Charles University in Prague

Third Faculty of Medicine

Hospital Bulovka

Department of Pneumology and Thoracic Surgery

Laboratories of Molecular and Cell Biology

Expression and function of serpinB9 in lung cancer cells

PhD Thesis

Ilona Rousalova, MD

Supervisor:

Evzen Krepela, MD PhD

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Statement of authorship

I certify hereby that the thesis represents valid work elaborated under the supervision of Evzen Krepela, MD PhD, and that neither this manuscript nor one with substantially similar content under my authorship has been submitted in support of an application for any other academical degree. My participation in the published papers is specified at the end of the comments to each paper.

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Ilona Rousalova, MD

Statement of co-authors

I certify herewith that Ilona Rousalova substantially contributed to the formation of the papers used as a basis of this thesis, and that her participation specified at the end of the comments to each paper is correct.

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Evzen Krepela, MD PhD

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This thesis is based on the following papers, referred to by their capital letters in the text as indicated here:

A. **Rousalova I**, Krepela E, Prochazka J, Cermak J, Benkova K.: *Expression of proteinase inhibitor-9/serpinB9 in non-small cell lung carcinoma cells and tissues*. Int J Oncol 36 (1): 275-283, 2010 (IF = 2.571)

B. **Rousalova I**, Krepela E.: *Granzyme B-induced apoptosis in cancer cells and its regulation (Review).* Int J Oncol 37 (6): 1361-1378, 2010 (IF = 2.571)

C. Moravcikova E, Krepela E, Prochazka J, **Rousalova I**, Cermak J, Benkova K.: *Down-regulated expression of apoptosis-associated genes APIP and UACA in non-small cell lung carcinoma.* Int J Oncol 40 (6): 2111-2121, 2012 (IF = 2.571)

The above papers are included in full in this PhD thesis. For the complete list of my published articles, see List of publications (Section 7).

LIST OF ABBREVIATIONS

Ad5	adenovirus type 5
ADC	5-aza-2'-deoxycytidine
AIF	inducing factor
AP-1	activator protein-1
Apaf-1	apoptotic protease-activating factor-1
Bid protein	BH3-interacting domain death agonist protein
CI-MPR	cation-independent mannose-6-phosphate receptor
CrmA	Cytokine response modifier A
CTL	Cytotoxic T lymphocyte
DFF	DNA fragmentation factor
DISC	Death-inducing signalling complex
DNA-PKCS	catalytic subunit of DNA-dependent protein kinase
Endo-G	endonuclease-G
DR	death receptor
E2	estradiol-17β
EGFR	epidermal growth factor receptor
ER	endoplasmatic reticulum
ER-α	estrogen receptor-α
ERE	estrogen response element

ERU	estrogen responsive unit
FADD	Fas-associated death domain adaptor protein
FGFR1	fibroblast growth factor receptor-1
GA	Golgi apparatus
GrB	granzyme B
hrGrB	human recombinant granzyme B
Hax-1	HS-1-associated protein X-1
HER2	epidermal growth factor receptor 2
HtrA2/Omi	high temperature requirement A2 serine protease
IAP	inhibitor of apoptosis protein
IL	interleukin
IMP-α	importin-α
IS	immunological synapse
JAK1	Janus kinase 1
LAC	lung adenocarcinoma
LAK	lymphokine activated killer
LCLC	large cell lung carcinoma
M6P	mannose-6-phosphate
MNEI	monocyte neutrophil elastase inhibitor
MOMP	mitochondrial outer-membrane permeabilization
NFAT	nuclear factor of activated T cell

NK	natural killer cell
NF-κB	nuclear factor-κB
NSCLC	non-small cell lung carcinoma
OMM	outer mitochondrial membrane
PC-9	procaspase-9
PFN	pore-forming protein perforin
PI-6	proteinase inhibitor-6
PM	plasma membrane
SB9	serpinB9
RCL	reactive centre loop
ROCK II	Rho-associated coiled coil-containing protein kinase 2
SAGA	Survivin and GrB-induced apoptosis
scFv	single-chain antibody fragment
SCLC	small-cell lung carcinoma
SG	serglycin
SLC	sarcomatoid lung carcinoma
Smac	second mitochondria-derived activator of caspases
SQCLC	squamous cell lung carcinoma lung
STAT	signal transducer and activator of transcription
Sur	survivin
TCR	T cell receptor

TNF-α	tumour necrosis factor-α
UNDIF	undifferentiated lung carcinoma
UV	ultraviolet
VEGF	vascular endothelial growth factor

CONTENT

1 INTRODUCTION

Susceptibility of cancer cells to enter apoptosis depends on their abilities to express the components of apoptosis pathways and efficiently activate them in response to extrinsic or intrinsic death stimuli (1). The death receptor- and the cytotoxic granule-induced pathways represent the extrinsic death pathways. Whereas, the mitochondrial, lysosomal and PIDDosome death pathways represent the intrinsic death mechanisms.

The death receptor pathway is initiated by the binding of a death ligand, such as FasL (also known as CD95L) and TRAIL (also known as APO-2L) to a specific transmembrane death receptors, Fas (also known as APO-1/CD95) and death receptor 4 and/or 5 (DR4, DR5), respectively (2,3). After binding of cytosolic Fas-associated death domain adaptor protein (FADD, also known as MORT1) to the liganded death receptors, DISCs (death inducing signalling complexes) are formed in the plasma membrane (PM) and PM-derived endocytic vesicles through the binding of initiator procaspase-8 and/or -10 monomers to the DR-bound FADD molecules (2-5). Once procaspase-8 and -10 monomers are activated within DISCs via homodimerization, active procaspase-8 and -10 homodimers are processed through interdimer proteolysis and the active caspase-8 and -10 molecules dissociate into the cytoplasm. In the cytoplasm, both caspase-8 and –10 cleave and activate procaspase-3 and -7 proteins and the cytosolic BH3-interacting domain death agonist (Bid) protein (2,3,6-9), thus activating the apoptosis effector and amplification steps, respectively. After binding of the C-terminal fragment of Bid, t(c)Bid, to mitochondria, OMM is permeabilized while holocytochrome-c (cyt-c) and other pro-apoptotic mitochondrial proteins are released into the cytoplasm (10).

The mitochondrial pathway is trigged in response to a variety of death stimuli such as DNA damage, chemotherapeutic agents or UV light (and see above). Under such conditions, the pro-apoptotic proteins Bax or Bak mediate MOMP and release several pro-apoptotic intermembrane mitochondrial proteins such as cyt-c, Smac, HtrA2/Omi serine protease, AIF and Endo-G (11-13). In the presence of dATP or ATP, cyt-c binds to Apaf-1 and induces its oligomerization into a large heptameric complex called apoptosome (14-16). The apoptosome apparatus recruits and activates procaspase-9 (17-20). The active apoptosome-bound caspase-9 then activates the zymogens of the apoptotic effector caspase-3 and -7 (21-23). Both Smac and HtrA2 neutralize the anti-apoptotic functions of

the IAPs (13,24), whilst AIF and Endo-G are involved in DNA fragmentation after translocation into the nucleus (11,25).

An another multiprotein complex called PIDDosome, which can activate initiator procaspase-2 (26-28), can be inducibly formed in the cytoplasm and/or in the cell nucleus in the response to diverse cellular stresses (28,29). The PIDDosome assembled in the cytosol is created of three layers of homooligomerized proteins. The proteins are an autoproteolytic carboxyterminal fragment of the p53-induced protein with a death domain (PIDD-CC), the receptor interacting protein (RIP)-associated ICH-1/CED-3 homologous protein with a death domain (RAIDD, also named CRADD), and procaspase-2 (27,29,30). The PIDDosome assembled in the nucleus is built of PIDD-C and PIDD-CC fragments, the catalytic subunit of DNA-PK_{CS} and procaspase-2 (28). The active caspase-2 is released from the complex and cleaves the BH3-only protein Bid and other protein substrates including Golgin 160, DFFA and some cytoskeletal proteins (31,32).

Lysosomal membrane permeability can be disrupted by many apoptotic stimuli, such as oxidative stress, TNF-α treatment, lysosomotropic agents, sphingosines, etoposide, UV light, FasL or TRAIL (33-35). Consequently, the partial release of several cathepsins including B, K, L, and S into the cytoplasm leads to the proteolytic fragmentation of Bid protein, which induces release of cyt-c and formation of the apoptosome (36-38).

Both the intrinsic and the extrinsic apoptosis pathways converge on the activation of the effector procaspase-3, -6, and -7 (23,35,39,40) and they can lead to MOMP through cleavage of Bid protein and/or the Mcl-1 protein component of the Mcl-1•Bim complex (7,41-45).

My thesis is focused mainly on the role and regulation of expression of the intracellular serpinB9 which serves as an irreversible inhibitor (inactivator) of the serine proteinase GrB in cancer cells and certain normal cells such as lymphocytes and endothelial cells. GrB-induced apoptosis seems to be the most important death pathway for clearance of intracellular pathogens infected cells, allogeneic and tumour cells (46-50). After the recognition and conjugation of target cells by CTLs and NK cells, the cytotoxic secretory granules of CTLs and NK cells are vectorially transported toward the conjugation zone and their content is released into a intercellular cleft called IS (51,52). The most abundant components of cytotoxic granules are GrB and PFN. GrB is considered to be a

major effector of NK cells (53). Furthermore, the cytotoxic granules of human CTLs and NK cells contain also other granzymes (A, H, K and M) (54), which may co-deliver alternative death signals that can operate synergistically with MOMP induction and procaspases activation (55-57) or independently of that (48,53,58-60). The precise mechanism of GrB translocation into the target cell is still unclear (61). Once in the target cell cytoplasm, GrB can trigger several apoptotic pathways through direct proteolytic activation of signalling or executioner components and cleaves multiple intracellular housekeeping proteins (Fig. 1).

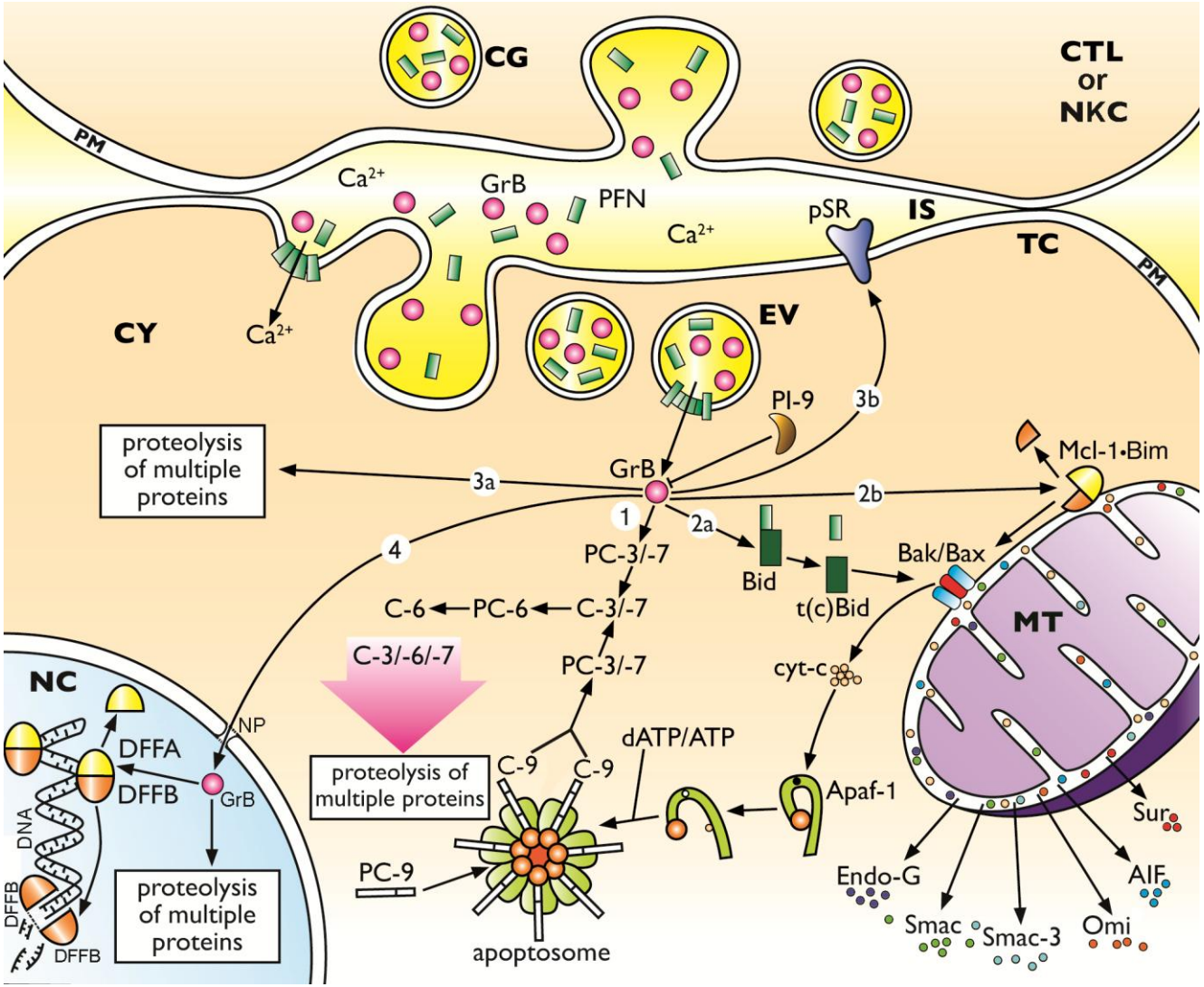

Figure 1. Granzyme B-induced death pathways in cancer cells. [1], Direct proteolytic processing and activation of the executioner procaspase-3 and -7, followed by the caspase-3-mediated activation of the executioner procaspase-6. The active executioner caspases cleave many intracellular proteins. [2], MOMP via the GrB-mediated proteolytic [2a] conversion and activation of protein Bid to the MOMP-inducing t(c)Bid fragment, and [2b] disruption of the Bim•Mcl-1 complex, involving fragmentation of the Mcl-1 component, and hence derepression of the MOMP-activity of Bim, followed by cytosolic release of several pro-apoptotic proteins (cyt-c, Smac, Smac-3, Omi/HtrA2, AIF and Endo-G) and the inhibitor-of apoptosis protein survivin (Sur). [3a], Proteolytic fragmentation of multiple housekeeping proteins in the cytoplasm (Table I), including [3b] the cytosol-facing signalling domains of some pro-survival plasma membrane receptors (pSR). [4], Translocation into the nucleus and proteolytic fragmentation of multiple intranuclear proteins (Table I), including the DFFA subunit of DNA fragmentation factor (DFFA•DFFB), which leads to the homodimerization-mediated activation of its deoxyribonuclease subunit DFFB. See the text for a more detailed description. CTL, cytotoxic lymphocyte; NKC, natural killer cell; CG, cytotoxic granule; IS, immunological synapse; PM, plasma membrane; TC, target cell; EV, endocytic vesicle; CY, cytoplasm; MT, mitochondrion; NC, nucleus; NP, nuclear pore; PC, procaspase; C, caspase.

