# In Vitro and In Vivo Evaluation of Bifunctional Bisthiosemicarbazone $^{64}$Cu-Complexes for the Positron Emission Tomography Imaging of Hypoxia

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The copper(II) bisthiosemicarbazonato complex, copper-diacetyl-bis($N^4$-methylthiosemicarbazonate) (Cu-ATSM), has been used clinically as a positron emission tomography (PET) tracer for the delineation of hypoxia. Six novel, asymmetric bis(thiosemicarbazones) derived from diacetyl-2-(4-*N*-methyl-3-thiosemicarbazone)-3-(4-*N*-amino-3-thiosemicarbazone) ($H_2$ATSM/A), one of which contained a nitroimidazole functionality, were radiolabeled with $^{64}$Cu ($t_{½}$ = 12.7 h, $\beta+$ = 19.3%). In vitro studies were performed on three of the compounds using EMT6 mammary carcinoma cells under hypoxic and normoxic conditions. All compounds displayed rapid cellular association and appreciable hypoxic selectivity with increased uptake under normoxic and hypoxic conditions when compared to $^{64}$Cu-ATSM. Biodistribution and small animal PET imaging studies were then carried out in vivo using two compounds in EMT6 tumor-bearing mice. The compounds showed high tumor uptake, but also substantial accumulation in the liver. These complexes demonstrate that $H_2$ATSM/A represents a novel and versatile synthetic platform that can be utilized to provide hypoxic cell selectivity through functionalization of the bisthiosemicarbazonate group.

## Introduction

The delineation of hypoxia in tissue has direct implications for the treatment of diseases including cancer, diabetic retinopathy, and arthritis. In the case of cancer, resistance to standard radiotherapy and chemotherapy treatments has, in part, been correlated to hypoxic areas in tumors.[1–3] The presence of hypoxic cells within tumors has been determined with an Eppendorf needle, which is invasive, technically demanding, and its use can be limited by tumor accessibility.[4] Therefore, there is an interest in developing noninvasive, quantitative imaging agents to measure hypoxia.[5]

Copper has a variety of potential isotopes for use in PET,[a] including $^{60}$Cu, $^{61}$Cu, $^{62}$Cu, and $^{64}$Cu.[6] Bis(thiosemicarbazonate) complexes with copper have been examined clinically as PET radiopharmaceuticals. Bis(thiosemicarbazonate) ligands derived from 1,2-diones react rapidly with copper to form stable, low molecular weight, neutral complexes capable of rapid cellular uptake. Copper diacetyl-bis($N^4$-methylthiosemicarbazonate) (Cu-ATSM) has been shown to be selective for hypoxic tissue[7–12] and has been used clinically for the detection of hypoxia in cancer.[13–16] Cu-PTSM, [copper pyruvaldehyde-bis($N^4$-methylthiosemicarbazonate)], which differs from Cu-ATSM only by a methyl group, has been used as blood perfusion tracer.[17] The difference in uptake profiles for these two compounds has been attributed to their reduction potential and p$K_a$.[18–21]

Bifunctional chelators derived from bisthiosemicarbazones have previously been prepared by Arano et al. and McPherson et al. and radiolabeled with $^{99m}$Tc, $^{64}$Cu, and $^{67}$Cu.[22–24] It has been suggested that complexes that retain the dimethylated backbone are less susceptible to reduction and, thus, more practical for use in delineating hypoxia.[18] To this end, bis(thiosemicarbazones) containing carboxylate[25] and hydrazine groups[26] at the N-terminus have been recently synthesized and conjugated to octreotide and glucose, respectively, though little biological and radiochemical data has been published on these complexes, so it is unknown how these modifications affect their hypoxic selectivity.

In this paper, we describe the copper-radiolabeling of six novel asymmetric bisthiosemicarbazone imine conjugates derived from $H_2$ATSM/A, including one which contains a nitroimidazole group, to ascertain whether this would improve the hypoxic selectivity compared to the parent Cu-ATSM. These conjugates have been radiolabeled with $^{64}$Cu and their partition coefficients were calculated. The cellular uptake of three of these compounds was then determined in EMT6 mammary carcinoma cells under hypoxic and normoxic conditions. Two complexes were then further examined in vivo by small animal PET imaging and acute biodistribution studies.

## Results

**Labeling of $^{64}$Cu Complexes.** Complexes were prepared either by the direct reaction of $^{64}$Cu(acetate)$_2$ with the corresponding proligand, or by transmetalation of the zinc complex (Figure 1).[26] Complexes were prepared at a specific activity range of 1–1000 mCi/$\mu$g ligand. All complexes were prepared in sufficient radiochemical purity (>95%, determined by radio-TLC and identity confirmed by direct comparison with the elution profile of the authentic nonradioactive analog) to undertake in vitro and in vivo studies. The $R_f$ values obtained for the $^{64}$Cu complexes were within ±3% of the values obtained for the corresponding cold copper complexes (Table 1).

