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ORIGINAL PAPER

Preparation and biological evaluation of [^{61}Cu]bleomycin complex as a possible PET radiopharmaceutical in normal and fibrosarcoma-bearing animals

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Abstract. [^{61}Cu]bleomycin ([^{61}Cu]BLM) was prepared using [^{61}Cu]$CuCl_2$ produced via ^{nat}Zn(p,x)^{61}Cu. [^{61}Cu]BLM was prepared under optimized conditions (room temperature, 45 min, 0.1 mg bleomycin for 92.5–370 MBq $^{61}CuCl_2$) with radiochemical purity over 98% shown by HPLC and RTLC. [^{61}Cu]BLM was administered into normal and tumor bearing rodents up to 210 min followed by biodistribution and co-incidence imaging studies. A significant tumor/non tumor accumulation was observed either by animal sacrification or an imaging method. [^{61}Cu]BLM can be a potential PET radiotracer for tumor imaging.

Key words: radiopharmaceutical • copper-61 • bleomycin • positron emission tomography • fibrosarcoma

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Introduction

Positron emission tomography is one of the latest diagnostic tools in nuclear medicine. The amazing physical characteristics of PET radioisotopes in connection with sophisticated PET cameras have provided clinicians with such a powerful diagnostic tool. According to our previous researches on the radiosynthesis and evaluation of non-fluorine PET radiopharmaceuticals [9, 21], we were interested in the production and application of Cu-61 tumor seeking radiopharmaceuticals.

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 min to 61.9 h suitable for imaging and/or radiotherapy. The most commonly used copper radioisotopes, ^{62}Cu and ^{64}Cu, provide very good physical properties suitable for therapeutic and/or diagnostic purposes. Few production methods of copper-61 have been reported for radiolabeling of biomolecules and other applications [17]. There are few literature reports on the medical applications of copper-61 [16]. 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 vs. ^{64}Cu [5]. Copper-61 has been used in radiolabeling of small imaging molecules [11, 14] for various diagnostic purposes.

Bleomycins (Fig. 1) are tumor seeking antibiotics that have been widely used in cancer chemotherapy since the 1970's. It is believed that bleomycin antibiotics interfere with DNA as false nucleotides, assuming the dithiazole moiety acts like a purine base [6]. On the other hand, these compounds are activated by a cation insertion as anti-neoplastic agents. The whole complex can then act like a peroxidase system, by producing hy-

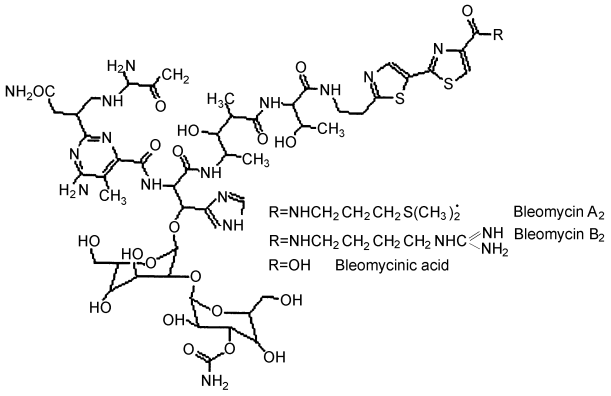

Fig. 1. Structures of bleomycin components in the pharmaceutical sample used.

drogen peroxide, resulting in DNA decomposition. Thus, labeling of bleomycin with bi/trivalent radioisotopes can produce pharmacologically active compounds carrying a diagnostic and/or therapeutic radioisotope [13].

Several radioisotopes have been used in radiolabeling of bleomycins for imaging and/or therapy of neoplastic tissues such as indium-111 [13], ruthenium [23] and rhodium-105 [3]. Recently, an [^{111}In]bleomycin complex has shown a high *in vitro* and *in vivo* stability and has been successfully used in human tumor detection and therapy [23].

In continuation of our works on the development and evaluation of radiolabeled bleomycins using cyclotron-derived radioisotopes [8, 10, 12], we were interested to develop copper-61 labeled BLM using our routine ^{61}Cu production already reported [22] as a positron emitter tracer for use in tumor imaging. We hereby report the preparation, stability tests, biodistribution studies in normal and tumor bearing animals as well as co-incidence imaging of tumor-seeking [^{61}Cu]bleomycin complex.

