Antitumor effects and drug interactions of the proteasome inhibitor bortezomib (PS341) in gastric cancer cells

Takeo Fujita[a], Hiroyoshi Doihara[a], Kazuhiro Washio[b], Hideo Ino[a], Masakazu Murakami[a], Minoru Naito[a] and Nobuyoshi Shimizu[a]

The proteasome inhibitor bortezomib (PS341) inhibits the function of the 26S proteasome and has been extensively investigated in the clinical setting of hematologic malignancies. Remarkable efficacy has been reported in the treatment of multiple myeloma, but there have been few studies of its use in the treatment of gastrointestinal malignancy, especially gastric cancer. Here, we demonstrate its efficacy, both alone and in combination with other cytotoxic agents, in gastric cancer cell lines. The human gastric cancer cell lines AZ521, MKN45 and NUGC3 were used as experimental models. Bortezomib produced significant growth inhibition in these cells (mean IC_{50} values: 1.26, 9.44 and 8.63 μmol/l, respectively) and was also observed to decrease the activity of the extracellular signal-regulated kinase 1/2 and Akt signal pathways, increasing the accumulation of p21. Cell-cycle analysis revealed that a low concentration of bortezomib (10–100 nmol/l) increased accumulation in the G_1 phase. Moreover, bortezomib showed synergistic growth inhibition in combination with the conventional cytotoxic agents 5-fluorouracil, paclitaxel, doxorubicin and SN-38, and also downregulates the activity of nuclear factor -κB, which is induced by these agents. Our results demonstrate that bortezomib could be an effective antitumor agent in the treatment of gastric cancer, both as single-agent therapy and in combination with conventional chemotherapeutic agents. *Anti-Cancer Drugs* 18:677–686 © 2007 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2007, **18**:677–686

Keywords: Akt, bortezomib (PS341, Velcade), ERK, gastric cancer, nuclear factor-κB

[a]Department of Cancer and Thoracic Surgery, Okayama University School of Medicine and [b]Department of Surgery, Okayama Rosai Hospital, Okayama, Japan

Correspondence to Hiroyoshi Doihara, MD/PhD, Department of Cancer and Thoracic Surgery, Okayama University School of Medicine, 2-5-1 Shikata-cho, Okayama 700-8558, Japan
Tel: +181 86 235 7265; fax: +181 86 235 7269;
e-mail: cqs03255@nifty.com

Received 26 July 2006 **Revised form accepted** 6 January 2007

Introduction

The proteasome inhibitor bortezomib (PS341), which inhibits the function of the 26S proteasome, has been extensively investigated in the clinical setting of hematologic malignancies [1–3]. There have been some reports of its efficacy for intestinal malignancy, especially colorectal cancer [4–6], but its application in gastric cancer remains relatively unstudied. Here, we demonstrate that bortezomib has significant growth inhibition effects against gastric cancer cell lines, both alone and in combination with the conventional chemotherapeutic agents 5-fluorouracil (5FU), paclitaxel, doxorubicin and SN-38. Activity of extracellular signal-regulated kinase (ERK) and phosphatidylinositol-3-kinase (PI3K)/Akt pathways were decreased. Moreover, bortezomib downregulates the activities of nuclear factor NF-κB that are induced by the conventional chemotherapeutic agents. Increase in the G_1 fraction of the cell cycle, however, was recognized with low-dose bortezomib and supposed to be partly due to accumulation of p21. Here, we estimate the influence of bortezomib on the activities of the ERK and PI3K/Akt signaling pathways, and discuss the possible use of this agent in the treatment of gastric cancer.

0959-4973

Materials and methods

Materials and cell culture

The human gastric carcinoma cell lines, MKN45, NUGC3 and AZ521, were obtained from the American Type Culture Collection (Manassas, Virginia, USA) and grown in RPMI 1640 medium (Sigma, St Louis, Missouri, USA) supplemented with fetal bovine serum (10% v/v), penicillin (100 IU/ml) and streptomycin (100 μg/ml), and incubated in 5% CO_2. Doxorubicin and 5FU were from Kyowa Hakko Kogyo (Tokyo, Japan), paclitaxel (Taxol injection) from Bristol-Myers Squibb (Tokyo, Japan), SN-38 from Aventis Pharma (Tokyo, Japan), bortezomib (Velcade) from Millennium Pharma (Cambridge, Massachusetts, USA). The 5FU concentration was chosen based on both our previous studies and drug information from Kyowa Hakko Kogyo, as follows. Assay of plasma 5FU in 23 patients receiving pharmacokinetic-modulating chemotherapy revealed that its serum concentration ranged from 88 to 1323 ng/ml (approximately 0.1–10 μmol/l) [7]. The drug information from Kyowa Hakko Kogyo indicated that the plasma concentration of 5FU reached 15.3 μg/ml (100 μmol/l), after a bolus injection (500 mg/body) and 0.6 μg/ml (5 μmol/l) during continuous infusion (60 mg/body/48 h). The paclitaxel

concentration was chosen based on the plasma concentrations obtained from clinical use cited in the drug information for taxol injection (paclitaxel) from Bristol-Myers Squibb. This information indicated that its plasma concentration reached 1–10 μg/ml (1–10 μmol/l) after an injection and 0.05–0.1 μg/ml (50–100 nmol/l) 24 h after a drip infusion (105–270 mg/m^2). The concentrations of doxorubicin and SN-38 were similarly chosen based on our previous study and drug information.

Analysis of growth inhibition with MTS assay

[MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.] Cells were seeded at 3×10^3 cells per well in flat-bottomed 96-well microplates. After 24 h, they were cultivated with the appropriate cytotoxic agent for the indicated time or were left untreated. After incubation, the viability of the cells was determined using Promega's CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, Wisconsin, USA).

Western blotting analysis

Cells ($5–10 \times 10^6$) were washed twice with phosphate-buffered saline (PBS), then lysed with a hypotonic *N*-2-hydroxyl piperazine-*N*′-2-ethane sulfonic acid buffer (250 μl; 10 mmol/l; pH 7.6) containing KCl (50 mmol/l), phenylmethylsulfonylfluoride (0.1 mmol/l), aprotinin (0.5 μl/ml) and sodium orthovanadate (0.1 mmol/l). Upon centrifugation, the supernatants were adjusted to contain equal amounts of protein, diluted with one volume of 5 × sodium dodecyl sulfate sample buffer and heated at 95°C for 5 min. Samples (30 μg protein) were run on 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and electroblotted onto polyvinylidene fluoride membrane. Blots were blocked overnight with 5% nonfat milk powder and 0.1% Tween 20 (Acros Organics, Belgium, New Jersey, USA) in PBS (blocking solution) at 4°C.

Afterwards, the blots were exposed to the appropriate antibody [against ERK1/2, p-ERK1/2, Akt (Santa Cruz Biotechnologies, Santa Cruz, California, USA), p-Akt or p21 (Cell Signaling, Danvers, Massachusetts, USA)] at 1000-fold dilution in blocking solution (1 h, room temperature). After extensive washing with blocking solution, blots were exposed to the appropriate secondary antibody at 10 000-fold dilution in blocking solution. After extensive washing with blocking solution, the blots were developed using the enhanced chemiluminescence detection method (ECL kit; Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

Cell cycle analysis with fluorescence-activated cell sorting

Measurement of cell cycle was measured by propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) analysis. Cells were plated in six-well plates (1×10^4 cells/well). The concentration of bortezomib used for the in-vitro studies (0–1000 nmol/l) was selected to ensure appropriate cell growth inhibition in the AZ521, MKN45 and NUGC3 gastric cancer cell lines. Following incubation with 0–1000 nmol/l bortezomib, cells were harvested, pelleted by centrifugation, and resuspended in PBS containing PI (50 μg/ml), Triton X-100 (0.1%) and RNase (5 μg/ml). Cells were incubated with the PI solution and flow cytometric analysis of stained cells was performed with a FACScan (Becton Dickinson, Mountain View, California, USA).

