

## Preparation, distribution, stability and tumor imaging properties of [<sup>62</sup>Zn] bleomycin complex in normal and tumor-bearing mice

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### ABSTRACT

**Backgrounds:** Bleomycin (BLM) has been labeled with radioisotopes and widely used in therapy and diagnosis. In this study BLM was labeled with [<sup>62</sup>Zn] zinc chloride for oncologic PET studies.

**Materials and methods:** The complex was obtained at the pH=2 in normal saline at 90°C in 60 min. Radio-TLC showed an overall radiochemical yield of 95-97% (radiochemical purity > 97%). Stability of complex was checked *in vitro* in mice and human plasma/urine.

**Results:** Preliminary *in vivo* studies performed to determine complex stability and distribution of [<sup>62</sup>Zn] BLM in normal and fibrosarcoma-bearing mice. [<sup>62</sup>Zn] BLM accumulated significantly in induced fibrosarcoma tumors in mice according to bio-distribution/imaging studies.

**Conclusion:** [<sup>62</sup>Zn] BLM can be used in PET oncology studies due to its suitable physico-chemical properties as a diagnostic complex *in vitro* and *in vivo*. Further studies should be performed for evaluation of the complex behavior in higher animals. *Iran. J. Radiat. Res.*, 2003; 1(1): 37 - 44.

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**Keywords:** PET, pharmacokinetic, biodistribution, zinc-62, bleomycin.

### INTRODUCTION

Several radiolabeled bleomycin derivatives have been developed for imaging and / or therapy of neoplastic tissues.

The most important compounds contain indium-111 (Umezawa *et al.* 1965), Cobalt - 57 (Umezawa *et al.* 1966), technetium - 99m (Naganawa *et al.* 1977) and radioferric salts (Burger *et al.* 1981). Technetium complexes of Bleomycin did not form any suitable tracers for

imaging due to their low radiochemical yield, while trivalent radioisotopes like Cobalt - 57 and Indium-111 afforded stable ones. Recently some other new complexes, like Rhodium-105, have been studied for therapeutic purpose (Brooks *et al.* 1999).

Zn-62 (HL=6.9 h, EC: 3 %, β<sup>+</sup>: 97%) is a rather long-half life PET radioisotope mostly used in preparation of <sup>62</sup>Zn/<sup>62</sup>Cu generators (Green, *et al.* 1990), but its direct use has not been reported in labeling or imaging studies. [<sup>62</sup>Zn] labeled bleomycin preparation had been once reported without further biological studies (Neirinckx 1977). The aim of this study was to investigate the possibility of labeling bleomycin

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with zinc-62 for use in positron emission tomography. Due to interesting properties and increasing importance of PET radiotracers, we optimized its complex formation conditions with bleomycin, in order to develop [ $^{62}\text{Zn}$ ] BLM as a tumor imaging agents. We report preparation, optimization, stability, bio-stability, formulation and tumor imaging studies of [ $^{62}\text{Zn}$ ]-Bleomycin complex.

## MATERIALS AND METHODS

Chemicals were purchased from Aldrich Chemical Company, Milwaukee, WI. Bleomycin sulfate (BLM-S) was a pharmaceutical sample purchased from Nippon Kayaku laboratories, Japan. Thin layer chromatography (TLC) was performed on silica gel polymer-backed (F 1500/LS 254, 20 x 20 cm, TLC Ready Foil, Schleicher and Schuell®). Methanol and normal saline used for labeling were of high purity. A mixture of ammonium acetate 10% methanol (1:1) was used as eluent. Radio-chromatography is performed by counting different 5 mm slices of polymer-backed silica gel paper using a Canberra™ high purity germanium detector (model GC1020-7500SL). All calculations and TLC counting was performed based on 511 KeV peak. Animal experiments were carried out in compliance with standard protocols and guidelines.

