Original Article

Preliminary Imaging Studies of [^{61}Cu]diacetyl-bis (N_4-methylthiosemi-carbazone) in Normal and Hypoxic Tumor Models

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ABSTRACT

Introduction: [^{61}Cu]diacetyl-*bis*(N^4-methylthiosemicarbazone) ([^{61}Cu]ATSM) is a well-established hypoxia imaging tracer with simple production and significant specifity. In this work the accumulation of the tracer is studied in wild-type, necrotic and hypoxic fibrosarcoma tumors.

Methods: [^{61}Cu]ATSM was prepared using ATSM ligand and [^{61}Cu]CuOAc followed by i.v. administration and imaging studies in wild-type rats and hypoxic fibrosarcoma-bearing mice.

Results: [^{61}Cu]ATSM with high radiochemical purity (>99%, HPLC, RTLC) was injected to wild-type rats as well as hypoxic and necrotic fibrosarcoma-bearing mice followed by imaging up to 3 hours.

Conclusion: [^{61}Cu]ATSM was mainly accumulated in liver, as well as kidney and bladder and less but still significant in brain of wild-type rats. A significant and hypoxia-specific tumor/non tumor ratio in hypoxic models was observed by co-incidence imaging 2 h post injection, while in necrotic and 12-week tumor-induced mice very slight tumor uptakes were detected. [^{61}Cu]ATSM is a positron emission tomography (PET) radiotracer for selective tumor hypoxia imaging from necrotic and proliferative tumors.

Keywords: [^{61}Cu]ATSM, Copper radiopharmaceuticals, Hypoxia, Co-incidence imaging, Fibrosarcoma

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INTRODUCTION

Hypoxia is an important determinant for biological behavior of malignant solid tumors. *In vitro* and *in vivo* studies have shown that tumor hypoxia is associated with an increased likelihood of local recurrence and distant metastasis, as well as resistance to radiation therapy and certain types of chemotherapy (1).

Preclinical studies have shown that some copper-bis thiosemicarbazones especially, Cu-ATSM accumulates avidly in hypoxic cells, but washes out rapidly from normoxic cell. Copper-61 is a positron emitter ($t_{1/2}$=3.33 h, β^+: 62%, EC: 38%), with excellent potentials for application in PET method and molecular imaging (2).

Few production methods of copper-61 have been reported for radiolabeling of biomolecules and other applications (3). Interestingly, it has been shown that the tomographic images obtained using ^{61}Cu are superior to those using ^{64}Cu, based on the larger abundance of positrons emitted by ^{61}Cu compared with ^{64}Cu (62% *vs.* 18%) (4). As an example of copper-labeled radiopharmaceutical, Cu-ATSM has been used as a PET hypoxia tracer for oncologic/cardiologic studies (5, 6).

Based on the interesting properties of copper-61 and the possibility of its production *via* ^{nat}Zn(p,x)^{61}Cu, we have already reported the production method (7), and in continuation of this work we hereby report the imaging studies of [^{61}Cu]ATSM in normal and hypoxic tumor-bearing rodents.

METHODS

Production of ^{61}Cu was performed at the Agricultural, Medical and Industrial Research School (AMIRS, Karaj, Iran), 30 MeV cyclotron (Cyclone-30, IBA, Belgium). Natural zinc chloride with a high purity of more than 99% was provided commercially (Merck, Germany).

All chemicals were purchased from Sigma-Aldrich Chemical Co. (U.K.). Radio-chromatography was performed by counting of polymer-backed silica gel paper thin layer sheets using a thin layer chromatography scanner, Bioscan AR2000 (France). Analytical HPLC to determine the specific activity was performed by a Shimadzu LC-10AT (Japan), armed with two detector systems, flow scintillation analyzer (Packard-150 TR, USA) and UV-visible (Shimadzu, Japan) using Whatman Partisphere C-18 column 250 x 4.6 mm, Whatman Co. (USA). Eluent, H_2O:CH_3CN (1:1), FR=1 mL/min. All calculations and RTLC counting were based on 283 keV peak.

All values were expressed as mean ± standard deviation and the data were compared using Student T-test. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd edn. 1987. The approval of NSTRI ethical Committee was obtained for conducting this research.

