

Preclinical studies of [^{61}Cu]ATSM as a PET radiopharmaceutical for fibrosarcoma imaging

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[^{61}Cu]diacetyl-*bis*-(N^4 -methylthiosemicarbazone) ([^{61}Cu]ATSM) was prepared using in house-made diacetyl-*bis*-(N^4 -methylthiosemicarbazone) (ATSM) ligand and [^{61}Cu]CuCl₂ produced *via* the $^{nat}\text{Zn}(p,x)^{61}\text{Cu}$ (180 μA proton irradiation, 22 MeV, 3.2 h) and purified by a ion chromatography method. [^{61}Cu]ATSM radiochemical purity was >98 %, as shown by HPLC and RTLC methods. [^{61}Cu]ATSM was administered into normal and tumor bearing rodents for up to 210 minutes, followed by biodistribution and co-incidence imaging studies. Significant tumor/non-tumor accumulation was observed either by animal sacrifice or imaging. [^{61}Cu]ATSM is a positron emission tomography (PET) radiotracer for tumor hypoxia imaging.

Keywords: radiopharmaceuticals, copper-61, [^{61}Cu]ATSM hypoxia, positron emission tomography, fibrosarcoma, imaging

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 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, accumulate avidly in hypoxic cells, but are washed out rapidly from normoxic cells.

Copper offers a unique selection of radioisotopes (^{60}Cu , ^{61}Cu , ^{62}Cu , ^{64}Cu , and ^{67}Cu) with half-lives ranging from 9.8 minutes to 61.9 hours, suitable for imaging and/or radiotherapy (2). The most commonly used copper radioisotopes, ^{62}Cu and ^{64}Cu , provide very good physical properties for therapeutic and/or diagnostic purposes. Copper-62 is usually provided by a $^{62}\text{Zn}/^{62}\text{Cu}$ generator, but has a short half life of 8 minutes (3). Copper-64 is used for diagnostic imaging as well as therapy (4).

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Copper-61 is a positron emitter ($t_{1/2} = 3.33$ h, β^+ : 62 %, EC: 38 %), with excellent potentials for application in the PET method and molecular imaging (5). Few production methods of copper-61 have been reported for radiolabeling of biomolecules and other applications (6). 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 to ^{64}Cu (62 % *vs.* 18 %) (7). As an example of copper-labeled radiopharmaceutical, Cu-ATSM has been used as a PET hypoxia tracer for oncologic/cardiologic studies (8, 9).

Based on the interesting properties of copper-61 and the possibility of its production *via* $^{\text{nat}}\text{Zn}(\text{p},\text{x})^{61}\text{Cu}$, we have already reported a production method (10), and in continuation of this work we hereby report the preclinical studies of [^{61}Cu]ATSM as an imaging agent in normal rodents and animals with hypoxic tumors.

EXPERIMENTAL

Materials and procedures

Production of ^{61}Cu was performed at the Agriculture, Medicine and Industrial Research School (AMIRS, Karaj, Iran), using a 30 MeV cyclotron (Cyclone-30, IBA, Belgium). Natural zinc chloride with high purity of more than 98 % was provided commercially (Merck, Germany). All chemicals were purchased from Sigma-Aldrich Chemical Co. Radio-chromatography was performed by counting polymer-backed silica gel paper thin layer sheets using a thin layer chromatography scanner, Bioscan AR2000 (Bioscan, France). Analytical HPLC to determine the specific activity was performed using a Shimadzu LC-10AT (Japan), armed with two detector systems, a flow scintillation analyzer (Packard-150 TR, USA) and UV-visible (Shimadzu), using Whatman Partisphere C-18 column 250×4.6 mm (Whatman Co., USA). Eluent was $\text{H}_2\text{O} : \text{CH}_3\text{CN}$ (1:1), flow rate = 1 mL min^{-1} . All calculations and RTLC counting were 283 keV peak based. All values were expressed as mean \pm standard deviation and the data were compared using Student's *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. Approval of the AMIRS Ethical Committee was obtained for the research.

