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Immuno-LipoTRAIL: Targeted Delivery of TRAIL-Functionalized Liposomal Nanoparticles

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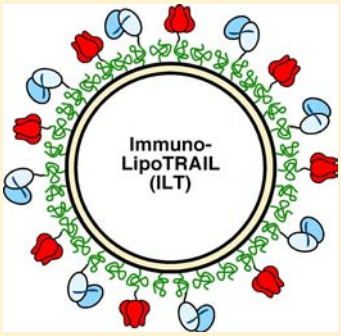
Supporting Information

ABSTRACT: The TNF-related apoptosis-inducing ligand (TRAIL) is a powerful inducer of apoptosis in tumor cells; however, clinical studies with recombinant soluble TRAIL were rather disappointing. Here, we developed TRAIL-functionalized liposomes (LipoTRAIL, LT) to mimic membrane-displayed TRAIL for efficient activation of death receptors DR4 and DR5 and enhanced induction of apoptosis, which were combined with an anti-EGFR single-chain Fv fragment (scFv) for targeted delivery to EGFR-positive tumor cells. These immuno-LipoTRAILs (ILTs) bound specifically to EGFR-expressing cells (Colo205) and exhibited increased cytotoxicity compared with that of nontargeted LTs. Compared to that of the soluble TRAIL, the plasma half-life of the functionalized liposomes was strongly extended, and increased antitumor activity of LT and ILT was demonstrated in a xenograft tumor model. Thus, we established a multifunctional liposomal TRAIL formulation (ILT) with improved pharmacokinetic and pharmacodynamic behavior, characterized by targeted delivery and increased induction of apoptosis due to multivalent TRAIL presentation.



INTRODUCTION

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily of homotrimer TNF ligands and is capable of inducting apoptosis by binding to the proapoptotic death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5).[1−4] Like most TNF ligands, TRAIL exists in two functional, bioactive forms: (i) as a membrane protein (mTRAIL), which is converted into (ii) a soluble protein (sTRAIL) by proteolytic cleavage.[5] Of note, mTRAIL and sTRAIL show differential activity. Thus, mTRAIL can efficiently activate TRAIL-R1 and TRAIL-R2, while sTRAIL signals mainly through TRAIL-R1.[6] Consequently, mTRAIL is more potent in inducing apoptosis than sTRAIL. Furthermore, TRAIL-R2 levels are elevated on the cell surface of many tumor cells, and a positive correlation between TRAIL-R2 expression and long-term survival has been established for some tumors, e.g., nonmetastatic colon carcinoma.[7]

TRAIL receptor overexpression by many tumor cells enables therapeutic strategies based on receptor activation, e.g., using agonistic antibodies or soluble TRAIL.[8] Clinical trials of soluble TRAIL (dulanermin) in combination e.g. with chemotherapy (paclitaxel, carboplatin) and antibody therapy (bevacizumab) established a good safety profile; however, no therapeutic improvements were reported.[9−11]

The therapeutic potential of sTRAIL can be improved by targeted delivery, e.g., through fusion to antibody moieties.[12] Examples include fusion of single-chain Fv molecules (scFv) directed against EGFR, HER2, EpCAM, and CD20 to the N-terminus of TRAIL.[13,14] We could recently show that the stability and activity of antibody-TRAIL fusion proteins can be further improved by generating single-chain variants of the homotrimeric ligands[6] and dimeric antibody fusion proteins containing two of these single-chain TRAIL variants, generated by utilizing a diabody format or the heavy chain domain 2 from IgE (EHD2) as homodimerization module, respectively.[15−17] This was attributed to an increased targeting effect, due to bivalent antigen binding, and an efficient activation of TRAIL-R1 and TRAIL-R2, due to multivalent TRAIL receptor binding.

We reasoned that the activity of mTRAIL might also be mimicked using liposomal carrier systems, exhibiting a prolonged circulation time and further allowing endowment with ligands for targeted delivery. Multifunctionalized and long-circulating liposomes were generated through the coupling of TRAIL molecules and EGFR-specific scFvs to the surface of PEGylated liposomes. These Immuno-LipoTRAIL nanoparticles exhibited increased cell death induction in a target-dependent manner and showed improved antitumor activities in xenograft mouse tumor models compared to that in immunoliposomes and nontargeted LipoTRAIL.

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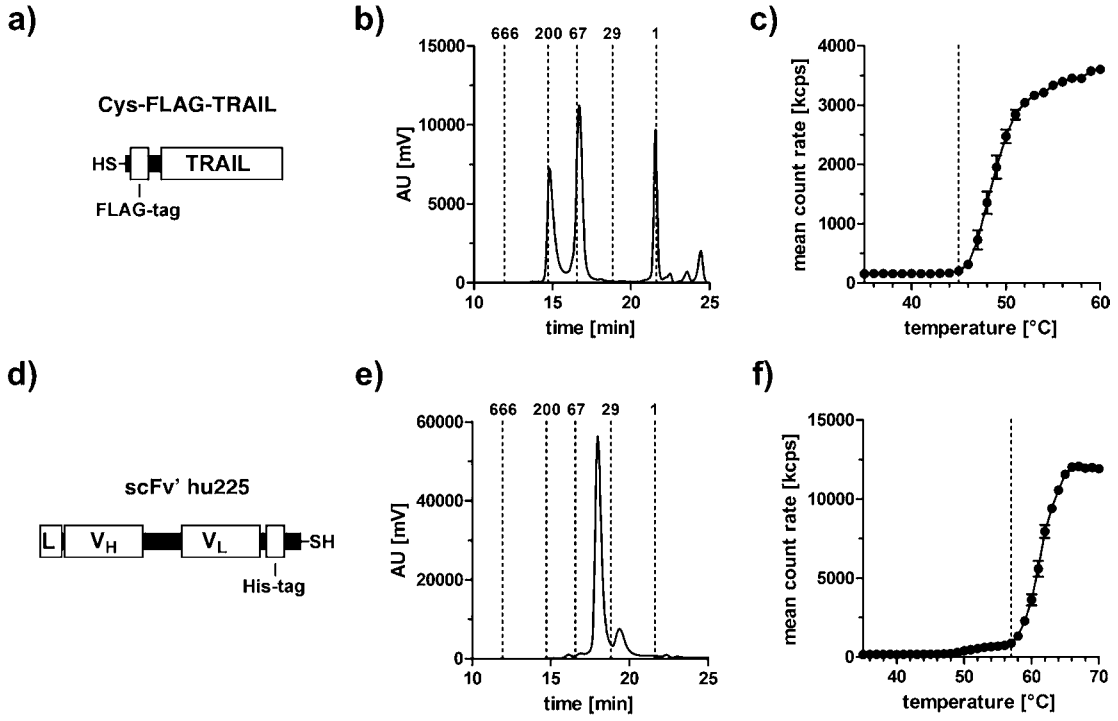

