Sulfonamide-Functionalized Gadolinium DTPA Complexes as Possible Contrast Agents for MRI: A Relaxometric Investigation

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Keywords: NMRD / MRI / Contrast agents / Carbonic anhydrase / Sulfonamides

A novel Gd-DTPA derivative with a built-in sulfonamide (SA) was synthesized as a contrast agent for MRI. The complex was designed to selectively target the enzyme carbonic anhydrase. It is shown that the longitudinal relaxation rates of aqueous solutions of Gd-DTPA-SA in the presence of carbonic anhydrase increase significantly. The binding constant is determined to be 15,000 ± 5,000 M^{-1}. This value ensures substantial formation of the carbonic anhydrase adduct at imaging concentrations of Gd-DTPA-SA. The complex interacts with erythrocytes, presumably due to a high affinity for the carbonic anhydrase present on the outer surface of the latter. This takes place even though the enzyme has a low abundance and is easily saturated by small amounts of Gd-DTPA-SA. The interaction of Gd-DTPA-SA with serum proteins is negligibly small. Therefore, the complex could potentially be tested as a selective contrast agent for compartments outside the blood pool.

Introduction

Contrast agents for MRI are characterized by their large nuclear relaxation capability for the protons of water molecules. In general, a complex of a paramagnetic metal ion, typically gadolinium(III) with seven unpaired electrons, is bound to at least one water molecule, which exchanges rapidly with bulk water. In imaging fields, the gadolinium complex relaxes the protons of the coordinated water at rates ranging from 10^5 s^{-1} for small molecules to 10^6 s^{-1} for macromolecules, whereas the protons of bulk water relax at about 0.4 s^{-1}. If the coordinated water exchanges at a rate of the order of 10^6 s^{-1} or faster, it transfers significant relaxation enhancements to bulk water. This enhancement is what is measured in relaxometry and determines the properties of the contrast agent.[1–3]

Besides low-toxicity requirements, the development of a good contrast agent aims at achieving two major objectives: (i) the increase of the rotational correlation time, τ_r, with respect to the value relative to small complexes, in order to increase the relaxation rates of protons in the perfused tissues, and (ii) the localization of the contrast agent with a larger concentration in some compartments with respect to others nearby. Both requirements can be satisfied by synthesizing a molecule with an efficient targeting system that is able to provide interactions between the agent and specific macromolecular receptors. Such interactions will be regulated by a binding constant, that should be high enough to maximize the effect of the contrast agent even if administered in low doses. Binding constants of the order of 10^4 M^{-1} are large enough to ensure good affinity. The ideal binding constant should also ensure a residence time that is long enough for the MRI acquisition but not so long as to appreciably increase toxicity, as it might happen if the binding constant were much higher.[4]

Organic moieties attached to the complex favor the interaction of the contrast agent with biological molecules or tissues. Three important examples of contrast agents of this class are Gd-BOPTA,[5,6] Gd-EOB-DTPA,[7] and MS-325,[8] which are able to bind noncovalently to serum albumin and can thus highlight the uptake of the gadolinium complex by hepatocytes. Contrast agents have also been proposed to be used as reagents for quantitative in vitro assays, as, for example, Gd-DTPA derivatives containing boronic residues, which should be able to bind to glycated proteins.[9]

Another strategy was used to detect regions in the body or cells where certain enzymes are concentrated. For example, if a gadolinium complex is covalently bound to a galactopyranose residue, so that the access of water to the paramagnetic center is sterically blocked, β-galactosidase, if present, cleaves this bond and thus may expose gadolinium to the solvent.[10] Wherever β-galactosidase is present, the magnetic resonance image is then improved by the contrast agent. If a contrast agent is functionalized with a molecule that is able to specifically react with an enzyme, the contrast agent targets the enzyme and any enzyme-rich tissue. Examples are sulfonamides, which are known to be specific inhibitors of carbonic anhydrase (CA) and have been widely used in medical therapy.[11,12] Carbonic anhydrase is a zinc enzyme present in various tissues in variable amounts.[11] Therefore, different tissues may exhibit different behavior depending on the presence of the enzyme and its accessibility, which could be altered during pathologic processes.[13] Therefore, by synthesizing a Gd-DTPA contrast agent with a sulfonamide (SA) at-

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tached, binding to carbonic anhydrase and relaxation enhancement may be expected.

