Nrf2- and ATF4-Dependent Upregulation of xCT Modulates the Sensitivity of T24 Bladder Carcinoma Cells to Proteasome Inhibition

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The ubiquitin–proteasome pathway degrades ubiquitinated proteins to remove damaged or misfolded protein and thus plays an important role in the maintenance of many important cellular processes. Because the pathway is also crucial for tumor cell growth and survival, proteasome inhibition by specific inhibitors exhibits potent antitumor effects in many cancer cells. xCT, a subunit of the cystine antiporter system \( \chi^- \), plays an important role in cellular cysteine and glutathione homeostasis. Several recent reports have revealed that xCT is involved in cancer cell survival; however, it was unknown whether xCT affects the cytotoxic effects of proteasome inhibitors. In this study, we found that two stress-inducible transcription factors, Nrf2 and ATF4, were upregulated by proteasome inhibition and cooperatively enhance human xCT gene expression upon proteasome inhibition. In addition, we demonstrated that the knockdown of xCT by small interfering RNA (siRNA) or pharmacological inhibition of xCT by sulfasalazine (SASP) or (S)-4-carboxyphenylglycine (CPG) significantly increased the sensitivity of T24 cells to proteasome inhibition. These results suggest that the simultaneous inhibition of both the proteasome and xCT could have therapeutic benefits in the treatment of bladder tumors.

The ubiquitin-proteasome system (UPS) is the major pathway for intracellular protein degradation (1–3). The proteasome selectively degrades ubiquitinated substrate proteins to eliminate damaged or misfolded proteins and recycles intracellular amino acids. Thus, proteasome activity is required for many important cellular processes, such as cell cycle progression, proliferation, and apoptosis (1–3). A number of different types of agents have been reported to be proteasome inhibitors, such as MG132, bortezomib (BTZ), epoxomicin (EPO), and carfilzomib (CFZ) (4–6). Because proteasome inhibitors induce cancer cell death via the induction of proteotoxicity, oxidative stress, and ER stress, some of these agents are considered anticancer drugs (4–6). For example, BTZ (also known as Velcade or PS-341) and CFZ have been approved by the FDA for the treatment of patients with mantle cell lymphoma (MCL) and multiple myeloma (7–9). However, differential sensitivities to BTZ were observed among cancer cell lines, though the lines were derived from the same tissues (9–14). To increase the efficacy of BTZ, combination therapies using BTZ and other chemotherapeutic agents or therapies have been studied (11, 12, 15). However, it is important to clarify the molecular mechanisms that affect the efficacy of proteasome inhibitors in cancer cells.

Bladder cancer is a common worldwide disease, especially in developed countries (16). In the United States, more than 70,000 cases were newly diagnosed in 2012, resulting in approximately 15,000 deaths, most of which occurred as a result of metastatic progression (17). Although approximately 70% of bladder tumors can be cured by surgical removal, approximately 60% to 70% of patients experience local or distant recurrences, and almost 20% to 30% of these relapsed tumors progress to higher grades or stages (18). In bladder cancer treatment, Mycobacterium bovis bacillus Calmette-Guérin (BCG) administration, irradiation, and chemotherapy are often beneficial, in addition to surgical resection (17–19). In chemotherapy, the recurrent tumors occasionally became more malignant and resistant to the anticancer drugs that were initially used. Proteasome inhibitors could be alternative agents for bladder cancer chemotherapy because strong cytotoxic effects were observed in some bladder carcinoma cell lines upon BTZ treatment (10). However, different levels of resistance to BTZ were also observed among these cell lines (10, 11).

The glutamate/cystine antiporter system \( \chi^- \) is an obligate sodium-independent amino acid antiporter that transports extra- cellular cystine into cells to maintain intracellular cysteine pools, and it also creates a reducing extracellular environment by the cystine/cysteine redox cycle (20–23). Cysteine plays an important role in glutathione (GSH) synthesis, which is indispensable for maintaining intracellular redox balance and drug metabolism (23–25). xCT is highly expressed in several human cancers, and its expression is associated with malignancy, drug resistance, and poor survival in patients (21, 25–28). In addition, a CD44 variant promotes tumor growth by stabilizing the xCT protein (29). Therefore, xCT has been considered a potential therapeutic target and a novel marker for predicting malignancy.

The expression of xCT is induced by various stimuli, including oxidative stress, amino acid deprivation, bacterial lipopolysaccharides, and nitric oxide (30–33). Upon oxidative stress, the oxidative stress-responsive transcription factor NF-E2-related factor 2 (Nrf2) mediates xCT induction (30). Nrf2 modulates the cytoprotective response and drug metabolism through the induction of its...
target genes, such as heme oxygenase 1 and glutathione S-transferase genes (34, 35). Under unstressed conditions, Nrf2 is constitutively degraded through the UPS and repressed. In response to oxidative stress, Nrf2 activates target gene expression in an antioxidant response element (ARE) (also referred to as the electrophile response element [EpRE])-dependent manner. Activating transcription factor 4 (ATF4) mediates xCT expression during amino acid deprivation and oxidative stress (31, 36). This protein is a bZip transcription factor that is activated by multiple stress signals, including amino acid deprivation, heme deficiency, and endoplasmic reticulum (ER) stress (37, 38, 39). Under stress conditions, ATF4 is selectively translated through the integrated stress response (ISR) pathway, and it upregulates its target genes by binding to the amino acid response elements (AAREs) in their regulatory regions. In the mouse xCT gene promoter, one ARE and two AAREs mediate oxidative stress- and amino acid deprivation-induced xCT gene expression, respectively (30, 31). However, the regulatory mechanism of the human xCT gene remains poorly understood. Interestingly, both Nrf2 and ATF4 are activated by proteasome inhibition (32).

