2.2(b) 温度調節器

温度調節器は、アズワン社製の卓上型温度調節気 T-550-P を用いた。

現在、放射性 Cu 精製装置に使用されているのと同じものである。T-550-P の仕様 Table.11 に示す。

Table.11. T-550-P の仕様

項目	T-550-P
操作スイッチ	POWER スイッチ及び温度調節計のアップダウンキー
電源電圧	AC100V 50/60Hz
許容電源電圧	上記電源電圧に対して±10%以内
出力	SSR による電圧出力, AC100V MAX, 10A(抵抗負荷)
入出力方法	裏面端子台による接続
温度制御方式	PID オートチューニングタイプ PIDS（オーバーシュート抑制型）
センサー	Pt100Ω
設定温度範囲度(℃)	0.0～199.9℃（0.1℃単位で設定）

2.2(c) テフロン(PTFE)試験管

テフロン試験管は相互理化学硝子製作所から購入した。

仕様を Table.12 に示す。

Table.12. テフロンチューブの仕様

容量(ml)	全長×胴外径×上部外径×肉厚(mm)	コード
85	100×40×42×3.0	355-07

2.2(d) テフロンハーフユニオンコネクター

テフロンハーフユニオンコネクターはフロン工業社製の F2112-02 を用いた。

2.2(e) テフロンチューブ

テフロンチューブは、フロン工業社製の外径 3.0mm、内径 2.0mm を用いた。

2.2(f) 試験管とヒーターの間の筒銅

ヒーターとテフロン試験管の間に入れる銅筒をアルサス工業社と共同研究で製作した。

2.2(g) Target 用ふた自動開閉装置

Target 用ふた自動開閉装置京藤樹脂技研社と共同研究で製作した。装置の全体図を Fig.11 に示す。

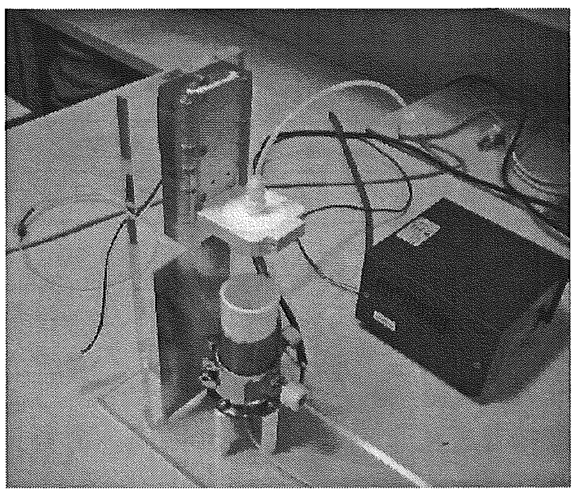

Fig.11. 装置の全体図

2.3 真空ポンプ

真空ポンプは旭テクノグラ社製の FTP-20A（フル・テフロン真空ポンプ）を用いた。

仕様を Table.13 に示す。

Table.13. FTP-20A の仕様

品名コード	FTP-20A
ポンプ型式	テフロンダイヤグラム真空ポンプ
接ガス部材質	ポンプヘッド/バルブボディー=PTFE ダイヤグラム=PTFE-COATED バルブ=CARLEZ, ホースコネクター=PVDA
吸引口径	φ10mm
電源	AC100V
定格電流値(A)	1.5A
排気速度(50/60Hz)min	17/20L
到達圧力(mbar)	8
到達圧力(Torr)	6
外寸法(W×D×Hmm)	312×10×207
重量(Kg)	9.3

2.4 イオン交換樹脂カラム

イオン交換樹脂カラムは、BIO-RED 社の Poly-Prep® Columns, AG® 1-X8 resin を用いた。

仕様を Table.14 に示す。

Table.14. Poly-Prep® Columns, AG® 1-X8 resin の仕様

Item	Poly-Prep® Columns, AG® 1-X8 resin
Matrix	Styrene divinylbenzene
Bed Volume	2 ml
Ion	Q (Quaternary ammonium)
pH Stability	0-14
Column Bore	8 mm
Column Length	4 cm
Barrel Material	polypropylene
Mesh Size	100–200
Ionic Capacity	1.2 meq/ml

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Basic characterization of ^{64}Cu-ATSM as a radiotherapy agent

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Abstract

^{64}Cu-diacetyl-bis(N^4-methylthiosemicarbazone) (^{64}Cu-ATSM) is a promising radiotherapy agent for the treatment of hypoxic tumors. In an attempt to elucidate the radiobiological basis of ^{64}Cu-ATSM radiotherapy, we have investigated the cellular response patterns in vitro cell line models. Cells were incubated with ^{64}Cu-ATSM, and the dose–response curves were obtained by performing a clonogenic survival assay. Radiation-induced damage in DNA was evaluated using the alkali comet assay and apoptotic cells were detected using Annexin V-FITC and propidium iodide staining methods. Washout rate and subcellular distribution of ^{64}Cu in cells were investigated to further assess the effectiveness of ^{64}Cu-ATSM therapy on a molecular basis. A direct comparison of subcellular localization of Cu-ATSM was made with the flow tracer analog Cu-pyruvladehyde-bis(N^4-methylthiosemicarbazone). In this study, ^{64}Cu-ATSM was shown to reduce the clonogenic survival rate of tumor cells in a dose-dependent manner. Under hypoxic conditions, cells took up ^{64}Cu-ATSM and radioactive ^{64}Cu was highly accumulated in the cells. In the ^{64}Cu-ATSM-treated cells, DNA damage by the radiation emitted from ^{64}Cu was detected, and inhibition of cell proliferation and induction of apoptosis was observed at 24 and 36 h after the treatment. The typical features of postmitotic apoptosis induced by radiation were observed following ^{64}Cu-ATSM treatment. The majority of the ^{64}Cu taken up into the cells remained in the postmitochondrial supernatant (the cellular residue after removal of the nuclei and mitochondria), which indicates that the β^- particle emitted from ^{64}Cu may be as effective as the Auger electrons in ^{64}Cu-ATSM therapy. These data allow us to postulate that ^{64}Cu-ATSM will be able to attack the hypoxic tumor cells directly, as well as potentially affecting the peripheral nonhypoxic regions indirectly by the β^- particle decay of ^{64}Cu.

