

Retention mechanism of hypoxia selective nuclear imaging/radiotherapeutic agent Cu-diacetyl-bis(*N*⁴-methylthiosemicarbazone) (Cu-ATSM) in tumor cells

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The retention mechanism of the novel imaging/radiotherapeutic agent, Cu-diacetyl-bis(*N*⁴-methylthiosemicarbazone) (Cu-ATSM) in tumor cells was clarified in comparison with that in normal tissue *in vitro*. With Cu-ATSM and reversed phase HPLC analysis, the reductive metabolism of Cu-ATSM in subcellular fractions obtained from Ehrlich ascites tumor cells was examined. As a reference, mouse brain was used. To determine the contribution of enzymes in the retention mechanisms, and specific inhibitor studies were performed. In subcellular fractions of tumor cells, Cu-ATSM was reduced mainly in the microsome/cytosol fraction rather than in the mitochondria. This finding was completely different from that found in normal brain cells. The reduction process in the microsome/cytosol was heat-sensitive and enhanced by adding exogenous NAD(P)H, an indication of enzymatic reduction of Cu-ATSM in tumor cells. Among the known bioreductive enzymes, NADH-cytochrome b5 reductase and NADPH-cytochrome P450 reductase in microsome played a major role in the reductive retention of Cu-ATSM in tumors. This enzymatic reduction was enhanced by the induction of hypoxia. Radiocopper labeled Cu-ATSM provides useful information for the detection of hypoxia as well as the microsomal bioreductive enzyme expression in tumor.

Key words: Cu-ATSM, hypoxia, bioreductive enzyme, microsome

INTRODUCTION

RADIOLABELED Cu-diacetyl-bis(*N*⁴-methylthiosemicarbazone) (Cu-ATSM) was originally proposed as a radiopharmaceutical for the imaging of hypoxic tissues by means of positron emission tomography (PET).^{1–3} Cu-ATSM is of suitable molecular size and lipophilicity to penetrate cell membranes from the blood stream. Once in the cell, it is selectively reduced to monovalent Cu in hypoxic but not in normoxic tissues.¹

Canine as well as human PET studies have been successfully performed with the clinically applicable ultra-shortlived radionuclides Cu-62 and Cu-60, and regions of unstable angina as well as lung tumors could be clearly visualized.^{4–6} Imaging of hypoxic tumor is considered to be useful for the planning of radiation therapy as well as in the bioreductive drug treatment of tumors. The former is because tumor resistance to radiation therapy can be correlated, in part, to the development of hypoxic regions,⁷ and the latter because bioreductive drugs can be activated in hypoxic tumors.⁸

Copper-64 ($t_{1/2} = 12.7$ hr; $E_{\beta^+max} = 0.663$ MeV (17.4%), $E_{\beta^-max} = 0.574$ MeV (40%)) is a readily available cyclotron-produced positron-emitting isotope with utility in diagnostic imaging and as a therapeutic radionuclide.⁹ Hypoxia-selective delivery of Cu-64 as Cu-ATSM would provide a non-invasive method to treat tumors with

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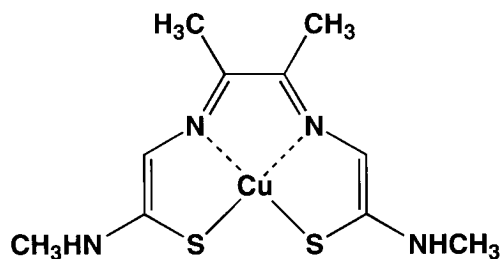


Fig. 1 Structure of Cu-diacetyl-(N^4 -methylthiosemicarbazone) (Cu-ATSM).

simultaneous radiation-dose monitoring by PET. Our recent study has indicated that Cu-64-ATSM showed exceptional promise for this purpose.¹⁰

