Chelating Agents and their Use in Radiopharmaceutical Sciences

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Abstract: Radiometal nuclides can serve as diagnostic markers in molecular imaging or can be used in therapeutic settings for a rising number of human afflictions. For the targeted delivery of these medically interesting ions, appropriate chelating agents forming stable complexes are of fundamental importance.

For different metal ions exhibiting different physical and chemical properties, resulting in different coordination chemistries and therefore differing requirements on the chelator used, a broad variety of chelating agents has been developed over the years. Not only the chemical properties of the metal ion determine the choice of the chelator, but also the desired *in vivo* behavior of the resulting molecular imaging or therapeutic compound influences the choice of the complexation agent. Furthermore, the conjugation chemistry for the introduction of the chelator into the biologically active compound and the complexation reaction of the metal ion can affect the choice of the appropriate chelator.

This review outlines chelating agents used in medicinal chemistry, their radiometal complexation behavior and their potential influence on the properties of the resulting drugs.

Keywords: Radiometal nuclides, chelator, complex stability, medicinal application, biodistribution.

1. INTRODUCTION

Radiopharmaceuticals for application in nuclear medicine have gained a central role in the diagnosis and therapy of diseases of various origins.

PET (positron emission tomography) and SPECT (single photon emission computed tomography) represent imaging modalities which enable the non-invasive detection of very early stages of diseases due to changes in metabolism and alterations of biological functions of abnormal tissues on the cellular level. Endoradiotherapy – the internal application of radioactive nuclides, decaying within the organism and depositing their therapeutic radiation in the target tissues – exhibits very favorable treatment properties by limiting systemic side effects as exerted by conventional chemo- or radiotherapy. This is achieved by a tumor-selective targeting vector that directs the radiopharmaceutical to the diseased tissues and is thus even suitable for treatment of disseminated disease. Another advantage of endoradiotherapy over conventional, systemic treatments is the so-called "crossfire-effect", since not only the cells to which the targeting vector binds are affected by the therapeutic, but also all cells that are within the range of the emitted radiation. Hence, malignant cells not expressing the receptor or surface protein in sufficient extent for targeting vector binding can nevertheless be affected by the therapeutic agent, considerably reducing the probability for a recurrent disease.

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Mainly naturally derived biologically active substances such as peptides, antibodies, oligonucleotides and other macromolecules are used as targeting vectors. They have found broad utilization in medicinal applications and are still in the focus of targeting vector development [1-9].

For diagnostic as well as therapeutic applications in nuclear medicine, the choice of the appropriate radionuclide is essential. For targeting vectors which are not "small molecules" but naturally derived, radiometal nuclides are particularly suitable for radiolabeling since they *i*) can much more easily be introduced into the molecule by complexation than nonmetallic nuclides that have to be introduced by covalent bond formation often resulting in multi-step and cumbersome radiosyntheses and *ii*) are differing in physical properties such as half-lives, radiation energy and tissue penetration (which enables the choice of the most appropriate radionuclide and thus an adapted disease monitoring or treatment). Nonmetallic radionuclides exhibiting favorable radiation energy characteristics but short half-lives (such as ^{11}C: $t_{1/2}$=20 min and ^{18}F: $t_{1/2}$=109 min) would for example not be appropriate when IgGs are used for targeting purposes as the biological half-life of the targeting vector would be much longer than that of the diagnostic radionuclide.

For diagnostic imaging and particularly for therapeutic applications, the stable introduction of the radiometal into the targeting vector is crucial as it would accumulate in non-target tissues when released from the complex. This results – in case of diagnostic compounds – in high background activities limiting the target visualization and – in case of therapeutic compounds – in radiation burden to healthy, potentially radiation-sensitive tissues. Thus, chelators forming stable complexes with radiometals intended for medicinal applications are necessary and highly sought after. This is

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also reflected by the ongoing research and excellent recent reviews in this field [10-15]. But not only the chelator itself, but also the linker – if applied – between targeting vector and chelator as well as the conjugation chemistry can have an impact on the *in vivo* biodistribution and pharmacodynamic behavior of the resulting radiotracer [16].

This review outlines successful recent advances in the field of chelator development and the state of the art of radiometal nuclide introduction into biologically interesting targeting vectors. In this regard, chelators for gallium, indium, yttrium, lutetium and copper nuclides are discussed. Chelators for technetium isotopes are – although ^{99m}Tc is of high interest in diagnostic SPECT imaging – not within the scope of this review. However, excellent reviews in this field are available [10, 17-19].

2. GENERAL CONSIDERATIONS FOR CHELATOR DESIGN AND COMPLEX STABILITY

The stability of a radiometal-chelate-complex is determined by the properties of the metal ion as well as the chelating agent and both have to be taken into consideration when developing a new complexation ligand. One important guideline is the so-called HSAB (hard and soft acids and bases) principle, allowing an estimation of complex stabilities [20-22].

Most of the currently used radiometal nuclides in biomedical applications such as Ga^{3+}, In^{3+}, Y^{3+} and Lu^{3+} are hard acids, requiring hard donor atoms such as oxygen and nitrogen atoms in the complex ligand. Such hard base donor atoms allow for strong interactions of central metal and coordination sphere and thus enable the formation of stable complexes. In contrast, Cu^{2+} is of "borderline" hardness with intermediate hard/soft acid properties. Cu^{2+} is not only an exception in terms of hardness, but is also the only of the mentioned radiometals susceptible to reduction processes *in vivo*. This reduction process is mainly observed in hypoxic tissues and not limited to "free" copper ions but can also occur with complexed Cu^{2+}, producing a soft acid (Cu^{+}) which is usually not stably bound by the same complex ligands as Cu^{2+}, which can result in the release of the radioisotope from the complex. Thus, a stable complex for Cu^{2+} should ideally be able to withstand reducing conditions so that no Cu^{+} is released from the complex accumulating in non-target tissues.

Another important factor influencing the thermodynamic stability of the complex is its geometry. Polydentate ligands comprising several donor atoms for example generate far more stable complexes than ligands with low or monodenticity due to the chelate effect: the higher complex stability is a result of the higher interaction probability of a donor atom with the central ion due to the rigid complex geometry caused by steric fixation of the donor atoms within the ligand. This results in enhanced and stable formation as well as impeded decomposition of the complex. In addition, macrocyclic ligands normally form more stable complexes than acyclic ligands because of the more fixed geometry. However, the high stability of complexes formed by macrocyclic ligands is accompanied by the requirement of harsh complexation conditions. This resulted in the recent efforts to develop macrocyclic ligands with pendant arms that are supposed to combine the relatively mild chelation chemistry of acyclic ligands with the high complex stability achieved by macrocyclic ligands [23-27]. The complex stability can furthermore be maximized by using a ligand with a cavity size adapted to the size of the central ion, completely encapsulating it without steric strain.

Although all these considerations affecting the thermodynamic stability of the complex are important principles regarding ligand design, the kinetic or solution stability is an important factor for *in vivo* applications as well. This kinetic stability can strongly influence the overall stability of the complex: Although exhibiting a high thermodynamic stability, a complex can be challenged under physiological conditions, resulting in an undesirable release of the radiometal.

Since the *in vivo* stability of a complex is not predictable by determining its thermodynamic stability constant, serum stability studies are commonly carried out for newly developed complexes, allowing at least a prognosis of its *in vivo* stability and biodistribution properties. However, data about the *in vivo* performance of a complex or its bioconjugates can only be obtained by accordant studies.

The following chapters outline recent advances and state of the art of coordination chemistry and radiometal nuclide introduction into interesting targeting vectors for gallium, indium, yttrium, lutetium and copper radioisotopes.

3. COORDINATION CHEMISTRY FOR SPECIFIC RADIOMETAL NUCLIDES

In medicinal applications, several radiometal nuclides can be used. The choice of the respective radionuclide depends on the individual application and the requirements of physical characteristics and accessibility. Currently, the mainly used diagnostic radiometal nuclides for PET are the β^{+}-emitters ^{64}Cu ($t_{1/2}$ = 12.7h), ^{68}Ga ($t_{1/2}$ = 68min) and ^{86}Y ($t_{1/2}$ = 14.7h). For SPECT, the γ-emitters ^{67}Ga ($t_{1/2}$ = 78.3h) and ^{111}In ($t_{1/2}$ = 2.83d) are generally used apart from ^{99m}Tc that is not discussed here.

