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Delivery of small-interfering RNA (siRNA) to the brain

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Abstract

Background—Two fundamental difficulties in the delivery of drugs to treat central nervous system (CNS) diseases are the systemic delivery of therapeutics across the bloodbrain-barrier (BBB), and the targeting of drugs to specific tissues or cells within the brain. With the advent and promise of RNA-based therapeutics that utilize RNA interference (RNAi) to trigger specific silencing of genes within diseased tissues, the necessity to surmount such obstacles has become even more urgent.

Objective—Most pre-clinical and clinical studies on delivery of RNAi to the CNS have utilized invasive, intra-cerebral delivery of RNA to the targeted tissue. Thus, methods need to be developed to facilitate delivery of therapeutically significant quantities of RNA to the CNS via the systemic route, and to elicit clinically significant RNAi effects within the CNS tissues.

Methods—Cell-penetrating-peptides (CPPs) are ‘molecular delivery vehicles’ that can traverse cell membranes and co-transport peptides or polynucleotides. The present invention examines 1) the utility of CPP-RNA duplexes for delivery of RNA to CNS tissues and, 2) cell-mediated release of the RNA payload once the CPP-RNA duplex is internalized by the CNS cells.

Conclusions—The invention and embodiments listed therein outline molecular tools that can be adapted for non-invasive, systemic delivery of therapeutic RNA to the CNS in a future clinical setting.

Keywords

messenger RNA; shRNA, microRNA; mRNA, miRNA, short-hairpin RNA; siRNA, short-interfering RNA

1. Introduction

RNA interference (RNAi) is caused by small (21 – 23 bases long) single-stranded RNA molecules (small-interfering RNA; siRNA), which trigger silencing of target genes in a sequence-specific manner [1,2]. The siRNA's sequence is ‘anti-sense’ (or complementary) to a specific stretch of the messenger RNA (mRNA) transcribed by the target gene. However, during both experimental and therapeutic approaches, the RNA molecule is delivered to target cells or tissues of interest as a more stable (thus more effective) double-stranded form – double-stranded RNA (dsRNA) or short-hairpin RNA (shRNA) – where the ‘anti-sense’ strand is

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Declaration of interest

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Patent details

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delivered annealed to its 'sense' strand [1]. Alternatively, the shRNA is generated within the target tissue or cells by introducing a DNA expression cassette, which transcribes the dsRNA moiety *in vivo* [3]. The latest to join the RNAi repertoire are microRNAs (miRNA), an endogenously transcribed family of RNA molecules that mediate expression of target genes in a manner that closely parallels the mechanism for RNA interference via siRNA [4,5]. miRNA are expressed from specific gene transcription units, or they can be expressed from introns of protein-coding genes.

Mechanistically, the RNA molecule interferes with translation of the target gene's mRNA by first binding to the complementary sequence region, followed by cleavage of the mRNA or via interfering with the translation process itself, based on complete or incomplete base-pairing between the target and the siRNA moiety, respectively [4].

Thus, RNAi presents itself as a promising yet novel therapeutic modality that is applicable against many CNS diseases, including brain tumors, neurotrauma, neuropsychiatric diseases, neuromuscular diseases, pain, and infections [6,7].

2. The brain as a 'privileged organ'

The blood vessels that traverse the brain are much more impermeable to solutes in blood than the vasculature elsewhere in the body. The blood-brain barrier (BBB) is formed by a tight arrangement of endothelial cells that line the brain capillaries. This physiological barrier protects the CNS from invading pathogens and toxins that enter the systemic circulation. A second barrier, formed by the epithelial cells of the choroids, controls the exchange of molecules between the systemic circulation and the cerebrospinal fluid (CSF). Thus, these tight junctions between endothelial cells that line the cerebral capillaries will ensure that the only molecules that will gain entry to the CNS are: i) lipid-soluble molecules of around 400 Da or less; ii) molecules, such as glucose or amino acids, which are internalized via carrier-mediated transport; and iii) much larger molecules, such as insulin and transferrin, which are facilitated entry via receptor-mediated transport [8]. Thus, the BBB will be essentially impenetrable to any RNA moiety used in a potential RNAi therapy, since the average mass of a therapeutic siRNA molecule will be approximately 14 kDa (dsRNA, or shRNA generating DNA expression cassettes will be much larger) [9,10].

