ORIGINAL ARTICLE

Inostamycin enhanced TRAIL-induced apoptosis through DR5 upregulation on the cell surface

Kohta Yamamoto¹, Masafumi Makino¹, Ramida Watanapokasin², Etsu Tashiro¹ and Masaya Imoto¹

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been considered as a possible therapeutic agent for cancer treatment. This is because of its selective cytotoxicity against various cancer cells without a detrimental effect on normal cells. However, recent studies have reported that the potential application of TRAIL in cancer therapy is limited, as many cancer cells have been found to be resistant to TRAIL. Therefore, small molecule compounds that potentiate the cytotoxicity of TRAIL would be strategic candidates for therapeutic applications in combination with TRAIL. Here we found that a combined treatment of inostamycin and TRAIL synergistically induced caspase-dependent apoptosis in HCT116 cells. Inostamycin upregulated DR5, and a knockdown of DR5 suppressed the apoptosis that was synergistically induced by co-treatment with inostamycin and TRAIL. Moreover, inostamycin increased the expression of DR5 on the cell surface. Therefore, inostamycin-increased cell surface expression of DR5 may have contributed to the enhancement of TRAIL-induced apoptosis. Our study suggests that combined treatment with inostamycin and TRAIL may offer a strategy to overcome TRAIL resistance in tumor cells.

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INTRODUCTION

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), also known as Apo2L, is a type II transmembrane protein. TRAIL was originally identified on the basis of sequence homology to the Fas ligand and tumor necrosis factor.^{1,2} TRAIL is hypothesized as being a potentially good therapeutic agent for cancer treatment. This is because TRAIL has been shown to induce apoptosis in a variety of tumor cell lines more efficiently than in normal cells.^{3–5} TRAIL exerts its function by binding its receptors, which are expressed on the surface of target cells. To date, four different types of membrane-bound death receptors for TRAIL have been identified: TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1 and TRAIL-R4/DcR2. Both DR4 and DR5 have a conserved cytoplasmic region called the 'death domain'. This is a homolog to Fas and tumor necrosis factor-R1, and is required for TRAIL-induced apoptosis.^{6–8} On the other hand, TRAIL also binds to DcR1 and DcR2 that sequester the ligand, but are unable to initiate an apoptosis signal. Thus, DcR1 and DcR2 are considered to be the decoy receptors.^{7,9} The apoptosis signal that is induced by TRAIL has been shown to be similar to that which is induced by Fas. The TRAIL homotrimer induces trimerization of DR5 or DR4 on the surface of target cells, which leads to the formation of death-inducing signaling complex. The trimerization of the death domains results in the recruitment of an adaptor molecule Fas-associated protein with death domain (FADD), which in turn

recruits and activates caspase-8. In type I cells, activation of caspase-8 is sufficient for the subsequent activation of effector caspase-3. On the other hand, in type II cells, amplification through the mitochondrial pathway is initiated by the cleavage of Bid by caspase-8. Truncated-Bid causes the loss of mitochondrial membrane potential and caspase-9 cleavage, resulting in the activation of caspase-3 and subsequent cellular apoptosis.

The potential application of TRAIL in cancer therapy is currently limited, as many cancer cells have been found to be resistant to the cytotoxic effects of TRAIL. This resistance may be because of the low expression of pro-apoptotic molecules: death receptors or caspase-8. Alternatively, the resistance may be because of the high expression of anti-apoptotic molecules: decoy receptors, FLICE-like-inhibitory protein, inhibitor of apoptosis protein and Bcl-2. Because of the limitations of TRAIL-induced cytotoxicity, the combination of TRAIL with other small molecule compounds has been postulated as strategy to potentiate the cytotoxicity of TRAIL and its therapeutic applications. Indeed, it was reported that several chemotherapeutic agents and natural products, such as CDDP,¹⁰ etoposide,^{10,11} doxorubicin,¹² PS-341 (bortezomib),¹³ tunicamycin,¹⁴ rottlerin,¹⁵ brandisianins,¹⁶ silibinin¹⁷ and sodium butyrate,¹⁸ were succeeded to cause the sensitization of TRAIL-resistant tumor cells to TRAIL-induced apoptosis.

