Monoclonal antibody (mAb)-induced down-regulation of RON receptor tyrosine kinase diminishes tumorigenic activities of colon cancer cells

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Abstract. Overexpression of the RON receptor tyrosine kinase contributes to pathogenesis of epithelial cancers and disruption of RON signals has potential for therapeutic intervention. Here, we report the inhibitory effects of monoclonal antibodies (Zt/g4, Zt/f2 and Zt/c9) on RON expression and tumorigenic activities in colon cancer cells. Persistent treatment of colon SW620 or other cells with Zt/g4 dramatically down-regulated RON expression as evident by Western blot and cell surface fluorescent analyses. The effect was both concentration and time-dependent and specific to RON but not to structure-related MET or -unrelated EGFR. The cause of reduction was antibody-induced receptor internalization followed by protein degradation through lysosome and proteasome-mediated pathways. Down-regulation of RON impaired intracellular signaling events. Phosphorylation of Erk1/2 and AKT was dramatically reduced after Zt/g4 treatment. Zt/g4 treatment also affects activities of DVL and GSK-3ß, which results in diminished ß-catenin nuclear translocation. Functional studies revealed that Zt/g4 treatment changes cellular morphology and affects colony formation in soft agar. It also increased the sensitivity of SW620 cells in response to gemcitabine-induced cytotoxicity. In this case, the death of SW620 cells was significantly increased when Zt/g4 was used in combination with gemcitabine. We conclude that persistent treatment of cancer cells with antibodies specific to RON extracellular domains results in down-regulation of RON expression. The reduced RON expression is accompanied with impaired signaling events, diminished tumorigenic activities and enhanced sensitivity towards cytotoxic drugs. Thus, Zt/g4-directed targeting could have therapeutic implication for controlling tumorigenic phenotypes of cancer cells.

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Introduction

Aberrant expression of receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR) and MET plays a significant role in pathogenesis of epithelial cancers (1,2). The mechanism by which RTKs regulate epithelial tumorigenesis is their ability to activate intracellular signals that control cell growth, survival, migration and invasion (3,4). These oncogenic activities are characterized as oncogenic dependency or addiction (5). Because of their tumorigenic potential and accessibility, RTKs have emerged as attractive targets for pharmaceutical intervention. The development of therapeutic monoclonal antibodies (mAbs) such as trastuzumab and cetuximab is a typical example (6,7). These mAbs are molecularly engineered proteins and highly specific to the EGFR family members (6,7). Antibodies targeting other RTKS including MET and VEGFR are also available. By regulating RTK expression, blocking their oncogenic signaling, and inducing cytotoxic or immune responses, therapeutic antibodies inhibit tumor growth, prevent malignant progression and improve clinical outcomes (8,9).

The RON receptor tyrosine kinase belongs to the MET family of RTKs (10). RON expression is highly altered in epithelial tumors including colon, breast and pancreatic cancers (11-13). Alterations are characterized by protein overexpression and spontaneous phosphorylation (11-13). Pathological analyses indicate that RON overexpression has prognostic value and correlates with disease progression and shortened survival rates (14,15). Altered RON expression is also accompanied with generation of RON variants by deletion, truncation, or insertion (16-18). One variant, known

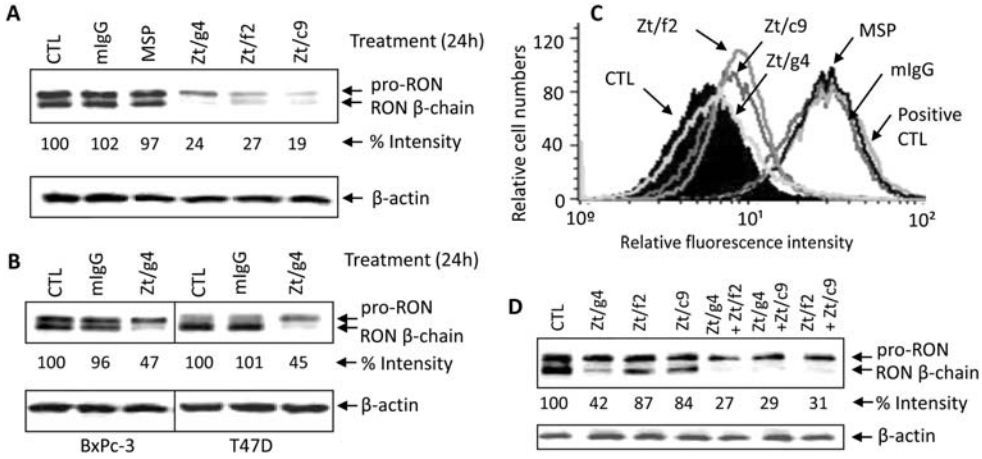

Figure 1. Inhibitory effect of anti-RON mAbs on RON expression by various types of cancer cells. (A) Inhibitory effect of individual mAbs on RON expression by colon SW620 cells. Cells ($2x10^6$ in a 60-mm culture dish) were treated with 10 μg/ml of individual mAbs for 24 h. Normal mouse IgG and MSP were used as controls. Western blot analysis was performed using proteins from cell lysates (50 μg per sample). The intensities of protein bands were determined by VersaDoc scanning software. The total reduction (pro-RON plus mature RON) was calculated. The membrane was also reprobed with IgG antibodies to ß-actin as the loading control. (B) Effect of Zt/g4 on RON expression by BxPC-3 and T-47D cells. Experimental conditions were similar to those described in (A). ß-actin was used as the controls. The intensities of total RON were calculated. (C) Immunofluorescent analysis of RON expression on the surface of SW620 cell surface. SW620 cells ($2x10^6$ in a 60-mm culture dish) were treated with or without 10 μg/ml of Zt/g4, Zt/c9, or Zt/f2 for 24 h. Normal mouse IgG served as the control. Cells were collected, washed with acid buffer to eliminate cell surface bound mAb, and then incubated with 1.5 μg/ml of anti-RON mAb Zt/c1 (34). FITC-coupled goat-anti mouse IgG (FITC-gm IgG) was used as the detecting antibody. Zt/c1 recognizes an epitope that does not compete with Zt/g4 or other two mAbs for RON binding (34). Positive control (CTL): cells were incubated at 4˚C with Zt/c1 followed by anti-mouse IgG coupled with FITC; MSP or mIgG: cells were incubated with MSP or mouse normal IgG, respectively, at 37˚C, acidic washing, incubated with Zt/c1 at 4˚C, and then FITC-gm IgG; Zt/g4, Zt/f9 or Zt/c9: cells were treated with individual mAb at 37˚C, acidic washing, incubated with Zt/c1, followed by FITC-gm IgG. Control (CTL): cells were incubated with normal mouse IgG at 4˚C followed by FITC-gm IgG. (D) Synergistic effect of different mAbs on RON expression. SW620 cells ($2x10^6$ per 60-mm culture dish) were treated with Zt/g4 alone or in combination with Zt/c9 or Zt/f2 for 6 h. Levels of RON expression were determined by Western blot analysis. The total reduction of RON was determined as described in (A). ß-actin was used as the controls. Data shown here are from one of three experiments with similar results.

as RON160, is oncogenic *in vivo* and capable of mediating invasive phenotypes (16,18). Animal studies further show that RON overexpression by transgenic techniques in lung epithelial cells results in formation of tumors (19). Inactivation of the RON tyrosine kinase domain by knockout methods has also revealed that RON activity is involved in skin and breast cancer development (20,21). *In vitro*, RON activation directs a cellular program featured by cell spreading, morphological changes and invasive growth (22,23). These activities resemble a phenotype known as epithelial to mesenchymal transition (EMT) (24,25). EMT is a distinct feature occurring during embryonic development and tumor progression towards metastasis (25,26).