Figure 1. (a) Schematic illustration of the Cys-FLAG-sTRAIL molecule (Cys-sTRAIL), including the N-terminal leader sequence with the N-terminal cysteine residue and FLAG-tag. (b) Size exclusion chromatography of the Cys-FLAG-sTRAIL molecule. The elution time and molecular mass of standard proteins are indicated. The peak of 1 kDa corresponds to the FLAG peptide used for protein elution. (c) Determination of the melting point Cys-FLAG-sTRAIL by dynamic light scattering. Melting point is indicated by the dashed line. (d) Schematic illustration of the scFv′ hu225 fragment, including the N-terminal leader sequence as well as the C-terminal hexahistidyl-tag and cysteine residue. (e) Size exclusion chromatography of the scFv′ hu225 fragment. The elution time and molecular mass of standard proteins are indicated. (f) Determination of the melting point of scFv′ hu225 by dynamic light scattering. The melting point is indicated by the dashed line.

RESULTS

LipoTRAIL (LT). For the generation of TRAIL-functionalized liposomes (LipoTRAIL), we produced a soluble TRAIL (aa 114−281) derivative containing an N-terminal cysteine residue (Cys-sTRAIL) for site-directed conjugation (Figure 1a). Thus, the homotrimeric TRAIL molecules exhibit three reactive thiol groups per molecule. Cys-sTRAIL was expressed in bacteria and purified from whole cell extracts by FLAG-affinity chromatography. In sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) under reducing conditions, the purified protein migrated with an apparent molecule mass of 20 kDa (see also Figure 2b). Size exclusion chromatography revealed the presence of two peaks corresponding in size to homotrimeric molecules with a molecular mass of approximately 65 kDa and a higher order complex with a molecular mass of approximately 200 kDa, the latter most likely representing disulfide-linked molecules (Figure 1b). A melting temperature of 45 °C was determined by dynamic light scattering (Figure 1c). Cys-sTRAIL retained the ability to induce cell death as shown for Colo205 cells in the absence or presence of the proteasome-inhibitor bortezomib, known to sensitize cells for TRAIL-induced apoptosis[19] (Figure 3a). Compared to unmodified soluble TRAIL, IC_{50} values of Cys-sTRAIL were slightly increased (5- to 6-fold), probably due to the presence of disulfide-linked dimeric molecules. Coupling of Cys-sTRAIL to maleimide-PEG_{2000}-DSPE (Mal-PEG_{2000}-DSPE) was achieved with an efficiency of 70% as revealed by SDS−PAGE analysis (Figure 2b). Lipid-conjugated Cys-sTRAIL was then inserted into preformed PEGylated liposomes by the postinsertion method (Figure 2a) using a temperature of 42 °C for the postinsertion step and between 0.01 to 0.3 mol % of lipid-conjugated Cys-sTRAIL. The average size of the LipoTRAIL formulations after postinsertion was around 90 nm. The LipoTRAIL formulations were then tested in vitro for their cell-death-inducing bioactivity using Colo205 cells. Here, a concentration-dependent induction of apoptosis was observed, which was dependent on the amount of inserted Cys-sTRAIL and was pronounced in the presence of bortezomib (Figure 3b). High bioactivity was observed for formulations containing between 0.03 to 0.3 mol % lipid-conjugated Cys-sTRAIL, corresponding to approximately 6 to 60 TRAIL molecules per liposomes. Plain liposomes (nt-liposomes) included as control showed no cell killing activity (not shown).

Anti-EGFR scFv-Immunoliposomes (IL). For the generation of anti-EGFR immunoliposomes, a humanized version of cetuximab (hu225) was expressed as an scFv′ molecule containing an additional C-terminal cysteine residue for site-directed coupling to maleimide groups (Figure 1d). The bacterially expressed scFv′ molecule was purified by IMAC. In SDS−PAGE under reducing conditions, the purified protein migrated with an apparent molecule mass of 29 kDa (see also Figure 2b). Size exclusion chromatography revealed the presence of a peak corresponding in size to dimeric molecules, most likely disulfide-linked molecules (Figure 1e). A melting temperature of 57 °C was determined by dynamic light scattering for scFv′ hu225 (Figure 1f). Flow cytometry experiments confirmed binding to EGFR-expressing cell lines (data not shown). ScFv′ hu225 was conjugated to micellar maleimide-PEG_{2000}-DSPE (Mal-PEG_{2000}-DSPE) with a cou-