Keywords: ^{64}Cu-ATSM; Tumor; Internal radiation therapy; Hypoxia; Apoptosis; PET

1. Introduction

Radionuclide therapy is one of the highly promising approaches for treating cancer and is widely investigated in basic research and clinical practice [1]. Radionuclide therapies using labeled antibodies or peptides targeting tumor-related antigens or receptors have been extensively studied especially in the field of hematological malignancies such as lymphoma and leukemia [2–4]. However, success of these agents in solid tumors has been limited mainly due to heterogeneous antigen expression and low overall tumor uptake. A variety of strategies have been employed to improve the tumor targeting and therapy effects of such methods including combination therapy with other modalities [5–8].

Conventionally, low linear energy transfer β-emitters such as ^{131}I, ^{90}Y and ^{186}Re have been widely used for radionuclide therapy [9]. ^{67}Cu also has been investigated as a candidate for radionuclide therapy. ^{67}Cu has excellent physical and biochemical properties for radionuclide therapy and ^{67}Cu-labeled monoclonal antibodies were reported to be useful for the treatment of lymphoma and colorectal carcinoma [9,10]. However, production of ^{67}Cu requires a large accelerator or nuclear reactor, limiting the availability of ^{67}Cu.

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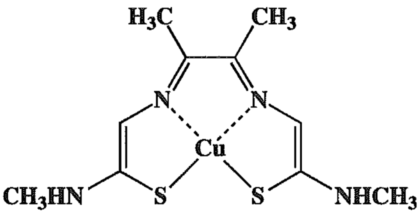

Fig. 1. Structure of Cu-ATSM.

^{64}Cu with a half-life of 12.7 h decays by electron capture (41%), β^- decay (0.573 MeV, 40%) and β^+ decay (0.656 MeV, 19%), accompanied by emission of annihilation radiation (0.511 MeV, 38%) and γ photons (1.34 MeV, 0.5%). It is a promising therapeutic radionuclide because of its favorable β^- particle emissions [11,12]. ^{64}Cu is reported to be as efficient as ^{67}Cu for tumor treatment [13,14] and can be produced using small biomedical cyclotrons in regular PET centers on a daily basis [15,16]. Furthermore, because of its multiple decay modes, ^{64}Cu can be used for real-time PET monitoring of regional drug concentration, kinetics and dosimetry during radiation therapy if it is used to label the therapeutic radiopharmaceuticals.

Copper-diacetyl-bis(N^4-methylthiosemicarbazone) (Cu-ATSM; Fig. 1) has been examined extensively by our group and others as a possible imaging agent to delineate hypoxia within tumors [12,17,18]. Cu-ATSM is taken up into tumor cells rapidly and efficiently and reduced by an enzymatic system of sequential electron transport chains, where monovalent Cu is released from the chelate to be retained subsequently [19]. Considering these characters, ^{64}Cu-ATSM has potential as a radiotherapy agent with an option of real-time PET monitoring. Indeed, as a therapy agent, ^{64}Cu-ATSM was reported to be useful for the treatment of colorectal carcinoma in vivo tumor model [20]. In the present report, we have investigated the molecular basis of ^{64}Cu-ATSM therapy using in vitro tumor cell models. The cell killing ability of ^{64}Cu-ATSM was evaluated by colony-forming assay. Some of the typical features of cell death derived by radiation were observed; namely, inhibition of the cell proliferative rate and apoptotic cell death. Subcellular localization of Cu-ATSM was studied and compared with Cu-pyruvladehyde-bis(N^4-methylthiosemicarbazone) (Cu-PTSM), a flow tracer [21]. We also discuss the fate of ^{64}Cu in relation to cell toxicity. This study may provide useful information for designing effective therapy strategies and improving the radiotherapy efficacy in cancer treatment.

2. Materials and methods

All chemicals were reagent grade. The ^{64}Cu at University of Fukui was produced on a 12-MeV biomedical cyclotron using previously reported methods [15,16]. The ^{64}Cu at Washington University was produced on a CS-15 biomedical cyclotron (Cyclotron) [15]. H_2ATSM was synthesized as described previously [22]. ^{64}Cu-ATSM was prepared by mixing 200 mM glycine buffer containing ^{64}Cu and H_2ATSM in dimethyl sulfoxide (20:1 by volume) [17]. Labeling efficiency was determined by radio-HPLC using conditions described previously [19]. Radiochemical purity and specific activity of ^{64}Cu-ATSM in all studies were >99% and >56,000 GBq/mmol, respectively.

2.1. Cell culture

Mouse Lewis Lung carcinoma LL/2 cells were purchased from Dai-Nippon Seiyaku (Japan) and were grown in a 5% CO_2-humidified atmosphere at 37 °C. Cells were routinely maintained in Dulbecco's modified Eagle Medium (DMEM) (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum.

2.2. Uptake of ^{64}Cu-ATSM into cells

Previous reports show that the uptake of ^{64}Cu-ATSM was dramatically increased under hypoxic conditions [23]; therefore, we followed the reported methods during the uptake phase. Briefly, cells were trypsinized and collected in a polypropylene tube (Falcon, Becton Dickinson, Lincoln Park, NJ). Cell numbers were counted with a hemocytometer and the cells were resuspended in serum-free DMEM to a concentration of 1×10^6 cells/ml. Ten million cells were transferred to a three-necked flask and hypoxic gas (95% N_2, 5% CO_2) was passed over the cells at 37 °C for 1.5 h. ^{64}Cu-ATSM was then added to the flask and incubated for 1 h. After incubation, aliquots of the cell suspension were removed and the cells were pelleted from the reaction media to calculate the percentage uptake of ^{64}Cu-ATSM. Additional aliquots of cells were transferred to a polypropylene tube and resuspended with fresh DMEM containing 10% fetal bovine serum for further experiments.

2.3. Clonogenic survival assay

The radiotherapy effect of ^{64}Cu-ATSM was measured by performing a clonogenic survival assay under normoxic condition [24,25] because continuous hypoxia treatment itself affected cell viability. The ^{64}Cu-ATSM-treated cells, resuspended in fresh medium, were counted using a hemocytometer and diluted properly. Cells were seeded into 60-mm dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) and cultured in a humidified 5% CO_2/95% air atmosphere at 37 °C. After 5 days of incubation, the cells were stained with 3% Giemsa solution, and colonies containing more than 50 cells were counted as survivors. The absolute plating efficiencies of control cells were 34.1±9.7%. The surviving fraction was determined as the ratio of live colonies in the ^{64}Cu-ATSM-treated cell populations relative to the glycine-treated control.