In this paper, we describe the synthesis of a contrast agent functionalized with the sulfonamide ethylene sulfanilamide (Gd-DTPA-SA). The molecule is shown to bind carbonic anhydrase effectively in vitro with a suitable binding constant and to display a sizable relaxation enhancement. Although not designed as a blood-pool reagent, Gd-DTPA-SA shows a small relaxation enhancement in intact blood with respect to serum, probably as a consequence of its interaction with the small amount of carbonic anhydrase bound to the outside of the erythrocyte membranes.[14]

Results and Discussion

NMRD Characterization of the Gd-DTPA-SA Complex

The paramagnetic relaxivity profile of Gd-DTPA-SA in an aqueous solution at 298 K is shown in Figure 1 (open circles). It is obtained by subtracting the diamagnetic contribution of the solvent relaxation rate in the absence of the paramagnetic compound, R_{1dia}, from the total relaxation rate of the solution, R_1, and normalizing to 1 mM Gd-DTPA-SA.

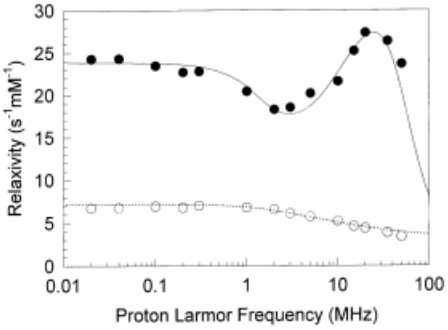

Figure 1. Proton ^{1}H-NMRD profiles of a Gd-DTPA-SA solution at 298 K in the absence (open circles) and in the presence (filled circles) of carbonic anhydrase (1.36 mM); the dotted and the solid lines represent the best fit profiles of the two sets of data

The relaxivity profile of Gd-DTPA-SA appears to be qualitatively and quantitatively very similar to the relaxivity profile of Gd-DTPA.[1] Only one inflection is present, centered at about 10 MHz, corresponding to the ω_s dispersion (where ω_s is the electron Larmor frequency in rad s^{-1}). The data were fitted by taking into account both the outer- and inner-sphere contributions; for comparative purposes, the resulting parameters are reported together with those of Gd-DTPA[1,15,16] (Table 1). The best fit profile is reported as a dotted line in Figure 1. The fit was made by keeping the parameters determining the outer-sphere contribution (the distance of closest approach, d, and the diffusion coefficient, D_{diff}) constant. Two protons were fitted at a distance of about 3 Å, the values of the other parameters being in agreement with those expected from the results of similar complexes,[5,7,15–22] including the increase in the exchange time between bulk and coordinated protons, τ_m, on passing from the binegative Gd-DTPA to the mononegative Gd-DTPA-SA complex.[23]

NMRD Characterization of the Gd-DTPA-SA–Carbonic Anhydrase Complex

Increasing concentrations of CA were added to the Gd-DTPA-SA solution in order to estimate the binding capability of the Gd-DTPA-SA complex. Relaxation measurements of such solutions were performed at 0.02, 0.15, 10, and 15 MHz. Monitoring the field dependence of water proton relaxation allows a better characterization of the parameters responsible for relaxation. Figure 2 shows the water proton longitudinal relaxation rates for solutions with different CA concentrations at 0.02 MHz. A 1:1 binding is assumed as sulfonamides are well known to interact specifically with the catalytic metal ion and the active site of the enzyme in a 1:1 fashion.[13] The data were fitted according to Equations 4–6, and a value for the binding constant of 15,000 ± 5000 M^{-1} was found. The relaxivity of the adduct, R_{add}, was found to be 25.8 s^{-1} mM^{-1} at 0.02 MHz.

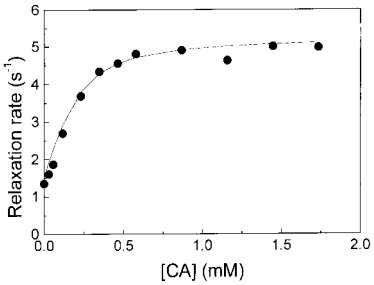

Figure 2. Titration curve of a solution of Gd-DTPA-SA (0.2 mM) with carbonic anhydrase at 0.02 MHz and 298 K

Then, the NMRD profile of a 0.2 mM Gd-DTPA-SA solution with a CA concentration of 1.36 mM (pH 8.1, 298 K) was obtained. This CA concentration ensured that the gadolinium complex was fully bound to CA. The relaxation rate values, after subtraction of the diamagnetic contribu-

