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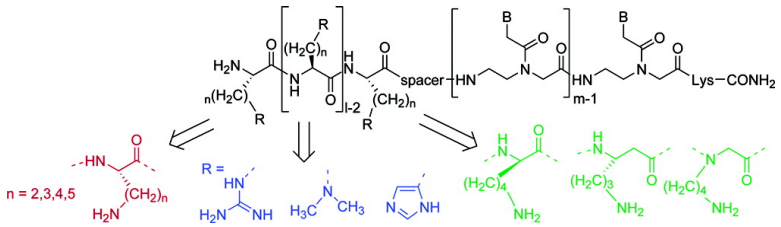

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Improving cellular uptake and biodistribution remains one of the major obstacles for a successful and broad application of peptide nucleic acids (PNAs) as antisense therapeutics. Recently, we reported the identification and functional characterization of an antisense PNA, which redirects splicing of murine CD40 pre-mRNA. In this context, it was discovered that a simple octa(L-lysine) peptide covalently linked to the PNA is capable of promoting free uptake of the conjugate into BCL_1 cells as well as primary murine macrophages. On the basis of this peptide motif, the present study aimed at identifying the structural features, which define effective peptide carriers for cellular delivery of PNA. While the structure−activity relationship study revealed some clear correlations, only a few modifications actually led to an overall improvement as compared to the parent octa(L-lysine) conjugate. In a preliminary PK/tissue distribution study in healthy mice, the parent conjugate exhibited relatively broad tissue distribution and only modest elimination via excretion within the time frame of the study.

Introduction

Peptide nucleic acids (PNAs) are oligonucleotide mimetics, in which the anionic sugar−phosphate backbone characteristic to nucleic acids and most nucleic acid analogues is replaced with an achiral, uncharged polyamide backbone.[1] PNA binds to complementary DNA or RNA with high binding affinity and specificity[2] and exhibits extraordinary stability against enzymatic degradation.[3] These features have spurred great interest in PNA for diagnostic as well as for therapeutic applications in the context of antisense and antigene drugs. However, limited solubility in physiological buffers, insufficient cellular uptake, and poor biodistribution due to rapid elimination and excretion have prevented a broad application of unmodified PNA as antisense inhibitors. On the other hand, PNA is more amenable to manipulations than most other nucleic acid analogues, and one obvious and synthetically straightforward approach to improve its physicochemical and biological properties lies in the conjugation of PNA to short synthetic peptide carriers.

In recent years, a large variety of synthetic cell-penetrating peptides (CPPs) have been developed and applied as membrane permeable delivery vehicles for a wide variety of compounds (for reviews, see refs 4−6). While most of the sequences have been derived from naturally occurring peptides or proteins, some of the peptides are artificial models designed to probe the physicochemical properties required for traversing biological membranes. The mechanism of internalization of CPPs and their cargo is not well-understood and has been the subject of speculation and controversy. Recently reported studies indicate that adsorptive endocytosis plays a significant role in the process of internalization for some cationic CPPs.[7] In another study, oligoarginines were shown to be internalized into mammalian cells predominantly through an endocytotic pathway, which is mediated through binding to cell surface heparan sulfates linked to proteoglycans (HSPGs).[8] Recently, the pathways of internalization and subcellular distribution of several cationic CPPs were investigated in great detail.[9] The authors report a pathway common for all of the CPPs investigated, which involves endocytosis followed by retrograde transport through the Golgi complex and endoplasmic reticulum. The escape from endosomal compartments and release into the cytoplasm was found to depend on the nature of the peptide, i.e., its membrane activity under endosomal pH and its stability against enzymatic degradation.

Since the discovery of CPPs for drug delivery, several peptide vehicles have been synthesized and evaluated for the cellular delivery of oligonucleotides and PNAs (for review, see refs 10 and 11). A well-characterized peptide vector, termed penetratin (pAntp), has been derived from the basic homeodomain of *Antennapedia*.[12,13] PNA conjugates of penetratin have been demonstrated to be efficiently internalized into mammalian cells in culture.[14] However, the constructs seemed to be localized mainly in vesicular structures and did not exhibit their intended antitelomerase activity. A chimeric peptide termed transportan, which combines the sequence derived from the N terminus of galanin with the highly membrane active wasp venom peptide toxin mastoparan, has been reported to be internalized into various cell types through a receptor-independent mechanism.[15] PNAs targeted against the galanin receptor and coupled to either transportan or penetratin have been shown to inhibit the expression of the receptor in vivo as indicated by a decreased binding of galanin in the dorsal horn of rats after

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intrathecal administration of the PNA-peptide constructs.[16] In a comprehensive study, two well-known basic CPPs, penetratin and pTat, a 14-amino acid fragment derived from the HIV protein Tat, were evaluated and compared in their ability to deliver PNA to a number of human cell lines.[17] The authors found a time-, temperature-, and concentration-dependent uptake of the conjugates into vesicular compartments suggesting an endocytotic process. They also reported profound differences in uptake patterns depending on the cell type. The existing data suggest that it is unlikely that any one peptide vector will be generally applicable for the cellular delivery of PNA. The best choice will depend on the target cell type, the sequence and length of the PNA cargo, and the site and mechanism of action, e.g., inhibition of transcription, redirection of splicing, or translational arrest.

Recently, we reported on the identification and functional characterization of an antisense PNA that redirects splicing of murine CD40 mRNA thereby inhibiting CD40 expression.[18] CD40 is a membrane protein, which is expressed on antigen-presenting cells such as B lymphocytes, dentritic cells, macrophages, endothelial and epithelial cells, and smooth muscle cells. It plays a key role in the initiation and propagation of inflammation, and various studies in cellular systems as well as in vivo suggest that inhibition of CD40 signaling could be therapeutically relevant for inflammatory and autoimmune diseases.[19,20]

On the basis of previous work using PNA conjugated to short lysine oligomers to correct aberrant splicing in cell culture and in vivo,[21,22] we discovered that a simple octa(L-lysine) motif is capable of promoting free uptake of the PNA conjugates into mouse BCL_1 cells as well as primary murine macrophages.[18] It was demonstrated that the presence of the basic peptide led to a 10-fold increase in activity under conditions of free uptake, i.e., without the aid of transfection agents or electroporation, while the activity of the conjugate was comparable to the unmodified PNA when cellular uptake was mediated through electroporation. This indicated that the peptide predominantly enhanced cellular uptake and possibly improved intracellular trafficking rather than influencing the PNA-mediated effect on pre-mRNA splicing.