2.4. Cell growth assay

After the treatment, cells were seeded in six-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ) and cultured

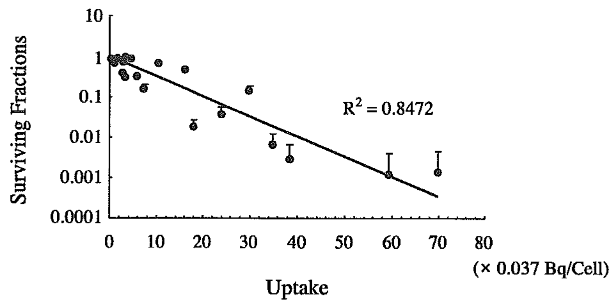

Fig. 2. Clonogenic survival of LL/2 cells treated with ^{64}Cu-ATSM. LL/2 cells were treated with different concentrations of ^{64}Cu-ATSM for 1 h under hypoxic conditions to trap ^{64}Cu metabolically into cells. After the treatment, surplus ^{64}Cu-ATSM was removed and cells were resuspended in fresh culture medium and the radioactivity taken up by the cells was measured. Cells were seeded in 6-cm dishes and cultured for 5 days, and the colonies containing more than 50 cells were counted. The surviving fractions determined as described in Materials and methods was plotted against the amount of ^{64}Cu taken up by the cells. Values are the mean and S.D. (n=6).

under normoxic condition. Cells were trypsinized and counted using the trypan blue dye exclusion test at the specified time points.

2.5. Comet assay (single cell gel electrophoresis)

To detect the damage to DNA induced by ^{64}Cu, the comet assay was performed after ^{64}Cu-ATSM treatment [26]. Glycine-treated control cells and ^{64}Cu-ATSM-treated cells were seeded in 100-mm dishes (Falcon, Becton Dickinson) and cultured under normoxic condition. Six hours after the treatment, cells were trypsinized and counted using the trypan blue dye exclusion test. Viability of cells was >90%. The cells were sedimented by centrifugation and adjusted to the concentration of 1×10^5 cells/ml with phosphate-buffered saline. The cells were treated with a

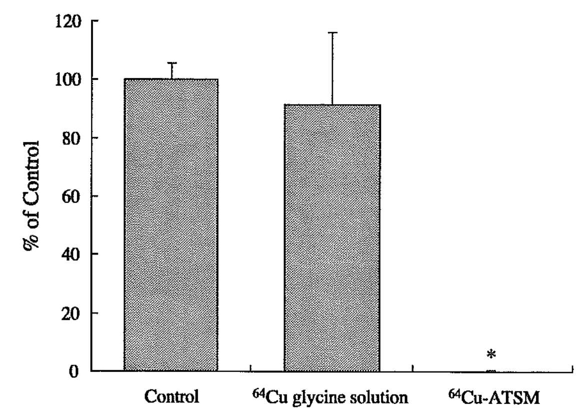

Fig. 3. The effect of carrier-free ^{64}Cu and ^{64}Cu-ATSM on the survival rate of LL/2 cells. A clonogenic survival assay was performed with a control group (treated with glycine solution), a ^{64}Cu-glycine solution-treated group (938.3±41.65 μCi added to 10^7 cells) and a ^{64}Cu-ATSM-treated group (896.7±35.84 μCi added to 10^7 cells). Viability compared with control group was 91.43±24.64% and 0.1247±0.2874%, respectively. *P<.0001, significant degrease compared with control (Student's t test, n=3).

Table 1
^{64}Cu uptake ratio and radioactivity in tumor cells after 1 h of treatment

	% Uptake	Radioactivity in tumor cells (Bq/cell)
^{64}Cu-glycine	2.47±0.46	0.0857±0.0038
^{64}Cu-ATSM	61.4±17.7	2.04±0.08

Data are expressed as the mean±S.D. (n=3).

CometAssay Kit (Trevigen, Gaithersvurg, MD) according to the instruction manual from the manufacturer. After electrophoresis, assay slides were treated with CometAssay Silver Staining Kit (Trevigen) to visualize the assay results. Data analysis was performed using Komet v. 4.0.2 software (Kinetic Imaging, Wirral, UK).

2.6. Measurement of apoptotic cell death

Early stages of apoptosis were identified using an Annexin V-FITC Apoptosis Detection Kit I (Becton Dickinson, San Jose, CA) [27]. After the uptake period, cells were seeded in six-well plates and cultured under normoxic condition. At the specified time points, cells were collected and stained with Annexin V-FITC/propidium iodide (PI) according to the manufacturer's protocol. Level of apoptosis was quantified using a Becton Dickinson FACSCalibur system and analyzed using CellQuest v. 3.1 software (Becton Dickinson, San Jose, CA). Cells that were Annexin

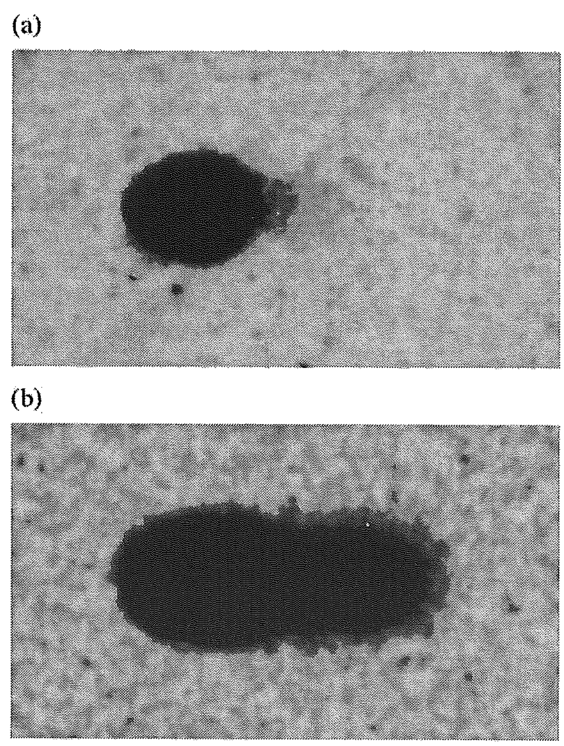

Fig. 4. The typical microscopic images following the comet assay at 6 h after treatment. Images of a normal cell (a) and DNA-damaged cell (b) are shown. In the ^{64}Cu-ATSM-treated groups, over 90% of total cells suffered from DNA damage and displayed damaged pattern as shown in b.

