Molecular imaging of angiogenic blood vessels in vulnerable atherosclerotic plaques with a mimetic of RGD peptide grafted to Gd-DTPA

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1. Purpose

In normal artery :

- microvascular network of vasa vasorum is confined to the adventitia and outer media.

In atherosclerotic artery ([esmrmb_mimrgd_fig1.jpg] Figure 1):

- microvascular networks become more abundant and extend into the intima.

Plaque vessels may recruit inflammatory cells into lesions and initiate a **positive feedback mechanism** because [1 – 3]:

- the vessels are often found in areas rich in macrophages, T cells and mast cells – **cell types that can activate angiogenesis** .
- **adhesion molecules** are expressed on the endothelium of plaque vessels.
- the supply of oxygen and nutrients provided via plaque vessels is a precondition for **growth beyond a certain stage** , after which diffusion from the artery lumen is insufficient to meet the metabolic demands of the plaque.

Recent evidence suggests that **alpha-v beta-3 integrin is a critical molecule** in several processes involved in atherosclerosis progression and in restenosis, eg, smooth muscle cell migration and angiogenesis. The microvessels in the adventitia as well as in the plaque prominently express the integrin alpha-v beta-3 [4].
The **noninvasive molecular imaging** of plaque-associated **angiogenesis** has been assessed with a low molecular weight **non-peptidic RGD mimetic** [2] grafted to Gd-DTPA (Gd-DTPA-g-mimRGD). The *in vivo* imaging evaluation has been performed on transgenic ApoE $^{-/-}$ mice, while the pharmacokinetic parameters were determined on Wistar rats.

2. Methods and Materials

Synthesis and physico-chemical characterization of Gd-DTPA-g-mimRGD
The RGD mimetic (mimRGD) was obtained as described by G.A.G. Sulyok [5]. Synthesis was performed on solid support (trityl chloride polystyrol resin) by the Fmoc strategy [6]. This molecule was grafted onto Gd-DTPA compound by reaction between C4-Bz-NCS-DTPA (Macrocyclics, Texas, USA) and the mimetic in aqueous solution (pH=10). DTPA-g-mimRGD was then complexed with $GdCl_3 x6H_2O$ to obtain Gd-DTPA-g-mimRGD ([esmrmb_mimrgd_fig2.jpg] Figure 2).
The chemical structure was confirmed by mass spectrometry. Mass spectra were obtained on a Q-tof 2 mass spectrometer (Micromass, Manchester, UK). Samples were dissolved in a MeOH/H_2O mixture (50/50) and injected at a rate of 5 µl/min.
In vivo evaluation: molecular targeting of atherosclerotic plaques by MRI
Female C57Bl ApoE ^{tm1unc} mice, aged ~15 months (n = 10, Charles River Laboratories, Brussels, Belgium), received a Western diet for three months before the MRI studies. For MRI experiments, the animals were anesthetized with 60 mg/kg b.w., i.p., of sodium penthobarbital (Nembutal, Sanofi, Brussels, Belgium). The contrast agents (Gd-DTPA-g-mimRGD and Gd-DTPA) were injected i.v. at a dose of 0.1 mmol/kg.
To confirm the specific targeting of angiogenic blood vessels, an *in vivo* experiment of competition (n = 4) was performed in the presence of Eu-DTPA-g-mimRGD, which was injected at a dose of 0.1 mmol/kg ~10 min before the administration of Gd-DTPA-g-mimRGD. All the images were acquired at the level of abdominal aorta.
MRI equipment and protocols
All the experiments were performed on a 200 MHz (4.7 T) Bruker imaging system (Bruker, Karlsruhe, Germany) equipped with a vertical magnet and the micro-imaging device. A test tube filled with a solution of 0.5 mM Gd-DTPA prepared in 2% gelatin was used as a reference.
The images were acquired with the following MRI protocols:

- **RARE:** TR/TE = 1048.5 / 4 ms, RARE factor = 4, NEX = 4, matrix = 256, FOV = 2.3 cm, slice thickness 0.8 mm, 20 slices, spatial resolution = 90 micrometers, TA = 4 min 28 sec.
- **MSME:** TR/TE = 695.8/8.9 ms, NEX = 2, FOV = 2.3x2.3 cm, matrix = 256x256, slice thickness = 1 mm, 20 axial slices, spatial resolution = 90x90 µm, TA = 5 min 56 sec.
- **3DTOF** was used with the aim to confirm the anatomical localization of the aorta in the image slice: TR/TE = 10/2 ms,

flip angle = 20°, NEX = 2, FOV = 4x2x4 cm, matrix = 256x128x64, slice thickness = 1 mm, 60 axial slices, spatial resolution = 156x156x625 micrometers, TA = 2 min 43 sec.

Image analysis

Signal intensity (SI) values for each time delay were measured within several regions of interest in the arterial wall of the abdominal aorta by using the OSIRIS image analysis software. SI enhancement (DSNR%) was calculated according to the following equation:

$DSNR\% = [[(SI_{post}/\text{Noise SD}) - (Si_{pre}/\text{Noise SD})] / (Si_{pre}/\text{Noise SD})]*100$

The data were averaged and the standard error was calculated for each time point.

