In vitro characterization of an Ab42 specific heptapeptide selected by phage display and preliminary MRI evaluation of Alzheimer's Disease - detection by functionalized USPIO

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Keywords: MRI, Amyloid-beta, Phage display, Alzheimer

1. Purpose

Alzheimer's disease (AD):

- fatal neurodegenerative pathology
- predominant cause of senile dementia
- substantial neuronal loss
- amyloid β (A β) amyloidosis (senile plaques, SP) and tauopathy (neurofibrillary tangles, NFTs).

Although the SP by themselves may not be the direct cause of AD symptoms, a noninvasive method to detect their presence in the brain will allow diagnosis and preventive treatment before occurrence of neurological symptoms and irreversible neurodegeneration.

MRI has a high spatial resolution:

Better suited to assess the SPs in AD, which are often reported to require a spatial resolution of less then 200 micrometers [1]. However, the image acquisition requires high magnetic fields (often higher than 7T) and long acquisition times (several hours) [2-5].

Phage display:

Molecular biology technique that identify high affinity peptides for any molecular target [6].

» This study reports preliminary investigations on a cyclic heptapeptide (P22), which was selected by phage display based on its affinity for A β_{42} [7], and the biochemical and biological characterization of this peptide conjugated to biotin (P22b) or to USPIO (USPIO-P22).

The P22 vectorization of USPIO particles may help to the SP detection by MRI, which has the advantage of a better spatial and anatomical resolution as compared to the imaging techniques of nuclear medicine.

2. Methods and Materials

Conjugation of P22

The disulfide constrained heptapeptide (P22) (NeoMPS, Strasbourg, France) was conjugated either to biotin (P22b) or to USPIO (USPIO-P22) with a linker (8-amino-3,6-dioxaoctanoyl).

Mass spectra were obtained on a Q-tof-2 (Micromass, UK). 0.1 mg of peptide was dissolved in 1 ml of a mixture of formic acid/ acetonitrile/ water (0.5%/ 50%/ 49.5%).

Biotinylated peptide synthesis:

The 8-amino-3,6-dioxaoctanoyl-P22 was purchased from NeoMPS (Strasbourg, France). The peptide was biotinylated as follows: 8 µmol of biotin-NHS (FLUKA, Begium) and 5 µmol of peptide were solubilized in 2 ml of DMF and stirred during 24h at room temperature. 5 ml of water were added, the solution was filtered and dialyzed (cut-off of the membrane: 500; Spectra/Por, VWR, Leuven, Belgium).

The electrospray mass spectrometry confirmed the structure of the biotinylated peptide.

ES-MS: $[M+H]^+$: 1439, $[M+Na]^+$: 1461

Peptide conjugation to USPIO:

The peptide was linked covalently in 2 steps: the reactive alkyl halogen end of epichlohydrin was first coupled to the hydroxyls of dextran surface of the Fe_3O_4 particles to give a terminal glycidyl ether derivative which can be used to link amine containing molecule ([Fig 1] Fig 1).

Relaxivity (s^{-1} mM^{-1}):

20 MHz and 37°C: r1 = 32.26 and r2 = 85.32 => r2/r1=2.64

60 MHz and 37°C: r1= 13.50 and r2= 83.75 => r2/r1=6.20

In vitro characterization

Evaluation of the dissociation constant (K_d) for A β_{42} , as the main component of SP:

ELISA:

- Immobilization of 100 mg/L of A β_{42} on a polystyrene surface.

- Incubation with a range of concentrations of P22b [1.25x10 $^{-6}$ M – 1.91x10 $^{-9}$ M].
- Detection with peroxydase-conjugated streptavidine (ABC complex) and measurement of OD $_{405}$ (nm).

Relaxometry at 60 MHz:

- Incubation of a range of A β_{42} concentrations [10 $^{-16}$ – 10 $^{-7}$ M] with 25 µM of USPIO-P22 in solution.
- Measurement of the transverse relaxation time (T $_2$) at 60 MHz (Bruker mq60 Minispec, Bruker, Karlsruhe, Germany) after 3 minutes of incubation at 37°C.

