BaxΔ2 sensitizes colorectal cancer cells to proteasome inhibitor-induced cell death

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Abstract
Proteasome inhibitors, such as bortezomib and carfilzomib, are FDA approved for the treatment of hemopoietic cancers, but recent studies have shown their great potential for treatment of solid tumors. BaxΔ2, a unique proapoptotic Bax isoform, promotes non-mitochondrial cell death and sensitizes cancer cells to chemotherapy. However, endogenous BaxΔ2 proteins are unstable and susceptible to proteasomal degradation. Here, we screened a panel of proteasome inhibitors in colorectal cancer cells with different Bax statuses. We found that all proteasome inhibitors tested were able to block BaxΔ2 degradation without affecting the level of Baxα or Bcl-2 proteins. Among the inhibitors tested, only bortezomib and carfilzomib were able to induce differential cell death corresponding to the distinct Bax statuses. BaxΔ2-positive cells had a significantly higher level of cell death at low nanomolar concentrations than Baxα-positive or Bax-negative cells. Furthermore, bortezomib-induced cell death in BaxΔ2-positive cells was predominantly dependent on the caspase 8/3 pathway, consistent with our previous studies. These results imply that BaxΔ2 can selectively sensitize cancer cells to proteasome inhibitors, enhancing their potential to treat colon cancer and other solid tumors.

Keywords
BaxΔ2; Cell death; Proteasome inhibitors; Cancer therapy; Bortezomib; Carfilzomib

1. Introduction
The 26S proteasome is a critical intracellular complex that is necessary for the degradation of many cytosolic proteins [1]. Alteration of cellular proteostasis by inhibition of the 26S proteasome has proven to be effective for targeting cancer cells. Unlike conventional chemotherapeutic drugs, proteasome inhibitors specifically target the 20S core particle of the 26S proteasome without affecting drug transporters [1,2]. These inhibitors can cause apoptosis, mainly through protein accumulation-mediated cellular stress or proteotoxicity [1,3,4]. Several proteasome inhibitors have been approved by the FDA or are undergoing clinical and pre-clinical studies for cancer therapy [5–10]. The primary applications of
proteasome inhibitors in cancer therapy are for the treatment of multiple myeloma or mantle cell lymphoma \[\text{2,3,11,12}\]. To date, no proteasome inhibitor has been approved for the treatment of solid tumors.

MG132 is a reversible 26S proteasome inhibitor commonly used in laboratories, that directly promotes apoptosis through various mechanisms, including production of radical oxygen species (ROS) and activation of caspase 8 \[\text{1,4,13,14}\]. However, MG132 is not FDA approved for clinical use due to its non-specific cytotoxicity. Bortezomib, the most common and first FDA approved proteasome inhibitor used for the treatment of multiple myeloma, belongs to the boronate class of proteasome inhibitors, and induces reversible inhibition \[\text{1,2,12}\]. While bortezomib is only approved to treat of multiple myeloma and mantle cell lymphoma, multiple studies have shown that the use of bortezomib in concert with other chemotherapeutics can significantly increase cell death in solid tumors \[\text{8–10,15,16}\].

Ixazomib is another reversible boronate proteasome inhibitor, which recently gained clinical approval for treatment of multiple myeloma, and has also shown the ability to inhibit proliferation and induce apoptosis in solid tumors \[\text{2,3,5,12}\]. On the other hand, carfilzomib, is a potent irreversible proteasome inhibitor approved for treatment of multiple myeloma, especially in patients who didn’t respond well to previous treatment with bortezomib \[\text{2,3,7,12}\]. Recent research has shown that carfilzomib can also suppress cell proliferation and promote apoptosis in solid tumors when used in combination with other chemotherapeutics \[\text{7,17,18}\]. As a precursor of carfilzomib, epoxomicin has also drawn interest due to its anti-neoplastic properties, however, its structure hindered it unsuitable to be used as a drug \[\text{19}\].

