Abstract. Aim: We investigated the effects of trastuzumab, an anti-HER2 humanized monoclonal antibody, on DNA breaks induced by SN-38, a topoisomerase-1 inhibitor, in gastric cancer cell lines positive or negative for HER2 expression. Materials and Methods: NCI-N87 (HER2+) and MKN74 (HER2−) cells were exposed to SN-38 in the presence or absence of trastuzumab. Trastuzumab was added either prior to or after SN-38. Effects of trastuzumab on the induction of gamma-H2AX, a marker of DNA double-strand breaks, the cytotoxicity of SN-38 and cell cycle progression were determined. Results: When trastuzumab was administered following SN-38, it increased γH2AX levels and cytotoxicity of SN-38 in NCI-N87 cells, but not in MKN74 cells. In contrast, pretreatment with trastuzumab reduced SN-38-induced γH2AX expression and cytotoxicity of SN-38 in NCI-N87 cells, but not in MKN74 cells. Trastuzumab delayed cell cycle progression in NCI-N87 cells only. Conclusion: Trastuzumab has opposing effects on SN-38-induced double-strand breaks and cytotoxicity depending on the order of administration of the two agents.

Gastric cancer is one of the most common malignant diseases in Asian countries. Around 20% of gastric carcinoma express HER2 (ERBB2/neu), a member of the epidermal growth factor receptor (EGFR) family (1). Gastric cancer with HER2 amplification has been associated with a poor prognosis (2). Trastuzumab, a humanized anti-HER2 monoclonal antibody, inhibits downstream signaling from HER2 receptors, resulting in the inhibition of growth of HER2-positive cells (3). While the efficacy of trastuzumab in the treatment of metastatic breast cancer with overexpression of HER2 is well known (4), the last decade has also seen many studies showing the in vitro and in vivo antitumor effects of trastuzumab in HER2-overexpressing gastric cancer (5-7). Furthermore, the 2009 phase III clinical trial, a study of trastuzumab in combination with chemotherapy compared with chemotherapy alone in patients with HER2-positive advanced gastric cancer (ToGA trial), also demonstrated that trastuzumab enhanced the therapeutic efficacy of antineoplastic agents for gastric cancer with HER2 expression (8). The mechanisms by which trastuzumab enhances the therapeutic efficacy of anticaner agents have been extensively studied. However, the role of trastuzumab on DNA breaks and cytotoxicity of antineoplastic agents in gastric cancer has not been fully elucidated.

Recent studies have found that EGFR family is associated with DNA repair and that inhibition of the EGF signaling pathway attenuates the repair of DNA breaks. EGFR facilitates the repair of DNA breaks induced by ionizing radiation (9), and promotes both non-homologous end-joining (NHEJ) and homologous recombination (HR) (10). We therefore considered that trastuzumab enhances the therapeutic efficacy of chemotherapy for HER2-positive cancer by attenuating the repair of DNA breaks induced by antineoplastic agents.

The HER2 signaling pathway stimulates cellular growth by promoting DNA replication and transcription in cells. Trastuzumab inhibits this cellular replication and transcription (11). However, most antineoplastic agents induce DNA double-strand breaks (DSBs) during replication or transcription. For example, SN-38, a topoisomerase-1 inhibitor, induces DNA DSBs in a replication-dependent manner (12). Accordingly, the inhibition of cellular replication by trastuzumab might theoretically reduce the chance of collision of replication forks and topoisomerase-1-DNA cleavage complex by SN-38, resulting in a decrease in the induction of replication-mediated DNA breaks by SN-38
in HER2-positive cells. These considerations raise the question of whether trastuzumab always works synergistically with antineoplastic agents that induce DNA breaks in a replication-dependent manner.

Here, to better understand the role of the order of trastuzumab administration in the processing and repair of replication-mediated DSBs, and the rationale for the sequential use of trastuzumab following antineoplastic agents in HER2-positive gastric cancer, we investigated the influence of pre- and post-treatment with trastuzumab on the replication-mediated generation of DSBs by a topoisomerase-1 inhibitor, SN-38, using gamma-H2AX (γH2AX) and its cytotoxic effect in human gastric cancer cells positive or negative for HER2 expression.

