Nephrogenic Systemic Fibrosis-Like Effects of Magnetic Resonance Imaging Contrast Agents in Rats with Adenine-Induced Renal Failure

Nathalie Fretellier,*† Nejma Bouzian,* Nadège Parmentier,* Patrick Bruneval,‡ § Gaëlle Jestin,* Cécile Factor,* Chantal Mandet,‡ Florence Daubiné,¶ France Massicot,‡ Olivier Laprévot,†|| Claire Hollenbeck,* Marc Port,* Jean-Marc Idée,* and Claire Corot*†

*Research Division, Guerbet, Roissy Charles de Gaulle Cedex, France; †Chemie Toxicologie Analytique et Cellulaire, Faculté des Sciences Pharmaceutiques et Biologiques, Université Paris Descartes, Sorbonne Paris Cité, Paris, France; ‡Department of Pathology, Hôpital Européen Georges Pompidou, Paris, France; §INSERM U970, Department of Pathology, Hôpital Européen Georges Pompidou, Paris, France; ¶Atlantic Bone Screen, Nantes, France; and ||Service de Toxicologie Biologique, Hôpital Lariboisière, Paris, France

†To whom correspondence should be addressed at Research Division, Guerbet, BP 57400, Roissy Charles de Gaulle 95943, France. Fax: +33 1 45 91 51 23. E-mail: nathalie.fretellier@guerbet-group.com.

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Nephrogenic systemic fibrosis (NSF) is a scleroderma-like disease associated with prior administration of certain gadolinium chelates (GCs). NSF occurs in patients with severe renal failure. The purpose of this study was to set up a rat model of GC-associated NSF to elucidate the mechanism of this devastating disease. Firstly, after characterization of the model, male Wistar rats received a 0.75% adenine-enriched diet for 8, 14, or 16 days to obtain various degrees of renal failure. Rats received five consecutive daily iv injections of saline or gadodiamide (2.5 mmol/kg/day). Secondly, the safety profile and in vivo propensity to dissociate of all categories of marketed GCs (gadoterate, gadobutrol, gadobenate, gadopentetate, and gadodiamide) were compared in rats receiving adenine-enriched diet for 16 days. Serial skin biopsies were performed for blinded histopathological study. Total Gd concentration in tissues was measured by Inductively Coupled Plasma Mass Spectrometry. Relaxometry was used to evaluate the presence of dissociated Gd in skin and bone. Gadodiamide-induced high mortality and skin lesions (dermal fibrosis, calcification, and inflammation) were related to adenine diet duration. No skin lesions were observed with other molecules. Unlike macrocyclic GCs, gadodiamide, gadopentetate, and gadobenate gradually increased the r1 relaxivity value, consistent with in vivo dissociation and release of soluble Gd (or, in the case of gadobenate, the consequence of protein binding). Gadodiamide-induced cutaneous and systemic toxicity depended on baseline renal function. We demonstrate in vivo dissociation of linear GCs, gadodiamide, and gadopentetate, whereas macrocyclic agents remained stable over the study period.

Key Words: nephrogenic systemic fibrosis; renal impairment; relaxometry; gadolinium; magnetic resonance imaging; contrast agents.

Nephrogenic systemic fibrosis (NSF) is a rare but serious systemic disease associated with prior administration of certain gadolinium chelates (GCs) used as magnetic resonance imaging contrast agents. This disease has been shown to occur in patients with severe or end-stage renal disease (ESRD) (Cowper et al., 2008). Skin involvement (thickening and hardening of the skin associated with plaques, papules, and nodules) is the first sign of the disease. Patients often report pain, associated with pruritus, paraesthesia, and burning (Cowper et al., 2008). Histologically, NSF is characterized by increased dermal cellularity, expression of CD34-positive cells with prominent collagen bundles, presence of spindle cells, septal involvement, and sometimes osseous metaplasia (Cowper et al., 2008). No single diagnostic test is available for NSF. However, a working definition including a consensus scoring system has recently been published (Girardi et al., 2011). This definition should serve as a working diagnostic standard and the basis for adjudicating borderline cases. Several authors have reported the involvement of many organ systems referring to the "systemic" aspect of the disease, which can eventually lead to death (Galan et al., 2006).

The recent recognition of a link between GCs and the development of NSF (Grobner, 2006) has revived the long-debated issue of the relevance of the thermodynamic stability of these agents (Morcos, 2008). In order to be used as contrast agents, the lanthanide element gadolinium must first be chelated with a polyaza-polycarboxylic ligand due to its intrinsic toxicity (Idée et al., 2008). Two structurally distinct categories of GCs are currently marketed, according to the structure of the ligand: (1) “macrocyclic” chelates (e.g., gadoterate or gadobutrol) in which Gd³⁺ ions are caged into a cavity of the ligand and (2) “linear”, open-chain chelates (e.g., gadopentetate, gadobenate, or gadodiamide). GC can also be either “nonionic” (where the number of carboxyl groups is reduced to 3, thereby neutralizing the 3 positive charges of Gd³⁺) or “ionic” (Port et al., 2008). GCs dramatically differ in terms of their thermodynamic
molecular stability. High kinetic stability (i.e., low dissociation rate over time) provided by the macrocyclic structure combined with high thermodynamic stability minimize the amount of free Gd\(^{3+}\) that can be gradually released from the parent chelate (Port et al., 2008). For lower stability GCs, excess free ligand is included in the pharmaceutical preparation to reduce possible release of Gd\(^{3+}\) during shelf life (Port et al., 2008). The vast majority of published cases of NSF were associated with the nonionic, linear GC gadodiamide and the ionic, less linear GC gadopentetate (Rodby, 2011).

