Journal of
Medicinal Chemistry

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J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.9b00386 • Publication Date (Web): 08 Jul 2019

Just Accepted

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Novel Meta-iodobenzylguanidine-based Copper Thiosemicarbazide-1-guanidinomethyl-benzyl Anticancer Compounds Targeting Norephinephrine Transporter in Neuroblastoma

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ABSTRACT

Meta-iodobenzylguanidine (MIBG) is a ligand with high affinity against norephinephrine transporter (NET) that has been used for diagnostic imaging and radionuclide therapy of NET-expressing tumors, such as neuroblastoma. We hypothesize that MIBG can be used as a ligand for development of new anticancer drugs targeting NET-expressing neuroblastoma (NB). To test our hypothesis, we synthesized two MIBG-based anticancer copper complexes [$Cu(m\text{-}TSBG)_2$ and $Cu(p\text{-}TSBG)_2$] by conjugating of a thiosemicarbazone-copper group onto MIBG ligand. Both $Cu(m\text{-}TSBG)_2$ and $Cu(p\text{-}TSBG)_2$ compounds showed potent anticancer activity against NB cells (BE2C and SK-N-DZ cells). The NB-specific anticancer activity of $Cu(m\text{-}TSBG)_2$ and $Cu(p\text{-}TSBG)_2$ was further demonstrated by the reduced anticancer activities when non-conjugated MIBG ligand was used to competitively block binding of $Cu(m\text{-}TSBG)_2$ or $Cu(p\text{-}TSBG)_2$ onto NET-expressing NB cells. Both $Cu(m\text{-}TSBG)_2$ or $Cu(p\text{-}TSBG)_2$ compounds hold potential

as promising new drugs for targeted therapy of neuroblastoma and other NET-expressing tumors.

INTRODUCTION

Neuroblastoma is the most common extracranial cancer in childhood and often occurs in very young children.[1, 2] Chemotherapy plays a major role in the treatment of high-risk neuroblastoma. Acquired resistance of NB to cisplatin and other anticancer drugs currently available results in poor prognosis of children diagnosed with late-stage disease of NB. Despite significant efforts devoted to development of effective drugs for treatment of NB, side effects from non-specific cytotoxicity of many of anticancer compounds hampered their clinical application.[3-5] There are continued efforts in developing new anticancer drugs with improved cancer cell targeting capability or improved *in vivo* pharmacokinetics.[6] Positron emission tomography (PET) could be used to study biodistribution and *in vivo* pharmacokinetics of new compounds by radiolabeling of anticancer compounds with a positron emitting radionuclide.[7]

Many of metal complexes were found to have potent anticancer activity and hold potentials as promising new anticancer drugs.[8-12] Copper is a nutritional metal required for many of physiological processes in human body. Many of copper complexes were found to have strong anticancer activity against NB and other tumors such as prostate cancer.[13] Copper complexes of 8-hydroxyquinoline-2-carboxaldehyde-thiosemicarbazide (CuHQTS) is an anticancer copper complex with potent anticancer activity against NB and prostate cancer cells.[14, 15] Recently, it was found that growth of human prostate

cancer xenograft tumors was inhibited in athymic nu/nu mice treated with CuHQTS.[16] However, significant weight loss of the mice treated with CuHQTS was observed, likely a side effect of non-specific toxicity on body metabolism due to lack of tumor-specific cytotoxicity of CuHQTS.[14-16]

Neuroblastoma cells are derived from the neural crest and reserve many properties such as synthesis, storage, release and reuptake of norephinephrine (NE).[17, 18] Benzylguanidine is a metabolically stable NE analogue derived from the adrenergic neuron-blocking agents, bretylium and guanethidine. Benzylguanidine can be specifically, actively and efficiently taken up by neuroblastoma cells through NE transporter (NET), a specific transport system located at neuroblastoma cell membrane.[19, 20] Meta-iodobenzylguanidine (MIBG), a small molecule, has been widely applied as an imaging agent in the diagnostic imaging and radionuclide therapy of neuroendocrine tumors, such as neuroblastoma, pheochromocytoma and carcinoid tumor, based on its high affinity binding to NET.[21-24] We hypothesize that MIBG may be used as a targeting ligand for synthesis of new anticancer drugs consisting of MIBG and an anticancer copper complex such as CuHQTS for targeted therapy of neuroblastoma.

This study aimed to test our hypothesis by synthesizing new CuTSBG anticancer compounds combining MIBG and CuHQTS for targeted therapy of NB. $Cu(m\text{-}TSBG)_2$ and $Cu(p\text{-}TSBG)_2$ (**Figure 1**), were designed to engineer a new compound consisting of both benzylguanidine and copper-thiosemicarbazone groups for NB specific delivery and cytotoxicity.

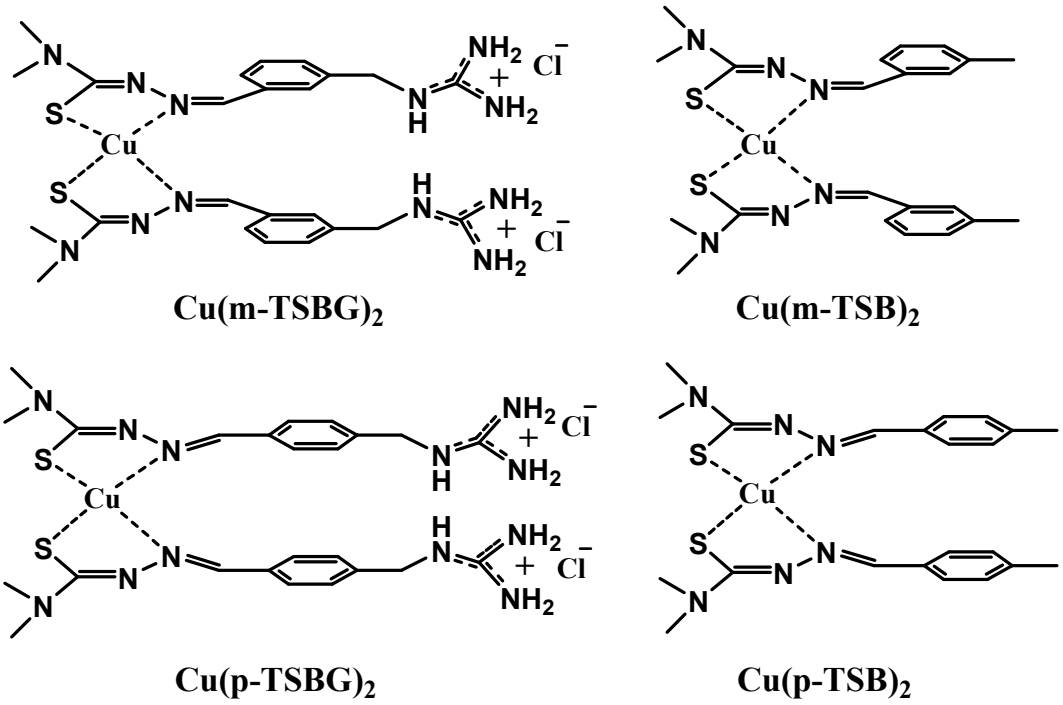

Figure 1. Schematic presentation of structure of MIBG-based anticancer copper compounds and control compounds.

