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Review Article

Targeting of protein expression in renal disease using siRNA – A review

Manal Ali Buabeid[1], Nihal Abdalla Ibrahim[1], Zelal Jaber Kharaba[2,3], Muhammad Ihtisham Umar[4], Ghulam Murtaza[4]*

[1]Department of Clinical Sciences, College of Pharmacy and Health Sciences, Ajman University, Ajman 346, [2]Department of Clinical Sciences, College of Pharmacy, Al Ain University of Science and Technology, Abu Dhabi, United Arab Emirates, [3]Honorary Associate Lecturer, Faculty of Medical Sciences, Newcastle University, Newcastle Upon Tyne, United Kingdom, [4]Department of Pharmacy, COMSATS University Islamabad, Lahore Campus, Pakistan

For correspondence: **Email: gmdogar356@gmail.com; **Tel:** +92-314-2082826; **Fax:** +92-992-383441*

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Abstract

The kidneys have rarely been used as a target in the systemic delivery of siRNA when compared to other tissues or organs in the body. This review article deals with various modalities adopted to deliver siRNA to the renal system under different normal and pathophysiological states. In this article, the authors have reviewed extensive clinical data that describe the use of siRNA for the treatment of renal diseases. Conventional and 3D modeling utilizes the existing genome-based RNA libraries, which facilitated the identification of molecular pathways involved in renal diseases.

Keywords: *siRNA, kidney disease, targeting proteins, signal pathways*

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INTRODUCTION

Since its exploration, RNA interference (RNAi) has been studied in biological and molecular researches more as an experimental tool in gene transfer [1]. This approach deals with the sequencing of mRNA by using double-stranded RNA that suppresses translation. It leads to reduced expression of target proteins. The libraries of short interfering RNA (siRNA) are the best source for disease identification and targeting [2]. The researchers use siRNA in the therapy of kidney transplants, various clinical diseases, including diabetic nephropathy and glomerular diseases [4]. The kidney is a good candidate for renal disease treatment because of its uptake of siRNA is rapid, which results in reduced targeted protein expression [5].

siRNA-based research is advantageous in studying the pathological signaling pathways without influencing the interactive pathways. This area of research is particularly advantageous in healing a pathology and preventing new damage to the kidney.

In recent experiments carried out on animals, the focus has been on systemic delivery and the therapeutic potential of RNAi in the treatment of renal diseases and injuries.

RNA interference (RNAi)

The mode of action of RNAi is pre-requisite for the understanding of its biomedical applications. As far as its mechanism is concerned, small fragments of double-stranded RNA are cleaved by ribonuclease and fixed with argonaute-2 for the unwinding of both strands of siRNA. Argonaute-2 is an RNA-provoked silencing complex (RPSC). Then, the RPSC degrades mRNA selectively [6]. After cleaving each mRNA, the RPSC begins a novel cascade that starts mRNA splintering that may last for several days or weeks. This approach is useful for the prevention of protein synthesis and any permanent effect [7].

In vivo study of siRNA, effects revealed that siRNA degradation exerts an inhibitory effect on protein expression [8]. The formation of short hairpin RNA begins with the separation of sense and antisense sequences via a non-coding loop under the effect of plasmid DNA, which contains 21 sense and antisense nucleotides. Self-binding of complementary nucleotide sequence and folding of RNA leads to the formation of regular short hairpin RNA (shRNA). At the same time, loop domain cleavage results in the formation of regular siRNA. The synthetic siRNA this formed is then directly loaded on to RPSC after injection into cells. The siRNA comprising 25–27 nucleotides, having been subjected to Dicer processing, exhibit an improved potency, and have a longer-lasting gene silencing effect than the 21-nucleotide siRNAs [9]. It results in the emergence of various off-target effects of siRNA that induces the inflammatory response, activates the angiogenic response, and regulates the non-targeted protein [10]. The immune system recognizes double-stranded DNA as an antigen and initiates an immunogenic response [11].