**Log *P*.** The partition coefficients (log *P*) values for the compounds are displayed in Table 1. All the compounds studied have similar log *P* values, which is unsurprising given the

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[a] Abbreviations: ATSM, diacetyl-bis($N^4$-methylthiosemicarbazone; ATSM/A, diacetyl-2-(4-*N*-methyl-3-thiosemicarbazone)-3-(4-*N*-amino-3-thiosemicarbazone); PET, positron emission tomography; PTSM, pyruvaldehyde-bis($N^4$-methylthiosemicarbazone).

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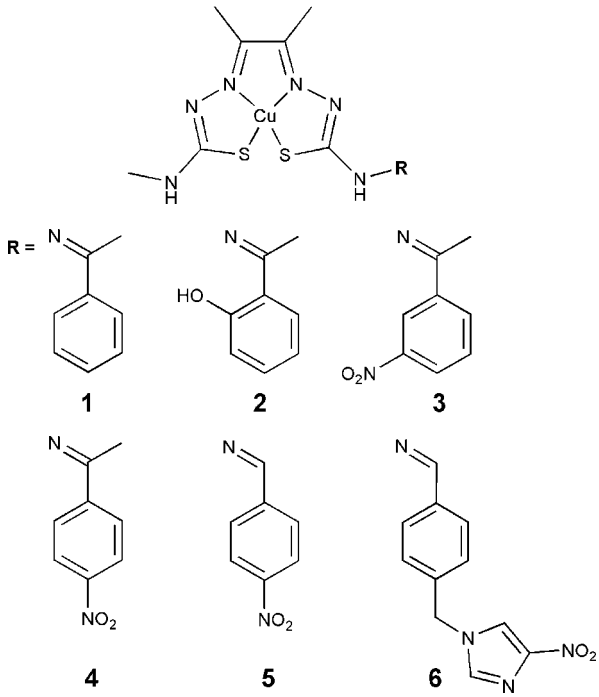


**Figure 1.** Cu(ATSM/A) imine derivatives (**1**−**6**) used in this study.

**Table 1.** Partition Coefficients (Log *P*) and $R_f$ Values[a] for $^{64}$Cu(ATSM/A) Imine Conjugates

| compound | log *P* | TLC $R_f$ (standard) | radio-TLC $R_f$ ($^{64}$Cu complex) |
|---|---|---|---|
| **1** | 2.30 ± 0.06 | 0.71 | 0.69 |
| **2** | 2.38 ± 0.08 | 0.77 | 0.75 |
| **3** | 2.28 ± 0.05 | 0.68 | 0.68 |
| **4** | 2.28 ± 0.02 | 0.69 | 0.70 |
| **5** | 2.38 ± 0.10 | 0.68 | 0.66 |
| **6** | 2.13 ± 0.04 | 0.54 | 0.57 |
| $^{64}$Cu-ATSM[b] | 1.85 ± 0.05 | 0.56 | 0.61 |

[a] Ethyl acetate/methanol (95:5) on silica TLC. [b] TLC performed on silica plates with EtAc as the eluent.

similarities in their structure. Compound **6**, which contains a nitroimidazole moiety, is the only compound with appreciably different lipophilicity (log $P$ = 2.13 ± 0.04). $^{64}$Cu-ATSM is more hydrophilic than the imine conjugates used in this study (log $P$ = 1.85 ± 0.05).

**Cell Uptake Studies.** Compounds **1**, **3**, and **6** were chosen for cell uptake studies to establish the influence of the bioreducible nitro groups on hypoxic selectivity. The uptake profiles under both anoxic and normoxic conditions over 60 min are displayed in Figure 2. Compound **1** shows very rapid uptake, reaching 75.4 ± 5.8% after 1 min under anoxic conditions. The uptake reached a maximum of 92.2 ± 0.4% after five min, after which time the cellular uptake decreased slightly to 89.8 ± 1.5% after 60 min. In contrast, the uptake in EMT6 cells after 1 min under normoxic conditions was 55.7 ± 1.0%, and the uptake steadily increased to 77.2 ± 1.4% after 60 min. The observed difference in cellular uptake was statistically significant at all time points ($P \leq 0.05$) Compounds **3** and **6** showed similar uptake profiles to **1** under anoxic conditions, with cellular uptake reaching a maximum at 5 min before remaining effectively constant for the remaining 55 min. As with **1**, the uptake under anoxic conditions for **3** was higher than under normoxic conditions at all time points, and, as with **1**, the relative difference between the uptake under hypoxic and normoxic conditions decreased over the time course of the experiment. The presence of the nitro group on the phenyl ring for **3** and the nitroimidazole moiety on **6** did not appear to improve the hypoxic selectivity of the copper complex.

