A novel humanized anti-human death receptor 5 antibody CS-1008 induces apoptosis in tumor cells without toxicity in hepatocytes

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Background: The antitumor activity of CS-1008, a humanized agonistic anti-human death receptor (DR) 5 antibody, was investigated in preclinical models.

Materials and methods: Cytotoxicity of CS-1008 was evaluated in a several human tumor cell lines as well as primary human hepatocytes in vitro. To evaluate antitumor efficacy, athymic nude mice were inoculated with human colorectal tumor COLO 205, pancreatic tumor MIA PaCa-2 or non-small-cell lung carcinoma NCI-H2122 and CS-1008 was i.v. administered. The combination effects of CS-1008 with gemcitabine or docetaxel (Taxotere) against MIA PaCa-2 or NCI-H2122 were evaluated in vivo, respectively.

Results: CS-1008 inhibited the growth of tumor cell lines with DR5 expression, including COLO 205, NCI-H2122, MIA PaCa-2 and renal cell adenocarcinoma ACHN in vitro with antibody cross-linkage. Using COLO 205, apoptosis induction was confirmed by annexin V staining. Weekly administration of CS-1008 resulted in the inhibition of COLO 205 tumor growth as well as MIA PaCa-2 in vivo. CS-1008 in combination with gemcitabine or docetaxel demonstrated enhanced antitumor activity against MIA PaCa-2 or NCI-H2122 cells, respectively. Unlike tumor necrosis factor-related apoptosis-inducing ligand, CS-1008 did not induce cell death in human primary hepatocytes.

Conclusion: CS-1008 has a selective toxicity toward tumor cells expressing DR5 and the potential for antitumor efficacy in human malignancies.

Key words: apoptosis, combination therapy, death receptor 5, humanized antibody

Introduction

Apoptosis plays a crucial role in many physiological processes and is essential to homeostasis in the normal tissues of the body, especially those of the gastrointestinal tract, immune system and skin. An imbalance between cell proliferation and death contributes to many diseases, including malignancy, autoimmune diseases, neurodegenerative diseases and viral infections [1]. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF ligand superfamily and induces apoptosis in many tumor cell lines by binding to death receptors (DRs) [2]. Among DRs, DR4 and DR5 contain cytoplasmic death domains capable of transducing an apoptotic signal on ligation with their endogenous ligand TRAIL. An X-ray crystallography study of soluble TRAIL confirmed its homotrimeric subunit organization of the ligand [3]. After the binding of TRAIL, DRs are trimerized and rapidly assemble a death-inducing signaling complex (DISC) that involves a Fas-associated death domain (FADD) and procaspase-8 [4]. The activated caspase-8 subsequently cleaves and activates caspases-3, -6 and -7 (extrinsic pathway). Bid is also cleaved by caspase-8 and induces the release of proapoptotic factors from mitochondria (intrinsic pathway) [5]. Thus, TRAIL induces apoptosis in cells by triggering both extrinsic and intrinsic apoptosis signals via DRs.

DR5 expression is detected with high frequency in tumor cell lines and clinical tumor specimens [6–9]. Relatively low level of cytoplasmic DR5 expression was observed in several normal tissues including liver; however, it is still unclear whether functional DR5 localizes on the cell membrane [9, 10]. The physiological function of DR5 is not yet fully understood, and it is speculated to play an essential role in the immune surveillance of malignancy by TRAIL. In spite of the limited expression of DR5 in normal tissues, there still remains the concern for side-effects especially hepatotoxicity when targeting DRs. Indeed, several reports have demonstrated hepatotoxicity in vitro induced by recombinant TRAIL [11].

TRA-8, a novel murine anti-human DR5 mAb, has been shown to induce apoptosis in several tumor cell lines and inhibited the growth of tumors xenografted in athymic nude mice [12–14]. TRA-8 was humanized by a complementarity-determining region (CDR) grafting method. In this study, we...
examined the efficacy of CS-1008, a humanized TRA-8 on the proliferation of several tumor cell lines in vitro and the efficacy of CS-1008 alone or in combination with chemotherapeutic drugs on the growth of colon, lung and pancreatic tumor xenografts in athymic nude mice. Furthermore, we assessed the safety profile of CS-1008 in human primary hepatocytes in vitro because TRAIL and other anti-DR5 antibodies are known to induce cytotoxicity [11, 15].

materials and methods

CS-1008 and antitumor agents

Murine monoclonal anti-human DR5 antibody (TRA-8) was generated by immunization of BALB/c mice with DR5-hIgG1 fusion protein and subsequently humanized by a CDR grafting method [12].

