

Preclinical Pharmacokinetic and Safety Studies of Copper-Diacetyl-Bis(N^4 -Methylthiosemicarbazone) (Cu-ATSM): Translational Studies for Internal Radiotherapy



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Abstract

Hypoxia plays important roles in the prognosis of malignant brain tumors such as glioblastoma because it causes drug delivery deficiencies and the induction of hypoxia-inducible factor-1 α in tumor cells. Extensive hypoxic areas are associated with poor prognosis of these fatal diseases. We previously reported that multiple administrations of the hypoxia-targeted internal radiotherapy agent ⁶⁴Cu-diacetyl-bis(N^4 -methylthiosemicarbazone) (⁶⁴Cu-ATSM), four times at intervals of 1 or 2 weeks, show antitumor effects in glioblastoma without treatment-related adverse events. Before initiating clinical trials, preclinical safety studies using Cu-ATSM composed of stable isotopes and its precursor ATSM were required to understand the potential risks of systemic and repeated chemical exposure of our investigational drug. In this study, the concentrations of Cu-ATSM and ATSM in mouse plasma after intravenous administration were determined by liquid chromatography–tandem mass spectrometry, and the half-lives were estimated to be 21.5 and 22.4 minutes for Cu-ATSM and ATSM, respectively. Based on this result, approach 2 of the current ICH M3 [R2] guideline was adopted, and a 7-day intravenous toxicity study was conducted in mice. Cu-ATSM and ATSM in a ratio of 2:25 mimicking our current investigational drug was used, and no adverse effects were observed when Cu-ATSM and ATSM were administered at 81 μ g/kg. These results and those of previous studies suggest that our current investigational drug formulation containing Cu-ATSM and ATSM at a dose of 15 μ g can be safely administered to patients once per week for 4 weeks for treatment with ⁶⁴Cu-ATSM.

Translational Oncology (2019) 12, 1206–1212

Introduction

Malignant brain tumors such as glioblastoma are very aggressive diseases with poor prognosis in humans [1]. Standard therapies are insufficient, and adverse events caused by these therapies lead to severe life-long morbidity. Extensive hypoxic areas are associated with the poor treatment outcomes of patients, as hypoxia promotes the malignant characteristics of cancer cells [2–5]. The expression of hypoxia-inducible factors-1 α (HIF-1 α) in malignant brain tumors promotes angiogenesis by inducing vascular endothelial growth factor; however, new vessels constructed during this process are not well organized [6]. Drug delivery deficiencies and hypoxia caused by

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Declaration of Interest H. M., B. A., and E. K. are employees of Nihon Medi-Physics Co., Ltd. All the other authors declare no competing financial interests.
Received 4 March 2019; Revised 22 May 2019; Accepted 23 May 2019

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1936-5233/19

<https://doi.org/10.1016/j.tranon.2019.05.017>

vascular stasis [6] make it difficult to effectively treat these malignant diseases.

Because of the important effects of hypoxia on the aggressiveness of malignant brain tumors, targeting hypoxia may improve patient outcomes. Therefore, we focused on ^{64}Cu -diacetyl-bis(*N*⁴-methylthiosemicarbazone) (^{64}Cu -ATSM), a promising internal radiotherapy agent that targets hypoxic regions in tumors [7–9]. ^{64}Cu shows β^+ decay (0.653 MeV, 17.4%), β^- decay (0.574 MeV, 40%), and electron capture (42.6%). β^- particles and Auger electrons emitted from this radionuclide can damage tumor cells [7–9]. Particularly, heavy damage to DNA in tumor cells occurs by high-linear energy transfer (LET) from Auger electrons [10]. Our previous preclinical study demonstrated that multiple administrations of ^{64}Cu -ATSM effectively inhibited tumor growth and prolonged the survival of mice bearing U87MG glioblastoma tumors [11]. Based on these findings, we initiated a phase 1 clinical trial of ^{64}Cu -ATSM with multiple doses as a new therapeutic option for malignant brain tumors. This study was conducted to justify the dosages of our planned phase 1 clinical trial.

