Published in final edited form as:

Graphene Technol. 2016 December ; 1(1): 17–28. doi:10.1007/s41127-016-0001-2.

Safety and Efficacy of A High Performance Graphene-Based Magnetic Resonance Imaging Contrast Agent for Renal Abnormalities

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Abstract

The etiology of renal insufficiency includes primary (e.g polycystic kidney disease) or secondary (e.g. contrast media, diabetes) causes. The regulatory restrictions placed on the use of contrast agents (CAs) for non-invasive imaging modalities such as X-ray computed tomography (CT) and magnetic resonance imaging (MRI) affects the clinical management of these patients. With the goal to develop a next-generation CA for unfettered use for renal MRI, here we report, in a rodent model of chronic kidney disease, the preclinical safety and efficacy of a novel nanoparticle CA comprising of manganese (Mn^{2+}) ions intercalated graphene coated with dextran (hereafter called Mangradex). Nephrectomized rats received single or 5 times/week repeat (2 or 4 weeks) intravenous (IV) injections of Mangradex at two potential (low = 5 mg/kg, and high = 50 mg/kg) therapeutic doses. Histopathology results indicate that Mangradex does not elicit nephrogenic systemic fibrosis (NSF)-like indicators or questionable effects on vital organs of rodents. MRI at 7 Tesla magnetic field was performed on these rats immediately after IV injections of Mangradex at one potential therapeutic dose (25 mg/kg, $[Mn^{2+}] = 60$ nmoles/kg) for 90 minutes. The results indicated significant (>100%) and sustained contrast enhancement in the kidney and renal artery at these low paramagnetic ion (Mn^{2+}) concentration; 2 orders of magnitude lower than the paramagnetic ion concentration in a typical clinical dose of long circulating Gd^{3+} -based MRI CA gadofosveset trisodium. The results open avenues for further development of Mangradex as a MRI CA to diagnose and monitor abnormalities in renal anatomy and vasculature.

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Conflict of Interest Disclosure: Stony Brook University, along with its researchers, has filed patents related to the technology reported in this article. If licensing or commercialization occurs, the researchers are entitled to standard royalties. Balaji Sitharaman has financial interest in Theragnostic Technologies Inc., which, however, did not support this work.

Keywords

Nephrogenic Systemic Fibrosis; Gadolinium; Mangradex; Chronic Kidney Disease; Contrast Agent; Magnetic Resonance Imaging

Introduction

Failure of the kidney to filter toxins and waste products from the blood is a consequence of renal or kidney failure (also referred to as renal insufficiency) [1, 2]. Renal insufficiency is associated with a significant increase in morbidity and mortality worldwide [1, 2]. Acute kidney injury (AKI) can result from an ischemic event due to blood loss or low flow to the kidney or an acute inflammatory process due to infections, drugs, or autoimmune diseases [1]. On the other hand, chronic kidney disease (CKD) is a consequence of repeated insults to the kidney either from frequent episodes of AKI or from slowly progressive systemic illnesses such as diabetes mellitus and hypertension. CKD has been divided into five stages in order of increasing severity (i.e., stage 1 is considered mildly diminished kidney function, and stage 5 signifies end-stage renal disease (ESRD)[2]. Every year, in the U.S., 0.6 million individuals are diagnosed with an acute kidney injury [1], and over 20 million people have chronic kidney disease [3], with over 0.7 million of these cases classified as either stage 4 or stage 5 [3]. Episodes of AKI as well as CKD contribute to a significant increase in morbidity and mortality with a significant burden on health care resources. In fact, in developed countries the expenditures related to ESRD has been estimated to be 2–3% of the overall healthcare costs and in the US consumes nearly 25% of the Medicare budget [4].

To date, the utilization of non-invasive imaging for renal specific pathologies as well as general imaging of patients with moderate to advanced renal disease presents a major challenge in the clinical practice. The estimated glomerular filtration rate (eGFR), calculated from serum creatinine, is the most frequently used method to measure renal function [5]. However, no information on the function of individual kidneys is obtained from these tests. Renal scintigraphy provides some information on individual kidney function, but reveals limited anatomical details and utilizes ionizing radiation. X-ray computed tomography (CT) is widely used for non-invasive monitoring of renal system anatomy especially at advanced stages, but such imaging remains a major challenge because iodine based CAs utilized to improve the diagnostic confidence of CT[6] are associated with nephrotoxicity in patients with renal failure [7].

