Effect of bortezomib in combination with cisplatin and 5-fluorouracil on 4T1 breast cancer cells

AZMI YERLIKAYA1, SAYIT ALTIKAT2, REYHAN IRMAK3, FATMA ZEHRA CAVGA3, SULTAN AYSUN KOCACAN4 and IHSAN BOYACI5

1Department of Medical Biology, Faculty of Medicine, Dumlupınar University, Kutahya 43100; 2Department of Biochemistry, Faculty of Medicine, Dumlupınar University, Kutahya 43100; 3Department of Biology, Dumlupınar University, Kutahya 43100; 4Kutahya Evliya Çelebi Education and Research Hospital, Department of Radiation Oncology, Dumlupınar University, Kutahya 43100; 5Istanbul Medipol University, Vatan Clinic, Istanbul, 34214, Turkey

Received March 1, 2013; Accepted April 29, 2013

DOI: 10.3892/mmr.2013.1466

Abstract. Bortezomib is a highly selective and reversible inhibitor of the 26S proteasome. It has been approved for the treatment of patients with relapsed and refractory multiple myeloma. A number of studies have been conducted to evaluate the activity and safety of bortezomib either alone or in combination with several cytotoxic agents and radiation. In the current study, the efficacy of bortezomib alone or in combination with cisplatin and 5-fluorouracil was evaluated in 4T1 breast cancer cells, a highly metastatic murine cancer cell line. Using MTT assay, IC50 values of cisplatin and 5-fluorouracil were determined to be 14.2 and 8.9 µM for cisplatin and 5-fluorouracil, respectively. The effects of different concentrations of cisplatin and 5-fluorouracil in combination with two different concentrations of bortezomib were examined in the 4T1 cells. Statistically significant differences were found when 1 or 5 µM cisplatin was combined with 10 or 50 nM bortezomib. Similarly, 1 µM 5-fluorouracil or 5 µM 5-fluorouracil in combination with 10 nM bortezomib caused significant cell death as compared to treatment with single agents. However, 1 or 5 µM 5-fluorouracil did not potentiate the effects of higher concentrations of bortezomib (50 nM). The effect of the combination of cisplatin, 5-fluorouracil and bortezomib was determined by soft agar assay. It was confirmed that a combination of cisplatin and bortezomib was more effective than each drug as a monotherapy. Therefore, the combination of cisplatin and bortezomib should be tested further in clinical settings.

Introduction

Bortezomib is a novel and highly selective proteasome inhibitor. It is a dipeptide boronic acid inhibitor, selectively blocking the chymotrypsin-related activity of the proteasome (1). Data from clinical studies showed that the 26S proteasome inhibitor bortezomib has therapeutic potential against various types of cancer including breast, colorectal, lymphoma, multiple myeloma, ovarian, pancreatic, prostate and squamous cell carcinomas (2). Findings of previous studies have shown that the proteasome inhibitor bortezomib exhibits antitumor activities by inducing the accumulation of pro-apoptotic proteins or cell cycle inhibitors (such as Phorbol-12-myristate-13-acetate-induced protein 1, BH3 interacting-domain death agonist, Bcl-2-associated X protein, p53, Bcl-2-associated death promoter and cyclin-dependent kinase inhibitors p21 and p27) depending on the cell line used (2-5). In addition, inhibition of the proteasome by bortezomib results in inhibition of the activation of nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) by preventing the degradation of IκB (an inhibitor of NFκB). NFκB is an important transcription factor for cell survival (6,7). Therefore, in addition to the stabilization of the above-mentioned pro-apoptotic proteins, bortezomib promotes the apoptosis of cancer cells through inhibition of the activation of NFκB.

