Do ASARM peptides play a role in Nephrogenic Systemic Fibrosis?

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Abstract

Nephrogenic systemic fibrosis (NSF) is a devastating condition associated with gadolinium (Gd\(^{3+}\)) based contrast agents (GBCAs) in patients with kidney disease. The release of toxic Gd\(^{3+}\) from GBCAs likely plays a major role in NSF pathophysiology. The cause and etiology of Gd\(^{3+}\) release from GBCAs is unknown. Increased Acidic Serine Aspartate Rich MEPE Associated peptides (ASARM peptides), induce bone mineralization abnormalities and contribute to renal phosphate-handling defects in inherited hypophosphatemic rickets and tumor-induced osteomalacia. The proteolytic cleavage of related bone matrix proteins with ASARM motifs results in release of ASARM peptide into bone and circulation. ASARM peptides are acidic, reactive, phosphorylated inhibitors of mineralization that bind Ca\(^{2+}\) and hydroxyapatite. Since the ionic radius of Gd\(^{3+}\) is close to that of Ca\(^{2+}\), we hypothesized that ASARM peptides increase risk for NSF by inducing release of Gd\(^{3+}\) from GBCAs. Here we show: 1) ASARM peptides bind and induce release of Gd\(^{3+}\) from GBCAs \textbf{in vitro} and \textbf{in vivo}; 2) a bioengineered peptide (SPR4) stabilizes the Gd\(^{3+}\)-GBCA complex by specifically binding to ASARM peptide \textbf{in vitro} and \textbf{in vivo}; and 3) SPR4 peptide infusion prevents GBCA induced NSF like pathology in a murine model with increased ASARM peptide (Hyp mouse). We conclude ASARM peptides may play a role in NSF, and SPR4 peptide is a candidate adjuvant for preventing or reducing risk of disease.
Introduction

Magnetic resonance imaging (MRI) is an invaluable imaging tool that can be markedly enhanced in some cases with use of gadolinium-based contrast agents (GBCAs). Nephrogenic systemic fibrosis (NSF) is a devastating disease described in patients with diminished renal function, and is associated with GBCA use. As a result, contrast-enhanced MRI has largely been avoided in patients with kidney disease (9, 11). Free gadolinium ($\text{Gd}^{3+}$) is present in tissues from patients with NSF and thought to be directly responsible for pathophysiology of NSF (9). Factor(s) that lead to release of $\text{Gd}^{3+}$ remain unknown although transmetallation has been proposed (8, 10).

Calcium and hydroxyapatite bind with high affinity to the bone-matrix peptide ASARM peptide. A detailed review describes the role of ASARM peptides in bone renal diseases (13). Importantly, ASARM-peptides are physiological substrates for PHEX (Zn metalloendopeptidase), potent inhibitors of mineralization and chiefly responsible along with renal phosphate wasting for the bone and teeth mineralization defects in inherited hypophosphatemic rickets diseases (x-linked and autosomal) (1, 2, 6, 12, 14-18). In summary, proteolytic cleavage releases ASARM peptides from MEPE and SIBLING proteins of the bone extracellular matrix. The ASARM motif when released as a peptide (ASARM peptide; 2.3 kDa), is highly reactive, acidic, phosphorylated and protease-resistant. We designed a small PHEX-related peptide (SPR4 peptide, 4.2 kDa) that binds with high affinity and specificity to ASARM peptides and motifs in vivo and in vitro (13, 16, 18). Ionized calcium ($\text{Ca}^{2+}$) and $\text{Gd}^{3+}$ share many biophysical properties. We predicted that ASARM peptide would destabilize GBCA and cause $\text{Gd}^{3+}$ release. To determine whether ASARM peptide is involved in early events in NSF we conducted the following experiments using SPR4 peptide as a control.

