Engineering of a bifunctional anti-Kv10.1 antibody for cancer therapy

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Here I declare that my doctoral thesis entitled **"Engineering of a bifunctional anti-Kv10.1 antibody for cancer therapy"** has been written independently with no other sources and aids than quoted.

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1 Abstract

Antibody-based cancer therapy uses the high specificity of antibodies to selectively destroy cancer cells. Therefore, a chief factor for an efficient antibody-based cancer therapy is the use of targeted antigens. The potassium channel Kv10.1 (ether-á-go-go) has been selected as a target because this surface protein is not detected in normal tissues outside of the central nervous system, but more than 70 % of tumors from different origins have been tested positive for Kv10.1 expression. I designed a fusion construct containing an single-chain antibody against an extracellular region of K_V10.1 (scFv62) and the human soluble TRAIL. The K_V10.1-specific scFv62 antibody-TRAIL fusion construct was expressed in CHO-K1 cells and tested for biological activity. I analyzed the apoptosis-inducing potential of scFv62-TRAIL on different Kv10.1-positive and Kv10.1-negative cancer cell lines. Because of the reported resistance of different cancer cells against TRAIL-induced apoptosis the cells were sensitized with different agents. In combination with CHX scFv62-TRAIL induced apoptosis only in K_V10.1-positive cancer cells, but not in non-tumor cells, regardless of Kv10.1 expression. The strongest apoptosis-induction was observed in the Kv10.1-positive prostate cancer cell line DU145, whereas the two Kv10.1-negative prostate cancer cell lines PC3 and LNCaP were not affected. scFv62-TRAIL induces apoptosis in an autocrine manner, but can also activate apoptosis in neighboring cells (paracrine). This so-called bystander effect was studied in mixed cultures of Kv10.1-positive prostate cancer cells, normal prostate cells and Kv10.1-negative prostate cancer cells. An effective apoptosis-induction was detected in K_V10.1-negative cancer cells, while normal prostate epithelia cells were not affected when present as bystander. In summary, scFv62-TRAIL selectively induces apoptosis in tumor cells, if K_V10.1-expressing cells are present, and represents a promising agent for combinational *in vivo* applications.

2 Introduction

2.1 Immunotherapy - basic principles and therapeutic strategies

Immunotherapy is by definition the “treatment designed to produce immunity to a disease or enhance the resistance of the immune system to an active disease process, as cancer” [1]. Antibodies and related products are the fastest growing class of therapeutic agents and the discovery of tumor-associated antigens (TAA) was a significant step in the development of antibody-based immunotherapies. The period between the discovery of the targeted antigen and the design of an antibody can take many years. For clinical applications the extensive characterization of therapeutic antibodies for specificity, binding affinity, effector function and toxicity is crucial. This section describes basic functions and structures of antibodies and antibody fragments and presents different antibody-based strategies for therapy.

2.1.1 Antibody structure, function and recombinant production

The immune system consists of a diverse selection of immunocompetent cells and inflammatory mediators that interact in complex networks. Antibodies, also known as immunoglobulines (Ig), represent an essential part of the immune response. They specifically identify and neutralize foreign objects, like viruses or bacteria.

All antibodies share a uniform structure and consist of two light and two heavy chains (Fig. 2.1). The light chains contain two domains, the variable (V_L) and constant (C_L) domain. The heavy chain composed of one N-terminal variable domain (V_H) and three C-terminal constant domains (C_{H1}, C_{H2}, C_{H3}). Based on their function antibodies can be subdivided into

two domains: the fragment of antigen-binding (Fab) and the constant region (Fc). The Fab contains the variable region, which consists of three hypervariable complementary-determining regions (CDRs) that form the antigen-binding site of the antibody and confer antigen specificity. The immune effector function of an antibody is mediated by the Fc region and is capable of initiating complement-dependent cytotoxicity (CDC) or antibody-dependent cell-mediated cytotoxicity (ADCC) [2].

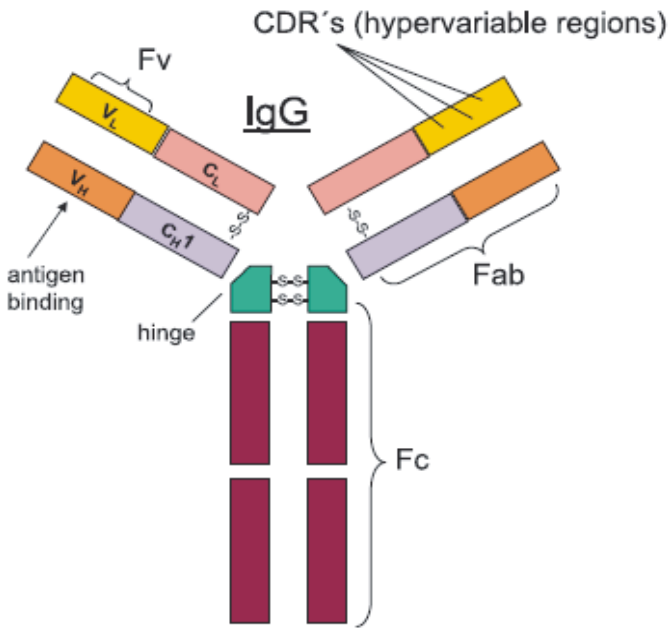

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Figure 2.1 Antibody structure

IgG, immunoglobulin G; Fab, fragment of antigen-binding; Fv, fragment variable; Fc, fragment constant; V_L/V_H, light and heavy variable region; C_L/C_H, light and heavy constant region; CDR´s, complementary-determining regions; -S-S-, disulfide bonds.

Antibodies are expressed and secreted by B cells, which are continuously produced in the bone marrow. Every B cell produces only one specific antibody, which is expressed on its surface. When one B cell gets in touch with the antigen, the cell becomes activated and starts

to produce and secret antibodies. These antibodies bind the antigen and designate it for elimination by the immune cells.

Antibodies for use in research are originally produced by immunization of mammals, e.g. mouse, rat or rabbit, with the specific antigen. The produced antibodies against the antigen can be purified from the serum. The obtained antibodies are polyclonal: a mixture of different antibodies from different B cells against the same antigen.

The development of hybridoma technology in 1975 revolutionized the antibody research field and clinical application of antibodies [4]. This technique allowed the efficient production of monoclonal antibodies by fusing one specific B cell with a myeloma cell. These fused cells are the so-called hybridoma cells. Later, the recombinant DNA technology enabled the production of different recombinant antibodies and antibody fragments (Fig. 2.2). The antigen binding ability of an antibody is contained in the V_L and V_H region. These domains can either be associated non-covalently (Fv fragment) by a disulfide bond (dsFv) or by a peptide linker (scFv, *single-chain fragment variable*).

The scFv fragment is the smallest and most popular recombinant antibody format. It has been shown that they have the same monomeric binding affinity as the parental monoclonal antibody. scFv antibodies offer several advantages compared to whole antibodies, like easier production or modification. Additionally, scFv antibodies with a molecular weight of 30kDa can penetrate solid tumors easier than large IgG molecules with 150kDa size [5]. The human anti-mouse immune response (HAMA) is another described limiting factor of whole monoclonal antibodies in therapeutic use [6]. Humanization of mouse immunoglobulins is one strategy to solve this problem. But also in scFv antibodies this high immunogenicity is reduced, because the Fc region and therefore a large part of the mouse antibody is missing.

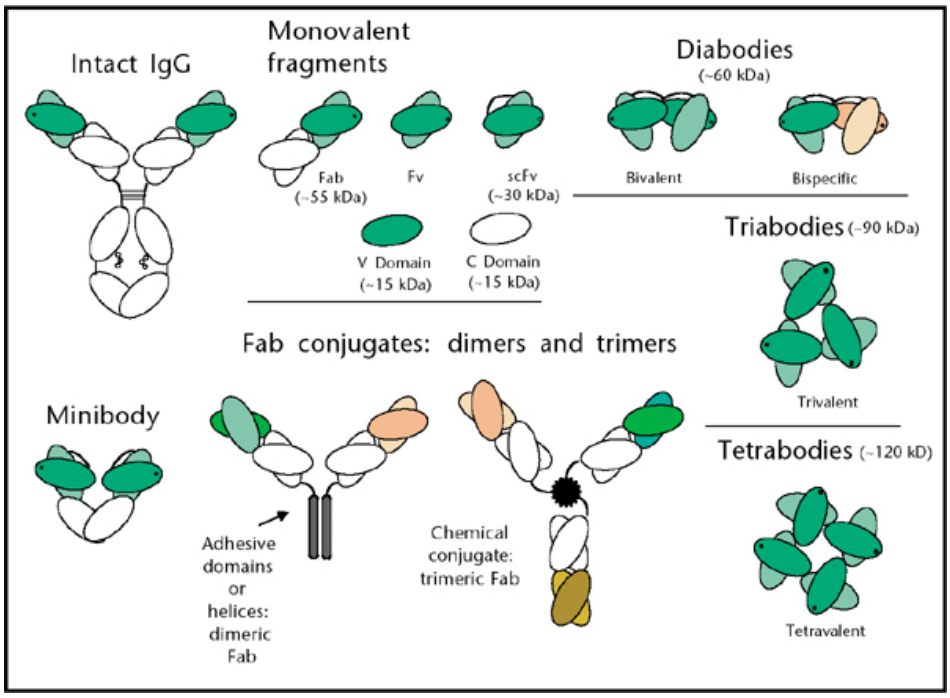

Figure 2.2 Recombinant antibodies and antibody fragments

scFv antibodies are not glycosylated and can be expressed recombinantly in various expression systems, including bacteria, yeasts, plants, insect and mammalian cell lines [7]. To generate a scFv fragment total RNA is isolated from the original hybridoma cells and translated into cDNA. Sequences encoding the heavy and light chain of the whole antibody will be amplified by choosing primers in the conserved region and subcloning the amplified PCR product with the peptide linker into the defined expression vector.

The scFv antibodies can be produced in bacteria, like *E.coli* at relative high expression levels. In addition, eukaryotic expression cell lines like chinese hamster ovary (CHO-K1) cells or human embryonic kidney cells (HEK-293) are also used for larger antibody constructs [8, 9].

2.1.2 Targeted therapy strategies

The use of antibodies for therapeutic applications is a rapidly progressing field. In the last 25 years a huge amount of antibodies and antibody derivatives have been analyzed for therapy. To date, 10 monoclonal antibodies have been approved by the Food and Drug Administration (FDA) for treatment of different cancer types. Rituximab was the first antibody approved for the treatment of Non-Hodgkin lymphoma and binds to the CD20 antigen, which is expressed on 95% of B cell lymphoma cells and normal B-lymphocytes [10]. This mouse-derived antibody underwent further genetic engineering and the constant region was replaced by the human constant region to generate a chimeric antibody. The cytotoxicity of this therapeutic antibody is mediated via the human Fc region which directly activates antibody-dependent cell-mediated cytotoxicity (ADCC) and an effector cell of the immune system lysates the tagged cell. This antibody and several other therapeutic antibodies like trastuzumab (Herceptin) or bevacizumab (Avastin) belong to the group of unconjugated or naked antibodies and act as enhancers of the immune response or inhibitor of signal pathways via blocking surface receptors [11, 12]. Beside the success of the first antibody-based therapy, naked antibodies show some limitations, like insufficient activation of effector function or targeting of normal cells.

Numerous strategies for improving the efficiency of antitumor antibodies have been suggested (summarized in Figure 2.3). The most widely explored strategy is the direct arming of antibodies with radionuclides or toxic payloads. A look backwards to the starting of cancer therapy, surgery and radiation were the only available treatments of cancer. However, in most cases this was not sufficient to control aggressive cancer. The fusion of radionuclides to antibodies allows a selective localization and more specificity. Radionuclides are cytotoxic over many cell diameters, which enable them to kill bystander cells. The strong bystander effect of this radioimmunoconjugates also results in targeting of non-antigen expressing

cancer and poorly perfused areas within the tumor mass, which is a benefit for eliminating solid tumors [13].

The design of immunotoxins is another therapeutic strategy of direct cytotoxicity. An immunotoxin consists of an antibody-based part, which is chemical or genetically linked to a toxin derived from plants or bacteria. The bacterial toxin *Pseudomonas* exotoxin (PE) and diphtheria toxin (DT) are single-chain proteins and preferentially used for design of immunotoxins. Some immunotoxins are already in clinical trials and represent promising candidates for further clinical evaluation [14].

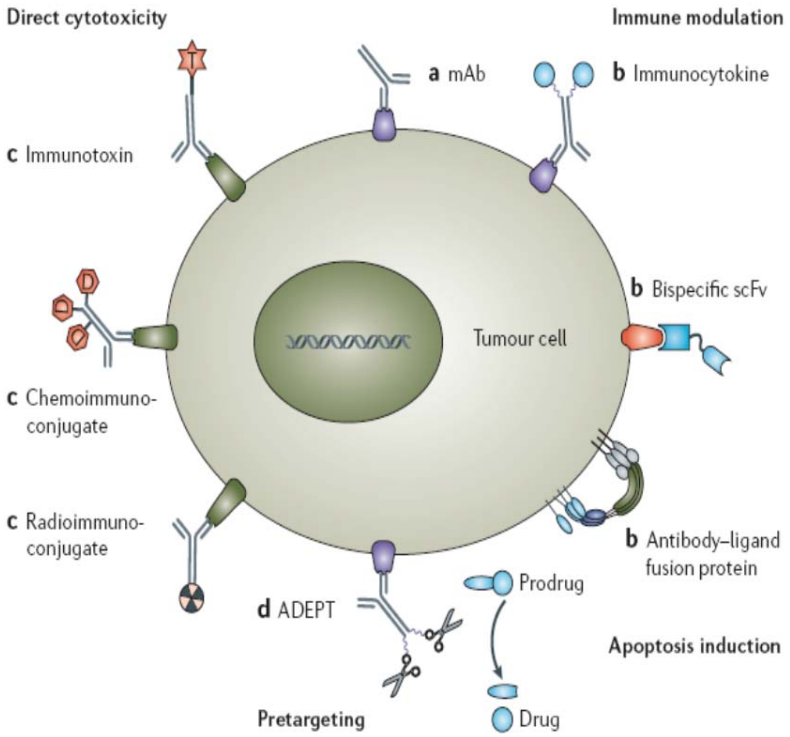

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Figure 2.3 Therapeutic strategies

a) Unconjugated antibodies can eliminate tumor cells by inducing antibody-dependent cellular cytotoxicity or activating the complement system. b) To modulate the immune response against the tumor antibodies can be conjugated with cytokines, another antibody or apoptosis-inducing ligands. c) A direct cytotoxicity can be maintained by conjugating antibodies to toxins (T), cytotoxic drugs (D) or radionuclides. d) In the antibody-directed enzyme prodrug therapy (ADEPT) concept the antibody is fused to an enzyme, which converts a prodrug into a highly toxic drug.

The knowledge about the development of tumors and structure of tumor tissues has tremendously increased over the last years. It has been emphasized that targeting of bystander cells strongly enhances immunotherapeutic treatment of cancer. The antibody-directed enzyme-mediated prodrug therapy (ADEPT) has been developed to address this prerequisite [16]. In ADEPT, the tumor specific antibody is fused to an enzyme. The antibody-enzyme construct is applied and after blood clearance the non-toxic prodrug is administered. The prodrug diffuses widely, but is only activated at the tumor site by the fused enzyme. Beside the amplification of the cytotoxic effect by the enzyme activity, also bystander cells are killed and the toxic agent is small enough to penetrate solid tumor tissue as well. ADEPT has proven highly effective in several different tumor xenograft studies, but the translation into clinic turned out to be difficult. The enzyme and the used prodrug/drug have to be selected carefully and the injected amounts and application times need to be accurately established.

The immune system is a very effective “search and destroy” system with an unlimited repertoire, extremely powerful effector functions and effector cells for attacking malignant cells. Beside the various immune modulating cytokines the immune system hold a complex apoptosis-inducing program. Antibody–cytokine fusion proteins enhance the direct antitumor effect of the antibody and concentrate the cytokine in the tumor microenvironment, without causing severe toxic side effects of systemic high-dose cytokine administration [17]. The tumor-necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) belongs to a special group of cytokines. TRAIL eliminates transformed cells by inducing apoptosis. TRAIL and its signaling pathway turned out to be a promising tool for targeted cancer therapy.

2.2 TRAIL apoptotic pathway

2.2.1 TRAIL signaling

TRAIL, also known as ApoL, was discovered as a powerful activator of programmed cell death and a high sequence homology with the extracellular domain of CD95 (Fas-L) and TNF was reported [18, 19]. As a member of the TNF superfamily, TRAIL is expressed on the membrane (memTRAIL) and is detected in most human tissues. In addition, a soluble form (sTRAIL) can be generated by alternative mRNA splicing or proteolytic cleavage of the extracellular domain [19]. TRAIL forms homo-trimers that bind receptors present on the cell surface. The TRAIL receptor system is very complex and consists of five different receptors, including the death receptors TRAIL-R1 (DR3) and TRAIL-R2 (DR4), and the decoy receptors TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) [20]. Only TRAIL-R1 and TRAIL-R2 transmit the apoptotic signal. These receptors belong to the so-called death receptors and contain the intracellular death domain (DD), which is important for mediating the apoptotic signal. The decoy receptors R3 and R4 lack a functional death domain and therefore they are not able to induce apoptosis. Beside these four membrane-bound receptors, TRAIL can also bind to the soluble receptor osteoprotegerin, but with low affinity [21]. This complex receptor system with 5 diverse receptors that differentially bind and interact with TRAIL, suggests a highly regulated TRAIL signaling.

Apoptosis can occur by the intrinsic and extrinsic apoptosis pathways. The intrinsic pathway is activated by cellular stress, like DNA damage, and can be induced by chemo- or radiotherapy. This pathway is also described as the mitochondrial pathway, because of the depolarization of the mitochondrial membrane that leads to release of a variety of pro-apoptotic factors into the cytosol. The release triggers the effector caspase activation and apoptotic cell death and can act as amplification loop of the extrinsic pathway.

The extrinsic pathway is also known as the death receptor pathway and the binding of a specific ligand to death receptor is the first step of this pathway. Binding of trimeric TRAIL to TRAIL-R1 or TRAIL-R2 leads to a conformation change, oligomerization and formation of the functional active receptor, which allows the binding of the Fas-associated protein with death domain (FADD) (Fig. 2.4). The resulting multi-protein complex is the Death-Inducing Signaling Complex (DISC) [22]. The death effector domain (DED) of FADD binds to the DED of pro-caspase-8/-10 resulting in its oligomerization and autoactivation [23]. The activated caspase-8/-10 activates and cleaves the effector caspase as well as numerous regulatory and structural proteins that execute the apoptotic program [24].

Caspase-8 can also cleave and activate the pro-apoptotic Bcl-2 protein (Bid), which initiates the intrinsic apoptotic pathway. Truncated Bid in turn activates Bax (Bcl-2 associated X protein) and Bak (Bcl-2 homologues antagonist killer) leading to their oligomerization and formation of pores in the outer mitochondrial membrane. Pro-apoptotic proteins like Cytochrome c and Smac/DIABLO (Second mitochondria-derived activator/direct inhibitor of apoptosis-binding protein with low pI) are released in the cytosol. Cytochrome c induces the assembly of the apoptosome, the activation-platform for caspase-9. Caspase-9 also feeds into the caspase cascade, providing a positive feedback loop to caspase-8/-10-induced apoptotic events.

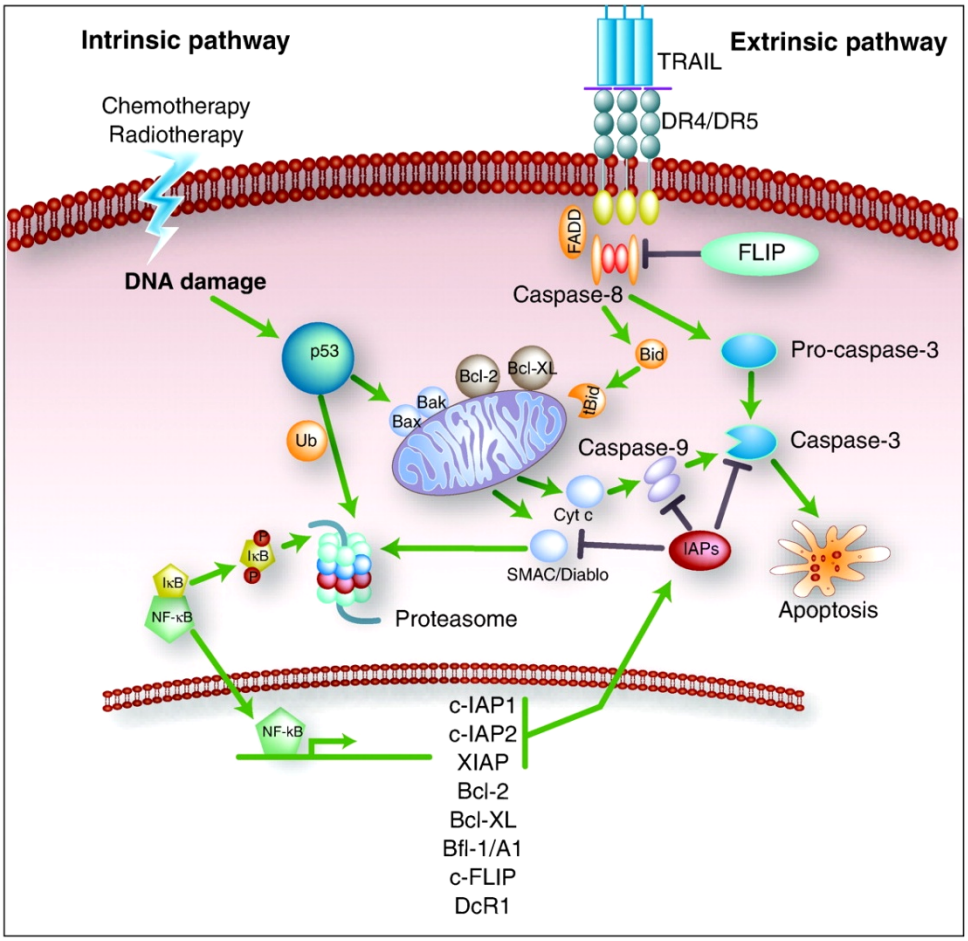

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Figure 2.4 The TRAIL signaling pathway

The apoptotic pathway is divided into the extrinsic and intrinsic pathway. Binding of TRAIL to one of the two death receptors (TRAIL-R1/DR4 or TRAIL-R2/DR5) activates a signaling cascade of the extrinsic pathway which results in apoptotic cell death. Apoptosis induction via death receptors can also activate the intrinsic pathway. Hence, TRAIL can activate both apoptotic pathways, whereas chemo- and radiotherapy induce apoptosis only via intrinsic pathway.

→ activation, ⊣ inhibition.

A number of studies show that TRAIL is not only involved in inducing apoptotic cell death but also able to initiate non-apoptotic pathways [26]. The formation of an alternative signaling complex that promotes the survival through NF-κB-mediated upregulation of various anti-apoptotic proteins or activation of additional survival pathways has been proposed [27,

28]. Studies by Walczak, Ashkenazi and colleagues attracted the interest of TRAIL in context with tumorigenesis [29, 30]. These studies showed *in vivo* the selective apoptosis induction of TRAIL in tumor cells, with no effects on normal cells.

2.2.2 TRAIL and cancer

Apoptosis, the programmed cell death, has the function to eliminate cells that are misplaced, damaged or no longer needed. Deficiency in apoptotic pathways is the key factor in development of cancer. One attractive feature of using apoptosis activation for the therapy of cancer is its potential to induce tumor regression rather than simply reduce tumor growth. Traditional cancer treatment uses chemo- and radiotherapy to induce apoptosis via the intrinsic pathway. In these treatments the risk of side effects is very high, because there is no discrimination between normal and malignant cells. Additionally the mutation of p53, which occurs in 50% of all cancer cells, leads to resistance against cellular stress-induced apoptosis by chemotherapy [31]. The TRAIL apoptosis pathway has been selected for cancer therapy, not only because of the p53 independence but also of selective targeting of cancer cells while preserving normal cells. Fewer side effects profile TRAIL as a better candidate for clinical applications over other death-inducing ligands like CD95L or TNF.

Different strategies for TRAIL delivery have been developed. Various recombinant soluble TRAIL constructs showed apoptotic effects on different cancer cells *in vitro* and *in vivo*. In animal experiments TRAIL treatment substantially inhibits the growth of a variety of human tumors [29]. Another strategy is the transfection of adenoviral vectors containing the TRAIL sequence [32]. However, the described high hepatotoxicity, the required high dose of active TRAIL and the low *in vivo* half-life have delayed the translation of TRAIL into the clinic [33, 34].

Furthermore, with the progress of this therapeutic strategy more and more cancer cells with resistance against TRAIL-induced apoptosis have been discovered, especially from highly malignant tumors. The mechanism of developed resistance is not completely understood and seems to be diverse for the various cancer types. Since the various TRAIL receptors have been identified they were analyzed for regulating TRAIL-induced apoptosis and involvement of resistance [20, 35]. A study with early breast cancer samples indicated that TRAIL-R2 expression was associated with a decreased survival rate in patients and high aggressiveness [36]. Changes in the death receptor signaling due to gene mutations or post-translational modifications can also generate resistant cancer cells [37, 38]. Additionally, resistance against TRAIL-induced apoptosis has been shown to be mediated by the decoy receptors with competitive binding or formation of dysfunctional receptor complexes with TRAIL-R1/R2 [39, 40]. However, other studies failed to show any correlation between resistance and expression levels of decoy receptors or death receptors [41]. A possible explanation for the lack of correlation is the additional presence of intracellular mechanism of TRAIL resistance and the diversity of different cancer cell types.

In addition to the TRAIL receptor system, the apoptosis pathway can also be influenced by over-expression of apoptosis pathway inhibitors, like c-FLIP, or reduced levels of initiator caspases-8. High levels of c-FLIP lead to insensitivity to TRAIL and have been observed in several cancer types including breast, lung and prostate cancer, malignant melanoma and leukemia [42, 43]. Beside the overexpression of anti-apoptotic Bcl-2 members, such as Bcl-X_L or Mcl-1, deficiency in pro-apoptotic Bax or Bak has been observed in TRAIL-resistant human prostate cancer and colon carcinoma cell lines [44, 45].

Several studies evaluated other molecular mechanisms involved in cellular resistance to TRAIL-induced apoptosis, like elevated Akt activity, constitutively active NF-κB and XIAP expression [32, 46-48]. In cancer cells, NF-κB controls various genes that contribute to many aspects of tumorigenesis, including cell growth and proliferation, anti-apoptosis, angiogenesis

and metastasis development. Therefore, NF-κB functions as an upstream regulator of IAP (inhibitor of apoptosis proteins) and negatively regulates TRAIL signaling.

In the following part various already developed concepts for combinational treatments of cancer cells to restore the TRAIL sensitivity are descripted.

2.2.3 Sensitizing strategies and TRAIL antibody fusion

Combinational treatments with sensitizing agents are used to make cancer cells more susceptible to TRAIL-apoptosis and furthermore to prevent the development of resistance. Figure 2.5 is an overview of different resistance mechanisms and potential therapeutic strategies to re-sensitize tumor cells to TRAIL [49]. Beside the use of TRAIL-R1 and R2-selective TRAIL mutants, blocking of anti-apoptotic proteins can enhance the death signaling. Chemotherapeutics and targeted agents have been shown to down-regulate c-FLIP and sensitized cancer cells for TRAIL-induced apoptosis [50, 51]. Downstream of the TRAIL apoptosis signaling several anti-apoptotic proteins, like Bcl-2 or XIAP, can be targeted with specific agents to avoid their blocking effect [49].