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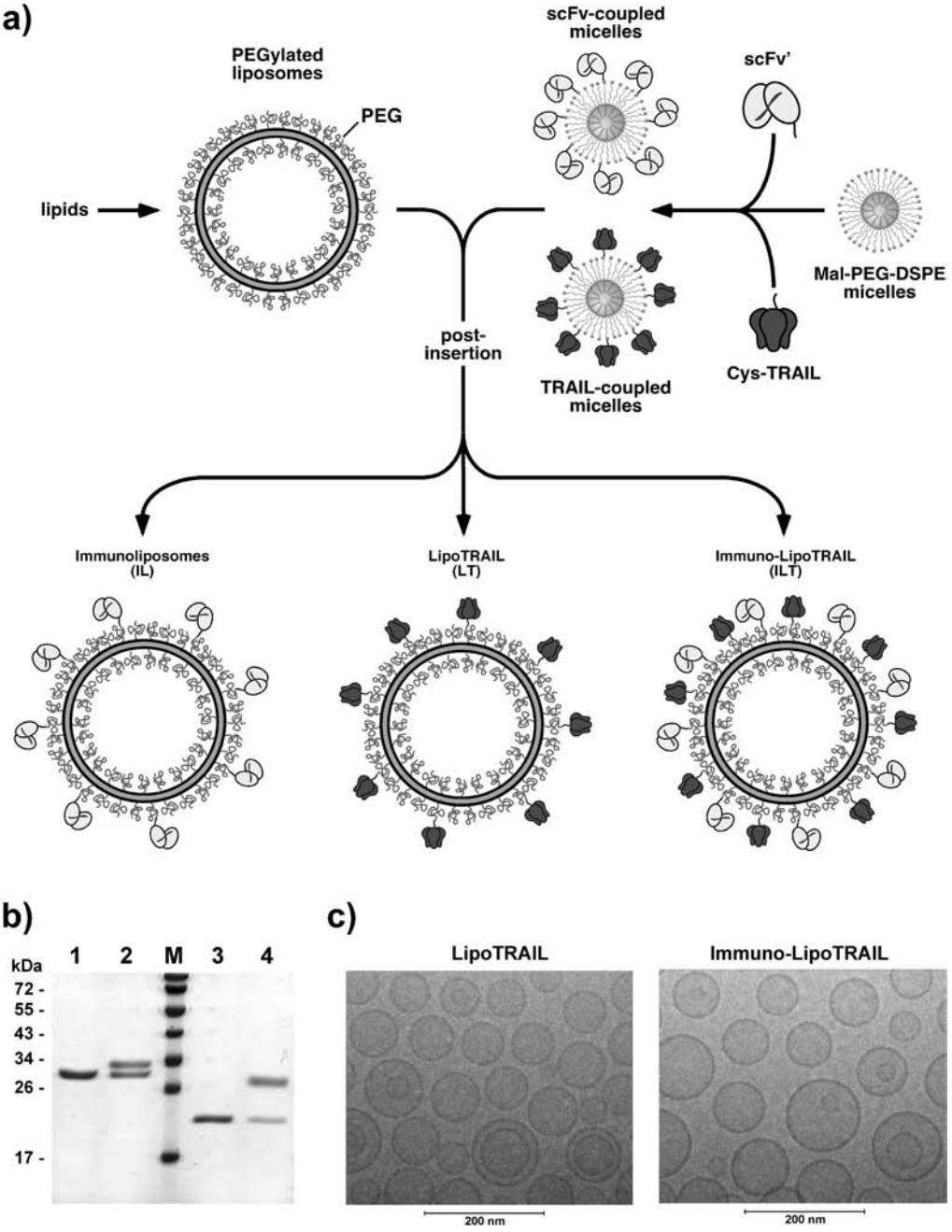

Figure 2. (a) Schematic illustration of the preparation of the functionalized liposomes by the postinsertion method, resulting in immunoliposomes (IL), LipoTRAIL (LT), and Immuno-LipoTRAIL (ILT). (b) SDS−PAGE analysis under reducing conditions of the scFv fragment (lanes 1 and 2) and Cys-sTRAIL molecule (lanes 3 and 4) before (lanes 1 and 3) and after (lanes 2 and 4) coupling to Mal-PEG_{2000}-DSPE. (c) Cryo-transmission electron microscopy of LipoTRAIL and Immuno-LipoTRAIL.

pling efficiency of approximately 60% as revealed by SDS−PAGE analysis (Figure 2b). The antibody−lipid conjugate was then inserted into preformed PEGylated liposomes at varying concentrations (0.03−0.3 mol % lipid) at a temperature of 42 °C using the postinsertion method (Figure 2a). All immunoliposomes (IL) showed a concentration-dependent and specific binding to EGFR-expressing Colo205 cells (Figure 4a). The strongest binding was observed with liposomes containing 0.1 mol % scFv-conjugated Mal-PEG_{2000}-DSPE corresponding to approximately 15 scFv molecules per liposome. No or only marginal binding was observed for nontargeted liposomes (nt-liposomes).

Immuno-LipoTRAIL (ILT). On the basis of the results obtained for immunoliposomes and LipoTRAIL, we generated TRAIL-functionalized immunoliposomes (Immuno-LipoTRAIL) inserting 0.1 mol % scFv-conjugated Mal-PEG_{2000}-DSPE and 0.1 mol % TRAIL-conjugated Mal-PEG_{2000}-DSPE into PEGylated liposomes (Figure 2a). After postinsertion, the various liposomal formulations exhibited a size of around 91 nm, similar to the value before postinsertion (84 nm) (Table 1). Cryo-TEM pictures revealed the presence of mainly unilamellar vesicles in the LipoTRAIL and Immuno-LipoTRAIL preparations (Figure 2c).

Binding of the functionalized liposomes was tested on EGFR-expressing Colo205 cells via flow cytometry. Liposomes

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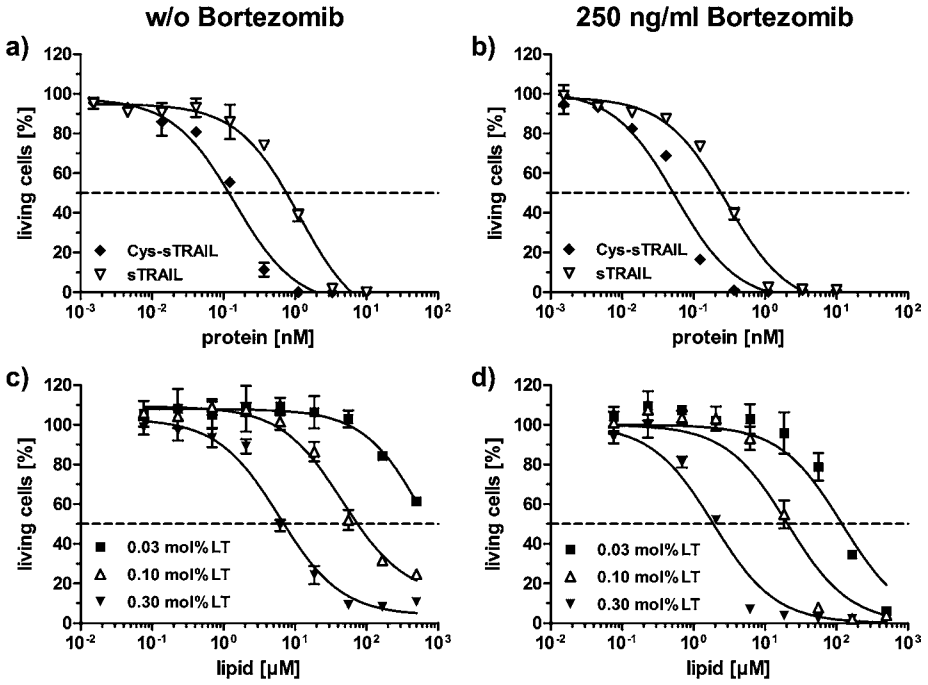