For therapeutic purposes, α- and β^{-}-emitting radionuclides can be applied. Among the available α-emitters, mainly ^{212}Bi ($t_{1/2}$ = 61 min), ^{213}Bi ($t_{1/2}$ = 46 min) and ^{225}Ac ($t_{1/2}$ = 10 d) are used [28-33]. Due to their high charge and mass α-particles exhibit a very short tissue penetration range of only about one cell diameter and a large LET (linear energy transfer) which can be understood as a measure of exerted damage in the penetrated tissue. Although the high LET of α-particles is highly desirable for therapy, the small tissue penetration range is often unfavorable as it limits the crossfire effect and necessitates a carrier that accumulates in direct vicinity of the tumor cell nucleus. Therefore, mainly β^{-}-emitters are used for tumor therapy as the emitted β^{-}-particles exhibit a comparably low mass and charge combined with an adequate LET, resulting in a much wider range of tissue penetration better-suited for the therapy of solid tumors.

The choice of the appropriate β^{-}-emitting nuclide thus depends on the application and the energy of the emitted radiation as this directly correlates with its tissue penetration

and thus the range of tissue damage. Suitable therapeutic β^--emitters used in tumor treatment are ^{67}Cu ($t_{1/2}$ = 2.58d, maximum β^--range: 1.8mm), ^{90}Y ($t_{1/2}$ = 2.66d, maximum β^--range: 12.0 mm) and ^{177}Lu ($t_{1/2}$ = 6.7d, maximum β^--range: 1.5 mm).

3.1. Chelators for Gallium Isotopes

As Ga^{3+} – which is the stable oxidation state of gallium under physiological conditions – exhibits a high charge density, it prefers donor atoms in its coordination sphere also exhibiting a high charge density, such as secondary amine and carboxylate functionalities. Due to its small ion radius, it is most frequently 6-coordinated even if a higher number of donor atoms is available [34]. Extensive reviews are available summarizing commonly applied as well as bi- to hexadentate non-medicinally applied ligands for gallium isotopes [35-36].

Unbound Ga^{3+}, when released from a complex *in vitro* or *in vivo*, is binding to the iron-transporting serum protein transferrin which can be explained by the similarity of Ga^{3+} and Fe^{3+} in terms of charge density and biological properties. This protein-binding is reflected *in vivo* in a high blood, liver and sometimes also lung accumulation of the radioactivity and demonstrates a low stability of the respective gallium chelate [37-38]. This effect was found for example for several recently developed acyclic chelators for gallium isotopes, which unfortunately showed a release of the metal ion from the complex and are thus not suitable for an *in vivo* application [39-41].

Nevertheless several bifunctional chelators – mainly based on macrocycles – are available for stable gallium incorporation and are summarized in Fig. (**1**).

Macrocyclic ligands in general form thermodynamically and kinetically more stable complexes than acyclic chelators. The first complex ligand that was used for routine gallium isotope introduction was DOTA (**1**, Fig. **1**). DOTA is still the most often used ligand in medicinal applications [42-46] and is in form of its gadolinium complex distributed as the MRI-contrast agent Dotarem® that is approved by the FDA as well as by European agencies. However, the cavity for the central ion is somewhat too large for the small Ga^{3+} ion, resulting in a participation of only two of the three carboxylic functionalities in complex formation (N_4O_2) [47] although a higher coordination would in theory be possible. Utilizing this fact, attempts have been made to synthesize drug dimers by reacting two of the four carboxylic acids of DOTA or by synthesizing DO2A-based (**2**, Fig. **1**) dimers, still showing an appropriate stability of the respective gallium complexes [48-49]. Gallium isotopes also exhibit slow complex formation kinetics with DOTA, resulting in the requirement of high temperatures for efficient complex formation. This limits the application of this chelator to non-heat-sensitive biomolecules or prelabeling approaches.

An alternative to DOTA is the use of the acyclic chelator HBED-CC (**3**, Fig. **1**) which was shown to be easily coupled to antibodies and antibody fragments and could efficiently be radiolabeled with ^{68}Ga under mild conditions [50-51]. Despite its favorable properties in the complexation of gallium isotopes, this chelator is rarely used which may be attributed to its intricate synthesis.

Another important alternative to DOTA is NOTA (**4**, Fig. **1**). The cavity of NOTA has an ideal size for gallium ions, resulting in thermodynamically much more stable complexes ($\log K_{NOTA\text{-}Ga}$ = 30.98 and $\log K_{DOTA\text{-}Ga}$ = 21.33) [52-53] in which all nitrogen and carboxylic groups of the ligand are involved in complex formation (N_3O_3) [54]. Furthermore, the complexation kinetics are much faster, allowing for gallium incorporation under mild conditions within short time-spans [55-57] which is a particular advantage when the short-lived

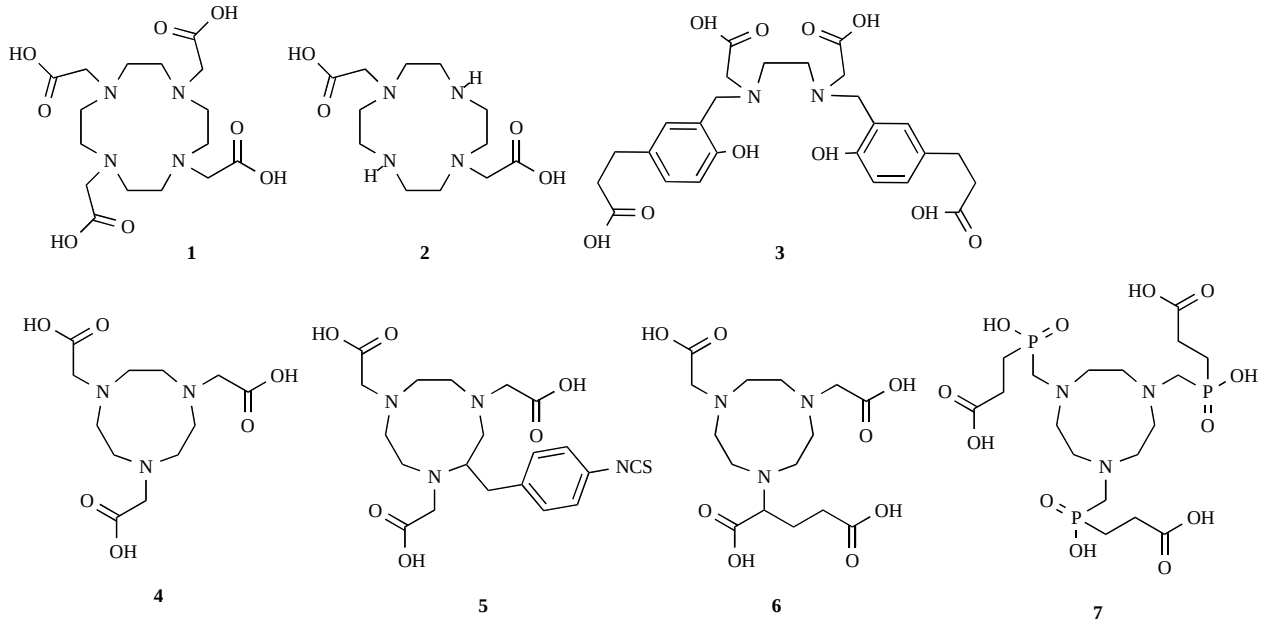


Fig. (1). Structures of the chelators DOTA (**1**), DO2A (**2**), HBED-CC (**3**), NOTA (**4**), *p*-SCN-Bn-NOTA (**5**), NODA-GA (**6**) and PrP9 (**7**) for gallium complexation.

gallium isotope ^{68}Ga is used ($t_{1/2}$ = 68 min). However, when NOTA is introduced into a biomolecule by transferring one of its carboxylic functionalities into an acid amide, the complex stability is reduced to a certain extent as the acid amide is not such a hard donor as the carboxylic oxygen [58-59]. In order to avoid this reduction of complex stability caused by bioconjugation, new NOTA derivatives were developed containing an additional functionality for bioconjugation. This additional functionality for coupling to the targeting vector can be attached to the backbone or to one pendant arm of the ligand (for example *p*-SCN-Bn-NOTA (**5**) and NODA-GA (**6**), Fig. **1**). These NOTA derivatives show very favorable complex formation properties such as short reaction times with the radionuclide under very mild conditions. Bioconjugates synthesized using these NOTA derivatives show besides favorable radiolabeling conditions and preserved complex stabilities also optimized biodistribution properties such as high target-to-background ratios and fast clearance from non-target tissues which enables an efficient target-visualization. Thus, these synthons are currently often used for biomolecule modification, subsequent gallium radiolabeling and *in vivo* application [47, 55-57, 60].