How can naked siRNA be delivered to the kidney?

One of the major concerns of using siRNA techniques *in vivo* is an effective knockdown at the target site. Moreover, the anticipated outcomes could be affected by unexpected toxicities. Several studies have revealed the effectiveness of siRNA in local drug delivery tools [11]. However, the current difficulties in system delivery need more work for their resolution. For systemic administration, along with spleen and liver, the kidney is a preferred site. It is preferred because nephrons readily absorb siRNA, and the kidneys play an important role in excretion from the body (Table 1).

Administration through intravascular route

The intravenous route is useful for the administration of exogenous nucleotides. For instance, a study described the efficient delivery of plasmid DNA through a hydrodynamic injection into the tail vein [12].

Several studies have described the introduction of siRNA via the intravenous route. However, the values of renal targeting rate varied significantly among these studies, probably, due to the use of different transfection reagents. A similar study described the systematic treatment of proximal tubule for proximal multidrug resistance protein isoform 2 (Mrp2) by using 150 µl single dose of siRNA. As a result of this application, siRNA underwent rapid renal distribution and revealed a reduction in Mrp2 activities after four days. The kidney eliminated a radiolabel substance at a higher rate than the radiolabelled siRNA [5]. A study reported the treatment of ischemia-reperfusion injury of mice by using Zag proteins. The siRNA as a dilute saline solution (200 µl) was injected daily for one week through the retro-orbital sinus, and the seventh-day aroused with the knock-down of the renal proteins [13].

Additionally, the hydrodynamic delivery of siRNA necessitated the use of a large volume of intravenous solution. The volume used in hydrodynamic delivery was 10% of body weight. It was much higher than the recommended mean volume of intravenous solution in the standard protocol, i.e., 50-100 µl. The increase in volume caused a transient capillary dysfunction, which caused the exposure of parenchymal tissue cells to siRNA. Furthermore, hepatotoxicity was another drawback of hydrodynamic delivery of siRNA [14]. This modality has the excellent potential of delivering siRNA to the kidney through glomerular filtration.

In various models used in the studies with mice, the hydrodynamic approach resulted in fast uptake of siRNA from the tubular lumen. In hydrodynamic delivery, the first action resulted in flush, and its alternation with the mentioned solution at the stated intervals resulted in the knockdown [15]. The animal model studies have revealed the use of tissues containing siRNA in heart transplant [16].

A comparative study disclaimed the uptake of fluorescent-labeled siRNA when administered via rectal and intraperitoneal routes. However, when administered hydrodynamically, fluorescent detection was significantly improved [17]. Though these studies facilitate the evaluation of siRNA targeting and uptake by tissue, such studies are

not useful for assessing target protein concentrations. It also showed that the high workload placed on the kidneys due to the overload of siRNA during these procedures might cause renal dysfunction [18].

Administration through routes other than intravenous

Several routes are beneficial for the intrarenal and localized delivery of siRNA. The renal artery and renal vein are advantageous for targeting glomeruli [19] and tubular interstitium [15], respectively. *In vivo,* electroporation is useful for enhancing siRNA delivery. According to a previous study, the use of electroporation to enhance the intra-arterial administration of a transgenic construct in rats led to siRNA expression in mesangial cells [21]. The siRNA administration through renal artery induced mild ischemia, which caused temporary occlusion of the renal artery. This outcome diminished vulnerability to neurological deficits due to reduced blood flow to the brain [22].

The intrapelvic injection in the renal medulla resulted in a transgenic expression that influenced the tubular epithelium. However, the inner region of the medulla did not exhibit such effects. An intraurethral injection of DNA enzyme resulted in the transgenic expression in the interstitial cells [23].

The subcapsular administration to kidney requires a surgical procedure, which adversely affects renal function. There are certain limitations to this technique. For example, there is leakage of a small fraction of the administered fluid via the puncture hole after needle retraction.

