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Targeted fibrillar nanocarbon RNAi treatment of acute kidney injury

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Abstract

RNA interference has tremendous yet unrealized potential to treat a wide range of illnesses. Innovative solutions are needed to protect and selectively deliver small interfering RNA (siRNA) cargo to and within a target cell to fully exploit siRNA as a therapeutic tool in vivo. Herein, we describe ammonium-functionalized carbon nanotube (fCNT)-mediated transport of siRNA selectively and with high efficiency to renal proximal tubule cells in animal models of acute kidney injury (AKI). fCNT enhanced siRNA delivery to tubule cells compared to siRNA alone and

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SUPPLEMENTARY MATERIALS

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Methods

Author contributions: M.R.M. and S.A. conceived the study; designed and executed the functionalization and characterization of fCNT, fCNT/siRNA binding studies, radiolabeling, and in vivo experiments; analyzed data; and wrote the paper. N.A. performed in vitro experiments, RT-PCR, Western blotting, and flow cytometry analysis, and contributed to the in vivo *EGFP* knockdown study, the ⁶⁴Cu study, the biodistribution, the cisplatin dose-response experiment, the cisplatin response experiment, and the survival experiment. D.L.J.T. contributed to the writing and provided Figs. 2A and 3D. K.B. assisted in the nonhuman primate imaging experiment. Y.R. and K.M.-T. contributed to the acquisition and analysis of IHC and confocal images. D.Q. performed RT-PCR experiments. B.J.B. provided settings for the PET/CT imaging and helped analyze the data. M.B. performed DLS and TEM analysis. D.A.S. discussed experimental design, analyzed the data, and helped write the manuscript.

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effectively knocked down the expression of several target genes, including *Trp53*, *Mep1b*, *Ctrl*, and *EGFP*. A clinically relevant cisplatin-induced murine model of AKI was used to evaluate the therapeutic potential of fCNT-targeted siRNA to effectively halt the pathogenesis of renal injury. Prophylactic treatment with a combination of fCNT/si*Mep1b* and fCNT/si*Trp53* significantly improved progression-free survival compared to controls via a mechanism that required concurrent reduction of meprin-1 β and p53 expression. The fCNT/siRNA was well tolerated, and no toxicological consequences were observed in murine models. Toward clinical application of this platform, fCNTs were evaluated for the first time in nonhuman primates. The rapid and kidney-specific pharmacokinetic profile of fCNT in primates was comparable to what was observed in mice and suggests that this approach is amenable for use in humans. The nanocarbon-mediated delivery of siRNA provides a therapeutic means for the prevention of AKI to safely overcome the persistent barrier of nephrotoxicity during medical intervention.

INTRODUCTION

RNA interference (RNAi) is acknowledged as an important technological advance promising new therapeutic strategies through gene regulation. One mechanism of interference involves sequence-specific targeting of messenger RNA (mRNA) with complementary small interfering RNA (siRNA) resulting in mRNA degradation. Accordingly, siRNA-based therapeutics can transiently mute genes that regulate disease or injury. However, siRNA has yet to overcome major obstacles in vivo primarily related to tissue- and cell-specific delivery of siRNA, untoward off-target effects, and poor serum stability (1, 2). Nano-molecular delivery platforms are being investigated as a means to overcome the obstacles to use siRNA in vivo (2–5). An ideal platform is expected to be biocompatible and nonimmunogenic, possess a capacity for transport of RNA cargoes to the target cell, and afford protection from ribonucleases. Nanoscale molecular transporters and synthetic modification of the RNA backbone may remedy some of these problems, and several lipid and polymer nanoparticle formulations have already entered clinical trial (2–5).

Carbon nanotubes have been investigated as siRNA delivery platforms (6, 7). Ammonium-functionalized single-walled carbon nanotubes (fCNTs) are a unique class of fibrillar macromolecules that can deliver drugs, proteins, and radioisotopes (8). Paradoxically, owing to their large aspect ratio, fCNTs have a very favorable renal glomerular filtration and elimination profile (9–12), unlike most of the globular particles that accumulate in the liver and/or do not clear. A fraction of filtered fCNT is reabsorbed at the kidney's proximal tubular cell (PTC) brush border and endocytosed (10). This provides the opportunity for fCNT to transport noncovalently bound siRNA (Fig. 1A) to and within the critical PTC physiological compartment (13) and thus treat kidney-related pathologies.

Acute kidney injury (AKI) is recognized as an unavoidable side effect of numerous medical treatments. These include nephrotoxic damage sustained by antibiotics, antivirals, and chemotherapy as well as surgical procedures, which deprive the kidney of oxygen (14, 15). Injury to this organ is exacerbated in the elderly, which make up the bulk of the cancer population. The result is protracted and expensive hospital care, and half of the elderly population with AKI will succumb. This severe morbidity limits the therapeutic window

because chemotherapeutic dosages must be titrated down, resulting in a reduced antineoplastic effect. The pathogenesis of AKI is a complex biological process (15), and the loss of proximal tubule cell polarity (16, 17) and apoptosis (18, 19) are critical early events. Currently, treatment of AKI is largely supportive after damage, and despite the large number of patients at risk, pharmacological therapies remain unavailable (20).

Meprin-1 β and p53 are key proteins in the depolarization and apoptotic processes of kidney injury, respectively. p53 promotes cell cycle arrest or apoptosis in response to cellular stress, whereas meprins are metalloproteinases localized to the brush border membrane of polarized epithelial cells, where they are able to hydrolyze peptides and extracellular proteins (21). A redistribution of meprin to the cytosol in response to an insult is associated with renal injury (22). In studies where meprin activity was inhibited, there was protection against AKI induced by ischemia-reperfusion (I/R) injury, cisplatin nephrotoxicity, and sepsis (23–25). Meprin-deficient mice were markedly resistant to kidney damage from I/R. A chemically modified siRNA targeted to p53 was previously investigated to prevent kidney injury (19) and was evaluated clinically but did not meet the primary endpoint in a phase 2 clinical trial (4). Knocking out p53 in mice has also been reported to improve survival in response to nephrotoxic insults (26). However, no study has looked at this combination RNAi to prevent AKI.

