Efficacy of Anti-RON Antibody Zt/g4–Drug Maytansinoid Conjugation (Anti-RON ADC) as a Novel Therapeutics for Targeted Colorectal Cancer Therapy

Liang Feng1,2, Hang-Ping Yao1, Wei Wang3, Yong-Qing Zhou4, Jianwei Zhou5, Ruiwen Zhang3, and Ming-Hai Wang1,2

Abstract

**Purpose:** The receptor tyrosine kinase RON is critical in epithelial tumorigenesis and a drug target for cancer therapy. Here, we report the development and therapeutic efficacy of a novel anti-RON antibody Zt/g4–maytansinoid (DM1) conjugates for targeted colorectal cancer (CRC) therapy.

**Experimental Design:** Zt/g4 (IgG1a/k) was conjugated to DM1 via thioether linkage to form Zt/g4–DM1 with a drug-antibody ratio of 4:1. CRC cell lines expressing different levels of RON were tested in vitro to determine Zt/g4–DM1-induced RON endocytosis, cell-cycle arrest, and cytotoxicity. Efficacy of Zt/g4–DM1 in vivo was evaluated in mouse xenograft CRC tumor model.

**Results:** Zt/g4–DM1 rapidly induced RON endocytosis, arrested cell cycle at G2–M phase, reduced cell viability, and caused massive cell death within 72 hours. In mouse xenograft CRC models, Zt/g4–DM1 at a single dose of 20 mg/kg body weight effectively delayed CRC cell-mediated tumor growth up to 20 days. In a multiple dose-ranging study with a five injection regimen, Zt/g4–DM1 inhibited more than 90% tumor growth at doses of 7, 10, and 15 mg/kg body weight. The minimal dose achieving 50% of tumor inhibition was approximately 5.0 mg/kg. The prepared Zt/g4–DM1 is stable at 37°C for up to 30 days. At 60 mg/kg, Zt/g4–DM1 had a moderate toxicity in vivo with an average of 12% reduction in mouse body weight.

**Conclusion:** Zt/g4–DM1 is highly effective in targeted inhibition of CRC cell-derived tumor growth in mouse xenograft models. This work provides the basis for development of humanized Zt/g4–DM1 for RON-targeted CRC therapy in the future. Clin Cancer Res; 20(23); 6045–58. ©2014 AACR.

Introduction

The RON receptor tyrosine kinase, a member of the MET proto-oncogene family (1, 2), has been implicated in epithelial tumorigenesis (3). Overexpression of RON exists in various primary tumors, including colorectal, breast, and pancreatic cancers (4–10). In colorectal cancers (CRC), RON is overexpressed in more than 50% of cases (4, 5). Aberrant RON expression also results in generation of oncogenic and constitutively active RON variants such as RONA160 (3, 5). The consequence of these abnormalities is the activation of various intracellular signaling pathways that facilitate CRC cell growth, invasion, and chemoresistance (3). Overexpression of RON in CRC also has prognostic value in predicting patient survival and clinical outcomes (11). Thus, aberrant RON expression is a pathogenic feature in CRC cells, which contributes to tumorigenic phenotype and malignant progression (3–5, 11–13).

The high frequency of CRC RON overexpression and the dependency of CRC cells on RON signaling for growth provide the rationale to target RON for therapy. Tyrosine kinase inhibitors (TKI) such as foretinib (14), BMS-777607 (15), and MK-2461 (16) that target RON and MET are currently under clinical trials (www.clinicaltrials.gov). Therapeutic monoclonal antibodies (TMA) specific to RON such as IMC-41A10, narnatumb (clinical trial ID: NCT01119456), and Zt/f2 also have been evaluated in preclinical models (17, 18). Results indicate that targeted inhibition of RON has a therapeutic effect on tumors mediated by colon, breast, and pancreatic cancer cells in animal models (17–19). However, efficacy is limited to only about 40% to 50% (17–19). Complete inhibition of tumor growth by a single RON-targeted TKI or TMA has not been observed (14–19). Thus, there is an
Translational Relevance

Aberrant RON expression is a pathogenic factor contributing to epithelial tumorigenesis. However, therapeutic antibodies or tyrosine kinase inhibitors targeting RON for cancer therapy have shown very limited efficacy. Thus, there is a need to develop RON-targeted therapeutics with improved efficacy. Here, we describe a novel therapeutics in the form of anti-RON antibody Zt/g4–drug maytansinoid conjugates (Zt/g4–DM1) for targeted cancer therapy.