Cargo of siRNA

The injected naked siRNA deplete target proteins. An experimental study involving green-fluorescent protein (GFP)-transgenic mice revealed that the administered siRNA diminished the renal protein expression [24], likely due to nuclease-based degradation of siRNA [8]. It is feasible to deliver siRNA to cells without using additional carriers. However, the efficiency of siRNA delivery can be enhanced by encapsulating it or making its complex with transfection reagents [4].

Use of transfection reagents

Transfection agents having lipid nature and bearing positive charge have been used to enhance the siRNA uptake by tubular epithelium. Also, liposomes promisingly deliver the biochemical agent. DOTAP liposomal transfection reagent is a promising agent for renal delivery of siRNA [25].

Table 1: The literature on siRNA targeting

Target	Concentration/Dose of siRNA	Volume of siRNA (µL)	Route of administration	Method of transfection	Reference
Glomerulus	50 µg	Not given	Renal artery	Electroporation, Phosphate buffered saline	19
Glomerulus	50 µg	500	Renal artery	Electroporation, Phosphate buffered saline	75
Tubules	50 µg	100	Renal vein	Phosphate buffered saline	15
Not specified	20 µg	200	Renal vein	DOTAP	17
Not specified	0.1 mg/kg	2500	Renal vein	Phosphate buffered saline	66
Tubules	0.33 mg/kg	300	Intravenous	Phosphate buffered saline	63
Not specified	20 µg	200	Intravenous	Phosphate buffered saline	18
Not specified	50 µg	200	Retro-orbital sinus	Phosphate buffered saline	13
Not specified	7 nmol	150	Intravenous	Phosphate buffered saline	5
Tubules	50 µg	1500	Intravenous	Phosphate buffered saline	24
Tubules	50 µg	50	Intraurethral vein	Phosphate buffered saline	20

Besides, 1,2-dioleoyl-sn-glycero-3-phosphatidyl-choline (DOCP) is a natural liposome that promotes siRNA uptake by different organs, including liver, kidney, and lungs [26]. The researchers have developed a novel agent, named as lipofectamine, to improve siRNA efficacy.

An injection containing 300 µl of the siRNA-lipofectamine complex was administered via a renal vein and observed the animal for 30 min to study the tubular uptake of siRNA [26]. Another study revealed that the complex of siRNA with polyethyleneimine (PEI) not only reduced siRNA degradation but also enhanced the siRNA uptake, especially into the cytoplasm [27]. Several studies have revealed the biodegradation of naked siRNA in the kidney. The degradation of the siRNA-PEI complex occurred in the renal tubules and allowed the release of siRNA [28]. A critical advantage of the PEI-siRNA complex is the endosomal disruption it causes, which facilitates the infusion of siRNA in the cytoplasm [29].

A copolymer of poly-L-Lysine and polyethylamine effectively delivered siRNA to a target site, resulting in an effective uptake of siRNA by renal cells [4]. Another strategy for the delivery of siRNA to specific cells is antibody-based targeting. For instance, complexes of IgG with different agents such as siRNA and neutravidin. Based on the findings acquired from this study, there was strong binding of the antibody with podocytes, but not with tubular cells that caused the glomerular-sclerotic injury.

Protein knockdown is an indicator of this specific targeting. Different tissues of the body, such as the liver, intestinal, muscles, and spleen, exhibited the presence of the antibodies that could be used in specific targeting [30]. Streptavidin-antibody conjugates undergo complex formation with siRNA. This complex showed better performance in vivo than other delivery systems, including PEGylation-based siRNA liposomes, antibody-siRNA-cationic lipid complex, and siRNA-protamine complex [31].