Zinc targetry and bombardment

The target was a layer of natural zinc, electroplated on copper plate which was coated with a 50-µm gold layer to prevent interference of the backing copper during radiochemical separation.

Cross section calculations by ALICE nuclear code (8) showed that the best proton energy range for ^{nat}Zn(p,x)^{61}Cu reaction is 22-12 MeV. The target had to be thick enough to reduce the proton energy from 22 MeV to about 12 MeV. The targets were irradiated in a glancing angle of 6º to achieve higher production yield. Stopping and Range of Ions in Matter (SRIM) code (9) was run to determine the best target thickness in the energy range.

Gold and zinc electrodeposition

A gold containing bath was prepared according to ref. (10) with slight modifications. As the 6° glancing angle reduces the required target thickness by 10 fold, electroplating a 75-µm thick target is good enough. The target was irradiated by 22 MeV (150 µA) protons with for 76 minutes.

Chemical separation

Chemical separation was carried out in no-carrier-added form. The irradiated target was dissolved in 10 molL^{-1} HCl (15 mL, H_2O_2 added). The solution was passed through a cation exchange resin (AG 50W, H^+ form, mesh 200-400, 1.3 x 10 cm) which had been preconditioned by passing 25 mL of 9 molL^{-1} HCl. The column was then washed by 25 mL of 9 molL^{-1} HCl at a rate of 1 mLmin^{-1} to remove copper and zinc ion contents. To the eluent water (30 mL) was added to about 100 mL of a 6 molL^{-1} HCl solution. The latter solution was loaded on another exchange resin (AG1X8 Cl^- form, 100-200 mesh, 25x1.7 cm) pretreated with 6 molL^{-1} HCl (100 mL).

Finally, ^{61}Cu was eluted using 2 molL^{-1} HCl (50 mL) in form of [^{61}Cu]$CuCl_2$. The whole process took about 60 min (11).

Quality control of the product

Gamma spectroscopy of the final sample was carried out by a high purity germanium (HPGe) detector coupled with a Canberra multi-channel analyzer (USA). The peaks were observed and the area under curve was counted for 5000 seconds. The formation of colored dithizone-zinc complex was measured using visible spectroscopic assay to determine zinc cation concentration (12). The amount of gold cation in the final solution was checked using color formation with acidic rhodamine B reagent reacting with gold dilutions based on a previously reported colorimetric method.

Preparation and quality control of [^{61}Cu]diacetyl-bis(N^4-methylthio-semicarbazone)([^{61}Cu]ATSM)

The labeling precursor (H_2ATSM) was prepared according the reported method with modifications (13) and [^{61}Cu]ATSM was prepared starting from H_2ATSM and copper acetate according to the reported method (7).

Briefly, [^{61}Cu]$CuCl_2$ (370 MBq) dissolved in acidic medium obtained above (about 2 mL) was transferred to a 5 mL-vial containing 3 molL^{-1} sodium acetate (4 mL) to prepare a [^{61}Cu]copper acetate solution. A mixture of ATSM (4 µg) in anhydrous DMSO (0.1 mL) was added to the copper acetate solution and vortexed at 50 °C for 1 min. The mixture (about 5 mL) was then cooled in an ice bath, and rapidly injected into a C_{18} Sep-Pak column pretreated with 5 mL of ethanol and 2 mL of water. The column was washed with water (4 mL) and purged with a stream of dry N_2. The labeled compound was finally eluted using 0.2 mL-portions of absolute ethanol and the fractions were counted in HPGe detector. The vial containing the maximum radioactivity was diluted to a 5% solution by addition of normal saline. The active solution was checked for radiochemical purity by polymer-backed silica gel layer chromatography using dry ethyl acetate as mobile phase. The final solution was then passed through a 0.22-µm filter and pH was adjusted to 5-7 by the addition of 3 molL^{-1} sodium acetate buffer. For Radio thin layer chromatography, a 5 µl sample of the final fraction was spotted on a chromatography silica gel sheet paper, and developed in a mixture of 10% ammonium acetate:methanol (1:1) as the mobile phase. Alternatively, 10 mmolL^{-1} diethylenetriaminepentaacetic acid (DTPA) solution can be used as another mobile phase to discriminate free copper from radiolabeled compound. High performance liquid chromatography (HPLC) was performed on the final preparation using a

mixture of water:acetonitrile 1:1(V/V) as the eluent (flow rate: 1 mLmin^{-1}) for 20 min, in order to elute low molecular mass components. Radiolabeled compound was eluted using reverse stationary phase. Any remaining free Cu^{2+} cation with chloride or acetate counter ion is eluted at the same time.

