Bioorganic & Medicinal Chemistry 20 (2012) 3219–3222

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Cellular uptake of covalent conjugates of oligonucleotide with membrane-modifying peptide, peptaibol

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ARTICLE INFO

Article history:
Received 1 March 2012
Revised 26 March 2012
Accepted 26 March 2012
Available online 1 April 2012

Keywords:
Peptaibol
α-Aminoisobutyric acid
Oligonucleotide
Antisense
Membrane-modifying property
Aib

ABSTRACT

Using the membrane-modifying peptide, trichorovin-XIIa (TV-XIIa), which is an 11-residual peptaibol isolated from the fungus *Trichoderma viride*, we synthesized covalent conjugates of 20-mer oligonucleotide with TV-XIIa to examine the potential use of TV-XIIa in cellular delivery. The results indicated that the conjugates were progressively taken up by human lung carcinoma A549 cells. Next, the antisense effects of the conjugates on p53 protein expression were examined. The p53 expression was significantly decreased by ca. 20–50% in the presence of the conjugates upon treatment with the transfection solution at the concentration of 5 μM.

1. Introduction

Antisense oligonucleotides (A-ODNs), short-interfering RNAs (siRNAs), antigenes, aptamers, and ribozymes are typically used for the regulation of gene expression.[1] These ODN-based therapeutics have shown promise for the treatment of a variety of diseases, such as cancer, viral infection, and genetic disorder. For ODNs to achieve highly efficient gene expression regulating effects, it is required that they diffuse across the plasma membrane at first. However, ODNs are large charged polar molecules that typically exhibit poor membrane permeabilization because of the hydrophobic membrane barrier, and are therefore inefficiently taken up by cells. Various vehicles have been developed to this end, including cationic lipoplexes,[2] cell-penetrating peptides (CPPs),[3] and polymers.[4] However, although numerous studies have been performed, no perfect vehicle has been developed for clinical use so far. It is vital to develop a new type of vehicle and new methodology for ODN-based therapeutics.

Peptaibols are a class of linear peptides produced by fungi,[5] and more than 400 reports of peptaibols have been published to date. They exhibit a broad range of bioactivities, such as the induction of characteristic voltage-dependent ion conduction in lipid bilayer membranes, the enhancement of catecholamine release from adrenal chromaffin cells, and the inhibition of amoeba cell multiplication.[6] All of those activities are related to their membrane-modifying properties. Trichorovin-XIIa (TV-XIIa), which belongs to a class of peptaibols, was isolated from the fungus *Trichoderma viride*, and its amino acid sequence is Ac-U-N-I-I-U-P-L-L-U-P-Iol (Ac: acetyl; U: α-aminoisobutyric acid; Iol: isoleucinol).[7] TV-XIIa can also form voltage-dependent ion channels in planar lipid bilayer membranes.[8] We have reported that the fluorescein-labeled TV-XIIa derivative permeates living cell plasma membrane via a non-endocytic pathway.[9] Then, utilizing the membrane-modifying property of 11-residual peptaibol, we explored the possibility of developing a useful vehicle for the delivery of ODNs. We considered two strategies, complexation and conjugation, for the delivery of ODNs by TV-XIIa. In the former strategy, complexes were formed between a cationic TV-XIIa derivative, which was TV-XIIa linked to a 10-mer of lysine, and ODN via electrostatic interaction, and this was followed by the treatment of cells with the formed complexes. Unfortunately, the complexes could not deliver 20-mer ODNs into human lung carcinoma A549 cells[10] and showed cytotoxicity when the concentration of the TV-XIIa derivative was increased (unpublished data). Thus, we examined the latter strategy: conjugation. In this strategy, TV-XIIa is covalently conjugated to ODNs through linkers and the conjugates interact with the plasma membrane. The entry of ODNs into cells is promoted by the membrane-modifying property of TV-XIIa. As the first step of our investigation of new peptaibol vehicles, we have firstly chosen a single-stranded A-ODN that has the lowest molecular weight among the ODNs described above, as cargo.[1]

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http://dx.doi.org/10.1016/j.bmc.2012.03.054