Table 1. Best-fit parameters of the ^{1}H-NMRD data on Gd-DTPA and Gd-DTPA-SA in aqueous solution and in a solution containing excess carbonic anhydrase (1.36 mM) at 298 K

Complex	τ_r[a] (10^{-12} s)	τ_v (10^{-12} s)	τ_m (10^{-6} s)	Δ_t cm^{-1}	n_H[b]	r Å	D (ZFS)[c] cm^{-1}	d[b] Å	D (diff. coeff.)[b] (10^{-5} cm^2s^{-1})
[Gd-DTPA][d]	58–73	18–25	0.30	0.034–0.037	2	3.1	0	3.6	2.6
[Gd-DTPA-SA]	64	17	0.50	0.042	2	3.1	0	3.6	2.6
[Gd-DTPA-SA]-CA	12,000	18	0.56	0.017	2	3.0	0.008	3.6	2.6

[a] In the adduct, this value is estimated from the Stokes' equation and kept constant in the fitting procedure. – [b] This value is kept constant in the fitting procedure. – [c] The transient ZFS parameter was introduced to fit the NMRD profile of the adduct only, as it was found not necessary to fit the free complex profile. – [d] From refs. [1, 15, 16]

tion of CA at the same temperature and normalization to 1 mM, are reported in Figure 1 (filled circles). The introduction of CA both increases the relaxivity values and modifies the shape of the profile. The profile now exhibits three inflections: the first (centered at about 1 MHz) can be attributed to the ω_s dispersion, the second (centered at about 10 MHz) is the result of the field-dependent electron relaxation (see below), and the third inflection, for frequencies greater than 30 MHz, is related to the ω_I dispersion (ω_I indicates 2π times the proton Larmor frequency). This behavior is due to a dramatic change in the correlation time for the proton relaxation, given by the shortest time among rotational (τ_r), exchange (τ_m), and electron relaxation (τ_e) time, according to Equation 1.[24]

$$\tau_c^{-1} = \tau_r^{-1} + \tau_m^{-1} + \tau_e^{-1} \quad (1)$$

The rotational correlation time can be estimated from the Stokes-Einstein equation (Equation 2).

$$\tau_r = \frac{4\pi a^3 \eta}{3kT} \quad (2)$$

where a is the radius of the spherical-modeled molecule, η is the viscosity of the solvent, k is the Boltzmann constant and T indicates the temperature. The value of τ_r varies from $\simeq$70 ps for the free complex to $\simeq$12,000 ps for the bound complex (MW $\simeq$30,000). Therefore, τ_r is the dominant correlation time for the nuclear relaxation (according to Equation 1) in the Gd-DTPA-SA solution,[21,25] but it loses its influence when the CA adduct is formed.[21,25] τ_m is likely to be longer, as verified a posteriori (see below). In this case, τ_c is given by the electron relaxation time only and is calculated according to the Bloembergen-Morgan equation (Equation 3).[15,26,27]

$$\tau_e^{-1} = \frac{2\Delta_t^2 (4S(S+1)-3)}{50}\left(\frac{\tau_v}{1+\omega_s^2\tau_v^2} + \frac{4\tau_v}{1+4\omega_s^2\tau_v^2}\right) \quad (3)$$

where ω_s is the electron Larmor frequency, S is the electron spin quantum number, τ_v is the correlation time for electron relaxation and Δ_t^2 is the average quadratic transient zero field splitting.[28] The decay of the electron relaxation rate as a function of the magnetic field is responsible for the peak relaxivity in the 10–50 MHz region of the proton relaxation rate. Therefore, the occurrence of this peak in the high field part of the NMRD profile confirms the presence of binding between Gd-DTPA-SA and CA. The profile was fitted (solid line in Figure 1) and the resulting best-fit parameters are reported in Table 1. With τ_r being fixed at such a large value that it is negligible in the calculation of the correlation time, the fitting procedure provides values of τ_v, τ_m, and Δ_t close to those obtained for other Gd-DTPA derivatives bound to HSA.[7,21,29,30] It should be noted, in particular, that the value of τ_m is almost the same after binding and, in any case, is longer than τ_e. From the fitting, τ_m is the limiting factor for the relaxivity peak between 10–50 MHz. As required for many protein–Gd complex adducts, the effect of a small static zero-field splitting (D) was introduced so that the profile could be reproduced better.[21]