Materials and Methods

Cell lines and reagents

CRC cell lines DLD1, LoVo, HCT116, HT29, and SW620 were from ATCC and authenticated in 2010 with cytogenetic analysis. HT29-luc2 and HCT116-luc2 cells expressing the firefly luciferase gene-2 were from PerkinElmer and authenticated in 2011 with DNA profiling and cytogenetics. Mouse anti-RON mAbs Zt/g4, Zt/c1, and rabbit IgG antibody to the RON C-terminal peptide were used as previously described (2). Goat anti-mouse IgG labeled with FITC or rhodamine was from Jackson ImmunoResearch. Maytansinoid (DM1) and N-succinimidyl-4-[maleimidomethyl] cyclohexane carboxylate (SMCC) were from Concoris.

Conjugation of anti-RON mAb with DM1 through thioether linkage

Conjugation was performed according to a protocol to achieve a drug-antibody ratio (DAR) of 4:1 (26–28). Briefly, Zt/g4 at 10 mg/mL was mixed with 10 mmol/L SMCC-DM1 in a conjugation buffer to form Zt/g4–SMCC-DM1 (designated as Zt/g4–DM1). The anti-RON mAb Zt/c1 also was conjugated with SMCC-DM1 to form Zt/c1–DM1. We also prepared the control ADC by conjugating normal mouse IgG (CmlG) with SMCC-DM1 to form CmlG-DM1 as described above. All conjugates were purified using a PC10 Sephadex G25 column, sterilized through a 0.22 μmol/L filter, and stored at 4°C.

Analysis of Zt/g4–DM1 conjugation and its stability

The conjugation of DM1 to Zt/g4 was verified by hydrophobic interaction chromatography (HIC) using a Varian Prostar 210 Quaternary HPLC system coupled with a TSK butyl-NPR 4.6 x 3.5 column (Tosoh Biosciences; ref. 29). The average DARs were calculated from the integrated areas of the DAR species. This method also was used to determine the stability of Zt/g4–DM1 at 37°C.

Assay for cell surface RON expression

Cell surface RON was quantitatively determined by the immunofluorescence assay using QIFKIT reagents from DAKO. Cells (1 x 10⁶ cells per mL in PBS) were treated with Zt/g4 at saturating concentrations followed by incubation in parallel with the QIFKIT beads and goat F(ab')₂ F0479. After establishing a calibration curve, the number of RON receptor on the cell surface was then determined by interpolation following the manufacturer’s instruction.

Western blot analysis of RON expression

Cellular proteins (50 μg per sample) were separated in an 8% SDS-PAGE under reduced conditions. Western blotting of RON expression was performed as previously described.
Membranes also were reprobed with anti-actin antibody to ensure equal sample loading.

**Detection of internalized RON**

Cells at $1 \times 10^5$ cells per well in a 6-well plate were treated with 5 μg/ml Zt/g4 or Zt/g4–DM1 for various times followed by goat anti-mouse IgG coupled with HTC or rhodamine. Nuclear DNAs were stained with 4′,6-diamidino-2-phenylindole (DAPI). Immunofluorescence was observed under an Olympus BK71 microscope equipped with DUS/fluorescent apparatus as previously described (30).
Cell viability and death assays
Cell viability 72 hours after Zt/g4–DM1 treatment was determined by the MTT assay (22). Viable or dead cells were determined by the Trypan blue exclusion assay. A total of 900 cells were counted from three individual wells to reach the percentages of dead cells.

Analysis of cell cycle
HT29, HCT116, and SW620 cells (1 × 10⁵ cells per dish) were incubated at 37°C with 5 μg/mL Zt/g4–DM1 for 24 hours, labeled with propidium iodide, and then analyzed by an Accuri Flow Cytometer. Cell-cycle changes were determined by measuring DNA contents as previously described (30).

