

Proteasome Inhibitors Counteract the Effect of Cisplatin in HPV-Positive Squamous Cell Carcinoma in Vitro

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Research article

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Abstract

Background A rapid increase in human papilloma virus (HPV)-positive oropharyngeal squamous cell carcinoma (OPSCC) is a global trend. Although HPV-positive patients have a more favorable prognosis, distant metastases occur, warranting new, systemic treatment options. The aim of this study was to investigate the effect of combining proteasome or MDM2 inhibitors with cisplatin on an HPV-positive oropharyngeal squamous cell carcinoma cell line (LU-HNSCC-26).

Methods The LU-HNSCC-26 cells were treated with proteasome inhibitor (bortezomib, carfilzomib or ixazomib) or MDM2 inhibitor (RG7112) in combination with cisplatin. Combinatorial effects were analyzed by isobolograms. Protein expression was investigated by Western blotting and cell cycle phase distribution by flow cytometry.

Results There was no synergy between the substances and cisplatin. All proteasome inhibitors displayed antagonistic effects while the MDM2 inhibitor was additive in combination with cisplatin. The expression of p53 was only marginally affected and apoptosis was not detected. The cell cycle progression was halted in G0/G1 with all inhibitors and in S phase with cisplatin. The expression of p21 increased by bortezomib or carfilzomib, ixazomib increased p21 in combination with cisplatin while RG7112 did not affect p21. There was no effect on ERCC1 with any of the substances.

Conclusions In the investigated HPV16-positive OPSCC cell line, proteasome inhibition decreased the effect of cisplatin. A possible mechanism for this includes low effects on p53 expression with concomitant increase in p21 expression and blocking of cell cycle progression in G0/G1 with preserved DNA damage repair. The combination of proteasome inhibition with ordinary cytotoxic treatment for HPV-positive OPSCC patients is thus questionable, and clinical trials should be preceded by thorough testing in adequate models.

Background

An increase in the number of oropharyngeal squamous cell cancer (OPSCC) has been a global trend over the last few decades. It is well known that human papillomavirus (HPV) infection is strongly associated with OPSCC. A recent meta-analysis showed that up to 70% of OPSCC were HPV-positive [1]. Of the approximately 12 high-risk HPV types, it is primarily HPV16 that is present in OPSCC [2]. Patients affected by HPV-positive OPSCC are generally younger than HPV-negative OPSCC patients and have no history of tobacco or alcohol overconsumption, both which are major risk factors for the classic HPV-negative head and neck cancers (HNC). HPV containing tumors also show high susceptibility to chemoradiotherapy and have a better prognosis than HPV-negative tumors. One major difference between HPV-negative and positive OPSCC is the key tumor suppressor protein p53. Many studies have shown that HPV-positive OPSCC often harbor wild type p53 in contrast to HPV-negative HNC which often carry mutant p53 [3]. High-risk HPV E6 proteins bind to p53 and cause continuous proteasomal degradation of p53, which inactivates p53 and promotes carcinogenesis by evasion of apoptosis. The different clinical outcomes

between HPV-positive and HPV-negative OPSCC have become generally accepted and raise the need to classify them differently to improve the concurrence of TNM-staging and prognosis. In the recently published 8th edition of TNM classification, a new stage classification for HPV-positive OPSCC was added [4, 5]. However, in general the same treatment is still used for both HPV-positive and HPV-negative OPSCC. Cisplatin is a standard radiosensitizer for treatment of OPSCC. It has been used for several decades although it has several undesired side effects, such as nephro- and oto-toxicity.. In addition, the pattern of distant metastases differs between HPV-positive and -negative OPSCC, with a significant higher rate of lung metastases for HPV-positive OPSCC [6]. For these patients, systemic antitumor drugs are vital. Therefore, it is desirable to identify new efficient antitumor drugs in order to provide alternative treatment options.

Inhibiting the proteasome is a new approach for cancer treatment that functions by preventing protein degradation by the ubiquitin-proteasome pathway. Bortezomib is a proteasome inhibitor approved by the United States of America Food and Drug Administration (FDA) for treatment of relapsed multiple myeloma and mantle cell lymphoma that has been successfully used for a decade. Recently, a second generation of proteasome inhibitors (carfilzomib, ixazomib) has been developed. These inhibitors have greater specificity and fewer side-effects. Several *in vitro* studies have shown effectiveness of these three substances on cell lines from different solid tumors including head and neck tumors [7–13]. Some of these cell lines upregulated p53 protein in response to treatment. It was concluded that stabilization of the p53 protein lead to p53-dependent apoptosis.

In many cancers, the murine double minute 2 (MDM2) protein is overexpressed. MDM2 is one of the E3 ubiquitin ligases which binds directly to the p53 and inactivates it by inhibiting proteasomal degradation. MDM2 overexpression can inhibit p53-mediated tumor suppressing activities. Therefore, MDM2 inhibitors constitute another family of emerging anticancer drugs that reduce proteasomal degradation of the p53 protein. We hypothesize that these substances might have higher specificity towards the p53 protein and therefore would increase the levels of functional p53 more efficiently than proteasome inhibitors.

