A Histone Deacetylase Inhibitor, OBP-801, and Celecoxib Synergistically Inhibit the Cell Growth with Apoptosis via a DR5-Dependent Pathway in Bladder Cancer Cells

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Abstract

The prognosis of muscle-invasive bladder cancer with metastasis is poor. There have been no therapeutic improvements for many years, and an innovative therapy for muscle-invasive bladder cancer has been awaited to replace the conventional cytotoxic chemotherapy. Here, we show a candidate method for the treatment of bladder cancer. The combined treatment with a novel histone deacetylase (HDAC) inhibitor, OBP-801, and celecoxib synergistically inhibited cell growth and markedly induced apoptosis through the caspase-dependent pathway in high-grade bladder cancer cells. Furthermore, the combined treatment induced expression of death receptor 5 (DR5). We identified that knockdown of DR5 by small interfering RNA (siRNA) significantly suppressed apoptosis by the combined treatment. Therefore, we conjectured that the apoptosis induced by OBP-801 and celecoxib is at least partially dependent on DR5. However, it was interesting that the combined treatment drastically suppressed expression of DR5 ligand, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL). These data suggest that there is no involvement of TRAIL in the induction of apoptosis by the combination, regardless of the dependence of DR5. Moreover, xenograft studies using human bladder cancer cells showed that the combined therapy suppressed tumor growth by upregulating expressions of DR5 and Bim. The inhibition of tumor growth was significantly more potent than that of each agent alone, without significant weight loss. This combination therapy provided a greater benefit than monotherapy in vitro and in vivo. These data show that the combination therapy with OBP-801 and celecoxib is a potential novel therapeutic strategy for patients with muscle-invasive bladder cancer. Mol Cancer Ther; 15(9); 2066–75. ©2016 AACR.

Introduction

The prognosis of muscle-invasive bladder cancer is poor with a 5-year overall survival of 48% to 62% after radical cystectomy (1). The efficacy of cisplatin-based chemotherapy for advanced bladder cancer is not adequate considering the limited response rate as well as the dose-limiting nephrotoxicity (2). Instead of conventional chemotherapeutic agents that often cause DNA damage in tumors and normal tissue, more successful chemotherapeutic agents are needed.

It has been reported that expression of COX-2 in high-grade bladder cancer was higher compared with that in low-grade bladder cancer (3, 4). Therefore, we focused on a selective COX-2 inhibitor celecoxib used for the treatment of several diseases. It has also been reported that celecoxib inhibited the cell growth of high-grade bladder cancer in vitro and in vivo (5), and that celecoxib could induce apoptosis in various cancer cells independent of COX-2 inhibitory activity (6). However, the underlying mechanisms still remain unclear. In order to enhance the effect of celecoxib against high-grade bladder cancer cells, we tried to identify the effective chemotherapeutic agents in the combination with celecoxib.

On the other hand, it was reported that expression levels of HDAC1 and HDAC2 were associated with high-grade bladder cancer, and that high-grade bladder cancer with high expression of HDAC1 showed a poor prognosis (7). Several studies have reported that HDAC inhibitors synergize with cisplatin (8) or gemcitabine (9) and could potentially increase clinical efficacy in bladder cancer (10). We previously reported that OBP-801/YM753, identified as a novel HDAC inhibitor by screening for p21WAF1/CIP1-inducing agents, had attractive pharmacodynamic and pharmacokinetic properties (11). OBP-801 exerted the most potent HDAC-inhibitory activity when compared with other HDAC inhibitors (11).

Consequently, we discovered that cotreatment with HDAC inhibitor OBP-801 and celecoxib drastically inhibited cell growth, and induced apoptosis against human bladder cancer.
cells. Moreover, we showed that the combination therapy significantly decreased tumor volume in a human high-grade bladder cancer T24 xenograft model. Taken together, combination therapy with OBP-801 and celecoxib can be one of the novel therapeutic applications for patients with muscle-invasive bladder cancer.

