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Synthesis and Characterization of a Doxorubicin–Gd(III) Contrast Agent Conjugate: A New Approach toward Prodrug–Procontrast Complexes

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We have prepared the first MRI prodrug–procontrast complex by conjugating doxorubicin to a Gd(III) chelate using an acid-labile linker. The relaxometric properties of the adduct and pH activation are reported.

Doxorubicin (adriamycin) is a widely used anticancer drug of the anthracycline class and was discovered more than 3 decades ago.[1] The mechanism of action of doxorubicin is not fully understood; however, at clinically relevant concentrations (micro- or submicromolar), a number of physiological effects have been identified. Doxorubicin interferes with DNA helicase, DNA unwinding, and DNA strand separation and appears to induce cell differentiation.[2] At peak plasma concentrations (usually 1−2 μm), doxorubicin interacts with topoisomerase II, which ultimately leads to apoptosis.[2] The efficacy of doxorubicin is limited by dose-dependent toxic side effects such as cardiotoxicity, myelosuppression, and extravasation.[3] One approach to overcoming these side effects and simultaneously increasing the therapeutic efficacy has been to prepare doxorubicin prodrugs.[4]

Several conjugates of doxorubicin with antibodies, proteins, or polymers using acid-labile linkers such as hydrazones or semicarbazones have been reported.[5] Following cellular uptake of the conjugate via endocytosis, the linker is cleaved by lysosomal enzymes, allowing intracellular drug release. However, a significant limitation of this approach is that the activation of the drug is measured indirectly by high-performance liquid chromatography or by cytotoxicity studies.

To overcome this limitation, we are investigating a class of prodrug−procontrast derivatives of doxorubicin. The complexes combine the therapeutic effects of doxorubicin with a bioactivated magnetic resonance (MR) contrast agent that can be noninvasively detected in whole organisms. The efficiency of a contrast agent is expressed by relaxivity (r_{1p}) and is described by the Solomon−Bloembergen−Morgan theory. Several parameters contribute to the observed relaxivity of a contrast agent and include the number of H_2O molecules coordinated to the metal center (q), the rotational correlation time of the complex (τ_r), and the mean residence lifetime of the coordinated H_2O (τ_M). Changes in these parameters are reflected in a change in relaxivity.[6]

Our laboratory pioneered the design of responsive MR contrast agents (q-modulated), which provided the first example of complexes that have two distinct relaxivity states with respect to inner-sphere H_2O coordination.[7,8] The complexes were synthesized with biologically active, removable protection groups that largely prevent access of H_2O to a paramagnetic center. By limitation of the access of H_2O, the unprocessed agent is an ineffective contrast agent.

Here, we report the synthesis and relaxometric characterization of a doxorubicin−Gd(III) contrast agent conjugate with an acid-labile hydrazone linker (**7**; Scheme 1). Several reports have appeared that exploit the conjugation of doxo-

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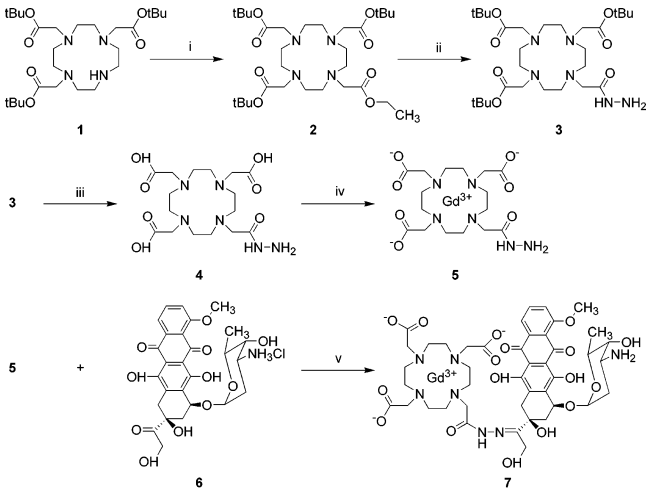
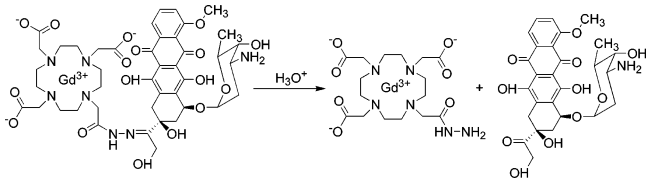
Scheme 1 [10]



Scheme 2



rubicin to macromolecules (monoclonal antibodies, human serum albumin, and polymers) via this linker.[5] We have covalently attached a MR contrast agent to doxorubicin at the C13 position through a hydrazone group to obtain **7**. This complex is designed to release doxorubicin when exposed to low pH and simultaneously undergo a change in relaxivity (Scheme 2).