One of the numerous hypotheses for the mechanism of NSF is that dissociation of less stable GC in at-risk patients may lead to gradual release of the free gadolinium ion Gd\(^{3+}\), which may subsequently activate trafficking of circulating CD34+ fibrocytes and/or directly activate resident fibroblasts (Idée et al., 2008). In vitro proliferative effects of gadolinium have been described (Li et al., 2010). An alternative hypothesis is a profibrotic effect of the GC molecule itself (Newton and Jiménez, 2009). Various rat models of NSF have been proposed to elucidate the mechanism of this disease (Siebert et al., 2009). The most common model consists of the administration of GCs in subtotally (five-sixth) nephrectomized rats (five-sixth SNx) (Fretellier et al., 2011a; Grant et al., 2009; Haylor et al., 2010; Pietsch et al., 2009). Overall, these studies concluded that fibrotic-like lesions can be induced in rats treated with gadodiamide. Gadodiamide has also been shown to increase Gd retention in rats, with a higher skin gadolinium concentration in rats receiving gadodiamide compared with animals receiving gadopentetate. The lowest values were observed in rats treated with macrocyclic GCs. However, this interesting and validated model presents certain disadvantages such as cost, limited reduction of glomerular filtration rate (GFR), and absence of hyperphosphatemia. Addition of adenine to the diet of rat has been reported to induce renal failure (Koeda et al., 1988). This model has several potential advantages over the five-sixth SNx model (high reproducibility, hyperphosphatemia, and early drop in GFR with potentially adjustable amplitude).

To our knowledge, no study has compared all marketed categories of GCs in a sensitized (renally impaired) in vivo model. This model would therefore appear to be of great interest. The aim of this study was to (1) characterize this new rat model of NSF, (2) investigate the role of baseline function in rats receiving the nonionic linear GC gadodiamide, and (3) compare the safety profile and in vivo propensity to dissociate of all categories of marketed GCs.

**MATERIALS AND METHODS**

**Animals**

A total of 84 male Wistar rats (Centre d’Elevage René Janvier, CERJ, Le Genest Saint Isle, France), aged 6 weeks, and weighing 285 ± 39 g at the time of the study were used. The animals were housed two per cage at an ambient temperature of 22°C ± 2°C, hygrometry of 45 ± 10%, with 12/12-h light/dark cycles. Rats were given ad libitum access to water and food (A04, SAFE, Augy, France) for 10 days of acclimatization before starting the experiments. At the start of the study (day 0), animals were given a diet containing 0.75% adenine (SAFE). Rats were housed individually from 1 week after the beginning of the study. All experimental procedures were performed in accordance with French regulations and in compliance with the European Economic Community Directive (2010/63/EU) on animal welfare.

**Characterization of the Adenine Rat Model**

Seven rats received 0.75% adenine-enriched diet for 4 weeks and were then fed a standard diet (A04) for another 3 weeks. Five rats (normal control group) were fed a normal diet. The animals were euthanized (exsanguination under isoflurane anesthesia) at the end of the experiment (i.e., on day 49 after the start of adenine diet). Blood (from sublingual vein) and 24-h urine samples were collected once or twice a week (days 0, 7, 14, 21, 28, 35, 42, and 49). Blood was used for routine hematological examinations using a MS4-5 automat (Melet-Schloessing, Osny, France). Plasma levels of total calcium, phosphorus, transferrin-bound iron, creatinine ( Vitrosoft II auto-analyzer), sodium, chloride, and potassium (direct potentiometry, Vitros-350 autoanalyzer, Ortho-Clinical Diagnostics Inc., Issy les Moulineaux, France) were also measured. Urine levels of creatinine were determined using a Vitrosoft-II analyzer. Creatinine clearance (ml/min/100 g bw) was calculated by a standard method.

At necropsy, kidneys and femurs were removed and fixed in 4% neutral buffered formalin. After dehydration, the femurs were embedded in glycol methacrylate resin, sectioned (4–6 µm thick), and stained with Von Kossa (mineralized bone structure) and Goldner’s Trichrome (osteoid, bone marrow, and bone structure) stains. After routine dehydration, the kidneys were embedded in paraffin, sectioned (5 µm thickness), and stained with hematoxylin-eosin-saffron (HES) for histological examination. Histopathological examinations were performed in a blinded fashion in accordance with the Society of Toxicologic Pathology guidelines (Crisman et al., 2004).

**Role of Baseline Renal Function on the Clinical and Biochemical Effects of Gadodiamide**

**Study design.** Based on the duration of the adenine diet, three levels of renal impairment were tested. Rats received 0.75% adenine-enriched diet for either 8 (study 1), 14 (study 2), or 16 (study 3) days (n = 4–11 rats per group). Rats were allocated to five consecutive daily iv injections (into the tail vein) of 0.5 (study 1) or 2.5 mmol/kg (studies 1, 2, and 3) of gadodiamide (Omniscan, GE Healthcare, Batch 11082864 in studies 1 and 3; Batch 10755384 in study 2) or saline from day 7 to 11 (studies 1 and 2) or from day 14 to 18 (study 3) (Fig. 1). Blood samples were collected for determination of hematological and biochemical parameters on day 0, the first day of gadodiamide treatment and at sacrifice. Skin biopsies were performed on the 1st and 8th (study 2) or saline from day 7 to 11 (studies 1 and 2) or from day 14 to 18 (study 3) (Fig. 1). Blood samples were collected for determination of hematological and biochemical parameters on day 0, the first day of gadodiamide treatment and at sacrifice. Skin biopsies were performed on the 1st and 8th (study 2) or saline treatment. Rats were euthanized 3 weeks after the last injection of gadodiamide or saline (corresponding to day 32 in studies 1 and 2; day 39 in study 3), and skin, the left kidney, and liver were removed.