We have previously established a HPV16-positive tonsil cell line with wild-type p53 named LU-HNSCC-26 (HN26) [14]. Except for this cell line, there are only eight reported HPV-positive HNSCC cell lines to our knowledge, and only one of those is of oropharyngeal origin but not from tonsil [15, 16]. In this study, we treated HN26 cells with cisplatin alone or in combination with three different proteasome inhibitors and an MDM2 inhibitor to see if proteasome or MDM2 inhibition could prevent p53 degradation and increase the effect of cisplatin.

Methods

Cell line

The cell line LU-HNSCC-26 (HN26) was previously isolated from a low-grade, oropharyngeal, p16 positive, squamous cell tonsillar stage II (T2N0M0 [17]) carcinoma [14]. The cells were cultured in R10 medium

(RPMI 1640 with stable glutamine supplemented with 1 mmol/L sodium pyruvate, 1 × MEM non-essential amino acids, 20 µg/mL gentamicin and 10% fetal bovine serum (FBS), all from GE Healthcare (Piscataway, NJ, USA)) in a humidified 37 °C incubator with 5% CO₂.

Substances

Bortezomib, carfilzomib, ixazomib and RG7112 were purchased from Selleck Chemicals (Houston, USA) and cisplatin from Sandoz AS (Copenhagen, Denmark).

Determination of drug sensitivity

Dose response curves were generated for each substance individually. Cells were seeded in 96-well plates (6,000 cells/well). After incubation for 48 h, they were treated with increasing concentrations of the drugs as indicated in the figures. After 5 days of incubation, the number of cells were measured by the sulforhodamine B (SRB) assay as previously described [18]. Dose response curves were generated by fitting the data to sigmoidal dose-response curves and half maximal inhibitory concentration (IC₅₀) values were calculated using GraphPad Prism version 7 (GraphPad Software, La Jolla, CA).

Combination treatment

For each experiment, cells were seeded in ten 96-well plates (6,000 cells/well). Each plate was first treated with one of the concentrations of cisplatin (0 and a serial dilution ranging from 0.05 to 50 µmol/L) for 1 h, and, after siphoning off the medium with cisplatin, with a serial dilution of the respective combination drug (bortezomib: 0.84 to 59.5 nmol/L, carfilzomib: 0.20 to 200 nmol/L, ixazomib: 0.40 to 400 nmol/L, RG7112: 0.010 to 100 µmol/L). Each combination of concentrations was repeated in 6 wells. The plates were incubated with the inhibitor for 5 days followed by the SRB assay to determine cell numbers. The STR result were fitted to sigmoidal dose-response curves, and IC₅₀ values with 95% confidence intervals were calculated using GraphPad Prism. These parameters were calculated for both substances in each combination pair, determining the IC₅₀ values for each substance in combination with each concentration of the other. Only data that fitted the sigmoidal equation with an R² coefficient higher than 0.95 were used in the final analysis.

Western blot analysis

HN26 cells were treated as indicated after which attached cells were washed once in cold PBS and lysed in radioimmunoprecipitation assay buffer: 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mmol/L NaCl, 2 mmol/L Na₃VO₄, 1 mmol/L NaF, 20 mmol/L Na₄P₂O₇, complete protease inhibitors with EDTA (Roche Applied Science, Basel, Switzerland), and 50 mmol/L Tris-HCl, pH 7.4. The protein concentration of the lysates was determined by the Micro Bicinchoninic Acid protein assay (Thermo Scientific, Rockford, IL). Equivalent quantities of protein were electrophoresed on 4–12% NuPAGE Bis-Tris gels (Invitrogen) and transferred to polyvinylidene fluoride membranes. Specific proteins were detected with the indicated antibodies and the ECL prime chemiluminescence detection system (GE Healthcare, Fairfield, CT). The antibodies used were anti-p53 (#2527), anti-p21 (#2947), anti-caspase-3 (#9665), and anti-ERCC-1 (#5437) from Cell Signaling Technology (Danvers, MA) and PARP1 (#sc-7150)

Santa Cruz Biotechnology (Santa Cruz, CA). To control for gel-loading, the membranes were stained with 0.1% Coomassie R-350 in 50% methanol followed by quantification of the total protein content in each lane by densitometry [19]. A C33A2 cell lysate was used as positive control for p53. The C33A2 cell line is derived from the HPV-negative cervical cancer cell line C33A and has been described previously [20].

Cell cycle analysis

Cells were seeded in cell culture flasks and treated with the different proteasome inhibitors and the MDM2-inhibitor in combination with cisplatin and then fixed at different time points as indicated. After treatment, attached cells were trypsinized and mixed with floating cells, collected by centrifugation, followed by fixation with ice cold 70% ethanol. Cells were then stained with 50 µg/mL propidium iodide and analyzed on a FACS Calibur flow cytometer (Becton, Dickinson, San Jose, CA, USA) connected to a computer running CellQuest (Becton Dickinson) data collection software. The respective cell cycle phases were evaluated using ModFit LT software, version 3.1 (Verity Software House, Topsham, ME, USA).

Results

Determination of IC₅₀ values for proteasome and MDM2 inhibitors

In order to determine the sensitivity of the HN26 cell line to the various proteasome and MDM2 inhibitors, the cells were treated with the drugs and analyzed using the SRB assay (Fig. 1A-D). Each experiment was repeated twice. The IC₅₀ values were calculated to be 8.9 nmol/L for bortezomib, 25 nmol/L for carfilzomib, 47 nmol/L for ixazomib, and 7.8 µmol/L for RG7112 (Table 1). The IC₅₀ for cisplatin was previously determined to be 0.99 µmol/L [14].

