RESPONSE OF HUMAN LEUKEMIA CELL UPON TREATMENT WITH BIOACTIVE EXTRACTS FROM TROPICAL MEDICAL MUSHROOMS

DISSERTATION

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Dedicated to my family
With love.

„Non sông Việt Nam có trở nên vẻ vang hay không, dân tộc Việt Nam có được vẻ vang sánh vai các cường quốc năm châu được hay không, chính là nhờ một phần rất lớn công học tập của các cháu …“

(„That the name of our country may be better known, that our nation prosperity may be compared with others, is greatly determined by your efforts in widening knowledge…”)

-Ho Chi Minh – Letter to pupils in the beginning of the first school year after The August Revolution-

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List of abbreviations

°C	-	Celsius grade
AO	-	acridine orange
Apaf1	-	apoptotic protease activating factor 1
APS	-	ammonium Persulfate
Bax	-	Bcl-2-associated X protein
Bcl-2	-	B-cell lymphoma 2
Bcl-X_L	-	B-cell lymphoma – extra large
Bcl-w	-	Bcl-2 like 2
BH	-	Bcl-2 homology
Bid	-	BH3 interacting domain death agonist
bidest.	-	bidistilled
BIR	-	baculoviral IAP repeat
bp	-	base pair
BSA	-	bovin serum albumin
C	-	control
cm^2	-	square centimetrer
cm^3	-	cubic centimeter
CPT	-	camptothecin
CyDye	-	cyanine dye
ddH_2O	-	double distilled water
DGCR8	-	DiGeorge syndrome critical region gene 8
DIGE	-	difference gel electrophoresis
DNA	-	deoxyribonucleic acid
DR	-	death receptor
EB	-	ethidium bromide
ECL	-	enhanced chemiluminscence
eIF	-	eukaryotic initiation factor
et al.	-	Lat. *et allii* and others
FACS	-	fluorescent-activated cell sorting
Fas	-	Fas receptor, TNF receptor superfamily, member 6
FasL	-	Fas ligand
FBS	-	fetal calf serum
FLIP	-	FLICE-like inhibitory protein
g	-	gram
G_1/S/G_2/M	-	Gap 1/Synthesis/Gap 2/Mitosis phase
GI_{50}	-	50% growth inhibition
GSH	-	glutathione (reduced state)
GST	-	glutathione S-transferase
GTP	-	guanosine triphosphate
h	-	hour(s)

H1	-	*Phellinus pachyphloeus* aqueous extract
hsa-miR-x	-	miRNA-x from *Homo sapiens*
HtrA2	-	HtrA serine peptidase 2
IAP	-	inhibitor of apoptosis
IEF	-	isoelectric focusing
IgG	-	Imunoglobulin G
kDa	-	kilodalton
M	-	molar
m	-	milli (10^{-3})
µ	-	micro (10^{-6})
mA	-	milliampere
MALDI	-	matrix assisted laser desorption/ionization
Mcl-1	-	myeloid cell leukeamia sequence 1(Bcl-2-related)
mg	-	milligram
µg	-	microgram
min	-	minute (s)
MK	-	marker
ml	-	milliliter
µl	-	microliter
mM	-	millimolar
µM	-	micromolar
MS	-	mass spectrometry
n	-	nano (10^{-9})
NF-κB	-	nuclear factor-kappa B
ng	-	nanogram
NL	-	non-linear
nm	-	nanometer
no.	-	number
nt	-	nucleotide
p53	-	tumour protein 53
PAGE	-	polyacrylamide gel electrophoresis
PARP	-	poly ADP-ribose polymerase
PBS	-	phosphate buffered saline
PCR	-	polymerase chain reaction
pH	-	power of hydrogen – negative logarithm of the concentation of hydrogen ions in solution
PI	-	propidium iodide
pRb	-	retinoblastoma protein
PVDF	-	polyvinylidenedifluoride
QR	-	quinone reductase
qRT-PCR	-	real-time Reverse transcription polymerase chain reaction
RNA	-	ribonucleic acid
RNAse A	-	ribonulease A

RPMI	-	Roswell Park Memorial Institute Medium
RQ	-	relative quantification
RT	-	room temperature
Smac	-	second mitochondria-derived activator of caspases
snRNA	-	small nuclear RNA
TBE	-	Tris-boric acid- EDTA
TNFaR	-	tumour necrosis factor receptor superfamily, member 1A
TOF	-	time of flight
TRAIL	-	TNF-related apoptosis-inducing ligand
UV	-	ultra-violet light
V	-	Volt
v/v	-	volume per volume
w/v	-	weight per volume
XIAP	-	X-linked Inhibitor of apoptosis protein

I. INTRODUCTION

I.1. The formation, prevention and treatment of cancer

I.1.1. General information

Cancer is a leading cause of death worldwide, it accounted for nearly 8 million deaths in 2007 and this number continues rising. Cancer can affect any tissue of the human body at all ages. One defining feature of cancer is the rapid and uncontrolled growth, invasion and spread of abnormal cells. By mortality worldwide, the most frequent types of cancer among males are lung, stomach, liver, colon-rectal, oesophagus and prostate cancer while among females they are breast, lung, stomach, colon-rectal and cervix cancer [WHO fact sheet N ° 297, 2008].

Cancer progression is the consequence of the correlation between genetic and exogenous factors, including: physical carcinogens (such as ultraviolet and ironising radiation), chemical carcinogens (such as asbestos, tobacco smoke, aflatoxin and arsenic) and biological carcinogens (such as infections from certain viruses, bacteria or parasites) [Ames & Gold, 1997]. Knowledge about interventions of cancer prevention, early detection, treatment and care becomes more extensive.

I.1.2. Cancer formation, prevention and treatment

Many different types of cancers are diagnosed. Hanahan and Weinberg suggested there are six capabilities which are shared in common by most if not all types of human tumours (Figure 1): self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [Hanahan & Weinberg, 2000].

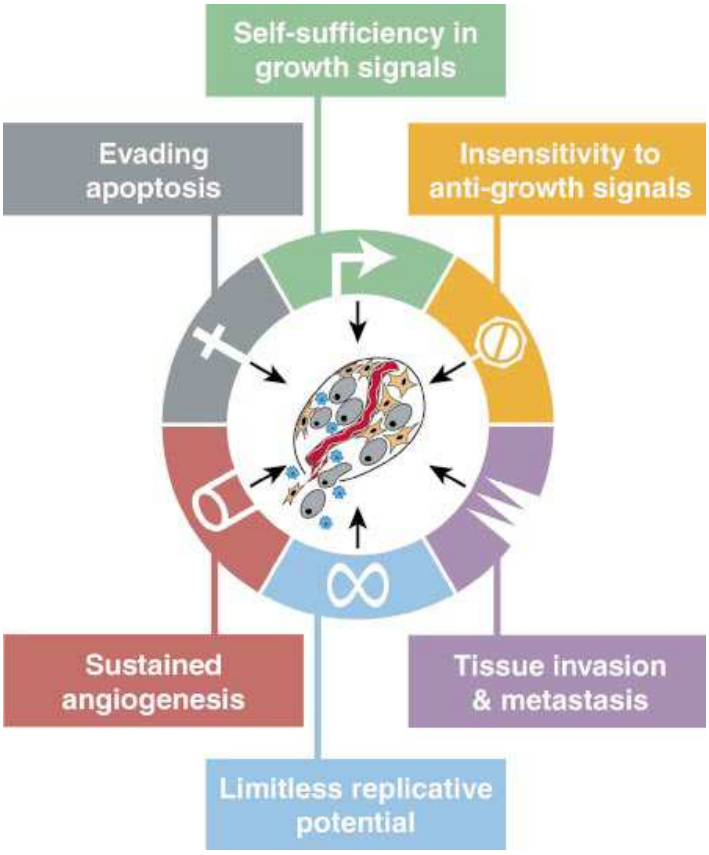


Figure 1: Acquired capabilities of cancer. Most if not all cancers have acquired the same set of functional capabilities during their development, although through various mechanistic strategies. (Adopted from Hanahan & Weinberg, 2000)

The transformation from normal human cells into malignant tumours is a multi-step process. At least three separate stages with certain differences at the molecular and biological level are involved: initiation, promotion and progression [Pitot, 1993]. Under the influence of endogenous and exogenous factors, initiation stage spontaneously results from an irreversible genetic damage. Initiated cells might be repaired or removed through apoptosis before they can evolve into a malignancy. The subsequent stage, promotion, is a reversible step resulting in the development of numerous benign lesions. The progression of benign tumours to malignant cancers occurs irreversibly spontaneously at a low frequency. During progression, additional genetic damage appears and the cell shows characteristics of a fully transformed phenotype, including invasion and metastasis.

Cancer prevention can be divided into three stages: primary, secondary and tertiary. Primary prevention refers to the complete prevention through inhibition of exposure to carcinogenic factors. Secondary prevention involves early detecting and the appropriate retarding the

disease progress after diagnosis. After a suitable therapy, tertiary prevention represents an attempt to prevent local recurrences, invasion and metastasis [De Flora *et al.*, 2001].

Chemoprevention of cancer is a means of cancer control in which the occurrence of the end stages of cancer (invasion and metastasis) are prevented by administration of one or several natural or synthetic compounds [Sporn & Suh, 2000]. Chemopreventive interventions with agents targeted in order to block, arrest or even reverse specific stages of carcinogenic process is really a promising strategy for cancer prevention. Natural products which have multiple mechanisms of action became a vast source of potent chemopreventive agents [Shureiqi *et al.*, 2000].

Nowadays a variety of therapeutic options for cancer are available. Besides standard modalities, including surgery, chemotherapy and radiation therapy, other treatment methods have been developed, such as immunotherapy, targeted therapy, hormonal therapy, gene therapy, adjuvant therapy, cancer vaccine therapy and cancer complementary and alternative medicine.

I.2. Apoptosis: a mechanism for cancer prevention and treatment

I.2.1. General information

Cell death can occur by many morphological, biological and functional distinct pathways, including apoptosis, autophagy, necrosis and mitotic catastrophe [Galluzzi *et al.*, 2007]. The term “apoptosis” had been coined and was first introduced in a publication in 1972 by Kerr, Currie and Wyllie [Kerr *et al.*, 1972] to describe a cell death process leading to controlled cellular self-destruction characterized by defined morphological changes, including cellular shrinkage, chromatin condensation, loss of nuclear membrane integrity, plasma membrane blebbing and eventually apoptotic body formation. Apoptosis plays a fundamental role for the development of all multicellular organisms whereby unwanted cells which are functionally redundant or potentially detrimental to the organism are eliminated. This mode of cell death is significant for variety of biological processes, such as morphogenesis of embryonic tissues as well as homoeostasis maintenance of architecture of adult organs and tissues, regulation and function of the immune system and removal of defect cells. Therefore inappropriate

regulation and function of apoptosis bring severe consequences. Failure of apoptosis can cause cancer, autoimmune diseases and spreading of viral infections, while excessive apoptosis results in neurodegenerative/neurodevelopmental disorders, acquired immune deficiency syndrome (AIDS) and ischaemic diseases [Rudin & Thompson, 1997]. Apoptosis has been found to occur in all multicellular animals studied so far [Ameisen, 2002], such as cnidaria [Böttger & Alexandrova, 2007], nematodes [Liu & Hengartnet, 1999], insects [Richardson & Kumar, 2002], amphibians [Nakajima *et al.*, 2005] and mammals [Ranger *et al.*, 2001].

I.2.2. Caspases activation

In mammalian cells, caspases, a family of cysteinyl aspartate-specific proteases, are the central regulators of apoptosis [Nicholson & Thornberry, 1997]. So far, 14 members of the caspase family have been identified in mammals. Based on their function in the proteolytic caspase cascade, they can be classified in three groups: initiator caspases, including caspase-2, -8, -9 and -10; effector caspases, such as caspase-3, -6, and -7 and caspases involved in control of inflammation, including caspase-1, -4, -5, -11, -12, -13, -14 [Nuñez *et al.*, 1998]. Caspases are synthesized in the cell as inactive zymogens, the procaspases that become mature by the autoproteolytic cleavage or the cleavage by other caspases. Initiator caspases are activated by a spectrum of apoptotic stimuli. Whereas effector caspases possess short prodomains, initiator caspases possess long prodomains containing either a death effector domains (DED) (in procaspase-8 and -10) or a caspase recruitment domains (CARD) (procaspase-2 and -9). Subsequently, activated initiator caspases can cleave and activate an overlapping set of effector caspases, which in turn execute apoptosis by cleaving cellular proteins following specific Asp residues.

There are two main pathways of caspase activation: ligation of death receptors following extracellular triggering (extrinsic apoptosis pathway) and release of cytochrom c from mitochondria following intracellular signals (intrinsic apoptosis pathway). In addition, two other apoptotic pathways are emerging: endoplasmic reticulum stress-induced apoptosis and caspase-independent apoptosis [Vermeulen *et al.*, 2005].

I.2.3. The death receptor-induced apoptosis pathway

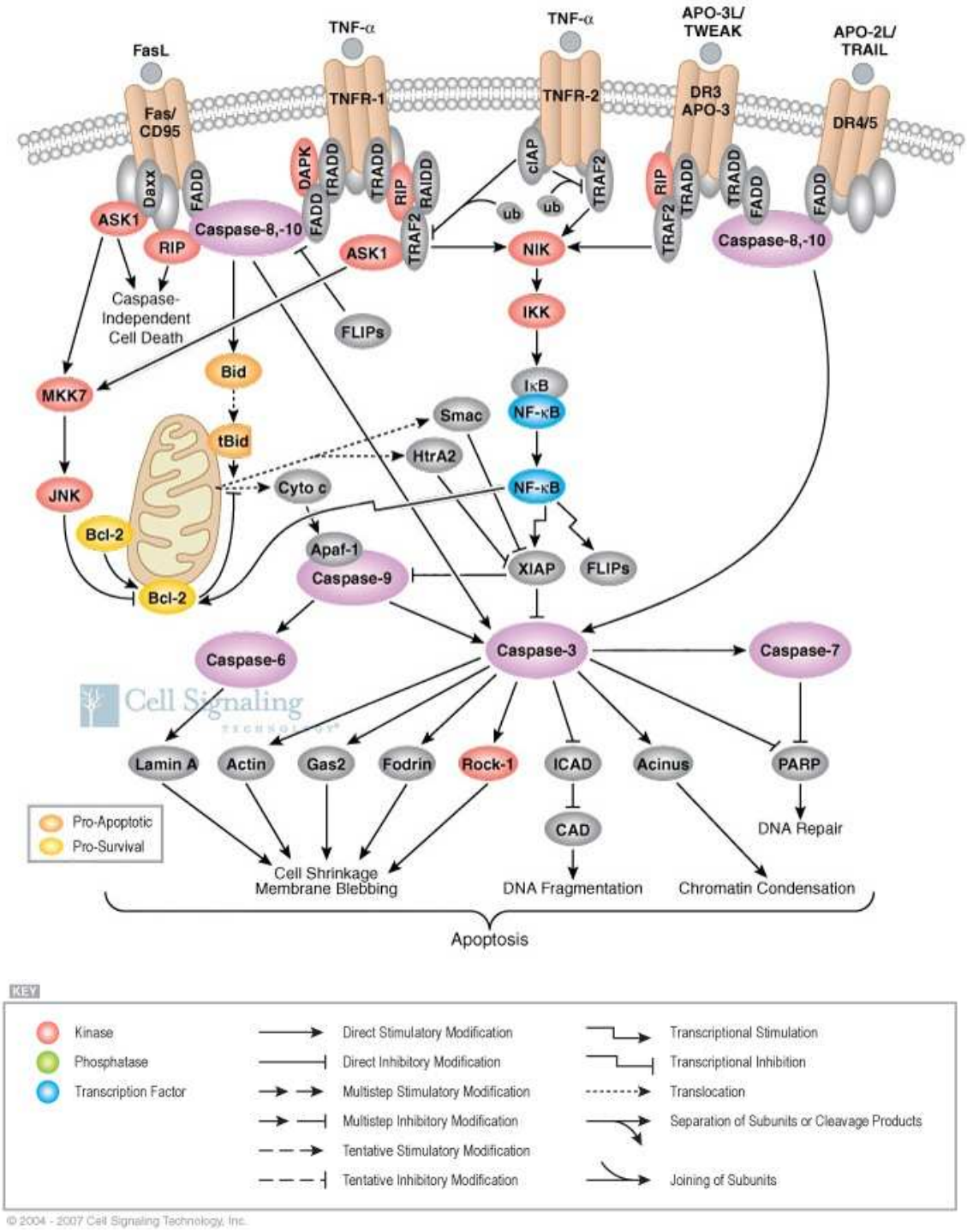


Figure 2: Death receptor pathway. Death factors such as FasL and TNF trigger apoptosis by binding on 'death receptors' such as Fas and TNFR-1. The death receptors recruit procaspase-8 by means of an adaptor protein, FADD. After cleavage the mature caspase-8 then directly activates caspase-3 or cleaves Bid. tBid interacts with Bax and Bak, then stimulates the release of cytC by the mitochondria and the activation of caspase-9. Effector caspases are activated leading to the degradation of cellular proteins necessary to maintain cell survival and integrity. Activation of caspases is negatively regulated at the receptor level by FLIP, which block caspase-8 activation, and at the mitochondria by BCL-2 family proteins and IAPs.

The death-receptor pathway is triggered by members of the death-receptor superfamily which are cell surface receptors that transmit apoptotic signals after ligation with respective ligands. Death receptors belong to the tumour necrosis factor receptor (TNFR) gene superfamily, including Fas/CD95, TNFαR, DR3, and the TRAIL receptors DR4 and DR5. All of them possess cysteine-rich extracellular domains and a cytoplasmic death domain (DD) [Ashkenazi, 2002]. The DD is crucial for the transduction of the apoptotic signal. Death receptor ligands initiate signaling via receptor oligomerization in the cell membrane, which leads to the recruitment of specialized adaptor proteins via DD interactions and activation of caspase cascades.

The recruitment of adaptor proteins results in the formation of a death inducing signalling complex (DISC). In this complex, adaptor protein such as Fas-associated protein with DD (FADD) interacts via its own DD with the DD of Fas. In addition, adapter protein FADD also contains a death effector domain (DED) which is homologous to the domain found in caspase-8. Therefore, binding of FADD to Fas receptor results in the recruitment and auto-activations of caspase-8. The activated caspase-8 stimulates apoptosis through two pathways. Activated caspase-8 can directly cleave and activate downstream effector caspases, which in turn subsequently execute apoptosis. Alternatively, it can cleave Bid, a Bcl-2 family member. Truncated Bid (tBid) translocates to mitochondria, and then the release of cytochrome c is induced and caspase-9 is activated. Activated caspase-9 initiates a caspase cascade by activating downstream effector caspases [Ashkenazi & Dixit, 1998; Luo *et al.*, 1998].

TNF-α and DR-3L can deliver pro- or anti-apoptotic signals. TNF-α, unlike FasL and TRAIL, is generally a poor inducer of cell death. Interaction of TNF-α with TNFαR might activate NF-κB pathway [Karin & Lin, 2002]. NF-κB induces expression of several anti-apoptotic genes, including Bcl-2, FLIP and XIAP. FLIP inhibits the activation of caspase-8. XIAP directly binds and inhibits effector caspases.

I.2.4. The mitochondria apoptosis pathway

The intrinsic apoptotic pathway is triggered by various death signals originating from inside the cell, including DNA damage, starvation and stress signals induced by irradiation and

chemotherapeutic drugs [Wang *et al.*, 2001]. In this pathway, various apoptosis-inducing signals suddenly induce mitochondrial outer membrane permeabilization (MOMP) [Green, 2004]. It results in the release of pro-apoptotic proteins normally found in the space between inner and outer mitochondrial membranes into cytoplasm, including cytochrome c (cytC), HtrA2/Omi, Smac/Diablo, apoptosis-inducing factor (AIF) and endonuclease EndoG. MOMP can also cause the metabolic or functional defect in the mitochondria which leads to the loss of the biochemical homeostasis of the cell and cell death. Cytochrome c after released from mitochondria assembles with the adaptor molecule APAF-1 and triggers apoptosome formation. Only the caspase-9 bound to apoptosome can cleave and activate downstream effector caspases, which in turn execute apoptosis. XIAP molecules bind and inhibit caspase-3 via their BIR domains. This inhibition is relieved by Smac/Diablo that binds to BIR domains of XIAP molecules and replaces caspases from XIAP and enables their activation [Verhagen *et al.*, 2000]. It has been known that HtrA2 protein, like Smac/Diablo, can prevent XIAP inhibition of active caspase-3 and promote cell death [Verhagen *et al.*, 2002]. The translocation of AIF results in caspase-independent chromatin condensation and DNA fragmentation [Susin *et al.*, 1999]. EndoG induces also caspase-independent DNA fragmentation once released from the intermembrane space [Li *et al.*, 2001]. Roles of AIF and EndoG in apoptosis indicate that apoptosis can proceed without caspases.

The mitochondrial permeability and the release apoptogenic molecules are regulated by Bcl-2 protein family. Bcl-2 was discovered as an oncogene that prevents apoptosis rather than promotes proliferation [Vaux *et al.*, 1988]. Up to date, at least members of Bcl-2 protein family have been identified which are characterized by the presence of the conserved sequence motif, the so-called Bcl-2 homology domains [Cory & Adams, 2002]. Besides Bcl-2, subfamily of pro-survival proteins consists of Bcl-X_L, Bcl-w, A1, and Mcl-1 which all possess BH1, BH2, BH3 and BH4 domains. There are two subfamilies of pro-apoptotic proteins: the Bax and the BH3-only subfamilies. Members of Bax subfamily, including Bax, Bak, Bok, possess BH1, BH2 and BH3 domains but proteins of BH3-only subfamily, such as Bik, Bad, Bid, Bim, Bmf, Hrk, Noxa, and Puma, have only the short BH3 motif. Cellular homeostasis might depend on the balance between the pro-survival and BH3-only proteins.

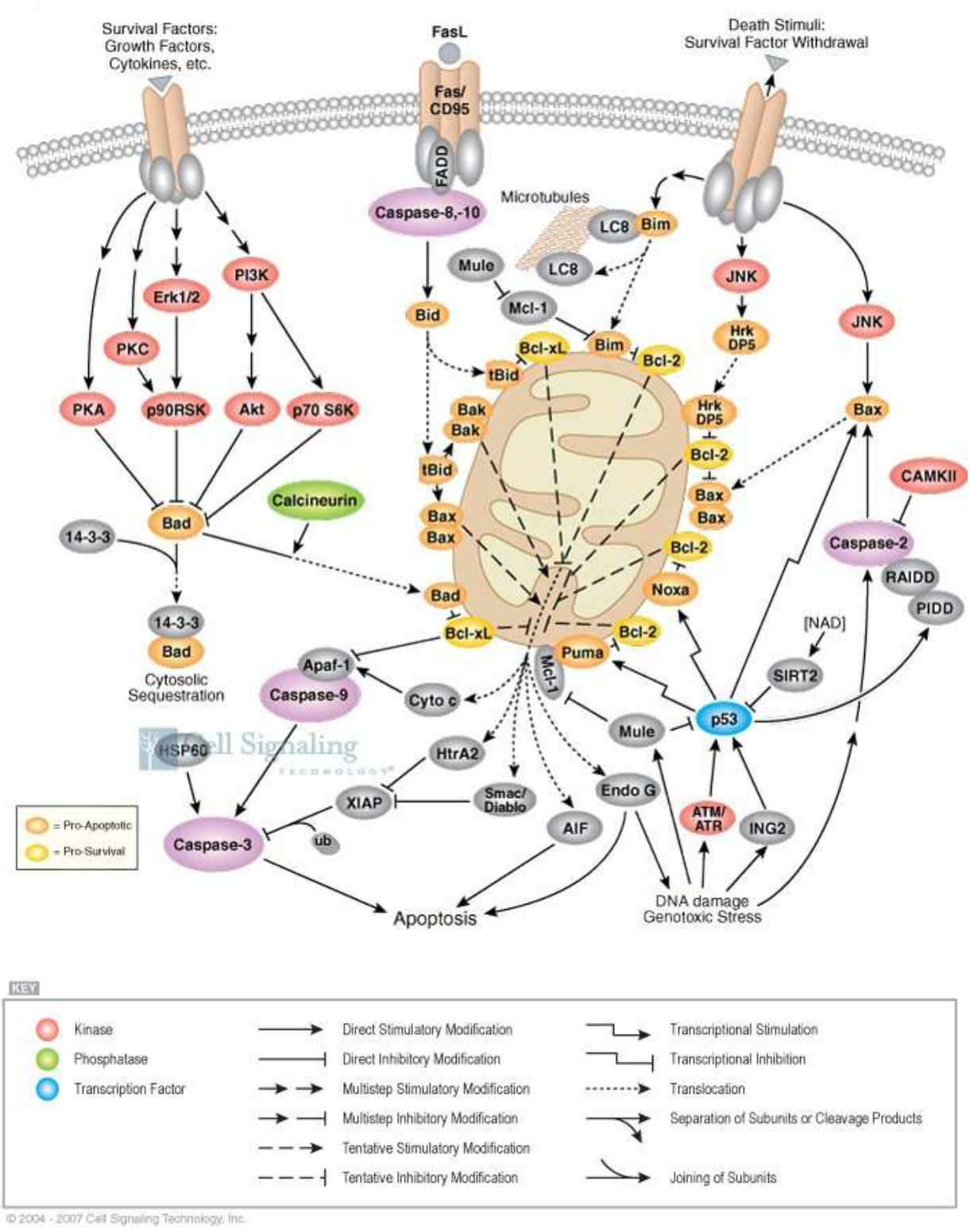


Figure 3: Mitochondrial pathway. The intrinsic pathway occurs when various apoptotic stimuli trigger the release of apoptogenic factors such as cytC (independently of caspase-8 activation), AIF, or Smac/Diablo from the mitochondrial inter-membrane space. The release of cytC into cytoplasm triggers caspase-3 activation through formation of the cytC/Apaf-1/caspase-9-containing apoptosome complex. The death-receptor and mitochondrial pathway can be interconnected at different levels.

