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Peptide–PNA Conjugates: Targeted Transport of Antisense Therapeutics into Tumors**

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Dedicated to Professor Harald zur Hausen

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Systemic toxicity is one of the major problems in chemotherapy. While the traditional chemotherapeutics generally affect all of the proliferating cells, new therapy modalities, in particular gene therapy, offer the possibility to selectively act in tumor cells. However, the high molecular weight of the active substances used in these treatment methods leads to unfavorable pharmacokinetic behavior that causes problems in the clinical application of these substances. Therefore, the success of these treatment methods depends on the development of new vector systems that enable transport of high molecular weight substances for example, oligonucleotides. Remarkable progress can be made here by chemical modifications [1].

Somatostatin-receptors (SSTRs) are found in numerous kinds of tumors (e.g., breast tumors, small-cell lung cancer). As the native peptide somatostatin is rapidly degraded in vivo, stabilized derivatives have been developed for clinical application. The most successful derivative is octreotide [2]. This peptide is able to induce endocytosis by binding to SSTRs and, because of the broad substrate specificity of these receptors, to mediate

internalization of conjugated substances. That this can be used for the transport of tumor therapeutics has been shown in vitro for conjugates of the spindle poison taxol [3].

Since, up to now, it has not been possible to selectively transport antisense oligonucleotides into tumor cells, the question arose whether it would be possible to use SSTR-affine peptides as carriers for oligonucleotides. The oligonucleotide target sequence chosen was the anti-*bcl*-2- sequence [4, 5]. It is quite difficult to generate the peptide– nucleic acid conjugates that are needed for this targeting concept by solid-phase synthesis. The standard methods for the synthesis of oligonucleotides and peptides are incompatible. Consequently, the conjugates are favorably generated by postsynthetic conjugation. We have recently reported on phosphorothioate oligonucleotides that are bound to a maleimido-modified SSTR-affine peptide through a thiol group at their 5'-end [6]. The conjugates showed high hybridization efficiency and a high affinity for SSTRs. In spite of the promising results that could be found in vitro, animal experiments revealed no significant enrichment in the tumors [7].

The pharmacokinetics of the phosphorothioates seemed to be dominated by strong unspecific interactions. Consequently, we chose the metabolically stable peptide nucleic acids (PNAs) for the experiments described herein [8]. The convergence of PNA and peptide synthesis enables the stepwise synthesis of PNA–peptide conjugates on the same polymeric support. Either 9-fluorenylmethoxycarbonyl (Fmoc) or *tert*-butyloxycarbonyl (Boc) chemistry are suitable for the synthesis of PNA oligomers. However, due to side reactions during deprotection of the Fmoc protecting group, the purity of the products produced by Boc chemistry is higher. To allow a convergent synthesis, the peptide part had to be built up by Boc chemistry as well. The PNA oligomers can be synthesized by using Boc chemistry in good yields, consequently this method is also suitable for the synthesis of PNA–peptide conjugates [9].

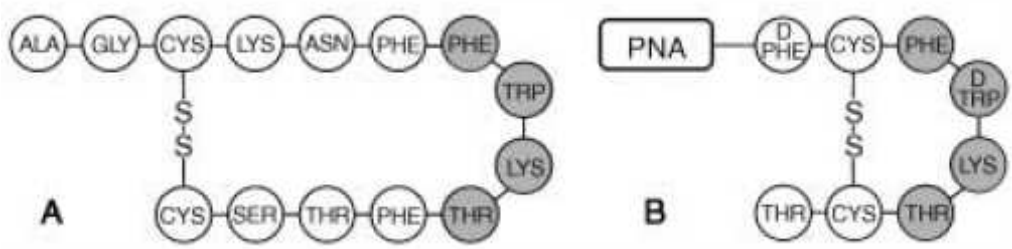
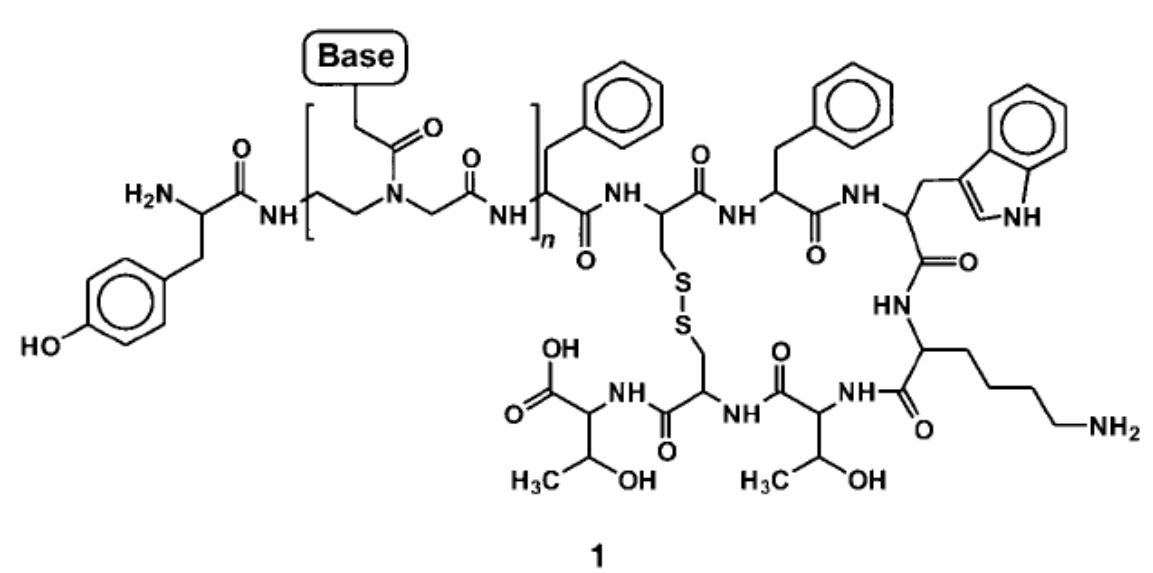


