

Bowman-Birk protease inhibitor from soybeans enhances cisplatin-induced cytotoxicity in human mesothelioma cells

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Received March 8, 2011; Accepted April 11, 2011

DOI: 10.3892/etm.2011.270

Abstract. Malignant mesothelioma (MM) is an aggressive cancer with no effective treatment options. Enforced expression of the gap junction (GJ) component connexin 43 (Cx43) increases the sensitivity of MM cells to cisplatin. Bowman-Birk protease inhibitor (BBI) induces the restoration of Cx43 in several types of tumor cells. In this study, we examined the capability of BBI to enhance the cytotoxic effect of cisplatin in MM cells via the induction of Cx43. Human MM H28 cells were used. Cell viability was evaluated by a WST-1 assay and proteasomal activity was determined by fluorometric analysis. Protein and mRNA levels were determined by immunoblot analysis and real-time RT-PCR, respectively. GJ function mediated by Cx43 was evaluated using the scrape-loading method. BBI effectively inhibited H28 cell growth in a dose-dependent manner (200-400 μ g/ml). In parallel with the growth inhibition, Cx43 levels (mRNA and protein) and GJ function were elevated by BBI treatment. Knockdown of BBI-induced Cx43 by an antisense nucleotide treatment almost cancelled the growth inhibition. BBI enhanced cisplatin-induced cytotoxicity in H28 cells, and down-regulation of Cx43 by the antisense nucleotide treatment abrogated the enhancing effect of BBI. The induction of Cx43 by BBI contributed to Src inactivation and subsequent induction of Bax. Furthermore, an Src inhibitor (SU6656) also enhanced cisplatin-induced cytotoxicity in H28 cells. These results suggest that BBI improves the cytotoxic efficacy of cisplatin in H28 cells via the inhibition of Src signaling.

Introduction

Malignant mesothelioma (MM) of the serosal membranes of body cavities is a particularly aggressive cancer characterized by rapid progression, late metastases and poor prognosis (1).

Although surgery, radiotherapy, chemotherapy and/or their combinations have been used as therapeutic modalities, median patient survival is 8-18 months (2). Cisplatin has been used in clinical MM therapy, and its chemotherapeutic effect as a single agent as well as in combination with other chemotherapeutic agents has been previously examined (3,4). However, most patients relapsed within 1 year after starting treatment. Therefore, new therapeutic approaches are required for MM patients.

Among the different types of cell-cell interactions in mammalian cells, gap junctional intercellular communication (GJIC) is considered to be the only route allowing free direct transfer of ions and hydrophilic molecules of up to 1,000-1,500 Da in size between cells, thereby maintaining electrical and metabolic cell homeostasis (5). The gap junction is made up of juxtaposed transmembrane hemichannels (connexons) provided by adjacent cells, and each connexon consists of six individual transmembrane proteins called connexin (Cx) (6). In general, it is well known that the Cx gene acts as a tumor-suppressor gene by maintaining homeostatic control in multicellular organisms via GJIC. Moreover, many transfection studies using Cx cDNAs revealed that Cx is a tumor suppressor in cells originating from tissues in which they are normally expressed (7). In line with this, we recently reported that Cx43 abrogates various malignant phenotypes in MM cells, such as chemoresistance (8).

Protease inhibitors are a class of well-established cancer chemopreventive agents due to their strong anticarcinogenic activity *in vivo* and *in vitro* in cancer model systems (9). The most predominant protease inhibitor in soybeans is the Bowman-Birk inhibitor (BBI) (9). BBI, a 71-amino acid protein (8 kDa) and a serine protease inhibitor with both trypsin and chymotrypsin inhibitory activities, was found to be a valid suppressor of carcinogenesis in a human phase IIa clinical trial (10). Although BBI has a broad spectrum of cancer-protective activities (10-12), knowledge of the exact mechanism(s) by which BBI exerts its anticarcinogenic effects remains limited. In our previous studies, we demonstrated that the induction of Cx43 by BBI contributes to the negative growth control of tumor cells *in vivo* as well as *in vitro* (13,14). Overall, it appears that BBI improves chemoresistance in MM cells via the induction of Cx43.

