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Preparation and Evaluation of Tumor-Targeting Peptide-Oligonucleotide Conjugates

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Abbreviations: Acm, acetamidomethyl; Boc, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; DIPEA, diisopropylethylamine; DTT, dithiothreitol; EDITH, 3-ethoxy-1,2,4-dithiazolin-5-one; Fmoc, 9-fluorenylmethoxy-carbonyl; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; ODN, oligodeoxynucleotide; SPPS, solid-phase peptide synthesis; SSTR, somatostatin receptor; TEAA, triethylammonium acetate; TIS, triisopropylsilane

Abstract.

Enormous progress has been made in the development of antisense oligodeoxynucleotides (ODNs) as therapeutic agents inhibiting gene expression. Unfortunately, the therapeutical application of ODNs is still held back because of the low cellular uptake and the lack of specific transport into particular cells. In this paper, we report a drug-targeting system using somatostatin receptors (SSTRs) which are overexpressed in various tumors. Phosphorothioate ODNs were covalently linked to Tyr^3- octreotate, an analogue of somatostatin. The peptide was assembled by solid-phase synthesis, oxidized to form the cyclic disulfide, and subsequently derivatized with a N-terminal maleimido functionality. 5'-Thiol derivatized phosphorothioate-ODNs directed against the protooncogene *bcl-2* were conjugated to this maleimido-modified peptide. Binding studies revealed that the conjugates retain specific binding with nanomolar affinities to SSTRs (IC_{50}-values between 1.83 and 2.52 nM). Furthermore, melting studies with complementary DNA revealed that the terminal conjugation of the ODNs did not significantly affect their hybridization affinity.

INTRODUCTION

Antisense therapy holds great promise for the treatment of gene-related disorders. Still, the major obstacle for an efficient antisense therapy is the low internalization rate into target cells. Even though ODNs are large,

negatively charged molecules, they are to some extent transported through the cellular membranes. This uptake is achieved by pinocytosis and processes which are known to be concentration and energy dependent. Depending on the ODN, the cell line and experimental conditions, the uptake rate ranges 1-20%.

Numerous attempts have been made to increase the intracellular bioavailability and to enable specific internalization into selected cell types. A highly efficient and versatile system for uptake enhancement is encapsulation into liposomes. Within liposomes, ODNs are preserved from serum nuclease degradation. Taking advantage from various coating modifications which increase stability and uptake, liposomes have become valuable shuttles for ODNs (*1*). ODNs attached to hydrophobic moieties, such as lipids (*2*), cholesterol (*3*), or geraniol (*4*), show increased antisense effects. However, these effects have always been controversially discussed because of sequence unrelated effects and the lack of a plausible mechanism. Cellular internalization of cationic molecules by adsorptive endocytosis has been exploited by several procedures. For example, cellular import can be increased with ODN-polylysine complexes (*5*) or ODNs conjugated to avidin (*6*). Conjugation of PNA with a monoclonal antibody to the rat transferrin receptor enabled the transport through the blood brain barrier (*7*). This approach led to a 28-fold uptake rate as compared to the unmodified ODN analogue. The complexes formed when mixing ODNs with porphyrin possess enhanced stability and cellular delivery by an unknown mechanism (*8*). The ubiquitously expressed receptors for vitamin B_6 can be addressed with ODNs conjugated to vitamin B_6 (*9*). These conjugates were believed to be taken up by potocytosis. A unique targeting system for liver cells has been developed using the asialo-glycoprotein receptors (*10*). Residues of β-D-galactose within the asialo-glycoprotein mediate the selective uptake of the conjugated ODN into the liver. These findings should stimulate the search for selective targeting vectors which enable enhanced receptor-mediated delivery into other tissues. Peptides which bind to integrins (*11*) and the EGF-receptor family (*12*) represent examples of peptide vectors which have been employed for ODN-transport. These peptides bind to distinct receptors, which upon binding, mediate endocytosis of the peptide-ODN complex. Conjugates of ODNs with penetratins (*13*) or signal peptides (*14*, *15*) acquire increased uptake rates due to the membrane translocation properties of these peptides. Hitherto no targeting system with selectivity for cancer cells and high capacity sufficient for antisense therapy has been found to be effective *in vivo*.