Rats were checked for macroscopic skin changes before the first injection and then daily throughout the study. On the 8th or 10th day of treatment and at sacrifice, the animals’ backs were carefully shaved to facilitate visualization of potential lesions.

**Histopathological examinations.** Skin, kidney, and liver samples were fixed in 4% neutral buffered formalin. After dehydration, samples were embedded in paraffin, sectioned (5 µm thickness), and stained with HES stain for histological examination. Immunohistochemistry was performed on skin samples at sacrifice in studies 2 and 3. Transforming growth factor β (TGFβ), a fibrotic marker, was detected using anti-TGFβ, mouse antibody (AbD Serotec, Colmar, France) and ED-1-positive macrophages (AbD Serotec) were detected using anti-ED-1 mouse antibody and an antimouse biotinylated antibody (AbCys, Paris, France) staining. Histopathological examinations were performed, in blinded fashion, by a certified pathologist.

**Biochemistry and hematoloy.** Hematological parameters were determined with a MS4-5 automat. Plasma levels of total calcium, phosphorus, blood urea nitrogen (BUN), iron (Vitrosoft DT60II, Ortho-Clinical Diagnostics,
Inc.), creatinine, sodium, chloride, and potassium (Vitros fusion 5.1, Ortho-Clinical Diagnostics, Inc.) were also determined. These parameters were measured on the first day of gadodiamide treatment (day 7 in studies 1 and 2; day 14 in study 3) and at sacrifice. All assays were performed in duplicate. Blood samples were collected from the sublingual vein.

**Relaxometry and gadolinium measurement.** Total Gd levels in skin samples were measured by inductively coupled plasma mass spectrometry (ICP-MS) on ELAN DRC Plus (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA) as described elsewhere (Fretellier et al., 2011b). The limit of quantification was 0.64nmol/L.

The presence of dissociated Gd in skin biopsies was assessed by a relaxometry technique (in vivo studies), as described elsewhere (Fretellier et al., 2011b, 2012). Dilutions of 1:11 were performed in D₂O/H₂O (90/10) mix to mask samples with a Preceelys homogenizer. Before measurement, the suspension was mixed to avoid sedimentation, then checked to ensure a homogeneous appearance. Longitudinal relaxation times (T₁) were measured on Bruker Minispec (Bruker Optics, Ettlingen, Germany) at 60 MHz (i.e., 1.42 T) and at 37°C. When the 1/T₁ − 1/T₁diamagnetic value was less than 20% of 1/T₁diamagnetic in Minispec (Bruker Optics, Ettlingen, Germany) at 60 MHz (i.e., 1.42 T) and at 37°C. When the 1/T₁ − 1/T₁kuppers value was less than 20% of 1/T₁kuppers in the absence of Gd precipitation (i.e., because of a low total Gd concentration in the sample), it was considered that r₁ relaxivity could not be determined. Total Gd was then determined in samples by ICP-MS.

Relaxivities (in vivo r₁ value) were calculated according to the formula: r₁ = (1/T₁sample − 1/T₁kuppers) × [Gd]sample, with relaxation rate (1/T₁) expressed in s⁻¹, Gd concentration in mM, and relaxivity r₁ in mM⁻¹ s⁻¹.

Relaxometry studies (in vitro spiking studies) on biological matrices were performed by spiking with gadodiamide (range of 0, 0.005, 0.01, 0.02, 0.04, 0.05, 0.1, 0.5, and 1mM) on D₂O/H₂O mix or rat skin from nontreated rats.

The term “in vitro studies” refers to all experiments performed by spiking GC on tissue matrices (to obtain the reference range of r₁ relaxivities), and “in vivo studies” refers to all experiments performed on test animals. Based on unpublished in vitro relaxometry studies, the uncertainty of relaxivity r₁ measurements (including variability of relaxation time measurement and Gd measurement by ICP-MS) was set at r₁ ± 23%. The in vitro r₁ relaxivity range therefore represents the in vitro r₁ relaxivity value (obtained from spiking studies) ± uncertainty of 23%.

**Comparison of All Categories of GCs in Rats Receiving a 16-day Adenine Diet**

**Study design.** Rats received 0.75% adenine-enriched diet for 16 days (starting on day 0). Rats were allocated to daily intravenous injections of 2.5 mmol/kg of gadodiamide (Dotarem, batch 10GD54A, Guerbet, Villepinte, France), gadodiamide (Omniscan, batch 11151244, General Electrics Healthcare, Chalfont St Giles, UK), dimeglumine gadobenate (MultiHance, batch 50P260A, Bracco, Milan, Italy), gadobutrol (Gadovist, batch 04023D, Bayer Healthcare, Berlin, Germany), gadopentetate (Magnevist batch 92083K, Bayer Healthcare) for 5 consecutive days starting on day 14 after starting the adenine diet (n = 4 or 6 rats per group). Skin biopsies were performed on days 14, 23, and 39 after the first administration of adenine.

**Histopathological examinations.** Histopathological examinations (skin, kidney, and liver; HES stain) were performed as previously described, in blinded fashion, by a certified pathologist. CD34, ED-1, and TGFβ₁-positive cells were detected in skin samples.

**Biochemistry and hematology.** Blood samples from the sublingual vein were collected on days 0, 7, 14, 16, 21, 29, and 39 after starting the adenine diet for routine hematological and biochemical examinations. Plasma levels of interleukin-1-β (IL-1-β), monocyte chemotactic protein (MCP-1), TGFβ₁, and macrophage inflammatory protein-1 β (MIP-1β) were measured by ELISA (Bender MedSystems, Vienna, Austria) on days 16 and 39. All assays were performed in duplicate.