Highly purified CS-1008, which contained monomer fractions of >99% purity, was used for the experiments. Human immunoglobulin G (IgG) 1 and human IgG were purchased from UK-Serotec Ltd. (Oxford, UK) and MP Biomedicals Inc. (Solon, OH), respectively, and used as isotype-matched controls. Phosphate-buffered saline (PBS) containing 0.01% polysorbate 80 was used as a diluent for antibodies.

Recombinant human TRAIL was purchased from R&D Systems Inc. (Minneapolis, MN) and stored at −20°C until use. Docetaxel hydrate (Taxotere®) and gemcitabine hydrochloride (Gemzar®) were purchased from Sanofi-aventis K.K. (Tokyo, Japan) and Eli Lilly Japan K.K. (Kobe, Japan), respectively.

cell lines and cell culture

Human tumor cell lines COLO 205 (colorectal adenocarcinoma), NCI-H2122 [non-small-cell lung cancer (NSCLC)], Mia PaCa-2 (pancreatic carcinoma), ACHN (renal cell adenocarcinoma) and U266B1 (myeloma) were purchased from American Type Culture Collection (Manassas, VA) and MKN-28 (gastric cancer) was purchased from Immuno-Biological Laboratories Co., Ltd (Takasaki, Japan). COLO 205, NCI-H2122, U266B1 and MKN-28 cells were cultured in RPMI-1640 (Invitrogen Corp., Carlsbad, CA) medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories Inc., South Logan, UT), Mia PaCa-2 and ACHN cells were cultured in Dulbecco’s Modified Eagle’s Medium (Invitrogen Corp.) and Minimum Essential Medium (Invitrogen Corp.), respectively, and supplemented with 10% heat-inactivated fetal bovine serum. All cell lines were cultured at 37°C under 5% CO₂.

reagents

Anti-human IgG goat polyclonal antibody was purchased from MP Biomedicals Inc. and used as a cross-linker for the in vitro proliferation assay. Phyceroerythrin (PE)-conjugated control mouse IgG1 and PE-conjugated anti-human DR5 antibody (eBioscience Inc., San Diego, CA) were used for flow cytometric analysis. Rabbit polyclonal antibodies to caspase-8, -9, -3, poly (ADP-ribose) polymerase (PARP) and horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signalling Technology Inc., Danvers, MA) were used for western blotting.

flow cytometry

Adherent cells were trypsinized and resuspended in culture medium and then washed once and resuspended in flowcytometry (FCM) wash buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Each test sample with 1.0 × 10⁶ cells was stained with 20 µl of PE-conjugated mouse IgG1 or PE-conjugated anti-human DR5 antibody for 30 min on ice. After washing twice with FCM wash buffer, the cells were analyzed using a flow cytometer (EPICS XL-MCL, Beckman Coulter Inc., Fullerton, CA). The mean fluorescence intensity in each sample was calculated using XL System II software ver. 3.0 (Beckman Coulter Inc.).

in vitro proliferation assay

Goat anti-human IgG antibody was added at a concentration of 50 µg/ml in 50 mM Tris buffer (pH 8.5) into each well of a 96-well flat-bottomed plate and was adsorbed onto the wells at 4°C for 24 h. The wells were washed with PBS and then CS-1008 solutions at various concentrations were added into each well. After 2 h of reaction at 4°C, the cell suspensions were plated into each well at the density of 1.0 × 10⁵ cells per well (COLO 205), 2.0 × 10⁵ cells per well (NCI-H2122 and Mia PaCa-2), 3.0 × 10⁵ cells per well (ACHN), 5.0 × 10⁵ cells per well (MKN-28) and 1.0 × 10⁶ cells per well (U266B1). The COLO 205, NCI-H2122, Mia PaCa-2, ACHN and U266B1 cells were incubated for 72 h, and the MKN-28 cells were incubated for 48 h at 37°C. The cell viabilities were determined by a3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt dye reduction assay (CellTiter 96 AQueous One Solution; Promega Corp., Madison, WI) for COLO 205, NCI-H2122, Mia PaCa-2, ACHN and U266B1 and by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; Dojindo Laboratories, Kumamoto, Japan dye reduction assay for MKN-28. The growth inhibitory concentration (IC₅₀) values were estimated by a sigmoid E₅₀ model using SAS System Release 8.2 (SAS Institute Inc., Heidelberg, Germany).