In vivo evaluation: plasma pharmacokinetics

Plasma pharmacokinetics were assessed on male Wistar rats (weight 200 g ± 50 g, Harlan, Horst, The Netherlands) injected with contrast agents (Gd-DTPA-g-mimRGD and Gd-DTPA) at a dose of 0.1 mmol/Kg b.w. Gadolinium content of the blood samples (collected through the carotid artery) was determined by relaxometry at 37°C and 60 MHz on a Bruker Minispec. A two-compartment distribution model was used to calculate the pharmacokinetic parameters: the elimination half-life ($T_{e1/2}$), the steady-state volume of distribution (VD_{ss}) and the total clearance (Cl_{tot}).

Immunohistochemistry

The presence of atherosclerotic plaques and of microvascular network was assessed on aortas collected from mice after MRI evaluation. Immediately after sacrifice, the aortas were dissected and fixed by immersion in Duboscq-Brazil fluid for 48 hours at room temperature. Fixed tissue specimens were rinsed in 70° ethanol, progressively dehydrated in graded ethanol solutions and in butanol, and embedded in Paraplast PlusÒ paraffin according to standard procedures. Paraffin sections of 4-5 µm thickness were cut on a Reichert Autocut 2040 microtome following a sagittal plane and placed on silane-coated glass slides. After rehydratation, some sections were stained with Masson's trichrome to allow the systemic examination of sections and mounted in a permanent medium.

CD31 was detected with rat anti-mouse PCAM-1 biotin-conjugated monoclonal antibody (Chemicon International, Heule, Belgium). Tissue sections were immunostained following a slightly modified version of the streptavidin-biotin immunoperoxidase method (ABC method) detailed in previous publications (Schaudies et al. 1993, Toubeau et al. 2001). The color was developed with 0.02% 3, 3'-diaminobenzidine - 0.01% H_2O_2 in PBS. The sections were finally counterstained with hemalun and Luxol fast blue and mounted in a permanent medium.

3. Results

Molecular targeting of atherosclerotic plaques by MRI

The specific interaction of mimRGD with integrins was previously assessed after grafting to USPIO (USPIO-g-mimRGD) [6]. The K^*_d for integrins was of $1.13\ 10^{-8}$ M in activated state and of $4.60\ 10^{-7}$ M in non-activated state; the pre-incubation with linear peptide GRGD inhibited the interaction at the receptor sites by 70%.

[esmrmb_mimrgd_fig3.jpg] Figure 3 shows axial slices at the level of abdominal aorta of an ApoE $^{-/-}$ mouse before and after the administration of Gd-DTPA-mimRGD. The external structures of the aortic wall (probably tunica media and adventitia) are strongly enhanced 10 min post-contrast; although weaker, this enhancement is still present at 55 min post-contrast. In the enlarged image of this anatomic slice ([esmrmb_mimrgd_fig4.jpg] Figure 4), the almost continuous contour of atherosclerotic wall is visible, while the more profound layers (toward the aortic lumen) of the aortic wall (possibly tunica media and intima) can be distinguished, which was not the case in the pre-contrast images. The aortic lumen seems to be restrained and distorted. It is remarkable that the spatial resolution of these images is in the micrometer range, i.e. 90 micrometers.

The same mouse was injected with Gd-DTPA and the comparative images are shown in [esmrmb_mimrgd_fig5.jpg] Figure 5 . The anatomic slices are almost in the same position, by taking into account the spinal cord as a reference (the other organs change relatively their position). As compared to Gd-DTPA-g-mimRGD, the enhancement produced by the non-specific contrast agent Gd-DTPA is rather diffuse and the aortic wall is not clearly outlined.

The aorta localization was possible due to the MRI protocol 3DTOF ([esmrmb_mimrgd_fig6.jpg] Figure 6 and [esmrmb_mimrgd_fig7.jpg] Figure 7), which has also evidenced the backward enhancement of the aortic wall (a clear spot

toward the spinal cord). The pre-contrast image clearly indicates that this particular enhancement was produced by the contrast agent.

An in vivo competition experiment was designed to validate the specific integrin targeting at the level of atherosclerotic plaques by blocking the receptor with an analogous compound, Eu-DTPA-g-mimRGD. The competitor was injected 10 min before the injection of integrin-targeted paramagnetic compound, Gd-DTPA-g-mimRGD. The results obtained in these experimental conditions (Figures 8 and 9) are compared to contrast enhancement in non-competing conditions ([esmrmb_mimrgd_fig8.jpg] Figure 8) and to the results obtained with the non-specific contrast agent Gd-DTPA ([esmrmb_mimrgd_fig9.jpg] Figure 9).

The signal enhancement of the aortic wall produced in competing or non-specific conditions is lower (40 – 90 % lower than with Gd-DTPA-g-mimRGD). This effect was either global, or restricted to a certain area of the aortic wall. This means that a certain fraction of the contrast agent (specific or non-specific) is free to circulate into the microvascular network of the aortic wall, which explains the relative signal enhancement produced even by the non-specific compound, Gd-DTPA. Even low, such a non-specific enhancement could represent a potential drawback for the specific diagnosis of atherosclerotic disease in this particular case. In clinical practice, this could be solved by subtracting the images obtained with non-specific compound from the ones produced by the specifically targeted contrast agent. In this way, the pathologic areas expressing the targeted receptor could be delineated.