In our previous study [11], ^{64}Cu -ATSM showed dose-dependent adverse events in BALB/c nude mice, while multiple administrations of ^{64}Cu -ATSM at 37 MBq per dose four times at intervals of a week or two showed no significant adverse events. The adverse events observed in the study were transient body weight loss and hematological toxicity caused by radiation from ^{64}Cu of ≥ 111 MBq. We also reported the 24-hour biodistribution and excretion of ^{64}Cu -ATSM in tumor-free BALB/c nude mice after intravenous injection [12]. Noticeable ^{64}Cu accumulation was observed in the liver, small intestine, and large intestine during the first 6 hours after injection. Large amounts of ^{64}Cu were excreted in the feces by 16 hours after injection, but little urinary excretion was observed in mice. Based on these results, the effective half-life (physical plus biological half-life) of ^{64}Cu -ATSM in the mouse body was estimated to be less than 24 hours.

Based on these findings, we designed our phase 1 study of ^{64}Cu -ATSM to administer a therapeutic dose to patients with malignant brain tumors once per week for 4 weeks. Before initiating this study, preclinical safety studies using copper-diacetyl-bis(*N*⁴-methylthiosemicarbazone) (Cu-ATSM) composed of a stable copper isotope and its precursor H₂-diacetyl-bis(*N*⁴-methylthiosemicarbazone) (ATSM) were required to understand the potential risks of systemic and repeated chemical exposure to our investigational drug. Lewis et al. reported several toxicology and pharmacology studies of Cu-ATSM and ATSM [13]. They observed no significant adverse events. However, our current formulation as an investigational drug for use in planned clinical trials contains sodium ascorbate as a stabilizer, while their formulation contained ethanol rather than sodium ascorbate. This difference in formulation suggested that the systemic exposure of the chemical substances contained in each formulation can be changed. Moreover, their preclinical studies were conducted for the investigational new drug application of ^{64}Cu -ATSM with single administration for positron emission tomography (PET) imaging. In contrast, based on our preclinical efficacy study [11] and biodistribution study [12], we plan to administer the therapeutic dose of ^{64}Cu -ATSM once per week for 4 weeks. Our current formulation of the investigational drug contains 15 μg of Cu-ATSM and ATSM in total; therefore, by combining these previously available data and considering the risks of administration, we adopted approach 2 of the current ICH M3 [R2] guidelines. This approach

uses the following definitions: “total cumulative dose ≤ 500 μg , maximum of five administrations with a washout between doses (six or more actual or predicted half-lives)”. However, the half-lives of Cu-ATSM and ATSM were not determined previously.

In this study, we determined the pharmacokinetics of Cu-ATSM and ATSM in mouse plasma to confirm that the half-lives of these compounds fulfill the definition of approach 2 of the current ICH M3 [R2] guidelines. A 7-day intravenous toxicity study in mice was conducted to determine the safety profile of the chemical substances contained in the investigational drug for initiating clinical trials of this promising therapeutic option for malignant brain tumors.

Materials and Methods

Ethics Statements

The experiments described in this paper were performed in accordance with the laws and guidelines for animal welfare of Japanese government and National Research Council. All animal experimental procedures were approved by the institutional animal ethics committee and conducted in accordance with institutional guidelines. Good Laboratory Practice regulations were observed in the 7-day intravenous toxicity study.

Compounds and Formulation

Cu-ATSM and ATSM were obtained from ABX Advanced Biochemical Laboratories GmbH (Radeberg, Germany). Cu-ATSM and ATSM were formulated with dimethyl-sulfoxide (DMSO), polysorbate-80, sodium L(+)-ascorbate, glycine, and sterile water. The sterile water used in this formulation was of pharmaceutical grade. The other chemicals used were of chemical grade. Cu-ATSM and ATSM were dissolved in DMSO, a solution containing polysorbate-80, sodium L(+)-ascorbate, and glycine was added, and this mixture was diluted with sterile water. The vehicle consisted of 20% DMSO, 5 g/l polysorbate-80, 4.4 g/l sodium L(+)-ascorbate, and 7.4 g/l glycine in sterile water. The current formulation of our investigational drug contains a total of 15 μg of Cu-ATSM and ATSM, and 0.2 ml of DMSO in a total volume of 10.2 ml. Since the doses of Cu-ATSM and ATSM tested in our toxicity study were insoluble in 10% DMSO, which is commonly used in toxicity studies, we used 20% DMSO to dissolve the target doses of Cu-ATSM and ATSM. DMSO >20% has been used as a solvent for poorly soluble lipophilic compounds in previous studies to evaluate their toxicity [14] and PK [15] in mice. In addition, the dose of DMSO we used here has been reported to have minimal effects (e.g., tolerable hemoglobinuria) in mice [16]. Based on these reasons, we used 20% DMSO to evaluate the potential risk of systematic Cu-ATSM and ATSM exposure. Polysorbate-80, which is not contained in our current formulation, was used as a solubilizer because it is generally used in toxicity studies. The dosing volume was 5 ml/kg in the pharmacokinetic study and 10 ml/kg in the 7-day intravenous toxicity study, and administration rates were 0.3 and 0.1 ml/min, respectively. The concentrations of Cu-ATSM and ATSM in the dosing formulation were confirmed by high-performance liquid chromatography (2695 Separation Module, Waters Corporation, Milford, MA) with a Luna C18(2) 100 Å column (4.6 mm I.D. \times 150 mm, 5 μm , Phenomenex, Inc., Torrance, CA).