The NMRD measurements show a good binding capability of Gd-DTPA-SA with carbonic anhydrase. The binding constant is 15,000 M^{-1}, compared to 300 M^{-1} for Gd-BOPTA and HSA, 700 M^{-1} for Gd-DTPA-EOB and HSA, or 1,700 for Gd-DOTA-$(BOM)_3$ and HSA.[30] This binding constant ensures that the carbonic anhydrase adduct is largely formed at imaging concentrations. As previous studies have shown,[13] the large value of the binding constant of sulfonamides for CA is accompanied by favorable life times of the gadolinium complex–protein adduct of the order (of a few hours).

The relaxivity of the gadolinium complex at 1 T increases from about 3.5 $mM^{-1}s^{-1}$ when free to about 25 $mM^{-1}s^{-1}$ when bound to the protein. Such an increase is comparable to that of other Gd complexes when they are completely bound to the HSA present in solution.[29] However, such an increase does not occur at the imaging concentration of other Gd complexes (Gd-BOPTA, Gd-EOB-DTPA) because of their relatively small binding constants.[6]

Measurements in Intact Blood

Experiments on human blood were performed to test whether it was possible to detect binding of Gd-DTPA-SA to the small amount of carbonic anhydrase present on the surface of the red cells. Different concentrations of Gd-DTPA-SA were added to human blood samples (and to human serum samples as controls) and relaxation measure-

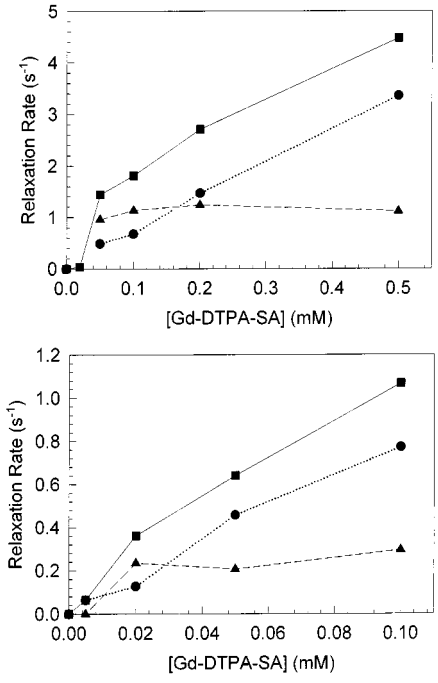

Figure 3. Paramagnetic enhancement in the proton relaxation rate measured in human blood (solid lines) and in serum (dotted lines) as a function of the Gd-DTPA-SA concentration at 0.02 MHz (A) and 50 MHz (B) at 298 K; the differences in relaxation rate between blood and serum are reported as dashed lines

ments at proton Larmor frequencies of 0.02, 0.15, 1.2, 35, and 50 MHz (corresponding to about 0.0005, 0.004, 0.03, 0.8, and 1.2 T) were performed. A small enhancement in proton relaxation was observed. The results obtained at two frequencies (0.02 and 50 MHz), decreased by the diamagnetic contributions, are shown in Figure 3, together with the difference between the paramagnetic enhancements in blood and serum. The low field measurements are more reliable, since the differences detected are much larger with respect to the instrumental precision. The measurements performed at the imaging fields are affected by larger errors, but provide the same indications as the low field experiments. The plots indicate that the Gd complex is bound in the blood sample, while it is free in the serum sample. This is shown by the higher initial increase of relaxation in the blood sample compared to the serum sample. The difference in the two samples levels off to an approximately constant value above Gd concentrations of 0.02–0.04 mM, where the increase in the relaxation rates is linear with the concentration in both blood and serum samples. Presumably, this behavior is a consequence of the saturation of the available carbonic anhydrase on the membranes of the red cells.

Conclusions

We have shown that a Gd-DTPA derivative targeted to bind to carbonic anhydrase in vivo has the desired fundamental properties in terms of binding constant and relaxation enhancements. We have also shown that the compound does not appreciably interact with any soluble macromolecule in serum, and that the amount of carbonic anhydrase present on the outer erythrocyte membranes is too small (although detectable through its enhancement) to block this reagent in the blood pool. Therefore, Gd-DTPA-SA is a potential candidate for in vivo tests for specificity in various tissues outside the blood pool.