In this study, we demonstrated a role for xCT in proteasome inhibitor-induced T24 bladder cancer cell cytotoxicity. Proteasome inhibition strongly upregulates xCT expression, and the knockdown of xCT by small interfering RNA (siRNA) or the pharmacological inhibition of xCT increased the sensitivity of T24 cells to proteasome inhibition. In addition, we found that proteasome inhibition induced human xCT gene expression in an Nrf2- and ATF4-dependent manner. These results suggest that xCT induction by proteasome inhibition might affect the sensitivity of T24 cells to proteasome inhibitors.

**MATERIALS AND METHODS**

**Materials.** BTZ was obtained from Cell Signaling Technology (Danvers, MA). EPO and MG132 were obtained from the Peptide Institute (Osaka, Japan). CFZ was obtained from Selleck Chemicals (Houston, TX). (S)-4-carboxyphenylglycine (CPG) was obtained from Tocris Bioscience (Bristol, United Kingdom). Dulbecco’s modified Eagle medium (DMEM), N-acetylg-l-cysteine (NAC), sultasazin, and tunicamycin (Tm) were obtained from Sigma-Aldrich (St. Louis, MO). tert-Butylhydroquinone (tBHQ) was obtained from Kanto Chemical (Tokyo, Japan). Dimethyl sulfoxide (DMSO) and 2-mercaptoethanol (2-ME) were obtained from Wako Pure Chemical (Osaka, Japan). Bovine serum albumin (BSA) was purchased from Nakalai Tesque (Kyoto, Japan). Anti-Nrf2 antibody (sc-722 or sc-13032), anti-ATF4 antibody (sc-200), and anti-lamin B antibody (sc-722 or sc-13032), anti-ATF4 antibody (sc-200), and anti-lamin B antibody (sc-722 or sc-13032) were purchased from Abcam (Cambridge, MA). Anti-α subunit of eukaryotic initiator factor 2 anti-elf2α and anti-phospho-elf2α (Ser51) antibodies were purchased from Cell Signaling Technology (Beverly, MA).

**Cell culture.** The human bladder cancer cell line T24, human cervix carcinoma cell line HeLa, human astrocytoma cell line U373MG, human glioblastoma cell line T98G, and human embryonic kidney cell line 293T were maintained in DMEM containing 10% fetal bovine serum (Gibco, Grand Island, NY) with 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). The cells were cultured at 37°C with 5% CO2 and saturated humidity.

**RNA preparation and RT-qPCR.** Total RNAs from T24 and HeLa cells were isolated using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. cDNAs were synthesized using the PrimeScript II 1st-strand cDNA synthesis kit (TaKaRa Bio, Otsu, Japan). The reverse transcription–quantitative-PCR (RT-qPCR) analyses were performed using SYBR Premix Ex Taq II (TaKaRa Bio) and the CFX real-time PCR detection system (Bio-Rad, Hercules, CA). The primers used for RT-qPCR were as follows: human xCT (forward, 5′-CCA TGA ACG AGC GTG GTG TGT T-3′; reverse, 5′-GAC CCT CTC GAG ACA C-3′), human GCLC (forward, 5′-TTG AGC ATA GAT AAA GAG ATC TAC GAA-3′; reverse, 5′-TCT CTA ATA AAG TGA TGA GCA ACA TGC-3′), human GCLM (forward, 5′-TGG GCA GAG GTA AAA CCA A-3′; reverse, 5′-CAG TCA AAT CTG GTG GCA TC-3′), human BIRC5 (forward, 5′-AGA AGC CCT GCC CTT TGG AA-3′; reverse, 5′-CAA GTC TGG CTC GTC CTT AGT C-3′), human HSPA2 (forward, 5′-GCG ACA AAT CAG ATG TGC-3′; reverse, 5′-GTC GTG TGT TTC CTC TTG AGT AGT G-3′), human PSMB5 (forward, 5′-GAG TCT CAG TGA TGG TCT GAG C-3′; reverse, 5′-GAC TCC ATG GGA GAA CTT GA-3′), and human cyclophilin A (forward, 5′-ATG CTG GAC CCA ACA CAA AT-3′; reverse, 5′-TCT TTC ACT TTG CCA AAC ACC-3′). Cyclophilin A was used as an internal control.

**Immunoblot analysis.** Whole-cell lysates were prepared by dissolving cell pellets directly in sample buffer (62.5 mM Tris–HCl [pH 6.8], 2% SDS, and 10% glycerol) and then sonicated to shear the DNA. Protein concentrations were quantified using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s protocol. After protein quantification, 2-mercaptoethanol (final concentration, 1%) and bromophenol blue (final concentration, 0.01%) were added to each sample, and the samples were incubated at 100°C for 5 min or at room temperature for 30 min (for xCT detection). Fifteen micrograms of protein per lane was separated by 6 to 10% SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were blocked with 1% skim milk–phosphate-buffered saline (PBS) with 0.1% Tween 20 (PBST) or 1% BSA–PBST (for xCT and elf2α blot) and then blotted with anti-Nrf2, anti-ATF4, anti-xCT, anti-elf2α, anti-phospho-elf2α, or anti-lamin B antibody. After washing with PBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and then visualized using ImmunoStar chemiluminescent reagent (Wako Pure Chemical).