On the other hand, biodegradable hydrogels promisingly deliver siRNA [32] as well as can carry plasmid DNA and siRNA for their prolonged release [33]. For the transdermal/topical delivery of siRNA, electroporation-based approaches have also been adopted [34]. Electroporation is a good technique for the swift delivery of siRNA. It enhances siRNA uptake and knockdown of targeted proteins in glomeruli [3]. However, electroporation stimulates the stress signaling of cells [35]. Thus, an alternative is a sonoporation to enhance siRNA uptake [36]. Studies have described a successful delivery of siRNA across the skin by using other approaches such as ultrasound-enhanced nucleotides. Still, this approach was not effective for siRNA delivery to the kidney [37].

Use of siRNA derivatives

The phosphodiester bond of a naked siRNA is sensitive to nucleases in the blood cells or serum. Chemically-altered siRNA is more stable and brings about improvement in renal targeting [38]. Chemical modification of phosphodiesters gives protection against the serum nucleases and exonucleases [39]. This modification is because of the replacement of nonbridging oxygen and prolonged half-life [40]. The *in vitro* investigations have revealed that phosphorothioate has low toxicity but high distribution after systemic administration; however, it proved toxic in the kidney because of some characteristic proteins [41]. The linkage of phosphorothioate with siRNA is generally resistant to enzymatic breakdown that is responsible for its greater accumulation in various sites, including skin and kidney [38]. In an experiment with mice, complexing with lipids resulted in an increase in cellular uptake of siRNA, accumulation in certain organs, and prolongation of half-life [42].

Toll-like receptors (TLRs) are expressed in the tubular epithelium and undergo binding with RNA. In a viral infection, TLRs induce characteristic signals [42], which activate immune response and degradation by endonucleases [43]. As a result of the modification, the obtained siRNA exhibited higher resistance to nucleases as compared to the unmodified form. However, the modified and unmodified siRNA showed a comparable potential to activate immune response [44].

The complexes of siRNA with polyethylamine (PEG) not only suppressed the immunogenic response but also improved stability of siRNA against nucleases, likely due to its readily soluble nature [45]. It resulted in a prolonged half-life due to PEGylation, which increased water-insolubility of siRNA, reduced its immunogenicity, and also helped in the formation of micelles for targeted delivery of drugs to the receptors [6].

Locked nucleic acids (LNAs) constitute a new category of nucleic acids that comprise a backbone having sugar and phosphate. Their synthesis is quite easy. The important characteristics of LNAs are their water solubility, the capability to produce a duplex with specific

RNAs after combining with RNA and DNA, enhanced degradation by serum nucleases, and increased expression of their related target proteins [7].

Meanwhile, morpholino oligonucleotide or nonionic DNA analog neither was affected by serum nucleases nor acted as an RNase substrate. Also, these analogs obstructed the expression of the glomerular protein in zebrafish embryos [4] as well as clogged the growth of microRNA and its activities but at low concentrations remain active for longer periods as compared to unmodified siRNA. Further, the off-target effects of siRNA are insignificant in targeting the kidney due to its improved stability after modification of MO and LNA to siRNA. Additionally, chemically-modified siRNA is steadily cleaved by the exonucleases produced by *Caenorhabditis elegans* [39].

Use of viral vector delivery shRNA

Vectors having shRNA-encoded expression cassettes such as plasmid DNA (pDNA) have been found as an alternative to siRNA administration [19] and employed for delivering a load to the nucleus to join transcription. The synthesis of pDNA vectors requires more time in comparison with siRNA synthesis. On the other hand, viral vectors are more efficient, owing to their greater stability [5]. Medullary epithelium, glomeruli, and tubule exhibited transgenic expression after adenoviral delivery in rats. Besides, severe toxicity limited its further use [23].

Adeno-associated virus (AAV) exhibited different immunogenicity than the adenoviral vector [9]. Thus, their transgenic expression was also different from one another [6]. A similar study described the delivery of recombinant AAV to the intrapelvic area of the kidney. It resulted in the transgene expression in epithelium and the medullary area [25].

