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The evaluations of ^{99m}Tc cyclopentadienyl tricarbonyl triphenyl phosphonium cation for multidrug resistance

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ABSTRACT

A triphenylphosphonium cation, [^{99m}Tc]Technetium cyclopentadienyltricarbonyl-6-hexanoyl-triphenylphosphonium cation (**[^{99m}Tc]3**) was prepared to target multidrug resistance (MDR). The radiotracer was evaluated in the MDR-negative MCF-7 and MDR-positive MCF-7/ADR cell lines *in vitro*, as well as animal models *in vivo*. **[^{99m}Tc]3** was proofed to be a substrate of P-glycoprotein and multidrug resistant protein 1, and showed a higher accumulation in the MDR-negative MCF-7 cells compared to ^{99m}Tc-sestamibi *in vitro*. The MCF-7 tumor-to-MCF-7/ADR tumor ratio of **[^{99m}Tc]3** was ~3 at 1 h p.i. in the biodistribution study. These results demonstrated the capability of the radiotracer to detect multidrug resistance in tumor cells.

MDR (multidrug resistance) is the main obstacle in cancer chemotherapy.[1,2] It is usually associated with the decrease of drug accumulation in tumor cells, as a result of the drug efflux mediated by the over-expressed ATP-binding cassette (ABC) transporter proteins. ABC proteins that attribute to drug resistance mostly include P-glycoprotein (P-gp), multidrug resistant protein 1 (MRP1) and breast cancer resistance protein (BCRP). These proteins are capable of removing numerous structurally unrelated chemicals, including many clinical anticancer drugs out of tumor cells,[3] which eventually leads to the failure in cancer chemotherapy. Thus, a noninvasive and effective way for tumor MDR detection is demanded to guide the individualized therapeutic treatment on patients.

P-gp is one of the principal MDR transporters in humans, and expresses at a high level in many carcinomas.[4] Clinical studies revealed that the level of P-gp expression was closely correlated with drug resistance in the breast cancer chemotherapy.[5] Therefore, P-gp has been considered to be a critical target in tumor MDR detection. Similar to P-gp, MRP1 is also a member of MDR proteins, which overexpresses in a number of cancer cells such as lung and gastric cancer cells.[4] The high expression level of MRP1 is recognized to be responsible for tumor resistance to many chemotherapeutic drugs.[4]

In the current clinic, single photon emission computed tomography (SPECT) with cationic imaging agents such as ^{99m}Tc-sestamibi (^{99m}Tc-MIBI) and ^{99m}Tc-tetrofosmin, have been used for noninvasive cancer early diagnose and tumor MDR detection. They were both characterized as substrates for P-gp and MRP1. Their accumulation in tumor is due to the increased mitochondrial membrane potential.[2,6] However, the high uptake in non-targeting organs such as heart and liver, limits their tumor diagnostic accuracy in chest and abdominal regions. Compared to ^{99m}Tc-sestamibi, lipophilic phosphonium cations seem to be more distinguished in detecting tumor MDR. For example, ^{3}H-TPP has a higher tumor accumulation and greater tumor-to-nontumor ratio than ^{99m}Tc-sestamibi, and ^{64}Cu(DO3A-xy-TPEP) has a greater uptake difference between U87MG (MDR negative) and MDR-positive tumors.[7,8] These promising results encourage us to design and synthesize ^{99m}Tc-labeled triphenylphosphonium cation radiotracer for tumor MDR detection.

In the current study, [^{99m}Tc]Technetiumcyclopentadienyltricarbonyl-6-hexanoyl-triphenylphosphonium cation (**[^{99m}Tc]3**) and the corresponding Re complex, Rheniumcyclopentadienyltricarbonyl-6-hexanoyl-triphenylphosphonium cation (**Re5**) were prepared. ^{99m}Tc was chosen as the radiolabeled isotope because of its optimal nuclear properties ($t_{1/2}$ = 6.02 h, $E\gamma$ = 141 keV), the availability through commercial ^{99}Mo/^{99m}Tc generator and the low cost for SPECT imaging. The cyclopentadienyltricarbonyl ^{99m}Tc/Re moiety was adopted due to the stability and compactness of the organometallic core, which makes it possible to substitute or

Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein; R123, rhodamine 123; Vrp, verapamil; CsA, cyclosporine A; CCCP, carbonyl cyanide m-chlorophenylhydrazone; MRP1, multidrug resistant protein 1.

