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Original Article

Lethality Assay of Radiopharmaceutical *bis*-Thiosemicarbazones Using Brine Shrimp (*Artemia salina*) Test

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Abstract

In the present study, aqueous solutions of some copper-complexing ligands were screened for their cytotoxicity using brine shrimp lethality test. Among the ligands tested, diacetyl-*bis*(N^4-methylthiosemicarbazones) (ATSM) proved to be the most safe and non-toxic compound (2% lethality at 10 ppm), while pyruvaldehyde *Bis*(N^4-methyl)thiosemicarbazone (PTSM) was shown to possess low toxicity (7% lethality at 10 ppm) and glyoxal-bis-thiosemicarbazone (GTS) proved to be a toxic compound even at 1 ppm (20% lethality). An interesting structure-toxicity relationship was observed for the ligands based on their water solubility leading to more toxicity which can be related to polysaccharide crustae of the shrimps. Considering the 1-10 ppm to be the maximum possible concentration of the ligands in the final pharmaceutical samples, the safety of these ligands are ATSM>PTSM>GTS.

Keywords: Brine shrimp; Cytotoxicity; Radiopharmaceutical; *bis*-Thiosemicar bazones.
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1. Introduction

Metal-based radiopharmaceuticals are major portion of radiopharmaceuticals used in the world. They usually consist of a cation and an organic moiety going through a complexation reaction. The pharmacological evaluation of residual starting ligands remained in the final preparation is of great importance. Copper *bis*-thiosemicarbazones (Cu-bTSC) have attracted attentions as possible positron emission tomography (PET) radiopharmaceuticals and have already been tested in various human studies. Among those, Cu-pyruvaldehyde *bis*(N^4-methyl)thiosemicarbazone (Cu-PTSM) is categorized as a perfusion agent [1, 2]. Another interesting radiotracer in this group, is Cu-diacetyl-bis(N^4-methylthiosemicarbazones) (Cu-ATSM) for imaging hypoxic tissue with PET [3, 4], determining biological behavior of malignant solid tumors for therapeutic modality determination in oncology [5]. Both compounds as well as many other research Cu-

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bTSC tracers are produced at the radiopharmaceutical grade by mixing the appropriate starting bTSC ligands with copper radionuclides in buffrers followed by solid-phase extraction using reverse phase column to remove free copper from the mixture. However there are no current starting ligand removal procedure in the practice and it is highly probable that ligand residuals are found in the final pharmaceutical solutions (Figure 1).

In order to study the toxicity of these residual ligands in final preparations, we performed brine shrimp lethality bioassay which based on the ability to kill laboratory cultured brine shrimp (*Artemia salina*). The brine shrimp assay was proposed by Michael *et al.* [6], and latter developed by Vanhaecke *et al.* [7]. The assay is considered a useful tool for preliminary assessment of toxicity and it has been used for the detection of fungal toxins, plant extract toxicity, heavy metals, pesticides and cytotoxicity testing of dental materials [8] and synthetic organic compounds [9].

The method is attractive because it is very simple, inexpensive and low toxin amounts are sufficient to perform the test in the microwell scale. In the present work, we report the cytotoxicity studies on some in-house synthesized bis-thiosemicarbazone radiopharmaceutical ligands and the results obtained were described.

2. Materials and methods

The starting materials were purchased from Aldrich Chemical Company. Proton NMR spectra were obtained on a Brucker FT-80 (80 MHz) or a Varian Unity plus (400 MHz) instrument with tetramethylsilane as internal standard. Mass spectra were recorded by a Finnigan Mat TSQ-70 Spectrometer. All chemicals were recrystallized repeatedly before use. Infrared spectra were taken on a Perkin-Elmer 781 spectrometer (KBr disks). Thin-layer chromatography (TLC) of products was run on silica gel polymer-backed (F 1500/LS 254, 20×20 cm, TLC Ready Foils Schleicher & Schuell) or glass plates (25×35 cm, E-Merck). Dimethyl sulfoxide (DMSO) used for labeling experiments were of `Sure-Seal` grade (Aldrich). Analytical HPLC to determine the purity of ligands was performed by a Shimadzu LC-10AT, armed with UV-visible (Shimadzu) using Si Kromasil 100, 5 μm 250×4.6 mm (M & W), Inchrom. A mixture of acetonitrile-chloroform (65:35) was used as eluent at a flow rate of 2 ml/min (Rt=7.5 min). Melting points were determined on a Reichert-Jung hot stage microscope and are uncorrected. Elemental microanalyses were within ±0.4% of theoretical values for C, H and N. brine shrimp eggs were purchased from INVE aquaculture, Thailand. Sea-water was from Texas, USA Noah Technologies. All stock solutions were passed through a 0.22 micron biological filter, Waters.

