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Evaluation of ^{64}Cu -Labeled Acridinium Cation: A PET Radiotracer Targeting Tumor Mitochondria

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Abstract

This report presents the synthesis and evaluations of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ ($\text{DO3A-xy-ACR} = 2,6\text{-bis(dimethylamino)-10-(4-((4,7,10\text{-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl)acridin-10-ium})$) as a radiotracer for imaging tumors in athymic nude mice bearing U87MG glioma xenografts by PET (positron emission tomography). The biodistribution data suggested that $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ was excreted mainly through the renal system with >65% of injected radioactivity being recovered from urine samples at 1 h post-injection (p.i.). The tumor uptake of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ was 1.07 ± 0.23 , 1.58 ± 0.55 , 2.71 ± 0.66 , 3.47 ± 1.19 , and 3.52 ± 1.72 %ID/g at 0.5, 1, 2, 4 and 24 h p.i., respectively. $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ had very high liver uptake (31.90 ± 3.98 , 24.95 ± 5.64 , 15.20 ± 4.29 , 14.09 ± 6.82 , and 8.18 ± 1.27 %ID/g at 0.5, 1, 2, 4 and 24 h p.i., respectively) with low tumor/liver ratios. MicroPET studies showed that the tumors were clearly visualized as early as 30 min p.i. in the glioma-bearing mouse administered with $^{64}\text{Cu}(\text{DO3A-xy-ACR})$. The high liver radioactivity accumulation was also seen. $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ had a relatively high metabolic stability during excretion via both renal and hepatobiliary routes; but it was completely decomposed in the liver homogenate. We explored the localization mechanism of $\text{Cu}(\text{DO3A-xy-ACR})$ using both U87MG human glioma and the cultured primary U87MG glioma cells. The results from the cellular staining assays showed that $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ is able to localize in the mitochondria of living U87MG glioma cells due to the enhanced negative mitochondrial potential as compared to normal cells. Although $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ is not an ideal PET radiotracer for tumor imaging due to its high liver uptake, the results from this study strongly suggest that ^{64}Cu -labeled acridinium cations are indeed able to localize in the energized mitochondria of tumor cells.

Keywords

^{64}Cu PET radiotracers; mitochondrial potential and tumor imaging

INTRODUCTION

Alteration in the mitochondrial potential ($\Delta\Psi_m$) is an important characteristic of cancer. It has been reported that the mitochondrial potential in carcinoma cells is significantly higher than that in normal epithelial cells (1–5). Since the mitochondrial potentials are negative, the delocalized organic cations with appropriate structures tend to accumulate inside the

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energized mitochondria of carcinoma cells (5). For example, rhodamine-123 and ^3H -tetraphenylphosphonium (^3H -TPP) have been used to measure mitochondrial potentials due to their preferential localization in tumor cells (6–13). The observation that the enhanced mitochondrial potential is prevalent in tumor cell phenotype provides the conceptual basis to develop mitochondrion-targeting anticancer drugs and molecule imaging probes (14–16).

We have been interested in ^{64}Cu -labeled phosphonium cations as PET (positron emission tomography) radiotracers for tumor imaging (17–20). Biodistribution studies in the athymic nude mice bearing U87MG glioma xenografts showed that $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$ (Figure 1: $\text{DO3Axy-TPEP} = 2\text{-(diphenylphosphoryl)ethyl)diphenyl(4-((4,7,10\text{-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl)phosphonium})$ had a good tumor uptake with better tumor-to-background (T/B) ratios than those of $^{99\text{m}}\text{Tc}$ -Sestamibi (19). Although *in vitro* assays indicated that ^{64}Cu radiotracers were able to localize in the tumor mitochondria, the evidence was not conclusive (17). That has led us to explore other alternatives with the fluorescent emission so that their mitochondrial localization can be visualized.

