TRAIL-based multivalent and multifunctional fusion proteins and liposomes for therapeutic applications

Von der Fakultät Energie-, Verfahrens- und Biotechnik der Universität Stuttgart zur Erlangung der Würde eines Doktors der Naturwissenschaften (Dr. rer. nat.) genehmigte Abhandlung

Vorgelegt von
Oliver Seifert
aus Stuttgart

Hauptberichter: Prof. Dr. Roland Kontermann
Mitberichter: Prof. Dr. Klaus Pfizenmaier

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Institut für Zellbiologie und Immunologie
Universität Stuttgart
2014
Für Anna

und meine Eltern
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<tbody>
<tr>
<td>Å</td>
<td>Ångström (10^{-10} m)</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AE</td>
<td>adverse events</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>amp</td>
<td>ampicillin</td>
</tr>
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<td>APS</td>
<td>ammonium persulfate</td>
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<tr>
<td>asn</td>
<td>asparagine</td>
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<tr>
<td>AUC</td>
<td>area under the curve</td>
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<tr>
<td>Bid</td>
<td>BH3-interacting death domain</td>
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<td>bortezomib</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<td>CDR</td>
<td>complementarity determining region</td>
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<tr>
<td>cFLIP</td>
<td>cellular FLICE inhibitory protein</td>
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<td>C_{H}</td>
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<td>cholesterol</td>
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<td>complement-mediated cytotoxicity</td>
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<td>complete response</td>
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<td>DSPE</td>
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<td>e.g.</td>
<td>exempli gratia / for example</td>
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<td>EC_{50}</td>
<td>mean effective concentration</td>
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<td>ethylenediaminetetraacetate</td>
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<td>EGFR</td>
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<td>EHD2</td>
<td>IgE heavy chain domain 2</td>
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<td>EPR</td>
<td>enhanced permeability and retention</td>
</tr>
<tr>
<td>f</td>
<td>femto (10^{-15})</td>
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<tr>
<td>F(ab')_{2}</td>
<td>antigen binding fragment dimerized via hinge region</td>
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<td>f.c.</td>
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<tr>
<td>Fc</td>
<td>fragment crystalizable</td>
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<td>FcyR</td>
<td>Fc gamma receptor</td>
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<td>FceR</td>
<td>Fc epsilon receptor</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FOLFIRI</td>
<td>folic acid (FOL), 5-fluorouracil (F), irinotecan (IRI)</td>
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<td>h</td>
<td>hour</td>
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<td>HER2</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>His_{6}</td>
<td>hexahistidyl</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
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<td>HRP</td>
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</tr>
<tr>
<td>hu</td>
<td>human</td>
</tr>
<tr>
<td>i.e.</td>
<td>id est / that is</td>
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<td>i.p.</td>
<td>intraperitoneal</td>
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<td>i.v.</td>
<td>intravenous</td>
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<tr>
<td>IAP</td>
<td>inhibitor of apoptosis</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IL</td>
<td>immunoliposomes</td>
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<td>ILT</td>
<td>Immuno-LipoTRAIL</td>
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<td>IMAC</td>
<td>immobilized metal ion affinity chromatography</td>
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<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>iKB</td>
<td>inhibitor of κB</td>
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<tr>
<td>LB</td>
<td>lysogeny broth</td>
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<tr>
<td>LT</td>
<td>LipoTRAIL</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>m</td>
<td>milli (10^{-3})</td>
</tr>
<tr>
<td>Mal</td>
<td>maleimide</td>
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<td>med PFS</td>
<td>median progression free survival</td>
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<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
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<td>MFI</td>
<td>mean fluorescence intensity</td>
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<td>MHD2</td>
<td>IgM heavy chain domain 2</td>
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<tr>
<td>mol%</td>
<td>substance amount fraction</td>
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<td>mPEG</td>
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<td>M_{r}</td>
<td>molecular mass</td>
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<td>MTD</td>
<td>maximum tolerated doses</td>
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<td>mTNF</td>
<td>membrane-bound TNF</td>
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<td>MW</td>
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<td>n</td>
<td>nano (10^{-9})</td>
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<td>NFκB</td>
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<td>nm</td>
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<td>NSCLC</td>
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<tr>
<td>nt</td>
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<td>OD</td>
<td>optical density</td>
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<td>OPG</td>
<td>osteoprotegerin</td>
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<td>ORR</td>
<td>overall response rate</td>
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Oliver Seifert
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<td>OS</td>
<td>overall survival</td>
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<tr>
<td>p</td>
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<td>PARA</td>
<td>pro-apoptotic receptor agonists</td>
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<td>PBS with BSA and azide</td>
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<td>PEG&lt;sub&gt;2000&lt;/sub&gt;</td>
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<td>PR</td>
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<td>reticuloendothelial system</td>
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<td>rpm</td>
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<td>room temperature</td>
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<td>scFv</td>
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<td>scFv'&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>scTRAiL</td>
<td>single-chain TRAIL</td>
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<td>SD</td>
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<td>sTRAil</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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<td>TNFR</td>
<td>TNF receptor</td>
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<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
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<td>TTRAILR</td>
<td>TRAIL receptor</td>
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<td>V&lt;sub&gt;L&lt;/sub&gt;</td>
<td>variable light chain domain</td>
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<tr>
<td>α</td>
<td>anti-</td>
</tr>
<tr>
<td>ε</td>
<td>molecular extinction coefficient</td>
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<td>µ</td>
<td>micro ($10^{-6}$)</td>
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Summary

The current state of the clinical studies in the field of pro-apoptotic receptor agonists (PARA) targeting the receptor of the TNF-related apoptosis-inducing ligand (TRAIL) has revealed disappointing results for the treatment of cancer until now. Based on the targeted and multivalent presentation of TRAIL, the therapeutic concept of this study provides an efficient induction of apoptosis in cancer cells by activating the two death receptors DR4 and DR5, thus mimicking the activity of the membrane-bound TRAIL (mTRAIL). These targeted multivalent TRAIL molecules were generated as: i) dimeric-assembled and targeted TRAIL fusion proteins utilizing the heavy chain domain 2 of IgE (EHD2) as homodimerization module, and ii) targeted TRAIL-functionalized liposomes. Furthermore, the heavy chain domain 2 of IgM (MHD2) was also tested as homodimerization module generating dimeric-assembled and targeted multivalent tumor necrosis factor (TNF) fusion proteins.

Antibody-MHD2 fusion proteins possessing mono or dual specificity were generated by fusing a single-chain fragment variable (scFv) directed against epidermal growth factor receptor (EGFR, EGFR1) or EGFR2 (HER2) to the N-terminus and/or the C-terminus of the MHD2 and showed an avidity-effect and a cooperative binding to tumor cells. By modifying the MHD2 with a single-chain derivative of the TNF (scTNF) molecule, a hexavalent TNF fusion protein was generated being able to activate TNF receptor 1 and 2, thus mimicking mTNF. Furthermore, the scFv-mediated delivery of the targeted multivalent TNF fusion protein to EGFR-positive cells further increased its potency of activating TNF receptors.

Fusing a scFv fragment directed against EGFR to the N-terminus and a single-chain derivative of TRAIL (scTRAIL) to the C-terminus of the EHD2, a targeted fusion protein was generated displaying multivalent TRAIL molecules (scFv-EHD2-scTRAIL). The in vitro cell death induction of the trivalent scTRAIL molecule was exceeded by the TRAIL fusion proteins, while the scFv-EHD2-scTRAIL fusion proteins exhibited an approximately 8- to 18-fold increased induction of apoptosis compared with the non-targeted EHD2-scTRAIL fusion protein. This resulted in a superior antitumoral activity in a xenograft Colo205 model in mice.

As an alternative, liposomes were functionalized with TRAIL molecules for multivalent display of TRAIL (LipoTRAIL, LT). Additionally, TRAIL-functionalized liposomes were equipped with scFv fragments directed against EGFR for a targeted delivery to EGFR-positive tumor cells (Immuno-LipoTRAIL, ILT). ILT bound specifically to EGFR-positive tumor cells and induced a more potent cell death compared with the untargeted LT. The functionalized liposomes exhibited a prolonged serum half-life compared with the soluble TRAIL molecule. Furthermore, ILT revealed a potent antitumoral response in a xenograft Colo205 model in mice.

In conclusion, the results of this study demonstrate that the fusion proteins and functionalized liposomes displaying multivalent TRAIL molecules and scFv fragments might overcome the limitation of the PARA currently tested in clinical studies.
Zusammenfassung

Der aktuelle Stand der klinischen Forschung bietet im Bereich der pro-apoptotischen Rezeptoragonisten, welche die TRAIL-Rezeptoren (TNF-related apoptosis-inducing ligand) ansteuern, für die Krebstherapie bisher keine zufriedenstellenden Ergebnisse. In dieser Doktorarbeit wurde ein therapeutisches Konzept entwickelt, das auf der zielgerichteten und multivalenten Präsentation von ebendiesem TRAIL basiert, und welches eine effektive Induktion des Zelltods in Krebszellen durch die Aktivierung der beiden Todesrezeptoren DR4 und DR5 vorsieht, indem die membrangebundene Form des TRAIL-Moleküls nachgeahmt wird. Folgende zielgerichtete und multivalente TRAIL-Moleküle wurden hergestellt: a) als Dimer angeordnete, zielgerichtete TRAIL-Fusionsproteine durch Verwendung des Homodimerisierungs-Moduls EHD2 (Domäne 2 der schweren Kette des Immunglobulins IgE), b) zielgerichtete und mit TRAIL-Molekülen modifizierte Liposomen. Darüber hinaus wurde MHD2 (Domäne 2 der schweren Kette des Immunglobulins IgM) als ein weiteres Homodimerisierungs-Modul genauer untersucht und als Dimer-angeordnete, zielgerichtete und multivalente TNF (Tumor Nekrose Faktor)-Fusionsproteine hergestellt. Durch Fusionierung eines einzelkettigen Antikörper-Fragments (scFv), welches entweder gegen EGFR (epidermal growth factor receptor, EGFR1) oder gegen HER2 (EGFR2) gerichtet ist, an den N- und/oder C-Terminus des MHD2 konnten mono- oder bi-spezifische Antikörper-MHD2-Fusionsproteine hergestellt werden. Diese wiesen einen Aviditätseffekt und eine kooperative Bindung gegenüber Tumorzellen auf. Indem man das MHD2 mit einem einzelkettigen Derivat des TNF (scTNF) modifizierte, konnte ein hexavalentes TNF-Fusionsprotein generiert werden, welches die NF-Rezeptoren 1 und 2 zu aktivieren vermag und demnach das membrangebundene TNF-Molekül nachahmen kann. Darüber hinaus, wurde durch den scFv-vermittelten Transport des zielgerichteten und multivalenten TNF-Fusionsproteins zu EGFR-positiven Zellen seine Aktivität der TNF-Rezeptor-Aktivierung weiter gesteigert.

Durch Fusionierung eines gegen EGFR gerichteten scFv-Fragments an den N-Terminus des EHD2 und eines einzelkettigen Derivats des TRAIL-Moleküls (scTRAIL) an den C-Terminus, konnte ein zielgerichtetes Fusionsprotein hergestellt werden, welches multivalente TRAIL- und scFv-Moleküle präsentiert (scFv-EHD2-scTRAIL). In vitro zeigte sich eine ausgeprägte tumorzellspezifische Induktion des Zelltods für die TRAIL-Fusionsproteine im Vergleich zu den trivalenten scTRAIL-Molekülen: Das scFv-EHD2-scTRAIL-Fusionsprotein wies sogar eine 8- bis 18-mal stärkere Apoptoseinduktion als das nicht-zielgerichtete EHD2-scTRAIL-Fusionsprotein auf. Diese Ergebnisse spiegelten sich auch in einer ausgezeichneten antitumoralen Aktivität im xenografen Colo205 Tumormodell bei Mäusen wieder. Alternativ wurden Liposomen mit TRAIL-Molekülen modifiziert, um diese multivalent präsentieren zu können (LipoTRAIL, LT). Außerdem wurden die TRAIL-funktionalisierten Liposomen mit an EGFR bindenden scFv-Fragmenten ausgestattet (Immuno-LipoTRAIL,

Die Ergebnisse dieser Arbeit deuten darauf hin, dass durch den Einsatz von Fusionsproteinen und funktionalisierten Liposomen, die durch multivale TRAIL-Moleküle und scFv-Fragmente modifiziert wurden, die Einschränkung der PARA, welche derzeit in klinischen Studien erprobt werden, überwindet werden könnte.
1 Introduction

In the last decades, two members of the TNF superfamily of cytokines, TNFα and CD95 ligand (CD95L), were investigated for the treatment of cancer. However, acute toxicity after systemic administration limited their use as therapeutic agents. Another member of the TNF superfamily is the TNF-related apoptosis-inducing ligand (TRAIL), which was reported to induce apoptosis on cancer cells in a wide variety via activation of death receptors, but simultaneously appeared to be well tolerated by normal cells. For these reasons, the TRAIL molecule and pro-apoptotic antibodies that activate the death receptors are of interest for generating novel therapeutics for the treatment of cancer by inducing apoptosis. However, the clinical studies of these therapeutics showed disappointing results for different kinds of cancers. For this reason, the purpose of this study was to develop concepts leading to novel therapeutic agents that utilize the apoptosis-inducing potential of TRAIL in a more effective manner.

1.1 Cellular apoptosis

Apoptosis is a cellular process that leads to cell death in order to remove damaged cells from the body. The induction of apoptosis is triggered by two pathways, namely the intrinsic pathway, which is activated via a cellular signal due to cell stress, e.g. damaging of cellular DNA via chemotherapy or radiotherapy, and the extrinsic pathway for transducing an extracellular signal into the cell via pro-apoptotic receptors, e.g. killing by immune cells. Both pathways end up with the activation of the effector caspases 3, 6, and 7, which then execute the apoptosis (Lavrik et al., 2005).

1.1.1 Intrinsic pathway

The induction of apoptosis via the intrinsic pathway depends strongly on the balance between pro- and anti-apoptotic proteins in the cell that control the release of the cytchrome c from the mitochondrion into the cytosol. Cytochrome c can interact with Apaf-1 and caspase 9 forming a signaling complex called apoptosome, which is able to induce the effector caspases 3, 6 and 7 leading to the execution of the cell (Henry-Mowatt et al., 2004). The pro- and anti-apoptotic proteins are classified into the Bcl-2 gene family. The pro-apoptotic proteins Bax, Bak, and Bok belong to the Bax subfamily and are responsible for the permeabilization of the mitochondrion leading to release of cytchrome c, whereas the members of the anti-apoptotic Bcl-2 subfamily (e.g. Bcl-2 and Mcl-1) are able to prevent this process. Besides cytchrome c, another pro-apoptotic protein is released from the mitochondrion into the cytosol, namely the Smac/DIABLO protein, which is able to induce
apoptosis indirectly by inhibiting the members of the subfamily inhibitor of apoptosis (IAP), e.g. XIAP, cIAP1, and cIAP2. The IAP subfamily is able to interact with the initiator caspase 9 and the effector caspases 3 and 7 resulting in an inhibition of these caspases and are E3 ligases (Falschlehner et al., 2007). More active effector caspases are present in the cell, when Smac/DIABLO inhibits the IAPs (Wiezorek et al., 2010). The tumor suppressor protein p53 regulates the expression of the genes of the pro-apoptotic proteins, e.g. Puma, Noxa, Bax, and Apaf-1, and thereby is mainly involved in the induction of apoptosis via the intrinsic pathway (Vousden and Lu, 2002).

Figure 1-1: The intrinsic and extrinsic pathway of the signaltransduction of apoptosis. The extrinsic pathway is triggered by the binding of apoptosis-inducing ligands to pro-apoptotic receptors resulting in oligomerization of the receptors. The death-inducing signaling complex (DISC) is composed of the Fas-associated death domain (FADD), which binds to the death domain (DD) of the receptor and recruits the procaspases 8 and 10 undergoing auto-activation by proteolytic cleavage (caspases 8 and 10). The initiator caspases 8 and 10 activate the downstream effector capsases 3, 6, and 7, which execute apoptosis in the cells. The intrinsic pathway is triggered by cellular stress, e.g. DNA damage by radiotherapy, resulting in a p53-dependent upregulation of pro-apoptotic proteins, e.g. Puma and Noxa. These proteins induce the permeabilization of mitochondrions resulting in a release of cytochrome c and Smac/DIABLO into the cytosol. The apoptosome is composed of Apaf-1, cytochrome c and caspase 9, exhibiting the activation of the effector caspases 3, 6, and 7. Smac/DIABLO also activates apoptosis indirectly by inhibiting the subfamily inhibitor of apoptosis (IAP). The cross-talk between the extrinsic and the intrinsic pathway is performed by the pro-apoptotic protein Bid, which can be cleaved and activated by caspase 8 into the truncated form tBid. This protein induces apoptosis by inhibiting the anti-apoptotic proteins Bcl-2 and Mcl-1 and by activating the pro-apoptotic proteins Bax and Bac. The mitochondrion is shown in brown, while the pro-apoptotic proteins are displayed in green and the anti-apoptotic proteins are colored in red.
1.1.2 Extrinsic pathway

The extrinsic pathway transduces an extracellular signal into the cellular level. Upon oligomerization of the death receptor (DR) by binding of the death-ligand, the Fas-associated death-domain (FADD) and the initiator pro-caspases 8 and 10 are recruited to the activated receptor. When the pro-caspases (zymogen) are auto-activated by proteolytic processing, the death-inducing signal complex (DISC) is accomplished. Consequently, the initiator caspases can activate the effector caspases 3, 6, and 7 (Bernardi et al., 2012) leading to the execution of apoptosis. Cells are classified into type 1 cells, that induce apoptosis via the extrinsic pathway upon activation of the DR, while the type 2 cells need an additional amplification step via the mitochondrial (intrinsic) pathway (Ozören and El-Deiry, 2002). The cross-talk between the extrinsic and the intrinsic pathway is performed by the Bid protein, which belongs to the pro-apoptotic BH3-only protein subfamily of the Bcl2 gene family. The Bid protein is caspase-8-dependent cleaved to a truncated form of Bid (tBid), which is able to inhibit the anti-apoptotic properties of the Bcl2 subfamily (Luo et al., 1998).

1.2 TNF-related apoptosis-inducing ligand (TRAIL)

The TNF-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily and is expressed as a transmembrane type II protein consisting of 281 amino acids. The bioactive form of TRAIL is composed of a homotrimeric structure (see Figure 1-2 A), whose coordination is dependent on the binding of an internal zinc ion (Lawrence et al., 2001). Apoptosis can be induced by binding to the pro-apoptotic receptors TRAIL-receptor 1 (TRAILR1; DR4) (Pan, 1997a) and TRAIL-receptor 2 (TRAILR2; DR5) (Pan, 1997b) (Hymowitz et al., 1999). The cytosolic segment of these death receptors consists of a death domain, which is able to build the DISC initiating the extrinsic pathway of apoptosis (see 1.1.2). The membrane-bound TRAIL (mTRAIL) can be converted into a soluble TRAIL (sTRAIL) via proteolytic cleavage (LeBlanc and Ashkenazi, 2003), which can induce apoptosis largely via activation of DR4 (Schneider et al., 2010), whereas the mTRAIL is able to activate DR4 and DR5 (see Figure 1-2 B) (Berg et al., 2007, 2009; Wajant et al., 2001). Besides the death receptors, TRAIL also binds to the decoy receptors that do not transduce apoptotic signals into the cells. The decoy receptors consist of TRAIL-receptor 3 (TRAILR3; DcR1) (Degli-Esposti et al., 1997b), which lacks the complete death domain, TRAIL-receptor 4 (TRAILR4; DcR2) (Degli-Esposti et al., 1997a; Marsters et al., 1997) harboring only a truncated death domain, and the soluble osteoprotegerin (OPG) (Emery et al., 1998).
1.2.1 Pro-apoptotic receptor agonists (PARA)

Apoptosis is a powerful mechanism to kill cells. However, about 50% of the cancers exhibit a dysfunction of apoptosis due to the mutation of the tumor suppressor protein p53 leading to a resistance against chemotherapy- or radiotherapy-induced apoptosis via the intrinsic pathway. For this reason, novel therapeutic strategies focus on the induction of apoptosis via a p53-independent manner, namely the activation of the death receptors transduced by the extrinsic pathway.

In general, the therapeutic strategy of the pro-apoptotic receptor agonists (PARA) is based on the activation of the apoptosis-inducing receptor CD95, DR4 and DR5 using either the recombinant human TRAIL (Ashkenazi et al., 1999), the CD95L protein, or agonistic IgG antibodies directed against either DR4 or DR5 (Chuntharapai et al., 2001; Ichikawa et al., 2001). CD95L showed potent cell death-inducing potential, but hepatotoxicity limited its usage (Nagata, 1997). In addition, the targeted scFv-CD95L fusion protein showed cell surface antigen-mediated activation of CD95 for the first time (Samel et al., 2003). Furthermore, a hexavalent CD95L fusion protein was administered locoregional and induced glioma cell death in vivo (Eisele et al., 2011). This fusion protein has entered a clinical study phase I dose finding study in patients with solid tumors, but no results were reported until now (see http://clinicaltrials.gov/ct2/show/NCT00437736). In contrast, experiments with a recombinant sTRAIL molecule showed strong cell death induction on a wide variety of cancer cells in vitro being well tolerated by normal cells (Ashkenazi et al., 1999). PARA that activate the DRs are listed in Table 1-1.
Introduction

Table 1-1: Pro-apoptotic receptor agonists (PARA) in clinical studies; adapted from (Bernardi et al., 2012).

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Developed by</th>
<th>Mechanism/Pharmacology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conatumumab</td>
<td>Amgen</td>
<td>Human mAb targeting DR5</td>
</tr>
<tr>
<td>Drozitumab</td>
<td>Genentech</td>
<td>Human mAb targeting DR5</td>
</tr>
<tr>
<td>Tigatuzumab</td>
<td>Daiichi Sankyo</td>
<td>Humanized version of murine DR5-targeting antibody</td>
</tr>
<tr>
<td>Mapatumumab</td>
<td>HGS GSK</td>
<td>Human mAb targeting DR4</td>
</tr>
<tr>
<td>Lexatumumab</td>
<td>HGS</td>
<td>Human mAb targeting DR5</td>
</tr>
<tr>
<td>LBY135</td>
<td>Novartis</td>
<td>Chimeric mAb targeting DR5</td>
</tr>
<tr>
<td>Dulanermin</td>
<td>Genentech/ Amgen</td>
<td>rhTRAIL targeting DR4 and DR5</td>
</tr>
</tbody>
</table>

HGS, Human Genome Science; GSK, Glaxo Smith Kline

Although dulanermin (recombinant human TRAIL molecule) and the agonistic antibodies showed similar cell death induction on a wide variety of cancer cells in the preclinical studies (Ashkenazi and Herbst, 2008), there are different properties for these different therapeutic agents. In case of dulanermin, half-life in human is 30 minutes (Herbst et al., 2006), whereas the antibodies exhibit half-lives from 6 to 21 days (Fox et al., 2010). Furthermore, dulanermin is composed of an homotrimeric form that is able to activate the receptors resulting in sufficient induction of apoptosis in vitro. Agonistic antibodies are characterized to bind only two receptor moieties per molecule. Effective induction of apoptosis in in vitro studies was achieved by adding a cross-linking agent (Adams et al., 2008; Motoki et al., 2005; Takeda et al., 2004). Tumor cell killing of antibodies in the absence of a cross-linker was sufficient in xenograft tumor models in mice, which led to the assumption that the Fcγ receptors of the immune cells were responsible for the cross-linkage (Chuntharapai et al., 2001). Recently, Wilson and coworkers (Wilson et al., 2011) showed the dependency of the tumor response of a DR5-directed antibody (Drozitumab) on FcγR-mediated activation of DR5 via clustering. In vitro assays showed an overall effective cell killing of tumor cells by cross-linking the antibody. The in vivo assays clearly displayed the dependency on Fcγ receptor cross-linking for potent antitumoral activity. It was assumed that the marginal antitumoral response of the DR5-directed antibody observed in clinical studies results from the high IgG blood concentration of patients leading to a competitive situation of the blood IgGs and the therapeutic IgGs towards binding to the Fcγ receptors on leucocytes. Since the IgG concentration in nude mice is rather low, the clustering of the DR4- or DR5-directed antibodies via the Fcγ receptors should be lower in patients compared with nude mice, thus resulting in an insufficient tumor response in patients (Gieffers et al., 2013).
1.2.2 Clinical studies of the PARA

The clinical trials of the pro-apoptotic receptor agonists (PARA) were overall safe and well-tolerated and the maximum tolerated doses (MTD) were not reached during the trials (Ashkenazi, 2008; Camidge et al., 2010). Generally, the clinical outcome of the monotherapy of all PARAs did not show sufficient tumor response neither for dulanermin nor for the antibodies directed against the death receptors (Bernardi et al., 2012; den Hollander et al., 2013). The first monotherapy in clinical phase I with dulanermin was conducted with 71 patients having either advanced solid tumors or non-Hodgkin lymphoma (NHL) and revealed partial response (PR) for only 1 patient with chondrosarcoma (Herbst et al., 2010). In order to achieve more potent antitumoral response, the treatment with dulanermin was combined with chemotherapeutics theoretically recruiting the intrinsic pathway of apoptosis. Preclinical data suggested an improved tumor response by combinatorial treatment of TRAIL with the chemotherapeutic irinotecan. Therefore, patients with metastatic colorectal cancer (CRC) were treated with dulanermin in combination with either irinotecan and the antibody cetuximab (Erbitux®, anti-EGFR) or FOLFIRI-regimes (combination of folic acid (FOL), 5-fluoruracil (F) and irinotecan (IRI)) in a clinical phase Ib study. The data indicated that dulanermin could be safely combined with the FOLFIRI-regime. However, no antitumoral activity was observed. The combinatorial treatment of TRAIL with irinotecan and cetuximab showed several side effects (Yee et al., 2009). Additionally, in another clinical phase Ib study the combination of dulanermin with chemotherapy (paclitaxel, carboplatin) and antibody therapy (bevacizumab, Avastin®, anti-VEGF) was tested on 24 patients with previously untreated advanced or recurrent non-small cell lung cancer (NSCLC) and revealed 1 complete response (CR) and 13 PR (overall response of 58 %) (Soria et al., 2010). Evaluating these results in a randomized clinical phase II study (213 patients with advanced or recurrent NSCLC), the outcome could not be improved compared with conventional chemotherapy (Soria et al., 2011). Furthermore, in a clinical phase Ib study, dulanermin was tested in combination with antibody therapy (rituximab, MabThera®, anti-CD20) on 7 patients with low grade NHL (follicular and small cell) who relapsed after rituximab treatment. This combination resulted in 2 CR and 1 PR (Yee et al., 2007). In another study, 12 patients with low grade NHL, who also relapsed after rituximab treatment, showed 3 CR and 3 PR (Fanale et al., 2008). The antitumoral response of this rituximab-dulanermin combination was tested in a clinical phase II study on 26 patients with follicular NHL (progression after first rituximab therapy). However, the outcome did not improve compared with a patient group that only received rituximab (n=22) (Belada et al., 2010). Recently, dulanermin was tested in combination with FOLFOX (combination of folic acid (FOL), 5-fluoruracil (F), and oxaliplatin (OX)) and bevacizumab on 23 patients with advanced, recurrent or metastatic CRC. Adverse events (AEs) were reported, but they were similar to the treatment with FOLFOX plus
bevacizumab only and did not change for the worse. PR could be observed for 9 patients and the results will be validated in a randomized clinical phase II study (Wainberg et al., 2013). The clinical studies of dulanermin are listed in Table 1-2. In summary, the clinical studies of dulanermin showed good tolerability as single agent or in combination with other approved therapeutic agents such as chemotherapeutics or antibodies. No objective responses were mediated by dulanermin as monotherapy. In contrast, in case of the combination of dulanermin with other approved therapeutics, tumor response could be observed in clinical phase I studies, but the outcomes were not confirmed in randomized clinical phase II studies.
### Table 1-2: Clinical studies of dulanermin, adapted from (den Hollander et al., 2013).

<table>
<thead>
<tr>
<th>Ph.</th>
<th>Tumortype</th>
<th>No.</th>
<th>Therapy</th>
<th>Response</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>advanced solid tumors or NHL</td>
<td>51</td>
<td>dulanermin</td>
<td>1 PR</td>
<td>(Herbst et al., 2006)</td>
</tr>
<tr>
<td>I</td>
<td>advanced cancer</td>
<td>71</td>
<td>dulanermin</td>
<td>2 PR, 31 SD med PFS 2.3 months</td>
<td>(Herbst et al., 2010)</td>
</tr>
<tr>
<td>Ib</td>
<td>relapsed low-grade NHL</td>
<td>7</td>
<td>rituximab + dulanermin</td>
<td>2 CR, 1 PR, 2 SD</td>
<td>(Yee et al., 2007)</td>
</tr>
<tr>
<td>Ib</td>
<td>relapsed low-grade NHL</td>
<td>12</td>
<td>rituximab + dulanermin</td>
<td>3 CR, 3 PR</td>
<td>(Fanale et al., 2008)</td>
</tr>
<tr>
<td>II</td>
<td>NHL</td>
<td>48</td>
<td>Arm1: rituximab + dulanermin</td>
<td>Arm1/2 ORR 61.5%/63.6%</td>
<td>(Belada et al., 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Arm2: rituximab</td>
<td>CR 5 (1 unc.)/5 PR 12/9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>paclitaxel, carboplatin, bevacizumab</td>
<td>1 CR, 13 PR ORR 58%</td>
<td>(Soria et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>advanced non-squamous NSCLC</td>
<td></td>
<td>+ dulanermin</td>
<td>med PFS 7.2 months</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>213</td>
<td>Arm1: paclitaxel (P) + carboplatin (C)</td>
<td>Arm1/2/3/4/5 ORR 39/38/50/40/40%</td>
<td>(Soria et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Arm2: P+C+dulanermin (D)</td>
<td>PFS:6.1/5.5/7.3/8.6/9.5 months</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(8mg/kg/5days)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Arm3: P+C+bevacizumab (B)</td>
<td>OS:10.1/9.8/15.1/13.914.3 months</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Arm4: P+C+B+D(8mg/kg/5days)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Arm5: P+C+B+D(20mg/kg/5days)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>35</td>
<td>irinotecan + cetuximab or FOLFIRI</td>
<td>no results</td>
<td>(Yee et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>metastatic CRC</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>advanced, recurrent or metastatic CRC</td>
<td>23</td>
<td>FOLFOX + bevacizumab + dulanermin</td>
<td>PR 13 (4 unc.) SD 7</td>
<td>(Wainberg et al., 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>med PFS 9.9 months</td>
<td></td>
</tr>
</tbody>
</table>

NHL, non-Hodgkin’s lymphoma; NSCLC, non-small cell lung cancer; CRC, colorectal cancer; CR, complete response; PR, partial response; SD, stable disease; ORR, objective response rate; med PFS, median progression free survival; OS, overall survival; unc., unconfirmed
1.2.3 Bortezomib as sensitizer for TRAIL-induced apoptosis

Bortezomib, also known as Velcade®, is a dipeptidyl boronic acid compound that is able to block reversibly the catalytic activity of the proteasome (Adams and Kauffman, 2004) and is FDA-approved for the treatment of multiple myeloma. In preclinical studies it was observed that bortezomib in combination with TRAIL induces apoptosis on TRAIL-resistant colon cancer cell lines (Zhu et al., 2005) or on hepatocellular carcinoma (Ganten et al., 2005). Many factors of the extrinsic and the intrinsic pathway of apoptosis are affected when treating the cells with bortezomib (de Wilt et al., 2013), e.g. the increased expression of DR4 and DR5 on the cell surface (Liu et al., 2010), the slower degradation of the inhibitor IκB resulting in lower activity of NF-κB followed by lower expressions of anti-apoptotic proteins like c-FLIP (Sayers et al., 2003), and the reduced degradation of the BH3-only protein tBid, which engages the intrinsic pathway after activation of the death receptors (Naumann et al., 2011). Two clinical studies were performed using anti-DR antibodies in combination with bortezomib. Conatumumab in combination with bortezomib was investigated in a clinical phase Ib study on patients with lymphoma and was well-tolerated (Younes et al., 2009), while mapatumumab in combination with bortezomib entered a phase II study treating 105 patients with refractory or relapsed multiple myeloma (see http://clinicaltrials.gov/ct2/show/NCT00315757; HGS1013-C1055), for which no results are reported.

1.2.4 TRAIL fusion proteins with improved cell death inducing activity

TRAIL is a homotrimeric protein and is able to bind to the death receptors and to induce apoptosis. The genetic fusion of three TRAIL subunits into one polypeptide chain via short peptide linker resulted in a single-chain derivative of TRAIL (scTRAIL; see Figure 1-3) with enhanced stability and antitumoral activity (Schneider et al., 2010), similar to the preparation of single-chain TNF (Krippner-Heidenreich et al., 2008). Furthermore, the bioactivity of the scTRAIL molecule was increased by tumor-targeted delivery, e.g. by fusing a single-chain Fv (scFv) fragment directed against HER2, resulting in increased cell death induction of HER2-positive tumor cells in vitro and in vivo in xenograft models using Colo205-bearing mice (scFv-scTRAIL; see Figure 1-3) (Schneider et al., 2010). The next generation of TRAIL fusion proteins was the controlled dimerization of scTRAIL by fusing a scTRAIL to each subunit of a diabody molecule resulting in a diabody-scTRAIL fusion protein (Db-scTRAIL; see Figure 1-3). In this case, the diabody moiety is responsible for dimerization of the scTRAIL moieties and for targeting of tumor-associated antigens. Using a diabody directed against EGFR resulted in increased in vitro and in vivo bioactivity of the Db-scTRAIL fusion protein compared with the scTRAIL and scFv-scTRAIL (Siegemund et al., 2012) fusion proteins.
1.3 Novel concepts for TRAIL therapeutics

A potent induction of apoptosis was reported for the Db-scTRAIL fusion protein, which is able to activate both death receptors DR4 and DR5 by displaying multivalent TRAIL molecules and thereby mimicking the activity of mTRAIL (Siegemund et al., 2012). The concept of multivalent TRAIL presentation was utilized for the generation of other fusion proteins using novel homodimerization modules and for the generation of TRAIL-functionalized liposomes.

1.3.1 Homodimerization domains

Homodimerization of therapeutic proteins such as antibodies, cytokines and growth factors generates bivalent molecules that generally display improved efficacy due to increased valency and improved pharmacokinetic properties because of increased molecular weight (Deyev and Lebedenko, 2008; Kontermann, 2011). Additionally, the combination of two different effector molecules allows the generation of fusion proteins with dual functionalities. Such molecules can be designed either by two different antibodies resulting in dual targeting or by one antibody and one effector moiety resulting in a bifunctional fusion protein (Kontermann, 2012). Various homodimerization and multimerization molecules have been described in literature including the Fc region (Jazayeri and Carroll, 2008), as well as the constant domains of the heavy chain (C_H3) (Hu et al., 1996) and the light chain (C_L) of immunoglobulins (Giersberg et al., 2010), but also other protein domains and short peptide sequences derived for example from streptavidin (Dübel et al., 1995), p53 (Rheinnecker et al., 1996), uteroglobin (Ventura et al., 2009), tenasin (Wüest et al., 2002), and collagen (Fan et al., 2008). However, many of these modules are either non-human and/or non-covalently connected by disulfide bonds. Thus, using these modules for the generation of therapeutic molecules could induce an immune response against the homodimerization module (Baker et al., 2010) and/or could result in an imperfect dimerization of the therapeutic molecule.
1.3.2 Novel developed homodimerization modules

The immunoglobulins (Ig) consist of light and heavy chains and represent a family of five different classes based on the sequence of their heavy chain constant domain: IgA, IgD, IgE, IgG, and IgM. These classes harbor different biological functions associated with various structures and biological properties. IgA, IgD, and IgG are composed of 3 domains of the heavy chain, which is covalently linked to the other heavy chain via the hinge-region. In contrast, IgM and IgE contain an additional domain (C_{H2}), which is also called 'hinge-domain' that connects the two heavy chains (see Figure 1-4). The heavy chain domain 2 of IgM (MHD2) consists of 111 amino acids and exhibits one N-glycan. The homodimer of MHD2 is covalently held together by one disulfide bond. The heavy chain domain 2 of IgE is composed of 106 amino acids and also exhibits one N-glycan. In contrast to the MHD2, the EHD2 homodimer is covalently linked by two disulfide bonds. Because of the central localization of the MHD2 and the EHD2 in the Ig molecule, the exchange of the flanked C_{H1} and C_{H3} domain with proteins should result in dimeric fusion proteins. Thus, the MHD2 and the EHD2 should be ideally suited for the usage as homodimerization modules.