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Key words: chemoresistance, cisplatin, Bowman-Birk protease inhibitor, mesothelioma, Src

In this context, we evaluated whether BBI enhances cisplatin-induced cytotoxicity in MM cells. The H28 human MM cell line was chosen to evaluate the inhibitory effect of BBI on MM cell growth, due to its resistance to cisplatin, a representative agent used to clinically treat MM.

Materials and methods

Chemicals. All culture chemicals and BBI were purchased from Gibco BRL (Tokyo, Japan) and Sigma (St. Louis, MO, USA), unless otherwise indicated. SU6656 (an Src inhibitor) was purchased from Calbiochem-Novabiochem (La Jolla, CA, USA). Anti-Cx43 antibody was from Zymed Laboratories (San Francisco, CA, USA). Other antibodies were purchased from Wako Pure Chemicals (Osaka, Japan) and BD Transduction Laboratories (Lexington, KY, USA).

Cell culture and treatment. Human mesothelioma H28 cell line was supplied by ATCC, and the cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 0.01 M HEPES buffer solution, 1 mM sodium pyruvate and 4.5 g/l glucose in a 5% CO₂ atmosphere at 37°C in a humidified incubator. BBI was dissolved in saline, and the cells were treated with BBI (200 and 400 µg/ml) or vehicle (saline) for indicated periods. Treatment conditions involving the other agents were performed as described in each figure legend.

Cell growth assay. Cells (2x10⁴) were seeded on a 96-well culture plate with the culture medium, and after overnight culture, the cells were treated with each agent. Following this, cell growth was determined with a cell proliferation assay kit using WST-1 reagent (Roche Japan, Tokyo, Japan).

Apoptosis analysis. Cells were trypsinization, washed with PBS, resuspended in 70% ethanol and maintained at 4°C for at least 30 min. Before analysis, the cells were washed again with PBS and resuspended and incubated for 30 min in PBS containing 0.05 mg/ml propidium iodide, 1 mM EDTA, 0.1% Triton X-100 and 1 mg/ml RNase A. The suspension was then passed through a nylon mesh filter and analyzed on a Becton Dickinson FACScan.

Measurement of GJIC. For measurement of GJIC, we used a scrape-loading/dye transfer method with some modification (15). H28 cells on 35-mm dishes were rinsed several times with PBS. The center of the dish was scraped by a surgical blade, and 2 ml of 0.05% Lucifer yellow CH (LY) in PBS was added to the dishes after scraping. LY is a small molecule (457 Da) that freely moves through gap junctions from loaded cells to neighboring ones. Five minutes after the dye treatment, the cells were rinsed several times with PBS to remove excess dye. The intensity of LY transfer was observed with an Olympus inverse microscope equipped with appropriate filters (Olympus, Tokyo, Japan), five points were photographed per dish and the cell layers into which LY had spread were counted.

Cx43 antisense phosphorothioate oligodeoxynucleotide treatment. Cx43 antisense phosphorothioate oligodeoxynucleotide

(AS-ODN) (5'-CTCCAGTCACCCATGTTG-3') was purchased from Sigma-Genosys (Hokkaido, Japan). The sequence encompassed the start codon of rat Cx43 mRNA. As a control for the non-specific effects of oligonucleotide treatment, the corresponding sense phosphorothioate ODN (S-ODN) (5'-CAACATGGGTGACTGGAG-3') was also obtained from Sigma Genosys. The cells were exposed to 2 µM AS-ODN or S-ODN and added to the culture medium at 2-day intervals. Under these conditions, the expression of Cx43 protein was almost diminished in the cells treated with AS-ODN, while S-ODN treatment at the equivalent dose did not affect the protein level.