### *Preparation of [ $^{62}\text{Zn}$ ] zinc chloride from natural copper solid target*

[ $^{62}\text{Zn}$ ] Zinc chloride was prepared by 30 MeV proton bombardment of a natural electroplated copper-target in a 30 MeV cyclotron (Cyclone-30, IBA) based on a method described previously with slight modification (Green *et al.* 1990). After dissolution of the irradiated target by 8N  $\text{HNO}_3$ , the solution was heated under a flow of nitrogen until a precipitate is formed. The residue was rinsed 2 times by distilled water (10 ml) and a portion of HCl 2N was added and mixed gently. The solution was passed through a cation exchange resin (Dowex 1X8) followed by

washing the column by HCl 2N solution. High purity zinc chloride solution was used directly in the labeling step.

### *Labeling of bleomycin with [ $^{62}\text{Zn}$ ] zinc chloride*

[ $^{62}\text{Zn}$ ] Zinc chloride (0.25-2.5 mCi) dissolved in acidic media obtained above (0.5-2 ml) was transferred to a 2 mL-vial and pH was adjusted using HCl 1M and/or NaOH 1M (pH=1-7). The mixture was evaporated by slight warming under a nitrogen flow. A mixture of BLM (0.25-2.5 mg) in normal saline (0.1 mL) was then added. This mixture was heated at different temperatures (25, 50, 80 and 100°C). The mixture was cooled in an ice bath and rapidly sent for use. The active solution was checked for radiochemical purity by polymer-backed silica gel layer using a mixture of ammonium acetate 10%-methanol as the mobile phase. Radio thin layer chromatography showed two major and distinct radio peaks at the  $R_{\text{fs}}$  of 0.4 and 0.70. The radiochemical yields (>95% in each case) was also determined by RTLC method. These analyses were carried out every 30 min after labeling step. The final solution was then passed through a 0.22  $\mu$  filter and pH was adjusted to 5-7 by the addition of sodium acetate (1M) buffer. The gamma spectroscopy of the final sample was obtained by a HPGe detector and showed a radio-nuclide purity higher than 98%. Pyrogen test was performed using a commercial LAL kit. Microbial-fungal tests showed a suitable pharmaceutical sterility.

### *Stability of [ $^{62}\text{Zn}$ ] BLM complex in final product*

A sample of [ $^{62}\text{Zn}$ ] BLM (0.5 mCi) was kept at room temperature for 48 h while checked by RTLC at various time intervals (2, 4, 8, 12 and 24). A micropipet sample (50  $\mu\text{L}$ ) was taken from the shaking mixture and the ratio of free radiozinc to [ $^{62}\text{Zn}$ ] BLM was checked by radio thin layer chromatography (eluent: 10%  $\text{NH}_4\text{OAc}$  buffer and methanol 1:1). The patterns for [ $^{62}\text{Zn}$ ]  $\text{ZnCl}_2$  and [ $^{62}\text{Zn}$ ] BLM were not changed in 24 h.

### Stability of [<sup>62</sup>Zn] BLM complex in human and mice serum in vitro

A mixture of 5 parts of serum and one part radiopharmaceutical (0.2 mCi) was shaken in a 37-degree incubator under nitrogen atmosphere. A micropipet sample (50 μL) was taken from the shaking mixture every 30 min. The ratio of free radiozinc [ (R<sub>f</sub>=0) to [<sup>62</sup>Zn]BLM (R<sub>f</sub>= 0.4 & 0.7)] was checked by radio thin layer chromatography (eluent: pH 5.6 NH<sub>4</sub>OAc buffer and methanol 1:1).

### Stability of [<sup>62</sup>Zn] BLM complex in human urine

A mixture of 5 parts of healthy human urine and one part radiopharmaceutical (0.2 mCi) was incubated at 37°C under nitrogen atmosphere. A micropipet sample (50 μL) was taken from the shaking mixture every 30 min. The ratio of free radiozinc [ (R<sub>f</sub>=0) to [<sup>62</sup>Zn]BLM (R<sub>f</sub>= 0.4 & 0.7)] was checked as above.

