Peptide Drugs

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An Apoptosis-Inducing Peptidic Heptad That Efficiently Clusters Death Receptor 5

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Abstract: *Multivalent ligands of death receptors hold particular promise as tumor cell-specific therapeutic agents because they induce an apoptotic cascade in cancerous cells. Herein, we present a modular approach to generate death receptor 5 (DR5) binding constructs comprising multiple copies of DR5 targeting peptide (DR5TP) covalently bound to biomolecular scaffolds of peptidic nature. This strategy allows for efficient oligomerization of synthetic DR5TP-derived peptides in different spatial orientations using a set of enzyme-promoted conjugations or recombinant production. Heptameric constructs based on a short (60–75 residues) scaffold of a C-terminal oligomerization domain of human C4b binding protein showed remarkable proapoptotic activity (EC_{50} = 3 nM) when DR5TP was ligated to its carboxy terminus. Our data support the notion that inter-ligand distance, relative spatial orientation and copy number of receptor-binding modules are key prerequisites for receptor activation and cell killing.*

Derived from Greek and meaning the falling off of leaves, the term "apoptosis" signifies a programmed set of events leading to cell death. In the human body, apoptosis is responsible for the elimination of aged, damaged, or self-reactive cells. This cell suicide can be induced by certain molecular triggers, with DNA damage and cell distress being internal signals that activate a pro-apoptotic cascade that leads to the mitochondrial release of apoptogenic factors into the cytosol.[1] As a result, the apoptosis-initiating protease caspase-9 is recruited and activated by the apoptosome complex, thus inducing a further cascade of executioner proteases (caspase-3, -6, and -7), which culminates in cell lysis.[1a,2]

Alternatively to intrinsic stimuli, external triggers initiate apoptosis upon binding to the so-called death receptors (DR) located on the cell surface, thus promoting their oligomerization. This causes the formation of the death-inducing signaling complex (DISC) and activation of caspase-8 and -10, which actuate a cascade of executioner caspases. Among these external agents, the tumor necrosis factor (TNFα) related apoptosis-inducing ligand (TRAIL) is of major importance as it promotes apoptosis in cancer cells upon binding to the death receptors 4 and 5 (DR4 and DR5). Native TRAIL is a type II transmembrane protein that possesses an architecture of a homotrimer. It binds apoptosis mediating DR4 and DR5, as well as three decoy receptors, namely DcR1, DcR2, and osteoprotegrin (OPG), which do not induce apoptosis.[3]

TRAIL specifically triggers apoptosis in tumor cells while sparing healthy cells and tissues.[4] This makes TRAIL and its receptors promising candidates for cancer therapy.

To date, several TRAIL-mimicking molecules and antibodies directed against DR4 or, more often, DR5 have been generated, among them several monoclonal antibodies.[5] To gain cytotoxic activity at least in cell culture experiments, these bivalent binders require a secondary cross-linking agent that mediates antibody oligomerization in vitro.[6]

Soluble recombinant TRAIL and its DR4- or DR5-specific engineered variants, as well as single-chain variable antibody fragments (scFvs), multimerized camelid single domain antibody domains (nanobodies), and several DR5-targeting oligopeptides have been reported to date (Supporting Information, Table S4).[1a,7] Using recombinant screening, a DR5-targeting peptide (DR5TP) was identified, which competes with TRAIL upon binding to the receptor.[8] This pentadecapeptide possesses an extended DR5 binding loop, which is stabilized by a disulfide bond between two cysteines (Scheme 1 and the Supporting Information).[8] Solitaire DR5TP does not induce apoptosis in cancer cells even at millimolar concentrations,[9] whereas its crosslinked bi- and trivalent derivatives (Figure S1 A–D) trigger this process in DR5-positive cells.[9] Interestingly, the hexavalent adamantane-based dendrons (Figure S1 D) showed no improvement of cytotoxic activity in cellular assays compared to the trimeric adamantane counterpart (Figure S1 B, Table S4).[10]

Herein, we report on DR5-specific constructs that induce apoptosis in tumor cells (Scheme 1). We designed macromolecular architectures bearing multiple DR5 binding motifs and assembled them using scaffold-assisted enzyme-promoted conjugation (Scheme 2). As a binding moiety we chose disulfide-linked DR5TP (Schemes 1 and 2) as this peptide revealed no binding to DR4, DcR1, or DcR2.[9] In this peptide, the *N*-terminal tryptophan, two residues within the disulfide loop and the *C*-terminus are crucial for binding to the receptor.[8]