Figure 1. Comparison of native somatostatin (A) with a conjugate consisting of a PNA and Tyr^3-octreotate (B). The amino acids that are essential for receptor binding are marked in gray.

Octreotate, which is nearly identical to octreotide except that it has a carboxy group at the *C* terminus and thus supports internalization (Figure 1B), was chosen as the peptide part of the conjugate. Boc-D-Phe-Cys(Acm)-Phe-D-Trp-Lys-Thr-Cys(Acm)-Thr-PAM resin was synthesized by application

of a modified in situ neutralization protocol [10] with HATU as activator. The average coupling efficiency, determined by ninhydrin assay, was 99.1%. To enable the introduction of radioactive iodine (^{125}I) an additional tyrosine residue was conjugated to the *N*-terminus, which led to the target molecule **1** (base sequence=Tyr- AGC GTG CGC CAT CCC- peptide).



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During chain elongation in peptide and PNA synthesis, aggregation of the growing oligomer chain can be caused by either intra- or intermolecular interactions. This may lead to a collapse of the solid support and thus to low coupling efficiencies. The tendency for aggregation increases with increasing chain length. Consequently, the syntheses of long PNA–peptide conjugates is especially prone to the appearance of "difficult sequences". Application of known synthesis protocols [11] did not lead to the anticipated success, thus, solvents, coupling times, the capping step, as well as the reaction conditions for the repeated coupling steps had to be optimized. The use of Ac_2O/pyridine in DMF in the capping step proved to be essential. This was followed by a washing step with DMF/ piperidine to cleave possible acetyl esters. The coupling cycles were performed with five equivalents of the Boc-protected PNA monomers using HATU as the activating agent. The exocyclic amino functions of the A, C, and G monomers were protected by benzyloxycarbonyl groups. The coupling cycle is shown schematically in Figure 2.