Nevertheless, the exact reductive retention mechanism of Cu-ATSM in tumor cells is still controversial. Petering et al. speculated that Cu-ketoxal-bis(N^4 -thiosemicarbazone), a member of the Cu-bisthiosemicarbazone complexes, might be chemically reduced by cytosolic thiol compounds.¹¹ On the other hand, Fujibayashi et al. clarified that Cu-pyruvaldehyde-bis(N^4 -methylthiosemicarbazone) (Cu-PTSM) as well as Cu-ATSM was enzymatically reduced by a mitochondrial electron transport chain.^{1,12} Shibuya et al. recently reported that the reduction site of Cu-PTSM in tumor cells was different from that in brain tissue.¹³ In the present study, the metabolic site of Cu-ATSM in tumor cells was studied by performing subcellular fractionation on Ehrlich ascites tumor cells in order to elucidate the hypoxia related reduction mechanism for Cu-ATSM and the possible involvement of microsomal bioreductive enzymes. Radioactive Cu-62 has too short a half-life for systematic metabolism studies, so that a metabolic analysis method with stable Cu was used.

MATERIALS AND METHODS

Cu-ATSM, its structure shown in Figure 1, was synthesized as previously described.¹⁴ In brief, ATSM ligand was dissolved in dimethylsulphoxide and mixed with an excess of cupric acetate solution. Crude precipitate was collected, dissolved in chloroform, and washed with distilled water. The remaining solvent was evaporated *in vacuo*. The chemical purity of Cu-ATSM was determined by elemental analysis and mass spectrometry. DdY male mice weighing 25 g were used for metabolic studies. These mice were fed a commercial diet and tap water *ad libitum*.

Subcellular fractionation

Subcellular fractionation of tumor cells and brain tissue were performed by slightly different procedures optimized for each cell type as follows. The activities of succinate dehydrogenase, NADPH-cytochrome c reductase and lactate dehydrogenase were measured as markers

of mitochondria, microsomes and cytosol, respectively, to confirm the efficiency of fractionation.¹⁵

Ehrlich ascites tumor cells: Ehrlich ascites tumor cells were maintained in male ddY mice and were withdrawn from the abdominal cavity 7 days after inoculation. Subcellular fractionation was done by a modification of the method reported previously.¹⁶ Three milliliters of the ascites was centrifuged at $500 \times g$ and the cells were washed twice with an isolation medium (0.25 M sucrose buffered to pH 7.4 with 10 mM HEPES). The cell pellet was suspended in 10 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, after which the P1 fraction (crude nuclear fraction) was obtained by centrifugation at $1,000 \times g$ for 5 min at 4°C . The supernatant was centrifuged at $8,000 \times g$ for 10 min at 4°C to yield the P2 (crude mitochondrial) and S2 (microsome/cytosol) fractions. The volumes of the P1 and P2 fractions resuspended in the isolation medium and that of the S2 fraction were adjusted to the initial homogenate volume. To obtain a concentrated microsome fraction, the S2 fraction was centrifuged at $105,000 \times g$ for 60 min and the precipitate was resuspended with the isolation medium to 1/3 the volume of the initial S1 fraction.

Brain: Subcellular fractionation of the murine brain was performed as described.¹⁷ Brains were isolated, weighed and homogenized with the isolation medium in a Potter-Elvehjem type homogenizer. Then the homogenate was centrifuged at $1,000 \times g$ for 5 min at 4°C . The supernatant (S1) was removed and the precipitate (P1) was resuspended in the medium. The S1 fraction was centrifuged at $10,000 \times g$ for 10 min at 4°C , and the supernatant (S2) was isolated and the precipitate resuspended in the medium (P2). The volumes of the P1, P2, and S2 fractions were adjusted to the initial brain homogenate volume.

