption was detected when samples from cytostatic drug-treated cells were compared with control extracts. Since this experiment was performed in parallel with a caspase-3 activity assay (Fig. 46) it was clearly excluded that activation of caspase-1 plays a role during drug-induced apoptosis in BJAB cells.

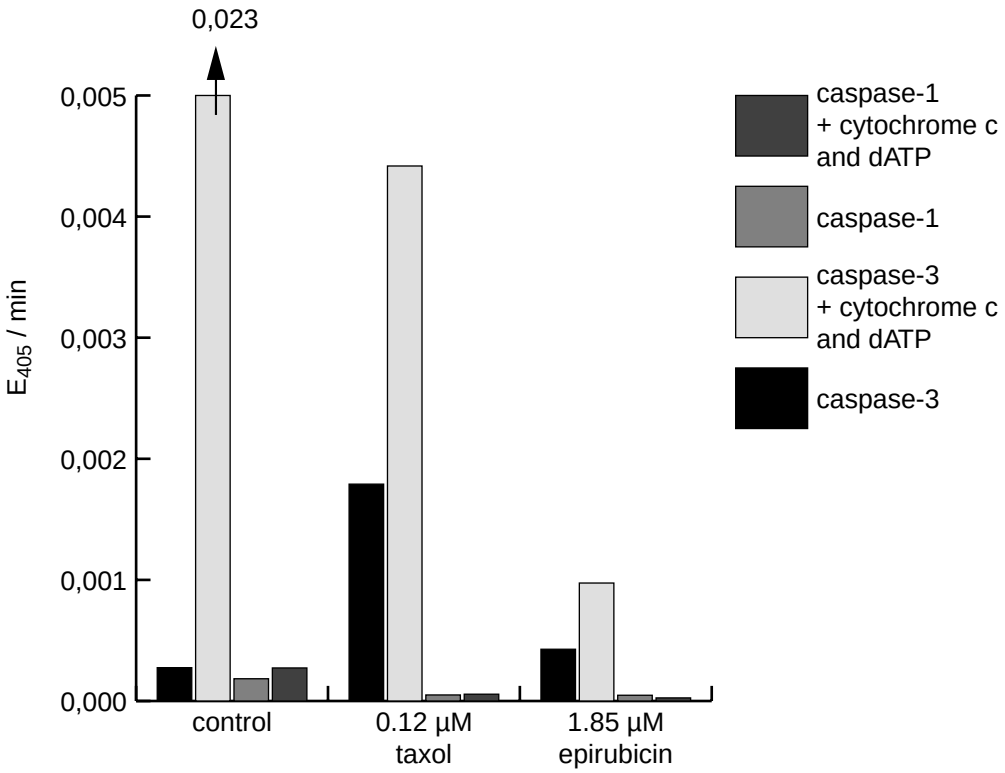

Fig. 46: Caspase-3-like and caspase-1-like activity assay after drug treatment. BJAB/mock cells were left untreated or incubated with 0.12 µM taxol or 1.85 µM epirubicin for 48 hs. Cell extracts were prepared and Ac-DEVD-pNA or Ac-WEHD-pNA were used for detection of caspase activities.

VII-8.6 Is D4-GDI Cleaved *in Vivo* and *in Vitro* by Caspase-3 or Caspase-1?

This question was answered using different methods. On the one hand, *in vivo* experiments using irreversible peptide inhibitors specific for caspase-1 and caspase-3 were performed. Second, the colorimetric substrate Ac-DEVD-pNA was used as a competitive inhibitor *in vitro,* and third, caspase-3 was depleted from cellular extracts prior to activation. Finally, the obtained results were confirmed using mass spectrometric methods to analyze the cleavage site *in vivo.*

Taxol-induced D4-GDI Cleavage is Blocked by Z-DEVD-fmk but not by Z-WEHD-fmk in Cultured BJAB/mock Cells

BJAB/mock cells were induced to undergo apoptosis by addition of 0.12 µM taxol in the presence or absence of Z-DEVD-fmk and Z-WEHD-fmk which specifically inhibit caspase-3 and caspase-1 activity, respectively. Cells were harvested after 48 hs of incubation. Subsequently, Western blot analysis was performed using the D4-/rhoGDI specific Ab. D4-GDI cleavage was nearly blocked in samples cultured in the presence of caspase-3 inhibitor whereas caspase-1 inhibitor had no significant effect (Fig. 47).

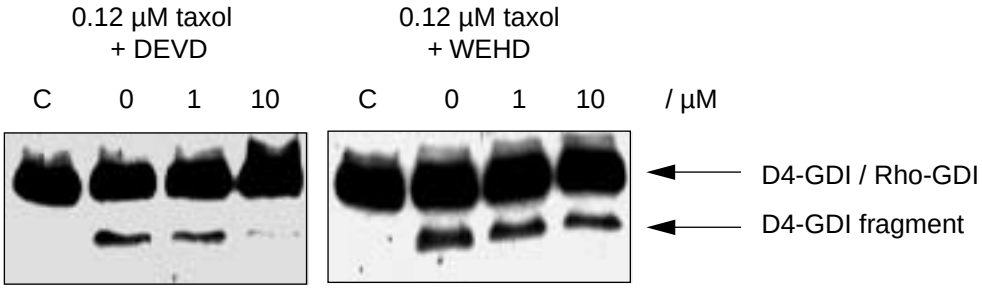

Fig. 47: Z-DEVD-fmk but not Z-WEHD-fmk blocks D4-GDI cleavage. BJAB/mock cells were incubated with 0.12 µM taxol in the presence or absence of Z-DEVD-fmk or Z-WEHD-fmk for 48 hs. Western blot analysis was performed using anti-rho-GDI 1 / D4-GDI Ab as described in Methods. The positions of D4-GDI / rho-GDI and D4-GDI fragment are indicated at the right margin.

Ac-*DEVD-pNA Blocks D4-GDI Cleavage In Vitro*

The specific caspase-3 colorimetric substrate Z-DEVD-pNA was used as competitive inhibitor in activated cell extracts of BJAB/mock control cultures. Sample preparation and *in vitro* activation of caspases were done as described. Western blot analysis using anti-rho-GDI 1 / D4-GDI Ab was performed and showed clear inhibition of D4-GDI cleavage by Ac-DEVD-pNA (Fig. 48). Thus, it is concluded that D4-GDI is a substrate of caspase-3, at least *in vitro.*

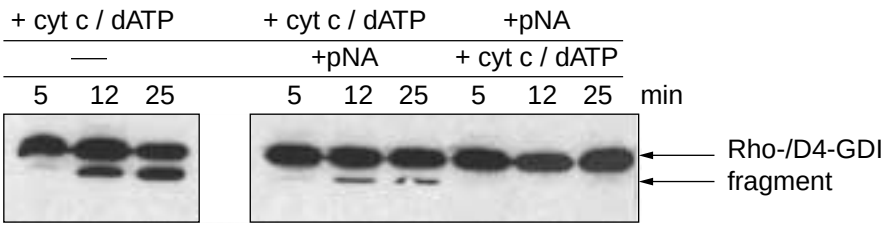

Fig. 48: Ac-DEVD-pNA inhibits cleavage of D4-GDI *in vitro*. Cellular extracts from BJAB/mock cells were prepared and *in vitro* activated before, after or without addition of Ac-DEVD-pNA. Samples obtained after the indicated periods of time were analyzed by Western blot analysis using anti-rho-GDI 1 / D4-GDI Ab. The positions of rho-/D4-GDI and the corresponding fragment are indicated at the right margin.

Immunoprecipitation of Caspase-3 in Vitro

This experiment was performed exactly as described under VII-3.3. Samples were analysed using anti rho-GDI 1 / D4-GDI Ab. Results are shown in Fig. 49 and confirm the necessity of active caspase-3 for D4-GDI cleavage, as already indicated by the inhibitor experiments.