### In vitro studies

Cell line of murine fibroblastoma were used for experiments. For each culture 1-2 × 10<sup>4</sup> cells were seeded into a 75 cm<sup>3</sup> flask containing 20 ml of medium supplemented with 10% fetal bovine serum and 1% glutamine. Cells were incubated at 37°C in 5% CO<sub>2</sub>. The cell line was maintained in exponential growth phase and passaged twice per week.

### Animal studies

Fibrosarcoma cells (about 10<sup>4</sup>) were injected SC to the dorsal area of Balb / C mice weighing 20-25 g. After 14 days the tumor weighed 0.7 g and was not grossly necrotic. The distribution of [<sup>62</sup>Zn] ZnCl<sub>2</sub> and [<sup>62</sup>Zn] BLM among tissues were determined for untreated mice and for mice with fibrosarcoma. A volume (0.1 ml) of final [<sup>62</sup>Zn] BLM solution containing 20-40 μCi radioactivity (≤ 6 μg bleomycin in 50 μL) was injected into the dorsal tail vein. The total amount of radioactivity injected into each mouse was measured by counting the 1-ml syringe before and after injection in a radiometer with a fixed geometry. The animals were sacrificed by

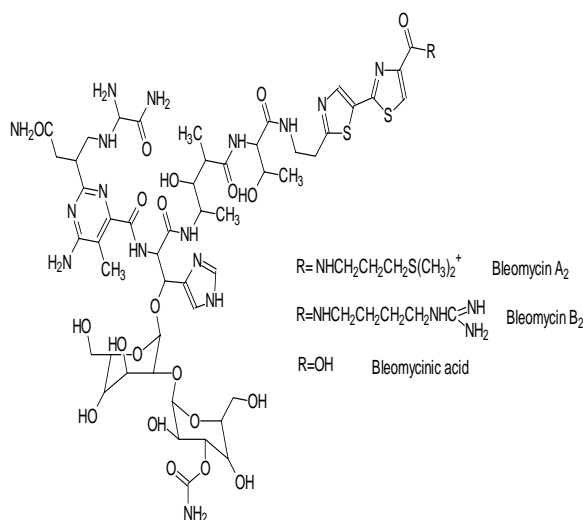
ether asyxplication at selected times after injection, the tissues weighed and their specific activities determined with a γ-ray scintillation as percentage of injected dose per gram of tissues (tables 1 & 2).

### Imaging of [<sup>62</sup>Zn] BLM in tumor bearing mice

Fibrosarcoma-bearing mice were used for tumor imaging when the tumors had reached a size of 1.5-2 cm at 2-3 weeks after it's induction. Images were taken 1, 2, 4, 6 and 8 h after administration of the radiopharmaceutical in the coincidence mode by a Dual-Head SPECT system (SMV, France, Sopha DST-XL). The mouse-to-high energy septa distance was 12 cm. Images were taken from both normal and tumor-bearing mice.

## RESULTS AND DISCUSSION

Bleomycin is an antineoplastic agent; widely used in therapy (Jaaskela-Saari *et al.* 1998) this compound produces suitable and stable complexes with cations like Mg<sup>2+</sup>, Ca<sup>2+</sup>, Fe<sup>2+</sup>, In<sup>3+</sup> (figure 1) (Umezawa *et al.* 1972).



**Figure 1.** Structures of commercial Bleomycin components

It is believed that these antibiotics interfere with DNA as false nucleotides assuming the

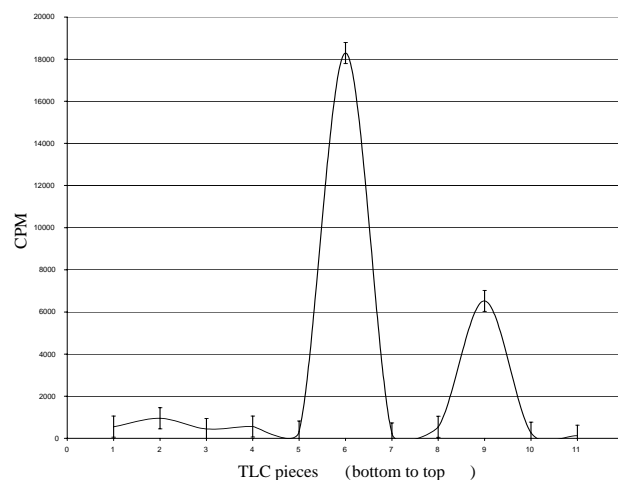
dithiazole moiety acts like a purine base (Hoehn *et al.* 2001). On the other hand, these compounds are activated by a cation insertion as an anti-neoplastic agent. The whole complex then can act like a peroxidase system by the production of hydrogen peroxide, resulting in DNA decomposition (Umezawa *et al.* 1966).