**siRNA transfection.** T24 cells and HeLa cells were transfected with anti-human Nrf2 siRNA (sense, 5′-GUU AGA AGG CAG CAG AUG UUA ATT-3′; antisense, 5′-UUA ACA UGU GUC UUC UUA CTT-3′), anti-human ATF4 (hATF4) siRNA (sense, 5′-GCC UAG GUC UAC UAG AUG ATT-3′; antisense, 5′-UCA UAC UAG ACA CUG CTT-3′) and anti-human xCT siRNA (sense, 5′-AGA GAC AGU CCC AGC CUG CTT-3′; antisense, 5′-GCA GCG UGG GCA UCU UTT-3′), or control siRNA (Qiagen, Hilden, Germany) using Lipofectamine RNAiMax (Life Technologies). Four hours after transfection, the medium was replaced with fresh medium. After 20 h of incubation, the transfected cells were treated with chemicals for the indicated times and subjected to immunoblot or RT-qPCR analysis.

**ChiP analysis.** Chromatin immunoprecipitation (ChiP) analysis was performed as previously described with a few modifications (40). In brief, T24 cells were incubated with 100 nM bortezomib for 6 h and then fixed with 1% formaldehyde for 10 min at 37°C. After fixation, the cells were collected and sonicated to prepare chromatin suspensions of DNA approximately 300 bp in length. Subsequently, the lysates were incubated with anti-Nrf2 (sc-13032) or anti-ATF4 (sc-200) antibody overnight at 4°C. Normal rabbit IgG (sc-2027) was used as a negative control. After antigen–antibody reaction, immunocomplexes were captured with rProtein A-Sepharose Fast Flow beads (GE Healthcare, Little Chalfont, United Kingdom), and coimmunoprecipitated DNA fragments were purified. The relative amounts of immunoprecipitated DNA fragments were evaluated by qPCR using the following primer pairs: human xCT gene promoter region, forward, 5′-TTG AGC AGG AAC CAC CTC CTC CT-3′, and reverse, 5′-CAA ACC AGG TCA GCT TCC TC-3′; human xCT gene intron 1 region, forward, 5′-ATT GCA GAG GTG TCT TT-3′, and reverse, 5′-TCA GAT TTT GCT GTG CCT GC-3′; human xCT gene intron 2 region, forward, 5′-AGA CAC TTT GCT GCC TCA CAA C-3′, and reverse, 5′-CTG ACC ACA AAC TGG CAC GGC-3′. 
Plasmid construction. To construct the human xCT gene promoter-luciferase reporter plasmid (pcXT pro-WT-Luc), an approximately 0.7-kb DNA fragment of the human xCT gene promoter was amplified by PCR using the following primers: forward, 5'-GGG TAG TCT TGC GTA GGT GTA AAT GAG TCT TCT C-3' and reverse, 5'-GGG AGA TCT ACA AAG CAG CTC AGC TCT TCT C-3' (underlines indicate restriction enzyme sites). The amplified DNA fragment was digested with NheI and BglII and then subcloned into the NheI/BglII sites of the pGL3 basic vector. The ARE mutant reporter plasmid (pcXT pro-mt1-Luc) was generated by site-directed mutagenesis using the following primer pair: forward, 5'-AAA GAG CTC AGC ACT GCT GGA GCC TTC TCA TGT GG-3'; reverse, 5'-CCA CAT GAG AAC CCT CCA GGA GTG CTC AGC TCT T-3'. The construct with mutations in both AREs (pXCT pro-mt2-Luc) was generated by site-directed mutagenesis using the following primer pairs: forward, 5'-GGG AGG CTC ATG TGG CCG GTG CAA ACC TGG AG-3' and reverse, 5'-CTC CAG GTT TGG ACC CGC CAT ATG AGA CCT AAG CTG CTG-3'.

The PCR-amplified Nrf2 cDNA fragments were subcloned into the BamHI/XhoI sites of pGEX-6P-2 vector. To clone the human ATF4, the corresponding human ATF4 cDNA was amplified by using the following primer sets (144 –269), and pGEX-hATF4 (270 –351). The amplified Nrf2 cDNA fragment was digested with BamHI and XhoI and subcloned into the BamHI/XhoI sites of pGEX-6P-2 vector. The expression plasmid pcDNA3-hNrf2 was prepared as previously described (40). pcDNA3-hATF4 was constructed by subcloning PCR-amplified human ATF4 cDNA into the BamHI/XhoI sites of the pcDNA3 vector as described previously. To construct the glutathione S-transferase (GST)–hATF4 fusion protein, the full-length human ATF4 cDNA was PCR amplified using primers 5'-GGG ATC CCC CAA CAT GAC CAA AAT GAG C-3' and 5'-GGA ATG TTT GTC TTA TGG AGA GGT GCT TAG CAG TAT TCA GTG TGT-3'. The expression plasmid pcDNA3-hNrf2 was prepared as described previously (40). pcDNA3-hATF4 was subcloned into pCMV-Flag vector. After 24 h of transfection, the cells were transfected with either control or xCT siRNA as described above. Twenty-four hours after transfection, the transfected cells were reseeded on 96-well plates at a density of 3.0 x 10^4 cells/well. After 24 h, the cells were transfected with 20 nM BTZ. Luciferase activity was measured using dual-luciferase assay kits (Promega) according to the manufacturer’s recommended protocol.

