IN VIVO AND *IN VITRO* ^{27}Al NMR STUDIES OF ALUMINUM(III) CHELATES OF TRIAZACYCLONONANE POLYCARBOXYLATE LIGANDS

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Keywords: aluminum chelates; triazamacrocycles; ^{27}Al NMR

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Abstract

The metallic radioisotope of a known radiopharmaceutical chelate, ^{67}Ga(NOTA) (NOTA=1,4,7-triazacyclonane-1,4,7-triacetic acid), used for tumor detection, was substituted by the chemically similar but non radioactive aluminum ion. Our aim was to detect and evaluate the *in vivo* behavior of the chelate. For this purpose, Al(NOTA) and the related chelate Al(NODASA) (NODASA=1,4,7-triazacyclononane-1-succinic acid-4,7-diacetic acid) were studied using *in vitro* and *in vivo* ^{27}Al NMR spectroscopy in rats. Both chelates showed high stability towards acid catalyzed dissociation and their ^{27}Al NMR resonances are characteristic of highly symmetrical species, with chemical shifts within the range for octahedral or pseudo-octahedral geometries. The thermodynamic stability constant of the novel chelate Al(NODASA) was estimated using ^{27}Al NMR. The obtained value suggested that the chelate does not undergo *in vivo* demetalation by transferrin. The *in vivo* spectroscopic studies and the analysis of blood and urine samples for Al(III) concentrations indicated that the chelates remain intact under physiological conditions and that they are mainly eliminated from the body through the kidneys.

1. Introduction

Metal chelates find wide applications both as diagnostic and therapeutic agents in biomedical sciences. Gadolinium chelates are being used as paramagnetic contrast agents for MRI [1-3]. Radiochelates of gamma or positron emitting metals find strong application in Nuclear Medicine, e.g, chelates of gallium-67/68, indium-111 or copper-64 in tumor detection, and chelates of yttrium-90 and rhenium-188 in internal tumor therapy [4, 5].

Radiochelates of Ga(III) are often used in scintigraphy for diagnostic procedures [6, 7]. The radioactive ^{67}Ga(NOTA) (NOTA=1,4,7-triazacyclonane-1,4,7-triacetic acid) is an interesting radiopharmaceutical agent [8]. Parker *et al* substituted the radioactive gallium in Ga(NOTA) by the NMR sensitive cold gallium ($^{69/71}$Ga) and performed an *in vivo* ^{71}Ga NMR study [9]. Recently Geraldes *et al* studied other gallium chelates of triazacyclononane ligands also using ^{71}Ga NMR spectroscopy [10]. We previously performed an *in vivo* ^{69}Ga NMR study with Ga(NODASA) (NODASA=1,4,7- triazacyclononane-1-succinic acid-4,7-diacetic acid) [11].

The main goal of this work was to detect and evaluate the *in vivo* behavior of Ga(NOTA)- like metal chelates. For this purpose we used Al(NOTA) (Figure1–a) and the closely related Al(NODASA) (Figure1-b) as models. These chelates were investigated by *in vitro* and *in vivo* ^{27}Al NMR spectroscopy in rats.

The non radioactive Al(III) ion, being magnetically active, has an NMR signal which may give information about the chemical structure and integrity of its chelates under *in vivo* conditions.

The quadrupole splitting of the Al(III) ion is small in a highly symmetrical environment but can be as large as several MHz in an asymmetrical one. Large

quadrupole splitting will broaden the resonance beyond detection; consequently, the observation of high resolution NMR will be limited to complexes in which the nucleus experiences a highly symmetrical environment [12].

^{27}Al has a 1.5 times higher NMR sensitivity than ^{71}Ga and its natural abundance is 100 % compared to 40 % of ^{71}Ga resulting in a 3.7 fold increase in absolute sensitivity [12]. Simple calculation using the spin numbers and the quadrupole moments of ^{27}Al and ^{71}Ga shows that for isostructural chelates the ratio of the linewidths of ^{27}Al and^{71}Ga resonances is about 0.4. Consequently, the observation of ^{27}Al resonances may be easier.

Aluminum possesses an NMR sensitivity of 21 % relative to the proton and its short T_1- relaxation time allows fast data sampling. In a previous work with aluminum containing drugs Seelig *et al* demonstrated that the Al- nucleus is suitable for MRI [13].

