

# DENDRIMER-LINKED NITROXIDES: MRI CONTRAST AGENTS FOR CARTILAGE

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## INTRODUCTION

With the advent of new pharmaceuticals aimed at decreasing or inhibiting progression of the cartilage degradation in osteoarthritis, there is a tremendous need to establish accurate, minimally invasive techniques to assess the efficacy of these agents.[1-5] Furthermore, early detection of osteoarthritic changes in cartilage may facilitate the ability of these new treatments to retard disease progression. In the past, assessment of disease progression has been limited to comparison of plain radiographs by measurements of joint space narrowing.[6-8] Other methods, such as high-definition microfocal radiography using iodinated contrast arthrography, also provide valuable information as to the extent of the disease.[9-11] However, a technique that permits assessment of cartilage matrix compositional alterations as well as structural changes, *i.e.*, fissuring and fibrillation, would not only provide verification of the mechanisms of action of the investigational drugs, but would also permit monitoring of the disease process at the cellular level.

Magnetic resonance imaging (MRI) provides an excellent, minimally invasive tool for visualization of connective tissues within the joint. It has potential for use in early detection of disease progression in arthritic joints and evaluation of treatment efficacy of new pharmaceutical agents.[12] Various techniques to optimize cartilage evaluation by MRI have been investigated.[13-17] While cartilage detection is possible, optimal

visualization permitting determination of minor changes in the extracellular matrix requires the use of contrast agents.[15] The most promising of these agents is the negatively charged contrast agent, gadopentetate dimeglumine (Gd-DTPA)[18-21]. This imaging technique takes advantage of the fixed charge density within cartilage that arises from the highly negatively charged glycosaminoglycans in the cartilage extracellular matrix. Because it is negatively charged, Gd-DTPA is repulsed by the glycosaminoglycan (GAG) within the cartilage and its equilibrium distribution is inversely proportional to the GAG concentration in cartilage [19]. Thus, enhancement by Gd-DTPA is an indication of cartilage GAG loss. MR images can be analyzed to estimate the amount of GAG at various sites of the joint. These data can be compared to data from prior images to determine disease progression. However, post-processing of the images is required as the low total Gd-DTPA concentration within the cartilage limits detection of abnormalities on standard images.

Alternatively, a positively charged contrast agent should diffuse preferentially into cartilage due to its attraction to the GAG in the cartilage matrix. This would result in a high concentration of the agent within normal extracellular matrix and therefore, enhancement of normal cartilage. Nitroxides are stable radicals with paramagnetic attributes as a result of an odd number of free electrons. In MRI, paramagnetic molecules increase the signal intensity of tissues and shorten the relaxation rate. This is accomplished by interaction of the paramagnetic ions with the protons in water within the tissues. In the magnetic field, protons align either parallel or anti-parallel to the field, which causes a wobbling effect termed "precession." Because alignment parallel to the external field requires less energy, there is a net magnetization in the direction of the magnetic field, *i.e.*, longitudinal magnetization. When a radiofrequency (rf) pulse is imposed, energy is absorbed and the protons shift alignment. Upon removal of the rf pulse, protons slowly return to the lower energy state. The relaxation rate is a measurement of the time required for the protons to return to their lower energy state or equilibrium. T1 relaxation relates to the longitudinal magnetization while T2 relaxation relates to the transverse magnetization, the shift produced by application of the rf pulse. The relaxivity of a contrast agent is a measure of its ability to increase the relaxation states. The positively charged nitroxide 3-trimethylaminomethyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy iodide has been shown to diffuse into cartilage, but the T1 relaxivity values are low and not use-

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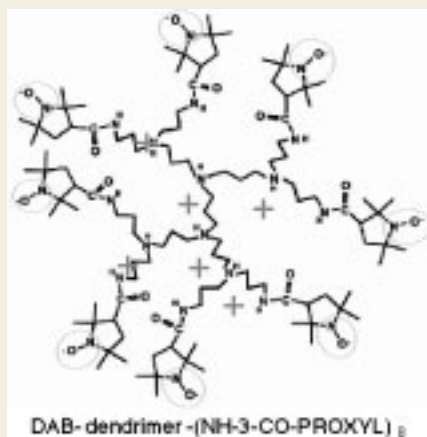
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**Figure 1:**  
Chemical structure of DAB Dendrimer with 8 nitroxides and 6 positive charges.

ful for imaging.[22] However, with an increase in the number of nitroxides there should be an increase in the relaxivity. One method of increasing the number of nitroxides per molecule is with the use of dendrimers, compounds that have been widely investigated as potential drug delivery systems.