In comparison, two control compounds, Cu(m-TSB)$_2$ and Cu(p-TSB)$_2$ (**Figure 1**), were designed with similar structures to Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$, but without benzylguanidine group. Benzylguanidine group may represent a critical functional group for NET-mediated cellular uptake and internalization of Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ (**Figure 2**).

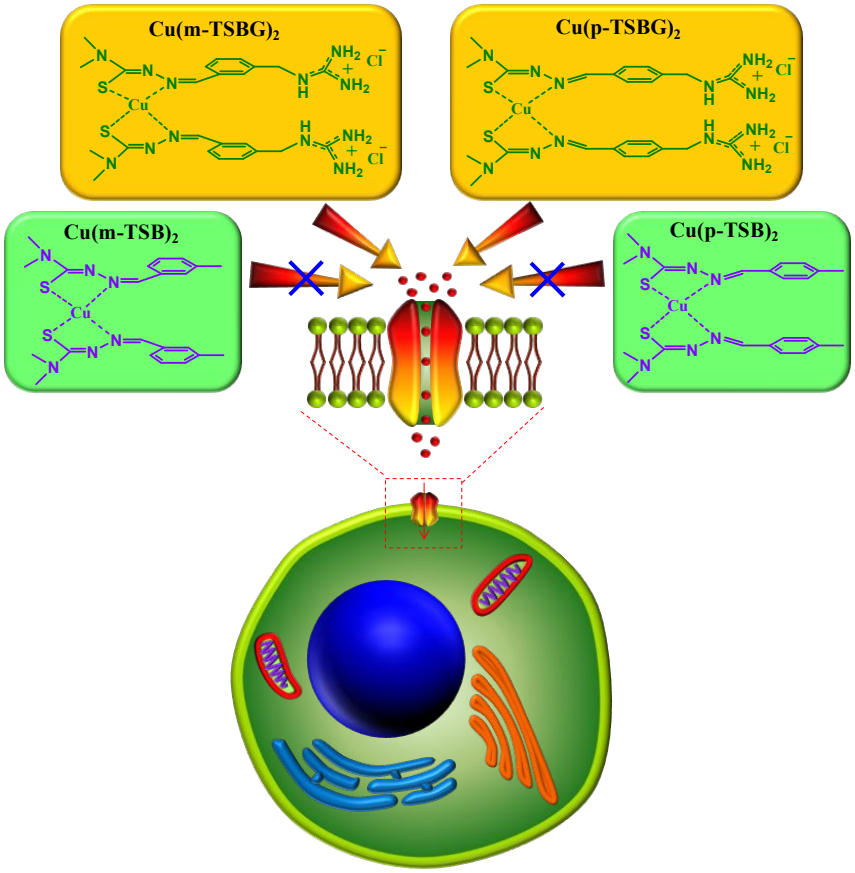

Figure 2. Schematic presentation of NB-specific uptake of MIBG-based anticancer copper complexes mediated by NET. Cellular uptake of Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ by NET-expressing NB cells, but absence of NB-specific cellular uptake of control Cu(m-TSB)$_2$ and Cu(p-TSB)$_2$ compounds without MIBG ligand.

Demonstration of anti-cancer activity of Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ compounds on NET-expressing NB cells, but not on NET-negative U87 glioblastoma cells or PC-3 prostate cancer cells, may provide evidence of feasibility to support further investigation of MIBG-mediated anticancer drug delivery.

RESULTS

$Cu(m\text{-}TSBG)_2$ and $Cu(p\text{-}TSBG)_2$ were synthesized through chelation of copper ions with 3-carbaldehyde-thiosemicarbazide-1-guanidinomethyl-benzyl (m-TSBG) and 4-carbaldehyde-thiosemicarbazide-1-guanidinomethyl-benzyl (p-TSBG) ligands, respectively. The m-TSBG and p-TSBG were synthesized through multi-step reactions as shown in **Supporting Information (Scheme 1)**. Commercially available isophthalaldehyde (**1**) and terephthalaldehyde (**2**) were used as starting materials to prepare monoaldehyde-monoalcohol (**3)** and (**4)** through $NaBH_4$ reduction.[25] Dimethylthiosemicarbazide in methanolic solution reacted with (**3)** or (**4)** to form (**5)** or (**6)** by direct condensation of amine and aldehyde.[14] Under Mitsunobu conditions (TPP, DIAD and THF),[21] the hydroxyls in (**5)** or (**6**) was further replaced with bis-Boc-guanidine to form Boc-protected guanidines products (**7)** or (**8)**. Boc groups in (**7)** or (**8)** were readily removed by treatment with HCl/dioxane to provide m-TSBG (**9**) and p-TSBG (**10**) ligands.[25] Under weak acidic conditions, dimethylthiosemicarbazide group in m-TSBG (**9**) or p-TSBG (**10**) could chelate copper (II) ion in methanol solution, and the resulting mixtures were concentrated and subjected to Sephadex LH-20 column to obtain pure copper compounds, $Cu(m\text{-}TSBG)_2$ (**11**) and $Cu(p\text{-}TSBG)_2$ (**12**).[26] Keeping the copper ion chelation reaction under weak acidic condition prevailed the copper coordination through dimethylthiosemicarbazide group rather than guanidine group, which was verified by IR spectra that showed C=S vibration in dimethylthiosemicarbazide group was significantly influenced while N-H vibration in benzylguanidine group remain unchanged.[14] $Cu(m\text{-}TSBG)_2$ and $Cu(p\text{-}TSBG)_2$ have very similar structures, and the only difference

between them was the substitution of copper-thiosemicarbazide group at benzylguanidine group where Cu(m-TSBG)$_2$ have meta- substitution while Cu(p-TSBG)$_2$ has para-substitution. Purities of Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ synthesized for this study were >95% as determined by ^{1}H NMR spectra of ligands and elemental analyses of compounds.