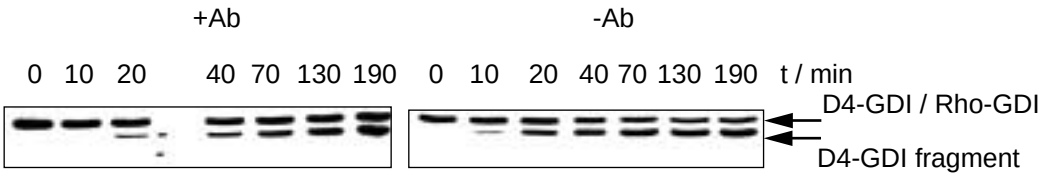

Fig. 49: Immunochemical depletion of procaspase-3 inhibits D4-GDI cleavage. Cell extracts were prepared as described and incubated with protein-A sepharose with (+Ab) or without (-Ab) procaspase-3 specific Ab prior to activation. Protein-A sepharose was sedimented by centrifugation. The clear supernatant was used for *in vitro* activation. Samples were removed after different periods of incubation at 30°C (as indicated) and analyzed by Western blot using a rho-GDI 1/D4-GDI specific Ab.

VII-8.7 Exact Determination of the D4-GDI Cleavage Site *In Vivo*

N-terminal Edman sequencing needs far more protein as compared with mass spectrometric methods. Furthermore, it is very difficult to obtain large amounts of protein from apoptotic cells. Therefore, mass spectrometry was chosen to identify the exact *in vivo* cleavage site of D4-GDI. Detailed analysis of peptide mass fingerprints from full length D4-GDI and the corresponding fragment was performed. Subsequent microsequencing of peptides by MS/MS revealed the presence of the peptide $E^{34}LQEMDKDDESLIK^{47}$ (see Fig. 50) in both samples. Since the cleavage site of caspase-3 is represented by $DELD^{19}S$ and the cleavage site of caspase-1 by $LLGD^{55}G$ the detection of this peptide, located between Lys^{33} and Tyr^{48} of D4-GDI, in the samples derived from D4-GDI fragment was only consistent with the caspase-3-mediated cleavage of D4-GDI after drug-induced apoptosis (see also Fig. 50).

D4-GDI:

```
  1 MTEKAPEPHV EEDDDDELDS KLNYKPPPQK SLKELQEMDK
 41 DDESLIKYKK TLLGDGPVVT DPKAPNVVVT RLTLVCESAP
 81 GPITMDLTGD LEALKKETIV LKEGSEYRVK IHFKVNRDIV
121 SGLKYVQHTY RTGVKVDKAT FMVGSYGPRP EEYEFLTPVE
161 EAPKGMLARG TYHNKSFFTD DDKQDHLSWE WNLSIKKEWT E
```

D4-GDI fragment:

```
  1 MTEKAPEPHV EEDDDDELDS KLNYKPPPQK SLKELQEMDK
 41 DDESLIKYKK TLLGDGPVVT DPKAPNVVVT RLTLVCESAP
 81 GPITMDLTGD LEALKKETIV LKEGSEYRVK IHFKVNRDIV
121 SGLKYVQHTY RTGVKVDKAT FMVGSYGPRP EEYEFLTPVE
161 EAPKGMLARG TYHNKSFFTD DDKQDHLSWE WNLSIKKEWT E
```

caspase-3 cleavage site caspase-1 cleavage site

Fig. 50: D4-GDI sequence and identified peptides. Peptides identified by mass spectrometric analysis are printed in bold. Identification of E^{34}LQEMDKDDESLIK^{47} revealed that the D4-GDI fragment resulted from cleavage by caspase-3 rather than caspase-1.

VII-9 Approaches to Identify Apoptosis Regulating Proteins

So far the role of caspases in drug-induced apoptosis, especially caspase-9, caspase-8, caspase-3 and caspase-1, had been well elucidated. Furthermore, D4-GDI was identified to be specifically cleaved by caspase-3 during this process while rho-GDI was not affected. On the basis of these results, additional attempts were made to identify yet unknown caspase substrates or proteins involved in regulation of drug-induced apoptosis in BJAB cells. Although it is possible to separate more than 10000 proteins in high resolution 2DE PAGE this method alone was obviously not suitable to obtain the desired results when entire cell lysates were used for analysis. This is reflected by the low copy numbers of regulatory proteins and detection of proteins by silverstain is limited to 1 ng - 10 ng of protein per spot. Thus, additional approaches had to be performed in order to study regulatory effects of cytostatic drug treatment on yet unknown proteins: (I) cell fractionation prior to 2DE PAGE on the basis of characteristics different from apparent molecular weight and isoelectric point was used and (II) the detection limit of protein analysis was lowered by metabolic radioactive labeling of proteins.

VII-9.1 Cell Fractionation using Magnetic Annexin V-Beads

In living cells, membrane assymmetry is maintained by the flipase which keeps phosphatidylserine (PS) on the inner side of the membrane by an ATP-dependent process. On the other hand, apoptosis is an active ATP-consuming process and breakdown of mitochondrial membrane potential abolishes ATP production. As a consequence, ATP is no longer provided for energy consuming proteins, i.e. the flipase, and membrane asymmetry breaks down resulting in exposure of PS to the outer membrane site. This effect can be used to enrich apoptotic cells or to deplete them from samples. To enrich

apoptotic cells, magnetic beads covalently coupled with Annexin V, a protein which specifically binds to PS, were used. Samples of BJAB/mock cells treated with 0.12 µM taxol or 1.85 µM epirubicin were incubated with Annexin V coupled magnetic beads and separation was then performed in the presence of a magnetic field. Cells bound to magnetic beads (apoptotic cells) were retained and unbound cells (non-apoptotic) cells passed the column. Finally, apoptotic cells were eluted by removal of the magnetic field. Cells which had been separated by this technique were analyzed by 2DE PAGE. Then, the silverstained gels were submitted to subtractive analysis. However, spots displaying differential intensity fitted those detected in whole cell lysates. However, these data revealed that purification of apoptotic cells did not sufficiently enhance specificity and sensitivity of the analysis (results not shown). Thus, cell fractionation by the use of Annexin-V coupled magnetic beads did not significantly improve the experimental approach.

VII-9.2 METABOLIC ^{35}S-LABELLING

In these experiments, BJAB/mock cells were starved by incubation in methionine/cysteine free medium and apoptosis was induced using taxol or epirubicin. Radioactive methionine and cysteine were added simultaneously to the apoptotic stimulus and the cells were incubated for different periods of time (6 - 24 hs). This short incubation times were chosen because regulatory effects should occur prior to activation of the apoptotic cascade, which had been shown to occur after 24 hs. Cells were harvested and samples were prepared for 2DE PAGE. After separation of proteins, gels were either fixed, dryed immediately and exposed to x-ray films or silverstained prior to exposure to x-ray-films. This was done to compare protein patterns resulting from autoradiography and normal protein staining.

At least one spot displaying significant difference in intensity after cytostatic drug-treatment could be visualized by this technique (Fig. 51). Protein staining revealed low abundance of the protein and despite several attempts the protein representing this spot has not been identified yet.

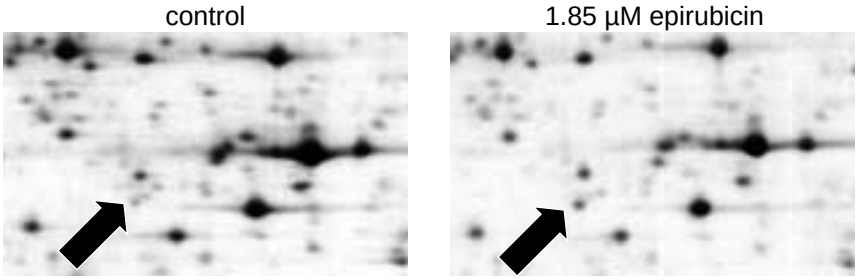

Fig. 51: Differentially expressed spot after epirubicin treatment. BJAB/mock cells were treated with epirubicin or control medium for 8 hs and proteins were labeled with ^{35}S-methionine/cysteine. 2DE PAGE analysis was performed and the resulting autoradiographies were submitted to subtractive analysis. The position of one spot displaying a significant change in intensity is indicated.