1.1. Granzyme B gene organization and regulation of expression

The human GrB gene *(GZMB)* is located in the "chymase locus" on chromosome 14q11.2 (62). There were find other three functional genes in this locus: granzymes H gene *(GZMH),* cathepsin G gene *(CTSG),* and mast cell chymase gene *(CMA1)*. *GZMB* gene is localized at the 5` end of the cluster, followed by *GZMH, CTSG,* and *CMA1* genes (63). *GZMB* gene is approximately 3.2 kb in length and is comprised of five exons and four introns (64). The signal (leader) sequence of GrB preproprotein is encoded by exon I, the amino acid residues forming the catalytic triad, i.e. His^{57}, Asp^{102}, and Ser^{195}, are encoded within the exons II, III and V, respectively (65).

The *GZMB* promoter contains consensus sequences for binding of several transcription factors, including NFAT, Ikaros and AP-1 (66,67). Huang et al. identified a novel NF-κB binding site outside of the *GZMB* gene, approximately 10 kb downstream from its transcription start point in NK cells (68). Recently, it has been identified a crucial role of IL-3 for inducible GrB expression in human plasmacytoid dendritic cells and the involvement of the JAK1, STAT3 and STAT5 in this process (69). Trotta and colleagues have demontrated a new link in human NK cells between IL-2/IL-5 receptor signalling, SET-PP2A interplay and transcriptional expression of GrB and PFN (70).

GZMB gene polymorphism has been described in a genetic study of individuals from various racial groups (71). Compared to the wild-type QPY allele of *GZMB* gene encoding GrB preproprotein with Gln^{48}, Pro^{90}, and Tyr^{247} in its sequence, the RAH allele of *GZMB* gene encodes GrB preproprotein containing Arg^{48}, Ala^{90}, and His^{247} (71). The occurence of RAH allele was at a frequency rate of 25-30% in each of the racial groups tested and it was demonstrated to represent a neutral *GZMB* gene polymorphism (72).

Initially, it was thought that the GrB expression is restricted to lymphoid cells (46,73,74). However, GrB can be expressed not only in normal haematopoietic cells, such as CD4+ T cells, mast cells, activated macrophages and Kupffer cells, neutrophils, basophils and dendritic cells, but also in normal cells of non-haematopoietic origin, including chodrocytes, keratinocytes, type II pneumocytes, Sertoli cells, primary spermatocytes, and cells of granulosa and syncytiotrophoblast (75-91) under certain pro-

inflammatory conditions, for instance at the presence of particular extracellular cytokines, under various receptors engagement, and the occurrence of CD4+ T lymphocytes.

1.2. Granzyme B biosynthesis, activation and subcellular localization

Schmid and Weissmann identified human preproGrB mRNA in 1987 (92) and one year later human GrB cDNA was cloned (93,94). The human GrB protein was first purified and characterized in 1991 (95). GrB and other known human granzymes (A, H, K and M) as well as the closely related myeloid serine proteases, such as cathepsin G, are members of the chymotrypsin superfamily. GrB is synthesised as a preproenzyme while its signal peptide (i.e. the 18 amino acid residues long N-terminal pre-part) is removed co-translationally. The signal peptide directs the nascent polypeptide chain of the protein into the ER. The resulting proGrB is covalently modified with a M6P group and transported in ER-derived vesicles to the GA (65,96). In GA, the M6P moiety serves as sorting and targeting tool for proGrB and other progranzymes to the GA-derived secretory granules (97). Primarily activation of proGrB is carried out inside the secretory granules by removal of the N-terminal dipeptide GlyGlu by co-segregated DPPI (also known as cathepsin C) (65,96,98,99). If removal of the GlyGlu propeptide fails, the formation of the GrB catalytic site is disrupted. Besides the major mode of proGrB activation by DPPI, other less defined mechanisms of proGrB proteolytic activation were described (100-102). D'Angelo and colleagues recently identified lysosomal cathepsin H as another activator of proGrB (103).

Within the secretory granules, active granzymes are stored in association with the chondroitin sulphate containing proteoglycan SG and are secreted in this macromolecular complex during the target cell killing (104-107). Two distinct molecular sizes of GrB●SG complexes were revealed. One complex contains ~ 4-8 molecules of GrB, whereas the other one holds as many as 32 molecules of GrB or other granule proteins (106). The proteolytic activity of GrB might be minimized by storage of GrB in a scaffolded form in the acidic interior of the secretory granules (106,107). The free GrB molecule has a high positive surface charge, but when GrB forms the complex with SG its charge may be considerably neutralized. Therefore, the free GrB molecule might interact with various negatively charged groups exposed on the cell surface, including phospholipid headgroups and those in glycosaminoglycans, compared to the SG-bound GrB.

The newly synthesised GrB is heterogeneously glycosylated. The N-linked oligosaccharide chains can be attached at two potential glycosylation sites at Asn^{51} and Asn^{84} of mature GrB molecule (108). The process of GrB glycosylation results in generation of two glycosylated forms of GrB - 32 kDa and 35 kDa. The 32 kDa GrB forms contain high mannose oligosaccharide moieties, accumulate in CTLs after TCR stimulation (109) and can be stored in the secretory granules of these cells. In contrast, the 35 kDa GrB forms possess only the complex oligosaccharide groups and are not stored in CTLs. These forms are secreted through the constitutive and less specific calcium-independent secretory pathway after TCR activation (109). When a CTL recognizes its target via the TCR, two events occur: the vectorial exocytosis of the secretory granules towards the IS, and the *de novo* biosynthesis of the lytic granule proteins, including granzymes and PFN.

1.3. Granzyme B structure and substrate specificity

Human GrB is a single chain and single domain serine protease. Its crystal structure was recently determined and gives some rationale for the substrate specificity (110,111). The GrB structure is folded into two six-stranded β-barrels, which are connected by three *trans*-domain segments. The regular secondary structure elements include a helical loop between Ala^{56} and Cys^{58}, a helix involving residues from Asp^{165} to Leu^{172}, and a long C-terminal helix from Phe^{234} to Arg^{244}. The peptide bond between Pro^{224} and Pro^{225} is in the *cis* conformation. The *cis*-conformation of these proline residues orients the positively charged Arg^{226} side chain into the S1 subsite (111).

Similar to caspases, GrB has a preference for cleaving peptide bonds immediately adjacent to Asp residues (95). This specificity is due to the structure of the GrB active site, which contains Arg^{226} residue in the S1 subsite. The tetrapeptide sequence IEPD was identified as the preferred P4-P3-P2-P1 recognition motif for GrB, although optimal susbstrate recognition may involve features beyond this tetrapeptide sequence (111-114). It has been showed that GrB requires an extended substrate sequence for specific and efficient binding of protein substrates corresponding to the P4-P4'positions (114).

1.4. Granzyme B delivery mechanism into the target cell cytoplasm

The effective mechanism of granzymes entry into the cytosol of target cells during killer cells attack has not been clarified so far. Upon formation of a temporary intercellular conjugation zone called IS, the lytic granules rapidly move and polarize towards it. (51,52). The IS functions as a conduit for the transportation of lytic granules content and other soluble factors between the CTLs and the target cell (115). The movement of lytic granules towards the target cell is directional and depends on an underlying Ca^{2+}-activated microtubule cytoskeleton and other less defined Ca^{2+}-required molecular events (116). Recently, it has been demonstrated that the Rab27a/Slp3/kinesin-1 transport complex is required for the terminal transport of cytotoxic granules and their secretion at the IS (117). Once arrived at the site of secretion, the membrane of secretory granules fuses with the plasma membrane and their content is released into a secretory cleft of IS. It is still under debate whether granzymes enter the target cell cytoplasm through PFN pores at the post-synaptic plasma membrane or whether both granzymes and PFN are first endocytosed and the granzymes are subsequently released from endosomes within the cytoplasm (105,118-131).

1.4.1. Granzyme B secretion

The precise mechanism how granzymes release from CTLs and NK cells into the extracellular space remains unclear, but several mechanisms seem to be involved. The LAK cells are stimulated to undergo granule exocytosis by phorbol myristic acetate and anti-CD2 monoclonal antibodies (104). A single CTL can cause the process of serial killing when free granzymes leak from the immunological synapse during the degranulation and move to another target (109,132). Extracellular granzymes may also arise from constitutive non-specific secretion after TCR activation and/or prolonged exposure to interleukin-2 (109,133). Stimulation of TCR triggers *de novo* granzyme synthesis, and a proportion of newly synthesized GrB is non-specifically secreted via a non-vectorial pathway. This is due to the absence of M6P in the glycan moiety of the secreted GrB which cannot be targeted to the lytic granules via the M6P receptor (109). Moreover, it has been recently found that a proportion of GrB secreted into the IS can be reabsorbed back into NK cells through clathrin-dependent endocytosis (134).

In the absence of target cell engagement, both CTLs and NK cells constitutively secrete a portion of GrB. In CTLs, the protease is primarily secreted in an inactive form, bypassing the granules. Whereas in NK cells, GrB is released in an active form via secretory granules (132). Whether the secreted proGrB can be proteolytically processed to the active GrB is not known so far. There is possibility that an active DPPI and/or cathepsin H, which are co-secreted by the CTLs or other adjacent cells, might remove the activation GlyGlu dipeptide en bloc. Additionally, two aminopeptidases acting sequentially might remove the amino acid residues of the N-terminal dipeptide in a step-by-step fashion.

1.4.2. Mechanism of granzyme B internalization into target cells

The process of granzymes and PFN entry into the cytoplasm of target cells is not fully understood. Several studies provided evidence that GrB can be taken up into the endosomal compartment of the cell. PFN or other endosomolytic agent is necessary for GrB translocation from these endosomal vesicles into the cytoplasn and/or nucleus (119-122).

It has been demonstrated that GrB binds to the target cell surface in the concentration-dependent and saturable manners and enters the cells through endocytosis (119). The CI-MPR was identified as the plasma membrane receptor of GrB in the model of receptor-mediated GrB endocytosis (123,128,135). The process of the CI-MPR-mediated GrB internalization is clathrin- and dynamin-dependent (123,136). Recently, several groups have showed that PFN triggers a reparative response of wounded plasma membrane, which is the clathrin- and dynamin-dependent endocytosis. This mechanism removes PFN and granzymes from the plasma membrane to early endosomes (125,131). Moreover, alternative mechanisms of GrB receptor-mediated cell entry have been proposed, e.g. via CD44 molecules with known affinity for serglycin (137) or via Hsp70 which can serve as a GrB receptor (138).

Trapani et al. showed that cells lacking CI-MPR are also readily killed by glycosylated GrB (123). Therefore, a different model for GrB cell uptake has been proposed. It suggests that selective GrB receptors may not be required. The positively charged free GrB (having the isoelectric point of approximately 9.5 - 10) absorbs to the

negatively charged surface structures of target mostly via non-specific electrostatic interactions (127,139). There is one difficulty with this model because the high positive charge of GrB is probably neutralized *in vivo* by SG, making absorption to the cell surface much less efficient. It is therefore to suppose that GrB exchanges from SG to more negatively charged elements on the cell surface, such as the phospholipid headgroups, sulphated lipids, gangliosides, or heparan sulphate proteoglycans, and is subsequently internalized by absorptive pinocytosis (124,125).

Since both uptake mechanisms of GrB entry into target cells, i.e. the receptor-mediated endocytosis and the receptor-independent absorptive pinocytosis, are supported by convincing experimental evidence, it is probable that they co-exist in the same target cell.

1.4.3. The role of perforin in granzyme B delivery in the target cell cytoplasm

There is strong evidence that GrB itself does not have a capability to bind and disrupt lipid membrane (130) and its internalization into a target cell depends on PFN (140). PFN is a pore-forming glycoprotein that can bind to phospholipid components of PM in the presence of Ca^{2+} ions and subsequently oligomerizes into pore-forming structures with a diameter of 5-20 nm (141-144). Compared to progranzymes, proteolytic processing of the PFN precursor occurs in the absence of DPPI activity (96). It has been found that secreted PFN can be recaptured by early endosomes of NK cells via a clathrin-dependent endocytosis after target cell stimulation (145). There are two well-supported hypotheses explaining the process of the PFN-assisted GrB entry into the cytoplasm of target cells. First, the pore entry hypothesis claims that GrB and other granzymes are primarily translocated from the cell exterior (e.g. IS) into the cytoplasm by diffusion through repairable plasma membrane pores (146). Nevertheless, recent data demonstrate that GrB probably does not enter the cytoplasm through PFN membrane pores since inhibition of the PFN-activated endocytosis increases the number of PFN pores persisting at the cell surface, but decreases the GrB uptake (131). Nonetheless, PFN oligomerization and transmembrane pore assembly is a prerequisite for the GrB-induced apoptosis (147). Second, the endosome permeabilization/endosomolysis entry hypothesis states that GrB and other granzymes are delivered into the cytoplasm of target cell after endocytosis and via a PFN-mediated disruptive escape from the endosomal compartment (119,122,125).

The precise mechanism of the perforin-assisted cytosolic translocation of GrB from this compartment has not been clarified yet (61).

1.5. Death pathways induced by granzyme B in cancer cells (Fig. 1)

After GrB delivery into the cytosol, GrB proteolytically attacks different protein substrates and initiates apoptosis. More than three hundreds intracellular and extracellular human proteins have been identified as potential GrB substrates (56,58,60). The list of the proteins cleaved by GrB during apoptosis is indicated in Table I. However, only for a few of them the physiological relevance of their cleavage in the process of cell demise was established.

1.5.1. Direct proteolytic activation of executioner procaspases

Similar to caspases, GrB has a preference for cleaving protein peptide bonds C-terminal to Asp residue (112,113). GrB directly cleaves and activates the executioner procaspase-3 and -7 (44,148-154,299) (Table I).

On the contrary, there are inconsistent reports on the direct procaspase-6 proteolytic activation (44,155,156). Moreover, the active GrB can proteolytically cleave initiator apoptotic procaspases such as procaspase-9, -2, -8, and -10 (44,150,156-160), but it cannot activate them. The initiator procaspase-9 is activated by a specific conformational change in apoptosome (20), whereas the initiator procaspases -2, -8 and -10 are activated exclusively by homodimerization in specific multiprotein activation platforms such as DISCs and PIDDosome (2,5,9,19,32).