The DSNR as a function of time is represented in [esmrmb_mimrgd_fig10.jpg] Figure 10 . Gd-DTPA-g-mimRGD produces the maximum signal enhancement 42 min post-contrast (147 %); subsequently, the signal decreases progressively, but it remaines at 97% 105 min post-contrast. In the presence of the non-paramagnetic competitor Eu-DTPA-g-mimRGD, DSNR does not surpass the level produced by the non-specific Gd-DTPA and ranges between 75 % and 15 %. These results plead for a specific integrin targeting of atherosclerotic plaque vessels produced by Gd-DTPA-g-mimRGD.

Pharmacokinetic characterization

A diminished blood clearance was observed for Gd-DTPA-g-mimRGD, as proven by the prolonged elimination half-life (61 min, compared to 15 min for Gd-DTPA) and the diminished total clearance (3.6 mL/kg/min, compared to 8.7 mL/kg/min for Gd-DTPA) ([esmrmb_mimrgd_fig11.jpg] Figure 11 and [esmrmb_mimrgd_table1.jpg] Table 1). The delayed blood clearance represents an advantage for molecular targeting of atherosclerotic blood vessels.

Immunohistochemistry

The presence of atherosclerotic plaques was confirmed on histologic sections ([esmrmb_mimrgd_fig12.jpg] Figures 12 and [esmrmb_mimrgd_fig13.jpg] 13). The thickened intima was positive for PECAM-1 expression.

4. Conclusion

The non-peptide RGD mimetic grafted to Gd-DTPA (Gd-DTPA-g-mimRGD) was designed for the targeting of alpha-v beta-3 integrin, which is an adhesion molecule over-expressed on angiogenic blood vessels in various pathologies, such as atherosclerosis and cancer. The new contrast agent could greatly contribute to the high-resolution in vivo molecular imaging methods, aimed at the localization and quantification of unstable atherosclerotic lesions.

Acknowledgements

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5. References

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7. Mediafiles:

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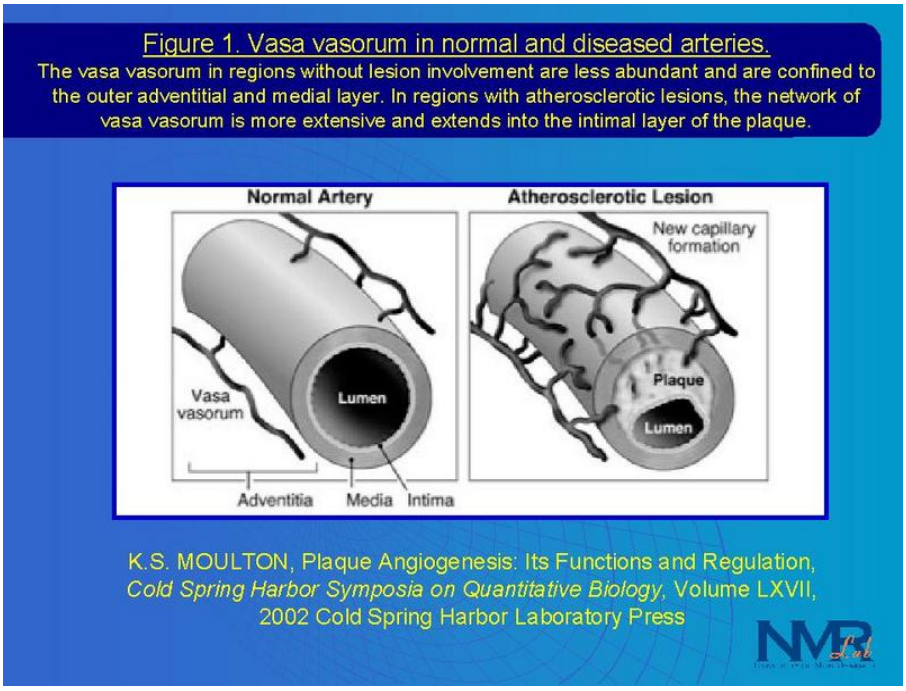

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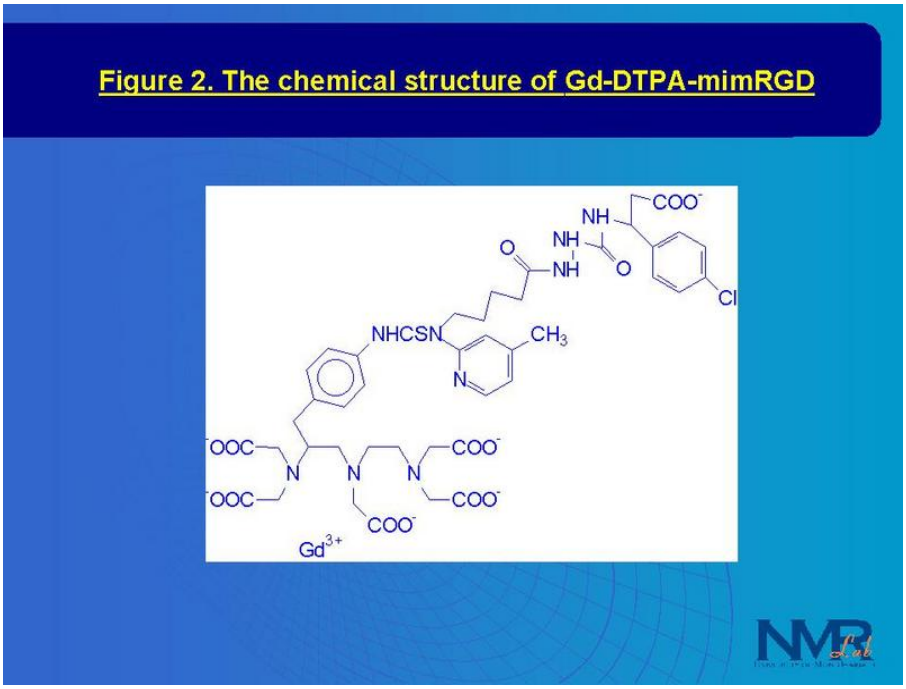

