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ORIGINAL ARTICLE

Luce Vander Elst · Florence Chapelle
Sophie Laurent · Robert N. Muller

Stereospecific binding of MRI contrast agents to human serum albumin: the case of Gd-(*S*)-EOB-DTPA (Eovist) and its (*R*) isomer

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Abstract The water proton relaxation rate enhancement of the hepatospecific Gd-(*S*)-EOB-DTPA (Eovist) and of its (*R*) isomer in aqueous solutions free of protein, in serum and in 4% human serum albumin solution, are compared. In the absence of proteins, both compounds exhibit, as expected, the same proton relaxivity, as measured by the nuclear magnetic relaxation dispersion (NMRD) profiles. In serum and albumin solution, non-covalent binding of the paramagnetic complexes to macromolecules is observed. Both isomers are likely to bind to the same site of human serum albumin, but the affinity of the (*S*) isomer is larger than for the (*R*) isomer.

Keywords Gd-EOB-DTPA · MRI contrast agent · Non-covalent binding · Human serum albumin

Introduction

In the context of MRI contrast media development, non-covalent binding of small paramagnetic complexes to endogenous macromolecules is a strategy used to improve the relaxation enhancement power by slowing down the rotational motion, and to reduce the extravasation. For example, MS-325 [AngioMARK, trisodium {4-(*R*)-[(4,4-diphenylcyclohexyl)phosphonooxymethyl]-3,6,9-triaza-3,6,9-tris(carboxymethyl)undecanedioato}gadolinium(III)], a derivative of Gd-DTPA (Magnevist), which binds non-covalently and efficiently to human serum albumin (HSA), has been reported to provide a very good enhancement of blood vessels in MRI [1, 2, 3, 4, 5, 6, 7, 8, 9, 10]. Another derivative of Gd-DTPA, MP-2269 [Gd(III) complex of 4-pentylbicyclo[2.2.2]octane-1-carboxyldi-L-*aspartyllysine-derived* DTPA] was also shown to have a high affinity for serum albumin (owing to its non-aromatic side chain) and to afford excellent vascular enhancement [11, 12, 13, 14]. Gd-(*S*)-EOB-DTPA [Eovist: (4*S*)-4-(4-ethoxybenzyl)-3,6,9-tris(carboxylatomethyl)-3,6,9-triazaundecanedioic acid, gadolinium complex, disodium salt] (Fig. 1), which was designed as a hepatobiliary contrast agent specifically taken up by the hepatocytes, is also known for its moderate non-covalent binding to HSA [15, 16, 17].

When MRI contrast agents bind non-covalently to proteins, their efficacy as described by their water proton relaxation rate enhancement is directly related to the amount of contrast agent bound to the macromolecules. The larger the association constant, the higher the efficacy for MRI studies and the lower the injected dose. Since HSA is known for its stereospecific binding to numerous endogenous and exogenous compounds, the presence of chiral carbons in the MRI contrast agents (as those cited above) can influence the strength of the interaction as well as the number of binding sites of each stereoisomer. Subsequently, one of the isomers could be much more efficient than

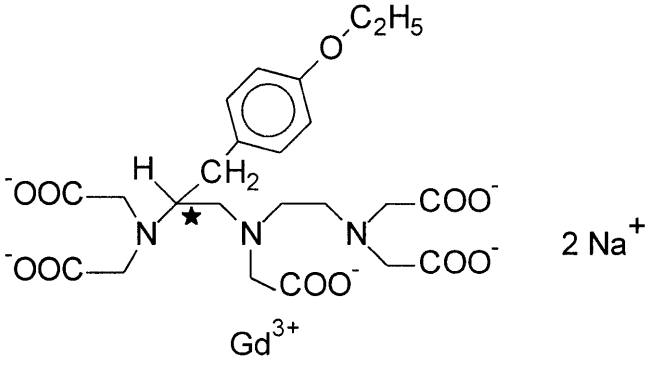

Fig. 1 Chemical structure of Gd-EOB-DTPA

L. Vander Elst · F. Chapelle · S. Laurent · R.N. Muller (✉)
NMR Laboratory, Department of Organic Chemistry,
University of Mons-Hainaut, 7000 Mons, Belgium
E-mail: robert.muller@umh.ac.be
Phone: +32-65-373520

the other ones, but to our knowledge no such study has been reported so far.