Blood pharmacokinetics

- Plasma pharmacokinetics were assessed on male Wistar rats anesthetized with 60 mg Nembutal/kg b.w., i.p. The rats were tracheotomized, and the left carotid artery was catheterized for blood collection.
- The contrast agent was injected as a bolus through the femoral vein at a dose of 0.1 mmol Fe/Kg b.w. USPIO has been used as a control.
- Blood samples (~0.3 mL, with saline replacement) were collected before and at 1, 2.5, 5, 15, 30, 45, 60, 90 and 120 min after injection. The iron content of the blood samples was determined by relaxometry at 60 MHz (Bruker mq60 Minispec).
- A two-compartment distribution model was used to calculate the pharmacokinetic parameters such as the elimination half-life (T $_{e1/2}$), the volume of distribution steady state (VD $_{ss}$) and the total clearance (Cl $_{tot}$).

Biodistribution

- The apparent biodistribution has been determined in rats 2h after a single i.v. injection of 0.1 mmol Fe/kg bw.
- The T $_2$ of the organs (liver, kidneys, heart, lungs and brain) was measured by relaxometry at 10 MHz on a Bruker minispec PC-110 (Bruker, Karlsruhe, Germany).
- The R $_2$ values were computed and normalized by the subtraction of R $_2$ values of rat organs that were not exposed to any contrast agent.

In vivo MRI evaluation

Animal model of AD:

- Twenty-two months old double transgenic mice APP[V717I] and PS1-A246E.
- The histology and MRI investigations indicated the presence of amyloid plaques both in the cortex and in the thalamic areas [5] .

Contrast agent administration:

- i.v. injection of 100 micromol Fe/kg of USPIO-P22.
- to open the blood-brain barrier, the mice were first injected with a solution of 25% mannitol i.v. (14mL/kg). The wild type (WT) control mice received the same treatment.

MRI protocol:

- 4.7 T Bruker AVANCE-200 system, vertical bore, micro-imaging device.
- MRI protocol: RARE, TR/TE = 5552/68 ms, NE = 32, matrix 128x128, slice thickness = 1.5 mm, FOV = 2.5 cm, spatial resolution = 195 µm.

Histology

- After MRI session, brain was dissected, fixed and paraffin-embedded, and slice of 5 µm were cut.
- Incubation with USPIO-P22 (30.4 mM).
- Iron staining with Perl's method (Prussian blue).
- Counter-staining with Sirius Red to localize the SP.

3. Results

In vitro characterization

ELISA:

- P22b has a k $_d$ of 4.13x10 $^{-7}$ M for A β_{42} ([Fig 2] Fig 2).

Relaxometry 60 MHz:

- USPIO-P22 has a K_d of 1.2×10^{-10} M ([Fig 3] Fig 3).

These results shown that the P22 has a very good affinity for its target. The relaxometry seems more sensitive than ELISA, but the difference between the two K_d can also be explained by the different chemical group linked to the peptide or because the A β_{42} is in solution when measured by relaxometry and is immobilized in the other case.

Blood pharmacokinetics

- Diminution ($p < 0.01$) of the blood clearance ($Te_{1/2}$ = 766 min, Cl_{tot} = 0.038 mL/kg/min) of USPIO-P22 as compared to USPIO ($Te_{1/2}$ = 255 min, Cl_{tot} = 0.153 ml/kg/min) ([Fig 4] Fig 4 and [Table 1] Table 1).

- Although not statistically significant, the low VD_{ss} value suggests a diminished diffusion into the interstitial space.

Biodistribution

- The apparent biodistribution of contrast agents was analyzed by measuring T_2 values at 10 MHz of the rat organs harvested after pharmacokinetic studies. Because of the intrinsic iron, a normalized R_2 (R_2^{Norm}) was computed by subtracting the R_2 of organs from rats that did not receive any contrast agent ([Fig 5] Fig 5).

- The contrast agents seem to be localized in liver and lungs, and more USPIO than USPIO-P22 is found in the liver, but the opposite is noticed in the other organs.

Hence, the results suggest a diminished USPIO-P22 uptake by the liver, which could prolong its half-life of elimination.