Table I. Granzyme B protein substrates

Protein	Function	Localization	Cleavage sites	References
Acetylcholin receptor ε subunit (AchRε)	Acetylcholine receptor	Plasma membrane	IDID[195]	301
Muscarinic acethylcholine receptor 3 (M3R)	Acethylcholine receptor	Plasma membrane	MDQD[330] , PSSD[387]	302
β-Actin	Structural protein	Cytosol	N.D.[a]	303,304
Alanyl tRNA synthetase (ARS)	Translation	Cytosol	VAPD[632]	305
Bcl-2-associated athanogene 1 (Bag-1L)	Co-chaperone	Nucleus, cytosol	VTRD[125], VVQD[172]	204
Bid	Pro-apoptotic sensor	Cytosol	IEAD[75]	164,306
CD3	Signal tranducer	Plasma membrane	Many	307
Centromere protein B (CENP-B)	Mitosis	Nucleus	VDSD[457]	305,308
Centromere protein C (CENP-C)	Mitosis	Nucleus	N.D.	305
DNA-dependent protein kinase catalytic subunit (DNA-PKcs)	DNA repair	Nucleus	VGPD[2698]	305,309
DNA ligase IV/XRCC4	DNA repair	Nucleus	SKDD[254]	310
Fibrillarin	rRNA processing	Nucleolus	VGPD[184]	305
Fibroblast growth factor receptor-1 (FGFR-1)	Membrane receptor	Plasma membrane	N.D.	200
Filamin	Cytoskeletal protein	Cytosol	Many	196
Focal adhesion kinase (FAK)	Signal transducer	Cytosol	VSWD[704], DQTD[772]	310
α-Fodrin	Cytoskeletal protein	Cytosol	IVTD[1554], AEID[1961]	197,302
Glutamate receptor subunit 3 (GluR3B)	Glutamate receptor	Plasma membrane	ISND[388]	312
Hip	Chaperone	Cytosol	IEPD[92], INPD[180]	203, 204
Histidyl tRNA synthetase (HRS/Jo-1)	Translation	Cytosol	LGPD[48]	305,282
Hop	Chaperone	Cytosol	LGVD[186]	202
Hsp27	Protein folding	Cytosol	VSLD[100]	204
Hsp70	Protein folding	Cytosol	INPD[366]	200
Hsp90α	Protein folding	Cytosol	Many	204
Hsp90β	Protein folding	Cytosol	Many	204
Isoleucyl tRNA synthetase	Translation	Cytosol	VTPD[983]	305
Ki-67	Proliferation	Nucleus	VCTD[1481]	305
Ku-70	DNA repair	Nucleus	ISSD[79]	305
La/SSB	RNA binding	Nucleus	LEED[220]	305
Lamin B	Structural protein	Nuclear lamina	VEVD[231]	199
Mi-2	DNA methylation, chromatin remodeling	Nucleus	VDPD[1312]	305,309
Mcl-1	Inhibition of MOMP[b]	Cytosol	PAAD[117], EELD[127], TSTD[157]	42
Notch1	Membrane receptor	Plasma membrane	N.D.	200
Nuclear mitotic apparatus protein 1 (NuMa)	Mitosis	Nucleus	VATD[1705]	305,280
Nucleolus organizing region 90 kDa (NOR-90/UBF)	Transcription factor	Nucleolus	N.D.	305

Table I. Granzyme B protein substrates - continues

Protein	Function	Localization	Cleavage sites	References
Nucleophosmin B23	rRNA processing	Nucleolus	LAAD^{161}, VEVD^{122}	314, 315
PMScl/EXOSC10	mRNA degradation	Cytosol	VEQD^{252}	305
Poly(ADP)ribose polymerase 1 (PARP1)	Ribosylation	Nucleus	VDPD^{537}	305,316
Postmeiotic segregation 1 (PMS1)	DNA mismatch repair	Nucleus	ISAD^{496}	305
Postmeiotic segregation 2 (PMS2)	DNA mismatch repair	Nucleus	VEKD^{493}	305
Procaspase-3	Apoptosis execution	Cytosol	IETD^{175}	150
Procaspase-7	Apoptosis execution	Cytosol	IQAD^{198}	150,151
Procaspase-8	Apoptosis initiation	Cytosol	N.D.	159,277
Procaspase-10	Apoptosis initiation	Cytosol	IEAD^{372}	150
Procaspase-9	Apoptosis initiation	Cytosol	PEPD^{315}	156
Procaspase-2	Apoptosis initiation	Cytosol, nucleus	N.D.	318
Pyruvate dehydrogenase complex E2 (PDC-E2)	Acetyl-CoA synthesis	Mitochondria	N.D	319
Rho-associated coiled coil-containing protein kinase 2 (ROCK II)	Plasma membrane blebbing (zeiosis)	Cytosol	IGLD^{1131}	198
RNA polymerase I (RNA Pol I)	Transcription	Nucleus	ICPD^{448}	305
RNA polymerase II (RNA Pol II)	Transcription	Nucleus	ITPD^{370}	305
Signal recognition particle 72 kDa (SRP-72)	Translation	Cytosol	VTPD^{573}	305
Subunit A of DNA fragmentation factor (DFFA/DFF45)	Chaperone and inhibitor of DFFB (DFF40) deoxyribonuclease	Nucleus, cytosol	DETD^{117}, VTGD^{6}	300,320
Topoisomerase I (Topo-1)	Transcription	Nucleus	IEAD^{15}	305
α-Tubulin	Microtubule polymerization/ aggregation	Cytosol	VGVD^{438}	195,304
U1small nuclear ribonucleoprotein 70kDa (U1-70 kDa)	RNA processing	Nucleus	LGND^{409}	305
UBF/NOR-90	Nucleolar transcription factor	Nucleolus	VRPD^{220}	305
Ubiquitin fusion degradation 2 (UFD2)	Ubiquitination	Nucleus	VDVD^{123}	321

1.5.2. Mitochondrial outer membrane permeabilization (MOMP)

It is not clear whether GrB preferentially initiates apoptosis through an alteration of the OMM rather than by the direct activation of the executioner caspases (161). GrB has a capability to disrupt OMM at least by two distinct mechanisms. First, GrB cleaves and activates the cytosolic BH3-only protein Bid. Its C-terminal proteolytic fragment, t(c)Bid, translocates to OMM and promotes t(c)Bid-mediated MOMP (162-167). After this event, several pro-apoptotic mitochondrial proteins are released into the cytoplasm and trigger the caspase-dependent and -independent death pathways (see above). Second, an alternative and/or parallel GrB-induced MOMP is proceeded by the proteolytic cleavage of the Mcl-1 component of a Mcl-1●Bim complex located at OMM, thus relieving the OMM-permeabilizing Bim activity (41,42). Moreover, it was recently discovered that GrB translocates into the mitochondria and cleaves Hax-1 protein. Its N-terminal proteolytic fragment then is responsible for mitochondrial depolarization (168).

After MOMP, holocytochrome-c is released into the cytoplasm and interacts with Apaf-1 monomer through its WD40 repeats, thus triggering, in the presence of (d)ATP, the formation of a heptameric Apaf-1 protein complex called apoptosome. Apaf-1 apoptosome becomes an allosteric activator of PC-9 and initiates a caspase activation cascade, terminating with proteolytic devitalizacion and apoptotic destruction of the cells (14,19,20). Regulation of Apaf-1 and PC-9 expression is important in predisposition of cells to activate the apoptosome apparatus (169-173). Moreover, a variety of negative and positive posttranslational regulations influence the processes of apoptosome assembly and functioning in cancer cells (174,175). On the other hand, impaired signalling in the apoptosome pathway, due to the lack of apoptosome core components or apoptosome dysfunction, contributes to tumourigenesis and progression of malignant tumours as well as to their chemo- and radioresistance (173,176-183).

The activation of apoptosome apparatus is frequently impaired in NSCLC cells and tissues (154,184). The molecular mechanism of apoptosome suppression in NSCLC is still unknown. There is evidence that suppression does not involve the segregatory binding of PC-9 to TUCAN (185) or direct inhibition of caspase-9 by XIAP (184,186). The Apaf-1 interacting protein (APIP/AIP) and the uveal autoantigen with coiled coil domains and ankyrin repeats (UACA)/nucling belong to the endogenous regulators of apoptosome

apparatus (174,175), but their role in NSCLC tumourigenesis and progression is not clear. APIP is a cytosolic protein which binds to the CARD domain of Apaf-1 and thus prevents PC-9 recruitment to the apoptosome (187,188). Furthermore, APIP triggers sustained activation of AKT and ERK1/2 kinases under hypoxic condition. These kinases directly phosphorylate PC-9 and thus inhibit its activation in the apoptosome (189). UACA/nucling was found in both cytoplasmic and perinuclear/nuclear localization (190-192). During proapoptotic stress, UACA/nucling interacts with Apaf-1 and induces its translocation into the nucleus (193). Moreover, UACA also interacts with the NF-κB and blocks its entry into the nucleus and thus reducing expression of the NF-κB-targeted genes (194).

1.5.3. Intracellular housekeeping protein substrates of granzyme B

GrB has a significant role in disrupting the cytoskeleton by cleaving of several its protein components such as α-tubulin (195), filamin (196) β-fodrin (197) and ROCK II (198).

Besides that, GrB and other granzymes can dismantle the compactness of the nuclear lamina by direct cleaving of lamin B (199). This facilitates its nuclear entry through nuclear pore complexes (199). Within the nucleus, GrB cleaves several nuclear protein substrates and initiates series of important intranuclear molecular processes (Table I).

Recently, other classes of GrB substrates have been identified, including the transmembrane receptors for growth factors, such as Notch1 and FGFR1 that transmit pro-survival and pro-proliferative signals from the extracellular environment (200,201), and members of the heat shock/stress response family (Hsp) including Hsp70, Hsp90, Bag1-L, Hsp70/Hsp-90-organizing protein (Hop), and Hsc70/ Hsp70-interacting protein (Hip) (202-204).

The high sequence homology and conserved primary cleavage specificity of human and mouse granzymes has led to widespread and interchangeable use of human and mouse enzymes in experimental conditions, usually without side-by-side comparisons being made (205-207). Human and mouse GrB exhibit substantial difference in their ability to cleave Bid, as well as several other protein substrates, such as DFFA and procaspase-8 (206). Thus, conclusions based on mixing human enzymes with mouse protein substrates and vice versa should be always interpreted with caution.

1.5.4. Intranuclear translocation and direct activation of DNA fragmentation factor

Once delivered into the cytoplasm of target cells, GrB is rapidly translocated to the nucleus (208-210). The precise mechanism of GrB translocation and accumulation within the nucleus and nucleolus is still unclear. Both unglycosylated and the high-mannose glycan moiety bearing GrB molecules can be imported into the nucleus. On the other hand, GrB molecules containing complex glycan moieties are excluded from the nuclear entry (208). The nuclear GrB import is independent of both ATP and GTP, but there is some evidence to be dependent on certain cytosolic factors including importin (IMP)-α (208-211). In the cytoplasm, GrB is a target of serpinB9 (SB9) which forms with GrB a covalent inhibitory complex (see below). The GrB●SB9 complex may prevent the nuclear import of active GrB by competing with free GrB for binding to IMP-α (211).

Once in the nucleus, GrB directly cleaves the subunit A of DFF (300). DFF is a heterodimer of the inhibitor/chaperone subunit A (DFFA) and the nuclease subunit B (DFFB) and it is prebound to DNA (11). After proteolytical fragmentation of DFFA subunit, the DFFB subunit becomes catalytically active via homodimerization and/or oligomerization and cleaves both strands of the genomic DNA (11,212). GrB can alternatively activate DFF in cancer cells which are unable to translocate the active caspase-3, the main DFF activator (212), into the nucleus (cf.213) or carry a loss-of-function mutation of the *CASP3* gene (214). Apart from DFF, GrB is able to cleave several other nuclear proteins, see Table I.

1.5.5. Detachment of cells from extracellular matrix and anoikis

Both newly synthesized GrB zymogen and active GrB are constitutively and non-specifically released from CTLs (132). Once secreted, the active GrB can cleave components of extracellular matrix such as vitronectin, fibronectin and laminin (215,216). This event can induce anoikis, the detachment-triggered cell death. (215-217). In general, changes of cell adhesion by GrB may have significant biological and pathobiological consequences. Due to extracellular matrix remodelling, GrB may contribute to migration of activated leukocytes through tissues. Moreover, the secreted GrB may either inhibit

tumourigenesis via inducing anoikis of tumour cells, or it may facilitate tumourigenesis through promoting tumour cell spreading, migration and invasion (217-220).

1.6. Regulation of granzyme B activity

It remains enigmatic how one CTL can kill multiple target cells over longer periods of time without self-destruction and why tumour cells or virus-infected cells became resistant to granule-mediated apoptosis. In the past period, several candidate regulators of GrB activity have been identified. First, the serine proteinase inhibitor serpinB9 (SB9) was identified and established as a powerful GrB inhibitor (221,222). Second, other granzymes, such as GrM and GrH, which are co-secreted with GrB have a potential to influence the activity of GrB indirectly. It has been showed that both granzymes can promote GrB activity through the direct cleavage and inactivation of its inhibitors. The GrM destroys SB9 in a variety of target cells (58) whereas the GrH attacks L4-100K in the virally infected cells (223). Moreover, certain cell surface-bound and/or secreted proteases, such as cathepsin B, might control the susceptibility of various tumour cells to the CTL-mediated killing via the proteolytic inactivation of PFN (cf.224).

1.6.1. Viral GrB inhibitors

Cytokine response modifier A (CrmA)

CrmA is a cowpox virus-derived 39 kDa serpin protein and plays an important role in regulation of the response associated with the cowpox virus infection via the inhibition of caspase-1 (225). Besides this anti-inflammatory activity, CrmA also showed the anti-tumour activity via targeting and inhibiting both the GrB- and caspase-8-initiated death pathways (226-229). CrmA is classified as a "cross-class" inhibitor because of the inhibition of GrB, a serine proteinase, and some caspases, which are cysteine proteinases, (230). Interestingly, CrmA and SB9 show extensive structural homology (221) and this suggests that SB9 might also function as a "cross-class" protein inhibitor of proteinases (see below).

Adenoviral L4-100K protein

Another member of the viral family of GrB inhibitors is the adenovirus assembly protein L4-100K/Ad5-100K. This protein is required for the life cycle of human adenovirus type 5 (Ad5), including virus assembly and activation of late viral protein synthesis (231,232). L4-100K is a substrate for GrB and inhibits the protease through an unclear mechanism involving interactions of this protein with both the active site and an exosite in the GrB molecule (232). It has been demonstrated that the inhibitory effect of L4-100K on GrB can be eliminated by GrH (223).

1.6.2 Non-viral granzyme B inhibitors

SerpinB9 (Proteinase inhibitor-9)

Human SB9 is a 42-kDa intracellular protein. It is a member of the serpin superfamily, an ovalbumin family serpin (i.e. a clade B serpin) (233,234). By definition, members of this family lack a classical secretory signal peptide (234,235). SB9 is very efficient and a highly specific physiological inhibitor of GrB (K_{ass} = 1.7 x 10^6 $M^{-1}\cdot s^{-1}$) that protects CTLs themselves as well as bystander cells from misdirected GrB (222,236,237).

SERPINB9 gene organization and expression, and subcellular localization and function of serpinB9

Human *SERPINB9* gene is localized to a gene cluster on the chromosome 6 at p25. Besides the *SERPINB9* gene, the 6p25 region contains also *SERPINB1* gene, encoding MNEI, and *SERPINB6* gene, encoding PI-6 (238-242). *SERPINB9* gene comprises of seven exons and six introns (242,243). The translation start site resides in exon 2 and the RCL, a region of the SB9 protein which is proteolytically attacked by GrB, is located in exon 7 (243).

SB9 is abundantly expressed in cells that produce high levels of GrB, i.e. in $CD8^+$ T cells and NK cells (222,237,244). Its major physiological function is to defend these cells against the misdirected autonomous GrB (237,245). Moreover, SB9 may also protect bystander cells or antigen-presenting cells likely to be exposed to GrB during an immune response (237,245). Consistent with such a role is the expression of SB9 in B cells (222),

monocytes (246), mast cells (247), endothelial and mesothelial cells (248), smooth muscle cells (249) and dendritic cells (237,250). Cells at immune-privileged sites, including the eye lens capsula, testes, ovary, placenta, and embryonic stem cells, also upregulate the SB9 expression (244,248,251).

