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Bortezomib decreases Rb phosphorylation and induces caspase-dependent apoptosis in Imatinib-sensitive and -resistant Bcr-Abl1 expressing cells

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Running title: Bortezomib in Imatinib resistant Bcr-Abl1 cells

Abstract

The use of *c-abl* specific inhibitors such as Imatinib or Dasatinib has revolutionized the treatment of Chronic Myeloid Leukemia. However, a significant percentage of patients become resistant to Imatinib. In this report, we have analyzed the possibility of using the proteasome as a molecular target in CML. Our results show that cells that express Bcr-Abl1 are more sensitive to the inhibition of the proteasome with Bortezomib than control cells. This treatment reduces the proliferation of Bcr-Abl1 expressing cells, by inactivating NF- κ B2 and decreasing the phosphorylation of Rb, eventually leading to an increase in caspase-dependent apoptosis. Furthermore, we show that Bortezomib also induces cell cycle arrest and apoptosis in cells expressing Bcr-Abl1 mutants that are resistant to Imatinib. These results unravel a new molecular target of Bortezomib, i.e. the Rb pathway, and open new possibilities in the treatment of CML especially for patients that become resistant to Imatinib due to the presence of the T315I mutation.

Keywords: Bortezomib, CML, Bcr-Abl1, Imatinib-resistance, Rb, apoptosis

Introduction

Chronic Myeloid Leukemia appears as a consequence of the reciprocal translocation between chromosomes 9 and 22, giving rise to the expression of the Bcr-Abl1 oncoprotein. Current treatment of the disease includes the use of the specific Tyrosine-Kinase inhibitor Imatinib. Even though treatment with Imatinib induces complete cytogenetic remission in more than 70% of CML patients, the appearance of Bcr-Abl1 mutants resistant to Imatinib remains a significant problem (Druker, 2008). This resistance arises mainly by point mutations in the ATP-binding pocket at the active site of the tyrosine kinase Abl, which reduces the affinity for Imatinib without changing the affinity for ATP (Shah & Sawyers, 2003). Second generation inhibitors, such as Dasatinib and Nilotinib, have been proven efficient against most Bcr-Abl1 mutants, but the T315I mutant remains insensitive to the new inhibitors (Ramirez & DiPersio, 2008).

We have previously reported that Bcr-Abl1 induces the degradation of the cell cycle inhibitor p27Kip1 by the proteasome, through the induction of the F-box protein Skp2 (Andreu et al., 2005). These results prompted us to explore the possibility of using the proteasome as a potential therapeutic target in the treatment of CML. The use of the proteasome inhibitor Bortezomib has been successful in the treatment of Multiple Myeloma and Mantle Cell Lymphoma (Chauhan et al., 2008; Wei & Roberts, 2008). Recently, it has also been shown that Bcr-Abl1 induces the activity of the proteasome, again supporting the idea of using the proteasome as a suitable target in Bcr-Abl1 expressing cells (Crawford et al., 2009).

In this report, we have analyzed the effect of Bortezomib on proliferation and survival of different cells expressing Bcr-Abl1, such as the murine BaF/3 cell line stably transduced with Bcr-Abl1, the human blast crisis-derived TCC-S cell line and primary CD34+ cells from CML patients. We have also used BaF/3 cells expressing mutant forms of Bcr-Abl1 that confer resistance to Imatinib, including the highly resistant T315I mutant. Our results show that Bcr-Abl1 expressing cells, including the Imatinib resistant

ones, are more sensitive to Bortezomib than control cells and that Bortezomib treatment reduces proliferation and induces caspase-dependent apoptosis. We have also analyzed the molecular mechanisms responsible for the effect of Bortezomib in these cells. Our results underscore the effect of Bortezomib on the Rb pathway of cell cycle control and support the use of Bortezomib in the treatment of CML, particularly for those patients that become resistant to Imatinib as a consequence to the T315I mutation.

Materials and Methods

Cell culture

BaF/3 control cells, Bcr-Abl1 expressing BaF/3-p210 cells and BaF/3 cells expressing Imatinib resistant Bcr-Abl1 mutants were a kind gift of Dr. Druker (Griswold et al., 2006). The TCC-S cell line derived from a CML patient in blast crisis and that expresses mainly the p210 form of Bcr-Abl1 and, to a lower extent, also the p190 form of the oncoprotein (Van et al., 2005), was obtained from the American Type Culture Collection (Manassas, VA, USA). These cell lines were grown in RPMI supplemented with 10% FCS, L-glutamine and penicillin-streptomycin. In the case of the BaF/3 cells, 10% WEHI-conditioned medium was added to the culture medium as a source of murine IL-3, whereas growth of Bcr-Abl1 expressing BaF/3 cells was independent of growth factors. Primary CML CD34⁺ cells and control CD34⁺ cells from umbilical cord blood, obtained after informed consent, were selected and cultured as described (Andreu et al., 2005). All media, serum and supplements were from Invitrogen Life Technologies (Paisley, UK). When indicated, cell lines were treated with the tyrosine kinase inhibitor Imatinib (IM, STI571, Gleevec, generously provided by Dr. Elisabeth Buchdunger, Novartis, Basel, Switzerland). The reversible proteasome inhibitor Bortezomib (Btz, PS341, Velcade) was kindly supplied by Millenium Pharmaceuticals (Cambridge, MA). Both drugs were administered as a single dose at the beginning of the experiment. To inhibit caspase activity, cells were pretreated for 1 h with 100 μ M of the pan-caspase inhibitor z-Val-Ala-DL-Asp(OMe)-fluoromethylketone (z-VAD) (Bachem, Bubendorf, Switzerland). Cell lines were seeded at a density of 1×10^5 /ml and viable cells (assessed by Trypan blue exclusion) were counted at different time points as indicated.

Analysis of the cell cycle and apoptosis by flow cytometry

Cell cycle profiles were obtained by Propidium Iodide (PI) staining using the DNA Prep™ Reagent System (Beckman Coulter, Fullerton, CA). Briefly, 5×10^5 cells were washed twice in cold PBS, fixed in ice-cold 80% ethanol and kept at -20°C until further analyzed. For a detailed analysis of the cell cycle distribution, cells were labelled for 30 min with $0.5 \mu\text{M}$ of the nucleotide analogue 5-ethynyl-2'-deoxyuridine (EdU) using the Click-iT™ EdU Flow Cytometry Assay (Invitrogen, Eugene, OR) following the manufacturer's instructions. The double staining with EdU and PI allows the quantification of the cell populations in the G0/G1, S and G2 phases of the cell cycle. Phosphatidylserine (PS) exposure was used as an indication of apoptosis and quantified by Annexin-V/PI staining with Annexin-V-FLUOS Staining kit (Roche, Mannheim, Germany). PI staining of un-fixed cells to quantify the subG1/G0 population was also used as a measure of apoptosis. Cells were analyzed in a FC500 flow cytometer (Beckman-Coulter, Fullerton, CA), using Cytomics™ RXP and WinMDI programs.

