RON Receptor Tyrosine Kinase as a Therapeutic Target for Eradication of Triple-Negative Breast Cancer: Efficacy of Anti-RON ADC Zt/g4-MMAE

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Abstract

Triple-negative breast cancer (TNBC) is a highly diverse group of malignant neoplasia with poor outcome. Currently, the lack of effective therapy has fostered a major effort to discover new targets to treat this malignant cancer. Here we identified the RON receptor tyrosine kinase as a therapeutic target for potential TNBC treatment. We analyzed RON expression in 168 primary TNBC samples via tissue microarray using anti-RON IHC staining and demonstrated that RON was widely expressed in 76.8% TNBC samples with overexpression in 76 cases (45.2%). These results provide the molecular basis to target RON for TNBC therapy. To this end, anti-RON monoclonal antibody Zt/g4-drug monomethyl auristatin E conjugate (Zt/g4-MMAE) was developed with a drug to antibody ratio of 3.29 and tested in a panel of TNBC cell lines with different phenotypes. In vitro, Zt/g4-MMAE rapidly induced RON internalization, resulted in cell-cycle arrest followed by massive cell death. The calculated IC50 values ranged from 0.06 to 3.46 μg/mL dependent on individual TNBC cell lines tested. Zt/g4-MMAE also effectively killed TNBC stem-like cells with RON+/CD105+/CD44+ phenotypes and RON-negative TNBC cells through the bystander effect. In vivo, Zt/g4-MMAE at 10 mg/kg in a Q12 × 2 regimen completely eradicated TNBC xenografts without the regrowth of xenograft tumors. In conclusion, increased RON expression is a pathogenic feature in primary TNBC samples. Zt/g4-MMAE is highly effective in eradicating TNBC xenografts in preclinical models. These findings lay the foundation for using anti-RON Zt/g4-MMAE in clinical trials as a novel strategy for TNBC treatment. Mol Cancer Ther; 17(12): 2654–64. ©2018 AACR.

Introduction

Triple-negative breast cancer (TNBC), defined by the absence of the estrogen receptor (ER), progesterone receptor (PR), and HER2, belongs to a highly malignant subgroup of breast cancer with poor prognosis and high mortality (1). Genetic and cellular profiling has revealed that TNBC is heterogeneous with different phenotypes including two basal-like, one immunomodulatory, one mesenchymal, one mesenchymal stem-like, and one luminal androgen receptor subtype (1, 2). These phenotypes constitute the molecular basis for various TNBC malignant behaviors, which are manifested through alterations at genetic and cellular levels (1–2). A major clinical challenge in managing TNBC is the lack of effective therapies (1–3). Currently, chemotherapy is the primary established treatment for patients with TNBC in both early and advanced-stages of the disease (1–3). However, the efficacy of chemotherapy is very limited, which leads to poor survival outcomes (1, 2). Targeted therapeutic agents have recently been applied to treat patients with advanced TNBC. The identified targets include androgen receptor, phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), trophoblast antigen-2, programmed cell death-1, programmed cell death ligand-1 receptors, and others (3, 4). In addition, inhibition of PARP is appealing in TNBC patients with a BRCA mutant phenotype (5, 6). Nevertheless, initial observations from different stages of clinical trials indicate that these targeted therapies exert only moderate efficacy against TNBC (1–6). In light of these observations, it is clear that additional exploration is necessary to understand the TNBC biology and to identify new drug targets for effective TNBC therapy.

The RON receptor tyrosine kinase, a member of the MET proto-oncogene family (7), has been implicated in breast cancer malignancy and chemoresistance (8–14). Studies in murine models have revealed that aberrant RON expression is critical for breast epithelial cell transformation, tumorigenesis, and distance metastasis (8). Clinically, increased RON expression has been found in more than 50% of primary breast cancer samples (15–18). RON overexpression also is an independent predictor for breast cancer...
distant relapse and disease-free survival (16). Molecularly, aberrant RON expression results in constitutive kinase phosphorylation, which activates various intracellular signaling cascades including the β-catenin pathway. This leads to increased breast cancer cell proliferation, migration, invasion, stemness, and drug resistance (8–14). In addition, aberrant RON expression has been validated as a therapeutic target for breast cancer therapy (18–22). Various therapeutics targeting RON including small molecule kinase inhibitors BMS777607, PHA665752, and therapeutic antibodies such as Zt/g4, 29B806, and nanatumab have been developed and undergoing preclinical and clinical evaluation (23, 24). Preliminary results indicate that suppressing RON signaling or targeting RON for drug delivery has the therapeutic potential for breast cancer treatment (18). Recently, a novel anti-RON antibody–drug conjugates (ADC) with significantly improved therapeutic index has been developed and used in preclinical studies (21, 25, 26), which open a new avenue for RON-targeted therapy of breast cancer.