Mouse xenograft CRC model and anti-RON ADC 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 × 10⁶ HT29-Luc2, HCT116-luc2, or SW620 cells in the subcutaneous space of the right flank as previously described.
Mice were randomized into different groups (5 mice per group). Treatment began when all tumors had reached an average bioluminescence of approximately $10^7$ (for HT29- and HCT116-luc2 cells) or a mean tumor volume of approximately 100 mm$^3$ (for SW620 cells). The single-dose group received a tail vein injection of 20 mg/kg Zt/g4–DM1 in 0.1 mL PBS followed by observation for 28 days. The multidose study was performed by treating mice with Zt/g4–DM1 at 1, 3, 7, 10, and 15 mg/kg every 4 days for a total of five injections. Bioluminescence from individual tumors was measured every 4 days using Caliper IVIS image system (PerkinElmer). Tumor volumes from SW620-derived tumors were measured according to a formula: $V = \pi/6 \times 1.58 \times (\text{length} \times \text{width})^{3/2}$ (18, 31).

Animals were euthanized when tumor volumes exceeded 2,000 mm$^3$ or if tumors became necrotic or ulcerated through the skin.

**In vivo toxicity studies**

Acute toxicity with MTD was determined in Balb/C mice (4 mice per dose) by a single tail vein injection of Zt/g4–DM1 at 20, 40, and 60 mg/kg body weight. Toxicity associated with different therapeutic doses was evaluated in athymic nude mice bearing HT29 tumor xenograft (5 mice per dose). Mice were observed for about 30 days. Toxicity was assessed by observing mouse behavior, weight loss, and survival.

**Statistical analysis**

GraphPad Prism 6 software was used for statistical analysis. Results are shown as mean ± SD. The data between control and experimental groups were compared using Student $t$ test. Statistical differences at $P < 0.05$ were considered significant.

**Results**

**Characterization of anti-RON ADC Zt/g4–DM1**

Zt/g4 was selected as a lead ADC candidate due to its ability to induce RON internalization in various cancer cells (Supplementary Table S1; refs. 21–23, 32). Zt/g4 only recognizes human RON but not mouse RON homologue (32) and by itself has no tumor agonistic effect in vivo (18). Structures of Zt/g4–DM1 are shown in Fig. 1A. A total of 250 mg Zt/g4 was conjugated to DM1 with conditions to achieve an average DAR of 4:1. Our
selection of this ratio was based on published observations of trastuzumab-emtansine (T-DM1) in which one IgG molecule coupling with four DM1 molecules achieves maximal therapeutic efficacy (26, 33). HIC analysis revealed average DARs of Zt/g4–DM1 at 3.724 (Fig. 1B). The percentages of conjugates with different DARs from the integrated areas of the conjugates also were determined (Fig. 1B and Supplementary Table S2). The major peak accounting for 39.05% was peak 4 with a DAR of 4:1. The prepared Zt/g4–DM1 with DARs at 5:1, 4:1,
RON molecules on the surface of a single CRC cell was based on a quantitative method (Fig. 2A). The calculated expression of RON receptors was measured on CRC cell surfaces by the QIFKIT fluorescence-conjugate method (11). We first determined the number of RON receptors by CRC cells (11). We found no difference in binding intensity between free Zt/g4 and Zt/g4–DM1 in all three CRC cell lines tested (Fig. 2B). This suggests that the conjugation does not impair the Zt/g4 binding capability.

We next studied Zt/g4–DM1-induced RON endocytosis, a process essential for delivering DM1 into CRC cells. Zt/g4–DM1 causes a progressive reduction of cell surface RON in a time-dependent manner in all three CRC cell lines tested (Fig. 2C). Less than 20% of RON remained on the cell surface after a 48-hour treatment. The time required for Zt/g4–DM1 to induce 50% RON reduction (internalization efficacy) was at 12.26, 11.02, and 12.30 hour for HCT116, HT29, and SW620 cells, respectively. In contrast, the time required for Zt/c1–DM1-induced 50% RON reduction in HCT116, HT29, and SW620 was at 19.11, 19.41, and 18.65 hour, respectively. Thus, Zt/g4–DM1 is more efficient and potent in induction of RON endocytosis.

We performed Western blotting to verify the effect of Zt/g4–DM1 on RON expression (Fig. 2D). Both pro-RON and mature RON (indicated by RON-β chain) were progressively reduced in all three CRC cell lines tested. Zt/g4–DM1 was effective in reducing mature RON expression, which resides on the cell surface. Less than 20% of the RON-β chain was detected 36 hours after Zt/g4–DM1 treatment. The kinetic reduction of mature RON was quite different among three cell lines (Fig. 2E). However, the patterns of Zt/g4–DM1-induced RON reduction were comparable with those of free Zt/g4-induced RON reduction, suggesting that the conjugation does not impair the ability of Zt/g4–DM1 to induce RON endocytosis.

