

# Bortezomib activity and in vitro interactions with anthracyclines and cytarabine in acute myeloid leukemia cells are independent of multidrug resistance mechanisms and p53 status

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## Abstract

**Purpose** The proteasome inhibitor bortezomib may be effective in combination with cytarabine and anthracyclines in the treatment of acute myeloid leukemia (AML) by virtue of targeting aberrantly activated NF- $\kappa$ B in AML stem cells. We tested whether bortezomib cytotoxicity is affected by multidrug resistance (MDR) proteins expressed in AML cells. We also tested whether bortezomib interactions with cytarabine and anthracyclines are affected by p53, because proteasome inhibition stabilizes p53 and may thus cause cell cycle arrest.

**Experimental design** Bortezomib sensitivity of cell lines overexpressing P-glycoprotein, multidrug resistance protein-1, breast cancer resistance protein and lung resistance protein was studied in the presence and absence of established modulators of these transport proteins. Drug interactions during simultaneous and sequential exposure to bortezomib and anthracyclines or cytarabine in diverse ratios were evaluated by isobologram and combination index analyses in AML cell lines with wild type and inactive p53 and were correlated with cell cycle perturbations induced by bortezomib.

**Results** Of the MDR mechanisms studied, only P-glycoprotein conferred resistance to bortezomib, and

resistance was only twofold. Interactions between bortezomib and anthracyclines and cytarabine changed from antagonistic to additive or synergistic with increasing drug activity levels and were not affected by p53 status. **Conclusions** MDR proteins and p53 do not affect bortezomib cytotoxicity or in vitro interactions with anthracyclines or cytarabine, but these interactions are concentration-dependent, and this concentration-dependency should be considered in the design of combination regimens.

**Keywords** Bortezomib · AML · P-glycoprotein · Multidrug resistance protein-1 · Breast cancer resistance protein · p53

## Introduction

Cure rates in acute myeloid leukemia (AML) remain low, and new treatments continue to be needed for this disease. Mechanisms known to contribute to treatment failure in AML include impaired cellular drug transport associated with expression of ATP-binding cassette (ABC) proteins, including P-glycoprotein (Pgp; ABCB1), multidrug resistance protein-1 (MRP-1; ABCC1) and breast cancer resistance protein (BCRP; ABCG2) [6, 20, 21, 32], and impaired cytoplasmic-nuclear drug transport associated with expression of the major vault protein lung resistance protein (LRP) [24]. In addition to impaired drug transport, aberrant activation of signal transduction proteins including the transcription factor nuclear factor-kappa B (NF- $\kappa$ B) has been implicated in treatment failure in AML [15].

Constitutive activation of NF- $\kappa$ B in AML stem cells [15] suggests that therapies inhibiting NF- $\kappa$ B have the

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potential to target AML stem cells, and lack of activation of NF- $\kappa$ B in normal hematopoietic stem cells [15] suggests that these therapies could be leukemia-specific. In *in vitro* studies, NF- $\kappa$ B inhibition specifically induces apoptosis in AML cells and potentiates the apoptotic response induced by doxorubicin, cytarabine and etoposide, while sparing normal CD34+ hematopoietic precursors [12]. Activation of NF- $\kappa$ B requires proteasome-dependent degradation of its inhibitor I $\kappa$ B [5, 19], and proteasome inhibitors thus have therapeutic potential in AML by virtue of targeting NF- $\kappa$ B.

Mechanisms of resistance to NF- $\kappa$ B inhibition have not been studied in AML cells. Bortezomib, the first proteasome inhibitor approved for use in humans [8], was designed to abrogate transport by Pgp, in contrast to the earlier generation of proteasome inhibitors, peptide aldehydes, whose efficacy was limited by Pgp-mediated transport [1, 18]. In contrast, transport by MRP-1, BCRP and LRP has not been studied. Moreover, in addition to its effects on NF- $\kappa$ B, bortezomib also stabilizes p53, resulting in cell cycle arrest (reviewed in Ref. [36]). Sensitivity to bortezomib and/or its interaction with cytotoxic chemotherapy drugs as a function of p53 status may be cell type-dependent (reviewed in Ref. [7]), with p53-dependence reported for breast [26] and lung [23] cancer and p53-independence reported for prostate [1] and colon [11] cancer and multiple myeloma [16]. We studied the effects of p53 status on the efficacy of bortezomib in combination with anthracyclines and cytarabine in AML cells, and correlated the effects of p53 status with bortezomib effects on cell cycle.

## Materials and methods

### Cell lines

Human AML cell lines included HL60, which is p53-null [20], ML1, which has wild-type p53 [22], and established drug-selected variants of HL60 cells with multidrug resistance mediated by Pgp (HL60/VCR) and MRP-1 (HL60/Adr). Because leukemia cell lines expressing BCRP and LRP are not available, we studied drug-selected 8226/MR20 multiple myeloma cells, which overexpress BCRP and LRP, and also studied parental 8226 cells, with low-level BCRP expression, and drug-selected 8226/Dox6 cells, with Pgp and low-level BCRP expression. Cell culture conditions and characterization of MDR protein expression and function in these cell lines were previously described [28, 31].

