

Synthesis and Feasibility Evaluation of a new Trastuzumab Conjugate Integrated with Paclitaxel and ^{89}Zr for Theranostic Application Against HER2-Expressing Breast Cancers

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The preparation and *in vitro* evaluation of a theranostic conjugate composed of trastuzumab, paclitaxel (PTX), and deferoxamine (DFO)-chelated ^{89}Zr have been reported. These compounds have potential applications against HER2 receptor positive breast cancers. We conjugated DFO and PTX to trastuzumab by exploiting simple conjugation chemistry. The conjugate (DFO-trastuzumab-PTX) showed excellent radiolabeling efficiency with ^{89}Zr and the labeled conjugate had high

in vitro stability in human serum. Furthermore, DFO-trastuzumab-PTX displayed comparable cytotoxicity with PTX and ^{89}Zr -DFO-trastuzumab-PTX exhibited HER2 receptor-mediated binding on HER2-positive MDA-MB-231 breast cancer cells. The results of our *in vitro* study indicate high potential of ^{89}Zr -DFO-trastuzumab-PTX to be utilized in the theranostic application against HER2-positive breast cancers.

1. Introduction

Theranostic agents, defined as a material capable of diagnosing and treating a disease simultaneously using a single platform composed of multiple functional units, have withdrawn tremendous interest for past decades from research community. These agents have potential to provide information about localization and biodistribution of a drug in a patient for better assessment of the drug as well as planning of treatment regimen.^[1] Furthermore, theranostic agents are capable of minimizing the inevitable difference in biodistribution and selectivity existing between diagnostic and therapeutic materials for a specific disease.^[2,3] Especially for cancer, which is a highly heterogeneous disease, application of theranostic agent is essential for better assessment of treatment options to different individuals to achieve improved prognosis.^[4,5] There can be variety of theranostic platforms for simultaneous accommodation of imaging and therapeutic moieties, and antibody conjugate would be a great choice since the formulation has been proven to be effective in several clinical cases.^[6,7]

Breast cancer is the most common cancer in women and its incidence is over 1.5 million cases worldwide every year and the

mortality rate is also very high.^[8,9] Researchers have put significant effort to understand novel characteristics of breast cancer and to develop appropriate therapeutic strategies. It was found that around 25–30% of all breast cancer and approximately 50% of breast cancer with bone metastasis overexpress HER2 receptor, which is a member of human epidermal growth factor receptor family, and its expression causes poor prognosis.^[10–12] Trastuzumab (Herceptin®), which is a FDA-approved humanized monoclonal antibody for the treatment of metastatic breast cancer, selectively targets the HER2 receptor and is known to block HER2-related cellular transduction pathway and cause antibody-dependent cell-mediated cytotoxicity.^[12] Trastuzumab's clinical efficacy was proven when used solely as well as in combination with chemotherapeutics, and its antibody drug conjugate version (Trastuzumab emtansine, T-DM1) has shown improved treatment outcome compared to sole treatment of trastuzumab.^[13] Therefore, trastuzumab is a pertinent choice for the development of theranostic platform when HER2-expressing breast cancer is considered.

Paclitaxel (PTX) is an effective chemotherapeutics used in a wide range of cancers including breast, ovarian, head and neck cancers, etc., and it stabilizes microtubules leading to apoptosis.^[14,15] However, the clinical use of PTX is limited by its poor water-solubility and high toxicity to normal cells necessitating the development of appropriate delivery systems for improved solubility and target selectivity.^[14]

Positron emission tomography (PET) is a sensitive and noninvasive molecular imaging technique utilizing positron-emitting radioisotopes and most frequently used option for cancer diagnostics. 2-deoxy-2-[^{18}F]-fluoroglucose (^{18}F -FDG), which exploits increased glucose metabolism of cancer, is the predominantly used as a PET agent for cancer imaging. However, it poses a crucial limitation such that ^{18}F -FDG is not cancer specific, i.e. it accumulates not only at cancer sites but