Methods:

Animals and Diets

Male (C57BL/6) mice (5 week) were housed at Kansas University Medical Center (KUMC) Department of Laboratory Animal Resources. The policies and procedures of the animal laboratory are in accordance with those detailed in the Guide for the Care and Use of Laboratory Animals published by the US Department of Health and Human Services. Male wild-type (WT) or mutant X-linked hypophosphatemic rickets mice (Hyp) were used for the study (N=6) and maintained on a 1% phosphorus and 2.4IU/g Vitamin-D3 diet (Harlan Teklad Rodent Diet 8604, Indianapolis, IN) (18). As reported previously Hyp mice had major increases in circulating ASARM peptide compared to WT (6, 13, 17, 18).
Osmotic infusion of GBCA and SPR4

Micro-osmotic pumps (Model 1003D; Durect Corporation) containing either: 1) vehicle (44 mM Tris pH7.4/132 mM NaCl/19.6 μM ZnCl₂; 2) gadobenate 32 nmoles/hr/kg; or 3) SPR4 peptide 32 nmoles/hr/kg were subcutaneously implanted and left for 3 days (total infusion over 3 days 2.2 umoles/kg and N=6 mice). Serum, urine, femurs, kidney and skin were collected for analysis as described previously (16, 18). SPR4 peptide was synthesized using standard techniques by Polypeptide Laboratories (San Diego, CA) as reported previously (12, 16, 18). Peptide purity was greater than 80% via HPLC and mass spectrometry. SPR4 peptide was dissolved as follows: 100 μL/1 mg of peptide of 25 mM acetic acid was first added to dissolve the peptide, then 900 μL of 50 mM Tris pH7.4/150 mM NaCl was added and after thorough mixing 20 μL of 1 mM ZnCl₂. Final buffer composition was 44 mM Tris pH7.4/132 mM NaCl/19.6 μM ZnCl₂. Zn is required for the Zn-binding motif of SPR4 peptide to structurally optimize SPR4 structure for binding to ASARM peptide (12, 16, 18).

Serum Analysis, RNA Isolation and Real Time PCR analysis

Blood samples were collected in serum-separator tubes and serum prepared as described previously (12, 16, 18). Gene expression was performed with specific primers using RNA extracted from femurs and whole kidneys (N=6 mice) as previously described (12, 16, 18).

High Pressure Liquid Chromatography (HPLC) & Inductively Coupled Plasma-Mass Spec (LC-ICP-MS):

ASARM and SPR4 peptides were synthesized as reported previously (12, 16, 18). A Jupiter-300TM 4μ proteo 90 A C18 reverse phase HPLC column (150 X 4.6) (Phenomenex) with a Bio-Rad HPLC/FPLC system (BioLogic DuoFlow) was used to resolve peptides and GBCAs. Measurements of free and bound Gd³⁺ were undertaken using LC-ICP-MS (4, 8); results shown graphically in Figure 1A. The compounds were resolved using an Agilent 7500e ICPMS detector (Collision Cell 2% He) linked to an Agilent HPLC. A Shodex-Ohpak HPLC column (6 μm CB802.5 HQ 80Å 8 X 300 mm) was used at 40ºC. The percentage change in free to bound Gd³⁺ in Omniscan samples treated with ASARM peptide, SPR4 peptide or both was measured by LC-ICMS and is discussed in the text.

Nuclear magnetic Resonance Studies (NMR); ¹⁵N/¹H

All peptides (including ¹⁵N isotopically labeled for NMR) were synthesized by Peptide Group (San Diego, CA as previously described (12, 16, 18). Confirmation of SPR4 binding to peptides was obtained using 2-dimensional ¹H/¹⁵N NMR (12).
Microcomputed tomography (µCT) for Bone and Soft Tissue

Microcomputed tomography (µCT) using a Scanco µCT 40 system was carried out as described previously with N=6 mice (5, 16). Mice bones, kidneys and skin samples (fixed and ethanol dehydrated) were scanned with a high-resolution µCT (µCT40; Scanco Medical, Southeastern, PA) as previously described (5, 16).

Magnetic resonance imaging (MRI): kidney.

