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Research Article

The correlation of serum protein association and cellular uptake with lipophilicity and polarity of the backbone substituents of the technetium-99m-amine-oxime chelates

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Abstract: Detection of tumor hypoxia is of importance because hypoxic cancer cells can resist radiation therapy and also induce molecules to help them surviving and metastasizing. Our previous study has reported the development of technetium-99m labeled amine-oxime compounds containing 2-nitroimidazoles to target hypoxic cells. In order to develop new generation of ^{99m}Tc, ^{64}Cu, and ^{67}Cu based amine-oxime chelates with improved selectivity on tumor hypoxia in the future, it is important to understand the correlation of some structural parameters such as lipophilicity and polarity of the backbone -constituents of a radiochelate with its serum protein binding potential and cellular-uptake-level. The correlation is investigated in this work by using five ^{99m}Tc-amine-oxime chelates containing 2-NI group and another five containing aniline group (as control). The results indicate that the level of protein-and cellular-binding of a radiochelate increased with their lipophilicity which generally correlates with the lengthening of the alkyl chains on the backbone of the radiochelates. The greater lipophilicity of a radiochelate, the higher percentage it bound to serum proteins and cellular membrane. Our study also indicates that, in addition to lipophilicity, polarity of the constituents is also an important factor of determining the levels of serum protein binding and cellular accumulation of a radiochelate. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: amine-oxime; lipophilicity; polarity; tumor hypoxia; technetium-99m

Introduction

Cancer cells caused by insufficient supply of oxygen can be found in solid tumors.[1,2] The problems of hypoxic cancer cells in clinic are their ability of resisting radiation therapy and chemotherapy.[3,4] Moreover, tumor hypoxia can promote the expression of hypoxia-inducible-factor (HIF-1α), an important molecule of regulating the development of tumor angiogenesis.[1,5] Measurement of tumor hypoxia is therefore clinically necessary and important. Many radiopharmaceuticals developed for nuclear medicine imaging tumor hypoxia are radioactive halogens or transition metallic ions labeled molecules such as [^{18}F]fluoromisonidazole.[6] [^{18}F](2-(2-nitro-1-H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide) (EP5),[7] [^{123}I]iodoazomycin arabinoside,[8] [^{99m}Tc](4,9-diaza-3,3,10,10-tetramethyldodecan-2,11-dione dioxime) ([^{99m}Tc]HL91),[9] [^{99m}Tc](3,3,9,9-tetramethyl-1-(2-nitro-1-H-imidazol-1-yl)-4,8-diazaundecane-2,10-dione dioxime) ([^{99m}Tc]BMS181321).[10] [^{99m}Tc](3,3,9,9-tetramethyl-6-[(2-nitro-1-H-imidazol-1-yl)methyl]5-oxa-4,8-diazadioxime) ([^{99m}Tc]BRU59-21),[11] and [^{64}Cu](diacetyl-bis(N^4-methylthiosemicarbazone)) ([^{64}Cu]ATSM).[12,13] Those neutral agents had exhibited selective accumulation in hypoxic cells *in vitro* and *in vivo*. It is believed that the tumor-hypoxia-selectivity of a radiolabel is usually electronically driven, and also correlates to its structural characters. However, the tumor hypoxia selectivity and pharmacologic effectiveness of a radiotracer in many cases can be diminished by its non-specific binding to proteins and normal organs *in vivo*,[14,15] while the level of those non-specific bindings is often determined by the same structural characters of the radiotracer as well. One of the important parameters is lipophilicity that in many circumstances influences the levels of a molecule binding to extracellular serum proteins,[16,17] the rates

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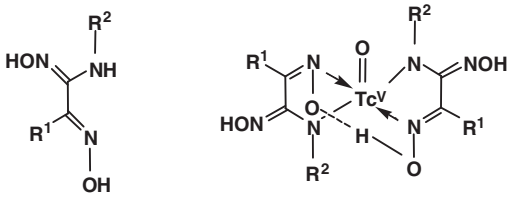
of penetrating through cellular membranes,[18,19] the affinity of associating to intracellular molecules such as enzymes and DNAs,[20] and the toxicity to cancer cells.[21] It needs to point out that in addition to lipophilicity the role of other parameters such as configuration, charge, and polarity of subsistent on the backbone of a radiotracer playing in cellular binding has not been well documented, while all of those structural characters and parameters can be related to the nature of the backbone substitutions of the radiotracer.

