Identification of novel therapeutic targets in mastocytosis: Inhibition of survival of neoplastic mast cells by targeting IAPs and TRAIL receptors

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Summary

Mastocytosis is a rare disease characterized by neoplastic accumulation of mast cells in various organs, particularly in skin and bone marrow. Sporadic mastocytosis is usually caused by somatic point mutations of the receptor tyrosine kinase KIT that occur in exon 17, codon 816, and activates KIT by affecting the intracellular enzymatic site of the molecule. Homeostasis of so altered mast cells is influenced on different levels. These cells show an elevated rate of proliferation and changes in cellular apoptosis. In this thesis the expression of the inhibitor of apoptosis protein (IAP) Survivin in neoplastic mast cells and its importance as a diagnostic marker or therapeutic target has been evaluated. For this purpose the Survivin content of normal and neoplastic mast cells was analyzed. Furthermore Survivin expression in mast cells of bone marrow samples from patients with mastocytosis and healthy donors was compared. The expression of Survivin protein in the neoplastic human mast cell line HMC-1 was downregulated by siRNA and apoptosis was subsequently induced with tyrosine kinase inhibitors or the death receptor ligand TRAIL. It is known from other hematopoietic neoplasia that Smac mimetics, inhibitors of the IAPs XIAP, cIAP1 and cIAP2, are potent therapeutics able to amplify the effect of classical cytotoxic therapies. Here the Smac mimetic LCL161 was used in combination with the tyrosine kinase inhibitor PKC412 and TRAIL to inhibit growth of the neoplastic mast cell line HMC-1.

The expression of the IAP Survivin is enhanced in neoplastic altered mast cells and can be detected in significantly higher amounts in mast cell infiltrates of the bone marrow from patients with mastocytosis compared to healthy donors underlining its value as a diagnostic marker. Downregulation of Survivin protein as well as the use of the Smac mimetic LCL161 inhibited the growth of the neoplastic mast cell line HMC-1 synergistically with tyrosine kinase inhibitors and the death receptor ligand TRAIL. These results emphasize the relevance of cellular regulation of apoptosis as a future target for the therapy of mastocytosis.

Zusammenfassung

Mastozytose ist eine seltene Erkrankung die durch eine neoplastische Akkumulation von Mastzellen in unterschiedlichen Organen, insbesondere der Haut und dem Knochenmark charakterisiert ist. Auslöser für die Mastozytose sind oftmals aktivierende somatische Punktmutationen in Exon 17, Codon 816 des Gens der Rezeptortyrosinkinase KIT, welche die intrazelluläre enzymatische Domäne des Rezeptors beeinflussen. Die Homöostase derartig veränderte Mastzellen ist auf mehreren Ebenen gestört. So konnte nicht nur eine erhöhte Proliferationsrate, sondern auch ein verändertes Apoptoseverhalten nachgewiesen werden. In dieser Arbeit wurde die Expression des *inhibitor of apoptosis* Proteins (IAP) Survivin in neoplastisch veränderten Mastzellen und seine Bedeutung als diagnostischer Marker sowie als therapeutisches Target untersucht. Hierzu wurden gesunde und neoplastisch veränderte Mastzellen auf ihren Survivingehalt hin überprüft sowie die Survivinexpression der Mastzellen in Knochenmarksstanzen von Mastozytosepatienten mit denen gesunder Probanden verglichen. Des weiteren wurde die Expression von Survivin in der neoplastischen Mastzelllinie HMC-1 mit Hilfe von siRNA herunterreguliert und Apoptose mithilfe von Tyrosikinaseinhibitoren und dem Todesrezeptorliganden TRAIL induziert. Aus anderen hämatopoetischen Neoplasien ist bekannt, dass sogenannte *Smac mimetics*, Moleküle, die die IAPs XIAP, cIAP1 und cIAP2 binden und inaktivieren können, potente Therapeutika sind, die in der Lage sind, die Wirkung klassischer zytotoxischer Therapien zu verstärken. Hier wurde das *Smac mimetic* LCL161 in Kombination mit dem Tyrosinkinaseinhibitoren PKC412 sowie TRAIL verwendet, um das Wachstum von HMC-1 Zellen zu hemmen.

Das IAP Survivin wird verstärkt in neoplastisch veränderten Mastzellen exprimiert und ist in Mastzellinfiltraten im Knochenmark von Mastozytosepatienten in signifikant höheren Mengen nachzuweisen als in denen gesunder Probanden, was den Wert des Proteins als diagnostischen Marker herausstellt. Die Herunterregulierung des Survivin Proteins, ebenso wie der Einsatz des *Smac mimetic* LCL161 hemmte das Wachstum der neoplastischen Mastzelllinie HMC-1 im Synergismus mit Tyrosinkinaseinhibitoren oder dem Todesliganden TRAIL. Dies unterstreicht die Bedeutung der zellulären Apoptoseregulation als zukünftiges Ziel der medikamentösen Therapie der Mastozytose.

1 Introduction

1.1 Mast cells

Mast cells were first described by Paul Ehrlich in 1878 as cells containing granules which can be stained red with aniline blue (Metachromasy) (Ehrlich 1878). He speculated that the cells were “fattened” (german: “gemästet”) by phagocytosis and so termed them mast cells. Today it is known that these granules contain preformed mediators that can be released after stimulation (Bradding 1996; Metcalfe, Baram et al. 1997) and that mast cells are important effector cells of the immune system. They can be found in tissue forming the inner and outer barrier of an organism like the skin or the mucosa of the lung and the gut. During a type-I allergic reaction, immunoglobine E (IgE) produced by B lymphocytes binds to the membrane bound IgE receptor I (FcεRI) on the mast cell. When antigen is crosslinking the receptors, mainly preformed mediators from the cytoplasmic granules like histamine, heparin, lipid mediators or cytokines are released.

1.1.1 Development of mast cells

Mast cell progenitors (MCP) arise from CD34+ hematopoietic stem cells in the bone marrow. These MCP emigrate via the blood circuit into different tissues where they maturate under the influence of stem cell factor (SCF) produced by different cells (i.e. fibroblasts). SCF binds to the mast cell receptor tyrosine kinase c-KIT (CD117). Mast cells are the only hematopoietic cells showing a high c-KIT expression during all stages of their development. MCP stimulation by SCF leads to formation of granules and maturation of mast cells (Kirshenbaum, Goff et al. 1992; Valent, Spanblochl et al. 1992). While human mast cells mainly depend on SCF for maturation and proliferation in the murine system other substances like interleukins (IL-3, IL-4 and IL-9) and nerve growth factor (NGF) have been described as regulators (Matsuda, Kannan et al. 1991; Rennick, Hunte et al. 1995). Different subtypes of mature mast cells can be distinguished by their localization and their histochemical properties. Three mast cell subtypes can be classified in humans by the protease content in their granules. Mast cells only containing tryptase (MC_T), tryptase and chymase (MC_{TC}) or chymase and cathepsin G (MC_C) can be distinguished (Metcalfe 2008). While mast cells of the MC_T subtype can be found mainly in the lung, the mucosa of the gut and the nose, the MC_{TC} subtype resides in the skin, tonsils and the submucosa of the gut. The microenvironment of the tissue in which mast cells reside determines their sensitivity towards activating stimuli independent of their subtype. While skin mast cells can be activated by poly-L-lysine, the neuropeptide substance P, compound 48/80 or morphine, lung mast cells are insensitive towards these stimuli (Lowman, Rees et al. 1988).

1.1.2 Mast cell functions

Mast cell degranulation, the exocytotic release of preformed or de-novo synthesized mediators can be triggered by various stimuli. The pattern of released substances is determined by the subtype and localization of the mast cell and the type and intensity of the stimulus. Preformed mediators can be substances like mast cell proteases, peptidoglycans, tumor necrosis factor (TNFα), vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2) while interferons, leukotrienes, chemokines can be synthesized upon activation (Marshall 2004). Besides activation via crosslinking of the FcεRI mast cell degranulation can be triggered by activation of complement receptors (C3a, C5a), Fcγ receptors, toll-like receptors and chemokine receptors. At the end the combination of triggers and their intensity determines the pattern of synthesized and released mediators from the mast cell (Gilfillan and Tkaczyk 2006). The physiological role of mast cells is their function in the innate as well as the acquired immunity in the control of infections with bacteria and parasites. It has been shown that mast cell released TNFα can help to control bacterial infection in a model of murine peritonitis (Echtenacher, Mannel et al. 1996) and nematode infections in mice lead to accumulation of mucosal mast cells in the gut with release of mast cell protease 1 (Knight, Wright et al. 2000).Toll-like receptors on mast cells are capable of binding microbial particles leading to release of cytokines like TNF, IL-6 and IL-8 stimulation immigration of effectors cells into infected tissues (Marshall 2004). Finally they are possibly linked to allograft tolerance (Lu, Lind et al. 2006) and tumor invasion (Gounaris, Erdman et al. 2007). In the context of allergy mast cells react to otherwise harmless antigen by release of several preformed mediators which directly influence the surrounding tissue. During the immediate hypersensitivity reaction, the enzyme tryptase, chymase, cathepsine G and carboxypeptidase activate a rebuilding of the connective tissue matrix by metalloproteases. Histamine and heparin are released, increase vessel permeability and trigger smooth muscle contractions. These effects are amplified by release of lipid mediators like leukotrienes C4, D4 and E4. These lipid mediators are also responsible for intensified mucoid secretion and maintenance of inflammatory reactions. The late onset reaction again is initiated by preformed and newly synthesized mast cell mediators. By secreting cytokines like TNFα, leukotriene B4 or histamine endothelial cells are activated which upregulate expression of adhesion molecules like P-selectine, E-selectine and ICAM-1 on their surface. To these molecules leucocytes like neutrophils, monocytes and CD4+ T cells can bind and migrate into the tissue by transmigration (Koh, Dupuis et al. 1993; Robinson, Hamid et al. 1993; Montefort, Gratziou et al. 1994).

1.2 Mastocytosis

The term Mastocytosis describes disorders caused by the pathogenic increase of mast cell numbers in different tissues. The consequences that arise from mast cell burden and accumulation of their associated mediators can be both locally and systemically. Local mast cell accumulation in organs like skin, gastrointestinal tract, bone marrow, spleen and lymph nodes can be associated with different hematologic disorders (Metcalfe 2008). In Cutaneous Mastocytosis (CM) mast cell infiltrates are restricted to the skin and variants of CM are defined by their clinical presentation. In addition in Systemic Mastocytosis (SM) one or more internal organs predominantly the bone marrow are infiltrated by mast cells (Valent, Horny et al. 2001) often associated with hematologic disorders. The pathological proliferation of mast cells in Mastocytosis and the release of their mediators can cause symptoms including pruritus, flushing, nausea, diarrhea and vascular instability and in systemic Mastocytosis severe symptoms like Osteoporosis and organ failure occur. The complex pattern of symptoms and their severity leads to a classification listed by the World Health Organization (WHO) and subsequent prognosis for each case of Mastocytosis (Horny, Sotlar et al. 2007):

Variant – Term (Abbreviation)	**Subvariants**
Cutaneous Mastocytosis (CM)	Urticaria Pigmentosa (UP) = Maculopapular CM (MPCM)
	Diffuse CM (DCM)
	Mastocytoma of Skin
Indolent Systemic Mastocytosis (ISM)	Smoldering SM (SSM)
	Isolated bone marrow Mastocytosis (BMM)
Systemic Mastocytosis with an associated clonal hematologic non-mast cell lineage disease (SM-AHNMD)	Systemic Mastocytosis with Acute Myeloid Leukemia (SM-AML)
	Systemic Mastocytosis with Myelodysplastic Syndrome (SM-MDS)
	Systemic Mastocytosis with Myeloproliferative Disorder (SM-MPD)
	Systemic Mastocytosis with Chronic Myelomonocytic Leukemia (SM-CMML)
	Systemic Mastocytosis with Non-Hodgkin's Lymphoma (SM-NHL)
	Systemic Mastocytosis with Hypereosinophilic Syndrome (SM-HES)
Aggressive Systemic Mastocytosis (ASM)	Lymphadenopathic SM with eosinophilia
Mast cell Leukemia (MCL)	Aleukemic MCL
Mast Cell Sarcoma (MCS)	
Extracutaneous Mastocytoma	

Table 1.1 WHO Classification of Mastocytosis

1.2.1 Clinical presentation

Cutaneous Mastocytosis (CM)

CM shows a wide variety of skin alterations (Rueff, Dugas-Breit et al. 2006). Even though all lesions in CM are due to an abnormal elevated mast cell number in the dermis the number and morphology of efflorescences varies (Longley, Duffy et al. 1995). The most common subvariant of CM presents as disseminated macular or maculopapular rash, and is (descriptively) termed urticaria pigmentosa (UP) (Wolff, Komar et al. 2001) (Hartmann and Henz 2002). Diffuse CM is less frequently diagnosed. The solitary localized mastocytoma (of the skin) is also rare, and has a benign clinical course. Thus, most mast cell tumors of the skin appear to be benign in most cases.

Indolent systemic mastocytosis (ISM)

The most common systemic variant of mastocytosis is the indolent systemic mastocytosis (ISM) wich involves the skin with limited increase of mast cell number in other organs, in most cases the bone marrow (Valent, Horny et al. 2001). With a common onset in adult age ISM shows a prolonged clinical course in almost all patients with survival times of two decades and more. Besides bone marrow infiltrates, more than 90% of patients show maculopapular cutaneous mastocytosis / urticaria pigmentosa. Level of serum tryptase is raised in comparison to cutaneous mastocytosis with levels of 20 to 200 µg/l. A rare variant of ISM is the so called smoldering systemic mastocytosis (SSM) with pronounced mast cell infiltration (>30% in bone marrow histology), organomegaly and tryptase levels above 200 µg/l.

Systemic mastocytosis with an associated clonal hematologic non mast cell lineage disease (SM-AHNMD)

A small subgroup of patients with systemic mastocytosis develops an additional clonal disease of hematologic non-mast cell lineage cells like the myelodysplastic / myeloproliferative syndrome or myeloid leukemia. Generally these are myeloid neoplasia while systematic lymphatic diseases like lymphoma or myeloma are extremely rare in these patients.

Aggressive systemic mastocytosis (ASM)

In rare cases an ISM can develop into an aggressive systemic mastocytosis (ASM) with progressive infiltration of immature mast cells and subsequent organ destruction and failure (Valent 1996). ASM is much less common than ISM comprising only about 5% of all SM patients. Possible symptoms are cytoplenias, malapsorption, bone fractures and signs of hepatopathy with loss of liver function (Horny, Sotlar et al. 2007). A rare subvariant of ASM with prominent eosinophilia of blood and tissues and generalized lymphadenopathy (clinically mimicking malignant lymphoma) has been described as lymphadenopathic mastocytosis with eosinophilia (Hauswirth, Sperr et al. 2002).

Mast cell leukemia (MCL)

In patient with mast cell leukemia smear preparations of the bone marrow show more than 20% mast cells (Horny and Valent 2001). In case of a typical mast cell leukemia numbers of circulating mast cell in the blood are generally above 10% while in cases of aleukemic mast cell leukemia the numbers are below 10%. Prognosis of MCL is poor, mostly with survival of less than two years.

Mast cell sarcoma (MCS)

Localized mast cell proliferations are also extremely rare and include both the extracutaneous mastocytoma (of the lung) and the “true” mast cell sarcoma. It is noteworthy that the mast cell sarcomas reported occurred in tissues not commonly involved by SM (larynx, colon, meningeal site). All cases showed rapid progression and generalization with the terminal phase resembling MC leukemia. (Horny, Sotlar et al. 2007).

1.2.2 Pathogenesis

The most important growth factor for human mast cells is the cytokine stem cell factor (SCF). SCF binds to the tyrosine kinase receptor KIT (CD117) encoded by the proto-oncogene *KIT*. By KIT activation SCF induces various cell functions in mast cells like proliferation, differentiation, mediator release, migration and survival (Tsai, Takeishi et al. 1991; Mekori, Oh et al. 1993). The majority of all adult patients with mastocytosis carry a somatic *KIT* mutation in exon 17 codon 816 with substitution of asparagine by valine (D816V) leading to an autonomous ligand independent activation of KIT (Nagata, Worobec et al. 1995) (Longley,

Tyrrell et al. 1996) (Longley, Metcalfe et al. 1999). In seldom cases substitutions by other amino acids in codon 816 i.e. D816Y, D816H and D816F (Feger, Ribadeau Dumas et al. 2002) or mutations in neighboring codons (D820G) (Liegl, Kepten et al. 2008) have been described. In contrast to adult patient only a subgroup of children with mastocytosis carry activating *KIT* mutations (Sotlar, Escribano et al. 2003) (Yanagihori, Oyama et al. 2005). To some extent pediatric patients show untypical (i.e. D816F, D816Y, R816K) or silent non activating (D816D) mutations. Despite these findings revealing the molecular cause of mastocytosis many questions concerning its pathogenesis still remain unanswered. In particular factors besides the activating KIT^{D816V} mutation affecting the heterogeneity of mastocytosis and factors which are active in cases without activating KIT mutations i.e. in pediatric patients still have to be discovered.

1.2.3 Therapy

Despite growing scientific knowledge no curative therapy for the treatment of mastocytosis is available (Escribano, Akin et al. 2002; Brockow 2004). Currently applied treatments base on reduction or antagonism of mediator release and reduction of mast cell numbers (Longley, Duffy et al. 1995; Golkar and Bernhard 1997; Marone, Spadaro et al. 2001). Since mastocytosis is a rare disease and treatment is not always necessary clinical studies with large patient numbers are currently not available. Thus recommended therapies base on isolated cases or case series.

In cases of cutaneous mastocytosis education of patients about diagnosis, prognosis and possible trigger factors and their avoidance is of capital importance (Allison and Schmidt 1997; Golkar and Bernhard 1997; Brockow 2004). Medication in most cases is symptomatically and in accordance with the patient's subjective condition. Commonly corticosteroids, antihistamines or a psoralen + UVA (PUVA) treatment have to be considered (Escribano, Akin et al. 2002; Worobec and Metcalfe 2002). In case of periodical condition i.e. weekly swelling of skin lesions or flushes, regular medication with H1 antihistamines should be prescribed. Gastrointestinal conditions can be treated by H2 antihistamines, cromoglycic acid, proton pump inhibitors and antacids. Systemic administered corticosteroids can be effective in patients with frequent anaphylactic reactions or severe systemic conditions like ascites, diarrhea or malapsorption (Metcalfe 1991). Calcium, vitamin D or bisphosphonate come into considerations in cases with osteopenia or osteoporosis (Laroche, Bret et al. 2007). In patients with progressed categories of systemic mastocytosis especially aggressive mastocytosis or mast cell leukemia cytoreductive therapies have to be considered. Interferon alpha for example can reduce mast cell numbers in patients with aggressive systemic or smoldering systemic mastocytosis (Kluin-Nelemans, Jansen et al. 1992; Lippert and Henz

1996; Worobec, Kirshenbaum et al. 1996; Hauswirth, Simonitsch-Klupp et al. 2004). Cladribin (2-chlorodeoxyadenosine, 2-CDA) according to first studies is reducing mast cell numbers in patients with aggressive mastocytosis (Tefferi, Li et al. 2001; Kluin-Nelemans, Oldhoff et al. 2003).

1.2.4 Tyrosine kinase inhibitors

During the last years the use of drugs targeting the tyrosine kinase (TK) KIT has been proposed and inhibitors have been developed. The TK inhibitor imatinib (= STI571) is of none or low effectiveness in patients with *KIT*^{D816V} mutation most common in patients with mastocytosis since the mutation is leading to a steric alteration in the receptor preventing imatinib from binding to the ATP binding domain of KIT (Ma, Zeng et al. 2002; Vendome, Letard et al. 2005). However in patients carrying uncommon *KIT* mutations in exons 9 or 10 imatinib leads to a reduction in mast cell numbers, lowered tryptase levels and improvement of clinical symptoms (Akin, Fumo et al. 2004; Zhang, Smith et al. 2006). Novel multi kinase inhibitors like midostaurin (PKC412), nilotinib (AMN107) or dasatinib (BMS354825) have been developed and undergo clinical testing (Gotlib, Berube et al. 2005; von Bubnoff, Gorantla et al. 2005; Gleixner, Mayerhofer et al. 2006). Reportedly midostaurin has remarkable effects on neoplastic mast cells in vitro as well as to induce remission in a patient with MCL carrying the activating D816V *KIT* mutation (Gotlib, Berube et al. 2005).