VIII DISCUSSION

Three major starting points of apoptosis are under discussion in the literature: (I) death receptors (DRs) (Schulze-Osthoff, 1998 and literature cited therein), (II) mitochondria (ER) (Liu, 1996, Loeffler and Kroemer, 2000) and (III) the endoplasmic reticulum. Much is known about DR-signalling as well as the death cascade induced by mitochondria whereas only little is known about death induced by ER-stress (Nakagawa, 2000).

DR-signalling in general is mediated by oligomerisation of the receptor. This induces binding of adaptor molecules on the inner membrane part of the receptor (formation of the DISC) thereby mediating recruitment of procaspases and subsequent autoproteolytic activation. Within the CD95/Fas-signalling pathway caspase-8 is believed to be the initiator caspase leading to downstream activation of the executioner caspases, which are caspase-3, -6 and -7.

A similar mechanism is published for the mitochondrial induction of the caspase cascade. BCL-2 family proteins are supposed to regulate loss of mitochondrial membrane potential and release of apoptogenic factors, like cytochrome c. Then, cytochrome c, APAF-1 and dATP build up the mitochondrial DISC, the initiator caspase, procaspase-9, is incorporated and autoproteolytically activated. Active caspase-9 then mediates activation of the effector caspases.

Only little is known about regulation of ER-stress induced apoptosis. However, procaspase-12 is thought to be the initiator-caspase within this signalling pathway.

Although the first two signalling pathways are quite well documented there is an ongoing debate about death induced by cytostatic drugs. On the one hand, some authors announce induction of FasL expression upon cytostatic drug treatment and autocrine or paracrine induction of apotosis mediated via CD95/Fas (Friesen et al., 1996, Friesen et al., 1997, Müller et al., 1997, Fulda et al., 1997, Fulda et al., 1998). On the other hand, FasL expression is not found by others upon cytostatic drug treatment and induction of apoptosis is claimed to be independent of CD95/Fas (McGahon et al., 1998, Adjei et al., 1998, Eischen et al., 1997, Micheau et al., 1999). Since most of the work on drug-induced apoptosis has been done in T cells, namely Jurkat cells, the present study aims to elucidate the signalling pathways involved in drug-induced apoptosis in B-lymphoid cells upon treatment with taxol and epirubicin.

VIII-1 THE CASPASE CASCADE

VIII-1.1 THE MITOCHONDRIAL SIGNALLING PATHWAY

Cytostatic drug-induced apoptosis is reported in several publications to be mediated via activation of mitochondria and subsequent release of cytochrome c either via outer membrane rupture or opening of a channel large enough for cytochrome c to pass through (Reed, 1997). This leads to formation of the mitochondrial DISC, consisting of

APAF-1, cytochrome c and dATP, inducing a conformational change in APAF-1 (Qin et al., 1999) thereby recruiting procaspase-9 to the complex and leading to autoproteolytic activation of procaspase-9 (Zou et al., 1999). Once activated, caspase-9 processes the predominant effector caspase-3. Besides caspase-3, caspase-6 and caspase-7 are also reported to be activated (Bratton et al., 2000). Further downstream events in cells undergoing apoptosis are represented by the cleavage of the prototypic caspase-3 substrate PARP resulting in the formation of a 84 kDa fragment and DNA fragmentation mediated by CAD (caspase activated DNase) or AIF (apoptosis inducing factor).
In order to examine effects of taxol and epirubicin-treatment on BJAB/mock cells several events associated with apoptosis were investigated. First, the outcome of drug treatment was investigated: Cell death. BJAB/mock cells were shown to die after treatment with taxol and epirubicin using the trypan exclusion assay which discriminates between dead and living cells. In order to provide evidence for apoptosis to occur rather than necrosis downstream events of the apoptotic cascade were investigated. It was shown by flow cytometric analysis that, in accordance with published data, both drugs induce DNA fragmentation at a significant level (50 % - 60 %). The experimental setup was further characterized by Western blot analysis and cleavage of PARP as well as proteolysis of procaspase-3 was shown to specifically occur in drug-treated BJAB/mock cells. These data confirmed that the chosen concentrations of taxol (0.12 µM) and epirubicin (1.85 µM) clearly induce apoptosis.
While the aim of this study was of more general concern it was shown that the reported upstream events of the mitochondrial apoptotic pathway are also characteristic for drug-induced apoptosis in BJAB/mock cells. Western blot analysis revealed that procaspase-9 is processed after drug treatment. This is reported to be mediated by formation of the mitochondrial DISC (Liu, 1996) after release of cytochrome c and depolarization of mitochondria (Bossy-Wetzel et al., 1998). It was therefore tested whether procaspase-9 is processed and depolarization of mitochondria can be detected in drug treated BJAB/mock cells. Western blot analysis confirmed caspase-9 proteolysis and flow cytometric analysis using the JC-1 dye showed significant loss of mitochondrial membrane potential specifically after drug-treatment of BJAB/mock cells reaching up to 60 % - 80 % after 72 hs. Taken together these data provide strong evidence for induction of the mitochondrial apoptotic pathway in BJAB cells upon treatment with taxol and epirubicin at the given concentrations.

VIII-1.2 The CD95/Fas Signalling Pathway

However, the involvement of the CD95/Fas signalling pathway in cytostatic drug-induced apoptosis had to be investigated as the role of CD95/Fas signalling in drug-induced apoptosis is discussed controversely. This is true for T-cell and B-cell apoptosis and it is a matter of debate if death receptor signalling is functionally or even involved at all in drug-induced apoptosis. One reason for controversy might be the origin of the published data with regard to the different cell types and apoptotic stimuli used. An attempt to include the contradictory results in one theory leads to the current model for induction of apoptosis which divides cells in two classes (Scaffidi et al., 1998):

- type I cells, in which apoptosis is mediated via Fas-ligation and subsequent activation of large amounts of caspase-8 directly processing effector caspases.
- type II cells, in which minor amounts of active caspase-8 induce apoptogenic activity of mitochondria, e.g. by cleavage of BID.

Further problems might arise from other characteristics of the established model systems. Drug-resistance, for example, just describes the phenomenon and can be mediated by overexpression of detoxifying proteins (MDR-type), overexpression of anti-apoptotic proteins, functionally inactive pro-apoptotic proteins or other mutations or deletions in yet unknown proteins associated with the apoptotic machinery. In line with this, it was also investigated whether CD95/Fas signalling is involved in taxol- and epirubicin-induced apoptosis in BJAB cells. This was necessary because death pathways in B-lymphoma cell lines, unlike in T-cells, have been less intensively characterized. BJAB cells do not express significant amounts of CD95 ligand mRNA after challenge with the cytotoxic drug epirubicin (Daniel, PT, unpublished data). This might indicate the exclusion of CD95/Fas induced apoptosis via paracrine or autocrine Fas-signalling. Additionally, epirubicin-induced cell death was not influenced by antagonistic anti-CD95 antibodies (Daniel, PT, unpublished data). By this means, BJAB cells represent a suitable model system to investigate CD95-independent drug-induced apoptosis and further manipulation, e.g. generation of restistant clones, is not necessary.