AAV-2 is useful for the delivery of shRNA, especially to target the mineralocorticoid receptor. This process induced significant down-regulation of the receptor, up to three weeks after infection. It prevented the loss of renal function in a rat model for hypertension-induced kidney injury. Tropism features could be modified by retargeting AAV by pseudotyping. Kinoshita and Hynynen (2005) described the AAV-2 delivery of shRNA. Their findings showed that the mineralocorticoid receptors were significantly down-regulated up to three weeks after inducing infection [36].

Lentiviral vectors could transfect various dividing and non-dividing cells by genomic integration. The pseudotyped lentivirus successfully delivered a gene through the urethra in mice. The function of this gene was to increase GFP to tubular epithelial cells [15]. In the course of renal transplantation, an undesired vital gene region appeared as a result of lentivirus-induced gene slicing [18].

Renal disease therapy using siRNA

Treatment of acute renal injury

The reactive oxygen species or apoptosis cause renal injury, thus, stress mediators are targeted in siRNA delivery. One of the crucial pro-inflammatory agents is Nf-kB. It infiltrates renal immune cells and induces acute renal injury leading to the secretion of various pro-inflammatory cytokines [5]. In this context, the intravenously administered siRNA significantly ameliorates the nephrotoxicity [36]. Additionally, apoptosis could be induced by infiltrated cytotoxic T cells in the kidney donor during the post-transplantation phase. Another study has described similar findings as a result of siRNA injection into a local renal vein of rat's kidney, likely due to C3 inhibition by siRNA. It resulted in reduced renal reperfusion and ischemia [7].

Treatment of chronic renal diseases

Chronic renal complications (CRCs) include allograft dysfunction, glomerulosclerosis, and diabetic nephropathy. One of the most prevalent CRCs is tubulointerstitial compartment fibrosis [2] that is caused by transforming growth factors [23]. According to previous studies, fibrosis [44] and glomerular matrix deposition [3] were significantly reduced by siRNA. The kidneys produce antibodies, which induce glomerulosclerosis in mice [5].

Assessment of siRNA libraries for *in vitro* identification of target

In genomic studies, high throughput screening procedures mainly depend on pathway-specific siRNA libraries. Where, *in vitro,* pre-screening is useful for establishing *in vitro* target leads, which could be useful for the prediction of signal pathway association with renal injury *in vivo*. The combined knowledge of cell image analysis with siRNA knockdown screens is valuable for the identification of the complex signal pathway association [19,35]. However, such types of studies require the optimized and programmed sample and data management.

Selection of model

The use of diseased animal models in the *in vitro* experiments has eased the procedures to attain the *in vivo*-related extrapolation. Previous studies have reported the screening of apoptosis-related proteins by assessing cell viability or cascade activity. Several studies in current times have reported the use of image screening modalities using live-cells [31,40].

Assessment of altered morphology

Monolayer cell cultures are largely useful in *vitro* cell studies. The native tissue stimulation is needed to enhance the interaction and differentiation of normal cells [29]. The *in vivo* modeling needs a 3D culture environment for the tubulogenesis or morphogenesis. The studies have described an improvement in RNAi screening by using such 3D cultures [1,29]. Furthermore, shRNA-based viral delivery is better than a naked siRNA-based knockdown, where a prolonged duration of culture is a pre-requisite for complex morphogenesis.

CONCLUDING REMARKS

Renal targeting by siRNA is relatively easier than targeting other tissues, such as muscles, brain, or tumor cells. Though PEGylation seems to not be very effective for targeted delivery to the tubules, likely due to its low level of immunogenic potential and its barrier property to nuclease-mediated degradation. Based on these features, siRNA could potentially be more valuable for managing kidney dysfunction. The siRNA, particularly its modified form, could be evidence used to target renal epithelium and glomerulus since the modification of siRNA improves its renal uptake. The siRNA is not only useful for targeting specific tissues but also obstructing various signaling routes. Furthermore, the study of siRNA and shRNA in the 3D culture environment is useful for the assessment of *in vivo* functions of new drug candidates.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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