BaxΔ2 is a unique pro-apoptotic member of the Bax family that was first identified in colon cancer tumors with microsatellite instability (MSI) \[\text{20–22}\]. This isoform is generated by the combination of an alternative splicing of Bax exon 2 and a mononucleotide deletion in the microsatellite region within exon 3 (G8 to G7). As with Baxα, BaxΔ2 is pro-apoptotic, forms homodimers, and interacts with Bcl2 \[\text{20}\]. However, the pro-death potency of BaxΔ2 is stronger than that of the parental Baxα, and unlike the majority of Bax isoforms, BaxΔ2 fails to target mitochondria due to the lack of exon 2, which encodes for the mitochondrial targeting signal \[\text{23–27}\]. Instead, BaxΔ2 induces cell death by formation of large aggregates in the cytosol and activation of caspase 8 \[\text{20,23,28}\]. Interestingly, soluble cytosolic BaxΔ2 monomers are very unstable and susceptible to degradation, which can be blocked by a common proteasome inhibitor, MG132 \[\text{28}\].

We wanted whether prevention of BaxΔ2 degradation could promote BaxΔ2-mediated cell death and increase the efficiency of chemotherapy. To test this, we screened a group of proteasome inhibitors in several colon cancer cell lines. We also used single-cell derived sublines which have a similar genetic background, but different statuses of Bax protein expression (such as Baxα-positive, BaxΔ2-positive, or Bax-negative) \[\text{28}\]. We compared the effect of these inhibitors in terms of accumulation of BaxΔ2 proteins and induction of cell death in these cell lines. We also monitored cellular morphological changes and analyzed a panel of caspase activities. Our study suggests that BaxΔ2-positive cancer cells are more sensitive to proteasome inhibitors than those expressing Baxα or no Bax, and that BaxΔ2 might serve as an indicator for selective chemotherapy.

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2. Materials and methods

2.1. Materials

Bortezomib, carfilzomib, epoxomicin, and ixazomib were obtained from ApexBio. Inhibitors against caspase 3 (Z-DEVD-FMK), caspase 6 (Z-VEID-FMK), caspase 8 (z-VETD-FMK), caspase 9 (z-LEHD-FMK), and caspase 12 (ATAD-FMK) were obtained from Calbiochem. Ethidium-homodimer-1 was obtained from Invitrogen. Antibody against BaxΔ2 was generated previously [20]. Antibodies against actin, cleaved caspase 8, LC3B, and Bcl-2 were obtained from Cell Signaling. Antibody against Baxα (N20) was from Santa Cruz Biotechnology, and HRP-conjugated secondary antibody was from Jackson.

2.2. Cell culture

Human colorectal cancer HCT116 cells were from ATCC. Three sublines, Baxα(+), BaxΔ2(+) and Bax(−), were derived from HCT116 cells as described previously [28]. Two other colorectal cancer cell lines, LS174T expressing BaxΔ2 and SW1116 expressing Baxα, were previously characterized [20]. All cell lines were maintained in DMEM with 10% FBS at 37 °C and 5.5% CO₂, and incubated in 6 or 24-well plates for 24 h, up to 60% confluency, before each experiment.

2.3. Cell viability assay

Cells were treated with the corresponding concentration (0–200 nM) of proteasome inhibitors for 48 h with DMSO as a control. Cells were then incubated with Ethidium-homodimer-1 for 15 min at 37 °C and 5.5% CO₂, harvested, and imaged using either light or fluorescence microscope. Results from at least three independent experiments were used for statistical analysis and fitted to a dose response sigmoid curve that was used to calculate the effective concentrations, EC50 and EC95.

2.4. Caspase inhibition assay

Cells were treated with individual caspase inhibitors (50 nM for each) as indicated in the text for 30 min. Then, 20 nM of bortezomib was added and cells were incubated for further 24–48 h. Finally, cells were incubated with Ethidium-homodimer-1 for additional 15 min at 37 °C and 5.5% CO₂ before harvesting for cell viability assay.

2.5. Western blot

BaxΔ2-positive cells were treated as indicated in the text and harvested for lysis. Equal amounts of protein for each sample were separated by a 15% SDS-PAGE gel and transferred onto a 0.2 μm PVDF membrane. Membranes were blotted with 5% BSA, followed by incubation with primary antibody against BaxΔ2 (1:200 dilution), Baxα (N20) (1:200), Bcl-2 (1:200), Cleaved Caspase 8 (1:200), or actin (1:3000) overnight at 4 °C, and then with a corresponding HRP-conjugated secondary antibody for 1 h. The protein bands were visualized on X-ray films with Pierce ECL Western Blotting Substrate developing kit (ThermoScientific).
2.6. Statistical analysis

All values shown represent the mean ± SD. Two-way ANOVA followed by t-test was performed using GraphPad Prism 7.03 (La Jolla, California, USA). P < .05 was considered significant.