HPLC analysis

Cu-ATSM metabolism was analyzed by HPLC (LC-6A, Shimadzu, Kyoto, Japan) on a reversed phase column (Cosmosil 5C18-AR, $4.6 \times 50 \text{ mm} + 4.6 \times 150 \text{ mm}$, Nacalai Tesque, Kyoto, Japan). Elution conditions were as follows: flow rate = 1 ml/min, buffer A = 10 mM potassium phosphate buffer (pH 7.4), buffer B = 10 mM potassium phosphate buffer (pH 7.4)/ethanol (50/50), linear gradient = 50% buffer B (0 min) – 100% buffer B (20 min) – 100% buffer B (25 min), UV detection = 313 nm. In this system, peaks of Cu-ATSM and metal-free ATSM were found at 12.2 min and 5.3 min, respectively. The reductive metabolism of Cu-ATSM was explored by determining the metal-free ATSM concentration in the collected fractions. The close correlation between the reduction of Cu-ATSM and increase in metal-free ATSM was confirmed by comparative analysis with electron spin resonance spectrometry (ESR)¹ and HPLC.

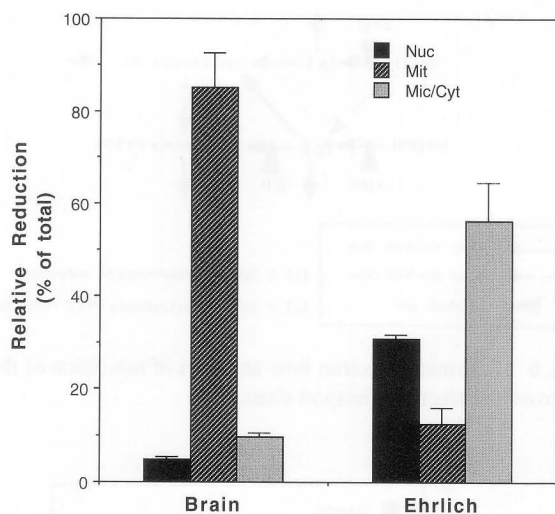


Fig. 2 Reduction of Cu-ATSM in subcellular fractions obtained from mouse brain and Ehrlich ascites tumor cells. % of total = activity found in each fraction/sum of activities found in nuclear, mitochondria and microsome/cytosol fractions \times 100. Average of 3–4 experiments (bar: 1 S.D.).

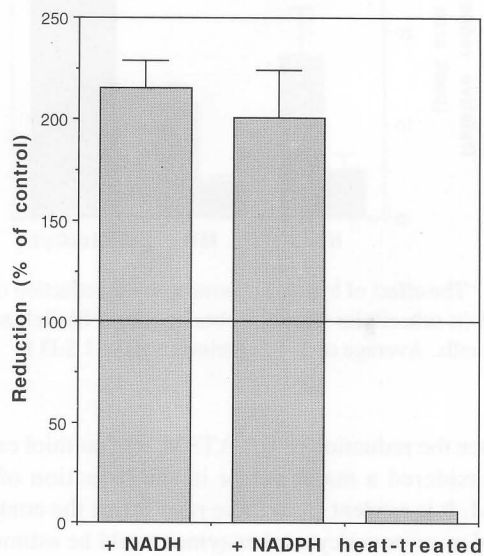


Fig. 3 The effect of NADH, NADPH and heat-treatment on the reduction of Cu-ATSM in the microsome/cytosol fraction obtained from Ehrlich ascites tumor cells. Average of 3–4 experiments (bar: 1 S.D.).

In vitro metabolic studies

Each subcellular fraction (100 μ l) was mixed with 98 μ l of the medium and, then 2 μ l of 250 μ M Cu-ATSM in DMSO. The mixture was incubated at 37°C for 60 min, then the reaction was stopped with the addition of 200 μ l of ice-cooled methanol. The mixture was mixed, stored at -20°C for a few minutes, then centrifuged at 8,000 \times g for 10 min. The supernatant (100 μ l) was analyzed by reversed phase HPLC as described above.

To determine the contribution of enzymes, the tumor microsome/cytosol fraction was heated to 100°C for 5 min and then metabolic studies were performed. To exclude the possibility of a contribution of thiol to the reduction of Cu-ATSM, thiol content of the fraction was compared before and after heat treatment, by the DTNB method.¹⁸ The effect of a biological electron donor, namely NADH or NADPH, on the reduction of Cu-ATSM in the tumor microsome/cytosol fraction was also studied at a concentration of 1 mM.