GST pulldown assay. The GST pulldown assay was performed by a conventional method. Briefly, Escherichia coli JM109 carrying a GST or GST fusion protein expression vector was incubated at 37°C for 90 min in the presence of 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The bacterial cell pellet was suspended in bacterial lysis buffer (40 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.5% Triton X-100 with 1X protease inhibitor cocktail [EDTA free; Roche, Basel, Switzerland]). After brief sonication, the bacterial lysate was cleared by centrifugation, and the supernatant was then incubated with a glutathione-Sepharose 4B bead slurry (GE Healthcare) for 1 h at 4°C. Each GST fusion protein-bound bead sample was divided into two equal aliquots, and one was subjected to SDS–PAGE and stained with BioSafe Coomassie stain (Bio-Rad) for GST or GST fusion protein visualization. The remaining GST fusion protein-bound beads were incubated with 293T whole-cell extract expressing FLAGx3-hATF4 or FLAXx3-hNrf2 for 4 h with washing buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1.5 mM MgCl2–10% glycerol). The pulled-down sample was separated with SDS-PAGE and subjected to immunoblot analysis. To prepare 293T whole-cell extract expressing FLAXx3-hATF4 or FLAXx3-hNrf2, 293T cells were transiently transfected with either FLAXx3-hATF4 or FLAXx3-hNrf2 expression plasmids. After 24 h of transfection, the cells were treated with 5 µM MG132 for 6 h, and then the cell pellet was collected. The cell pellet was dissolved in cell lysis buffer (50 mM Tris-HCl [pH 8], 300 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1.5 mM MgCl2, 10% glycerol with 1X EDTA-free protease inhibitor cocktail and 1X PhosStop [Roche]) and incubated for 30 min on ice. After centrifugation, the supernatant was diluted with an equal volume of dilution buffer (50 mM Tris-HCl [pH 8], 1 mM EDTA–1% Triton X-100–1.5 mM MgCl2–10% glycerol with 1X EDTA-free protease inhibitor cocktail and 1X PhosStop) and kept as the whole-cell extract.

Cell viability analysis. T24 cells were plated on 6-well plates at a density of 3.0 x 10^4 cells/well. The next day, the cells were transfected with control or xCT siRNA as described above. Twenty-four hours after transfection, the transfected cells were reseeded on 96-well plates at a density of 3.0 x 10^4 cells/well and incubated overnight. The following day, 5 to 100 nM BTZ or 10 nM EPO was added to the transfected cells and incubated for an additional 48 h. For SASP or CPG treatment experiments, T24 cells were seeded on 96-well plates at a density of 5.0 x 10^4 cells/well. After 24 h, the culture media were replaced with media containing 0.3 mM SASP or 0.2 mM CPG for 6 h or 30 min, and then the cells were administered increasing doses of proteasome inhibitors for an additional 48 h. In some experiments, 0.2 µM 2-ME was added. Cell viability was evaluated using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer’s recommended protocol.

Intracellular cysteine and GSH measurements. T24 cells were plated on 6-well plates at a density of 3.0 x 10^4 cells/well and incubated overnight. The following day, the cells were transfected with either control or anti-xCT siRNA as described above. Twenty-four hours after transfection, the cells were transfected with either control or anti-xCT siRNA as described above. Twenty-four hours after transfection, the cells were transfected with either control or anti-xCT siRNA as described above. Twenty-four hours after transfection, the cells were transfected with either control or anti-xCT siRNA as described above.
an additional 24 h. Intracellular cysteine and GSH levels were measured as previously described (30).

Statistical analysis. The data are presented as means and standard errors of the mean (SEM). The Student t test or one-way analysis of variance (ANOVA) with the Bonferroni post hoc test were used to determine significant differences between means. A P value of < 0.05 was considered significant.

RESULTS Proteasome inhibitors induce xCT expression in T24 cells. In a preliminary study, we found that xCT mRNA is highly induced in T24 human bladder carcinoma cells by BTZ. To investigate the profile of xCT expression by proteasome inhibitors in T24 cells, we first analyzed xCT induction by proteasome inhibitors by RT-qPCR. RT-qPCR analysis revealed that xCT mRNA expression was strongly upregulated (9.8-, 13.2-, and 15.9-fold by 20, 50, and 100 nM BTZ, respectively) and significantly upregulated at 6 to 24 h by 20 nM BTZ (Fig. 1A and C). In accordance with these results, marked xCT protein induction was observed in BTZ-treated T24 cells in a dose- and time-dependent manner (Fig. 1D and E). Different types of proteasome inhibitors, EPO and MG132, also increased xCT mRNA and protein levels in T24 cells (Fig. 1B and D and data not shown). These results indicate that xCT expression is strongly enhanced by proteasome inhibition in T24 cells.

A proteasome inhibitor induces xCT expression in a Nrf2- and ATF4-dependent manner. Nrf2 and ATF4 modulate xCT gene expression in murine cells (30, 31, 36). To investigate the roles of Nrf2 and ATF4 in proteasome inhibitor-induced xCT expression, we analyzed Nrf2 and ATF4 expression in T24 cells. All the examined proteasome inhibitors strongly induced both Nrf2 and ATF4 expression in T24 cells, although Nrf2 induction was decreased at a high EPO concentration (Fig. 2A). Both Nrf2 and ATF4 mRNAs and proteins were upregulated in a time-dependent manner (Fig. 2B and C). However, the induction of Nrf2 and ATF4 proteins by BTZ peaked earlier than each mRNA induction, suggesting that Nrf2 and ATF4 induction by BTZ might be mainly due to protein stabilization (Fig. 2C). Consistent with this hypothesis, eIF2α phosphorylation, which enhances selective translation of ATF4, was not increased by BTZ treatment (Fig. 2B). To evaluate the requirement for Nrf2 and ATF4 in BTZ-inducible xCT expression, we next knocked down Nrf2 and/or ATF4 using siRNAs. Nrf2 and ATF4 protein levels after siRNA transfection were detected by immunoblot analysis (Fig. 2E). As shown in Fig. 2D, Nrf2 and ATF4 knockdown attenuated xCT mRNA induction by 20 or 100 nM BTZ treatment, and the simultaneous knockdown of both Nrf2 and ATF4 more effectively decreased xCT mRNA expression than the single knockdowns. In accordance with the RT-qPCR results, BTZ-induced xCT protein expression was diminished in Nrf2 and ATF4 knockdown cells and further decreased in Nrf2/ATF4 double-knockdown cells (Fig. 2F). To address the individual effects of Nrf2 and ATF4 activation, T24 cells were treated with Nrf2- or ATF4-specific inducers. Treatment with the Nrf2-specific inducer tBHQ activated Nrf2 and xCT expression in T24 cells (Fig. 2G and I, lanes 2 from left). Similarly, the ATF4-specific inducer Tm also induced xCT expression (Fig. 2G and J, lanes 2 from left). Simultaneous tBHQ and Tm treatment additively enhanced xCT expression (Fig. 2H to J). These results suggest that Nrf2 and ATF4, induced by proteasome inhibition, coordinately modulate xCT gene expression in T24 cells.