3. Results

Colorectal cancer HCT116 cells contain mixed populations of wild type and mutant Bax microsatellite. We previously established several single-cell derived sublines expressing either Baxα (Baxα(+)) with Bax G7/G8 microsatellite, BaxΔ2 (BaxΔ2(+)) with G7/G7 microsatellite, or no Bax (Bax(−)) with G7/G8 microsatellite (Fig. 1A) [28]. The growth profiles are similar under normal culture conditions (Fig. 1B), as well as the morphology (Fig. 1C top panel). We previously showed that degradation of BaxΔ2 could be blocked by a commonly used proteasome inhibitor, MG132 [28]. Treatment with MG132 caused all three sublines to accumulate intracellular vacuole-like structures, and a great number of cells rounded up after 24 h (Fig. 1C bottom panel). MG132 treatment was able to accumulate a significant amount of BaxΔ2 proteins in the BaxΔ2(+) cells, a very low amount in the Baxα(+) cells, and none in the Bax(−) cells (Fig. 1D). Importantly, neither Baxα nor Bcl-2 protein levels were altered by MG132 (Fig. 1D). To test whether accumulation of BaxΔ2 is sufficient to sensitize colon cancer cells to cell death, we treated all three sublines with MG132 for 24–48 h. All sublines underwent significant cell death with no difference among each other at 24 h. However, at 48 h, there was a moderately higher increase of cell death in BaxΔ2(+) cells than in Baxα (+) or Bax(−) cells (Fig. 1E). The non-specific toxicity of MG132 may have masked the effect of BaxΔ2, but it gave us a hint that accumulation of BaxΔ2 by proteasomal inhibition may potentiate cell death.

The preliminary results with MG132 led us to test a group of proteasome inhibitors which are either approved by the FDA (bortezomib, carfilzomib and ixazomib) or are well studied (epoxomicin, potent precursor of carfilzomib) [2,5–7,19]. To test whether these drugs could differentially kill BaxΔ2(+) cells, a dose range from 0 to 200 nM for each drug was used to treat HCT116 BaxΔ2(+), Baxα(+), and Bax(−) sublines for 48 h, and cell viability was analyzed. Effective concentrations, EC50 and EC95, were calculated for each inhibitor in all three cell sublines. We found that all proteasome inhibitors tested showed lower EC50 values for the BaxΔ2(+) cells than for the other two sublines, while inducing higher levels of cell death (Fig. 2A and B). Among them, bortezomib and carfilzomib showed higher differential sensitization between the three sublines. For example, bortezomib at 20 nM induced ~70% of cell death in BaxΔ2(+) cells, ~45% in Baxα(+) cells, and less than 10% in Bax(−) cells. A similar profile was observed for carfilzomib, but the differences for epoxomicin and ixazomib were far less significant. These results indicate that, in colon cancer cells with a similar genetic background, loss of Bax results in lower sensitivity to proteasome inhibitors, while expression of BaxΔ2 could differentially sensitize the cells, especially to bortezomib and carfilzomib.

In the process of analyzing the cellular behaviors and mechanisms underlying proteasome inhibitor-induced cell death in BaxΔ2(+) cells, we noticed that all inhibitors tested also induced morphological changes like those observed with MG132 (Fig. 1C). Bortezomib and
carfilzomib (20 nM each) treated cells had more accumulation of vacuole-like structures in BaxΔ2(+) cells than in the other two sublines, while epoxomicin (20 nM) and ixazomib (100 nM) presented similar levels of vacuole-like structures in all three sublines (Fig. 3A). We wondered whether these vacuole-like structures could be autophagosomes. To test this, BaxΔ2(+) cells were stained with antibodies against BaxΔ2 and autophagy marker LC3B. We found that BaxΔ2 aggregates and moderate LC3B positive staining could be detected in many cells, but none of them was colocalized with the vacuole-like structures (Fig. 3B). Interestingly, the presence of vacuole-like structures appears consistent with the cell viability (Figs. 2 and 3), in which bortezomib and carfilzomib caused more vacuoles and higher percentage of cell death in BaxΔ2-positive cells.

