

Cytosolic/microsomal redox pathway: a reductive retention mechanism of a PET-oncology tracer, Cu-pyruvaldehyde-bis(N^4 -methylthiosemicarbazone) (Cu-PTSM)

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Objective: To clarify the retention mechanism of a PET imaging agent Cu-pyruvaldehyde-bis(N^4 -methylthiosemicarbazone) (Cu-62-PTSM) in tumor cells, reductive metabolism of non-radioactive Cu-PTSM in five cultured tumor cell lines, a tumor specimen and non-tumor tissues *in vitro* was evaluated by electron spin resonance spectrometry (ESR).

Results: In the brain, mitochondrial electron transport enzyme reduced Cu-PTSM specifically. On the other hand, Cu-PTSM was not reduced in tumor mitochondria. The mitochondrial electron transport enzyme in tumor cells was not damaged, but NADH was considered to be depleted. In compensation for that, the tumor cells acquired complementary reduction activity in the microsome/cytosol. The reduction was enzymatic and NADH-dependent, possibly similar to the activation mechanism of bioreductive anticancer drugs.

Conclusion: Cu-PTSM and its derivatives are considered to be used as a marker for microsome/cytosol redox ability in PET oncology, although the physiological role of the redox enzyme system in tumor cells has not been clarified. The change in electron (NADH) flow in tumor cells might be a mechanism supporting aerobic glycolysis in tumor cells.

Key words: tumor, electron transport, Cu-PTSM, NADH, Complex I

INTRODUCTION

IN OUR PREVIOUS WORK, we found that Cu-pyruvaldehyde-bis(N^4 -methylthiosemicarbazone) (Cu-PTSM), labeled with short half-lived positron emitting radionuclide for nuclear medicine (Cu-62, Cu-60, Cu-64), can be used as a functional marker of the mitochondrial electron transport chain in normal tissues *in vivo*.¹⁻⁵ The complex easily penetrates through membranes including the blood-brain barrier and shows reductive retention in cells. In normal

tissues, Cu-PTSM is reduced specifically at the Complex I site in mitochondria in an NADH-dependent manner.² This characteristic provides new insight into cerebral ischemia-reperfusion injury as a hyperfixation phenomenon of Cu-PTSM in lesions, an indication of enhanced reductive retention caused by disturbed electron flow in the lower part of the mitochondrial electron transport chain (Complex III) in the brain.⁶

Cu-PTSM shows very high accumulation in tumors and can be used as a tumor imaging agent,^{7,8} but, our previous basic study on Ehrlich ascites cells has indicated that the actual intracellular reduction site of Cu-PTSM in tumor cells is different from that in the normal brain.⁹ If the reduction site and/or mechanism in tumors is different from those in normal tissues, Cu-PTSM images of tumors might provide us with new information on tumor-specific redox conditions.

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In the present study, non-radioactive Cu-PTSM and electron spin resonance spectrometry (ESR) were used to detect the redox reaction of Cu-PTSM and clarify the metabolic changes in tumor cells. As for tumor models and spontaneous tumors, five experimental cell lines including those of human and mouse origin, and a fresh human glioblastoma specimen obtained by surgery were used. Based on the metabolic changes in tumor cells observed, the possibility of new diagnostic criteria for tumors in nuclear medicine was discussed.

MATERIALS AND METHODS

Reagents

Cu-PTSM was prepared as described previously.³ Reagents for all experiments were purchased from Nacalai Tesque (Kyoto, Japan).

Tumor cell lines

SCCVII, EMT-6, HT1080, HeLa and N3 cell-lines, Ehrlich ascites cells, murine brain tissue and human brain tumor (glioblastoma) were used in this experiment. The murine tumor cell lines SCCVII and EMT-6¹⁰ were cultured in Eagle's minimal essential medium (MEM) supplemented with 12.5% fetal bovine serum (FBS) under humidified conditions at 37°C with 5% CO₂. HT1080 and HeLa are human tumor cell lines, obtained from the Health Science Research Resources Bank, Osaka, Japan, and were cultured in α -MEM with 10% FBS. N3 were human fibroblast cells, cultured in Dulbecco's MEM with 10% FBS. Ehrlich ascites tumor cells were maintained in male ddY mice and were withdrawn from the peritoneal cavity 7 days after inoculation.

