SUMOylation plays a role in gemcitabine- and bortezomib-induced cytotoxicity in human oropharyngeal carcinoma KB gemcitabine-resistant clone

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Abstract

Bortezomib, a novel dipeptide boronic acid proteasome inhibitor, has been shown in previous studies to be synergistic with gemcitabine; however, the molecular mechanisms are not fully understood. Because post-translational modification of proteins, such as ubiquitination and SUMOylation, plays a critical role in governing cellular homeostasis, we explored this further by treating human oropharyngeal carcinoma KB wild-type (KBwt) and gemcitabine-resistant (KBGem) cells with gemcitabine and bortezomib in a time-dependent and sequence-dependent manner. Treatment with bortezomib at 4 to 8 hours post-gemcitabine significantly induced cell death in KBwt cell lines. However, in KBGem cells, bortezomib alone was just as cytotoxic. Using reporter assays, nuclear factor-κB (NF-κB) activity was found to be 5-fold higher in KBGem cells than that in KBwt cells, and the combination treatment decreased NF-κB activity by 44% in KBwt cells and 28% in KBGem cells, respectively. By Western blot analyses, treatment with gemcitabine and bortezomib resulted in a cleavage of NF-κB in KBwt but not in KBGem cells. SUMOylation capacity was modulated by transducing KBwt and KBGem cells with lenti-SUMO-1 or the unconjugatable lenti-SUMO-1aa followed by drug treatment. The expression of cyclins A, D1, and E was differentially regulated by SUMOylation capacity in KBGem but not in KBwt cells. We report herein that the activation of NF-κB signaling plays a critical role in eliciting KBwt cell survival against gemcitabine, whereas the role of SUMOylation in modulating the steady-state levels of key cell cycle regulator proteins seems more significant in KBGem cells.

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Introduction

The 26S proteasome, found in the cytoplasm and nuclei of all eukaryotic cells, functions to degrade proteins via the ubiquitin pathway. Many of the key regulatory proteins that govern cell division, growth, activation, signaling, and transcription are degraded by the ubiquitin-proteasome system. This ordered degradation is required for the cell to progress through the cell cycle and undergo mitosis. Cellular proteins that are targets of ubiquitin-mediated protein degradation include p21 and p27, which can induce G1 cell cycle arrest by inhibiting cyclin A, cyclin D, and cyclin E–dependent kinases, the tumor suppressor gene p53, various transcriptional factors (e.g. c-myc, m-fos, and c-jun), and several members of the apoptosis regulatory family, such as Bax (1–4). Disruption of this ubiquitination-dependent cellular homeostasis control leads to cellular apoptosis. Bortezomib (also known as PS341), a novel dipeptide boronic acid proteasome inhibitor, is a promising chemotherapeutic agent, which has antitumor effects on several human cancers (5).

Gemcitabine, a nucleoside analogue, has also shown significant antitumor effects against many solid tumors, and its mechanism of action has been extensively studied. Gemcitabine is first phosphorylated to dFdCMP by deoxycytidine kinase and subsequently phosphorylated to its diphosphate and triphosphate forms. The incorporation of the active triphosphate metabolite dFdCTP rather than dCTP into DNA causes masked chain termination (6, 7). Gemcitabine diphosphate also directly inhibits ribonucleotide reductase activity (8). Previously, we showed a time and sequence dependence between gemcitabine and hydroxyurea with the greatest enhancement of cytotoxicity occurring when gemcitabine was added at 8 hours after hydroxyurea treatment. This enhancement was associated with a decrease in the hRRM2 RNA protein and activity from hydroxyurea treatment (9). A time and sequence dependence between gemcitabine and bortezomib has also been shown in the pancreatic MIA-PaCa-2 cell line (10). Gemcitabine followed by bortezomib induced the greatest amount of apoptosis and inhibition of cell growth. The mechanism of action is thought to be due to the induction of prolonged cell cycle arrest, leading to cell death. However, the expression of p21, p27, and BCL-2 after drug treatment was not found to correlate...
with cellular effects (10). Our lab has extensively worked with human oropharyngeal carcinoma KB wild-type (KBwt) cells and also developed gemcitabine-resistant (KBGem) cells. We tested this combination in KBwt cells, which allowed us to examine the differences in KBwt versus gemcitabine-resistant cells.