Zinc targetry and bombardment

The target was a layer of natural zinc, electroplated on a copper plate coated with a 50- μm gold layer to prevent interference of the backing copper during radiochemical separation. Cross section calculations by the ALICE nuclear code (11) showed that the best proton energy range for $^{\text{nat}}\text{Zn}(\text{p},\text{x})^{61}\text{Cu}$ reaction was 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 6° to achieve a higher production yield. Stopping and Range of Ions in Matter (SRIM) code (12) 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. 13 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 for 76 minutes.

Chemical separation

Chemical separation was carried out in no-carrier-added form. The irradiated target was dissolved in 10 mol L^{-1} HCl (15 mL, H_2O_2 added). The solution was passed through a cation exchange resin (AG 50 W, H^+ form, mesh 200–400, 1.3×10 cm), which had been preconditioned by passing 25 mL of 9 mol L^{-1} HCl. The column was then washed by 25 mL of 9 mol L^{-1} HCl at a rate of 1 mL min^{-1} to remove copper and zinc ions. Water (30 mL) was added to the eluent to about 100 mL of a 6 mol L^{-1} HCl solution. The latter solution was loaded on another exchange resin (AG1X8 Cl^- form, 100–200 mesh, 25×1.7 cm) pretreated with 6 mol L^{-1} HCl (100 mL). Finally, ^{61}Cu was eluted using 2 mol L^{-1} HCl (50 mL) in the form of [^{61}Cu]CuCl₂. The whole process took about 60 min (14).

Quality control of the product

Gamma spectroscopy of the final sample was carried out by a high purity germanium (HPGe) detector coupled with a CanberraTM multi-channel analyzer (Canberra, USA). The peaks were observed and the area under the curve was counted for 4 hours. Formation of the colored dithizone-zinc complex was measured using a visible spectroscopic assay to determine the zinc cation concentration (15). The amount of gold cation in the final solution was checked using color formation with an acidic rhodamine B reagent reacting with gold dilutions based on a previously reported colorimetric method (15).

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

[^{61}Cu]ATSM was prepared starting from H_2ATSM and copper acetate (16). H_2ATSM was prepared according to the reported method with modifications (12, 17). Briefly, [^{61}Cu]CuCl₂ (370 MBq) dissolved in acidic medium obtained above (about 2 mL) was transferred to a 5 mL-vial containing 3 mol L^{-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 $^\circ\text{C}$ for 1 min. The mixture (about 5 mL) was then cooled in an ice bath, and rapidly injected into a C₁₈ 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 fractions were counted in an HPGe detector. The vial containing 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 the mobile phase. The final solution was then passed through a 0.22- μm filter and pH was adjusted to 5–7 by adding 3 mol L^{-1} sodium acetate buffer.

Quality control of ⁶¹Cu-ATSM

Radio thin layer chromatography. – A 5- μ L sample of the final fraction was spotted on chromatography silica gel sheet paper and developed in a mixture of 10 % ammonium acetate/methanol (1:1) as the mobile phase. Alternatively, 10 mmol L⁻¹ diethylenetriaminepentaacetic acid (DTPA) solution can be used as another mobile phase to discriminate free copper from the radiolabeled compound.

High performance liquid chromatography. – HPLC was performed in the final preparation using a mixture of water/acetonitrile 1:1 (V/V) as the eluent (flow rate: 1 mL min⁻¹, pressure: 1275 N m⁻²) for 20 min, in order to elute low molecular mass components. Radiolabeled compound was eluted using the reverse stationary phase. Any remaining free Cu²⁺ cation with chloride or acetate counter ion was eluted at the same time.

Stability of [⁶¹Cu]ATSM complex

Stability tests were based on previous studies performed for radiolabeled copper complexes (18). A sample of [⁶¹Cu]ATSM (185 MBq) was kept at room temperature for 5 hours and checked by RTLC every half-an-hour. A micropipet sample (5 μ L) was taken from the shaking mixture and the ratio of free radiocopper to [⁶¹Cu]ATSM was checked by radio thin layer chromatography (eluent: dry ethyl acetate).

