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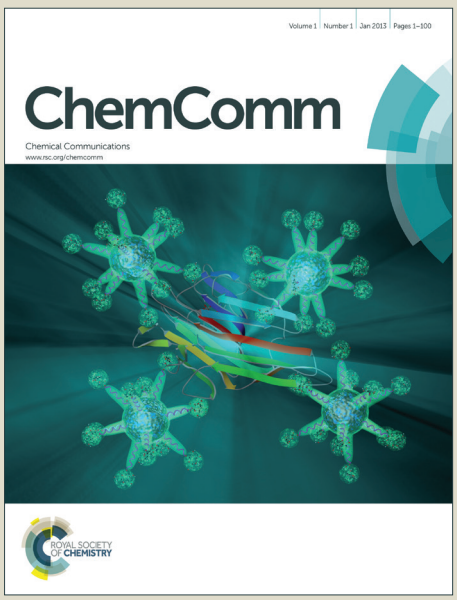

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Designed Cell Penetrating Peptide Dendrimers Efficiently Internalize Cargo Into Cells †

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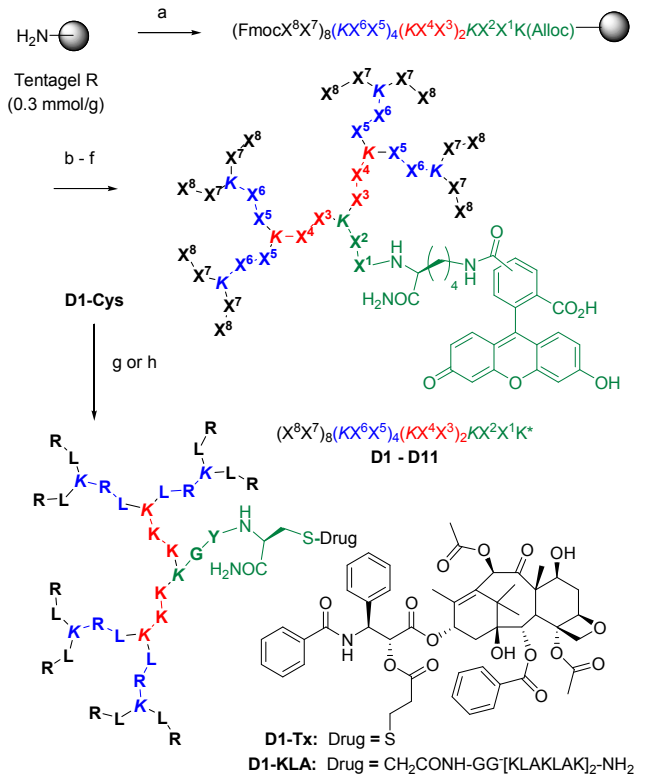
Redesigning linear cell penetrating peptides (CPPs) into a multi-branched topology with short dipeptide branches gave cell penetrating peptide dendrimers (CPPDs) with higher cell penetration, lower toxicity and hemolysis and higher serum stability than linear CPPs. Their use is demonstrated by delivering a cytotoxic peptide and paclitaxel into cells.

The cell membrane poses an impermeable barrier for a large number of compounds, in particular peptides, limiting their use as drugs. Nevertheless the discovery of the cell penetrating properties of the HIV-1 Tat protein[1] led to the discovery of a variety of cell penetrating peptides (CPPs),[2] an effect which was later also reported for synthetic oligomer analogs of linear peptides,[3] cyclic peptides,[4] and various organic dendrimers.[5] CPPs can serve as carriers for drug delivery, however they suffer from the typical metabolic instability of linear peptides and tend to be hemolytic and cytotoxic. Interestingly the cell internalization of CPPs can be enhanced by grafting them onto multivalent scaffolds, but their toxicity is usually also increased in such constructs despite the fact that their folding propensity is retained.[6] We showed recently that peptide dendrimers containing very short mono- or dipeptide branches,[7] while not able to form stable secondary structures,[7b] are generally more resistant to proteolytic degradation compared to linear peptides,[7c] and show almost no cytotoxicity when used for DNA transfection[7f] and only very weak hemolysis when designed as antimicrobials,[7e] which is probably a benefit of their particular molten globule conformation enforced by the dendritic topology.[7a] Although no increase in cellular uptake was reported with branched octaarginines compared to linear $(Arg)_8$,[8] we asked the question whether a broader survey of CPPs redesigned in multi-branched topology might lead to cell penetrating peptide dendrimers (CPPDs) with higher cellular uptake, metabolic stability and lower toxicity compared to linear CPPs, and potential use as drug delivery agents.