Activated in response to oncogene activation or DNA damage, p53, a tumour suppressor protein, induces the expression of pro-apoptotic proteins, such as Bax, Noxa and Puma or represses the transcription of pro-survival genes, such as bcl-2, bcl-X_L or surviving [Chipuk & Green, 2006].

I.2.5. Apoptosis: a novel target for the chemoprevention of cancer

A variety of chemopreventive agents has been shown to trigger apoptosis in pre-malignant and malignant cells *in vitro* and *in vivo* [Sun *et al.*, 2004]. Some chemopreventive agents, such as triterpenoids and retinoids induce the expression of death receptors [Sun, 2005]. Several other cancer chemopreventive agents can induce apoptosis via mitochondrial pathway by proceeding through the regulation of Bcl-2 family members or through induction of the mitochondrial permeability transition [Hail, 2005]. The strategy to use chemopreventive agents that induce apoptosis might be an effective means in order to abate or control cancer.

I.3. Mushroom as a source of anti-tumour agents

I.3.1. Medical mushrooms with anti-tumour and immuno-modulatory activities

Many edible and non-edible mushrooms have long been used worldwide, especially in oriental countries, for medical purposes. Medical mushrooms have become more and more widely used for prevention and treatment of a variety of diseases, including cancer. In the last decades, medical mushrooms have been intensively investigated *in vivo* and *in vitro* for medical properties. Wasser has indicated that at least 651 species of mushroom classes Hetero- and Homobasidiomycetes contain anti-tumour or immunostimulatory compounds [Wasser, 2002]. Pretrova *et al.* demonstrated that fungal metabolites can be used as novel cancer chemopreventive agents [Petrova *et al.*, 2008]. Until now, several pharmaceutical products have been derived from medical mushrooms: Ganopoly -polysaccharide extract from *Ganoderma lucidum* and *Cordyceps sinensis,* Lentinan from fruit bodies of *Lentinula edodes,* Schizophyllan from liquid cultured broth product of *Schizophyllum commune,* PSP

(polysaccharide peptide) and PSK (or Krestin, protein-bound polysaccharide) from cultured mycelial biomass of *Coriolus versicolor,* Grifon-D from *Grifola frondosa,* Mesima from *Phellinus linteus*, etc.

Selected examples of medical mushrooms with anti-tumour and immuno-modulatory activities were listed in Table 1. Subsequently evidences about pharmaceutical activities of some of medical mushrooms were analyzed.

Agaricus blazei is an edible mushroom, native to Brazil, widely cultivated for its culinary and medical uses. This mushroom has been used in folk medicine against various diseases, such as chronic hepatitis, diabetes, arteriosclerosis, hyperlipidaemia and cancer [Wasser & Weis, 1999]. Its immuno-modulatory property has shown in many *in vitro* and *in vivo* studies but the molecular mechanisms and chemical substances involved in its pharmaceutical activities remain unclear [Firenzuoli *et al.*, 2008]. Extracts from *A. blazei* have been reported to have anti-tumour [Mizuno *et al.*, 1999; Ohno *et al.*, 2001; Takaku *et al.*, 2001; Kimura *et al.*, 2004] , anti-angiogenic [Kimura *et al.*, 2004] and anti-mutagenic effects [Delmanto *et al.*, 2001; Guterrez *et al.*, 2004]. It has been reported that a 10% powdered *A. blazei* diet has a chemopreventive influence on the promoting phase of the rat chemical hepatocarcinogenesis process [Pinheiro *et al.*, 2003]. Aqueous extract of *A. blazei*, induced a specific G_2/M phase arrest through cdc2 and cyclin B1 and triggered apoptosis via the regulation of Bax and the activation of caspase-3 in human gastric epithelial AGS cells [Jin *et al.*, 2006]. In another study, people from the same group demonstrated that apoptosis induced by aqueous extract of *A. blazei* was associated with the PI3K/Akt pathway, the reduction of Bcl-2 levels, caspase-3 activation and PARP degradation [Jin *et al.*, 2007].

Coriolus versicolor has been commonly used in China. Many reports based on *in vivo* and *in vitro* preclinical and clinical studies have revealed the therapeutic values of extracts from *C. versicolor* in the treatment of cancer [Chu *et al.*, 2002]. The chemopreventive potential of polysaccharides from *C. versicolor* and *Ganoderma lucidum* was demonstrated using biomarkers for carcinogenesis [Kim *et al.*, 1999]. Polysaccharide peptides isolated from *C. versicolor*, such as PSP and PSK, have long been shown to manifest immuno-modulatory anti-tumour effects. PSP was able to diminish side effects of radiotherapy and chemotherapy; therefore, it might be used as adjunct for cancer treatment [Ng, 1998]. Extracts from this mushroom were found to selectively and dose-dependently inhibit the proliferation of various tumour cell lines via arrest of cell cycle and induction of apoptosis [Hsieh *et al.*, 2006; Jeménez-Medina *et al.*, 2008].

Inonotus obliquus is a white rot fungus, widely distributed over Europe, Asia and North America. Its imperfect form, sclerotium, called ‘Chaga’ has been used as a folk medicine in Russia and western Siberia since the 16th century [Saar, 1991]. Recently, many bioactive compounds such as polyphenols [Cui *et al.*, 2005] and triterpenoids, including lanosterol, inotodiol and trametenolic acids [Nakata *et al.*, 2007] have been found in ‘Chaga’. These compounds exhibited various biological activities, such as anti-tumour, anti-oxidant [Song *et al.*, 2008], anti-inflammatory activites [Kim *et al.*, 2007]. 3,4-Dihydroxybenzalacetone, one of the most potent polyphenol derived from ‘Chaga’, could enhance apoptosis and inhibit proliferation and invasion through the inhibition of NF-κB activation and NF-κB-regulated gene expression [Sung *et al.*, 2008]. It has been reported that aqueous extract from ‘Chaga’ induced the growth inhibition, G_0/G_1 phase arrest and apoptosis in human hepatoma HepG2 cells [Youn *et al.*, 2008]. This phenomenon was closely associated with the down-regulation of p53, pRb, p27, cyclins D1, D2, E, cyclin-dependent kinase (Cdk) 2, Cdk 4 and Cdk6 expression.

Ganoderma mushrooms have been used in oriental countries as medical remedy for longevity and health promotion since centuries [Paterson, 2006]. Extracts and even substances from *Ganoderma* mushrooms rapidly emerged as promising immunotherapeutic agents in treatment of many diseases.

Ganoderma lucidum was the most extensively studied species and has been used to prevent or treat various human diseases [Sliva, 2006]. Recent studies showed that both the polysaccharides fraction and the triterpene fraction of *G. lucidum* possessed anti-tumour and immuno-modulatory properties through mechanisms involving the inhibition of activities of replicative DNA polymerases [Mizushina *et al.*, 1998], the stimulation of cytokine release and apoptosis [Wang *et al.*, 1997] and the inhibition of angiogenesis and invasion [Lin & Zhang, 2004]. Previous reports revealed that *G. lucidum* extract inhibited the cell proliferation and induced apoptosis in a dose- and time-dependent manner in several cancer cell lines [Hong *et al.*, 2004; Hu *et al.*, 2002; Müller *et al.*, 2006]. Differentiation protein and gene expressions upon treatment of extracts from *G. lucidum* have already investigated to get a deeper look into its molecular mechanisms [Ma *et al.*, 2008; Cheng *et al.*, 2007].

Crude extracts from another species, *G. tsugae*, have been demonstrated to stimulate splenic natural killer cell activity and serum interferon production in mice [Won *et al.*, 1992]. It has been reported that a lanostanoid ester glucoside and a steroid, both isolated from *G. tsugae*, mediate their cytotoxicity through cell cycle regulation and apoptosis [Gan *et al.*, 1998].

Mushroom scientific name	Potential compounds	References
Agaricus blazei Murr.	(1→6)-β-D-glucan, heteropolysaccharides, polysaccharide-protein complex	[Itoh *et al.*, 1994; Kim *et al.*, 2005]
Agrocybe aegerita (Brit.) Sing.	lectin	[Zhao *et al.*, 2003]
Auricularia auricula-judae (Bull.) Wettst.	(1→3)-β-D-glucan	[Misaki *et al.*, 1981]
Coriolus versicolor (L.:Fr.) Quel.	polysaccharide peptides	[Ng, 1998]
Flammulina velutipes (Curt.:Fr.) P.Karst.	glycoprotein	[Ohkuma *et al.*, 1982]
Fomes fomentarius (L.:Fr.) Fr.	exopolysaccharide	[Chen *et al.*, 2008]
Ganoderma applanatum (Pers.) Pat.	polysaccharide	[Sasaki *et al.*, 1971]
Ganoderma lucidum (Curt.:Fr.) P. Karst.	β-D-glucans, heteropolysaccharides, glycoproteins	[Hong *et al.*, 2004; Hu *et al.*, 2002; Müller *et al.*, 2006]
Ganoderma tsugae Murr.	polysaccharide-protein complex	[Zhang *et al.*, 1994]
Grifola frondosa (Dick.:Fr.) S. F. Gray	(1→3)-β-D-glucan	[Suzuki *et al.*, 1989]
Hericium erinaceus (Bull.:Fr.) Pers.	polysaccharide	[Wang *et al.*, 2001]
Inonotus obliquus (Pers.:Fr.) Bond.et Sing.	polysaccharide	[Song *et al.*, 2008]
Lentinula edodes (Berk.) Pergler	(1→3)-β-D-glucan with (1→6)-β-D-glucosyl branches	[Zhang & Cheung, 2002]
Phellinus linteus (Berk.&Br.) Teng	polysaccharides	[Sasaki *et al.*, 1971]
Phellinus rimosus (Berk.) Pilat	ethyl aceate, methanol and aqueous extracts	[Ajith & Janardhanan, 2003]
Pleurotus ostreatus (Jacq.:Fr.) Kumm.	β-glucan, heteroglucan	[Gern *et al.*,2008]
Poria cocos Wolf	β-glucan, heteropolysaccharide	[Wang *et al.*, 2004; Jin *et al.*, 2003]
Schyzophyllan commune Fr.:Fr.	(1→3)-β-D-glucan with (1→6)-β-D-glucosyl branches	[Usui *et al.*, 1994]
Tremella fuciformis Berk.	glucuronoxylomannans	[Kiho *et al.*, 1994]
Tricholoma mongolicum Imai	lectins	[Wang *et al.*, 1996]
Volvariella volvacea (Bull.:Fr.) Sing.	β-glucan	[Kishida *et al.*, 1989]

Table 1: Selected examples of medical mushrooms with anti-tumour and immuno-modulatory activities

The anti-tumour and immuno-modulatory effects of a perennial representative of *Ganoderma* genus, *G. applanatum*, have been also demonstrated in mice [Jeong *et al.*, 2008].

Phellinus is another large and widely distributed genus. In oriental folk medicine, several species of *Phellinus* were known to improve health and to prevent and remedy various diseases. In the last decade, many pharmacological properties of *Phellinus* species have been elucidated.

Extract from *P. igniarius* has been demonstrated to possess anti-mutagenic activities, likely by inducing QR and GST activities and increasing GSH level [Shon & Nam, 2001]. It has been reported that the butanol extract of *Phellinus igniarius* induced relaxation of the phenylephrine-procontracted rat aorta in a dose-dependent manner, and its effect is abolished by the removal of functional endothelium [Kang *et al.*, 2006]. In addition, ethanol extract of *P. igniarius* has been shown to exert anti-proliferative and anti-metastatic effects on human hepatocarcinoma SK-Hep-1 cells and rat heart vascular endothelial cells, partly by suppressing the secretion of matrix metalloproteinase-2 and vascular endothelial growth factor [Song *et al.*, 2008].

Ajith and Janardhanan pointed out that ethyl acetate, methanol and aqueous extracts of *P. rimosus* showed remarkable anti-inflammatory, anti-oxidant and anti-tumour effects [Ajith & Janardhanan, 2001; Ajith & Janardhanan, 2002; Ajith & Janardhanan, 2003]. They also suggested that aqueous extract of *P. rimosus* possessed significant chemopreventive properties [Ajith & Janardhanan, 2006].

P. linteus is another non-edible mushroom which gained important medical usage since centuries in China and Korea. Recently, *P. linteus* has been paid great attention due to its anti-tumour effect [Han *et al.*, 2006; Guo *et al.*, 2007; Sliva *et al.*, 2008] and other medical values, including antioxidant [Song *et al.*, 2003], anti-inflammatory [Kim *et al.*, 2004], anti-microbial activities [Yeo *et al.*, 2007]. Studies of different groups have shown that extracts of *P. linteus* rendered several human cancer cell lines susceptible to apoptosis [Choi *et al.*, 2004; Li *et al.*, 2004; Zhu *et al.*, 2007]. Furthermore, it has been reported that *P. linteus* was able to enhance activities of doxorubixin, a conventional chemotherapeutic drug [Collins *et al.*, 2006]. These evidences indicated that *P. linteus* might be an alternative anti-tumour agent or a synergizer for existing anti-tumour drugs [Zhu *et al.*, 2008].

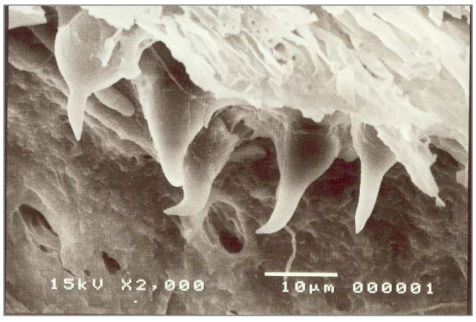
Although many pharmaceutical properties, emphatically the anti-tumour properties, of several medical mushrooms were extensively investigated, sufficient scientific studies have not been

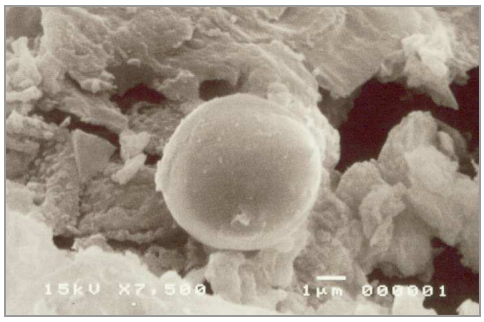
performed to characterize the mechanisms by which medical mushrooms exert their activities. Moreover, the majority of these observations often lack standardization and are still based on phenomenology [Borchers *et al.*, 2008]. So considerable understanding in molecular level remains to be determined.

I.3.2. *Phellinus pachyphloeus* (Pat.) Pat.

A. 

B. 

C. 

D. 

E. 

F. 

Figure 4: *Phellinus pachyphloeus*. A. Natural perennial ungulate basidiocarp. B. Layers of tubes separate from tissue layer. C. Hymenial setae through scanning electron microscope (SEM). D. Basidiospore through SEM. E. Formation of the immature fruit body on potato dextrose agar medium. F. Formation of cicatricial, immature fruit body on sawdust culture medium.

Phellinus pachyphloeus (Pat.) Pat. is a wood-decaying polypore macro-fungus species; belongs to the family Hymenochaetaceae of Basidiomycetes: lives on both deadwood and living trees, on fern roots, flowering plants, specially Fagaceae, Moraceae, Euphobiaceae and Mangiferaceae; and causes white rot [Larsen & Cobb-Poulle, 1990; Wagner & Firscher, 2002]. This species is abundant and widely distributed. Basidiocarp of this species is up to 65 × 125 × 50 cm, applanate to ungulate, sessile and usually thick (as depicted in Figure 4. A, B). Distinct structures for this species are the up-to-300 × 17-µm dark ferruginous tramal setal hyphae and the sharp-pointed hymenial setae with straight tips (Figure 4.C). As shown in Figure 4. E, F, we were successful in cultivating this species on potato dextrose agar medium and on sawdust culture medium and in both medium, immature fruit body were formed. The medicine values of its related species, such as *Phellinus linteus* or *Inonotus obliquus*, were well-documented, as remarked above. Water-soluble extract of *P. pachyphloeus* is increasingly used to treat a wide variety of disease processes. It therefore became necessary to attempt to determine its medical properties.

I.4. miRNA

I.4.1. General information

Cells contain numerous classes of small RNAs which mediate gene regulation and function for several pathways for gene silencing [Chu & Rana, 2007]. Small RNAs can be divided into different groups, including microRNAs (miRNAs), small-interfering RNAs (siRNAs), trans-acting siRNAs (tasiRNAs), small-scan RNAs (scnRNAs), repeat-associated siRNAs (rasiRNAs) and Piwi-interacting RNAs (piRNAs). One major class of small RNAs - miRNAs - was first introduced in 2001 [Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee & Ambros, 2001]. miRNAs are endogenous ~22-25 nt RNAs that play key regulatory roles by targeting mRNAs for cleavage or translational repression. Currently, hundreds of miRNAs have been identified in several metazoans by cloning or bioinformatics and this number is still increasing.

I.4.2. Genomics of miRNAs

Many miRNA genes are identified in clusters in the genome that can be transcribed as multi-cistronic primary transcripts [Bartel, 2004]. The miRNAs within a genomic cluster are often related to each other. The miRNA genes are located in intergenic regions, introns of non-coding genes, introns of coding genes in the sense or anti-sense orientation and in exons of non-coding genes [Rodriguez *et al.*, 2004]. Many of miRNAs are evolutionarily conserved though the copy number of miRNA genes can vary among orthologs. The *let-7* miRNA family has four members in *Caenohabditis elegans*, 15 in human but only one in *Drosophila melanogaster* [Aravin *et al.*, 2003; Lim *et al.*, 2003; Pasquinelli *et al.*, 2000].

Many modern approaches, including Northern blotting, microarray, real-time RT-PCR and *in situ* hybridisation have been used to identify miRNA expression profiles. Expression of miRNAs might be specific for a certain development stage or for a distinct tissue or cell type.

I.4.3. Biogenesis of miRNAs

Biogenesis of miRNAs is a multi-step process. miRNAs are transcribed by RNA polymerase II (Pol II) as pri-miRNAs, precursor molecules with 3’ poly-A tails and 5’ capping structures. These long transcripts are then cleaved by Drosha-DGCR8 complex to produce pre-miRNAs, ~ 70 nt stem-loop precursors with a 5’ phosphate end and a 2-nt 3’ overhang. Subsequently, pre-miRNAs are transported through nuclear pores into the cytoplasm by Ran-GTP and the export receptor Exportin-5. Pre-miRNAs are then processed by Dicer to produce ~ 22 nt duplexes of mature miRNA and an miRNA passenger strand (miRNA/miRNA*) with 2-nt 3’ overhang. Following processing, the miRNA duplex is loaded into miRNA-induced silencing complex (miRISC). During RISC assembly only one strand of the miRNA/miRNA* duplex might be incorporated, always the one whose 5’ end is less tightly paired, while the other one is degraded [Snøve & Rossi, 2006].

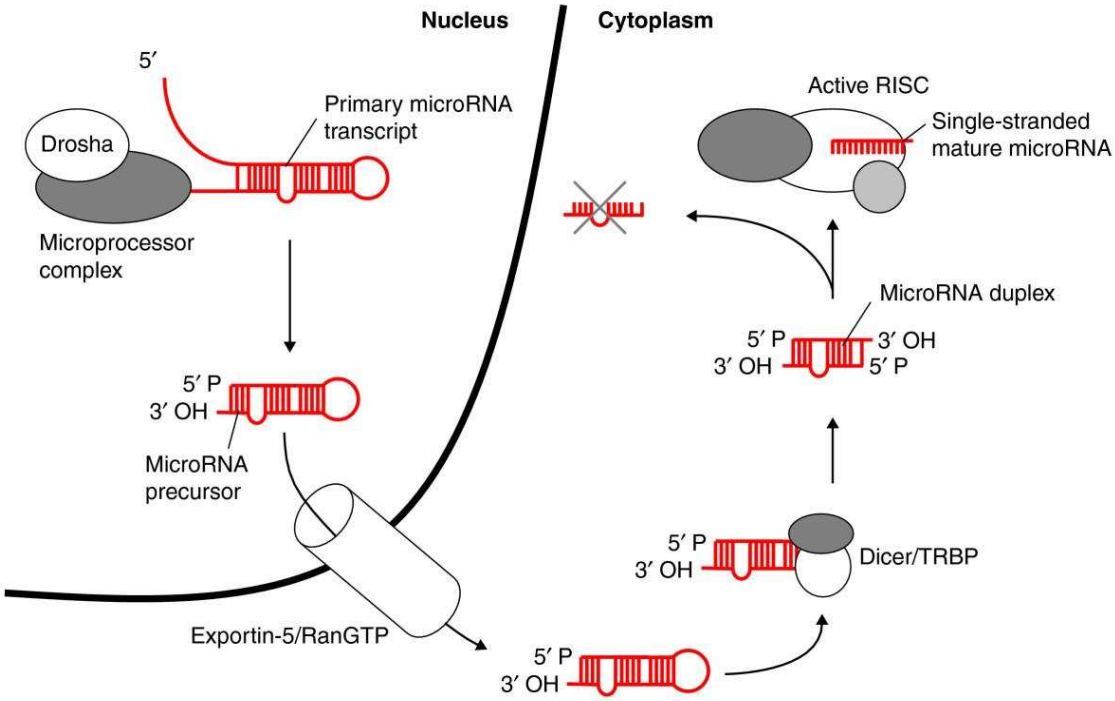


Figure 5: miRNA biogenesis. Primary miRNA transcripts are recognized and cleaved in the nucleus by the RNase III enzyme Drosha, resulting in hairpin precursors named pre-miRNA. Pre-miRNA is exported from the nucleus to the cytoplasm by Exportin-5 and is further processed by another RNase enzyme named Dicer, resulting in a ~ 22 nt duplex. Only one strand of this duplex is incorporated into a RNA induced silencing complex (RISC). (Adopted from Snøve & Rossi, 2006).