In a number of preclinical murine tumor models, bortezomib was identified to exhibit promising antitumor activities as a single agent. LeBlanc et al (8) examined the efficacy, toxicity and in vivo mechanism of action of bortezomib using a human plasmacytoma xenograft mouse model. They observed that the median overall survival was significantly prolonged compared with controls. Their results showed that bortezomib has significant in vivo antimyeloma activity at well-tolerated doses in a murine model. The antitumor activity of bortezomib in combination with other therapies has also been evaluated. For example, Denlinger et al (9) observed that a combined treatment with histone deacetylase inhibitor suberoylanilide hydroxamic acid and bortezomib induced greater reactive oxygen species...
generation and more apoptosis than either drug alone. Teicher et al (10) also evaluated the efficacy of bortezomib in combination with 5-fluorouracil, cisplatin, taxol and adriamycin. Results of that study showed that bortezomib produced primarily additive tumor growth delays against the EMT-6/parent mouse mammary carcinoma grown as a solid tumor subcutaneously in the flanks of female Balb/c mice. The combinations were also highly effective against metastasis to the lungs.

The purpose of this study was to determine the cytotoxic effects of bortezomib, cisplatin and 5-fluorouracil as monotherapies or in combination in a highly metastatic and p53-null 4T1 breast cancer cell line. The results obtained demonstrated that additional studies should be performed on the combination of bortezomib and cisplatin in highly aggressive and metastatic cancers bearing mutated p53 gene.

Materials and methods

Materials. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI-1640 cell culture media, fetal bovine serum (FBS), trypsin and penicillin/streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Agarose was from Life Sciences Advanced Technologies, Inc. (St. Petersburg, FL, USA). The Stericup vacuum filtration system was obtained from Millipore Inc., (St. Quentin, France).

Cell culture maintenance. 4T1 breast cancer cells were cultured in RPMI-1640 with 10% FBS, 10 mM Heps, 4.5 g/l glucose, 1 mM sodium pyruvate, 0.15% sodium bicarbonate, 100 µg/ml streptomycin and 100 U/ml penicillin. Cells were incubated at 37°C with 5% CO₂. Stock cultures were grown in 25 cm² corning plates and experimental cultures were plated in 60x15 or 35x10 mm corning plates.

IC₅₀ determination. Fifty thousand cells were seeded in 35x10 mm plates. After 48 h of plating, cells were treated with various doses of cisplatin or 5-fluorouracil (0.01, 0.1, 0.5, 1, 10, 50, 100 or 200 µM) for 24 h. The cells were incubated for 4 h with RPMI-1640 media containing 0.5% FBS + 0.5 mg/ml MTT at 37°C with 5% CO₂ to determine the number of surviving cells. After removing the medium containing MTT, the cells were lysed with 3% SDS and 40 mM HCl/isopropanol for 15 min. The lysate was pipetted well to dissolve the MTT-formazan crystals completely and centrifuged at 11,000 x g for 5 min. Absorbance at 570 nm was recorded with a Bio-Rad (Hercules, CA, USA) Smartspec Plus spectrophotometer (5,11). The IC₅₀ values of each agent were determined with a Prism 3.03 program.

Soft agar assay. Similarly, 100,000 cells were seeded in 60x15 mm petri dishes. At logarithmic generation, the cells were treated with 10 nM bortezomib, 1 µM cisplatin, 1 µM 5-fluorouracil or in combination (1 µM cisplatin + 10 nM bortezomib or 1 µM fluorouracil + 10 nM bortezomib) for 24 h. After the drug treatment, the cells were counted and 0.75 ml of 2X RPMI-1640 + 20% FBS (containing 5,000 cells) was mixed with 0.75 ml of 0.7% agarose in a tube for each plate. The mixture was added to the base agar containing 0.5% agar + 1X RPMI-1640 + 10% FBS. Plates were incubated for 3 weeks at 37°C in a humidified incubator and fed twice a week (12). Plates were then stained with 0.1% crystal violet in 10% ethanol for 1 h and washed extensively with PBS until colonies become apparent for counting.