Nrf2 and ATF4 coordinately upregulate xCT gene expression. One functional ARE and two AAREs have been identified in the mouse xCT gene promoter and are responsible for xCT gene expression (30, 31). Sequence data analysis revealed that these cis elements are also conserved in the human xCT gene promoter (Fig. 3A). To investigate whether these ARE and AARE elements mediate BTZ-inducible xCT expression, we constructed a series of
FIG 2. Proteasome inhibitor induces xCT expression in an Nrf2- and ATF4-dependent manner. (A) T24 cells were exposed to 50 or 100 nM BTZ or EPO or 5 μM MG132 for 6 h, and Nrf2 and ATF4 protein expression levels were analyzed by immunoblot analysis. (B) T24 cells were exposed to 20 nM BTZ for 1 to 24 h, and elF2α phosphorylation and ATF4 and Nrf2 protein expression levels were assessed by immunoblot analysis. (C) Nrf2 and ATF4 mRNA expression by 20 nM BTZ was evaluated by RT-qPCR analysis. Differences between groups (versus the 0-h control) were assessed by one-way ANOVA with a Bonferroni post hoc test. **, P < 0.01. (D) T24 cells were transfected with Nrf2 and/or ATF4 siRNA as described in Materials and Methods. At 24 h posttransfection, the T24 cells were treated with DMSO, 20 nM BTZ, or 100 nM bortezomib for another 6 h, and xCT mRNA expression was evaluated by RT-qPCR analysis. Differences between groups (versus control siRNA) were assessed with one-way ANOVA with a Bonferroni post hoc test. **, P < 0.01. (E and F) Immunoblot analysis of siRNA-transfected T24 cells. The arrowheads indicate Nrf2 or ATF4, and the asterisks indicate nonspecific bands. (G) HeLa cells were exposed to tBHQ or Tm, as indicated, for 24 h. The expression of Nrf2 and ATF4 was assessed by immunoblot analysis. (H) HeLa cells were treated with tBHQ and/or Tm, as indicated, for 24 h. xCT mRNA expression was analyzed by RT-qPCR and normalized with CypA expression. The data are presented as means and SEM from at least three independent experiments. Differences between groups were assessed by one-way ANOVA with a Bonferroni post hoc test. *, P < 0.05. (I and J) xCT protein induction by tBHQ and/or Tm treatment (24 h) in T24 cells was analyzed by immunoblot analysis.

human xCT gene promoter-luciferase reporter constructs and analyzed their responsiveness to BTZ (Fig. 3B) (see Materials and Methods). As shown in Fig. 3B, the wild-type (WT) reporter gene (pxCT-pro WT-Luc) showed strong constitutive and BTZ-inducible reporter activities. However, both the constitutive and BTZ-inducible reporter activities were decreased in the ARE mutant and the AARE double-mutant reporter genes (mt1-Luc and mt2-Luc genes, respectively) (Fig. 3B). Notably, both constitutive and
BTZ-inducible reporter activities were almost abolished when both the ARE and the two AAREs were mutated (mt3-Luc) (Fig. 3B). Ectopically expressed Nrf2 and ATF4 were also able to activate the wild-type reporter (Fig. 3C). When ARE was mutated (mt1-Luc), Nrf2 lost the ability to induce reporter gene activity, whereas ATF4 was still able to activate the reporter. Interestingly, although there is no Nrf2 binding site in the reporter construct, Nrf2 further activated ARE mt-Luc (mt1-Luc) in the presence of ATF4 (Fig. 3C). Similarly, the AARE double-mutant reporter (mt2-Luc) was not activated by ATF4, but in the presence of Nrf2, ATF4 further activated reporter gene activity, although this difference was not significant (Fig. 3C). Neither Nrf2 nor ATF4 was able to activate the reporter when both ARE and the two AAREs were mutated (mt3-Luc) (Fig. 3C).

