Functionalised copper-64 complexes as precursors of potential PET imaging agents for neurodegenerative disorders

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A range of unsymmetrical bis(thiosemicarbazonato)zinc(II) and copper(II) complexes functionalised with structural features of the neuroactive agents dopamine and tropinone have been synthesised. The new ligands and complexes have been characterised by using mass spectrometry, elemental analysis, NMR, infrared and electronic absorption spectroscopy techniques. Transmetallation reactions of the zinc(II) analogues with $^{64}Cu(OAc)_2$ in aqueous solution were found to be a rapid and efficient method for preparing ^{64}Cu-radiolabelled complexes for potential use as *in vivo* tracers in positron emission tomography (PET). Oxygen-dependent cellular association assays in EMT6 murine carcinoma cells revealed that despite conjugation of the tropinone group and reduced lipophilicity, the bis(thiosemicarbazonato)copper(II) complex retains hypoxia-selectively *in vitro*. The results demonstrate that recognised neuroactive pharmacophores can be conjugated to the bis(thiosemicarbazonato)copper(II) core as potential precursors of ^{64}Cu-radiopharmaceuticals for imaging of neurodegenerative disorders.

Introduction

Molecular imaging techniques such as single-photon computerised tomography (SPECT) and positron emission tomography (PET) have the potential to revolutionise clinical management and treatment of disease.[1] Cancer imaging has provided the main driving force for advances in technology.[2–8] However, the ability of PET and SPECT to image tissue physiology has recently been exploited in the design of imaging agents targeting either striatal dopaminergic terminals or dopamine-active transporters (DAT) for early-stage diagnosis of neurodegenerative disorders including essential tremor (ET), Alzheimer's disease (AD), and Parkinson's disease (PD) (Fig. 1).[9–13]

PD is progressive and is characterised by decreased physical movement (bradykinesia) and reaction times, speech impairment and, in later stages of development, memory loss and dementia. The majority of PD cases are described as 'idiopathic' in that they arise spontaneously from an obscure or unknown cause and even after a patient's death, only 75% of PD cases are confirmed by post-mortem.[14] Other factors include genetic defects, neurotoxins or cerebral anoxia resulting from head trauma. In the latter case, symptoms can include areas of ischaemic hypoxia.[10,15]

Most PD imaging agents have structural motifs based on dopamine- or tropane-ring derivatives such as the dopamine analogue, 6-[^{18}F]-fluoro-L-DOPA (Fig. 1).[16–22] However, 6-[^{18}F]-fluoro-L-DOPA is rapidly metabolised to 6-[^{18}F]-fluorodopamine and other fluorine-18 metabolites, which leads to striatal accumulation of the radionuclide, exposing the patient to relatively high radiation doses.[16,22] It has also been reported that the use of 6-[^{18}F]-fluoro-L-DOPA may lead to an overestimate of the number of striatal dopaminergic terminals in early-stage PD, preventing accurate diagnosis.[16] This led Madras *et al.* to explore the use of phenyltropane analogues of cocaine, including carbomethoxy-3β-(4-fluorophenyl) tropane (CFT), as DAT-selective binding agents.[23–27] Brownell *et al.* compared the *in vivo* specific binding affinities of ^{11}C-radiolabelled CFT derivatives in caudate and putamen striatal tissue and concluded that [^{11}C]-CFT is clinically useful in monitoring dopamine neuronal degeneration.[16] However, the short half-life of ^{11}C ($t_{1/2}$ = 20.38 min) means that these radiotracers require in-house cyclotron facilities. Other radiopharmaceuticals including the SPECT agent DaTSCAN[9,11,28] and ^{99m}Tc-TRODAT derivatives have also been investigated.[29] However, as images acquired using SPECT are non-quantitative PET remains the technique of choice for developing radiotracers.

The work presented here describes initial efforts towards the synthesis of a new ^{64}Cu-radiolabelled tracer for potential quantitative PET imaging of DAT receptors *in vivo*. Our experience with the well-established copper(II) complexes of bis(thiosemicarbazonato) ligands [CuBTSCs] led us to investigate the possibility of functionalising these complexes with pendent groups based on dopamine or tropane rings that are structural analogues of known DAT-targeting

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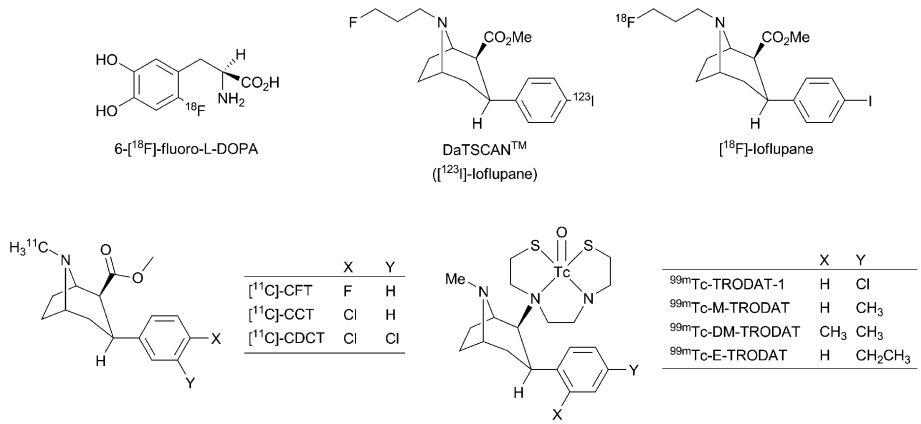


Fig. 1 Structures of a range of SPECT and PET radiopharmaceuticals used in the clinical imaging and diagnosis of PD.[16–22]