This study was to determine RON expression in primary TNBC samples and to evaluate the efficacy of a novel anti-RON ADC for targeted TNBC therapy. The objective was to establish proof-of-concept that anti-RON ADC has the therapeutic value for TNBC therapy. As described above, the pathogenesis of RON in breast cancer has been extensively studied. However, the role of RON in TNBC tumorigenesis and therapy has not been investigated. Currently, knowledge about aberrant RON expression and signaling in TNBC are unknown. Moreover, inhibition of RON signaling as a therapeutic potential for TNBC has not been thoroughly investigated. Considering these facts, we reason that understanding RON expression in TNBS could help dissect the molecular mechanisms underlying TNBC malignancy and find a new strategy for TNBC treatment.

Materials and Methods

TNBC microarrays and IHC staining

TNBC tissue microarrays containing 168 samples with certain clinical information were from US Biomax, Inc. All samples were negative for ER, PR, and HER2 expression (www.biomax.us). The IHC staining of RON was performed using a mouse anti-RON mAb Zt/f2 followed by DAKO EnVision System as previously described (27). The intensity of RON-mAb Zt/f2 followed by DAKO EnVision System was determined as negative, weak, moderate, or strong as previously described (27). The intensity of RON-mAb Zt/f2 followed by DAKO EnVision System as previously described (27). The intensity of RON-mAb Zt/f2 followed by DAKO EnVision System was determined as negative, weak, moderate, or strong as previously described (27). Two pathologists blinded to clinical information independently scored RON positivity in each sample. The combined scores that were determined as previously described (27). The intensity of RON-mAb Zt/f2 followed by DAKO EnVision System was determined as negative, weak, moderate, or strong as previously described (27). Two pathologists blinded to clinical information independently scored RON positivity in each sample. The combined scores that were determined as previously described (27). Two pathologists blinded to clinical information independently scored RON positivity in each sample. The combined scores that were determined as previously described (27).

Assays for Zt/g4-MMAE-mediated cell surface RON internalization

Zt/g4-MMAE-mediated RON internalization by TNBC cells was determined as previously described (26). Residual RON remained on cell surface after Zt/g4 treatment was detected by the immunofluorescence assay using anti-RON mAb Zt/g4 (26). A time required to achieve 50% reduction of cell surface RON (internalization efficiency, IE50) was calculated for individual cell lines. Detection of intracellular RON was performed using immunofluorescence staining as previously described (26). Nuclear DNAs were stained with 4',6-diamidino-2-phenylindole (DAPI). Lysosomal-associated membrane protein (LAMP)-1 was detected by goat anti-mouse IgG coupled with Alexa Fluor-647. Cellular immunofluorescence was observed under an Olympus microscope equipped with DUS/fluorescent apparatus.

Analysis for Zt/g4-MMAE-mediated changes in cell cycle, viability, death, and colony formation

Individual TNBC cell lines (1 × 10^6 cells per dish) were treated with different amounts of Zt/g4-MMAE for various times. Measurement of cell-cycle changes was performed by labeling cells with propidium iodide and analyzed by measuring DNA contents as previously described (21). Cell viability 96 hours after Zt/g4-MMAE treatment was determined by the MTS assay (26). Levels of dead cells were determined by using the trypan blue exclusion assay (26). TNBC cell colony formation and Zt/g4-MMAE treatment was performed as previously described (20).
Western blot detection of RON proteins and cellular apoptotic markers

TNBC cellular proteins (20 μg per sample) were separated in a 12% SDS-PAGE under reduced conditions. RON and its phosphorylated proteins were detected using rabbit anti-RON IgG antibody or phospho-anti-RON antibody as previously described (26). Cyclin B1 and PARP fragment were detected in Western blotting using specific antibodies, respectively. Membranes were reprobed with anti-actin antibody to ensure equal sample loading.

Assay of cellular caspase-3 and -7 activities for cellular apoptosis

TNBC HCC1937, HCC2185, MDA-MB-468, and SUM52PE cell lines (10,000 cells per well in a 96-well plate in triplicate) were treated with 5 μg/mL Zt/g4-MMAE for 48 hours. Cells treated with control ADC CmIgG MMAE served as the control. Activities of caspase-3 and -7 were measured using the Caspase-Glo 3/7 assay system according to the manufacturer’s instruction (www.promega.com). Results were expressed as fold induction of control (caspase-3/7 activities from control cells were set as 0).