A 9.4 Tesla 31cm horizontal bore Varian system was used for all MRI measurements as described previously (12). A customized RF probe (two-turn solenoid coil, diameter = 7 mm) was used to increase the filling factor, thereby increasing the signal-to-noise ratio. Spin-echo pulse sequence was used to acquire T1-weighted MR images (FOV = 2 cm, resolution = 153 x 153 x 400 µm³, TE/TR = 4 / 140 ms).

Statistical analyses

Statistical analyses were performed using PRISM5 (GraphPad Software, La Jolla, CA) as described previously (16, 18).

Results

ASARM peptide induces release of Gd³⁺ from GBCA and this is prevented by SPR4 peptide

HPLC linked to inductively coupled plasma mass spectrometry (LC-ICP-MS) was used to measure free and bound gadolinium in physiologically buffered aqueous solution containing mixtures of GBCA, ASARM peptides and SPR4 peptides (4, 8). Figure 1A shows ASARM peptide-induced release of Gd³⁺ from gadodiamide \textit{in vitro}. Addition of excess SPR4 peptide prevented Gd³⁺ release. This data confirms ASARM peptides bind gadodiamide, induces de-sequestration of Gd³⁺, an effect prevented by SPR4 peptide. We then used HPLC to resolve both molecules and complexes (Figure 1B) after mixing equimolar concentrations of gadodiamide and ASARM peptide. An ASARM-gadodiamide complex peak appeared that eluted later than the free ASARM peptide and gadodiamide peaks. This finding provided additional evidence that ASARM peptide binds to gadodiamide. We then used NMR to study this further (Figure 1C and D). Our previous published studies using 2-dimensional $^1$H/$^{15}$N nuclear magnetic resonance (2D-NMR) and surface plasmon resonance (SPR) showed ASARM peptides bind to PHEX and SPR4 \textit{in vitro and in vivo} (12, 13, 16, 18). Figure 1C and 1D show $^1$H-NMR spectra and $^{15}$N-HSQC edited $^1$H-NMR spectra of the ASARM peptide and SPR4 peptide binding complex and the effects of gadodiamide on T2 relaxation. SPR4 and ASARM peptides are of similar molecular size making Gd³⁺-induced T2 effects directly comparable. In the presence of gadodiamide, the $^1$H-spectrum with 5.8 fold molar excess ASARM peptide induced major spectral T2-relaxation (Figure 1C). Specifically,
broadening and quenching of spectral peaks occurred at even at low concentrations of gadodiamide (Figure 1D). The $^1$H spectrum in Figure 1C represents ASARM peptide signal because of the vast excess of ASARM peptide relative to $^{15}$N-labeled SPR4 peptide (5.8 fold molar excess). The $^{15}$N-HSQC edited spectra with $^{15}$N-labeled SPR4 peptide confirmed this assertion (Figure 1D). The same interactions analyzed using $^{15}$N-HSQC edited spectra showed vastly reduced SPR4 line-broadening effects (Figure 1D). Notably, the $^{15}$N-HSQC edited spectra represent the $^{15}$N labeled $^{15}$N-SPR4 peptide and not unlabeled ASARM peptide. From this we infer the spectral T2 relaxation for the GBCA markedly affects ASARM peptide but has no effect on the $^{15}$N-labeled SPR4 peptide. Therefore the GBCA is closer to the ASARM peptide providing compelling evidence that ASARM peptide binds with gadodiamide but not $^{15}$N-SPR4 peptide.