NOTA, with its three carboxylates and the three nitrogen atoms in the cyclononane moiety, is a suitable chelator to form a highly symmetrical octahedral Al(III) complex affording a narrow ^{27}Al NMR signal. In spite of its narrow signal the sensitivity is low due to the low solubility of Al(NOTA) in water at room temperature. For this reason we aimed at the synthesis of a new ligand based on NOTA but with an extra carboxylic acid group (NODASA). Solutions of Al(NOTA) and Al(NODASA) were intravenously administrated to rats and the aluminum signals of the chelates were observed by *in vivo* ^{27}Al NMR spectroscopy.

2. Experimental

2.1 - Synthesis

NOTA. This ligand was synthesized according to a procedure published elsewhere [14,15]. Crystals of Al(NOTA) were prepared also according to a method described in the literature [16].

NODASA. This ligand was prepared according to our method previously published [11]. Crystals of Al(NODASA) were prepared using 0.130 g of NODASA ($2.88x10^{-4}$ mol) and 0.106 g of $Al(NO_3)_3.9H_2O$ ($2.88x10^{-4}$ mol) which were dissolved in 4 mL of water. The pH was adjusted to 3-4 with the addition of 1 M NaOH(aq) and the solution was heated at 70 ^oC during 1 hour. The small amount of white precipitate formed was filtrated off and by slow evaporation of water small crystals were obtained which were recrystallized from water.

^1H NMR (400 MHz, D_2O, pD=4.4): δ 2.65-3.05 (overlapping m's, –CHC<u>H</u>$_2$COOH, 2H and ethylenic ring protons, 2H); 3.05-3.15 (m, ethylenic ring protons, 2H); 3.15-3.25 (m, ethylenic ring protons, 4H); 3.35-3.45 (m, ethylenic ring protons, 2H); 3.45-3.55 (m, ethylenic ring protons, 2H); 3.75 (m, –C<u>H</u>$_2$COO$^-$, 4H); 4.10 (m, –C<u>H</u>CH$_2$COOH, 1H).

^{13}C NMR (90 MHz, D_2O, pD=4.4): δ 32.0 (C12); 45.0 (C2, 9); 52.0 (C3, 5, 6, 8); 62.5 (C10, 10'); 66 (C11); 175 (carboxylates).

m/z (ESI^-): 384.0 ($M-H^-$, 100)

IR (cm^{-1}): 1303 (COO^- *sy*); 1665 (COO^- *as*); 1715 (COOH)

2.2 - Preparation of solutions

Al(NOTA) and Al(NODASA) solutions for *in vitro* ^{27}Al NMR experiments were prepared by dissolving the respective crystals in the appropriate volume of water for a final concentration of 10 mM.

The chelate solutions for *in vivo* studies were prepared by dissolving the respective crystals in NaCl isotonic solution in order to obtain saturated solutions at room temperature (20.75 mg of Al(NOTA)/5 mL and 40.30 mg of Al(NODASA)/5 mL).

2.3 - Stability of the Chelates

For the estimation of the thermodynamic stability constant of Al(NODASA) we used a batch method consisting of several samples which contained a fixed concentration of Al(III) of 19.8 mM ($Al(NO_3)_3.9H_2O$ was used). The ligand concentration was different from sample to sample (the ratio [NODASA]/[Al(III)] was varied from 0.10 to 2.0). The samples were made up to a volume of 0.8 mL and the pH of each sample was adjusted to 2.0±0.1 with 1 M HCl to prevent the hydrolysis and polymerization of the aluminum ion. The solutions were allowed to equilibrate at 20±0.5 °C and ^{27}Al NMR spectra were acquired periodically.

The stability of the chelates towards alkaline and acidic hydrolysis was tested using solutions obtained by dissolving the respective crystals in water.

2.4 - Animal Studies

The *in vivo* experiments were carried out with well-fed male Sprague-Dawley rats (230 and 290 g) that had free access to a standard laboratory rat diet and drinking water *ad libidum*. For the experiments, the individual rats were anesthetized with 1.8% isoflurane in N_2O/O_2 (3:1) and cannulated in the tail vein. The animals were placed in prone position on a specially designed plexiglas® holder, so that the liver was in the sensitive volume of the NMR antenna. The animals were positioned on the holder in the center of the magnet and the magnetic field was shimmed to a halfwidth of 85 Hz

(protons). Thereafter the coil was tuned to the ^{27}Al resonance. A water operated warming pad was placed on the back of the rat to maintain normal body temperature (measured rectally) throughout the experiment. Subsequently, Al(NOTA) or Al(NODASA) solutions were infused for 72 min at rates of 3.0 and 6.3 μmol/min/kg body weight, respectively. Concomitantly, the abdominal region was assessed by *in vivo* ^{27}Al NMR spectroscopy. At the end of the experiment, the animals were sacrificed by an overdose of Nembutal® and samples of blood and urine were taken.

