Journal of Peptide Science
J. Pept. Sci. 2008; **14**: 455–460
Published online 30 January 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.979

JOURNAL OF PEPTIDE SCIENCE

Review

Arginine-rich cell penetrating peptides: Design, structure–activity, and applications to alter pre-mRNA splicing by steric-block oligonucleotides‡

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Received 9 July 2007; *Revised 8 October 2007*; *Accepted 22 October 2007*

Abstract: Rerouting the splicing machinery with steric-block oligonucleotides (ON) might lead to new therapeutic strategies in the treatment of diseases such as β-thalassemia, Duchenne muscular dystrophy, or cancers. Interfering with splicing requires the sequence-specific and stable hybridization of RNase H-incompetent ON as peptide nucleic acids (PNA) or phosphorodiamidate morpholino oligomers (PMO). Unfortunately, these uncharged DNA mimics are poorly taken up by most cell types and conventional delivery strategies that rely on electrostatic interaction do not apply. Likewise, conjugation to cell penetrating peptides (CPPs) as Tat, Arg_9, Lys_8, or Pen leads to poor splicing correction efficiency at low concentration essentially because PNA– and PMO–CPP conjugates remain entrapped within endocytotic vesicles. Recently, we have designed an arginine-rich peptide $(R\text{-Ahx-R})_4$ (with Ahx for aminohexanoic acid) and an arginine-tailed Penetratin derivative which allow sequence-specific and efficient splicing correction at low concentration in the absence of endosomolytic agents. Both CPPs are undergoing structure–activity relationship studies for further optimization as steric-block ON delivery vectors. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cell penetrating peptides; steric-block oligonucleotides; splicing correction; delivery

OLIGONUCLEOTIDES AS GENE REGULATION TOOLS: AN OVERVIEW

Synthetic oligonucleotides (ON) provide invaluable tools to regulate gene expression through sequence-specific interactions with RNA (antisense ON, ribozymes, siRNA, DNAzymes), DNA (triple helix-forming ON) or proteins (decoy ON, immunostimulatory ON, aptamers). Binding to an RNA target may lead to its specific ablation through the sequence-specific recruitment of RNase H (as amply demonstrated for oligodeoxyribonucleotides and a few of their analogs) or of the RNA-induced silencing complex (RISC) associated endonuclease for the now very popular small interfering RNA (siRNA). This mechanism of action is advantageous since it leads to target RNA destruction and allows ON to act in a catalytic mode. However, many ON modifications that lead to increased metabolic stability and increased affinity for the RNA target are incompatible with RNase H recruitment, thus, restricting such antisense ON to a steric-block mode of action [1–4]. Steric-block ON analogs have been used, for example, to interfere sequence-specifically with the assembly of a translation initiation complex or of splicing complexes. Although acting at best on a stoichiometric basis, steric-block ON might be advantageous for applications aimed at regulating pre-mRNA splicing [5,6] or at promoting therapeutic exon skipping [7]. As an example, some forms of β-thalassemia are associated with intronic mutations leading to the production of a cryptic splice site and to incomplete removal of the mutated intron. Hybridization of a steric-block ON masks the mutated site, reorients the splicing machinery toward the complete removal of the intron, and leads to the production of a functional, fully-spliced mRNA [8]. Potential applications of this strategy might be numerous, since the majority of human genes undergo alternative splicing and also dysfunctioning of the splicing machinery has already been demonstrated in several human diseases [9,10]. Despite the great potential of ON-based strategies to control gene expression and a large number of clinical trials, only few ON-based drugs are presently available [11]. A major issue has been poor bioavailability of ON when administered as free entities as has mostly been the case up to now. This was not unexpected, since polyanionic nucleic acids do not cross lipid bilayers and their uptake by endocytosis is not efficient in most cell types. Neutral ON analogs, such as

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‡ This article is part of the Special Issue of the Journal of Peptide Science entitled "2nd workshop on biophysics of membrane-active peptides".