Figure 3. (a,b) Killing of Colo205 cells in vitro by sTRAIL and Cys-sTRAIL in the absence (w/o) or presence of 250 ng/mL bortezomib. (c,d) Killing of Colo205 cells in vitro by LipoTRAIL formulations containing varying amounts of coupled Cys-sTRAIL (0.03, 0.1, and 0.3 mol % of micellar lipid) in the absence or presence of 250 ng/mL bortezomib.

that were functionalized with the scFv fragment (IL and ILT) showed strong binding to EGFR-positive Colo205, while no binding was seen with the nontargeted liposomes (nt-liposomes, LT) (Figure 4b). Specificity of EGFR binding was demonstrated by preincubating cells with an excess amount of cetuximab, which recognizes the same epitope as scFv hu225 (Figure 4b). Preincubation with cetuximab strongly reduced the binding of the scFv-functionalized liposomes (IL and ILT).

Simultaneous binding of the ILTs to EGFR and TRAIL receptors was shown in a sandwich-ELISA. Immobilized EGFR-Fc or TRAIL-R2-Fc was incubated with the liposomal formulations (nt-liposomes, IL, LT, and ILT) and subsequently with either an anti-FLAG-tag or an anti-His-tag secondary antibody. Signals were revealed when ILTs were bound to EGFR-Fc (through the scFv) and detection was with an anti-FLAG-tag antibody (detecting liposomal TRAIL) or when ILTs were bound to TRAILR2-Fc (through TRAIL) and detection was with an anti-His-tag antibody (detecting liposomal scFv)(Figure 4c). Using these settings, no binding was observed for IL and LT. Thus, these experiments confirmed that scFv and TRAIL molecules are displayed on the same liposome.

Cell Death Mediated by Immuno-LipoTRAIL. The bioactivity of the functionalized liposomes was determined with Colo205 by incubating the cells with functionalized liposomes for 16 h in the presence or absence of bortezomib. In the absence of bortezomib, neither plain liposomes (nt-liposomes) nor immunoliposomes (IL) showed cytotoxic effects against Colo205 cells in the analyzed concentration range (500 μM to 76 nM lipid). In contrast, the TRAIL-functionalized liposomes induced cell death with an IC_{50} of 20.2 μM lipid of the nontargeted LipoTRAIL (LT) and 8.0 μM lipid of the targeted Immuno-LipoTRAIL (ILT) (Figure 5a and Table 2). Thus, the targeting moiety improved apoptosis induction of TRAIL-functionalized liposomes by a factor of 2.5. In the presence of bortezomib, the dose−response curve of the TRAIL-functionalized liposomes was shifted to the left, which indicates a further improvement of TRAIL-dependent apoptosis. Similarly, the cytotoxic effect of the targeted TRAIL-functionalized liposomes (2.6 μM lipid) was increased compared to the nontargeted TRAIL-functionalized liposomes (LT) by a factor of 2.9 (Figure 5c and Table 2). In contrast, primary fibroblasts isolated from the vicinity of a lung tumor showed not apoptosis induction with LT and ILT in the absence of bortezomib and only some degree of apoptosis induction in the presence of bortezomib, not reaching 50% cell death, with no significant differences between LT and ILT (Figure 5b,d).

Next, we analyzed LT and ILT on Jurkat cells known to express DR5 but not DR4 and thus are not sensitive against treatment with soluble TRAIL.[17] Incubation of Jurkat cells with LT and ILT at different concentrations resulted in approximately 25−40% cell death at 500 μM lipid, indicating that the liposomal TRAIL induced DR5-mediated apoptosis (Supporting Information, Figure 1). In further experiments, we analyzed the bioactivity of the liposomal TRAIL (LT and ILT) on the EGFR-positive cell line NCI-H460 and the EGFR-negative cell lines HepG2.[17] Strong binding of ILT to NCI-H460 was demonstrated by flow cytometry analysis, while LT showed some weak binding at the highest concentrations. Similarly, LTs exhibited only very weak binding on HepG2 and also only weak binding of ILTs. Increased apoptosis induction by ILT in comparison with LT was found with NCI-H460, while for HepG2 no differences between LT and ILT were observed. This finding indicates that targeted delivery to EGFR by displayed scFv improves the bioactivity of the liposomal TRAIL (Supporting Information, Figure 2).

Pharmacokinetics. The serum half-lives of the liposomal formulations were analyzed in CD1 mice receiving a single dose of DiR-labeled liposomes (1 μmol lipid). The liposomal

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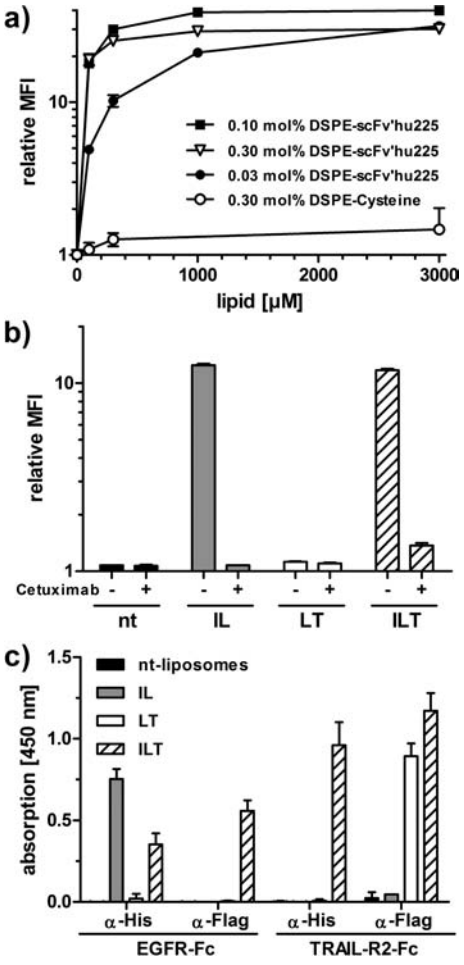

Figure 4. (a) Binding of functionalized immunoliposomes to Colo205 cells analyzed by flow cytometry. Different formulations containing varying amounts of DSPE-PEG_{2000}-Mal-scFv hu225 were tested. Plain liposomes (nt-liposomes) prepared from DSPE-cysteine were included as the negative control. (b) Binding of the functionalized liposomes (nt = nontargeted liposomes, IL = 0.1 mol % inserted DSPE-PEG_{2000}-Mal-scFv, LT = 0.1 mol % inserted DSPE-PEG_{2000}-Mal-Cys-sTRAIL, and ILT = 0.1 mol % inserted DSPE-PEG_{2000}-Mal-scFv and 0.1 mol % inserted DSPE-PEG_{2000}-Mal-Cys-sTRAIL) to Colo205 cells by flow cytometry at a lipid concentration of 1 mM. Specific binding to EGFR-positive cells was demonstrated by blocking with cetuximab (5 μM). (c) Binding of the functionalized liposomes (see b) to EGFR-Fc or TRAIL-R2-Fc by ELISA. Coupled proteins on the surface of the liposomes were detected either with an anti-His-tag antibody (for scFv) or with an anti-FLAG-tag antibody (for TRAIL).