Somatostatin is a cyclic tetradecapeptide found in the hypothalamus. It is an important regulating peptide which exerts inhibitory effects on the secretion of growth hormones. The SSTRs are known to be overexpressed by a variety of neoplastic tissues such as small cell lung cancers, breast tumors, brain tumors, and many other (mainly endocrine) tumors. Therefore, the membrane-associated SSTRs represent potential molecular targets for selective delivery into tumor cells. Because of the short biological half-live of the natural somatostatin (<3 min) in vivo, many analogues have been developed. Octreotide, a cyclic octapeptide with an improved specificity and a half-live of about 90 min (*16*) has become the most successful analogue. Recently, it has turned out that octreotate, the carboxylic acid derivative of octreotide, offers improved pharmacological

properties and should therefore be an interesting alternative to octreotide (*17*).

A large number of tumor types show aberrant gene expression of the protooncogene *bcl-2*. These abnormalities result in an overexpression of *bcl-2* protein. This protein is known to inhibit apoptosis. Antisense ODNs have been used to inhibit *bcl-2* expression in cellular studies (*18*). The model ODN used in the present studies is a 20-mer that corresponds to a selected part of the coding sequence within the genome of human *bcl-2* cDNA (*19*). Besides their high enzymatic stability, phosphorothioates have been shown to be highly potent in inhibiting *bcl-2* expression (*18*). Therefore, the ODN conjugates were prepared as phosphorothioates.

Even though octreotate contains only eight residues, the synthesis is complicated by several structural features. In particular, cyclization of the disulfide bond does not converge with the introduction of amine- and thiolreactive groups necessary for conjugation. Hence, a synthetic protocol, which allows the solid-phase formation of the disulfide bond and subsequent attachment of the reactive group has been developed.

In addition to the synthesis, we report herein data on binding specificity to the SSTRs and binding specificity to the corresponding ODNs. These experiments were performed to scrutinize whether the ODN-Tyr^3-octreotate conjugates fulfill the major prerequisites for receptor mediated uptake and antisense effect.

EXPERIMENTAL PROCEDURES

General. The peptides were analyzed and separated by liquid chromatography (HPLC) on a Gyncotech P-580 system (Germering, Germany) equipped with a variable SPD 6-A UV detector and a C-R5A integrator (both Shimadzu, Duisburg, Germany). The columns used were Nucleosil C18, 5 μm, 250 x 4 mm (Macherey & Nagel, Düren, Germany) and LiChrosorb RP-select B 10 μm, 250 x 10 mm (Merck, Darmstadt, Germany). The ODNs were purified on a Waters liquid chromatography system on PRP-1 material 7 μm, 305 x 7 mm (Hamilton, Bondauz, Switzerland). UV measurements as well as the melting studies were performed with a computer interfaced Varian Cary 13 UV-vis spectrophotometer. Mass spectrometry analysis of the peptides and of the oligonucleotides was performed on a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-1, Kratos Instruments, England). ^{1}H and ^{13}C NMR spectra of compound **4** were recorded on a Bruker AM 250 spectrometer and are expressed as δ units relative to CD_3OD (δ) 49.3 for ^{13}C). The peptides were synthesized manually with an in-house manufactured SPPS reactor. The oligodeoxynucleotides were synthesized on an Applied Biosystems model 394 DNA synthesizer or on an PerSeptive Expedite 8900 synthesizer. Lyophilization was performed on a Christ (Osterode, Germany) R1-2 lyophilizator. Membrane-binding experiments were performed using an in-house manufactured filtration apparatus.