Because of its importance in cancer pathogenesis, RON has emerged as a potential drug target (27). Various approaches have been evaluated to inhibit RON signaling (28-32). Small interfering RNA and dominant negative RON variants have shown inhibition of RON and its associated tumorigenic activities (28,30). Small chemical inhibitors such as Compound 1 from Amgen and PHA665752 from Pfizer have been synthesized and tested in various *in vitro* and *in vivo* tumor models (32,33). Although these chemicals are not specific only to RON, they block RON-mediated signals and tumor growth *in vivo* (32,33). Recently, mAbs specific to the extracellular domains of RON have been generated and tested *in vitro* and *in vivo* animal models (31). The obtained data indicate that mAb-direct RON targeting has potential to be developed for clinical evaluation.

The present work focused on the utilization of mAb specific to RON extracellular domains to determine if mAb-directed down-regulation of RON expression disrupts the dependency of cancer cells towards RON signals and diminishes tumorigenic phenotypes. Colon cancer cells were used as the main model. Three mAbs recognizing different epitopes on RON extracellular domains were evaluated. Among them, Zt/g4 was selected for detailed analyses. Zt/g4 is a highly sensitive mAb specific to an antigenic epitope on RON extracellular sema domain (34). By persistent treatment of colon, breast and pancreatic cancer with Zt/g4, we observed a progressive loss of RON expression. This effect was mediated by Zt/g4-induced internalization of RON followed by intracellular protein degradation. Zt/g4 induced down-regulation also impaired RON phosphorylation and attenuated RON-mediated downstream signaling, which significantly diminishes tumorigenic activities of colon cancer cells. Thus, Zt/g4-directed down-regulation of RON expression impairs tumorigenic phenotypes of colon cancer cells, which has potential to be developed for targeted cancer therapy.

Materials and methods

Cell lines, antibodies and reagents. Human colon SW620, pancreatic BxPC-3 and breast T-47D cells were from ATCC (Manassas, VA). Human macrophage-stimulating protein (MSP) was from Dr E.J. Leonard (National Cancer Institute, Frederick, MD). Mouse mAbs specific to RON extracellular domains (Zt/g4, Zt/c9, Zt/f2 and Zt/c1) and rabbit IgG antibodies to RON C-terminus were used as previously described (34). Mouse mAb (PY-100) to phosphotyrosine,

goat or rabbit IgG antibodies specific to regular or phosphor Erk1/2 (p44/42), AKT and other proteins were from Cell Signaling Inc (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Concanamycin A and lactacystin were from CalBiochem (La Jolla, CA); Gemcitabine was from Chemiceuticals (Indianapolis, IN). Endocytic inhibitor filipin was from Fisher Scientific (Pittsburgh, PA).

Immunofluorescent assay. Cells (2x10^6 per sample) were treated at 37˚C with 10 μg/ml of Zt/g4 or normal mouse IgG for 60 min. Cells were then washed with acid buffer (150 mM NaCl, pH 2.5) to remove antibodies bound to the cell surface. Resulting cells were then incubated at 4˚C with 1.5 μg/ml of Zt/c1 for 45 min followed by goat anti-mouse IgG coupled with fluorescein isothiocyanate (FITC). Zt/c1 recognizes a RON extracellular epitope and has no competition with Zt/g4 or other mAb (34). Fluorescent intensities were determined by FACScan (Becton-Dickinson).

Immunoprecipitation and Western blotting. These methods were performed as previously described (11). Proteins were separated in 10% SDS-PAGE under reduced conditions and detected by Western blotting using specific antibodies. The reaction was developed with enhanced chemiluminescent reagents and analyzed by VersaDoc Imaging system (Bio-Rad).

Colony formation assay. The assay was performed as previously described (28). SW620 cells were treated with Zt/g4 for 24 h and then suspended in culture medium containing 0.3% agarose. Zt/g4 (10 μg/ml) was added to the plate after initiation of incubation. Number of colonies were counted and photographed after a 12-day incubation.

Cytotoxicity assay. The assay was performed as previously described (34). SW620 cells (1x10^4 cells/well in a 96-well plate) were cultured in triplicate with various amounts of gemcitabine in the presence or absence of Zt/g4. After incubation for 3 days, cells were washed, stained with Diff-Quik and lysed in 1% SDS buffer. Color intensity was measured at 570 nm wavelength using an ELISA plate reader. Cell numbers were calculated with reference to a standard curve.

Results

Down-regulation of RON expression in cancer cells by a panel of mAb. Zt/g4, Zt/f2 and Zt/c9 were used to determine their effects on RON expression. Zt/g4 and Zt/c9 recognize different epitopes on the RON sema domain and Zt/f2 reacts with an epitope on immunoglobulin, plexins and transcriptional factor (IPT) domains (34, and our unpublished data). SW620 cells, expressing high levels of RON and responsive to MSP stimulation (16,28), were treated for 24 h with 10 μg/ml of mAb followed by Western blot analysis using rabbit anti-RON C-terminal antibodies. As shown in Fig. 1A, MSP and normal mouse IgG had no effect on RON expression. In contrast, Zt/g4, Zt/c9 and Zt/f2 treatment caused significant reduction of RON expression. As judged by protein intensities, levels of RON ß-chain were dramatically reduced. Interestingly, the mAb treatment also resulted in the decrease

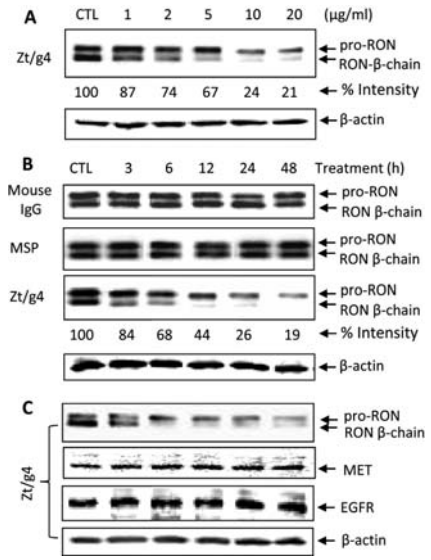

Figure 2. Dose-dependent and kinetic activities of Zt/g4 and its specificity in down-regulation of RON expression. (A) Concentration-dependent down-regulation by Zt/g4 of RON expression. SW620 cells (2x10^6 per 60-mm culture dish) were treated with different amounts of Zt/g4 for 12 h and then subjected to Western blot analysis using anti-RON antibodies to C-terminal peptide. Membranes were also reprobed with IgG antibody to ß-actin as the loading control. The total reduction of RON was determined as detailed in Fig. 1A. (B) The kinetic effect of Zt/g4 on RON expression by SW620 cells. Cells were treated with Zt/g4 for various time intervals followed by Western blot analysis as described in Fig. 1A. Normal mouse IgG was used as a negative control. Human MSP at 5 nM was used to determine if it has regulatory effect on RON expression. ß-actin was used as the controls. (C) Specific activities of Zt/g4 on RON expression. Breast T-47D cells were used as the model because they simultaneously express RON, MET and EGFR. Experimental conditions were similar to those in Fig. 1A. After incubation with Zt/g4 for various time intervals, the levels of RON, MET and EGFR were determined by Western blot analysis using individual antibodies. ß-actin was used as the controls. Data shown here are from one of two experiments with similar results.

of pro-RON expression, which is not expressed on the cell surface. These results suggest that mAb treatment not only does it decrease RON expression but also affects pro-RON expression although the underlying mechanisms are unknown. The effect of Zt/g4 was not limited to colon cancer cells because RON expression was also decreased in breast T-47D and pancreatic BxPC-3 cells after Zt/g4 treatment (Fig. 1B).