I.4.4. Mechanism of miRNA-mediated gene regulation

Once incorporated into miRISC, miRNA can recognize its target mRNA at the 3' UTR and down-regulate gene expression by repressing protein translation or by facilitating mRNA degradation and sequestrating mRNA to storage compartments. At least three models for the mechanism of miRNA-mediated gene silencing have been suggested. The first possible mechanism proposes a post-initiation repression mechanism primarily due to ribosome drop off during elongation of translation [Petersen *et al.*, 2006]. After ribosomes are removed, miRSC containing transcripts locate to processing bodies (P-bodies) for storage or RNA destruction. The second model indicates that upon the regulation of miRNA, the mRNA translation is inhibited at the initiation step. Human let-7 miRNA inhibits translation initiation of its mRNA target in an m^7G cap-dependent manner [Pillai *et al.*, 2005]. Kiriakidou *et al.*

proposed that Agonaute protein 2 repressed the translation initiation by binding to the m^7G cap of mRNA targets, therefore likely avoiding the recruitment of eIF4E, a translation initiation factor [Kiriakidou *et al.*, 2007]. In another study, it has been shown that human RISC associated with a multi-protein complex containing eIF6, a ribosome-associated protein known to prevent the assembly of the 80S ribosome. The third model has been found by Bagga and colleagues [Bagga *et al.*, 2005]. They revealed that mRNAs containing partial miRNA complementary sites can be targeted for degradation *in vivo*. mRNA decay might be caused by the accelerated deadenyltation [Yu *et al.*, 2005]. mRNA targets and miRISC after the translation repression are localized to P-bodies [Liu *et al.*, 2005].

Any miRNA might target different mRNAs and any mRNA might be targeted by several distinct miRNAs. Potential miRNA targets have been predicted and identified by bioinformatics approaches [Lewis *et al.*, 2003]. These data should be further validated by experiments.

I.4.5. Biological functions of miRNAs

Up to date, functions of only a few miRNAs have been identified. miRNAs involve in numerous biological processes and have crucial regulatory functions. In *C. elegans*, miRNAs affect the developmental timing [Moss *et al.*, 1997] and the asymmetric expression of chemoreceptors in embryos [Chang *et al.*, 2004]. miRNAs have been shown to suppress apoptosis, induce cell proliferation [Brennecke *et al.*, 2003] and regulate fat metabolism in *D. melanogaster* [Xu *et al.*, 2003]. In *Mus musculus*, miRNAs have been found to regulate developmental patterning [Yekta *et al.*, 2004], proliferation and differentiation of several cell type [Chen, 2004 *et al.*; Chen *et al.*, 2006], insulin secretion [Poy *et al.*, 2004] and spermatogenesis [Yu *et al.*, 2005]. Human miRNAs have been demonstrated to regulate adipocyte differentiation [Esau *et al.*, 2004], mediate antiviral defence [Lecellier *et al.*, 2005].

I.4.6. miRNAs and cancer

Recent studies support the tight connection of miRNAs with the initiation and progression of human malignancies. Calin *et al.* have been first indicated the relation of miRNAs to cancer

[Calin *et al.*, 2002]. They showed that both *miR15* and *miR16* genes lie in a 30-kb region at chromosome 13q14 and are frequently down-regulated or even deleted in patients with B cell chronic lymphocytic leukaemia, mantle cell lymphoma, multiple myeloma or prostate cancers. Surprisingly, a large number of miRNAs is located at fragile sites and cancer-associated genomic regions [Calin *et al.*, 2004]. It has been shown that miRNAs behave as tumour suppressor genes and oncogenes in many types of cancer [Zhang *et al.*, 2007] and miRNA expression profiles might classify tumours [Lu, 2005] and predict the clinical outcome for patients [Calin *et al.*, 2006]. Normal levels of miRNAs with the tumour suppressor function prevent uncontrolled cell proliferation. Examples of this group are miR-15a, miR16 [Cimmino *et al.*, 2005], and let-7 [Yanaihara *et al.*, 2006]. The oncogene miRNAs promote tumour development by negatively inhibiting tumour suppressor genes or genes that control cell differentiation or apoptosis. The miRNAs that belong to miR-17-92 cluster are over-expressed in several tumours, such as small-cell lung cancer and human B-cell lymphomas [He *et al.*, 2005; Hayashita *et al.*, 2005]. The additional examples of oncogenic miRNAs are miR-21 [Iorio *et al.*, 2005], miR-106a [Volinia *et al.*, 2006], miR-155, miR-372 and miR-373 [Voorhoeve *et al.*, 2006]. Increasing knowledge of regulatory functions of miRNAs might lead to promising diagnostic and therapeutic strategies.

I.5. Aims of the study

Aqueous extracts of the mushroom are a source containing highly promising anti-tumour and immuno-modulatory compounds. The genus *Phellinu*s whose all members are perennial has been used in oriental countries to prevent and treat many diseases since centuries. The species *Phellinus pachyphloeus* Pat. (Pat.) is widely distributed and has also long been used for medical purposes in eastern traditional folk medicine and ethno-pharmacology. Mechanisms of anti-tumour, immuno-modulatory and other properties of medical mushrooms in general and *P. pachyphloeus* in particular are only partially examined and remain to be fully ascertained. Therefore, the aim of this study was to elucidate molecular mechanisms responsible for anti-tumour and immunostimulatory properties in human leukaemia cell line HL-60 upon treatment with water-soluble extract of *P. pachyphloeus*.

Like starting studying other natural products, before developing an understanding about these mechanisms, we first sought to clarify whether the aqueous extract of *P. pachyphloeus*

possessed cytotoxic and anti-proliferative effects and whether the metal content of this extract might play a role in its activities. Apoptosis induction might involve in these mechanism thus one purpose of this study was to investigate in case apoptosis is trigged by our mushroom extract by valuable tools, such as FACS and ‘DNA laddering’ analysis. Apoptosis can occur via two main pathways, including the mitochondrial and the death-receptor pathway. In order to determine which pathway involves in apoptosis, the activation of the caspase cascade and the expression of other apoptotic/anti-apoptotic regulatory proteins were examined.

Proteomics help to see behind the curtain the highly regulated network of protein expression, protein modifications and protein interactions. Therefore, the investigation on the differences on the protein level in human leukaemia HL-60 cells upon the treatment of our mushroom extract and the identification of apoptosis-associated proteins might provide an insight into the apoptotic process.

Another goal of this thesis entails an investigation into miRNA expression in human leukaemia HL-60 due to treatment of our mushroom extract. In pursuit of this purpose, microarray and real-time qRT-PCR were performed. Significant modifications in miRNA expression have been figured out gave us a better look into molecular mechanisms of the anti-tumour and immuno-modulatory properties of aqueous extract of the medical mushroom *P. pachyphloeus*.

II. MATERIALS AND METHODS

II.1. Materials

II.1.1. Mushroom and extract preparation procedure

II.1.1.1. Mushroom and extract preparation procedure

All the samples of *Phellinus pachyphloeus* (Pat.) Pat. used in this research were collected in Vietnam and classified by Prof. Dr. Sc. Trinh Tam Kiet (Centre of Biotechnology, Vietnam National University, Hanoi) referring to Larsen & Cobb-Poulle [Larsen & Cobb-Poulle, 1990].

As previously described by Choi and colleagues [Choi *et al.*, 2004], mycelium H1 extracts of *P. pachyphloeus* were prepared by boiling 300 g of 2-cm^3 slices of fruiting body in 2 l deionized water at 100 °C for 6 hs. Boiling was done three times, and the volume of extract was reduced to about 60 ml. The extract was filtered with the Whatman no. 1 filter paper. Ethanol (96%) was added in a 3:1 ratio of ethanol and extract, and the mixture was stored for 18 hs at 4 °C. Precipitated materials were recovered by centrifugation at 50000 ×g for 1 h. Precipitants were then resolubilized in 30 ml of distilled water. The sample was lyophilized until dry to obtain polysaccharides. Upon determination of the dry weight, the freeze – dried extract was reconstituted in cell culture media, RPMI 1640 medium supplemented with 10% heat – inactivated fetal bovine serum and 20 μg/ml gentamicin (stock solution: 50 mg/ml), was then centrifuged once at 5000 ×g for 1 h. The supernatant was taken, sterilized by filtration through a 0.22 μm Millex filter and subsequently stored in the dark at -20 °C for the next experiments.

II.1.1.2. Metal content analysis of mushroom extract

The freeze-dried extract sample was sent to FOOD GmbH (Jena) and there the content of heavy metals in mushroom extract was analyzed, in details, tests for arsenic, lead, cadmium, quicksilver, iron, chromium, manganese, nickel, copper, selenium, zinc, and cesium were performed.

II.1.2. Cell culture

II.1.2.1. Cell lines

All cell lines were kindly provided by Dr. Hans-Martin Dahse (Department of Infection biology, HKI, Jena)

- HL-60: human acute myeloid leukeamia (DSMZ no.: ACC 3)
- Hela: human cervix carcinoma (DSMZ no.: ACC 57)

II.1.2.2. Cell culture media

- DMEM with 4.5 g/l Glucose, Pyruvat, GlutaMAX^{TM}-I
- RPMI 1640 with GlutaMAX^{TM}-I

Addtion for complete culture medium: 10% FBS; 20 µg/ml gentamicin

Freezing medium: 70% (v/v) DMEM or RPMI 1640), 20% FBS, 10% DMSO

II.1.3. Chemicals

All chemmicals used were of the highest quality available.

- RNaseZap® (Ambion)

- trypan blue (0.5 % (w/v) in physiological saline) (Biochrom AG)
- gentamicin sulfate (BioWhittaker)
- ammonium sulfate, monosodium phosphate - dihydrate (Calbiochem)
- Tween-20, Nonidet P-40 (NP-40) (Fluka)
- ECL Plus Western blot detection reagents (GE Healthcare)
- DMEM, RPMI-1640, gentamicin (Invitrogen)
- magnesium chloride (Merck)
- fetal bovine serum (Perbio)
- Complete^{TM} Protease-Inhibitor-Cocktail-Tablet (PIC) (Roche Diagnostics)
- acetone, boric acid, butanol, Coomassie Brilliant Blue G-250, acetic acid, Diethylpyrocarbonate (DEPC), ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), ethanol, ethidium bromide, glycine, glycerol, hydrochloric acid, isopropanol, methanol, milk powder, natrium hydroxide, orthophosphoric acid, 2-Propanol, Rotiphorese Gel 30, sodium dodecyl sulfate (SDS), Tris-base, sodium azide, sucrose, urea (Roth)
- ammonium persulfate (APS) (Serva)
- agarose, bovine serum albumin (BSA), camptothecin, 3-(3-cholamidopropyl) dimethylammonio-1-propansulfonate (CHAPS), digitonin, dimethylsulfoxide (DMSO), dithiothreitol (DTT), (4-(2-Hydroxyethyl)-1- piperazineethanesulfonic acid (HEPES), iodoacetamide (IAA), ß-mercaptoethanol, Orange G, phenylmethanesulfonylfluoride (PMSF), tetramethylethylenediamine (TEMED) (Sigma-Aldrich)

II.1.4. Solutions

Acrylamide gel solution for SDS-PAGE (for 4 gels)	125 ml Rotiphorese Gel 30, 75 ml 1.5 M TrisCl pH 8.8, 300 mg SDS, 96 ml bidest. water, 3 ml 10 % APS, 42 µl TEMED

APS		10% (w/v) APS in bidest. water
Bradford solution	Stock solution	100 ml 96% ethanol, 200 ml 88% orthophosphoric acid, 350 mg Coomassie Brilliant Blue G-250
	Working solution	15 ml 96% ethanol, 30 ml 88% orthophosphoric acid, 425 ml
Blocking solution		5% (w/v) milk powder in 1×TBST
Bromophenol blue solution		1% (w/v) bromophenol blue, 0.6% (w/v) Tris-base in bidest. water
Coomasie staining solution		30% (v/v) methanol, 10% (v/v) acetic acid, 0.25% Coomassie Brilliant Blue G-250 in bidest. water
Coomasie destaining solution		30% (v/v) methanol, 10% (v/v) acetic acid in bidest. water
Displacing solution		0,375 M TrisCl, pH 8.8, 50% (v/v) glycerol, trace bromophenol blue in bidest. water
DTT working solution		1% DTT in SDS equillibration solution
EDTA		0.5 M EDTA in 1% DEPC-water
Fixing solution for SDS-PAGE		20% (v/v) methanol, 1.3% (v/v) orthophosphoric acid in bidest. water
IAA working solution		2.5% IAA in SDS equillibration solution
Lysine		10 mM lysine in bidest. water
Nonidet NP-40 (0,25%) in distilled water		Nonidet NP-40 (0.25%, v/v) in bidest. water
50× propidium iodide solution		2 mg/ml propidium iodide in 38 mμ sodium citrate pH 7.0

Propidium iodide/RNase A solution	40 µg/ml propidium iodide, 250 µg/ml Rnase A in 1× PBS
1 mg/ml proteinase K	1 mg/ml proteinase K in bidest. water
RNase A solution	100mg/ml RNase A in bidest. water
SDS equillibration solution	50 mM TrisCl (1.5 M, pH 8.8), 6 M Urea, 30% (v/v) Glycerol (87%), 2% (w/v) SDS, trace Bromphenol blue in bidest. water
Sealing solution	0.25 mM Tris-base, 192 mM glycine, 0.1% SDS, trace bromophenol blue, 0.5% (w/v) agarose in bidest. water
Sodium azide	0.02% (w/v) sodium azide in bidest. water
Sodium hydroxide	50 mM NaOH in bidest. water
Solution B	1.5 M TrisCl pH 8.9, 0.4% (w/v) SDS, in bidest. water
Solution C	0.5 M TrisCl pH 6.8, 0.4% (w/v) SDS, in bidest. water
Staining solution A	2% (v/v) orthophosphoric acid, 10 % (w/v) ammonium sulfate in bidest. water
Staining solution B	5% (w/v) Coomassie Brilliant Blue G-250 in bidest. water
Staining solution for 2D-DIGE, 2D-PAGE	per gel: 200 ml Staining solution A, 5 ml Staining solution B, 50 ml methanol
Stripping solution	62.5 mM TrisCl pH 6.8, 100 mM ß-mercaptoethanol, 2% SDS in bidest. water
Tris-Glycine (13 %) resolving gel	6.5 ml Rotiphorese Gel 30, 4.64 ml bidest. water, 3.75

solution		ml Solution B, 15 µl TEMED, 90 µl 10% APS
Tris-Glycine (4 %) stacking gel solution		0.65 ml Rotiphorese Gel 30, 3.045 ml bidest. water, 1.25 ml Solution C, 5 µl TEMED, 25 µl 10% APS
Washing solution for microarray	Washing solution 1	2× SSC 0.2% SDS in DEPC-water
	Washing solution 2	2× SSC in DEPC-water
	Washing solution 3	0.2× SSC in DEPC-water
Water-saturated butanol		50 ml butanol, 5 ml bidest. water

II.1.5. Buffers

DEPC water	0.2% (v/v) DEPC in bidest. water
Lysis buffer for 2D-DIGE	4% (w/v) CHAPS, 8 M urea, 10-30 mM TrisCl pH 8.0- 9.0, 5 mM magnesium acetate, 1 PIC tablet/10 ml in bidest. water
Lysis buffer for 2D-PAGE	9 M urea, 4% (w/v) CHAPS, 40 mM Tris-base, 1% (65 mM) DTT, 1 PIC tablet/10 ml, 2% (v/v) Pharmalyte, 0.002% (w/v) BPB in bidest. water
10× PBS buffer	1.4 M sodium chloride, 27 mM potassium chloride, 100 mM disodium hydrogen phosphate, 18 mM potassium dihydrogen phosphate in bidest. water
Phosphate-citrate (PC) buffer	24 parts of 0.2 M disodium hydrogen phosphate and 1 part of 0.1 M citric acid (pH 7.8)
4× Protein loading buffer for SDS-PAGE	250 mM TrisCl pH 6.8, 8% (w/v) SDS, 20% (v/v) glycerol, 0.012% (v/v) BPB, 1.8% (v/v) ß-

		mercaptoethanol in bidest. water
2× Sample buffer for 2D-DIGE	Stock solution	8 M urea, 4% (w/v) CHAPS
	Working solution	2.5 ml sample buffer stock solution, % (v/v) Pharmalyte, 2 % (w/v),130 mM DTT
Rehydration buffer		8 M urea, 0.5% (w/v) CHAPS, 40 mM Tris-base, 0.2% (w/v) DTT, 0.5% (v/v) Pharmalyte, 0.002% (w/v) BPB, in bidest. water
10× SDS electrophoresis running buffer		250 mM Tris-base, 192 mM glycine, 1% (w/v) SDS in bidest. water
20× SSC		3 M sodium chloride, 0.3 sodium citrate, pH 7.0 in bidest. water
TE buffer		10 mM TrisCl pH 8.0, 1mM EDTA
20× TBS		400 mM TrisCl pH 7.5, 3 M sodium chloride
1× TBST		0.1% (v/v) Tween-20 in 1× TBS
TNN lysis buffer		40 mM TrisCl pH 8.0, 120 mM sodium chloride, 0.5% NP-40, 1 PIC tablet/10 ml in bidest. water
Transfer buffer		48 mM Tris-base, 39 mM glycine, 0.0375% (w/v) SDS, 20% (v/v) methanol in bidest. water

II.1.6. Markers and protein standards

- Novex® Sharp Protein Standard, BenchMark™ Protein Ladder (Invitrogen)
- Prestained Protein Marker, Broad Range (New England BioLabs)
- FastRuler^{TM} DNA ladder (Fermentas)

II.1.7. Apparatuses

- Agilent 2100 Bioanalyzer, RNA 6000 Nano LabChip® (Agilent)
- StepOne real-time PCR system (Applied Biosystems)
- wash container (Array It)
- FACSCalibur flow cytometer, Falcon™ polystyrene - cell culter flasks (BECTON DICKINSON Biosciences)
- spectrophotometer DUR640 (Beckmann)
- Mini-PROTEAN® 3 Cell-System, blot appratus Trans-Blot SD Semi-Dry
- transfer cell (BioRad)
- RNase-free pipettor tips (Biozym)
- 96-microtitrer plate (Brand)
- heater (Eltret)
- pipettors, pipettor tips, Eppendorf tubes, centrifuge 5702 R, 5415 C, Speed Vac 3301, hybridization block (Eppendorf)
- 24x 60 LifterSlips™ (Erie Scientific Company)
- freezer (Forma Scientific Inc.)
- Amersham Hyperfilm™ ECL, CyDye™DIGE Cy™2 minimal dye, CyDye™DIGE Cy™3 minimal dye, CyDye™DIGE Cy™5 minimal dye, DALT blank cassette, DALT glass plates, DALT separator sheets, Ettan™ IPGphor™ IEF system, Ettan™ DALT*six* electrophoresis vertical system, Ettan™ DALT*twelve* gel caster, Immobiline™ DryStrip pH 3-11 NL, 24 cm; Immobiline™ DryStrip reswelling tray, Pharmalyte 3-11 NL, PlusOne Immobiline™ DryStrip cover fluid, Typhoon Image scanner (GE Healthcare)
- spectrophotometer Spectronix 20 (Genesys Spectronic Instruments)
- water bath (GFL)
- micro test plate (Greiner Bio-One)

- cell culture incubator BB 6060, Laminar-Box LaminAir® HB2472 (Heraeus Instruments)
- shaking machine KS250 *basic*, thermomixer (Ika- Labortechnik)
- film X-OMAT^{TM}AR, XAR 5 (Kodak)
- centrifuge tubes (LabCon)
- Immobilon Transfer membrane filter type PVDF (Millipore)
- GenePix® 4000B microarray scanner (Molecular Devices)
- cryo 1°C freezing container, NYL FilterUnit (Nalgene)
- NanoDrop® 100R (Nanodrop technologies)
- spectrophotometer 1420 Multilabel Counter Victor 3V (Pelkin Elmer)
- Neubauer-improved counting chamber, cuvettes, gloves, Rotilabo® syringe filter sterile (0.22 µm), pipettor tips (Roth)
- balance Talent TE1502S, Expert LE1003S-OCE (Sartorius)
- slab gel dryer (Savant Intruments)
- electronic cell analyser system CASY 1 (SCHÄRFE)
- vortexer VF2, ultrasonic apparatus Labsonic U (Schütt Labortechnik)
- microwave (Siemens)
- centrifuge Sigma 4K15 (Sigma)
- GelVue UV-transilluminator (Syngene)
- SUNRISE micro plate reader (TECAN)
- concentrator SC210A Speed Vac™ Plus (Thermo Savant)
- serological pipettes (5, 10, 25 ml) (TPP)
- autoclave Type 500 (Varioklav)
- light microscope Axionvert25 (Zeiss)

II.1.8. Kits, commercial solutions and commercial master mixes

- MirVana™ miRNA Isolations Kit (Ambion)
- RNA 6000 Nano Assay Kit (Agilent)
- TaqMan® microRNA assay, TaqMan® microRNA Reverse Transcription Kit, Taqman® Universal PCR Master Mix, No AmpErase® UNG(Applied Biosystems)
- Quant-iT ™ RiboGreen® RNA Assay Kit, NCode™ miRNA Labeling System, NCode™ Multi-Spezies miRNA Microarray V2, PureLink™ miRNA Isolations kit (Invitrogen)
- CASYTON (SCHÄRFE)

II.1.9. Antibodies

- sheep anti-mouse IgG, peroxidase-conjugate (whole antibody) [NXA931] (Amersham)
- monoclonal mouse anti-Caspase-8 (Ab-3) IgG [AM46] (Calbiochem)
- polyclonal rabbit anti-Caspase-6 IgG [9762], polyclonal rabbit anti-cleaved Caspase-6 (Asp162) IgG [9761], polyclonal rabbit anti-Caspase-7 IgG [9492], polyclonal rabbit anti-Caspase-3 IgG [9662], polyclonal rabbit anti-cytochrom c IgG [4272], polyclonal rabbit anti-Caspase-9 IgG (Cell Signalling Technology)
- AffiniPure *goat* anti-rabbit IgG
- (H+L) peroxidase-conjugate [111-035-003] (Jackson Immunoresearch Laboratories Inc.)
- monoclonal mouse anti-actin (C-2) IgG [sc-8432], polyclonal rabbit anti-Ly-GDI (Santa Cruz)
- monoclonal mouse anti-ß-actin (AC-74) IgG [A 5316] (Sigma)

II.1.10. Software

- Agilent 2100 Expert Software (Agilent)
- CellQuest (Becton Dickinson)
- Labscan, DeCyder™ Differential Analysis Software, ImageQuant (GE Healthcare)
- GenePix® Pro 4.0 and 6.0 (Molecular devices)
- CASYSTAT (SCHÄRFE)
- Magellan (TECAN)

II.2. Methods

II.2.1. Cell culture

The cells were maintained in DMEM or RPMI 1640 supplemented with 10% heat – inactivated fetal bovine serum and 20 µg/ml gentamicin and grown in an incubator at 37 °C with 5% CO_2. All experiments were performed on exponentially growing cell cultures. The doubling time of HL-60, at densities of 1-3 $\times$ 10^5 cells/ml, was 24-25h. All experiments were repeated at least once with essentially identical results.

Before treatment, cells were seeded at concentration 1 $\times$ 10^5 cells/ml in cell culture flask and incubated over night. In the next day, cell number was checked again by counting with haemocytometer. When reaching the concentration 2 $\times$ 10^5 cells/ml, the cells were cultured in the presence or absence of variable concentrations of mycelium H1 extract.

II.2.2. Antiproliferative and cytotoxic assay [Dahse *et al.*, 2001]

Approximately 1 $\times$ 10^5 cells were seeded with 0.1 ml RPMI 1640, containing 25 µg/ml gentamicin sulphate, but without HEPES, per well of the 96-well micro plate. Cells were then

incubated for 72 hours at 37°C in a humidified atmosphere and 5% CO_2 with or without the extract.

Suspension cultures of HL-60 in micro plates were analysed by an electronic cell analyser system CASY 1 with the software CASYSTAT for data evaluation. In detailed, the 0.2 ml content of each well in the micro plate was diluted 1:50 with CASYTON. Every count/ml was automatically calculated from the arithmetic mean of three successive counts of 0.4 ml each. From the dose response curves the GI_{50} values were calculated with CASYSTAT. The GI_{50} value was defined as being where the concentration-response curve intersected the 50% line, determined by means of the cell counts/ml, compared to control. The essential parameters for the estimation of growth inhibition and for changes in diameter distribution curves are expressed as diagrams.

The other cells were fixed by glutaraldehyde and stained with a 0.05% solution of methylene blue for 15 min. After gently washing the stain was eluted by 0.2 ml of 0.33 N HCl in the wells. The optical densities were measured at 660 nm in SUNRISE micro plate reader. Comparisons of the different values were performed with software Magellan.

II.2.3. Apoptotic detection methods

II.2.3.1. Camptothecin-induced apoptosis as control

As control for all apoptotic detection methods, apoptosis was induced in HL-60 cells by adding camptothecin with the end-concentration 5 μM into the culture medium.