The effect of hypoxia on the reduction of Cu-ATSM in the microsome/cytosol fraction was evaluated with an isolation medium saturated with nitrogen gas for the subcellular fractionation as well as by *in vitro* metabolic studies under a nitrogen atmosphere, as described.¹⁹ As for the normoxia control, an isolation medium saturated with oxygen gas was used.

In vitro enzyme inhibition studies

A 100 μ l aliquot of the tumor microsome/cytosol fraction was mixed with 98 μ l of the inhibitor solution followed by 2 μ l of 250 μ M Cu-ATSM in DMSO. The metabolic study was then performed as described above. As inhibitors of cytosolic DT-diaphorase and xanthine oxidase, dicoumarol (1 μ M–100 μ M)^{20–22} and allopurinol (1 μ M–1 mM)^{21,23} were used, respectively. Diphenyliodonium chloride (DPIC, 10 nM–1 mM)^{24,25} and *p*-hydroxy-mercuribenzoate sodium salt (pHMB, 4 μ M–500 μ M)^{24,26} were used as a microsomal flavin enzyme inhibitor and a microsomal thiol enzyme inhibitor, respectively. The concentration range of each inhibitor used was taken from the references given.

In the concentrated microsome fraction, the contribution of microsomal electron transport enzymes to the reduction of Cu-ATSM was studied. Adenosine 2'-monophosphate (AMP, final conc. = 5 mM) and 5-propyl-2-thiouracil (PTU, final conc. = 5 mM) were used as specific inhibitors of NADPH: cytochrome P450 reductase^{24,27} and NADH: cytochrome b5 reductase,^{24,28} respectively. The activity of the cytochromes was suppressed by gentle bubbling with carbon monoxide (CO) gas for 5 min.^{23,24} The concentration ranges of the inhibitors used were taken from the references indicated.

RESULTS

The subcellular distribution of the ability to reduce Cu-ATSM is shown in Figure 2. In tumor homogenate, most of the Cu-ATSM reduction occurred in the microsome/cytosol fraction with little reductive activity in the mitochondria. On the other hand, Cu-ATSM was reduced exclusively in the brain mitochondria, as previously reported.¹

The effect of NADH or NADPH addition on the reduction of Cu-ATSM in the microsome/cytosol fraction is shown in Figure 3. The reduction was significantly en-

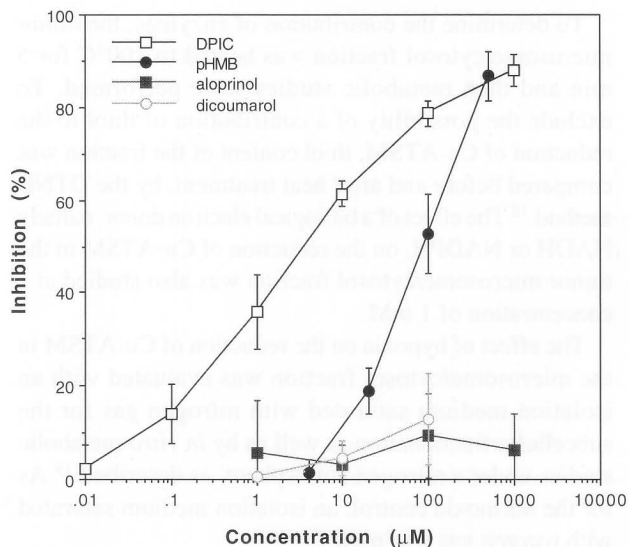


Fig. 4 The effect of redox-enzyme inhibitors on the reduction of Cu-ATSM in the microsomes/cytosol fraction obtained from Ehrlich ascites tumor cells. Average of 3–4 experiments (bar: 1 S.D.).