Here, we demonstrate specific delivery of *Trp53*- and *Mep1b*-targeted siRNA to proximal tubule cells using an fCNT platform to prophylactically mitigate AKI in animal models. The fCNT-facilitated siRNA delivery prevented renal injury after a nephrotoxic insult that subsequently reduced fibrosis and immune cell infiltration and resulted in progression-free survival. Our data also provide strong evidence for the role of meprin in AKI. Toward clinical application of fCNT/siRNA for targeted prophylactic AKI treatment, we also evaluated fCNT biodistribution in nonhuman primates. A clinical strategy using the fibrillar nanocarbon platform could enable targeted siRNA protection of the kidney safely and effectively and prevent AKI in those on chronic pharmacological regimens, especially the elderly and cancer patients.

RESULTS

Characterization of the fCNT/siRNA complex

The fCNTs were prepared and characterized as previously reported via covalent cycloaddition of azomethine ylides with single-walled CNT (SWCNT) (9–13). fCNT had an amine content of 0.3 mmol/g (fig. S1A) and chemical purity >99%. Dicer-validated RNA sequences (27) were designed to silence enhanced green fluorescent protein (*EGFP*), murine copper transport protein 1 (*Ctr1*), meprin-1 β (*Mep1b*), and p53 (*Trp53*); a nonspecific scrambled sequence (*Scram*) was used as a control. The noncovalent binding affinity between fCNT and siRNA was ~5 nM (Fig. 1B), and up to four siRNAs could be loaded per fCNT (Fig. 1C) under physiological conditions (13). Transmission electron microscope (TEM) imaging of solid fCNT and fCNT/si*EGFP* (1:1 molar ratio) showed an average length of 300 nm (Fig. 1D). Both fCNT samples were water-soluble up to 10 g/liter, could be resolved chromatographically, and were rapidly renally filtered in mice, as has been shown previously (9–11). The molecular lengths of fCNT and fCNT/si*EGFP* (at 1:1 molar

ratio) were comparable, with mean diameters ($\pm$ SD) of 356.2 ± 14.2 and 332.7 ± 10.6 nm, respectively (fig. S1B). High-performance liquid chromatography (HPLC) showed a single peak (330 nm) (fig. S1C).

Kinetics of fCNT-mediated siRNA transport in vitro

Cellular internalization of fCNT/siEGFP-Cy3 (1:1) was investigated with EGFP-expressing HeLa cells (EGFP⁺ HeLa) using time-lapse confocal microscopy. In accordance with the loading/off-loading mechanism for fCNT/siRNA [when concentration is greater than the dissociation constant (K_d), then the two species remain bound together with nanomolar affinity; at concentrations below the K_d (such as when the complex is internalized inside the cell and the local concentration is lower than the extracellular milieu), the siRNA will dissociate from the nanotube (13)], fCNT/siEGFP-Cy3 did not fluoresce due to cyanine emission quenching by fCNT. siRNA-Cy3 emission or siRNA-[¹¹¹In] signal was detected at 2 hours and progressively increased over the 5-hour time course experiment (fig. S2A), as the fCNT complex was internalized and the siRNA off-loaded intracellularly. siEGFP alone exhibited negligible internalization. The internalization radioassay indicated that 10^4 molecules of siRNA were delivered by fCNT per cell, whereas siRNA alone demonstrated minimal uptake (fig. S2B).

fCNT-mediated RNAi of EGFP in vitro

Gene and protein knockdown was evaluated in EGFP⁺ HeLa cells. Time-lapse confocal microscopy images were collected over 60 hours, and region of interest (ROI) analyses showed that fCNT/siEGFP and a Lipofectamine/siEGFP (Lf/siEGFP)–positive control produced a significant decrease in fluorescence; control siEGFP alone was visibly less effective (Fig. 2A). The fCNT-mediated siEGFP reduced fluorescence expression by 70% at 24 hours and by 92% at 60 hours, whereas control siEGFP alone reached maximal inhibition of about 40% by 60 hours (Fig. 2A). Several cell division cycles were imaged and confirmed both cell viability and biocompatibility of the fCNT transfection reagent (fig. S3A). Confirmation of fCNT-mediated interference was acquired at 1, 2, and 3 days using flow cytometry (Fig. 2B), Western blot (Fig. 2C), or reverse transcription polymerase chain reaction (RT-PCR) (Fig. 2D); each demonstrated a decrease in expression compared to control groups. Notably, fCNT/siEGFP reduced EGFP expression significantly more than Lf/siEGFP. Kinetic analysis indicated that the maximum RNAi occurred at 48 hours (fig. S3B). Cytotoxicity was evaluated as a function of increasing dose of fCNT (or controls); after 72 hours of constant exposure, there was no significant difference compared to the Lipofectamine group up to a concentration of 100 mg/liter (fig. S4).

The pharmacokinetic profile of fibrillar nanocarbon and siRNA cargo

Specific renal targeting of fCNT/siRNA was substantiated by evaluating the pharmacokinetic (PK) fate in mice. As the main tissue targeted, kidneys accumulated about 22% of the injected dose of fCNT/siEGFP-[¹¹¹In]DOTA per gram within 1 hour (Fig. 3A and fig. S5), indicating that the siRNA remained bound to the fCNT in vivo until it reached the kidney. fCNT-mediated delivery resulted in a 10-fold increase of siRNA kidney accumulation compared to control, and the fraction of dose that was not delivered to the

kidney was rapidly (<1 hour) eliminated (fig. S5B). fCNT also protected siRNA cargo from serum degradation (fig. S6).

