Protein–Protein Interactions

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Design and Synthesis of Highly Potent and Plasma-Stable Dimeric Inhibitors of the PSD-95–NMDA Receptor Interaction**

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Protein–protein interactions (PPIs) mediate numerous regulatory pathways, which are vital for normal biological processes and disease development.[1] Inhibitors of PPIs are therefore of great value to unravel complex cellular phenomena and as potential therapeutics.[2] Postsynaptic density protein-95/discs large/zonula occludens-1 (PDZ) domains are part of scaffold and adaptor proteins involved in the assembly of cellular signaling complexes, typically by recognizing the C terminal of the interacting proteins.[3] They consist of about 90 amino acids and are present in great number in multicellular organisms; in humans 256 different PDZ domains are found in 142 different proteins.[4] Inhibition of PDZ-domain-mediated PPIs represents a promising strategy for specific therapeutic intervention of signaling events rather than targeting entire signaling cascades by receptor antagonists.[5]

Extensive activation of the *N*-methyl-D-aspartate (NMDA) receptor by glutamate,[6] as seen in excitotoxicity, plays a key role in several brain diseases. However, development of drugs that directly modulate the NMDA receptor has been difficult.[5c,6,7] Instead, inhibition of the ternary complex of the NMDA receptor, neuronal nitric oxide synthase (nNOS), and postsynaptic density protein-95 (PSD-95) attenuates glutamate-induced cell death by impairing the functional link between Ca^{2+} entry and NO production without affecting vital synaptic transmission (Figure 1a).[5e,8] Thus, PSD-95 constitutes a promising target for neuroprotective drugs. Uncoupling of PSD-95 and the NMDA receptor subunit, GluN2B, has been achieved by a 20-mer peptide (Tat-N2B), which mimics the C terminal of GluN2B and thereby inhibits the nNOS–NMDA receptor linkage by binding to PDZ1 or PDZ2 of PSD-95 (Figure 1). This peptide is currently in clinical trials as a potential treatment of stroke.[8,9]

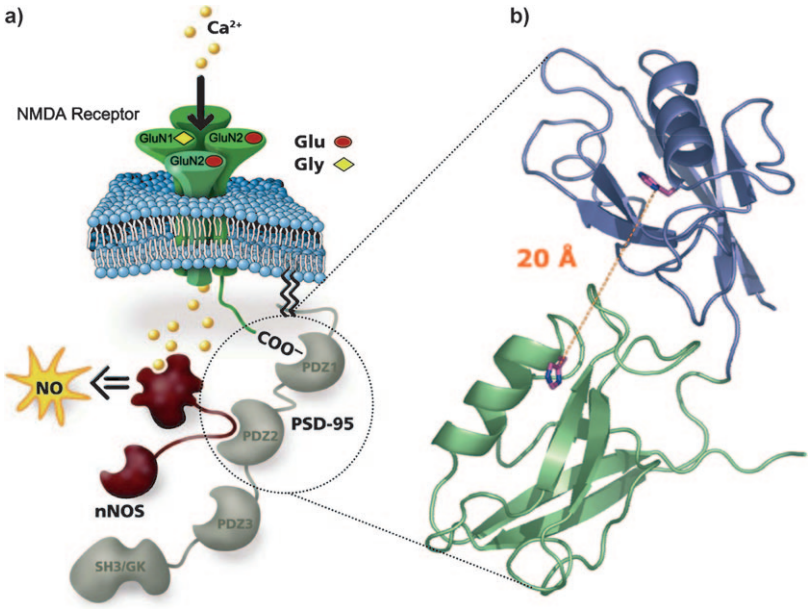

Figure 1. a) PSD-95 facilitates Ca^{2+}-mediated excessive NO production during excitotoxicity, which can be impaired by inhibition of the PSD-95–NMDA receptor interaction. b) Structural model of PDZ1-2, where two conserved histidine residues, important for ligand binding, and their relative distance are highlighted.[11] SH3/GK = src homology 3/guanylate kinase; src proteins cause sarcomas.