Compound **1** was chosen as the lead compound for oxygen-dependent cell uptake studies as it demonstrated the most promising initial in vitro results. The oxygen-dependent study performed 15 min after incubation with **1** showed an appreciable increase in cellular uptake with decreased oxygen concentration (Figure 3). Compound **1** showed very high uptake at 0% oxygen (87.6 ± 0.4%) and 0.1% oxygen (83.9 ± 0.2%), and the difference in uptake between normoxic and hypoxic conditions is statistically significant (87.6 ± 0.4 at 0% $O_2$, 58.3 ± 2.1 at 20% $O_2$, $P$ = 0.003). The uptake profile of **1** was compared to those obtained for $^{64}$Cu-ATSM, $^{64}$Cu(OAc)$_2$, and **3**. Compound **3** was specifically chosen to establish whether the substitution of a hydrogen on the exocyclic aromatic system of **1** for a nitro group had any influence on the cellular uptake at intermediate (0.1, 1, and 5%) oxygen concentrations. Similarly to **1**, compound **3** showed significant hypoxic selectivity (90.8 ± 0.9% at 0% $O_2$, 69.5 ± 0.8 at 20% $O_2$, $P$ = 0.001). The similarity in the uptake profiles of **1** and **3** suggests that the presence of the nitro group on the exocyclic phenyl group has minimal effect on the in vitro behavior. While there are statistically significant differences in uptake between **1** and **3** at 0% (87.6 ± 0.4 and 90.8 ± 0.9% for **1** and **3**, respectively, $P$ = 0.04) and 0.1% (83.9 ± 0.2 and 82.7 ± 0.1% for compounds **1** and **3**, respectively, $P$ = 0.02) oxygen, it is only under normoxic conditions (200000 ppm $O_2$) that there is any appreciable difference in the mean cellular accumulations (58.3 ± 2.1 and 69.5 ± 0.8% for **1** and **3**, respectively, $P$ = 0.001).

The uptake of the $H_2$ATSM/A derivatives studied here is higher than $^{64}$Cu-ATSM at all oxygen concentrations (73.9 ± 1.7 and 36.3 ± 0.3%, respectively under anoxic and normoxic conditions), but the inflection points in uptake profiles for the imine conjugates occur at the same place as $^{64}$Cu-ATSM (between 5000 and 50000 ppm $O_2$). The control $^{64}$Cu(acetate)$_2$, in contrast, shows minimal uptake, irrespective of the air composition, and is unselective for hypoxic cells.

**Serum Stability.** Compounds **1** and **3** were incubated for 60 min in fresh mouse serum, with samples taken at various time points. After 1 min, approximately 20% of the activity was bound to proteins. This remained constant over the 60 min experimental period. Compound **3** displayed similar results, with 75% of the activity remaining unbound at 1 min; a level that remained effectively constant for the remaining 60 min. These results are comparable to what was observed for Cu-ATSE in mouse serum.[27]

**Acute Biodistribution Studies.** The biodistribution data obtained for **1** and **3** from BALB/c mice at 5, 20, 40, and 60 min is shown in Table 2. Compounds **1** and **3** showed very similar organ uptake and accumulation in their biokinetic profiles. Both were initially cleared rapidly from the blood (1.86 ± 1.97%ID/g and 2.45 ± 0.99%ID/g for **1** and **3**, respectively) at 5 min, however, after 60 min, 2.91 ± 0.58%ID/g **1** and 2.20 ± 0.10%ID/g **3** were still in the blood pool. This is comparable for what has been observed for $^{64}$Cu-ATSM and $^{64}$Cu-ATSE in previous studies but is lower than what was observed for the bis(selenosemicarbazonates), which showed much lower serum stability.[10,27] The liver uptake for both compounds was very high. For **1**, 74.68 ± 4.46%ID/g had been extracted into the liver after 5 min. This uptake decreased over 60 min to 52.91 ± 3.45%ID/g, with a corresponding increase in uptake in the kidneys (10.56 ± 6.41%ID/g and 12.44 ± 3.03%ID/g at 5 and

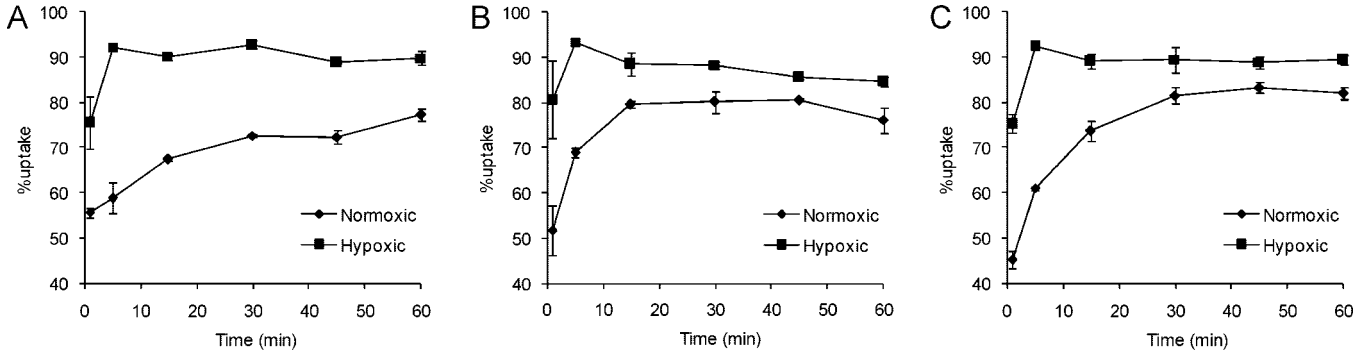


**Figure 2.** Percentage uptake of compounds **1** (A), **3** (B), and **6** (C) into suspended EMT6 cells over time. Errors, if not indicated, are within symbols. At 15 min, significant differences [$P = 0.0004$ (**1**), $P = 0.036$ (**3**), $P = 0.013$ (**6**)] were observed between cells incubated under anoxic conditions and those incubated under normoxic conditions.

