Mitochondria-Selective Reduction of $^{62}$Cu-Pyruvaldehyde Bis(N$^4$-methylthiosemicarbazone) ($^{62}$Cu-PTSM) in the Murine Brain; a Novel Radiopharmaceutical for Brain Positron Emission Tomography (PET) Imaging

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The retention mechanism of $^{62}$Cu-pyruvaldehyde bis(N$^4$-methylthiosemicarbazone) ($^{62}$Cu-PTSM) in the murine brain was evaluated. For this purpose, stable $^{62}$Cu-PTSM was subjected to electron spin resonance spectrometry (ESR) and high performance liquid chromatography (HPLC) analysis to determine the valence state, coordination structure and tissue metabolism. In murine brain homogenate, ESR and HPLC analysis indicated the reduction and cleavage of Cu(II)-PTSM to Cu(I). This virtually irreversible reduction was specifically initiated by the mitochondrial enzymatic system in the murine brain.

Keywords $^{62}$Cu-PTSM; brain; retention mechanism; perfusion imaging; mitochondria; reduction

Introduction

As the low molecular weight neutral complex, $^{62}$Cu-labeled bis(thiosemicarbazone) (Cu-DTS), indicates high brain accumulation, various neutral Cu-DTS compounds have been surveyed by Green et al. Of the ligands studied, Cu-pyruvaldehyde bis(N$^4$-methylthiosemicarbazone) (Cu-PTSM, Fig. 1) has been found to exhibit the most potential as a brain imaging agent with high brain extraction and excellent retention rates on cerebral perfusion.

The elution of $^{62}$Cu under physiologically mild conditions with a new Cu-62 generator system has provided a simple and easy labeling procedure. Briefly, $^{62}$Cu (positron emitter, half-life = 9.8 min) is eluted as a neutral $^{62}$Cu-glycine solution, and PTSM labeling can be complemented by mixing the $^{62}$Cu-eluante with a PTSM solution. This simple kit can be applied for preparations of various DTS-containing radiopharmaceuticals. The typical preparation of human serum albumin-DTS conjugate for plasma distribution imaging is one such example. The simple elution and labeling procedure coupled with the short half-life of $^{62}$Cu allow clinical studies to be repeated easily. In the normal brain, the retention of $^{62}$Cu-PTSM has been almost fully dependent on regional blood flow. Thus, this reagent is proposed as an agent for the measurement of the cerebral perfusion rate.

Unlike Cu-pyruvaldehyde bis(4,4-bismethylthiosemicarbazone) (Cu-PTSM2), which exhibits high brain accumulation with poor retention, Cu-PTSM manifests a high brain extraction rate with effective retention. As a result, Cu-PTSM2 shows a rapid decrease in the brain/blood ratio on cerebral perfusion. The high extraction of Cu-complexes in the brain is attributed to their appropriate lipophilicity and small molecular size. Hitherto, despite displaying an excellent brain/blood ratio, the retention mechanism of Cu-PTSM remains unclear.

In our present approach, the brain retention mechanism of Cu-PTSM was studied using the non-radioactive Cu and $^{64}$Cu, with two analytical methods: electron spin resonance spectrometry (ESR) and high performance liquid chromatography (HPLC). The former would evaluate the structure of the divalent Cu-complex and the valence state of Cu, whereas the latter could shed light on the ligand behavior of the complex. Cu-PTSM2 was employed as a reference compound for comparative purposes.

Materials and Methods

PTSM and PTSM2 (Fig. 1) were synthesized by a previously reported method. These Cu complexes were prepared by mixing excessive Cu(CH$_3$COO)$_2$, (dissolved in water) with the corresponding ligands (dissolved in dimethylsulfoxide (DMSO)). Precipitated Cu-complex was collected by filtration, dissolved in CHCl$_3$ and washed with water to remove the excess Cu. The Cu-complex was procured by evaporating the organic solvent. Anal. Cu-PTSM: Calcd for C$_6$H$_{13}$CuNO$_2$: C, 27.30; H, 2.92; N, 27.29. Found: C, 27.73; H, 4.02; N, 27.02. MS: 307 (parent peak).

C$_6$H$_{12}$CuN$_2$: C, 32.17; H, 4.80; N, 25.01. Found: C, 31.80; H, 4.72; N, 25.02. MS: 335 (parent peak). $^{64}$Cu was obtained from the Japan Atomic Energy Research Institute (Japan) as a Cu-acetate solution (17TBq/g Cu). $^{64}$Cu-PTSM and PTSM2, prepared according to a previously reported method, were obtained in an ethanol-saline solution (1:9, v/v).