Statistical analysis. Data were analyzed and presented using GraphPad Prism 3.03 program. One-way ANOVA with the Bonferroni post-test or two-tailed Student's t-test was used to evaluate the statistical significance. P<0.05 was considered to indicate a statistically significant difference.

Results

Determination of IC₅₀ values. In previous studies, we determined the p53 status of 4T1 breast cancer cells and verified that they are p53-deficient by examining the induction of both p53 and its downstream target p21 in response to various concentrations of the proteasome inhibitor bortezomib (5,13). Additionally, we identified the IC₅₀ value of bortezomib in 4T1 cells as 71 nM, indicating that these p53-deficient cells are sensitive to the inhibition of the proteasome (5). In the present study, we first determined whether the chemotherapeutic agents (cisplatin and 5-fluorouracil) were also cytotoxic to the highly metastatic and p53-null 4T1 breast cancer cells. Results showed that these drugs are highly cytotoxic to 4T1 cells (Fig. 1). Using these data, we calculated the IC₅₀ values of cisplatin and 5-fluorouracil to be 14.2 and 8.9 µM.

Effect of bortezomib and cisplatin combination on cell viability. The effect of the combination of each drug with bortezomib was investigated. Based on the IC₅₀ values, the cells were first treated with two different doses of cisplatin and bortezomib as well as the combinations of each dose tested. As shown in Fig. 2, 1 µM cisplatin treatment did not significantly reduce cell survival (P>0.05 vs. control) after 24 h of treatment and 5 µM treatment of cisplatin caused a 31% reduction in the cell number (P<0.001 vs. control).
By contrast, 10 nM bortezomib reduced the cell number by ~40% (P<0.001 vs. control), and 50 nM bortezomib resulted in a 66% reduction in the cell viability (P<0.001 vs. control), altogether indicating that bortezomib is a more cytotoxic agent than cisplatin in this highly metastatic breast carcinoma cell line. When cells were treated with 10 nM bortezomib + 1 µM cisplatin, a statistically significant cell death was observed as compared with single drug treatments (for example, P<0.05 vs. 10 nM treated bortezomib). When a higher concentration (5 µM) of cisplatin was combined with 10 nM bortezomib, again more cytotoxicity was detected (P<0.001 vs. 10 nM bortezomib). In cells treated with

By contrast, 10 nM bortezomib reduced the cell number by ~40% (P<0.001 vs. control), and 50 nM bortezomib resulted in a 66% reduction in the cell viability (P<0.001 vs. control), altogether indicating that bortezomib is a more cytotoxic agent than cisplatin in this highly metastatic breast carcinoma cell line. When cells were treated with 10 nM bortezomib + 1 µM cisplatin, a statistically significant cell death was observed as compared with single drug treatments (for example, P<0.05 vs. 10 nM treated bortezomib). When a higher concentration (5 µM) of cisplatin was combined with 10 nM bortezomib, again more cytotoxicity was detected (P<0.001 vs. 10 nM bortezomib). In cells treated with

50 nM bortezomib + 1 µM cisplatin, no significant results were observed when compared with 50 nM bortezomib as a monotherapy (P>0.05). However, when cells were treated
with 50 nM + 5 μM cisplatin, a statistically significant result was obtained as compared with 50 nM bortezomib-treated cells (P<0.01) (Fig. 2).

Effect of combination of bortezomib and 5-fluorouracil on cell viability. As can be seen in Fig. 3, 1 or 5 μM 5-fluorouracil caused significant cell death as compared to the control group (P<0.001 in both cases). However, a 10 or 50 nM concentration of bortezomib was more effective compared with 5-fluorouracil. Results showed that 1 μM 5-fluorouracil potentiated the cytotoxicity of 10 nM bortezomib when used in combination (P<0.001 vs. 10 nM bortezomib). Similarly, 10 nM bortezomib + 5 μM 5-fluorouracil was also more cytotoxic (P<0.001 vs. 10 nM bortezomib). However, the effect of 50 nM bortezomib + 1 μM 5-fluorouracil was not statistically different compared with the effect of 50 nM bortezomib as a monotherapy (P>0.05). Similarly, 50 nM bortezomib + 5 μM 5-fluorouracil did not produce significant cell death as compared with 50 nM bortezomib-treated group (P>0.05).