Experimental Section

Materials: Carbonic Anhydrase (lyophilized powder from bovine erythrocytes, MW 30000) was purchased from Sigma. *N,N*-Bis(2-{bis[2-(1,1-dimethylethoxy)-2-oxoethyl]amino}ethyl)glycine was supplied by Bracco S.p.A. Human blood and serum were treated with lithium-heparin to prevent coagulation.

Synthesis of Ligands: A schematic summary of the synthesis of Gd-DTPA-SA is reported in Figure 4. *p*-Ethylaminesulfonamide II (236 mg, 1.18 mmol) was added to 20 mL of a DMF solution of *N,N*-bis(2-{bis[2-(1,1-dimethylethoxy)-2-oxoethyl]amino}ethyl)-glycine I (730 mg, 1.18 mmol). Upon the dropwise addition of 0.2 mL of DEPC [$(EtO)_2P(O)CN$] (215 mg, 1.32 mmol), the reaction mixture was maintained at 3 °C using an ice bath. Triethylamine (0.204 mL, 149 mg, 1.48 mmol) was then added slowly to the solution. The reaction was followed by TLC by observing the depletion of the free sulfonamide (R_f = 0.1) and the appearance of the product (R_f = 0.45) with the eluent $CH_2Cl_2/CH_3OH/NH_4OH$ (9:1:0.1). After 10 minutes the amine had fully reacted and the solution was warmed up and allowed to stand at room temperature for 30 minutes. DMF was then removed by vacuum evaporation. The crude product was washed and phase separated in ethyl acetate (33 mL) with a sodium bicarbonate (2 × 25 mL) and saturated sodium chloride solution (2 × 20 mL). Product III (838 mg, yield 89%) was obtained. Its purity was confirmed by using TLC (R_f = 0.45). Its ^{1}H-NMR spectrum was recorded in $CDCl_3$. – ^{1}H NMR: δ = 1.40 (s, 36 H, tBu), 2.4–2.7 (m, 8 H, N–CH_2–CH_2–N), 2.95 (s, 2 H, Ph–CH_2), 3.05 (s, 2 H, N–CH_2–CO), 3.3 (s, 8 H, N–CH_2–COO), 3.5–3.6 (m, 2 H, N–CH_2), 5.6 (s, 2 H, SO_2NH_2), 7.3–7.9 (m, 4 H, Ph), 8.2–8.3 (m, 1 H, NH).

Product III (838 mg, 1.05 mmol) was dissolved in 6 N HCl (8 mL). The mixture was stirred at room temperature for 1 hour and was then washed with CH_2Cl_2 (2 × 10 mL). The aqueous solution was dried and 671 mg (0.98 mmol, yield 93%) of pure compound IV were obtained. An ^{1}H-NMR spectrum was recorded in D_2O. – ^{1}H NMR: δ = 2.8–2.9 (m, 4 H, N–CH_2–CH_2–N), 3.1–3.0 (m, 4 H, N–CH_2–CH_2–N), 3.4–3.3 (m, 2 H, CH_2–Ph), 3.4–3.6 (m, 4 H, N–CH_2–CO–N–CH_2), 4.0 (s, 8 H, N–CH_2–CO), 7.4–7.9 (m, 4 H, Ph).