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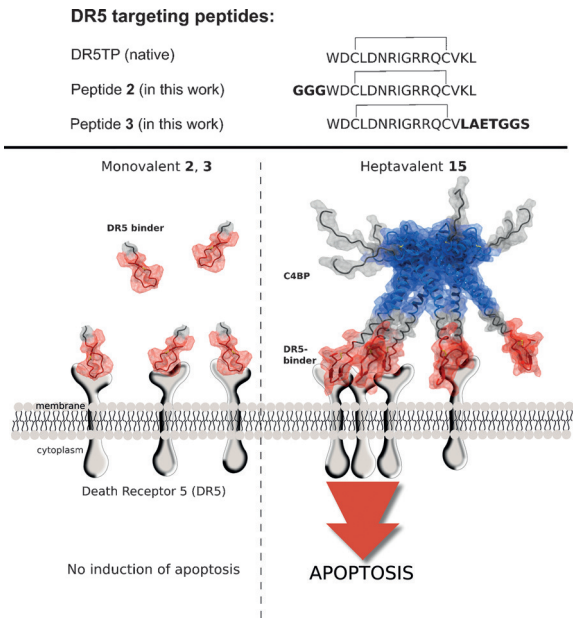
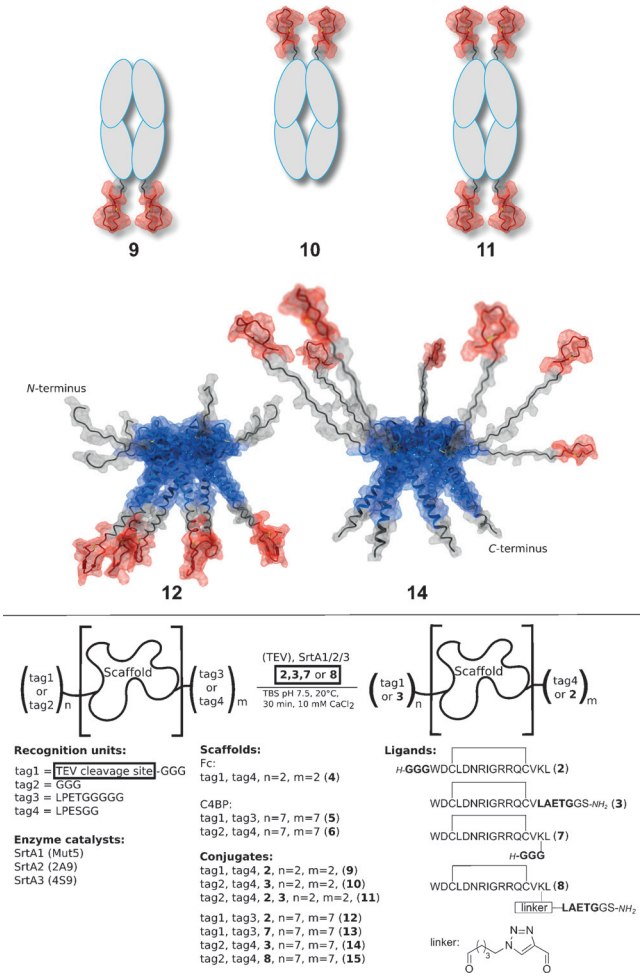


Scheme 1. Binding of DR5TP to DR5 on cancer cells. Left: Monovalent DR5TP (sequence is indicated) is not able to induce apoptotic signaling. Right: DR5TP attached to the *C*-terminus of C4BP binds to DR5 in a heptavalent manner and induces apoptosis.



Scheme 2. Scaffolds used in this work. Top: Representation of bi- and tetravalent Fc conjugates **9**, **10**, and **11** and heptads **12** and **14**. Bottom: Conjugation of synthetic DR5TP-based ligands **2** and **3** onto scaffolds **4**, **5**, and **6** using sortase A-mediated ligation. For *N*-terminal ligation tag1 is cleaved by TEV protease, liberating tag2, which is then conjugated with peptide **3** under SrtA2 catalysis.