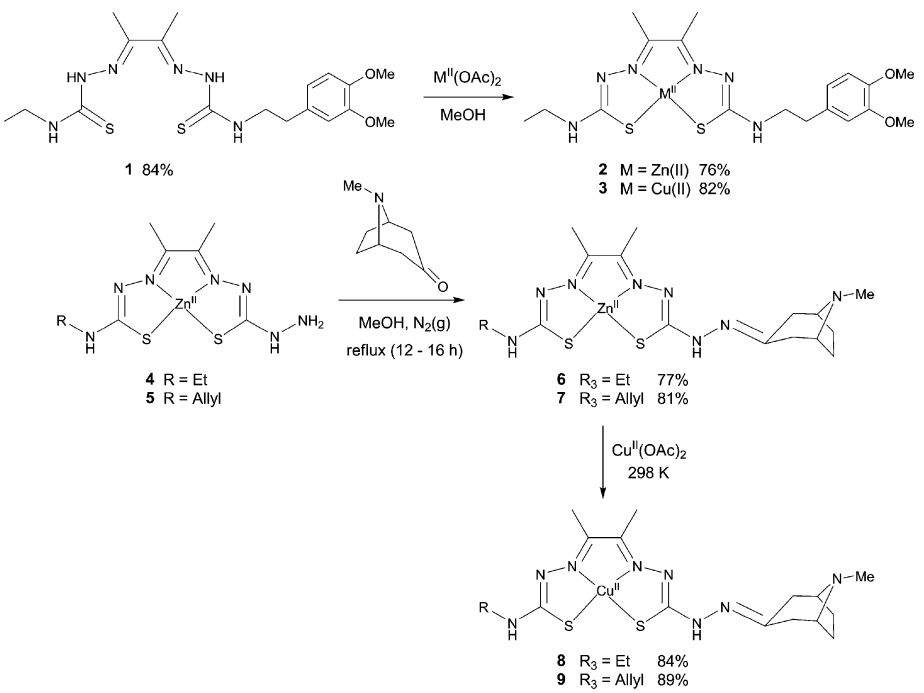
radiotracers. The results presented describe initial synthetic efforts towards the development of a [^{64}CuBTSC] radiotracer for PD imaging *in vivo*.

Results and discussion

Structures of the compounds synthesised are shown in Scheme 1. Proligand **1** was synthesised in 84% yield as a colourless microcrystalline solid using standard procedures.[30] Metal complexation with either zinc(II) or copper(II) diacetate in refluxing methanol proceeded with high yields to give the expected complexes **2** and **3** as bright yellow and dark red–brown microcrystalline solids (76% and 82% yield), respectively.

Compounds **1–3** contain the bis(methylether) protected catechol ring in which the amine functional group has been incorporated into the BTSC ligand. This structural modification will undoubtedly affect the binding of the ligand to DAT receptors. However, the aim of this project was to demonstrate that dopamine-like moieties could be conjugated to the BTSC core without negating the biological properties of the copper complex. Further synthetic efforts towards preparing additional analogues are under way.

The synthesis of zinc(II) complexes **4** and **5** has been reported previously.[30] Functionalisation of the reactive hydrazinic NH_2 group by the formation of imine, amide and *N*-glycosidic bonds has also been reported.[30,31] In this work, the [Zn^{II}ATSR/A] complexes **4** and **5** were reacted with 8-methyl-8-azabicyclo[3.2.1]octan-3-one (3-tropinone) under reflux in an inert nitrogen atmosphere in methanol for 12–16 h. Zinc(II) complexes **6** and **7** were isolated as dark yellow–orange amorphous solids in 77% and 81% yield, respectively. Complexes **6** and **7** gave molecular ion base peaks in the high resolution positive ion electrospray mass



Scheme 1 Reaction schemes showing the structures of compounds **1–9** synthesised in this work as precursors of potential ^{64}Cu-PET imaging agents.

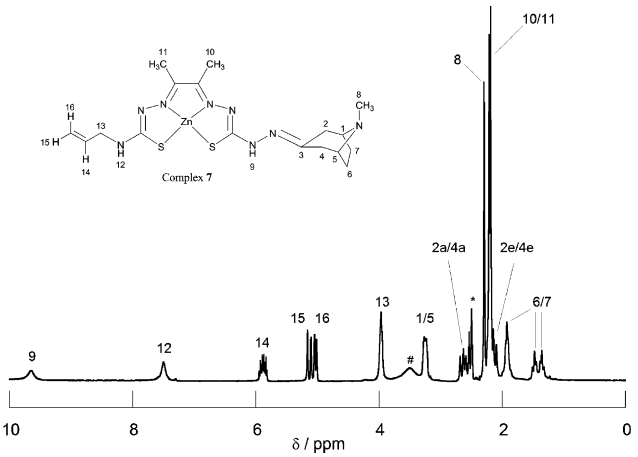


Fig. 2 ^{1}H NMR spectrum (300 MHz, DMSO-d_6, 298 K) of complex **7**. The spectrum was referenced with respect to the residual DMSO solvent peak labelled '*' at δ 2.50 ppm. The broad peak labelled '#' at $\delta \approx 3.30$ ppm is due to the presence of water in the NMR solvent. (inset) The structure of complex **7** with hydrogen atoms labelled 1–16 is shown.[32]

spectrometry (HRMS-ES$^+$) at m/z 459.1096 and 471.1102. Elemental analyses consistent with the molecular formulae $C_{16}H_{26}N_8S_2Zn$ and $C_{17}H_{26}N_8S_2Zn$ were obtained for complexes **6** and **7**, respectively. In addition, single peaks at 7.81 and 8.05 min were observed in the reverse phase high performance liquid chromatography (HPLC) of complexes **6** and **7**, respectively. The water and acetonitrile mobile phase solvents both contained 0.1% trifluoroacetic acid (TFA) and gradient elution methods were employed.

