Stabilization of an 211At-Labeled Antibody with Sodium Ascorbate

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ABSTRACT: 211At, an α-particle emitter, has recently attracted attention for radioimmunotherapy of intractable cancers. However, our sodium dodecyl sulfate polyacrylamide gel electrophoresis and flow cytometry analyses revealed that 211At-labeled immunoconjugates are easily disrupted. Luminol assay revealed that reactive oxygen species generated from radiolysis of water caused the disruption of 211At-labeled immunoconjugates. To retain their functions, we explored methods to protect 211At-immunoconjugates from oxidation and enhance their stability. Among several other reducing agents, sodium ascorbate most safely and successfully protected 211At-labeled trastuzumab from oxidative stress and retained the stability of the 211At-labeled antibody and its cytotoxicity against antigen-expressing cells for several days.

INTRODUCTION

Radioimmunotherapy (RIT) is defined as targeted radionuclide therapy using radiolabeled antibodies. RIT has expanded the applications of radiotherapy from focusing on local tumors to targeting scattered tumors, such as distant metastases and disseminated lesions. As for β-particles, two kinds of radiopharmaceuticals, which target the CD20 molecule on the surface of lymphoma cells,90Y-labeled rituximab and 131I-labeled rituximab, have already shown clinical benefits against CD20-positive non-Hodgkin B-cell lymphoma.1 Compared with β-particles, α-particles have more potent linear energy transfer (LET) and a shorter path range. Owing to their high-energy emission within a short path length, α-particles have more potent linear energy transfer (LET) and a shorter path range. Owing to their high-energy emission within a short path length, α-particles can selectively eliminate target cells with minimal radiation damage to the surrounding normal tissues when delivered selectively to tumor tissues. These properties render α-particles an attractive tool for treating intractable tumors.2–5 211At is an α-emitter with a short half-life (7.2 h) and does not yield cytotoxic daughter isotopes during its decay; the first branch (58.2%) decays through electron capture to 211Po (half-life: 516 ms), which decays through α-particle emission to 207Bi (half-life: 31.55 y). The 211At-labeled anti-Tenascin mAb 81C6 was administered locally to 18 patients with recurrent malignant brain tumors,11 and the 211At-labeled MX35 F(ab’)2, targeting the sodium-dependent phosphate transport protein 2B, was intraperitoneally administered to nine patients with ovarian cancer.12 In addition to the promising results obtained by these studies, the procedure for the production of 211At-labeled antibodies under the current Good Manufacturing Practices (cGMP) toward clinical application was recently reported.19 To maximize the functions of 211At-labeled antibodies, the quality of the conjugates must be validated. Therefore, in the present study, we evaluated the quality of an 211At-conjugated antibody.

bodies were reportedly tested to deliver highly cytotoxic 211At to the target in preliminary investigations and preclinical situations.11–18 The 211At-labeled anti-Tenascin mAb 81C6 was administered locally to 18 patients with recurrent malignant brain tumors,11 and the 211At-labeled MX35 F(ab’)2, targeting the sodium-dependent phosphate transport protein 2B, was intraperitoneally administered to nine patients with ovarian cancer.12 In addition to the promising results obtained by these studies, the procedure for the production of 211At-labeled antibodies under the current Good Manufacturing Practices (cGMP) toward clinical application was recently reported.19 To maximize the functions of 211At-labeled antibodies, the quality of the conjugates must be validated. Therefore, in the present study, we evaluated the quality of an 211At-conjugated antibody.

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suppressed by quenching ROS by addition of sodium ascorbate (SA), a safe (or non-cytotoxic) reducing agent.

■ RESULTS AND DISCUSSION

Evaluation of the Effects of SA Concentration on the Stability of $^{211}$At-labeled Trastuzumab by SDS–PAGE, Autoradiography, and Flow Cytometry Assay. $^{211}$At-labeled trastuzumab was prepared according to the previously described procedures.13,25,26 The effects of different concentrations of SA on the stability of $^{211}$At-labeled trastuzumab were evaluated using SDS–PAGE, and autoradiography was performed on the day of $^{211}$At labeling and on the following day (Figure 1). Even on the day of $^{211}$At labeling, $^{211}$At-labeled trastuzumab was slightly disrupted in the absence of SA (Figure 1a,b). On the following day, $^{211}$At-labeled trastuzumab was completely disrupted in the presence of less than $6 \times 10^{-4}$ mg/mL SA (Figure 1c,d). These results indicate that the astatinated antibodies were disrupted in a time-dependent manner. In contrast, $^{211}$At-labeled trastuzumab in the presence of more than $6 \times 10^{-2}$ mg/mL SA was still stable on the following day, thus indicating the concentration-dependent protective effects of SA.27