Magnetic Resonance Imaging (MRI), another imaging modality routinely used in the clinical diagnosis of renal failure [8], has the potential to provide both functional [9, 10] and anatomical information about individual kidneys [11]. In individuals with renal failure or with a kidney transplant, the use of MRI is more attractive than imaging modalities such as CT and nuclear imaging since it does not require use of ionizing radiation or nephrotoxic iodinated contrast media. MRI allows for non-invasive rendering of anatomical details of soft and hard tissues; thereby improving the diagnostic accuracy [12]. CAs are routinely employed to improve the diagnostic yield of clinical MRI. In fact, CAs account for approximately 44% of the 65 million clinical procedures globally [13]. Specifically,

gadolinium (Gd^{3+})-based CAs that currently dominate the market [14], are used to differentiate renal cysts and detect vascular abnormalities from background parenchyma [15]. However, when administered to patients with AKI or CKD ($eGFR < 30 \text{ mL/min/1.73m}^2$), some (Gd^{3+})-based CAs (gadodiamide, gadopentetate dimeglumine, gadoversetamide) have been demonstrated to induce a rare but potentially fatal disease called nephrogenic systemic fibrosis (NSF) [8, 16–18]. Early clinical features of NSF include the onset of limb edema accompanied by red or violaceous cutaneous papules and plaques overlying dermal, subcutaneous fat fibrosis, and when fully developed, results in limb pain, contractures, and loss of mobility [19]. Furthermore, it can cause fibrotic damage to internal viscera such as esophagus, heart, skeletal muscles, lungs and kidneys [19–21]. Thus, in the United States, Europe, and Japan, these CAs cannot be administered in patients at risk of NSF [22]. The American College of Radiology specifically does not recommend the use of these agents in patients with an $eGFR < 45 \text{ mL/min}$ or in patients with known or suspected kidney injury (irrespective of GFR values) [23]. Apart from the above-mentioned three (Gd^{3+})-based CAs, all other clinical (Gd^{3+})-based CAs (gadoteridol, gadobenate dimeglumine, gadofosveset trisodium, gadoxetate disodium) can only be used in patients with renal insufficiency at risk of NSF for critical diagnostic information not possible with MRI (without exogenous CAs) or other imaging modalities. The Food and Drug Administration (FDA) requires manufacturers to include a mandatory black box warning about the potential risks in the use of Gd^{3+} -based MRI CAs in patients with renal dysfunction [24, 25]. Further, recently concerns have been raised on lingering accumulation of residual gadolinium in the brain and bones of patients, even in those with normal renal function and further warnings and restrictions are expected for gadolinium-based CAs [26]. Consequently, there is a critical need for a next-generation T_1 MRI CA with a negligible nephrotoxicity with better efficacy than currently used Gd^{3+} -based CAs. This novel CA could be utilized to non-invasively monitor malignant lesions in the kidney after nephron-sparing surgery, renal vasculature secondary to carcinoma, nephron-sparing surgery or post kidney transplantation [11]. Additionally, such a MRI CA could also be used for non-invasively imaging and monitoring of structural and vascular changes in other organs in the setting of renal dysfunction.

Recently, manganese-based MRI CAs garnered attention as a possible alternative to overcome some of the above safety concerns associated with gadolinium-based MRI CAs [27]. Unlike lanthanoid gadolinium, manganese is a natural cellular constituent resembling Ca^{2+} , and often functions as a regulatory cofactor for enzymes and receptors. Normal daily dietary requirements for manganese are 32–41 μmol [28] while normal serum levels are 0.072–0.272 $\mu\text{mol/l}$ [29]. Manganese toxicity, resulting in neurological symptoms, is rare and has only been reported following long-term exposure or at high concentrations [27]. Two manganese (II)-based agents; Mn-DPDP (Mangafodipir trisodium) and an oral contrast containing manganese (II) chloride that were initially approved for clinical use have been discontinued [27].

We have developed a novel carbon nanostructure-based MRI CA comprised of disc-shaped graphene nanostructure known as graphene nanoplatelets (diameter $\sim 40 \text{ nm}$, thickness 2–3 nm, 5–7 sheets of graphene) intercalated (chemical species inserted and trapped in the voids between two graphene sheets), and coordinated with trace amounts of manganese (0.1 %

w/w (w = weight)) and functionalized with FDA approved natural polymer dextran to impart water dispersibility (hereafter called Mangradex; Representative transmission electron microscope (TEM) image and digital image of the formulation included in the supplementary information (Figure S1))[30, 31]. We have previously reported that this formulation is highly water dispersible (up to 100 mg/ml) and stable in blood and biological fluids [30]. We have performed *in vitro* and *in vivo* (normal healthy rats) safety studies that indicate that these nanoparticles demonstrate good hemodynamic characteristics [32], low acute toxicity (lethal dose LD₅₀ value > 500 mg/kg) and maximum tolerable dose 100 mg/kg (Table S1–3 summarizes experiments and salient results of those studies) [33]. *In vitro* r_1 relaxivity (an important measure of efficacy) measurements showed values ~10 times greater than current clinical MRI CAs [30]. Preliminary small animal (normal mice) MRI at 7 Tesla showed significant and persistent (up to 2 hours) contrast enhancement even at low dosages suggesting possibilities for development of this CA as a clinical extended-residence-intravascular agent (blood pool), and tissue (organ)-specific, and pre-clinical molecular imaging CA[34]. Based on these previous results, herein we have further investigated the suitability of the GNP-Dex MRI CA for renal imaging in a small animal model of renal failure at potential therapeutic dosages. We report the first preclinical safety and efficacy of a novel high-performance carbon nanostructure-based magnetic resonance imaging (MRI) contrast agent (CA) in a rat model of chronic kidney disease.