Experimental

Materials

Production of ^{61}Cu was performed at the Agricultural, Medical and Industrial Research School (AMIRS) using a 30 MeV cyclotron (Cyclone-30, IBA). Natural zinc chloride of high purity (more than 98%) was provided commercially (Merck chemical company, Darmstadt, Germany). All other chemicals were purchased from Sigma-Aldrich Chemical Co. U.K. Bleomycin sulfate (BLEO-S) was a pharmaceutical sample purchased from Nippon Kayaku Laboratories, Japan. Radiochromatography was performed by counting polymer-backed silica gel paper thin layer sheets using a thin-layer chromatography scanner, Bioscan AR2000, Paris, France. Analytical HPLC to determine the specific activity was performed by a Shimadzu LC-10AT, armed with two detector systems, a flow scintillation analyzer (Packard-150 TR) and UV-visible (Shimadzu) using a Whatman Partisphere C-18 column 250 × 4.6 mm, Whatman Co. NJ, USA. Eluent, H_2O:CH_3CN (1:1), FR = 1 ml/min. All calculations and RTLC counting were based on the 283 keV peak. All values were expressed as mean ± standard deviation (Mean ± SD) and the data were compared using the Student t-test. Statistical significance was defined as $P < 0.05$. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd ed.

Procedures

Labeling of bleomycin with [^{61}Cu]$CuCl_2$

[^{61}Cu]$CuCl_2$, obtained from natural zinc irradiation on a gold-plated support in two-step cation exchange chromatography, was prepared according to the reported method [22]. Briefly, [^{61}Cu]$CuCl_2$ (92.5–370 MBq) dissolved in an acidic medium (0.5–2 ml) was transferred to a 2 ml-vial and the solution was evaporated by slight warming under nitrogen flow. A mixture of BLM (0.1 mg) in normal saline (0.1 ml) was then added and heated at 25°C. The active solution was checked for radiochemical purity by polymer-backed silica gel layer chromatography using a 1:1 mixture of 10% ammonium acetate and methanol as the mobile phase every 15 min. The final solution was then passed through a 0.22 μ filter and pH was adjusted to 5–7 by addition of 1 M sodium acetate buffer.

Quality control of ^{61}Cu-BLM

Radio-thin-layer chromatography: A 5 μl sample of the final fraction was spotted on a chromatography Si sheet paper, and developed in a mixture of 10% ammonium acetate:methanol (1:1) as the mobile phase. Alternatively, 10 mM 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 ml/min, pressure: 130 kgF/cm^2) for 20 min, in order to elute low molecular weight components. The radiolabeled compound was eluted using the reverse stationary phase. Any remaining copper cations (such as Cu^{2+} and $CuCl_4^{2-}$, …) with chloride counter-ion is eluted at the same time.

Stability of [^{61}Cu]BLM complex in the final product

Stability tests were based on previous studies performed for other radiolabeled bleomycins [7].

A sample of [^{61}Cu]BLM (18.5 Bq) was kept at room temperature for 5 h while checked by RTLC every 30 min. A micropipet sample (5 μL) was taken from the shaking mixture and the ratio of free radio-copper to [^{61}Cu]BLM was checked by radio-thin-layer chromatography (eluent: 10% NH_4OAc and methanol 1:1).

Serum stability studies

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

Induction of fibrosarcoma tumors in mice

Tumor induction was performed by the use of poly aromatic hydrocarbon injection in rodents as reported previously [4]. For tumor model preparation, 10 μl 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.

Biodistribution of [^{61}Cu]$CuCl_2$ and [^{61}Cu]BLM in normal and fibrosarcoma bearing animals

[^{61}Cu]$CuCl_2$ and [^{61}Cu]BLM were administered to separate normal rat groups. A volume (50 μl) of [^{61}Cu]BLM or [^{61}Cu]$CuCl_2$ solutions containing radioactivity (1.48 MBq for rats and 0.37 MBq for mouse) were injected intravenously via their tail veins. The animals were sacrificed at exact time intervals (1 and 2 h for [^{61}Cu]$CuCl_2$ and 30–210 min for [^{61}Cu]BLM), and the ID/g % of different organs was calculated as percentage of injected dose (based on the area under the curve of the 283 keV peak) per gram using an HPGe detector.