Combination index and fractional effects

Drug effect analysis was performed using Biosoft (Great Shelford, Cambridge, UK) computer software [8,9]; details of this methodology have been published previously [10,11]. The combination index (CI) was calculated on the basis of the most conservative assumption of mutually nonexclusive drug interactions. CI values significantly lower than 1.0 indicate synergy, values significantly higher than 1.0 indicate antagonism and values approximately equal to 1 indicate additivity. The CI values we determined ranged from 0.3 to 0.7 of fractional effect.

Preparation of nuclear extracts and nonisotopic electrophoretic mobility shift assay

Nuclear extracts from stimulated (5FU, paclitaxel, doxorubicin and SN-38 with bortezomib) and unstimulated (5FU, paclitaxel, doxorubicin and SN-38 without bortezomib) AZ521 cells (1×10^7 cells) were prepared using the method of Dignam *et al.* [12] with slight modification. The NF-κB (5′-AGTTGAGGGGACTTT CCCAGGC-3′) (Santa Cruz Biotechnology) primers were annealed by incubation with an equal molar concentration of each single-stranded oligonucleotide in Tris–HCl (10 mmol/l; pH 8.0), ethylenediaminetetraacetic acid (1 mmol/l) and NaCl (200 mmol/l) at 95°C for 10 min, and then the mixture was allowed to cool to room temperature. The DNA-binding assay was performed using the DIG-Gel Shift Kit (Boehringer Mannheim, Indianapolis, Indiana, USA). Binding reactions were carried out at room temperature for 20 min: reaction mixtures contained nuclear protein (0.3 ng), *N*-2-hydroxyl piperazine-*N*′-2-ethane sulfonic acid (20 mmol/l, pH 7.6), KCl (30 mmol/l), $MgCl_2$ (5 mmol/l), ethylenediaminetetraacetic acid (1 mmol/l), dithiothreitol (1 mmol/l), Tween 20 (0.2%), poly(dI–dC) (50 mg/ml) and approximately 0.3 pmol of specified probe labeled with DIG-DDUTP using terminal dexoxynucleotidyl transferase (Boehringer Mannheim). Protein–DNA complexes were separated from protein-free DNA by nondenaturing electrophoresis in 5% polyacrylamide gel. Gels were run at a constant voltage (8 V/cm) and electroblotted onto positively charged nylon membranes. The membranes were baked at 80°C for 15 min, washed with 0.3% Tween 20 in Buffer I and hybridized with the diluted

anti-DIG-alkaline phosphatase (1 : 10 000) in Buffer II for 30 min. After two 15-min of washes with 0.3% Tween 20 in Buffer I, the membranes were equilibrated in alkaline buffer for 2 min. After incubation of the membranes at 37°C for 15–20 min, they were exposed to Hyperfilm-ECL (Amersham) for detection of the chemiluminescent signal.

Statistical analysis

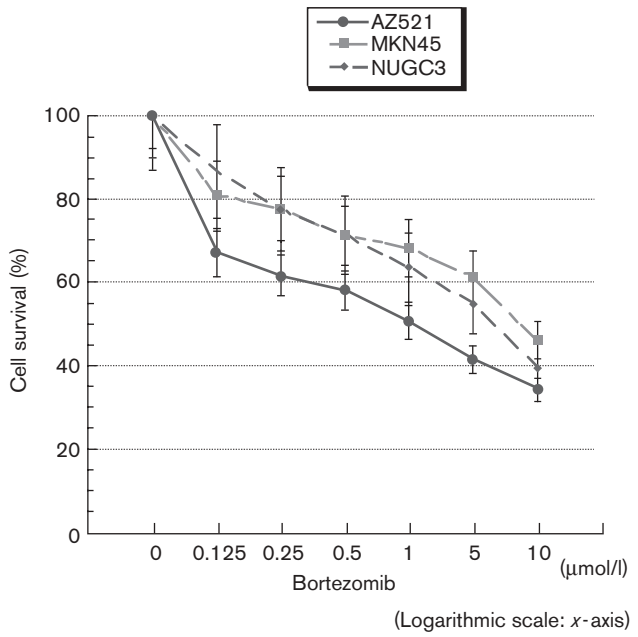
Levels of statistical significance were evaluated using data from at least three independent experiments, using a two-tailed Student's *t*-test and analysis of variance; $P < 0.05$ was considered to be statistically significant. All data were analyzed using Stat-View for Windows (SAS Institute, Cary, North Carolina, USA).

Results

Growth inhibition of bortezomib in the gastric cancer cell lines

Bortezomib was cytotoxic for the three cell lines, both alone, and in combination with 5FU, paclitaxel, doxorubicin and SN-38. We analyzed growth inhibition of each agent for gastric cancer cells independently three times. The mean IC_{50} values of bortezomib in the AZ521, MKN45 and NUGC3 cells were 1.26, 9.44 and 8.63 μmol/l, respectively (Fig. 1). The mean IC_{50} values of 5FU, paclitaxel, doxorubicin and SN-38 for these cells were as follows: AZ521: 5FU, 122.2 μmol/l; paclitaxel, 22.3 μmol/l; doxorubicin, 2.47 μmol/l; SN-38, 514.7 μmol/l. MKN45: 5FU, 517.3 μmol/l; paclitaxel, 58.0 μmol/l; doxorubicin, 13.1 μmol/l; SN-38, 590.5 μmol/l. NUGC3: 5FU, 235.1 μmol/l; paclitaxel, 28.96 μmol/l; doxorubicin, 52.69 μmol/l; SN-38, 435.1 μmol/l (Fig. 2). When the agents were combined, the mean IC_{50} values were statistically significantly lower than either agent alone in all three cell lines ($P < 0.05$) (Fig. 2).

Fig. 1



IC_{50} values of bortezomib (PS341) for the AZ521, MKN45 and NUGC3 gastric cancer cell lines. Cells were seeded at 3×10^3 cells per well in flat-bottomed 96-well microplates. After 24 h, cells were cultivated with bortezomib for 24 h or left untreated, following which time their viability was determined using Promega's CellTiter 96 Aqueous One Solution Cell Proliferation Assay.

Combination index and fractional effect of bortezomib under the treatment with conventional agents

Growth inhibition effects of bortezomib were analyzed in combination with 5FU, paclitaxel, doxorubicin and SN-38. Dose–response curves were constructed for each drug and combination at fixed molar ratios, defined according to the results of the growth-inhibition assay: doxorubicin : bortezomib = 10 : 1, 5FU : bortezomib = 100 : 1, paclitaxel : bortezomib = 100 : 1 and SN-38 : bortezomib = 1000 : 1. To evaluate the interaction between combination partners, CI values were calculated across all combinations and doses tested. Figure 3 summarizes the drug-effect data for each of the drug combinations in each cell line. Data points represent CI values (mean of three separate experiments) and fractions of unaffected cells. Each agent's interactions with bortezomib were strongly dose-related, with synergistic (CI < 1.0) or additive (C = 1.0) interaction effects.