Results

Table 1 summarizes the various groups and any mortality for single and repeat dose study. During the 2 weeks study, no deaths occurred and there were no outward signs of clinical disorder. During the 4 weeks study, a rat that deceased under anesthesia at 5 mg/kg dose on day 24 before injection was taken out of the cohort as the cause of mortality was not related to the dosage of Mangradex. No instances of outwardly obvious pain or severe signs of distress were observed in either 2 weeks or 4 weeks group. No significant changes in food consumption were noted. There was also no significant difference in changes in body weights compared with the controls (Figure S2).

Figure 1 shows histology of lungs, liver and kidney showing minor diagnostic abnormalities 4 weeks after injection. Acute inflammation was observed in the kidneys of both sham and treated animals that likely reflect short-term consequences of the surgical nephrectomy procedure. There were no other diagnostic abnormalities observed after injection. Histopathology of the major organs in experimental groups revealed presence of brown pigment deposition in the pulmonary alveolar macrophages and microvascular spaces and in hepatic Kupffer cells. All other major organs (brain, heart, spleen) did not have any diagnostic abnormality in sham or experimental groups (results not shown).

Figure 2A displays the histomorphometric analysis of the thickness of H&E stained dorsal skin sections with no overt differences among groups. Also, shown for comparison in Figure S3, are the skin thickness measurements of the folded dorsal skin using digital calipers in the animals administered with multiple doses of 50 mg/kg of Mangradex in the 2 weeks group. The results show no statistically significant differences between the baseline control and experimental group. Figure 2B shows trichrome stained images of skin sections used to

determine collagen fiber concentration. Figure 2C shows the dorsal skin collagen area density performed by quantitative analysis using Image J. The results show no statistically significant differences among the groups. There was no histopathological evidence of fibrosis in systemic organs - lungs, liver, muscles and heart.

Figure 3A shows MRI phantom of Mangradex solution at graded concentration from 1 mg/ml to 20 mg/ml. Also, shown for comparison the concentration of manganese ions obtained from inductively coupled plasma with mass spectrometry (ICP-MS) at the given concentration of Mangradex. Images show noticeable concentration dependent contrast enhancement in phantom images. Figure S4 shows representative plot of the relaxation rate $1/T_1$ as a function of Mn^{2+} ion concentration. Based on the plot, the r_1 relaxivity of the Mangradex formulation at 7T magnetic field was calculated to be $61 \text{ mM}^{-1}\text{S}^{-1}$.

Figure 3B shows representative T_1 -weighted MRI images of the renal system of 5/6 Nephrectomized rats at 7 Tesla magnetic field pre and 25 minutes post injection of gadofosveset trisodium (dose = 455 nmoles/kg of Gd^{3+} ions), or pre and 25, 50 and 85 minutes post injection of Mangradex (dose = 25 mg/kg or 455 nmoles/kg Mn^{2+}) (To provide context, representative MRI images of normal rat showing both kidneys intact, 5/6 Nephrectomized rats (without contrast enhancement) are shown in supplementary information Figure S5). As clearly seen in Figure 3a, which shows the coronal view of the kidney, it is difficult to discern the organ prior to contrast agent administration. Subtle tissue contrast changes were noted 25 minutes post administration of the gadofosveset trisodium (Figure 3B (b)), however the signal decayed (data not shown) at the later times (50 and 85 minutes) and contrast enhancement was not adequate. Conversely, the administration of Mangradex leads to significant contrast enhancement in the kidney with Mangradex (Figure 3B (d-f) which was persistent up to 85 minutes. Further, qualitatively, comparing Figures 3B c-f, it can be discerned that the MR signal intensity from the kidney increases 25 minutes post Mangradex injection, and gradually decreases at 50 and 85 minutes (Figures 3B (e,f)). Figure 3B (g-h) shows representative MR angiogram images of the kidney before and after administration of Mangradex (dose = 25 mg/kg or 455 nmoles/kg Mn^{2+}). The intravascular space is difficult to distinguish before CA injection (Figure 3B (g)). On the other hand, as evident from Figure 3B (h)), a clear demarcation of renal blood vessels from surrounding tissue was not observed 25 minutes post Mangradex injection.