1.3 Targeting of the inhibitor of apoptosis protein (IAP) family and TRAIL death receptors in cancer

1.3.1 Apoptosis

The ability of multicellular organisms to control cell number is the prerequisite for many physiological processes. The targeted destruction of cells is executed by an active molecular suicide program so called programmed cell death. So acting as a counterpart to cell division, apoptosis enables the organism to regulate cell number and to specifically kill harmful cells. Therefore the programmed cell death is of central importance in developmental processes and tissue homeostasis. A dysregulation with reduced or excessive cell death may lead to severe illnesses like cancer, autoimmune diseases or neurological disorders like Alzheimer or Parkinson disease (Thompson 1995).

Described early under varying names in 1972 the programmed cell death was finally named apoptosis by Kerr et al describing an uniformly cell death characterized by defined morphological changes of dying cells throughout various cell types and tissues (Kerr, Wyllie et al. 1972). Early characteristics of apoptosis are shrinking of the cell, condensation of chromatin and bubble formation of the cell wall. Later DNA is fragmented by endonucleases and the whole cell collapses. Cell debris is packed into apoptotic vesicles and taken up by phagocytotic cells. Since the cell membrane remains intact during the whole process no cellular content is released into the cells surrounding. Therefore the apoptotic cell death does not trigger inflammation in contrast to cells dying by necrosis. Necrosis is a type of cell death induced by mechanical stress or other environmental factors. Here the plasma membrane is disrupted and cell content is released. This can trigger an immune response and often is accompanied by inflammations which can harm surrounding tissues (Edinger and Thompson 2004).

1.3.2 The extrinsic pathway of apoptosis

The extrinsic or receptor mediated pathway of apoptosis for is used for example in cells of the immune system to eliminate infected or tumorigenic cells (Igney and Krammer 2002). It is triggered by binding of ligands to specific death receptors (CD95, TNFR1, TRAIL-R1 and TRAIL-R2) on the target cells surface. The binding of the ligand leads to the recruitment of adaptor molecules on the cytosolic domain of the activated receptor. These bind to initiator caspase 8 and 10 leading to dimerisation of caspases leading to autocatalysis and activation of initiator caspases. This signaling complex is called “death-inducing signaling complex” (DISC). The initiator caspases subsequently cleave and activate effector caspases 3, 6 and 7 (Ashkenazi 2002; Lavrik, Golks et al. 2005).

1.3.3 The intrinsic pathway of apoptosis

The intrinsic or mitochondrial pathway of apoptosis is triggered by DNA damage, oncogene activation or other intracellular apoptotic signals. The outer mitochondrial membrane is permeabilized and cytochrome C as well as other apoptotic factors is released into the cytosol. The permeabilization of the mitochondrial membrane is regulated by an interaction of anti- and pro-apoptotic molecules of the Bcl-2 family of proteins. After the release of mitochondrial proteins into the cytosol a multi-protein complex of Apaf-1 and cytochrome C molecules is formed. The so called apoptosome binds and activates the initiator caspase 9. In turn caspase 9 cleaves and activates effector caspases 3 and 7 (Riedl and Shi 2004; Gogvadze and Orrenius 2006). The intrinsic pathway as well can be activated by death

receptors. The Bcl-2 family protein BID can be cleaved by caspase 8. Cleaved BID activates the pro-apoptotic proteins Bak and Bax leading to permeabilization of the mitochondria (Korsmeyer, Wei et al. 2000).

The effector caspase activated by both apoptotic pathways cleave a variety of cellular substrates leading to the characteristic biochemical and morphological changes of the apoptotic cell like chromatin condensation, DNA fragmentation or shrinking and fragmentation of the cell (Fischer, Janicke et al. 2003).

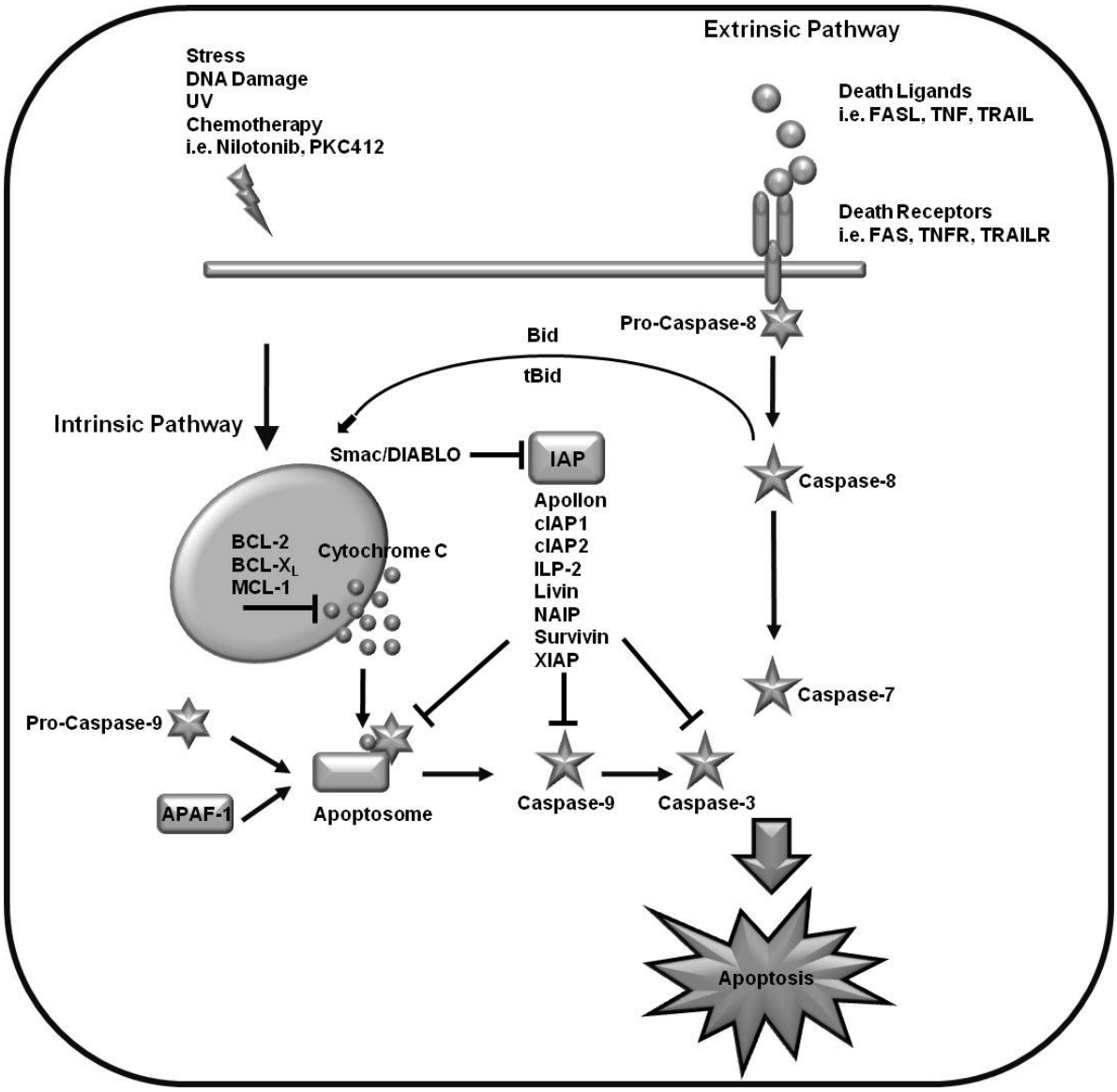

Fig. 1.1 Apoptotic pathways:
Apoptosis is initiated via two main pathways. The extrinsic pathway is triggered by binding of ligands to their respective death inducing receptor and following activation of caspase 8. Release of pro-apoptotic proteins and cytochrome C from the mitochondria following different stress signals initiates the intrinsic pathway of apoptosis leading to activation of pro-caspase 9. The activation of both initiator caspases leads to processing of effector caspases with subsequent cleavage of target proteins and cell death. The extrinsic and intrinsic pathways are connected via the cleavage of Bid to tBid by caspase 8. Depending on the cell type activation of death receptors directly leads to activation of effector caspases (type I cells) or indirectly via the mitochondria (type II cells).

1.3.4 TRAIL receptors

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor (TNF) superfamily of proteins, that was discovered in 1995 (Wiley, Schooley et al. 1995) and since then is under investigation as a biologically targeted anti-tumor protein since it induces apoptosis in a variety of human cancer cell lines while leaving normal cells unaffected (Walczak, Miller et al. 1999). In humans TRAIL can bind to four known membrane bound receptors, death receptor 4 (DR4)/TRAIL receptor-1 (Pan, O'Rourke et al. 1997), death receptor 5 (DR5)/TRAIL receptor-2 (Pan, Ni et al. 1997; Walczak, Degli-Esposti et al. 1997), TRAIL receptor without an intracellular domain (TRID)/decoy receptor 1 (DCR1)/TRAIL receptor-3 (Degli-Esposti, Smolak et al. 1997; Pan, Ni et al. 1997; Sheridan, Marsters et al. 1997), and TRAIL receptor-4/decoy receptor 2 (DCR2)/TRAIL receptor with a truncated death domain (TRUNDD) (Degli-Esposti, Smolak et al. 1997; Marsters, Sheridan et al. 1997; Pan, Ni et al. 1998). TRAIL receptor-3 and TRAIL receptor-4 do not possess an intracellular domain and have been proposed as competitive inhibitors of TRAIL induced apoptosis (Sheridan, Marsters et al. 1997). TRAIL receptor 1 and TRAIL receptor 2 on the other hand contain a cytoplasmic death domain capable of activating the extrinsic pathway of apoptosis by recruiting the death-inducing signaling complex (DISC) upon ligand binding and trimerization of the receptor (Hymowitz, Christinger et al. 1999). DISC includes FAS-associated death domain protein (FADD), pro-caspase 8, and possibly cellular FADD-like IL-1β-converting enzyme inhibitory protein (cFLIP) (Kischkel, Lawrence et al. 2000; Sprick, Weigand et al. 2000). After autocatalytic cleavage and activation of pro-caspase 8 at the DISC downstream effector caspases are activated in type I cells (Kischkel, Lawrence et al. 2000). In type II cells intrinsic apoptosis can be triggered by cleavage of Bid (Barnhart, Lee et al. 2003). Finally TRAIL can also bind to osteoprotegerin (Emery, McDonnell et al. 1998), a soluble receptor for Receptor Activator of NF-κB Ligand (RANKL) (Fig. 1.2).

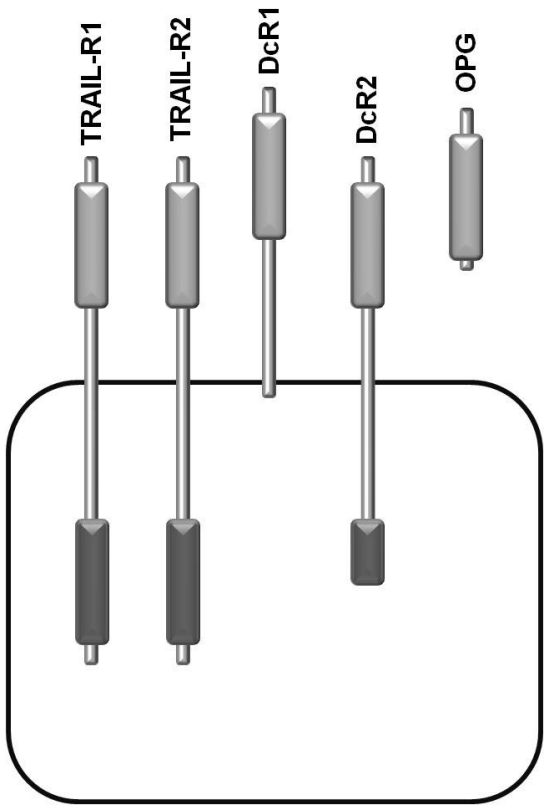

Fig. 1.2 The human TRAIL receptors:
Human TRAIL can bind to five different receptors. TRAIL receptor 1 and TRAIL receptor 2 can signal apoptosis via their cytoplasmatic death domain. Decoy receptor 1 is lacking a death domain while decoy receptor 2 has a truncated death domain unable to signal apoptosis. Osteoprotegerin is a soluble receptor for TRAIL.

The different sensitivity of normal and neoplastic cells towards TRAIL induced apoptosis may be due to several reasons. Messenger RNA of all TRAIL receptors is expressed throughout a wide variety of cells and tissues (Wiley, Schooley et al. 1995). Therefore the expression pattern of apoptosis signalling versus non signalling TRAIL receptors has been proposed as a way of regulation of sensitivity to TRAIL-induces apoptosis (Marsters, Sheridan et al. 1997; Pan, Ni et al. 1997; Pan, Ni et al. 1998) and has been confirmed for primary tumors of the gastrointestinal tract (Sheikh, Huang et al. 1999) and human breast cancer cell lines (Sanlioglu, Dirice et al. 2005). Another finding suggests that the cell cycle phase determines cell sensitivity towards TRAIL as was shown in human colon and lung cancer cell lines arrested in the G_0/G_1phase of the cell cycle (Jin, Dicker et al. 2002) may be due to differential expression of pro- and anti-apoptotic proteins during different cell cycle phases as has been shown in activated T cells (Algeciras-Schimnich, Griffith et al. 1999). The most prominent theories concerning regulation of TRAIL-induces apoptosis involve the differential expression of pro- and anti-apoptotic proteins within the targeted cells such as cFLIP, Bcl-2 family members, the ERK and Akt signaling pathways and the inhibitor of apoptosis proteins (IAPs). (Griffith, Chin et al. 1998; Shiiki, Yoshikawa et al. 2000; Nesterov, Lu et al. 2001; Fulda, Meyer et al. 2002; Vaculova, Hofmanova et al. 2006; Kim, Ricci et al. 2008). Since cFLIP binds competively to FADD its suppression can sensitize some cancer cells to TRAIL-induced apoptosis (Geserick, Drewniok et al. 2008). In many tumor cell lines XIAP has been observed to be highly expressed and it may lead to TRAIL resistance by directly inhibiting caspases 3, 7 and 9, even in the presence of Smac/DIABLO (Deveraux, Takahashi et al. 1997; Schimmer, Welsh et al. 2004). Anti-apoptotic members of the Bcl-2 family regulate sensitivity towards TRAIL as has been shown for Bcl-2 and Bcl-xL (Hinz, Trauzold et al. 2000; Sinicrope, Penington et al. 2004) as well as for myeloid cell leukemia-1 protein (Mcl-1) (Clohessy, Zhuang et al. 2006).

1.3.5 Inhibitor of apoptosis protein (IAP) family

Originally IAP proteins were identified in baculoviruses. These insect viruses express IAP proteins to inhibit the apoptosis initiated by the infected host cell (Crook, Clem et al. 1993). Primarily IAPs were thought to function by regulating caspases, which are cysteine proteases that are involved in apoptosis. However IAPs also influence a multitude of other cellular processes, such as ubiquitin (Ub) –dependent signaling events that regulate activation of nuclear facor-κB (NF-κB) transcription. Until now eight human IAPs have been identified: XIAP, ILP-2, cIAP1, cIAP2, ML-IAP (Livin), NAIP, Survivin and Apollon. The defining feature of an IAP protein is the presence of the baculovirus IAP repeat (BIR) domain, a zinc-binding fold of approximately 70 amino acid residues that mediates protein-protein interactions

(Birnbaum, Clem et al. 1994; Hinds, Norton et al. 1999; Sun, Cai et al. 2000). IAPs of which there are eight in humans, carry between one and three copies of this domain (Fig. 1.3).

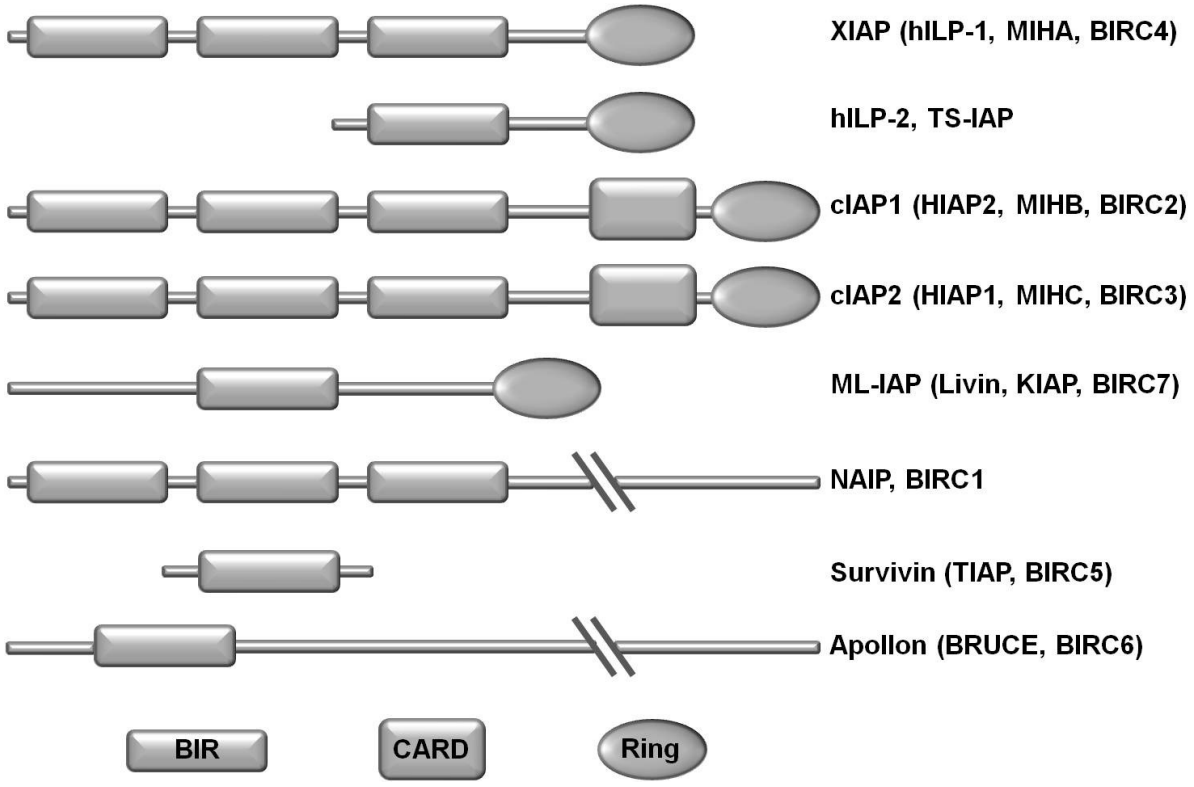

Fig. 1.3 Inhibitor of apoptosis proteins (IAPs):
The eight human inhibitor of apoptosis proteins are characterized by at least one BIR domain. BIR: Baculovirus IAP repeat, CARD: caspase recruitment domain, RING: really interesting new gene, ILP: IAP-like protein, cIAP cellular IAP. ML-IAP: melanoma IAP, NIAP: neuronal IAP, XIAP: X-linked IAP.

Additionally XIAP, cIAP1, cIAP2 and Livin have a RING domain that provides them with Ub ligase (E3) activity (Yang, Fang et al. 2000) that can link target proteins to one or more ubiquitin residues. Moreover they carry an Ub-associated (UBA) domain through which they interact with ubiquitylated proteins (Gyrd-Hansen, Darding et al. 2008; Blankenship, Varfolomeev et al. 2009). Monoubiquitination of target proteins leads to functional changes and polyubiquitination in most cases leads to proteolytic degradation. Polyubiquitinated proteins are degraded in the proteasome, a multi protein complex (Hicke 2001; Vaux and Silke 2005). Only cIAP1 and cIAP2 carry a caspase recruitment domain (CARD) located between the BIR and the RING domain. Its function is currently unknown.