Co-incidence imaging studies

0.1 ml volumes of the final [^{61}Cu]BLM solution containing 1.85 MBq activity were injected into the dorsal tail vein of healthy rats. The total amount of radioactive material injected into each rat was measured by counting the 1-ml syringe before and after injection in an activity meter with fixed geometry. The animals were relaxed by diethyl ether and fixed in a suitable probe. Images were taken 1, 2 and 3 h after administration of the radiopharmaceutical in co-incidence mode of a Dual-Head SPECT system (SMV, France, Sopha DST-XL). The useful field of view (UFOV) was 540 × 400 mm. The spatial resolution in the co-incidence mode was 10 mm FWHM at the CFOV, and the sensitivity was 20 Kcps/37 kBq/cc. Sixty four projections were acquired for 30 s per view with a 64 × 64 mm matrix. Each rat was studied for 3 h during which images were taken every 60 min.

Results

The oxidation-reduction potential of Cu(II)-bleomycin has been measured at 25°C, pH 7.0 and the data suggested that the potentials were within the range that would allow the reduction of Cu(II)-bleomycin to take place in a cell [19]. Thus, the incorporation of the whole complex into cells are possible especially at the high thiol levels as has been reported for many tumor cells containing metallothioproteins [1] while Cu(II)BLM has been reported to be kinetically and thermodynamically stable in ligand substitution processes and is only slowly reduced and dissociated by sulfohydryl reagents. On the other hand it also has been shown that the complex is stable in human plasma [1].

All these data support the possibility of development of an interesting radio-copper tracer with positron emitting properties. The suggested structure for copper-bleomycin complex has been proposed as shown in Fig. 2 reported in the literature [15].

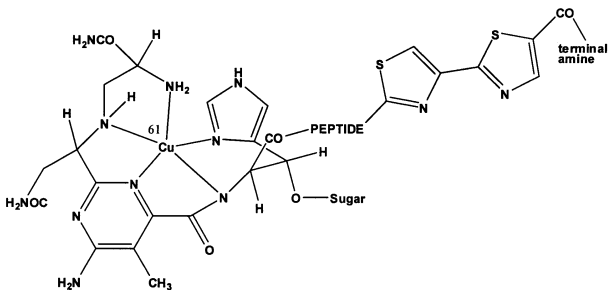

Fig. 2. The proposed structure of [^{61}Cu]BLM complex based on early reports [15].

The optical spectrum of a pH 6–7 solution of the 1:1 complex between copper(II) and bleomycin has been already characterized by spectroscopic methods [2, 4] suggesting a 1:1 Cu-BLM complex in this study.

It has been shown that some metal-bleomycin complexes become more non-toxic towards cultured mammalian cells in comparison to free bleomycin such as cobalt(III) bleomycin and do not cause any detectable DNA damage, while the effects of copper(II) bleomycin on intact cells is similar to metal free bleomycin with regard to both cytotoxicity and DNA damage [20].

Because of the several polar functional groups in its structure, labeling of bleomycin with a cation does not greatly affect its chromatographic properties. Thus, the labeled and unlabeled bleomycin migrate to almost the same R_f using RTLC. The more polar bleomycin fraction, i.e. bleomycin A_2, correlates with the smallest R_f, while the other polar fractions come close R_fs (bleomycin B_2). According to the tumor-seeking properties of all bleomycins, separation of the above labeled species was not intended.

As shown in Fig. 1 the pharmaceutical sample is mainly composed of 3 components with reported ratio mixture [18], considering the molar ratio, a mean molecular weight of 1495.22 can be calculated, resulting in a specific activity of 50–55 GBq/mM using optimized radiolabeling conditions.

The labeling step took about 45 min. In all radiolabeling procedures (n = 5), the integral ratio of the two bleomycin chromatogram peaks were constant (B_2:A_2, 0.27), showing the isomeric ratio of the peaks. The labeling yield was greater than 99%. The ratio of the sum of two Cu-BLM peaks at R_fs 0.3 and 0.7 to free the Cu^{2+} radiopeak (R_f:0.0) was considered as the radiochemical yield (Fig. 3).

For optimization of the labeling conditions, at a random temperature of 25°C for instance, the best pH for the labeling step was 5.5–7. In basic conditions, the radiochemical yield decreased drastically due to the degradation of bleomycin to less soluble compounds [24].