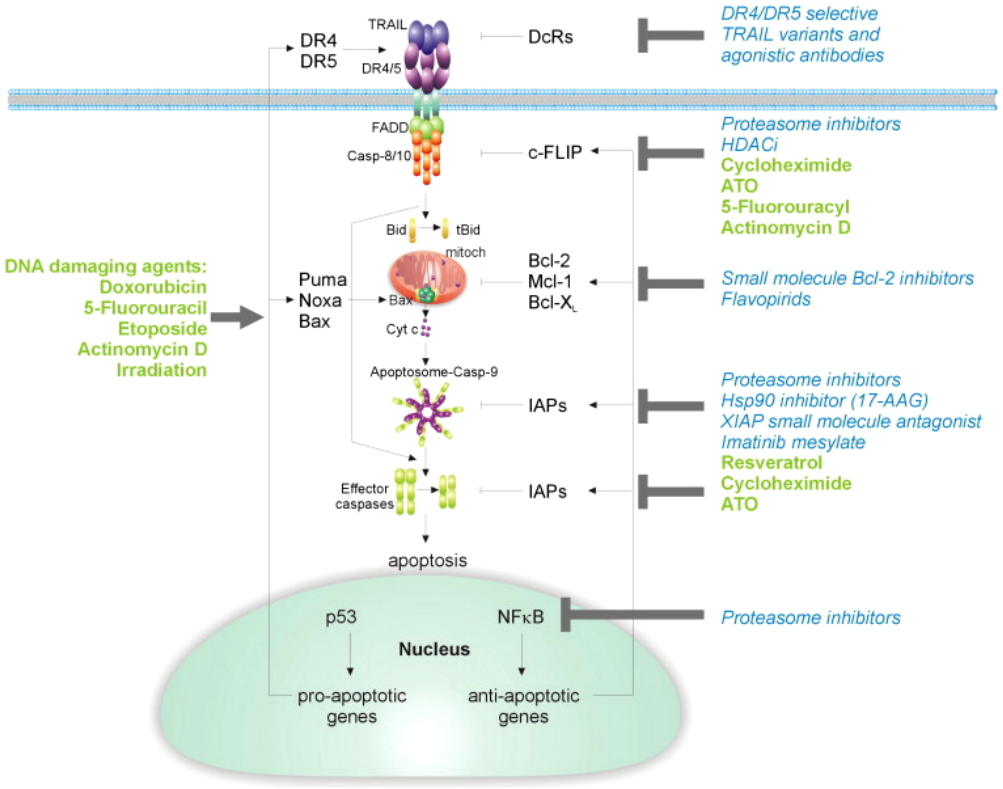

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Figure 2.5 Sensitization strategies to overcome TRAIL-resistance

Different cancer cells developed resistance against TRAIL-induced apoptosis for example by expressing anti-apoptotic proteins or down-regulating the death receptors. This resistance can be overcome by sensitization with different chemotherapeutics (green) and targeted agents (blue).

In some tumor cells, TRAIL itself can induce pro-survival signals and activate the NF-κB transcription factor that regulates the transcription of anti-apoptotic genes [28, 52]. Various proteasome inhibitors have been used to overcome TRAIL resistance mediated by NF-κB [53, 54]. Several tumor cells can also be sensitized by activating the intrinsic pathway and up-regulating pro-apoptotic proteins. Another interesting discovery regarding the TRAIL sensitivity was shown by Jin and colleagues [55]. They found a correlation between the cell cycle and TRAIL sensitivity of cancer cells and observed enhanced sensitivity of G_0/G_1-phase

arrested cells in comparison to cells in late G1, S or G2/M phase. Therefore, these findings suggest a cancer treatment with a combination of TRAIL and agents that induce arrest in G_0/G_1 phase.

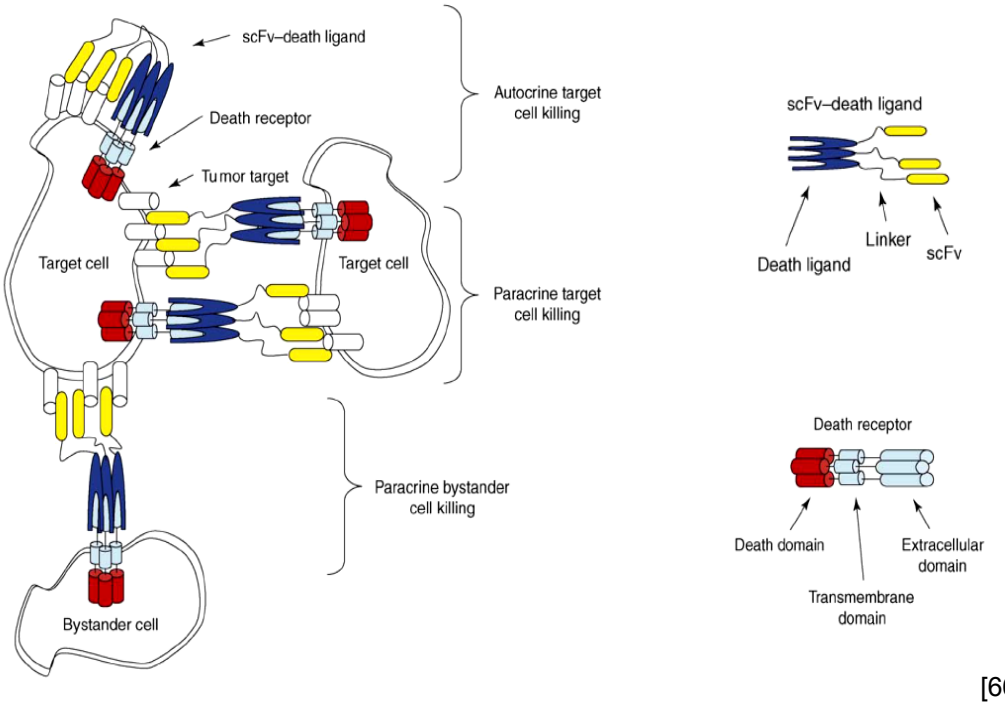
Table 2.1 scFv:TRAIL fusion proteins reported

Fusion protein	Target antigen	Target tumor	*In vivo*	Combination with other anti-cancer therapeutics
MBOS4-TRAIL [56]	Fibroblast activation protein (FAP)	Tumor stroma	No	-
scFvC54:sTRAIL [57, 58]	Epithelial cell adhesion molecule (EpCAM)	Carcinoma	Yes[a]	-
scFv425:sTRAIL [59]	Epidermal growth factor receptor (EGFR)	Glioblastoma, ovarian cancer, colon cancer, lung cancer	Yes [60]	Iressa [59], Valproic acid and Cisplatin [61],
scFv425:sTRAIL-mR1-5 [61]	Epidermal growth factor receptor (EGFR)	Glioblastoma, ovarian cancer, colon cancer, lung cancer	No	Valproic acid and Cisplatin [61]
scFvCD7:sTRAIL [62]	CD7	T-cell leukemia	No	UCN01, Vincristin and Cyclohexamide [62]
scFvCD19:sTRAIL [63]	CD19	B-cell leukemia	Yes [63]	Valproic acid [63]
scFvCD33:sTRAIL [64]	CD33	Acute myeloid leukemia (AML)	No	Mitoxantrone, Vaplroic acid, 17-AAG and Gleevec [64]

[a] Unpublished data.

Combinational therapies enhance TRAIL-induced apoptosis through diverse regulation of anti-apoptotic and pro-apoptotic proteins. In addition, several studies emphasized the importance of the TRAIL construction properties. Important findings are the different affinity of TRAIL-R1 and -R2 to sTRAIL and the diverse cross-linking requirements, e.g. TRAIL-R2 is less sensitive to sTRAIL [65]. The reason why TRAIL-R2 fails to respond to the binding of the soluble ligand form is currently not understood, but there is evidence that secondary aggregation of ligand-bound receptors plays a role [56]. Fusing the ligand to an antibody can turn the soluble ligand into a membrane-bound form and therefore overcome these limitations [57, 61, 64]. Table 2.1 shows some examples of scFv antibody-TRAIL fusion proteins. Importantly, the use of tumor-specific antibodies allows tumor-localized activation and potentially a decrease in side effects. Cell surface antigen-bound TRAIL fusion proteins effectively induce apoptosis by binding to TRAIL receptors on the same cell (Fig. 2.6), also

called autocrine effect. This can happen also in the paracrine manner and thus has the potential to kill neighboring antigen-negative tumor cells. This process is called bystander effect, which offers a benefit especially in heterogenic tumors containing cells with different tumor antigen expression levels.



[66] modified

Figure 2.6 Bystander effect

Specific binding of scFv:TRAIL results in accretion at the tumor cell surface. Afterwards, apoptosis can be induced in an autocrine manner by binding to death receptors on the same cell. Apoptosis can also be induced in a neighboring cell in the so-called paracrine targeting. The paracrine apoptosis induction can also target and eliminate surrounding cells without antigen-expression. This effect of the scFv:TRAIL fusion proteins is called bystander effect.

2.3 Kv10.1 as target for antibody-based cancer therapy

Tumorigenesis is defined as multistep processes from normal to malignant cells including several cellular and molecular changes. Hanahan et al. suggested seven essential

alliterations during the development of a cancer cell [67]. Those hallmarks are self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, avoidance of immune surveillance and tissue invasion and metastasis [67, 68]. Since ion channels have been found to be involved in cell signaling they also became interesting regarding cancer development. In particular the complex implication of ion channels in cancer has been repeatedly highlighted [69].

More than 10 years ago the voltage-gated potassium channel Kv10.1 (Eag1, Ether-á-go-go) was associated with an oncological potential for the first time [70]. In general potassium channels are complexes of transmembran proteins primarily involved in controlling the resting potential and excitability of electrically excitable cells, and in many basic cellular processes, e.g. cell cycle or proliferation. Kv10.1 consists of six transmembran domains and complex intracellular N- and C-termini. Kv10.1 was first cloned from *Drosophila melanogaster* (Fig. 2.7). Overexpression of the human channel induces significant features characteristic of malignant cells, e.g. faster growth, loss of contact inhibition and loss of growth factor and substrate dependence [70, 71]. The ion flux through the pore might be necessary for modulation of different signaling pathways, but a mutation of the pore region which eliminates ion flux did not significantly reduce of proliferation [72]. Using mouse fibroblasts transfected with *Drosophila* Kv10.1 is has been shown that even in the absence of K^+ flux, Kv10.1 acts as voltage sensor and regulates the activity of the mitogen-activated protein kinase (MAPK) pathway [72]. The activated MAPK signaling leads to upregulation of a number of transcription factors that regulate apoptosis, proliferation and migration, chemotaxis, immune response and RNA biosynthesis [73].

In rodents and humans, Kv10.1 is mostly expressed in brain, mainly in the olfactory bulb, cerebral cortex, hippocampus, hypothalamus and cerebellum, albeit the role of the channel is still unknown [74]. Outside of the CNS Kv10.1 is not detected in normal tissue, although 70% of tumor cells from different origin have been tested positive for Kv10.1

expression [75-78]. This relatively high frequency of ectopic expression in primary tumors suggests that tumor cells have an advantage when expressing Kv10.1. Concomitantly, an upregulation of hypoxia-inducible factor 1 (HIF-1) and vascular endothelia growth factor (VEGF) secretion has been shown in Kv10.1-expressing cells [79]. Both signaling factors are important for cancer development and the correlation with Kv10.1 expression strengthen the involvement of the channel in tumor formation.

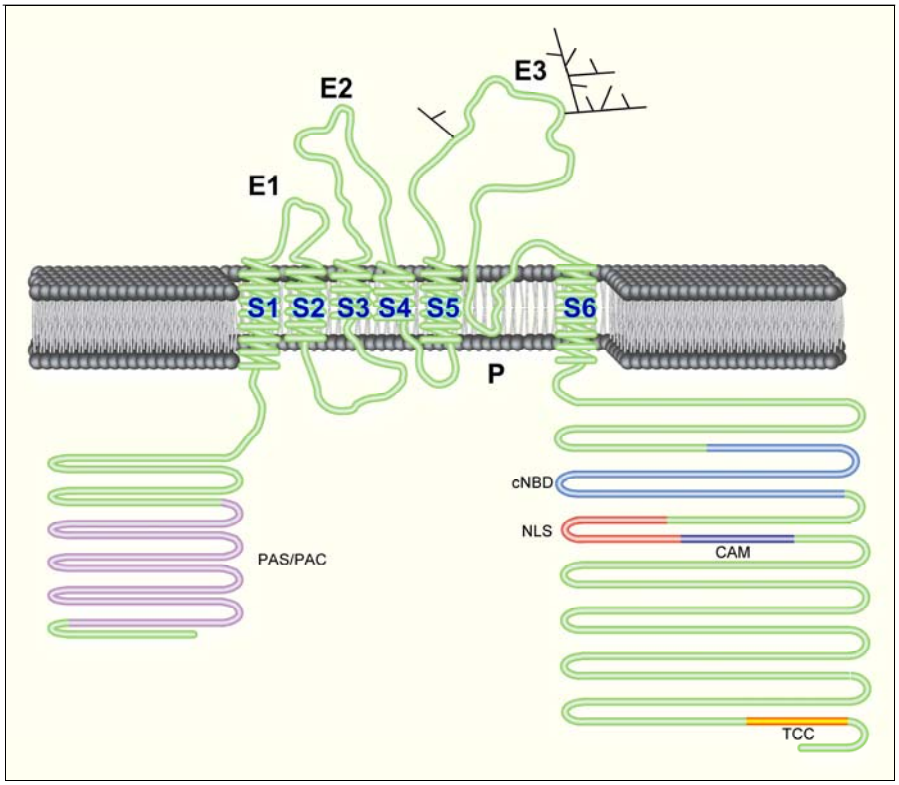


Figure 2.7 Schematic structure of Kv10.1

Kv10.1 consists of six transmembrane regions (S1-6), three external loops (E1-3) and a pore region (P). The intracellular located N- and C-terminus contains different functional domains: CaM (calmodulin binding domain), cNBD (cyclic nucleotide binding domain), NLS (nuclear localization signal), TCC (tetramerized coiled-coil), PAS/PAC (Per-Arnt-Sim).

Moreover, it has been shown that the inhibition of Kv10.1 by blocking or downregulation of the expression using RNA interference leads to a decreased proliferation

rate of tumor cells and impaired tumor growth *in vivo* [80-82]. Therefore, blocking agents of Kv10.1 represents a potential tool for therapy. Imipramine and astemizole have been shown to block Kv10.1 and also reduce the proliferation rate in cancer cells [82, 83]. Unfortunately, these agents have side effects because they are unspecific and also block human-Eag related (HERG) channels. Furthermore, specific inhibition of proteins using siRNA *in vivo* is encountering practical difficulties.

The tumor-cell restricted expression and accessibility from the external environment turn Kv10.1 into a perfect target for antibodies and open the field for developing new antibody-based cancer therapies.

3 Aim of the study

The voltage-gated potassium channel Kv10.1 has been proposed as tumor marker and appears to be a promising novel target for cancer therapy. As a transmembran expressed protein the channel can be easily targeted from the external cell environment. Channel blockers have been used to decrease proliferation of cancer cells, but a Kv10.1-selective strategy that also leads to tumor regression is still missing. This study was designed to investigate the applicability of a Kv10.1-specific antibody-based cancer therapy by generating a single-chain antibody fusion to TRAIL. It has been shown that recombinant fusion constructs consisting of TRAIL and surface antigen specific single-chain antibodies form fully active membrane-bound ligands. By using the Kv10.1-specific antibody the therapeutic efficiency can be increased because of tumor-selective accumulation of TRAIL.

4 Results

4.1 Construction and expression of scFv62-TRAIL

In comparison to whole antibodies, single-chain antibodies offer many advantages, like better tissue penetration, less immunogenicity and easier production and/or labeling. To prove the concept of antibody-based cancer therapy for Kv10.1 I fused an anti-Kv10.1 single-chain antibody to the apoptosis inducing ligand TRAIL.

Two different expression systems for producing the scFv62-TRAIL fusion construct were used: an inducible prokaryotic expression system using *E.coli* and an eukaryotic expression system using HEK-293 and CHO-K1 cells. Figure 4.1 shows a schematic structure of the scFv62-TRAIL fusion protein. The two variable chains of the whole antibody are linked by a glycine-serine peptide-linker and to the human soluble TRAIL in the same manner. In the active form TRAIL forms a trimer, this would also lead to a trimerization of our scFv62-TRAIL antibody (Fig. 4.1b).

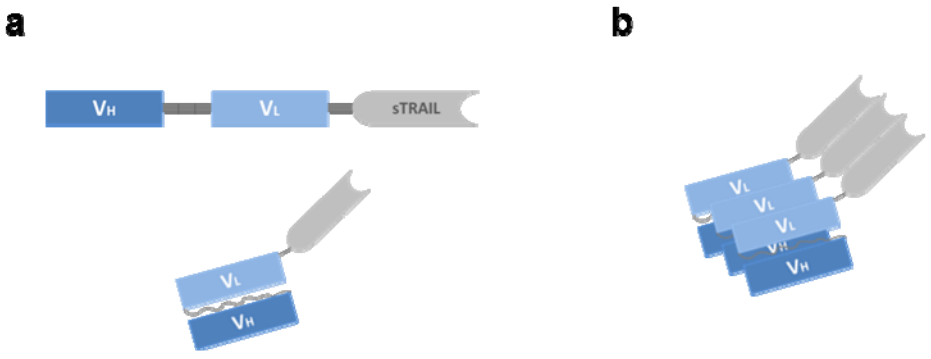


Figure 4.1 scFv62-TRAIL structure

a: Schematic structure of the recombinant scFv62-TRAIL construct, scFv62 is genetically linked through a flexible linker to soluble TRAIL and has a predicted molecular weight of 51kDa as a monomer; b: active trimeric structure with a molecular weight of ~150kDa.

4.1.1 Prokaryotic expression system

Bacterial expression systems with a high expression level are commonly used for the expression of single-chain antibodies, because these small fragments do not contain posttranslational modifications. I constructed the bacterial expression plasmid pASK-IBA2-scFv62-TRAILHis containing a C-terminal 6xHis-tag and transfected it into the *E.coli* protein expression strain BL21. Upstream of the scFv62-TRAILHis sequence the plasmid contains the OmpA signal sequence, which directs the expressed protein into the periplasmic space and is cleaved during translocation process. Analysis of the bacterial protein expression detected a strong protein signal at approximately 50kDa in the soluble fraction (periplasmic space, Fig. 4.2a, lane 1), but also in the non-soluble fraction (inclusion bodies; Fig. 4.2a, lane 2). The expected molecular weight of the scFv62-TRAIL monomer is 51 kDa and an immunoblot using an anti-6xHis antibody confirmed the scFv62-TRAILHis expression in the soluble (Fig. 4.2b, lane 1) and non-soluble fractions (Fig. 4.2b, lane 2).

Therefore, during expression in *E.coli* a high amount of scFv62-TRAILHis construct was packed into inclusion bodies. I used a denaturing procedure to solubilize the antibody and performed a subsequent dialysis to refold the protein. Both the refolded sample and the soluble fraction were submitted to nickel-affinity purification based on the 6xHis-tag. Figure 4.3 shows a protein gel analysis of the different fractions detecting high amounts of unbound (lane 2 and 8) and washed-out scFv62-TRAIL (lane 3, 4 and 9, 10). Only a weak signal of the scFv62-TRAILHis could be detected in the elution of the refolded preparation (Fig. 4.3, lane 5) with high amount of unspecific proteins and no signal in the eluates of the periplasmic fraction (Fig. 4.3, lane 11).

Because of the high amounts of unbound scFv62-TRAILHis and some residual proteins in the elution, I generated an antigen-tagged column and performed an affinity chromatography. The soluble and refolded scFv62-TRAIL samples were loaded on the

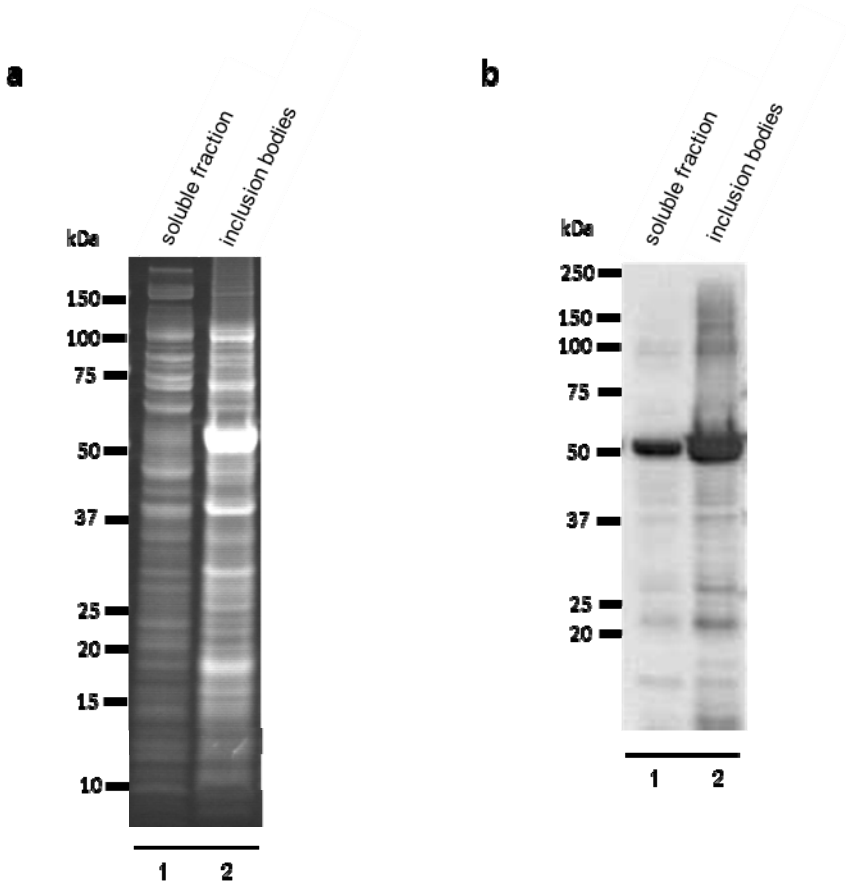


Figure 4.2 Bacterial expression of scFv62-TRAIL

a: *E.coli* BL21 cells were transfected with pASK-IBA2-scFv62-TRAIL vector and expression was induced as described under Methods. 20 µl of soluble (periplasmic space) and insoluble (inclusion bodies) fractions were analyzed on a 4-12% reducing SDS-PAGE stained with SyproRuby; b: analysis of protein expression by immunoblot using anti-6xHis-tag antibody.

antigen-coupled column and eluted by low pH. Analysis of the elution peaks on protein gel did not show a scFv62-TRAILHis signal (Fig. 4.4a, lane 2). Therefore, to allow a better binding of scFv62-TRAILHis to the affinity column I performed a size-exclusion chromatography under denaturing conditions to remove unspecific proteins. Under these denaturing conditions the scFv62-TRAILHis antibody with size of 51kDa was expected in a single peak. The chromatogram showed no defined peaks; therefore I analyzed single fractions on protein

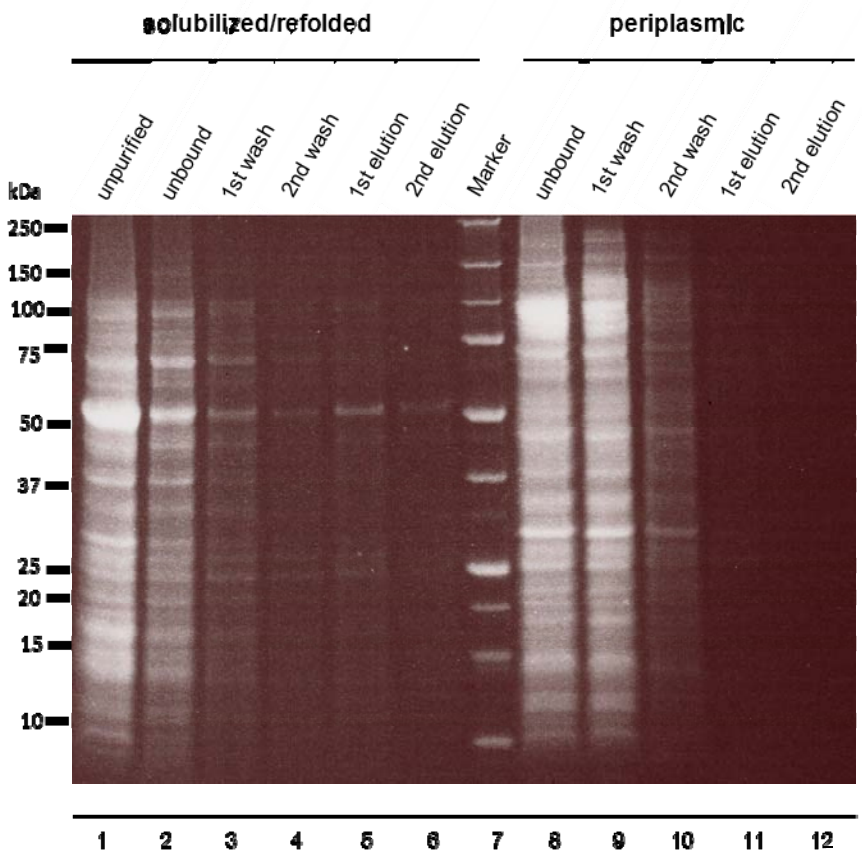


Figure 4.3 Ni-affinity purification

Solubilized/refolded and periplasmic scFv62-TRAIL preparations were purified using Ni-NTA agarose as described under methods. 20µl of each fraction were analyzed on 4-12% SDS-PAGE and SyproRuby staining under reducing conditions.

gel (Fig. 4.4b). The scFv62-TRAILHis construct could be detected in fractions 8-25 (Fig. 4.4b, lane 2-7), but also many larger and smaller proteins.

Together, I was able to express the scFv62-TRAILHis with high efficiency in *E.coli.* During the different purification steps I could not completely clean up the antibody from the residual bacterial proteins. Possible explanation for the ineffective purification is the presence of aggregates, which has been described for scFv antibodies especially at high concentration [84, 85].

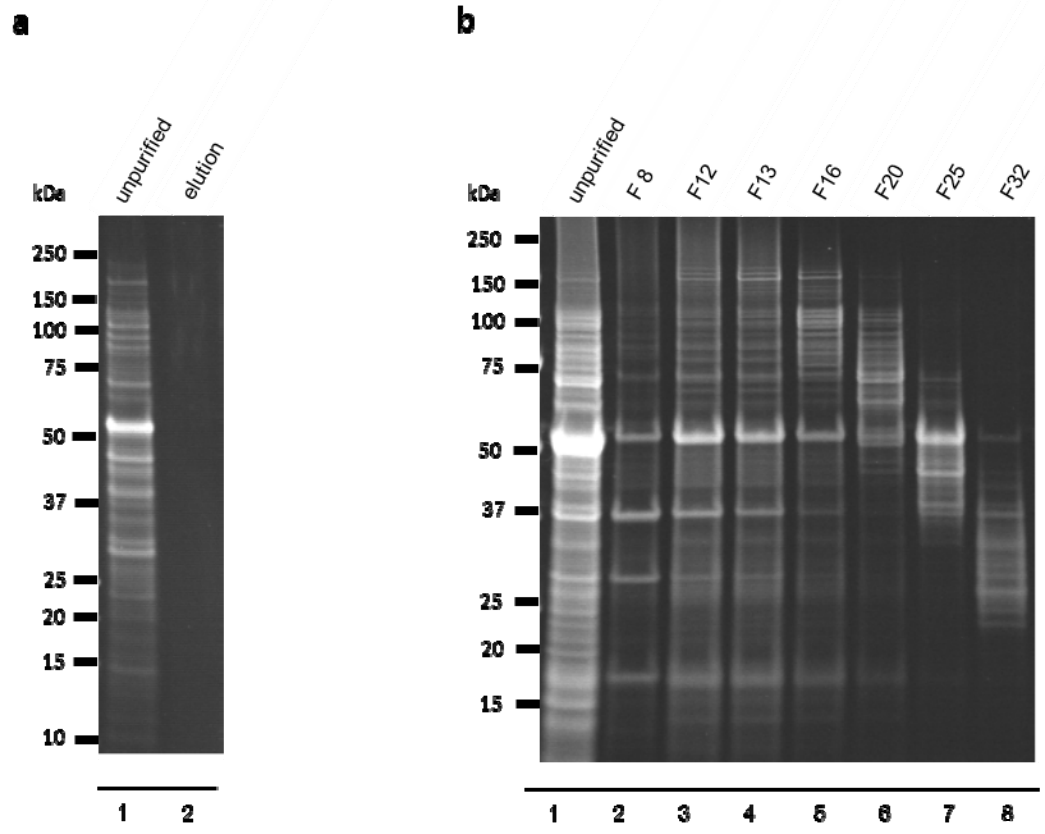


Figure 4.4 Size exclusion chromatography of scFv62-TRAIL under denaturing conditions

a: The h1x-affinity column was prepared and tested as described under material and methods; 5ml of *E.coli* expressed scFv62-TRAIL preparations were loaded on the h1x-affinity column; after washing, bound proteins were eluted by pH reduction. The elution peak was collected and neutralized with 1M Tris-HCl pH 8 and analyzed on 4-12% reducing SDS-PAGE with SyproRuby staining. b: The inclusion bodies preparation of the scFv62-TRAIL overexpression was solubilized in 5ml 100mM Tris-HCl/100mM NaCl pH 8 containing 8M urea; 4.5ml of that preparation was loaded on a Superdex200 size exclusion column, the peaks were collected in 1ml fractions and analyzed on 4-12% SDS-PAGE with SyproRuby staining.