Recently, a novel NOTA-based ligand – PrP9 (**7**, Fig. **1**) – was described [61], containing three arms, each comprising a phosphinic acid and a distant carboxylic acid functionality. For this new chelator, favorable radiolabeling properties could be shown. It is assumed that the distant carboxylic acid functionalities may act as "pre-coordination sites" and could combine the favorable stability of macrocyclic ligands with the fast complex formation kinetics of acyclic ligands. However, the thermodynamic stability constant is somewhat lower than for NOTA ($\log K_{PrP9\text{-}Ga}$ = 26.24 and $\log K_{NOTA\text{-}Ga}$ = 30.98) [53, 61] and no conjugation to biologically interesting molecules or *in vivo* application was shown so far. Nevertheless, it can be anticipated that this chelator type will be often used in medicinal applications in the future due its favorable properties.

3.2. Chelators for Indium Isotopes

In^{3+} exhibits a high charge density comparable to Ga^{3+}, preferring hard donor atoms in the complex coordination sphere. Its similarity to Ga^{3+} is also reflected by its incorporation into endogenous transferrin when released from a complex *in vivo*. However, due to its larger ion radius, In^{3+} is not 6-coordinated in complexes as Ga^{3+}, but 7- or 8-coordinated, depending on the individual complex ligand [62-63]. This difference results in a different complex geometry and the need for chelators providing at least 7 donor atoms for the formation of stable complexes.

DOTA offers high thermodynamic complex stabilities with indium ($\log K_{DOTA\text{-}In}$ = 23.9) [52], and produces radiotracers with good clearance and biodistribution properties and is therefore often used in ^{111}In-labeling of biologically interesting molecules [64-67]. Recently, it could be shown that an ^{111}In-labeled tumor-targeting peptide containing DOTA-GA [68] (a DOTA derivative exhibiting an additional

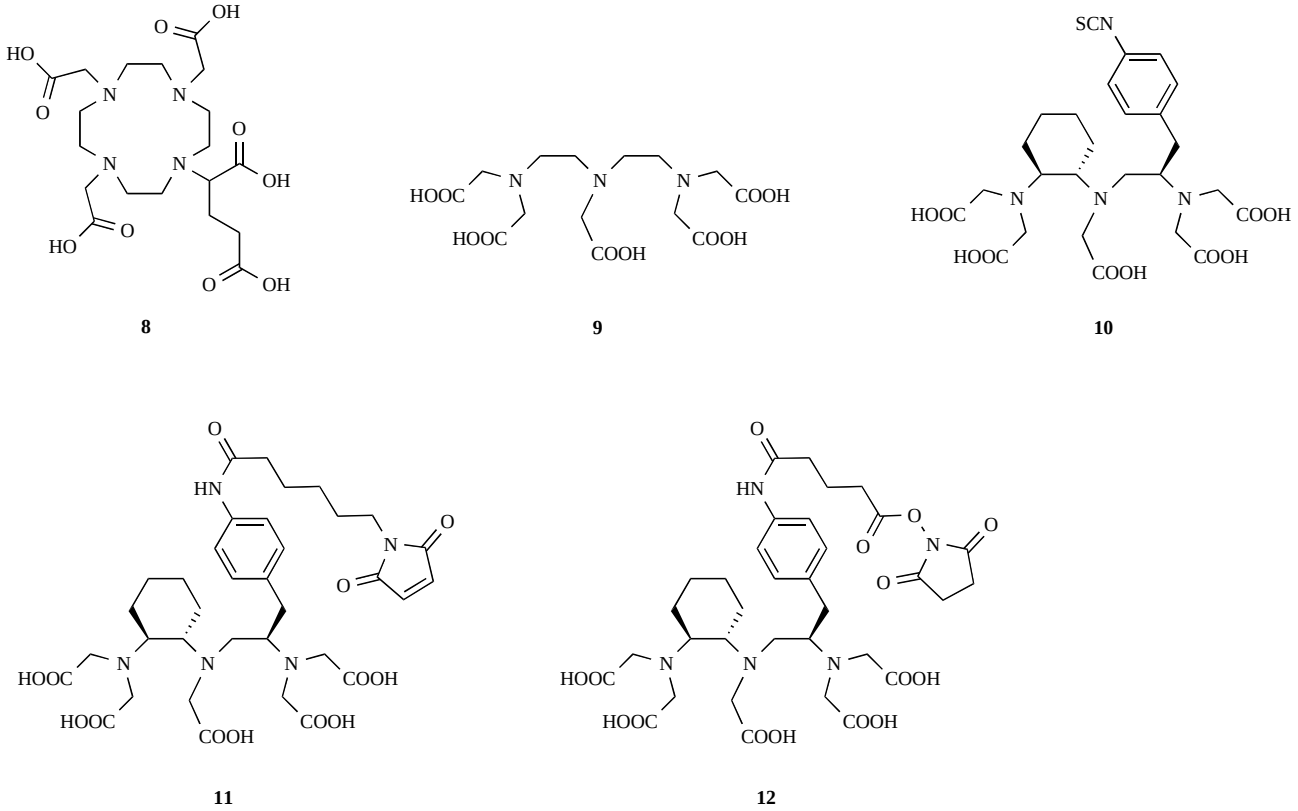


Fig. (2). Structures of chelators DOTA-GA (**8**), DTPA (**9**), CHX-A''-DTPA-isothiocyanate (**10**), CHX-A''-DTPA-maleimide (**11**) and CHX-A''-DTPA-glutaric acid NHS ester (**12**) commonly used for biomolecule modification and subsequent complexation with indium isotopes.

arm for biomolecule conjugation (**8**, Fig. **2**)) as chelator showed an even higher *in vivo* stability than the same peptide comprising DOTA or DTPA (**9**, Fig. **2**) as chelators [69]. This demonstrates the high suitability of complex ligands with a pendant arm for biomolecule conjugation in terms of stability and favorable biodistribution properties.

However, since the complexation reaction of indium using DOTA derivatives requires elevated temperatures between 60 and 99°C, this chelator is not suitable for the radiolabeling of heat-sensitive biomolecules such as most antibodies or other proteins. In order to enable ^{111}In-radiolabeling of heat susceptible biomolecules, DTPA derivatives are used, as they allow for efficient radionuclide incorporation even at room temperature. Due to these favorable properties, the complex of DTPA with ^{111}In (distributed as MPI Indium DTPA In-111® or [^{111}In]Indium-Pentetate®) and a DTPA-peptide conjugate for ^{111}In-labeling (distributed as Octreoscan®) are approved by the FDA and European agencies.

Lately, underivatized DTPA is rarely applied as complexation agent [70-71] as it could be shown in a comparative study using isothiocyanatobenzyl-DTPA and CHX-A"-DTPA-isothiocyanate (**10**, Fig. **2**) for affibody-conjugation and subsequent radiolabeling that the CHX-A"-DTPA-modified proteins could be radiolabeled in much higher efficiency with ^{111}In. Furthermore, using CHX-A"-DTPA for derivatization, the products could be obtained in higher radiochemical purities while at the same time exhibiting comparable *in vivo* biodistribution properties of the radiotracers [72]. The much higher radiolabeling efficiency of CHX-A"-DTPA compared to DTPA-derivatized compounds is attributable to the pre-organized configuration of the chelator that is achieved by the cyclohexyl backbone modification. This in comparison to DTPA much more rigid steric alignment on the one hand accelerates the complexation kinetics without the need for higher reaction temperatures and on the other hand results in a higher stability of the formed complexes, making this ligand a virtually ideal candidate for biomolecule conjugation and subsequent radiolabeling. This is reflected in the high number of described applications and synthesized derivatives comprising different functionalities for introduction into biomolecules (for example maleimide (**11**) and NHS-ester (**12**), Fig. **2**) [72-76].

3.3. Chelators for Yttrium Isotopes

Y^{3+} is often referred to as a "pseudo-lanthanide" as it exhibits a similar ion radius and charge density as for example Lu^{3+}. Due to their large ion size, Y^{3+} and lanthanide ions are mostly 8- or 9-fold coordinated in stable complexes [34, 62]. A high complex stability is of course of crucial importance as non-bound ^{90}Y and ^{177}Lu isotopes, producing high-energetic β^--radiation, accumulate in the bone and can produce bone marrow ablation during endoradiotherapy [77-79]. Thus, the formation of thermodynamically and kinetically stable complexes is even more important for these therapeutic radionuclides than for diagnostic ones.