Acridines are used as duplex DNA intercalators or “chemiluminescent agents” in many bioassays (21). Recently, it was reported that the $\text{Re}(\text{CO})_3$ complex $[\text{Re}(3,6\text{-bis(dimethylamino)-10-(4-isocyanobutyl)acridinium})(2,4\text{-pyridinedicarboxylato})(\text{CO})_3]^+$ ($[\text{Re}(\text{CN-C4-ACR})(2,4\text{-pydic})(\text{CO})_3]^+$) was able to localize in the nuclei of tumor cells by binding to duplex and triplex DNAs (22). Although the *in vivo* data were not reported, these promising results from *in vitro* assays have inspired us to prepare the complex $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ (Figure 1: $\text{DO3A-xy-ACR} = 2,6\text{-bis(dimethylamino)-10-(4-((4,7,10\text{-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl)acridin-10-ium})$. In this report, we present evaluation of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ as a potential PET radiotracer for tumor imaging in athymic nude mice bearing U87MG glioma xenografts. We were interested in this tumor-bearing animal model because the xenografted U87MG glioma tissues have low expression of multidrug resistance P-glycoproteins (MDR Pgps) and multidrug resistance-associated proteins (MRPs) (20, 23–26). $\text{Cu}(\text{DO3A-xy-ACR})$ was also prepared as a fluorescent probe for the cellular staining assay to demonstrate the intracellular location of ^{64}Cu -labeled ACR cations in tumor cells.

EXPERIMENTAL SECTION

Materials and Instruments

Acridine orange (ACR) and α,α' -dibromo-p-xylene were purchased from *Sigma/Aldrich* (St. Louis, MO). $\text{DO3A}(\text{O}Bu\text{-}t)_3$ (1,4,7,10-tetraazacyclododecane-4,7,10-tris(*tert*-butyl acetate)) was obtained from Macrocyclics Inc. (Dallas, TX). NMR data were obtained using Bruker ARX 300 MHz FT NMR spectrometer. Chemical shifts are reported as δ in ppm relative to TMS. The ESI (electrospray ionization) mass spectral data were collected on a Finnigan LCQ classic mass spectrometer, School of Pharmacy, Purdue University. ^{64}Cu was produced using a CS-15 biomedical cyclotron at Washington University, School of Medicine by the $^{64}\text{Ni}(\text{p,n})^{64}\text{Cu}$ nuclear reaction.

HPLC Methods

The semi-prep HPLC method (Method 1) used a LabAlliance system equipped with a UV/vis detector ($\lambda=254\text{ nm}$) and Zorbax C_{18} semi-prep column ($9.4\text{ mm} \times 250\text{ mm}$, $100\text{ \AA}$ pore size; Agilent Technologies, Santa Clara, CA). The flow rate was 2.5 mL/min and the mobile phase was isocratic with 60% A (0.1% TFA in water) and 40% B (0.1% TFA in methanol) at 0–5 min, followed by a gradient mobile phase going from 40% B at 5 min to 100% B at 20 min. The radio-HPLC method (Method 2) used the LabAlliance system equipped with a β -ram IN/US detector (Tampa, FL) and Vydac protein and peptide C_{18} column ($4.6\text{ mm} \times$

250 mm, 300 Å pore size; Grace Davison Discovery Sciences, Hesperia, CA). The flow rate was 1 mL/min with the gradient mobile phase being isocratic with 90% solvent A (25 mM NH₄OAc, pH = 6.8) and 10% solvent B (acetonitrile) at 0 – 5 min, followed by a gradient mobile phase going from 10% B at 5 min to 90% B at 20 min. Method 3 used the LabAlliance HPLC system equipped with a UV/vis detector (λ = 254 nm), a β -ram IN-US detector and a Superose™ 12 10/300 GL size-exclusion column. The flow rate was 0.4 mL/min. The aqueous mobile phase was isocratic with 20 mM HEPES and 150 mM NaCl.

10-(4-(Bromomethyl)benzyl)-3,6-bis(dimethylamino)acridin-10-ium Bromide (ACR-xy-Br)

Acridine orange base (737 mg, 2.08 mmol) and α,α' -dibromo-p-xylene (500 mg, 1.89 mmol) were dissolved in 30 mL of toluene. The resulting mixture was refluxed for 20 h. After cooling to room temperature, the solid was filtered, and washed with toluene (5 mL) and ether (5 mL) three times, respectively, and then dried under vacuum overnight. The yield was 580 mg (~70%). ¹H NMR (in CDCl₃): 3.17 (s, 12H, NCH₃), 4.42 (s, 2H, CH₂Br), 6.25 (s, 2H, NCH₂), 6.66 (s, 2H, aromatic C₆H₃), 6.95 (d, 2H, aromatic C₆H₃), 7.27–7.40 (m, 4H, aromatic C₆H₄), 7.89 (d, 2H, aromatic C₆H₃), 8.27 (s, H, aromatic NC₅H₁).