Influence of bortezomib on the cell cycle in gastric cancer

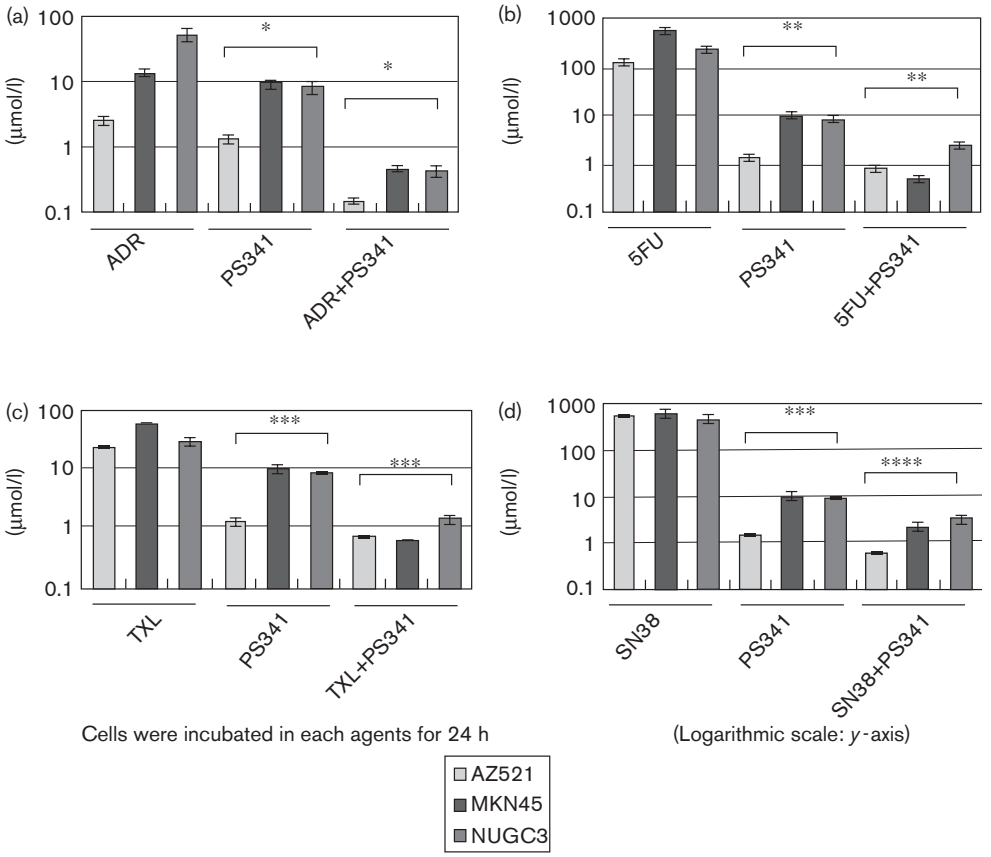
To determine the influence of bortezomib on the cell lines, we analyzed the cell cycle profile. Each cell line was treated with various concentrations (0–100 nmol/l) of bortezomib for 12 h. The results showed that bortezomib treatment increased accumulation in the G_0/G_1 phase of the cell cycle: the accumulation is marked upon treatment with 100 nmol/l bortezomib (Fig. 4 and supplementary data).

Evaluation of ERK and Akt signaling status under treatment with bortezomib

Western blotting analysis was performed to analyze the expression of ERK1/2 and phosphorylated ERK1/2 (p-ERK1/2), Akt and phosphorylated Akt (p-Akt) in the gastric cancer cell lines. Cells were exposed to each concentration of bortezomib and incubated for 24 h, then harvested. Expression of both p-ERK1/2 and p-Akt decreased dose dependently with bortezomib treatment (Fig. 5), whereas p21 expression increased in a dose-dependent manner.

We analyzed the influence of bortezomib in combination with the conventional chemotherapeutic agents 5FU, paclitaxel, doxorubicin and SN-38 on the expression of ERK and Akt. As a single agent, these agents showed little influence on either ERK1/2 or Akt signaling (data

Fig. 2



IC_{50} values of 5-fluorouracil (5FU), doxorubicin (ADR), paclitaxel (TXL) and SN-38, alone and in combination with bortezomib (PS341). Cells were treated with the appropriate cytotoxic agents for the indicated time or left untreated and viability was determined. IC_{50} values of combination treatment (PS341+5FU, ADR, TXL and SN-38 incubated concurrently for 24 h) were calculated with a 1 : 1 ratio of each agent on the basis of three independent experiments. (a) IC_{50} values of doxorubicin (ADR) and bortezomib (PS341) show similar impact on AZ521 and MKN45 gastric cancer cells; however, combinatorial treatment with these drugs exhibits a significantly lower IC_{50} value compared with isolated treatment. (b) Noticeable cytotoxic effects are observed using 5FU and bortezomib (PS431) on AZ521, MKN45 and NUGC3 cells, with MKN45 cells showing the most significant effect. (c) Combinatorial treatment using paclitaxel (TXL) and bortexomib (PS431) shows adequate cytotoxic effect on MKN45 and NUGC3 cells. A similar effect is also observed using SN-38 (d). Each chemotherapeutic agent exhibits a statistically significant growth inhibitory effect on cells in combination with PS341 (*, **, ***, ****: $P<0.05$).

not shown). When combined with bortezomib, expression of both p-ERK1/2 and p-Akt were decreased in most cases: no significant differences in p-Akt expression were recognized in combination with 5FU (Fig. 6a and b).