4.1.2 Eukaryotic expression of scFv62-TRAIL construct

Protein yields in eukaryotic expression systems are lower in comparison to bacterial expression systems, but in return the expressed proteins are properly folded and have a reduced tendency to aggregate. For optimizing protein quality I decided to use eukaryotic cell

systems for further protein production. Additionally, in the industry mammalian cell lines are important host cells for pharmaceutical protein fabrication. Since different protein tags also influence the protein folding, the scFv62-TRAIL was expressed without any C- or N-terminal tag.

HEK-293 and CHO-K1 cells were transiently transfected with the pSecTag2A-scFv62-TRAIL vector containing a leader sequence for secretory protein expression using either Lipofectamine 2000 or FuGene. Afterwards I analyzed the medium for protein expression by immunoblot using an anti-TRAIL antibody. The expressed protein was detected in the medium supernatant with the expected size of 51kDa for the monomeric scFv62-TRAIL (Fig. 4.5). Comparing the scFv62-TRAIL yields in the medium supernatant, CHO-K1 cells transfected with FuGene showed the highest scFv62-TRAIL expression rate (Fig. 4.5, lane 3).

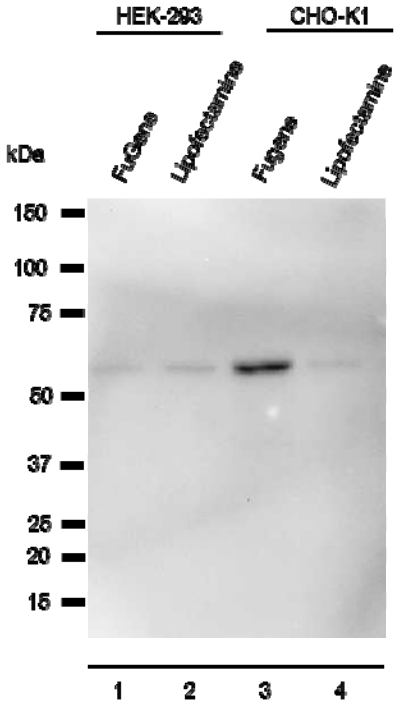


Figure 4.5 Optimization of eukaryotic scFv62-TRAIL expression

Transient transfected HEK-293 and CHO-K1 cells with pSecTag2A-scFv62-TRAIL and either Lipofectamine 2000 or FuGene; after 3 days medium was collected and analyzed using an immunoblot with anti-TRAIL antibody detection.

The protein yields in transient transfections can vary between different preparations, whereas stable cell transfections enable a constant protein production and the highest protein amount when selected for efficient clones. Therefore, transfected CHO-K1 cells were selected with Zeocin for single clones and scFv62-TRAIL expression was analyzed (Fig. 4.6, lane 1-4, 6 and 7). I discovered clone F5 with the highest scFv62-TRAIL expression rate (Fig. 4.6, lane 3).

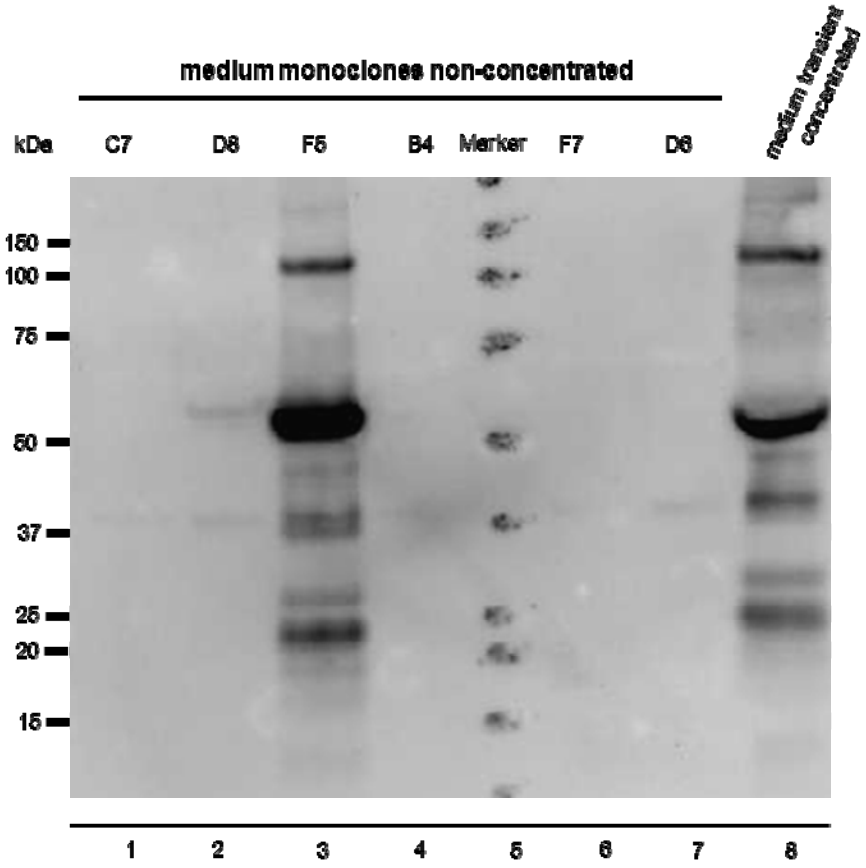


Figure 4.6 Selection of monoclonal CHO-K1 clones expressing scFv62-TRAIL

CHO-K1 cells stable transfected with pSecTag2A-scFv62-TRAIL were selected in 96-well plates for single clones. The different clones were cultured and seeded in 6-well plates; after incubation in 2ml Panserin C6000 medium for 4 days medium was analyzed using an immunoblot with anti-TRAIL antibody detection. Concentrated medium of a transient transfection was used as control.

The normal CHO-K1 medium contains many growth factors and serum proteins. To make the protein purification easier CHO-K1 cells were cultured in an optimized protein- and serum-free medium. Different factors can influence the efficiency of protein expression, for example the transfection reagent -as described earlier- or the incubation temperature. During expression cells were incubated at different temperatures for 5 days (Fig. 4.7). Compared to normal conditions, I detected an increase in protein expression at 30°C (Fig. 4.7, lane 1 and 4).

In summary, for improving the scFv62-TRAIL production I established an eukaryotic expression system using stable transfected CHO-K1 cells and optimized protein expression conditions.

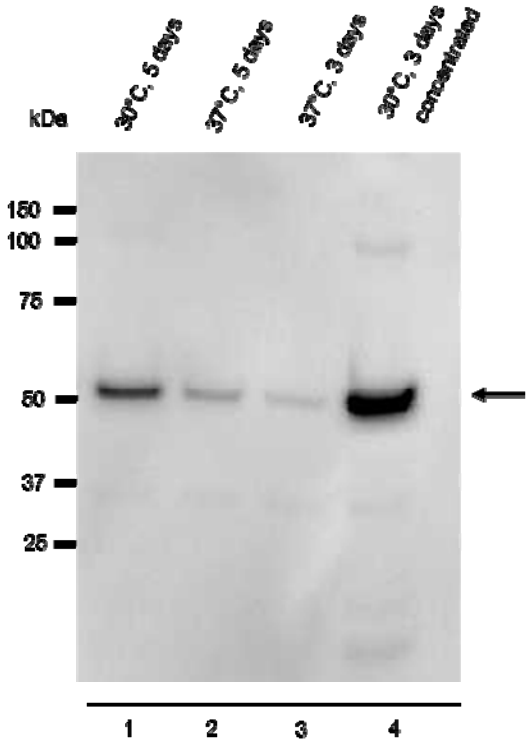


Figure 4.7 Temperature dependent protein expression

scFv62-TRAIL-expressing CHO-K1 clones were seeded on cell culture flasks; after allowing cell attachment normal medium was changed into serum- and protein-free Panserin C6000 medium and cells were incubated at 37°C or 30°C. After 3 or 5 days medium was analyzed using an immunoblot with anti-TRAIL antibody detection.

4.1.3 Analysis and purification of eukaryotic expressed scFv62-TRAIL

The normal active form of TRAIL is a trimeric structure. The trimeric expected size for scFv62-TRAIL fusion construct is 150kDa. To determine the native molecular weight of the product, scFv62-TRAIL preparations expressed in CHO-K1 cells and *E.coli* were analyzed on native gel electrophoresis followed by immunoblot detection with anti-TRAIL (Fig. 4.8a). In both preparations a clear signal was detected in the high molecular weight range. The signal was stronger in the more concentrated *E.coli*-produced sample (Fig. 4.8a, lane 1). However, the native SDS-PAGE did not provide clear information about the scFv62-TRAIL structure or presence of aggregates.

As an alternative to native gel electrophoresis scFv62-TRAIL I analyzed the scFv62-TRAIL preparation on denaturing gel electrophoresis under non-reducing conditions (Fig. 4.8b). Under non-reducing conditions, a clear signal at 150kDa was detected which matches to the expected size of trimeric scFv62-TRAIL (Fig. 4.8b, lane 1). Additionally to the signals of the trimeric antibody at 150 kDa, smaller fragments were also present on the immunoblot in the lane of the non-reducing sample. To test whether those fragments correspond to non-active scFv62-TRAIL monomers or degraded proteins, I used size exclusion chromatography and subsequently immunoblotting on the peaks separated by size (Fig. 4.9). The scFv62-TRAIL antibody was detected only in the first peak (Fig. 4.9, lane 2), without signals of proteins with lower molecular weight in further fractions (Fig. 4.9, lane 3-9).

The scFv62-TRAIL construct expressed in eukaryotic cells does not contain any tag for purification; therefore an antigen-based affinity chromatography was performed to purify the antibody from the supernatant. This approach did not succeed to purify the scFv62-TRAIL, which did not bind to the affinity column and could only be detected in the elution front and not in the purified fractions (Fig. 4.10, lane 2). For further analysis and applications supernatant medium containing the scFv62-TRAIL was collected, concentrated and sterile filtered.

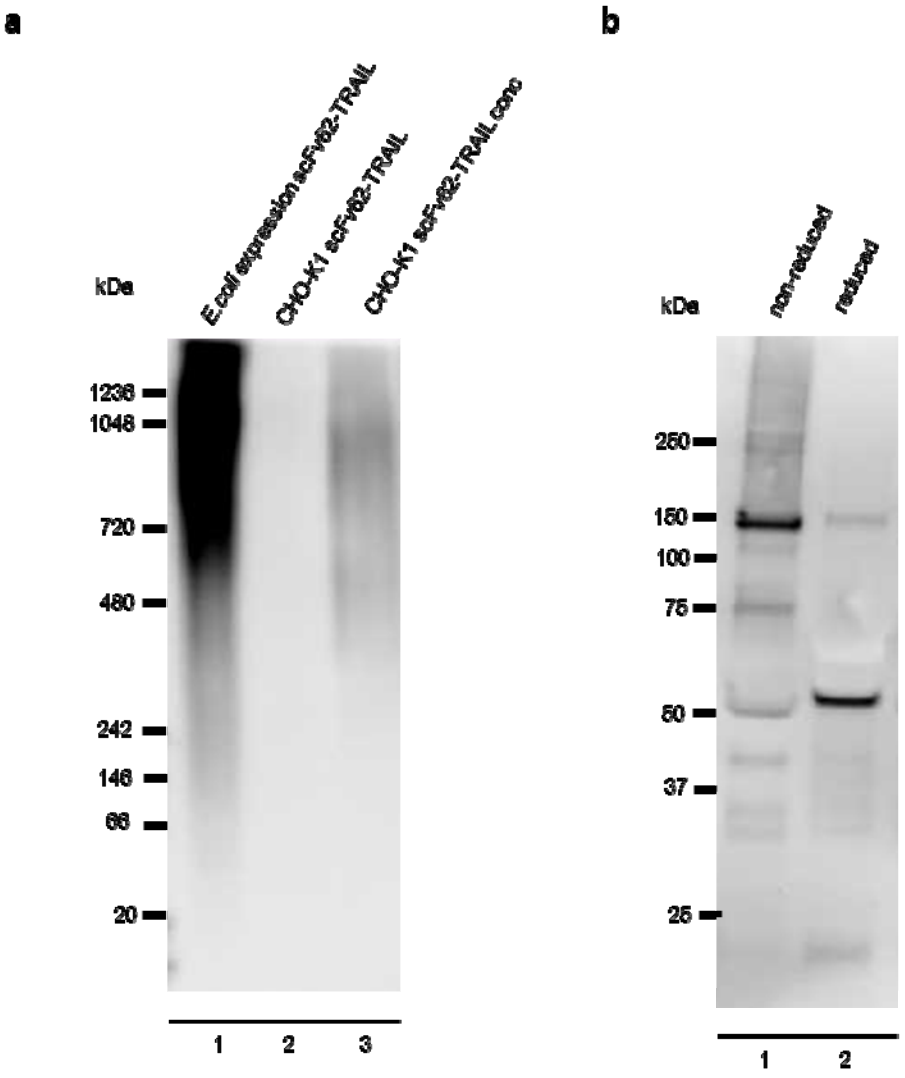


Figure 4.8 Native and non-reducing gel electrophoresis

a: 5µl samples of scFv62-TRAIL expressed in *E.coli* and CHO-K1 cells (concentrated and non-concentrated) preparations were analyzed on native western blot using anti-TRAIL antibody for detection. b: 20µl of CHO-K1 cell expressed scFv62-TRAIL analyzed on immunoblot (anti-TRAIL antibody) under reducing and non-reducing conditions, with or without reducing agent in the samples.

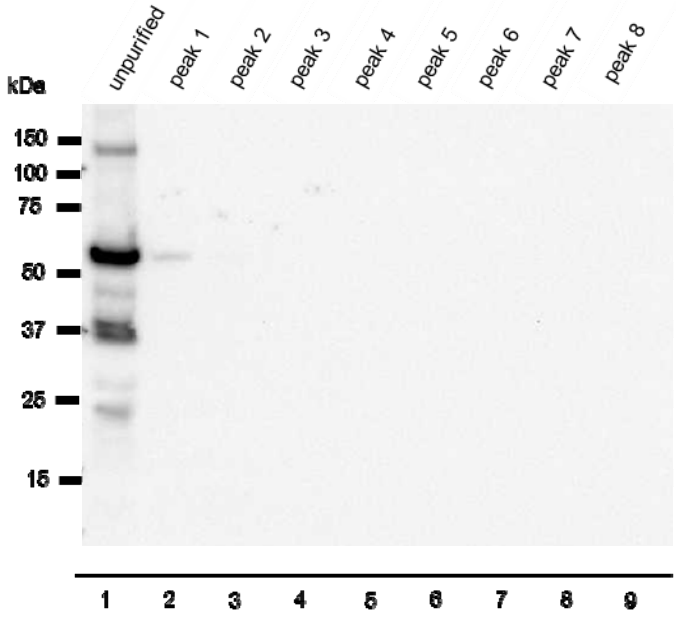


Figure 4.9 Size exclusion purification of scFv62-TRAIL

3ml CHO-K1 cell medium containing scFv62-TRAIL were loaded on Superdex200 size exclusion column; the peaks were collected in 1ml fractions and analyzed using immunoblot and anti-TRAIL antibody detection.

The concentration of active scFv62-TRAIL was determined by sandwich ELISA using the recombinant fusion protein containing the epitope as antigen and detecting by anti-TRAIL antibody. Due to the fact that I have to use two different secondary antibodies the data of the standard antibody does not necessarily correlate with the concentration of scFv62-TRAIL. Therefore, I calculated scFv62-TRAIL in U/ml (units/ml).

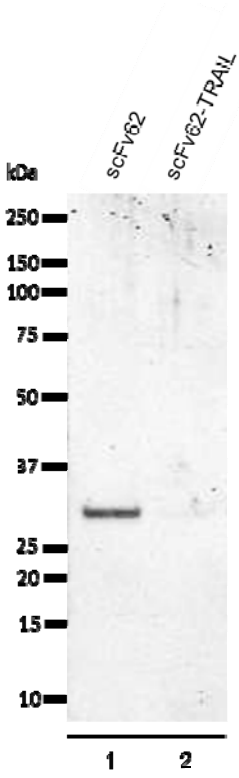


Figure 4.10 Antigen-affinity chromatography

20ml supernatant of the CHO-K1 cells expressing scFv62 or scFv62-TRAIL was loaded on the h1x-affinity column. After washing, bound proteins were eluted by pH reduction. The elution peaks were collected and neutralized with 1M Tris-HCl pH 8 and analyzed on 4-12% reducing SDS-PAGE with SyproRuby staining.

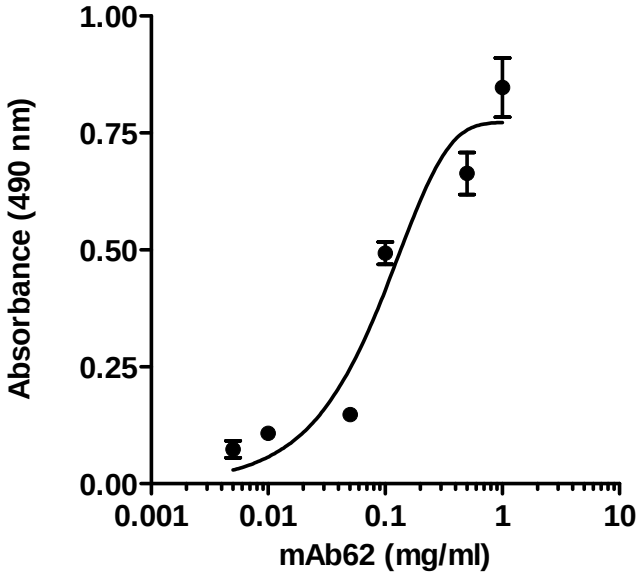


Figure 4.11 ELISA standard curve

The scFv62-TRAIL concentration was determined by ELISA using an antigen-coated 96-well plate and the mouse monoclonal anti-Kv10.1 mAb62 as standard. For detecting the scFv62-TRAIL an anti-TRAIL antibody was used.

In summary, I expressed the scFv62-TRAIL antibody using a prokaryotic and eukaryotic protein expression system. Despite the different purifications methods, I was not able to completely purify the antibody. Therefore I decided to use the concentrated CHO-K1 cell medium containing the scFv62-TRAIL construct for further activity studies.

4.2 Analysis of Kv10.1 expression in different tumor cell lines

Kv10.1 is normally expressed in the brain of humans and rodents and is involved in signal transduction and repolarization of the cell. Analysis of tumor tissue form different origins showed a significant overexpression of the channel with a frequency of 70%. Outside of the

CNS, the tumor-restricted expression of Kv10.1 could be used to selectively target cancer cells by scFv62-TRAIL fusion protein.

Hence, I analyzed different cancer cells for their expression of Kv10.1 using real-time PCR. The mRNA levels were normalized to the reference genes human transferrin and beta-actin (Fig. 4.12). HEK-293 cells transfected with a vector containing human Kv10.1 were used as positive control and HEK-293 wild type cells as negative control.

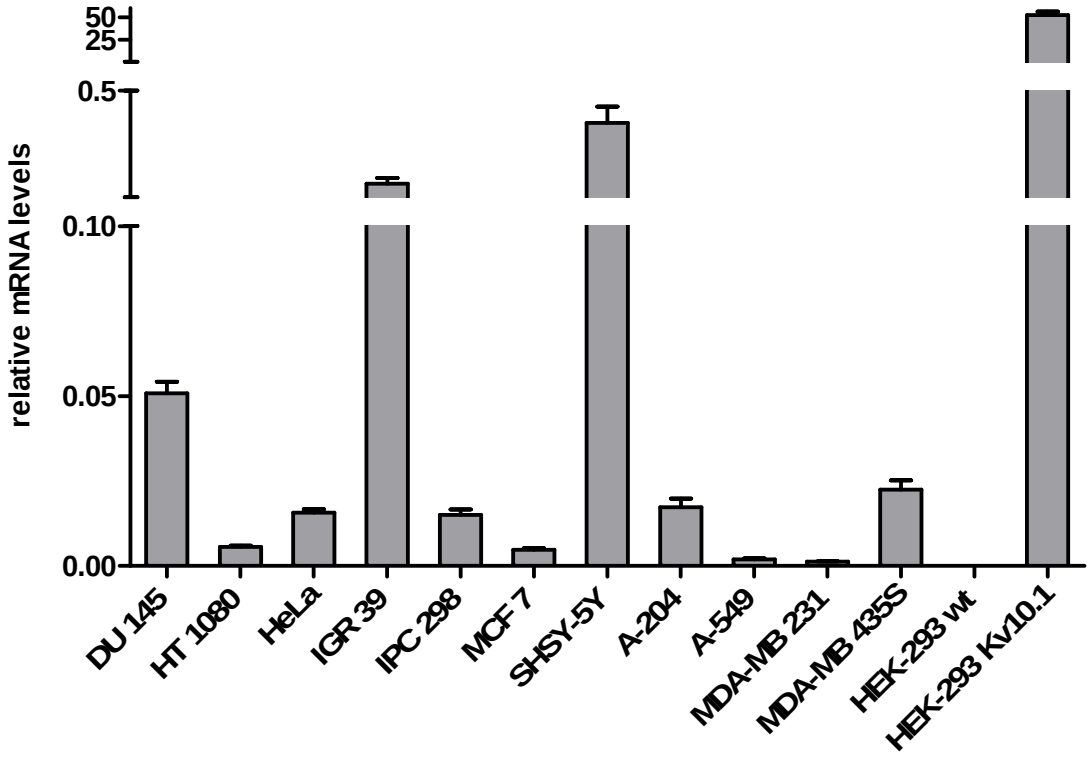


Figure 4.12 Kv10.1 expression analysis

RNA was isolated from $2x10^6$ cells of different cell lines and 5µg total RNA were used for cDNA synthesis; quantitative real-time PCR was performed using the human transferrin receptor and beta-actin as reference.

Kv10.1 expression could be detected in all cancer cell line (Fig. 4.12). In comparison I observed the highest expression level in DU145 (prostate cancer), IGR39 (melanoma) and SHSY-5Y (neuroblastoma) cells.

These data are consistent with the previously described overexpression of Kv10.1 in cancer cells from different origins. Additionally, I found differences in Kv10.1 expression levels between the cancer cell lines, even if they derived from the same tumor type.

4.3 Effect of scFv62-TRAIL on cancer cells

The binding of TRAIL to its death receptors TRAIL-R1 or R2 on the cell surface induces apoptosis. Depending on the type of cancer the cells show different sensitivity against TRAIL-induced apoptosis and some develop resistance towards apoptosis induction, which can be overcome by various sensitizing strategies. I analyzed the effect of scFv62-TRAIL alone as single application and also in combinational treatments with chemotherapeutics.

Apoptosis, the programmed cell death, is a highly regulated multi-step process, which can be experimentally followed by several cellular characteristics. Two different apoptosis assays based either on caspase activity or loss of phospholipid membrane asymmetry were used to analyze the effect of scFv62-TRAIL on cancer cells.

4.3.1 Caspase activity in scFv62-TRAIL preparations

Caspase-3 activation plays a key role in initiation of cellular events during the early apoptotic process. Its activity can be tested using a luminescent caspase 3/7 substrate. To analyze the effect of scFv62-TRAIL, the different cancer cells were seeded in 96-well-plates and treated with different amounts of scFv62-TRAIL for 24h. Unfortunately, I detected caspase-3 activity in the scFv62-TRAIL preparation without cells indicating the presence of active caspase-3 in the medium supernatant of scFv62-TRAIL (Fig. 4.13). This caspase-3 activity could be a side-product of protein expression or released from lysed CHO-K1 cells. To remove the low molecular weight proteins including activated caspase-3 of about 19kDa, a dialysis with a

100kDa cut-off membrane was performed. scFv62-TRAIL preparations samples before and after dialysis and the scFv62 preparation medium were analyzed by immunoblotting using anti-caspase-3 antibody. The presence of caspase-3 was confirmed by the immunoblot, but it was not removed by dialyzing the scFv62-TRAIL preparation (Fig. 4.14).

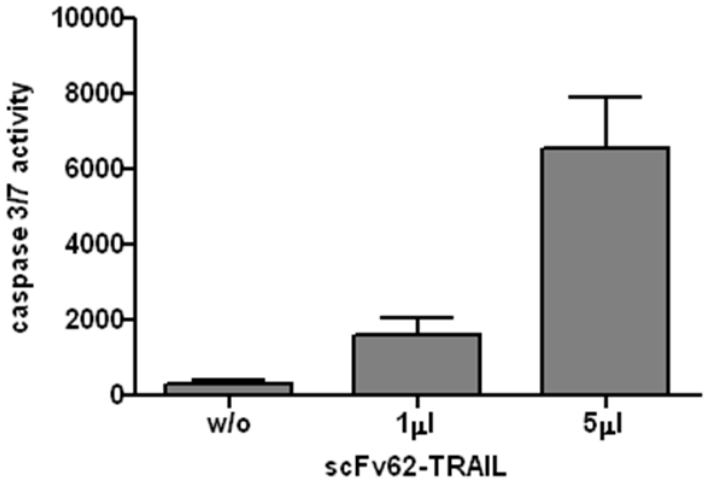


Figure 4.13 Caspase-3/7 activity in scFv62-TRAIL preparation

1µl and 5µl scFv62-TRAIL in medium without cells were assayed for caspase3/7 activity. A strong luminescence signal was measured.

In summary, CHO-K1 supernatant with the scFv62-TRAIL antibody contains active caspase-3, which resulted in non-specific signals in the caspase-3-based apoptosis assay. Free active caspase-3 appears to be integrated in high-molecular-weight complexes, because it could not eliminate it by dialysis. To avoid unspecific signals, I did all subsequent apoptosis measurements by flow cytometry and Annexin/PI staining protocols, which are independent of caspase-3 activity.

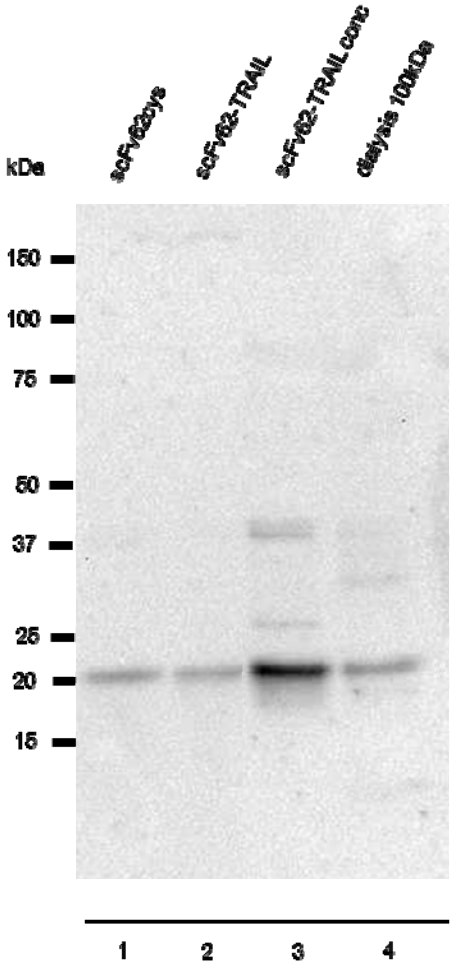


Figure 4.14 Immunoblot for caspase-3 detection

CHO-K1 cells supernatants containing scFv62 or scFv62-TRAIL were concentrated or dialyzed with a 100kDa MWCO membrane, 20µl of each preparation were analyzed using immunoblotting with anti-active caspase-3 antibody.