detection of apoptotic cells

Goat anti-human IgG antibody was adsorbed onto six-well plates as described above. Then, CS-1008 and hIgG1 solutions at concentrations of 1 or 2 µg/ml were added into each well and incubated at 37°C for 1 h. COLO 205 cells were plated at a density of 1.0 × 10⁵ cells per well and incubated at 37°C for 6–9 h. Apoptosis was determined by flow cytometric analysis using an annexin V fluorescein isothiocyanate (FITC) apoptosis detection kit I (BD Biosciences, San Jose, CA). Apoptosis signal induction was determined by western blot analysis using antibodies to caspase-8, -9, -3 or PARP.

Fifteen microgram of cell extract was used for the analysis in each lane. Detection was carried out with horseradish peroxidase-conjugated anti-rabbit IgG using diamobenzidine method.

in vitro hepatotoxicity assay

Primary human hepatocytes from three donors (HH-182, -185 and -189) were purchased from BD Biosciences, and for the preparation and incubation of the hepatocytes, a medium set (Biopredic International, Rennes, France) was used. The hepatocytes were preincubated at 3.5 × 10⁴ cells per well in a collagen-coated 96-well plate for 4 h. After further 18 h of incubation with fresh medium, the cells were used for an experiment. COLO 205 cells were used as a positive control. The hepatocytes and COLO 205 cells were treated with a mixture of CS-1008 solution at various concentrations and goat anti-human IgG antibody or TRAIL solution at various concentrations for 6 h at 37°C. Adenosine triphosphate detection assay was carried out using ATPlite-M Luminescence Assay System (PerkinElmer Inc.). The IC₅₀ values were estimated by linear regression analysis using the two common logarithmic-transformed concentrations closest to either side of the 50% mean cell viability.

purification of RNA and quantitative RT-PCR

Total RNA was extracted from a human hepatocyte (donor: HH-182) and COLO 205 cells using RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan). To avoid contamination of genomic DNA, the RNA samples were treated with deoxyribonuclease I (Invitrogen Corp.). Using these RNAs, the expression level of DR4 and DR5 were measured by real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) using the Quantitect Probe RT-PCR kit (Qiagen) and an ABI PRISM 7900 HT machine (Applied Biosystems, Foster City, CA). Commercially available probe and primer pairs were used (DR4: DR4_1 probe and primers, DR5: DR5_2 probe and primers; Applied Biosystems). An equal amount of RNA (10 ng) in each
in vivo xenograft models

For the xenograft tumor model, female BALB/cA Jcl-nu mice, aged 6 weeks, were purchased from CLEA Japan, Inc (Tokyo, Japan). COLO 205 cells were inoculated s.c. into right axillary region of nude mice at 2.0 x 10^6 cells per mouse. Seven days after tumor inoculation, the tumor-bearing mice were divided into experimental groups on the basis of the tumor volume, resulting in 104–108 mm³ of the mean tumor volumes. CS-1008, vehicle and hlgG solutions were injected i.v. into the mice on days 7, 14 and 21 at dosages of 0.1, 0.3, 1, 3, 10 and 30 mg/kg for CS-1008 and 30 mg/kg for hlgG.

NCI-H2122 cells were inoculated s.c. into the right axillary region of the nude mice at 3.0 x 10^6 cell per mouse. Fourteen days after the tumor inoculation, the tumor-bearing mice were assigned into groups as above, and the initial mean tumor volumes among experimental groups were 155–159 mm³. CS-1008 was injected i.v. into the mice on days 14, 21, 28 and 35, at a dose of either 0.3 or 3 mg/kg. Docetaxel was injected i.v. into the mice on day 14 at a dose of 20 mg/kg.