**ASARM peptide associated NSF-like pathology is prevented by SPR4 peptide**

We next used a murine model of X-linked hypophosphatemic rickets (HYP) that has elevated ASARM peptides levels in circulation and bone (12, 16, 18). Since ASARM peptides are small (4.2 kDa), acidic, reactive, highly phosphorylated with a low pI we hypothesized the excess levels in Hyp mice should contribute to reduced GBCA stability and increase the risk of developing NSF-like pathology. To test this theory we infused gadobenate with and without SPR4 peptide at concentrations known to neutralize excess circulating ASARM peptides (12, 13, 16, 18). SPR4 peptide prevented gadobenate-induced hyperphosphatemia and hypercalcemia (Table 1A). The hyperphosphatemia and its prevention by SPR4 was confirmed by increased renal Na-dependent phosphate co-transporter (NPT2A and NPT2C) mRNA expression (Table 1B). SPR4 peptide treatment also prevented a gadobenate-induced drop in bone sclerostin expression (Table 1B). Of note, a recent study showed a link with serum phosphorus in renal patients with NSF (3). This case control study shows NSF patients with chronic kidney disease have significantly lower phosphorus levels compared with controls. Also, other investigators have shown serum phosphate is responsible for sensitizing rats to the profibrotic effects of gadodiamide (7). This is consistent with the known phosphaturic effect of ASARM peptides shown by us and others and supports the ASARM model (13). Thus, exposure to GBCA in renal patients with high ASARM peptide levels (relatively hypophosphatemic) is proposed to induce release of Gd$^{3+}$. A transient hyperphosphatemia due to released Gd$^{3+}$ likely occurs but as the disease progresses high ASARM peptide levels reestablish a relative hypophosphatemia. Fibrosis with organ failure then follows with classic NSF pathologies. The data in table 1 therefore show compelling evidence for a link with ASARM peptide bioactivity, GBCA effects and SPR4 in vivo. Further studies are required to confirm a direct link with NSF pathology.

We then tested the effects on bone by scanning resected femurs removed from Hyp mice using µCT (Figure 2A). We found significant decreased bone-volume/tissue-volume (BV/TV) after 3 days of infusion. Figure 2A illustrates these changes and the drop in volumetric mineral density in gadobenate-treated mice.
conclude that acute infusion of GBCA and high ASARM peptide levels in Hyp mice induced release of Gd$^{3+}$ that then displaced Ca$^{2+}$ and PO$_4$ from bone even after 3 days. SPR4 peptide co-treatment prevented the gadobenate-induced changes (Figure 2A). SPR4 peptide likely indirectly prevented the gadobenate-mediated altered mineral content by binding to ASARM peptides. Also, μCT scans of kidneys and dermal sections demonstrate metastatic calcification in gadobenate treated mice (Figure 2B & C). The micro-calcified nodules were absent in mice co-treated with SPR4 peptide. This suggests that co-treatment with SPR4 peptide prevents gadobenate-induced NSF-like pathology in Hyp mice.

**ASARM peptide destabilizes GBCA in vivo and this is blocked by SPR4 peptide**

To determine whether ASARM peptides destabilize GBCA *in vivo* we carried out the following experiment using Hyp mice: mice were 1) injected (intraperitoneal) with gadodiamide; or 2) pretreated with a bolus of SPR4 peptide (129 nmoles) and then treated 30 min later with gadodiamide. After 2 hours mice were sacrificed and kidneys removed and scanned by T$_1$-weighted MRI. The high intensity MRI signal reflects intact Gd$^{3+}$-gadodiamide complex and lower MRI signal reflects a breakdown of the complex, Fig. 3A. We saw a marked quenching of the gadodiamide signal in Hyp mice compared to WT. Hyp mice pretreated with SPR4 peptide showed a striking restoration of signal intensity (Figure 3A). This is consistent with binding of SPR4 peptide with the excess ASARM peptides with resulting increased stability of Gd$^{3+}$-gadodiamide complex. Although the precise biophysical mechanism requires validation, this experiment suggests SPR4 peptide binds to excess ASARM peptide thereby indirectly increases the stability of the Gd$^{3+}$-gadodiamide complex.