Highly variable levels of SB9 expression were also detected at in human cancer cells of carcinomas of the breast, cervix, nasopharynx, esophagus, stomach, colon, and lung, and melanomas (252-257). The mechanisms responsible *in vivo* for the differential expression of SB9 in cancer cells are unknown so far.

In a wide spectrum of cells, SB9 protein is expressed in both the cytoplasm and the nucleus (258). This nucleocytoplasmic distribution protects the SB9-expressing cells against the GrB-mediated damage of the target proteins inside of these compartments. The exact mechanisms of SB9 nuclear import is not known so far. Conversely, sensitivity of SB9 nuclear export to leptomycin B points to the involvement of Crm1 protein (258). This finding is consisted with the presence of a functionally conserved nuclear export signal in SB9 protein (259).

Expression of SB9 can be up-regulated or induced in CTLs, a NK cell line YT-N10, endothelial cells, dendritic cells, human hepatocytes, hematoma cell lines HepG2 and Huh-7, and gastric cancer cells by several cytokines and inflammatory mediators, such as IL-1β, IL-18, TNF-α, interferon-α and -β, 12-O-tetradecanoylphorbol-13-acetate and lipopolysaccharide (237,247,248,256,260-262). It has been demonstrated that an AP-1 binding site and two NF-κB binding sites in the *SERPINB9* gene promoter play a role in the IL-1β-mediated SB9 expression (260). Moreover, there is evidence that estradiol-17β and other oestrogens can induce the expression of SB9 in human hepatocytes and in the ER-α-positive hepatoma cell line HepG2-ER7 (263,264). The oestrogen-mediated induction of SB9 expression in HepG2-ER7 cells and MCF-7 protect these cells against the CTLs- and NK cells-triggered apoptosis (265,266,267). A unique ERU located approximately 200 nucleotides downstream of the transcription start site is responsible for the oestrogen-triggered and ERα-mediated induction of *SERPINB9* gene transcription. It consists of an imperfect palindromic ERE being immediately adjacent to a direct repeat containing two consensus ERE half-sites separated by 13 nucleotides (DR13) (268).

It has been proven that up-regulated expression of SB9 in cancer cells may contribute to their resistance against the immune mediated killing and thus it can promote tumour growth and progression (255,267,269). To date executed studies showed that high levels of SB9 are associated with poor therapeutic response and prognosis in lymphomas and melanomas (255,270).

Proteinase inhibitor-9 structure and mechanism of inhibition

SB9 is comprised of 376 amino acids and its tertiary structure is composed of nine α-helices (denoted A-I) and three β-sheets (denoted A-C) (233,243). The regions important for protease inhibition are located on β-sheet A and the RCL. The RCL of SB9 acts as a pseudosubstrate and contains a GrB cleavage site P1-P1' which equals to the residues E^{340}-C^{341} (114). The RCL segment $VVAE^{340}$-CCME constitute an extended P4-P4' region important for interaction with GrB, while the P4' residue E^{344} is necessary for efficient binding of SB9 and GrB (114). SB9 is a direct and irreversible GrB inhibitor with a stoichiometry of inhibition of 1:1 (222). SB9 represents a suicide substrate for GrB because the GrB-mediated proteolytic cleavage of SB9 RCL causes a rapid conformational change in the serpin, resulting in the formation of a stable serpin-proteinase covalent complex.

Recent studies demonstrate that SB9 can inhibit not only the GrB/perforin-mediated death pathway but also death pathways triggered with TNFα, TRAIL and FasL (271,272). It seems that SB9 directly interact with the intermediate active forms of caspase-8 and -10 (272). Thus SB9 can be classified as a "cross-class" proteinase inhibitor.

Other granzyme B inhibiting serpins

Another member of the intracellular serpin family was isolated from the rat pituitary gland and it is termed raPIT5a (273,274). This protein is expressed also in other rat tissues (274). raPIT5a has a high amino acid sequence similarity to the sequence of SB9 (273) and its incubation with human GrB lead to the formation of an SDS-stable enzyme-inhibitor complex (273,274).

Recently, it was discovered that a mouse serpin3n is an inhibitor of mouse and human GrB (275). This serpin is expressed and secreted by Sertoli cells and forms an SDS-

stable enzyme-inhibitor complex with GrB (275). Moreover, it was also indicated in this study that serpin3n can protect Sertoli cells from harmful GrB-mediated immune reactions.

2 MATERIALS AND METHODS

All methods and techniques, which are commonly used in our laboratories, including isolation and quantification of total RNA, real-time RT-PCR analysis, Western blot analysis, enzyme analyses and cell culture techniques, are described in the selected publications (see section 9). Since the immunocytochemical analysis has been recently introduced to our laboratory by the author of this thesis, this method will be described in details.

2.1 Immunocytochemistry of estrogen receptor-α

Immunocytochemical staining for ERα in ten human NSCLC cell lines and four human breast cancer cell lines (two $ER\alpha^+$ and two $ER\alpha^-$ cell lines) was performed using the avidin-biotin peroxidase method. The cells grown in cell culture chambers (Lab-Tek II Chamber Slides, Nunc, Denmark) were fixed with 3% paraformaldehyde in PBS (phosphate buffer saline) for 10 min. The cells were permeabilized by 0.2% (v/v) Triton-X100 in PBS using incubation for 10 min at room temperature and then were washed in 0.2% (v/v) Tween-20. To reduce non-specific background staining, the cells were incubated in a blocking solution (85.5% methanol - 3% H_2O_2) for 5 min and washed in distilled water. The cells were then incubated with a primary anti-ERα antibody (Moloclonal Mouse Anti-Human Estrogen Receptor alfa, clone 1D5; DAKO, Denmark; cat. No. M7047), which was diluted 1:50 using the Dako Antibody Diluent (cat. No. S0809). Incubation with mouse IgG1 (DAKO, cat. No. X0931) was carried out in parallel in negative controls.

The cell samples were then washed twice in distilled water and with a wash buffer (DAKO; cat. No. S3006), incubated with the EnVision system reagents (Envision ™+/HRP; DAKO, cat. No. K4000) for 30 min at room temperature and washed again. 3,3´-Diaminobenzidine solution (Liquid DAB+; DAKO, cat. No. K3467) was applied for 15 min as a chromogen. The stained cells were then washed twice in distilled water and were mounded in Ultramount, a mounting medium for microscopy (DAKO, cat. No. S1964). The mounted preparations of stained cells were photographed in phase contrast on an inverted light microscope (IX71, Olympus, Tokyo, Japan) fitted with a digital camera

(Zoom C5050, Olympus), which was operated from within the Quick PhotoMicro software (Olympus).

3 HYPOTHESIS AND AIMS OF THE THESIS

Granzyme B is a key proapoptotic secretory protease of CTLs and NK cells. Its specific proapoptotic effects in cancer cells can be blocked by increased expression of serpinB9. *SerpinB9* gene expression can be transcriptionally up-regulated by some interleukins and by the oestrogen activated oestrogen receptor-α (ERα) in cells which express ERα protein.
The specific aims of this thesis regarding the regulation of granzyme B - induced apoptosis were:

1. To evaluate the expression of SB9 and to examine its inhibitory activity against exogenous active granzyme B in non-small cell lung carcinoma cell lines and tissues.
2. To analyse the expression status of granzyme B mRNA in non-small cell lung carcinoma cell lines and tissues.
3. To investigate the role of estradiol-17β (E2), selected ILs and DNA methylation in regulation of SB9 expression in non-small cell lung carcinoma cells.

The apoptosome apparatus is a cell death signalling platform, which recruits and activates the apoptosis initiator procaspase-9. It is assembled in the cytosol via the cyt-c- and (d)ATP-mediated formation of an Apaf-1 heptameric complex. Activation of the apoptosome apparatus is often impaired in various types of cancer but the molecular basis of its suppression is still unknown. APIP and UACA/nucling belong to the endogenous regulators of apoptosome apparatus.
The specific aims of this thesis regarding the regulation of the apoptomosome apparatus was:

4. To investigate whether DNA methylation is involved in the transcriptional regulation of expression of *APIP* and *UACA* genes in non-small cell lung carcinoma cell lines.

4 RESULTS

4.1 Publication A: Expression of proteinase inhibitor-9/serpinB9 in non-small cell lung carcinoma cells and tissues

The aim of this study was to investigate expression status of SB9 in lung carcinoma cells and tissues and to verify the inhibitory activity of the endogenous SB9 protein on the exogenous active granzyme B.

First, the expression of SB9 mRNA was detected in all studied NSCLC cell lines but its level was highly variable (Fig. 2, Fig. 3A). The SB9 mRNA expression was strong in six of ten examined NSCLC cell lines and it was weak in the remaining four ones (Fig. 3A). I also analysed the expression of SB9 mRNA in SCLC cell lines which was lower, but not significantly, as compared to NSCLC cell lines (P = 0.305; Mann-Whitney test). The median/range values of the β-actin mRNA-normalized expression of SB9 mRNA ($2^{-\Delta C_T}$) for NSCLC cell lines (n = 10) and SCLC cell line (n = 7) were as follows: 0.0167/0.00008 - 0.0404 and 0.0037/0.0013 - 0.0118, respectively. Moreover, we could quantitate the expression of SB9 mRNA in two SCLC tumours which was higher ($2^{-\Delta C_T}$ = 0.0319 and 0.0140) than in the examined SCLC cell lines.

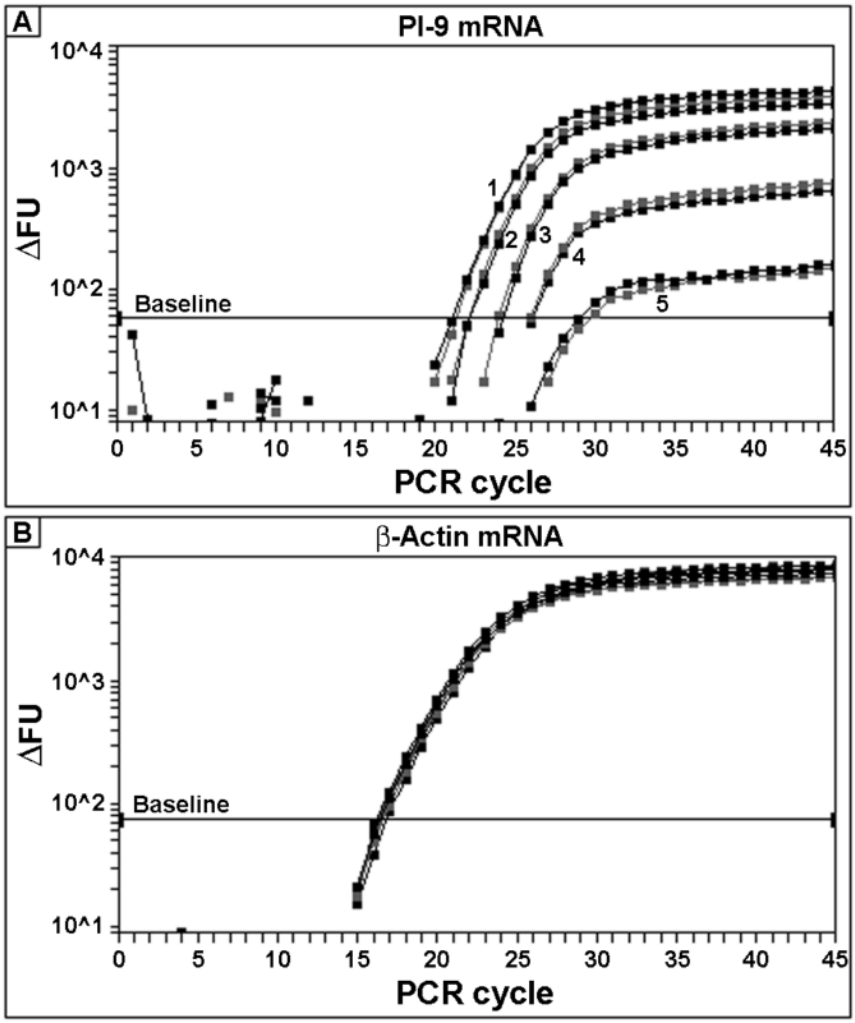

Figure 2. Real time RT-PCR quantitation of expression of SB9 mRNA and β-actin mRNA. Real time RT-PCR quantitation SB9 mRNA expression (A) and β-actin mRNA expression (B) (an endogenous reference transcript) in NSCLC cell lines (1 = LXF-289, 2 = CALU-1, 3 = NCI-H1299, 4 = SKLU-1, 5 = COLO-699). All real time RT-PCR assays were performed with an input of 200 ng of total RNA and were run in duplicate.

Second, the expression level of SB9 protein, which was detected by Western blot analysis in the detergent-containing extracts or in cytosols from all studied NSCLC cell lines, was also highly variable (Fig. 3B and 3C, Fig. 4B). A significant positive correlation was confirmed between the expression of SB9 mRNA and protein in the investigated NSCLC cell lines (Fig. 3D). Among the studied NSCLC cell lines, I could distinguish high and low expressors of both SB9 mRNA and protein (Fig. 3D). They showed significant difference in the expression of SB9 transcript as well as SB9 protein (P = 0.0095, Mann-Whitney test).