MIA PaCa-2 tumors had been maintained in vivo as solid tumors and were implanted s.c. into the nude mice using trocars. Ten days after tumor implantation, the tumor-bearing mice were assigned into groups as above, and the initial mean tumor volumes among experimental groups were 315–320 mm³. CS-1008 was injected i.v. into the mice on days 10, 17, 24 and 31 at a dose of either 0.3 or 3 mg/kg. Gemcitabine was injected i.v. into the mice on day 14 at a dose of 20 mg/kg.

The tumor volumes were calculated according to the following equation.

\[
\text{TUMOUR VOLUME (mm}^3\text{)} = 1/2 \times (\text{length}) \times (\text{width})^2.
\]

Tumor growth inhibition (TGI) was calculated according to the following equation.

\[
\text{TGI(\%)} = \left(1 - \frac{T}{C}\right) \times 100.
\]

Where \( T \) indicates the mean tumor volume (mm³) of the test groups and \( C \) indicates the mean tumor volume (mm³) of the vehicle-treated group.

All experiments were carried out in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Daiichi Sankyo Co. Ltd.

statistical analysis

In the xenograft experiment using COLO 205 tumors, the tumor growth rate (increase in mm³ of tumor volume per day) of each mouse was estimated as the slope of the tumor growth by a linear regression analysis of the volume. The tumor growth rates of the CS-1008-treated mice were compared with those of the vehicle-treated mice by a Dunnett test, and the tumor growth rates of the hlgG-treated mice were compared with those of the vehicle-treated mice by a Student’s t-test.

For the NCI-H2122 and MIA PaCa-2 xenograft models, the doubling time (DT) was calculated as the number of days in which the tumor doubled in volume relative to the tumor volume on the grouping day (day 14 for NCI-H2122 and day 10 for MIA PaCa-2). The median DT (MDT) and 95% confidence interval were estimated by the Kaplan–Meier method. The DT values of the test substance-treated groups were compared with those of the vehicle-treated control group by a log-rank test. To study the combination effects, the DT values of the combination treatment groups were also compared with those of the each single-agent treated group by a log-rank test. All calculations, estimations and statistical analyses described above were carried out using SAS System Release 8.2.

results

effects of CS-1008 on apoptosis in human tumor cell lines

The DR5 expression and cytotoxic activity of CS-1008 were analyzed in several human tumor cell lines as follows: colorectal adenocarcinoma COLO 205, non-small cell lung cancer (NSCLC) NCI-H2122, pancreatic carcinoma MIA PaCa-2, renal cell adenocarcinoma ACHN, myeloma U266B1 and gastric cancer MKN-28. All of the cell lines, with the exception of the U266B1, were revealed to express detectable levels of cell surface DR5 by a flow cytometric analysis (Figure 1A). Treatment of these cell lines with CS-1008 induced cell death in COLO 205, NCI-H2122, MIA PaCa-2 and ACHN cells in a dose-dependent manner (Figure 1B). Although several cell lines, for example, MIA PaCa-2 and NCI-H2122 were not sensitive to CS-1008 without cross-linking with anti-hlgG antibody, their sensitivities were remarkably augmented by cross-linking. No correlation was observed between the expression level of DR5 and the sensitivity to CS-1008 with cross-linking (Table 1). CS-1008 did not induce cell death in U266B1 and MKN-28 cells (Figure 1B). These results indicate that CS-1008 induces cell death in human tumor cell lines and that detectable DR5 expression is required but not sufficient for CS-1008 to induce cell death.