1.3.6 The IAP Survivin

With a molecular size of 16.5 kDa Survivin is the smallest known member of the IAP family of anti-apoptotic proteins. Survivin contains a single BIR domain but no RING finger or other identifiable domain typically found in IAPs. In healthy tissues its expression is found during embryogenesis and in adults in vascular endothelial cells (Mesri, Morales-Ruiz et al. 2001) (Blanc-Brude, Mesri et al. 2003), polymorphonuclear cells (Altznauer, Martinelli et al. 2004), T cells (Okada, Bakal et al. 2004; Xing, Conway et al. 2004) and haematopoietic progenitor cells (Xing, Conway et al. 2004; Gurbuxani, Xu et al. 2005; Leung, Xu et al. 2007). Survivin has the ability to counteract with extrinsic and intrinsic mediators of apoptosis with effecting mechanisms such as withdrawal of IL-3 (Fukuda and Pelus 2004), stimulation of death receptors of the TNFR family like FAS (CD95) (Tamm, Wang et al. 1998), TNF-related apoptosis-inducing ligand (TRAIL) (Chawla-Sarkar, Bae et al. 2004). By binding to Smac/DIABLO (Sun, Nettesheim et al. 2005) it may regulating apoptosis directly by sequestering SMAC away from XIAP (Song, Yao et al. 2003) or preventing altogether its release from mitochondria (Ceballos-Cancino, Espinosa et al. 2007). Besides its anti-apoptotic functions survivin plays a key role in modulating cell cycle dependent mechanisms such as cell division, cell stress response and cell cycle checkpoints with physical localisation to the mitotic spindle apparatus (Pennati, Folini et al. 2008). Therefore its properties tend to increase cell proliferation of tumor cells and also mutant cancer cells making survivin an attractive therapeutic target and diagnostic marker in a wide spectrum of malignancies.

1.3.7 Smac/DIABLO

The second mitochondria-derived activator of caspase (Smac) or direct IAP binding protein with low isoelectric point (pI) DIABLO was identified simultaneously by two groups in 2000 (Du, Fang et al. 2000; Verhagen, Ekert et al. 2000). It resides within the mitochondrial intermembrane space and is subsequently released into the cytosol through semiselective permeability together with other mitochondrial proteins such as Omi, adenylate kinase-2, cytochrome c and apoptosis-inducing factor upon the induction of apoptosis. Bcl-family proteins are pivotal in this process as they may facilitate (i.e. Bid (Du, Fang et al. 2000; Li, Zhao et al. 2002), Bax and/or Bak (Du, Fang et al. 2000; Kandasamy, Srinivasula et al. 2003) or inhibit (such as Bcl-2 or Bcl-xL) (Du, Fang et al. 2000; Sun, Bratton et al. 2002) the release of apoptotic mediators. There are different ways in which Smac/DIABLO contributes to the apoptotic process. Smac can bind many different IAPs (i.e. XIAP, cIAP1, cIAP2 and Survivin) (Du, Fang et al. 2000). It is able to interact with IAPs by binding to Survivins BIR1 (Gao, Zhang et al.; Sun, Nettesheim et al. 2005) as well as BIR2 and BIR3 domains of XIAP,

cIAP1 and cIAP2 (Chai, Du et al. 2000) and so preventing their interaction with caspases 3 and 7 and in case of XIAP to caspase 9 (Srinivasula, Hegde et al. 2001). Smac/DIABLO can alter levels of certain IAPs. For example it can enhance autoubiquitination of cIAP1 and cIAP2 resulting in their proteasomal degradation (Varfolomeev, Blankenship et al. 2007; Vince, Wong et al. 2007). Furthermore, Smac/DIABLO increases tumor necrosis factor alpha (TNFα) mRNA expression leading to autocrine stimulation of the extrinsic apoptotic pathway (Varfolomeev, Blankenship et al. 2007; Vince, Wong et al. 2007).

1.3.8 Smac mimetics

Determination of the specific biochemical structures involved in the IAP-Smac interaction has facilitated the development of Smac-like molecules. The crucial *N*-terminus of Smac has four residues (Ala-Val-Pro-Ile) that contact the BIR3 domain of XIAP, and this structure is stabilized by electrostatic and hydrophobic forces (Liu, Sun et al. 2000; Wu, Chai et al. 2000). Once this structure was elucidated, the development of Smac mimetics, including effective nonpeptic mimetics that have advanced bioavailability and stability (Liu, Sun et al. 2000; Wist, Gu et al. 2007), provided a variety of molecules that could serve as IAP antagonists. Several different groups of Smac mimetics can be distinguished: fusion peptides of the last four to eight *N*-terminal residues of Smac with various modifications, plasmid based polynucleotides coding for full-length Smac and peptic or nonpeptic small molecules mimicking Smac structure.

1.3.9 Preclinical and clinical studies

TRAIL

The tumoricidal activity of TRAIL has been described in multiple preclinical trials carried out *in vitro* and *in vivo* using recombinant human TRAIL (rhTRAIL) on cell lines derived from both solid and hematologic malignancies, either alone or in combination with various chemotherapy agents or irradiation (Ashkenazi, Pai et al. 1999; Gazitt 1999; Kelley, Harris et al. 2001; Marini, Schmid et al. 2005). A way of directing TRAIL towards tumor cells is the creation of cell specific fusion proteins (Stieglmaier, Bremer et al. 2008). Combination therapy with conventional chemotherapy or radiotherapy where successful to overcome TRAIL resistance of tumors (Keane, Ettenberg et al. 1999; Mizutani, Yoshida et al. 1999; Nimmanapalli, Perkins et al. 2001). Bortezomib, a proteasome inhibitor approved for the treatment of multiple myeloma has been shown to have a pro-apoptotic effect in combination

with TRAIL possibly by its ability to increase p53 and TRAIL receptor-2 expression while decreasing cFLIP expression (Johnson, Stone et al. 2003; Sayers, Brooks et al. 2003; Williams and McConkey 2003). Bcl-xL, Mcl-1 and cFLIP have been downregulated by the kinase inhibitor sorafenib in several human leukemia sensitizing them to TRAIL-induced apoptosis while normal CD34+ bone marrow cells stay unaffected (Rosato, Almenara et al. 2007). In human prostate cancer cells BH3I-2' a Bcl-2 inhibitor induces apoptosis synergistically in the presence of TRAIL (Ray, Bucur et al. 2005).

To date clinical phase II trials for monoclonal TRAIL-R1 antibody TRM-1 (Mapatumumab) for treatment of non-small cell lung cancer and Non-Hodgekin's lymphoma have been completed and the clinical phase II trial of recombinant human TRAIL AMG 951 for treatment of small cell lung cancer is still ongoing (http://clinicaltrials.gov/).

Survivin

Strong survivin expression is observed in the vast majority of cancers (Altieri 2003) and in patients with haematologic malignancies (Cong and Han 2004). In cancer elevated survivin is commonly associated with enhanced proliferative index (Sui, Dong et al. 2002; Takai, Miyazaki et al. 2002; Fields, Cotsonis et al. 2004), resistance to chemotherapy (Tran, Master et al. 2002; Zaffaroni, Pennati et al. 2002) and rate of tumor recurrence (Swana, Grossman et al. 1999).

Besides classical diagnostic methods like reverse transcription polymerase chain reaction (RT-PCR), and flow cytometric analysis several new tools have been developed to establish survivin as a diagnostic biomarker like molecular beacon technology (Yang, Cao et al. 2005), recombinant nucleic acid-reporter gene-based assays (Caldas H, Altura RA WO2006066451;2006) and LabMAP technology (Lokshin A. US20070042405; 2007).

Prominent strategies in targeting survivin in disease involve adenoviral expression of mutant survivin (Mesri, Wall et al. 2001), small hammerhead RNA (shRNA) (Caldas, Holloway et al. 2006) or small inhibitory RNA (siRNA) (Uchida, Tanaka et al. 2004). Combinational treatment in association with chemotherapeutics like gemcitabine, peclitaxel or doxorubicin results in marked downregulation of survivin expression and tumor regression (Patents reviewed in (Kanwar, Kamalapuram et al. 2010)).

A Phase II study with LY2181308 a modified antisense oligonucleotide for treatment of acute myeloid leukemia and an evaluation study for survivin mRNA as a marker in bladder cancer patients has been completed. The transcriptional survivin repressors YM155 finished phase II studies for treatment of solid tumors, melanoma and prostate cancer and EM-1421 finished phase I trials for several tumor types (http://clinicaltrials.gov/).

Smac mimetics

Over the past few years evidence for the use of Smac mimetics in the treatment of neoplastic malignancies continues to accumulate. At this stage only a few small molecule Smac mimetics have entered Phase I clinical trial like AT-406, TL32711, HGS1029 and LCL161 for treatment of advanced solid tumors or GDC-0152 for non-Hodgkin's lymphoma (http://clinicaltrials.gov/) but the effectiveness of a multitude of substances has been proven *in vitro* and *in vivo*. For example peptides successfully induce apoptosis in human breast cancer cells in combination with paclitaxel, etoposide or doxorubicin (Arnt, Chiorean et al. 2002) and augmented TRAIL induced apoptosis in JURKAT cells (Guo, Nimmanapalli et al. 2002). Polynucleotides increased the leukemia cell lines K562 and CEM sensitivity to both UV light-induced and TRAIL-induced apoptosis (Jia, Patwari et al. 2003) and sensitized JURKAT cells to TRAIL- and epothilone-induced apoptosis (Guo, Nimmanapalli et al. 2002). Small molecules sensitized human glioblastoma T98G cells to TRAIL induced apoptosis and HeLa cells to TNFα-induced apoptosis (Li, Thomas et al. 2004). Finally in female C.B-17-*Prkdc*^{scid} mice Smac mimetic treatment was able to significantly reduce tumor growth rate (Eiseman, Lan et al. 2005).

1.4 Objectives

Aim of the work is the identification and characterization of possible therapeutic targets and diagnostic markers in mastocytosis with special attention to pro- and anti-apoptotic proteins. Therefore the human neoplastic mast cell lines HMC-1.1 and HMC-1.2 carrying one respectively two activating mutations in the tyrosine kinase receptor KIT (CD117) were analyzed for the expression of members of the inhibitor of apoptosis protein (IAP) family and the expression of the pro-apoptotic TNF-related apoptosis-inducing ligand receptors (TRAIL-R1 and TRAIL-R2) which could be of future use as diagnostic markers or therapeutic targets in mastocytosis. With respect to ongoing research with recently generated mice carrying a mutant KIT receptor and so resembling the situation in human mastocytosis patients (Gerbaulet, Wickenhauser et al. 2010) the biological function of the murine TRAIL-R on murine bone marrow derived mast cells was characterized in this work. In two different approaches the combinational targeting of members of the IAP family of proteins and the use of either TRAIL or tyrosine kinase inhibitors as stimuli for the extrinsic respectively intrinsic pathway of apoptosis in neoplastic human mast cells were analyzed to make way for future investigation of treatment options of neoplastic mast cells diseases.

2 Results

2.1 Expression of pro- and anti-apoptotic molecules in mast cells

Identification of pro- or anti-apoptotic molecules differentially expressed in neoplastic mast cells compared to healthy tissue could be a step in enhancing existing therapies for mastocytosis patients. Since the constitutive activation of the tyrosine kinase KIT is a hallmark of neoplasia in mastocytosis the two neoplastic mast cell lines HMC-1.1 and HMC-1.2 carrying one respectively two mutations in the *KIT* gene were analyzed for expression of pro- and anti-apoptotic molecules.

The IAP Survivin was found to be stronger expressed in HMC-1.2 cells indicating a link between KIT activation and Survivin expression. Further primary human mast cells generated from umbilical cord blood or isolated from skin were compared to different mast cell and non-mast cell lines for Survivin expression on the mRNA and protein level showing very low expression in the primary healthy mast cells. Comparison of bone marrow samples of mastocytosis patients and healthy donors revealed a significantly higher Survivin expression in mast cell infiltrates of the mastocytosis patients while expression in infiltrates of healthy donors was almost undetectable. This pattern highlights Survivin expression as to be more disease-related than for example the recently analyzed anti-apoptotic bcl-2 family protein Mcl1 (Aichberger, Mayerhofer et al. 2007) which is expressed in normal and neoplastic transformed mast cells.

In a second approach human neoplastic mast cells were analyzed for the expression of the TNF-related apoptosis-inducing ligand receptors (TRAIL-R1 and TRAIL-R2). Both cell lines show expression of both receptors on mRNA and protein level, and their expression on the cell surface was confirmed by flow cytometric analysis. With regard to a recently generated mouse model of mastocytosis murine mast cells were analyzed for expression of the murine TRAIL receptor and its regulation after KIT- and FcεR-activation. The murine TRAIL receptor is expressed on murine bone marrow derived mast cells and in accordance with findings in human mast cells (Berent-Maoz, Piliponsky et al. 2006; Berent-Maoz, Salemi et al. 2008) its expression is upregulated after IgE-dependent activation. Here a dose dependent regulation of the murine TRAIL receptor expression after activation of KIT with stem cell factor has been shown.

2.1.1 Expression of TRAIL receptors in mast cells

2.1.1.1 Expression of TRAIL and its receptors in human neoplastic mast cells

The expression of the TRAIL receptors TRAIL-R1 and TRAIL-R2 was analyzed in both clones of the human mast cell line HMC-1. To identify a potential differential expression both cell lines were analyzed for mRNA- and protein-expression of the receptors. The surface expression of both TRAIL receptors on HMC-1 cells was determined by flow cytometry. Furthermore the expression of TRAIL mRNA and protein was measured since recent findings showed TRAIL expression in human neoplastic mast cells with still unclear mechanisms of regulation, release or function (Berent-Maoz, Salemi et al.). Both cell lines showed comparable expression levels with no significant differences of TRAIL, TRAIL-R1 and TRAIL-R2 mRNA, protein and surface expression of the receptors.

The human neoplastic mast cell lines HMC-1.1 and HMC-1.2 were analyzed for expression of mRNA of TRAIL and its receptors TRAIL-R1 and TRAIL-R2. Both cell lines were seeded in complete medium and harvested during logarithmic growth. The mRNA was extracted and subjected to RT-PCR. Both cell lines express mRNA of TRAIL and the receptors TRAIL-R1 and TRAIL-R2. The densitometric analysis of several PCR gels showed no significant difference between PCR products of both cell lines (Fig. 2.1).

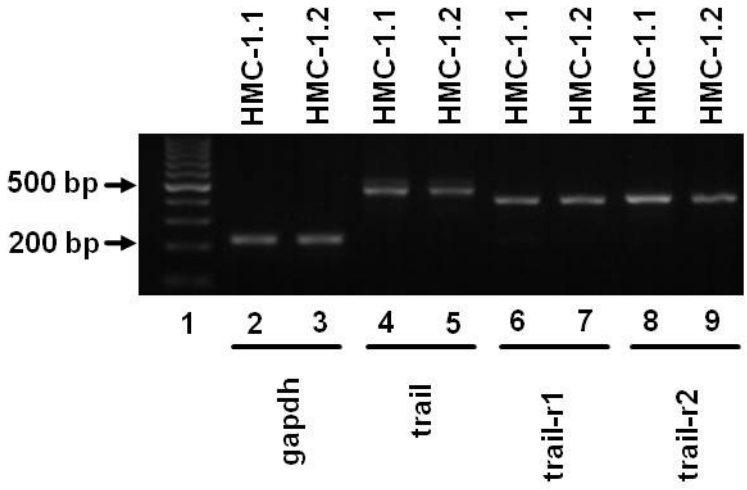

Fig. 2.1 Human neoplastic mast cells show RNA expression of trail and its receptors:
RT-PCR products of RNA isolated from HMC-1.1 and HMC-1.2 cells separated on a 2% agarose gel. (1, 100 bp Ladder; 2+3, gapdh 226 bp; 4+5, trail 510 bp; 6+7, trail-r1 415 bp; 8+9, trail-r2 437 bp). Data are representative of three independent experiments, all of which had similar results.

Both neoplastic mast cell lines were subjected to Western blot analysis with specific antibodies against TRAIL and its receptors TRAIL-R1 and TRAIL-R2. Expression of all three proteins can be detected in cell lysates of both cell lines harvested during logarithmic growth (Fig. 2.2).

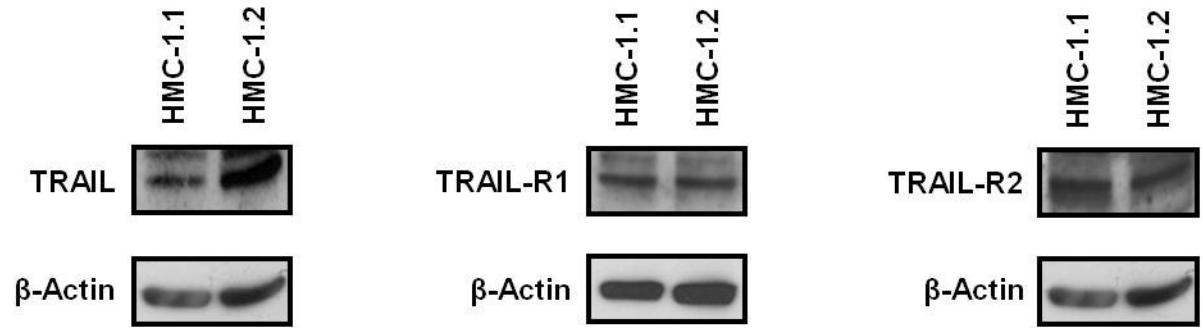

Fig. 2.2 Expression of TRAIL and its receptors can be detected in cell lysates of human neoplastic mast cells:
Western blot analysis of HMC-1.1 and HMC-1.2 cell lysates was performed with specific antibodies against TRAIL, TRAIL-R1 and TRAIL-R2. β-Actin was used as a loading control.
Data are representative of three independent experiments with similar results.

Surface expression of TRAIL-R1 and TRAIL-R2 was shown on HMC-1.1 and HMC-1.2 cells by staining both cell lines with fluorescent antibodies specific for the receptors (Fig. 2.3).

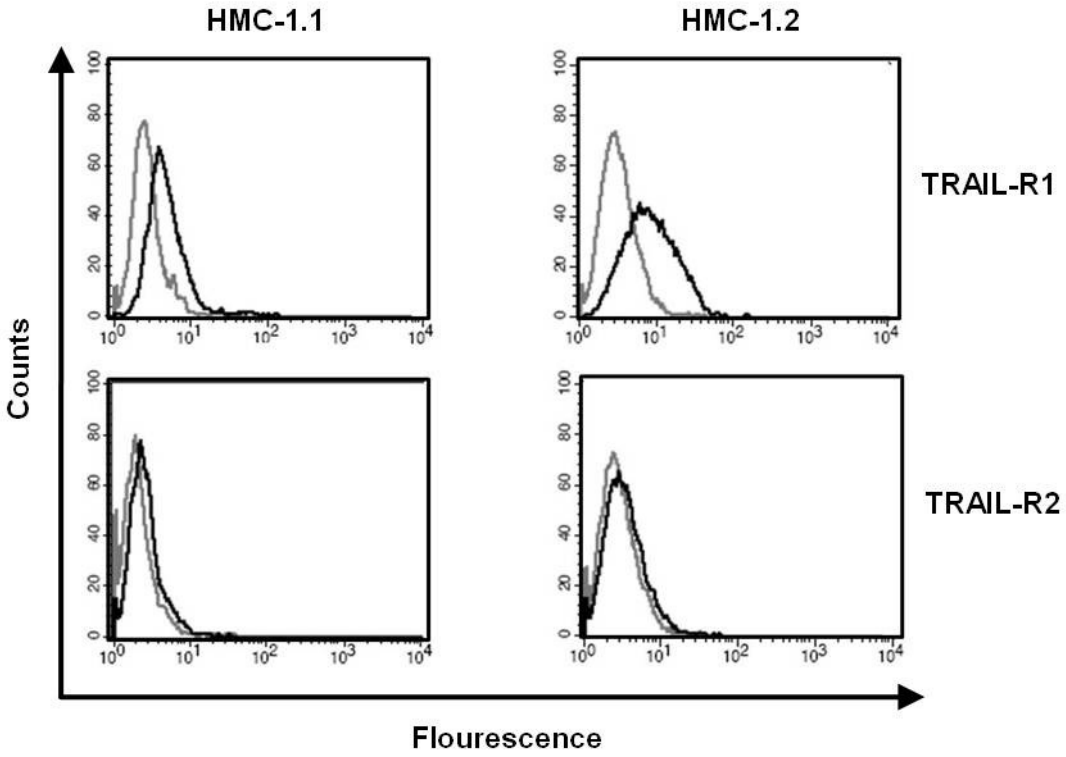

Fig. 2.3 Human neoplastic mast cells express TRAIL receptors on the cell surface:
HMC-1.1 and HMC-1.2 cells where labeled with PE-conjugated antibodies against TRAIL-R1, TRAIL-R2 (black line) or PE-conjugated isotype controls (grey line). Cells were analyzed by flow cytometry. Data are representative of three independent experiments with similar results.

