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Differential mechanism of retention of Cu-pyruvaldehyde-bis(N^4-methylthiosemicarbazone) (Cu-PTSM) by brain and tumor: A novel radiopharmaceutical for positron emission tomography imaging

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The reductive retention of ^{62}Cu-PTSM was comparatively studied in the brain and Ehrlich ascites tumor cells by electron spin resonance spectrometry and nonradioactive Cu-PTSM. In the brain, only the mitochondrial fraction showed the ability to reduce Cu-PTSM, and the other subcellular fractions did not. In contrast, the cytosolic fraction of Ehrlich ascites tumor cells was the specific site of Cu-PTSM reduction. It was therefore considered that the retention of Cu-PTSM in the brain is closely related to mitochondrial reduction, most probably involving the mitochondrial electron transport system.

Key words: Cu-PTSM, reduction, metabolism, brain, tumor

INTRODUCTION

GENERATOR-PRODUCED positron-emitting ^{62}Cu-labeled copper(II)-pyruvaldehyde-bis(N^4-methylthiosemicarbazone) (Cu-PTSM) has been proposed as a radiopharmaceutical for positron emission tomography imaging of the brain, heart kidneys and tumors,[1,2] but, the mechanism of its retention is still unclear. Green et al. postulated that prolonged tissue retention of copper is due to the reductive decomposition of Cu-PTSM by reaction with ubiquitous intracellular sulfhydryl (SH) groups.[3] Their proposal is based on the findings of Petering et al. regarding the reaction of Cu(II)-α-diketone-bis(thiosemicarbazone) (DTS) complexes in Ehrlich ascites tumor cells.[4,5] On the other hand, Fujibayashi et al. have reported the selective mitochondrial reduction in Cu-PTSM in the murine brain, presumably by an enzymatic reaction.[6] It is therefore likely that there are several mechanisms involved in the retention of Cu-PTSM.

In the present study, Cu-PTSM reduction in brain and tumor tissues was investigated. With subcellular fractions as well as tissue homogenates, the metabolism of Cu-PTSM was studied, and the activity of marker enzymes of the fractions and the SH concentration of each fraction were determined.

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MATERIALS AND METHODS

Cu-PTSM was prepared as described previously.[6,7] BCA protein assay reagent was obtained from Pierce Ltd. (Illinois, USA). Reagents for enzyme assays and DTNB were purchased from Nacarai Tesque (Japan). DdY male mice weighing 25 g were used for the metabolic studies. These mice were fed a commercial diet and tap water ad libitum.

Subcellular fractionation

Brain: Subcellular fractionation of the murine brain was performed by the method reported previously.[8] Brains were isolated, weighed and homogenized with an isolation medium (1.5 g wet tissue/m*l*, 0.25 M sucrose buffered to pH 7.4 with 10 mM HEPES) in a Potter-Elvehjem type homogenizer. Then the homogenate was centrifuged at 1,000 × 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 10,000 × g for 10 min at 4°C, and the

Table 1 Marker enzymes in the brain*

	Homogenate	Nucleus	Mitochondria	Cytosol
Succinate dehydrogenase (mitochondria)				
units/g protein	69.08	15.86	90.14	10.01
	(11.0)	(6.93)	(18.39)	(5.65)
units/*l*	1039	37.00	767.9	55.95
	(174)	(29.63)	(180.4)	(32.40)
% of total	—	4.4	89.4	6.2
		(3.2)	(5.4)	(3.0)
NADH-cytochrome c reductase (microsome)				
units/g protein	3.54	0.61	2.91	5.7
	(1.21)	(1.06)	(0.77)	(1.61)
units/*l*	53.56	1.81	24.37	31.90
	(19.66)	(3.13)	(4.69)	(9.94)
% of total	—	2.4	42.9	54.7
		(4.1)	(7.2)	(4.1)
Lactate dehydrogenase (cytosol)				
units/g protein	396.7	148.2	296.6	634.0
	(41.6)	(46.5)	(48.5)	(74.9)
units/*l*	5979	334.8	2524	3539
	(861)	(228.4)	(456)	(523)
% of total	—	5.1	39.7	55.3
		(3.2)	(7.7)	(5.1)

*Data are the average (1 S.D.) of five experiments.

supernatant (S2, crude microsomal and soluble fractions) was isolated, and the precipitate was resuspended in the medium (P2, crude mitochondrial fraction). The volumes of the P1, P2, and S2 fractions were adjusted to the initial of the brain homogenate volume.