As described, cytostatic drug treatment of BJAB/mock cells induces several characteristics of the mitochondrial apoptotic pathway. Nonetheless, as evidence for cross-talk between the two main apoptotic pathways exists, it could not be excluded that the primary cause for apoptosis is FasR ligation. Activation of the CD95/Fas pathway leading to cleavage of procaspase-8 after recruitment to the CD95/Fas DISC was investigated by Western blot analysis of caspase-8. Surprisingly, caspase-8 processing was clearly detected after challenging BJAB/mock cells with taxol or epirubicin. Since caspase-8 is reported to be the predominant initiator caspase in CD95/Fas-mediated apoptosis further investigation had to be made and experiments using BJAB cells stably transfected with a dominant negative mutant of FADD, FADDdn, were performed. FADD is the

adaptor molecule necessary for formation of the CD95/Fas DISC and associates with the trimerized FasR (Walcac and Krammer, 2000) thereby recruiting caspase-8. Similar to caspase-9 in the mitochondrial DISC caspase-8 autoproteolyses and active caspase-8 heads for effector caspases to start the apoptotic machinery.
The results obtained in FADDdn-transfected cells were similar to those derived from BJAB cells. DNA fragmentation, caspase-8 and caspase-3 processing and loss of mitochondrial membrane potential was readily detected using the suitable assays. This shows that FasL signalling, either autocrine or paracrine, is not involved in drug-induced apoptosis in BJAB cells. No significant differences in the drug-induced apoptosis pathway in mock transfectants compared with FADDdn transfected cells could be detected and CD95/Fas signalling therefore can not be necessary for induction of apoptosis by cytostatic drugs in this experimental setup.

VIII-1.3 Caspase-8 as Downstream Target

Caspase-8 activation after drug treatment of BJAB cells apart from CD95/Fas signalling was shown for the first time. However, the presented data clearly confirmed results showing that Fas-resistent Jurkat T cells are still succeptible to drug-induced apoptosis (Wesselborg et al., 1999). Nevertheless, further experiments were carried out to elucidate the circumstances of caspase-8 processing in drug-induced apoptosis. It is reasonable to assume that caspase-8 is processed as downstream target in the mitochondrial apoptotic pathway, probably leading to an amplification loop in the caspase cascade. To address this question, BJAB cells were treated with the respective cytostatic drugs in the presence or absence of the specific caspase inhibitors Z-DEVD-fmk and Z-WEHD-fmk. As a positive control PARP cleavage was shown to be substantially inhibited by Z-DEVD-fmk whereas Z-WEHD-fmk showed no effect on PARP cleavage indicating that Z-DEVD-fmk clearly blocks caspase-3-like cleavage activity. In line with the hypothesis of caspase-8 processing as a downstream event, Z-DEVD-fmk also blocked formation of the 18 kDa mature subunit of caspase-8 after drug-induced apoptosis in BJAB/mock cells. This clearly indicates that caspase-3-like activity is necessary for caspase-8 processing in drug-induced apoptosis of BJAB cells. Furthermore, it can be reasoned that caspase-8 and caspase-3 are cytoplasmatic proteins and this cleavage is in line with experiments performed by Slee et al (Slee et al., 1999). Within their publication, Slee et al. report caspase-8 processing downstream of caspase-3, at least in T cell extracts *in vitro*.
But, as caspase inhibitors are specific rather than selective, this positioning of caspase-8 downstream of caspase-3 in drug-induced apoptosis of BJAB/mock cells had to be confirmed. This was done using a cell free extract assay system. In this system, the mitochondrial apoptotic cascade can be induced by addition of cytochrome c and dATP leading to formation of the mitochondrial DISC and subsequent caspase-9 and

caspase-3 activation. In this experimental setup, a time-course experiment revealed that caspase-8 processing and activation followed a significant slower kinetic than caspase-3 activation. This shows that a) procaspase-8 is processed after mitochondrial induction of the caspase cascade in the cell free system, b) this processing is accompanied by caspase-8-like cleavage activity and c) because of the slower kinetics caspase-8 activity might follow caspase-3 activation. In another experiment procaspase-3 was immunochemically depleted from the cell free extract prior to activation of the caspase cascade. As assumed caspase-3-like cleavage activity in the depleted extracts was significantly reduced showing that caspase-3 removal was succesfull. Moreover, caspase-8 processing was diminished. This shows that processing of procaspase-8 in cell free extracts relies on active caspase-3, confirming the observation in drug-treated BJAB/mock and BJAB/FADDdn cells.

VIII-1.4 Procaspase-8 Processing in NALM6 and REH Cells

As all the experiments were performed in only one cell line the question arose whether procaspase-8 processing after drug-induced apoptosis is a general phenomenon or is just restricted to BJAB cells. In order to answer this question, two B-lymphoma cell lines, namely NALM6 and REH, were used to examine the effects of drug treatment in these cells. It was shown that, after challenged with taxol or epirubicin, both cell lines show significant DNA fragmentation confirming induction of the apoptotic machinery. Loss of mitochondrial membrane potential after drug treatment was also assayed and clearly detected as soon as 24 hs after application of the respective cytostatic drugs. Procaspase-8 processing in NALM6 and REH cells after treatment with 0.12 µM taxol and 1.85 µM epirubicin after 24 hs and 48 hs was investigated by Western blot analysis. The mature p18 subunit of caspase-8 was detected in REH cells after 24 hs exposure to epirubicin and after 48 hs exposure to taxol. NALM6 cells were less susceptible to drug-induced caspase-8 processing and the 18 kDa subunit was only detected after 48 hs of epriubicin treatment. This reflects the results obtained in a caspase-8 activity assay using the caspase-8-specific colorimetric substrate Ac-IETD-pNA. Caspase-8 activity was clearly detected in REH cells treated with epirubicin for 24 hs and with taxol for 48 hs. NALM6 did not show enhanced caspase-8 activity as compared with controls at these points of time. This confirms the correlation of caspase-8 processing detected in Western blot analysis with Ac-IETD-pNA cleavage activity. However, processing of procaspase-8 after drug-induced apoptosis in NALM6 and REH was hereby confirmed as both cell lines showed appearance of the 18 kDa subunit of caspase-8 after epirubicin-induced apoptosis, although taxol-induced procaspase-8 processing was less efficient.

VIII-1.5 Future Considerations

Although active caspase-3 can not be rendered directly responsible for caspase-8 processing in the *in vitro* mitochondrial apoptotic cascade it is obviously necessary for downstream processing of procaspase-8. Nevertheless, additional experiments should elucidate the apoptotic cascade in BJAB cells in more detail. For example, it should be investigated whether caspase-3 itself or caspase-6 or caspase-7 mediate procaspase-8 processing. Using specific inhibitors it should be possible to investigate the substrate specificity of the acting caspase, Z-VEHD-fmk for caspase-6 and Z-DEVD-fmk for caspase-3 and -7. Unfortunately, caspase-3 and -7 display the same preferred cleavage sequence and this experiment would only allow differentiation between caspase-6 and caspase-3 or caspase-7. Even determination of the exact cleavage site in caspase-8 would not be sufficient. It can be speculated that caspase-7 is unlikely to cleave caspase-8 because caspase-7 in cells undergoing apoptosis is located in the microsomal fraction (Zhivotovsky, 1999). Furthermore, immunoprecipitation of caspase-3 prior to activation of the mitochondrial apoptotic cascade *in vitro* blocked caspase-8 processing whereas caspase-7 is activated in parallel with caspase-3 *in vitro*. On the basis of the performed experiments it can be concluded that processing of caspase-8 after drug-induced apoptosis is most likely mediated by caspase-6 as shown *in vitro* by Slee et. al. (1999).

It would be interesting to further investigate upstream events in drug-induced apoptosis, e.g. BID cleavage or even signalling proteins located upstream of mitochondrial depolarization, as well as downstream events, e.g. other caspases, not involved in mitochondrial induced apoptosis, or caspase substrates. Additionally, a caspase-8 deficient cell line or functionally inactive mutant of caspase-8 could provide evidence for implications of caspase-8 activation after drug-induced apoptosis, although this could also be adressed by the use of Z-IETD-fmk as a specific caspase-8 inhibitor.

