Eeyarestatin causes cervical cancer cell sensitization to bortezomib treatment by augmenting ER stress and CHOP expression

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HIGHLIGHTS

► Eeyarestatin enhances the cytotoxic effect of bortezomib in cervical cancer cells.
► Bortezomib and eeyarestatin induce a mitochondria-independent cell death mechanism.
► Bortezomib and eeyarestatin cause upregulation of the pro-apoptotic protein CHOP.

ARTICLE INFO

Article history:
Received 11 July 2012
Accepted 20 October 2012
Available online 26 October 2012

Keywords:
Eeyarestatin
Bortezomib
Cervical cancer
Endoplasmic reticulum stress
Heat shock protein

ABSTRACT

Objective. The proteasome inhibitor bortezomib is currently being tested in clinical trials against refractory cervical cancer. However, high doses of bortezomib are associated with adverse effects, which may lead to treatment abrogation or to the use of lower, ineffective doses. We investigated combination drug treatments that could enhance the efficacy of low bortezomib concentrations on cervical cancer cells.

Methods. The cervical cancer cell lines CaSki, HeLa and SW756 were treated with various combinations of bortezomib and eeyarestatin. Treated cells were analyzed for cell viability by clonal assays and the MTT assay, and for expression of pro-apoptotic proteins and cell stress markers by immunofluorescence, immunoblots and RT-PCR analysis.

Results. Cotreatment of bortezomib with eeyarestatin markedly enhanced cell death in cervical cancer cells, allowing reduction of the bortezomib concentration necessary for efficient cell death to as low as 5 ng/ml. Combination of bortezomib with eeyarestatin resulted in a massive induction of the endoplasmic reticulum stress reaction, small and large heat shock protein activation, autophagy, and upregulation of pro-apoptotic CHOP.

Conclusion. Eeyarestatin is a small molecule recently shown to cause endoplasmic reticulum stress by inhibiting the endoplasmic reticulum-associated degradation pathway, which directs misfolded cytotoxic proteins to proteasomal degradation. Concomitant inhibition of both pathways markedly enhances the efficacy of bortezomib against cervical cancer cells and thus may be applied to reduce the bortezomib dosage required for efficient cervical cancer treatment.

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Introduction

Forced pharmacological induction of the endoplasmic reticulum (ER) stress reaction or the unfolded protein response (UPR) is a recently developed strategy to induce cell death in cancer cells. Bortezomib, HSP90 inhibitors and nelfinavir are examples of clinically applied drugs that successfully utilize this mechanism [1]. Proteasome inhibition with drugs like bortezomib causes the accumulation of poly-ubiquitinated proteins within a cell that disturb cellular functions, and eventually result in cytotoxic proteins and protein aggregates [2–4]. To prevent formation of these detrimental proteins in the absence of a functional proteasomal system, cellular heat shock proteins become activated to interfere with protein aggregation or to support protein degradation [5,6]. Further, the mechanism of autophagy can be induced to mediate bulk protein degradation [7,8]. Other drugs that disturb intracellular protein homeostasis, such as nelfinavir, cause an accumulation of misfolded proteins within the ER, which must be degraded by the autophagy-related mechanism of reticulophagy [9,10] or by a retranslocation process of misfolded ER proteins to cytosolic proteasomes via the ER-associated degradation mechanism (ERAD) [2,11]. ERAD inhibitors such as eeyarestatin are known to induce ER stress by themselves and to enhance the cytotoxic activity of bortezomib in leukemia cells [12]. Based on these findings, we investigated whether eeyarestatin could be applied in the treatment of solid cancer, preferentially against cervical cancer, and whether eeyarestatin could be used to sensitize cervical cancer cells to the
cytotoxic effects of nelfinavir and bortezomib. Both nelfinavir and bortezomib are highly active even on chemoresistant ovarian [13] and cervical [14] cancer cells, and are currently being tested in clinical studies on ovarian [15,16] and cervical cancer patients (NCT00106262; NCT00329589; NCT01485731; www.clinicaltrials.gov).

