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Preparation, quality control and biodistribution of [^{61}Cu]-doxorubicin for PET imaging

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Abstract This work was conducted for radiolabeling of an anticancer antibiotic, i.e. doxorubicin with ^{61}Cu for production of possible tracer used in PET oncology. ^{61}Cu was prepared with natural zinc target and 22 MeV150 μA protons via ^{nat}Zn(p, xn)^{61}Cu reaction with a yield of 123.2 MBq·μA^{-1}·h^{-1}. Optimization reactions were performed for pH, temperature and concentration. Biodistribution of the tracer was studied in normal and fibrosarcoma bearing mice. At the optimized conditions, ITLC showed that radiochemical purity was over 97% with a specific activity of 2.22×10^{3}MBq ·mmol^{-1}·L^{-1}. This was kept unchanged even with presence of human serum as well as room temperature for 5 h. Biodistribution of the tracer in fibrosarcoma bearing mice demonstrated significant tumor uptake after 2 h. This tracer can be used in the detection of various tumors responding to doxorubicin chemotherapy using PET scan and/or determination of tumor therapy response to doxorubicin chemotherapy.

Key words Copper-61, Doxorubicin, Radiolabeling, Biodistribution

1 Introduction

As part of our researches on radiosynthesis and evaluation of non-fluorine PET radiopharmaceuticals[1,2], we are interested in the production and application of ^{61}Cu tumor seeking radiopharmaceuticals. ^{61}Cu is a positron emitter ($t_{1/2}$=3.33 h, β^+: 62%, EC: 38%), with excellent potentials for PET and molecular imaging applications[3].

Few production methods of ^{61}Cu have been reported for radiolabeling of biomolecules and other applications[4,5]. Also, 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[6], leading to the radiolabelling of small molecules[7,8] for various diagnostic purposes using this radionuclide.

Doxorubicin (Fig. 1) is a tumor seeking antibiotic that has been widely used in cancer chemotherapy[9], mainly activated by a cation insertion as anti-neoplastic agent[10]. The whole complex can then act like an intercalating moiety[11], and/or a hydrogen peroxide producing agent, resulting in DNA decomposition. Thus, labeling of doxorubicin with bi/trivalent radioisotopes can produce pharmacologically active com pounds carrying a diagnostic and/or therapeutic radioisotope for copper-doxorubicin complex[12,13].

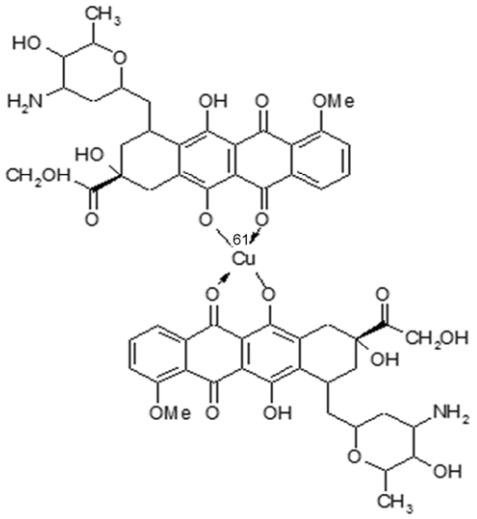

Fig. 1 Possible structure of ^{61}Cu-doxorubicin complex according to literature[14,15].

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It has been shown that copper forms a complex with doxorubicin in 1:2 molar ratio[14] while the EPR spectrum of this complex show that the Cu^{2+} ion is bound at the carbonyl and phenolate oxygen in the 1,4-dihydroxyanthraquinone moiety and the amino nitrogen in the sugar part does not seem to participate in the coordination to the metal ions[15].

In continuation of our works on the development and evaluation of radiocopper tracers, including small molecules[16,17], antibodies[1] and thiosemicarbazones[18], and vast applications of doxorubicin antibiotic in the treatment of fibrosarcomas[19], lymphomas[20], etc, we were interested to develop copper-61 labeled doxorubicin (DXR) using our routine ^{61}Cu production already reported[21] as a positron emitter tracer for use in tumor imaging. We hereby report the preparation, stability tests and biodistribution studies in normal and tumor bearing mice of [^{61}Cu] doxorubicin complex.