The conjugate **7** was prepared from *tert*-butyl-protected DO3A (**1**).[9] Alkylation of the secondary amino group with ethyl bromoacetate afforded **2** in high yield. The ethyl ester was converted to the hydrazide **3** by use of a large excess of hydrazine monohydrate. Deprotection of the carboxylic acid arms was achieved within 2 h by reaction with HCl. Complex **5** was obtained by combining **4** and gadolinium chloride in an aqueous solution at reflux, and the Eu(III) analogue of **5** was prepared following the same procedure. The final step was accomplished using doxorubicin hydrochloride (**6**) in MeOH with 1% (v/v) trifluoroacetic acid (TFA). The purification of **7** was accomplished by dialysis and was obtained in 27% yield in five steps (Scheme 1).

To elucidate the coordination geometry of the target complex, we employed ab initio methods to investigate the relative stability of two isomers of **5** (Figure 1). In one isomer, the hydrazide ligand coordinates to the Gd(III) center via the O (OGd) and via the secondary N in the second isomer (NGd). The X-ray diffraction structure of GdDOTA[11] (Cambridge ID JOPJIH) with appropriate substitutions was

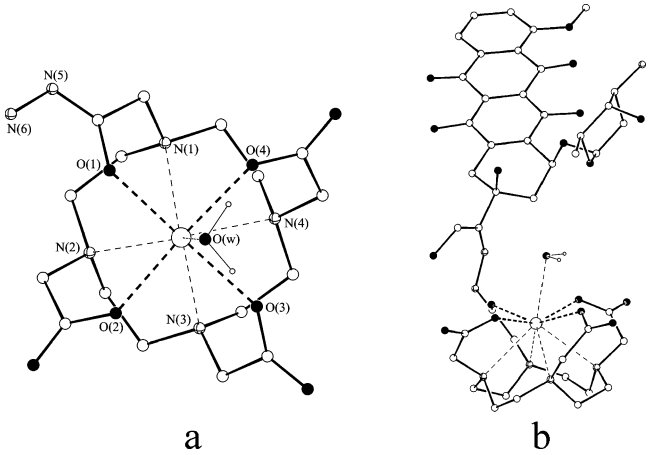


Figure 1. Calculated structures of the most stable OGd coordination isomer of **5** (a) and of **7** (b). H atoms have been omitted for clarity.

used as a starting point for the molecular modeling of **5**. For Ln complexes of DOTA derivatives, the macrocyclic ring adopts a [3333][12] conformation. The torsion angle θ between the square formed by N1−N4 and a second square made up of O1−O4(N5) defines the coordination geometry of the complex. This geometry is a capped square antiprism (SAP) with a large torsion angle of about 39° or a capped twisted SAP for a smaller torsion angle of about 25°.[13] The optimized structures for the species O−Gd and N−Gd show a monocapped SAP geometry about the Ln ion with a common torsion angle θ of 36.8°. As expected, the coordination via the O results in a complex more stable by 0.5 kcal mol^{-1}. On the basis of these calculations, it appears that the doxorubicin moiety is directed away from the tetraazamacrocycle and may not affect hydration.

The relaxometric properties of **5** and **7** were measured at 37 °C and 60 MHz. **5** has a relaxivity of 2.9 s^{-1} mM^{-1}, while **7** shows a relaxivity that is dependent upon the concentration. Below a concentration of ca. 1 mM, the relaxivity of **7** is 5.0 s^{-1} mM^{-1}, while above 1 mM, the relaxivity increases, indicating a transition to an aggregated form with a longer rotational correlation time. The relaxivity of **5** remains constant between pH 3 and 12, suggesting a stable structure in this pH range (see the Supporting Information). The small variation observed at pH 5 may reflect the deprotonation equilibrium of the hydrazide. The more pronounced increase in the relaxivity at pH < 3 may be due to dissociation of the acetate ligands.