Assays for Zt/g4-MMAE-mediated bystander killing of TNBC cells

HCC1806 cells were labeled with CellTrace CFSE (Thermo Fisher Scientific) according to manufacturer’s instructions. Labeled cells were then mixed at 1:1 ratio with RON-positive HCC1937, MDA-MB231, or SUM52PE cells and incubated at 1 × 10^5 cells per well in a six-well plate in duplicate. After treatment with 1 μg/mL CmIgG-MMAE or Zt/g4-MMAE for 96 hours, cells were collected and labeled with 7-AAD (Thermo Fisher Scientific) to identify dead cells. Fluorescence intensities were determined using the BD C6 Accuri flow cytometer. The presence of CFSE-labeled HCC1806 cells facilitate the detection and gating between the RON-negative HCC1806 cells and the RON-positive unlabeled TNBC cells. Dead cells were identified by gating cells according to 7-AAD staining. The percentage of viable cells was calculated on the basis of the total cell population. Viable cells plus dead cells are set as 100%.

Isolation of breast stem-like CD44+/CD24− TNBC cells for cytotoxic study

Existence of CD44+/CD24− cells in seven TNBC cell lines were evaluated by flow cytometric analysis using FITC-conjugated anti-CD44 and PE-conjugated anti-CD24 mAbs (BD Biosciences), respectively. MDA-MB231, MDA-MB468, and SUM52PE cells with a relatively high population of CD44+/CD24− cells were selected for isolation TNBC stem-like cells using a magnetic cell-sorting method as previously described (30). Briefly, TNBC cells (5 × 10^5 cells/mL) were incubated with anti-CD24 mAb followed by anti-mouse IgG dynal magnetic beads (Thermo Fisher Scientific) to eliminate CD24− cells in the cell population. Cells were then incubated with anti-CD44 mAb and goat anti-mouse IgG dynal beads to obtain CD44+ cells. Cells with RON+/CD44+/CD24− phenotypes (designated as TNBCSLC) were confirmed by flow cytometric analysis.

TNBC xenograft in mouse models and Zt/g4-MMAE treatment

All experiments on mice were approved by the institutional animal care committee. Female athymic nude mice at 6 weeks of age (Taconic) were injected with 5 × 10^6 cancer cells in the subcutaneous space of the right flank and randomized into different groups (five mice per group). Treatment began when tumors reaches a mean tumor volume of ~100 to 150 mm^3. Zt/g4-MMAE was injected through the tail vein at 10 mg/kg in a Q12 × 2 schedule. Tumor volumes were measured every 4 days and calculated as previously described (26). Mice were monitored for tumor growth up to 68 days. Animals were euthanized when tumor volumes exceeded 2,000 mm^3 or if tumors became necrotic or ulcerated through the skin.

Statistical analysis

GraphPad Prism 7 software was used for statistical analysis. Results are shown as mean ± SD. The data between control and treatment groups were compared using Student t test. Statistical differences at P < 0.05 were considered significant.

Results

RON expression profile in human primary TNBC samples

Clinical features of 168 TNBC samples were summarized as follows: The age of patients ranged from 30 to 79 years old with an average of 51.37 ± 10.96 years old. One hundred fifty-one cases were confirmed as invasive ductal carcinoma. Ten cases were invasive lobular carcinoma. The remaining seven cases were medullary carcinoma. Majority of the patients (134 cases, 79.76%) had clinical stage II tumors. Twenty-six cases (15.48%) were at stage III and eight cases at stage I. Tumor size distributions were: T1, 8 cases; T2, 115 cases; T3, 21 cases; and T4, 24 cases. Tumor differentiation status included: grade 1, 7 cases; grade 2, 97 cases; and grade 3, 38 cases. The grade for remaining 26 cases was unknown. Lymph node involvement was observed in 44 cases (26.19%). Distance metastasis was confirmed in only one patient (0.59%).

Immunoreactivity of RON by Zt/f2 IHC staining with different intensities is shown in Fig. 1A. The patterns of RON expression from individual TNBC samples were different with heterogeneous and homogeneous staining appearances (Fig. 1B), which are similar to the IHC staining patterns of RON in other epithelial cancers (16, 17). In addition, analysis of IHC staining identified RON residing in different cellular compartments including: predominant membrane localization, predominant cytoplasmic localization, and mixed membrane and cytoplasmic localization (Fig. 1B). Such appearances probably indicate the maturation process of RON moving from intracellular compartment to cell surface. Particularly, it is the reflection of differential RON expression with intrinsic heterogeneous nature of individual TNBC cells.