Western blot analysis

For analysis of protein expression, cell extracts were prepared and analyzed as previously described (Poch et al., 2007). We used primary antibodies against the following proteins: p27Kip1 (C19 and F8), p21Cip1 (C19), c-Myc (N262), cyclin A (C19) and NF- κ B2 (k27); from Santa Cruz Biotechnology (Santa Cruz, CA); p45Skp2 (2B12), from Zymed (San Francisco, CA); Rb (G3-245), from BD Biosciences-Pharmingen (San Jose, CA); and β -tubulin from Sigma (St Louis, MO). Horseradish peroxidase-conjugated secondary antibodies were from Pierce (Rock-Ford, IL). Blots were developed using the enhanced chemiluminescence system (ECL Plus, Amersham Bioscience, Little Chalfont, UK). Rb phosphorylation was analyzed as previously described (Andreu et al., 2005). Samples were separated in 20x20 cm PAGE gels. We

used the QuantityOne software (Bio-Rad, Hercules, CA) to quantify the intensity of the bands.

Electrophoretic mobility shift assay (EMSA)

To obtain nuclear extracts, 10×10^6 cells were collected, washed in PBS and centrifuged at 500xg at 4°C. The pellet was resuspended in cytosol extraction buffer containing 5 mM Na₂HPO₄ pH 7.4, 50 mM NaCl, 150 mM sucrose, 5 mM KCl, 2 mM DTT, 1 mM MgCl₂, 0.5 mM CaCl₂ and 0.1 mM PMSF (phenylmethylsulphonyl fluoride) with 0.1% NP-40, were left on ice for 3 min and centrifuged at 1000xg, 10 min at 4°C in a refrigerated Eppendorf 5417 R centrifuge with a fixed angle rotor. The pellet was gently resuspended again in the same buffer without NP-40 and deposited on a solution containing 30% sucrose, 2.5 mM Tris-HCl pH 7.4, and 10 mM NaCl, prepared fresh for each sample and centrifuged at 1000xg, 10 min at 4°C. The pellet was resuspended in nuclear extraction buffer (50 mM Tris-HCl pH 7.4, 300mM NaCl and 0.5% Triton X-100). Once resuspended, the samples were incubated on ice 20-30 min, subsequently centrifuged at 10000xg, 10 min at 4°C and the supernatant corresponding to the nuclear fraction was collected and quantified with the DC protein assay (Bio-Rad). The DNA binding activity of NF-κB in these extracts was analyzed by EMSA using the DIG Gel Shift Kit, 2nd Generation (Roche, Mannheim, Germany) and following the manufacturer's instructions. The nuclear extracts (10 μg) were incubated with Digoxigenin-labelled double-stranded DNA oligonucleotides corresponding to the consensus binding site for NF-κB 5'-AGTTGAGGGGACTTTCCAGGC-3' (Dai et al., 2004).

Statistical analysis

Microsoft Excel and GraphPadPrism were used to analyze the data and calculate IC₅₀ values from dose-response plots. To analyze the possible synergism between Imatinib

and Bortezomib on BaF/3-p210 cells, we used the CalcuSyn software (Biosoft, Cambridge, UK), based on the Chou and Talalay method (Chou & Talalay, 1984).

Results

Bortezomib inhibits proliferation of Bcr-Abl1 expressing cells

In order to test the possibility of using the proteasome as a therapeutic target in the treatment of Chronic Myeloid Leukemia (CML), we treated control BaF/3 cells and BaF/3 cells that stably express wt Bcr-Abl1 p210 (BaF/3-p210) with different doses of the proteasome inhibitor Bortezomib (Btz), ranging from 0 to 100 nM. Btz is lethal above 10 nM on both cell lines (not shown). The dose-response analysis between 0 and 10 nM Btz reveals that the Bcr-Abl1 expressing BaF/3 cells are more sensitive to Btz than the parental BaF/3 cells (**Fig. 1A**). The difference between BaF/3-p210 and control BaF/3 cells is statistically significant ($p < 0.05$) at 5, 6 and 7 nM Btz. The IC_{50} values are 6.2 nM and 4.7 nM at 48h and 7.2 nM and 5.3 nM at 72 h for BaF/3 and BaF/3-p210 cells, respectively. As the maximal difference between BaF/3 and BaF/3-p210 at 72 h is seen with the dose of 6nM, this is the dose we decided to use for the rest of the experiments.

We also wanted to analyze the sensitivity of human cells to Btz. We chose the TCC-S cell line, which has been previously used as a model of Ph⁺ cells (Kano et al., 2007; Roman-Gomez et al., 2007). These cells derive from a CML patient in blast crisis and express mainly the p210 form of Bcr-Abl1. This cell line is more sensitive than the other two cell lines tested, with IC_{50} values of 2.8 nM at 48 h and 3.7 nM at 72 h (**Fig. 1A**).

We then compared the effect of a 6 nM dose of Btz on the growth curve of BaF/3 and BaF/3-p210 cells. As shown in **Fig. 1B**, Btz treatment initially reduces the proliferation of control BaF/3 cells at 24-48 h, but they recover in the 48-72 h period. However, Bcr-Abl1 expressing BaF/3 cells show a permanent growth arrest for the length of the experiment. The smaller effect of Btz on BaF/3 compared to BaF/3-p210 cells could be due to the fact that control cells are cultured in the presence of IL3. However, when we add IL3 to the BaF/3-p210 cells, the effect of Btz is indistinguishable from that shown in

the absence of IL3 (**Fig. 1B**). Therefore, the sensitivity to Btz is not a consequence of the different culture conditions but most likely due to the expression of Bcr-Abl1. In the case of the TCC-S cell line, we compared the effect of Btz with that of Imatinib (IM). First, we calculated the IC_{50} of IM for this cell line, getting a value of 0.2 μ M (not shown). **Fig. 1C** shows that the treatment with 6 nM Btz causes the same growth inhibition as the treatment with 0.3 μ M IM. The difference between untreated and Btz treated cells is statistically significant ($p < 0.01$) both at 24 and 48 h in the BaF/3-p210 and TCC-S cell lines but not in the parental BaF/3 cell line (**Fig. 1D**). These results show that Bcr-Abl1 expressing cells are more sensitive to Btz treatment than control cells.

We then explored the possible synergy between Btz and IM. We used a fix ratio of doses ranging from 1 to 5 nM Btz and from 0.1 to 0.5 μ M IM, around the IC_{50} for each one of them in the BaF/3-p210 cell line. The Combination Index (CI) obtained using the Chou-Talalay method indicates that there is an additive effect of both drugs but no synergism, at least under our experimental conditions (data not shown). As IM and Btz have additive effects, this would suggest that the activation of the proteasome in Bcr-Abl1 expressing cells is a consequence of Bcr-Abl1 kinase activity.