### Drugs

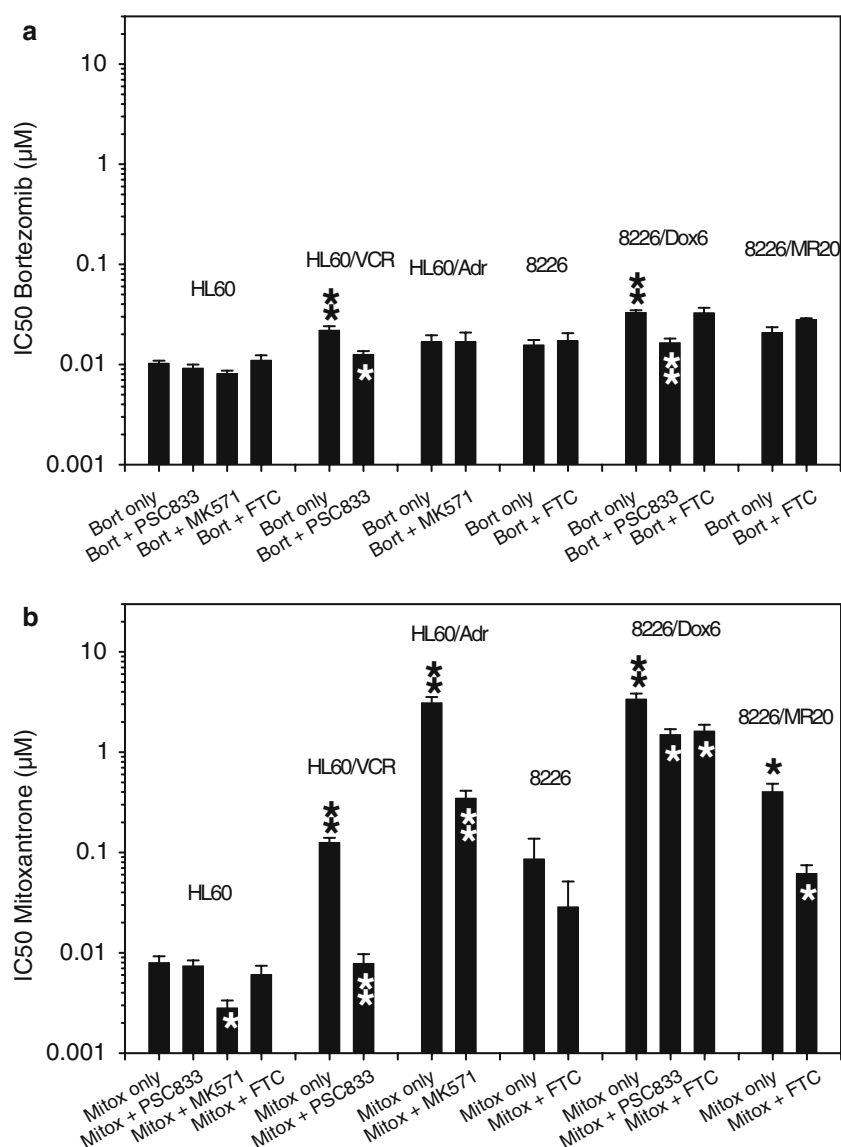
Bortezomib (Velcade) was obtained from Millennium Pharmaceuticals Inc., Cambridge, MA, USA, and stored at  $-20^{\circ}\text{C}$  as a 50  $\mu\text{M}$  stock solution in phosphate-buffered saline (PBS). Doxorubicin, daunorubicin and idarubicin were purchased from Sigma-Aldrich Corp., St. Louis, MO, USA and stock solutions of 1 mg/ml in PBS were stored at  $-20^{\circ}\text{C}$ . Mitoxantrone was also purchased from Sigma-Aldrich, and a stock solution of 19.3 mM in PBS was stored at  $-20^{\circ}\text{C}$ . Cytarabine was purchased from The Upjohn Company, Kalamazoo, MI, USA and stored as a 358 mM stock solution at  $-20^{\circ}\text{C}$ . MDR modulators used in comparison studies included PSC-833, which is Pgp-specific, obtained from Novartis, East Hanover, NJ, USA, MK-571, which is MRP-1-specific, purchased from Calbiochem, San Diego, CA, USA, and fumitremorgin C (FTC), which is BCRP-specific, obtained from Dr. Susan Bates, National Cancer Institute, Bethesda, MD, USA, at concentrations of 2.5, 15 and 10  $\mu\text{M}$ , respectively [31].

### Cytotoxicity assay

To study cytotoxicity of drugs alone or in combination with fixed non-cytotoxic concentrations of MDR modulators, cell lines were treated with drug at 11 logarithmically spaced concentrations (five-log range) in triplicate in 96-well plates. Cells were seeded at a starting density of 10,000/well and drugs were added to the cell cultures from frozen stocks to achieve the final desired concentrations in 100  $\mu\text{l}$  final volumes. Cells were then cultured for 96 h, and viability was quantified by the WST-1 colorimetric assay (Roche Diagnostics, Mannheim, Germany), as described previously [31]. Briefly, 10  $\mu\text{l}$  WST-1 were added to each well and culture plates were allowed to develop for 2–4 h in the incubator to achieve the desired absorbance in the control wells. Absorbance at 450 nm (A450) and 600 nm (A600, background) was read for each well using a plate reader (Beckman Coulter, Fullerton, CA, USA). The drug concentrations resulting in 50% decrease of viable cell numbers compared to the non-drug-treated controls ( $\text{IC}_{50}$ ) were determined through weighted curve-fitting, as described previously [14].  $\text{IC}_{50}$  concentrations in different treatment groups were compared by non-paired *t*-test statistics.