To investigate whether apoptosis is the principal mechanism of cell death induced by CS-1008, COLO 205 cells were treated with CS-1008 or control IgG with cross-linking for 6 h, and the apoptotic cells were detected by an annexin V method. When COLO 205 cells were treated with CS-1008, 61.3% of cells were labeled as early apoptotic cells, whereas only 2.45% of such cells were detected in the hlgG1-treated control cells (Figure 2A). Similar results were obtained from three independent experiments. Furthermore, the activation of caspases and the processing of PARP were analyzed by western blotting after CS-1008 treatment. In COLO 205 cells treated with CS-1008, cleavage of caspase-8, -9 and -3 and PARP was detected (Figure 2B). We concluded that CS-1008 induces cell death in DR5-positive human tumor cells by apoptosis.

effect of CS-1008 on induction of cell death in human hepatocytes

TRAIL and other anti-DR antibodies have been reported to induce cell death in human hepatocytes, raising the concern that DR-targeted drugs might cause hepatotoxicity [11, 15]. We assessed the hepatocellular toxicity of CS-1008 using...
primary human hepatocytes in vitro. Among human hepatocytes from three donors (HH-182, -185 and -189), TRAIL, which was a bioactive homotrimer, induced cell death in the hepatocytes from two donors, HH-182 and -185 (Figure 3A). In contrast, at the concentrations tested, CS-1008 had little, if any, cytotoxicity against these hepatocytes. In the control COLO 205 tumor cells, both TRAIL and CS-1008 induced cell death. Using one donor of hepatocytes (HH-182) that was shown to be sensitive to TRAIL but resistant to CS-1008-induced apoptosis, the expression of DR4 and DR5 was confirmed by a quantified RT–PCR method (Figure 3B), indicating that lack of DR5 expression in the hepatocytes was not responsible for negligible level of cytotoxicity after exposure to CS-1008. These results indicated that CS-1008 may have a much safer liver toxicity profile than TRAIL in vitro.

**Figure 1.** Flow cytometric analysis of death receptor (DR) 5 and effect of CS-1008 on COLO 205, NCI-H2122, MIA PaCa-2, ACHN, U266B1 and MKN-28 cells. (A) Cells (1 × 10^6 cells) were stained with phycoerythrin (PE)-conjugated mouse immunoglobulin G (IgG) 1 (filled histogram) or PE-conjugated anti-human DR5 antibody (open histogram) and analyzed by flow cytometry. (B) Tumor cells were cultured with CS-1008 with (filled circle) or without (open circle) cross-linking by anti-human IgG antibody (n = 4), and cell viabilities after 72 h of culture were measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt except for MKN-28. For MKN-28, cell viability was measured by a 3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide assay.

**effect of CS-1008 on the growth inhibition of COLO 205**

Since CS-1008 induced apoptosis in tumor cells in vitro, we evaluated the antitumor effects of this antibody in xenograft experiments, using tumor cell lines that both express DR5 and show in vitro sensitivity to CS-1008-induced cell death.

CS-1008 in the dose range of 0.1–30 mg/kg and control IgG at the dose of 30 mg/kg were i.v. administered to the mice on days 7, 14 and 21. As a result, the TGI by CS-1008 at the doses of 3 and 10 mg/kg was above 70%. In particular, slight tumor shrinkage was demonstrated at these two dosages after the first administration of CS-1008 (Figure 4). The statistically significant decrease in tumor growth rates was observed in the dose range between 0.3 and 30 mg/kg (P < 0.05 versus vehicle treatment). We used human IgG as an isotype-matched control.
and it appeared to produce minor inhibition of tumor growth when compared with the vehicle control.

**Table 1.** DR5 expression and CS-1008 sensitivity in several human tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor type</th>
<th>DR5 expression</th>
<th>IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLO 205</td>
<td>Colorectal adenocarcinoma</td>
<td>12.40</td>
<td>0.053</td>
</tr>
<tr>
<td>NCI-H2122</td>
<td>Non-small-cell lung cancer</td>
<td>7.75</td>
<td>0.232</td>
</tr>
<tr>
<td>Mia PaCa-2</td>
<td>Pancreatic carcinoma</td>
<td>5.21</td>
<td>0.146</td>
</tr>
<tr>
<td>ACHN</td>
<td>Renal cell adenocarcinoma</td>
<td>10.07</td>
<td>0.139</td>
</tr>
<tr>
<td>U266B1</td>
<td>Myeloma</td>
<td>1.10</td>
<td>&gt;30</td>
</tr>
<tr>
<td>MKN-28</td>
<td>Gastric cancer</td>
<td>4.16</td>
<td>&gt;1</td>
</tr>
</tbody>
</table>

DR5 expression was calculated as a relative mean fluorescent intensity (MFI) using MFI of control IgG stained and anti-DR5 antibody-stained cells. DR5, death receptor 5; IC50, growth inhibitory concentration; IgG, immunoglobulin G.