PDZ domains are known for their promiscuous ligand recognition, and together with their plentiful representation in the human genome it is likely that compounds directed towards individual PDZ domains of PSD-95 are not very selective.[10] Herein, we focus on dimeric ligands that bind PDZ1 and PDZ2 of PSD-95 simultaneously. Dimerization should not only increase affinity[11,12] but also enhance selectivity,[5d] since the tandem PDZ1-2 motif (Figure 1b) is structurally more unique than individual PDZ domains.

We have previously shown that a pentapeptide (**1**, IESDV), which corresponds to the extreme GluN2B C-terminal sequence, is sufficient to bind the PDZ1 and PDZ2 domains of PSD-95 with similar affinity as the GluN2B

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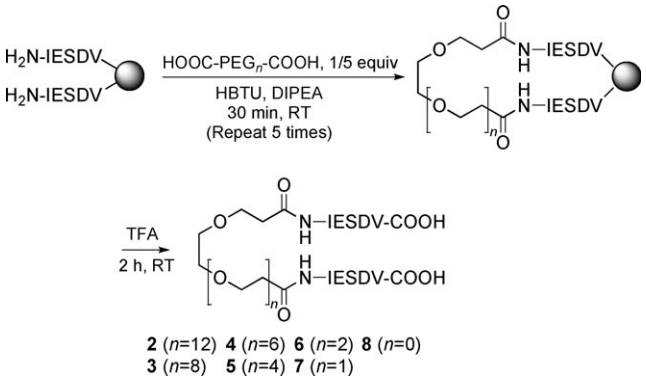
undecapeptide, while further truncation impairs binding.[13] PDZ1 and PDZ2 have similar selectivity profiles,[10,13] and we therefore focused on designing symmetric dimeric ligands. As a starting point we used pentapeptide **1** as the ligand, and selected monodisperse polyethylene glycol (PEG) linkers for dimerization, since PEG linkers are structurally flexible and known to impose favorable pharmacokinetic properties.[14] Previously, dimeric peptide ligands have been synthesized by activating polydisperse PEG diacids as pentafluorophenyl (Pfp) esters followed by cross-linking of the peptides by addition of Pfp-PEG diester in the presence of 1-hydroxybenzotriazole, which was repeated five times over five days.[15] In our hands, the synthesis of dimeric ligands by this method with monodisperse PEG diacids resulted in an average yield of 21%. However, we found that direct coupling of PEG diacids to the peptides using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) allowed much shorter reaction times (5×30 min) and gave increased yields (30%; Scheme 1).



Scheme 1. Synthesis of dimeric compounds **2**–**8**. DIPEA = *N*,*N*-diisopropylethylamine, TFA = trifluoroacetic acid.

Initially, we synthesized dimeric ligand **2** by cross-linking resin-bound peptide **1** with PEG12 diacid ($n = 12$; Scheme 1). This linker has a linear distance of about 50 Å, which we considered to be a suitable starting point for enabling simultaneous ligand binding to both PDZ domains (Figure 1b).[16] The ligand was evaluated for binding affinity in an in vitro fluorescence polarization (FP) assay, which measures displacement of a peptide probe (Cy5-GluN2B) by the compound of interest.[13] A dramatic increase in affinity was observed for dimeric ligand **2** (inhibition constant $K_i \leq 0.1$ μM) compared to **1** ($K_i = (2.9 \pm 0.10)$ μM) when tested at the tandem PDZ1-2 construct. In contrast, **2** showed affinities similar to **1** at the individual PDZ1 and PDZ2 domains (Figure 2 and Table S1 in the Supporting Information), which suggests that **2** binds as a dimer towards PDZ1-2.