FIG 3 Reporter analysis of the human xCT gene promoter. (A) Schematic representation of the human xCT gene. The putative ARE (ARE-pro) and the two AAREs (AARE-F and AARE-R) are indicated by solid and open triangles, respectively. (B) T24 cells were transfected with either a wild-type reporter gene (pxCT pro WT-Luc) or mutated reporter genes and then incubated for 6 h in the presence of DMSO or 100 nM BTZ. Reporter activities were measured as described in Materials and Methods. (C) HeLa cells were cotransfected with each reporter plasmid in combination with the Nrf2 and/or ATF4 expression vector. After 24 h of incubation, the transfected cells were subjected to a luciferase assay. Luciferase activities were normalized with Renilla luciferase activities, and the bars represent the means and SEM from at least three independent experiments. Differences between groups were assessed by one-way ANOVA with a Bonferroni post hoc test. *, $P < 0.05$; **, $P < 0.01$; NS, not significant.
In addition to the above-mentioned cis elements in the human xCT gene promoter, we found a novel ARE consensus sequence in the second intron of the human xCT gene (6,654 nucleotides from the transcription start site) by in silico analysis (Fig. 3A) (see Materials and Methods). To investigate the function of this ARE sequence (named intronic ARE, or ARE-int), we constructed WT and mutant reporter genes (see Materials and Methods). Interestingly, BTZ treatment and Nrf2 overexpression separately enhanced WT reporter activity but not mutant reporter activity (Fig. 4A and B). These results suggest that Nrf2 modulates human xCT gene expression not only through the promoter ARE but also through the intronic ARE.

To evaluate Nrf2 or ATF4 recruitment to the human xCT gene in response to BTZ, we performed ChIP assays. The PCR-amplified regions used in the ChIP assay are shown in Fig. 5A. After BTZ treatment, both Nrf2 and ATF4 were recruited to the promoter region of the human xCT gene (Fig. 5B and C, left), but neither Nrf2 nor ATF4 was recruited to the first intron region (negative-control region) (Fig. 5B and C middle). Notably, Nrf2 bound to the ARE in the second intron upon BTZ treatment (Fig. 5B, right). Interestingly, ATF4 was also recruited to the intronic ARE by BTZ, although there are no ATF4 binding sites in this region (Fig. 5C, right). It is noteworthy that Nrf2 knockdown did not affect ATF4 recruitment to the promoter ARE, but it completely abolished ATF4 recruitment to the intronic ARE upon BTZ treatment (Fig. 5D). This ChIP result and reporter analysis result (Fig. 3C) strongly suggest that Nrf2 and ATF4 may interact during xCT gene regulation. As expected, Nrf2 and ATF4 interaction was observed by GST pulldown experiments (Fig. 5E to H). GST pull-down experiments also revealed that both the C-terminal bZip and N-terminal transactivation domains of ATF4 associate with Nrf2 protein (Fig. 5E). On the other hand, the C-terminal domain of Nrf2, which contains CNC-bZip and Neh3 domains, conferred ATF4 interaction (Fig. 5F). These results indicate that Nrf2 and ATF4 physically interact to cooperatively activate human xCT gene expression upon BTZ treatment.

**xCT inhibition increases bortezomib sensitivity.** To investigate whether upregulated xCT affects the BTZ sensitivity of T24 cells, we next suppressed xCT function by using xCT siRNA or pharmacologic xCT inhibitors. As shown in Fig. 6D, the constitutive and BTZ-inducible xCT expression levels were effectively decreased by xCT siRNA. Intriguingly, enhanced sensitivity to BTZ was observed in xCT knockdown cells compared to control cells (Fig. 6A). Similarly, a 6-h pretreatment with a pharmacologic xCT inhibitor, SASP (41), significantly enhanced the BTZ sensitivity of T24 cells without affecting xCT induction by BTZ (Fig. 6B and E). In addition, this sensitization was abolished by cotreatment with 2-ME, which bypasses cystine transport by reducing extracellular cystine to cysteine and allowing the cellular uptake of cysteine via the L system, a transporter for neutral amino acids, such as leucine (Fig. 6B) (42). Another xCT inhibitor, CPG, also enhanced BTZ sensitivity (Fig. 6C). These results indicate that xCT has a protective role against BTZ-induced cytotoxicity in T24 cells. Because xCT is crucial for the maintenance of intracellular cysteine and GSH levels, we analyzed intracellular cysteine and GSH levels in cells with xCT knocked down. BTZ treatment increased the intracellular cysteine level approximately 2-fold in control cells. However, the intracellular cysteine level was not increased in xCT knockdown cells (Fig. 7A). Similarly, the cellular GSH level was increased by BTZ treatment and significantly decreased in xCT knockdown cells (Fig. 6C). Nearly the
same effects were observed in SASP-treated T24 cells, although SASP more severely decreased the intracellular GSH level (Fig. 7C and D). We next analyzed the effect of the antioxidant NAC on BTZ-induced cytotoxicity, because NAC is an antioxidant itself and functions as a source of cysteine and GSH (43). Interestingly, BTZ-induced cytotoxicity was attenuated by NAC in a dose-dependent manner (Fig. 7E). These results indicate that BTZ treatment decreases T24 cell viability in part through oxidative stress and that upregulated xCT at-
tenuates oxidative stress by increasing the intracellular cysteine and GSH levels.

xCT knockdown and SASP increase the sensitivity of T24 cells to other proteasome inhibitors. To investigate whether xCT inhibition affects the cytotoxic effect of proteasome inhibitors other than BTZ, we next analyzed the effects of xCT inhibition on EPO-, MG132-, and CFZ-induced cytotoxicity in T24 cells. As shown in Fig. 8A and B, xCT knockdown and SASP significantly increased the EPO sensitivity of T24 cells. In addition, the cytotoxic effects of MG132 and CFZ also increased with SASP treatment. The cytotoxicities of these proteasome inhibitors were also enhanced by CPG in T24 cells (data not shown). These results indicate that xCT generally protects T24 cells from the cytotoxic effect of proteasome inhibitors.

xCT knockdown increases BTZ sensitivity of other cancer cell lines. It is unknown whether xCT affects BTZ sensitivity in cancer cell lines other than T24 cells. By using T98G glioblastoma and U373MG astrocytoma cells, we found that xCT was induced by BTZ treatment and xCT knockdown increased BTZ sensitivity (Fig. 9A to D). However, several cancer cell lines, such as HeLa and MCF7, were severely damaged by xCT knockdown only, and we were not able to assess the effect of xCT knockdown on BTZ sensitivity (data not shown). These results indicate that xCT generally protects T24 cells from the cytotoxic effect of proteasome inhibitors.