**Figure 2.** Apoptosis induction by CS-1008 in COLO 205 cells. (A) COLO 205 cells were incubated with 1 µg/ml CS-1008 or immunoglobulin G (IgG) for 6 h with cross-linking by anti-hIgG antibody and analyzed for annexin V binding using flow cytometry. Bottom left quadrants, viable cells; bottom right quadrants, early apoptotic cells; top right quadrants, late apoptotic/necrotic cells; top left quadrants, dead cells. Representative data in three independent experiments are shown in this figure. (B) Cleavage of caspases and poly (ADP-ribose) polymerase was detected by western blotting method. After incubation with 2 µg/ml CS-1008 or IgG for 9 h with cross-link by anti-hIgG antibody, protein of COLO 205 cells was extracted and subjected to western blotting analysis.

**Figure 3.** Effect of CS-1008 and TRAIL on human primary hepatocytes. (A) Human hepatocytes from three donors (HH-182, HH-185 and HH-189) and COLO 205 cells were incubated with CS-1008 with cross-linking by anti-hIgG antibody (open circle) and TRAIL (filled diamond) for 6 h (n = 3), and cell viabilities were measured by a adenosine triphosphate detection assay. (B) Death receptor (DR) 4 and DR5 messenger RNA expressions were relatively quantified by reverse transcriptase–polymerase chain reaction (RT–PCR) in HH-182 and COLO 205 cells. Total RNA was extracted, and RT–PCR was carried out using a RT–PCR Quantitect Gene Expression Assay kit, as described in ‘materials and methods’.

we chose docetaxel for NSCLC and gemcitabine for pancreatic tumor studies.

After NCI-H2122 was established in athymic nude mice, tumor-bearing mice were treated with CS-1008 at the doses of 0.3 and 3 mg/kg on days 14, 21, 28 and 35 and with docetaxel at the dose of 20 mg/kg on day 14 (Figure 5A). Treatment with CS-1008 alone at the doses of 0.3 and 3 mg/kg prolonged the MDT by 0.5 and 2.0 days compared with the vehicle control group, respectively. Docetaxel alone significantly prolonged the MDT of the vehicle-treated control group by 12.0 days (P = 0.0178). The combination treatment of either 0.3 or 3 mg/kg CS-1008 with docetaxel synergistically enhanced MDT prolongations to 22.0 and 35.0 days, respectively (P < 0.0001). In these combination groups, tumor shrinkage

**Antitumor effects of CS-1008 in combination with chemotherapeutic agents in human tumor xenograft models**

We examined the effects of CS-1008 in combination with chemotherapeutic agents in human NSCLC and pancreatic tumor xenograft models. For use in combination with CS-1008,
was also observed. In the combination group of 0.3 mg/kg of CS-1008 with docetaxel, one mouse showed complete regression with no palpable tumors. These results demonstrated the synergistic enhancement of antitumor effects of CS-1008 in combination with docetaxel in a human NSCLC xenograft model (Table 2).

Subsequently, we examined the efficacy of CS-1008 combined with gemcitabine in human pancreatic tumor xenograft. MIA PaCa-2 tumors were established in athymic nude mice, and the mice were treated with CS-1008 at the doses of 0.3 and 3 mg/kg and with gemcitabine at the dose of 400 mg/kg, on days 10, 17, 24 and 31 (Figure 5B). CS-1008 alone prolonged the MDT of the vehicle-treated control group at the doses of 0.3 and 3 mg/kg in a dose-dependent manner by 3.0 and 7.0 days, respectively. Gemcitabine alone prolonged MDT by 2.0 days only. The combination treatment significantly inhibited the tumor growth with MDT prolongations of 5.5 and 27.0 days (*P = 0.0017 and <0.0001, respectively). In the combination group of 3 mg/kg CS-1008 with gemcitabine, two mice showed complete regression with no palpable tumors. These results demonstrate the synergistic combination effect of CS-1008 with gemcitabine in exhibiting antitumor activity (Table 2).