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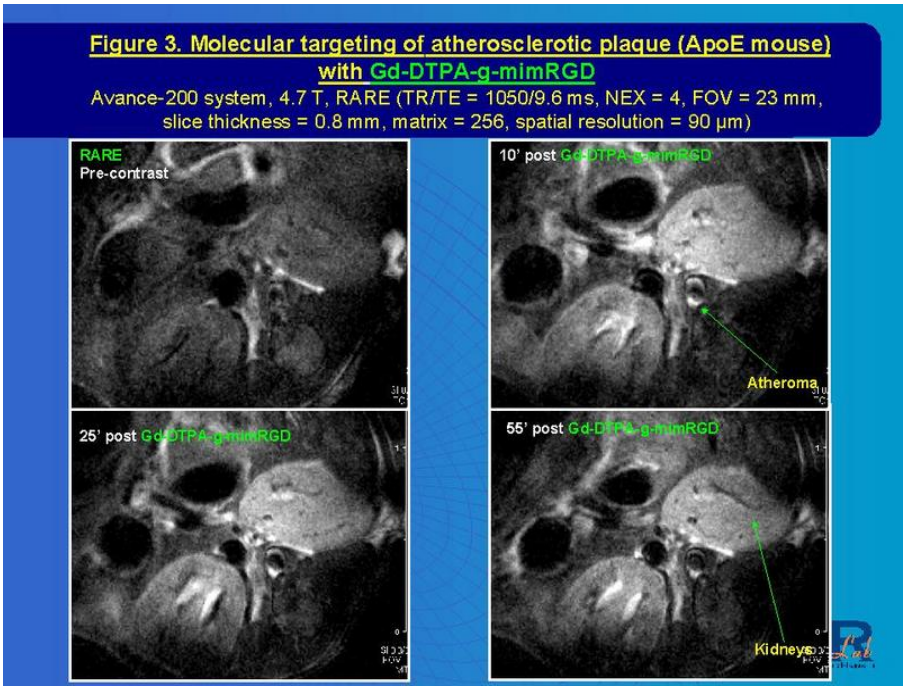

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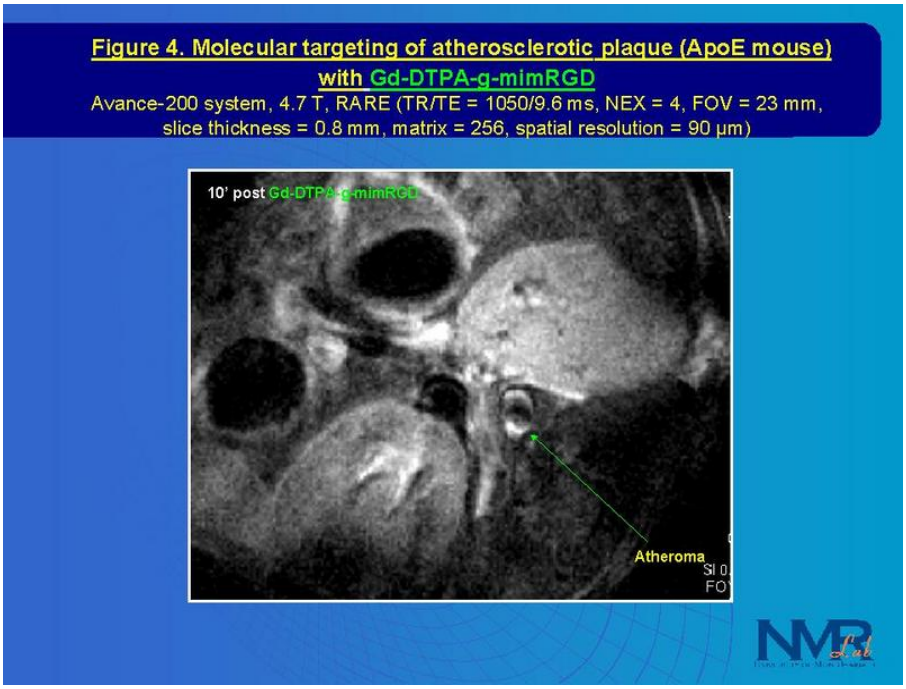