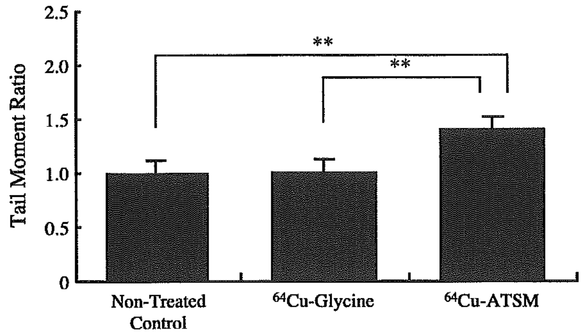

Fig. 5. Tail moment ratio in the comet assay. Forty cells were selected ad libitum and then analyzed for the tail moment using the Comet v. 4.0.2 software. Data are presented as a ratio to the nontreated control groups (bar, 1 S.E.). $**P<.01$, significant increase (Student's t test).

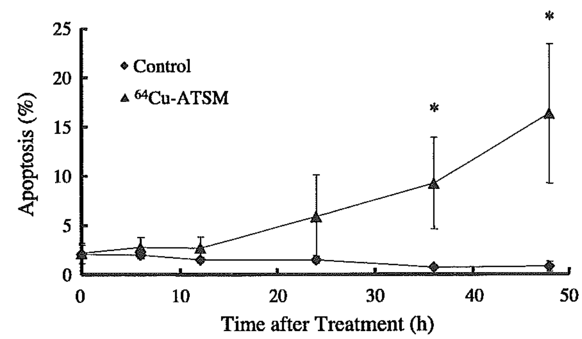

Fig. 7. Apoptosis of LL/2 cells after ^{64}Cu-ATSM treatment. Floating and adherent cells were collected, stained with Annexin V-FITC and PI, then analyzed by FACS using CellQuest software. A significant increase in apoptosis was observed with ^{64}Cu-ATSM-treated cells from 36 h after the treatment. Data points are the average percentage of apoptotic cells and S.D. ($n=3$). $*P<.05$, significant increase (Student's t test).

V-FITC-positive and PI-negative were identified as apoptotic cells as described previously [28,29].

2.7. Retention and intracellular distribution of ^{64}Cu-ATSM and ^{64}Cu-PTSM in LL/2 cells

Cells were incubated in hypoxic gas (95% N_2, 5% CO_2) for 1.5 h at which point either ^{64}Cu-ATSM or ^{64}Cu-PTSM was added to the media. Following a 1-h incubation, the media was removed and the cells in fresh medium were seeded in 100-mm dishes, cultured under normoxic condition and recollected at 0, 1, 3, 6, 12 and 24 h after the treatment. Cells were washed twice with an ice-cold isolation medium (0.25 M sucrose buffered to pH 7.4 with 10 mM HEPES). Aliquots of cells were separated and the radioactivity in the cells was counted using a γ-counter (ARC-2000, ALOKA, Japan). Another aliquot of the cells was resuspended in ice-cold lysis buffer (0.005% SDS buffered to pH 7.4 with 10 mM HEPES) and homogenized. After homogenization, the P1 fraction (crude nuclear fraction) was obtained by centrifugation at $1000\times g$ for 5 min at 4 °C. The supernatant was centrifuged at $8000\times g$ for 10 min at 4 °C to yield the P2 (crude mitochondrial) and S2 (the postmitochondrial supernatant which contains the cellular residue after removal of the nuclei and mitochondria) fraction. Radioactivity in each fraction was measured.

3. Results

Fig. 2 shows the radiation dose–response curve for the LL/2 cells treated with ^{64}Cu-ATSM. Clonogenic survival was decreased in a dose-dependent manner. ^{64}Cu-ATSM uptake of 1.50 Bq/cell produced 99% killing of the cells. Mock treatment with H_2ATSM or nonradioactive Cu-ATSM did not affect the survival ratio of LL/2 cells (data not shown), which also indicates that hypoxia treatment during uptake phase did not affect the cell viability by itself.

The effect of "free" ^{64}Cu ion and ^{64}Cu-ATSM on tumor cells was also compared using the clonogenic survival assay. When approximately 34.8 MBq of ^{64}Cu-glycine solution was inoculated, the survival rate of cells was not affected, whereas the equivalent amount of ^{64}Cu-ATSM treatment produced nearly complete killing of LL/2 cells (Fig. 3). The ^{64}Cu uptake ratio in each treatment was 2.47±0.459% and 61.4±17.7%, respectively, which means the radioactivity

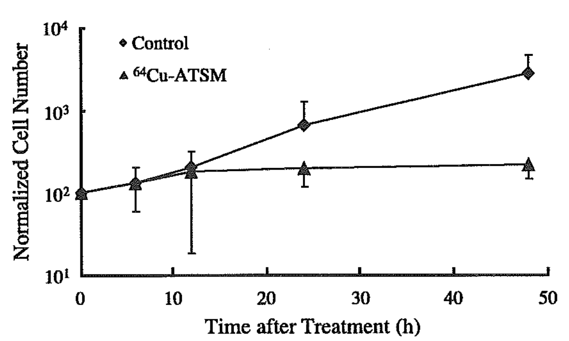

Fig. 6. Growth curve of LL/2 cells after ^{64}Cu-ATSM treatment. Proliferation of the cells was inhibited 24 h after the treatment. Data are presented as normalized cell number ± S.D. relative to time 0 ($n=3$).