Reagents. All standard synthesis reagents were purchased from Merck (Darmstadt, Germany). The chemicals for peptide synthesis were obtained from Novabiochem (La¨ufelfingen, Switzerland). *N*-Maleimido-6-caproic acid, thallium(III)trifluoroacetate, and TIS were obtained from Fluka

(Buchs, Switzerland). The chemicals for the oligonucleotide synthesis were obtained from Perkin- Elmer Applied Biosystems (Norwalk, Connecticut) or PerSeptive Biosystems (Hamburg, Germany). The sulfurization- reagent EDITH was from PerSeptive Biosystems. The thiol linker phosphoroamidite was purchased from Glen research (Sterling, Minnesota). Anhydrous solvents were obtained from Merck, PerSeptive Biosystems, and SDS (Peypin, France). NAP-10 gel filtration columns were from Pharmacia (Uppsala, Sweden). Protein was determined using the Bradford assay (Sigma). Water was purified by a Milli-Q water system from Millipore (Eschborn, Germany). Tyr^3-octreotide was prepared by SPPS. Radioisotope $Na^{125}I$ was purchased from Amersham Pharmacia Biotech (Freiburg, Germany). [^{125}I]Tyr^3-octreotide was prepared by iodination of Tyr^3-octreotide using the chloramine-T method according to the method of Bakker et al. (*20*). The product was purified by HPLC and stored at -80 °C. The complementary unmodified phosphodiester and phosphorothioate ODNs for the melting temperature analysis 5'- GTT CTC CCA GCG TGT GCC AT-3' (antisense) and 5'- ATG GCA CAC GCT GGG AGA AC-3' (sense) were synthesized by standard procedures.

Synthesis of the Maleimido-Peptide 4. The peptide was assembled by Fmoc chemistry on 1 g of Fmoc-Thr-(tBu)-Wang resin (0.61 mmol/g). NR-Fmoc amino acids with the following side chain protecting groups were employed: Cys(Acm), Lys(Boc), Thr(tBu), D-Trp(Boc), and Tyr(tBu). All couplings were performed in DMF. The peptide chain was constructed manually according to a modified in situ neutralization cycle (*21*). Briefly, this cycle consisted of a 2-fold decoupling (1 and 5 min) with 50% piperidine in DMF and 10 min coupling with 4 equiv of the Fmoc-amino acid (0.4 M in DMF, incubated for 5 min with 3.9 equiv of HBTU and 6 equivDIPEA). After completion, the resin (1.75 g dry weight) was treated with piperidine in DMF to deprotect the terminal R-amino group of the peptide. An aliquot was cleaved and analyzed by HPLC indicating formation of **1** with a yield of >90%. A total of 200 mg of the resin-bound peptide **1** was cyclized at room temperature with a 2-fold molar excess of thallium (III) trifluoroacetate in DMF. As determined by analysis of a small aliquot, formation of **2** was essentially complete within 1 h. After thorough washing, *N*-maleimido-6-caproic acid was coupled as described above, the resin was washed and dried under vacuum overnight. Cleavage was performed with 5 mL of 37:1: 1:1 TFA/H2O/phenol/TIS for 2 h at room temperature. The resin was filtered and washed. The peptide was precipitated by the gradual addition of *tert*-butyl-methyl ether at 4 °C. Purification was accomplished by reversed phase HPLC on the RP-selectB column using a gradient of 20% B f 50% B in 7.5 min and 50 f 100% B in 5 min (A) H_2O and B) acetonitrile, both containing 0.1% TFA), flow rate) 4 mL/min. Under these conditions, the peptide was eluted at 10.2 min. After lyophilization, 44 mg of **4** (43.6% overall yield) was obtained as a fluffy powder. The purified peptide was characterized by mass spectrometry. Anal. calcd for $C_{59}H_{75}N_{11}O_{15}S_2$ [M + H]$^+$: *m/z* 1243.4. Found: 1244.2. ^{13}C NMR (CD_3OD) *ä*) 20.02 (q), 20.58 (q), 22.89 (t), 26.28 (t), 27.14 (t), 27.70 (2 C) (t), 29.24 (t), 31.42 (t), 36.75 (t), 38.40 (t), 39.34 (t), 40.19 (t), 40.65 (t), 46.45 (t), 46.52 (t), 53.86 (d), 54.16 (d), 54.43 (d), 55.18 (d), 56.42 (d), 57.68 (d), 59.68 (d), 60.55 (d), 68.55 (d), 69.09 (d), 110.41 (s), 112.33 (d), 116.23 (2 C) (d), 128.66 (s), 128.91 (s), 129.34 (2 C) (d), 130.66 (2 C) (d), 131.54 (2 C)

