Abstract. Background: 4-[3,5-Bis(trimethylsilyl)benzamido] benzoic acid (TAC-101) is a novel retinobenzoic acid derivative which has a specific binding affinity to the retinoic acid receptors (RAR)α and RARβ. Using time-dependent FACScan analysis, it was observed that TAC-101 induced apoptosis in a DLD-1 human colon cancer cell line. In this study, the induction of apoptosis-related proteins and the activities of caspasas in a DLD-1 cell line under medication with TAC-101 were investigated. Materials and Methods: DLD-1 cells were cultured with different concentrations of TAC-101 for 12, 24 and 48 h. The expressions of Fas, TNF-R1, DR3, bcl-2, Bax and Bid were measured using a Western blot analysis. The activities of caspase-3, -8 and -9 were measured using a colorimetric protease assay kit. Results: The Western blot analysis showed that TAC-101 had almost no effect on the level of Bcl-2, Bax or Bid protein. Although TAC-101 did not change the expression of TNF-R1 and DR3, TAC-101 increased the expression of Fas in both a time- and a dose-dependent manner. A 3-fold increase in caspase-3 activity and a 1.5-fold increase in caspase-8 activity were observed in cells treated with TAC-101 in comparison to the control cells (p<0.01). Conclusion: Our data indicate that the death receptor root of the apoptotic signal transduction in DLD-1 cells mainly participates in the apoptotic induction of TAC-101. Because the compounds inducing apoptotic activity are frequent targets of cancer therapy, TAC-101 may be a good candidate for use in the treatment of colon cancer.

The reduction of hepatic metastasis would lead to much better prognoses for patients with advanced colon cancer. Recent studies have indicated that several synthetic retinoic acid derivatives (RAs) might have anticancer potential against colon cancer cell lines (1-4). The mechanism of the antitumor effects of RA appears to be related to its effects on the proliferation, differentiation, apoptosis and angiogenesis of several cancer cells (5-11). In particular, the all-trans-retinoic acid (ATRA) has been clinically used as a therapeutic agent for acute promyelocytic leukemia (APL), because of its appropriate effects on differentiation and apoptosis (12). RA also indicated an apoptotic effect through the activation of the caspase pathway (13).

The initiation and execution of apoptosis requires the activation of a complex network of caspases (14-16). There are two main groups of caspases involved in apoptosis; caspase-8 and -10 initiate the extrinsic pathway, which is activated upon ligation between the death receptors (such as Fas/CD95 and the TNF receptor) on the cell surface and its corresponding ligands (15). Caspase-9 is activated in the intrinsic or mitochondria-initiated apoptosis pathway (16). The initiator or upstream caspases, which include caspase-8, caspase-10, and caspase-9, activate the effector or downstream caspases which include caspase-3, caspase-6, and caspase-7.

TAC-101 is a novel benzoic acid derivative, a type of synthetic retinoid, which has a binding affinity for the retinoic acid receptors (RAR)α and RARβ (17). Several reports have documented the antitumor and anti-metastatic activity of TAC-101 in in vivo and in vitro experiments (18-28). The previous studies have shown that TAC-101 possesses various...
biological activities, including differentiation-induction (24), apoptosis induction (19-21, 24, 27, 28), anti-angiogenesis (22, 26, 29) and life-prolonging effects (18, 20, 23-25, 27) in various cancer cells or experimental metastatic models.

Several studies have demonstrated that TAC-101 induces apoptotic cell death in several cancer cell lines via using DNA fragmentation (19-21, 24, 27) and this apoptosis was inhibited by Z-VAD-FMK, a potent and broad inhibitor of caspase (24, 27). Moreover, using FACScan, we previously observed that TAC-101 induced apoptosis in DLD-1 human colon cancer cells (28). It was also indicated that TAC-101 induces the apoptosis and increased Fas expression in hepatic metastatic tumors in a rat experimental model (28).

However, the alteration of the caspase activities under medication with TAC-101 has not been evaluated. The detailed mechanisms of apoptosis induced by TAC-101 also remain unclear. Therefore, the induction of apoptosis-related proteins and activities of caspasas was investigated in a DLD-1 cell line under medication with TAC-101.

### Materials and Methods

**Reagents, cell culture and antibodies.** TAC-101 was synthesized and kindly donated by Taiho Pharmaceutical Co., Ltd. (Hannou, Japan). TAC-101 was dissolved in DMSO at a concentration of 10 mM for the stock solutions and kept at −20°C until use. The final concentration of DMSO was less than 0.2%.