The above qualitative results were further quantitatively corroborated by analyzing T_1 relaxation time, signal to noise (SNR) and contrast to noise (CNR) of the kidney of the same rat (Figures 3C and D; raw data of all rats used in the MRI studies included in supplementary information Table S4). 25 minutes post administration of gadofosveset trisodium, the T_1 relaxation time of the region of interest (kidney) showed 23% decrease, and corresponding 36% increase in the SNR and 236% increase in the CNR respectively (Figure 3C). The SNR of the renal artery obtained from the MR angiogram also showed 6.2% increase (Figure 3D). After 50 minutes, the values of these parameters were similar to the baseline (pre-injection) values. The T_1 relaxation time in the region of interest Mangradex showed a significant decrease at all the time points (Figure 3C). This decrease led to a corresponding increase in the SNR (up to ~196%) and CNR (up to ~900%). The

SNR (Figure 3D) of the renal artery obtained from the MR angiogram also showed ~50% increase.

Discussion

The overall objective of this study was to investigate the suitability of the Mangradex MRI CA for the diagnosis of renal failure at potential clinical dosages. We conducted single and repeat dose safety studies and proof-of-principle efficacy studies in a rat model of chronic renal failure [35–38] to accomplish the objective.

FDA guidelines suggest performing special toxicity studies of a new pharmaceutical or diagnostic technology under development in an animal model relevant to its intended clinical indication [39]. Thus, we conducted single and repeat dose toxicity studies of Mangradex in a widely used chronic renal failure model 5/6 nephrectomized rats. Additionally this animal model has been widely employed to investigate the propensity of Gd^{3+} -based MRI CAs to induce NSF. It could be argued that NSF has been attributed to Gd^{3+} -based MRI CAs and never been reported for Mn^{2+} -based MRI CAs. A reason for this discrepancy could be that clinically Gd^{3+} -based MRI CAs have been used most of the time in clinic. As mentioned above, the clinically approved Mn^{2+} -based MRI CAs were discontinued. Thus, the propensity of gadolinium-free MRI CAs synthesized using other elements such as manganese and iron to induce NSF have never been thoroughly examined. Thus, it is prudent that preclinical safety studies of any novel MRI CA (including Mangradex) should include studies that investigate its nephrotoxicity. Previous NSF studies on this animal model for clinically approved MRI CAs were performed for 5 days to 4 weeks [35, 40]; hence, to investigate short and mid-term effects of Mangradex exposure, the time points chosen in this study are 2 weeks and 4 weeks. The nephrectomized rats were administered with single or multiple IV injections of Mangradex at two escalating doses (low - 5 mg/kg, and high - 50 mg/kg). The maximum dose (50 mg/kg) selected was based on the outcomes of previous dose ranging, expanded acute and sub-acute toxicity studies for Mangradex formulation [33], where we found the maximum tolerated dose (MTD) for Mangradex was 50 mg/kg. Hence, in this study, we have assessed short and long-term toxicity effects at doses 50 mg/kg. Mannitol (which was used to control osmolality of the Mangradex formulation) was selected as the control. The blood biochemical analysis of kidney function in our previous safety pharmacological studies [33, 34] indicated no adverse effects on kidney function. Those studies provided evidence that Mangradex at the potential diagnostic doses does not induce contrast-induced nephropathy. Thus, in this study, which mainly focused on histopathological analysis of major organs, investigating the propensity of Mangradex to induce NSF-like symptoms and assessing its renal anatomical imaging efficacy, additional blood and urine biochemical analysis of Mangradex as a way of quantifying the kidney functionality of the 5/6 nephrectomized rats was not performed.

The major organs (lung, liver, kidney, brain, heart, spleen) of animals were examined by histological analysis for any questionable effects due to the test article and we did not notice any adverse inflammatory response in these organs. Mn^{2+} toxicity after IV administration, resulting in neurological symptoms, is rare and has only been reported following long-term exposure or at high concentrations [27]. The absence of notable accumulation or