**Relaxometry and gadolinium measurement.** In skin, liver, kidney, and femur samples, total Gd was measured by ICP-MS using an Elan DRC Plus instrument (PerkinElmer Life and Analytical Sciences Inc.). The plasma-dissociated Gd⁺⁺ concentration was measured by high-pressure liquid chromatography linked to an ICP-MS system as described elsewhere (Fretellier et al., 2011b). The LOQ of the method was 0.5nmol/L.

The presence of dissociated Gd in skin biopsies, liver, and femoral epiphysis was assessed by relaxometry (in vivo studies). Relaxometry studies (in vitro spiking studies) on biological matrices were performed by spiking with GC (from commercial solutions) (range of 0, 0.005, 0.01, 0.02, 0.04, 0.05, 0.1, 0.5, and 1mM) on D₂O/H₂O mix or rat skin from nontreated rats.

The term “in vitro studies” refers to all experiments performed by spiking GC on tissue matrices (to obtain the reference range of r₁ relaxivities), and “in vivo studies” refers to all experiments performed on test animals. Based on unpublished in vitro relaxometry studies, the uncertainty of relaxivity r₁ measurements (including variability of relaxation time measurement and Gd measurement by ICP-MS) was set at r₁ ± 23%. The in vitro r₁ relaxivity range therefore represents the in vitro r₁ relaxivity value (obtained from spiking studies) ± uncertainty of 23%.

**Statistical Analyses**

Data are expressed as mean ± SD. SD values are given only for n ≥ 2 rats. The effects of test solutions on body weight and biochemical and hematological parameters were compared by ANOVA with repeated measures (effects of time and treatment). The 95% confidence intervals of in vivo r₁ relaxivity were calculated (mean ± 1.96 SD) and compared with in vitro relaxivity r₁ values ± 23% (in vitro r₁ range) (Fretellier et al., 2011b). When the confidence interval for mean on the in vivo relaxivity r₁ was included in the in vitro relaxivity r₁ range, the in vivo Gd state was considered equivalent to the in vitro Gd state (chelated, dissociated, or precipitated), according to the respective relaxivity r₁ value (Fretellier et al., 2011b), or were otherwise considered to be different. Assuming a normal distribution of values, Gd concentration measurements were tested globally with ANOVA. In the case of a significant difference, pairwise comparisons were performed using a Bonferroni’s test. Statistical analyses were carried out using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Differences were considered significant for p < 0.05.
RESULTS

Characterization of the Adenine Rat Model

Clinical findings, weight, biochemistry, and hematology. A decrease in body weight was observed in the group receiving adenine diet compared with the control group over the adenine diet period (202.7 ± 22.1 g vs. 432.3 ± 17.1 g, p < 0.05 on day 28). An abrupt decrease in creatinine clearance was observed in adenine-fed rats from the first week of the adenine period and creatinine clearance then remained stable until the end of the study (Fig. 2). A gradually increasing high mortality was observed in the adenine-fed group from the third week of adenine diet onwards (Fig. 2). An increase in plasma phosphorus (peak value on day 21: 4.1 ± 1.0 vs. 2.4 ± 0.2 mmol/l in controls, p < 0.01) associated with a decrease in plasma total calcium (peak value on day 28: 2.0 ± 0.1 mmol/l vs. 2.5 ± 0.2 mmol/l in controls, p < 0.05) was observed during the adenine period. An increase in urinary total proteins was observed in adenine-fed rats (peak value on day 7: 523 ± 144 mg/l vs. 12 ± 4 mg/l in controls, p < 0.01). At necropsy, kidneys of adenine-treated rats were pale and hypertrophied with a higher mean weight than that of kidneys from control rats (1.0 ± 0.05 g/100 g bw vs. 0.29 ± 0.01 g/100 g bw, p < 0.05).

Histopathology of kidneys and femurs. Adenine induced major tubular lesions with intratubular lithiasis-like precipitates and renal interstitial fibrosis with few inflammatory cells (HES) and no glomerular and vascular changes (Fig. 3A). A marked decrease in bone tissue mineralization in femoral epiphyses and/or diaphyseal bone-degrading cell infiltrate was observed in adenine-fed rats (Von Kossa’s stain) (Fig. 3B). These lesions were associated with a marked decrease in bone marrow density (Goldner’s Trichrome). Fibrous structures were observed in bone marrow of adenine-fed rats (Fig. 3C).

Role of Baseline Renal Function on the Clinical and Biochemical Effects of Gadodiamide

Clinical findings and biochemistry. On day 0 (i.e., before adenine diet), mean plasma creatinine was 20.9 ± 3.7 mmol/l. On the first day of gadodiamide administration, plasma creatinine was equivalent in studies 1 and 2, but was higher in study 3 (p < 0.001 vs. studies 1 and 2) (Table 1). At sacrifice, plasma creatinine was lower in study 1 than in studies 2 and 3 (p < 0.05). A decrease in plasma creatinine compared with the first day of gadodiamide was observed at sacrifice in studies 1 and 3, but remained stable in study 2.

On the first day of gadodiamide treatment, the severity of renal failure estimated by BUN was consistent with that estimated by plasma creatinine (Table 1). BUN at sacrifice was not a reliable parameter, as the duration of standard diet differed between the three studies, and this difference may have interfered with BUN levels.

Gadodiamide administration was associated with high mortality (10/11 rats) in rats in study 3 (i.e., rats with severe renal failure) (Table 2). In study 1 (low renal impairment), one rat receiving a dose of 2.5 mmol/kg was found dead on the second day of gadodiamide injection (corresponding to day 8) and in study 2, one rat had to be euthanized on day 16 for ethical reasons (loss of bodyweight, prostration, and loss of locomotor’s activity). Five of the six rats from study 2 (intermediate renal impairment) presented epidermal skin lesions (from day 14) and severe ulcerative and squamous skin eruptions were observed in two gadodiamide-treated rats in study 3 (i.e., severe renal failure) from day 22. No macroscopic skin lesions were observed in rats in study 1, corresponding to low-grade renal failure.