Information about RON expression in TNBC samples are summarized in Supplementary Table S1. A total of 129 samples expressed RON (76.79%) with variable intensities and patterns. The remaining 39 cases were RON negative (23.21%). Among RON positive samples examined, 38 cases showed low level expression (combined score: 1–2, 22.62%); 15 cases moderate expression (combined scores: 3–4, 8.93%); and 76 cases overexpression (combined scores: 5–6, 45.24%). Because of incomplete pathologic and clinical information, RON expression in correlation with pathologic parameters was not performed. Nevertheless, the IHC staining confirmed that RON was broadly expressed in primary TNBC samples. The fact that RON was overexpressed in ~45% cases suggests that aberrant RON...
expression is a TNBC pathogenic feature, which renders it a suitable therapeutic target.

Generation and characterization of Zt/g4-MMAE

To target RON for TNBC treatment, we conjugated Zt/g4 with MMAE through cathepsin B-sensitive dipeptide linker to generate Zt/g4-MMAE (Fig. 2). The conjugation achieved the average DAR of 3.29 as confirmed by HIC analysis. The percentages of conjugates with different DARs from the integrated areas of the conjugates are shown in Supplementary Fig. S1A. Major peaks included peak 2 (33.02%) with a DAR of 2:1 and peak 4 (46.16%) with a DAR of 4:1. Zt/g4-MMAE with DARs at 2:1, 4:1, and 6:1 accounted for 87.07% of the total conjugates. DARs for CmIgG-MMAE were 3.54.

We determined the stability of Zt/g4-MMAE in PBS, which reflects the storage condition. Zt/g4-MMAE appeared to be stable at 37°C in PBS as evident by HIC analysis (Supplementary Fig. S1B). On day 28, Zt/g4-MMAE had an average DAR of 3.06, which represented only ~7.0% reduction from DAR of 3.29 on day 0. The major changes appeared to be peak 4 and 6. From day 0 to day 28, peak 4 increased from 46.16% to 55.32% and P6 decreased from 7.89% to 0.00%. These results suggest that Zt/g4-MMAE is stable in storage condition in PBS.

Induction by Zt/g4-MMAE of cell surface RON internalization

Pathologic features of several TNBC cell lines are summarized in Supplementary Table S2. Levels of RON expression were determined by flow cytometric analysis using Zt/g4 as the primary antibody (Fig. 3A). T-47D cells expressing 15,756 ± 314 RON molecules per cell were used as the control. Specific binding was not observed in BT-20 or HCC1806 cells (<50 RON molecules per cell), which served as the negative control. Zt/g4-MMAE treatment caused a progressive reduction of cell surface RON in a time-dependent manner in RON-positive TNBC cell lines tested (Fig. 3B). Less than 30% of RON remained on the cell surface after a 36-hour treatment. The calculated IC50 values were 10.21 hours for HCC1937, 10.25 hours for HCC2185, 10.88 hours for MDA-MB231, 22.86 hours for MDA-MB468, and 19.86 hours SUM52PE cells, respectively.

Cellular immunofluorescence analysis confirmed Zt/g4-MMAE-induced RON internalization in three TNBC cell lines tested (Fig. 3C). RON was detected on the cell surface at 4°C. Internalization occurred at 37°C after Zt/g4-MMAE treatment. Moreover, cytoplasmic RON was colocalized with LAMP-1 in TNBC cells. Thus, results presented in Fig. 3 demonstrate that Zt/g4-MMAE is highly effective in induction of RON internalization by RON-expressing TNBC cells.
Effect of Zt/g4-MMAE in vitro on TNBC cell cycle, growth, death, and colony formation

We first determined sensitivities of individual TNBC cell lines to free MMAE using the MTS assay. TNBC cells were sensitive to free MMAE regardless levels of RON expression (Supplementary Table S2). We then studied the effect of Zt/g4-MMAE on cell cycles (Fig. 4A). Changes in cell cycle were observed as early as 6 hours after addition of Zt/g4-MMAE in RON-positive TNBC cell lines with a significant reduction in G0–G1 phase, a decrease in S phase, and a dramatic increase in G2–M phase. RON-negative HCC1806 cells did not respond to Zt/g4-MMAE. These results indicate that Zt/g4-targeted delivery of MMAE has a profound effect on cell cycles of RON-expressing TNBC cells.

The effect of Zt/g4-MMAE on TNBC cell viability is shown in Fig. 4B. A significant reduction in cell viability was observed in a Zt/g4-MMAE dose-dependent manner in all RON-positive TNBC cell lines tested. The IC50 values were 1.42 μg/mL for HCC1937, 0.06 μg/mL for HCC2185, 3.46 μg/mL for MDA-MB231, 0.98 μg/mL for MDA-MB468, and 1.26 μg/mL for SUM52PE cells, respectively. BT-20 and HCC1806 cells were insensitive to Zt/g4-MMAE. The kinetic effect of Zt/g4-MMAE caused a time-dependent reduction in cell viability among TNBC cell lines tested. Thus, Zt/g4-MMAE reduces cell viability in both dose- and time-dependent manners in RON-positive TNBC cells.