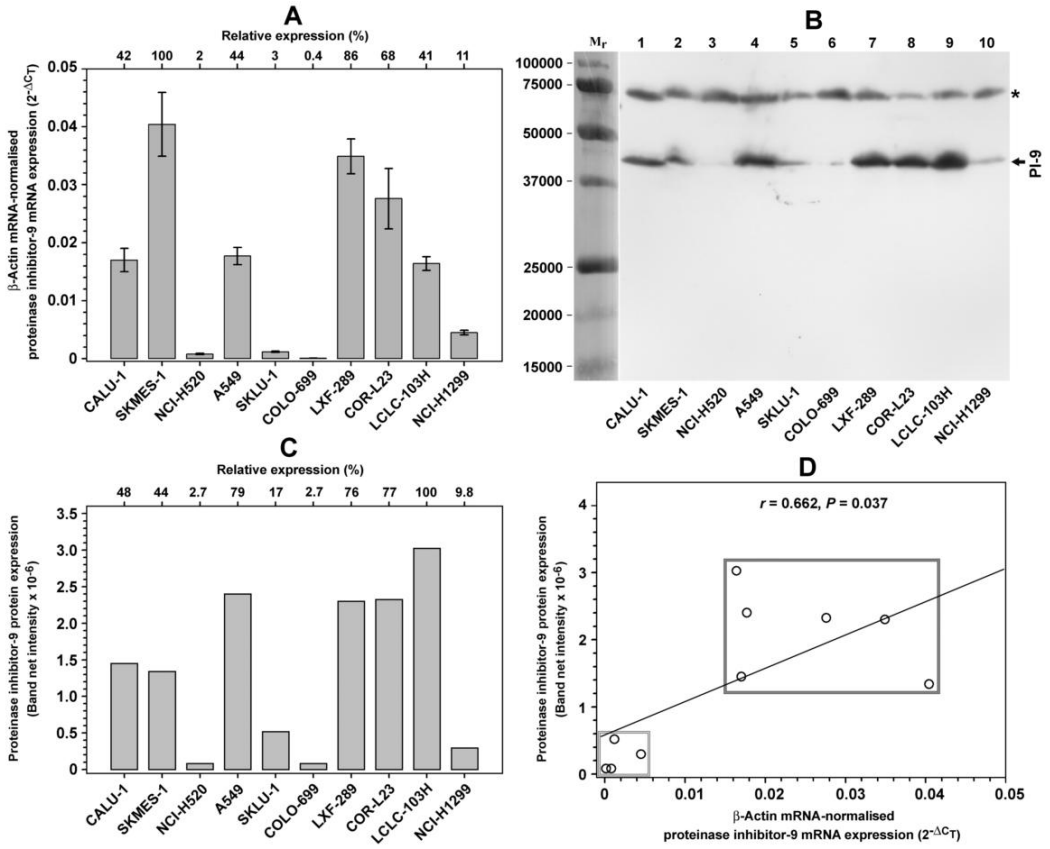

Figure 3. Analysis of SB9 expression in non-small cell lung carcinoma cell lines.
(A) Relative expression of SB9 mRNA in the cell lines as quantitated by real time RT-PCR. Data indicated as mean ± SEM from three independent experiments. (B) Expression of SB9 protein ($M_r \approx 43000$) in the cell lines as analysed by SDS-PAGE and immunoblotting. The asterisk denotes an unknown immunoreactive protein ($M_r \approx 71300$). (C) Relative expression of SB9 protein as determined by image analysis of the immunogram shown in (B). (D) Correlation analysis of mRNA and protein expression for SB9, respectively, in the cell lines. The rectangles define two groups of NSCLC cell lines: the high and low SB9 expressors. The Pearson linear correlation coefficient *r* and its *P* value are indicated.

Third, it is known that many serpins, including SB9, form SDS-stable covalent complexes with serine proteases, including GrB (222,248,233,276). For this reason, I attempted to detect formation of a complex between hrGrB and the endogenous SB9 protein expressed in cell-free cytosol from NSCLC cell lines, using denaturing SDS-PAGE and immunoblotting. The incubation of the cytosols with hrGrB led to a marked shift of the molecular mass of hrGrB to higher values (Fig. 4A) and to a substantial decrease of the intensity of SB9 protein band in parallel (Fig. 4B). Unfortunately, proving the molecular mass shift of SB9 to higher values was spoiled in the experiments by the occurrence of an unknown protein reacting with the anti-SB9 antibody PI9-17 and having M_r of approximately 71300 (Fig. 3B, Fig. 4B). A similar unknown protein was observed earlier in melanomas (255).

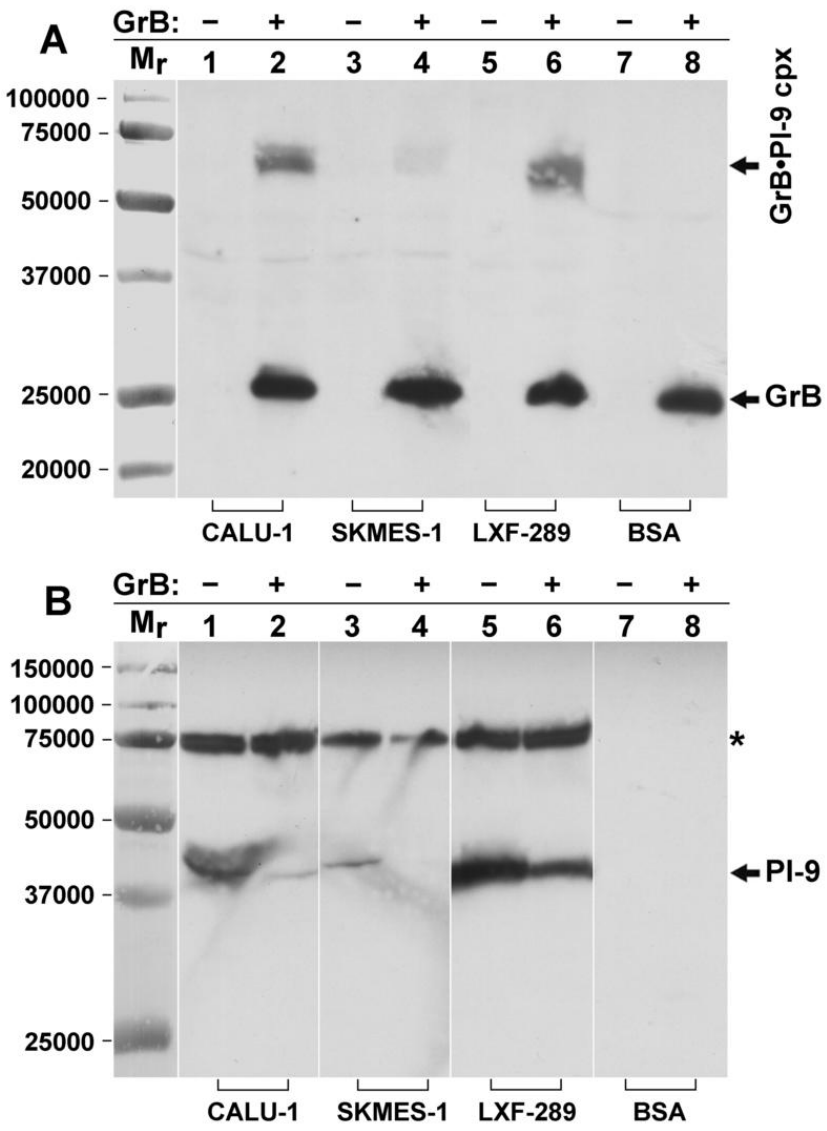

Figure 4. Interaction of human recombinant GrB with SB9 in NSCLC cell cytosols. Cytosol samples from several NSCLC cell lines were incubated with (+) and without (-) hrGrB and the reaction mixtures were analysed by denaturing SDS-PAGE and immunoblotting. As a negative control, a mixture of human recombinant GrB and bovine serum albumin (BSA) was used (lane 8 in A and B). (A) The membrane with transferred proteins was incubated with an anti-GrB antibody (2C5). Arrows show an SDS-resistant GrB·SB9 complex (cpx) and free GrB. (B) The membrane with transferred proteins was probed with an anti-SB9 antibody (PI9-17). The asterisk denotes an unknown immunoreactive protein.

Our laboratories showed previously that GrB cleaves and activates procaspase-3 in extracts from NSCLC cells and tissues (154). To support the hypothesis that the endogenous SB9 can contribute to the resistance of NSCLC cells against the GrB-mediated apoptosis, the relationship between the SB9 protein and the GrB-induced caspase-3-like activity levels were studied. The results showed that the SB9 expression level negatively correlates with the GrB-induced caspase-3-like activity in NSCLC cell extracts (Fig. 5).

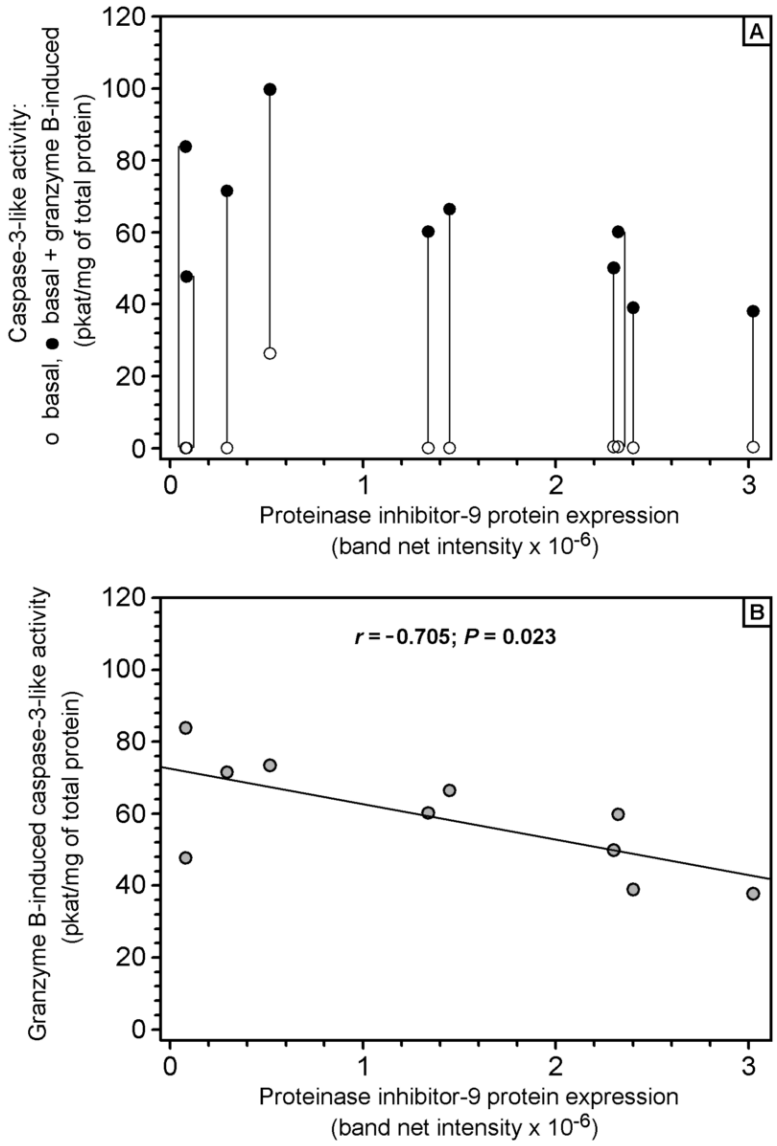

Figure 5. Relationship between the expression of SB9 protein and the granzyme B-induced caspase-3-like activity in extracts from NSCLC cell lines. (A) Basal and total induced (i.e. basal + granzyme B-induced) caspase-3-like activity. (B) Correlation analysis of the granzyme B-induced caspase-3-like activity and the level of SB9 protein expression in NSCLC cell lines. The Pearson linear correlation coefficient *r* and its *P* value are indicated.

Moreover, we examined the expression status of SB9 mRNA in the tumours and matched lungs from surgically treated patients to reveal the relationship between SB9 expression in NSCLC tumours and the clinicopathological data of NSCLC patients. All studied NSCLC tumour types and lung tissues showed the expression of SB9 mRNA (Fig. 6), but there was no statistically significant difference in the SB9 mRNA expression between the tumours and lungs (Table II). However, in 26 (17%) of 150 studied NSCLC

patients the tumours had more than twofold higher level of SB9 mRNA as compared to matched lungs. The SB9 mRNA expression in NSCLC tissues (Table II) and NSCLC cells (see the data above) was not significantly different (P = 0.222; Mann-Whitney test). Moreover, NSCLC tumours of various histopathological types showed comparable levels of SB9 mRNA (Table II). The expression of SB9 mRNA was not significantly affected by patients ‘gender, smoking status and tumour stage (Table III, IV). On the other hand, SB9 mRNA expression was significantly higher in the less-differentiated tumours (grade 3) as compared to the well-differentiated ones (grade 1+2) (Table III, IV). Nonetheless, when SQCLCs and LACs were considered separately, the expression of SB9 mRNA remained significantly higher in the less-differentiated LACs, but not in the less-differentiated SQCLCs (P = 0.002 and P = 0.591, respectively; Mann-Whitney test).

My contributions to this work were culturing of NSCLC cell lines, management and coordination of tissue samples collection from surgically treated NSCLC patients and of total RNA isolation, accomplishment of real time RT-PCR assays, preparation of protein extracts and cytosols, protein determination, Western blot analysis, analysis of SB9●granzyme B complexes, and procaspases activation by granzyme B.

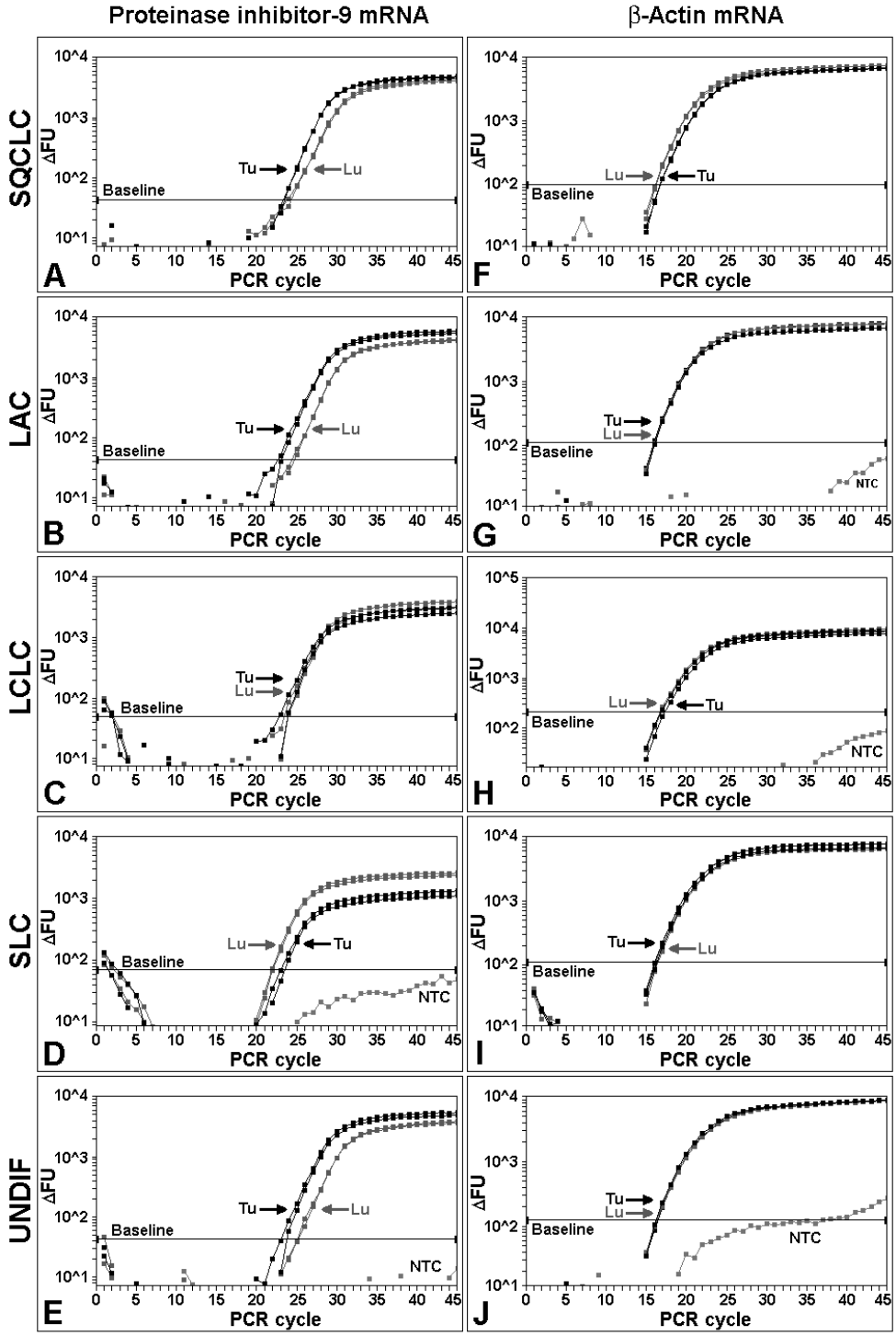

Figure 6. Real time RT-PCR quantitation of expression of SB9 and β-actin mRNAs in non-small cell lung carcinoma tissues and matched lungs. The set of PCR amplification plots, representing the coupled real time RT-PCR assays with an input of 200 ng of total RNA and running in duplicate, shows the expression of SB9 (A – E) and b-actin (F – J) mRNAs in five tumour (Tu)-lung (Lu) matched pairs. The tumours were: squamous cell lung carcinoma (SQCLC), lung adenocarcinoma (LAC), large cell lung carcinoma (LCLC), sarcomatoid lung carcinoma (SLC), and undifferentiated lung carcinoma (UNDIF). NTC = no template control. DFU = background-subtracted fluorescence intensity (in arbitrary units) of the released reporter dye (6-FAM or VIC).