Table 1
Sensitivity of HN26 to bortezomib, carfilzomib, ixazomib, and RG7112

Substance	IC ₅₀ (95% confidence interval)
Bortezomib	8.9 (8.6–9.2) nmol/L
Carfilzomib	25 (22–28) nmol/L
Ixazomib	47 (41–53) nmol/L
RG7112	7.8 (7.1–8.5) µmol/L
Cisplatin	0.99 (0.89–1.1) µmol/L ^a
^a From ref. [14]	

Effects of bortezomib, carfilzomib, ixazomib, and RG7112 in combination with cisplatin

To assess the effect of combining the various proteasome and MDM2 inhibitors with cisplatin, IC_{50} values for each drug and cisplatin as well as different combinations of those were determined and isobolograms were constructed. Each experiment was repeated three times with similar results. The combinations of cisplatin with the proteasome inhibitors bortezomib, carfilzomib and ixazomib all resulted in several data points significantly above the line interconnecting the IC_{50} values of the pure substances, indicating antagonistic effects when combining the drugs (Fig. 2A–C). For the MDM2 inhibitor RG7112, the results were indicative of an additive effect between RG7112 and cisplatin with all data points close to the interconnecting line (Fig. 2D). Thus, there was no synergy detected between any of the substances and cisplatin.

Molecular and cellular effects

To investigate the lack of synergy between proteasome or MDM2 inhibitors and cisplatin, the levels of p53 and other proteins involved in cell cycle regulation, apoptosis and DNA repair were determined after treatment with the various inhibitors alone, and in combination with cisplatin. Initially, the time dependence was tested using one of the substances, bortezomib. The cells were analyzed between 1 and 96 h after treatment start with bortezomib, cisplatin or a combination of the two drugs. The largest effects were seen at the 24 h time point (Fig. 3) and this time point was chosen for comparison of the different compounds.

The p53 protein was only detected at very low levels in untreated HN26 cell as expected (Figs. 3 and 4). Incubation of HN26 cells with bortezomib, ixazomib, and RG7112, induced detectable, but low, levels of p53 (Fig. 4).

The p21 protein was expressed in the untreated HN26 cells but increased substantially in the presence of bortezomib and carfilzomib (Fig. 4). Ixazomib and RG7112 did not increase the p21 expression, while cisplatin exposure resulted in decreased expression (Fig. 4). However, ixazomib restored the cisplatin-induced reduction of p21, an effect that was not seen with RG7112 (Fig. 4).

The cleavage of 116 kDa PARP1 to its 85 kDa fragment and of the 35 kDa caspase-3 to its 17/19 kDa fragment were monitored as markers of the apoptotic process. We could not detect cleavage of any of the proteins by Western blotting, indicating that apoptosis was not efficiently induced with the substances analyzed (Fig. 4).

The excision repair cross-complementation group 1 (ERCC1) protein expression was also analyzed in response to cisplatin and the proteasome inhibitors since this protein has an important role in repair of cisplatin-induced DNA damage [21, 22]. The ERCC1 protein was present in untreated HN26 cells, but only marginal effects on ERCC1 levels were seen with the different treatments (Fig. 4).

Cell cycle analysis

Cell cycle distribution was analyzed using flow cytometry. With all inhibitors, there was an accumulation of cells in G1/G0-phase with concomitant decrease of cells in S-phase. This was particularly pronounced with bortezomib and ixazomib (Fig. 5). Cisplatin treatment, on the other hand, resulted in an accumulation of cells in S-phase with lower percentage in G1/G0. When cisplatin was combined with bortezomib, this effect of cisplatin was reversed to the distribution seen in the control.

Discussion

In this study, we treated the HPV-positive OPSCC cell line HN26 with three proteasome inhibitors and one MDM2-inhibitor in combination with cisplatin. The main objective was to determine if there could be synergistic effects when combining cisplatin with the potentially p53 enhancing substances when treating HPV-positive tonsil cancer. Such synergistic effects have previously been documented in both uterine cervical cancer and HPV-positive head and neck squamous cell carcinoma cell lines [13, 23] and have been proposed based on the high proportion of wild-type *TP53* in HPV-positive cancers and the importance of p53 removal by the HPV E6 protein for carcinogenesis.

In our experiments, all three proteasome inhibitors displayed antagonistic effects in combination with cisplatin while additive effects were seen with the MDM2 inhibitor. Thus, no synergy could be detected with any of the combinations. This implies that the combination of proteasome inhibition with cisplatin might result in negative effects for HPV positive OPSCC patients rather than improved treatment outcomes. Possibly this could be different for MDM2 inhibition, though only additive effects were detected.

To us, these findings were unexpected. Our original hypothesis was that the proteasome and MDM2 inhibitors would increase the expression of p53 leading to increased apoptosis and a synergistic effect on cell killing in combination with cisplatin. Instead, the effects on p53 protein expression were barely above the detection level of the Western blot for bortezomib, ixazomib and RG7112 and below it for carfilzomib (Figs. 3 and 4). This contrasted with an earlier study in which bortezomib induced a substantial increase in p53 expression in three HPV positive HNSCC cell lines [13]. We also investigated if there was increased expression at other time points after treatment start for bortezomib, but the peak of expression was at 24 h also in combination with cisplatin (Fig. 3).