The DLD-1 human colon cancer cell line was also kindly supplied by Prof. Kohno (University of Occupational and Environmetal Health, Japan). The cells were maintained in RPMI-1640 medium (GIBCO BRL, Gaithersburg, MD, USA) containing 10% heat-inactivated fetal bovine serum (FBS; ICN Pharmaceuticals, Aurora, OH), 100 IU/ml penicillin and 60 μg/ml kanamycin, at 37°C in a humidified chamber with 5% CO2/95% air atmosphere.

Anti-FAS (sc-715-G), anti-TNF-R1 (sc-8436), anti-DR3 (sc-7909) and anti-BID (sc-11423) antibodies were purchased from Daco (Carpinteria, CA, USA), according to the protocol recommended by the manufacturer. The cell pellets were counted and the cytosol extracts from the 2x10⁶ DLD-1 cell culture were diluted to 100 μg protein per 50 μL cell lysis buffer. The protease activity was measured using tetrapeptide p-nitroanilide substrates. Asp-Glu-Val-Asp-pNA (DEVD-pNA), Ile-Glu-Thr-Asp-pNA (IETD-pNA) and Leu-Glu-His-Asp-pNA (LEHD-pNA) were used in the caspase-3,-8 and -9 assay, respectively. After incubation with these substrates at 37°C for 2 h, the absorbance of each well was measured at 405 nm using a microplate reader.

**Statistical analysis.** All data are expressed as the mean±SD. The differences between groups were assessed for statistical significance using Student’s t-test. P-values less than 0.05 were considered statistically significant.

### Results

**Effect of TAC-101 on Fas, TNF-R1 and DR3.** TAC-101 increased the expression of Fas in a time-dependent manner (Figure 1A), and in a dose-dependent manner (Figure 1B). On the other hand, TAC-101 had no effect on the level of TNF-R1 or DR3.

**Effect of TAC-101 on Bcl-2, Bax and Bid.** Western blot analysis showed that TAC-101 had almost no effect on the levels of Bcl-2, Bax or Bid proteins (Figure 2A, B).

**Effect of TAC-101 on the activity of caspase-3, caspase-8 and caspase-9.** Forty-eight hours after incubation with 20 μM TAC-101, a 3-fold increase in caspase-3 activity and a 1.5-fold increase in caspase-8 activity were observed in the treated cells in comparison to the control cells (p<0.01; Figure 3A, B). The increase in caspase-3 and caspase-8 activities was also significantly different.

**Western blot analysis of Fas, TNF-R1, DR3, Bcl-2, Bax and Bid.** The cell pellets were treated with an extraction buffer containing 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10% glycerol and Complete Mini (Roche Diagnostics, Mannheim, Germany). The cellular debris was separated from the extract by centrifugation, and the supernatant was stored at −70°C. The protein concentrations of the whole-cell extracts were determined using the Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Tokyo, Japan). The whole-cell extracts were separated on 10 or 15% SDS-PAGE gels (Bio-Rad Ready Gels J, Bio-Rad Laboratories, Tokyo, Japan). After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (ATTO, Tokyo, Japan) using a semidry blotter. A Western blot analysis was performed using an appropriate dilution of Anti-FAS (sc-715-G), anti-TNF-R1 (sc-8436), anti-DR3 (sc-7909), or anti-BID (sc-11423) antibodies, followed by visualization using the ECL plus kit (Amersham Biosciences Corp., NJ, USA) with enhanced chemiluminescence.
compared to 24 h incubation with 20 μM TAC-101 ($p<0.01$; Figure 3A) and to 48 h incubation with 10 μM TAC-101 ($p<0.05$; Figure 3B). TAC-101 increased the activity of caspase-3 and caspase-8 in both a time- and a dose-dependent manner.

In contrast, TAC-101 had no significant effect on the activity of caspase-9 (Figure 3A, B).

**Discussion**

Several synthetic retinoids have been created and evaluated as potent chemoprevention and chemotherapeutic agent. Each retinoid exhibits individual binding affinity to RARs and/or RXRs and has different effects on apoptotic-related factors. 4-Amino-2-(butyryl-amino)phenyl(2E,4E,6E,8E)-
Figure 3. Activity of caspase-3, -8 and -9. The total cell lysates from DLD-1 cells treated with (A) 20 µM TAC-101 for 0, 24, 48 h and (B) 0, 10, 20 µM TAC-101 for 48 h were analyzed using caspase colorimetric protease assay kits. The data points and error bars represent the mean values and standard deviation, respectively. *p<0.05, **p<0.01.
3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6,8-non-tetraenoate (ABPN) activated all three RAR isotypes to an extent similar to all-trans-retinoic acid (ATRA)-induced natural RA (4). Adapalene (ADA) (6-[3-(1-adamantanyl)-4-methoxyphenyl]-2-napthoic acid) had high affinity for RARβ and RARγ and only weak affinity for RARα, but does not bind to members of RXR (30). 4-(N-hydroxyphenyl) retinamide (4-HPR) (31) and 6-[3-(1-adamantanyl)-4-hydroxyphenyl]-2-napthalene carboxylic acid (CD437) (32) mainly bind and activate RARγ. The binding affinity of 9-cis-retinoic acid (CRA), a natural retinoid, is best for RXRα (33, 34). In contrast, TAC-101 has a strong binding affinity for RARα and a weak binding affinity for RARβ. The pattern of TAC-101 binding affinity may be specific and different from other synthetic RAs.