We further confirmed Zt/g4–DM1-induced RON endocytosis by immunofluorescence analysis of cytoplasmic RON using HT29 cells as the model (Fig. 2F). Cells stained for lysosomal-associated membrane protein 1 (LAMP1) were used as a marker for colocalization of internalized RON. At 4°C, RON is detected on the cell surface. The intracellular localization of internalized RON occurred at 37°C after Zt/g4–DM1 treatment. Also, the cytoplasmic RON was colocalized with LAMP1 in HT29 cells, indicating that internalized RON resides within lysosomes. In contrast, RON endocytosis was minimal in cells treated with CmlIgG1–DM1. Colocalization of RON with LAMP1 was not observed in these cells. Thus, results from Fig. 2 demonstrate that Zt/g4–DM1 is effective in induction of RON endocytosis by CRC cells.

Effect of Zt/g4–DM1 on CRC cell cycle, growth, and death
DM1 acts on microtubules to cause cell-cycle arrest at G2–M phase followed by cell death (27, 34, 35). Zt/g4 intracellular delivery of DM1 results in cell-cycle changes. The changes in cell-cycle profile were observed as early as 3

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**Figure 3.** (Continued.) C, increased cell death: cells were treated with different amount of Zt/g4–DM1 for 72 hours. Morphologic changes were observed under the Olympus BK-41 inverted microscope and photographed. Images showing cell death are presented. D, cell death percentages were determined by the Trypan blue exclusion method. The IC50 values for cell viability or death at 72 hours from individual groups were calculated using the GraphPad Prism 6 software. Results shown here are from one of three experiments with similar results.

The stability of Zt/g4–DM1 was determined by incubating the conjugates in vitro at 37°C for 30 days. DAR changes were measured by HIC from different time-points. Zt/g4–DM1 seems to be stable at 37°C for up to 30 days (Fig. 1C and Supplementary Table S2). At day 30, it has an average DAR of 3.484, which represents only a 6.4% reduction from the DAR of 3.724 at day 0. The major changes appeared to be 3:1, and 2:1 accounted for more than 92% of the total conjugates. DARs for Zt/c1–DM1 and CmlIgG1–DM1 were 3.91 and 4.01, respectively.

The changes in cell-cycle profile were observed as early as 3 hours after Zt/g4–DM1 treatment. The time required for Zt/g4–DM1 to induce 50% RON reduction (internalization efficacy) was at 12.26, 11.02, and 12.30 hour for HCT116, HT29, and SW620 cells, respectively. In contrast, the time required for Zt/c1–DM1-induced 50% RON reduction in HCT116, HT29, and SW620 was at 19.11, 19.41, and 18.65 hour, respectively. Thus, Zt/g4–DM1 is more efficient and potent in induction of RON endocytosis.

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hours after addition of Zt/g4–DM1, featuring a significant reduction in G0–G1 phase, a decrease in S phase, and a dramatic increase in G2–M phase (Fig. 3A). These changes were present in all three CRC cell lines tested. Quantitative measurement of cell-cycle changes at 24 hours is shown in Supplementary Table S3. CmlgG-DM1 treatment had minimal effect on cell cycles compared with those from the Zt/g4–DM1-treated cells. Thus, Zt/g4-targeted delivery of DM1 affects cell cycles in CRC cells.