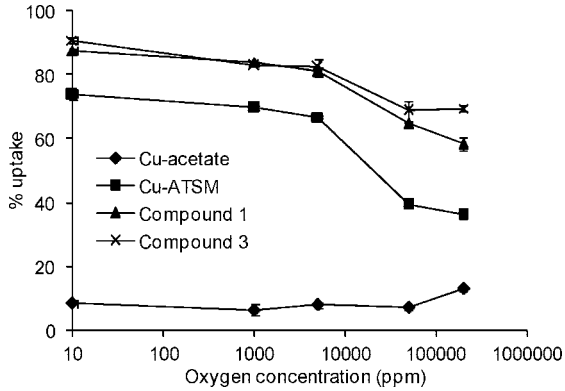


**Figure 3.** Percentage uptake of compounds **1**, **3**, $^{64}$Cu-ATSM, and $^{64}Cu(OAc)_2$ into EMT6 cells at varying oxygen concentrations. Errors, if not indicated, are within symbols. Differences in uptake between anoxic and 20000 ppm $O_2$ were significant for compounds **1** ($P = 0.003$), **3** ($P = 0.001$, and Cu-ATSM ($P = 0.002$).

**Table 2.** Biodistribution (%ID/g ± SD, $n = 4$) at 5, 20, 40, and 60 min of **1** and **3** in BALB/c Mice Bearing the EMT6 Mammary Tumor

| tissue | 5 min | 20 min | 40 min | 60 min |
|---|---|---|---|---|
| | | Compound **1** | | |
| blood | 1.86 ± 1.97 | 3.62 ± 0.24 | 3.14 ± 0.19 | 2.91 ± 0.58 |
| lung | 19.87 ± 3.02 | 20.03 ± 3.23 | 18.49 ± 1.19 | 15.16 ± 4.21 |
| liver | 74.68 ± 4.46 | 61.29 ± 6.45 | 58.77 ± 5.69 | 52.91 ± 3.45 |
| spleen | 18.99 ± 6.98 | 13.97 ± 3.01 | 11.15 ± 0.63 | 8.82 ± 1.08 |
| kidney | 10.56 ± 6.41 | 13.28 ± 2.75 | 13.52 ± 1.00 | 12.44 ± 3.03 |
| heart | 4.25 ± 3.35 | 5.46 ± 0.63 | 4.84 ± 0.50 | 4.52 ± 0.25 |
| brain | 2.54 ± 1.40 | 2.80 ± 0.28 | 2.43 ± 0.17 | 2.58 ± 0.25 |
| bone | 1.37 ± 0.98 | 2.45 ± 0.40 | 2.44 ± 0.60 | 2.63 ± 0.70 |
| tumor | 2.69 ± 0.49 | 5.65 ± 1.76 | 5.56 ± 1.89 | 4.17 ± 0.37 |
| intestines | 7.21 ± 0.31 | 13.31 ± 2.92 | 18.56 ± 4.60 | 18.60 ± 8.93 |
| | | Compound **3** | | |
| blood | 2.45 ± 0.99 | 3.10 ± 0.24 | 2.90 ± 0.82 | 2.20 ± 0.10 |
| lung | 26.92 ± 5.44 | 22.49 ± 4.96 | 17.73 ± 1.65 | 12.82 ± 0.48 |
| liver | 51.03 ± 8.61 | 65.94 ± 12.05 | 72.62 ± 5.28 | 44.99 ± 6.97 |
| spleen | 9.56 ± 1.66 | 9.21 ± 2.77 | 7.98 ± 2.11 | 4.60 ± 0.57 |
| kidney | 12.62 ± 2.59 | 17.88 ± 1.79 | 18.48 ± 2.21 | 11.69 ± 0.85 |
| muscle | 2.36 ± 0.50 | 3.21 ± 0.88 | 2.31 ± 0.87 | 1.30 ± 0.23 |
| heart | 5.85 ± 1.55 | 6.29 ± 0.89 | 5.58 ± 0.22 | 3.82 ± 0.24 |
| brain | 1.92 ± 0.29 | 2.54 ± 0.52 | 2.37 ± 0.11 | 1.46 ± 0.02 |
| bone | 2.32 ± 0.47 | 2.69 ± 0.58 | 2.97 ± 0.74 | 1.92 ± 0.23 |
| tumor | 1.78 ± 0.28 | 4.77 ± 2.07 | 4.42 ± 0.49 | 3.38 ± 0.15 |
| intestines | 5.71 ± 2.42 | 12.14 ± 1.99 | 20.41 ± 3.96 | 16.71 ± 0.37 |