During early apoptosis, the plasma membrane loses its asymmetry. Phosphatidylserine (PS) is translocated from the cytoplasmic to the external face and can be detected by using Annexin V. In the late apoptotic state the impermeability of the plasma membrane becomes compromised. Propidium iodide (PI), an additional marker, which binds to DNA, is used to distinguish between competent and permeable membranes (dead, early and late apoptotic cells). Annexin V labeled with FITC or Alexa680 and PI were measured by flow cytometry.

First, the effect of scFv62-TRAIL alone on different Kv10.1-positive cancer cells was investigated. According to the Kv10.1 expression analysis the following Kv10.1-positive cancer cells were used: MDA-MB453S (breast cancer), DU145 (prostate cancer), HeLa (epithelia carcinoma), IGR39 and IPC298 (melanoma), HT1080 (fibrosarcoma), SHSY-5Y (neuroblastoma) and Kv10.1 transfected HEK-293 cells. The different cell lines were treated with 50U/ml scFv62-TRAIL for 24h and analyzed with an Annexin/PI staining for apoptosis induction (Fig. 4.15). Comparing the non-treated and treated cells no, or only weak, changes in the amount of apoptotic cells could be detected.

These first experiments did not show any pro-apoptotic effect of scFv62-TRAIL to Kv10.1-positive cancer cells nor to Kv10.1-overexpressing HEK-293 cells.

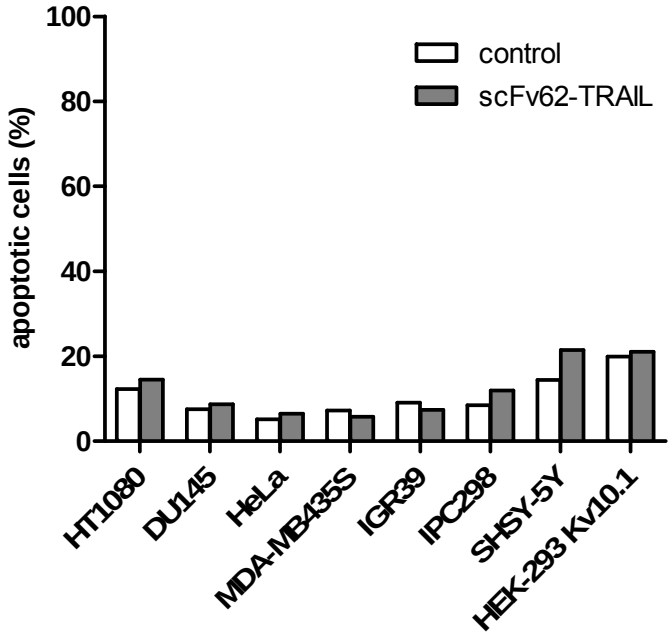


Figure 4.15 Apoptosis induction by scFv62-TRAIL

$1x10^5$ cells from different lines were plated in 12-well plates and cultured overnight. Treatment was done with or without 50U/ml scFv62-TRAIL for 20h. Cells were stained with Annexin V-FITC/PI and measured by flow cytometry. Apoptotic cells are presented in percentage of total cells: non-treated (white bars) and treated (grey bars). Measurement was performed in duplicates.

4.3.2 Combinational scFv62-TRAIL treatments of cancer cells

Many cancer cells developed resistance against TRAIL-induced apoptosis. In general, different strategies are used to overcome the resistance and make cells more susceptible to TRAIL-induced apoptosis. Inhibition of protein synthesis is one of the sensitizing strategies; therefore cycloheximide (CHX) in combination with scFv62-TRAIL was used *in vitro*. CHX has been often used in prostate cancer as sensitizer, because it inhibits the cellular caspase-8 (FLICE)-like inhibitory protein (c-FLIP) and other anti-apoptotic proteins (IAP).

To study the sensitizing effect of CHX, cancer cells and HEK-293-Kv10.1 cells were treated with or without 50U/ml scFv62-TRAIL in presence of 5µg/ml CHX for 20h. Apoptosis induction was measured with Annexin V-FITC/PI staining and analyzed by flow cytometry (Fig.4.16). In some cancer cell lines a strong increase in the apoptosis induction was observed when using combinational treatment with CHX in comparison to single treatments with scFv62-TRAIL. HT1080 and HeLa cells showed an apoptosis induction of around 40% and DU145 80% of apoptotic cells, whereas the other cancer cells responded only slightly or showed no effect. In the Kv10.1-expressing HEK-293 cells no change in apoptosis after treatment with scFv62-TRAIL and CHX was observed. CHX was subsequently used as sensitizer for further *in vitro* studies due to its low toxicity within the tested time window.

Because of the strong effect of scFv62-TRAIL treatment in combination with CHX on the prostate cancer cell line DU145, the apoptosis induction was investigated in more detail in a dose-response analysis treating DU145 cells with 5µg/ml CHX and various amounts of scFv62-TRAIL (Fig. 4.17). The treatment resulted in a dose-dependent apoptosis induction with a calculated IC_{50} of ~5U/ml. Furthermore, the apoptosis induction over a time period of 20h was analyzed (Fig. 4.18). The cells progressed from viable over early apoptosis to non-viable cells within 20h. At the end of this period, 80% of the cells were apoptotic and already one half of them showed non-competent plasma membrane (Fig. 4.18b).

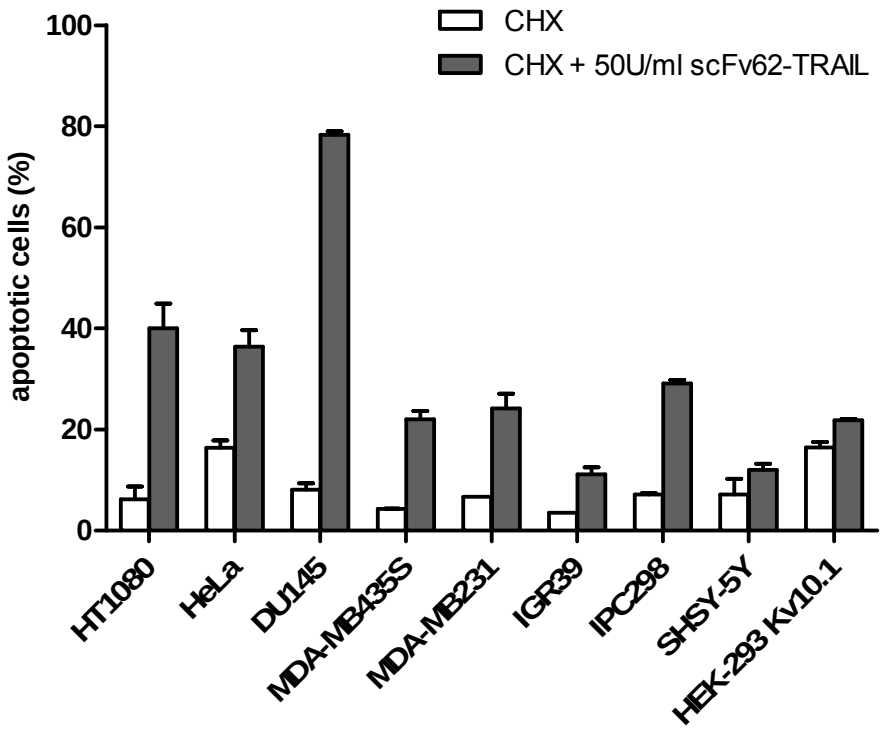


Figure 4.16 scFv62-TRAIL treatment in combination with CHX

Different cancer cell lines were treated in 12-well plates ($2x10^5$ cells/well) with or without 50U/ml scFv62-TRAIL in presence of 5µg/ml CHX. HEK-293 cells transfected with Kv10.1 were used as control. Apoptosis was determined by Annexin V-FITC/PI staining and flow cytometry. Treatments with 5µg/ml CHX alone (white) and in combination with 50U/ml scFv62-TRAIL (grey) were performed in duplicates.

These data showed that CHX was able to sensitize cancer cells for scFv62-TRAIL-induced apoptosis, whereas normal cells expressing Kv10.1 were not affected. The apoptosis induction of scFv62-TRAIL was dose-dependent. However, some Kv10.1 cancer cell lines were still resistant against scFv62-TRAIL-induced apoptosis.

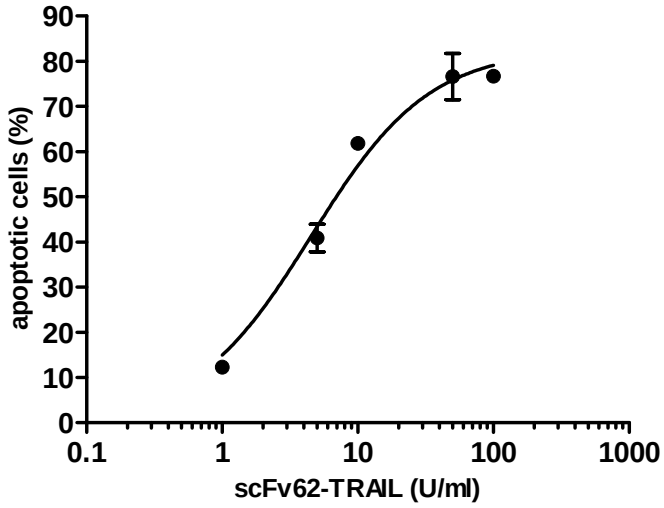


Figure 4.17 Dose-response curve of scFv62-TRAIL effect in the presence of CHX

DU145 cells were treated with different amounts of scFv62-TRAIL in presence of 5µg/ml CHX. Apoptosis was measured by using Annexin V-FITC/PI staining and flow cytometry. Measurements were done in triplicates.

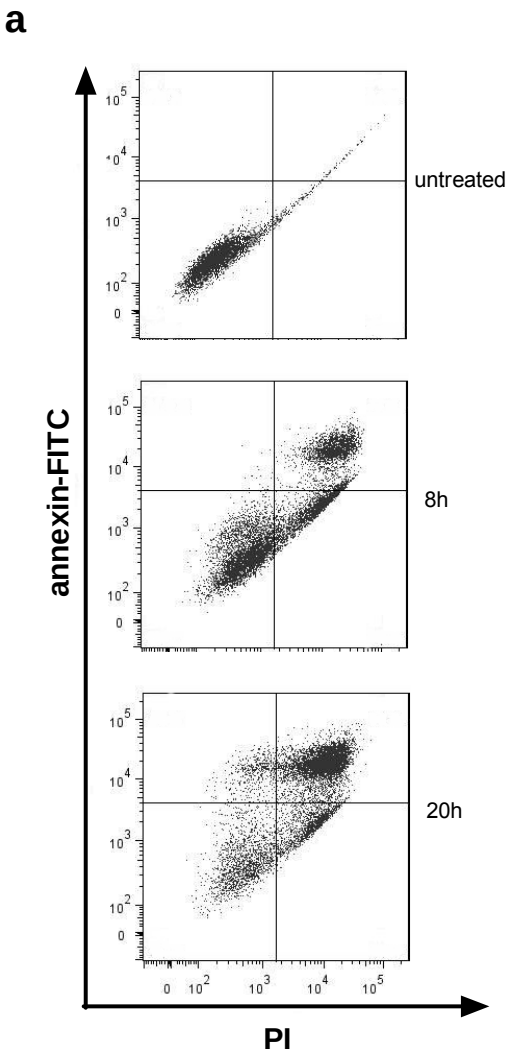


Figure 4.18 Time course of apoptosis progression

DU145 cells were treated with 50U/ml scFv62-TRAIL in combination with 5µg/ml CHX. Apoptosis progression was monitored for 20h. a: flow cytometry measurement (dot plots) b: graphic analysis of apoptosis progression: live cells (white), early apoptotic (grey) and late apoptotic (black).

4.3.3 Effect of CHX on DU145 cells

CHX inhibits protein synthesis by blocking the translocation process. I was able to show that CHX can sensitize cancer cells for scFv62-TRAIL induced apoptosis. The inhibition of the synthesis of anti-apoptotic proteins is one explanation for the restored sensitivity to apoptosis. Additionally, it has been described that cells that are in the G1 phase of the cell cycle are more susceptible to TRAIL apoptosis. Therefore I wanted to study the influence of CHX on the cell cycle.

For this purpose DU145 cells were cultured for 24h in their normal medium supplemented with or without 5µg/ml CHX. Cell cycle analysis was performed by PI-staining solution and flow cytometry. As shown in Figure 4.19a, treatment with CHX results in decrease of G2 phase and enrichment of arrested cells in G1 phase. DU145 cells cultured under normal conditions show a balanced cell cycle phase distribution; whereas treatment with CHX affects an increase up to 72% of G1 arrested cells (Fig. 4.19b).

In summary, these data show an influence of CHX on the cell cycle with an enrichment of the cells in the G1 phase.

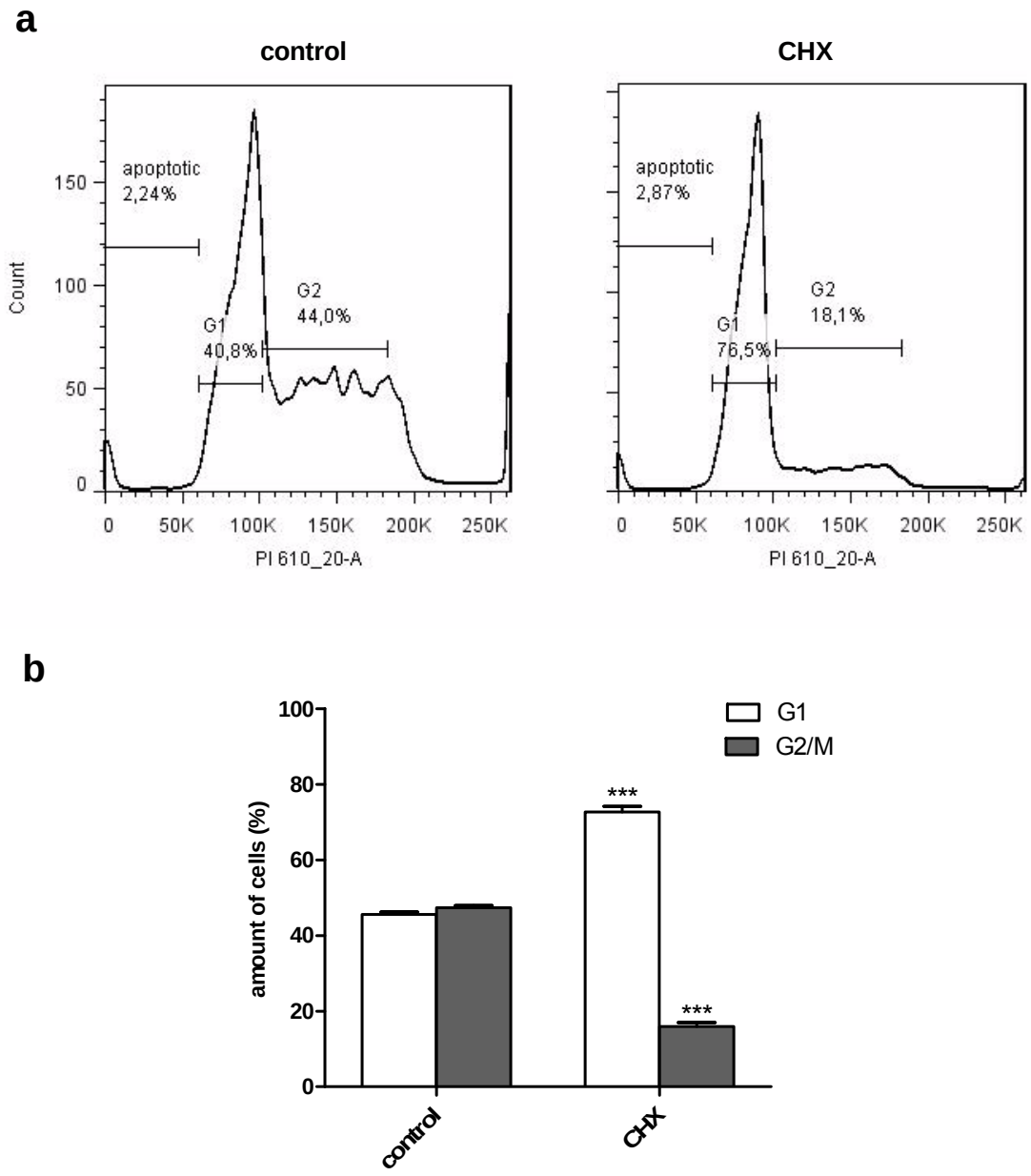


Figure 4.19 Influence of CHX on cell cycle

DU145 cells were incubated for 24h in normal culture medium supplemented with or without 5µg/ml CHX and analyzed using cell-cycle staining and flow cytometry. a: histogram flow cytometer b: diagram amount of cell in G1 and G2/M phase.

4.3.4 Apoptosis induction by scFv62-TRAIL requires Kv10.1

TRAIL naturally exists in two forms, soluble and membrane-bound in immune cells, but only the membrane form of TRAIL is effective in inducing apoptosis. These observations were the starting point of attempts to fuse TRAIL to cancer specific antibodies and of turning soluble TRAIL into the membrane-bound form. The Kv10.1-positive prostate cancer cell line DU145 showed the highest susceptibility to scFv62-TRAIL induced apoptosis; therefore this cell line was used for further analysis.

The scFv62-TRAIL was designed to bind to Kv10.1 and induce apoptosis specifically on K_V10.1-expressing cancer cells. To confirm the specificity, scFv62-TRAIL was incubated either with antigen to block the antibody part or with an anti-TRAIL antibody to block the death ligand.

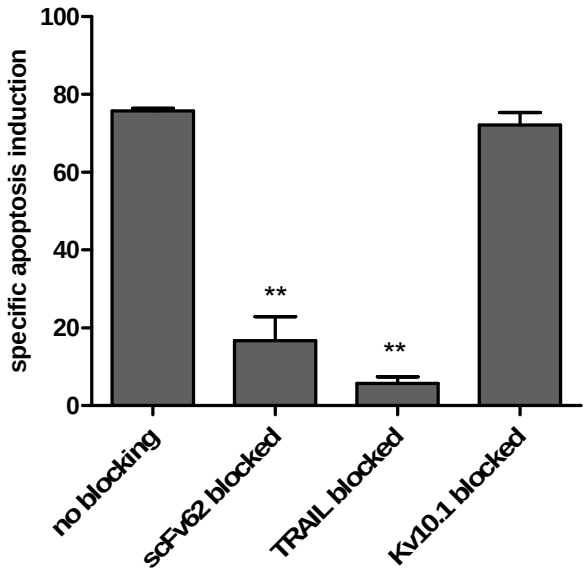


Figure 4.20 Specificity of scFv62-TRAIL induced apoptosis

50U of scFv62-TRAIL were incubated either with 50ng antigen h1x or 1µg mouse monoclonal anti-TRAIL antibody for 1h. DU145 cells were treated with blocked scFv62-TRAIL samples or with 50U/ml scFv62-TRAIL for 18h. $1x10^5$ DU145 cells were incubated for 1h with 100ng scFv62 and then treated with 50U/ml scFv62-TRAIL for 18h. Cells were analyzed by using Annexin V-FITC/PI staining and flow cytometry. Specific apoptosis induction was calculated as followed: Specific apoptosis = (experimental apoptosis - spontaneous apoptosis) / (100 - spontaneous apoptosis).

I calculated the specific apoptosis induction by subtraction of spontaneous apoptosis (Fig. 4.20). A strong reduction on apoptotic cells was observed by blocking the antibody and apoptosis was completely inhibited by blocking TRAIL. Furthermore, I tested if the binding site of scFv62-TRAIL on K_V10.1 on the cell surface can be blocked and inhibit the effect of scFv62-TRAIL. I incubated the DU145 cells with scFv62 antibody for 1h and afterwards treated them with scFv62-TRAIL and CHX. Apoptosis induction of scFv62-TRAIL treatment was not affected by preincubation with Kv10.1 antibody (Fig. 4.20).

Furthermore, I wanted to study the effect of scFv62-TRAIL on Kv10.1-negative cancer cells and normal cells, because it has been described that TRAIL only induces apoptosis on cancer while sparing normal cells. For this purpose, I analyzed the Kv10.1 expression in different prostate cell lines: LNCaP and PC3 are prostate cancer cell lines and PNT2 is an

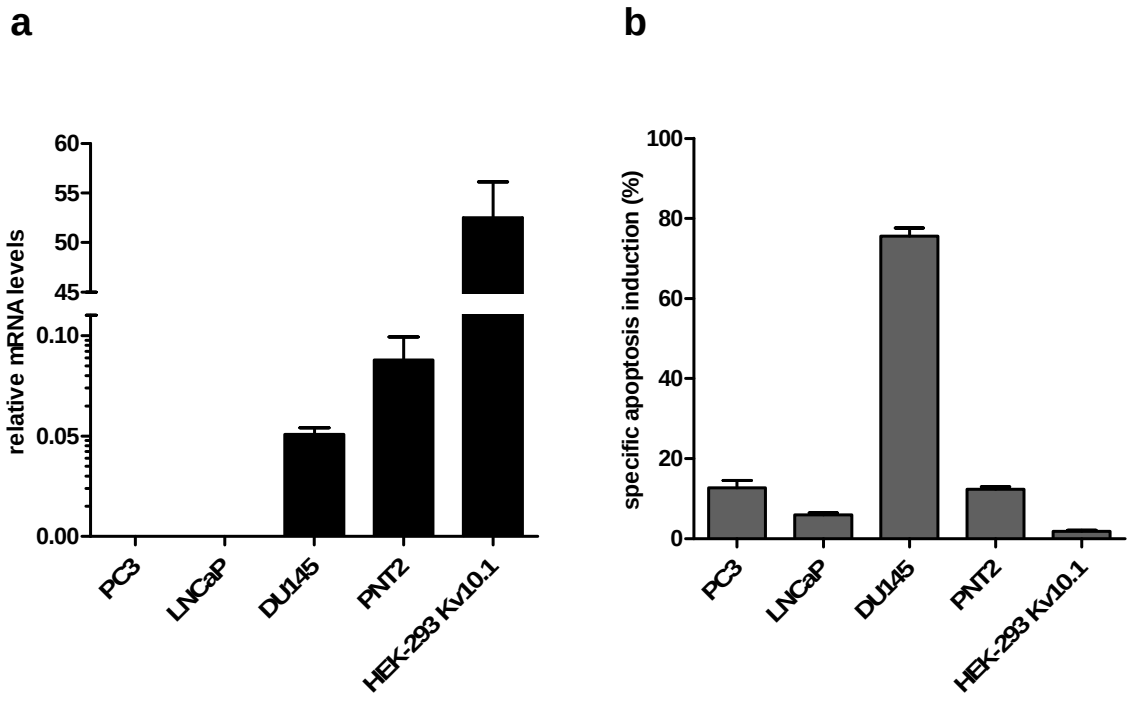


Figure 4.21 Kv10.1-specific apoptosis induction

a: Quantitative real-time analysis of Kv10.1 expression in different cell lines. b: Different cell lines were treated with 50U/ml scFv62-TRAIL in combination with 5µg/ml CHX. Apoptosis induction was analyzed by Annexin V-FITC/PI staining and flow cytometry.

immortalized normal prostate epithelial cell line (Fig. 4.21a). In contrast to DU145 cells, LNCaP and PC3 are Kv10.1-negative cancer cells. Kv10.1 mRNA could be detected in PNT2 cells, a non-cancer cell line, even in a higher level than DU145 cells. With these data I was interested to see, if scFv62-TRAIL affects cancer without Kv10.1 expression and if normal Kv10.1-expressing cells undergo apoptosis. I treated the different cancer cells with scFv62-TRAIL in combination with CHX and analyzed the apoptosis induction (Fig. 4.21b). As described earlier, HEK-293 Kv10.1 transfected cells were not sensitive to scFv62-TRAIL-induced apoptosis, and also PNT2 showed only a weak increase in apoptotic cells. In comparison to the intense apoptosis induction in DU145, the Kv10.1-negative cancer cells lines PC3 and LNCaP responded only modestly to scFv62-TRAIL treatment.

In summary, these experiments confirmed the specificity of scFv62-TRAIL and strongly indicated that both binding to Kv10.1 to the cell surface and an active TRAIL are required to induce apoptosis. Furthermore, I demonstrated that scFv62-TRAIL selectively kills Kv10.1-positive cancer cells, while not affecting normal cells whether they express Kv10.1 or not.

4.4 Analysis of TRAIL receptor expression

The TRAIL receptor system consists of 4 different membrane located receptors: two death-receptors TRAIL-R1 and TRAIL-R2 mediating the apoptosis signal and two decoy receptors TRAIL-R3 and TRAIL-R4 lacking the intracellular death-domains. The complexity of this receptor system suggests a highly regulated TRAIL-signaling and deregulation in cancer cells.

4.4.1 TRAIL receptor expression in different cell lines

TRAIL can bind to four membrane-associated receptors, whereas the apoptosis signal can only be mediated by the receptors TRAIL-R1 and R2. The TRAIL receptor expression pattern is one supposed reason for the developed TRAIL resistance in different cell lines [40]. Therefore, I analyzed the expression levels of the four TRAIL receptors in different cell lines by real-time PCR. The data were normalized to the housekeeping genes human transferrin receptor and beta-actin.

Concerning the decoy receptors the TRAIL-R3 was not expressed in PC3, DU145, PNT2, MDA-MB435S or SHSY-5Y cells and weakly expressed in LNCaP, hTERT-RPE1, HEK-293 wild type and Kv10.1 transfected, HT1080, HeLa, MDA-MB231, IGR39 and IPC298 cells (Fig. 4.22). In contrast TRAIL-R4 was detected at diverse expression levels with relatively high expression in the non-cancer cell lines hTERT, HEK-293 wild type and Kv10.1 transfected cells. In the cancer cell lines LNCaP, DU145, HeLa and MDA-MB231 TRAIL-R4 was expressed in same level as the two death receptors (Fig. 4.22). TRAIL-R4 mRNA was not detected in PC3, HT1080, MDA-MB435S, IGR39, IPC298 and SHSY-5Y cells (Fig. 4.22).

TRAIL-R2 was detected in all analyzed cell lines and in PC3, LNCaP, DU145, PNT2, hTERT-RPE1, HeLa, MDA-MB231, MDA-MB435S and IP298 cells expressed together with TRAIL-R1. Whereas TRAIL-R1 was slightly higher expressed in PC3, DU145 and MDA-MB231 cells than TRAIL-R2 and slightly lower expressed in PNT2, HeLa and IPC298 cells. MDA-MB435S cells showed equal amounts of TRAIL-R1 and R2 mRNA. The cell lines HT1080, SHSY-5Y and HEK-293 showed no TRAIL-R1 mRNA. Especially HT1080 cells exclusively showed high TRAIL-R2 expression without expressing any other TRAIL receptor (Fig. 4.22b).