DOTA forms very stable complexes with a broad variety of medically interesting radiometal nuclides [80], but its cavity size is almost ideal for the complexation of yttrium and lanthanide ions and thus DOTA is often used as ligand for peptides requiring ^{90}Y-labeling [34]. However, due to its high steric rigidity and closed structure DOTA necessitates elevated temperatures for metal ion complexation, which limits its application to radiolabeling of non heat-sensitive biomolecules. An opportunity to overcome this problem is the prelabeling approach, a technique consisting of two steps: *i*) The radiolabeling of the complex ligand and *ii*) the subsequent conjugation of the formed complex to the biomolecule. This method is feasible if the bioconjugation step proceeds efficiently, and has been shown to be applicable in the ^{90}Y-labeling of antibodies and oligonucleotides [81-83].

As DTPA enables an efficient complexation of indium ions at room temperature, its use was also proposed for the radiolabeling with yttrium isotopes. However, the formed complexes showed an insufficient stability resulting in the release of yttrium [84]. Therefore, several backbone-substituted and thus sterically more rigid DTPA derivatives – such as 1M3B-, 1B3M-, 1B4M- (**13**, Fig. **3**), CHX-A- and CHX-B-DTPA – were developed and evaluated regarding their thermodynamic and kinetic complex stabilities for yttrium. Among these, CHX-A"-DTPA (**14**, Fig. **3**) was found to form the most suitable yttrium complexes displaying an acceptable complex stability during the time course of a potential therapy. Thus, mostly ^{90}Y-CHX-A"-DTPA-conjugates can be found in recent applications [85-86]. Zevalin®, consisting of an antibody-1B4M-DTPA-conjugate, is FDA approved for ^{90}Y-labeling and utilization in tumor therapy. Nevertheless, all complexes based on DTPA derivatives were found to release yttrium to some extent [23, 87-88].

A recent strategy to overcome the problems of elevated temperatures required for complexation with closed ring chelators and less than optimal complex stabilities exhibited by open ring chelators is the development of compounds combining a high *in vivo* stability and fast complexation kinetics at low temperatures. Representatives of this new family of chelators are NETA, C-NETA and C-NE3TA (**15**, **16** and **17**, Fig. **3**). In studies evaluating their stability and complex formation kinetics, it could be shown that NETA exhibits much faster complexation kinetics with Y^{3+} than DOTA and forms a complex that displays a similar stability to that formed by DOTA [23-24, 26]. ^{90}Y-complexes of the unconjugated ligands were prepared and evaluated regarding their *in vitro* serum stability, showing no release of the radioisotope over 11 days. However, the radiolabeling had to be performed at 80°C for 12h which is comparable to reaction conditions used in DOTA labeling, meaning no considerable improvement in terms of complexation reaction or complex stability [26]. Thus, further studies have to decide on the potential superiority of this new class of complex ligands for yttrium introduction into biomolecules.

3.4. Chelators for Lutetium Isotopes

For Lu^{3+}, which exhibits a similar ion radius and charge density as Y^{3+}, the same chelators are used for the introduction of this radiometal ion into biomolecules in principle. Since ^{177}Lu is an emitter of therapeutic high-energetic β^--radiation, high complex stabilities are a crucial prerequisite for an *in vivo* application. Free lutetium accumulates in the bone as it acts as Ca^{2+} mimetic or is taken up into the liver

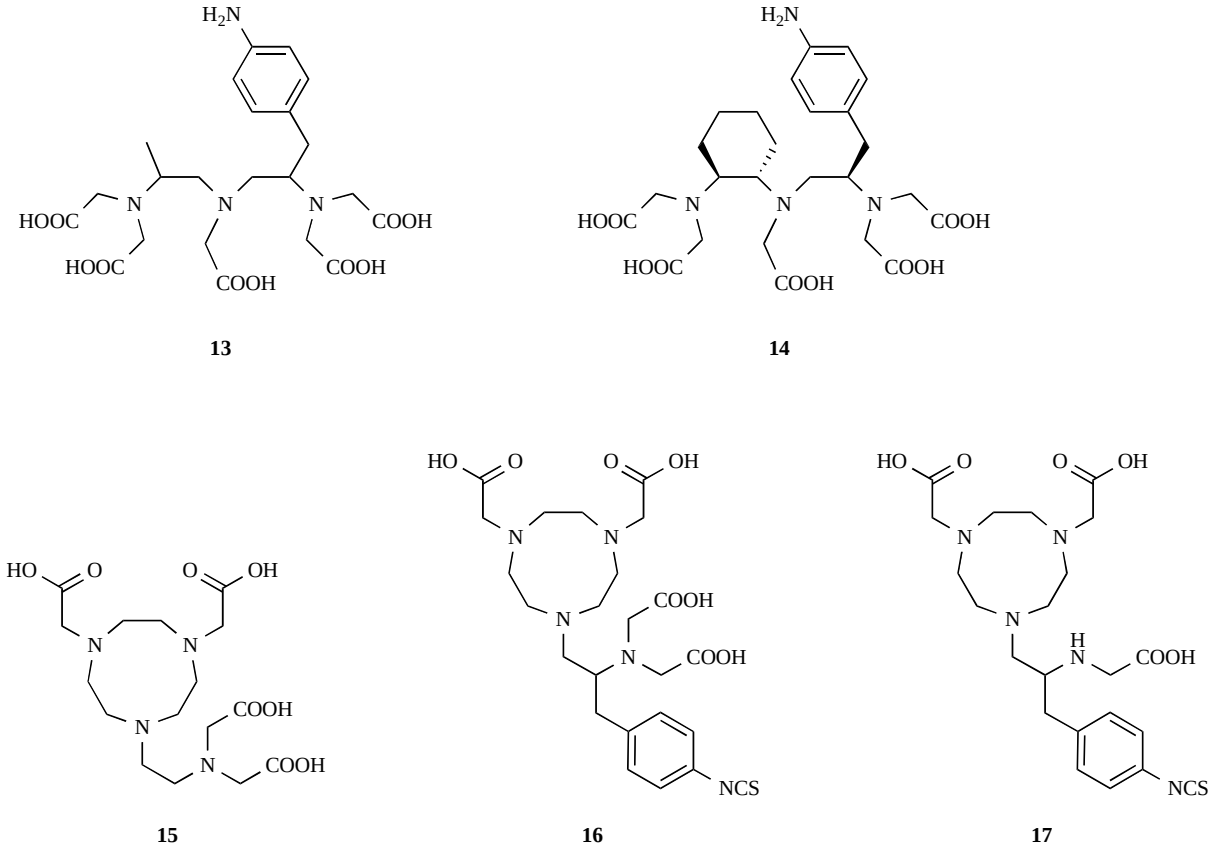


Fig. (3). Structures of 1B4M-DTPA (**13**), CHX-A''-DTPA (**14**), NETA (**15**), C-NETA (**16**) and C-NE3TA (**17**).

because of $Lu(OH)_3$ colloid formation at physiological pH and can thus result in high doses to sensitive non-target organs [89-92].

DOTA-derivatives – mainly applied in form of the NHS-ester – are often used for ^{177}Lu-introduction into antibodies and peptides for *in vivo* application due to the high stability of the resulting complex [63, 93-95]. However, as the radiolabeling of heat-sensitive proteins with Lu^{3+} has to be performed in a time-consuming reaction step which requires an incubation with the radionuclide for 2 – 3h at 37°C and results in comparably low metal incorporation yields, CHX-A"-DTPA is also frequently used for protein modification and Lu-radiolabeling [96-97].

Recently, two studies were carried out evaluating different macrocyclic and acyclic complex ligands – 1B4M-DTPA, CHX-A"-DTPA, C-DOTA, MeO-DOTA and PA-DOTA isothiocyanates (**18**, **10**, **19**, **20** and **21**, Figs. **2** and **4**) – for antibody conjugation and subsequent radiolabeling with ^{177}Lu. In these studies, the acyclic ligands showed superior labeling characteristics whereas the macrocyclic ligands showed higher *in vivo* stabilities and thus lower bone uptakes in biodistribution studies [98-99]. Among the tested derivatives, the MeO-DOTA-antibody-conjugate exhibited the most favorable biodistribution properties of fast clearance and high tumor-to-background ratios and at the same time provided a high complex stability [99]. Thus, MeO-DOTA seems to be a very promising chelator for ^{177}Lu-introduction that should be further evaluated.