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Andrey Arzumanov. Born in 1961, he graduated from Moscow Institute of Fine Chemical Technology in 1986. He worked as a research scientist (1986–1998) in V.A. Engelhardt Institute of Molecular Biology (Russian Academy of Sciences, Moscow), completing a Ph.D degree in 1995. His research was focused on phosphate modifications in anti-HIV-1 nucleotides and on the study of DNA polymerases' nucleotide selectivity. In 1998 he moved to Cambridge, UK, first for a postdoctoral fellowship in MRC Laboratory of Molecular Biology, and then continued to work in Dr Mike Gait's group as a research assistant. In the following years his scientific interests transformed from chemistry to biochemistry and cellular biology, exploring the field of oligonucleotides and their peptide conjugates as potential therapeutics against HIV-1, muscular dystrophy and other diseases.

Hong M. Moulton was born in Chongqing, China, did B.S. in Chemistry from Chongqing Normal University in 1985, taught Chemistry in Chongqing College of Education and moved to USA in 1991 to pursue biochemistry and biophysics research and received Ph.D from Portland State University in 1996, and has been doing research in industry since 1996. Current research area: peptide-mediated drug delivery.

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Gabriela Dimitrova Ivanova. has a Ph.D in Bioorganic chemistry on the mechanism of ribosomal peptide bond formation from the Bulgarian Academy of Sciences, Sofia, Bulgaria. As a young postdoctoral researcher she joined the European Network on the Development of Artificial Nucleases (ENDEVAN) and worked with Prof. Roger Strömberg at Karolinska Institutet, Stockholm. Since 2004 she has been working in the Laboratory of Molecular Biology, MRC, Cambridge with Dr. Mike Gait on developing novel oligonucleotide therapeutics for various applications. Her current project involves design and synthesis of PNA-peptide conjugates, which are able to re-direct pre-mRNA splicing.

Michael J. Gait. Obtained his Ph.D in 1973 in Chemistry from the University of Birmingham, UK. 1973–75: Research Associate at M.I.T., Cambridge, Massachusetts, working on gene synthesis with H. Gobind Khorana. Since 1975: Staff Scientist at MRC, Laboratory of Molecular Biology in Cambridge, UK, receiving tenure in 1980 and being promoted to Senior Staff Scientist in 1987 and to a MRC Programme Leader in 1994. Known initially for his work on the development of solid-phase DNA and RNA synthesis methodology, he was also the first to clone and express the gene for T4 RNA ligase. In the 1980s and 1990s, he applied synthetic RNA analogues in studies of the hammerhead and hairpin ribosomes and the interactions of the HIV proteins Tat and Rev with viral TAR RNA. More recently, he developed steric block antisense oligonucleotide analogues for inhibition of Tat-dependent trans-activation and as potential antiviral agents and has worked on cellular delivery of siRNA. Currently, he is developing PNA-peptide conjugates for splicing redirection in cells and in~vivo and as inhibitors of micro-RNA action. He is a Fellow of the Royal Society of Chemistry and former chair of the RSC Nucleic Acids Group. He won the RSC 2003 Award in Nucleic Acids Chemistry. He was elected to EMBO in 2006 and is Senior Executive Editor of the journal Nucleic Acids Research. He is also well known as editor of "Oligonucleotides and Analogues: A Practical Approach" (1984) and co-editor of "Nucleic Acids in Chemistry and Biology" (with G. M. Blackburn and others 1990, 1996 and 2006).

peptide nucleic acids (PNA) or phosphorodiamidate morpholino oligomers (PMO), do not behave differently since they are too large to diffuse across lipid bilayers [12].

Many chemical modifications have been proposed to improve the pharmacological properties of ON. Very few have addressed cellular uptake with the exception of ON that carry a cationized backbone. Although

DOI: 10.1002/psc

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encouraging, reported data with such ON derivatives remain very preliminary [13].

Most efforts to improve ON bioavailability have concerned the many existing delivery strategies that have been adapted to ON delivery. Electroporation [14] and complexation to cationic lipids as lipoplexes [15] have become the most popular strategies to transfect ON but they essentially remain as laboratory tools and they cannot be used for neutral ON analogs. Various alternative strategies including lipid conjugation, liposomes encapsulation, or nanoparticles association are actively explored to improve ON delivery *in vivo* [16]. Chemical conjugation or physical association of charged or uncharged ON analogs to peptidic delivery vectors is becoming increasingly popular and will be described in more details in following sections [13].