Serum stability studies

To 36.1 MBq of [⁶¹Cu]ATSM, 500 μ L of freshly prepared human serum was added and the resulting mixture was incubated at 37 °C for 5 hours. Aliquots (5- μ L) were analyzed by radio-TLC after 0, 0.25, 0.5, 1, 2 and 3 hours of incubation to determine the stability of the complex.

Biodistribution of [⁶¹Cu]acetate and [⁶¹Cu]ATSM in normal rats

Mature male NMRI rats (Razi Institute, Iran), weighing 150–250 g were used in experiments. The animals were kept in groups of ten, in cages under constant temperature (24 °C) and 12 h light/dark schedule. They had free access to the standard rat diet and water except during the experiment. On the day of the experiment, animals were randomly transferred to individual cages and allowed to acclimatize for 30 min before injection of tracer and free copper cation. [⁶¹Cu]acetate and [⁶¹Cu]ATSM were administered to separate normal rat groups. A volume (50 μ L) of [⁶¹Cu]ATSM or [⁶¹Cu]acetate solutions containing 37 MBq radioactivity were injected intravenously to rats *via* tail veins. The animals were sacrificed at exact time intervals (1 and 2 h for [⁶¹Cu]acetate and 30–210 minutes for [⁶¹Cu]ATSM), and the injected dose ID was calculated as percentage (based on area under the curve of 283 keV peak) per gram of different tissue using an HPGe detector.

Co-incidence imaging studies

The final [⁶¹Cu]ATSM solution of 1.85 MBq activity (0.1 mL) was injected into the dorsal tail vein of healthy rats. The total amount of radioactive material injected into each rat was measured by a counting 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 the coincidence mode of a Dual-Head SPECT system (SMV, Sopha DST-XL, Sopha, France). 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) in the central field of view (CFOV), and sensitivity was 0.320 counts s⁻¹ Bq⁻¹ mL⁻¹. Sixty four projections were acquired for 30 seconds per view with a 64 × 64 matrix. Each rat was studied for 3 hours during which time images were taken every 60 minutes.

RESULTS AND DISCUSSION

For 76-min bombardment of the ^{nat}Zn target with 22 MeV proton, 150 μA the resulting activity of ⁶¹Cu 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 (4.2 %), 282.96 (12.2 %), 373 (2.2 %), 511 (122.9 %), 656 (10.8 %), 1186 (3.8 %) keV γ-rays from ⁶¹Cu and showed a radionuclidic purity of 99 % at the end of synthesis (EOS). The rest of activity was attributed to ⁶⁰Cu (0.23 %). In order to check the chemical purity, concentrations 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.

[⁶¹Cu]ATSM is a neutral, lipophilic compound that can penetrate all the cells with perfusion. However its structure changes due to the reduction of Cu²⁺ cation to Cu⁺ cation in hypoxic conditions resulting in hypoxic cell accumulation. Fig. 1 shows the chemical structure of [⁶¹Cu]ATSM.

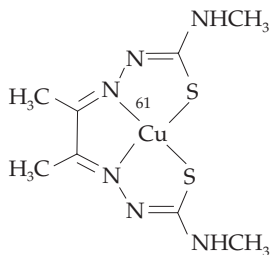


Fig. 1. Structural formula for [⁶¹Cu]ATSM.

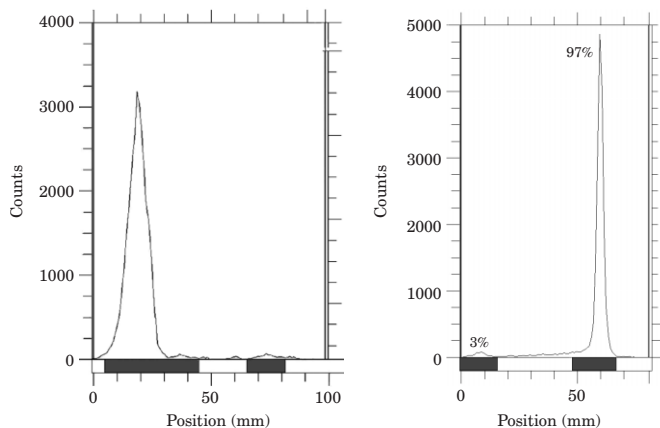


Fig. 2. RTLC of the starting $[^{61}\text{Cu}]\text{CuOAc}$ (left) and $[^{61}\text{Cu}]\text{ATSM}$ (right).