To study combinations of bortezomib with anthracyclines or cytarabine, experiments were conducted with the same procedures as for single agent studies, but in quintuplicate 96-well plates. Each plate

**Fig. 1** In vitro sensitivity of cell lines overexpressing Pgp (HL60/VCR, 8226/Dox6), MRP-1 (HL60/Adr), BCRP (8226/MR20, 8226/Dox6) and LRP (8226/MR20) to **a** bortezomib and **b** mitoxantrone in the absence and presence of the MDR protein-specific modulators PSC833 (Pgp), MK571 (MRP-1) or FTC (BCRP). Parental HL60 cells do not express any of these MDR transport proteins, whereas parental 8226 cells express a low level of BCRP. Each bar represents the mean IC<sub>50</sub> concentration of triplicate experiments and error bars represent the standard errors of the mean. The black \* and \*\* above the bars denote  $p < 0.01$  and  $0.001$ , respectively, for differences between IC<sub>50</sub>'s of drug-resistant and parental cell lines treated with drug (bortezomib or mitoxantrone) alone. The white \* and \*\* inside the bars denote  $p < 0.01$  and  $0.001$ , respectively, for differences between IC<sub>50</sub>'s of cells treated with drug (bortezomib or mitoxantrone) in the absence and presence of modulator



contained each single agent alone at 11 concentrations and drug combinations at five constant ratios (1:1 at the IC<sub>50</sub>, 2:1, 1:2, 4:1, 1:4) at 11 different concentrations. Drug interactions were then evaluated by isobologram analysis, as previously described [14], and by combination index analysis according to the method of Chou and Talalay [9], using CalcuSyn Software (Biosoft, Cambridge, UK).

#### Cell cycle distribution

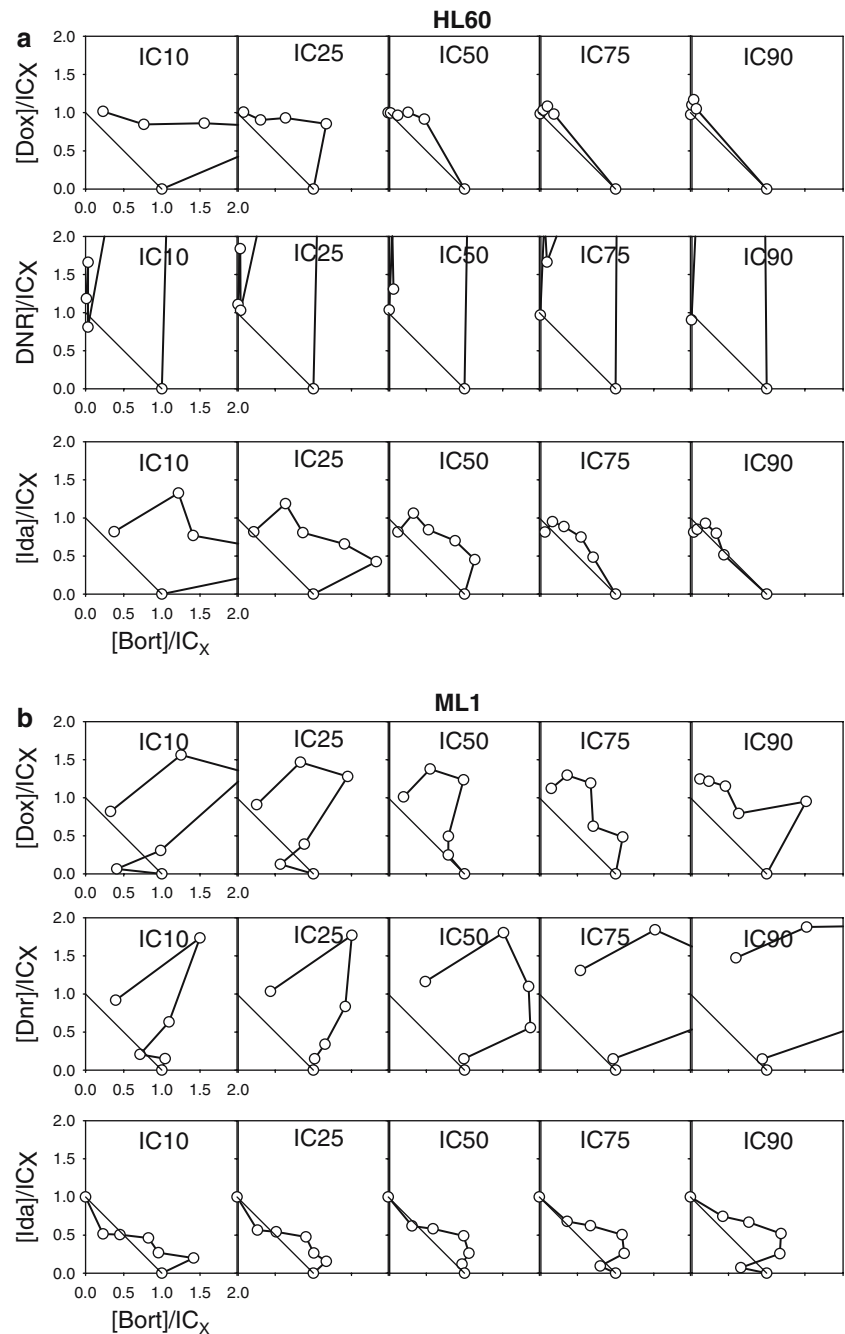
To study cell cycle distribution before and during bortezomib exposure,  $0.5 \times 10^6$  cells at each time point were fixed in 70% ice-cold ethanol for at least 1 h, then washed with PBS, stained with propidium iodide (PI) and analyzed on a FacScan flow cytometer as previously described [38].