3.5. Chelators for Copper Isotopes

Of the possible oxidations states of copper, Cu^{2+} is the most stable in aqueous solutions and it is therefore commonly used in medical applications. Since Cu^{2+} shows a borderline hardness, not only "hard" donors such as secondary amines and carboxylate functionalities can be used for the ligand design, but also borderline donors such as phosphines or thioethers, which expands the field of possible ligand structures.

Copper ions released from a complex get instantaneously incorporated and quenched by two different proteins *in vivo*: superoxide dismutase, which is mainly present in the cytosol of liver, kidneys and red blood cells or ceruloplasmin, which is a copper-containing serum protein [100-101]. A radioactivity accumulation in blood, liver and lung is therefore an indication for released or unstably bound copper nuclides.

For the radiometal nuclides discussed so far, the main factors influencing the thermodynamic and kinetic stability and thus the *in vivo* applicability of a complex are the size of the metal ion, the size of the cavity formed by the ligand, an appropriate number of donor atoms and similar HSAB properties of the metal ion and the donor atoms of the ligand.

In contrast to this, the stability of copper complexes depends on some additional factors. One of the most important is the relatively easy reducibility of Cu^{2+} to Cu^{+} in tissues featuring reducing conditions such as hypoxic tissues [102]. The Cu^{+} ion, however, can normally not be stably bound by

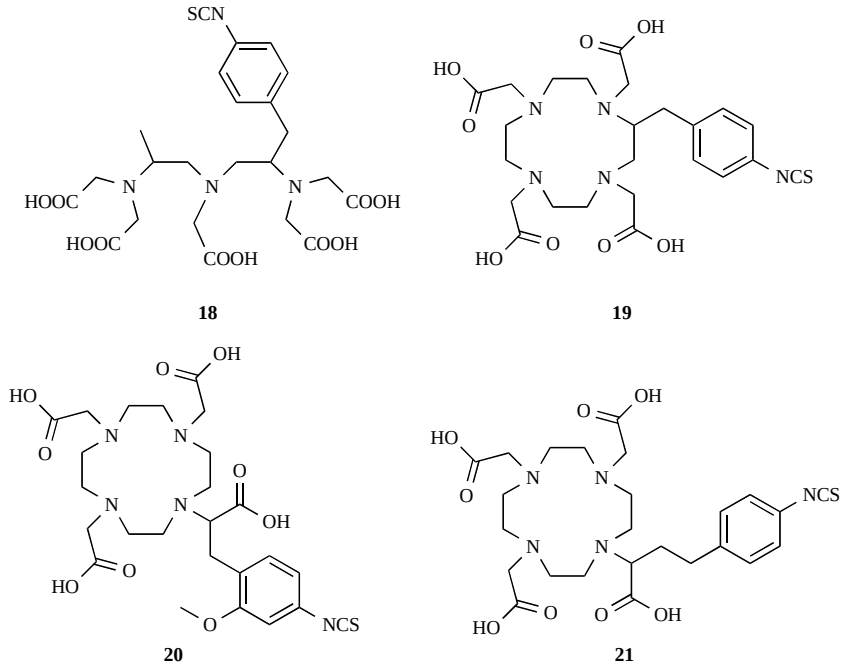


Fig. (4). Structures of 1B4M-DTPA- (**18**), C-DOTA- (**19**), MeO-DOTA- (**20**) and PA-DOTA-isothiocyanate (**21**).

a ligand that stably binds Cu^{2+}, resulting in the release of the copper ion from the complex. Thus, a copper complex suitable for *in vivo* applications has not only to exhibit exceptionally high thermodynamic and kinetic stabilities but also has to minimize the probability for central metal ion reduction and should ideally also allow for the re-oxidation of the central Cu^{+} ion to Cu^{2+}. Given that the factors influencing the stability of a copper complex *in vivo* are manifold and often difficult to predict, more approaches for complex ligands applied in copper introduction into biomolecules are in use than for the other radiometal nuclides discussed so far.

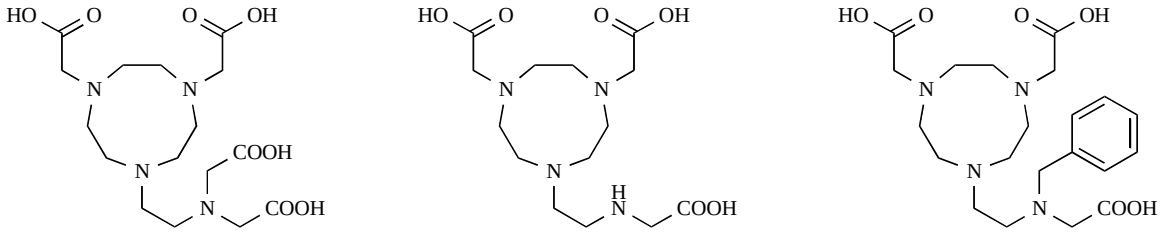
Chelators Based on DOTA

DOTA and its derivatives are mainly used for the modification and radiolabeling of peptides and peptide derivatives since harsh labeling temperatures are needed for quantitative ^{64}Cu incorporation [16, 103]. The radiolabeled compounds were successfully used for tumor imaging although a slight copper release could be found which was reflected in a higher radioactivity uptake into the liver compared to the biodistribution of the respective ^{86}Y-labeled compound [103]. Although high temperatures are required for Cu^{2+} complexation using DOTA, not only ^{64}Cu-radiolabeled peptides, but also labeled antibodies have been described [104-106]. However, these mostly heat-sensitive biomolecules had to be labeled under much milder conditions between 37 and 40°C for 1.5 to 3 hours, resulting in varying incorporation yields of 85 to 92%. A slight release of the ^{64}Cu ion from the complex could be observed also in these cases, although the tumor imaging was nevertheless successful.

Chelators Based on NOTA

As Cu^{2+} has a relatively small ion radius and prefers an octahedral complex geometry, NOTA derivatives were used for copper introduction into tumor-affine biomolecules such as bombesin, cRGD and heat-stable enterotoxin derivatives [107-110]. Although they could be labeled under mild conditions that would allow for the efficient Cu-radiolabeling not only of peptides but also of heat-sensitive biomolecules, the resulting radiolabeled peptides were shown in two of these studies to exhibit *i*) a significantly higher background accumulation at different time-points in kidneys, liver and blood than the respective DOTA-derivatized compounds [108] and *ii*) a relatively higher radioactivity accumulation in liver and kidneys when compared to the respective ^{68}Ga-labeled compounds [109], pointing to a release of copper from the complex and a lower *in vivo* stability of the ^{64}Cu-NOTA compared to the ^{64}Cu-DOTA or ^{68}Ga-NOTA complexes. However, another study directly comparing the influence of the chelators NOTA, DOTA and TETA on the biodistribution of tumor-affine heat-stable enterotoxin interestingly provided contrary results, showing the highest tumor-to-background-ratios and the fastest clearance properties for the ^{64}Cu-labeled NOTA-peptide one hour post injection, a high liver accumulation for the DOTA and a high kidney accumulation for the TETA derivative [110].

Based on NOTA, new chelators were developed that should allow for higher *in vivo* complex stabilities with Cu^{2+}. This higher stability was intended to be achieved by the use of less "hard" donor atoms and a combination of macrocyclic and acyclic ligand properties. These attempts should result in more favorable donor-radiometal interactions and thus more favorable biodistribution properties of the resulting radiotracers [25, 100]. Some of these new chelators (NETA (**15**), NE3TA (**22**) and NE3TA-Bn (**23**), Fig. **5**) showed promising *in vitro* serum stabilities, but their coupling to biologically active molecules, their radiolabeling with copper isotopes and a suitable stability and biodistribution of the resulting radiotracers for *in vivo* applications remains to be shown.



15 **22** **23**

Fig. (5). Structures of NETA (**15**), NE3TA (**22**) and NE3TA-Bn (**23**).