Subcellular fractionation

Exponentially growing tumor cells (5×10^7) from monolayer cultures were isolated and washed twice with an isolation medium (0.5 g/2.5 ml, 0.25 M sucrose buffered to pH 7.4 with 10 mM HEPES). Subcellular fractionation of the cells was done by a modification of the method reported previously (free-cell method).¹¹ The cells were suspended in 4 ml of a lysis buffer (0.005% sodium dodecyl sulfate buffered to pH 7.4 with 10 mM HEPES). The suspension was homogenized with a Dounce homogenizer and centrifuged at $1,000 \times g$ for 5 min at 4°C. The supernatant (S1) was removed, and the precipitate (P1, crude nuclear fraction) was resuspended in the medium. The S1 fraction was centrifuged at $9,000 \times g$ for 10 min at 4°C, and the supernatant (S2, crude microsomal and soluble fractions) was isolated, and the precipitate was resuspended in the medium (P2, crude mitochondrial fraction). The P1 and P2 fractions were resuspended in the isolation medium. The volumes of P1, P2 and S2 fractions were adjusted to the initial homogenate volume. To evaluate the effect of cell growth, similar studies with SCCVII cells in a confluent condition were performed.

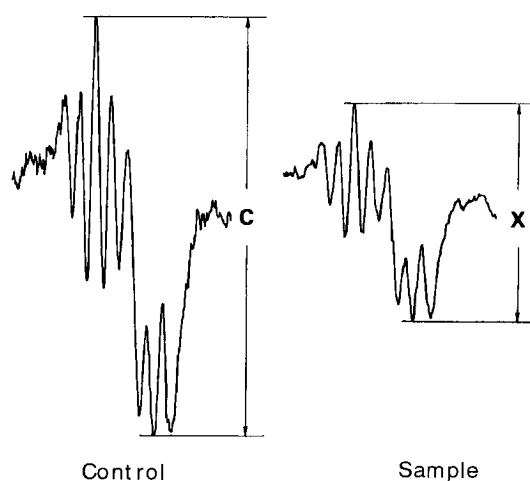


Fig. 1 A typical ESR spectrum of non-radioactive Cu-PTSM. Reduction rate (%) of Cu-PTSM was calculated as $(C - X)/C \times 100$. C: ESR signal of the control, X: ESR signal of the sample.

Subcellular fractionation of the murine brain tissue was performed by the method reported previously (solid-tissue method).¹² Male ddY mice (25 g body weight) were killed by decapitation. The brain was removed, weighed and immediately homogenized with the isolation medium in a Potter-Elvehjem-type homogenizer (0.15 g wet tissue/ml). The homogenate was then centrifuged at $1,000 \times g$ for 5 min at 4°C, and the P1 fraction was resuspended in the medium. The S1 fraction was centrifuged at $10,000 \times g$ for 10 min at 4°C to yield the S2 and P2 fractions. The volumes of P1, P2 and S2 fractions were adjusted to the initial brain homogenate volume with the isolation medium.

The activities of succinate dehydrogenase, NADPH-cytochrome c reductase and lactate dehydrogenase were measured as markers of mitochondria,¹³ microsomes¹⁴ and cytosol,¹⁵ respectively, to confirm the appropriateness of the two fractionation methods. In every case including tumor cells and brain, approximately 90% succinate dehydrogenase activity was found in the P2 fraction. NADPH-cytochrome c reductase activity was found in both the P2 and S2 fractions, indicating that microsomes were distributed in both fractions. In the tumor cells, most of the lactate dehydrogenase was found in the S2 fraction. Only 55% of the lactate dehydrogenase in the brain homogenate was in the S2 fraction; the remaining activity was in the P2 fraction.

The subcellular fractionation of the human brain tumor was performed by the same method as that used for the murine brains (solid-tissue method).

ESR spectrometry of Cu-PTSM

An aliquot (1.8 ml) of the homogenate, P1, P2 or S2 fraction was mixed with 200 μ l of Cu-PTSM solution (0.2 mM) and incubated at 37°C for 15 min. After incubation, 300 μ l of the mixture was put into an ESR tube, and the

tube was frozen in liquid nitrogen. The ESR signal was then measured. Before incubation, 300 μ l of the mixture was collected as a control. ESR spectra were obtained with an X-band spectrometer (JES-FE3XG, Japan Electron Optic Laboratory, Tokyo, Japan) under conditions appropriate to obtain the specific spectrum for Cu(II)-PTSM (microwave power, 5 mW; modulation amplitude, 6.3 gauss; modulation frequency, 100 kHz; microwave frequency, 9.25 GHz; magnetic field, 3300 \pm 500 gauss; and temperature, 77 K. Figure 1 shows a typical ESR spectrum of non-radioactive Cu-PTSM. The Cu-PTSM was ESR-active, and its spectrum indicated that the Cu-complex was in a divalent state. This spectrum displayed all of the characteristic structures reported in the original Cu-dithiosemicarbazone complex.¹⁶ Any Cu(I) complexes were ESR-inactive. Change in the strength of the spectrum ($C - X/C \times 100$) was therefore calculated as reductive metabolism of Cu(II)-PTSM, as described previously.²