## Results

### Activity of bortezomib in cell lines overexpressing MDR proteins

Sensitivity of multidrug resistant HL60/VCR, HL60/ADR, 8226/Dox6 and 8226/MR20 cells and of parental HL60 and 8226 cells to bortezomib was tested in the absence and presence of the MDR protein-specific modulators PSC-833 (Pgp), MK571 (MRP-1) and FTC (BCRP) (Fig. 1a). HL60/VCR and 8226/Dox6, the two cell lines expressing Pgp, displayed a low but significant level of resistance to bortezomib (1.8- and 2.0-fold resistance, respectively) and resistance was significantly modulated by the Pgp-specific modulator PSC-833. The HL60/ADR and 8226/MR20 cell lines, expressing MRP-1 and BCRP, respectively, were not

**Fig. 2** Isobologram analysis of interactions between bortezomib and doxorubicin (*top row*), daunorubicin (*middle row*) or idarubicin (*bottom row*) under simultaneous drug exposure conditions in 96-h cultures. For each drug combination, the isobols at the IC<sub>10</sub>, IC<sub>25</sub>, IC<sub>50</sub>, IC<sub>75</sub> and IC<sub>90</sub> drug activity levels are shown. **a** Results for the p53-null cell line HL60 and **b** results for the wild-type p53 cell line ML1

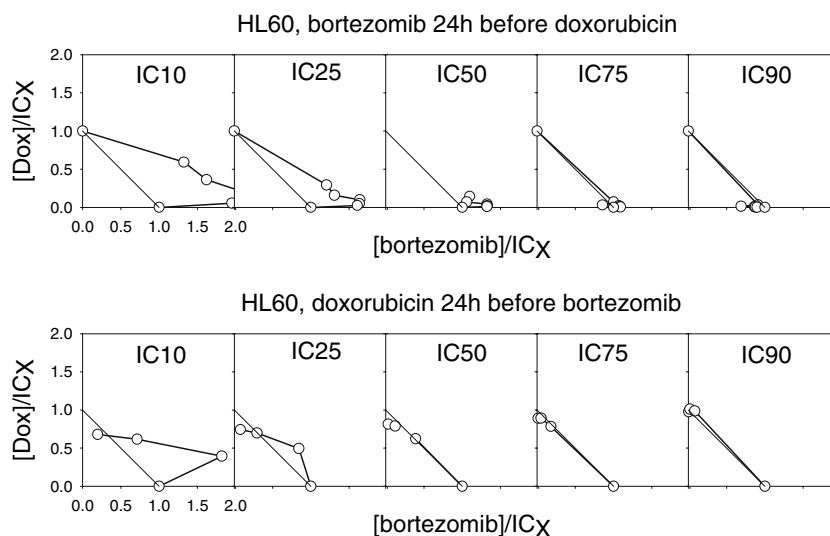


resistant to bortezomib, in relation to parental cells, and the MRP-1- and BCRP-specific modulators MK571 and FTC had no effect on bortezomib sensitivity. Of note, since 8226/MR20 cells also overexpress LRP [31], the data indicate that bortezomib is also not subject to LRP-mediated resistance.

As a control, sensitivity of the same cell lines to the MDR protein substrate drug mitoxantrone was determined in the absence and presence of modulators (Fig. 1b). Multidrug resistant HL60/VCR, HL60/

ADR, 8226/Dox6 and 8226/MR20 cells were 15.8-, 390-, 39- and 4.7-fold resistant to mitoxantrone, respectively, and were sensitized by the relevant MDR modulators. FTC sensitization of parental 8226 cells was consistent with their known low-level BCRP expression [28]. MK571 unexpectedly modulated sensitivity of the HL60 parental cell line to mitoxantrone 2.8-fold, but this modulator had a much greater (ninefold) effect on mitoxantrone sensitivity of HL60/Adr cells.

**Fig. 3** Isobologram analysis of interactions between bortezomib and doxorubicin with bortezomib exposure started 24 h before doxorubicin exposure (*top panels*) and doxorubicin 24 h before bortezomib (*bottom panels*). For each drug combination, the isobols at the IC<sub>10</sub>, IC<sub>25</sub>, IC<sub>50</sub>, IC<sub>75</sub> and IC<sub>90</sub> drug activity levels are shown



Thus all of the MDR mechanisms studied, including Pgp, MRP-1, BCRP and LRP, confer resistance to mitoxantrone, but only Pgp conferred resistance to bortezomib, and resistance was low-level (twofold).

#### Effect of p53 status on interaction between bortezomib and anthracyclines in AML cells

To determine the effect of p53 status on the interaction between bortezomib and anthracyclines in AML cells, the effect of simultaneous exposure to bortezomib and doxorubicin, daunorubicin or idarubicin at fixed constant drug concentration ratios was studied and compared to the activity of each drug as a single agent in HL60 (p53 null) and ML1 (wild-type p53) cells. Figure 2 shows the isobologram analysis of the interactions between bortezomib and doxorubicin, daunorubicin and idarubicin at five different drug activity levels (IC<sub>10</sub>, IC<sub>25</sub>, IC<sub>50</sub>, IC<sub>75</sub> and IC<sub>90</sub>). In each plot, the diagonal represents the additivity line, and data below and above the additivity line signify synergistic and antagonistic interactions, respectively. In HL60 cells the interaction between bortezomib and the anthracyclines was generally antagonistic, in particular at lower drug activity levels, but for doxorubicin and idarubicin the degree of antagonism decreased with increasing drug activity levels (Fig. 2a). The interaction between bortezomib and anthracyclines was also generally antagonistic in ML1 cells, and the degree of antagonism decreased with increasing drug activity levels for doxorubicin, but antagonism increased with

increasing drug activity levels for daunorubicin and idarubicin (Fig. 2b).