Soft agar assay for the determination of cytotoxicity. The effect of the combination of cisplatin or 5-fluorouracil with bortezomib was examined using soft agar assay. Of note, in contrast to the MTT assay results, cells formed a low number of colonies when treated with 1 μM cisplatin or 1 μM 5-fluorouracil alone (Fig. 4A). The colony numbers were lower than those in the plate treated with bortezomib alone. Additionally, the number of colonies in the 10 nM bortezomib + 1 μM cisplatin-treated group was significantly lower than that of colonies in the plate treated with 1 μM cisplatin alone (P=0.0317), indicating that cisplatin potentiated the effect of bortezomib (Fig. 4B). By contrast, the number of colonies in the 10 nM bortezomib + 1 μM 5-fluorouracil-treated plate was not significantly lower than that in the 1 μM 5-fluorouracil alone-treated plate (P=0.1271), a result consistent with those obtained with the MTT assay.

Discussion

The IC₅₀ value of cisplatin, and 5-fluorouracil was previously determined as 43.5 and 10 μM, respectively, in MCF cells (14-16), which are comparable to the values obtained in this study with the 4T1 breast cancer cells. Since we have previously shown that 4T1 cells are p53-null cells (5), the present results indicate that these anticancer chemotherapeutic agents are able to induce cell death in 4T1 cells in a p53-independent manner. In terms of the combination treatments, the results have shown that cisplatin + bortezomib is more potent than the combination of bortezomib with 5-fluorouracil since 5 μM cisplatin potentiated the cytotoxic effects of 50 nM bortezomib. By contrast, 5 μM 5-fluorouracil did not affect the degree of toxicity of 50 nM bortezomib significantly. Cisplatin is a widely used anticancer agent. To the best of our knowledge, combination of the cisplatin and bortezomib has not been previously tested in this metastatic 4T1 breast cancer cell line, a cell line commonly used in animal tumor models. In addition, the effect of bortezomib and cisplatin combination has not been widely tested in other breast cancer cells. However, in a study with the EMT-6 murine mammary carcinoma cell line, Teicher et al (10) showed that bortezomib (also known as Velcade or PS-341) increased the tumor cell killing of cisplatin. Based on the results of the MTT assay, we expected more colonies in cisplatin- or 5-fluorouracil alone-treated plates (with ~14 or 9 times lower concentrations of cisplatin or 5-fluorouracil than the IC₅₀ values, respectively) in soft agar assay. Of note, we observed only a 24% colony formation in cisplatin-treated plates and 17% colony formation in 5-fluorouracil-treated plates, which may be due to the irreversibility of cisplatin or 5-fluorouracil to the cell targets. In 10 nM bortezomib-treated plates, we observed 72% colony formation, which was consistent with the results obtained with the MTT assay. Since bortezomib is known to be a highly selective and reversible inhibitor of the 26S proteasome (17), the discrepancies between the number of colonies in soft agar assay and MTT results obtained for cisplatin and 5-fluorouracil may therefore be explained by the reversibility of the drugs. Similar results may be obtained with other irreversible drugs and the assays used may be taken into account when comparing the efficacy of the drugs.

The data presented in this study suggest that p53-null 4T1 cells can be used to delineate the mechanism of p53-independent induction of apoptosis by cisplatin and 5-fluorouracil and bortezomib. The studies also suggest that the combination of cisplatin + bortezomib is more effective and that additional investigations should be conducted in clinical settings.

Acknowledgements

We would like to thank Prof. Dr. Engin Ulukaya (Uludağ University, Bursa, Turkey) for providing bortezomib.

References