2. Results and discussion

2.1. Synthesis of conjugates

The chemical structures of the conjugates are shown in Figure 1. TV-XIIa with cysteine amide linked to the C-terminus, which has a reactive thiol group, was prepared by Fmoc-based solid-phase synthesis on Rink amide resin; this method was the same as that previously reported.[10] The ODN used is a 20-mer A-ODN complementary to positions 1071–1090 of exon 10 of the *p53* gene (5′-CCCTGCTCCCCCCTGGCTCC-3′), and the control ODN is 5′-GGAGCCAGGGGGGAGCAGGG-3′.[11] Both ODNs are fully phosphorothioated in their backbones. The conjugates were synthesized by forming two different linkages: one is a thioether bond (conjugates **1**–**3**) that is stable within cells, and the other is a disulfide linkage (conjugate **4**) that is easily reduced within cells to release ODN to cytosol.[12] The maleimide-activated and the 2-pyridylthio-activated ODNs were reacted with an excess of peptide harboring a Cys residue to give peptide-ODN conjugates **1**–**4** in good yields. 3′-Fluorescein-labeled conjugate **1** was also synthesized to monitor the internalization of the conjugates by fluorescence analyses.

2.2. Cellular uptakes into cells

The uptake of fluorescein-labeled conjugate **1** by A549 cells was studied by confocal laser-scanning microscopy (CLSM) and fluorescence spectroscopy. In the CLSM experiments, when cell cultures were treated with conjugate **1** and fluorescein-labeled 20-mer ODN alone at concentrations of 1.0–4.0 μM at 37 °C for 2–24 h, conjugate **1** efficiently traversed A549 cell membrane and diffused into the cytoplasm, whereas, as expected, ODN alone was poorly taken up by the cells (Fig. 2). The intensity of the green fluorescence appeared to increase in proportion to the conjugate concentration and the incubation time, indicating a dose- and time-dependent uptake. To estimate the amount of conjugate **1** taken up in terms of fluorescence intensity, fluorescence analyses of the cell lysates were conducted. Cells were incubated with either conjugate **1** or 20-mer ODN alone at the concentration of 1.0 μM for 2, 4, 8, and 24 h (Fig. 3). Cells treated with ODN alone at the concentration of 1.0 μM showed very low fluorescence intensities over time (~24 h). In contrast, cells treated with conjugate **1** for 2, 8, and 24 h showed fluorescence intensity increase by approximately 4-, 7-, and 14-fold relative to the fluorescence intensity of cells treated with ODN alone, respectively. The large increase in the fluorescence intensity over time (~24 h) indicated the continuous cellular uptake of conjugate **1**. Thus, we expected that the conjugate could induce gene expression in the cytosol.

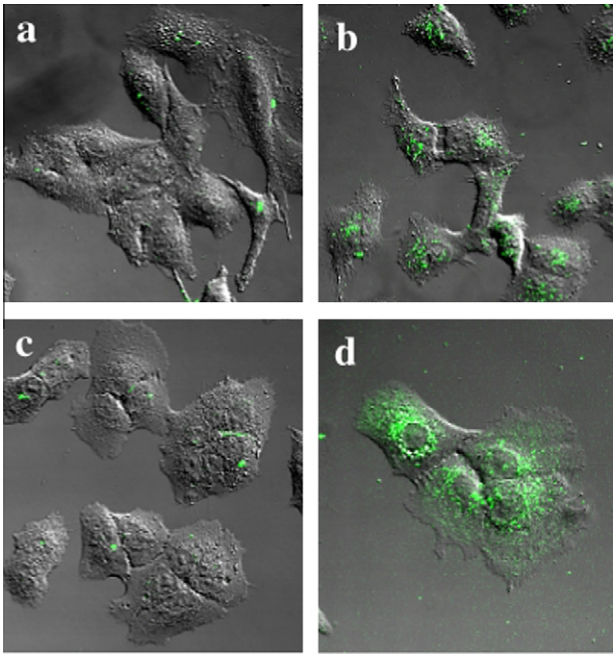
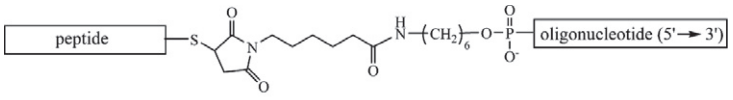


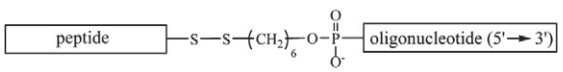
Figure 2. Confocal microscopy images of A549 cells. Fluorescence images and images from transmitted light (differential interference constant) were overlaid for all the images. The cells were treated with oligonucleotide alone (a and c) and conjugate **1** (b and d) at the concentration of 1.0 μM at 37 °C for 2 h (a and b) and 24 h (c and d).