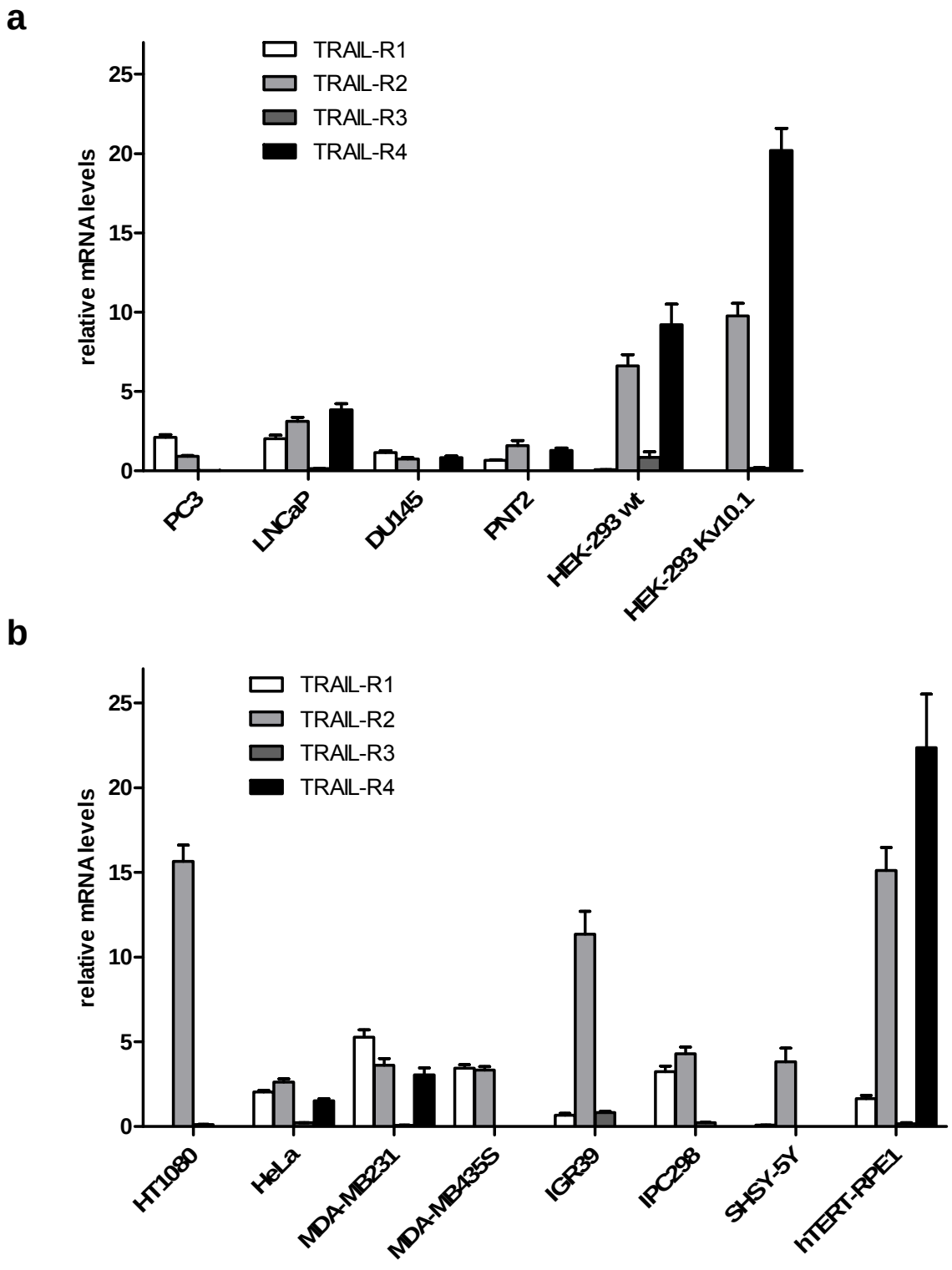


Figure 4.22 Analysis of TRAIL receptor expression in different cell lines

a: and b: RNA was isolated from different cell lines and real-time PCR was performed by using the UPL System from Roche and human transferrin receptor and beta-actin as reference genes.

Comparing the different prostate cancer cells, the expression levels of the two death receptors were relative low (Fig. 4.22a). TRAIL-R4 was only expressed in DU145 and LNCaP with higher expression level in LNCaP cells. I was not able to detect a strong difference between the normal prostate cell line PNT2 and the prostate cancer cells (Fig. 4.22a). Among non-cancer cells, HEK-293-Kv10.1 and hTERT-RPE1 cells showed very high TRAIL-R2 and TRAIL-R4 expression compared to the cancer cell lines (Fig. 4.22). Additionally, I observed increase in receptor expression in HEK-293 cells when transfected with Kv10.1 (Fig. 4.22a).

4.4.2 Effect of Kv10.1 downregulation on TRAIL receptor expression

Analysis of TRAIL receptor expression of HEK-293 Kv10.1 transfected and wild type cells showed an increase in the Kv10.1-overexpressing cells. To investigate the possible effect of Kv10.1 to TRAIL receptor expression DU145 and HT1080 cells were transfected with siRNA against Kv10.1. I confirmed by real-time PCR analysis a clear downregulation of Kv10.1 expression (Fig. 4.23a), but there was no or only a weak effect detectable on the TRAIL receptor mRNA (Fig. 4.23b).

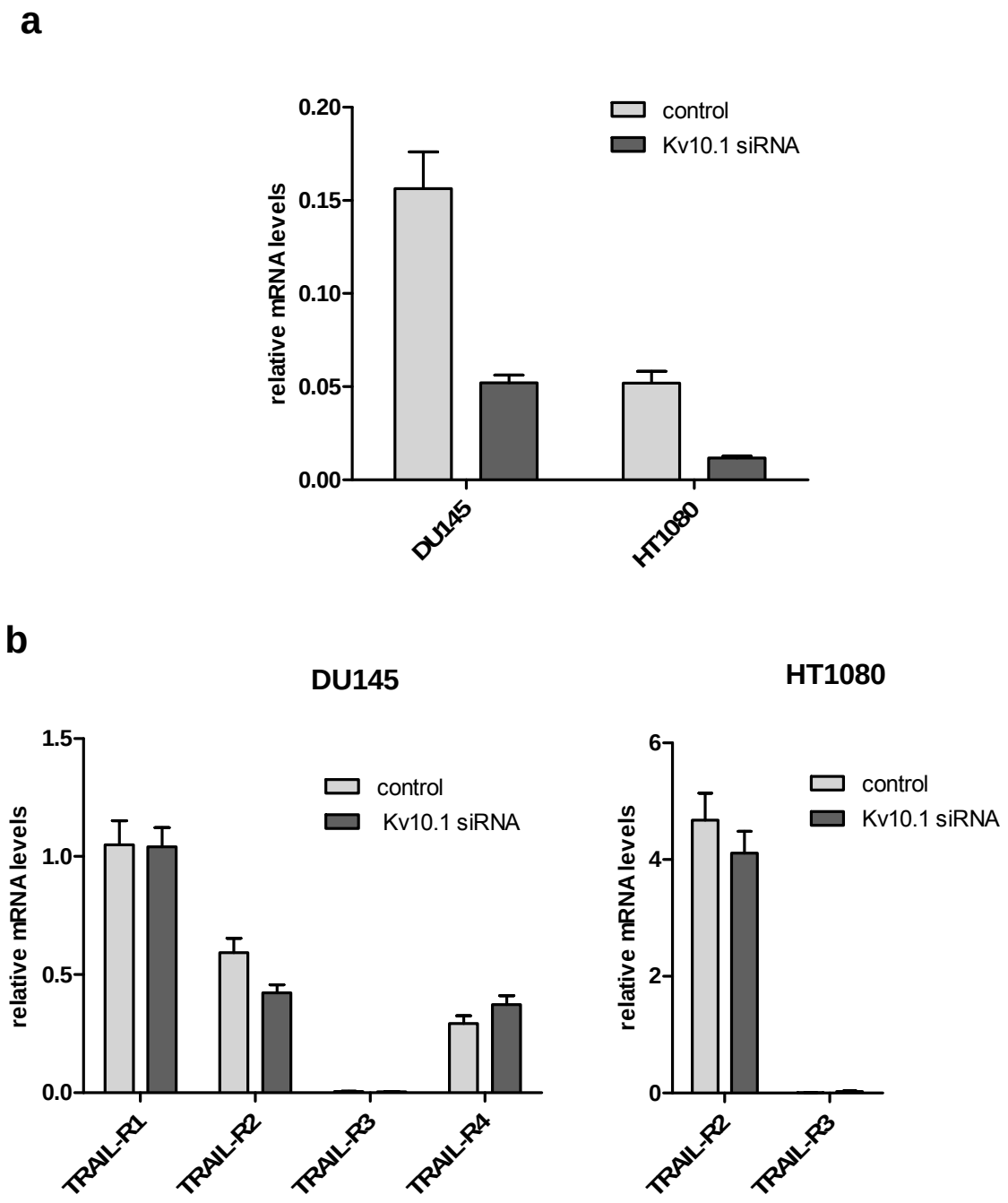


Figure 4.23 Effect of TRAIL receptor expression after Kv10.1 downregulation

a: 5 x 10^5 DU145 and HT1080 cells were transfected with anti-Kv10.1 siRNA, after 24h efficiency of Kv10.1 downregulation was determine by real-time PCR. b: Analysis of mRNA levels of the TRAIL-receptors after Kv10.1 downregulation.

4.4.3 Analysis of death receptor-mediated apoptosis induction

TRAIL-induced apoptosis can be mediated by binding to the death receptor TRAIL-R1 or TRAIL-R2. Therefore, I wanted to study whether scFv62-TRAIL induced apoptosis is mediated by TRAIL-R1, TRAIL-R2 or by both receptors.

I pre-incubated DU145 cells with 2µg of blocking antibodies against TRAIL-R1, TRAIL-R2 or both in combination and then treated them with scFv62-TRAIL and CHX. After this I analyzed the apoptotic cells using Annexin V-FITC/PI staining and flow cytometry (Fig. 4.24). Blocking of TRAIL-R1 reduced apoptosis induction by scFv62-TRAIL by 20%; blocking of TRAIL-R2 and both receptors resulted in a 30% apoptosis reduction. These effects

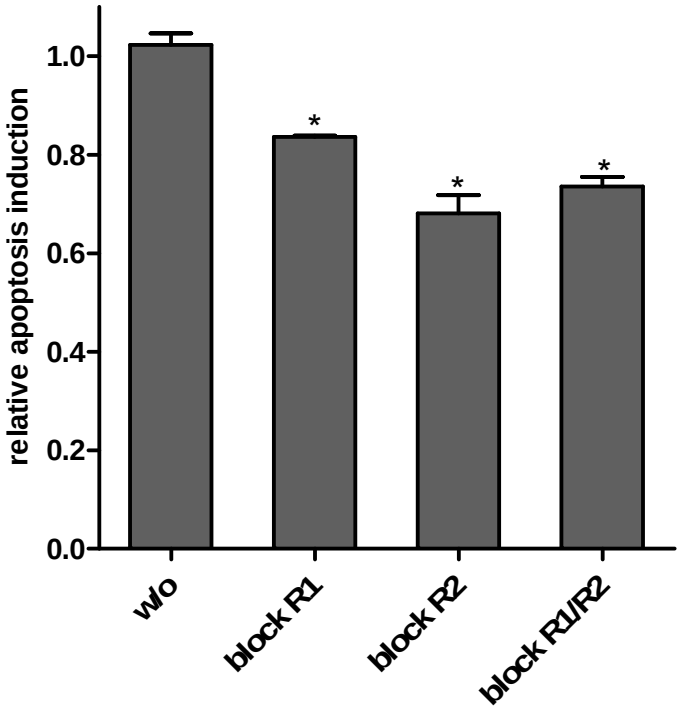


Figure 4.24 Blocking of TRAIL receptors

1 x 10^5 DU145 cells were incubated with 2µg mouse anti-TRAIL-R1, anti-TRAIL-R2 or both for 1h at 37°C and afterwards treated for 18h with 50U/ml scFv62-TRAIL in presence of 5µg/ml CHX. Apoptosis was analyzed by Annexin V-FITC/PI staining and flow cytometry. Relative apoptosis induction was calculated.

suggest that apoptosis induced by scFv62-TRAIL can be mediated by both receptors. However, the slight reduction of the apoptosis rate can also be a sign for incomplete blocking of the TRAIL-receptors using the specific antibodies. For this reason I decided to use siRNA to downregulate the expression of the two death receptors.

DU145 cells were transfected with siRNA against TRAIL-R1, TRAIL-R2 or both receptors. After 24h and 48h, the mRNA levels of the different receptors were determined using real-time PCR (Fig. 4.25). Effective downregulation of both receptors was already observed after 24h. DU145 cells were treated with scFv62-TRAIL in combination with CHX 24h after TRAIL receptors downregulation. Apoptosis induction was analyzed by Annexin V/PI-staining and flow cytometry (Fig. 4.26a). The apoptosis induction was reduced by 30% after downregulating TRAIL-R1 or both death receptors, whereas downregulation of TRAIL-R2 weakly affected the apoptotic signal.

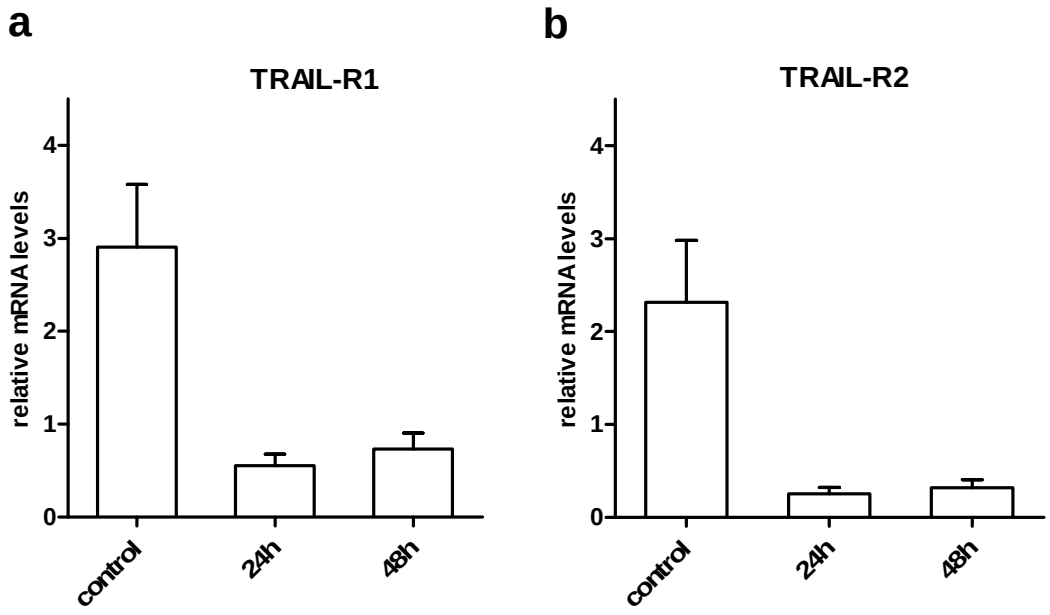


Figure 4.25 Analysis of TRAIL receptor downregulation

1 x 10^5 DU145 cell were transfected with siRNA against TRAIL-R1 (a) or TRAIL-R2 (b) using Lipofectamine RNAiMax and incubated for 24h and 48h. Analysis of protein expression was performed by real-time PCR.

Because of apoptosis reduction by blocking TRAIL-R2 with an antibody (Fig. 4.24) I expected also stronger reduced apoptosis induction after downregulating the TRAIL-R2. Therefore, I wanted to elucidate an influence of siRNA-mediated inhibition of protein synthesis on the expression of other death receptor (Fig. 4.26b). I detected an upregulation of TRAIL-R1 when TRAIL-R2 expression was downregulated and a slight reduction of TRAIL-R2 after downregulation of TRAIL-R1. The total amount of death receptors in cells with TRAIL-R2 downregulation is almost the same than in the control cell, which would explain the weak reduction in the apoptosis induction.

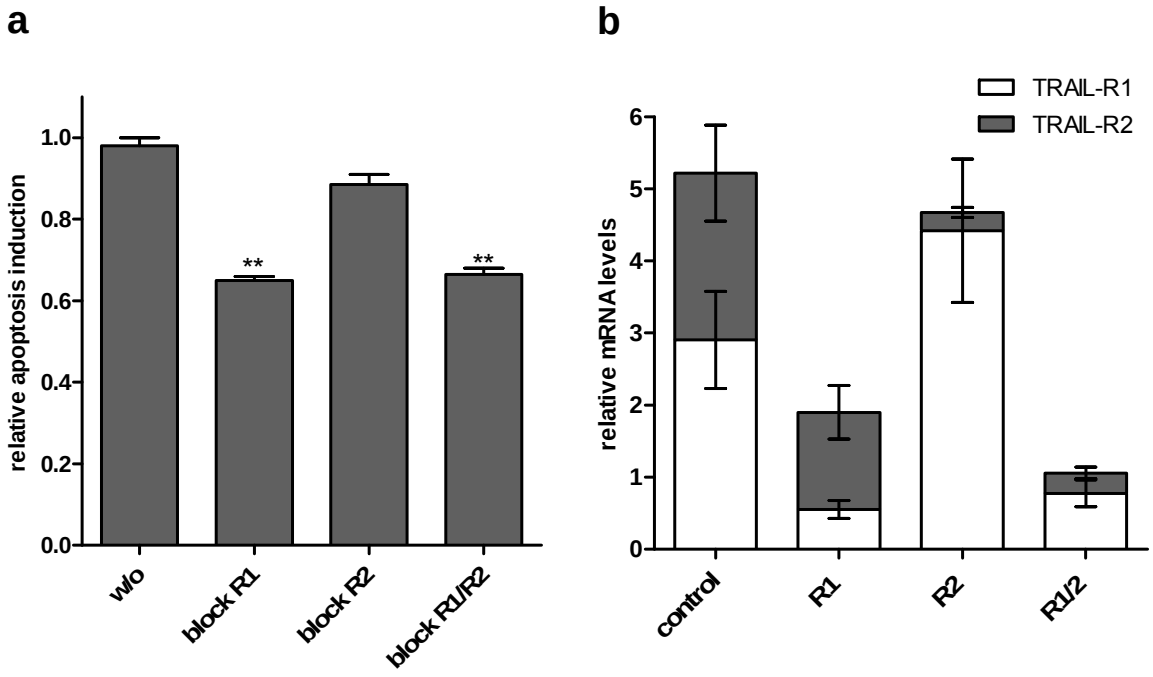


Figure 4.26 Analysis of death receptor mediated apoptosis induction

$2x10^5$ DU145 cells were transfected with siRNA against TRAIL-R1, TRAIL-R or both. a: After 24h cells were treated with 50U/ml scFv62-TRAIL in combination with 5µg/ml CHX and analyzed for apoptosis induction. b: Real-time PCR analysis of TRAIL-R1 and TRAIL-R2 24h after siRNA transfection.

4.4.4 Bystander effect of scFv62-TRAIL

Binding of scFv62-TRAIL to K_V10.1 results in accretion at the cell surface. Apoptosis can be induced in autocrine manner by binding to TRAIL receptors on the same cell or by binding to receptors of a neighboring cell, resulting in paracrine cell apoptosis. In this way also neighboring tumor cells devoid or with low expression of the target antigen can be effectively eliminated by the so-called bystander effect (Fig. 4.27).

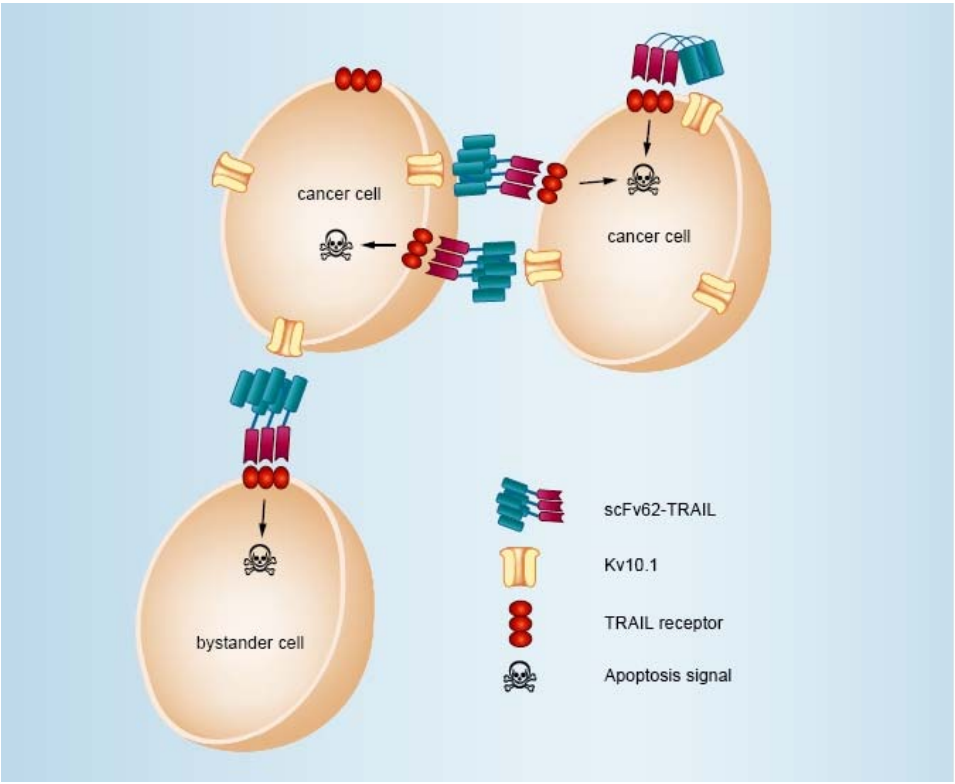


Figure 4.27 Illustration of the scFv62-TRAIL bystander effect

Binding of scFv62-TRAIL to Kv10.1 results in the membrane-bound form of TRAIL (memTRAIL). Thereby, it is possible to induce apoptosis on the same cell (paracrine) or in a neighboring cell (autocrine) independent of Kv10.1 expression. This targeting of Kv10.1-negative cells is referred as bystander effect.

To study this effect a DU145 cell line stable expressing the fluorescent protein Venus was generated. DU145-venus was co-cultured with PC3 (Kv10.1-negative prostate cancer cells) or PNT2 (Kv10.1-positive prostate epithelia cells) and the cells were treated with scFv62-TRAIL in combination with CHX. With the 488nm laser and special filter settings of the flow cytometer I could distinguish between the venus-positive target cells and venus-negative bystander cells (Fig. 4.28a). Apoptosis induction was analyzed using Annexin V-Alexa680/PI and flow cytometry. In the co-cultures the DU145 cells underwent apoptosis with similar intensity than in single culture (Fig. 4.28b). The non-cancer cell line PNT2 remained insensitive to scFv62-TRAIL also when co-cultured with DU145 cells (Fig. 4.28b). In contrast, by co-culturing the Kv10.1-negative cancer cell line PC3 with DU145 cells the amount of apoptotic PC3 cells was doubled (Fig. 4.28b).

In summary, these data indicate that scFv62-TRAIL induces apoptosis in Kv10.1-positive cells and in bystander cancer cells that do not express Kv10.1, but leaving normal cells intact, independently of Kv10.1 expression.

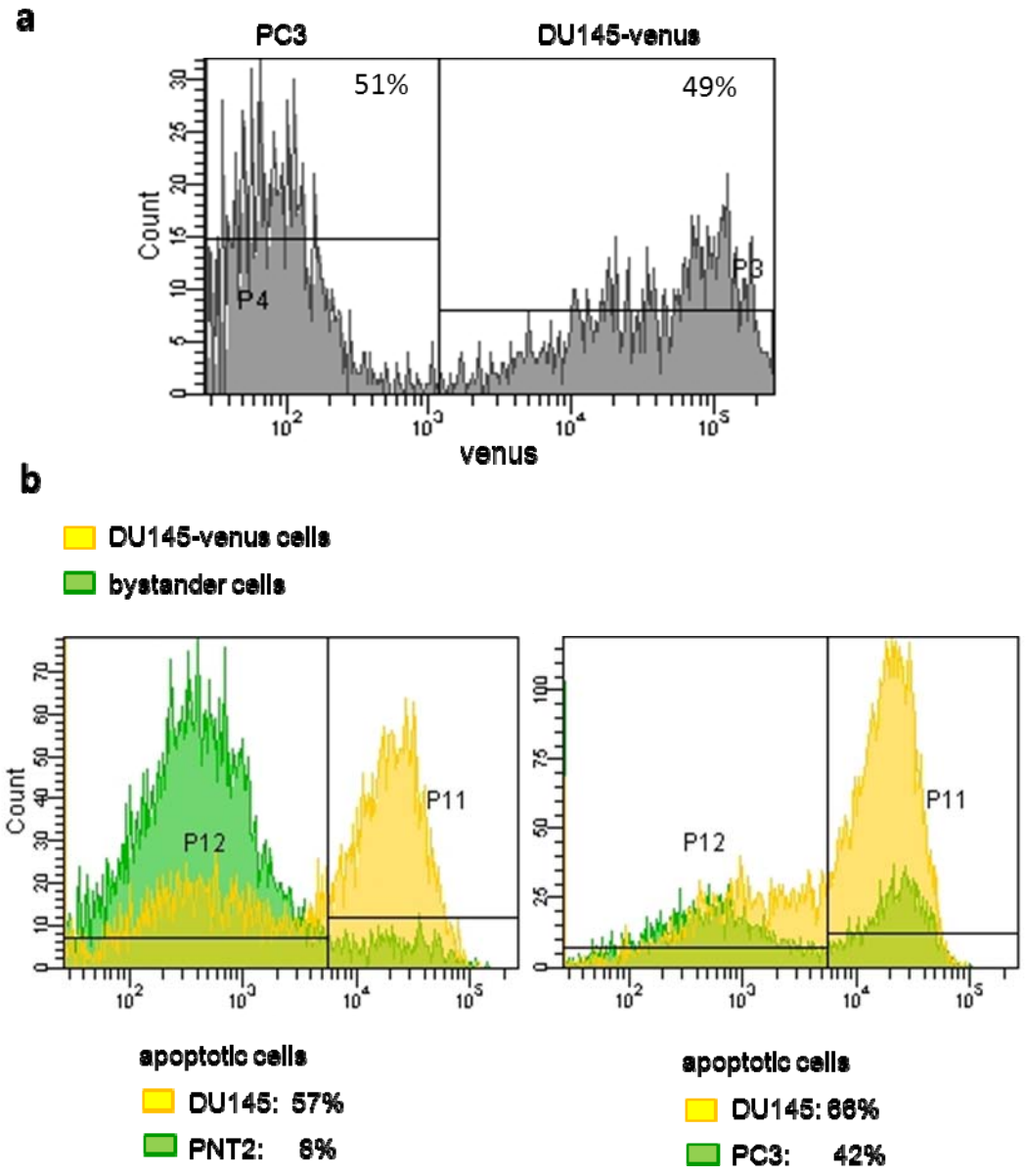


Figure 4.28 Potent bystander effect of scFv62-TRAIL

a: Venus flow cytometry histogram. DU145-venus cells were co-cultured with PC3 cells and analyzed for Venus fluorescence by flow cytometry. b: $1x10^5$ PNT2 and PC3 cells were co-cultured with $1x10^5$ DU145 cells and treated with 50U/ml scFv62-TRAIL in combination with 5µg/ml CHX for 18h.

4.5 Evaluation of scFv62-TRAIL application *in vivo*

There are special requirements for an efficient *in vivo* therapeutic agent and it is important to characterize a novel antibody to confirm suitability and safety for further clinical development. The main part of my thesis was the cloning of the scFv62-TRAIL antibody and establishment of an optimal antibody expression system. With the CHO-K1 cells I obtained enough scFv62-TRAIL to analyze the activity and potent apoptosis induction potential on cancer cells *in vitro*. The following part evaluates the scFv62-TRAIL for *in vivo* application.

4.5.1 Stability of scFv62-TRAIL

Agents for *in vivo* applications need to be stable and functionally active under *in vivo* conditions to reach the target cells or tissues and develop their function. To study the stability of the scFv62-TRAIL construct, aliquots of the antibody solution were incubated up to 72h at 37°C in mouse serum. The biological activity of the resulting material was tested on DU145 cells (Fig. 4.29). After 48h and 72h of incubation at 37°C without serum, a reduction of 5-10% in the apoptosis induction potential was observed (Fig. 4.29a). The presence of mouse serum reduced the activity of scFv62-TRAIL to 50% after 48h and to 40% after 72h (Fig. 4.29b).