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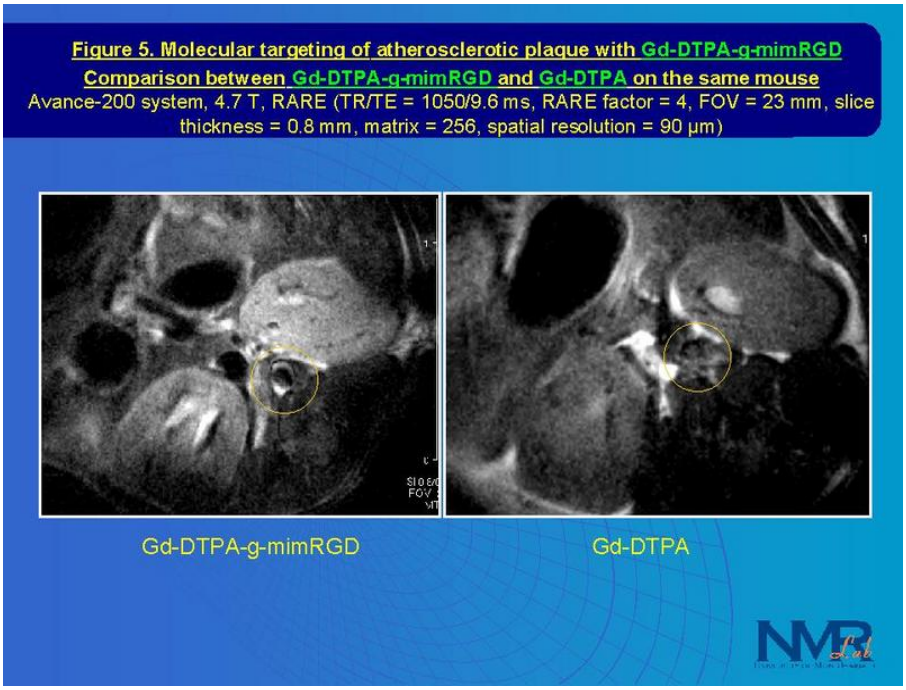

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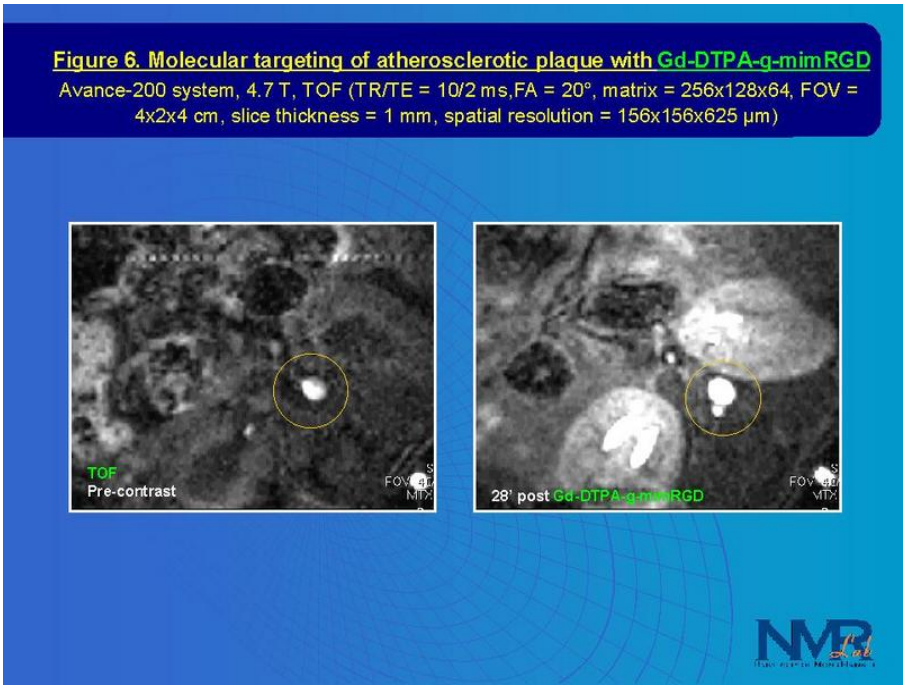

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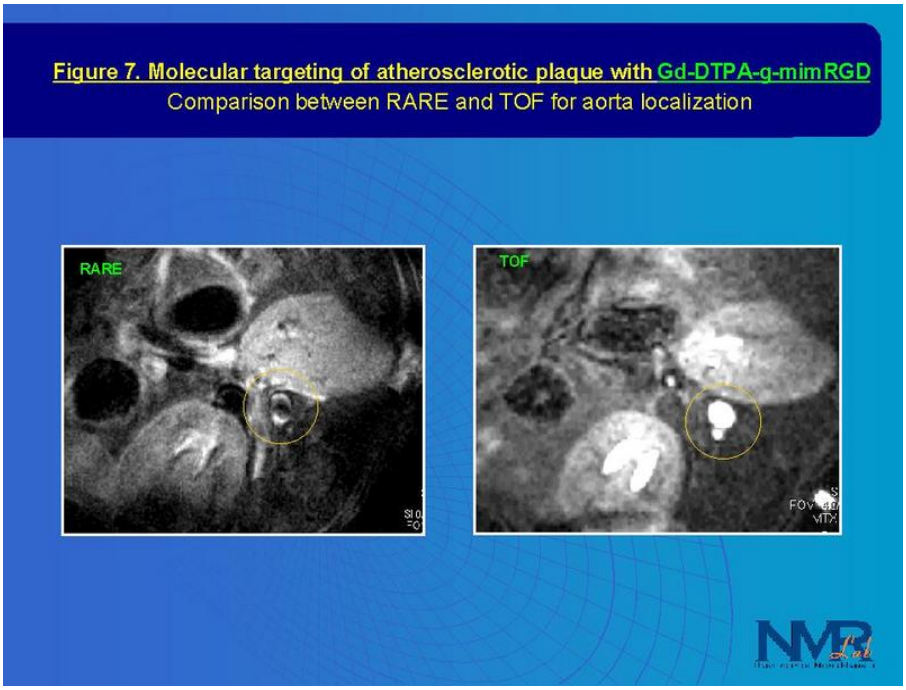