In this study, we have screened candidate small molecule compounds that synergistically induce apoptosis in the presence of

¹Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, Yokohama, Japan and ²Department of Biochemistry, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand

Correspondence: Dr E Tashiro, Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, 223-8522, Japan.

E-mail: tashiro@bio.keio.ac.jp

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Figure 1 Co-treatment with inostamycin (INM) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) synergistically induced apoptosis in HCT116 cells. **(a)** Structure of inostamycin.¹⁹ **(b)** Co-treatment with INM and TRAIL induced a synergistic cytotoxicity in HCT116 cells, HeLa cells, HT29 cells and LNCaP cells, but not in Ms-1 cells. HCT116 cells, HeLa cells, HT29 cells, LNCaP cells or Ms-1 cells were treated with the indicated concentration of TRAIL and 0.0 (square), 0.01 (triangle), 0.1 (diamond-shaped) or 1 (circle) μg ml⁻¹ of INM for 18 h. The cytotoxicity was evaluated using a Trypan Blue Exclusion assay. **(c)** The cytotoxicity induced by the co-treatment with INM and TRAIL was completely suppressed by z-VAD-fmk. HCT116 cells were pre-treated with or without 100 μM of z-VAD-fmk, and then treated further with 10 ng ml⁻¹ of TRAIL and 1 μg ml⁻¹ of INM. After an 18-h incubation period, the sub-G₁ population was measured by flow cytometer. **(d)** Co-treatment with INM and TRAIL activated caspase. HCT116 cells were treated with or without 10 ng ml⁻¹ of TRAIL and 1 μg ml⁻¹ of INM for the indicated periods. Cell extracts were prepared for western blotting to detect the expression of caspase-8, caspase-9, caspase-3 and poly(ADP-ribose) polymerase (PARP).

TRAIL. As a result, we found that co-treatment with inostamycin and TRAIL synergistically induced caspase-dependent apoptosis in colorectal cancer HCT116 cells. Inostamycin was isolated from *Streptomyces* species. MH816-AF15;¹⁹ its structure was shown in Figure 1a. Inostamycin weakly upregulated DR4 and strongly upregulated DR5 expression. Additionally, the synergistic apoptosis that was induced by this co-treatment was completely suppressed in DR5 small interfering RNA (siRNA)-transfected but not in DR4 siRNA-transfected HCT116 cells. Furthermore, inostamycin increased cell surface expression of DR5. Taken together with these results, upregulation of cell surface DR5 by inostamycin possibly contributes to the enhancement of TRAIL-induced apoptosis.

MATERIALS AND METHODS

Materials

Inostamycin was prepared as previously described,¹⁹ TRAIL was purchased from Millipore (Billerica, MA, USA). Tunicamycin and z-VAD-fmk were purchased from SIGMA (St. Louis, MO, USA). Rabbit monoclonal anti-poly(ADP-ribose) polymerase, rabbit polyclonal anti-caspase-9, anti-caspase-3, anti-Bcl-xL and mouse monoclonal anti-caspase-8 were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal anti-survivin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti-X-linked inhibitor of apoptosis protein (XIAP) was purchased from BD Transduction Laboratories (San Diego, CA, USA). Mouse monoclonal anti-Bcl-2 was purchased from Dako (Carpentaria, CA, USA). Rabbit polyclonal anti-DR5 was purchased from Proscience (Poway, CA, USA). Rabbit polyclonal anti-DR4 was purchased from Millipore. Mouse monoclonal anti-C/EBP homologous protein (CHOP) was purchased from Thermo scientific (Rockford, IL, USA). Mouse monoclonal anti-DR5 phycoerythrin (PE)-conjugated was purchased from eBioscience (San Diego,

CA, USA). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG secondary antibodies were purchased from GE Healthcare (Little Chalfont, UK).