Immunofluorescent analysis confirmed that Zt/g4 treatment causes diminished RON expression on the cell surface (Fig. 1C). In this case, fluorescent intensities of cells treated with Zt/g4 or the other two mAbs were significantly reduced as compared to those from controls. Similar results were also observed with T-47D and BxPC-3 cells, although the levels of reduction varied (data not shown).

Results in Fig. 1D demonstrated synergistic activities of mAbs on RON expression. Zt/g4 in combination with Zt/f2 caused further reduction of RON expression. Similar results were also seen in cells treated with Zt/g4 plus Zt/c9 or Zt/f2 plus Zt/c9. These results, together with those in Fig. 1A, B and C, demonstrate that Zt/g4, Zt/c9 and Zt/f2 down-regulate RON expression by different types of cancer cells and that synergistic activity exists among mAbs.

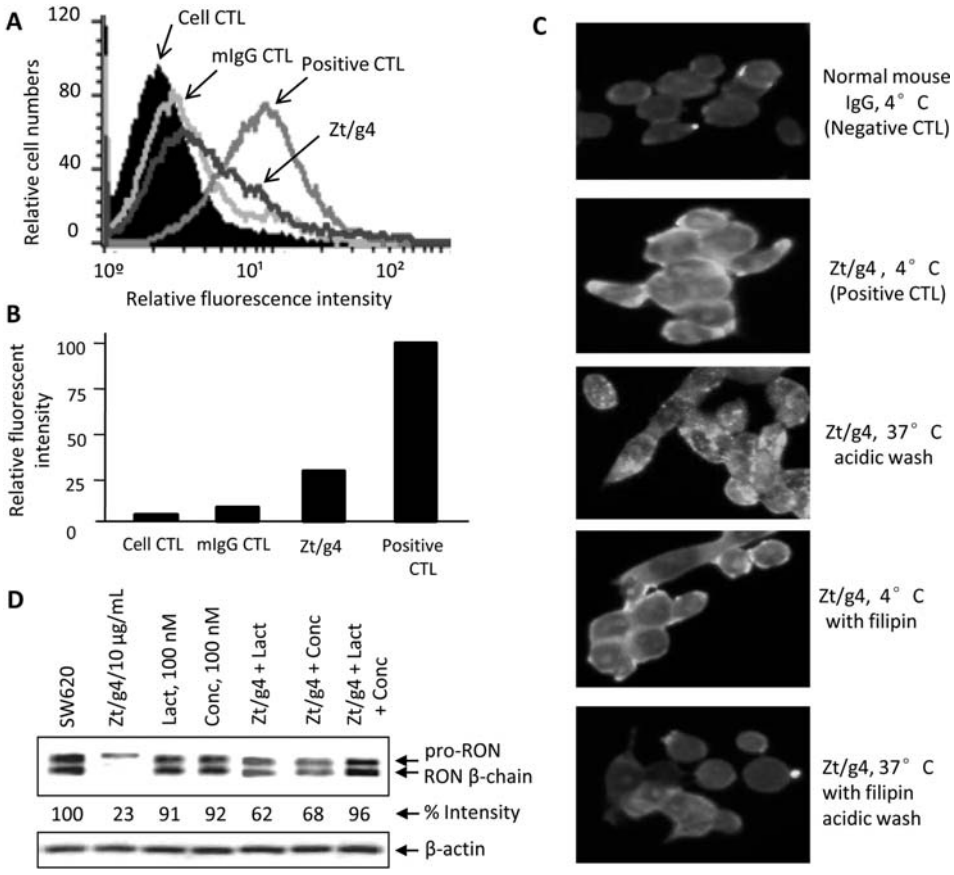

Figure 3. RON internalization induced by Zt/g4 and its subsequent degradation. (A) Zt/g4-induced RON internalization in SW620 cells. Cells ($2x10^6$ per sample) were cultured at 37˚C and treated with 10 μg/ml of Zt/g4 for 60 min. Normal mouse IgG (mIgG) were used as the control. Cells were then washed with acidic buffer to eliminate cell surface bound IgG and re-incubated with Zt/c1, which binds to different epitopes on RON extracellular domains (34). Positive controls (CTL) were cells incubated with Zt/c1 at 4˚C to minimize the temperature-induced internalization. Rabbit anti-mouse IgG conjugated with FITC was used as the detecting antibody. (B) Relative fluorescent intensities of individual samples in (A). (C) Confocal analysis of mAb-induced RON internalization. SW620 cells grown on glass surface were incubated with Zt/g4 at 4˚C followed by addition of FITC-coupled anti-mouse IgG. Cells were then incubated at 4˚C or switched to 37˚C for 60 min. Endocytic inhibitor filipin (10 μg/ml) (37) was added simultaneously. After incubation, cells from 37˚C were washing with acidified buffer to remove cell-surface bound antibody. Nucleus was stained with propidium iodide. FITC-fluorescence was examined under an Olympus DSU confocal microscope. (D) Effect of lysosomal or proteasomal inhibitors on Zt/g4-induced RON degradation. SW620 cells ($2x10^6$ in 60-mm dish) were treated with 10 μg/ml of Zt/g4 alone or in combination with 100 nM of concanamycin A (conc), 100 nM of lactacystine (lact), or both for 24 h. Levels of RON in the cell lysates (50 μg/sample) were detected by Western blot analysis as described in Fig. 1A. ß-actin was used as the controls. Data shown here are from one of three experiments with similar results.

Concentration, kinetic activity and specificity of Zt/g4-induced down-regulation of RON expression. The dose-dependent down-regulation of RON expression by Zt/g4 is shown in Fig. 2A. The significant reduction of RON ß-chain expression was seen when 2 μg/ml of Zt/g4 was used. When 10 μg/ml of Zt/g4 were added, pro-RON expression was also reduced. Interestingly, the increase in concentration of Zt/g4 (up to 20 μg/ml) did not further cause RON reduction. Similar results were also observed when Zt/c9 and Zt/f2 were used (data not shown). Thus, the maximal effect induced by Zt/g4 was at the range of 10 μg/ml of mAb. This concentration was chosen as the standard for further experiments.

The kinetic effect of Zt/g4 was shown in Fig. 2B. Prolonged treatment of SW620 cells for up to 48 h with normal mouse IgG or MSP had no effect on levels of RON expression. In contrast, significant reduction of RON ß-chain after Zt/g4 treatment was seen as early as 3 h and lasted up to 48 h (Fig. 2B). After 24 h stimulation, the levels of pro-RON were also reduced progressively. These results suggest that Zt/g4-induced down-regulation acted rapidly and the effect was long-lasting.

Cross-talk between RON and MET or EGFR exists in cancer cells (35,36). To determine if Zt/g4-induced RON reduction affected MET or EGFR expression, breast T-47D cells were selected since they express RON, MET and EGFR (SW620 cells do not express EGFR). As shown in Fig. 2C, Zt/g4-treatment caused the progressive reduction of RON but had no effect on MET expression. The levels of MET expression remained unaffected up to 48 h after mAb treatment. In addition, Zt/g4 had no effect on EGFR expression (Fig. 2C). Similar results were also observed when Zt/f2 or Zt/c9 was used (data not shown). Thus, Zt/g4-induced RON reduction was specific to RON and had no effect on the family member MET or non-family member EGFR.