In TLC studies, the more polar free compound and free copper correlate to smaller R_f s ($R_f = 0.1\text{--}0.2$) (Fig. 4), while the $[^{61}\text{Cu}]\text{ATSM}$ complex migrates with higher R_f ($R_f = 0.9$). In all radiolabeling runs ($n = 9$) (Fig. 5), the integral ratio of the Cu-ATSM and free Cu peaks was constant (98:2), showing the high radiochemical purity and consistency of the labeling method.

In order to obtain the best labeling reaction conditions, the complex formation was studied for temperature dependence. Heating the reaction mixture to 50 °C did not increase the yield, but resulted in various unknown radiochemical impurities. Thus, 25 °C was considered as the optimum temperature.

In HPLC studies, the fast eluting compound was shown to be hydrophilic $[^{61}\text{Cu}]\text{Cu}^{2+}$ cation (2.01 min), while the lipophilic $[^{61}\text{Cu}]\text{ATSM}$ complex was eluted a few minutes later (6.22). In various studies, $n = 9$, the purity of both radiochemical species was shown to be 100 %, as shown in Fig. 3.

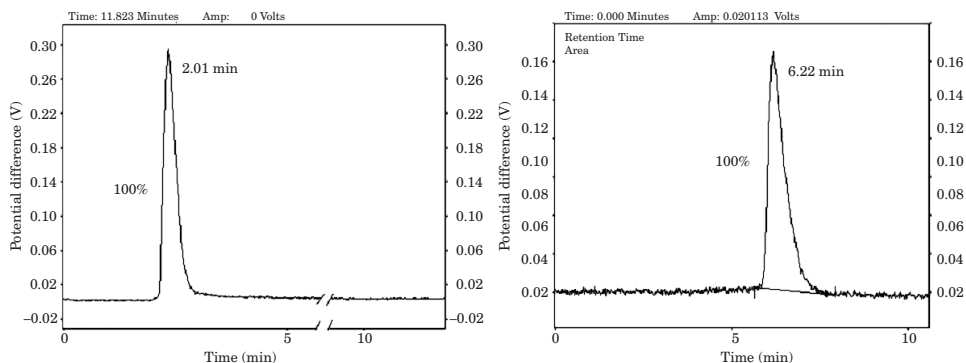


Fig. 3. HPLC for $[^{61}\text{Cu}]\text{Cu}^{2+}$ (in acetate and chloride form, left) and $[^{61}\text{Cu}]\text{ATSM}$ (right) using a reverse phase column with a mixture of $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ (1:1) as eluent.

The final radiolabelled complex diluted in normal saline was then passed through a 0.22- μm (Millipore, USA) filter for sterilization. Due to its thermal instability, $[^{61}\text{Cu}]\text{ATSM}$ preparation could be totally degraded and left detectable amounts of free copper after autoclaving. Incubation of $[^{61}\text{Cu}]\text{ATSM}$ in freshly prepared human serum for 3 hours at 37 °C showed no loss of ^{61}Cu from the complex at least for 2.5 hours.

RTLC was performed, using a mixture of dry ethyl acetate as the mobile phase to control the radiochemical purity of the product.

Total labeling and formulation of $[^{61}\text{Cu}]\text{ATSM}$ took about 5 minutes, with a yield of 98 %. No labeled by-products were observed upon RTLC analysis of the final preparations after C_{18} solid phase purification. $[^{61}\text{Cu}]\text{ATSM}$ was stable in aqueous solutions for at least 24 h as detected by RTLC (radiochemical purity 98 %).