Effect of proteasome inhibitors on nuclear factor-κB binding activities

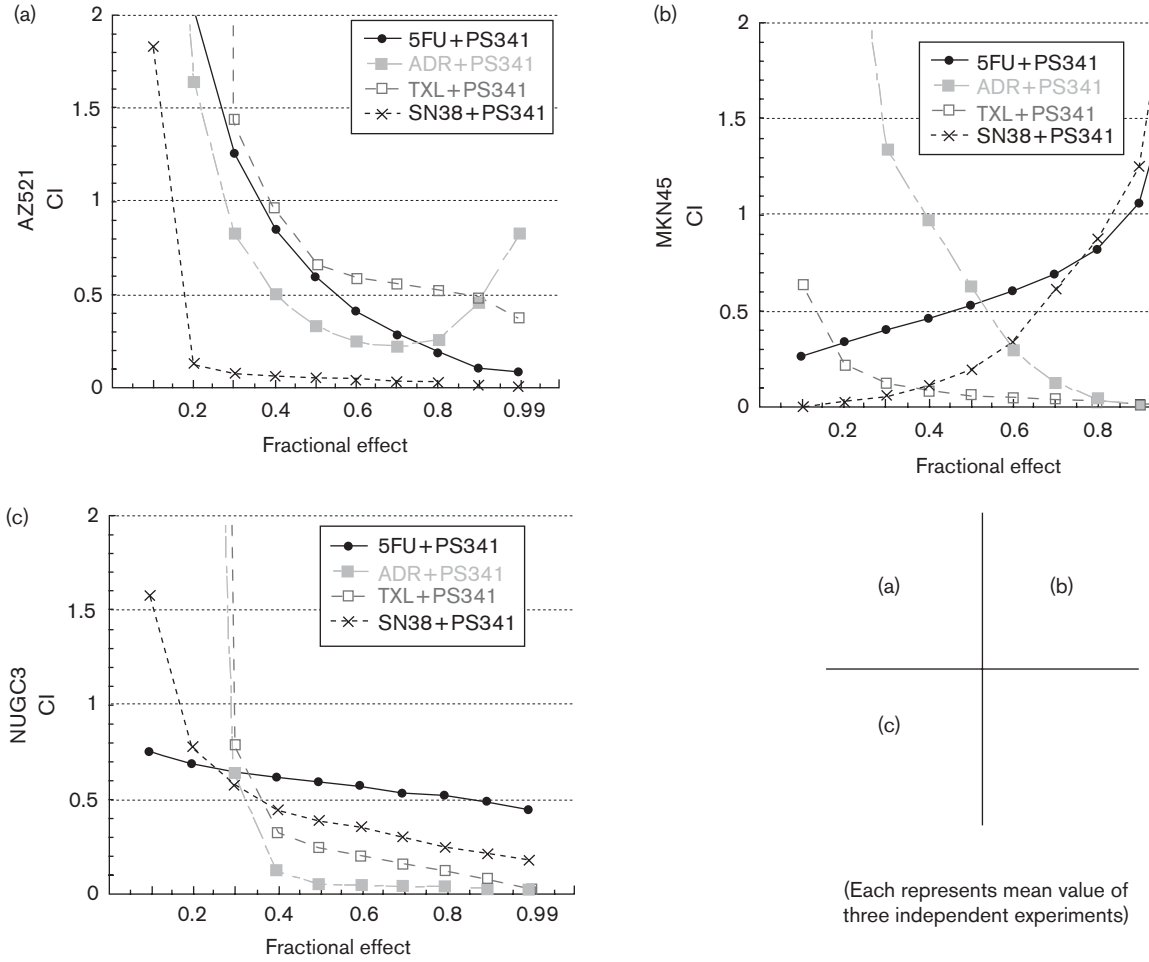
To determine whether proteasome inhibitors would inhibit the DNA-binding activity of NF-κB, AZ521 cells were treated with 5FU, paclitaxel, doxorubicin and SN-38, alone or in combination with bortezomib, for 12 h.

The concentration of each drug was chosen based on the results of the previous growth inhibition assay. Nuclear extracts were prepared and evaluated by electrophoretic mobility shift assay. Bortezomib inhibited the DNA-binding function of NF-κB in AZ521 cells. Activation of NF-κB, one of the cell survival factors, was increased by treatment with single-agent conventional cytotoxic drugs, especially 5FU and doxorubicin. The induction of NF-κB activity by cytotoxic agents was inhibited by the addition of bortezomib (Fig. 7, right panel). Similar tendencies were recognized in MKN45 and NUGC3 gastric cancer cells (data not shown).

Discussion

The ubiquitin–proteasome pathway plays an important role in regulating the cell cycle, neoplastic growth and metastasis [13] of cancer cells. A number of key regulatory proteins are degraded during the cell cycle by the ubiquitin–proteasome pathway; ordered degradation of these proteins is required for the cell to progress though the cell cycle and undergo mitosis. Therefore, proteasome inhibitors can inhibit tumor growth, tumor

Fig. 3



Combination index (CI) and fractional effects. The CI was calculated on the basis of the most conservative assumption of mutually nonexclusive drug interactions. In this experiment, drug-combination ratios [doxorubicin : bortezomib = 10 : 1, 5-fluorouracil (5FU) : bortezomib = 100 : 1, paclitaxel : bortezomib = 100 : 1 and SN-38 : bortezomib = 1000 : 1] were determined based on the results of the growth inhibition assay described previously. The CI values were estimated within the range from 0.3 to 0.7. The synergistic effect achieved with bortezomib in combination with most conventional chemotherapeutic agents falls within the fractional range of 0.3–0.7. (a) For AZ521 cells, the synergistic combinatorial effect (CI < 1) using doxyrubicin (ADR) or SN-38 is observed in the fractional range of 0.3–0.99. 5FU and paclitaxel (TXL) also exhibit a synergistic effect in the range of 0.4–0.99 when used in combination with bortezomib (PS341). (b) Combinatorial treatment with PS341 and doxorubicin (ADR) exhibits a synergistic effect in the fractional range of > 0.4. Synergistic effects are noticeable in combination with 5FU, paclitaxel (TXL) or SN38 (fractional range < 0.8 in 5FU and SN-38, 0.0–0.99 in TXL). (c) For NUGC3 cells, a synergistic cytotoxic effect is noticeable when all conventional agents are used in combination with bortezomib (PS342) in the fractional range of > 0.3.

spread and angiogenesis through multiple mechanisms [14–17]. This offers a potential new approach to treating cancer, and laboratory findings support this hypothesis.

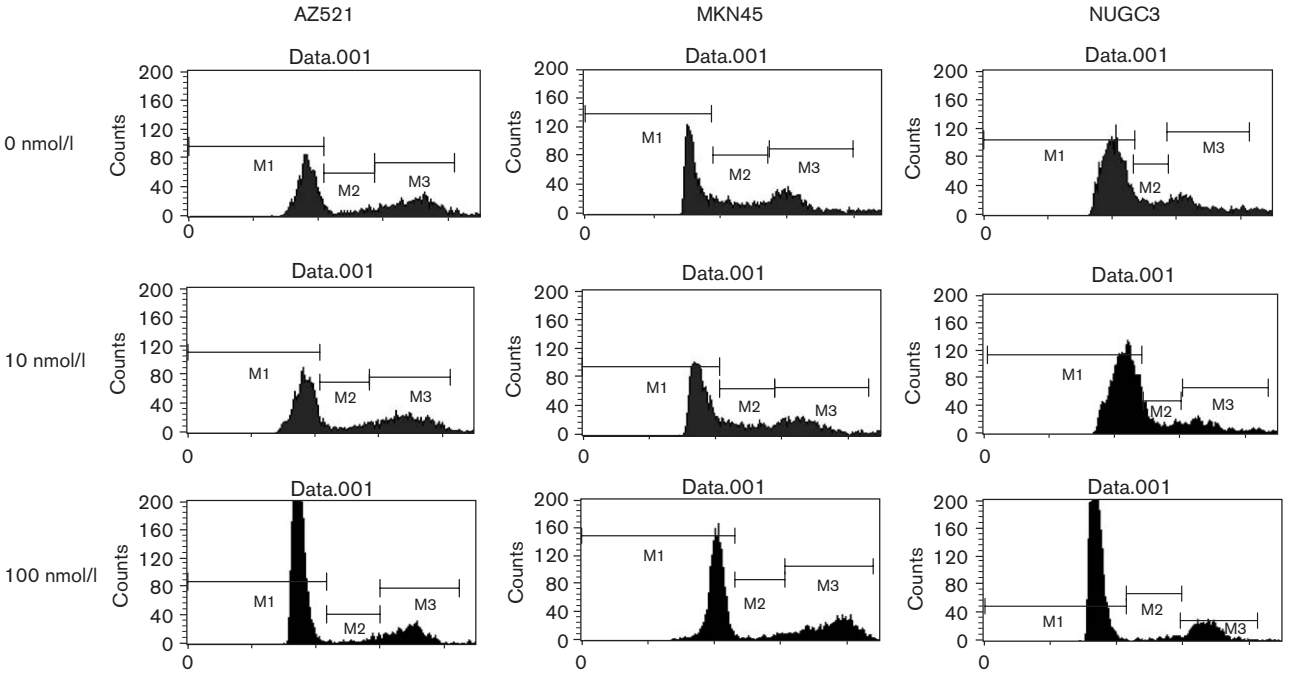
The efficacy of proteasome inhibitors has been vigorously investigated in hematologic malignancies and combination with such agents is reported to enhance the cytotoxic activity of conventional chemotherapeutic agents. Mitsiades *et al.* [18] investigated the mechanism of this chemosensitizing effect in multiple myeloma using oligonucleotide gene microarray analysis. They demonstrated that a proteasome inhibitor (bortezomib) could downregulate the transcripts for several effectors of the protective cellular response to genotoxic stress (topoisomerase II-β), which relaxes DNA torsion on replication, transcription and cell division, and is inhibited by doxorubicin [19]. Previous proteomic analysis, however, has revealed that bortezomib decreases the expression of Bcl-2, cellular inhibitor of apoptosis-2, X-linked inhibition of apoptosis and Fas-associated death domain protein-like interleukin 1β-converting enzyme-inhibitory protein [20].

