**Internal radiotherapy with copper-64-diacetyl-bis (N^4 -methylthiosemicarbazone)
reduces CD133⁺ highly tumorigenic cells and metastatic ability of mouse colon
carcinoma**

Abbreviated title: ^{64}Cu -ATSM treatment reduces CD133⁺ cells

Yukie Yoshii¹, Takako Furukawa², Yasushi Kiyono¹, Ryo Watanabe³, Tetsuya Mori¹,
Hiroshi Yoshii⁴, Tatsuya Asai^{1,3}, Hidehiko Okazawa¹, Michael J. Welch⁵ and Yasuhisa
Fujibayashi^{1,2}

¹*Biomedical Imaging Research Center, University of Fukui, Eiheiji, Fukui 910-1193,
Japan*

²*Molecular Imaging Center, National Institute of Radiological Sciences, Inage, Chiba
263-8555, Japan*

³*Faculty of Engineering, University of Fukui, Fukui 910-8507, Japan*

⁴*Faculty of Medical Sciences, University of Fukui, Eiheiji, Fukui 910-1193, Japan*

⁵*Mallinckrodt Institute of Radiology, Washington University School of Medicine, St.
Louis, Missouri 63110, USA*

*Corresponding author: Yasuhisa Fujibayashi PhD, Molecular Imaging Center, National
Institute of Radiological Sciences, 4-9-1, Anagawa, Inage, Chiba 263-8555, Japan. E-
mail: yfuji@nirs.go.jp. TEL: +81-43-206-3423. FAX: +81-43-206-4079.

Key words: cancer stem cell, CD133, ^{64}Cu -ATSM, highly tumorigenic cells, hypoxia, internal radiotherapy

Abstract

Introduction: ^{64}Cu -diacetyl-bis (N^4 -methylthiosemicarbazone) (^{64}Cu -ATSM) is an imaging agent for positron emission tomography (PET) that targets hypoxic tumors. ^{64}Cu -ATSM is also reported to be a potential agent for internal radiotherapy. In a mouse colon carcinoma (Colon-26) model, we have shown that ^{64}Cu -ATSM preferentially localizes in intratumoral regions with a high density of CD133^+ cells, which show characteristics of cancer stem cells or cancer stem cell-like cells (CSCs). In this study, we evaluated the therapeutic effect of ^{64}Cu -ATSM in relation to CD133 expression using this model.

Methods: Systemic administration of 37 MBq ^{64}Cu -ATSM or saline was conducted twice within a 1-week interval to mice bearing 1-week-old Colon-26 tumors (day 0–7). At day 19, tumor size measurement, flow cytometry analysis and experimental lung metastatic assay were performed. The therapeutic effect of ^{64}Cu -ATSM on sorted CD133^+ and CD133^- Colon-26 cells was also examined *in vitro*.

Results: *In vivo* studies showed that ^{64}Cu -ATSM treatment inhibited tumor growth. The percentage of CD133^+ cells and metastatic ability in ^{64}Cu -ATSM treated tumors was decreased compared with that in control animals. *In vitro* studies demonstrated that ^{64}Cu -ATSM accumulated in cells under hypoxic conditions and incorporation of ^{64}Cu -ATSM under hypoxia caused cell death in both CD133^+ and CD133^- cells in a similar extent.

Conclusions: ^{64}Cu -ATSM administration reduced tumor volume as well as percentage of CD133^+ cells and the metastatic ability of Colon-26 tumors. Together with our data, it is suggested that ^{64}Cu -ATSM accumulates in regions high in CD133^+ highly tumorigenic

cells and kills such regions by radiation resulting in a decrease of the percentage of CD133⁺ cells.

1. Introduction

Hypoxic tumor is related to the malignant behaviours of tumors, such as poor outcome in radiotherapy/chemotherapy and metastasis (1). Thus, it is of great significance to develop methods for diagnosis and therapy of hypoxic tumors.