In Vitro Biodistribution Studies Male ddY mice (20–25 g body weight, n = 4) were intravenously injected with $^{62}$Cu-PTSM or $^{62}$Cu-PTSM2 (0.17 MBq/0.1 ml carrier Cu = 55 μM) and sacrificed by ether anesthesia at 1, 5 and 30 min post-injection. Blood was collected by heart puncture, organs (brain, heart, lung, liver, kidneys) were isolated and weighed, and their radioactivity levels were determined with a well-type scintillation counter (ARC-300, Aloka, Japan). Radioactivity accumulation was calculated as % dose/g tissue.

In Vitro Metabolism Studies A male ddY mouse (25 g body weight) was sacrificed by decapitation. The brain was promptly removed, weighed and homogenated with a 7-fold volume of 50 mM HEPES buffer (pH 7.4; ref. 8) at 4 °C. A 900 μl aliquot of the homogenate was mixed with 100 μl of either Cu-PTSM or Cu-PTSM2 solution (0.2 mm) and

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incubated at 37°C for 1, 1.5 and 5 min. At the end of incubation, 300 µl of the mixture was put into an ESR tube, frozen in liquid nitrogen and the ESR signal was measured at 77 K, accordingly. After incubation, the mixture was agitated with an equal volume of acetonitrile, and the precipitated protein was removed by centrifugation at 1700 g for 5 min at 4°C. A sample volume of 100 µl of the supernatant was uniformly defined for HPLC analysis. For HPLC analysis, a 50-fold diluted homogenate was used, because the reaction rate found in the ESR studies was considered to be too fast to be detected in the HPLC studies.

Further, chemical stability in mouse plasma was also examined. In a manner similar to that mentioned above, blood was collected from the rodents using heparinized syringes. The plasma, isolated by centrifugation (see above), was diluted with a 9-fold volume of HEPES buffer, and stored at ~20°C before use.

The homogenate was inactivated by heating at 60°C for 1.5 min.

**Subcellular Fractionation**

Subcellular fractionation of the murine brain was performed by a modified method described previously. Briefly, the mouse brain was isolated, weighed and homogenized with a 9-fold volume of 0.25 M sucrose (buffered to pH 7.4 with 10 mM HEPES) in a Potter-Elvehjem type homogenizer. The homogenate was centrifuged at 2000 g for 3 min at 4°C. The supernatant (S1) was removed and the precipitate (P1, crude nuclear fraction) was resuspended with the medium. The S1 fraction was then centrifuged at 12500 x g for 8 min at 4°C, and the supernatant (S2, crude microsomal and soluble fractions) was isolated with a resuspension of the precipitate (P2, crude mitochondrial fraction) in the medium. Volumes of P1, P2 and S2 were subsequently adjusted to the initial value of brain homogenate volume.

**ESR Spectrometry**

ESR spectra of Cu-complexes were portrayed with an X-band spectrometer (JEOL-JE3XG, Japan Electron Optic Laboratory, Japan). Spectrometry conditions were 5 mW microwave power, 6.3 gauss modulation amplitude, and 100 kHz modulation frequency, 9.25 GHz microwave frequency with a magnetic field of 3300±500 gauss at 77 K.

**HPLC Studies**

HPLC analysis was performed with the LC-6A system (Shimadzu Co., Ltd., Japan) attached to an ODS column (Cosmosil 4.6 x 50 x 4.6 x 150 mm, Nacalai Tesque Co., Ltd., Japan). The elution phase was water-acetonitrile (1:1). Optical absorbance was detected at 345 nm.

### Results

Table I depicts the biodistribution of ⁶⁴Cu-PTS1 and ⁶⁴Cu-PTS2 in mice; the former indicated high brain accumulation and retention, whereas the latter displayed poor retention rate, rendering a rapid decrease in the brain/blood ratio with time.

Figure 2 shows the ESR spectrum of non-radioactive Cu-PTS1. The Cu-PTS1 was ESR active with its spectrum indicating that the Cu-complex was in a divalent state. This ESR spectrum displayed all the characteristic structures reported in the original Cu-DTS compound, Cu-3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazone) (Cu-KTS). Using the signal strength of the spectrum, quantification of the divalent Cu-PTS1 complex was performed. Signal intensity of the Cu-PTS1 correlated well with the concentration of Cu-PTS1. Because of high specificity and possible quantification of the ESR, metabolic studies of Cu-PTS1 were attempted.

Brain tissue contains approximately 3 µg/g tissue of endogenous Cu. However, the brain homogenate itself did not display any ESR signal in the region of divalent Cu-complexes (data not shown). Thus, it was clear that endogenous Cu does not interfere with the determination of Cu-PTS1 concentration using ESR.

**In vitro** metabolic studies of Cu-PTS1 using the murine brain homogenate indicated the prompt conversion of Cu-PTS1 to the ESR-inactive form; namely, monovalent Cu in brain homogenate (Table II). However, such a reduction of Cu-PTS1 to monovalent Cu was not observed in the plasma (data not shown). Unlike Cu-PTS1, Cu-PTS2 maintained a high degree of stability in both the brain homogenate and plasma.