Reactivity of submitochondrial particles (SMP)

The mitochondrial fraction was isolated as described above and sonicated for 90 s (15 s \times 6 times) (Sonicator, Model W-220F, Heatsystem-Ultrasonics, Inc., New York, USA) at 4°C. A 1.8 ml aliquot of SMP was mixed with 1.0 ml of NADH solution (final: 1 mM) and 200 ml of Cu-PTSM solution (0.2 mM), and the mixture was incubated at 37°C for 15 min. After incubation, the ESR signal was measured at 77 K. An aliquot of the mixture before incubation was retained as a control.

RESULTS

Table 1 shows the reduction of Cu-PTSM due to the subcellular fractions of the brain tissue, cultured tumor cells, intra-peritoneally maintained tumor cells and the cultured human fibroblasts (N3). In the brain homogenate, most of the reducing activity was found in the P2 fraction, rather than the P1 or S2 fraction. In contrast, the cultured tumor cell line SCCVII showed most of its reducing activity in the S2 fraction. The other four tumor cell lines showed results similar to that of the SCCVII. The distribution of reducing ability was completely different in the normal murine brain and tumor cells. The reduction activity in the P2 fraction of the fibroblasts was not as high as that of the brain, but was higher than that of the tumor cells.

In the present study we used exponentially growing cells. Because it is possible that there were metabolic differences in the state of cell growth, we investigated the metabolic activity of cells in a confluent condition with SCCVII. The reduction rate was 4.70% in the P1 fraction, 1.20% in the P2 fraction and 78.9% in the S2 fraction. There was no difference between the distribution patterns of the cells with exponential growth and those with confluent condition.

Table 1 Contribution of each subcellular fraction to the reduction of Cu-PTSM

	P1 (nucleus)	P2 (mitochondria)	S2 (microsome/ cytosol)
Normal brain	3.01 \pm 3.9	69.8 \pm 6.7	4.33 \pm 3.9
SCCVII	15.7 \pm 8.9	10.9 \pm 14.0	62.9 \pm 13.0
EMT-6	3.61 \pm 4.1	2.83 \pm 3.0	51.4 \pm 7.8
HeLa	9.41 \pm 4.4	8.23 \pm 9.6	85.8 \pm 22.0
HT1080	8.36 \pm 4.8	9.80 \pm 7.0	69.2 \pm 12.0
Ehrlich ascites cells	11.0 \pm 5.9	4.26 \pm 1.1	67.4 \pm 12.0
N3 fibroblast	9.67 \pm 5.9	29.8 \pm 4.7	58.2 \pm 21.0

Reduction rate (%) of Cu-PTSM, data are the average and 1 S.D. of five experiments.

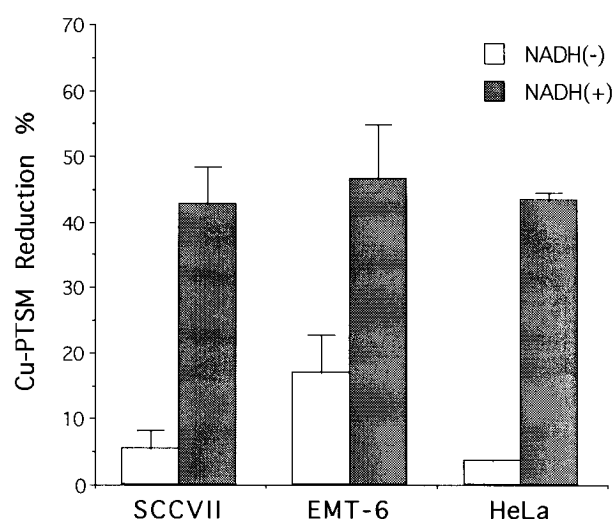


Fig. 2 The Cu-PTSM reduction ability of SMP (submitochondrial particles) in the presence or absence of exogenous NADH. Data are the average of reduction % (1 S.D.) of four experiments.

In the case of the human brain tumor, the reduction rate was 4.6% in the P1 fraction, 0.1% in the P2 fraction and 21.0% in the S2 fraction, so that most of the reducing activity was found to be in the S2 fraction.