Our previous work has reported the development of amine-oxime chelating agents bearing a polar 2-nitroimidazole (2-NI) group to chelate ^{99m}Tc for tumor hypoxia imaging.[22] Since an amine-oxime chelator contains a vicinal dioxime group, it may be able to chelate copper ions to form a stable chelates as well.[23] If it is the case, this series of chelators can be used to chelate ^{64}Cu and ^{67}Cu to produce agents for either tumor hypoxia PET imaging or tumor radiotherapy. In order to develop new radiochelates with improved selectivity for hypoxic cancer cells, it is important to understand the correlation of some structural parameters with the protein- and cellular-binding-potentials of a radiochelate. This work reports the investigation of how the nature of the backbone substitutions such as alkyl chains, polar 2-NI, and less polar aniline correlate with lipophilicity and with serum protein and cellular binding of the ^{99m}Tc-amine-oxime chelates.

Results and discussion

In our previous work of developing ^{99m}Tc labeled amine-oxime containing 2-NI for tumor hypoxia targeting,[22] it had been noted that the accumulation level (a combination of selective- and non-selective-uptakes) in hypoxic Chinese hamster ovary (CHO) cells of the radiochelates had shown a correlation to their lipophilicity. It has been well known that lipophilicity is an key parameter that plays significant role in extracellular protein binding and in cellular uptake of a drug.[16–20] However, according to our observation in the experiment,[22] polarity of a substituent on the backbone of a molecule may also play a significant role as does the lipophilicity in protein and cellular binding. In order to investigate the roles of these two structural parameters, this work uses two series of amine-oxime chelates, which at one end of the backbone has an attachment of alkyl chains with different lengths and at the other end either a 2-NI or an aniline group (Figure 1).[22] As reported previously,[22] two molecules of an amine-oxime chelator can stably chelate one ^{99m}TcO^{v} to form a neutral chelate. The radiochemical



Amine-o xime
1a-e, **2**a-e

^{99m}Tc complexes

Amine-oxime	R^1	R^2	
		1a-e	**2**a-e
1a, **2**a	CH_3-	-C_2H_4-2NI	-C_6H_5
1b, **2**b	$CH_3CH_2CH_2$-	-C_2H_4-2NI	-C_6H_5
1c, **2**c	C_6H_5-	-C_2H_4-2NI	-C_6H_5
1d, **2**d	$(CH_3)_2CH_2CH_2$-	-C_2H_4-2NI	-C_6H_5
1e, **2**e	$C_6H_5CH_2CH_2$-	-C_2H_4-2NI	-C_6H_5

Figure 1 The proposed structures of [^{99m}Tc](**1**a–e)$_2$ and [^{99m}Tc](**2**a–e)$_2$ in the cis arrangement.

purity of the [^{99m}Tc](**1**a–e)$_2$ and [^{99m}Tc](**2**a–e)$_2$ chelates ranged from 87 to 100%. As it has been indicated before that the lipophilicity of the chelators correlated with the hydrophobicity of the backbone substitutions, while the hydrophobicity of the substitutions is roughly proportional to the length of the alkyl chains such as Me, Pr, Ph, *i*Bu, and 2-PhEt groups. Upon chelating a ^{99m}TcO^{v}, the length of the alkyl substitutions remained to be an important element in determining the lipophilicity of the radiotracer. The lipophilicity of [^{99m}Tc](**1**a–e)$_2$ and [^{99m}Tc](**2**a–e)$_2$ were represented by their partition coefficients (*PC*s) between *n*-octanol and phosphate buffered saline (PBS), while the *PC*s roughly correlated with length of the alkyl chains and exhibited an order as: Me < Pr < Ph < *i*Bu < 2-PhEt. The five chelates of [^{99m}Tc](**2**a–e)$_2$ containing two less polar groups of aniline were more lipophilic than those of the [^{99m}Tc](**1**a–e)$_2$ counterparts containing two polar substituents of 2-NI, suggesting that the aniline had made greater contribution to lipophilicity of the radiochelates over the 2-NI. By comparison, the PCs ratios of the five [^{99m}Tc](**2**a–e)$_2$ over their counterparts of [^{99m}Tc](**1**a–e)$_2$ were: 7.6 ([^{99m}Tc](**2**a)$_2$/[^{99m}Tc](**1**a)$_2$), 3.7 ([^{99m}Tc](**2**b)$_2$/[^{99m}Tc](**1**b)$_2$), 2.6 ([^{99m}Tc](**2**c)$_2$/[^{99m}Tc](**1**c)$_2$), 2.0 ([^{99m}Tc](**2**d)$_2$/[^{99m}Tc](**1**d)$_2$), and 1.1 ([^{99m}Tc](**2**e)$_2$/[^{99m}Tc](**1**e)$_2$) respectively, indicating that the contribution of aniline to the lipophilicity decreased with the increase of molecular weight of the radiochelates.