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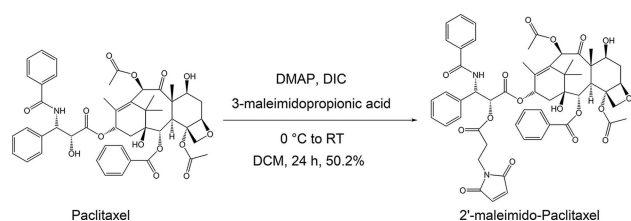
also at the sites with increased glucose metabolism, such as infectious and inflammatory sites, leading to false-positive result.^[16] To overcome the limitation of ¹⁸F-FDG, development of monoclonal antibody labeled with a positron emitting radioisotope has attracted a lot of attention.^[17,18] Accordingly, various positron emitting radioisotopes including ¹²⁴I, ⁸⁶Y, ⁶⁴Cu and ⁶⁸Ga have been explored to be labeled to the antibodies. However, they have some drawbacks that hamper the optimal construct of immuno-PET agents. For instance, *in vivo* dehalogenation of PET agent using ¹²⁴I might cause radiation damage to healthy tissue, and the short half-lives of ⁶⁴Cu (12.7 h), ⁶⁸Ga (67.7 min), and ⁸⁶Y (14.7 h) do not match with *in vivo* half-life (2–3 days) of typical antibodies.^[19] On the other hand, ⁸⁹Zr has optimal physical characteristics that can be exploited in PET imaging application of cancer. Its decay half-life (78.4 h) is in line with physiological half-life of typical antibodies, and the methods for production with high radiochemical purity and specific activity are well developed.^[18–21] Furthermore, suitable chelator (Deferoxamine, DFO) for ⁸⁹Zr is already commercially available fueling the investigation of ⁸⁹Zr as an immuno-PET agent.^[22–24]

In the present study, we first synthesized a new antibody conjugate composed of trastuzumab, PTX, and DFO by sequential conjugation of DFO and PTX with trastuzumab. Following characterization of the conjugate, radiolabeling efficiency using ⁸⁹Zr and serum stability of ⁸⁹Zr-labeled complex were assessed. Furthermore, *in vitro* cell binding study and cytotoxicity evaluation were done using HER2-expressing (HER2-transduced) MDA-MB-231 human breast cancer cell line.

2. Results and Discussion

2.1 Synthesis of 2'-Maleimido-PTX

Synthetic route of 2'-maleimido-PTX is outlined in scheme 1. Steglich esterification using DIC/DMAP as coupling reagents



Scheme 1. Synthesis of maleimide coupled PTX by utilization of Steglich esterification with DMAP/DIC coupling reagents. DMAP, 4-(Dimethylamino)pyridine; DIC, *N,N*-Diisopropylcarbodiimide.

was preferentially done at the 2'-hydroxyl group with 50.2% yield due to steric hindrance at 7'-hydroxyl group of PTX. NMR analysis indicated that C-2' proton peak was shifted from 4.78 ppm in unmodified PTX to 5.47 ppm in maleimide-coupled PTX, and there was the appearance of proton signal from ethylene in maleimide at 6.49 ppm. Furthermore, retention time shift from 6.77 min to 10.99 min on HPLC (Supporting Informa-

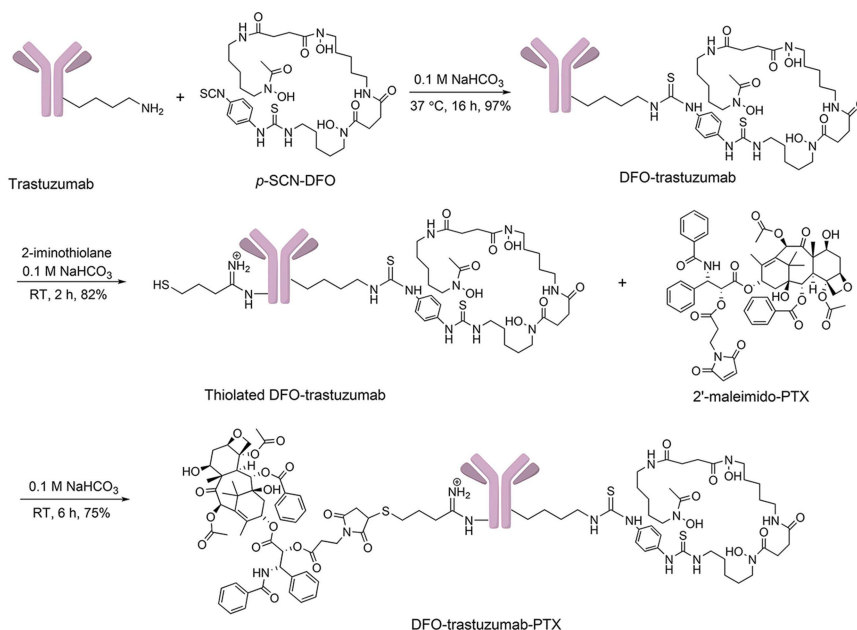
tion) evaluation using reversed-phase C-18 column validates the coupling of maleimide group to PTX, and clean single peak also confirms the purity of the compound. Mass analysis via MALDI ($[M + Na]^+$ calculated/found: 1027.35/1027.49) further confirms the synthesis of 2'-maleimido-PTX.