Materials and methods

Cells and cell culture

The cervical adenocarcinoma cell line HeLa (ATCC CCL-2), cervical squamous carcinoma cell line CaSki (ATCC CRL-1550), and cervical squamous cell carcinoma cell line SW756 (ATCC CRL-10302) represent established human cervical cancer cell lines and were purchased from ATCC (LG&C Standards, Wesel, Germany). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics at 37 °C in a humidified atmosphere with 5% CO2. All cell culture reagents were from PAA (Pasching, Austria).

Drugs and drug treatment

Bortezomib was purchased from Selleck Chemicals (Boston, USA). Nelfinavir and eeyarestatin were purchased from Sigma (Munich, Germany). Geldanamycin and 17-AAG were provided by Axxora (Lörrach, Germany). All drugs were kept at −20 °C in stock solutions of 20 mg/ml in DMSO, except bortezomib (1 mg/ml in PBS) and eeyarestatin (5 mg/ml in ethanol).

MTT assay

A total of 5 × 10^5 cells per well were seeded in 96-well cell culture dishes and incubated for 72 h under cell culture conditions. For the MTT assay analysis, 20 μl of an MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma, Germany) stock solution (5 mg/ml PBS) in 200 μl of cell culture medium was added to viable cells and incubated for 1 h under cell culture conditions. The water-insoluble precipitate was dissolved in 100 μl of DMSO and analyzed by an ELISA reader at 595 nm. All experiments were performed in triplicate.

Mitochondrial membrane potential analysis

For analysis of mitochondrial membrane potential, the MitoCapture Mitochondrial Apoptosis Detection Kit (Axxora, Lörrach, Germany) was used according to the manufacturer’s instructions. Cells were stained and photographed as viable cells by means of a Zeiss Axiophot fluorescence microscope (Zeiss, Germany).

Western blot analysis

Cell extracts of cervical cancer cells cultured in cell culture plates were prepared with RIPA-buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% doxycholate, 0.1% SDS) and 20 μg of protein (BioRad Bradford Assay, BioRad, Munich, Germany) were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to PVDF membranes in a BioRad Mini Protein II Cell (BioRad, Munich, Germany) at 1 mA/cm2 membrane in 10% methanol, 192 mM glycine, 25 mM Tris, pH 8.2. Membranes were blocked with 4% non-fat milk powder in PBS-0.05% Tween for 4 h. Primary antibodies were applied in blocking buffer and incubated at room temperature overnight. Antibodies against cyclin D3, LC3B, mcl1, phospho-mcl1, HSP40, HSP90, phospho-HSP27, ubiquitin (monoclonal), and ub-H2A were all purchased from Cell Signaling Technology (NEB, Frankfurt, Germany). Antibodies against BIP (H-129), ATF3 (C-19), ATF4 (C-20) and β-actin (C4) were from Santa Cruz Biotech (Heidelberg, Germany). Antibodies against HSP27 and HSP70 were from LabVision (Thermo Scientific, Dreieich, Germany), and bcl-2 antibodies were from Biozol (Eching, Germany). Secondary, alkaline phosphatase (AP)-coupled antibodies against the corresponding primary antibodies were from Dianova, Hamburg, Germany. AP detection was performed by the chromogenic BCIP/NBT assay (Promega, Mannheim, Germany).

PCR analysis

The PCR-based analysis of CHOP and ATF3 expression and XBP1 splicing was performed as previously described [17]. In brief, 5 × 10^5 HeLa cells were seeded in 6-well cell culture plates and incubated for 16 h with the indicated drugs. RNA preparation and cDNA synthesis was performed with the RNA extraction kit (Macherey-Nagel, Düren, Germany) and MMLV-reverse transcriptase (Promega, Mannheim, Germany), according to the manufacturer’s recommendations.

Immunofluorescence analysis

For immunofluorescence analysis, cells were seeded on glass cover slips and incubated under cell culture conditions with bortezomib or eeyarestatin. Cells were fixed with 5 min with ice-cold methanol and incubated with either a 1:200 dilution of a monoclonal anti-ubiquitin antibody (Cellsignal, Hamburg, Germany), a 1:100 dilution of a monoclonal anti-CHOP antibody (Cellsignal, Hamburg, Germany), or a 1:40 dilution of a monoclonal anti-HSP70 antibody (LabVision, Dreieich, Germany) for 2 h at room temperature. For detection of primary antibodies, 1:400 dilutions of either a Cy3-conjugated or Alexa488-conjugated secondary anti-mouse antibody (Dianova, Hamburg, Germany) were applied.