questionable effects on brain tissue in this study and our previous studies [33, 34] that the administration protocols employed for Mangradex should not elicit neurological (manganism) effects. However, additional long term chronic exposure studies are necessarily to obtain a complete picture of the neurological effects Mangradex. The animals were also examined for signs of NSF or other debilitating conditions such as weight loss, morbidity or mortality. A noticeable hallmark feature of human NSF is skin thickening caused by increased dermal fibrosis in the presence of unregulated collagen density [25, 41, 42]. Consequently, skin thickness and collagen concentration in skin were measured in the animals given the repeat high dose (50 mg/kg) in 2 and 4 weeks groups. Using two distinct methods; caliper measurements and histomorphometry, we found no differences in the thickness of the skin of the experimental animals and the controls. Furthermore, digital image analysis of skin collagen densities showed no significant differences between the experimental and control animal groups. NSF is also known to cause fibrosis of the lungs, liver, muscles and heart. We also did not find any histopathological evidence of fibrosis in any of these organs. These results suggest that Mangradex at dosages 50 mg/kg does not, at least in the short or mid-term, cause immediate NSF after single or repeat injections. Despite these positive outcomes, we remain circumspect. One reason is the unavailability of a validated animal model to test the risk or study the mechanism of human NSF. The 5/6 nephrectomized rats have been used widely to investigate the propensity of Gd^{3+} -based clinical MRI CAs to induce NSF [35, 43, 44], and proposed as a model to understand the causal relationship [35, 45]. However, there is still no broad consensus on its suitability to predict the risk of NSF. In a preclinical study conducted by Sieber *et al.* in healthy rats with normal renal function administered multiple doses of 6 different commercially available gadolinium based contrast agents, NSF like lesions were found in animals treated with gadodiamide or gadoversetamide [40]. Whereas, Grant *et al.*, in naïve and 5/6 nephrectomized rats administered Gd^{3+} -based MRI CAs known to cause NSF, skin lesions, observed skin lesions without the presence of dermal fibrosis [35].

At 7T magnetic field, the r_1 relaxivity ($61 \text{ mM}^{-1}\text{s}^{-1}$) of the Mangradex is significantly higher than clinical MRI CAs ($< 6 \text{ mM}^{-1}\text{s}^{-1}$) [46]. This relaxivity value is lower than the values of Mangradex at lower fields (r_1 at 1.5 Tesla magnetic field = $92 \text{ mM}^{-1}\text{s}^{-1}$ [30, 31]). The drop is not surprising since various theoretical and experimental studies have clearly established that longitudinal relaxivity of MRI CAs decreases with increase in magnetic field [47] [48]. However, the value is in the range predicted by theoretical simulations for an “ideal” high field agent [47]. Our previous relaxometric studies indicate that key molecular parameters: hydration number (q) i.e the number water molecules that can simultaneously coordinate with the intercalated Mn^{2+} ions, the residence lifetime (τ_M) of the coordinated water molecules, and the rotational correlation time (τ_R) of Mangradex are modulated [31]. The values of these parameters are in the optimal range predicted by simulations to achieve improved relaxivities at higher fields [47].

The dosage of Mangradex (25 mg/kg) for the *in vivo* MRI studies was determined based on the results of the *in vitro* and *in vivo* (including the repeat dose) safety studies in normal rodents [30, 32, 33]. Our previous studies indicated that the no-observed adverse effect level (NOAEL) was 50 mg/kg, and thus, the potential therapeutic dose selected for *in vivo* MRI was less than half of the NOAEL. The clinical Gd^{3+} -based blood pool agent gadofosveset

trisodium was used as control. The paramagnetic ion (Gd^{3+} ions) concentration in a typical clinical dose of gadofosveset trisodium is 0.03 mmoles/kg. Thus, paramagnetic ion concentration (Mn^{2+} ions) of the injected Mangradex dose was 66 times lower than the paramagnetic ion concentration in a typical clinical dose. Numerous clinical studies have demonstrated the efficacy of gadofosveset trisodium for MRA examinations [49, 50]. Thus, due to its extended circulation times (its intravascular distribution half-life is 29 minutes, and mean elimination half-life is ~16.3 hours) [51] we chose gadofosveset trisodium as control. We noted vascular enhancement after the administration of gadofosveset trisodium as demonstrated by other studies [51]. However, at the 25 minute time point, the positive contrast enhancement obtained by Mangradex was significantly higher than that provided with gadofosveset trisodium at equi-molar concentration ($[Mn^{2+}] = [Gd^{3+}] = 455$ nmoles/kg). It is important to note that 25 minute time maybe sub-optimal for image acquisition after administration of gadofosveset trisodium since this time close to its blood half-life. Additionally, differences in pharmacokinetics of gadofosveset trisodium and Mangradex restrict us from making direct comparison between the two compounds based on our MRI results. Nevertheless, at the Mn^{2+} /kg dose used in this study, the estimated Mn^{2+} steady state blood concentration in rat (weight = 200 grams; total blood volume = 12.7 ml) after the first pass would be ~ 165 femtomoles /voxel ($75\ \mu m \times 75\ \mu m \times 75\ \mu m$ voxel). The low Mn^{2+} /kg dose (66X and 220X lower than clinical dose of gadofosveset trisodium (Gd^{3+} /kg dose = 0.03 mmol/kg [49]) and gadopentetate dimeglumine (Gd^{3+} /kg dose = 0.1 mmol/kg [52]), is also in the range of average daily dietary intake of manganese (1.8–2.3 mg or 33–42 micromoles per day) [53]. The decrease in signal intensity and contrast to noise ratio at the later time points can be due to slower elimination of Mangradex. The bright enhancement of bladder (Figure 3h) indicates renal excretion as a route of elimination. Our previous study also suggest biliary pathway as another route of clearance [33]. Other rodent studies with intravenously injected covalently or non-covalently functionalized graphene formulations have shown similar pharmacokinetics with prolonged recirculation, and biliary and renal modes of excretion.[54, 55]