In HPLC studies the fast eluting compound was shown to be the hydrophilic [^{61}Cu]Cu^{2+} cation (2.84 min), while the [^{61}Cu]BLM high molecular weight complex was eluted couple of minutes after (15.33 min). In various studies, n = 9, the purity of both radiochemical spieces were shown to be almost 95% as shown in Fig. 4.

At the optimum reaction pH, the yield reached a maximum within 45 min, and stayed constant for lon-

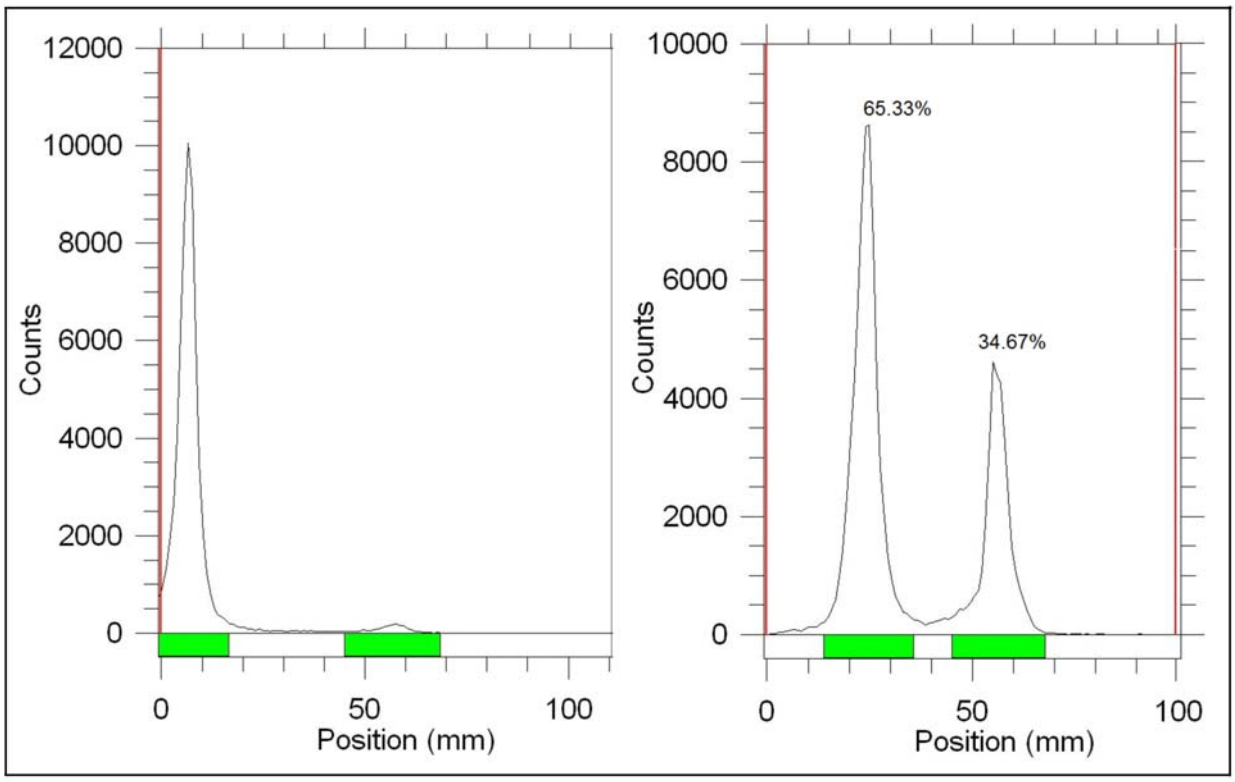

Fig. 3. Radiochromatogram of free Cu^{2+} cation (left) and [^{61}Cu]BLM (right) in 10% ammonium acetate:methanol (1:1) under optimized conditions (n = 5).

ger reaction times. Increasing the ratio of bleomycin to radioactivity increased the labeling yield, presumably due to a more available chelate in the solution (data not shown). The final radiolabeled complex diluted in normal saline was then passed through a 0.22 micron (Millipore) filter for sterilization. Incubation of [^{61}Cu]BLM in freshly prepared human serum for 5 h at 37°C showed no loss of ^{61}Cu from the complex at least for 1 h.