![Diagram of immunoglobulins](image)

**Figure 1-4: Schematic illustration of the immunoglobulins.** The variable domains are displayed in gray (V_{L}, V_{H}) and the constant domains are shown in white (C_{L}, C_{H1}, C_{H2}, C_{H3}, and C_{H4}). The domains of the light chain are indicated as L, while the domains of the heavy chain are labeled as H. The interdomain or interchain disulfide bonds are indicated as orange lines. Hinge region and 'hinge-domain' are highlighted in the schemes.

1.4 Liposomal nanocapsules

Liposomes are capsular structures based on lipid bilayers. The fundamental idea of preparing liposomes was the encapsulation of toxic agents lowering the side effects after application. Since the first liposomes, also called conventional liposomes (see Figure 1-5), exhibited a short plasma half-life (Gregoriadis and Ryman, 1972), polyethylene glycol (PEG) modified liposomes are commonly used, due to their prolonged pharmacokinetic properties.
Introduction

(Allen and Hansen, 1991; Gref et al., 1994). These liposomes, also called stealth liposomes (see Figure 1-5), have been described to accumulate in the tumor site via the enhanced permeability retention effect (EPR) (Maruyama, 2011). This phenomenon can be explained by a leaky tumor endothelial system with pores sized up to 400 to 600 nm (Allen and Cullis, 2004; Gullotti and Yeo, 2009) and by a reduced lymphatic drainage of the tumor tissue (Jain, 1987). Incorporating a targeting moiety into the liposomal surface was expected with a further accumulation in the tumor site. These liposomes, also called immunoliposomes (see Figure 1-5), were initially modified with a whole antibody. However, the half-life was reduced due to the recognition of the Fc-part of the antibody by the reticuloendothelial system (RES) (Koning et al., 2001, 2003). Therefore, antibody fragments lacking the Fc part were used for the preparation of liposomes, namely fragment antigen binding (Fab') or a single-chain fragment variable (scFv) (see Figure 1-5) (Kontermann, 2006).

![Schematic illustration of the different types of liposomes.](image)

**Figure 1-5: Schematic illustration of the different types of liposomes.** The conventional liposomes are composed of a lipid bilayer. The stealth liposomes exhibit additionally a PEG chain on the surface of the membrane. The immunoliposomes are further functionalized with a targeting moiety on the liposomal surface, e.g. a single-chain fragment variable (scFv; shown in white).

One approach to generate scFv-coupled immunoliposomes is the post-insertion method, in which scFv fragments are firstly coupled to micelles composed of functionalized lipids and then inserted into the outer layer of pre-formed liposomes in a temperature- and time-dependent manner (see Figure 1-6) (Ishida et al., 1999). The post-insertion method has several advantages: i) the coupling and the preparation of liposomes (including the encapsulation of drugs) are separated and can be independently optimized, and ii) different proteins can be separately coupled to micelles and can be accurately inserted into the liposomes. A site-directed coupling of the scFv fragment with the micelle can be performed by introducing an additional cysteine residue into the scFv fragment (scFv'), whose free sulfhydryl group can react with the maleimide group of a functionalized lipid to a stable thioether bond (Messerschmidt et al., 2008).
Figure 1-6: Schematic illustration of the post-insertion method. Cysteine-modified scFv fragment (scFv') is coupled to micelles composed of DSPE-PEG-Mal resulting in scFv-coupled micelles. These micelles can be incorporated into preformed PEGylated liposomes in a temperature- and time-dependent manner (post-insertion method) resulting in immunoliposomes.

Park and coworkers could show an increased antitumoral activity of doxorubicin-loaded and scFv-modified immunoliposomes (directed against HER2) on HER2-expressing cells compared with non-targeted doxorubicin-loaded liposomes and free doxorubicin (Park et al., 2002). Similar results were observed for anti-EGFR Fab'-modified and doxorubicin-loaded immunoliposomes on EGFR-expressing tumor cells (Mamot et al., 2005). However, both investigations revealed that the increased bioactivity did not correlate with the amount of liposomes at the tumor site, due to similar accumulation of immunoliposomes and non-targeted liposomes. Therefore, the targeting moiety may not be crucial for the transport to the tumor, but for the bioactivity at the tumor site. The binding of immunoliposomes to various tumor cells expressing different antigens can be improved by a dual targeting strategy (Kontermann, 2012). For example, dual-targeted immunoliposomes were generated by incorporating two scFv fragments directed against EGFR and CEA via a two-step post-insertion approach, resulting in binding to both antigens in in vitro assays (Mack et al., 2012). Instead of the scFv fragments, the liposomal surface can also be modified with other molecules, e.g. with TRAIL. Recent studies described that the decoration of the liposomal surface with TRAIL results in increased cell death induction compared with the unconjugated soluble TRAIL (Martinez-Lostao et al., 2010; De Miguel et al., 2013).
1.5 Aim of this study

The aim of this study was the generation of multivalent and multifunctional therapeutic molecules based on TNF-related apoptosis-inducing ligand (TRAIL) for the induction of apoptosis and a single-chain fragment variable (scFv) for the targeting of tumor cells. The concept of the therapeutic molecules implies the scFv-mediated delivery to the tumor cell and the TRAIL-induced apoptosis by activating both death receptors DR4 and DR5. In a first approach, dimeric fusion proteins should be generated by utilizing the homodimerization modules IgE heavy chain domain 2 (EHD2) and IgM heavy chain domain 2 (MHD2). A comparison of EHD2 and MHD2 should be performed investigating the preferred module with regard to stability. Thus, the antibody-TRAIL fusion protein was generated by fusing a single-chain derivative of human TRAIL (scTRAIL) and a scFv fragment directed against EGFR to the EHD2 resulting in the scFv-EHD2-scTRAIL fusion protein. In a second approach, cysteine-modified soluble TRAIL molecules (Cys-sTRAIL) and scFv fragments directed against EGFR (scFv’) were coupled to liposomes resulting in Immuno-LipoTRAIL (ILT). These multivalent and multifunctional therapeutic molecules (scFv-EHD2-scTRAIL and ILT) should be initially tested in vitro on their potential of inducing cell death in cancer cells in combination with the proteasome-inhibitor bortezomib, which is known to sensitize tumor cells to TRAIL-induced apoptosis. Furthermore, in vivo studies in mice should be performed investigating the pharmacokinetic properties and the antitumoral activity of the scFv-EHD2-scTRAIL and the ILT.

Additionally, the MHD2 should also be utilized for the generation of dimeric fusion proteins. For this purpose, bispecific antibody-MHD2 as well as bifunctional antibody-MHD2-scTNF fusion proteins were generated by fusing either scFv fragments directed against EGFR and HER2, or a scFv fragment directed against EGFR and a single-chain derivative of the human tumor necrosis factor (scTNF) to the MHD2. The dimeric-assembled fusion proteins should be tested on binding to tumor cells in case of the antibody-MHD2 fusion protein and on the potential of activating TNF receptors in case of the antibody-MHD2-scTNF fusion proteins.
2 Materials and methods

2.1 Materials

2.1.1 Instruments and implements

Balances 440-39N, 440-333N and ALJ 120-4
[Kern, Balingen, Germany]

Blotter TransBlot SD, Semidry transfer cell
[Bio-Rad, Munich, Germany]

Centrifuges Eppendorf 5415C, 5810R [Eppendorf, Hamburg, Germany]
J2-MC with rotors JA10, JA14, JA20, JA30.5
[Beckman Coulter, Krefeld, Germany]
Avanti J-30I [Beckman Coulter, Krefeld, Germany]
Optima™ TL with rotor TLA 100.3
[Beckman Coulter, Krefeld, Germany]

Concentrator Vivaspin MW cutt-off 10 kDa (2ml) and 30 kDa (10 ml)
[GE Healthcare, Freiburg, Germany]

Electrophoresis systems Mini-PROTEAN 3 Electrophoresis Cell System
[BioRad, Munich, Germany]
Ready Agarose Precast Gel System
[BioRad, Munich, Germany]

ELISA plate reader Tecan infinte M200 [Tecan Austria, Grödig, Austria]

Extruder LiposoFast. Basic [Avestin, Ottawa, Canada]

Film developing machine Film Processor Curix 60 [Agfa, Düsseldorf, Germany]

Flow cytometer Cytomics FC 500 [Beckman Coulter, Krefeld, Germany]

Gel documentation Transilluminator, Gel documentation system Felix
[Biostep, Jahnsdorf, Germany]

Heat block HBT-1-131 [HLC BioTech, Bovenden, Germany]
### Materials and methods

<table>
<thead>
<tr>
<th>Equipment Type</th>
<th>Model and Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC systems</td>
<td>Waters 2695 Separation Module, Waters 2489 UV/Visible detector [Waters Cooperation, Milford, USA]</td>
</tr>
<tr>
<td>Incubator for bacteria</td>
<td>BD 53 [Binder, Stuttgart, Germany]</td>
</tr>
<tr>
<td>Incubator for cell culture</td>
<td>CO₂ Inkubator 2424-2 [Zapf, Sarstedt, Germany]</td>
</tr>
<tr>
<td>LI-COR</td>
<td>Odyssey reader [LI-Cor Bioscience, Bad Homburg, Germany]</td>
</tr>
<tr>
<td>Magnetic stirrer</td>
<td>MR 3001K 800W [Heidolph Instruments, Nürnberg, Germany]</td>
</tr>
<tr>
<td>Microscope</td>
<td>CK2 [Olympus, Hamburg, Germany]</td>
</tr>
<tr>
<td>PCR Cycler</td>
<td>RoboCycler 96 [Stratagene, La Jolla, USA]</td>
</tr>
<tr>
<td>Sonicator</td>
<td>Sonoplus HD200; MS73D [Bandelin, Berlin, Germany]</td>
</tr>
<tr>
<td>Spectrophotometer</td>
<td>NanoDrop Spectrophotmeter ND-1000 [PEQLAB, Erlangen, Germany]</td>
</tr>
<tr>
<td>Sterile bench</td>
<td>Variolab Mobilien W90 [Waldner-Laboreinrichtungen, Wangen, Germany]</td>
</tr>
<tr>
<td>Vacuum rotary evaporator</td>
<td>LABORATA 4000, VAC control automatic, ROTAVAC valve control [Heidolph, Schwalbach, Germany]</td>
</tr>
<tr>
<td>Vortexer</td>
<td>Sky Line [Elmi Ltd., Riga, Latvia]</td>
</tr>
<tr>
<td>Waterbath</td>
<td>MA6 [Lauda, Lauda-Königshofen, Germany]</td>
</tr>
<tr>
<td>ZetaSizer</td>
<td>ZetaSizer Nano ZS [Malvern Instruments, Herrenberg, Germany]</td>
</tr>
</tbody>
</table>

#### 2.1.2 Special implements

All plastic implements for bacterial and mammalian use were ordered from Greiner [Bio-One, Frickenhausen, Germany].

<table>
<thead>
<tr>
<th>Equipment Type</th>
<th>Model and Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatography columns</td>
<td>Poly-Prep® columns [Bio-Rad, Munich, Germany]</td>
</tr>
<tr>
<td>Counting chamber</td>
<td>Neubauer 0.0025 mm² [Marienfeld, Lauda-Königshofen, Germany]</td>
</tr>
</tbody>
</table>
Materials and methods

Dialyser
D-Tube™ Dialyzer Mini (MW cut-off 6 - 8 kDa)
[Calbiochem, Merck, Darmstadt, Germany]

Dialysis membranes
23 mm (cut-off 12.4 kDa)
[Sigma-Aldrich, St. Louis, Germany]

ELISA plates
Microlon high binding ELISA plate
[Greiner Bio-One, Frickenhausen, Germany]

HPLC columns
BioSep-SEC-S2000, -S3000 or Yarra SEC-2000
[Phenomenex, Aschaffenburg, Germany]

IMAC affinity beads
Protino® Ni-NTA agarose
[Machery-Nagel, Düren, Germany]

Nitrocellulose membrane
BioTrace™ NT Nitrocellulose Transfer Membrane
[Pall Life Sciences, East Hills, USA]

Photo films
BioMax® MR film [Kodak, Stuttgart, Germany]

Polycarbonat filter membrane
Armatis (pore diameter: 50 nm; diameter: 19 mm) Liposofast
[Avestin, Ottawa, Canada]

Protein A beads
TOYOPEARL® AF rProtein A-650F
[Tosoh Bioscience, Stuttgart, Germany]

Quartz cuvette
12mm square glass cell for 90 sizing (PCS8501)
[Malvern Instruments, Herrenberg, Germany]

Sepharose
Sepharose™ 4CLB
[Amersham-Biosciences, Munich, Germany]

Syringe filters
Acrodisc® 13 mm with 0.2 µM HT Tuffryn® membrane
[Pall Corporation, Dreieich, Germany]

Whatman filter paper
Whatman® chromatography paper 3 mm
[A.Hartenstein Laborbedarf, Würzburg, Germany]

2.1.3 Chemicals and lipids

All chemicals were purchased from Roth [Karlsruhe, Germany], Merck [Darmstadt, Germany], Roche [Basel, Switzerland], and Sigma-Aldrich [St. Louis, USA]. It is stated when chemicals were purchased from other companies.
### Materials and methods

<table>
<thead>
<tr>
<th>Material/Chemical</th>
<th>Source/Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bond-Breaker® TCEP</td>
<td>0.5 M [Thermo Scientific, Rockford, USA]</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>Velcade®, kindly provided by Dr. Thomas Mürdter</td>
</tr>
<tr>
<td></td>
<td>[Institute of Clinical Pharmacology, Dr. Margarete Fischer-Bosch Foundation, Stuttgart, Germany]</td>
</tr>
<tr>
<td>Coomassie Brilliant Blue G250</td>
<td>[SERVA Electrophoresis, Heidelberg, Germany]</td>
</tr>
<tr>
<td>All lipids</td>
<td>were dissolved in chloroform and stored at -20 °C.</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>[Calbiochem, Darmstadt, Germany]</td>
</tr>
<tr>
<td>DiI</td>
<td>1,1'-dioctadecyl-3,3',3',3'-tetramethylindocarbocyanine perchlorate [Sigma-Aldrich, St. Louis, USA]</td>
</tr>
<tr>
<td>DiR</td>
<td>1,1'-dioctadecyl-3,3',3'-tetramethylin dotricarbocyanine iodide [Sigma-Aldrich, St. Louis, USA]</td>
</tr>
<tr>
<td>DSPE-mPEG&lt;sub&gt;2000&lt;/sub&gt;</td>
<td>1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-</td>
</tr>
<tr>
<td></td>
<td>[methoxy(poly-(ethylene glycol))-2000] (ammonium salt) [Avanti Polar Lipids, Alabaster, USA]</td>
</tr>
<tr>
<td>DSPE-PEG&lt;sub&gt;2000&lt;/sub&gt;-Mal</td>
<td>1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-</td>
</tr>
<tr>
<td></td>
<td>[maleimide(poly-(ethylene glycol))-2000] (ammonium salt) [Avanti Polar Lipids, Alabaster, USA]</td>
</tr>
<tr>
<td>EPC</td>
<td>Egg phosphatidylcholine [Lipoid, Ludwigshafen, Germany]</td>
</tr>
</tbody>
</table>

### 2.1.4 Media and supplements

**Bacterial culture**

<table>
<thead>
<tr>
<th>Material</th>
<th>Source/Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100 mg/ml in H&lt;sub&gt;2&lt;/sub&gt;O [Roth, Karlsruhe, Germany]</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside, 1 M in H&lt;sub&gt;2&lt;/sub&gt;O</td>
</tr>
<tr>
<td></td>
<td>[Gebru Biochemicals, Gaiberg, Germany]</td>
</tr>
<tr>
<td>LB&lt;sub&gt;Amp,Glc&lt;/sub&gt; agar plates</td>
<td>LB-medium, 2.0 % (w/v) agar, after autoclaving added ampicillin (f.c.: 100 µg/ml) and 1 % (w/v) glucose</td>
</tr>
<tr>
<td>LB-medium (Amp)</td>
<td>1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 0.5 %</td>
</tr>
</tbody>
</table>
Materials and methods

(w/v) NaCl in H₂O (for selection 100 µg/ml ampicillin)

TY-medium (Amp)  1.6 % (w/v) pepton, 1 % (w/v) yeast extract, 0.5 % (w/v) NaCl in H₂O (for selection 100 µg/ml ampicillin)

Cell culture

Eosin solution  0.4 % (m/v) eosin G, 0.02 % (w/v) NaN₃ in sterile 1x PBS, pH 7.4

Fetal calf serum (FCS)  HyClone® research grade fetal bovine serum

Lipofectamine  Lipofectamine™ 2000 [Invitrogen, San Diego, USA]

Opti-MEM®  GIBCO® [Invitrogen, San Diego, USA]

Penicillin/streptomycin (100x)  10,000 U/ml / 10,000 µg/ml (100x) GIBCO®

RPMI 1640  + 2 mM glutamine GIBCO®

Trypsin/EDTA  0.5 % (w/v) trypsin, 5.3 mM EDTA (10x)

Zeocin™  100 mg/ml in H₂O [Invitrogen, San Diego, USA]

2.1.5 Solutions

Bradford reagent  BioRad protein assay [BioRad, Krefeld, Germany]

Competent cell solution A  0.1 M CaCl₂ in 1x PBS

Competent cell solution B  20 % (v/v) glycerol, 50 mM CaCl₂ in 1x PBS

Coomassie staining solution  0.008 % (w/w) Coomassie Brilliant Blue G-250, 35 mM HCl in H₂O

Crystal violet staining solution  20 % (v/v) methanol, 0.5 % (m/v) crystal violet in dH₂O

DNA loading buffer (5x)  25 % (v/v) glycerol, 0.02 % (w/v) bromphenol blue in 5x TAE buffer
<table>
<thead>
<tr>
<th>Materials and methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA – blocking solution 2 % (m/v) dry milk powder in 1x PBS</td>
</tr>
<tr>
<td>ELISA – developing solution 0.1 mg/ml TMP, 100 mM sodium acetate buffer pH 6.0, 0.006 % (v/v) ( H_2O_2 )</td>
</tr>
<tr>
<td>ELISA – stopping solution 1 M ( H_2SO_4 )</td>
</tr>
<tr>
<td>ELISA – washing solution 0.05 % (v/v) Tween20 in 1x PBS</td>
</tr>
<tr>
<td>Freezing solution 10 % (v/v) DMSO in FCS</td>
</tr>
<tr>
<td>IMAC elution buffer 250 mM imidazole in 1x sodium phosphate buffer</td>
</tr>
<tr>
<td>IMAC wash buffer 35 mM imidazole in 1x sodium phosphate buffer</td>
</tr>
<tr>
<td>Laemmli sample buffer (5x) Non-reducing: 10 % (w/v) SDS, 25 % (v/v) glycerin, 0.05 % (w/v) bromphenol blue in 312.5 mM Tris-HCl pH 6.8; Reducing: non-reducing buffer, 25 % (v/v) ( \beta )-mercaptoethanol</td>
</tr>
<tr>
<td>L-cysteine 1 mM L-cysteine in dH( _2 )O, pH 5.5</td>
</tr>
<tr>
<td>PBA 2 % (v/v) FBS, 0.02 % (w/v) ( NaN_3 ) in 1x PBS</td>
</tr>
<tr>
<td>Periplasmic preparation buffer (PPB) 30 mM Tris-HCl pH 8.0, 1 mM EDTA, 20 % (w/v) sucrose in H( _2 )O</td>
</tr>
<tr>
<td>Phosphat-buffered saline (PBS, 10x) 80.6 mM ( Na_2HPO_4 \cdot 2H_2O ), 14.7 mM ( KH_2PO_4 ), 1.37 M NaCl, 26.7 mM KCl (10x); used as 1x PBS diluted in dH( _2 )O</td>
</tr>
<tr>
<td>Protein A – elution buffer 100 mM glycine, pH 3.0</td>
</tr>
<tr>
<td>Protein A – neutralization buffer 1 M Tris-HCl, pH 8.0</td>
</tr>
<tr>
<td>Protein A – washing buffer 100 mM Tris-HCl, pH 7.0</td>
</tr>
<tr>
<td>scFv-coupling buffer (10x) 10 mM ( Na_2HPO4/NaH_2PO4 ) buffer, 0.2 mM EDTA, 30 mM NaCl, pH 6.7</td>
</tr>
<tr>
<td>SDS running buffer (10x) 1.92 M glycine, 0.25 M Tris, 1 % (w/v) SDS in dH( _2 )O, pH 8.3</td>
</tr>
<tr>
<td>Sodium phosphate buffer (5x) 210 mM (Na_2HPO_4 \cdot 2H_2O ), 40 mM ( NaH_2PO_4 \cdot H_2O ), 1.25 M</td>
</tr>
</tbody>
</table>
Materials and methods

NaCl in dH₂O, pH 7.5

TAE buffer (50x) 2 M Tris, 1 M glacial acetic acid, 50 mM EDTA in dH₂O, pH 8.0

TRAIL-coupling buffer (10x) 10 mM Na₂HPO₄/NaH₂PO₄ buffer, 30 mM NaCl, pH 6.7

Western Blot – blocking solution 5 % (w/v) milk powder, 0.1 % (v/v) Twenn 20 in 1x PBS

Western Blot - blotting buffer 20 % (v/v) methanol, 192 mM glycine, 25 mM Tris, pH 8.3

Western Blot – solution A 0.1 M Tris, 0.25 mg/ml luminol, pH 8.6

Western Blot – solution B 0.11 % (w/v) p-coumaric acid in DMSO

Western Blot – washing solution 0.05 % (v/v) Tween 20 in 1x PBS

2.1.6 Markers

GeneRuler™ DNA ladder mix [Fermentas, St. Leon-Rot, Germany]

PageRuler™ prestained protein ladder [Fermentas, St. Leon-Rot, Germany]

2.1.7 Antibodies

Table 2-1: Antibodies used for ELISA, flow cytometry (FACS) analysis and Western Blot (WB)

<table>
<thead>
<tr>
<th>Name</th>
<th>Origin</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-EGFR-PE</td>
<td>Mouse monoclonal IgG2b [Santa Cruz Biotech., Santa Cruz, USA]</td>
<td>1:100 (FACS)</td>
</tr>
<tr>
<td>Anti-FLAG-HRP</td>
<td>Mouse monoclonal IgG1 [Sigma-Aldrich, St. Louis, USA]</td>
<td>1:15,000 (ELISA), 1:2,000 (WB)</td>
</tr>
<tr>
<td>Anti-HER2-PE</td>
<td>24D2, mouse monoclonal IgG1 [Santa Cruz Biotech., Santa Cruz, USA]</td>
<td>1:100 (FACS)</td>
</tr>
<tr>
<td>Anti-His₆-FITC</td>
<td>Mouse monoclonal IgG1 [dianova, Hamburg, Germany]</td>
<td>1:100 (FACS)</td>
</tr>
<tr>
<td>Anti-His₆-HRP</td>
<td>Mouse monoclonal IgG1, 200 µg/ml [Santa Cruz Biotechnology, Santa Cruz, USA]</td>
<td>1:1000 (ELISA, WB)</td>
</tr>
</tbody>
</table>
### Materials and methods

<table>
<thead>
<tr>
<th>Name</th>
<th>Origin</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-His&lt;sub&gt;6&lt;/sub&gt;-PE</td>
<td>Clone GG11-8F3.5.1 MACS molecular [Miltenyi Biotec, Bergisch Gladbach, Germany]</td>
<td>1:100 (FACS)</td>
</tr>
<tr>
<td>Anti-human IgG (Fc specific)-HRP</td>
<td>Polyclonal [Sigma-Aldrich, St. Louis, USA]</td>
<td>1:5000 (ELISA, WB)</td>
</tr>
<tr>
<td>Anti-IgG2b-PE</td>
<td>K-isotype control, clone MPC-11 [Biolegend, San Diego, USA]</td>
<td>1:100 (FACS)</td>
</tr>
<tr>
<td>Anti-mouse IgG (Fc specific)-HRP</td>
<td>Polyclonal [Sigma-Aldrich, St. Louis, USA]</td>
<td>1:5000 (ELISA, WB)</td>
</tr>
<tr>
<td>Anti-mouse IgG1-FITC</td>
<td>Clone 203 [ImmunoTools, Friesoythe, Germany]</td>
<td>1:100 (FACS)</td>
</tr>
<tr>
<td>Anti-mouse IgG1-PE</td>
<td>Mouse monoclonal IgG1 [ImmunoTools, Friesoythe, Germany]</td>
<td>1:100 (FACS)</td>
</tr>
<tr>
<td>Anti-TNFR1</td>
<td>H398, Mouse monoclonal IgG2b [kindly provided by Dr. Olaf Maier]</td>
<td>2.5 µg/ 500 µl (ELISA)</td>
</tr>
<tr>
<td>Anti-TNFR2</td>
<td>Mr1-2, Mouse monoclonal IgG1 [kindly provided by Dr. Olaf Maier]</td>
<td>1 µg / 500 µl (ELISA)</td>
</tr>
<tr>
<td>Anti-TRAIL</td>
<td>Mouse monoclonal IgG1 [R&amp;D Systems, Wiesbaden, Germany]</td>
<td>1:50 (FACS)</td>
</tr>
<tr>
<td>Cetuximab</td>
<td>Erbitux®, kindly provided by Dr. Thomas Mürdter,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[Institute of Clinical Pharmacology, Dr. Margarete Fischer-Bosch Foundation, Stuttgart, Germany]</td>
<td></td>
</tr>
<tr>
<td>IgG1 isotyp control</td>
<td>Monoclonal from murine myeloma [Sigma-Aldrich, St. Louis, USA]</td>
<td>1:50 (FACS)</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>Herceptin®, kindly provided by Prof. Hans-Heinrich Heidtmann</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[St. Joseph Hospital, Bremerhaven, Germany]</td>
<td></td>
</tr>
</tbody>
</table>

### 2.1.8 Enzymes

- **Lysozyme**: Muramidase from hen egg white [Roche Diagnostics, Mannheim, Germany]
## Materials and methods

<table>
<thead>
<tr>
<th>Enzyme/Reagent</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI</td>
<td>10 U/µl</td>
<td>[Fermentas, St. Leon-Rot, USA]</td>
</tr>
<tr>
<td>BshTI (Agel)</td>
<td>10 U/µl</td>
<td>[Fermentas, St. Leon-Rot, USA]</td>
</tr>
<tr>
<td>DreamTaq™ Green PCR Master Mix</td>
<td>2x</td>
<td>[Fermentas, St. Leon-Rot, USA]</td>
</tr>
<tr>
<td>EcoRI</td>
<td>10 U/µl</td>
<td>[Fermentas, St. Leon-Rot, USA]</td>
</tr>
<tr>
<td>Fast alkaline phosphatase</td>
<td>1 U/µl</td>
<td>[Fermentas, St. Leon-Rot, USA]</td>
</tr>
<tr>
<td>HindIII</td>
<td>10 U/µl</td>
<td>[Fermentas, St. Leon-Rot, USA]</td>
</tr>
<tr>
<td>KpnI</td>
<td>10 U/µl</td>
<td>[Fermentas, St. Leon-Rot, USA]</td>
</tr>
<tr>
<td>N-Glycosidase F</td>
<td>PNGaseF</td>
<td>[Roche Diagnostics, Mannheim, Germany]</td>
</tr>
<tr>
<td>NheI</td>
<td>10 U/µl</td>
<td>[Fermentas, St. Leon-Rot, USA]</td>
</tr>
<tr>
<td>NotI</td>
<td>10 U/µl</td>
<td>[Fermentas, St. Leon-Rot, USA]</td>
</tr>
<tr>
<td>Pfu-DNA-polymerase (native)</td>
<td>2.5 U/µl</td>
<td>[Fermentas, St. Leon-Rot, USA]</td>
</tr>
<tr>
<td>SfiI</td>
<td>10 U/µl</td>
<td>[Fermentas, St. Leon-Rot, USA]</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>5 U/µl</td>
<td>[Fermentas, St. Leon-Rot, USA]</td>
</tr>
</tbody>
</table>

### 2.1.9 Kits

- NucleoBond® Xtra Midi [Machery-Nagel, Düren, Germany]
- NucleoSpin® Gel and PCR Clean-up [Machery-Nagel, Düren, Germany]
- NucleoSpin® Plasmid [Machery-Nagel, Düren, Germany]
- Interleukin-8 Sandwich ELISA kit [Immunotools GmbH, Friesoythe, Germany]
- BD OptEIA™ human TRAIL ELISA Set [BD Biosciences, Heidelberg, Germany]
- MaxDiscovery™ ALT Enzymatic Assay Kit [BIOO Scientific, Austin, USA]

### 2.1.10 Bacterial Strains

*Escherichia coli* were cultivated at 37 °C shaking with 170 rpm. TG1 strain was used for cloning and periplasmic production of scFv fragments, whereas the BL21-DE3 strain, which
is transfected with the \(\lambda\)-phage, was used for cytosolic production of the Cys-sTRAIL molecule.

\textit{E. coli} BL21-DE3 \quad F^{-} \text{ompT gal dcm lon hsdS}_{\text{B}}(\text{r}_{\text{B}} \text{- } \text{m}_{\text{B}}) \lambda(\text{DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]})

\textit{E. coli} TG1 \quad \text{supE thi-1} \Delta(lac-proAB) \Delta(mcrB-hsdSM)5 (rK- mK-)[F’ traD36 proAB lacIqZ\DeltaM15] [Stratagene, La Jolla, USA]

\subsection*{2.1.11 Eucaryotic cell lines}

Eucaryotic cells were cultivated at 37 °C in a humidified incubator with 5 % CO\(_2\) atmosphere. All cell lines grew adherently.

\textbf{Table 2-2: Cell lines used in the experiments}

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Culture medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>Human epidermoid carcinoma</td>
<td>RPMI 1640 + 5 % (v/v) FCS</td>
</tr>
<tr>
<td>Colo205</td>
<td>Human colon adenocarcinoma</td>
<td>RPMI 1640 + 5 % (v/v) FCS</td>
</tr>
<tr>
<td>HCT116</td>
<td>Human colon carcinoma</td>
<td>RPMI 1640 + 10 % (v/v) FCS</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney</td>
<td>RPMI 1640 + 5 % (v/v) FCS</td>
</tr>
<tr>
<td>HepG2</td>
<td>Human hepato carcinoma</td>
<td>RPMI 1640 + 10 % (v/v) FCS</td>
</tr>
<tr>
<td>HT1080</td>
<td>Human fibrosarcoma</td>
<td>RPMI 1640 + 5 % (v/v) FCS</td>
</tr>
<tr>
<td>Huh-7</td>
<td>Human hepatic carcinoma</td>
<td>RPMI 1640 + 10 % (v/v) FCS</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
<td>RPMI 1640 + 5 % (v/v) FCS</td>
</tr>
<tr>
<td>NCI-H460</td>
<td>Human lung carcinoma</td>
<td>RPMI 1640 + 5 % (v/v) FCS</td>
</tr>
<tr>
<td>SKBR3</td>
<td>Human breast adenocarcinoma</td>
<td>RPMI 1640 + 10 % (v/v) FCS</td>
</tr>
</tbody>
</table>

\subsection*{2.1.12 Animal models}

CD1 mouse \quad \text{RjOrl:SWISS [Janvier, Saint Berthevin Cedex, France]}

NMRI nu/nu \quad \text{Rj:NMRI-Foxn1\textsuperscript{nu}/Foxn1\textsuperscript{nu}} [Janvier, Saint Berthevin Cedex, France]
2.1.13 Plasmids

Table 2-3: Plasmids used in this study

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Cloned by</th>
</tr>
</thead>
<tbody>
<tr>
<td>694</td>
<td>pSecTagA-MHD2</td>
<td>Nadine Heidel</td>
</tr>
<tr>
<td>707</td>
<td>pSecTagA-scFvhu225-MHD2</td>
<td>Nadine Heidel</td>
</tr>
<tr>
<td>708</td>
<td>pSecTagA-MHD-scFv4D5</td>
<td>Nadine Heidel</td>
</tr>
<tr>
<td>709</td>
<td>pSecTagA-scFvhu225-MHD2-scFv4D5</td>
<td>Nadine Heidel</td>
</tr>
<tr>
<td>959</td>
<td>pSecTagA-MHD2-scTNF</td>
<td>Oliver Seifert</td>
</tr>
<tr>
<td>746</td>
<td>pSecTagA-scFvhu225-MHD2-scTNF</td>
<td>Sylvia Messerschmidt</td>
</tr>
<tr>
<td>878</td>
<td>pSecTagA-MHD2-C337S</td>
<td>Oliver Seifert</td>
</tr>
<tr>
<td>879</td>
<td>pSecTagA-MHD2-N332Q</td>
<td>Oliver Seifert</td>
</tr>
<tr>
<td>880</td>
<td>pSecTagA-MHD2-N332Q-C337S</td>
<td>Oliver Seifert</td>
</tr>
<tr>
<td>966</td>
<td>pSecTagA-GHD3</td>
<td>Oliver Seifert</td>
</tr>
<tr>
<td>509</td>
<td>pAB1-scFvhu225</td>
<td>Anja Nusser</td>
</tr>
<tr>
<td>510</td>
<td>pABC4-scFvhu225</td>
<td>Anja Nusser</td>
</tr>
<tr>
<td>532</td>
<td>pAB1-scFv4D5</td>
<td>Anja Nusser</td>
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<tr>
<td>1064</td>
<td>pSecTagA-EHD2</td>
<td>Oliver Seifert / Aline Plappert</td>
</tr>
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<td>1083</td>
<td>pSecTagA-scFvhu225-EHD2</td>
<td>Oliver Seifert / Aline Plappert</td>
</tr>
<tr>
<td>1084</td>
<td>pSecTagA-EHD2-scTRAIL</td>
<td>Oliver Seifert / Aline Plappert</td>
</tr>
<tr>
<td>1086</td>
<td>pSecTagA-scFvhu225-EHD2-scTRAIL</td>
<td>Oliver Seifert / Aline Plappert</td>
</tr>
<tr>
<td>1191</td>
<td>pSecTagA-TRAILR1-Fc</td>
<td>Oliver Seifert</td>
</tr>
<tr>
<td>1192</td>
<td>pSecTagA-TRAILR2-Fc</td>
<td>Oliver Seifert</td>
</tr>
<tr>
<td>1193</td>
<td>pSecTagA-TRAILR3-Fc</td>
<td>Oliver Seifert</td>
</tr>
<tr>
<td>1194</td>
<td>pSecTagA-TRAILR4-Fc</td>
<td>Oliver Seifert</td>
</tr>
<tr>
<td>516</td>
<td>pET15b-Cys-sTRAIL</td>
<td>Nadine Pollak</td>
</tr>
</tbody>
</table>
2.1.14 Primers

All primers were synthesized by Sigma-Aldrich [St. Louis, USA], dissolved in dH₂O (f.c.: 50 µM) and stored at -20 °C.

