RESEARCH ARTICLE

Combined anti-metastasis therapy of an siRNA-based medicine and ABT-263 in orthotopically xenografted prostate cancer model mice

Yoshifumi Takei[1,2,*], Yuan Yuan,[2,3], Akiko Suzuki[2], Keichiro Mihara[4]

Authors' affiliations:

[1] Department of Medicinal Biochemistry, School of Pharmacy, Aichi Gakuin University, Nagoya, Japan;

[2] Division of Disease Models, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, Nagoya, Japan;

[3] Department of Lifelong Sports and Health Sciences, Chubu University, Kasugai, Japan;

[4] Department of Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan

[*]**Corresponding author:** Yoshifumi Takei, Ph.D., Department of Medicinal Biochemistry, School of Pharmacy, Aichi Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya 464-8650, Japan, E-mail: takei@dpc.agu.ac.jp

Abstract

Prostate cancer is a major public health problem among elderly men. In the United States, it is the second leading cause of cancer-related death among men. We investigated the therapeutic potential of combining siRNA-based medicine and ABT-263 in a prostate cancer mouse model (an orthotopic PC-3 transplanted nude mouse model). Both medicines target an anti-apoptotic protein, Bcl-xL. To deliver the siRNA-based medicine, we used a biomaterial atelocollagen as a delivery vehicle specific to tumors. Atelocollagen shows great advantages as a carrier for siRNA systemic delivery. We previously reported that an siRNA targeting human Bcl-xL showed that atelocollagen-mediated systemic delivery of the siRNA significantly suppressed tumor progression in a PC-3 orthotopic tumor model. Thus, we decided to investigate whether the therapeutic potential of the Bcl-xL siRNA complexed with atelocollagen could be increased by further inhibiting Bcl-xL by ABT-263. The intravenous injection of Bcl-xL siRNA mixed with atelocollagen (50 μg siRNA/shot) plus oral administration of ABT-263 (50 mg/kg) significantly and synergistically inhibited tumor growth in the PC-3 orthotopic model compared with each single administration. A luciferase-expressing PC-3 cell line was used to evaluate liver metastasis. The combined treatment of the siRNA and ABT-263 almost completely inhibited liver metastasis. The combined administration of Bcl-xL siRNA and ABT-263 indicated a synergistic therapeutic effect, suggesting that our proposed therapy has excellent potential to treat prostate cancers.

Keywords: prostate cancer, apoptosis, Bcl-xL, siRNA-based medicine, anti-metastasis therapy

1. Introduction

With the identification of new oncogenes and signaling pathways essential for tumor progression, RNA interference mediating sequence-specific gene silence provides an exhilarating strategy for cancer treatment. The main challenge for siRNA-based cancer therapy is to overcome the inconvenient properties of siRNA, such as large molecular weight, negative charges, and short half-life in blood circulation, in order to develop a method for systemic delivery of siRNA *in vivo*.[1,2] Numerous siRNA delivery methods have been developed to protect siRNAs from nuclease degradation, promote the tumor-specific accumulation of siRNAs, and increase cancer cells' uptake of siRNA molecules.[1,3,4]

For around 20 years, we have been studying a tumor-specific siRNA delivery method mediated by atelocollagen, a functional biomaterial, purified from bovine dermal collagen.[5-8] Atelocollagen shows neither antigenicity nor toxicity, because it is free from antigenic telopeptides with pepsin digestion.[5-8] Atelocollagen possesses basicity (a positive charge) under physiological conditions, whereas siRNA has acidity (a negative charge). Therefore, atelocollagen and siRNA form a rigid complex by electrostatic binding.[5-8] Atelocollagen functions in increasing siRNA stability in serum, sustaining the siRNA release, promoting tumor-specific delivery of siRNA, and preventing the siRNA-induced immune response.[5-9] Thus, atelocollagen is an effective carrier vehicle for systemic tumor-specific delivery of siRNA/miRNA, plasmid DNA, and antisense oligodeoxynucleotide.[10-12]