We next studied the effect of Zt/g4–DM1 on cell viability. Sensitivity of CRC cells to free DM1 was shown in Supplementary Fig. S1 with IC50 values at 4.1 nmol/L for HCT116, 4.4 nmol/L for HT29, and 3.2 nmol/L for SW620 cells, which suggests high sensitivity to DM1. We then treated cells with Zt/g4–DM1. A significant reduction in cell viability was observed in a time and dose-dependent manner (Fig. 3B). The IC50 value of Zt/g4–DM1 at 72 hours was 1.64 µg/mL for HT29, 2.16 µg/mL for HCT116, and 4.03 µg/mL for SW620 cells, respectively. The effect of Zt/c1–DM1 was relatively weak with IC50 values at 6.26 µg/mL for HT29, 4.64 µg/mL for HCT116, and 4.36 µg/mL for SW620 cells, respectively. Both Zt/g4–DM1 and Zt/c1/DM1 had no effect on RON-negative LoVo cells. DLD1 cells showed a slight reduction in cell viability with IC50 value at 20.36 µg/mL (Supplementary Fig. S2). This suggests that anti-RON ADC is ineffective in CRC cells expressing low levels of RON (below 5,000 sites per cell). A comparison of the Zt/g4–DM1 efficacy among four CRC cell lines with the different number of RON receptor per cells is shown in Supplementary Fig. S3. Thus, Zt/g4–DM1 is more efficient than Zt/c1–DM1 in reducing viability of CRC cells expressing high levels of RON.

Morphologic observation indicated a massive cell death 72 hours after cells were exposed to Zt/g4–DM1 (Fig. 3C). More than 50% cell death was observed 72 hours after cells were treated with 7.5 mg/mL Zt/g4–DM1 (Fig. 3D). The IC50 value ranged at 5 to 7 µg/mL in all three CRC cell lines tested. We also counted viable cells 72 hours after incubation of 1 × 10^6 CRC cells per well in the presence of Zt/g4–DM1. Zt/g4–DM1 treatment results in a significant reduction in the number of viable cells (Supplementary Fig. S4). Thus, Zt/g4–DM1 not only causes cell-cycle arrest and reduces cell viability, but also reduces viable cell numbers and induces massive CRC cell death.

**Therapeutic activity of Zt/g4–DM1 in mouse xenograft tumor model**

We first determined the efficacy of a single dose of Zt/g4–DM1 at 20 mg/kg body weight on tumors derived from HCT116, HT29, and SW620 cells. Tumor growth by HT116-luc2 and HT29-luc2 cells was measured by bioluminescence emitted from tumor cells. SW620-mediated tumors were evaluated by tumor volume (18, 34). A single dose of Zt/g4–DM1 at 20 mg/kg is sufficient to delay tumor growth caused by all three CRC cell lines (Fig. 4A and B). This time-dependent inhibition was statistically significant. Images of tumors obtained at day 16 are shown in Fig. 4C. More than 95% inhibition, measured by average bioluminescence intensity, was achieved in both HT29 and HCT116 tumor models. We observed similar results in mice bearing SW620 tumors. In this case, an average 82% inhibition in tumor volume was documented (Fig. 4C). Tumor regrowth was observed at day 20 and thereafter. An accelerated phase was observed from day 24 to 28 (Fig. 4A and B). It is known that mouse IgG1 has a half-life of approximately 6 days in vivo (36). Thus, our results indicate that maintenance of Zt/g4–DM1 at about 5 mg/mL in vivo is required to delay tumor growth (Supplementary Fig. S5). Nevertheless, by measuring the average tumor weight at day 28, we still found a significant delay in tumor growth in the single-dose experiment. The inhibition rate was 50.98% for HT29, 58.0% for HCT116, and 61.9% for SW620 tumors, respectively (Fig. 4D). Thus, a single dose of 20 mg/kg Zt/g4–DM1 is effective and displays long-lasting activity in inhibition of tumor growth initiated by all three CRC cell lines.