Encouraged by this substantial increase in potency, we explored the importance of linker length, and peptide **1** was dimerized with PEG_n diacids where $n = 8$, 6, 4, 2, 1, and 0 to give compounds **3**–**8**, respectively (Scheme 1). Testing of **3**–**8** in the FP assay using PDZ1-2 revealed that compounds **3**–**5** displayed affinities similar to that of **2**, whereas **6**–**8** showed a stepwise increase in K_i (Figure S1 and Table S1 in the Supporting Information). Thus, PEG linkers with $n = 12$, 8, 6, or 4 are favored over those with $n = 2$, 1, or 0. However, the affinities measured in the FP assay for compounds **2**–**5** were too high for determining accurate binding constants (K_i values).[17]

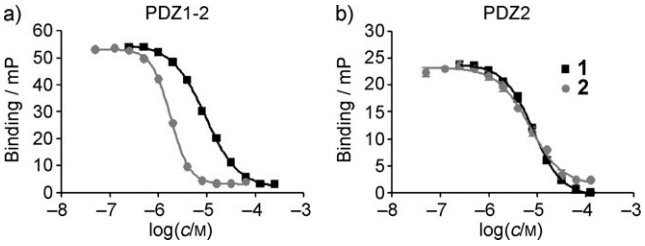


Figure 2. FP inhibition curves for compounds **1** and **2** towards PDZ1-2 (a) and PDZ2 (b) of PSD-95.

To preclude the possibility that the PEG linker contributes to the increased affinity through a direct interaction with PDZ, we first verified that the dimeric ligand containing the nonbinding pentapeptide IEAAA (**9**) was inactive. Moreover, dimerization of nonbinding peptide IESDD with **1** to provide the asymmetric dimeric ligand **10** resulted in a two- to fourfold decrease in affinity relative to **1**. Identical results were obtained for the mono-PEGylated derivative of **1**, compound **11**. These data clearly suggest that the PEG linker itself possesses no affinity towards the PDZ domains of PSD-95 (Table S1 in the Supporting Information).

Since the affinities of **2**–**5** were too high for accurate determination of K_i values by FP, we performed isothermal titration calorimetry (ITC), which provided reliable dissociation constant (K_d) values as well as information on thermodynamic parameters (enthalpy ΔH, entropy ΔS, and Gibbs energy ΔG) and the stoichiometry between ligand and protein. The ITC experiments confirmed that compounds **2**–**5** were significantly more potent than **6**–**8** and established PEG4 as the optimal linker. Hence, compound **5** displayed the lowest K_d value of only (32 ± 1.7) nM and is thereby an exceptionally potent inhibitor of PPIs in general and 100-fold more potent than **1**. Compound **4** (PEG6 linker) was almost equipotent to **5**, whereas **2** and **3** (PEG12 and PEG8, respectively) were slightly less potent (Figure 3 and Table S2 in the Supporting Information). In general, these results support a recent finding that increasing the PEG-linker length beyond its optimum value only results in a

a)

Compd.	K_d
1	3200 ± 160
2	90 ± 15
3	128 ± 16
4	39 ± 0.22
5	32 ± 1.7
6	223 ± 6
7	463 ± 22
8	1400 ± 55
10	10700 ± 2000
11	9750 ± 2200

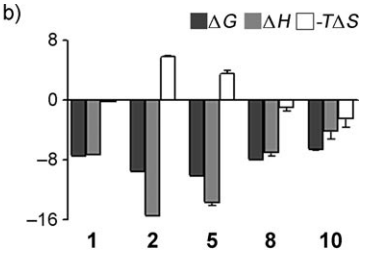


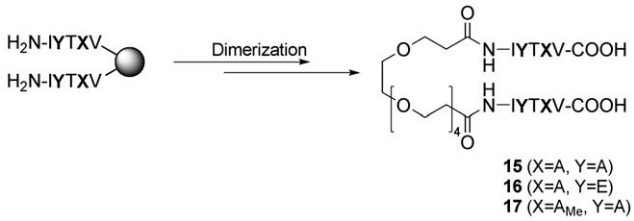
Figure 3. a) K_d values in nM from ITC shown as mean ± standard error of the mean (SEM). b) Thermodynamic bar graphs for selected compounds (ΔH, $-T\Delta S$, ΔG; all in kcal mol^{-1}) towards PDZ1-2.

relatively weak effect on affinity, whereas shortening the linker leads to a more substantial reduction in affinity.[16]