3. Chemistry and biology

As a result, most current preclinical strategies, and the single clinical study reported thus far [11], which have utilized siRNA for CNS diseases have relied on invasive introduction of siRNA into the target tissue within the brain via surgical penetration of the BBB. Among these, application of siRNA in its 'naked' form, or sequestered within liposomes (to enhance internalization by the target cells) have been tested *in vivo* in both animal studies and in a single clinical trial [11-15]. Delivery of the liposomal 'payload' has been further improved by incorporating ligands (that bind receptors on the target cells) to the surface of liposomes in attempts to enhance tissue specificity. However, liposomal methods suffer from inherent toxicity towards the target cells, particularly when neurons are targeted. An alternative approach to enhancing the cellular uptake of siRNA in the absence of liposomes is the direct coupling of the RNA molecule to cell-penetrating-peptides (CPPs) [16] or to ligands (those with corresponding receptors on target cells) [17], two methods which deliver the siRNA molecules into the cells with minimal tissue toxicity. The current patent application describes one of the first such studies to target neurons *in vitro* via CPP.

Further improvements to both liposome- and CPP-mediated methods have now enabled researchers to deliver siRNA moieties in a non-invasive manner via the systemic route, by: i) incorporating ligands or antibodies that target both the BBB and the target tissue in the CNS

onto the liposomes; or ii) direct coupling of ligands that target both the BBB and the target tissue within the CNS to the siRNA (described below in detail).

4. Expert opinion

RNA interference is a powerful molecular tool for silencing aberrant gene expression in diseased tissues. Thus, the technique has elicited considerable interest as a method to target human disease. Despite its promise as a novel mode of therapy, the primary difficulty in utilizing this technique has been the delivery of therapeutic quantities of siRNA to the tissue *in vivo*. This problem is exacerbated when CNS diseases are considered, as the only therapeutically significant method for drug delivery is invasive application by surgically penetrating the BBB. The difficulty is even more pronounced when RNAi therapies are considered, as the molecules are not only of significantly higher mass but carry an overall negative ionic charge due to the nature of ribonucleotides. Thus, most investigational therapies of RNAi against CNS disease have involved direct application of RNA drugs via surgical intervention, which most commonly has involved convection-enhanced delivery (CED) [18, 19] of the RNAi molecules to the target site. In fact, in the single clinical trial reported for RNAi delivery to brain tumor (which targeted tenascin-C), the siRNA was injected directly into infiltrative (inoperable) regions of brain tumor [11].

Steady progress in tissue-targeting methods has enabled researchers to deliver siRNA to diseased tissues or organs via the systemic route with positive outcomes, including the brain. This challenge has been met from several angles as outlined below.

Two modalities have come to the forefront in delivering RNAi to target sites within the CNS via the systemic route. The first involves chemically modified liposomal complexes (which carry the therapeutic RNA as a payload), which are coupled to ligands that target specific receptors on the BBB, as well as to additional ligands that can 'home-in' on the diseased tissue once the liposomal complex is internalized by the CNS. The second modality involves direct coupling of siRNA to ligands, where the ligand first targets the BBB, followed by 'homing-in' on the target tissue once internalized into the CNS.

Most current strategies that have been examined on delivery of RNAi to the brain via the systemic route have utilized receptor-mediated transport of therapeutic RNA across the BBB. In one example, RNAi delivery vectors that target epidermal growth factor receptor (EGFR), which are expressed by a majority of aggressive brain tumors, have been encapsulated in PEGylated immunoliposomes (poly-ethylene-glycol 'encapsulated' liposomes) and targeted in an orthotopic brain tumor animal model. The liposomes were surface modified with monoclonal antibodies against the insulin receptor (to target and penetrate the BBB) and against the transferrin receptor (to target glioma) [20,21]. The same group has also tested direct conjugation of transferrin-receptor-targeting antibodies to siRNA, where the antibody was coupled to the RNA molecules via a biotin-streptavidin linker [13].

Recently, short peptides derived from rabies virus glycoprotein (RVG), which binds to the acetylcholine receptor, have been utilized for the successful delivery of siRNA across the BBB to target neuronal cells [22]. Here, dsRNA was coupled to RVG and delivered systemically. As the nicotinic acetylcholine receptor (AChR) is present both on neurons and the vascular endothelium of the BBB, the dsRNA-RVG duplex is effectively internalized across the BBB by receptor-mediated endocytosis and then targeted to the neuronal tissues in the CNS. Thus, a single peptide with corresponding receptors on both the BBB and the target cell type (neurons) has been used in a 'one pot' strategy to elicit RNAi in the target tissue via the systemic route. However, the exact mechanism by which RVG penetrates the BBB, and the cell-specific targeting within the CNS, remain to be elucidated (as cells other than neurons also express the AChR).