Chelators Based on Bisthiosemicarbazones

Another group of chelators that was developed in order to complex Cu^{2+} and also stabilize Cu^{+} ions that can be generated from Cu^{2+} in hypoxic tissues is that of bisthiosemicarbazones. One of the best known representatives of this group is Cu-ATSM (**24**, Fig. **6**) that was shown to accumulate in hypoxic tissues [111-112]. Based on this lead structure, several derivatives were synthesized containing potential tumor-tar-geting small molecules such as glucose or 2-nitroimidazole (**25** and **26**, Fig. **6**) [111, 113-115]. 2-Nitroimidazole is known to accumulate in hypoxic tissues and for the bisthiosemicarbazones-2-nitroimidazole conjugates, it could be shown that the hypoxia-selectivity was enhanced compared with Cu-ATSM alone. However, the high lipophilicity of the compounds resulted in a high background activity *in vivo*, limiting an efficient tumor-visualization [113]. In addition to the small molecule-comprising bisthiosemicarbazones, several derivatives containing functional groups for conjugation to biomolecules were synthesized [111, 116-117]. It was found that the stability of the complex as well as the resistance to reduction of the central copper ion are highly dependent on the substituents of the diimine backbone and that the best results can be achieved by changing the substituents on the exocyclic nitrogen atoms [111, 117]. Of the group of functionalized bisthiosemicarbazones, two acid-comprising derivatives (**27** and **28**, Fig. **6**) were successfully coupled to bombesin derivatives and the resulting peptide-conjugates could be radiolabeled with ^{64}Cu within 30 minutes at ambient temperature in quantitative yields [116-117]. Unfortunately, the conjugate (when evaluated *in vivo*) showed – although exhibiting a high receptor affinity – a very high background activity and an unfavorable tumor-to-liver-ratio of 1:2 after 24h, pointing to a considerable limitation of the use of bisthiosemicarbazones: their high lipophilicity and a potentially insufficient *in vivo* stability of the copper complex.

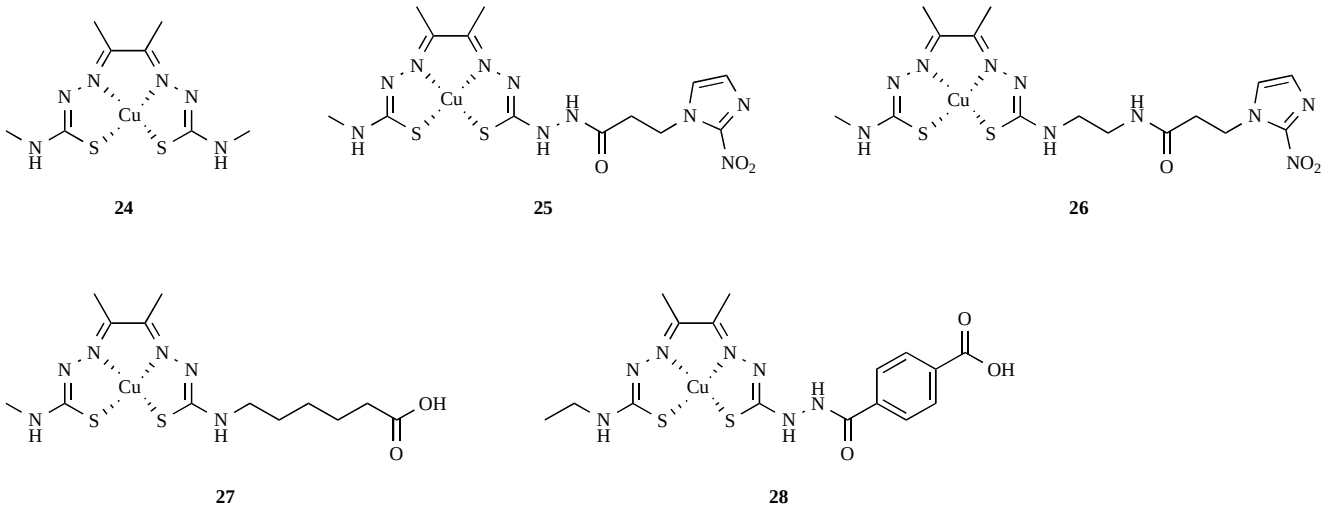
Chelators Based on Cyclam and TETA

For cyclam and its derivatives, an extensive review covering the mechanism of complexation, complex stabilities and applications of cyclam complexes in medicine is available [118].

In order to determine the most appropriate chelator for Cu^{2+} complexation, the backbone-substituted chelators 2-(4-nitrobenzyl)-cyclen (**29**), 2-(4-nitrobenzyl)-cyclam (**30**), 2-(4-benzamidobenzyl)-NOTA (**31**), 2-(4-nitrobenzyl)-DOTA (**32**), 2-(4-nitrobenzyl)-TETA (**33**) and 1-(4-nitro)-B4M-DTPA (**34**) (Fig. **7**) were radiolabeled with ^{67}Cu and their *in vitro* stability in serum was determined [119]. The following order of decreasing stability of the chelators was found: **30** ≈ **29** ≈ **32** › **31** » **33** » **34**. The low stability of the DTPA-derivative was attributed to the acyclic form of the chelator resulting in a lower complex stability compared to macrocyclic ligands. In the TETA derivative complex, the two free carboxylic functionalities that do not participate in complex formation are supposed to interact with other metal ions from the solution and thus enhance the probability for metal exchange, also resulting in relatively low complex stability. This assumption has been supported by a very recent study comparing complex stabilities and biodistribution properties of TETA and TE2A (**35** and **36**, Fig. **8**). In this study, it was shown that the metal ion was far more difficult to reduce in the Cu-TE2A complex, thus decreasing the risk of copper release *in vivo*. This could be confirmed in acid decomplexation, serum stability as well as *in vivo* biodistribution studies where the Cu-TE2A complex showed a much higher stability and inertness than the corresponding TETA complex [120]. Interestingly, a higher stability of copper complexes using backbone-substituted TETA can be found when the tetraazacyclotetradecane-ring is modified in 6-position instead of the 2-position [119].

The relatively low stability found for the NOTA (**31**) complex is in accordance with the findings described before and is assumed to be due to the incomplete envelopment of the copper ion by the ligand, resulting in increased probability of ion reduction and release from the complex [102]. Interestingly, the backbone-substituted cyclen (**29**) and cyclam (**30**) showed relatively high serum stabilities although only four of the ideal six coordination sites can be occupied by these ligands.

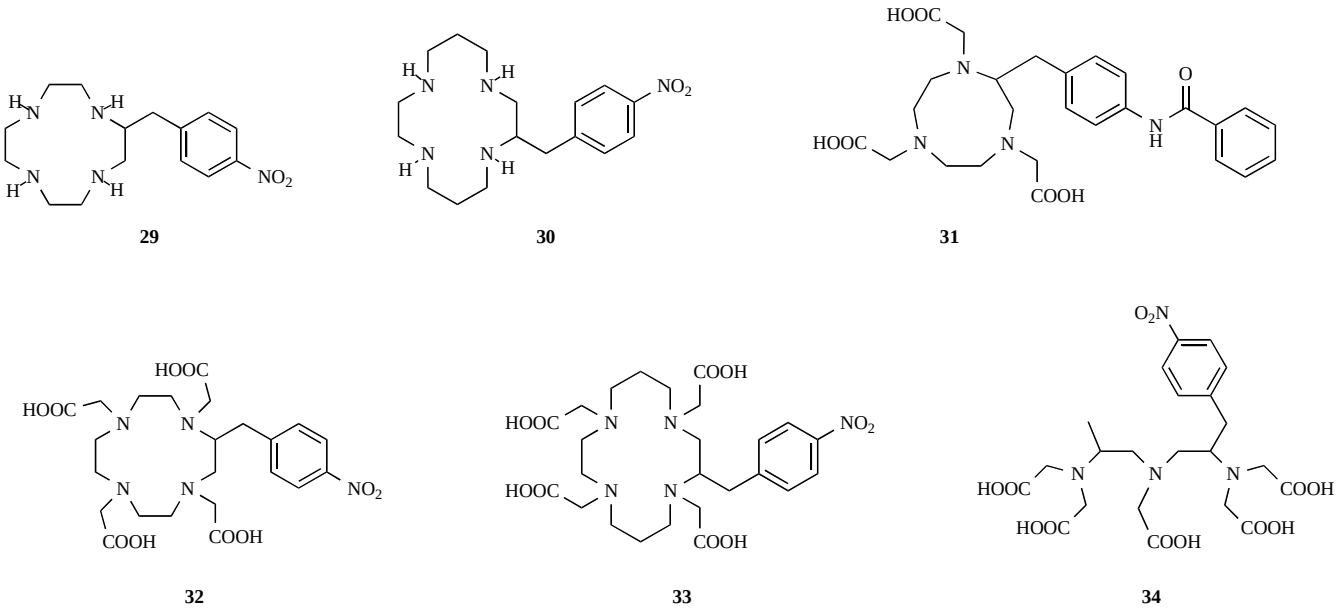
Thus, cyclam and its derivatives have also been used for the introduction of copper isotopes into biomolecules [121-123]. In a recently published study, five cyclam-based complex ligands were directly compared regarding the complex formation with ^{64}Cu under mild conditions (37°C for 30 minutes) and their biodistribution properties. Among these derivatives, only two, namely 1-(4-nitrobenzyl)-cyclam and 6-(4-nitrobenzyl)-cyclam (**37** and **38**, Fig. **8**) showed quantitative copper complexation rates within 30 minutes at 37°C [121]. Due to the favorable radiolabeling results, the corresponding amino-derivate of **38** was introduced into an antibody targeting colorectal and ovarian cancer cells using EDC as coupling reagent and evaluated *in vivo*. Unfortunately, although a significant tumor uptake was achieved, high non-