Ehrlich 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.[4] Three milliliters of the ascites was centrifuged at 500 × g and the cells were washed twice with the isolation medium. The cell pellet was suspended in 10 m*l* 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 was obtained by centrifugation at 1,000 × g for 5 min at 4°C. The supernatant was centrifuged at 8,000 × g for 10 min at 4°C to yield the P2 and S2 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.

In vitro *metabolic studies*

One hundred microliters of the Cu-PTSM solution (200 μM in 0.25 M sucrose buffered to pH 7.4 with 10 mM HEPES containing 10% DMSO) was mixed with 900 μl of the homogenate or subcellular fraction, and then the mixture was incubated for 15 min at 37°C. After incubation, 300 μl of the mixture was placed in a sample tube for electron spin resonance spectrometry (ESR) and was frozen in liquid nitrogen so that the ESR signal could be measured at 77°K. Samples without incubation were used as controls.

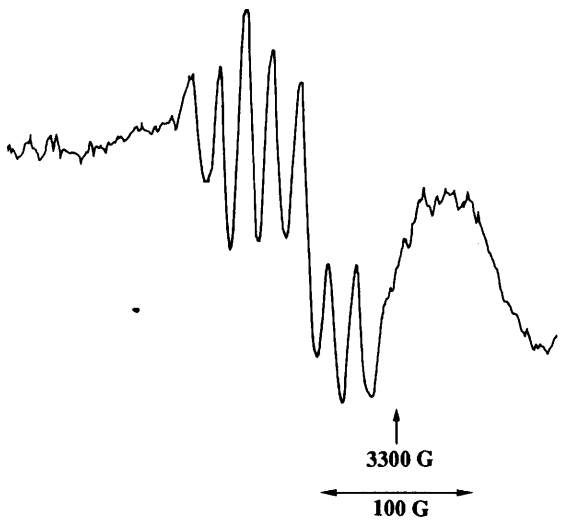

Fig. 1 A typical ESR spectrum of Cu-PTSM.

Assays for protein content and marker enzymes

Fractions were diluted with 10 mM phosphate buffer (pH 7.5), if necessary, and the protein concentration was measured with BCA Protein Assay Reagent. The activities of succinate dehydrogenase, NADH-dehydrogenase

Table 2 Marker enzymes in Ehrlich ascites tumor cells*

	Homogenate	Nucleus	Mitochondria	Cytosol
Succinate dehydrogenase (mitochondria)				
units/g protein	26.55	4.38	120.90	2.25
	(9.01)	(3.85)	(31.60)	(1.65)
units/*l*	148.80	4.76	108.00	8.93
	(62.40)	(4.19)	(26.70)	(7.78)
% of total	—	3.6	89.7	6.7
		(3.2)	(5.1)	(3.8)
NADH-cytochrome c reductase (microsome)				
units/g protein	6.78	3.28	15.54	5.95
	(2.01)	(1.23)	(4.40)	(0.12)
units/*l*	36.71	3.55	13.73	21.97
	(4.63)	(1.2)	(2.06)	(3.17)
% of total	—	9.0	35.0	55.9
		(2.7)	(5.8)	(7.5)
Lactate dehydrogenase (cytosol)				
units/g protein	568.8	240.5	93.43	830.7
	(99.5)	(9.0)	(5.34)	(156.4)
units/*l*	3133	269.5	85.22	3011
	(271)	(57.4)	(18.42)	(232)
% of total	—	8.0	2.5	89.4
		(1.8)	(0.4)	(2.1)

*Data are the average (1 S.D.) of five experiments.

and lactate dehydrogenase were measured as markers of mitochondria,[9] microsome[10] and cytosol,[11] respectively.