Renal cortex uptake of fCNT was confirmed using confocal microscopy (Fig. 3B). Organelle trafficking was investigated, and representative images of the early endosome, Golgi, and lysosomes in the cortex all costained for fCNT; early endosome signal was evidenced at 5 min, whereas Golgi and lysosome staining was more pronounced at 1 hour (Fig. 3C). These data, along with our previous reports (10, 28), support a clathrin-mediated endocytic uptake mechanism by the PTC (29, 30).

The biodistribution of tracer-labeled [^{86}Y]fCNT was determined in a large animal model (naïve nonhuman primates) using positron emission tomography–computed tomography (PET/CT). fCNT exhibited similar blood clearance, tissue biodistribution, and renal elimination in a 5-kg cynomolgus monkey (*Macaca fascicularis*). Intravenous [^{86}Y]fCNT (1 mg/kg) had a blood half-life of 7 min. Majority of the dose was rapidly eliminated in the urine, with a fraction accumulated in the kidneys (standardized uptake value was 16) (Fig. 3D).

Mitigating AKI with kidney-targeted fCNT-mediated RNAi

A therapeutically effective dose of fCNT/siRNA in mice was determined to be 1.6-mg fCNT + 0.087-mg siRNA per kilogram per day for 3 to 5 days (Fig. 1B) (13). These doses of fCNT/siRNA were sufficient to achieve knockdown of target mRNAs and were well tolerated by the host. Our regimen achieved prophylaxis with a cumulative dose of 0.4 mg siRNA/kg. We first demonstrated renal tubule–specific gene knockdown using fCNT and siRNA targeting *EGFP* (31). fCNT/si*EGFP* yielded a significant 75% decrease in renal cortical fluorescence versus controls receiving si*EGFP* alone, a scrambled siRNA, or vehicle control (Fig. 4, A to C). Western blot confirmed fCNT-mediated knockdown (Fig. 4D).

EGFP knockdown mediated by fCNT/siRNA provided the in vivo proof of principle. We therefore attempted to prophylactically ameliorate AKI by down-regulating *Ctr1*, a transmembrane protein responsible for the cellular uptake of copper that has been implicated as the key mediator of cisplatin uptake into the renal tubule (32). The quantification of renal accumulation of copper-64 served as a functional readout of *Ctr1* expression. Animals treated with fCNT/si*Ctr1* showed a significant decrease in renal copper uptake at 1 hour after injection compared to the untreated group and the si*Ctr1* alone (Fig. 4E). si*Ctr1* cargo administered without fCNT transport was unable to decrease copper uptake.

Safely targeting two genes involved in AKI pathogenesis

Meprin-1 β and p53 proteins play key roles in the depolarization and apoptotic processes of kidney injury, respectively. We aimed to target the genes encoding these proteins, *Mep1b* and *Trp53*, to establish feasibility of fCNT-mediated RNAi in AKI and to evaluate biocompatibility of the siRNA delivery system. Each animal received the fCNT/siRNA constructs or the siRNA alone at a dose of 0.087-mg siRNA $\pm$ 1.6-mg fCNT per kilogram per day for three consecutive days. Immunohistochemistry (IHC) revealed that the fCNT-mediated RNAi reduced the expression of both target proteins in the cortex (Fig. 5A). Quantitative ROI analysis of the IHC images revealed a significant decrease in p53

expression in the fCNT/si *Trp53* group versus si *Trp53* alone and vehicle. Similarly, meprin-1 β expression was significantly reduced by fCNT/si *Mep1b*, but not when receiving si *Mep1b* alone or vehicle (Fig. 5B).

Neither the nanocarbon vehicles nor the siRNA adversely affected renal health or tissue morphology. Renal function was assessed using a metabolic panel of blood urea nitrogen, serum creatinine, and phosphorus as biomarkers. No statistical changes were observed for any of the biomarkers, indicating that prophylactic fCNT and/or siRNA components were biocompatible (table S1). Tissue morphology was examined and scored (fig. S7) with no structural abnormalities.

Combination si *Trp53* and si *Mep1b* minimized mRNA and protein expression in kidneys challenged with a nephrotoxic insult

Mice were prophylactically treated for five consecutive days with a combination dose of fCNT/si *Trp53*/si *Mep1b* or scrambled control (si *Scram*) and then presented with a single nephrotoxic dose of cisplatin to induce AKI (10 mg/kg) on the third day. Mice were sacrificed on day 5, and IHC staining of paraformaldehyde (PFA)-fixed kidney tissues showed low p53 and meprin-1 β signals in the naïve controls and elevated protein expression in the fCNT/si *Scram* group. The combination drug maintained both p53 and meprin-1 β at baseline expression (Fig. 6A). Protein quantification in kidney cortexes via pan enzyme-linked immunosorbent assay (ELISA) (Fig. 6B) and mRNA expression (Fig. 6C and fig. S8) confirmed the IHC data demonstrating simultaneous *Trp53* and *Mep1b* knockdown. Fluorescence in situ hybridization (FISH) microscopy imaging permitted more accurate identification of mRNA in the renal cortex, and representative images of *Trp53*, *Mep1b*, and *dapB* mRNA in mouse cortical PTCs are shown in fig. S8A.

Simultaneously targeting *Trp53* and *Mep1b* reduced renal injury, fibrosis, and immune infiltration

Fibrillar nanocarbon-mediated RNAi treatment successfully minimized renal injury from a nephrotoxic cisplatin dose and improved progression-free survival in mice by targeting *Mep1b* and *Trp53* in the PTC. RNAi treatment or control was administered daily for five consecutive days, commencing on day -2, as outlined in the experimental timeline in Fig. 7A. A single dose of cisplatin (10 mg/kg) was administered on day 0. The cisplatin dose was selected on the basis of a dose-response study (fig. S9), whereas the nanotube-RNAi drug dosage was established on the basis of the binding affinity (Fig. 1B) and a dose escalation study (fig. S10). Animals were monitored for acute injury days 1 through 11 after cisplatin and then longitudinally over 6 months for chronic or adverse effects (tables S2 and S3). Kidneys obtained from randomly selected mice were histologically examined at 11 and 180 days for fibrosis and immune cell infiltration.