2.2 Preparation of Trastuzumab Conjugate

Preparation of DFO-trastuzumab-PTX is shown in scheme 2. Conjugation of DFO with trastuzumab was done via thiourea linkage between lysine residues from trastuzumab and isothiocyanate group from DFO. Solution of *p*-SCN-DFO was prepared in DMSO and %v/v of DMSO (< 1.0%) in a reaction buffer (0.1 M NaHCO₃, pH 9.0) was carefully controlled to minimize possible antibody aggregation. It was found between 0.45 to 0.55 DFO was conjugated (Supporting Information) to a molecule of trastuzumab after 16 h of reaction at 37 °C. Following DFO conjugation, thiolation of trastuzumab in DFO-trastuzumab was carried out using 2-iminothiolane (Traut's reagent), after which conjugation with 2'-maleimido-PTX was done. The solution of 2'-maleimido-PTX was prepared in DMSO, and conjugation with thiolated DFO-trastuzumab was progressed in the reaction buffer for 6 h at RT, and it was found between 3.8 to 4.2 PTX was conjugated (Supporting Information) to a molecule of DFO-trastuzumab via thioether linkage. Although more extensive investigation needs to be done to find the best conditions for conjugation of antibody with DFO and PTX, especially when considering they are not water soluble, the number of conjugated DFO or PTX to trastuzumab was good enough to test *in vitro* feasibility for the development of theranostic agent.

2.3 Radiolabeling with ⁸⁹Zr and Serum Stability of ⁸⁹Zr-Labeled Complex

The complexation kinetics of the new antibody conjugate (DFO-trastuzumab-PTX) with ⁸⁹Zr was highly efficient (100% at 1 min, supporting information), and the labeling efficiency was comparable with that of DFO-trastuzumab. As DFO is an excellent chelator for ⁸⁹Zr,^[25,26] it was expected that DFO-trastuzumab would have a good radiolabeling efficiency with ⁸⁹Zr. However, it was not unreasonable to assume that complexation kinetics of DFO-trastuzumab-PTX would be compromised due to more intricate conjugation status. Nonetheless, DFO-trastuzumab-PTX instantly grabbed ⁸⁹Zr at RT with specific activity of 0.457 Ci/μmol. Since rapid chelation of radionuclide in mild condition is an important prerequisite for the development of antibody-based radiopharmaceuticals due to the concern for antibody's integrity, the labeling result of DFO-trastuzumab-PTX with ⁸⁹Zr indicated it has the potential to be a theranostic agent utilizing antibody and radionuclide. Stability of ⁸⁹Zr-labeled DFO-trastuzumab-PTX and DFO-trastuzumab was evaluated by incubating the complexes in human serum at 37 °C (Supporting Information). As human serum has variety of proteins and trace metals, trans-chelation would occur if the complex is not very stable, thereby incubation of labeled



Scheme 2. Preparation of a new antibody conjugate composed of DFO, trastuzumab and PTX.

complex in human serum is a good way to evaluate stability of the labeled complex. Stability of ^{89}Zr -labeled DFO-trastuzumab-PTX was assessed up to 7 days and it was shown that no significant amount of ^{89}Zr was dissociated from the labeled complex, and the result was comparable with that of ^{89}Zr -labeled DFO-trastuzumab indicating that the addition of PTX did not affect the stability of labeled complex.