II.2.3.2. DNA fragmentation analysis [Gong *et al.*, 1994]

About 2×10^6 cells untreated or treated cells were collected by centrifugation at 80g for 4 min, washed once in 10 ml cold PBS. The cell pellet was carefully resuspended in 1 ml PBS and then while vortexing, 10 ml of ice cold 70% ethanol was put drop by drop into tubes containing the cell suspension. The cells were stored in the fixative at –20 °C generally for 24-72 hs. The cells were then centrifuged at 800 ×g for 5 min and the ethanol was thoroughly

removed. The cell pellets (1-2 × 10^6 cells), were resuspend in 40 µl of phosphate-citrate (PC) buffer, at room temperature, for at least 30 min. After centrifugation at 1000 ×g for 5 min, the supernatant was transferred to new tubes and concentrated by vacuum in a SpeedVac concentrator for 15 min. A 5-µl aliquot of 0.25% Nonidet NP-40 was then added followed by 5 µl of a solution of 1mg/ml RNase A. After 30 – 60 min incubation at 37 °C in a thermomixer, 5 µl of a solution of 1 mg/ml proteinase K was added and the extract was incubated for an additional 30 – 60 min at 37 °C in the thermomixer. After the incubation, DNA concentration and pureness were measured by Nanodrop. If another DNA purification step was necessary, 2 volume ice cold 96% ethanol and 0.1 volume 3M sodium acetate were added and the tube was incubated in an ethanol bad at –20 °C for 2 hs for DNA precipitation, then centrifuged, the pellet was dried with Speed Vac concentrator and 15 µl TE-buffer was added and DNA concentration and pureness are measured again by Nanodrop. 2 µl of 6× loading buffer was added and the entire content of the tube was transferred to the gel. Horizontal 1% agarose gel electrophoresis was performed at 5 V/cm until the bromophenol blue front runs through two third of the gel. Because ethidium bromide was added before into the gel solution while casting, DNA in the gels was visualized under UV light.

II.2.3.3. DNA content analysis by flow cytometry [Gong *et al.*, 1994]

Pellets of cells removed from the PC buffer described above were washed once with PBS and then resuspended with fresh PI/RNase A solution. The cells were incubated for 30 min in the dark at room temperature. Each sample was transferred from a microfuge tube to a FACS tube, mix by syringes. The red fluorescence of individual nuclei was measured with a FACSCalibur flow cytometer. 10 × 10^3 events were collected for each sample, and the data were acquired and analyzed using CELLQUEST software. The cell cycle distribution (including the percentage of apoptotic cell) was also analyzed.

II.2.4. Analysis of protein expression

II.2.4.1. Protein extraction

II.2.4.1.1. Protein extraction for SDS – polyacrylamide gel electrophoresis

Untreated or treated cells were collected by centrifugation at 80 ×g for 4 min, washed twice with cold PBS and immediately the NP-40 lysis buffer was added and incubated for 30 min. The cell lysate was sonicated 3 times on ice, 0.5 s impulse, 50% power level, 10 s interval and then centrifuged at 16000 ×g for 30 min. The supernatant was taken as protein extract.

II.2.4.1.2. Protein extraction for two-dimensional gel electrophoresis

After 30 h incubation with or without mycelium extract of *P. pachyphloeus*, HL-60 cells were collected by centrifugation at 80 ×g for 4 min, washed twice with cold PBS. 500 µl cold lysis buffer was added and incubated for 30 min at room temperature. The cell lysate was sonicated 3 times on ice, 0.5 s impulse, 50% power level, 10 s interval and then centrifuged at 16000 ×g for 30 min. The supernatant was taken as protein extract.

II.2.4.2 Protein quantification (Bradford assay)

The spectrophotometer was warmed up 15 min before use. Seven standard solutions containing 0, 1, 2, 5, 10, 20, 25 µg/ml BSA were prepared and filled to 100 µl in 1.5 ml Eppendorf tubes. 100 µl sample containing around 1 – 20 µg protein was prepared. 900 µl of dye reagent was added to the tubes containing standard solutions and protein samples, gently mixed and incubated for 5 min at room temperature. The mixtures were transferred into cuvettes and the absorbance at 595 nm of the spectra of the standard solutions and protein samples were examined. A graph of absorbance at 595 nm versus protein concentration for the protein standards was prepared. The Bradford assay gives a hyperbolic plot for absorbance versus protein concentration but within a range of relatively low protein concentrations, the

hyperbolic standard curve can be approximated reasonably well by a straight line. To determine the protein concentration of a sample from it absorbance, use the standard curve to find the concentration of standard that would have the same absorbance as the sample.

II.2.4.3. Immunoblot analysis of proteins

II.2.4.3.1. One-dimensional SDS – Polyacrylamide gel electrophoresis of proteins

Aliquots containing 30 – 80 μg of protein samples were mixed with loading buffer and the mixtures were heated to 95 °C for 5 min for denaturation, chilled on ice, short centrifuged and subjected to one – dimensional electrophoresis (1-D PAGE) in 12% gel in SDS. For prerun, a voltage of 0.8 – 1 mA/cm^2 was applied to the gel. After the dye front moved into the resolving gel, the voltage was increased to 1.5 – 2 mA/cm^2 until the bromophenol blue front reached the bottom of the resolving gel. Proteins in gel could be stained with Coomassie brilliant blue or used to establish an immunoblot. For staining with Coomassie, the gel was soaked at first for 30 min in Coomassie staining solution and then in Coomassie destaining solution until the protein bands became obviously clear.

II.2.4.3.2. Immunoblot analysis

After separated by SDS – polyacrylamide gel electrophoresis, proteins were transferred to a pre-wetted nitrocellulose or a PVDF membrane at 23 V for 1 h. In the case of nitrocellulose membrane, the membrane was pre-wetted by soaking in transferring buffer for at least 15 min. In the case of PVDF membrane, the membrane was pre-wetted by soaking in methanol for 1 min and then in transferring buffer for at least 15 min.

After blotting, the membranes were blocked with 5% nonfat milk in TBST (blocking solution) for 1 h at room temperature or over night at 4 °C. Membranes were washed several times with TBST buffer and incubated for 3 hs with the primary antibody (diluted to 1:500 to 1:2000 diluted in blocking solution) at room temperature. After several wash with TBST buffer, the membranes were incubated with horseradish peroxidase – conjugated secondary antibodies for 1 h at room temperature (diluted to 1:1000 to 1:2000 in blocking solution). The

membranes were washed with TBST buffer several times. The antigens were then detected using the enhanced chemiluminescence Western blotting detection system ECL Plus according to the manufacturer instruction and visualized by autoradiography on X – ray film.

II.2.4.3.3. Stripping

For detection of different proteins on a membrane, antibodies after the first handling could be removed. The membrane was soaked and shaken in 55 °C – pre-warmed stripping solution for 30 min. After that, the membrane was intensively washed with TBST several times. For the second time detecting with antibodies, the membrane must be blocked again.

II.2.4.4. Two-dimensional Gel Electrophoresis (2D-GE)

II.2.4.4.1. Conventional two-dimensional gel electrophoresis (2D-PAGE)

Rehydration and strip loading

The immobilized pH gradient (IPG) gel strips with a non-linear separation range of pH 3-11 (24cm) were rehydrated overnight at room temperature in the Immobiline DryStrip Reswelling Tray. 1 mg of total protein lysate in a total volume of 450 µl of the rehydration solution was applied to the reservoir slots of the Reswelling Tray, and then the IPG strips were soaked individually. Strips were prevented from dehydration and oxidation by covering with mineral oil.

Isoelectric Focusing

The rehydrated IPG strips were placed in the Ettan IPGphor Manifold. Thereafter, the proteins were separated according to their pI. The isoellectric focusing was carried out at 20 °C with the IPGphor system, covered with mineral oil.

Homogeneous SDS Gel casting

Laboratory-made gels were casted in glass cassettes using Ettan DALT*twelfe*^{TM} system caster. The inner-side of the glass plates was carefully cleaned to remove any dust. The cassettes alternating with separator sheets were assembled into the caster, the offset edges of the cassettes up. Each 75 ml gel solution was prepared per 1-mm glass cassette. At first the hydrostatic balance chamber was filled with 100 ml of the displacing solution.

Equilibration / S-S bond reduction and alkylation

Prior to separation in the second dimension, the IEF gels (IPGs) were equilibrated in equilibration solution, first for 30 min with an addition of 65 mM DTT (reduction step), and finally for 15 min with 135 mM IAA (alkylation step). Excess of equilibration solution was removed by briefly dipping the IEF gels into deionized water.

SDS Electrophoresis /Second dimension (SDS-PAGE)

A separation according mainly to the molecular mass was obtained after denaturation of the protein by SDS, an anionic detergent, giving the proteins a negative charge and a similar shape by removing the secondary structure.

The casting cassettes (containing the polymerized gels) were shortly washed with deionized water to remove gel pieces. Thereafter, the IPG strips were placed, acidic end on the left, directly onto the acrylamide gel surface and remaining air bubbles between both gels were removed with a spatula. Strips were covered to be free of air bubble with 60 °C – heated agarose – sealing solution.When agarose was gelatinized, the gel cells were inserted into the Ettan DALTsix filled with 1× electrophoresis buffer. Finally, the 2D gels were overlaid with 2× electrophoresis buffer. Electrophoresis of the SDS-polyacrylamide gels were performed at 20 °C using a two-step increase of current until the bromophenol blue dye reached the bottom of the glass plates.

II.2.4.4.2. Fluorescence difference gel electrophoresis

CyDye DIGE Fluor minimal dyes (Cy2, Cy3, Cy5) were reconstituted in 99.8% anhydrous dimethylformamide (DMF) giving a concentration of 1 mM and stored at –20 °C. Routinely 50 μg protein was labelled in each reaction. 1/3 of each of the untreated and treated samples were mixed together to create an internal standard sample and then labelled with Cy2. The remaining 2/3 of the untreated sample was labelled with Cy3 and the remaining 2/3 of the treated sample was labelled with Cy5.

The differently labelled protein samples and the unlabeled protein from untreated and treated samples (each 425 μg) were combined into a single microfuge tube and mixed. 225 μl of 2× sample buffer and rehydration buffer were added to have total volume 450 μl and then the mixture was pipetted directly into the slot of the Immobiline dry strips reswelling tray for rehydration. All labelling reactions were performed in the dark to avoid the exposure of CyDyes to light.

Rehydration, first dimension IEF and second dimension SDS-PAGE were carried out in the same way as in the conventional 2D-PAGE experiment but the exposure of proteins labelled with CyDyes, the strips when running the first dimension and the gels when running second dimension to all light sources were kept to a minimum.

2D-gels of CyDye-labelled samples were scanned with the Typhoon variable mode imager 9400. Scan parameters were set followed the protocol from manufacturer. DIGE images were cropped using ImageQuant TL software and then analyzed using DeCyder 2D software version 6.5. The protein spots were detected and quantified by DIA (Differential In-gel Analysis) module on a set of images, from the same gel. After the detection, the DIA files were imported and analyzed by BVA (Biological Variation Analysis) module. Multiple images from different gels were matched to get statistical data with the p-value from the Student’s t-Test from different protein abundance levels between untreated and treated samples.

II.2.4.4.3. Fixation, Staining and destaining

The gels were fixed for 1 h with fixing solution, stained with colloidal blue G-250 for 24 – 36 hs, and then destained with water to decrease background. Gels were scanned (300 dpi resolution) with Image Scanner UMAX and Magic Scan software.

II.2.4.4.4. Spot picking and digestion

A transferring plan for gel piece arrangement in micro plates was prepared. Finally, the protein spots shown to be different between untreated and treated samples were carefully excised out of the gels by using pipette tips cut with the tube cutter (position 3) with caution to avoid keratin contaminations and stored at –20 °C until mass spectrometry analysis.

II.2.4.4.5. In-gel digestion

Destaining, digestion and peptide extraction were done completely automaticcally on Ettan digester. The standard program included 4 washing steps with 70 µl 50 mM ammoniumbicarbonate in 50% acetonitrile (ACN), 3 washing steps with 70 µl 70% ACN (time each step: 20 min) and the peptide transfer with 2 times addition and transfer of 25 µl 0.1% TFA in 50% ACN. After dehydration with ACN, the proteins were digested in gel with 8 µl of 6.25 ng/µl of porcine trypsin buffered with 50 mM ammoniumbicarbonate (freshly diluted) at 37 °C for 22 – 24 hs. The generated peptides were extracted. The peptide extracts were used for MALDI-TOF-MS analysis

II.2.4.4.6. MALDI measurement

Matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) measurements were carried out on MALDI micro MX. 1 µl of the peptide extracts were manually spotted on a polished steel target plate and co-cristallized in 1 µl α-cyano-4-

hydroxycinnamic acid matrix for the MALDI-TOF-MS analysis. For lock mass human [glu] - fibrinopeptide b was used. An internal calibration using 2 BSA sample spots on the Waters 96 formate low mass sample plate was performed.

II.2.5. Analysis of miRNA expression

II.2.5.1. Total RNA isolation procedure

Total RNA was isolated using mirVana miRNA isolation kit following the protocol of the manufacturer. Briefly, untreated or treated cells were collected by centrifugation, washed twice with cold PBS and then lysed in a denaturing lysis solution which stabilized RNA and inactivated Rnases. The lysate was then extracted once with acid-phenol: chloroform which removes most of the other cellular components, leaving a semi-pure RNA sample. This was further purified over a glass-fibre filter with solutions that specifically formulated for miRNA retention to avoid the loss of small RNAs to yield total RNA.

II.2.5.2. RNA quantification and quality assessment

RNA concentration and pureness were roughly estimated by measuring with Nanodrop. Then total RNA was analyzed by using Agilent RNA kits with the Agilent 2100 bioanalyzer. RNA concentration and integrity were determined at least in duplicate.

II.2.5.3. Microarray

II.2.5.3.1. miRNA labelling and hybridization [Landers, 2007]

microRNA-enriched samples were prepared using PureLink^{TM} miRNA isolation kit (Invitrogen) and then enzymatically labelled with the Ncode miRNA Labelling System. Labelled miRNAs were then hybridized to Ncode Multi-Species miRNA microarray slides.

II.2.5.3.2. Image analysis and data processing

Microarray data was obtained using the Axon GenePix 4000B scanner and associated GenePix Pro 6.0 software. Each of the data sets used was filtered using signal-to-noise criteria. Quality data was defined to have signal-to-noise ratio more than 3 in both channels.

The data were then exported to GenePix Results GPR Files and converted using Express Converter Version 1.7 (Institute for Genomic Research, USA). The Microarray Data Analysis System (MIDAS) version 2.19 (Institute for Genomic Research, USA) program [Saeed, 2003] was used to normalise raw experimental array data. In brief, three types of normalisation were performed. Firstly, total intensity normalisation was performed, followed by Lowess normalisation and finally, in-slide replicate normalisation [Quackerbush, 2002].

The Multi-experimental Viewer (MeV) Version 4.1 (Institute of Genomic Research, USA) program was used to identify genes and expression patterns of interest.

II.2.5.4. miRNA qRT-PCR [Chen *et al.*, 2005]

Expression of hsa-miR-338, hsa-miR-378 were verified by quantitative real-time PCR. Purified RNA was reverse transcribed and then PCR-amplified using TaqMan microRNA assays in StepOne real-time PCR system. Amplification of the U6 snRNA transcript was performed in every sample and the expression of each miRNA relative to U6 RNA was determined using the $2^{-\Delta CT}$ method [Livak & Schmittgen, 2001]. Experiments for both miRNAs were done with 3 differently biological samples, each sample in triplicates.

III. RESULTS

In the present study, we report the first time some investigations about molecular mechanisms responsible for the anti-tumour property in human leukeamia cell line HL-60 upon treatment with water-soluble extract of medical mushroom *Phellinus pachyphloeus*.

III.1. Metal content of H1 mushroom extract

Many mushroom species are able to accumulate relatively high concentrations of metals of which many are toxic and can be harmful for human health, including arsenic, lead, cadmium, mercury as well as cesium. It is well known that a wide variety of metals, depending on cell type, exposure concentrations and time, act as mutagenic and carcinogenic agents or might trigger apoptosis [Pulido & Parrish, 2003]. Therefore, before validating any anti-tumour properties, it is necessary to determine whether the metal content of the promising mushroom is above allowable limit or not.

In Table 2 we present elemental concentrations ($\mu g.g^{-1}$, dry weight) of our mushroom extract after lyophilized and molar concentrations (nM) of the same extract after resuspended in culture medium.

When studying the apoptosis induction mechanism in adult rat hepatic stem cells by arsenic trioxide and lead acetate, Agarwal and colleagues treated cells with As and Pb at 40µM concentration upon 6h [Agarwal *et al.*, 2008]. Their treatment resulted in 20% death of the isolated oval cells and the activation of caspase-3, caspase-9 and the dephosphorylation of survival factors like p38 and Erk play essential roles in the induction of apoptosis. It has previously been shown that the low concentration 0.5 µM arsenic trioxide As_2O_3 failed to induce caspase activation. In HL-60, it has been shown that caspase-9 activation observed in response to 4.0 µM As_2O_3 upon 48 h treatment and signalling through mitochondria pathway is the major route for As_2O_3-induced apoptosis [Jiang *et al.*, 2008]. Therefore it was obvious that arsenic is present in our mushroom extract at a non-toxic level. Lead could also not be the apoptosis-caused factor.

Previous study demonstrated that treatment of HL-60 cell with 12.5 µM concentrations of Cr and Cd ions for 48 h produced significant induction of the apoptotic cell population and resulted in the increase in cytochrome c reduction [Baqchi *et al.*, 2000]. In this study, concentrations of Cd and Cr in the extract used to treat HL-60 cells were less than potential concentrations for apoptosis. These results suggested that these two cations exhibited no dramatic effects on HL-60 cells.

Metal	Concentration ($\mu g.g^{-1}$, dry weight)	Concentration in culture medium containing mushroom extract (nM)	Apoptosis-inducing concentration (nM)		References
			In HL-60	In other cell lines	
As	1,2	16,017	4000	Rat hepatic stem cell: 40000	[Jiang *et al.*, 2008; Agarwal *et al.*, 2008]
Pb	0,17	0,820		Rat hepatic stem cell: 40000	[Agarwal *et al.*, 2008]
Cd	0,08	0,712	12500		[Baqchi *et al.*, 2000]
Hg	0,053	0,264	30000		[Araragi *et al.*, 2003]
Fe	108	1933,924			
Cr	1,1	21,155	12500		[Baqchi *et al.*, 2000]
Mn	270	4914,627		U87 (one of the most sensitive): 1000000	[Puli *et al.*, 2006]
Ni	1,9	32,372		T cell hybroma: 80000	[Kim *et al.*, 2002]
Cu	37	582,255	250000		[Ma *et al.*, 1998]
Se	0,19	2,406	20000		[Kim *et al.*, 2001]
Zn	12	183,514	25000		[Kondoh *et al.*, 2005]
Cs	3,6	26,277			

Table 2: Concentrations of heavy metals in H1 mushroom extract ($\mu g.g^{-1}$, dry weight) and concentrations of extract (which was resuspended in culture medium) used to treat cells (nM). Apoptosis-inducing concentration (nM) in HL-60 and other cell lines currently reported on the scientific literature were shown for comparison.

It is well known long ago that mercurial compounds induce apoptosis in immune system cell [Pollard & Hultman, 1997]. Araragi and colleagues reported the induction of apoptosis via mitochondrial pathway upon treatment with 30 µM mercuric chloride $HgCl_2$ at 9 h [Araragi *et*

al., 2003]. Our finding indicated that it is impossible that the amount of Hg in our extract could cause apoptosis in HL-60 cells.

Although being an essential trace metal, manganese (Mn) intake in excess results in toxicity and CNS is among one of the most sensitive tissues. As previously demonstrated, human glioblastoma U87 cells underwent apoptosis upon treatment 1mM Mn after 72h [Puli *et al.*, 2006]. In our observation, Mn content was about 200 times and treatment time was two times less compared to the above study and leukaemia cells are not so sensitive to Mn as CNS cells therefore in our extract, Mn was still at the harmless level.

As previously reported, apoptosis is induced via activation of caspase in HL-60 cells treated with zinc (Zn) at 25 µM [Kondoh *et al.*, 2005]. Various forms of selenium compounds have been also reported to be potent inducers of apoptosis and exert a cytotoxic effect in a dose-dependent manner in HL-60 cells [Kim *et al.*, 2001]. Both treatments with 50 µM Se-methylselenocysteine (MSC) and with 20 µM selenite induced apoptosis in HL-60 cells and selenite did not show the full range of apoptotic features. Nickel acetate treatment at the concentration 80 µM rapidly induces apoptosis in T cell hybridoma cells through an increase in FasL protein levels and transient activation of caspase-3 [Kim *et al.*, 2002]. Ma *et al.* found that cupric nitrilotriacetate (Cu-NTA), which was added to HL-60 cells to give a final concentration of copper of 250 µM, induced oxidative DNA damage and apoptosis [Ma *et al.*, 1998]. Comparing with the elemental amounts of Zn, Se, Ni and Cu in our extract (see Table 2); we demonstrate that contents of Zn, Se, Ni and Cu play no role in the cellular apoptosis induced by our mushroom extract.

III.2. H1 Mushroom extract treatment of HL-60 cells

In a first set of experiments, HL-60 cells were incubated with different concentrations of mushroom extract for different periods of time. Untreated HL-60 cells were used as control. The range of tested concentrations was 0.5 mg/ml to 2 mg/ml and time incubation was 12 hs, 24 hs, 36 hs and 48 hs. It turned out that 1 mg/ml mushroom extract efficiently induced apoptosis after 36 hs of treatment and this concentration and time point were therefore used as standard condition.

III.3. Anti-proliferative effects of H1 mushroom extract

The activities of mushroom extracts were evaluated in various bioassay test systems including cytotoxic (Hela cells) and anti-proliferative assays (HL-60 cells). Cells were incubated in the presence or absence of H1 mushroom extract.

Cytotoxicity assay was performed in human cervical (Hela) cell line. As shown in Figure 6, populations of treated cells and cells left untreated diverged from one another. In detail, H1 extract up to the concentration of 1 mg/ml did not exhibit any detectable cytotoxicity towards Hela. Percentage of treated cells was about 35 % after 72 hs of treatment with 1 mg/ml mushroom extract.

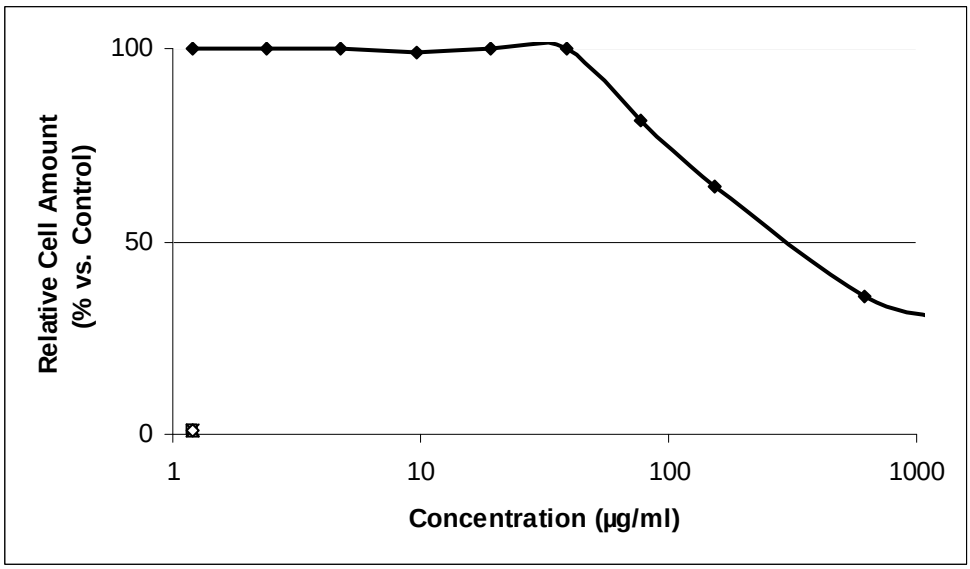


Figure 6: Dose response curve of Hela cells after 72h-treatment with H1 mushroom extract

Incubation of human acute myeloid leukeamia HL-60 led to a dose-dependent inhibition of cell proliferation. GI_{50} values (the concentration that inhibits cell growth by 50%) obtained after 36 h of treatment was 0.84 ± 0.064 mg/ml (Figure 7). Effectiveness of H1 extract increased with elongated treatment times. Collectively, our mushroom extract has no cytotoxic effect and shows anti-proliferative effect at high concentrations. For the next experiments, HL-60 was only treated with extract at concentration: 0.5 mg/ml, 1.0 mg/ml and 1.5 mg/ml.