Lipophilic radiochelates usually exhibit significant serum protein binding that may cause negative

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Table 1 The partition coefficients of $[^{99m}Tc](\mathbf{1}a–e)_2$ and $[^{99m}Tc](\mathbf{2}a–e)_2$ between *n*-octanol and PBS

^{99m}Tc chelates containing 2-NI		^{99m}Tc chelates containing aniline	
Chelates	PC	Chelates	PC
$[^{99m}Tc](\mathbf{1}a)_2$	0.11 (± 0.01)	$[^{99m}Tc](\mathbf{2}a)_2$	0.84 (± 0.03)
$[^{99m}Tc](\mathbf{1}b)_2$	5.54 (± 0.26)	$[^{99m}Tc](\mathbf{2}b)_2$	19.8 (± 1.0)
$[^{99m}Tc](\mathbf{1}c)_2$	10.6 (± 0.7)	$[^{99m}Tc](\mathbf{2}c)_2$	28.0 (± 1.2)
$[^{99m}Tc](\mathbf{1}d)_2$	32.6 (± 2. 0)	$[^{99m}Tc](\mathbf{2}d)_2$	64.2 (± 0.4.0)
$[^{99m}Tc](\mathbf{1}e)_2$	193 (± 17)	$[^{99m}Tc](\mathbf{2}e)_2$	210 (± 12)

Each value is mean of 3 determinations ± SEM.

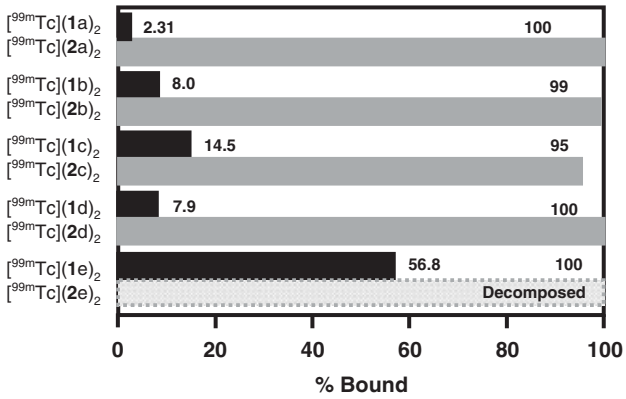

Figure 2 The binding tests of $[^{99m}Tc](\mathbf{1}a–e)_2$ (black bars) and $[^{99m}Tc](\mathbf{2}a–e)_2$ (grey bars) in 0.73 mM BSA. Percentage of the binding was measured by HPLC with Tosohass TSK-GEL G3000XWXL 7.8 × 300 mm column and with 0.05 M pH 6.7 phosphate buffer as eluent. Flow rate: 0.7 ml/min.