The aim of this work was thus to assess the influence of the chiral carbon configuration on the efficacy of each isomer of Gd-EOB-DTPA in HSA and other protein-containing solutions. Since binding to macromolecules modifies the water proton relaxation rate enhancement through the prolongation of the rotational correlation time (τ_R) of the paramagnetic center, proton relaxometry is a well-suited methodology for such a study [9, 10, 17, 18, 19, 20, 21, 22]. The evolution of the water proton paramagnetic relaxation rate as a function of the relative concentrations of the contrast agent and of the protein can indeed be analysed in term of the association constant [9, 10, 17, 18, 19, 21, 22].

Materials and methods

Products

Non-defatted HSA and serum Kontrollogen L were respectively purchased from Sigma (Bornem, Belgium) and from Behring (Marburg, Germany). Gd-(*S*)-EOB-DTPA, the commercial isomer (Eovist), Gd-(*R*)-EOB-DTPA and the ligand (*S*)-EOB-DTPA were provided by Schering (Berlin, Germany). La-(*S*)-EOB-DTPA was synthesized by stirring an aqueous solution containing 2 equiv of the ligand and 1 equiv of lanthanum oxide (La_2O_3, Aldrich, Bornem, Belgium) at room temperature and pH 5.5–6.5. After 6 h, acetone was added and the solid complex was filtered.

Proton relaxometry

Nuclear magnetic relaxation dispersion (NMRD) data were obtained at 310 K on 0.6 mL samples on a field cycling system (FCS) relaxometer operating at proton Larmor frequencies ranging from 0.02 MHz to 50 MHz. Additional points were obtained at 300 MHz on a AMX-300 spectrometer (Bruker, Karlsruhe, Germany).

The protein binding studies were performed by measuring the proton relaxation time on a spin analyzer operating at 0.47 T and 310 K (PC-20 Minispec, Bruker). In these experiments, a constant concentration of HSA equal to 4% w/v was used, whereas the concentration of both isomers of Gd-EOB-DTPA was varied from 0.05 to 6.25 mM.

Data analysis

Classical equations describing the inner- and the outer-sphere interactions have been used [23, 24, 25] to analyze the proton NMRD data. Fitting of the ^{1}H NMRD curves was performed with previously described software using minimization routines (Minuit, CERN Library) [26, 27]. In the fittings, some parameters were fixed to the values obtained in a previous study of Eovist [17], i.e. the number of water molecules in the first coordination sphere (q=1), the residence time of the coordinated water molecule (τ_M=83 ns), the mean distance between the proton of the coordinated water molecule and the gadolinium ion (r=2.81 Å), the distance of closest approach of outer-sphere water molecules (d=3.6 Å [28, 29]) and the relative diffusion constant (D=3.5×10^{-9} m^2 s^{-1}) [30]. The rotational correlation time (τ_R) and the parameters describing the electronic relaxation rates (τ_{SO} and τ_V) were fitted.

For the protein solutions, the relaxation rate enhancements which correspond to the paramagnetic proton relaxation rates are obtained by subtracting the diamagnetic proton relaxation rates (0.40 s^{-1} for 4% HSA solution and 0.58 s^{-1} for serum solution at 0.47 T and 310 K) from the observed relaxation rates.