Table 1. Physicochemical Properties of Liposomal Formulations[a]

formulation	scFv-lipid (mol %)	sTRAIL-lipid (mol %)	zeta-average (nm)	PDI
nt			85.1 ± 0.2	0.06 ± 0.01
IL	0.1		86.8 ± 0.8	0.06 ± 0.02
LT		0.1	90.1 ± 2.0	0.10 ± 0.02
ILT	0.1	0.1	91.7 ± 0.3	0.06 ± 0.02

[a]nt = nontargeted, IL = immunoliposomes, LT = LipoTRAIL, and ILT = immuno-LipoTRAIL.

formulations exhibited terminal half-lives between 10.8 and 11.9 h. Compared to nonfunctionalized liposomes (nt-liposomes), the AUC_{0-72h} of the functionalized liposomes was increased approximately 2.5-fold. Soluble TRAIL (sTRAIL), exhibiting a molecular mass of approximately 63 kDa, showed a rapid clearance with a terminal half-life of 2.3 h (Figure 6a and Table 3).

Antitumor Activity. The functionalized liposomes were tested for the antitumoral activity in NMRI nude mice bearing subcutaneous Colo205 xenograft tumors. Mice received three i.v. injections of functionalized liposomes (1 μmol lipid/per application) and three i.p. injections of bortezomib (5 μg) every other day (Figure 6b). A statistically significant reduction of tumor growth was observed for the targeted TRAIL-functionalized liposomes (ILT) in comparison to that of mice treated with nt-liposomes and immunoliposomes (IL) (Figure 6c). The nontargeted TRAIL-functionalized-liposome (LT)-treated group had only marginal effects on the reduction of tumor growth (Figure 6c). In a previous study, we already demonstrated that soluble TRAIL has no antitumoral activity in a Colo205 tumor model.[17]

DISCUSSION

Through attachment of a cysteine-modified derivative of soluble TRAIL as an effector moiety and an scFv directed against EGFR as the targeting moiety to the surface of PEGylated liposomes, we have generated a long-circulating multifunctional and multivalent nanocarrier system with improved antitumoral activity. This finding is in support of recent work showing that decoration of the surfaces of liposomes and other nano- or microparticles results in increased bioactivity, presumably through mimicking the activity of cell-surface-displayed TRAIL.[20−25]

Various strategies for surface modification with recombinant TRAIL were employed in recent studies, including noncovalent adsorption[22,21,26] and chemical coupling, e.g., through amino groups exposed in TRAIL.[22−24] In the present study, we developed a modified soluble TRAIL molecule (Cys-sTRAIL) exhibiting an additional cysteine residue inserted at the N-terminus of the TRAIL polypeptide chain allowing a site-directed and defined conjugation without interference with TRAIL activity. Because of its homotrimeric nature, the modified TRAIL molecules exhibit three additional thiol groups per homotrimer, all accessible for conjugation. Theoretically, coupling of one Mal-PEG_{2000}-DSPE lipid chain per TRAIL molecule is sufficient for insertion into the lipid bilayer. SDS−PAGE analysis revealed approximately 70% coupling efficacy, indicating that on average two lipid chains are present per homotrimeric TRAIL molecule, which does not interfere with the display of active TRAIL molecules on the liposomal surface.

Additional functionalization of the TRAIL-functionalized liposomes with an anti-EGFR scFv for tumor targeting further increased the bioactivity of such bifunctional liposomes. Flow cytometry studies indicate that cell binding is mainly mediated by the scFv molecule and not through TRAIL, reflecting the rather low levels of TRAIL receptors on the tested cells. The increased bioactivity of ILTs is presumably caused by (i) increasing the concentration of cell membrane-bound particles and (ii) further stabilizing the TRAIL−TRAIL receptor interactions. Similar effects were recently observed for bivalent antibody-scTRAIL fusion proteins.[15,17] Because TRAIL-R2 (DR5) is supposed to be the major death receptor of several tumor cells and is preferentially activated by membrane-displayed TRAIL,[6] such as, e.g., in the Colo205 tumor cell line used here, we assume that TRAIL-functionalized liposomes

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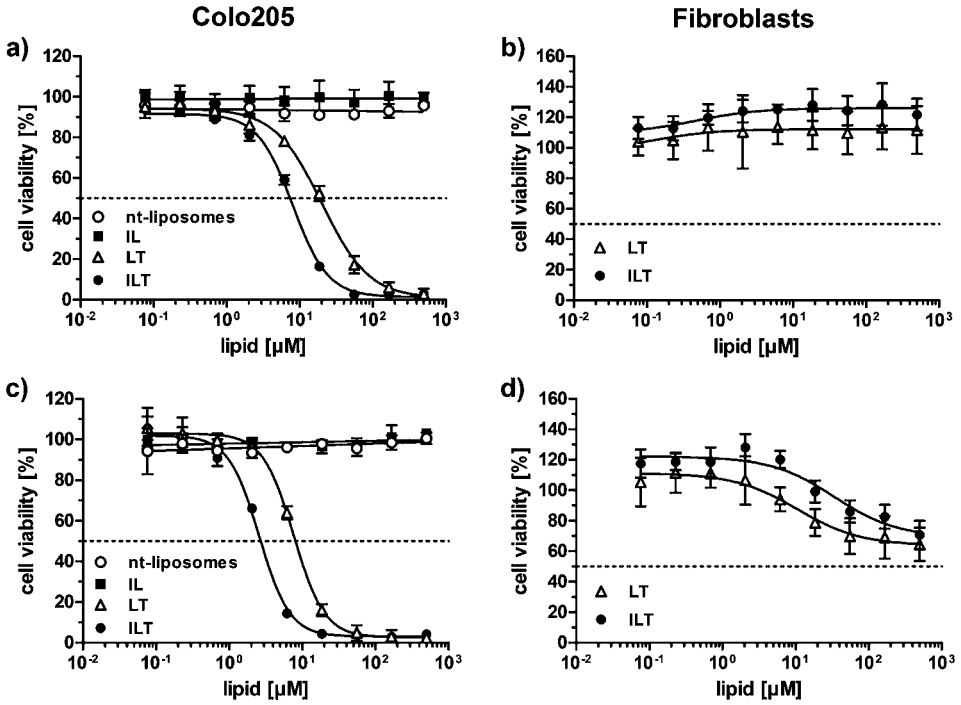