The ^{1}H and ^{13}C{^{1}H} NMR spectra of zinc(II) complexes **2**, **6** and **7** are consistent with the proposed structures (Fig. 2). The ^{13}C{^{1}H} NMR spectrum of complex **7** shows a broad weak resonance at 176.1 ppm, assigned to the two quaternary *C*=S carbon atoms. Two peaks were also observed at 151.9 and 147.0 ppm, assigned to the two quaternary imine carbon atoms of the ligand backbone. The three resonance peaks corresponding to the carbon atoms, CH_2=*C*H–CH_2–, *C*H_2=CH–CH_2–, and CH_2=CH–*C*H_2–, of the allyl group were observed at chemical shifts of 135.7, 115.1 and 44.6 ppm. The two methyl groups of the ligand backbone were observed at 14.0 and 13.8 ppm. The carbon atoms of the tropane-ring system have been labelled in accordance with the structure shown in Fig. 2 (inset). Carbon atom *C*-3 of the imine group gives a broad resonance peak at 145.3 ppm. The two bridgehead carbon atoms *C*-1 and *C*-5 are bonded to the electron withdrawing NCH_3 group and are shifted downfield, with resonance peaks at 60.1 and 59.2 ppm. Carbon atoms *C*-2 and *C*-4 are adjacent to the imine group and are deshielded with peaks at 39.3 and 38.3 ppm (partially obscured by the residual solvent septet centred at 39.43 ppm). Carbon atom *C*-8 of the *N*-methyl group gives a resonance peak at 33.3 ppm and the two remaining carbon atoms *C*-6 and *C*-7 give resonance peaks at 27.1 and 26.3 ppm.

The copper(II) complex **3** can be synthesised either by direct metallation of proligand **1** with $Cu(OAc)_2$ in refluxing methanol or by rapid and facile transmetallation from the corresponding zinc(II) complex **2**.[30] In contrast, complexes **8** and **9** could only be synthesised by transmetallation at room temperature from complexes **6** and **7**, respectively. All attempts to synthesise copper(II) complexes by transmetallation of complex **4** or **5** or by direct metallation of the precursors were unsuccessful. Upon transmetallation of complexes **6** and **7** with $Cu(OAc)_2$ in methanol, single peaks with longer HPLC retention times (R_t = 8.69 and 9.64 min) were observed for complexes **8** and **9**, for which the calculated lipophilicity (log P_c) values are 0.83 ± 0.23 and 1.02 ± 0.25, respectively.[33] The low lipophilicity (log P_c) values are due to the protonation of the NCH_3 bridgehead nitrogen atom of the tropane ring in 0.1% TFA and the formation of mono-cationic complexes. Due to the hydrophobic nature of the metal(II)–BTSC complex, the true lipophilicities of complexes **8** and **9** are likely to be considerably higher, potentially facilitating transport of the complexes across the blood–brain barrier.

The electronic absorption spectra of complexes **2**, **3**, **6–9** in DMSO showed features indicative of the N_2S_2 square-planar coordination geometry of the [CuBTSC] system.[30,34,35]

Copper-64 radiolabelling experiments were also performed. We have previously used the transmetallation reaction in the one-step preparation and purification of ^{64}Cu-radiolabelled complexes for use in small animal PET studies.[36,37] Fig. 3(a)

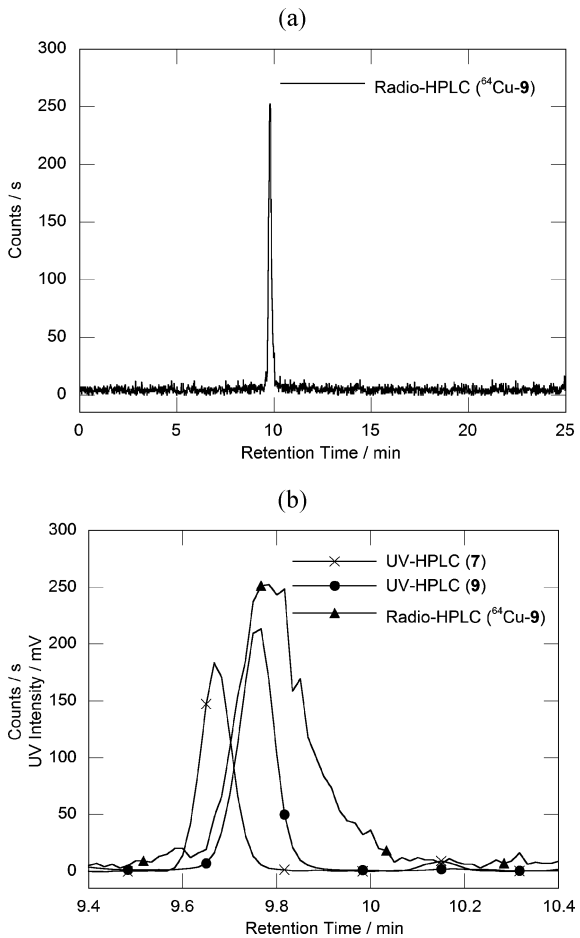


Fig. 3 Radio- and UV-HPLC chromatograms of (a) ^{64}Cu-**9** prepared in aqueous solution by transmetallation from complex **7** and (b) an overlay of the UV-HPLC and radio-HPLC chromatograms of complexes **7**, **9** and ^{64}Cu-**9**.

and (b) show the radio- and UV-HPLC chromatograms of complexes **7**, **9** and the radiolabelled complex ^{64}Cu-**9**, prepared in aqueous solution by transmetallation of complex **7** with ^{64}Cu(OAc)$_2$(aq.). Radiolabelling was rapid and efficient with >98% radiochemical yield achieved after stirring the reaction mixture at room temperature for 10 min. The ^{64}Cu-**3** complex was also prepared in high radiochemical yield (>80%) and purity (>95%). The ability to radiolabel these complexes with copper-64 demonstrates their potential for use as PET imaging agents. In addition, *in vitro* ligand challenge experiments revealed that the ^{64}Cu-**3** complex is stable with respect to demetallation in the 0.1 M solution of disodium ethylenediaminetetraacetic acid (EDTA) during incubation at pH 7.4 at 30 °C for 24 h. Less than 3% loss of the ^{64}Cu^{II} to EDTA measured by radio-HPLC was observed.