**Table 2-4: Primer for PCR screening and sequencing**

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Sequence 5'-3'</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>89</td>
<td>pET-Sq1</td>
<td>TAA TAC GAC TCA CTA TAG GG</td>
<td>pSecTagA</td>
</tr>
<tr>
<td>91</td>
<td>pSec-Sq2</td>
<td>TAG AAG GCA CAG TCG AGG</td>
<td>pSecTagA</td>
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</table>

**Table 2-5: Primer for cloning of MHD2 mutants and fusion proteins**

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Sequence 5'-3'</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>304</td>
<td>NotI-scFv4D5-for</td>
<td>ATA GTT TAG CGG CCG CAC GCT TGA TCT CGA CCT TCG T</td>
<td>4D5</td>
</tr>
<tr>
<td>426</td>
<td>BamHI-scFv4D5-back</td>
<td>CGC GGA TCC GGC GGA GAA GTG CAG CTC GTC GAA AGT GGC</td>
<td>4D5</td>
</tr>
<tr>
<td>439</td>
<td>NotI-scTNF-for</td>
<td>TAA AGC GGC CGC TAG ATA TCA TCA CAG AGC</td>
<td>scTNF</td>
</tr>
<tr>
<td>451</td>
<td>EcoRI-scTNF-back</td>
<td>CCG GAA TTC ATG AGA GGA TCG CAT CAC CAT</td>
<td>scTNF</td>
</tr>
<tr>
<td>851</td>
<td>KpnI-MHD2-C337S-for</td>
<td>TCC GGT ACC TCC GCC GGA CCC TCC GCC GTC GGG CAC GGA CAT AGA GCT GGC GTT CTG CTG GAA GGT CAG GC</td>
<td>MHD2</td>
</tr>
<tr>
<td>852</td>
<td>KpnI-MHD2-N332Q-for</td>
<td>TCC GGT ACC TCC GCC GGA CCC TCC GCC GTC GGG CAC GCA CAT AGA GCT GGC CTG CTG CTG GAA GGT CAG GC</td>
<td>MHD2</td>
</tr>
<tr>
<td>853</td>
<td>KpnI-MHD2-N332Q-C337S-for</td>
<td>TCC GGT ACC TCC GCC GGA CCC TCC GCC GTC GGG CAC GGA CAT AGA GCT GGC CTG CTG CTG GAA GGT CAG GC</td>
<td>MHD2</td>
</tr>
</tbody>
</table>
Materials and methods

Table 2-6: Primer for cloning of GHD3

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Sequence 5'-3'</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>773</td>
<td>HindIII-CH3-back</td>
<td>CCC AAG CTT GGG GCA GCC CCG AGA ACC AC</td>
<td>GHD3</td>
</tr>
<tr>
<td>774</td>
<td>KpnI-CH3-for</td>
<td>GGG GTA CCT TTA CCC GGA GAC AGG G</td>
<td>GHD3</td>
</tr>
</tbody>
</table>

Table 2-7: Primer for cloning of EHD2 fusion proteins

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Sequence 5'-3'</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>801</td>
<td>NotI-EHD2-back</td>
<td>GAA TGC GCC CGC aGG AGG CTC TGG CGG TGA TTT CAC CCC CCC CAC</td>
<td>EHD2</td>
</tr>
<tr>
<td>802</td>
<td>EcoRI-EHD2-for</td>
<td>CGG AAT TCA CCG CCG CTT CCC CCG TTG CTG TCG</td>
<td>EHD2</td>
</tr>
<tr>
<td>803</td>
<td>SfiI-EHD2-back</td>
<td>AAA GGC CCA GCC GGC CGA TTT CAC CCC CCC CA</td>
<td>EHD2</td>
</tr>
</tbody>
</table>

Table 2-8: Primer for cloning of TRAIL-receptor-Fc fusion proteins

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Sequence 5'-3'</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>897</td>
<td>SfiI-TRAILR1-back</td>
<td>AAA GGC CCA GCC GGC CGC GAG TGG GAC AGA GGC</td>
<td>TRAIL-R1</td>
</tr>
<tr>
<td>898</td>
<td>NotI-TRAILR1-for</td>
<td>TTT GCG GCC GCA TTA TGT CCA TTG CCT GAT TC</td>
<td>TRAIL-R1</td>
</tr>
<tr>
<td>899</td>
<td>AgeI-TRAILR2-back</td>
<td>AAA ACC GGT GAG TCT GCT CTG ATC ACC</td>
<td>TRAIL-R2</td>
</tr>
<tr>
<td>900</td>
<td>NotI-TRAILR2-for</td>
<td>TTT GCG GCC GCT GAG AGA CAG GGA GAG</td>
<td>TRAIL-R2</td>
</tr>
<tr>
<td>901</td>
<td>AgeI-TRAILR4-back</td>
<td>AAA ACC GGT GCC ACC ATC CCC CGG C</td>
<td>TRAIL-R4</td>
</tr>
<tr>
<td>902</td>
<td>NotI-TRAILR4-for</td>
<td>TTT GCG GCC GCG TAG TGA TAG GGA GAG GC</td>
<td>TRAIL-R4</td>
</tr>
</tbody>
</table>

2.1.15 Vectors

pAB1 Vector for prokaryotic protein expression into the periplasm of *E. coli* TG1 (Kontermann et al., 1997)
Materials and methods

pABC4 Vector for prokaryotic expression of cysteine modified proteins into the periplasm of *E. coli* TG1 (Baum et al., 2007)

pSecTagA Vector for eukaryotic protein secretion [Invitrogen, Karlsruhe, Germany]

pSecTagA-Fc Originated from pSecTagA; containing hinge region, C\textsubscript{H}2 and C\textsubscript{H}3 of human Fc\gamma1 chain

2.1.16 Proteins

A humanized anti-human epidermal growth factor receptor (EGFR) 1 scFv (hu225) was generated from the antibody C225 (Goldstein et al., 1995) by complementarity-determining region (CDR) grafting. The anti-human EGFR 2 (HER2) scFv 4D5 was reproduced from published sequences (Carter et al., 1992).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Db\textsubscript{EGFR}-scTRAIL</td>
<td>Kindly provided by Dr. M. Siegemund (Siegemund et al., 2012)</td>
</tr>
<tr>
<td>EGFR-Fc</td>
<td>Kindly provided by Sina Fellermeier; (aa 25-645)</td>
</tr>
<tr>
<td>HER2-Fc</td>
<td>Kindly provided by Sina Fellermeier, (aa 23-652)</td>
</tr>
<tr>
<td>HER3-Fc</td>
<td>Kindly provided by Sina Fellermeier (aa 20-643)</td>
</tr>
<tr>
<td>scF\textsubscript{EGFR}-scTRAIL</td>
<td>Kindly provided by Dr. M. Siegemund (Siegemund et al., 2012)</td>
</tr>
<tr>
<td>scTNF</td>
<td>Kindly provided by Prof. Dr. Peter Scheurich (Krippner-Heidenreich et al., 2008); L1-linker</td>
</tr>
<tr>
<td>scTRAIL</td>
<td>Kindly provided by Dr. M. Siegemund (Siegemund et al., 2012)</td>
</tr>
<tr>
<td>TNFR1-Fc</td>
<td>Kindly provided by Fabian Richter; (aa 30-211)</td>
</tr>
<tr>
<td>TNFR2-Fc</td>
<td>Kindly provided by Fabian Richter; (aa 23-257)</td>
</tr>
</tbody>
</table>

2.1.17 Programs and online tools

<table>
<thead>
<tr>
<th>Tool</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone Manager Professional 7</td>
<td>[Scientific &amp; Educational software, Carey, USA]</td>
</tr>
<tr>
<td>ExPASy Prot Param Tool</td>
<td><a href="http://web.expasy.org/protparam/">http://web.expasy.org/protparam/</a></td>
</tr>
<tr>
<td>GraphPad Prism 5.01</td>
<td>[GraphPad software, La Jolla, USA]</td>
</tr>
<tr>
<td>ImageJ</td>
<td><a href="http://rsb.info.nih.gov/ij/">http://rsb.info.nih.gov/ij/</a></td>
</tr>
</tbody>
</table>
2.2 Cloning

2.2.1 Cloning strategy of MHD2 derivatives

The DNA sequence of the MHD2 was synthetically produced (Geneart, Regensburg, Germany) and cloned into the vector pSecTagA (no. 118) by digestion with SfiI/NotI. The three different mutants were generated by mutating the cysteine residue at position 337 into a serine and/or the asparagine residue at position 332 into a glutamine. Therefore, mutated MHD2-DNA was amplified by PCR using pSecTagA-MHD2 (no. 694) as template and primers no. 89 in combination with primer no. 851 (MHD2-C337S), 852 (MHD2-N332Q), or 853 (MHD2-N332Q-C337S). PCR products were digested with KpnI/Nhel and cloned into the vector pSecTagA-scFv-MHD2 (no. 707) digested with KpnI/Nhel. Used primers are listed in Table 2-5 and sequences of the constructs are shown in 6.1.

2.2.2 Cloning strategy of MHD2 fusion proteins

The single-chain fragment variable (scFv) was cut from pAB1-scFvhu225 (no. 509) digested with SfiI/HindIII and ligated into pSecTagA-MHD2 (no. 694) resulting in pSecTagA-scFv_{EGFR^{•}}MHD2 (no. 708). The scFv_{HER2} was amplified via PCR using the plasmid pAB1-scFv4D5 (no. 532) and the primer 304/426. PCR product was digested with BamHI/NotI and ligated into pSecTagA-MHD2 (no. 694) also digested with BamHI/NotI resulting in pSecTagA-MHD2-scFv_{4D5} (no. 709). For the cloning of pSecTagA-scFv_{EGFR^{•}}MHD2-scFv_{HER2} (no. 710), scFv_{EGFR} (no.708) was digested from pSecTagA-scFv_{EGFR^{•}}MHD2 with SfiI/HindIII and inserted into pSecTagA-MHD2-scFv_{HER2} (no. 709) also digested with SfiI/HindIII. Single-chain TNF (scTNF, aa 80-233) was amplified via PCR using the primer 439/451 digested with EcoRI/NotI and ligated into pSecTagA-scFv_{EGFR^{•}}MHD2 (no. 708) resulting in pSecTagA-scFv_{EGFR^{•}}MHD2-scTNF (no. 746). ScTNF was cut from pSecTagA-scFv_{EGFR^{•}}MHD2-scTNF (no. 746) with EcoRI and NotI and ligated into EcoRI/NotI digested vector pSecTagA-MHD2 (no. 694) resulting in pSecTagA-MHD2-scTNF (no. 959). Used primers are listed in Table 2-5 and sequences of the constructs are shown in 6.1.
2.2.3 Cloning strategy of GHD3

The CH3 domain of IgG1 (GHD3) was amplified by PCR using pSecTagA-CH3 (no. 312) as template and primers 773/774. Together with pSecTagA-MHD2 (no. 694), PCR product was digested with HindIII and KpnI and ligated to pSecTagA-GHD3 (no. 966). Used primers are listed in Table 2-6 and the sequence of the construct is shown in 6.2.

2.2.4 Cloning strategy of TRAIL-receptor-Fc fusion proteins

The DNA encoding for the extracellular domain of the TRAIL-receptors (TRAIL-R) 1 (aa 24-239), 2 (aa 52-212), and 4 (aa 56-212) was amplified via PCR from the plasmids (TRAIL-R1: no. 140; TRAIL-R2: no. 252; TRAIL-R4: no. 451; kindly provided from Peter Scheurich’s lab) with the respective primers (TRAIL-R1: 897/898; TRAIL-R2: 899/900; TRAIL-R4: 901/903). DNA encoding for the extracellular domain of TRAIL-R3 (aa 25-240) was purchased from Geneart (Regensburg, Germany). DNA was digested with SfiI/NotI (TRAIL-R1) or AgeI/NotI (TRAIL-R2-4) and cloned into the vector pSecTag-Fc (no.1126) digested with the respective enzymes. Used primers are listed in Table 2-8 and sequences of the constructs are shown in 6.4.

2.2.5 Cloning strategy of EHD2 fusion proteins

Proteins were cloned corporately with Aline Plappert (diploma student). DNA encoding EHD2 was ordered from Geneart (Regensburg, Germany) and was digested with HindIII and KpnI into pSecTagA-vector (no. 118) resulting in pSecTagA-EHD2 (no. 1064). Subsequently, pSecTagA-scFvhu225-MHD2 (no. 707) was digested with SfiI/HindIII and cut DNA fragments of scFvhu225 were cloned into pSecTagA-EHD2 resulting in pSecTagA-scFvhu225-EHD2 (no. 1083). For the generation of EHD2-TRAIL (aa 95-281) and scFv-EHD2-scTRAIL (aa 95-281) fusion proteins, EHD2 was amplified by PCR using template pSecTagA-EHD2 (no. 1064) and primers 801/802 or 803/802 and cloned into pSecTagA-scFvhu225-MHD2-scTRAIL (no. 1040) digested either with NotI/EcoRI resulting in pSecTagA-scFvhu225-EHD2-scTRAIL (no. 1086) or with SfiI/EcoRI resulting in pSecTagA-EHD2-scTRAIL (no. 1084). Used primers are listed in Table 2-7 and sequences of the constructs are shown in 6.3.

2.2.6 Chemical competent E. coli cells

A fresh overnight culture of E. coli TG1 or BL21-DE3 was used to inoculate 1 l of LB medium (dilution 1:100). Cells were grown at 37 °C (shaking at 170 rpm) to an OD_{600} 0.5 to 0.6 and chilled on ice for 15 minutes. After centrifugation step (4000x g, 5 minutes, 4 °C), supernatant was discarded and cell pellet was resuspended in ice-cold 50 ml competent cell solution A (0.1 M CaCl₂ in 1x PBS) and incubated for 30 minutes on ice. Cells were
centrifuged again (4000x g, 5 minutes, 4 °C) and cell pellet was resuspended in ice-cold 10 ml competent cell solution B (20 % (v/v) glycerol/50mM CaCl$_2$ in 1x PBS). Finally, the competent cells were quick-frozen in liquid nitrogen and stored in 300 µl aliquots at -80 °C.

2.2.7 Polymerase chain reaction

Polymerase chain reaction (PCR) with pfu-DNA-polymerase was used to amplify desired DNA fragments from vector templates for further cloning steps. A typical PCR mixture contained 50 µl:

\[
\begin{align*}
10x \text{pfu-polymerase buffer + MgSO}_4 & \quad 5 \mu l \\
\text{Forward primer (10 pmol/µl)} & \quad 1 \mu l \\
\text{Reverse primer (10 pmol/µl)} & \quad 1 \mu l \\
d\text{NTPs (5 mM each nucleotide)} & \quad 2 \mu l \\
\text{pfu-DNA-polymerase (2.5 U/µl)} & \quad 1 \mu l \\
dH_2O & \quad 40 \mu l
\end{align*}
\]

The amplification of DNA was performed using the PCR program as describe in Table 2-9.

<table>
<thead>
<tr>
<th>PCR step</th>
<th>Temperature [°C]</th>
<th>Time [minutes]</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94</td>
<td>5</td>
<td>1x</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>dependent</td>
<td>0.5</td>
<td>33x</td>
</tr>
<tr>
<td>Elongation</td>
<td>72</td>
<td>dependent</td>
<td></td>
</tr>
<tr>
<td>Final elongation</td>
<td>72</td>
<td>5</td>
<td>1x</td>
</tr>
</tbody>
</table>

Elongation time depended on the length of the PCR product, due to the limited synthesis rate of pfu-DNA-polymerase with 500 nucleotides per minute. The annealing temperature (Tm) of the primer was calculated using the following formula:

\[
T_{m} = (\text{number}_{\text{adenine or thymine}} \cdot 2 ^{\circ C}) + (\text{number}_{\text{cytosine or guanine}} \cdot 4 ^{\circ C})
\]

The PCR product was loaded on an agarose gel and separated from primers and template DNA.
2.2.8 Restriction digestion

Restriction digestion was performed in a total volume of 40 µl, containing either 3 µg of vector DNA or complete extraction of DNA from agarose gel. Restriction enzymes (each 1 µl) were added to the mixture and incubated with the corresponding buffer (10x; 4 µl) according to the manufacturer’s protocol for 2 to 3 hours. Exchange of buffer was performed using NucleoSpin® Gel and PCR Clean-up kit. After the digestion step, vector DNA was dephosphorylated adding Fast alkaline phosphatase (1 µl) and incubated for 1 hour at 37 °C. Digested DNA fragments were separated from vector DNA using agarose gel.

2.2.9 Agarose Gel and DNA Gel Extraction

Digested or amplified DNA fragments were analyzed and purified from vector DNA using horizontal agarose gel electrophoresis. Depending on the size of the analyzed DNA fragment, 0.7 to 2.0 % (w/v) agarose was dissolved in 1x TAE buffer by heating. DNA fragments were mixed with 5x DNA loading buffer and loaded on an agarose gel containing 1 µg/ml ethidium bromide. The agarose gel was run in 1x TAE buffer at 100 V for 30 minutes and analyzed with UV light. Relevant DNA bands were excised from gel and extracted with a NucleoSpin® Gel and PCR Clean-up kit according to the manufacturer’s protocol. DNA was eluted in 30 µl dH₂O.

2.2.10 Ligation

Insert and dephosphorylated vectors were mixed at a molar ratio of 3:1 and incubated with T4 DNA ligase according to the manufacturer’s protocol in a total volume of 40 µl. 20 µl were used for the transformation of chemical competent E. coli cells.

2.2.11 Transformation of E. coli cells

The competent E. coli cells were thawed on ice. For re-transformation of plasmids, 20 µl of competent cells were mixed with 1 µl of plasmid DNA. For the transformation of ligated plasmids, 100 µl of competent cells were mixed with 20 µl of the ligation mixture and incubated on ice for 10 minutes. Accordingly, cells were incubated for 1 minute at 42 °C (heat shock) and placed back on ice to add 1 ml of LB medium. Cells were incubated for 20 minutes at 37 °C developing antibiotic resistance. Finally, cells were harvested (3000x g, 3 minutes, room temperature) and streaked in reflux on LB agar plates containing the selective antibiotic and incubated overnight at 37 °C.

2.2.12 Screening of clones

For the screening of clones, DreamTaq™ Green PCR Master Mix was used according to the manufacturer’s protocol containing the primers no. 89 and 91 (see Table 2-4). Single
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colonies were picked from the LB<sub>Amp,Glu</sub> agar plate and were dispensed in 20 µl of PCR mixture. Subsequently, 100 µl of LB medium containing the respective antibiotic were inoculated with the same tip and incubated at 37 °C. PCR products were analyzed on an agarose gel identifying correct clones with predicted DNA fragment size. One to two clones were cultivated overnight at 37 °C for plasmid extraction.

2.2.13 Plasmid-DNA isolation (Mini, Midi)

One clone of the PCR screening or from a LB<sub>Amp,Glu</sub> plate was used for an overnight culture in 5 ml (for Mini-preparation) or 150 ml (for Midi-preparation) LB medium containing respective antibiotic. Plasmid DNA was purified either with the NucleoSpin® Plasmid kit for the Mini-preparation or with the NucleoBond® Xtra Midi kit for the Midi-preparation according to the manufacturer’s protocol. In case of the Midi-preparation the DNA pellet was dissolved in 150 µl dH<sub>2</sub>O. DNA concentration was determined using NanoDrop.

2.2.14 Sequence analysis

DNA sequencing was performed by GATC Biotech AG (Konstanz, Germany) using the light run labels. DNA and primers were mixed according to the protocol of GATC Biotech AG using the primers no. 89 or 91 (see Table 2-4). The DNA sequence alignment was performed using the online program ‘nucleotide blast’ of NCBI (see 2.1.17). Virtual cloning was performed using the program Clone Manager 7.

2.3 Cell culture

All eukaryotic cell lines were cultivated at 37 °C with humidity and 5 % CO<sub>2</sub> in the appropriate medium (see Table 2-2) and were split every two to three days, when medium changed color into yellow. Then cells were detached by incubation with 1x Trypsin/EDTA and splitted approximately 1:10. Determining cell number, detached cells were resuspended in the respective medium, 12 µl of the cell suspension and 12 µl of eosin solution (staining of dead cells) were mixed and cells were counted using a Neubauer counting chamber.

For long-term storage, detached cells were resuspended in the respective medium and centrifuged (500x g, 5 minutes, 4 °C). Harvested cells were resuspended in freezing solution (10 % (v/v) DMSO in FCS) and aliquoted in cryo vials. Cells were slowly frozen to -80 °C using a cryobox filled with isopropanol. For defrosting cells, cryo vials were incubated at 37 °C and cells were resuspended in appropriate medium removing DMSO. After centrifugation (500x g, 5 minutes, 4 °C), cells were transferred to a cell culture flask containing the respective culture medium.
2.3.1 Transfection

For the eukaryotic expression of proteins the encoding DNA plasmid was transfected into HEK293 cells. The day before transfection, $1 \cdot 10^6$ cells per well were seeded in a 6-well plate and incubated in 2 ml culture medium overnight at 37 °C. Then, culture medium was discarded and exchanged by 1.5 ml serum-free medium (Opti-MEM). 170 µl Opti-MEM and 7 µl lipofectamin were gently mixed and incubated for 5 minutes at room temperature. Meanwhile, 170 µl Opti-MEM were mixed with 3 µg plasmid DNA. Both solutions were mixed and incubated for 20 minutes at room temperature. Subsequently, the lipofectamin-DNA solution was dropped into the medium of the seeded well and cells were incubated overnight at 37 °C. The next day, Opti-MEM was discarded, cells were transferred into a culture flask and incubated in culture medium for 4 hours at 37 °C. Finally, zeocin was added to the culture medium (f.c.: 300 µg/ml) to select stably transfected cells. Selection medium containing zeocin was refreshed twice a week until cells were 80 % confluent. Three stocks of cells were long-term conserved at -80 °C.

2.4 Expression and purification of recombinant proteins

2.4.1 Periplasmic protein expression in *E. coli* TG1

All scFv fragments were produced in the periplasm of *E. coli* (TG1 strain). One transformed colony was picked from a LB$_{\text{Amp.Glu}}$ agar plate, inoculated in 20 ml of 2x TY medium (1 % (w/v) glucose, 100 µg ampicillin / ml medium) and incubated overnight at 37 °C while shaking (170 rpm). The next day, one liter of 2x TY medium (0.1 % (w/v) glucose, 100 µg ampicillin / ml medium) was inoculated with 10 ml (1:100 dilution) of the overnight culture and cells were grown at 37 °C while shaking (170 rpm) to an OD$_{600}$ of 0.8 to 1.0. The production of the scFv fragments was induced by adding IPTG (f.c.: 1 mM) to the cell suspension and incubating at room temperature for 3 hours on a shaker (170 rpm). The cells were harvested (6,000x g, 10 minutes, 4 °C) and the cell pellet was resuspended in 50 ml periplasmic preparation buffer. The bacterial cell wall was lysed by adding lysozyme (f.c.: 50 µg/ml) and incubating for 20 minutes on ice. The stability of the spheroblast was increased by addition of MgSO$_4$ (f.c.: 10 mM). After centrifugation (10,000x g, 10 minutes, 4 °C) the periplasmic preparation (supernatant) was dialyzed in 5 liter of 1x PBS overnight at 4 °C using a magnetic stirrer. The dialyzed periplasmic preparation was centrifuged (10,000x g, 10 minutes, 4 °C) to pellet aggregations and the scFv fragments were purified by Ni-NTA-IMAC as descried in 2.4.4.
2.4.2 Cytosolic protein expression in *E. coli* BL21DE3

The FLAG-tagged Cys-sTRAIL protein was produced in the cytosol of *E. coli* (BL21DE3 strain). One transformed colony of a LB<sub>Amp,Glu</sub> plate was inoculated in 20 ml LB medium (100 µg ampicillin / ml medium) and incubated overnight at 37 °C while shaking (170 rpm). The next day, one liter of LB medium (100 µg ampicillin / ml medium) was inoculated with 1 ml (1:1000 dilution) of the overnight culture and cells were grown at 37 °C while shaking (170 rpm) to an OD<sub>600</sub> of 0.8 to 0.9. The production of the Cys-sTRAIL protein was induced by adding IPTG (f.c.: 1mM) and ZnCl<sub>2</sub> (f.c.: 50 µM) to the cell suspension and incubating at 37 °C for 1.5 hours on a shaker (170 rpm). The cells were harvested (6,000x g, 10 minutes, 4 °C) and cell pellet was resuspended in 20 ml 1x PBS. The bacterial cell wall was lysed by adding lysozyme (f.c.: 50 µg/ml) and incubating for 20 minutes on ice. Meanwhile, cells were sonificated (40 % cycle) six times for 30 seconds followed by 30 seconds cooling break. The cytosolic preparation could be achieved by several centrifugation steps (twice with 3,500x g, 10 minutes; 15,000x g, 10 minutes; 30,000x g, 30 minutes; all centrifugation steps at 4 °C). The Cys-sTRAIL protein was purified by FLAG-agarose as described in 2.4.5.

2.4.3 Mammalian cell expression in HEK293 cells

For eukaryotic protein production, HEK293 cells were transfected with the plasmid DNA encoding for the protein of interest, as described in 2.3.1. After the selection of stably transfected cells, zeocin selection pressure was reduced (f.c.: 50 µg/ml) and cells were expanded in triple tissue flasks to a confluence of approximately 80 %. Culture medium was exchanged by Opti-MEM (100 ml) and refreshed three times a week until cells detached from the flasks. For TRAIL-fusion proteins, ZnCl<sub>2</sub> (f.c.: 50 µM) was added to Opti-MEM. The supernatant was centrifuged (500x g, 5 minutes, 4 °C) and collected at 4 °C. Proteins were precipitated by adding ammonium sulfate ((NH₄)₂SO₄) slowly to the supernatant (390g/l) and stirring for 30 minutes at 4 °C. After centrifugation (11,250x g, 30 minutes, 4 °C), precipitated proteins were pelleted and resuspended in 20 ml 1x PBS. It must be mentioned that the ammonium sulfate precipitation was not performed for the TRAIL-fusion proteins. Proteins were either purified by Immobilized Metal Affinity Chromatography (2.4.4) for His<sub>6</sub>-tagged proteins, by FLAG affinity chromatography (2.4.5) for FLAG-tagged proteins, or by protein A affinity chromatography in case of the TRAIL-receptor-Fc fusion proteins (2.4.6).

2.4.4 Purification by Immobilized Metal Affinity Chromatography (IMAC)

All recombinant proteins harboring a His<sub>6</sub>-tag were purified using IMAC. For the preparation of the Ni-NTA-agarose beads, 1 ml of the beads was loaded on a column and equilibrated with 8 ml 1x PBS. In a batch purification, beads were either mixed with ammonium sulfate for precipitation and resuspended (in case of the eukaryotic expressed proteins) (2.4.3) or they
were mixed with the dialyzed periplasmic preparation and incubated rolling at 4 °C for at least two hours (in case of the scFv fragments) (2.4.1). Unspecific bound proteins were washed with IMAC wash buffer (1x IMAC Na-phosphat buffer with 35 mM imidazole) until the collected wash fractions were free of proteins. Protein content of the fractions was tested using qualitative Bradford assay (90 µl 1x Bradford reagent and 10 µl of the fractions), which colored to blue in the presence of proteins. Bound proteins were eluted from the beads using IMAC elution buffer (1x IMAC Na-phosphate buffer with 250 mM imidazole). Fractions of 300 µl volume were collected and tested by qualitative Bradford assay of the presence of proteins. Fractions with a high amount of proteins were pooled and dialyzed against 5 l of 1x PBS overnight at 4 °C using magnetic stirrer. Flow through, wash, and dialyzed elution fractions were analyzed by SDS-PAGE and Western Blot.

2.4.5 Purification by FLAG Affinity Chromatography

All recombinant proteins harboring a FLAG-tag were purified by anti-FLAG antibody agarose. For the preparation of the anti-FLAG antibody agarose beads, 3 to 4 ml of the beads were loaded on a column washed firstly three times with 8 ml of low pH buffer (100 mM glycine pH 2.2) and secondly five times with 8 ml of 1x PBS to recover physiological pH conditions. In batch purification, beads were mixed either with the cell culture supernatant of the eukaryotic expressed proteins (TRAIL fusion proteins) or with the cytosolic preparation of prokaryotic expressed molecules (Cys-sTRAIL) and incubated rolling at 4 °C for at least two hours. Unspecific bound proteins were washed with 1x PBS until the collected washing fractions were free of proteins. The protein content was analyzed by qualitative Bradford assay as described in 2.4.3. Bound proteins were eluted from the beads using 10 ml of FLAG-peptide (f.c.: 0.1 mg/ml). The elution was concentrated using a Vivaspin column concentrator (MW cut-off 10 kDa for Cys-sTRAIL; 30 kDa for EHD2-scTRAIL and scFv-EHD2-scTRAIL). The concentrated proteins were dialyzed against 5 l of 1x PBS overnight at 4 °C using magnetic stirrer. Flow through, wash, and dialyzed elution fractions were analyzed by SDS-PAGE and Western Blot.

2.4.6 Purification by protein A Affinity Chromatography

The TRAIL-receptor-Fc fusion proteins were purified via protein A Affinity Chromatography because of specific interaction of the Fc part of the fusion protein with protein A. The ammonium sulfate precipitated and resuspended protein (2.4.3) was incubated with protein A beads rolling overnight at 4 °C and was loaded into a protein purification column. The solution was run through the column and the beads were washed with 5 ml protein A wash buffer (100 mM Tris-HCl, pH 7.0). The protein content was tested with a qualitative Bradford assay, as described in 2.4.3. The washing step was repeated until no protein could be detected anymore in the flow through. After the washing steps, the bound proteins were...
eluted from the protein A beads by adding 500 µl fractions of protein A elution buffer (10 mM glycin, pH 3.0) until no protein could be detected anymore by qualitative Bradford assay. In adding 50 µl of neutralization buffer (1 M Tris-HCl, pH 8.0), the sour milieu of the elution fractions were neutralized. All purification fractions (flow-through, washing steps and elution fractions) were analyzed on SDS-PAGE and Western Blot.

2.5 Protein Characterization

2.5.1 Determination of protein concentration

The protein concentration was calculated using the absorbance measured at 280 nm with a spectrophotometer (NanoDrop).

\[
c\left[\frac{\mu g}{\mu l}\right] = M\left[\frac{mol}{l}\right] \cdot MW\left[\frac{g}{mol}\right] = \frac{OD_{280}}{\varepsilon} \cdot MW
\]

\[
\varepsilon = \left(\text{number}_{\text{tryptophan residues}} \cdot 5540\right) + \left(\text{number}_{\text{tyrosine residues}} \cdot 1480\right)
\]

Molecular weight was determined by using the online tool ‘ProtParam’ (see 2.1.17).

(c, concentration; M, molarity; MW, molecular weight \(\left[\frac{g}{mol}\right] = Da\); \(\varepsilon\), molar extinction coefficient)

2.5.2 SDS-PAGE and Coomassie staining

Concerning molecular mass and purity, Proteins were analyzed by SDS-PAGE. Protein samples (2 µg for Coomassie staining or 1 µg for Western Blot) were mixed either in reducing or non-reducing 5x laemmli loading buffer (f.c.: 1x) and boiled at 94 °C for 5 minutes. Samples and the protein standard for the determination of the molecular mass were loaded on gel, which was operated at 45 mA per gel for approximately 55 minutes. After washing the gel in boiling water, it was stained with Coomassie staining solution for 1 hour on a shaker and finally destained in water. Different concentrations of acrylamide were used for the preparation of the gels (see Table 2-10) dependent on the different molecular masses of the analyzed proteins.
Table 2-10: Solutions of SDS-polyacrylamid gels

<table>
<thead>
<tr>
<th>Substances</th>
<th>Stacking Gel</th>
<th>Separating Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 %</td>
<td>10 %</td>
</tr>
<tr>
<td>dH\textsubscript{2}O</td>
<td>2.1 ml</td>
<td>2.95 ml</td>
</tr>
<tr>
<td>30 % Acrylamide</td>
<td>500 µl</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>1.5 M Tris, pH 8.8</td>
<td>-</td>
<td>1.9 ml</td>
</tr>
<tr>
<td>1.0 M Tris, pH 6.8</td>
<td>380 µl</td>
<td>-</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>30 µl</td>
<td>75 µl</td>
</tr>
<tr>
<td>10 % APS</td>
<td>30 µl</td>
<td>75 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

2.5.3 Deglycosylation of proteins

Proteins (10 µg) were diluted in 1x PBS to a volume of 50 µl and denatured at 94 °C for 5 minutes. After cooling, 2 units of N-glycosidase F was added to the protein solution and incubated overnight at 37 °C. The deglycosylated proteins were compared with the untreated protein in SDS-PAGE under reducing conditions.

2.5.4 Western Blot Analysis

Protein samples were separated by SDS-PAGE and identified by Western Blot. The transfer of the proteins from the acrylamide gel onto the nitrocellulose membrane was performed at amperage of 110 mA for 40 minutes in a semidy blot using the Western Blot - blotting buffer. The voltage was limited to 18 V. Remaining binding sites of the membrane were blocked with Western Blot - blocking solution (5 % (m/v) dry milk in 1x PBS) for 30 minutes at room temperature on a shaker and incubated with HRP-conjugated antibodies (2.1.7, also dissolved in blocking solution) either at room temperature for 2 hours or at 4 °C overnight. Subsequently, the membrane was washed three times in Western Blot - washing solution (0.05 % (v/v) Tween20 in 1x PBS) and once in PBS each for 5 minutes and incubated with developing solution (4 ml Western Blot - solution A + 400 µl Western Blot - solution B + 1.4 µl 30 % H\textsubscript{2}O\textsubscript{2}) for 2 minutes. Finally, the membrane was exposed to a radiographic film for 15 seconds up to 2 minutes and developed by using an automatic radiographic film processor.
2.5.5 Determination of the protein melting point

The melting points of proteins were determined by measuring the thermal denaturation with ZetaSizer Nano ZS (Malvern). Approximately 100 µg (for scFv fragments and proteins with higher molecular masses) or 200 µg (for EHD2, MHD2 and GHD3) of purified proteins was diluted in 1x PBS to a total volume of 1 ml and sterile filtered into a quartz cuvette. Dynamic laser light scattering (mean count rate) was measured while the temperature was increased in 1 °C intervals from 35 to 92 °C with 2 minutes equilibration time for each temperature step. The melting point was defined as the temperature at which the mean count rate increased.

2.5.6 Size exclusion chromatography

High Performance Liquid Chromatography (HPLC) was performed with different columns for size exclusion chromatography of proteins, namely with a BioSuite 250 column, a BioSep SEC-S2000, -S3000, or Yarra SEC-2000. 20 µl of protein sample concentrated from 0.2 to 0.6 µg/µl was injected into the HPLC system and analyzed with a flow rate of 0.5 ml/min (1x PBS as mobile phase). The following standard proteins were used to determine the molecular mass of the analyzed proteins: thyreoglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), aprotinin (6.5 kDa), and FLAG peptide (1 kDa).

2.6 Functionalization of liposomes

Liposomes were functionalized either with only a targeting moiety, namely scFv, resulting in immunoliposomes (IL), or with only an effector moiety, namely TRAIL, resulting in LipoTRAIL (LT), or with both, targeting and effector moiety, resulting in Immuno-LipoTRAIL (ILT). Additionally, non-functionalized plain liposomes (nt-liposomes) were prepared (see Figure 2-1).
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Figure 2-1: Schematic illustration of the preparation of functionalized liposomes. Functionalized liposomes were generated by using the post-insertion method. Therefore, scFv'\textsubscript{EGFR}-coupled and/or Cys-sTRAIL-coupled micelles were incorporated into preformed PEGylated liposomes at 42 °C for 2 hours resulting in immunoliposomes (IL), LipoTRAIL (LT), and Immuno-LipoTRAIL (ILT).