Dendrimers are molecules synthesized by the addition of highly branched monomers to the core molecule. They have reactive sites on the terminal end of each branch so that each generation of added monomers doubles the number of reactive sites. In this case, nitroxides were linked to the termini of the dendrimer (Figure 1).

Our studies investigated the use of specifically designed dendrimer-linked nitroxides which are positively charged at physiological pH. These agents were evaluated *in vitro* to determine their affinity for cartilage and diffusivity through the cartilage matrix. Preliminary *in vivo* studies investigated the pharmacokinetics of several dendrimer-linked nitroxides

by MRI after intraarticular (IA) injection into the rabbit stifle (knee) joint, and provided histologic samples to determine their effects on cartilage and synovial tissue.

#### **EFFECT OF INCREASING THE NUMBER OF NITROXIDES ON RELAXIVITY**

Several generations of two different dendrimers, polypropyleneimine (DAB) and polyamidoamine (PAMAM), were synthesized with nitroxides linked to the terminal amino groups (Figure 1). Each generation increases the number of terminal amines to which nitroxides can be attached thereto (Table 1). Solutions of dendrimer-linked nitroxides were imaged using a standard quadrature head coil in a 1.5T magnet (Signa: General Electric Medical Systems). The T1 values for the solutions were measured with an inversion recovery spin echo imaging sequence with repetition times (TR) of 6000msec, inversion times of 50, 100 200, 400, 700 and 1400msec and an echo time (TE) of 15msec. The T2 values were obtained using the Carr-Purcell-Meiboom-Gill imaging sequence. The relaxivities, r1 and r2, were calculated and compared to those of Gd-DTPA.[23] By increasing the number of nitroxides attached to the dendrimer, the relaxivity values for the resultant dendrimer-linked nitroxides increased, some of which exceeded Gd-DTPA (Table 1). As expected, the two classes of dendrimer-linked nitroxides with the same number of nitroxides had the same relaxivities.

#### **EFFECT OF MOLECULAR WEIGHT ON CARTILAGE DIFFUSIVITY**

Cartilage slices 4 to 5 mm thick were dissected from 3 bovine patellae and placed either in DAB-16, DAB-32, PAMAM-32, Gd-DTPA or phosphate buffered saline (PBS, control group). Samples were allowed to equilibrate for 5 days at 4°C. MRI of the cartilage slices in the above solutions were obtained and the T1 relaxivities from the bath (each dendrimer-linked nitroxide solution) and from the cartilage were measured. The ratio of the T1 relaxivity measurements describes the affinity

Dendrimer	Number of nitroxides	Mean T1 relaxivity (s-1mM-1) ± SD	Mean T2 relaxivity (s-1mM-1) ± SD
DAB	4	0.78 ± 0.01	0.88 ± 0.02
DAB	8	1.57 ± 0.03	1.82 ± 0.002
DAB	16	2.93 ± 0.07	3.53 ± 0.2
DAB	32	5.17 ± 0.08	5.81 ± 0.02
PAMAM	16	2.93 ± 0.07	3.24 ± 0.01
PAMAM	32	5.00 ± 0.12	5.68 ± 0.2
Gd-DTPA	-	4.76 ± 0.2	5.42 ± 0.2

**Table 1:** T1 and T2 Relaxivities of Dendrimer-linked Nitroxides (Data from Winalski, *et al.*, 2002.)

Saline	Gd-DTPA	DAB-16	DAB-32	PAMAM-32
0:0	0.4:1	16:1	42:1	0.04:1

**Table 2:** Cartilage to Bath Ratios at 5 days

and diffusivity of the agent into cartilage. Table 2 demonstrates the increased affinity for cartilage with increasing number of nitroxides but also shows the effect of molecular weight on diffusivity. While DAB-32 had a ratio more than twice that of DAB-16, this difference was limited to the cartilage surface, as the higher molecular weight dendrimer-linked nitroxide had not diffused through the entire depth of the cartilage by 5 days. Indeed, the dendrimer-linked nitroxide with the highest molecular weight (12,285 daltons), PAMAM-32, was excluded from the cartilage slices whereas the DAB-32 with the same number of nitroxides but a molecular weight of 8,890 daltons had a surface cartilage to bath ratio of 42:1 and a deep zone to bath ratio of 4:1 at 5 days.

#### EFFECT OF CHARGE ON CARTILAGE AFFINITY

With an increase in the number of positive charges on the dendrimer-linked nitroxides, there was an increase in the ratio of cartilage to bath distribution. As expected, the negatively charged Gd-DTPA (-2) had a higher concentration in the bath than the cartilage while the DAB-16 with 14 positive charges had a ratio of 16:1 and the DAB-32 with 30 positive charges had a cartilage to bath ratio of 42:1.