Table II. Real time RT-PCR analysis of serpinB9 mRNA expression in non-small cell lung carcinomas and lungs.

Tumour type	n [a]	β-Actin mRNA-normalised expression of SB9 mRNA ($2^{-\Delta C_T}$) [b]		Statistical difference (*P*) of SB9 mRNA expression in Tu *versus* Lu [c]	Tu/Lu ratio of SB9 mRNA expression [b]	Number of patients with Tu/Lu SB9 mRNA expression ratio ≥ 2 and ≤ 0.5
		Tumours (Tu)	Lungs (Lu)			
NSCLC	150	0.0172 (0.0002 – 0.3737)	0.0173 (0.0003 – 0.4506)	0.816	0.9 (0.09 – 23.3)	26 (17%) and 28 (19%)
SQCLC	69	0.0172 (0.0002 – 0.2132)	0.0138 (0.0003 – 0.4506)	0.811	0.9 (0.16 – 23.3)	13 (19%) and 12 (17%)
LAC	56	0.0163 (0.0008 – 0.3737)	0.0294 (0.0015 – 0.2774)	0.275	0.8 (0.09 – 5.3)	8 (14%) and 16 (29%)
LCLC	7	0.0144 (0.0078 – 0.0988)	0.0082 (0.0056 – 0.0960)	0.382	1.8 (0.95 – 2.6)	3 (43%) and 0
SLC	4	0.0455 (0.0150 – 0.0643)	0.0499 (0.0274 – 0.0587)	0.885	1.1 (0.35 – 1.2)	0 and 1 (25%)
UNDIF	11	0.0146 (0.0024 – 0.1267)	0.0067 (0.0017 – 0.0802)	0.308	1.5 (0.53 – 4.6)	2 (18%) and 0

[a] Total of 150 NSCLC patients was studied including 69 patients with SQCLC, 56 patients with LAC, 3 patients with SQCLC+LAC mixed type tumours, 7 patients with LCLC, 4 patients with SLC, and 11 patients with UNDIF.

[b] Data indicated as median with the range in parentheses.

[c] Statistical difference of the β-actin mRNA-normalised PI-9 mRNA expression in Tu versus Lu was calculated by Mann-Whitney test.

Table III. Impact of gender, smoking, tumour grade, and tumour stage on serpinB9 mRNA expression in non-small cell lung carcinomas.

Category		n [a]	β-Actin mRNA-normalised expression of SB9 mRNA ($2^{-\Delta C_T}$) [b]	Statistical difference (*P*) [c]
Gender	**Men**	110	0.0163 (0.0002 – 0.2365)	0.162
	Women	40	0.0237 (0.0008 – 0.3737)	
Smoking	**Non-smokers**	20	0.0155 (0.0002 – 0.1486)	0.746
	Smokers	130	0.0178 (0.0005 – 0.3737)	
Tumour grade	**Grade 1+2**	57	0.0131 (0.0007 – 0.2132)	0.023
	Grade 3	67	0.0240 (0.0002 – 0.3737)	
Tumour stage	**Stage I**	83	0.0163 (0.0002 – 0.2365)	0.125
	Stage II+III	63	0.0192 (0.0008 – 0.3737)	

[a] The number, n, of examined NSCLC tissues belonging to the particular category is indicated.
[b] Data indicated as median with the range in parentheses.
[c] Statistical difference was calculated by Mann-Whitney test.

Table IV. Impact of gender, smoking, tumour grade, and tumour stage on serpin9 mRNA expression in non-small cell lung carcinomas.

Category		n [a]	β-Actin mRNA-normalised expression of SB9 mRNA ($2^{-\Delta C_T}$T) [b]	Statistical difference (*P*) [c]
Gender	**Men**	110	0.0163 (0.0002 – 0.2365)	0.162
	Women	40	0.0237 (0.0008 – 0.3737)	
Smoking	**Non-smokers**	20	0.0155 (0.0002 – 0.1486)	0.746
	Smokers	130	0.0178 (0.0005 – 0.3737)	
tumour grade	**Grade 1+2**	57	0.0131 (0.0007 – 0.2132)	0.023
	Grade 3	67	0.0240 (0.0002 – 0.3737)	
tumour stage	**Stage IA**	21	0.0107 (0.0009 – 0.1088)	0.476
	Stage IB	62	0.0172 (0.0002 – 0.2365)	
	Stage II+III	63	0.0192 (0.0008 – 0.3737)	0.126

[a] The number, n, of examined NSCLC tissues belonging to the particular category is indicated.
[b] Data indicated as median with the range in parentheses.
[c] Statistical difference between NSCLC tissues belonging to the particular category was calculated using Mann-Whitney test. The levels of SB9 mRNA expression in stage IB tumours and stage II+III tumours were compared, respectively, with those in stage IA tumours.

4.2 Publication B: Granzyme B-induced apoptosis in cancer cells and its regulation (Review)

Although this publication was mainly a review, I presented here also novel original data on the expression status of granzyme B mRNA in NSCLC cells and tumours as compared to lungs.

Surprisingly, there is some evidence that GrB can be expressed in cancer cells of primary human breast carcinomas (277,278), lung carcinomas (277), urothelial carcinomas (279) and in the nasal-type NK/T-cell lymphoma (280). Furthermore, GrB expression was revealed by immunohistochemistry in cancer cells of oral squamous cell carcinoma (281).

Considering these observations, we analysed the expression status of GrB mRNA in NSCLC cell lines and NSCLC tumours and matched lungs from surgically treated patients using uncoupled real time RT-PCR. All examined NSCLC cell lines expressed GrB mRNA but its level was quite low and variable (Fig. 7A). However, compared to NSCLC cell lines, the expression of GrB mRNA in NSCLC tumours was substantially higher (Fig. 7B). In addition, there was no statistically significant difference in the GrB mRNA expression in NSCLC tumours and matched lungs (Fig. 7B). These results indicate that the lung cancer cells themselves are not the major source of GrB expression in the lung tumours. This notion is further supported by undetectable expression of endogenous GrB protein in NSCLC cell lines (257).

My contributions to this work were participation on manuscript writing and on the experimental part, including culturing of NSCLC cell lines, management and coordination of tissue samples collection from surgically treated NSCLC patients, total RNA isolation, and accomplishment of real time RT-PCR assays.

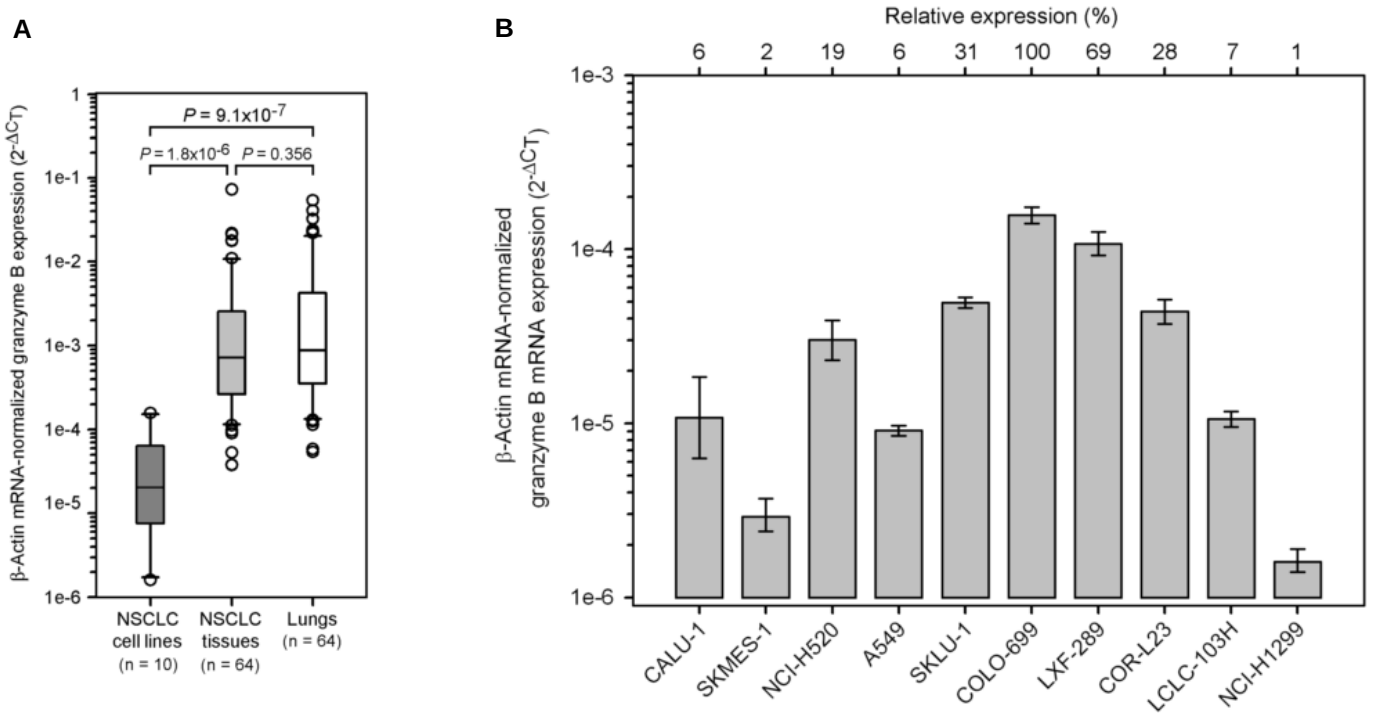

Figure 7. Granzyme B mRNA expression in NSCLC cell lines (A), cell lines and tissues and lungs (B). Expression of granzyme B mRNA in non-small cell lung carcinoma (NSCLC) cell lines and NSCLC tissues and lungs as analysed by uncoupled real-time RT-PCR. The sequences of the forward primer, the reverse primer, and the fluorogenic TaqMan probe used for quantification of granzyme B mRNA expression were, respectively: 5'-CTACTGCAGCTGGAGAGAAAGG-3', 5'- CAGCCGGCCACACTGCATGTCT-3', and 5'-(6FAM)GTACTGTCGTAA TAATGGCGTAAGTC(TAMRA)-3'. The sequences of the forward primer, the reverse primer, and the fluorogenic TaqMan probe used for quantification of ß-actin mRNA (an endogenous reference transcript) expression were, respectively: 5'-CTGGCACCCAGCACAATG-3', 5'-GGGCCGGACTCGTCATAC-3', and 5'-(VIC)AGCCGCCGATCCACACGGAGT(TAMRA)-3'. (A), Relative levels of expression of ß-actin mRNA-normalized granzyme B mRNA in NSCLC cell lines. Data indicated as mean ± SEM from three independent experiments. (B), Comparison of the ß-actin mRNA-normalized granzyme B mRNA expression in NSCLC cell lines and NSCLC tissues and matched lungs from surgically treated patients. In the box plot, the upper and the lower boundary of the box and the line within the box indicate the 75th and 25th percentiles and the median, respectively. The error bars above and below the box indicate the 90th and 10th percentiles. Statistical difference (P) between the granzyme B mRNA expression levels was calculated by Mann-Whitney test.

4.3 Publication C: Down-regulated expression of apoptosis-associated genes APIP and UACA in non-small cell lung carcinoma

The objective was to determinate whether DNA methylation is involved in the transcriptional regulation of APIP and UACA expression in NSCLC cell lines For investigation of the expression of APIP and UACA mRNAs in NSCLC cells lines we used coupled real-time RT-PCR to quantify the level of these transcripts and β-actin mRNA (an endogenous reference transcript).

To analyse whether DNA methylation is involved in the transcriptional regulation of APIP and UACA expression in NSCLC cell lines, we cultured the tumour cells for 72 hours in the presence and the absence of 5-aza-2'-deoxycytidine (decitabine, ADC), a DNA methyltransferase inhibitor and demethylating agent (282-284). Although the statistically significant increase of APIP and UACA mRNAs expression in several NSCLC cell lines cultured in the presence of ADC was detected (Fig. 8A and 8B), the ADC-induced up-regulation of transcript expression higher than two-fold was observed only for UACA mRNA in CALU-1 cells (a 3.9-fold increase; Fig. 8B).

My contributions to this work were culturing of NSCLC cell line in the presence and absence of ADC, total RNA isolation, and accomplishment of real time RT-PCR assays.