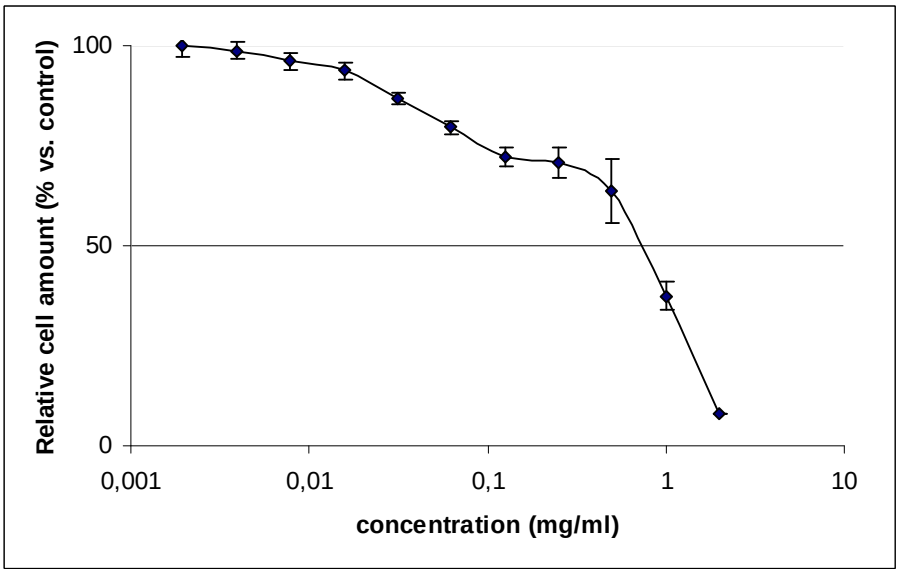


Figure 7: Dose response curve of HL-60 cells after 36h-treatment with H1mushroom extract. The viability percentage of HL-60 cells treated for 36h with different concentrations H1 mushroom extract as estimated by the assay. The results were represented as the percentage of the number of treated cells versus the number of control untreated cells.

III.4. Cell cycle regulation

To get first hints about the mechanisms responsible for the anti-tumour effect of *P. pachyphloeus* extract, we focused initially our study on how the effect of H1 extract on cell cycle regulation would be related to the observed anti-proliferative activity. To distinguish between necrotic and apoptotic cell death, a cell cycle analysis was performed. Cell cycle distribution was assessed by flow cytometry after staining fixed cells with propidium iodide and thereby cells in different phases of cell cycle were discriminated: G_1, S, G_2/M and hypodiploid cells. Because apoptosis leads to DNA strand breaks due to the activation of DNA digesting enzymes, this method can be used to determine the number and percentage of apoptotic cells in a cell population.

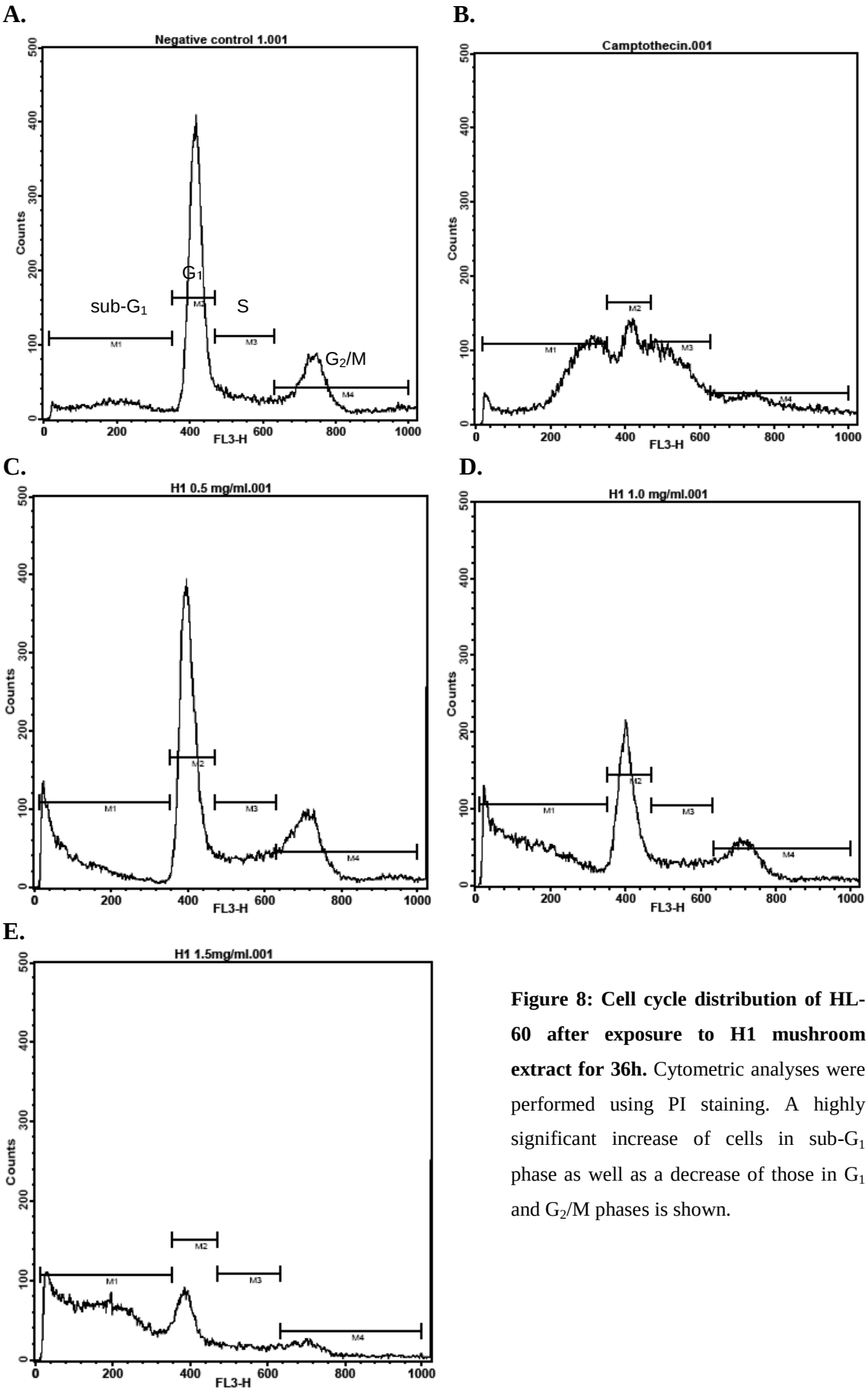


Figure 8: Cell cycle distribution of HL-60 after exposure to H1 mushroom extract for 36h. Cytometric analyses were performed using PI staining. A highly significant increase of cells in sub-G_1 phase as well as a decrease of those in G_1 and G_2/M phases is shown.

Figure 8 illustrated the DNA content which indicated the different distributions of phases of cell cycle in treated and control cells. The control group showed a prominent G_1 phase whereas; 1.0 mg/ml and 1.5 mg/ml of mushroom extract significantly reduced the portion of cells in G_1-phase and simultaneously decreased the percentage of cells in S/G2M-phase over a period of 36 hours.

The reduced DNA content of apoptotic nuclei resulted in an unequivocal hypodiploid DNA (sub-G1) peak which was easily discriminable from the narrow peak of cells with normal (diploid) DNA content. Untreated cells exhibited a minimal amount of cells in sub-G1 phase consistent with the low background level of apoptosis. However, upon treatment with H1 extract, cells exhibited a significantly greater amount of cells in sub-G1 compared to control cells. The presence of a distinct sub-G1 peak was depicted in Figure 9. The percentage of apoptotic nuclei was quantified and shown in this figure. After 36 hs the percentage of cells in sub-G1 increased from 12.84 % in untreated cells to 37.81% and 63.41% with increasing concentrations from 1.0 to 1.5 mg/ml of mushroom extract, respectively. This result indicated that the mushroom extract induces apoptosis.

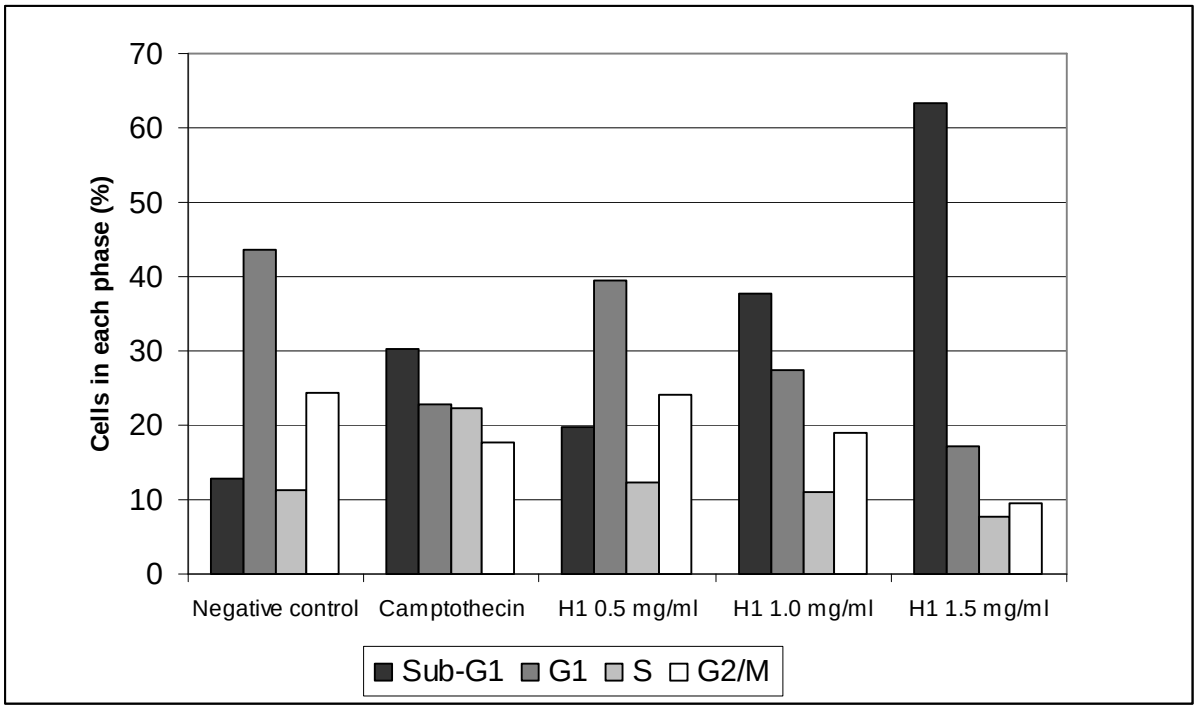


Figure 9: Histogram of cell cycle arrest and cell apoptosis. Histogram showed the percentages of cells at various phases of cell cycle of HL-60 cells with and without mushroom extract treatment: sub-G_1 phase (black bar), G_1 phase (dark gray bar), S phase (light gray bar) and G_2/M phase (white bar).

III.5. DNA fragmentation analysis

The cleavage of DNA between nucleosomes by endonucleases produced fragments in multiples of approximately 180 bp. DNA fragmentation can be visualized by agarose gel electrophoresis, thereby a typical DNA-ladder pattern formed by separation of DNA fragments can be observed. Since several compounds leading to cell cycle progression subsequently induce DNA fragmentation and apoptosis, the DNA integrity was examined after exposing HL-60 cells to H1 extract at increasing concentrations 0.5 mg/ml, 1.0 mg/ml, and 1.5 mg/ml for 36 hours and results were depicted in Figure 10. An enhanced DNA fragmentation in to mono- and oligonucleosomes, a typical hallmark of late apoptosis, was obvious at the concentrations 1.0 and 1.5 mg/ml. Therefore it was firmly established that the anti-proliferative effect of H1 mushroom extract was mediated by activation of the apoptosis pathways.

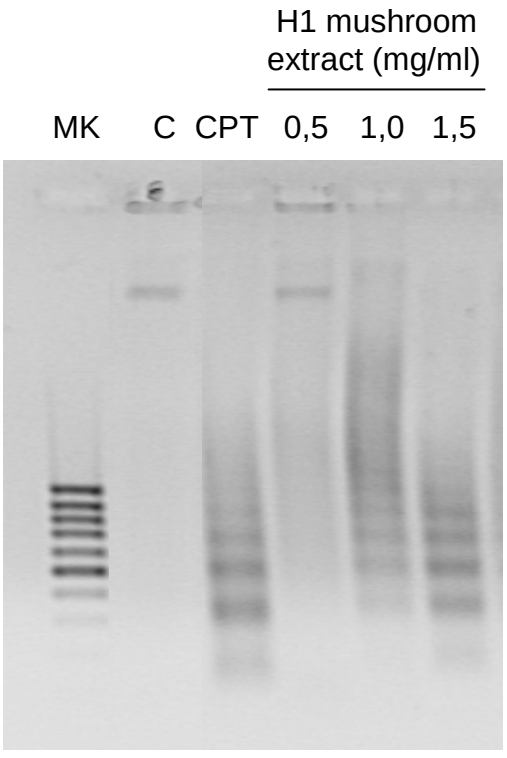


Figure 10: Representative results of a qualitative analysis of fragmented DNA separated on agarose gels. Lane 1: DNA marker; lane 2: negative control; lanes 3: cells exposed to 5 µM Camptothecin; lane 4-6: cells incubated with increasing concentrations of H1 mushroom extract.

III.5. Cleavage and activation of caspases in H1 mushroom extract-treated cells

It is well known that a family of cysteinyl proteases, the so-called caspases, is involved in apoptotic cell death. Caspases are synthesized as inactive zymogen and converted to an active complex composed of several heterodimeric subunits. To confirm the apoptosis-inducing potential of H1 extract, we analyzed cleavage and activation of caspases. The processing of procaspases was monitored in immunoblot analyses using antibodies which are specific to individual proteases.

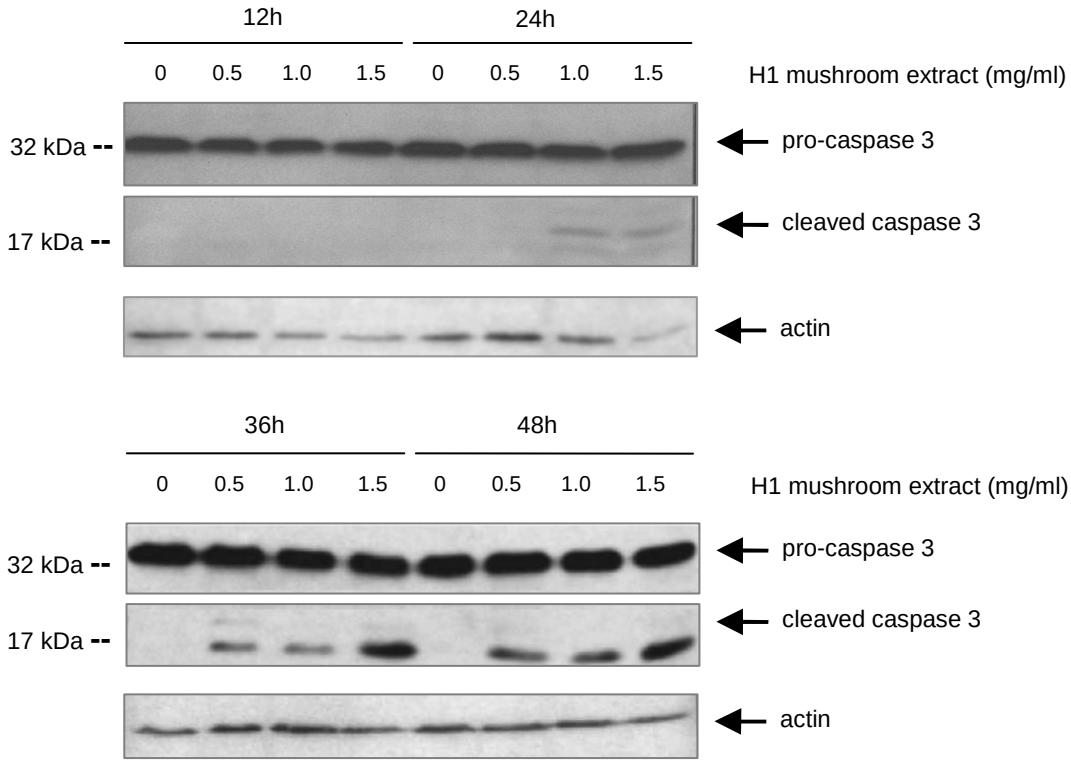


Figure 11: H1 mushroom extract induces activation of caspase-3. HL-60 cells were incubated with the indicated concentrations of mushroom extract for 12 hs, 24 hs, 36 hs and 48 hs. Western blot analysis showed the activation of caspase-3 resulting in time-dependent and concentration dependent appearance of the p17 subunit. After being exposed, the membrane was stripped and blotted with anti-Actin antibody as a loading control.

Treatment of HL-60 cells with H1 mushroom extract resulted in the conversion of the inactive 32-kDa caspase-3 precursor to the proteolytically cleaved p17 subunit, indicating that

caspase-3 was activated during mushroom extract-induced apoptosis. In detail, H1 extract did not affect the expression of pro-caspase-3 protein however the active form of caspase-3 (p17 subunit) was concentration-dependently increased by mushroom treatment, as shown in Figure 11. After 24 h of treatment, H1 extract at a concentration of 1 mg/ml, as well as 1.5 mg/ml induced caspase-3 cleavage. After 36 h, caspase-3 cleavage was markedly induced by H1 extract. These results show that our mushroom extract at the 1mg/ml concentration induces apoptosis rather than necrosis.

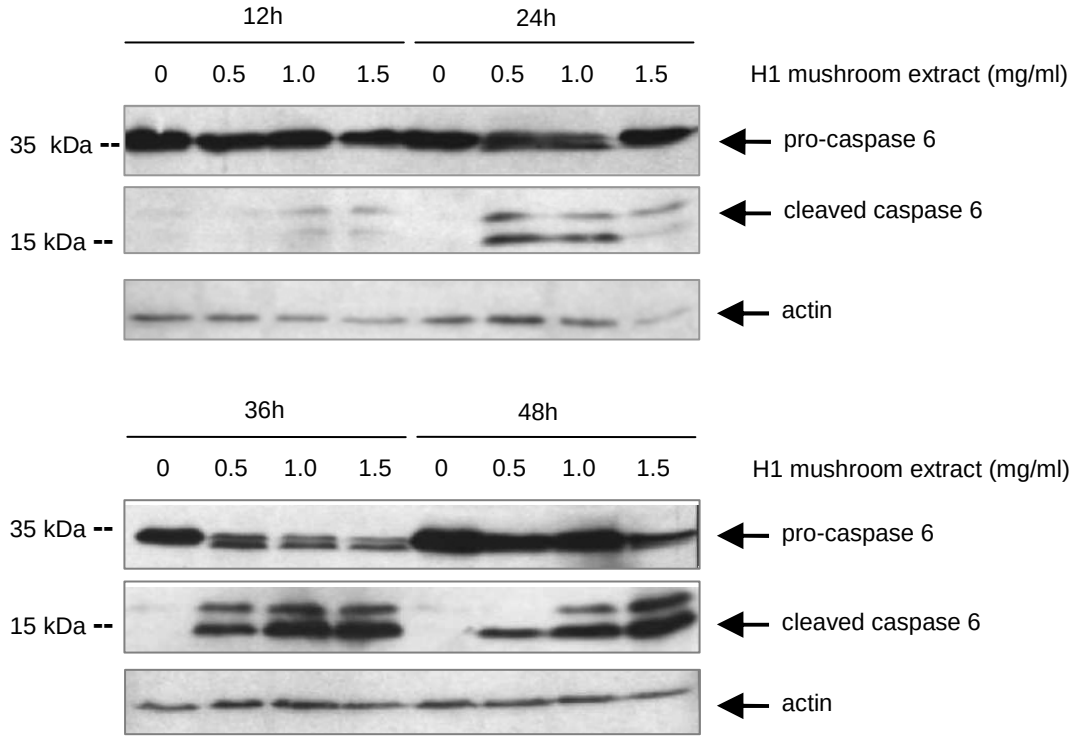


Figure 12: H1 mushroom extract induces activation of caspase-6. HL-60 cells were incubated with the indicated concentrations of mushroom extract for 12 hs, 24 hs, 36 hs and 48 hs. Western blot analysis showed the activation of caspase-6 resulting in time-dependent and concentration-dependent appearance of the p18 subunit. After being exposed, the membrane was stripped and blotted with anti-Actin antibody as a loading control.

H1 mushroom extract was also able to induce a highly significant increase of caspase-6 and caspase-7 activities. Figure 12 showed that the p18 subunit of the active caspase-6 was formed upon treatment of HL-60 cells with H1 mushroom extract. Extract-induced activation of caspase-6 already occurred after 12 hs and reached its maximum after 36 hs.

Activation of caspase-7 was later induced as depicted in Figure 13. HL-60 cells incubated with mushroom extract got no signal of the mature subunit after 24 hs. A small amount of the cleaved active form of caspase-7 was present at 36 h and increased further after 48 hs. These data suggested that our mushroom extract induced apoptotic cell death via not only the activation of caspase-3 but also via the activation of caspase-6 and caspase-7.

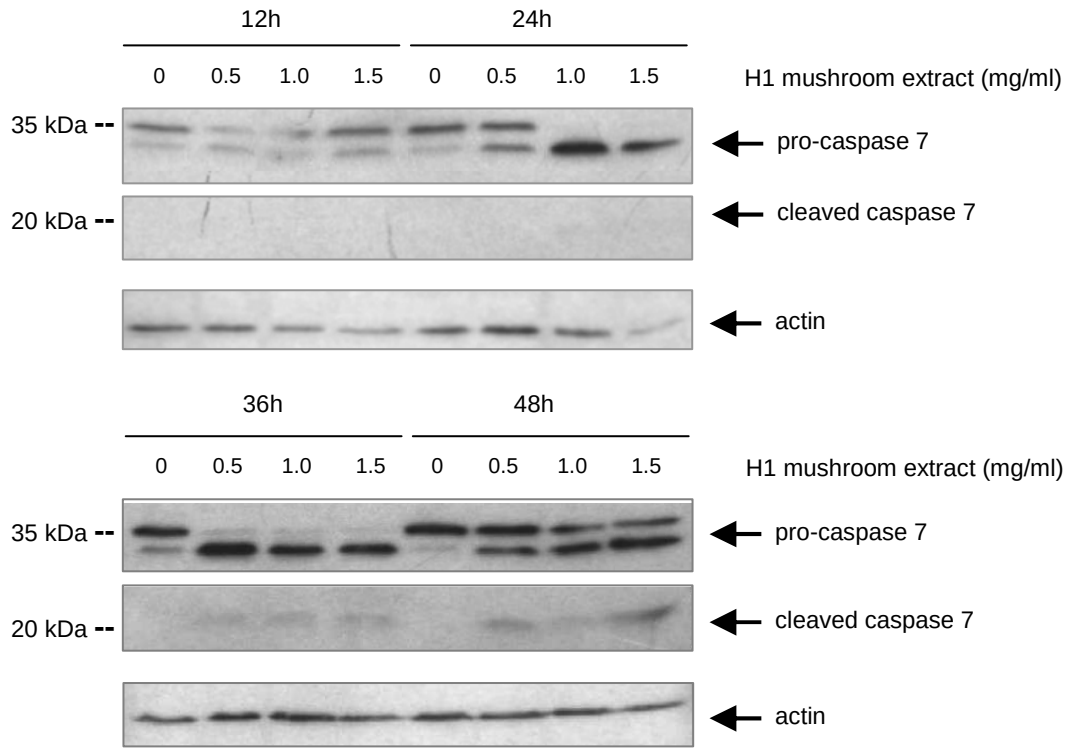


Figure 13: H1 mushroom extract induces activation of caspase-7. HL-60 cells were incubated with the indicated concentrations of mushroom extract for 12 hs, 24 hs, 36 hs and 48 hs. Western blot analysis showed the activation of caspase-7 resulting in time-dependent and concentration-dependent appearance of the p20 subunit. After being exposed, the membrane was stripped and blotted with anti-Actin antibody as a loading control.