2.1.1.2 Expression of TRAIL-R in murine mast cells

TRAIL-R is expressed on murine mast cells and its expression is upregulated by stem cell factor and IgE dependent activation

To analyze the effect of c-KIT activation and IgE-dependent activation on TRAIL-R-expression of murine mast cells, bone marrow-derived mast cells (BMMC) were either incubated with SCF or stimulated with IgE/anti-IgE or both for 24 h. Cells were harvested and stained with TRAIL-R specific or isotype antibodies and analyzed by flow cytometry (Fig. 2.4 A). Mean fluorescence was calculated and plotted as a percentage of the control (Fig. 2.4 B), showing that incubation with SCF of IgE-dependent stimulation increases TRAIL-R expression on BMMC but a combination of both has no additive effect.

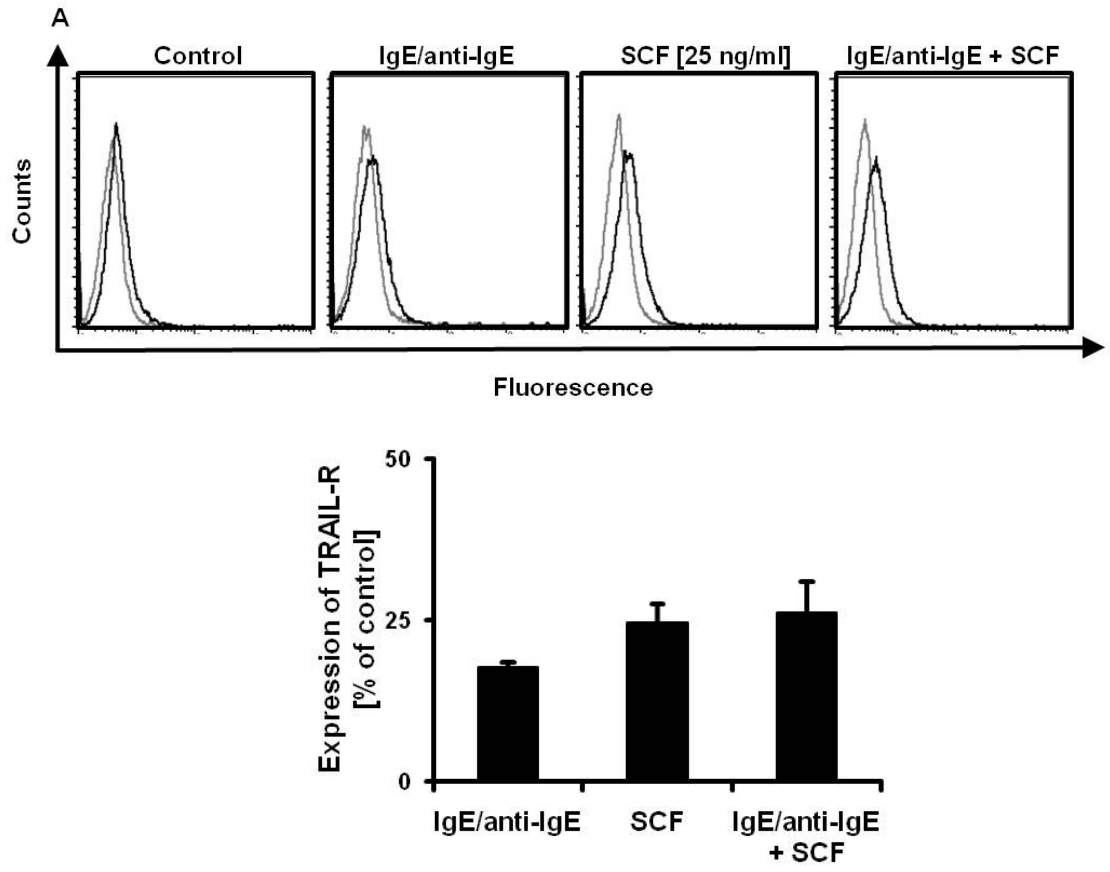

Fig. 2.4 TRAIL-R expression on BMMC is upregulated by SCF and IgE dependent activation: (A) BMMC were incubated with SCF, activated by 1 µg/ml IgE/anti-IgE or both for 24 h. TRAIL-R expression was measured by flow cytometry after staining of cells with PE conjugated TRAIL-R specific antibodies (black graphs) or PE conjugated isotype control (grey line). Data are representative of three independent experiments, all of which had similar results. (B) Mean fluorescence of stimulated and unstimulated cells was calculated. Results are presented as percent expression of TRAIL-R on stimulated cells compared to unstimulated cells (defined as 0%) and expressed as mean +/- SEM of three independent experiments.

SCF dependent upregulation of TRAIL-R is dose dependent

To confirm the first flow cytometric results, RT-PCR from mRNA isolated from SCF treated BMMC after 24 h was performed and a clear increase of PCR product was observed (Fig.2.5 A). Further, BMMC were incubated with SCF for 24 h and analyzed by flow cytometry. When BMMC were incubated with increasing concentrations of SCF for 24 h an increase of TRAIL-R expression was detected showing a correlation between receptor expression and level of c-KIT activation (Fig. 2.5 B, C).

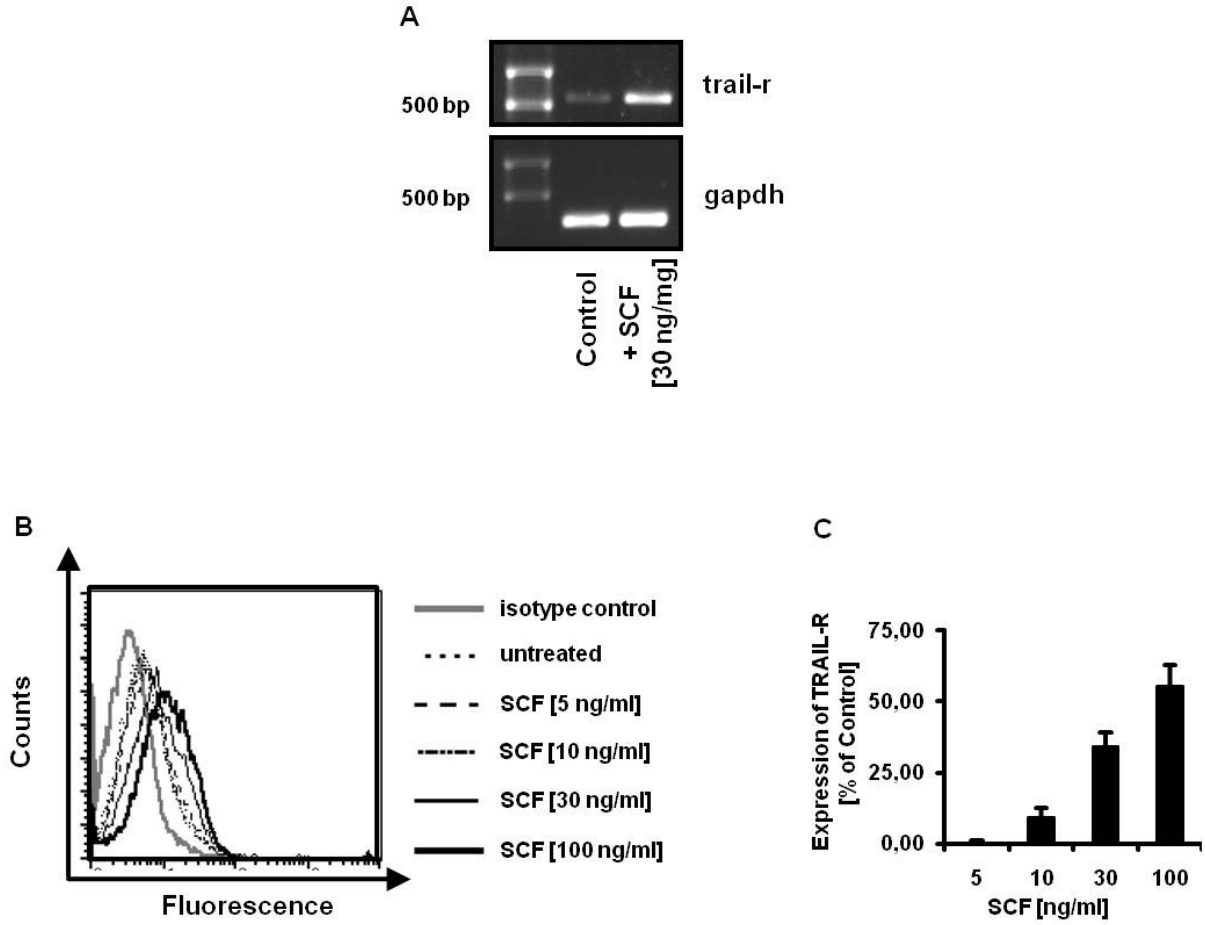

Fig. 2.5 Upregulation of TRAIL-R on BMMC by SCF is dose dependent:
RT-PCR was performed of RNA isolated from BMMC incubated with SCF (30 ng/ml) for 24 h compared to untreated control cells (A). PCR products where analyzed on a 1% agarose gel (TRAIL-R 579 bp, GAPDH 226 bp).
BMMC where incubated with different concentrations of SCF (0, 5, 10, 30 and 100 ng/ml) for 24 h and surface expression of TRAIL-R was analyzed by flow cytometry after staining of cells with PE-conjugated antibody against TRAIL-R (black lines) or PE-conjugated isotype control antibody (grey line) (B).
Mean fluorescence of stimulated and unstimulated cells was calculated (C). Results are presented as percent expression of TRAIL-R on stimulated cells compared to unstimulated cells (defined as 0%) and expressed as mean +/- SEM of three independent experiments.

2.1.2 Human neoplastic mast cells express inhibitor of apoptosis proteins (IAPs)

To determine proteins involved in apoptosis which are differently expressed under the control of the active tyrosine kinase KIT, the two human neoplastic mast cell lines HMC-1.1 and HMC-1.2 were analyzed. Both cell lines where seeded in complete medium, harvested during logarithmic growth and subjected to Western blot analysis with antibodies specific for X-linked inhibitor of apoptosis (XIAP), cellular inhibitor of apoptosis 1 and 2 (cIAP1, cIAP2) and Survivin, the second mitochondria-derived activator of caspase (Smac), the bcl-2 family members Mcl1 and Noxa, the cellular FLICE-like inhibitory protein (cFLIP) and β-Actin as loading control. Resulting films were analyzed densitometrically.

The expression of almost all proteins analyzed except Survivin was found to be equal in both HMC-1 subclones. Slight differences in single blots like seen below for Noxa (Fig. 2.6) could not be confirmed over the whole range of experiments. A pronounced expression of the IAP Survivin in HMC-1.2 cells was observed in all repeated experiments and was highly significant after densitometric analysis.

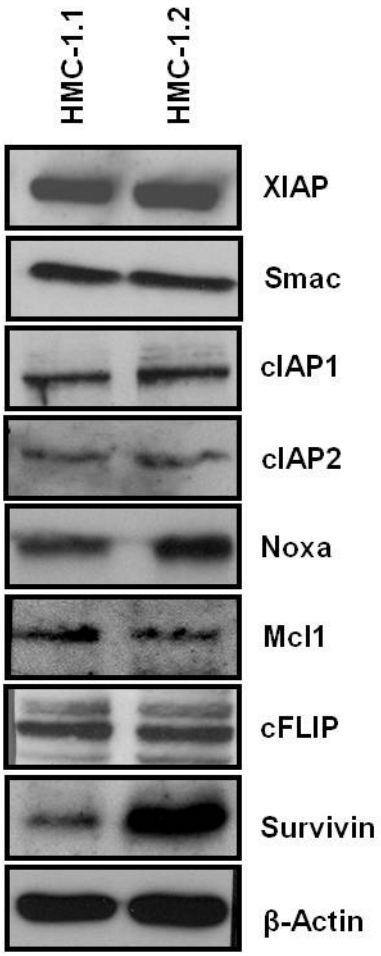

Fig. 2.6 Expression of IAPs in neoplastic human mast cells: Cell lysates of HMC-1.1 and HMC-1.2 cells were analyzed with specific antibodies against XIAP, Smac, cIAP1, cIAP2, Noxa, Mcl1, cFLIP and Survivin. β-Actin was used as a loading control. Data are representative of at least four independent experiments with similar results.

2.1.3 Expression of Survivin in human mast cells

2.1.3.1 Expression of Survivin mRNA and protein in human mast cells

Different human mast cells were analyzed for expression of survivin mRNA (Fig. 2.7 A) and protein (Fig. 2.7 B) to study a possible link between Survivin expression and neoplasia.

Human skin mast cells, human umbilical cord blood-derived mast cells and the mast cell line LAD-2 which is stem cell factor dependent and carries no activating KIT mutations showed low expression of survivin mRNA expression compared to HMC-1.2 cells, the basophilic KU812 cell line and JURKAT T cells. Western blot analysis performed with antibodies specific for Survivin showed low expression of Survivin protein in human umbilical cord blood-derived mast cells and LAD-2 cells compared to HMC-1.2, KU812 and JURKAT T cells.

Survivin is expressed in all cells analysed. The amount of mRNA and protein correlates with the rate of proliferation.

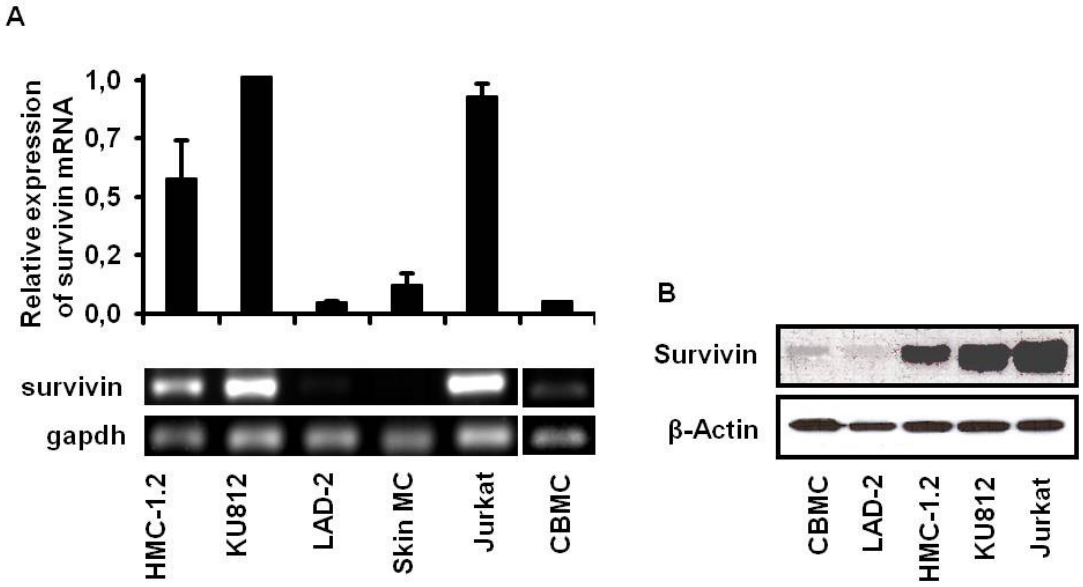

Fig. 2.7 Increased expression of survivin mRNA (A) and protein (B) in human neoplastic mast cells compared to primary mast cells:
RT-PCR was performed of mRNA isolated from different mast cell lines (HMC-1.2, KU812, LAD-2), primary human skin mast cells (skin MC) and the control cell line JURKAT with primers specific for survivin and gapdh (A). Fluorescence of ethidium bromide-stained RT-PCR products was measured by densitometry (upper graph). Results are presented as relative expression of survivin mRNA compared to the most pronounced expression KU812 cells (defined as 1.0) of at least three independent experiments with similar results.
Western blot analysis was performed of cell lysates of umbilical cord blood-derived mast cells (CBMC), different mast cell lines (LAD-2, HMC-1.2, KU812) and the control fell line JURKAT with a Survivin-specific antibody. β-Actin served as a loading control (B). Data are representative of three independent experiments with similar results.

2.1.3.2 Expression of Survivin protein in mast cells infiltrating the bone marrow of patients with Mastocytosis

To verify the relevance of upregulated survivin expression in vivo, bone marrow sections with mast cell infiltrates from Mastocytosis patients were screened for co-expression of mast cell tryptase and Survivin. In all samples from Mastocytosis patients Survivin expression was strongly pronounced compared to samples from healthy donors with reactive bone marrow infiltrates (Fig. 2.8).

Mast cells in bone marrow-infiltrates of patients with mastocytosis do not show elevated staining of the interphase marker Ki67 but significant increase of the marker of G_1 arrest p21 compared to mast cells in reactive bone marrow infiltrates (Baldus, Zirbes et al. 2004). Further p21 expression in mast cells is linked to c-KIT activity (Panzenbock, Bartunek et al. 1998), so elevated Survivin expression could be due to the neoplastic state of the cells not their higher rate of proliferation. Serial sections of bone marrow specimen where stained with antibodies specific for tryptase and survivin to analyze a possible co-expression. The double staining revealed no co-localization of tryptase and Survivin in bone marrow sections of healthy patients while most mastocytosis patients showed Survivin expression in at least 30% of infiltrates (Fig. 2.8 A-D).

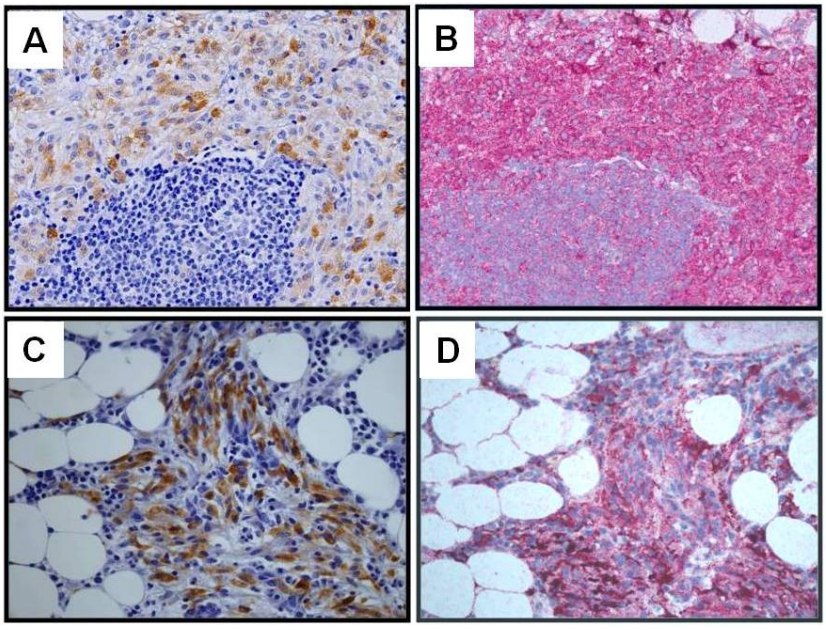

Fig. 2.8 Expression of Survivin protein in mast cells infiltrating the bone marrow of patients with mastocytosis:
Paraffin embedded bone marrow sections of mast cell infiltrates from patients with Mastocytosis were stained for mast cell tryptase (A, C) and Survivin (B, D).