We have previously shown that BaxΔ2 induces cell death through activation of caspase 8 [23,28]. To determine whether the proteasome inhibitor-induced cell death could also be through BaxΔ2-mediated activation of caspase 8, BaxΔ2(+) cells were treated with proteasome inhibitors for 24 h, and analyzed for BaxΔ2 expression and caspase 8 activation by immunoblotting. When compared with the control group (DMSO), all proteasome inhibitors induced accumulation of BaxΔ2, especially bortezomib (Fig. 4A). Of note, the accumulation of BaxΔ2 appears to be specific, as the levels of Bcl-2 and actin remained unaltered. Regarding caspase 8, cleaved fragments (P41 and P18) could be detected for all proteasome inhibitors (Fig. 4B), including MG132, which is known to directly activate caspase 8 even in the absence of Bax [4]. Furthermore, based on the caspase inhibition assay, cell death appeared to be caspase 8-dependent, as it could be significantly blocked by caspase 8 inhibitor, as well as its downstream caspase 3 inhibitor, but not by mitochondrial pathway caspase 9 inhibitor (Fig. 4C). Caspase 12 inhibitor also appeared to be effective, consistent with the fact that bortezomib mediates endoplasmic reticulum stress [29,30]. The results from the inhibition of caspase 6 could be explained by the fact that caspase 6 is known to be activated by caspase 3 [31]. These results together suggest that bortezomib-induced cell death in BaxΔ2(+) cells is, to a significant extent, through activation of caspase 8.

Finally, to determine whether the BaxΔ2-mediated sensitization to proteasome inhibitors observed in HCT116 cells would also occur in other colorectal cancer cell lines, we tested LS174T cells (G7/G7), which express BaxΔ2, and SW1116 cells (G8/G8), which express Baxα [20]. Both cell lines were treated with a range of concentrations of bortezomib. The effective dose analysis showed that, although the EC50 values for both cell lines were similar, the levels of cell death at EC95 were significantly different (61.86% for LS174T, 38.51% for SW1116). Statistically significant differences between these two cell lines start at 20 nM (Fig. 4D and E). These results indicate that BaxΔ2-mediated sensitization of colorectal cancer cells to proteasome inhibitors could have a broad implication, rather than being cell line specific.

4. Discussion

Proteasome inhibitors have been extensively studied for cancer therapy. Some are FDA approved for the treatment of hemopoietic malignancies, while several are being tested for the treatment of solid tumors [5–7,16]. In this study, we demonstrated that the pro-apoptotic
Bax isoform, BaxΔ2, can sensitize colorectal cancer cells to proteasome inhibitor-based therapeutics. All proteasome inhibitors tested promote accumulation of BaxΔ2, without altering either Baxα or Bcl-2 protein levels (Figs. 1D and 4A). However, only bortezomib and carfilzomib showed higher differential killing at low concentration in BaxΔ2(+) cells (Fig. 2). Furthermore, bortezomib-induced cell death in BaxΔ2(+) cells is predominantly dependent on the caspase 8/3 pathway (Fig. 4), consistent with our previous findings [20,23,28]. BaxΔ2-mediated sensitization of colon cancer cells is not cell line-specific, as a similar behavior was also observed in other colorectal cancer cell lines (Fig. 4). Our results suggest that the presence of BaxΔ2 can sensitize cancer cells to proteasome inhibitors, highlighting their potential to treat solid tumors, like colorectal cancer.

During the study, we noted that the cell death induced by these inhibitors appears morphologically different from conventional apoptosis. These cells swelled and accumulated cytosolic vacuole-like bodies before rounding up and blebbing (Fig. 3). Furthermore, the amount of the vacuoles appears consistent with the potency of the inhibitors, especially for bortezomib and carfilzomib. One possible explanation for the formation of these vacuole is from the process of autophagy, but our results showed that neither LC3B nor BaxΔ2 were colocalized with the vacuoles. The results from many other studies regarding proteasome inhibitor-mediated autophagy have often been controversial and inconsistent [29,32–34], therefore the association between proteasome inhibitors and autophagy still remains to be further explored.