2.4 Binding Assay of ^{89}Zr -Labeled Theranostic Agent

To evaluate binding property of ^{89}Zr -labeled DFO-trastuzumab-PTX, *in vitro* binding assay was performed on HER2-positive MDA-MB-231 cells (Figure 1). After 5 h incubation at 4 °C, 49.5 ± 5.3% of the ^{89}Zr -labeled DFO-trastuzumab-PTX treated were remained bound to cells, while blocking with excess trastuzumab reduced the cell bound percentage significantly (p -value =

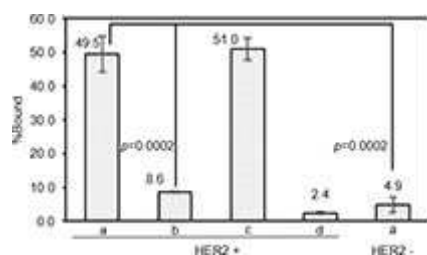


Figure 1. Binding assay of ^{89}Zr -labeled trastuzumab conjugates on HER2-positive and negative MDA-MB-231 cells [a: (^{89}Zr)DFO-Tras-PTX; b: Blocking; c: (^{89}Zr)DFO-Tras; d: ^{89}Zr]. ^{89}Zr -labeled trastuzumab conjugate (1 μg , ~700kcpm) was incubated on the cells (1×10^6) for 5 h at 4 °C, after which the cells were washed with cold PBS ($3 \times 1 \text{ mL}$) and radioactivity remained on the cell pellets was measured using gamma counter. The evaluation was done in triplicate (mean ± standard deviation %).

0.0002) showing cell binding was receptor mediated. ^{89}Zr -labeled DFO-trastuzumab also had similar binding percentage ($51.0 \pm 3.3\%$) indicating additional conjugation of PTX did not alter biological activity of trastuzumab. Free ^{89}Zr was also incubated with the cells to ensure %bound of the ^{89}Zr -labeled conjugates was not due to adsorption of dissociated free ^{89}Zr , and the uptake of the free ^{89}Zr was only $2.4 \pm 0.3\%$ confirming intact complexes of ^{89}Zr -labeled conjugates were bound to the cells. ^{89}Zr -labeled DFO-trastuzumab-PTX was also incubated with HER2-negative MDA-MB-231 cells, and it showed significantly lower binding ($4.9 \pm 2.2\%$, p -value = 0.0002) validating receptor-mediated uptake of ^{89}Zr -labeled DFO-trastuzumab-PTX.

2.5 In Vitro Toxicity Evaluation

Cytotoxicity screening (50 μM , PTX equimolar) of DFO-trastuzumab-PTX was done using MTT assay on HER2-positive and negative MDA-MB-231 cells and compared with PTX itself (Figure 2). Approximately half of the HER2-positive MDA-MB-231 cells were dead after 72 h incubation with DFO-trastuzumab-PTX, which was similar with the effect of PTX treatment. Exposure of trastuzumab only on the cells did not exert any cytotoxic effect, as expected, since it is known trastuzumab requires other effectors to trigger anti-cancer effect and would not result in cell death *in vitro*.^[27] On the HER2-negative cells, PTX also had almost the same level of cytotoxic effect (55.5% vs. 56.4%) as it is not a targeted therapeutics. However, DFO-trastuzumab-PTX also showed quite similar cytotoxic effect on the HER2-negative cells compared to HER2-positive cells (62.4% vs. 55.8%), which was not expected, since less anti-cancer effect was anticipated due to the lack of HER2 receptor. It was

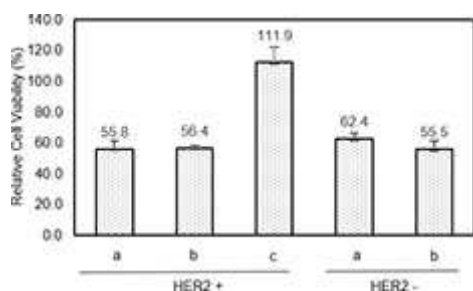


Figure 2. *In vitro* toxicity evaluation of DFO-trastuzumab-PTX on HER2-positive and negative MDA-MB-231 cells [a: DFO-Tras-PTX; b: PTX; c: Tras]. 50 μ M (PTX equimolar) of the conjugate was incubated for 72 h, and relative cell viability was measured using MTT assay. The evaluation was done in triplicate (mean \pm standard deviation %).