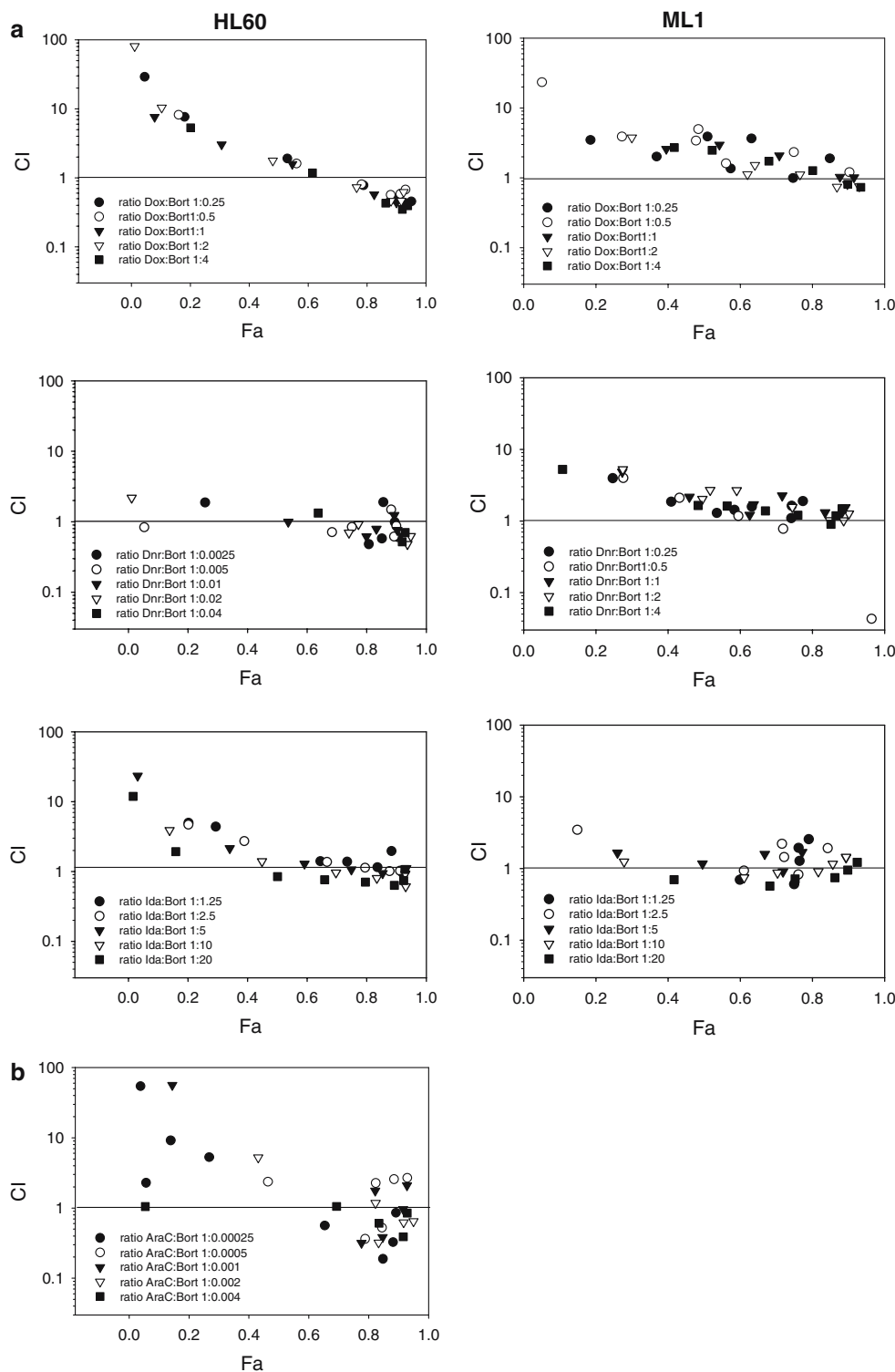
#### Effect of drug sequence on interaction between bortezomib and anthracyclines

Since other reports have indicated that interactions between bortezomib with anthracyclines may be schedule-dependent [4, 29], the effect of a 24-h delay between the start of drug exposures was studied, with doxorubicin as a representative anthracycline. The isobols in Fig. 3 demonstrate that in HL60 cells, the interaction between doxorubicin and bortezomib was independent of the sequence of drug exposure.