For many years we have been selecting Bcl-xL, an anti-apoptotic Bcl-2 family protein, as a target gene for cancer therapy against prostate cancers.[7,14] Bcl-xL, which is commonly upregulated in many types of cancers, including prostate cancers, protects tumor cells from mitochondrial-mediated apoptosis and confers multi-drug resistance.[13] Our previous study revealed that Bcl-xL siRNA mixed with atelocollagen significantly inhibited tumor growth and metastasis to liver in a PC-3 (a human prostate cancer cell line) orthotopic tumor model.[14] Although nucleic acid-based medicine such as siRNA or miRNA, which is systemically delivered into tumors with some biodegradable delivery vehicle, commonly may take charge of inevitable fate. For example, our tumor-specific systemic siRNA-delivery method via atelocollagen, at the very best, reduced the target gene expression in tumors by no more than half.[14] Indeed, regarding orthotopic tumors,[14] we showed that our Bcl-xL siRNA/atelocollagen complex administration halved the expression level of Bcl-xL.[14] Accordingly, we considered whether a more distinct therapeutic effect would be achieved by further inhibiting the effect of Bcl-xL. Therefore, in this article we investigated the effect of the Bcl-xL siRNA/atelocollagen complex in combination with ABT-263, a small molecule inhibitor of Bcl-xL.

ABT-263 is a potent antagonist for Bcl-xL. As a BH3 (Bcl-2 homology domain 3) mimetic, ABT-263 diminishes the anti-apoptotic effect of Bcl-xL by binding to the BH3 groove of Bcl-xL and impeding its association with pro-apoptotic proteins. It was reported that ABT-263 specifically induced apoptosis in Bcl-2/Bcl-xL dependent cancer cells and showed remarkable an-

ti-cancer effects in xenograft mouse models for acute lymphoblastic leukemia (ALL) and small-cell lung cancer (SCLC), especially via a unique p.o. administration.[15-17]

Here, we showed that the combined treatment of Bcl-xL siRNA/atelocollagen complex and ABT-263 induced additional and synergistic tumor regression and completely suppressed metastasis to liver in a PC-3 orthotopic model.

2. Materials and methods

1.1. Cell culture and reagents The human prostate cancer cell line PC-3 was supplied by American Type Culture Collection (Manassas, VA, USA). PC-3-Luc (JCRB 1406), a PC-3 cell line with stable expression of luciferase, was obtained from the Japanese Collection of Research Bioresources Cell Bank (Ibaraki, Japan). These cells were maintained in F12K medium (Kaighn's Modification of Ham's F-12 Medium, Invitrogen, Carlsbad, CA, USA), containing 10% FBS at 37℃ with 5% CO_2. ABT-263 was purchased from Selleck Chemicals (Munich, Germany).

1.2. siRNA We have already reported an siRNA targeting human Bcl-xL and a scrambled control siRNA (Bcl-xL siRNA-SCR), both of whose designs were based on the sequence of the human Bcl-xL gene.[7,14] All of the siRNAs were purchased from Dharmacon (Lafayette, CO, USA) as reported previously.[5,6]

2.3. Atelocollagen Atelocollagen was obtained from Koken Co. Ltd. (Tokyo, Japan). To eliminate the antigenicity of the collagen molecule, atelocollagen was prepared from the pepsin-treated tropocollagen in which the antigenic telopeptide was removed. Positively charged atelocollagen combined efficiently with siRNAs, carrying negative charges to form a stable complex.

2.4. Preparation of siRNA/atelocollagen complex To make siRNA/atelocollagen nanoparticles, we stirred siRNA solution in an equal volume of atelocollagen (0.1%) slowly with a rotator at 4℃ for 20 min.[7-9,14]

2.5. PC-3 orthotopic tumor model Eight-week-old male BALB/c athymic mice were purchased from Japan SLC (Hamamatsu, Japan). Somnopentyl (Kyoritsu Seiyaku Corp., Tokyo, Japan) diluted in saline was used to anesthetize the mice via i.p. injection (5 mg/kg). Then an incision was made in the lower abdomen with a surgical scissors. PC-3 cells ($5x10^5$ cells) in 20 μl FBS-free F12K medium was injected into the ventral lobe of the prostate gland, which is located under the bladder, using a 29-gauge needle (Terumo Clinical Supply, Tokyo, Japan). After the incision was sewn up with 5-0 surgical sutures (Alfresa Holdings, Tokyo, Japan), the mice were placed under a heating lamp to keep them warm. The details of the model preparation were reported previously.[14]

2.6. Evaluation of metastasis in PC-3 orthotopic model Orthotopic inoculation of PC-3-luc cells was performed according to the protocol described above. Four weeks later, the mice were sacrificed and their livers were harvested. The tissues were homogenized in a mixed solution consisting of CelLytic MT Mamalian Tissue Lysis/Extraction Reagent (Sigma Aldrich, St. Louis, MO, USA) and protease inhibitor mixtures (Sigma). After centrifugation, the supernatant was collected and luciferase

activity was measured by a dual-luciferase reporter assay kit (Promega, Madison, WI, USA) as reported previously.[14]

2.7. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay We performed TUNEL staining of frozen sections of the excised tumors using a MEBSTAIN® Apoptosis Kit II according to the manufacturer's protocol (MBL, Nagoya, Japan). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) to count the cells. TUNEL-positive cells were counted under a fluorescence microscope (Olympus) as reported previously.[6,7,14]

2.8. Statistical analysis The Mann–Whitney U-test was used to determine statistical differences, and *P*-values below 0.05 were considered significant.