Imatinib-resistant Bcr-Abl1 expressing cells are also sensitive to Bortezomib

To see whether Btz could have an effect on cells expressing IM resistant forms of Bcr-Abl1, we used BaF/3 cells expressing four different mutants of Bcr-Abl1: T315I, Q252H, Y253F and E255K. Q252H is only partially resistant to IM and is affected by the drug at high doses. The highly resistant T315I mutation affects the direct contact between IM and Bcr-Abl1, therefore preventing this interaction (Shah & Sawyers, 2003). We cultured these cell lines in parallel with the one expressing wt Bcr-Abl1 (BaF/3-p210) and treated them with a single 6 nM dose of Btz. As shown in **Fig. 2**, Btz inhibits proliferation of all these Bcr-Abl1 expressing cell lines to the same extent, indicating that Btz is equally effective against IM sensitive and resistant Bcr-Abl1

expressing cells. One way ANOVA followed by Tukey's analysis revealed that all the mutant cell lines behave the same as the BaF/3-p210 one, and that there are statistically significant differences between untreated and treated groups at 48 and 72 h ($p < 0.01$).

Bortezomib inhibits S-phase entry in Bcr-Abl1 expressing cells

We then wanted to characterize the effect of Btz on the cell cycle of these cells. DNA profiles of BaF/3 control and BaF/3-p210 cells shows that 48 h after the addition of Btz there is an accumulation of the Bcr-Abl1 expressing cells in the G0/G1 phase of the cell cycle, whereas control cells are minimally affected (**Fig. 3A**). We also analyzed the cell cycle profile of mutant Bcr-Abl1 expressing cells (**Fig. 3A**). In all cases, there is a reduction of the proliferating populations (S+G2/M) and an accumulation of cells in G0/G1. The DNA profiles also show that Btz induces cell death, as there is an increase in the subG0/G1 population.

To characterize the nature of the cell cycle arrest in more detail and quantify the percentage of cells in each of the cell cycle phases more precisely, we performed an EdU incorporation assay. **Fig 3B** shows an example of this analysis with BaF/3 and BaF/3-p210 cells. In BaF/3 cells, there is a slight reduction of the S-phase population at 24 h, but the cells have recovered at 48 h after the addition of Btz. In contrast, there is a big decrease of the S-phase population in the BaF/3-p210 cells that is permanent. Quantification of the EdU incorporation experiments (**Fig. 3C**) reveals that 48 h after Btz addition, the cell cycle profile of control BaF/3 cells is not statistically different from untreated cells. In contrast, all the cell lines expressing Bcr-Abl1, including the mutant forms that are IM resistant, display a G0/G1 arrest when treated with Btz. The differences between Btz treated cells and the untreated controls are statistically significant in the G0/G1 accumulation ($p < 0.05$ in all cases) and also in the S phase reduction ($p < 0.01$ in all cases).

Bortezomib treatment reduces the phosphorylation and the activity of Rb in Bcr-Abl1 expressing cells

At the molecular level, Rb phosphorylation regulates the transition from G1 into S phase (Khidr & Chen, 2006). Therefore, we analyzed the effect of Btz on the phosphorylation status of Rb by western blot. As shown in **Fig. 4A**, Btz treatment causes a reduction in the total level of Rb protein, both in BaF/3 and in BaF/3-p210 cells. However, in the case of the BaF/3-p210 and not the BaF/3 cells, a faster migrating band, corresponding to the hypo-phosphorylated form of Rb (Rb), can be detected. At the same time, there is a decrease in the intensity of the slower migrating bands, corresponding to hiper-phosphorylated Rb (P-Rb). This change in the phosphorylation status of Rb can be detected as early as 12 h and is more evident at 24h (**Fig. 4A**). If we represent the ratio between the hypo-phosphorylated and the hiper-phosphorylated forms of the protein (Rb/P-Rb), the increase in this ratio is statistically significant at 12 and 24 h after Btz addition compared to time 0 h ($p < 0.05$, **Fig. 4C**). We compared this effect with that of the Bcr-Abl1 kinase inhibitor IM. As previously reported (Andreu et al., 2005), IM also increases the level of hypo-phosphorylated Rb, finally inducing the disappearance of the hiper-phosphorylated forms of the protein (**Fig. 4A**).

Rb regulates the expression of cyclins such as Cyclin A (CycA), through the E2F family of transcription factors. Therefore, the expression of CycA reflects the activity of Rb. As shown in **Fig. 4A**, the amount of CycA protein decreases as the levels of P-Rb also decrease upon Btz or IM treatment, confirming that the effect of these drugs on Rb is also functional. Rb phosphorylation is mediated by Cyclin/Cdk complexes active during G1 (Murray, 2004). The activity of these complexes is in turn negatively regulated by inhibitors of the Cip/Kip family, such as p21Cip1 (p21) and p27Kip1 (p27) (Sherr & Roberts, 2004). As shown in **Fig. 4A**, Btz treatment results in the accumulation of both cell cycle inhibitors. p21 accumulates both in control and Bcr-Abl1 expressing cells.

However, in BaF/3 cells there is only a slight accumulation of p27 at 12 h and not later on, whereas in BaF/3-p210 cells, p27 accumulation increases with time. In contrast, IM treatment only induces the accumulation of p27, and causes a reduction in p21 expression.

In the case of IM-resistant Bcr-Abl1 expressing BaF/3 cells, Btz also induces a decrease in the level of total Rb, but also of P-RB (**Fig. 4B**). In fact, the increase of the Rb/P-RB ratio is higher than the decrease of total Rb (**Fig. 4C**). In every case, the P-Rb decrease is detected at 12 h and the effect is more evident at 24 h (**Fig. 4B,C**). Similar to the BaF/3-p210 cells, the mutant Bcr-Abl1 expressing cell lines also display a decrease in CycA expression and an increase in p21 and p27 protein levels (**Fig. 4B**). Therefore, the effect of Btz at the molecular level on the cell cycle regulatory machinery of cells expressing Bcr-Abl1 is the same regardless to the IM sensitivity of the cells.

Bortezomib prevents the activation of NF- κ B

One of the known targets of Btz in different models is the transcription factor NF- κ B (Galimberti et al., 2008). We assayed the effect of Btz on the activity of this protein by EMSA, and found that Btz reduces the DNA binding activity of NF- κ B on BaF/3 and BaF/3-p210 cells with no significant differences between both cell types (**Fig. 5A**). Therefore, the activity of this transcription factor does not seem to be responsible for the cell cycle arrest that we see only in BaF/3-p210 cells. As the initial level of NF- κ B activity is higher in BaF/3-p210 cells, we cannot rule out the possibility that the disappearance of NF- κ B activity has a bigger impact on these cells. There are two alternative pathways of NF- κ B activation, the canonical and the non-canonical pathways (Pomerantz & Baltimore, 2002). The second one involves the proteasome-dependent activation of NF- κ B2 from a 100 kDa to a 52 kDa form (Heusch et al., 1999). Because Btz could be affecting this activation, we analyzed the processing and activation of NF- κ B2 in our experimental system by western blot. As shown in **Fig. 5B**,

BaF/3-p210 cells exhibit a higher level of processed, active p52-NF- κ B2 protein than BaF/3 control cells. However, upon Btz treatment, there is a marked reduction in the level of the 52 kDa active form of NF- κ B2.