Previous study has demonstrated that the substitution at meta- or para-position of benzylguanidine will not influence the capability of benzylguanidine targeting to neuroblastoma cells.[19, 20, 22] Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ were expected to show specific antiproliferative activity to NB cells because benzylguanidine group could act as a targeting ligand to direct the whole molecule specifically internalized into neuroblastoma cells through NE transporter, while enhancing anticancer activity of copper-thiosemicarbazone group delivered into NB cells. To further demonstrate the critical role of benzylguanidine group, two non-targeting control compounds, Cu(m-TSB)$_2$ and Cu(p-TSB)$_2$ (**Figure 1**), were synthesized as shown in **Scheme 2 in Supporting Information,** for comparing the anti-proliferative activity of non-targeting Cu(m-TSB)$_2$ and Cu(p-TSB)$_2$ without a benzylguanidine group with the anti-proliferative activity of NET-targeting Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ compounds.

The anti-proliferative activity of Cu(m-TSBG)$_2$, Cu(p-TSBG)$_2$, Cu(m-TSB)$_2$ and Cu(p-TSB)$_2$ was examined in NB cells (BE2C and SK-N-DZ cells) and NET-negative glioblastoma cells (U87 cells) and prostate cancer cells (PC-3 cells) by MTT assay. Non-NET-expressing U87 glioblastoma cells were selected as control cells based on their similarity to NB cells,

while PC-3 prostate cancer cells were selected as control cells based on prior use for testing anticancer activity of CuHQTS.[15,16] After 24 h treatment, Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ showed strong anti-proliferation activity on NB cells (BE2C and SK-N-DZ cells) with IC_{50} of 0.87±0.11 and 0.55±0.08 μM for BE2C cells as well as 1.09±0.24 and 0.83±0.13 μM for SK-N-DZ cells. In contrast, weak inhibition activity on non-neuroblastoma cells was observed with IC_{50} of 4.6±0.32 and 4.8±0.24 μM for U87 glioblastoma cells as well as 5.0±0.35 and 4.7±0.21 μM for PC-3 prostate cancer cells (**Figure 3A,** and **Table S1 in supporting information**).

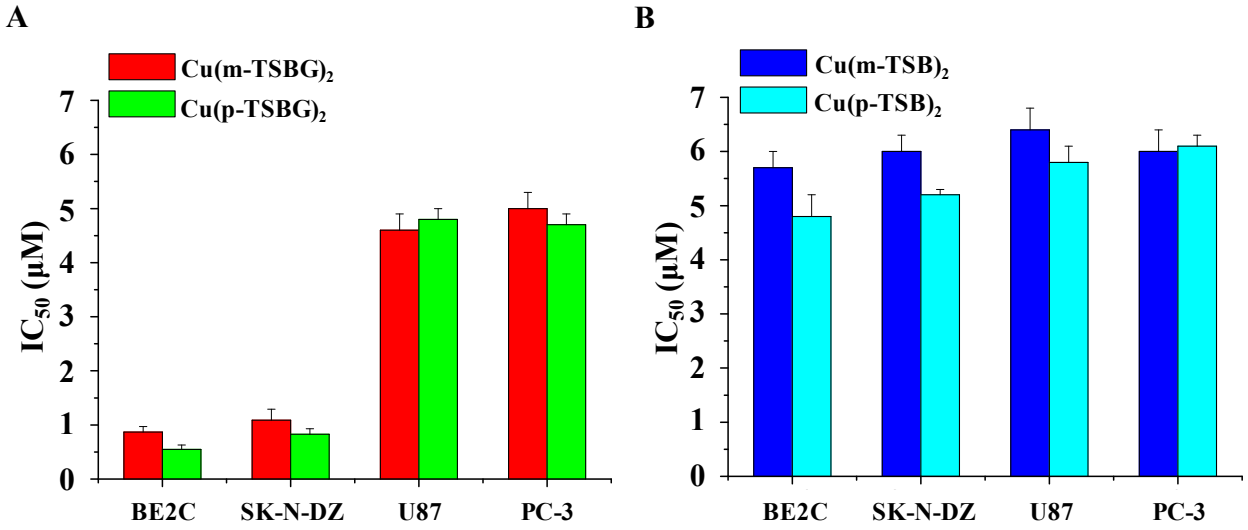

Figure 3. NB specific anti-proliferative activity of CuTSBG compounds by comparison of IC_{50} values of compounds on growth of NB cells (BE2C, SK-N-DZ) and non-NB cells (U87 and PC-3 cells). A) IC_{50} values of Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$; B) IC_{50} values of Cu(m-TSB)$_2$ and Cu(p-TSB)$_2$.

Similar results were also obtained by evaluation for cytopathological effects of these cells treated with Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ (**Figure S1 in supporting information**). The

data demonstrated that Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ had strong NB-specific anti-proliferative activity. In contrast, non-targeting Cu(m-TSB)$_2$ and Cu(p-TSB)$_2$ showed weak inhibition of the proliferation of both NB and non-NB cells with IC_{50} of 5.7±0.33 and 4.8±0.45 μM for BE2C cells, 6.0±0.31 and 5.2±0.13 μM for SK-N-DZ cells, 6.4±0.42 and 5.8±0.32 μM for U87 cells, 6.0±0.44 and 6.1±0.26 μM for PC3 cells, in which the difference of anti-proliferative activity between NB and non-NB cells were not significant (**Figure 3B,** and **Table S1 in supporting information**).

Since Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ have similar structures to Cu(m-TSB)$_2$ and Cu(p-TSB)$_2$, respectively, except the presence of a benzylguanidine group, their differential activity profiles can only be attributed to the role of benzylguanidine group. Where the existence of benzylguanidine groups at Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ will avail Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ to show specific anti-proliferative activity against NB cells while the elimination of this group at Cu(m-TSB)$_2$ and Cu(p-TSB)$_2$ will dramatically impair the specificity of anti-proliferative activity on NB cells. We also noticed that the precursors (m-TSBG, P-TSBG, m-TSB, P-TSB) without copper ions have minimal anti-proliferative activity on both NB and non-NB cells, with IC_{50} between 23.3±1.2 to 142.1±5.7μM (**Table S1 in supporting information**). The results of these experiments have demonstrated essential role of copper ions accounting for anti-proliferative activity of these MIBG-based anticancer compounds.

Benzylguanidine and their derivatives have been known capable of being specifically internalized into cells by NET expressed on tumor cell membrane.[17, 19-22] Since most of NB cells have high NET expression, and Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ have benzylguanidine groups, the NB-specific anti-proliferative activity of Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ was probably achieved by targeted cellular uptake by NB cells. To verify this hypothesis, ^{64}Cu-radiolabeled Cu(m-TSBG)$_2$, Cu(p-TSBG)$_2$, Cu(m-TSB)$_2$ and Cu(p-TSB)$_2$ were prepared to assess their cellular uptake by both NB cells (BE2C and SK-N-DZ cells) and non-NB cells (U87 and PC-3 cells). Radiolabeling of these compounds with radioactive ^{64}Cu radionuclide was performed in a protocol modified from the method reported by Lewis et al.[27] Cells were incubated with ^{64}Cu-radiolabeled copper complexes for 3 hours and the cellular ^{64}Cu radioactivity was determined by gamma counting using a γ-counter. The results indicated that the ^{64}Cu radioactivity of the NB cells incubated with ^{64}Cu(m-TSBG)$_2$ or ^{64}Cu(p-TSBG)$_2$ were higher than the ^{64}Cu radioactivity of the non-NB cells incubated with these two radioactive compounds (**Figure 4A**). There was no significant difference of ^{64}Cu radioactivity between the NB cells or non-NB cells after incubation with ^{64}Cu(m-TSB)$_2$ or ^{64}Cu(p-TSB)$_2$ compounds (**Figure 4B**).