The fCNT/si *Trp53*/si *Mep1b* combination prolonged injury-free survival significantly compared to all controls (Fig. 7B). Eighty-eight percent of the animals treated with the combination fCNT/siRNA survived injury-free for the full 11 days after cisplatin, whereas <40% of those treated with fCNT/si *Mep1b*, si *Mep1b*, and fCNT/si *Trp53* survived that long. Median times to injury and the comprehensive results of statistical analyses are reported in

table S4. These data describe a prophylactic therapeutic intervention for acute renal injury that depends on fCNT-mediated delivery of siRNA targeting two early pathologic events. A forest plot of the hazard ratios strongly favored the fCNT/si *Trp53*/si *Mep1b* combination drug in minimizing renal injury (Fig. 7C). There was no therapeutic advantage in the separate use of fCNT/si *Mep1b* or fCNT/si *Trp53*, and the siRNA vectors alone were ineffective presumably because of degradation and/or low delivery efficiency. The fCNT/si *Ctrl* therapy was also ineffective in minimizing injury and improving survival (Fig. 7B).

Histological analysis of kidneys from fCNT/si *Trp53*/si *Mep1b*- and fCNT/si *Scram*-treated mice was performed at 11 and 180 days after nephrotoxic injury. Hematoxylin and eosin (H&E)-stained tissue from mice that received the combination drug showed tissue morphology consistent with healthy control mouse tissue (Fig. 7D). Kidney fibrosis was not observed at 11 days in any animal, but the interstitial fibrotic level was significantly higher for the fCNT/si *Scram* group at 180 days (Fig. 7E). Lymphocyte and macrophage infiltration occurs in both the early and later phases of cisplatin-induced AKI (33, 34); therefore, immune infiltration was assessed using IF staining for CD45⁺ leukocytes, CD3⁺ T lymphocytes, and Iba1⁺ macrophages. fCNT/si *Trp53*/si *Mep1b* treatment significantly reduced T cell, lymphocyte, and macrophage infiltration as early as 11 days after cisplatin injury, with the cell populations remaining at lower densities than scrambled controls at 180 days (Fig. 7, F to H).

DISCUSSION

Here, we deployed CNTs to deliver bioactive siRNA to renal PTC as a pharmacological intervention to prevent nephrotoxic injury. Despite great potential, use of therapeutic RNAi is hindered by ineffective delivery strategies, degradation, and off-target effects. Currently, there is no U.S. Food and Drug Administration–approved pharmaceutical for the prevention or treatment of AKI, which is associated to very high rates of mortality and morbidity (20). Other platforms for RNA delivery often involve complex aggregate structures, synthetic RNAi modification, intricate formulations, and multiple purification steps. The fCNT platform is a single molecule that can be loaded with RNA in a predetermined stoichiometry with nanomolar binding affinity (13). Assembly is rapid (<1 min) and no separations are necessary. Looking forward to clinical use, this drug could also be assembled with a tracer-labeled siRNA component to PET image and assess biodistribution and clearance to determine RNAi dose delivered to tissue.

fCNT is an ideal delivery vehicle for siRNA because the unique molecular platform protects noncovalently loaded siRNA from serum degradation. It is even more ideal for RNAi in kidney-related diseases because the soluble fCNT naturally traffics to the kidney, with rapid clearance in vivo (9, 10). These nanotubes rapidly cleared from the blood ($t_{1/2}$ = 6 min) and were readily filtered by the glomerulus. Thus, for the first time, a macromolecular-sized nanomaterial platform can be used to specifically deliver effective doses of siRNA to the kidney, whereas other nanomaterials are hepatically sequestered. PK profiling of fCNT-bearing radiotracer-labeled siRNA showed that 10-fold more siRNA cargo was distributed to the kidney compared to the siRNA alone.

The siRNA-laden construct remained intact, and the fCNT directed clearance and distribution of the cargo. Cell binding data demonstrated that the siRNA cargo was internalized and ultimately released in accordance with our proposed off-loading mechanism (13). Confocal imaging data also described PTC internalization and trafficking of fCNT in vivo that was consistent with a clathrin-mediated endocytic uptake after capture at the brush border (28–30). PET/CT imaging analyses of ^{86}Y tracer-labeled fCNT in nonhuman primates showed a PK profile that was nearly identical to the distribution and clearance in mice, suggesting that this molecular platform could be translated for human use. Other than targeting the kidney, the fCNT delivery vehicle improved the RNAi process compared with free siRNA because fCNT/siRNA was able to knock down 75% of *EGFP* as well as *Ctr1*, a copper-ion transporter in vivo.

Epithelial cells lose their polarity and apoptose after nephrotoxic insult (15–19). PTCs are known to accumulate siRNA, and several studies have proposed RNAi as a strategy to counteract AKI pathogenesis (19), highlighting the potential of systemically administered siRNA for the treatment of kidney disorders in clinical practice (35, 36). Stabilized siRNA that inhibits *P53* expression is in early development for AKI therapy ([ClinicalTrials.gov: NCT00554359](https://clinicaltrials.gov/ct2/show/study/NCT00554359) and [NCT00802347](https://clinicaltrials.gov/ct2/show/study/NCT00802347)) and is one of the first systemically administered siRNA drugs to enter clinical trials. Furthermore, the involvement of meprin in kidney injury has been shown in knockout mice and through meprin inhibitors, both providing protection against AKI and improving renal function (21). Our strategy was to deliver siRNA to the kidney to reduce the expression of two proteins with demonstrated roles in AKI, meprin-1 β and p53 (17–19, 21–26).