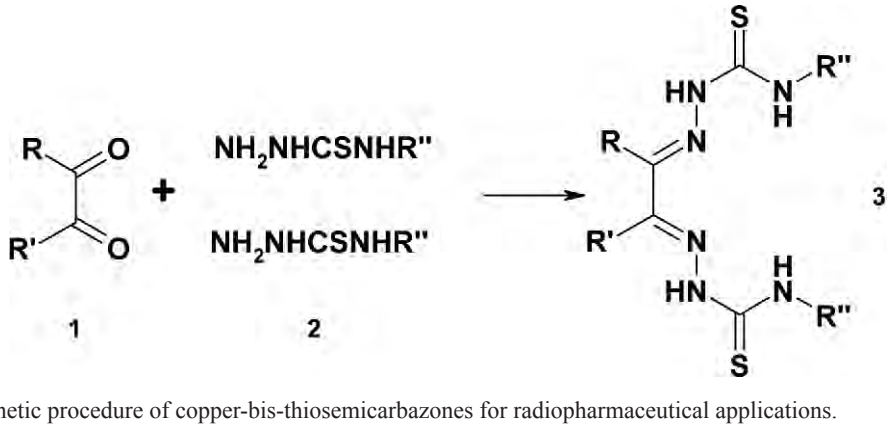

Figure 1. Synthetic procedure of copper-bis-thiosemicarbazones for radiopharmaceutical applications.

2.1. bis-thiosemicarbazone ligands

bTScs were prepared according the method for production of thiosemicarbazones starting N_4-substituted thiosemicarbazide and di-carbonyl compound which was consistent with results of the previous study reported by Gingras *et al.* [10]. Briefly, to a transparent stirring mixture of N_4-substituted thiosemicarbazide 2 in 5% acetic acid at 50 °C, was added drop wise freshly distilled carbonyl compound 1 during 5 min. The mixture was stirred for another 30 min at 50 °C. The reaction mixture was cooled down in an ice bath and the precipitate was filtered. The precipitate was washed with water (10 ml), ethanol (20 ml) and finally dried in oven at 70-80 °C for at least 8 h. The residue can be further purified by refluxing the mixture of the precipitate in 80% acetic acid at 50-70 °C for 10-14 h. The filtered mass was heated in an oven at 80 °C and finally crystallized from hot ethanol to give a light yellow powder (60-70%). The spectroscopic data on the ligands have been reported previously [11, 12].

2.2. Preparation of samples

The chemicals were dried in oven overnight under vacue and 1 mg/ml stock solutions were prepared for the preparation of serial dilutions, the solutions were filtered through the Millex filters (0.22 micron) and kept in fridge and darkness.

2.3. Cytotoxicity bioassay

Brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of synthesized ligands. Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a conical shaped vessel (1 L), filled with sterile artificial seawater (prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1 N NaOH) under constant aeration for 48 h. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Twenty nauplii were drawn through a glass capillary and placed in each vial containing 4.5 ml of brine solution. In each experiment, 0.5 ml. of the compound stock solutions in DMSO was added to 4.5 ml of brine solution and maintained at room temperature for 24 h under the light and surviving larvae were counted. Experiments were conducted along with control (DMSO treated), different concentrations (1, 10 and 100 ppm) of the test substances in a set of three tubes per dose.

2.4. Lethality concentration determination

The percentage lethality was determined by comparing the mean surviving larvae of the test and control tubes. Values were obtained from the best-fit line plotted concentration verses percentage lethality. Taxol was used as a positive control in the bioassay.

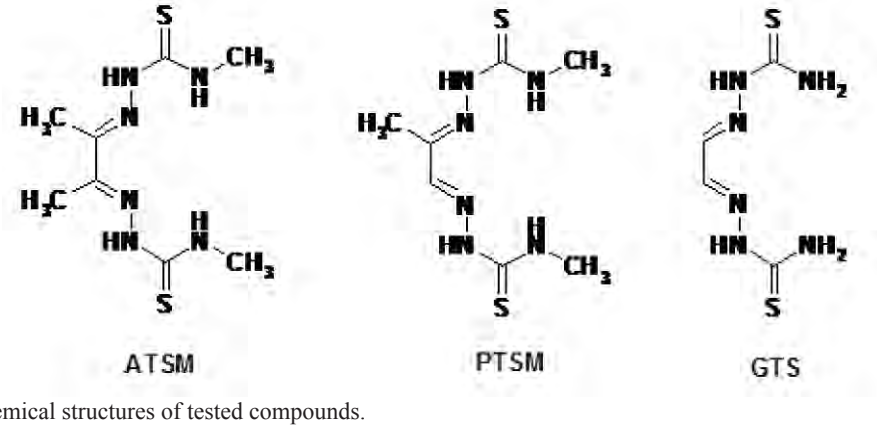

Figure 2. Chemical structures of tested compounds.