Materials and Methods

Reagents

OBP-801 (Oncolyts BioPharma), celecoxib (Selleckchem), and zVAD-fmk (R&D Systems) were dissolved in dimethyl sulfoxide (DMSO). Soluble recombinant human TRAIL/Apo2L was purchased from PeproTech. The human recombinant DR5/Fc chimera was purchased from R&D Systems.

Cell culture

Human bladder cancer T24, UM-UC-3, and HT1376 cells were purchased from the American Type Culture Collection in September 2013, and UM-UC-11 cells were purchased from the European Collection of Animal Cell Cultures in January 2014. Cell line authentications were not carried out by the authors within the last 6 months. T24, UM-UC-11, UM-UC-3, and HT1376 cells were cultured in RPMI-1640 supplemented with 10% FBS, L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO2.

Cell proliferation assay

The number of viable cells was determined using the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer’s instructions (Dojindo). After the incubation of cells for 72 hours (T24, UM-UC-3, HT1376) or 96 hours (UM-UC-11) with the indicated concentrations of OBP-801 or celecoxib, the kit reagent CCK-8 was added to the medium, and it was incubated for 4 hours. The absorbance of samples (450 nm) was determined using a scanning multiwell spectrophotometer (Multiskan FC, Thermo Scientific).

Combination index

Combination index (CI) values were analyzed using CalcuSyn software (Biosoft). Synergism is defined as more than the expected additive effect with CI<1.

Analysis of cell cycle and detection of apoptosis

Cells were treated with the agents and harvested from culture dishes. After washing with PBS, the cells were treated with PBS containing 0.1% Triton X-100 and the nuclei were stained with propidium iodide (Sigma). The DNA content was measured using FACScalibur (Becton Dickinson). For each experiment, 10,000 cells were analyzed. Cell Quest software (Becton Dickinson) and ModFit LT V2.0 software package (Verity Software House) were used to analyze the data. DNA fragmentation was quantified by the percentage of hypodiploid DNA (sub-G1).

Western blotting

Cells were lysed in lysis buffer (50 mMol/L Tris–HCl, 1% SDS, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 0.5 mMol/L phenylmethylsulfonyl fluoride, and 1 mMol/L dithiothreitol). The lysate was sonicated and centrifuged at 15,000 rpm for 20 minutes at 4°C, and the supernatant was collected. Xenograft tumor samples were snap-frozen in liquid nitrogen and stored at −80°C. Frozen samples were powdered in liquid nitrogen by using a Cryo-Press (Microtec Nichion), and then lysed with sample buffer (50 mMol/L Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.002% bromophenol blue). The protein extract was loaded onto a 10% or 12.5% SDS-polyacrylamide gel for electrophoresis, and blotted onto polyvinylidene difluoride membranes (Millipore). The following antibodies were purchased from the indicated sources: rabbit monoclonal antibodies for anti-Bim (Abcam), anti-Bcl-xL, and anti-PARP (Cell Signaling Technology); rabbit polyclonal antibodies for anti-survivin (R&D Systems), anti-DR5 (Prosci or Cell Signaling Technology), anti-DR4 (Prosci), anti-caspase-3, anti-cleaved caspase-3, anti-Histone H4, anti-acetyl-Histone H4, and anti-Bid (Cell Signaling Technology); mouse monoclonal antibodies for anti-caspase-8, and anti-caspase-9 (MBL), anti-FLIP (Enzo Life Sciences), anti-TRAIL (Novus), and anti-GAPDH (HyTest). The blots were incubated with HRP-conjugated secondary antibody (GE Healthcare), and signals were detected using the Chemilumino-One chemiluminescent kit (Nacalai Tesque) or Chemiluminescent HRP Substrate (Millipore).

RNA analysis

Total RNA was isolated from T24 or UM-UC-11 cells treated with agents using Sepasol-RNA I (Nacalai Tesque) according to the manufacturer’s instructions. Total RNA (2 μg) was reversely transcribed to cDNA in a 25 μL reaction volume with Moloney murine leukemia virus reverse transcriptase (Promega) and oligo (dT) primers (Toyobo) according to the manufacturer’s instructions. Quantitative real-time RT-PCR was carried out using a StepOnePlus system (Applied Biosystems) to quantify the expression level of FLIP, survivin, DR5, and TRAIL mRNA normalized to β2-microglobulin (β2MG) RNA. Real-time RT-PCR primer probes (FLIP, Hs00153353_m1, survivin; Hs00153353_m1, DR5; Hs00187196_m1, TRAIL; Hs00921974_m1, β2MG, Hs00984230_m1) were purchased from Applied Biosystems.