Animals and Housing

Five-week-old male and female BALB/c mice were obtained from Japan Charles River (Atsugi, Japan). Mice were housed with up to

Table 1. Schedule of Endpoint Collections and Study Timeline

Day	1	2	3	4	5	6	7	8
Dosing	X	X	X	X	X	X	X	
Cage-side observations ^a	X	X	X	X	X	X	X	
Body weight	X			X			X	
Food consumption	X			X			X	
Urinalysis						X		
Hematology, and serum biochemistry								X
Sacrifice ^b								X

^a General conditions such as external appearance, nutritional status, posture, behavior and abnormalities in excrement were observed twice per day (predosing and 1 hour after dosing)

^b Organ weights, necropsy, and histopathological observations.

four other animals of the same sex in polycarbonate bins containing appropriate bedding. A 12-hour light/dark cycle was applied, and mice were housed at a room temperature of 22°C–23°C with a relative humidity of 46%–50%. Solid food (Oriental Yeast Co., Ltd., Tokyo, Japan) and municipal tap water were provided ad libitum. After more than 7 days of quarantine, the mice were weighed and randomized for analysis. There were no known contaminants in the food or water that interfered with the conduct of the studies.

Pharmacokinetic Study in Mouse Plasma

Thirty male mice were randomized into 10 groups (three animals per group). Five groups were intravenously injected with single doses of 25 µg/kg Cu-ATSM, while the other five groups were intravenously injected with single doses of 25 µg/kg ATSM. These doses were considered to provide sufficient exposure to measure the pharmacokinetic parameters in mice and were approximately 1375- and 110-fold higher than the clinical doses on a µg/kg basis. Blood was collected by cardiac puncture under isoflurane anesthesia at 2 minutes, 30 minutes, 1 hour, 4 hours, and 24 hours after injection, and mice were sacrificed at the end of study. Blood was centrifuged, and then plasma was collected and mixed with ice-cold DMSO and acetonitrile. The Cu-ATSM and ATSM content of the supernatant was measured using a liquid chromatography–tandem mass spectrometry (LC–MS/MS) system from Waters Corporation, which consisted of LC (ACQUITY UPLC I-Class Systems) and MS/MS (Xevo TQ-XS, MRM type, ES+ mode). Chromatographic separation was conducted using an InertSustain C18 column (2.1 mm I.D. × 50 mm; 5 µm; GL Sciences, Tokyo, Japan), eluting with an acetonitrile–water gradient of 10 mmol/l ammonium acetate. The elution of Cu-ATSM, ATSM, and the internal standard (niflumic acid) was monitored using the positive electrospray ionization multiple-reaction monitoring mode with the *m/z* transitions of 322.0 (Q1) >> 248.9 (Q3), 261.1 (Q1) >> 156.1 (Q3), and 283.1 (Q1) >> 245.1 (Q3), respectively. The calibration range was 1–1000 ng/ml, and the lower limit of quantification was < 1 ng/ml in plasma.