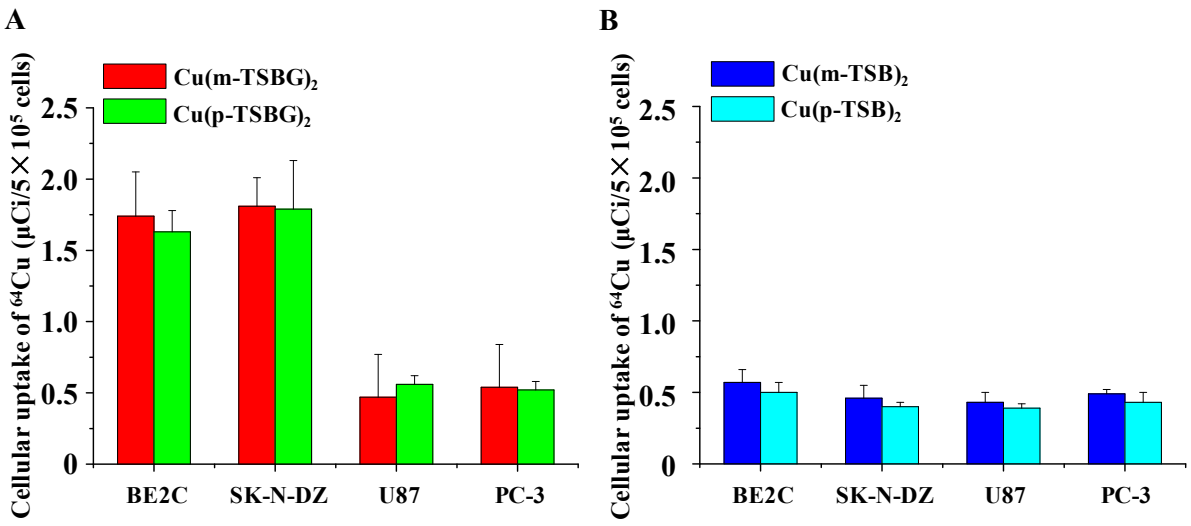

Figure 4. Cellular uptake of ^{64}Cu-labeled compounds by NB cells (BE2C, SK-N-DZ) and non-NB cells (U87 and PC-3 cells). A) Cellular uptake of ^{64}Cu(m-TSBG)$_2$ or ^{64}Cu(p-TSBG)$_2$ following incubation of cells with ^{64}Cu(m-TSBG)$_2$ or ^{64}Cu(p-TSBG)$_2$ (10 μCi/mL) for 3 h, respectively. B) Cellular uptake of ^{64}Cu(m-TSB)$_2$ or ^{64}Cu(p-TSB)$_2$ following incubation of cells with ^{64}Cu(m-TSB)$_2$ or ^{64}Cu(p-TSB)$_2$ (10 μCi/mL) for 3 h, respectively.

Moreover, ^{64}Cu radioactivity of NB cells incubated with ^{64}Cu(m-TSBG)$_2$ and ^{64}Cu(p-TSBG)$_2$ was significantly higher than the ^{64}Cu radioactivity of NB cells incubated with ^{64}Cu(m-TSB)$_2$ and ^{64}Cu(p-TSB)$_2$. The findings from these experiments suggested that NB-specific anti-proliferative activity of Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ was most likely due to presence of benzylguanidine group and specific cellular uptake of these two compounds mediated by NET expressed on NB cells. The benzylguanidine groups at Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ compounds could be recognized by NET on the NB cell membrane, and

probably could work like the “Trojan horse” to guide the whole Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ compounds to be internalized into cells, leading to the high cellular uptake and enhanced NB-specific cytotoxicity. In comparison, Cu(m-TSB)$_2$ and Cu(p-TSB)$_2$ compounds without a benzylguanidine group showed low NB cellular uptake due to lack of NET-mediated uptake or internalization.

To further confirm the role of NET-mediated cellular uptake in NB-specific anticancer activity of Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$, MIBG blocking assay was performed to determine whether the cellular uptake of ^{64}Cu(m-TSBG)$_2$ and ^{64}Cu(p-TSBG)$_2$ could be blocked by MIBG to reduce anti-proliferative activities of Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ on NB cells.

NB cells were incubated with ^{64}Cu(m-TSBG)$_2$ or ^{64}Cu(p-TSBG)$_2$ and MIBG ligand for 3 hours, and ^{64}Cu radioactivity of the cells was determined by gamma counting using a γ-counter. As expected, ^{64}Cu radioactivity of the NB cells (BE2C and SK-N-DZ) incubated with ^{64}Cu(m-TSBG)$_2$ or ^{64}Cu(p-TSBG)$_2$ in the presence of MIBG blocking ligand (**Figure 5A**) were significantly lower than the ^{64}Cu radioactivity of the NB cells (BE2C and SK-N-DZ) incubated with ^{64}Cu(m-TSBG)$_2$ or ^{64}Cu(p-TSBG)$_2$ without presence of MIBG blocking ligand (as shown in **Figure 4A**). In comparison, there was no difference of ^{64}Cu radioactivity of the cells incubated with ^{64}Cu(m-TSB)$_2$ or ^{64}Cu(p-TSB)$_2$ in the presence of MIBG blocking ligand (**Figure 5B**) or in the absence of MIBG blocking ligand (**Figure 4B**). Furthermore, no effect of MIBG blocking ligand on cellular uptake of these radiolabeled compounds by non-NB cells (**Figure 5,** and **Figure S2 in supporting information**).