In vivo MRI evaluation

- The preliminary MRI data on a double transgenic APP/PS1 mouse show a signal decrease in the brain of about 50% after the administration of USPIO-P22 ([Fig 6] Fig 6).

- The effect produced by USPIO-P22 is of only 15% on a control mouse in the same conditions and is comparable to that produced by USPIO.

- The resolution of these images is not adequate to detect individual SP ([Fig 7] Fig 7), but the color map reveals the presence of several dark spots in the thalamic areas and cortex, which could correspond to SP.

Histology

- The SP on histologic sections are stained in red with Sirius Red ([Fig 8] Fig 8).

- On the slices incubated with USPIO-P22, a blue iron staining is visible around the cores of SP.

- The negative control with USPIO does not show any blue iron staining around the SP.

Therefore, USPIO-P22 seems to interact specifically with amyloid fibers. The core of SP is too dense to allow the penetration of the contrast agent deep inside.

4. Conclusion

The contrast agent USPIO-P22 has a good affinity for A β_{42} and seems to be specific to SP. It could represent a potential tool for AD diagnosis after further characterization.

5. References

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6. Personal Information

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7. Mediafiles:

Fig 1

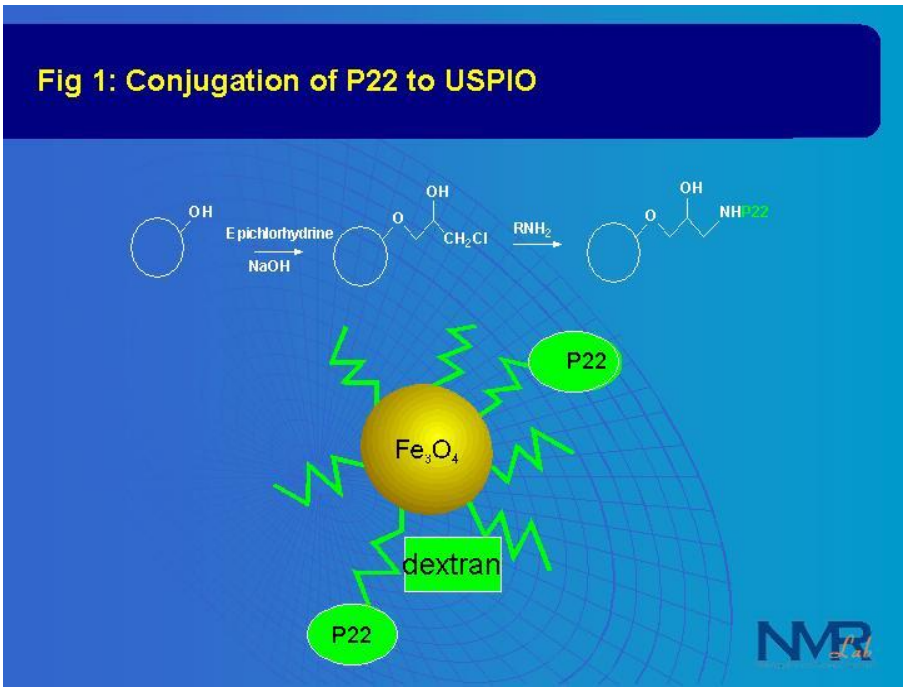

Fig 2

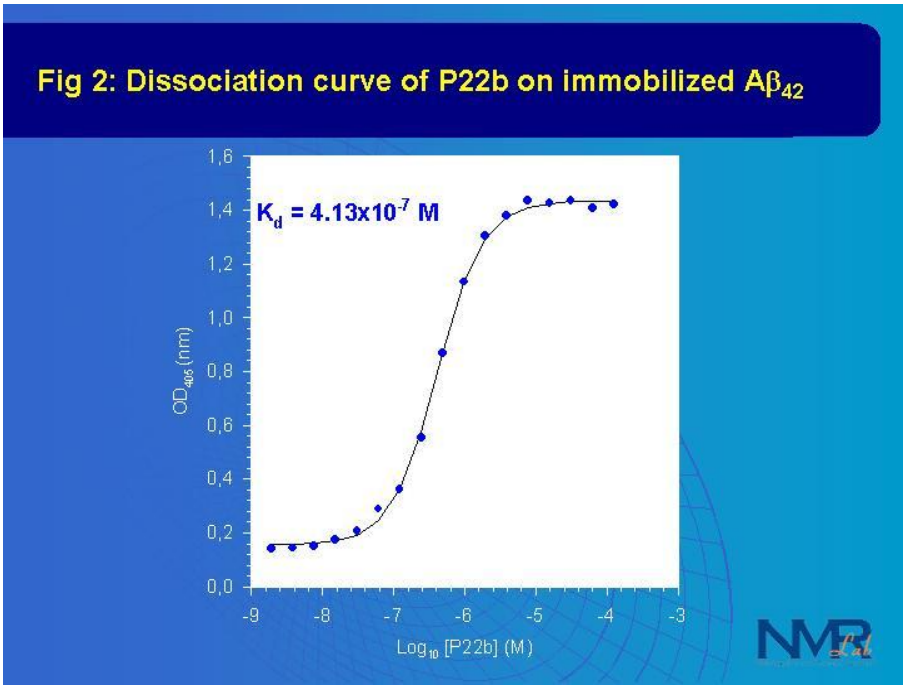
Fig 2: Dissociation curve of P22b on immobilized Aβ42
Kd = 4.13x10-7 M
OD405 (nm)
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
-9
-8
-7
-6
-5
-4
-3
Log10 [P22b] (M)

Fig 3

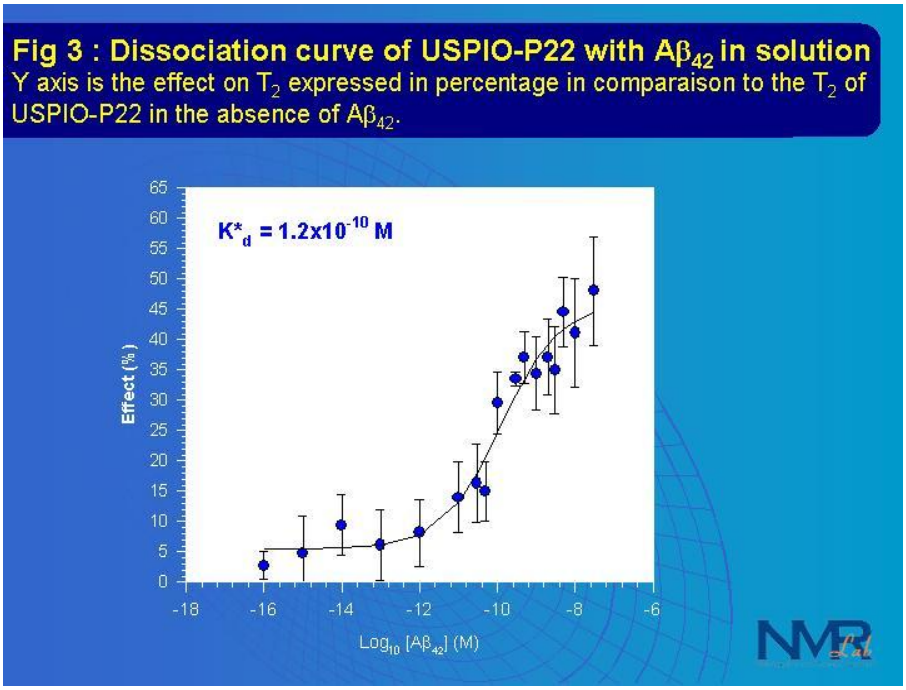
Fig 3 : Dissociation curve of USPIO-P22 with Aβ42 in solution
Y axis is the effect on T2 expressed in percentage in comparaison to the T2 of USPIO-P22 in the absence of Aβ42.
K*d = 1.2x10-10 M
Effect (%)
65
60
55
50
45
40
35
30
25
20
15
10
5
0
-18
-16
-14
-12
-10
-8
-6
Log10 [Aβ42] (M)