Other investigators have utilized coated nanoparticles (reviewed in [23]) for the delivery of encapsulated siRNA or siRNA-generating expression vectors through the BBB. These nanoparticles are thought to mimic low-density lipoproteins (LDL) and thus interact with the LDL receptors on the endothelium resulting in their uptake across the BBB [24].

An alternative technique has utilized the ‘loosening’ of the tight junctions of the BBB capillary endothelium by first targeting cell-adhesion molecules within the barrier. siRNA-mediated targeting of claudin-5 in the tight junctions enhanced permeability of the BBB to larger molecules [25].

In contrast to the above-described non-invasive strategies for delivery of shRNA or siRNA to the CNS via receptor-mediated targeting or as liposome-based ‘payloads’, an alternative method (that can replace the cell- or tissue-specific ligands) is the use of CPP for targeting of siRNA [16,26]. CPP are mainly cationic peptides 10 – 30 amino acids in length, which can cross the plasma membrane of mammalian cells. To deliver the siRNA, CPP can be non-covalently complexed with siRNA, or be covalently linked for greater effectiveness. However, a couple of obstacles are evident in this approach: i) the anionic siRNA can condense with the cationic CPP, effectively neutralizing the ability of the CPP to penetrate the cell membrane; ii) as the CPP are targeted to the nucleus, the CPP–siRNA link needs to be ‘uncoupled’ once the complex is internalized (as the siRNA mediates its effect in the cytoplasm). Thus, an optimal chemical linkage would be made of thiol groups for separation of the siRNA via reductases in the cytoplasm, one of the preferred embodiments in the current patent. Several reports following this approach have been published thus far. Two CPPs – penetratin and transportan – were coupled to siRNA duplexes (capable of silencing luciferase or enhanced green fluorescent protein in engineered mammalian cells) via disulfide linkages and delivered to cells, which were shown to silence the targeted genes [27]. A second study utilized another CPP, Tat, to deliver siRNA against enhanced green fluorescent protein or cyclin-dependent kinase-9, again with the siRNA being coupled to CPP via thiol linkages [28].

In the present patent application (and the publications related to the application; see ref. [26]), the inventors use a thiol-based linkage to deliver CPP (penetratin)-coupled siRNA to neurons *in vitro* and to the CNS in an *in vivo* rat model. Multiple siRNA targets, mainly related to regulation of cellular apoptotic cascades, were involved in the study. Preferred embodiments listed within the application also describe additional siRNA targets, as well as other CPPs not investigated in the primary research publication related to the patent. The study demonstrates the efficacy of CPP conjugated siRNA duplexes in targeting primary neurons *in vitro* and the CNS tissue *in vivo* (however, this is via invasive administration of the siRNA directly to the brain tissue by convection enhanced delivery). The approach has minimal toxicity towards the targeted cells, in contrast to reported toxicities with liposome-based siRNA application strategies. Thus, several issues remain to be resolved before the method can be deemed applicable in a future strategy to target siRNA to the CNS via the (non-invasive) systemic route: i) in a pattern similar to other studies that utilized CPP–siRNA, the duplexes were not separated and characterized before testing, thus, it is not certain whether an siRNA/CPP (non-covalent) complex was responsible for the observed cell-internalization and silencing effects, or, as outlined in the application, whether siRNA–CPP duplexes were directly responsible; ii) although effective *in vitro*, it is not certain whether only neurons were targeted in the *in vivo* studies, as the complexes were administered directly to one brain hemisphere in the animal model. Furthermore, although penetratin is suggested to traverse the BBB [29], it is unclear which other organs would be reached by the therapeutic complex in a future systemic application. In this case, the bioavailability of the CPP–siRNA to the brain would be reduced. Whereas the CPP–siRNA duplex might traverse the BBB and enter the CNS, its specific target (s) (tissue and cells) within the brain also remain to be elucidated.

In conclusion, the present invention outlines a novel approach for delivery of siRNA to the CNS in a future clinical strategy. The success of the method will provide both researchers and clinicians with an additional repertoire of molecules to direct therapeutic RNA to diseased tissue within the CNS. However, several hurdles remain to be overcome before this strategy can become widely applicable. These include the discovery of additional CPP (or characterization of currently available CPP) as to their tissue (or cell) specificity, demonstration of the therapeutic efficacy of the ability of CPP to traverse the BBB via the systemic route, and the ability of individual CPP to 'home-in' on specific target tissues or cells within the CNS to deliver their siRNA payload.

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