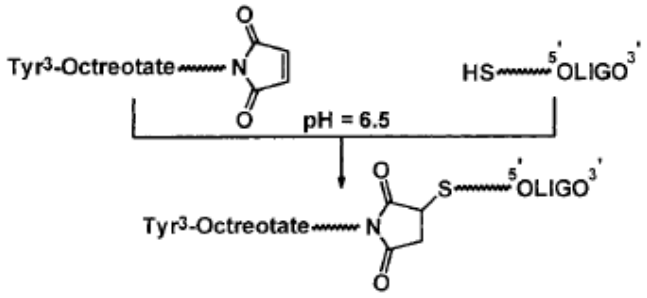
(d), 135.32 (2 C) (d), 137.99 (s), 138.56 (s), 157.35 (s), 171.26 (s), 172.11 (s), 172.60 (2 C) (s), 172.74 (s), 173.26 (s), 173.57 (s), 174.20 (s), 174.35 (s), 175.00 (s), 175.36 (s).

Synthesis and Purification of the 5′-Thiol ODNs. The all-phosphorothioate ODNs, 5′-GTTCTCCCAGCGTGTGCCAT- 3′ (antisense), 5′-ATGGCACACGCTGGGAGAAC- 3′ (sense), and 5′-TACCGTGTGCGACCCTCTTG-3′ (nonsense), were synthesized using β-cyanoethyl phosphoramidite chemistry at the 1 *í*mol scale. Acetylation was performed by 0.1 M acetic anhydride/tetrahydrofuran (THF) and 0.1 M imidazole/THF. Sulfurization was affected by using the EDITH reagent. The commercially available six-carbon thiol linker phosphoroamidite (1-*O*-dimethoxytrityl-hexyl-disulfide-1′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (Glen Research) was coupled to the 5′ end. The final coupling was followed by an acetonitrile wash. The resin was dried under a stream of argon and treated with concentrated ammonia containing 0.1 M DTT at 55 °C for 12 h to simultaneously affect deprotection of the thiol protection as well as cleavage from the resin (*22*). The resin was removed by filtration and rinsed with concentrated ammonia. Rotary evaporation of the resultant solution afforded a clear residue which was dissolved in sterile water. To remove excess DTT, the solution was passed through a NAP-10 gel filtration column. The fractions containing the ODN were immediately used for conjugation to the peptide.

Synthesis of the Tyr3-Octreotate-Oligodeoxynucleotide Conjugates. The 5′-thiol-ODNs in eluent buffer were added to a solution of the maleimido-peptide **4** (5-fold excess) in aqueous 0.1 M TEAA, pH 6.5, containing 20% DMF. The pH was adjusted by adding 1 M TEAA, pH 6.5. The mixture was incubated at room temperature for 4 h after which time analytical HPLC indicated complete conjugation. Buffers A, 5% acetonitrile in 0.1 M TEAA, pH 6.5, and B, 70% acetonitrile in 0.1 M TEAA, pH 6.5, were employed for purification of the conjugates by RPHPLC. A linear gradient of 0 to 100% B (2 mL/min) in 30 min was used. The conjugates eluting at 22.3 min were collected and lyophilized. Yields were between 34 and 42% (based on the amount of the starting 5′-thiol-ODNs). The conjugates were characterized by MALDI-TOF analysis using 3-hydroxy-picolinic acid as matrix and a sample preparation essentially as described by Langley et al. (*23*): **5**, *m*/*z*) 7822.9 $[M + H]^+$ ($C_{258}H_{335}N_{79}O_{120}P_{20}S_{23}$ calcd, 7819.96 g/mol); **6**, *m*/*z*) 7936.8 $[M + H]^+$ ($C_{260}H_{331}N_{95}O_{112}P_{20}S_{23}$, calcd, 7936.07 g/mol); and **7**, *m*/*z*) 7823.1 $[M + H]^+$ ($C_{258}H_{335}N_{79}O_{120}P_{20}S_{23}$, calcd 7819.96 g/mol).