VIII-2 D4-GDI in Drug-induced Apoptosis

VIII-2.1 Discussion of Presented Results

Within this study D4-GDI was identified to be specifically proteolyzed by caspase-3 after drug treatment of BJAB/mock cells with taxol or epirubicin. This was achieved in the following order: First, significant decrease in intensity of the spot representing D4-GDI in BJAB/mock cells incubated in the presence of taxol or epirubicin was detected after high resolution 2DE PAGE. The identitiy of this spot was examined using mass spectrometric analysis and data bank search and identified as D4-GDI. Then, conventional Western blot analysis of samples from a time course experiment revealed the appearance of a distinct band with a lower apparent molecular weight upon drug treatment of BJAB/mock cells. In order to elucidate the identity of this band, Western blot analysis

of protein samples separated by 2DE PAGE was performed resulting in detection of three spots. According to the specificity of the used Ab one of these spots was assumed to represent D4-GDI, as already identified by mass spectrometry, another immunoreactive spot was assigned to rho-GDI 1 and the third spot should represent a fragment of either rho-GDI 1 or, more likely, of D4-GDI, as D4-GDI was already detected to display decreased concentration upon drug treatment. Computer aided image processing allowed correlation of results from Western blot analysis and 2DE PAGE and lead to the identification of the spot representing rho-GDI 1. Furthermore, the observed fragment was definitively assigned to D4-GDI as shown by mass spectrometric analysis. This showed clearly, that D4-GDI is specifically proteolyzed during drug-induced apoptosis of BJAB/mock cells. Additionally, as no significant decrease in the intensity of the spot representing rho-GDI 1 was detected, degradation of rho-GDI 1 during apoptosis was excluded. Despite high homology to D4-GDI, rho-GDI 1 seems to be cleavage resistant thereby confirming that the apoptotic machinery selectively targets different proteins in the cell. Nonetheless, caspase-3 (Na et al., 1996) and caspase-1 (Danley et al., 1996) had been reported to mediate D4-GDI cleavage during apoptosis. Thus, it was further investigated whether caspase-1 or caspase-3 was responsible for appearance of the D4-GDI fragment. Western blot analysis was performed to examine activation of caspase-1 upon challenge of BJAB/mock cells with taxol or epirubicin. Although decrease of procaspase-1 in this samples was detected no significant increase of the band detected with the Ab specific for the mature subunit of caspase-1, p20, was seen. Additionally, caspase-1 like cleavage activity was neither detected in drug-treated BJAB/mock cells nor in cell free extracts activated by addition of cytochrome c, indicating that caspase-1 is not activated in the mitochondrial apoptotic cascade. This is in accordance with the *in vitro* ordering of the caspase cascade by Slee et al.(Slee et al., 1999) *In vitro* activation of extracts clearly showed specific cleavage of D4-GDI thereby providing evidence for caspase-3-mediated cleavage of D4-GDI. Taken together, these results strongly indicated caspase-3-mediated cleavage of D4-GDI in BJAB/mock cells challenged with taxol or epirubicin and in cell free extracts activated by addition of cytochrome c and dATP. This assumption was further confirmed using the caspase-3 and caspase-1 specific peptide inhibitors Z-DEVD-fmk and Z-WEHD-fmk. In these experiment exposure of BJAB/mock cells to the caspase-3 specific inhibitor during drug-treatment resulted in significant decrease in intensity of the D4-GDI-fragment band in contrast to exposure to the caspase-1-specific inhibitor Z-WEHD-fmk. Furthermore, immunochemical depletion of cell free extracts using a caspase-3 specific Ab and application of the caspase-3-specific colorimetric substrate as competitive inhibitor in *in vitro* experiments diminished D4-GDI cleavage. These results clearly indicate caspase-3-mediated cleavage of D4-GDI in BJAB/mock cells after exposure to cytostatic drugs and upon *in vitro* activation of the mitochondrial apoptotic

cascade. Finally, mass spectrometric analysis of the resulting D4-GDI fragment revealed the presence of the peptide containing the published caspase-1 cleavage site thereby undoubtedly demonstrating caspase-3 as the acting protease. In detail, caspase-1 cleaves D4-GDI at aa-position 59 whereas caspase-3 acts on aa-position 19 and the D4-GDI fragment represents the C-terminal part of the protein. Thus, the presence of the peptide containing the caspase-1 cleavage site excludes caspase-1 mediated proteolysis of D4-GDI.

VIII-2.2 Implications of D4-GDI cleavage in Drug-induced Apoptosis

Cleavage of the GDP dissociation inhibitor D4-GDI has been demonstrated in CD95/ Fas-induced (Na et al., 1996), anti-IgM mediated (Rickers et al., 1998), etoposide- and staurosporine-induced (Krieser and Eastman, 1999) apoptosis and the relevance of this cleavage for cytoskeletal changes has been discussed. Three GDP dissociation inhibitors are known so far: rho-GDI 1, rho-GDI 2 (D4-GDI), and rho-GDI 3. Rho-GDI 3 is not expressed in haematopoietic tissues in contrast to rho-GDI 1 and D4-GDI. Rho-GDI and D4-GDI both are reported to associate with cdc42Hs, rhoA, rac1 and rac2 *in vitro* but in contrast, after microanalytic liquid chromatography of human myelomonocytic cytosol none of this proteins was found in the 45 kDa - 50 kDa complex containing D4-GDI (Gorvel et al., 1998). This indicates that D4-GDI primarily acts on a yet unidentified protein. Interestingly, the existence of two GTPases belonging to the rho subgroup, TTF (Dallery et al., 1995) and rhoE (Foster et al., 1996), has been described. At least TTF interaction with D4-GDI is reasonable because TTF is expressed in a restricted manner in hematopoietic cells (Dallery et al., 1995) and D4-GDI can solubilize overexpressed TTF from membranes (Gorvel et al., 1998). But detailed knowledge about D4-GDI regulating activities remains elusive.

However, rho-GTPases are involved in the regulation of integrin activity and in the organization of the actin cytoskeleton (Hall, 1994, Narumiya, 1996) and three types of regulator proteins are known to act on the different rho-GTPases: GTPase-activating Proteins (gaps), guanine-nucleotide exchange factors (gefs) and GDP-dissociation inhibitors (GDIs). Additionally, at least for the rab-cycle, a protein has been discovered that mediates dissociation of the GDI from the GTP-binding protein, called GDF (GDI dissociation factor). Although the complex signalling pathways and molecular interactions within the family of small GTP-binding proteins has not yet been completely elucidated, the small GTP binding proteins have in common, that they act as molecular switches. The GTP-bound state is the ’active’ state and they are ’inactive’ in the GDP-bound state. This is especially true for ras which is involved in cell-cycle regulation. What impact D4-GDI cleavage has on regulation of the cytoskeleton is not clear. It has been shown that the N-terminal domain of rho-GDI 1 blocks exchange of guanosine-nucleotide whereas interaction with rho-proteins is restricted to the C-terminal part. De-

letion of the first 42 aa in rho-GDI 1 lead to abrogation of its ability to inhibit GDP exchange and release of cdc42 from membrane bilayers whereas deletion of the first 25 aa showed no effect. Correlated with D4-GDI, cleavage by caspase-1 corresponds to deletion of the larger aminoterminal part leading to defective GDP-exchange regulation and cleavage by caspase-3 can be compared to deletion of the first 25 aa in rho-GDI 1. Interestingly, the D4-GDI fragment resulting from caspase-3 mediated cleavage is reported to translocate to the nucleus but overexpression of an uncleavable mutant did not show any influence on cell death as determined by staining with Hoechst 33342 (Krieser and Eastman, 1999). Therefore, further studies to investigate the role of D4-GDI and its cleavage during apoptosis should be performed, because it is unlikely that specific cleavage and translocation of the D4-GDI fragment to the nucleus is just an epiphenomenon. Especially the influence of GDI-cleavage on the organisiation of the cytoskeleton should be investigated on basis of the facts given above.