speculated that the ester linkage between PTX and trastuzumab was broken due to its susceptibility to hydrolysis even *in vitro*,^[28,29] and PTX was released during the incubation period leading to cell death. Therefore, the cytotoxicity exhibited by DFO-trastuzumab-PTX on MDA-MB-231 breast cancer cells, whether they express HER2 receptor or not, was indeed the result of premature release of PTX rather than the receptor-mediated delivery of PTX. Therefore, smarter conjugate strategies linking PTX and trastuzumab should be exploited for improved performance of targeted delivery and tumor-selective action of the drug. Furthermore, the 2'-hydroxyl group of PTX should be intact after release from the conjugate to properly interfere with tubulin.^[30,31] Consequently, a linker that is stable enough in human plasma but hydrolyzed within cancer cells releasing PTX without linker residues should be applied. Recently, alcohol modification chemistry using acetal pyrophosphate ester, which satisfies above mentioned requirements, to conjugate an anti-cancer drug with an antibody was developed,^[32] and it would be interesting to apply the acetal pyrophosphate ester into our conjugate system in the future.

3. Conclusions

A new antibody conjugate (DFO-trastuzumab-PTX) was prepared via simple modification of PTX and sequential conjugation of DFO and the modified PTX with trastuzumab. DFO-trastuzumab-PTX showed efficient labeling with ^{89}Zr , and the labeled complex was highly stable in human serum. Furthermore, DFO-trastuzumab-PTX exhibited comparable cytotoxicity with PTX *in vitro*, and ^{89}Zr -labeled DFO-trastuzumab-PTX had receptor-specific binding on HER2-positive MDA-MB-231 cells. Although improved linker chemistry needs to be applied to the conjugation of PTX with trastuzumab for better selective anti-cancer effects, our *in vitro* results present promising potential of ^{89}Zr -labeled DFO-trastuzumab-PTX as a theranostic agent against HER2-positive breast cancers.

Experimental Section

Instruments and Materials: ^1H NMR spectrum was obtained using a Bruker 300 instrument, and chemical shifts are reported in ppm on the δ scale relative to TMS. Matrix-assisted laser desorption ionization mass (MALDI-MS) was obtained using Applied Biosystems ABI5800 Plus MALDI-TOF/TOF analyser (Korea Drug Development Platform using Radio-isotope, Korea Institute of Radiological and Medical Sciences, Seoul, Korea). Analytical HPLC was performed on a Waters 2998 system (Waters, Milford, MA, USA) equipped with photodiode array detector ($\lambda = 230$ nm) using Atlantis[®] dC18, 5 μ m, 3.0 \times 150 mm column with solvent system consisting of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B), where isocratic eluent profile 0–20 min, 50% A, 50% B (0.5 mL/min) was applied. All measurements of protein concentration were obtained using Thermo Scientific Nanodrop 2000 (Waltham, MA, USA). Radioactive Thin layer chromatography (TLC) measurements was done using Eckert & Ziegler TLC scanner B-AR2000-1 (Eckert & Ziegler, Valencia, CA, USA). For measurements of radioactivity in cell binding assay, PerkinElmer 2480 Automatic Gamma Counter (Waltham, MA, USA) was used. RT-PCR was performed using 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) and fluorescence images were obtained using Olympus IX71 (Olympus, Tokyo, Japan). 4-(Dimethylamino)pyridine (Cat# 522805), *N,N'*-Diisopropylcarbodiimide (Cat# D125407) and 2-iminothiolane hydrochloride (Cat# I6256) were obtained from Sigma Aldrich (St. Louis, MO, USA). 3-maleimidopropionic acid (Cat# M1962), paclitaxel (Cat# P106869) and *p*-SCN-Bn-Deferoxamine (Cat# B-705) was obtained from Tokyo Chemical Industry (Tokyo, Japan), Aladdin Industrial Corporation (City of Industry, CA, USA), and Macrocyclics (Plano, TX, USA), respectively. HERBB2 inducible lentiviral particle (Cat# LVP504) was purchased from GenTarget Inc. (San Diego, CA, USA).