Assessment of cell surface DR5 expression

T24 cells (8 × 105 cells) and UM-UC-11 cells (1.6 × 105 cells) treated with agents for 48 hours (T24) or 72 hours (UM-UC-11) were harvested, washed once with ice-cold PBS, and resuspended in 100 μL PBS with 1% BSA. Then, phycoerythrin (PE)-conjugated mouse anti-human DR5 mAb (eBioscience) was added. To assess nonspecific staining, PE-labeled IgG isotype control (eBioscience) was applied. After a 30-minute incubation on ice, the cells were washed twice in ice-cold PBS, resuspended in 100 μL of ice-cold PBS, and were then analyzed by FACScalibur.

Small interfering RNA transfection

The DR5 siRNA and the negative control (NC) siRNA were purchased from Invitrogen and Ambion (Life Technologies). The Bim siRNA and the NC siRNA were purchased from Sigma. The DR5 siRNAs (HS112940 by Invitrogen, siRNA ID# s16756 by Ambion) and Bim siRNA (ACGU1ACGAGCAAGGUGUIC) were used for the transfection. At the same time as the
transfection, $6 \times 10^4$ of T24 cells and $1.2 \times 10^5$ of UM-UC-11 cells were seeded per well in 6-well plates without antibiotics. The D53 siRNA (0.25 nmol/L) and Bim siRNA (20 nmol/L) for T24 cells, and D53 siRNA (40 nmol/L) and Bim siRNA (5 nmol/L) for UM-UC-11 cells were transfected into cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. Twenty-four hours after the transfection, the cells were treated with agents for 48 hours (T24) or 72 hours (UM-UC-11) and then harvested.

Enzyme-linked immunosorbent assay (ELISA)

T24 cells were treated with agents for 48 hours. Concentrations of soluble TRAIL in the supernatants were measured by a Human TRAIL ELISA kit (Diaclone).

DR5 immunoprecipitations

T24 cells treated with agents for 24 hours were harvested, washed once with ice-cold PBS, and resuspended in IP lysis buffer containing 1% of NP-40, 20 mmol/L of Tris–HCl (pH 7.5), 150 mmol/L of NaCl, and 10% of glycerol. For positive control, recombinant TRAIL was incubated with T24 cells for 30 minutes at $37^\circ$C before lysis. Lysates were precleared with nProtein A Sepharose 4 Fast Flow (GE Healthcare) and incubated overnight at 4°C with either 0.5 μg of rabbit anti-DR5 (Cell Signaling Technology) or isotype-matched antibody (Cell Signaling Technology). After the addition of nProtein A Sepharose 4 Fast Flow, each sample was further incubated for 2 hours at 4°C. The complexes were subsequently washed four times with IP lysis buffer and eluted by SDS sample buffer for Western blotting.

Mouse xenograft models

Female SHO-Pkrdc scid mice (5-week-old) were purchased from Charles River. All experiments and procedures were done in accordance with the institutional animal care and use committee guidelines. The present study was also approved by the Committee for Animal Research of Kyoto Prefectural University of Medicine (permission No. M26-226). T24 cells (5 × 10^7 cells) mixed with high-concentration Matrigel (BD Biosciences) were inoculated into the back of the mice by subcutaneous injection. The tumor volume was calculated using the following formula: $1/2 \times (\text{length}) \times (\text{width})^2$. When the tumor volume was approximately 130 to 200 mm³, the mice were randomly divided into four groups (seven mice per group) and treatment was initiated. The mice were injected 2 times a week for 2 weeks (on days 1, 4, 8, and 11) with diluent only, or OBP-801 (8 mg/kg). The mice were also orally administered 5 times a week for 2 weeks (on days 1, 4, 5, 6, 7, 8, 11, 12, 13, and 14), with diluent only, or celecoxib (25 mg/kg). OBP-801 was dissolved in 20% hydroxypropyl-β-cyclodextrin/saline and injected into the tail vein. Celecoxib was dissolved in polyethylene glycol/saline (diluent, 2:1) and provided for oral injection. The tumor size was measured 3 times a week (on days 1, 4, 6, 8, 11, 13, and 15). On day 15, the tumors were excised from the euthanized mice.