2 Experimental

2.1 Materials

Production of ^{61}Cu was performed on a 30 MeV cyclotron (Cyclone-30, IBA) at the Agricultural, Medical and Industrial Research School (AMIRS). Natural zinc chloride in purity of > 98% was from Merck Co., Germany. Doxorubicin was a pharmaceutical sample from Pharmacia laboratories, Italy. Other chemicals were from Sigma-Aldrich Chemical Co., U.K. Instant thin layer chromatography was performed by counting of Whatman No.2 paper thin layer sheets using a thin layer chromatography scanner, Bioscan AR2000, Paris, France. Biodistribution data were acquired by counting normal saline washed tissues after weighting on a Canberra™ HPGe detector (GC1020-7500SL). Radionuclidic purity was checked with the same detector. For activity measurement of the samples, a CRC Capintech Radiometer (USA) was used. All calculations and tissue counting were based on the 283 keV peak. 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.

2.2 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[22] 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. SRIM code[23] was run to determine the best target thickness in the energy range.

2.3 Gold and zinc electrodeposition

A gold containing bath was prepared according to Ref.[24] 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 min.

2.4 Chemical separation

Chemical separation was carried out in no-carrier-added form. The irradiated target was dissolved in 10 mol/L 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 cm×10 cm) which had been preconditioned by passing 25 mL of 9 mol/L HCl. The column was then washed by 25 mL of 9 mol/L HCl with a rate of 1 mL/min to remove copper and zinc ion contents. To the eluent 30 mL water (30 mL) was added to about 100 mL of a 6 mol/L HCl solution. The latter solution was loaded on another exchange resin (AG1X8 Cl^- form, 100~200 mesh, 25cm×1.7 cm) pretreated with 6 mol/L HCl (100 mL). Finally, ^{61}Cu was eluted by 2 mol/L HCl (50mL) in form of [^{61}Cu]$CuCl_2$. The whole process took about 120 min[25].

2.5 Quality control of the product

Gamma spectroscopy of the final sample was carried out with the HPGe detector coupled to a Canberra™ MCA. The peaks were counted for 4 hours. The formation of colored dithizone-zinc complex was measured using visible spectroscopic assay to determine zinc cation concentrations[26] using dithizone organic reagent (0.002% in CCl_4). The amount of gold cation in the final solution was checked using color formation

of acidic rhodamine B reagent reacting with gold dilutions based on a previously reported colorimetric method[27].

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

[^{61}Cu]$CuCl_2$ (37~370 MBq) dissolved in acidic medium obtained above (0.5~2 mL) was transferred to a 2 mL-vial and the mixture was evaporated by slight warming under a nitrogen flow. A mixture of DXR (0.1 mg) in normal saline (0.1 mL) was then added and was kept at 25°C (pH 5~6). The final solution was then passed through a 0.22 μm filter and pH was adjusted to 5~7 by addition of 1 mol/L sodium acetate buffer. The active solution was checked for radiochemical purity by ITLC using a 1:1 mixture of 10% ammonium acetate and 10% methanol as the mobile phase every 15 minutes.

2.7 Quality control of ^{61}Cu-DXR

Radio thin layer chromatography: A 5 μL sample of the final fraction was spotted on Whatman No. 2 paper, and developed in a mixture of 10% ammonium acetate: methanol (1:1) as the mobile phase. Alternatively, 10mmol/L DTPA solution can be used as another mobile phase to discriminate free copper from radiolabeled compound.

2.8 Stability of [^{61}Cu]DXR in the final product

Stability tests were based on previous studies performed for other radiolabeled antibiotics[28]. A sample of [^{61}Cu]DXR (18~180 MBq) was kept at room temperature for 5 hours while checked by ITLC every 30 minutes. A micropipet sample (5 μL) was taken from the shaking mixture and the ratio of free radio-copper to [^{61}Cu]DXR was checked by radio thin layer chromatography (eluent: 10% NH_4OAc and methanol 1:1).

2.9 Serum Stability Studies

The 36.1 MBq of [^{61}Cu]DXR 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 incubation to determine stability of the complex.

2.10 Induction of fibrosarcoma tumors in mice

Tumor induction performed by the use of poly aromatic hydrocarbon injection in rodents as reported previously[29]. 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.

2.11 [^{61}Cu]$CuCl_2$ and [^{61}Cu]DXR biodistribution in normal and fibrosarcoma bearing animals

[^{61}Cu]$CuCl_2$ and [^{61}Cu]DXR were administered to separate normal rat groups. A volume (50 μL) of [^{61}Cu]DXR or [^{61}Cu]$CuCl_2$ solutions containing radioactivity (3.7 MBq for mice and 1 MBq for mouse) were injected intravenously *via* their tail veins. The animals were sacrificed at exact time intervals (1 and 2h for [^{61}Cu]$CuCl_2$ and 30~210 min for [^{61}Cu]DXR), and the %ID/g of different organs was calculated as percentage of injected dose (based on area under the curve of 283 keV peak) per gram using the HPGe detector.