The binding activity (binding activity = $\text{MI} - \text{MINC}$) of $^{211}$At-labeled trastuzumab to high (SK-BR-3) and low (MCF-7) human epidermal growth factor receptor 2 (HER2)-expressing cell lines was investigated using flow cytometry, where MI is defined as median intensities of samples incubated with trastuzumab, Sn-trastuzumab, or $^{211}$At-trastuzumab and MINC is median intensities of negative control, which is the sample incubated with only the secondary antibody. The flow cytometry analysis was performed 6 d after $^{211}$At labeling.
tuzumab), and astatinated trastuzumab ($^{211}$At-trastuzumab) in phosphate-buffered saline (PBS) for SK-BR-3 cells were 2.01 × $10^5$, 1.98 × $10^5$, and 3.91 × $10^5$, respectively (Figure 2). Binding activities of $^{211}$At-trastuzumab in 6 × $10^{-4}$, 6 × $10^{-5}$, and 6 mg/mL SA to SK-BR-3 cells were 1.29 × $10^5$, 1.33 × $10^5$, and 1.59 × $10^5$, respectively. In the presence of more than 6 × $10^{-3}$ mg/mL SA, the binding activity of $^{211}$At-trastuzumab was maintained. The binding affinities of trastuzumab and functionalized trastuzumab to MCF-7 cells were weak because of their low HER2 expression levels. 

**In Vitro Cytotoxicity Evaluation of $^{211}$At-Labeled Trastuzumab.** The cytotoxicity of $^{211}$At-labeled trastuzumab was evaluated using the WST-8 assay. The cytotoxic effects of $^{211}$At-labeled trastuzumab on cancer cells depended on HER2 expression levels. Astatinated trastuzumab killed SK-BR-3 cells more efficiently than free $^{211}$At. However, the differences in the cytoidal effects on MCF-7 cells between free $^{211}$At and the immunoconjugates were minor. Based on the protective effects on the binding activities of $^{211}$At-trastuzumab, SA contributed to the cytoidal effects of the immunoconjugates in a dose-dependent manner (Figure 3). $^{211}$At-trastuzumab in 6 × $10^{-2}$ and 6 mg/mL SA exerted greater cytoidal effects on SK-BR-3 cells than $^{211}$At-trastuzumab in PBS and 6 × $10^{-4}$ mg/mL SA.

**Detection of ROS Using Luminol Assay.** We speculated that antibody damage was caused by ROS generated through the interaction of water molecules and $\alpha$-particles emitted from $^{211}$At. ROS can be detected using chemiluminescent luminol assay. The luminol assay can measure the global levels of ROS, such as $H_2O_2$, $O_2^-$, and $OH^-$, with high sensitivity under physiological conditions and can be sensitized by the addition of horseradish peroxidase. We speculated that antibody damage was caused by ROS generated through the interaction of water molecules and $\alpha$-particles emitted from $^{211}$At. ROS can be detected using chemiluminescent luminol assay. The luminol assay can measure the global levels of ROS, such as $H_2O_2$, $O_2^-$, and $OH^-$, with high sensitivity under physiological conditions and can be sensitized by the addition of horseradish peroxidase.

**Scope of Reducing Agents.** The scope of reducing agents, in addition to SA, potentially applicable for reducing ROS levels was investigated. We used agents with other mechanisms of reduction, namely, l-cysteine, sodium hydrosulfite, and maltose. The reducing ability of l-cysteine and sodium hydrosulfite is because of the reduct potential of the sulfur atom, whereas that of maltose is inherent in its hemiacetal structure. The concentrations of these reducing agents were set to 6 × $10^{-2}$ mg/mL based on the SA threshold. We performed a luminol assay to assess the ROS-quenching abilities of various reducing agents in solutions of free $^{211}$At and $^{211}$At-labeled trastuzumab (Figure 5). The order of the reducing abilities of the agents in the $^{211}$At-labeled trastuzumab solution was SA > l-cysteine ≥ sodium hydrosulfite > maltose (Figure 5b). We found that 6 × $10^{-2}$ mg/mL l-cysteine did not completely quench ROS in the $^{211}$At-labeled antibody solution, although it efficiently quenched ROS in the solution of free $^{211}$At (Figure 5a). At present, the reason for the difference in ROS concentration in the solutions of free $^{211}$At and $^{211}$At-labeled antibody in the presence of 6 × $10^{-2}$ mg/mL l-cysteine is unclear.