Production of ^{89}Zr Chloride: ^{89}Zr was produced as previously described^[33] with little modification. Briefly, nuclear reaction of ^{89}Y (p,n) ^{89}Zr on an enriched yttrium target (^{89}Y , 200 \times 200 \times 0.025 mm, 99.9%) was carried out with a bombardment by the proton beam (about 20 MeV) of medical cyclotron (MC50, Scanditronix co., 1985). To obtain ^{89}Zr with high radiochemical purify, the solution of radioactive yttrium target melted by hydrochloric acid (6.0 N, 10 mL) was passed through a hydroxamate resin column. ^{89}Zr trapped in the column was recovered by oxalate solution (1.0 N, 2.0 mL), and the ^{89}Zr -oxalate solution was slowly loaded into a QMA Sep-Pak column (Waters co.). To remove oxalate, the column was slowly washed using distilled water (100 mL). The product left in the column was obtained by hydrochloric acid (1.0 N, 1.0 mL), and the radiochemical yield of ^{89}Zr chloride was found to be 6.5 ± 1.5 mCi/h with over 99% radionuclide purity.

Establishment of HER2-Expressing MDA-MB-231 Breast Cancer Cell: Lentivirus vectors carrying *HER2* and red fluorescent protein (*RFP*) fused with blasticidin deaminase (*Bsd*) genes (200 μ L, 1×10^7 IFU/mL in DMEM medium) were transduced into MDA-MB-231 cells. HER2-expressing cells were selected by blasticidin for 4 weeks, and confirmation of HER2 expression was done by RT-PCR and immunofluorescence imaging (Supporting Information).

Cell Culture: HER2-positive and negative MDA-MB-231 human breast cancer cell lines were cultured in a humidified atmospheric condition with 5% CO_2 , at 37 $^\circ\text{C}$ using RPMI-1640 culture medium containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic.

Synthesis of 2'-Maleimido-PTX: 4-(Dimethylamino)pyridine (0.70 mg, 0.006 mmol), 3-maleimidopropionic acid (10.9 mg, 0.06 mmol) and *N,N'*-Diisopropylcarbodiimide (7.4 mg, 0.06 mmol) were sequentially added to PTX (50 mg, 0.06 mmol) in dichloro-

methane (5 mL) at 0 °C. The temperature was gradually increased to room temperature, and the reaction mixture was reacted for 24 h under argon atmosphere while the progress was monitored by TLC. After the reaction was complete, the mixture was washed using saturated solution of NH₄Cl (2 × 15 mL) and NaHCO₃ (2 × 15 mL), followed by NH₄Cl (3 × 15 mL). The organic layer was dried over MgSO₄, filtered, and evaporated to obtain a crude product. The crude product was purified by auto column chromatography (0% B/3 min, 0–2% B/3–5 min, 2% B/5–10 min; solvent A=dichloromethane, solvent B=MeOH) and dried *in vacuo* to provide pure product (29.6 mg, 50.2%). The product was characterized by ¹H NMR (300 MHz, CDCl₃),²⁸ HPLC (*t*_R = 10.99 min and 6.77 min for 2'-maleimido-PTX and unmodified PTX, respectively, Supporting Information), and MALDI-MS [M+Na]⁺ (calculated/found: 1027.35/1027.49).

Conjugation of DFO with Trastuzumab: To a solution of trastuzumab (19.5 mg) in 0.1 M NaHCO₃ (pH 9.0, 5 mL) was added 20-fold excess *p*-SCN-DFO in DMSO (44.6 μL). The reaction mixture was agitated in a thermomixer at 37 °C for 16 h, after which unreacted *p*-SCN-DFO was removed using Amicon Ultra centrifugal filters (Millipore, Ultracel-30K, UFC803024) while washing with 0.1 M NaHCO₃ (3 × 2 mL). DFO-trastuzumab was recovered (18.6 mg, 97.0%) using 0.1 M NaHCO₃ (pH 9.0, 1 mL), and the number of DFO conjugated to a molecule of trastuzumab was found to be 0.45–0.55 by MALDI-Mass analysis (Supporting Information).

Thiolation of Trastuzumab: To a solution of DFO-trastuzumab (18.6 mg) in 0.1 M NaHCO₃ (pH 9.0, 1 mL) was added 20-fold excess 2-iminothiolane. The reaction mixture was agitated in a thermomixer at RT for 2 h, after which unreacted 2-iminothiolane was removed using Amicon Ultra centrifugal filters (Millipore, Ultracel-30K, UFC803024) while washing with 0.1 M NaHCO₃ (3 × 2 mL). Thiolated DFO-trastuzumab was recovered (15.2 mg, 82.0%) using 0.1 M NaHCO₃ (pH 9.0, 0.9 mL).