#### IN VIVO KINETICS OF DENDRIMER-LINKED NITROXIDES

Small molecular weight solutes and particles have been shown to egress the joint space rapidly. For dendrimer-linked nitroxides to be clinically relevant, these compounds must remain in the joint space with adequate time to diffuse into the cartilage. To determine the pharmacokinetics of these compounds in synovial joint fluid, interval MRI was performed after intraarticular injections into the stifle joints of 14 male New Zealand white rabbits. For each animal the two joints were taped together and placed in a 12 cm quadrature radiofrequency (rf) coil with the knees in extension. T1-weighted images were acquired at 2 Tesla with a 10 cm field of view, 2 mm slice thickness, 256 x 256 matrix, TR of 500ms, TE of 14.4 ms and imaging time of 8.5 minutes. Images were obtained every 20 minutes and the signal intensities of the synovial fluid for each joint at each time point were measured in the same region of interest. From these data the half-life of the compounds in joint fluid was calculated.

Both the 16 and 32 nitroxide DAB dendrimers had longer half-lives than Gd-DTPA (2.8 hours vs 1.6 hours) while the PAMAM-32 had the longest half-life of the compounds tested

(3.2 hours). Cartilage enhancement results were similar to the *in vitro* studies in that both DAB 16 and 32 showed a bright band on the cartilage surface while PAMAM-32 had no cartilage enhancement (Fig.2) [23].

#### IN VIVO EFFECTS OF DENDRIMER-LINKED NITROXIDES ON SYNOVIUM AND CARTILAGE

An important consideration for any compound that is injected into the joint space is its potential toxicity to local tissue. To assess any adverse short-term effects on the rabbit joints injected with nitroxides, synovial tissue and cartilage samples were dissected from each joint approximately 24 hours post-injection. All samples were placed in 10% phosphate buffered formalin for paraffin embedment and subsequent microtome sectioning for histologic staining.

On dissection, there were no gross indications of inflammation or necrosis. By histologic examination, there was no difference between groups. The tissues exposed to the dendrimer-linked nitroxides were similar to tissue from joints injected with phosphate buffered saline.

#### DISCUSSIONS AND CONCLUSIONS

These preliminary studies have demonstrated the potential applicability of dendrimer-linked nitroxides as contrast agents for cartilage imaging (Figure 2). Of particular interest is the design of new dendrimer-linked nitroxides that demonstrate improved specificity for cartilage and short-term retention within the joint space. From our studies we have seen that molecular weight of these agents has a profound effect on cartilage affinity as the number of positive charges is increased but, at the same time, as the size of the molecule is increased, the diffusion rate decreases substantially. These results may be explained by the effective pore size of the cartilage matrix, approximately 3-6 nm, and the interaction of positively charged dendrimers with the GAG in the matrix.

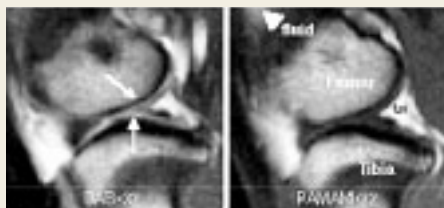
Considerations to be addressed are the *in vivo* half-life of these agents within cartilage and the effects of their bioreduction on the tissue over time. From an imaging standpoint, long-term partial sequestration of these agents within the cartilage might confound future imaging and comparison of two sessions would be compromised. Finally, an *in vivo* analysis of disease progression in an animal model of osteoarthritis will confirm the applicability of dendrimer-linked nitroxides as contrast agents for MRI of cartilage.

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**Figure 2:** T1-weighted images of rabbit stifle joints injected with DAB or PAMAM dendrimers each containing 32 nitroxides. Cartilage is enhanced with DAB-32 but not by PAMAM-32. (From Winalski, *et al.* 2002.)