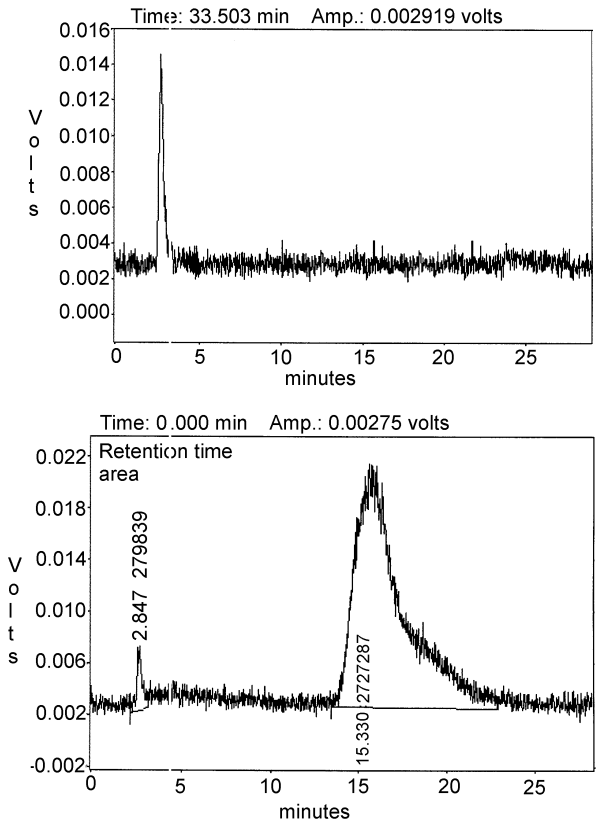

Fig. 4. HPLC chromatogram for free copper cation (up), and [^{61}Cu]BLM (down).

In order to investigate biodistribution of [^{61}Cu]BLM in our animal models, we had to obtain the biodistribution data for free copper cation in our hands, thus after injection of 1.48 MBq of the [^{61}Cu]$CuCl_2$ 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.

The major content of copper is washed out by kidneys and consequently through the urinary tract due to high water solubility of the cation. The uptakes of the rest of tissues are not significant. Copper is also partly accumulated in the liver as a reservoir for many metals transferred by serum ceruloplasmin.

GI accumulation, especially in the first hour, is expressed as a result of liver secretion via hepatobilliary excretion, while it is not significant after 2 h (Fig. 5).

The radiolabeled bleomycins have a similar biokinetics to that of the free BLMs. The major route of excretion for the tracer is the urinary tract similar to BLM, i.e., 70% of the tracer is excreted from kidneys in the first 24 h [25]. As shown in the figure, the urinary tract is almost the major uptake organ up to 3 h. The other significant organ concerning accumulation is the liver and, naturally, the intestine and stomach (Fig. 6).

The uptake of free copper cation must be checked in fibrosarcoma-bearing animals in order to validate the real [^{61}Cu]BLM uptake and not the released ^{61}Cu cation from BLM complex in the case of biodegradation. The tumor uptake in various parts of the tumor

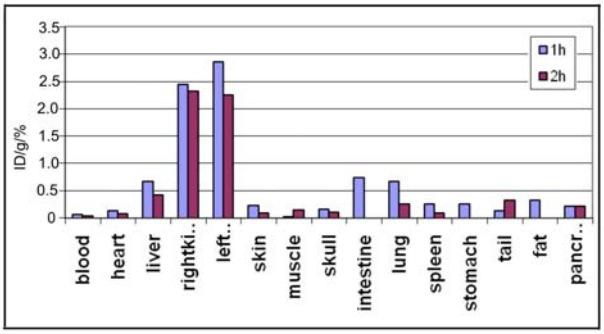

Fig. 5. Calculated ID/g% of [^{61}Cu]$CuCl_2$ 60–120 min post injection of 1.48 MBq IV of the tracer in normal rats.