In a separate experiment, inhibition studies on the reduction of Cu-PTS1 in rodent brain homogenate was conducted (Table III). At 37°C, Cu-PTS1 was completely reduced to the ESR-inactive monovalent Cu.

### Table 1. Biodistribution of ⁶⁴Cu-PTS1 and ⁶⁴Cu-PTS2 in Male ddY Mice

<table>
<thead>
<tr>
<th>Organ</th>
<th>⁶⁴Cu-PTS1</th>
<th>⁶⁴Cu-PTS2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 min</td>
<td>5 min</td>
</tr>
<tr>
<td>Blood</td>
<td>4.01</td>
<td>2.73</td>
</tr>
<tr>
<td>Brain</td>
<td>8.23</td>
<td>7.15</td>
</tr>
<tr>
<td>Heart</td>
<td>21.64</td>
<td>14.94</td>
</tr>
<tr>
<td>Lung</td>
<td>26.21</td>
<td>22.26</td>
</tr>
<tr>
<td>Liver</td>
<td>6.43</td>
<td>2.77</td>
</tr>
<tr>
<td>Kidney</td>
<td>12.75</td>
<td>13.90</td>
</tr>
<tr>
<td>Brn/Bld</td>
<td>2.05</td>
<td>2.64</td>
</tr>
</tbody>
</table>

- a) % dose/g tissue, mean (S.D.) of 4 animals.

**Fig. 2. Electron Spin Resonance (ESR) Spectrum of Cu-PTS1**

The M1 = 3/2 line is shown at an increased gain (left, inside). From this spectrum, ESR parameter, g (parallel), g (perpendicular) and A (parallel) of Cu-PTS1 were calculated at 2.1428, 2.03116 and 0.0187, respectively. Cu-PTS1, 1 mm, temp., 77 K; R.F., 10 mW, field, 2500-3500 G.

**Fig. 3. The Standard Curve for Cu-PTS1 Determination**

Data are means of duplicates. ESR signal intensity was well correlated with the logarithm of Cu-PTS1 concentration.
TABLE II. Chemical Stability of Cu-PTSM and Cu-PTSM2 in the Brain Homogenate

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Cu-PTSM</th>
<th>Cu-PTSM2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>100.0 (11.2)</td>
<td>100.0 (7.7)</td>
</tr>
<tr>
<td>1.5 min</td>
<td>51.6 (4.1)</td>
<td>98.5 (0.9)</td>
</tr>
<tr>
<td>5.0 min</td>
<td>Not detectable</td>
<td>84.6 (11.7)</td>
</tr>
</tbody>
</table>

a) % compared to value at 0 min (control), mean (S.D.) of 4 experiments.

TABLE III. Effects of Temperature on Reduction of Cu-PTSM in the Murine Brain Homogenates

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>0 min</th>
<th>1.5 min</th>
<th>5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C incubation</td>
<td>100.0 (11.2)</td>
<td>51.6 (4.1)</td>
<td>Not detectable</td>
</tr>
<tr>
<td>4°C incubation</td>
<td>100.0 (5.2)</td>
<td>106.9 (6.1)</td>
<td>97.5 (6.9)</td>
</tr>
<tr>
<td>Heat pretreatment</td>
<td>100.0 (8.3)</td>
<td>99.2 (5.6)</td>
<td>97.7 (4.8)</td>
</tr>
</tbody>
</table>

4°C incubation + 37°C incubation

a) % compared to value at 0 min (control), mean (S.D.) of 4 experiments.

Conversely, the reduction was markedly inhibited without any change in the signal intensity of Cu-PTSM when incubation was performed at 4°C.

The effect of homogenate on the reduction of Cu-PTSM to Cu(I) before heat treatment is illustrated in Table III. The reducing property of the homogenate was completely suppressed. This result indicated the contribution of a heat-sensitive substance to the reduction of Cu-PTSM to Cu(I).

To elucidate the reduction site of Cu-PTSM, subcellular fractionation was attempted. As illustrated in Fig. 4, reduction of the Cu-PTSM signal was found in the whole homogenate, S1 and P2 (crude mitochondria) fractions, but not in P1 (nucleus) and S2 (cytosol) fractions. Further density fractionation of mitochondria from the crude mitochondrial fraction with Ficoll indicated reduction activity in the mitochondria (data not shown). Moreover, no reduction activity was found in the mitochondria-free fraction. Thus, it was concluded that Cu-PTSM was reduced by an enzymatic system in the mitochondria.