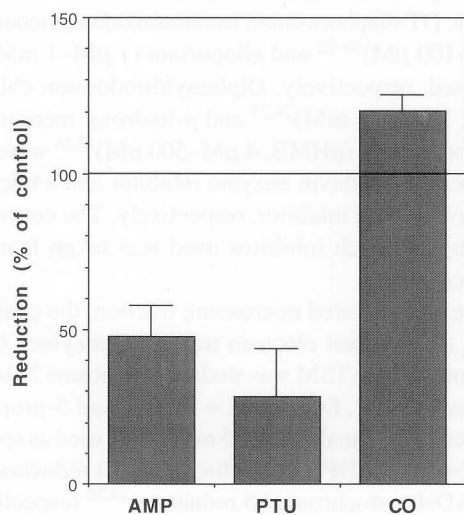


Fig. 5 The Effect of microsomal electron transport enzyme inhibitors on the reduction of Cu-ATSM in the microsomes/cytosol fraction obtained from Ehrlich ascites tumor cells. Average of 3–4 experiments (bar: 1 S.D.).

hanced by the presence of both NADH and NADPH. In our preliminary studies, we confirmed that neither NADH nor NADPH alone was able to reduce Cu-ATSM.¹ In addition, the reduction was completely suppressed by heat treatment. Thiol content in the microsomes/cytosol fraction, a possible non-enzymatic reducing agent, was not decreased but slightly increased after heat treatment (control: $443 \pm 132 \mu\text{M}$, heat-treated: $639 \pm 158 \mu\text{M}$, average and 1 SD of three experiments). If thiol acted as a reducing agent of Cu-ATSM, heat-treatment should

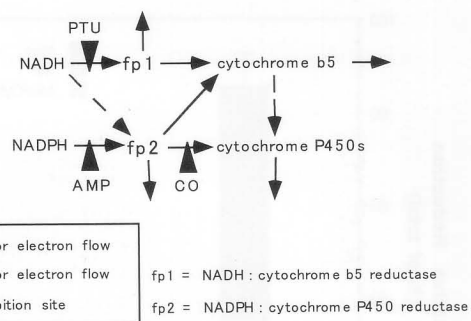


Fig. 6 Schematic electron flow and sites of inhibition of the microsomal electron transport chain.

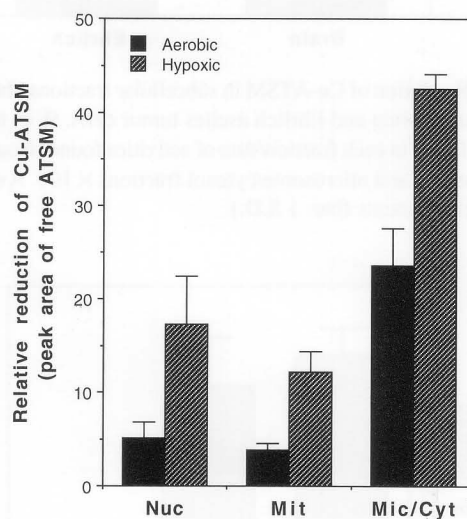


Fig. 7 The effect of hypoxic treatment on the reduction of Cu-ATSM in subcellular fractions obtained from Ehrlich ascites tumor cells. Average of 3–4 experiments (bar: 1 S.D.).

enhance the reduction of Cu-ATSM, so that thiol cannot be considered a major factor in the reduction of Cu-ATSM. It is evident from these results that the contribution of microsomes/cytosol enzymes could be estimated.

To determine the redox enzyme(s) responsible for the reduction of Cu-ATSM in the tumor microsomes/cytosol fractions, specific inhibitors for known reductases were selected and their effect on the reduction of Cu-ATSM was evaluated (Fig. 4). Dicoumarol and allopurinol, specific inhibitors of cytosolic DT-diaphorase and xanthine oxidase, respectively, did not cause any significant inhibition. On the other hand, DPIC, a flavin-enzyme inhibitor, and pHMB, a thiol-enzyme inhibitor, caused strong inhibition in a dose-dependent manner. These two inhibitors are known to act on the microsomal electron transport chain. These results strongly suggest that microsomal enzymes contribute to the reduction of Cu-ATSM in tumor cells.