60 min, respectively) and intestine (7.21 ± 0.31 and 18.60 ± 8.93%ID/g at 5 and 60 min, respectively). In contrast, **3** had slightly lower liver uptake at 5 min (51.03 ± 8.61%ID/g), but the uptake increased to 72.62 ± 5.28%ID/g at 40 min. The kidney uptake of **3** was comparable to that of **1**. The biodistribution of both compounds differs from that of the known hypoxia imaging agent, $^{64}$Cu-ATSM, and perfusion tracer, $^{64}$Cu-PTSM, studied in the same EMT6 tumor model.[10] These previous studies showed that $^{64}$Cu-PTSM in BALB/c mice had a liver uptake of 12.32%ID/g and 24.70%ID/g at 5 and 40 min, respectively. The more lipophilic hypoxic tracer, $^{64}$Cu-ATSM (log $P = 1.85$), showed uptake in the liver of 20.84%ID/g and 29.83%ID/g at the same time points.[10]

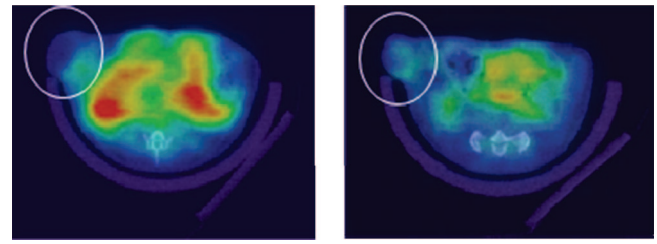

**Figure 4.** Representative transaxial slice of microPET/CT coregistered images of BALB/c mice bearing EMT6 tumors on the right flank. Mice were injected with 300 $\mu$Ci of $^{64}$Cu-labeled **1** (left) and **3** (right). White circle denotes tumor.

The uptake in the lung for **1** and **3** was high (19.87 ± 3.02%ID/g and 26.92 ± 5.44%ID/g after 5 min for **1** and **3**, respectively), and there is appreciable retention over the 60 min period. Both compounds show maximum tumor uptake at 20 min (5.65 ± 1.76%ID/g and 4.77 ± 2.07%ID/g for **1** and **3**, respectively), which is comparable with the maximum tumor uptake previously observed for $^{64}$Cu-ATSM (4.78 ± 1.00%ID/g) but lower than $^{64}$Cu-ATSE (7.71 ± 0.46%ID/g).[10,27]

**Small Animal PET.** Two of the labeled compounds were examined in vivo in a mouse model of breast cancer-EMT6 mammary carcinoma (Figure 4). The uptake in nontarget organs mirrored the acute biodistribution results with high uptake in the liver, kidneys, and lungs. Average tumor SUVs were 0.36 ± 0.05 for compound **1** and 0.41 ± 0.08 for compound **3**. Average muscle SUVs were 0.06 ± 0.08 for compound **1** and 0.08 ± 0.04 for compound **3**.

## Discussion

Copper complexes of bisthiosemicarbazones are a class of compounds that have been intensively investigated for potential as imaging agents for hypoxia and blood flow.[8–10,13,15,16,28–30] Recently, bisthiosemicarbazones containing pendant carboxylic acid and hydrazinic groups off the N-terminus have been

synthesized, but little biological data on these molecules has been published. The formation of imines via condensation between ketones and the hydrazinic nitrogen of $H_2$ATSM/A provides a versatile method of functionalization of bisthiosemicarbazones without significantly perturbing the reduction potential of the complex by altering the dimethyl backbone.

The $^{64}$Cu−imine conjugates in this study were prepared either directly from the free asymmetric bisthiosemicarbazone ligand or via transmetalation of the corresponding zinc complex. All complexes could be radiolabeled cleanly via transmetalation, but occasionally radiolabeling from the free ligand resulted in complexes of lower radiochemical yield. These preliminary results suggest that the zinc bis(thiosemicarbazones) provide a useful precursor for the synthesis of copper bis(thiosemicarbazone) radiopharmaceuticals.

The apparatus used in these in vitro experiments has been used in previous hypoxic selectivity studies[10,27] and is specifically designed to keep variables such as cell concentration, temperature, and pH constant so that the cellular association of the radiolabeled compounds is entirely based on the dissolved $pO_2$ in the cell media. The in vitro studies showed rapid cellular uptake of compounds **1**, **3**, and **6**. Rapid cellular uptake has previously been observed for small, neutral, lipophilic, square planar compounds.[31,32] In contrast, $^{64}$Cu(OAc)$_2$ showed minimal uptake, which is consistent with what has previously been observed. The association profiles of **1**, **3**, and $^{64}$Cu-ATSM after 15 min at varying oxygen concentrations were similar, with all three complexes displaying inflection points in their uptake profile between 5000 and 50000 ppm $O_2$. The major difference between the compounds studied here and $^{64}$Cu-ATSM is the overall cellular uptake under both normoxic and hypoxic conditions. While **1** and **3** had higher uptake than $^{64}$Cu-ATSM at low oxygen concentrations, they also displayed increased uptake under normoxic conditions. Crystallographic studies have shown that the imine conjugates of $H_2$ATSM/A are essentially planar,[33] and, thus, the higher cellular uptake of the new conjugates is most likely a result of their higher lipophilicity. The hypoxic selectivity index (HSI),[19] calculated at 15 min from the oxygen-dependent uptake study, was 0.70 and 0.64 for **1** and **3**, respectively. The HSI calculated for $^{64}$Cu-ATSM in this study was 0.70. It has been noted previously that reassociation of the dissociated copper and ligand may occur in vitro, because the cells are kept in a static medium and, therefore, the HSI of these compounds in vivo may be higher. This is because the culture system cannot account for the possible reassociation of the ligand with $^{64}$Cu outside the hypoxic cells. If dissociation occurs intracellularly and the free ligand then escapes the cell, then it could reassociate with $^{64}$Cu and reenter the cell. However, this process could not occur in vivo, as the liberated ligand would be removed by the circulation.[27]