Histopathology. No histological abnormalities were observed on skin samples from treated rats in study 1 (Table 2). The skin was considered to be abnormal in four of the six gadodiamide-treated rats in study 2: gradual dermal fibrosis associated with hypercellularity and/or inflammation. Marked foci of early calcification with or without low-grade fibrosis and few inflammatory cells were observed in 5 of the 11 gadodiamide-treated rats in study 3. ED-1 and TGFβ1-positive cells were also observed in rats included in study 2 and in the surviving rat included in study 3 (Fig. 4). Blinded analysis of the kidneys (and livers) at sacrifice did not reveal any treatment-related abnormality in gadodiamide-treated rats. Kidney histopathological data were therefore pooled according to the study (“low,” “medium,” and “high” renal failure) (Table 1).

Major tubular lesions including marked lithiasis, inflammation, and interstitial fibrosis were observed in the kidneys of rats from studies 2 and 3, whereas lithiasis was rarely observed in study 1 animals (foci of tubular lesions associated with inflammation and fibrosis were sometimes observed) (Table 1).

Total gadolinium measurement and relaxometry studies. In all studies, a gradual increase in in vitro R1 relaxivity value, exceeding the in vitro R1 range, was observed in skin samples of gadodiamide-treated rats (Fig. 5).
FIG. 3. Histological findings in kidneys (A) and femurs (B and C) of normal or renally impaired rats. HES stain of kidneys (A), Von Kossa stain (black)/Ponceau-Fuchsin (pink) (B), and Masson’s Trichrome stain (C) of femurs were performed 49 days after starting adenine diet. In kidneys (A), interstitial fibrosis associated with a few inflammatory cells was observed in rats fed with adenine diet. Major tubular lesions, with intratubular lithiasis-like precipitate (black arrow) associated with granuloma, normal glomeruli, and vessels were also observed in this group. In femurs (B), cell infiltrate in the diaphysis (black arrow) degrading bone surface, and decreased trabeculae were observed in adenine-fed rats. The bone marrow (brown/red) appears less dense compared with physiological bone and fibrous areas (red arrow) were observed in the trabecular bone and/or diaphyseal bone (C).
TABLE 1
Plasma Creatinine and BUN Levels Measured on the First Day of 2.5 mmol/kg of Gadodiamide Treatment and/or at Sacrifice, and Histopathology of Kidneys at Sacrifice (HES Stain) in Rats With Low-Grade, Intermediate, or Severe Renal Failure (8, 14, or 16 Days of Adenine-Enriched Diet)

<table>
<thead>
<tr>
<th>Study</th>
<th>Degree of renal failure</th>
<th>Plasma [creatinine] (µmol/l)</th>
<th>Plasma [BUN] (mmol/l)</th>
<th>Histopathology of kidney (number of rats)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Low-grade</td>
<td>Absence</td>
<td>12/15/15</td>
<td>7/15/15</td>
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<td></td>
<td>First day of gadodiamide</td>
<td>Day 7: 48.2±3.2</td>
<td>Mild</td>
<td>1/15/15</td>
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<td></td>
<td>Sacrifice</td>
<td>Day 32: 21.5±0.7</td>
<td>Moderate</td>
<td>1/15/15</td>
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<tr>
<td></td>
<td></td>
<td>Day 7: 14.5±3.6</td>
<td>Severe</td>
<td>1/15/15</td>
</tr>
<tr>
<td>2</td>
<td>Intermediate</td>
<td>Absence</td>
<td>0/10/10</td>
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<td>Day 7: 59.3±7.2</td>
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<td>0/10/10</td>
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<td>Day 32: 66.6±10.2</td>
<td>Moderate</td>
<td>1/10/10</td>
<td>1/10/10</td>
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<td></td>
<td></td>
<td>Day 7: 14.9±6.6</td>
<td>Severe</td>
<td>9/10/10/10</td>
</tr>
<tr>
<td>3</td>
<td>Severe</td>
<td>Absence</td>
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<td></td>
<td>Day 14: 139.9±11.7</td>
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<td>0/5/5/5</td>
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<td></td>
<td>Day 39: 56.9±12.1</td>
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<td></td>
<td></td>
<td>Day 14: 25.6±4.2</td>
<td>Severe</td>
<td>3/5/5/5/5</td>
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<td>Statistical analysis</td>
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</table>

Note. “Mild lesions” corresponding to lesions < 25% of slide surface; “moderate” lesions corresponding to lesions between 25 and 50% of slide surface; “severe” lesions corresponding to lesions > 50% of slide surface.