2.5 - ^{27}Al NMR spectroscopic studies

In vivo and *in vitro* ^{27}Al NMR spectroscopy was performed on a Bruker BioSpec 70/20® equipped with a 7 Tesla horizontal bore magnet (^{27}Al resonance frequency of 78.2 MHz). For *in vitro* spectroscopy, a home-built 5-turn solenoid coil (10 mm inner diameter) that accomodates 0.5 mL micro tubes in vertical position with a snug fit was used. The temperature was controlled with a thermostated water jacket. The relative concentrations of free aluminum and complexed aluminum in solution were quantified with ^{27}Al NMR spectroscopy in the pulse-acquire mode whereas T_1 and T_2 relaxation were measured with a standard inversion-recovery and a spin-echo sequence, respectively. *In vivo* ^{27}Al NMR spectra of intact rats were obtained using a surface coil for signal excitation and acquisition. A home-built single-turn coil with a diameter of 3.6 cm was used for the *in situ* assessment of Al(NOTA) whereas a 2-turn 1.8 cm coil was employed for a more localized detection of Al(NODASA) in rat liver. Spectra were collected in the pulse-acquire mode in blocks of 2000 scans (6 min 10 sec) with a repetition time (*TR*) of 512 ms and 20.5 ppm spectral width. For deeper penetration, the excitation pulse (50 μs) was adjusted to 180° at the coil center by

minimizing the signal of a reference consisting of $[Al(H_2O)_6]^{3+}$ (0 ppm). Shimming was performed on the proton resonance of water using the ^{27}Al coils. ^{27}Al spectra were processed with a 40 Hz apodisation prior to Fourier transformation.

3. Results and discussion

3.1 - *In vitro* experiments

The ^{27}Al resonances of both chelates appear as single peaks with chemical shifts within the region of octahedral or pseudo-octahedral aluminum chelates. Al(NOTA) has its resonance at 49 ppm [17] with a linewidth of about 60 Hz. Al(NODASA) appears at 47 ppm with a halfwidth of 80 Hz (Figure 2). This result is consonant with the structure of the analogous Ga(NODASA), previously determined by us using X-ray diffraction [11]. In this work we demonstrated that the chelate is electrically neutral and the metal ion is fully chelated in a slightly distorted octahedral environment. The β-carboxylate from the succinate pendant arm remains protonated and does not participate in the complexation of the metal ion. The X-ray structure of Al(NOTA) [20] similarly showed that the metal ion is also chelated by the 3 nitrogen atoms of the triazacyclononane and by the 3 carboxylate oxygens, in an almost octahedral arrangement.

Both chelates show a linear increase of the T_1 and T_2 relaxation time with temperature (T_1 and T_2 relaxation times at T= 37 °C are about 20 ms).

Al(NOTA) and Al(NODASA) were shown to be stable in a pH range between 0.8-8. For pH values above 9 a second peak corresponding to the formation of $Al(OH)_4^-$could be detected at 80 ppm. After 7 months in HCl (pH 0.8) the chelates were still almost intact (less then 1% of the Al(III) ion could be detected by ^{27}Al NMR

in the form of $[Al(H_2O)_6]^{3+}$, which demonstrates their extremely high resistance against acid catalyzed dissociation.

The integration of the ^{27}Al NMR signal of $[Al(H_2O)_6]^{3+}$ and Al(NODASA) at pH 2.1 enabled a quantitative measurement of both the free metal ([Al(III)]) and the bound metal ([Al(NODASA)]). The intensity of the aquaaluminum ion signal decreased whereas the intensity of the Al(NODASA) signal increased with time (Figure 3). After *ca* 10 months the solutions were equilibrated and the intensity of the ^{27}Al NMR signals remained unchanged which allowed the estimation of the conditional stability constant of the complex, K_{cond}=[Al(NODASA)]/([Al(III)].[NODASA]). The value found was K_{cond}=6.80±0.06 M^{-1} to which corresponds the thermodynamic stability constant of log K_{ML}= 18.50±0.01.[§] This value for the thermodynamic stability constant of Al(NODASA) indicates that the chelate might remain intact towards aluminum transfer to human serum transferrin (Tf) (the two macroscopic binding constants of Al-Tf are logK_1=13.5 and logK_2=12.5 at pH 7.4 [18]. The binding of Al(III) in the chelate prevents the metal from having a toxic effect [19].