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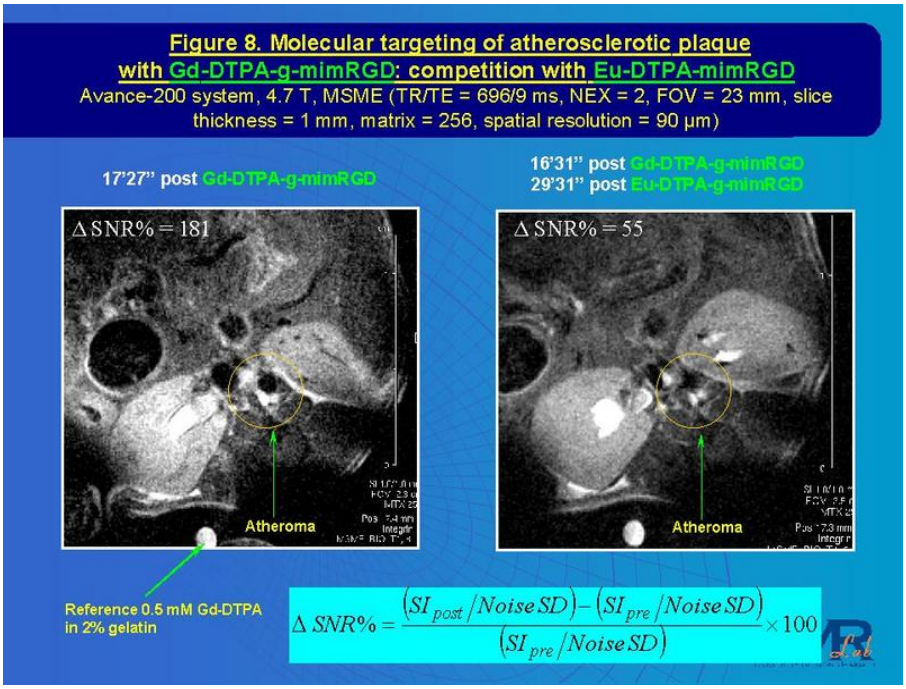

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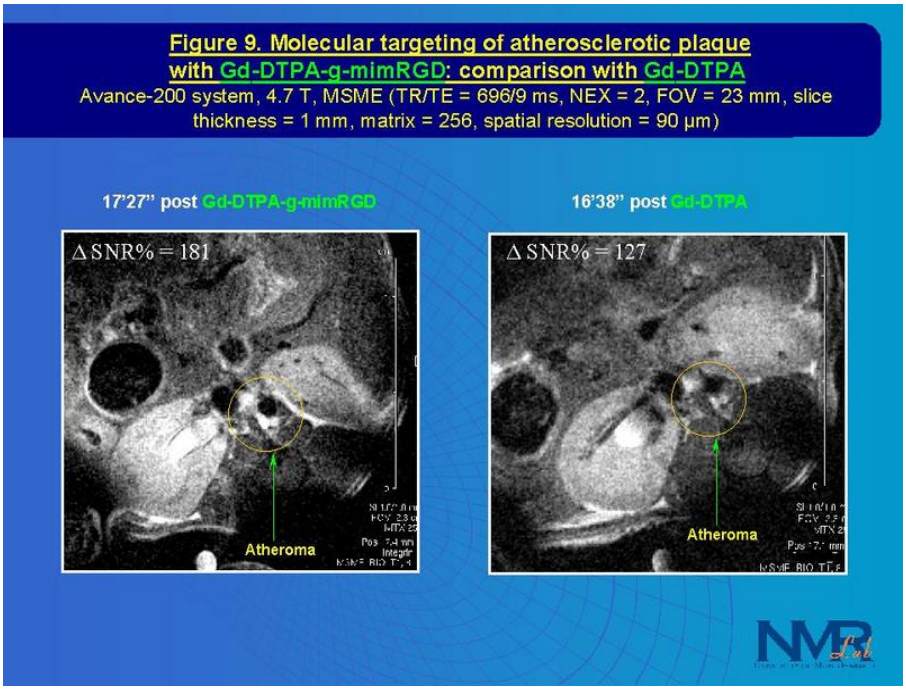