Induction of fibrosarcoma tumors in mice

Tumor induction performed by the use of poly aromatic hydrocarbon injection in rodents as reported previously (14). For tumor model preparation, 10µl of 3-methyl cholanthrene solution in extra-virgin olive oil (4 mg/ml) was injected SC to the dorsal area of the mice. After 14-16 weeks the tumor weighed 0.2-0.4 g and was not grossly necrotic. Tumor tissues of some random animals were sent for pathological tests and were diagnosed as fibrosarcoma.

Co-incidence imaging studies

The final [^{61}Cu]ATSM solution of 1.85 MBq activity (0.1 mL) was injected into the dorsal tail vein of wild-type rats. For fibrosarcoma-bearing mice about 0.6 MBq activity (30-50 uL) was injected. The total amount of radioactive material injected into each animl was measured by counting the 1-mL syringe before and after injection in an activity meter with fixed geometry. The animals were relaxed by halothane and fixed in a suitable probe. Images were taken 1, 2 and 3 hours after administration of the radiopharmaceutical in coincidence mode of a Dual-Head SPECT system (SMV, France, Sopha DST-XL). The useful field of view (UFOV) was 540 mm × 400 mm. The spatial resolution in the coincidence mode was 10 mm full width at half maximum (FWHM) at the central field of view (CFOV), and sensitivity was 320 Kcps/MBq/mL. Sixty four projections were acquired for 30 seconds per view with a 64 × 64 matrix. Each rat was studied for 3 hours during which images were taken every 60 minutes.

RESULTS AND DISCUSSION

Although the hypoxia imaging property of ^{64}Cu-ATSM has been verified by various research group, but the long half-life Cu-64 radionuclide allows enough time for the accumulation as well as imposing radiation dose related to β^- particles. According some advantages of Cu-61 radionuclides, such as high positron emission ratio, short half-life as well as feasibility of production using natural targets, the need for verification of copper-61 ATSM as practical hypoxia imaging agent was still needed. In this work the hypoxia imaging ability of ^{61}Cu-ATSM especially in fibrosarcoma tumors is well justified.

Copper-61 production

For 76-min bombardment of the ^{nat}Zn target with 22 MeV proton, 150 µA , the resulting activity of ^{61}Cu was 222 GBq at the end of bombardment (EOB) and the production yield was 440 MBq.µA^{-1}h^{-1}. Yield from the radiochemical separation was more than 95%. Quality control of the product was performed in two steps. Radionuclidic control showed the presence of 67.41 (4.23%), 282.96 (12.2%), 373 (2.15%), 511 (122.9%), 656 (10.77%), 1186 (3.75%) keV γ-rays from ^{61}Cu and showed a radionuclidic purity of 99% at the end of synthesis (EOS). The rest of activity was attributed to ^{60}Cu (0.23%). In order to check the chemical purity, concentration of zinc (from target material) and gold (from target support) were determined using visible colorimetric assays. The presence of zinc cations was checked by visible colorimetric assays. Even at 1 mg/kg^{-1} of standard zinc concentration, the pinkish complex was visible by naked eye, while the test sample remained similar to the blank. The colorimetric assay demonstrated that the zinc cation

concentration was far below the maximum permitted levels, i.e. 5 mg/kg^{-1} (less than 1 mg/kg^{-1} zinc). The gold concentration was less than 0.9 mg/kg^{-1}.

Preparation and quality control of the tracer

[^{61}Cu]ATSM is a neutral, lipophillic compound which can penetrate all the cells with perfusion; however the structure changes due to the reduction of Cu^{2+} cation to Cu^{+} cation in hypoxic conditions results in hypoxic cell accumulation. Figure 1, shows the chemical structure of [^{61}Cu]ATSM.