Statistical analysis

Data were expressed as the mean ± SD of three determinations. Statistical analysis was performed using a Student t test. Samples were considered significantly different at $P < 0.05$.

Results

Combined treatment with OBP-801 and celecoxib synergetically inhibits cell growth and induces apoptosis in human bladder cancer cells

To investigate the antiproliferative effects of OBP-801 or celecoxib alone, we assessed the viable cell number of T24, UM-UC-11, UM-UC-3, and HT1376 cells after 72 or 96 hours of treatment with the indicated concentrations of the agents. Each agent was effective against cell growth in a dose-dependent manner (Fig. 1A). Interestingly, cotreatment with low-dose OBP-801 and celecoxib markedly inhibited cell growth when compared with the single treatment of each agent (Fig. 1B). Moreover, the combination index values for 6 nmol/L OBP-801 and 50 μmol/L celecoxib in T24 cells were <1.0, indicating a synergistic effect for the inhibition of cell growth (Table 1).

Combined treatment with OBP-801 and celecoxib induces caspase-dependent apoptosis in human bladder cancer cells

To clarify the mechanisms of synergistic inhibitory effects on cell growth from the combination of OBP-801 and celecoxib, we next performed cell-cycle analysis using flow cytometry after treatment for 48 or 72 hours. Celecoxib slightly induced G1 cell-cycle arrest in T24 cells (Fig. 1C). OBP-801 or celecoxib alone only weakly induced apoptosis, but the cotreatment with OBP-801 and celecoxib drastically induced apoptosis in T24, UM-UC-11, UM-UC-3, and HT1376 cells (Fig. 1D). The pan-caspase inhibitor zVAD-fmk effectively blocked apoptosis induced by cotreatment with OBP-801 and celecoxib (Fig. 1D). These results suggest that the combined treatment with OBP-801 and celecoxib synergistically inhibits cell growth and induces caspase-dependent apoptosis in human bladder cancer cells.

Concurrent treatment with OBP-801 and celecoxib causes apoptosis via intrinsic and extrinsic pathways in human bladder cancer cells

We examined the expression of apoptosis-related proteins in the combined treatment. As shown in Fig. 2A, caspase-3, caspase-8, caspase-9, and PARP were clearly cleaved by the concurrent treatment. We confirmed the increased acetylation of histone in T24 and UM-UC-11 cells by OBP-801 (Fig. 2B, and C). The cotreatment upregulated the expression of proapoptotic proteins, Bim and DR5, and downregulated the expression of antiapoptotic proteins survivin and FLIP, (Fig. 2B and C). These results suggest that the combined treatment with OBP-801 and celecoxib induced apoptosis through the activation of both intrinsic and extrinsic pathways in human bladder cancer cells. To examine how the cotreatment regulated the expression of antiapoptotic proteins, we next investigated the expression of mRNA by real-time RT-PCR. The combined treatment with OBP-801 and celecoxib substantially downregulated FLIP and survivin mRNA expression compared with the single treatment in both T24 and UM-UC-11 cells (Supplementary Fig. S1), suggesting that the cotreatment downregulated the expression of survivin and FLIP at the transcriptional level. DR5 mRNA expression in T24 cells was not proportionate to upregulation of DR5 protein (Supplementary Fig. S2A). On the other hand, the combined treatment induced DR5 mRNA expression in UM-UC-11 cells (Supplementary Fig. S2A). We next
Figure 1.
Combination treatment with OBP-801 and celecoxib synergistically inhibits cell growth and induces apoptosis in human bladder cancer cells. A and B, human bladder cancer T24, UM-UC-11, UM-UC-3, or HT1376 cells were treated with the indicated concentrations of OBP-801 and/or celecoxib. After incubation for 72 hours (T24, UM-UC-3, and HT1376 cells) or 96 hours (UM-UC-11 cells), cell counts were assessed by the CCK-8 assay. Columns, means of triplicate data; bars, SD; †, P < 0.05; ‡, P < 0.01. C, representative histogram patterns in T24 cells. D, T24, UM-UC-11, UM-UC-3, and HT1376 cells were treated with the indicated concentrations of OBP-801 and/or celecoxib with or without the pan-caspase inhibitor zVAD-fmk for 48 hours (T24 and UM-UC-3 cells) or 72 hours (UM-UC-11 and HT1376 cells). Sub-G₁ populations were analyzed by flow cytometry. Columns, means of triplicate data; bars, SD; †, P < 0.05, ‡, P < 0.01.
investigated the cell surface expression of DR5 by flow cytometry. The cell surface expression level of DR5 in T24 cells was highest in the combined treatment (Supplementary Fig. S2B), but not in UM-UC-11 cells. The regulation of DR5 expression by the combination differs from cell lines.