Cell culture

Human colorectal carcinoma HCT116 cells, HT29 cells and human small cell lung cancer Ms-1 cells were maintained in Roswell Park Memorial Institute medium (Nissui, Tokyo, Japan) supplemented with 5% fetal bovine serum. Human cervical cancer HeLa cells were maintained in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supplemented with 8% fetal bovine serum. Human prostate adenocarcinoma LNCaP cells were maintained in Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum—Roswell Park Memorial Institute medium.

Trypan blue exclusion assay

HCT116 cells, HT29 cells, HeLa cells, LNCaP cells or Ms-1 cells were seeded in 48-well plates and incubated overnight, before experiments were conducted. The cells were treated with TRAIL with or without inostamycin for 18 h. Floating and adherent cells were collected and resuspended in phosphate-buffered saline (PBS⁻). Cells were mixed with trypan blue and counted under the microscope.

Propidium iodide staining

Propidium iodide staining and flow cytometry were used to determine the degree of cellular apoptosis. HCT116 cells were seeded in 6-well plates and incubated overnight before experiment. The cells were treated with TRAIL with or without inostamycin for 18 h. Floating and adherent cells were collected and resuspended in PBS⁻. Cold ethanol was added in a dropwise manner while vortexing to fix the cells. Fixed cells were collected and resuspended in PBS⁻ containing 50 μg ml⁻¹ propidium iodide. Flow cytometry was done using

EPICS (Beckman Coulter, Fullerton CA, USA). The percentage of sub-G₁ cells was used to quantify apoptotic cells.

Western blotting

Cells were lysed with a lysis buffer containing 25 mM HEPES (pH 7.8), 1.5% Triton-X 100, 1.0% sodium deoxycholate, 0.1% SDS, 0.5 M NaCl, 5 mM EDTA, 50 mM NaF, 100 μ M Na₃VO₄, 0.1 mg ml⁻¹ leupeptin and 1 mM phenylmethylsulfonyl fluoride. Proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore). After the membranes had been incubated with primary and secondary antibodies, the immune complexes were detected with an Immobilon Western kit (Millipore), and the luminescence was detected with a LAS-1000 mini (Fujifilm, Tokyo, Japan).

Real-time reverse transcriptase (RT)–PCR analysis

Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed with M-MLV reverse transcriptase (PROMEGA, Madison, WI, USA) according to the manufacturer's instructions. Real-time reverse transcription (RT)–PCR was performed using SYBR Premix Ex Taq (Takara, Shiga, Japan). Primer sequence was as follows: DR5, 5'-CACCA GGTGTGATTCAAGTG-3' (sense) and 5'-TACGGCTGCAACTGTGACTC-3' (antisense); CHOP, 5'-GCGCATGAAGGAGAAAGAAC-3' (sense) and 5'-TCACCATTCGGTCAATCAGA-3' (antisense); glyceraldehyde-3-phosphate dehydrogenase, 5'-AGGTCGGAGTCAACGGATT-3' (sense) and 5'-TAGTTG AGGTCAATGAAGGG-3' (antisense).

RNA interference

siRNA for control (12935-300), CHOP (5'-CCUCACUCUCCAGAUUCCA GUCAGA-3') DR4 (HSS112945) and DR5 (HSS112939) were purchased from Invitrogen. HCT116 cells were transfected with siRNA using HiPerFect (QIAGEN, Hilden, Germany), according to the manufacturer's instructions.

Cell surface staining of DR5

HCT116 cells were seeded in 6-well plates and incubated overnight before the experiment. Cells were then treated with inostamycin for 12 h. Cells were then collected and resuspended in PBS⁻ including PE-conjugated DR5 antibody. After incubation for 1 h at 4 °C, the cells were washed with PBS⁻ twice and resuspended in PBS⁻. Flow cytometry analysis was performed with EPICS (Beckman Coulter).