Isolation of total RNA and real-time PCR. Total RNA was isolated by using the SV Total RNA isolation system (Promega, Madison, WI, USA), and cDNA was synthesized as previously described (8). Real-time PCR was performed by using ABI Prism 7000 sequence detection system (Applied Biosystems Japan Ltd., Tokyo, Japan) and SYBR Premix Ex Taq™ (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. The primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), accession no. (BC023632), sense (nucleotides 737-756), antisense (nucleotides 916-897); Cx43, accession no. (NM_000165), sense (nucleotides 257-276) and antisense (nucleotides 433-414).

Immunoblot analysis. Immunoblotting was performed as previously described (14). Briefly, the cell lysate was prepared in cell lysis/extraction reagent (Sigma), and 10 µg total protein extract from each sample was loaded onto 8% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes. The blots were incubated with each antibody. Each immunoreactive band was detected using the ECL system (Amersham) and a cooled CCD camera-linked Cool Saver system (Atto, Osaka Japan). Molecular sizing was carried out using Rainbow MW marker (Amersham). Protein concentrations were determined using the DC protein assay system (Bio-Rad, Hercules, CA, USA).

Assay for proteasome activity. H28 cells were seeded on a 12-well plate (5x10⁴ cells/well) overnight. These cells were then treated with the specific concentration of BBI for 24 or 72 h, followed by the addition of 20 µM of fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (for chymotrypsin-like activity) at 37°C for 2 h. Afterwards, 100 µl of the cell medium was collected and diluted with 1X PBS to 400 µl. Measurement of free AMC groups was performed as previously described (16).

Statistical analysis. Data were analyzed by one-way ANOVA followed by the Student's t-test, Dunnett's multiple-range test or Tukey-Kramer test. P≤0.05 was considered statistically significant.

Results

Effect of BBI on cell growth, proteasomal activity and C43 expression in H28 cells. To evaluate the effect of BBI on cell growth, proteasomal activity and Cx43 expression level,

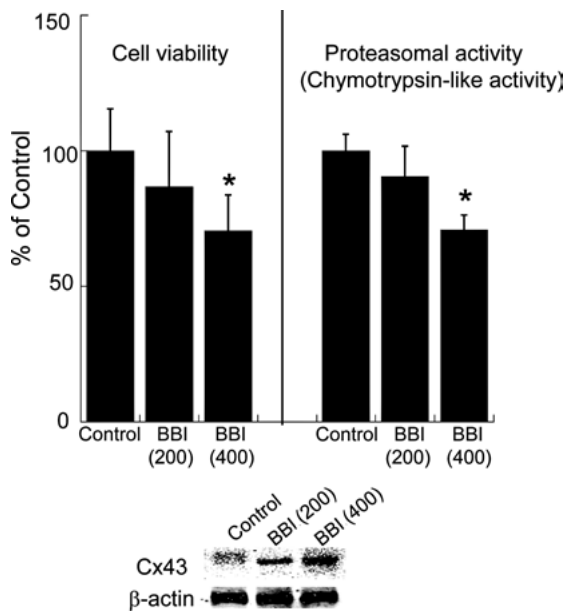


Figure 1. Effects of BBI on cell viability, proteasomal activity of chymotrypsin-like activity and the Cx43 protein level of H28 cells. The cells were treated with BBI (200 $\mu\text{g/ml}$) and BBI (400 $\mu\text{g/ml}$) for 5 days (cell viability) and 3 days (proteasomal activity and Cx43 protein level), respectively. Each value is the mean from five determinants, and vertical lines indicate SD (cell viability and proteasomal activity). *Significant difference from control. The level of Cx43 protein was determined by immunoblot analysis, and β -actin was used to confirm equal loading between each group. This result is representative of two independent experiments. BBI (200), BBI 200 $\mu\text{g/ml}$; BBI (400), BBI 400 $\mu\text{g/ml}$.