If we assume a non-covalent binding which involves one site exhibiting a much larger affinity than the other ones [31], then the longitudinal relaxation rate enhancement of water protons ($R^P_{1\,obs}$) is given by Eq. 1:

$$R^P_{1\,obs} = 1000 L_0 r_1^F + \frac{1000(r_1^B - r_1^F)\left[(P_0 + L_0 + K_a^{-1}) - \sqrt{(P_0 + L_0 + K_a^{-1})^2 - 4L_0P_0}\right]}{2} \quad (1)$$

where r_1^F and r_1^B are the proton relaxivities (L s^{-1} mmol^{-1}) of the free and the bound paramagnetic complex respectively, L_0 and P_0 are the initial concentrations of Gd-EOB-DTPA and HSA (mol L^{-1}), and K_a is the association constant characterizing the equilibrium between the paramagnetic complex and the binding site on the protein. The fitting of the data obtained using this equation and minimization routines (Minuit, CERN Library) allows, in principle, the estimation of the association constant K_a and r_1^B. During the fitting procedure, r_1^F was allowed to fluctuate by 10% around the value obtained in pure water. The error given by the fitting procedure corresponds to the square root of the diagonal elements of the covariance matrix.

For N equivalent binding sites, an equation equivalent to Eq. 1 can be used where P_0 is replaced by NP_0.

Results and discussion

Proton NMRD profiles in water

The proton longitudinal relaxivities r_1 (defined as the proton longitudinal relaxation rate enhancement induced by 1 mM of the complex) of both gadolinium complexes in water at proton Larmor frequencies ranging between 0.02 and 300 MHz are, as expected, similar (Fig. 2) and r_1 at 20 MHz is equal to 5.3–5.4

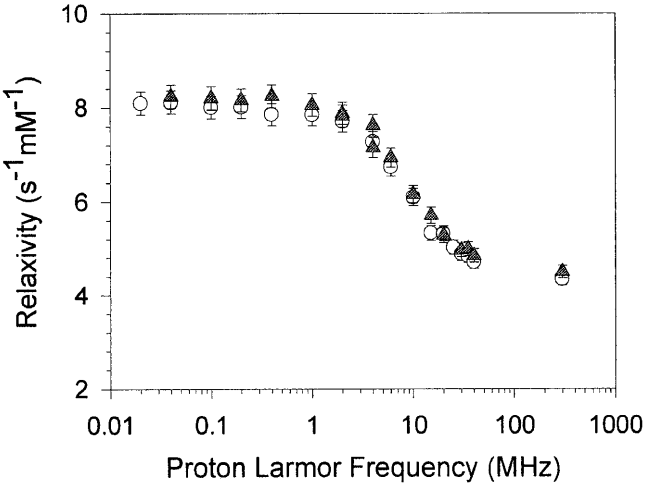

Fig. 2 Proton NMRD profiles of Gd-(*S*)-EOB-DTPA (*open circles*) and Gd-(*R*)-EOB-DTPA (*closed triangles*) at 310 K in water solution free of protein

Table 1 Values of τ_R, τ_{SO} and τ_V obtained from the fitting of the proton NMRD data of both isomers of Gd-EOB-DTPA in water at 310 K

	Gd-(*S*)-EOB-DTPA	Gd-(*R*)-EOB-DTPA
τ_R (ps)	59.6±1.2	57.1±1.2
τ_{SO} (ps)	59.7±1.4	59.1±1.4
τ_V (ps)	16.2±1.2	15.7±1.16

s^{-1} mM^{-1}. The values obtained for τ_R, τ_{SO} and τ_V by the theoretical fitting of the data are shown in Table 1.

Proton relaxometry in protein-containing media

In the absence of significant interaction between the contrast agent and the seric components, a slight increase of the paramagnetic relaxation rates (increase $\leq$30%) of a solution of Gd complex (1 mM) is expected as compared to the value obtained in water solution. This small difference can be related to a reduction of the rotational motion induced by the higher viscosity of serum (1.15 cP for serum versus 0.69 cP for water at 310 K) or by non-specific and weak interactions with serum components. On the other hand, when a gadolinium complex binds to macromolecules, a significant and concentration-dependent increase of the paramagnetic relaxation rates is observed as a result of the slower rotational motion upon binding to macromolecules. At constant protein concentration, the lower the concentration of gadolinium complex, the larger the proportion of bound gadolinium complex and, consequently, the larger the ratio between the paramagnetic relaxation rates observed in the presence and in the absence of proteins. This leads to a non-linear evolution of R_1^p for concentrations of the paramagnetic complex of the order of magnitude of the protein concentration.