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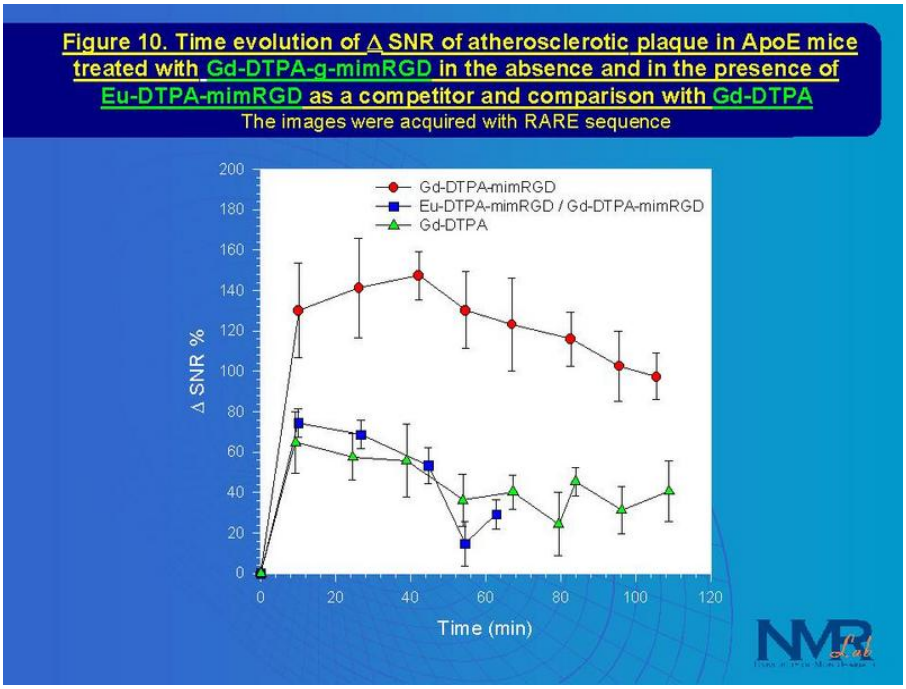

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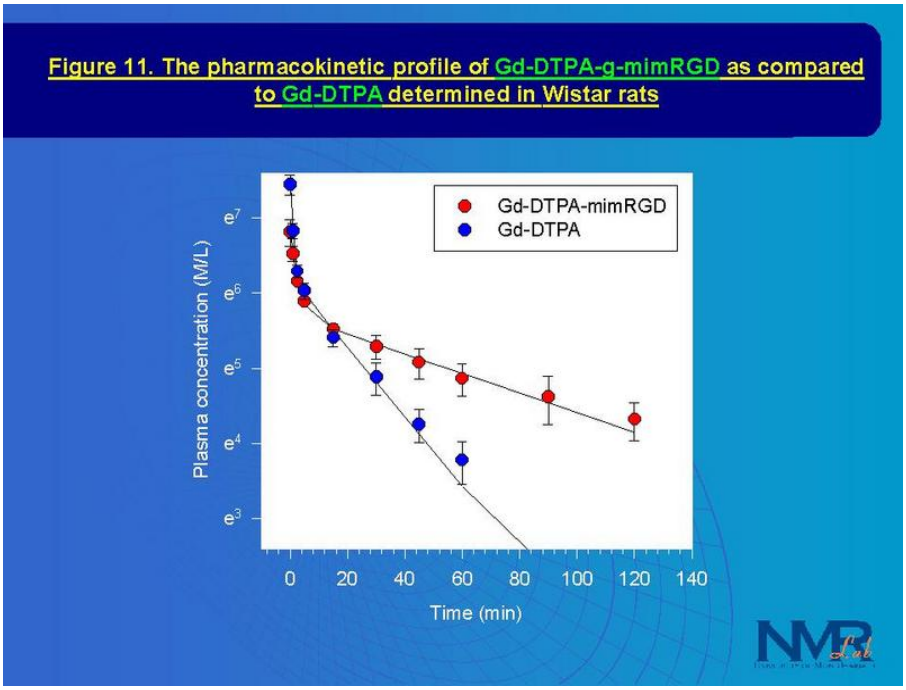

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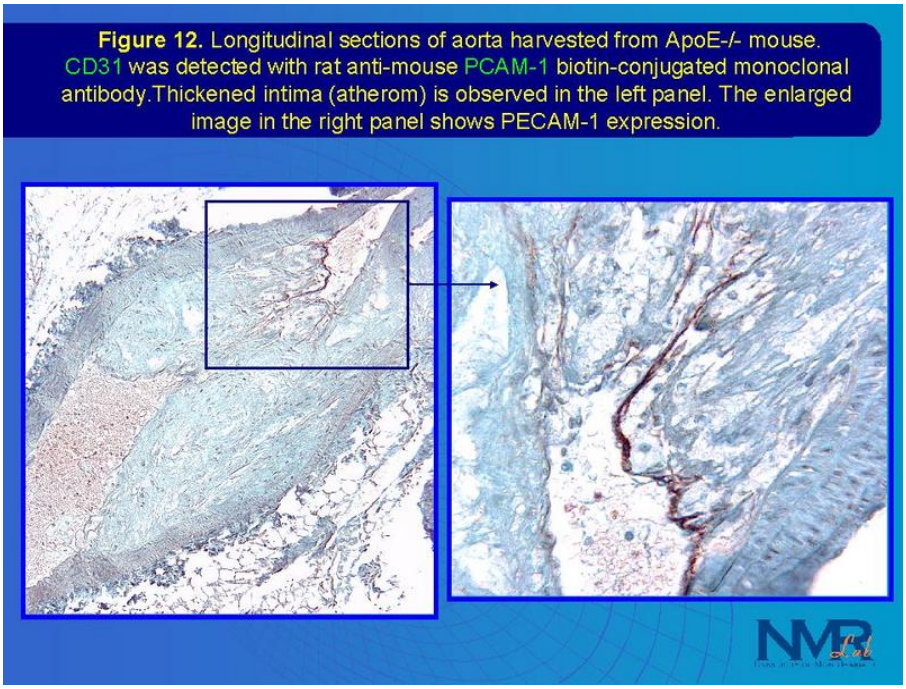