Gadolinium complexes with carbon nanostructures such as fullerenes, nanotubes, and graphene, have been proposed as T_1 CAs for MRI [56–59]. Even though these MRI CAs show higher relaxivity compared to clinical MRI CAs, and preliminary efficacy of some of these complexes have been assessed in rodents [57, 60], their acute or sub-acute toxicity, MTD, and NOAEL levels still need to be established. Additionally, their nephrotoxic assessment at potential diagnostic dosages still needs to be completed. The above results open avenues for the translation of Mangradex for clinical use, specifically for renal imaging, and provide a foundation for preclinical studies to subsequently initiate first-in-human trials. Upon complete development, Mangradex could be employed as FDA-approved MRI CA specifically for non-invasive imaging and monitoring of renal abnormalities, thereby overcoming the significant limitation of currently available clinical CT and MRI CAs such as performing risk benefit analysis and follow up dialysis[22, 61]. Additionally, such an MRI CA could also be used for non-invasive imaging and monitoring of other organs in patients (typically diabetics or patients with high blood pressure or cardiovascular diseases) with renal failure. Further, it could be employed as an alternative off-label MRI CA to currently existing T_1 MRI CAs used for clinical diagnostic applications. Due to

Mangradex's high relaxivity and possibility to functionalize it with molecular and cellular targeting moieties it could also be suitable for preclinical molecular MRI of animal models to study the progression of kidney diseases and other pathologies. Further, since this new MRI CA could substantially improve disease detection by increasing sensitivity and diagnostic confidence, it could enable earlier treatment of disabling kidney diseases, conditions associated with renal failure and other diseases, pathologies or lesions in patients with renal failure making overall care less expensive yet more effective.

Conclusion

Mangradex doses up to 50 mg/kg doses are potentially safe for single and repeated intravenous administration in rodent models of chronic kidney disease. Short (2 weeks) and mid-term (4 weeks), single or sub-acute administration of Mangradex does not elicit adverse effects on vital organs in histopathology nor induce NSF-related indicators. Mangradex at low Mn^{2+} ion concentrations allows significant and sustained contrast enhancement of the kidney and renal vasculature. The results taken together suggest exciting possibilities towards the development of Mangradex for preclinical and clinical renal imaging.

Materials and Method

Animal preparation

Adult male and female Wistar Kyoto (WKY) rats (200 g) with 5/6 Nephrectomy were used in this study, following the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Stony Brook University. The nephrectomy procedures were similar to those described in literature ([35, 43]). Animals were anesthetized with 5% mixture of air: isoflurane in a 1:1 air - oxygen mixture. Sterilized Mangradex formulation at 5 and 50 mg/kg ($n = 24/\text{dose}$) were injected by tail vein. 12 animals at each dose (6 M/6 F) were injected once during the start of the study and observed for vital parameters, behavior and toxicity related adverse effects for 2 weeks ($n=8$) or for 4 weeks ($n=4$). Remaining 12 animals at each dose (6 M/6 F) were injected 5 times/week on consecutive days for 2 weeks ($n=8$) or for 4 weeks ($n=4$) and observed for vital parameters, behavior and toxicity related adverse effects. Sham (untreated) and control (injected with 55 mg/ml mannitol) animals were treated with the same dose regimen. At the end of 2 or 4 weeks, the animals were euthanized by inhalation using 100% CO_2 . All major organs (heart, lungs, liver, brain, spleen) including patches of the dorsal skin from each animal were harvested and prepared for histopathology analysis.

Histology and slide digitization

All major organs (heart, lungs, liver, brain, spleen) including patches of dorsal skin were fixed in 10% formalin for 24–48 hours, embedded in paraffin, sectioned onto glass slides and stained for both H&E for histopathology interpretation and Masson's Trichrome for collagen quantification using standard procedures. As described below, histomorphometry was utilized for skin thickness measurements. Exact placement of the dorsal skin samples during sectioning was crucial for accurate histomorphometric measurements. As such, paraffin blocks with the embedded skin samples were precisely oriented to allow sectioning

at 90° to the axis of the skin surface. Subsequently, all slides were imaged at 2.5X for subsequent histomorphometry and quantitative collagen analysis using digitized bright field microscope. For the collagen quantitative images, the exact microscope settings were maintained for all images.