To determine a statistical significance of these findings the bone marrow sections were scored for their level of Survivin antibody reactivity (Table 2.1): 0, mastocytosis: no reactivity of dense focal infiltrates of mast cells, reactive bone marrow (RBM): no reactivity of mast cells; 1, mastocytosis: reactivity in <30% of infiltrates, RBM: reactivity in <30% of mast cells; 2, reactivity in 30% to 70% of infiltrates or mast cells; 3, reactivity in >70% of infiltrates or mast cells. While a high Survivin expression (score 1-3) was only observed in patients with mastocytosis, Survivin expression score was 0 in healthy (RBM) donors. The serum tryptase level of the patients has a tendency to correlate with the degree of survivin expression but this was not statistical significant.

No.	Age [y]	Sex	Diagnosis	Mast cell infiltrates [%]	Tryptase [µg/ml]	Survivin [score]
1	53	M	RBM	5	ND	0
2	72	M	RBM	5	ND	0
3	47	F	RBM	1	ND	0
4	32	F	ISM	15	169.9	3
5	55	M	ISM	5	60.3	3
6	35	F	ISM	10	28.1	2
7	43	F	ISM	5	71.1	3
8	51	F	ISM	5	24.5	1
9	57	F	ISM	10	42.5	3
10	37	M	ISM	5	16.0	2
11	48	F	ISM	15	93.3	3
12	50	M	ISM	5	74.6	2
13	63	M	ISM	15	125.0	3
14	47	M	ISM	15	99.2	2
15	45	F	ISM	30	108.0	3
16	41	F	ISM	15	64.5	3
17	49	F	ISM	2	ND	3
18	46	F	ISM	5	72.3	2
19	62	M	ASM	20	257.0	3
Mean score (ISM and ASM cases)						2.56
Standard deviation						0.61

Table 2.1 Expression of Survivin in human Mastocytosis:
Paraffin-embedded bone marrow sections of 16 patients with mastocytosis were analyzed by immunohistochemistry and compared with 3 control patients with reactive bone marrow (RBM). The diagnosis of mastocytosis was made on the basis of published criteria. Out of the 16 patients, 15 patients had indolent systemic mastocytosis and one patient had aggressive systemic mastocytosis. Informed consent was obtained from all before bone marrow biopsies were performed.

2.2 Control of mast cell survival by pro- and anti-apoptotic molecules

After identifying Survivin as an anti-apoptotic protein being differentially expressed in human neoplastic mast cells depending on the level of KIT activation and TRAIL receptors being expressed in both subclones and murine mast cells, the susceptibility of these cells to TRAIL induced apoptosis was evaluated. HMC-1.1 and HMC-1.2 cells were incubated with recombinant human TRAIL and exhibited comparable low but significant loss of viability and an increasing number of apoptotic cells was detectable after at least 24 h of incubation.

In murine bone marrow-derived mast cells TRAIL alone failed to induce apoptosis. Only co-incubation with stem cell factor raised their susceptibility and significant apoptosis was measurable at 72 h of incubation.

In two approaches anti-apoptotic proteins were targeted in neoplastic human mast cells before apoptosis was induced by either tyrosine kinase inhibitors or TRAIL.

First the Survivin expression of HMC-1.2 cells was downregulated by Survivin-specific siRNA. This alone abolished proliferation and reduced their viability. Furthermore downregulation of Survivin combined with either tyrosine kinase inhibitor treatment or TRAIL induced apoptosis resulted in a synergistic loss of viability.

In a second approach the Smac mimetic LCL161 was used to target the IAPs XIAP, cIAP1 and cIAP2 in HMC-1.1 and HMC-1.2 cells. LCL161 alone led to accumulation of HMC-1 cells in the G_1 phase of the cell cycle and to inhibited proliferation. Combination of LCL161, the tyrosine kinase inhibitor PKC412 or TRAIL reduced viability and increased the rate of apoptotic cells in a synergistical manner after 24 h.

2.2.1 TRAIL reduces viability of human neoplastic mast cells and induces apoptosis

To investigate a dose dependent sensitivity of HMC-1 cells towards TRAIL induced apoptosis human neoplastic mast cells were incubated with human recombinant FLAG-tagged TRAIL at concentrations of 250 and 500 ng/ml. Furthermore a difference in sensitivity between both subclones should be examined. The percentage of viable cells was determined by propidium iodide uptake (Fig 2.9 A) and the rate of apoptosis by binding of Annexin-V (Fig. 2.9 B). After 48 h of incubation cells were analyzed by flow cytometry. Both the loss of viability and the increase of apoptotic cells were significant and dose dependent in HMC-1.1 and HMC-1.2 cells but there was no significant difference in loss of viability or rate of apoptosis between both cell lines.

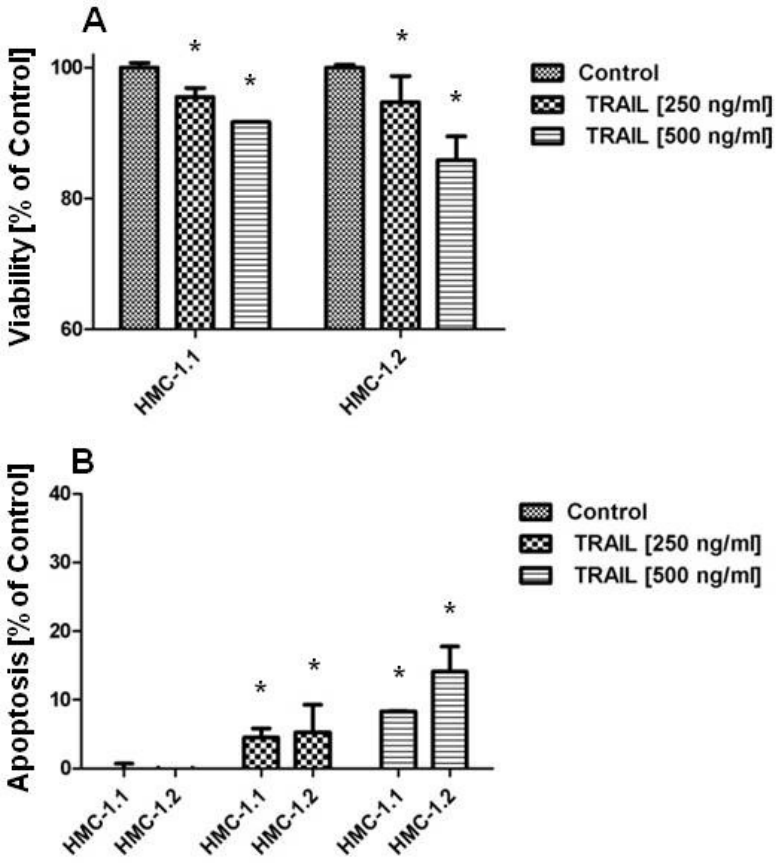

Fig. 2.9 TRAIL induces apoptosis in neoplastic human mast cells and reduces their viability:
HMC-1.1 and HMC-1.2 cells were incubated with TRAIL-FLAG for 48 h. Viability and apoptosis was measured by FACS analysis after staining with APC-labeled Annexin-V and propidium iodide.
Data are representative of at least three independent experiments.
P values ≤ 0.05 where considered as significant (*).

2.2.2 TRAIL induced loss of viability of BMMC is increased by SCF

To examine the effect of TRAIL on primary murine mast cells, bone marrow-derived mast cells were incubated with 500 ng/ml TRAIL, 40 ng/ml of the inhibitor or RNA synthesis actinomycin D, a combination of both or medium alone for up to 72 h. Actinomycin D at low doses has been described to sensitize mast cells to Fas mediated apoptosis with only a slight impact on their viability (Hartmann, Wagelie-Steffen et al. 1997).

While TRAIL alone was capable of reducing viability of BMMC to a small amount this effect was more pronounced under co-treatment with actinomycin D. Incubation of BMMC with SCF enhanced viability loss of BMMC (Fig. 2.11).

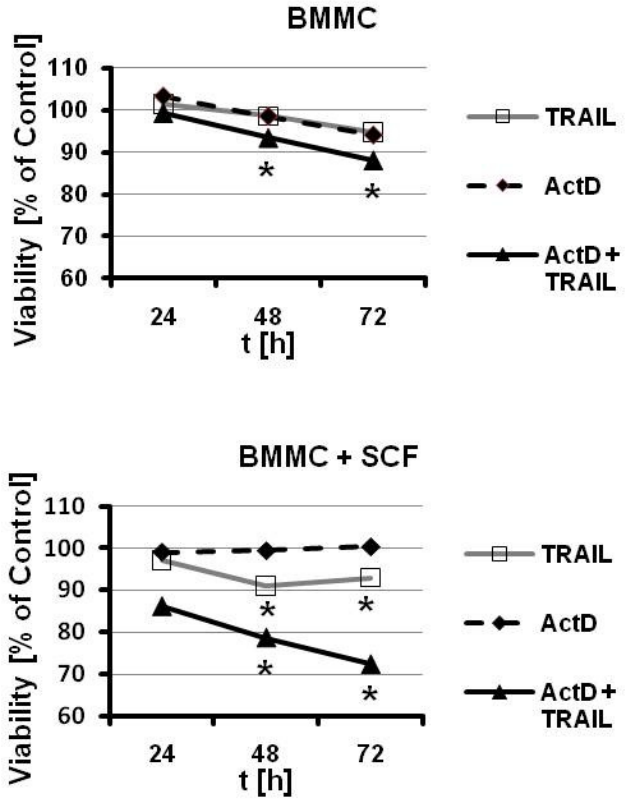

Fig. 2.11 TRAIL induced loss of viability of BMMC is increased by SCF:
BMMC were incubated with medium alone, TRAIL-FLAG (500 ng/ml), Actinomycin D (40 ng/ml) or a combination of TRAIL-FLAG and Actinomycin D (40 ng/ml) for up to 72 h. Cells were stained with propidium iodide. Viability was measured by flow cytometric analysis.
Data are representative of three independent experiments.
P values ≤ 0.05 where considered as significant (*).

2.2.3 TRAIL-induced apoptosis of BMMC is increased by SCF

When BMMC were incubated with FLAG-tagged recombinant TRAIL for three days and apoptosis was measured by flow cytometry after Annexin-V and propidium iodide staining, significant increase of apoptosis was detectable after 48 h. However the rate of apoptosis is very low compared to human neoplastic mast cells used in this thesis. Co-treatment of BMMC with the inhibitor of transcription actinomycin D increased the rate of apoptosis slightly. When BMMC were incubated in medium plus SCF, a marked increase of apoptotic BMMC under TRAIL treatment could be observed, even more pronounced when co-treated with actinomycin D (Fig. 2.10).

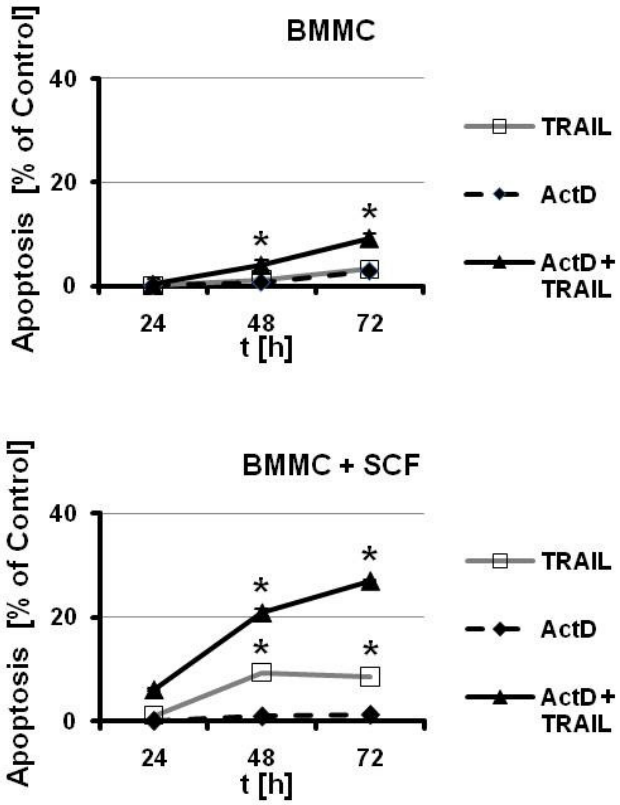

Fig. 2.10 TRAIL-induced apoptosis of BMMC is increased by SCF:
BMMC were incubated with medium alone, TRAIL-FLAG (500 ng/ml), Actinomycin D (40 ng/ml) or a combination of TRAIL-FLAG and Actinomycin D for up to 72 h. Cells were stained with Annexin-V. The rate of apoptosis was measured by flow cytometric analysis.
Data are representative of three independent experiments.
P values ≤ 0.05 where considered as significant (*).

2.2.4 Downregulation of Survivin increases mast cells sensitivity towards apoptotic stimuli

HMC-1.2 cells were transfected with the Survivin-specific siRNA expressing plasmid pSuper-Survivin (Lens, Wolthuis et al. 2003) or the Luciferase-specific siRNA expressing plasmid pSuper-Luc (Butz, Ristriani et al. 2003). After 24 h cells were harvested and subjected to Western blot analysis which showed the marked downregulation of Survivin protein in pSuper-Survivin transfected cells (Fig. 2.12). Downregulation of Survivin abolished HMC-1.2 proliferation as determined by cell count on day 1 and 2 post transfection of three independent transfections with 3 respective 6 µg of pSuper-Survivin or pSuper-Luc.

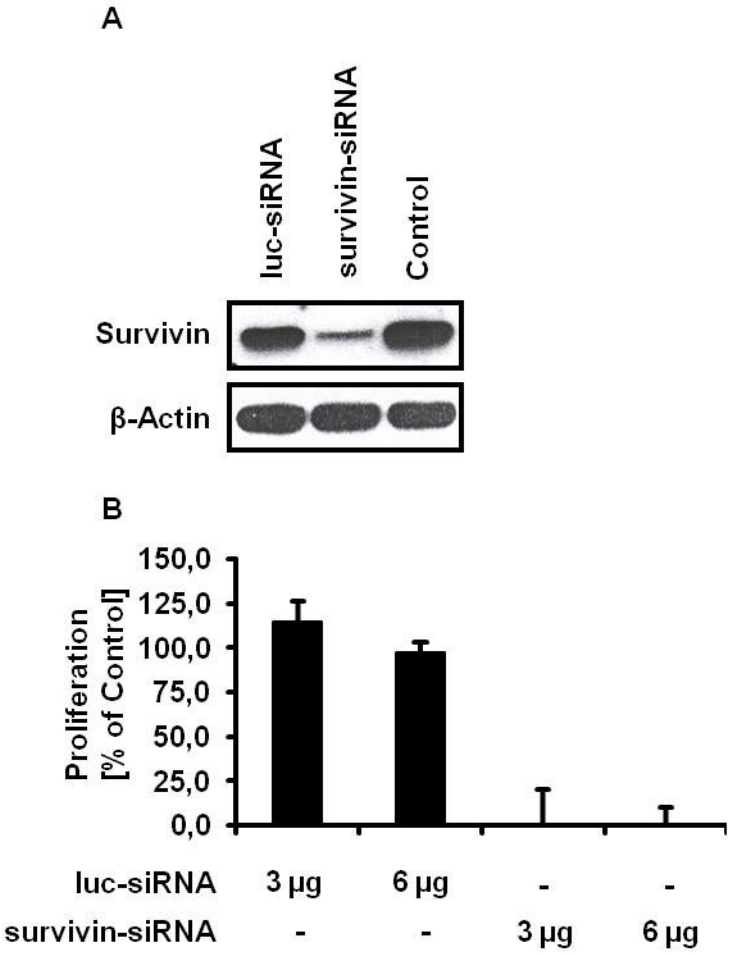

Fig. 2.12: Survivin protein expression in HMC-1.2 cells is reduced in pSuper-Survivin transfected HMC-1.2 cells (A). Rate of proliferation of pSuper-Survivin transfected cells is reduced compared to pSuper-Luc transfected cells (B):
Western blot analysis of HMC-1.2 cell lysates was performed with a specific antibody against Survivin. β-Actin served as a loading control.
Proliferation was calculated for each transfection by counting cells of three independent transfections after 24 and 48 h. The increase of cell number of cells transfected with 6 µg pSuper-Luc was defined as 100%.
Data are representative of three independent experiments.

HMC-1.2 cells transfected with 3 µg pSuper-Survivin or pSuper-Luc were incubated with 250 ng/ml TRAIL-FLAG, the tyrosine kinase inhibitors Nilotinib (7 µM) and PKC412 (1 µM) or medium alone. Propidium iodide uptake of cells was measured by flow cytometry after 48 h of culture. The loss of viability in pSuper-Survivin transfected cells was significantly increased compared to pSuper-Luc transfected cells (Fig. 2.13).

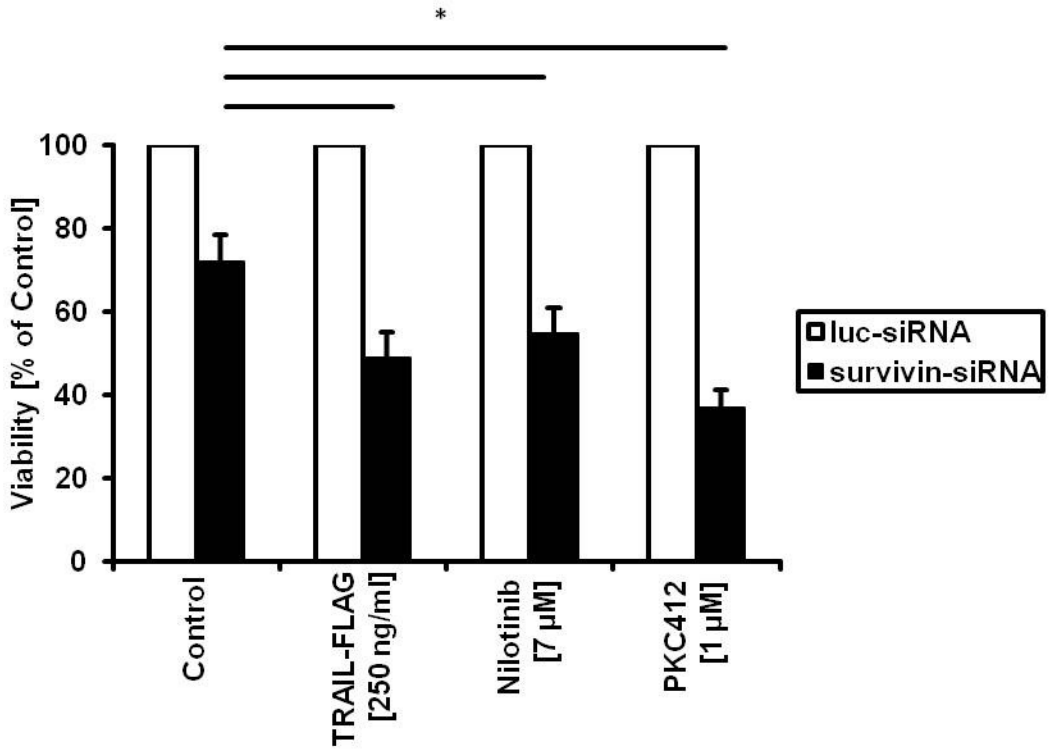

Fig. 2.13: pSuper-Survivin transfected HMC-1.2 cells show reduced viability compared to pSuper-Luc transfected cells:
HMC-1.2 cells were transfected with 3 µg of pSuper-Survivin or pSuper-Luc. After 48 h incubation with TRAIL-FLAG, Nilotinib, PKC412 or medium alone, cells were harvested. Cells were stained with propidium iodide and their viability was measured by flow cytometric analysis.
Data are representative of three independent experiments.
P values ≤ 0.05 where considered as significant (*).