Scheme 1. Synthesis of CPPD. Conditions: a) Fmoc solid-phase peptide synthesis (SPPS), X^i = amino acid, *K* = branching lysine; b) $PhSiH_3$, $Pd(PPh_3)_4$ c) 5(6)-carboxyfluorescein, HOBt, DIC; d) piperidine/DMF; e) optional: Ac_2O; f) $CF_3CO_2H/iPr_3SiH/H_2O$, then preparative HPLC; g) $ClCH_2CO$-GG[KLAKLAK]$_2NH_2$, DIEA; h) PTX-PDP, DIEA. See supporting information for details.

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Fourteen peptide dendrimers were prepared by SPPS with sequence $(X^8X^7)_8(KX^6X^5)_4(KX^4X^3)_2KX^2X^1K^*$ (*K* = branching lysine) and labeled with 5(6)-carboxyfluorescein (CF) attached to the ε-amino group of the core lysine residue (K*) (Scheme 1). Variable residues (X) were assigned to amino acids found in the linear cationic CPP Tat[1] or the amphipathic CPP TP10[9] and pVEC[10] to form dendrimers with Arg and Lys (**D1-D4** and **D11**) or only Lys (**D5-D10**) as cationic residues, featuring various ratios and distribution of cationic and hydrophobic side chains in the branches and optional acetylation of *N*-termini to reduce the number of positive charges (Table 1).

CD spectra of CPPDs gave random coil signals independent of amino acid composition consistent with the disordered conformation of branched peptides and similar to Tat and pVEC (Figure 1a and S1). CPPD uptake by HeLa, CHO and Jurkat cells was evaluated by flow cytometry after 1h incubation of the cells with 1 µM or 10 µM compounds (Table 1, Figure 1b-d and S2). A strong fluorescence increase was observed with arginine containing dendrimers **D1** and **D2** (staining 10-15 times higher than the parent linear Tat), **D11** and **AcD11** (staining comparable to the parent linear pVEC). Exchanging the eight leucines in the G2-branches of **D2** for polar asparagines to form **D3** decreased the MFI average in spite of the same total number of cationic residues (8 Arg and 4 Lys), highlighting the importance of hydrophobic residues for uptake. **D5** and **AcD7** with only lysine as cationic residues also showed strong fluorescence comparable to the parent linear TP10, implying that arginine is not necessary for cellular uptake of CPPD. The remaining CPPDs showed MFI average comparable to linear Tat. Hydrophobic **D10** with only one cationic side chain was the least efficient CPPD in the library.

Table 1. Synthesis and cellular uptake of CF-labelled CPP and CPPD.