We have elucidated the drug interactions of proteasome inhibitor bortezomib in gastric cancer cells, and showed effective growth inhibition both as a single agent and in

Fig. 4

	AZ521			MKN45			NUGC3		
Bortezomib	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
0 nmol/l	48.29	12.03	35.96	40.57	26.21	32.6	55.32	21.67	23.01
10 nmol/l	52.47	13.38	33.78	43.29	23.45	33.2	58.93	17.30	23.60
100 nmol/l	77.06	3.44	16.23	72.81	4.79	22.21	80.67	3.67	15.17

Summary of data (%)

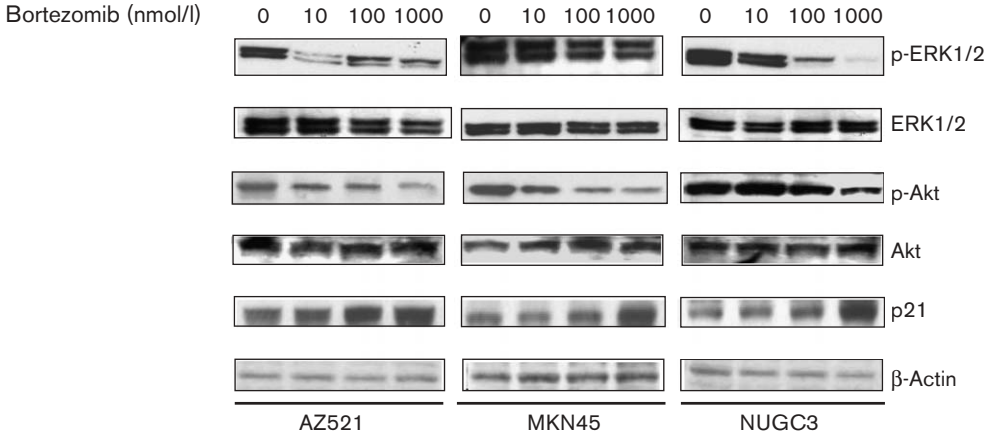


Cell-cycle analysis with fluorescence-activated cell sorting. Cells were plated in six-well plates (1×10^4 cells/well) and treated with bortezomib (0–100 nmol/l). After 24 h of incubation, cells were harvested. DNA fragmentation was measured by propidium iodide staining and flow cytometric analysis of stained cells was performed with a FACScan. Accumulation of the G_0/G_1 phase of the cell cycle was prominently seen with 10–100 nmol/l bortezomib. Supplementary data summarize the percentage distribution of cells (data were the mean of three different experiments).

combination with the conventional chemotherapeutic drugs 5FU, paclitaxel, doxorubicin and SN-38. In most gastrointestinal malignancies, esophageal carcinoma or colorectal carcinoma, 5FU has been one of the most well-established and effective chemotherapeutic agent for decades, and it remains the major chemotherapeutic component in the treatment regimen for these malignancies. Recently, taxane and irinotecan have been widely recognized as effective treatments for the advanced disease [21,22]. Gastric cancer, however, is able to develop resistance to chemotherapy, and their efficacy as a single agent is not sufficient enough in the aggressive stage of disease. Therefore, novel chemotherapeutic agents, effective both alone and in combination with conventional drugs, are required.

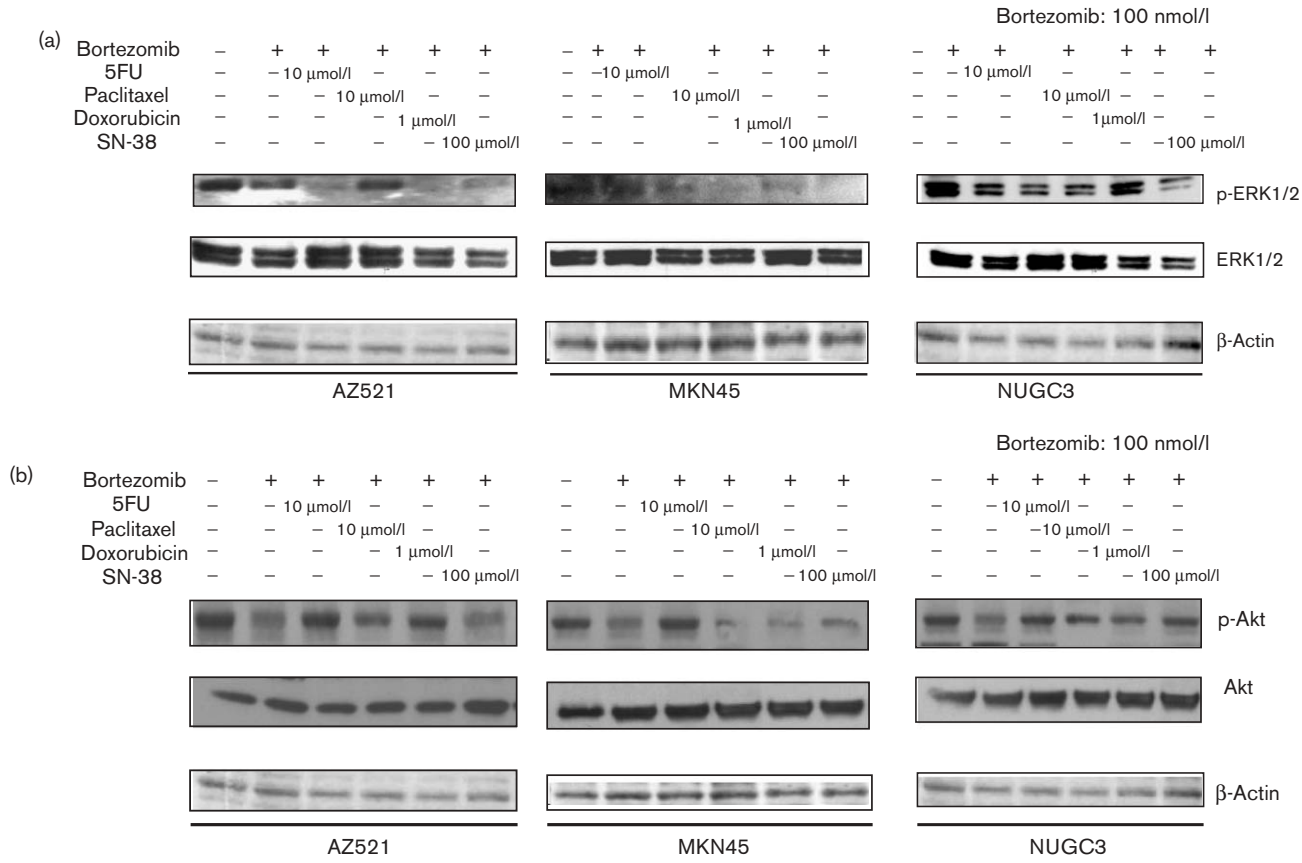
The results of our study suggest that bortezomib is a possible adjuvant treatment for gastric carcinoma. Moreover, we have demonstrated that bortezomib successfully inhibits the activity of the ERK1/2 and Akt-mediated signaling pathways. Both MEK/ERK1/2 and PI3K/Akt-mediated signaling are highly associated with cell survival, proliferation and progression of cancer cells [8,9,23–25]. Aberrant activation of the mitogen-activated protein kinase pathway is implicated in controlled proliferation, metastasis and inhibition of apoptosis. Therefore, inhibition of these signaling cascades significantly affects the behavior of cancer cells: inhibition has been investigated in both preclinical and clinical setting. Phase I and II clinical testing of a second-generation oral MEK inhibitor is currently underway [26,27]. ERK1/2 has also been suggested to be related to the development of gastric lesions via *Helicobacter pylori* infection [28–30]. Indeed, Kim *et al.* [30] demonstrated an effect of *H. pylori* on urokinase plasminogen activator receptor expression, which is closely related to gastric cancer invasion via