SH assay

Fractions were diluted with 1% SDS, and then the SH concentration was determined by the DTNB method.[12]

ESR spectrometry

The ESR spectrum of Cu-PTSM was obtained with an X-band spectrometer (JES-FE3Xg, Japan Electron Optic Laboratory, Japan) under the following conditions; microwave power, 5 mW; modulation amplitude, 6.3 gauss; modulation frequency, 100 kHz; microwave frequency, 9.25 GHz; magnetic field, 3300 ± 500 gauss; and temperature, 77°K. Figure 1 shows a typical ESR spectrum of Cu-PTSM. This spectrum was specific for Cu(II)-PTSM and any Cu(I) complexes were ESR inactive. The strength of the spectrum was used for determination of the Cu(II)-PTSM concentration, as described previously.[6]

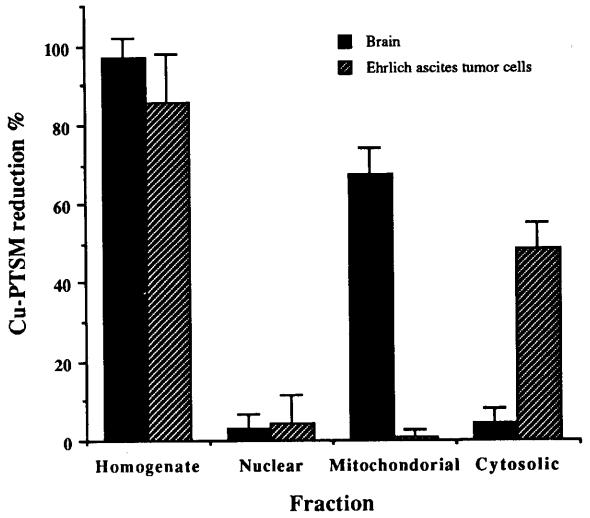

Fig. 2 Reduction of Cu-PTSM in the subcellular fraction of brain tissue and Ehrlich ascites tumor cells. Data are the average and 1 S.D. of five experiments.

RESULTS

Tables 1 and 2 show the marker enzyme activities in the subcellular fractions of the brain and Ehrlich ascites tumor cells. Although the reported procedures for subcellular fractionation were different, marker enzymes for the mitochondria, microsome and cytosol were rather similarly distributed. Succinate dehydrogenase, a mitochondrial enzyme, was found mainly in the mitochondrial fractions (ca. 80–90% of the total) of the tissues. In the case of lactate dehydrogenase, a cytosolic enzyme, almost 90% of its activity was found in the cytosolic fractions of Ehrlich ascites tumor cells, but the level fell to only 55% in the brain cytosolic fractions. In both tissues, microsomal NADH-cytochrome C reductase was distributed in both the cytosolic and mitochondrial fractions, but showed slightly higher levels in the cytosol.

To obtain a similar 90–95% Cu-PTSM reduction under

Table 3 Protein and SH concentrations*

	Homogenate	Nucleus	Mitochondria	Cytosol
Brain				
Protein	15.04	3.10	8.51	5.57
(mg/ml)	(0.81)	(0.84)	(0.80)	(0.17)
SH	1.28	0.25	0.68	0.43
(mM)	(0.13)	(0.07)	(0.04)	(0.07)
SH	85.45	50.68	80.47	77.51
(nmol/mg protein)	(12.32)	(5.60)	(3.49)	(13.62)
Ehrlich ascites tumor cells				
Protein	5.76	1.24	0.97	3.70
(mg/ml)	(1.12)	(0.09)	(0.06)	(0.60)
SH	0.68	0.09	0.08	0.43
(mM)	(0.10)	(0.01)	(0.02)	(0.10)
SH	122.90	82.24	84.83	116.10
(nmol/mg protein)	(19.20)	(16.59)	(20.22)	(7.80)

*Data are the average (1 S.D.) of 5 experiments.

the conditions shown above, each homogenate was processed as indicated in Materials and Methods. The SH and protein concentrations of the homogenates were determined (Table 3). The SH concentration of brain homogenate was twice that found in the Ehrlich ascites tumor cells (1.28 and 0.68 mM, respectively). Accordingly, the reduction abilities normalized for the SH and protein concentrations were completely different, while the relative concentrations of SH (nmole SH/ mg protein) were rather similar. Figure 2 shows the reduction in Cu-PTSM due to the subcellular fractions of the brain tissue and Ehrlich ascites tumor cells. In the brain homogenate, most of the reducing activity was found in the mitochondrial fraction, rather than the nuclear or cytosolic fractions. On the other hand, Ehrlich ascites cells had more than 80% of their reducing activity in the cytosolic fraction.