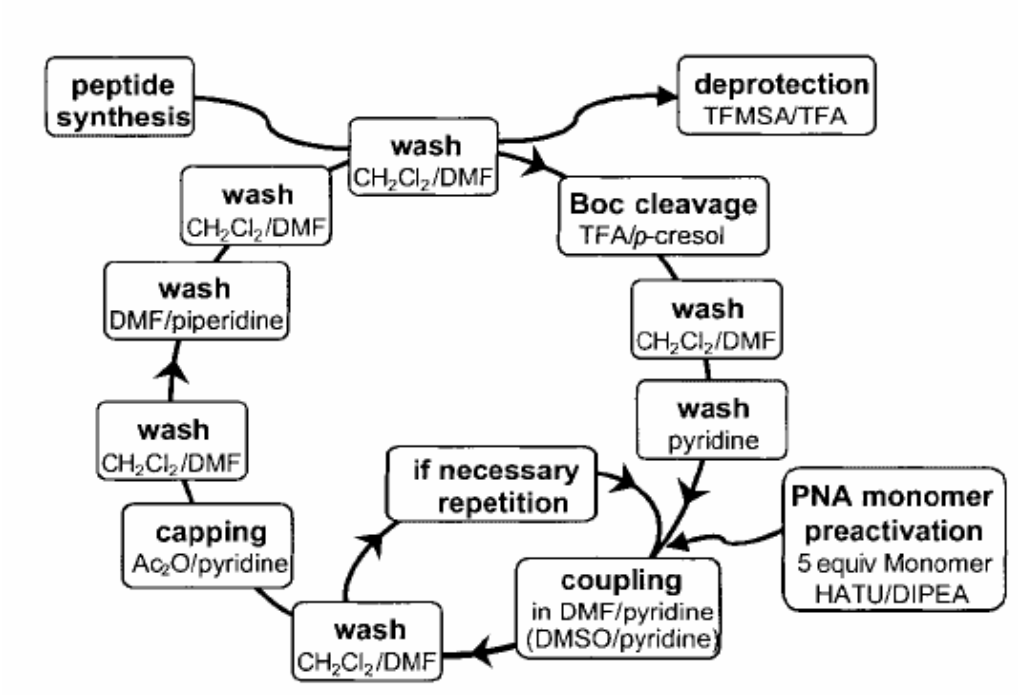
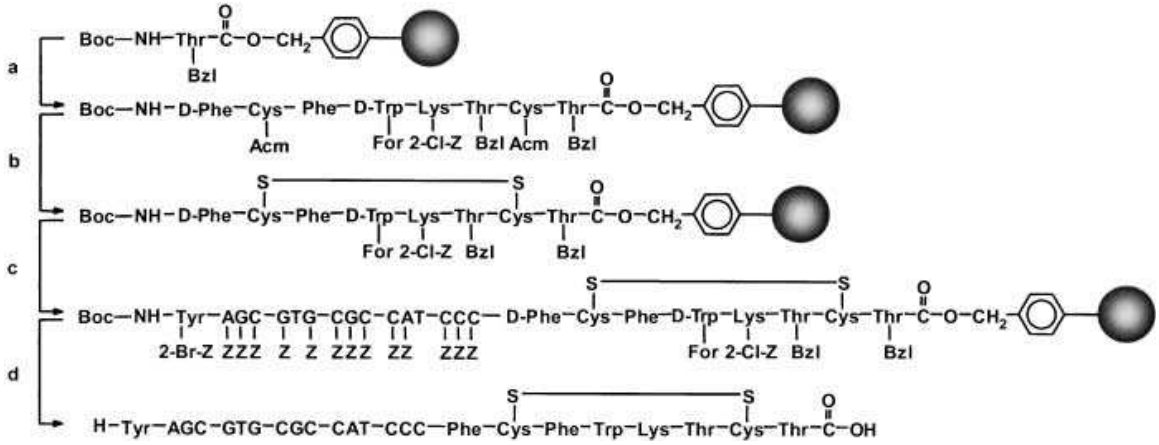


Figure 2. Flow diagram of the synthesis cycle used for conjugating the PNA part (Boc chemistry). The abbreviations are defined in Scheme 1.



Scheme 1. Flow diagram of the synthesis of the PNA–octreotate conjugate using Boc chemistry. a) Synthesis cycles with HATU/DIEA/Boc cleavage with 5% *p*-cresol in TFA; b) $Tl(TFA)_3$; c) synthesis cycles according to Figure 3; d) TFA /*p*-cresol/TFMSA. Protecting groups: Acm= acetamidomethyl, 2-Br-Z=2-bromobenzyloxycarbonyl, 2-Cl-Z=2-chlorobenzyloxycarbonyl, Bzl= benzyl, For= formyl, Z= benzyloxycarbonyl, TFA= trifluoroacetic acid, TFMSA=trifluoro-methanesulfonic acid, DIPEA= *N*,*N*-diisopropylethylamine, HATU= *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluoro-phosphate.

Above a critical length of the oligomers, HPLC analysis of cleaved aliquots revealed strongly decreasing coupling efficiencies: after the 9th monomer, in particular when G monomers were conjugated, coupling efficiencies decreased to about 50%. By elongation of coupling times and by performing repeated couplings, the critical monomers could finally be coupled with coupling efficiencies of more than 95%. Using these optimized coupling conditions, we obtained the target conjugate in about 75% yield (according to HPLC analysis). However, it was not possible to cyclize this conjugate on the solid support, because the Tl^{3+} ions cause side reactions with the nucleobases. Owing to the size of the molecule, the course of the alternative air oxidation cannot be analyzed by HPLC. Therefore, the PNA oligomer was built up on the peptide which had been cyclized beforehand. Scheme 1 shows the synthesis of the octreoate–PNA conjugate from the peptide that was assembled by Boc chemistry and then cyclized by using Tl(TFA)3. After conjugation of the PNA monomers, a homogeneous product was obtained, which was deprotected with TFMSA in TFA using *p*-cresol as the scavenger. After precipitation with diethyl ether and subsequent purification by RP-HPLC, **1** was isolated in about 40% yield.