In order to investigate biodistribution of $[^{61}\text{Cu}]\text{ATSM}$ in our animal models we had to obtain the biodistribution data for free copper cation. Thus after injection of 1.48 MBq of the $[^{61}\text{Cu}]\text{CuCl}_2$ pre-formulated by the normal saline (pH. 6.5–7), through the tail vein of adult rats, the biodistribution of the cation was checked in various vital organs. How-

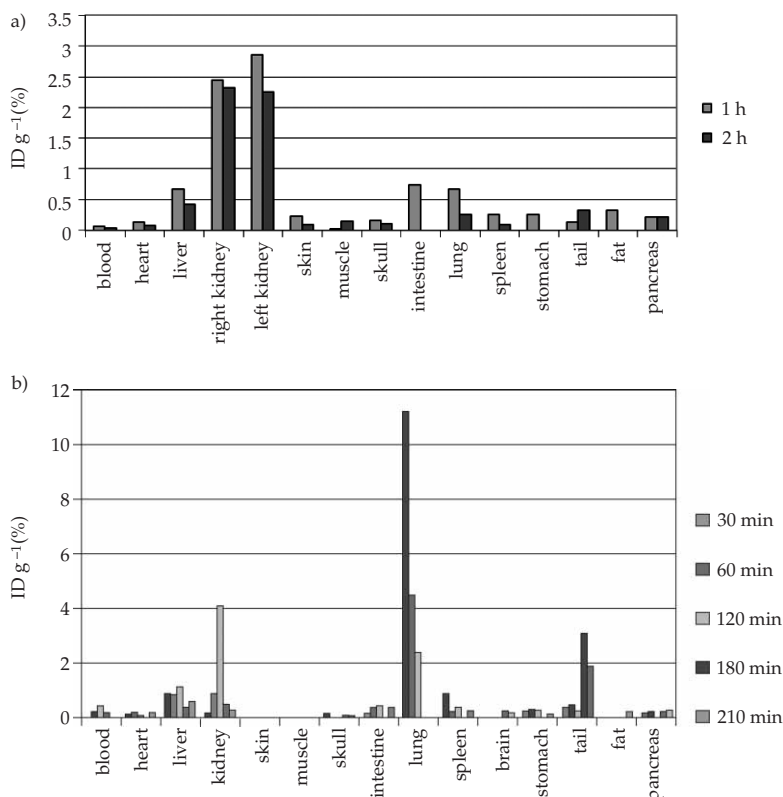


Fig. 4. Calculated ID g^{-1} (%) of: a) $[^{61}\text{Cu}]\text{CuCl}_2$ and b) $[^{61}\text{Cu}]\text{-ATSM}$ in normal rats ($n = 5$).

ever, 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 liver secretion *via* hepatobiliary excretion, while it is not significant after 2 hours. The major content of copper is washed out by kidneys and consequently urinary tract. The uptakes of rest of the tissues are not significant.

As shown in Fig. 4, 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 for most of Cu-thiosemicarbazones.

Interestingly, ^{61}Cu -ATSM was highly accumulated in the lungs starting from 30 minutes up to 210 minutes (Fig. 4).

Regarding the lung as a metabolic organ, the presence of various reductases and especially matrix metalloproteinases in this tissue can be a cause of tracer retention since the $\text{Cu}^{2+}/\text{Cu}^{+}$ reduction can produce $\text{Cu}(\text{I})\text{ATSM}^{-}$ in charged form, without the ability of crossing the phospholipid bilayer of the cell membrane (19).

Administration of the tracer to fibrosarcoma-bearing rats showed high uptake in the liver, stomach, intestine, suggesting a hepatobiliary excretion model. The lung uptake was significant as discussed in normal animal distribution.

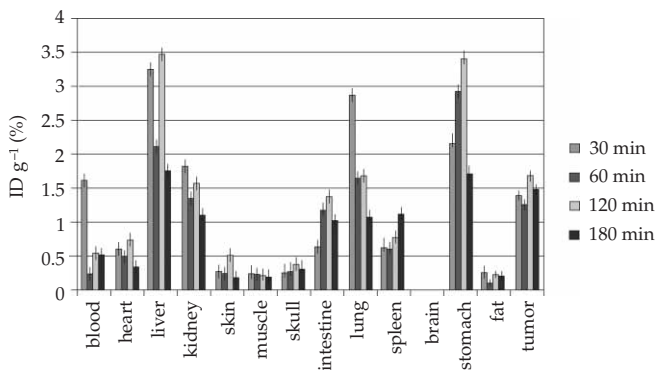


Fig. 5. Calculated $\text{ID g}^{-1} (\%)$ of [^{61}Cu]ATSM 30–180 minutes post tracer injection in fibrosarcoma-bearing rats ($n = 5$).