Histomorphometry and collagen image analysis

Using Image J and the digitized images, both histomorphometric analysis of dorsal skin thickness and collagen quantification of histology sections were obtained. For histomorphometry, 10 digitized photomicrographs (10X; 1290 × 1018 pixels) of the H&E stained skin sample of each animal with 50 mg/kg multiple dose were utilized. For each photomicrograph, the dermis and epidermis were identified and 20 thickness measurements were taken using the line tool of Image J® (National Institute of Health, MD, USA). The average of these 20 measurements for each animal represented its unique skin thickness. Using quantitative image analysis, collagen content of dorsal skin samples and other major organs at 50 mg/kg multiple dose and control groups were measured. For this analysis, percentage of plaque type collagen content was determined by quantifying the area of the distinctive blue color of collagen fibers by Masson's Trichrome staining in skin tissue sections photomicrographs. These images were converted to 8-bit greyscale and segmented to separate background from noise using an upper threshold value of 115. These values ensured that less than 1 in 1000 of the pixels in the collagen free background would be visible. The % collagen area in the image was calculated as the number of pixels representing collagen divided by total number of pixels x 100.

Statistical Analysis

Statistical analysis was performed using xlstat statistical software (version 2013.4.03). The results were analyzed by One-way ANOVA single factor with $p < 0.05$ as the criteria of statistical significance.

In vitro and *In vivo* MRI

The MRI phantoms ($n = 2$) were prepared at 1, 5, 7.5, 10, 20 mg/ml concentration of Mangradex in distilled deionized (DDI) water. The manganese concentrations in Mangradex solutions were measured by Inductive coupled plasma *mass spectrometry* (ICP-MS) (Model). The plot of relaxation rate ($1/T_1$ in s^{-1} , y-axis) vs. concentration (mM of Mn^{2+} , x-axis), was fit to a linear least-square regression line. The slope of this line provided the relaxivity r_1 value of the Mangradex.

A total of 4 animals were used for the *in vivo* MRI studies; 2 animals were administered with Mangradex and 2 with gadofosveset trisodium. *In vivo* T_1 -weighted MRI was performed using a 7 T Bruker Biospec 7.0T/20-cm USR horizontal magnet (Bruker, Billerica, Massachusetts). 5/6 Nephrex WKY rats were positioned in 72 mm quadrature volume coil (Bruker BioSpin Corporation, Billerica MA.) and maintained in anesthesia at 1.5–2.5% isoflurane. Temperature and respiration rate (RR) of subject was monitored through the experiment; the respiration rate was maintained at 50 ± 5 breaths per minute. Mangradex formulation at 25 mg/kg (60 nmoles/kg) was injected intravenously via *tail vein infusion catheter*. Gadofosveset trisodium at the same molar concentration (60 nmoles/kg)

was used as control. T_1 relaxation time was measured using RAREvtr (variable TR) pulse sequence with 5 TR values 293, 635, 1114, 1926, 6000 milliseconds. 5 axial slices of 1 mm thickness with 256×256 in plane resolution were collected. T_1 measurements were computed using Paravision 5.1 software by fitting absolute signal at particular TR to inversion recovery equation. The T_1 value scans were then segmented using ITK snap (www.itksnap.org). T_1 value for each region was computed by calculating average T_1 value for all the voxels in that region of interest (ROI).

Quantitative analysis

Using T_1 weighted scan with TE =12.5 mSec TR = 635 mSec, and by taking the signal (S_k) to be the mean pixel intensity value in a region of interest (kidney), and the noise to be the standard deviation (σ) in pixel intensity of ROI in background air (free of ghosting artifacts), signal to noise ratio (SNR) was calculated using the following equation.

$$\text{SNR} = \frac{S_k}{\sigma}$$

By taking muscle tissue (internal/external oblique) as outside tissue signal (S_t), contrast to noise ratio (CNR) was calculated as

$$\text{CNR} = \frac{S_k - S_t}{\sigma}$$

The T_1 , SNR and CNR values for each rodent is provided separately. No statistics was performed for these measures between the animals that received Mangradex or Gadofosveset trisodium.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Wallace H Coulter Foundation Translational Research Award, Fusion Award from the Stony Brook School of Medicine and the Office of the Vice President for Research, Technology Accelerator Fund from the Research Foundation for SUNY, and the National Institute of Health (1R41DK100205-01A1 and 2R44DK100205-02).

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Figure 1.

Representative high (400X) magnification photomicrographs illustrating histopathology of major organs from 5/6 Nephrex animals for sham, and treated with single and multiple dose of 50 mg/kg of GNP-Dex. **Lungs** a) sham - without diagnostic abnormality, b) single dose - pulmonary parenchyma with aggregates of granular brown pigment (arrow) within alveolar macrophages and microvascular spaces, c) multiple doses - focal aggregates (arrow) within alveolar vascular space. **Liver** d) sham - presence of micro abscess (arrow) in liver, potentially related to reactive/inflammatory changes resulting from the 5/6 Nephrex surgery (micro abscesses were not detected in other sham or treated animals), e) single dose - without diagnostic abnormality, f) multiple doses - pigments (arrows) in Kupffer cells suggestive of the presence of graphene nanoparticles. **Kidney** - g) sham, h) single dose – showing acute inflammation (by presence of neutrophils) related to 5/6 Nephrex surgery, and i) multiple dose -showing acute inflammation related to 5/6 Nephrex surgery along with the presence of brown pigments (arrows).

