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The synthesis and toxicity of tripodal tricarbonyl rhenium complexes as radiopharmaceutical models

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Abstract

We report the synthesis and toxicity of a series of rhenium(I) tricarbonyl complexes incorporating the trisaminomethylethane (TAME) ligand. Compounds with the (TAME)Re(CO)₃⁺ cation were synthesized via several routes, including by use of Re(CO)₅X precursors as well as the aqueous cation Re(CO)₃(H₂O)₃⁺. Salts of the formula [(TAME)Re(CO)₃]X where X = Br[−], Cl[−], NO₃[−], PF₆[−] and ClO₄[−] were evaluated using two cell lines: the monoclonal S3 HeLa line and a vascular smooth muscle cell line harvested from mice. All compounds have isostructural cations and differ only in the identity of the non-coordinating anion. None of the complexes exhibited any appreciable toxicity in the HeLa line up to the solubility limit. In the vascular smooth muscle cell line, the bromide salt exhibited some cytotoxicity, but this observation most likely results from the presence of bromide anion, which has been shown to have limited toxicity.

Keywords

Rhenium; technetium; trisaminomethylethane; Re(CO)₃⁺; toxicity

1. Introduction

Non-invasive imaging techniques play an increasing role in the diagnosis of disease states, and this has spurred the continuing development of new imaging agents. Radiological techniques, such as single photon emission computed tomography (SPECT) and positron emission tomography (PET) based methods, require the use of radionuclide-based drugs, and the most common nuclide currently used in the clinic is the ^{99m}Tc isotope [1–4]. Since the development of Cardiolite® kit for the in situ production of ^{99m}Tc-sestamibi, new generations of ^{99m}Tc drugs have appeared in the clinic, and current research is focusing on

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developing the chemistry of the $\text{Tc}(\text{CO})_3^+$ unit as the nuclide containing moiety [5-8]. Imaging agents containing this fragment can be readily generated in aqueous solution from the $[\text{Tc}(\text{H}_2\text{O})_3(\text{CO})_3]^+$ ion, which can be synthesized in one step from TcO_4^- solutions by use the IsoLink® kit developed by Mallinkrodt. However, in spite of the potential importance of this species, much of the fundamental chemistry and biological processing of $[\text{Tc}(\text{H}_2\text{O})_3(\text{CO})_3]^+$ and $\text{Tc}(\text{CO})_3^+$ -based compounds has yet to be elucidated.

One advantage for the development of $\text{Tc}(\text{CO})_3^+$ based drugs is that the chemistry of this moiety can be readily modeled by using the analogous, non-radioactive $\text{Re}(\text{CO})_3^+$ fragment [9]. Although the chemistry of the two elements do differ slightly, the chemistries of both metals in the +1 oxidation state are similar, and this is particularly true for the $\text{M}(\text{CO})_3^+$ moieties [10]. Both ions form facial octahedral geometries upon complexation, and the resultant complexes are typically quite inert to substitution. We have recently been investigating the fundamental chemistry of the $\text{Re}(\text{CO})_3^+$ fragment, and have developed complexes that exhibit remarkable stabilities in aqueous solution, even in the presence of competing biological ligands, such as histidine and cysteine [11-13]. We have reported on the structures and stabilities of several tripodal complexes of $\text{Re}(\text{CO})_3^+$, and have found that a complex incorporating the 1,1,1-trisaminomethylethane (TAME) ligand, $[\text{Re}(\text{TAME})(\text{CO})_3]^+$ (Figure 1) showed no degradation under biologically relevant challenge conditions [14].

The chemical inertness of the $[\text{Re}(\text{TAME})(\text{CO})_3]^+$ ion indicates that this complex should exhibit little or no toxicity. Since the complex is inert to substitution and unreactive to either histidine or cysteine, we wanted to continue to examine the effect of this complex on living cells. In general, the toxicity of radionuclide-containing compounds is not as important as in other imaging agents [15]. Typically, the doses of the drug are so low that only the radioactivity is of concern, and clearly the diagnostic benefit of the imaging technique outweighs the radiological risk. However, any demonstrable lack of toxicity due to inertness does bode well for rapid and complete clearance from the patient, which is a requirement for any radionuclide-base imaging agent [16].