2.6.1 Preparation of liposomes

The liposomes were prepared using the film-hydration-extrusion method and were composed of EPC/cholesterol/DSPE-mPEG\textsubscript{2000} at a molar ratio of 6.77:3:0.23. Additionally, all liposomes contained either 0.03 mol% Dil (for in vitro studies) or 0.03 mol% DiR (for in vivo studies) as fluorescent dye. Lipids and dyes were dissolved in chloroform and stored at -20 °C. Using a rotary evaporator, a thin lipid film was formed in a glass flask and resuspended in 10 mM HEPES buffer, pH 7.4, (f.c.: 10 nmol lipid/µl) by vortexing. To create unilamellar liposomes, the multilamellar suspension was pushed through a polycarbonate membrane (50 nm pores) using a LipoFaster extruder.

2.6.2 Coupling of scFv'\textsubscript{EGFR} and Cys-sTRAIL

The cysteine modified proteins were coupled to DSPE-PEG\textsubscript{2000}-Maleimide micelles. To achieve this, chloroform was removed from the lipids and suspended in ddH\textsubscript{2}O (f.c.: 10 mg/ml) for 5 minutes at 60 °C. The day before coupling, proteins were reduced by adding TCEP (f.c.: 5 mM) and incubating for 2 hours at room temperature. Then TCEP was removed by dialysis in D-Tube\textsuperscript{TM} Dialyzer Mini (MW cut-off 6 - 8 kDa) against nitrogen-saturated 1x
scFv-coupling buffer (for scFv'\textsubscript{EGFR}) or 1x TRAIL-coupling buffer (Cys-sTRAIL) at 4 °C overnight using a magnetic stirrer. Reduced proteins were mixed with DSPE-PEG\textsubscript{2000}-Mal-micelles in a molar ratio of 1:5 (protein : micellar lipid) and incubated for 1 hour at room temperature. Avoiding a re-oxidation of the proteins, incubation was overlaid with nitrogen and mixed by quick vortexing. Finally, non-coupled and reactive maleimide groups were quenched by adding L-cysteine (f.c.: 100 µM) for 10 minutes at room temperature.

2.6.3 Analysis of coupling efficiency

The scFv- and TRAIL-coupled micelles were analyzed by SDS-PAGE under reducing conditions and visualized using Coomassie staining solution. The coupling efficiency was calculated by dividing the intensity of the band of the coupled protein (upper band) by the sum of the intensity of the coupled and the non-coupled (lower band) protein using the software ImageJ.

2.6.4 Calculation of the number of proteins in the liposomal surface

As described in Table 3-7, the average diameter of the functionalized liposomes is approximately 90 nm. Yuan and coworkers described the length of a monolayer with approximately 27 Å (Yuan et al., 2007), thus the length of a bilayer results in approximately 54 Å. Using the equation for the calculation of the surface of a sphere ($A = 4\pi r^2, r_{outer\ layer} = 450$ Å, $r_{inner\ layer} = 396$ Å), the liposomal surface possesses a surface of approximately $2.55 \cdot 10^6$ Å$^2$ for the outer layer and approximately $1.97 \cdot 10^6$ Å$^2$ for the inner layer. The surface of one phosphatidylcholine molecule tightly packed in cholesterol was reported with approximately 38 Å$^2$ (Forge et al., 1978). Hence, the outer layer consists of approximately $6.71 \cdot 10^4$ molecules and the inner layer of approximately $5.18 \cdot 10^4$ molecules. In conclusion, one liposome sized with 90 nm in diameter consists of approximately $1.19 \cdot 10^5$ molecules. Concerning the ratio of functionalized lipid and protein (DSPE-PEG\textsubscript{2000}-Mal:protein; 5:1), the amount of inserted protein-coupled micelles (0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-protein) and the coupling efficiency (60 % for the scFv'\textsubscript{EGFR}), one liposome was functionalized with approximately 24 TRAIL molecules and/or 14 scFv fragments.

2.6.5 Post-insertion method

Functionalized liposomes were generated by the post-insertion method, which was performed with scFv-coupled and/or TRAIL-coupled micelles and pre-formed PEGylated liposomes for 2 hours at 42 °C. Unbound proteins were removed by gel filtration (sepharose 4CLB). The collected samples of functionalized liposomes were centrifugated (135,000x g, 1 hour, 4 °C) and pelleted liposomes were dissolved in 10 mM HEPES, pH 7.4, (f.c.: 10 nmol lipid/µl).
2.6.6 Characterisation of liposomes

Size and homogeneity (polydispersions index; PDI) of liposomes were analyzed using a Zetasizer Nano ZS. Therefore, liposomes were diluted in sterile filtered 1x PBS and transferred into a cuvette.

2.7 Flow cytometry

The different fusion proteins and functionalized liposomes were tested for their binding to different cell lines. Cells were cultured in culture flasks in the appropriate medium (2.1.11) at 37 °C in a humidified incubator. For flow cytometry analysis, cells were firstly washed with 1x PBS and secondly detached using 1x Trypsin/EDTA. The cell number was determined as described in 2.3 and the respective amount of cells was pelleted by centrifugation (500x g, 5 minutes, 4 °C) and resuspended in PBA (f.c.: 5 · 10⁶ cells/ml). Finally, 50 µl of the PBA cell suspension was pipetted per well on a V-shaped 96-well plate and incubated with the fusion protein or the functionalized liposome in a total volume of 100 µl. The flow cytometer possessed an extinction-wavelength of 488 nm. Obtained data were analyzed with the software WinMDI, version 2.9, and the mean fluorescence intensity (MFI) was relativized as follows:

\[
\text{relative } MFI = \frac{MFI_{\text{construct}} - (MFI_{\text{detecion control}} - MFI_{\text{cells}})}{MFI_{\text{cells}}}
\]

2.7.1 Expression of EGFR and HER2 on cell lines

For the analysis of the EGFR- and HER2-expression of different cell lines, PE-conjugated antibodies (see 2.1.7) were incubated with the cells for 1 hour at 4 °C. Subsequently, cells were washed three times with 100 µl PBA each (centrifugation step: 500x g, 5 minutes, 4 °C), resuspended in 300 µl PBA, transferred into FACS tubes, and analyzed in the flow cytometer measuring FL2 (Emission of PE: 575 nm).

2.7.2 Binding of the MHD2 fusion proteins

In case of the MHD2 fusion proteins, either 300 nM of the antibody fusion protein (scFvEGFR-MHD2, MHD2-scFvHER2, scFvEGFR-MHD2-scFvHER2) was titrated (1:3 dilution) on A431, SKBR3, NCI-H460 and Colo205, or 50 nM of the antibody-scTNF fusion protein (scFvEGFR-MHD2-scTNF) was mixed with A431 or HT1080 cells in the absence or presence of cetuximab and trastuzumab (blocking experiments). For this purpose, antibodies were pre-incubated in excess amount with the cells. Fusion proteins were incubated with cells for 1 hour at 4 °C and were washed three times with 100 µl PBA each as described in 2.7.1. The
detection of all bound MHD2 fusion proteins was performed using a FITC-conjugated anti-His<sub>6</sub>-antibody (see 2.1.7). After washing, the measurements (FL1) were performed as described above (Emission of FITC: 525 nm). For detection control, only FITC-conjugated anti-His<sub>6</sub>-antibody was incubated with the cells.

### 2.7.3 Binding of the EHD2 fusion proteins

Cells (Colo205, NCI-H460, HCT116, and HepG2) were incubated with 20 nM of the EHD2 fusion protein and incubated for 1 hour at 4 °C. After washing three times with PBA, bound scTRAIL-conjugates (EHD2-scTRAIL, scFv-EHD2-scTRAIL) were detected with anti-TRAIL mouse antibody and PE-conjugated anti-mouse IgG antibody (see 2.1.7) (incubation for 1 hour at 4 °C). After each incubation step, cells were washed three times with 100 µl PBA as described in 2.7.1. Cells were resuspended in 300 µl PBA, transferred in FACS tubes and analyzed in the flow cytometer measuring FL2 (Emission of PE: 575 nm). For the detection control, only anti-TRAIL antibody and PE-conjugated anti-mouse-antibody was incubated with the cells. The His<sub>6</sub>-tagged scFv-EHD2 fusion protein was detected using PE-conjugated anti-His<sub>6</sub>-antibody (2.1.7). Measurements (FL2) were performed as described above. For detection control, only PE-conjugated anti-His<sub>6</sub>-antibody was incubated with the cells.

### 2.7.4 Binding of functionalized liposomes

Due to incorporation of Dil into the liposomal membrane, the binding of the functionalized liposomes to the cells could be detected by flow cytometer without adding additional conjugated antibody. Dil could be measured in FL2 at a wavelength of 575 nm. Consequently, the calculation of the relative mean fluorescence intensity for the liposomes changed as follows:

\[
\text{relative MFI} = \frac{MFI_{\text{liposome}}}{MFI_{\text{cells}}}
\]

The binding of liposomes was tested on Colo205, NCI-H460 and HepG2 cells. Liposomes were titrated (1:3 dilution) on the cells starting at 3000 µM lipid and were incubated for 1 hour at 4 °C. After washing the cells three times with 100 µl PBA (centrifugation step: 500x g, 5 minutes, 4 °C), they were resuspended in 300 µl PBA and transferred into FACS tubes. Analysis was performed in the flow cytometer measuring FL2. In a blocking experiment, cetuximab (5 µM) was pre-incubated with the cells prior incubating them with 1000 µM lipid of liposomes. Non-functionalized plain liposomes (nt-liposomes) were used as an unconjugated reference.
2.8 Enzyme-linked immunosorbent assay (ELISA)

Proteins dissolved in a solution can be specifically detected via ELISA. All proteins, functionalized liposomes and antibodies used in ELISA were dissolved in ELISA blocking solution (2 % (w/v) dry milk in 1x PBS). The developing of all ELISA was performed using 100 µl/well of ELISA developing solution (0.1 mg/ml TMB, 100 mM sodium acetate buffer pH6.0, 0.006 % H₂O₂). The enzymatic reaction was stopped by adding 50 µl ELISA stopping solution (1 M H₂SO₄). The absorbance of the wells at 450 nm was measured in an ELISA reader.

2.8.1 TRAIL-receptor-Fc ELISA

TRAIL-receptor-Fc fusion proteins (TRAIL-R1-Fc, TRAIL-R2-Fc, TRAIL-R3-Fc, TRAIL-R4-Fc in 1x PBS) were coated (200 ng/well) on an ELISA plate overnight at 4 °C and remaining binding sites were blocked with ELISA blocking solution (2 % (w/v) dry milk in 1 xPBS). Fusion proteins were titrated starting from 10 mM (diluted 1:3) in duplicates and incubated for one hour at room temperature. Subsequently, plates were washed five times in ELISA washing solution (0.05 % (v/v) Tween20 in 1x PBS). Bound proteins were detected either with HRP-conjugated anti-His₆-tag mouse antibody (see 2.1.7) for scFv-EHD2 fusion proteins or with HRP-conjugated anti-FLAG mouse antibody (see 2.1.7) for all scTRAIL fusion proteins. Developing and measuring was performed as described above (2.8). Additionally, the coating step of the Fc-fusion proteins was controlled by incubating wells (only blocked with ELISA blocking solution) with HRP-conjugated anti-human IgG (Fc-specific) antibody (2.1.7).

2.8.2 EGFR/HER2/HER3-receptor-Fc-ELISA

EGFR-Fc, HER2-Fc, or HER3-Fc fusion proteins were coated (100 ng/well) on an ELISA plate overnight at 4 °C and remaining binding sites were blocked with ELISA blocking solution (2 % (w/v) dry milk in 1x PBS). For binding assays, the MHD2 fusion proteins (scFᵥEGFR-MHD2, MHD2-scFᵥHER2, scFᵥEGFR+MHD2-scTNF, MHD2-scTNF) were concentrated with 10 mg/ml. For the avidity ELISA assay, the MHD2 fusion proteins (scFᵥEGFR, scFᵥEGFR-MHD2, scFᵥHER2, MHD2-scFᵥHER2) and the EHD2 fusion proteins (scFᵥEGFR-EHD2, scFᵥEGFR) were concentrated with 1 µM and titrated (1:3 dilution) in duplicates. All fusion proteins were incubated with the Fc fusion proteins for 1 hour at room temperature. In case of pharmacokinetic studies of scFv-EHD2, dilutions of the serum samples were diluted in ELISA blocking solution to the expected protein levels. In addition, each serum sample was diluted 1:5 in ELISA blocking solution again. All samples were incubated for one hour on the ELISA plate. After washing (see 2.8.1), bound proteins were detected with HRP-conjugated mouse anti-His₆-tag antibody (2.1.7), developed and
measured as described above (2.8). Additionally, the coating step was controlled according to 2.8.1. The absorption values of the different time points were interpolated to the respective protein content using a standard curve of titrated scFv-EHD2 fusion protein.

2.8.3 TNF-receptor-Fc-ELISA

TNF-receptor-Fc fusion proteins (TNFR1-Fc, TNFR2-Fc in 1x PBS) were coated (100 ng/well) on an ELISA plate overnight at 4 °C and remaining binding sites were blocked with ELISA blocking solution (2 % (w/v) dry milk in 1x PBS). The MHD2 fusion proteins (scFvEGFR-MHD2, MHD2-scTNF and scFvEGFR-MHD2-scTNF) were incubated in triplicates for one hour at room temperature. Washing steps and the detection of bound protein with HRP-conjugated anti-His6-tag antibody (see Table 2-1) and of coated protein with HRP-conjugated anti-human IgG antibody were performed as described in 2.8.1. Developing and measuring of ELISA plates were performed according to 2.8.

2.8.4 Liposomes-ELISA

EGFR-Fc or TRAILR2-Fc fusion proteins were coated (200 ng/well) on an ELISA plate overnight at 4 °C and remaining binding sites were blocked with ELISA blocking solution (2 % (w/v) dry milk in 1x PBS). 1000 µM lipid of the respective liposomes were incubated for one hour at room temperature. Subsequently, plates were washed five times in 1x PBS. Proteins attached to the bound liposomes were detected either with HRP-conjugated anti-His6-tag antibody for scFv fragments or with HRP-conjugated mouse anti-FLAG antibody (see Table 2-1) for TRAIL molecules and developed as described above (see 2.8.1). Analogously, the coating step of Fc-constructs was also tested. Developing and measuring of the ELISA plate was performed as described in 2.8.

2.9 Interleukin-8 secretion assay

HT1080 (2·10^4 cells per well) cells were grown in 100 µl culture medium (see Table 2-2) in 96-well plates overnight. The next day, the culture medium was exchanged to remove constitutively produced IL-8. Cells were treated with serial dilutions (starting from 200 nM; 1:10 diluted) of the MHD2-constructs (scFvEGFR-MHD2, MHD2-scTNF, scFvEGFR-MHD2-scTNF) or scTNF in duplicates. After 18 hours of incubation at 37 °C, plates were centrifugated (500x g, 5 minutes, 4 °C) and induction of IL-8 production, respectively the release of IL-8 into the culture supernatant was measured via an IL-8-Sandwich ELISA according to manufacturer's protocol. Additionally, cells were treated with 20 pM of fusion proteins in absence or presence of the monoclonal antibodies cetuximab or trastuzumab (100 µg/ml) and analyzed for IL-8 secretion after incubation for 18 hours at 37 °C. To compensate the daily variability of the IL-8 production, the block shift correction method was performed:
Materials and methods

$$X'_n = X_n - (A_n - A)$$

($X'_n$, corrected value $X$ of the experiment $n$; $X_n$, value $X$ of the experiment $n$; $A_n$, average of the duplicate values of $X_n$; $A$, average of the $X$ values from all experiments)

2.10 Cell death induction assay

2.10.1 Cell death induction of MHD2 fusion proteins

Testing the potential of activating TNFR1 and/or TNFR2 for the MHD2 fusion proteins, mouse embryonic fibroblasts (MEF) were used, expressing the chimeric receptor fusion protein on the cell membrane by a stable transfection with TNFR1-Fas (MEF-TNFR1-Fas) or TNFR2-Fas (MEF-TNFR2-Fas) (Krippner-Heidenreich et al., 2002). 2 \cdot 10^4 cells per well were grown in 100 µl culture medium in 96-well plates overnight at 37 °C. Cells were treated with serial dilutions of scTNF, scF_{EGFR}-MHD2, and MHD2-scTNF starting from 100 nM (1:10 dilution). After 16 hours of incubation at 37 °C, culture medium was discarded and cell viability was analyzed by adding 50 µl of crystal violet staining solution (20 % (v/v) methanol, 0.5 % (m/v) crystal violet in dH2O) for 20 minutes at room temperature. After washing (H2O) and air-drying the wells, crystal violet was dissolved in 50 µl methanol and the absorbance at 550 nm was measured in an ELISA reader.

2.10.2 Cell death induction of EHD2 fusion proteins

Colo205 (5 \cdot 10^4 cells per well), NCI-H460 (2 \cdot 10^4 cells per well), HepG2 (2 \cdot 10^4 cells per well) or HCT116 (1 \cdot 10^4 cells per well) cells were grown in 100 µl culture medium in 96-well plates overnight. Cells were treated with serial dilutions (starting from 10 or 3 nM; 1:3 diluted) of the EHD2-constructs (scFv-EHD2, EHD2-scTRAIL, scFv-EHD2-scTRAIL), diabody-scTRAIL (Db-scTRAIL), or scTRAIL in duplicates. Cell death induction assays were performed in the absence or presence of bortezomib (f.c.: 250 ng/ml) which was added 30 minutes prior the serial dilutions of the fusion proteins to sensitize the cells to TRAIL-induced apoptosis. After 16 hours of incubation at 37 °C, the culture medium was discarded and viable cells were analyzed and measured as described in 2.10.1.

2.10.3 Cell death induction of functionalized liposomes

Colo205 (5 \cdot 10^4 cells per well), NCI-H460 (2 \cdot 10^4 cells per well), HepG2 (2 \cdot 10^4 cells per well) and Huh-7 (2 \cdot 10^4 cells per well) cells were grown in 100 µl culture medium in 96-well plates overnight. Cells were treated with serial dilutions of functionalized liposomes or TRAIL proteins in triplicates. Cell death induction assays were performed in the presence or absence of bortezomib (f.c.: 250 ng/ml) or cyclohexamide (f.c.: 2.5 µg/ml). Before adding the...
serial dilutions of functionalized liposomes or proteins, bortezomib or cyclohexamidine were pre-incubated with the cells for 30 minutes at 37 °C to sensitize the cells to TRAIL-induced apoptosis. After 16 hours of incubation, supernatant was discarded and cell viability was analyzed by crystal violet staining as described in 2.10.1.

2.11 *In vivo* assays

All working with experimental animals was conducted according to federal guidelines. Mice were at least 8 week old at the starting of the experiments and were cultivated for at least two weeks in our animal facilities for acclimation.

2.11.1 Pharmacokinetic studies

All pharmacokinetic studies for determining the serum half-life of the fusion proteins and the functionalized liposomes were performed in female SWISS mice ordered from Janvier. Three animals were used to determine each serum half-life of the fusion proteins or functionalized liposomes. Fusion proteins or functionalized liposomes were intravenously injected and blood samples (50 µl) were collected from the tail in the time intervals of 2 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 1 day, and 3 days and incubated on ice for 10 minutes. Clotted blood was centrifugated (16,100x g, 20 minutes, 4 °C) and serum samples were stored at 4 °C.

2.11.1.1 Pharmacokinetics of EHD2 fusion proteins

SWISS mice received an i.v. injection of 25 µg of fusion proteins dissolved in 1x PBS to a total volume of 100 µl. Time intervals of taking blood samples and the preparation of the serum was performed as described in 2.11.1. TRAIL fusion proteins were analyzed with the TRAIL ELISA Set according to manufacturer’s protocol. The scFv-EHD2 construct was detected by ELISA using an HRP-conjugated anti-His6-tag mouse antibody as described in 2.8.2. To allow comparisons, the 2-minute-value was set to 100%. Terminal half-life ($t_{1/2}$) and bioavailability (area under the curve) were calculated with Excel.

2.11.1.2 Pharmacokinetics of functionalized liposomes and sTRAIL

SWISS mice received an i.v. injection of 1 µg of TRAIL molecule or 1 µmol of functionalized liposomes dissolved in 1x PBS in a total volume of 100 µl. Blood samples and preparation of serum was performed as described above (2.11.1). The serum concentration of TRAIL molecules was analyzed with the TRAIL ELISA Set according to manufacturer’s protocol. The serum concentrations of functionalized liposomes containing DiR-dye was analyzed via LI-COR Odyssey reader. For this purpose, serum samples were diluted in 1x PBS in a total volume of 110 µl. The samples were transferred (50 µl) into a 96-well plate and fluorescence
(700 nm) was measured in duplicates. The concentration of the samples was calculated by interpolating the fluorescence values with a standard curve of DiR-labeled liposomes (dilution factor was multiplied). To allow comparisons, the 2-minute-value was set to 100%. Terminal half-life ($t_{1/2}$) and bioavailability (area under the curve) were calculated with Excel.

### 2.11.2 ALT assay

Female SWISS mice (Janvier, CD1, 8 weeks old, 3 mice per construct) received an intravenous i.v. injection of 1 nmol of scFv-EHD2-scTRAIL in combination with an intraperitoneal (i.p.) injection of 5 µg bortezomib each dissolved in 150 µl 1x PBS. The control group only received an i.v. injection of 150 µl 1x PBS. Blood samples (100 µl) were collected from the tail after 4 and 24 hours. The preparation of serum samples was performed as described in 2.11.1 and stored at -20 °C. The activity of alanine transaminase (ALT) was measured using an enzymatic assay kit.

### 2.11.3 Antitumor activity

All pharmacodynamic studies for testing the antitumor activity of the fusion proteins and functionalized liposomes were performed in female nude NMRI mice ordered from Janvier. Nude NMRI mice received subcutaneous injection at the left and right dorsal side each with $3 \cdot 10^6$ Colo205 cells in a total volume of 100 µl 1x PBS. For this purpose, adherent cells were detached from the culture flasks using working solution of trypsin/EDTA and resuspended in 50 ml of the respective cell medium. Certain amounts of cells were harvested (500x g, 5 minutes, 4 °C), washed with 50 ml 1x PBS and pelleted again. Finally, the cells were resuspended in 1x PBS adjusting $30 \cdot 10^6$ cells per ml in 1x PBS. During the injection of the cells, the mice were anesthetized with isoflurane. The treatment with the fusion proteins was initiated when the tumor volume reached about 100 mm³.

#### 2.11.3.1 Antitumor activity of EHD2 fusion proteins

In a first experiment, Colo205-bearing mice received in total 4 i.v. injections of either 0.35 nmol EHD2-scTRAIL or scFv-EHD2-scTRAIL, or 0.70 nmol of scTRAIL dissolved in 100 µl 1x PBS every fourth day starting from day 7 (day 7, 11, 15, 19). Additionally, the mice received an i.p. injection of 5 µg bortezomib dissolved in 100 µl 1x PBS every other day starting from day 7 (day 7, 9, 11, 13, 15, 17, 19, 21). In a second experiment, Colo205-bearing mice received in total 4 i.v. injections of 1 nmol of scFv-EHD2 or scFv-EHD2-scTRAIL in a total volume of 100 µl 1x PBS every other day starting from day 8 (day 8, 10, 12, 14). Additionally, the mice received an i.p. injection of 5 µg bortezomib dissolved in 100 ml 1x PBS every other day starting from day 8 (day 8, 10, 12, 14). In both experiments, the control groups received i.v. injections of 100 µl 1x PBS and i.p. injections of 5 µg bortezomib dissolved in 100 µl 1x PBS.
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PBS at the respective time points. Tumor growth was monitored with a caliper and tumor volume was calculated as follows:

\[
tumor \, volume = \frac{(L \cdot W^2)}{2}
\]

(L, longitudinal diameter of tumor; W, transverse diameter of tumor)

2.11.3.2 Antitumor activity of functionalized liposomes

Colo205-bearing mice received in total 3 i.v. injections of 1 µmol functionalized liposomes in a total volume of 100 µl 1x PBS in combination with an i.p. injection of 5 µg bortezomib dissolved in 100 µl 1x PBS every other day starting from day 7 (day 7, 9, 11). The control group received three i.v. injections of 1 µmol plain liposomes in a total volume of 100 µl 1x PBS (nt-liposomes) instead of the functionalized liposomes. Tumor monitoring and calculations were performed as described in 2.11.3.1.

2.12 Statistical analysis

The error value of the in vitro assay corresponded to the standard deviation (SD), whereas the 95 % confidential interval (95%CI) was used for in vivo experiments. Significances of experiments were calculated by Graph Pad Prism 5.0.1 and results were compared either via unpaired t-tests (in vitro assays) or via one-way ANOVA followed by Tukey’s multiple comparison test (post-test) (in vivo assays).
3 Results

3.1 Multivalent fusion proteins

In the first part of this study, the homodimerization modules IgM heavy chain domain 2 (MHD2, see Figure 3-1) and IgE heavy chain domain 2 (EHD2, see Figure 3-16) were utilized for the generation of dimeric fusion proteins. MHD2 was used as model to generate fusion proteins composed of a scFv fragments directed against EGFR and HER2 and/or of a single-chain derivative of the human TNF (scTNF). The TRAIL fusion proteins were generated by fusing a scFv fragment directed against EGFR to the N-terminus and/or a single-chain derivative of the human TRAIL (scTRAIL) to the C-terminus of the EHD2.

3.1.1 IgM heavy chain domain 2 (MHD2) and MHD2 derivatives

IgM is composed of the light chain, consisting of one variable (V_L) and one constant (C_L) domain, and the heavy chain, also consisting of one variable (V_H), but of four constant (C_H1-C_H4) domains (see Figure 1-4) (Perkins et al., 1991). The C_H2 of IgM (MHD2) is centrally located in the heavy chain and consists of 111 amino acid residues resulting in a total molecular weight of 12.2 kDa. The MHD2 covalently connects the two heavy chains by an interdomain disulfide bond formed between the cysteine residues 337 of each domain (see Figure 3-1). The domain itself is stabilized by an intradomain disulfide bond between cysteine residues 261 and 321. Furthermore, a potential N-glycosylation site is located at asparagine residue 332. The exchange of the flanked domains of the MHD2 (C_H1 and C_H3) with proteins should result in the generation of a dimeric fusion protein. The high segmental flexibility of the MHD2 further affirms this domain as an operative homodimerization module (Roux et al., 1998). Thus, the MHD2 should be ideally suited as homodimerization module for the generation of fusion proteins.

Figure 3-1: The human IgM heavy chain domain 2 (MHD2). A Protein sequence of the human MHD2. The potential N-glycosylation site at asparagine residue 332 (NAS highlighted in gray), the intradomain disulfide between cysteine residues 261 and 321, and the interdomain disulfide residue 337 are marked. B Structure of the mouse MHD2 (from pdb entry 4JVU) (Müller et al., 2013). The two domains are colored in red and blue. Cysteine residues are shown in yellow spheres for the blue domain and in orange spheres for the red domain. Numbering of the residues according to the EU index (Kabat et al., 1991).
The MHD2 (aa 229-340) was produced in stably transfected mammalian cells as a C-terminal hexahistidyl (His₆)-tagged protein. SDS-PAGE analysis showed two bands under reducing conditions in the range of approximately 20 kDa, corresponding most likely to the non-glycosylated (lower band) and to the N-glycosylated version (upper band) of the monomeric MHD2 (see Figure 3-2). Under non-reducing conditions, bands in the range of the dimeric MHD2 with approximately 37 kDa were observed. The two major bands correspond most likely to the disulfide-linked dimer modified either with two (upper band) or with one (middle band) N-glycan, as well as a very thin band (lower band) which correspond to the non-glycosylated dimer. Only a small fraction (less than 5 %) was the monomeric MHD2. These results indicat an almost complete covalent assembly of the MHD2 under denaturing conditions. The melting point of the MHD2 was determined at 55 °C by dynamic light scattering. The influence of the interdomain disulfide-bond (cysteine residue 337) and the N-glycan (asparagine residue 332) of the MHD2 concerning the thermo stability of the domains was also tested. For this purpose, three derivatives of the MHD2 were generated, one lacking the interdomain disulfide-bond due to a substitution of the cysteine residue 337 by a serine (MHD2-C337S), one lacking the N-glycosylation site due to the substitution of the asparagine residue 332 by a glutamine (MHD2-N332Q), and a last variant combining both substitutions (MHD2-N332Q-C337S). The MHD2 as well as the derivatives were produced in a similar range varying between 0.8 – 2.7 mg/l supernatant.

Figure 3-2: Biochemical analysis of the MHD2 and its derivatives. A Schematic illustration of the MHD2 and its derivatives. The MHD2 is shown in gray, N-glycans attached to the MHD2 are shown as black hexagon and the interdomain disulfide bond is indicated as black line. B SDS-PAGE analysis of the MHD2 and its derivatives under reducing (1) and non-reducing (2) conditions (M, protein standard marker). C Determination of the melting points of the MHD2 and its derivatives via dynamic light scattering. The measured melting point is indicated as dotted line.
The absence of the interdomain disulfide bond (MHD2-C337S) was analyzed via SDS-PAGE analysis. Under both conditions, reducing and non-reducing, the MHD2-derivative revealed bands in the range of monomeric MHD2, comparable with the bands of the wild-type MHD2 under reducing conditions. Under reducing and non-reducing conditions, the non-glycosylated derivative MHD2-N332Q showed one band corresponding to the lower band of the wild-type MHD2 under reducing respectively non-reducing conditions. Under both conditions, reducing and non-reducing, the combinatorial derivative MHD2-N332Q-C337S showed only one band correlating to the lower band of reduced wild-type MHD2. These SDS-PAGE experiments confirm the assumption that the lower band of MHD2 in SDS-PAGE belongs to the non-glycosylated version of MHD2. The thermal stability of the MHD2 derivatives was reduced compared with that of the wild-type MHD2. The stability of the non-glycosylated version MHD2-N332Q was reduced by 3 °C to 52 °C, whereas the absence of the intradomain disulfide-bond decreased the stability by 6 °C to 49 °C. The melting point of the combinatorial derivative MHD2-N332Q-C337S was reduced by 10 °C to 45 °C, indicating that both, interdomain disulfide-bond and N-glycan contribute to the stability of the MHD2.

### 3.1.2 Multivalent antibody-MHD2 fusion proteins

For the generation of targeted molecules with increased valency, the MHD2 was used as homodimerization module and fused to single-chain Fv fragments (scFv). Two bivalent antibody-MHD2 fusion proteins were generated by fusing either a humanized anti-EGFR scFv to the N-terminus of the wild-type MHD2 (scFv\textsubscript{EGFR}-MHD2) or an anti-HER2 scFv to the C-terminus of the wild-type MHD2 (MHD2-scFv\textsubscript{HER2}) (see Figure 3-3). Additionally, a tetravalent and bispecific fusion protein was produced by fusing the scFv\textsubscript{EGFR} to the N-terminus and the scFv\textsubscript{HER2} to the C-terminus of the wild-type MHD2 (scFv\textsubscript{EGFR}-MHD2-scFv\textsubscript{HER2}). All constructs were C-terminally His\textsubscript{6}-tagged and produced in stably transfected HEK293 cells with yields between 1.5 – 2.5 mg/ml supernatant.

**Figure 3-3**: Schematic illustration of the antibody-MHD2 fusion proteins. 

A Schematic illustration of the antibody-MHD2 fusion proteins directed against EGFR and/or HER2, including the N-terminal leader sequence and C-terminal hexahistidyl (His\textsubscript{6})-tag. 

B Schematic illustration of the antibody-MHD2 fusion proteins fusing a scFv\textsubscript{EGFR} to the N-terminus and/or a scFv\textsubscript{HER2} fragment to the C-terminus of the MHD2. The MHD2 is shown in gray, N-glycans attached to the MHD2 are shown as black hexagon and the interdomain disulfide bond is indicated as black line.
The antibody-MHD2 fusion proteins were analyzed via SDS-PAGE investigating their monovalent and dimeric assembly under denatured conditions (see Figure 3-4). Under reducing conditions, the SDS-PAGE analysis of all antibody-MHD2 fusion proteins showed one band at the expected monomeric molecular mass with approximately 50 kDa for the scFv\textsubscript{EGFR}-MHD2 and MHD2-scFv\textsubscript{HER2} fusion proteins and approximately 75 kDa for the scFv\textsubscript{EGFR}-MHD2-scFv\textsubscript{HER2} fusion protein. Under non-reducing conditions, all antibody-MHD2 fusion proteins showed one band corresponding to the dimeric molecule, although another band correlating to the monomeric molecule was also revealed. Concerning the monospecific antibody-MHD2 fusion proteins, approximately 80 – 90 % of the molecules were dimerized via disulfide-linkage, while approximately 50 % of the bispecific antibody-MHD2 fusion proteins were disulfide-linked.

The melting points of the antibody-MHD2 fusion proteins were determined via dynamic light scattering (see Figure 3-5). The melting point of the scFv\textsubscript{EGFR}-MHD2 fusion protein was 61 °C, while the melting point of the MHD2-scFv\textsubscript{HER2} fusion protein was 65 °C. The bispecific scFv\textsubscript{EGFR}-MHD2-scFv\textsubscript{HER2} fusion protein has its melting point at 59 °C similar to that of the bivalent scFv\textsubscript{EGFR}-MHD2 fusion protein.

![SDS-PAGE analysis of the antibody-MHD2 fusion proteins.](image1)

![Determination of the melting points of the antibody-MHD2 fusion proteins.](image2)
The selectivity of the antibody-MHD2 fusion proteins was analyzed by ELISA using Fc fusion proteins of the extracellular region of EGFR, HER2, and HER3 (see Figure 3-6). The scFv\textsubscript{EGFR}-MHD2 fusion protein bound specifically to the EGFR-Fc fusion protein and the MHD2-scFv\textsubscript{HER2} fusion protein to the HER2-Fc fusion protein, while the bispecific scFv\textsubscript{EGFR}+MHD2-scFv\textsubscript{HER2} fusion protein showed specific binding to both EGFR-Fc and HER2-Fc fusion proteins. No binding of the antibody-MHD2 fusion proteins to the HER3-Fc fusion protein could be observed, which was applied as negative control.

![Figure 3-6: Binding of the antibody-MHD2 fusion proteins to EGFR and HER2 via ELISA. Binding of 10 μg/ml scFv\textsubscript{EGFR}-MHD2, MHD2-scFv\textsubscript{HER2}, scFv\textsubscript{EGFR}+MHD2-scFv\textsubscript{HER2} was tested on EGFR-Fc and HER2-Fc fusion protein via ELISA. HER3-Fc fusion protein was included as negative control. The antibody-MHD2 fusion proteins were detected using an HRP-conjugated anti-His\textsubscript{6}-tag antibody. An HRP-conjugated anti-human Fc antibody was used for the coating control of the Fc fusion proteins.](image)

Furthermore, the receptor binding of the monospecific and bivalent antibody-MHD2 (scFv\textsubscript{EGFR}-MHD2, MHD2-scFv\textsubscript{HER2}) fusion proteins was compared with that of the monovalent counterpart scFv\textsubscript{EGFR} and scFv\textsubscript{HER2} by ELISA (see Figure 3-7). The scFv\textsubscript{EGFR}-MHD2 fusion protein bound to EGFR-Fc with an EC\textsubscript{50} of 0.5 nM, while the binding of the scFv\textsubscript{EGFR} fragment was decreased by a factor of approximately 3 to an EC\textsubscript{50} value of 1.5 nM. Similar results were observed for the MHD2-scFv\textsubscript{HER2} fusion protein, whose binding to the HER2-Fc fusion protein was determined with 1.7 nM compared with 4.7 nM for the scFv\textsubscript{HER2} fragment, resulting in a 2.8-fold increased binding for the bivalent construct.
Results

Figure 3-7: Avidity-ELISA of the antibody-MHD2 fusion proteins binding to EGFR and HER2. Binding of titrated scF_{EGFR}-MHD2 and scF_{EGFR} or MHD2-scF_{HER2} and scF_{HER2} to the EGFR-Fc or HER2-Fc fusion protein. All fusion proteins were detected using an HRP-conjugated anti-His-6-tag antibody.