2,6-Bis(dimethylamino)-10-(4-((4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl)acridin-10-ium (DO3A-xy-ACR)

DO3A(OBu-t)₃ (22.8 mg, 44 μ mol) and ACR-xy-Br (23.5 mg, 44 μ mol) were dissolved in DMF (3 mL). To the mixture was added triethylamine (35 μ L, 250 μ mol). The reaction mixture was stirred at room temperature overnight. Upon removal of volatiles, the residue was dissolved in 3 mL of 12N HCl. After being stirred at room temperature for 2 h, volatiles were completely removed. The residue was dissolved in 2 mL of 50% DMF/water mixture, and the solution was subjected to HPLC purification (Method 1). Fraction at 14.4 min was collected and lyophilized to give orange powder (19.7 mg, 63%). ESI-MS: m/z = 714.3 for [M + H]⁺ (714.4 calcd. for [C₃₉H₅₂N₇O₆]⁺).

Cu(DO3A-xy-ACR)

To a clean vial containing DO3A-xy-ACR (16.9 mg, 22 μ mol) and Cu(OAc)₂·H₂O (9.2 mg, 45 μ mol) was added 2.5 mL of NH₄OAc (0.5 M, pH = 5.5). The resulting solution was heated at 100 °C water bath for 30 min. After cooling it to room temperature, the product was isolated by HPLC (Method 1). Fraction 14 min was collected and lyophilized to give orange powder. The yield was 12.8 mg (75%). ESI-MS: m/z = 775.1 for [M + H]⁺ (775.3 calcd. for [C₃₉H₄₉CuN₇O₆]⁺). Anal. Calcd. for C₃₉H₄₉CuN₇O₆·2.5H₂O: C, 57.09; H, 6.63; N, 11.95. Found: C, 56.94; H, 6.71; N, 11.70.

⁶⁴Cu-Labeling and Dose Preparation

To a clean Eppendorf tube was added 40 μ g of DO3A-xy-ACR dissolved in 0.3 mL of 0.1 M NaOAc buffer (pH = 6.5) and 20 μ L of ⁶⁴CuCl₂ solution (~ 500 μ Ci) in 0.05 N HCl. The reaction mixture was then heated at 100 °C for 20 min in a water bath. After heating, the vial was allowed to stand at room temperature for ~5 min. The radiochemical purity was > 95%. For biodistribution studies, ⁶⁴Cu(DO3A-xy-ACR) was prepared and purified by HPLC (Method 2). Volatiles in the HPLC mobile phase were removed on a rotary evaporator. Doses were prepared by dissolving the purified ⁶⁴Cu(DO3A-xy-ACR) in saline to ~30 μ Ci/mL. The resulting solution was filtered with a 0.20 micron Millex-LG filter before being injected into animals. For the imaging study, ⁶⁴Cu(DO3A-xy-ACR) was prepared and the reaction mixture was then diluted to ~5 mCi/mL with saline. The log P value of ⁶⁴Cu(DO3A-xy-ACR) was determined according to the literature procedure (19).

Animal Model

Biodistribution studies were performed using athymic nude mice bearing U87MG human glioma xenografts in compliance with the NIH animal experiment guidelines (*Principles of Laboratory Animal Care*, NIH Publication No. 86-23, revised 1985). The animal protocol was approved by the Purdue University Animal Care and Use Committee (PACUC). U87MG cells were cultured in the Minimum Essential Medium, Eagle with Earle's Balanced Salt Solution (non-essential amino acids sodium pyruvate) (ATCC, Manassas, VA) in humidified atmosphere of 5% CO₂, and were supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin solution (GIBCO, Grand Island, NY). Female athymic nu/nu mice (5 – 6 weeks) were purchased from Harlan (Indianapolis, IN). Each mouse was implanted with 5×10^6 tumor cells subcutaneously into the left and right upper shoulder flanks. Four weeks after inoculation, the tumor size was 0.1 – 0.4 g, and animals were used for biodistribution studies.