Observation of cell morphology under a microscope indicated massive cell death after Zt/g4-MMAE treatment (Supplementary Fig. S2B). Dose-dependent cell death was documented among TNBC cell lines tested after a 96-hour treatment (Fig. 4C). The calculated IC50 values were 1.81 μg/mL for MDA-MB468, 3.92 μg/mL for HCC1937, 0.15 μg/mL for HCC2185, and 3.64 μg/mL for SUM52PE cells, respectively. Flow cytometric analysis confirmed increased apoptotic cell death in RON-expressing TNBC cell lines tested (Fig. 4D). Moreover, we performed Western blot analysis to determine Zt/g4-MMAE-caused cyclin B1 expression and proteolytic activation of PARP (Fig. 4E and F), both served as indicators for cellular apoptosis (31). Western blot analysis indicated spontaneous RON activation as evident by the detection of phosphorylated RON in six cell lines tested (Supplementary Fig. S3). Increased cyclin B1 expression and fragments of activated PARP were evident after Zt/g4-MMAE treatment. Increased caspase-3 and -7 activities in Zt/g4-MMAE-treated TNBC cell lines also were documented (Fig. 4G). These observations provide the evidence of apoptotic cell death caused by Zt/g4-MMAE. Finally, we observed the inhibitory effect of Zt/g4-MMAE on TNBC cell colony formation (Fig. 4H). Zt/g4-MMAE at 1 μg/mL dramatically inhibited the colony formation by three RON-expressing TNBC cell lines. Thus, results shown in Fig. 4 demonstrate that Zt/g4-MMAE is capable of arresting cell cycles, decreasing cell viability, reducing viable cell numbers, inhibiting clonogenic growth, and inducing massive apoptotic cell death.

Cytotoxic effect of Zt/g4-MMAE on bystander TNBC cells and stem-like TNBC cells

To explore the possibility of Zt/g4-MMAE in killing RON-negative bystander TNBC cells, we cocultured RON-negative HCC1806 cells with one of the following RON-positive TNBC cells, HCC1937 (RON 3+), MDA-MB231 (RON 1+), or SUM52PE cells (RON 2+) in the presence of CmIgG-MMAE, Zt/g4-DM1, or Zt/g4-MMAE. The reason we include Zt/g4-DM1 formed with noncleavable linker was to determine if Zt/g4-MMAE with the cleavable linker was superior in killing bystander cancer cells. As shown in Fig. 5A, viable HCC1806 cells remained at high levels in the presence of CmIgG-MMAE but decrease significantly in response to Zt/g4-MMAE. In contrast, high levels of viable HCC1806 cells were observed in the presence of Zt/g4-DM1. Similar results were observed when HCC1806 cells were cocultured with SUM52PE and HCC1937 cells. We further confirmed by using the MTS assay that HCC1806 cells were highly sensitive to Zt/g4-MMAE cytotoxicity but resistant to Zt/g4-DM1 action when they were cocultured with MDA-MB231, SUM52PE, or HCC1937 (Fig. 5B). In these cases, levels of HCC1806 cell...
survival were significantly reduced in the presence of Zt/g4-MMAE but not Zt/g4-DM1. These results indicate that Zt/g4-MMAE formed with the cleavable linker is superior to Zt/g4-DM1 with noncleavable linker due to the reduction of viability of bystander RON-negative TNBC cells.

Another interesting finding was that the survival levels of HCC1806 cells were significantly lower in the presence of TNBC cells expressed high levels of RON (SUM52PE or HCC1937 cells) than those in the presence of TNBC cells expressed low levels of RON (MDA-MB231, Fig. 5A and B). The killing of bystander cells