24 **25** **26**

27 **28**

Fig. (6). Structures of Cu-ATSM (**24**), Cu-diacetyl-(4'-methyl-4-N-ethyl-3-(2-nitro-1H-imidazol-1-yl)propanamide-bis-thiosemicarbazone (**25**), Cu-diacetyl-4'-methyl-4-N-ethyl-3-(2-nitro-1H-imidazol-1-yl)propanamide-bis-thiosemicarbazone (**26**), Cu-diacetyl-4-hexanoic acid-4'-methyl-bis-thiosemicarbazone (**27**) and Cu-diacetyl-2-(4-N-ethyl-3-thiosemi-carbazonato)-3-[4-N-(amino)-4-carboxybenzamide)-3 thiosemicarbazone] (**28**).



29 **30** **31**

32 **33** **34**

Fig. (7). Structures of 2-(4-nitrobenzyl)-cyclen (**29**), 2-(4-nitrobenzyl)-cyclam (**30**), 2-(4-benzamidobenzyl)-NOTA (**31**), 2-(4-nitrobenzyl)-DOTA (**32**), 2-(4-nitrobenzyl)-TETA (**33**) and 1-(4-nitro)-B4M-DTPA (**34**).

target uptakes were observed in blood, heart, liver and kidneys, indicating a limited applicability of this complex ligand for antibody modification and tumor imaging. However, in another attempt at tumor imaging using a cyclam-containing ^{64}Cu-labeled cRGD-tetramer, a successful tumor visualization could be achieved although a relatively high kidney uptake was observed [122].

The complex ligand that is for now most commonly used for radiocopper introduction into tumor-affine peptides and peptide derivatives is CB-TE2A (**39**, Fig. **8**) [58, 124-130]. This can be attributed to the extraordinarily high stability of the resulting Cu-complex which is reflected in higher tumor uptakes, higher tumor-to-background-ratios, very favorable clearance kinetics and low radioactivity accumulation in blood, liver and kidneys (due to copper release from the complex) when compared to the corresponding TETA and DOTA radiotracers [124-126, 131]. This high stability of Cu-CB-TE2A complexes is the result of several different factors determined in three studies comparing the complex stabilities of CB-TE2A (**39**), CB-DO2A (**40**), TETA (**35**), DOTA (**1**) and two CB-TE2A and CB-DO2A derivatives with prolonged pendant arms (**41** and **42**, Fig. **8**) [102, 132-133]. In these studies, the complex stabilities were determined in serum, rat liver and *via* biodistribution. CB-TE2A showed by far the highest *in vivo* stability, followed by CB-DO2A and TETA. The lowest stability was observed for DOTA. The extraordinarily high stability of CB-TE2A com-

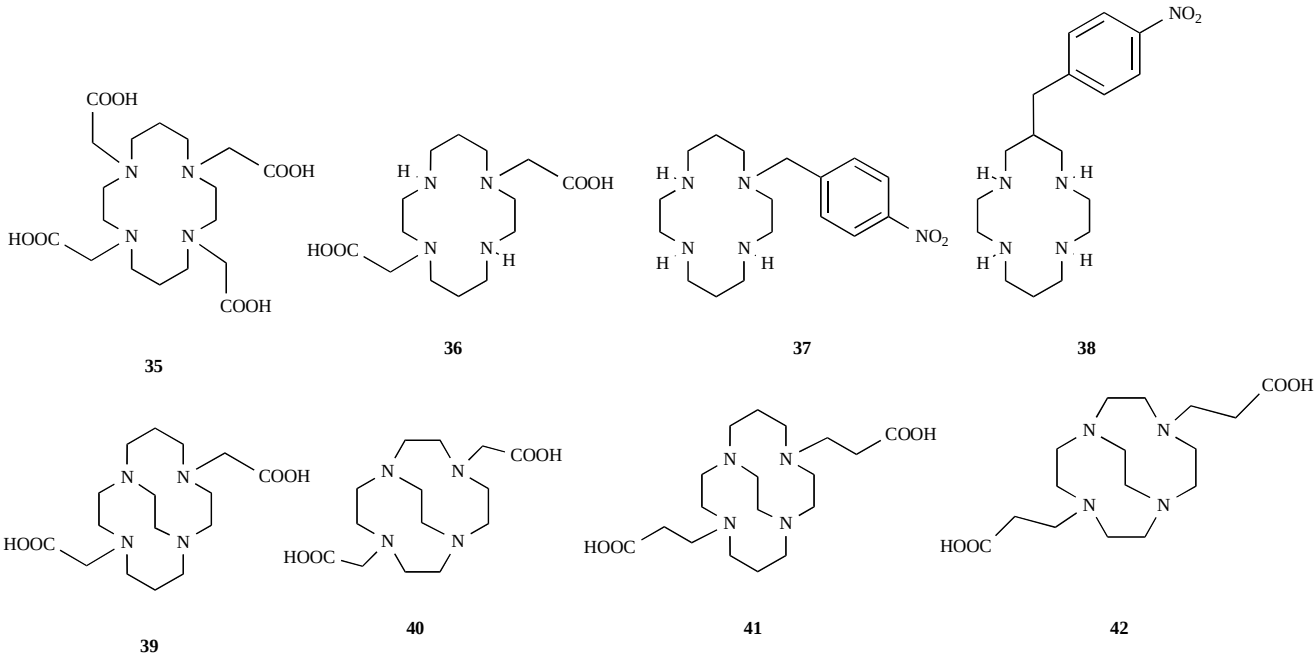


Fig. (8). Structures of TETA (**35**), TE2A (**36**), 1-(4-nitrobenzyl)-cyclam (**37**), 6-(4-nitrobenzyl)-cyclam (**38**), CB-TE2A (**39**), CB-DO2A (**40**), 3,3'-(1,4,8,11-tetraazabicyclo[6.6.2]hexadecane-4,11-diyl)dipropanoic acid (**41**) and 3,3'-(1,4,7,10-tetraazabicyclo[5.5.2]tetradecane-4,10-diyl)dipropanoic acid (**42**).

pared to the other chelators was attributed to *i*) the cross-bridging that supposedly stabilizes the steric configuration of the donor atoms even if a protonation of an oxygen or nitrogen atom occurs, thus facilitating the reformation of the metal-donor atom-interaction [133]. *ii*) The ideal cavity size of CB-TE2A for the Cu^{2+} ion (verifiable by crystal structures) that results in short donor atom-copper ion distances that reduce the probability for protonation of the donor atoms and enhance the kinetic stability (a cleft in the ligand envelopment was observed for the Cu-CB-DO2A complex, enhancing the probability of transmetallation and detachment of donor atoms) [102, 133]. *iii*) CB-TE2A allows for a reversible reduction of the Cu^{2+} ion and is able to complex also Cu^{+} with a certain stability, thus allowing for an enhanced probability of reformation of the Cu^{2+}-CB-TE2A complex [102, 133]. *iv*) The carboxymethyl pendant arms exhibit an ideal length for complete envelopment of the central copper ion whereas carboxyethyl pendant arms result in decreased stability due to less ideal encapsulation of the copper ion and therefore stronger susceptibility to central metal reduction [102].