In line with the marginal effects on p53 expression, we could not detect any apoptosis as measured by PARP1 or caspase-3 cleavage. This was true for all substances and combinations with cisplatin (Fig. 4) – also tested at different time points for bortezomib (Fig. 3). Obviously, the expected increase in p53 mediated apoptosis leading to an enhanced effect of cisplatin did not occur in the investigated cell line. The reason for this cannot be deduced from our results, but the fact that the MDM2 inhibitor did not increase p53 expression and that p21 expression was increased by the proteasome inhibitors indicated that it was not failure to inhibit the proteasome activity that caused the lack of effect. An ubiquitin-independent E6 promoted degradation of p53 has been described and could possibly explain our results [24].

The *TP53* gene in HN26 is homozygous for arginine at codon 72 which has been shown to make p53 more susceptible for E6 mediated degradation [25]. Potentially, this might affect the ability of proteasome inhibition to substantially increase the levels of p53 protein. But on the other hand, at least one of the cell lines (UD-SCC-2) used by Li and Johnson [13] was shown to be homozygous for arginine at codon 72 [26] while one of the other cell lines (UPCI:SCC090) was heterozygous for proline and arginine at this site [27], while still displaying bortezomib induced up-regulation of p53. Thus, rendering it unlikely that the effect on HN26 cells was due to arginine at position 72.

A large number of different proteins are regulated by proteasome degradation, for example, in apoptosis and cell cycle control. In the absence of p53 up-regulation with resulting increase in apoptosis as a response to proteasome inhibition, a multitude of different outcomes are therefore possible. We investigated if cell cycle progression was affected by proteasome inhibition in the HPV-positive cell line and found accumulation of cells in G1/G0 and fewer cells in S phase after incubation with all proteasome inhibitors, while incubation with cisplatin resulted in an increase of cells in S-phase. When combining bortezomib with cisplatin, the cisplatin induced S-phase increase was reversed (Fig. 5). Thus, a possible explanation for the reduced efficacy of cisplatin when combined with inhibitors could be the G1-arrest caused by the inhibitors, giving the cells time to repair cisplatin-induced DNA-damages before migration in the cell cycle, and hence becoming less prone to succumb from mitotic catastrophe.

Bortezomib and carfilzomib both induced a high increase in p21 expression, whereas ixazomib increased p21 in combination with cisplatin as compared with cells treated with cisplatin alone. As p21 is regulated by proteasome degradation, this could be one mechanism responsible for the observed cell cycle alterations.

However, also the MDM2 inhibitor RG7112 affected cell cycle progression to an extent comparable with the proteasome inhibitors, although not antagonizing the effects of cisplatin (Fig. 5). Thus, cell cycle regulation per se does not seem to suffice in explaining the antagonistic effect of the proteasome inhibitors. As RG7112 did not affect p21 expression, neither with nor without cisplatin, one possibility is that other p21-regulated mechanisms are involved, or that other proteins that are affected by proteasome inhibition play an important role in HN26 cells.

Conclusions

To summarize, we did not find evidence for synergism between proteasome or MDM2 inhibitors and cisplatin in this study. On the contrary, the effect of combining proteasome inhibitors with cisplatin was antagonistic. The results indicate a lack of pronounced p53 up-regulation with resulting increase of apoptosis. Instead, there were changes in cell cycle distribution that could facilitate increased DNA damage repair. One possible regulator of the cell cycle modifications could be p21, which was upregulated by the proteasome inhibitors. MDM2 inhibition also failed to increase p53 expression but did not antagonize the effect of cisplatin. These observations raise concerns regarding future clinical studies exploring proteasome inhibitors in combination with cisplatin and other cytostatic drugs for HPV-positive

OPSCC patients. One such phase I trial combining bortezomib with cetuximab and radiation was prematurely terminated due to early tumor progression. These results were assumed to depend on effects of proteasome inhibition on EGFR and cell survival signaling [28]. Our results do not contraindicate MDM2 inhibition in combination with cisplatin, though the lack of synergy could decrease the rationale for clinical testing of this combination.

Thus, thorough pre-clinical testing in appropriate HPV positive cell and animal models is warranted for proteasome inhibiting substances in combination with cytostatic drugs prior to new clinical trials. In fact, it might even be contraindicated to further exploit proteasome inhibition to boost the activity of other cytostatic drug for HPV positive OPSCC patients. An alternative approach previously suggested by us could be to exploit substances that inhibit the transcription of the HPV16 E6 and E7 genes as the E6 and E7 mRNA and proteins are labile and quickly degraded, p53 levels are restored and cause apoptosis of the cancer cells [29].

Abbreviations

HPV

human papilloma virus, OPSCC:oropharyngeal squamous cell carcinoma, HNC:head and neck cancer, MDM2:murine double minute 2, SRB:sulforhodamine B, IC₅₀:half maximal inhibitory concentration, BTZ:bortezomib, CFZ:carfilzomib, IXZ:ixazomib, NER:nucleotide excision repair.

Declarations

Ethics approval and consent to participate

The cell line was established earlier and used after approval by Lund University regional ethical review board (LU 376-01).

Consent for publication

Not applicable

Availability of data and material

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study. Any raw data not included in the article is available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

NS designed, performed and analyzed the experiments, and drafted the manuscript.

GA took part in the cell cycle analyses and revised the manuscript.

SS conceived of the study and drafted the manuscript.