Biodistribution Protocol

Twenty five tumor-bearing mice (20 – 25 g) were randomly divided into five groups. Each animal was administered with $\sim 3 \mu\text{Ci}$ of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ by tail vein injection. Five animals were sacrificed by sodium pentobarbital overdose ($\sim 200 \text{ mg/kg}$) at 0.5, 1, 2, 4 and 24 h p.i. Blood samples were withdrawn from the heart of tumor-bearing mice. The tumor, brain, eyes, heart, spleen, lungs, liver, kidneys, muscle and intestine were harvested, dried with absorbent tissue, weighed, and counted in a γ -counter (Perkin Elmer Wizard – 1480, Shelton, CT). The organ uptake was calculated as %ID/g and %ID/organ. Biodistribution data (%ID/g) and T/B ratios are reported as an average with the standard deviation based on results from 5 tumor-bearing mice (10 tumors) at each time point. Comparison between two radiotracers was made using the two-way ANOVA test (GraphPad Prim 5.0, San Diego, CA). The level of significance was set at $p < 0.05$.

MicroPET

MicroPET scans were performed using an Inveon DPET scanner (Siemens Medical Solutions). The tumor-bearing nude mice ($n = 3$) were imaged in the prone position in the microPET scanner. Each mouse was injected with $\sim 250 \mu\text{Ci}$ of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ via the tail vein, then anesthetized with 2% isoflurane and placed near the center of the FOV. Multiple static scans were obtained at 0.5, 1, 2, 4 and 24 h p.i. The images were reconstructed by a three-dimensional ordered subsets expectation maximum (3D-OSEM) algorithm, and no correction was applied for attenuation or scatter. Image analysis was done using ASI Pro VMTM software.

Metabolic Stability

Normal athymic nude mice were used to study the metabolic stability of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$. Each mouse was administered with $\sim 100 \mu\text{Ci}$ $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ via tail vein. Urine samples were collected at 30 min and 120 min p.i. by manual void, and were mixed with equal volume of 50% acetonitrile aqueous solution. The mixture was centrifuged at 8,000 rpm. The supernatant was collected and passed through a $0.20 \mu\text{m}$ Millex-LG filter. The filtrate was analyzed by radio-HPLC (Method 2). Feces samples were collected at 120 min p.i. and suspended in 50% acetonitrile aqueous solution. The mixture was vortexed for 5 – 10 min. After centrifuging at 8,000 rpm, the supernatant was collected and passed through a $0.20 \mu\text{m}$ Millex-LG filter unit. The filtrate was analyzed by radio-HPLC (Method 2). The percentage radioactivity recovery was $>90\%$ for both urine and feces samples. The liver tissue was harvested at 120 min p.i., counted in a γ -counter, cut into small pieces, and then homogenized. The homogenate was mixed with 2 mL of 50% acetonitrile aqueous solution. The mixture was vortexed for ~ 5 min. After centrifuging at 8000 rpm for 5 min, the

supernatant was collected, and counted in a γ -counter. The radioactivity recovery was ~35% from the liver homogenate. After filtration through a 0.20 μm Miley-LG filter unit to remove the precipitate and particles, the filtrate was then analyzed by radio-HPLC (Method 2).

Cellular Culture

All cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). U87MG human glioma cells were cultured in the Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS, ATCC) and 1% penicillin and streptomycin (GIBCO) solution. Human fibroblasts were cultured in fibroblast dermal media supplemented with low serum growth kit (ATCC, including 7.5 mM L-glutamine, 5 ng/ml rh FGF β , 5 $\mu\text{g/ml}$ insulin, 1 $\mu\text{g/ml}$ Hydrocortisone, 50 $\mu\text{g/ml}$ Ascorbic acid and 2% serum). The primary U87MG glioma cells were extracted from xenografted U87MG tumors. Briefly, tumor tissues were cut and immersed immediately in completed Minimum Essential Medium (ATCC) media. The tumor tissues were rinsed twice with the Hanks' solution and dissected into small pieces with razor blades. Small tumor pieces were digested with 0.25% Trypsin (1 mM EDTA, without Ca^{2+} and Mg^{2+}) solution at 37 $^{\circ}\text{C}$ for 30 min, followed by vigorous pipetting and filtration through a 40 μm mesh nylon screen. After centrifugation at 1000 rpm for 5 min, the pellet was re-suspended in the culture media. All cells were grown at 37 $^{\circ}\text{C}$ in a humidified atmosphere containing 5% CO_2 .