**Discussion**

Our study provides compelling evidence that ASARM peptides bind to GBCAs, induce release of Gd$^{3+}$ and SPR4 peptide prevents this *in vitro* and *in vivo*. Figure 3C provides a scheme that explains this model. Importantly, our study has limitations since the associated *in vivo* biological changes we described are insufficient to confirm NSF pathology and could be interesting phenomena unrelated to NSF. Also, our study did not investigate a representative range of GBCA’s with differing thermodynamic stabilities (Ktherm). Consequently, the implications of these new findings for NSF on pathology, etiology, prevention and treatment need further investigation. Specifically, further studies should address the following questions: 1) Is there a subset of patients with CKD-MBD who have increased circulating or tissue levels of ASARM peptides and are they at increased risk of developing NSF; 2) Are patients with inherited or tumor acquired bone–mineral disorders with increased ASARM peptide levels such as autosomal and X-linked hypophosphatemic rickets and tumor induced osteomalacia (13) at increased risk for NSF; 3) Since SPR4 peptide prevents release of free Gd$^{3+}$ and indirectly increases GBCA stability is this peptide an ideal adjuvant for reducing or preventing
NSF in susceptible patients; 4) Is the current ELISA test for ASARM peptide measurement in urine and sera a useful prescreen for patients at risk for NSF; and 5) Can we reduce the risk of NSF in MRI scanned patients by neutralizing ASARM peptides with SPR4 peptide followed by dialysis immediately after the scan?

Acknowledgements:

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Legends:

Figures

Figure 1:

SPR4 competitively inhibits binding of ASARM peptides to GBCA in vitro: A) ASARM induced Gd3+ release from Omniscan is prevented by SPR4 peptide. Percentage change in free to bound Gd3+ in Omniscan samples treated with ASARM peptide, SPR4 peptide or both were measured by High Pressure Liquid Chromatography linked to Inductively Coupled Plasma-Mass Spectrometry (HPLC-ICP-MS) in vitro. Specifically, a 3x fold molar excess of SPR4 peptide relative to ASARM peptide (2.9 molar and 0.96 molar respectively). Omniscan was added at 0.49 molar, see figure for combinations. We have shown previously the ratio of ASARM peptide to SPR4 peptide is optimal for binding in vitro and in vivo (12, 16, 18). The Y-axis represents the percentage ratio difference of free to bound gadolinium relative to vehicle. The total free and bound gadolinium for control (vehicle and Omniscan) and experimental conditions were not significantly different (17400 ppm (µg/g) SD ± 8508). For all experimental conditions including vehicle we detected a significant amount of free Gd³⁺. The ratios of “free/bound” gadolinium for gadodiamide treated with vehicle, ASARM peptide, SPR4 peptide and SPR4 + ASARM peptide were 0.58, 0.76, 0.59 and 0.60 respectively; B) ASARM peptide binds to gadodiamide: HPLC separation of peptides on a phenomenex C18 5µ Jupiter column. Traces A and D show buffer gradient profiles (buffer A; 0.1% TFA/H2O, buffer B; 100% acetonitrile/0.1% TFA). ASARM-gadodiamide complex resolves from free ASARM and gadodiamide; C) Our previous published studies using 2-dimensional ¹H/¹⁵N nuclear magnetic resonance (2D-NMR) and Surface Plasmon Resonance (SPR) showed ASARM peptides bind to PHEX and SPR4 in vitro and in vivo (12, 16, 18). The ¹H-NMR
spectra of the ASARM peptide and SPR4 peptide binding complex and the effects of gadodiamide (Omniscan®) on T2 relaxation are shown. A 5.8 fold molar excess of ASARM peptide (2.73 mM) to [15N-labelled]-SPR4 peptide (0.48 mM) was used as previously described (12). Broadening and quenching of spectral peaks as measured using 1H NMR even at low concentrations of Gd3+ contrast-agent “gadodiamide” occurred; and D) In contrast, 15N-HSQC edited spectra of the same sample-run shows vastly reduced [15N-labeled]-SPR4 peptide line broadening effects. For further discussion and explanation see text.