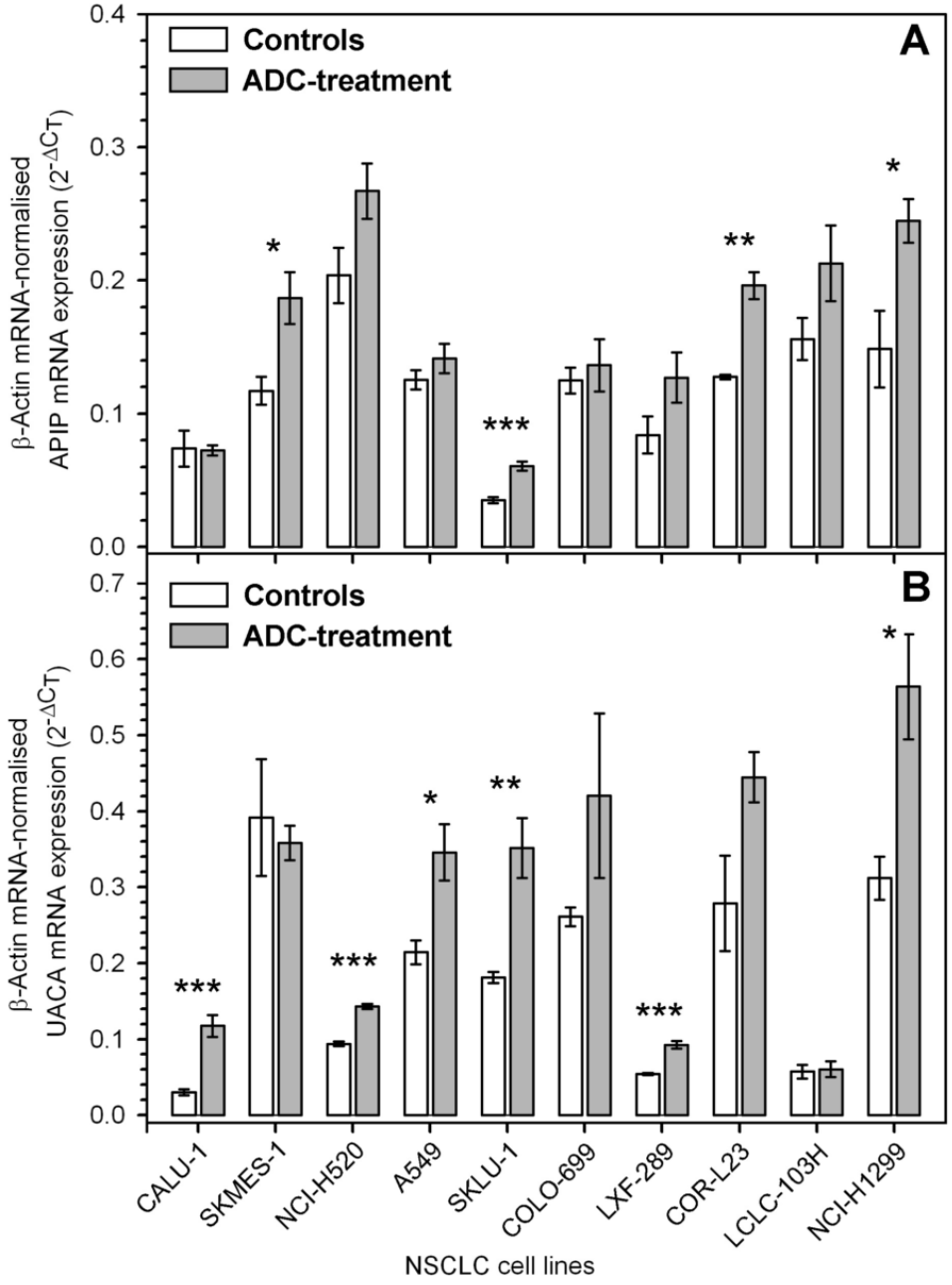

Figure 8. Effect of 5-aza-2'-deoxycytidine (ADC) on the expression of APIP mRNA (A) and UACA mRNA (B) in cultured NSCLC cell lines. Data are indicated as the mean ± SEM from three independent experiments. The asterisks indicate statistically significant up-regulation of transcripts expression in the ADC-treated cells: ***, $P < 0.01$; **, $P < 0.02$; *, $P < 0.05$ (t-test).

4.4 Unpublished results

To investigate the effect of E2 and IL-1β, IL-6, IL-18 on the SB9 expression in NSCLC cell lines, the cells were cultured in the presence and the absence of E2 (10 nM for 4 and 24 h), IL-1β, IL-6 and IL-18 (10, 50, 30 ng/ml, respectively for 24 h). The effect of E2 and the ILs on SB9 mRNA expression in NSCLC cell lines was rather weak and variable (Figs. 9 and 10).

The nuclear expression of ERα in NSCLC cell lines was examined by immunocytochemistry. Some positivity of the immunocytochemical staining, revealing only very low nuclear expression of ERα, was detected in 3 of 10 tested NSCLC cell lines: H520, COLO-699, COR-L23 cells (Figs. 11I, 11K and 11M). On the contrary, a strong positive immunocytochemical staining of nuclei was found in the $ER\alpha^{+}$ breast cancer cell lines MCF-7 and T47D (Fig. 11A and 11B).

Surprisingly, the treatment with 10 μM of ADC for 72 hours induced a significant up-regulation of SB9 mRNA expression in 6 of 10 tested NSCLC cell lines (Fig. 12). A strong up-regulation of SB9 mRNA expression was revealed in 4 NSCLC cell lines (Fig. 12). These NSCLC cell lines were previously shown to be low expressors of SB9 mRNA and protein (Fig. 3C and 3D).

My contribution to these results were cultivation of NSCLC cell with E2, ILs, and ADC, mRNA isolation and real-time RT-PCR analysis of SB9 mRNA levels in NSCLC cell lines.

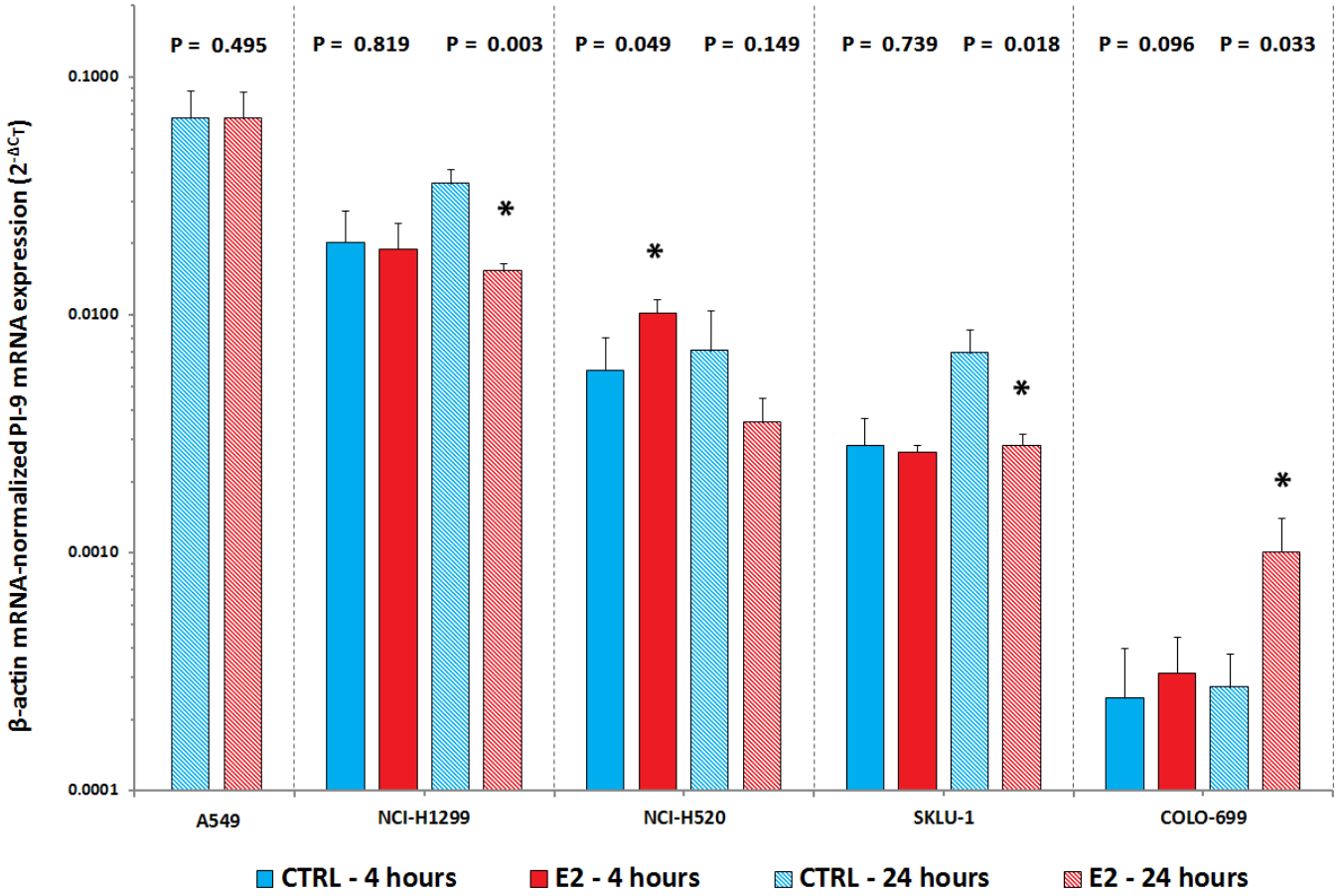

Figure 9. Effect of estradiol-17β on the expression of SB9 mRNA in in cultured NSCLC cell lines. Data are indicated as the mean ± SEM from three independent experiments. The asterisks demonstrate statistically significant up-regulation of transcripts expression in the ADC-treated cells: *, $P < 0.05$ (t-test).

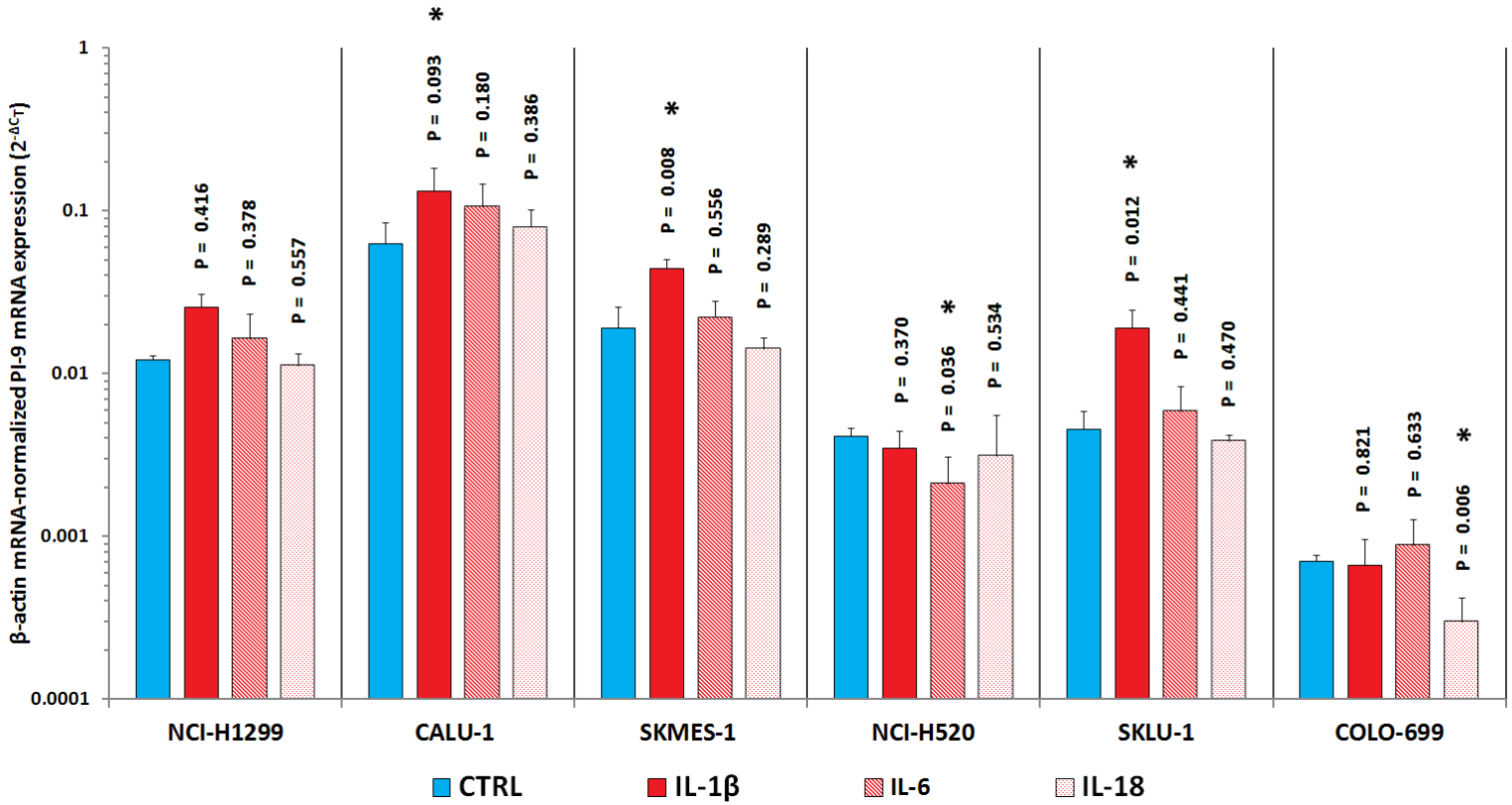

Figure 10. Effect of interleukins on the expression of SB9 mRNA in cultured NSCLC cell lines. Data are demonstrated as the mean ± SEM from three independent experiments. The asterisks indicate statistically significant up-regulation of transcripts expression in the ADC-treated cells: *, $P < 0.05$ (t-test).

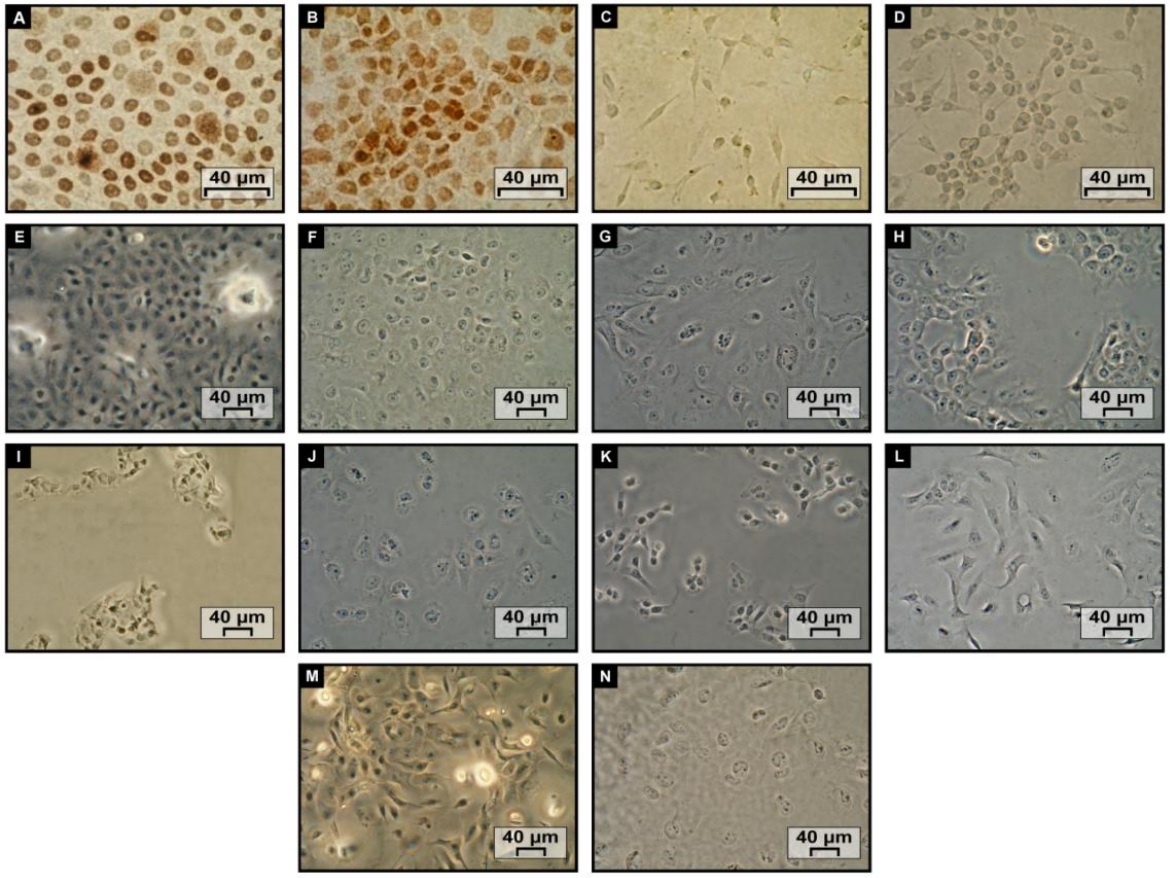

Figure 11. Immunocytochemistry staining of ER-α in NSCLC cell lines. E2 positive breast cancer cell lines (A) MCF-7, (B) T47D. E2 negative breast cancer cell lines (C) MDA-MB-231, (D) SK-BR3. (E) A549, (F) NCI-H1299, (G) CALU-1, (H) SKMES-1, (I) NCI-H520, (J) SKLU-1, (K) COLO-699, (L) LXF, (M) COR-L23, (N) LCLC. Weak positivity of E2 was detected in: H520, COLO-699, COR-L23 cells.