Cpd.	Sequence[a)]	Yield mg (%)[d)]	+[b)]	Arg	Lys	Hyd.[c)]	MFI av. (1 µM) (10µM)[e)] HeLa, CHO, Jurkat		Hemolysis [f)] µg.mL^{-1} / µM
Tat	*-YGRKKRRQRRR	90.9 (26)	8	6	2	1	2, 2, 11	14, 23, 65	125 / 44
D1	$(RL)_8(KRL)_4(KKK)_2KGYK^*$	14.7 (3)	24	12	4	13	11, 10, 49	214, 114, 1450	31 / 4
D2	$(RL)_8(KLL)_4(KKK)_2KGYK^*$	9.6 (6)	20	8	4	17	20, 22, 108	158, 175, 631	31 / 4
D3	$(RL)_8(KNN)_4(KKK)_2KGYK^*$	23.3 (5)	20	8	4	9	5, 4, 40	64, 48, 210	125 / 17
D4	$(GY)_8(KKR)_4(KQQ)_2KRRK^*$	13.2 (2)	18	6	4	12	3, 4, 27	19, 51, 112	63 / 9
AcD4	$(AcGY)_8(KKR)_4(KQQ)_2KRRK^*$	4.7 (1)	10	6	4	12	3, 2, 9	15, 25, 65	250 / 38
TP10	*-AGYLLGKINLKALAALAKKIL	51.3 (15)	4	-	4	15	7, 6, 23	152, 186, 385	31 /10
D5	$(AL)_8(K\,I\,K)_4(KLA)_2KK\,I\,K^*$	33.4 (5)	13	-	5	25	4, 9, 28	46, 122, 118	63 / 11
D6	$(LA)_8(KKL)_4(KKL)_2KYAK^*$	55.1 (8)	14	-	6	24	2, 5, 7	17, 21, 31	1000 / 163
AcD6	$(AcLA)_8(KKL)_4(KKL)_2KYAK^*$	9.0 (7)	6	-	6	24	2, 7, 14	35, 40, 64	n.d.
D7	$(I\,K)_8(KLA)_4(KLA)_2KK\,I\,K^*$	6.4 (1)	17	-	9	21	5, 14, 29	33, 51, 355	125 / 19
AcD7	$(Ac\,I\,K)_8(KLA)_4(KLA)_2KK\,I\,K^*$	3.0 (1)	9	-	9	21	5, 14, 190	33, 259, 862	63 / 10
D8	$(LA)_8(K\,I\,K)_4(KLA)_2KK\,I\,K^*$	11.1 (2)	13	-	5	25	2, 4, 11	20, 30, 71	63 / 11
D9	$(LA)_8(KLA)_4(K\,I\,K)_2KK\,I\,K^*$	8.2 (2)	11	-	3	27	2, 2, 10	14, 14, 61	125 / 22
D10	$(LA)_8(KLA)_4(KLA)_2KK\,I\,K^*$	12.3 (3)	9	-	1	29	3, 2, 5	9, 8, 36	250 / 48
pVEC	*-LLIILRRRIRKQAHAHSK	94.3 (26)	6	4	2	8	36, 71, 84	126, 210, 292	4 / 1
D11	$(L\,I)_8(KRK)_4(KRA)_2KHSK^*$	15.0 (2)	18	6	4	18	3, 12, 58	60, 106, 968	500 / 70
AcD11	$(AcL\,I)_8(KRK)_4(KRA)_2KHSK^*$	7.0 (5)	10	6	4	18	4, 8, 26	44, 105, 385	n.d.

[a)] One letter codes for amino acids. Branching diamino acids are shown in italics. * = 5(6)-carboxyfluorescein amidated to the Lys side chain (K*) or the N-terminus (*-) added as the last coupling step, see supporting information for details. Ac = acetyl. All peptides are carboxamide ($CONH_2$) at the C-terminus. [b)] + = Number of cationic amino acid side chains (Lys and Arg) and free N-termini. [c)] Hyd = Hydrophobic residues: Ala, Ile, Leu and Tyr. [d)] Yields given for RP-HPLC purified products as TFA salts. [e)] Flow cytometry data. MFI average = average of the mean fluorescence intensity of the tested peptides over background. Three independent experiments are used to get values. [f)] Hemolysis assay detailed in supporting information. n.d. = not determined.

Fixed confocal microscopy images with HeLa and CHO cells confirmed the trends seen in flow cytometry and revealed the cellular localization of peptide dendrimers (Figure S3). For the Arg containing dendrimers **D1, D2**, **D3** and **D11**, and **D5** with only Lys as cationic residue, images showed a strong staining of the cytoplasm similar to Tat in both cell lines. In contrast to PAMAM dendrimers,[5b] acetylation of N-terminal amino groups did not block cellular uptake of CPPDs, although differences of subcellular localization were visible between **D11** (homogeneous staining) and **AcD11** (punctuated patterns). Live confocal microscopy experiments were performed with **D1** and **D11** to confirm cellular uptake and distribution (Figure 1e, S5 S6). After 1h, dendrimers were found inside cells localized in the cytoplasm in a punctuated pattern suggesting confinement in endosomes.

Both energy-dependent endocytosis and energy-independent or direct translocation across the cell membrane have been proposed as uptake mechanisms for linear CPPs.[11] The uptake of dendrimer **D1** by HeLa cells was reduced by chlorpromazine, indicating clathrin dependent uptake, and to a lesser extent by rottlerin (macropinocytosis inhibitor) and nystatin (caveola/lipid raft-dependent endocytosis inhibitor) (Figure 1f). Uptake was only 30% lower at 4°C compared to 37°C, suggesting that direct membrane translocation also takes place for **D1**. Dendrimer **D11** showed a similar pattern however with reduced uptake at 4°C, indicating lower self-translocating ability probably due to its smaller number of positive charges and different distribution of Arg residues in the branches compared to **D1.** Results with CHO cells (Figure S6) show primarily a clathrin dependent uptake for both **D1** and **D11** and

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a reduced uptake at 4°C (energy-dependent uptake mostly) in line with previous observations that the cell type plays a critical role in the internalization mechanism of CPPs. In the case of CPPDs, clathrin mediated uptake appears to be the major process of internalization taking place in both cell lines.