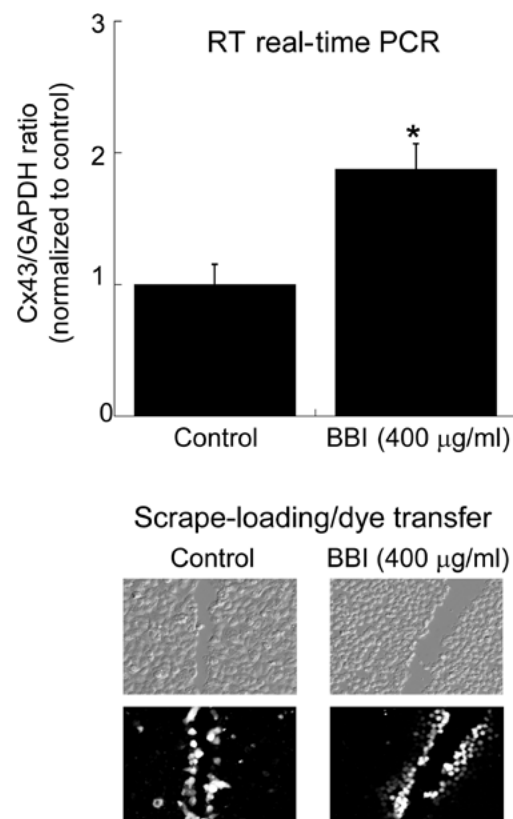


Figure 2. Restoration of Cx43 mRNA expression and GJIC in H28 cells treated with BBI. The cells were treated with BBI (400 $\mu\text{g/ml}$) for 3 days. The level of Cx43 mRNA was determined by real-time RT-PCR. Each value is the mean from five determinants, and vertical lines indicate SD. *Significant difference from control. GJIC was estimated by scrape-loading/dye transfer method. Original magnification, x200. This result is representative of two independent experiments.

dose-dependent changes in cell viability, chymotrypsin-like activity involved in the proteasome and the protein level of Cx43 after BBI exposure for each time period were examined. Since the elevation of Cx43 by BBI partly depends on the inhibition of chymotrypsin-like activity in the proteasome (14), we assessed the activity in the proteasome to confirm the effect of BBI. As shown in Fig. 1, BBI decreased cell viability and suppressed proteasomal chymotrypsin-like activity in a dose-dependent manner, whereas the expression level of Cx43 protein was dose-dependently elevated by BBI treatment. Next, the effect of BBI on the Cx43 mRNA level and GJIC in H28 cells was investigated (Fig. 2). BBI treatment up-regulated the Cx43 mRNA level and in parallel caused the restoration of GJIC estimated by the scrape-loading assay. Finally, to confirm the contribution of the BBI-mediated elevation of Cx43 to the negative growth effect in H28 cells, we ascertained whether the knockdown of Cx43 by AS-ODN abrogates the BBI-dependent negative growth effect in H28 cells. As shown in Fig. 3, Cx43 AS-ODN almost completely abolished BBI-mediated growth inhibition in H28 cells upon down-regulation of Cx43.

Combination effect of BBI and cisplatin on the growth of H28 cells. We previously reported that enforced expression of the Cx43 gene improved the resistance of cisplatin to H28 cells (8); therefore, we investigated whether BBI enhances cisplatin-induced cytotoxicity and apoptosis in H28 cells. As shown in Fig. 4, cisplatin treatment (10 μM) had no effect on cell viability in H28 cells, but the combination of BBI with cisplatin significantly decreased the cell viability compared