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mimic a benzene ring of TPP by integrated approach.[9,10] And the long hexanoyl linker was used to reduce the interaction between the cyclopentadienyltricarbonyl ^{99m}Tc/Re chelating group and the phosphonium cation moiety.

As shown in Scheme 1, intermediates **1** and **4** were synthesized through a Friedel-Crafts reaction according to the previous protocol[10] from the ferrocene and (cyclopentadienyl)tricarbonyl rhenium reactants, respectively. Then they could react with triphenylphosphine to obtain the precursor ferrocenyl compound **2** and rhenium complex **Re5** at a modest yield respectively. The aimed compounds were characterized by ^{1}H NMR, ^{13}C NMR, and HRMS (as shown in the Supporting Information). According to literature method,[11,12] the precursor ferrocenyl compound **2** directly reacted with the *fac*-[^{99m}Tc$(H_2O)_3(CO)_3$]$^+$ at 145 °C for 45 min to yield **[^{99m}Tc]3** (radiochemical yield: 40–50%, n = 9). Taking the corresponding rhenium complex **Re5** as identification, **[^{99m}Tc]3** was purified by semi-preparative HPLC (Supporting Information), and the radiochemical purity of **[^{99m}Tc]3** was >98%. The radiotracer was stable *in vitro*, and the radiochemical purity was >95% both in saline at room temperature and in mouse serum at 37 °C for 4 h. The partition coefficient (log *P*) in octanol and phosphate-buffered saline (0.05 mol·L^{-1}, pH = 7.4) of **[^{99m}Tc]3** was 1.20 ± 0.02 (n = 3), suggesting that it was a lipophilic complex.

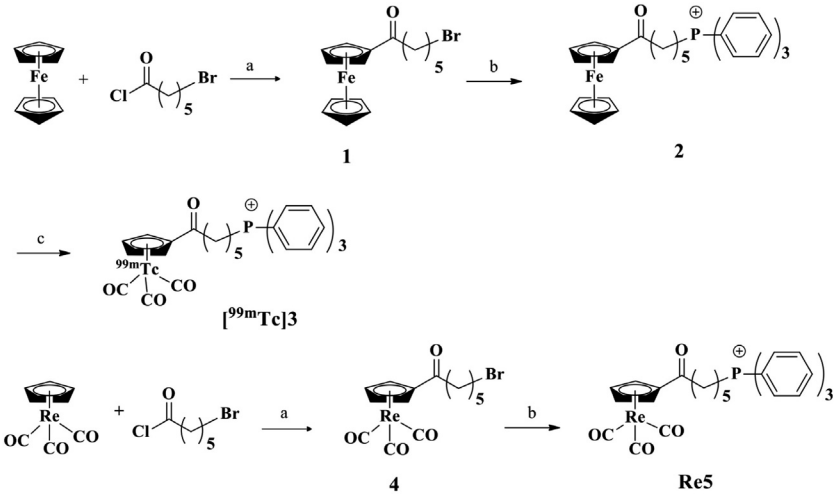
The rhodamine 123 (R123) is a fluorescent probe that can accumulate in cells and selectively transported by P-gp.[13,14] In order to reveal the different P-gp expression level between the MCF-7 (human breast cancer cells) and MCF-7/ADR (an MDR subline) cell lines, the rhodamine 123 accumulation assay was conducted in this study (Supporting Information). As shown in Fig. 1, obviously brighter fluorescence staining was visualized in MCF-7 cells than in MCF-7/ADR cells, which indicated the high level of P-gp expression in MCF-7/ADR cells compared to MCF-7 cells.