To investigate whether BTZ induces these genes in T24 cells, we analyzed their expression. RT-qPCR analysis revealed that all of the genes except PSMB5 are increased by BTZ treatment (Fig. 9E). In addition, GCLC and GCLM, which increase cellular GSH biosynthesis, were also upregulated by BTZ treatment (Fig. 9E). These results indicate that several genes other than xCT may also contribute to T24 cell survival during proteasome inhibition.

DISCUSSION

In bladder cancer treatment, many cytotoxic, anti-angiogenic, epigenetic agents have been tested in chemotherapy (16–19). To increase the efficacy of anticancer drugs, it is important to understand what types of mechanisms confer chemoresistance on tumor cells.

In this study, we revealed the role of xCT in T24 sensitivity to proteasome inhibitors, and the molecular mechanisms by which proteasome inhibitors induce xCT are summarized in Fig. 10. Because of its fundamental importance in cellular processes, the proteasome has been considered a promising target of anticancer chemotherapy (5, 6). Many types of proteasome inhibitors have been developed, and some of them have been approved as anticancer drugs or are currently undergoing phase II or III trials. BTZ is the first proteasome inhibitor approved for clinical treatment (5–7). BTZ shows cytotoxic effects in many cancer cells, including myeloma, mantle cell lymphoma, head and neck carcinoma, and bladder cancer cells. However, similar to other anticancer drugs, intrinsic and acquired resistance to BTZ have been observed (9).
For example, BIRC5/survivin expression and p53 status affect BTZ sensitivity in several cancer cell lines (14). BTZ-induced HSPA2/HSP72 protects human bladder carcinoma cells from BTZ-induced cell death (10). Mutations in the proteasome subunit PSMB5 and PSMB5 overexpression confer BTZ resistance on human myelomonocytic THP1 cells (44). The upregulation of the insulin-like growth factor 1 signaling pathway also affects BTZ resistance in multiple myeloma (45). As shown in Fig. 9E, HSPA2 and PSMB5 were upregulated by BTZ, indicating the possibility that these genes affect BTZ cytotoxicity in T24 cells. These data

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**FIG 7** xCT knockdown or SASP treatment decreases intracellular cysteine and GSH levels. (A and B) Intracellular cysteine and GSH levels in control or xCT siRNA-transfected T24 cells. Twenty-four hours after siRNA transfection, the cells were exposed to DMSO or 20 nM BTZ for 18 h, and then cellular cysteine and GSH levels were measured as described in Materials and Methods. (C and D) T24 cells were pretreated with 0.3 mM SASP for 6 h and then incubated in the presence or absence of 20 nM BTZ for another 24 h. Intracellular cysteine and GSH levels were analyzed as described in Materials and Methods. (E) T24 cells were pretreated with 0.5 or 1 mM NAC for 30 min and then treated with 100 nM BTZ. After 48 h of incubation, cell viability was measured. The value for untreated cells was arbitrarily set as 100%, and the means of relative values are presented with SEM. Differences between groups were assessed by one-way ANOVA with a Bonferroni post hoc test. *, P < 0.05; **, P < 0.01; NS, not significant.

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**FIG 8** Effects of xCT inhibition on T24 cell sensitivity to other proteasome inhibitors. (A) T24 cells were transfected with xCT siRNA as described in the legend to Fig. 6A and then treated with 10 nM EPO. Cell viability was evaluated after 48 h by using CCK-8. (B) T24 cells were pretreated with 0.3 mM SASP in the presence or absence of 66 μM 2-ME for 30 min and then exposed to 20 to 60 nM EPO. After 48 h of incubation, cell viability was measured using CCK-8. (C) The effect of SASP on EPO-induced xCT expression was assessed by immunoblot analysis. The arrowhead indicates xCT. (D and E) T24 cells were pretreated with 0.3 mM SASP for 30 min and then treated with different concentrations of MG132 or CFZ. After 48 h of incubation, cell viability was measured as described for panels A and B. The data are expressed relative to the corresponding value for untreated cells. Differences between groups (versus the respective controls) were assessed by one-way ANOVA with a Bonferroni post hoc test. **, P < 0.01. The data are presented as means and SEM.
emphasize the importance of clarifying the cellular pathway that affects the efficacy of BTZ in cancer cells. xCT has been considered a potential target of cancer treatment. The pharmacological inhibition of xCT function by SASP disrupts the proliferation of lymphoma, glioma, prostate cancer, and hepatocellular carcinoma (41, 46, 47, 48). It was also reported that xCT inhibition represses esophageal cancer cell metastasis and that both siRNA-mediated xCT knockdown and SASP treatment induce autophagic cell death in hepatocellular carcinoma (48, 49). Because xCT is highly induced by BTZ treatment and because xCT knockdown by siRNA increases the BTZ cytotoxicity of T24 cells (Fig. 6D), we conclude that xCT is one of the mediators of BTZ resistance in T24 cells. In hepatocellular carcinoma, xCT inhibition abrogates GSH synthesis and increases ROS (48). In our experiments, xCT knockdown and SASP pretreatment decreased intracellular cysteine and GSH levels (Fig. 7A to D). These results indicate the association between BTZ cytotoxicity and oxidative stress. The attenuation of BTZ cytotoxicity by NAC cotreatment supports this idea (Fig. 7E). However, since the GSH decline was not so severe in xCT knockdown, it is possible that BTZ-induced Nrf2 and ATF4 maintain intracellular GSH levels even in the absence of xCT, because Nrf2 and ATF4 coordinately modulate the