7-Day Intravenous Toxicity Study in Mice

Fifty male and 50 female mice were randomized into single-sex groups for administration of daily doses of saline, vehicle, 27 µg/kg mixture of Cu-ATSM and ATSM (Cu-ATSM/ATSM), 81 µg/kg Cu-ATSM/ATSM, or 135 µg/kg Cu-ATSM/ATSM. These doses were expected to provide appropriate exposure multiples against human systemic exposure at therapeutic doses. Given that 15 µg of ATSM was labeled with 10 GBq of ⁶⁴Cu and based on our recent results for the specific activity of ⁶⁴Cu, the chemical ratio of Cu-

ATSM on ATSM was predicted to be 2:25 in our current formulation of the investigational drug for the planned clinical trials. Therefore, we mixed Cu-ATSM and ATSM at this ratio, and 27, 81, and 135 µg/kg were considered as approximately 110-, 320-, and 540-fold higher than the clinical doses on a µg/kg basis. Prior to administration, all animals were weighed, and randomization was stratified by body weight. Mice were treated daily for 7 days according to the approach 2 of current ICH M3 [R2] guidelines.

The schedule of study assessments is summarized in Table 1. Cage-side observations were performed daily at predosing and 1 hour postdosing. Evaluation of the predosing body weight and food consumption was performed at days 1, 4, and 7. Urine samples were collected at day 6, and blood samples were collected at 1 day after the last dosing to assess clinical pathology parameters including urinalysis, hematological assessment, and serum chemistry. Urinalysis was performed using an automatic urine analyzer (CLINITEK 500, Siemens Healthineers, Erlangen, Germany). Hematological parameters were measured using a hematological analyzer (ADVIA 2120i, Siemens Healthineers). Biochemical parameters in the serum were measured using a blood biochemical analyzer (TBA 120FR, Toshiba Medical Systems, Tokyo, Japan). Animals were sacrificed at 1 day after the last dosing, and necropsy was performed. The brain, heart, lung, liver, kidney, and testes were excised and weighed.

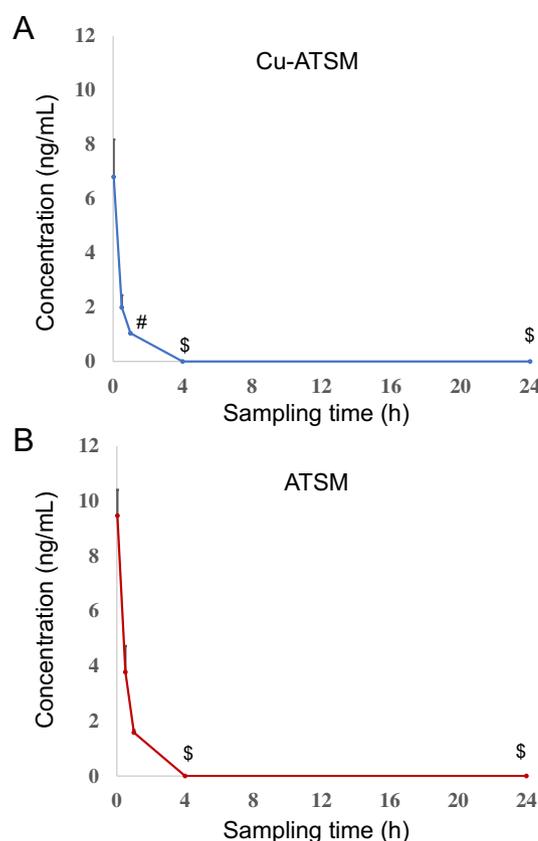


Figure 1. Concentrations of Cu-ATSM and ATSM in mouse plasma after intravenous administration. The average concentrations of (A) Cu-ATSM and (B) ATSM in mouse plasma after intravenous administration. Values are shown as the mean ± SD of three animals. \$: Below the lower limit of quantification (< 1 ng/ml). #: One data point of three mice was below the lower limit of quantification, and therefore, SD was not calculated.

Table 2. Pharmacokinetic Parameters of Cu-ATSM and ATSM in Mouse Plasma

	Cu-ATSM	ATSM
C_{max} (ng/ml)	6.80	9.47
T_{max} (h)	3.33×10^{-2}	3.33×10^{-2}
$T_{1/2}$ (h)	3.58×10^{-1}	3.74×10^{-1}
AUC _{all} (ng·h/ml)	4.61	7.13

Histopathological evaluations were performed for the cerebrum, cerebellum, heart, lung, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, kidney, testis/ovary, femur, administration site (tail), and macroscopically abnormal site.