JW conceived of the study and revised the manuscript.

LE conceived of the study, designed and evaluated the experiments, and drafted the manuscript.

All authors have read and approved the manuscript.

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Figures

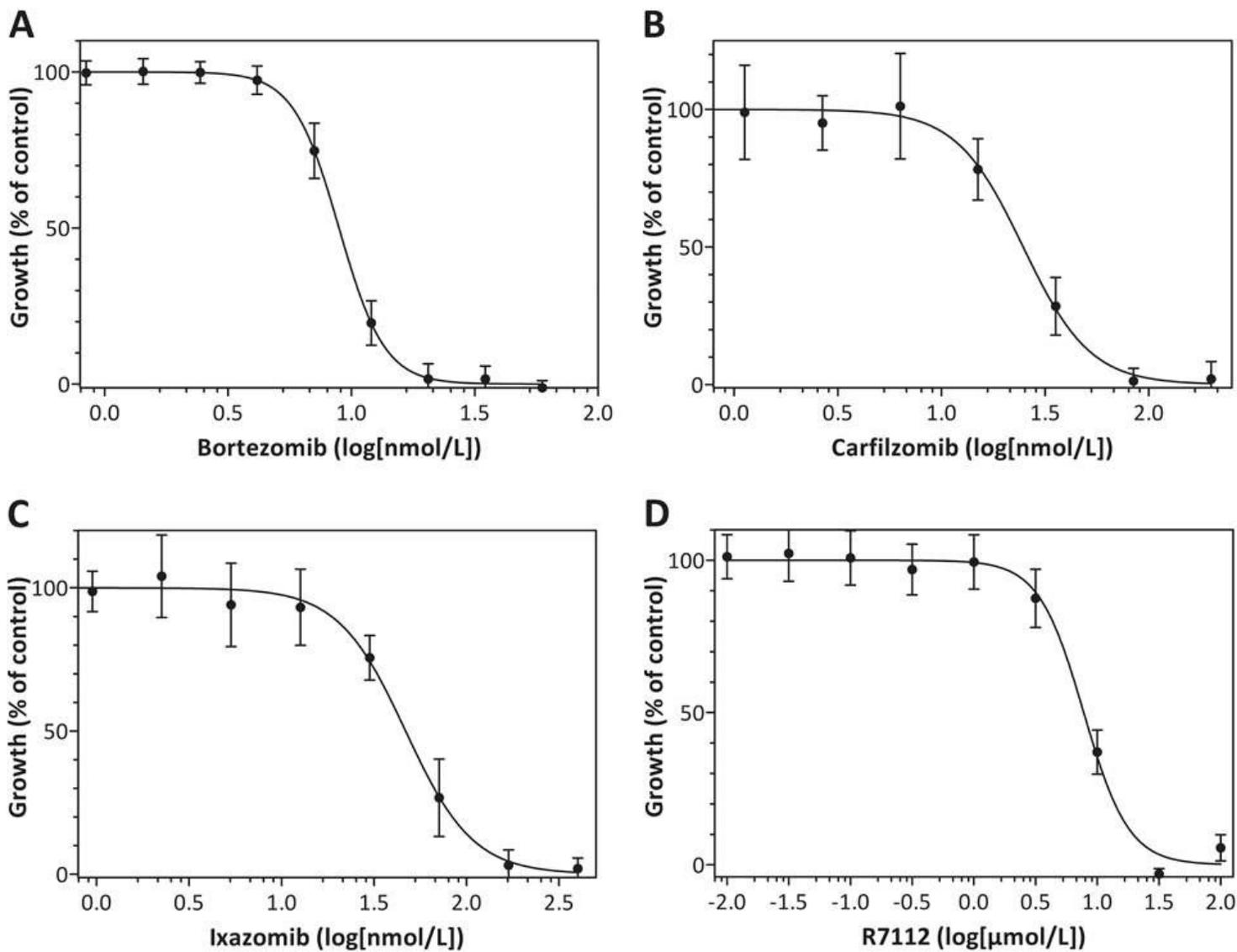


Figure 1

Determination of IC₅₀ for A. bortezomib, B. carfilzomib, C. ixazomib, and D. R7112 on HN26 cells. Cells were seeded in 96-well plates, incubated for five days and then analyzed by the SRB assay. Each experiment was repeated twice with six determination per drug concentration. Error bars show 95% confidence interval.