Cellular Localization Study

Cells were allowed to culture in Lab-TekTM 8-well glass chamber slides for at least 24 h before being used. Cu(DO3A-xy-ACR) was added into the culture media to achieve a concentration of 20 μM . Hoechst 33342 was added directly into the media to achieve 1 μM at 5 min before completion of incubation with Cu(DO3A-xy-ACR). After incubation for the indicated time, the cells were washed three times with phenol red-free Minimum Essential Medium. Lissamine (10 μM) was incubated along with Cu(DO3A-xy-ACR) to determine the mitochondrial localization. The fluorescence was visualized immediately with an Olympic BX51 fluorescence microscope (Olympus America Inc., Center Valley, PA) under 400x magnification. Images were acquired using a Hamamatsu digital CCD camera ORCA-R² (Hamamatsu Photonics K.K., Japan) with Olympus MetaMorph software.

Supporting Information Available: The HPLC concordance (Figure SI1) for ⁶⁴Cu(DO3A-xy-ACR) and Cu(DO3A-xy-ACR), microscopic images (Figure SI2) of the U87MG human glioma cells stained with 20 μM of Cu(DO3A-xy-ACR), microscopic images (Figure SI3) of the cultured primary U87MG glioma cells stained with various concentration (1 – 100 μM) of Cu(DO3A-xy-ACR), and microscopic images (Figure SI4) of the cultured primary U87MG glioma cells stained with 20 μM of Cu(DO3A-xy-ACR) at different time points (1, 2, 4 and 24 h) are all in word document. These materials are available free of charge at <http://pubs.acs.org>.

RESULTS

DO3A-xy-ACR and Cu(DO3A-xy-ACR)

DO3A-xy-ACR was prepared in two steps. First, ACR-xy-Br was allowed to react with DOTA(OBu-t)₃ in the presence of excess triethylamine in DMF. The intermediate DO3A(OBu-t)₃-xy-ACR was then hydrolyzed to give DO3A-xy-ACR in ~40% overall yield. Cu(DO3A-xy-ACR) was prepared by reacting DO3A-xy-ACR with one equivalent of Cu(II) acetate in 0.5 M NH_4OAc (pH = 5.5). Since phosphonium and acridinium cations share very similar structures, it is reasonable to believe that Cu(DO3A-xy-ACR) will exist in

its Zwitterion form, which has been established for ^{64}Cu -labeled phosphonium cations (27). This statement is completely supported by the elemental analysis data.

Radiochemistry

$^{64}\text{Cu}(\text{DO3A-xy-ACR})$ was prepared by reacting DO3A-xy-ACR with $^{64}\text{CuCl}_2$ in 100 mM NH_4OAc buffer (pH = 5.0) at 100 °C for 10 min. The radiochemical purity was >95% without HPLC purification with the specific activity of ~50 Ci/mmol. It had the log P value of -2.0 ± 0.2 , which was more hydrophilic than $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$ (log P = -1.7 ± 0.1) (19). $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ was stable for >24 h in the presence of excess EDTA (3 mg/mL in 25 mM phosphate buffer, pH = 7.4). Since $\text{Cu}(\text{DO3A-xy-ACR})$ and $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ share almost identical HPLC retention time in the concordance experiment (Figure S11), it is reasonable to believe that they have the same chemical composition in solution.

Biodistribution Characteristics

Selected biodistribution data for $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ are listed in Table 1. Figure 2 compares the organ uptake (%ID/g) and T/B ratios between $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ and $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$ in the same glioma-bearing animal model. $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$ was used only for comparison purposes since it has the best tumor uptake and T/B ratios among the ^{64}Cu -labeled phosphonium cations evaluated under the same conditions (17–20). While its biodistribution data were obtained from our previous report (19), the biodistribution data of $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$ at 4 h p.i. were added in this study. In general, $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ was excreted mainly through the renal route with >65% of injected radioactivity being recovered from urine samples at 1 h p.i. The tumor uptake of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ was 1.07 ± 0.23 , 1.58 ± 0.55 , 2.71 ± 0.66 , 3.47 ± 1.19 , and 3.52 ± 1.72 %ID/g at 0.5, 1, 2, 4 and 24 h p.i., respectively. The tumor uptake of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ was significantly ($p < 0.05$) lower than that of $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$ at <1 h p.i., but this difference disappeared at 2–4 h p.i. The liver uptake of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ was very high (31.90 ± 3.98 , 24.95 ± 5.64 , 15.20 ± 4.29 , 14.09 ± 6.82 , and 8.18 ± 1.27 %ID/g at 0.5, 1, 2, 4 and 24 h p.i., respectively). As a result it had very low tumor/liver ratios. The tumor/heart ratios (Figure 2) of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ were comparable to those of $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$; but it had better tumor/lung ratios (Figure 2) than $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$.