Statistical Analysis

Data were expressed as the means with corresponding standard deviations (SDs). Pharmacokinetic parameters in plasma were determined with Phoenix WinNonlin 6.4 software (Certara, Princeton, NJ). To compare the vehicle group vs. Cu-ATSM/ATSM-treated groups, Bartlett and Dunnett tests were performed. In the case of heterogeneous group variances, the Steel test was performed. *P* values less than .05 were considered statistically significant.

Results

Pharmacokinetic Parameters of Cu-ATSM and ATSM in Mouse Plasma

Figure 1 shows the concentrations of Cu-ATSM and ATSM in the plasma after intravenous administration into mice. The pharmaco-

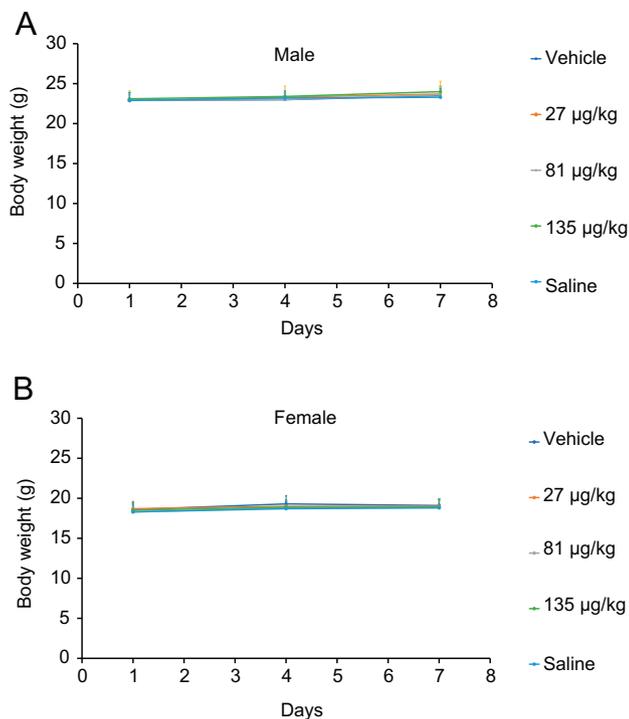


Figure 2. Changes in body weights of mice after intravenous administration of Cu-ATSM and ATSM. Plots of the average body weight of (A) male and (B) female mice in each treatment group in the 7-day intravenous toxicity study are shown. Values are shown as the mean ± SD of 10 animals except for the 135-µg/kg-treated group of males on day 7 with nine animals.

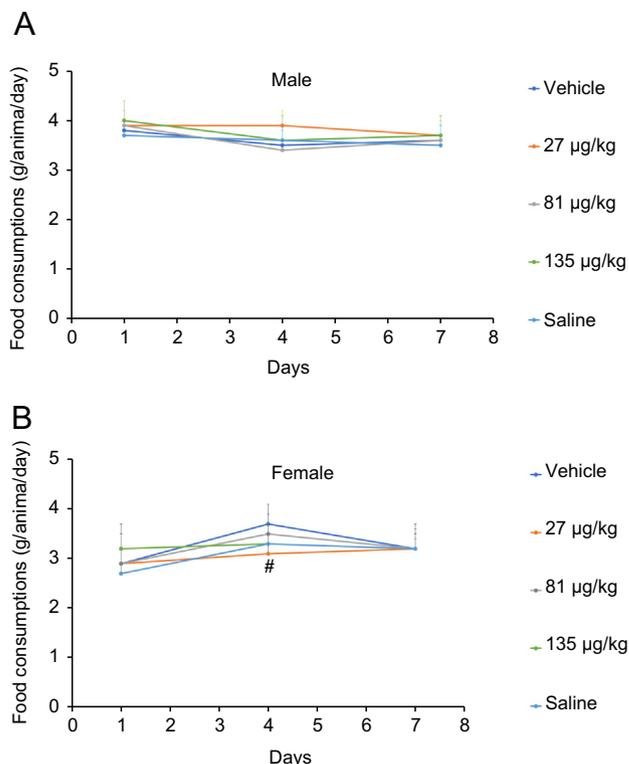


Figure 3. Changes in food consumption of mice after the intravenous administration of Cu-ATSM and ATSM. (A) Male. (B) Female. Plots of average food consumption for each treatment group in the 7-day intravenous toxicity study are shown. Values are shown as the mean ± SD of the consumed food grams by each animal per day of 10 animals except for the 135-µg/kg-treated group of males on day 7 with nine animals. #*P* > .05 between 27-µg/kg-treated group vs. vehicle control.