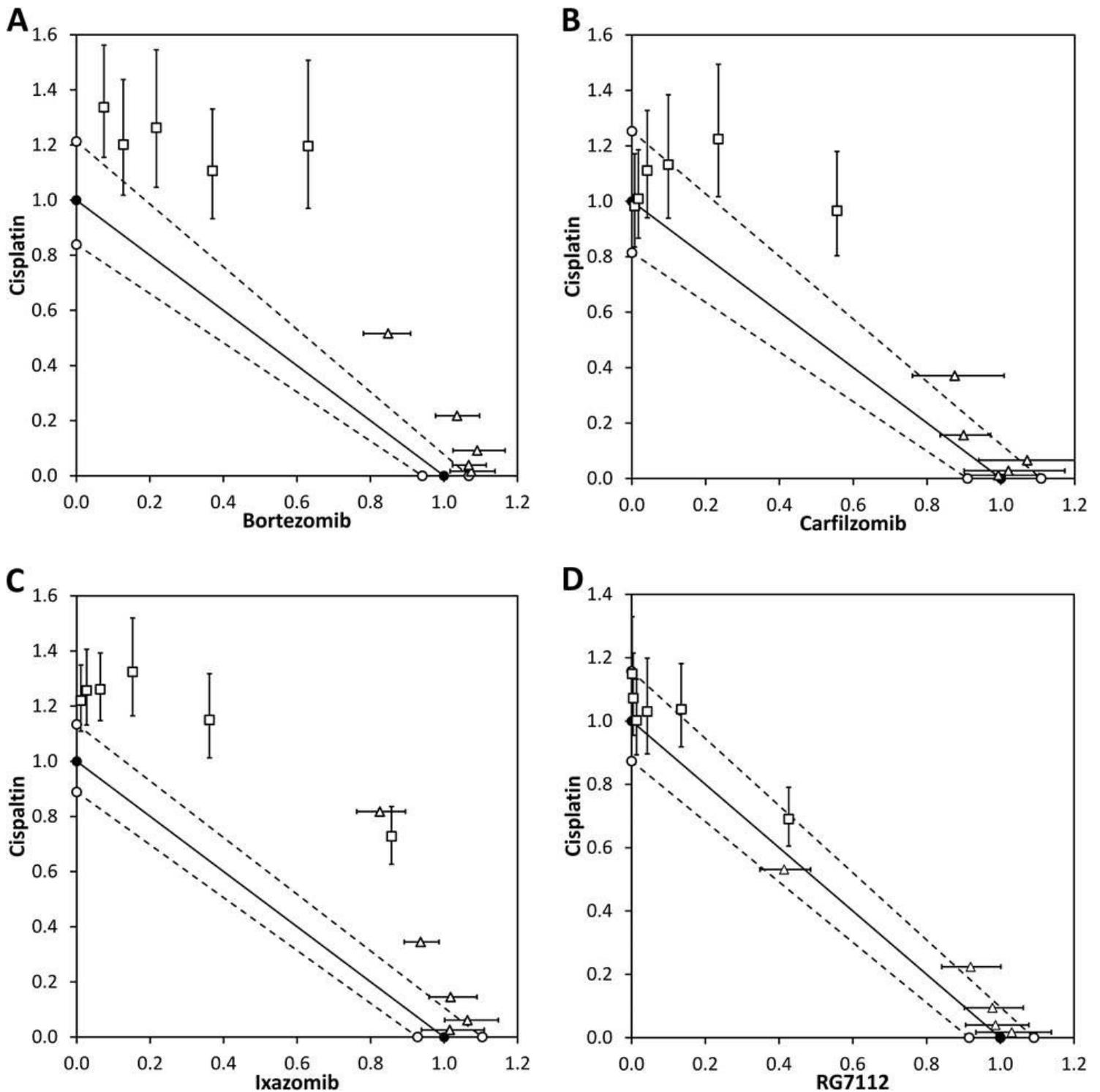


Figure 2

Representative relative isobolograms for the interactions between cisplatin and the different substances on HN26 cells. All IC₅₀ values for each graph were determined in single experiments to avoid cell batch and dilution variations (as detailed in the Materials and Methods section) and plotted as fractions of the respective single substance values. The cells were incubated for one hour with varying concentrations of cisplatin and then for five days with varying concentrations of the other substances. A solid line was drawn between the IC₅₀ values of both substances in single treatment (filled circles) with the dotted lines

indicating the 95% confidence interval (open circles). Squares indicate the IC50 values for cisplatin at different concentrations of the other substance and triangles the IC50 value of the other substance at different cisplatin concentrations. Error bars indicate 95% confidence interval. The analyses were repeated 3 times with similar results. All experiments were performed with 0–50 $\mu\text{mol/L}$ cisplatin in combination with (A) bortezomib, 0–59.5 nmol/L, (B) carfilzomib, 0–200 nmol/L, (C) ixazomib, 0–400 nmol/L, and (D) RG7112, 0–100 $\mu\text{mol/L}$.