The cellular uptake and efflux experiments of **[^{99m}Tc]3** were performed on the MCF-7 and MCF-7/ADR cell lines, using ^{99m}Tc-sestamibi as a reference (Supporting Information). Similar to ^{99m}Tc-sestamibi, the cellular uptake of **[^{99m}Tc]3** at 37 °C was time-dependent at both cell lines incubating for 0.5, 1, 1.5, 2, 3 and 4 h (Fig. 2**A**), and the uptake values of radiotracers were always higher in MCF-7 cell line than the data obtained in MCF-7/ADR cell line. The cellular uptake of **[^{99m}Tc]3** in MCF-7 and MCF-7/ADR cell lines reached a peak at 3 h (280.98 %uptake/mg protein) and 1.5 h (78.72 %uptake/mg protein) respectively, while the uptake of ^{99m}Tc-sestamibi rose with time. Furthermore, the uptakes of **[^{99m}Tc]3** at every time point were all higher than those of ^{99m}Tc-sestamibi in both cell lines. But the uptake differences of **[^{99m}Tc]3** between the two cell lines were less than those of ^{99m}Tc-sestamibi. The cellular uptake of ^{99m}Tc-sestamibi in MCF-7 was 6.7-fold higher than that in MCF-7/ADR, while it was 5.6-fold of **[^{99m}Tc]3** at 3 h incubation. As shown in Fig. 2**B**, the efflux rate of **[^{99m}Tc]3** in MCF-7/ADR cell line was significantly higher than that in the MCF-7 cell line. These results suggested that the cellular uptake of the radiotracers was associated with the expression level of MDR proteins. The over expression of MDR proteins contributed to the efflux of radiotracer, and reduced the radioactivity accumulation in the MCF-7/ADR cell line.

To further investigate whether **[^{99m}Tc]3** was a substrate of P-gp/MRP1, inhibiting study was performed by monitoring the radiotracer accumulation in MCF-7/ADR cell line (P-gp and MRP1 overexpression).[19] Verapamil is the modulator of P-gp. Cyclosporine A is the modulator of P-gp and MRP1. And MK571 is the modulator of MRP1.[19] As shown in Fig. 3, the pre-treatment of modulators verapamil (Vrp, 20 μM), cyclosporine A (CsA, 20 μM) and MK571 (20 μM) could block the P-gp/MRP1 mediated transportation of the **[^{99m}Tc]3** or ^{99m}Tc-sestamibi, and significantly increased the radio-accumulation in cells. These results demonstrated that the radiotracer **[^{99m}Tc]3**, similar to ^{99m}Tc-sestamibi, could be transported both by P-gp and MRP1.

To determine whether the radiotracer **[^{99m}Tc]3** localized in the tumor cells through mitochondrial membrane potential, the cellular assay was conducted on MCF-7 cell line using carbonyl cyanide m-chlorophenylhydrazone (CCCP) to depolarize the mitochondrial membrane potential.[15] As shown in Fig. 4, **[^{99m}Tc]3** and ^{99m}Tc-sestamibi displayed 22% and 23% uptake decrease in the MCF-7 cells in the presence of CCCP (10 μM, 30 min), respectively. It suggested that **[^{99m}Tc]3** permeated into tumor cells through the mitochondrial membrane potential as ^{99m}Tc-sestamibi.[16,17]

The *in vivo* biodistribution study of **[^{99m}Tc]3** was performed in nude mice bearing MCF-7 or MCF-7/ADR tumor xenografts (Supporting Information). As demonstrated in Table 1, the uptake in MCF-7 tumor was 1.44 ± 0.09 %ID/g at 1 h p.i., approximately 3-fold higher than that in MCF-7/ADR tumor (0.42 ± 0.06 %ID/g). The significant accumulation difference agreed well with the cellular uptake data of **[^{99m}Tc]3** in the two cell lines. High level of MDR proteins expression resulted in a rapid excretion of radiotracer from tumor cells and lower uptake in MCF-7/ADR tumor. However, **[^{99m}Tc]3** also displayed a high radioactivity accumulation and



Scheme 1. (a) anhydrous CH_2Cl_2, anhydrous $AlCl_3$, 0 °C to room temperature; (b) triphenylphosphine, acetonitrile, 100 °C, reflux; (c) [^{99m}Tc$(H_2O)_3(CO)_3$]$^+$, DMF/H_2O = 1/1, pH = 1, 145 °C, 45 min.