VIII-2.3 The Cytokine Reponse Modifier A (crmA)

Experiments were also performed with BJAB cells stably transfected with the cytokine response modifier A. CrmA belongs to the serpin family of protease inhibitors (Pickup et al., 1986) and has been shown to block mature caspase-8 and caspase-1 activity (Tewari et al., 1995). Therefore, it was first surprising to detect strong effects of crmA on cytostatic drug-induced apoptosis in BJAB/crmA cells as compared to vector control transfected cells although caspase-1 activation was excluded (see above) and caspase-8 activation has also been shown to be a downstream event in this experimental setup. However, speculations can be made regarding this outcome. On the one hand these results rise the possibility that crmA also significantly influences activity of caspases involved in the mitochondrial apoptotic pathway, although the inhibitory constant for inhibition of caspase-9 activity is about two orders of magnitude higher than that for inhibition of caspase-8 (Ekert, 1999). On the other hand, it might be concluded that caspase-8 activation, although representing a downstream event in drug-induced apoptosis of BJAB cells, is important for progression of the apoptotic program for example by leading to additional caspase-3 activation in an amplification loop. Nevertheless, with respect to the presented results it can not be concluded conceivably whether crmA blocks the mitochondrial apoptotic pathway by inhibition of mature caspase-9 or caspase-3 itself or by abrogation of an amplification loop due to downstream processing of caspase-8. This question could be answered by the use of caspase-8 specific inhibitors or BJAB cells overexpressing an inactive mutant of caspase-8. Additionally, *in vitro* experiments could be performed using cell extracts immunochemically depleted of procaspase-8 prior to activation. This would further elucidate the influence of caspase-8 processing on downstream events in the mitochondrial apoptotic cascade.

VIII-3 APOPTOSIS ASSOCIATED PROTEINS

Using high resolution 2DE PAGE some apoptosis associated proteins have been identified using subtractive analysis within this study. Procaspase-3 concentration is shown to decrease after drug-induced apoptosis of BJAB/mock cells which is consistent with data presented in the literature and this study. Moreover, specific cleavage of D4-GDI, as shown by decreased intensity of the spot representing D4-GDI and increased intensity of the resulting fragment, was detected in high resolution 2DE PAGE gels (as already discussed above). The remaining proteins, which have been detected by subtractive analysis of gels derived from drug-treated and untreated BJAB/mock cells are discussed in detail below. These are β-Actin, which has also been reported as a caspase-3 substrate during apoptosis, cyclophilin A (cypA), t-complex binding protein subunit δ (TCPD), desoxyuridine 5'-triphosphate nucleotide hydrolase (DUT), nucleolin and RNA polymerase B transcription factor 3 (BTF3).

VIII-3.1 PROCASPASE-3 (ICE3)

Although processing and activation of caspase-3 as the major effector caspase is well documented the identification of the spot #32 representing procaspase-3 in high resoltution 2DE PAGE gels is a great advantage for future investigations. Most of the marker proteins associated with apoptosis identified on high resolution 2DE gels so far represent caspase substrates and are therefore presumably located downstream of effector caspases in the apoptotic cascade. Caspase-3 activation is at least one step upstream of degradation of several of the already identified caspase substrates and decreased spot intensity of the spot representing procaspase-3 in 2DE PAGE gels now can be interpreted as induction of apoptosis in these cells. Moreover, it now might be possible to discriminate between apoptosis related variation of spot intensity in general and changes accompanied by decrease of procaspase-3 concentration. This gives further information about the mechanisms responsible for changes in spot intensity detected by subtractive analysis.
However, a procaspase-3 concentration above the detection limit of analytical silver-staining is a precondition for this implications.

VIII-3.2 β-ACTIN (ACTB)

The cytoskeleton consists of microtubules (α- and β-tubulin), actin filaments (β- and γ-actin) and intermediate filaments. Actin filaments are located within the plasmamembrane and mediate changes of plasma membrane shape together with actin-binding proteins such as myosin (Michal, 1999).
In 2DE PAGE gels two spots were identified as actin fragments, either N-terminal or C-terminal. The appearance of the C-terminal fragment (spot #6) was readily detected in lysates derived from cytostatic drug-treated BJAB/mock cells after 2DE PAGE and af-

ter induction of the mitochondrial caspase cascade in cell-free extracts. This indicates that formation of the C-terminal actin fragment is located downstream of caspase activation and caspases therefore represent the enzyme family responsible for actin proteolysis. Proteolysis of β-actin after drug-induced apoptosis in BJAB/mock cells is in accordance with data presented in the literature. Apoptosis is accompanied by dramatic reorganization of the cytoskeleton, which is followed by morphological changes and cellular fragmentation (Brancolini et al., 1995). It has been suggested that the microfilament is involved in morphological changes in apoptosis by some inhibitor studies (Cotter et al., 1992). Several cytoskeletal proteins (such as Gas2, gelsolin, β-catenin, fodrin, actin, PAK-2) are reported to be proteolytically cleaved by caspases during apoptosis (Brancolini et al., 1995, Brancolini et al., 1997, Cryns et al., 1996, Kayalar et al., 1996, Kothakota et al., 1997, Martin et al., 1995, Mashima et al., 1995, Rudel and Bokoch, 1997). However, in some systems, the morphological changes were shown to be independent of the apoptotic proteases (McCarthy et al., 1997, Mills et al., 1998, Toyoshima et al., 1997). Mashima et al. showed that cytoskeletal actin is the substrate of caspases (Mashima et al., 1995; Mashima et al., 1995). Furthermore, they report on the induction of morphological changes by expression of the 15 kDa C-terminal fragment of β-actin (Mashima et al., 1999).

The detection of a N-terminal fragment at slightly higher apparent molecular weight (spot #11) is restricted to samples derived from *in vitro* activation of cell extracts. This is in clear contrast to *in vivo* proteolysis of β-actin where it is shown that cleavage only results in the detected 15 kDa C-terminal fragment and a 31 kDa N-terminal fragment (Mashima et al., 1999). These data might reflect that *in vitro* activated extracts are an artificial system which does not reflect exactly the *in vivo* situation during apoptosis. Additional cleavage of the 31 kDa fragment during *in vitro* activation might arise from membrane disruption during extract preparation. However, another conclusion is, that *in vitro* cleavage of proteins by distinct caspases at distinct cleavage sites might not always occur *in vivo.* Thus, experiments carried out with purified recombinant proteins, which are even more artificial than experiments using cellular extracts, should always be confirmed under *in vivo* conditions.

VIII-3.3 CYCLOPHILIN A (CYPH)

Cyclophilin was first identified as the intracellular receptor for the immunosuppressant drug cyclosporin A (Handschumacher et al., 1984). Cyclophilins comprise a highly conserved, ubiquitous family of proteins and range in size from 15 kDa to 40 kDa (Kieffer et al., 1992, Hornbogen and Zocher, 1995). These proteins are localized to the cytoplasm (Handschumacher et al., 1984), mitochondria (Rassow et al., 1995) the nucleus (Ryffel et al., 1991, McDonald et al., 1992) and endoplasmic reticulum (Price et al., 1991). Various activities have been reported for cyclophilins, including action as chaperones (Freskgard et al., 1992, Baker et al., 1994), as chemotactic agents (Xu et al., 1992) and as stress response proteins (Sykes et al., 1993, Marivet et al., 1994). Moreover, cyclophilin A is reported to bind to the p55 gag protein of human immunodeficiency virus (Luban et al., 1993) and appears to be necessary for successful infection (Thali et al., 1994, Franke et al., 1994). Additionally Montague et al (Montague et al., 1994) supposed a nuclease activity associated with the cyclophilin proteins based on sequence and structural similarities between nuc18 and the cyclophilin family. This assumption was later experimentally confirmed (Montague et al., 1997).
In this context, it is interesting that after drug-induced apoptosis an additional spot representing cyclophilin (spot #13) had been detected on 2DE PAGE gels. This spot is located at the same apparent molecular weight as compared with the two high abundant spots also representing cyclophilin A (Otto et al., 1998).