It has been shown that the NF- κ B2 subunit is relevant for the regulation of Skp2 and c-Myc expression (Barre & Perkins, 2007). It is known that these two proteins are among the many factors that regulate the levels and activity of the cell cycle inhibitors p21Cip1 and p27Kip1 (Kossatz et al., 2004; Perez-Roger et al., 1999). On the other hand, Bcr-Abl1 kinase activity inhibition with IM results in the downregulation of both Skp2 and c-Myc, indicating that these two proteins are molecular targets of Bcr-Abl1 (Andreu et al., 2005; Sawyers, 1993). Therefore, we analyzed the effect of Btz on Skp2 and c-Myc and found that both show lower levels in Btz treated cells expressing Bcr-Abl1, with no significant change in BaF/3 control cells (**Fig. 5B**).

Bortezomib induces caspase-dependent apoptosis of Bcr-Abl1 expressing cells

Treatment with Btz in different tumor models induces apoptosis *in vitro* (Brignole et al., 2006; Galimberti et al., 2008; Perez-Galan et al., 2006; Stapnes et al., 2007; Strauss et al., 2007; Zhao et al., 2008). As noted previously, Btz induces an increase of the sub-G0/G1 population (**Fig. 3A**). In order to evaluate the effect of Btz on the survival of Bcr-Abl1 expressing cells, we double-stained the cells with Annexin-V and Propidium Iodide (PI) to distinguish between apoptosis and necrosis and analyzed them by flow cytometry. The results shown in **Fig. 6A** indicate that Btz treatment induces apoptosis in Bcr-Abl1 expressing cells, as indicated by the increase in the Annexin V positive population. In the case of BaF/3 cells, there is an initial increase in the apoptotic population at 24 h, but then it decreases at 48 and 72 h. The difference between BaF/3-p210 and control BaF/3 cells is statistically significant at 48 and 72 h ($p < 0.01$). To test the caspase dependency of the apoptosis induced by Btz in our model, we used the pan-caspase inhibitor z-VAD and analyzed apoptosis by measuring the appearance of the sub-G0/G1 apoptotic population in the DNA profile (**Fig. 6B**). Pre-

treatment of the cells with this inhibitor partially abrogates the cell death induced by Btz, suggesting that caspase activation is at least in part necessary for the induction of apoptosis by Btz. In the IM-resistant Bcr-Abl1 expressing cells Btz also induces apoptosis and it can be partially prevented by z-VAD pre-treatment (**Fig. 6B**). Quantification of the sub-G0/G1 apoptotic population gives a statistically significant difference at 24 and 48 h after Btz treatment compared to time 0 h in all cell lines. Also, the anti-apoptotic effect of z-VAD is significant in all cases (**Fig. 6C**).

Finally, we tested whether Btz could have an effect on primary CD34+ cells from a CML patient. As a control, we used primary non-CML CD34+ cells from umbilical cord. We treated the cells with 6 and 10 nM Btz and analyzed the cell cycle profile by PI staining (**Fig. 7**). Even though control cells show some cell death, the CML CD34+ cells are more sensitive, with a marked decrease in proliferation and an increase in the sub-G0/G1 population (20% in control to 60% at 6 nM) whereas control CD34+ cells show a smaller effect (30% cell death at 6 nM). This result confirms that Bcr-Abl1 expressing cells are more sensitive to the proteasome inhibitor Btz.

Discussion

In this report, we have explored the possibility of inhibiting the proteasome in the treatment of Chronic Myeloid Leukemia and analyzed the changes induced by Btz on the molecular mechanisms controlling cell proliferation and survival. Our results show that Btz induces a cell cycle arrest and also apoptosis in Bcr-Abl1 expressing cells and not in control cells. The event of apoptosis can be partially prevented with the pan-caspase inhibitor z-VAD indicating the implication of caspases in Btz induced apoptosis in the cell models we have used. Regarding the effect of Btz on proliferation, this inhibitor blocks entry into S phase. This is demonstrated by our cell cycle analysis, but also by preventing apoptosis with the caspase inhibitor z-VAD: the cells treated with this inhibitor display an absence of cells in S phase, with the accumulation of the cells in G0/G1 and in G2/M. This indicates that the effects of Btz on proliferation and survival can be separated and suggests that the apoptosis could be a consequence of the cell cycle arrest.

At the molecular level, the effect of Btz is characterized by a decrease in the phosphorylation status and later processing of Rb, the latter being related to the apoptosis induced by Btz. We propose that the G1 cell cycle arrest induced by the inhibition of the proteasome by Btz is the consequence of a series of events involving the failure in the activation of NF- κ B2 with a decrease in the expression of c-Myc and Skp2 that results in the accumulation of p21 and p27, inhibitors of the kinase activity of G1 Cyclin/Cdk complexes. As a result, Rb is not correctly maintained in its phosphorylated form and keeps E2F sequestered and inactive, reducing the expression of Cyclin A and preventing S phase entry. On the other hand, Btz induces the activity of caspases involved in apoptosis and these proteases are also responsible for the proteolytic cleavage of Rb inactivating the antiapoptotic function of this protein (Janicke et al., 1996).

There are some works reporting that Btz inhibits growth of Bcr-Abl1 expressing cells. Most of them use Btz in combination with other drugs (Dai et al., 2004; Dasmahapatra et al., 2006; Yan et al., 2007; Yu et al., 2003) or lack the use of control cells (Gatto et al., 2003). In our case, we have used Btz alone at a dose that has no effect on BaF/3 cells that do not express Bcr-Abl1, showing that Btz alone is sufficient to arrest the cell cycle and induce apoptosis specifically in Bcr-Abl1 expressing cells. The use of control cells reinforces the idea of the higher sensitivity of tumour cells to the inhibition of the proteasome (Joazeiro et al., 2006; Vink et al., 2006). We also show that Btz is able to induce the same effects on cells expressing mutant forms of Bcr-Abl1 that confer resistance to IM. These effects are indistinguishable from the ones seen with IM-sensitive Bcr-Abl1 expressing cells. Moreover, Btz is able to induce apoptosis also in the kinase inhibitor-resistant mutant T315I.

We also show that Btz is effective in primary CD34+ cells from a CML patient. Very recently, Heaney and co-workers have also shown that Btz induces apoptosis of primary CD34+ cells and also the more primitive CD34+38- cells from CML patients. These authors show that Btz reduces 1) the capacity of these cells to form colonies in long-term cultures and 2) the engraftment potential of these human CD34+ CML cells (Heaney et al., 2010). This indicates that Btz can target CML stem cells that are resistant to IM treatment.

NF- κ B is commonly inactive in Btz treated cells (Adams, 2001; Galimberti et al., 2008; Markovina et al., 2008; Ocio et al., 2008; Sartore-Bianchi et al., 2007; Yang et al., 2008). In our case, Btz reduces the DNA binding activity of NF- κ B in control and Bcr-Abl1 expressing cells. Therefore, this protein does not seem to be responsible for the effects we see only in Bcr-Abl1 expressing cells. However, there is a difference in the effect of Btz on the activation of NF- κ B2 that could be responsible for the cell cycle arrest and, perhaps, also the caspase-dependent apoptosis that we observe. We show the inactivation of NF- κ B2 by Btz in a direct way, by measuring the level of processing

from the inactive 100 kDa form to the active 52 kDa form, and also indirectly, by analyzing the expression levels of two known targets of NF- κ B2, namely Skp2 and c-Myc. This result establishes a new link between Bcr-Abl1 and the NF- κ B2 signaling pathway that is sensitive to proteasome inhibition in CML cells.