The number of ligand copies was specified by the biomolecular template taken for its oligomerization (Scheme 2). First, we considered the Fc part of human IgG (Scheme 2), which has been reported to serve as a scaffold for di- and tetramerization of peptides.[11] This framework retains desirable attributes of antibodies, that is, prolonged plasma half-life and high apparent affinity due to its bivalent avidity. Another candidate, a scaffold based on the α-chain of the *C*-terminal oligomerization domain of human C4b binding protein[12] (C4BP, Scheme 1 and 2), which is a plasma component without biological function due to the lack of complement control proteins (CCP),[13] allows assembly of seven DR5TP units. This construct can be easily produced recombinantly as a thioredoxin fusion along with spontaneous formation of seven disulfide bridges, which yields a stable heptavalent architecture (Section S1.4, Figure S4).[12] As an additional advantage, conjugation with this scaffold that consists of seven α-helical units, each comprising approximately 60 residues, results in a significantly enhanced plasma half-life of ligated molecules.[12,14]

Generally, the choice of an oligomerization scaffold is of major significance as upon binding to DR5 valence and spatial positioning of the ligand seems to play the key role for efficient death signaling.[8–10] Thus, the selected biomolecular frameworks, namely an Fc antibody domain **4** and C4BP derivatives **5** and **6**, were tailored for the decoration with two, four, or seven copies of DR5TP-derived ligands **2** and **3** (Scheme 2). To localize the tethered binding units in the target oligomers at defined positions, we introduced enzyme recognition sites and attached DR5TP-based fragments by an enzyme-catalyzed coupling (Scheme 2). For this purpose we applied engineered sortases referred to as SrtA1-3 (Table S1), which catalyze formation of an amide bond between glycine and β-hydroxy-bearing amino acids (Ser, Thr) located, respectively, at the *N*-terminal oligoglycine and the *C*-terminal $LX_iEX_{ii}G$ counterparts (Scheme 2, Figure S2).[15] The used sortases were tailored to recognize certain peptide tags enabling modularity of ligand attachment (Section S1.6, Figure S2, S3, and Table S2). Thus, the Fc scaffold **4** was designed to carry two payloads either *C*- or *N*-terminally (conjugates **9**, **10**, Figure 2, Table S3), and 4 payloads (two at the *C*- and two at the *N*-terminus; construct **11**, Figure S3). The scaffold of C4BP was loaded with seven DR5-binding motifs, either *C*- or *N*-terminally (constructs **5, 6**, **12–15**; Figure 2, Table S3).

Tagged DR5-binding ligands (Scheme 2, Table S3) were assembled by Fmoc-SPPS followed by disulfide bond formation (**1–3**, **7** and **8**; Figure S9–S14, Table S3). To study the influence of the anchoring position on the efficiency of enzyme-catalyzed coupling and bioactivity, in peptides **7** and **8** conjugation tags were installed via the side chain of lysine (Sections S1.5 and S4).

Covalent grafting onto scaffolds **4**–**6** lead to formation of oligomeric constructs **9**–**15**. Enzyme-promoted reactions were monitored by SDS-PAGE (Figures S15–S18) and showed quantitative conversion within 30 min at 22 °C for constructs **9**–**13** (Figures S15–S17). Conjugates were isolated by size-exclusion chromatography. Multivalent constructs **9**–**13, 16**, and **17** as well as biotinylated monomer **1** were examined for DR5 binding using enzyme-linked immunosorbent assay (ELISA, Figure 1). In our hands, **1** bound DR5 with impaired binding ($K_d = 2.1$ μM, Table 1) due to an unfavorable orientation of ligands. Accordingly, in the case when a sortase tag was connected with the binding motif via the side chain of an additional lysine (peptides **7** and **8)**, their conjugation with scaffold **6** was hindered to a large extent and resulting constructs **13** and **15** showed low or no affinity to DR5 receptor (Table 1). This indicates that the orientation of the conjugated peptide plays a crucial role upon multivalent binding.