From the paramagnetic relaxation rates observed for both isomers of Gd-EOB-DTPA in serum and HSA solutions at 0.47 T (Fig. 3), it is obvious that they interact with serum proteins and that the phenomenon is more pronounced for the (*S*) isomer than for the (*R*) isomer. For a concentration of 1 mM in contrast agent, the paramagnetic relaxation rates in serum are increased by 7.9 s^{-1} (150%) and 4.7 s^{-1} (90%) for the (*S*) and (*R*) isomers, respectively, as compared to the values observed in the aqueous solution free of protein. The difference between the isomers can be explained either by a binding of both isomers to the same site(s) but with a stronger interaction for the (*S*) isomer or, less likely, by different distribution of the isomers among various sites.

Since the (*S*) isomer is known to bind mainly to serum albumin [17], the increase of relaxation rate observed in serum must therefore result, at least in part, from the interaction with this protein. A similar

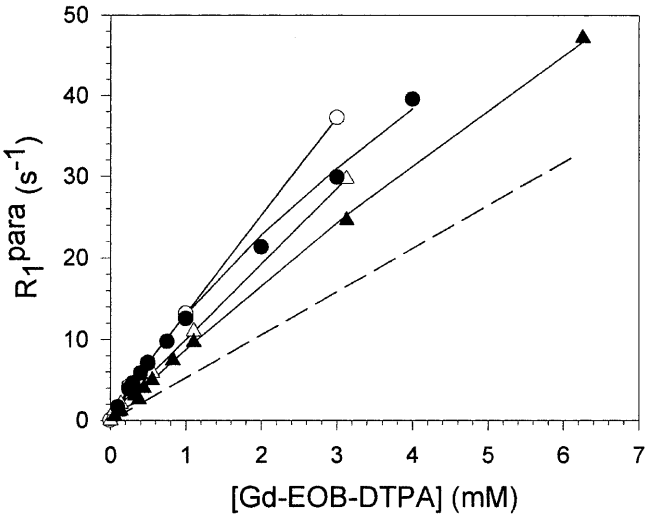

Fig. 3 Proton paramagnetic relaxation rate in solutions containing, on the one hand, serum Kontrollogen L and increasing amounts of Gd-(*S*)-EOB-DTPA (*open circles*) or Gd-(R)-EOB-DTPA (*open triangles*) and, on the other hand, 4% HSA and Gd-(*S*)-EOB-DTPA (*closed circles*) or Gd-(*R*)-EOB-DTPA (*closed triangles*) at 310 K and 20 MHz. The *dashed line* represents the paramagnetic relaxation rate of both isomers in water