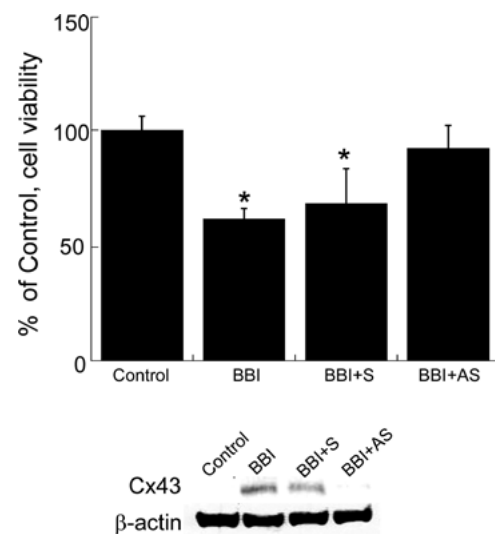


Figure 3. Change in cell viability in BBI-treated H28 cells upon the down-regulation of Cx43 by AS-ODN. The cells were treated with BBI (400 $\mu\text{g/ml}$) and/or AS-ODN (2 M) for 5 days. After the treatment, cell viability was determined by WST-1. Each value is the mean from four determinants, and vertical lines indicate SD. *Significant difference from control. Under the same treatment condition, the Cx43 protein level in each group was estimated by immunoblot analysis. This result is representative of two independent experiments. S-ODN was used as a negative control. BBI, BBI treatment; BBI+S, BBI + sense phosphorothioate oligodeoxynucleotide treatment; BBI+AS, BBI + Cx43 antisense phosphorothioate oligodeoxynucleotide treatment.

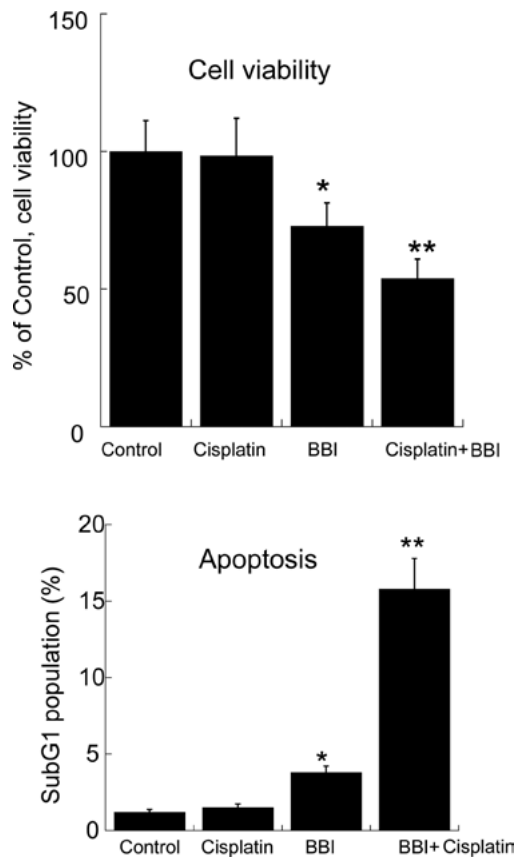


Figure 4. Effect of BBI on cell viability and apoptosis in H28 cells treated with cisplatin. The cells were pre-treated with BBI (400 μ g/ml) for 3 days and subsequently co-treated with BBI and/or cisplatin (10 μ M) for 2 days. After the treatment, cell viability was determined by WST-1, and apoptosis was evaluated as the subG1 population. Each value is the mean from four determinants, and vertical lines indicate SD. *Significant difference from control and BBI-treated group and **significant difference from other three groups.

to BBI treatment alone. Also, the induction of apoptosis, estimated as the subG1 population, by the combination of BBI and cisplatin was significantly increased compared to that with BBI or cisplatin alone (Fig. 4). These results indicate that BBI abrogates the resistance to cisplatin in the cells. Furthermore, in order to ascertain whether BBI-driven restoration of Cx43 is closely related to the improvement of chemoresistance in H28 cells by BBI, we investigated whether AS-ODN for Cx43 cancels BBI-enhanced cytotoxicity in H28 cells treated with cisplatin. As a result, the enhanced effect of BBI was almost abolished by the AS-ODN treatment (Fig. 5).