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2.2.5 The Smac mimetic LCL161 inhibits proliferation of neoplastic human mast cells

When incubated with the Smac mimetic LCL161 proliferation of HMC-1.1 and HMC-1.2 cells is inhibited in a dose dependent manner as was determined by cell count during two days of incubation (Fig. 2.14 A,B). While concentrations up to 10 µM of LCL161 only had moderate influence on both HMC-1 clones' proliferation, it was inhibited at a concentration of 50 µM. When incubated with the Smac mimetic LCL161 for 24 h the cell cycle of HMC-1.2 cell shifts from the G_2 phase towards the G_1 and sub-G_1 phase (Fig. 2.14 C).

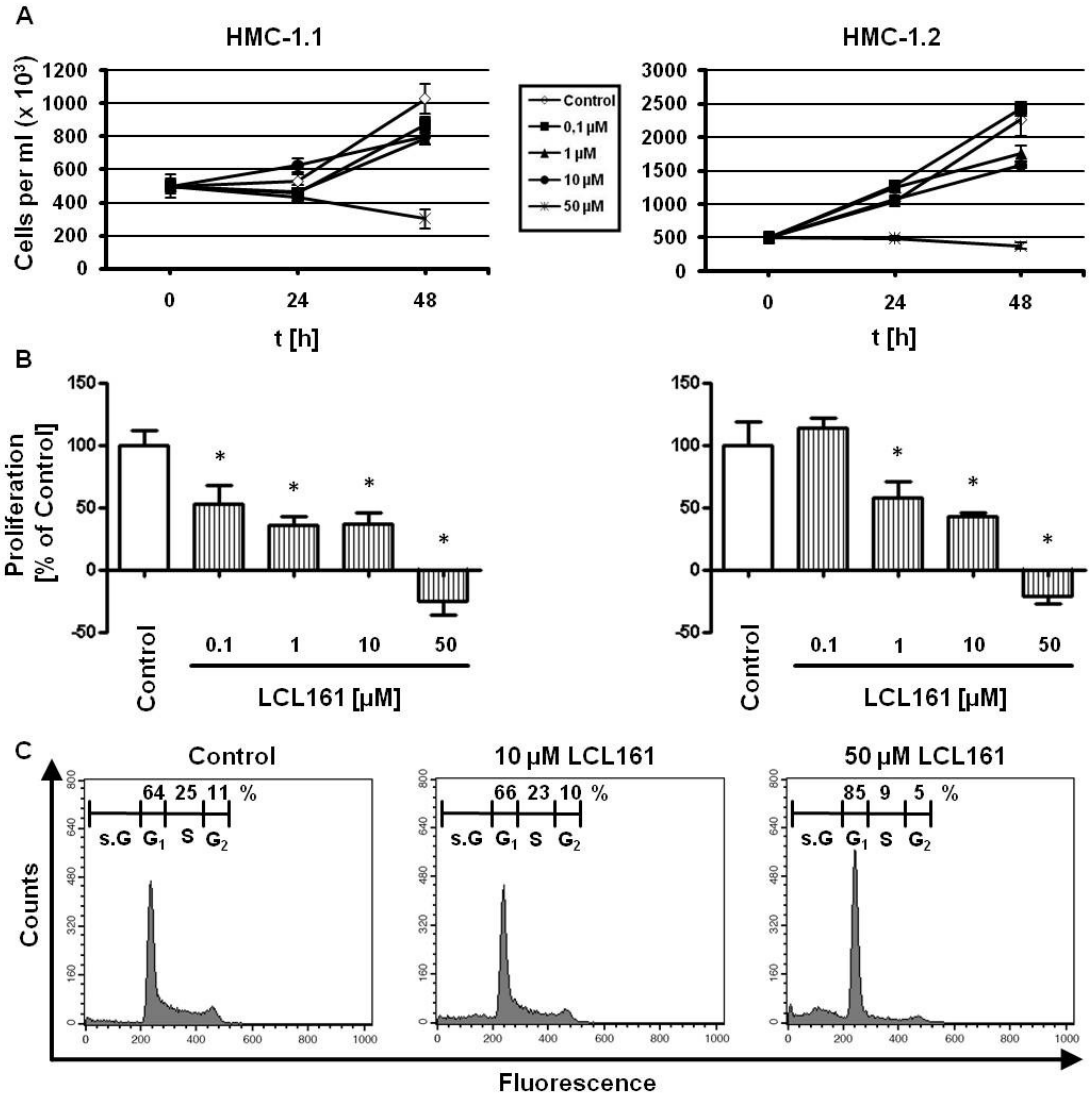

Fig. 2.14 LCL161 inhibits proliferation of HMC-1 cells in a dose-dependent manner:
HMC-1.1 and HMC-1.2 were incubated with various concentrations of LCL161 for 48 h. Cell numbers and rate of proliferation were determined by manual cell count at time points indicated (A). Rate of proliferation was calculated from cell count at 0 and 48 h (B).
For the cell cycle analysis cells where incubated with various concentrations of LCL161 for 24 h. Cells were fixed with ethanol and stained with propidium iodide to determine cell cycle phases by flow cytometry. (Cell cycle phases are indicated by bars in the diagrams: s.G: sub G peak; G_1: Gap 1 phase; S: Synthesis phase; G_2: Gap 2 phase) (C).
Data are representative of three independent experiments.

2.2.6 LCL161 does sensitize neoplastic human mast cells to TRAIL and PKC412

HMC-1.1 and HMC-1.2 cells were incubated with LCL161, Flag-tagged recombinant TRAIL or a combination of both for 72 h. Loss of viability was determined by flow cytometry. Both stimuli alone were sufficient to reduce viability in both cell lines and their combination resulted in a synergistic effect.

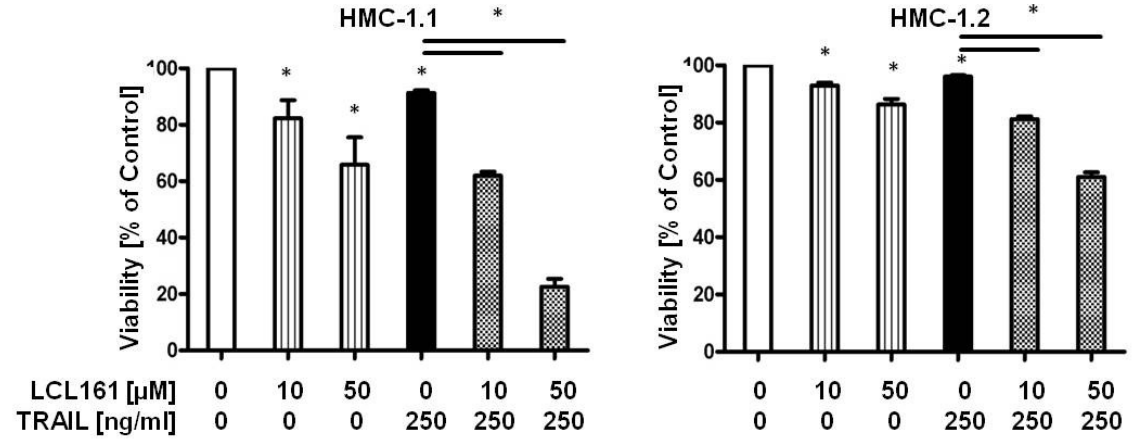

Fig. 2.16 LCL161 and TRAIL reduce viability of HMC-1 cells in a synergistic manner:
HMC-1 cells were incubated with LCL161 and TRAIL-FLAG for 72 h. Cells were stained with propidium iodide and their viability was measured by flow cytometric analysis.
Data are representative of three independent experiments.
P values ≤ 0.05 where considered as significant (*).

Both HMC-1 subclones were incubated with LCL161, Flag-tagged recombinant TRAIL or a combination of both for 72 h and the rate of apoptosis was determined by flow cytometry. In both cell lines TRAIL and LCL161 were able to induce apoptosis and their combination resulted in a synergistic effect.

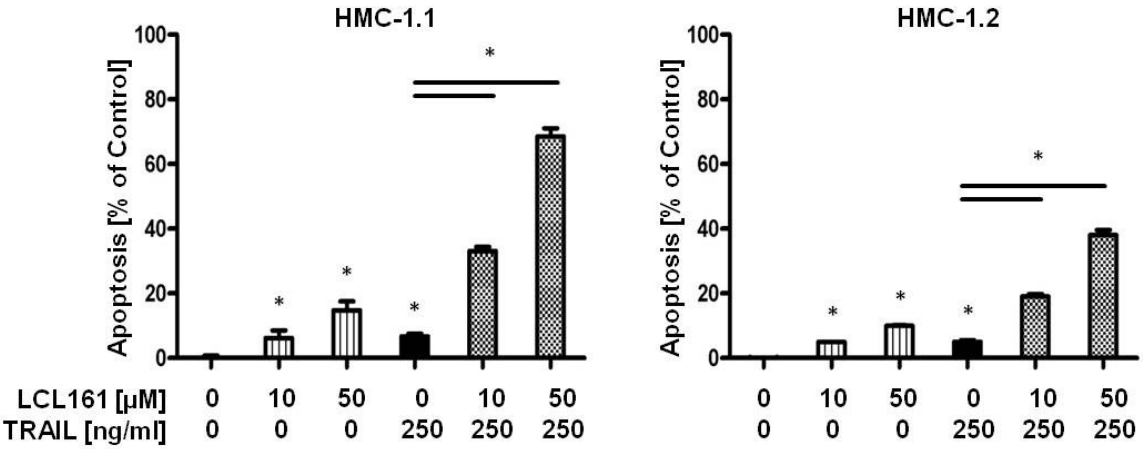

Fig. 2.17 LCL161 and TRAIL induces apoptosis of HMC-1 cells in a synergistic manner:
HMC-1 cells were incubated with LCL161 and TRAIL-FLAG for 72 h. Cells were stained with propidium iodide and Annexin-V. The rate of apoptosis was measure by flow cytometric analysis.
Data are representative of three independent experiments.
P values ≤ 0.05 where considered as significant (*).

HMC-1.1 and HMC-1.2 cells were incubated with LCL161, PKC412 or a combination of both for 72 h and the loss of viability was determined by flow cytometry. Both stimuli alone were sufficient to reduce viability in both cell lines and their combination resulted in a synergistic effect.

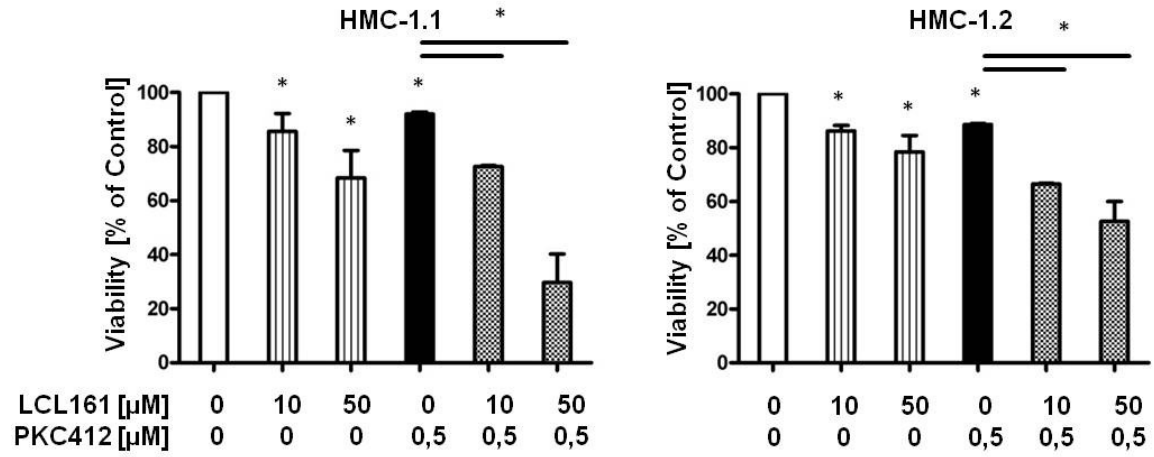

Fig. 2.18 LCL161 and PKC412 reduce viability of HMC-1 cells synergistically:
HMC-1 cells were incubated with LCL161 and TRAIL-FLAG for 72 h. Cells were stained with propidium iodide. Viability was analyzed by flow cytometric analysis.
Data are representative of three independent experiments.
P values ≤ 0.05 where considered as significant (*).

Both HMC-1 subclones were incubated with LCL161, PKC412 or a combination of both for 72 h and the rate of apoptosis was determined by flow cytometry. In both cell lines TRAIL and LCL161 were able to induce apoptosis and their combination resulted in a synergistic effect.

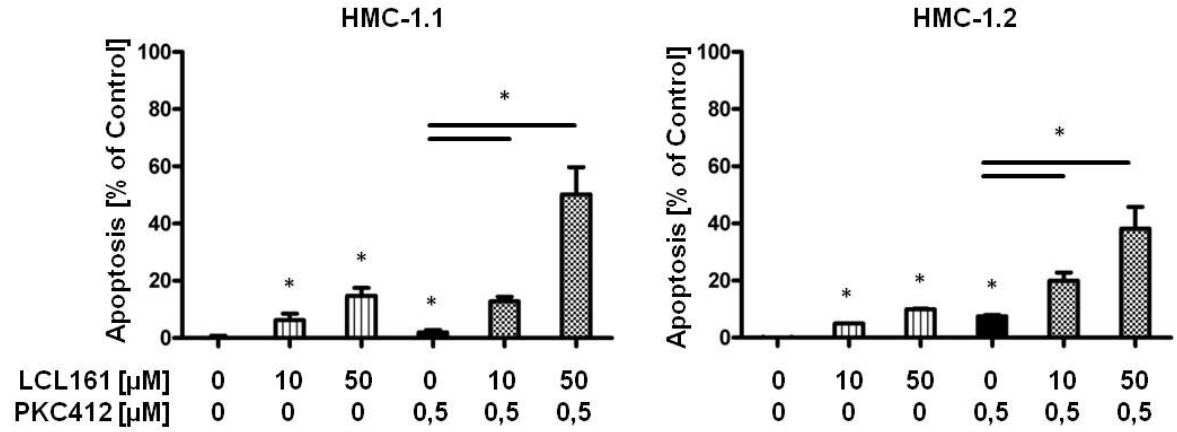

Fig. 2.19 LCL161 and PKC412 induce apoptosis of HMC-1 cells synergistically:
HMC-1 cells were incubated with LCL161 and PKC412 for 72 h. Cells were stained with propidium iodide and Annexin-V. The rate of apoptosis was analyzed by flow cytometric analysis.
Data are representative of three independent experiments.
P values ≤ 0.05 where considered as significant (*).

3 Discussion

Mastocytosis is a rare human disease characterized by the pathological accumulation of mast cells in various tissues. Different variations of mastocytosis can be distinguished by localization and severity of mast cell infiltration and proliferation. While in cutaneous mastocytosis mast cell infiltrates are restricted to the skin, mast cells in systemic mastocytosis can additionally infiltrate internal organs in particular the bone marrow. Increased release of mast cell mediators in patients with mastocytosis can cause pruritus, nausea, diarrhea or dizziness. In cases of systemic mastocytosis progressive mast cell infiltration can lead to severe symptoms like osteoporosis or organ failure. According to the WHO an important criterion for diagnosis of systemic mastocytosis is the detection of the activating D816V point mutation of the c-KIT receptor. Since no curative treatment for Mastocytosis is available yet, therapy options focus on inhibition of mediator release from mast cells and on control of mast cell numbers. Because increase of mast cell numbers of mastocytosis patients results from altered cell proliferation and dysregulated apoptosis, targeting the apoptotic pathways could provide an additional tool alongside conventional treatment with tyrosine kinase inhibitors.

This work presents novel approaches for inhibiting the survival of neoplastic mast cells by targeting the cells' apoptotic apparatus. Investigation of anti-apoptotic molecules showed a distinct correlation of expression of a single molecule with the state of disease. Immuno-staining of mast cells from bone marrow infiltrates of patients with mastocytosis have been shown to express elevated levels of the anti-apoptotic protein Survivin compared to those of healthy donors. Higher c-KIT activation in neoplastic mast cell lines is associated with higher Survivin expression. The regulation of expression of the pro-apoptotic TRAIL receptor by c-KIT activation has been shown on murine mast cells. By use of siRNA the expression of Survivin in neoplastic mast cells was downregulated. Treatment with tyrosine kinase inhibitors or a soluble TRAIL-R ligand lead to markedly enhanced loss of viability of neoplastic mast cells compared to mast cells with normal levels of Survivin. When neoplastic mast cells were pre-treated with LCL161, a mimetic of the pro-apoptotic protein Smac the induction of apoptosis and reduction of viability by treatment either with the tyrosine kinase inhibitor PKC412 or soluble TRAIL was increased in a synergistic manner. These results point out the importance of Survivin as a molecular marker of neoplastic transformed mast cells and the effectiveness of intervention in apoptosis regulation for the treatment of systemic mastocytosis providing a promising new therapeutic approach.

3.1 Identification of novel therapeutic targets in mastocytosis: Inhibition of survival of neoplastic mast cells by targeting of IAPs and TRAIL

Altered expression of pro- and anti-apoptotic proteins has been observed in many neoplastic malignancies. It has been reported that bone marrow mononuclear cells of patients with mastocytosis express higher amounts of TRAIL-R mRNA (D'Ambrosio, Akin et al. 2003) and neoplastic human mast cells express functional TRAIL receptors (Berent-Maoz, Piliponsky et al. 2006). This thesis reports that activation of c-KIT enhances TRAIL-R expression and mast cells susceptibility towards TRAIL induced apoptosis which is even more pronounced in combination with tyrosine kinase inhibitors or inhibition of anti-apoptotic proteins. Further the inhibitor of apoptosis protein Survivin was found to be highly expressed in neoplastic mast cells *in vitro* and *in vivo* making it an interesting candidate for a diagnostic marker. Survivin downregulation enhanced the effect of tyrosine kinase inhibitors and TRAIL as did the inhibition of XIAP, cIAP1 and cIAP2 by the Smac mimetic LCL161.

3.1.1 Choice of models for mast cell investigation

For the investigation of normal human mast cells *in vitro* it is necessary to obtain a pure culture either by isolation of mature mast cells from tissue or by generation of mast cells from their immature progenitors. Mature human mast cells can be isolated for example from the skin, the intestine or the lung. Isolation of mast cells generally does not yield high cell numbers and the cells generally do not divide or survive for a longer time. To generate mast cells from the pools of progenitor cells for example from umbilical cord-blood or peripheral blood is extremely time consuming and since culture conditions are not clearly standardized different media compositions used produce mast cells with different phenotypes. Therefore the use of permanent cell lines still is the first choice for the *in vitro* study of human mast cell functions. Available today are the stem cell factor dependent mast cell line LAD-2 (Kirshenbaum, Akin et al. 2003) and the stem cell factor independent mast cell line HMC-1 with their subclones HMC-1.1 and HMC-1.2 carrying one respective two activating c-KIT mutations. In this work the HMC-1 cell lines isolated from a case of mast cell leukemia where used. These cells represent the standard tool for investigation of neoplastic mast cells resembling mast cells in mastocytosis patient and are widely used for the development of therapies. As a control cell line the basophilic KU812 cell line was used. KU812 cells share characteristics with basophiles like IL-3 expression and mast cells like expression of tryptase. KU812 cells express functional c-KIT and are sensitive towards tyrosine kinase inhibitors.

For the murine system two immature mast cell lines are used. The P-815 cell line is carrying an activating c-KIT mutation resembling the situation in the HMC-1.1 line. The cell line C57 grows independently of c-KIT activity and therefore is unsuitable for the investigation of c-KIT dependent mast cell disorders. On the other hand the generation of primary mast cells from mice is well established and the standard protocols for the generation of bone marrow-derived and peritoneal mast cells are less time consuming and generate high yields of mast cells of reproducible properties. Here BMMC from wild type animals were used for functional studies which were taken from animals of the same age and all cell preparations were routinely checked for uniform morphology and unvarying expression of mast cells surface markers.