Cu–BTSC complexes have been studied *in vivo* as both blood perfusion tracers ([Cu^{II}PTSM])[38] and tumour hypoxia-selective radiopharmaceuticals ([Cu^{II}ATSM])[39] and show high uptake in the brain (>10% ID g^{-1}).[40] The presence of two methyl substituents on the backbone of the BTSC ligand has also been shown to confer hypoxia-selective cellular uptake and retention of the corresponding copper(II) complexes.[41–43] Therefore, in order to assess the effect of tropinone conjugation on the biological properties of the [Cu^{II}BTSC] system, pO_2-dependent *in vitro* cellular association of ^{64}Cu-**9** in EMT6 mouse carcinoma cells was measured (Fig. 4). Data were collected in triplicate at five time points (1, 5, 15, 30 and 60 min) during incubation in a 50 mL EMT6 cell suspension at a cell concentration of 5×10^6 cells per mL for 1 h at 37.0 °C. The results show that after 1 h, ^{64}Cu-**9** displays high cellular association under both hypoxic (0.5% pO_2) and normoxic (20.0% pO_2) conditions. Cellular association is more rapid under hypoxic conditions but reaches a plateau at around 88% between 5 and 10 min incubation time. Approximately 20% difference between hypoxic and normoxic cellular association within the first 10 min is observed. At longer incubation times cellular association under normoxic conditions continues to increase, and by 60 min, the difference between hypoxic and normoxic uptake is less than 5%. However, even at 60 min there is still a statistically significant difference between normoxic and hypoxic uptake ($P < 0.05$). In comparison to the established tumour hypoxia-selective marker, [^{64}Cu^{II}ATSM], ^{64}Cu-**9** shows higher overall cellular association over 60 min (89% hypoxic, 83% normoxic) but a lower hypoxic-to-normoxic ratio (1.07 compared to >1.40 for [^{64}Cu^{II}ATSM] in the same cell line).[40]

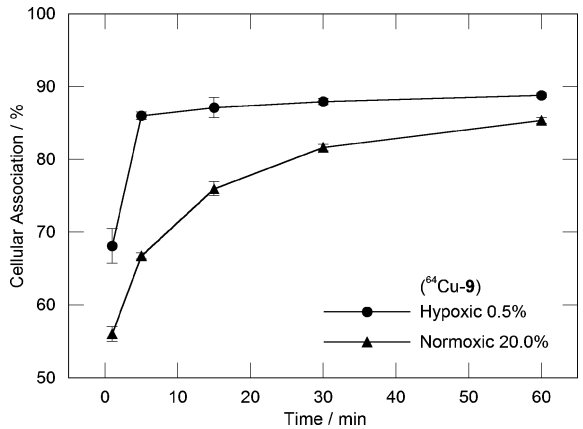


Fig. 4 Percentage cellular association of ^{64}Cu-**9** in EMT6 murine mammary carcinoma cells. Uptake was measured over 60 min using 5×10^6 cells per mL, incubated at 37 °C under normoxic (20.0%) and hypoxic (0.5%) oxygen concentrations. Control experiments revealed that ^{64}Cu-**9** did not adhere to the tube.

The convergence of percentage cellular association of ^{64}Cu-**9** under hypoxic and normoxic conditions at incubation times >60 min, suggests that functionalisation of complexes **4** and **5** with tropinone may alter the mechanism of cellular uptake.[40] However, the fact that ^{64}Cu-**9** remains partially hypoxia-selective (hypoxic-to-normoxic ratio of around 1.30 and 1.18 at 5 and 10 min, respectively) is an encouraging result which demonstrates that functionalisation does not completely negate the pO_2-dependent behaviour conferred by the [Cu^{II}BTSC] group. Indeed if *in vivo* DAT targeting and intracellular localisation of the radiotracer are found to be rapid, the higher cellular uptake and retention observed would potentially improve image contrast in comparison to established imaging agents.

Further synthetic efforts aimed at producing complexes with pendent groups that are closer analogues of dopamine and cocaine-cogeners are under way, and *in vitro* and *in vivo* studies are planned.

Summary and conclusions

In conclusion, the synthesis and ^{64}Cu-radiolabelling of [Cu^{II}BTSC] complexes functionalised with dopamine- and tropinone-like groups have been achieved. Although the structural simplifications are likely to alter the binding of the complexes to neuro-receptors, these complexes serve as precursors of potential ^{64}Cu-based radiopharmaceuticals and demonstrate the versatility of the BTSC ligand. The radiolabelling experiments also demonstrate that transmetallation from the corresponding zinc(II) precursor complexes is a viable route for the preparation of ^{64}Cu-radiotracers.

The *in vitro* cellular association experiments demonstrate that the hypoxia-selective properties conferred to the molecule by the [^{64}Cu^{II}BTSC] group are modified but not completely negated by conjugation. Indeed, the higher cellular association and rapid delineation of hypoxia observed *in vitro* indicate that the control of important properties such as binding affinity can be achieved and represent very promising results towards the goal of developing a ^{64}Cu-radiotracer for PD imaging.

Experimental

General

All reagents and solvents were obtained from commercial sources (Sigma-Aldrich and Lancaster) and, unless otherwise stated, were used as received. Elemental analyses were performed by the microanalysis service of the department at the University of Oxford. NMR spectra were recorded on a Varian Mercury VX300 spectrometer, (^{1}H at 300 MHz,

$^{13}C\{^1H\}$ at 75.5 MHz) using the residual solvent signal as an internal reference. High resolution mass spectra were recorded on a micromass LCT time-of-flight mass spectrometer using the positive ion electrospray (ES^+) technique. Where possible, accurate masses are reported to four decimal places using tetraoctylammonium bromide (466.5352 g mol^{-1}) as an internal reference. Electronic absorption spectra were recorded on a Perkin Elmer Lambda 19 UV/VIS/NIR spectrometer. High performance liquid chromatography (HPLC) was conducted by using a Gilson HPLC machine equipped with a Hamilton PRP-1 reverse phase column and UV/VIS detection was conducted at 254 nm. HPLC grade water (>18.2 MΩ cm^{-1}) and acetonitrile with 0.1% trifluoroacetic acid were used as mobile phase solvents at a flow rate of 1.0 mL min^{-1}. The gradient method used was as follows: *t* min^{-1}, %B(acetonitrile): 0, 5%; 15, 95%; 20, 95%; 25, 5% and 30, 5%.