Antibody-induced RON internalization followed by protein degradation. To determine mechanisms underlying Zt/g4-induced down-regulation, we first tested if Zt/g4 induced

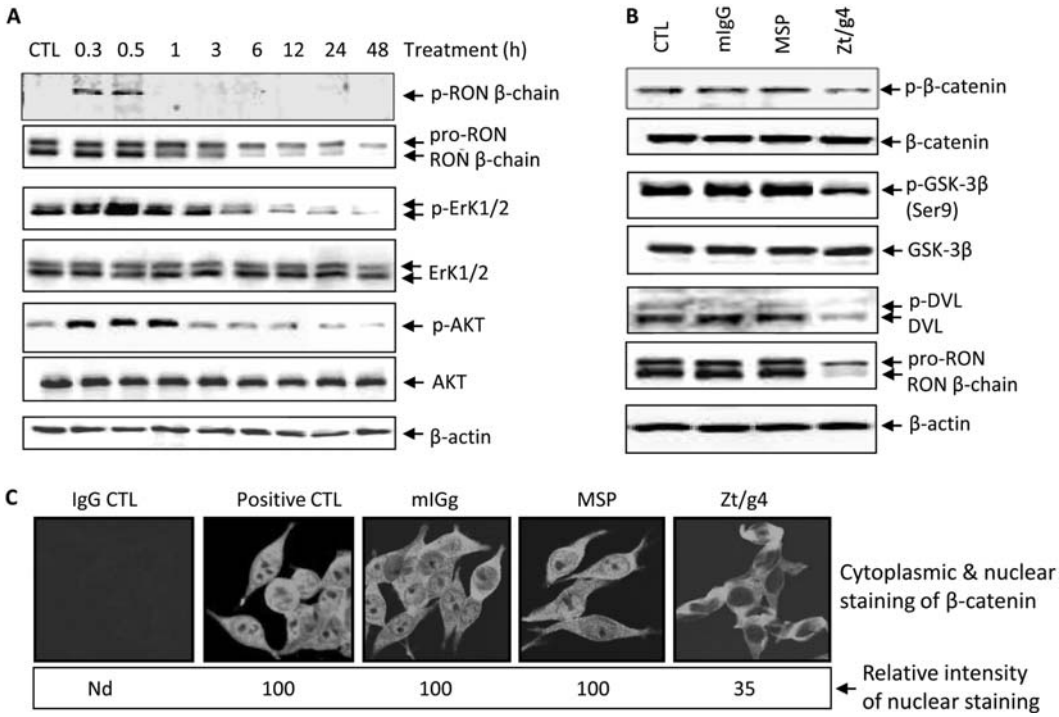

Figure 4. Effect of Zt/g4 on RON phosphorylation, activation of downstream signaling molecules and ß-catenin nuclear translocation. (A) Effect of Zt/g4 on RON phosphorylation. SW620 cells ($2x10^6$ per 60-mm culture dish) were serum-starved overnight and then treated with 10 μg/ml of Zt/g4 for various time intervals. Proteins from cell lysates were subjected to immunoprecipitation using Zt/c1 followed by Western blot analysis using PY100 to detect RON phosphorylation. The same membrane was also reprobed with rabbit anti-RON antibody to determine the levels of RON. Regular or phosphorylated Erk1/2 or AKT in cellular proteins (50 μg/sample) were determined by Western blot analysis using individual antibodies. ß-actin was used as the loading control. (B) Regulation by Zt/g4 of DVL or GSK-3ß phosphorylation. SW620 cells were treated with 10 μg/ml of Zt/g4 for 24 h. Cellular proteins (50 μg/sample) were subjected to Western blot analysis. Regular and phosphorylated GSK-3ß at Ser9 residue or DVL were detected by using corresponding antibodies, respectively. ß-actin was used as the loading control. (C) Immunofluorescent analysis of ß-catenin in cytoplasm and nucleus. SW620 cells were grown in glass chamber, treated with or without 10 μg/ml of Zt/g4 or 5 nM of MSP for 24 h. Cells treated with or without normal mouse IgG were used as the controls. Cell fixation with cold acetone and immunofluorescent detection was performed using rabbit IgG antibodies to ß-catenin as previously described (41). Relative fluorescent intensities of nuclear ß-catenin were calculated as previously described (41). Data shown here are from one of two experiments with similar results.

RON ectodomain shedding, leading to reduced RON expression. RON ectodomain was not detected in cell culture fluids from repeated experiments (data not shown). Ectodomain shedding results in the formation of truncated RON with intact C-terminal tail, which should be detected in cell lysates by Western blotting using rabbit antibodies to RON C-terminus. However, we did not find truncated proteins from Zt/g4 or other mAb-treated cells (data not shown). We then tested if RON reduction was caused by Zt/g4-induced receptor internalization. Cells were treated with Zt/g4 at 37˚C for 60 min to induce RON internalization. Results in Fig. 3A showed that levels of RON on the cell surface were dramatically reduced. More than 70% of cell surface RON was reduced within 60 min after Zt/g4 treatment (Fig. 3B). Similar results were also obtained when Zt/c9 and T-47D cells were used (data not shown), although levels of reduction varied among mAbs and cells. These results indicate that Zt/g4 treatment reduced levels of RON expressed on the cell surface.

To determine if mAb-induced RON reduction is mediated through internalization, confocal analysis was performed. As shown in Fig. 3C, fluorescence associated with cell membrane was observed when cells were incubated Zt/g4 at 4˚C. Upon incubation at 37˚C, cytoplasmic fluorescence was detected after elimination of cell surface-bound Zt/g4. However, cytoplasmic fluorescence was not observed in cells treated with filipin, an endocytic inhibitor (37). Similar results were also observed when other cells were used (data not shown). These results demonstrated that Zt/g4 treatment causes RON internalization.

Chemical inhibitors, concanamycin A and lactacystin that inhibit lysosome and proteoasome-mediated protein degradation (38,39), respectively, were used to determine RON degradation in Zt/g4-treated SW620 cells. Lactacystin or concanamycin A alone had no effect on RON expression. However, when added to cell cultures, lactacystin or concanamycin A prevented Zt/g4-induced down-regulation of RON. Moreover, when both inhibitors were combined, >95% of RON expression (pro-RON plus RON ß-chain) was recovered (Fig. 3D). These results, together with those described above, demonstrated that Zt/g4-induced down-regulation was mediated by receptor internalization and not by ectodomain shedding. The internalized RON was degraded by a combination of proteoasome and lysosome-mediated mechanisms.

Effect of mAb-induced receptor down-regulation on RON-mediated signaling. The kinetic effect of mAbs on RON phosphorylation is shown in Fig. 4A. In SW620 cells, Zt/g4 treatment resulted in transient RON phosphorylation and

peaked at 30 min. Phosphorylation was then diminished in the persistent presence of mAb. The reduction was associated with diminished RON protein expression.

We then studied the effect of Zt/g4 on RON-mediated downstream signaling with focus on the MAP kinase and PI-3-AKT pathways. As shown in Fig. 4A, Erk1/2 was spontaneously phosphorylated in SW620 cells. Zt/g4 treatment transiently induced Erk1/2 phosphorylation, which peaked at ~30 min. However, prolonged treatment progressively reduced Erk1/2 phosphorylation to the minimal level. The patterns of AKT phosphorylation were similar to those in Erk1/2. It was transiently phosphorylated and then progressively diminished (Fig. 4A). Similar results were also seen in BxPC-3 cells and T-47D cells (data not shown). These results demonstrate that activation of Erk1/2 or AKT pathways in SW620 cells was impaired after persistent Zt/g4 treatment.