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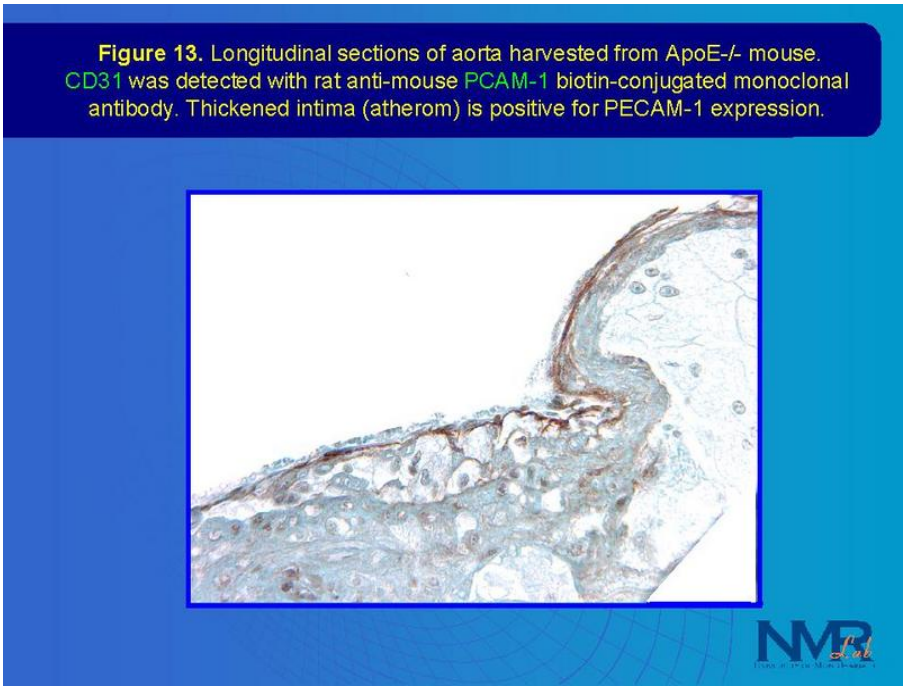

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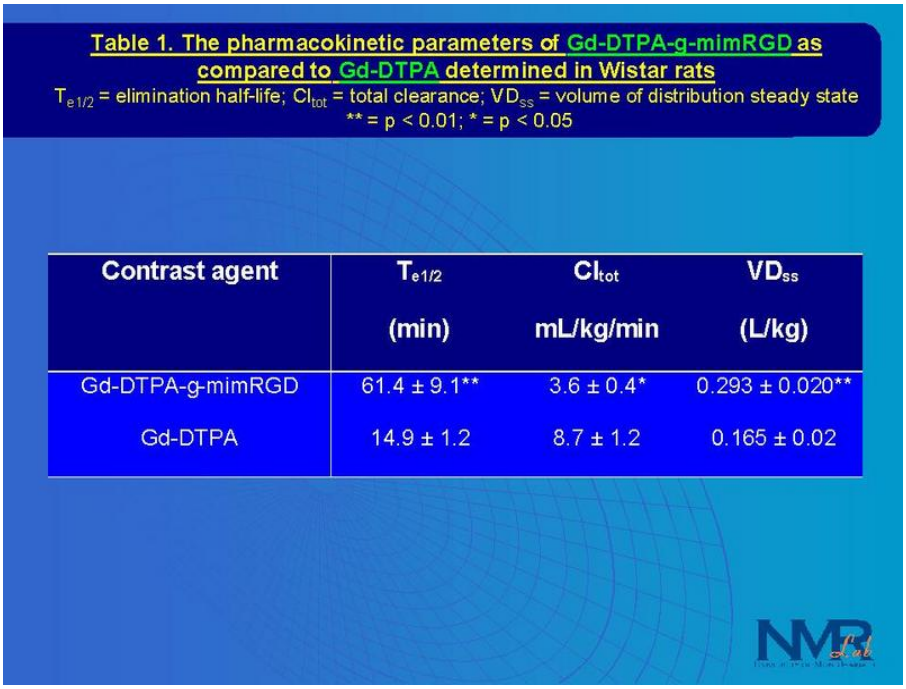

Table 1. The pharmacokinetic parameters of Gd-DTPA-g-mimRGD as compared to Gd-DTPA determined in Wistar rats

$T_{e1/2}$ = elimination half-life; Cl_{tot} = total clearance; VD_{ss} = volume of distribution steady state
** = p < 0.01; * = p < 0.05

Contrast agent	$T_{e1/2}$ (min)	Cl_{tot} mL/kg/min	VD_{ss} (L/kg)
Gd-DTPA-g-mimRGD	61.4 ± 9.1**	3.6 ± 0.4*	0.293 ± 0.020**
Gd-DTPA	14.9 ± 1.2	8.7 ± 1.2	0.165 ± 0.02