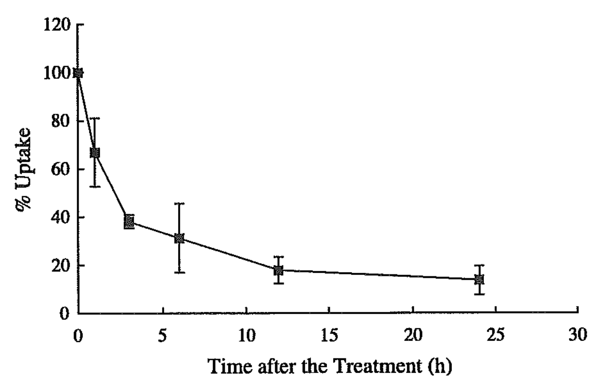

Fig. 8. Washout rate of ^{64}Cu from LL/2 cells; 1×10^7 cells were treated with ^{64}Cu-ATSM and radioactivity in the cell fractions was measured at each time point. Data are shown as ratio to the initial radioactivity after the decay correction. Each data point is the average ± S.D. ($n=3$).

taken up into tumor cells was 0.0857 ± 0.0038 and 2.04 ± 0.08 Bq/cell, respectively. The result suggests that intracellular uptake is the major factor in the efficient tumor cell killing by ^{64}Cu-ATSM (Table 1).

To elucidate the mechanism of cell killing by ^{64}Cu-ATSM, the comet assay was performed 6 h after the ^{64}Cu-ATSM treatment. Fig. 4 shows a typical microscopic image of the results. In the ^{64}Cu-ATSM-treated groups, over 90% of the total cells showed comet tails. The tail moment ratio was significantly increased in the ^{64}Cu-ATSM-treated groups compared with ^{64}Cu-glycine and nontreated control groups (Fig. 5) (nontreated group, P=.0085; ^{64}Cu-glycine-treated group, P=.0085). These results indicated that the radiation emitted from the intracellular ^{64}Cu caused DNA strand breaks, with the maximum recoil energy from the transmutation of ^{64}Cu to its highly charged daughter nucleus potentially increasing the cell killing ability. This damage in the DNA is thought to be one of the major triggers of cell death pathways. Fig. 6 shows the proliferation rate of LL/2 cells after the treatment. In the ^{64}Cu-ATSM-treated group, cell proliferation was completely inhibited after 24 h and a significant apoptotic fraction was seen 36 h after the treatment (P<.05) (Fig. 7).

Both the washout rate and subcellular distribution of ^{64}Cu were investigated in this study. Also, a direct comparison of the subcellular localization was made between ^{64}Cu-ATSM and ^{64}Cu-PTSM. With ^{64}Cu-ATSM, defining the amount of radioactivity associated with the cells at the time of resuspension as 100%, 38.0% of the radioactivity remained at 3 h posttreatment and 31.2% at 6 h posttreatment (Fig. 8). Considering the half-life of ^{64}Cu, the effect of radiation emitted from ^{64}Cu up to 12 h after treatment dominates in this study: about 14,200 decays occurred in one cell up to 12 h after treatment (Fig. 9). In the subcellular fractionation comparison, the distribution of ^{64}Cu from both ^{64}Cu-ATSM and ^{64}Cu-PTSM was very similar (Fig 10). For both ^{64}Cu-ATSM and ^{64}Cu-PTSM, the majority of the radioactivity remained in the S2 fraction over the 24 h. A transition of the radioactivity from the S2 and mitochondria fraction to the nuclear fraction was noted over the 24-h period (Fig. 10).

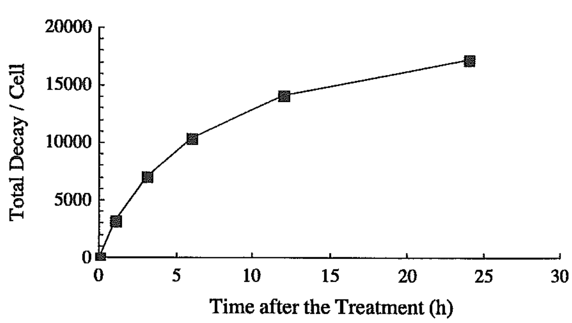

Fig. 9. Total decay frequency of intracellular ^{64}Cu. Based on the washout result shown in Fig. 8, the total decay frequency was calculated for a cell taking up a 99% cell killing dose of 1.50 Bq/cell. Radioactivity at each time point was corrected for a half-life of 12.7 h and the area under the curve was calculated.

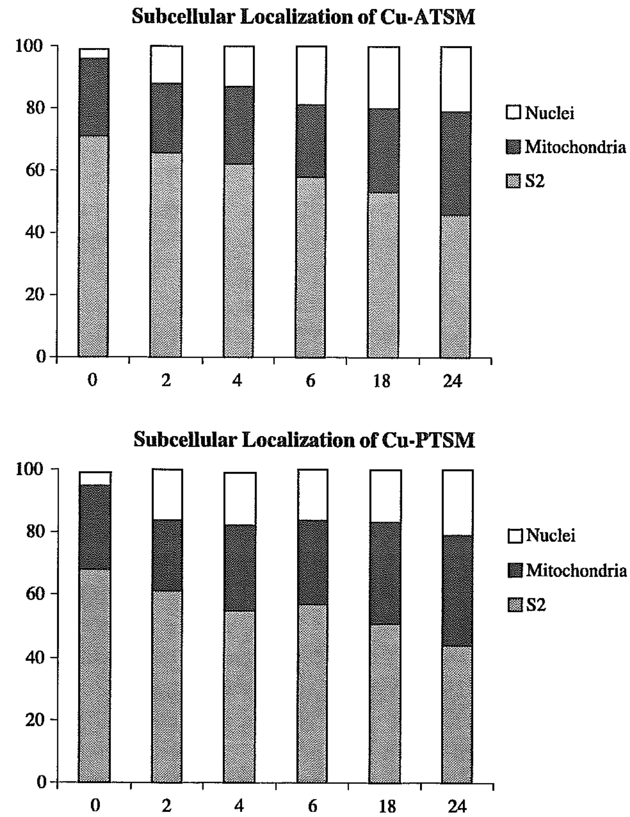

Fig. 10. Subcellular distribution of ^{64}Cu following ^{64}Cu-ATSM or ^{64}Cu-PTSM treatment. After ^{64}Cu-ATSM and ^{64}Cu-PTSM were metabolized in the cells, the majority of the ^{64}Cu was retained in the microsomal/cytosol fraction. A transition of radioactivity from the mitochondria and S2 fractions to the nuclear fractions was noted. Data are expressed as the mean percentage of total cellular radioactivity (n=4).