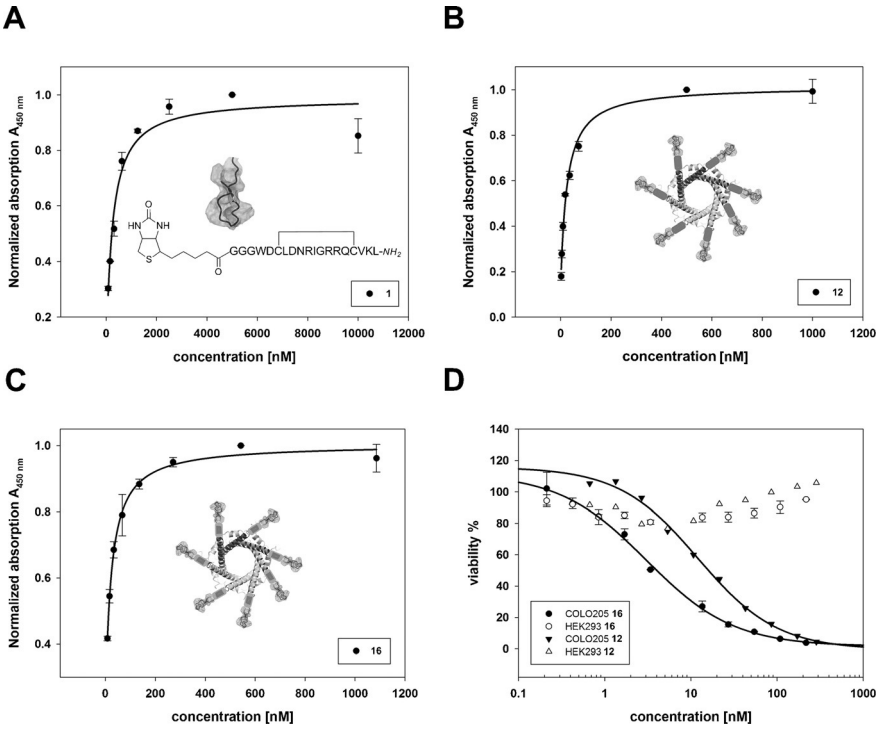


Figure 1. ELISA binding studies and cytotoxicity assay. A) Binding of monovalent **1** to DR5 ($K_d = 240$ nM). B) Binding of sortase A conjugated heptavalent construct **12** to DR5 ($K_d = 25$ nM). C) Binding of fusion protein 16 to DR5 ($K_d = 23$ nM). D) Cytotoxic activity of **12** ($EC_{50} = 12$ nM) and **16** ($EC_{50} = 3$ nM) on COLO205 cells (HEK293 cells were used as negative control).

a dissociation constant (K_d) of 240 nM (Figure 1 A; reported K_d of 129 nM was determined for the unlabeled peptide by surface plasmon resonance).[9] Compared to **1**, bivalent conjugate **9** with peptide ligands located at the *C*-termini of an antibody Fc fragment showed an almost 3-fold improved affinity ($K_d = 92$ nM), while the binding capacity of its *N*-terminal counterpart **10** was slightly decreased ($K_d = 134$ nM), and the tetravalent conjugate **11** demonstrated comparable affinity ($K_d = 84$ nM, Table 1, Figure S5).

With its K_d of 25 nM, conjugate **12** bearing seven copies of peptide **2** which are located at the *C*-termini showed the strongest potency upon binding to DR5 receptor (Figure 1, Table 1). This construct along with the *N*-terminally functionalized variant was also recombinantly produced in *E. coli* cells resulting in genetically fused compounds **16** (C4BP-DR5TP) and **17** (DR5TP-C4BP) (Figure S17).

A dissociation constant of 23.1 nM for **16** accords with that observed for enzymatically ligated **12** (Table 1, Figure 1 C). In contrast, the *N*-terminally fused **17** exhibited strongly

The ability to induce apoptosis was examined using colorectal cancer cell line COLO205 (Figure 1). Even at the concentration of 1000 μM no induction of apoptosis was observed for monovalent **2.** C4BP-based heptads bearing fused or ligated DR5-binders at the *C*-termini exhibited good cytotoxic activity (Figure 1 D), while their *N*-terminal counterparts did not induce apoptosis (data not shown). Interestingly, recombinantly produced heptamer **16** was more potent compared to the sortase-conjugated heptad **12** (EC_{50} of 3 nM vs. 12 nM, respectively; Figure 1 D). As assumed, the conjugates that showed impaired binding to DR5 (**13, 14, 17**) did not induce apoptosis in vitro. Surprisingly, no apoptotic signaling was observed for the Fc-based bi- and tetravalent DR5TP constructs (**9**–**11**) even in the presence of cross-linking agents (protein G and *anti*-Fc IgG, data not shown). Compound **12** that possessed, compared to the other heptameric constructs, the closest arrangement of its seven receptor-binding peptides induced the strongest response. In view of this finding it is tempting to speculate that dense multivalent local clustering of a DR5 targeting peptide is a prerequisite for efficient receptor activation.