Figure 3. Expression of RON and its internalization by TNBC cells: A, Individual TNBC cell lines (1 x 10^6 cells/mL) in 1 mL PBS in duplicate were incubated at 4°C with 5.0 μg Zt/g4 (red line) for 60 minutes. Isotope-matched mouse IgG (black line) was used as the control. Cell surface RON was quantitatively determined by immunofluorescence analysis using QIFKIT (DAKO) as previously described (17). B, Zt/g4-induced internalization of cell surface RON was determined using anti-RON mAb Zt/c1 immunofluorescence analysis as previously described (21). Immunofluorescence from cells treated with Zt/c1 at 4°C was set as 100%. Among six RON-positive cell lines tested, the percentages of RON remaining on the cell surface at 24 and 36 hours were significantly lower than those at 0 hour (P < 0.05). C, Detection of internalized RON using the immunofluorescence staining method was performed as previously described (21). Nuclear DNAs were stained with DAPI. LAMP-1 was used as a marker for protein cytoplasmic localization. Enlarged images were from the areas indicated with yellow arrows. Scale bar: 10 μmol/L.
was more prominent in the presence of TNBC cells overexpressed RON after treatment of Zt/g4-MMAE. These data suggest that Zt/g4-MMAE is capable of killing the RON-negative bystander TNBC cells and its effectiveness is associated with levels of RON expressed by TNBC cells.

Next, we studied the effect of Zt/g4-MMAE in reducing viability of TNBC SLC cells with CD44\(^+\)/CD22\(^−\)/CD0\(^−\) phenotypes. Breast cancer with CD44\(^+\)/CD22\(^−\)/CD0\(^−\) phenotypes are known to be tumor-initiating stem-like cells (32, 33). We isolated RON\(^+\)/CD44\(^+\)/CD24\(^−\)/CD0\(^−\) TNBC SLC cells from MDA-MB231, MDA-MB468, and SUM52PE cell lines expressed different levels of RON. The flow cytometric analysis confirmed RON coexpression with CD44/CD24 TNBC SLC cells (Supplementary Fig. S4). All three TNBC SLC populations displayed an increase

Figure 4.
Effect of Zt/g4-MMAE on TNBC cell-cycle change, viability, death, and colony formation. A, Cell-cycle changes caused by Zt/g4-MMAE were conducted by flow cytometric analysis after labeling with propidium iodide as previously described (21). RON-negative HCC1806 cells were used as the control. Changes in cell cycles were marked with arrows. B, Cell viability was determined by the MTS assay as previously described (20). CmIgG-MMAE served as the control. The individual IC\(_{50}\) values derived from six RON-positive cell lines treated with Zt/g4-MMAE were significantly lower than those from control cells treated with CmIgG-MMAE (P < 0.05). C, Death of TNBC cells were determined by treating cells with different amounts of Zt/g4-MMAE for 96 hours. Percentages of cell death were determined by the trypan blue exclusion method. All IC\(_{50}\) values derived from four RON-positive cell lines treated with Zt/g4-MMAE were significantly lower than those from control cells treated with CmIgG-MMAE (P < 0.05). Data shown here are derived from one of three experiments with similar results. D, Treatment of cells with Zt/g4-MMAE was performed as described in C. Cell apoptotic death was also determined by flow cytometric analysis after labeling cells with markers as detailed in the Materials and Methods. Percentages of cell death obtained from HCC1937 and SUM52PE cells treated with Zt/g4-MMAE were significantly higher than those from control cells treated with CmIgG-MMAE (P < 0.05). E and F, Western blot analysis of Zt/g4-MMAE-induced cyclin B1 expression (E) and fragmentation of PARP (F) was performed using specific antibodies as previously described (25). Same membranes were used to detect both proteins with actin as the loading control. G, Zt/g4-MMAE-mediated activation of caspase-3 and -7 in four TNBC cell lines were determined using the caspase-Glo 3/7 assay system as detailed in the Materials and Methods. Cells were treated with Zt/g4-DM1 or Zt/g4-MMAE. Caspase-3/7 activities from control TNBC cells treated with CmIgG-MMAE served as the baseline (zero-fold). Increased caspase-3/7 activities expressed as the fold induction of control were statistically significant in comparison with those from controls. H, The assays to determine the effect of Zt/g4-MMAE on TNBC cell colony formation was performed as previously described (20). After incubation of cells with Zt/g4-MMAE for 20 days, cells were fixed and stained. Colonies consisting of at least 50 cells were counted using a stereo-microscope as previously described (20). The number of colonies in controls was set as 100%. Percentages of colony in ADC-treated cells were calculated as: [(colonies from ADC treated plate × 100%)/colonies from control plate]. * Student t test with P < 0.05.
in chemoresistance in response to a panel of chemoagents when compared with their corresponding parental cell lines (Supplementary Table S3). Zt/g4-MMAE significantly decreased cell viability in a dose-dependent manner among three TNBC\textsuperscript{SLC} populations tested. The observed IC\textsubscript{50} values for cell viability were in the range of 0.9 to 2.0 µg/mL similar to those from their parental cells (Fig. 5C). These results suggest that the Zt/g4-MMAE reduces the viability of CD44\textsuperscript{+}/CD24\textsuperscript{-} RON-negative TNBC cells and RON-positive stem-like TNBC cells.