The ITC data also revealed a number of important features of the dimeric ligands. First, the dimeric ligands bind in a 1:1 relationship with PDZ1-2, whereas monomeric peptide **1** binds in a 2:1 relationship as expected (Table S2 in the Supporting Information). Second, dimerization of **1** with appropriate PEG linkers, compounds **2**–**5**, favors PDZ binding by a substantial enthalpy decrease, which, however, is associated with a considerable entropy penalty. A reduced decrease in the enthalpy is seen for compounds **6**–**8**, and although the entropy penalties are concomitantly smaller, binding affinities are reduced compared to those of **2**–**5**, as also observed in the FP assay. Third, it is noticed that the entropy penalty in general increases with linker length (Figure 3, and Figure S2 and Table S2 in the Supporting Information).

To further characterize the binding mechanism, we performed kinetic studies by using fluorescence intensity measurements. By applying PDZ1-2 constructs with a tryptophan residue introduced in either PDZ1 (PDZ1*-2) or PDZ2 (PDZ1-2*), we determined the kinetic rate constants by measuring the altered fluorescence properties as a result of ligand binding.[18] It was observed that the dimeric ligand **5** increased the off-rate constants in the individual PDZ domains two- to threefold, but **5** dramatically decreased the off-rate constants by six- to ninefold compared to **1** at PDZ1-2 (Figure S3 and Table S3 in the Supporting Information). On-rate constants were increased for **5** relative to **1**, for both PDZ2 and PDZ1-2 to a similar degree (data not shown). The increased affinity of dimeric ligands compared to monomeric ligands can therefore be explained by the decreased dissociation rate constants, which supports the view that **5** simultaneously binds to both PDZ1 and PDZ2 in PDZ1-2. In agreement with the dissociation kinetics and the ITC measurements, compound **5** binds PDZ1-2 in a 1:1 stoichiometry as determined by fluorescence endpoints from the kinetic data (Figure S4 in the Supporting Information).

Having optimized the linker length and verified the binding mechanism, we focused on optimization of the ligand moiety. We have previously carried out structure–activity relationship (SAR) studies of monomeric penta- and tetrapeptides, which, for example, revealed that substitution of serine with a threonine moiety increases the affinity twofold.[13] Based on these studies, the monomeric ligands IATAV (**12**), IETAV (**13**), $IATA_{Me}V$ (**14**), and their corresponding dimeric ligands (**15**–**17**) were synthesized (Scheme 2) and tested in the FP assay. As demonstrated for compounds **2**–**5**, no change in affinity



Scheme 2. Synthesis of dimeric compounds **15**–**17**.

towards individual PDZ domains was seen, but a dramatic increase in affinity towards PDZ1-2 was observed for dimeric ligands **15**–**17** (Figure 4a and Table S1 in the Supporting Information).

The affinity of compound **16** was too high to be accurately quantified by FP, hence ITC was performed, which revealed an exceptionally high affinity towards PDZ1-2 with a K_d value of (9.8 ± 1.6) nM (Figure 4b and Table S2 in the Supporting Information). This corresponds to a 145-fold increase compared to monomeric ligand **13**, and compound **16** is to the best of our knowledge the most potent PDZ domain inhibitor yet described. Furthermore, a strong linear correlation with slope > 1 between the logarithmic K_d values for monomeric versus dimeric ligands was observed (Figure 4c), which means that the effect (fold-change) of dimerization increases with increasing affinity of the monomeric ligand (Figure 4c and Table S4 in the Supporting Information).