evolution of the paramagnetic relaxation rates measured at the same magnetic field is indeed observed for each isomer in HSA solutions (4% w/v) (Fig. 3). At a concentration of 1 mM of contrast agent, the increase is 7.3 s^{-1} (137%) and 3.5 s^{-1} (67%) for the (*S*) and (*R*) isomers, respectively. As already observed in serum, the enhancement is larger for the (*S*) isomer but the overall effect is of a smaller extent (Fig. 3). The differences between both media can be attributed to the higher viscosity of serum (1.15 cP and 0.84 cP for serum and 4% HSA solution, respectively) and/or, as mentioned above, to a binding to other serum components. The fitting of the data obtained in HSA solutions was performed using Eq. 1 with the assumption of one binding site. Association constants of 772± 195 L mol^{-1} for the (*S*) isomer and 177±84 L mol^{-1} for the (*R*) isomer were obtained. This K_a value for the (*S*) isomer is comparable to those obtained previously by proton relaxometry (909 L mol^{-1}), dialysis (333 L mol^{-1}) and ^2H NMR of the deuterated ligand (250 L mol^{-1}) [17]. The fitted values of r_1^F and r_1^B are similar for both isomers: r_1^F=5.4±0.3 L s^{-1} $mmol^{-1}$, r_1^B=39.0± 4.4 L s^{-1} $mmol^{-1}$ for the (*S*) isomer and r_1^F=5.9±0.5 L s^{-1} $mmol^{-1}$, r_1^B=35.6±6.1 L s^{-1} $mmol^{-1}$ for the (*R*) isomer. Additional fittings were performed assuming that *N*, the number of equivalent binding sites, was free to fluctuate from 0.1 to 4. This procedure gives values of *N* close to 1 [0.81±0.14 and 1.22±0.54 for the (*S*) and (*R*) isomers, respectively] and values of K_a comparable to those obtained above [778±139 L mol^{-1} for the (*S*) isomer and 118±54 L mol^{-1} for the (*R*) isomer]. Also, the relaxivities in the free and bound

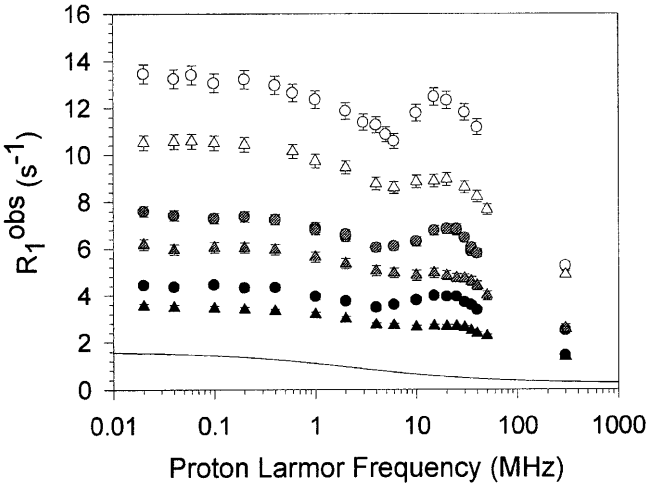

Fig. 4 Proton NMRD profiles of solutions containing 4% HSA and increasing amounts of Gd-(*R*)-EOB-DTPA (*triangles*) and Gd-(*S*)-EOB-DTPA (*circles*) at 310 K (*black circles*, *black triangles* 0.25 mM; *grey circles*, *grey triangles* 0.5 mM; *white circles, white triangles* 1 mM). The *solid line* represents the NMRD profile of a 4% HSA solution free of contrast agent