Syntheses

Diacetyl-2-[4-*N*-(3,4,-dimethoxyphenethyl)-3-thiosemicarbazido]-3-(4-*N*-ethyl-3-thiosemicarbazide), (1). Compound **1** was synthesised in accordance with previously published procedures.[30,44] Compound **1** was isolated as an off-white microcrystalline solid (0.95 g, 2.24 mmol, 84%). Mp 195–199 °C (decomp.). Elemental analysis (%). Found (calcd) for $C_{18}H_{28}N_6O_2S_2$: C, 50.9 (51.0); H, 6.6 (6.7); N, 19.7 (19.8). ^1H NMR (300 MHz, DMSO-d_6): δ/ppm 10.30, (1H, s, N*H*); 10.18, (1H, s, N*H*); 8.41 (1H, t, $^3J_{HH}$ = 5.8 Hz, N*H*Et); 8.29 (1H, $^3J_{HH}$ = 5.8 Hz, N*H*CH_2CH_2Ar); 6.86 (1H, $^3J_{HH}$ = 8.1 Hz, *H*-5); 6.81 (1H, d, $^4J_{HH}$ = 2.1 Hz, *H*-2); 6.72 (1H, d, $^3J_{HH}$ = 8.1, $^4J_{HH}$ = 2.1 Hz, *H*-6); 3.76 (2H, obscured quartet, NHC$H_2$$CH_3$); 3.73 (3H, s, OC$H_3$); 3.72 (3H, s, OC$H_3$); 3.59 (2H, quintuplet, $^3J_{HH}$ = 7.2 Hz, NHC$H_2$$CH_2$Ar); 2.83 (2H, t, $^3J_{HH}$ = 7.2 Hz, NHCH$_2$CH_2Ar); 2.21 (3H, s, CH_3C=N); 2.12 (3H, s, CH_3C=N); 1.13 (3H, t, $^3J_{HH}$ = 7.2 Hz, NHCH$_2$CH_3).$^{13}C\{^1H\}$ NMR (75.5 MHz, DMSO-d_6): δ/ppm 177.5 (*C*=S), 177.3 (*C*=S); 148.6 (*C*-3 or *C*-4); 148.0 (*C*=N); 147.6 (*C*=N); 147.2 (*C*-3 or *C*-4); 131.4 (*C*-1); 120.4 (*C*-6); 112.3 (*C*-2 or *C*-5); 111.9 (*C*-2 or *C*-5); 55.5 (OCH_3); 55.3 (OCH_3); 45.2 (NH$CH_2$$CH_2$Ar); 38.5 (NH$CH_2$$CH_3$); 34.0 (NHCH$_2$$CH_2$Ar); 14.3 (NHCH$_2$$CH_3$); 11.7 ($CH_3$C=N); 11.5 ($CH_3$C=N). HRMS-$ES^+$: *m*/*z* (calcd) 447.1599 (447.1607) = {M + Na^+}. λ_{max} (DMSO)/nm 345 (ε/mol^{-1} dm^3 cm^{-1} 26 681), 336 (27 390) and 282 (8080). ν_{max}/cm^{-1} (KBr disc) 558m, 808w, 1027m, 1135s, 1217s, 1234s, 1262s, 1311m, 1488s, 1515s, 1530s, 2878w, 3216m, 3350m.

Diacetyl-2-[4-*N*-(3,4,-dimethoxyphenethyl)-3-thiosemicarbazonato]-3-(4-*N*-ethyl-3-thiosemicarbazonato) zinc(II), (2). Complex **2** was synthesised in accordance with previously published procedures.[30] Complex **2** was isolated as a light-yellow microcrystalline solid (0.43 g, 0.89 mmol, 76%). Mp 94–97 °C. Elemental analysis (%). Found (calcd) for $C_{18}H_{26}N_6O_2S_2Zn{\cdot}0.5H_2O{\cdot}0.5MeOH$: C, 43.3 (43.0), H, 5.8 (5.7), N, 16.4 (16.1). ^1H NMR (300 MHz, DMSO-d_6): δ/ppm 7.28 (2H, m, 2 × N*H*); 6.85 (1H, d, $^3J_{HH}$ = 8.1 Hz, *H*-5); 6.81 (1H, d, $^4J_{HH}$ = 2.1 Hz, *H*-2); 6.73 (1H, d, $^3J_{HH}$ = 8.1, $^4J_{HH}$ = 2.1 Hz, *H*-6); 3.74 (3H, s, OCH_3); 3.71 (3H, s, OCH_3); 3.50 (2H, m, NHC$H_2$$CH_3$); 3.35 (2H, m, NHC$H_2$$CH_2$Ar); 2.78 (2H, t, $^3J_{HH}$ = 7.5 Hz, NHCH$_2$CH_2Ar); 2.22 (3H, s, CH_3C=N); 2.19 (3H, s, CH_3C=N); 1.10 (3H, t, $^3J_{HH}$ = 7.2 Hz, NHCH$_2$CH_3). $^{13}C\{^1H\}$ NMR (75.5 MHz, DMSO-d_6): δ/ppm 175.5 (br, 2 × *C*=S); 148.5 (*C*-3 or *C*-4); 147.1 (*C*-3 or *C*-4); 145.2 (br, 2 × *C*=N); 120.3 (*C*-6); 112.4 (*C*-2 or *C*-5); 111.8 (*C*-2 or *C*-5); 55.5 (OCH_3); 55.3 (OCH_3); 44.2 (NH$CH_2$$CH_2$Ar); 36.9 (NH$CH_2$$CH_3$); 34.5 (NHCH$_2$$CH_2$Ar); 14.6 (NHCH$_2$$CH_3$); 13.83 ($CH_3$C=N); 13.78 ($CH_3$C=N). HRMS-$ES^+$: *m*/*z* (calcd) 487.0917 (487.0923) = {M + H^+}. λ_{max} (DMSO)/nm 437 (ε/mol^{-1} dm^3 cm^{-1} 11 655), 316 (11 390) and 278 (9952). ν_{max}/cm^{-1} (KBr disc) 764w, 807w, 837w, 1027m, 1086m, 1140m, 1156m, 1233s, 1261s, 1344m, 1420s, 1513s, 2872m, 2931w. HPLC: R_t 9.47 min.