Fig 4

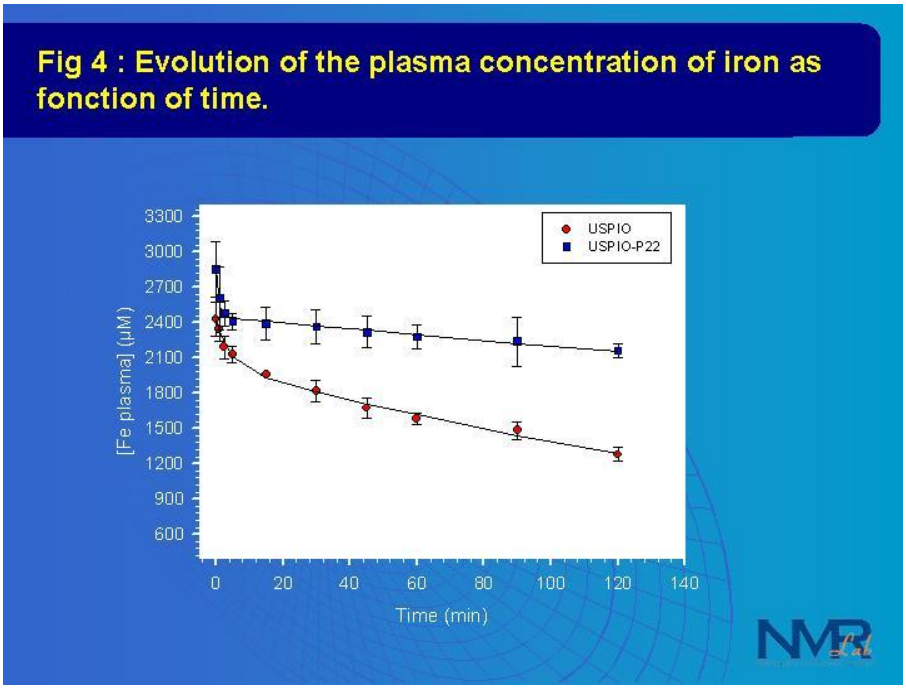
Fig 4 : Evolution of the plasma concentration of iron as fonction of time.
[Fe plasma] (µM)
3300
3000
2700
2400
2100
1800
1500
1200
900
600
0
20
40
60
80
100
120
140
Time (min)
USPIO
USPIO-P22

Fig 5

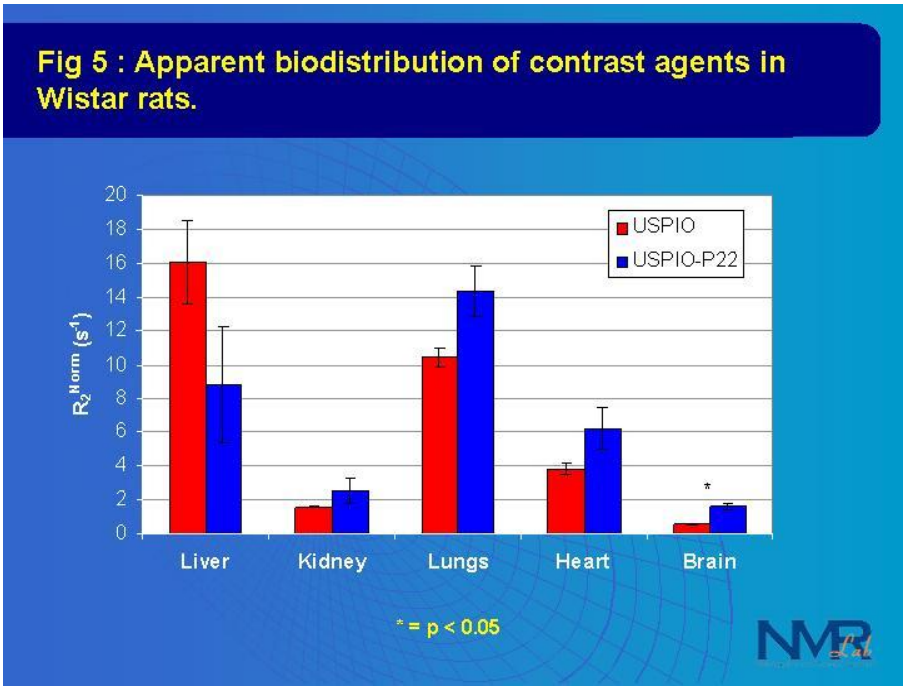
Fig 5 : Apparent biodistribution of contrast agents in Wistar rats.
R_2^{Norm} (s^{-1})
20
18
16
14
12
10
8
6
4
2
0
USPIO
USPIO-P22
Liver
Kidney
Lungs
Heart
Brain
*
* = p < 0.05