Figure 2 shows the Cu-PTSM reducing ability of SMP prepared from SCCVII, EMT-6 and HeLa, in the presence or absence of exogenous NADH. The reducing activity of the SMP was very low, but was significantly increased when NADH was added to the medium.

To evaluate the possible contribution of enzymes to the reduction of Cu-PTSM in the tumor S2 fraction, the effect of heat treatment was studied in Ehrlich ascites tumor cells. After heat treatment (95°C, 5 min), the S2 fraction of the tumor cells lost a large part of the reducing ability (Fig. 3). In this condition, no decrease in thiol (SH) content was found after the treatment.

The effect of the addition of NADH on the reduction in Cu-PTSM in the tumor S2 fraction was also studied in Ehrlich ascites tumor cells (Fig. 3). The addition of

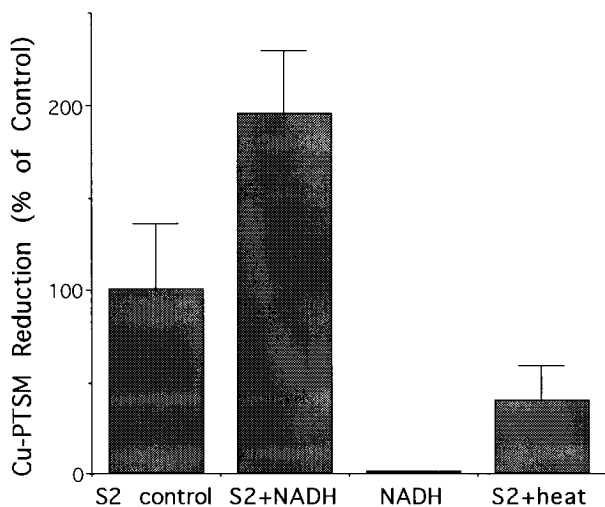


Fig. 3 The Cu-PTSM reduction ability of the tumor S2 fraction (microsome/cytosol), S2 with NADH, NADH alone and S2 after heat treatment. Data are the average of four experiments.

NADH to the S2 fraction significantly increased the reduction in Cu-PTSM. NADH alone showed no reducing effect on Cu-PTSM. As a reference, Cu-PTSM was incubated with brain S2 fraction in the absence or presence of exogenous NADH (1 mM), but there was very little increase in the rate of reduction of Cu-PTSM (4.3% and 6.3%, respectively).

DISCUSSION

Structural changes in tumor mitochondria are well known, but it is still unclear how the structural and/or functional changes in mitochondria contribute to aerobic glycolysis and other metabolic abnormalities in tumor cells.^{17,18} In the present study, we observed that the P2 (crude mitochondria) fraction of the experimental tumor cells as well as the tumor specimen from a human patient lost the ability to reduce Cu-PTSM. In mitochondria, the reduction of Cu-PTSM requires two factors, NADH dehydrogenase (Complex I) and NADH.² The present results therefore indicate the loss of the enzyme activity and/or depletion of NADH in mitochondria. Complex I is on the inner-matrix of mitochondria, and available NADH could be expected to be in mitochondria. The use of SMP, i.e., an inside-out mitochondrial membrane preparation, allowed us to control the concentration of available NADH for Complex I in the metabolic studies, and it was shown that all of the SMP prepared from three different tumor cell lines could reduce Cu-PTSM when exogenous NADH was added. Complex I system itself is therefore considered to be intact, but available NADH is depleted in tumor mitochondria.

In non-tumor tissues, NADH is synthesized by the reduction of NAD⁺ in cytosolic glycolysis as well as in the mitochondrial TCA cycle. Under normoxic conditions,

NADH derived from glycolysis is transported into mitochondria by a malate-aspartate shuttle¹⁹ and used as a fuel for ATP synthesis. Under hypoxic conditions, in contrast, NADH cannot be consumed in mitochondria, and as a result, NADH is abnormally accumulated in the cells,^{20,21} both in the cytosol and intra-mitochondria. It has been reported that the NADH concentration in tumor cells is extremely high,¹⁹ as it is in ischemic non-tumor tissue, but tumor cells show signs of high glucose utilization but low oxygen consumption even under normoxic conditions, the so-called "aerobic glycolysis",²² when compared with normal cells with similar growth rates. At this point, tumor metabolism does not seem to be the same as hypoxic metabolism in non-tumor tissues. The depletion of intra-mitochondrial NADH in tumor cells indicates that disturbed transport from the cytosol as well as the suppressed production of NADH in the TCA cycle in mitochondria should occur simultaneously. The high NADH concentration in tumor cells reported¹⁹ might be a result of accelerated glycolysis and suppressed NADH transport into mitochondria. NADH transport from the cytosol to mitochondria is governed by a malate-aspartate shuttle, and the transformation of embryonal cells with Rous sarcoma virus or Simian virus 40 leads to abnormalities in this malate-aspartate shuttle.¹⁹ The most important point is that this is not a result of hypoxia but is rather a characteristic of tumors.