Materials and Methods

Cell culture and cell cycle synchronization. The human gastric cancer-derived cell line NCI-N87 (N87) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the human gastric cancer cell line MKN74 from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). They were cultured at 37°C in the presence of 5% CO₂ in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen Co., Carlsbad, CA, USA). 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Co.) were used. HER2 expression was strong in N87 cells, but undetectable in MKN74 cells, as established by western blotting (data not shown).

Cell cycle synchronization was performed after incubation of an appropriate number of cells. Cells were initially seeded at a cell density of 1×10⁵ cells/ml and incubated at 37°C in the presence of 5% CO₂ in RPMI-1640 medium. Aphidicolin (1 μM) was then added for 18 h to synchronize cells at the G₀ phase. After incubation with aphidicolin, the cells were then suspended in fresh medium containing FBS and allowed to progress synchronously with or without treatment with trastuzumab or control immunoglobulin G (IgG; 10 mg/ml).

Drugs, chemicals and antibodies. We used SN-38 as a cytotoxic antineoplastic agent. SN-38 is an active metabolite of irinotecan and induces DNA DSBs in cells in a replication-dependent manner (12). SN-38 was kindly provided by Yakult Pharmaceutical Industry Co. (Tokyo, Japan), and trastuzumab by Chugai Pharmaceutical Co. (Tokyo, Japan). Aphidicolin and control IgG1 (purified immunoglobulin) were purchased from Sigma-Aldrich Chemical Co. (Tokyo, Japan). Aphidicolin and control IgG1 were pelleted from the supernatant by adding a 1/4 volume of 100% (w/v) H₂SO₄, and centrifuged at 15490 ×g (13000 r/min). Histones were dissolved in ultrapure water and evaluated for protein concentration (Bio-Rad, Hercules, CA, USA). An aliquot corresponding to 10 μg of protein was boiled in SDS sample buffer (Tris-glycine SDS sample buffer (2x); Invitrogen Co.) and loaded onto the 4-20% Tris-glycine precast gel (Invitrogen Co.). The separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore) for 45 min at 15 V. The membrane was blocked with TBST (10 mM Tris-Cl pH7.4, 200 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk for 1 h, and had prior incubation with anti-γH2AX antibody (1:2000 dilution) for 2 h. The

MTT assay. Cells were seeded into 96-well microplates at 5000 cells/80 μl in each well and incubated for 3-6 days to an appropriate degree of confluence. The cells were then treated with or without the indicated concentrations of SN-38 for 24 h. Trastuzumab was added before or after incubation with SN-38 and the cells were then incubated for a further 72 h. Following incubation, 10 μl of MTT (Sigma-Aldrich Chemical Co.) solution (5 mg/ml) was then added to each well, followed 6 h later by aspiration of the medium and the addition of 100 μl Dimethyl sulfoxide (DMSO) to each well. The optical density of each well (wavelength 595 nm) was measured with a microplate reader (MTP-800AFC/Lab; Corona Electric Co. Ltd. Ibaraki, Japan) and the survival of cells at each drug concentration was expressed as the percentage (%) ratio of the optical density of untreated cells. The means of four independent experiments were used.

Immunohistochemistry. The two cell types were seeded into 4-well chamber plates at 1×10⁵ cells/ml in each well, incubated for 3-6 days to an appropriate degree of confluence, then treated with either or both SN-38 and trastuzumab as indicated in the protocol. Following incubation, the medium was removed and the cells were washed twice with phosphate buffered saline (PBS). The cells were then fixed with 2% paraformaldehyde (PFA) for 10 min. After removal of PFA, they were washed twice with PBS for 10 min and permeabilized with 100% methanol for 20 min at –20°C. After removal of methanol, cells were again washed twice with PBS.