Because caspase-3 is the most prominent downstream effector caspase, caspase-3 processing could happen via two different signaling pathways: (i) the death receptor-mediated signaling or (ii) the activation of mitochondria. We next examined these two possibilities by detecting caspase-9 subunit (p20) since detection of caspase-9 subunits would indicate processing of caspase-3 via the mitochondria signaling pathway. As shown in Figure 14, procaspase-9 was partly cleaved and the level of its active form increased time-dependently in HL-60 cell lines,

being especially significant after 36 hs and 48 hs, indicating that induction of the apoptotic cascade occurs via the mitochondrial signaling pathway.

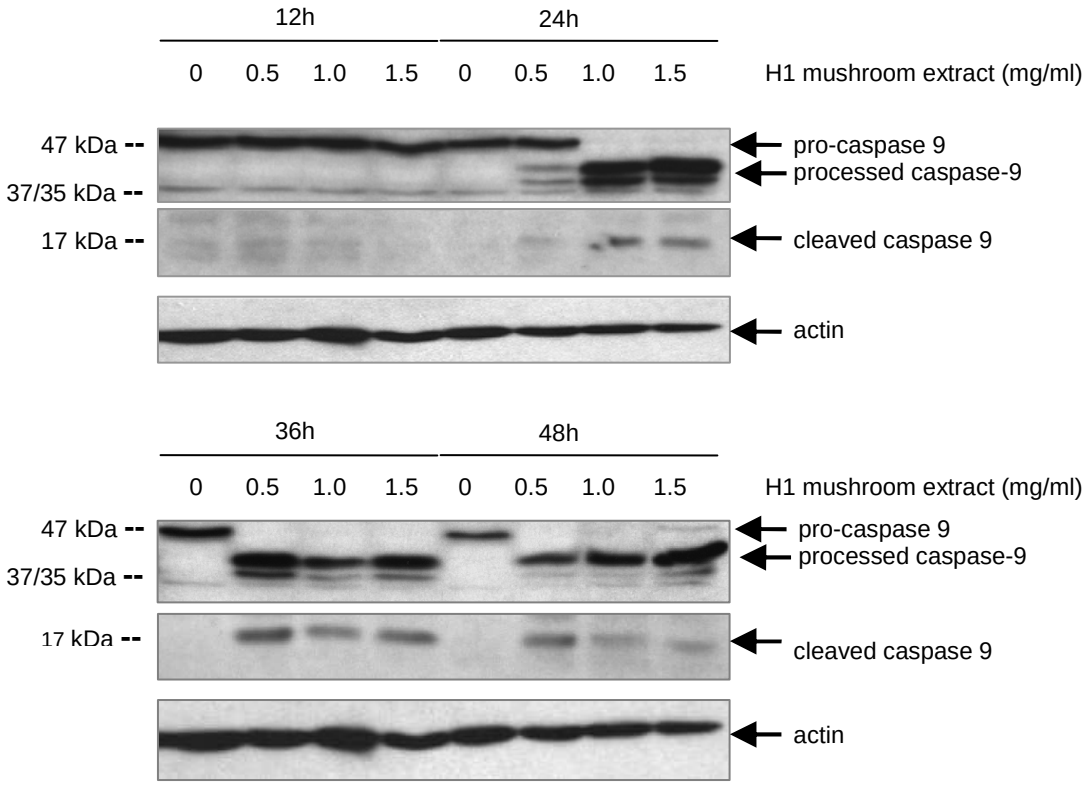


Figure 14: H1 mushroom extract induces activation of caspase-9. HL-60 cells were incubated with the indicated concentrations of mushroom extract for 12 hs, 24 hs, 36 hs and 48 hs. Western blot analysis showed the activation of caspase-9 resulting in time-dependent and concentration-dependent appearance of the p17 subunit. After being exposed, the membrane was stripped and blotted with anti-Actin antibody as a loading control.

To confirm this, mitochondrial potential alteration was examined upon mushroom treatment. Cytosolic-specific, mitochondria-free protein lysates were prepared. The release of cytochrom c from mitochondrial to cytoplasm was showed by visualising protein level of cytochrom c of cytosolic fraction by Western blot. As illustrated in Figure 15, treatment with H1 extract caused an increase of cytochrom c in the cytosolic fraction after 24 hs and more pronounced after 36 hs and 48 hs. Taking together, these results indicate that H1 extract potentially induces the mitochondrial, intrinsic pathway of apoptosis in HL-60 leukeamia cell line.

Bcl-2 expression was also analyzed but neither increase nor reduction of bcl-2 expression was detected (data not shown).

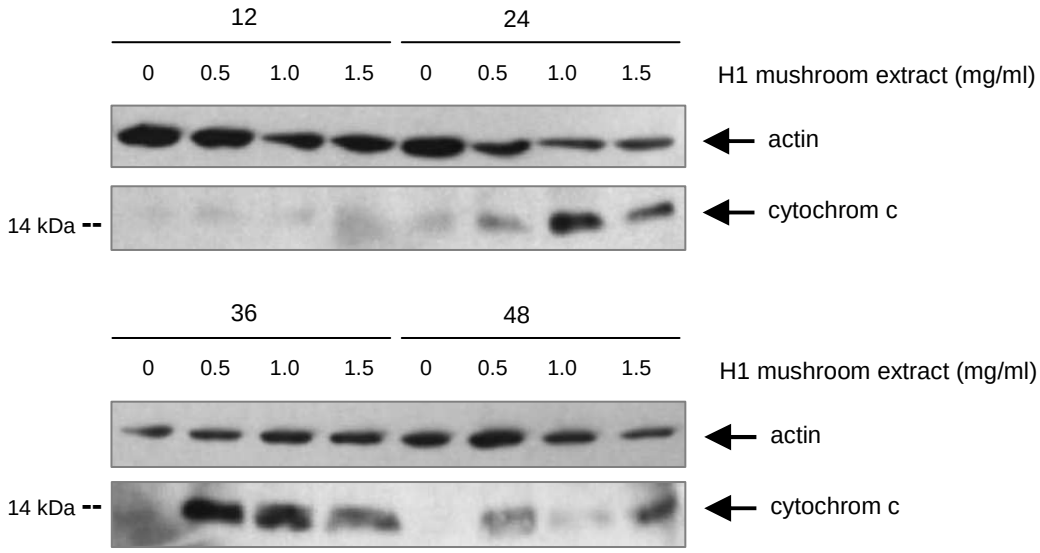


Figure 15: The cytochrome c protein level of cytosolic fractions of HL-60 cells treated with H1 mushroom extract. HL-60 cells were incubated with the indcated concentrations of H1 extract for 12 h, 24 h, 36 h and 48 h. Western blot analysis shows the presence of cytochrom c in cytosolic fractions after release of cytochrom c from mitochondria.

However, 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 death receptor pathway. Caspase-8 is the initiator caspase in this pathway therefore activities of caspase-8 was determined by Western blot analysis (Figure 16). Caspase-8 is synthesized as an inactive precursor of 55 kDa, which was detected as a double protein band, representing the isoforms procaspase-8a and procaspase-8b, and due to the induction of apoptosis, it was followed by the formation of a 43-kDa, 41-kDa intermediate cleavage product, processed to a p18 heterodimer. Interestingly, here in our case, the formation of intermediate cleavage products p43/p41 was evident while no mature caspase-8 subunit p18 was observed.

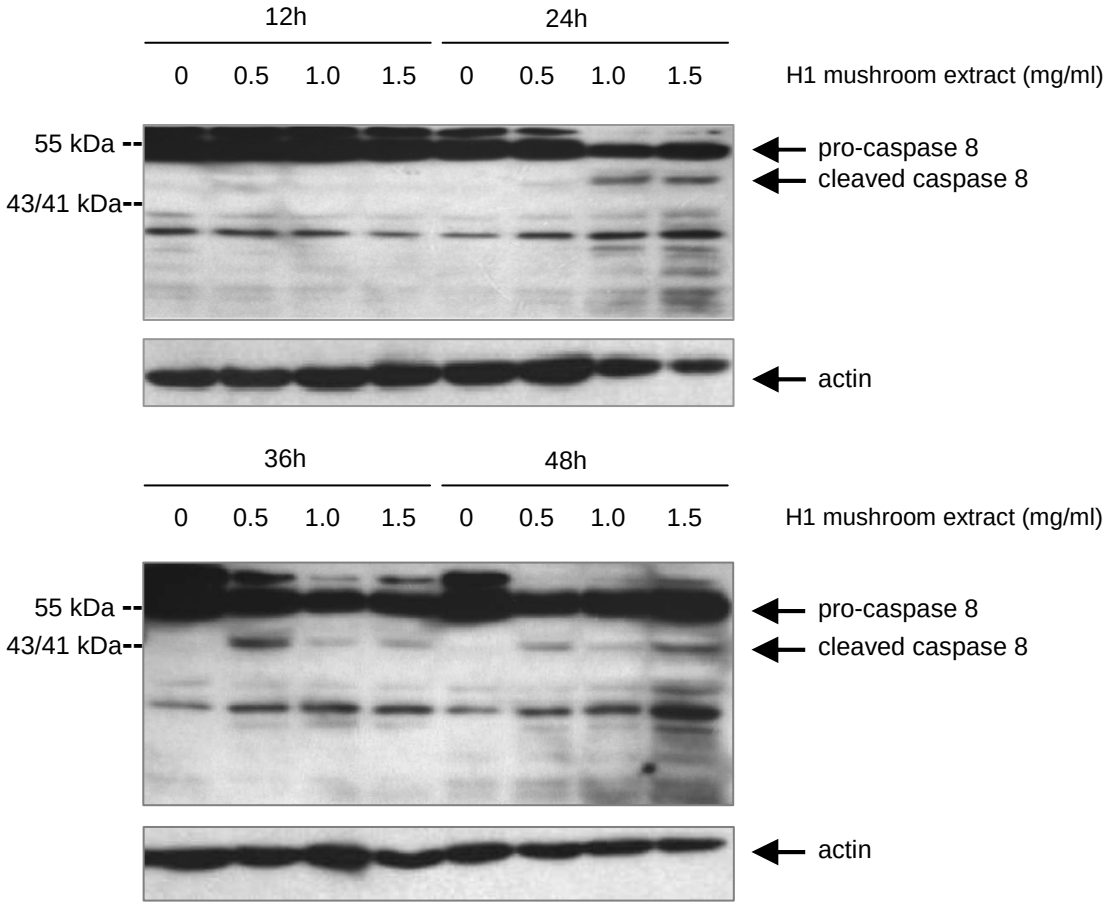


Figure 16: H1 mushroom extract induces activation of caspase-8. HL-60 cells were incubated with the indicated concentrations of mushroom extract for 12 hs, 24 hs, 36 hs and 48 hs. Western blot analysis showed the activation of caspase-8 resulting in time-dependent and concentration-dependent appearance of the p42/p41 subunit. After being exposed, the membrane was stripped and blotted with anti-Actin antibody as a loading control.

III.6. Proteome analysis of apoptosis induced by H1 mushroom extract in HL-60 cells

High resolution 2DE-PAGE and DIGE were used to identify proteins associated with apoptosis and other cellular pathways in HL-60 cells upon treatment of our mushroom extract. The basic requirement for reliable image analysis is the reproducibility of a series of good-quality 2-DE gels with low background staining. 2-DE gels of both protein lysates of untreated and treated cells from at least 3 different biological samples were prepared and compared.

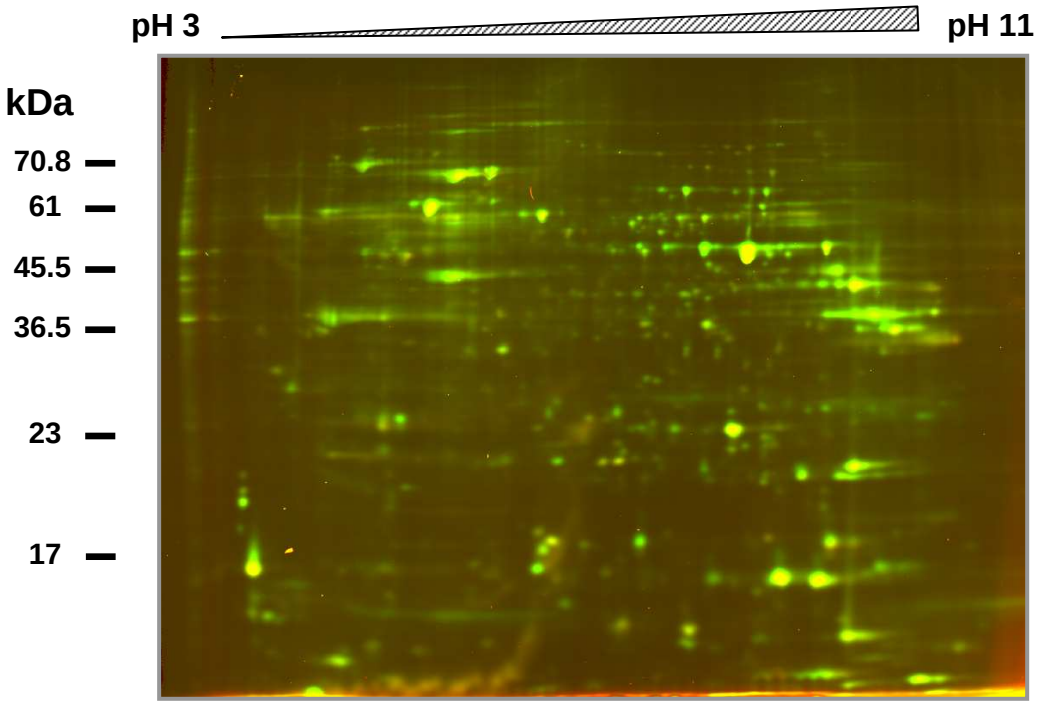


Figure 17: DIGE analysis of proteins from mushroom extract treated cells. Proteins of HL-60 cells treated with 1mg/ml H1 extract (Cy5, *red*) were compared with those of control sample (Cy3, *green*) by 2D-DIGE using 24-cm pH 3-11 IPG strips and a 12.5 % SDS-PAGE gel. Orientation of the pH gradients are indicated on the horizontal axes and approximately apparent molecular mass ranges are indicated along the vertical axes. Proteins increased upon treatment appear *red* and those repressed by H1 extract appear *green*, whereas those unaffected appear *yellow*.

The visual evaluation aimed for obviously intensity-different spots, especially spot which appeared on gels of one sample but not the other sample because such differences were more likely associated with apoptosis and were not the consequence of differences in sample

concentration or in the staining process. Therefore, only spots consistently regulated in all preparations were considered differentially expressed and chosen for further analysis.

Abundance ratio (as reported in table 3) was acquired as changes between Cy5 (treated) and Cy3 (untreated) gel images relative to the internal reference (Cy2).

Paired treated and control samples were combined and separated on the same 2D gel. Each gel was then sequentially scanned at the wavelengths specific for each fluorescent label and the images were overlaid digitally. The overlaid pattern (Cy3, Cy5 and Cy2) of protein spots generated in a non-linear pH 3-11 gradient immobilized pH gradient (IPG) strip was shown in Figure 17. This might be considered as a general picture of the merged proteomic profile of HL-60 control cell and HL-60 cell treated with H1 extract. Fluorescent green and red spots indicated spots of repressed and increased proteins upon treatment, respectively. Yellow spots reflect no significant change in protein regulation. Significant changes (+/- 1.4 fold in our case) in spot intensity were manually validated. The expression pattern was highly reproducible, however, minor differences in spot intensity were observed for few spots from gel to gel.

Several significantly altered spots have been detected in both untreated and treated cells. Altered spots were excised from preparative gels, digested with trypsin and mass spectrometric analysis was performed. Results of data bank searches and their degree of regulation were given in Table 3 and positions of corresponding spots on gels are shown in Figure 18. Enlarged 2D-view and 3D-view of certain altered spots are shown in Figures 19, 20 and 21.

The majority of spots were successfully identified only a few spots were unidentified due to low protein abundance. Among spots which were reproducibly detected, 29 were changed by H1 mushroom extract in comparison to the control (greater or equal to 1.4 fold). These 29 differently expressed proteins sequenced by MALDI-TOF-MS are listed in Table 3. Significant decrease of intensity was detected for heat shock protein beta 1, nucleoside diphosphate kinase whereas spot intensity was obviously increased for fragments of alpha-enolase, heterogeneous nuclear ribonucleaoprotein F, Rho GDP dissociation inhibitor 2 (D4-GDI) and phosphatidylethanolamine-binding protein 1.

Protein (1)	SwissProt Accession no. (2)	Description (3)	mW (Da) (4)	pI (pH) (5)	PLGS score (6)	Probability (%) (7)	Peptides (8)	Coverage (%) (9)	Abundance Ratio (10)	Biological function (11)
NDKA	P15531	Nucleoside diphosphate kinase A	17137	5,7662	11,4181	34,44	7	51,9737	-2,9	Antimetastatic activity
PEBP1	P30086	Phosphatidylethanolamine-binding protein 1	21043	7,3889	12,4482	96,48	9	58,2888	2,72	Binding protein
GDIS	P52566	Rho GDP dissociation inhibitor 2	22973	4,8983	8,1	1,25	5	29,8507	3,41	Cell communication & signal tranduction
HSP7C	P11142	Heat shock cognate 71 kDa protein	70854	5,1998	10,4181	12,67	18	33,5913	2,1	Cellular transport & chaperoning
HSPB1	P04792	Heat shock protein beta 1	22768	5,9588	12,484	100	7	37,561	-2,36	Cellular transport & chaperoning
TBB5	P07437	Tubulin beta chain	49638	4,5903	10,2614	10,83	12	29,0541	-1,7	Cell structure and related proteins
VIME	P08670	Vimentin	53619	4,8629	11,9875	60,86	31	60,9442	-1,69	Cytoskeleton conformation
ENOA	P06733	Alpha-enolase	47139	7,1713	12,0106	62,28	16	43,318	2,25	Glycolysis, ATP generation & transport
ALDOA	P04075	Fructose-bisphosphate aldolase A	39395	8,0645	10,8009	18,58	14	43,1319	1,64	Glycolysis, ATP generation & transport
PGAM1	P18669	Phosphoglycerate mutase 1	28785	6,7861	11,2638	29,52	10	57,0866	1,83	Glycolysis, ATP generation & transport
G3P	P04406	Glyceraldehyde-3-phosphate dehydrogenase	36030	8,6966	11,8621	53,69	10	35,2239	1,5	Glycolysis, ATP generation & transport
PRDX6	P30041	Peroxiredoxin-6	25019	5,9572	11,4671	36,17	11	52,6786	1,99	Lipid degradation
AK1A1	P14550	Alcohol dehydrogenase NADP	36549	6,3384	12,4684	98,45	9	30,1538	1,64	Metabolism, energy pathway
NUP50	Q9UKX7	Nucleoporin 50 kDa	50113	6,6951	10,7506	17,67	11	33,1197	-1,6	Nucleoctoplasmic transport
HNRH1	P31943	Heterogeneous nuclear ribonucleoprotein H	49198	5,8513	11,7884	49,88	16	44,098	-1,88	RNA metabolism
HNRPF	P52597	Heterogeneous nuclear ribonucleoprotein F	45642	5,2414	11,6551	43,65	12	40,9639	-1,41	RNA metabolism
HNRPL	P14866	Heterogeneous nuclear ribonucleoprotein L	60149	6,6826	12,4819	99,79	16	29,7491	1,55	RNA metabolism
ROA1	P09651	Heterogeneous nuclear ribonucleoprotein A1	38822	9,4587	11,3823	33,23	9	30,1075	-1,82	RNA metabolism
ROA3	P51991	Heterogeneous nuclear	39570	9,2212	11,3836	33,27	11	31,2169	-1,85	RNA metabolism

		rebinucleoprotein A3								
(1)	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**	**(7)**	**(8)**	**(9)**	**(10)**	**(11)**
HNRPM	P52272	Heterogeneous nuclear ribonucleoprotein M	77464	9,0037	12,3299	85,72	21	31,3699	-1,41	RNA metabolism
STIP1	P31948	Stress-induced-phosphoprotein 1	62599	6,3858	12,3634	88,64	10	23,0203	1,61	Stress response
CH60	P10809	60 kDa heat shock protein mitochondrial precursor	61016	5,5503	12,4685	98,46	19	40,8377	1,7	Stress response
PDIA3	P30101	Protein disulfide isomerase A3 precursor	56746	5,9299	11,3866	33,37	19	36,6337	1,47	Stress response
F10A1	P50502	Hsc70-interacting protein	41305	4,9966	11,4949	37,19	10	27,6423	1,87	Stress response
TCPE	P48643	T-complex protein 1 subunit epsilon	59632	5,3036	11,476	36,49	20	32,1627	1,29	Stress response
CATA	P04040	Catalase	59718	6,9716	12,4711	98,72	16	34,1556	1,53	Stress response, antioxiant enzyme
FUBP1	Q96AE4	Far upstream element binding protein 2	67518	7,4264	12,4785	99,45	17	29,6584	-1,56	Transcription
IF32	Q13347	Eukaryotic translation initiation factor 3 subunit 2	36478	5,2615	11,5989	41,27	11	38,4615	-1,6	Translation initation
MDHM	P40926	Malate dehydrogenase mitochondrial precursor	35508	8,8202	11,6093	41,7	14	51,4793	1,81	Tricarboxylic, acid cycle

Table 3: Altered spots identified by mass spectrometric analysis.

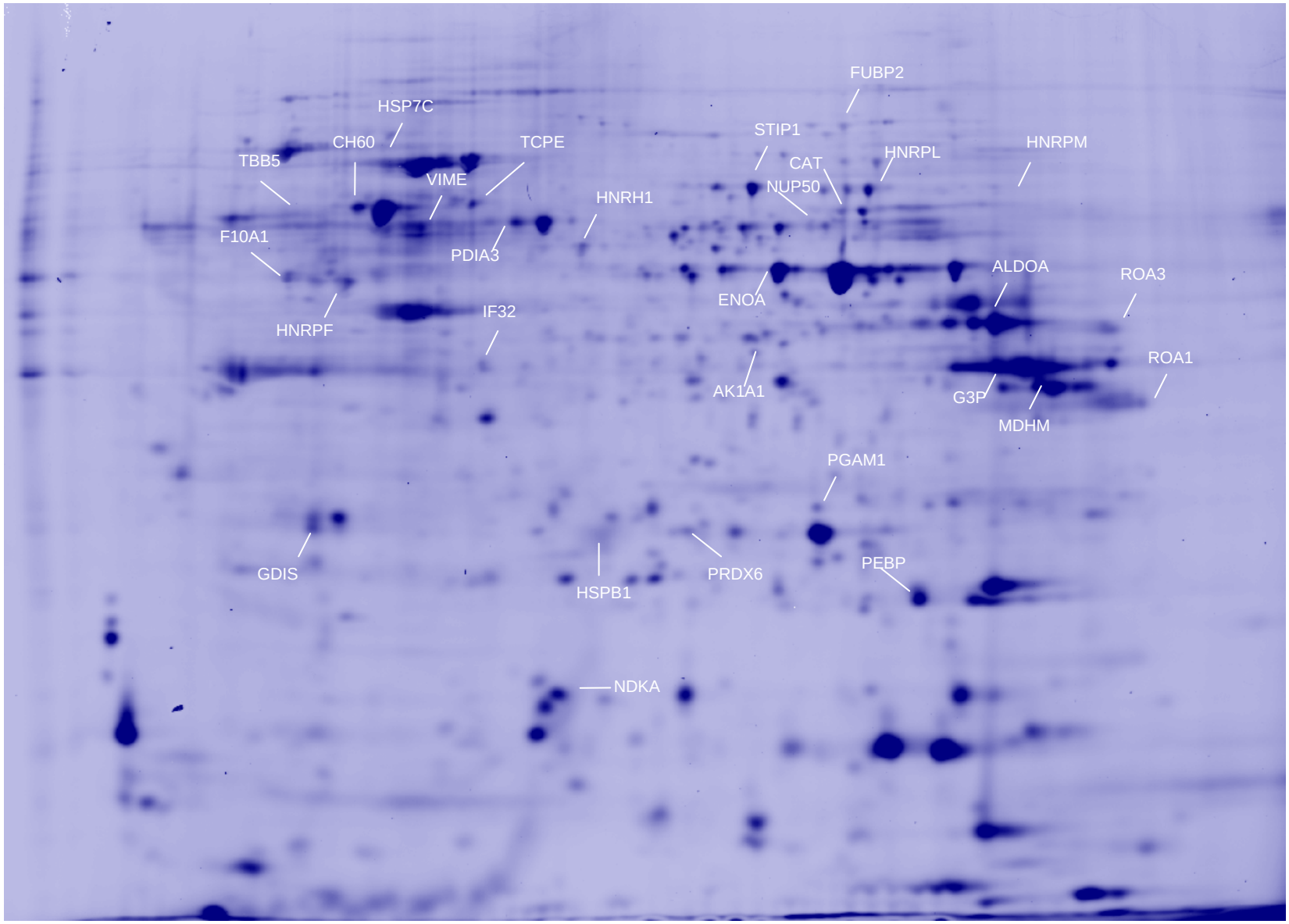


Figure 18: Proteome maps indicating the position of altered proteins identified.

