Stable Bifunctional Chelates of Metals Used in Radiotherapy

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Abstract

Monoclonal antibody technology allows the specificity of an antibody for its antigen to be used in targeting cancer cells. The conjugation of metals, particularly radionuclides such as Y or Cu, to monoclonal antibodies results in agents for radioimmunotherapy and other medical applications. Chelators that can hold radiometals with high stability under physiological conditions are essential to avoid excessive radiation damage to nontarget cells. Derivatives of polyazamacrocycles (bearing a C-substituted functional group for antibody attachment) can exhibit remarkable kinetic inertness; for example, the copper complex of the 14-membered 6-(p-nitrobenzyl)-1,4,7,10-tetraazaacyclocadecane-N,N',N'',N'''-tetraacetic acid is very stable in human serum under physiological conditions, and a conjugate of this complex with a monoclonal antibody has tested well in tumor-bearing mice. Desreux and coworkers [Loncin, M. F., Desreux, J. F., and Merciny, E. Inorg. Chem., 25: 2646-2648, 1986] have shown that complexes of lanthanides with 1,4,7,10-tetraazaacyclocadecane-N,N',N'',N'''-tetraacetic acid have formation constants that are several orders of magnitude higher than that of 1,4,8,11-tetraazaacyclocadecane-N,N',N'',N'''-tetraacetic acid; thus the 12-membered macrocycle is the favored target for binding trivalent yttrium. We have developed a new synthetic route to these macrocycles via peptide synthesis and intramolecular tosylamide ring closure. Incubation of the 90Y(III) complex of 2-p-nitrobenzyl-1,4,7,10-tetraazaacyclocadecane-N,N',N'',N'''-tetraacetic acid for 18 days in serum results in loss of so little Y(III) from the complex (less than 0.5%) that the rate of loss cannot be measured under these conditions.

Introduction

To develop chelating agents for radioimmunodiagnostics and radioimmunotherapy, we have directed our efforts toward the synthesis of novel ligands that can stably hold metal ions in vivo. Our approach includes strategically incorporating a functionalized side chain into known ligands such as the polyamino-polycarboxylates, via new synthetic routes. The resulting molecule is known as a BCA.1 These agents were first developed by Sundberg et al. (1, 2) and Yeh et al. (3) in the 1970s. In addition, Krejcarek and Tucker (4) developed an activated DTPA analogue via mixed anhydride which can be linked to protein. Later, Hnatowich et al. (5) used the cyclic anhydride of DTPA for the same purpose. Although these noncycligens form stable complexes with a variety of metal ions (6, 7) such as Fe(III), Co(III), In(III), etc., their complexes with other metals such as Cu(II) are labile (8). In response to the need for stronger chelating agents, we have developed a new class of ligands known as MBCAs. The compounds 2-p-nitrobenzyl-1,4,7,10-tetraazaacyclocadecane-N,N',N'',N'''-tetraacetic acid (compound 1); and 6-p-nitrobenzyl-1,4,8,11-tetraazaacyclocotadecane-N,N',N'',N'''-tetraacetic acid (compound 2) (Fig. 1) are examples.

MBCAs are synthetic molecules consisting of a chemically modifiable moiety and a macrocyclic metal-chelating moiety. The nitrobenzyl group is very useful for this purpose, because it can be reduced easily to the amine when desired. In turn, this amine can be transformed into useful functionalities for attachment to biomolecules (9). The structure of the metal-chelating moiety is made up of a tetraaza macrocycle containing N-carboxymethyl groups. Stetter et al. first reported the complexation properties of this type of ligand; the thermodynamic stability constants of their complexes with alkali, alkaline earth, transition metals (10), and certain lanthanides (11) are remarkably high. More importantly, the macrocyclic structure of the ligand imposes a degree of rigidity which is thought to be responsible for their slow rate of complex formation and dissociation. Thus, the metal complexes of these macrocycles are expected to have kinetic stability as well. It is this basic feature that makes these macrocycles highly attractive for in vivo applications.