After fixation, the cells were blocked with blocking solution (1% bovine serum albumin, 5% goat serum) for 1 h. The blocking solution was then removed and the primary antibody treatment was performed for 2 h at room temperature or in the refrigerator overnight. After removal of the primary antibody, the cells were washed three times with PBS for 10 min and treated with secondary antibody (1/200 dilution) for 1 h in darkness. For propidium iodide (PI) staining, the cells were washed three times with PBS for 10 min after removal of the secondary antibody and then incubated with PBS containing PI and RNase (PI 25 μg/ml and RNase 1-2U/1 ml) for 30 min in an incubator (37°C). After removal of the solution, the cells were covered with a cover slip using mounting medium (Vector Laboratories, Burlingame, CA, USA).

Western blot analysis for γH2AX. Cells were grown to 50-80% confluence when treated with different agents. After treatment, the cells were scraped and pelleted by centrifugation at 4°C at 370 xg (2000 r/min) for 10 min, re-suspended with 1 ml PBS and pelleted again by centrifugation at 4°C at 830 xg (3000 r/min) for 10 min. The pellets were washed twice in PBS, homogenized in 0.2 M H₂SO₄, and centrifuged at 15490 xg (13000 r/min). Histones were pelleted from the supernatant by adding a 1/4 volume of 100% (w/v) trichloroacetic acid. The pellets were suspended in 100% ethanol overnight and centrifuged again at 15490 xg. The pellets were dissolved in ultrapure water and evaluated for protein concentration (Bio-Rad, Hercules, CA, USA). An aliquot corresponding to 10 μg of protein was boiled in SDS sample buffer (Tris-glycine SDS sample buffer (2x); Invitrogen Co.) and loaded onto the 4-20% Tris-glycine precast gel (Invitrogen Co.). The separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore) for 45 min at 15 V. The membrane was blocked with TBST (10 mM Tris-Cl pH7.4, 200 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk for 1 h, and had prior incubation with anti-γH2AX antibody (1/2000 dilution) for 2 h. The
blots were washed in TBST, incubated with horseradish peroxidase-conjugated anti-mouse antibody (1/1000 dilution), and visualized by chemiluminescence using the ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). All data shown were confirmed in independent experiments.

**Western blot analysis for pMAPK and pAKT.** Cells were grown to 50-80% confluence when subjected to treatment with the different agents. They were harvested by scraping, washed twice with PBS, then incubated on ice for 30 min in lysis buffer [0.3% NP-40, 1 mM Na3VO4, 25 mM NaF, 150 mM NaCl, 2 mM EGTA, 1 mM EDTA, 0.2% Triton X-100, 50 mM Tris-HCl (pH 7.4), 1 mM Phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 100 μg/ml leupeptin, and 2 mM Aminoethyl benzylsulfonyl fluoride]. Cell debris was removed by centrifugation at 15490 ×g for 15 min at 4˚C. Supernatant was evaluated for protein concentration and either used immediately for assays or stored at −80˚C.

Cell lysates containing 50 μg of total protein were electrophoresed in Tris-glycine precast gels (Invitrogen Co.) after boiling with SDS sample buffer, and then electrophoretically transferred to Immobilon™-P membranes for 45 min at 15 V. The membrane was blocked with 5% nonfat milk for 1 h prior to incubation with anti-p-AKT and p-MAPK antibodies (1/2000 dilution) for 2 h. After washing three times with TBST for 5 min, the membranes were incubated with secondary antibody (1/1000 dilution) for 1 h and visualized by chemiluminescence using the ECL Plus Western Blotting Detection System. All data shown were confirmed in independent experiments.

**Flow cytometry.** For cell cycle analyses, control and treated cells were fixed in 70% ethanol at −20˚C, washed, and treated with RNase A for 30 min at 37˚C. The cells were then stained with PI and subjected to flow microfluorimetry on a flow cytometer (Epics XL; Beckman Coulter, Tokyo, Japan).