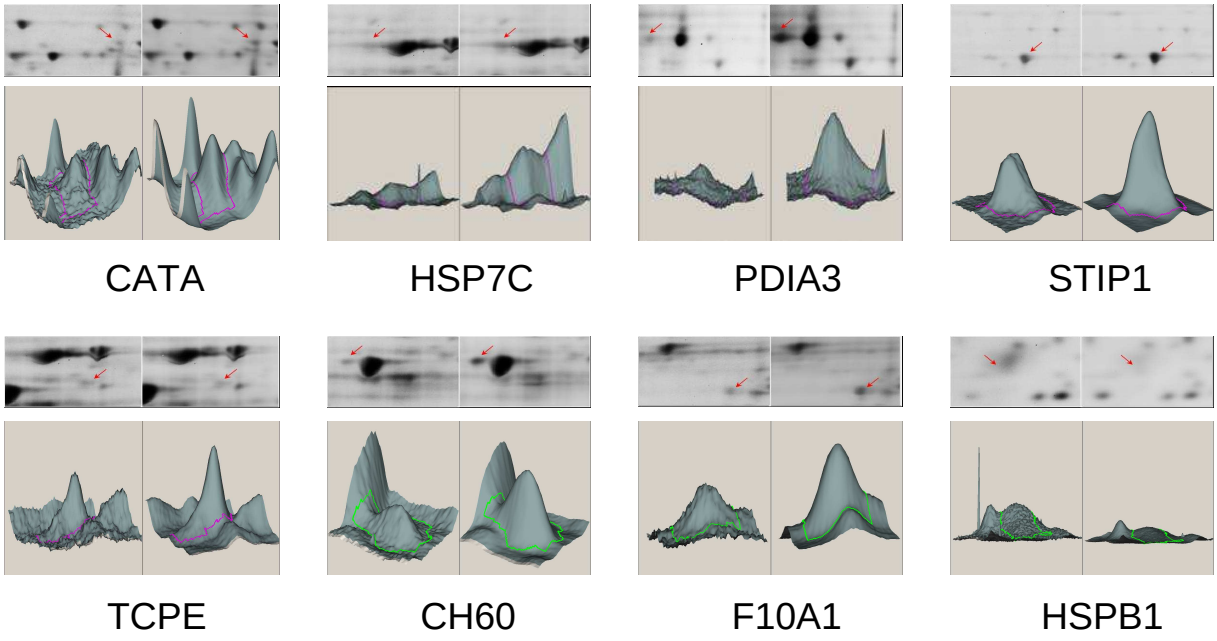


Figure 19: Enlarged 2D-view and 3D-view of altered spots of proteins which have stress reponse or chaperone function

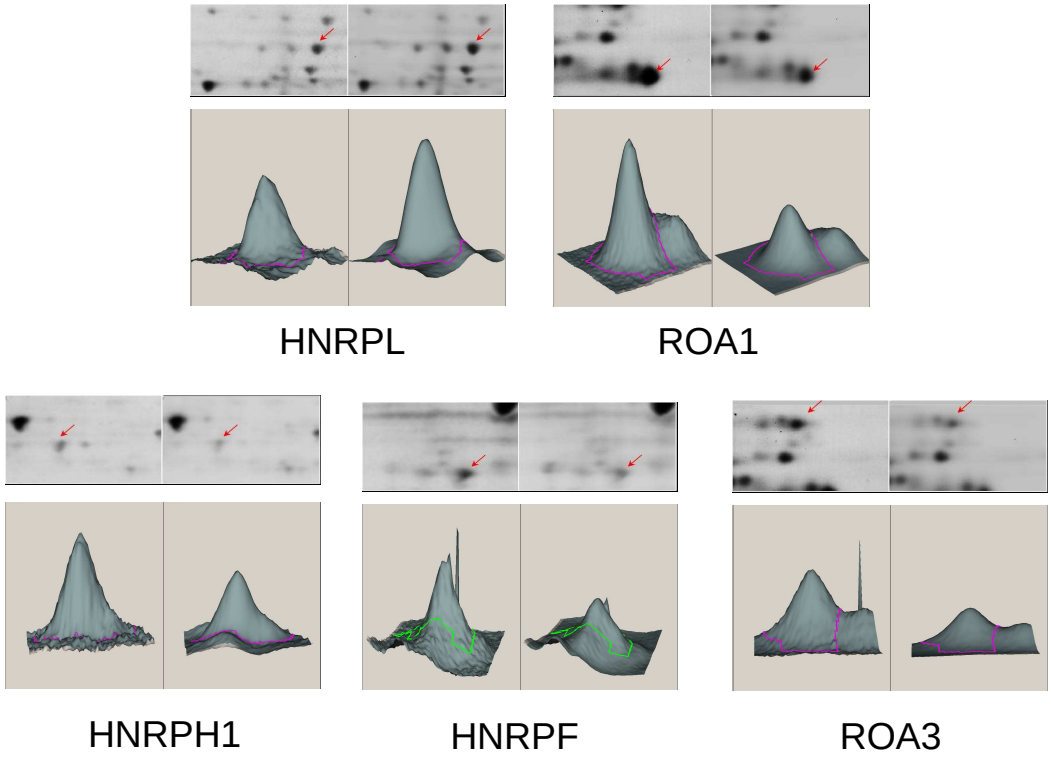


Figure 20: Enlarged 2D-view and 3D-view of altered spots of proteins which belong to HNRP protein family.

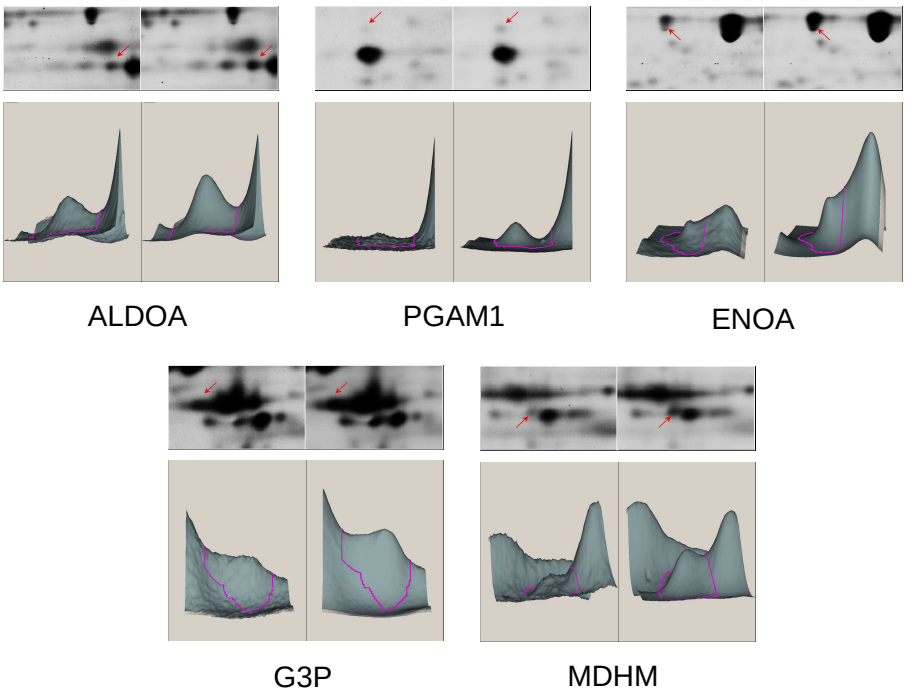


Figure 21: Enlarged 2D-view and 3D-view of altered spots of proteins which involved in glycolysis and tricarboxylic acid cycle.

Differently expressed proteins identified are involved in various cellular processes. Heat shock cognate 71 kDa protein (HSP7C), heat shock protein beta 1 (HSPB1), stress-induced-phosphoprotein 1 (STIP1), 60 kDa heat shock protein mitochondrial precursor (CH60), Protein disulfide isomerase A3 precursor (PDIA3), Hsc70-interacting protein (F10A1), T-complex protein 1 subunit epsilon (TCPE) and catalase (CATA) play roles in stress response, cellular transport and chaperone (Figure 19). Involved in RNA metabolism are the Heterogeneous nuclear ribonucleoprotein family including the heterogeneous nuclear ribonucleoprotein H, F, L, A1, A3 and M (Figure 20). Another large group is the group of proteins essential for glycolysis, ATP generation and transport, such as alpha-enolase (ENOA), fructose-bisphosphate aldolase A (ALDOA), phosphoglycerate mutase 1 (PGAM1) and glyceraldehydes-3-phosphate dehydrogenase (G3P) (Figure 21); protein metabolism; DNA repair; cell structure; cell communication and signal transduction, etc.

III.7. Cleavage of D4-GDI upon treatment with H1 mushroom extract

One of the most variable spots was identified as D4-GDI (GDIS). This alteration was reproducible although searches on data bank showed very low probability as shown in Table 3. The effect of mushroom extract on the expression of Rho guanine nucleotide dissociation inhibitor (GDI) 2 found in proteomic study was confirmed by western blotting using a rabbit polyclonal antibody. As illustrated in Figure 22, in the untreated sample, the 27/28-kDa band was obvious while nearly no signal was detected for the 23-kDa band. D4-GDI cleavage was induced after 36 hs upon treatment with H1 mushroom extract. The 27-kDa D4-GDI band was specifically cleaved to a 23-kDa fragment. The uncleavable 28-kDa Rho-GDI and the uncleaved 27-kDa D4-GDI appear as a single band in the Western blot, which can only be resolved by short exposure time.

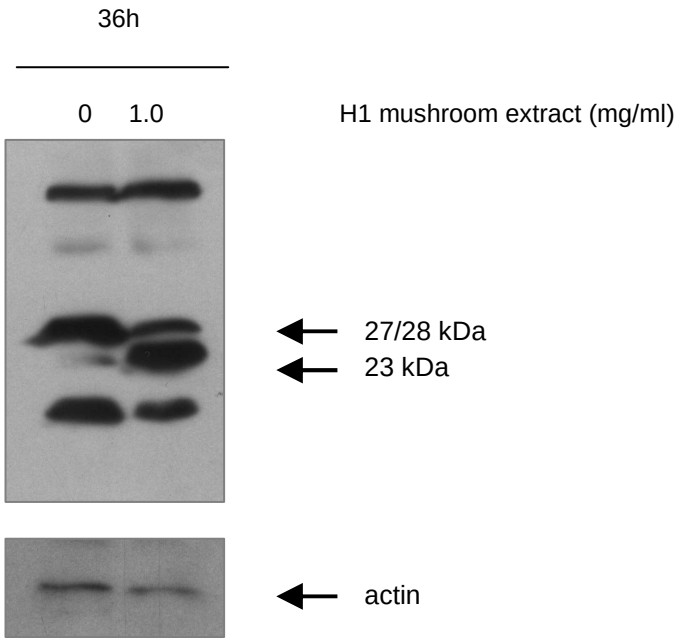


Figure 22: H1 mushroom extract induces cleavage of D4-GDI. HL-60 cells were incubated with the concentration 1mg/ml of mushroom extract for 36 hs. Cell lysates were prepared and analyzed by Western blotting using a Rho-GDI-specific polyclonal antiserum (which cross-reacts with D4-GDI). The first arrow on the right pointed out the 27-kDa D4-GDI and the 28-kDa Rho-GDI band. Western blot analysis showed the cleavage of D4-GDI resulting in the appearance of the 23-kDa cleavage product. After being exposed, the membrane was stripped and blotted with anti-Actin antibody as a loading control.

III.8. miRNA expression level analysis in HL-60 treated with H1 mushroom extract

After getting information about the proteomic profile of HL-60 cells upon treatment with H1 mushroom extract, in order to gain insights into the signaling cascades, it is essential to understand the regulatory machine in which miRNAs act as one of the most crucial part. MicroRNAs represent a new class of gene products, which are believed to specifically control the translation and stability of target mRNAs based on (partial) sequence complementarity. They play an important role in many biological processes. There are up to date about more than 800 miRNAs identified in *Homo sapiens* (according to miRBase), but biological roles of not many of them have been experimentally validated. Determining specific expression patterns for miRNAs of cells in different conditions would provide important evidences regarding their regulatory functions. Moreover, data about distinct miRNA expression patterns would be very useful in investigating predicted mRNA targets of different miRNAs and also the interrelation between the differentiation in protein expression and miRNA expression.

In order to obtain the differential expression information for miRNAs from human leukeamia HL-60 cells upon treatment with mushroom extract, miRNA expression was evaluated by micro array and real-time RT-PCR analysis. Total RNA which was isolated from untreated cells and cells treated with mushroom extract was prepared and probed by real-time RT-PCR or used for miRNA enrichment for further screening by micro array.

Comparison between H1 mushroom extract-treated sample and untreated sample resulted in large differences in miRNA expression profile. Utilising NCode Multi-Species miRNA micro array, 50 miRNAs were detected (as shown in Figure 23). Strikingly, expression of 16 miRNAs in the mushroom-treated versus untreated sample has been changed by more than 2-fold.

One of the features of miRNAs is that they can occur in clusters on the genomic DNA that are co-expressed [Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001]. Our micro array analysis revealed that such miRNAs were also co-expressed and found in clusters: such as 15b-cluster (including hsa-miR-15b and hsa-miR-16-2), 16-1-cluster (including hsa-miR-16-1 and hsa-miR-15a), 25-cluster (including hsa-miR-25, hsa-miR-93 and hsa-miR-106b) and 17-cluster (including hsa-miR-17, hsa-miR-18, hsa-miR-19a, hsa-miR-19b, hsa-miR-20 and hsa-miR-92). Expression patterns of individual miRNA in each cluster are relatively similar indicating that these clustered miRNA families are not regulated upon mushroom extract treatment.

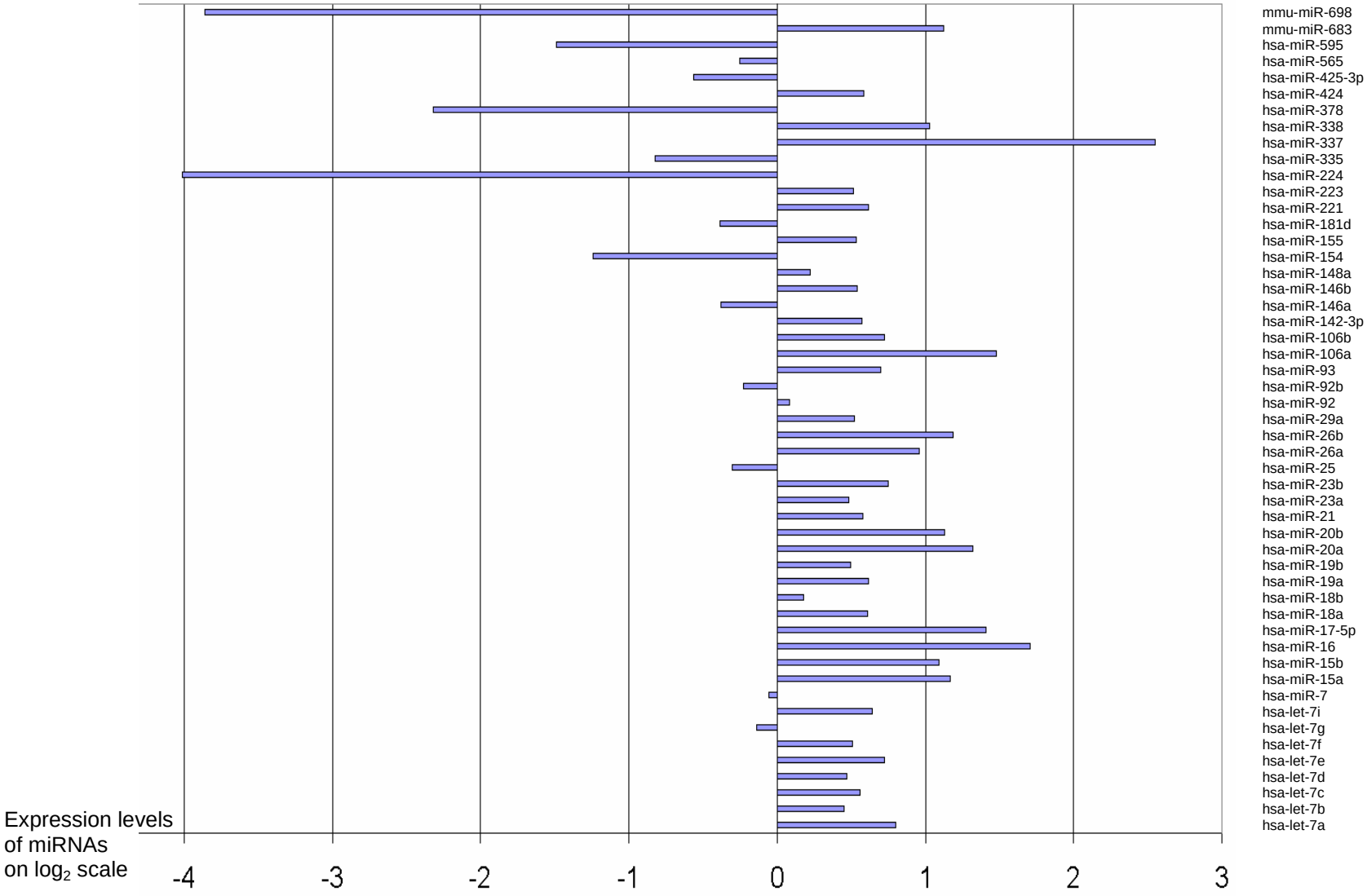


Figure 23: Comparative expression of miRNAs in HL-60 (mushroom extract-treated versus untreated sample). Bars represent deviations from mean expression levels for each miRNA on a $\log_2$ scale.

It has been reported that several miRNAs were strongly expressed in specific tissues, for instance, miR-1 variants in heart, miR-122 variants in liver or miR-124 variants in brain [Lee, 2001]. miRNAs which are specific for cells which belong to haematopoietic tissue, including miR-142-3p, miR-155, miR-181, and miR-223 [Ramkissoon *et al.*, 2006] usually expressed at high levels in our micro arrays. In addition, let-7 miRNA variants, miR-16, miR-21, miR-26a which are abundant in many tissues or cells [Lagos-Quintana *et al.*, 2003] also appeared in our micro arrays.

hsa-miR-224, hsa-miR-337, hsa-miR-378 and mmu-miR-698 exhibited a relatively high level of alteration in mushroom-treated versus untreated sample. U6 was used as a control to indicate ubiquitous miRNA expression.

A.

```
   g  c                ugu      ccu
agg cu cugacuccaggucc   guguua   a
||| || ||||||||||||||   ||||||
ucc ga gacugagguucagg   cacgau   g
   g  a                --u      aaa
```

B.

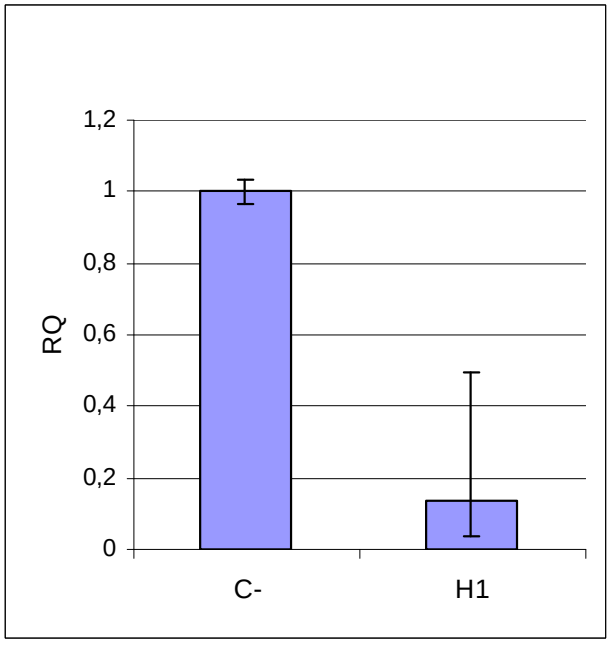


Figure 24: hsa-miR-378 expression upon treatment of H1 mushroom extract in human leukeamia cell HL-60. A. *Homo sapiens* miR-378 stem-loop with mature sequence in red. B. Expression of has-miR-378 in untreated (sample C-) and treated (sample H1) human leukeamia cell HL-60 assessed by miRNA-specific real-time qRT-PCR.

To verify miRNA expression, selected miRNAs were chosen and their relative expression levels were quantified using real-time RT-PCR. Notably, a close relation between real-time RT-PCR and microarray was found. The differences in expression of hsa-miR-338 and hsa-miR-378 evaluated by real-time RT-PCR were shown here.

As depicted in Figure 24, hsa-miR-378 (previously called has-miR-422b) has been identified as the most abundant miRNA cloned from human leukeamia HL-60 cells [Kasashima *et al.*, 2004]. hsa-miR-378 was expressed strongly in untreated HL-60 cells whereas only quite weak signal was detected upon treatment with mushroom extract.

A.

```
   c      u   -         -   gauga
ucu caacaa auc cuggugcug agu     c
||| |||||| ||| |||||||||| |||     u
aga guuguu uag gacuacgac uca     c
   a      u   u         c   gcgga
```

B.

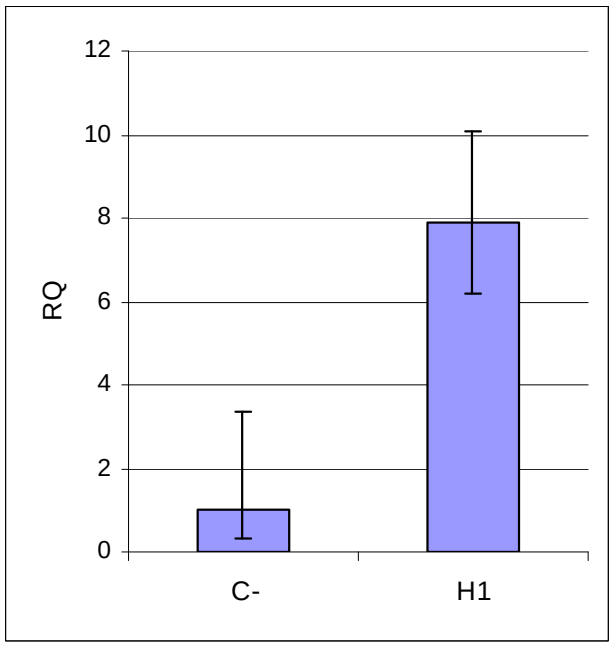


Figure 25: hsa-miR-338 expression upon treatment of H1 mushroom extract in human leukeamia cell HL-60. A. *Homo sapiens* miR-338 stem-loop with mature sequence in red. B. Expression of has-miR-338 in untreated (sample C-) and treated (sample H1) human leukeamia cell HL-60 assessed by miRNA-specific real-time qRT-PCR.

The difference in expression of hsa-miR-338 was also confirmed by real-time qRT-PCR. In contrast, the level of expression of hsa-miR-338 was over-expressed upon mushroom treatment, as depicted in Figure 25.

In summary, 16 differently expressed miRNAs were detected in an effort to provide a more comprehensive view into molecules of the regulatory machine of HL-60 cells upon treatment with H1 mushroom extract. This is important to understand the posttranscriptional regulation of gene expression.