Altered RON activation increases cytoplasmic ß-catenin accumulation and nuclear translocation, which is regulated through disheveled (DVL) and glycogen synthase kinase (GSK)-3ß (40,41). As shown in Fig. 4B, Zt/g4 treatment moderately inhibited ß-catenin phosphorylation but had no effect on cytoplasmic ß-catenin accumulation. Similarly, the phosphorylation levels of GSK-3ß (ser49) and DVL were reduced after Zt/g4 treatment. Interestingly, Zt/g4 treatment reduced DVL protein expression, but had no effect on GSK-3ß. The inhibitory effect of Zt/g4 on phosphorylation of ß-catenin, GSK-3ß and DVL was accompanied with the reduction of RON expression. Results in Fig. 4C were the analysis of ß-catenin nuclear translocation by immunofluorescent staining. ß-catenin existed in both cytoplasmic and nuclear compartments. Addition of normal mouse IgG or MSP had no effect on ß-catenin distribution. However, after Zt/g4 treatment, the amount of nuclear ß-catenin was significantly reduced as judged by fluorescent intensity. These results, together with those from Fig. 4A and B demonstrate that Zt/g4-induced down-regulation of RON expression was accompanied with reduced phosphorylation of ß-catenin, DVL and GSK-3ß. Zt/g4 also decreased the ß-catenin accumulation in the nucleus, although the total amount was not significantly changed.

Effect of Zt/g4 on tumor cell morphology, colony formation and drug sensitivity. The effect of Zt/g4 on cell morphology is shown in Fig. 5A. SW620 cells treated with or without normal mouse IgG displayed typical epithelial morphologies. MSP slightly induced cell elongation and scattering. However, after Zt/g4 treatment, the majority of SW620 cells displayed rounded up morphology and only a small fraction of cells maintained epithelial cell appearance. Moreover, Zt/g4-treated cells did not attach firmly to the surface of the culture dish. Similar results were also seen in Zt/g4-treated BxPC-3 cells (data not shown). These observations suggest that Zt/g4 treatment affected cell adhesion and spreading.

The effect of Zt/g4 on anchorage-independent growth was examined in the soft agar assay. As shown in Fig. 5B, SW620 cells formed numerous colonies in soft agar. Normal mouse IgG had no effect on the colony formation and MSP only slightly increased the number of colonies. However, in the presence of Zt/g4, the number of colonies was significantly reduced. Moreover, the size of the colonies was much smaller

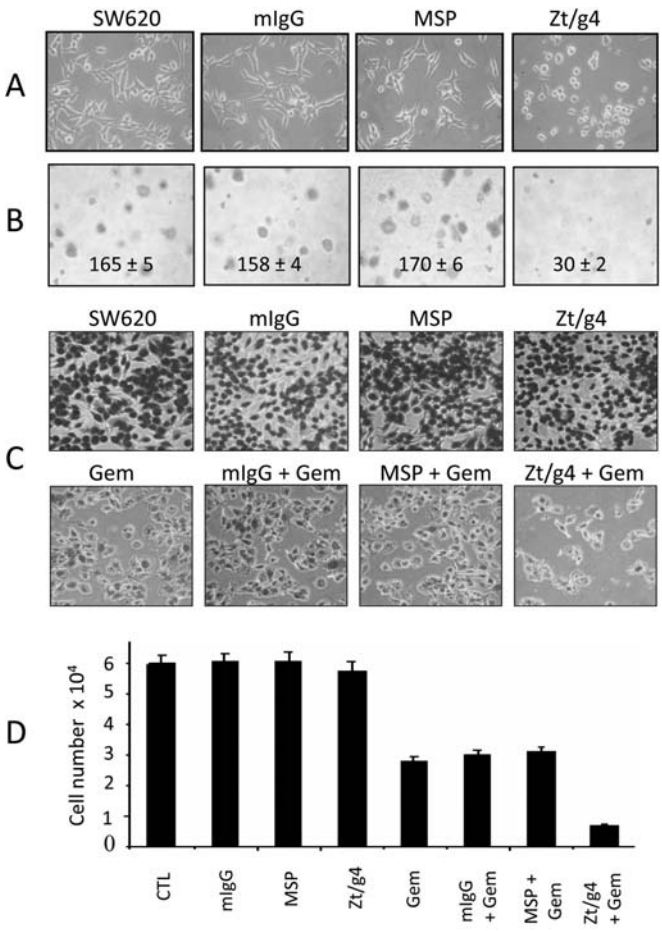

Figure 5. Effects of Zt/g4 treatment on cellular morphology, colony formation and sensitivity towards gemcitabine cytotoxicity. (A) Effect of Zt/g4 on morphological changes of SW620 cells. Cells ($2x10^6$ in 60-mm culture dish) were treated with 10 μg/ml of Zt/g4 or 5 nM of MSP for 24 h. Cells treated with normal mouse IgG were used as the controls. Morphological changes were observed under Olympus X-71 inverted microscope and photographed. (B) Inhibitory effect of Ztg4 on colony formation of SW620 cells. Cells ($2x10^6$ per dish) were first treated with or without 10 μg/ml of Zt/g4 for 24 h. Cells were collected, suspended in medium containing 0.3% agar, and then seeded in a 24-well plate (2000 cells/well) in duplicate in the top layer of soft agar containing 10 μg/ml of Zt/g4, normal mouse IgG, or 5 nM of MSP. After incubation for 12 days, numbers of colonies in individual wells were counted and photographed. (C) Increased sensitivity of Zt/g4-treated SW620 cells towards gemcitabine-induced cytotoxicity. SW620 cells ($1x10^4$ cells/well in a 96-well plate) were treated with 10 μg/ml of Zt/g4 in triplicate for 24 h and then added with 2 μM of gemcitabine for 3 days. Cells were fixed with Diff-quick solution and photographed. (D) Effect of Zt/g4 on gemcitabine cytotoxicity. Survival cells from different groups as described in C were collected and counted to determine the differences in cell numbers among different groups. Data shown here are from one of two experiments with similar results.

than the control groups. Similar results were also found in BxPC-3 cells (data not shown). These results suggest that Zt/g4 treatment affected anchorage-independent growth of SW620 cells in soft agar.

Since Zt/g4 treatment regulates SW620 cell adhesion and growth and may affect the cellular responsiveness towards chemotherapeutic drugs, the effect of Zt/g4 on sensitivity of SW620 cells towards gemcitabine was studied. Cells were incubated with Zt/g4 (10 μg/ml) for 24 h and then treated

with gemcitabine (2 μM, IC_{50} value for SW620 cells) for 72 h. As shown in Fig. 5C and D, Zt/g4 treatment did not cause cell death. The number of cells was comparable to the control cells. Gemcitabine induced cytotoxic death of SW620 cells, which was not affected by addition of normal mouse IgG or MSP. However, Zt/g4 enhanced cytotoxic activities of gemcitabine. The percentage of surviving cells was significantly reduced when gemcitabine and Zt/g4 were used together. These results indicate that Zt/g4 increased the sensitivity of SW620 cells towards cytotoxic activities of gemcitabine.