Developing methods to circumvent this resistance relies on understanding the mechanisms underlying its resistance. In KBGem cells, the hRRM2 promoter was up-regulated almost 5-fold via NF-Y transactivation compared with KBwt cells (11). This transcriptional activation of the hRRM2 gene resulted in an increased steady-state level of hRRM2, whereas the expression of hRRM1 and p53R2 was not up-regulated. Hence, the ribonucleotide reductase activity in KBGem cells was significantly higher. Another mechanism of chemoresistance is the inducible nuclear factor-κB (NF-κB) activation, resulting in an inhibition of the apoptotic response. Phosphorylation of the inhibitor protein IκB leads to its degradation via the ubiquitin-proteasome pathway. Subsequently, NF-κB translocates to the nucleus and regulates the transcription of a variety of genes, including those necessary to suppress apoptosis (12–15). In the human colorectal cancer cell line LOVO, combining bortezomib with SN-38, the active metabolite of CPT-11, resulted in a 94% decrease in tumor size. There was also a significant increase in growth inhibition (64–75%) compared with cells treated with bortezomib alone (20–30%; ref. 16).

SUMOylation is an evolutionarily conserved pathway that has emerged as an important regulator of gene expression by altering the subcellular localization of proteins or antagonizing other modifications, such as ubiquitination (17–20). The covalent attachment of small ubiquitin-like modifier (SUMO) to its targets involves the formation of an isopeptide bond between the COOH terminus of glycine of SUMO and the ε-amino group of a lysine residue in the target protein. This reaction is ATP dependent and requires the E1-activating enzyme, the E2-conjugating enzyme, and the E3 ligase (21, 22). Because NF-κB activation requires ubiquitination and proteasome-mediated degradation of IκBα SUMO-1 conjugation to Lys21, which is also used for ubiquitination prevents IκBα from being targeted for degradation. Thus, SUMO-1 inhibits the activation of NF-κB (23). SUMOylation may also have a role in regulating transcription factor activity. In this report, we showed that treatment with gemcitabine (0.5 μmol/L) and bortezomib (0.03 μmol/L) was significantly more cytotoxic than each drug alone. In both the KBwt and KBGem cells, the maximal effect by the combination treatment on reducing cell viability may be in a time-dependent and sequence-dependent manner. Moreover, we found that SUMOylation played a critical role in gemcitabine/bortezomib–mediated cell death in KBGem cells as well as expression of key cell cycle regulatory proteins. These findings provide novel insight into molecular mechanisms underlying chemoresistance towards gemcitabine-dependent and gemcitabine/bortezomib–dependent cell killing in human oropharyngeal carcinoma KB cells. Oropharyngeal cancer treatment mainly relies on surgery or radiation. Responses to chemotherapy are rapid but usually short lived. Examining drug combinations with novel mechanisms of action may help to direct future care for this disease.

Materials and Methods

Cell Culture

Human oropharyngeal carcinoma KB cells (American Type Culture Collection, Manassas, VA) were cultured in a 5% CO2 atmosphere at 37°C on plastic tissue culture plates in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The drug-resistant cell line KBGem was selected from KB cells by stepwise exposure to increasing concentrations of gemcitabine and maintained in the presence of 8 μmol/L gemcitabine.

Cell Viability Assay

Cell concentration was determined using KOVA Glasstic slides and 2.5 × 104 cells were plated in each well (Falcon 24-well plates). KB cells were grown overnight and treated with gemcitabine and/or bortezomib in a time and sequence manner. Cell viability was determined by spectrophotometry after incubation with drug for 72 hours. KBGem cells were maintained in the presence of 8 μmol/L gemcitabine until the cells were plated in 24-well plates and were grown overnight without any gemcitabine. Statistical analysis by Student’s t test was done on triplicate experiments.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assays for Measurement of Cell Viability

The cells were treated with gemcitabine and/or bortezomib and medium was changed daily for 3 days; 0.2 mL of 0.1 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma, St. Louis, MO) in Opti-MEM I (Invitrogen, Carlsbad, CA) was added to each well, and the plate was incubated at 37°C for an additional 1.5 hours. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution was then aspirated, and 0.2 mL isopropanol was added to each well to dissolve the formazan crystals. Contents of each well were transferred to 96-well plates, and absorbance was read immediately at 540 nm in a scanning multiwell spectrophotometer. Statistical analysis by Student’s t test was done on triplicate experiments.

Cell Cycle Analysis

The suspended cells were fixed in 70% ethanol and stored at −20°C until analysis. The cell suspension was centrifuged, and the ethanol was removed by washing with PBS. The cell pellet was resuspended in 1 mL propidium iodide/Triton X-100 solution with RNase A and incubated at room temperature for 30 minutes. Cell fluorescence was measured by flow cytometry.