Figure 2:
SPR4 infusion reverses GBCA induced pathology in Hyp mice in vivo (N=6): A) High resolution µCT (6 µM) scans of femurs from Hyp mice infused with vehicle, gadobenate or gadobenate + SPR4 peptide. Note significant reduction in mineral density mg cm⁻³ in GBCA treated mice compared to vehicle and GBCA + SPR4 peptide treated mice. Tabulated BV/TV shown below the scheme; a=P<0.05 significant difference to HYP + Vehicle. b=P<0.05 significant difference to HYP + GBCA + SPR4. Index: 1. GBCA used was gadobenate (MultiHance®). 2. HA = hydroxypatite, 3. BV= Bone Volume, 4. TV= Tissue Volume; B) High resolution (6 μm) scans of kidney and dermal sections (mid dorsal region) removed from “gadobenate” and “gadobenate + SPR4 peptide” infused Hyp mice (osmotic pumps). For more detailed description see text. Note metastatic calcifications in gadobenate infused mice that are absent in mice infused with gadobenate + SPR4 peptide; and C) Mineral-content (mg HA cm⁻³) of whole kidneys measured using µCT at 6 µm resolution (N=6). Note that with mice treated with the GBCA MultiHance®, an increased mineral density occurs relative to HYP vehicle mice and mice co-infused with SPR4 peptide and GBCA (see also B). This indicates renal ectopic deposition of mineral that is likely bone-derived (see also A).

Figure 3:
ASARM induced Gd³⁺ release from Omniscan is prevented by SPR4 peptide and increases GBCA stability in renal MRI scans of Hyp mice ex vivo (N=6): A) Magnetic Resonance Images (ex-vivo) of representative kidneys resected 2 hours after intra-peritoneal injection with Omniscan (Gd-OMN) or Gd-OMN + SPR4 peptide as indicated in the scheme. Photo A shows a wild type mouse (WT) and photos B and C Hyp mice (Hyp mice overexpress ASARM peptides). Note in Hyp mice treated only with gadodiamide (photo B; Figure 3A) the MRI signal is quenched compared to WT mice (photo A; Figure 3A) and Hyp mice treated with Gd-OMN + SPR4 peptide (photo C; Figure 3A). This indicates preferential de-sequestration and release of Gd3+ ion from the gadodiamide vehicle in Hyp mice due to excess ASARM peptides (photo B; Figure 3B). This is prevented in Hyp mice pretreated with SPR4 peptide (compare photos B and C; Figure 3A). Thus, SPR4 peptide indirectly stabilizes Omniscan (gadodiamide) by binding to ASARM peptide. Note, the contrast images for WT mice, Hyp mice and Hyp+SPR4 treated mice were identical to Hyp mice treated with Gd-OMN. Thus the quenching of the
contrast signal by Gd-OMN is quite marked. See text for more detailed description; and B) Model illustrating
the proposed ASARM peptide induced release of toxic Gd$^{3+}$ from GBCA resulting in NSF. SPR4 peptide may
prevent this by binding to and neutralizing ASARM peptide. Note that free Gd$^{3+}$ as well as inducing
organ/tissue toxicity is also reported to displace Ca$^{2+}$ and PO$_4$ from bone and alter expressions of FGF23 and
PTH. This results in hyperphosphatemia, hypercalcemia and soft tissue calcification.

Tables

Table 1

SPR4 prevents GBCA induced hyperphosphatemia & hypercalcemia in Hyp mice (N=6): A) Serum chemistry
measurements for HYP male mice (N=6; 5-wks age) infused with vehicle, gadobenate or gadobenate + SPR4
peptide for 3 days. Superscripts a, b and c indicate significant difference (p<0.05) for vehicle (a), gadobenate
(b) and gadobenate + SPR4 peptide (c) respectively; and B) shows “fold” mRNA expression levels
(quantitative RT/PCR) for “vehicle versus gadobenate” and “vehicle versus gadobenate + SPR4 peptide”. Both
bone and kidney results are shown. Expression analyses were carried out as described previously and PCR
efficiencies calculated for each primer set and transferrin used as a housekeeping gene (16, 18). Asterisks (*)
indicate significant difference (p<0.05) calculated using Wilcoxon signed rank test (theoretical median = 1).
(NPT2a & c are renal Na dependent phosphate co-transporters). The pharmaceutical name for gadobenate is
MultiHance®. Note as stated in the title for both tables (A and B) ± values represent standard deviations (SD).