To elucidate further the structural features, which are required for those simple cationic CPPs to be effective vehicles for cellular delivery of antisense PNAs, amino acids with modified side chains, cationic headgroups, or backbones have been introduced at various positions in the peptide and the corresponding conjugates have been evaluated in vitro. The antisense activity of the PNA conjugates, as measured by the inhibition of CD40 protein expression, has been used as the primary end point of the study and compared to the effect of the peptide modifications on enzymatic stability and cell viability. The parent octa(L-lysine) conjugate, as a representative example of the series of conjugates described in this paper, has been evaluated for its PK/tissue distribution in healthy mice.

Results

The antisense PNA used in this study targets the 3′-end of exon 6 of the primary murine CD40 transcript. We have previously established that the unmodified version bearing just a single L-lysine residue at the C terminus (**1**) can promote the omission of exon 6 from CD40 pre-mRNA in a sequence- and dose-dependent fashion, which leads to a frame shift in the downstream codons of exons 7, 8, and 9 and truncates the protein due to an in-frame stop codon in exon 8.[18] The induced splice variant matches an alternatively spliced transcript of murine CD40 (type 2), which occurs naturally at low abundance and lacks the sequence encoding for the transmembrane domain.[23] Our preliminary experiments showed that conjugating **1** to short oligolysine peptides significantly improved activity under conditions of free cellular uptake, i.e., without the use of facilitated delivery such as electroporation. In particular, the potency of an octa(L-lysine) conjugate **2** was comparable to the parent PNA **1** delivered by electroporation.

We used this simple octa(L-lysine) motif as a starting point to investigate the effects of amino acid modifications on the biological and biochemical properties of the corresponding PNA-peptide conjugates and to elucidate the structural requirements for efficient cellular uptake and nuclear accumulation. The antisense activity, i.e., the ability of the conjugates to induce alternative splicing of CD40 pre-mRNA, was the primary end point of the study. The PNA-peptide conjugates were screened under conditions of free uptake in BCL_1 cells, a murine B-cell line that constitutively expresses high levels of CD40. CD40 cell surface expression was analyzed by flow cytometry.

Cell viability was assessed by WST-1 assay. Cytotoxicity was considered significant if the level of viable cells dropped below 50% of the untreated control following 3 days of compound exposure. The enzymatic stability of the conjugates was evaluated in serum-containing cell culture medium and 25% mouse serum. The compounds were analyzed by HPLC after removal of serum proteins by precipitation as outlined in the Experimental Section. To determine the variability of the assay, the parent oligo(L-lysine) conjugate **2** was analyzed multiple times using different batches of mouse serum (Experimental Section). The major degradation products for several representative compounds were determined by LC-MS.

Number of Basic Amino Acids and C- vs N-Terminal Positioning of the Peptide. Conjugates **1**−**9** carrying between zero and eight lysine residues at the N terminus were evaluated for their ability to reduce CD40 expression in BCL_1 cells under conditions of free uptake (Figure 1a,b). As compared to the parent compound **1**, the activity increased significantly with the length from the hexa- to the octapeptide, while no significant increase in activity could be observed for conjugates carrying 1−5 lysines.

A similar trend was observed for oligo(L-lysine) peptides with two (**10**), four (**11**), and eight lysines (**12**) attached to the C-terminal end of the PNA (Figure 2). The latter, however, exhibited a slightly lower activity than its N-terminal analogue (**2**). Interestingly, the inhibitory activity was reduced to a level similar to **11** bearing a single tetra(L-lysine) chain if the octa(L-lysine) motif was split into two tetra(L-lysine) peptides, one attached to the C and the other to the N terminus (**13**).

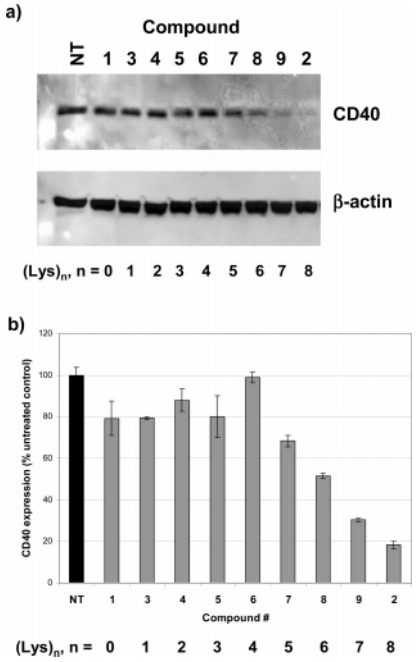

Figure 1. Effect of the number of N-terminal lysine residues on the reduction of CD40 expression in mouse BCL_1 cells treated with either no compound (NT) or PNA conjugates at 10 μM concentration. After 3 days of incubation, cells were harvested and CD40 expression was measured by (a) western blot and (b) flow cytometry. The values shown in part b represent the averages ($n = 3$ per group) and their corresponding standard deviations.

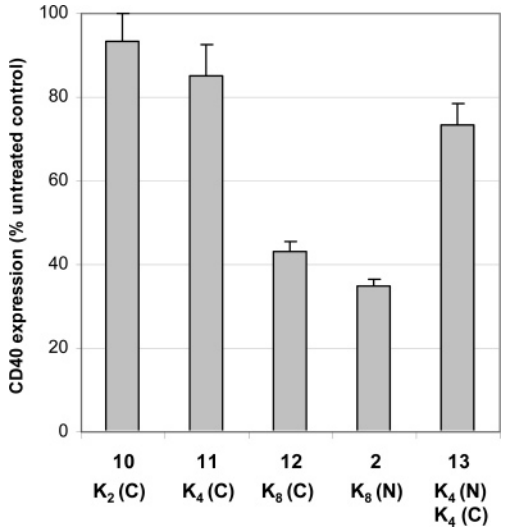

Figure 2. Effect of C-terminally (C) conjugated as well as both C and N terminally conjugated L-lysine peptides on the inhibition of CD40 expression as compared to the N-terminal octa(L-lysine) conjugate 2. BCL_1 cells were treated with PNA conjugates at a concentration of 10 μM. After 3 days of incubation, cells were harvested and CD40 expression was measured by flow cytometry. The values shown represent the averages ($n = 3$ per group) and their corresponding standard deviations.

The C-terminal conjugate **12** showed a significantly lower enzymatic stability as compared to its N-terminal analogue **2** (Scheme 1, Supporting Information). LC-MS analysis of the digested samples revealed that the C-terminal peptide was almost exclusively cleaved between the first and the second lysine residues adjacent to the PNA thereby removing the major part of the peptide in one cut while the N-terminal analogue is cleaved more or less sequentially from its N terminus. Provided the observed differences in serum stability and degradation pattern correlate with the intracellular degradation, it could explain the reduced activity observed for the C-terminal conjugate.