Western Blot Analysis

Radioimunoprecipitation assay buffer with freshly added phenylmethylsulfonyl fluoride, aprotinin, and
sodium orthovanadate was used to extract protein. Equal amount of protein was loaded on 10% Tris-glycine gel. After separation, they were transferred either to polyvinylidene difluoride membranes or Kodak multiblot membranes. After blocking, the membranes were incubated at room temperature with the primary antibody, diluted 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes. Secondary antibodies were diluted 1:5,000. CSPD ready-to-use substrate (Applied Biosystems, Foster City, CA) was added to the membrane, which was then exposed to radiographic film.

Measurement of NF-κB Activity

pGL2-NF-κB luciferase reporter construct harboring an interleukin-6 promoter and two NF-κB binding sites was constructed by Dr. Jian Jian Li's lab (Previously City of Hope National Medical Center). KB and KBGem cells were transfected by electroporation. About 1 to 3 × 10⁶ cells were suspended in 400-μL Eppendorf electroporation cuvettes (gap, 2 mm) in hyposmolar electroporation buffer with 15 μg desired reporter plasmid DNA and 10 μg β-galactosidase control plasmid (Promega, Madison, WI). Electroporation was done at 800 V for 75 microseconds. After electroporation, cells were recultured for 48 hours with about 50% cell viability and then harvested. Luciferase activity was detected using the Promega assay system.

Gel Shift Assay

Nuclear protein was extracted from KBwt, KBGem, and KBHUR (hydroxyurea-resistant KB clone). Using the Promega gel shift assay system, consensus oligonucleotide sequences for NF-κB end-labeled with 32P were incubated with protein and analyzed on a nondenaturing polyacrylamide gel. Competition experiments were done with excess unlabeled oligonucleotides.

Construction of SUMO Lentivirus Expression Vector

Lentivirus with EGFP-SUMO-I and EGFP-SUMO-1aa was constructed in Dr. David Ann's lab at the University of Southern California. EGFP-SUMO-1 and EGFP-SUMO-1aa cDNA was cloned into the PinAI/HincII sites of pRRLsin.hCMV156, downstream of the cytomegalovirus promoter. By calcium phosphate precipitation, 293T cells were transfected with plasmids that create the nonreplicating lentivirus. Titer of the lentiviral stock was determined by fluorescence-activated cell sorting (Fig. 1C). Compared with vehicle control cells, the KBwt cells treated with bortezomib or gemcitabine plus bortezomib (simultaneously) showed a small increase in sub-G₁ cells. The greatest percentage increase in sub-G₁ cells occurred when KBwt cells were treated with gemcitabine followed by bortezomib 8 hours later. This increase in preapoptotic cells correlated with the reduction in cell viability. By contrast, there was a very modest, at best, enhancement in the percentage of sub-G₁ cells after sequential drug treatment in KBGem cells compared with KBwt cells.

NF-κB Activity Differs between KBwt and KBGem Cells

To determine the effect of sequential drug treatment on NF-κB activity, a PGL2-based luciferase reporter with two NF-κB binding sites was transiently transfected into KBwt and KBGem cells, respectively. There was a 5-fold increase of NF-κB activity in KBGem cells than that in KBwt cells (Fig. 2A). Treatment with gemcitabine (0.5 μmol/L) followed by bortezomib (0.03 μmol/L) 8 hours later reduced luciferase activity by 44% and 28% in KBwt and KBGem cells, respectively (Fig. 2A).

With drug treatment, cell viability was significantly decreased in both cell lines although there was a 5-fold increase in NF-κB activity in KBGem cells. Because this difference in NF-κB activity may be due to transcriptional
regulation, we examined the NF-κB DNA binding activity by gel shift assays in KBwt and KBGem cells. As illustrated in Fig. 2B, an enhanced intensity (arrowhead b) and an additional species (arrowhead c) were observed in resting KBGem cells (lane 3), which was markedly reduced in wild-type KB cells (lane 2). Both signals (arrowheads b and c) were effectively competed out with an excess of unlabeled corresponding oligonucleotides (Fig. 2B, lane 6). This finding correlates with the robust NF-κB activation in KBGem cells.