2.3. Antisense effects

Next, the antisense effects of conjugates **2** and **4** on p53 protein expression were examined. Serum-free reaction solutions containing conjugates **2** and **4** at concentrations of 1 and 5 μM were incubated with A549 cells for 8 h at 37 °C, followed by additional culture in a serum-containing medium for 24 h. p53 protein expression in A549 cells was measured by Western blot analysis, as shown in Figure 4. Both conjugates **2** and **4** had no influence on p53 protein expression when they were incubated at the concentration of 1 μM.



conjugates **1**~**3**



conjugate **4**

conjugate	peptide [a]	oligonucleotide (5'→3')
1	Ac-UNIIUPLLUPIC-NH_2	CCCTGCTCCCCCCTGGCTCC-**Fl** [b, c]
2	Ac-UNIIUPLLUPIC-NH_2	CCCTGCTCCCCCCTGGCTCC [c]
3	Ac-UNIIUPLLUPIC-NH_2	GGAGCCAGGGGGGAGCAGGG [d]
4	Ac-UNIIUPLLUPIC-NH_2	CCCTGCTCCCCCCTGGCTCC [c]

Figure 1. Chemical structures of conjugates. [a]One-letter codes are used (Ac: acetyl; U: α-aminoisobutyric acid). C-terminal cysteine amide was linked to oligonucleotide to form the conjugates. [b]Fl: fluorescein. [c]The oligonucleotide is an antisense phosphorothioate oligonucleotide complementary to positions 1071–1090 of exon 10 of the *p53* gene. [d]The oligonucleotide is the sense phosphorothioate oligonucleotide and is used as control.

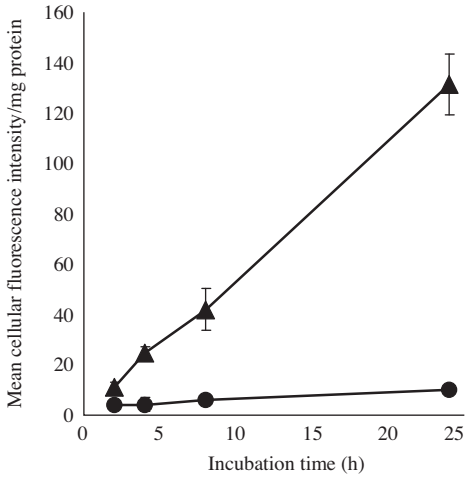


Figure 3. Uptake of conjugate **1** by A549 cells. A549 cells were incubated with conjugate **1** (▲) and 20-mer ODN alone (●) at 37 °C for different incubation times.

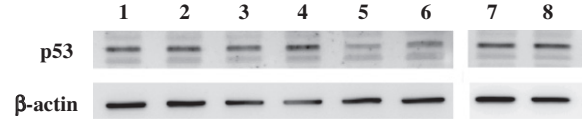


Figure 4. Western blot analysis of the effects of conjugates **2**, **3**, and **4** on p53 protein expression in A549 cells. lanes 1, 4, and 7, no treatment; lanes 2 and 3, 1 μM conjugates **2** and **4**, respectively; lanes 5, 6, and 8, 5 μM conjugates **2**, **4**, and **3**, respectively.

However, at the concentration of 5 μM, conjugate **2** showed significant suppression of p53 protein expression whereas conjugate **4** showed slight suppression compared to control conjugate **3**. To quantify knockdown efficiency, the relative amount of p53 was compared to that of the control protein, β-actin. Whereas all control experiments produced relatively constant ratios between p53 and β-actin, p53 expression was significantly decreased by 50% in the presence of conjugate **2** and 20% in the presence of conjugate **4** upon treatment with the transfection solution at the concentration of 5 μM. The effect of conjugate **2** was stronger than that of conjugate **4**. In addition, cytotoxicity was monitored by measuring the release of lactate dehydrogenase (LDH), a cytoplasmic enzyme (Fig. 5), that was treated with the conjugates **1**, **2**, and **4** at the concentration of 5 μM for 2 h and 24 h, and the results supported the non-toxic A-ODN delivery by TV-XIIa.

3. Conclusions

In conclusion, we have demonstrated that covalent conjugates of A-ODN with TV-XIIa could be taken up by A549 cells to suppress p53 protein expression without any noticeable toxic effects, supporting the idea that TV-XIIa is a promising carrier for ODN delivery. Further studies, including cell entry mechanism and cell type dependence, are required to optimize delivery. To our knowledge, this is the first report of the possibility of using peptaibol for the delivery of ODN into cancer cells where the conjugate exerts its antisense effect.