3. Results

3.1. The combined treatment of Bcl-xL siRNA mixed with atelocollagen and ABT-263 showed significant and synergistic anti-cancer effects in the PC-3 orthotopic tumor model.

The tumor-bearing mice were treated with Bcl-xL siRNA/atelocollagen complex via i.v. injection and ABT-263 via oral administration at the indicated time points in Figure 1, and on day 28 the tumors were weighed to evaluate the anti-cancer effect (Figure 2). The single treatment of the Bcl-xL siRNA/atelocollagen complex alone or ABT-263 alone showed a medium effect in suppressing tumor growth (Figure 2). The anti-cancer effect was significantly enhanced by combining the Bcl-xL siRNA complex with ABT-263 ($p<0.01$). On the other hand, the Bcl-xL siRNA-SCR/atelocollagen complex or atelocollagen alone did not show any anti-cancer effect at all (Figure 2).

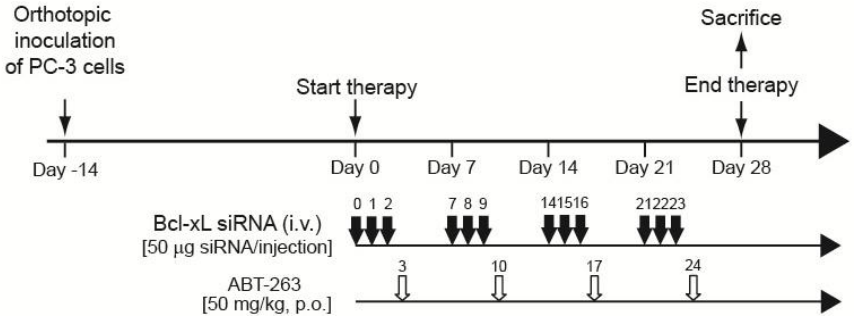

Figure 1. The procedure for therapeutic experiment.

After 14 days following PC-3 orthotopic inoculation, Bcl-xL siRNA mixed with atelocollagen (50 μg siRNA/injection, 0.05% atelocollagen) was intravenously injected into tumor-bearing mice for 3 consecutive days. ABT-263 (50 mg/kg) was orally administered on the fourth day. This drug regimen was repeated four times (one drug set per week), as indicated in the figure. On day 28 (therapy endpoint), all of the mice were sacrificed and each tumor was removed.

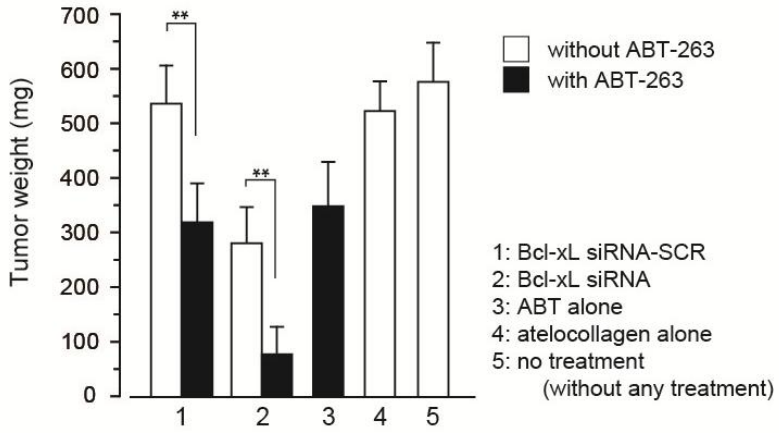

Figure 2. Significant and synergistic therapeutic effects of Bcl-xL siRNA/atelocollagen complex in combination with ABT-263 on PC-3 orthotopic tumors.
The anti-cancer effects of these reagents were evaluated by measuring the tumor weight at the end of therapy (on day 28). White bars, without ABT-263; black bars, with ABT-263. The results are means±SD (n=6 mice). ***P*< 0.01.

3.2. Our combined treatment significantly increased apoptotic cell death (TUNEL-positive cells) in orthotopic tumors.

On day 28 (the endpoint of the therapy), we sacrificed all of the remaining mice, obtained orthotopic PC-3 tumors from them, and prepared frozen sections. The sections were used for TUNEL staining according to our previous report.[6,7,14] It should be noted that the combined therapy significantly increased apoptotic cell death (TUNEL-positive cells) as shown in Figure 3 ($p<0.01$).