Melting Temperature Analysis of Hybridization. Melting studies were performed in stoppered quartz cells (1 cm path length) at 260 nm. Samples were prepared as 0.5 OD solutions of the two complementary oligomers in 1 mL of buffer. The buffer was 50 mM Tris/HCl, pH 7.5, containing 0.15 M NaCl. The melting curves were measured using a temperature gradient from 30 to 90 °C with a ramp rate of 0.5 °C/min. Prior to analysis, all samples were annealed by heating to 90 °C for 5 min and subsequently cooled slowly to room temperature. Analysis was carried out using the Varian thermal software. All *T*m values were calculated from the first derivative of the melting curve and represent the average

((standard deviation) of several analyses. Uncertainty in the T_m data is estimated at (0.5 °C based on repetitions of experiments.



Scheme 1. Preparation of the Tyr3-Octreotate-ODN Conjugates

SSTR Binding Assays. The concentration of the conjugates was determined using an estimated extinction coefficient. The extinction coefficient of the conjugate was assumed to be the sum of ε_{ODN} and $\varepsilon_{peptide}$: ε_{260nm}) = Σ– [(*n*A x 15.4 + *n*C x 7.3 + *n*G x 11.7 + *n*T x 8.8) x 0.9] + (*n*Trp x 3.8 + *n*Tyr x 0.5 + *n*Phe x 0.2). Using this equation, the following ε-values were obtained: **5**) 1.8 x 10^5, **6**) 2.1 x 10^5, and **7**) 1.8 x 10^5. For the binding assays, rat cortex membranes were resuspended at a protein concentration of 500 μg/mL in incubation buffer. Per assay, 100 μg of protein were employed. The incubation buffer was 10 mM HEPES, pH 7.6, containing 5% BSA fraction V, $MgCl_2$ (10 mM), and bacitracin (20 *í*g/ mL). The cell membranes (200 μL) were mixed with 3 μL of incubation buffer containing increasing concentrations of the competitor (conjugates **5**-**7**) (10^{-5}-10^{-10} mol/ L). Approximately 20.000 cpm [^{125}I]Tyr^3-octreotide (ca. 20 pM) in 70 μL of incubation buffer was added. After 1 h at room temperature, the incubation was stopped by quick filtration trough Whatman GF/B glass fiber filters prewetted with buffer containing 1% BSA. The filters were washed with ice-cold buffer (10 mM Tris, 150 mM NaCl), and the bound radioactivity was counted in a *ç* counter. Nonspecific binding, as determined by measuring binding in the presence of excess unlabeled octreotide (10-6 mol/L), was approximately 10-20% of the total binding. Specific binding was defined as total binding minus nonspecific binding. Results are expressed as specific binding obtained from triplicate experiments.

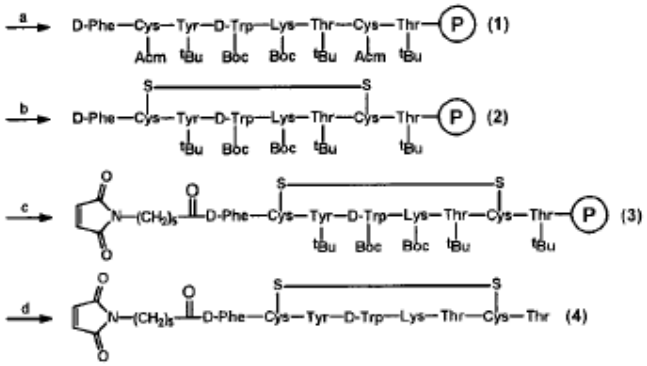
RESULTS

Synthesis Strategy. To obtain targeted analogues of ODNs, conjugation to the SSTR affine Tyr^3-octreotate was performed. Therefore, the peptide was functionalized with a maleimido group and the ODNs were modified with a sulfhydryl group. Upon reaction, a stable thioether bond is formed (Scheme 1).

According to the Merrifield strategy, Tyr^3-octreotate was synthesized on a solid support (Scheme 2). The tyrosine was introduced to enable radioactive labeling with iodine. Conjugation of the maleimido moiety to the peptide prohibited subsequent cyclization of the disulfide. Therefore, the

success of this peptide synthesis was mainly depending on the elaboration of a protocol for on resin cyclization.