Discussion

The purpose of this study was to determine the effect of anti-RON mAbs on RON expression by colon cancer cells. Special attention was focused on Zt/g4 because of its unique binding specificity to the RON extracellular domains. Altered RON expression exists in large numbers of colon cancer samples (11,42) increasing tumorigenic activities (22,27). Clearly, targeting RON signaling has potential for therapeutic purpose (27,43). Recent studies using therapeutic mAbs and small molecule inhibitors have shown that inhibition of RON has clinical relevance in the treatment of colon and other epithelial cancers (31,32). Our current studies demonstrate that mAb-induced RON reduction decreases tumorigenic activities of colon cancer cells. We showed that Zt/g4, Zt/c9, or Zt/f2 significantly diminish RON expression by colon cancer cells. These effects were mediated by antibody-induced RON internalization, which facilitates protein degradation. Moreover, we found that Zt/g4 treatment attenuates RON-mediated signaling cascades, which leads to altered cell morphology, impaired cell growth and enhanced sensitivity towards gemcitabine cytotoxicity. Thus, Zt/g4-directed RON reduction has profound impact on tumorigenic activities of colon cancer cells. Further evaluation of Zt/g4 and other mAbs should establish the experimental foundation for the development of RON targeted cancer therapy.

Regulation of RON expression in epithelial tissues, particularly in cancer cells, is a complicated process (44,45). RON expression is low or moderate in normal epithelial cells. However, upon transformation, RON expression is dramatically increased in certain types of cancers including those from colon, breast and pancreatic tissues (11). Various mechanisms including transcriptional and translational regulation are involved in controlling RON expression (44,45). At protein level, RON is mainly regulated by cellular ubiquitylation and degradation systems (46,47). In normal epithelial cells, RON physically forms a protein-protein complex with the E3 ligase c-Cbl (46,47). Upon activation, RON is internalized and degraded through protein degradation pathways (46,47). However, this pathway is often dysregulated in cancerous cells. For example, RON mutants are highly resistant to endocytosis and ubiquitylation (46,47). Such changes result in abnormal accumulation and activation of RON in cancer cells.

Results in this study provide evidence showing that antibodies including Zt/g4, Zt/c9 and Zt/f2 are highly effective in down-regulation of RON expression by colon, breast and pancreatic cancer cells. These activities are of biological significance. It provides the mechanistic explanation underlying the therapeutic activities of mAbs. Moreover, it confirms the feasibility of the use of mAb to overcome resistance of RON towards internalization and degradation. By analyzing Zt/g4 activities, the following features were observed. First, the effect of Zt/g4 and other two mAbs on RON expression is not limited to a particular type of cancer cells. Zt/g4 also down-regulates RON expression by pancreatic and breast cancer cells (Fig. 1). These data suggest that a common mechanism is trigged by three mAbs that promote RON internalization and subsequent degradation. Second, the binding of mAbs to the epitopes either on sema or IPT domains is sufficient to cause RON reduction. These data indicate that Zt/g4 or other two mAbs act as a trigger that facilitates RON internalization and degradation. It is possible that epitopes on sema or IPT domains are critically important in regulating RON stability on the cell surface. One interesting observation is the down-regulation of pro-RON after mAb treatment (Figs. 1A and 2B). Pro-RON resides in cytoplasm and requires proteolytic conversion to be expressed on the cell surface (48). Since pro-RON is not in direct contact with mAb, the mechanism underlying its reduction is currently unknown. It is reasoned that internalized mAb may act on intracellular pro-RON and trigger it degradation. Third, synergistic activities exist between two mAbs regardless of their difference in epitope binding. Zt/g4 plus Zt/f2 or Zt/c9 displayed enhanced activity in down-regulation of RON. Similarly, Zt/f2 in combination with Zt/c9 showed similar synergistic activity (Fig. 1D). A noteworthy observation is that synergistic activities were seen only during the early but not later stages of mAb treatment. Nevertheless, these data suggest that the binding of two mAbs to their corresponding epitopes accelerates the process of RON internalization and degradation. Fourth, the effect of Zt/g4 is both concentration and kinetic-dependent. Significant reduction was seen when 2 μg of Zt/g4 per ml were used and the maximal effect was at the range of 10 μg per ml. Further increase of Zt/g4 up to 20 μg per ml did not yield additional effect (Fig. 2A). Kinetic analysis showed that the effect of Zt/g4 occurs as early as 3 h after Zt/g4 treatment and lasted up to 48 h (Fig. 2B), indicating that Zt/g4-induced RON reduction is a rapid and progressive process. Considering these data, we believe that maximal effect is related to the amounts of mAb that bind to RON extracellular domain. Since the binding of Zt/g4 to RON achieved saturation levels at ~10 μg per ml per $2x10^6$ SW620 cells, it is reasoned that Zt/g4-saturated binding is required to reach the maximal effect. We did not see the effect of MSP on RON expression as displayed in Fig. 2B (second panel). Previous studies using RON cDNA-transfected cells have shown that MSP is capable of inducing RON reduction through receptor internalization (46,47). However, our repeated experiments did not observe these changes in colon and other cancer cells. It is possible that cellular events responsible for MSP-induced RON reduction are altered in tumor cells. Finally, the effect of Zt/g4 is specific to RON. It has no effect on the structurally-related MET or unrelated EGFR, even though both are known to form a protein-protein complex with RON and cause biological activities (35,36).

Antibody-induced receptor internalization and degradation is a mechanism that diminishes cell surface RTK expression (4). The therapeutic activity of trastuzumab is mediated by inducing HER2 internalization that reduces its cell surface expression followed by protein degradation (49). Similar mechanism seems to be utilized by Zt/g4 and two other mAbs to reduce RON expression. Zt/g4 reduces the density of RON expressed on the SW620 cell surface (Fig. 3), which is caused by Zt/g4-induced RON internalization because receptor ectodomain shedding was not observed. By studying the effect of chemical inhibitors on RON degradation, we observed that lysosomal and proteasomal degradation pathways are both involved in RON reduction (Fig. 3C). Lactacystin is a selective inhibitor of the proteasome events (39) and concanamycin A inhibits the lysosomal degradation pathway (38). Individually, both chemicals were able to partially prevent Zt/g4-induced RON degradation. Their combination exerted more potent activities, which almost completely prevents Zt/g4-induced RON degradation. These results suggest that Zt/g4-induced RON reduction follows the common pathways employed by other therapeutic antibodies (4). Both lysosomal and proteasomal degradation pathways are involved in the process.

Overexpression of RON in colon cancer cells is featured by activation of multiple signaling cascades including the MAP kinase and ß-catenin pathways (22,27), which are important in RON-mediated tumorigenic activities (22,27). We observed that Zt/g4 treatment transiently increases RON phosphorylation. However, such activation was progressively lost due to diminished RON expression. Transient activation of Erk1/2 and AKT was also observed. Again, such increase was progressively diminished to the minimal levels (Fig. 4A). These results indicate that the effect of Zt/g4 on RON has two phases: transient activation followed by prolonged inactivation. A biochemical link exists between phosphorylation of RON and activation of Erk1/2 and AKT during the early stages of Zt/g4 treatment. However, persistent Zt/g4 treatment reduced RON expression leading to impairment of Erk1/2 and AKT activation. Thus, Zt/g4-induced RON reduction disrupts the downstream signaling events, which ultimately affects tumorigenic activities.