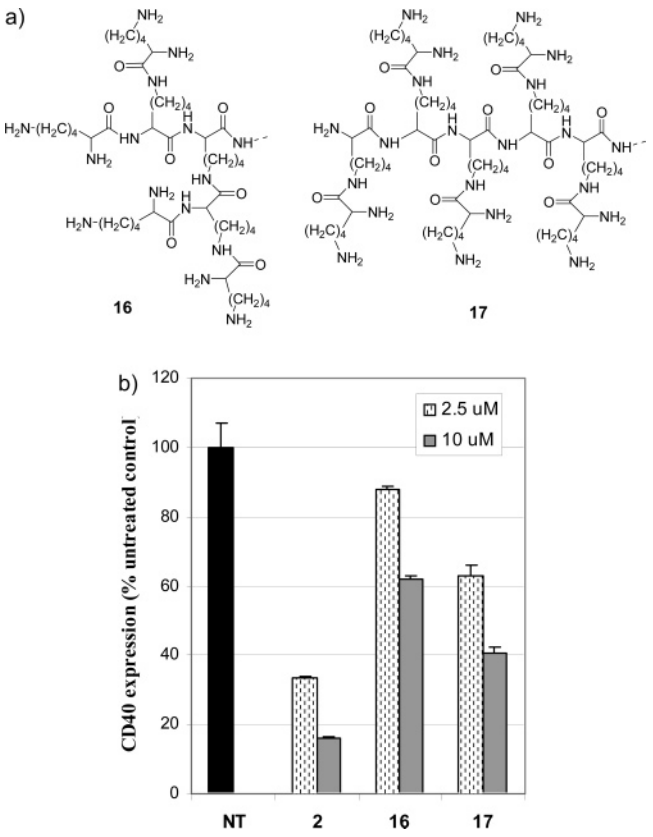

Figure 3. (a) Structure of branched lysine conjugates and (b) their effect on CD40 expression in mouse BCL_1 cells treated with PNA conjugates at concentrations of 2.5 and 10 μM and analyzed by flow cytometry. The values shown represent the averages ($n = 3$ per group) and their corresponding standard deviations.

To evaluate further the role of positive charges in the peptide motif, two of the lysine residues of **2** were replaced at positions 3 and 6 by either glycine (**14**) or citrulline residues (**15**), bearing no side chain or a side chain with an uncharged urea headgroup, respectively (Supporting Information). Both sequence modifications exhibited a level of activity comparable to conjugate **8** containing the truncated hexa(L-lysine) peptide, which clearly confirms the correlation between the number of positively charged headgroups and the activity in this motif.

Lysine Branching. Various branched lysine motifs were prepared to investigate how the spatial arrangement of the positively charged amino groups influences the activity of the conjugates. Two representative compounds (**16** and **17**) are shown in Figure 3. The results indicate that for both constructs, even for **17** carrying 11 free amino groups, the inhibition of CD40 expression was significantly lower than for the parent conjugate **2** with the linear octapeptide (Figure 3).

Cationic Headgroup Modification. The effect of the peptide side chain functionalities on the activity was evaluated with a series of conjugates containing amino acids with structurally different cationic headgroups,

Table 1. Headgroup-Modified Conjugates: Effect on Antisense Activity, Cell Viability, and Enzymatic Stability

		activity (%UTC)[a]				half-life ($t_{1/2}$)	
compd	peptide sequence (N to C)	10 μM	3 μM	1 μM	cell viability (μM)[b]	10% FBS	25% MS
2	K_8 (N), K (C)	34 ± 2	72 ± 4	88 ± 2	20	≫7 days[c]	6.7 h ± 1.4
18	R_8 (N), K (C)	60 ± 6	81 ± 9	102 ± 4	>20	20 h	2−3 h
19	KKRKKRKK (N), K (C)	43 ± 1	76 ± 3	90 ± 10	10−20	ca. 90 h	2−3 h
20	$(hR)_8$ (N), K (C)	20 ± 3	45 ± 1	91 ± 4	5	≫7 days	15 h
21	KK-hR-KK-hR-KK (N), K (C)	36 ± 2	75 ± 6	92 ± 1	10	≫7 days	n/a[d]
22	$(dmK)_8$ (N), K (C)	71 ± 4	89 ± 3	94 ± 3	10	≫7 days	>48 h
23	$(dmK)_2$-K-$(dmK)_2$-K-$(dmK)_2$ (N), K (C)	63 ± 2	89 ± 4	91 ± 4	>20	≫7 days	n/a
24	$(K\text{-}dmK)_4$ (N), K (C)	53 ± 1	89 ± 0	96 ± 2	20	≫7 days	n/a
25	KK-dmK-KK-dmK-KK (N), K (C)	41 ± 4	78 ± 1	96 ± 8	5	≫7 days	22 h
2	K_8 (N), K (C)	33 ± 4	58 ± 6	79 ± 10		see above	
26	$(H)_8$ (N), K (C)	64 ± 6	74 ± 3	81 ± 5	>20	≫7 days	44 h
27	$(K\text{-}H)_4$ (N), K (C)	73 ± 5	87 ± 6	88 ± 6	>20	≫7 days	6 h
28	KK-H-KK-H-KK (N), K (C)	52 ± 4	73 ± 6	86 ± 7	10−20	≫7 days	6 h

[a] Inhibition of CD40 expression in BCL1 cells treated with PNA conjugates at concentrations of 10, 3, and 1 μM and analyzed by flow cytometry. The values shown represent the averages ± SD of percent untreated control ($n = 3$). [b] Concentration at which cell viability (measured by WST-1 assay) dropped below 50% of untreated control. [c] No degradation observed within the period of time indicated. [d] No data available.