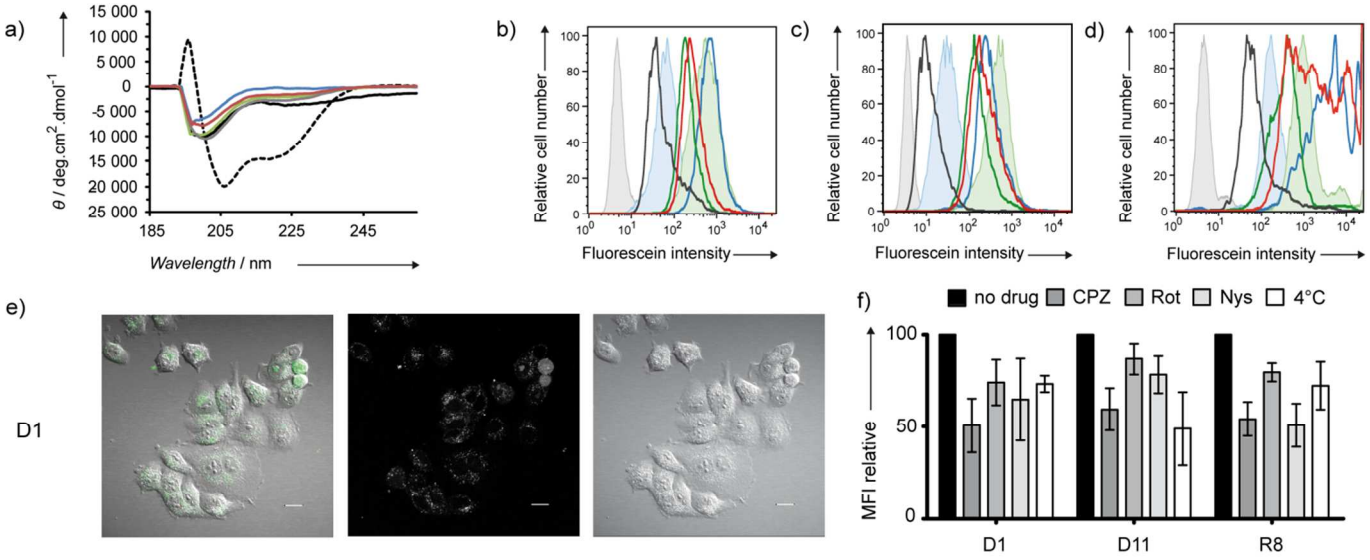

Figure 1. **A**. CD measurements of **D1** (blue), **D5** (green), **D10** (grey), and **D11** (red). **Tat** (black), **TP10** (doted black) in PBS buffer (pH 7.4). **B, C, D** Flow cytometry histograms for HeLa (**B**), CHO (**C**) and Jurkat (**D**) cells after 1 h incubation with 10 µm of peptide dendrimers at 37 °C. **D1** (blue), **D5** (green), **D10** (black), and **D11** (red). Untreated cells (grey), **Tat** (filled blue), **TP10** (filled green). **E.** Observed uptake of **D1** (10µM) after 1h incubation at 37°C on HeLa cells in live confocal microscopy of the differential interference contrast (DIC, left panel) and the CF fluorescence at 525 nm (middle panel). Left panels are the merged of the DIC and fluorescence pictures. White bar = 20 µm. See Fig. S5 for data with CPPD **D11**. **F.** Uptake levels of **D1** and **D11** in HeLa cells in the presence of various inhibitors. Cells were pretreated 30 min with Chlorpromazine 50 µM (CPZ), Rottlerin 20 µM (Rot), Nystatin 25 µg/mL (Nys) or cooled down at 4°C prior to 1 h incubation with 10 µM CPPD or linear octaarginine (R8) in the presence of inhibitor. Error bars represent the SD of three independent experiments. See also Fig. S6 for CHO cells.

CPPDs were not significantly toxic to HeLa or CHO cells (up to 10 µM, 24 h, Figure S4). However less than 50% viable Jurkat cells remained after 24 h exposure to 10 µM **pVEC**, **D1**, **D2**, **D7**, **D11** or**AcD11**, which belong to the most cell penetrating compounds. While hemolysis was very strong for the linear CPP pVEC (4 µg.mL^{-1}), CPPDs were less hemolytic including those with strong cell penetrating properties (30-500 µg.mL^{-1}, Table 1).