In summary, this work shows that Btz reduces the proliferation and survival of Bcr-Abl1 expressing cells, regardless of their sensitivity to IM and including the highly resistant mutant T315I. Our results also show that the inhibition of the proteasome affects the Rb pathway through the inactivation of NF- κ B2. This, in turn, reduces the level of Skp2 and c-Myc. The central roles of these two potent oncoproteins in regulating the cell cycle indicates that Btz is affecting key pathways that are usually altered in tumor cells, suggesting that this drug could have potential uses not only in CML but also in other types of tumors.

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Figure legends

Figure 1: Bortezomib inhibits proliferation of Bcr-Abl1 expressing cells. (A) Dose response of Btz on Ba/F3, BaF/3-p210 and TCC-S cell lines. Cells were seeded at a density of 1×10^5 /ml and treated with a single dose of Btz ranging from 0 to 10 nM. Viable cell counts at 72 h were used to plot the dose response effect. Cell counts relative to control (100%) from four different experiments are represented as Mean \pm SD. Differences between BaF/3 and BaF/3-p210 cells are statistically significant at 5, 6 and 7 nM, as revealed by a one way ANOVA analysis followed by a Tukey's multiple comparison test ($p < 0.01$). Note that the maximal difference between BaF/3 and BaF/3-p210 is at 6 nM Btz. **(B)** Effect of Btz on the proliferation of BaF/3 and BaF/3-p210. Growth of untreated (C) and Btz treated (6 nM, Btz) cells was followed by viable cell count every 24 h. Mean \pm SD from four different experiments is shown. Addition of 10% WEHI-conditioned medium to the culture medium of BaF/3-p210 cells as a source of murine IL-3 (p210 IL3 Btz) does not prevent the effect of Btz on these cells. **(C)** The effect of 6 nM Btz is similar to that of 0.3 μ M IM on the human derived TCC-S cell line. Cells were treated and counted as in **(B)**. The dose of 0.3 μ M IM was selected from a previous dose-response experiment (not shown). **(D)** Differences between untreated (C) and Btz treated (Btz) cells from **(B)** and **(C)** at 48 and 72 h are plotted as columns (Mean+SD). Student's *t* test analysis revealed statistically significant differences between Btz and C at 48 and 72 h only in Bcr-Abl1 expressing cells but not in BaF/3 cells ($p < 0.01$).

Figure 2: Bortezomib inhibits proliferation of cells expressing Bcr-Abl1 mutants that confer resistance to IM. BaF/3 expressing wt Bcr-Abl1 (p210), or different mutants of the protein (T315I, Q252H, Y253F and E255K) untreated (C) or treated with 6 nM Btz (Btz) were seeded and counted as in **Fig. 1**. Mean \pm SEM is represented from a total of 8 different experiments. There are no statistically differences among control cells or among treated cells, but in each cell line the difference between treated and

untreated is significant, as revealed by a one way ANOVA analysis followed by a Tukey's multiple comparison test ($p < 0.01$).

Figure 3: Bortezomib induces a G1 cell cycle arrest in Bcr-Abl1 expressing cells.

(A) BaF/3 cells and derivatives expressing wt Bcr-Abl1 (p210) and different IM resistant mutants (T315I, Q252H, Y253 and E255K), untreated (C, left) or treated with 6 nM Btz (Btz, right) for 48 h were fixed, stained with PI and analyzed by flow cytometry as described in Materials and Methods. The experiment shown is a representative example from six different experiments **(B)** and **(C)** To quantify the different cell cycle populations in each case, cells received a 30 min pulse of the nucleotide analog EdU. EdU/PI double staining allows for an accurate quantification of cells in G0/G1, S and G2/M. An example of this kind of analysis for BaF/3 and BaF/3-p210 cells at 0, 24 and 48 h after the addition of 6 nM Btz is shown in **(B)**. Quantification of such experiments ($n=3$) with BaF/3, BaF/3-p210 and the IM resistant Bcr-Abl1 expressing BaF/3 cells at 48 h is represented in **(C)** as Mean+SD. There are no significant differences between C and Btz treated BaF/3 cells (the G0/G1 – S – G2/M values are: 24 ± 11 – 58 ± 7 – 16 ± 5 , for untreated cells and 32 ± 4 – 45 ± 2 – 20 ± 7 , for Btz-treated cells). In all other cases, Student's *t* test reveals that there are statistically significant differences indicating an increase in G0/G1 ($p < 0.05$) and a decrease in S phase ($p < 0.01$) in Btz treated cells compared to each control (as an example, for the BaF/3-p210 cells, the G0/G1 – S – G2/M values are: 39 ± 7 – 54 ± 7 – 5.4 ± 0.8 , for untreated cells and 68 ± 9 – 6 ± 2 – 7 ± 3 , for Btz-treated cells).

Figure 4: Bortezomib treatment decreases the phosphorylation and the function of Rb in Bcr-Abl1 expressing cells. (A) Control BaF/3 (left) and Bcr-Abl1 expressing BaF/3-p210 (right) cells were treated with 2 μ M IM or 6 nM Btz for 12 and 24 h or untreated (C) and cell extracts were prepared and analyzed by western blot with the appropriate primary antibodies, as indicated. In the Rb blot (top) the faster migrating band corresponds to the hypo-phosphorylated form of Rb (Rb), whereas the slower

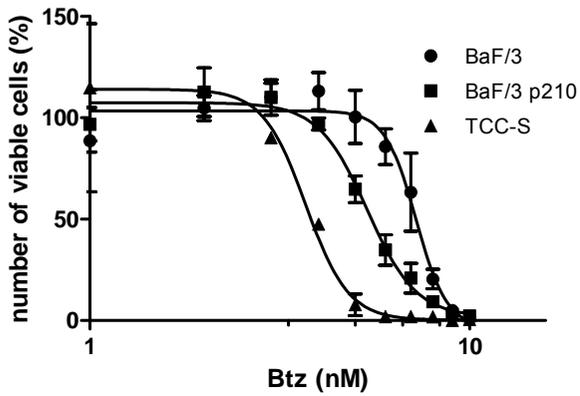
migrating bands correspond to the hyper-phosphorylated forms of the protein (P-Rb). β -tubulin is used as a loading control. Shown is a representative blot of three independent experiments. **(B)** Similar analysis as in **(A)** was performed with extracts from untreated (C) and treated with 6 nM Btz for 12 and 24 h BaF/3 cells expressing four different Bcr-Abl1 mutants that confer IM resistance. A single band is detected in the p27 blot because a different antibody was used that does not detect the lower band seen in **(A)**. **(C)** Btz treatment reduces the amount of total Rb as well as its phosphorylation. P-Rb and total Rb bands were quantified using the QuantityOne software (Bio-Rad). Columns represent Mean+SD relative to the values at t=0 (value of 1) from three independent experiments as the ones shown in **(A,B)**. Both total Rb and the Rb/P-RB ratio are represented. Student's *t* test reveals that the differences between Btz and control cells are significant as early as 12 h in most cases (* $p < 0.05$; ** $p < 0.01$).