Our data confirmed the ability of fCNT to effectively deliver si*Mep1b* and si*Trp53* to PTCs and significantly reduce the expression of these two target genes and their respective proteins. These experiments were also designed to assess safety and showed that the fCNT and siRNA were biocompatible with no observed renal (or other) toxicity. The fCNT/si*Trp53*/si*Mep1b* combination was able to maintain the message and protein expression for p53 and meprin-1 β at baseline levels after a nephrotoxic insult and pharmacologically spare mice from renal injury. We identified a pharmacological intervention for a dose of nephrotoxic cisplatin that improved progression-free survival, reduced fibrosis, and decreased immune cell infiltration more than control treatments. Kidneys from animals treated with fCNT/si*Trp53*/si*Mep1b* showed significantly lower levels of macrophage, leukocyte, and T cell infiltration within the kidney cortex at 11 and 180 days after cisplatin injury, which indicates not only immediate disease resolution but also a durable effect. Combination fCNT-mediated RNAi also reduced chronic fibrosis.

The mechanism of drug action required the simultaneous targeting and down-regulation of si*Mep1b* and *Trp53* expression in the renal proximal tubule cells to significantly reduce kidney injury and prolong survival. The ability of fCNT-mediated delivery of a combination of si*Mep1b* and si*Trp53* to protect mice from renal injury resulted from targeted mRNA degradation of two genes that contribute to loss of epithelial cell polarity and apoptosis; the up-regulation of either can initiate injury. Individually, each siRNA (with or without fCNT) was insufficient to limit injury at the cumulative 0.4 mg siRNA/kg doses that we used in this work. These findings suggest that the loss of polarity and apoptosis in PTC were distinct co-

events that can each contribute to injury, but the coadministration of si*Mep1b* and si*Trp53* provided a synergistic therapeutic effect.

Therapeutic single gene (*P53*) interference was reported to mitigate cisplatin injury in rat kidneys (19). However, in our study, si*Trp53* (delivered with or without fCNT) was unable to control injury, whereas the combination with si*Mep1b* delivered by the fCNT was effective. We attribute the differences in these two studies to the following: siRNA doses differing 90-fold, with cumulative dose of 36 mg/kg in (19) and 0.4 mg/kg in our study; our use of natural RNA versus stabilized, synthetic RNA in (19); animal model [8-week-old rats in (19) versus 49- to 52-week-old mice in our study]; and degree of injury, where we used 10 mg/kg cisplatin versus 7.5 mg/kg in (19).

Because drug-induced nephrotoxicity is responsible for nearly one-fifth of AKI cases (37), we investigated the use of dual RNAi in a rodent model of cisplatin injury in a prophylactic treatment setting. Although cisplatin is often used in the context of cancer, one limitation of our study was the use of healthy but elderly animals. Although it is possible that tumors could alter the PKs of the fCNT platform (that is, by nonspecific accumulation in tumor), we do not expect this to be a major impact owing to the lack of a targeting moiety on the fCNT/siRNA (11, 12, 28). We also investigated only one model of renal injury, others such as I/R injury should be studied to assess the broad impact of this siRNA therapeutic approach.

Nano-mediated RNAi as a treatment for AKI seeks to transform the way in which a nephrotoxic renal insult could be managed clinically to avoid injury. Our fibrillar nanocarbon agent with dual siRNA significantly improved progression-free survival in a clinically relevant model of cisplatin-induced AKI and was safe in nonhuman primates. fCNT therefore represents a nanomedicine tool to enable robust prophylactic therapy to mitigate and minimize AKI with widespread application to patients receiving pharmaceutical regimens with nephrotoxic side effects. Such an approach may also afford the capacity to increase the therapeutic index while protecting the kidney (which is commonly a dose-limiting organ). Moreover, this technology can serve as a precision tool in the study of biological pathways in the nephron and aid in selecting appropriate targets to facilitate the drug design process. The translation of this work into a preventive therapeutic opportunity for patients will require the cGMP (current Good Manufacturing Practices) production of fCNT, a comprehensive cGMP toxicity evaluation of the fCNT/siRNA drug in rodents and larger animal models, the investigation of different models of AKI, and clinical trials to assess safety, compatibility, and PKs in patients at high risk of nephrotoxic injury.

MATERIALS AND METHODS

Study design

The purpose of this study was to investigate the therapeutic effect of fibrillar nanocarbon (fCNT)-mediated RNAi in a rodent model of nephrotoxic AKI by targeting two genes that are overexpressed in humans in response to renal injury, *P53* and *MEP1B*. The use of fCNT was expected to selectively target the proximal tubule cells and reduce the siRNA dose, avoid off-target effects, and protect siRNA drug cargo. This model could be translated into humans as a preventive therapy in anticipation of a nephrotoxic insult. Age-related

morbidity was introduced by using 49- to 52-week-old Balb/c mice. Mice were randomized and treated first with the fCNT/RNAi drug or controls 3 days before renal injury was induced by administering cisplatin intraperitoneally. Blood was collected on days 1, 5, 8, and 11 (after cisplatin administration) to assay biomarkers of kidney injury; weight changes were recorded daily; and observations of lethargy or death were noted. Progression-free survival was analyzed using the Kaplan-Meier method to score outcomes of weight loss ($> 20\%$ of initial mass), renal biomarker values (> 3 SDs relative to untreated group mean), severe lethargy, or death. Three representative mice from each group were sacrificed on day 11 (endpoint for acute injury in survival experiments) and the remainder on day 180 (chronic injury time point). Kidneys were harvested, fixed, and embedded in paraffin for histological analysis of fibrosis, T cell, lymphocyte, and macrophage infiltration. Weights, serum samples, and ROIs for IHC and IF images were recorded and analyzed blindly. Sample size was determined on the basis of our preliminary data (fig. S9) and on previous studies (19, 38, 39). After set endpoints for the survival experiment were reached, the weights increased and blood chemical parameters returned to normal for all the surviving animals. No outliers were selected in any of the experiments.