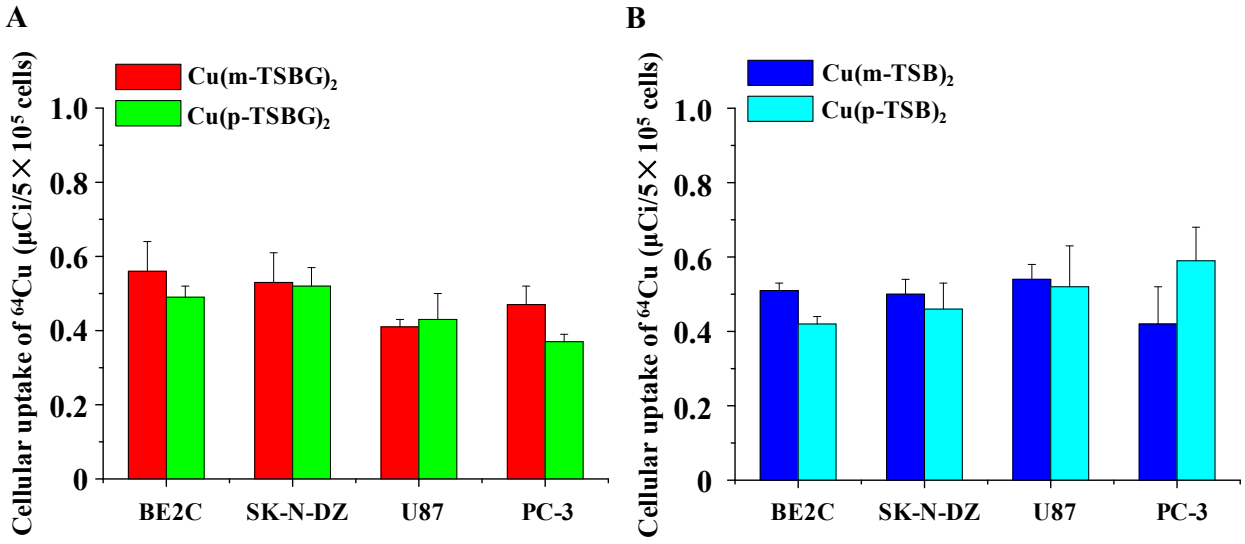

Figure 5. MIBG-blocking of NB cellular uptake of ^{64}Cu(m-TSBG)$_2$ or ^{64}Cu(p-TSBG)$_2$ compounds. A) Cellular uptake of ^{64}Cu(m-TSBG)$_2$ or ^{64}Cu(p-TSBG)$_2$ (10 μCi/mL) by NB cells (BE2C, SK-N-DZ) and non-NB cells (U87 and PC-3 cells) in the presence of 10 ng MIBG ligand (20 ng/mL) ; B) Cellular uptake of ^{64}Cu(m-TSB)$_2$ or ^{64}Cu(p-TSB)$_2$ (10 μCi/mL) by NB cells (BE2C, SK-N-DZ) and non-NB cells (U87 and PC-3 cells) in the presence of MIBG ligand (20 ng/mL).

Additionally, MIBG blocking assay were performed to assess the influence of MIBG on anti-proliferative activity of Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$. Following addition of MIBG at various concentrations to NB cell culture medium, NB cells (BE2C, SK-N-DZ) were treated with Cu(m-TSBG)$_2$ or Cu(p-TSBG)$_2$ at their IC_{50} concentrations for 24 h, and anti-proliferative activity of the compounds was assessed by MTT assay. Significant reduction of anti-proliferative activity of Cu(m-TSBG)$_2$ or Cu(p-TSBG)$_2$ on NB cells was observed, in the presence of gradually increased concentration of MIBG competitive

blocking ligand. In the presence of 2 μM of MIBG, more than 80% of NB cells were found to be viable after treatment with IC50 dose of Cu(m-TSBG)$_2$ or Cu(p-TSBG)$_2$ for 24 hours.

DISCUSSION AND CONCLUSIONS

Children suffered advanced stage neuroblastoma have a poor prognosis due to acquired resistance to many of anticancer drugs currently available. There is an urgent need of new effective drugs for targeted treatment of children with drug-resistant NB metastasis. Continuing from our efforts in developing new anticancer copper complexes for treatment of cisplatin-resistant NB tumors, we synthesized new anticancer compounds targeting NET-expressing NB cells by combining MIBG targeting ligand and CuHQTS anticancer copper complexes that were demonstrated to have potent anticancer activity on NB and prostate cancer cells. Two novel Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ compounds, consisting of CuHQTS (anticancer copper complexes) and MIBG ligand, were found to have MIBG-mediated NB-specific anticancer activity. The findings from this study demonstrated that potent anticancer activity of Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ on NB cells was most likely due to NET-mediated NB cellular uptake and internalization.

. It was observed that Cu(p-TSBG)2 exhibited higher inhibition activity over Cu(m-TSBG)2 against NB cells, that could be due to different strength of functional group of CuHQTS following its connection to MIBG ligand, not due to difference in cellular binding and internalization of these two compounds. Indeed, we did not observed positive correlation between inhibition activity and cellular uptake suggesting no difference of these two compounds in MIBG-ligand mediated cellular uptake (Figure 4). Both Cu(m-TSBG)2

and Cu(p-TSBG)2 displayed higher cellular uptake in NB cells (BE2C and SK-N-DZ cells) over non-NB cells (U87 and PC-3 cells). It was most likely due to difference in NET expression (high level expression in NB and low level expression in U87 and PC-3 cells), not due to difference in cellular uptake of free ^{64}Cu radionuclide disassociated from the copper compounds, based on previously demonstrated increase of carrier-free $^{64}CuCl_2$ uptake by PC-3 prostate cancer cells, [28, 29] U87 glioblastoma cells, [30, 31] and NB cells [32].

Radiolabeled MIBG ligand is clinically used for diagnostic imaging of neuroblastoma [21, 24]. NB-specific cellular uptake of $^{64}Cu(m\text{-}TSBG)_2$ or $^{64}Cu(p\text{-}TSBG)_2$ demonstrated in this study (**Figure 4**) support further study of $^{64}Cu(m\text{-}TSBG)_2$ or $^{64}Cu(p\text{-}TSBG)_2$ compounds as novel theranostic agents for targeted imaging and therapy of NB in children. Moreover, the findings of this study invite investigation of $^{64}Cu(m\text{-}TSBG)_2$ or $^{64}Cu(p\text{-}TSBG)_2$ for diagnostic imaging and therapy of other NET-expressing neuroendocrine tumors such as carcinoid.

In conclusion, two novel $Cu(m\text{-}TSBG)_2$ and $Cu(p\text{-}TSBG)_2$ compounds, consisting of MIBG (targeting ligand) and CuHQTS (anticancer copper complexes), were synthesized and tested for MIBG-mediated NB-specific anticancer activity. Potent anticancer activity of $Cu(m\text{-}TSBG)_2$ and $Cu(p\text{-}TSBG)_2$ compounds on NB cells were demonstrated, suggesting that these two novel $Cu(m\text{-}TSBG)_2$ and $Cu(p\text{-}TSBG)_2$ compounds are promising new drugs for treatment of children suffering advanced stage NB refractory to cisplatin or other chemotherapeutic drugs currently available.