Herein, we report the synthesis, characterization, and *in vivo* cellular studies of a series of (TAME) rhenium(I) salts. The salts incorporate both halide (chloride and bromide) and non-coordinating anions (nitrate, hexafluorophosphate, and perchlorate). Our characterizations of these salts indicate that the identity of the anions does not affect the structures of the rhenium complex cations. In two separate cell line studies, no appreciable toxicity was observed, with the exception of the bromide salt. The bromide salt did increase mortality in the vascular smooth muscle cells, but we propose that this toxicity resulted from the halide anion rather than the complex itself.

2. Experimental

2.1 General Materials/Methods

All reagents and solvents were purchased from Sigma, Aldrich, Acros Organics or Strem and used without further purification. Compound **1** was prepared as previously described. Sterile cell culture implementation was obtained from VWR and used without further sterilization. High resolution mass spectrometry experiments were performed at the Mass Spectrometry and Proteomics Facility of Ohio State University on a Micromass electrospray ionization time of flight (ESI-ToF™) II (Micromass, Wythenshawe, UK) mass spectrometer equipped with an orthogonal electrospray source (Z-spray) operated in positive ion mode. Sodium iodide was used for mass calibration for a calibration range of m/z 100-2000. Samples were prepared in a solution containing acidified methanol and infused into the electrospray source at a rate of 5-10 $\mu\text{L min}^{-1}$. Optimal ESI conditions were: capillary

voltage 3000 V, source temperature 110 °C and a cone voltage of 55 V. The ESI gas was nitrogen. Data was acquired in continuum mode until acceptable averaged data was obtained. Elemental analysis was conducted at the University of Illinois, School of Chemical Sciences Microanalysis Laboratory. Cells were obtained from ATCC. All media and staining assays were obtained from Invitrogen. Additional vessels and reagents were obtained from VWR. Cell viability was visualized using fluorescent microscopy (Axiovert 200, Carl Zeiss) and imaged with a CCD camera (AxioCam HRm, Carl Zeiss)

Single crystal X-ray diffraction data was collected at 100 K (Bruker KRYO-FLEX) on a Bruker SMART APEX CCD-based X-ray diffractometer system equipped with a Mo-target X-ray tube ($\lambda = 0.71073 \text{ \AA}$) operated at 2000 watts power. The detector was placed at a distance of 5.009 cm from the crystal. Integration and refinement of crystal data was done using Bruker SAINT software package and Bruker SHELXTL (version 6.1) software package, respectively [17]. Absorption correction was completed using the SADABS program. Crystals were placed in Paratone oil upon removal from the mother liquor and mounted on a plastic loop in the oil. Data collection and refinement parameters for crystals of **2** through **5** are shown in Table 1 (crystal data for **1** were previously published).

2.2 Syntheses

2.2.1 Synthesis of $[\text{Re}(\text{CO})_3(\text{TAME})]\text{Cl}$ (2**)**—100 mg of $\text{Re}(\text{CO})_5\text{Cl}$ (2.46 mmol) was refluxed in approximately 30 mL of methanol until dissolved and then a 1.1 molar equivalent of the TAME ligand (33 mg) was added. The mixture was allowed to reflux for 5 hours, and then the solvent was allowed to evaporate overnight. The resulting white crystals were then recrystallized from water. Yield: 86.9 mg (74% yield) High res. ESI MS (positive ion): 388.0674 M/z (M+) (calculated: 388.0671 M/z) CHN Analysis Calc. for $\text{ReC}_8\text{H}_{19}\text{N}_3\text{O}_5\text{Cl}$: C, 21.23%; H, 3.58%; N, 8.85%. Found: C, 21.69%; H, 4.32%; N, 9.49%. IR (CO stretch, cm^{-1}): 2023, 1884. Although the elemental analyses data for “H” and “N” are somewhat unsatisfactory, the ESI-MS results reasonably support the formula.