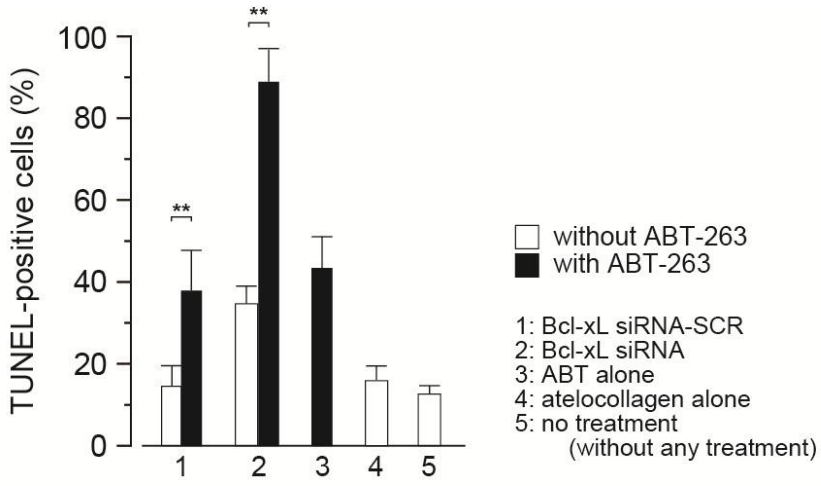

Figure 3. Quantification of TUNEL-positive cancer cells in PC-3 orthotopic tumors treated with Bcl-xL siRNA/atelocollagen complex in combination with ABT-263.

Frozen sections from the tumors at the endpoint (on day 28) were examined by TUNEL staining. The stained sections were photographed via fluorescence microscopy, and the TUNEL-positive

cells on the sections were quantified according to the method of our previous reports. White bars, without ABT-263; black bars, with ABT-263. The results are means±SD (n=6 tumors). ***P*<0.01.

3.3. Bcl-xL siRNA/atelocollagen complex in combination with ABT-263 almost completely inhibited liver metastasis from PC-3-luc orthotopic tumors.

In clinical cases of prostate cancer, metastasis usually occurs in bone, lymph nodes, liver, lung, and brain.[18] In our PC-3-luc orthotopic model, metastasis to the liver was frequently observed after 4 weeks following inoculation.[14] Liver metastasis was evaluated by determining the luciferase activity (relative light units) in liver tissue lysates prepared at the end of therapy (day 28). Either Bcl-xL siRNA complex or ABT-263 significantly inhibited the metastasis compared to the control groups (Bcl-xL-SCR, atelocollagen alone, and no treatment), in which no anti-metastasis effect was observed at all (Figure 4). Liver metastasis was almost completely inhibited by the Bcl-xL siRNA complex in combination with ABT-263 (Figure 4). We also observed the inhibition of lung metastasis by our therapy method (Takei Y et al., unpublished results).

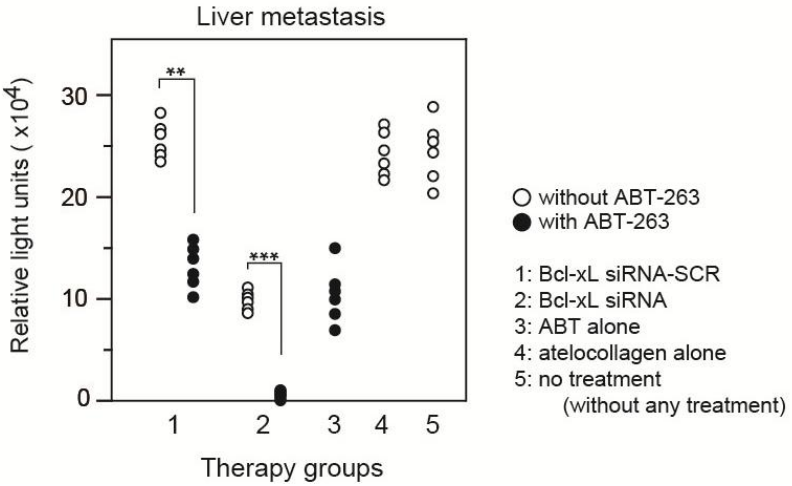

Figure 4. Significant anti-metastasis effect of the combination of Bcl-xL siRNA/ atelocollagen complex and ABT-263.