EXPERIMENTAL SECTION

1. Chemical Synthesis

All chemicals were purchased from Sigma-Aldrich unless otherwise noted. Dried THF was obtained by refluxing with sodium and distilling. Dried CH_2Cl_2 was obtained by refluxing with CaH_2 and distilling. Other reagents were all commercial and used without further purification. The synthesis of Cu(m-TSBG)$_2$, Cu(p-TSBG)$_2$, Cu(m-TSB)$_2$ and Cu(p-TSB)$_2$ compounds were described in **Supporting Information.** ^{1}HNMR spectra and elemental analysis were used to determine purity of ligands and elemental analyses was used to determine purity of copper compounds, along with use of IR spectra for characterization. Purity of copper compounds ($\geq$ 95%) were confirmed by elemental analysis. IR spectra were recorded from 4000 to 400 cm^{-1} as KBr pellets on a Tensor 27 FT-IR spectrophotometer. ^{1}HNMR spectra were measured using Varian 400 mHz instruments. ESI mass spectra were measured in a triple quadrupole Micromass QuattroLC spectrometer with an electroscopy/APCI source. Elemental analyses were performed by Midwest Microlab (Indianapolis, IN).

2. MTT Cell Proliferation Assay

MTT assay was performed to evaluate antiproliferative activity of the compounds using a MTT assay kit from Chemicon (Temecula, CA), as described previously.[14, 33] Briefly, BE2C, SK-N-DZ, U87 and PC-3 cells from ATCC (Manassas, VA) were cultured in EMEM:F12K (BE2C), DMEM (SK-N-DZ), RP1640 (U87 and PC-3) medium (Biosource International, Camarillo, CA), respectively, supplemented with fetal bovine serum (10%), penicillin (100 units /ml), streptomycin (100mg/ml), and glutamine (100 mg/ml), at 37°C in an atmosphere of 5% CO_2. The cells were seeded in a 96-well plate (1×10^4 /0.1 ml/well)

and were treated with compounds at noted concentration for 24 h. Upon end of drug treatment, MTT solution was added and optical density (*OD*) was measured at the wavelength of 570 nm with a reference wavelength of 630nm. The results of the MTT assay were recorded as inhibition of cell proliferation (%) calculated from a formula = (*OD* of the cells in treatment group/ *OD* of the cells in negative control group) ×100%. Each experiment was repeated at least three times and each point was determined in triplicate. MIBG inhibitory assays were performed to evaluate the inhibitory effects of MIBG on the anti-proliferative activity of compounds. Briefly, the cells were seeded in a 96-well plate (1×10^4 /0.1 ml/well) for 12 h, and then were treated with varied MIBG concentrations for 24 h in the presence of compounds at their IC_{50} concentrations, respectively. The anti-proliferative activity was evaluated by MTT assay as described above.

3. ^{64}Cu Radiolabeling of Compounds

$^{64}Cu(m\text{-}TSBG)_2$, $^{64}Cu(p\text{-}TSBG)_2$, $^{64}Cu(m\text{-}TSB)_2$ and $^{64}Cu(p\text{-}TSB)_2$ were prepared according to literature methods.[28-30] In brief, a 10 µl aliquot of m-TSBG, p-TSBG, m-TSB or p-TSB (1 mg/ml DMSO) was mixed with 150 µl of ^{64}Cu (1M NaOAc, pH 4.0) and vortexed for 30 min. The mixture was added to an ethanol-water preconditioned C-18 SepPak Light column. $^{64}Cu(m\text{-}TSBG)_2$, $^{64}Cu(p\text{-}TSBG)_2$, $^{64}Cu(m\text{-}TSB)_2$ or $^{64}Cu(p\text{-}TSB)_2$ was eluted off with 500 µl of ethanol after the initial 150 µl fraction. The radiochemical purity of compounds were determined by Instant Thin Layer Chromatography using ITLC ^{TM} SG Impregnated Glass Fiber Sheets (Pall Life Sciences, Ann Arbor, MI) as the stationary phase and MeOH/10% NaOAc as the eluent, found to exceed 99%. Through saline dilution, final concentration of $^{64}Cu(m\text{-}TSBG)_2$, $^{64}Cu(p\text{-}TSBG)_2$, $^{64}Cu(m\text{-}TSB)_2$ or $^{64}Cu(p\text{-}TSB)_2$ was provided with 0.5

μCi/μl.

4. Cellular Radioactivity Assay

To test cellular uptake of $^{64}Cu(m\text{-}TSBG)_2$, $^{64}Cu(p\text{-}TSBG)_2$, $^{64}Cu(m\text{-}TSB)_2$ and $^{64}Cu(p\text{-}TSB)_2$, NB cells (BE2C, SK-N-DZ) and non-NB cells (U87 and PC-3 cells) were seeded in 6-well plates (5×10^5 /2 ml/well), respectively. After incubated for 12 h, cells were treated with $^{64}Cu(m\text{-}TSBG)_2$, $^{64}Cu(p\text{-}TSBG)_2$, $^{64}Cu(m\text{-}TSB)_2$ or $^{64}Cu(p\text{-}TSB)_2$ at 10 μCi/mL for 3 h. After treatment, cells were washed with PBS for 3 times, then harvested and counted in a γ-counter (Packard). MIBG blocking assays were performed to evaluate the inhibitory effects of MIBG on cellular uptake of these radioactive compounds. Cells were treated with ^{64}Cu-labeled compounds (10 μCi/mL) for 3 h in the presence of MIBG (20 ng/mL). After treatment, cells were washed with PBS for 3 times, then harvested and counted in a γ-counter (Packard).

5. Statistical analysis

A series of two-factor analysis of variance (ANOVA) models were employed to examine differences in mean values (IC50 or cellular uptake) between study groups by concentration and cell types. Statistically significant differences were considered achieved at a P-value ≤0.05, two-tailed. All analyses were conducted using SPSS Version 15.

ASSOCIATED CONTENT

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Author Contributions

H.Z. synthesized and characterized chemical compounds, and performed radiolabeling and biological assays. F.X. performed biological assays. F.P. analyzed data of radiolabeling and biological assays. The manuscript was written through the contributions of all authors (H.Z., F.X., M.C., and F. Peng). All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CuHQTS, Copper complex of 8-hydroxyquinoline-2-carboxaldehyde-thiosemicarbazide; $Cu(m\text{-}TSB)_2$, 3-(hydrazinecarbothioamide)-1-benzyl-copper (II) chlorate; $Cu(p\text{-}TSB)_2$, 4-(hydrazinecarbothioamide)-1-benzyl-copper (II) chlorate; $Cu(m\text{-}TSBG)_2$, 3-carbaldehyde-thiosemicarbazide-1-guanidinomethyl-benzyl-copper (II) chlorate; $Cu(p\text{-}TSBG)_2$, 4-carbaldehyde-thiosemicarbazide-1-guanidinomethyl-benzyl-copper (II) chlorate; DIAD, diisopropylazodicarboxylate; MIBG, Meta-iodobenzylguanidine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; $NaBH_4$, sodium borohydride; NB, Neuroblastoma; NE, norephinephrine; NET, norephinephrine transporter; TPP, triphenylphosphine.