Radiolabeled Cu-diacetyl-bis (*N*⁴-methylthiosemicarbazone) (Cu-ATSM) has been developed as an imaging agent targeting hypoxic tumors for use with positron emission tomography (PET) (2-8). Previous studies have shown that Cu-ATSM uptake is dependent upon the level of hypoxia, *in vitro* and *in vivo*. The mechanism of Cu-ATSM accumulation in hypoxic regions has been reported (4, 9-12): under highly reduced intracellular conditions such as hypoxia, Cu(II) in Cu-ATSM is reduced to Cu(I), instantly released from the ATSM ligand and trapped in the cells (4, 9-11). It has been also shown that Cu-ATSM uptake is associated with a tumor's therapeutic resistance and metastatic potential in several tumors including cervical carcinoma and rectal carcinoma (2, 8, 13, 14).

Among the Cu radioisotopes available (⁶⁰Cu, ⁶¹Cu, ⁶²Cu, ⁶⁴Cu and ⁶⁷Cu), ⁶⁴Cu has several advantages, including the fact that it can be readily produced with an in-hospital small cyclotron and can be used not only for imaging but also internal radiation therapy (15-18). ⁶⁴Cu decays by β^+ decay (0.66 MeV, 19 %), β^- decay (0.58 MeV, 40 %) and electron capture (44 %), which means that this nuclide can be detected by PET but also act as a therapeutic nuclide due to β^- particle emission and Auger electron emission. In addition, the half-life of ⁶⁴Cu ($t_{1/2} = 12.7$ h) is appropriate for both diagnostic and therapeutic use. Copper-64 has been reported to have similar lethality to ⁶⁷Cu for tumor treatment (19). The therapeutic effect of ⁶⁴Cu-ATSM has been demonstrated in both *in*

vitro (17) and *in vivo* studies (16, 20). In these studies, Obata et al. (17) showed that ^{64}Cu -ATSM reduced the clonogenic survival rate of tumor cells and caused postmitotic apoptosis *in vitro*. Lewis et al. (16) showed that treatment with ^{64}Cu -ATSM increased the survival time of tumor-bearing hamsters. Thus, ^{64}Cu -ATSM is a potential agent for internal radiotherapy of tumors.

Recently the concept of “cancer stem cells” has been developed and provides a new paradigm to understand tumor biology (21, 22). Based on published data, it is shown that a certain type of cancer cells called cancer stem cells or cancer stem cell-like cells (collectively referred here as CSCs) are present in small numbers within tumors, but contribute to difficulties in eradicating tumors by possessing high therapeutic resistance and metastatic ability (21, 22). CD133 (Prominin-1) is a frequently used marker to identify the CSCs in various human and murine cancers, such as brain, prostate, breast, liver and colon (23-29). It has also been reported that CD133^+ cancer cells, rather than CD133^- cancer cells, show high colony-forming ability and high tumor-initiating ability and possess survival advantage under hypoxia (23-33). CD133^+ cells are also recognized as possessing resistance to radiotherapy/chemotherapy and metastatic potential of tumors (34, 35). Therefore, it is considered that CD133^+ cells contribute to difficulties in eradicating tumors due to their therapeutic resistance, although the CD133^+ cells are present in a small population within tumors. In a previous study (34), using a mouse colon carcinoma (Colon-26) model, we demonstrated that ^{64}Cu -ATSM preferentially accumulated in intratumoral regions with a relatively high density of CD133^+ cells by immunohistochemistry and autoradiography analyses: in the study, we classified tumor sections into several areas by ^{64}Cu -ATSM uptake and examined the density of CD133^+

cells in each area. Also, our previous study showed that the CD133⁺ Colon-26 cells possess the typical characteristics of CSCs, such as high colony-forming ability, high tumor-initiating ability and increased survival under hypoxia. Namely, since the CD133⁺ cells are in a small population, but possess malignant characteristics within tumors, it is important to decrease the number of CD133⁺ cells present within tumors in tumor treatment. In this study, we have developed a therapy technique that decreases the number of CD133⁺ cells present within the tumor. We hypothesized that internal radiotherapy with ⁶⁴Cu-ATSM will reduce the number of CD133⁺ cells present within tumors by accumulating in CD133⁺ cell-rich regions. In this study, we examined the therapeutic effect of ⁶⁴Cu-ATSM in relation to CD133 expression, with Colon-26 model *in vivo* and *in vitro*.

2. Materials and methods

2.1. Cell lines and growth

We used a mouse colon carcinoma cell line, Colon-26 (TKG 0518; Cell Resource Centre for Biomedical Research, Tohoku University, Sendai, Japan). The cells were incubated in a humidified atmosphere of 5% CO₂ in air at 37 °C. Dulbecco's modified Eagle's medium (DMEM 11995-065; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and antibiotics were used for cell growth. Exponentially growing cells were used for the study. The cells were trypsinized to detach them from the plates and were assayed using the trypan blue dye-exclusion method for cell counting.