4. Discussion

^{64}Cu-ATSM has distinct accumulation mechanisms that are based on hypoxic tumor-selective metabolism by some enzymes [19]. Equipped with the radionuclidic characteristics of ^{64}Cu and the high and specific accumulation in tumor cells, ^{64}Cu-ATSM is making a new type of radiotherapy agent that provides a new strategy for the treatment of tumors [20]. In this study, we investigated the cytotoxic effect of ^{64}Cu-ATSM using clonogenic survival assays in an in vitro cell culture model. ^{64}Cu-ATSM decreased the clonogenic survival of LL/2 cells in a dose-dependent manner where 99% cell killing occurred when 1.50 Bq/cell of ^{64}Cu was taken up into the cells.

The nonligand binding form of ^{64}Cu (Cu-glycine) did not affect the survival rate of LL/2 cells, whereas the equivalent amount of radioactivity of ^{64}Cu-ATSM significantly reduced the survival rate to about 0.1% (P<.0001). In this experiment, the incubation medium containing ^{64}Cu was exchanged into fresh culture medium after the treatment, meaning that the ^{64}Cu taken up into tumor cells prior to the exchange was the only source of the cell killing. The ^{64}Cu taken up into the cells was dramatically different between the ^{64}Cu-glycine-treated groups and the ^{64}Cu-ATSM-treated groups, 0.0857±0.0038 and 2.04±0.08 Bq/cell, respectively. This may be the major cause for the differences in the subsequent survival rates. Furthermore, considering that there was no difference in the survival rate between the nontreated control and ^{64}Cu-glycine groups, the radioactive emissions from outside of the cells during the 1-h treatment in the flask did not affect the tumor cell survival. To be brief, the most effective character of ^{64}Cu-ATSM is to accumulate radioactive ^{64}Cu into the cells and as such produces the ability to kill tumor cells.

Radiation causes direct and/or indirect effects on irradiated cells. One of the well-known direct effects is damage to DNA. DNA damage by ionizing radiation predominantly consists of single-strand breaks, but also includes double-strand breaks, alkali-label sites, and oxidized purines and pyrimidines [30–32]. We confirmed with the alkali comet assay that a significant increase in DNA damage was observed with ^{64}Cu-ATSM-treated groups at 6 h after treatment. Comet assay allows us to detect the DNA fragmentation in apoptotic and/or necrotic cells [33]. However, we could not detect significant increases in the number of apoptotic cells as well as necrotic and/or dead cells using the Annexin V-FITC/PI staining method at the same time point. For this reason, the DNA damage observed after the treatment was considered to be caused directly by the radiation from intracellular ^{64}Cu and not by apoptosis.

Radiation-induced DNA damage elicits a variety of cellular responses including apoptosis as one of the major mechanisms of cell death in radiation therapy [34–36]. In this study, apoptosis marker-positive cells significantly increased in number ($P<.05$) 36 h after the treatment when detected by the Annexin V-FITC/PI staining method, preceded by the inhibition of cellular proliferation 24 h after the treatment. In an X-irradiation study, high-dose irradiation was reported to induce a rapid and strong apoptotic response, whereas low-dose irradiation induced a slow and mild apoptosis; each type of apoptosis was defined as premitotic apoptosis and postmitotic apoptosis, respectively [37]. In the postmitotic apoptosis, cell death occurs after one or a few cell divisions, not immediately after the radiation, and cell cycle arrest is observed at the G_2/M phase. In our preliminary study, we also observed increase of G_2/M phase cells, namely, G_2/M arrest, in the ^{64}Cu-ATSM-treated groups at 12–24 h after the treatment. These findings indicated that the typical cell death after ^{64}Cu-ATSM treatments is through postmitotic apoptosis.

It is conceivable that ^{64}Cu-ATSM would be able to exert stronger cytotoxicity via premitotic apoptosis if a higher radiation dose of ^{64}Cu-ATSM was given to the cells. However, considering radiotherapy in clinical setting, doses should be reduced to avoid adverse effects on nontarget tissues. In this regard, the ability of ^{64}Cu-ATSM to induce mild but steady cytotoxicity and postmitotic apoptosis confirmed in this study is desirable as a radiotherapy agent.

Recently, it has been reported that the damage to DNA by ^{64}Cu might be derived from Auger electrons because about 40% of ^{64}Cu decays by electron capture emitting Auger electrons with high linear electron transfer [20]. Auger electrons are expected to bring very high toxicity if they are in close proximity to DNA. The tissue penetration range of Auger electrons emitted from ^{64}Cu is about 5 μm [20] and is shorter than the diameter of most tumor cells, so it is well recognized that the radiotoxicity of Auger electron emitters depends strongly on their distribution within the cell. The most severe effects have been observed when the Auger emitter is localized in the nucleus [38]. Therefore, also in the case of ^{64}Cu, it is necessary for the radiocopper to localize in the nucleus and around the DNA to exert cytotoxicity through Auger electrons. In this study, we investigated the subcellular distribution of ^{64}Cu after uptake into tumor cells and showed that the radioactivity existed mainly in the S2 fraction with the radioactivity slowly migrating into the nucleus over time. Furthermore, nonradioactive Cu-ATSM did not affect the survival rate, which indicated the Cu ions themselves were not cytotoxic at the concentrations used in this study. Based on these findings, β^- particles emitted from intracellular ^{64}Cu as well as the Auger emissions of ^{64}Cu in the nuclear fraction are considered to be the major cytotoxic sources in ^{64}Cu-ATSM therapies. Moreover, the maximum recoil energy from the transmutation of ^{64}Cu to its highly charged daughter nucleus may also increase the cell killing ability. The multiplicity of decay mode of ^{64}Cu seems to be working in favor of the antitumor effect of ^{64}Cu-ATSM. Previous reports showed a significant portion of ^{67}Cu-pyruvaldehyde-bis(N^4-methylthiosemicarbazone), an analog of the series of Cu(II)-bis(thiosemicarbazones) compounds, is delivered to the cell nucleus [21] and our current studies have demonstrated similar uptake patterns.

The tissue penetration range of β^- particles emitted from ^{64}Cu is up to several hundred of micrometers. For this reason, the cytotoxic effect of the β^- particle of ^{64}Cu can range not only to the single cell that take up ^{64}Cu but also to the surrounding cells that do not take up ^{64}Cu, which could increase the antitumor effect in vivo ^{64}Cu-ATSM therapy. This feature becomes important when treating in vivo tumor masses because aggregation of the cells with heterogeneous characters is expected in vivo tumors.