ACKNOWLEDGEMENT

This study was partly funded by a faculty research development fund awarded to F. P. by

Carman & Ann Adams Department of Pediatrics, School of Medicine, Wayne State University, Detroit, MI 48201, United States and a faculty research development fund awarded to F. P. by Harold C. Simmons Comprehensive Cancer Center, University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States.

Supporting Information Availability

1. **Schemes** for synthesis of Cu(m-TSBG)$_2$, Cu(p-TSBG)$_2$, Cu(m-TSB)$_2$ and Cu(p-TSB)$_2$ and characterization. Scheme 1, Synthesis of Cu(m-TSBG)$_2$; Scheme 2, Synthesis of Cu(p-TSBG)$_2$; and Scheme 3, Synthesis of Cu(m-TSB)$_2$
2. **Table S1**. IC_{50} values of copper complexes, ligands, and MIBG in BE2C, SK-N-DZ, U87 and PC-3 cell lines.
3. Microscopic examination for cytotoxicity on NB cells (**Figure S1**)
4. Cellular uptake of radiolabeled compounds (**Figure S2**)
5. Molecular Formula Strings (CSV) is available in supporting information

REFERENCES

1. Olshan A. F, Bunin G. R (2000) In: Brodeur GM, Sawada T, Tsuchida Y, Voute PA (eds) Neuroblastoma, Elsevier, New York, p33–37
2. Maris J. M, Recent Advances in Neuroblastoma, N. Engl. J. Med., 2010, 362, 2202-2211.
3. Raji M. A, Management of chemotherapy-induced side-effects, Lancet Oncol., 2005, 6, 357.
4. Staff N.P., Grisold A, Grisold W. and Windebank A.J. Chemotherapy-induced

peripheral neuropathy: A current review. Ann. Neurol., 2017, 81, 772-781.

5. Ahmad S.S, Reinius M. A, Hatcher H. M. and Ajithkumar T. V. Anticancer chemotherapy in teenagers and young adults: managing long term side effects, The BMJ, 2016, 354, i4567.

6. Chong P.Y, Shotwell J.B, Miller J, Price D.J, Maynard A, Voitenleitner C, Mathis A, Williams S, Pouliot J.J, Creech K, Wang F, Fang J, Zhang H, Tai VW, Turner E, Kahler K.M, Crosby R, Peat A.J. Design of N-Benzoxaborole Benzofuran GSK8175-Optimization of Human Pharmacokinetics Inspired by Metabolites of a Failed Clinical HCV Inhibitor. J. Med. Chem. 2019, 62, 3254-3267.

7. Zmuda F, Blair A, Liuzzi MC, Malviya G, Chalmers AJ, Lewis D, Sutherland A, Pimlott SL. An ^{18}F-Labeled Poly(ADP-ribose) Polymerase Positron Emission Tomography Imaging Agent. J. Med. Chem., 2018, 61, 4103 - 4114.

8. Vellaisamy K, Li G, Wang W, Leung CH, Ma DL. A long-lived peptide-conjugated iridium(iii) complex as a luminescent probe and inhibitor of the cell migration mediator, formyl peptide receptor 2. Chem. Sci., 2018, 9, 8171-8177.

9. Yang G.J, Wang W, Mok S.W.F, Wu C, Law B.Y.K, Miao X.M, Wu K.J, Zhong H.J, Wong C.Y, Wong V.K.W, Ma D.L, Leung C.H. Selective Inhibition of Lysine-Specific Demethylase 5A (KDM5A) Using a Rhodium(III) Complex for Triple-Negative Breast Cancer Therapy. Angew Chem. Int. Ed. Engl., 2018, 57, 13091 - 13095.

10. Coverdale JPC, Bridgewater HE, Song JI, Smith NA, Barry NPE, Bagley I, Sadler PJ, Romero-Canelón I. In Vivo Selectivity and Localization of Reactive Oxygen Species (ROS) Induction by Osmium Anticancer Complexes That Circumvent Platinum

Resistance. J. Med. Chem., 2018, 61, 9246 - 9255.

11. Wu C, Wu KJ , Liu JB , Zhou XM , Leung CH , Ma DL . A dual-functional molecular strategy for in situ suppressing and visualizing of neuraminidase in aqueous solution using iridium(iii) complexes. Chem. Commun (Camb), 2019, 55, 6353 - 6356.
12. Liu C, Zhang R, Zhang W, Liu J, Wang YL, Du Z, Song B, Xu ZP, Yuan J. "Dual-Key-and-Lock" Ruthenium Complex Probe for Lysosomal Formaldehyde in Cancer Cells and Tumors. J. Am. Chem. Soc. 2019, 141, 8462 - 8472.
13. Santini C, Pellei M, Gandin V, Porchia M, Tisato F. and Marzano C. Advances in Copper Complexes as Anticancer Agents, Chem. Rev., 2014, 114, 815-862.
14. Zhang H, Thomas R, Oupicky D. and Peng F. Synthesis and characterization of new copper thiosemicarbazone complexes with an onns quadridentate system: Cell growth inhibition, s-phase cell cycle arrest and proapoptotic activities on cisplatin-resistant neuroblastoma cells, J. Biol. Inorg. Chem., 2008, 13, 47-55
15. Xie F. and Peng F. Anti-Prostate Cancer Activity of 8-Hydroxyquinoline-2-Carboxaldehyde-Thiosemicarbazide Copper Complexes by Fluorescent Microscopic Imaging. J. Fluoresc., 2017, 27, 1937–1941
16. Xie F. Cai H. and Peng F. Anti-prostate cancer activity of 8-hydroxyquinoline-2-carboxaldehyde-thiosemicarbazide copper complexes in vivo by bioluminescence imaging, J. Biol. Inorg. Chem., 2018, 23, 949-956.
17. Jaques S, Tobes M.C, Sisson J. C, Baker J. A and Wieland D. M. Comparison of the sodium dependency of uptake of meta-Iodobenzylguanidine and norepinephrine into cultured bovine adrenomedullary cells. Mol. Pharmacol., 1984, 26, 539-546.

18. Streby K. A, Shah N, Ranalli M. A, Kunkler A. and Cripe T.P. Nothing but NET: a review of norepinephrine transporter expression and efficacy of 131I-mIBG therapy., Pediatr. Blood Cancer, 2015, 62, 5-11.