2.2. Radioisotope production and radiochemical synthesis

^{64}Cu was prepared with an RDS eclipse biomedical cyclotron (Siemens, Knoxville, TN) according to a method previously reported (15). The separation of ^{64}Cu and the synthesis of ^{64}Cu -ATSM was carried out using a previously reported procedure (15, 36).

2.3 Animal studies

All animal experiments and procedures were conducted in compliance with the Animal Treatment Regulations of the University of Fukui, Japan. BALB/c male mice (6 weeks of age, 20 to 25 g of body weight) were obtained from Japan SLC (Shizuoka, Japan). Before the experiments, the mice were kept undisturbed for at least 1 week.

2.4 In vivo ^{64}Cu -ATSM treatment

Colon-26 cells suspended in phosphate-buffered saline (PBS) (at 1×10^7) were subcutaneously injected into the flanks of the mice. One week after implantation (tumor grew to approximately 3 mm diameter), 37 MBq (1 mCi) of ^{64}Cu -ATSM (^{64}Cu -ATSM treatment) or saline (control) were administered intravenously into each tumor-bearing mouse ($n = 6$) (day 0). On day 7, 37 MBq of ^{64}Cu -ATSM (^{64}Cu -ATSM treatment) or saline (control) were injected intravenously to each mouse. The radioactivity dosage of ^{64}Cu -ATSM treatment used here was determined based on previous studies (16, 20). On day 19, the tumors were removed and the size was measured using precision callipers. The tumor volume was calculated using the method reported by Janik et al. (37). In the study of tumor volume, saline containing ATSM in equal amounts to the radioactive studies was also evaluated.

2.5 Flow cytometry of treated tumors

The isolated ^{64}Cu -ATSM-treated and control tumors on day 19 were cleaned with saline, dissected, enzymatically digested and passed through a 100 μm nylon mesh. The isolated cells were subjected to flow cytometry and experimental lung metastatic assay. Flow cytometry analysis was performed using a method previously reported (36). Briefly, the cells were labelled with the rabbit anti-mouse CD133 antigen antibody (1:100 dilution; ab16518, Abcam, Cambridge, UK) and Alexa Flour 488-conjugated secondary antibody (1:100 dilution) (Molecular Probes, Eugene, OR). The labelled cells were analyzed by the Beckman Coulter EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA).

2.6 Experimental lung metastatic assay

The cells isolated from ^{64}Cu -ATSM-treated and control tumors were also used for the experimental lung metastatic assay. In this assay, the 5×10^4 isolated cells in 100 μl PBS were intravenously inoculated into mice. After 16 days the mice were sacrificed and their lungs were isolated and fixed in 70 % ethanol. The number of lung metastases was counted using a dissecting microscope.

2.7 Separation of CD133^+ and CD133^- cells

Cultured Colon-26 cells were stained by rabbit anti-mouse CD133 antigen antibody and Alexa Flour 488-conjugated secondary antibody as described above. For

separation the cells were sorted using a EPICS ELITE cell sorter (Beckman Coulter, Fullerton, CA).