Effect of BBI on Src signaling in H28 cells. We previously reported that inactivation of the Src family of protein tyrosine kinases is involved in enhancing the effect of Cx43 on cisplatin-induced cytotoxicity in H28 cells (8). Therefore, we speculated that the enhancing effect of BBI on cytotoxicity is due to suppression of Src signaling via the induction of Cx43. To confirm this hypothesis, we evaluated whether BBI-enhanced Cx43 expression improves the efficacy of cisplatin to H28 cells via the inactivation of Src signaling. As shown in Fig. 6, BBI suppressed the activation of Src (phosphorylation on Tyr416), and knockdown of Cx43 by AS-ODN treatment almost completely cancelled this BBI-mediated

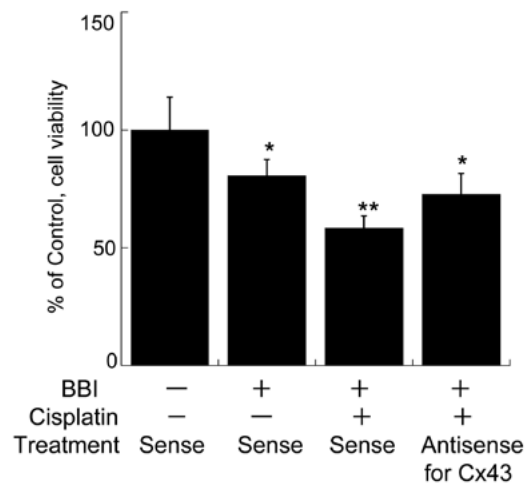


Figure 5. Effect of Cx43 AS-ODN on cisplatin-induced cytotoxicity in H28 cells treated with BBI. The cells were treated with Cx43 AS-ODN (2 μ M) and BBI (400 μ g/ml) for 3 days, and subsequently co-treated with cisplatin (10 μ M) for 2 days. Each value is the mean from four determinants, and vertical lines indicate SD. *Significant difference from control and **significant difference from other three groups.

inactivation of Src. In our previous study, Bax, a representative pro-apoptotic factor, was shown to be induced by enforced expression of Cx43 and to play an important role in the enhancement of cisplatin-mediated damage in H28 cells (8); therefore, we evaluated the contribution of Bax to the enhancing effect of BBI in H28 cells. In the present study, BBI treatment up-regulated Bax and, upon knockdown of Cx43 by AS-ODN treatment, the Bax level was almost restored to the level of that in the control (Fig. 6). Additionally, the Src inhibitor, SU6656, elevated the Bax level, indicating that inhibition of Src leads to the induction of Bax. Finally, in order to confirm that Src signaling plays a crucial role in the BBI-induced enhancement of cisplatin-mediated cytotoxicity, we evaluated the combination effect of SU6656 and cisplatin on the growth of H28 cells. As shown in Fig. 7, when the cells were treated with both SU6656 and cisplatin, cell viability was significantly inhibited as compared to that when the cells were treated with either SU6656 or cisplatin alone.

Discussion

Management of MM continues to be a difficult clinical issue. The relative clinical resistance to chemotherapy and other conventional treatments strongly necessitate the development of new potential therapeutic strategies for MM to establish viable MM treatment protocols. In our previous study, enforced expression of Cx43 in MM cells exhibiting resistance to cisplatin significantly reduced this resistance (8), indicating that successful restoration of Cx43 by a treatment agent rather than Cx43 gene transfection may lead to the establishment of a novel effective MM therapy. Fortunately, in regards to the restoration of Cx43 expression in tumor cells, we and other research groups previously found that BBI effectively caused the induction of Cx43 in several types of tumor cells and that this induction is closely associated with negative growth control of tumor cells (14,17). Based on these reports, we speculated that BBI improves the efficacy of cisplatin in MM