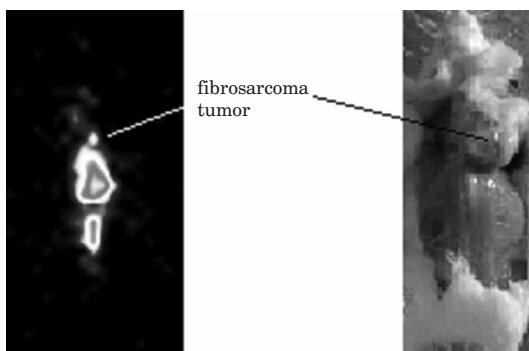


Fig. 6. Co-incidence images for ^{61}Cu -ATSM uptake of a fibrosarcoma-bearing rat 60 minutes post injection.

Biodistribution of the tracer was checked in normal and tumor-bearing animals for up to 3 hours. Significant accumulation also took place in kidneys and fibrosarcoma in all animals.

As mentioned earlier Cu-ATSM is a hypoxic cell targeting agent. The tumor uptake was high in some parts at all time intervals and the level of uptake remained constant. Since all tumor parts may not necessarily be hypoxic in the counting experiments, the tumors were cut into pieces and various parts were counted.

The distribution of more than 1.5 % could be enough to perform imaging studies (Fig. 5). The best time period for scanning was found to be 1 to 2 hours post injection; up to 2 hours post injection rather high accumulation was observed in the liver and GI system of the rats.

In order to visualize the tissue and tumor uptake in the administered mice, a co-incidence image was taken, while the deceased animal body was shown with the tumor body shown on the right (Fig. 6). While the whole tumor volume was 10 cm³, only slight parts of tumor demonstrated the hypoxic imaging agent uptake. Thus, 60–120 min was confirmed to be the best acquisition time post tracer injection.

CONCLUSIONS

[⁶¹Cu]ATSM is a PET radiopharmaceutical for hypoxia imaging with an intermediate half-life. The co-incidence imaging in the tumor-bearing animals showed the specific tumor uptake of copy in parts of the tumor. Our future experiments on this radiopharmaceutical will focus on other tumor models and finally PET studies in humans.

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S A Ž E T A K

Predkliničke studije [⁶¹Cu]ATSM kao PET radiofarmaka za snimanje fibrosarkoma

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[⁶¹Cu]diacetyl-bis(N⁴-metiltiosemikarbazon) ([⁶¹Cu]ATSM) dobiven je iz liganda diacetyl-bis(N⁴-metiltiosemikarbazona) (ATSM) pripravljenog u vlastitom laboratoriju i [⁶¹Cu]CuCl₂ dobivenog iz ^{nat}Zn(p,x)⁶¹Cu (180 μA protonskim zračenjem, 22 MeV, 3,2 h).

[⁶¹Cu]ATSM je čišćen ionskom kromatografijom. Prema HPLC i RTLC radiokemijska čistoća bila je > 98 %. [⁶¹Cu]ATSM je davan zdravim glodavcima i glodavcima s tumorom tijekom 210 minuta te je praćena biodistribucija. Žrtvovanjem testiranih životinja te snimanjem primijećena je značajna razlika u akumulaciji [⁶¹Cu]ATSM u tumorskom tkivu u odnosu na zdravo tkivo. [⁶¹Cu]ATSM je pogodan za dijagnostiku hipoksije tumora pozitron emisijskom tomografijom (PET).

Ključne riječi: radiofarmak, bakar-61, [⁶¹Cu]ATSM, hipoksija, pozitronska tomografija, fibrosarkom, snimanje

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