2.2.2 Synthesis of $[\text{Re}(\text{CO})_3(\text{TAME})]\text{X}$, $\text{X} = \text{ClO}_4^-$, NO_3^- and PF_6^- (3**, **4**, **5**)**—1, (50 mg 1.06 mmol) was added to 25 mL of water and heated with stirring until completely dissolved. A molar equivalent of a solution of AgX ($\text{X} = \text{ClO}_4^-$, NO_3^- and PF_6^-) in 10 mL water was then added and the resultant mixture was stirred for at least 3 hours in the dark to ensure the reaction went to completion. The resulting AgBr precipitate was removed by filtration with a 0.2 μm syringe filter. The solvent water was then allowed to evaporate resulting in large white crystals (Note: Caution should be used when handling the perchlorate salt, since it is potentially shock sensitive).

3: Yield: 59.6mg (57.2% yield) ESI MS (positive ion): 388.1 M/z (M+) (calculated: 388.1 M/z) CHN Analysis Calc. for $\text{ReC}_8\text{H}_{15}\text{N}_3\text{O}_7\text{Cl}$: C, 19.74%; H, 3.11%; N, 8.63%. Found: C, 19.88; H, 2.87%; N, 8.29%. IR (CO stretch, cm^{-1}): 2021, 1875.

4: Yield: 63.2 mg (65.7% yield) ESI MS (positive ion): 388.7 M/z (M+) (calculated: 388.1 M/z) CHN Analysis Calc. for $\text{ReC}_8\text{H}_{15}\text{N}_4\text{O}_6$: C, 21.38%; H, 3.36%; N, 12.47%. Found: C, 21.41%; H, 3.03%; N, 12.51%. IR (CO stretch, cm^{-1}): 2015, 1875, 1859

5: Yield: 84.9mg (74.5% yield) ESI MS (positive ion): 388.1 M/z (M+) (calculated: 388.1 M/z) CHN Analysis Calc. for $\text{ReC}_8\text{H}_{15}\text{N}_3\text{O}_3\text{PF}_6$: C, 18.05%; H, 2.84%; N, 7.89%. Found: C, 18.25%; H, 2.94%; N, 7.91%. IR (CO stretch, cm^{-1}): 2025, 1870

2.3 General Biology

2.3.1 Spontaneously Hypertensive Rat (SHR) vascular smooth muscle

cultures—The vascular smooth muscle cell cultures used were isolated from thoracic aorta explants from spontaneously hypertensive rats using a modified form of the Ross procedure for explants [18]. The rats were obtained from the SHR breeding colony that has been maintained on Standard Purina Laboratory Chow and tap water, *ad lib*, in the animal facility at the University of Akron and treated according to NIH guidelines. The vascular smooth muscle cells were subcultured and plated on 6 well plates in a 50:50 mixture of Dulbecco's modified eagle medium (DMEM) and Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer, 7.5% (w/v) NaHCO₃ and 1% antibiotic/antimycotic mixture. The cells were incubated at 37°C and 5% CO₂ and were 40% confluent when the compounds were added. The cells were returned to the incubator for 24 h before being washed with 0.4% PBS (phosphate buffer solution) and treated with 0.025% trypan blue for 3 m. After being washed again with PBS, the cells were manually counted, in four fields, from each quadrant of the well, under 20× magnification, ensuring that over 400 cells total were counted.

2.3.2 Hela Culture—HeLa-S3 cells were obtained from ATCC, grown in Ham's F12-K media supplemented with 10% FBS, 10 mM HEPES buffer, 7.5% (w/v) NaHCO₃ and 1% antibiotic/antimycotic mixture on 100 mm plates. The cells were subcultured every 72 h. Cells were seeded onto 24 well plates, after counting in a hemacytometer, to ensure that approximately 20,000 cells were plated per well. Cells were incubated overnight to allow them to adhere to the plates before being treated with the complexes in media. After treatment cells were incubated for 24 h before being stained with Invitrogen's LIVE/DEAD® Viability Assay. The cells were excited at 488 nm and fluorescence was observed at 530 and 570 nm. Photos were taken of the wells such that counts of 400 cells in each well were observed.