The washout rate of ^{64}Cu from tumor cells is relatively high in our experimental model. At 3 h after the treatment, radioactivity in the cells was reduced to 38.0% of the total uptake at the end of the treatment. However, in the biodistribution study using tumor-bearing animal models, accumulation of ^{64}Cu-ATSM into tumor cells was reported to be highest at 4 h after injection [20]. High washout rate observed in our assay system might be limited only to in vitro model and actual accumulation of ^{64}Cu in the tumor might continue for much longer periods. If so, the therapeutic effectiveness of ^{64}Cu-ATSM might be higher in the in vivo tumors.

Based on the washout rate and half-life of ^{64}Cu, we calculated the total number of atomic disintegrations of ^{64}Cu occurring in the tumor cells when 1.50 Bq/cell of ^{64}Cu, a 99% cell killing dose, was taken up into cells (Fig. 9). Under these conditions, approximately 10,000 atomic disintegrations occurred within 6 h after the treatment, and about 14,000 within 12 h. That is, 10,000–14,000 atomic disintegrations per cell might be enough for killing tumor cells. D37 value (37% cell death) [39] was ca. 4500 decay per cell when biological washout from the cells was taken into account for the calculation, which was one fourth of the value previously reported (16,000 decay per cell) [40]. The actual area under time–radioactivity curve in the present study (Fig. 8) was about one fourth of the estimated value from nonwashout model, so that the present result is considered to be more accurate as actual retention pattern was included into the calculation.

In this study, the cells were kept under hypoxic condition during uptake phase of ^{64}Cu-ATSM to realize high retention of ^{64}Cu for 1 h, then cultured for 5 days at the maximum in normoxic condition to evaluate solely the effect of radiation, excluding the effect of hypoxia on cell viability. As hypoxia is considered to lessen the radiation toxicity in general, the cell killing ability determined in this study in normoxic tumor cells might be higher than that expected in continuously hypoxic tumor cells. On the other hand, the present results can be used for the estimation of cell toxicity in normoxic nontumor cells. Hypoxia selectivity of ^{64}Cu-ATSM is critical for realizing tumor-selective treatment. In our human studies using ^{62}Cu-ATSM, the highest accumulation was found in lung tumor (tumor/blood=3.00±1.50), followed by liver (liver/blood=2.45±1.03), whereas other normal tissues showed relatively low accumulation (heart/blood=1.84±0.35, lung/blood=0.43±0.09) [41]. High abdominal accumulation observed in mice [23] might be a result of hepatobiliary secretion of ^{64}Cu after metabolism. From these findings, cytotoxicity in the liver and, to a lesser extent, in the intestine arise as a possible problem in ^{64}Cu-ATSM treatment. Roughly 75% of the blood entering the liver is from portal vein and 25% from hepatic artery. Thus, considerable part of the liver seems to be hypoxic and it might be responsible for the rather high uptake of ^{64}Cu-ATSM in the liver. If so, radiation effect of ^{64}Cu in the liver could be estimated in a similar manner as in hypoxic tumor cells. Physiological excretion route of Cu is from liver to duodenum. Radioactive ^{64}Cu injected as Cu-ATSM is also expected to follow this route after released from the chelate. As shown in the present study, extracellular ^{64}Cu did not show significant cell toxicity, so that radioactivity in the duodenum is less likely to become a source of adverse effect in the treatment.

In vitro cell uptake study reported previously, Cu-ATSM showed some uptake also in normoxic tumor cells [23]. However, in our PET studies in normal human, selective Cu accumulation was found only in the liver but no other tissues (unpublished data). Thus, the uptake in the normoxic tumor cells might be from a characteristic of tumor cells. In fact, tumor cells expressed microsomal electron transport enzymes and they played a major role in reductive retention of Cu-ATSM in tumor cells, especially under hypoxic conditions [19]. Thus, Cu-ATSM can be evaluated mainly as a marker of hypoxia and, in some part, as a marker of tumor-selective gene expression by means of microsomal enzyme expression, in clinical practice.

5. Conclusion

^{64}Cu-ATSM has been shown to be effective in the treatment of tumors. ^{64}Cu-ATSM efficiently delivered radioactive ^{64}Cu into hypoxic tumor cells and the emitted β^- particles caused lethal damage to DNA leading to postmitotic apoptosis and mild but steady cell death. Since Cu-ATSM showed low but considerable uptake in normoxic cells in vitro and may be taken up in normoxic cells in vivo, the therapeutic index need to be carefully considered. In order to ensure the safety and effectiveness of ^{64}Cu-ATSM treatment, contribution of hypoxia on both ^{64}Cu accumulation and radiation sensitivity of target and/or nontarget cells and possible adverse effects to the excretion route should be clarified.

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資料(3)

Automatic Synthesis of 16α-[^{18}F]fluoro-17β-estradiol ([^{18}F]FES) Using a Cassette Type FDG Synthesizer

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ABSTRACT

16α-[^{18}F]fluoro-17β-estradiol ([^{18}F]FES) is a radiotracer for imaging estrogen receptors by positron emission tomography. We developed a clinically applicable automatic preparation system for [^{18}F]FES by modifying a cassette type FDG synthesizer. Two mg of 3-O-methoxymethyl-16,17-O-sulfuryl-16-epiestriol in acetonitrile was heated at 105 °C for 10 min with dried [^{18}F]fluoride. The resultant solution was evaporated and hydrolyzed with 0.2N HCl in 90% acetonitrile/water at 95 °C for 10 min under pressurized condition. The neutralization was carried out with 2.8% $NaHCO_3$ and then the HPLC purification was performed. The desired radioactive fraction was collected and the solvent was replaced by 10 ml of saline, and then passed through a 0.22 μm filter into a pyrogen-free vial as the final product. The HPLC purification data demonstrated that [^{18}F]FES was synthesized with a yield of 76.4 ± 1.9% (n=5). The yield as the final product for clinical use was 42.4 ± 3.2% (n=5, decay corrected). The total preparation time was 88.2 ± 6.4 min including the HPLC purification and the solvent replacement process. The radiochemical purity of the final product was >99% and the specific activity was more than 111 GBq/μmol. The final product was stable for more than 6 h in saline containing sodium ascorbate. This new preparation system enables us to produce [^{18}F]FES safe for clinical use with high and reproducible yield.