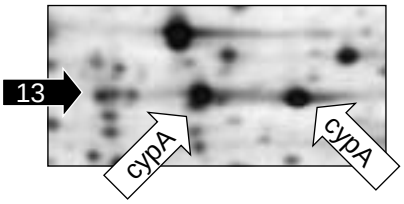

Fig. 52: Cyclophilin A in 2DE PAGE gels of apoptotic cells. The position of cyclophilin A on 2DE PAGE gels is indicated as well as the position of spot #13 specifically appearing after drug-induced apoptosis of BJAB/mock cells (compare Fig. 39). cypA = cyclophilin A.

This is in agreement with a supposed alteration of the post-translational modification in cyclophilin A during apoptosis which is probably necessary for activation of the DNase activity (Montague et al., 1997). However, the exact kind of post-translational modification has not been unraveled yet. Mass spectrometric analysis of spot #13 might thus reveal the presence of a post translational modification in cyclophilin A upon drug treatment. Another possibility is loss of a distinct modification which might be present in the two other spots representing cyclophilin A. However, this supposed modification is far more reasonable than proteolysis by caspases while spot #13 was not detected in 2DE PAGE gels derived from *in vitro* activated extracts of BJAB/mock cells (see Fig. 39).

VIII-3.4 T-COMPLEX BINDING PROTEIN SUBUNIT δ (TCPD)

Spot #28 was identified as tcpd (t-complex binding protein, subunit δ) located at an apparent molecular weight of ~25 kDa. This is in strong contrast to the theoretical molecular weight calculated from the aa-sequence, which is 57.8 kDa (swissprot: p50991). It is therefore assumed that spot #28 represents a fragment of the δ-subunit of the t-complex binding protein. Intensity of the spot representing this fragment specifically increases after drug-induced apoptosis in BJAB/mock cells as compared with the intensity of surrounding spots. An increased intensity of this spot is also observed upon caspase activation in cell free extracts after addition of cytochrome c and dATP (see Fig. 38). This indicates that formation of the tcpd-fragment is an apoptosis-related event and results from caspase activation. Mass spectrometric analysis of the spot exclusively detected N-terminal peptides (representing aa-residues 66-79, 80-89, 161-174 and 194-206). This suggeests that the assumed proteolytic cleavage occurs upstream of aa-position 206 because identified peptides.

Tcpd belongs to the tcp-1 family of proteins which act as molecular chaperones for tubulin, actin and probably some other proteins (Rommelaere et al., 1993). These proteins are significantly related to the groEL chaperonin family, which is known to assist in protein folding in an ATP-consuming manner. However, not much is known about these proteins and therefore it can only be stated that appearance of the tcpd-fragment on the basis of the given data is coupled to drug-induced apoptosis in BJAB/mock cells as well as induction of the mitochondrial caspase cascade in cell free extracts of these cells. With regard to the endorsed function of tcp-1 family proteins its proteolytic degradation during apoptosis might play a role for a) the reorganization of the cytoskeleton (Brancolini et al., 1995) and b) the degradation of ATP-consuming proteins (e.g. PARP).

VIII-3.5 DESOXY URIDINE 5'-TRIPHOSPHATE NUCLEOTIDE HYDROLASE (DUT)

The nuclear isoform of dut (desoxyuridin 5' triphosphate nucleotide hydrolase) is represented by spot #17. This spot displayed decreased intensity after drug treatment of BJAB/mock cells but no significant changes were detected in *in vitro* acitvated cell free extracts in comparison with non-activated extracts (Fig. 36). On this basis, it is appropriate to assume that decrease occurs due to degradation rather than proteolysis by caspases. Nevertheless, in the context of apoptosing cells, degradation of DUT does make sense.

dUTPase removes dUTP from the DNA biosynthetic pathway by catalysing its hydrolysis to dUMP and PPi. Simultaneously, this reaction provides dUMP for de novo synthesis of thymidylate (Lindahl, 1982). dUTPase is important for prokaryotic, eukaryotic and viral systems while it is essential for the viability of E.coli and S.cerevisiae and efficient DNA replication in viral systems (el-Hajj et al., 1988; Gadsden et al., 1993; Pyles

et al., 1992; Threadgill et al., 1993). Furthermore, dUTPase provides dUMP for the thymidylate synthesis by thymidylate synthase (TS) and blocking of this enzyme results in accumulation of excess dUTP (Beck et al., 1986; Curtin et al., 1991; Ingraham et al., 1986; Sedwick et al., 1981). This leads to misincorporation of dUMP into DNA during DNA replication and repair, resulting in DNA fragmentation and subsequent cell death (Curtin et al., 1991).

Taken together degradation of DUT once more reflects the general character of apoptosis, but in a slightly different manner. Since DUT appears to be degraded rather than specifically proteolyzed it can be argued that DUT protein concentration is no longer maintained in apoptosing cells. DUT mRNA levels and protein content are tightly regulated and coincide with nuclear DNA replication status (Ladner and Caradonna, 1997). Degradation of DUT should therefore be a fast and continuous process. This process of degradation presumably is maintained during apoptosis whereas biosynthesis of DUT protein is no longer initiated. These two conditions in combination may lead to the observed decrease in DUT content in cells undergoing apoptosis.

VIII-3.6 NUCLEOLIN (NUCL)

Spot #12 was identified as nucleolin by sequencing two peptides located in the C-terminal part of the full length protein. As the calculated molecular weight for nucleolin is ~77 kDa and the examined spot is located in the low molecular weight part of the 2DE PAGE gels the identified spot clearly represents a fragment of this multifunctional protein (apparent MW < 15 kDa). Comparison of protein patterns derived from separated samples of drug-treated BJAB/mock cells and *in vitro* activated cell free extracts shows that this fragment not only appears upon drug-induced apoptosis but additionally after *in vitro* initiation of the mitochondrial caspase cascade. This is important with respect to the fact, that nucleolin inherits an autoproteolytic activity (Srivastava and Pollard, 1999). Specific increase of the nucleolin-fragment content after *in vitro* activation indicates that this event is mediated or initiated by caspase activation.

Nucleolin is a high abundant nuclear, non-histone phosphoprotein (Bugler et al., 1982; Shaw and Jordan, 1995) and active nucleolin comprises about 5 % of nuclear protein in dividing eukaryotic cells (Srivastava and Pollard, 1999). It exerts numerous distinct biochemical activities including organization of nuclear chromatin (Erard et al., 1990), packaging of preRNA, transcription of rDNA (Borer et al., 1989; Bouche et al., 1987; Caizergues-Ferrer et al., 1989; Herrera and Olson, 1986; Jordon, 1987) and ribosome assembly (Jordon, 1987). In addition, nucleolin exhibits autodegradation (Chen et al., 1991; Fang and Yeh, 1993; Lapeyre et al., 1987; Olson et al., 1990), DNA and RNA helicase activities (Tuteja et al., 1995) and DNA-dependent ATPase activity (Tuteja et al., 1997). Nucleolin belongs to a large family of RNA binding proteins including heterogenous nuclear ribonucleoproteins (hnRNPs), mRNA polyadenylate binding protein

(SS-B), small nuclear RNPs (Chan et al., 1989) and human alternative splicing factors ASF and SF2 (Ge et al., 1991; Krainer et al., 1991). For hnRNP C1/C2 which is related to nucleolin and which also is involved in pre-mRNA processing, specific cleavage has been reported (Wang et al., 1996; Waterhouse et al., 1996). Nucleolin is physiologically cleaved by granzyme A. Truncated nucleolin then activates autolytic endonucleases finally leading to DNA fragmentation (Arends et al., 1990, Smith et al., 1994). The specific cleavage of nucleolin after drug-induced apoptosis is further underlined by the identification of nucleolin to be associated with anti-IgM induced apoptosis in another human Burkitt-lymphoma cell line (Brockstedt et al., 1998). In proliferating cells nucleolin content is regulated by the expression of a proteolysis inhibiting protein that prevents self-degradation (Chen et al., 1991). It therefore might be possible that the caspases do not directly act on nucleolin but proteolyse this inhibitor.