TABLE 2
Clinical Signs and Skin Histopathology After Treatment With Gadodiamide in Rats With Low-Grade, Intermediate, or Severe Renal Failure (8, 14, or 16 Days of Adenine-Enriched Diet)

<table>
<thead>
<tr>
<th>Study</th>
<th>Gadodiamide dose</th>
<th>N (rats)</th>
<th>Mortality</th>
<th>Morbidity/Macrosopic skin lesions</th>
<th>Skin histopathology</th>
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</thead>
<tbody>
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<td>5 x 1.0 mmol/kg</td>
<td>6</td>
<td>No</td>
<td>No</td>
<td>No abnormalities</td>
</tr>
<tr>
<td></td>
<td>5 x 2.5 mmol/kg</td>
<td>6</td>
<td>1 found dead(day 8)</td>
<td>No</td>
<td>No abnormalities</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>4</td>
<td>0/4</td>
<td>No</td>
<td>No abnormalities</td>
</tr>
<tr>
<td>2 (intermediate renal failure)</td>
<td>5 x 2.5 mmol/kg</td>
<td>6</td>
<td>1 euthanized (Day 16)</td>
<td>4/5: skin lesions (2: ++ Euthanized rat: minor abdominal lesions)</td>
<td>Two rats: no lesions</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>4</td>
<td>0</td>
<td>No</td>
<td>No abnormalities</td>
</tr>
<tr>
<td>3 (severe renal failure)</td>
<td>5 x 2.5 mmol/kg</td>
<td>11</td>
<td>10</td>
<td>Prostration, piloerection, loss of body weight, and skin lesions (two rats)</td>
<td>Five rats: Low-grade fibrosis at the time of euthanasia, few inflammatory foci, and TGFβ+ immunostaining. Early calcification foci</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>5</td>
<td>0</td>
<td>No</td>
<td>No abnormalities</td>
</tr>
</tbody>
</table>

Total gadolinium concentration in skin at sacrifice (25 days after the first injection of gadodiamide) was higher in the single surviving rat from study 3 (216.6 nmol/g) than in rats included in study 2 (168.4 ± 105.2 nmol/g) and rats with low-grade renal failure (69.0 ± 43.1 nmol/g). Interestingly, in Study 2, the skin total Gd concentration measured in the only rat without skin lesions was around 7-fold lower than in the rats with skin lesions (31.3 vs. 202.7 ± 83.1 nmol/g). A significant positive linear correlation was observed between skin total Gd and plasma creatinine concentrations (Fig. 6).

Comparison of All Categories of GCs in Rats Receiving a 16-Day Adenine Diet

Clinical findings. Three of four gadodiamide-treated rats were either found dead or had to be euthanized for ethical reasons (loss of body weight > 20%, prostration, and marked loss of...
locomotor activity). Transient epidermal lesions were observed between day 17 and 18 on the abdomen of four of the six gadopentetate-treated rats. No macroscopic skin lesions were observed for other treatments except for wrinkled skin in the gadodiamide-treated survivor. No significant difference in body weight changes was observed between the test groups. One rat in the gadoterate and gadobenate-treated groups died, but death was unrelated to injection of the product (due to anesthesia).

Biochemistry and hematology. No significant differences were observed between groups in terms of plasma creatinine, sodium, potassium, chloride, phosphorus, calcium, and iron levels or hematological parameters (data not shown).

On day 16, plasma MCP-1 levels were significantly higher (13.2±1.8 pg/ml vs. 4.4±1.4 pg/ml in the control group; p < 0.001 vs. other groups) for gadodiamide-treated rats. However, no differences between treatments were observed on day 39. No significant changes in the plasma levels of the other

FIG. 4. Histological skin lesions in the dermis: HES staining to analyze cellular lesions, TGFβ1 staining, and ED-1 staining to detect macrophages were performed at the end of the studies (day 32 for studies 1 and 2; day 39 for study 3).

FIG. 5. In vivo relaxivity $r_1$ values (60 MHz, 37°C) in skin samples from rats fed an adenine diet for 8 (study 1), 14 (study 2), or 16 (study 3) days and treated with five consecutive daily injections of gadodiamide (2.5 mmol/kg). Gray bars correspond to in vitro $r_1$ range of gadodiamide in dorsal skin.

FIG. 6. Positive linear correlation between plasma creatinine and skin gadolinium concentration at sacrifice in rats receiving five consecutive daily injections of gadodiamide (2.5 mmol/kg) following adenine diet for either 8 days (study 1) or 14 days (study 2). Dotted lines indicate 95% confidence limits.
cytokines and the enzyme TIMP-1 were observed, regardless of the GC tested (data not shown).

**Histopathology.** Skin biopsies were studied on days 14, 23, and 39. No abnormalities were observed on skin biopsies from treated groups except for the gadodiamide-treated survivor rat, in which mild inflammation of the dermis without fibrosis was observed at day 39. Positive immunostaining for TGF-β1, TIMP-1, and ED-1 was observed in the dermis of the gadodiamide-treated survivor at sacrifice. No increase in the density of CD34- and prolyl-4-hydroxylase-positive cells was observed in the dermis, whatever the groups. At sacrifice, no abnormalities were observed in the lungs, kidney, or liver samples in any of the treatment groups.

**Gadolinium Measurement**

**Plasma-dissociated Gd**\(^{3+}\) **levels.** The plasma Gd\(^{3+}\) concentration at day 21 was below the limit of detection for the ionic macrocyclic GC gadoterate and the nonionic macrocyclic GC gadobutrol. At this time point, it was 79.3\(\mu\)M in the single gadodiamide-treated survivor. In one rat from the gadobenate and gadopentetate-treated groups with measurable Gd\(^{3+}\) levels, the dissociated Gd\(^{3+}\) concentrations were 0.44 and 3.0\(\mu\)M, respectively.

**Skin total gadolinium levels.** No significant differences in skin total Gd concentration on day 23 were observed between treatment groups, except for gadobenate-treated rats, in which the total Gd concentration was significantly lower than in the other groups (\(p < 0.001\)). A dramatic decrease in the skin total Gd concentration on day 39 (vs. day 23) was observed in the gadoterate, gadopentetate, gadobenate, and gadobutrol-treated groups. The total Gd concentration in dorsal skin on day 39 was higher in the gadodiamide-treated survivor (128.8 nmol/g) than in the other GC-treated groups (Fig. 7).