impacts such as high background and poor resolution on tumor imaging. This work used bovine serum albumin (BSA) as the representative of human serum proteins to test the binding potentials of the radiochelates with various lipophilicity. The binding was measured by size-exclusion HPLC, while the binding percentage was calculated by dividing the radioactivity moved with BSA over that injected. The results were summarized in Figure 2. The binding of the five $[^{99m}Tc](\mathbf{1}a–e)_2$ radiochelates to 0.73 mM BSA was 2.3% for $[^{99m}Tc](\mathbf{1}a)_2$ (PC 0.11), 8.0% for $[^{99m}Tc](\mathbf{1}b)_2$ (PC 5.54), 14.5% for $[^{99m}Tc](\mathbf{1}c)_2$ (PC 10.6), 7.9% for $[^{99m}Tc](\mathbf{1}d)_2$ (PC 32.6), and 56.8% for $[^{99m}Tc](\mathbf{1}e)_2$ (PC 193), respectively. Despite of the difference in *PC*s, $[^{99m}Tc](\mathbf{1}c)_2$ (PC 10.6) had displayed greater protein binding percentage over that of $[^{99m}Tc](\mathbf{1}d)_2$ (PC 32.6) suggesting that the non-polar phenyl group right on the backbone of $[^{99m}Tc](\mathbf{1}c)_2$ made more significant contribution to serum protein binding than the long alkyl chain of iso-butyl group of $[^{99m}Tc](\mathbf{1}d)_2$. With the exception of $[^{99m}Tc](\mathbf{1}c)_2$, the BSA binding potential of the rest four $[^{99m}Tc](\mathbf{1}a\text{-}e)_2$ increased with the elevation of their PCs. The role of polarity of the substituents played in serum protein binding of the radiochelates can also be observed in the experiment. The results (Figure 2) exhibited that all the five $[^{99m}Tc](\mathbf{2}a–e)_2$ radiotracers containing two aniline groups showed over 95% binding to BSA under the same condition of $[^{99m}Tc](\mathbf{1}a–e)_2$. In BSA solution with even lower concentration (0.029 mM), 20% of $[^{99m}Tc](\mathbf{2}c)_2$ and 100% of $[^{99m}Tc](\mathbf{2}e)_2$ were bound respectively, suggesting that (1) the binding of the radio analogues was BSA concentration dependent; (2) the binding correlated to the lipophilicity of the analogues. No binding of $[^{99m}Tc](\mathbf{2}c)_2$ to *h*IgG of 0.013 mM was detected, which implies that the binding might be taken place mainly between the radiochelates and the serum proteins. By comparing the serum protein binding levels of the $[^{99m}Tc](\mathbf{1}a–e)_2$ and $[^{99m}Tc](\mathbf{2}a–e)_2$, it was noted that, in addition to lipophilicity, polarity of backbone substitutions of a radiochelate was also an important factor in determining the binding level in serum proteins. For example, with a PC of only 0.84, 100% $[^{99m}Tc](\mathbf{2}a)_2$ was bound to BSA (Figure 2), 12.5 fold greater than that of the $[^{99m}Tc](\mathbf{1}b)_2$ (PC 5.54). The nitro group of 2-NI in the radiochelates is a polar group because of its stronger electronegativity compared to the aniline. It was speculated that the binding of the neutral compounds to BSA might be occurred in a hydrophobic environment of the proteins. It needs to point out that the $[^{99m}Tc](\mathbf{1}e)_2$ was unstable when incubated in the BSA at 37°C, suggesting that a radiochelate with longer and larger side chain could be destructed in BSA solution.

In addition to serum protein binding, lipophilicity is also an influential parameter in non-specific cellular uptake of a radiochelate. The correlation of non-specific cellular accumulation with lipophilicity of $[^{99m}Tc](\mathbf{1}a–e)_2$ and $[^{99m}Tc](\mathbf{2}a–e)_2$ was tested in aerobic CHO cells where the accumulation of all the radiotracers was supposed to be non-specific. The results (Figure 3) indicated that cellular uptakes of the $[^{99m}Tc](\mathbf{1}a–e)_2$ and $[^{99m}Tc](\mathbf{2}a–e)_2$ increased with their lipophilicity, suggesting a higher level of accumulation in cellular membrane and intracellular proteins for a radiochelate with greater lipophilicity. It might be due to the stronger serum protein binding potential, $[^{99m}Tc](\mathbf{2}a–e)_2$ of greater PCs had contrarily shown smaller uptake level in CHO cells (suspending in α-MEM containing 10% (v/v) fetal bovine serum) than their counterparts of the $[^{99m}Tc](\mathbf{1}a–e)_2$ with smaller *PC*s at both 5 min (panel A, Figure 3) and 6 h (panel B) incubation time. It is summarized that: (1) lipophilicity of a charge-neutral chelate correlated with