#### Effect of method of analysis

In order to determine whether the observed drug interactions were affected by the method of analysis, all of the data were additionally analyzed using the combination index method of Chou and Talalay [9]. In the plots shown in Fig. 4, a combination index (CI) value of 1 equates with drug additivity, while CI values below or above 1 indicate synergistic or antagonistic drug interactions, respectively. Whereas with the isobologram analysis the drug interactions at different drug activity levels are plotted individually, in the CI plots interactions at different drug activity levels (Fa) are combined in one plot. The data in Fig. 4 demonstrate that, in agreement with the isobologram analyses, interactions between bortezomib and the anthracyclines were generally antagonistic in both HL60 (p53-null) and ML1 (wild-type p53) cells, regardless of the constant ratio

**Fig. 4 a** Combination index (CI) analysis (Chou and Talalay method) of interactions between bortezomib and doxorubicin (*top*), daunorubicin (*middle*) or idarubicin (*bottom*) under simultaneous drug exposure conditions (96-h cultures). For each drug combination, CI's at various drug activity levels (Fa) and various constant drug concentration ratios (as indicated in each plot) are shown. Results for the p53-null cell line HL60 are shown on the *left* and results for the wild-type p53 cell line ML1 are shown on the *right*. **b** Combination index (CI) analysis (Chou and Talalay method) of the interactions between bortezomib and cytarabine (Ara-C) in HL60 cells under simultaneous drug exposure conditions in 96-h cultures



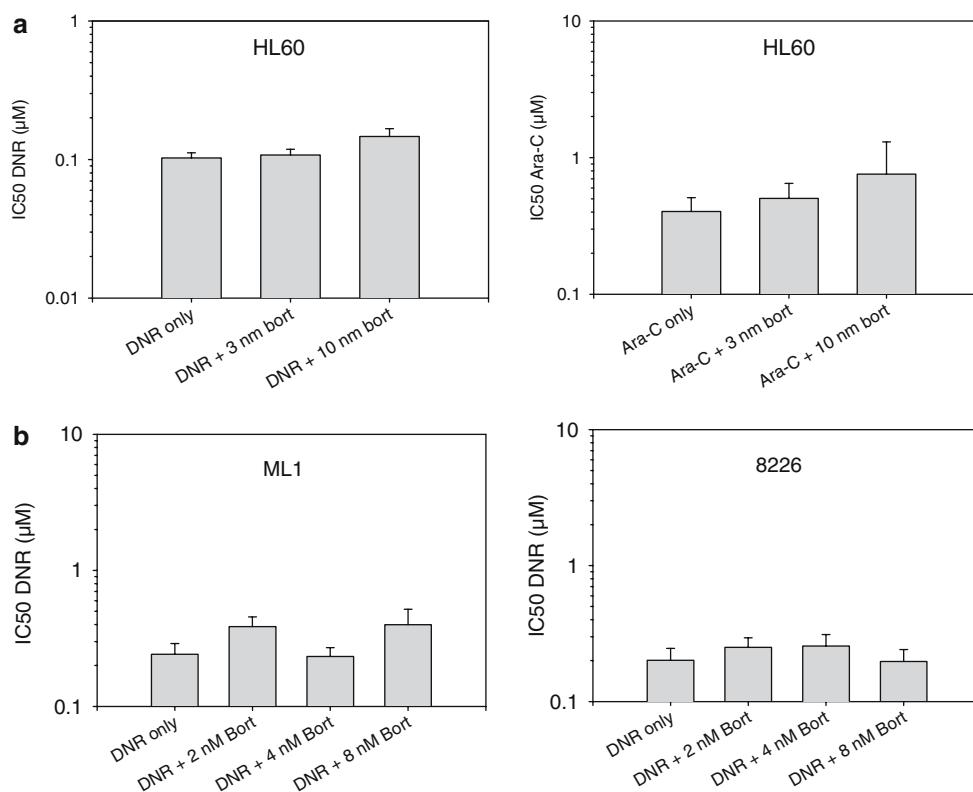
between the partner drugs. The combination of bortezomib with idarubicin in ML1 cells was the only exception, showing a generally additive effect. The CI data demonstrated a general trend of gradual change of drug interactions from antagonism to additivity and synergy with increasing drug activity levels.

#### Drug interaction between bortezomib and cytarabine

The interaction between bortezomib and cytarabine was studied in the HL60 cell line. Like the anthracyclines, cytarabine also had an antagonistic interaction



**Fig. 5** Effect of pre-incubation with fixed concentrations of bortezomib on sensitivity of HL60 cells to daunorubicin or cytarabine. **a** Cells were exposed to bortezomib 24 h before and during exposure to daunorubicin or cytarabine. The  $IC_{50}$  concentrations were determined as the survival of the cells exposed to the drug combinations relative to cells exposed to bortezomib alone at the corresponding concentrations. Each bar represents the mean  $IC_{50}$  concentration of triplicate experiments and error bars represent the standard errors of the mean.  $IC_{50}$  concentrations did not change significantly as a function of bortezomib pre-incubation. **b** The same approach was applied to daunorubicin sensitivity in ML1 and 8226 cells



with bortezomib, particularly at lower drug activity levels (Fig. 4b).