MicroPET

Figure 3 shows microPET images of a glioma-bearing mouse administered with ~250 μCi of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ at 0.5, 1, 2, 4 and 24 h p.i. The tumor was clearly visualized with excellent T/B contrast as early as 30 min p.i. High liver uptake was seen at all time points. After normalization, the tumor uptake of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ was calculated to be 0.8 ± 0.1 , 1.0 ± 0.2 , 1.4 ± 0.3 , 2.27 ± 0.8 , and 3.40 ± 0.25 %ID/g at 0.5, 1, 2, 4 and 24 h p.i., respectively. These data were completely consistent with that observed in the biodistribution study (Figure 2).

Metabolic Stability

Figure 4 shows radio-HPLC chromatograms of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ in the urine at 30 min (A) and 120 min p.i. (B), in the feces at 120 min p.i. (C) and in the liver homogenate at 120 min p.i. (D). In general, $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ was stable in the urine sample over 2 h study period while ~80 % of it remained intact in the feces sample. We also found that only ~35 % of the liver radioactivity was recovered from the liver homogenate. The HPLC analysis showed only one radiometric peak at 4 min (Figure 4: D) with no intact $^{64}\text{Cu}(\text{DO3A-xy-ACR})$, and strongly suggested that $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ underwent complete transchelation in the liver during the 2 h study period.

Localization in Mitochondria

Figure 5 shows microscopic images of the cultured primary U87MG glioma and normal fibroblast cells stained with 20 μ M of Cu(DO3A-xy-ACR). The human fibroblasts were used as the “negative control” to demonstrate the tumor selectivity of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$. Lissamine was used as the “positive control” since it is a well-known mitochondrion-targeting agent. Nuclear staining was achieved with Hoechst 33342 (1 μ M). The orange color in the overlay image indicated that Cu(DO3A-xy-ACR) was able to co-localize with Lissamine in the tumor mitochondria. It was clear that Cu(DO3A-xy-ACR) (Figure 5A) localize mainly in the mitochondria of U87MG glioma cells. There was very little localization of Cu(DO3A-xy-ACR) in the nuclei of tumor cells. Similar results were also obtained with U87MG human glioma cells (Figure SI2). Although Cu(DO3A-xy-ACR) can localize in mitochondria of normal human fibroblasts, the fluorescent intensity was too low to be seen clearly using the same brightness and contrast as those for U87MG glioma cells. It is very interesting to note that the mitochondrial fluorescent intensity was dependent on the concentration (Figure SI3) and the incubation time (Figure SI4) of Cu(DO3A-xy-ACR). After 1 h incubation with 20 μ M of Cu(DO3A-xy-ACR), the fluorescence was weak while the fluorescence in the U87MG glioma cells became much more intense after 24 h incubation (Figure SI4).

$[\text{Re}(\text{CN-bn-ACR})(2,4\text{-pydic})(\text{CO})_3]^+$ was reported to localize in the tumor nuclei (22). Since $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ and $[\text{Re}(\text{CN-bn-ACR})(2,4\text{-pydic})(\text{CO})_3]^+$ share the same ACR cation, we decided to perform staining studies using both U87MG human glioma cells and the cultured primary U87MG glioma cells with $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ as a fluorescent probe. We found that if U87MG glioma cells were not fixed with 4% formalin, the fluorescent signal was concentrated inside tumor mitochondria (Figure 6: left). If U87MG glioma cells were fixed with 4% formalin, the majority of fluorescence was inside the tumor cell nucleus while the remaining fluorescence was evenly distributed inside cytoplasm of tumor cells (Figure 6: right). This distribution pattern was similar to that reported for $[\text{Re}(\text{CN-C4-ACR})(2,4\text{-pydic})(\text{CO})_3]^+$ in B16-F1 and PC-3 cells fixed with 4% formalin (22); but it was completely different from the “spotted” patterns in the tumor mitochondria without fixation (Figure 6: left). Obviously, the intracellular distribution patterns of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ depend largely on formalin fixation.

DISCUSSION

In this study, we found that $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ and $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$ shared similar tumor uptake (Figure 2) at 2 – 4 h p.i. Even though $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ ($\log P = -2.0 \pm 0.2$) is more hydrophilic than $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$ ($\log P = -1.7 \pm 0.1$) (19), its liver uptake was very high with much lower tumor/liver ratios than those of $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$, suggesting that the radiotracer liver uptake is not completely due to its lipophilicity. Because of its lower liver uptake, $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$ is a better PET radiotracer than $^{64}\text{Cu}(\text{DO3A-xy-ACR})$.

