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Knockdown of a G protein-coupled receptor through efficient peptide-mediated siRNA delivery

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ABSTRACT

In recent years, therapeutic applications of siRNAs have come into the focus of pharmaceutical research owing to their potential to specifically regulate gene expression. However, oligonucleotides have to overcome a series of extracellular and intracellular barriers which is why delivery systems helping to overcome these barriers are desperately needed. A promising approach to transport nucleic acids beyond cellular membranes is the use of cell-penetrating peptides (CPPs), which are able to autonomously cross the plasma membrane. Recently, we synthesized branched derivatives of truncated human calcitonin (hCT) and identified them as efficient vehicles for non-covalent gene delivery. Here we describe two novel branched hCT-derivatives that are optimized for efficient intracellular delivery of siRNA by conjugation with either a fatty acid or an endosomolytic peptide sequence. As target we chose the human NPY Y_1 receptor (NPY1R), which belongs to the family of G protein-coupled receptors and thus constitutes a model for complex therapeutic targets related to various disorders. For instance, knockdown of Y_1 receptor expression offers a potential therapy for osteoporosis. We present a read-out system that allows for the quantitation of the induced knockdown of receptor expression on the protein as well as on the mRNA level. As a result of this study, we could show that the herein presented cell-penetrating peptides effectively transport siRNA into HEK-293 cells without inducing cytotoxicity and that the knockdown rates are comparable to those obtained by lipofection.

1. Introduction

G protein-coupled receptors (GPCRs) comprise a superfamily of seven-transmembrane receptors that are ubiquitously expressed in eukaryotic organisms. GPCRs are key players in cellular signaling processes and allow tissues to respond to a wide array of extracellular signaling molecules [1,2]. Most of their endogenous ligands are small and can be mimicked or blocked by synthetic analogs. Since they are involved in a tremendous variety of physiological processes, they make up nearly half of the pharmacological targets of current drugs [3]. One strategy that is growing ever more important for the elucidation of signal transduction pathways and the therapy of difficult-to-cure diseases is the knockdown of receptor expression by means of small interfering RNA (siRNA). In recent years, therapeutic applications of oligonucleotides in general (ON) have come more and more into the focus of pharmaceutical research, offering highly specific interference with disease-related genes. Among those strategies, RNA interference (RNAi) is of particular interest, having shown its great potential in the treatment of cardiovascular diseases, central nervous system disorders, cancer, acquired immune deficiency syndrome (AIDS), and hepatic disorders, e.g. [4]. RNAi is ubiquitous in eukaryotic cells and leads to degradation of the target mRNA upon loading of the siRNA into the RNA-induced silencing complex (RISC) located in the cytoplasm [5]. However, efficient siRNA delivery suffers from the incapacity of the highly negatively charged nucleic acids to efficiently cross the plasma membrane. Hence, in order to improve their cellular delivery, several techniques have been developed based on viral vectors [6,7], cationic polymers and lipids [8,9], nanoparticles [10] and electroporation [11]. Yet, these strategies are often subject to severe drawbacks like immunogenicity, cytotoxicity or restriction to *in vitro* application. By way of overcoming those obstacles, a different approach adopts the use of cell-penetrating peptides (CPPs) [12,13]. Those are short peptides with usually less than 30 amino acid residues which commonly feature a high positive net charge. CPPs have garnered widespread attention due to their ability

Abbreviations: CPP, cell-penetrating peptide; CQ, chloroquine; EYFP, enhanced yellow-fluorescent protein; GPCR, G protein-coupled receptor; hCT, human calcitonin; HEK-293, human embryonic kidney cells; NPY1R, neuropeptide Y1 receptor; siRNA, small interfering ribonucleic acid; RNAi, RNA interference; RT-PCR, reverse transcription polymerase chain reaction.