**DR5 is involved in apoptosis by the cotreatment with OBP-801 and celecoxib**

The cotreatment with OBP-801 and celecoxib upregulated the expression of DR5 in T24 and UM-UC-11 cells (Fig. 2B and C). Previously, Martin and colleagues reported that COX-2 inhibition is responsible for the clustering of DR5 (12). Accordingly, we next examined whether DR5 contributed to the induction of apoptosis by the cotreatment with OBP-801 and celecoxib. The effects of DR5 knockdown were confirmed by Western blotting (Fig. 3A and B). As shown in Figure 3A and B, DR5 siRNA significantly suppressed the apoptosis by the cotreatment in T24 and UM-UC-11 cells. These results suggest that the cotreatment with OBP-801 and celecoxib causes apoptosis at least partially through the upregulation of DR5 in bladder cancer cells.

**Combined treatment with OBP-801 and celecoxib stimulates binding of caspase-8 to DR5 in human bladder cancer T24 cells**

As shown in Fig. 2B and C, the cotreatment upregulated the expression of DR5 and downregulated the expression of FLIP. Day and colleagues reported that c-FLIP interacts with DR5 and caspase-8 to form an apoptotic inhibitory complex that inhibits spontaneous DISC signaling (13). Therefore, we next examined whether the cotreatment encouraged DISC signaling. Caspase-8 was immunoprecipitated using an anti-DR5 monoclonal antibody from whole cell lysates of T24 cells treated with each agent for 24 hours (Supplementary Fig. S3A). As results, the combined treatment with OBP-801 and celecoxib enhanced the binding of caspase-8 to DR5.

**Bim is also concerned with apoptosis by the combined treatment in bladder cancer cells**

The combined treatment upregulated the expression of Bim in bladder cancer cells (Fig. 2B and C). We examined the contribution of Bim in the combination treatment. The effects of Bim knockdown were confirmed by Western blotting (Supplementary Fig. S3B and S3C). Bim siRNA significantly suppressed the apoptosis by the combination (Supplementary Fig. S3B and S3C). Therefore, the combined treatment with OBP-801 and celecoxib causes apoptosis partially through the upregulation of Bim in bladder cancer cells.

**Apoptosis induced by OBP-801 and celecoxib is independent of TRAIL**

We next examined the expression of TRAIL, a ligand of DR5. TRAIL expression of supernatants and whole cell lysate were also significantly downregulated by celecoxib alone and the combined treatment in T24 cells. (Fig. 4A and B). Furthermore, TRAIL mRNA was drastically repressed by celecoxib alone and the combined treatment (Fig. 4C; Supplementary Fig. S4A). and DR5/Fc chimera failed to rescue this apoptosis in T24 and UM-UC-11 cells (Fig. 4D; Supplementary Fig. S4B). In the case of UM-UC-11 cells, the concentration of TRAIL in supernatants was too low to be measured in all of samples (data not shown). These results suggest that the contribution of TRAIL is very low in the apoptosis induced by the cotreatment with OBP-801 and celecoxib in bladder cancer cells.