Conjugation of 2'-Maleimido-PTX with DFO-Trastuzumab: To a solution of thiolated DFO-trastuzumab (7.0 mg) in 0.1 M NaHCO₃ (pH 9.0, 6 mL) was added 30-fold excess 2'-maleimido-PTX in DMSO (28.8 μL). The reaction mixture was agitated in a thermomixer at RT for 6 h, after which unreacted 2'-maleimido-PTX was removed using Amicon Ultra centrifugal filters (Millipore, Ultracel-30K, UFC803024) while washing with 0.1 M NaHCO₃ (3 × 2 mL). DFO-trastuzumab-PTX was recovered (5.2 mg, 75.0%) using 0.1 M NaHCO₃ (pH 9.0, 0.7 mL), and the number of PTX conjugated to a molecule of trastuzumab was found to be 3.8–4.2 by MALDI-Mass analysis (Supporting Information).

Radiolabeling of DFO-Linked Trastuzumab Conjugates with ⁸⁹Zr Chloride: Radiolabeling reaction buffer (0.25 M NH₄OAc, pH 7.0) was prepared by using 99.999% trace metals basis NH₄OAc (Sigma Aldrich, Cat# 372331) and treated with Chelex-100 resin (Biorad, Cat# 1422842) to remove any trace metals possibly existing in the solution. To a buffer solution (17.96–18.32 μL) in a capped microcentrifuge tube was sequentially added a solution of either DFO-trastuzumab-PTX (10 μg) or DFO-trastuzumab (10 μg) and a solution of ⁸⁹Zr chloride (30 μCi, 1.5 μL, 0.1 N HCl). The reaction mixture was agitated in a thermomixer (1000 rpm) at RT. An aliquot of the mixture was withdrawn at designated time points (1, 10, 30, 60 min), spotted on a TLC plate and eluted using 20 mM EDTA in 0.15 M NH₄OAc for measurement of labelling efficiency by radio-TLC scanner.

Serum Stability Evaluation of ⁸⁹Zr-Labeled Complexes: ⁸⁹Zr-labeled complexes were prepared as described in the radiolabelling section. After complete complexation of the DFO-trastuzumab conjugates was confirmed by radio-TLC, human serum (40 μL) was added to the ⁸⁹Zr-labelled conjugate (40 μL), and the mixture was incubated

in a thermomixer at 37 °C. ⁸⁹Zr bound percentage was assessed for 7 consecutive days using radio-TLC (20 mM EDTA in 0.15 M NH₄OAc as an eluent).

In Vitro Binding Assay: ⁸⁹Zr-labeled DFO-trastuzumab-PTX and DFO-trastuzumab were prepared as described in the radiolabelling section. An aliquot of ⁸⁹Zr-labeled conjugate (1 μg, ~700 kcpm) was added to HER2-positive or negative MDA-MB-231 breast cancer cells (1 × 10⁶) suspended in PBS with 1% BSA (0.5 mL) in a sterile polystyrene round-bottom tube (BD Falcon, Cat# 352052) and incubated at 4 °C with vigorous stirring for 5 h. The radioactivity of each tube was measured using gamma counter, after which the cell pellets were washed with cold PBS (3 × 1 mL). The radioactivity after the wash was measured again, and the percentage bound was calculated by comparing the radioactivity before and after the wash. For blocking experiment, 5000-fold excess of trastuzumab was added to the cells prior to the addition of ⁸⁹Zr-labeled conjugate.

Cytotoxicity Evaluation: HER2-positive and negative MDA-MB-231 breast cancer cells (1 × 10⁴) were seeded on a 96-well plate and allowed for them to attach for 24 h. PTX (50 μM), DFO-trastuzumab-PTX (50 μM, PTX equimolar), and trastuzumab (12.5 μM, trastuzumab concentration resulted when PTX to trastuzumab ratio was considered for 50 μM PTX in DFO-trastuzumab-PTX) were treated on the cells and incubated for 72 h. After the incubation, relative cell viability was obtained by comparing the viabilities between untreated control cells and treated ones by using MTT assay.

Statistical Analysis: Data were analysed by unpaired, two-tailed Student's *t*-test using GraphPad Prism 8.0.2 (GraphPad Software Inc.), and the difference with *p*-value of <0.05 (95% confidence level) was considered as statistically significant.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: theranostics · trastuzumab · paclitaxel · ⁸⁹Zr · breast cancer

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