Together, the determined stability of scFv62-TRAIL at 37°C and in mouse serum is sufficient for *in vivo* applications.

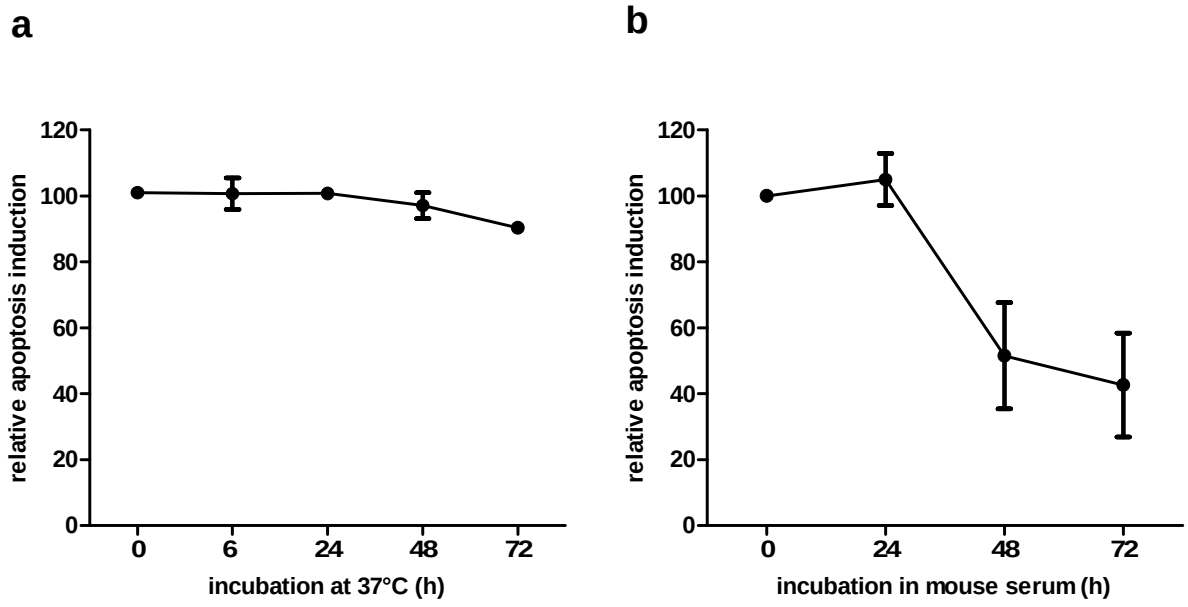


Figure 4.29 Stability of scFv62-TRAIL

a: scFv62-TRAIL was incubated at 37°C or b: mixed 1:1 with mouse serum and incubated at 37°C for different time periods. Remaining apoptosis induction was analyzed on DU145 cells using Annexin V-FITC/PI staining and flow cytometry.

4.5.2 Combination of scFv62-TRAIL with different sensitizing agents

In the previous *in vitro* experiments I was able to show that scFv62-TRAIL induces potent apoptosis only in presence of CHX. Because of the described high toxicity of CHX in *in vivo* animal models, I further analyzed various common used chemotherapeutics as potential sensitizers for scFv62-TRAIL treatment.

For this purpose DU145 cells were treated with cisplatin, 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG), actinomycin D, doxorubicin, etoposide, roscovitine or 5-fluorouracil and scFv62-TRAIL for 18h and the amount of apoptotic cells were analyzed by flow cytometry (Fig. 4.30). The combinational treatments with cisplatin and 17-AAG did not

induce increased apoptosis. Actinomycin D, doxorubicin and roscovitine increased apoptotic cells by 10% when applied in combination with scFv62-TRAIL, but actinomycin D alone also induced apoptosis. The strongest effect of scFv62-TRAIL was observed in combination with etoposide (62%) and 5-fluorouracil (35%).

Furthermore, I wanted to know if the various agents affect the expression of the TRAIL receptors or Kv10.1 and thereby sensitizing the cancer cells for scFv62-TRAIL induced apoptosis. To verify this DU145 cells were treated with different chemotherapeutics and analyzed the mRNA levels of Kv10.1 and the TRAIL receptors (Fig. 4.31). Cell treatment with etoposide increased the expression of TRAIL-R1 and R2 after 20h (Fig. 4.31a und b). Beside doxorubicin, which increased TRAIL-R2 expression, no significant effect of the other chemotherapeutic agents was observed.

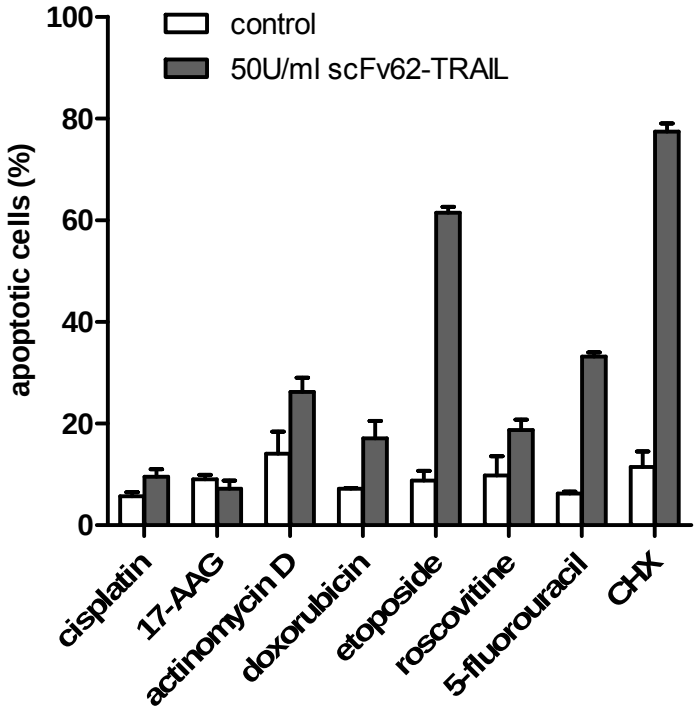


Figure 4.30 scFv62-TRAIL in combination with different chemotherapeutics

DU145 cells were treated for 18h with or without 50U/ml scFv62-TRAIL in combination with different chemotherapeutics: cisplatin (10µM), 17-AAG (5µM), actinomycin (800nM), doxorubicin (1.8µM), etoposide (75µM), roscovitine (10µM), 5-fluorouracil (100µM), cycloheximide (CHX) (5µg/ml). Apoptosis induction was analyzed using Annexin V-FITC or Alexa648/PI staining and flow cytometry.

Surprisingly, a downregulation of Kv10.1 expression was detected 4h and 20h after treatment of DU145 cells with etoposide and an even stronger downregulation after doxorubicin treatment (Fig. 4.31c). In contrast, treatment with 5-fluorouracil and roscovitine resulted in increased Kv10.1 mRNA expression levels.

The highest apoptosis induction was observed with scFv62-TRAIL in combination with etoposide. The real-time analysis showed an increase in death receptor expression after incubation with etoposide which can explain the restored sensitivity to TRAIL-induced apoptosis in combinational treatment. Taking this into account I asked whether a pre-treatment with etoposide can intensify the apoptosis induction. Therefore, DU145 cells were pre-treated with a lower concentration of etoposide 12h before adding the scFv62-TRAIL antibody. After 18h the apoptosis induction was analyzed with Annexin V-FITC/PI staining and flow cytometry (Fig. 4.32a). An increase in the total amount of apoptotic cells when etoposide was applied 12h before scFv62-TRAIL treatment could be observed. Comparing the apoptotic stages the late apoptotic cells were strongly increased in the pre-treated sample suggesting a faster onset of the apoptotic signaling. Furthermore, various time periods of etoposide treatment were analyzed (Fig. 4.32b). Pre-treatment with etoposide and following incubation with scFv62-TRAIL induces an increasing apoptosis induction showing the strongest effect after 24h.

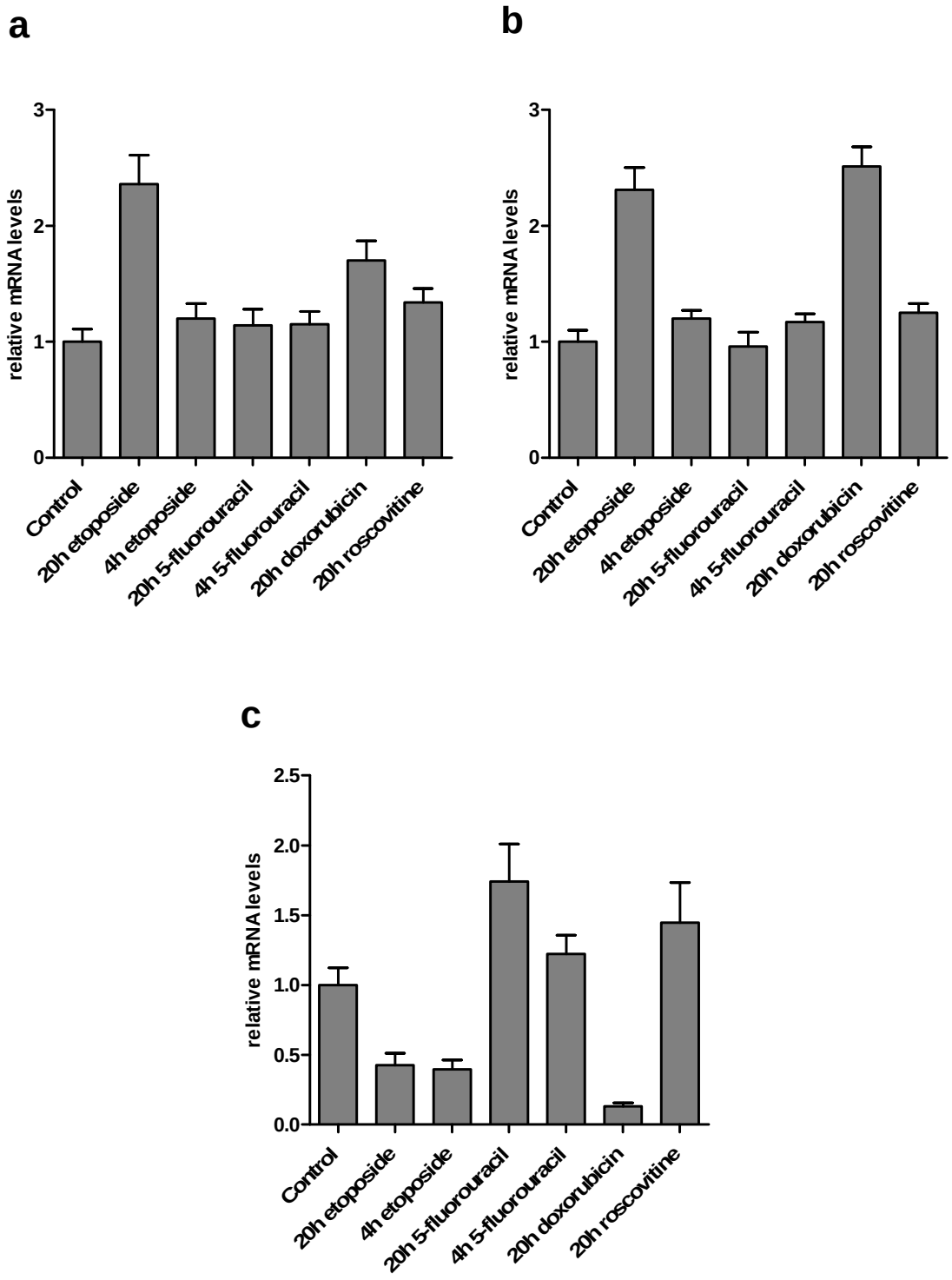


Figure 4.31 Effect of chemotherapeutics on TRAIL-R1, TRAIL-R2 and Kv10.1 expression

After treatment of DU145 cells for 4h or 20h with etoposide (50µM), 5-fluorouracil (100µM), doxorubicin (1.8µmM) and roscovitine (10µM), cells were analyzed with real-time PCR for expression of a: TRAIL-R1, b: TRAIL-R2 and c: Kv10.1.

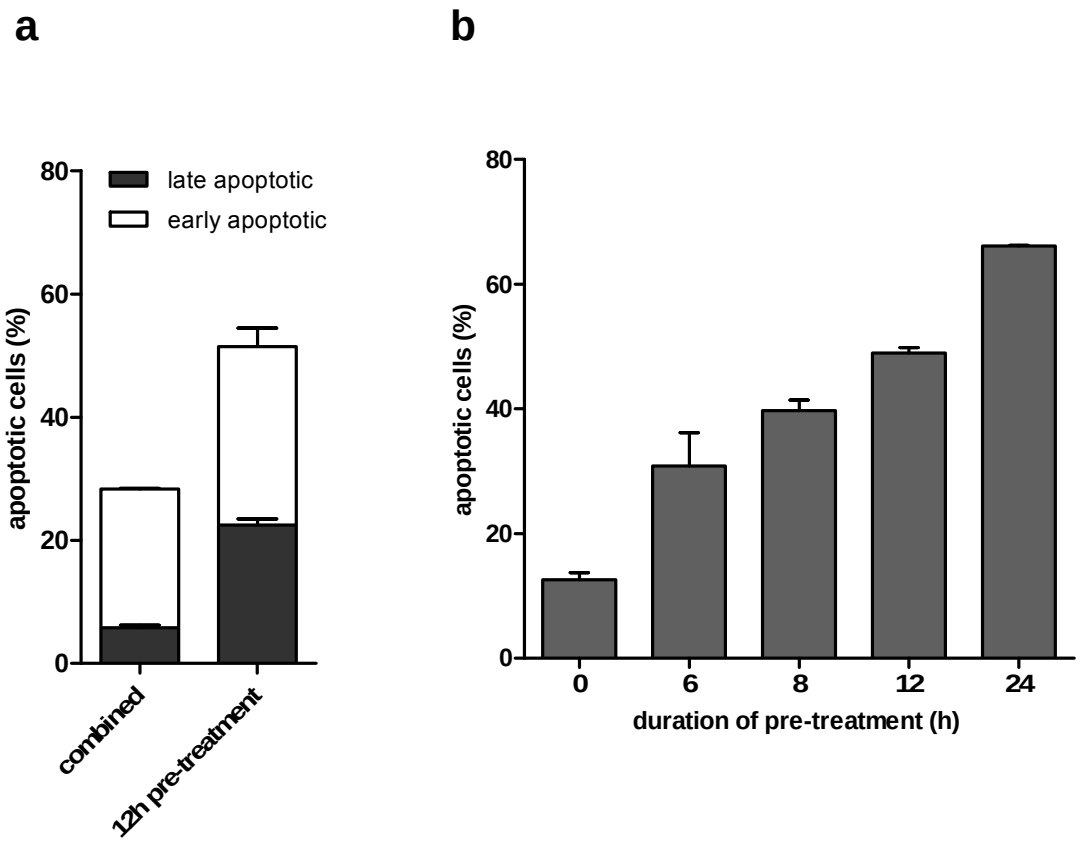


Figure 4.32 Pre-treatment with etoposide

a: 2x10^5 DU145 cells were treated with 5U/ml scFv62-TRAIL in combination with 50µM etoposide or 12h pre-treatment with 50µM etoposide. Amount of early and late apoptotic cells were analyzed after 20h using Annexin V-FITC/PI staining and flow cytometry. b: 2x10^4 DU145 cells were pre-treated with 50µM etoposide at different time points before scFv62-TRAIL (50U/ml) treatment. Amount of apoptotic cells was analyzed using Annexin V-FITC/PI staining and flow cytometry.

In summary, various chemotherapeutic agents were analyzed and etoposide resulted in a potent sensitizing agent for combinational treatment with scFv62-TRAIL that can be used *in vivo*. The effect of etoposide on the expression of TRAIL-R1 and R2 can explain the restored susceptibility to scFv62-TRAIL-mediated apoptosis and the increasing apoptosis after etoposide pre-treatment.

In this last part I described the stability properties of scFv62-TRAIL and different combinational treatments with chemotherapeutic agents related to *in vivo* applications. The determined stability at 37°C and in mouse serum is sufficient for *in vivo* use. As sensitizing agent CHX exhibit a high toxicity, but etoposide was identified as a potent sensitizing agent for combinational treatments with scFv62-TRAIL that can also be used *in vivo*. Further studies using tumor-bearing mice need to be done to clearly confirm the selective anti-tumor activity of scFv62-TRAIL.

5 Discussion

Since many years the concept of ion channels as therapeutic targets and prognostic biomarkers has attracted increasing interest. More than 10 years ago the voltage-gated potassium channel Kv10.1 was found to be expressed on cancer cells [70]. Today, Kv10.1 is referred as a tumor marker even though the function and involvement of this channel in cancer progression is not completely elucidated [86]. However, the cancer cell selective expression turns Kv10.1 into a potential target for cancer therapy. The aim of this study was the design of a Kv10.1-specific therapeutic strategy using a single-chain antibody fused to the apoptosis-inducing ligand TRAIL.

5.1 Antibody therapy

Antibodies are essential tools in research and clinical application. The specificity and high affinity to the antigen are the main attractive features of antibodies. Advances in the antibody research field and recombinant protein technology allow the generation and production of antibodies or fragments practically against any protein. Important for an effective antibody-based therapy is not only the targeted antigen but also antibody structure and the feature of an optional payload.

5.1.1 Antibody engineering for improved cancer therapy

The search of effective cancer therapeutics is greater than ever. In comparison to conventional chemotherapy, antibody-based therapy is more effective and has fewer side effects because of the selectivity and specificity of this approach. However, since antibodies

are mainly derived from mouse hybridomas the clinical application has been greatly restricted due to production of human anti-mouse antibody (HAMA) in patients during therapy [6]. This immunogenicity of mouse antibodies mainly relies in its constant region (Fc) and can be reduced by generating humanized antibodies. An alternative method is the construction of single-chain antibodies (scFv), were the Fc region is removed and thereby the immunogenicity reduced. Additionally, scFv fragments offer other advantages which make them attractive for therapeutic use, like better tissue penetration, reduced uptake in the liver mediated by specific Fc-receptors and that they can be easier produced or labeled [5]. Therefore, I used a single-chain antibody for analyzing different fusion constructs for use in cancer therapy.

The idea of killing cancer cells using antibodies presupposes that tumor cells have target molecules on their surface, which are absent in normal cells. Many tumor-associated antigens are overexpressed on tumor cells, but also present on normal cells even at lower expression levels. The resulting side-effects against non-tumor cells narrowed the promising potential of antibody-based therapy.

In this study I wanted to prove a new anti-cancer therapy by combining a basic therapeutic antibody strategy with a novel established tumor marker.

5.1.2 Kv10.1 as a target for antibody-cancer therapy

The voltage-gated potassium channel K_V10.1 is normally expressed in the CNS and is involved in signal transduction and controlling the resting potential of neurons [74]. It has been found that Kv10.1 also controls many basic cellular processes, including cell cycle and cell proliferation [71, 87]. Moreover the analysis of various cancer cell lines and tumor tissues demonstrated Kv10.1 expression with frequency of 70% [75-77, 88-91]. Different blocking experiments and prognostic studies confirmed the oncogenic potential of Kv10.1 [78, 80].

Additionally, a current study by Lin and colleagues demonstrated the negative influence of the tumor suppressor gene p53 on Kv10.1 expression [92]. Consequently, mutation of p53 in nearly all cancer cells results in oncogenic overexpression of Kv10.1 as an effect of p53 inactivation [31, 92, 93]. The described upregulation of the hypoxia-inducible factor 1 (HIF-1) and vascular endothelia growth factor (VEGF) emphasize a growth advantage of Kv10.1-expressing cells because of increased vascularization and therefore better nutrient and oxygen supply [79]. In my work I used different tumor cell lines and confirmed the Kv10.1 expression by real-time PCR. Interestingly, by focusing more on prostate cancer K_V10.1 was detected also in normal prostate epithelia cells. I assume that the expression of Kv10.1 in these cells is an effect of the SV40 virus transfection, which is described to influence p53 and thereby up-regulate K_V10.1.

The idea of targeting Kv10.1 for therapy is not new, since this channel has been designated as a tumor marker. Due to its surface expression this ion channel can be easily targeted from the external cell environment by e.g. channel blockers. Astemizole and imipramine are Kv10.1 blockers, which were shown to decrease tumor cell proliferation *in vitro* and *in vivo* [82]. Unfortunately, the pore region of Kv10.1 and Kv11.1 (HERG) are similar, so that all known Kv10.1 blockers are also effective blockers of Kv11.1, which represents a cardiac risk. The development of a specific antibody, which selectively blocks Kv10.1, should solve the problem of unwanted side-effects. This antibody showed inhibition of tumor cell growth *in vitro* and *in vivo* [80]. Nevertheless, for effective elimination of tumors it is not sufficient to just reduce the tumor progression, but induction of tumor regression clearly improves the therapy. Therefore, I wanted to design an antibody-mediated strategy to selective destroy cancer cells using Kv10.1 as a target.

The monoclonal antibody mAb62, which binds to the pore region of Kv10.1 was selected. This antibody has been characterized intensively and recognizes human, rat and mouse Kv10.1, since a conserved epitope was used. The single-chain antibody scFv62 fused

to the enzyme phosphatase A was analyzed and already used for expression studies in neuronal and tumor tissue [74, 75]. Experiments with Cy5.5-labeled scFv62 in tumor-bearing mice confirmed the stability and specificity of this antibody *in vivo* [94]. Hence, I decided to use the scFv62 for construction of therapeutic antibody fusions.

5.1.3 Therapeutic antibody-TRAIL fusion

The direct activation of the apoptotic machinery in cancer cells has become an attractive therapeutic strategy. Beside the other members of the TNF family, TRAIL has generated great enthusiasm because it selectively induces apoptosis in various malignant cells, while sparing normal cells. Cytotoxicity and enhanced survival or even proliferation of resistant tumor cells hampered the clinical used of soluble TRAIL. Additionally, the short half-life and rapid blood clearance are drawback of sTRAIL *in vivo* [33, 34]. Agonistic anti-TRAIL-R mAbs against TRAIL-R1 (mapatumumab) or TRAIL-R2 (lexatumumab) have been designed to overcome these limitations, because in general mAb have a longer half-life than recombinant proteins [95, 96]. Another approach is the fusion of TRAIL to a tumor-selective antibody as a strategy for antibody-mediated cancer therapy.

Several features make the human TRAIL to a promising candidate for antibody-mediated cancer therapy, e. g. tumor-selectivity. Additionally, in comparison to antibody-fused toxins, human TRAIL is less immunogenic, because it will be not detected as foreign substance by the immune system. There is also no internalization necessary and the TRAIL receptors are ubiquitously expressed.

5.1.4 Expression and purification of scFv62-TRAIL construct

Recombinant antibody fusions have been produced in various expression systems, such as bacteria, yeast, plants, insect cells or mammalian cells [7]. The expression strategy needs to be optimized for each antibody. Single-chain antibodies consist only of the variable light (V_L) and heavy chain (V_H) of a whole antibody and they do not contain posttranslational modifications. Therefore scFv antibodies are mostly expressed in bacterial expression systems, like *E.coli*, because it is the easiest and cheapest protein expression system. V_L and V_H are normally not covalently linked and therefore peptide linkers are used to connect the chains. Peptide linkers range from 10 to 25 amino acids in length and typically include hydrophilic amino acids. The scFv62 contains the most common used $(Gly_4Ser)_3$ linker. Glycin and serine as non-charged amino acids allow a high flexibility. The length of 15 amino acids allows optimal refolding, shorter linker would prevent V_H interaction with V_L [97].

It has been shown that C-terminal fusion to scFv62 does not change binding capacity to Kv10.1 [75]. Therefore, I genetically linked the C-terminus of the antibody to the N-terminus of TRAIL with a serine-glycine peptide linker (SSGSG). The soluble form of TRAIL is a single-chain protein consisting of around 190 amino acids. The recombinant scFv62-TRAIL fusion construct has a size of around 51kDa without posttranslational modifications, which allows production in *E.coli*. For effective expression scFv62-TRAIL has to be correctly folded and stabilized by intramolecular disulfide bonds. I used a special expression vector that contains the OmpA signal sequence. This signal sequence is expressed in frame with the antibody and leads to secretion into the periplasmic space, where it is cleaved off by bacterial enzymes. The periplasma has an oxidizing milieu and contains chaperones, which inhibit aggregation and support the formation of disulfide bonds. Additionally, proteins can be easier purified just by opening the bacterial cell membrane.

The scFv62-TRAIL construct expressed in bacteria contains 6xHis-tag to facilitate the purification. Interestingly, I found scFv62-TRAIL protein mainly packed in inclusion bodies

and just a small portion expressed in the periplasmic space. Therefore, for purifying proteins from inclusion bodies a denaturing and extensive refolding procedure by a stepwise dialysis was performed. The most occurring problem of scFv antibodies during refolding is the formation of aggregates. There are different strategies to optimize protein folding and reduce protein aggregation in solubilized inclusion bodies. I performed a stepwise dialyzing procedure based on the protocol of Umetsu and colleagues, where the dialysis buffer are supplemented with L-arginine and oxidized glutathione [85]. Whereas the oxidized glutathione supports and stabilizes disulfide bond formation, the theoretical basis of L-arginine is unknown. But it has been suggested, that it inhibits the association of partial folded proteins.

I performed different purification methods alone or in combination to clean up the scFv62-TRAIL from the bacterial expressed preparation, but it was not possible to remove all residual bacterial proteins. Especially the antigen-coupled column did not show any binding of scFv62-TRAIL construct. Possible explanations could be the presence of aggregates. Furthermore, TRAIL in its active form has a trimeric structure and the scFv62-TRAIL trimer with a size of around 150kDa can be too large for binding to the column. The size exclusion chromatography under denaturing conditions is another evidence for the presence of aggregates, because the inclusion bodies seemed to be not completely solubilized. Because I could not find any solution to overcome the purification problems of the bacterial expression system I decided to switch to eukaryotic protein expression.

In comparison to *E.coli* a smaller yield of product is achieved when using eukaryotic cells, but is compensated by a higher quality of the product, proper folding and decreased tendency to aggregate. To produce the scFv62-TRAIL in mammalian cells I used the pSecTag2A expression vector, which carries the murine kappa light-chain leader peptide upstream of the MCS resulting in excretion of the protein to the culture supernatant. I decided to express scFv62-TRAIL without any tags, since the C-terminus of TRAIL seems to be important for binding to its receptor.

Many factors can influence the expression efficiency of proteins in mammalian cells. For this reason I analyzed two cell lines, CHO-K1 and HEK-293, and different reagents for transfecting the expression vector. Both cell lines are used for antibody expression in general, but I found a better expression in transient transfected CHO-K1 cells. Since other studies demonstrated higher protein expression in HEK-293 cells or equal protein amount in both cell lines, the optimized expression cell needs to be evaluated for every recombinant protein. Differences in protein expression levels depend on the transfection agent, which is described in many expression studies. Consequently it is no surprising that higher expression with FuGene could be observed. To avoid batch-to-batch variations CHO-K1 cells were stable transfected and selected for monoclones with high scFv62-TRAIL expression. I analyzed the influence of temperature on protein expression based on a study, where lowering the culture temperature increased the productivity of cells [98]. During expression the CHO-K1 cells were incubated at 30°C and I observed an increase of scFv62-TRAIL production. Shi and colleagues found that lowering the culturing temperature leads to increased lag time so that the growth rate is reduced and thereby increasing the protein production [98]. Other studies suggested that cell growth and antibody production in hybridoma cells can be separated and demonstrated as well as an inverse correlation between the growth rate of cells and secretion of antibodies [99]. In addition to lowering the temperature I used a special serum- and protein-free medium, which simplifies the purification but also results in reduced proliferation during the first days.