Further studies with the concentrated microsomes fraction were performed. In the presence of PTU (5 mM) or

AMP (5 mM), specific inhibitors for microsomal NADH: cytochrome b5 reductase (fp1) and NADPH: cytochrome P450 reductase (fp2), respectively, microsomal reduction of Cu-ATSM was largely prevented (Fig. 5). In contrast, pretreatment with carbon monoxide, blocking the electron flow at cytochrome P450, enhanced the reduction of Cu-ATSM. From these results, the reduction of Cu-ATSM in the microsomal electron transport chain (Fig. 6) is considered to occur at fp1 and fp2.

The effect of the oxygen concentration on the reduction of Cu-ATSM in tumor cells was studied (Fig. 7). Hypoxia enhanced the reduction of Cu-ATSM in all fractions, with most of the activity being found in the microsomal fraction under both aerobic and hypoxic conditions.

DISCUSSION

Cu-bis(thiosemicarbazone) complexes (Cu-BTSs), originally developed as antitumor agents, are considered to become cytotoxic on bioreduction from divalent to monovalent Cu.^{11,29,30} In normal tissues, for example the brain, we have found that Cu-pyruvaldehyde-bis(*N*⁴-methylthiosemicarbazone) (Cu-PTSM), a Cu-BTS with good membrane permeability and tissue retention characteristics *in vivo*, was specifically reduced by a mitochondrial electron transport enzyme, NADH dehydrogenase in Complex I.^{12,31} But in comparative studies on experimental tumor cells as well as tumor specimens from patients, we found that the reduction of Cu-PTSM occurred not in the mitochondria but in the microsome/cytosol fraction,¹³ so that the bioreduction systems in normal brain and in tumors, by which Cu-BTSs are reduced, are considered to be different.

The novel hypoxia imaging agent, Cu-ATSM, also demonstrated different patterns of reduction in normal brain and tumor cells, similar to Cu-PTSM. In tumor cells, most of the reduction activity was found in the microsome/cytosol fraction, with little reductive activity seen in the mitochondrial fraction. In addition, the reducing ability of the microsome/cytosol fraction was heat-sensitive and dependent on the presence of electron donors such as NADH or NADPH. These results suggest that bioreductive enzymes in the microsomes or cytosol contribute to the reduction of Cu-ATSM in tumor cells. It can also be stated that the microsomal reduction system also plays a major role in the enhanced reductive retention of Cu-ATSM in hypoxic tumors.

Bioreductive drugs include cytotoxins as well as hypoxia markers. These drugs are activated by intracellular reductase enzymes under hypoxic conditions.³² A wide variety of reductases found in the microsome and/or cytosol have been reported as enzymes for bioreductive drug activation.³³ The reduction of Cu-ATSM was also considered to be caused by some of these microsomal and/or cytosolic enzymes. We selected five major enzymes known to contribute to the reduction of bioreductive drugs

in microsome/cytosol. Among the enzymes studied, Cu-ATSM was selectively reduced by microsomal enzymes, especially by NADH: cytochrome b5 reductase and NADPH: cytochrome P450 reductase. These two enzymes are known to be one-electron reductases involved in the bioactivation of certain kinds of hypoxia sensitive agents,^{24,34} such as tirapazamine (SR4233).¹⁹ Cu-ATSM might be useful for the classification of tumors, from the viewpoint of the enzyme expression-based drug selection.

In conclusion, radiocopper labeled Cu-ATSM will provide useful information not only for the detection of hypoxia, but for the characterization of tumors by means of microsomal bioreductive enzyme expression. For years in drug design, the focus of the medicinal chemist has been on organic compounds and natural products, with little attention being given to inorganic compounds such as metal complexes.¹¹ In the development of hypoxic tumor-seeking agents, most attention was focused on organic compounds such as nitroimidazole-containing compounds. Cu-ATSM shows that even simple metal complexes can act as artificial substrates for biological enzyme systems as well as diagnostic/therapeutic drugs in clinical medicine.

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