**Results**

**Effects of SN-38 and trastuzumab on phosphorylation of AKT and MAPK.** We first assessed the effects of trastuzumab with or without SN-38 on phosphorylation of AKT and MAPK in N87 cells. Phosphorylation of AKT and MAPK (pAKT and pMAPK) was observed in untreated N87 cells and was reduced by trastuzumab administration (Figure 1). Phosphorylation of AKT and MAPK was enhanced by SN-38 and was similarly reduced by trastuzumab. These decreases in the phosphorylation of AKT and MAPK by trastuzumab were not observed in MKN74 cells.

**Effects of the order of trastuzumab administration on the cytotoxic effects of SN-38 in N87 and MKN74 cells.** We examined the effect of trastuzumab given either prior to or following SN-38 in N87 and MKN74 cells by MTT assay. N87 and MKN 74 cells were treated with different doses of SN-38 for 24 h. They were then treated with different doses of trastuzumab for 72 h and subjected to MTT assay. When administered following SN-38, trastuzumab enhanced the cytotoxicity of SN-38 to N87 cells in a dose-dependent manner (Figure 2A), but not in MKN74 cells (Figure 2B). In contrast, when administered for 72 h prior to SN-38, trastuzumab reduced the cytotoxic effects of SN-38 to N87 cells in a dose-dependent manner (Figure 2C), but not that to MKN74 cells (Figure 2D).

We next examined the effect of order of administration on γH2AX expression induced by SN-38. Cells were treated by the same protocol as above and subjected to western blot analysis. When administered following SN-38, trastuzumab enhanced the induction of γH2AX by SN-38 in N87 cells, but not in MKN74 cells (Figure 3A). When administered prior to SN-38, in contrast, trastuzumab reduced the induction of γH2AX by SN-38 in N87 cells, but not in MKN74 cells (Figure 3B).

We histochemically examined the effect of trastuzumab administration order on the formation of SN-38-induced foci of γH2AX in N87 and MKN74 cells. When administered following SN-38, trastuzumab enhanced focus formation in N87 cells (compare left and middle panels in Figure 4A), but not in MKN74 cells (compare the left panel and the middle panels in Figure 4B), and the incidence of cells with dense γH2AX staining was high (Figure 4A middle panel). When administered prior to SN-38, however, this focus was decreased in N87 cells (compare the left and middle panels in Figure 4C), but not in MKN74 cells (Figure 4D).

**Effect of trastuzumab on cell cycle progression in N87 and MKN74 cells.** SN-38 induces DSBs in a replication-dependent manner. Given that trastuzumab administered prior to SN-38 reduced the cytotoxicity of SN-38 in N87 cells, we then examined the effect of trastuzumab on cell cycle progression in N87 and MKN74 cells.

We first synchronized cells at the G1 phase using aphidicolin, then assessed the effect of trastuzumab on cell

![Figure 1. Effect of trastuzumab on phosphorylation of AKT and MAPK. Trastuzumab inhibited phosphorylation of AKT and MAPK in untreated N87 cells. SN-38 increased the phosphorylation of AKT and MAPK, but this effect was reduced by trastuzumab. Tmab, Trastuzumab.](image-url)
Figure 2. Enhancement and attenuation of the cytotoxic effect of SN-38 by trastuzumab in HER2-positive cells. Trastuzumab administered following SN-38 increased the cytotoxic effect of SN-38 in a dose-dependent manner in N87 cells (A), but not in MKN74 cells (B). However, trastuzumab administered prior to SN-38 attenuated the cytotoxic effect of SN-38 in a dose-dependent manner in N87 cells (C), but not in MKN74 cells (D). The means of the results of four independent experiments are shown. Tmab, Trastuzumab.
Results showed that trastuzumab delayed the progression of N87 cells (compare Figures 5B and C), but did not affect that of MKN74 cells (compare Figures 5D and E).