IV. DISCUSSION

Here in our study, many evidences about molecular mechanisms of apoptosis induced in HL-60 upon treatment of water-soluble extract of tropical medical mushroom *Phellinus pachyphloeus* are elucidated.

Medical mushrooms such as *Ganoderma lucidum*, *Lentinus edodes* and many others have historically been used to remedy different diseases in eastern traditional folk medicine and ethno-pharmacology. Many traditionally used mushrooms have been explored for novel medical properties and have evoked considerable interest as anti-tumour agents especially in the context of apoptosis induction [Wasser, 2002].

Apoptosis is a defined program of cell death that is markedly influenced both positively and negatively by a variety of genes, many of which are mutated and/or dysfunctionally regulated in human cancers [McGill & Firscher, 1997] and therefore is a very important aspect in cancer treatment. There are many reports regarding the ability of mushrooms to induce apoptosis in various tumour models [Hsieh *et al.*, 2006; Sliva *et al.*, 2008; Müller *et al.*, 2006].

Within the framework of our project we focused on *Phellinus pachyphloeus* which is an abundant tropical mushroom [Wagner & Firscher, 2002]. Many species of the *Phellinus* genus such as *P. igniarius*, *P. robustus* and *P. linteus* are reported to possess anti-tumour activities [Song *et al.*, 2008; Ajith & Janardhanan, 2007; Choi *et al.*, 2004]. *Phellinus pachyphloeus* has often been used in the form of aqueous extract in eastern traditional folk medicine but no attempt has been made so far to elucidate its molecular properties. Therefore, in this study we investigated the molecular action of identical aqueous extracts in HL-60 human leukeamia cell line. Of special interest was the elucidation of the molecular action of crude water-soluble extract from *P. pachyphloeus* to govern anti-proliferation, cell cycle and apoptosis in HL-60 human promyelocytic leukaemia cells. Moreover, expression of an abundance of specific molecular targets concerning these processes such as apoptogenic/anti-apoptotic and extracellular signalling regulatory proteins and related miRNAs upon treatment with mushroom have unveiled.

Crude water-soluble extract, not fractionated compounds and substances, have been long used in eastern traditional medicine. Results from many investigations suggested that constituent

compounds from crude extracts could potentially have synergistic effects in the prevention and treatment of cancer while their action could also interfere with each other's [Borschers *et al.*, 2004]. In our study, we also used crude water-soluble extract of *P. pachyphloeus* without further fractionation.

Many investigators doubt that wild mushrooms may be contaminated with toxic metals such as arsenic, lead, cadmium as well as radioactive substances because many mushrooms have the ability to accumulate relatively high concentrations of heavy metals [Borschers *et al.*, 2004]. Therefore, before performing all other experiments, our extract was tested for the heavy metals' content. Amounts of heavy metals at even the highest extract concentration we used for experiments were much lower than allowed levels. Extract in the concentration range of treatment showed no cytotoxic effect in HL-60 and other cell lines such as Hela, Vero-B4, etc. (data not shown). But it could significantly dose-dependently suppress the proliferation of HL-60 cells *in vitro*.

There are many reports showing that crude water extract of many medical mushrooms inhibit cell proliferation of several kinds of cell lines through apoptosis induction [Wasser, 2002]. Aqueous extract from *Agaricus blazei* inhibited the growth of human gastric epithelial AGS cells through cell cycle arrest at the G2/M phase and the induction of caspase-3-dependent apoptosis [Jin *et al.*, 2006]. *Ganoderma lucidum* inhibited the growth of MDA-MB-231 breast cancer cells by modulating Akt/NF-κB signaling [Jiang, 2004]. *Phellinus linteus*, another well-known medical mushroom which belongs to the same genus as ours, induced apoptosis in human neuroblastoma cell by caspase-3 activation through an up-regulation of bax [Choi *et al.*, 2004]. From results reported here it was clear that antiproliferative activity of our mushroom extract was manifested by induction of apoptosis determined by the presence of sub-G1 peak with flow cytometry measurement and by DNA laddering.

We attempted to clarify which pathway – the death receptor (extrinsic) or the mitochondrial (intrinsic) pathway – was involved in *P. pachyphloeus* extract – induced apoptosis. In the intrinsic pathway, apoptosis inducers cause release of cytochrome c from mitochondria which consequently activate caspase-9 and caspase-3, the latter then triggers apoptosis. The extrinsic pathway is caused by the direct interaction between so-called "death ligands" and "death receptors", in which caspase-8 is activated [Debatin, 2004]. It seems that apoptosis caused by extracts from mushrooms is regulated through intrinsic pathway [Kim *et al.*, 2007]. Aqueous extracts of I'm-Yunity (PSP) inhibited cell proliferation and induced apoptosis in HL-60 and

U-937 cells accompanied by the expression of anti-apoptotic proteins bcl-2 and survivin, the increasing in apoptogenic proteins bax and cytochrome c, and the cleavage of poly (ADP-ribose) polymerase (PARP) [Hsieh *et al.*, 2006]. In HL-60 cells, the activation of caspase-3, caspase-6, caspase-7, caspase 8, and caspase-9 was observed upon treatment with *P. pachyphloeus* mushroom extract. So both the activation of a proximal caspase, such as caspase-8 for extrinsic receptor-mediated apoptosis and caspase-9 for intrinsic mitochondrial-mediated apoptosis were observed. Western blotting analysis also revealed the release of cytochrome c. This further supports our current suggestion that apoptosis induction by our mushroom extract is mediated via both intrinsic and extrinsic pathways.

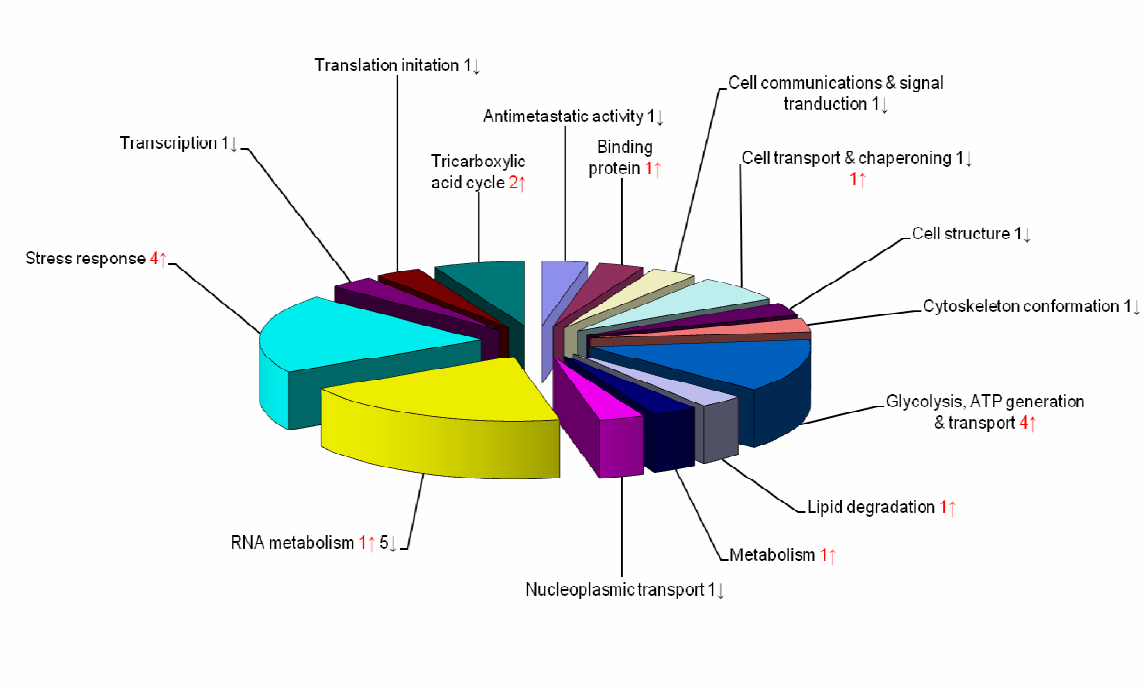


Figure 26: Funtional classification of differentially expressed proteins identified from cells upon treatment with H1 mushroom extract. For each category the numbers of up- & down-regulated proteins are indicated by arrows.

To understand the mechanism of apoptosis induced by mushroom extract, we checked the possible molecular targets upon treatment with the extract in HL-60 by proteomics experiments. The DIGE technique has been proved to be a highly sensitive and dynamic method which facilitated the detection of significantly regulated proteins from HL-60 cells upon treatment with H1 mushroom extract. Our proteomic study revealed 29 proteins with differentially regulated expression levels in cells treated with mushroom extract and the control. These proteins play important roles in variety of pathway including stress response,

cytoskeleton, ATP synthesis, RNA metabolism, etc (Table 3). The data about functions of these proteins was represented as a pie chart above (Figure 25). This was based on i) the extent to which the protein was up- or down-regulated and ii) a detailed investigation into their currently known roles as described in the scientific literature.

The most differently expressed protein spots of cells treated with mushroom extract and untreated cells were identified as various heterogeneous nuclear ribonucleoprotein (hnRNPs) and Rho guanine nucleotide dissociation inhibitor (GDI) 2.

Rho-GDI 2 was identified up-regulated in the mushroom extract-treated sample comparing to untreated one. Rho-GDI 2 is a GDP dissociation inhibitor for the Ras-related Rho family GTPase in haematopoietic cells during apoptosis and the cleavage of Rho-GDI 2 has been demonstrated during CD95/Fas-induced apoptosis and occurs by a caspase-3-dependent mechanism [Na, 1996]. Rho-GDI 2 was found more than 2 fold increase of expression as well in mouse spleen mononuclear cells treated with polysaccharides from spores of *Ganoderma lucidum* [Ma *et al.*, 2008]. Rho-GDI 2 is regulated during apoptosis induced by daunorubicin, an anti-cancer drug, in HL-60 cells through the activation of caspase-3 [Kwon *et al.*, 2002]. Owing to its crucial role as a modulator of Rho GTPase, this might in turn have a significant impact on the mechanisms that induce the cytoskeletal and morphological changes in apoptotic cells.

Heterogeneous nuclear ribonucleoproteins (hnRNP) have central roles in DNA repair, cell signalling and in regulating gene expression at both transcriptional and translational levels and changes of balance among hnRNPs can contribute to apoptosis induction [Carpenter *et al.*, 2006]. Upon treatment with our mushroom extract, six hnRNPs was identified to be altered after induction of apoptosis: hnRNP L were up-regulated and hnRNP A1, A3, F, H and M were down-regulated. Involvement of hnRNP proteins have been demonstrated for the hnRNPs A0, A1, A2/B1, A3, C1/C2, R and U [Brockstedt *et al.*, 1998; Waterhouse *et al.*, 1996; Thiede *et al.*, 2001]. HnRNP A1 is a dephosphorylated protein that might be cleaved into different cleavage products after apoptosis induction [Hermann *et al.*, 2001].

Treatment with extract from *P. pachyphloeus* also caused the up-regulation of several proteins such as alpha anolase, phosphoglycerate mutase 1, phosphoglycerate kinase 1, and adenylate kinase isoenzyme 2 which play a role in glycolysis, ATP generation and transport [López-Pedrera *et al.*, 2006]. It is known that increased rates of glycolysis are generally found in tumour cells [Przybytkwoski *et al.*, 2007]. But here we monitored the opposite direction of

regulation. This might be explained due to the large content of polysaccharides that are present in our extract.

There are some common aspects between apoptosis induced by our mushroom extract and apoptosis induced by staurosporine. Apoptosis induction by both resulted in up-regulation of heat shock cognate 71, stress-induced phosphoprotein 1 and protein disulfide isomerase A1 precursor and in down-regulation of vimentin [Short *et al.*, 2007]. Protein disulfide isomerase A3 precursor and stress-induced phosphoprotein 1 play important roles in stress response. Heat shock cognate 71 (hsp70) and protein disulfide isomerase are endoplasmic reticulum chaperone. Several reports emphasize chaperones have a complex role in apoptosis. Although, the cytoprotective effect of hsp70 is related to their ability to disable apoptosis [Garrido *et al.*, 2006], our study indicates that there is an inverse relationship between the level of hsp70 and the rate of apoptosis induced by HL-60 upon treatment of mushroom extract. There are also reports that hsp70 can promote receptor-mediated apoptosis [Liossis *et al.*, 1997]. On the other hand, in non-muscle cells, vimentin is one of the characteristic proteins of intermediate filaments [Izdebsja, 2006]. Vimentin is rapidly proteolyzed by multiple caspases into similar sized fragments during apoptosis induced by many stimuli [Byzn *et al.*, 2001]. Caspase cleavage of vimentin disrupts its cytoplasm network of intermediate filaments and coincides temporally with nuclear fragmentation [Morishima, 1999]. Moreover, it has become increasingly clear that the induction of apoptosis in tumour cells can occur by at least three different pathways involving the cell surface receptors, the mitochondria and the endoplasmic reticulum [Cory & Cory, 2007]. Taken together, our findings suggested that endoplasmic reticulum stress pathway also might involve in mushroom extract-induced apoptosis.

Also, although the 2D DIGE separation technology is capable of resolving several thousand individual protein spots on a single gel, not all proteins were resolved because of extremes of size or pI, or because of gel feature overlap in some areas. Furthermore, many low-abundance proteins were presumably not detected. Some of these limitations could be addressed by altering separation techniques to increase resolution (e.g., by using narrow-range pI separations or various acrylamide concentrations). Finally, some of the proteins might have been detected on the gels but not identified by MS. Improvements in the sensitivity of MS techniques and/or improved database searching algorithms should reduce identification failures in the future.

Furthermore, we have identified several miRNAs differentially expressed in human leukaemia HL-60 cells upon treatment with H1 mushroom extract.

MicroRNAs (miRNAs) belong to a new class of regulators of gene expression. Initially discovered as regulators of developmental timing in invertebrates [Lee *et al.*, 1993], miRNAs have subsequently been implicated in a variety of biological processes including proliferation, differentiation and also in apoptosis [Alvarez-Garcia & Miska, 2005; Wienholds & Plasterk, 2005; Carthew, 2006]. Several recent studies have shown many evidences for the involvement of miRNAs in cancers and also indicated that miRNAs play important role in cancer formation and regulate cell growth and apoptosis [Stefani, 2007].

As reviewed by Park and Peter [Park & Peter, 2008], studies in many tissues have revealed that there are miRNAs with pro- or anti-apoptotic functions. While miR-34 [Chang *et al.*, 2007], miR-15a, miR-16-1 [Cimmino *et al.*, 2005] and miR-29 [Mott *et al.*, 2007] represent examples of miRNAs which show pro-apoptotic function; miR-21 [Chan *et al.*, 2005], part of miR-17-92 cluster [Matsubara *et al.*, 2007] have been proposed to inhibit apoptosis. In our micro array screening, these miRNAs expressed but expression levels of them were not much changed. Furthermore, in present study, miRNAs which are specific for hematopoietic cells such as miR-142, miR-155, miR-181, miR-223 [Ramkissoon *et al.*, 2006] also expressed at high level in human leukeamia HL-60 cells.

Upon treatment of our mushroom extract, while miR-337 over-expression has been clearly observed, the most highly down-regulated miRNAs were miR-224, miR-378 and miR-698. These regulation differences were shown in our microarray experiment and then confirmed by real-time PCR. When performing searching capable targets for these miRNAs in microRNA.org [Betel *et al.*, 2008], we found that some of our differently expressed proteins are also their targets. Phosphoglycerate mutase 1 and Fructose – biophosphate aldolase A which have been found over-expressed are targets of the down-regulated miR-378. Catalase and heterogeneous nuclear ribonucleoprotein K are negatively regulated by miR-224. Heterogeneous nuclear ribonucleoprotein A3 which shows down-regulated has target sequence for both miR-338 and miR-224.

Murakami *et al.* [Murakami *et al.*, 2006] investigated miRNA expression profiles of hepatocellular carcinoma (HCC) and adjacent nontumourous tissue and found that miR-224 was significantly over-expressed in HCC compared with nontumour tissues. miR-224 expression was further found to be inversely correlated with apoptosis inhibitor-5 (API-5) expression in HCC patients [Wang *et al.*, 2008]. miR-224 was also shown to be over expressed in prostate tumours with perineural invasion compared to those without it [Prueitt *et al.*, 2008]. Our finding identified and validated miR-224 down-regulation in HL-60 after

treatment with mushroom extract. This outcome strongly suggests that miR-224 plays important role in proliferation and differentiation of several cancers.

The involvement of miR-378 in HL-60 was previously indicated by Karashima *et al.* [Karashima *et al.*, 2004], in this study, miR-378 was firstly named miR-422b. 12-O-tetradecanoylphorbol-13-acetate (TPA) was first found in the croton plant, is diester of phorbol and being studied as a drug in treatment of heamatologic cancer [Ito *et al.*, 2001; Laouar *et al.*, 2001]. However, miR-378 was detected almost equally in the TPA (-) and TPA (+) samples. Expression of miR-378 has been also reported to enhance cell survival, reduce caspase-3 activity and promote tumour growth and angiogenesis [Lee *et al.*, 2007]. So miR-378 may play important role in caspase-3-dependent apoptosis fashion in HL-60 treated with mushroom extract.

In summary, the expression of many miRNAs was regulated in HL-60 upon treatment of *P. pachyphloeus* aqueous extract. Both up-regulated and down-regulated expression patterns were observed, depending on the miRNA in question. Precise mechanism concerning this regulation remains unknown and warrants further investigation.

Outlook

In present study, aqueous extract from *Phellinus pachyphloeus* was shown to induce apoptosis in human leukaemia cell line through the activation of the caspase cascade via both death receptor and mitochondrial signalling pathway. Many bioactive compounds and substances, including glucans, glycoproteins, lectins, etc., have been found from aqueous extracts of many other tropical medical mushrooms. But there are also opinions that compounds in the extracts act synergistically to potentiate their bioactivity. Considerably more work is needed to be done to investigate whether constituent compounds in our mushroom extract have synergistic effects or there are individual compounds which possess anti-tumor properties. Further fractionations of the extract and bioassay tests remain to be done.

On the other hand, what is now also needed are further studies to establish more in detail about the correlation between proteins and miRNAs which have been found to differentially expressed upon treatment with our mushroom extract. These studies will help us to see more precisely behind the curtain about the entire mechanisms of response of human cells upon treatment with extracts from tropical medical mushrooms, including *Phellinus pachyphloeus*.

V. SUMMARY

Despite the availability of several therapeutic options, a safer and more effective approach is urgently sought for treatment of cancer. Numerous novel anticancer agents are being discovered from eastern traditional folk medicine and ethno-pharmacology. Besides plants, mushrooms represent a significant source of bioactive compounds for the development of therapeutics by the pharmaceutical industry. Presently, studies on the bioactive components of medical mushrooms are mainly focused on polysaccharides, triterpenes and steroid compounds which contain in water- or alcohol-soluble extracts. Although many pharmaceutical properties, emphatically the anti-tumour properties, of several medical mushrooms were extensively investigated, sufficient scientific studies have not been performed to characterize the molecular mechanisms by which medical mushrooms exert their activities. Many reports showed that mushrooms possess the ability to stimulate mononuclear cell activation, spleen cell proliferation, and cytokine expression; to modulate anti-proliferative effects on many cancers; to regulate cell cycle and induce apoptosis in cancer cell lines. However, the majority of these observations often lack standardization and still based on phenomenology. So considerable understanding in molecular level remains to be determined.

In the present study, pharmaceutical properties of a tropical and subtropical, abundant, wood-decaying mushroom *Phellinus pachyphloeus* (Pat.) Pat. were investigated by means of classical and modern molecular analyses using human leukeamia HL-60 cells.

It has been proven that heavy metal bioaccumulation of *P. pachyphloeus* was at nontoxic level for human cell lines and played no role on activities of this mushroom. The next step towards further understanding was determining the anti-proliferative effect of the aqueous extract of this mushroom. At certain concentration, aqueous mushroom extract showed no cytotoxic effect but anti-proliferative effect due to induction of apoptosis. Strikingly, our findings indicate that aqueous extract of *P. pachyphloeus* induces apoptosis in HL-60 through the activation of the caspase cascade through both death receptor pathway and mitochondrial pathway.

The molecular mechanisms responsible for anti-tumour and immune-stimulatory properties in human leukaemia cell line HL-60 upon treatment with aqueous extract of *P. pachyphloeus* were further elucidated. The expression of many apoptotic/anti-apoptotic regulatory proteins

has been also ascertained by using proteomics. We also figured out the significant changes in miRNA expression due to our treatment.

VI. ZUSAMMENFASSUNG

Obwohl es bereits verschiedene Möglichkeiten der Behandlung von Krebs gibt, wird immer noch nach neuen, sichereren und effektiveren Methoden gesucht. Eine Reihe von neuen antitumoralen Substanzen wird aus der östlichen Medizin und Ethnopharmakologie entwickelt. Neben den Pflanzen stellen vor allem die Pilze eine wichtige Quelle von bioaktiven Substanzen für die Entwicklung von Therapien in der pharmazeutischen Industrie dar. Zurzeit fokussiert sich die Forschung von biologisch aktiven Metaboliten aus Pilzen, die in Wasser – sowie Ethanol – löslichen Extrakten enthalten sind. Obgleich viele pharmazeutische Eigenschaften, insbesondere die anti-Tumoraktivität von einigen medizinischen Pilzen intensiv untersucht wurden, fehlen wissenschaftliche Untersuchungen zu den molekularen Mechanismen der Pilzwirkung. Viele Publikationen zeigen, dass die biologisch aktiven Substanzen aus Pilzen in Tumorzelllinien die Aktivierung der mononuklearen Zellen, die Hemmung der Zellproliferation sowie die Cytokine-Expression stimulieren, die Zellteilung regulieren und Apoptose induzieren. Es fehlen aber Standardtisierungen und Untersuchungen auf molekularem Niveau.

In der vorliegenden Arbeit wurde ein tropischer, holzzerstörender Pilz *Phellinus pachyphloeus* (Pat.) Pat. unter Verwendung klassischer und moderner molekularer Analytik auf seine Wirkung auf die Leukämiezelllinie HL-60 untersucht. Zunächst wurde festgestellt, dass die Bioakkumulation von Schwermetallen durch *Phellinus pachyphloeus* keinen negativen Einfluss auf die menschliche Zelllinie hat.

Im nächsten Schritt wurden die antiproliferativen Effekte der bioaktiven Komponenten aus Wasserextrakten dieses Pilzes untersucht. Dosis-Wirkungsversuche bestätigten eine antiproliferative Wirkung der bioaktiven Pilzextrakte. Diese wurde als apoptotischer Effekt eingegrenzt. Unsere Ergebnisse zeigten, dass die Wasserextrakte aus *Phellinus pachyphloeus* keinen cytotoxischen sondern einen apoptotischen Effekt in humanen Leukämiezellen induzieren könne. Die induzierte Apoptose erfolgte sowohl über den äußeren, Rezeptor-vermittelten, als auch den mitochondrialen Signalweg.

Der Einfluss der Extrakte von *Phellinus pachyphloeus* auf menschliche Zellen wurde hinsichtlich antitumoraler und immunstimulierender Wirkung weiter evaluiert. Dabei wurden unter Verwendung von 2D-Proteingel-Elektrophorese Veränderungen in der Expression

Apoptose-relevanter Proteine gefunden. Darüber hinaus wurden damit korrelierende Veränderungen in der Expression regulatorischer miRNAs festgestellt.

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Publications

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8. Mediation of apoptogenic/anti-apoptotic and signalling regulatory protein expression by aqueous extract of *Phellinus pachyphloeus*.

9. MicroRNA expression changes of human leukaemia cell upon treatment with aqueous extract of *Phellinus pachyphloeus*.

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Posters

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2. **Kiet T. T., Bao T. T. T., Saluz H. P.** (2007). Research on taxonomy of the perennial

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Selbständigkeitserklärung

Ich erkläre hiermit, dass mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität bekannt ist.

Ich habe die vorliegende Dissertation selbständig verfasst und alle Hilfsmittel, persönlichen Mitteilungen und Quellen in der Arbeit angegeben.

Sämtliche Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben, sind in der Danksagung genannt.

Ich habe weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte unmittelbar oder mittelbar geldwerte Leistungen von mir für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen.

Ich habe die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Ferner habe ich nicht versucht, die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation einzureichen.

Jena, December 2008

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