Fig. 5



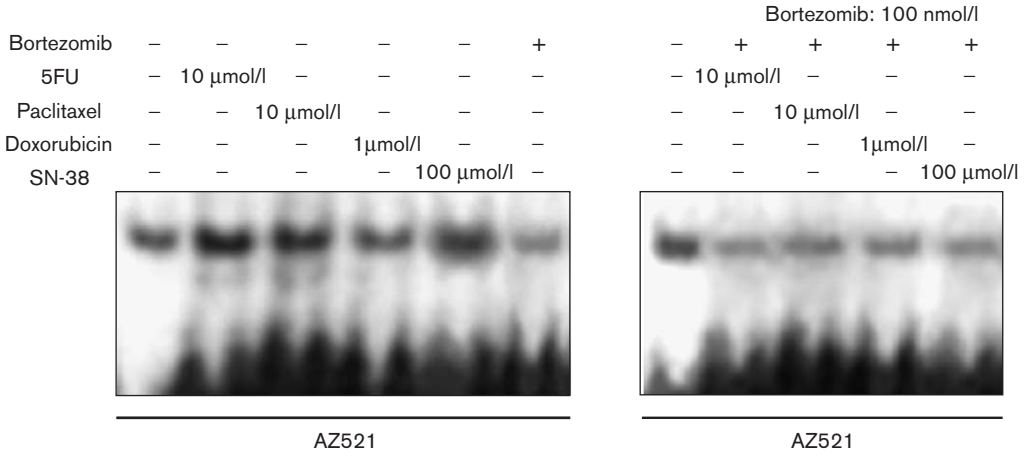
Expression profiles of ERK1/2, p-ERK1/2, AKT, p-Akt and p21. Cells were treated with 0.01–1 μmol/l bortezomib for 24 h and expression profiles were detected by Western blotting analysis. Under the treatment of bortezomib (PS341), p-ERK1/2 expression was decreased in a dose-dependent manner (upper panel). p-Akt expression was also decreased with bortezomib (middle panel). On the other hand, p21 is recognized to accumulate in a dose-dependent manner (lower panel).

Fig. 6



(a and b) Expression profiles of ERK1/2, p-ERK1/2, Akt and p-Akt with concurrent treatment of bortezomib (PS341) and conventional agents. Cells were treated for 24 h with 0.1 μmol/l bortezomib both alone and in combination with 5-fluorouracil (5FU), doxorubicin (ADR), paclitaxel (TXL) and SN-38 as indicated. Expression profiles were detected by Western blotting analysis. Expression of p-ERK1/2 and p-Akt was significantly inhibited with the combination treatment.

Fig. 7



Result of DNA binding activity with electrophoretic mobility shift assay. AZ521 cells were treated with 5-fluorouracil (5FU), doxorubicin (ADR), paclitaxel (TXL), SN-38 and bortezomib (PS341) at the indicated dose for 12 h. The concentration of each drug was chosen on the basis of our previous results of growth inhibition assay. Nuclear extracts were prepared and evaluated. Left panel: Bortezomib (PS341) inhibited the DNA-binding activity of nuclear factor (NF)-κB in AZ521 cells (right-most lane). Activity of NF-κB was remarkably increased under the treatment of single-agent conventional cytotoxic drugs [especially 5FU, doxorubicin (ADR) and SN-38] (lanes 1–5). Right panel: in combination with PS341, the DNA-binding activity of NF-κB was remarkably decreased (lanes 2–5).

MEK/ERK1/2 signaling pathway. Also, Mitsuno *et al.* [31] revealed that *H. pylori*-induced transactivation of SRE and AP-1 is mediated through the ERK/mitogen-activated protein kinase cascade.

The role of PI3K/Akt signaling is reported to be highly associated with cell survival and proliferation in many human cancers [8,9,25]. Activation of the PI3K/Akt pathway can occur by a ligand binding to the extracellular domain of receptor tyrosine kinases such as the epidermal growth factor receptor, which is amplified or mutated in various human cancers. Growth-factor-receptor-mediated signal transduction has also been implicated in conferring resistance to conventional chemotherapy in cancer cells. In gastric carcinoma, the PI3K/Akt pathway plays a critical role in regulating cell proliferation. Oki *et al.* [8] have described a pathway that involves Akt/PI3K to mediate chemoresistance in gastric cancer patients. Therefore, targeting Akt could be possible therapies or chemosensitivity tests that improve the outcomes of patients with gastric cancer. These hypotheses support our observations that bortezomib inhibits the ERK1/2 and Akt signaling pathways when used both as a single agent or combination with conventional chemotherapeutic agents, and induces additional growth inhibition in these gastric cancer cell lines *in vitro*.

Considering cell-cycle control in gastric carcinoma, previous studies have suggested an association of cell-cycle regulation with the ubiquitin–proteasome pathway. An in-vitro study showed that MKN45 gastric cancer cells treated with a specific blockade of the PI3K/Akt pathway inhibited cell proliferation via induction of G_0–G_1 arrest [32]. It is well known that p27/Kip1, a substrate of activated Akt, and phosphorylated-p27/Kip1 are exported from the nucleus to the cytoplasm and degraded by the ubiquitin–proteasome system [33]. Moreover, several cyclin-dependent kinase inhibitors of $p16^{INK4}$ and $p21^{Cip1}$ are regulated by the ubiquitin–proteasome system. Indeed, in this study, accumulation of p21 was dose-dependent. Therefore, we suggest that the G_0–G_1 arrest was partly due to the accumulation of these cell-cycle regulators via proteasome inhibition.

Bortezomib has been previously demonstrated to have significant combination effects with conventional chemotherapeutic agents in certain cancers. These effects might be due, at least in part, to inhibition of NF-κB activity [34]. NF-κB is one of the most important regulators of apoptosis and can be constitutively activated in several cancers. NF-κB activity protects cells from cascade triggered by cytotoxic agents [35–37], tumor necrosis factor, interleukin-1 and other stimuli. In addition, NF-κB can promote cell growth by activating the transcription of genes that encode the G_1 cyclin. Moreover, NF-κB regulates cell adhesion molecules and angiogenesis [38].