We additionally investigated the most potent heptad **16** in an AnnexinV FACS assay, which allows for the discrimination of apoptotic and necrotic cells (Figure 2 A–D, Section S1.9).

Table 1: Binding affinity (K_d) towards DR5 and apoptotic potency (EC_{50}) of synthesized constructs.

Construct	Conjugate	Valence	K_d [nM]	EC_{50} [nM]
1	–	1	240	$>1\times10^6$
9	Fc-DR5TP	2	92	>1400
10	DR5TP-Fc	2	133	>1000
11	DR5TP-Fc-DR5TP	4	84	>1150
12	C4BP-DR5TP	7	25	12
13	C4BP-DR5TP	7	1061	>500
14	DR5TP-C4BP	7	–	>580
15	DR5TP-C4BP	7	–	n.d.
16	C4BP-DR5TP	7	23	3
17	DR5TP-C4BP	7	2100	>4000

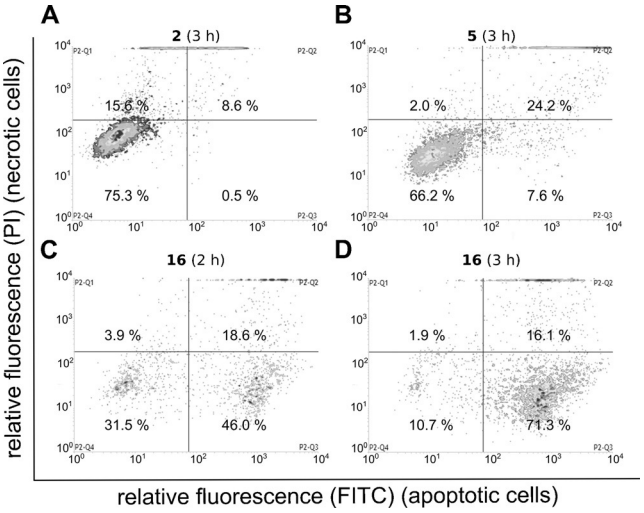


Figure 2. AnnexinV-FITC and propidium iodide staining of COLO205 cells. A) Cells incubated with monovalent **2** for 3 h. B) Cells treated with heptavalent **5** for 3 h. C) and D) Cells treated with fusion protein **16** for 2 h and 3 h, respectively; negative control: HEK293 cells (Figure S7).

We found that in presence of **16** 40 % of the examined cells underwent apoptosis after 2 h (Figure 2C), and after 3 h the portion of apoptotic cells reached 70 % (Figure 2D). However, it required higher concentration of DR5-binding agent compared to the natural ligand TRAIL (Figure S6).

To conclude, heptavalent constructs **12** and **16** efficiently induced apoptosis without a secondary antibody often required to crosslink DR5- or DR4-specific antibodies.[6] To the best of our knowledge, for the first time oligomerization on recombinant scaffolds improved the cytotoxic activity of DR5TP. Our data indicate that crosslinking of DR5 strongly depends on the orientation and spatial organization of the ligands on the scaffold. We show that C4BP could be used as a platform of human origin to oligomerize target-binding peptides in a modular way using an efficient enzymatic approach. Additionally, we demonstrated that the most potent candidates can be easily produced recombinantly without sacrificing activity. This ranges the C4BP-based symmetric, compact and stable multivalent scaffolds as viable platforms for the enhancement of bioactivity of small binders, especially when multiple valence is of peculiar interest (as in the case of targets inducing apoptosis upon crosslinking of the receptors, that is, DR4 and DR5). For additional experiments with C4BP-fused alternative DR5 binders, see Figure S8.

Recently, apoptosis-inducing oligomeric DR5-targeting nanobodies were developed (EC_{50} at the subpicomolar range; Table S4).[7b] However, their clinical study was terminated due to unexpected liver toxicity.[16] Several factors might have contributed to this effect, among them immunogenicity and induction of DR5 expression on hepatocytes due to the particularly high potency of the nanobody leading to an undesired activation of hepatocyte apoptosis.[7b,16] These findings indicate that fine-tuning of potency and immunogenicity requires special consideration upon the design of next-generation apoptosis-inducing compounds. It will be interesting to elucidate whether our modular approach may open new avenues for the development of potent and safe modulators of tumor growth.

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Keywords: apoptosis · C4BP · death receptor 5 · oligomerization · TRAIL mimicking peptide

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