**The effect of celecoxib with TRAIL is weaker than that of celecoxib with OBP-801 in bladder cancer cells.**

TRAIL alone was not effective against suppression of cell growth in T24 and UM-UC-11 cells (Fig. 4E; Supplementary Fig. S4C). On the other hand, the combined treatment of celecoxib and TRAIL inhibited cell growth and induced additional apoptosis compared with celecoxib alone in bladder cancer cells. However, the combined effects of celecoxib and TRAIL were less potent than those of celecoxib and OBP-801 in bladder cancer cells (Fig. 4E and F; Supplementary Fig. S4C and S4D).

**Combined therapy with OBP-801 and celecoxib inhibits tumor growth in vivo**

To examine the potential effects of combination with OBP-801 and celecoxib in vivo, we evaluated the antitumor activity of the combined therapy in SCID mice xenograft models inoculated with T24 cells. Mice were treated with vehicle, OBP-801, celecoxib, or their combination. After treatment for 15 days, neither OBP-801 nor celecoxib treatment alone significantly inhibited the growth of T24 tumors. However, the combined therapy drastically suppressed the tumor growth when compared with both control and monotherapy (Fig. 5A). None of the treated mice showed any evidence of significant weight loss (Fig. 5B). Furthermore, we examined the expressions of apoptosis-related proteins in T24 tumors. As shown in Figure 5C, caspase-3 was clearly cleaved, and the expressions of DR5 and Bim were upregulated by the concurrent treatment. These results suggest that the combined therapy with OBP-801 and celecoxib is more effective than monotherapy in vivo and induces apoptosis via upregulation of DR5 and Bim as observed in vitro.

**Discussion**

As mentioned above, more effective chemotherapeutic agents are needed for the treatment of high-grade bladder cancer. In this study, we focused on COX-2 and HDAC as therapeutic targets, both of which were highly expressed in high-grade bladder cancer (3, 4, 7). Previously, Peulen and colleagues reported that combined treatment with the HDAC inhibitor MS-275 and the COX-2 inhibitor celecoxib induced G1 cell-cycle arrest against pancreatic cancer cells in vitro (14). On the other hand, in the present study, the combined treatment with a novel HDAC inhibitor, OBP-801, and celecoxib synergistically inhibited cell growth of high-grade bladder cancer cells in vivo by enhancing apoptosis (Fig. 1), inconsistent with the previous report. The same combination therapy also showed the similar effect in vivo (Fig. 5). Consequently, we investigated the mechanisms of significant apoptotic effects by the cotreatment with OBP-801 and celecoxib.

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**Table 1.** The combination index of OBP-801 and celecoxib was calculated in T24 cells

<table>
<thead>
<tr>
<th>OBP-801 (nmol/L)</th>
<th>Celecoxib (µmol/L)</th>
<th>Combination index</th>
</tr>
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<tbody>
<tr>
<td>3</td>
<td>25</td>
<td>0.751</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>0.668</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>0.802</td>
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As the molecular mechanisms of the combination, it was suggested that DR5, FLIP, Bim, and survivin participated in the apoptosis by the combined treatment. Previous reports showed that HDAC inhibitors alone or COX-2 inhibitors alone at high concentrations induced a variety of proapoptotic genes, thereby inducing apoptosis (6, 15). Especially, the results of knockdown of DR5 showed that the cooperative effects of OBP-801 and celecoxib occurred at least partially through DR5 in both bladder cancer cells (Fig. 3).

In the present study, the combination with OBP-801 and celecoxib induced the expression of DR5 mRNA in UM-UC-11 cells but not in T24 cells (Supplementary Fig. S2A). From the results of Western blotting (Fig. 2B), we speculate that the combination might contribute the stabilization of DR5 protein in T24 cells.
cells. On the other hand, the cell surface expression level of DR5 in T24 cells was highest in the combined treatment, but not in UM-UC-11 cells (Supplementary Fig. S2B). Regardless of no clear enhancement of the cell surface expression of DR5 in UM-UC-11 cells, DR5 was weakly but significantly involved in apoptosis by the combination (Fig. 3B). We speculate that one of the reasons might be due to regulation of several molecules downstream of the DR5 pathway, such as suppression of FLIP and induction of Bim in the present study.