19. Vaidyanathan G, Shankar S, Affleck D. J, Welsh P. C, Slade S. K. and Zalutsky M. R. Biological evaluation of ring- and side-chain-substituted m-iodobenzylguanidine analogues., Bioconjug. Chem., 2001, 12, 798-806

20. Vaidyanathan G, Shankar S. and Zalutsky M. R. Synthesis of ring- and side-chain-substituted m-iodobenzylguanidine analogues. Bioconjug. Chem., 2001, 12, 786-797.

21. Wieland D. M, Wu J, Brown L. E, Mangner T. J, Swanson D. P. and Beierwaltes W. H. Radiolabeled adrenergi neuron-blocking agents: adrenomedullary imaging with [131I]iodobenzylguanidine., J. Nucl. Med.,1980, 21, 349-353.

22. Vaidyanathan G. Meta-iodobenzylguanidine and analogues: chemistry and biology., Q. J. Nucl. Med. Mol. Im., 2008, 52, 351-368.

23. DuBois S. G, Allen S, Bent M, Hilton J. F, Hollinger F, Hawkins R, Courtier J, Mosse Y. P, and Matthay K. K. Phase I/II study of (131)I-MIBG with vincristine and 5 days of irinotecan for advanced neuroblastoma. Br. J. Cancer, 2015, 112, 644-649.

24. Zhang H, Huang R, Cheung N. K, Guo H, Zanzonico P.B, Thaler H. T, Lewis J. S, and Blasberg R. G. Imaging the norepinephrine transporter in neuroblastoma: a comparison of [^{18}F]-MFBG and 123I-MIBG. Clin. Cancer Res., 2014, 20, 2182-2191.

25. Han G, Tamaki M, and Hruby V. J. Fast, efficient and selective deprotection of the tert-butoxycarbonyl (Boc) group using HCl/dioxane (4 m). J. Pept. Res., 2001, 58,

338-341.

26. Toyota E, Sekizaki H, Itoh K. and Tanizawa K. Synthesis and evaluation of guanidine-containing Schiff base copper(II), zinc(II), and iron(III) chelates as trypsin inhibitors., Chem. Pharm. Bull., 2003, 51, 625-629.

27. Lewis J, Laforest R, Buettner T, Song S, Fujibayashi Y, Connett J. and Welch M. Cu-64-ATSM as a radiotherapeutic agent: Extended survival in gw39 tumor-rearing hamsters, Proc. Natl. Acad. Sci. USA, 2001, 98, 1206-1211.

28. Peng F, Lu X, Janisse J, Muzik O, Shields AF. PET of human prostate cancer xenografts in mice with increased uptake of 64CuCl2. J. Nucl. Med., 2006, 47, 1649 - 1652.

29. Cai H, Wu JS, Muzik O, Hsieh JT, Lee RJ, Peng F. Reduced 64Cu uptake and tumor growth inhibition by knockdown of human copper transporter 1 in xenograft mouse model of prostate cancer. J. Nucl. Med, 2014, 55, 622 - 628.

30. Ferrari C, Asabella AN, Villano C, Giacobbi B, Coccetti D, Panichelli P, Rubini G. Copper-64 Dichloride as Theranostic Agent for Glioblastoma Multiforme: A Preclinical Study. Biomed. Res. Int., 2015, 129764.

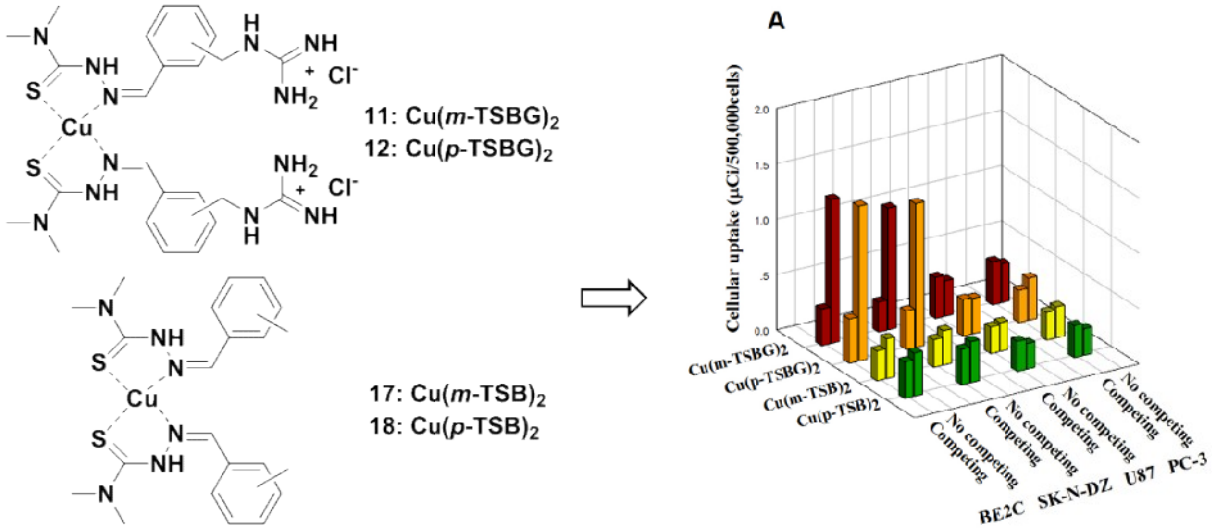
31. Panichelli P, Villano C, Cistaro A, Bruno A, Barbato F, Piccardo A, Duatti A. Imaging of Brain Tumors with Copper-64 Chloride: Early Experience and Results. Cancer Biother. Radiopharm., 2016, 31, 159 - 167.

32. Parmar A, Pascali G, Voli F, Lerra L, Yee E, Ahmed-Cox A, Kimpton K, Cirillo G, Arthur A, Zahra D, Rahardjo G, Liu GJ, Lengkeek N, Saletta F, Charil A, Kavallaris M, Vittorio O. In vivo [64Cu]CuCl2 PET imaging reveals activity of Dextran-Catechin on

tumor copper homeostasis. Theranostics. 2018, 8, 5645 - 5659.

33. Shakya R, Peng F, Liu J, Heeg M. J. and Verani C. N. Synthesis, structure, and anticancer activity of gallium(iii) complexes with asymmetric tridentate ligands: Growth inhibition and apoptosis induction of cisplatin-resistant neuroblastoma cells, Inorg. Chem., 2006, 45, 6263 - 6268.

Table of Contents Graphic



NB-specificity of novel Cu(m-TSBG)$_2$ and Cu(p-TSBG)$_2$ anticancer copper complexes demonstrated by differential cellular uptake of radioactive ^{64}Cu(m-TSBG)$_2$, ^{64}Cu(p-TSBG)$_2$, ^{64}Cu(m-TSB)$_2$ and ^{64}Cu(p-TSB)$_2$ compounds by NB cells (BE2C, SK-N-DZ) and non-NB cells (U87 and PC-3) with or without competitive blockage of MIBG binding to NET.