Table 2. Side Chain-Modified Conjugates: Effect on Antisense Activity, Cell Viability, and Enzymatic Stability

		activity (%UTC)[a]				half-life ($t_{1/2}$)	
compd	peptide sequence (N to C)	10 μM	3 μM	1 μM	cell viability (μM)[b]	10% FBS	25% MS
2	K_8 (N), K (C)	39 ± 3	71 ± 0	90 ± 4	10−20	≫7 days[c]	6.7 h ± 1.4
29	$(Dab)_8$ (N), K (C)	77 ± 4	93 ± 4	94 ± 4	5	≫7 days	ca. 60 h
30	$(Dab)_2$-K-$(Dab)_2$-K-$(Dab)_2$•(N), K (C)	64 ± 4	81 ± 1	96 ± 2	2.5−5	≫7 days	n/a[d]
31	$(K\text{-}Dab)_4$ (N), K (C)	52 ± 7	68 ± 2	95 ± 1	2.5	≫7 days	n/a
32	KK-Dab-KK-Dab-KK (N), K (C)	38 ± 5	62 ± 2	80 ± 6	2.5−5	≫7 days	40 h
2	K_8 (N), K (C)	51 ± 2	86 ± 5	102 ± 1		see above	
33	$(Orn)_8$ (N), K (C)	77 ± 3	86 ± 1	104 ± 2	5	≫7 days	115 h
34	Orn-Orn-KKKK-Orn-Orn (N), K (C)	52 ± 6	69 ± 2	97 ± 0	5	≫7 days	n/a
35	$(K\text{-}Orn)_4$ (N), K (C)	42 ± 4	61 ± 0	94 ± 2	5	≫7 days	n/a
36	KK-Orn-KK-Orn-KK (N), K (C)	34 ± 2	65 ± 0	94 ± 4	10−20	≫7 days	18 h
37	$(hK)_8$ (N), K (C)	31 ± 2	65 ± 1	87 ± 4	10	>7 days	17 h
38	$(hK)_2$-KKKK-$(hK)_2$ (N), K (C)	34 ± 1	66 ± 2	86 ± 1	>20	ca. 120 h	n/a
39	$(K\text{-}hK)_4$ (N), K (C)	32 ± 1	61 ± 1	81 ± 3	>20	>7 days	n/a
40	KK-hK-KK-hK-KK (N), K (C)	31 ± 1	61 ± 1	76 ± 2	>20	>7 days	ca. 10 h

[a] Inhibition of CD40 expression in BCL1 cells treated with PNA conjugates at concentrations of 10, 3, and 1 μM and analyzed by flow cytometry. The values shown represent the averages ± SD of percent untreated control ($n = 3$). [b] Concentration at which cell viability (measured by WST-1 assay) dropped below 50% of untreated control. [c] No degradation observed within the period of time indicated. [d] No data available.

namely, arginine (**18**, **19**), homoarginine (**20**, **21**), *N*-ϵ-dimethyllysine (**22**−**25**), and histidine (**26**−**28**), and compared to the parent conjugate **2** (Table 1). Clearly, the replacement of lysine against N-ϵ-dimethyllysine or histidine reduced the activity of the corresponding PNA conjugates depending on the number of modified amino acids introduced. For the histidine-containing peptides, the reduced activity can be attributed to a lower charge density at neutral pH (pK_a 6.1). On the contrary, the *N*,*N*-dimethylamino headgroup (pK_a ≈ 9.8) can be expected to be almost fully protonated under physiological conditions; therefore, the observed decrease in activity should not be due to a reduced number of positively charged headgroups.

The lower activity observed for the uniform arginine analogue **18** was surprising considering the previously reported rapid and efficient cellular uptake of oligoarginines.[24,25] On the other hand, the incorporation of arginine residues was found to dramatically decrease the enzymatic stability of the conjugates in cell culture medium as well as mouse serum. LC-MS analysis of the cleavage products revealed that cleavage preferentially occurred between residues close to the PNA (Scheme 1, Supporting Information). Furthermore, the activity of the PNA conjugates could be maintained or even enhanced by replacing lysines with homoarginine residues (**20** and **21**), which were found to stabilize the peptide against degradation in mouse serum.

The cell viability after the 3 day exposure with compound was not significantly affected in the concentration range tested, except for the uniform homoarginine conjugate **20** and the *N*-ϵ-dimethyllysine containing conjugate **25**, both of which showed evidence of cytotoxicity at 5 μM concentration.

Side Chain Length. The lysine homologues 2,4-diaminobutyric acid (**29**−**32**), ornithine (**33**−**36**), and homolysine (**37**−**40**) with two, three, and five carbon side chains, respectively, were utilized to elucidate the effect of the amino acid side chain length on antisense activity (Table 2). Shortening the side chain uniformly by replacing all eight lysines with 2,4-diaminobutyric acid (**29**) or ornithine (**33**) led to a significant reduction in antisense activity, which could be restored to the level of the parent compound with four ornithine (**34** and **35**) or two 2,4-diaminobutyric acid substitutions (**32**). Replacement of two lysine residues with ornithine (**36**) produced a slight but reproducible increase in activity over the parent compound **2**. Both modifications stabilized the peptide against enzymatic degradation. The presence of two ornithines in **36**, for instance, increased the half-life in mouse serum approximately 2−3-fold.

Table 3. Backbone-Modified Conjugates: Effect on Antisense Activity, Cell Viability, and Enzymatic Stability

		activity (%UTC)[a]				half-life ($t_{1/2}$)	
compd	peptide sequence (N to C)	10 μM	3 μM	1 μM	cell viability (μM)[b]	10% FBS	25% MS
2	K_8 (N), K (C)	51 ± 2	86 ± 5	102 ± 1	10−20	≫7 days[c]	6.7 h ± 1.4
41	(DK)$_8$ (N), K (C)	54 ± 0	81 ± 0	97 ± 4	10−20	≫7 days	≫48 h[c]
42	KK-DK-KK-DK-KK (N), K (C)	62 ± 1	87 ± 2	102 ± 3	10−20	≫7 days	≫48 h
2	K_8 (N), K (C)	48 ± 3	66 ± 3	88 ± 3		see above	
43	(βK)$_8$ (N), K (C)	66 ± 1	81 ± 5	94 ± 3	5−10	≫7 days	≫48 h
44	KK-βK-KK-βK-KK (N), K (C)	61 ± 3	82 ± 3	98 ± 3	5−10	≫7 days	n/a[d]
2	K_8 (N), K (C)	37 ± 1	66 ± 2	87 ± 6		see above	
45	G-(pK)$_8$ (N), K (C)	56 ± 3	80 ± 6	92 ± 6	10−20	≫7 days	≫48 h
46	(K-pK)$_4$ (N), K (C)	70 ± 2	96 ± 6	111 ± 11	10−20	≫7 days	n/a
47	KK-pK-KK-pK-KK (N), K (C)	60 ± 3	88 ± 12	104 ± 1	10−20	≫7 days	29 h

[a] Inhibition of CD40 expression in BCL1 cells treated with PNA conjugates at concentrations of 10, 3, and 1 μM and analyzed by flow cytometry. The values shown represent the averages ± SD of percent untreated control (n = 3). [b] Concentration at which cell viability (measured by WST-1 assay) dropped below 50% of untreated control. [c] No degradation observed within the period of time indicated. [d] No data available.