CELL PENETRATING PEPTIDES: BRIEF DESCRIPTION AND MECHANISM OF CELL UPTAKE

Conjugation to polypeptides containing basic amino acids, such as poly (L-lysine) (PLL), has been proposed long ago as a tool to promote the cellular internalization of biomolecules. Our own group has documented the cellular delivery of PLL-conjugated antisense ON in several *in vitro* models. Complement activation and cytotoxicity of these long-chain heterodisperse polycationic formulations did, however, preclude further developments toward clinical applications [17,18].

A revived interest in basic amino acid-rich peptides as delivery vectors emerged with the discovery of cell penetrating peptides (CPP), also named protein transduction domains (PTD) [19]. Virologists had described the unexpected properties of the HIV-Tat protein. Addition of the purified Tat protein was able to trans-activate a HIV-1 LTR promoter, meaning that it was able to cross cellular membranes to access nuclei [19]. Structure–activity studies in our group have delineated a short peptide, often named Tat 48–60 peptide (sequence GRKKRRQRRRPPQ), which appeared to behave as the parent Tat protein [20,21]. Remarkable features of Tat 48–60 include high content of basic amino acids, the presence of a GRKKR nuclear localization signal, and a seemingly receptor- and energy-independent mechanism of nuclear translocation. Several of these properties were shared with the prototypic Penetratin CPP isolated from the *Antennapedia* Drosophila transcription factor [22,23]. More importantly CPPs, and Tat (48–60) in particular, rapidly gave rise to many potentially important biotechnological applications. Chemical conjugation to low molecular weight drugs and peptides, fusion constructions with full-size proteins, and more surprisingly, grafting to nanoparticles or liposomes gave rise to improved delivery of the transported payload in many cell types (including difficult to transfect primary cells) [19]. Interestingly, little has been documented concerning antisense ON delivery and our own efforts remained unsuccessful for a long time.

As briefly mentioned above, most early studies on the mechanism of cell uptake of CPPs and their conjugates to various payloads concluded that there is a direct translocation across the plasma membrane. Whatever the exact mechanism, avoiding endocytosis was considered advantageous for a delivery vector and provided much excitement [24]. A parallel between CPPs and some antimicrobial peptides has sometimes been made both in terms of peptide organization (for example, amphipathic organization and richness in basic amino acids) and of mechanism of cellular internalization. However, major differences between these two groups of peptides might be pointed. Antimicrobial peptides have been engineered as defense mechanisms aimed at destabilizing bacterial membranes and eventually leading to cell killing. Protein transduction domains originate from eucaryotic proteins and are possibly involved in information shuttling between cells. Moreover, in our opinion, for aiming at clinical applications one should avoid delivery vectors that lead to even transient membrane-destabilization or pore formation [25].

DOI: 10.1002/psc

Initial data concerning CPP mechanism of uptake have been reconsidered when artefacts specific to these strongly basic peptides were unravelled. By use of new protocols (the avoidance of cell fixation for fluorescence microscopy observation, inclusion of a proteolytic treatment to eliminate cell-bound material), an energy-dependent mechanism involving binding to cell-surface glycosaminoglycans followed by endocytosis was proposed, at least for arginine-rich CPPs [26]. Whether clathrin-coated pit endocytosis (as also documented for the full-size Tat protein), macropinocytosis, or another endocytic route is used is still a matter of debate and the route might differ with cell type, nature of the payload, or other factors [26,27]. Whatever the case, most recent studies do agree on the accumulation of CPP-payload conjugates within endocytic vesicles and not in nuclei as previously thought. As a consequence, escape from endocytic vesicles is now considered as the major limitation of CPP-mediated biomolecules delivery [28,29].

CPP DELIVERY OF STERIC-BLOCK OLIGONUCLEOTIDE ANALOGS

As pointed in previous sections, CPP-based delivery of ON has been poorly documented, despite early promising data with PNA–transportan conjugates targeting galanin receptors [30].