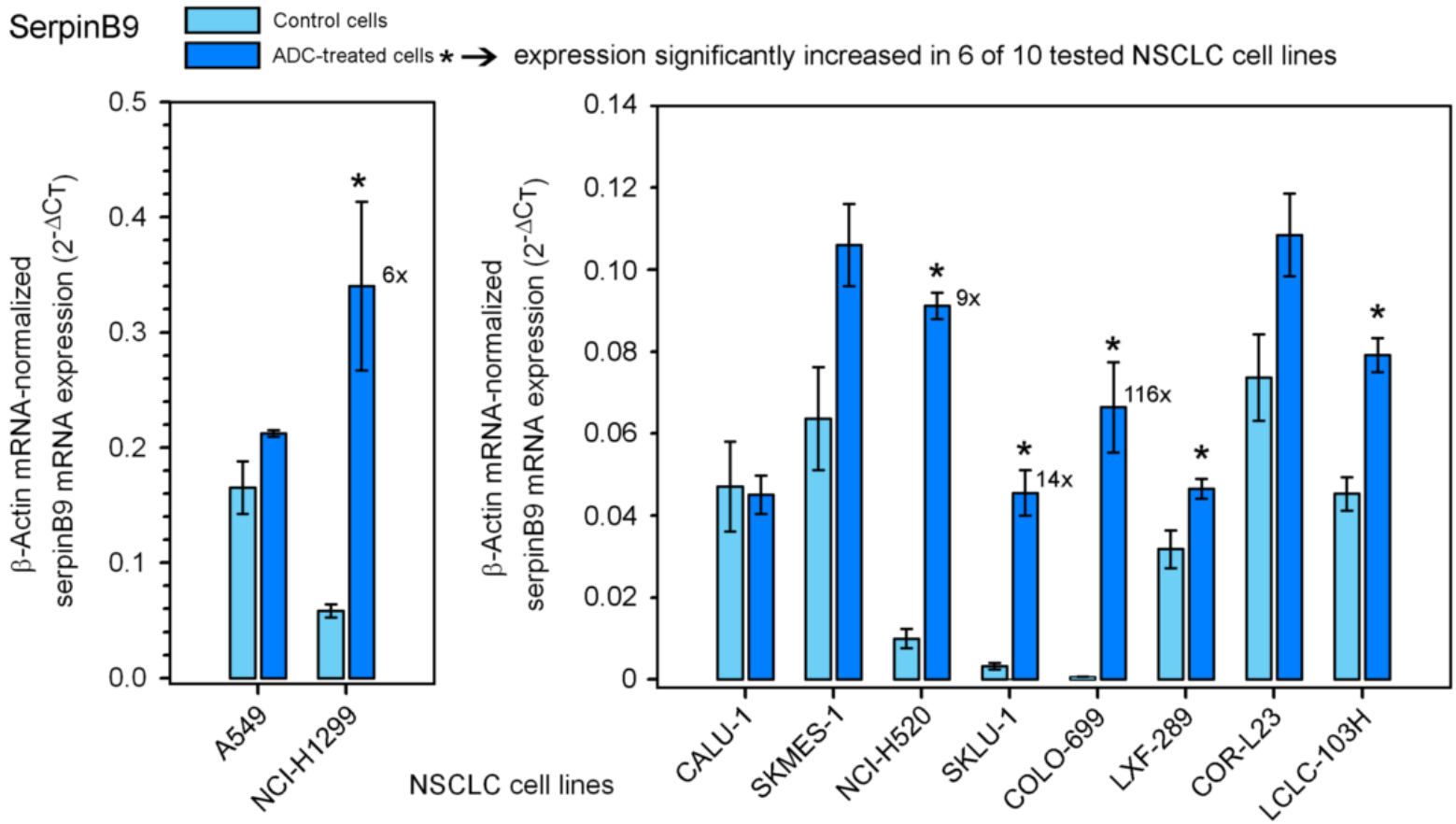

Figure 12. Effect of 5-aza-2'-deoxycytidine (ADC) on the expression of SB9 mRNA in cultured NSCLC cell lines. Data are indicated as the mean ± SEM from three independent experiments. The asterisks show statistically significant ($P < 0.05$, t-test) upregulation of SB9 mRNA expression in the ADC-treated cells. The numbers at the top of some dark blue columns incicate the degree of upregulation (fold increase) of SB9 mRNA expression in the ADC-treated NSCLC cells as compared to the same NSCLC cells cultured in the absence of ADC (controls).

5 DISCUSSION

In the presented publication A (257), it was demonstrated for the first time that both SB9 mRNA and protein are expressed in NSCLC cells. Surprisingly, it was also revealed that SB9 mRNA is expressed in SCLC cells at levels which were lower, but not significantly, as compared to NSCLC cells. There was found a positive correlation between the levels of SB9 mRNA and protein in NSCLC cells and, according to the differential abundance of SB9 mRNA and protein expression, the cells could be classified as low and high SB9 expressors. Highly variable levels of SB9 mRNA expression were already reported in cancer cells and tissues including breast, cervical, oesophageal, gastric, colon, and nasopharyngeal carcinomas, and melanomas and Ewing sarcomas (252-256,285). The mechanisms responsible *in vivo* for the differential expression of SB9 in malignant tumours of the same type are not known so far. There is evidence that they may involve transcriptional up-regulation of *SERPINB9* gene expression, mediated by NF-κB (260,286), the oestrogen activated ERα (266,267) and/or the AP-1 transcription factors (260,287). However, neither selected ILs nor E2 markedly up-regulated the transcriptional expression of SB9 in NSCLC cells (Fig. 9 and Fig. 10). Further immunocytochemical analysis using a panel of ten NSCLC cell lines revealed that only three NSCLC cell lines (NCI-H520, COLO-699, and COR-L23) showed occasional and weak positivity of ERα expression in their nuclei. Although probably not involved in NSCLC tumours, the strong up-regulation of transcriptional expression of *SERPINB9 gene* by various oestrogens (265,266,267) deserves further systematic investigation in the oestrogen-responsive tumours bearing ERα, especially in breast, uterine and ovarian carcinomas. The reason for these studies is to clarify whether the overexpression of SB9 may predict more aggressive and therapeutically resistant tumours (cf.255). Since c-Jun and c-Fos, the major constituents of AP-1 transcription complex, seem to play an important role in tumourigenesis of NSCLCs (288-290), there is possible that the AP-1 transcription complex might be involved in the regulation of transcriptional expression of *SERPINB9* gene in NSCLC cells. Finally, because of profound variability of SB9 expression in tumours of the same histopathological type, ranging from very high to barely detectable SB9 mRNA levels (252,257), it is important to determine whether the *SERPINB9* gene is a target for epigenetic reprogramming in cancer cells (291). Surprisingly, our recent data

indicate that DNA methylation can down-regulate the expression of SB9 in a subset of NSCLC cell lines (Fig. 12). This suggests that DNA demethylating drugs might desensitize NSCLC tumours against the granzyme B-induced apoptosis through induction of SB9 expression.

In order to confirm the interaction between SB9 and GrB in the cytosol from NSCLC cells, hrGrB was incubated with the cytosols and searched for molecular mass shift of hrGrB and SB9 using denaturing SDS-PAGE and immunoblotting. We revealed a marked shift of hrGrB to higher M_r-values and a concurrent decrease of SB9 protein band intensity. The M_r-shift of SB9 protein could not be reliably detected in our experiments due to the presence of an unknown immunoreactive protein which migrated to the same M_r-region as the hrGrB• SB9 complex. Previously, the anti-SB9 antibody PI9-17 was used for immunohistochemical detection of SB9 protein expression *in situ* in normal and tumour cells (244,253,255). Since this antibody is not completely specific for SB9 protein, the immunohistochemical data obtained with it should be interpreted with caution.

We observed a significant negative correlation between the level of SB9 protein expression and the GrB-induced caspase-3-like activity in extracts from NSCLC cell lines. This result supports the evidence that the overexpression of SB9 can contribute to the resistance of NSCLC cells against the GrB-mediated apoptosis. In fact, the SB9-dependent inactivation of GrB can block the GrB-catalysed proteolytic processing and activation of the executioner procaspase-3 and -7 and the fragmentation of multiple housekeeping proteins in the cytoplasm and of the cytosol-facing signalling domains of some pro-survival plasma membrane receptors (see Table I). Moreover, the inhibition of GrB by SB9 prevents induction of MOMP and the nuclear translocation of certain mitochondrial proteins (AIF and endonuclease G) and fragmentation of multiple intranuclear proteins (154,200, 205,206;Fig. 1). Mahrus and colleagues (58) showed that SB9 protein can be proteolytically inactivated by granzyme M (GrM). Therefore, during the apoptotic attack, the internalised GrM into the cytosol of SB9- expressing cancer cell might attenuate the inhibitory effect of SB9 on the delivered GrB.

Although the expression of SB9 mRNA in NSCLC tumours and lungs was not significantly different, and patients' gender and smoking status and tumour stage did not significantly affect the expression of SB9 mRNA in the tumours, the less-differentiated

SQCLCs and LACs showed significantly higher expression of SB9 mRNA as compared to the well-differentiated tumours. Thus, it is likely that poorly differentiated NSCLC tumours with up-regulated SB9 expression might be more resistant to the GrB-mediated immune deletion. To overcome such immunoresistance, the cancer cell-specific overloading of GrB would be required. Taking advantage of survivin (*BIRC5)* gene overexpression in lung cancer cells (292,293), the tumour cell-specific overexpression of GrB can be achieved *via* targeted delivery of recombinant DNA constructs consisting of a fusion of the *BIRC5* promoter to the coding sequence of active GrB (Caldas et al. 2006). Soriano and colleagues observed increased expression of SB9 protein in NSCLC cells and tissues using immunocytochemical and immunohistochemical methods, respectively (295). Some questions arise from this *in situ* study concerning the antigenic specificity of the used antibody for SB9 (cf. 257), including the target antigen immunoselectivity of the used antibody with respect to free SB9 or the complex SB9·GrB or both.

In the presented publication B (322), it was demonstrated that NSCLC cells express very low and variable levels of GrB mRNA. However, in another study, the presented publication A (257), we were unable to detect GrB protein expression in NSCLC cells using Western blotting analysis. On the contrary, Kontani and colleagues, examining NSCLC tumour tissue sections by immunocytochemistry, observed GrB protein positivity in the cytoplasm of NSCLC cancer cells rather than in the tumour-infiltrating lymphocytes (277). Although the later study seems to detect the tumour cell origin of GrB protein, it is much more probable that the simultaneous presence of GrB in tumour cells and its absence in the tumour-infiltrating lymphocytes reflects the tumour cells internalization of captured exogenous GrB along with the tumour-infiltrating lymphocytes in the post-degranulation phase.

In the presented publication C (296), it was demonstrated that *APIP* and *UACA* genes are expressed at both mRNA and protein levels in NSCLC cell lines and NSCLC tumours and lungs interestingly, different histopathological type of NSCLC tumours showed significantly lower expression of both APIP and UACA mRNAs and proteins as compared to matched lungs. Especially, the expression of UACA mRNA was down-regulated with a high frequency in NSCLC tumours. Moreover, although the expression of APIP mRNA in NSCLC and SCLC cells was comparable, the expression of UACA mRNA

in SCLC cells was significantly lower as compared to NSCLC cells. These results suggest that down-regulation of *UACA* gene expression might bring certain survival advantage to lung cancer cells. This view is supported by the recent experimental data showing that nucling-knockout mice are not only resistant to the neurotoxin-induced apoptosis (297) but also often develop hepatocellular carcinoma (298). The lower level of UACA mRNA in stage IA of NSCLC tumours as compared to higher stages suggests that the down-regulation of *UACA* gene expression is of particular importance during the early period of NSCLC development. The weak increase of expression of both APIP and UACA mRNAs in the ADC-treated NSCLC cell lines indicates that mechanisms other than DNA methylation are involved in regulation of *APIP* and *UACA* genes expression in these cancer cells. Contrary to the weak up-regulation of APIP and UACA mRNAs expression, the expression of serpinB9 mRNA was strongly induced in several ADC-treated NSCLC cell lines (Fig. 12).

6 CONCLUSION

Concerning the specific aims of this thesis, the following conclusions can be formulated:

- NSCLC cells express both serpinB9 (SB9) mRNA and protein and there is a subset of NSCLC cell lines and tumours with up-regulated SB9 mRNA and protein expression. SB9 protein expressed in NSCLC cells is functional as it can interact with the active granzyme B (GrB) via forming a stable enzyme-inhibitor complex GrB•SR9. Analysis of SB9 mRNA expression in NSCLC tumours from surgically treated patients showed that the expression of this transcript is particularly up-regulated in the less-differentiated LACs.

- Estradion-17β and interleukins -1β, -6, and -18 do not markedly up-regulate the transcriptional expression of SB9 in NSCLC cells. On the contrary, however, DNA methylation can profoundly down-regulate the expression of SB9 in a subset of NSCLC tumours. This suggests that DNA demethylating drugs might desensitize NSCLC cells, through a strong induction of SB9 expression, against the granzyme B-induced apoptosis.

- NSCLC cells and tumours which are high expressors of SB9 may be protected, via the constitutively or inducibly high levels of SB9, against the GrB-mediated apoptosis during the immune attack executed by cytotoxic lymphocytes and NK cells.

- Contrary to its participation in the regulation of transcriptional expression of *SERPINB9* gene, DNA methylation is not significantly involved in the regulation of transcriptional expression of *APIP* and *UACA* genes in NSCLC cells.

7 LIST OF PUBLICATIONS

A. **Rousalova I**, Krepela E, Prochazka J, Cermak J, Benkova K.: *Expression of proteinase inhibitor-9/serpinB9 in non-small cell lung carcinoma cells and tissues*. Int J Oncol 36 (1): 275-283, 2010 (IF = 2.571)

B. **Rousalova I**, Krepela E.: *Granzyme B-induced apoptosis in cancer cells and its regulation (Review).* Int J Oncol 37 (6): 1361-1378, 2010 (IF = 2.571)

C. Moravcikova E, Krepela E, Prochazka J, **Rousalova I**, Cermak J, Benkova K.: *Down-regulated expression of apoptosis-associated genes APIP and UACA in non-small cell lung carcinoma.* Int J Oncol 40 (6): 2111-2121, 2012 (IF = 2.571)

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