Figure 5. In vitro cytotoxicity of functionalized liposomes on Colo205 cells (a,c) and primary fibroblasts (b,d). Cytotoxicity was analyzed in the absence (a,b) or presence (c,d) of bortezomib (250 ng/mL). Cells were incubated for 16 h with the functionalized liposomes (nt = nontargeted liposomes, IL = 0.1 mol % inserted DSPE-PEG$_{2000}$-Mal-scFv, LT = 0.1 mol % inserted DSPE-PEG$_{2000}$-Mal-Cys-sTRAIL, ILT = 0.1 mol % inserted DSPE-PEG$_{2000}$-Mal-scFv, and 0.1 mol % inserted DSPE-PEG$_{2000}$-Mal-Cys-sTRAIL), and viable cells were determined by crystal violet staining.

Table 2. In Vitro Cell Death Induction (n = 3)[a]

formulation	EC$_{50}$ (μM lipid)	
	w/o bortezomib	with bortezomib
nt		
IL		
LT	20.2 ± 0.8	7.6 ± 0.2
ILT	8.0 ± 0.4	2.6 ± 0.1

[a]nt = nontargeted, IL = immunoliposomes, LT = LipoTRAIL, and ILT = immuno-LipoTRAIL.

mimic this process leading to efficient activation of both apoptosis-inducing TRAIL receptors, DR4 and DR5.

It is well-established that various substances, including the proteasome inhibitor bortezomib but also chemotherapeutic drugs and small molecule inhibitors, are capable of sensitizing tumor cells for apoptosis induction by TRAIL.[19,27] Here, we could show that bortezomib also increases the cell-death-inducing activity of liposomal TRAIL. Liposomes are established carrier systems for drugs encapsulated into the liposomal interior or inserted into the lipid bilayer.[28,29] Consequently, it should be possible to furnish our TRAIL-functionalized liposomes with TRAIL-sensitizing drugs, thus combining all properties within one pharmaceutical formulation. Feasibility was shown recently for HSA-nanoparticles surface-modified with TRAIL and transferrin and loaded with doxorubicin exhibiting synergistic cytotoxic effects[23] as well as for doxorubicin-loaded PLGA microparticles with surface-attached TRAIL.[26]

Using liposomes as a carrier system, the pharmacokinetic properties of TRAIL were dramatically increased, with an approximately 5-fold prolonged serum half-life and an approximately 13-fold increased bioavailability compared with that of soluble TRAIL. The size of the functionalized liposomes of approximately 90 nm should be small enough to enable efficient extravasation into the tumor tissue. An enhanced permeability and retention (EPR) contributes to increased accumulation and enhanced drug delivery of nanoparticulate carrier systems within tumors.[30] Importantly, the TRAIL-functionalized liposomes do not rely on release of the effector molecules from the carrier and intracellular delivery for tumor cell killing, as for most of the therapeutic drugs, but activate cell death by binding to cell-surface death receptors. The in vivo experiment indicates that targeted delivery of liposomal TRAIL indeed leads to increased antitumor activities. Further studies are required to establish efficient dosing regiments and to evaluate the safety of the TRAIL-functionalized liposomes. An analysis of a bivalent EGFR-targeting antibody-TRAIL fusion protein with intact explants from hepatocellular carcinoma (HCC) and healthy liver tissues already demonstrated selective cytotoxicity for HCC and no toxicity in tumor-free liver tissues.[16] The modular composition of the established multifunctional liposomal formulation (ILT), combining long circulation with targeted delivery and increased TRAIL-induced apoptosis by multivalent TRAIL presentation, allows to further adopt the applications to other tumor entities, e.g., by using different ligands, and furthermore to implement additional therapeutic and/or diagnostic compounds for improving the therapy of cancer.

■ CONCLUSIONS

In this study, we established a liposomal formulation composed of surface-displayed TRAIL and antibody fragments, retaining the different functional properties, i.e., target delivery mediated by the antibody fragment, efficient apoptosis induction by TRAIL, and long circulation due to PEGylation. First experiments indicate that the targeted delivery of liposomal

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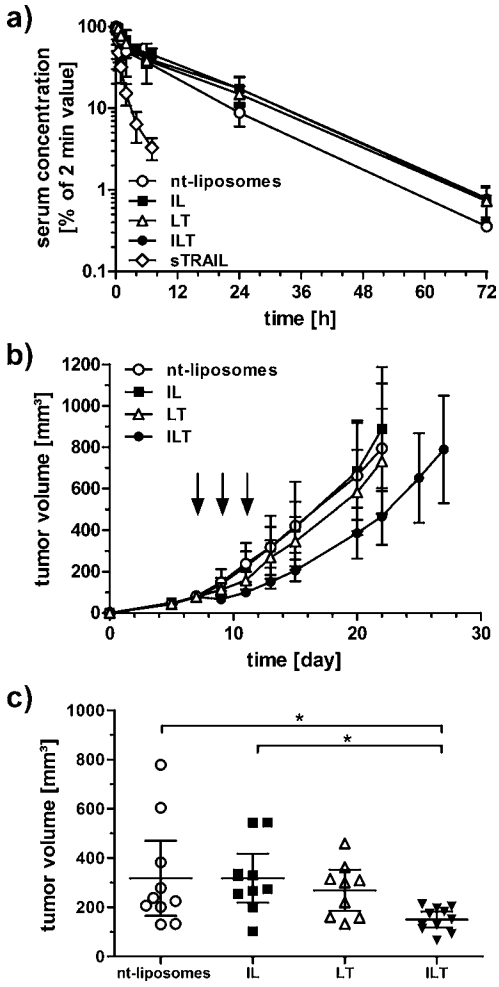