SDS–PAGE revealed that maltose or sodium hydrosulfite could not adequately protect the astatinated antibodies (Figure 6). However, SA and l-cysteine protected the immunoconjugates from oxidative stress. These results are in agreement with the ROS concentration measured by luminol assay. Accordingly, $^{211}$At-labeled trastuzumab solution containing l-cysteine or SA displayed high binding activity to SK-BR-3 and MCF-7 cells (Figure 7), depending on the surface HER2 expression levels, and these results were comparable to those obtained for the naked and linker-attached trastuzumab before astatination. However, compared with the $^{211}$At-labeled trastuzumab protected with SA or l-cysteine, the astatinated antibodies with maltose or sodium hydrosulfite did not retain their binding activity. The binding activities of trastuzumab and Sn-trastuzumab to SK-BR-3 cells were 1.50 × $10^5$ and 1.42 × $10^5$, respectively. Binding of $^{211}$At-trastuzumab in PBS, $^{211}$At-trastuzumab in 6 × $10^{-2}$ mg/mL sodium hydrosulfite, and $^{211}$At-trastuzumab in 6 × $10^{-2}$ mg/mL maltose to SK-BR-3 cells became weak, with activities of 1.84 × $10^5$, 7.77 × $10^4$, and 7.57 × $10^4$, respectively. $^{211}$At-trastuzumab in 6 × $10^{-2}$ mg/mL SA and $^{211}$At-trastuzumab in 6 × $10^{-2}$ mg/mL l-cysteine retained binding activities of 6.61 × $10^4$ and 7.77 × $10^4$, respectively. In MCF-7 cells, the binding activities of trastuzumab, Sn-trastuzumab, $^{211}$At-trastuzumab in PBS, $^{211}$At-trastuzumab in 6 × $10^{-2}$ mg/mL l-cysteine, $^{211}$At-trastuzumab in 6 × $10^{-2}$ mg/mL sodium...
hydrosulfi te, and $^{211}$At-trastuzumab in $6 \times 10^{-2}$ mg/mL maltose were $8.11 \times 10^3$, $7.87 \times 10^3$, $2.96 \times 10^3$, $6.78 \times 10^3$, $7.28 \times 10^3$, $1.33 \times 10^2$, and $1.08 \times 10^3$, respectively. Regarding cytotoxicity, $^{211}$At-labeled trastuzumab with 1-cysteine had the same potency as $^{211}$At-labeled trastuzumab with SA, and the astatinated antibodies with these protectants exerted greater cytotoxic effects than the immunoconjugate without the protectant (Figure 7). However, the astatinated antibodies with maltose or sodium hydrosulfi te displayed antitumor activities that were less than or similar to those of the immunoconjugate without the protectant.

Figure 4. Detection of ROS using luminol assay. (a) SA was added at different concentrations to $^{211}$At in PBS. The lower (boxed) graph is an expansion of the 6 mg/mL SA addition protocol. (b) SA was added at different concentrations to $^{211}$At-labeled trastuzumab in PBS. The lower (boxed) graphs are expansions of $6 \times 10^{-2}$ and 6 mg/mL SA addition protocols. RLU = relative luminescence units. Data are presented as mean ± SD values.
CONCLUSIONS

In this study, we longitudinally investigated the quality of conjugates after labeling of antibodies with $^{211}$At, a promising $\alpha$-emitter applicable for targeted alpha therapy. Our results indicate that the radioimmunoconjugates were severely degraded within 1 day of labeling with $^{211}$At. Although these devastating effects of $\alpha$-particle emitters on macromolecules, such as proteins, have been reported previously, the mechanism underlying the destruction of macromolecules including antibodies in vivo has not been clarified as of now. Here, we...
particularly focused on the high LET of $^{211}\text{At}$. High-LET particles can strongly induce radiolysis of exposed materials. When the water is irradiated, numerous types of radicals, primarily ROS in water solutions, are produced. In this study, using luminol assay, SDS-PAGE, flow cytometry, and cytotoxicity assays, we clearly show that $^{211}\text{At}$-labeled trastuzumab was degraded by ROS generated from the radiolysis of water.