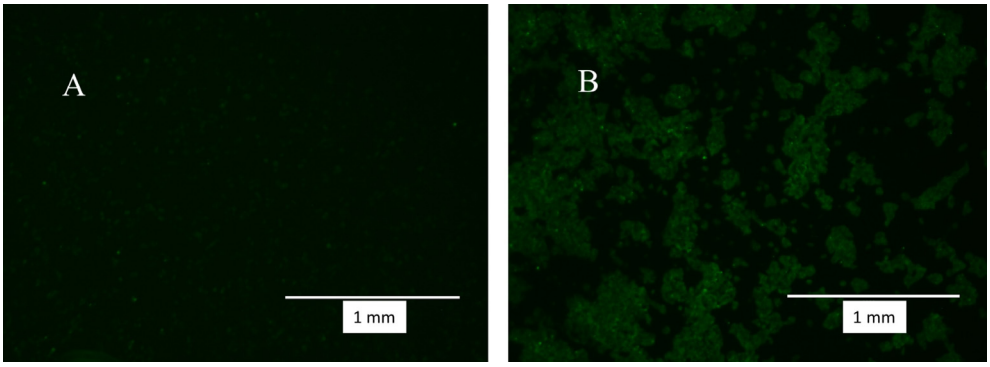


Fig. 1. The level of P-glycoprotein expression was assessed by the accumulation of R123 using a fluorescence microscopy. Significantly brighter fluorescence staining was visualized in MCF-7 cells (B) than that in MCF-7/ADR cells (A). Scale bar denotes 1 mm.

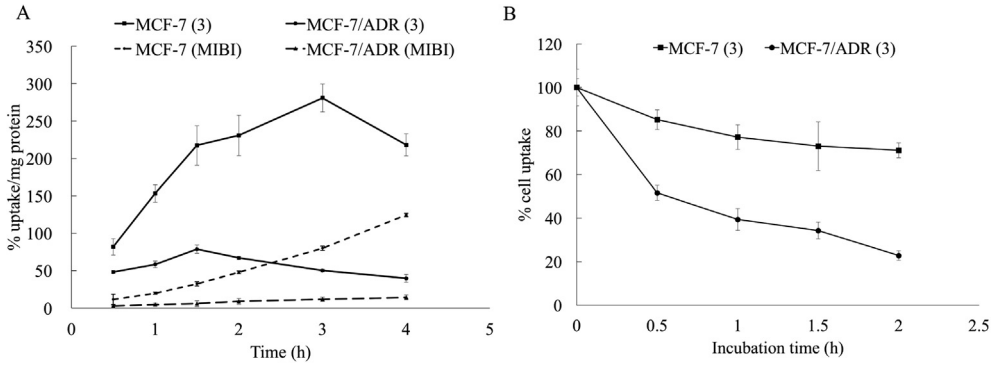


Fig. 2. (A) Cellular uptake of **[^{99m}Tc]3** and ^{99m}Tc-sestamibi (MIBI) in MCF-7 and MCF-7/ADR cell lines. Results are expressed as percentage of uptake per mg protein (mean ± SD; n = 3). (B) Efflux of **[^{99m}Tc]3** in MCF-7 and MCF-7/ADR cell lines.

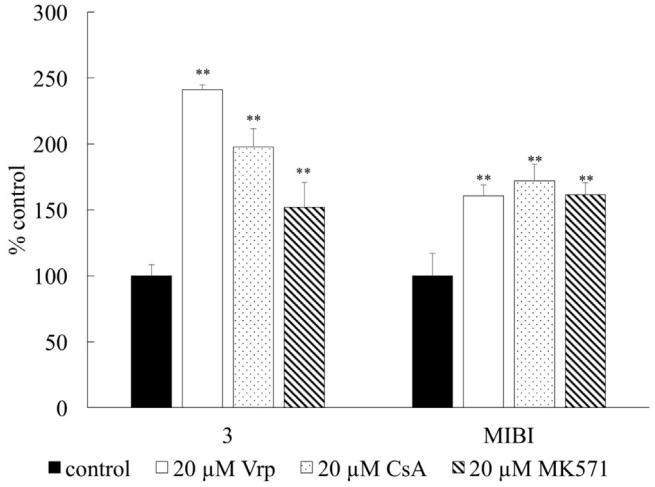


Fig. 3. Radio accumulation of **[^{99m}Tc]3** and ^{99m}Tc-sestamibi (MIBI) in MCF-7 and MCF-7/ADR cell lines in the presence or absence of known modulators, Vrp (verapamil, P-gp inhibitor, 20 μM), CsA (cyclosporine A, P-gp and MRP1 inhibitor, 20 μM) and MK571(MRP1 inhibitor, 20 μM). Taking the cellular uptake in the absence of modulators as control, the data were expressed as (accumulation activity/control) × 100%. $^{**}p < 0.01$ v.s. control, n = 3.