With the exception of **36**, the most active compound of the ornithine series, the replacement of lysine against ornithine or 2,4-diaminobutyric acid led to significantly reduced cell viability. In the case of **30**−**32**, viability dropped below 50% of untreated control at concentrations as low as 2.5 μM.

Extending the side chain by one methylene group (**37**−**40**) did not significantly alter the activity regardless of the number of homolysine residues incorporated. This is in sharp contrast to the effects obtained with the shorter homologues ornithine or 2,4-diaminobutyric acid. Because the parent compound **2** was not included in this experiment, a subsequent head-to-head comparison between the parent octa(L-lysine) and the uniform homolysine conjugate **37** was performed, in which both compounds showed very similar reduction of CD40 expression (Supporting Information). Similarly, the presence of homolysine in conjugates **37**−**40** led to only moderate changes in enzymatic stability.

Backbone Modification. Peptide backbone modifications, namely, D- (**41**, **42**) and β-lysines (**43**, **44**) as well as lysine peptoids (**45**−**47**), were employed to evaluate the influence of chirality and hydrogen bonding along the peptide backbone on the biological properties of the conjugates (Table 3). As expected, all modifications led to a significant increase in enzymatic stability. For instance, two D-lysines were sufficient to completely stabilize the octapeptide of **42** against degradation in 25% mouse serum for the duration of the experiment (48 h). Generally, however, the increase in stability did not translate into improved antisense activity. The presence of β-lysines in **43** and **44** lysine peptoid residues in **45**−**47** even produced a significant loss in activity as compared to the parent compound **2**, albeit with no clear correlation to the number of residues introduced.

Cell viability in the applied concentration range was only compromised by the β-lysine-containing compounds **43** and **44**, which reduced the number of viable cells to 50% of untreated control at concentrations between 5 and 10 μM.

Spacer Length and Hydrophobicity. To address the question whether spatial separation between the peptide carrier and the PNA had an effect on the biological properties of the conjugates, linear alkyl spacers of various length and hydrophobicity ranging from β-alanine (**48**), 6-aminohexanoic acid (**49**), and 8-aminooctanoic acid (**50**, **51**) to ω-aminododecanoic acid (**52**) were incorporated into the octa(L-lysine)-PNA conjugate and compared to the ethylene glycol-based O-spacer (**53**, **54**) as well as the parent compound **2** without a spacer (Table 4). While the two shortest linkers, β-Ala (**48**) and ahx (**49**), did not elicit any significant effect, a decrease in activity could be observed with the longer, more hydrophobic tethers aoc (**50**, **51**) and addc (**52**). In contrast, the incorporation of the more polar O-spacer, which has the same number of atoms as 8-aminooctanoic acid, did not cause any appreciable change in activity (**53**, **54**).

Table 4. Effect of Spacer Length and Hydrophobicity on Antisense Activity

	spacer		activity (%UTC)[a]		
compd	type	length (atoms)	10 μM	3 μM	1 μM
2	none	0	44 ± 2	76 ± 4	90 ± 6
48	β-Ala	4	43 ± 4	73 ± 6	95 ± 7
49	ahx	7	48 ± 2	79 ± 3	101 ± 8
50	aoc	9	54 ± 2	85 ± 3	111 ± 4
51	2 × aoc	18	62 ± 7	88 ± 14	97 ± 3
52	addc	13	68 ± 2	85 ± 6	102 ± 6
53	O-spacer	9	44 ± 4	82 ± 2	99 ± 2
54	2 × O-spacer	18	46 ± 2	83 ± 1	107 ± 3

[a] Inhibition of CD40 expression in BCL1 cells treated with PNA conjugates at concentrations of 10, 3, and 1 μM and analyzed by flow cytometry. The values shown represent the averages ± SD of percent untreated control (n = 3).

Biodistribution of an Octa(L-lysine) Conjugate in Mice. The parent compound **2**, which exhibits in vitro activity under conditions of free uptake combined with moderate stability against enzymatic degradation ($t_{1/2}$ = 6.7 ± 1.4 h in 25% mouse serum), was chosen to be evaluated for its PK/tissue distribution in healthy Balb/c male mice. Therefore, the compound was radiolabeled by introducing ^{3}H-acetyl at the ϵ-amino group of the C-terminal lysine (**55**). Prior to the in vivo study, the stability of this modification was evaluated with "cold" sample in mouse plasma and no evidence of deacetylation was found within 24 h.

The plasma profile indicates that **55** was cleared very rapidly from circulation. Five minutes after i.v. injection, only about 1% of the injected dose could be detected in the plasma (Figure 4, Supporting Information). Table 5 shows the average tissue concentrations after 24 h for i.v. and s.c. administration. The results demonstrate that the octa(L-lysine)-PNA conjugate distributed relatively broadly with highest concentrations reached in liver, kidney, and spleen. Only very low amounts,

Table 5. Average Tissue Concentration of Conjugate **2**[a]

tissue	concentration (μg equiv/g) i.v.	s.c. dosing
liver	11.47 ± 4.79	5.44 ± 0.51
kidney	12.74 ± 3.33	11.55 ± 0.98
spleen	3.68 ± 0.29	1.51 ± 0.22
pancreas	0.60 ± 0.04	0.20 ± 0.03
lungs	0.22 ± 0.03	0.10 ± 0.02
MLN	1.94 ± 0.16	0.76 ± 0.13
heart	0.18 ± 0.11	0.07 ± 0.05
skin	1.25 ± 0.07	0.75 ± 0.30
skeletal muscle	0.29 ± 0.03	0.20 ± 0.01
testes	0.38 ± 0.06	0.23 ± 0.01
small intestine	1.31 ± 0.27	0.26 ± 0.03
large intestine	2.07 ± 0.35	0.31 ± 0.05
injection site		20.00 ± 9.96

[a] The values shown represent averages ± SD ($n = 3$) measured 24 h after i.v. or s.c. dosing (3 mg/kg).

however, could be detected in lungs, heart, skeletal muscle, and testes. Furthermore, i.v. dosing as compared to s.c. administration yielded 2−6-fold higher concentrations in most of the tissues except kidney. The overall lower concentration is probably due to a slower distribution for the latter as indicated by the large residual amount at the injection site. For both modes of administration, less than 10% of the administered dose was found to be excreted within 24 h (Figure 5, Supporting Information).