A

B

C

Figure 2.

A) Thickness of dorsal skin in 5/6 Nephrex rats given multiple doses of 50 mg/kg for 2 weeks or 4 weeks by histomorphometric image analysis using Image J. Analysis shows no statistically significant differences in collagen concentration in skin among test and control animals (mean $\pm$ standard deviation for $n=20$; ($p < 0.05$)). **B)** Histology of 5/6 Nephrex rat skin stained with Masson's Trichrome (MT) staining. This stains collagen fibers brilliant blue and is commonly used to determine collagen fiber concentration, tissue fibrosis or abnormal deposition of collagen fibers. a) female rat given multiple doses of 50 mg/kg of Mangradex; b) male rat given multiple doses of 50 mg/kg of Mangradex, c) control rat given multiple doses of mannitol. **C)** Quantification of collagen in 5/6 Nephrex rats given multiple doses of 50 mg/kg for 2 weeks or 4 weeks using Masson's trichrome staining. Analysis shows no statistically significant differences in collagen concentration in skin among test and control animals (mean $\pm$ standard deviation for $n=20$; ($p < 0.05$)).

A

Concentrations Mangradex	1 mg/ml	5 mg/ml	7.5 mg/ml	10 mg/ml	20 mg/ml
Manganese	2 nM	10 nM	18 nM	25 nM	46 nM

B

C

<i>T_I</i> relaxation time calculated from the region of interest in Figure 3B						
	Ablavar			Mangradex		
Time point	<i>T_I</i> in (ms)	**Std Dev	% decrease in <i>T_I</i> time	<i>T_I</i> in (ms)	Std Dev	% decrease in <i>T_I</i> time
Pre	2803	1413	0	3250	179	0
25 minutes	2148	855	23	808	270	75
50 minutes	2516	1297	10	925	251	72
85 minutes	NA	NA	NA	998	416	69

** Higher Std. Dev. is due to noise introduced by respiration.

NA – Not available

D

MR images parameters calculated from the region of interest in Figure 3B. The raw data of the SNR and CNR values are for Table S4 supplementary information. The % increase were calculated using the equation $[(\text{Post})-(\text{Pre})/\text{Pre}] \times 100$.				
MRI Parameter	Pre	Post Ablavar increase	Pre	Post Mangradex increase
SNR (kidney)	8.0	10.9 (25 min.); 36% 10.0 (50 min.); 25%	7.8	23.1 (25 min.); 196% 17.8 (50 min.); 128 % 15.6 (85 min); 100%
CNR (kidney)	0.7	1.0 (25 min.); 42% 0.7 (50 min.) 0%	1.8	17.5 (25 min.); 872% 7.1 (50 min.); 294% 8.4 (85 min.); 366%
SNR (Angio-renal artery)	6.4	7.1 (25 min.); 11%	6.3	64.5 (25 min.); 923%

Figure 3.

A) At 7T magnetic field T_1 weighted MRI phantom images of Mangradex at concentrations 1, 5, 7.5, 10 and 20 mg/ml. Also shown for comparison are corresponding manganese concentrations. B) *In vivo* MRI (a–f) Representative T_1 weighted MR images of pelvic region (coronal view) that show the subtotally nephrectomized (SNx) rat kidney (red arrow) before (a) and 25 minutes after (b) injection of Ablavar (control); before (c) and 25 (d), 50 (e) and 85 (f) minutes after injection of Mangradex. (g,h) Representative MR angiograms (head of the rat is on the right side) of pelvic region that show the renal artery (red arrows) before (g) and 25 (h) minutes after injection of Mangradex. C) Table showing *in vivo* T_1 relaxation time and % decrease in the region of interest before and 25, 50 and 85 minutes after injection of Mangradex at 25 mg/kg (60 nmoles/kg of manganese) and Ablavar (60 nmoles/kg of gadolinium). D) A table showing % increase in T_1 relaxation time post injection of Ablavar and Mangradex.

Mangradex dose regimen in nephrectomized rats. Each animal received IV injections of Mangradex either once on the first day of the study or five times per week (Monday to Friday) for two weeks.

Table 1

Group	Dose	Total animals single injection			Total animals multiple injection		
		M		F	M		F
		2 wk	4 wk	2 wk	2 wk	4 wk	4 wk
Mangradex	5 mg/kg 50 mg/Kg	4 4	2 2	4 4	2 4	4 2	2 4
Control Sham	Mannitol	2 2	- -	2 2	2 2	- -	2 2