Thus, labeling of Bleomycins with bi/trivalent radioisotopes produces pharmacologically active compounds carrying a diagnostics and/or therapeutic radioisotope (Korppi-Tommola *et al.* 1999). In-111 labeled Bleomycin ( $^{111}\text{In}$ -BLM) has been widely used as a therapy/diagnostic agent since 1970's up to now (Jekunen *et al.* 1996, Kairemo *et al.* 1997). Zinc cation coordinates with at least five nitrogen atoms of bleomycin, based on NMR studies (Williamson *et al.* 1990, Vanbelle *et al.* 2000). This coordination forms a rather stable complex. Cell toxicity of Zn-Bleomycin has been studied and tested in human and different animals (Sausville *et al.* 1978). The antitumor activity of Zn-Bleomycin complex has been elucidated in some human tumor models (Lyman *et al.* 1986) suggesting the possibility of application of radiozinc-bleomycin complexes in human tumor imaging.

### Labeling

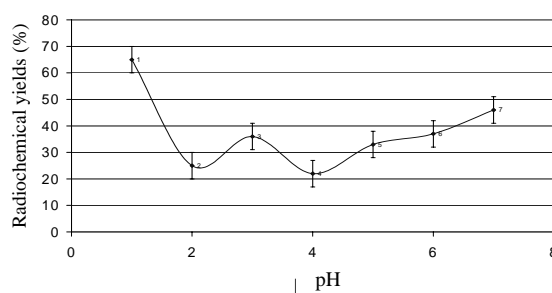
Because of several polar functional groups in its structure, labeling of bleomycin with a cation does not affect its chromatographic properties, so that the labeled and unlabeled bleomycin almost migrate to the same  $R_f$ . The more polar bleomycin fraction, i.e., bleomycin  $A_2$  correlates to the less  $R_f$ , the two other polar fractions come at the close  $R_f$ s (bleomycin  $B_2$  and bleomycinic acid). In all radiolabeling procedures ( $n=5$ ), the area under curve ratio of two peaks was constant ( $C_1: C_2 \& C_3, 0.7:0.3$ ), showing the isomeric ratio of the two bleomycin chromatogram peaks (figure 2).

In order to obtain the best labeling reaction conditions, the complex formation was optimized for pH, temperature, time, and the amount of bleomycin.



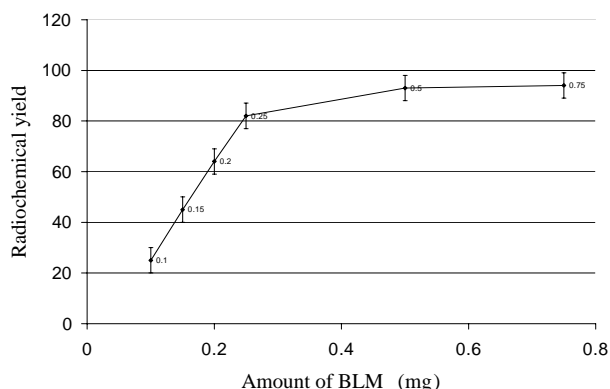
**Figure 2.** Radio chromatogram of a  $[^{62}\text{Zn}]$  BLM sample at optimized condition.

At a random temperature ( $80^\circ\text{C}$  for instance), the best pH for the labeling step was 2, while at higher pHs (5-6) the radiochemical yield is increasing again due to the formation of different labeled species (figure 3). At basic conditions the radiochemical yield decreased drastically due to degradation of bleomycin to less soluble compounds.