Li and colleagues reported that bladder cancer patients with high expression of DR5 had a significantly longer postoperative recurrence-free rate than those with low expression of DR5 during 10 years of follow-up (16). It is suggested that DR5 might play an important role in regulating the malignant potential of bladder cancer and upregulation of DR5 by chemotherapy or molecular-targeted drugs will be beneficial to bladder cancer patients.

DR5 is one of the TRAIL-specific receptors and initiates apoptosis by forming trimer (17). There are many reports that the upregulation of DR5 induces apoptosis through the enhancement of TRAIL sensitivity in several cancer cells (18, 19). However, the combined treatment drastically suppressed the expression of TRAIL (Fig. 4A–C; Supplementary Fig. S4A), in spite of the induction of apoptosis through DR5 upregulation. Furthermore, DR5/Fc chimera, which blocks the binding of DR5 and TRAIL, failed to rescue the apoptosis by the combined treatment (Fig. 4D; Supplementary Fig. S4B). Previously, Cazanave and colleagues and Eckhardt and colleagues reported that DR5 upregulation induced apoptosis in a TRAIL-independent manner (20, 21). Therefore, we conclude that the combined treatment with OBP-801 and celecoxib mediates DR5 upregulation and induces apoptosis in a TRAIL-independent manner in bladder cancer cells.

Previous studies have reported that celecoxib induced apoptosis via upregulation of DR5 in several cancer cells (22, 23). However, in this study, celecoxib alone did not induce apoptosis (Fig. 1C and D) in spite of the upregulation of DR5 in human bladder cancer cells (Fig. 2B and C). In this study, celecoxib increased the expression of FLIP (Fig. 2B and C) and suppressed the expression of TRAIL (Fig. 4A–C and Supplementary Fig. S4A). Therefore, it was suggested that the upregulation of FLIP and downregulation of TRAIL may contribute to the inhibition of apoptosis induced by celecoxib alone in bladder cancer cells.

To demonstrate more convincing evidence of the mechanisms of the apoptosis by the combination, we investigated the involvement of Bim, because Bim was drastically upregulated by the combination (Fig. 2B and C). We performed the experiment of Bim knockdown using Bim siRNA in bladder cancer cells.
Figure 4.
OBP-801 and celecoxib induce apoptosis in a TRAIL-independent manner in T24 cells. 

A–C, T24 cells were treated with the indicated concentrations of OBP-801 and/or celecoxib for 48 hours. A, supernatants were collected, and TRAIL was quantified by ELISA. Columns, means of triplicate data; bars, SD; *, P < 0.05; **, P < 0.01. B, Western blotting of TRAIL. GAPDH was a loading control. C, quantitative real-time RT-PCR of TRAIL mRNA. The internal control was β2MG. Values show fold change in TRAIL mRNA expression compared with control (DMSO treatment). Data represent the means of triplicates with SD.

D–F, T24 cells were treated with the indicated concentrations of OBP-801 and/or celecoxib with or without DR5/Fc chimera. After incubation for 48 hours, sub-G1 populations were analyzed by flow cytometry. D, T24 cells were treated with the indicated concentrations of TRAIL or PBS, followed by the treatment with OBP-801 and/or celecoxib at the indicated concentrations. After incubation for 72 hours, cell counts were assessed by the CCK-8 assay. Columns, means of triplicate data; bars, SD; **, P < 0.01.
As a result, the apoptosis induced by the combination was significantly inhibited by the Bim knockdown. The results suggest that Bim might act as one of key molecules downstream of DR5 for induction of apoptosis by the combination in bladder cancer cells.