FIG 9 xCT knockdown increases BTZ sensitivity of other cancer cell lines. (A and B) Cell viability of xCT siRNA-transfected T98G and U373MG cells after 48-h BTZ treatment. The data represent means and SEM from three independent experiments. Differences between groups were assessed with Student’s t test. *, P < 0.05. (C and D) T98G or U373MG cells were transfected with either control or xCT siRNA. After 24 h of transfection, the cells were exposed to BTZ for 6 h, and the whole-cell lysates were subjected to immunoblot analysis. (E) T24 cells were treated with 20 nM BTZ for 3 to 24 h, and then GCLC, GCLM, HSPA2, PSMB5, and BIRC5 mRNA expression was evaluated by RT-qPCR and normalized with cyclophilin A (CypA) expression levels. The data represent means and SEM from three independent experiments. Differences between groups (versus the respective 0-h controls) were assessed with one-way ANOVA and a Bonferroni post hoc test. **, P < 0.01.

FIG 10 Hypothetical model for xCT induction by proteasome inhibitors and its effect on proteasome inhibitor–induced cytotoxicity in T24 cells. Proteasome inhibitor–induced xCT protects the cells from the cytotoxic effects of the proteasome inhibitor. See the text for details.
expression of many genes involved in the glutathione synthetic pathway, except for xCT (50). Further analysis is required to clarify the correlation between BTZ-induced xCT and GSH synthesis. Considering that xCT attenuates oxidative stress by creating a reducing extracellular environment via cystine/cysteine cycle up-regulation, the xCT-dependent, but GSH-independent, anti-oxidative system may also affect the BTZ resistance of T24 cells (23, 51).

SASP is a sulfa drug used for the treatment of inflammatory bowel diseases and rheumatoid arthritis, and it is also a potent xCT inhibitor (41). A metabolotropic glutamate receptor agonist/antagonist (CPG) also functions as an inhibitor of xCT (52). In T24 cells, SASP or CPG pretreatment increased BTZ sensitivity (Fig. 6B and C). Although a 6-h SASP or CPG pretreatment was required for BTZ sensitization, a 30-min SASP pretreatment was sufficient to increase sensitivity to other proteasome inhibitors in T24 cells (Fig. 8 and data not shown). Currently, it is unknown what causes this difference between BTZ and other proteasome inhibitors; however, cotreatment with these xCT inhibitors might improve proteasome inhibitor-based cancer chemotherapy. The FDA approved carfilzomib, a derivative of EPO, for the treatment of multiple myeloma in 2012 (53). Further studies are required to understand the effective drug combinations that simultaneously target xCT and the proteasome.

The mechanisms of regulation of the mouse xCT gene promoter by Nrf2 or ATF4 are well established, and one ARE and two AAREs in the promoter region are responsible for Nrf2 and ATF4 binding, respectively (30, 31). In this study, we found a novel Nrf2-responsive ARE in the second intron of the xCT gene, which is conserved among mammalian species (Fig. 4). The evidence that an xCT gene promoter-luciferase construct with ARE mutated was strongly activated by ATF4 and Nrf2 coexpression (Fig. 3C) and that BTZ-induced ATF4 recruitment to the intronic ARE was diminished by Nrf2 knockdown (Fig. 5D) indicates that Nrf2 and ATF4 interact with each other and cooperatively activate BTZ-induced xCT gene expression. He et al. reported that the Nrf2/ATF4 heterodimer binds to the stress response element (StRE) of the HO-1 gene enhancer and activates StRE-dependent transcription (54). To bind the StRE, Nrf2 and ATF4 likely interact through their bZIP domains. In accordance with this, the C-terminal regions of Nrf2 and ATF4, which contain a bZIP domain, conferred interaction between Nrf2 and ATF4 (Fig. 5E and F). However, the ATF4 transactivation domain was also associated with Nrf2 (Fig. 5E). Since the ATF4 activation domain contains a second leucine zipper, it is possible that this second Zip domain interacts with the Nrf2 bZip domain. It is also possible that ATF4 and Nrf2 interact indirectly through coactivators, because the Nrf2 C-terminal region contains an Neh3 transactivation domain (55). Further study is required to clarify what kinds of Nrf2-ATF4 interaction regulate xCT gene induction.

Both Nrf2 and ATF4 play crucial roles in cancer cell survival and growth (36, 56–59). Nrf2 activates the antioxidative pathway by inducing a variety of antioxidant proteins in addition to xCT. Also, Nrf2 upregulates phase II and phase III drug-metabolizing enzymes, which enhance the excretion of anticancer drugs from cancer cells (34, 35). Nrf2 also activates cancer cell growth through metabolic reprogramming (58). ATF4 is involved in many stress pathways, including amino acid metabolism, redox homeostasis, and ER stress response (37, 38). Because most of these stresses are unfavorable for cancer cells, ATF4 plays an important role in cancer cell growth and survival. Further, ATF4 increases chemoresistance in hepatocellular carcinoma (59). It is likely that other cytoprotective pathways driven by the Nrf2 or ATF4 pathway, such as GCLC and GCLM, also contribute to the resistance of T24 cells to BTZ. Further studies are needed to clarify this issue.

The present study has revealed that xCT induction confers resistance to T24 bladder carcinoma cells upon proteasome inhibition. It is plausible that cotreatment with an xCT inhibitor may improve proteasome inhibitor efficacy in bladder cancer chemotherapy.

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