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to cross the plasma membrane in an autonomous and receptor-independent fashion, mostly showing a favorable cytotoxic and immunogenic profile. They have been used for the delivery of various kinds of cargo, among which oligonucleotides and especially siRNA play a prominent role. Beside covalent conjugation of the siRNA to the peptide [14], non-covalent strategies have emerged, employing derivatives of HIV-Tat [15,16], MPG [17–19], transportan [20] and other, newly designed CPPs [21,22]. A recent review on CPP-mediated delivery of nucleic acids is presented by Hassane et al. [23].

In our laboratory we focus on cell-penetrating peptides derived from human calcitonin (hCT), a peptide hormone consisting of 32 amino acids, which is synthesized in the thyroid gland and involved in calcium homeostasis. We identified truncated *C*-terminal fragments of hCT [24,25], which allowed for efficient membrane translocation of molecules as diverse as proteins [26], organometallic complexes [27] and quantum dots [28] to various cell lines via an endocytotic pathway without eliciting cytotoxicity themselves. Recently, we reported on the successful delivery of plasmid DNA into primary cell lines using novel branched hCT-derived CPPs which are able to form stable electrostatic complexes with nucleic acids [29]. However, for effective non-covalent siRNA delivery, we needed to further improve the peptides in terms of stability of the electrostatic complexes and efficient siRNA internalization into the cell with particular focus on enhanced cytosolic distribution. Thus, we designed two variants of hCT(18–32)-k7, JH1 and JH2, the first bearing a fatty acid. Lipidation was shown in various studies to effectively enhance CPP-mediated ON delivery due to enhanced lipid bilayer interaction, including that of the endosomes, ultimately leading to enhanced cytosolic uptake [30–32]. JH2 was obtained by conjugation with a fusogenic peptide, N-E5L, derived from the HA2 domain of influenza virus that was recently shown to enhance cytosolic uptake [33].

The aim of this study was to elucidate whether our modified hCT(18–32)-k7 peptides are able to efficiently deliver siRNA into the cells. As target we chose the human NPY Y_1 receptor (NPY1R), which belongs to the family of G protein-coupled receptors [34], thus constituting a model for complex therapeutic targets related to various disorders. This is the first time, to our knowledge, that the expression of a GPCR is regulated by CPP-mediated siRNA delivery. The particular therapeutic significance of the hY_1 receptor lies in its putative involvement in breast and ovarian cancer tumorigenesis [35]. Furthermore, it was shown that down-regulation of the Y_1 receptor in osteoblasts leads to increased bone mass in mice [36].