Binding experiments with the PNA conjugates using rat cortex membranes showed that the conjugates bind to SSTRs with a high affinity similar to that for octreotide, as confirmed by the IC_{50} values (octreotate–PNA oligomer 1.52, octreotide 1.98 nM). Determination of the melting temperatures revealed that the PNA–conjugates hybridize with high affinity to the complementary phosphodiester–DNA oligonucleotides. The melting temperature of the conjugate was 82 ºC and thus much higher than that of a phosphodiester duplex of the same sequence (Tm=71.4 ºC). This shows that due to the high hybridizing affinity the PNA–conjugate should be suitable as an antisense drug.

Organ distribution was evaluated in Lewis rats carrying the SSTR-affine pancreatic tumor CA20948 (Figure 3). After deprotection and cleavage from the solid support, the PNA conjugate and a control PNA with the same sequence were labeled selectively with ^{125}I at the tyrosine residue by using the Chloramine-T method [12]. In contrast to the phosphorothioate conjugate, for the ^{125}I-labeled PNA–peptide conjugate, a selective enrichment in the tumor tissue was obtained. The physicochemical properties of the PNA–peptide conjugate are different to those of the Tyr^3–octreotide. These properties influence factors like the interaction with serum proteins and result in different organ uptake, in particular in the kidney, the liver, and the lung. The modification of the PNA with the peptide part led to an about tenfold increase in tumor uptake.

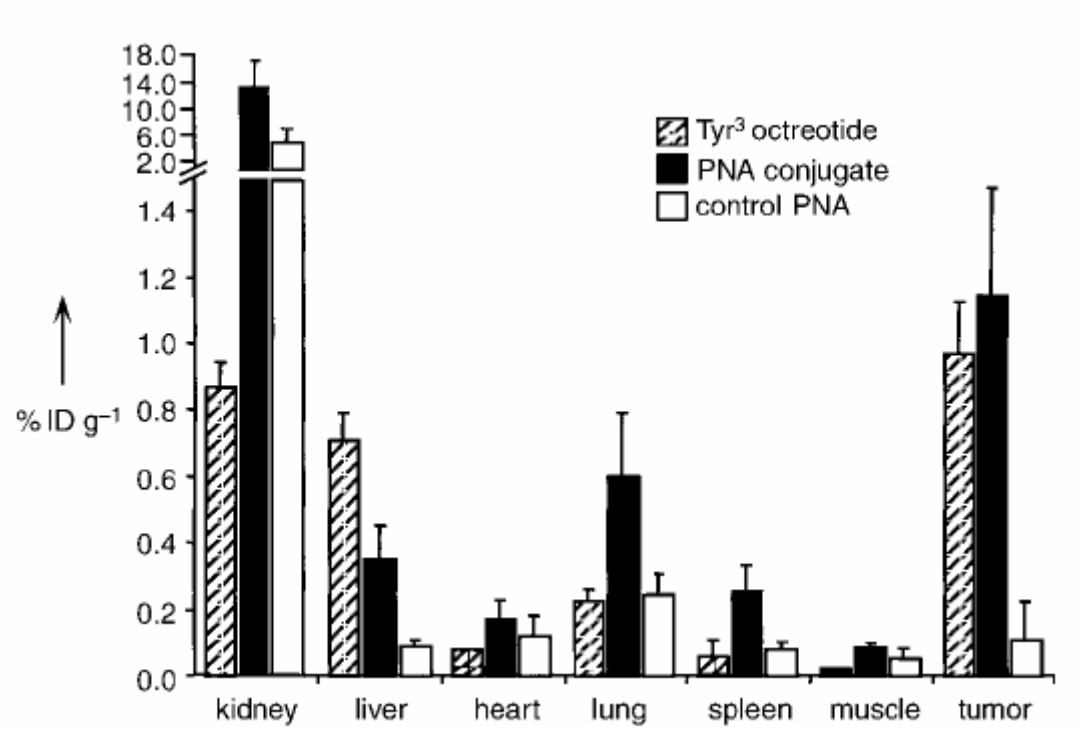


Figure 3. Organ distribution data for tumor-bearing Lewis rats, given in percent of the injected dose per gram of tissue (%IDg^{-1}) ± standard deviation 1 h after intravenous injection (average values from three or six animals, compounds were labeled with ^{125}I). The conjugation of the peptide moiety causes a strongly increased accumulation of the PNA oligomer in the tumor tissue (statistical significance in students t-Test: p=0.021).