RESULTS AND DISCUSSION

Co-treatment with inostamycin and TRAIL synergistically induced apoptosis in HCT116 cells

In this study, we first found that co-treatment with inostamycin and TRAIL synergistically induced cytotoxicity in HCT116 cells. As shown in Figure 1b, although neither 10 ng ml⁻¹ TRAIL nor 1 μ g ml⁻¹ inostamycin alone showed cytotoxicity in HCT116 cells after 18 h treatment, the cell viability was decreased to about 40% when HCT116 cells were treated with inostamycin and TRAIL in combination. The synergistic cytotoxicity occurred in inostamycin and TRAIL dose-dependent manner. This synergistic effect is cell type-dependent, and is observed in HeLa cells, HT29 cells and LNCaP cells but not in Ms-1 cells (Figure 1b). Flow cytometer analysis demonstrated that the synergistic increase of the Sub-G₁ population was observed in co-treatment with inostamycin and TRAIL in HCT116 cells (Figure 1c). Furthermore, a one hour preincubation with the pan-caspase inhibitor 100 μ M z-VAD-fmk suppressed the cell death that was induced by co-treatment with inostamycin and TRAIL (Figure 1c). We also observed the cleavage of caspase-8, caspase-9, caspase-3 and poly(ADP-ribose) polymerase, a well known substrate of caspase-3, at 6 h after inostamycin and TRAIL co-treatment (Figure 1d). In contrast, the cleaved caspase-8, caspase-9, caspase-3 and poly(ADP-ribose) polymerase were only weakly detected when

HCT116 cells were treated with 10 ng ml⁻¹ of TRAIL alone. Inostamycin alone also could not activate caspase-8, caspase-9 or caspase-3 in HCT116 cells (Figure 1d). These results suggested that co-treatment with inostamycin and TRAIL induced caspase-dependent apoptosis.

Inostamycin enhanced TRAIL-induced apoptosis possibly through DR5 upregulation on the cell surface

TRAIL-stimulated death signal is initiated by the binding of TRAIL to DR4 or DR5, which resulted in the subsequent activation of caspase-8. As shown in Figure 2a, western blotting analysis using an anti-DR4 or anti-DR5 antibody showed that inostamycin strongly upregulated the DR5 protein in HCT116 cells, it also slightly upregulated the DR4 protein (Figure 2a). Similarly, inostamycin-induced DR5 upregulation was also observed in HT29 cells and HeLa cells, in which co-treatment with inostamycin and TRAIL induced synergistic cytotoxicity (Figure 2a). Inostamycin-induced DR5 protein was detected as two bands, which were likely products of alternative splice variants of the DR5 gene^{20,21}. Furthermore, under the condition where inostamycin-upregulated DR4 or -DR5 protein was efficiently reduced by transient transfection of DR4 siRNA or DR5 siRNA (Figure 2b), apoptosis induced by co-treatment with inostamycin and TRAIL was completely suppressed in DR5 siRNA-transfected HCT116 cells, but not in DR4 siRNA-transfected HCT116 cells (Figure 2c). These results indicated that DR5 is indispensable for the apoptosis induced by co-treatment with inostamycin and TRAIL. Moreover, inostamycin induced cell surface expression of DR5 in a time-dependent manner (Figure 2d). On the other hand, the protein expression of four anti-apoptotic molecules, Bcl-2, Bcl-xL, XIAP and survivin, was unaffected by inostamycin in HCT116 cells (Figure 2e). Considering that it increases the cell surface expression of DR5, it is likely that inostamycin-increased cell surface expression of DR5 contributes to TRAIL sensitization.

Inostamycin-induced transcription of DR5 was regulated by CHOP.