The mechanism underlying antibody denaturation upon $^{211}\text{At}$ labeling provides insights into the protection against damage caused by radioactive conjugated antibodies. Certain reducing agents or radial scavengers can suppress ROS upon $^{211}\text{At}$ conjugation.

Our assays were performed 4–6 days after $^{211}\text{At}$ conjugation, with and without the reducing agents. $^{211}\text{At}$-conjugated trastuzumab was stable in the presence of SA for several days. SDS-PAGE, flow cytometry, and cytotoxicity assays revealed that the minimum concentration of SA for protection of $^{211}\text{At}$-labeled trastuzumab is $6 \times 10^{-2}$ mg/mL. Protected $^{211}\text{At}$-labeled trastuzumab maintained its binding activity and potent antitumor effects on antigen-expressing cells. Although numerous $^{211}\text{At}$-labeled antibodies have been reported so far, the stability of $^{211}\text{At}$-labeled immunoglobulin G has remained largely unclear, except in studies using large quantities of $^{211}\text{At}$-conjugated samples. Overall, our results clearly indicate that protection from oxidative stress is required for $^{211}\text{At}$ immunoconjugation.

The stability and biodistribution of $^{211}\text{At}$-labeled antibodies are important considerations for $\alpha$-particle conjugation; hence, the structure of the conjugated antibody needs to be maintained, as observed upon the addition of SA. In the presence of $6 \times 10^{-2}$ mg/mL SA, the $^{211}\text{At}$-labeled antibody was stable for several days.

The selection of reducing agents is important in RIT using $^{211}\text{At}$-labeled antibodies. In this case, reducing agents should have an efficient ROS-quenching ability and less toxicity. The results of our experiments indicate that SA and L-cysteine are good candidates for use as reducing agents. Because SA is frequently added as a stabilizing agent in clinically approved formulations, SA addition is highly practicable. The toxicity of reducing agents is also important when clinical application is considered. LD$_{50}$ (rat) of SA is 11,900 mg/kg, whereas that of L-cysteine is 1890 mg/kg. Considering that the LD$_{50}$ of SA is
extremely high, SA is currently the most effective and safest candidate as a ROS scavenger in the view of toxicity as well.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00684.

General Procedure, 211At generation, preparation of N-[2-(maleimido)ethyl]-3-(trimethylstannyl)benzamide-conjugated trastuzumab, 211At labeling of trastuzumab by Sn−211At exchange reaction, detection of ROS using luminol assay, SDS–PAGE, analysis of 211At-labeled antibodies, flow cytometry analysis, in vitro cytotoxicity assay, and 1H NMR spectrum of N-[2-(maleimido)ethyl]-3-(trimethylstannyl)benzamide (PDF)

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Figure 7. Cytotoxic effects of 211At-labeled trastuzumab in breast cancer cell lines. The cytotoxic effects of 211At-labeled trastuzumab in the presence of reducing agents, SA and L-cysteine, and on breast cancer cell lines with different expression levels of human epidermal growth factor receptor 2 (HER2) were determined using the WST-8 assay. SK-BR-3: high HER2 expression; MCF-7: low HER2 expression. SA: sodium ascorbate; Cys: L-cysteine; SHS: sodium hydrosulfitel; Mal: maltose. N = 4. Data are presented as mean ± SD values.
Author Contributions
S.M. and Y.M. conceived and designed the project. Y.K. and R.T. conjugated N-[2-(maleimido)ethyl]-3-(trimethylstannyl)benzamide to trastuzumab. H.T., T.Y., H.H, and S.M. carried out $^{211}$At labeling. H.T., K.O., and N.I. performed TLC analysis. K.O. measured ROS by the luminol assay. H.T., K.O., R.T., and N.I. evaluated the astatinated antibodies by SDS-PAGE. H.T. performed flow cytometry. H.T. and Y.K. evaluated cytotoxicity of the conjugated antibodies. T.Y., K.Y., W.Y., D.M., and H.H. prepared $^{211}$At and measured radioactivity. H.F., M.Y., and Y.M. supervised the project. S.M., H.T., and K.O. wrote the major part of the paper. All authors analyzed and discussed results and assisted in paper preparation.

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Notes
The authors declare no competing financial interest.

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Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| cGMP | current Good Manufacturing Practices |
| LET | linear energy transfer |
| PBS | phosphate-buffered saline |
| RIT | radioimmunotherapy |
| SA | sodium ascorbate |
| SDS-PAGE | sodium dodecyl sulfate—polyacrylamide gel electrophoresis |
| SHS | sodium hydrosulfite |

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