2.8 In vitro characterization of uptake and therapeutic effect of ^{64}Cu -ATSM in sorted cells

To characterize ^{64}Cu -ATSM uptake in CD133^+ and CD133^- Colon-26 cells under normoxic and hypoxic conditions, ^{64}Cu -ATSM uptake study was performed with CD133^+ and CD133^- Colon-26 cells sorted by the cell sorter as described above. For the ^{64}Cu -ATSM uptake study, the sorted cells were preincubated under normoxia for normoxic uptake or hypoxia (1% O_2) for hypoxic uptake, for 2 h in the growth medium. In this experiment, hypoxia (1% O_2 , 94% N_2 and 5% CO_2) was achieved with a hypoxic chamber (Personal Multi Gas Incubator; Astec, Fukuoka, Japan). The preincubated cells of CD133^+ and CD133^- Colon-26 cells (5×10^3) were suspended in a growth medium containing ^{64}Cu -ATSM at 5 $\mu\text{Ci}/\text{ml}$ and incubated for 1 h under normoxic or hypoxic conditions, respectively. After 1 h incubation, the cells were collected and washed twice with ice-cold PBS and the radioactivity was counted by a γ -counter (Wizard 1480, PerkinElmer Wallac, Turku, Finland). Next, we evaluated therapeutic effect of ^{64}Cu -ATSM on CD133^+ and CD133^- Colon-26 cells. In this assay, to make cells incorporate ^{64}Cu -ATSM, the sorted CD133^+ and CD133^- cells were treated under hypoxia in the same manner as in uptake study: cells were incubated in a growth medium containing ^{64}Cu -ATSM at 5 $\mu\text{Ci}/\text{ml}$ for ^{64}Cu -ATSM treatment or that without radioactivity for control. The resultant cells were seeded in 2 ml growth medium in 35-mm dishes (BD Falcon, Franklin Lakes, NJ) to form colonies in accordance with (17, 36). The medium was

changed every 3 days. At day 7, colonies were stained with Diff-Quik kit (Sysmex, Kobe, Japan) and the number of colonies containing more than 50 cells was counted to determine the survival fractions.

2.9 Statistical analysis

Data are expressed as the means and standard deviations. *P* values were calculated by the two-sided *t*-test for comparison between two groups or ANOVA for comparison between multiple groups. *P* values < 0.05 were considered statistically significant.

3. Results

3.1 Tumor volume after ^{64}Cu -ATSM treatment in vivo <Please locate Fig. 1 here.>

To investigate the effectiveness of the ^{64}Cu -ATSM therapy, therapeutic doses of ^{64}Cu -ATSM were injected into mice bearing a Colon-26 tumor. In this study, 1 mCi of ^{64}Cu -ATSM or saline was administered twice within a 1 week interval to 1-week-old tumor bearing mice from day 0 ($n = 6$). Images of tumors treated by ^{64}Cu -ATSM and control tumors on day 19 were shown in Fig. 1A. The volume of tumors treated by ^{64}Cu -ATSM was 14-fold smaller than that of control tumors ($P < 0.02$) (Fig. 1B). There was no significant difference between administrations of saline only and saline containing ATSM (data not shown), which was similar to our previous report (16). The remaining tumors after each treatment were converted to single cell suspensions and subjected to the flow cytometry analysis and experimental lung metastatic assay and will be described later.

3.2 Percentage of CD133⁺ cells after ⁶⁴Cu-ATSM treatment in vivo <Please locate Fig. 2 here.>

We performed flow cytometry analysis to investigate the content of CD133⁺ cells in Colon-26 tumors treated by ⁶⁴Cu-ATSM and control tumors. The analysis by flow cytometry of cell suspensions from ⁶⁴Cu-ATSM treated tumors and control tumors is shown in Fig. 2. The percentage of CD133⁺ cells in tumors treated by ⁶⁴Cu-ATSM was significantly reduced ($0.16\% \pm 0.09\%$), compared with control tumors ($0.88\% \pm 0.14\%$) ($P < 0.01$).

3.3 Experimental lung metastatic assay <Please locate Fig. 3 here.>

To evaluate the metastatic potential of the Colon-26 tumors treated with ⁶⁴Cu-ATSM and controls, we performed experimental lung metastatic assay with the cell suspension derived from these tumors. In this study, we intravenously injected cell suspension (5×10^4 cells) into mice ($n = 6$). Fig. 3A shows images of lungs isolated from mice established by the experimental lung metastatic assay after 16 days of the cell injection and Fig. 3B shows the data summary. In mice injected with control cancer cells the number of lung metastasis was 27.5 ± 12.67 , whereas, in mice injected with ⁶⁴Cu-ATSM-treated cancer cells, the number of lung metastasis was 2.00 ± 0.82 . There was a significant decrease in the number of lung metastasis in mice injected ⁶⁴Cu-ATSM-treated cancer cells, compared with mice injected with control cancer cells ($P < 0.01$).