Figure 5: Bortezomib inhibits the activation of NF- κ B2 in Bcr-Abl1 expressing cells. **(A)** A digoxigenin-labelled double stranded DNA oligonucleotide with the consensus sequence for NF- κ B was incubated with nuclear extracts from control BaF/3 (top) and Bcr-Abl1 expressing BaF/3-p210 (bottom) cells treated with 6 nM Btz for the times indicated. The DNA binding activity of NF- κ B in those extracts was analyzed by EMSA. O: labeled oligonucleotide without extract. C: binding was competed with a 50-fold molar excess of unlabeled oligonucleotide. The graph on the right shows the quantification of five independent assays as the one shown on the left. Columns represent the Mean+SD of the intensity of the bands relative to time 0h. **(B)** BaF/3 and BaF/3-p210 cells were treated with 6 nM Bortezomib for the indicated times and cell extracts were prepared and analyzed by western blot with the indicated antibodies. The antibody against NF- κ B2 recognizes both the 100 kDa (inactive) and the 52 kDa (active) forms of the protein. β -tubulin is used as a loading control. A representative blot of three different experiments is shown.

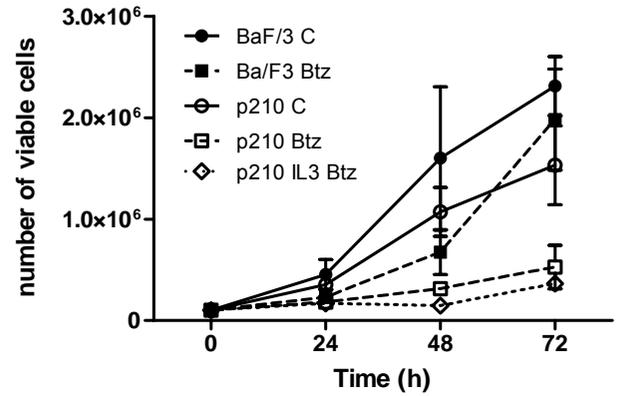
Figure 6: Bortezomib induces caspase dependent apoptosis in Bcr-Abl1 expressing cells. (A) Control BaF/3 (top row) and Bcr-Abl1 expressing BaF/3-p210 (low row) cells were treated with 6 nM Btz for 0, 24, 48 and 72 h and stained with Annexin-V and Propidium Iodide (PI). Flow cytometry analysis reveals viable cells (negative for Annexin-V and PI staining, bottom left square) early apoptotic (Annexin-V positive, bottom right square) and late apoptotic-necrotic (Annexin-V and PI positive, top-right square) cell populations. Quantification of the apoptotic, Annexin-V positive populations from three different experiments as the one shown above (bottom) reveals that Btz induces more apoptosis in Bcr-Abl1 expressing cells than in control BaF/3 cells at 48 and 72 h ($p < 0.01$). (B) BaF/3 cells expressing wt and mutant forms of Bcr-Abl1 were untreated (C), treated with 6 nM Btz (Btz) or pre-treated with z-VAD for 1 h before the addition of Btz. 48 h later, cells were stained with PI to analyze and quantify the apoptotic sub-G0/G1 population. The quantification of three different such experiments is shown in (C), showing that Btz induces apoptosis at 24 and 48 h compared to untreated controls and that z-VAD reduces this apoptosis also at 24 and 24 h compared to the Btz column for each cell line (* $p < 0.05$; ** $p < 0.01$).

Figure 7: Bortezomib induces cell death in primary CD34+ cells from a CML patient. Control CD34+ cells from umbilical cord (Control CD34+, left) and CD34+ cells from a CML patient (CML CD34+, right) were seeded at 1×10^6 cells/ml and treated with 6 and 10 nM Btz, or untreated (C). After 48 h cells were stained with PI and the cell cycle profile was analyzed by flow cytometry.

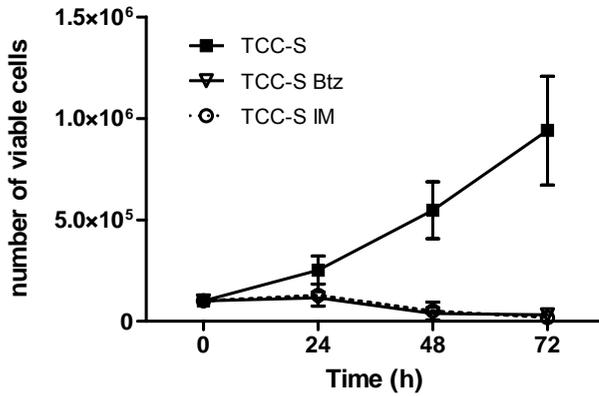
1A



1B



1C



1D

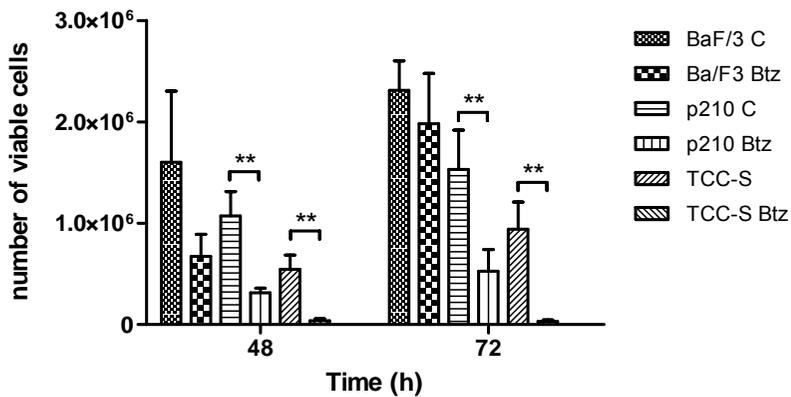


Figure 1: Bortezomib inhibits proliferation of Bcr-Ab11 expressing cells.

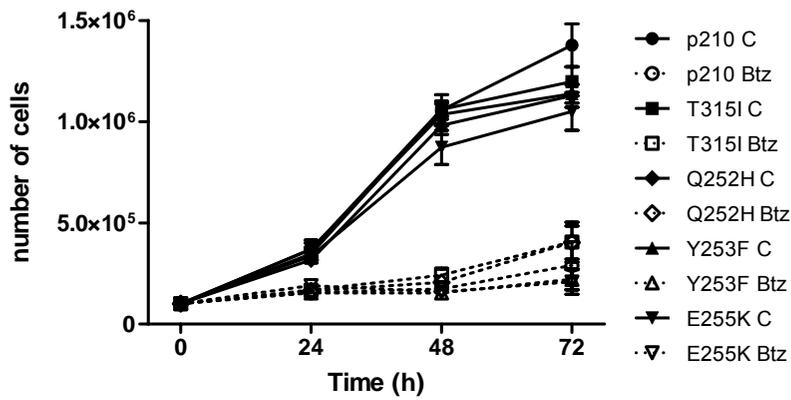


Figure 2: Bortezomib inhibits proliferation of cells expressing Bcr-Abl1 mutants that confer resistance to IM.