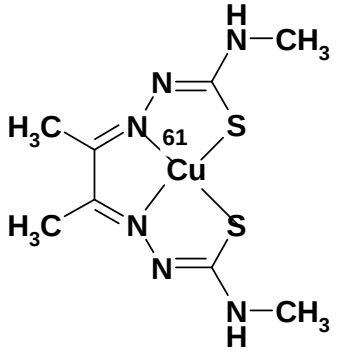

Figure 1. Structure formula for [^{61}Cu]ATSM.

In TLC studies, the more polar free compound and free copper, correlate to smaller R_fs (R_f =0.1-0.2), while [^{61}Cu]ATSM complex migrates with higher R_f (R_f=0.9). In all radiolabeling runs (n=9), the integral ratio of the Cu-ATSM and free Cu peaks were constant (97:3), showing the high radiochemical purity and consistency of the labeling method.

In HPLC studies the fast eluting compound was shown to be hydrophilic [^{61}Cu]Cu^{2+} cation (2.01 min), while the lipophillic [^{61}Cu]ATSM complex was eluted couple of minutes after (6.22). In various studies, n=9, the purity of both radiochemical species were shown to be 100% (data not shown).

Biodistribution studies

The biodistribution of [^{61}Cu]ATSM in animal models as well as free copper cation has already been reported in details by our group recently (15).

It was well shown that the copper is partly accumulated in liver as a reservoir for many metals especially copper by serum ceruloplasmin. Gastrointestinal (GI) accumulation especially in the first hour is expressed as a result of secretion via hepatobilliary excretion, while it was not significant after 2 hours. The major content of copper is washed out by kidneys and consequently urinary tract due to high water solubility of the cation. The uptakes of rest of the tissues are not significant. Due to lipophilicity of [^{61}Cu]ATSM, it is rapidly washed out from blood stream while easily penetrates into phospholipid bilayer of cells. The compound has a slight liver uptake which is common with most of Cu-thiosemicarbazones (15).

The administration of the tracer to fibrosarcoma-bearing mice showed high uptake in liver, stomach, intestine proposing a hepatobilliary excretion model. As mentioned earlier Cu-ATSM is a hypoxic cell targeting agent. The distribution of more than 1.5% can be enough to perform imaging studies. The best time period for scanning showed to be up to 2 hours post injection with normally developed tumor (12-15 weeks after induction) (15).

Imaging studies

The planar images from the tracer obtained in wild-type rats demonstrated the accumulation was majorly in liver as expected for most of copper *bis*-thiosemicarbazones. This makes liver a dose-limiting organ in future studies and further possible studies should probably focus on the development of more hydrophilic tracers from this group with various hydrophilic groups such as peptides as well as sugar moieties. The other

consideration could be the use of bile-secreting agents accelerating the bile excretion to lower liver radiation (Figure 2).

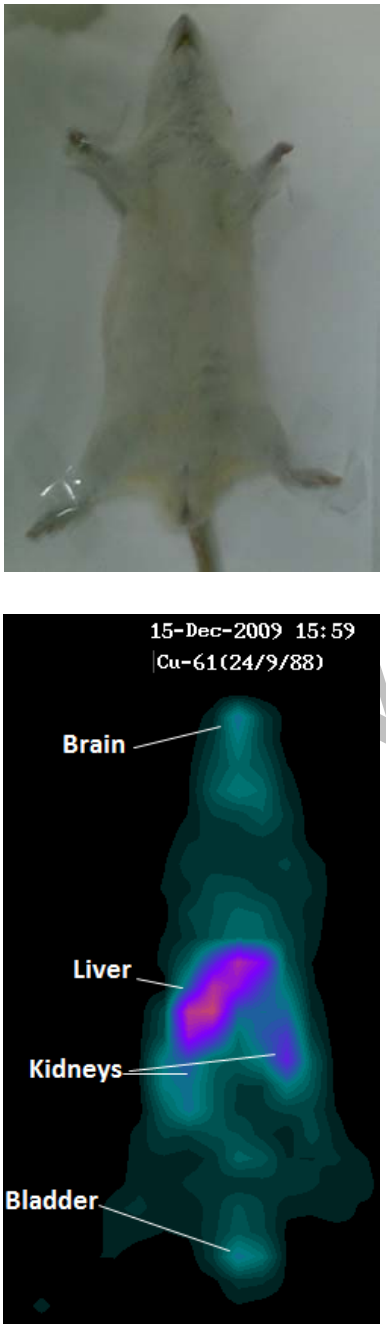

Figure 2. The planar image of the wild-type rat one hour post i.v. injection of ^{61}Cu-ATSM (1.8 MBq).