The antibody-MHD2 fusion proteins were further analyzed for binding to different cell lines expressing different amounts of EGFR and HER2 on the membrane by flow cytometry (see Figure 3-8) (see Table 3-1).

Table 3-1: Expression of EGFR and HER2 on different cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EGFR expression</th>
<th>HER2 expression</th>
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</thead>
<tbody>
<tr>
<td>A431</td>
<td>+++</td>
<td>(+)</td>
</tr>
<tr>
<td>Colo205</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>HCT116</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>HepG2</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>HT1080</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>NCI-H460</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>SKBR3</td>
<td>(+)</td>
<td>+++</td>
</tr>
</tbody>
</table>

_+ = low expression; ++ = moderate expression; +++ = high expression; - = negative; ND = not determined_

The high EGFR-expressing human epidermoid cell line A431 showed strong binding of the scF_{EGFR}-MHD2 and scF_{EGFR}-MHD2-scF_{HER2} fusion proteins, while only marginal binding to the MHD2-scF_{HER2} fusion protein was detected (see Figure 3-8). Vice versa, strong binding of the MHD2-scF_{HER2} and scF_{EGFR}-MHD2-scF_{HER2} fusion proteins was measured for the high HER2-expressing human breast adenocarcinoma cell line SKBR3, whereas only weak binding was measured for the scF_{EGFR}-MHD2 fusion protein. Only weak binding of the bivalent scF_{EGFR}-MHD2 and MHD2-scF_{HER2} fusion proteins was observed for the lung cancer cell line NCI-H460 expressing low amounts of EGFR and HER2, but the bispecific
Results

scFv\textsubscript{EGFR}-MHD2-scFv\textsubscript{HER2} fusion protein bound strongly to these cells. Similar results could be revealed for the human colon carcinoma cell line Colo205, expressing also low amounts of EGFR and HER2, which showed weak binding for the scFv\textsubscript{EGFR}-MHD2 and MHD2-scFv\textsubscript{HER2} fusion proteins but an increased binding of the scFv\textsubscript{EGFR}-MHD2-scFv\textsubscript{HER2} fusion protein.

Figure 3-8: Binding of the antibody-MHD2 fusion proteins to different cell lines via flow cytometry. Binding of titrated scFv\textsubscript{EGFR}-MHD2, MHD2-scFv\textsubscript{HER2} and scFv\textsubscript{EGFR}-MHD2-scFv\textsubscript{HER2} fusion proteins to the cell lines A431, SKBR3, NCI-H460 and Colo205 analyzed via flow cytometry. All antibody-MHD2 fusion proteins were detected via FITC-conjugated anti His\textsubscript{6}-tag antibody. These flow cytometer analyses were performed by Nadine Heidel.

3.1.3 Multivalent antibody-TNF MHD2 fusion proteins

Antibody-cytokine MHD2 fusion proteins were designed by fusing a targeting moiety and a cytokine moiety to each end of the MHD2 (see Figure 3-9). The antibody-cytokine MHD2 fusion protein scFv-MHD2-scTNF was generated by fusing the scFv\textsubscript{EGFR} fragment to the N-terminus of the MHD2 and a single-chain tumor necrosis factor (scTNF) to the C-terminus of the MHD2. A non-targeted cytokine-MHD2 fusion protein (MHD2-scTNF) was generated lacking the scFv\textsubscript{EGFR} fragment on the N-terminus. Both constructs were equipped with a His\textsubscript{6}-tag located between the MHD2 and the scTNF moiety and were produced in stably transfected HEK293 cells with yields of 5 to 17 mg/l supernatant.
Results

Figure 3-9: Schematic illustration of the cytokine-MHD2 fusion proteins. A Schematic illustration of the cytokine-MHD2 fusion proteins (MHD2-scTNF and scFvEGFR-MHD2-scTNF) including the N-terminal leader sequence and the His6-tag located between the MHD2 and the scTNF moiety. B Schematic illustration of the cytokine-MHD2 fusion proteins generated by fusing scFvEGFR to the N-terminus and/or scTNF to the C-terminus of the MHD2. The scFvEGFR is shown in white, the MHD2 in middle gray, and the scTNF molecules in dark gray. N-glycans attached to the N-terminus are shown as black hexagons and the interdomain disulfide bond is indicated as black line.

Dimeric assembly of the antibody-cytokine MHD2 fusion proteins was analyzed via SDS-PAGE analysis (see Figure 3-10). Under reducing conditions, SDS-PAGE analysis revealed single bands at 70 kDa for the MHD2-scTNF fusion protein and 100 kDa for the scFv-MHD2-scTNF fusion protein corresponding to the monomeric size. Under non-reducing conditions, dimeric assembly was detected for about 40 % of the MHD2-scTNF fusion protein and for 90 % of the scFv-MHD2-scTNF fusion protein. Serving as comparative value, scTNF and scFvEGFR-MHD2 were tested in the following experiments. SDS-PAGE analysis of scTNF showed a single band at approximately 50 kDa that corresponds to the monomeric size. The scFvEGFR-MHD2 fusion protein showed similar bands as described in 3.1.2.

Figure 3-10: SDS-PAGE analysis of the cytokine-MHD2 fusion proteins. SDS-PAGE analysis of the purified fusion proteins under reducing conditions (1-4) and non-reducing condition (5-8) (1 and 5, scTNF; 2 and 6, scFvEGFR-MHD2; 3 and 7, MHD2-scTNF; 4 and 8, scFvEGFR-MHD2-scTNF; M, protein standard marker).

Size exclusion chromatography (SEC) of the proteins was performed determining the size of the antibody-cytokine MHD2 fusion proteins under native conditions (see Figure 3-11 A). The scTNF molecule revealed a major peak at approximately 38 kDa indicating a monomeric form of this protein. The dimeric assembly of the cytokine-MHD2 fusion proteins was confirmed via SEC showing a major peak at the apparent molecular mass of approximately
180 kDa for the MHD2-scTNF fusion protein and at 230 kDa for the scFv-MHD2-scTNF fusion protein.

![Figure 3-11: Biochemical characterization of the cytokine-MHD2 fusion proteins. A Size exclusion chromatography (SEC) of scTNF, MHD2-scTNF and scFvEGFR-MHD2-scTNF. Separation was performed using a BioSep-SEC-S2000 column. B Determination of the melting points of scTNF, MHD2-scTNF and scFvEGFR-MHD2-scTNF by dynamic light scattering. The measured melting point is indicated by a dotted line.](image)

Melting points of the antibody-cytokine MHD2 fusion proteins were also determined via dynamic light scattering (see Figure 3-11 B). The melting points of the scTNF molecule and the MHD2-scTNF fusion protein were similar with 76 °C respectively 77 °C, while the bifunctional fusion protein scFvEGFR-MHD2-scTNF revealed two melting points, one at 63 °C similar as described for scFvEGFR-MHD2 and one at 77 °C, which corresponds to the melting point measured for the MHD2-scTNF fusion protein.

The binding of the cytokine-MHD2 fusion proteins to EGFR was tested via ELISA (see Figure 3-12 A). Only the targeted scFv-MHD2-scTNF fusion protein showed specific binding to an EGFR-Fc fusion protein, while there was not any binding of the MHD2-scTNF fusion protein detected lacking the targeting moiety. Additionally, there was not any binding of the cytokine-MHD2 fusion proteins to the HER2-Fc and HER3-Fc fusion proteins observed, which were included as negative controls. The coating control shows that same amounts of the different receptor-Fc fusion proteins were coated.
Figure 3-12: Binding of the cytokine-MHD2 fusion proteins to EGFR-Fc fusion protein and EGFR-expressing cell lines. A Binding of the scFvEGFR-MHD2-scTNF and MHD2-scTNF fusion proteins (10 µg/ml) was tested on coated EGFR-Fc fusion protein via ELISA. Bound cytokine-MHD2 fusion proteins were detected with HRP-conjugated anti-His<sub>6</sub> antibody. The HER2-Fc and HER3-Fc fusion proteins were included as negative control. An anti-human Fc antibody was used for the coating control. B Binding of the scFvEGFR-MHD2-scTNF fusion protein (10 µg/ml) to the cell lines A431 and HT1080 in the presence or absence of monoclonal antibodies (cetuximab, anti-EGFR; trastuzumab, anti-HER2) analyzed via flow cytometry (gray, cells alone; bold line, cells incubated with scFvEGFR-MHD2-scTNF; thin line, cells incubated with scFvEGFR-MHD2-scTNF in the presence of cetuximab or trastuzumab). The bound fusion protein was detected using a FITC-conjugated anti-His<sub>6</sub>-tag antibody.

The binding to EGFR of the scFv-MHD2-scTNF fusion protein was further tested via flow cytometry analysis using high EGFR-expressing (A431) and low EGFR-expressing cell lines (HT1080) (see Table 3-1, Figure 3-12 B). Strong binding to A431 cells could be observed, while moderate binding was detected to HT1080 cells. The binding of the scFv-MHD2-scTNF fusion protein could be blocked for both cell lines by pre-incubating the cells with an excess amount of cetuximab, which binds to the same epitope of EGFR as the scFv fragment of the fusion protein. No or only marginal blocking effects could be observed when pre-incubating the cells with trastuzumab, whose epitope is located in HER2.

Additionally, the binding of the cytokine-MHD2 fusion proteins to the TNFR1-Fc and TNFR2-Fc fusion proteins were analyzed via ELISA (see Figure 3-13). All molecules, harboring a TNF moiety, exhibited binding to TNFR1-Fc and TNFR2-Fc fusion proteins. The scFv<sup>EGFR</sup>-MHD2 fusion protein lacking the scTNF moiety did not reveal any binding neither to the TNFR1-Fc nor to the TNFR2-Fc fusion protein. There was not any binding of a fusion protein to the control HER3-Fc fusion protein observed. The coating control showed that at least the TNFR1- and TNFR2-Fc fusion proteins were coated with the same amount.
Results

Figure 3-13: Binding of the MHD2-fusion proteins to TNFR1 and TNFR2 via ELISA. Binding of 10 µg/ml scTNF, MHD2-scTNF, scFvEGFR-MHD2-scTNF and scFvEGFR-MHD2 to coated TNFR1-Fc and TNFR2-Fc fusion proteins analyzed via ELISA. The MHD2-fusion proteins and scTNF were detected using the HRP-conjugated anti-His6 antibody. HER3-Fc fusion protein was included as negative control. An HRP-conjugated anti-human Fc antibody was used for coating control.

The MHD2 fusion proteins were tested for triggering cell death on mouse embryonic fibroblasts (MEF) expressing TNFR-Fas chimeric receptors either with TNFR1 (MEF-TNFR1-Fas) or with TNFR2 (MEF-TNFR2-Fas) on the cell surface (see Figure 3-14) (Krippner-Heidenreich et al., 2002). Both receptors were fused to the transmembrane and the cytosolic part of the Fas-receptor, thus, apoptosis is induced upon activation of the TNF-receptor. TNFR1 is activated by both, soluble TNF and membrane-bound TNF (mTNF), while the activation of TNFR2 required the binding of mTNF. Using these two cell lines, cell death was induced depending on the monomeric or dimeric assembly of the MHD2 fusion proteins. A strong cell death induction on MEF-TNFR1 was shown by scTNF and MHD2-scTNF killing approximately 85 % of the cells. For the scTNF molecule the cell death induction was reduced at a concentration of 1 fM killing 60 % of the MEF-TNFR1-Fas cells, while the cell death induction of MHD2-scTNF was not reduced over the analyzed range (1 fM to 100 nM). Apoptosis on MEF-TNFR2-Fas cells was only induced by dimeric MHD2-scTNF killing approximately 65 % of the cells, while no cell death inducing effect could be observed for the monomeric scTNF construct. The scFvEGFR-MHD2 construct, which was included as negative control, lacking the scTNF moiety, showed no cell death inducing activity neither on the MEF-TNFR1-Fas cells nor on the MEF-TNFR2-Fas cells.
Results

Figure 3-14: Cell death induction assay on mouse embryonic fibroblasts (MEF) expressing TNFR-Fas chimeric receptors either TNFR1-Fas or TNFR2-Fas. Cell death induction of scFv<sub>EGFR</sub>-MHD2, scTNF and MHD2-scTNF on TNFR1-Fas and TNFR2-Fas transfected MEF cells incubated with increasing concentration for 16 hours (n=3, ± SD). Viable cells were stained with crystal violet.

The bioactivity of the cytokine-MHD2 fusion proteins was further analyzed measuring the IL-8 release of HT1080 cells upon activation of TNF receptors (see Figure 3-15 A). The scTNF molecule and the MHD2-scTNF fusion protein induced secretion of IL-8 in a concentration-dependent manner with EC<sub>50</sub> values of approximately 200 pM, whereupon the bioactivity of the MHD2-scTNF fusion protein was only slightly increased compared with that of the scTNF molecule. A strongly increased bioactivity was observed for the scFv-MHD2-scTNF fusion protein with an optimum at 20 pM. With increasing concentration of scFv-MHD2-scTNF the IL-8 release was reduced to approximately 25% of the optimum. The scFv<sub>EGFR</sub>-MHD2 construct lacking the scTNF moiety was used as negative control and showed no stimulatory effect. By pre-incubating the cells with excess amounts of cetuximab, which is directed against the same epitope as the scFv<sub>EGFR</sub> of the various MHD2 fusion proteins, the strong IL-8 release of scFv-MHD2-scTNF (at 20 pM) could be almost completely blocked to the secretion level of the non-targeted scTNF molecule and the MHD2-scTNF fusion protein (see Figure 3-15 B). Only marginal reduction of the secretion level triggered by scFv-MHD2-scTNF was detected using trastuzumab directed against an epitope of HER2. Finally, the expression of the TNF receptors of the HT1080 cells was analyzed via flow cytometry analysis. Only TNFR1 was detected on the surface of the HT1080 cells, while no signal was revealed for the TNFR2 (see Figure 3-15C).
Results

Figure 3-15: IL-8 release assay using MHD2 fusion proteins on HT1080 cells. A IL-8 secretion of HT1080 cells by incubating with increasing concentrations of scFvEGFR-MHD2-scTNF, MHD2-scTNF and scTNF (blockshift corrected). The scFvEGFR-MHD2 fusion protein was included as negative control (n=4, ± SD). B Blocking of the scFvEGFR-MHD2-scTNF induced IL-8 release at 20 pM by adding anti-EGFR antibody cetuximab (0.6 µM). Trastuzumab (anti-HER2) was included as negative control. C Expression of TNF receptor 1 (TNFR1) and 2 (TNFR2) of HT1080 analyzed via flow cytometry (gray, cells incubated with FITC-conjugated antibody; bold line, cells incubated with mouse anti-TNFR1 or anti-TNFR2 antibody and detected with FITC-conjugated anti-mouse antibody).

3.1.4 IgE heavy chain domain 2 (EHD2)

The IgE heavy chain domain 2 (EHD2) acts as the dimerization domain connecting the two heavy chains of IgE (see Figure 1-4) (Beavil et al., 1995; Wan et al., 2002). EHD2 is composed of 106 residues and possesses a molecular weight of 11.6 kDa (see Figure 3-16 A). Similar in size and homologous in amino acid sequence to MHD2 (see Figure 3-16 B), EHD2 also contains a potential N-glycosylation position at asparagine residue 275 (Asn275, EU index). In contrast to the MHD2, whose dimeric formation is stabilized by one disulfide link, the two EHD2 domains are covalently connected by two disulfide bonds formed by the cysteine residues Cys247 and Cys337 of each domain (Wan et al., 2002).

Figure 3-16: The human IgE heavy chain domain 2 (EHD2). A Sequence of the human IgE heavy chain domain 2 (EHD2) including the marked inter- and intradomain disulfide bonds (yellow) and the potential N-glycosylation site (green). B Sequence-alignment of the EHD2 with the corresponding human IgM heavy chain domain 2 (MHD2). Cysteine residues (yellow) and N-glycosylation sites (green) are marked. C Structure of the EHD2 (from pdb entry 1O0V) (Wan et al., 2002). The two domains are colored in red and blue and cysteine residues in the two domains are indicated as spheres (yellow for red domain; orange for blue domain). The potential N-glycosylation site is shown in green. Numbering of the residues according to the EU index (Kabat et al., 1991).
The EHD2 (aa 234-341) was produced in stably transfected HEK293 cells and purified via IMAC. The purified EHD2 was analyzed in SDS-PAGE investigating monomeric and dimeric assembly of the domain (see Figure 3-17). In SDS-PAGE under reducing conditions, the EHD2 revealed two bands with apparent molecular masses of 14 and 16 kDa corresponding to monomeric size (see Figure 3-17 B). Under non-reducing conditions, three bands with apparent molecular masses of 26, 38 and 45 kDa were detected, confirming the disulfide linked dimeric assembly of the EHD2. The two bands observed under reducing conditions and the three bands observed under non-reducing conditions were probably caused by diverse degrees of N-glycosylation. The dimeric assembly of the EHD2 was further confirmed by size exclusion chromatography (SEC) showing a major peak at an apparent molecular mass of approximately 49 kDa (see Figure 3-17 C). The melting point of EHD2 was 80 °C via dynamic light scattering (see Figure 3-17 D). The biochemical characterization of EHD2 was compared with those of MHD2 and IgG heavy chain domain 3 (GHD3) (see Figure 3-17 A). In SDS-PAGE analysis, the MHD2 showed two bands with apparent masses of 13 and 15 kDa under reducing conditions and three bands with apparent masses between 38 and 42 kDa under non-reducing conditions, while for GHD3 only one band with an apparent molecular mass of 12 kDa could be revealed for each condition (see Figure 3-17 B). The dimeric formation of MHD2 and GHD3 was confirmed by SEC, showing a single peak at an apparent molecular mass of 41 kDa for MHD2 and 31 kDa for GHD3 (see Figure 3-17C). For MHD2 a rather low melting point at 55 °C was determined by dynamic light scattering, while GHD3 exhibited a melting point of approximately 75 °C (see Figure 3-17D).
3.1.5 Multivalent antibody-TRAIL EHD2 fusion proteins

Since the EHD2 is connected via two disulfide bonds and exhibits the highest thermal stability, it was used for the generation of multivalent TRAIL fusion proteins (see Figure 3-18). Fusing a humanized anti-EGFR scFv fragment as targeting moiety to the N-terminus of the EHD2, a bivalent scFv-EHD2 fusion protein was generated exhibiting a His6-tag at the C-terminus for purification. Besides the targeting moiety, an effector molecule, namely the cytokine single-chain TNF-related apoptosis-inducing ligand (scTRAIL) was also fused to the C-terminus of the EHD2 resulting in EHD2-scTRAIL. Additionally, fusing a scFvEGFR fragment to the N-terminus of the EHD2 and a scTRAIL molecule to the C-terminus of the EHD2, a multivalent and bifunctional fusion protein (scFv-EHD2-scTRAIL) was generated. Both TRAIL fusion proteins (EHD2-scTRAIL and scFv-EHD2-scTRAIL) were equipped with a FLAG tag at the N-terminus for purification. All fusion proteins were produced in stably transfected
Results

HEK293 cells with yields of 7.9 mg/l supernatant for scFv-EHD2, 2.8 mg/l supernatant for EHD2-scTRAIL and 4.6 mg/l supernatant for scFv-EHD2-scTRAIL.

Figure 3-18: Schematic illustration of the various EHD2 fusion proteins. 

A Schematic illustration of the EHD2 fusion proteins with the N-terminal leader sequence and the following FLAG tag for EHD2-scTRAIL and scFvEGFR-EHD2-scTRAIL, or the C-terminal hexahistidyl tag for scFvEGFR-EHD2. 

B Schematic illustration of the EHD2 fusion proteins fusing scFvEGFR fragment to the N-terminus and/or the scTRAIL moiety to the C-terminus of the EHD2. The scFvEGFR is shown in white, the EHD2 in middle gray, and the scTRAIL molecules in dark gray. N-glycans attached to the EHD2 are shown as black hexagons and the disulfide bonds are indicated as black lines.

The EHD2 fusion proteins were analyzed by SDS-PAGE investigating their dimeric assembly under denaturing conditions (see Figure 3-19 A). SDS-PAGE analysis under reducing conditions revealed two bands with apparent molecular masses of approximately 43 and 46 kDa for the scFv-EHD2 fusion protein, while only one band with an apparent mass of 85 or 115 kDa, respectively, was observed for EHD2-scTRAIL or scFv-EHD2-scTRAIL. The two bands of scFv-EHD2 most likely belong to the glycosylated and non-glycosylated fusion protein, while the glycosylated and non-glycosylated version of EHD2-scTRAIL and scFv-EHD2-scTRAIL were represented as one band due to insufficient separation of the proteins. The N-glycosylation of the scFv-EHD2 fusion protein was confirmed by incubating the protein with PNGaseF and analyzing on SDS-PAGE under reducing conditions (see Figure 3-19 B). The deglycosylated scFv-EHD2 fusion protein revealed only one band that corresponds with the faster migrated band observed for the untreated protein. The dimeric formation of all EHD2 fusion proteins was confirmed by SDS-PAGE analysis under non-reducing conditions (see Figure 3-19 A). For scFv-EHD2 only one band was observed at approximately 90 kDa. Under non-reducing conditions, the TRAIL fusion proteins revealed multiple bands derived most likely from the scTRAIL molecule. Approximately 30 % of the EHD2-scTRAIL fusion protein and 80 % of the scFv-EHD2-scTRAIL fusion protein were assembled as dimer.
Results

Figure 3-19: SDS-PAGE analysis of the EHD2 fusion proteins. A SDS-PAGE analysis under reducing conditions (lane 1-3) and non-reducing conditions (lane 4-6) analyzing scFv-EHD2 (lane 1 and 4), EHD2-scTRAIL (lane 2 and 5), and scFv-EHD2-scTRAIL (lane 3 and 6) (M, protein standard marker). B SDS-PAGE analysis under reducing conditions investigating the deglycosylated scFv-EHD2 fusion protein incubated with N-glycosidase F (lane 1) and the untreated fusion protein (lane 2) (M, protein standard marker).

Size exclusion chromatography (SEC) of the EHD2 fusion proteins confirmed the correct assembly into dimeric molecules under native conditions (see Figure 3-20). The scFv-EHD2 fusion protein was eluted in a single peak at an apparent molecular mass of approximately 95 kDa, while the EHD2-scTRAIL molecule revealed an apparent molecular mass of approximately 140 kDa. The bifunctional fusion protein scFv-EHD2-scTRAIL showed a major peak at an apparent molecular mass of approximately 190 kDa.

Figure 3-20: Size exclusion chromatography of the EHD2 fusion proteins. The molecular masses of scFv\textsubscript{EGFR}-EHD2, EHD2-scTRAIL and scFv\textsubscript{EGFR}-EHD2-scTRAIL were measured under native conditions via size exclusion chromatography. Separation was performed using a Yarra-SEC-2000 column.

The melting points of the EHD2 fusion proteins were measured via dynamic light scattering (see Figure 3-21). The melting point of scTRAIL was 46 °C, which is identical to that of the recombinant human sTRAIL molecule (Peprotech). A slightly increased thermal stability at 50 °C was measured for the EHD2-scTRAIL fusion protein. The scFv-EHD2 had a melting point at 63 °C, which is identical to that of the monomeric counterpart scFv\textsubscript{EGFR} fragment. The bifunctional scFv-EHD2-scTRAIL fusion protein revealed two melting points, one at 50 °C
corresponding to that of the EHD2-scTRAIL fusion protein, and the other one at 66 °C corresponding to that of the scFv-EHD2 fusion protein.

![Graphs showing melting points of different proteins](image_url)

**Figure 3-21: Determination of the melting points of the EHD2 fusion proteins.** Melting points of the scFvEGFR-EHD2, EHD2-scTRAIL and scFvEGFR-EHD2-scTRAIL fusion proteins and their counterparts sTRAIL (ordered from Peprotech), scTRAIL and scFvEGFR (scFv hu225) were determined using dynamic light scattering. The measured melting point is indicated with the dotted line.

The binding functionality of the EHD2 fusion proteins was tested on an immobilized EGFR-Fc fusion protein via ELISA (see Figure 3-22). Binding of the targeted fusion proteins (scFv-EHD2, scFv-EHD2-scTRAIL) was observed to the EGFR-Fc fusion protein, while the non-targeted EHD2-scTRAIL fusion protein revealed no binding (see Figure 3-22 A). The HER2-Fc fusion protein as negative control did not show any binding to the EHD2 fusion proteins. A serial dilution of the monovalent scFvEGFR fragment and the bivalent scFvEGFR-EHD2 fusion protein was tested for binding to an immobilized EGFR-Fc fusion protein via an avidity ELISA (see Figure 3-22 B). The monomeric scFvEGFR fragment revealed an EC<sub>50</sub> value of 0.84 nM, while the bivalent scFvEGFR-EHD2 fusion protein showed an EC<sub>50</sub> value of 0.27 nM resulting in an approximately 3-fold increased binding of the scFvEGFR-EHD2 fusion protein to immobilized EGFR-Fc fusion protein compared with that of the scFvEGFR fragment.
Results

Figure 3-22: Binding of the EHD2 fusion proteins to EGFR-Fc fusion proteins via ELISA. A Binding of the scF_{EGFR}-EHD2, EHD2-scTRAIL and scF_{EGFR}-EHD2-scTRAIL fusion proteins (10 µg/ml) to the EGFR-Fc fusion protein via ELISA. HER2-Fc fusion protein was included as negative control. The scF_{EGFR}-EHD2 fusion protein was detected using an HRP-conjugated anti-His$_6$-tag antibody, while EHD2-scTRAIL and scF_{EGFR}-EHD2-scTRAIL were detected with an HRP-conjugated anti-FLAG-tag antibody. This ELISA experiment was performed by the former diploma student Aline Plappert. B Binding of the titrated scF_{EGFR}-EHD2 fusion protein to EGFR-Fc fusion protein in comparison with the counterpart scF_{EGFR} fragment. Both proteins were detected using an HRP-conjugated anti-His$_6$-tag antibody.

Furthermore, binding of the EHD2 fusion proteins to the different TRAIL receptors (TRAILR) was tested on immobilized TRAILR1-, TRAILR2-, TRAILR3- and TRAILR4-Fc fusion protein via ELISA (see Figure 3-23).

Figure 3-23: Binding of the EHD2 fusion proteins to the TRAILR-Fc fusion proteins via ELISA. Binding of the titrated scF_{EGFR}-EHD2, EHD2-scTRAIL and scF_{EGFR}-EHD2-scTRAIL fusion proteins on TRAILR1-Fc, TRAILR2-Fc, TRAILR3-Fc and TRAILR4-Fc fusion proteins via ELISA. The scF_{EGFR}-EHD2 fusion protein was detected with HRP-conjugated anti-His$_6$ antibody, while EHD2-scTRAIL and scF_{EGFR}-EHD2-scTRAIL was measured using HRP-conjugated anti-FLAG antibody.
Results

A serial dilution of the EHD2 fusion proteins revealed EC\textsubscript{50} values in the low nM range for all four TRAIL receptor-Fc fusion proteins (see Table 3-2). Both TRAIL fusion proteins bound to the TRAILR1-Fc fusion proteins with an EC\textsubscript{50} value of approximately 3 nM, while the binding of the EHD2-scTRAIL and scFv-EHD2-scTRAIL fusion proteins to the TRAILR2-Fc fusion protein revealed an EC\textsubscript{50} value of approximately 2 nM and 1 nM, respectively. The TRAIL fusion proteins bound to the TRAILR3-Fc or TRAILR4-Fc fusion proteins with approximately 1 nM or 2 nM, respectively. The scFv-EHD2 fusion protein, which was included as negative control, showed no binding to the TRAIL receptor Fc-fusion proteins.

Table 3-2: Binding of EHD2 fusion proteins to TRAIL receptors.

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<thead>
<tr>
<th>Construct</th>
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<tr>
<td>TRAILR4-Fc</td>
<td>-</td>
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<td>1.40 ± 0.01</td>
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</tbody>
</table>

Additionally, the binding of the EHD2 fusion proteins was investigated on Colo205, NCI-H460, HCT116 and HepG2 cells (see Table 3-1) via flow cytometry analysis (see Figure 3-24). Binding of the scFv-EHD2 fusion protein to the low EGFR-expressing Colo205, NCI-H460, and HCT116 cells was observed, while no or only marginal binding was detected for HepG2 cells, which can be considered as EGFR-negative (see Figure 3-24 B). Only weak binding of the EHD2-scTRAIL fusion protein was measured for Colo205, NCI-H460 and HepG2, indicating a rather low expression of TRAIL receptors (see Figure 3-24 A). Strongest binding of the EHD2-scTRAIL fusion protein was observed to the HCT116 cells. The binding of the scFv-EHD2-scTRAIL fusion protein to Colo205, NCI-H460 and HCT116 cells was stronger compared with that of the EHD2-scTRAIL fusion protein, while both fusion proteins bound equally to the target-negative cell line HepG2 was identic for both fusion proteins. Thus, the scFv-moiety as well as the TRAIL-moiety are responsible for the binding of the constructs to the cells. Vice versa the EGFR and/or TRAIL receptor of the cells are bound by the EHD2 fusion proteins. Binding of the EGFR-specific diabody(Db)-scTRAIL fusion protein to the cell lines Colo205, NCI-H460, and HepG2 was also tested, which was similar as measured for the scFv-EHD2-scTRAIL fusion protein. However, the binding of the Db-scTRAIL fusion protein to HCT116 was slightly decreased compared with that of the scFv-EHD2-scTRAIL fusion protein.
Results

Figure 3-24: Binding of the EHD2 fusion proteins to cells via flow cytometry. A Binding of the EHD2-scTRAIL, scFvEGFR-EHD2-scTRAIL and diabody(Db)-scTRAIL fusion proteins to Colo205, NCI-H460, HCT116 and HepG2 analyzed by flow cytometry. Fusion proteins were detected using mouse anti-TRAIL antibody and a FITC-conjugated anti-mouse antibody. B Binding of the scFvEGFR-EHD2 fusion proteins to Colo205, NCI-H460, HCT116 and HepG2 analyzed by flow cytometry using FITC-conjugated anti-His-tag antibody.

3.1.5.1 Cell death induction of multivalent TRAIL fusion proteins *in vitro*

TRAIL fusion proteins (EHD2-scTRAIL and scFv-EHD2-scTRAIL) were tested of the potential of inducing cell death on the EGFR-expressing Colo205, NCI-H460, and HCT116 cell lines as well as on the EGFR-negative HepG2 cell line and compared with that of the monomeric scTRAIL molecule. The cell death induction assays were performed in the absence or in the presence of the proteasome-inhibitor bortezomib, which is known to sensitize the cells for TRAIL-induced apoptosis. The EHD2-scTRAIL and the scFv-EHD2-scTRAIL fusion proteins consist of hexavalent TRAIL molecules due to dimeric assembly of two scTRAIL moieties, whereas the scTRAIL molecule consists of only trivalent TRAIL molecules. It is important to mention that indicated protein concentration refers to the monomeric molecular mass of EHD2-scTRAIL and scFv-EHD2-scTRAIL resulting in an adequate TRAIL molecule ratio compared with the scTRAIL molecule. In the absence of bortezomib, scTRAIL did not induce 50 % cell death over the analyzed concentration range from 1 pM – 10 nM. However, the hexavalent EHD2-scTRAIL fusion protein caused cell death with an EC$_{50}$ value of 220 pM for NCI-H460, 570 pM for Colo205 and 930 pM for HCT116, while only marginal cell death was observed for HepG2 even when using the highest concentration of protein. The cell death inducing effect of the targeted scFv-EHD2-scTRAIL fusion protein was further increased compared with that of the non-targeted EHD2-scTRAIL fusion protein resulting in EC$_{50}$ values of 28 pM for NCI-H460, 31 pM for Colo205 and 43 pM for HCT116. To sum up, concerning the EC$_{50}$ values, the cell death on EGFR-expressing cells induced by the scFv-EHD2-scTRAIL was increased 8-fold for NCI-H460, 18-fold for Colo205 and 21-fold for HCT116 compared with the EHD2-scTRAIL fusion protein, indicating the importance of the targeted delivery of TRAIL molecules for efficient induction of apoptosis. Similar to the cell death inducing effect of the EHD2-scTRAIL fusion protein on HepG2 cells, only marginal cell death
Results

was induced by the scFv-EHD2-scTRAIL fusion protein even at the highest concentration of used fusion protein.

Figure 3-25: Cell death induction assay of the EHD2 fusion proteins in the absence of bortezomib. *In vitro* cell death induction of the EHD2-scTRAIL and the scFvEGFR-EHD2-scTRAIL fusion proteins in comparison with the scTRAIL molecule. Cell death induction was analyzed on NCI-H460, Colo205, HCT116 and HepG2 cell lines in the absence of bortezomib. Cells were incubated 16 hours with the fusion proteins and viable cells were measured using crystal violet staining (n=3, ± SD). Concentrations of EHD2-scTRAIL and scFvEGFR-EHD2-scTRAIL refer to their monomeric mass.

In the presence of bortezomib, the dose response for all TRAIL fusion proteins was left-shifted, showing the sensitization of cells for TRAIL-induced apoptosis. The scTRAIL molecule caused cell death with *EC$_{50}$* values of 270 pM for NCI-H460, 3.47 nM for Colo205 and 97 pM for HCT116, while almost 50 % cell death was measured for HepG2 at the highest concentration at 10 nM. The bivalent EHD2-scTRAIL fusion protein showed an increased cell death inducing effect with 22 pM for NCI-H460, 140 pM for Colo205, 8 pM for HCT116 and 476 pM for HepG2 compared with that of the scTRAIL molecule. Again, the strongest cell death induction was observed for the targeted scFv-EHD2-scTRAIL fusion protein with 5 pM for NCI-H460, 14 pM for Colo205 and 2 pM for HCT116. Compared with the EHD2-scTRAIL fusion protein, the multivalent scFv-EHD2-scTRAIL showed an increased cell death inducing effect on EGFR-expressing cell lines by the factor of 4 for NCI-H460, of 10 for Colo205 and of 4 for HCT116 in the presence of bortezomib. The differences between the *EC$_{50}$* values of scTRAIL, EHD2-scTRAIL and scFv-EHD2-scTRAIL within one cell line treated with (see Figure 3-26) or without (see Figure 3-25) bortezomib were statistically significant. The cell death inducing effect of the targeted scFv-EHD2-scTRAIL fusion protein
on the EGFR-negative cell line HepG2 with an EC$_{50}$ value of 337 pM showed only marginal increase of cell death induction compared with the non-targeted EHD2-scTRAIL (1.4-fold).

Figure 3-26: Cell death induction assay of the EHD2 fusion proteins in the presence of bortezomib (250 ng/ml). *In vitro* cell death induction of the EHD2-scTRAIL and the scFv$_{\text{EGFR}}$-EHD2-scTRAIL fusion proteins in comparison with the scTRAIL molecule. Cell death induction was analyzed on NCI-H460, Colo205, HCT116 and HepG2 cell lines in the presence (250 ng/ml) of bortezomib. Cells were incubated 16 hours with the fusion proteins and viable cells were measured using crystal violet staining (n=3, ± SD). Concentrations of EHD2-scTRAIL and scFv$_{\text{EGFR}}$-EHD2-scTRAIL refer to their monomeric mass.