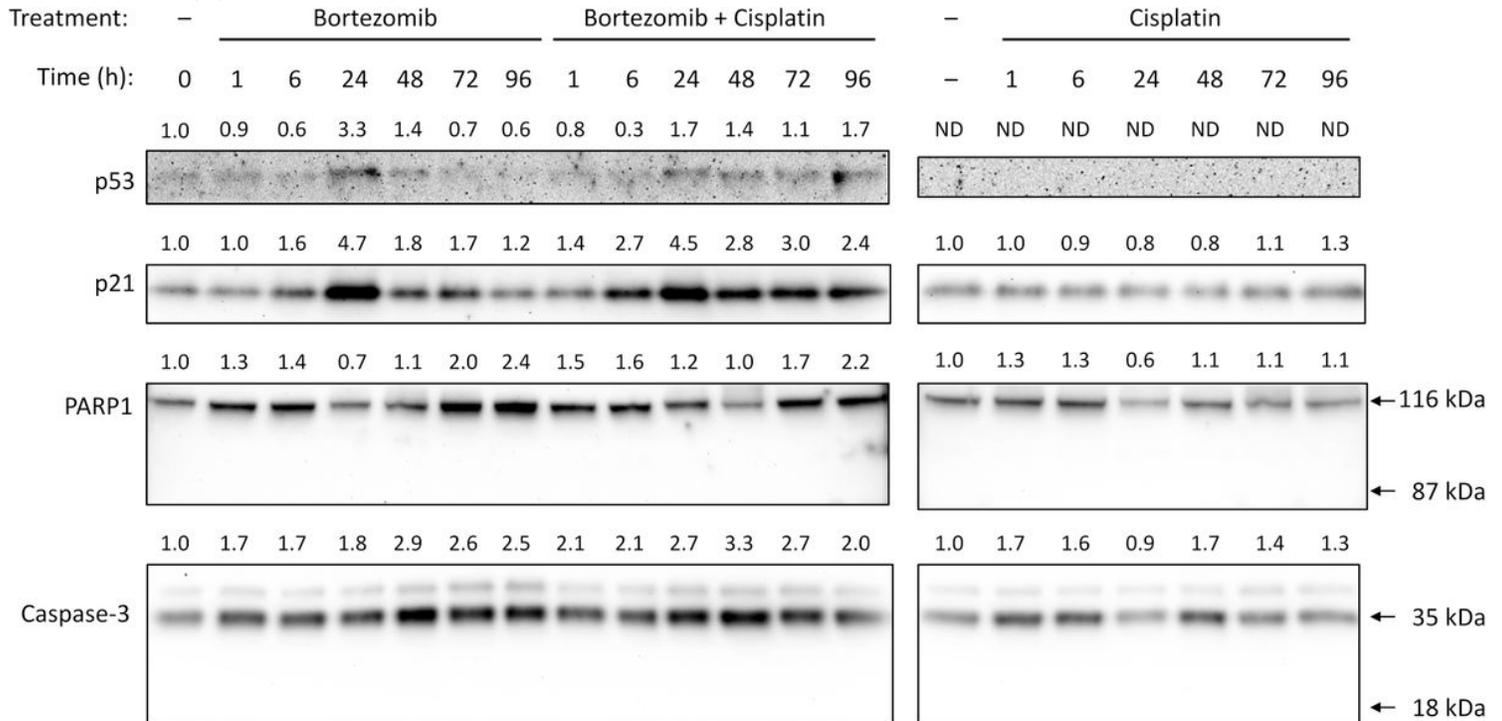


Figure 3

HN26 cells (50–80% confluent) were treated with the IC50 concentrations of bortezomib (8.9 nmol/L) and cisplatin (0.99 $\mu\text{mol/L}$ [12]). For bortezomib alone the cells were treated with the inhibitor for the indicated times. For combination treatments with the two drugs, the cells were first treated with cisplatin for 1 h and then with bortezomib for the indicated times. For cisplatin alone, the cells were treated with cisplatin for 1 h and then with R10 medium for the indicated times. Each expression was normalized according to protein load and the relative expression, in comparison with control, calculated and indicated above the protein bands.