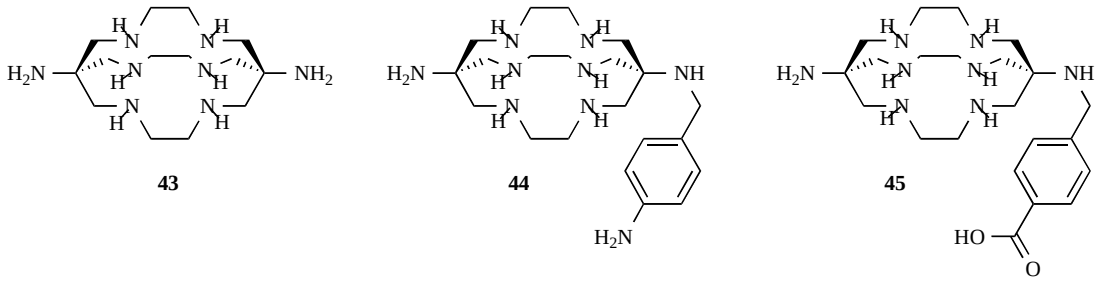
The only disadvantage of this favorable chelator is its slow complex formation kinetics that requires relatively long reaction times of one hour at high temperatures (between 75 and 99°C) for quantitative complex formation [58, 131]. A possible solution could be the implementation of this complex ligand in a prelabeling approach to make use of the advantages of this very favorable chelator for heat-susceptible biomolecules to be radiolabeled with copper isotopes.

Chelators Based on Sarcophagine Ligands

Recently, the new class of sarcophagine chelators was developed in order to obtain copper complexes exhibiting a high stability as well as fast complex formation kinetics. For introduction into medically interesting compounds, derivatives suitable for biomolecule-conjugation – DiAmSar (**43**), SarAr (**44**) and AmBaSar (**45**) – were synthesized (Fig. **9**). They could be introduced into tumor-affine peptides and antibodies and the resulting conjugates were labeled with ^{64}Cu within 5 to 60 minutes at ambient temperature in nearly quantitative yields of ≥95% [127, 134-137]. However, the *in vitro* stability and biodistribution studies revealed a considerable degradation of the complex associated with a copper release, pointing to an insufficient stability under physiological conditions [134, 136-137].

In a recent study, ^{64}Cu-labeled conjugates of the tumor-affine cRGD peptide comprising CB-TE2A and DiAmSar as chelating agents were directly compared. The CB-TE2A required, as described before, harsh conditions for the radiolabeling step of 95°C for one hour (in contrast to this, the DiAmSar-conjugate was radiolabeled at ambient temperature within the same time) but achieved a higher tumor-uptake and far more favorable tumor-to-background-ratios together with faster clearance kinetics in the biodistribution studies of both tracers [127]. These findings were confirmed by a study comparing different chelating agents for antibody derivatization, subsequent ^{64}Cu-labeling and the *in vivo* biodistribution characteristics of the obtained radiotracers. Among the investigated chelators used (one NOTA, one TETA, two DOTA and one sarcophagine derivative), the sarcophagine-derivatized antibody showed the lowest tumor-to-background-ratios of all antibody-chelator conjugates indicating a limited stability of the complex *in vivo* [138].

As can be inferred from the studies described in this paragraph, CB-TE2A and its derivatives seem to still represent the most suitable chelating agents for the *in vivo* appli-



43 **44** **45**

Fig. (9). Structures of DiAmSar (**43**), SarAr (**44**) and AmBaSar (**45**).

cation of copper radionuclides due to the extremely high stability of their complexes.

4. CONCLUSION

Great efforts have been made to develop new radiopharmaceuticals for the diagnosis and treatment of various diseases. For this purpose, target-specific agents have to be found, synthesized and derivatized for use as diagnostic markers or therapeutic agents and the research in this field is still underway.

One prerequisite for the successful use of such a biomolecule exhibiting favorable properties as radiopharmaceutical is certainly the stable binding of an appropriate radioisotope to the targeting vector. In case of radiometal nuclides intended for imaging or therapy, the targeting vector has to be derivatized with a chelator that forms a highly stable complex with the respective radiometal ion. The high complex stability is mandatory for an *in vivo* utilization as it enables high target tissue-to-background-ratios and thus high image quality in diagnostic applications as well as a maintainable radiation burden for non-target organs in therapeutic applications. In addition to high thermodynamic and *in vivo* stabilities, the chelator has furthermore to provide efficient labeling kinetics and only minor influence on the biodistribution of the targeting vector.

For some of the currently most commonly used radiometal nuclides, the stable complexation using chelating agents is still challenging, whereas for others, suitable ligands have been found that allow for a stable introduction of the metal ion. Thus, research in the field of targeting vector design and chelator chemistry for radiometal nuclides is still ongoing, providing increasingly suitable radiopharmaceuticals with designed properties for medical applications.

ACKNOWLEDGEMENT

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ABBREVIATIONS

1B3M-DTPA	= 1-benzyl-3-methyl-diethylenetriamine-pentaacetic acid
1B4M-DTPA	= 1-benzyl-4-methyl-diethylenetriamine-pentaacetic acid
1M3B-DTPA	= 1-methyl-3-benzyl-diethylenetriamine-pentaacetic acid
AmBaSar	= 4-((8-amino-3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosan-1-ylamino)methyl)benzoic acid
Bn-DTPA	= 2-benzyl-diethylenetriamine-pentaacetic acid
CB-TE2A	= 1,4,8,11-tetraazabicyclo[6.6.2]hexadecane-4,11-diacetic acid
CB-DO2A	= 1,4,7,10-tetraazabicyclo[5.5.2]tetradecane-4,10-diacetic acid
C-DOTA	= 2-(4-nitrobenzyl)-DOTA
C-NE3TA	= 2,2'-(7-(2-(carboxymethylamino)-2-(4-isothiocyanatobenzyl)ethyl)-1,4,7-triazonane-1,4-diyl)diacetic acid
C-NETA	= 2,2'-(2-(4,7-bis(carboxymethyl)-1,4,7-triazonan-1-yl)-(2-(4-isothiocyanatoben-zyl))ethylazanediyl)diacetic acid
Cu-ATSM	= Cu-diacetyl-4,4'-dimethyl-bis-thiosemicarbazone
Cyclam	= 1,4,8,11-tetraazacyclotetradecane
Cyclen	= 1,4,7,10-tetraazacyclododecane
DiAmSar	= 3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane-1,8-diamine
DO2A	= 1,4,7,10-tetraazacyclododecane-1,7-diacetic acid
DOTA	= 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DOTA-GA	= 2-(4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)pentanedioic acid
DTPA	= Diethylenetriamine-pentaacetic acid

HBED-CC = 3,3'-(3,3'-(ethane-1,2-diyl-bis((carboxymethyl)azanediyl)) bis(methylene) bis(4-hydroxy-3,1-phenylene))dipropanoic acid

MeO-DOTA = 2,2',2''-(10-(1-carboxy-2-(4-isothiocyanato-2-methoxy)phenylethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid

NE3TA = 7-(2-(carboxymethylamino)ethyl)-1,4,7-triazonane-1,4-diacetic acid

NE3TA-Bn = (7-(2-(ben-zyl(carboxymethyl)amino)ethyl)-1,4,7-triazonane-1,4-diacetic acid

NETA = (2-(4,7-bis(carboxymethyl)-1,4,7-triazonan-1-yl)ethylazane-diacetic acid

NOTA = 1,4,7-triazonane-1,4,7-triacetic acid

NOTA-GA = 2-(4,7-bis(carboxymethyl)-1,4,7-triazonan-1-yl)pentanedioic acid

PA-DOTA = 10-(1-carboxy-3-(4-isothiocyanato)phenylpropyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid

PrP9 = 1,4,7-triazonane-1,4,7-tri(2-carboxyethyl)methyl phosphinic acid

SarAr = N-(4-aminobenzyl)-3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane-1,8-diamine

p-SCN-Bn-NOTA = 2-(4-isothiocyanatobenzyl)-NOTA

p-SCN-CHX-A"-DTPA = 2*S*-(4-isothiocyanatobenzyl)cyclohexyl-diethylenetriamine-pentaacetic acid

p-SCN-CHX-B"-DTPA = 2*R*-(4-isothiocyanatobenzyl)cyclohexyl-diethylenetriamine-pentaacetic acid

TE2A = 1,4,8,11-tetraazacyclo-tetradecane-1,8-diacetic acid

TETA = 1,4,8,11-tetraaza-cyclotetradecane-1,4,8,11-tetraacetic acid

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