CNT generation and characterization

High-pressure carbon monoxide–produced SWCNTs were processed to yield SWCNT-NH₂ as previously described (9–13). Purity and identity of the fCNT were assessed by ultraviolet-visible spectroscopy, HPLC, TEM, and DLS (dynamic light scattering) as described (9, 10). Complete details are reported in the Supplementary Materials.

siRNA sequences

Dicer-validated RNA sequences designed (27) to target enhanced green fluorescent protein (*EGFP*), murine copper transport protein 1 (*Ctr1*), meprin-1 β (*Mep1b*), and p53 (*Tip53*) were obtained from Integrated DNA Technologies Inc. along with a nonspecific scrambled sequence (*Scram*) and are reported in the Supplementary Materials.

Animals

All animal studies were approved by the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center (MSKCC). The experiments used female Balb/c mice (Taconic) aged 6 to 7 weeks or 49 to 52 weeks; male *nu/nu* aged 8 to 12 weeks (Taconic); and female C57BL/6 *Tip53* null and female C57BL/6 wild type (Jackson Labs) 6 to 8 weeks old. The β -actin-*EGFP* transgenic C57BL/6 mice were provided by the Joyce laboratory at MSKCC (female, 8 to 10 weeks old). Male cynomolgus monkeys (Charles River Laboratories) were 3 years old.

Cell culture

HeLa cells expressing *EGFP* (EGFP⁺ HeLa, Cell Biolabs) were cultured at 37°C and 5% CO₂ in high-glucose Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies), 0.1 mM MEM nonessential amino acid solution (Life Technologies), 2 mM L-glutamine (Life Technologies), and blasticidin (0.010 mg/ml) (Life Technologies).

Kinetics of EGFP⁺ HeLa cell internalization of fCNT/siRNA

Internalization kinetics of fCNT/siEGFP was evaluated and quantified in EGFP⁺ HeLa cells. Confocal microscopy was used to image internalization in real time and utilized the fluorescent siEGFP-Cy3 sequence; radionuclide-based internalization of siEGFP-[¹¹¹In]DOTA was quantified using a cell-stripping assay.

Proximal tubule cell trafficking experiments

Mice received fCNT-(AF488)(AF680)(DOTA) or controls which included vehicle, only the AF488 dye. Harvested tissue was fixed overnight in 4% PFA at 4°C, embedded in paraffin, and sectioned to obtain 0.005-mm-thick samples. IF staining details are described below and in table S5. Widefield microscopy was performed with an Axioplan2 imaging microscope equipped with AxioCam MRm camera (Zeiss), using filter cubes for DAPI, AF488, and TRITC (tetramethylrhodamine isothiocyanate). Confocal microscopy was performed using an Inverted Leica TCS SP5 microscope (Leica). All three-dimensional (3D) rendering has been done with Imaris (Bitplane).

fCNT-mediated RNAi in vitro

EGFP⁺ HeLa cells were used to investigate fCNT/siEGFP silencing in vitro using flow cytometry, confocal microscopy, Western blot analyses, and quantitative RT-PCR (qRT-PCR) versus controls. Flow cytometry was used to investigate the change in green fluorescence intensity in cells. Time-lapse microscopy was used to image the change in green cell fluorescence in real time. Western blot analysis was used to measure EGFP protein expression. qRT-PCR analysis was used to measure EGFP expression.

In vitro cytotoxicity experiment

EGFP⁺ HeLa cells were treated with fCNT or controls, and viability was evaluated by flow cytometry using propidium iodide (Life Technologies) to detect dead cells.

Immunohistochemical and immunofluorescence staining

Kidney tissue sections were stained using a Discovery XT processor (Ventana Medical Systems) in the MSKCC Molecular Cytology Core Facility. Reagents and protocol details are listed in table S5.

PK studies of fCNT/siEGFP-[¹¹¹In]DOTA and [⁸⁶Y]fCNT

Biodistribution studies of fCNT/siEGFP-[¹¹¹In]DOTA versus siEGFP-[¹¹¹In]DOTA alone examined the heart, kidneys, lung, spleen, liver, stomach, intestine, muscle, bone, blood, and urine. Standards of the injected formulation were counted to determine the %ID and %ID per gram of tissue. Samples of the injected formulations and urine samples from each group were analyzed by HPLC. Dynamic PET/CT was performed with a Siemens Biograph mCT PET/CT system (40). Images in Fig. 3 (D and E) were elaborated as 3D volume rendering of PET data overlaid onto surface-rendered CT data. [⁸⁶Y]fCNT was prepared and characterized as previously described (31).

fCNT persistence in the kidneys as a function of time

Animals (4- to 6-week-old female Balb/c mice) received an intravenous dose of 0.1 ml of fCNT-AF488 (0.25 g/liter in PBS). Mice were sacrificed at 1, 3, 7, or 30 days after injection, and kidneys were fixed in PFA and paraffin-embedded for IF staining.

Dose escalation and renal accumulation of fCNT/siEGFP-[¹¹¹In]DOTA

The kidney accumulation of fCNT/siEGFP-[¹¹¹In]DOTA was investigated as a function of dose and schedule.

EGFP knockdown in vivo

This experiment used β -actin-EGFP transgenic C57BL/6 mice that received fCNT/siEGFP or controls. Each animal per group received a daily 0.22-ml intravenous injection of the respective drug or control for three consecutive days. Mice were sacrificed 1 day after the last injection. Tissues were harvested and fixed-frozen for histological studies. Images were acquired with an inverted fluorescence microscope (Nikon Ti Eclipse run with NIS-Elements Ar) and processed with Fiji (41). ROI analysis was done on $\times 20$ magnification of 0.010-mm-thick sections imaged with white light (DIC-like), DAPI, and GFP channel. About 50 tubules per experimental or control image were quantified (more than 300 cells per group).