Investigating the benefit from the scFv-mediated targeting for cell death induction, the cell death induction assays were repeated with the EHD2-scTRAIL and scFv-EHD2-scTRAIL fusion proteins on the cell lines NCI-H460 and Colo205 in the absence or the presence of bortezomib adding 200-fold molar excess of cetuximab (see Figure 3-27). The EC$_{50}$ values of the non-targeted EHD2-scTRAIL with 280 pM or 14 pM for NCI-H460 and 530 pM or 160 pM for Colo205 in the absence or the presence of bortezomib, respectively, were hardly influenced by the addition of cetuximab. However, the addition of cetuximab decreased the cell death induction of the scFv-EHD2-scTRAIL fusion protein with EC$_{50}$ values of 240 pM or 9 pM for NCI-H460 and 610 pM and 156 pM for Colo205 in the absence or the presence of bortezomib, respectively, assimilating to the EC$_{50}$ values observed for the non-targeted EHD2-scTRAIL fusion protein. The differences of the EC$_{50}$ values between the EHD2-scTRAIL fusion protein and the scFv-EHD2-scTRAIL fusion protein were no longer statistically significant when cells were treated with cetuximab.
Figure 3-27: Cell death induction assay of the EHD2 fusion proteins in the presence of cetuximab. In vitro cell death induction of the EHD2-scTRAIL and the scFVEGFR-EHD2-scTRAIL fusion proteins in comparison the scTRAIL molecule on NCI-H460 and Colo205 in the absence (w/o) or the presence (250 ng/ml) of bortezomib and by adding cetuximab (anti-EGFR, 200-fold molar excess). Cells were pre-incubated 30 minutes with cetuximab and subsequently incubated 16 hours with the fusion proteins. Viable cells were measured using crystal violet staining (n=3, ± SD). Concentrations of EHD2-scTRAIL and scFVEGFR-EHD2-scTRAIL refer to their monomeric mass.

In addition, the cell death inducing effect of both cetuximab only and of the scFv-EHD2 fusion protein only was also tested on NCI-H460 and Colo205 cells in the absence or the presence of bortezomib (see Figure 3-28). Neither for cetuximab nor for the scFv-EHD2 fusion protein any cell death induction could be observed on both cell lines. The EC$_{50}$ values of the in vitro cell death induction assays incubating the EHD2 fusion proteins on NCI-H460, Colo205, HCT116, and HepG2 are listed in Table 3-3.
Results

Figure 3-28: Cell death induction assay of cetuximab and of the scFvEGFR-EHD2 fusion protein in the absence or the presence of bortezomib. In vitro cell death induction of cetuximab (anti-EGFR) and the scFvEGFR-EHD2 fusion protein on NCI-H460 and Colo205 in the absence (w/o) or the presence (250 ng/ml) of bortezomib. Cells were incubated 16 hours with the fusion proteins and viable cells were measured using crystal violet staining (n=3, ± SD).

Table 3-3: In vitro cell death induction of the EHD2 fusion proteins.

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>Bortezomib</th>
<th>Cetuximab</th>
<th>EC50 (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NCI-H460</td>
<td>Colo205</td>
<td>HCT116</td>
</tr>
<tr>
<td>scTRAIL</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>270 ± 40</td>
</tr>
<tr>
<td>scFv-EHD2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>EHD2-scTRAIL</td>
<td>-</td>
<td>-</td>
<td>220 ± 90</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>280 ± 10</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>22 ± 6</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>14 ± 2</td>
</tr>
</tbody>
</table>
Results

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>Bortezomib</th>
<th>Cetuximab</th>
<th>EC\textsubscript{50} (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NCI-H460</td>
</tr>
<tr>
<td>scFv-EHD2-scTRAIL</td>
<td>-</td>
<td>-</td>
<td>28 ± 2</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>240 ± 50</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>5 ± 1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>9 ± 0</td>
</tr>
</tbody>
</table>

*Bortezomib: +, 250ng/ml; -, without; Cetuximab: +, 200-fold molar excess; -, without; EC\textsubscript{50} values: ND, not determined; -, EC\textsubscript{50} could not be measured*

Furthermore, the induction of the bifunctional scFv-EHD2-scTRAIL fusion protein was compared with that of another bifunctional protein, namely the scFv-scTRAIL fusion protein. Both proteins are composed of a targeting (scFv) moiety and an effector (scTRAIL) moiety. However, in case of the scFv-scTRAIL fusion protein, each moiety is present in a monomeric form, while each moiety is dimeric regarding the scFv-EHD2-scTRAIL fusion protein. It is important to mention that indicated protein concentration refers to the monomeric molecular mass of the scFv-EHD2-scTRAIL fusion protein resulting in an adequate TRAIL molecule ratio compared with the scFv-scTRAIL fusion protein. Cell death induction assays were performed incubating the scFv-scTRAIL and the scFv-EHD2-scTRAIL fusion proteins on the cell lines NCI-H460 and Colo205 in the absence or the presence of bortezomib (see Figure 3-29). In the absence of bortezomib, the scFv-EHD2-scTRAIL fusion protein induced cell death with EC\textsubscript{50} values of 51 pM for NCI-H460 and 52 pM for Colo205, while for the scFv-scTRAIL fusion protein 50 % cell death was only reached for NCI-H460 at a concentration of approximately 1 nM. In the presence of bortezomib, the induction of cell death for both fusion proteins was increased resulting in EC\textsubscript{50} values of the scFv-scTRAIL fusion protein of 17 pM for NCI-H460 and 146 pM for Colo205 and of the scFv-EHD2-scTRAIL fusion protein of 6 pM for NCI-H460 and 26 pM for Colo205. The EC\textsubscript{50} values of the scFv-scTRAIL and the scFv-EHD2-scTRAIL fusion protein, treating the cells either with or without bortezomib, were significantly different ($p < 0.01$). The EC\textsubscript{50} values of the \textit{in vitro} cell death induction assays using the scFv-scTRAIL and the scFv-EHD2-scTRAIL fusion protein are listed in Table 3-4.
Results

Figure 3-29: Cell death induction assay of the scFvEGFR-EHD2-scTRAIL fusion protein in comparison with the scFvEGFR-scTRAIL fusion protein. In vitro cell death induction of the scFvEGFR-scTRAIL and the scFvEGFR-EHD2-scTRAIL fusion proteins. Cell death induction was analyzed on NCI-H460 and Colo205 cell lines in the absence (w/o) or the presence (250 ng/ml) of bortezomib. Cells were incubated 16 hours with the fusion proteins and viable cells were measured using crystal violet staining (*n=3, ± SD*). Concentrations of scFvEGFR-EHD2-scTRAIL refer to its monomeric mass.

Table 3-4: *In vitro* cell death induction of scFvEGFR-scTRAIL in comparison with scFvEGFR-EHD2-scTRAIL.

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>Bortezomib</th>
<th>EC_{50} (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NCI-H460</td>
</tr>
<tr>
<td>scFvEGFR-scTRAIL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>scFvEGFR-EHD2-scTRAIL</td>
<td>-</td>
<td>52 ± 17</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6 ± 1</td>
</tr>
</tbody>
</table>

*Bortezomib: +, 250ng/ml; -, without; EC_{50} values: -, EC_{50} could not be measured*

Finally, the induction of apoptosis of two bifunctional fusion proteins, namely the scFv-EHD2-scTRAIL and diabody(Db)-scTRAIL fusion proteins, were compared. Both moieties of each fusion protein are dimeric. The targeting moiety of the Db-scTRAIL fusion protein is directed against the identical epitope as the targeting moiety of the scFv-EHD2-scTRAIL fusion protein. It is important to mention that indicated protein concentration refers to the monomeric molecular mass of each fusion protein enabling a simple comparison with the other cell death induction assays. The cell death induction assays were performed using Db-scTRAIL and scFv-EHD2-scTRAIL on the cell lines NCI-H460, Colo205 and HCT116 in the absence or the
presence of bortezomib (see Figure 3-30). In the absence of bortezomib, scFv-EHD2-scTRAIL induced apoptosis with EC$_{50}$ values of 74 pM for NCI-H460, 28 pM for Colo205 and 48 pM for HCT116, while Db-scTRAIL caused cell death with EC$_{50}$ values of 360 pM for NCI-H460, 120 pM for Colo205 and 200 pM for HCT116. Again, the addition of bortezomib increased the cell death induction of the fusion proteins. The cell death inducing effect of scFv-EHD2-scTRAIL was observed with EC$_{50}$ values of 4 pM for NCI-H460, 8 pM for Colo205 and 2 pM for HCT116, while cell death of Db-scTRAIL was measured with 9 pM for NCI-H460, 30 pM for Colo205 and 4 pM for HCT116. All differences of the EC$_{50}$ values comparing the bioactivity of the scFv-EHD2-scTRAIL fusion protein with that of the Db-scTRAIL fusion protein were not statistically significant for the NCI-H460 and Colo205 cells. A significant difference was calculated for the HCT116 cells in the absence of bortezomib (p=0.02) and in the presence of bortezomib (p=0.03). The EC$_{50}$ values of the *in vitro* cell death induction assays incubating the scFv-EHD2-scTRAIL and the Db-scTRAIL fusion protein on NCI-H460, Colo205, and HCT116 cells are listed in Table 3-5.
In vitro cell death induction of the diabody(\text{Db})_{\text{EGFR}}-\text{scTRAIL} and the scFv_{\text{EGFR}}-\text{EHD2-scTRAIL} fusion protein. Cell death induction was analyzed on NCI-H460, Colo205 and HCT116 cell lines in the absence or the presence (250 ng/ml) of bortezomib. Cells were incubated 16 hours with the fusion proteins and viable cells were measured using crystal violet staining (n=3, ± SD).

Table 3-5: In vitro cell death induction of the scFv-EHD2-scTRAIL fusion protein in comparison with the diabody(\text{Db})_{\text{EGFR}}-\text{scTRAIL} fusion protein.

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>Bortezomib</th>
<th>NCI-H460 (pM)</th>
<th>Colo205 (pM)</th>
<th>HCT116 (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\text{Db}_{\text{EGFR}}-\text{scTRAIL}</td>
<td>-</td>
<td>360 ± 380</td>
<td>120 ± 80</td>
<td>200 ± 50</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>9 ± 2</td>
<td>30 ± 20</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>scFv_{\text{EGFR}}-\text{EHD2-scTRAIL}</td>
<td>-</td>
<td>74 ± 75</td>
<td>28 ± 13</td>
<td>48 ± 46</td>
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<tr>
<td></td>
<td>+</td>
<td>4 ± 3</td>
<td>8 ± 3</td>
<td>2 ± 0.2</td>
</tr>
</tbody>
</table>

*Bortezomib: +, 250ng/ml; -, without; \text{EC}_{50} values: -, \text{EC}_{50} could not be measured*
3.1.5.2 In vivo studies of multivalent TRAIL fusion proteins

For the determination of the half-life properties of the EHD2 fusion proteins, SWISS mice received a single i.v. injection of 25 µg of the scFv-EHD2, EHD2-scTRAIL, scFv-EHD2-scTRAIL, diabody(Db)-scTRAIL fusion proteins or the scTRAIL molecule (see Figure 3-31). The three EHD2 fusion proteins exhibited prolonged pharmacokinetic properties with terminal half-lives ($t_{1/2}$) of 9.4 hours for the scFv-EHD2, 6.5 hours for the EHD2-scTRAIL and 7.2 hours for the scFv-EHD2-scTRAIL fusion proteins compared with the monomeric scTRAIL molecule with $t_{1/2}$ of 3.0 hours. Thus, the half-lives of the EHD2-scTRAIL and scFv-EHD2-scTRAIL fusion proteins were increased 2-fold, and of the scFv-EHD2 fusion protein 3-fold. The bifunctional Db-scTRAIL fusion protein revealed a terminal half-life of 3.5 hours. The area under the curve (AUC; %·hour) in the range from 0 to 24 hours, which describes the bioavailability of the respective construct in the blood for 24 hours, was calculated with 127 %·hour for the scTRAIL molecule. The AUC of scFv-EHD2 (358 %·hour), EHD2-scTRAIL (388 %·hour) and Db-scTRAIL (326 %·hour) was increased by the factor of approximately 3 compared with that of the scTRAIL molecule, while the AUC of the bifunctional scFv-EHD2-scTRAIL fusion protein (508 %·hour) was increased 4-fold. Differences of half-lives and AUC between scTRAIL and the EHD2 fusion proteins were statistically significant, whereas differences of the values of half-lives and AUC of the fusion proteins were not statistically significant.

The pharmacokinetic properties of the EHD2 fusion proteins, scTRAIL molecule and the Db-scTRAIL fusion protein are listed in Table 3-6.
Results

Table 3-6: Pharmacokinetic properties of the EHD2 fusion proteins.

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>$M_r$ (kDa)</th>
<th>$S_r$ (nm)</th>
<th>$t_{1/2}$ (h)</th>
<th>AUC$_{0\text{-}24\text{hours}}$ (%$\cdot$h)</th>
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</thead>
<tbody>
<tr>
<td>scTRAIL</td>
<td>67.5</td>
<td>3.3</td>
<td>3.0 ± 0.3</td>
<td>127 ± 30</td>
</tr>
<tr>
<td>scFv-EHD2</td>
<td>82.8</td>
<td>4.4</td>
<td>9.4 ± 0.5</td>
<td>358 ± 89</td>
</tr>
<tr>
<td>EHD2-scTRAIL</td>
<td>164.6</td>
<td>4.6</td>
<td>6.5 ± 0.4</td>
<td>388 ± 135</td>
</tr>
<tr>
<td>scFv-EHD2-scTRAIL</td>
<td>218.6</td>
<td>5.1</td>
<td>7.2 ± 0.9</td>
<td>508 ± 112</td>
</tr>
<tr>
<td>Db-scTRAIL</td>
<td>187.0</td>
<td>5.0</td>
<td>3.5 ± 0.3</td>
<td>326 ± 29</td>
</tr>
</tbody>
</table>

$M_r$, molecular mass; $S_r$, stokes-radius; $t_{1/2}$, terminal half-life; AUC$_{0\text{-}24\text{hours}}$, area under the curve of first 24 hours

Furthermore, the antitumor activity of the EHD2 fusion proteins was tested in nude mice bearing subcutaneous EGFR-expressing Colo205 tumors (see Figure 3-32). Investigating the antitumor effect of scTRAIL and of the non-targeted and targeted multivalent TRAIL molecules, mice received four i.v. injections of scTRAIL, EHD2-scTRAIL or scFv-EHD2-scTRAIL every fourth day (see Figure 3-32 A). The treatment with the fusion proteins was initiated when tumors reached a volume of approximately 100 mm$^3$ (day 7 after inoculation). Doses with 0.70 nmol of scTRAIL and 0.35 nmol of EHD2-scTRAIL and scFv-EHD2-scTRAIL were administered and bortezomib was injected i.p. every second day starting at day 7 over a period of 12 days. The control group also received the i.p. injections of bortezomib, but PBS was i.v. injected instead of the fusion protein. The treatment with scTRAIL showed no antitumor activity when compared with the control group. The treatment with EHD2-scTRAIL revealed only marginal effects. In contrast, the bifunctional scFv-EHD2-scTRAIL fusion protein showed strong antitumor activity resulting in partial remission of the tumor volume shortly after initiation of treatment. However, a regrowth of the tumor was observed on day 13 during the period of treatment. Nevertheless, the scFv-EHD2-scTRAIL treated Colo205 bearing mice benefited compared with the control group, with the scTRAIL-treated ones and with the EHD2-scTRAIL-treated ones resulting in significant differences of tumor volume, e.g. on day 19 (see Figure 3-32 B).
Results

Figure 3-32: In vivo antitumor activity of the EHD2-scTRAIL and scFv-EHD2-scTRAIL fusion proteins in comparison with scTRAIL. A Colo205-bearing nude mice received four intravenous injections of the proteins (0.70 nmol of scTRAIL and 0.35 nmol of EHD2-scTRAIL and scFvEGFR-EHD2-scTRAIL) every fourth day represented by an arrow as well as eight intraperitoneal injections of bortezomib (Brt; 5 µg per injection) every second day displayed by an asterix (n=11 tumors for Brt-treated group and 12 tumors for other groups, ± 95%CI). B Tumor volumes of the different treated groups at day 19. [*p<0.05; **p<0.01; ***p<0.001]

In a second experiment, the treatment of the scFv-EHD2-scTRAIL fusion protein was compared with the treatment of the scFv-EHD2 fusion protein investigating the contribution of EGFR-blocking on the therapeutic activity of the fusion proteins (see Figure 3-33). Obtaining only partial remission in the former in vivo studies by treating mice with 0.35 nmol scFv-EHD2-scTRAIL every fourth day, this experiment was modified in applying doses of 1 nmol of the fusion proteins every second day (see Figure 3-33 A). Again, bortezomib was administered as i.p. injection every second day during the treatment with the fusion protein. This was initiated when the tumor had a volume of approximately 100 mm³ (day 8 after tumor inoculation). The control group received the i.p. injections of bortezomib, but i.v. injected PBS instead of the fusion proteins. A superior antitumor activity was observed for the bifunctional scFv-EHD2-scTRAIL fusion protein showing macroscopically complete remission, while the scFv-EHD2 fusion protein showed no additional antitumor effect compared with that of the control group. On day 21, only one of twelve tumors started to regrow in the scFv-EHD2-scTRAIL treated group, while no differences were observed between the scFv-EHD2 treated group and the control group (see Figure 3-33 B). Regrowth of the tumors was observed starting on day 27, while one of the twelve tumors was still not detectable macroscopically at the end of the experiment on day 43.
Results

Figure 3-33: In vivo antitumor activity of the scFvEGFR-EHD2-scTRAIL fusion protein in comparison with scFvEGFR-EHD2. A Colo205-bearing nude mice received four intravenous injections of the fusion proteins (1.0 nmol) every other day represented by an arrow as well as four intraperitoneal injections of bortezomib (Brt; 5 µg per injection) every second day displayed by an asterix (n=12 tumors per group, ± 95%CI). B Tumor volumes of the different treated groups at day 21. [**p<0.01]

Finally, the in vivo tolerability of the scFv-EHD2-scTRAIL fusion protein was investigated measuring the alanine aminotransferase (ALT) activity, which correlates with liver toxicity, after a single i.v. injection of the fusion protein in combination with an i.p. injection of bortezomib. The ALT activity of blood samples taken after 4 and 24 hours was compared with that of a control group receiving only an i.v. injection of PBS. Both groups, the scFv-EHD2-scTRAIL treated and the control group revealed similar values of the ALT activity with approximately 35 U/L lying below the threshold of 50 U/L, which was defined for liver toxicity (see Figure 3-34).

Figure 3-34: Liver toxicity of the scFv-EHD2-scTRAIL fusion protein. Activity of alanine aminotransferase (ALT) was measured 4 and 24 hours after injection of PBS or scFvEGFR-EHD2-scTRAIL (i.v.; 1 nmol) in combination with bortezomib (i.p.; 5 µg). Threshold was defined at 50 (U/L) indicating liver toxicity (n=3, ± 95%CI).
3.2 Targeted delivery of multivalent TRAIL-functionalized liposomes

As described in 3.1.5, the antibody-scTRAIL fusion proteins exhibiting the delivery of multivalent TRAIL molecules are more potent in induction of apoptosis in \textit{in vitro} (3.1.5.1) and \textit{in vivo} (3.1.5.2) assays compared with the scTRAIL molecules displaying only trivalent TRAIL molecules. These properties are ascribed on the one hand to an increased targeting effect, due to bivalent antigen binding, and on the other hand to an efficient activation of TRAIL receptor 1 and 2, due to multivalent binding of TRAIL to the receptors mimicking membrane TRAIL (mTRAIL). Liposomal nanocarrier systems exhibiting a prolonged half-life in blood were used modifying the liposomal surface. The functionalized liposomes were generated by coupling cysteine-modified EGFR-specific scFv’ fragments and cysteine-modified TRAIL molecules to the surface of PEGylated liposomes exhibiting targeted delivery and the potential to mimic mTRAIL.

3.2.1 Biological activity of scFv’ and Cys-sTRAIL

For the site-directed coupling of the proteins to the liposomal surface, the proteins were modified with an additional cysteine residue providing a reactive sulfhydryl group (see Figure 3-35 A). For this purpose, the scFv\textsubscript{EGFR} fragment was modified with an additional cysteine at the C-terminus for coupling to a maleimide group. The His\textsubscript{6}-tagged scFv’ fragment was expressed in the periplasm of bacteria and purified by IMAC with yields of 0.5 mg/l cell suspension. The soluble TRAIL (sTRAIL) derivative (aa 114-281) was modified with a cysteine residue at the N-terminus for site-directed conjugation, thus one homotrimeric TRAIL molecule exhibits three reactive sulfhydryl groups. The FLAG-tagged Cys-sTRAIL molecule was expressed in the cytosol of bacteria and purified via FLAG affinity chromatography with yields of 0.8 mg/l cell suspension. In SDS-PAGE analysis under reducing conditions one band with an apparent molecular mass of approximately 29 kDa for the scFv’ fragment and 20 kDa for the Cys-sTRAIL molecule was observed corresponding to monomeric size (see Figure 3-35 B). Under non-reducing conditions, each protein revealed two bands, one faster migrating band corresponding to the monomeric size, and the other with an apparent molecular mass of approximately 57 kDa for the scFv’ fragment and 43 kDa for the Cys-sTRAIL molecule. Approximately 20 % of the proteins were existent as dimers under non-reducing conditions.
Figure 3-35: Biochemical investigation of cysteine-modified proteins for the functionalization of liposomes. A Schematic illustration of the scFv'EGFR (scFv'hu225) fragment including the N-terminal leader sequence (L) and C-terminal hexahistidyl tag (His6) followed by the additional cysteine residue (SH). The sTRAIL molecule is also illustrated, including the N-terminal located cysteine residue (SH) and FLAG-tag. B SDS-PAGE analysis under reducing conditions (1) and non-reducing conditions (2) of the scFv'EGFR fragment and the Cys-sTRAIL molecule (M, protein standard marker). C Size exclusion chromatography of scFv'EGFR fragment and Cys-sTRAIL molecule. The elution time and the molecular masses of the standard proteins are indicated. Concerning Cys-sTRAIL, the peak at 1 kDa correlates to FLAG peptide, which was used for protein elution. D Size exclusion chromatography of scFv'EGFR fragment and Cys-sTRAIL molecule in combination with the reduction agent TCEP. The elution time and the molecular masses of the standard proteins are indicated. Concerning Cys-sTRAIL, the peak at 1 kDa correlates to FLAG peptide, which was used for protein elution. E Determination of the melting points of scFv'EGFR fragment and Cys-sTRAIL molecule by dynamic light scattering. The determined melting point is indicated by the dashed line.

Size exclusion chromatography (SEC) showed one peak with a molecular mass of approximately 40 kDa for the scFv' fragment corresponding most likely to the disulfide-linked dimer (see Figure 3-35 C). By incubating the scFv' fragment with a reducing agent (TCEP), SEC revealed two major peaks, the first peak corresponding to the dimeric assembly as observed for the non-reduced sample, and the other peak at a molecular mass of approximately 22 kDa according to the monomeric protein (see Figure 3-35 D). Cys-sTRAIL revealed three peaks, corresponding to the FLAG-peptide at 1 kDa, to the trimeric molecule at 65 kDa and most likely to higher ordered TRAIL molecules of approximately 190 kDa (see Figure 3-35 C). Reducing the disulfide bonds by adding TCEP, SEC revealed only two bands at molecular masses of 1 kDa corresponding to FLAG-peptide and approximately at 65 kDa according to trimeric TRAIL molecules (see Figure 3-35 D). Melting points were determined at 57 °C for the scFv' fragment and at 45 °C for the Cys-sTRAIL molecule by dynamic light scattering (see Figure 3-35 E). These melting points helped to define the temperature for the post-insertion performed at 42 °C preventing denaturation of the proteins.

The bioactivity of the scFv' fragment and the Cys-sTRAIL molecule might be interfered by the insertion of an additional cysteine. In order to test whether the bioactivity of the cysteine-modified proteins is retained, the scFv' fragment was tested for binding to EGFR and for cell death induction of the Cys-sTRAIL molecule. The binding of the scFv'EGFR' fragment to the
Results

EGFR-positive cell line NCI-H460 (see Table 3-1) was tested via flow cytometry. Binding of the scFv\textsubscript{EGFR} to the EGFR-expressing NCI-H460 cells could be observed (see Figure 3-36 A). The in vitro induction of cell death of the Cys-sTRAIL molecule was tested on Colo205 cells and compared with the unmodified sTRAIL molecule in the absence or presence of the proteasome-inhibitor bortezomib, which is known to sensitize the cells to TRAIL-induced apoptosis (see Figure 3-36 B). In the absence of bortezomib, the Cys-sTRAIL molecule showed an increased induction of apoptosis with an EC\textsubscript{50} value of 0.14 nM, the sTRAIL molecule revealed an EC\textsubscript{50} value of 1.14 nM. In the presence of bortezomib, the bioactivity of the proteins was increased resulting in EC\textsubscript{50} values of 0.06 nM for the Cys-sTRAIL molecule and of 0.29 nM for the sTRAIL molecule. Thus, the EC\textsubscript{50} values of the modified Cys-sTRAIL molecule were slightly increased by the factor of 5 and 8 compared with the unmodified sTRAIL protein, probably due to the disulfide-linked multimers of Cys-sTRAIL.

![Figure 3-36: Functionality of the cysteine-modified proteins scFv\textsuperscript{EGFR} and Cys-sTRAIL. A Binding of the scFv\textsuperscript{EGFR} (10 µg/ml) fragment to the EGFR-positive cell line NCI-H460 was analyzed via flow cytometry (gray, cells alone; bold line, cells incubated with scFv\textsuperscript{EGFR} and detected with FITC-conjugated anti-His\textsubscript{6} antibody; thin line, cells incubated with FITC-conjugated anti-His\textsubscript{6} antibody). B In vitro cell death induction of the Cys-sTRAIL molecule in comparison with the sTRAIL (Peprotech) molecule. Cell death induction was analyzed on Colo205 cell line in the absence (w/o) or the presence (250 ng/ml) of bortezomib. Cells were incubated 16 hours with the proteins and viable cells were measured using crystal violet staining (n=2, ± SD).](image)

3.2.2 Preparation of functionalized liposomes

The cysteine-modified proteins were coupled to the maleimide conjugated PEGylated phospholipids (DSPE-PEG\textsubscript{2000}-Mal). The coupling of the proteins to the DSPE-PEG\textsubscript{2000}-Mal lipid could be analyzed via SDS-PAGE, due to the shift of mobility of the coupled proteins (see Figure 3-37). SDS-PAGE analysis revealed the conjugation of the scFv\textsubscript{EGFR} to DSPE-PEG\textsubscript{2000}-Mal with an efficiency of approximately 60 %, while the coupling of the Cys-sTRAIL molecule to DSPE-PEG\textsubscript{2000}-Mal showed an efficiency of approximately 70 %.
Results

Figure 3-37: SDS-PAGE analysis showing the coupling efficiency of the scFv'_{EGFR} fragment and the Cys-sTRAIL molecule to DSPE-PEG\textsubscript{2000}-Mal. SDS-PAGE analysis under reducing conditions of the scFv'_{EGFR} fragment (lanes 1 and 2) and the Cys-sTRAIL molecule (lanes 3 and 4) before (lanes 1 and 3) and after (lanes 2 and 4) coupling to DSPE-PEG\textsubscript{2000}-Mal (M, protein standard marker).

For further investigation about the functionalization of the liposomes, the impact of the amount of PEG in the pre-formed liposomes was observed concerning the efficiency of incorporating scFv'_{EGFR}-coupled micelles and the binding of the generated immunoliposomes to an EGFR-expressing cell line (see Figure 3-38). Firstly, the influence of the PEG level on the pre-formed liposomes was examined. For this purpose, it had to be checked whether there is a sufficient incorporation of the micelles into the membrane of the liposome during the post-insertion step (42 °C, 2h). Constant amounts of 0.1 mol% scFv'-coupled DSPE-PEG\textsubscript{2000}-Mal were inserted into PEGylated liposomes comprising different levels of PEGylation (1.5, 2.5, 5.0, 7.5 and 10 mol% DSPE-mPEG) and the incorporation was tested by Western Blot (see Figure 3-38 A). Micelle-coupled scFv’ fragments were detected in the pre-formed liposomes composed of 1.5 and 2.5 mol%, while only a marginal signal was observed for the 5.0 mol% PEGylation. There were not any signals observed for the liposomes containing 7.5 and 10 mol% PEGylation corresponding to the scFv’-coupled micelles.

Figure 3-38: Efficiency of the post-insertion step using different formulations of PEGylated liposomes. A Immunoblot analysis of 200 nmol immunoliposomes composed of 1.5 mol% (lane 1), 2.5 mol% (lane 2), 5.0 mol% (lane 3), 7.5 mol% (lane 4), or 10.0 mol% (lane 5) DSPE-mPEG, which were modified with 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-scFv'_{EGFR} micelles. Post-insertion step was performed at 42 °C for 2 hours. Non-targeted liposomes containing 5.0 mol% DSPE-mPEG were included as negative control (lane 6). Uncoupled scFv'_{EGFR} fragment (1 µg) was included as control, showing potential non-coupled proteins in the liposomal preparations (lane 7). Detection of scFv'_{EGFR} fragments was performed via HRP-conjugated anti-His\textsubscript{6} tag antibody. B Binding of the immunoliposomes (IL) composed of different PEGylated liposomes (1.5 mol%, 2.5 mol%, 5.0 mol%, 7.5 mol%, and 10.0 mol% DSPE-mPEG), which were modified with 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-scFv'_{EGFR}, to NCI-H460 cells analyzed by flow cytometry. Immunoliposomes were incubated with the cells at 37 °C for 1 hour. Non-targeted liposomes (nt) composed of 5.0 mol% DSPE-mPEG were included as negative control. Cells and functionalized liposomes were incubated at 4 °C for one hour. Dil-labeled liposomes were measured in FL2.
These prepared anti-EGFR immunoliposomes were tested of binding to EGFR-positive NCI-H460 cells at 37 °C. Analogously to the results of the Western Blot, binding of immunoliposomes composed of low level of DSPE-mPEG (1.5 mol%, 2.5 mol%, and 5.0 mol%) to the cells was observed, while the high level PEGylated immunoliposomes (7.5 mol% and 10.0 mol%) revealed no binding to the cells (see Figure 3-38 B). For the generation of functionalized liposomes exhibiting a sufficient incorporation of the protein-coupled micelles and showing a maximal stealth effect regarding prolonged half-life properties, 2.5 mol% PEGylated preformed liposomes were utilized.

### 3.2.3 Binding studies of immunoliposomes

The best formulation of incorporated scFv'EGFR-coupled micelles in the pre-formed liposomes providing maximal binding properties was investigated (see Figure 3-39). Different amounts of scFv'EGFR-coupled micelles were incorporated into preformed liposomes. Subsequently, they were tested for binding to EGFR-positive NCI-H460 and Colo205 cell lines via flow cytometry. A saturation curve was observed including a rapid increasement of binding at low amounts of lipids and a plateau effect at higher amounts of liposomes. The formulation incorporating 0.10 and 0.30 mol% of scFv'EGFR-coupled micelles revealed best binding at low amounts of liposomes for both cell lines, while at higher lipid concentrations the formulation with 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-scFv'\textsubscript{EGFR} showed best binding properties. Worst binding was observed for the formulation inserting the lowest amount of scFv'EGFR-coupled micelles, namely the 0.03 mol% formulation. The non-targeted liposomes (nt-liposomes) modified with 0.30 mol% DSPE-PEG\textsubscript{2000}-Mal-cysteine were included as negative control and showed no or only marginal binding at the highest concentration of lipids. Consequently, the amount of incorporated scFv'EGFR-coupled micelles with best binding properties was defined as 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-scFv'EGFR.

![Figure 3-39: Binding of immunoliposomes to cells via flow cytometry analysis.](image)

Figure 3-39: Binding of immunoliposomes to cells via flow cytometry analysis. Binding of immunoliposomes modified with different amounts of DSPE-PEG\textsubscript{2000}-Mal-scFv'\textsubscript{EGFR} (0.03 mol%, 0.10 mol%, and 0.30 mol%) to NCI-H460 and Colo205 cells were analyzed via flow cytometry. Non-targeted (nt) liposomes composed of 0.30 mol% DSPE-PEG\textsubscript{2000}-Mal-cysteine were included as negative control. Cells and functionalized liposomes were incubated at 4 °C for one hour. DiI-labeled liposomes were measured in FL2.
3.2.4 In vitro studies of functionalized liposomes in combination with cycloheximide

An optimal amount of incorporated TRAIL-coupled micelles showing best cell death inducing effects on NCI-H460 cells was investigated. These experiments were performed in combination with cycloheximide (CHX), which is an inhibitor of translation sensitizing the cells to apoptosis. For the generation of TRAIL-functionalized liposomes (LipoTRAIL), between 0.003 and 0.030 mol% of TRAIL-coupled micelles were incorporated into pre-formed liposomes by the post-insertion method at 42 °C for 2 hours. The various LipoTRAIL formulations were tested in vitro for their cell-death inducing activity using NCI-H460 cells in combination with CHX (see Figure 3-40). The induction of apoptosis was dependent on the concentration of the TRAIL-functionalized liposomes and also correlated with the amount of inserted TRAIL-coupled micelles. The highest bioactivity was observed for the 0.030 mol% formulation with an EC_{50} value of 5.4 µM lipid, while the bioactivity of the 0.010 mol% formulation was measured with an EC_{50} value of 71.2 µM lipid. Consequently, the bioactivity of the TRAIL-functionalized liposomes decreases with a decreasing amount of inserted TRAIL-coupled micelles. Plain liposomes (nt-liposomes) composed of 0.030 mol% DSPE-PEG_{2000}-Mal-cysteine were included as negative control. The 0.003 mol% formulation showed no or only marginal cell killing activity.

![Cell death induction assay of different LipoTRAIL formulations on NCI-H460 cells in the presence of cycloheximide](image)

Based on the results obtained from the preparation of immunoliposomes and of LipoTRAIL, TRAIL-functionalized immunoliposomes (ImmunolipoTRAIL; ILT) were generated by incorporating 0.10 mol% DSPE-PEG_{2000}-Mal-scFv’_{EGFR} (see Figure 3-39) and 0.10 mol% DSPE-PEG_{2000}-Mal-Cys-sTRAIL (see Figure 3-40) into preformed liposomes containing 2.4 mol% DSPE-PEG_{2000} (see Figure 3-38). For comparison, immunoliposomes and LipoTRAIL were generated by inserting 0.10 mol% of scFv’_{EGFR}-coupled or Cys-sTRAIL-coupled micelles
into the pre-formed liposomes, respectively. Binding of the functionalized liposomes to the EGFR-expressing cell line NCI-H460 (see Table 3-1) as well as to the EGFR-negative cell line HepG2 was tested via flow cytometry analysis (see Figure 3-41 A). Strong binding of the scFv'-functionalized liposomes (IL and ILT) was observed for the EGFR-positive cell lines NCI-H460, while only marginal binding was detected for the EGFR-negative HepG2 cells. For both cell lines, no or only marginal binding was shown for the non-targeted liposomes, namely the LipoTRAIL and the plain liposomes (nt-liposomes). The binding of IL and ILT could be blocked by pre-incubating the cells with excess amounts (1.8 µM) of uncoupled anti-EGFR scFv fragments (see Figure 3-41 B).