The complexation properties of these macrocyclic ligands are determined by several factors: (a) the nature of the donor atoms. Each terminal amine nitrogen in the ring is sp3 hybridized. One of the sp3 orbitals is filled with an unshared pair of electrons. It is this pair of electrons that is responsible for the ligating properties of the amine. Also, the negatively charged carboxylate oxygens are strong electron donors for "hard" metals such as Y(III), Ga(III), etc.; (b) the protonation constants. In addition to being a donor atom for metals, nitrogen can share its lone-pair electrons with hydrogen ion, to form an ammonium species. This protonated species can no longer serve as a metal ligand. Therefore, the competition of protons for the nitrogen can greatly affect the complexation properties of the amine; (c) the structure of the chelator; the macrocyclic structure can provide a preformed metal binding site.

In this article the chelation properties of the MBCAs and linear BCAs with 67Cu and 90Y, two metals that have useful nuclear properties, are described. The properties of 67Cu are potentially useful for diagnosis and therapy. Its nuclear decay results in emission of β-particles (577, 486, and 395 KeV) (12). It also emits γ-radiation (93 and 184 KeV) which allows it to be traced in the body for imaging and dosimetry. The inorganic chemistry of Cu(II) has been studied extensively. A transition metal, Cu(II) has the electronic configuration [Ar] 3s23d9. Its complexes generally assume square planar or distorted octahedral geometry. For many complexes of copper the predominant feature is elongation of the bonds along the z axis to a tetragonal structure, with four short in-plane metal-ligand bonds and two long metal-ligand bonds perpendicular to them. In the absence of steric factors, Cu(II) favors nitrogen donors over oxygen donors (13).

The radionuclide 90Y has a pure β-particle emission which is useful in radiotherapy. In comparison to 131I, a currently used therapeutic nuclide which has an emission energy of 0.6 MeV, 90Y has a higher energy β-emission of 2.3 MeV. The absence of any γ-radiation makes 90Y a particularly attractive therapeutic agent for outpatient use. The inorganic chemistry of Y2+ falls in the category of the hard Lewis acids and is much like that of the lanthanides. The bonding interactions are mainly electro-
The chelators 1-benzyl-EDTA and DTPA-NH-Bu were prepared according to the methods of Meares et al. (16) and Eckelman et al. (17) respectively. The 14-membered macrocycle, nitrobenzyl-TETA (compound 2) was prepared by the methods of Moi et al. (18, 19).

Carrier-free $^{67}$Cu (in 3 M HCI) was obtained from Brookhaven National Laboratory. Preparation of Radiochelates. The radiochelates were prepared by incubating the chelator (approximately 10 mM) with carrier-free $^{67}$Cu in 0.1 M ammonium acetate buffer, pH 7. The mixture was incubated for 6 h and analyzed for binding by TLC. Consequently, 5 µl of the mixture were diluted 100-fold with 0.1 M ammonium acetate buffer, pH 7. Then 5 µl aliquots of this diluted solution were mixed with 2 ml of human serum to give a concentration of 140 nM.

Preparation of $^{88}$Y-DTPA-NH-Bu in Serum. A stock solution of carrier-free $^{88}$YCl$_3$ (6 µl in 12 M HCl) was quickly mixed with 4 µl of 2 M ammonium hydroxide. Then a stock solution of 2-$\beta$-nitrophenylalanylglycylglycine (21) was prepared by Dr. S. M. Yeh (20); the compound 2-$\beta$-nitrobenzyl-DOTA (Fig. 1, compound 1) was prepared from the tetrapeptide nitrophenylalanylglycylglycine (21).

Bio-Gel P-60 gel filtration resin (polyacrylamide beads, 100–200 mesh; fractionation range, M, 3000–6000 for proteins) was used. The eluting buffer was 0.1 M ammonium acetate, pH 7.

Preparation of $^{88}$Y-1-$\beta$-Nitrobenzyl-DTPA in Serum. A stock solution of carrier-free $^{88}$YCl$_3$ (2 µl in 0.1 M ammonium acetate buffer, pH 5, adjusted with 2 M ammonium hydroxide) was added to stock solution of 1-$\beta$-nitrobenzyl-DTPA (2 µl of 30.8 mM, 0.25 pmol) was added. Subsequently, 10 µl of 0.1 M ammonium acetate buffer, pH 7, were added. The pH was checked to be 7. After 3 h, the mixture was analyzed for binding by TLC. Then 5 µl of the mixture were diluted 100-fold with 0.1 M ammonium acetate buffer, pH 7. Finally, 5.7 µl of this diluted solution were mixed with 2 ml of human serum to give a concentration of 140 nM.