kinetics parameters of Cu-ATSM and ATSM determined from these data are shown in Table 2. The maximum plasma concentrations (C_{max}) were observed 2 minutes after the administration of both compounds. Thereafter, both compounds were rapidly cleared from the blood. The estimated half-lives ($T_{1/2}$) of Cu-ATSM and ATSM in the plasma were 21.5 and 22.4 minutes, respectively. Based on this result, approach 2 of the current ICH M3 [R2] guideline was adopted, and a 7-day intravenous toxicity study was conducted in mice.

7-Day Intravenous Toxicity Study in Mice

In-Life Observations. In the 135-µg/kg-treated group, one male mouse and one female mouse died immediately after dosing at days 5 and 7, respectively. These animals showed no abnormalities in cage-side observations, body weight, and food consumption during their survival. Necropsies and histopathological observations of these animals revealed injection-derived minimal hemorrhage and inflammation at the injection site only.

For the remaining surviving animals, no treatment-related clinical signs of toxicity were observed during the study. There were no significant differences in body weight (Figure 2) between the treated groups vs. vehicle control group. Food consumption in each group is shown in Figure 3. A significant difference was found in the female 27-µg/kg-treated group on day 4. However, this was observed only

Table 3. Results of Urinalysis

Sex	Male					Female					
	Dose ($\mu\text{g}/\text{kg}$)	0	27	81	135	Saline	0	27	81	135	Saline
No. of animals	6	6	6	6	6	6	6	6	6	6	6
Proteins											
(-)	0	0	0	0	0	0	0	0	1	0	
(+/-)	0	0	0	1	0	0	1	1	0	2	
(1+)	2	1	1	2	5	2	2	2	2	4	
(2+)	2	2	3	3	1	1	1	1	2	0	
(3+)	2	3	2	0	0	3	2	2	1	0	
Glucose											
(-)	2	2	2	4	6	0	0	2	2	2	
(1+)	4	4	4	2	0	4	5	4	4	4	
(2+)	0	0	0	0	0	2	1	0	0	0	
Uric blood											
(-)	3	2	1	4	6	2	3	2	2	6	
(+/-)	0	0	0	0	0	0	0	1	1	0	
(1+)	0	0	0	0	0	1	0	0	0	0	
(2+)	0	1	3	0	0	0	0	0	0	0	
(3+)	3	3	2	2	0	3	3	3	3	0	
Color											
Light yellow	0	0	0	0	0	0	0	0	0	0	
Yellow	3	2	1	4	6	2	3	3	4	6	
Dark yellow	0	0	0	0	0	0	0	0	0	0	
Brown	3	4	5	2	0	4	3	3	2	0	

on day 4 in females and was not a dose-dependent phenomenon, and thus, finding was considered as incidental.

Clinical Pathology (Urinalysis, Hematology, and Serum Biochemistry). Table 3 summarizes the urinalysis findings. Treatment-related changes were not observed in all animals. Urine with brown color was observed in several animals in the vehicle control group and treated groups. These animals had uric blood scores of 1+ or higher, while microscopic examination revealed no red blood cells in the urinary sediments of these animals. Additionally, compared to the saline group, animals in the vehicle control and treated groups showed increased protein and glucose levels. Because DMSO causes intravascular hemolysis and hemoglobinuria [16,17], the changes observed in the urine were considered to be caused by DMSO contained in the vehicle.

There were no significant changes in hematological parameters between any treated groups vs. the vehicle control group. Compared to the saline group, red blood cells, hemoglobin, and hematocrit were increased in the vehicle control groups and treated groups. Therefore, these hematological changes were considered as caused by the vehicle. The hematological parameters of each group are summarized in Supplemental Table S1-1 and 1-2.