Figure 3-41: Binding of functionalized liposomes to cells via flow cytometry analysis. The functionalized liposomes were modified with 0.10 mol% DSPE-PEG\(_{2000}\)-Mal-scFv'\(_{\text{EGFR}}\) (immunoliposomes, IL), with 0.01 mol% DSPE-PEG\(_{2000}\)-Mal-Cys-sTRAIL (LipoTRAIL, LT), or with 0.10 mol% DSPE-PEG\(_{2000}\)-Mal-Cys-sTRAIL (Immuno-LipoTRAIL, ILT). Plain liposomes (nt-liposomes) modified with 0.11 mol% DSPE-PEG\(_{2000}\)-Mal-cysteine were included as negative control. Cells and functionalized liposomes were incubated at 4 °C for one hour. DiI-labeled liposomes were measured in FL2. A Binding of functionalized liposomes to EGFR-positive cell line NCI-H460 and EGFR-negative cell line HepG2 analyzed via flow cytometry. DiI-labeled liposomes were measured in FL2. B Binding of IL and ILT to NCI-H460 or HepG2 cells was analyzed via flow cytometry. Specificity of binding was demonstrated by pre-incubating the cells with anti-EGFR scFv fragment (1.8 µM) (+), which binds to the same epitope as the scFv'\(_{\text{EGFR}}\) fragment.

The bioactivity for inducing cell death of the functionalized liposomes in combination with cycloheximide was tested on NCI-H460 (see Figure 3-42). Neither the plain liposomes (nt-liposome) nor the scFv-functionalized immunoliposomes induced cell death in the analyzed concentration range (500 µM to 76 nM lipid). In contrast, the TRAIL-functionalized liposomes (LT) induced cell death with an EC\(_{50}\) value of 166 µM lipid, while the bioactivity of the targeted TRAIL-functionalized liposomes (ILT) was increased to an EC\(_{50}\) value of 5.9 µM lipid. Thus, the bioactivity of ILT was increased 28-fold compared with that of LT by adding the targeting moiety to TRAIL-functionalized liposomes.
Results

Figure 3-42: Cell death induction assay of functionalized liposomes in the presence of cycloheximide. Cell death induction of functionalized liposomes (immunoliposomes (IL) modified with 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-scF VGFR, LipoTRAIL (LT) modified with 0.01 mol% DSPE-PEG\textsubscript{2000}-Mal-Cys-sTRAIL, and Immuno-LipoTRAIL (ILT) modified with 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-scF VGFR and 0.01 mol% DSPE-PEG\textsubscript{2000}-Mal-Cys-sTRAIL) was tested on NCI-H460 cells in the presence of cycloheximide (CHX; f.c.: 2.5 µg/ml). Cells were incubated for 16 hours with the functionalized liposomes and viable cells were measured using crystal violet staining. Plain liposomes (nt-liposomes) modified with 0.11 mol% DSPE-PEG\textsubscript{2000}-Mal-cysteine were included as negative control (n=3, ± SD).

3.2.5 \textit{In vitro} studies of functionalized liposomes in combination with bortezomib

The following experiments were performed in combination with the proteasome-inhibitor bortezomib, which is known to sensitize cells to TRAIL-induced apoptosis. In contrast, TRAIL-functionalized liposomes (LipoTRAIL; LT) were generated by inserting 0.03, 0.10, and 0.30 mol% DSPE-PEG\textsubscript{2000}-Mal-Cys-sTRAIL into pre-formed liposomes composed of 2.4 mol% DSPE-mPEG (see Figure 3-38) by post-insertion at 42 °C for 2 hours. The bioactivity of LipoTRAIL for inducing cell death was tested \textit{in vitro} on Colo205 cells in the absence or presence of bortezomib (see Figure 3-43). Colo205 cells were chosen for these experiments due to the established expertise for \textit{in vivo} studies investigating antitumor effects of TRAIL.

In the absence of bortezomib cell death inducing effects of the different LipoTRAIL formulations were observed in a concentration-dependent manner with EC\textsubscript{50} values of 37.1 µM lipid for 0.10 mol% and 6.0 µM lipid for the 0.30 mol% inserted TRAIL-coupled micelle. The 0.03 mol% LipoTRAIL formulation did not induce cell death of 50% of the cells in the analyzed range (500 µM to 76 nM lipid). In the presence of bortezomib, the dose-response curve was left-shifted showing increased cell death induction of the LipoTRAIL liposomes. All three LipoTRAIL formulations induced sufficient cell death over the analyzed range, with EC\textsubscript{50} values of 118 µM lipid for the 0.03 mol%, 18.0 µM lipid for the 0.10 mol%, and 2.1 µM for the 0.30 mol% DSPE-PEG\textsubscript{2000}-Mal-Cys-sTRAIL liposomes. Thus, the bioactivity of LipoTRAIL correlates with the amount of inserted TRAIL-coupled micelles.
Results

![Graphs showing cell viability with and without Bortezomib](image)

Figure 3-43: Cell death induction assay of different LipoTRAIL formulations on Colo205 cells in the absence or presence of bortezomib. Cell death induction of different LipoTRAIL formulations modified with different amounts of DSPE-PEG2000-Mal-Cys-sTRAIL (0.03 mol%, 0.10 mol%, and 0.30 mol%) was tested on Colo205 cells in the absence (w/o) or the presence (250 ng/ml) of bortezomib. Cells were incubated for 16 hours with the functionalized liposomes and viable cells were measured using crystal violet staining (n=2, ± SD).

Based on the results from binding of immunoliposomes to Colo205 cells (see Figure 3-39) and the bioactivity of TRAIL-functionalized liposomes on Colo205 cells in the absence or the presence of bortezomib (see Figure 3-43), targeted TRAIL-functionalized liposomes (Immuno-LipoTRAIL) were generated by inserting 0.10 mol% of DSPE-PEG2000-Mal-scFvEGFR and 0.10 mol% DSPE-PEG2000-Mal-Cys-sTRAIL into pre-formed liposomes by post-insertion method, performed as described for the LipoTRAIL preparation. Size and homogeneity of the functionalized liposomes was measured via light scattering method. Size changed from 84 nm of liposomes prior the post-insertion step to approximately 85 nm for plain liposomes (nt-liposomes), 87 nm for immunoliposomes (IL), 90 nm for LipoTRAIL (LT), and up to approximately 92 nm for Immuno-LipoTRAIL (ILT) by a similar low polydispersionsindex (PDI), which confirmed a homogenous population of liposomes (see Table 3-7).

Table 3-7: Physiochemical properties of functionalized liposomes.

<table>
<thead>
<tr>
<th>formulation</th>
<th>scFv-lipid (mol%)</th>
<th>TRAIL-lipid (mol%)</th>
<th>Zeta-average (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>nt</td>
<td>-</td>
<td>-</td>
<td>85.1 ± 0.2</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>IL</td>
<td>0.1</td>
<td>-</td>
<td>86.8 ± 0.8</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>LT</td>
<td>-</td>
<td>0.1</td>
<td>90.1 ± 2.0</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>ILT</td>
<td>0.1</td>
<td>0.1</td>
<td>91.7 ± 0.3</td>
<td>0.06 ± 0.02</td>
</tr>
</tbody>
</table>

*nt = non-targeted, IL = immunoliposomes, LT = LipoTRAIL, ILT = Immuno-LipoTRAIL*

Furthermore, the cryogenic transmission electron microscopy (Cryo-TEM) pictures revealed the presence of homogeneous and mainly unilamellar liposomes with a size of approximately 100 nm for the LipoTRAIL and Immuno-LipoTRAIL preparations (see Figure 3-44).
Results

Figure 3-44: Cryo-transmission electron microscopy of LipoTRAIL and Immuno-LipoTRAIL. Functionalized liposomes (LipoTRAIL modified with 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-Cys-sTRAIL and Immuno-LipoTRAIL modified with 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-scFv\textsuperscript{EGFR} and 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-Cys-sTRAIL) were analyzed via cryo-transmission microscope operated at 120 kV and viewed under low dose conditions. Cryo-TEM was performed by Dr. Frank Steiniger.

In addition, the binding of the functionalized liposomes on EGFR-expressing Colo205 cells was analyzed via flow cytometry (see Figure 3-45 A). The scFv-modified liposomes, namely immunoliposomes (IL) and Immuno-LipoTRAIL (ILT), showed strong binding to the EGFR-positive Colo205 cells, while there was no binding observed for the non-targeted liposomal preparations, neither for the plain liposomes (nt-liposomes) nor for the LipoTRAIL (LT). The specificity of the binding was confirmed by blocking experiments using excess amounts of the anti-EGFR antibody cetuximab, which recognizes the same epitope as the coupled scFv\textsuperscript{EGFR} fragment. Pre-incubation of the Colo205 cells with cetuximab (5 µM) strongly reduced the binding of IL and ILT.

Figure 3-45: Binding of functionalized liposomes analyzed via flow cytometry and ELISA. The functionalized liposomes were modified with 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-scFv\textsuperscript{EGFR} (immunoliposomes, IL), with 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-Cys-sTRAIL (LipoTRAIL, LT), or with 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-scFv\textsuperscript{EGFR} and 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-Cys-sTRAIL (Immuno-LipoTRAIL, ILT). Plain liposomes (nt-liposomes) modified with 0.20 mol% DSPE-PEG\textsubscript{2000}-Mal-cysteine were included as negative control. A Binding of functionalized liposomes to EGFR-positive Colo205 cells was analyzed via flow cytometry. Specificity of binding was demonstrated by pre-incubating the cells with cetuximab (5 µM) (+), which binds to the same epitope as the scFv\textsuperscript{EGFR} fragment. Cells and functionalized liposomes were incubated at 4 °C for one hour. Dil-labeled liposomes were measured in FL2. B Binding of 1000 µM lipid functionalized liposomes to the EGFR-Fc and TRAILR2-Fc fusion proteins via ELISA. Coupled proteins on the liposomal surface were detected either with HRP-conjugated anti-His\textsubscript{6}-tag (for scFv\textsuperscript{EGFR}) or HRP-conjugated anti-FLAG-tag (for Cys-sTRAIL).

Finally, the simultaneous binding of the ILT to EGFR and TRAIL receptor was investigated using a sandwich-ELISA (see Figure 3-45 B). Either EGFR-Fc or TRAILR2-Fc fusion proteins were coated on an ELISA plate and incubated with the various functionalized liposomes (nt-
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liposomes, IL, LT, ILT). The coupled proteins on the liposomal surface were detected using HRP-conjugated antibodies directed against either His\textsubscript{6}-tag or FLAG-tag. The IL revealed signals when the liposomes were incubated with coated EGFR-Fc fusion proteins and when the scFV\textsubscript{EGFR}-coupled proteins were detected on the liposomal surface via anti-His\textsubscript{6}-tag antibody. Vice versa, a signal for LT could be observed when the liposomes were incubated with coated TRAILR2-Fc fusion protein and when the TRAIL-coupled proteins were detected on the liposomal surface via anti-FLAG-tag antibody. Signals for ILT were measured when liposomes were incubated with coated EGFR-Fc or TRAILR2-Fc fusion proteins and when the coupled-proteins (scFV\textsubscript{EGFR} fragment and Cys-sTRAIL molecules) were detected with either anti-His\textsubscript{6}-tag or anti-FLAG-tag antibodies. In case of ILT, this experiment proves the presence of the scFV\textsubscript{EGFR} fragment and the Cys-sTRAIL molecule on the same liposome. The plain liposomes (nt-liposomes) revealed no signal.

The bioactivity of functionalized liposomes was tested on Colo205 cells incubating the cells with the liposomes for 16 hours in the absence or the presence of bortezomib (see Figure 3-46). In the absence of bortezomib, neither plain liposomes (nt-liposomes) nor the immunoliposomes (IL) showed any cell death inducing effect. In contrast, the TRAIL-functionalized liposomes showed concentration-dependent cell death inducing effects on Colo205 cells with an EC\textsubscript{50} value of 17.2 µM lipid for the LipoTRAIL (LT) and 6.5 µM lipid for the targeted TRAIL-functionalized liposomes ImmunoLipoTRAIL (ILT). Consequently the targeting moiety improved the TRAIL-functionalized liposomes by the factor of 2.6. In the presence of bortezomib, the cell death inducing effect of the TRAIL-functionalized liposomes was further increased showing a left-shift of the dose-response curve. The EC\textsubscript{50} value of the targeted TRAIL-functionalized liposomes ILT with 2.8 µM was increased 3.1-fold compared with the EC\textsubscript{50} value of the non-targeted TRAIL-functionalized liposomes LT.

![Figure 3-46: Cell death induction assay of functionalized liposomes in the absence or the presence of bortezomib.](image-url)

Figure 3-46: Cell death induction assay of functionalized liposomes in the absence or the presence of bortezomib. Cell death induction of functionalized liposomes (immunoliposomes (IL) modified with 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-scFV\textsubscript{EGFR}, LipoTRAIL (LT) modified with 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-Cys-sTRAIL, and Immuno-LipoTRAIL (ILT) modified with 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-scFV\textsubscript{EGFR} and 0.10 mol% DSPE-PEG\textsubscript{2000}-Mal-Cys-sTRAIL) was tested on EGFR-positive Colo205 cells in the absence (w/o) or the presence (250 ng/ml) of bortezomib. Cells were incubated for 16 hours with the functionalized liposomes and viable cells were measured using crystal violet staining. Plain liposomes (nt-liposomes) modified with 0.20 mol% DSPE-PEG\textsubscript{2000}-Mal-cysteine were included as negative control (n=2, ± SD).
3.2.6 *In vivo* studies of functionalized liposomes

For *in vivo* studies, the liposomes were functionalized with the protein-coupled micelles as described for the liposomes in 3.2.5, which were investigated in combination with bortezomib. For the determination of the serum half-lives of the functionalized liposomes (nt-liposomes, IL, LT, ILT) and the sTRAIL molecule (lacking cysteine residue on the N-terminus), SWISS mice received a single i.v. injection of 1 µmol of the functionalized liposomes or 1 µg sTRAIL. The functionalized liposomes revealed terminal half-lives of 11.9 hours for nt-liposomes, 10.8 hours for IL, 11.3 hours for LT and 11.5 hours for ILT (see Figure 3-47).

![Figure 3-47: Pharmacokinetic studies of the functionalized liposomes in mice.](image)

Interestingly, the AUC in the range from 0 to 72 hours of the protein-modified liposomes (IL, LT, ILT) was increased by the factor of 2.5 compared with that of nt-liposomes resulting from a decreased initial half-life of the nt-liposomes. The sTRAIL molecule exhibited a terminal half-life of approximately 2.3 hours. The pharmacokinetic properties of the functionalized liposomes and the sTRAIL molecule are listed in Table 3-8.

**Table 3-8: Pharmacokinetic properties of the functionalized liposomes and sTRAIL.**

<table>
<thead>
<tr>
<th>formulation / protein</th>
<th>t½ [h]</th>
<th>AUC₀⁻₇₂h [%·h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>sTRAIL</td>
<td>2.3 ± 0.1</td>
<td>113 ± 31</td>
</tr>
<tr>
<td>nt</td>
<td>11.9 ± 0.9</td>
<td>561 ± 55</td>
</tr>
<tr>
<td>IL</td>
<td>10.8 ± 0.6</td>
<td>1394 ± 337</td>
</tr>
<tr>
<td>LT</td>
<td>11.3 ± 1.0</td>
<td>1216 ± 157</td>
</tr>
<tr>
<td>ILT</td>
<td>11.5 ± 0.3</td>
<td>1443 ± 396</td>
</tr>
</tbody>
</table>

*sTRAIL = soluble TRAIL, nt = non-targeted, IL = immunoliposomes, LT = LipoTRAIL, ILT = Immuno-LipoTRAIL*
Subsequently, the antitumor activity of the functionalized liposomes was tested in nude mice bearing subcutaneous Colo205 tumors (see Figure 3-48 A). Mice received three i.v. injections of 1 µmol of the respective functionalized liposome in combination with three i.p. injections of bortezomib every other day. The treatment with the functionalized liposomes was initiated when tumors reached a volume of approximately 100 mm³ (day 7). Treatment with nt-liposomes was indicated as control group. The treatment with IL showed no additional antitumor activity compared with the control group. The treatment with LT revealed only marginal effects. Treating the mice with ILT, tumor growth stopped after the first injection, but regrowth was observed at day 11. In conclusion, a potent antitumoral activity of the ILT was observed. Statistically significant reduction of tumor volume was observed for the ILT-treated mice compared with the treatment of nt-liposomes and IL at day 13 (see Figure 3-48 B).

Figure 3-48: In vivo antitumor activity of the functionalized liposomes. A Colo205-bearing nude mice received three intravenous injections of the functionalized liposomes (nt-liposomes, immunoliposomes (IL), LipoTRAIL (LT), Immuno-LipoTRAIL (ILT) (1 µmol per injection) and three intraperitoneal injections of bortezomib (5 µg per injection) every other day represented by arrows (n=10 tumors per group, ± 95%CI). B Tumor volumes of the different treated groups at day 13. [*p<0.05]
4 Discussion

In this work, TRAIL-based multivalent and multifunctional fusion proteins and liposomes were generated for therapeutic applications. In the first part of this study, dimeric assembled fusion proteins were generated utilizing the novel homodimerization modules EHD2 and MHD2. These fusion proteins retained their bioactivity. The MHD2-derived TNF fusion proteins were able to activate both TNF receptors and the scFv-mediated delivery further increased their potency of activating TNF receptors. The multivalent TRAIL EHD2 fusion proteins showed an increased cell death induction of tumor cells in combination with the proteasome-inhibitor bortezomib compared to the trivalent scTRAIL molecule. The delivery to tumor cells via the scFv-targeting moiety further increased the induction of apoptosis. The in vivo studies of the TRAIL fusion proteins revealed a prolonged serum half-life and a superior antitumoral activity of the scFv-EHD2-scTRAIL fusion protein in a xenograft Colo205 model in mice including excellent tolerability. In the last part, scFv- and TRAIL-functionalized liposomes (Immuno-LipoTRAIL) showed potent induction of apoptosis of tumor cells in in vitro assays and exhibited excellent pharmacokinetic properties and a potent tumor response in a xenograft Colo205 model in mice.

4.1 MHD2 and EHD2 as homodimerization modules

The MHD2 and EHD2 are small N-glycosylated domains of the human IgM and IgE, respectively, and contribute to the dimerization of the two heavy chains of each molecule. In this study, the MHD2 and the EHD2 were used as homodimerization modules to generate dimeric fusion proteins exhibiting improved bioactivity, e.g. increased binding due to avidity. The dimeric assembly of the MHD2 and the EHD2 fusion proteins was confirmed via SEC showing only one major peak, which corresponds to the dimer of the respective fusion proteins. This was also confirmed by the ELISA binding studies of the antibody MHD2 and EHD2 fusion proteins (scFv\textsubscript{EGFR}\textsuperscript{MHD2}, scFv\textsubscript{EGFR}\textsuperscript{EHD2} and MHD2-scFv\textsubscript{HER2}) in comparison with the monomeric counterpart (scFv\textsubscript{EGFR} and scFv\textsubscript{HER2}). The binding of the dimeric fusion proteins was increased approximately 3-fold compared with the monovalent counterpart (scFv\textsubscript{EGFR} and scFv\textsubscript{HER2}) due to avidity effects (Crothers and Metzger, 1972). The EC\textsubscript{50} value for the binding of the EGFR to the scFv\textsubscript{EGFR}\textsuperscript{MHD2} fusion protein with 0.50 nM and to the scFv\textsubscript{EGFR}\textsuperscript{EHD2} with 0.27 nM perfectly matches with the K\textsubscript{D} value of cetuximab (0.3 to 0.39 nM) (Mendelsohn and Baselga, 2003; Tijink et al., 2008). However, the K\textsubscript{D} value of the antibody trastuzumab to HER2 with 0.1 nM (Baselga, 2001) was decreased approximately 17-fold compared with the EC\textsubscript{50} value of the MHD2-scFv\textsubscript{HER2} binding to HER2 with 1.7 nM. This decrease of binding of the MHD2-scFv\textsubscript{HER2} is most likely attributed to decreased binding
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properties of the scFv fragment directed against HER2. Furthermore, the TRAIL fusion proteins (EHD2-scTRAIL and scFv-EHD2-scTRAIL) bound to the four different TRAIL receptor Fc fusion proteins with EC$_{50}$ values in the low nM range, which is in accordance with published data (Gasparian et al., 2009; van der Sloot et al., 2006; Tur et al., 2008). Thus, the fusion partners of the EHD2 or the MHD2 are displayed in an active form.

In contrast to the results of the SEC, the SDS-PAGE analysis of these fusion proteins under non-reducing conditions showed only partial dimeric molecules and this indicates that the MHD2 and the EHD2 are dimerized at least partly by non-covalent bonds. Concerning the MHD2, this is in accordance with the results described for the mutated IgM molecule, in which the cysteine residue 337 was substituted for a serine (IgM-Ser337), for which reason the intermolecular disulfide bond was not formed (Davis et al., 1989). Analysis of the sedimentation rates of IgM-Ser337 under non-reducing conditions showed approximately 65% of pentameric/hexameric formation. These formations were further analyzed via SDS-PAGE and the denaturing milieu revealed, besides dimers and some higher assembled forms, monomers. For this reason, it was concluded that the pentameric and hexameric forms must be held together at least partly by non-covalent bonds (Davis and Shulman, 1989). This result was recently confirmed for the mouse MHD2. Analyzing the crystal structure of the mouse MHD2, the dimeric assembly of the mouse MHD2 as well as for its derivative (C337S) was demonstrated (Müller et al., 2013). Besides the interdomain disulfide bond, the crystal structure of the mouse MHD2 revealed that the interface of both domains is dominated by a hydrophobic core and further stabilized by six hydrogen bonds and one salt bond (Müller et al., 2013). They concluded that dimeric assembly of the mouse MHD2-C337S derivative is enabled by these stabilizing forces. In contrast to the MHD2, the intradomain disulfide bond was described to be necessary for the dimeric assembly of the EHD2 (McDonnell et al., 2001).

MHD2 and the EHD2 are suitable for the use as dimerization modules. Since there are naturally occurring interdomain disulfide bonds, one in the case of MHD2 (cysteine residue 337 of each domain) and two in the case of EHD2 (cysteine residues 247 and 337 of each domain), there is no necessity to introduce an additional cysteine residue to form stable dimers as it was described for the small immune proteins (SIP), which are based on the C$_{r}4$ domain of IgE (Borsi et al., 2002). The biochemical characterization of the MHD2 derivatives demonstrated that the interdomain disulfide bond and the N-glycan at asparagine residue 332 contribute to the stability of the MHD2. It was reported that the intradomain disulfide bond of the mouse MHD2 contributes also to the thermal stability of this MHD2 (Müller et al., 2013). Furthermore, the stability of MHD2 and EHD2 is enforced by the intradomain disulfide bond between the cysteine residues 261 and 321. An essential parameter for the generation
Discussion

of fusion proteins is the thermal stability of the MHD2 and EHD2 homodimerization modules. The EHD2 exhibited an increased thermal stability with a melting point at 80 °C (55 °C for MHD2) accompanying with an increase of overall stability, which is probably ascribed to the two interdomain disulfide bonds. EHD2 also exceeded the stability of the non-covalently linked Cα3 domain of the IgG (GHD3). Hence, the covalently-linked EHD2 is the preferred dimerization module. Regarding the EHD2 fusion proteins, the thermal stability of the EHD2 is much higher compared with that of the fusion partners (scFvEGFR at 63 °C and scTRAIL at 50 °C). Thus, the thermal stability of the fusion proteins is not influenced by the EHD2, but is dependent on the scTRAIL moiety in case of the TRAIL fusion proteins and on the scFv moiety in case of the scFv-EHD2 fusion protein.

Concerning the structure of immunoglobulins, the MHD2 and EHD2 are located in the center of the IgM or IgE heavy chain, respectively, and are flanked by Cα1 and Cα3 with the N- and C-terminus on opposite sides. As described above, in this study it was shown that proteins can be fused either to the N-terminus or to the C-terminus or simultaneously to both sides of the domains resulting in stable dimeric fusion proteins. Furthermore, the fusion partners of the fusion proteins retained their active form. In contrast, it was shown that other modules, for example the triplex-forming collagen-like scaffold peptide (collabodies), are only able to trimerize when either the N-terminus or the C-terminus is fused to one molecule, but not to both ends simultaneously (Fan et al., 2008). In other dimerization modules, for example the uteroglobin, both termini are located on the same side of the module, for which reason the fusion partners are displayed in a rather crowded manner (Ventura et al., 2009). Furthermore, uteroglobin interacts with progesterone (Peter et al., 1989), which limits its usage as therapeutic module for the therapy of inflammatory diseases (Mukherjee et al., 2007). In contrast, EHD2 and MHD2 originate from IgE and IgM and are present in the blood. Therefore, they should be biologically inert. A major function of the IgM in the body is the recruitment of the complement system to pathogens via its Fc part. The binding interface of C1q to IgM was identified in Cα3 and Cα4 excluding the MHD2 from complement recruitment (Perkins et al., 1991) (Davis and Shulman, 1989). For IgE, the Fc part is described to interact with the Fcε receptors, which are expressed on cells of the immune system. The main interface of the interaction could be identified in the Cα3 domain of IgE for FcεRI (Garman et al., 2000; Wurzburg and Jardetzky, 2002) and FcεRII (Hibbert et al., 2005; Sutton et al., 2000). In another study, a binding of the EHD2 to FcεRI was identified, but only with a very low affinity resulting in a $K_D$ value of 200 µM, which is much lower (2.7·10⁵-fold) compared to the $K_D$ value of the binding of the whole IgE to FcεRI ($K_D = 750$ pM) (McDonnell et al., 2001). Additionally, no interaction between the EHD2 and the FcεRII could be detected. It was concluded that the EHD2 is mainly responsible for the dissociation of the whole IgE molecule from the FcεRI. Consequently, it is unlikely that the EHD2 itself or as a component of a fusion
protein is able to interact with any of the Fcε receptors in the blood of patients. Furthermore, due to the presence of IgM and IgE in the blood, MHD2 and EHD2 should be non-immunogenic, i.e. should not induce a neutralizing antibody response (Baker et al., 2010; De Groot and Scott, 2007). This is not assumed for the use of other dimerization domains that do not originate from humans, e.g. modules using streptavidin (Dübel et al., 1995) or collabodies (Fan et al., 2008).

4.2 Multivalent TRAIL fusion proteins

By fusing a scFv fragment directed against EGFR and a single-chain derivative of human TRAIL (scTRAIL) to the EHD2, a multivalent and bifunctional fusion protein (scFv-EHD2-scTRAIL) was generated, exhibiting improved cell death-inducing activity in vitro and superior antitumoral response in in vivo assays. As described above, the dimeric assembly of the EHD2 fusion protein was confirmed by SEC resulting in TRAIL fusion proteins displaying multivalent TRAIL molecules.

The EHD2-scTRAIL fusion protein showed an increased cell death inducing effect against various tumor cell lines (NCI-H460 (DR4⁺,DR5⁺; (Siegemund et al., 2012; Zhang et al., 2013)), Colo205 (DR4⁺,DR5⁺; (Gieffers et al., 2013)), HCT116 (DR4⁺, DR5⁺; (Zhou et al., 2011)), and HepG2 (DR4⁵⁺, DR5⁺; (Kojima et al., 2011)) compared with that of the trivalent scTRAIL molecule, varying from 12-fold to 25-fold. In the absence of bortezomib, the trivalent scTRAIL molecule induces no cell death in the HepG2 cell line, while the EHD2 fusion protein showed cell death-inducing activity. Thus, it is most likely that the hexavalent EHD2-scTRAIL fusion protein is able to mimic the activity of the mTRAIL molecule and activate both DRs (Wajant et al., 2001), whereas the trivalent scTRAIL molecule is able to induce cell death predominantly via DR4. Furthermore, scFv-mediated binding of the scFvEGFR-EHD2-scTRAIL fusion protein to EGFR-expressing cell lines (NCI-H460, Colo205, and HCT116) revealed further increased cell death induction compared with the non-targeted EHD2-scTRAIL varying from 8-fold to 21-fold. Binding to cells may stabilize and enforce the oligomerization of the TRAIL receptors on the cellular surface leading to an increased DISC-assembly (Delmas et al., 2004; Song et al., 2007). The benefit of the scFvEGFR-EHD2-scTRAIL fusion protein was not observed on the EGFR-negative cell line HepG2, revealing a similar cancer cell death induction of the targeted and the non-targeted fusion protein. Accordingly, the improved induction of apoptosis might contribute to two structural properties: i) a covalently linked dimerization of the scTRAIL molecules, displaying hexavalent TRAIL molecules, and ii) the targeted delivery of the fusion protein to tumor-associated antigens, improving the TRAIL-TRAILR interaction and maintaining the TRAIL receptor activation. This
assumption was investigated by analyzing the induction of cell death of the monomeric scTRAIL molecule (trivalent TRAIL) in comparison with that of the targeted monomeric scFv-scTRAIL molecule (trivalent TRAIL) and with that of the targeted dimeric scFv-EHD2-scTRAIL fusion protein (hexavalent TRAIL) on EGFR-expressing cell lines (NCI-H460 and Colo205). In the absence of bortezomib, only the scFv-EHD2-scTRAIL revealed induction of cell death. In the presence of bortezomib, the scFv-scTRAIL was more potent in inducing cell death compared with the scTRAIL (16-fold for NCI-H460, 24-fold for Colo205). Additionally, the scFv-EHD2-scTRAIL revealed increased cell death induction compared with the scFv-scTRAIL fusion protein (3-fold for NCI-H460 and 7.5-fold for Colo205). In accordance with these results, it is reported for the scFv-scTRAIL and the scFv-sTRAIL fusion proteins, that upon binding to the cells via the scFv fragments, the fusion protein mimics the function of mTRAIL enabling the activation of both death receptors. This resulted in an increased cell death induction of the scFv-TRAIL fusion proteins compared with the scTRAIL molecule due to the activation of both DRs (Bremer et al., 2005; ten Cate et al., 2009; Schneider et al., 2010). This high efficacy of the scFv-TRAIL fusion proteins is caused by the ability to affect the DRs in cis as well as in trans including the bystander effect (Wajant et al., 2013). The scFv-EHD2-scTRAIL fusion protein should also be able to act in the same manner (see Figure 4-1). Obviously, the targeted multivalent scFv-EHD2-scTRAIL fusion protein exhibits a further increased induction of apoptosis compared with the targeted scFv-scTRAIL fusion protein, probably due to an increased oligomerization of the DRs by the multivalent TRAIL molecules. Similar results were reported for the scFv-scTRAIL fusion protein compared with the Db-scTRAIL fusion protein (Siegemund et al., 2012).

In general, the induction of apoptosis of the fusion proteins was strongly increased in the presence of the proteasome-inhibitor bortezomib, which is known to sensitize the cells for TRAIL-induced apoptosis on different levels (see 1.2.3). Other sensitizers for the TRAIL-induced apoptosis might be useful in combination with the EHD2 fusion proteins, including kinase inhibitors, smac mimetics and chemotherapeutic drugs (Bevis et al., 2010; Lecis et al., 2010; Manzoni et al., 2012; Wu et al., 2004).

All EHD2 fusion proteins obtained approximately 2 to 3.5-fold prolonged half-life compared with the scTRAIL molecule, probably due to an increased hydrodynamic radius and a reduced clearance of the protein by renal filtration (Kontermann, 2011). The bioavailability (AUC) is consequently increased and might influence the efficacy of the fusion proteins. Compared with therapeutic antibodies (median half-life of cetuximab of approximately 7 days, (Baselga et al., 2000)), the terminal half-lives of the EHD2 fusion proteins are rather short, which requires further concepts to increase the pharmacokinetic properties, e.g. PEGylation (Stork et al., 2009), fusion to an albumin or Fc part (Stork et al., 2007), or fusion
to an immunoglobulin-binding domain (Hutt et al., 2012). However, these strategies also affect the hydrodynamic radius of the fusion protein, thus might alter their tissue penetration and receptor binding. The N-terminal PEGylation of TRAIL is already described in literature resulting in decreased activation of the DRs, but in increased terminal half-life of approximately 18 hours. Experiments in xenograft tumor models in mice showed increased antitumoral effects of the PEGylated proteins compared with the natural protein (Kim et al., 2011). Recently, Hill and coworkers published a fusion protein fusing a scTRAIL molecule C-terminally to the Fc-part of human IgG1 (Gieffers et al., 2013). The fusion protein is dimeric assembled via the Fc-part displaying hexavalent TRAIL molecules (APG350), but non-targeted. Due to the recycling of the Fc-part via the neonatal receptor, the terminal half-life of this fusion protein was prolonged to 28 hours. In contrast to the DR-directed antibodies (see 1.2.1), it was demonstrated that the activity of APG350 is independent on the binding to the leucocyte Fcγ receptors. However, this binding also exhibits the induction of ADCC and CMC, which might result in reduced serum concentrations of the fusion proteins in patients. Both fusion proteins, APG350 and scFv-EHD2-scTRAIL, showed tumor response in vitro and in vivo. Since the scFv-EHD2-scTRAIL and the APG350 fusion proteins display multivalent TRAIL molecules, it is assumed that the induction of apoptosis by the EHD2 fusion protein should also be independent on the Fcγ receptors. Finally, the potential of inducing cell death of the scFv-EHD2-scTRAIL fusion protein should be compared with a targeted version of APG350, e.g. by fusing a scFv fragment to the Fc-part for identifying the fusion protein exhibiting the strongest antitumoral activity.

In this study, bortezomib was also used for the in vivo studies testing the antitumoral activity of the EHD2 fusion proteins in a xenograft tumor model in mice. The EHD2-scTRAIL treated Colo205-bearing mice showed slightly increased antitumoral effects compared with the control group, while the targeted scFv-EHD2-scTRAIL fusion protein revealed superior tumor response in a dose-dependent manner demonstrating the importance of scFv-mediated binding of the fusion protein to tumor cells. After the termination of the treatment with fusion proteins, tumor regrowth was observed at day 27. More effective tumor response might be achieved by different therapeutic regimes, e.g. increasing dose, extending the period of applications and/or shortening the intervals. Studies with artificial cross-linked (Ganten et al., 2006) or aggregated (Lawrence et al., 2001) TRAIL molecules showed unwanted side effects, for which reason it was essential to test systemic tolerability of the scFv-EHD2-scTRAIL fusion protein. The fusion protein was well-tolerated in mice up to a concentration of 7 mg/kg (in combination with 0.17 mg/kg bortezomib). There was no acute hepatotoxicity as judged from baseline serum ALT values.
The scFv-EHD2-scTRAIL fusion protein exhibits superior antitumoral response in combination with good tolerability and shows prolonged serum half-life compared with the scTRAIL molecule. In comparison with the TRAIL molecule dulanermin, which shows no substantial therapeutic effect in clinical studies, the scFv-EHD2-scTRAIL fusion protein exhibits different properties that might overcome these disappointing results. Firstly, although dulanermin possesses the potential to bind to both DRs, it is assumed to activate predominantly DR4, while the targeted multivalent scFv-EHD2-scTRAIL fusion protein should be able to activate both DRs. A correlation of the expression level of the DRs with the responsiveness of the tumor cells to TRAIL-induced apoptosis has not been described yet, which leads to the assumption of a cell-line-specific induction of apoptosis by activating either DR4 or DR5, or both receptors simultaneously (Ashkenazi et al., 2008; Kelley et al., 2005; Kriegl et al., 2012). Therefore, the concept of the targeted multivalent TRAIL fusion protein should result in molecules that are able to activate sufficiently both DRs. Secondly, the pharmacokinetic properties of the scFv-EHD2-scTRAIL fusion protein are improved compared with dulanermin resulting in an increased bioavailability of the EHD2 fusion protein.