4. Experimental

4.1. General

The oligonucleotides (ODNs) used are 20-mer antisense all phosphorothioate ODNs complementary to positions 1071–1090 of exon 10 of the *p53* gene (5′-CCCTGCTCCCCCCTGGCTCC-3′), and

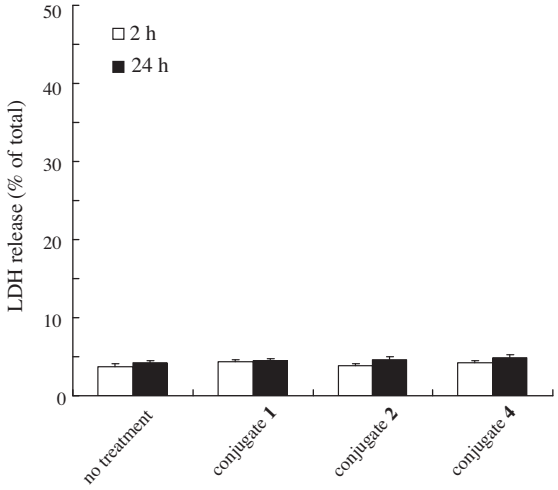


Figure 5. Effects of conjugate **1**, **2**, and **4** on lactate dehydrogenase (LDH) release in A549 cells. The cells were treated with conjugates **1**, **2**, and **4** at the concentration of 5.0 μM at 37 °C for 2 h (white) and 24 h (black).

the sense phosphorothioate ODN is 5′-GGAGCCAGGGGGGAG-CAGGG-3′ as control. The 3′-amino- or 3′-thiol-modified ODN and the 5′-amino-modified and 3′-fluorescein-labeled ODN were synthesized by Japan Bio Services Co., Ltd, Saitama, Japan or Sigma–Aldrich, Hokkaido, Japan. HPLC: Waters Delta-600 or Shimadzu LC-6A apparatus equipped with a COSMOSIL 5C18 PAQ column [Nacalai Tesque Co., Ltd]. MALDI-TOF MS: Applied Biosystems Voyager-DE STR; in *m*/*z*; matrix: solution A: solution B = 1:1 (v/v). Solution A: saturated 2′,6′-dihydroxyacetophenone in 50% aqueous methanol, and solution B: saturated diammonium hydrogen citrate in 50% aqueous methanol.

4.2. Synthesis of conjugates 1–3

The same procedure was used for the synthesis of conjugates **1**–**3**. A solution of *N*-6-maleimidocaproyloxysuccinimide (EMCS) (1.1 mg, 3.5 μmol) in acetonitrile was added to a solution of 5′-amino-modified ODN (6.83 OD, 44.3 nmol) in 0.1 M phosphate buffer (pH 8.0), and the reaction mixture was left to stand for 2 h at 30 °C. To remove excess EMCS, the reaction mixture was passed through a reversed-phase C18 cartridge (Sep-pak C18 light cartridge, Waters) to give maleimido-activated ODN (6.26 OD, 40.7 nmol, 91.7%). A solution of TV-XIIa harboring a Cys residue (0.53 mg, 407 nmol) in acetonitrile was added to the activated ODN (6.26 OD, 40.7 nmol) in 0.1 M phosphate buffer (pH 7.0), and the reaction mixture was left to stand for 3 h at 30 °C, after which time analytical HPLC indicated complete conjugation. Solvents A (50 mM triethylammonium acetate, pH 7.2) and B (acetonitrile) were employed for purification of the conjugates by reversed-phase HPLC. A linear gradient of 10–55% B in 45 min was used. The eluted conjugates were collected and lyophilized (4.25 OD, 27.6 nmol, 68%), and then characterized by MALDI-TOF-analysis. Conjugate **1**: *m*/*z* = 8442.1 $[M+H]^+$ ($C_{291}H_{401}N_{79}O_{134}S_{20}P_{20}$, calcd 8441.5); conjugate **2**: *m*/*z* = 7871.9 $[M+H]^+$ ($C_{264}H_{377}N_{78}O_{123}S_{20}P_{20}$, calcd 7872.0); conjugate **3**: *m*/*z* = 8311.9 $[M+H]^+$ ($C_{274}H_{373}N_{110}O_{115}S_{20}P_{20}$, calcd 8308.3).