Fig 6

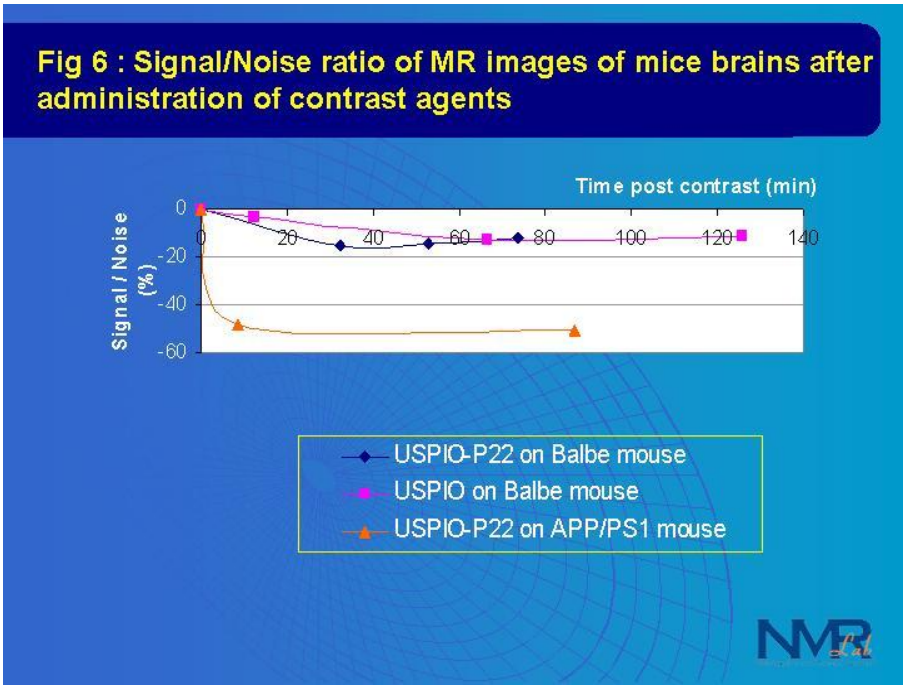
Fig 6 : Signal/Noise ratio of MR images of mice brains after administration of contrast agents
Time post contrast (min)
Signal / Noise (%)
0
-20
-40
-60
0
20
40
60
80
100
120
140
USPIO-P22 on Balbe mouse
USPIO on Balbe mouse
USPIO-P22 on APP/PS1 mouse