## References

1. Lequesne M, Cadet C. [Do 'chondroprotective agents' agents exist in osteoarthritis? Required proof]. *Therapie*. 1998; 53(1): 7-16.
2. Rosier RN, O'Keefe RJ. Hyaluronic acid therapy. *Instr Course Lect*. 2000; 49: 495-502.
3. Brief AA, Maurer SG, DiCesare PE. Use of glucosamine and chondroitin sulfate in the management of osteoarthritis. *J Am Acad Orthop Surg*. 2001; 9(2): 71-8.
4. Stove J, Puhl W. [Overview of clinical and experimental use of hyaluronic acid in gonarthrosis]. *Z Orthop Ihre Grenzgeb*. 1999; 137(5): 393-9.
5. Altman RD. Design and conduct of clinical trials in osteoarthritis. *Scand J Rheumatol Suppl*. 1990; 81: 24-7.
6. LaValley MP, McAlindon TE, Chaisson CE, Levy D, Felson DT. The validity of different definitions of radiographic worsening for longitudinal studies of knee osteoarthritis. *J Clin Epidemiol*. 2001; 54(1): 30-9.
7. Boegard T, Rudling O, Petersson IF, et al.: Postero-anterior radiogram of the knee in weight-bearing and semiflexion. Comparison with MR imaging. *Acta Radiol*. 1997; 38(6): 1063-70.
8. Boegard T, Jonsson K. Radiography in osteoarthritis of the knee. *Skeletal Radiol*. 1999; 28(11): 605-15.
9. Spring MW, Buckland-Wright JC. Contrast medium inhibition in osteoarthritic cartilage. *Br J Radiol*. 1990; 63(754): 823-5.
10. Buckland-Wright JC. Quantitative radiography of osteoarthritis. *Ann Rheum Dis*. 1994; 53(4): 268-75.
11. Bennett LD, Buckland-Wright JC. Meniscal and articular cartilage changes in knee osteoarthritis: a cross-sectional double-contrast macroradiographic study. *Rheumatology*. 2002; 41(8): 917-23.
12. Jacobson HG. Musculoskeletal applications of magnetic resonance imaging. *JAMA*. 1989; 262: 2420-7.
13. Waldschmidt JG, Braunstein EM, Buckwalter KA. Magnetic resonance imaging of osteoarthritis. *Rheum Dis Clin North Am*. 1999; 25(2): 451-65.
14. Hohe J, Faber S, Stammberger T, Reiser M, Englmeier KH, Eckstein F. A technique for 3D in vivo quantification of proton density and magnetization transfer coefficients of knee joint cartilage. *Osteoarthritis Cartilage*. 2000; 8(6): 426-33.
15. Burstein D, Bashir A, Gray ML. MRI techniques in early stages of cartilage disease. *Invest Radiol*. 2000; 35(10): 622-38.
16. Shapiro EM, Borthakur A, Gougoutas A, Reddy R. <sup>23</sup>Na MRI accurately measures fixed charge density in articular cartilage. *Magn Reson Med*. 2002; 47(2): 284-91.
17. Regatte RR, Kaufman JH, Noyszewski EA, Reddy R. Sodium and proton MR properties of cartilage during compression. *J Magn Reson Imaging*. 1999; 10(6): 961-7.
18. Bashir A, Gray ML, Boutin RD, Burstein D. Glycosaminoglycan in articular cartilage: in vivo assessment with delayed Gd(DTPA)(2-)-enhanced MR imaging. *Radiology*. 1997; 205(2): 551-8.
19. Bashir A, Gray ML, Burstein D. Gd-DTPA2- as a measure of cartilage degradation. *Magn Reson Med*. 1996; 36(5): 665-73.
20. Bashir A, Gray ML, Hartke J, Burstein D. Nondestructive imaging of human cartilage glycosaminoglycan concentration by MRI. *Magn Reson Med*. 1999; 41(5): 857-65.
21. Burstein D, Velyvis J, Scott KT, et al. Protocol issues for delayed Gd(DTPA)(2-)-enhanced MRI (dGEMRIC) for clinical evaluation of articular cartilage. *Magn Reson Med*. 2001; 45(1): 36-41.
22. Bacic G, Liu KJ, Goda F, Hoopes PJ, Rosen GM, Swartz HM. MRI contrast enhanced study of cartilage proteoglycan degradation in the rabbit knee. *Magn Reson Med*. 1997; 37(5): 764-8.
23. Winalski C, Shortkroff S, Mulkern R, Schneider E, Rosen G. Magnetic Resonance Relaxivity of Dendrimer-Linked Nitroxides. *Magn Res Med*. 2002; 48: 965-72.



The 'Tomes: (back row, from left) Phani Dantuluri, "Hutch the Touch" Huddleston, Karl Schultz, Don Bae, Erich McBride, (front row, from left) Bob O'Toole, Jim O'Holleran, David "above the rim" Kim, Frank Paganelli



Pediatric orthopaedic fellows (from left) Paul Moroz, Danielle Katz, and Edward Sun work with Dr. Lawrence Karlin during a thoracoscopic surgical lab



Former resident Tom Myers with Vince Carter of the Toronto Raptors.