Dendrimers **D1** and **D11** combined efficient uptake into cells with moderate toxicity and were further investigated. Degradation experiments in human serum showed that 40% of **D1** and **D11** were still unchanged after 12h while linear Tat was completely degraded, in line with previous reports that peptide dendrimers are more resistant to proteolysis than linear peptides (Figure S7).[7d-f] To evaluate the ability of **D1** and **D11** to deliver cargos into cells, dendrimers were conjugated via a thioether bridge to the α-helical tetradecapeptide [KLAKLAK]$_2$ (**KLA**), an antimicrobial peptide that does not penetrate mammalian cells but induces cell death by disrupting the mitochondrial membrane if delivered into the cytosol.[12] The resulting conjugates **D1-KLA** and **D11-KLA** (Scheme 1) were found significantly more cytotoxic to HeLa and CHO cells compare to **Tat-KLA** at 10µM (Figures S8 and S9), while **KLA** alone or ungrafted dendrimer were not toxic (Table 2, Figure S10). Covalent conjugation to CPPD does not change the bioactivity of **KLA** since disulfide bridged conjugates to both **D1** and **D11** showed cytotoxicity similar to the thioether adducts (Figure S11, Table S1).

Table 2. Cytotoxicity of CPPD-drug conjugates.[a)]

Cpd.	IC_{50} HeLa cells	IC_{50} CHO cells
KLA	>> 20 µM (100 %)[b)]	>> 20 µM (93 %)[b)]
D1-KLA	4.6 ± 0.2 µM	7.8 ± 0.6 µM
D11-KLA	6.0 ± 0.3 µM	8.9 ± 0.6 µM
Tat-KLA	10.1 ± 0.5 µM	~ 20 µM (48%)[b)]
Paclitaxel (PTX)	8.4 ± 0.6 nM[c)]	~ 4 µM (60%)[b)]
D1-PTX	47 ± 15 nM	~ 4 µM (57%)[b)]
D11-PTX	41 ± 9 nM	~ 4 µM (46%)[b)]

[a)] Cell survival measured after 24 h (KLA series) or 72 h (paclitaxel series) with the WST-8 assay; [b)] % of surviving cells at the indicated concentration, which was the highest measured. No IC_{50} is given when the full inhibition curve could not be measured; [c)] with 1.6 % DMSO added to solubilise paclitaxel.

In a further example, **D1** and **D11** were conjugated to paclitaxel (PTX), a notoriously insoluble anticancer drug whose solubility and targeting can be significantly improved by incorporation into a variety of nanocarriers including dendrimers and peptides.[13] CPPD conjugates **D1**-PTX and **D11**-PTX were prepared by disulfide bond formation between **D1-Cys** or **D11-Cys** and paclitaxel-2'-(3-(2-pyridyldithio)) propionate (PTX-PDP, Scheme 1). The dithiopropionyl linker has been reported for PTX-octaarginine[14] and for dendrimer drug conjugates[15] and is susceptible to intracellular reductive cleavage. Our CPPD-PTX conjugates were water soluble and stable in cell

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culture over the time of the experiment, yet retained most of the selective cytotoxicity of PTX to HeLa cells over CHO cells (Table 2, Figure S12).

Conclusions

Redesigning CPPs into G3 peptide dendrimers with short dipeptide branches gave CPPDs with stronger cellular uptake, lower cytotoxicity and hemolysis, and higher stability towards serum degradation compared to their linear counterpart. Cellular uptake was observed in a diversity of sequences containing either Arg or Lys as cationic residues and a balanced ratio of hydrophobic residues. Dendrimer **D1** inspired by the Tat peptide and **D11** inspired by pVEC efficiently localized in the cytoplasm and delivered cytotoxic cargo into cells. The low intrinsic toxicity and hemolysis of CPPDs might result from their inability to fold into amphipatic α-helical membrane lytic aggregates. The higher cellular uptake of CPPDs compared to linear CPPs probably reflects in part their larger size, which is remarkably obtained without increased synthetic complexity since the described CPPDs are obtained in only 12 SPPS coupling steps warranting low production costs. Due to their ease of synthesis and favorable properties CPPDs represent a promising and versatile new class of cell penetrating devices.

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Notes and references

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† Electronic Supplementary Information (ESI) available: details of synthesis and cellular experiments. See DOI: 10.1039/c000000x/

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