Attempts to synthesize **2** using trityl protecting groups for cystein were impeded by incomplete couplings. This failure was probably due to the nonpolar aggregations of the lipophilic trityl groups. Using the smaller and polar Acm-protecting group, these problems could be circumvented. As determined by deprotection and HPLCanalysis of an aliquot, **1** was formed in SPPS in more than 90% yield. The deprotection/oxidation was attempted by different protocols [$AgBF_4$ followed by DMSO/ HCl (*24*), I_2 (*25*), and $Tl(TFA)_3$ (*26*)]. The oxidation with $Tl(TFA)_3$ using a 2-fold molar excess proved to produce the highest yields. The Trp-residue is known be susceptible to modifications during disulfide bond formations. Boc-protection of the D-Trp-moiety was found to be inevitable to obtain **2** in high yields.



[a] (a) Stepwise elongation, (b) $Tl(TFA)_3$, (c) Mal-$(CH_2)_5$COOH, HBTU, DIPEA, (d) TFA, H_2O, phenol, TIS.

Scheme 2. Preparation of the Maleimido Modified Tyr^3-Octreotate[a]

Edwards et al. (*26*) have observed severe problems with the on-resin cyclization of octreotide. To definitely confirm the integrity of **4**, NMR analysis was performed. Both the ^{13}C NMR spectrum as well as the ^{1}H NMR spectrum (data not shown) were in accordance with the structure of **4**, no byproducts could be observed.

Upon oxidation, the ^{13}C NMR resonance of the β-carbon atoms of cystein is shifted considerably downfield (e.g., from $\delta = 25.5$ to $\delta = 38.9$ for cystein in glutathione). Consequently, this chemical shift can be used to determine the oxidation status of disulfide bridged peptides. The ^{13}C NMR signals of the two C_β of cystein in compound **4** appeared at 46.4 and 46.5 ppm, indicating formation of the disulfide bond. The chemical shift of carbons attached to disulfides is sensitive to changes in solvent and pH (*26*), to which this particular high shift can be attributed.

Three 20-mer phosphorothioate ODN sequences complementary to *bcl-2* (sense, antisense, and nonsense sequence) were synthesized on solid support. The six-carbon thiol linker (1-*O*-dimethoxytrityl-hexyl-disulfide-1'-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite was attached to the 5' end. Cleavage was performed in aqueous NH_3 containing 0.1 M DDT at 55 °C overnight. Under these conditions, deprotection of the disulfide protection of the thiol-linker as well as cleavage from the resin was

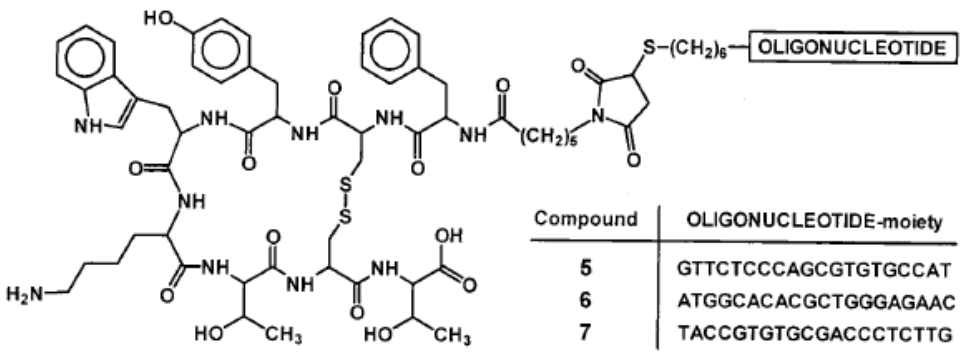
simultaneously achieved. Excess DTT was removed by gel filtration using double-distilled water as eluent. To prevent dimer-formation, the thiol ODNs were immediately conjugated to the maleimido-peptide. Failure to do that led to strongly varying coupling yields. In addition to the thiolated compound, the formation of an ODN byproduct which was not reactive to the maleimido peptide (probably the dimer) was observed and could not be suppressed.

Purification of this ODN and incubation with DTT resulted in slow conversion to the reactive ODN. Attempts to substitute DTT by tris(2-carboxyethyl)phosphine (*27*) led to complex mixtures containing little or no product. Large excess of DTT was found to afford the lowest rate of dimer formation, resulting in reliable good coupling yields.