The mitochondrial fraction of the brain tissue had 9-fold higher levels of protein and SH than the same fraction of Ehrlich ascites tumor cells (Table 3). In contrast, the cytosolic SH concentrations of the brain and Ehrlich ascites cells were similar and the levels in the nuclei fraction were slightly lower. All fractions contained stoichiometrically excessive SH concentrations (final: $74 - 615\ \mu M = 82 - 683\ \mu M \times 0.9$) with regard to the reduction in Cu-PTSM added to the reaction mixture (final concentration: 20 μM).

DISCUSSION

Copper-DTS complexes were originally developed as anticancer agents and most of the studies on the reactions of these complexes have been performed with tumor cells.[4,5,7] These studies have indicated that the reduction in Cu-DTS occurs by interaction with intracellular SH. The present findings in Ehrlich ascites tumor cells agree with this proposal. A large part of the intracellular SH was located in the cytosolic fraction and consequently Cu-PTSM was mostly reduced in the cytosol. Interestingly, no reduction in Cu-PTSM occurred in the mitochondrial fraction, although the SH concentration in the mitochondria was one fifth of that in the cytosol. The nuclear fraction had a similar SH concentration to the mitochondrial fraction, but had a rather higher reducing ability. With our fractionation method, one tenth of the lactate dehydrogenase activity, a cytosolic enzyme, was found in the nuclear fraction, but little was noted in the mitochondrial fraction. Thus, SH derived from the cytosol contaminated the nuclear fraction to some extent and might have contributed to the reduction in Cu-PTSM. If the reduction data are correlated with the marker enzyme levels, then the reduction in Cu-PTSM attributed to various fractions can be corrected and reassigned as shown in Table 4. This analysis indicates that Cu-PTSM was specifically reduced in the cytosol of Ehrlich ascites cells.

On the other hand, brain tissue showed a completely different pattern. The cytosolic fraction of the brain had no ability to reduce Cu-PTSM, although it contained the same SH concentration as that of Ehrlich ascites cells. This indicated that SH can be classified into Cu-PTSM-reducing and Cu-PTSM-nonreducing varieties. The former is found in the cytosol of Ehrlich ascites cells, and the latter is found in the cytosol of the brain. Interestingly, the reduction in Cu-PTSM in the brain occurred mainly in the mitochondrial fraction. This closely matched the subcellular distribution of succinate dehydrogenase, a mitochondrial marker, but not that of NADH-cytochrome c reductase or lactate dehydrogenase. From the corrected analysis shown in Table 4, it can be seen that the reduction in Cu-PTSM in the brain tissue was a mitochondria-specific phenomenon. The high affinity of Cu-PTSM for the mitochondria has been described by Petering et al. and the cytotoxicity of Cu-bisthiosemicarbazone complexes is

Table 4 Contribution of each subcellular fraction to the reduction of Cu-PTSM*

	Mitochondrial	Microsomal	Cytosolic
Ehrlich ascites tumor cells	1.01	0.14	−0.15
Brain	0.02	−0.08	1.06

Values shown are the solutions of the following equations:

	Marker enzyme content (% of total)			Reduction (% of total)**
	Mitochondria	Microsome	Cytosol	
Ehrlich ascites tumor cells				
Nuclear fraction	$3.6X+$	$9.0Y+$	$8.0Z=$	7.82
Mitochondrial fraction	$89.7X+$	$35.0Y+$	$2.5Z=$	1.86
Cytosolic fraciton	$6.7X+$	$55.9Y+$	$89.4Z=$	90.32
Brain				
Nuclear fraction	$4.4X+$	$2.4Y+$	$5.1Z=$	4.01
Mitochondrial fraction	$89.4X+$	$42.9Y+$	$2.5Z=$	90.39
Cytosolic fraciton	$6.2X+$	$54.7Y+$	$55.3Z=$	5.61

*Contribution of the mitochondria, microsomes and cytosol (abbreviated as X, Y and Z in the equation, respectively; $X+Y+Z=1$).