**Figure 5.**
Cytotoxic effect of Zt/g4-MMAE on RON-negative TNBC cells and RON-positive stem-like TNBC cells. **A**, The assay to determine the bystander killing effect of Zt/g4-MMAE on a panel of TNBC cell lines was detailed in the Materials and Methods. Both Zt/g4-MMAE and Zt/g4-DM1 were used for comparison. CmIgG-MMAE was used as the control. **B**, Experimental conditions were similar to those in (A). Cell survival was determined by flow cytometric analysis as detailed in the Materials and Methods. The Co-Culture set is a measurement of HCC1806 cells. **C**, The viability of TNBC stem-like cells after Zt/g4-MMAE treatment was determined by the MTS assay. Parental TNBC cells were used for comparison. The IC\textsubscript{50} values derived from three paired TNBC cell lines showed no statistical significance (P > 0.05). Data shown here are from one of three experiments with similar results.
tumor-initiating TNBC cells regardless their chemoresistant phenotype.

Therapeutic activity of Zt/g4-MMAE in TNBC xenograft tumor model

Among eight TNBC cell lines tested, HCC1806, HCC1937, and MDA-MB468 cells were able to grow in athymic nude mice and selected for therapy studies. It is noticed that some TNBC cell lines such as SUM52PE and HCC2185 were known to cause tumor growth (34). However, we were unable to reproduce these results. To test the therapeutic activity, Zt/g4-MMAE at 10 mg/kg with a Q12 × 2 schedule was used for in vivo studies. The rationale to use this treatment regimen was based on the terminal half-life (t1/2: 6.01 days) of anti-RON ADCs in mice (25). As shown in Fig. 6A, HCC1806 cell-initiated TNBC xenografts grew rapidly and were insensitive to Zt/g4-MMAE treatment. No statistical differences were observed between control and Zt/g4-MMAE treated mice. In contrast, Zt/g4-MMAE was highly effective in blocking tumor growth caused by HCC1937 and MDA-MB468 cell lines. It is worth to note that the growth rates of xenografts initiated by HCC1937 and MDA-MB468 cells were different. Nevertheless, Zt/g4-MMAE was able to completely inhibit tumor growth initiated by both cell lines. The effect of Zt/g4-MMAE in vivo was long lasting as measured by average tumor volume. After the second ADC injection at day 24, tumor growth was completely blocked for both HCC1937 and MDA-MB468 cell-derived TNBC xenografts. In both cases, the regrowth of tumors was not observed.

A comparison of average tumor weight between the control and Zt/g4-MMAE treated mice from the end of experiment are shown in Supplementary Fig. S5A and S5B. Consistent with those shown in Fig. 6A, Zt/g4-MMAE did not exhibit an inhibitory effect on the HCC1806-mediated xenograft. An exciting finding was the complete eradication by Zt/g4-MMAE of both HCC1937 and MDA-MB468-mediated TNBC xenografts. Detailed examination in the cell injection site did not find any traces of tumor residues. Thus, Zt/g4-MMAE as a single agent at 10 mg/kg in the Q12 × 2 regimen is highly effective not only in inhibition but also in eradication of TNBC xenografts.

To exclude any possibilities that reduction of tumor was related to in vivo general toxicity of Zt/g4-MMAE, we monitored animal behavior, food consumption, daily activity, and body weight in...
both control and Zt/g4-MMAE-treated mice. All mice behaved normally with regular activity and food consumption. The average body weight from mice injected with HCC1806, HCC1937, and MDA-MB468 cells were not affected by Zt/g4-MMAE treatment (Fig. 6B). Also, Zt/g4-MMAE treatment did not halt mouse growth. The average body weight of Zt/g4-MMAE treated groups was comparable to that of control mice with no statistical differences. Thus, Zt/g4-MMAE at 10 mg/kg in the Q12 × 2 schedule had no negative impact on mouse bodyweight, implying that tumor reduction was not related to the general toxicity of Zt/g4-MMAE on mice.