It is worth mentioning that the cell death induced by bortezomib seems to be not solely caspase-dependent. There is still ~20% of cell death that cannot be inhibited by individual caspase inhibitors (Fig. 4C). This is consistent with our previous finding that ectopic expression of BaxΔ2 results in formation large cytosolic aggregates, which serve as platform for recruiting caspase 8 [20,23,28]. Here, we were able to detect BaxΔ2 aggregates in cells treated with the proteasome inhibitors. Therefore, it is possible that BaxΔ2 accumulation and aggregation lead to direct cytotoxicity through other types of cell death.

High microsatellite instability (MSI-H) is a hallmark of many solid tumors, such as endometrial, stomach, and colorectal cancers. In general, MSI-H tumors are resistant to conventional chemotherapeutic drugs, like 5-Fluorouracil. Contrary to that trend, in a significant number of cases, tumors with mutations in the Bax microsatellite show better prognosis [35–37]. We have previously shown that BaxΔ2(+) cancer cells are more sensitive to certain chemotherapeutic agents [28] and here, we demonstrated that BaxΔ2 can also sensitize cancer cells to proteasome inhibitors, such as bortezomib and carfilzomib. Although the detailed underlying mechanisms remains to be further explored, the cumulative results from our BaxΔ2 studies imply that BaxΔ2 might be a useful biomarker for selection of personalized chemotherapeutics for cancer treatments.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

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Fig. 1. MG132 blocks BaxΔ2 degradation and moderately promotes cell death
A. Schematic representation of the generation of single cell-derived sublines: BaxΔ2(+), G7/G7 microsatellite expressing BaxΔ2; Baxα(+), G8/G7 expressing Baxα; Bax(−), G8/G7 expressing no Bax protein. B. Growth rate analysis for HCT116 sublines. C. Phase contrast imaging of the three sublines treated with or without MG132 (10 μM) for 24 h. Scale bar is 10 μm. D. Immunoblotting of sublines treated with or without MG132 for 24 h using antibodies against BaxΔ2, Baxα, Bcl-2, and actin. E. Cell Death Assay of the sublines treated with MG132 for 24 h and 48 h. DMSO was used as control.
Fig. 2. BaxΔ2-positive cells are more sensitive to proteasome inhibitors

A. BaxΔ2(+), Baxα(+), and Bax(−) sublines were treated with bortezomib, carfilzomib, epoxomicin, and ixazomib, with a concentration range of 0–200 nM for 48 h. Results from the cell viability assay were fitted to dose-response sigmoid curves. B. Analysis from (A) for EC50, EC95, and the EC95 cell death values for all proteasome inhibitors in each subline.
Fig. 3. BaxΔ2-positive cells have high accumulation of vacuole-like bodies when treated with proteasome inhibitors

A. BaxΔ2(+), Baxα(+), and Bax(−) sublines were treated with bortezomib (Bort, 20 nM), carfilzomib (Carf, 20 nM), epoxomicin (Epox, 20 nM), and ixazomib (Ixaz, 100 nM) for 24 h and imaged with phase contrast microscopy. DMSO was used as control. B. BaxΔ2(+) cells were treated with bortezomib and carfilzomib (20 nM each) for 24 h, stained with antibodies against BaxΔ2 (green) and LC3B (red), and imaged using a fluorescence and bright-field microscope. DAPI nuclear staining (blue). Scale bars are 10 μm. (For
interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
Fig. 4. Proteasome inhibitor-induced cell death in BaxΔ2-positive cells is caspase 8/3-dependent and not cell line-specific

A and B. Immunoblotting of cell lysates from BaxΔ2(+) cells treated with MG132 (10 μM), bortezomib (20 nM), carfilzomib (20 nM), epoxomicin (20 nM), and ixazomib (100 nM) for 24 h, using antibodies against BaxΔ2, Bcl-2, and actin (A), or cleaved caspase 8 (B). C. Cell death assay of BaxΔ2(+) cells treated with bortezomib (20 nM) for 24–48 h in the absence or presence of inhibitors for caspase 3 (C3I, 50 μM), caspase 6 (C6I, 50 μM), caspase 8 (C8I, 50 μM), caspase 9 (C9I, 50 μM), and caspase 12 (C12I, 50 μM). D. Human colon cancer LS174T and SW1116 cell lines were treated with bortezomib (0–200 nM) for 48 h. Results from cell death assay were fitted to dose-response sigmoid curves. E. Analysis from (D) for EC50, EC95, and the EC95 cell death values in each cell line.