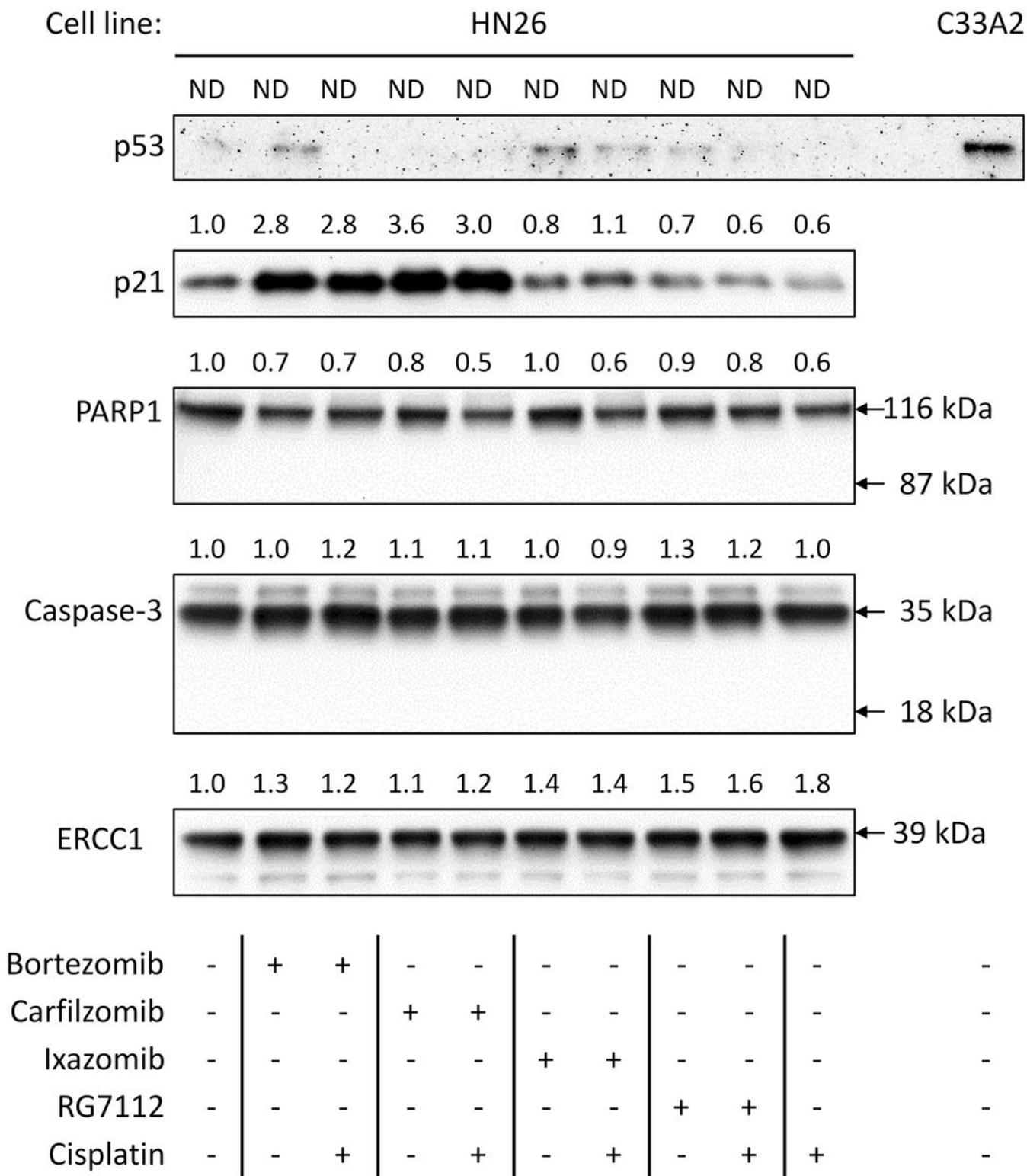


Figure 4

HN26 cells (50–80 % confluency) were treated with the IC₅₀ concentration of each inhibitor for 24 h: 8.9 nmol/L bortezomib, 25 nmol/L carfilzomib, 47 nmol/L ixazomib, 7.8 μmol/L RG7112 (Table 1). For cisplatin, the cells were treated with IC₅₀ concentration (0.99 μmol/L [12]) for 1 h and then incubated with R10 medium for 24 h. For the combination treatments, the cells were first incubated with cisplatin for 1 h and then with the inhibitors for 24 h. Each expression was normalized according to protein load and the

relative expression, in comparison with control, calculated and indicated above the protein bands. C33A2 cells were used as positive control for p53 (1/10 of the protein amount in the HN26 lanes was loaded to enable comparable detection intensities).

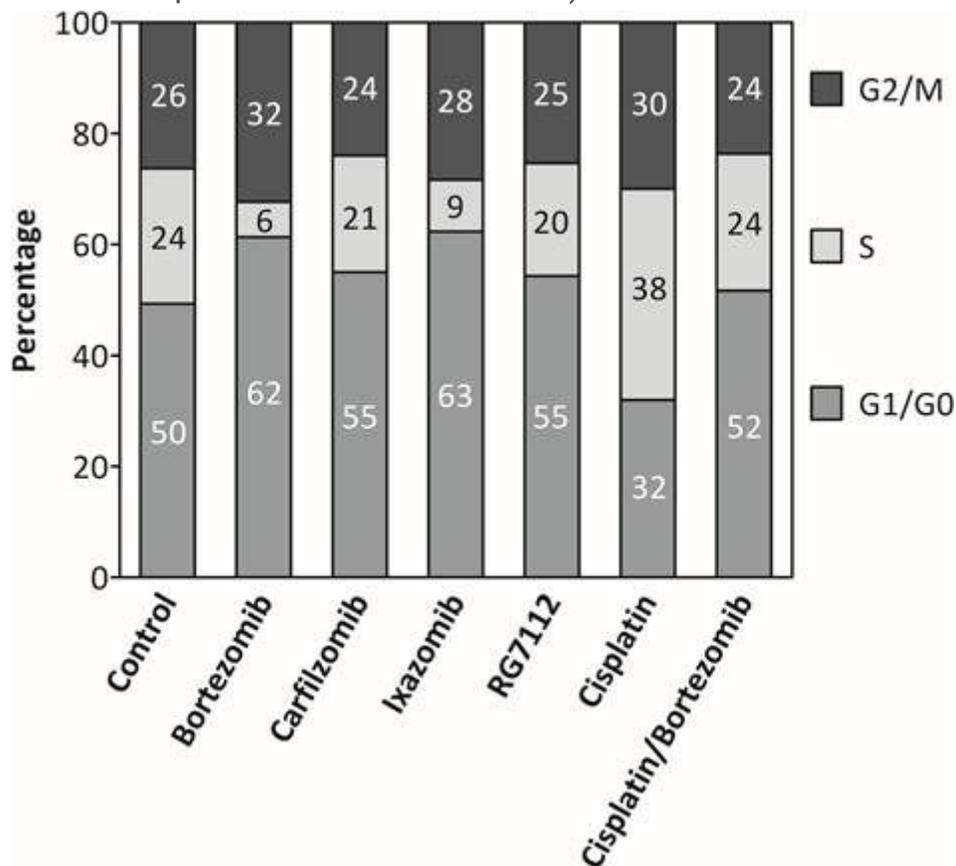


Figure 5

HN26 cells (40–60% confluent) were treated with IC50 concentrations of each substance (8.9 nmol/L bortezomib, 25 nmol/L carfilzomib, 47 nmol/L ixazomib, 7.8 μ mol/L RG7112 (Table 1) for 24 h after which the cell cycle distributions were determined by flow cytometry. For cisplatin, the cells were incubated with 0.99 μ mol/L substance [12]) for 1 h and then with R10 medium or bortezomib for 24 h before flow cytometry measurement. The treatments were performed in triplicate.