Figure 6. (a) Pharmacokinetic studies of functionalized liposomes (nt = nontargeted liposomes, IL = 0.1 mol % inserted DSPE-PEG_{2000}-Mal-scFv, LT = 0.1 mol % inserted DSPE-PEG_{2000}-Mal-Cys-sTRAIL, and ILT = 0.1 mol % inserted DSPE-PEG_{2000}-Mal-scFv and 0.1 mol % inserted DSPE-PEG_{2000}-Mal-Cys-sTRAIL) and sTRAIL molecule in mice. Functionalized liposomes (1 μmol lipid per animal) and sTRAIL (1 μg per animal) were injected i.v. into CD1 mice (n = 3). Serum concentrations of functionalized liposomes, containing DiR, were analyzed by fluorescence measurements and serum concentrations of TRAIL molecules were determined via ELISA. (b) In vivo antitumor activity of functionalized liposomes in combination with bortezomib. NMRI nude mice bearing s.c. Colo205 xenograft tumors received three i.v. injections of the functionalized liposomes (1 μmol lipid) and three i.p. injections of bortezomib (5 μg) every second day (indicated by arrows). (c) Tumor volumes at day 13. Asterisks indicate significant differences (* $p < 0.05$).

Table 3. Pharmacokinetic Properties of Liposomal Formulations (n = 3)[a]

formulation/protein	$t_{1/2}\beta$ (h)	AUC_{0-72h} (% h)
sTRAIL	2.3 ± 0.1	113 ± 31
nt	11.9 ± 0.9	561 ± 55
IL	10.8 ± 0.6	1394 ± 337
LT	11.3 ± 1.0	1216 ± 157
ILT	11.5 ± 0.3	1443 ± 396

[a]nt = nontargeted, IL = immunoliposomes, LT = LipoTRAIL, and ILT = immuno-LipoTRAIL.

TRAIL results in increased antitumor activities in a xenograft mouse model, probably attributable to antibody-mediated delivery and binding to tumor cells as well as to potent activation of TRAIL receptors 1 and 2 by mimicking the activity of cell surface-displayed TRAIL. The modular composition makes these multifunctional liposomes a versatile formulation for the treatment of different tumor entities, e.g., by using different ligands for target recognition, and further allows one to increase the therapeutic efficacy through additional drugs provided separately or encapsulated into the liposomal carrier.

EXPERIMENTAL PROCEDURES

Materials and Cell Lines. All lipids were purchased from Avanti Polar Lipids (Alabaster, USA). DiI (λ_{ex} = 549 nm; λ_{em} = 565 nm), and DiR (λ_{ex} = 750 nm; λ_{em} = 780 nm) were purchased from Sigma-Aldrich (Taufkirchen, Germany). The cell lines (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium (Life Technologies, Darmstadt, Germany) supplemented with 5% (Colo205, NCI-H460) or 10% (Jurkat, HepG2, fibroblasts) fetal calf serum (FCS; HyCLone, South Logan, UT, USA). EGFR-Fc and TRAIL-R2-Fc fusion proteins were purified from stably transfected HEK 293 cells.[17] Bortezomib and primary fibroblasts were kindly provided by Dr. T. Mürdter (Institute of Clinical Pharmacology, Margarete Fischer-Bosch Foundation, Stuttgart, Germany). Recombinant soluble TRAIL (sTRAIL; aa 114−281) was purchased from PeproTech (Hamburg, Germany) and cycloheximide (CHX) from Sigma-Aldrich (Steinheim, Germany).

Production of scFv′ and Cys-sTRAIL. DNA encoding the scFv fragment (pAB1 vector) and Cys-sTRAIL (pET15b vector) was transformed into bacteria (TG1 for scFv and BL21DE3 for Cys-sTRAIL). The humanized anti-EGFR scFv′ fragment, containing an additional cysteine residue at the C-terminus, was produced in the periplasm of the bacteria and purified by IMAC.[17] A TRAIL molecule possessing an additional cysteine residue at the N-terminus (Cys-sTRAIL) was produced in the bacterial cytosol using the expression plasmid pET15b. Protein expression was induced by adding IPTG (0.1 mM final concentration) and 50 μM $ZnCl_2$. After 3 h, cells were harvested by centrifugation (6,000g, 10 min, 4 °C), the pellet was resuspended in PBS, and cells were lysed by sonification. The supernatant was cleaned from cell debris by centrifugation. Protein was purified by M2 FLAG affinity chromatography (Sigma-Aldrich, Steinheim, Germany) as described elsewhere.[15] Protein was dialyzed against PBS containing 50 μM $ZnCl_2$ overnight at 4 °C and concentrated using Vivaspin devices (PES membrane with 10 kDa molecular weight cutoff; Sartorius Stedim, Aubagne, France). The protein concentration was measured with a spectrometer (NanoDrop) using the calculated molar extinction coefficient. Size exclusion chromatography was performed as described.[17] Aliquots were stored at −80 °C.

Generation of Liposomal Formulations. Functionalized liposomes were generated by postinsertion of scFv-coupled and/or TRAIL-coupled micelles into preformed PEGylated liposomes. The liposomes were prepared by the film-hydration-extrusion method and were composed of EPC/cholesterol/DSPE-$mPEG_{2000}$ at a molar ratio of 6.77:3:0.23. Additionally, all liposomes contained either 0.03 mol % DiI (for in vitro studies) or 0.03 mol % DiR (for in vivo studies) as fluorescent dye. Lipids and dyes were dissolved and stored in chloroform. Using a rotary evaporator, a thin lipid film was formed in a glass flask and rehydrated in 10 mM HEPES buffer at pH 7.4. The

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multilamellar vesicle dispersion was extruded through a 50 nm polycarbonate membrane using a LiposoFast extruder (Avastin, Ottawa, Canada). For the preparation of maleimide-DSPE-PEG_{2000}-micelles, chloroform was removed at RT, and the lipids were dissolved in ddH_2O. The day before, proteins (scFv′ and/or Cys-sTRAIL) were reduced in 5 mM tris(2-carboxylethyl)phosphine (TCEP; Thermo Scientific, Ulm, Germany) for 2 h at RT and dialyzed against oxygen-free 10 mM Na_2HPO_4/NaH_2PO_4 buffer and 30 mM NaCl (plus 0.4 mM EDTA in case of scFv′) at pH 6.7 at 4 °C overnight. Micellar lipids and reduced proteins were mixed in a molar ratio of 5:1 and incubated for 1 h at RT. Free maleimide groups of the lipids were quenched by adding L-cysteine (1 mM final concentration). The postinsertion step was performed with scFv-coupled and/or TRAIL-coupled micelles and PEGylated liposomes at 42 °C for 2 h. Unbound proteins were removed by gel filtration (Sepharose CL4B column; Amersham, Braunschweig, Germany). Liposomes were sedimented by centrifugation (135,000*g*, 1 h, 4 °C) and dissolved in 10 mM HEPES (pH 7.4) to a concentration of 10 nmol lipid per μL. Liposome size and polydispersion index (PDI) were measured using a Zetasizer Nano ZS (Malvern, Herrenberg, Germany).