There were no significant changes in serum biochemical parameters between any treated groups vs. the vehicle control group. A significant difference in the glucose level was found in the female 81- $\mu\text{g}/\text{kg}$ -treated group. However, this effect was not dose-dependent, and therefore, this finding was considered as incidental. The serum biochemical parameters in each group are summarized in Supplemental Table S2-1 and 2-2.

Organ Weights, Necropsy, and Histopathological Observations. There were no significant changes in organ weights between any treated groups vs. the vehicle control group. The brain weight of the male 135- $\mu\text{g}/\text{kg}$ -treated group was significantly higher than that of the vehicle control group; however, the relative difference was negligible. The organ weights of each groups are summarized in Supplemental Table S3-1 and 3-2. There were no treatment-related observable changes in the organs of each group according to necropsy.

Histopathological examinations were conducted for a group treated with the highest dose, 135 $\mu\text{g}/\text{kg}$, and compared the findings in the vehicle control group. There were no treatment-related histopathological changes in the 135- $\mu\text{g}/\text{kg}$ -treated group compared to the vehicle control group. Calcification in the heart, chronic inflammation in the rectal serosa, liver cell infiltration, regenerative renal tubules in the kidney, and cardiac arteritis were observed in the 135- $\mu\text{g}/\text{kg}$ -treated group and vehicle control group. Thrombosis, bleeding, and inflammation at the administration site were observed in both groups. Therefore, these histopathological changes were considered as caused by the vehicle. Because there were no significant differences in histopathological observations between the 135- $\mu\text{g}/\text{kg}$ -treated group and vehicle control group, histopathological observations in the 27- and 81- $\mu\text{g}/\text{kg}$ -treated groups were omitted. Histopathological findings in the 135- $\mu\text{g}/\text{kg}$ -treated group and vehicle control group are summarized in Table 4.

Discussion

This study was conducted to investigate the pharmacokinetic parameters and safety profiles of Cu-ATSM and ATSM to assess the potential risk of systemic exposure by chemical substances contained in our investigational drug ^{64}Cu -ATSM for internal radiotherapy of glioblastoma and other malignant brain tumors. Hypoxic areas in malignant brain tumors are known to be resistant to chemo- and radiation therapy [2–5], and ^{64}Cu -ATSM targets the overreduced state in tumors under hypoxic conditions [7,9,18–24]. In our previous study, we demonstrated that the intravenous administration of ^{64}Cu -ATSM effectively treated mice bearing U87MG glioblastoma without treatment-related adverse events [11].

In our pharmacokinetics study, the maximum observed plasma concentrations (C_{max}) were 6.80 and 9.47 ng/ml for Cu-ATSM and ATSM, respectively, and occurred at the first sampling time, i.e., 2 minutes after their intravenous administration. Our data demonstrated a rapid distribution phase followed by a terminal phase in which plasma concentrations fell below 1 ng/ml within 4 hours of intravenous administration (Figure 1). By fitting these data with a single-exponential curve, the estimated half-lives ($T_{1/2}$) of Cu-ATSM and ATSM in the plasma were 21.5 and 22.4 minutes, respectively. In our previous study, ^{64}Cu -ATSM rapidly accumulated in tumor tissues after intravenous administration to HT-29 tumor-bearing mice, with tumor uptake showing a plateau by 1 hour [12]. This rapid distribution of ^{64}Cu -ATSM into tumor tissues has been reported to be associated with a tumor overreduced status and resulted in antitumor effects [18,25]. The half-lives of Cu-ATSM and ATSM estimated in this study were consistent with those of previous biodistribution and efficacy studies and suggest that the chemical substances contained in the ^{64}Cu -ATSM investigational drug formulation did not accumulate in the body at the 1-week dosing intervals as scheduled in our planned phase 1 trial.

Based on the pharmacokinetic results, we adopted approach 2 of the current ICH M3 [R2] guidelines and conducted a 7-day intravenous toxicity study. A mixture of Cu-ATSM and ATSM was administered once per day for 7 days into BALB/c mice intravenously, and general toxicities were compared to those of the vehicle and saline controls. Based on these results, the no-observed-adverse-effect-level of the mixture of Cu-ATSM and ATSM was determined to be 81 $\mu\text{g}/\text{kg}$. This dose is approximately 320-fold higher on a $\mu\text{g}/\text{kg}$ basis than the chemical doses of Cu-ATSM and ATSM, by a total of 15 μg per dose, in our current investigational drug formulation.