3.2 - *In vivo* experiments

The infusion of Al(NOTA) in isotonic NaCl solution through tail vein injection in a rat started with a rate of 4 mL/h and a dead time of 7 min. In the following period a spectrum was acquired every 7 min. After 15 min a ^{27}Al NMR signal appears at 45 ppm. Figure 4 depicts the time course of the spectra. After 72 min the infusion was stopped.

Al(NODASA) in isotonic NaCl solution was also infused into the tail vein of a rat at a rate of 4 mL/h. The ^{27}Al NMR signal appeared at 46.3 ppm after 20 min p.i. (Figure 5).

A broadening of the signal was observed *in vivo* which may be explained by higher viscosity with concomitant increase of the rotational correlation time. The infusion was stopped after 72 min.

Atomic absorption spectroscopy for aluminum showed that the blood value was 140 μM and the urine level was >8 mM. In a control rat 0.3 μM of aluminum in blood and 1.3 μM in urine were found. ^{27}Al NMR spectroscopy of a urine sample collected over a period of 24 hours only showed the characteristic signal of the chelate (Figure 2-b), which clearly indicates that the chelate is not metabolized during the elimination through the kidneys. The high elimination rate via the kidneys explains the relatively low blood concentration.

4 - Conclusions

In vivo ^{27}Al NMR of aluminum macrocyclic chelates was shown to be feasible. The low *in vivo* ^{27}Al NMR signal intensity indicated low blood concentration of the chelates [13], which is due to the high clearance rate through the kidneys. The low NMR signal intensity does not allow MR- imaging within a reasonable acquisition time although a *in vivo* dynamic spectroscopic observation of the metallic chelates is possible.

The short T_1 time allows a fast acquisition and the relatively small half-width of the signal renders these aluminum chelates potential compounds for studies of biomedical interest. The possibility of coupling NODASA-metal chelates to biomolecules through the free pendant arm [11, 21] might render possible the tunning of their solubility and bioselectivity thus changing their pattern of biodistribution in organs and tissues.

Footnotes

§ We used the expression $K_{cond}=\alpha K_{ML}$, where $\alpha^{-1}= (1 + [H^+]/K^1 + [H^+]^2/K^1K^2 + ...+ [H^+]^n/K^1K^2...K^n)$; K^1, K^2, ...,K^n refer to the stepwise deprotonation constants of the ligand, determined by potentiometric titration [11].

Acknowledgements

J. P. A. and H. R. M. acknowledge the Swiss National Science Foundation for financial support and NOVARTIS for MS and NMR spectra.

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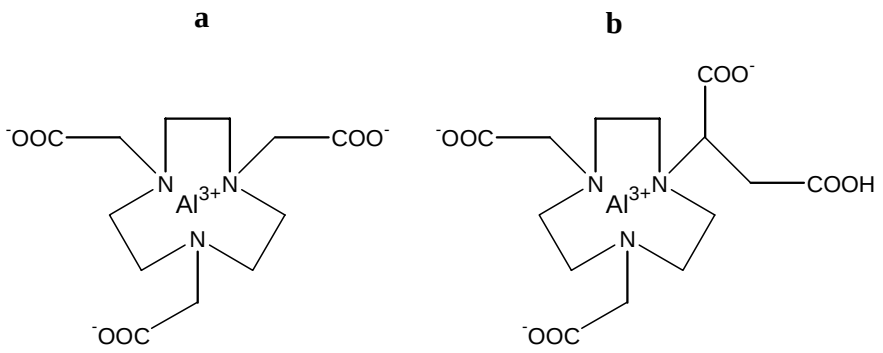

Figure 1- Chemical structures of Al(NOTA) **(a)** and Al(NODASA) **(b)**.

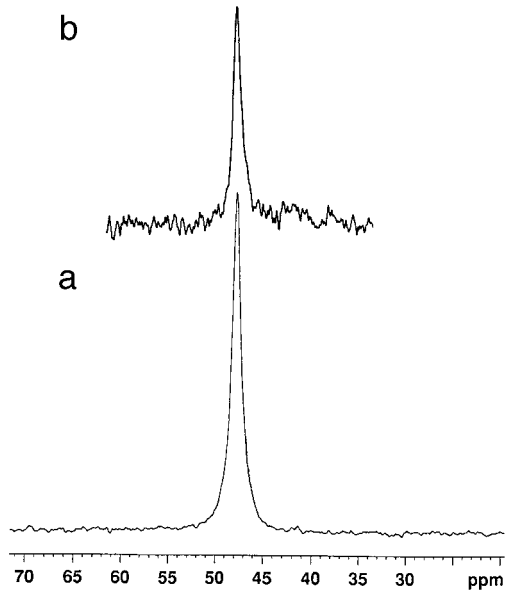

Figure 2 (a) *In vitro* ^{27}Al NMR spectrum of Al(NODASA), (H_2O, 10 mM, pH = 5.5, T = 20 ^{o}C); the signal appears at 47 ppm with a half width of 80 Hz (200 scans).