We then selected the HT29-Luc2 xenograft tumor model for the dose-ranging study. Mice were injected with different doses of Zt/g4–DM1 once every 4 days for a total of five injections. Zt/g4–DM1 at 1 or 3 mg/kg showed no inhibition of tumor growth (Fig. 5A). Significant Inhibition was observed in mice treated with 7 mg/kg Zt/g4–DM1after the third injection. In this case, more than 80% inhibition, calculated by the average photon emission, was obtained from day 19 to day 43. The efficacy was more prominent in mice treated with 10 and 15 mg/kg Zt/g4–DM1. In both cases, tumor growth was dramatically delayed after the second injection. Repeated injections at both doses kept tumor growth at minimal levels during the entire period of therapy. By analyzing the average photons at day 31, the IC50 dose for this multidose study was 5.01 mg/kg body weight (Fig. 5B). Images of tumors from different groups at day 31 are shown in Fig. 5C. In mice treated with Zt/g4–DM1 at 7, 10, and 15 mg/kg, inhibition was in a dose-dependent manner. More than 95% inhibition in mice treated with 10 and 15 mg/kg Zt/g4–DM1 was achieved compared with that of control mice (Fig. 5C). We also compared the average tumor weight from the control mice and the mice treated with 15 mg/kg Zt/g4–DM1 at day 31 to determine the rate of inhibition. A 90% inhibition at average tumor weight was observed (Fig. 5D). Tumors were collected at day 33 (for 1 and 3 mg/kg groups) and day 43 (for 7 and 10 mg/kg groups) and compared with tumors from control group. Significant inhibition was still observed for mice treated with 7 and 10 mg/kg Zt/g4–DM1. Thus, Zt/g4–DM1 at the regimens of 7, 10, and 15 mg/kg Q 4 days is highly effective in delaying HT29 cell-mediated tumor growth in mouse xenograft models. To determine whether cell death occurs in xenograft tumors, HT29 cell-derived tumor samples collected at day 31 from both control and 15 mg/kg-treated mice were processed for histologic analyses. Analysis by hematoxylin and eosin (H&E) staining revealed cell death in different regions in all Zt/g4–DM1-treated tumors but not in
control samples (Fig. 5E). An average percentage of dead areas in a tumor mass were 65% ± 7.4. Western blot analysis using cell lysates from tumor samples also showed that RON expression in Zt/g4-DM1-treated tumors (16.44% ± 5.75) was dramatically reduced compared with that in control samples (100% ± 15.56; Fig.
Figure 5. Evaluation of different doses of Zt/g4-DM1 on tumor growth and RON expression. A, effect of multi-dose of Zt/g4-DM1 on tumor growth was tested in HT29 cell-induced tumors. Tumor-bearing mice were treated with different doses of Zt/g4-DM1 every 4 days for a total of five injections (i). Tumor growth was determined by the average bioluminescence intensity. B, an IC50 value based on the average bioluminescence intensity from individual groups at day 31 was calculated using GraphPad Prism 6 software. C, bioluminescence images of individual tumors from each group at day 31 are shown. The percentages of inhibition were calculated from the average photon emission. The color scale from minimal to maximal is set at 300 to 35,000 photons per second. D, individual tumors from different groups were collected and weighed at day 31, 35, and 43, respectively. The percentages of inhibition were calculated as detailed in Fig. 4C. (Continued on the following page.)
Thus, Zt/g4–DM1 causes cells death in CRC xenograft tumors, which is associated with elimination of CRC cells overexpressing RON.

**Toxic effect of Zt/g4–DM1 on mice**

Three experiments using two different types of mice were performed to study Zt/g4–DM1 on animal behavior and body weight. The first experiment addressed the impact of multidoses of Zt/g4–DM1. Athymic nude mice were injected five times with 1, 3, 5, 7, 10, 15 mg/kg of Zt/g4–DM1 and monitored every 4 days for a total period of 31 days. All mice behaved normally during the entire observational period. The average body weight of experimental groups was comparable with that of control mice with no differences (Fig. 6A). The second experiment observed the effect of a single dose of Zt/g4–DM1 at 20 mg/kg in nude mice. Densitometry analysis was performed to determine the levels of RON expression.

**Figure 6.** Toxicity of Zt/g4–DM1 in vivo. Body weight was measured every 4 days during the period of Zt/g4–DM1 treatment. A, effect of multiple doses of Zt/g4–DM1 on mouse body weight was determined by administration of Zt/g4–DM1 at 1, 3, 7, 10, 15 mg/kg every 4 days with a total of 5 injections. Mice were weighed and monitored for a total of 31 days. B, effect of a single dose of Zt/g4–DM1 on mouse body weight was determined using mice bearing HT29, HCT116, or SW620-derived tumors. Body weight was monitored up to 28 days. C, effect of high doses of Zt/g4–DM1 on mouse body weight was analyzed by tail vein injection at 20, 40, and 60 mg/kg to Balb/c mice. Mice were euthanized at day 21. In all cases, the average body weight of mice before Zt/g4–DM1 injection was 19.8 ± 3.6 grams (5 mice per group) and set as 100%.
mice bearing tumors derived from HT29, HCT116, and SW620 cells. No changes in behavior or body weight were observed (Fig. 6B). The third involved a single-dose injection of Zt/g4–DM1 at 20, 40, and 60 mg/kg in Balb/c mice monitored for 24 days (Fig. 6C). Moderate distress was observed in mice administered with 60 mg/kg Zt/g4–DM1. Also, a moderate reduction of about 6% body weight was observed within the first 4 days after 60 mg/kg Zt/g4–DM1 injection. Although the average body weight from this group of mice slowly recovered during the observation period, the overall average remained lower than that of control mice with a 19% difference compared with that of control mice at day 24. Thus, Zt/g4–DM1 at the multiple-dose regimen seemed to be well tolerated. However, a single-dose of Zt/g4–DM1 at 60 mg/kg showed a toxic effect on mouse behavior and body weight.