3.1.2 Detection of apoptosis

Several methods for the detection of apoptosis are available. Qualitative analysis of apoptosis of *in situ* samples can be done by identification of cytoplasmic condensation, chromatin condensation and segregation with a transmission electron microscope. Light microscopic analysis of cells can reveal morphological changes like membrane blebbing or nucleus fragmentation which can be visually enhanced by additional staining with unspecific DNA dyes like DAPI (4,6-diamidino-2-phenylindole) or apoptosis specific staining of fragmented DNA by terminal-uridine-nick-end-labeling (TUNEL). Quantitative analysis of apoptosis in tissue samples is usually done by counting of apoptotic versus non-apoptotic cells. Western blot analysis of cell lysates with antibodies specific for phosphorylated caspase 3 can reveal the activity of apoptosis related signaling cascades. In a DNA ladder assay isolated DNA is cut enzymatically and analyzed on an agarose gel after staining with ethidium bromide. DNA fragments characteristic for apoptotic cells can be quantified. Isolated cells and cell lines in this work were analyzed either by analysis of nucleus fragmentation (“subG_1-peak”) or an Annexin-V test: due to fragmentation of the DNA and the nucleus, propidium iodide stained apoptotic cells show DNA content lower than in haploid cells and so can be separated in a FACS histogram. Annexin-V binds to phospatidylserine which is translocated to the outer cell membrane of apoptotic cells. Double staining of cells with Annexin-V and propidium iodide and subsequent flow cytometric analysis can discriminate viable, necrotic and apoptotic cells. These two methods were performed with standardized routine protocols and yield highly reproducible results at high sample throughput.

3.1.3 The IAP Survivin is highly expressed in neoplastic mast cells

Survivin plays an important role in the proliferation and maturation of normal hematopoietic cells (Fukuda, Foster et al. 2002; Fukuda, Mantel et al. 2004; Leung, Xu et al. 2007). Furthermore its expression in cells of this lineage seems to be linked to the expression and activity of the receptor tyrosine kinase c-KIT (Fukuda, Mantel et al. 2004; Jalal Hosseinimehr, Inanami et al. 2004; Fukuda, Singh et al. 2009).

Analysis of bone marrow-derived mast cells, skin mast cells and the stem cell factor dependent mast cell line LAD-2, revealed low Survivin mRNA expression compared to HMC-1 cells carrying an activating c-KIT mutation. The same pattern was found for Survivin protein expression in CBMCs and LAD-2 cells when compared to HMC-1 cells. Survivin specific staining of bone marrow from patients with mastocytosis and donors with reactive bone marrow infiltrates showed high expression of Survivin protein in the mastocytosis group while no Survivin was expressed in samples of the control group. The finding of higher Survivin expression during the comparison of IAP expression in both HMC-1 clones gives another hint on the connection between c-KIT activation and Survivin expression in mast cells. Although a strong link between Survivin expression and severity of common human neoplasms exists (Altieri 2003; Kanwar, Kamalapuram et al. 2010) no direct correlation between Survivin expression in mast cells of mastocytosis patients, severity of disease and other factors tested like serum tryptase level could be observed at a statistical significant level. Due to the small number of patients included in this study and the semi-quantitative analysis of histological data further studies with higher number of patients will be necessary possibly including other disease-relevant factors and the use of quantitative methods of analysis.

Since elevated expression of Survivin seems to be a feature of neoplastic transformed mast cells and bone marrow sections are still required for establishing a pathological diagnosis, staining for Survivin could be an additional tool to solidify diagnosis, and in case of further studies linking different c-KIT mutations with levels of Survivin expression, a prognostic marker. The downregulation of Survivin in neoplastic HMC-1.2 mast cells presented in this work shows the possible value of an anti-Survivin therapy in combination with approved therapies of mastocytosis like the use of tyrosine kinase inhibitors or possible new approaches like the activation of the apoptosis inducing TRAIL receptors.

3.1.4 TRAIL receptors in HMC-1 cells are differently expressed and murine bone marrow-derived mast cells TRAIL-R expression and function is influenced by c-KIT activity

As has been previously shown (Berent-Maoz, Piliponsky et al. 2006) TRAIL-R1 and TRAIL-R2 are expressed on human lung-derived mast cells, cord blood-derived mast cells and HMC-1.2 cells. In contrast to Fas mediated apoptosis all tested mast cells were sensitive to TRAIL-FLAG. TRAIL-R1 and TRAIL-R2 expression was compared in CBMC of different donors showing pronounced surface expression of TRAIL-R2 protein.

The results presented here show a pronounced surface expression of TRAIL-R1 protein on both HMC-1 subclones and activation of TRAIL-R1 alone was sufficient to induce apoptosis in both cell lines (data not shown). Still this work confirmed the overall moderate sensitivity of HMC-1 cells towards TRAIL induced apoptosis compared to those of highly sensitive cells like JURKAT T cells. Several studies have linked the predominant TRAIL-R1 expression to more aggressive state of disease like in breast cancer (McCarthy, Sznol et al. 2005) and colon cancer (van Geelen, Westra et al. 2006). Further studies will be made to analyze TRAIL-R expression on mast cells from mastocytosis patients and healthy donors to check for a possible connection between differential TRAIL-R expression and disease state.

IgE-dependent activation increases human cord blood-derived mast cells sensitivity towards TRAIL-induced apoptosis (Berent-Maoz, Salemi et al. 2008). IgE increased the expression of the anti-apoptotic molecules FLIP and MCL-1 and the pro-apoptotic molecule BIM. TRAIL reversed the increase of FLIP expression and induced apoptosis.

BMMC were used to show functional TRAIL-R expression on murine mast cells and to study its regulation on primary cells with regards to future experiments with a recently generated mouse model of mastocytosis (Gerbaulet, Wickenhauser et al. 2010). To analyze the effect of stem cell factor on TRAIL-R expression the murine system was chosen since primary murine mast cells are viable and can be cultivated in SCF free medium while human mast cells instantly undergo apoptosis under SCF deprivation. Here the analysis of mouse derived BMMC revealed a dose dependent upregulation of TRAIL-R upon activation with SCF comparable with the upregulation following IgE-dependent activation. Quantitative PCR analysis showed no increase in FLIP mRNA nor did Western blot analysis show increased FLIP protein expression upon SCF activation while protein expression of MCL-1 was increased (data not shown). Combination of IgE-dependent activation and stimulation with SCF did not result in an additive effect. By which signaling pathway SCF is regulating TRAIL-R expression still has to be investigated. Further incubation of BMMC with SCF increased their sensitivity towards TRAIL-induced loss of viability and increase of apoptosis.

As a control TRAIL-R$^{-/-}$ BMMC were analyzed. These were generated by crossing TRAIL-R-floxed mice with Deleter-cre mice. Isolated BMMC did not contain TRAIL-R DNA as was shown by specific PCR and showed no expression of TRAIL-R protein as was analyzed by flow cytometry. Incubation with TRAIL alone or TRAIL and SCF did not change their rate of viability nor resulted in apoptosis. Additionally our group is analyzing mice specifically lacking TRAIL-R on mast cells generated by crossing TRAIL-R floxed mice to mice expressing mast cell specific cre recombinase (Scholten, Hartmann et al. 2008). To date no differences in mast cell numbers, differential mediator release from mast cells or sensitivity towards anaphylaxis has been shown.

These results are in contrast to data published for erythroid progenitors which show reduced sensitivity towards TRAIL induced apoptosis following stimulation with SCF (Schmidt, van den Akker et al. 2004).

This data indicates a biological function of the TRAIL-R on BMMC since its expression is linked to SCF, the most important mast cell cytokine responsible for mast cell functions like proliferation, migration and cytokine release. Since the TRAIL concentrations used to induce apoptosis are very high, other functions of TRAIL i.e. in combination with SCF or IgE-dependent activation will be subject of future investigation by analyzing mice lacking TRAIL-R on mast cells.

3.1.5 Downregulation of Survivin increases neoplastic human mast cells sensitivity towards TRAIL and tyrosine kinase inhibitors

Targeting the small inhibitor of apoptosis protein Survivin in neoplastic disorders seems to be a most promising approach since this molecule is not only sharply differentially expressed in disease (Altieri 2006) but has the role of a nodal protein being involved in multiple signaling mechanisms controlling cell division, modulation of apoptotic and non-apoptotic cell death, stress response and cell survival (Altieri 2006; Lens, Vader et al. 2006). A global deregulation of the *survivin* gene mediated by oncogenes, including STAT3 (Kanda, Seno et al. 2004) and the phosphatidylinositol 3-kinase – Akt axis (Asanuma, Torigoe et al. 2005) accounts for the selective expression of Survivin. Since all mutant c-KIT receptors investigated to date signal via the STAT3 (Chaix, Lopez et al. 2010) and STAT5 – PI3K – Akt (Harir, Boudot et al. 2008) pathway this could explain the high expression of survivin in mastocytosis. Compared to other myeloid cells such as basophiles, mast cells are extremely long-lived but factors responsible for their long term survival are largely unknown.

The data provided here shows that Survivin is a critical survival molecule in neoplastic mast cells. The downregulation of Survivin in HMC-1 cells leads to decreased survival and increased sensitivity towards tyrosine kinase inhibitors or TRAIL-induced apoptosis. Survivin is a chromosomal passenger protein (Adams, Carmena et al. 2001) and the decreased proliferation of HMC-1 cells after Survivin-specific siRNA plasmid transfection is most likely due to inhibition of the cell cycle as own preliminary data and others (Lens, Wolthuis et al. 2003) have shown. Survivin has a complex role in the inhibition of apoptosis connecting to multiple parallel pathways that regulate gene expression, protein-protein interactions and mitochondrial functions. Survivin exhibits parallel interactions with other members of the IAP gene family. It binds to XIAP resulting in increased XIAP stability against proteasomal degradation and inhibition of apoptosis by synergistical inhibition of caspase 3 and 9 (Dohi, Beltrami et al. 2004) and a Survivin-cIAP1 complex might antagonize the function of survivin in late-stage cell division (Samuel, Okada et al. 2005). In mitochondria Survivin binds Smac and so preventing it from binding to XIAP which is subsequently hindered to inhibit caspases (Sun, Nettesheim et al. 2005). Taken together these effects of Survivin seem to be sufficient to explain the combined effect of Survivin downregulation with enhanced intrinsic apoptosis induced by tyrosine kinase inhibitors and the increased sensitivity to extrinsic apoptosis initiated by TRAIL.

3.1.6 LCL161 does sensitize neoplastic human mast cells to TRAIL and PKC412

In a second approach the small molecule Smac mimetic LCL161 was used to target multiple IAPs, namely XIAP, cIAP1 and cIAP2, while initiating apoptosis by the tyrosine kinase inhibitor PKC412 and the death receptor ligand TRAIL. LCL161 (Weisberg, Ray et al. 2010) is a structural analog of the previously developed Smac mimetic LBW242 (Weisberg, Kung et al. 2007) a 3mer based on the NH_2-terminal seven amino acids of Smac designed to neutralize the BIR3 domain of XIAP and to enhance the activity of different pro-apoptotic pathways (Galban, Hwang et al. 2009).

The combined treatment of Smac mimetics resembling the *N*-terminal residues of Smac together with TRAIL has been shown to potentiate the apoptotic effect on JURKAT cells compared to treatment with TRAIL alone (Guo, Nimmanapalli et al. 2002). This increase in apoptosis was accompanied by downregulation of XIAP, cIAP1, cIAP2 and Survivin and commensurate activation of caspase 3 and 8. Enhanced TRAIL induced apoptosis after small molecule Smac mimetic treatment has been shown for example in glioblastoma cells (Li, Thomas et al. 2004), breast cancer cells (Bockbrader, Tan et al. 2005) and ovarian

cancer cells (Petrucci, Pasquini et al. 2007). LBW242 acted in positive synergy with TRAIL to induce apoptosis in multiple myeloma cells (Weisberg, Kung et al. 2007) and LCL161 synergistically inhibited cellular proliferation of murine leukemic pro-B cells *in vitro* and *in vivo* with PKC412 (Weisberg, Ray et al. 2010).

Incubation with LCL161 alone resulted in reduced proliferation demonstrated by cell cycle analysis showing accumulation of HMC-1 cells in the G_1 phase as wells as increased apoptosis of the cells as shown by Annexin-V staining. Co-incubation with PKC412 and TRAIL increased the rate of apoptosis in a synergistical manner. This result could be an indication that the combined treatment of neoplastic mast cell diseases with Smac mimetics showing a low toxicity profile in normal hematopoietic progenitors (Servida, Lecis et al. 2010) and tyrosine kinase inhibitors or TRAIL could be a future treatment option.

3.1.7 Conclusion and Outlook

Interference with components of the cellular apoptosis pathways could be a beneficial way of enhancing therapy of mastocytosis. Mast cells generation time and survival rate within tissues is mainly controlled by the presence of stem cell factor. In absence of SCF mast cells undergo apoptosis (Iemura, Tsai et al. 1994). Current therapy of mastocytosis uses tyrosine kinase inhibitors to interfere with the survival signaling of the KIT receptor. Human mast cells express functional TRAIL receptors and their neoplastic transformation in mastocytosis renders them even more vulnerable towards TRAIL induced apoptosis (Berent-Maoz, Piliponsky et al. 2006). Downregulation or inhibition of anti-apoptotic molecules in neoplastic mast cells is a favorable way of enhancing induction of apoptosis either by intrinsic apoptotic stimuli like inhibition of KIT signaling by TK inhibitors or by targeting the extrinsic apoptotic pathway with TRAIL. The IAP Survivin is highly expressed in neoplastic mast cells compared to normal mast cells thus making it an excellent diagnostic marker in mastocytosis. Moreover its downregulation resulted in inhibition of mast cell proliferation and acted synergistically with TK inhibitors and TRAIL in reducing their viability. The promising effect of Smac mimetics as new cancer drugs was also seen in neoplastic mast cells. LCL161 inhibited growth of HMC-1 cells and amplified the reduction of viability and the apoptosis induced by PKC412 and TRAIL showing that targeting XIAP, cIAP1 and cIAP2 in mastocytosis is a beneficial option. Recently the link between both approaches becomes more obvious. Not only has been shown that Survivin and Smac/DIABLO form a complex promoting apoptosis (Song, Yao et al. 2003) but also Survivin and XIAP form a complex in which XIAP is stabilized by Survivin, protected from proteasome-mediated degradation and synergistically induces apoptosis (Dohi, Okada et al. 2004; Hu, Liu et al. 2010) and also participates in tumor cell invasion and metastasis (Mehrotra, Languino et al. 2010). These finding will direct

attention to investigation of combined inhibitors for Survivin and Smac/DIABLO (Oikawa, Unno et al. 2010) as well as Survivin and XIAP (Obiol-Pardo, Granadino-Roldan et al. 2008) allowing more efficient treatment of neoplastic malignancies like mastocytosis where combined approaches with induced apoptosis and simultaneous reduction of apoptosis inhibition can open new therapeutic perspectives.

4 Materials and Methods

4.1 Chemicals and enzymes

Unless otherwise specified, all standard chemicals were purchased from Roth (Karlsruhe), Merck Biosciences (Darmstadt), Serva (Heidelberg) or Sigma-Aldrich (München).

4.2 Mice

4.2.1 Mouse strains

All mice were on the C57BL/6 background and crossed exclusively to C57BL/6 mice. Following mouse lines were used in this thesis: Mice conditionally deficient for the *TRAIL-r* gene *loxP* sites flanking exon 2 so that upon expression of the Cre recombinase, a frame shift and an early stop codon is introduced resulting in a transcript devoid of TRAIL-binding cysteine-rich domains and an apoptosis-inducing death domain (Grosse-Wilde, Voloshanenko et al. 2008). Cre deleter mice, a cre-transgenic mouse strain for the ubiquitous deletion of *loxP*-flanked gene segments including deletion in germ cells (Schwenk, Baron et al. 1995). Mice were kept under specific pathogen free (SPF) conditions and all experiments were done according to institutional guidelines.

4.3 Cells and cell culture

4.3.1 Cell culture media and reagents

Penicillin/Streptomycin: 100 U/ml penicillin, 0.1 mg/ml streptomycin (final concentrations, Biochrom AG, Berlin)

L-glutamine: 2 mM final concentration (Biochrom AG, Berlin)

DMEM (Invitrogen, Darmstadt): supplemented with 10% FCS (Biochrom AG, Berlin), 50 µM β-mercaptoethanol (Sigma-Aldrich, München), L-glutamine, penicillin/streptomycin.

IMDM (Invitrogen, Darmstadt): supplemented with 10% FCS (Biochrom AG, Berlin), 1.2 mM α-thioglycerol (Sigma-Aldrich, München), L-glutamine, penicillin/streptomycin.

1 x PBS: 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.6 mM KCl, 136 mM NaCl, pH 7.4

RPMI-1640 (Invitrogen, Darmstadt): supplemented with 10% FCS (Biochrom AG, Berlin), L-glutamine, penicillin/streptomycin.

StemPro-34 (Invitrogen, Darmstadt)

WEHI-3B conditioned medium: Supernatant of IL-3 producing WEHI-3B cells purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig). The medium was prepared by culturing $1x10^5$ WEHI-3B cells in complete IMDM containing 10% FBS, 25 µM β-mercaptoethanol (Sigma-Aldrich, München), L-glutamine and penicillin/streptomycin for 3-4 days until cell density reached about $1x10^6$ cells/ml. Cells were carefully mobilized from the culture dish bottom using a cell scraper, centrifuged and the supernatant was collected. 50 ml aliquots were stored at -20 °C.

4.3.2 HMC-1

The HMC-1 cell lines have been established in 1988 by Butterfield et al. from peripheral blood from patients with mast cell leukemia (Butterfield, Weiler et al. 1988). The ultra structure of the cultivated cells shows characteristics of immature mast cells (Nilsson, Blom et al. 1994). HMC-1 cells can be stained by toluidine-blue. Like in mature mast cells histamine, tryptase, heparin and chondroitin sulfate can be detected within the cells. In contrast to primary human mast cells, no surface expression of the Fcε-receptor is detectable. HMC-1.1 cells carry one mutation at codon 560 in the juxtamembrane region of the *c-KIT* gene causing a substitution of Gly-560 for Val. In contrast only HMC-1.2 cells carry the mutation at codon 560 and a mutation at codon 816 causing an Asp→Val substitution in the intracellular kinase domain. Both mutations lead to KIT phosphorylation on tyrosine residues and are associated with phosphatidylinositol 3'-kinase (PI 3-kinase) activation but no constitutive activation of Akt or extracellular regulated protein kinase (ERK), which are signaling molecules normally activated by the interaction of stem cell factor (SCF) with KIT (Furitsu, Tsujimura et al. 1993; Kanakura, Furitsu et al. 1994; Weber, Babina et al. 1996; Sundstrom, Vliagoftis et al. 2003). HMC-1 cell can spontaneously release IL-1α, IL-1β, IL-6, IL-8 and TNF-α, while secretion of IL-2, IL-3, IL-4 and IL-7 has not been shown (Grabbe, 1994). HMC-1 cells were cultivated in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, 50 µg/ml streptomycin, 1.2 mM α-thioglycerol in a humidified incubator (at 37°C, 5% CO_2) and were passaged every 3-4 days.

4.3.3 KU812

The cell line KU812 has been isolated in 1985 from a patient with chronic myeloid leukemia during a blast crisis. It is Philadelphia chromosome positive and shares characteristics with basophiles (i.e. expression of the IL-3 receptor) and mast cells (i.e. tryptase expression) (Kishi 1985). In contrast to HMC-1 cells KU812 do not carry KIT mutations and they express the FcεR (Hamann, Grabbe et al. 1994). Cells were cultivated in RPMI-1640 medium supplemented with 10% calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin under the same conditions as the HMC-1 cells.