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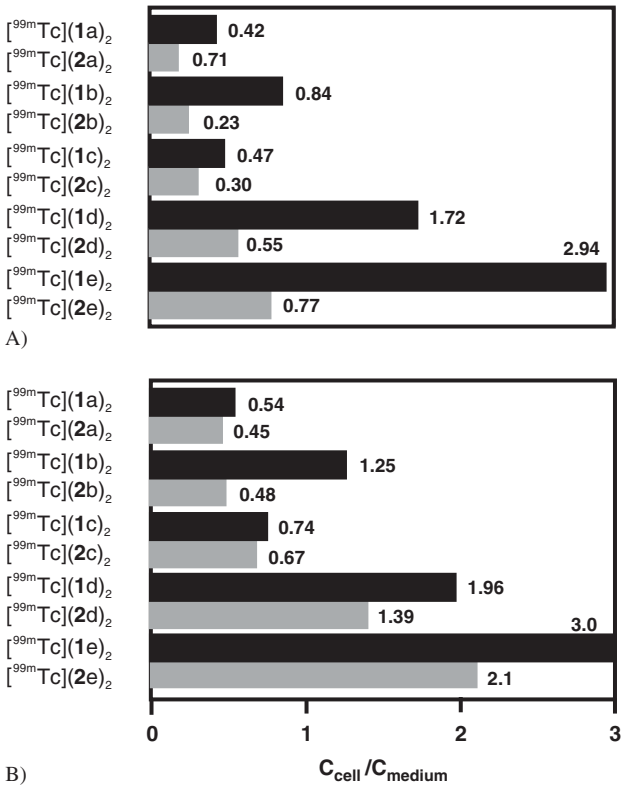

Figure 3 Accumulation of [^{99m}Tc](**1**a–e)$_2$ and [^{99m}Tc](**2**a–e)$_2$ in Chinese hamster ovarian (CHO) cells. The cells were suspended in α-minimal essential medium containing 10% (v/v) fetal bovine serum and incubated with each radiochelate under aerobic condition at 37°C for a given time of 5 min (panel A) and 360 min (panel B). The radioactivity in the cells (C_{cell}) and in equal volume of medium (C_{medium}) were counted and the ratios of C_{cell}/ C_{medium} were used as an indication of the cellular-accumulation-level of the radiochelates.

the hydrophobicity of the backbone substitution, while the hydrophobicity of the substitution was usually proportional to the length of the alkyl chains; (2) a radiochelate with shorter side-chain generally exhibits greater stability in solution compared to its analogues with longer one; (3) both lipophilicity and polarity of the backbone substituents of a radiochelate are important elements of influencing its serum protein binding potential and cellular accumulation level; (4) in order to design a radiopharmaceutical with improved cancer-selectivity and pharmacologic effectiveness, compromises between target-molecule-binding affinity, molecular configurations, lipophilicity, charge, and polarity of backbone substitutions need to be considered.

Experimental

Synthesis of amine-oxime chelators: The synthesis and structural confirmation by ^{1}H-NMR and ES-MS of the amine-oxime chelators containing 2-NI and aniline groups have been reported previously.[22]

Techetium-99m labeling of the amine-oxime chelators: ^{99m}Tc labeling of the chelating agents of **1**a–e and **2**a–e were carried out in an established manner).[22] Briefly, at room temperature, 40–100 μg (0.12×10^{-6}–0.52×10^{-6} mol) of a chelator in 10–25 μl of methanol was mixed with 200 μl 10 mg/ml pentasodium diethylenetriaminepentacetate ($\sim 4 \times 10^{-6}$ mol), 2–1000 MBq of $Na^{99m}TcO_4$ (0.1×10^{-12}–51×10^{-12}mol) in 2–200 μl saline, and 19.2 μg $SnCl_2$ (0.1×10^{-6} mol) in 50 μl 10 mg/ml pentasodium DTPA ($\sim 1 \times 10^{-6}$mol). The chelation was fast and can be completed in a few minutes at room temperature. The yield and purity of the labeling were tested by HPLC on a Beckman ODS 4.6 × 250 mm 5 μm C18 column with H_2O/MeOH as mobile phase, gradient 50% H_2O to 10% H_2O over 20 min, flow rate 1.0 ml/min.