Diacetyl-2-[4-*N*-(3,4,-dimethoxyphenethyl)-3-thiosemicarbazonato]-3-(4-*N*-ethyl-3-thiosemicarbazonato) copper(II), (3). Complex **3** was synthesised in accordance with previously published procedures.[30] The solid was purified by being taken up in CH_2Cl_2, and then passed through a plug of Florisil® and the solvent removed *in vacuo* to afford complex **3** as a dark purple–black microcrystalline solid (0.163 g, 0.34 mmol, 82%). Mp 205–209 °C. Elemental analysis (%). Found (calcd) for $C_{18}H_{26}N_7O_2S_2Zn$: C, 44.4 (44.5); H, 5.3 (5.4); N, 17.2 (17.3). HRMS-ES^+: *m*/*z* (calcd) 485.0859 (4885.0849) = {M + H^+}. λ_{max} (DMSO)/nm 472 (ε/mol^{-1} dm^3 cm^{-1} 3955), 313 (11 393) and 278 (11 918). ν_{max}/cm^{-1} (KBr disc) 619w, 764w, 806w, 844w, 1026s, 1140s, 1156s, 1235s, 1261s, 1340m, 1463s, 1514s, 2926m. HPLC: R_t 12.07 min, ^{64}Cu-**3** >95% radiochemical yield.

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazonato)-3-(4-*N*-amino-3-thiosemicarbazonato) zinc(II), [Zn(II)ATSE/A], (4). Complex **4** was synthesised in accordance with previously published procedures.[30] Complex **4** was isolated as a light-yellow amorphous solid (1.75 g, 5.2 mmol, 95%). Mp >230 °C (decomp.). Elemental analysis (%). Found (calcd) for $C_8H_{15}N_7S_2Zn$: C, 28.4 (28.4); H, 4.6 (4.5); N, 28.6 (28.9); S, 19.1 (18.9); Zn, 18.9 (19.3). ^1H NMR (300 MHz, DMSO-d_6): δ/ppm 8.22 (1H, s, N*H*NH_2); 7.29 (1H, br s, CH_3CH_2N*H*); 4.57 (2H, s, NHNH_2); 3.36 (2H, m obscured by residual HDO, assigned from COSY, CH_3CH_2NH); 2.22 (3H, s, CH_3C=N); 2.19 (3H, s, CH_3C=N); 1.10 (3H, t, $^3J_{HH}$ = 7.2 Hz, CH_3CH_2NH). HRMS-ES^+: *m*/*z* (calcd) 338.0190 (338.0200) = {M + H^+}. λ_{max} (DMSO)/nm 433 (ε/mol^{-1} dm^3 cm^{-1} 12 337). ν/cm^{-1} (KBr disc) 617m, 1035w, 1080w, 1184mw, 1215m, 1317m, 1369m, 1431s, 1506m, 1544w, 1618s, 1639s, 3417s. HPLC: R_t 8.10 min.

Diacetyl-2-(4-*N*-allyl-3-thiosemicarbazonato)-3-(4-*N*-amino-3-thiosemicarbazonato) zinc(II), [Zn(II)ATSAllyl/A], (5). Complex **5** was synthesised in accordance with previously published procedures.[30] Complex **5** was isolated as a bright-yellow amorphous solid (0.30 g, 0.9 mmol, 83%). Mp >230 °C (decomp.). Elemental analysis (%). Found (calcd) for $C_9H_{15}N_7S_2Zn$: C, 30.5 (30.8); H, 4.5 (4.3); N, 27.8 (28.0); S, 18.5 (18.3); Zn, 18.5 (18.6). ^1H NMR (300 MHz, DMSO-d_6): δ/ppm 8.27 (1H, s, N*H*NH_2); 7.44 (1H, br s, CH_2N*H*); 5.88 (1H, m, CH_2=C*H*CH_2); 5.18–5.00 (2H, m, CH_2=CH-CH_2); 4.48 (2H, s, NHNH_2); 3.96

(2H, m, CH_2=CH-CH_2NH); 2.22 (3H, s, CH_3C=N); 2.19 (3H, s, CH_3C=N). HRMS-ES$^+$: m/z (calcd) 350.0188 (350.0200) = {M + H$^+$}. λ_{max} (DMSO)/nm 433 (ε/mol^{-1} dm^3 cm^{-1} 11 416) and 314 (10 424). ν/cm^{-1} (KBr disc) 613m, 925mw, 1026m, 1064m, 1089m, 1182m, 1213s, 1274s, 1353m, 1431s, 1496s, 1544m, 1616s, 1637s. HPLC: R_t 8.17 min.

General procedure A: imine condensation reactions

3-Tropinone (1.0 equivalent) was added to a stirred solution of [Zn(II)ATSR/A] (complex **4** or **5**) in 30 mL methanol and the reaction mixture was heated under reflux for 12–16 h under a nitrogen atmosphere. The reaction mixture was then allowed to cool slowly to room temperature and the orange–yellow precipitate was collected by filtration, washed with cold methanol (2 × 20 mL) and diethyl ether (5 × 30 mL), then dried *in vacuo* at 80 °C to give the corresponding [Zn(II)ATSR/A–tropinone] conjugates **6** and **7**.