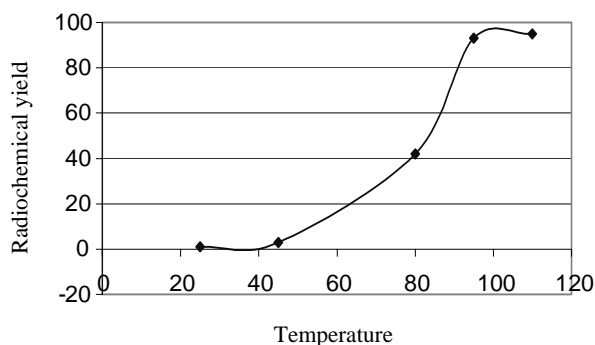
**Figure 3.** Effect of pH on radiochemical yield of  $[^{62}\text{Zn}]$  BLM at  $80^\circ\text{C}$ .

At the optimum reaction temperature and pH, the yield reached a maximum within 25 min, and stayed constant for longer reaction times. Increasing the ratio of bleomycin to radioactivity increased the labeling yield, presumably due to more available chelate in solution (figure 4).



**Figure 4.** Effect of amount of BLM on radiochemical yield of [<sup>62</sup>Zn] BLM at 80°C.

Heating the reaction mixture to 90°C increased the yield which remained constant for temperatures up to 100°C. Further heating reduced the radiochemical yield due to decomposition of bleomycin and/or product (figure 5).



**Figure 5.** Effect of temperature on radiochemical yield of [<sup>62</sup>Zn] BLM at optimized conditions.

Twenty five to forty percent of the activity remained on 0.22 millipore filters when filtration was used to sterilize the product. The thermal stability of [<sup>62</sup>Zn] BLM was excellent so that autoclaving a [<sup>62</sup>Zn] BLM preparation showed no change in the amount of free zinc present. The biological stability of [<sup>62</sup>Zn] BLM was high enough to perform scanning due to high stability

presence of 3-5% free zinc on the RTLC before and after autoclaving indicates that the preparation may be sterilized by this technique. Due to decay of zinc-62 to copper-62 in 9h, [<sup>62</sup>Zn] BLM complex produces the stable complex, [<sup>62</sup>Cu] BLM, which retains tumor affinity. The biological stability of [<sup>62</sup>Zn] BLM was high enough to perform scanning due to high stability of the final product in presence of murine/human blood serum and urine so that RTLC showed no change in the amount of free zinc up to 6 h. The presence of 3-5 % free zinc on the RTLC even after 6 h was unchanged.

#### **Biodistribution in animal tissues**

Final [<sup>62</sup>Zn] BLM solution was injected into the dorsal tail vein of test animals. The animals were sacrificed by ether asphyxiation at selected times after injection, the tissues weighed washed with saline and their specific activities (percentage of injected dose per gram) determined by  $\gamma$ -ray scintillation method.

Liver and spleen uptake increased 2-4 h after administration of [<sup>62</sup>Zn] BLM. Lung uptake increased after 4 h. After 2 h the radioactivity of bladder and kidney increased and maintained constant for the next few hours, like that of unlabeled bleomycin, suggesting the stable incorporation of Zn-62 into bleomycin core.

These observations were quite different from the biodistribution of [<sup>62</sup>Zn] ZnCl<sub>2</sub> which shows rapid washout from kidneys in the first 2-4 h. A late increase in liver uptake was observed that can be due to the accumulation of metalloproteins in this tissue (tables 1 and 2). Our results were similar in some aspects with *in vivo biodistribution* experiments previously done for [<sup>111</sup>In] bleomycin. [<sup>62</sup>Zn] Bleomycin is rapidly tagged in tumor and scanning can be done in rather short times after I.V. injection. Lower half-life of Zn-62 in contrast to In-111 is another important advantage leading to less radiation exposure to patients.