Although the RA modulating factors concerning invasion and metastasis, such as matrix metalloproteinases (9, 10) and growth factors (8) are relatively well understood, the RA inducing factors concerning apoptosis and differentiation are not clear. Several factors concerning apoptosis induced by RA were evaluated. In previous studies, we indicated that TAC-101 markedly increased the expression of Fas in hepatic metastatic tumors in an in vivo model (28). In our present study herein, we reconfirm that TAC-101 induce the expression of Fas in DLD-1 cells. The previous study indicated that Fas expression increased in a SW480 colon cancer cell line upon treatment with ATRA (11). ATRA also modulated the Fas expression in human medulloblastoma Med-3 cells (35) and enhanced Fas expression in an MCF-7 human breast cancer cell line with interferon-γ (36). Moreover, CD-437 induced apoptosis-involved induction of death receptor Fas, CD4 and CD-5 in lung cancer cell lines H460 and H292 (37). Although the Fas-inducing mechanism of RAs is unclear, TAC-101 and other synthetic retinoids indeed increased the expression of Fas.

The induction of apoptosis essentially needs the activation of caspases. According to the studies concerning caspase activities, several distinct pathways exist resulting in the induction of apoptosis by synthetic retinoids. There are some studies which denoted the apoptotic induction through the activation of caspase-3 and -8 by RA. 4-HPR induces apoptosis by activating caspase-3 or -8 (38, 39). CD-437 induces apoptosis by activating caspase-3 or -8 in DU145 human prostate cancer cells (40). ABPN induced apoptosis through the cleavage and activation of caspase-3 and -8, which may result in the cooperative cleavage of PARP in an HCT166 colon cancer cell line (4). In our present study, it was directly measured and indicated that TAC-101 induced the activities of caspase-3 and -8, but did not induce the activity of caspase-9 in DLD-1 cells (Figure 3). These data may indicate that the death receptor root of the apoptotic signal transduction in DLD-1 cells participates mainly in the apoptotic induction of TAC-101.

On the other hand, TAC-101 had almost no effect on the level of Bcl-2, Bax or Bid protein (Figure 2). Therefore, the mitochondrial root of apoptotic signal transduction in DLD-1 cells may not participate in the apoptotic induction of TAC-101.

In this study, it was indicated that TAC-101 increased the expression of Fas in a DLD-1 cell line both in a time- and a dose-dependent manner (Figure 1). Our previous study also indicated that TAC-101 markedly increased the expression of Fas in hepatic metastatic tumors in an in vivo model (28). According to the reports regarding the other RAs, ATRA can enhance the chemosensitivity of human medulloblastoma Med-3 cells presumably via modulation of the Fas expression pattern (35). The combination of ATRA and IFN-γ also acted synergistically in MCF-7 breast cancer cells to induce the expression of Fas mRNA and Fas protein in a time-dependent and dose-dependent manner (36). Several studies have indicated the induction of Fas ligand by several anticancer drugs, such as doxisorbidin, cisplatinum and VP-16 (41-43). In addition, the combination treatment of TAC-101 and CDDP caused a marked anti-tumor and life-prolonging effects in Lewis lung carcinoma (23) and A549 lung cancer cells (25). This combination therapy may also result in synergistic activity, even though the mechanisms of action of TAC-101 and CDDP are considered to be independent of each other. The combination therapy, both the induction of the Fas expression in the tumor cells by TAC-101 and the up-regulation of the Fas ligand by chemotherapeutic agents in a micro-environment including tumor cells, will be considered for subsequent studies.

Conclusion

The potent apoptotic mechanism of TAC-101 has been demonstrated in a DLD-1 human colon cell line. Because the compounds inducing apoptotic activity are frequent targets of cancer therapy, TAC-101 may be a good candidate for use in the treatment of colon cancer.

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