To further confirm that the transactivation by NF-κB was different between KBwt and KBGem cells, we employed Western blot analyses to examine cellular changes of NF-κB steady-state level after drug treatment. When KBwt cells were treated with gemcitabine plus bortezomib, we observed cleavage of NF-κB (Fig. 3A, lanes 2 and 3). Giving the drugs simultaneously versus sequentially resulted in similar cleavage of NF-κB. This phenomenon was not seen when KBGem cells were treated in the same manner (Fig. 3A). To determine if the observed NF-κB cleavage was a result of the combination of gemcitabine and bortezomib, cells were washed with PBS and the media changed at either 3 or 6 hours after gemcitabine was given. Under these conditions, no cleavage of NF-κB was observed (Fig. 3A, lanes 5 and 6). Moreover, simultaneous treatment of the cells followed by washing with PBS after 3 hours also resulted in disappearance of the NF-κB cleavage (Fig. 3A, lane 7). For KBwt and KBGem cells, both IkB-β and IkB-α levels were not changed by the treatment (Fig. 3B; data not shown). Because the cleavage of NF-κB only occurred with combination drug treatment in KBwt cells, this suggests that the antitumor activity of combination drug treatment occurs by different mechanisms in KBwt and KBGem cells.

Changes in Cell Cycle Proteins Differ between KBwt and KBGem Cells after Drug Treatment

The steady-state level of p21 increased in both KBwt and KBGem cells treated with gemcitabine and bortezomib (Fig. 4A, lanes 4 and 6 versus lanes 1 and 3), suggesting that bortezomib stabilized p21 protein. In contrast, only a moderate change on p27 was detected (data not shown). To examine changes in cyclins A, B1, D1, and E, Western blot analyses were done on protein lysates harvested at 24 hours after treatment. As shown in Fig. 4B, a decreased cyclin B1 expression was the only consistent change observed in both KBwt and KBGem cells with the greatest decrease occurring in KBGem cells. Treatment with gemcitabine and bortezomib resulted in a decreased expression of cyclin A, no change in cyclin D1 and an increased expression of cyclin E in KBwt cells, whereas there was no marked change in cyclins A, D1, and E after drug treatment in KBGem cells. This study further suggests differences in cellular responses to the combination therapy between KBwt and KBGem cells.

SUMOylation Plays a Role in Gemcitabine-Induced and Bortezomib-Induced Cytotoxicity of KBGem Cells

For the cells transduced with SUMO-1, Western blot analyses showed multiple bands compared with the cells transduced with the unconjugatable form of SUMO-1aa (Fig. 5A). For KBwt cells transduced with SUMO-1 and

Figure 1. Cell viability in KBwt and KBGem cells after treatment with gemcitabine and bortezomib (PS341). A, determination of bortezomib dose for experiments by cytotoxicity assays. KBwt cells were treated with gemcitabine (0.5 μmol/L) plus varying concentrations of bortezomib. B, KB cells were treated with gemcitabine (0.5 μmol/L) alone, gemcitabine (0.5 μmol/L) plus bortezomib (PS341, 0.03 μmol/L) simultaneously, and gemcitabine (0.5 μmol/L) followed by bortezomib (0.03 μmol/L) at hours 4, 8, and 16. After 72 h of incubation, the percentage of viable cells was determined (as described in Materials and Methods). KBGem cells were treated under the same conditions. Representative of three independent experiments. Bars, range. C, cell cycle analysis by flow cytometry. KBwt and KBGem cells were treated with bortezomib alone, gemcitabine plus bortezomib simultaneously, and gemcitabine followed by bortezomib 8 h later. Twenty-four hours after initial drug treatment, the cells were harvested, and percentage of sub-G1 cells determined by fluorescence-activated cell sorting.
SUMO-1aa, there was no significant decrease in cyclins A, D1, and E after drug treatment, and cyclin B1 level increased slightly compared with the control (Fig. 5B). In the KBGem cells transduced with SUMO-1, there was a significant decrease in cyclins A, D1, and E, especially in cells treated with bortezomib alone or simultaneous combination of gemcitabine and bortezomib. There was also a slight decrease when the drugs were given sequentially 8 hours apart. However, in the KBGem cells transduced with SUMO-1aa, cyclin A, B1, and E expression did not significantly change, and only cyclin D1 decreased after treatment. There was no noticeable modulation of NF-κB level by SUMO-1 or SUMO-1aa overexpression (Fig. 5C).

To further evaluate the role of SUMOylation with drug treatment in KBwt and KBGem cells, 10 μmol/L of cell permeable peptide containing SUMO-binding motif was delivered into cells and treated with varying concentrations of gemcitabine (Fig. 6A and B). A control scrambled peptide was introduced into cells and treated under the same conditions. In KBwt, cell viability decreased with increasing concentration of gemcitabine. There was not a significant decrease in cell viability in cells with SUMO-binding motif compared with control. However, in KBGem cells at 40 μmol/L concentration of gemcitabine, cell viability decreased by 20% in cells with 10 μmol/L SUMO-binding motif compared with control. This supports our results that the role of SUMOylation in modulating the steady-state levels of key cell cycle regulator proteins seems more significant in KBGem cells.