2.5. Statistical analysis

The percentage of lethality was calculated from the mean survival larvae of extracts treated tubes and control. Values were obtained by best-fit line method.

3. Results

The compounds are synthesized according to the published procedure and their structures are presented in Figure 2. All of the compounds were recrystalized and dehydrated according to the procedures and carefully weighed and the stock solutions were prepared under sterile conditions. The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxic and anti-tumor properties. In the present study, the brine shrimp lethality of extracts of four copper radiopharmaceutical precursors which can be found in the final pharmaceutical preparations.

The lethality values of the brine shrimp obtained for these compounds and that of the positive control, taxol, are given in Figure 3.

The toxicity of taxol is linear at various concentrations (1-100 ppm). For the two pharmaceutical ATSM and PTSM ligands almost a linear toxicity is observed. In case of GTS, the toxicity is much higher and is not linear and looks like the mechanism of toxicity is different from the other 2 ligands and stays at 20% of lethality in all cases.

For the control studies DMSO and brine itself showed no toxicity and that is assuring the working environments in this study. At 1 ppm ATSM demonstrates no toxicity.

An interesting trend of toxicity is observed among the ligands, where the more lipophilic the ligand is the less toxicity they show. This is possibly due to the polysaccharidic structure of the crustaceans which allow more conveniently the incorporation of hydrophilic compounds.

According to the radiopharmaceutical preparation process usually a 1 mg/ml solution of PTSM or ATSM in DMSO is prepared as stock solution followed by the use of 0.05-0.1 ml of this stock solution added to radiocopper followed by the solid-phase concentration and reconstitution of the ethanolic eluent in normal saline (5 ml), thus the final formulation has a maximum concentration of 1-10 ppm ATSM or PTSM, considering no loss of ligand is occured in the procedure.

Thus the toxicity of the final solution is negligible or very rare. However, considering the use of these radiotracers in nuclear medicine studies a better purification process for the deletion of ligand residue from the formulations is expected.

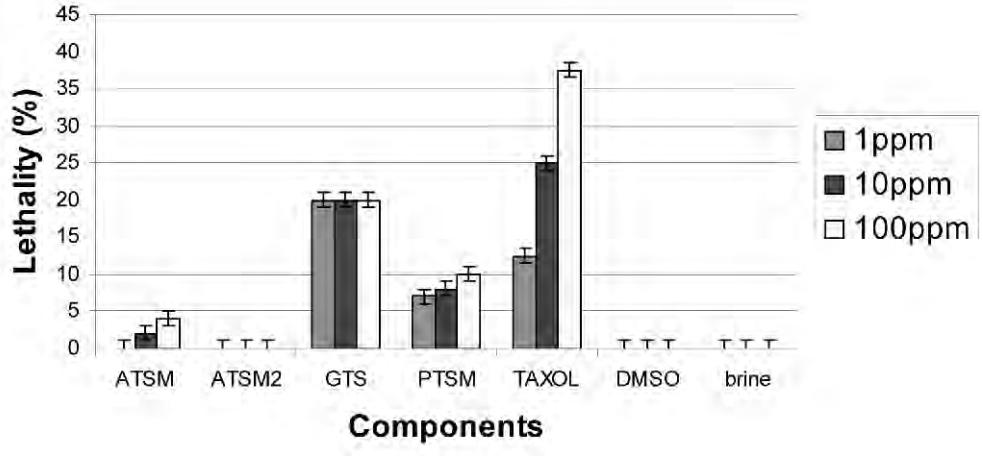

Figure 3. The percentage of brine shrimp lethality at various concentrations for test compounds and controls.

4. Discussion

Although the brine shrimp lethality assay is rather inadequate regarding the elucidation of the mechanism of action, it is very useful to assess the bioactivity of the chemical compounds and pharmaceutical impurities. In the course of our studies, the brine shrimp lethality assay actually has proven to be a convenient system for monitoring biological activities of several radiopharmaceutical ligands that are used in nuclear medicine. Out of the several ligands screened for toxicity against the brine shrimp, ATSM proved to be the safest and non toxic compound (2% lethality at 10 ppm), while PTSM was shown to possess low toxicity (7% lethality at 10 ppm). On the other hand, GTS proved to be a toxic compound even at 1 ppm (20% lethality). An inetersting structure-toxicity relationship was observed for the ligands based on their water solubility leading to more toxicity. Considering 1-10 ppm to be the maximum possible concentration of the ligands in the final pharmaceutical samples the safety of these ligands are ATSM>PTSM>GTS.

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