Another interesting finding of this study is that the targeting moiety has significant impact on metabolic stability of ^{64}Cu radiotracers. For example, $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$ has no metabolism during excretion from both renal and hepatobiliary routes (19). However, $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ has ~20% metabolism during hepatobiliary excretion (Figure 4C). We examined the liver stability of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$, and found that there was no intact $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ in liver homogenate (Figure 4D). Its HPLC profile is similar to that of $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$ in the liver homogenate (19). Since Anderson's group has clearly demonstrated the in vivo instability of ^{64}Cu -labeled tetraazamacrocycles (28–30), we believe that the observation of no intact $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ in the liver

homogenate is most likely caused by transchelation of ^{64}Cu from $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ to the proteins in the liver.

The mitochondrial localization of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ has been clearly demonstrated using $\text{Cu}(\text{DO3A-xy-ACR})$ as a fluorescent probe (Figure 5). Since $\text{Cu}(\text{DO3A-xy-ACR})$ can also localize in normal human fibroblasts with much less fluorescent intensity, the tumor selectivity of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ is caused by the increased negative mitochondrial potential in U87MG glioma cells as compared with that in normal fibroblasts. The time-dependence (Figure S14) of the mitochondrial fluorescent intensity for $\text{Cu}(\text{DO3A-xy-ACR})$ is consistent with the fact that $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ has a slow increase in the tumor uptake over the first 4 h p.i.

It must be noted that the cell fixation has a significant impact on the intracellular localization of fluorescent probes targeting mitochondria. For example, the fluorophore concentrates inside the tumor mitochondria with typical “spotted” patterns (Figure 6: left) if U87MG glioma cells are not fixed with 4% formalin. In the presence of formalin fixation, however, the intracellular distribution patterns (Figure 6: right) of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ are very similar to those of $[\text{Re}(\text{CN-bn-ACR})(2,4\text{-pydic})(\text{CO})_3]^+$ in B16-F1 and PC-3 cells (22). While there is no doubt that $[\text{Re}(\text{CN-bn-ACR})(2,4\text{-pydic})(\text{CO})_3]^+$ is able to bind to duplex and triplex DNAs in the in vitro binding assays (22), we believe that the nuclear localization (Figure 6: right) of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ is most likely the artifacts caused by formalin fixation. Similar fixation artifacts were reported with the cell-penetrating peptides (31–33). Cellular fixation with 3 – 4% formalin can cause significant damage in the mitochondrial membrane, which would result in the collapse of mitochondrial potential, and lead to the localization of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ inside cytoplasm and nuclei. Therefore, it is strongly recommended that living cells are used for studies related to the mitochondrial function.

CONCLUSION

In summary, we evaluated the potential of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ as PET radiotracer for tumor imaging. Although tumors could be clearly visualized in the athymic nude mice bearing U87MG human glioma xenografts, $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ is not as good a PET radiotracer as $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$ because of its much higher liver uptake. Even though it was reported that $[\text{Re}(\text{CN-C4-ACR})(2,4\text{-pydic})(\text{CO})_3]^+$ was able to localize in the nuclei of tumor cells by binding to the duplex and triplex DNAs (22), our results from cellular staining assays show that $\text{Cu}(\text{DO3A-xy-ACR})$ is localized in the mitochondria of living U87MG glioma cells. Due to the similarity between ACR and TPEP cations with respect to their structure and overall molecular charge (Figure 1), $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$ also targets the tumor mitochondria, where negative mitochondrial transmembrane potential is significantly elevated as compared with normal cells of the surrounding tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Proposed Chemdraw structures of $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$ and $^{64}\text{Cu}(\text{DO3A-xy-ACR})$. Like 2-(diphenylphosphoryl)ethylphenylphosphonium (TPEP), the acridinium (ACR) cation is used as the mitochondrion-targeting molecule to carry ^{64}Cu into tumor cells, where the negative mitochondrial transmembrane potential is elevated.

Figure 2. Comparison of the uptake (%ID/g) and T/B ratios for $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ and $^{64}\text{Cu}(\text{DO3A-xy-TPEP})$ in selected organs of the athymic nude mice bearing U87MG human glioma xenografts.