#### Effect of bortezomib pre-treatment on cytarabine and daunorubicin sensitivity

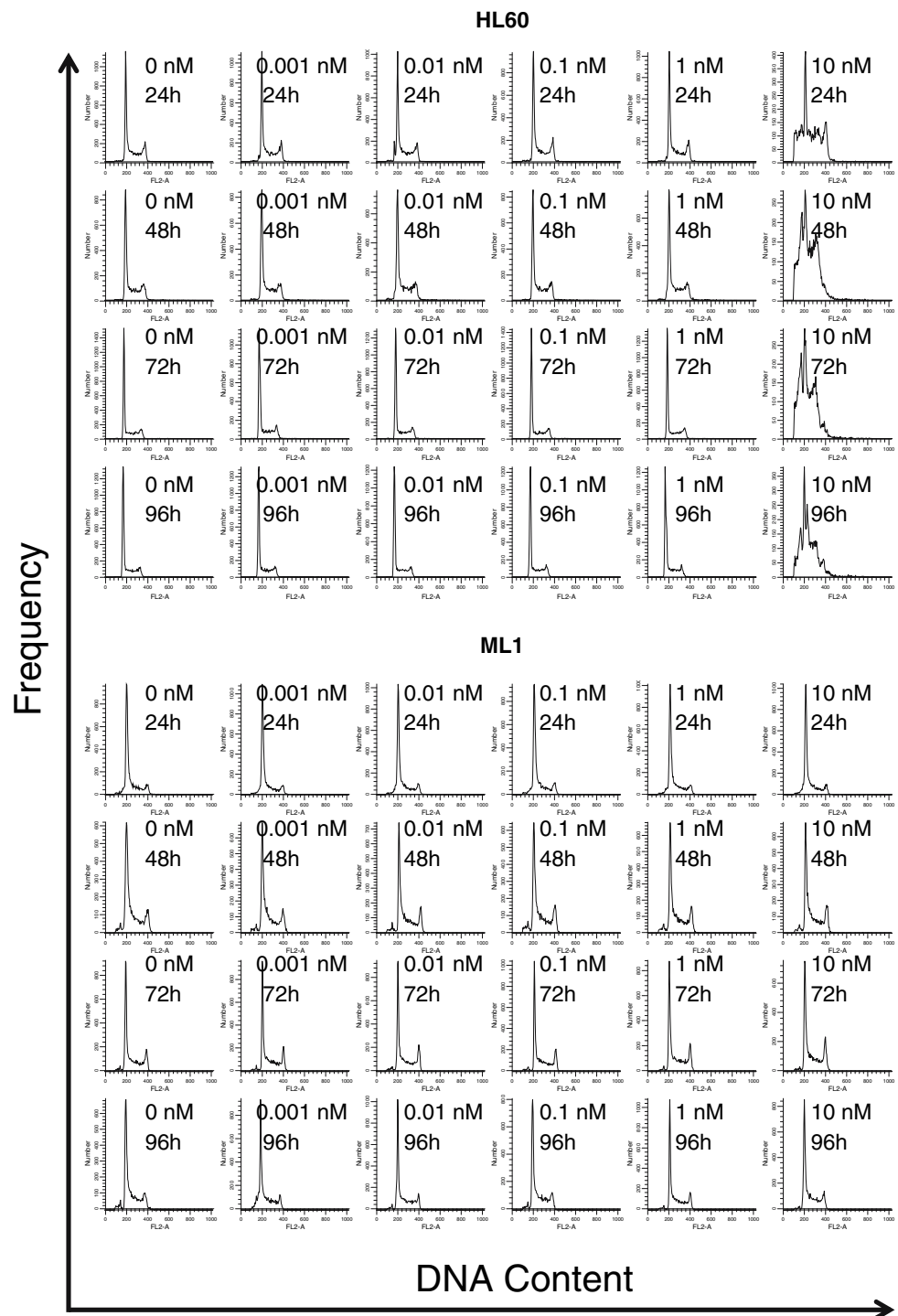
In a previous study of interactions between bortezomib and anthracyclines, the effect of 24-h pre-incubation with a fixed concentration of bortezomib on the  $IC_{50}$  of the second drug was studied [29]. To mimic these conditions, HL60 cells were exposed to bortezomib at a non-cytotoxic concentration (3 nM) or at its approximate  $IC_{50}$  concentration (10 nM). Cytarabine or daunorubicin was then added to the cultures after 24 h at a range of concentrations and the cell cultures were continued for 4 days in the presence of the drug combinations, after which cell viability was analyzed with the WST-1 assay and  $IC_{50}$  concentrations were determined using cells exposed to bortezomib alone as controls. As shown in Fig. 5 a, bortezomib did not enhance cytarabine or daunorubicin sensitivity under these conditions. In order to determine whether this lack of sensitization was particular to HL60 cells, we also treated ML1 cells and 8226 cells with bortezomib for 24 h, then added daunorubicin for 24 h, but, as in HL60 cells, bortezomib did not enhance daunorubicin

cytotoxicity in either of these two additional cell lines (Fig. 5b).

#### Bortezomib-induced cell cycle perturbations in p53-null and wt p53 cells

Cell cycle distributions of HL60 cells (p53-null) and ML1 cells (wt p53) before and during exposure to bortezomib are shown in Fig. 6. Cell cycle effects of bortezomib were both concentration- and p53-dependent. Bortezomib in concentrations up to 1 nM did not affect the cell cycle distributions in HL60 or ML1 cells, but at its approximate  $IC_{50}$  concentration of 10 nM, bortezomib induced an accumulation of cells in S-phase in the HL60 cells, which are p53-null [33], whereas no difference in cell cycle distribution was observed in ML1 cells, which have wild-type p53 [22]. Thus the antagonistic interactions observed at bortezomib activity levels below its  $IC_{50}$  concentration of 10 nM are not explained by cell cycle perturbations. On the other hand, the fact that bortezomib induces cell cycle arrest in HL60 cells at higher drug activity levels indicates that the additive or synergistic interactions observed at these drug concentration levels in these cells are independent of active cell cycle traverse.