Fig 7

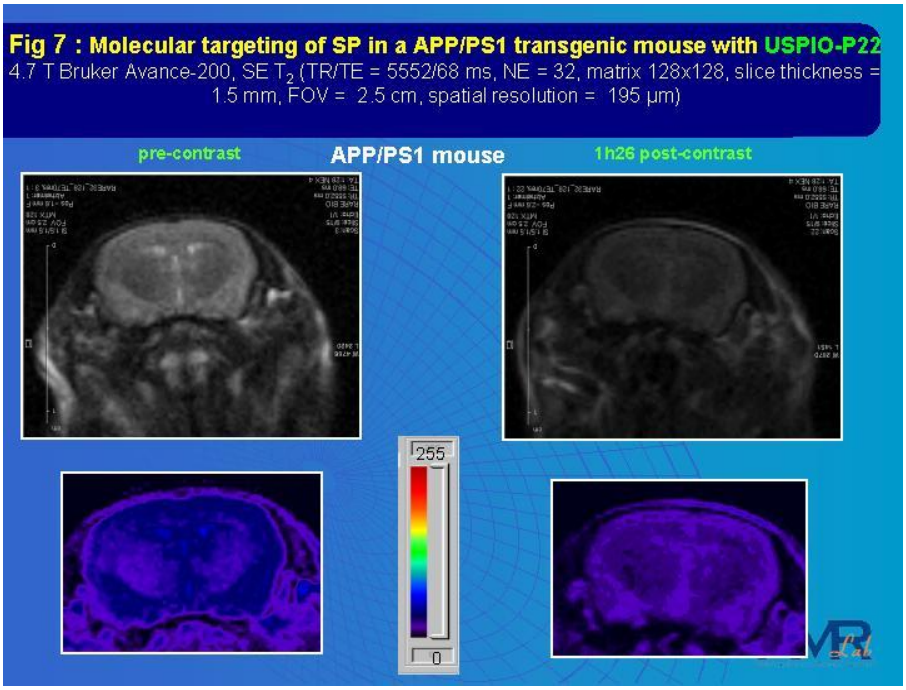
Fig 7 : Molecular targeting of SP in a APP/PS1 transgenic mouse with USPIO-P22
4.7 T Bruker Avance-200, SE T2 (TR/TE = 5552/68 ms, NE = 32, matrix 128x128, slice thickness = 1.5 mm, FOV = 2.5 cm, spatial resolution = 195 µm)
pre-contrast
APP/PS1 mouse
1h26 post-contrast
255
0

Fig 8

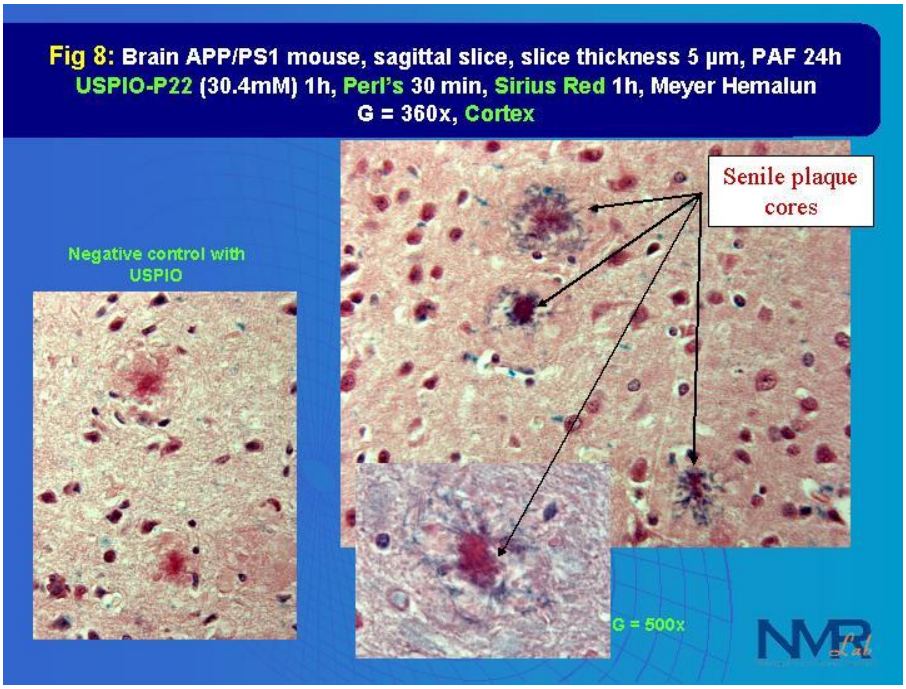

Table 1

Table 1 : Blood pharmacokinetic parameters of USPIO-P22 and USPIO determined on Wistar rats.

Contrast agent	$T_{e1/2}$ (min)	Cl_{tot} mL/kg/min	VD_{ss} (L/kg)
USPIO-P22	776 ± 92 *	0.038 ± 0.0036 *	0.047 ± 0.009
USPIO	255 ± 54	0.153 ± 0.025	0.053 ± 0.0035

* = $p < 0.05$

$T_{e1/2}$ = half-life of elimination
Cl_{tot} = total clearance
VD_{ss} = volume of distribution steady state