2. Materials and methods

2.1. Materials

N^{α}-Fmoc-protected amino acids were purchased from IRIS Biotech (Marktredwitz, Germany), the following side chain protecting groups were chosen: tert-butyl (tBu) for Ser, Thr, Tyr, Asp and Glu; trityl (Trt) for Asn, Gln, and His; tert-butyloxycarbonyl (Boc) and 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) for Lys. 1-Hydroxybenzotriazole (HOBt) and 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy (Rink amide) resin were obtained from Novabiochem (Darmstadt, Germany). Diisopropylcarbodiimide (DIC), the resazurin-based *in vitro* toxicology assay kit and ethidium bromide (EtBr) were purchased from Sigma-Aldrich (Taufkirchen, Germany). *O*-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), *N*,*N*-diisopropylethylamine (DIEA), thioanisole, *p*-thiocresole, piperidine, ethanedithiole, trifluoroacetic acid, Trypan blue, 5(6)-carboxyfluorescein (CF), and chloroquine (CQ) were purchased from Fluka (Taufkirchen, Germany). *N*,*N*-Dimethylformamide (DMF), dichloromethane, and diethyl ether were obtained from Biosolve (Valkenswaard, Netherlands). Acetonitrile was from Merck (Darmstadt, Germany). Decanoic acid was purchased from Acros Organics (Geel, Belgium). Agarose was from Bioline (Berlin, Germany), deoxynucleoside triphosphates were from MBI Fermentas (St. Leon-Rot, Germany). The RNeasy Mini Kit and QuantiTect® Probe PCR Master Mix were from Qiagen (Hilden, Germany). Lipofectamine™ 2000, 5× RT buffer, SuperScript™ II Reverse Transcriptase (200 U/μL), Oligo(dT) primers (0.5 μg/μL), Block-iT™ Alexa Fluor® Red dsRNA, Negative Universal Control™ siRNA and siRNA targeting the human Y_1 receptor were purchased from Invitrogen (Carlsbad, USA). The sequence of the hY1 siRNA was: 5′-GCC UUU CCU GAU CUA CCA AdTdT-3′ (sense strand) and 5′-UCU UUG UAC GCA UCA AGU GdTdT-3′ (antisense strand). The Taqman® Probe Gene Expression Assay was purchased from Applied Biosystems (Carlsbad, USA). For cell culture, the following media and supplements were used: Dulbecco's modified Eagle's medium (DMEM), Ham's F12 (without L-glutamine), OptiMEM®, Hank's balanced salt solution (HBSS), Dulbecco's phosphate buffered saline (PBS) without calcium and magnesium, fetal bovine serum (FBS), L-glutamine and trypsin/EDTA (all purchased from PAA (Linz, Austria) or Gibco Life Technologies (Karlsruhe, Germany), respectively. Cell culture flasks (75 cm^2) and 96-well plates were from TPP (Trasadingen, Switzerland), 48-well plates were purchased from Greiner Bio-One (Frickenhausen, Germany) and 8-well μ-Slides were from ibidi (Martinsried, München Germany). MCF-7 and HEK-293 cell lines and the EYFP_hY_1-pvitro plasmid (5847 bp) encoding the enhanced yellow fluorescent protein (eYFP)-tagged hY_1 receptor were kindly provided by Prof. Dr. A. G. Beck-Sickinger.

2.2. Peptide synthesis

The peptides used were synthesized by automated peptide synthesis on a multiple Syro II peptide synthesizer (MultiSynTech, Witten, Germany) following Fmoc/^{t}Bu-strategy utilizing a double coupling procedure and *in situ* activation with HOBt/DIC as described previously [29]. For manual coupling of the fatty acid, 5 eq. capric acid/HOBt/DIC in DMF was added to the peptide on solid support and the reaction mixture stirred for 3 h at room temperature. 5(6)-carboxyfluorescein (CF) was coupled by the same procedure, CF-polymers were subsequently cleaved by treatment with 20% piperidine in DMF for 45 min. All peptides were prepared as *C*-terminal amides using Rink amide resin as solid support. Cleavage from the resin was achieved using TFA/thioanisole/*p*-thiocresole (90:5:5 (v/v/v)) for 3 h at room temperature, followed by precipitation by addition of ice-cold diethyl ether. The peptides were purified and analyzed according to Rennert et al. [29]. For peptide sequences see Table 1. CF-labels were introduced at the *N*-terminus of the k7 peptide sequence located at the side chain of Lys^{18}.

2.3. Cell culture

HEK-293 cells (human embryonic kidney epithelium transformed with adenovirus 5 DNA) were grown to subconfluency in 75 cm^2 culture flasks at 37 °C and 5% CO_2 in a humidified atmosphere using DMEM/Ham's F12 (without L-glutamine) which contained 15% heat-inactivated FBS. MCF-7 cells (human breast adenocarcinoma) were

Table 1
Cell-penetrating peptides derived from human calcitonin that were used in this study. Capr = capric acid (n-decanoic acid).

CPP	Sequence	M.W.	Net charge
hCT(18–32)-k7	K^{18}FHTFPQTAIGVGAP-NH_2 KKRKAPKKKRKFA	3163.9	+11
JH1	GLLEALAELLEK^{18}FHTFPQTAIGVGAP-NH_2 KKRKAPKKKRKFA	4315.6	+8
JH2	Capr-K^{18}FHTFPQTAIGVGAP-NH_2 KKRKAPKKKRKFA	3318.0	+10