FP, ITC, and kinetic studies demonstrated that the remarkable affinities of the dimeric ligands result from linking two ligands together, thereby enabling simultaneous

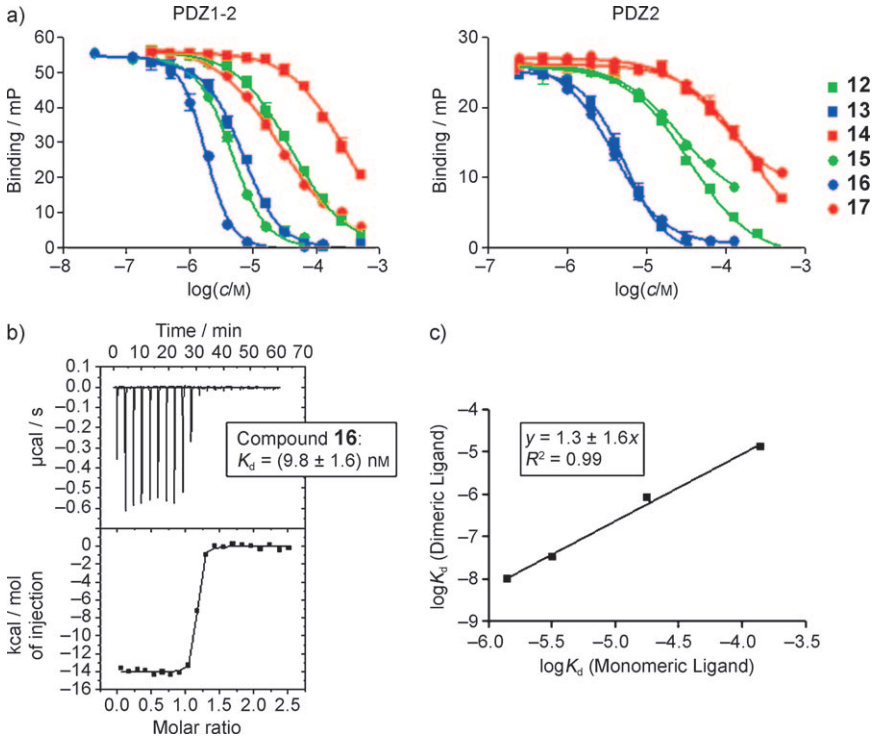


Figure 4. a) FP inhibition curves for compounds **12**–**17** towards PDZ1-2 (left) and PDZ2 (right) of PSD-95. b) ITC data for compound **16** towards PDZ1-2. c) Relationship between log K_d values for monomeric ligands and the corresponding dimeric ligands.

binding to both PDZ domains. Moreover, the PEG linker itself has no apparent affinity towards the PDZ domains. To examine this in a structural context, we performed molecular modeling studies. Two models of the PDZ1-2 structure, with and without peptide ligand, have been generated by NMR structure elucidation combined with modeling and molecular dynamics simulations.[11,19] We observed that compound **2** with the long PEG12 linker is well accommodated into both models, with a curled linker in the ligand-free model and an extended linker in the other (Figure 5a). In contrast, the more

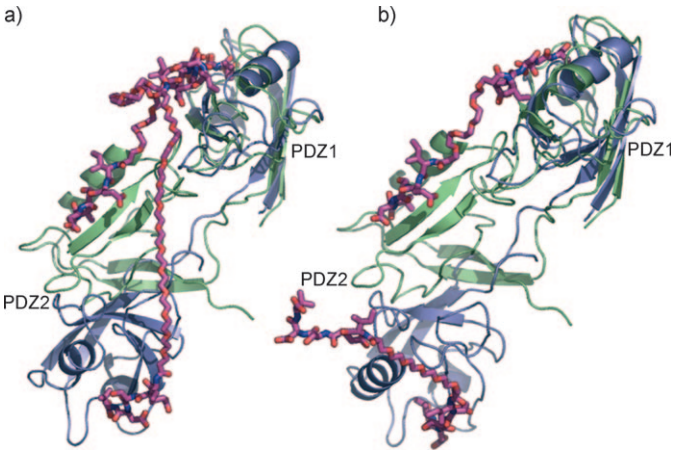


Figure 5. Modeling studies of dimeric ligands. a) Compound **2** and b) compound **5** in association with two different models of PDZ1-2. Ligands are shown in magenta, the ligand-free model[11] in green, and the ligand-bound model[19] in blue. In each case the PDZ1 domains of the two models have been aligned.

potent ligand **5** with the shorter PEG4 linker only fits into the ligand-free model with an extended linker, but is too short to bind both PDZ1 and PDZ2 in the ligand-bound model (Figure 5b). Thus, modeling confirms that the dimeric ligands can bind both PDZ domains in PDZ1-2, which explains the observed increase in affinity and decrease in enthalpy, as well as the decreased off-rate constants compared to monomeric ligands. Also, the accessible binding cavity of PDZ1-2 allows binding of the peptide ligand without any apparent accompanying interactions with the PEG linker (Figure 5).