3 Results and discussion

3.1 Targetry and irradiation

For 76 min bombardment of the ^{nat}Zn target with 22 MeV 150 μA protons, the activity of ^{61}Cu was 222 GBq at the end of bombardment (E.O.B.) and the production yield was 440 MBq·μA^{-1}·h^{-1}. Yield from the radiochemical separation was over 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% (E.O.S.). 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 1×10^{-6} mg/kg 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 maxi-

mum permitted levels, i.e. 5×10^{-6} mg/kg (less than 1×10^{-6} mg/kg zinc). The gold concentration was less than 9×10^{-5} mg/kg.

3.2 Radiolabeling

Because of the polar functional groups in doxorubicin, labeling it with a cation does not greatly affect its chromatographic properties. Thus the labeled and unlabeled doxorubicins remain almost at the origin (R_f.0.0). As shown in Fig. 1. The pharmaceutical sample is majorly composed of one component with molecular weight of 579.99 (as hydrochloride), leading to a specific activity of 0.06 Ci/(mmol·L^{-1}) using optimized radiolabeling conditions.

The labeling step took about 120 min. The ratio of Cu-DXR peak at R_f of 0.0 to free Cu^{2+} radiopeak (R_f: 0.6) was considered as the radiochemical purity (Fig.2).

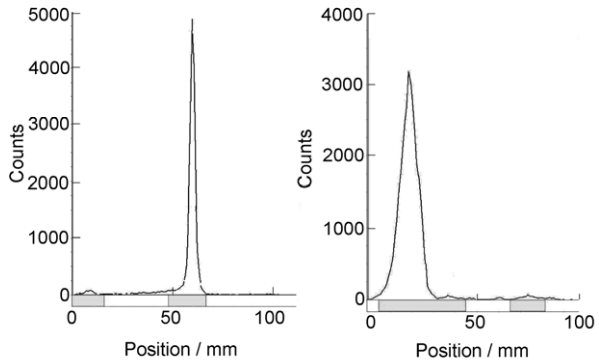

Fig. 2 Radio chromatogram of free Cu^{2+} cation (left) and [^{61}Cu]DXR (right) in 10% ammonium acetate: methanol (1:1) at the optimized conditions (n=5).

For labeling conditions, the best pH for the labeling step was 5~6 at 25°C. The structural studies on the copper-DXR complex already performed have demonstrated a stable complex at above conditions[14,15]. Thus we chose these conditions to avoid the formation of any other new species.

At the optimum reaction pH, the radiochemical purity reached a maximum within 100~120 minutes, and stayed constant for long reaction time. However a solid phase system could be used for the separation of the free cation from the radiolabeled compound in order to decrease the reaction time. Increasing the ratio of doxorubicin to radioactivity could increase the labeling yield, presumably, due to more available chelate in solution (data not shown). The final radiolabelled complex diluted in normal saline was then passed through a 0.22 micron (Millipore) filter for sterilization. In case of autoclaving, [^{61}Cu]DXR preparation could totally be degraded and left detectable amounts of free copper due to its thermal instability. Incubation of [^{61}Cu]DXR in freshly prepared human serum for 5 hours at 37°C showed no loss of ^{61}Cu from the complex. At the optimized conditions, ITLC showed radiochemical purity of 97%.

3.3 Biodistributions

3.3.1 [^{61}Cu]$CuCl_2$ in normal mice

In order to investigate biodistribution of [^{61}Cu]DXR in animal models, we had to obtain the biodistribution data for free copper cation. Thus injection of 3.7 MBq of the [^{61}Cu]$CuCl_2$ pre-formulated by the normal saline (pH. 6.5~7) through the tail vein of adult mice the bidistribution of the cation was checked in various vital organs.

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. Copper is also partly accumulated in liver as a reservoir for many metals transferred by serum ceruloplasmin (Fig. 3).