Discussion

This report is about the development of anti-RON ADC Zt/g4–DM1 for targeted cancer therapy. We showed that Zt/g4–DM1 retains its specificity to RON after conjugation with DM1. The conjugates were stable at 37°C with minimal dissociation of DM1 from antibody. Binding of Zt/g4–DM1 to CRC cells causes a rapid endocytosis of cell surface RON. Internalized Zt/g4–DM1 results in cell-cycle arrest in G1–M phase, followed by cell viability reduction, and massive cell death. Studies from mouse xenograft tumor models confirmed that a single dose of Zt/g4–DM1 at 20 mg/kg is sufficient to inhibit tumor growth with a long-lasting effect up to 20 days. The multiple-dose-ranging studies further demonstrated that the therapeutic regimen at 7, 10, 15 mg/kg, every 4 days × 5 with a total dose of 35, 50, and 75 mg, respectively, displays strong efficacy in tumor growth inhibition. Furthermore, we showed that Zt/g4–DM1 at doses up to 40 mg/kg has no toxic effect on mouse behavior or body weight. Thus, Zt/g4–DM1 is a novel biotherapeutics with enhanced efficacy for RON-targeted cancer therapy. Humanization of Zt/g4 is currently under way.

Zt/g4 was conjugated to DM1 at appropriate DARs through the thioether linkage (25, 26, 33). Consistent with previous reports (26, 33, 27), Zt/g4–DM1 has a favorable conjugation profile. Most conjugates have DARs ranging from 2:1 to 5:1 with the major peak at 4:1. Such a profile is the typical pattern of ADCs using the thioether linkage technology (29). Zt/g4–DM1 is relatively stable. Incubation of Zt/g4–DM1 at 37°C for 30 days resulted in only 6.5% reduction in DARs of DM1. These data are consistent with previous reports showing that antibodies conjugated with DM1 through thioether linkage are highly stable both in vitro and in vivo under various conditions (29, 31). Although we did not test the stability of Zt/g4–DM1 under in vivo conditions, it is expected that the conjugates have a similar stability profile due to the similar conjugation method (29, 31). The efficacy of in vitro studies using a single dose of Zt/g4–DM1 at 20 mg/kg seems to support this notion. In this case, a single injection is sufficient to inhibit tumor growth for almost 3 weeks, implying that Zt/g4–DM1 is relatively stable in vivo to exert a long-lasting effect. Clearly, the use of thioether linkage provides the practical basis for future development of humanized Zt/g4–DM1.

The selection of Zt/g4 as the leading candidate for DM1 conjugation is based on its unique features. Zt/g4 is a mAb highly specific and sensitive to RON, and recognizes an epitope in the RON sema domain (32). The binding of Zt/g4 to RON results in a rapid and efficient RON internalization process. The internalized RON colocalizes with LAMP1, suggesting that the endocytosis could be mediated through a clathrin-dependent pathway (37). Significantly, more than 80% of cell surface RON is internalized within 48 hours after addition of Zt/g4–DM1. In the case of HT29 cells expressing approximately 18,800 RON molecules per cell, it translates into 15,000 RON receptors that are internalized within 48 hours. This is equivalent to 60,000 DM1 molecules within a single cell, sufficient to cause cell-cycle arrest. It is noticed that the kinetics of RON internalization among three CRC cell lines are quite different after addition of Zt/g4–DM1, suggesting the importance of the rate of endocytosis in regulating efficacy of Zt/g4–DM1. Clearly, Zt/g4–DM1-induced RON endocytosis facilitates intracellular delivery of DM1 to exert cytotoxic activity. Moreover, Zt/g4 has no agonistic activities in CRC cells expressing RON (18).