The modeling studies also provide insights into the basis for the entropy penalties observed for the dimeric ligands, which could arise from restraining the ligand, the protein, or both. It has been suggested that binding of monomeric peptide ligands induces interdomain mobility and protein flexibility.[11,19] This interdomain mobility can only be facilitated for PDZ1-2 when bound to **2** but not **5**, according to the modeling studies, which in terms of protein flexibility suggests that **2** binds more favorably than **5**. However, ITC experiments reveal that the entropy penalty paid by **5** is smaller than that for **2**, thus indicating that confinement of the ligand, and not PDZ1-2, is more decisive in the observed entropy penalties of the binding reaction. This is important not only from a mechanistic point of view, but also in the design of future dimeric ligands.

Peptide-based ligands are generally subject to enzymatic cleavage by proteases in vivo, which often is the major limiting factor for advanced biological studies.[14] We therefore evaluated the stability of the ligands in blood plasma, and found that the dimeric ligands showed superior stability (Figure 6 and Table S5 in the Supporting Information). The

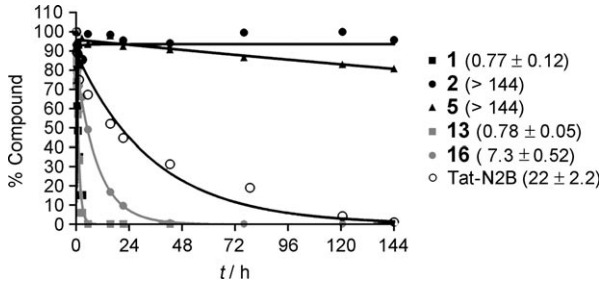


Figure 6. In vitro stability in human blood plasma at 37 °C for key dimeric (**2**, **5**, and **16**) and monomeric ligands (**1** and **13**) and Tat-N2B. Half-lives ($T_{1/2}$ [h]) are shown in parentheses (± SEM).

monomeric peptides (**1** and **12**–**14**) were degraded relatively fast with half-lives ($T_{1/2}$) of less than 1 h, but dimerization with PEG linkers led to a seven- to ninefold increase in $T_{1/2}$ in blood plasma (**15**, **16**) or even complete resistance towards degradation as measured over a time period of 6 days (**2**–**5**, **17**). The PEGylated monomeric ligand (**11**) demonstrated an approximately 14-fold increase in $T_{1/2}$ relative to **1**; thus, the PEG linker is the primary factor in mediating the increased stability. However, since compounds **2**–**5** are still significantly more stable than **11**, the dimerization per se contributes to stability. Thus, both the presence of PEG linkers and the dimerization of ligands promote plasma stability.

In conclusion, we have designed and synthesized remarkably potent dimeric inhibitors of the PSD-95–NMDA receptor interaction by linking pentapeptide ligands with monodisperse PEG linkers. Optimization of linker length and the peptide moiety guided by FP and ITC assays led to the identification of compound **16** as the most potent compound with a K_d value of (9.8 ± 1.6) nM, which is an unprecedented affinity for a PDZ-mediated PPI. Furthermore, this compound represents a 1000-fold improvement in terms of affinity compared to the clinical candidate, Tat-N2B.[13] These dimeric ligands could serve as excellent molecular tools for studying interdomain flexibility of the PDZ1-2 domain of PSD-95, and are candidates for further exploration towards development of in vivo neuroprotective compounds. Finally, our studies represent a general and versatile strategy for targeting tandemly arranged PDZ domains while achieving high potency, selectivity, and blood-plasma stability.

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