Despite the low concentration of other proteins in the medium supernatant of the CHO-K1 cells expressing scFv62-TRAIL, the purification procedure using antigen-coupled affinity column failed. Since I could determine the concentration of scFv62-TRAIL in the medium using antigen-based ELISA, I could exclude, that the antibody binding capacity is altered. Therefore, I conclude that the size or the steric configuration of the active scFv62-TRAIL trimer inhibit the binding to the column during flow-through. By gel filtration I did not

detect monomers or dimers of scFv62-TRAIL and I saw a clear TRAIL signal on the non-reduced immunoblot, compatible with the size of trimeric scFv62-TRAIL. Purification of the scFv62-TRAIL construct still allows significant improvement. For the purpose of this study, I decided to focus more on the characterization of the construct and to prove the therapeutic strategy. As it has been described previously for other antibody-TRAIL constructs I used the concentrated medium containing scFv62-TRAIL for further analysis [63, 100].

5.2 Analysis of scFv62-TRAIL – cancer selectivity and resistance

The TRAIL apoptosis pathway acts independently of p53, which makes it a potentially effective weapon against chemoresistant or radioresistant tumors. Different cancer types developed resistance against TRAIL by downregulating the death receptors or by upregulation of anti-apoptotic proteins. I analyzed the effect of scFv62-TRAIL in combination with different chemotherapeutics for sensitizing on various cancer cell lines.

5.2.1 Sensitizing to overcome TRAIL resistance in cancer cells

Apoptosis is a highly regulated process consisting of different signaling pathways. An apoptotic cell is characterized by specific features, which can be used to identify them. Induction of apoptosis via death receptors typically results in activation of the caspase cascade. I initially used an active caspase-3/7 assay, which can be performed in 96-well format and allow high-throughput screenings. Unfortunately, this method turned out to be unsuccessful because of the non-specific presence of caspase-3 in the scFv62-TRAIL preparation, apparently integrated in high-molecular-weight complexes. It is suspected that during production of proteins, many CHO-K1 cells undergo normal apoptosis strengthened by incubation at lower temperatures and minimal medium. Apoptotic proteins of lysed cells are

then released into the medium. Caspase-3 production is not related to TRAIL in the scFv62-TRAIL expression, because it is also detected in the scFv62 preparation without TRAIL fusion. This effect is important and needs be considered in all other studies using similar protein preparations and caspase assays. To avoid unspecific signals, further apoptosis measurements were performed using Annexin V/PI-staining and flow cytometry. This is an active-caspase-3 independent method.

TRAIL selectively kills a variety of tumor cells while sparing normal cells from apoptosis. I selected different Kv10.1-positive tumor cell lines derived from various tumor types. For most of them it is known that they are resistant against sTRAIL treatment. All tested cell lines are relative resistant against low doses (50U/ml) of scFv62-TRAIL as single agent. This has been previously reported for other antibody-TRAIL constructs [101-105]. Several studies highlight the need of sensitizing agents for effective TRAIL-induced apoptosis and requirement to prevent development of resistance [51, 106]. Resistance of cancer cells is likely to be mediated by multiple defects in the TRAIL signaling pathway. Proteasome inactivation has been shown to block NF-κB activity and thus reduce the NF-κB-mediated transcription of anti-apoptotic proteins including c-FLIP [54, 107]. This strategy has been used to successfully overcome TRAIL resistance in breast, colon, pancreatic, melanoma and leukemia cell lines [54].

I treated the different cell lines with scFv62-TRAIL in combination with CHX, an effective protein synthesis inhibitor, and detected apoptosis-induction within 20h in different Kv10.1-positive cancer cells, whereas Kv10.1-negative cancer and normal control cells remained unaffected. Depending on the Kv10.1-positive cancer cell line, I observed different sensitivity to scFv62-TRAIL in combination with CHX, but could not detect a correlation between sensitivity and Kv10.1 expression level. The cause of resistance to TRAIL is a combination of diverse alterations in the TRAIL signaling of the particular tumor cell, therefore optimized combinational treatment needs to be investigated for every cancer type. The

Kv10.1-positive prostate cancer cell line DU145 showed the highest apoptosis induction and was used for further analysis of the scFv62-TRAIL effect.

The TRAIL-induced extrinsic apoptosis pathway can be amplified by the mitochondrial intrinsic pathway. Cells are divided in two different types depending on the intrinsic pathway activation [108]. The mitochondria play an essential role in TRAIL-induced apoptosis in type II cells. In these cells, mutation or deletion of Bax results in resistance to TRAIL [44, 109]. Several studies highlighted the resistance of DU145 cells against common chemotherapeutic and TRAIL therapy as a result of p53 mutation and non-allelic frameshift mutation in the Bax gene leading to loss of Bax expression [110, 111]. Re-expression of Bax could restore the sensitivity to TRAIL. These findings explain on one hand the resistance of DU145 against scFv62-TRAIL as single application and on the other hand the strong apoptosis induction by combinational treatment by restoring the interrupted cross-talk between intrinsic and extrinsic apoptosis pathway. In general, effective treatment of DU145 cells with scFv62-TRAIL always requires sensitization.

Additionally, I found another effect of CHX that can be responsible for the restored TRAIL sensitivity. By inhibiting the protein synthesis CHX also leads to a cell cycle arrest in G1 phase, which confers a significant higher susceptibility to TRAIL-induced apoptosis compared to cells in the G2/M or S phase [55].

Several standard chemotherapeutic agents have been tested in combination with TRAIL for their efficiency in treatment of cancer [51, 106, 112-114]. Etoposide is a topoisomerase-II inhibitor and induces double- and single-strand breaks in DNA. In combination with other cytotoxic agents etoposide has shown promising effects in the treatment of hormone refractory prostate cancer [115-118]. Between the relative low or weak effects of the different analyzed agents, the treatment of DU145 cells with scFv62-TRAIL in combination with etoposide resulted in strong apoptosis induction comparable to the effect of CHX. Etoposide sensitizes cancer cells for TRAIL-induced apoptosis by upregulation of

TRAIL-R1, TRAIL-2, Bax and Bak [44]. Furthermore, etoposide itself induces caspase-3 and therefore amplifies the effect of TRAIL [119]. The described activation of the intrinsic pathway and upregulation of the death receptors explain the restored sensitivity to scFv62-TRAIL-induced apoptosis, but treatment with etoposide alone did not increase the amount of apoptotic cells.

The influence of TRAIL receptors is discussed in more detail in the next section. In comparison to some other antibody-TRAIL fusions equal or even lower amounts of single-chain TRAIL construct were required for potent apoptosis induction [57, 59, 62, 64].

5.2.2 Influence of TRAIL receptor expression

The TRAIL receptor system is very complex consisting of 5 different receptors, the two death receptors TRAIL-R1 and TRAIL-R2, and the putative decoy receptors TRAIL-R3 and TRAIL-R4 [20]. TRAIL can also bind to the soluble osteoprotegerin with low affinity. Different studies suggest the influence of TRAIL receptor expression levels on TRAIL sensitivity of tumor cells [21]. The genes coding for the four membrane receptors TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4 are located on the 8p21-22 chromosome [120, 121]. In various cancer types including lung, colon, breast, prostate and ovarian cancer this chromosome often contains deletions and is frequently subjected to allelic loss [122-125]. The resulted downregulation of the death receptors leads to resistance of cancer cells against TRAIL-induced apoptosis. I analyzed the relative mRNA expression of the four TRAIL receptors various cancer cell lines including prostate carcinoma, mammary carcinoma, melanoma, fibrosarcoma, cervix carcinoma, neuroblastoma. Comparing the cancer cell lines I found completely different expression levels of the receptors, whereas TRAIL-R3 was not or only weak expressed. In the literature the function of the decoy receptors is controversial. In general, as a decoy receptor, TRAIL-R3 has been suggested to compete with the death receptor for TRAIL

binding and therefore this receptor mediates resistance [40]. But it has also been reported that cancer cells often lack both decoy receptors and for this reason determines the sensitivity to TRAIL-apoptosis and explain the selectivity of TRAIL for transformed cells.

TRAIL-R4 mRNA was found in two prostate and in one breast cancer cell line, but not in the other analyzed cancer cell lines. As a non-apoptosis inducing receptor TRAIL-R4 stimulates the NF-κB pathway and high NF-κB levels leads to TRAIL resistance of prostate cells [28, 32]. Using CHX as protein synthesis inhibitor the NF-κB-induced protein expression would be inhibit and restore the sensitivity to TRAIL-induced apoptosis in DU145 cells. I was not able to sensitize the other cancer cells with CHX, independently of TRAIL-R4 expression. This indicates the presence of other resistance mechanisms and the need for different sensitizing strategies according to cancer type.

TRAIL-R4 and TRAIL-R2 could be involved in the resistance against TRAIL-induced apoptosis in normal cells, because HEK-293-Kv10.1 and hTERT-RPE1 show high mRNA levels for both receptors. In contrast, analyses of tumor samples from breast cancer patients have shown a correlation of TRAIL-R2 and TRAIL-R4 expression and bad prognosis [126]. Furthermore, it has been described especially for breast and prostate cancer that more aggressive and higher differentiated tumors express higher TRAIL-R2 levels [36, 126, 127]. Even though DU145 cells are androgen-independent and therefore higher differentiated cancer cells than LNCaP and PC3 cells, TRAIL-R2 expression is even lower in them. Comparing all cancer cell lines the TRAIL-R2 expression levels in the two cancer cell lines HT1080 and IGR39 were the highest. This can be an indication that these cell lines are derived from higher differentiated tumors.

The two death receptors TRAIL-R1 and TRAIL-R2 have distinct cross-linking requirements; TRAIL-R1 can be activated via sTRAIL or memTRAIL, whereas TRAIL-R2 can be activated only by memTRAIL [65]. This observation may explain the low efficacy of sTRAIL against prostate cancer cells described by other groups [128]. Preclinical studies

demonstrate that many resistant tumors can be sensitized to TRAIL-induced apoptosis by various chemotherapeutic agents [129, 130]. Additionally, triggering of TRAIL-R2, but not of TRAIL-R1, results in efficient apoptosis induction of resistant cancer cells [126]. By fusing TRAIL to the anti-Kv10.1 single-chain antibody the death ligand is presumably converted into the potent TRAIL-R2 activating form. If scFv62-TRAIL needs to be membrane-bound to induce apoptosis via TRAIL-R2, this can be an explanation for resistance of HT1080 against scFv62-TRAIL apoptosis, because the Kv10.1 expression in these cells is relatively low and TRAIL-R2 is strongly expressed.

By blocking the death receptors I wanted to investigate if scFv62-TRAIL mediates apoptosis via TRAIL-R1 or TRAIL-R2. It is not completely clear which death receptor or if even both receptors are important for the apoptosis induction via scFv62-TRAIL. However, the expression of TRAIL-R1 and TRAIL-R2 seem to be connected, because siRNA-mediated downregulation of TRAIL-R2 in DU145 cells dramatically increases TRAIL-R1. This explains why I observed no decrease in apoptosis induction after down-regulating TRAIL-R2, because increased TRAIL-R1 expression compensated the TRAIL-R2 downregulation. Furthermore, this effect suggests also an involvement of both death receptors in the scFv62-mediated apoptosis induction.

The analysis of common chemotherapeutics in combinational treatment with scFv62-TRAIL validates the sensitizing effect of etoposide. Etoposide overcomes the resistance in DU145 cells by increasing the expression levels of TRAIL-R1 and TRAIL-R2. The upregulation of the death receptors seems to be not the only effect of etoposide, because doxorubicin also increases TRAIL-R1 and TRAIL-R2 but does not sensitize DU145 for scFv62-TRAIL-induced apoptosis. Ashkenazi and colleagues described besides the upregulation of TRAIL-R2, also an activation of caspase-8 and upregulation of Bak in colon carcinoma cells after etoposide treatment and suggested that these effects restore the sensitivity to TRAIL [44]. Bak is homolog to Bax and increased amounts of Bak can affect the

mitochondria despite of the absence of Bax in DU145 cells. In summary, the upregulation of death receptors and activation of the intrinsic pathway explain restored sensitivity to scFv62-TRAIL-induced apoptosis in DU145 cells.

Many chemotherapeutics, which are used to sensitize cancer cells for TRAIL-induced apoptosis, affect TRAIL receptor expression by upregulating one or both death receptors. The influence of TRAIL receptor expression in development of cancer is not clear, but there is evidence for correlations between TRAIL-R1, tumor grade and breast carcinogenesis. Interestingly, I observed an upregulation of TRAIL-R2 and TRAIL-R4 in HEK-293 cells transfected with Kv10.1 compared to wild type cells. Comparing the Kv10.1 expression levels with the TRAIL receptor expression levels I did not observe a correlation in cancer cells. Also, siRNA mediated downregulation of Kv10.1 did not change the expression levels of the receptors. Therefore, the TRAIL receptor upregulation in Kv10.1-overexpressing HEK-293 cells does directly not affect the channel, but can be an influence of other cellular changes caused by Kv10.1. Interestingly, the chemotherapeutic agents etoposide and doxorubicin induced an upregulation of TRAIL-R1 and TRAIL-R2 and at the same time downregulate Kv10.1 expression. The supposed influence of TRAIL receptors on Kv10.1 expression need to be further investigated.

5.3 Evaluation of scFv62-TRAIL for *in vivo* application

The translation of *in vitro* studies into *in vivo* application is always challenging. For high therapeutic efficacy the agents need to be stable, less immunogenic and selective for the elimination of tumor cells. The use of a Kv10.1-selective antibody provides the required selectivity for the antibody-based cancer therapy. The anti-Kv10.1 scFv62 originally derived

from a mouse monoclonal antibody, but compared to whole mouse antibodies the immunogenicity of scFv antibodies is strongly reduced.

The *in vivo* efficacy of a therapeutic antibody is not only dependent on the used antibody but also on the effector fusion protein. TRAIL is a potent apoptosis inducing ligand and as I could show with cancer-specific scFv62 antibody fusion, selectively eliminates cancer cells. As a part of the human immune system TRAIL is non-immunogenic and should not cause an immune response as is described for immunotoxins [131-133]. However, some studies reported toxicity of TRAIL in *in vivo* experiments, although this effect seems to be a problem of high molecular weight aggregates deriving from bacterial expressed proteins [34]. By using CHO-K1 cells as expression system for of scFv62-TRAIL I was able to express active, correctly folded and non-aggregated protein. Beside the insufficient apoptosis induction, short half-life and rapid blood clearance are additional drawbacks of sTRAIL [29]. The scFv62-TRAIL antibody showed a half-life of ~72h in mouse serum at 37°C, sufficient for *in vivo* use. Because of the affinity to Kv10.1 scFv62-TRAIL tends to accumulate at the tumor tissue and should not be cleared so fast.

In comparison to other therapeutic antibody strategies the efficacy of scFv62-TRAIL can be increased by the so-called bystander effect [58]. Apoptosis can be induced in an autocrine manner by binding to TRAIL receptors on the same cell or in a paracrine manner by binding to receptors on a neighboring cell (Fig. 4.27). Since tumor tissue consist of tumor cells having also neighboring tumor cells devoid or with low expression of Kv10.1, the bystander effect leads to more efficient tumor elimination. In cell cultures I could detect potent bystander effect of scFv62-TRAIL against Kv10.1-negative cancer cells, whereas normal prostate epithelia cells are not affected.

In summary, the scFv62-TRAIL fusion construct is a promising agent for antibody-based cancer therapy. In combination with etoposide, an already established chemotherapeutic, I could overcome resistance of prostate cancer and the observed

bystander effect can enhance the efficiency of this therapeutic approach *in vivo.* However, the effect of scFv62-TRAIL needs further investigations in mouse tumor models.

6 Summary and conclusions

In summary, I describe in this study a novel therapeutic strategy targeting the tumor specific expressed potassium channel Kv10.1 with an antibody fused to the apoptosis-inducing ligand TRAIL. Apoptosis resistance in some cancer cell lines treated with scFv62-TRAIL could be overcome by using CHX as sensitizing agent. The Kv10.1-positive prostate cancer cell line DU145 showed the strongest apoptosis-induction after sensitization and therefore this cell line was used for further analysis. The selectivity of the scFv62-TRAIL construct to Kv10.1-expressing tumor cells was confirmed by using different Kv10.1-positive, Kv10.1-negative cancer cell lines and non-cancer cell lines. Expression analysis of the different TRAIL receptors did not present a correlation to Kv10.1 expression or sensitivity to scFv62-TRAIL. However, a correlation between the expression of the two death receptors TRAIL-R1 and TRAIL-R2 was found in DU145 cells indicating that scFv62-TRAIL can mediate apoptosis via both death receptors.

Effective cancer therapy requires the elimination of all tumor cells to avoid cancer recurrence, but complex tumors often consist of heterogenic cell populations. By the so-called bystander effect scFv62-TRAIL can induce apoptosis not only in Kv10.1-positive cancer cells, but also in surrounding cancer cells with low or even without Kv10.1 expression. This paracrine apoptosis-induction was observed in mixed cultures having a strong apoptotic effect against Kv10.1-negative cancer cells, while normal cells were not affected.

Analysis of different common used chemotherapeutics in combinational treatment with scFv62-TRAIL identified etoposide as a potential agent for *in vivo* application in combination with scFv62-TRAIL. Since Kv10.1 is frequently expressed on tumor cells further *in vitro* and *in vivo* studies with different cancer types and different sensitizing agents are necessary to confirm the potential of scFv62-TRAIL in cancer therapy.

7 Material and Methods

7.1 Material

7.1.1 Equipment

Centrifuges	XL-90, Beckman
	Optima TLX 120, Beckman
	5415 C, Eppendorf (Table-centrifuge)
	5402, Eppendorf (Cooling-centrifuge)
EppiMotion	Eppendorf
FASCaria	BD Bioscience
GelDoc	Bio-Rad
Incubator	Heraeus instruments
Mastercycler	Eppendorf
Microscope Axiovert 100	Zeiss
NuPAGE Bis-Tris Electrophoresis System	Invitrogen
Photometer	Eppendorf
pH meter	Knick, pH-Meter 766 Calimatic
Qiacube	Qiagen
Thermo block	Eppendorf
Vision Workstation	Applied Biosystems
Wallac Victor^2 Multilabel counter 1420	
Water Purification System	Sartorius

7.1.2 Chemicals

ABTS-Solution	Roche
Agar	Gibco/BRL
Agarose	Gibco/BRL
Anhydrotetracyclin	IBA
AlamarBlue	Biosoure
Ampicillin	Roche
Bromphenolblue	Merck

BSA	Sigma
Chloroform	Merck
Coomassie Brilliant Blue R-240	Bio-Rad
Diethyl pyrocarbonate (DEPC)	Sigma
Dimethylsulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
Acetic acid	Merck
Ethanol	Merck
Ethidiumbromide	Sigma
Filter 0,22 µm	Milipore
FuGENE 6	Roche
Glucose	Merck
Glycine	Merck
Glycerol	Merck
Hybond ECL Nitrocelulose Membrane	Amersham Biosciences
Imidazol	Fluka
Isopropanol	Merck
Luria Broth	Invitrogen
Milk powder	Bio-Rad
ß-mercaptoethanol	Fluka
Methanol	J.T.Backer
Natrium azide	Merck
Natrium chloride	Sigma
Natriumdihydrogen phosphate	Merck
Natrium hydroxide	Merck
POROS EP 20 µm Self Pack Media	Applied Biosystems
Potassium chloride	Invitrogen
Sodium Dodecyl Sulfate (SDS)	Sigma
SyproRuby	Invitrogen
Tris-Base	Sigma
Tris-HCL	Merck
Tween 20	Merck
Whatman filter paper	Schleicher & Schüll
Yeast extracts	Becton Dickinson
Zink chloride	Sigma

7.1.3 Antibodies

The following antibodies were used:

Table 7.1 Primary and secondary antibodies

Antibody	Application	Reference
polyclonal rabbit anti-TRAIL	ELISA	Abcam
monoclonal mouse anti-TRAIL	WB, ELISA	Sigma
monoclonal mouse anti-TRAIL receptor 1-4, antibody set	FACS, Cell culture	Enzo life science
polyclonal rabbit anti-cleaved caspase-3	WB, IH	Cell Signaling
monoclonal rabbit anti-mouse HRP labeled	WB, ELISA	Calbiochem
goat anti-rabbit HRP labeled	ELISA	GE Healthcare
mAb62	ELISA	Hemmerlein et. al (2006)

7.1.4 Oligonucleotides

Table 7.2 UPL Probes (real-time PCR)

number	5´-Sequence-3´	Gene
18	5´-FAM- TCC TGC TG -Dark Quencher Dye-3´	human TRAIL receptor 1-3
61	5´-FAM CTG GGC AA Dark Quencher Dye-3´	human transferrin receptor, human Kv10.1
64	5´-FAM CCA GGC TG Dark Quencher Dye-3´	human beta-actin
74	5´-FAM- CTG CTG CC -Dark Quencher Dye-3´	human TRAIL receptor 4

The UPL probes for real-time PCR were obtained from Roche Diagnostics.

Table 7.3 Oligonucleotides

Oligo	5´-Sequence-3´	Direction	Application
hTRAIL1	GGGTCCACAAGACCTTCAAGT	F	real-time PCR
hTRAIL1	TGCAGCTGAGCTAGGTACGA	R	real-time PCR
hTRAIL2	AGACCCTTGTGCTCGTTGTC	F	real-time PCR
hTRAIL2	TTGTTGGGTGATCAGAGCAG	R	real-time PCR
hTRAIL3	CCCTAAAGTTCGTCGTCGTC	F	real-time PCR
hTRAIL3	TGGTGGCAGAGTAAGCTAGGA	R	real-time PCR
hTRAIL4	AAGTTCGTCGTCTTCATCGTC	F	real-time PCR
hTRAIL4	GATGGTGGCAGAGTCACC	R	real-time PCR
insert stop codon	CAGTCACCGTCTCCTCATGAGGGGGAGGCTCTGGAGGTGGAGG	F	QuikChange
insert stop codon	CCTCCACCTCCAGAGCCTCCCCCTCATGAGGAGACGGTGACTG	R	QuikChange
insert XmaI	GGAGGTAGTGGGGGAGGGGCCCGGGATGTTGTGATGACCC	F	QuikChange
insert XmaI	GGGTCATCACAACATCCCGGGCCCCTCCCCCACTACCTCC	R	QuikChange
amplify scFv62-TRAIL	TTATATATGGCCCAGCCGGCCCGGGATGTGCAGCTT	F	Cloning
amplify scFv62-TRAIL	CGCGGGCCCTCAAGATCTAGAGCCAACTAAAAAGG	R	Cloning

7.1.5 Vectors

Table 7.4 Vectors

vector	company
pcDNA3-venus	Invitrogen
pSecTag2A	Invitrogen
pASK-IBA2	IBA
pGEM-T easy	Promega

7.1.6 Commercial cell lines

Cell lines were obtained from the DSMZ or ATCC. Each cell line was cultured in their respective media supplemented with FCS at 37°C in humidified 5% CO_2 atmosphere.

CHO-K1

Chinese hamster ovary

Subclone from parental CHO-K1 cell line that was initiated from an ovary biopsy of an adult Chinese hamster in 1957

medium: F-12, 10% FCS

DU145

human prostate carcinoma

Established from the tumor tissue removed from the metastatic central nervous system lesion of a 69-year-old man with prostate carcinoma in 1975

medium: DMEM, 10% FCS

HEK-293

human embryonic kidney

established from a human primary embryonal kidney transformed by adenovirus type 5

medium: DMEM/F-12, 10% FCS

HELA

human cervix carcinoma

Established from the epitheloid cervix carcinoma of a 31-year-old black woman in 1951

medium: RPMI, 19% FCS

hTERT RPE-1

human retina epithelia

immortalized with hTERT

medium: RPMI, 10% FCS

HT-1080

human fibrosarcoma

Established from the biopsy from a fibrosarcoma of a 35-year-old Caucasian man in 1972

medium: DMEM, 10% FCS

IGR39

human melanoma

Established from th primary cutaneous tumor of a 26-year-old man with malignant melanoma

medium: DMEM, 15% FCS

IPC-298

human melanoma

Established from the primary tumor of a 64-year-old woman with cutaneous melanoma

medium: RPMI, 10%FCS

LNCAP

human prostate carcinoma

Established from the left supraclavicular lymph node metastasis of a 50-year-old man with prostate carcinoma in 1977

medium: RPMI, 10% FCS

MCF-7

human breast adenocarcinoma

Established from the pleural effusion of a 69-year-old Caucasian female with metastatic mammary carcinoma in 1970

medium: RPMI, 10% FCS, 1mM sodium pyruvate, 10µg/ml bovine insulin, non-essential amino acids

MDA-MB231

human breast carcinoma

Established from mammary gland tumor tissue of a 51-years-old woman

medium: DMEM, 10% FCS

MDA-MB435S

human breast carcinoma

Established from a effusion of a 48-year-old female with breast carcinoma in 1976

medium: RPMI, 10% FCS

PC-3

human prostate carcinoma

Established from the bone marrow metastasis isolated post-mortem from a 62-year-old Caucasian man with grade IV prostate cancer

medium: DMEM, 10% FCS

SH-SY5Y

human neuroblastoma

Clonal subline of the neuroepthelioma cell lien SK-N-SH that had been established in 1970 from the bone marrow biopsy of a 4-year-old girl with metastatic neuroblastoma

medium: RPMI, 15% FCS

7.1.7 Bacterial strains

Table 7.5 Bacterial strain

***E.coli* strain**	**Genotype**
BL21	*E.coli* B, F-, *dcm*, *omp*T, *hsd*S(rB- mB-), galλ (DE3)
DH5α	80*lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K-,m_K+), *sup*E44, *rel*A1, *deo*R, Δ(*lac*ZYA-*arg*F)U169
XL10-Gold ultra competent cells (Stratagene)	Tet^RΔ(mcrA) 183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F´proAB $lacI^q$ZΔM15Tn10(Tet^R) Amy Cam^R]^α

7.1.8 Buffers and media

7.1.8.1 Buffers

Table 7.6 Buffers

Buffers	Composition
Dialysis solution I	100mM Tris-HCl, 100mM NaCl, 8M urea, pH 8.0
Dialysis solution II	100mM Tris-HCl, 100mM NaCl, 4M urea, pH 8.0
Dialysis solution III	100mM Tris-HCl, 100mM NaCl, 2M urea, 400mM arginine, 375µM L-glutathione, pH 8.0
Dialysis solution IIII	100mM Tris-HCl, 100mM NaCl, 1M urea, 400mM arginine, 375µM L-glutathione, pH 8.0
Dialysis solution IV	100mM Tris-HCl, 100mM NaCl, pH 8.0
DNA loading buffer (5x)	20% Ficoll, 100mM EDTA, 0.25% Bromphenolblue, 0.25%Xylencyanol, pH 8.0
Lysis buffer	50mM Tris, 150mM NaCl, 1mM EDTA, pH 7.4
PBS (10x)	20mM KH_2PO_4, 100mM Na_2HPO_4, 1.54M NaCl, pH 7.4
RF1 buffer	100mM RbCl, 50mM $MnCl_2$, 30mM potassium acetic, 10mM $CaCl_2$, 15% (w/v) glycerin, pH 5.8
RF2 buffer	100mM MOPS, 10mM RbCl, 75mM $CaCl_2$, 15% (w/v) glycerin, pH 6.8
TBE (10x)	600mM Tris-Base, 600mM bore acid, 1mM EDTA, pH 7.5
TBS (10x)	1.4M NaCl, 200mM Tris-HCl, pH 7.5
Transfer buffer	20% methanol, 0.01% SDS,

7.1.8.2 Cell culture media

The commercial cell culture media used in this study are listed in Tab. 7.7.

Table 7.7 Cell culture media

Medium	Obtained from
DMEM/F-12	Invitrogen
DPBS	Invitrogen
FCS	Biochrom
F-12	Invitrogen
non-essential amino acids	Biochrom
OptiMEM	Invitrogen
Panserin C6000	PAN Biotech
RPMI	Invitrogen
Sodium pyruvate	Biochrom
Trypsin/EDTA	Biochrom

7.1.9 Commercial kits

Commercial kits used in this study are listed in Tab. 7.8.