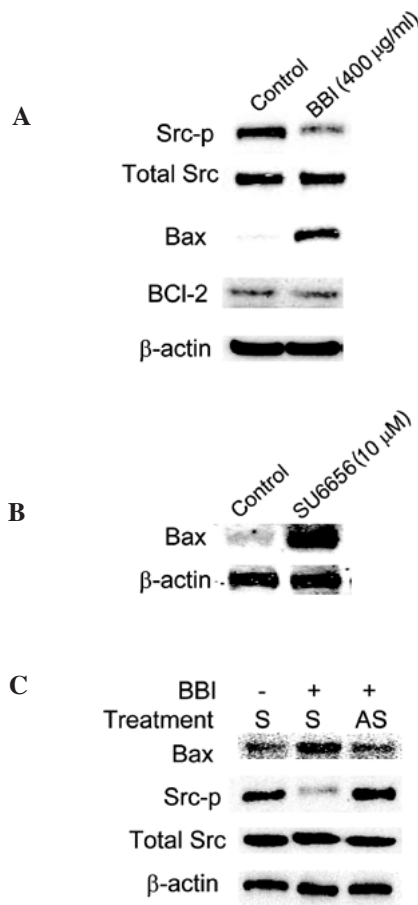


Figure 6. Contribution of Cx43 to the suppression of Src signaling in H28 cells treated with BBI. The cells were treated with (A) BBI (400 μ g/ml) for 3 days, (B) SU6656 (10 μ M) for 2 days, and (C) Cx43 AS-ODN (2 μ M) and BBI (400 μ g/ml) for 3 days. After each treatment, the level of each protein was determined by immunoblot analysis. This result is representative of two independent experiments. S, sense phosphorothioate oligodeoxynucleotide treatment; AS, Cx43 antisense phosphorothioate oligodeoxynucleotide treatment.

cells via restoration of Cx43 expression. Thus, the present study was carried out to confirm this hypothesis.

We previously reported that the Cx gene (the molecule being Cx32) enhances the sensitivity of chemotherapeutic agents to renal and lung cancer cells (18). Similarly, we demonstrated that enforced Cx43 expression in H28 cells improves the effect of cisplatin against H28 cells (14). These reports strongly suggest that, in part, Cx-dependent tumor suppressive effects mitigate chemoresistance in several types of tumor cells. In the present study, we showed that BBI treatment caused the restoration of Cx43 expression, in part, due to the suppression of Cx43 degradation by chymotrypsin-like activity in the proteasome, and that the restored Cx43 induced negative growth control in H28 cells. Finally, these events caused by BBI treatment led to an improvement in the efficacy of cisplatin. Thus, the combination of BBI and cisplatin may be a promising new therapy against cisplatin-resistant MM cells.

Through GJIC-dependent cell coupling, dying cancer cells communicate cell death signals to adjacent cells which then also die by apoptosis. These death messages which pass through the GJ to kill cells are very likely calcium ions (19).

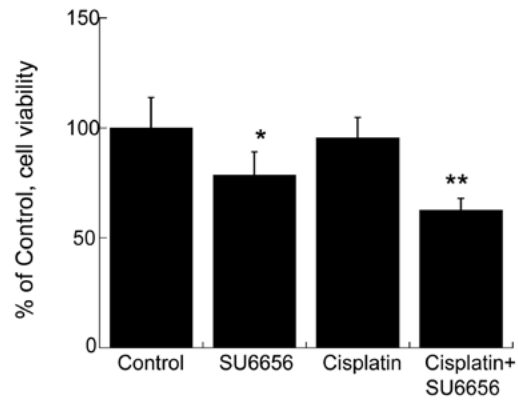


Figure 7. Enhancing effect of SU6656 on cisplatin-induced cytotoxicity in H28 cells. The cells were treated with SU6656 (10 μ M), cisplatin (10 μ M) and both for 2 days. After the treatment, cell viability was determined by WST-1. Each value is the mean from five determinants, and vertical lines indicate SD. *Significant difference from control and cisplatin-treated group and **significant difference from the other three groups.