4.3.4 C57.1

The C57.1 murine mast cell line has been isolated in 1987 from bone marrow of a C57BL/6J mouse. The cells grow IL-3 independent, contain histamine and express the FcεR (Young, Liu et al. 1987). C57.1 cells were cultivated in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, 50 µg/ml streptomycin, 1.2 mM α-thioglycerol under the same conditions as the HMC-1 cells.

4.3.5 Cord Blood Derived Mast Cells (CBMC)

The cultivation of immature mast cells from human cord blood is an established model for the generation of primary human mast cells (Dvorak, Furitsu et al. 1992; Mitsui, Furitsu et al. 1993). These cells were generated from umbilical cord blood mononuclear cells cultured in medium containing SCF. After eight weeks of culture of mononuclear cells from one umbilical cord, cultures of more than 90% mast cells can be generated, determined by flow cytometric detection of FcεR and KIT. The KIT expressing cells stain tryptase and partially chymase positive. As studies have shown CBMC do not carry mast cell typical crystal-granules and their histamine release pattern differs from primary human mast cells (Amano, Kurosawa et al. 2000; Matsushima, Ishikawa et al. 2000). Heparinized cord blood was mixed 1:1 with Hank's Balanced Salt Solution (HBBS). 20 ml were loaded on 12 ml Ficoll in a falcon tube and centrifuged for 20 min at 2000 rpm. Mononuclear cells were extracted, washed twice with HBBS and cultured in RPMI 1640 medium containing 20% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin and 100 ng/ml SCF. Cells were incubated in a humidified incubator (at 37°C, 5% CO_2) for 8 to 16 weeks with a weekly change of medium and maturation was determined by flow cytometric analysis of KIT (CD117) and tryptase.

4.3.6 Bone Marrow- Derived Mast Cells (BMMC)

Immature murine mast cells can be grown from murine bone marrow cultured in IL-3 containing medium (Yung, Eger et al. 1981). Bone marrow from femurs of 4-8 week-old mice was placed in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin and 20% conditioned medium from WEHI-3 cells containing IL-3. BMMC were enriched by discarding adherent cells. The isolated BMMC were maintained in culture at 0.5-1.0 x 10^6 cells/ml for 4-6 weeks. Before use, BMMC purity was routinely monitored by flow cytometry to detect KIT and FcεRI.

4.3.7 Skin Mast Cells

Skin mast cells were isolated from human foreskin and purified by sorting using the monoclonal anti-c-KIT antibody YB5B8 and a secondary magnetic microbead-coupled antibody as previously detailed described (Grutzkau, Henz et al. 2000).

4.3.8 JURKAT cells

The JURKAT human T cell line was established from a patient with acute lymphocytic T cell leukemia (Schneider, Schwenk et al. 1977). The cell line was grown in suspension in in RPMI 1640 medium supplemented with 2 x 10^{-3} M L-glutamine, penicillin (10 U/ml), streptomycin (0.1 mg/ml) and 10% heat-inactivated fetal calf serum (FCS). The cells were maintained in a 37°C, 5% CO_2 incubator in sealed flasks and were subcultured every 2 days.

4.3.9 LAD-2 cells

The LAD-2 cell represents a recently established MC sarcoma cell line (Kirshenbaum, Akin et al. 2003). The cells closely resemble primary cultures of CD34+-derived human mast cells, respond to rhSCF and have functional FcεRI and FcγRI receptors. LAD-2 cells survive in culture without SCF, and double in numbers in approximately 3 weeks in the presence of 100 ng/ml SCF. The slower doubling time reflecting cell proliferation and death, in contrast to 3-5 days for some tumorigenic cell cultures, may allow LAD-2 cells to exhibit a more mature phenotype. Cells were routinely cultured in StemPro-34 in the presence of 100 ng/ml rhSCF.

4.4 Stimulation of cells

4.4.1 Materials

Enhance for Ligands (Axxora, San Diego, CA): Stock solution: 1 mg/ml

LCL161 (Novartis, Basel): Stock solution 10 nM

Nilotinib (AMN107) (Novartis, Basel): Stock solution 10 nM

PKC412 (Midostaurin) (Novartis, Basel): Stock solution 10 nM

Flag-tagged recombinant human TRAIL (Axxora, San Diego, CA): Stock solution: 100 µg/ml

4.4.2 Methods

For siRNA transfection, 2 x 10^6 HMC-1 cells in exponential growths were suspended in 100 µl Nucleofector solution V and transfected with 3 µg of pSuper-Luc or 3 µg of pSuper-Survivin using a Nucleofector I, program T-030 (Amaxa Biosystems), following the manufacturer's instructions. After transfection cells were resuspended in medium containing tyrosine kinase inhibitors Nilotinib (10 µM) or PKC412 (500 nM), recombinant human TRAIL (250 ng/ml) and Enhancer for Ligands (2 µg/ml) or medium alone. Cells were incubated for 24 to 72 hours before measurement of apoptosis.

For LCL161 studies 5 x 10^5 HMC-1 cells were suspended in 1 ml medium containing indicated concentrations of LCL161, tyrosine kinase inhibitors, recombinant human TRAIL and 2 µg/ml Enhancer for Ligands for up to 72 h before further analysis.

4.5 Analysis of cells

4.5.1 Materials

Annexin-V - APC (eBiosciences, San Diego, CA): 20 x Stock solution

10 x Annexin-V Binding Buffer (eBiosciences, San Diego, CA): diluted 1 + 9 with ddH_2O

FACS buffer: 1 x PBS, 0,5% (w/v) BSA (Serva, Heidelberg), 2mM EDTA in ddH_2O

Lysis buffer for Western blot: 50 mM Tris-Cl pH 7.4, 1% NP-40 (Pierce, Rockford, USA), sodium deoxycholate 0.25%, 150 mM NaCl, 1 x Complete protease inhibitor cocktail (Roche; Mannheim), 1 x PhosSTOP phosphatase inhibitor cocktail (Roche; Mannheim)

Human Fc Receptor Binding Inhibitor (eBiosciences, San Diego, CA): 5 x stock solution

PBS 1x: 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.6 mM NaCl (all Sigma-Aldrich, München), pH 7.4

Propidium iodide (Sigma-Aldrich, München): 1 mg/ml Stock solution in ddH2O, working dilution 1:100, final concentration 0.5 µg/ml

RNase A (Qiagen, Hilden): 100 mg/ml Stock solution

RNA-Safe-Buffer: 0.05% Tween 20 (Roche, Mannheim), 0.05% sodium azide (Sigma-Aldrich, München)

Sample buffer for Western blot: 250 mM Tris-Cl pH 6.8, 20% glycerol, 2% (w/v) Sodium Dodecyl Sulfate, 5% β-mercaptoethanol, 0.5‰ bromphenol blue,

TAE 1 x: 40 nM Tris-Cl, 20 nM NaAc, 1 mM EDTA (all Sigma-Aldrich, München)

TBS-T: 25 nM Tris-Cl, 150 nM NaCl, 0.1% (v/v) Tween 20 (Serva, Heidelberg), pH 7.6

4.5.2 Reverse Transcription – PCR

RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden) and was stored at -80 °C in RNA-Safe-Buffer and concentration was determined by measuring absorbance in a spectrophotometer at 260 nm. Polyadenylated RNA was transcribed with the Superscript III First Strand Synthesis Kit (Invitrogen, Darmstadt) using oligo-dT primers following manufacturer's instructions. Synthesized cDNA was used as PCR template after RNAse H (Roche, Mannheim) digestion.

100 ng DNA was added to 50 µl 1 x reaction buffer (Fermentas, St. Leon-Roth) with 200 nM of each primer (Metabion, Martinsried), 200 µM dNTP Mix, 1.5 mM $MgCl_2$ and 0,025 U/µl Taq DNA polymerase (all Fermentas, St. Leon-Roth). In a first step the reaction mixture is heatet at 95 °C for 2 min for denaturation of the template followed by 30-40 repeats of following steps:

1. Denaturation: 95 °C for 30 s
2. Annealing of primers at specific temperature t_A for 45 s
3. Elongation of products at 72 °C for 1 min/kb.

A final elongation step of 72 °C for 7 min is performed for each protocol.

The PCR products were separated by electrophoresis on a 1-2% (m/v) gel composed of agarose (BioBudget, Krefeld) in 1 x TAE buffer, stained with 30 ng/ml ethidium bromide (Merck, Darmstadt) and visualized under UV light. Predicted product size was checked with the GeneRuler™ DNA Ladder Mix (Fermentas, St. Leon-Roth).

4.5.2.1 Isolation of genomic tail DNA

Mouse tail tips, about 0.5 cm in length, were incubated overnight at 55 °C rocking in 500 µl lysis buffer (200 nM TrisCl, 100mM NaCl, 1% SDS (w/v), 50 mM EDTA) supplemented with 100 µg Proteinase K (Fermentas Life Sciences). Undigested material was sedimented by centrifugation and the supernatant was poured into a fresh 1.5 ml microcentrifuge tube containing 500 µl isopropanol. The samples were mixed by inverting the tubes several times. The precipitated DNA was pelleted by centrifugation by centrifugation and washed once with 70% ethanol. The air dried pellet was dissolved in TE buffer by incubating at 55 °C shaking for at least 2 h.

4.5.2.2 Genotyping protocols

Mice were genotyped by using the standard PCR program (see section 4.5.2) with varying annealing temperatures (T_A) and cycle numbers. Primers for genotyping are listed in table 4.1.

4.5.3 Immunohistochemistry

For analysis of mastocytosis lesions in bone marrow specimens were fixed in formalin over night and embedded in paraffin. 6 µm sections were first stained with anti-tryptase antibody (DAKO, Glostrup, Denmark) and costaining of mast cell infiltrates with an antibody against survivin (R&D Systems, Wiesbaden) was then assessed in serial sections. Before exposure to antibodies, antigen unmasking was performed by incubation the bone marrow sections in 10 mM Na-citrate buffer pH 6 at 95 °C. Detection followed by secondary antibody staining and use of the Vectastain Elite ABC Kit.

4.5.4 Western blot analysis

2 x 10^5 cells per sample were harvested and lysed in 20 µl lysis buffer on ice for 30 minutes. 20 µl of sample buffer was added and sample was denaturized at 95 °C for 5 minutes. Samples were separated on a 10 – 15% SDS-gel and electroblotted on a nitrocellulose membrane (Amersham, GE Healthcare, München).

Membranes were blocked one hour with TBS-T containing 10% milk powder and 0.1 % BSA. Primary antibodies were diluted in either TBS-T + 5% (m/v) milk powder or TBS-T + 5% (m/v) BSA and incubated either 1h at room temperature or over night at 4 °C according to the manufacturer's instructions. Secondary antibodies swine-anti-rabbit-peroxidase, rabbit-anti-mouse-peroxidase and rabbit-anti-goat-peroxidase (all DAKO, Glostrup, Denmark) were diluted in TBS-T + 5% (m/v) milk powder according to manufacturer's instructions and incubated at room temperature for one hour. The blocking step and each antibody incubation were followed by 30 minutes of washing with TBS-T at room temperature.Signals were detected with ECL reagent on Hyperfilm ECL both GE Amersham.

4.5.5 Flow cytometry

For antigen staining 1 x 10^5 cells were harvested and washed twice with FACS buffer. Cells were resuspended in human Fc Receptor Binding Inhibitor and were stored on ice for 20 minutes. Hereafter cells again were washed twice with cold FACS buffer and incubated with an appropriate antibody dilution (see table 4.3) in FACS buffer and incubated on ice for 30 minutes in the dark. After washing the cells again twice with FACS buffer they were stored on ice resuspended in 200 µl FACS buffer until flow cytometric analysis.

For apoptosis detection 1 x 10^5 cells were harvested and washed once with PBS and once with Annexin-V Binding Buffer. Cells were resuspended in 100 µl Binding Buffer and 5 µl Annexin-V-APC was added. Cells were incubated for 15 minutes at room temperature in the dark. After cells were washed again in Binding Buffer they where resuspended in 200 µl

Binding Buffer and propidium iodide (final concentration 0.5 µg/ml) was added 5 minutes before cell analysis.

For cell cycle analysis 1 x 10^6 cells were fixed with 70% ethanol (-20 °C) and resuspended in PBS. RNA was digested with RNase A (50 µg/ml) at 37 °C for 30 minutes in the dark. Cells where stained with propidium iodide (final concentration = 50 µg/ml) five minutes before flow cytometric analysis.

Cells were analyzed with a FACS Calibur (BectonDickinson, Heidelberg) with a 488 and 633 nm laser. Data was acquired and analyzed with CellQuest Pro Software Version 5.2.1.

4.5.6 Cell proliferation

Cell proliferation was measured with the CellTiter 96® AQ_{ueous} One Proliferation Assay (Promega, Mannheim).

Briefly, quadruplicates of 2 x 10^4 cells per condition were seeded in 100 µl of culture medium in wells of a 96 well plate. 20 µl of CellTiter 96® AQ_{ueous} Solution Reagent was added to each well and the plate was incubated at 37 °C in a humidified incubator with 5% CO_2 for 1 h. Absorbance at 490 nm was measured in a 96 well plate reader.

4.6 Oligonucleotides

All oligonucleotides were produced by Metabion (Martinsried) and purified by HPLC.

Name	**Sequence (5'-3')**	**PCR product**	**T_A and cycle number**
Deleter Fwd	GAAAGTCGAGTAGGCGTGTACG	600 bp	58 °C, 35 cycles
Deleter Rev	CGCATAACCAGTGAAACAGCAT		
GAPDH fw	ATGGGGAAGGTGAAGGTCG	226 bp	62 °C, 30 cycles
GAPDH rev	GGGGTCATTGATGGCAACAATA		
mTRAIL-R fw	CGGGCAGATCACTACACCC	147 bp	63°C, 30 cycles

Name	Sequence (5'-3')	PCR product	T_A and cycle number
mTRAIL-R rev	TGTTACTGGAACAAAGACAGCC		
mTRAIL fw	ATGGTGATTTGCATAGTGCTCC	193 bp	62°C, 30 cycles
mTRAIL rev	GCAAGCAGGGTCTGTTCAAGA		
Survivin fw	GTTTCCAGCGAAGCTGTAACA	473 bp	65 °C, 40 cycles
Survivin rev	GGTCCCCGCTTTCTTTGGAG		
TRAIL-R1 fw	CCGCGGCCACACCCAGAAAGT	415 bp	61 °C, 30 cycles
TRAIL-R1 rev	GTACATGGGAGGCAAGCAAACAAA		
TRAIL-R2 fw	GCGCCCACAAAATACACCGACGAT	437 bp	61 °C, 30 cycles
TRAIL-R2 rev	GCAGCGCAAGCAGAAAAGGAG		
TRAIL-R flox:			
P813	GAGCCATCTTTTTAAGTCTTGACT	300 bp = wt	59 °C, 35 cycles
P814	GACGATTATGGGCTGGGTTAGCTG	470 bp = flox	
TRAIL-R knockout			
P813	GAGCCATCTTTTTAAGTCTTGACT	235 bp = knockout	59 °C, 35 cycles
P815	CGAACACAGCTGGTTTCCATGG	800 bp = wt	

Table 4.1 Primers used for PCR analysis:
fw = forward primer (3'-5'), rev = reverse primer (3'-5')

4.6.1 Plasmid based siRNA

pSuper-Luc: pSuper-Luc coding for siRNA complementary to a target sequence in the luciferase gene of the american firefly (*Photinus phyralis*) here used as a control siRNA (Butz, Ristriani et al. 2003).

pSuper-Survivin: pSuper-Survivin coding for siRNA complementary to a target sequence in the human survivin gene (Lens, Wolthuis et al. 2003).

4.7 Antibodies for Immunohistochemistry

Antigen	Clone	Dilution	Manufacturer
Survivin	Rabbit, polyclonal IgG	1:40	R&D Systems
Tryptase	AA1	1:50	Dako

Table 4.4 Antibodies for Immunohistochemistry:
Dako, Glostrup, Denmark; R&D Systems, Wiesbaden.

4.8 Antibodies for Western blot analysis

Antigen	Host/Isotype	Dilution	Manufacturer
β-Actin	Mouse polyclonal	1:5000	Sigma-Aldrich
c-IAP1	Rabbit polyclonal	1:1000	Cell Signaling
c-IAP2	Rabbit IgG	1:1000	Cell Signaling
cFLIP	Rabbit IgG	1:500	Abnova
Mcl-1	Rabbit IgG	1:500	BioLegend
Noxa	Mouse IgG1	1:1000	Abcam
Phospho-Akt (Ser 473)	Rabbit polyclonal IgG	1:1000	Cell Signaling
Phospho-ERK1/2 (Thr202/Tyr204)	Mouse IgG1	1:1000	Cell Signaling
Phospho-Stat5 (Tyr694)	Rabbit IgG	1:1000	Cell Signaling
Smac/Diablo	Mouse IgG1	1:1000	Cell Signaling
Survivin	Rabbit, polyclonal IgG	1:400	R&D Systems
TRAIL	Rabbit IgG	1:200	Novus Biologicals
XIAP	Rabbit IgG	1:1000	Cell Signaling

Table 4.2 Antibodies for Western blot analysis:
Abnova, Heidelberg; Abcam, Cambridge; Cell Signaling, Danvers, MA; R&D Systems, Wiesbaden; Sigma-Aldrich, München.

4.9 Antibodies for flow cytometric analysis

Antigen	Host/Isotype	Clone	Dilution	Dye	Manufacturer
CD117	Rat IgG2b,κ		1:500	APC	eBiosciences
FcεRIα	Hamster IgG		1:300	Phycoerythrin	eBiosciences
Isotype	Mouse IgG1,κ		1:300	Phycoerythrin	eBiosciences
Isotype	Rat IgG2a,κ		1:300	Phycoerythrin	eBiosciences
mTRAIL-R	Rat IgG2a,κ	N2B2	1:300	Phycoerythrin	eBiosciences
TRAIL-R1	Mouse IgG1,κ	DJR1	1:300	Phycoerythrin	eBiosciences
TRAIL-R2	Mouse IgG1,κ	DJR2-4	1:300	Phycoerythrin	eBiosciences

Table 4.3 Antibodies for flow cytometric analysis:
eBiosciences, San Diego, CA.

4.10 Statistical analysis

To determine the level of significance in differences found in data evaluation, the paired Student t test was applied. Results were considered significantly different at $P < .05$. To determine synergistic effects of TRAIL-FLAG and drugs, combination index values were calculated based on the method of Chou et al. 1984 (Chou and Talalay 1984) and were calculated as a combination index (CI) with $CI < 1$, $CI = 1$ or $CI > 1$ representing synergism, additivity or antagonism, respectively.

5 Abbreviations

BMMC	bone marrow-derived mast cells
bp	base pair
BSA	bovine serum albumin
CM	Cutaneous mastocytosis
ddH_2O	bidestilled water
DMEM	Dulbecco's modified Eagle medium
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleoside triphosphate
E.coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetate
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescent activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
FcεRI	high-affinity IgE receptor
g	gravitational acceleration, 9.81 ms^{-1}
h	hours
HPLC	High-performance liquid chromatography
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's Modified Dulbecco's Media
ISM	indolent systemic mastocytosis
kb	kilobases
l	liters

MACS	magnetic activated cell sorting
MCL	mast cell leukemia
mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PI	propidium iodide
rpm	revolutions per minute
RT	reverse transcription
SCF	stem cell factor
SDS	sodium dodecylsulfate
SM	systemic mastocytosis
SM-AHMND	systemic mastocytosis with additional hematopoietic non-mast cell disease
T_A	annealing temperature
TAE	Tris acetate EDTA buffer
TE	Tris-EDTA
TNFα	tumor necrosis factor α
TRAIL	TNF-related apoptosis-inducing ligand
Tris	Tris-(hydroxymethyl-)aminomethan
U	unit
v/v	volume per volume
w/v	weight per volume
wt	wild type
WHO	World Health Organization

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8 Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen –, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Manolis Pasparkis betreut worden.

Köln, den 26. April 2010

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Stefan Grotha