HPLC analysis was then combined with tissue metabolic studies to trace the fate of the ligand in the murine brain (Fig. 5). As the absorbance peak of Cu-PTSM decreased with incubation time, the free PTSM ligand absorbance peak increased. This was a good indication of the cleavage of Cu-PTSM to monovalent Cu as a result of Cu reduction in the brain homogenate.

Discussion

Since copper-DTS complexes have been reported to serve as plausible antineoplastic agents, attention is currently focused on the initial reaction of Cu-DTS complexes with viable Ehrlich ascites tumor cells because this reaction stage is a determining factor for the mechanism of the antitumor action of these agents. In Ehrlich cells, Cu has been found to be retained by reduction, and thiol-containing substances have been involved in the reaction. In addition, Cu accumulation in cell components displays non-specific localization. Based on these results, the retention mechanism of Cu-PTSM in the brain has been speculated to involve non-specific reduc-

Fig. 4. Typical ESR Spectrum of Cu-PTSM in Subcellular Fractions of the Brain
P1, P2, S2 represent nucleus, crude mitochondria and cytosol fractions, respectively.

Fig. 5. HPLC Profiles of Cu-PTSM in the Brain Homogenate
Cosmosil ODS column (4.6 x 50 + 4.6 x 150 mm, Nacalai Tesque Co., Ltd., Japan) was eluted with water-acetonitrile (1:1). Peaks of Cu-PTSM and free PTSM were detected at 345 nm.

In contrast to Cu-PTSM2, high brain retention was portrayed by Cu-PTSM, a finding that coincides well with the results of Green et al. Moreover, this divergent feature corresponded well with the reduction tendency of Cu-complexes to monovalent Cu in the brain homogenate; Cu(II)-PTSM was quickly reduced and cleaved in the brain to Cu(I), whereas Cu-PTSM2 was not affected. As the PTSM-ligand was kept intact during this process, the reduction of Cu(II) was considered to be a major retention mechanism of Cu-PTSM. With regards to the reduction of Cu(II), the initial reaction of Cu-PTSM seemed to resemble that indicated by Cu-DTS complexes with Ehrlich ascites tumor cells.

However, the subcellular reduction site in the brain was completely different from the non-specific reduction sites in Ehrlich cells. Among the subcellular components of the brain homogenate, the reduction of Cu(II)-PTSM to Cu(I) was found only in the mitochondrial fraction. As such, our present finding depicting the accumulation mechanism...
of Cu-PTSM in the brain is completely different from any of the previously proposed contrivances.12–15)

Petering et al.16) have reported the reactions of Cu-DTS complexes with isolated mitochondria; these complexes inhibit the respiration of Ehrlich ascites tumor cells and state 3 oxidative phosphorylation in mitochondria. The former qualitatively resembles the disruption of oxidative phosphorylation in bovine heart mitochondria. At low concentrations, Cu-DTS is highly specific on a site in the first energy coupling mechanism between the electron transport chain and the ATPase of ATP synthesis.16) It is considered that oxidation of the thiol groups coincides with the inhibition of the same compound. Thus, the reduction of Cu(II)-PTSM to Cu(I) in the brain was probably caused by the mitochondrial energy coupling system. In our study, the temperature-dependent and heat-sensitive reduction of Cu(II)-PTSM to Cu(I) was influenced by subcellular enzymatic system(s), most probably the thiol-containing proteins in mitochondria.

Green et al. found that the subcellular distribution of $^{65}$Cu in the brain following i.v. injection of $^{65}$Cu-PTSM reflected the distribution of endogenous intracellular Cu, mainly in cytosol and nuclei + membrane fractions, but only 10–20% in mitochondria (personal communication). These findings indicate that radioactive Cu injected as Cu-PTSM was also stored in the endogenous Cu storage pool. This helped confirm that a large part of the endogenous Cu (brain tissue contains 2.8 µg/g of Cu)11) is stored as an ESR-inactive form. In contrast, most of the subcellular fractions of the brain, except for the mitochondrial fraction, showed no ability to reduce the ESR-signal strength of injected Cu-PTSM. Thus, the reduction site of Cu-PTSM and storage site of Cu are considered to be different.

From these findings, the retention mechanism of the Cu-PTSM is summarized in Fig. 6. Divalent Cu-PTSM first accumulates in the brain, followed by a reduction of Cu(II) to monovalent Cu by enzymatic system(s) in the mitochondria. This Cu(II)→Cu(I) reduction might coincide with thiol-oxidation in the energy coupling system, a reaction most probably accomplished via the thiol-containing enzymes in the mitochondria of the brain. Following that, Cu is stored following the presence of endogenous Cu, as monovalent Cu.

Cu-PTSM may serve as a blood flow tracer in brain metabolic studies, as long as the mitochondrial energy coupling system remains normal. In addition, if there were injuries inflicted in this system, encephalic Cu-PTSM accumulations would be altered which could then indicate mitochondrial dysfunction.

References