Preparation of $^{88}$Y-1-$\beta$-Nitrobenzyl-DTPA in Serum. A stock solution of carrier-free $^{88}$YCl$_3$ (6 µl in 12 M HCl) was quickly mixed with 4 µl of 2 M ammonium hydroxide. Then a stock solution of 2-$\beta$-nitrobenzyl-DOTA (8.1 M! of 30.8 mM, 0.25 µmol) was added. Subsequently, 10 µl of 0.1 M ammonium acetate, pH 5, were added and the pH was adjusted to 5 using 6 µl of 2 M ammonium hydroxide. The mixture was incubated at room temperature for 24 h and the mixture was analyzed by TLC for binding. Then a 5 µl aliquot of the mixture was diluted 100-fold with 0.1 M ammonium acetate, pH 5. Finally, 2.89 µl of this diluted solution were mixed with 2 ml of human serum to give a concentration of 100 nM.

Preparation of the Bio-Gel P-60 Column. The resin was soaked in 0.1 M ammonium acetate buffer, pH 7, for 4 h. Then the resin slurry was packed in a 1-ml tuberculin syringe [bottom fitted with a polypropylene frit (16)]. The final resin bed volume was 1 ml.

Calibration of the Bio-Gel P-60 Column for Elution Profile of $^{88}$Y. A solution of unchelated $^{88}$Y(III) in serum (50 µl) was loaded on the column. Then the column was eluted with 200-µl aliquots of 0.1 M ammonium acetate buffer, pH 7. The effluent was collected in microcentrifuge tubes (0.5-ml capacity). A total of 10 fractions were collected and counted in the gamma well counter.

Analysis of Serum-Chelate Mixtures. At appropriate time intervals, 50-µl aliquots of the incubation mixtures were analyzed following the procedure above.

Polyacrylamide Gel Electrophoresis of the Chelate-Serum Samples under Non-denaturing Conditions. Following the procedure described (22), 50-µl aliquots each of 1-$\beta$-nitrobenzyl-DOTA and Y-DTPA-NH-Bu were analyzed by nondenaturing polyacrylamide gel electrophoro-
Results and Discussion

Stability Studies of Bifunctional Chelates in Human Serum under Physiological Conditions. In the application of MBCs as radiopharmaceuticals, one of the main factors is the stability of the radiometal chelate in the body. In general, any loss of the radiometal would result in the accumulation of radioactivity in nontarget organs. Stability of the radiometal chelate is affected by a variety of factors such as the pH of the medium, presence of metal-binding proteins, and metabolic processes. At the normal pH of blood, 7.4, hydrogen ions can effectively compete with the chelated metal ion for the ligand via protonation. In the case of polyniminopolycarboxylate ligands, protonation occurs at the basic nitrogens having pKₐ > 7.4. As a direct consequence, the stability of the chelate can be drastically affected.

Serum Stability Studies of the Copper Chelates. In the evaluation of copper chelates as potential radiopharmaceuticals, a major concern is the transcomplexation of copper from the chelate to albumin under normal physiological conditions. Albumin (M, 67,000) is known to be the protein in blood that binds free copper ions. At its amino terminal, albumin has a specific copper-binding site, Asp–Ala–His. This binding site involves the α-nitrogen of the amino-terminal aspartic acid, two intervening peptide nitrogens, and the imidazole nitrogen of histidine (residue 3) (23). In terms of the effects of the hydrogen ion concentration on the stability of the chelates, at pH 7.4 copper-albumin has a stability constant of 10¹⁵.². In comparison, copper complexes of TETA, EDTA, and DTPA have stability constants of 10¹¹.⁶, 10⁸.⁸, and 10⁷.⁴, respectively (Table 1). Fig. 2 shows the pH dependence of the conditional stability constants of the copper chelates.