Keywords: [^{18}F]FES, estrogen receptors, automatic synthesis, radiopharmaceutical, PET, cassette type FDG synthesizer

INTRODUCTION

The estrogen receptor (ER) status is an important prognostic factor in breast cancer, because the ER positive cancers are less aggressive in clinical course and likely to respond to hormonal therapy [1]. The ER expression can be estimated with biopsied material, however, sampling errors may occur when patients have large or multi-site tumors. 16α-[^{18}F]fluoro-17β-estradiol ([^{18}F]FES) has been developed as a tracer for imaging ER by positron emission tomography (PET) [2, 3]. In clinical studies, the uptakes of [^{18}F]FES were proportional to the ER concentration in both primary and metastatic breast cancers [4-7]. FES-PET information may predict the response of the advanced breast cancer to hormonal therapy and be useful for guiding better treatment. Therefore, [^{18}F]FES is one of the most promising radiopharmaceutical for monitoring of ER status in breast cancer, however, it is not widely used because of the difficulty of the synthesis. The first synthesis of [^{18}F]FES was reported by Kiesewetter et al. [8] and the application of robotic synthesis was evolved [9]. Their procedure involved reduction by $LiAlH_4$ and liquid N_2. Another procedure was reported which uses 3-O-methoxymethyl-16,17-O-sulfuryl-16-epiestriol (**1**) as a precursor [10, 11]. The advantages of the latter procedure are that the nucleophilic substitution by [^{18}F]fluoride proceeded rapidly with good yield and this precursor is commercially available. It is also advantageous that the hydrolysis reactions were performed under mild condition [12]. Although the automatic synthesis of [^{18}F]FES using [^{18}F]fluorodeoxyglucose (FDG) synthesis module was reported [13], reproducible yield of [^{18}F]FES could not be realized because of the complex reaction conditions and purification processes.

FDG synthesis has become a standard practice in clinical PET centers for diagnosis of cancer. To make a radiotracer to be widely used, it is necessary to establish a system which can produce high quality product with the operation easy enough for routine work. We chose TRACERlab MX_{FDG} as the module for [^{18}F]FES synthesis because it is widely used as a simple automatic FDG synthesizer and it has the flexibility in programming and accepts modification to the cassette. However, this module does not have a cooling function and the cassette has low chemical resistance. In this study, we studied the hydrolysis condition on this module and performed the synthesis without changing the hardware, and also established a formula of high quality product for clinical use.

MATERIALS AND METHODS

Reagents and Equipments

All chemicals were obtained from commercial sources and used without further purification. The precursor, 3-O-methoxymethyl-16,17-O-sulfuryl-16-epiestriol (**1**) and authentic 16α-fluoro-17β-estradiol were purchased from ABX GmbH (Radeberg, Germany). The sodium bicarbonate solution and the sodium ascorbate solution used were of pharmaceutical grade. The other reagents and solvents were obtained from Sigma-Aldrich Co. (St. Louis, MO). The synthesis was performed using TRACERlab MX_{FDG} (GE Medical Systems, Milwaukee, WI) which is one of the automatic FDG synthesizers. The high performance liquid chromatography (HPLC) system was consisted of a pump (LC-10AD, Shimadzu, Kyoto, Japan), a UV detector (SPD-10AVP, Shimadzu) and a NaI(Tl) radioactive detector (RLC-700, Aloka, Tokyo, Japan).

Process of [^{18}F]FES Preparation

The total procedure of [^{18}F]FES preparation consisted of four steps as follows; (a) Fluorination (b) Hydrolysis (c) HPLC purification (d) Formulation (Figure 1). The automatic synthesis of [^{18}F]FES including (a) and (b) were performed on TRACERlab MX_{FDG}. The diagram of the [^{18}F]FES preparation system is shown in Figure 2.

Synthesis Module: TRACERlab MX_{FDG} is a disposable cassette type module realizing easy modification of chemical process as well as easy operation as routine work without requiring expert knowledge in chemistry. The synthesis program consists of a Microsoft Excel file defining synthesis parameters such as temperature, time, vacuum pressure, 3-way cocks and syringe positions.

Preparation of Reagents and Cassette: The reagents for [^{18}F]FES synthesis were contained in a set of six vials. The content of each vial was as follows: A; 22 mg of Kryptofix 2.2.2 and 7 mg of potassium carbonate mixture in 50% acetonitrile/water (0.6 ml). B; Anhydrous acetonitrile (2 ml). C; 2 mg of the precursor in anhydrous acetonitrile (2 ml). D; 0.2N hydrochloric acid in 90% acetonitrile/water (2 ml). E; 2.8% sodium bicarbonate in water (2 ml). F; 70% ethanol/water (2 ml). The reagents except for A were sealed in standard 10 ml glass vials. The reagent A in 1.2 ml vial was the same to the one used in FDG synthesis.

The cassette was prepared from the FDG synthesis cassette with a little modification keeping aseptic condition (Figure 2). The alumina N and two C18 Sep-Pak cartridges, unnecessary for [^{18}F]FES synthesis, were removed from the cassette. The water bag connection tube, attached at position 7, was utilized for the central and right manifold connection (the position 10 and 11) after adjustment of the tube length. To add another vial, the male and male connector which had been attached to the C18 Sep-Pak was moved to position 7 and connected with a 18G needle. Prior to the synthesis, an activated QMA Sep-Pak light cartridge and two 30 ml polyethylene syringes were jointed to the cassette.

Synthesis of [^{18}F]FES: The synthesis of [^{18}F]FES was modified from the method of Romer et al. [12]. Fluorine-18 was produced by the ^{18}O(p,n)^{18}F reaction using a ultra small cyclotron OSCAR (JFE P&S/Oxford, Yokohama, Japan) or RDS eclipse RD/HP (Siemens/CTI, Knoxville, TN, USA) in the PET center of University of Fukui. The irradiated ^{18}O-water, which contained carrier free [^{18}F]fluoride, was transferred to the module automatically and passed through the QMA cartridge. The trapped [^{18}F]fluoride on the cartridge was eluted by A into the reaction vessel and dried three times adding small amount of B. The precursor in acetonitrile, C,