Korkolopoulou and colleagues reported that expression of FLIP in bladder cancer has been associated with advanced stage and grade (24). Therefore, it is expected that FLIP might be as an indicator of prognosis (25). Several studies have also shown that HDAC inhibitors can reduce the expression of FLIP (26, 27). In this study, the downregulation of FLIP protein by OBP-801 was not so obvious in bladder cancer cells (Fig. 2B and C). On the other hand, celecoxib induced the expression of FLIP as mentioned above (Fig. 2B and C). Interestingly, OBP-801 almost completely suppressed the induction of FLIP by celecoxib (Fig. 2B and C). The mechanisms of the downregulation of FLIP have been reported by several studies, showing the induction of proteasomal degradation and the inhibition of transcriptional regulation (28).

In this study, the expression of FLIP mRNA was drastically suppressed by the combined treatment (Supplementary Fig. S1A). Furthermore, the expression of survivin mRNA was also significantly suppressed by the combination in the same manner as FLIP (Supplementary Fig. S1B). Antiapoptotic factor IAPs, including FLIP and survivin, have been reported to be regulated by NF-κB at the transcriptional level in cancer cells (29, 30). Therefore, there is a possibility that the combined treatment with OBP-801 and celecoxib might inhibit common signal pathways such as NF-κB in bladder cancer cells.

Previous reports have shown that COX-2 inhibitors (12) or HDAC inhibitors (31, 32) were responsible for upregulation of DR5, improvement of TRAIL sensitivity, and concentrating DISC components. Furthermore, Day and colleagues reported that c-FLIP knockdown induced ligand-independent and DR5-dependent apoptosis in breast cancer cells (13). In this study, we showed that the combined treatment with OBP-801 and celecoxib stimulated the binding of pro-caspase-8 to DR5 in T24 bladder cancer cells (Supplementary Fig. S3A). Taken together, we speculate that the combined treatment induces not only the expression of DR5 but also the TRAIL-independent death signaling by downregulation of FLIP in bladder cancer cells.

Furthermore, our preclinical mice data showed that combined therapy with OBP-801 and celecoxib was effective in causing a marked reduction of bladder cancer without significant weight loss (Fig. 5). There is no widely recognized second-line therapy for the treatment of advanced bladder cancer. Therefore, the combined therapy with OBP-801 and celecoxib is a promising novel therapy for human bladder cancer patients.

Disclosure of Potential Conflicts of Interest

T. Sakai reports receiving commercial research grant from Oncoly Bio-Pharma Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: S. Toriyama, M. Horinaka, T. Sakai
Development of methodology: S. Toriyama, M. Horinaka, T. Sakai
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Toriyama, M. Horinaka, S. Yasuda, T. Taniguchi, Y. Aono, T. Takamura, Y. Morioka
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Toriyama, M. Horinaka, T. Sakai

Figure 5.
Combined therapy with OBP-801 and celecoxib inhibits tumor growth and induces apoptosis in a xenograft model. T24 cells (2 × 10⁷ cells) were injected into the flanks of SHO-Pikdcsid female mice. When the subcutaneous tumors reached a size of 130 mm³, mice were selected for treatment with a vehicle, OBP-801 (8 mg/kg, intravenous infusion), celecoxib (25 mg/kg, oral administration), or their combination. A, tumor growth curves per treatment group with the vehicle, OBP-801, celecoxib, or their combination. Points, means of tumor volumes (n = 6–7); bars, SD; *, P < 0.05; **, P < 0.01, compared with combined therapy. B, the body weight of the mice undergoing treatment in the four groups was measured 3 times a week. The body weight of each group was compared with that of the control group. Points, means of body weight (n = 6–7); n.s., not significant. C, Western blotting of cleaved caspase-3, DR5, and Bim. GAPDH was a loading control in all blots.
Grant Support

T. Sakai received the grant/research funding (1152001034) from Oncolys BioPharma Inc. (Tokyo, Japan).

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Received January 14, 2016, revised May 25, 2016; accepted June 19, 2016, published OnlineFirst July 12, 2016.

References


Molecular Cancer Therapeutics

A Histone Deacetylase Inhibitor, OBP-801, and Celecoxib Synergistically Inhibit the Cell Growth with Apoptosis via a DR5-Dependent Pathway in Bladder Cancer Cells

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doi:10.1158/1535-7163.MCT-16-0010

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