Another important consideration is that the concentrations of these chelates under clinical conditions are much smaller (typically in the nanomolar range) than that of albumin (0.5 mM) (24). Therefore, at equilibrium, the conditions would favor the complete loss of copper from any of the metal chelates to albumin. The important factor to be considered is the kinetic stability of these chelates. Kinetic stability governs the rate at which the copper is lost from the chelate. The rate of loss of copper from the chelates is shown in Fig. 3. The difference in the rate of decomposition between the linear and the cyclic chelates is very dramatic. Both Cu-1-p-nitrobenzyl-EDTA and Cu-DTPA-NH-Bu lost their metal very rapidly. At day 1, only 14 and 23% of intact chelate were observed for Cu-1-p-nitrobenzyl-EDTA and Cu-DTPA-NH-Bu, respectively.

In sharp contrast, the Cu-6-p-nitrobenzyl-TETA remained almost intact, with a loss of about 1% per day. Thus, the prediction based solely on thermodynamics is opposite to what was observed. Among the factors contributing to its kinetic stability, the macrocyclic structure may be predominant. The 14-membered tetraazaamacrocyle is more favorable to accommodating the copper ion than 13- or 15-membered rings. As shown by X-ray crystal structure studies on the copper complex of Cu-6-p-nitrobenzyl-TETA, the copper ion is held in the center of the macroyclic ring with all of its coordination sites occupied by the nitrogens and oxygens from the chelator (19). Therefore, it is less likely that any foreign ligands in serum will participate in the coordination of the metal. The situation is different in the case of both Cu-EDTA and Cu-DTPA, in which one or more of the copper coordination sites are occupied by solvent molecules (25).

Serum Stability Studies of Yttrium Chelates. Yttrium is a trivalent metal ion which is metabolized similarly to the lanthanides, indium(III) and the well-studied iron(III). In blood the main carrier protein for these metals is transferrin (26). Human serum transferrin (M, 81,000) is an iron transport protein which simultaneously binds two atoms of iron and two anions such as bicarbonate ions (27). In addition to binding Fe(III), transferrin can also form complexes with rare earth, other trivalent, and even divalent metals (28–31). Similar to the case of copper, yttrium chelates are affected by protonation; this can be illustrated by considering the more thoroughly studied gadolinium chelates. For example, Gd-DOTA has a

<table>
<thead>
<tr>
<th>Chelator</th>
<th>Log K₉₅₄</th>
<th>Log K₉₆₄</th>
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<tbody>
<tr>
<td>EDTA</td>
<td>15.9</td>
<td>14.5</td>
</tr>
<tr>
<td>DTPA</td>
<td>17.0</td>
<td>18.2</td>
</tr>
<tr>
<td>DOTA</td>
<td>12.8</td>
<td>21.6</td>
</tr>
<tr>
<td>TETA</td>
<td>12.6</td>
<td>12.9</td>
</tr>
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Values are calculated from Stetter et al. (33), Loncin et al. (11), and Martelli and Smith (7). Note that the values calculated are not corrected for the hydrolysis of the metal ion (34).
conditional stability constant of 10^{21.6} at pH 7.4, compared to 10^8 for its thermodynamic equilibrium constant (11). Fig. 4 shows the pH dependence of the conditional stability constants of the gadolinium chelates.

Fig. 5 shows the rate of decomposition of the yttrium chelates in serum under physiological conditions over a 18-day period. It is clear that Y-2-p-nitrobenzyl-DOTA is the most stable chelate in serum. Over the 18-day period no measurable loss of yttrium from the macrocyclic chelator was observed. Y-1-p-Nitrobenzyl-DTPA underwent decomposition of about 0.57%/day. This rate of decomposition is small compared to that of Y-DTPA-NH-Bu, which lost its yttrium to the serum protein exponentially with a half-life of 2.5 days. In order to determine which serum proteins are involved in the scavenging of the yttrium ions, nondenaturing gel electrophoresis was performed on the serum samples. The polyacrylamide gel electrophoresis analysis of the Y-DTPA-NH-Bu serum mixture at day 8 shows that most of the radioactivity was bound by transferrin.

The remarkably high stability of Y-2-p-nitrobenzyl-DOTA is superior to currently available chelates such as the Y-DTPA-NH-Bu and Y-1-p-nitrobenzyl-DTPA for use in vivo. Independent studies by Hnatowich et al. (32) have shown that, in agreement with our results, the Y-DTPA-NH-antibody lost its yttrium to serum proteins at an average rate of about 12%/day.

References

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