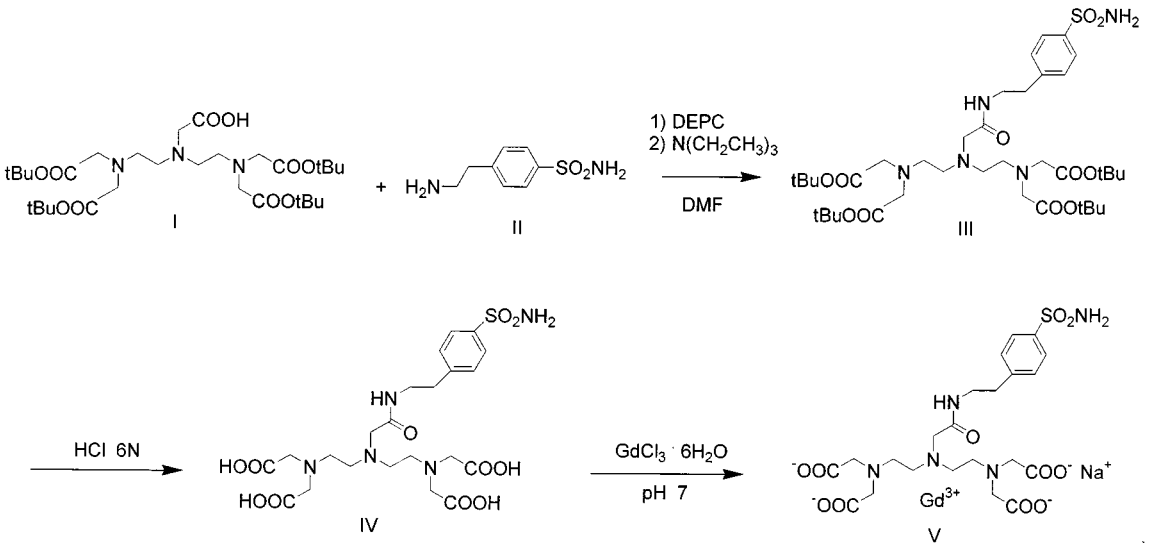

Figure 4. Schematic summary for the synthesis of Gd-DTPA-SA

Complexation and Purification of IV: The complexation and purification procedures follow a protocol already reported.[31] Compound IV (671 mg, 0.98 mmol) was dissolved in 27 mL of distilled water. To this solution 1 N NaOH was added until pH 7 was reached. An aqueous solution (5 mL) of $GdCl_3 \cdot 6H_2O$ (379 mg, 0.98 mmol) was then added slowly, maintaining a pH of 7 by addition of further aliquots of 1 N NaOH. The final crude product V (1.0 g) was obtained by evaporating the solution.

It was dissolved in 10 mL of distilled water and then loaded onto an Amberlite® XAD 1600 resin column (100 mL) and eluted with a CH_3CN/H_2O (9:1) gradient. The fractions containing the complex were evaporated to give 674 mg (0.86 mmol, yield 88%) of the crystalline white product V [negative ion FAB 729 $(M - Na)^-$].

The data collected on a large number of mono and bisamide derivatives of DTPA indicate that these compounds are remarkably stable with respect to dissociation in vivo. Therefore, the same stability is expected for our compound.[4]

Nuclear Magnetic Relaxation Dispersion Experiments: ^1H Nuclear Magnetic Relaxation Dispersion (NMRD) profiles were obtained by plotting the proton relaxation rates, R_1, as a function of the applied magnetic field. The NMRD profiles and all longitudinal water-proton relaxation rate measurements were collected with a Stelar prototype fast field cycling relaxometer or with a Koenig-Brown field cycling relaxometer[25,32] in the 0.01–50 MHz proton Larmor frequency range. Both instruments provide measurements with an error of about ±1%.

NMRD data, with the diamagnetic contribution subtracted, were analyzed according to existing theories, using a program described in ref.[33]

Determination of the Binding Constant: The observed relaxivity R_{obs} is the sum of the contributions from the free GdL complex and the GdL-CA adduct[30,34]

$$R_{obs} = \frac{C_{GdL} - [GdL - CA]}{C_{GdL}} R_{free} + \frac{[GdL - CA]}{C_{GdL}} R_{add} \quad (4)$$

with

$$[GdL\text{-}CA] = \frac{1 + K_A(C_{GdL} + C_{CA}) - \sqrt{(1 + K_A(C_{GdL} + C_{CA}))^2 - 4K_A^2 C_{GdL} C_{CA}}}{2K_A} \quad (5)$$

and

$$K_A = \frac{[GdL\text{-}CA]}{(C_{GdL} - [GdL\text{-}CA])(C_{CA} - [GdL\text{-}CA])} \quad (6)$$

where K_A is the binding constant, C_{GdL} and C_{CA} represent the initial concentrations of both GdL and CA, and R_{free} and R_{add} are the relaxivity of the free complex and the fully bound complex, respectively. Both K_A and R_{add} have been obtained through a two-parameter fitting of the R_{obs} data measured at various CA concentrations. The concentration of the gadolinium complex was 0.2 mM at pH 8.1.

Acknowledgments

For financial support to MF we thank Bracco S.p.A., Italy. For financial support, we also thank MURST ex 40%, Italy, the European Union, TMR-LSF contract ERB FMGE CT950033, and CNR, Progetto Finalizzato Biotecnologie, Italy. We also thank Dr. Claudiu Supuran for discussions and suggestions in the initial stage of this project.

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Received September 17, 1999
[I99327]