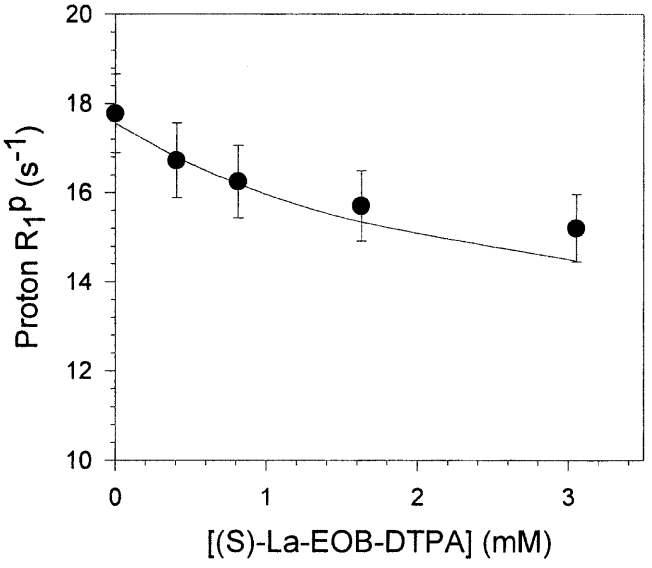

Fig. 5 Evolution of the proton relaxation rate at 20 MHz and 310 K of a solution containing 4% HSA, 2.06 mM Gd-(*R*)-EOB-DTPA and increasing amounts of La-(*S*)-EOB-DTPA. The fitting has been performed using values of r_1^B and r_1^F equal to 40 and 6 mM^{-1} s^{-1}, respectively, and association constants of 772 M^{-1} for the lanthanum complex of the (*S*) isomer and 177 M^{-1} for the gadolinium complex of the (*R*) isomer

states were similar for both isomers and comparable to those obtained with *N* fixed to 1 [r_1^F=5.6±0.3 L s^{-1} $mmol^{-1}$, r_1^B=44.1±4.0 L s^{-1} $mmol^{-1}$ for the (*S*) isomer and r_1^F=5.6±0.4 L s^{-1} $mmol^{-1}$, r_1^B=44.6±1.0 L s^{-1} $mmol^{-1}$ for the (*R*) isomer].

As expected for paramagnetic compounds binding to macromolecules, the proton NMRD profiles of solutions containing 4% HSA (0.0004–7.05 T) and increasing amounts of (*R*)- and (*S*)-Gd-EOB-DTPA show a hump at Larmor frequencies around 20–30 MHz (Fig. 4). Confirming the observations made at 0.47 T, the (*S*) isomer exhibits larger relaxation rates over the whole magnetic field range and for each contrast agent concentration (Fig. 4).

The potential competition of the isomers for the same binding site(s) was studied through the addition of the "magnetically silent" La-(*S*)-EOB-DTPA to a solution containing 4% HSA and 2.06 mmol L^{-1} of Gd-(*R*)-EOB-DTPA. A decrease of the amount of bound (*R*) isomer and the subsequent decrease of the paramagnetic relaxation rate of the solution will indicate a competition between the diamagnetic (*S*) isomer and the paramagnetic (*R*) isomer for the same binding site. If the isomers do not compete for the same site and if their binding sites do not influence each other, no change of the paramagnetic relaxation rates should be observed.

The experimental data show that the addition of diamagnetic La-(*S*)-EOB-DTPA results in a marked decrease of the proton paramagnetic relaxation rate (Fig. 5).

Conclusions

In aqueous solution free of protein, the configuration of the asymmetric carbon of Gd-EOB-DTPA has, as expected, no influence on the proton relaxivity. As indicated by the significant and specific enhancements of the proton relaxation rates in serum, it is however obvious that both isomers bind to serum components and that the interaction is more pronounced for the (*S*) isomer than for the (*R*) isomer. Similar results were obtained in solutions containing 4% HSA. The competition experiment performed on the system HSA/Gd-(*R*)-EOB-DTPA with added La-(*S*)-EOB-DTPA supports the assumption that both isomers compete for the same binding site. The similarity of the behavior of both contrast agents in serum and HSA media confirms that, among serum macromolecules, albumin plays a predominant role in the non-covalent binding of such small MRI contrast agents carrying lipophilic chains.

The differences in affinity observed for both isomers point out the importance of the configuration of stereoisomers when non-covalent interaction between MRI contrast agent and HSA is involved. Consequently, enantioselective resolution of racemic mixtures or stereoselective synthesis are well worth considering for the design and the development of new contrast agents, since the extent of their protein binding and consequently their efficacy, pharmacology and pharmacokinetics are very likely to be governed by their stereoisomerism. It is also interesting to

underline that the relaxivity calculated for the bound species of each isomer is similar, showing that either the anchorage of the ligand does not selectively influence the magnetic interaction between the paramagnetic ion and the water protons, or that the efficiency of this interaction is limited by other factors. Finally, it is to be stressed that Eovist, the (*S*) isomer of Gd-EOB-DTPA which was developed for clinical applications, has a higher affinity for albumin than the (*R*) isomer.

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