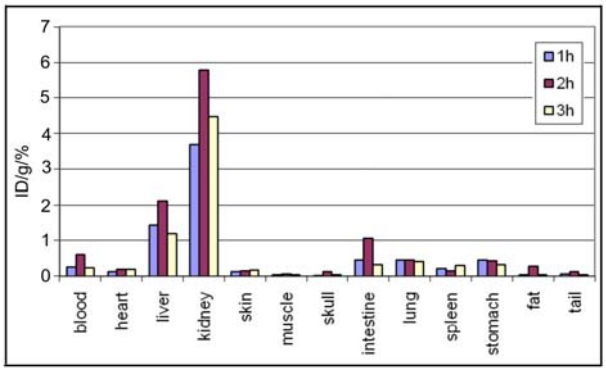

Fig. 6. Calculated ID/g% of [^{61}Cu]BLM 60–180 min post injection of 1.48 MBq IV of the tracer in normal rats.

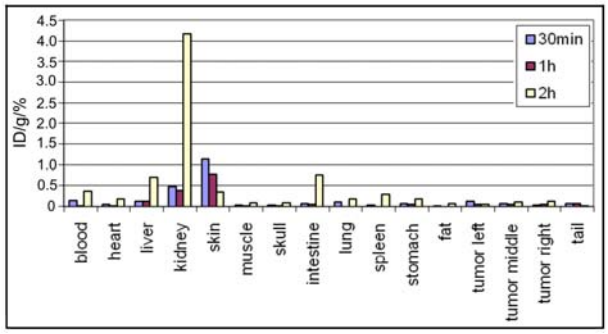

Fig. 7. Calculated ID/g% of [^{61}Cu]$CuCl_2$ 30–120 min post injection of 1.48 MBq IV of the tracer in fibrosarcoma-bearing rats.

were less than 0.1% at all time intervals (30–120). Mean while the kidney and liver demonstrate the excretion after 3 h (Fig. 7).

Figure 8 demonstrates the tracer uptake in tumor--bearing animals. Kidney uptake was removed from the diagram to ease comparison of the organ accumulation. The best accumulation is seen after 3 h since most of the background and circulating tracer have been deleted and the accumulation of the vital organs is obvious. Tumor uptake is significant after 3 h. The uptake can possibly be higher at longer time intervals, however due to the half-life limitation of the tracer (3.3 h) the study was performed at up to 3 h intervals.

The co-incidence imaging was performed for [^{61}Cu]$CuCl_2$ and most of the tracer was accumulated in kidneys as shown by the scarification studies. Thus, the biodistribution studies were validated using imaging. Figure 9 demonstrates the images of the normal rats receiving [^{61}Cu]$CuCl_2$ up to 120 min post injection. The tracer was accumulated in GI, kidneys in the first 20 min,

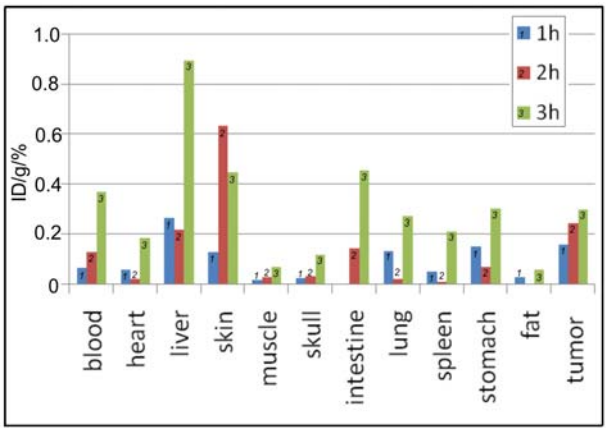

Fig. 8. Calculated ID/g% of [^{61}Cu]BLM 60–180 min post injection of 1.48 MBq IV of the tracer in fibrosarcoma-bearing rats.

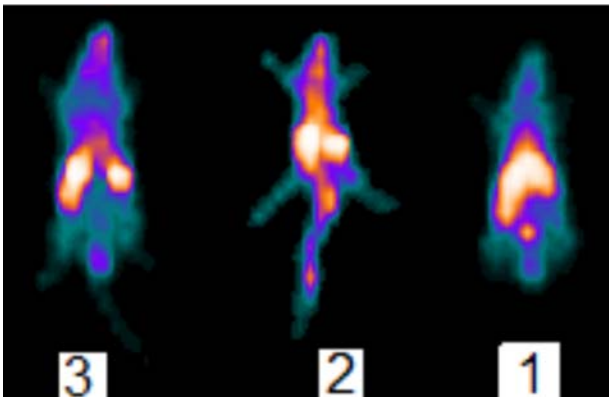

Fig. 9. Co-incidence images for [^{61}Cu]$CuCl_2$ uptake in normal rats 20 (1), 45 (2) and 120 (3) min post injection in normal rats.

while after 45 min the activity was mostly incorporated in kidneys and to a smaller extent in GI system. After 120 min, the activity can only be observed in kidneys with an insignificant tracer uptake in other organs.