It was decided that the initial in vivo experiments would be performed in mice bearing EMT6 tumors so that comparisons could be drawn with the previously studied analogues.[10,27] The EMT6 cell line has been used previously to investigate hypoxic selective agents because it can be used in vitro and in vivo.[34,35] This cell line has been used extensively to investigate hypoxic agents, as the cells cultured as spheroids or as solid tumors in vivo in BALB/c mice can contain a hypoxic fraction dependent on size and age.[36,37] Compound **1** was chosen for as the lead compound for in vivo study, as it demonstrated the most promising in vitro results. Compound **3** was used as a comparison to establish whether the substitution of a hydrogen on the exocyclic aromatic system for a nitro group had any influence on the biodistribution.

Compound **1** (log $P = 2.30 \pm 0.06$) and compound **3** (log $P = 2.28 \pm 0.05$) demonstrated high liver accumulation. It is likely that increased liver uptake of these compound is in someway related to the greater lipophilicity of these compounds. However, $^{64}$Cu-ATSE (log $P = 2.34 \pm 0.08$)[27] has similar lipophicility to the compounds studied herein but has a biodistribution more closely resembling $^{64}$Cu-ATSM.[10,27] While the imine bond has been shown to be stable over 48 h under aqueous conditions,[33] it is possible that some hydrolysis of this bond occurs in vivo. The small animal PET/CT images obtained show very high uptake in the liver, which is consistent with the results for the acute biodistribution results.

The behavior of the imine conjugates used in this study are very similar both in vitro and in vivo. This is not unexpected, as the differences between the imine conjugates are relatively subtle (a nitro group on the 3-position on the exocyclic phenyl ring) and the hypoxic selectivity and biodistribution of the complexes has previously been related to the reduction potential of the copper and the lipophilicity of the complexes, both of which are largely unaffected by this substitution. While the high liver uptake of these compounds means they are not viable as PET tracers, the in vitro results demonstrate that the $H_2$ATSM/A ligand provides a potential platform for functionalization of bis(thiosemicarbazoates), which is, to our knowledge, the first published results of hypoxic selectivity in functionalized bis(thiosemicarbazonates). Future studies will focus on the synthesis of more hydrophilic $H_2$ATSM/A derivatives with the idea of reducing the accumulation in the liver and uptake in normoxic cells.

## Conclusion

The studies carried out demonstrate that the ATSM/$H_2$ ligand provides a means for functionalizing copper-64 bis(thiosemicarbazonates), while maintaining hypoxic selectivity. All derivatives used in the study radiolabeled cleanly and efficiently, and those investigated in vitro demonstrated strong hypoxic selectivity in EMT6 cell lines. In addition, in vivo studies showed promisingly high tumor uptake values. Very little variation is observed between the complexes in vitro and in vivo, which is not unexpected due to the relatively subtle variations between the different complexes. While the compounds studied here are not clinically viable due to their high liver uptake, it is promising that the replacement of the exocyclic-methyl of $^{64}$Cu-ATSM with a hydrazinic nitrogen group results in compounds with comparable HSI values to that $^{64}$Cu-ATSM. It is hoped that the nontarget organ uptake can be limited in the future by reducing the log $P$ or by functionalizing via amide bonds, and investigations on the latter of these have commenced.

## Experimental Section

**Materials.** All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Water was distilled and then deionized (18 MΩ/cm$^2$) by passing through a Milli-Q water filtration system (Millipore Corp., Milford, MA). Radio-thin-layer chromatography (TLC) was performed on silica gel plates (7.5 × 2.5 cm) using ethyl acetate/methanol (95:5) as the mobile phase. TLC plates were analyzed on a BIOSCAN System 200 imaging scanner (Washington, DC). $^{64}$Cu was produced on a CS-15 biomedical cyclotron at the Washington University School of Medicine using published methods.[38] Radioactivity was counted with a Beckman Gamma 8000 counter containing a NaI crystal (Beckman Instruments, Inc., Irvine, CA).