3.4 In vitro ⁶⁴Cu-ATSM uptake and therapeutic effect of ⁶⁴Cu-ATSM in sorted CD133⁺ and CD133⁻ Colon-26 cells <Please locate Fig. 4, 5 here.>

To characterize ^{64}Cu -ATSM uptake in CD133^+ and CD133^- Colon-26 cells, we performed the ^{64}Cu -ATSM uptake study with CD133^+ and CD133^- cells sorted using a cell sorter. Fig. 4 indicates ^{64}Cu -ATSM uptake in sorted CD133^+ and CD133^- Colon-26 cells under normoxic or hypoxic conditions. The uptake of ^{64}Cu -ATSM was not significantly different between CD133^+ and CD133^- Colon-26 cells under these conditions. However, the ^{64}Cu -ATSM uptake was significantly increased under hypoxia, compared with normoxia, in both CD133^+ and CD133^- cells ($P < 0.01$); this is a 2.0-fold increase in CD133^+ cells and 2.3-fold increase in CD133^- cells.

Colony formation assay was also performed to evaluate the therapeutic effect of ^{64}Cu -ATSM on CD133^+ and CD133^- Colon-26 cells, which accumulated ^{64}Cu -ATSM under hypoxic conditions. Fig. 5 demonstrates the relative percentage of survival fraction of ^{64}Cu -ATSM-treated CD133^+ and CD133^- Colon-26 cells, compared with that of the control CD133^+ and CD133^- cells, respectively. In both CD133^+ and CD133^- Colon-26 cells, the survival fraction was significantly decreased after ^{64}Cu -ATSM treatment ($P < 0.01$) and there was no significant difference in the amount of reduction between CD133^+ and CD133^- cells. Both control CD133^+ and CD133^- cells were spindle-shaped and well-divided, whereas we observed giant cell formation in CD133^+ and CD133^- cells treated by ^{64}Cu -ATSM (data not shown).

4. Discussion

In this study, we found that ^{64}Cu -ATSM treatment reduced not only tumor size but also the proportion of CD133^+ cells and the metastatic potential in Colon-26 tumors. Our previous study demonstrated that, in Colon-26 tumors, ^{64}Cu -ATSM accumulates in

low-vascular regions with relatively high density of CD133⁺ cells and the CD133⁺ Colon-26 cells possess distinctive characteristics of CSCs, such as high colony-forming ability, high tumor-initiating ability and increased survival under hypoxic conditions (36). In addition, our *in vitro* study showed a therapeutic effect of ⁶⁴Cu-ATSM on both CD133⁺ and CD133⁻ Colon-26 cells under hypoxic conditions. These data suggest that ⁶⁴Cu-ATSM can accumulate in low-vascular regions where CD133⁺ cells preferentially localize within tumors and ⁶⁴Cu can affect such regions by radiation, which leads not only to decrease of tumor volume but also reduction of the proportion of CD133⁺ cells and metastatic potential of tumors.

Lewis et al. demonstrated that ⁶⁴Cu-ATSM treatment extended the survival of tumor-bearing hamsters (16). Aft et al. indicated that ⁶⁴Cu-ATSM injection into mouse mammary carcinoma EMT-6 tumor-bearing mice reduces the tumor volume (20). In the present study, we showed systemic ⁶⁴Cu-ATSM administration into Colon-26 tumor-bearing mice reduced tumor volume. Hence, we confirmed that ⁶⁴Cu-ATSM treatment can inhibit tumor growth in agreement with the data of Aft et al. (20).

In addition, we observed that ⁶⁴Cu-ATSM treatment decreased the percentage of CD133⁺ cells and the metastatic potential of Colon-26 tumors. Our previous study has also demonstrated that CD133⁺ Colon-26 cells possess malignant characteristics, which are typically seen in CSCs (36). Therefore, these data indicate the additional usefulness of ⁶⁴Cu-ATSM treatment, that is, ⁶⁴Cu-ATSM treatment can decrease the content of CD133⁺ cancer cells and reduce the metastatic potential in tumors. It has been so far demonstrated that the CD133⁺ cancer cells possess high metastatic ability in tumors (34, 35). Therefore, it is reasonable to assume that reduction of the proportion of CD133⁺ cells might be one

of the possible reasons of lowering the metastatic potential after ^{64}Cu -ATSM treatment in this study.

In colon carcinoma, recent reports revealed that CD133 is a useful marker for cancer cells linked with CSCs in human colon cell lines (38) and clinical colon cancer specimens (24, 25). On the other hand, conflicting data has been reported, an example is that CD133 is expressed ubiquitously in clinical colon carcinoma and both CD133⁺ and CD133⁻ cells displayed characteristics of CSCs (39). Although this issue is still under discussion, we report that CD133⁺ Colon-26 cells rather than CD133⁻ cells possess typical characteristics of CSCs. Therefore, in a Colon-26 model, CD133⁺ cells might be able to be considered CSCs.