In compensation for the loss of the reduction of Cu-PTSM in mitochondria, tumor cells acquired complementary activity in the S2 (microsome/cytosol) fraction. The reducing ability seemed to be enzymatic, namely heat-sensitive and NADH-dependent. This kind of reduction did not occur in the brain S2 fraction even when exogenous NADH was added, so that these results might indicate the tumor-specific or -selective expression of enzyme(s) with a reduction of Cu-PTSM in the tumor microsome and/or cytosol.

The fibroblast cells, showing a high growth rate but non-tumorous characteristics, had a metabolic pattern for Cu-PTSM intermediate between those of the tumor cells and brain tissue. It has been reported that the NADH/NAD ratio in the fibroblasts in the logarithmic phase is 2- to 3-fold higher than that in the same cells in the confluent phase.²³ Interestingly, the NADH/NAD ratio in transformed fibroblasts remained high even after they grew to the confluent condition.²⁴ In light of this, the intermediate metabolic pattern of the fibroblasts may be an indication of a transient shift to tumor-like metabolism in the fibroblasts in the logarithmic phase.

Enzymatic reduction in tumor cell cytosol/microsome is well known as an activation mechanism of bioreductive anticancer drugs.²⁵ DT-diaphorase, NADPH: cytochrome P-450 reductase, NADH: cytochrome b₅ reductase,²⁵ aldehyde oxidase, xanthine oxidase,²⁶ and others are considered candidates for cytosolic as well as microsomal bioreductive enzymes. It has been reported that the

expression levels of these types of reductive enzymes are higher in tumor cells than in normal cells.²⁷ Our preliminary study indicated the possible contribution of NADH-enzymes to the reduction of Cu-PTSM in the cytosol/microsome in tumor cells. Interestingly, Cu-bisthiosemicarbazone complexes, including Cu-PTSM, were originally developed as anticancer agents,²⁸ and bioreduction has been considered an essential step in the anticancer activity of metal complexes.^{28,29}

The physiological role of the cytosolic/microsomal redox enzyme system in tumor cells has not been clarified, although the system is known to activate bioreductive drugs. It is unlikely that tumor cells express this system in order to activate bioreductive anticancer drugs. It has been reported that some oncogene products have the ability to enhance glycolysis.^{24,30} In addition, aerobic glycolysis is thought not to be a simple result of hypoxia caused by an extremely high cell growth rate, but is rather an essential or important change for tumor growth.¹⁹ The alteration in electron (NADH) flow in tumor cells, namely the suppression of the import/synthesis of NADH in mitochondria and the expression of an alternative NADH-dependent redox system in microsome/cytosol, might be a mechanism supporting aerobic glycolysis in tumor cells.

In this work, the site of reduction of Cu-PTSM in tumor cells was clarified, but not the site of retention of Cu. In the brain the subcellular distribution of radioactive Cu in the brain after i.v. injection of Cu-PTSM is considered to reflect the distribution of endogenous intracellular Cu but not to be reduction site-selective.² The present results are also not considered to be a direct reflection of radioactive Cu distribution in tumors. Cu-PTSM itself is extremely stable in the divalent state, and can be easily redistributed into the circulation, so that retention of radioactive Cu should be initiated only by enzymatic reduction in the cells. Cu-retention could therefore be considered as a reducing ability of the tissues, but not a distribution of endogenous intracellular Cu.

Cu-PTSM shows high retention both in normal and tumor tissues but by different retention mechanisms, namely mitochondrial and microsomal/cytosolic reduction, respectively. Cu-PTSM is therefore not considered to be a selective tumor imaging agent, but a plausible marker for the microsome/cytosol-dominant redox system in tumor cells when using PET. We recently found that Cu-diacetyl-bis(*N*⁴-methylthiosemicarbazone) (Cu-ATSM), a derivative of Cu-PTSM with a lower redox potential, showed signs of selective accumulation in tumor tissues but not in normoxic non-tumor tissues. Further studies on the mechanism of reduction of Cu-PTSM and its derivatives in tumor cytosol/microsome are necessary to clarify the clinical usefulness of these compounds in PET oncology.

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