**Chemistry.** Diacetyl-bis($N^4$-methylthiosemicarbazonate) [$H_2$ATSM] was prepared by a previously reported procedure.[39] The ligands and copper complexes were synthesized by simple adaption of similar methods previously reported.[26]

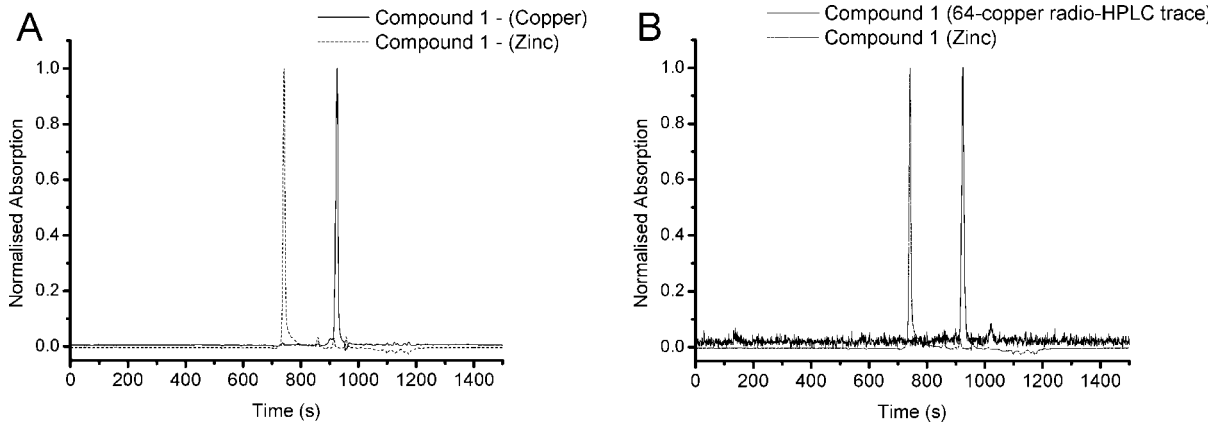


**Figure 5.** HPLC analysis of (A) the zinc complex of **1** with the corresponding nonradioactive copper complex of **1** and (B) the zinc complex of **1** with the corresponding radioactive trace associated with the $^{64}$Cu complex of **1** following transmetalation.

**$^{64}$Cu Complexation of Ligands.** All ligands were labeled with $^{64}$Cu in a similar fashion. The ligands or zinc complexes were dissolved in DMSO to give an overall concentration of 0.1 mg/mL. A total of 200 $\mu$L (20 $\mu$g) of this solution was then added to 200 $\mu$L (10 mCi) of $^{64}$Cu in NaOAc buffer (0.05 M, pH 5.5), which was typically supplied at a specific activity of around 200 mCi/$\mu$g. This solution was then stirred for 30 min at 37 °C, after which it was loaded onto a C18 SepPak-Light (Waters Corporation, Milford, MA), which had been pre-equilibrated with 5 mL of ethanol and 5 mL of water. After loading the sample, 15 mL of water was passed through to remove the DMSO and any unreacted $^{64}$Cu. The labeled complexes were then eluted in 350 $\mu$L of ethanol (following a 150 $\mu$L ethanol elution for the void volume). The purity of the labeled material was determined by radio-TLC using silica gel plates with ethyl acetate/methanol (95:5) as the mobile phase, and $R_f$ values were compared to those of the previously prepared, and fully characterized, cold copper compounds, which were coeluted with the radioactive analogs. In addition, a radio-HPLC was run on one of the complexes (**1**) and compared with the UV-HPLC of the cold copper complex (Figure 5). The radiochemical yield for all radiolabeling reactions was 80–85% and all were purified to >95% purity. On occasion, radiolabeling from the free ligand resulted in lower overall yields of complexes (55–62%). Therefore, radiolabeling via transmetalation became the reaction conditions of choice.

**Radio-HPLC Analysis.** The analysis was performed using an Agilent 1100 Series HPLC instrument with a 250 mm × 4.6 mm Phenomenox Primesphere 5 C18-HC 110H column (S/N 324707). Both UV detection ($\lambda_{obs}$ = 254 nm) and NaI scintillation crystal detection were used in series with a delay time of approximately 10 s. A 25 min gradient elution method was employed (MeCN/$H_2O$): start, 5% MeCN; gradient till 10 min, to 95% MeCN; hold till 15 min at 95% MeCN; reverse gradient till 20 min, to 5% MeCN; and hold till 25 min at 5% MeCN.

**Partition Coefficients.** Octanol/water partition coefficients were calculated as log $P$ = log(counts in octanol/counts in PBS buffer (pH 7.4)) and were determined in triplicate as described previously.[40]

**Serum Stability.** To measure the stability of the labeled complexes, 5 $\mu$Ci of the required $^{64}$Cu complex was incubated in 1 mL of fresh mouse serum at 37 °C, where at selected time points, the amount of intact complex was determined by the ethanol precipitation method. Briefly, to determine the amount of protein-bound $^{64}$Cu, 50 $\mu$L fractions were removed from the serum at the selected time points, and 200 $\mu$L of ethanol was added to precipitate the proteins present. The sample was then centrifuged until the precipitated proteins formed a pellet. The supernatant was then carefully removed from the pellet, the pellet was washed with 500 $\mu$L of ethanol, and the sample was centrifuged again. The combined supernatants and the pellet were counted on a gamma counter to determine the amount of protein-bound activity.