**Effet of AKT and MAPKK inhibitors on the cytotoxic effects of SN-38 in N87 and MKN74 cells.** The PI3K/Akt and MAPK pathways are the two major downstream signaling pathways of HER2. We examined which pathway was associated with enhancement of the cytotoxic effects of SN-38 using the AKT inhibitor AKT Inhibitor III (Calbiochem) and MAPKK inhibitor PD98059 (Calbiochem).

In MTT assay, AKT and MAPKK inhibitors alone or together did not affect the viability of either of N87 or MKN74 cells (Figures 6A and B), whereas these AKT and MAPKK inhibitors each increased the cytotoxic effects of SN-38 in N87 and MKN74 cells (Figures 6C and D). Administration of both of these inhibitors also appeared to further increase the cytotoxic effects of SN-38 in both N87 and MKN74 cells (Figures 6C and D). We also investigated the effects of the AKT and MAPKK inhibitors on the induction of γH2AX by SN-38 using western blot analysis.

While neither inhibitor increased γH2AX expression when given alone (Figures 7A and B), they did increase its induction in N87 and MKN74 cells when given in combination (Figure 7C).

**Discussion**

In this study, we found that trastuzumab exacerbated the cytotoxic effects of SN-38 in HER2-positive cells when sequentially given following SN-38, but abrogated them when given prior to SN-38. This phenomenon was not observed for HER2-negative cells. These findings indicate that the order of administration of trastuzumab influences the cytotoxic effects of SN-38 in HER2-positive cells.

SN-38, a representative topoisomerase-1 inhibitor, induces DSBs in a replication-dependent manner (12). Because DSBs are generated when the replication fork collides with the topoisomerase1-DNA cleavage complex (13), this effect can only occur when the cell cycle is in progress. Aphidicolin, a DNA polymerase inhibitor, disturbs replication, which in turn reduces the induction of DSBs by topoisomerase-1 inhibitor (13). In the present study, we found that...
pretreatment with trastuzumab reduced γH2AX induction by SN-38 and that the cytotoxic effect of SN-38 was reduced by pretreatment with trastuzumab. We also examined the effect of trastuzumab on cell cycle progression using flow cytometry and found that trastuzumab delayed cell cycle progression in HER2-positive but not HER2-negative cells. We therefore assume that the decrease in the cytotoxic effects of SN-38 in HER2-positive cells might be associated with the delay in cell cycle progression induced by trastuzumab pretreatment.

Figure 4. Opposing effects of trastuzumab on the focus formation of gamma-H2AX (γH2AX) induced by SN-38 in HER2-positive cells. Trastuzumab administered following SN-38 increased the induction of γH2AX induced by SN-38 in N87 cells (A) but not in MKN cells (B). However, trastuzumab administered prior to SN-38 reduced focus formation of γH2AX by SN-38 in N87 cells (C). In contrast, it did not reduce DNA breaks in MKN74 cells (D). Trastuzumab alone slightly induced γH2AX. Tmab, Trastuzumab. (×600).
Figure 5. Influence of trastuzumab on cell cycle progression in HER2-positive and -negative cells. Cells were synchronized at G1 phase after incubation with aphidicolin for 18 h. After medium change, the effect of trastuzumab on the cell cycle progression in N87 and MKN74 cells was assessed by flow cytometry at the indicated time point (A). Trastuzumab delayed cell cycle progression in N87 cells (compare B and C). However, trastuzumab did not affect cell cycle progression in MKN74 cells (compare D and E).
In contrast, we also demonstrated that post-treatment with trastuzumab enhanced the cytotoxic effect of SN-38 in HER2-positive cells, and increased the induction of γH2AX. The checkpoint mechanism stops cell cycle progression soon after the induction of DNA breaks (14), indicating that the inhibitory effect of trastuzumab on progression might not affect the efficacy of SN-38. Recent studies have shown that EGFR signaling pathways are involved in the repair of DNA damage, and that inhibition of these pathways using anti-EGFR antibodies disturbs DNA repair. In the present study,