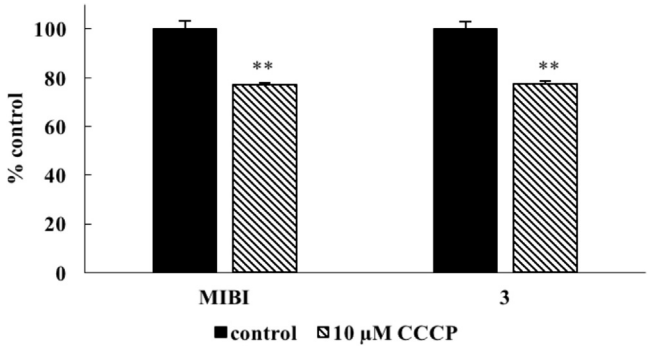


Fig. 4. Cellular uptake of **[^{99m}Tc]3** and ^{99m}Tc-sestamibi (MIBI) in MCF-7 cell lines in absence and presence of 10 μM CCCP (known to depolarized mitochondrial membrane potential). Taking the cellular uptake in the absence of CCCP as control, the data were expressed as (accumulation activity/control) × 100%. $^{**}p < 0.01$ v.s. control, n = 3.

delay clearance in the non-targeting organs, such as heart (2.64 ± 1.35 %ID/g at 1 h p.i.), liver (38.30 ± 7.14 %ID/g at 1 h p.i.) and kidneys (46.37 ± 8.84 %ID/g at 1 h p.i.). It might be due to the high lipophilicity (Log P = 1.20 ± 0.02) of the radiotracer, as well as the high density of mitochondrion in these organs.[18] The high accumulation in non-targeting organs is a drawback, which will cause a high radioactivity burden to patients, and interfere the tumor imaging quality. Studies are currently underway to optimize the pharmacokinetic of the radiotracer by modifying the chemical structure. For example, methoxy functional group is introduced to accelerate the liver clearance and enhance the MDR protein recognition to radiotracer.[20] Hydrophilic linker such as PEGs[21] can be employed to substitute the hexanoyl linker to reduce lipophilicity and accumulation in non-targeting organs.

In summary, a novel clopentadienyltricarbonyl triphenylphosphonium ^{99m}Tc based on tetraphenyl phosphonium cation has been developed for multidrug resistance detection. The radiotracer **[^{99m}Tc]3** tended to localized in tumor cells due to the increased

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Table 1
Biodistribution data of **[^{99m}Tc]3** in BALB/c nude mice bearing MCF-7 or MCF-7/ADR tumor xenografts. The data were reported as average ± standard deviation (%ID/g), n = 3.

Organ	[a] 1 h	2 h	4 h
Heart	2.64 ± 1.35	2.59 ± 0.55	2.11 ± 0.62
Liver	38.30 ± 7.14	34.13 ± 1.95	27.48 ± 7.52
Spleen	2.50 ± 1.30	1.61 ± 0.36	0.69 ± 0.26
Lung	1.84 ± 0.53	1.27 ± 0.22	0.84 ± 0.26
Kidneys	46.37 ± 8.84	56.81 ± 5.47	38.62 ± 2.24
Stomach	10.37 ± 3.66	8.68 ± 0.24	6.09 ± 1.50
Muscle	0.80 ± 0.15	0.65 ± 0.17	0.53 ± 0.19
Intestine	7.70 ± 2.82	4.37 ± 1.63	3.57 ± 1.28
Blood	0.97 ± 0.07	0.88 ± 0.07	0.61 ± 0.13
Thyroid (%ID)	0.18 ± 0.09	0.25 ± 0.11	0.13 ± 0.07
Brain	0.11 ± 0.06	0.12 ± 0.07	0.09 ± 0.02
MCF-7 tumor	1.44 ± 0.09	0.77 ± 0.07	0.67 ± 0.08
MCF-7/ADR tumor	0.42 ± 0.06**	–	–

** $p < 0.01$ v.s. MCF-7 tumor uptake.
[a] n = 6, except tumor.

mitochondrial membrane potential. Compared to ^{99m}Tc-sestamibi, **[^{99m}Tc]3** showed a higher accumulation in the MDR-negative MCF-7 cell *in vitro*. **[^{99m}Tc]3** exhibited a P-gp/MRP1-dependent manner, and displayed a significantly lower uptake in MDR-positive MCF-7/ADR than that in MCF-7 both *in vitro* and *in vivo*, thus indicating the feasibility of integrated approach for the MDR-detection probes design. Further investigation towards novel ^{99m}Tc-labelled triphenylphosphonium cations with optimal biodistribution properties is warrant.

Conflict of interest

The authors declare no conflicts of interest.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.05.053.

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