**Discussion**

Oropharyngeal cancer is potentially curable with surgery and/or chemoradiation; however, once metastatic disease develops, prognosis is poor due to eventual chemoresistance. This has been a major limitation in the treatment of malignancies, and combinations of chemotherapy that may act synergistically have been tried to achieve better results. Previously, a time and sequence dependence between gemcitabine and bortezomib has been shown in the pancreatic MIA-PaCa-2 cell line (10). Gemcitabine followed by
bortezomib induced the greatest amount of apoptosis and inhibition of cell growth. The mechanism of action was thought to be due to the induction of prolonged cell cycle arrest, leading to cell death. This may be specific to the pancreatic cell line because we did not see a statistically significant change in cell viability between treating the KBwt and KBGem cells simultaneously or sequentially with drug. To confirm this, additional cytotoxicity assays should be done at lower concentrations. This concentration of bortezomib was chosen because it approximately induced an additional 50% cell death when combined with gemcitabine alone. At lower concentrations and with minimal cytotoxicity, we may not see any differences in cell cycle proteins with post-translational modification. We did see a significant cytotoxic effect with the combination of gemcitabine and bortezomib. Interestingly, KBGem cells treated with bortezomib alone was as cytotoxic as the combination treatment. Currently, clinical trials have an ongoing investigation of the optimal use of this novel chemotherapeutic agent bortezomib.

Cells have many redundant pathways that allow it to survive when exposed to stressors, such as chemotherapy. The constitutive activation of nuclear transcription factor NF-κB, which regulates the transcription of various genes necessary to suppress apoptosis, has been implicated as one mechanism for chemoresistance. In LEVO human colorectal cancer cell lines, bortezomib circumvents drug resistance to be inhibiting the inducible activation of NF-κB (16), supporting the notion that this pathway is affected by proteasome inhibition. We have shown a 5-fold increase of NF-κB activity as well as robust NF-κB activation in KBGem cells compared with KBwt. However, when we looked at protein expression by Western blot analysis, we did not observe a significant change in NF-κB with treated KBGem cells; but in KBwt cells, we observed cleavage of NF-κB when the cells were exposed to both gemcitabine and bortezomib simultaneously. The mechanism of this cleavage is not fully understood and may represent an alternative pathway to prevent activation of NF-κB in KBwt cells. Previous studies have shown that bortezomib induces...
Bcl-2 phosphorylation and cleavage that is associated with \(G_2\)-M phase arrest and apoptosis (24). In KBwt cells, the cleavage only occurred via interaction of the drugs together.

Post-translational modification by SUMO protein is reported to elicit diverse consequences in regulating target protein function. SUMOylation can cause the relocation of RanGAP1 from the cytoplasm to the nuclear pore complex, and SUMOylation of IκB prevents ubiquitination and subsequent degradation. Recent studies have further supported the role of SUMOylation in regulating transcription factor activity (25–27). For example, SUMOylation of hsTAF5 interferes with binding of transcription factor IID to promoter DNA. The role of SUMOylation in governing cell homeostasis is complex and affects many cellular processes, including nuclear cytoplasmic trafficking, transcriptional control, and antagonizing other modifications, leading to altered function. In KBGem cells, SUMOylation differentially regulated cell cycle protein expression, which was not observed in KBwt cells. Because SUMOylation seems to play an important role in regulating cell death in KBGem cells, we plan on transducing KB and KBGem cells with lenti-EGFP-SUMO-1 and lenti-EGFP-SUMO-1aa, which will produce a stable clone. Then using the luciferase reporter vector, we will analyze the effect of SUMOylation on NFκB.

Thus, our data revealed the pathways leading to cell death are different between KBwt and KBGem cells. The role of NFκB seems to be more important for KBwt, whereas SUMOylation plays a more significant role in KBGem cells. Currently, doxorubicin-induced genotoxicity and SUMO-dependent protein interaction is being studied. In SUMO-1-overexpressing HS578T-S1 cells, more pronounced cell killing was observed compared with HS578T cells. Further studies are warranted examining the differences underlying SUMOylation in gemcitabine-resistant cells. This may help to develop future therapies to circumvent drug resistance.

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