3.3.2 [^{61}Cu]DXR in normal mice

The radiolabeled doxorubicin has similar biokinetic to the free DXR. The major route of excretion for the tracer is urinary tract similar to DXR, i.e., 38% of the tracer is excreted from kidneys in first 24 h[30]. As shown in Fig. 3 urinary tract is almost the major uptake organ up to 3 h. The other signifcat organ with accumulation is liver and naturally in intestine and stomach, however this accumulation is not related to the doxorubicin metabolism, but is mostly due to the release and/or metabolism of free copper from the antibiotic[28]. In order to determine [^{61}Cu]DXR biodistribution in normal mice, experiments were also performed by the injection of tracer. [^{61}Cu]DXR was accumulated in lung, heart and spleen as well as liver and kidneys as excretory organs. There are several reports on the cardiotoxicity and cardial uptake of doxorubicin antibiotic in the literature based on interfering with mitochondrial oxido/redox system, explaining the myocardial uptake of the tracer (Fig. 3).

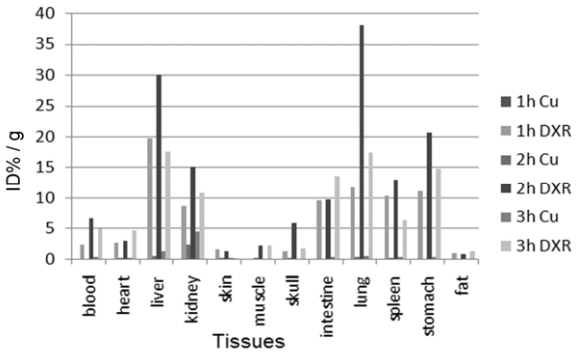

Fig. 3 Calculated %ID/g of [^{61}Cu]$CuCl_2$ and [^{61}Cu]DXR 60~180 minutes post injection of 3.7 MBq i.v. of the tracer in normal mice.

3.3.3 [^{61}Cu]$CuCl_2$ in fibrosarcoma-bearing mice

The uptake of free copper cation must be checked in fibrosarcoma-bearing animals in order to validate the real [^{61}Cu]DXR uptake and not the released ^{61}Cu cation from DXR complex in case of biodegradation. The tumor uptake in various parts of the tumor were less than 0.1% in all time intervals. While kidney and liver demonstrate the excretion after 3 h (Fig. 4).

3.3.4 [^{61}Cu]DXR in fibrosarcoma-bearing mice

Fig.4. demonstrates the tracer uptake in tumor-bearing animals. Kidney uptake was removed from the diagram to ease the comparison of the organ accumulation. The best accumulation was observed after 2 h since most of background and circulating tracer has been deleted and the accumulation of the vital organs is obvious (Fig. 4).

4 Conclusion

The method used in this research for the production and chemical separation of ^{61}Cu was quite simple and cost effective. Total labeling and formulation of [^{61}Cu]DXR took about 120 minutes, with a yield of higher than 97%. The radio-labeled complex was stable in aqueous solutions for at least 5 hours and no significant amount of other radioactive species were detected by ITLC, 5 hours after labeling. Trace amounts of [^{61}Cu]$CuCl_2$] (≈2%) were detected by ITLC which showed that radiochemical purity of the [^{61}Cu]DXR was higher than 97%. The biodistribution of tracer was checked in normal and tumor-bearing animals up to 4 hours and a significant accumulation took place in liver and kidneys, while significant fibrosarcoma uptake was observed in all animals at 2 h.

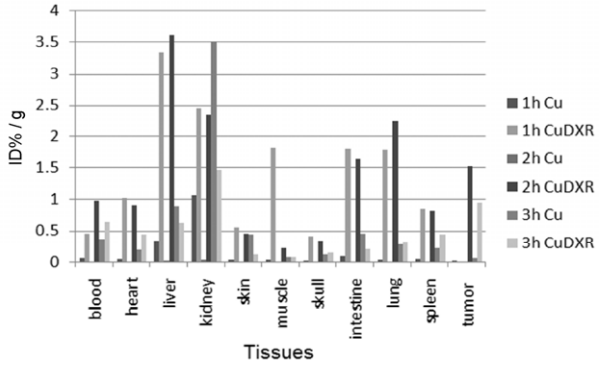

Fig. 4 Calculated %ID/g of [^{61}Cu]$CuCl_2$ and [^{61}Cu]-DXR 60~180 minutes post injection of 3.7 MBq i.v. of the tracer in fibrosarcoma-bearing mice.

[^{61}Cu]DXR can be a PET tracer with an intermediate half life, and our experiments on this tracer have shown satisfactory quality, suitable for future PET studies.

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