Ctr1 knockdown and copper-64 uptake into kidneys in vivo

Mice received fCNT/siCtr1 or controls every day for three consecutive days. On the third day, every animal received an intravenous injection of 133 kBq of ⁶⁴CuCl₂ (Washington University) and were sacrificed 1 hour later. The kidneys, liver, heart, and blood were harvested and weighed, and radioactivity was measured on a γ -counter. The % ID/g was evaluated by comparison with known standards.

fCNT-mediated knockdown of p53 and meprin-1 β in vivo

Female Balb/c mice were grouped as follows: (i) fCNT/siMep1b ($n = 7$); (ii) fCNT/siTrp53 ($n = 7$); (iii) siMep1b ($n = 7$); (iv) siTrp53 ($n = 7$); and (v) PBS vehicle ($n = 3$). Each animal received a daily 0.10-ml intravenous injection of 0.032 mg of the 1:1 (mol/mol) fCNT/siRNA constructs, 0.002 mg of the siRNA alone, or the PBS vehicle for three consecutive days. Renal health was assessed on day 4 using a metabolic panel that assayed blood urea nitrogen, serum creatinine, phosphorus, and magnesium. Kidneys were harvested on day 4, fixed, sectioned, and stained with H&E to examine tissue morphology as a function of treatment. Tissue morphology was examined and scored blindly by an institutional veterinary pathologist. The expression of meprin-1 β and p53 in the renal cortex was evaluated using IHC and quantitative ROI analysis.

mRNA and protein expression levels in mice that received fCNT/RNAi prophylaxis and cisplatin

Mice were treated for five consecutive days with a daily dose of fCNT/siTrp53/siMep1b (1.6-mg fCNT + 0.087-mg siRNA per kilogram), fCNT/siScram, or PBS. Cisplatin (10 mg/kg dose) was intraperitoneally administered at day 3 to the fCNT/siTrp53/siMep1b and fCNT/siScram groups. Animals were sacrificed on day 6; kidneys were harvested, and half

of each kidney was sectioned and fixed in PFA for IHC and FISH (Affymetrix) analyses. The remaining half was homogenized in a TissueLyser II (Qiagen) with radioimmunoprecipitation assay buffer and protease inhibitor to extract protein. *Trp53* and *Mep1b* expression levels were measured in kidney cortex homogenates using a pan p53 ELISA kit (Roche Applied Science) and pan meprin-1 β ELISA kit (MyBioSource), respectively, according to the manufacturer's instructions. mRNA was extracted and purified with the RNeasy Plus Mini kit (Qiagen) according to the manufacturer's protocol. Expression of the *Trp53* and *Mep1b* was assayed by qRT-PCR.

Progression-free survival after fCNT/RNAi prophylaxis

Progression-free survival was evaluated in mice prophylactically treated to silence the renal expression of *Trp53*, *Mep1b*, and *Ctrl*. Each animal received a daily dose of 1.6-mg fCNT + 0.087-mg siRNA per kilogram (1:1 mol/mol) or 0.087 mg siRNA/kg or PBS vehicle in 0.10 ml by intravenous injection for 5 days. Cisplatin (Sigma; 10 mg/kg in normal saline solution) was administered on day 3. Here, nine groups of female Balb/c mice were arranged as follows: (i) PBS vehicle ($n = 5$); (ii) fCNT/si*Mep1b* ($n = 8$); (iii) fCNT/si*Trp53* ($n = 8$); (iv) fCNT/si*Scram* ($n = 8$); (v) si*Mep1b* ($n = 8$); (vi) si*Trp53* ($n = 8$); (vii) a combination of fCNT/si*Trp53*/si*Mep1b* ($n = 8$); (viii) a combination of si*Trp53*/si*Mep1b* ($n = 8$); and (ix) fCNT/si*Ctrl* ($n = 7$). Parameters and analysis of the progression-free survival are described in Study design.

Statistical analysis

GraphPad Prism 6 was used for all statistical analyses. Student's unpaired *t* test was used for statistical comparisons between groups. Log-rank (Mantel-Cox) test was used for the survival curve comparison, and 95% confidence intervals from the Mantel-Haenszel test were used for the hazard ratios. *P* values of <0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Assembly of the CNT siRNA construct

(A) An illustration of the noncovalent bonding interactions between an fCNT and a siRNA to yield the molecular RNAi construct (n.b. not to scale). (B) Plot of the fluorescence quenching titration of siEGFP-Cy3 with fCNT and fitted binding isotherm (dashed line). (C) Relative fluorescence intensity as a function of siEGFP-Cy3/fCNT molar ratio and graphical interpolation of the curve (dashed lines) to yield the siEGFP/fCNT loading stoichiometry. (D) Representative TEM images of solid-state fCNT and fCNT/siEGFP (1:1 complex). Scale bars, 500 μm .

Fig. 2. In vitro functional study of fCNT-mediated siEGFP delivery to EGFP⁺ HeLa cells
(A) Representative time-lapse confocal microscopy images at 1, 24, and 60 hours. Fluorescence quantification from three ROIs of the cells. Scale bars, 50 μ m. **(B)** Flow cytometry histogram overlay. **(C and D)** Western blot (C) and RT-PCR analysis (D) of *EGFP* expression by cells isolated at day 3 after transfection. Data in (A) and (D) are means $\pm$ SEM ($n = 3$). P values were determined by unpaired t test. h, hours; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Fig. 3. PK profile of fibrillar nanocarbon and siRNA in mice, PTC organelle trafficking, and PET/CT imaging of [⁸⁶Y]fCNT in a nonhuman primate

(A) Tissue biodistribution of fCNT/siEGFP-[¹¹¹In]DOTA and siEGFP-[¹¹¹In]DOTA at 1 hour after injection. The y axis shows the percent of the injected dose per gram (%ID/g) of tissue. Data are means ± SEM (n = 5). P value determined by unpaired t test. (B) Representative immunofluorescence (IF) images of 5-μm kidney sections stained for Alexa Fluor 488 to mark fCNT (green) and DAPI (4',6-diamidino-2-phenylindole) to mark nuclei (blue) of mice administered with fCNT-AF488 and sacrificed after 1, 3, 7, and 30 days.