Table 7.8 Commercial kits

Kit	Application	Obtained from
QuikChange Site-Directed Mutagenesis Kit	mutations	Stratagene
NucleoSpin Plasmid Kit	DNA isolation	Macherey-Nagel
NucleoSpin Spin Kit	DNA purification	Macherey-Nagel
EndoFree Plasmid Maxi Kit	DNA isolation	Qiagen
Caspase-Glo 3/7 Assay	apoptosis measurement	Promega
CytoGLO Annexin V-FITC Apoptosis detection kit	apoptosis measurement	Imgenex
Quentix Western Blot Signal Enhancer Kit	WB	Thermo Scientific
ECL Developing	WB development	Millipore
RNeasy Mini Kit	RNA isolation	Qiagen
SuperScript First-Strand Synthesis System	cDNA synthesis	Invitrogen
TaqMan PCR Reagent Kit	quantitative real-time PCR	Applied Biosystems
Ni-NTA agarose	Protein purification	Qiagen

7.1.10 Enzymes, antibiotics and inhibitors

In this study different enzymes, antibiotic and reagents (Tab. 7.9) were used for molecular and biochemical methods.

Table 7.9 Enzymes, antibiotics and inhibitors

Agent	Company
17-AAG	Sigma
Actinomycin	Sigma
Ampicillin	Sigma
Anhydrotetracyclin	IBA
Aspirin	Sigma
BSA	Sigma
Cisplatin	Sigma
Cycloheximide	Sigma
dNTP mix	New England Biolabs
Doxorubicin	Sigma
Etoposide	Sigma
5-Fluorouracil	Sigma
G418	Invitrogen
Resveratol	Sigma
Restriction enzymes + Buffers	New England Biolabs
Roscovitine	Sigma
T4-Ligase + Buffer	New England Biolabs
Taq polymerase	Roche
Zeocin	Invivogen

7.1.11 siRNA constructs

Table 7.10 siRNA

Target	Obtained from
human Kv10.1	Ambion
human TRAIL-R1	Santa Cruz
human TRAIL-R2	Santa Cruz

7.1.12 Antigen h1x

The antigen h1x consists of two amino acid chains, the sequence of the pore region and C-terminal part of Kv10.1, linked by a peptide linker containing a 6xHis-tag.

Amino acid sequence of h1x:

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQN
PGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSGHMHHHHHHSSGL
VPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMGDYEIFDEDTKTIPTTAGCPTSDGHW
PPYQFNGSGSRKWEGGPSKNSVYISSLDFTMTSLTSV

7.1.13 Standards

Precision Plus Protein Standards All Blue	NEB Biolabs
Precision Plus Protein Standards Unstained	NEB Biolabs
100 bp DNA Ladder	Invitrogen
1 Kb DNA Ladder	Invitrogen

7.2 Molecular methods

7.2.1 Polymerase chain reaction

Polymerase chain reaction (PCR) is a molecular technique to amplify DNA fragments with sequence specific oligonucleotides (primers). The basic PCR requires DNA template containing the targeted DNA region, specific primers, desoxynucleoside triphosphates (dNTPs) and a heat-stable DNA polymerase. Beside DNA amplification PCR can also be used for insert mutations or DNA quantification.

One PCR cycle consists of denature, annealing and elongation step and the number of cycle vary between 20 and 40. PCRs were performed in 50 µl in the following composition:

10-100ng	plasmid DNA
10 pmol	forward primer
10 pmol	reverse primer
1 µl	nucleotide mix (200 µM of each dNTP)
1 µl	PCR buffer with MgCl2
1 U	Taq DNA polymerase
x µl	ddH2O

A 5 min initial denaturing incubation step at 94°C was followed by 30-40 cycles of:

30 sec	94°C (denaturing)
30 sec	50-60°C (annealing)
30-120 sec	68-72°C (elongation)

The last cycle followed by a final elongation step at 72°C for 10 minutes.

7.2.2 Site-directed mutagenesis

The Quik Change Site directed Mutagenesis Kit (Stratagene) was used to create point mutations or amino acid switches in plasmid DNA. The site-directed mutagenesis method was performed with complementary primers containing the mutation and the PfuTurbo polymerase. During temperature cycling the oligonucleotides generate the mutated plasmid. DpnI is used to digest the parental DNA template and to select for mutation-containing synthesized plasmid. The digested DNA was than transformed into *E.coli* XL-Blue cells.

QuikChange mutagenesis PCR (50µl):

5µl	10x reaction buffer
100ng	DNA
125ng	forward primer
125ng	reverse primer
1µl	dNTP mix
x µl	H_2O

Following cycling parameters were used:

Segment	cycles	temperatur	time
1	1	95°C	30 sec
2	18	95°C	30 sec
		55°C	1 min
		68°C	1min/1kb plasmid

After the last cycle reaction was cooled to 4°C. The parental dsDNA was removed in each amplification reaction by 1h incubation at 37°C with 1µl of DpnI (10 U/µl). For the amplification of the mutated DNA, 2-5 µl of each digested PCR products were transformed into XL1-Blue *E.coli*.

7.2.3 Agarose gel electrophoresis

1% (w/v) agarose and Ethidiumbromide (5µl/100ml) in TBE buffer was boiled in the microwave and poured into a horizontal gel chamber. Gels were then placed into an electrophoresis chamber filled with TBE buffer. The DNA samples were mixed with DNA-loading buffer (5x), applied on gel and run at 100V.

7.2.4 Production of competent bacterial cells

Competent bacterial cells were produced by modification of the cell wall, which mediates DNA uptake. One single bacterial colony from a LB plate was taken up in 100ml LB- medium and was incubated at 37°C on a shaker until the bacterial suspension reached an OD_{600} value of 0.6. Then bacterial suspension was cooled on ice and centrifuged at 1500xg for 15min at 4°C. Pellet was resuspended in 34ml of buffer RF1, incubated for 10min and centrifuged (1500xg, 30 min, 4°C). Supernatant was discarded and pellet resuspended in 2ml buffer RF2. Aliquots of 100µl were snap frozen in liquid nitrogen and stored at -80°C.

7.2.5 Transformation of bacteria

Chemically competent bacteria are able to uptake plasmids following a heat shock at 42°C. One aliquot of competent bacteria were thawed on ice and were incubated with up to 100ng plasmid DNA or 10µl ligation reaction. Following incubation on ice for 30min and a heat shock at 42°C for 45 sec was performed. After 2min incubation on ice 500µl LB-medium was added and incubated for 1h at 37°C with gentle agitation. Finally, cells were plated on LB-agar plates containing appropriate antibiotics and grown overnight at 37°C.

7.2.6 Plasmid mini preparation

MACHEREY-NAGEL NucleoSpin Plasmid Kit was used for plasmid mini preparations. Single *E.coli* colony was inoculated in 5ml LB medium containing the appropriated antibiotic and incubated for 10-16h at 37°C, 225 rpm. Bacterial cultures were centrifuged for 5min at 5000xg and the pellet was treated according to the manufacturer's protocol. DNA was eluted with 50µl of H_2O.

7.2.7 Endofree plasmid maxi preparation

For transfection of eukaryotic cells endotoxin-free DNA in large scale is necessary. A pre-culture of 5ml with a single colony was incubated for 6-8h at 37°C (225 rpm) and inoculated in 100ml of LB medium with appropriate antibiotic and incubated overnight at 37°C with vigorous shaking (225rpm). The plasmid DNA was isolated with the EndoFree Plasmid Maxi Kit (Qiagen) according to the user's manual.

7.2.8 Isolation of DNA fragments from agarose gels

For the isolation of DNA fragments from agarose gels NucleoSpin Extract kit (Macherey-Nagel) was applied. After separation of DNA on agarose gels, the desired DNA band was excised with a razor blade, weighed and treated as described in the user's manual. After isolation samples were analyzed on agarose gels and further used for cloning procedures.

7.2.9 Concentration determination of DNA and RNA

DNA concentration as well as DNA purity was measured spectrometrically by NanoDrop at 260nm. The quotient of extinction at 260nm and 280nm (E260/E280) determines purity and should have a value between 1.8 and 2.

$c_{DNA} = A_{260} \times 50\ \mu g/ml$

$c_{RNA} = A_{260} \times 40\ \mu g/ml$

7.2.10 Total RNA isolation of cells

For total RNA isolation 2-3x10^6 cells were resuspended in 600µl RTL Buffer (supplemented with 1% ß-mercaptoethanol and filtered through QiaShredder columns and a centrifugation step (14000xg, 2min).

7.2.11 cDNA preparation

For cDNA preparation the SuperScript First-Strand Synthesis System from Invitrogen was used. As standard 5µg RNA were used and the procedure was done according to the

manual. At the end cDNA was diluted to a concentration of 0.1µg/µl and used for quantitative real-time PCR.

7.2.12 Quantitative real-time PCR

The real-time PCR was performed using the TaqMan system in the Light Cycler (Roche). The TaqMan probes contain a reporter dye (FAM) at the 5´end of the probe and a quencher dye (TAMRA) at the 3´end of the probe. The AmpliTaq Gold DNA polymerase cleaves the TaqMan probe during PCR, therefore the reporter and quencher dyes are separated, which results in increased fluorescence of the reporter. The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR, so nonspecific amplification is not detected.

For analyzing the expression of the different TRAIL receptors and Kv10.1 the Universal Probe Library from Roche was used. Real-time PCR was performed with 100µg cDNA and as a control of RNA integrity and quantification human beta-actin and human transferrin were used as house-keeping genes. The number of PCR cycles to reach detection threshold was used to determine specific mRNA content.

7.2.13 Cloning

The expression of the scFv62-TRAIL antibody was done in two different expression systems. For this two vectors containing the scFv62 and TRAIL sequence were constructed; the pASK-IBA2-scFv62-TRAIL vector for the expression in *E.coli* and the pSecTagscFv62-TRAIL vector for expression in CHO-K1 cells. All cloning steps were performed with enzymes and buffers from New England Biolabs.

7.2.13.1 pASK-IBA2-scFv62-TRAIL vector

Construction of the pASK-IBA2-scFv62-PhoA vector has been described before [75]. The sequence of phosphatase A was removed by inserted XbaI and SacI sites. The vector pEGFP-TRAIL containing the sequence of the soluble TRAIL was purchased from Addgene [134]. TRAIL was amplified with two primers, one containing the XbaI site and the other containing the SacI site and a C-terminal 6xHis-tag and cloned as fusion to scFv62. The obtained pASK-IBA2-scFv62-TRAILHis vector was transfected into *E.coli* BL21 cells for overexpression.

7.2.13.2 pSecTagscFv62-TRAIL vector

The scFv62-TRAIL was amplified from pASK-IBA2-scFv62-TRAIL with SfiI and ApaI restriction sites and a C-terminal stop codon and cloned into the multiple cloning site of the pSecTag2A vector (Invitrogen). The pSecTagscFv62-TRAIL vector was transfected into CHO-K1 or HEK-293 cells for secretory expression. ScFv62-TRAIL was expressed without any N- or C-terminal tag.

7.2.13.3 pSecTagscFv62 vector

scFv62 was cloned from pASK-IBA-PhoA vector [75] by inducing ApaI restriction site. The sequence was cloned with SfiI and ApaI into pSecTag2A without any tag.

7.2.14 Prokaryotic expression system

A colony of *E.coli* BL21 cells containing the plasmid pASK-IBA2scFv62-TRAIL was picked and inoculated in 50ml LB-Amp medium and incubated at 37°C on orbital shaker. On the next

day, 6 x 2 liter flasks were filled with 500ml LB-Amp medium and 5ml of overnight culture (1:100 dilutions) were inoculated. The cultures were incubated until the OD 600 of 0.6 at 37 ° C and 180rpm. Each flask was treated with the following substances: 50µl AHT (2mg/ml), 2ml 1M $CaCl_2$, 40µl 0.1M $ZnCl_2$ and 250µl ampicillin (1mg/ml). The cultures were incubated for 4h at 30°C and 125rpm. Cells were pelleted, resuspended in 3ml 50 mM Tris-HCL, 200mM NaCl pH 8 and lysed by sonification. After a centrifugation step (10000xg, 30min, 4°C) supernatant (soluble fraction) was transferred in a new tube and pellet (inclusion bodies) was resuspended in 3ml 50 mM Tris-HCL, 200 mM NaCl pH 8, 8M urea. The solubilized pellet was transferred in a dialysis column (15kDa MWCO) and dialysis starting with Dialysis solution I till Dialysis solution IV. Solutions were changed every 12h. After the last dialysis step, scFv62-TRAIL preparation was stored at 4°C.

7.3 Cell biological methods

7.3.1 Culturing of cell lines

Cells were cultured in an incubator at 37°C and 5% CO_2. Media were supplemented, if not indicated differently, with 10% [v/v] heat-inactivated FCS. Media were prewarmed at 37°C prior use. The medium of stable transfected cell lines was supplemented with the appropriated antibiotic.

7.3.2 Cell counting

For counting cell suspension was mixed 1:1 with 0.4% trypan blue solution. Trypan blue is an acidic dye that binds to cellular protein. Due to the defective cell membrane of dead cells, the

dye may penetrate into the cytosol, and these cells stained deep blue. Living cells appear under the microscope bright. This allows the distinction between living and dead cells. Using a Neubauer counting chamber was the viable cell number determination.

7.3.3 Cyrostorage of cells

Established cell lines were frozen in liquid nitrogen for long-time storage. Cells were pelleted, resuspend in freezing medium and frozen as 1ml aliquots of $2x10^6$ cells in cryotubes. Cyrotubes were placed at -80°C in a freezing container filled with isopropanol to allow a constant decrease in the temperature at a rate of 1°C per minute. Finally, the tubes were stored in liquid nitrogen.

7.3.4 Thawing of cells

The thawing procedure of cells was performed as fast as possible to avoid the toxic side effects of DMSO in freezing medium. Cryotubes were placed in the water bath at 37°C until a little piece of ice clot was still visible in the cryotube. Cell suspension was transferred to 10ml of prewarmed supplemented cell culture medium. After centrifugation at 1200rpm for 1min, supernatant was removed and pellet was carefully resuspended in fresh medium in a new culture flask. Media were changed 24h later and cells were passaged after 1-2 days depending on cell growth rate.

7.3.5 Transfection of eukaryotic cells

For transfection I used Lipofectamine 2000 (Invitrogen) or FuGene (Roche) according to the user´s protocol. For production of stable cell lines Lipofectamine 2000 was used and Zeocin or G418 was added after 48h to the culture medium (50-100µg/ml) for selection.

7.3.6 Downregulation of protein expression by siRNA

RNA interference can be used to silence the expression of target genes in a variety of cell types. Small interfering RNAs (siRNA) are short duplexes of 19-21 nucleotides, which are designed to knock down target-specific gene expression. The specific siRNAs were purchased from Ambion or Santa Cruz and transfection was performed with Lipofectamine RNAiMAX (Invitrogen) and 30nM siRNA concentration according to the instruction. The affectivity of downregulation was determined by real-time PCR.

7.3.7 Expression of scFv62 antibody in eukaryotic cell lines

Expression of the scFv62 and scFv62-TRAIL construct was done in CHO-K1 or HEK-293 cells using the pSecTag2A expression vector. The antibodies were expressed either in transient or stable transfected cells. The vector pSecTag2A contains a murine kappa leader sequences, which leads to secretory protein expression. Transient transfections were done on T75 cell culture flasks with $1x10^6$ CHO-K1 or HEK-293 cells and Lipofectamine 2000 or FuGene. After the incubation with the DNA and transfection reagent solution for 4h medium was changed into protein and serum-free medium Panserin C6000 (PAN Biotech) for

expression. After incubation of 3-5 days medium was collected, concentrated with Centricon YM-100, sterile filtered and stored at 4°C.

For stable transfection CHO-K1 cells were transfected in 6-well plates with pSecTag2-scFv62 or pSecTag2-scFv62-TRAIL and Lipofectamine 2000. Transfected cells were selected with 500µg/ml zeocin and monoclones were analyzed for stable secretion of scFv62-TRAIL fusion protein. For protein expression stable expressing monoclones were seeded on flasks with 80% confluence. After cell attachment Panserin C6000 medium was added and the plates were incubated 3-5 days at 37°C or 30°C in humidified 5% CO_2 atmosphere. Medium was collect and handled as described.

7.3.8 Apoptosis assay

7.3.8.1 Caspase-3 activity assay

The caspase-3 is key effector caspase in apoptosis pathway of mammalian cells. The Caspase-Glo 3/7 Assay is a luminescent assay that measures caspase-3 and -7 activities and is used in the multiwell-plate format. The assay provides a luminogenic caspase-3/7 substrate, which releases after cleavage a luminescence signal. Luminescence is proportional to the amount of caspase activity.

Cells were seeded in 96-well plates ($2x10^3$ cells/well) in 100µl medium and treated as indicated. For measuring the caspase-3 activity Caspase-Glo® substrate was mixed with Caspase-Glo buffer and 100µl were added to each well. After 1h incubation at room temperature luminescence was measured.

7.3.8.2 Annexin staining and flow cytometry

Phosphatidylserine (PS) is one phospholipid which normally found in the inner leaflet of the plasma membrane. During apoptosis PS translocated to the outer leaflet, thereby exposing PS at the external surface of the cell. This effect occurs in the early phase of apoptotic cell death. The CytoGLO Annexin V-FITC Apoptosis detection kit is based on utilizing Annexin V to detect the cell surface exposure of PS.

For the apoptosis assay and flow cytometer analysis $1x10^5$ cells were plated on 12-well plate and treated as indicated. The cells were collected and washed with 1ml cold 1xPBS. After resuspension in 100µl 1x Annexin-Binding Buffer 5µl Annexin-FITC and 5µl propidium iodide (PI) has been added and incubated at room temperature for 20min in the dark. After the incubation 400µl 1x Annexin-Binding Buffer was added and analyzed by flow cytometry. The FACSaria cytometer (BD Bioscience) with the appropriate laser and filters has been used for cell analysis.

For the bystander effect analysis and the doxorubicin treatment Annexin V-Alexa648 was used instead of Annexin V-FITC.

7.4 Biochemical methods

7.4.1 SDS-PAGE

Protein analysis was done with The NuPAGE Bis-Tris Electrophoresis System pre-cast polyacrylamide mini-gel system (Invitrogen) and Bis-Tris NuPAGE gradient gels 4-12%. The samples were prepared and loaded according to the instruction manual. Depending on the analysis method the SDS-gels were stained for protein detection of further used for immune blot analysis.

7.4.2 Protein gel staining with SyproRuby

SyproRuby protein gel stain is a ready–to–use, sensitive, fluorescent stain for detecting proteins separated by polyacrylamide gel electrophoresis (PAGE). The SDS gel with the separated proteins was fixed in 10ml SyproRuby fix solution (50% methanol, 7% acetic acid) for 30min and then stained overnight in 5ml SyproRuby staining solution. After 30min washing in 10ml SyproRuby wash solution (10% methanol, 7% acetic acid) gel was rinse with water and detected under UV light.

7.4.3 Immunoblot

After electrophoresis separated proteins are transferred from the protein gel to a nitrocellulose membrane and detected by specific antibodies and horse radish peroxidase conjugates (HRP). The western blot transfer buffer was prepared and gel and the nitrocellulose membrane were equilibrated for 5min. A transfer ‘sandwich’ (sponge, 2 sheets

of paper, running gel, nitrocellulose membrane, 2 sheets of paper, sponge) held together by a plastic cassette, was assembled and inserted into buffer tank completely filled with transfer buffer. The transfer was run for 10min at 10V, 10min at 20V, 10min at 30V, 10min at 40V and 35min at 50V. Blot was disassembled and membrane washed in water and dried overnight.

On the next day membrane was equilibrate in water and treated with the Quentix^{TM} Western Blot Signal Enhancer Kit (Thermo Scientific) according to the instruction manual. Unspecific binding sites were covered by blocking the membrane with 0.1% casein in 1xTBS. After 3 washing steps with water the membrane was incubated with the primary antibody (directed against the antigen of interest) diluted in 1xTBS for 2h. Membrane was washed again three times in water and incubated with the appropriated HRP conjugated secondary antibody for 1h. Finally, the proteins were visualized by chemiluminescence. Detection was performed using the ECL detection system (Millipore) as recommended by the manufacturer and a CCD camera.

7.4.4 Cell lysate preparation

Cells (~1x10^6) were harvested and washed with 1xPBS. After centrifugation for 2min at 1200rpm cell pellet was resuspended in 100µl ice-cold lysis buffer and incubated for 30min on ice. The cell debris were removed by a centrifugation step (15min, 14000xg, 4°C) and supernatant was transferred in an new tube an stored as whole cell lysate at -20°C.

7.4.5 ELISA

The enzym-linked immunoabsorbant assay (ELISA) is a biochemical method to detect the presence of an antibody or an antigen in a sample. Beside the diagnostic approaches ELISA is used to determine the concentration of antibodies in solutions. 96-well plate was coated

with 500ng h1x in 100µl 1xTBS buffer and incubated overnight at 4°C. Unspecific binding sites were blocked with 3% BSA in TBS medium for 1h at room temperature. Aliquots of 100µl scFv62-TRAIL in different dilutions was added and incubated for 2h at room temperature. The monoclonal mouse antibody 62 was used as standard and detected with an anti-mouse peroxidase-conjugated antibody. After 3 washing steps with TBS, anti-TRAIL polyclonal rabbit antibody (1:2000 dilutions) was added and incubated for 1h. The secondary anti-rabbit antibody conjugated with horseradish peroxidase was incubated in 100µl TBS for 1h. After 3 washing steps with TBS the plate was developed by addition of 100µl ABTS substrate. Measurement was done in the Victor Wallac plate reader.

7.4.6 Gel filtration

Gel filtration separates proteins with differences in molecular size. This technique was used to analyze the scFv62-TRAIL preparation for the presence of trimers and smaller structures.

The HiLoad 16/60 Superdex 200 column from Amersham Bioscience was connected to Vision Workstation HPLC system. The column was equilibrated with 1xPBS and the separation was performed with a flow rate of 1ml/min. 1ml scFv62-TRAIL preparation were loaded and the separated samples were collected in 200µl factions. The different fractions were analyzed with an immune blot and anti-TRAIL detection.

7.4.7 Affinity chromatography

Affinity chromatography can be used for purifying antibodies with an antigen-coupled column. For purification of the scFv62 antibody an h1x-coupled column was generated. The column packing and purification procedure was done with the Vision Workstation. According to the instruction sheet Self Pack POROS 20EP media was activated, incubated with h1x overnight

and packed into a column. Capacity and function of the h1x-column has been tested with the monoclonal Ab62. Packed columns were stored in 1xPBS supplemented with 0.1% sodium acid at 4°C.

The scFv62 and scFv62-TRAIL were purified by loading the CHO-K1 cell medium supernatant containing the antibodies to the affinity column. PBS was used as running buffer during the purification. The loading procedure was done in 20 steps with 2.5ml volume per step. The column was washed with running buffer to remove unspecific bound proteins. To elute the bound antibodies the pH was changed with running a 5min gradient from pH 7.2 of PBS to pH 2.5 of the elution buffer. The elution peak was collected and neutralized with 1M Tris-HCl pH 8.0. Fractions were analyzed on SDS-PAGE and with ELISA.

7.5 Statistical analysis

Statistical analysis was performed with GraphPad Prism. For descriptive statistics, mean values and standard deviation of a data set were calculated and illustrated. To compare two independent groups with sample sets showing normal distribution and equal variance, the parametric Student's t-test was used. Depending on the p value data groups were stated as follow:

< 0.001	extremely significant	***
0.001 to 0.01	very significant	**
0.01 to 0.05	significant	*
>0.05	not significant	ns

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9 Appendix

9.1 Abbrevations

aa	amino acid
Ab	antibody
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ADCC	antibody-dependent cell-mediated cytotoxicity
ADEPT	antibody-directed enzyme prodrug therapy
AHT	Anhydrotetracyclin
Akt	protein kinase B
Amp	ampicillin
AML	Acute myeloid leukemia
ATCC	American type culture collection
Bak	Bcl-2 homologues antagonist killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bid	pro-apoptotic Bcl-2 protein
bp	base pair
BSA	bovine serum albumin
CaM	calmodulin binding domain
cDNA	complementary DNA/ copy DNA
CDC	complement dependent cytotoxicity
CDR	complementarity determining region
c-FLIP	Cellular caspase-8 (FLICE)-like inhibitory protein
CHO	chinese hamster ovary
cNBD	cyclic nucleotide binding
CNS	Central nervous system
Da	dalton
DD	death domain
DED	death effector domain
DISC	Death-inducing signaling complex
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	2’-deoxynucleoside-5’-trisphosphate

dsFv	disulfide bound single fragment variable
DT	diphtheria toxin
Eag1	ether-á-go-go, Kv10.1
E.coli	*Escherichia coli*
ECL	Enhanced chemical luminescence
EDTA	ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzym-linked immunoabsorbant assay
env	envelope; gene encoding glycoprotein160
EP	epoxide
EpCAM	Epithelial cell adhesion molecule
F	forward
FACS	fluorescence-activated cell sorter
Fab	fragment antigen binding
FADD	Fas-associated protein with death domain
FAM	Fluorescein
FAP	Fibroblast activation protein
Fc f	ragment crystalline
FcR	Fc receptor
FCS	fetal calf serum
FDA	Food and Drug Administration
FITC	fluoresceinisothiocyanat
g	gram
g	acceleration of gravity
GFP	green fluorescent protein
Gly	glycin
h	hour
HAMA	human anti-mouse immune response
HEK	human embryonic kidney
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid
HERG	human eag-related gene
HIF-1	hypoxia-inducible factor 1
His	Histidin
HPLC	high-performance liquid chromatography
HRP	horse radish peroxidase conjugates
IAP	inhibitor of apoptosis proteins
Ig	immunoglobulin
IPTG	isopropyl-β-D-thiogalactopyranoside

k	kilo
Kan	kanamycin
Kv10.1	voltage-gated potassium channel
L	liter
LB	Luria Broth
m	milli
M	molar
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
memTRAIL	membrane-bound TRAIL
min	minute
MWCO	molecular weight cut-off
n	nano
NF-κB	nuclear factor for κ gene in B lymphocytes
NLS	nuclear localization signal
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAS/PAC	Per-Arnt-Sim
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	Pseudomonas exotoxin
PhoA	phosphatase A
PI	propidium iodide
PS	phosphatidylserine
R	reverse
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
s	second
scFv	single-chain fragment variable
SDS	sodium dodecylsulfate
Ser	serin
siRNA	small interfering RNA
Smac/DIABLO	second mitochondria-derived activator/direct inhibitor of apoptosis-binding protein with low pI
sTRAIL	soluble TRAIL
t	time
T	temperature

TAA	tumor associated antigen
TAE	Tris acetate EDTA buffer
TAMRA	Tetramethyl-6-Carboxyrhodamine
TBE	Tris borate EDTA buffer
TBS	Tris-buffered saline
TCC	tetramerized coiled-coil
Tm	melting temperature
TNF	tumor-necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
µ	micro
U	unit
UPL	universal probe library
UV	ultraviolet
V	volt
VEGF	vascular endothelia growth factor
v/v	volume per volume
w/o	without
w/v	weight per volume
wt	wild type
XIAP	X-linked inhibitor of apoptosis protein

9.2 Curriculum vitae

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Publications

Hartung F, Stühmer W, Pardo L A, Tumor cell-selective apoptosis induction through targeting of Kv10.1 via bifunctional TRAIL antibody. Molecular Cancer, 2011. under revision

Stühmer W, Alves F, Hartung F, Zientkowska M, Pardo L A, The role of EAG1, a potassium channel, in cancer. FEBS Letters, 2006. **580**:2850-2852.

Poster and Talk

June 2011	ScieTalk Congress “Approaches in Molecular Biology and Experimental Medicine”, Göttingen, Talk
March 2010	2nd International Meeting “Ion channel and Cancer” Florence, Talk
April 2009	4th Fabisch-Symposium for Cancer Research and Molecular Cell Biology, 2nd Targeted Tumor Therapy, Berlin, Poster