3A

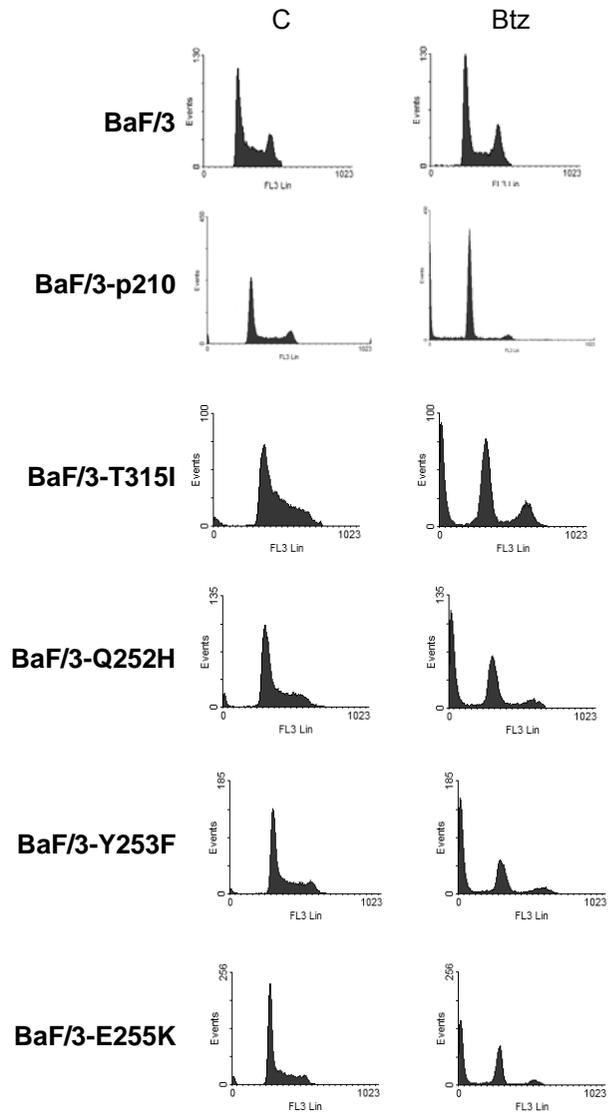


Figure 3: Bortezomib induces a G1 cell cycle arrest in Bcr-Abl1 expressing cells.

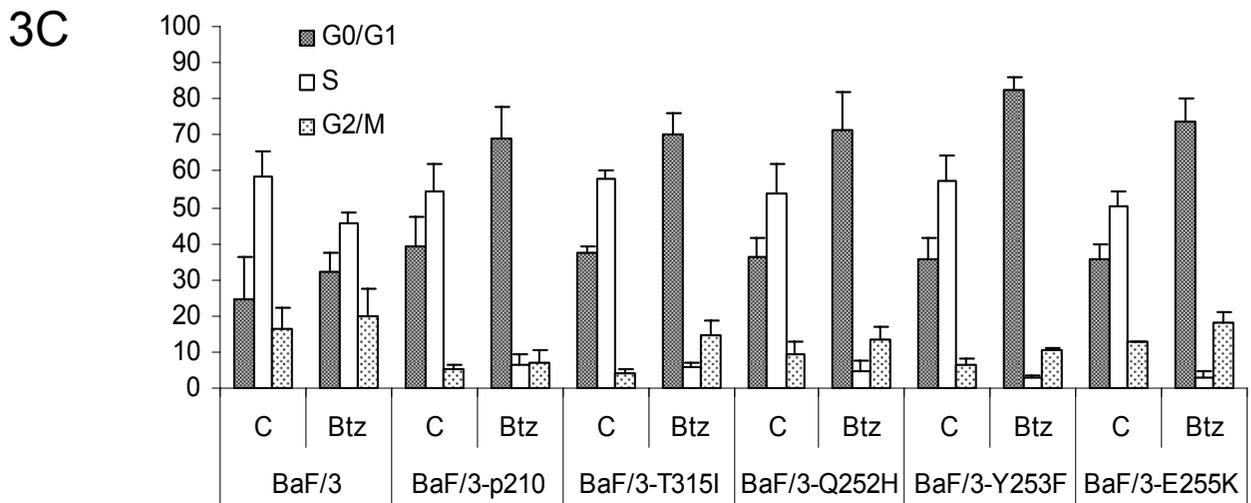
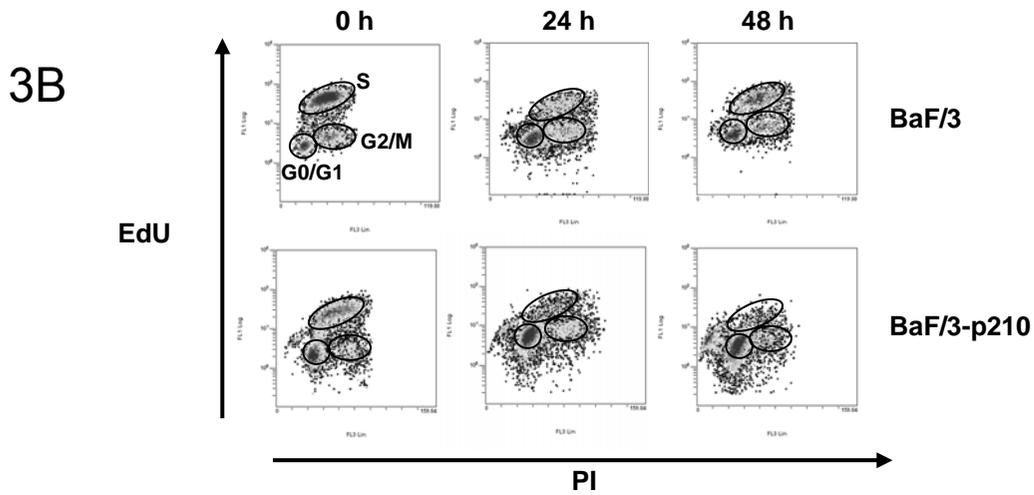


Figure 3: Bortezomib induces a G1 cell cycle arrest in Bcr-Abl1 expressing cells.