Scale bars, 100 μm . The panel also includes the quantification of the AF488 signal. Data are means $\pm$ SEM ($n = 6$ ROIs). (C) Confocal microscopy images of fCNT colocalization with different organelle staining (red) at 5 min, 20 min, 60 min, and 24 hours from mouse tissue. Images of the early endosome, Golgi apparatus, and lysosomes were obtained with EEA-1, GM130, and LAMP-1 co-staining, respectively. Scale bars, 5 μm . (D) Fused PET/CT image of a representative 5-kg cynomolgus monkey (*M. fascicularis*) that received an intravenous bolus of [^{86}Y]fCNT (1 mg/kg) and quantitative analysis of the standard uptake value (SUV) in the kidney and bladder. The PET data are represented across the frames using the identical semiquantitative blue (low) to red (high) color scale. (E) PET/CT image of transverse kidney section taken where the white dotted lines are in (D).

Fig. 4. In vivo proof of concept of nanocarbon-mediated RNAi

(A) Representative images of kidney cryosections from an *EGFP* transgenic mouse model treated for three consecutive days with the indicated siRNA or vehicle control, sacrificed and imaged at day 4. Scale bars, 500 μ m. (B) Quantification of EGFP-positive cells and number of nuclei in individual tubule cells. (C) Ratio of EGFP-positive cells to total cells in the PTC as a function of treatments. Data in (B) and (C) are means $\pm$ SEM ($n = 300$ cells per group). (D) Western blots of the kidney cortex tissues confirming fCNT/siEGFP knockdown of protein expression. (E) In vivo proof of principle for fCNT-mediated RNAi of transporter function. Copper transporter loss of function in Balb/c mice treated with fCNT/siCtrl1, siCtrl1 alone, or vehicle control evaluated through renal $^{64}\text{CuCl}_2$ uptake at 1 hour after administration and expressed as % ID/g. Data are individual kidneys with means $\pm$ SEM ($n = 9$). P values in (C) and (E) were determined by unpaired t test.

Fig. 5. Reduction in renal cortex expression of p53 and meprin-1β after fCNT-mediated RNAi Mice ($n = 3$) were treated daily for three consecutive days with siRNA targeting *Trp53* or *Mep1b*. Animals were sacrificed on day 4, and tissues were stained for protein expression. (A) Representative IHC images of p53 and meprin-1β in cortical kidney sections, including immunoglobulin G (IgG) negative control. Scale bars, 20 μm. (B) Quantitative ROI analysis of p53 and meprin-1β IHC images. Data are means ± SEM ($n = 15$ ROIs per section). *P* values were determined by unpaired *t* test.

Fig. 6. Combination fCNT/siRNA minimizes mRNA and protein expression in vivo during cisplatin-induced renal injury

Mice were treated for five consecutive days with PBS (phosphate-buffered saline), fCNT/siScram, or fCNT/siTrp53/siMep1b. On day 3, fCNT-treated mice also received cisplatin (10 mg/kg), whereas controls received saline. All animals were sacrificed on day 6. (A) Representative p53 and meprin-1β IHC images of cortical kidney sections. Scale bars, 20 μm. (B) Quantitative analysis of the pan ELISA assay for p53 and meprin-1β. Data are means ± SEM ($n = 3$ for the naïve groups and 4 for the other groups). (C) Quantitative

analysis of the FISH for *Trp53* and *Mep1b* mRNA. Data are means $\pm$ SEM ($n = 9$). *P* values were determined by unpaired *t* test.

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Fig.7. Kidney-targeted fCNT/siRNA improves survival and minimizes fibrosis and immune cell infiltration after a drug-induced injury

(A) Timeline of the progression-free survival experiment detailing the RNAi prophylaxis treatments, cisplatin administration, serum sampling for blood chemistry analysis, weight loss assessment, and the time of the histology evaluations. (B) Kaplan-Meier plot of the percent survival as a function of time from cisplatin administration. Data are from $n = 8$ for all groups except for PBS ($n = 5$) and fCNT/siCt1 ($n = 7$). P values are in table S4. (C) Forest plot of the hazard ratios of the various prophylactic groups versus the combination

fCNT/si *Trp53*/si *Mep1b*. **(D)** Comparison of the H&E staining at day 11 of the kidney cortex of a representative mouse receiving fCNT/si *Trp53*/si *Mep1b* and cisplatin-induced nephrotoxic insult versus a naïve control mouse. Scale bars, 50 μ m. **(E)** Quantification of picrosirius red staining for kidney fibrosis at day 11 or 180 after cisplatin administration and representative images. Data are means $\pm$ SEM ($n = 10$ at day 11; $n = 12$ for fCNT/si *Scram* and $n = 8$ for fCNT/si *Trp53*/si *Mep1b* at day 180). Scale bars, 200 μ m. **(F)** CD3 immunostaining for T cell infiltration and representative images. Data are means $\pm$ SEM ($n = 9$ for all groups except for fCNT/si *Trp53*/si *Mep1b* at day 180 when $n = 6$). **(G)** Analysis of the lymphocyte infiltration by CD45 staining. Data are means $\pm$ SEM ($n = 9$ for all groups). **(H)** Analysis of macrophage infiltration by Iba1 staining. Data are means $\pm$ SEM ($n = 9$ for all groups). For (F) to (H), P values were determined by unpaired t test. Scale bars, 20 μ m.