discussion

The anti-DR5 antibody CS-1008 induced cell death in various DR5-expressing human tumor cell lines. The underlying mechanism of cell death induced by CS-1008 was apoptosis induction, as demonstrated by annexin V FITC staining and caspase and PARP cleavage. These results strongly indicate that CS-1008 is an agonistic antibody to DR5 and that it stimulates the apoptosis pathway mediated by DR5. The study also demonstrated that the induction of apoptosis by CS-1008 was remarkably augmented by cross-linkage with anti-human IgG secondary antibody. This result is consistent with the mechanism of DR5-mediated apoptosis induction by TRAIL.

The initial step for apoptosis signal transduction by TRAIL is the multimerization of DR5 molecules after TRAIL stimulation [2]. Then, the assembled DR5 molecules form DISC with FADD and procaspase-8, which sequentially stimulate caspase cascade and cytochrome C release from mitochondria. The role of a secondary antibody is thought to artificially facilitate DR5 oligomerization to induce apoptotic signal in tumor cells, although the molecular mechanism how CS-1008 induces apoptosis should be clarified in more detail.

In addition to in vitro cytotoxicity, CS-1008 monotherapy and combination therapy demonstrated significant antitumor activities in various human tumor xenografts in athymic nude mice. Among cell lines that are sensitive to CS-1008 in vitro, two cell lines, COLO 205 and MIA PaCa-2, were shown to be sensitive to CS-1008 in xenograft experiments, although NCI-H2122 showed only marginal sensitivity to CS-1008 alone. Because these cell lines express DR5, it was very likely that the antitumor activity of CS-1008 in xenograft models was derived from induction of DR5-mediated apoptosis induction, although the contribution of the antibody-dependent cell-mediated cytotoxicity mechanism could not be excluded. With regard to apoptosis induction in vivo, we speculate that
In our studies, MKN-28 cells, which express sufficiently high levels of DR5 to be measured in vitro, were resistant to CS-1008. Our preliminary investigation also revealed that several other tumor cell lines were resistant to CS-1008 in spite of detectable DR5 expression (data not shown). These results indicate that there is a need to address resistance mechanisms in the development of DR5-targeting agents since DR5 expression does not always predict the sensitivity of tumor cells to these agents. Similar discordance was observed for other anti-DR antibodies and TRAIL [6, 18]. Thus far, no reported mutations or single nucleotide polymorphisms in a DR5 molecule explain this resistance. These variations in sensitivity may correlate to the expression level and/or activation status of various intracellular apoptotic factors including c-FLIP, caspases, Bax, Bid, cIAP, Bcl-2 and Bcl-xL [19–24]. Interestingly, some chemotherapeutic agents have been reported to counteract these resistance mechanisms by modifying the status of these apoptosis-regulating factors. For example, doxorubicin is known to sensitize cells to DR-mediated apoptosis [13, 25]. This enhancement is likely associated with the activation of caspases, Bid and JNK/p38 and increase in DISC formation as well as proapooptotic protein release from mitochondria [26–28] In addition, doxorubicin is also known to increase DR5 expression [25]. Paclitaxel has been reported to enhance TRAIL-induced apoptosis by increasing Bax, Bak, DR4 and DR5 expression or decreasing inhibitor of apoptosis family expression [25]. Gemcitabine has been indicated to enhance TRAIL-induced apoptosis by activating caspase-8 and Bid and decreasing Bcl-xL [29]. In our results, it was demonstrated that the antitumor activity of CS-1008 was enhanced by combined treatment with either docetaxel or gemcitabine in vivo. The mechanism of these combination effects is currently being investigated.

In spite of the tumoricidal activity of CS-1008, it did not induce cell death in human primary hepatocytes, while TRAIL induced cell death in two out of the three lots of hepatocytes investigated. We confirmed the expression of both DR4 and DR5 messenger RNA in one of the three lots of hepatocytes that showed a differential sensitivity to CS-1008 and TRAIL by RT–PCR. Although the mechanism of cytotoxicity in hepatocytes by TRAIL is controversial, the difference between CS-1008 and TRAIL did not seem to depend on the lack of DR5 expression in those primary hepatocytes. Mori et al. reported that among mAbs raised against DR5, some antibodies induced cell death in hepatocytes and others did not [15]. These antibodies’ varying hepatotoxicities were due to epitope variations rather than differences in their affinity to DR5 [15]. Whether the epitope of CS-1008 determines this selectivity to tumor cells or not remains to be determined. In any case, our results indicate that DR-mediated apoptosis by CS-1008 does not result in clinically significant hepatotoxicity.