The action of DM1 delivered through Zt/g4 was clearly displayed in CRC cells. First, we showed by flow-cytometric analysis that the delivery of DM1 results in cell-cycle arrest in G1–M phase, which is a feature of DM1 that impairs microtubule dynamics (35). This effect was observed as early as 3 hours after addition of Zt/g4–DM1, which is characterized by progressive reduction of the G1 phase and the accumulation of cells at the G2–M phase. Second, we observed that targeted delivery of DM1 progressively decreases cell viability. More than 80% reduction in cell viability 72 h after treatment was achieved among the three CRC cell lines tested. Finally, we documented a massive cell death in Zt/g4–DM1-treated CRC cells in a dose-dependent manner with IC50 values in the range of 5 to 7 μg/mL Zt/g4–DM1. These evidences suggest that DM1 is effectively delivered by Zt/g4 through a targeted pathway, which results in cell-cycle arrest, viability reduction, and cell death.

Results from mouse xenograft CRC models prove that Zt/g4–DM1 is highly efficient in inhibition of tumor growth. This conclusion is supported by mouse models using two treatment regimens. The single-dose therapy using 20 mg/kg Zt/g4–DM1 was designed to determine whether this dose is sufficient to inhibit tumor growth and, if so how long the effect will last. Indeed, Zt/g4–DM1 at 20 mg/kg was highly effective in delaying xenograft tumor growth with a long-lasting effect of almost 2 weeks. It is known that mouse IgG1 has a half-life of approximately 6 days in vivo (36). Administration of 20 mg/kg Zt/g4–DM1 should allow us to monitor its efficacy in a four-half-life cycle within 24 days. The obtained results confirmed that the efficacy of Zt/g4–DM1 lasts up
to 12 days without signs of tumor regrowth (from day 4 to day 16 as shown in Fig. 4A). By calculation, the amount of Zt/g4–DM1 in vivo required to inhibit tumor growth is about 5 mg/kg (Supplementary Fig. S5). In other words, a dose of 5 mg/kg Zt/g4–DM1 maintains a balance between tumor growth and inhibition.

The multiple dose-ranging studies were designed to determine the minimum dose required to inhibit xenograft tumor growth. Zt/g4–DM1 at 7 mg/kg in the regimen of Q 4 days × 5 with a total dose of 35 mg/kg achieves a significant inhibition. An increase of Zt/g4–DM1 up to 10 and 15 mg/kg in a similar regimen results in a superior therapeutic index. In both cases, the total amount of Zt/g4–DM1 was at 50 and 75 mg/kg, respectively. These results provide us the IC50 value of 5.01 mg/kg (calculated according to the repeated Zt/g4–DM1 administration and the estimated antibody half-life), which is consistent with the estimated values of 5 mg/kg from the single-dose study. Thus, results from multiple dose regimens should help us to design an optimal treatment strategy for the potential use of humanized Zt/g4–DM1 in future clinical settings.

Analysis of the toxic profile in two types of mice indicates that Zt/g4–DM1 is relatively safe at therapeutic doses with minimal impact on animal’s behavior and body weight. Because Zt/g4 does not recognize mouse RON, the observed low toxicity suggest a very limited dissociation of the Zt/g4–DM1 conjugates in vivo. However, a single dose of Zt/g4–DM1 at 60 mg/kg has a negative impact on mouse highlighted by an average of 6% to 19% reduction of body weight during the entire period of study. This suggests that during the administration of multiple doses of Zt/g4–DM1, the accumulated Zt/g4–DM1 in vivo should not exceed the 60 mg/kg limitation. This dose limitation should be a valuable reference for the use of humanized Zt/g4–DM1 in human subjects in the future.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: H.-P. Yao, Y.-Q. Zhou, J. Zhou, R. Zhang, M.-H. Wang
Development of methodology: H.-P. Yao, Y.-Q. Zhou, M.-H. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Wang, M.-H. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Feng, H.-P. Yao, M.-H. Wang
Writing, review, and/or revision of the manuscript: Y.-Q. Zhou, J. Zhou, R. Zhang, M.-H. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.-H. Wang
Study supervision: W. Wang, M.-H. Wang

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References


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Liang Feng, Hang-Ping Yao, Wei Wang, et al.


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