Our *in vitro* analysis demonstrated that CD133⁺ Colon-26 cells incorporated ^{64}Cu -ATSM under hypoxia as well as CD133⁻ cells and that incorporated ^{64}Cu -ATSM can damage both CD133⁺ and CD133⁻ cells. It has been reported that cancer cells under hypoxic conditions incorporate ^{64}Cu -ATSM and that ^{64}Cu -ATSM treatment inhibits cell growth (5, 10, 17). Obata et al. observed that ^{64}Cu is localized in the cell nuclei (20 % after 18 h) and the radiation induces DNA damage and cell apoptosis (17). In fact, we observed the giant cell formation, which usually occurs by DNA damage through radiation (40), in CD133⁺ and CD133⁻ cells treated by ^{64}Cu -ATSM. This indicates that ^{64}Cu -ATSM could damage the DNA of both CD133⁺ and CD133⁻ cells. Considering the facts that ^{64}Cu -ATSM treatment reduced percentage of CD133⁺ cells of remaining tumors and ^{64}Cu -ATSM attacked both CD133⁺ and CD133⁻ cells to a similar extent, it is reasonable to assume that ^{64}Cu -ATSM can decrease percentage of CD133⁺ cells of tumors by damaging both CD133⁺ and CD133⁻ cells in ^{64}Cu -ATSM-accumulating regions

with relatively high density of CD133⁺ cells within tumors, not by targeting CD133⁺ cells alone. In addition, it has been reported that CD133⁺ cells possess radioresistance and chemoresistance, while CD133⁻ cells do not (34, 35) and therefore CD133⁺ cells are considered to contribute to difficulties in eradicating tumors due to their therapeutic resistance. In the present study, the therapeutic effect of ⁶⁴Cu-ATSM did not show any significant difference between CD133⁺ and CD133⁻ cells. This indicates the possibility that radiation by ⁶⁴Cu-ATSM can damage CD133⁺ cells despite the therapeutic resistance and that ⁶⁴Cu-ATSM treatment could be more effective than other kinds of therapy, including radiotherapy and chemotherapy, in reducing the number of CD133⁺ cells. Namely, this study suggests that ⁶⁴Cu-ATSM treatment is of potential interest in eradicating treatment of tumors. Further studies will be necessary to examine dose dependency of ⁶⁴Cu-ATSM treatment and the efficacy in various kinds of malignant tumors. Also, it will be important to consider the combination use of ⁶⁴Cu-ATSM treatment with other tumor therapies.

Our previous study has shown that, in the Colon-26 tumor model, ⁶⁴Cu-ATSM preferentially localizes in intratumoral regions with the relatively high density of CD133⁺ cells (1.08% ± 0.33% in ⁶⁴Cu-ATSM high uptake regions and 0.68% ± 0.28% in ⁶⁴Cu-ATSM middle uptake regions), while density of CD133⁺ cells is low in ⁶⁴Cu-ATSM low uptake regions (0.23% ± 0.16%), which was determined by semiquantitative immunohistochemical analysis (34). In the present study, Figure 2, which was observed by flow cytometry analysis, showed that the percentage of CD133⁺ cells in tumors treated by ⁶⁴Cu-ATSM was low, as seen in ⁶⁴Cu-ATSM low uptake regions in the previous study.

These evidences might support the idea that ^{64}Cu -ATSM therapy preferentially damaged regions with the relatively high density of CD133^+ cells within tumors in this model.

Although ^{64}Cu -ATSM treatment has not yet done in human, Lewis et al. showed that an estimated dose of <7500 mCi of ^{64}Cu -ATSM would be possible in clinical therapy use based on the absorbed dose estimates for ^{64}Cu -ATSM using extrapolation from a hamster model (16). The authors also demonstrated that dose-fractionation is as effective as single dose injection to increase survival time and reduces the absorbed doses to non-target organs. In this study, we injected 1 mCi twice with one week interval into 25 g mice and that is estimated to be $3000 \text{ mCi} \times 2$ in humans, which will be a possible dose for clinical use. Therefore, the results of this study may indicate a potential for clinical use of ^{64}Cu -ATSM to decrease the content of CD133^+ cancer cells and reduce the metastatic potential of tumors in tumor treatment.