Finally, the results of this report strongly support the clinical use of CS-1008 in cancer therapy on the basis of the findings of its selective cytotoxicity against DR5-expressing tumor cells. The frequency of DR5 expression is relatively high in a variety of tumor types whereas only a limited amount of DR5 expression has been reported in normal tissues [6–10]. This selective expression profile of DR5 makes it an attractive target for antitumor agents, and clinical trials for several DR5-targeted molecules have been initiated. It is anticipated that these novel agents will provide clinical benefits and novel treatment opportunities to cancer patients.

### Table 2. Combination effect of CS-1008 with chemotherapeutic drugs in tumor xenograft models

<table>
<thead>
<tr>
<th>Group</th>
<th>MDT (95% CI) (day)</th>
<th>MDT prolongation (day)</th>
<th>TGI</th>
<th>CR</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NCI-H2122 xenografts)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>4.5 (4.0–5.0)</td>
<td>–</td>
<td>–</td>
<td>0/10</td>
</tr>
<tr>
<td>CS-1008, 0.3 mg/kg</td>
<td>5.0 (4.0–6.0)</td>
<td>0.5</td>
<td>12</td>
<td>0/10</td>
</tr>
<tr>
<td>CS-1008, 3 mg/kg</td>
<td>6.5 (6.0–8.0)</td>
<td>2.0</td>
<td>24</td>
<td>0/10</td>
</tr>
<tr>
<td>Docetaxel, 20 mg/kg</td>
<td>16.5 (16.0–17.0)</td>
<td>12.0</td>
<td>65</td>
<td>0/10</td>
</tr>
<tr>
<td>Docetaxel + CS-1008, 0.3 mg/kg</td>
<td>26.5 (21.0–34.0)↑</td>
<td>22.0</td>
<td>83</td>
<td>1/10</td>
</tr>
<tr>
<td>Docetaxel + CS-1008, 3 mg/kg</td>
<td>39.5 (37.0–44.0)abc</td>
<td>35.0</td>
<td>93</td>
<td>0/10</td>
</tr>
<tr>
<td>(MIA PaCa-2 xenografts)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>3.0 (2.0–4.0)</td>
<td>–</td>
<td>–</td>
<td>0/10</td>
</tr>
<tr>
<td>CS-1008, 0.3 mg/kg</td>
<td>6.0 (4.0–9.0)</td>
<td>3.0</td>
<td>27</td>
<td>0/10</td>
</tr>
<tr>
<td>CS-1008, 3 mg/kg</td>
<td>10.0 (4.0–21.0)</td>
<td>7.0</td>
<td>53</td>
<td>0/10</td>
</tr>
<tr>
<td>Gemcitabine, 400 mg/kg</td>
<td>5.0 (4.0–5.0)</td>
<td>2.0</td>
<td>35</td>
<td>0/10</td>
</tr>
<tr>
<td>Gemcitabine + CS-1008, 0.3 mg/kg</td>
<td>8.5 (6.0–18.0)</td>
<td>5.5</td>
<td>69</td>
<td>0/10</td>
</tr>
<tr>
<td>Gemcitabine + CS-1008, 3 mg/kg</td>
<td>30.0 (13.0–64.0)↑</td>
<td>27.0</td>
<td>84</td>
<td>2/10</td>
</tr>
</tbody>
</table>

| Significantly different from vehicle-treated mice. |
| Significantly different from CS-1008-treated mice. |
| Unable to calculate due to statistically insufficient number of mice whose tumor volumes reached double. |

MDT, median doubling time; CI, confidence interval; TGI, tumor growth inhibition; CR, complete regression.

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**References**


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1066 | Yada et al. | Annals of Oncology | June 2008 | Volume 19 | No. 6