4.3. Synthesis of conjugate 4

5′-Thiol-modified ODN (7.00 OD, 45.4 nmol) in 0.1 M phosphate buffer (pH 5.5) was mixed with a 50-fold excess of 2,2′-dipyridyl disulfide (0.50 mg, 2.3 μmol) in acetonitrile, and the reaction mixture was kept overnight at rt. To remove excess 2,2′-dipyridyl

disulfide, the reaction mixture was passed through a reversed-phase C18 cartridge (Sep-pak C18 light cartridge, Waters) to give 2-pyridylthio-activated ODN (3.92 OD, 25.4 nmol, 56%). A solution of TV-XIIa harboring a Cys residue (0.3 mg, 254 nmol) in acetonitrile was added to the activated ODN (3.92 OD, 25.4 nmol) in 0.1 M phosphate buffer (pH 5.5), and the reaction mixture was left overnight at rt, after which time analytical HPLC indicated complete conjugation. The conjugate was purified by reversed-phase HPLC under the same conditions as above. The eluted conjugate was collected and lyophilized (1.80 OD, 11.7 nmol, 46%), and then characterized by MALDI-TOF-analysis. Conjugate **4**: m/z = 7694.7 $[M+H]^+$ ($C_{254}H_{365}N_{76}O_{120}S_{21}P_{20}$, calcd 7695.8).

4.4. Cell culture

A549 cells were obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University) and cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C in a humidified incubator containing 5% CO_2. All studies were performed using asynchronous log-phase cultures.

4.5. Confocal laser-scanning microscopy (CLSM)

Exponentially growing cells were dissociated with trypsin–EDTA, plated at 50% confluence on glass microscope slides (Matsunami Glass Industries, Ltd), and cultured overnight. The culture medium was discarded and the cells were washed with FBS-free Dulbecco's modified Eagle's medium (DMEM) (3×). The cell monolayers were incubated for 2–24 h at 37 °C with 3′-fluorescence-labeled conjugate **1** and 5′-fluorescein-labeled ODN. Subsequently, the cells were rinsed with phosphate-buffered saline (PBS) (3×) for observation of living cells. The fluorescence distribution of 3′-fluorescence-labeled conjugate **1** and 5′-fluorescein-labeled ODN was analyzed on a Carl Zeiss LSM510 confocal laser-scanning microscope equipped with an Ar/Kr laser.

4.6. Fluorescence analysis

A549 cells (5×10^4 cells/mL) were plated onto a 24-well culture plate. After treatment with the same procedures as those described above, the cells were rinsed with PBS (3×), trypsinized, and centrifuged at 1000 rpm at 4 °C for 5 min. The cell pellets obtained were resuspended in RIPA buffer (50 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% (v/v) NP40, 0.5% (v/v) deoxycholate, 0.1% (w/v) SDS, pH 7.5) for lysis. Finally, the lysates were centrifuged at 8200 rpm at 4 °C for 10 min and then fluorescence was measured with a microplate reader.

4.7. Detection of p53 protein by Western blot analysis

A549 cells (1.6×10^5 cells) were plated onto a 35-mm cell culture dish and incubated overnight at 37 °C. The conjugates were incubated with the cells in displaced culture medium to serum-free medium for 8 h at 37 °C, followed by additional culture in a serum-containing medium for 24 h. The cells were washed with PBS and solubilized in lysis buffer (CelLytic™ M, Sigma–Aldrich, Inc.). The protein was then collected after centrifugation at 13,000 rpm for 20 min at 4 °C. The resulting supernatant was separated on 10% SDS–PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon™-P, Millipore Corporation). The membranes were blocked with Blocking One™ (Nacalai Tesque Inc.) for 1 h at room temperature before being incubated for 1 h with primary antibodies specific for p53 (BD Transduction Laboratories™) and β-actin (Sigma–Aldrich, Inc.) diluted with Tris-buffered saline (TBS)-0.1% Tween 20. Then, the membranes were washed with TBS-0.1% Tween 20 and incubated with horseradish-peroxidase-conjugated secondary antibodies in TBS-0.1% Tween 20 for 1 h. After successive washes, the membranes were visualized with an enhanced chemiluminescence kit (ECL Plus Western Blotting Detection Reagents [GE Healthcare]) and measured with a Luminoimage Analyzer (LAS-3000, Fujifilm, Japan). Relative protein expression levels were quantified by analysis software (Image Gauge, Fujifilm).

4.8. Release of cytoplasmic lactate dehydrogenase (LDH)

Cells (1×10^4 cells/mL) were incubated at 37 °C for 2 and 24 h in a 96-well culture plate with conjugates **1**, **2**, and **4**. After incubation, the cell-growth medium was examined for LDH release by spectrophotometric assay using a commercial LDH kit (Wako Pure Chemical, Co., Osaka, Japan). UV/VIS absorbance at 560 nm was recorded with a BioRad-680 microplate reader.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (22590109) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan. We thank the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University) for providing A549 cells.

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