In Fig. 10, the images of [^{61}Cu]$CuCl_2$ and [^{61}Cu]BLM are compared and it can obviously be observed that [^{61}Cu]BLM is mostly accumulated in the liver as a radiolabeled peptide antibiotic, while the free Cu cation is washed out through the kidneys showing more urinary accumulation as well as blood circulation through the animal body.

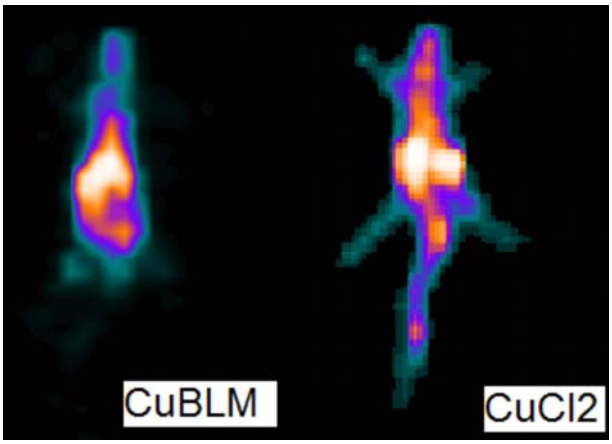

Fig. 10. Comparative co-incidence images for [^{61}Cu]$CuCl_2$ and [^{61}Cu]BLM uptake in normal rats 120 min post injection.

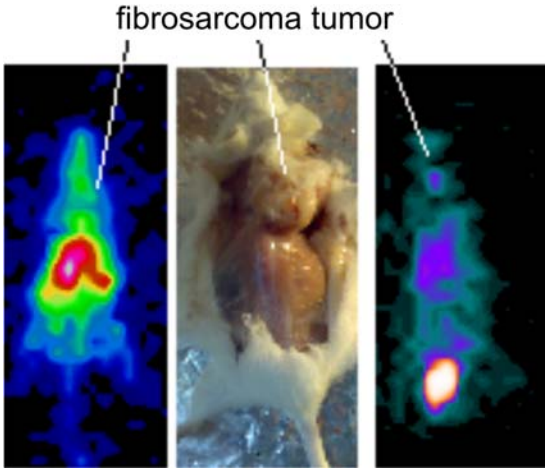

Fig. 11. Co-incidence images for [^{61}Cu]$CuCl_2$ (left) and [^{61}Cu]BLM (right) uptake in a fibrosarcoma-bearing rat (middle) 180 min post injection.

As observed in biodistribution studies in Fig. 8, the best tumor imaging time was 3 h post injection, thus imaging was performed at this time in tumor-bearing animals using free copper as well as [^{61}Cu]BLM. Figure 11 shows the interesting different selective tumor uptake using the two preparations. The major accumulation in the case of [^{61}Cu]BLM is the bladder, while in the ^{61}Cu cation case is the liver and GI.

Discussion

Total labeling and formulation of [^{61}Cu]BLM took about 45 min. The radio-labeled complex was stable in aqueous solutions for at least 2 h and no significant amount of other radioactive species were detected by RTLC, 2 h after labeling. Trace amounts of [^{61}Cu]$CuCl_2$ ($\approx 2\%$) were detected by RTLC which showed that radiochemical purity of the [^{61}Cu]BLM was higher than 98%. The biodistribution of tracer was checked in normal and tumor-bearing animals up to 3 h and a significant accumulation took place in the liver and kidneys, while a significant fibrosarcoma uptake was observed in all animals after 3 h.

The co-incidence imaging of the tracer in the tumor-bearing animals was studied and significant images with [^{61}Cu]BLM were obtained showing the specific tumor uptake in parts of the tumor.

[^{61}Cu]BLM is a potential PET compound with an intermediate half-life, and our experiments on this compound have shown a satisfactory quality, suitable for future animal PET studies.

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