Discussion

For PNA-peptide conjugates to be useful antisense therapeutics, which operate through modulation of splicing, they must be able to (i) bind to the cell surface and trigger cellular uptake, (ii) cross the lipid bilayer and enter the cytoplasm, (iii) translocate to the nucleus, and (iv) bind to their target pre-mRNA and inhibit the splicing machinery at a specific site. In contrast to the majority of studies involving CPPs, the purpose of the present study was to identify the structural features required for a peptide-based carrier to efficiently deliver PNA through the plasma membrane to its pre-mRNA target in the nucleus. Therefore, the primary end point of this study was the antisense activity of the conjugates, as measured by their ability to inhibit production of full length protein product.

The correlation between the number of positively charged headgroups and the activity has been demonstrated by varying the length of the oligo(L-lysine) peptide and confirmed with peptides containing uncharged amino acids (Gly, Cit) or residues, which are partially charged at neutral pH (His). The spatial arrangement of the cationic groups has an influence on the activity with a preference for the linear evenly spaced over the branched configuration, which would be expected if binding through electrostatic interactions to negatively charged groups on the cell surface is required to initiate uptake. Similarly, the significant loss in activity observed for the N-ϵ-dimethyllysine modification suggests that the two methyl groups may sterically interfere with the binding to the cell surface and the process of uptake and/or endosomal release. Alternatively, the headgroup modification could affect the ability of the peptide carrier to effectively mediate nuclear transport of the conjugates.

Contrary to reports of others, we observed a decrease in activity for the uniform oligo(L-arginine) conjugate as compared to its lysine analogue. Cellular uptake of cargo-free arginine-rich peptides or guanidinylated peptide mimetics has been reported to be a saturable but rapid process for which the enzymatic stability of the peptide should be less of a concern.[26] However, if the PNA-peptide conjugates enter the cells predominantly via endocytosis followed by a slow release from the endosomes into the cytoplasm, enzymatic degradation can be expected to play a more crucial role, which would explain the observed decrease in antisense activity of the more labile octa(L-arginine)-conjugate. This hypothesis is further strengthened by the observation that introduction of the more stable homoarginines led to the restoration and even improvement in activity.

Shortening of the side chain length can significantly impact activity. At this point, it is unclear whether it affects cell surface binding or nuclear translocation. On the other hand, no significant effect on the activity could be observed by extending the side chain through incorporation of homolysine. The results indicate that the four carbon side chain of the parent lysine residue provides favorable spacing of the cationic headgroups, which seems to be required to maintain activity. However, replacement of lysine at certain positions is tolerated and, in the case of ornithine, even leads to an improvement in activity, which might be due to the stabilizing effect of this modification against proteolytic degradation.

Altering the structure of the backbone with the exception of the uniform octa(D-lysine) conjugate caused significant reduction of the activity. This is in striking contrast to previously published work showing improved cellular uptake of various cationic peptide mimetics such as β-peptides,[27,28] peptoids,[26] and oligocarbamates.[29] From those studies, it is evident that such enzymatically stabilized peptide analogues are able to bind to the plasma membrane and efficiently enter the cells. Therefore, they should be well-suited to survive and eventually escape the hypothetical endosomal pathway. On the other hand, it is likely that the nature of the peptide carrier not only determines the efficiency of cellular uptake but also influences the intracellular trafficking and in our case the accumulation of the antisense PNA at its site of action in the nucleus.

In general, separating the peptide carrier from the antisense PNA with a linear alkyl tether did not improve but rather decreased the activity of the octa(L-lysine) conjugate. The impact of the spacer seemed to depend on its length and nature. The activity was not affected by the use of short (β-Ala, ahx) or more hydrophilic spacer (O-spacer).

Some of the compounds, in particular the ones containing the shorter side chain substitutions 2,4-diaminobutyric acid or ornithine, but also the uniform homoarginine or β-lysine-containing conjugates significantly affected cell viability in the concentration range used for this study. However, it should be pointed out that cell viability was determined after a 3 day compound exposure, in which the untreated cells go through at least two cell divisions. Therefore, the assay provides a very sensitive readout of cell viability albeit without distinguishing between cytotoxic and cytostatic effects.

In general, the study did not indicate a correlation between the antisense activity and the effects of the compounds on cell viability. It has been previously reported that cationic lysine-tagged PNAs can exhibit nonspecific electrostatic interactions with DNA under low-salt conditions, which causes the rates of sequence specific binding to slow substantially.[30] This effect, however, has been found to be negligible under higher (physiological) salt concentrations. Nevertheless, nonspecific binding to polyanionic DNA or RNA cannot be completely excluded if highly cationic PNAs, such as the ones described in this work, are used for biological applications and it could in part relate to the observed cytotoxicity.

To evaluate the suitability of cationic peptide-PNA conjugates for in vivo applications, the parent octa(L-lysine) conjugate was radiolabeled and screened for its biodistribution in mice. The results demonstrate that the conjugate was cleared rapidly from circulation and that it distributed to a variety of tissues with the highest concentrations found in liver, kidney, and spleen while excretion was found to be below 10% of the administered dose. While these preliminary findings are encouraging, further PK/PD studies are required to determine whether those simple peptides can be suitable delivery vehicles for systemic administration of PNA-based antisense therapeutics.

Conclusion

Numerous cationic carriers based on a simple oligolysine motif were investigated for their ability to promote cellular uptake and elicit antisense activity with a PNA pentadecamer, which redirects splicing of murine CD40 transcript. While some peptides showed activity comparable to the parent conjugate, only two compounds were found to be superior, an analogue with two lysines replaced by ornithines and a uniform homoarginine conjugate. Both compounds exhibited substantially increased stability against proteolytic degradation. The homoarginine reduced cell viability, while the ornithine-containing conjugate showed no significant effects in the concentration range applied. Overall, the present study does not provide evidence that the observed antisense activities of the PNA-peptide conjugates are correlated with their respective effects on cell viability. The results suggest, however, that intracellular trafficking and specifically nuclear localization are influenced by the nature of the peptide carrier. When administered to healthy mice, the parent oligolysine conjugate showed rapid distribution to a variety of tissues together with only modest elimination via excretion within the time frame of the experiment. This indicates that conjugation to simple basic peptides not only provides a viable strategy to improve cellular uptake but could also be used as a potentially versatile tool for modulating the in vivo biodistribution of antisense PNA.