Figure 6. Effect of MAPKK and AKT inhibitors on the cytotoxic effects of SN-38. AKT and MAPKK inhibitors alone and together, without SN-38 did not affect the viability of N87 (A) or MKN74 (B) cells. MAPKK inhibitor and AKT inhibitor increased the cytotoxicity of SN-38 in both N87 (C) and MKN74 (D) cells. When MAPKK inhibitor and AKT inhibitor were dosed together, further enhancement of the cytotoxicity of SN-38 was observed in both N87 and MKN74 cells (C and D). Means of the results of four independent experiments are shown. Tmab, Trastuzumab.
we found that phosphorylation of AKT and MAPK was increased by SN-38, suggesting that AKT and MAPK, which are key proteins of the EGFR signaling pathways, may play a role in the response to DNA breaks. Kriegs et al. (9) reported that the repair of DNA breaks induced by ionizing radiation was disturbed by the inhibition of EGF signaling. When dosed following SN-38, inhibition of downstream signaling from HER2 by trastuzumab might have attenuated the repair of SN-38-induced DNA DSBs, and thereby enhanced the cytotoxicity of SN-38.

Downstream signaling from HER2 consists of two major pathways, that of AKT and MAPK (11). We examined which was more closely associated with protection against DNA damage and repair, and found that inhibitors of both enhanced the cytotoxic effect of SN-38 but did not increase the induction of γH2AX. When AKT and MAPKK inhibitors were dosed simultaneously, however, γH2AX induction was enhanced. We therefore assume that the AKT and MAPK pathways are both involved in the protection or repair (or both) of DSBs by SN-38, and work complementarily with each other in protecting against DNA damage. Once DSBs are generated, moreover, the two pathways work synergistically in the repair of DNA damage. Each pathway is necessary for the repair of SN-38-induced DSBs. Because trastuzumab inhibits both pathways, the induction of γH2AX was enhanced, which in turn meant that the induction of DSBs was increased and the cytotoxicity of SN-38 was enhanced. The overall result of these effects is that trastuzumab disturbs the repair of DSBs induced by SN-38.

Our results should be interpreted within the limitations of the study. Firstly, we used only one cell line each for HER2-positive and -negative cells. Established HER2-positive gastric cancer cell lines are difficult to source and it was therefore difficult to obtain cell lines other than N87. However, we confirmed the presence of a similar phenomenon in HER2-positive and -negative breast cancer

Figure 7. Effect of AKT and MAPKK inhibitors on the induction of gamma-H2AX (γH2AX). Administration of either an AKT inhibitor (A) or MEK inhibitor (B) alone following SN-38 did not increase the induction of γH2AX in both of N87 and MKN74 cells. However, administration of the two inhibitors together increased γH2AX induction by SN-38 in both cells (C).
cell lines. Secondly, we were unable to assess the antibody-dependent cell-mediated cytotoxicity (ADCC) activity of trastuzumab, which represents one of its major antitumor mechanisms. Our results should therefore be considered preliminary, and requiring in vitro and in vivo verification in other HER2-positive and -negative gastric cancer cell lines.

In conclusion, this study shows that the order of administration of trastuzumab affects the cytotoxic efficacy of antitumor agents such as SN-38 in HER2-positive gastric cancer cells. Pretreatment with trastuzumab reduces cell cycle progression, resulting in a decrease in the induction of DNA DSBs by topoisomerase-1 inhibitors, which induce DSBs in a replication-dependent manner. In contrast, post-treatment with trastuzumab enhances the cytotoxic effects of SN-38. Although preliminary, our results indicate that the order of trastuzumab administration is critical when used together with antitumor agents that induce DNA breaks in a replication-dependent manner in the treatment of patients with HER2-positive cancer. Further studies are needed to verify whether the order of trastuzumab administration is associated with the therapeutic efficacy of antitumor agents in patients with HER2-positive gastric cancer.

Conflict of interest

None of the Authors had any conflict of interest related to this study.

Acknowledgements

This work was supported by a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (23590913).

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Received October 31, 2011
Revised December 2, 2011
Accepted December 5, 2011