The hydration of the Eu(III) analogue of **5** was determined using a method developed by Supkowski and Horrocks[14] and subsequently modified by Beeby et al.[15] When the differences in the decay rates of the Eu(III) complexes in H_2O and D_2O

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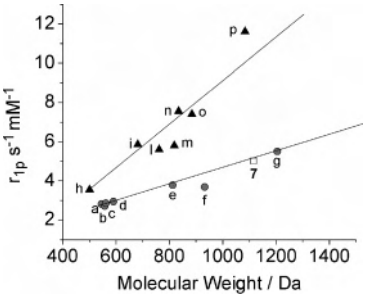

Figure 2. Millimolar relaxivities of selected complexes at 60 MHz and 37 °C as a function of their molecular weight compared with compound **7** (□). Complexes with $q = 1$ and 2 are indicated by circles and triangles, respectively: (a) GdDTPA, (b) GdDOTA, (c) compound **2**,[8] (d) compound **5**, (e) compound **1**,[16] (f) compound **1**,[8] (g) [pip{Gd(DO3A)(H_2O)$_2$}],[17] (h) GdDO3A, (i) GdTTAHA,[18] (l) Gd[TREN-Me-3,2-HOPOSAM],[19] (m) Gd-[TREN-Me-3,2-HOPOIAM],[19] (n) Gd[TREN-Me-3,2-HOPOTAM],[19] (o) Gd-TREN-Me-3,2-HOPO,[20] (p) Gd_2(mX(DTTA)$_2$)(H2O)$_4$.[21] Lines are guides for the eye.

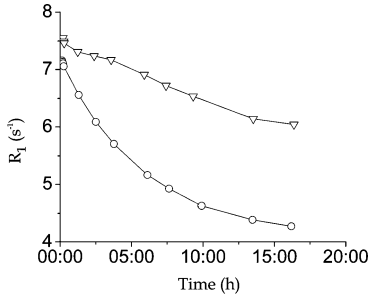

Figure 3. Time dependence of the relaxation rate of a 1.3 mM buffered solution of **7** at pH 7.4 (MOPS; 50 mM; triangles) and at pH 4.5 (acetate buffer; 50 mM; circles).

are measured and empirical corrections for unbound H_2O molecules (0.25 s^{-1}) and for NH oscillators are applied, the number of coordinated H_2O molecules can be determined.

The correction required for the NH oscillators of a hydrazide group has not been reported, and we therefore used that for amide NH oscillators (0.08 s^{-1}). Only one NH oscillator was considered because the distance of the closest approach of the NH_2 hydrogen measured on a molecular model is long enough (4.45 Å) to make this contribution negligible, and we calculated $q = 1.0$ for **5**. The hydration of **7** can be inferred by comparing its relaxivity on the low concentration limit with that of other complexes of known hydration at the same magnetic field and temperature (Figure 2).

For low-molecular-weight Gd(III) complexes, the relaxivity is primarily dependent on q and τ_r, which is, in turn, dependent on the molecular weight. Thus, for complexes with the same q, the relaxivity can be expected to be approximately linearly dependent on the molecular weight. The relaxivity of **7** is consistent with a single H_2O molecule coordinated to the Gd(III) ion.

The hydrolytic stability of **7** at pH 7.4 and 4.5 was investigated by relaxometry at 37 °C and 60 MHz. As a consequence of the hydrolysis, the molecular mass of the agent is reduced by approximately half and the rotational correlation time of the free complex is shortened. The resulting decrease in the relaxivity was followed over time (Figure 3). The decrease in the relaxivity observed at pH 4.5 shows that 90% of doxorubicin is released within 16 h, and the hydrolysis product was confirmed to be doxorubicin by reversed-phase high-performance liquid chromatography. The slower decrease in the relaxivity observed at pH 7.4, which is accompanied by the formation of a red precipitate, can be ascribed to the inherent instability of doxorubicin (under these conditions), which causes slow precipitation.[22]

In conclusion, we have presented the first example of a T_1-MRI contrast agent conjugated to an anthracycline anticancer drug. Modulation of the relaxivity following activation of the procontrast−prodrug agent is observed as a consequence of the reduced molecular weight of the hydrolysis products. As a result of the experiments described here, we are exploring new designs with the intent of obtaining (a) agents with higher relaxivities to be detectable at the low concentrations used during doxorubicin administration and (b) agents that upon activation increase their hydration state and, consequently, undergo an increase in relaxivity while simultaneously producing an active form of an anticancer drug.

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Supporting Information Available: Detailed experimental and modeling procedures are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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