5. Conclusion

This study shows that ^{64}Cu -ATSM treatment can reduce not only the tumor volume, but also the proportion of CD133^+ cells and the metastatic potential in Colon-26 tumors. Our *in vitro* study also showed that ^{64}Cu -ATSM treatment was effective to both CD133^+ and CD133^- Colon-26 cells under hypoxia. Our previous data demonstrated that ^{64}Cu -ATSM accumulates into low vascular-hypoxic regions with a high density of CD133^+ cells in Colon-26 tumors and the CD133^+ Colon-26 cells show typical characteristics of CSCs. Together with these data, it is suggested that ^{64}Cu -ATSM accumulates in rich regions of CD133^+ cells and affects such regions by radiation, which leads to reduction of percentage of CD133^+ cells, in Colon-26 tumor model. Although

^{64}Cu -ATSM does not target CD133⁺ cell itself, ^{64}Cu -ATSM could be a potential internal radiotherapy agent affecting the tumor's low vascular-hypoxic regions with a high density of CD133⁺ cells, designated as CSCs. In order to clarify its precise usefulness, further studies with other kinds of malignant tumors would be needed.

Acknowledgments

We thank members of the Biomedical Imaging Research Centre of the University of Fukui, Japan, for discussions, and S. Nakakoji, J. Yamamoto, H. Takagi and H. Maeda for technical help. This work was supported in part by the Grants-in-Aid for Young Scientists (B) from the Japan Society for the Promotion of Science, Japan (JSPS) (to Y.Y.); the Joint Research Grant from the Wakasa Wan Energy Research Center, Japan (to Y.F. & Y.Y.); the Research for Promoting Technological Seeds from Japan Science and Technology Agency, Japan (to Y.Y.); the 21st Century Centers of Excellence (COE) program from the JSPS (to Y.F.); and the Grant-in-Aid for Tumor Research from the Ministry of Health, Labor and Welfare, Japan 15-2 (to Y.F.).

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Figure Legends

Fig. 1. Tumor volume after ^{64}Cu -ATSM treatment in Colon-26 model *in vivo*. (A) Examples of tumors from control and ^{64}Cu -ATSM-treated mice. (B) Volume of control and ^{64}Cu -ATSM-treated tumors (mean $\pm$ s.d., $n = 6$). Asterisk indicates statistical significance ($*P < 0.02$).

Fig. 2. Percentage of CD133^+ cancer cells after ^{64}Cu -ATSM treatment in Colon-26 model. Data indicate percentage of CD133^+ cells in control and ^{64}Cu -ATSM-treated tumors, which is analyzed by flow cytometry (mean $\pm$ s.d., $n = 6$). Asterisk indicates statistical significance ($*P < 0.01$).

Fig. 3. Metastatic potential of cells after ^{64}Cu -ATSM treatment. (A) Representative images of isolated mouse lungs obtained by experimental lung metastatic assay. Lung metastasis was established by injection of cell suspension including 5×10^4 cells, which was derived from control and ^{64}Cu -ATSM-treated tumors, into a mouse. (B) Number of lung metastasis (mean $\pm$ s.d., $n = 6$). Asterisk indicates statistical significance ($*P < 0.01$).

Fig. 4. ^{64}Cu -ATSM uptake of CD133^+ and CD133^- Colon-26 cells *in vitro*. Data indicate ^{64}Cu incorporation in CD133^+ and CD133^- Colon-26 cells that are isolated by cell sorting, respectively (mean $\pm$ s.d., $n = 4$). There are statistically significant differences between different letters ($P < 0.01$).

Fig. 5. Therapeutic effect of ^{64}Cu -ATSM to CD133^+ and CD133^- Colon-26 cells that accumulate ^{64}Cu -ATSM under hypoxia. Data indicate percentage of survival fraction of ^{64}Cu -ATSM-treated cells, compared with that of control cells, in sorted CD133^+ and CD133^- cells, respectively (mean $\pm$ s.d., $n = 4$), which is analyzed by colony formation assay. Asterisks indicate statistically significant differences ($*P < 0.01$).

A

B

A

B

Figure(s)-Fig4

Figure(s)-Fig5