In fibrosarcoma-bearing mouse model, with larger hypoxic tumors, the radiotracer was administered and interestingly the tumor uptake was excessively high. The only significant accumulating tissue was shown to be tumor tissue in the imaging fields. These observations were confirmed by sacrificing the animal and counting the tumor mass and the rest of carcass. The tumor:whole body count ratio was excessively high (>40) (Figure 3).

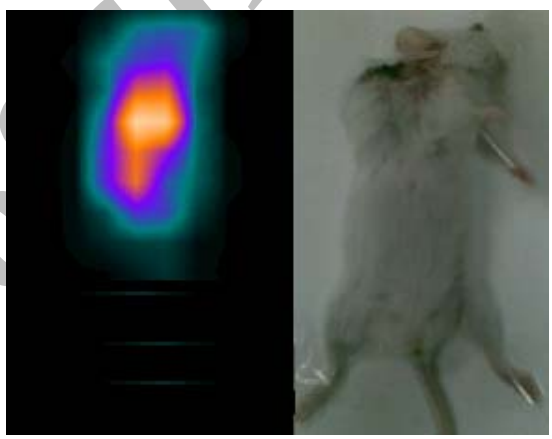

Figure 3. The planar image of hypoxic fibrosarcoma tumor in mouse one hour post i.v. injection of ^{61}Cu-ATSM (1.8 MBq).

In order to better demonstrate the tissue and tumor uptake in the animals, image was taken while the deceased animal body with the tumor was shown at the right. While the entire tumor has a volume of 1-1.5 cm^3, only slight parts of tumor demonstrated the hypoxic imaging agent uptake. The best acquisition time was confirmed to be 60-120 min post injection (Figure 4).

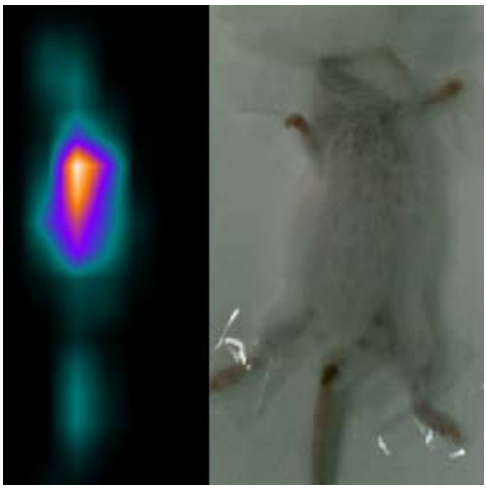

Figure 4. The planar image of a 12-week fibrosarcoma-induced mouse, one hour post i.v. injection of ^{61}Cu-ATSM (1.8 MBq).

CONCLUSION

Total labeling and formulation of [^{61}Cu]ATSM took about 10 minutes, with a radiochemical purity of >99% using HPLC. No labeled by-products were observed upon RTLC analysis of the final preparations after C_{18} solid phase purification. The biodistribution of tracer was checked in normal and tumor-bearing rodents up to 3 hours and a significant accumulation took place in liver, kidneys and brain. Significant fibrosarcoma uptake was observed in animal with tumors at hypoxic conditions, while low tumor uptake was noted for tumors at necrotic and proliferating stages using co-incidence imaging. [^{61}Cu]ATSM is a specific PET radiopharmaceuical for hypoxia imaging with an intermediate half life, for distinguishing the hypoxic/anoxic and normoxic tissues from each other. Our future experiments on this radiopharmaceutical would focus on other tumor models and finally PET studies in human.

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