Regarding the in vitro and in vivo results of the fusion proteins, the cell death induction of the scFv-EHD2-scTRAIL fusion protein using a humanized scFv fragment of cetuximab was increased compared with the non-targeted EHD2-scTRAIL fusion protein. It was demonstrated that inhibition of proliferation via cetuximab-mediated blockage of EGFR is only performed in a bivalent manner, e.g. as complete antibody, but not in a monovalent form (Fan et al., 1994). Thus, the dimeric scFv-EHD2 and the scFv-EHD2-scTRAIL fusion proteins are theoretically able to inhibit cell growth, which could contribute to the increased cell death-inducing effect of scFv-EHD2-scTRAIL. Therefore, it was investigated whether the blockage of EGFR contributes to the improved bioactivity of the scFv-EHD2-scTRAIL fusion protein. When pre-incubating EGFR-positive cells (NCI-H460 and Colo205) with cetuximab prior incubating them with the fusion proteins, the induction of apoptosis of the targeted fusion protein was reduced to that of the non-targeted fusion protein. Consequently, the cell death induction of the scFv-EHD2-scTRAIL fusion protein could not be mimicked using a cooperative effect by incubating the cells with both cetuximab and the hexavalent EHD2-scTRAIL fusion protein. Furthermore, neither the scFv-EHD2 fusion protein nor the cetuximab antibody showed any cytotoxic or growth inhibiting effect on the cell lines (NCI-H460 and Colo205) in in vitro assays. This demonstrates at least for this short-term assays that on the one hand the benefit of the scFv-EHD2-scTRAIL is based on the binding to the tumor cells. On the other hand the blocking of EGFR does not influence these cell lines due to the same cell death induction of the non-targeted EHD2-scTRAIL in the absence or in the presence of cetuximab. The cell lines NCI-H460, Colo205, and HCT116 are described as
Discussion

non-responder to cetuximab treatment due to mutations in the KRAS (HCT116 (Little et al., 2011), NCI-H460 (Mitsudomi et al., 1991)) or BRAF (Colo205 (Di Nicolantonio et al., 2008)). In contrast, the EGFR signaling pathway of A431 cells was rapidly inactivated by treating with a scFV_{EGFR}-sTRAIL fusion protein resulting in an increased sensitivity towards TRAIL-induced apoptosis (Bremer et al., 2005). Thus, the EGFR wild-type tumors might be more sensitive targets to the scFv-EHD2-scTRAIL fusion protein due to the additional inhibition of the EGFR signaling pathway.

The activity of the scFv-EHD2-scTRAIL fusion protein was compared with that of the Db-scTRAIL fusion protein (Siegemund et al., 2012) testing the induction of apoptosis and the binding to different EGFR-expressing cell lines. The scFv-EHD2-scTRAIL and the Db-scTRAIL fusion protein share similar features: i) the targeting moiety mediates binding to the tumor cell and stabilizes the TRAIL-TRAIL-receptor complex, and ii) the multivalent presentation of the TRAIL molecules enables the activation of both DRs by mimicking the activity of mTRAIL. Accordingly, both fusion proteins should be able to induce apoptosis of the tumor cells in both cis and trans (see Figure 4-1), including the potential to kill neighboring target-negative tumor cells (bystander effect), as it was reported for the scFv-TRAIL fusion proteins (Bremer et al., 2004, 2008; Wajant et al., 2013). The bioactivity of both fusion proteins was similar for the NCI-H460 and Colo205 cells, however, there was a significant difference in the bioactivity of the fusion proteins for the HCT116 cell line in the absence (p=0.02) and the presence of bortezomib (p=0.03): scFv-EHD2-scTRAIL was more potent than Db-scTRAIL. This could probably be explained by the different binding strengths on HCT116 cells as observed in flow cytometer analysis. These different binding properties might correlate with the different flexibilities of the fusion proteins due to their different format. In the scFv-EHD2-scTRAIL fusion protein, the modules for targeting (scFv), for dimeric assembly (EHD2), and for effector activity (scTRAIL) are spatially separated from each other leading to an enhanced flexibility of the fusion protein. In contrast, in the Db-scTRAIL fusion protein, the dimerization module and the targeting moiety are combined in the bivalent diabody, which may limits the flexibility of the scFv and TRAIL moieties.

The EHD2 homodimerization module was used for the generation of TRAIL fusion proteins showing an effective induction of cell death in vitro and potent antitumoral response in vivo. Besides the EHD2, the MHD2 was also used for the generation of targeted TNF fusion proteins. The experiments with the MEF cells expressing chimeric TNFR-Fas receptors enable to distinguish between a trivalent TNF molecule (scTNF), which is only able to activate TNFR1, and a multivalent TNF molecule, which is able to activate both TNF receptors (Grell et al., 1995, 1998). The scTNF molecule and the MHD2-scTNF fusion protein were able to induce a potent cell death in MEF-TNFR1-Fas cells, with the MHD2-
scTNF fusion protein being slightly more potent. It must be emphasized that only the MHD2-scTNF fusion protein, which displays hexavalent TNF molecules, was able to induce apoptosis in the MEF-TNFR2-Fas cell, thus mimicking the activity of the mTNF, while the trivalent scTNF molecule did not induce cell death. Approximately 35% of the MEF-TNFR2-Fas cells remained viable after treating with the MHD2-scTNF fusion protein. However, this is in accordance with the published data reporting of a subset (30%) of TNFR2-Fas negative population within the MEF-TNFR2-Fas cells (Krippner-Heidenreich et al., 2002).

Furthermore, the bifunctional antibody-cytokine MHD2 fusion protein scFv\textsubscript{EGFR}-MHD2-scTNF showed binding to EGFR-positive tumor cells, while the specificity of the binding could be confirmed by blocking assays pre-incubating the cells with cetuximab. On HT1080 cells the bifunctional scFv\textsubscript{EGFR}-MHD2-scTNF fusion protein showed strong increased bioactivity compared with the non-targeted MHD2-scTNF and scTNF molecule, while MHD2-scTNF was slightly more potent than the scTNF molecule similar to the results of MEF-TNFR1-Fas cells. This could be explained by a TNF receptor expressing profile of HT1080 cells measuring only TNFR1 on the cell surface, but no TNFR2. Thus, the IL-8 secretion is enabled via the activation of the TNFR1 by both scTNF and mTNF. These results are in accordance with published data testing the expression of TNF receptors of HT1080 cells (Gerspach et al., 2006). The strong bioactivity of the targeted scFv-MHD2-scTNF fusion protein confirms the importance of the scFv-mediated binding of the fusion protein to cells. Since the blocking experiments with cetuximab reduced the IL-8 release of the targeted bifunctional scFv\textsubscript{EGFR}-MHD2-scTNF fusion protein to that of the non-targeted MHD2-scTNF fusion protein and the scTNF molecule, it was confirmed that the accumulation of the fusion protein on the target cell (HT1080) is responsible for an efficient activation of the TNF receptors and not the blocking of the EGFR, at least for this short-term assay.

### 4.3 Multivalent TRAIL-functionalized liposomes

A multivalent and multifunctional liposomal nanocarrier system with improved antitumoral activity was generated by coupling scFv fragments directed against EGFR as targeting moiety and TRAIL molecules as effector moiety on the surface of PEGylated liposomes. This concept is supported by the recent work with TRAIL-displayed liposomes (Martinez-Lostao et al., 2010; De Miguel et al., 2013) and other nano- and microparticles (Bae et al., 2012; Kim et al., 2013a, 2013b; Lee et al., 2011; Perlstein et al., 2013; Wheatley et al., 2012) resulting in increased bioactivity. In pharmacodynamic experiments, the carriers were injected either into the joint space for the treatment of rheumatoid arthritis (Martinez-Lostao et al., 2010), or next to the tumor for the treatment of cervical cancer (Kim et al., 2013a) and glioma (Perlstein et
al., 2013), or were applied as an inhalation treatment of metastatic lung cancer (Kim et al., 2013b). In this study, the functionalized liposomes were applied systematically for the investigation of pharmacodynamics, as this is the usual application form of targeted cancer therapeutics, e.g. therapeutic antibodies (cetuximab or trastuzumab).

The TRAIL-functionalized liposomes LipoTRAIL (LT) showed a potent induction of apoptosis on NCI-H460 and Colo205 cells in this work, most likely due to multivalent displaying of TRAIL molecules mimicking efficiently the mTRAIL activity. By coupling a scFv fragment directed against EGFR to the LT, the cell death induction of the targeted Immuno-LipoTRAIL (ILT) liposome was further increased. Analogously to the EHD2 fusion proteins, it is assumed that the scFv-mediated binding of the ILT to the cells increases its bioactivity i) by increasing the concentration of the functionalized liposomes on the surface of the target cell and ii) by stabilizing the TRAIL-TRAIL-receptor complex. Furthermore, the functionalized liposomes should be able to induce apoptosis on tumor cells in both cis and trans including the bystander effect (see Figure 4-1).

**Figure 4-1: Mode of action of the EHD2 fusion protein and of the functionalized liposomes.** The scFv-EHD2-scTRAIL fusion protein and the Immuno-LipoTRAIL should be able to induce apoptosis on tumor cells in both, cis (targeting and inducing of apoptosis on the same cell) and trans (targeting via one cell and inducing of apoptosis on another cell). The induction in trans includes the bystander effect.

Binding of the functionalized liposomes to the EGFR-expressing NCI-H460 and Colo205 cells was observed for the scFv-functionalized liposomes, namely IL and ILT, via flow cytometry analysis. The specificity of the binding was demonstrated by pre-incubating the cells with cetuximab leading to an almost removed binding. Only marginal binding could be detected for LT via flow cytometry analysis, reflecting the low density of TRAIL receptors on the cellular surface compared with the EGFR density. This TRAIL-TRAIL receptor interaction might not be sufficient for the detection of liposomes using the flow cytometry. In contrast,
ELISA binding studies, providing high concentrations of coated proteins, showed binding of the liposomes functionalized with the scFv fragment and/or the TRAIL molecule to the EGFR-Fc or TRAILR2-Fc fusion protein, respectively. Furthermore, regarding the ILT, it could be demonstrated that the targeting moiety (scFv fragment) as well as the effector moiety (TRAIL molecule) is displayed simultaneously on one liposome.

By using the proteasome-inhibitor bortezomib as sensitizer for TRAIL-induced apoptosis, the cell death induction of the TRAIL-functionalized liposomes was increased on Colo205 cells. Liposomes are capsule-shaped and able to encapsulate and deliver drugs to the tumor site (Paszko and Senge, 2012; Slingerland et al., 2012). Hence, it should be possible to encapsulate bortezomib or other apoptosis sensitizers, e.g. smac mimetics (Manzoni et al., 2012) or chemotherapeutics (Wu et al., 2004), into the liposome delivering all components to the tumor site simultaneously as one formulation. Concerning TRAIL-displaying particles, it was reported that doxorubicin-loaded HSA-nanoparticles surface-modified with TRAIL and transferrin showed synergistic cell death inducing effects in vitro (Bae et al., 2012), as it was for doxorubicin-loaded PLGA microparticles with surface-attached TRAIL (Kim et al., 2013b).

The functionalized liposomes exhibited increased pharmacokinetic properties with a 5-fold prolonged serum half-life and an approximately 12-fold increased bioavailability (AUC) compared with the soluble TRAIL molecule. Furthermore, compared with the plain liposomes, the functionalized liposomes did not exhibit an altered terminal half-life, which was also described for anti-HER2 immunoliposomes (Park et al., 2002). Recently, it was reported that liposomes were decorated with TRAIL molecules that could be applied as therapeutic agents (Martinez-Lostao et al., 2010; De Miguel et al., 2013). However, hexahistidyl-tagged TRAIL molecules were non-covalently adsorbed by the Ni²⁺-NTA modified liposomal system, for which it was reported that it lacks stability for in vivo applications (Rüger et al., 2005, 2006). In this study, the proteins are covalently coupled to the liposomal surface preventing their removal from the liposomal surface in in vivo studies. The functionalized liposomes sized with approximately 90 nm should enable the accumulation of the liposomes in the tumor tissue via the EPR effect (Maruyama, 2011). For the in vivo experiments, mice received 1 µmol functionalized liposomes per injection, which correlates with a TRAIL concentration of only 0.2 nmol. With regard to this low concentration, the treatment with ILT revealed excellent antitumoral activity. However, further in vivo studies are necessary using different application regiments to receive further improved results and to demonstrate the safety of the functionalized liposomes. It is important to mention that functionalized liposomes induce cell death via TRAIL-TRAIL-receptor binding not requiring the release of the loaded therapeutics and/or their intracellular release as it is described for other liposomal approaches (Guo et al., 2011, 2012).
The site-directed coupling of proteins to lipids was enabled by inserting a cysteine residue either C-terminally into the scFv_{EGFR} fragment or N-terminally into the soluble TRAIL (sTRAIL). SDS-PAGE analysis under non-reducing conditions and size exclusion chromatography revealed a dimeric assembly of the cysteine-modified proteins demonstrating the accessibility of the additional cysteine residues. The bioactivity of the proteins was not affected by the additional cysteine residue showing binding to the EGFR-positive cell line NCI-H460 in flow cytometry analysis in case of the scFv'_{EGFR} fragment or showing a cell death inducing effect on Colo205 cells in case of the Cys-sTRAIL molecule. The cysteine-modified proteins were covalently coupled to the maleimide-modified lipid in a defined and site-directed way resulting in reproducible coupling efficiencies. As the TRAIL molecules are existent as homotrimeric molecules, the coupling of one DSPE-PEG_{2000}-Mal chain per TRAIL molecule should be sufficient for the insertion of the whole molecule into the liposomal surface. Based on a coupling efficiency of approximately 70% for the TRAIL molecule, it is assumed that two DSPE-PEG_{2000}-Mal chains are coupled to one protein in average. As the melting point of the Cys-sTRAIL protein was 45 °C, the post insertion method was performed at 42 °C for 2 hours inserting the protein-coupled micelles into the pre-formed PEGylated liposomes. The PEG density of the pre-formed liposomes was defined at 2.5 mol% due to sufficient insertion of protein-coupled micelles during the post insertion step including sufficient binding of the immunoliposomes to target-positive cells. An optimal PEG chain density is described in the range between 2 to 5 mol% leading to an efficient masking of the liposomes from serum proteins and the RES (Sofou and Sgouros, 2008).

In the present study, the cell death induction of LT exhibited an EC_{50} value of 17.2 µM lipid on Colo205 cells in the absence of bortezomib. Concerning firstly the ratio of the functionalized lipid to the TRAIL protein while coupling (DSPE-PEG_{2000}-Mal : Cys-sTRAIL; 5:1), secondly the amount of inserted TRAIL-coupled micelles (0.10 mol% DSPE-PEG_{2000}-Mal-Cys-sTRAIL), and thirdly the coupling efficiency, the correlating amount of TRAIL displayed on the liposomal surface is approximately 2.4 nM. The induction of apoptosis of the comparable fusion protein EHD2-scTRAIL revealed an EC_{50} value of 0.57 nM, which is approximately 4-fold reduced compared with that of the LT. This might be attributed to the distribution of the approximately 24 TRAIL molecules on a whole liposome, probably not guaranteeing the simultaneous activation of the TRAIL receptors by all 24 TRAIL molecules. However, the bioavailability of the TRAIL-functionalized nanocarrier was approximately 3-fold increased compared with the EHD2-scTRAIL fusion protein. Thus, both the fusion protein and the functionalized liposome, exhibit the potential to induce apoptosis via activating both DRs, but the fusion protein is equipped with stronger cell death-inducing activity, and the nanocarrier is featured with more prolonged pharmacokinetic properties.
4.4 Summary and Outlook

In summary, the EHD2 and the MHD2 are homodimerization modules enabling the fusion of proteins to the N-terminus and/or to the C-terminus and retaining the bioactivity of the proteins. The interdomain disulfide bonds of the modules stabilize and enforce their dimeric assembly and, furthermore, the modules should be biologically inert and non-immunogenic in patients. The combination of controlled dimerization via the EHD2 homodimerization module, of tumor targeting by the scFv fragment and induction of cell death via the TRAIL molecule resulted in the targeted multivalent scFv-EHD2-scTRAIL fusion protein with superior antitumoral activity in vitro and in vivo including excellent tolerability after systemic application in mice. Additionally, the functionalized liposomes (ILT) combining the multivalent display of TRAIL molecules and of scFv fragments with the prolonged pharmacokinetic properties also showed potent cell death induction of tumor cells in vitro and in vivo. The increased cell death inducing activity of both strategies is attributed to the activation of both DRs due to i) the multivalent presentation of TRAIL molecules as well as ii) the scFv-mediated binding of the scFv-EHD2-scTRAIL fusion protein and the ILT to the cells. Although the blocking of the EGFR was not relevant for the Colo205 model in the pharmacodynamic studies of this work, it might be important for in vivo studies using cetuximab-responsive tumor resulting in more potent cell death induction. Furthermore, the combination of bortezomib with other sensitizers for TRAIL-induced apoptosis, e.g. smac mimetics and chemotherapeutics, might further improve the tumor response of the scFv-EHD2-scTRAIL fusion protein and of the ILT. In case of the liposomes, these sensitizers could be encapsulated into the liposomal carrier combining all components into one formulation. With respect to the complex and extensive preparation of the functionalized liposomes and to the excellent data obtained from the pharmacodynamic studies of the scFv-EHD2-scTRAIL fusion protein, the EHD2 fusion protein should be used for potential clinical application. However, prior entering the clinical studies, several properties of the EHD2 fusion proteins have to be modified concerning the manufacturing processing and the downstream processing. The manufacturing processing includes, for instance, i) the establishment of production, e.g. by changing the production cell line or screening for a high-productive clone, and ii) protein optimizing, e.g. by modifying length and composition of the linkers including insertion of glycosylation sites or by shortening the length of the TRAIL subunit (aa 95-281) of the scTRAIL molecule as it was reported for the APG350 fusion protein using aa 121-281 (Gieffers et al., 2013). The downstream processing includes, for example, tag-less purification by using a preparative SEC. The modular organization of the functionalized liposomes, and of the EHD2 and MHD2 fusion proteins allows exchanging the targeting and/or the effector moiety. Concerning the targeting moiety different options are feasible: i) changing the target to address other cells, as it is described for the scFv-TRAIL fusion.
protein (e.g. EpCam or FAP (Bremer et al., 2004; Wajant et al., 2001)) or for the immunoliposomes (e.g. HER2 (Kontermann, 2006; Park, 2002)), ii) adding an additional targeting moiety to achieve dual specificity (e.g. single-chain Db construct in case of the fusion proteins or two scFv fragments directed against EGFR and CEA in case of the liposomes (Kontermann, 2012; Mack et al., 2012)), and/or iii) changing the targeting format including other antibody formats (e.g. nanobodies, Fab; (Kontermann, 2010)), novel scaffold proteins (Löfblom et al., 2011; Swers et al., 2013), or peptides (Shadidi and Sioud, 2003). Additionally, the effector moiety could also be exchanged, e.g. by using a TNFR2-selective (Loetscher et al., 1993) derivative of scTNF, which binds specifically to the TNFR2, whose activation is linked to tissue regeneration. Fischer and coworkers generated a nonavalent TNFR2-selective agonist by fusing a TNFR2-selective derivative to the trimerization domain tenascin c (TNC-scTNF_R2). They reported that after having induced cell death of neurons, they could rescue them by treating with TNC-scTNF_R2 (Fischer et al., 2011). Analogously, the MHD2- or EHD2-derived fusion protein (MHD2-scTNF_R2 or EHD2-scTNF_R2) should also be able to activate specifically TNFR2 due to the hexavalent displaying of TNFR2-selective molecules. Hence, the targeted multivalent and multifunctional fusion proteins and functionalized liposomes exhibit a promising therapeutic concept for the treatment of cancer based on TRAIL.
5 References


References


References


References


References


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6 Sequences

6.1 MHD2 and MHD2 fusion proteins

**MHD2 in pSecTagA**

SfiI

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1 atggagacag acacactcct gctatgggta ctgctgtctct gggctcaggg ttcacactgt gccgcccagcc aaccggccag

161 gcttctcagg caacccccaga aaagccacag cgtatgtctgt gccgcccagcc aaccggccag

312 ctacaaagt gccgccccag tcgctggtgt gccgcccagcc aaccggccag

BamHI

---

81 ggcggctcag gccgcccagcc aacgccagct ctatgtctgt gccgcccagcc aaccggccag

241 ctgctgctgt gccgcccagcc aaccggccag

332 gggcgccccag gc gggcgccccag

**MHD2-N332Q in pSecTagA**

NotI

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1 atggagacag acacactcct gctatgggta ctgctgtctct gggctcaggg ttcacactgt gccgcccagcc aaccggccag

161 gcttctcagg caacccccaga aaagccacag cgtatgtctgt gccgcccagcc aaccggccag

312 ctacaaagt gccgccccag tcgctggtgt gccgcccagcc aaccggccag

**MHD2-C337S in pSecTagA**

1 atggagacag acacactcct gctatgggta ctgctgtctct gggctcaggg ttcacactgt gccgcccagcc aaccggccag

161 gcttctcagg caacccccaga aaagccacag cgtatgtctgt gccgcccagcc aaccggccag

312 ctacaaagt gccgccccag tcgctggtgt gccgcccagcc aaccggccag

481 ttcgccccag ccacactcag ttcacactcag ttcacactcag ttcacactcag ttcacactcag ttcacactcag ttcacactcag

h h h h h
Sequences

MHD2-N332Q-C337S in pSecTagA

scF\textsubscript{EgFR}-MHD2 in pSecTagA
Sequences

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MHD2-scFvHER2 in pSecTagA

\[ \text{SfiI} \]

\[ \text{HindIII} \]

\[ \text{BamHI} \]

\[ \text{NotI} \]

\[ \text{EcoRI} \]

\[ \text{HindIII} \]

\[ \text{BamHI} \]

\[ \text{NotI} \]

\[ \text{EcoRI} \]

\[ \text{HindIII} \]

\[ \text{BamHI} \]

\[ \text{NotI} \]

\[ \text{EcoRI} \]
sequences

SCFEGFR-MHD2-sCFVHER2 in pSecTagA

1 atgagacag acaacactct gctatgggta ctcgcgctct ggcgtccagg tctactcctgg gcagcggccc agccggccat

81 gcggcagagt cacgtggtg ttcggtcgg gcgtctggtg gctgctgtgg gcgtgtgtgg gcgtgtgtgg

121 gcgggagacag aagccggccc ggcgtccagg tctactcctgg gcagcggccc agccggccat

>scFV4D5.>>
eikr

>>...His-tag...>>

h h h h h

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ScFv4D5.>>

scFv4D5.......................................>
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>>.....................MHD2......................>
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>........................................scTNF........................................>
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>........................................MHD2.........................................>
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>....................................>
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>........................................scTNF........................................>
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>>...........................IgK leader............................>>
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>........................................MHD2.........................................>
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>........................................MHD2.........................................>
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>........................................scFv4D5.......................................>
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>.......................................scFv4D5.......................................>
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>.......................................scFv4D5.......................................>
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>.......................................scFv4D5.......................................>
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>........................................scTNF........................................>
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>........................................scTNF........................................>
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>........................................scTNF........................................>
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scFv EGFR-MHD2-scTNF in pSecTagA

1 atggagacag aaccactctc gtcttggtta ctggctgctg ttctcggtg acgccgccttg agcggcccttg

Sequence 1

1  cggtggtgca aaccgcccag ctgaaagctg cgctggtcag

Sequence 2

1  cagccggaag attttgcgac ctattattgc cagcagaaca acaactggcc gaccaccttt ggtgcgggc

Sequence 3

1  ggcggaagtg cagctggttg aagc

Sequence 4

1  atggagacag acacactcct

Sequence 5
Sequences

6.2 GHD3

GHD3 in pSecTagA

```
1 atggagacag acacactctct gtatgggta ctgctgtctct gggttccagg ttccactggt gacgcggccc agccggccag
2 gqppreppqv yttlp sree mtk
81 cgctggcgcc ggaagcttag gcggctctgg cggcgatttc acccccccca a cagtgaagat cctccagagc agctgtgacg
81 df tpp tv kils scd
161 ggtggagatgt ctgctgtctct ggctctggta ctgctgctct gggttccagg ttccactggt gacgcggccc agccggccat
161 df tpp tv kils scd
241 cgctggcgcc ggaagcttag gcggctctgg cggcgatttc acccccccca a cagtgaagat cctccagagc agctgtgacg
241 df tpp tv kils scd
321 cgctggcgcc ggaagcttag gcggctctgg cggcgatttc acccccccca a cagtgaagat cctccagagc agctgtgacg
321 df tpp tv kils scd
```

6.3 EHD2 and EHD2 fusion proteins

EHD2 in pSecTagA

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2 gqppreppqv yttlp sree mtk
81 cgctggcgcc ggaagcttag gcggctctgg cggcgatttc acccccccca a cagtgaagat cctccagagc agctgtgacg
81 df tpp tv kils scd
161 ggtggagatgt ctgctgtctct ggctctggta ctgctgctct gggttccagg ttccactggt gacgcggccc agccggccat
161 df tpp tv kils scd
241 cgctggcgcc ggaagcttag gcggctctgg cggcgatttc acccccccca a cagtgaagat cctccagagc agctgtgacg
241 df tpp tv kils scd
321 cgctggcgcc ggaagcttag gcggctctgg cggcgatttc acccccccca a cagtgaagat cctccagagc agctgtgacg
321 df tpp tv kils scd
```

scFv_{EGFR}-EHD2 in pSecTagA

```
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2 gqppreppqv yttlp sree mtk
81 cgctggcgcc ggaagcttag gcggctctgg cggcgatttc acccccccca a cagtgaagat cctccagagc agctgtgacg
81 df tpp tv kils scd
161 ggtggagatgt ctgctgtctct ggctctggta ctgctgctct gggttccagg ttccactggt gacgcggccc agccggccat
161 df tpp tv kils scd
241 cgctggcgcc ggaagcttag gcggctctgg cggcgatttc acccccccca a cagtgaagat cctccagagc agctgtgacg
241 df tpp tv kils scd
321 cgctggcgcc ggaagcttag gcggctctgg cggcgatttc acccccccca a cagtgaagat cctccagagc agctgtgacg
321 df tpp tv kils scd
Sequences

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>......................................scFvhu225......................................>

| 321  | tctgcagatg  | aacagc  |
|      | ctcgccggtc  | tacccggaag   |

>......................................scFvhu225......................................>

| 401  | ttcgctatgg | ggcaccaggg  |
|      | ctcggctgctg | cgtctggcctg   |

>......................................scFvhu225......................................>

| 481  | ggtacgctgta | ttcagctgcc  |
|      | ccaagcctgc | gacgtctgcgt   |

>......................................scFvhu225......................................>

| 561  | aacgcagacgc | attcactcttg  |
|      | tacccggaag   | ccctccgagc   |

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| 641  | gcgcagacgc | attcactcttg  |
|      | cctccgagc   | ccctccgagc   |

>......................................scFvhu225......................................>

| 721  | cgcgaagatc | ttcagctgcc  |
|      | ccaagcctgc | gacgtctgcgt   |

>......................................scFvhu225......................................>

| 801  | aattagcttg | gcgcagatcc  |
|      | cccctccagc | cctccgagc   |

>......................................scFvhu225......................................>

| 881  | gcgcagacgc | attcactcttg  |
|      | cctccgagc   | ccctccgagc   |

>......................................scFvhu225......................................>

| 961  | cttccgagc   | cttccgagc   |
|      | cttccgagc   | cttccgagc   |

>......................................scFvhu225......................................>

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>......................................scFvhu225......................................>

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|      | cttccgagc   | cttccgagc   |

>......................................scFvhu225......................................>

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|      | cttccgagc   | cttccgagc   |

>......................................scFvhu225......................................>

| 1521 | cttccgagc   | cttccgagc   |
|      | cttccgagc   | cttccgagc   |

>......................................scFvhu225......................................>

| 1601 | cttccgagc   | cttccgagc   |
|      | cttccgagc   | cttccgagc   |

>......................................scFvhu225......................................>

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| 2001 | cttccgagc   | cttccgagc   |
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|      | cttccgagc   | cttccgagc   |

>......................................scFvhu225......................................>

| 2161 | cttccgagc   | cttccgagc   |
|      | cttccgagc   | cttccgagc   |

>......................................scFvhu225......................................>

| 2241 | cttccgagc   | cttccgagc   |
|      | cttccgagc   | cttccgagc   |

>......................................scFvhu225......................................>

| 2321 | cttccgagc   | cttccgagc   |
|      | cttccgagc   | cttccgagc   |

>......................................scFvhu225......................................>

| 2401 | cttccgagc   | cttccgagc   |
|      | cttccgagc   | cttccgagc   |

>......................................scFvhu225......................................>
scFvEGFR-EHD2-scTRAIL in pSecTagA

1 atggagacag acaactacct gctatggtta cgtctgtcat ggtttcaggg ttttcgggt g

m e a s f f g a f l v g

Oliver Seifert

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6.4 TRAIL receptor Fc fusion proteins

TRAILR1 in pSecTagA-Fc
TRAILR3 in pSecTagA-Fc

Sequences

AgeI
-------------

1 atggagagc accaactctct gctatggtta gctgttcgct ggtgctcacc ggtgcaagct cgggagaccct

-------------

NetI
-------------

1 gacgccccgc tcccgcca ggaagacta tgtgtgtgtgt tgtgtgtgtgt tgtgtgtgtgt tgtgtgtgtgt tgtgtgtgtgt

-------------
TRAILR4 in pSecTagA-Fc

```plaintext
TRAILR4 extracellular
```

```plaintext
AgeI
```

```plaintext
TRAILR4 extracellular
```

```plaintext
NotI
```

```plaintext
TRAILR4 extracellular
```

```plaintext
TRAILR4 extracellular
```

```plaintext
TRAILR4 extracellular
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```plaintext
TRAILR4 extracellular
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TRAILR4 extracellular
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TRAILR4 extracellular
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TRAILR4 extracellular
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```plaintext
TRAILR4 extracellular
```
6.5 Proteins for liposomal modification

**pABC4-scFv'EGFR**

```
1  atgaaatgcc tagtgcctac gcagccggt cgatgtgatt tacctgcggc ccagccggcc atggcggaag tgtgcagctggt
   >>.pelB leader...........................>>
   m k y l l p t a a a a g l l l l a a q p a m a
   >>.scFvhu225..>
   s v g l
81  tgaagccgcc gtggcatctcg ttacgccggt tgtgacctgt ctgctgggag cgccctttagc ctgaccaact
   >.scFvhu225.>
   v e s g p v q g q s l r l s c a a s g f s l t n
161  atgctgctga tcgggtctcg ccaacgccct caaacgtggt ggccggggt gcacccctgt
   >.scFvhu225.>
   y y v h w y r q a p k g l e w l y v i w s g n t d
241  tataaccccc cggttttcac attaccgctt atacaccaaa aacaccccttg tattcgcaaa tcacaccaac stkncacctg
   >.scFvhu225.>
   n t i d t a v y y c a r a l t y y d y y e f a y w q
401  gcacccagct tagtgcctag agcgctgggc tgtgacccgt tgttggggagt ctgcccacgtg atggccaggg
   >>.scFvhu225.>
801  acatcatcac catcaccacg gcggagca tgttgggttc tcatgttgggt gcggagga
   >>....FLAG....>>
   d y k d d d d k
```

**pET15b-Cys-sTRAIL**

```
1  atggcctgtg gtgggtctag cgattctaaa atagatggtg ataaaggttg tggatcctgt ctgacaagcg gcggcgacgc
   Cys
   >>.sTRAIL.>
   v r e r g p q
81  tggccgacag cattattacag gcacccggtg ctcgggtcaat acctgcagc gccggtagat caaacatgac aagcaagtgtg
   >>.sTRAIL.>
   r v a a h i t g t r g r n t l s p n s k n e k a l
161  gtggcacaat ctagtcgct gcacccgtg acctgtgctg acctggagac gttgagcttgc
   >>.sTRAIL.>
   v i h e k g f y y i s q y t f r f q e i k e n t
321  aaaggttg ccagataaat ctaatggctgt tcacgccgct ctcgtgctct gcggctgatcc
   >>.sTRAIL.>
   k n d k m v q y k t s y p d p i l l k m k s a r
401  atagatggtg gcagagagat gcgagattag gctgagcggtg ctgagcggtg ctgagcggtg caagagat
```
Sequences

>...............................sTRAIL.................................>
ns c w s k d a e y g l y s i y q g g i f e l k e n d
481  cgcatctttg ttagctgac caacgaacat ctgatgata tggatcatga agccagcttt tttgtgcat ttcgtttgg
>...............................sTRAIL.................................>
ri f v s v t n e h l i d m d h e a s f f g a f i v
561  ttaa
>..>> sTRAIL
g -
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Declaration

I hereby assure that I performed the present study independently without further help or other material than stated.

Oliver Seifert
Stuttgart, 22th of January 2014
Curriculum Vitae

Personal details

<table>
<thead>
<tr>
<th>name and surname:</th>
<th>Oliver Seifert</th>
</tr>
</thead>
<tbody>
<tr>
<td>address:</td>
<td>Adalbert-Stifter-Straße 6, 70437 Stuttgart, Germany</td>
</tr>
<tr>
<td>date of birth:</td>
<td>October, 10th, 1984</td>
</tr>
<tr>
<td>place of birth:</td>
<td>Stuttgart, Germany</td>
</tr>
<tr>
<td>nationality:</td>
<td>German</td>
</tr>
<tr>
<td>status:</td>
<td>married, 2 daughters</td>
</tr>
<tr>
<td>mobile:</td>
<td>+49 176 41390635</td>
</tr>
<tr>
<td>e-mail:</td>
<td><a href="mailto:oliver.seifert@gmx.de">oliver.seifert@gmx.de</a></td>
</tr>
</tbody>
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Education

<table>
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<tr>
<th>Date</th>
<th>Programme and Details</th>
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<tr>
<td>October 2010 to</td>
<td>Doctorate at the University of Stuttgart</td>
</tr>
<tr>
<td>January 2014</td>
<td>Institute of Cell Biology and Immunology Biomedical Engineering Group of Prof. Roland E. Kontermann ‘TRAIL-based multivalent and multifunctional fusion proteins and liposomes for therapeutic applications’</td>
</tr>
<tr>
<td>October 2006 to</td>
<td>Diploma in Biology (technical orientation)</td>
</tr>
<tr>
<td>April 2010</td>
<td>University of Stuttgart, overall grade: excellent (1.0) major subject: Biomedical Engineering and Immunology minor subjects: Bioprocess Engineering and Nucleic Acid Technology Compulsory optional subject: Biochemistry</td>
</tr>
<tr>
<td>July 2009 to</td>
<td>Diploma thesis at the University of Stuttgart</td>
</tr>
<tr>
<td>April 2010</td>
<td>Institute of Industrial Genetics Group of Prof. Jens Kurreck ‘Identification of RNA-G-quadruplex binding proteins’</td>
</tr>
<tr>
<td>August 2008 to</td>
<td>Student research project at Cancer Research UK</td>
</tr>
<tr>
<td>February 2009</td>
<td>London Research Institutes, Group of Helle Ulrich, PhD ‘Analysis of function and ubiquitin-binding properties of the protein Brp2 from <em>Saccharomyces cerevisiae</em>’</td>
</tr>
<tr>
<td>October 2004 to</td>
<td>Intermediate Diploma in Biology</td>
</tr>
<tr>
<td>April 2006</td>
<td>University of Hohenheim, overall grade: good (1.7)</td>
</tr>
<tr>
<td>September 1995 to</td>
<td>General qualification for university entrance</td>
</tr>
<tr>
<td>June 2004</td>
<td>Eschbach-Gymnasium, Stuttgart-Freiberg, Germany</td>
</tr>
<tr>
<td>September 1991 to</td>
<td>Grade school</td>
</tr>
<tr>
<td>June 1995</td>
<td>Rosenschule, Stuttgart-Zuffenhausen, Germany</td>
</tr>
</tbody>
</table>
Publications