Statistical analysis

Independent sample group analysis was performed using the non-parametric Mann–Whitney U rank-sum test (SPSS version 20.0; SPSS Inc., Chicago, IL, USA). Values were plotted as mean values (from triplicates) and significance was assumed at p ≤ 0.05 using the two-tailed test. Significant relations between cell cultures treated in the presence or absence of eeyarestatin have been indicated in the figures with an asterisk.

Results

Eeyarestatin sensitizes cervical cancer cells to bortezomib treatment

HeLa cervical cancer cells were treated in cell culture with different eeyarestatin concentrations combined with different concentrations of bortezomib, nelfinavir or HSP90 inhibitors geldanamycin or 17-AAG (17-allylamino-17-demethoxygeldanamycin). After treatment, analysis of the remaining cell viability by an MTT assay indicated that the efficacy of bortezomib, even at concentrations that revealed no apparent effects on cell viability when applied as a single agent (5 ng/ml), was markedly increased by eeyarestatin supplementation (Fig. 1A). By contrast, the efficacy of nelfinavir was not improved by addition of eeyarestatin, whereas a slight cooperative effect was observed between eeyarestatin and the HSP90 inhibitors geldanamycin and 17-AAG (Fig. 1A). To test whether the observed combination effects could also be achieved in other cervical cancer cell lines, HeLa, SW756 and CaSki cells were tested with defined concentrations of eeyarestatin and bortezomib, nelfinavir, or 17-AAG (Fig. 1B). The efficacy of bortezomib was enhanced by eeyarestatin in SW756 and CaSki cells, whereas the combination effect of eeyarestatin with HSP90 inhibitors appeared to be restricted to HeLa cells, since additional application of 17-AAG to eeyarestatin-treated CaSki or SW756 cells did not further reduce cancer cell viability (Fig. 1B). Therefore, further attention was focused on the analysis of the combination of bortezomib and eeyarestatin.
The combination of eeyarestatin with bortezomib enhances ER stress and causes accumulation of poly-ubiquitinated proteins

To analyze the biochemical mechanisms leading to the enhanced efficacy of bortezomib in the presence of eeyarestatin, Western blot analyses of cell extracts from HeLa cells incubated with bortezomib (5 ng/ml) or eeyarestatin (0.75 μg/ml), or with the combination of both, were performed (Fig. 2A). For comparative purposes, eeyarestatin was further tested as a single agent, by applying elevated concentrations (Fig. 2B). The combination of bortezomib and eeyarestatin led to a marked increase in the expression of the ER-resident molecular chaperone BiP/GRP78 (Fig. 2A), indicating specific induction of ER stress by this drug combination. When applied as single agents at 5 ng/ml (bortezomib) or 0.75 μg/ml (eeyarestatin), neither eeyarestatin nor bortezomib induced a pronounced upregulation of BiP (Fig. 2A), but when applied at higher concentrations, massive induction of ER stress was achieved with eeyarestatin only (Fig. 2B). Occurrence of ER stress was associated with cell cycle arrest and induction of autophagy, as indicated by downregulation of cyclin D3 (Fig. 2A/B) and upregulation of autophagy marker LC3B (Fig. 2A/B). Analysis of cytosolic heat shock proteins after treatment with the combination of eeyarestatin and bortezomib further revealed phosphorylation and activation of the small heat shock protein HSP27, and a marked upregulation of the large heat shock protein HSP70 and HSP40, which interacts with HSP70 (Fig. 2A). Interestingly, upregulation of HSP70 was also observed after treatment with 5 ng/ml bortezomib, indicating that proteasome inhibition and enhanced accumulation of misfolded proteins in the cytosol had already occurred under conditions of sub-lethal concentrations of bortezomib, and suggesting that low concentrations of bortezomib may be managed and successfully tolerated, at least transiently, by the cell. Elevated levels of proteasomal target proteins ATF4 and mcl-1 further indicated reduced proteasome activity in the presence of 5 ng/ml bortezomib, although additional pathways such as ER stress induction (ATF4 translation) and protein phosphorylation (mcl-1 stabilization) may also contribute to enhanced ATF4 and mcl-1 expression. Both bortezomib and eeyarestatin have been shown to lead to an accumulation of poly-ubiquitinated proteins that can be detected by Western blot analysis [18,19]. Immunoblot analysis performed on the cell extracts presented in Fig. 2A/B confirmed
Combination of eeyarestatin with bortezomib enhances ER stress and the accumulation of poly-ubiquitinated proteins. A) HeLa cells were treated for 24 h with 0.75 μg/ml eeyarestatin (Ee) and 5 ng/ml bortezomib (B) either alone or in combination (Ee/B), and cell extracts were analyzed by Western blot analysis for the expression of cell stress-associated proteins. B) HeLa cells were treated for 24 h with the indicated concentrations of eeyarestatin (Ee) and cell extracts were analyzed by Western blot analysis. C) Cell extracts generated as described in subpanels (A) and (B) were additionally subjected to Western blot analysis with anti-ubiquitin and anti-ubiquitinyl-H2A (histone 2A) antibodies. In addition, cell lysates of HeLa cells treated for 8, 16, and 24 h with 5 μg/ml eeyarestatin were generated and probed with anti-ubiquitin antibodies by Western blot analysis.