The conjugation reaction was performed on the basis of the synthesis technique previously described (*28*). Thus, the thiol modified ODNs were added to a solution of **4** in buffer containing DMF at pH 6.5. After 4 h, conjugation had reached completeness, as determined by HPLC. The chromatogram of the crude product mixture showed four peaks. These peaks corresponded to the truncated sequences and the unthiolated oligonucleotide at 16.8 min, the ODN-dimer byproduct at 19.2 min, the conjugate at 21.1 min, and excess maleimido peptide at 24.8 min (Figure 1). Due to the significant differences in size and polarity, the conjugates could be easily separated by HPLC from the side products and excess **4**.

Melting Temperature Analysis. Melting temperature analysis was utilized to determine influences of the peptide moiety on the hybridization efficiency of the antisense ODN-peptide conjugate for its complementary strand. The dissociation of duplexes formed from equimolar concentrations of the ODN conjugates and an unmodified 20-mer ODN target was examined. Measurements were carried out in triplicate. The T_m of the parent unmodified 20-mer phosphodiester sequence was found to be 73.1 (0.2 °C. As compared to this, the T_m values of the conjugates **5** (65.0 (0.0 °C) and **6** (63.8 (0.5 °C) appeared to be relatively low. The T_m of a phosphorothioate ODN is known to be decreased by about 0.5 °C per nucleotide as compared to a corresponding phosphodiester ODN (*29*). Therefore, the T_m curves were typical of those obtained with unmodified ODNs. To prove this, the analogous unmodified phosphorothioate ODNs were prepared. The resulting T_m values of the antisense (64.8 (0.5 °C) and the sense strand (66.2 (0.5 °C) confirmed that the prediction was correct. In Figure 2 the T_m curves of the duplex containing the conjugates **5**, **6**, the unmodified phosphorothioate ODNs, and the unmodified duplex are compared. These results indicate that the peptide moiety at the 5'-terminus of the 20-mer ODN does not interfere with the hybridization efficiency.

Receptor Binding. To allow examination of competitive displacement, the concentrations of the conjugates were determined accurately using their extinction coefficients. Figure 3 shows the progressive displacement of [^{125}I]Tyr^3-octreotide from rat cortex membranes. The three conjugates investigated bound with high affinity in the low nanomolar range. The IC_{50} values of **5**, **6**, and **7** were 1.83 (0.17, 2.52 (0.43, and 1.88 (0.47 nM, respectively. The similar affinities suggest that the sequence of the ODN does not significantly affect the receptor affinities.



Compound	OLIGONUCLEOTIDE-moiety
5	GTTCTCCCAGCGTGTGCCAT
6	ATGGCACACGCTGGGAGAAC
7	TACCGTGTGCGACCCTCTTG

Scheme 3. Chemical Structure of the Tyr^3-Octreotate-ODN Hybrid Molecules

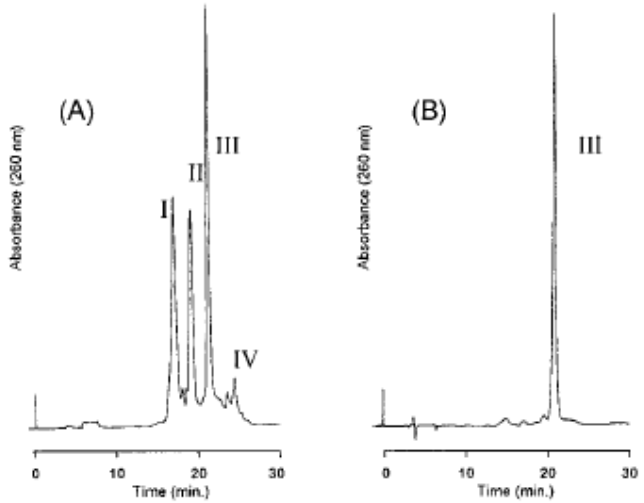


Figure 1. Reversed-phase HPLC chromatogram: Conjugation of a thiol-modified oligonucleotide with maleimido-peptide **4**. (A) Reaction mixture after 4 h. (I) Truncated sequences and the unthiolated oligonucleotide; (II) ODN-dimer byproduct, (III) conjugate, and (IV) excess maleimido peptide. (B) Purified conjugate **6**.