Experimental Section

Abbreviations. Ahx, 6-aminohexanoic acid; aoc, 8-aminooctanoic acid; addc, ω-aminododecanoic acid; Boc, *tert*-butyloxycarbonyl; t-Bu, *tert*-butyl; Cbz/Z, benzyloxycarbonyl; Cit, L-citrulline; CPP, cell-penetrating peptide; Dab, 2,4-diaminobutyric acid, DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMS, dimethyl sulfide; ESI-MS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; Gly, glycine; Arg, L-arginine; hArg, L-homoarginine; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HFBA, heptafluorobutyric acid; Lys, L-lysine; hLys, L-homolysine; D-Lys, D-lysine; β-Lys, β-L-lysine; HOBt, *N*-hydroxybenzotriazole; 2′-*O*-MOE, 2′-*O*-methoxyethyl; MBHA, *p*-methylbenzhydrylamine; MLN, mesentary lymph nodes; NHS-ester, *N*-hydroxysuccinimide ester; NMP, *N*-methylpyrrolidinone; Orn, L-ornithine, O-spacer, 1-amino-3,6-dioxaoctanoic acid; PNA, peptide nucleic acid; pK, lysine peptoid; SD, standard deviation; TEA, triethylamine; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; THF, tetrahydrofuran; dmK, *N*-ϵ-dimethyllysine.

Reagents and Solvents. The solvents used were purchased from Aldrich or J. T. Baker in the highest grade available. Amino acids, the resins used for solid phase synthesis, HBTU, and HOBt were purchased from Novabiochem. PNA monomers (Boc-protected), and HATU and the O-spacer (Boc or Fmoc-protected) were obtained from Applied Biosystems. Soluene-350 was purchased from Beckman, and Econosafe was obtained from RPI. Cell culture reagents were obtained from Invitrogen. All other reagents were purchased from Sigma-Aldrich Corporation.

Lys-peptoid Monomer Synthesis (56). *N*-Cbz-1,4-diaminobutane (5 g, 19.3 mmol) was dissolved in 200 mL of dry pyridine, and 20 mL of DMSO was added. To this solution triethylamine (9.24 mL, 66.5 mmol) was added. Methylbromoacetate (0.871 mL, 9.5 mmol) was diluted in 50 mL of dry DMF and added dropwise to the mixture over 3 h, which was then stirred for another 16 h. Di-*tert*-butyl dicarbonate (6.33 g, 29 mmol dissolved in 20 mL of DCM) was added dropwise under stirring and was allowed to react overnight. The ester was extracted with ethyl acetate, and after the solvents were evaporated, was saponified with LiOH (0.5 M, THF/MeOH/H_2O 1:1:1). The solution was acidified with HCl (3 M), and **56** was extracted with DCM. R_f 0.25 (DCM/MeOH, 95:5). ESI-MS: 380.2 (calcd 380.19).

Synthesis, Purification, and Characterization of PNA-Peptide Conjugates. For the N-terminal peptide conjugates, the PNA part of the conjugates with the sequence CACAGATGACATTAG-Lys (**1**) was assembled on MBHA polystyrene resin, preloaded with Boc-Lys(2-Cl-Z) (loading: 175 μmol/g) using a 433 A Peptide Synthesizer (Applied Biosystems) according to previously published procedures for PNA synthesis.[31,32] The peptide part of the conjugate was synthesized manually and in parallel either with Fmoc or Boc chemistry using custom fabricated glass columns, equipped with a glass frit, a stopcocked outlet, and an argon inlet. C-terminal peptide conjugates were assembled manually and in parallel using Boc chemistry throughout the synthesis. Deprotection and cleavage were generally carried out using a low/high TFMSA treatment, and the duration of the treatment was determined by the side chain protecting groups present.

The conjugates were purified by RP-HPLC using a Gilson HPLC system including a 306 Piston Pump System, a 811C Dynamic Mixer, a 155 UV/vis detector, and a 215 Liquid Handler together with the Unipoint Software on Zorbax SB300 C_3 columns (Agilent). HFBA (0.1%) in H_2O (A) and CH_3CN (B) were used as the solvent systems. The applied gradient was dependent on the length and sequence of the conjugate. Dual wavelength detection was carried out at 220 and 260 nm, and the column temperature was kept at 60 °C.

The purified conjugates were analyzed by analytical HPLC (generally the purity was greater 90%) and mass spectrometry (ESI-MS) and stored at −20 °C until prior to use.

Synthesis of L-Homoarginine-Containing PNA-Peptide Conjugates (20, 21). The conjugates were assembled by solid phase peptide synthesis as described above using Boc-L-lysine(Fmoc)-OH as a precursor for the L-homoarginine residues. Prior to acidic deprotection and cleavage, the Fmoc-protecting groups of the lysine side chains were selectively removed with 20% piperidine in DMF and the free amino

groups were guanidinylated by adding a 1.8 M solution of 1*H*-pyrazole-1-carboxamidine-HCl in DMF/DIEA (2:1) to the resin. After shaking at 55 °C for 24 h, the PNA-peptide conjugates were deprotected and cleaved from the resin, purified, and characterized as described above.

Preparation of ^{3}H-Acetyl-Labeled PNA-Peptide Conjugate 55. The PNA-peptide conjugate **2** was synthesized with a C-terminal Lys(Fmoc) residue. After completion of solid phase synthesis, the Fmoc group was removed with 20% piperidine in DMF, and the resin was washed with DMF and DCM and dried in vacuo. Labeling, cleavage, and purification of the compounds were carried out at Moravek Biochemicals (Brea, CA) according to the following protocol. The resin containing the PNA-peptide conjugate (ca. 1 μmol) was placed in a custom fabricated glass column equipped with a glass frit, a stopcocked outlet, and an argon inlet and allowed to swell in DCM for about 2 h. After it was washed with DCM, a mixture of 5.0 μmol of acetyl chloride (methyl, ^{3}H) and 25 μmol of DIEA in 0.3 mL of dry DCM was added and allowed to react for 1 h under shaking. The resin was washed thoroughly with DCM, pyridine, and again with DCM, and a mixture of 2% acetic anhydride in anhydrous pyridine/DMF was added to the resin and allowed to react for 5 min under shaking. Again, the resin was washed with pyridine, DMF, and DCM. After the resin was washed twice with TFA, a fresh solution of TFA/TFMSA/thioanisole/*m*-cresol (8:1:1:1) was added and the cleavage/deprotection was allowed to proceed for about 3 h at r.t. Workup and HPLC purification were performed according to the standard protocols. To remove HFBA salt adducts, the purified compound **55** was passed through an anion exchange resin (Dowex 2X8−200, Aldrich 428639) and the final product was analyzed by analytical HPLC including radiochemical detection. To confirm the identity of the sample by HPLC analysis, the "cold" standard was prepared from an aliquot of the same conjugate-containing resin under identical protocol but with "cold" acetyl chloride and analyzed by ESI-MS.