**Mitochondria-independent cell death and CHOP upregulation induced by bortezomib/eeyarestatin**

Upregulation of cytoprotective mitochondrial membrane protein mcl-1 by bortezomib and by the combination of bortezomib with eeyarestatin (Fig. 2A) indicates a possible mechanism of mitochondrial membrane stabilization that could counteract mitochondria-dependent apoptosis often caused by chemotherapeutics. In fact, analysis of the mitochondrial membrane potential in viable HeLa cells treated with low concentrations of eeyarestatin and bortezomib was performed (Fig. 6). Long-term treatment of HeLa and CaSki cells with bortezomib at low concentrations revealed no pronounced effects on cell clone development (Fig. 6). By contrast, the combination of low concentrations of bortezomib with eeyarestatin efficiently suppressed clonal growth of cervical cancer cells (Fig. 6).

**Discussion**

Bortezomib is a highly effective drug against certain types of leukemia [21], and is currently being tested in clinical studies of various types of solid cancer, including cervical cancer [NCT00106262; NCT00329589]. However, bortezomib treatment is associated with the occurrence of severe adverse effects [22], driving the need for dose reduction. Unfortunately, reduced bortezomib doses yield dissatisfying...
treatment efficiency or development of drug resistance. In this report, eeyarestatin treatment is shown to sensitize cervical cancer cells to lower bortezomib concentrations that displayed no anti-tumoral effects when used alone. The combination of eeyarestatin with bortezomib primarily enhanced the endoplasmic reticulum stress reaction, as revealed by BiP and CHOP expression and XBP1 splicing analysis.

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**Fig. 3.** Accumulation of poly-ubiquitinated protein aggregates in bortezomib-treated HeLa cells. HeLa cells grown on glass cover slips were treated for 24 h with the indicated concentrations of bortezomib and eeyarestatin, and analyzed by immunofluorescence analysis by using a monoclonal anti-ubiquitin antibody, followed by a fluorescent Alexa488-conjugated anti-mouse antibody.