As shown in Figure 3a, inostamycin-induced increases in DR5 mRNA levels were evaluated by real-time RT–PCR analysis. This data suggests that inostamycin activates transcription of DR5 in HCT116 cells. Recent studies have reported that tunicamycin-induced¹⁴ and MG132-induced²² CHOP (C/EBP homologous protein) upregulation activates DR5 transcription through CHOP-binding site in DR5 promoter region. This results in a sensitization of TRAIL-induced apoptosis. As we also found that 1 μ g ml⁻¹ inostamycin increased CHOP protein expression with similar time-course kinetics to DR5 upregulation (Figure 3b), we next examined whether this inostamycin-induced CHOP expression regulated DR5 transcription. When CHOP was successfully silenced by transient transfection of siRNA for CHOP (siCHOP) (Figure 3c), inostamycin-increased DR5 mRNA was markedly suppressed (Figure 3d). Furthermore, as consistent with previous report, tunicamycin-increased expression of DR5 mRNA was also completely suppressed in siCHOP-transfected HCT116 cells (Figure 3d). Next, we examined whether inostamycin-induced DR5 protein upregulation was also suppressed in CHOP-knockdown HCT116 cells. As shown in Figures 4a and b, neither inostamycin-induced DR5 protein expression nor inostamycin-increased cell surface expression of DR5 was suppressed under the conditions where tunicamycin-induced DR5 protein expression was completely suppressed in CHOP-knockdown cells. Furthermore, the transient transfection with siCHOP could not suppress apoptosis induction by the synergistic effect of co-treatment with inostamycin and TRAIL (Figure 4c).

Figure 2 Inostamycin (INM) increased cell surface expression of DR5 in HCT116 cells. **(a)** Effect of INM on the expression of DR4 and DR5 in HCT116 cells, and DR5 in HeLa cells and HT29 cells. HCT116 cells, HeLa cells and HT29 cells were treated with 1 $\mu\text{g ml}^{-1}$ of INM for the indicated periods. Cell extracts were prepared for western blotting to detect the expression of DR4 and DR5. **(b)** Transient transfection of small interfering RNA (siRNA) against DR4 or DR5 suppressed INM-induced DR4 or DR5 expression in HCT116 cells. HCT116 cells were transiently transfected with the indicated concentration of DR4 siRNA (siDR4) or DR5 siRNA (siDR5). After incubation for 24 h, the transfected cells were treated with 1 $\mu\text{g ml}^{-1}$ of INM for 12 h. Cell extracts were prepared for western blotting to detect the expression of DR4 and DR5. **(c)** Knockdown of DR5 suppressed synergistic apoptosis induction by co-treatment with INM and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). HCT116 cells were transiently transfected with 20 nM DR4 siRNA (siDR4) or siDR5. After incubation for 24 h, the transfected cells were treated with or without 1 $\mu\text{g ml}^{-1}$ INM together with 10 ng ml $^{-1}$ TRAIL for more 24 h. The cell viability was evaluated using a Trypan Blue Exclusion assay. **(d)** INM increased the cell surface expression of DR5 in HCT116 cells. HCT116 cells were treated with 1 $\mu\text{g ml}^{-1}$ of INM for 6 or 12 h. The cell surface DR5 were stained with or without PE-conjugated DR5 antibody and then further detected with flow cytometer. **(e)** INM did not affect the expression of anti-apoptotic proteins in HCT116 cells. HCT116 cells were treated with 1 $\mu\text{g ml}^{-1}$ of INM for the indicated periods. Cell extracts were prepared for western blotting to detect the expression of Bcl-2, Bcl-xL, XIAP and survivin. * indicates non-specific band.