Table 4. Histopathological Findings

Sex	Male		Female	
	Dose (μg/kg)	135	0	135
No. of animals	10	9	10	9
Heart				
Mineralization	1	1	1	1
Minimal	0	0	1	0
Mild	1	1	0	1
Arteritis	0	0	1	0
Mild	0	0	1	0
Rectum				
Inflammation, chronic, serosa	2	2	1	1
Minimal	1	0	1	1
Mild	1	2	0	0
Liver				
Cell infiltration	3	2	3	1
Minimal	3	2	3	1
Kidney				
Regeneration, tubular	1	1	0	0
Minimal	1	1	0	0
Injection site (tail)				
Thrombus	4	4	3	1
Minimal	1	1	1	1
Mild	3	3	2	0
Hemorrhage	3	5	8	3
Minimal	2	4	4	2
Mild	1	1	4	1
Inflammation	10	7	10	8
Minimal	5	4	7	5
Mild	5	3	3	3

In the 135-μg/kg-treated group, one male and one female mouse died immediately after dosing at days 5 and 7, respectively. These animals showed no abnormalities in cage-side observations, body weight, and food consumption during their survival. Necropsies and histopathological observations of these animals showed injection-derived mild inflammation at the injection site only. Because Cu-ATSM and ATSM are soluble in DMSO and the dose was approximately 540-fold higher than the chemical dose of Cu-ATSM and ATSM contained in our current investigational drug formulation, the potential risk of chemical exposure at this level is quite low. For the remaining surviving animals, no treatment-related changes in cage-side observations, body weights, food consumption, urinalysis, hematological and serum biochemical parameters, necropsy, organ weights, and histopathological examinations were observed during the study.

Lewis et al. conducted the preclinical toxicology and pharmacology analyses of a Cu-ATSM formulation using standard *in vitro* and *in vivo* assays, as well as 14-day toxicity studies in both rats and rabbits [13], before initiating their clinical trial of ⁶⁴Cu-ATSM for PET imaging. Their formulation used in toxicology studies contained ATSM (69.73%), Cu-ATSM (4.98%), CoCl₂ (0.38%), and NiCl₂ (24.91%). These four solid materials were dissolved in DMSO (1%), ethanol (5%), and saline (94%) before use. Mutagenicity was determined in the *in vitro* *Salmonella* reverse-mutation plate-incorporation assay, *in vitro* L5178Y/TK^{+/-} mouse lymphoma mutation assay, and *in vivo* micronucleus assay in rats. Safety pharmacology studies were performed by cardiovascular and pulmonary safety testing in beagle dogs, and neurologic safety assessment was conducted in rats. A 14-day toxicity study of the toxicology formulation in rats and rabbits was also performed. The

results showed that the formulation has an appropriate margin of safety for clinical use. They submitted an investigational new drug application to the U.S. Food and Drug Administration to initiate clinical trials of ⁶⁴Cu-ATSM by single-dose intravenous administration for PET imaging of patients with cervical cancer. Our current formulation contains sodium ascorbate as a stabilizer rather than ethanol. Additionally, our dosing schedule was once per week for 4 weeks rather than single administration. However, the results of their studies were very supportive for preparing our investigational new drug application of ⁶⁴Cu-ATSM for treating malignant brain tumors.

In conclusion, this study demonstrated that after intravenous administration, the half-lives of Cu-ATSM and ATSM in the plasma of mice were 21.5 and 22.4 minutes, respectively, and the no-observed-adverse-effect-levels of Cu-ATSM and ATSM were 81 μg/kg in a 7-day intravenous toxicity study in mice. These results and those of previous studies suggest that intravenous administration of Cu-ATSM and ATSM at 15 μg per dose is safe in patients once per week for 4 weeks to treat glioblastoma and other malignant brain tumor in patients by ⁶⁴Cu-ATSM.

Funding

This study was supported by Japan Agency for Medical Research and Development (AMED) programs of Practical Research for Innovative Cancer Control (grant number 18ck0106373h0002) and Project of Translational and Clinical Research Core Centers (grant number 18lk1403002h0001).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tranon.2019.05.017>.

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