3. Results and discussion

3.1 Synthesis and characterization

We previously reported the synthesis and *in vitro* stability of [Re(CO)₃(TAME)]Br (**1**) in comparison to other tripodal complexes based on the *fac*-Re(CO)₃⁺ core.[8] Because of the superior stability of **1** in comparison to the other compounds, we felt that it would be an excellent candidate for an imaging agent model. Since bromide has been reported to have some genotoxicity [19], we synthesized the chloride salt (**2**). This second complex was generated by refluxing Re(CO)₅Cl and the TAME ligand in methanol and then recrystallizing the product in water. Salts with the non-coordinating anions perchlorate (ClO₄[−]) (**3**), nitrate (NO₃[−]) (**4**), and hexafluorophosphate (PF₆[−]) (**5**) salts were synthesized via anion metathesis from **1**. A molar equivalent of the corresponding silver salt was added to **1** in aqueous solution, and the resulting precipitate was filtered away and the solvent was evaporated to give solid crystals of **3**, **4**, and **5**. Each compound was fully characterized by electrospray MS, elemental analysis, IR spectroscopy and single crystal X-ray diffraction.

The structures of compounds **2-5** are shown in Figure 1. The structure of the bromide salt (**1**) was reported previously in a preliminary communication [14]. All five complexes have essentially isostructural cations where the Re(I) ion adopts an octahedral coordination environment. The carbonyls adopt a facial coordination mode, and the TAME ligand binds to the metal in a facial tridentate mode similar to that seen in scorpionate ligand binding. The Re-C and C-O bond lengths for all of the complexes are identical and are in agreement with those observed in other Re(CO)₃⁺ complexes. The Re-N bonds range between 2.0 and 2.3 Å, with an average value of ~2.22 Å. The C-Re-C angles around the metal are close to

the ideal 90° values for an octahedral coordination geometry, but the N-Re-N angles of the TAME ligand are more acute and closer to 80° . The ligand adopts a chiral twist in the X-ray structures, resulting in a lower symmetry (C_3) than the expected C_{3v} . However, we believe this is a product of the solid state packing and not a fundamental aspect of the molecule's structure; all of the space groups have inversion centers, indicating that both enantiomers are present in the unit cell.

The spectroscopies for all three compounds are essentially identical. The IR spectra for these complexes show two intense C-O stretching frequencies. In chloroform solution, the three complexes have identical CO stretching frequencies at 2024 and 1902 cm^{-1} , but in the solid state compounds **1-5** show some differences. The CO stretching frequencies for dried samples of compounds **1-5** are shown in Table 1. As in the solution IR spectrum, due to the C_3 axis, these stretches show the expected a_1 and e symmetries observed at $\sim 2020\text{ cm}^{-1}$ and $\sim 1880\text{ cm}^{-1}$ respectively. Additional shoulder peaks were observed in compounds **1** and **4**, which result from carbonyl interactions with the anion in the solid state and from hydrogen bonding to neighboring NH groups on adjacent rhenium complexes. A similar interaction is observed in crystals of **2**, which exhibit two solvent water molecules per asymmetric unit. Prior to drying, a peak appears at 1915 cm^{-1} resulting from hydrogen bonding interactions with these water molecules

3.2 Partition Coefficient Determination

The partition coefficient of **1** was determined by using the shake flask method employing water and octanol as the hydrophilic and hydrophobic phases respectively [20]. The concentration of **1** in both phases was determined by ICP elemental analysis and the log P was found to be -1.84 . This partition coefficient is as expected for an ionic species, in spite of the hydrophobic nature of the organometallic cation. This log P value is less negative than that observed for simple salts [21], and is equivalent to those observed for some nucleotides and amino acids [22]. Due to the negative value of the log P for **1**, this species most likely would not diffuse across cell membranes and would have to enter the cell through either a channel or via receptor mediated endocytosis [23]. It is important to note that pH will most likely not affect the log P value, since the cation of **1** does not engage in acid-base chemistry over the physiological pH range.