**Table 1.** Biodistribution of [<sup>62</sup>Zn] BLM in organs of tumor-bearing mice (n=5) (%ID/g tissue), Avg.: average, SD: standard deviation

Organ	Time (hr)							
	1		2		4		8	
	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD
Blood	2.49	0.44	3.92	0.54	4.46	0.26	4.99	0.27
Liver	3.1	0.23	2.99	0.6	3.44	0.71	3.96	0.78
Kidney	1.0	0.06	3.66	0.88	7.30	1.12	7.86	1.15
Stomach	1.3	0.08	2.7	0.54	4.7	0.84	5.12	1.01
Colon	0.44	0.05	0.85	0.05	1.21	0.05	1.34	0.09
Stool	1.62	0.07	1.92	0.1	2.18	0.13	3.52	0.19
Bladder	1.12	0.06	2.39	0.15	3.48	0.21	4.84	0.35
Sternum	0.81	0.02	0.78	0.02	0.73	0.05	0.67	0.02
Lung	1.1	0.03	1.74	0.55	2.20	1.94	2.64	0.54
Skin	1.65	0.09	1.75	0.12	1.76	0.15	1.81	0.12
Muscle	7.26	1.11	5.12	0.91	3.32	0.42	1.87	0.25
Tumor	4.33	0.85	5.32	0.99	5.34	0.92	5.77	1.01

**Table 2.** Bio distribution of [<sup>62</sup>Zn]-ZnCl<sub>2</sub> in organs of tumor-bearing mice (n=5) (%ID/g tissue), Avg.: average, SD: standard deviation

Organ	Time (hr)							
	1		2		4		8	
	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD
Blood	1.33	0.04	1.02	0.14	0.35	0.06	0.19	0.07
Liver	0.12	0.03	0.19	0.06	0.34	0.02	0.56	0.18
Kidney	1.0	0.06	1.16	0.18	1.30	0.12	0.86	0.15
Stomach	0.63	0.08	2.7	0.54	1.7	0.44	1.12	0.01
Colon	0.64	0.05	1.25	0.05	0.84	0.15	0.34	0.09
Stool	0.62	0.07	1.92	0.1	0.58	0.13	0.52	0.09
Bladder	3.12	0.56	2.79	0.15	2.48	0.21	1.84	0.15
Sternum	0.80	0.02	0.73	0.02	0.79	0.05	0.72	0.02
Lung	1.02	0.03	0.94	0.25	0.80	0.04	0.64	0.14
Skin	0.63	0.09	0.75	0.12	0.75	0.15	0.81	0.16
Muscle	0.26	0.11	0.32	0.11	0.29	0.02	0.17	0.05

### Imaging

[<sup>62</sup>Zn] BLM imaging was performed with a Dual-Head SPECT equipped with a coincidence detection system. The useful field of view (UFOV) was 540 mm × 400 mm.

The spatial resolution in the coincidence mode was 10 mm FWHM at the CFOV, and sensitivity was 20 Kcps/μCi/cc. Sixty four projections were acquired for 30 seconds per view with a 64 × 64 matrix.



**Figure 6.** Co-incidence images of fibrosarcoma-bearing mice 2 h after I.V. injection of [<sup>62</sup>Zn] BLM

### Conclusion

Total labeling and formulation of [<sup>62</sup>Zn] BLM took about 60 min, with a yield of 95-97%. A suitable specific activity product was formed via insertion [<sup>62</sup>Zn] zinc cation. No unlabelled and/or labeled by-products were observed upon TLC or HPLC analysis of the final preparations. The radio-labeled complex was stable in aqueous solutions at least for 24 hours, no significant amount of other radioactive species was detected by HPLC 24 hours after labeling. Trace amounts of [<sup>62</sup>Zn] zinc chloride (<3%) were detected by paper chromatography. HPLC and TLC showed that radiochemical purity of the [<sup>62</sup>Zn] labeled components was >95%. In contrast to other labeled bleomycins, [<sup>62</sup>Zn] bleomycin, has a lower half life causing less undesirable irradiation and it also benefits from PET radiopharmaceutical advantages. Its rather higher half life in contrast to other PET radioisotopes and high chemical stability of radiopharmaceutical form makes it a suitable possible PET tracer for use in neighborhood PET centers.

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