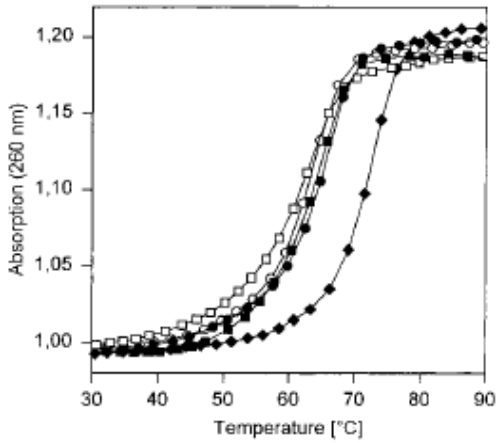


Figure 2. Melting curves of modified and unmodified 20-mer ODNs in 50 mM Tris/HCl (pH 7.5) and 0.15 M NaCl. Duplexes prepared from

complementary phosphorodiester strands with antisense*bcl-2* phosphorothioate (O), sense *bcl-2* phosphorothioate (b), ODN conjugate **5** (0), ODN conjugate **6** (9), and controlstrand antisense *bcl-2* phosphorodiester ([). For clarity only every tenth point is plotted.

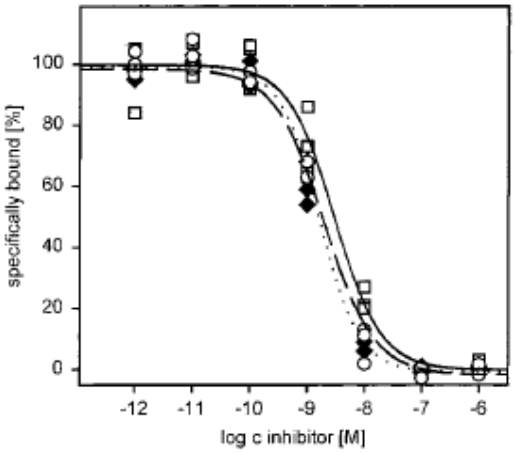


Figure 3. Displacement of [^{125}I]Tyr^{3}-octreotide from rat cortex membranes (triplicate measurements). The ordinate represents the percent specific binding which is total binding minus binding in the presence of the ODN conjugates **5** (0, straight line), **6** (O, dotted line), and **7** ([, dashed line).

DISCUSSION

The receptors of the hormone somatostatin are known to be overexpressed in various tumors. The ODN conjugates reported herein were designed to be targeted to these plasma membrane receptors. The SSTRs exhibit a high capacity as transport vehicles, e.g., for cargos as big as gold particles (*30*). Furthermore, they show high specificity, as known from octreoscan ([^{111}In]-DTPA-D-Phe1-octreotide), a radiopharmaceutical for tumor scintigraphy (*31*). Therefore, analogues of somatostatin are ideal transport vehicles for the targeting of tumor cells. These assumptions have very recently been supported by the successful conjugation of cytotoxic compounds to somatostatin derivatives (*32*, *33*). Using a postsynthetic approach, the conjugation of disulfide bridged peptides such as octreotide is complicated. This problem could be solved by coupling of thiol modified ODNs with a maleimido- derivatized peptide. To our knowledge this is the first report of a synthesis which enables the production of stable conjugates of ODNs with disulfide-bridged peptides.

The major targets of conjugates targeted to SSTRs are the receptor subtypes 2 and 5, which are predominantly expressed in SSTR-positive tumors. The feasibility of the ODN conjugates to target SSTRs was demonstrated by receptor binding studies with rat cortex membranes, which mainly express SSTR2. The IC_{50} values of the conjugates were in the low nanomolar range, thus, demonstrating high affinity binding to SSTRs. Investigation of the hybridization properties revealed that the peptide at the 5′-terminus of the 20-mer ODN does not influence the binding to complementary ODNs. These results provide a basis for the improvement of

selective uptake of ODNs into tumors and therefore hold promise for further development and application of antisense therapy. Further development of these conjugates investigating their biodistribution, stability, and bioactivity *in vivo* is in progress.

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