Partition coefficients (PCs) measurement of the ^{99m}Tc chelates: The lipophilicity of a radiochelate was represented by its partition coefficient between *n*-octanol and PBS, while the two phases were mutually saturated before use. Briefly, 10 μl of a label in the labeling solution was added to 1.0 ml of PBS which was equilibrated with 1.0 ml *n*-octanol. The mixture was vortexed for 1 min and then centrifuged at 12 000 g for 2 min. After the separation of the phases, 3 × 100 μl aliquots from each phase were removed for radioactivity counting in a Picker Pace-1 γ well counter. The PC vale of a radiolabel was the ratio of the mean counts in *n*-octanol over that in the PBS.

Stability test of the ^{99m}Tc chelates in BSA solution: 100 μl of each ^{99m}Tc chelate of the [^{99m}Tc](**1**a–e)$_2$ and [^{99m}Tc](**2**a–e)$_2$ was mixed with 500 μl BSA (Sigma) PBS solution with concentration of either 50 mg/ml or 2 mg/ml and incubated at 37°C for a period up to 24 h. 100 μl of the mixture was then injected into the HPLC with Tosohass TSK-GEL G3000XWXL 7.8 × 300 mm column, 0.05 M pH 6.7 phosphate buffer as eluent, and with a flow rate of 0.7 ml/min. The percentage of the ^{99m}Tc chelates binding to BSA was calculated by dividing the radioactivity of the chelates shifted to the position of BSA over that injected.

Stability test of [^{99m}Tc](2c)$_2$ in hIgG solution: 200 μl of [^{99m}Tc](**2**c)$_2$ was mixed with 250 μl 2 mg/mL *h*IgG (Sigma) PBS solution and incubated at 37°C for 3 h. 100 μl of the mixture was then injected into SE HPLC (with a similar condition as stated above) to calculate the binding percentage of the chelate to the *h*IgG.

Accumulation of the ^{99m}Tc chelates in the aerobic CHO cells. The *in vitro* cellular accumulation of the

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radiochelates were tested by an established model used previously.[22] CHO cells incubation under aerobic condition was used in this work to investigate the correlation of non-specific cellular-uptake-levels of the radiochelates containing 2-NI and aniline groups. Briefly, CHO cells suspension (with a concentration of $(1–2) \times 10^6$ cells/mL) in α-MEM containing 10% (v/v) of fetal bovine serum were incubated at 37°C under aerobic atmosphere containing 5% CO_2. 2–10 MBq ^{99m}Tc label was added to the suspension and aliquots were removed at a time intervals of 5 min and 6 h. 3×100 µl of aliquots were transferred to microcentrifuge tubes containing 1 ml of an oil mixture of phthalic acid dinonyl ester:dibutyl phthalate with a ratio of 2:3 and centrifuged at 12 000 g for 2 min. The radioactivity of the aliquots of supernatant (3×30 µl from each tube) was counted. After being isolated from the oil and the supernatant by aspiration and washed by minimum volume of cold PBS, the cell pellets in the tip of the tube were clipped into counting tubes for radioactivity counting. The ratios of the counts in the cell pellets (C_{cell}) over those in the equivalent volume of supernatants (C_{medium}) were used as a measurement of the cellular-uptake-level of the radiochelates.

Conclusion

The level of cellular- uptake and extracellular proteins binding had shown a direct correlation with the two parameters of lipophilicity and polarity of the substituents on the backbone of the ^{99m}Tc chelates. The protein-binding potential of the 10 radiochelates tested in this work had exhibited an increase with their lipophilicity, which was elevated by lengthening the alky chains on the backbone of the molecules. Since the radiochelates containing aniline group had shown greater protein binding levels compared to those containing 2-NI, it can be concluded that the polar substituents on the backbone of amine-oxime chelates may decrease their binding potential to serum proteins. In summary, the stability, lipophilicity, overall polarity, protein binding potential and cellular accumulation level of a radiochelate depends on the nature of the backbone substitution.

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