**Calculated from the data shown in Figure 2.

primarily due to the inhibition of cellular respiration,[13] especially at coupling site I between NADH dehydrogenase and coenzyme Q[14] in Complex I. From these results, it appears that the reduction in Cu-PTSM in the brain was not caused by ubiquitous SH, but by mitochondria-specific compound(s) and/or mechanism(s).

The present studies were done with non-radioactive Cu-PTSM, but a rather low concentration of Cu-PTSM could be used when compared with the intracellular SH concentration because of the high sensitivity of ESR. The present results could not therefore be affected by carrier Cu, and might be able to be extrapolated to Cu-62 studies.

In conclusion, it was shown that the mechanism of Cu-PTSM reduction varied according to the type of tissue, so that data obtained with one tissue cannot be extrapolated to other. In the brain, Cu-PTSM was specifically reduced in the mitochondria and this reduction process was completely different from that in Ehrlich ascites tumor cells.

REFERENCES

1. Green MA, Klippenstein DL, Tennison JR. Copper(II) bis(thiosemicarbazone) complexes as potential tracers for evaluation of cerebral and myocardial blood flow with PET. *J Nucl Med* 29: 1549–1557, 1988.
2. Barnhart AJ, Voorhees WD, Green MA. Correlation of Cu(PTSM) localization with regional blood flow in the heart and kidney. *Nucl Med Biol* 16: 747–748, 1989.
3. Green MA. The potential for generator-based PET perfusion tracers. *J Nucl Med* 31: 1641–1645, 1990.
4. Menkel DT, Petering DH. Initial reaction of 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazonato) copper(II) with Ehrlich ascites tumor cells. *Cancer Res* 38: 117–123, 1978.
5. Menkel DT, Saryan LA, Petering DH. Structure-function correlations in the reaction of bis(thiosemicarbozonato) copper(II) complexes with Ehrlich ascites tumor cells. *Cancer Res* 38: 124–129, 1978.
6. Fujibayashi Y, Wada K, Taniuchi H, Yonekura Y, Konishi J, Yokoyama A. 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. *Biol Pharm Bull* 16: 146–149, 1993.
7. Petering HG, Burskirk HH, Underwood GE. The antitumor activity of 2-keto-3-ethoxybutyraldehyde bis(thiosemicarbazone) and related compounds. *Cancer Res* 24: 367–372, 1964.
8. Voss DO, Camplleo AP, Bacila M. The respiratory chain and the oxidative phosphorylation of rat brain mitochondria. *Biochem Biophys Res Commun* 4: 48–51, 1961.
9. Pennington RJ. Biochemistry of dystrophic muscle; mitochondrial succinate-tetrazolium reductase and adenosine triphosphatase. *Biochem J* 80: 649–654, 1961.
10. Omura T, Takasue S. A new methods for simultaneous purification of cytochrome b_5 and NADPH-cytochrome c reductase from rat liver microsomes. *J Biochem* 67: 249–257, 1970.
11. Kornberg A. Lactate dehydrogenase of muscle. *In* Methods in Enzymology, vol. 1, pp. 441–443, 1955.
12. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 82: 70–77, 1959.
13. Van Giessen GJ, Crim JA, Petering DH, Petering HG. Effect of heavy metals on the *in vitro* cytotoxicity of 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazone) and related compounds. *J Natl Cancer Inst* 51: 139–146, 1973.
14. Chan-Stier CH, Minkel D, Petering DH. Reactions of bis(thiosemicarbazonato) copper(II) complexes with tumor cells and mitochondria. *Biochem* 6: 203–217, 1976.