3.3 Cellular Toxicity Studies

3.3.1 HeLa-S3 toxicity studies—In addition to being analogs for technetium chemistry, rhenium complexes incorporating radioactive isotopes of the metal can be used to both image and treat cancer cells [24-25]. For this reason among others, HeLa-S3 cells were chosen as a robust cell line in which to probe anion toxicity [26]. Data from the concentration studies are shown in Figure 2. The cells were grown in 24-well plates and incubated for 24 hours before being treated with aqueous solutions of the complexes in cell media. After treatment, the cells were incubated for another 24 hours before being stained with a fluorescent live/dead cell assay, photographed, and counted to determine percent viability. The cells were exposed to 10^{-8} M to 10^{-3} M concentrations of **1-5**. We compared the toxicity of the rhenium complexes to that of the common OTC analgesics acetaminophen (**6**) and acetylsalicylic acid (**7**) by exposing cells to aqueous solutions of these compounds at similar concentrations. Aqueous sodium bromide (**8**) was used to probe bromide toxicity in the same manner. Additional cells were treated with water or 2 mM hydrogen peroxide to provide a positive and negative control, respectively. Each study was repeated three times on three separate days. After the live:dead cell ratio was determined, the results were averaged to determine the percent viability of the cells. Errors associated with these measurements were calculated to be less than 4%. No significant toxicity was observed even at the highest concentration of every complex. Some slight toxicity is observed in compound **2**, although

even at the highest concentration of **2** cell viability remains greater than 95%. Attempts were made to increase the concentration of the complexes in solution, in order to determine an IC₅₀, but thus far these experiments have been unsuccessful due to their limited solubility in aqueous solution.

Figure 3 shows micrographs of the positive and negative controls of the S3 HeLa cells as well as cells exposed to 10⁻³ M concentrations of compounds **1**, **6** and **7**. The positive control shows predominantly healthy squamous cells as indicated by their morphology and red fluorescence. The negative control was carried out by exposing cell to 2 mM H₂O₂ for 4 h at 37° C. The cells were fixed to the plate with cold methanol, and the live/dead® assay exhibits the expected green fluorescence of dead cells resulting from incorporation of SYTOX® green stain. Cells exposed to 10⁻³ M compound **1** are nearly identical in appearance to the positive control, and exhibit no obvious morphological changes. Cells exposed to 10⁻³ M acetaminophen and aspirin also show predominantly living cells, but closer inspection of morphology shows a higher concentration of rounded cells that are detached from the plate surface, indicating some stress induced by these OTC medications.

3.3.2 Rodent cell toxicity studies—Because one of the intended targets of the technetium analogs of our rhenium complexes is a perfusion imaging agent, we also conducted preliminary toxicity studies in rat vascular smooth muscle cell cultures, isolated from the thoracic aorta and donated by Dr. Milsted's research group [27]. The cells were plated and incubated for 24 hours before being treated with aqueous solutions of **1**, **4**, and **5** in cell media. The treated cells were incubated for another 24 hours before being stained with 0.025% trypan blue solution and analyzed to determine the percent of viable cells. Figure 4 shows the results from this study. Significant cell death was present in the control cells since these cells were more sensitive to variations in cell concentration versus the S3 HeLa cell line. A lower percentage of viable cells was found in the cells treated with **1** at 1 × 10⁻⁴ M, which we attribute to the toxicity of the bromide ion, and not the complex itself. The number of viable cells did not vary significantly from the control cells to the treated cells at all other concentrations of **1**, **4**, and **5** tested.