VIII-3.7 RNA Polymerase B Transcription Factor 3 (BTF3)

One of the spots most sensitive to induction of apoptosis was identified to represent the RNA polymerase transcription factor 3 (BTF 3, spot #16, Fig. 39). This protein migrates on 2DE gels at ~17 kDa. In response to drug treatment of BJAB/mock cells this protein spot nearly disappears. The same is true for BTF3 concentration in 2DE PAGE gels derived from *in vitro* activated extracts of BJAB/mock cells. In conclusion, this response is presumably due to proteolytic cleavage of BTF3 by caspases. This is in accordance with published data (Brockstedt et al., 1999) in which it was shown that BTF3 is specifically degraded after anti-IgM induced apoptosis in a subclone of the human B-cell lymphoma cell line BL-60. Data presented in this study additionally assigns this degradation to accompany mitochondrial activation of the caspase cascade rendering one of the caspases activated after *in vitro* activation responsible for BTF3 degradation, either caspase-9 or one of the effector caspases. Studies on a) which caspase mediates degradation of BTF3, b) what the resulting fragments are and c) what consequences rapid degradation of BTF3 bears for apoptotic cells would be promising aims for further investigations.

VIII-4 General Comments about 2DE PAGE and Protein Identification

2DE PAGE is a widely used method for the separation of complex protein mixtures. Especially due to the commercial availability of 2DE PAGE systems and precast gels, it has become a famous technique in diversive fields of life scienes. Because of its ability to resolve more than 10000 polypeptides (Klose, 1995) in one integrated method, 2DE PAGE has also become the heart of proteome analysis. Although 2DE PAGE probably represents the most powerful technique for protein separation it also bears some severe obstacles. These obstacles need to be overcome in the future to make it a commonly used procedure leading to results which are comparable among different laboratories. Some of these problems also arose during the investigations presented above and are discussed in detail below. These include sample preparation, gel preparation, reproducibility and sensitivity. In this study exclusively the system purchased by WITA GmbH was used and therefore references to other 2DE PAGE systems are merely of theoretical nature.

Although some problems arose concerning the reproducibility of 2DE PAGE gels this method displays numerous advantages which make it an outstanding technique. Besides the high resolution power it can be combined with Western blot analysis. This allows application of 2DE PAGE to answer questions of general concern as well as specific questions, as done in this work by immunochemical detection. In a specific kind of proteomic approach it was investigated whether proteins are expressed/degraded specifically after drug-induced apoptosis. Some of these proteins, which mostly are assumed to be caspase substrates, have been identified. In a more specific case, the identity of an immunoreactive band was unraveled using 2DE PAGE. Although extensive computer aided image processing had to be performed to correlate results obtained by Western blot analysis with those obtained by silverstaining of 2DE PAGE gels applicability of this approach was clearly demonstrated. As a further result it can be suggested that in the first and second dimension PAGE problems arise from the lability of the polyacrylamide matrix. Swelling and shrinking of the second dimension during the blotting or staining procedure should be minimized probably by using an inert, flexible and to some extent permeable support, e.g. a nylon mesh or membrane. This would probably abolish changes in gel-size during these procedures and increase reproducibility and gel matching.

Some kind of flexible support would also be helpful in handling the first dimension. IEF-gels consist of 4 % acrylamide and handling must be performed with much care. In most cases, changes in gel length can not be prevented. Since this stretching during transfer to the second dimension SDS-PAGE gel is not proportional over the whole gel length it results in varying spot patterns. This, in turn, probably represents one of the problems leading to inefficient automatic gel processing.

Further advantages could arise from using ready made staining solutions and an automated gel stainer apparatus. The gel staining procedure according to Heuckeshofen (Heuckeshofen and Dernick, 1985) takes about 3 hs. This is a time-consuming technique and automated staining would not only diminish laborous gel handling but also enhance reproducibility.

Mass spectrometry is one of the most sensitive methods for protein identification and characterisation. However, sample preparation hampers efficient application of this method. Although sensitive staining methods allow detection of spots with very low protein content and this amount of protein would theoretically be enough for mass spectrometric identification this aim often is not achieved. This can be attributed to difficulties in handling samples with low protein content, e.g. adherence of protein to the tubes. Therefore, even lowering the detection limit by application of. radioactive labeling might not be a successful approach to investigate unknown proteins. Even if correlation of autoradiographic images and analytical silverstaining would be achieved a large number of 2DE PAGE gels would have to be performed in order to pool sufficient material for identification. To solve this problem, fractionation of samples prior to 2DE PAGE seems to be the most promising attempt. A suitable fractionation procedure should separate proteins on the basis of characteristics different from pI and molecular weight. Subcellular fractionation would be an encouraging methodology, as well as fractionation on the basis of hydrophobic interactions. Unfortunately, reproducibility of sample preparation would be reduced by this additional steps. Cell sorting still seems to represent another method for fractionation. Although it was stated that enrichment of apoptotic cells did not lead to the desired enhancement of apoptosis-specific differences in 2DE PAGE gels, other characteristics than PS-exposure could be taken advantage of, e.g. loss of mitochondrial membrane potential. Another useful method to obtain specific samples is, of course, immunochemical precipitation of protein complexes.

VIII-4.1 Cytostatic Drug Treatment

However, increased sensitivity might only be necessary with regard to distinct questions. For example increased sensitivity would be of great value to examine drug-induced effects on concentration of regulatory proteins. This is reasonable since standard methodologies didn’t reveal other than downstream apoptosis-related proteins.

Albeit this, regulatory effects probably could be detected by tuning the experimental and not the analytical system. Cytostatic drug-induced biochemical events are mostly coupled to the cell cycle. As compared with more physiological death-inducing stimuli, e.g. anti-IgM or FasL, cytostatic drug treatment takes a long time to induce apoptotic changes. This is one more difficulty in detecting regulatory proteins. The cells have to

pass a certain phase of the cell cycle for the drugs to take effect. Then, presumably repair mechanisms are activated and after those the apoptotic signal is given from the nucleus to the mitochondria. Therefore, several steps of signalling are located between drug-induced damage and onset of the apoptotic events, leading to diversification of the cells' states. Thus, it would be an advantageous endeavor to synchronize the cells prior to cytostatic drug treatment. In this case, momentum concentrations of regulatory proteins should be above those in unsynchronised cells because all cells are captivated in the same phase of the cell cycle. This increased concentration of regulatory proteins would probably be sufficient for detection in 2DE PAGE gels.

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X APPENDIX

X-1 CURRICULUM VITAE

Name:	Frank Eßmann
Date of Birth:	13.12.1971 in Waltrop, Germany
Matriculation:	June 1991, Theodor-Heuss-Gymnasium in Waltrop, Germany
Military Service:	July 1991 - June 1992
Study of Chemistry:	October 1992 - October 1997, Universität Dortmund, Germany
Experimental work:	January 1997 - October 1997 at the Max-Planck-Institut für Molekulare Physiologie, Dortmund, Germany
Diploma in Chemistry:	October 1997
Experimental work on Doctoral Thesis:	since November 1997 at the IGT-Labor/Robert-Rössle-Klinik/Charité, Berlin, Germany and Dep. of Protein Chemistry/Max-Delbrueck-Centrum, Berlin, Germany

X-2 PUBLICATIONS

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Endogenous Expression of Bax as a Regulator of Cellular Drug Sensitivity to Doxorubicin, Cyclophosphamide and Chlorambucile but not Corticosteroids in Chronic Lymphocytic Leukemia. Manuscript in preparation.

X-3 ABSTRACTS/POSTER

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