In all of the organs except for the kidney, the enrichment of the octreotate conjugate resembles the accumulation of the free peptide. This indicates that the PNA–peptide conjugates in the serum are nonspecifically associated to a lower degree than the phosphorothioate conjugates, and are consequently better accessible for the binding to the receptors. The fact that 79%of the tumor uptake can be inhibited by coinjection of an excess of nonlabeled octreotide provided further evidence for the receptor specificity of this uptake.

Although there are some promising in vitro studies with PNA conjugates [13], none of the methods described could be successfully applied for the targeting of tumors in vivo. The use of SSTR-affine carrier molecules for the first time enables the targeted transport of oligonucleotides in tumor tissue. This demonstrates that targeting of high molecular weight drugs is possible by using receptor-specific peptides.

Experimental Section

Solid-phase syntheses were performed manually in a batch-procedure using Boc-protected amino acids (Bachem, Germany) as well as PNA monomers (Applied Biosystems, Germany) on a 4-(oxymethyl)-phenylacetamidomethyl-(PAM) resin. Analyses and purifications by RP-HPLC were performed on LiChrosorb RP-selectB columns (Merck KGaA, Germany) with gradient elution (water/acetonitrile (0.1%TFA)). Electrospray ionization mass spectrometry was carried out on a Finnigan MAT TSQ 7000 System (Thermo Finnigan, San Jose, USA). The radioisotope ^{125}I was purchased from Amersham Pharmacia Biotech (Freiburg, Germany).

Synthesis of the PNA–peptide conjugate: First, the following synthesis cycle was carried out to obtain H-D-Phe-cyclo[Cys-Phe-D-Trp(For)-Lys(2-Cl-Z)-Thr(Bzl)-Cys]-Thr(Bzl)-O-PAM: The resin (100 mg; initial loading=0.72 mmol g^{-1}) was agitated with 5% *p*-cresol in TFA (two times for 2 min), and then washed with DMF, combined with a mixture of the carboxylic acid (4 equiv), HATU (3.9 equiv) and diisopropylethylamine (DIPEA; 10 equiv) which had been pre-incubated for 2 min in DMSO, agitated for 2 min and again washed with DMF. The synthesis cycle for conjugating the PNA monomers to this resin was carried out as follows: a) agitation with TFA/*p*-cresol (95:5, v/v) two times for 2 min; b) incubation of the PNA monomers with HATU (4.9 equiv) and DIPEA (10 equiv) for 2 min (final concentration 0.1M); c) washing with DMF/CH_2Cl_2 (1:1, v/v) and then with pyridine; d) agitation with the preactivated PNA monomer for 15 min; e) washing with DMF/ CH_2Cl_2 (1:1, v/v); f) agitation with Ac_2O/pyridine/DMF (1:10:10, v/v/v) for 5 min; g) washing with DMF/ CH_2Cl_2 (1:1, v/v); h) agitation with DMF/ pyridine (95:5, v/v) for 2 min; i) washing with DMF/ CH_2Cl_2 (1:1, v/v). After the coupling of Boc-Tyr(2-Br-Z) the resin was washed and dried. The loaded resin (30 mg) was mixed with 5% *p*-cresol (500 μL) in TFA. After the addition of TFMSA (50 μL), the resin was agitated for 2 h, filtered off, and the raw product was precipitated with diethyl ether. The raw product was resolubilized in acetonitrile/water (1:1) and incubated for 24 h at room temperature. The sample was purified by HPLC. After lyophilization, H-Tyr-AGC GTG CGC CAT CCC-D-Phe-cyclo[Cys-Phe-D-Trp-Lys-Thr-Cys]-Thr-OH was obtained as a white powder (overall yield: 16.8 mg; ca. 40%). The integrity and purity of the product was confirmed with ESI-MS (m/z for $C_{218}H_{270}N_{96}O_{58}S_2$ calcd: 5203 $gmol^{-1}$, found: 5205 $gmol^{-1}$ ($[M+H]^+$) and analytical HPLC (purity >95%).

The control PNA (H-Tyr-AGC GTG CGC CAT CCC-Lys-NH_2) was synthesized analogous to the PNA–peptide conjugate.

Organ distribution studies: A cell suspension of the CA20948 tumor in a nutrient mixture was subcutaneously administered into the nape of the neck of male Lewis rats. After about 10 days, the tumors were grown to a volume of about 5 mL. The ^{125}I-labeled compound was injected into the tail vein of the animals (groups of three animals). After 1 h, the animals were sacrificed and the activity concentration of the dissected organs was determined in a γ-counter.

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