**Total gadolinium levels in the femur.** Total Gd concentrations in the femur on day 39 were higher in the gadopentetate-treated groups than in the gadoterate, gadobenate, and gadobutrol groups (\(p < 0.001\)) (Fig. 8). A high total Gd concentration was measured in the gadodiamide-treated survivor (207.8 nmol/g).

**Total gadolinium levels in kidneys and liver.** No significant differences in total Gd concentrations in the kidneys and liver were observed between treatment groups (data not shown).
Relaxometry Studies

The in vitro $r_1$ relaxivity values obtained in D$_2$O/H$_2$O, skin, liver, and femoral epiphysis matrices are shown in Table 3.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Gadoterate</th>
<th>Gadopentetate</th>
<th>Gabotenate</th>
<th>Gadobutrol</th>
<th>Gadodiamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>D$_2$O/H$_2$O (90/10 vol/vol)</td>
<td>3.3 ± 23%</td>
<td>3.8 ± 23%</td>
<td>4.8 ± 23%</td>
<td>3.8 ± 23%</td>
<td>3.7 ± 23%</td>
</tr>
<tr>
<td>Femoral epiphysis</td>
<td>3.8 ± 23%</td>
<td>2.9 ± 23%</td>
<td>3.7 ± 23%</td>
<td>2.9 ± 23%</td>
<td>2.9 ± 23%</td>
</tr>
<tr>
<td>Dorsal skin</td>
<td>3.0 ± 23%</td>
<td>3.6 ± 23%</td>
<td>4.8 ± 23%</td>
<td>3.5 ± 23%</td>
<td>3.5 ± 23%</td>
</tr>
<tr>
<td>Liver</td>
<td>4.1 ± 23%</td>
<td>4.4 ± 23%</td>
<td>5.7 ± 23%</td>
<td>4.3 ± 23%</td>
<td>4.0 ± 23%</td>
</tr>
</tbody>
</table>

Note. The uncertainty of relaxivity $r_1$ measurements was set at $r_1 ± 23%$.

FIG. 9. In vivo relaxivity $r_1$ values (60 MHz, 37°C) in skin samples of rats fed an adenine diet for 16 days and treated with each GC (5 × 2.5 mmol/kg) on day 23 or 39 (i.e., sacrifice) after starting adenine diet. Mean and individual values are given. Gray bars correspond to in vitro $r_1$ range of each GC in dorsal skin ($r_1 ± 23%$). **p < 0.01 between day 14 and day 39 in gadopentetate-treated group. LOQ, limit of quantification.

Relaxometry values in the skin. In in vivo studies, the skin $r_1$ relaxivity value increased from 4.0 ± 0.1mM$^{-1}$·s$^{-1}$ on day 14 to 10.6 ± 3.8mM$^{-1}$·s$^{-1}$ on day 39 in the gadopentetate group ($p < 0.01$), and from 5.1 ± 0.2mM$^{-1}$·s$^{-1}$ on day 14 to 11.1mM$^{-1}$·s$^{-1}$ (two rats had measurable $r_1$ values) on day 23 in the gadodiamide group ($p < 0.1$). The skin $r_1$ values on days 23 and 39 were higher in the gadodiamide group than the skin $r_1$ values on day 14, whereas no significant changes were observed in the other groups (Fig. 9).

The $r_1$ relaxivity values measured in skin samples of the gadopentetate group were higher than those of the in vitro range on days 23 and 39. The $r_1$ relaxivity values measured in skin samples of the gadoterate and gadobutrol groups were situated within the in vitro range, but no conclusions could be reached for the $r_1$ values measured in the gadobenate and gadodiamide groups due to the insufficient number of animals.

Relaxometry values in the femur. The in vivo $r_1$ relaxivity values in the femoral epiphysis were higher in the gadobenate group (15.9mM$^{-1}$·s$^{-1}$, two rats with measurable levels) and gadopentetate group (8.8 ± 1.8mM$^{-1}$·s$^{-1}$), than in the gadoterate group (no rats with calculable $r_1$ value, as the 1/T1 - 1/T$_{1\text{diamagnetic}}$ value less than 20% of 1/T1$_{\text{diamagnetic}}$ due to a low total Gd concentration). One rat in the gadobutrol-treated group had a measurable $r_1$ value (6.2mM$^{-1}$·s$^{-1}$). The $r_1$ relaxivity values measured in femoral epiphysis samples of the gadopentetate group were higher than those of the in vitro range (Fig. 10A).

Relaxometry values in the liver. No significant differences were observed between groups in terms of in vivo $r_1$ value in the liver. The in vivo $r_1$ relaxivity values measured in liver samples from the gadopentetate and gadobenate groups were higher than the respective in vitro ranges (Fig. 10B).
DISCUSSION

Clinically relevant preclinical models are crucial to elucidate the mechanism of this devastating disease. The five-sixth SNx rat model is frequently used to mimic human renal failure (Fretellier et al., 2011a; Grant et al., 2009; Haylor et al., 2010; Pietsch et al., 2009). This study used the adenine-enriched model of renal failure in rats, developed by Yokozawa et al. (1986). Basically, adenine is converted to AMP. However, in the presence of excess of adenine, an alternative pathway is activated, leading to 2,8-dihydroxyadenine urolithiasis and eventually to renal failure (Koeda et al., 1988; Okada et al., 1999). Modulation of the duration of adenine diet resulted in various degrees of renal failure, allowing maintenance of renal function for a defined period, in agreement with Okada et al. (1999). Hyperphosphatemia associated with osteodystrophy (osteitis fibrosa) was observed under our study conditions. Osteitis fibrosa is common in patients with chronic renal failure (Coladonato, 2005). The most severe renal impairment was observed when the adenine-containing diet was given for 4 weeks. However, progressively increasing mortality was also observed, making a 4-week protocol unsuitable for further studies.