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A. \[\% \Delta \text{Ratio of (Free/Bound) Gd}^{3+}\]

<table>
<thead>
<tr>
<th></th>
<th>%\Delta (Free/Bound) Gd$^{3+}$</th>
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<tbody>
<tr>
<td>Omniscan</td>
<td>+</td>
</tr>
<tr>
<td>ASARM</td>
<td>+</td>
</tr>
<tr>
<td>SPR4</td>
<td>+</td>
</tr>
</tbody>
</table>

B.  
\[\text{Figure 1}\]

C. $^1$H-NMR spectra showing T2 relaxation effects of Gadodiamide on ASARM-peptide.

D. $^{15}$N-HSQC edited $^1$H spectra showing T2 relaxation effects of Gadodiamide.
Figure 2

A.

-0.69 to 4.90  

<table>
<thead>
<tr>
<th></th>
<th>Hyp Vehicle</th>
<th>Hyp + GBCA</th>
<th>Hyp + GBCA + SPR4</th>
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<tr>
<td>Mean</td>
<td>0.2028</td>
<td>0.1640 *sb</td>
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<tr>
<td>Std. Deviation</td>
<td>0.01258</td>
<td>0.006236</td>
<td>0.01233</td>
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<tr>
<td>Std. Error of Mean</td>
<td>0.005134</td>
<td>0.002546</td>
<td>0.005033</td>
</tr>
</tbody>
</table>

BV/TV [1] Cancellous Bone (N=6)

a = significantly different to Hyp Vehicle
b = significantly different to Hyp + GBCA + SPR4

B. μCT Scans

C. mg HA cm³

<table>
<thead>
<tr>
<th></th>
<th>HYP+GBCA</th>
<th>HYP+VE</th>
<th>HYP+SPR4+GBCA</th>
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<tr>
<td><strong>0.0003</strong>*</td>
<td>0.003***</td>
<td>0.0003 **</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3

A.

A. WT
B. HYP
C. HYP

Intense Signal

Gd-OMN + + +
SPR4 - - +

B.

(CKD-MBD)

ASARM

SPR4

SPR4 + ASARM

GBCA

Gd$^{3+}$

GBCA

Soft tissue calcification

PO$_4^{3-}$ Ca$_{2+}$

Toxicity
Nephrogenic Systemic Fibrosis
### Table 1

**A. Hyp mice serum chemistry (mg/dL & SD; N=6)**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Gadobenate</th>
<th>Gadobenate + SPR4</th>
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<tbody>
<tr>
<td>Serum PO4</td>
<td>4.36 ± 0.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.95 ± 0.48&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>4.12 ± 0.57&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Serum Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>9.73 ± 0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.64 ± 1.13&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>9.70 ± 0.35&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

Superscripts a, b and c indicate significant difference (p<0.05) for: vehicle (a), gadobenate (b) and gadobenate + SPR4 peptide (c)

**B. Hyp mice (mRNA Fold Expression & SD; N=6)**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Gene</th>
<th>Vehicle versus Gadobenate</th>
<th>Vehicle versus (Gadobenate + SPR4)</th>
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</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>NPT2A</td>
<td>1.84 ± 0.51*</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>NPT2C</td>
<td>1.92 ± 0.43*</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>1-α-Hydroxylase</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Bone</td>
<td>SOST (Sclerostin)</td>
<td>-2.86 ± 0.19*</td>
<td>NS</td>
</tr>
</tbody>
</table>

* = Significantly different p<0.05 vehicle; NS = Not significant.