**Fig. 6** Cell cycle perturbations induced by bortezomib in AML cells with intact (ML1) and non-functional (HL60) p53. DNA distribution histograms were determined in cell populations fixed at the indicated time points during continuous exposure to bortezomib at the indicated concentrations



## Discussion

Bortezomib is an active drug in the treatment of multiple myeloma, and its activity in AML is currently being investigated [4, 10]. We sought to determine the effects of drug resistance mechanisms prevalent in AML on the cytotoxic activity of bortezomib and to study interactions between bortezomib and chemo-

therapy drugs used in the treatment of AML, including anthracyclines and cytarabine, in AML cells with active and inactive p53.

We demonstrated that the presence of Pgp, which conferred more than tenfold resistance to mitoxantrone in the cell line models studied, resulted in approximately twofold resistance to bortezomib. A recent study of the *in vivo* sensitivity of Pgp-expressing



multidrug resistant neuroblastoma cells to bortezomib similarly showed a lack of resistance in relation to drug-sensitive parental cells [27], consistent with our finding that the effect of Pgp on bortezomib activity is small and is thus likely to be negligible *in vivo*. We also demonstrated here for the first time that the MDR proteins MRP-1, BCRP and LRP do not confer resistance to bortezomib.

We found that simultaneous combination of bortezomib with anthracyclines or cytarabine generally results in antagonistic or, at best, additive drug interactions at low to intermediate drug activity levels. These results are in agreement with some previous studies of the same drug combinations [2, 4, 17], but contradict others [25, 29]. Simultaneous combinations with drugs other than anthracyclines or cytarabine have also resulted in antagonistic interactions with some drugs [13, 17], but synergistic interactions with others [17, 25]. The apparent inconsistency in the literature also applies to the importance of sequence of administration in drug interactions. For example, in studies combining bortezomib with doxorubicin or paclitaxel, synergistic drug interactions were only seen when doxorubicin or paclitaxel was added to cultured cells 24 h after bortezomib, whereas the reverse combination or simultaneous exposure resulted in additive or antagonistic interactions [2]. In contrast, in a study combining bortezomib with SN-38, the active metabolite of irinotecan, synergy was only seen when bortezomib followed SN-38, while the reverse sequence resulted in antagonism [35]. Collectively, these inconsistent reports suggest that findings with regard to interactions between bortezomib and cytotoxic chemotherapy drugs might depend on the choice of drug used in combination, the cell line studied and the method used to assess the drug interactions.

The role of p53 in sensitivity to bortezomib and/or chemosensitization by bortezomib may also be cell type-dependent [7]. In the AML cell lines studied and in the bortezomib concentration range studied, p53 status did not affect drug interactions, although bortezomib effects on cell cycle differed in the p53 wild type and p53-null cell lines.

In the cell lines studied, bortezomib's interactions with anthracyclines and cytarabine appeared to be independent of cell cycle perturbations, as no cell cycle perturbations were observed following exposure to bortezomib in the ML-1 cells with wild-type p53, and complete inhibition of cell cycle traverse was observed in the HL60 cells, which were p53-null, but only following exposure to the highest bortezomib concentration tested (10 nM), which was associated with additive

or synergistic drug interactions in the drug combination assays. The antagonistic interactions observed at lower bortezomib activity levels may be associated with bortezomib's inhibitory effect on NF- $\kappa$ B, as activity of both anthracycline [3] and cytarabine [34] is known to be NF- $\kappa$ B-dependent.

With regard to clinical applicability, an important consideration is that the highest concentrations of bortezomib, which resulted in greater than 90% cell kill in our *in vitro* studies (0.1–0.2  $\mu$ M), are readily attainable in patients. Although the pharmacokinetics of bortezomib as a single agent have not been fully characterized at the recommended dose of 1.3 mg/m<sup>2</sup> in multiple myeloma patients, the median estimated maximum plasma concentration following intravenous administration at this dose is 509 ng/ml (1.32  $\mu$ M), with a range of 109–1,300 ng/ml (0.28–3.38  $\mu$ M) [18]. Even if the 83% binding to human plasma proteins [8] is considered, levels of bioavailable drug would still range from 0.047 to 0.574  $\mu$ M. Thus the synergistic interactions at high drug activity levels that we and others [17] have found are clinically relevant. However, the peak plasma concentrations of bortezomib are short-lived. Initial distribution half-lives of bortezomib in doses of 1.45–2.0 mg/m<sup>2</sup> ranged from 0.22 to 0.46 h and were dose-independent, followed by a more sustained terminal elimination half-life of more than 10 h [30]. The near steady-state plasma concentration in the latter studies was approximately 1 ng/ml (0.0026  $\mu$ M), which corresponds to the low to intermediate drug activity levels that resulted in antagonistic drug interactions in our *in vitro* studies. Thus the pharmacokinetic profile of bortezomib needs to be considered in choosing schedules for combination studies with anthracyclines or cytarabine.

Lack of synergistic interactions in *in vitro* assays does not rule out therapeutic benefit of drug combinations *in vivo*, since beneficial effects on factors pertinent to tumor environment are not measured in the *in vitro* studies. A recent study reporting that the combination of tipifarnib and bortezomib overcomes cell adhesion-mediated drug resistance in multiple myeloma and AML attests to potential beneficial effects of bortezomib on the tumor environment in leukemia [37].

In conclusion, bortezomib has the potential to be an effective drug in the treatment of AML since effective drug concentrations are readily attainable in patients and drug resistance mechanisms known to be relevant in AML do not affect its activity. Dose-dependent drug interactions between bortezomib and drugs with known efficacy in AML should be taken into consideration, along with pharmacokinetic profiles, in the design of combination regimens.

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