PC-3-luc cells were orthotopically inoculated into the nude mice as well. Fourteen days later, the tumor-bearing mice were administered with Bcl-xL siRNA/atelocollagen complex (i.v.) alone or in combination with ABT-263 (p.o.). On the 28th day after the first administration, the mice were sacrificed, and each liver tissue was removed. The tissue lysate was prepared for a dual luciferase assay. The assay was performed to detect liver metastases from the orthotopic prostate tumors. The luciferase activity was normalized to the total protein concentration. White circles, without ABT-263; black circles, with ABT-263. The results are means±SD (n=6 mice). ***P*< 0.01, ****P*< 0.001.

4. Discussion

Atelocollagen is a valuable tumor-specific siRNA delivery vehicle, especially in a systemic route.[7,10,14] In our previous study, we showed significant anti-cancer and anti-metastasis effects of a complex of atelocollagen and the siRNA targeting human Bcl-xL in a PC-3 orthotopic model.[14] In the present study, we investigated whether the therapeutic effect of the siRNA complex could be increased by further inhibiting Bcl-xL. We used ABT-263, a unique, orally bioavailable antagonist of Bcl-xL. ABT-263 reduced the anti-apoptotic effect of Bcl-xL by decreasing the interaction between Bcl-xL and the activator BH3-only proteins, such as BIM, to release those proteins, which activate BAX or BAK and finally lead to apoptosis. ABT-263 treatment alone induced apoptosis via a mitochondria-dependent pathway and significantly inhibited tumor growth in xenograft models of ALL and SCLC.[15-17,19] Furthermore, ABT-263 increased the sensitivity of tumor cells to anti-cancer reagents, including rituximab, R-CHOP, and bortezomib, in B-cell lymphoma and multiple myeloma models.[15,20,21]

We performed a therapeutic experiment by administering Bcl-xL siRNA complex (i.v.) and ABT-263 (p.o.) to tumor-bearing mice and evaluated the anti-cancer and anti-metastasis effects of this combined treatment at the end of therapy. We showed that the combined treatment of the siRNA complex and ABT-263 significantly increased the anti-cancer effect (Figure 2) and completely inhibited liver metastasis (Figure 3) compared with treatment using the siRNA complex alone or ABT-263 alone. The therapeutic effect of the siRNA and ABT-263 was synergistic, suggesting the target, Bcl-xL, was truly excellent for combating prostate cancers.

Elevating the tumor-specific siRNA delivery mediated by atelocollagen is one way to increase the knockdown efficiency of the target gene. Therefore, we have investigated the mechanism underlying tumor-specific delivery of the siRNA/atelocollagen complex. An enhanced permeability and retention (EPR) effect is usually considered important for the delivery of macromolecules to tumors. Blood vessels in tumors are more permeable, compared to the vessels in normal tissues, due to the weak associations between endothelial cells. Accordingly, macromolecules such as the siRNA/atelocollagen complex can easily pass through the walls of tumor vessels but not those of normal vessels. On the other hand, the lack of lymphatic vessels in tumors increases retention, because these molecules cannot leave the tumors via the lymphatic stream.[22-24] In this way, the macromolecules accumulate specifically in tumors.[25,26] Indeed, when we inhibited the EPR effect by blocking vascular endothelial growth factor-A (VEGF-A) by administering a neutralizing antibody *in vivo*, both the siRNA delivery efficiency to tumors and the knockdown efficiency of the target gene were significantly reduced.[14] This is a possible mechanism underlying atelocollagen-mediated siRNA delivery specific to tumors, although it is a “passive delivery mode” of atelocollagen.

For a “active delivery mode” of atelocollagen, we considered that Endo180, an endocytic receptor for collagen,[27,28] can be involved in the tumor-specific delivery of the

siRNA/atelocollagen complex, especially in the final stage of cellular uptake. We found that Endo180 was quite overexpressed in PC-3 cells compared to normal human prostate cells, PNT-1A and PNT-2, and that the neutralized blocking of Endo180 *in vivo* reduced the delivery efficiency of siRNA (Takei Y et al., unpublished results). This suggested that Endo180 might be associated with the siRNA complex uptake to PC-3 cell-derived tumors. In the future, the detailed mechanism underlying the cellular uptake of the siRNA/atelocollagen complex will be better elucidated, as will the delivery efficiency (for example, using guide antibody against Endo180). At that time, we will be able to treat PC-3 orthotopic tumors in nude mice via Bcl-xL siRNA/atelocollagen alone, without the inhibitor ABT-263. We hope our proposed method will provide researchers and clinicians with a useful approach to the treatment of prostate cancers.

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