ß-catenin plays a unique role in RON-mediated tumorigenic activities in colon cancer cells (40,41). The pathogenesis of ß-catenin is by its abnormal cytoplasmic accumulation followed by nuclear translocation, which facilitates the transcription of target genes. Proteins such as DVL and GSK-3ß regulate ß-catenin stability (40,41). Altered RON expression activates DVL and inactivates GSK-3ß through phosphorylation (41), which causes the increased ß-catenin accumulation and nuclear translocation (40,41). Results in Fig. 4 demonstrated that ß-catenin is highly expressed and localized in both cytoplasmic and nuclear compartments of SW620 cells, which is accompanied by spontaneous phosphorylation of DVL and GSK-3ß Ser9 residue. However, upon Zt/g4 treatment, phosphorylation of GSK-3ß at Ser9 residue was reduced. Zt/g4 also decreased the levels of DVL phosphorylation and reduced DVL protein expression. Since RON forms a protein-protein complex with DVL increasing DVL phosphorylation (40,41), we reasoned that Zt/g4 treatment impairs RON interaction with DVL, which affect DVL phosphorylation and expression. Consistent with these results, we observed that Zt/g4 treatment reduces the levels of ß-catenin accumulated in nucleus (Fig. 4C). The decreased DVL expression and activation is a known factor contri-buting to the ß-catenin nuclear translocation. Thus, Zt/g4 treatment had profound impact on intracellular signaling events. The disruption of RON-mediated signaling could be a mechanism underlying the therapeutical activities of Zt/g4.

The biological consequences after Zt/g4 treatment are presented in Fig. 5. These results serve as the functional link to the observed biochemical changes described in Fig. 1 and 4. Zt/g4 treatment impairs cell adhesion and spreading in the early stages of cell culture with rounded up morphology. However, these impairments were completely recovered after prolonged incubation. No cell death or growth reduction was observed. Although the significant reduction in cell proliferation was not seen in Zt/g4 treated cells, anchorage-dependent growth of SW620 cells in soft agar was dramatically affected. SW620 cells are tumorigenic and their growth was not affected when suspended in soft agar (Fig. 5B). However, Zt/g4 treatment impairs SW620 colony formation. The number of colonies was reduced significantly although not eliminated completely. Moreover, the size of colonies was relatively smaller as compared to those from controls. Clearly, Zt/g4-induced RON reduction affects anchorage-independent growth of SW620 cells.

Aberrant expression of RON contributes to the development of malignant phenotypes (22), which is reflected by increased survival of cancer cells against hostile environment (22,27). Overexpression of RON increases drug resistance of tumor cells (12). Considering the effect of Zt/g4 on RON expression, we tested the possibility that Zt/g4 attenuates the resistant phenotype of SW620 cells in response to gemcitabine. Gemcitabine is a nucleoside analog used to treat various carcinomas. SW620 cells are relatively resistant *in vitro* to gemcitabine with IC_{50} values at ~2 μM (0.6 μg/ml per $1x10^6$ cells). Addition of MSP has little effect on protection of cancer cells from killing. However, Zt/g4 treatment dramatically increased the cytotoxic activities of gemcitabine. The percentage of cell death reached ~88% with only a small fraction of cells still survived. These results indicate that Zt/g4 increases the sensitivity of SW620 cells towards cytotoxicity of gemcitabine. Currently, we do not know the exact mechanisms underlying the role of Zt/g4 in increasing gemcitabine cytotoxicity. One possible explanation is that Zt/g4-induced RON reduction followed by attenuation of downstream signals such as inactivation of the Erk1/2 pathway may render cells more susceptible to gemcitabine-induced cytotoxicity. In addition, the inhibitory effect of Zt/g4 on ß-catenin nuclear translocation as shown in Fig. 4C may also play a role in regulating the cellular sensitivity towards gemcitabine. Regardless of the involved mechanisms, these results demonstrate that Zt/g4 is beneficial in combination with chemotherapeutic agents to kill cancer cells. Clinically, therapeutic antibodies in combination with chemodrugs provide better responsiveness in cancer treatment (9). With these observations in mind, further investigation of Zt/g4 in combination with chemodrugs is warranted.