**In Vitro Hypoxic Selectivity.** The apparatus and procedures used for these in vitro experiments are based on those previously described.[10,18] The apparatus controls the in vitro incubation conditions of temperature, humidity, pH, and oxygen tension. The EMT6 mammary carcinoma cell suspension (10 mL) at a concentration of 5 × $10^6$ cells/mL was equilibrated in a three-necked, glass, round-bottomed flask at 37 °C at the desired oxygen concentration (0, 0.1, 0.5, 5, or 20%) by passing a continuous flow of warmed humidified gas over the cells, with all remaining variables kept constant. The gases used were 5% $CO_2$, the desired percentage of $O_2$, and the balance of $N_2$. After 30 min, when the vessels had reached equilibrium, 50 $\mu$Ci of the radiopharmaceutical was added. As a control, the same experiment was performed in tandem with $^{64}$Cu-ATSM and $^{64}$Cu(acetate)$_2$. Then at 15 min, triplicate samples of 200 $\mu$L of cell suspension were removed and centrifuged to pellet the cells. The percentage accumulation of the compound into the cells was then calculated. As a control, the compounds were put through the identical methods without cells present to determine the extent to which the compounds adhere to vials due to the lipophilicity of the compounds. No appreciable adherence of any of the compounds was noted. A protein assay was not required as the cell studies were all performed on the same homogeneous cell mixture of precisely known cellular concentration. Internalization studies were not performed, and so these studies are only able to determine the amount of tracer associated with the cells: from these data, it is not possible to state whether the activity is on the cell surface, partitioned in the membrane, or internalized within the cell.

**Time Course Studies.** The EMT6 mammary carcinoma cell suspension (10 mL), taken from a homogeneous 50 mL cell suspension at a concentration of 5 × $10^6$ cells/mL, was equilibrated in a three-necked, glass, round-bottomed flask at 37 °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, with all remaining variables kept constant. After 30 min, when the vessels had reached equilibrium, 50 $\mu$Ci of the radiopharmaceutical was added. As a control, the same experiment was performed in tandem with $^{64}$Cu(acetate)$_2$. Then at 1, 5, 15, 30, 45, and 60 min, triplicate samples of 200 $\mu$L of cell suspension were removed, the suspension was centrifuged, and the percentage uptake of the compound into the cells was calculated, as described above.

**Biodistribution.** All animal experiments were conducted in compliance with the Guidelines for the Care and Use of Research Animals established by Washington University's Animal Studies Committee. The biodistribution studies were conducted in female BALB/c mice (20 g, aged 5–6 weeks; Charles River Laboratories, Wilmington, MA) that had been implanted with 6 × $10^5$ EMT6 cells in 100 $\mu$L into the right flank. The tumors were allowed to grow for 12 days post-implantation, at which time the animals

received 10 $\mu$Ci of the desired complex in 100 $\mu$L of a saline/ethanol mix (ethanol <5%) via lateral tail-vein injection. The animals were euthanized at desired time points (5, 20, 40, and 60 min, $n = 4$ per group), and the organs were harvested (blood, lung, liver, spleen, kidney, heart, brain, bone, tumor, and intestines). Once the tissues and organs of interest were removed, they were weighed and the radioactivity was measured in a $\gamma$-counter. The percent dose per gram (%ID/g) and percent dose per organ (%ID/organ) were then calculated by comparison to known standards.

**Imaging Studies.** Small animal PET images in EMT6 tumor-bearing mice were obtained on a microPET-Focus 220[41] (Concorde MicroSystems Inc., Knoxville, TN) and were coregistered with CT images from a MicroCAT II System (ImTek Inc., Knoxville, TN). Isoflurane (1–2%) was used as an inhaled anesthetic to induce and maintain anesthesia during imaging. The animals received 300 $\mu$Ci of the desired complex in 100 $\mu$L of saline/ethanol (ethanol <5%) via lateral tail-vein injection. Mice were imaged individually or in pairs in a supine position in a specially designed bed. Imaging was performed in 60 min dynamic sessions.

Small animal PET images were evaluated by analysis of the standardized uptake value (SUV) of the tumor and nontarget organ (muscle) using the software ASIPRO (Concorde MicroSystems, Inc.) as well as Amira (TGS, Inc.; San Diego, CA) for coregistered PET/CT images. The average radioactivity concentration within the tumor or tissue was obtained from the average pixel values reported in nCi/cc within a volume of interest (VOI) drawn around the entire tumor or tissue on multiple, consecutive, transaxial image slices. SUVs were calculated by dividing this value, the decay-corrected activity per unit volume of tissue (nCi/mL), by the injected activity per unit of body weight (nCi/g).

**Statistical Methods.** To compare the differences in cell uptake, biodistribution, and SUV, the Student's *t*-test was performed. Differences at the 95% confidence level ($P < 0.05$) were considered significant.

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