Analysis of Coupling Efficiency. The scFv- and TRAIL-coupled micelles were analyzed by SDS−PAGE under reducing conditions and stained with Coomassie Brilliant Blue R250. The efficiency was quantified by dividing the intensity of the band of coupled proteins (upper band) by the intensity of the bands of both coupled (upper band) and noncoupled (lower band) bands using the software ImageJ.

Determination of Melting Points. Melting points of the proteins were determined by measuring thermal denaturation with the ZetaSizer Nano ZS (Malvern, Herrenberg, Germany). Approximately 100 μg of purified protein was diluted in PBS to a total volume of 1.0 mL and sterile filtered into a quartz cuvette. Dynamic laser light scattering intensity was measured while the temperature was increased in 1 °C intervals from 35 to 70 °C with 2 min equilibration for each temperature step. The melting point was defined as the temperature at which the light scattering intensity increased.

Cryo-Transmission Electron Microscopy. One droplet of the liposomal dispersions was applied to a copper grid covered by holey carbon film (Quantifoil R3.5/1 Micro Tools GmbH, Jena, Germany). An excess of liquid was blotted automatically for 3 s between two strips of filter paper. Subsequently, the samples were rapidly plunged into liquid ethane (cooled to ∼ −180 °C) in a cryobox (Carl Zeiss NTS GmbH, Oberkochen, Germany). Excess ethane was removed with a piece of filter paper. The samples were transferred with a cryo-transfer unit (Gatan 626-DH) into the precooled cryo-transmission electron microscope (Philips CM 120, Eindhoven, The Netherlands) operated at 120 kV and viewed under low dose conditions. The images were recorded with a 1K CCD Camera (FastScan F114, TVIPS, Gauting, Germany).

Flow Cytometry. Binding of liposomes to cells was determined by flow cytometry. Cells were incubated with an increasing concentration of liposomes for 1 h at 4 °C. Cells were then washed with PBS, 2% FBS, and 0.02% NaN_3 (PBA), and bound liposomes containing DiI were measured in FL2. The targeting effect was demonstrated by adding cetuximab (5 μM) to the cells. The relative mean fluorescence intensity (MFI) plotted in the binding experiment analysis was calculated according to the formula: relative MFI = MFI_{sample}/MFI_{cells}.

ELISA. ELISA studies using liposomes were performed as described.[18] Functionalized liposomes (1 mM) were added for 1 h at RT to wells coated with either EGFR-Fc fusion protein or TRAILR2-Fc fusion protein. Detection of conjugated molecules (scFv or TRAIL) on the liposomal surface was performed for the scFv fragment with HRP-conjugated anti-His-tag antibody (Santa Cruz Biotechnology) and for the TRAIL molecule with HRP-conjugated anti-FLAG-tag antibody (Sigma-Aldrich).

Cell Death Induction. Colo205 cells (5×10^4 cells per well), primary fibroblasts (1×10^4 cells per well), HepG2 cells (2×10^4 cells per well), NCI-H460 cells (2×10^4 cells per well), and Jurkat cells (1×10^5 cells per well) were grown in 100 μL of culture medium in 96-well plates for 24 h, followed by treatment in triplicate with serial dilutions of recombinant TRAIL or the functionalized liposomes. Assays were performed in the absence or presence of 250 ng/mL bortezomib or 2.5 μg/mL CHX. Before adding the serial dilutions of recombinant TRAIL or functionalized liposomes, cells were preincubated with bortezomib or CHX for 30 min at 37 °C to sensitize them to TRAIL-induced apoptosis. After 16 h of incubation, cell death was determined with crystal violet staining or MTT assay.

Pharmacokinetics. Female CD1 mice (Janvier, 8 weeks old, 3 animals per construct) received an intravenous (i.v.) injection of 1 μg of sTRAIL molecule or 1 μmol of functionalized liposomes in a total volume of 100 μL. Blood samples (50 μL) were taken at different time intervals and incubated on ice for 10 min. Clotted blood was centrifuged at 16,100*g* for 20 min at 4 °C, and serum samples were stored at 4 °C. Serum concentration of TRAIL molecules were analyzed with BD OptEIA Human TRAIL ELISA Set (BD, Heidelberg) according to the manufacturer's protocol. Serum concentrations of the functionalized liposomes containing DiR were analyzed via an odyssey reader (LI-COR Biotechnology GmbH, Bad Homburg, Germany). For comparison, the first value (2 min) was set to 100%. The terminal half-life ($t_{1/2}\beta$) and area under the curve (AUC) were calculated with Excel.

Tumor Models. Female NMRI nu/nu mice (Janvier, 8 weeks old) were injected subcutaneously (s.c.) at the left and right dorsal side each with 3×10^6 Colo205 cells in 100 μL of PBS. Treatment with functionalized liposomes was started when tumors had reached a volume of approximately 100 mm^3. Mice received 3 i.v. injections of 1 μmol of functionalized liposomes every second day (days 7, 9, and 11). Additionally, mice received 5 μg of bortezomib in 100 μL of PBS i.p. every second day (days 7, 9, and 11). Tumor growth was monitored and calculated as described.[6] Statistical analysis was performed with the ANOVA test and Tukey post-test.

■ ASSOCIATED CONTENT

Supporting Information

In vitro cytotoxicity of TRAIL-functionalized liposomes on Jurkat cells in the presence of cycloheximide and in vitro cytotoxicity and binding of TRAIL-functionalized liposomes on HepG2 and NCI-H460. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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