The evaluation of antisense ON has been obscured by off-target effects that are often difficult to distinguish from bona fide antisense effects. We (and an increasing number of laboratories in the field) are now using the splicing correction assay proposed by R. Kole and his colleagues [31]. It capitalizes on the β-thalassemia intronic mutation described in Section on Oligonucleotides as Gene Regulation Tools. The mutated β-globin intron has been inserted in the coding region of reporter genes (luciferase or EGFP) and these constructions have been stably transfected into HeLa cells. Expression of a functional luciferase or EGFP is strictly dependant on the hybridization of the splice correcting ON. The assay is easy to implement, since splicing correction can easily be quantitated by luciferase luminescence or by a RT-PCR assay that discriminates aberrant and correctly spliced products. Moreover, the assay has a low background and a large dynamic response. Several conjugates between conventional CPPs (Penetratin, Tat 48–60, oligoArg, oligoLys) and splice correcting ON (2′OMet RNA, PNA, PMO) have been compared in this assay with rather disappointing data. Significant luciferase expression was obtained only at high doses (5–10 μM), e.g. at concentrations leading to cell permeabilization.

Co-treatment with endosomolytic agents (such as chloroquine or high sucrose) largely increased splicing correction efficiency in keeping with segregation of the conjugates in endocytic vesicles [28,32,33]. Recently, we have described two arginine-rich CPP that allow significant and sequence-specific splicing correction in this assay [5,34].

The first CPP named (R-Ahx-R)$_4$ R is composed of arginine residues interspersed with nonnatural aminohexanoic acid. Indeed, structure–activity studies by J. Rothbard and his colleagues had established that the spacing of guanidinium groups in oligoarginine was a key factor for cellular internalization [35] and (R-Ahx-R)$_4$ R appeared to be one of the most active CPPs in this respect [5]. Conjugates of (R-Ahx-R)$_4$ with PMO- or PNA ON analogs indeed led to a dose-dependent and strictly sequence-specific splice correction [5]. Complete correction was achieved at micromolar concentration with EC50 values (derived from RT-PCR data) at submicromolar concentrations [36].

In vivo efficacy of an (R-Ahx-R)$_4$–PMO conjugate to alter pre-mRNA splicing has been demonstrated in the *mdx* dystrophic mouse model.

Systemic administration of a conjugate at 5 mg/kg resulted in effective exon skipping and the production of functional dystrophin in the skeleton muscles of the *mdx* mice [37].

Importantly, no significant membrane alteration has been observed (as monitored by FACS analysis of propidium iodide cell uptake) in this concentration range [5].

Likewise, R6Pen in which an hexaarginine stretch has been appended to Penetratin efficiently corrects splicing at submicromolar concentration when conjugated to PNA, while Penetratin itself is very poorly active in this assay. In both cases, sequence-specificity and efficiency have been quantitated by luciferase and RT-PCR assays [34].

Structure–activity studies are underway with both (R-Ahx-R)$_4$–PMO and R6Pen–PNA conjugates with the aim to improve activity and to understand which parameters are the most important for efficient nuclear delivery of the ON payload.

Although these studies have not been completed yet, a few interesting observations can already be made. First, cellular uptake and intracellular distribution (as monitored by FACS analysis and fluorescence microscopy of FITC-conjugated material) is not predictive of efficient nuclear delivery. As an example, FITC-labeled (R-Ahx-R)$_4$ – PMO is less efficiently taken up in cells than Tat–or Arg$_9$–PMO conjugates but is more active in splicing correction. Moreover, FITC-labeled (R-Ahx-R)$_4$ – PMO conjugates remain for a large part associated with endocytic vesicles, thus, suggesting that the endosomal release of a small percentage of the entrapped material is sufficient to achieve complete splicing correction [38]. Hopefully, therefore, improved analogs of these two lead peptides might

DOI: 10.1002/psc

become active at even lower submicromolar concentrations.

Acknowledgements

We thank Paul Prevot for his help with fluorescence microscopy experiments, David Owen and Donna Williams for peptides and PNA synthesis, and Philippe Clair for helpful discussions. Work has been supported by 5th PCRD EEC (BL and MG) and CEFIPRA (BL) grants. S. Abes and R. Abes are financed by the Ligue Régionale contre le Cancer and by the Région Languedoc-Roussillon, respectively.

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DOI: 10.1002/psc