Enzymatic Stability of PNA-Peptide Conjugates. Solutions (20 μM) of the PNA-peptide conjugates in either 10% FBS-containing cell culture medium (RPMI 1640, Gibco, Invitrogen Inc.) or mouse serum stabilized with 10 mM Hepes and diluted to 25% with PBS (pH 7.1−7.4) were prepared and incubated at 37 °C. Aliquots of 150 μL were removed at 0, 1, 3, 6, 24, 72, and 168 h and 0, 1, 3, 6, 24, and 48 h for 10% FBS-containing and 25% mouse serum-containing solutions, respectively, and an equal volume of a solution of 10% DCA in H_2O/CH_3CN was added. The samples were agitated for ca. 30 s on a Mini Vortexer (VWR) and allowed to stand for ca. 30 min at −20 °C. The precipitated serum proteins were separated by centrifugation (14000 rpm, 8 min), 200 μL of the supernatant was carefully withdrawn, and 10 μL of a 0.1 mM solution of a PNA 6mer (H-CATTAG-Lys-NH_2) was added as a standard for HPLC analysis. The samples were analyzed on a Gilson HPLC system as described above using a Zorbax SB300 C_3 (150 mm × 4.6 mm) column (Agilent) and 0.1% HFBA in H_2O (A) and CH_3CN (B) as the solvent system. The column temperature was kept at ca. 60 °C. To determine the $t_{1/2}$ values of the PNA-peptide conjugates, the normalized peak areas of the full length products (A_{PNA}/A_{Std}) were calculated and plotted as percent full length at time zero as a function of the incubation time. The linearity of the assay was confirmed in the concentration range of interest (1−20 μM) using the PNA-$(Lys)_8$ conjugate **12** as a representative example by plotting the normalized peak areas of the extracted samples against the applied sample concentration ($R^2 = 0.9936$). The variability of the assay was estimated by screening conjugate **2** as a representative example multiple times with different samples of mouse serum. The average half-life was found to be 6.7 h with a standard deviation of ± 1.4 h. For LC-MS analysis of the degradation products, fractions from the HPLC analysis were collected, evaporated, and redissolved in deionized water. The samples were analyzed in positive mode on a MSD 1100 ESI-MS system connected to a HPLC system (Agilent). The HPLC conditions were as follows: column, Vydac C_{18} MS (2.1 mm × 50 mm); column temperature, 50 °C; eluent system, 0.1% acetic acid (A), 95% aqueous CH_3CN (B); flow, 0.3 mL/min; gradient, 0−10 min: 0−22% B; 10−12 min: 22−50% B.

Cells. BCL_1 cells were obtained from the American Type Culture Collection and grown in normal growth medium (Dulbecco's modified Eagle medium, supplemented with 10% fetal bovine serum and antibiotics). Cells were incubated in a humidified chamber at 37 °C, containing 5% CO_2. PNA conjugates were added to cells at the indicated final concentrations. Cells were exposed to compound for 3 days. All experiments were done in triplicate.

Cell Viability. BCL_1 cells were seeded at 2000 cells per well into 96 well plates. Following 3 days of compound exposure, wells were analyzed for relative number of viable cells using WST-1 assay (Roche) according to manufacturer's protocol. Compound-related effects on cell proliferation and viability were considered to be significant if the average absorbance of WST-1 at a wavelength of 450 nm dropped below 50% of untreated control.

Flow Cytometry Analysis. Cells were detached from culture plates with 0.25% trypsin. Trypsin was neutralized with an equal volume of normal growth medium, and cells were pelleted. Cell pellets were resuspended in 200 μL staining buffer (phosphate buffered saline containing 2% bovine serum albumin and 0.2% NaN_3) containing 1 μg of either FITC-labeled isotype control antibody or FITC-labeled anti-CD40 antibody (clone HM40-3, BD Biosciences). Cells were stained for 1 h, washed once with staining buffer, and resuspended in PBS. CD40 surface expression level was determined using a FACScan flow cytometer (Becton Dickinson).

Western Blot. Cells were harvested in RIPA buffer (phosphate-buffered saline containing 1% NP40, 0.1% SDS, and 0.5% sodium deoxycholate). Total protein concentrations were determined by Lowry assay (BioRad), and equal quantities were precipitated with cold acetone by centrifugation. Protein pellets were vacuum-dried and resuspended in load dye (Invitrogen) containing 5% β-mercaptoethanol. Samples were heated to 92 °C for 10 min prior to gel loading. Protein samples were separated on 10% PAGE Tris-glycine gels and transferred to PVDF membranes. Membranes were blocked with blocking solution (TBS-T containing 5% nonfat dry milk) and blotted with appropriate antibody. The polyclonal CD40 antibody was obtained from Calbiochem. β-Actin monoclonal antibody was obtained from Sigma. HRP-conjugated secondary antibody was obtained from Jackson Immunoresearch. Protein bands were visualized using ECL-Plus (Amersham-Pharmacia).

PK/Tissue Distribution. Radiolabeled conjugate **2** was administered either i.v. or s.c. into male Balb/c mice ($n = 3$) at a dose of 3 mg/kg (0.5 mg/mL in PBS with 12% EtOH, pH 7.1). Mice were sacrificed after 5 min, 1, 4, and 24 h and samples from blood, feces, urine, heart, lung, liver, kidney, spleen, mesentery lymph nodes, pancreas, small/large intestine, testes, skeletal muscle, and furless skin were collected and dissolved in tissue solubilizer (Soluene-350). The samples were then added to liquid scintillation buffer (Econo-safe), and radioactivity was measured in a liquid scintillation counter (Beckman, LS6000IC).

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Supporting Information Available: Tabular analytical data (HPLC and ESI-MS) for PNA-peptide conjugates, representative example of HPLC and ESI-MS analysis data for compound **2**, scheme showing the pattern of enzymatic cleavage in 25% mouse serum for the parent conjugate **2**, the C-terminal octa(L-lysine) conjugate **12** and the octa(L-arginine) conjugate **18**, data of the antisense activity of glycine- (**14**) and citrulline-containing conjugates (**15**), direct comparison of the activity of the octa(L-lysine) conjugate **2** and the octa(homolysine) conjugate **37**, and plasma profile and excretion values of radiolabeled octa(L-lysine) conjugate **55**. This material is available free of charge via the Internet at http://pubs.acs.org.

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