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazoato)-3-(4-*N*-tropimine-3-thiosemicarbazonato)zinc(II) [Zn(II)ATSE/A-tropimine] (6). As per general procedure A, 3-tropimine (0.20 g, 1.47 mmol) was added to a stirred solution of [Zn(II)ATSE/A] (**4**) (0.50 g, 1.47 mmol) in 30 mL methanol. Complex **6** was isolated as an orange amorphous solid (0.52 g, 1.13 mmol, 77%). Mp >210 °C (decomp.). Elemental analysis (%). Found (calcd) for $C_{16}H_{26}N_8S_2Zn$: C, 41.4 (41.8); H, 5.6 (5.7); N, 24.0 (24.4); S, 14.4 (13.9); Zn, 14.3 (14.2). Please refer to Fig. 2 (inset structure) for the tropimine ring labelling. ^{1}H NMR (300 MHz, DMSO-d_6): δ/ppm 9.59 (1H, br s, N*H*-N=C-3(tropimine)); 7.23 (1H, br s, CH_3CH_2NH); 3.53 (2H, q, $^3J_{HH}$ = 7.3 Hz, CH_3CH_2NH); 3.24 (2H, br m, C*H*-1 and C*H*-5); 2.73–2.51 (2H, br m, C*H*-2a and C*H*-4a); 2.33 (3H, s, CH_3-8-N); 2.21 and 2.18 (6H, s, 2 × CH_3C=N); 2.14–2.04 (2H, br m, C*H*-2e and C*H*-4e). The CH_2 groups on tropimine ring positions 6 and 7 give a complex pattern of multiplets at 1.99 (2H, br m) and 1.55–1.33 (2H, br m); 1.21 (3H, t, $^3J_{HH}$ = 7.3 Hz, $CH_3$$CH_2$NH). ^{13}C{^{1}H} NMR (75.5 MHz, DMSO-d_6): δ/ppm 175.3 (br, 2 × *C*=S); 152.4 (*C*=N); 146.3 (*C*=N); 145.6 (*C*-3); 61.7 and 60.2 (*C*-1 and *C*-5); 40.2 and 38.6 (*C*-2 and *C*-4, partially obscured by the residual solvent septet at 39.43 ppm); 36.7 (CH_3CH_2); 33.4 (*C*-8 of NCH_3); 27.4 and 26.1 (*C*-6 and *C*-7); 15.3 (CH_3CH_2); 13.7 and 12.9 (2 × CH_3C=N). HRMS-ES$^+$: m/z (calcd) 459.1096 (459.1092) = {M + H$^+$}. λ_{max} (DMSO)/nm 431 (ε/mol^{-1} dm^3 cm^{-1} 8069) and 314 (7843). HPLC: R_t 7.81 min.

Diacetyl-2-(4-*N*-allyl-3-thiosemicarbazoato)-3-(4-*N*-tropimine-3-thiosemicarbazonato)zinc(II) [Zn(II)ATSAllyl/A-tropimine] (7). As per general procedure A, 3-tropimine (0.12 g, 0.86 mmol) was added to a stirred solution of [Zn(II)ATSAllyl/A] (**5**) (0.30 g, 0.86 mmol) in 30 mL methanol. Complex **7** was isolated as an orange amorphous solid (0.36 g, 0.75 mmol, 81%). Mp >210 °C (decomp.). Elemental analysis (%). Found (calcd) for $C_{17}H_{26}N_8S_2Zn$: C, 43.3 (43.3); H, 5.6 (5.55); N, 23.9 (23.7); S, 13.9 (13.6); Zn, 13.6 (13.8). Please refer to Fig. 2 (inset structure) for the tropimine ring labelling. ^{1}H NMR (300 MHz, DMSO-d_6): δ/ppm 9.65 (1H, br s, N*H*-N=C-3(tropimine)); 7.50 (1H, br s, CH_2=CH-CH_2N*H*); 5.89 (1H, m, CH_2=C*H*CH_2); 5.19–4.99 (2H, m, CH_2=CH-CH_2); 3.97 (2H, m, CH_2=CH-CH_2NH); 3.25 (2H, br m, C*H*-1 and C*H*-5); 2.71–2.52 (2H, br m, C*H*-2a and C*H*-4a); 2.30 (3H, s, CH_3-8-N); 2.21 and 2.19 (6H, s, 2 × CH_3C=N); 2.15–2.07 (2H, br m, C*H*-2e and C*H*-4e). The 2 × CH_2 groups on tropimine ring positions 6 and 7 give a complex pattern of multiplets at 1.96 (2H, br m) and 1.53–1.30 (2H, br m). ^{13}C{^{1}H} NMR (75.5 MHz, DMSO-d_6): δ/ppm 176.1 (br, 2 × *C*=S); 151.9 (*C*=N); 147.0 (*C*=N); 145.3 (*C*-3); 135.7 (CH_2=*C*HCH_2); 115.1 (CH_2=CHCH_2); 60.1 and 59.2 (*C*-1 and *C*-5); 44.6 (CH_2=CH*C*H$_2$); 39.3 and 38.3 (*C*-2 and *C*-4, partially obscured by the residual solvent septet at 39.43 ppm); 33.3 (*C*-8 of NCH_3); 27.1 and 26.3 (*C*-6 and *C*-7); 14.0 and 13.8 (2 × *C*H$_3$C=N). HRMS-ES$^+$: m/z (calcd) 471.1102 (471.1092) = {M + H$^+$}. λ_{max} (DMSO)/nm 427 (ε/mol^{-1} dm^3 cm^{-1} 7980) and 313 (7768). HPLC: R_t 8.05 min.