Discussion

The study presented here is our attempt to determine the feasibility of anti-RON ADCs for TNBC treatment. Anti-RON IHC staining confirmed RON overexpression in ~45% TNBC samples, which provide the rationale to target RON for TNBC treatment. ADCs conjugated with maytansinoid such as Zt/g4-DM1 have been previously used for RON-targeted cancer therapy (21, 25, 26). However, the efficacy of Zt/g4-DM1 in vivo is relatively weak, which lacks the tumor-eradicating activities (21, 25, 26). Thus, we produced Zt/g4-MMAE with goals to improve its stability and to increase its therapeutic index. Both in vitro and in vivo studies demonstrated that Zt/g4-MMAE effectively induced RON internalization by TNBC cells, which leads to massive apoptotic cell death. Moreover, we show that Zt/g4-MMAE was capable of killing RON-negative or low-expressive TNBC cells through the bystander effect and RON-positive TNBC stem-like cells. Results from xenograft studies in mice further confirmed that Zt/g4-MMAE at 10 mg/kg in the Q12 × 2 regimen not only inhibited but also eradicated TNBC xenografts with the long-lasting effect. This thoroughly shows that the RON receptor is a suitable target for TNBC therapy using anti-RON ADC Zt/g4-MMAE.

The IHC staining of RON expression in primary breast cancer samples has been studied with increased RON expression in the range of 30% to 80% (15–17). However, the detailed analysis of RON expression in TNBC samples has not been performed. This study was our first attempt to profile RON expression in TNBC samples, which shows that RON was expressed in ~75% of primary TNBC samples with overexpression in ~45% cases. This expression pattern is comparable to those from previous studies showing increased RON expression in breast cancer samples. Therefore, aberrant RON expression can be characterized as a pathogenic feature in this particular subtype of breast cancer. Moreover, increased RON expression renders it a suitable target for therapeutic application.

Identification of RON as the target opens a new avenue for TNBC treatment. The following evidences support this notion. First, Zt/g4 was effective in induction of RON internalization by TNBC cells. We observed that the kinetics of Zt/g4-induced RON internalization among five TNBC cell lines tested are different with IE50 values varied from 10 to 23 hours regardless their levels of RON expression. However, Zt/g4-induced RON internalization was highly effective with more than 70% of cell surface RON internalized within 36 hours. In the case of HCC2185 cells with cell surface RON molecules per cell at ~30,000, it was estimated 21,000 cell surface RON receptors internalized within 36 hours. This is equivalent to ~67,200 MMAE molecules delivered into a single cell, sufficient to cause cell-cycle arrest. Thus, Zt/g4-induced RON internalization facilitated intracellular delivery of MMAE in TNBC cells overexpressing RON.

Second, Zt/g4-MMAE had a profoundly cytotoxic impact on TNBC cells. We showed by flow cytometric analysis that Zt/g4-MMAE caused cell-cycle arrest in G1–M phase, which is a feature of MMAE that impairs microtubule dynamics. The effect was observed as early as 6 hours after the addition of Zt/g4-MMAE and characterized by progressive reduction of the G1 phase and the accumulation of cells at the G2–M phase. Moreover, we found that Zt/g4-MMAE progressively decreased cell viability. More than 80% reduction in cell viability was observed 96 hours after treatment among TNBC cell lines tested. Decreased viability was accompanied with massive cell death in a Zt/g4-MMAE dose-dependent manner. Analysis of cellular apoptotic markers confirmed Zt/g4-MMAE induced cyclin B1 expression and generation of PARP fragments. Finally, we confirmed that Zt/g4-MMAE killed RON-negative TNBC cells through the bystander effect and stem-like TNBC cells. These activities indicate that Zt/g4-MMAE is highly effective in elimination of TNBC cells.

Third, the therapeutic efficacy of Zt/g4-MMAE is confirmed in mouse TNBC xenograft models as evident in the 10 mg/kg in the Q12 × 2 schedule. This dosing-schedule regimen was designed to determine the capability of Zt/g4-MMAE at 10 mg/kg in a two terminal half-life cycle to inhibit TNBC xenograft growth. We show that Zt/g4-MMAE effectively inhibited TNBC xenograft growth mediated by HCC1937 and MDA-MB468 cells but had no effect on HCC1806 cell-mediated outgrowth. By monitoring Zt/g4-MMAE concentrations based on its terminal half-life, we confirmed that Zt/g4-MMAE displayed the long-lasting tumor-inhibitory activity up to 6 weeks without signs of tumor regrowth (from day 24 to day 68, Fig. 6A). Significantly, we demonstrated that Zt/g4-MMAE not only inhibited but also eradicated TNBC xenografts in both TNBC models tested. This effect is important since anticancer biotherapeutics often exert their activities by inhibition not by eradication. Finally, we observed that Zt/g4-MMAE at the therapeutic dose and regimen in vivo had no major toxic activities in animals, evident by the increase in average body weight in Zt/g4-MMAE treated mice. Considering these facts, it is suggested that Zt/g4-MMAE is effective and safe in the targeted treatment of TNBC xenograft tumors. Furthermore, these findings lay the foundation for transition of Zt/g4-MMAE into clinical trials in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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