(**b)** The *in vitro* ^{27}Al NMR spectrum of Al(NODASA) in the urine of a rat collected over 24 hours after the chelate injection shows the same chemical shift.

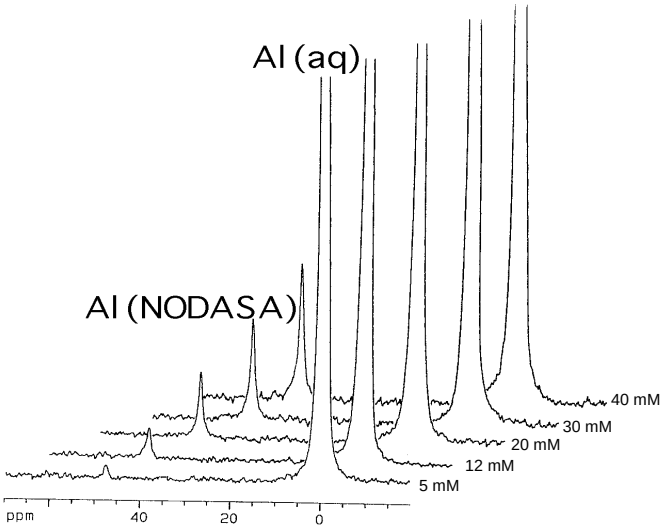

Figure 3 – Estimation of the thermodynamic stability constant of Al(NODASA) by ^{27}Al NMR spectroscopy.

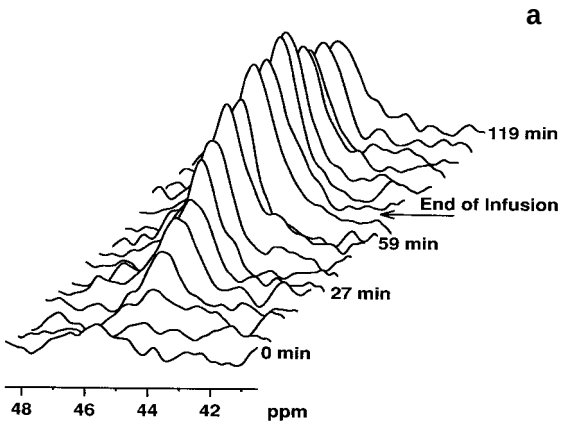

Figure 4 (a) Stack plot of the ^{27}Al NMR spectra of the *in vivo* experiment with Al(NOTA) in a rat. For one spectrum 2000 scans were accumulated. Prior to Fourier transformation the spectral data were treated with a 40 Hz Gauss filter. The linewidth was (unfiltered) 95 Hz. The *in vivo* signal appears *circa* 4 ppm lower (45 ppm) than *in vitro*. As marked with an arrow the infusion was stopped and the signal intensity decreased. **(b)** Time course of the signal intensity (integrated area) of the spectra. The linear decrease of the signal intensity demonstrates a constant clearence rate.

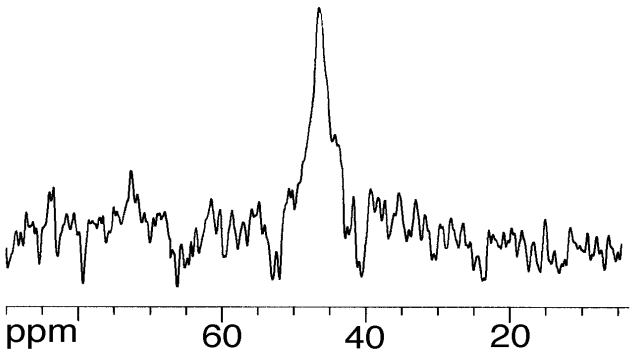

Figure 5 *In vivo* ^{27}Al NMR experiment with Al(NODASA) in a rat. The spectrum was the sum of 3000 scans. Also in this case the signal appears a few ppm lower than *in vitro.*