Diacetyl-2-(4-*N*-ethyl-3-thiosemicarbazoato)-3-(4-*N*-tropimine-3-thiosemicarbazonato)copper(II) [Cu(II)ATSE/A-tropimine] (8). $Cu(OAc)_2{\cdot}H_2O$ (0.08 g, 0.40 mmol, 1.2 eq.) was added to a stirred solution of complex **6** (0.15 g, 0.33 mmol) in 30 mL methanol at room temperature. Upon addition of $Cu(OAc)_2{\cdot}H_2O$ the reaction became dark red–brown. The mixture was then heated at 50 °C for 4 h and cooled overnight at 4 °C. The precipitate was isolated by filtration, washed with water (2 × 10 mL) and dried *in vacuo* at 60 °C to give complex **8** as a red–brown amorphous solid (0.13 g, 0.27 mmol, 84%). Elemental analysis (%). Found (calcd) for $C_{16}H_{26}N_8S_2Cu$: C, 42.1 (42.0); H, 5.7 (5.7); N, 24.1 (24.5); S, 14.4 (14.0); Cu, 13.6 (13.9). MS-ES$^+$: m/z (calcd) 458.1072 (458.1096) = {M + H$^+$}. λ_{max} (DMSO)/nm 522sh (ε/mol^{-1} dm^3 cm^{-1} 3753), 476 (6580), 350sh (11 090) and 312 (20 135). HPLC: R_t 8.69 min. Calcd log P_c: 0.83 ± 0.23.

Diacetyl-2-(4-*N*-allyl-3-thiosemicarbazoato)-3-(4-*N*-tropimine-3-thiosemicarbazonato)copper(II) [Cu(II)ATSAllyl/A-tropimine] (9). $Cu(OAc)_2{\cdot}H_2O$ (0.08 g, 0.4 mmol, 1.3 eq.) was added to a stirred solution of complex **7** (0.15 g, 0.32 mmol) in 30 mL methanol at room temperature. Upon addition of $Cu(OAc)_2{\cdot}H_2O$ the reaction became dark red–brown. The mixture was then heated at 50 °C for 4 h and cooled overnight at 4 °C. The precipitate was isolated by filtration, washed with water (2 × 10 mL) and dried *in vacuo* at 60 °C to give complex **9** as a red–brown amorphous solid (0.13 g, 0.28 mmol, 89%). Elemental analysis (%). Found (calcd) for $C_{17}H_{26}N_8S_2Cu$: C, 43.5 (43.4); H, 5.6 (5.6); N, 23.6 (23.8); S, 13.2 (13.6); Cu, 14.0 (13.5). MS-ES$^+$: m/z (calcd) 470.1114 (470.1096) = {M + H$^+$}. λ_{max} (DMSO)/nm 525sh (ε/mol^{-1} dm^3 cm^{-1} 3960), 478 (6890), 350sh (10 178) and 313 (19 934). HPLC: R_t 9.64 min. Calcd log P_c: 1.02 ± 0.25.

Radiolabelling

The copper-64 radiolabelled complex, ^{64}Cu-**9**, was prepared in aqueous solution by transmetallation of the corresponding zinc(II) complex (**7**) with ^{64}Cu(OAc)$_2$(aq.). The precursor complex (1.0 mg) was dissolved in DMSO (1.0 mL). An aliquot of the precursor solution (50 μL) was then diluted with deionised water (200 μL), and ^{64}Cu(OAc)$_2$(aq.) (250 μL, 350 μCi) added. After 10 min of stirring at room temperature, radio-HPLC analysis showed 98% conversion of ^{64}Cu(OAc)$_2$

(R_t = 2.62 min) to ^{64}Cu-**9** (R_t = 9.73 min). The unreacted $^{64}Cu(OAc)_2$ was removed by using a Sep-pak (C18) cartridge. The cartridge was conditioned with ethanol (8.0 mL) and deionised water (5.0 mL) before loading the reaction mixture. Deionised water (2.0 mL) was passed through to remove unreacted $^{64}Cu(OAc)_2$. Following a 0.15 mL void ethanol volume, the ^{64}Cu-**9** radiolabelled complex was eluted with ethanol (2 × 0.1 mL) and diluted with saline solution (0.9%, 1.8 mL) for use in the *in vitro* cellular association experiments. The radiochemical yield and purity of the ^{64}Cu-**9** complex were both >98% as confirmed by radio-HPLC. ^{64}Cu-**3** was also prepared by using the same procedure. Specific activities of the labelled complexes were typically in the range 5–14 μCi μg^{-1} of precursor ligand or zinc complex. These values of specific activity are estimates prior to purification using the C18 cartridges and as such represent a low boundary. The efficiency of the C18 cartridge in separating corresponding zinc and copper BTSC complexes is unknown. *In vitro* stability measurements were conducted in accordance with previously reported protocols.[45]

Cellular association assays

Cellular association measurements on ^{64}Cu-**9** in EMT6 murine mammary carcinoma cells were performed in accordance with previously reported procedures.[46] The EMT6 mammary carcinoma cell suspension (10 mL), taken from a homogenous 50 mL cell suspension at a cell concentration of 5 × 10^6 cells per mL, was equilibrated in a three-necked, glass round-bottomed flask at 37.0 °C under anoxic (95% N_2, 5% CO_2) and normoxic (75% N_2, 20% O_2, 5% CO_2) conditions by passing a continuous flow of warmed humidified gas over the cells. All other variables remained constant. After 30 min equilibration time, a 50 μCi sample of ^{64}Cu-**9** was added. Then at 1, 5, 15, 30 and 60 min, triplicate samples of 200 μL of cell suspension were removed by pipette. The suspension was centrifuged and the percentage uptake of the compound into the cells calculated. As a control, the same experiments were performed in the absence of cells to determine the extent to which the compounds adhere to vials due to the lipophilicity of the compounds. No appreciable adherence of the complexes to the vials was observed. A protein assay was not required as the cell studies were all performed on the same homogenous cell mixture of known cellular concentration. Internalisation studies were not performed and the results only reflect the amount of copper-64 activity associated with the cells. The differences in cellular association were compared by using the Student *t*-test. Differences at 95% confidence level ($P < 0.05$) were considered significant.

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