We subsequently investigated the relationship between the amplitude of renal failure and the clinical effects of gadodiamide. Most reported cases of NSF are associated with this agent (Rodby, 2011). High mortality and macroscopic skin lesions were observed in two rats with severe renal impairment. Microscopic lesions (i.e., fibrosis and calcification) were observed in 5 of the 11 rats from this group. In rats with intermediate renal failure, the majority of rats developed ulcerative and squamous skin eruptions associated with fibrosis, hypercellularity, and inflammation. Overexpression of the profibrotic...
marker TGFβ1, the collagenase inhibitor TIMP-1, and an increased number of ED-1-positive macrophages in the skin were observed. These findings are in agreement with a previously published report (Fretellier et al., 2012) and with those observed in NSF patients (Jiménez et al., 2004; Kelly et al., 2008, 2010). No toxicity was observed in rats with low-grade renal impairment. Under these conditions, skin profibrotic lesions and systemic toxicity (including mortality) induced by gadodiamide were therefore inversely correlated with baseline renal function. These findings are clinically relevant because the majority of cases have occurred in patients with ESRD, and about 20% were reported in patients with acute kidney injury or stage 4 chronic kidney disease (CKD) (Abu-Alfa, 2011). There have been no definitive NSF cases reported in patients with stage 3 CKD to date, with the exception of one possible case (Abu-Alfa, 2011). Pietsch et al. (2009) reported that renal failure in rats induced prolonged GC circulation time, as observed in patients with severe renal impairment, which is consistent with the positive correlation between plasma creatinine levels and skin total Gd concentrations found in this study and elsewhere (Haylor et al., 2010). Pietsch et al. also reported skin lesions in rats receiving gadodiamide. Interestingly, lesions occurred in the animals with the highest skin Gd concentration. We observed similar findings: the only rat without skin lesions and intermediate renal failure had a lower skin Gd concentration than rats with macroscopic skin lesions. Our findings support the hypothesis that skin lesions may be dependent on the amount of Gd retained in the tissues. Interestingly, High et al. (2010) showed gradients of Gd deposition correlated to fibrosis and hypercellularity in the skin of two NSF patients.

The purpose of our third study was to compare all categories of GCs in rats with impaired renal function. High mortality was observed after administration of gadodiamide, consistent with the results of our first study. However, no skin lesions were observed in this group, in contrast with the literature (Grant et al., 2009; Fretellier et al., 2011a, 2012; Pietsch et al., 2009). This discrepancy can be explained by early death of the animals. Transient punctate skin lesions were found in four out of six gadopentetate-treated rats during the injection week. To our knowledge, this has never been previously reported. It is noteworthy that gadopentetate, a linear, ionic GC, like gadobenate, belongs to the high-risk category of GCs according to both the European Medicines Agency and the FDA (Rodby, 2011; Thomsen, 2011). However, no histopathological lesions were observed in this group. The first skin biopsy was performed 5 days after the last GC injection, which might explain the absence of histological lesions; epidermal lesions were transient and occurred earlier.

Skin Gd levels gradually declined in all groups except in the gadodiamide group, in which the survivor had a high skin total Gd concentration. High Gd levels have already been reported for this compound in the skin (Pietsch et al., 2009; Sieber et al., 2008) and in the femur (Sieber et al., 2008). Interestingly, a high total Gd concentration was also observed in the femur of gadopentetate-treated rats. In rats, 50% of gadobenate is excreted by the liver (Lorusso et al., 1999), unlike other GCs which are excreted exclusively by the kidneys (Idée et al., 2009). This may explain the relatively low total Gd concentrations found in tissues in gadobenate-treated rats.

To our knowledge, this is the first study to compare the in vivo stability of all categories of GCs. Relaxometry can be used to determine longitudinal relaxation rates of tissues. Gadolinium, present in paramagnetic GCs, accelerates relaxation times (Caravan et al., 1999). Relaxometry is therefore a useful tool to investigate the in vivo dissociated vs. chelated state of Gd following systemic administration of CG (Fretellier et al., 2011b). Our results suggest gradual in vivo dissociation, with release of soluble Gd" from gadodiamide and from the linear, ionic GC gadopentetate, whereas the more stable macrocyclic GCs gadoterate and gadobutrol remained stable throughout the study. In vivo dissociation of gadodiamide has already been reported in the literature (Fretellier et al., 2011b, 2012), but in vivo dissociation of gadopentetate has never been previously reported, to our knowledge. The ionic and linear GC, gadobenate, induced increased $r_1$ relaxivity values in the skin (day 23) and femur, which may suggest gradual in vivo dissociation of this compound, but which would also be consistent with protein binding (4%) which is known to increase the $r_1$ relaxivity value (Port et al., 2005).

We therefore report in vivo dissociation of linear GCs, gadopentetate and gadodiamide, with gradual release of soluble, dissociated, Gd". Macrocyclic GCs have higher kinetic stability than linear GCs (Port et al., 2008), as demonstrated directly (Frenzel et al., 2008) or indirectly (Laurent et al., 2001). Free Gd" has been reported to induce fibroblast proliferation (Li et al., 2010), but our data do not allow us to draw conclusions on a causal link between dissociated Gd" and skin lesions and mortality.

In summary, cutaneous and systemic toxicity induced by gadodiamide depends on baseline renal function in an adenine rat model of NSF. In vivo dissociation of gadodiamide with gradual release of soluble Gd" was observed, whereas macrocyclic agents remained stable over the study period. For the first time, gradual dissociation was suspected for the ionic, linear GC gadopentetate. Further studies are required to investigate the molecular potential effect of nonchelated Gd".

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