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References

1. Zandi R, Larsen AB, Andersen P, Stockhausen MT and Poulsen HS: Mechanisms for oncogenic activation of the epidermal growth factor receptor. Cell Signal 19: 2013-2023, 2007.
2. Gao CF and Vande Woude GF: HGF/SF-Met signaling in tumor progression. Cell Res 15: 49-51, 2005.
3. Porter AC and Vaillancourt RR: Tyrosine kinase receptor-activated signal transduction pathways which lead to oncogenesis. Oncogene 17: 1343-1352, 1998.
4. Bache KG, Slagsvold T and Stenmark H: Defective down-regulation of receptor tyrosine kinases in cancer. EMBO J 23: 2707-2712, 2004.
5. Weinstein IB and Joe A: Oncogene addiction. Cancer Res 68: 3077-3080, 2008.
6. Fischgräbe J and Wülfing P: Targeted therapies in breast cancer: established drugs and recent developments. Curr Clin Pharmacol 3: 85-98, 2008.
7. Vincenzi B, Schiavon G, Silletta M, Santini D and Tonini G: The biological properties of cetuximab. Crit Rev Oncol Hematol 68: 93-106, 2008.
8. Shepard HM, Brdlik CM and Schreiber H: Signal integration: a framework for understanding the efficacy of therapeutics targeting the human EGFR family. J Clin Invest 118: 3574-3581, 2008.
9. Tortora G, Ciardiello F and Gasparini G: Combined targeting of EGFR-dependent and VEGF-dependent pathways: rationale, preclinical studies and clinical applications. Nat Clin Pract Oncol 5: 521-530, 2008.
10. Ronsin C, Muscatelli F, Mattei MG and Breathnach R: A novel putative receptor protein tyrosine kinase of the met family. Oncogene 8: 1195-1202, 1993.
11. Wang MH, Lee W, Luo YL, Weis MT and Yao HP: Altered expression of the RON receptor tyrosine kinase in various epithelial cancers and its contribution to tumourigenic phenotypes in thyroid cancer cells. J Pathol 213: 402-411, 2007.
12. Thomas RM, Toney K, Fenoglio-Preiser C, Revelo-Penafiel MP, Hingorani SR, Tuveson DA, Waltz SE and Lowy AM: The RON receptor tyrosine kinase mediates oncogenic phenotypes in pancreatic cancer cells and is increasingly expressed during pancreatic cancer progression. Cancer Res 67: 6075-6082, 2007.
13. Maggiora P, Marchio S, Stella MC, *et al*: Overexpression of the RON gene in human breast carcinoma. Oncogene 16: 2927-2933, 1998.
14. Lee WY, Chen HH, Chow NH, Su WC, Lin PW and Guo HR: Prognostic significance of co-expression of RON and MET receptors in node-negative breast cancer patients. Clin Cancer Res 11: 2222-2228, 2005.
15. Lee CT, Chow NH, Su PF, Lin SC, Lin PC and Lee JC: The prognostic significance of RON and MET receptor coexpression in patients with colorectal cancer. Dis Colon Rectum 51: 1268-1274, 2008.
16. Wang MH, Kurtz AL and Chen YQ: Identification of a novel splicing product of the RON receptor tyrosine kinase in human colorectal carcinoma cells. Carcinogenesis 21: 1507-1512, 2000.
17. Collesi C, Santoro MM, Gaudino G and Comoglio PM: A splicing variant of the RON transcript induces constitutive tyrosine kinase activity and an invasive phenotype. Mol Cell Biol 16: 5518-5526, 1996.
18. Lu Y, Yao HP and Wang MH: Multiple variants of the RON receptor tyrosine kinase: biochemical properties, tumorigenic activities, and potential drug targets. Cancer Lett 257: 157-164, 2007.
19. Peace BE, Toney-Earley K, Collins MH and Waltz SE: Ron receptor signaling augments mammary tumor formation and metastasis in a murine model of breast cancer. Cancer Res 65: 1285-1293, 2005.
20. Chen YQ, Zhou YQ, Fisher JH and Wang MH: Targeted expression of the receptor tyrosine kinase RON in distal lung epithelial cells results in multiple tumor formation: oncogenic potential of RON in vivo. Oncogene 21: 6382-6386, 2000.
21. Chan EL, Peace BE, Collins MH, Toney-Earley K and Waltz SE: Ron tyrosine kinase receptor regulates papilloma growth and malignant conversion in a murine model of skin carcinogenesis. Oncogene 24: 479-488, 2005.
22. Wagh PK, Peace BE and Waltz SE: Met-related receptor tyrosine kinase Ron in tumor growth and metastasis. Adv Cancer Res 100: 1-33, 2008.
23. Camp ER, Liu W, Fan F, Yang A, Somcio R and Ellis LM: RON, a tyrosine kinase receptor involved in tumor progression and metastasis. Ann Surg Oncol 12: 273-281, 2005.
24. Wang D, Shen Q, Chen YQ and Wang MH: Collaborative activities of macrophage-stimulating protein and transforming growth factor-beta1 in induction of epithelial to mesenchymal transition: roles of the RON receptor tyrosine kinase. Oncogene 23: 1668-1680, 2004.
25. Acloque H, Adams MS, Fishwick K, Bronner-Fraser M and Nieto MA: Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. J Clin Invest 119: 1438-1449, 2009.
26. Turley EA, Veiseh M, Radisky DC and Bissell MJ: Mechanisms of disease: epithelial-mesenchymal transition - does cellular plasticity fuel neoplastic progression? Nat Clin Pract Oncol 5: 280-290, 2008.
27. Wang MH, Yao HP and Zhou YQ: Oncogenesis of RON receptor tyrosine kinase: a molecular target for malignant epithelial cancers. Acta Pharmacol Sin 27: 641-650, 2006.
28. Xu XM, Wang D, Shen Q, Chen YQ and Wang MH: RNA-mediated gene silencing of the RON receptor tyrosine kinase alters oncogenic phenotypes of human colorectal carcinoma cells. Oncogene 23: 8464-8474, 2004.
29. Wang J, Rajput A, Kan JL, *et al*: Knockdown of Ron kinase inhibits mutant phosphatidylinositol 3-kinase and reduces metastasis in human colon carcinoma. J Biol Chem 284: 10912-10922, 2009.
30. Wang MH, Lao WF, Wang D, Luo YL and Yao HP: Blocking tumorigenic activities of colorectal cancer cells by a splicing RON receptor variant defective in the tyrosine kinase domain. Cancer Biol Ther 6: 1121-1129, 2007.
31. O'Toole JM, Rabenau KE, Burns K, *et al*: Therapeutic implications of a human neutralizing antibody to the macrophage-stimulating protein receptor tyrosine kinase (RON), a c-MET family member. Cancer Res 66: 9162-9170, 2006.
32. Zhang Y, Kaplan-Lefko PJ, Rex K, *et al*: Identification of a novel recepteur d'origine nantais/c-met small-molecule kinase inhibitor with antitumor activity in vivo. Cancer Res 68: 6680-6687, 2008.
33. Christensen JG, Schreck R, Burrows J, *et al*: A selective small molecule inhibitor of c-Met kinase inhibits c-Met-dependent phenotypes in vitro and exhibits cytoreductive antitumor activity in vivo. Cancer Res 63: 7345-7355, 2003.
34. Yao HP, Luo YL, Feng L, Cheng LF, Lu Y, Li W and Wang MH: Agonistic monoclonal antibodies potentiate tumorigenic and invasive activities of splicing variant of the RON receptor tyrosine kinase. Cancer Biol Ther 5: 1179-1186, 2006.
35. Follenzi A, Bakovic S, Gual P, Stella MC, Longati P and Comoglio PM: Cross-talk between the proto-oncogenes Met and Ron. Oncogene 19: 3041-3049, 2000.
36. Peace BE, Hill KJ, Degen SJ and Waltz SE: Cross-talk between the receptor tyrosine kinases Ron and epidermal growth factor receptor. Exp Cell Res 289: 317-325, 2003.
37. Torgersen ML, Skretting G, van Deurs B and Sandvig K: Internalization of cholera toxin by different endocytic mechanisms. J Cell Science 114: 3737-3747, 2001.
38. Petrelli A, Circosta P, Granziero L, Mazzone M, Pisacane A, Fenoglio S, Comoglio PM and Giordano S: Ab-induced ectodomain shedding mediates hepatocyte growth factor receptor down-regulation and hampers biological activity. Proc Natl Acad Sci USA 103: 5090-5095, 2006.

39. Orlowski RZ: The role of the ubiquitin-proteasome pathway in apoptosis. Cell Death Differ 6: 303-313, 1999.
40. Danilkovitch-Miagkova A, Miagkov A, Skeel A, Nakaigawa N, Zbar B and Leonard EJ: Oncogenic mutants of RON and MET receptor tyrosine kinases cause activation of the beta-catenin pathway. Mol Cell Biol 21: 5857-5868, 2001.
41. Xu XM, Zhou YQ and Wang MH: Mechanisms of cytoplasmic (beta)-catenin accumulation and its involvement in tumorigenic activities mediated by oncogenic splicing variant of the receptor originated from Nantes tyrosine kinase. J Biol Chem 280: 25087-25094, 2005.
42. Zhou YQ, He C, Chen YQ, Wang D and Wang MH: Altered expression of the RON receptor tyrosine kinase in primary human colorectal adenocarcinomas: generation of different splicing RON variants and their oncogenic potential. Oncogene 22: 186-197, 2003.
43. Dussault I, Bellon SF. From concept to reality: the long road to c-Met and RON receptor tyrosine kinase inhibitors for the treatment of cancer. Anticancer Agents Med Chem 9: 221-229, 2009.
44. Thangasamy A, Rogge J and Ammanamanchi S: Recepteur d'origine nantais tyrosine kinase is a direct target of hypoxia-inducible factor-1alpha-mediated invasion of breast carcinoma cells. J Biol Chem 284: 14001-14010, 2009.
45. Zalcenstein A, Weisz L, Stambolsky P, Bar J, Rotter V and Oren M: Repression of the MSP/MST-1 gene contributes to the antiapoptotic gain of function of mutant p53. Oncogene 25: 359-69, 2006.
46. Germano S, Barberis D, Santoro MM, Penengo L, Citri A, Yarden Y and Gaudino G: Geldanamycins trigger a novel Ron degradative pathway, hampering oncogenic signaling. J Biol Chem 281: 21710-21719, 2006.
47. Penengo L, Rubin C, Yarden Y and Gaudino G: c-Cbl is a critical modulator of the Ron tyrosine kinase receptor. Oncogene 22: 3669-3679, 2006.
48. Wang MH, Julian FM, Breathnach R, *et al*: Macrophage stimulating protein (MSP) binds to its receptor via the MSP beta chain. J Biol Chem 272: 16999-7004, 1997.
49. Hudis CA: Trastuzumab - mechanism of action and use in clinical practice. N Engl J Med 357: 39-51, 2007.