**Fig. 4.** Stabilization of the mitochondrial membrane potential by bortezomib/eeyarestatin combination. HeLa cells were treated for 24 h with combined bortezomib and eeyarestatin (5 ng/ml BTZ plus 0.75 μg/ml Ee), and analyzed for mitochondrial membrane polarization using the MitoCapture kit (Alexis, Lörrach, Germany). Yellow-red fluorescence indicates accumulation of staining dye in mitochondria with intact membrane potential; green fluorescence is derived from residual monomeric dye. Staining was performed on viable cells.
Eeyarestatin [23] was recently shown to induce the ER stress reaction by inhibiting the activity of the ER membrane-associated ATPase p97/VCP and Sec61 complex [12,24–26]. The Sec61 complex translocates nascent proteins into the ER, and may also take part in retranslocating misfolded, and subsequently poly-ubiquitinated proteins, from the ER back into the cytosol [25,27]. P97/VCP (valosin-containing protein) is associated with the translocation machinery that dislocates misfolded proteins into the cytosol [24,27]. Accordingly, eeyarestatin may interfere with the ERAD pathway by different, though closely related mechanisms. In addition to the induction of the ER stress reaction, a massive upregulation of heat shock proteins in eeyarestatin- and eeyarestatin/bortezomib-treated cervical cancer cells was observed. The combination of bortezomib with eeyarestatin markedly enhanced expression of the large heat shock protein HSP70, and HSP70-interacting HSP40. Phosphorylation of HSP27 further indicates activation of this small and abundant heat shock protein [28] from its cytosolic precursor pool. These observations, together with the described accumulation of poly-ubiquitinated proteins and paranuclear protein aggregates, indicate accumulation of misfolded proteins within the cytosol, whereas upregulation of ER-resident chaperone BiP indicates a concomitant accumulation of misfolded proteins within the ER. Thus, the combination of eeyarestatin and bortezomib causes a multi-compartmental accumulation of potentially cytotoxic proteins and protein aggregates in cancer cells, eventually leading to cell death. Interestingly, low concentrations of bortezomib (5 ng/ml) caused accumulation of poly-ubiquitinated proteins and enhanced HSP70 expression without affecting BiP expression (Fig. 2), suggesting that co-induction of the ER stress reaction by supplementation with eeyarestatin is necessary to induce efficient cancer cell death when low concentrations of bortezomib are applied. Under short-term conditions, cervical cancer cells are able to cope with low concentrations of bortezomib by depositing potentially cytotoxic proteins into paranuclear protein aggregates [20], whereas cancer cells appear to be poorly capable of coping with even transient physiological disturbances in the homeostasis of the endoplasmic reticulum system. Therefore, induction of the ER stress reaction can be regarded as the main mechanism of cancer cell death induced by treatment with the combination of eeyarestatin and bortezomib.

The exact mechanism of eeyarestatin-induced cell death is still not precisely understood, but is likely to rely on the same mechanisms previously described for apoptotic pathways identified for the general ER stress reaction [29,30]. For example, Wang et al. [12] previously identified upregulation of noxa as a key element in eeyarestatin-induced apoptosis in leukemia cells. In cervical cancer cells, the pro-apoptotic transcription factor CHOP was observed to be upregulated by eeyarestatin. CHOP and noxa are both known key effectors of ER stress-mediated apoptosis [29,30]. The observed upregulation of the anti-apoptotic mitochondrial protein mcl-1 appears to be contradictory to the pro-apoptotic effect of eeyarestatin and bortezomib, but was recently described to be mediated by other ER stress-inducing drugs as well [31], leading to a mitochondria-independent cell death mechanism that could be exploited for its use against chemo-resistant cervical cancer cells.

The applicability of bortezomib is often limited by the occurrence of adverse effects or resistance mechanisms [32]. Drug combination treatments are often applied to reduce the overall drug concentration and to avoid development of drug resistance. The combination of bortezomib with eeyarestatin may thus be suited to overcome the clinical limitations.
of bortezomib. However, it cannot be excluded that the combination of both drugs may induce other toxicities or may be associated with independent adverse effects. These have to be carefully monitored in cases that eeyarestatin is combined with bortezomib in clinical trials on refractory cervical cancer or other solid cancer entities.

In summary, our data indicate that eeyarestatin and bortezomib, both effective drugs against cervical cancer cells when applied as single agents, can be markedly enhanced in their efficacy when administered in combination. Bortezomib is being tested in clinical studies of cervical cancer patients, and eeyarestatin has been shown to be applicable in vivo, reducing the growth of cancer xenografts in mice [33]. Thus, clinical studies with a combination of both drugs are warranted, including studies on cervical cancer patients.

Conflict of interest statement
No conflict of interest.

Fig. 6. Long-term effect of eeyarestatin and bortezomib. A total of 250 HeLa (A) and CaSki cells (B) were seeded in six-well cell culture plates and incubated for 14 days in the presence of the indicated eeyarestatin and bortezomib concentrations. Cell clones were stained with crystal violet.

Figures and Images

References