Several of these studies indicated that the inhibition of NF-κB could increase response of cytotoxic drugs [35,37] and also that NF-κB regulates the expression of P-glycoprotein [39,40]. In gastric carcinoma, activity of NF-κB is highly associated with resistance to chemotherapy

[41]. Adversely, previous studies have revealed that 5FU also causes dose-dependent activation of NF-κB [41,42], as do other drugs and chemotherapeutic agents including taxol, doxorubicin, daunorubicin, etoposide, vincristin, vinblastin, cytarabine, anathralin, AZT, cisplatin, tamoxifen and camptothecin [43]. Similarly, Uetsuka *et al.* [42] suggested that 5FU chemoresistance can be overcome by the inhibition of inducible NF-κB activation and that use of a specific NF-κB inhibitor combined with 5FU treatment could be a new molecular therapeutic strategy aimed at treating gastric cancer resistance to 5FU.

On the basis of these ideas, we predict that inhibition by NF-κB could reduce the effective dose of chemotherapeutic agents. Thus, combinatorial therapy using inhibitors of NF-κB with chemotherapeutic agents could provide a more effective treatment for gastric cancer than chemotherapeutic agents alone. Indeed, the results of our study demonstrate that the activity of NF-κB is remarkably reduced in treatment comprising bortezomib (Fig. 7). Moreover, previous report suggests that the inhibitory effect of bortezomib could depend on the time of treatment [40,44,45]. On the other hand, the combinatorial effect with bortezomib could be due to the fractional effects of agents. In fact, in most cases, the cytotoxic effect of this drug seems to be prominent at the higher fractional range (Fig. 3). Taken together, the inhibition of NF-κβ could be the explanation behind the synergistic effect achieved when bortezomib is used in combination with other agents. To achieve sufficient synergistic cytotoxicity, a necessary dosage of bortezomib might be needed rather than treatment at a relatively low dose. In addition, the timing of administration (sequential or concurrent) might affect the efficacy of bortezomib although no noticeable difference is observed in this study (data not shown).

Phase II clinical studies to determine response rates, toxicities, progression-free survival and overall survival in patients with advanced gastric adenocarcinoma receiving bortezomib alone or in combination with irinotecan are ongoing [46,47]. The final results of this study have not yet been released, but will be important to evaluate the clinical significance of this novel proteasome inhibitor.

In summary, we have demonstrated that the proteasome inhibitor bortezomib (PS341) showed growth inhibition in the AZ521, MKN45 and UNGC3 gastric cancer cell lines. Several mechanisms are supposed to be involved in the effect, one of which is decreased activation of the ERK and Akt pathways. Downregulation of these pathways is thought to be associated with several signal transduction protein factors and cell-cycle regulators, of which NF-κB is considered to be the most important. In addition, our results provide a biochemical basis for clinical application of the proteasome inhibitor bortezomib (PS341) alone or in combination with conventional antineoplastic agents in treating gastric cancers.

Acknowledgements

We acknowledge Mayumi Okada for her support of the experiment, also appreciate George Wu for his assistance in manuscript preparation.

References

1 Richardson PG. Bortezomib: a novel therapy approved for multiple myeloma. *Clin Adv Hematol Oncol* 2003; **1**:596–600.
2 Vandenbroucke JP, Kroep JR. Bortezomib in multiple myeloma. *N Engl J Med* 2005; **353**:1297–1298.
3 Berenson JR, Jagannath S, Barlogie B, Siegel DT, Alexanian R, Richardson PG, *et al.* Safety of prolonged therapy with bortezomib in relapsed or refractory multiple myeloma. *Cancer* 2005; **104**:2141–2148.
4 Adams J, Kauffman M. Development of the proteasome inhibitor velcade (bortezomib). *Cancer Invest* 2004; **22**:304–311.
5 Mani A, Gelmann EP. The ubiquitin–proteasome pathway and its role in cancer. *J Clin Oncol* 2005; **23**:4776–4789.
6 Mackay H, Hedley D, Major P, Townsley C, Mackenzie M, Vincent M, *et al.* A phase II trial with pharmacodynamic endpoints of the proteasome inhibitor bortezomib in patients with metastatic colorectal cancer. *Clin Cancer Res* 2005; **11**:5526–5533.
7 Yoshikawa R, Kusunoki M, Yanagi H, Noda M, Furuyama JI, Yamamura T, *et al.* Dual antitumor effects of 5-fluorouracil on the cell cycle in colorectal carcinoma cells: a novel target mechanism concept for pharmacokinetic modulating chemotherapy. *Cancer Res* 2001; **61**:1029–1037.
8 Oki E, Baba H, Tokunaga E, Nakamura T, Ueda N, Futatsugi M, *et al.* Akt phosphorylation associates with LOH of PTEN and leads to chemoresistance for gastric cancer. *Int J Cancer* 2005; **117**:376–380.
9 Baoping Y, Guoyong H, Jieping Y, Zongxue R, Hesheng L. Cyclooxygenase-2 inhibitor nimesulide suppresses telomerase activity by blocking Akt/PKB activation in gastric cancer cell line. *Dig Dis Sci* 2004; **49**:948–953.
10 Chou TC, Talalay P. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984; **22**:27–55.
11 Pegram M, Hsu S, Lewis G, Pietras R, Beryt M, Sliwkowski M, *et al.* Inhibitory effects of combinations of HER-2/*neu* antibody and chemotherapeutic agents used for treatment of human breast cancers. *Oncogene* 1999; **18**:2241–2251.
12 Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 1983; **11**:1475–1489.
13 Orlowski RZ, Dees EC. The role of the ubiquitination–proteasome pathway in breast cancer: applying drugs that affect the ubiquitin–proteasome pathway to the therapy of breast cancer. *Breast Cancer Res* 2003; **5**:1–7.
14 Banerjee D, Lifeshitz A. Potential of the proteasome inhibitor MG-132 as an anticancer agent, alone and in combination. *Anticancer Res* 2001; **21**:3941–3948.
15 Adams J. Preclinical and clinical evaluation of proteasome inhibitor PS-341 for the treatment of cancer. *Curr Opin Chem Biol* 2002; **6**:493–500.
16 Papandreou CN, Daliani DD, Nix D, Yang H, Madden T, Wang X, *et al.* Phase I trial of the proteasome inhibitor bortezomib in patients with advanced solid tumors with observations in androgen-dependent prostate cancer. *J Clin Oncol* 2004; **22**:2108–2121.
17 Lenz HJ. Clinical update: proteasome inhibitors in solid tumors. *Cancer Treat Rev* 2003; **29**:41–48.
18 Mitsiades N, Mitsiades CS, Richardson PG, Poulaki V, Tai YT, Chauhan D, *et al.* The proteasome inhibitor PS-341 potentiates sensitivity of multiple myeloma cells to conventional chemotherapeutic agents: therapeutic applications. *Blood* 2003; **6**:2377–2380.
19 Hazlehurst LA, Valkov N, Wisner L, Storey JA, Boulware D, Sullivan DM, *et al.* Reduction in drug-induced DNA double-strand breaks associated with beta1 integrin-mediated adhesion correlates with drug resistance in U937 cells. *Blood* 2001; **98**:1897–1903.
20 Mitsiades N, Mitsiades CS, Poulaki V, Chauhan D, Fanourakis G, Gu X, *et al.* Molecular sequelae of proteasome inhibition in human multiple myeloma cells. *Proc Natl Acad Sci U S A* 2002; **22**:14374–14379.
21 Tanaka R, Ariyama H, Qin B, Shibata Y, Takii Y, Kusaba H, *et al.* Synergistic interaction between oxaliplatin and SN-38 in human gastric cancer cell lines in vitro. *Oncol Rep* 2005; **3**:683–688.