Figure 3.

The decay-corrected whole-body coronal microPET images of the athymic nude mice bearing U87MG glioma xenografts administered with $\sim 250 \mu\text{Ci}$ of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$. Arrows indicate the presence of glioma tumors.

Figure 4. Representative radio-HPLC chromatograms of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ in urine at 30 min (**A**) and 120 min p.i. (**B**), in feces at 120 min p.i. (**C**), and in the liver homogenate (**D**).

Figure 5.

Representative microscopic images (magnification: 400x) of the cultured primary U87MG glioma cells (**A**) and normal human fibroblast cells (**B**) stained with 20 μM of $\text{Cu}(\text{DO3A-xy-ACR})$ (green color). The nuclei were stained with 1 μM of Hoechst 33342 (blue color). Normal fibroblast cell line was used as the “negative control” to demonstrate the tumor selectivity of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$. The co-localization experiment was performed using U87MG glioma cells stained with 40 μM of $\text{Cu}(\text{DO3A-xy-ACR})$ (**C**) and Lissamine Rhodamine B (**D**; red color), which was used as the “positive control” for mitochondrial staining of tumor cells. The orange color in the overlay image (**E**) indicates that $\text{Cu}(\text{DO3A-xy-ACR})$ is indeed able to selectively localize in the mitochondria of U87MG tumor cells.

Figure 6. Microscopic images (magnification: 400x) of cultured primary U87MG (top) glioma cells and U87MG human cell line (bottom) stained with 40 μ M of Cu(DO3A-xy-ACR) for 1 h with (right) and without (left) formalin fixation. All images were adjusted with the same brightness and contrast.

Table 1

Selected biodistribution data of $^{64}\text{Cu}(\text{DO3A-xy-ACR})$ in the athymic nude mice ($n = 5$) bearing U87MG human glioma xenografts.

Organ (% ID/gram)	0.5 h	1 h	2 h	4 h	24 h
Blood	0.90 ± 0.22	0.60 ± 0.17	0.70 ± 0.08	0.70 ± 0.19	0.53 ± 0.05
Brain	0.04 ± 0.02	0.05 ± 0.01	0.06 ± 0.02	0.10 ± 0.03	0.13 ± 0.07
Heart	0.66 ± 0.25	0.66 ± 0.08	0.70 ± 0.11	0.89 ± 0.13	1.14 ± 0.33
Intestine	10.81 ± 5.99	8.05 ± 6.61	3.46 ± 0.69	2.69 ± 1.02	1.93 ± 0.17
Kidney	2.89 ± 0.90	2.53 ± 0.37	2.63 ± 0.42	2.18 ± 0.39	2.38 ± 0.41
Liver	31.90 ± 3.98	24.95 ± 5.64	15.20 ± 4.29	14.09 ± 6.82	8.18 ± 1.27
Lungs	1.63 ± 0.28	1.61 ± 0.39	1.75 ± 0.35	2.00 ± 0.75	1.83 ± 0.17
Muscle	0.29 ± 0.18	0.24 ± 0.11	0.20 ± 0.13	0.33 ± 0.06	0.34 ± 0.17
Spleen	0.97 ± 0.53	0.97 ± 0.46	0.79 ± 0.05	0.73 ± 0.17	1.58 ± 0.37
U87MG	1.07 ± 0.23	1.58 ± 0.55	2.71 ± 0.66	3.47 ± 1.19	3.52 ± 1.72
U87MG/Blood	1.23 ± 0.29	2.82 ± 1.04	3.96 ± 1.29	5.40 ± 3.66	8.76 ± 2.03
U87MG/Heart	1.79 ± 0.60	2.46 ± 0.98	3.99 ± 1.28	3.94 ± 2.17	3.28 ± 1.85
U87MG/Kidney	0.41 ± 0.16	0.61 ± 0.16	1.05 ± 0.29	1.63 ± 0.92	1.53 ± 0.83
U87MG/Liver	0.03 ± 0.01	0.07 ± 0.02	0.20 ± 0.09	0.31 ± 0.25	0.44 ± 0.25
U87MG/Lung	0.66 ± 0.10	0.97 ± 0.23	1.55 ± 0.26	1.89 ± 0.70	1.92 ± 0.87
U87MG/Muscle	4.90 ± 2.56	7.52 ± 3.91	17.82 ± 8.66	11.54 ± 8.06	12.26 ± 8.60