This finding suggests that chemoresistance to anticancer agents by tumor cells is reduced due to the propagation of cell death signals from dying cells to surrounding living cells via GJ. We previously reported that inhibition of Cx-driven GJIC by a known inhibitor towards GJ functions (18-glycyrrhetic acid) partly abrogated chemotherapeutic agent-induced cytotoxicity in cancer cells (18). However, in this study we did not find that inhibition of GJIC by the inhibitor negates the enhanced effect of BBI on cisplatin-induced cytotoxicity in H28 cells (data not shown), indicating that BBI-mediated restoration of GJIC does not affect cisplatin-induced cytotoxicity. Apart from the GJIC-dependent effect of Cx, other data showed that Cx affects cellular homeostatic balance independently of GJIC, and that this GJ-independent effect plays an important role in regulating abnormal growth of cancer cells (5,20). Moreover, we demonstrated that the Cx32 gene acts as a tumor suppressor gene against renal cancer cells in a GJIC-independent mechanism (21). Based on these reports, we speculated that the GJIC-independent effect contributes to the potentiation of cisplatin-induced cytotoxicity in H28 cells.

We previously reported that one of the GJIC-independent tumor-suppressive effects in renal cancer cells depends on the inactivation of Src caused by Cx32 (21). It has been well-established that Src plays a critical role in the survival, proliferation, invasion and metastasis of solid tumors (22), and also, the activation of Src contributes to the appearance of malignant phenotypes in MM cells (23). Furthermore, Src directly and indirectly phosphorylates Cx43 at tyrosine and serine residues, leading to loss of Cx43-mediated functions (24). On the contrary, we observed that overexpression of Cx43 induces, not only dephosphorylation, but also down-regulation of Src in MM cells (8). These reports suggest that these two molecules affect their respective function based on the expression level of each molecule. Thus, BBI-induced elevation of Cx43 may improve the toxicity of cisplatin to H28 cells via the inactivation of Src in a GJIC-independent manner. We confirmed that the knockdown of Cx43 in BBI-treated H28 cells by AS-ODN abrogated the enhancing effect of BBI on cisplatin-induced cytotoxicity, and that Src inhibition by a

specific inhibitor caused the potentiation of the cytotoxicity. These observations completely support the above speculation.

In general, the existence of anti-apoptotic molecules and pro-apoptotic molecules is well known (25). Bcl-2 is a major anti-apoptotic protein which protects cells from a wide variety of apoptotic stimuli (26). By contrast, Bax is a Bcl-2-like protein that binds to and antagonizes the protective effect of Bcl-2, rendering cells more sensitive to apoptosis (27). In the present study, we found that only the level of Bax in H28 cells was significantly elevated by BBI treatment. As a result, the balance between pro- and anti-apoptotic factors was altered towards the induction of apoptosis. Any approach that alters the balance in favor of apoptosis may thus confer a therapeutic benefit. Overall, our present results suggest that Cx43 induced by BBI treatment influences the balance between pro- and anti-apoptotic factors in the direction of apoptosis, possibly contributing to the improved sensitivity of H28 cells to cisplatin. At present, the reason why BBI enhanced the level of Bax remains unclear. From the present data, we can only speculate that the inactivation of Src signaling caused by BBI-induced elevation of Cx43 was associated with the up-regulation of Bax. Since it is known that Bax is located downstream in the Src signal pathway, it appears that the inhibition of Src signaling indirectly induces Bax in H28 cells treated with BBI. In order to clarify the mechanism by which the enhancing effect of BBI on cisplatin-induced cytotoxicity in H28 cells is activated, further study is warranted.

Acknowledgements

This study was supported by a research grant for Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation (KHC1023), and a grant-in-aid for Science Research from the Ministry of Education, Culture and Sciences of Japan (no. 22500754) to Yano T.

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