4. Conclusions

In conclusion, we have shown that complexes of the formula the [Re(TAME)(CO)₃]X exhibit little or no toxicity to both an immortalized cell line (HeLa-S3) and a harvested animal cell line (vascular smooth muscle from spontaneously hypertensive rats). We believe that this lack of biological activity results from the inert nature of the complex. This is in marked contrast to many third row transition metal complexes which typically exhibit moderate to high toxicity [28-31]. The cells examined in this report are able to tolerate high concentrations of [Re(TAME)(CO)₃]X salts with no observable morphological changes and at much higher doses than the OTC drugs acetaminophen and acetosalicylic acid (aspirin). We are continuing this work with investigations into other tripodal Re(CO)₃ complexes as well as bifunctional chelating agents incorporating the Re(TAME)(CO)₃⁺ moiety.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
The structures of the $[\text{Re}(\text{CO})_3(\text{TAME})]\text{X}$ salts where $\text{X} = \text{Cl}^-$ (**2**, top left), ClO_4^- (**3**, top right), NO_3^- (**4**, bottom left) and PF_6^- (**5**, bottom right) with 35% thermal ellipsoids. Hydrogen atoms and the solvate waters in **2** have been omitted for clarity.

Figure 2. Toxicity of complexes **1-8** in HeLa-S3 at concentrations of 10^{-8} to 10^{-3} M. Errors on these measurements are approximately $\pm 3\%$.

Figure 3.
Micrographs of S3 HeLa cells: positive control (A), negative control (2 mM H_2O_2 for 4 h at 37° C, B), 10^{-3} M $[\text{Re}(\text{TAME})(\text{CO})_3]\text{Br}$ (**1**, C), 10^{-3} M acetylsalicylic acid (**6**, D) and 10^{-3} M acetaminophen (**7**, D)

Figure 4.

Toxicity results for rat vascular smooth muscle cells exposed to compounds **1**, **4** and **5** at concentrations of 10^{-5}M - 10^{-9}M . Compound **1** shows limited toxicity at the highest concentration as compared to the untreated control wells. Average errors for measurements made for the three compounds were $\sim\pm 8\%$ for **1**, $\sim\pm 3\%$ for **4**, and $\sim\pm 4\%$ for **6**.

Table 1
Crystallographic Data

	2	3	4	5
Formula	ReC ₈ H ₁₉ N ₃ O ₅ Cl	ReC ₈ H ₁₅ N ₃ O ₇ Cl	ReC ₈ H ₁₅ N ₄ O ₆	ReC ₈ H ₁₅ N ₃ O ₃ PF ₆
formula weight	458.91	486.83	449.44	532.40
Cryst. syst.	Monoclinic	Monoclinic	Monoclinic	Monoclinic
space group	P2(1)/n	Cc	Cc	Cc
a, Å	6.8635(18)	13.579(4)	7.778(4)	14.396(7)
b, Å	12.951(3)	8.441(3)	13.745(6)	8.675(4)
c, Å	15.970(4)	11.878(4)	12.281(5)	11.829(6)
β, deg	93.803(4)	98.193(4)	92.507(7)	97.877(8)
vol, Å ³	1416.5(6)	1347.5(7)	1311.7(10)	1463.2(12)
Z	4	4	4	4
ρ(calc), Mg/m ³	2.152	2.370	2.276	2.417
μ, mm ⁻¹	8.783	9.250	9.293	8.495
<i>R</i> (000)	880	904	856	1008
Reflns. collected	11742	5493	5340	5799
Indep. reflns.	3210	2819	2709	3060
GOF on <i>F</i> ²	1.074	0.869	1.016	0.889
<i>R</i> [<i>I</i> > 2σ(<i>I</i>)]	0.0300/0.0658	0.0245/0.0490	0.0305/0.0649	0.0328/0.0605
<i>R</i> (all data)	0.0366/0.0677	0.0271/0.0501	0.0329/0.0656	0.0384/0.0620

Data collection and refinement parameters for crystals of **2** through **5** (crystal data for **1** was previously published)

Table 2**IR Data**

Compound	CO stretching frequency (cm ⁻¹)
1	2018, 1888, 1853(sh)
2	2023, 1884
3	2021, 1875
4	2015, 1875, 1859
5	2025, 1870

Solid state CO stretching frequencies for compounds **1-5**