22 Emoto T, Yoshikawa K, Fujii M, Nezu R, Dousei T, Fujikawa M, *et al*. Clinical study of weekly administration of paclitaxel for advanced and metastatic gastric cancer. *Gan To Kagaku Ryoho* 2003; **6**:809–815. [Japanese].

23 Liang B, Wang S, Zhu XG, Yu YX, Cui ZR, Yu YZ. Increased expression of mitogen-activated protein kinase and its upstream regulating signal in human gastric cancer. *World J Gastroenterol* 2005; **11**:623–628.

24 Kang W, Nielsen O, Fenger C, Leslie G, Holmskov U, Reid KB. Induction of DMBT1 expression by reduced ERK activity during a gastric mucosa differentiation-like process and its association with human gastric cancer. *Carcinogenesis* 2005; **26**:1129–1137.

25 Lee BL, Lee HS, Jung J, Cho SJ, Chung HY, Kim WH, *et al*. Nuclear factor-κβ activation correlates with better prognosis and Akt activation in human gastric cancer. *Clin Cancer Res* 2004; **11**:2518–2525.

26 Lorusso PM, Adjei AA, Varterasian M, Gadgeel S, Reid J, Mitchell DY, *et al*. Phase I and pharmacodynamic study of the oral MEK inhibitor CI-1040 in patients with advanced malignancies. *J Clin Oncol* 2005; **23**:5281–5293.

27 Rinehart J, Adjei AA, Lorusso PM, Waterhouse D, Hecht JR, Natale RB, *et al*. Multicenter phase II study of the oral MEK inhibitor, CI-1040, in patients with advanced non-small-cell lung, breast, colon, and pancreatic cancer. *J Clin Oncol* 2004; **22**:4456–4462.

28 Juttner S, Cramer T, Wessler S, Walduck A, Gao F, Schmitz F, *et al*. *Helicobacter pylori* stimulates host cyclooxygenase-2 gene transcription: critical importance of MEK/ERK-dependent activation of USF1/-2 and CREB transcription factors. *Cell Microbiol* 2003; **5**:821–834.

29 Zhu Y, Zhong X, Zheng S, Du Q, Xu W. Transformed immortalized gastric epithelial cells by virulence factor CagA of *Helicobacter pylori* through Erk mitogen-activated protein kinase pathway. *Oncogene* 2005; **24**:3886–3895.

30 Kim MH, Yoo HS, Chang HJ, Hong MH, Kim HD, Chung IJ, *et al*. Urokinase plasminogen activator receptor is upregulated by *Helicobacter pylori* in human gastric cancer AGS cells via ERK, JNK, and AP-1. *Biochem Biophys Res Commun* 2005; **333**:874–880.

31 Mitsuno Y, Yoshida H, Maeda S, Ogura K, Hirata Y, Kawabe T, *et al*. *Helicobacter pylori* induced transactivation of SRE and AP-1 through the ERK signalling pathway in gastric cancer cells. *Gut* 2001; **49**:18–22.

32 Osaki M, Kase S, Adachi K, Takeda A, Hashimoto K, Ito H. Inhibition of the PI3K-Akt signaling pathway enhances the sensitivity of Fas-mediated apoptosis in human gastric carcinoma cell line, MKN-45. *J Cancer Res Clin Oncol* 2004; **130**:8–14.

33 Viglietto G, Motti ML, Bruni P, Melillo RM, D'Alessio A, Califano D, *et al*. Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ by PKB/Akt-mediated phosphorylation in breast cancer. *Nat Med* 2002; **8**:1136–1144.

34 Hideshima T, Chauhan D, Richardson P, Mitsiades C, Mitsiades N, Hayashi T, *et al*. NF-κB as a therapeutic target in multiple myeloma. *J Biol Chem* 2002; **19**:16639–16647.

35 Camp ER, Li J, Minnich DJ, Brank A, Moldawer LL, Mackay SL, *et al*. Inducible nuclear factor-κB activation contributes to chemotherapy resistance in gastric cancer. *J Am Coll Surg* 2004; **199**:249–258.

36 Jung M, Dritschilo A. NF-κB signaling pathway as a target for human tumor radiosensitization. *Semin Radiat Oncol* 2001; **11**:346–351.

37 Muerkoster S, Arlt A, Witt M, Gehrz A, Haye S, March C, *et al*. Usage tpdel of the NF-κB inhibitor sulfasalazine as sensitizing agent in combined chemotherapy of pancreas cancer. *Int J Cancer* 2003; **104**:469–476.

38 Yang HH, Ma MH, Vescio RA, Berenson JR. Overcoming drug resistance in multiple myeloma: the emergence of therapeutic approaches to induce apoptosis. *J Clin Oncol* 2003; **21**:4239–4247.

39 Bentires-Alj M, Barbu V, Fillet M, Chariot A, Relic B, Jacobs N, *et al*. NF-κB transcription factor induced drug resistance through MDR1 expression in cancer cells. *Oncogene* 2003; **22**:90–97.

40 Fujita T, Washio K, Takabatake D, Takahashi H, Yoshitomi S, Tsukuda K, *et al*. Proteasome inhibitors can alter the signaling pathways and attenuate the P-glycoprotein-mediated multidrug resistance. *Int J Cancer* 2005; **117**:670–682.

41 Camp ER, Li J, Minnich DJ, Brank A, Moldawer LL, MacKay SL, *et al*. Inducible nuclear factor-κB activation contributes to chemotherapy resistance in gastric cancer. *J Am Coll Surg* 2004; **199**:249–258.

42 Uetsuka H, Haisa M, Kimura M, Gunduz M, Kaneda Y, Ohkawa T, *et al*. Inhibition of inducible NF-κB activity reduces chemoresistance to 5-fluorouracil in human stomach cancer cell line. *Exp Cell Res* 2003; **289**:27–35.

43 Pahl HL. Activators and target genes of Rel/NF-κB transcription factors. *Oncogene* 1999; **18**:6853–6866.

44 Mortenson MM, Schlieman MG, Virudachalam S, Bold RJ. Effects of the proteasome inhibitor bortezomib alone and in combination with chemotherapy in the A549 non-small-cell lung cancer cell line. *Cancer Chemother Pharmacol* 2004; **54**:343–353.

45 Cardoso F, Durbecq V, Laes JF, Badran B, Lagneaux L, Bex F, *et al*. Bortezomib (PS-341, Velcade) increases the efficacy of trastuzumab (herceptin) in HER-2-positive breast cancer cells in a synergistic manner. *Mol Cancer Ther* 2006; **12**:3042–3051.

46 Ocean AJ, Schnoll-Sussman F, Chen X, Holloway S, Wright J, Wadler S. Phase II study of PS-341 (VELCADE, bortezomib) with or without irinotecan in patients (pts) with advanced gastric adenocarcinomas (AGA). *Proc Am Soc Clin Oncol 2005*. 2005 Gastrointestinal Cancers Symposium, abstract 31.

47 Shah MA, Holen K, Singh D, Kemeny M, Levner A, Cox L, *et al*. A multicenter, two-stage, Phase II study of PS-341 in patients with unresectable or metastatic gastric or gastroesophageal junction (GEJ) Adenocarcinoma. *Proc Am Society Clin Oncol* 2005. 2005 Gastrointestinal Cancers Symposium, abstract 41.