Figure 3 Inostamycin (INM) activated DR5 transcription through CHOP upregulation. (a) INM induced DR5 upregulation of mRNA levels in HCT116 cells. HCT116 cells were treated with $1 \mu\text{g ml}^{-1}$ of INM for the indicated periods. The cells were collected and RNA was extracted. DR5 mRNA levels were evaluated by real-time RT-PCR. DR5 mRNA was normalized with the mRNA levels of glyceraldehyde-3-phosphate dehydrogenase. Data are the means $\pm$ s.d. among three independent experiments. (b) Effect of INM on the expression of CHOP in HCT116 cells. HCT116 cells were treated with $1 \mu\text{g ml}^{-1}$ of INM for the indicated periods. Cell extracts were prepared for western blotting to detect the expression of CHOP. (c) Transient transfection of small interfering RNA (siRNA) against CHOP suppressed INM-induced CHOP expression in HCT116 cells. HCT116 cells were transiently transfected with 20 nM siCHOP. After 24 h incubation, the transfected cells were treated with $1 \mu\text{g ml}^{-1}$ of INM for 4, 8 or 12 h. Cell extracts were prepared for western blotting to detect the expression of CHOP. (d) Knockdown of CHOP suppressed INM- and tunicamycin (Tm)-induced DR5 upregulation at mRNA levels. HCT116 cells were transiently transfected with 20 nM siCHOP. After incubation for 24 h, the transfected cells were treated with $1 \mu\text{g ml}^{-1}$ of INM for 4, 8 or 12 h or $10 \mu\text{g ml}^{-1}$ of Tm for 12 h. The cells were collected and RNA was extracted. DR5 mRNA levels were evaluated by real-time RT-PCR. DR5 mRNA was normalized with the mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are the means $\pm$ s.d. among three independent experiments. * $P < 0.05$ and ** $P < 0.005$.

When considered together, our results suggest that inostamycin enhanced TRAIL-induced apoptosis possibly because of a CHOP-independent upregulation of DR5 on the cell surface. At present, the mechanism by which inostamycin upregulates DR5 protein expression remains unclear. However, these results were consistent with our conclusion that the inostamycin-increased cell surface expression of DR5 possibly contributes to the enhancement of TRAIL-induced apoptosis.

In a previous study, various small molecule compounds, such as tunicamycin,¹⁴ were reported to transactivate the DR5 gene by the upregulation of CHOP. The inostamycin-induced increase in DR5 mRNA was suppressed in CHOP-knockdown HCT116 cells; however, DR5 protein expression was not suppressed. Furthermore, knockdown of CHOP by siCHOP suppressed only inostamycin-

increased CHOP-dependent DR5 mRNA expression, but constitutively expressed DR5 mRNA levels were not affected (Figure 3d). Therefore, in siCHOP-transfected HCT116 cells, inostamycin is considered to increase the protein levels of DR5 translated from constitutively expressed DR5 mRNA by posttranslational modulation. Various studies have reported the mechanism of the transcriptional activation of DR5 gene. However, little is known about the mechanism of translation, posttranslational modification, localization or degradation of DR5. Because of the data presented in this study, inostamycin may be a good bioprobe for use in investigating the regulatory mechanism of DR5 protein. Furthermore, elucidation of the mechanism by which inostamycin increases the expression of DR5 on the cell surface may provide a new molecular target for inducing DR5 upregulation to overcome TRAIL resistance.

Figure 4 Inostamycin (INM) enhanced tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis possibly through CHOP-independent cell surface DR5 upregulation. (a) Knockdown of CHOP suppressed tunicamycin (Tm)-induced DR5 upregulation but did not suppress INM-induced DR5 upregulation at protein levels. HCT116 cells were transiently transfected with 20 nM siCHOP. After incubation for 24 h, the transfected cells were treated with $1 \mu\text{g ml}^{-1}$ of INM for 4, 8, 12 h or $10 \mu\text{g ml}^{-1}$ of Tm for 12 h. Cell extracts were prepared for western blotting to detect the expression of DR5. (b) Knockdown of CHOP did not suppress the INM-induced upregulation of cell surface DR5. HCT116 cells were transiently transfected with 20 nM siCHOP. After incubation for 24 h, the transfected cells were treated with $1 \mu\text{g ml}^{-1}$ of INM for 12 h. The cell surface DR5 was stained with PE-conjugated DR5 antibody and then detected using a flow cytometer. (c) Knockdown of CHOP did not suppress the induction of apoptosis by co-treatment with INM and TRAIL. HCT116 cells were transiently transfected with 20 nM siCHOP. After incubation for 24 h, the transfected cells were treated with or without $1 \mu\text{g ml}^{-1}$ INM together with 10 ng ml^{-1} TRAIL for 18 h. The cells were stained with propidium iodide (PI), and sub- G_1 phase cells were detected using a flow cytometer.

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