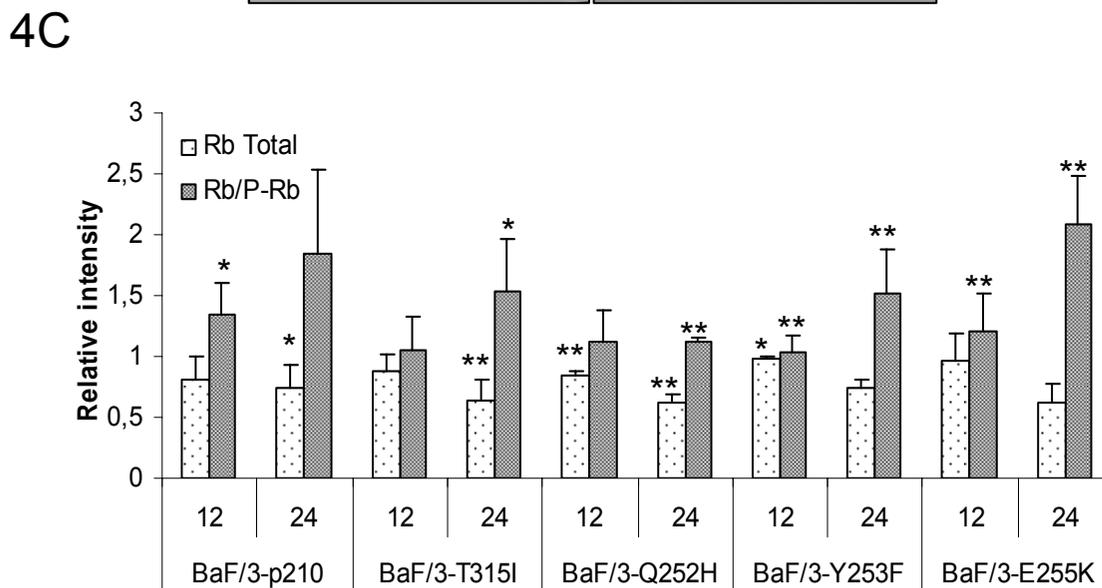
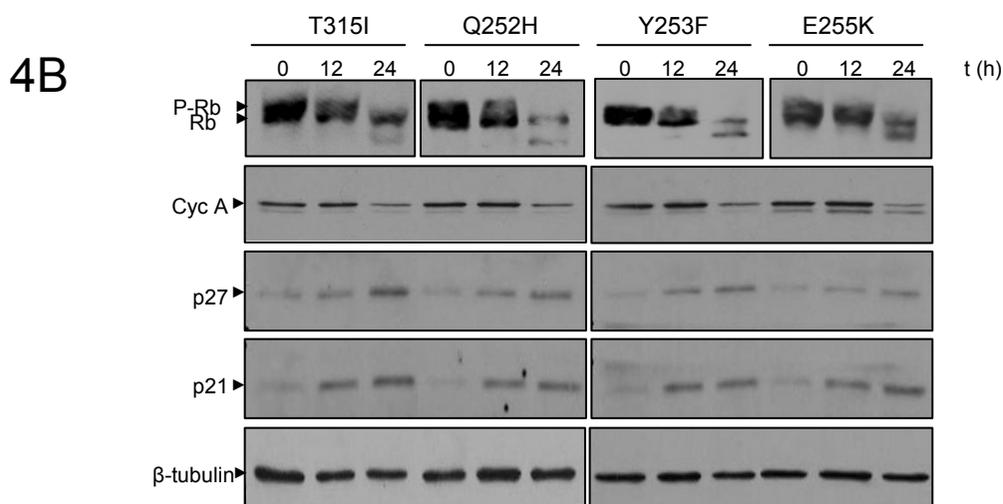
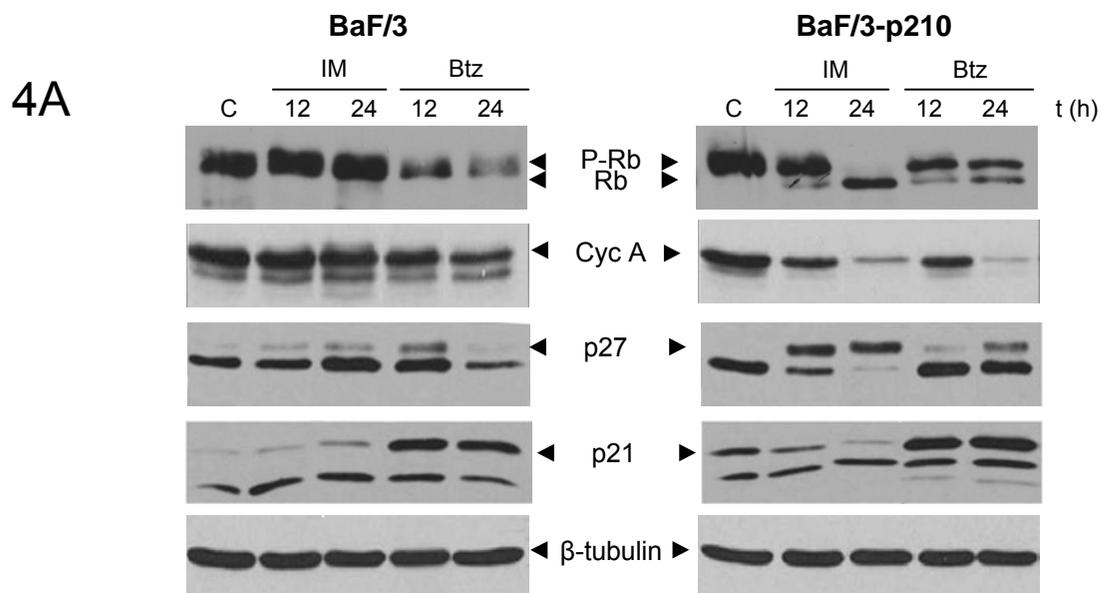
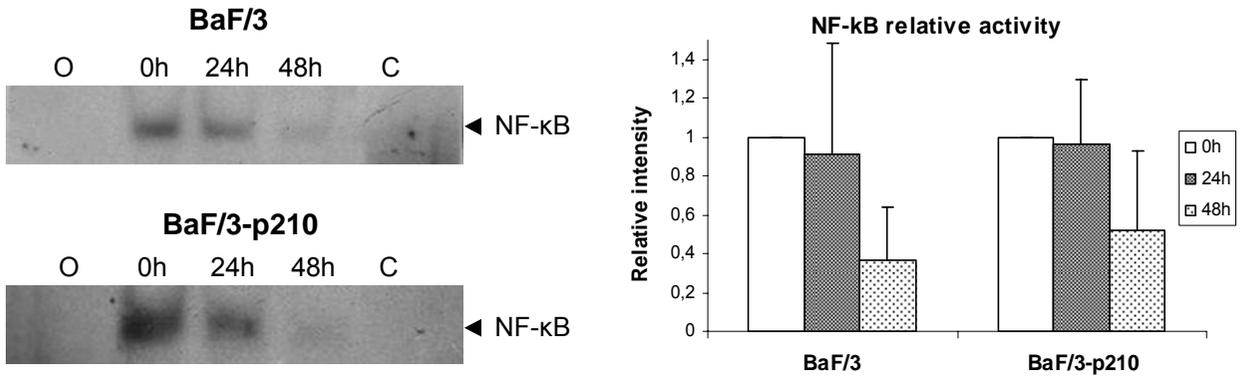


Figure 4: Bortezomib treatment decreases the phosphorylation and the function of Rb in Bcr-Abl1 expressing cells.

5A



5B

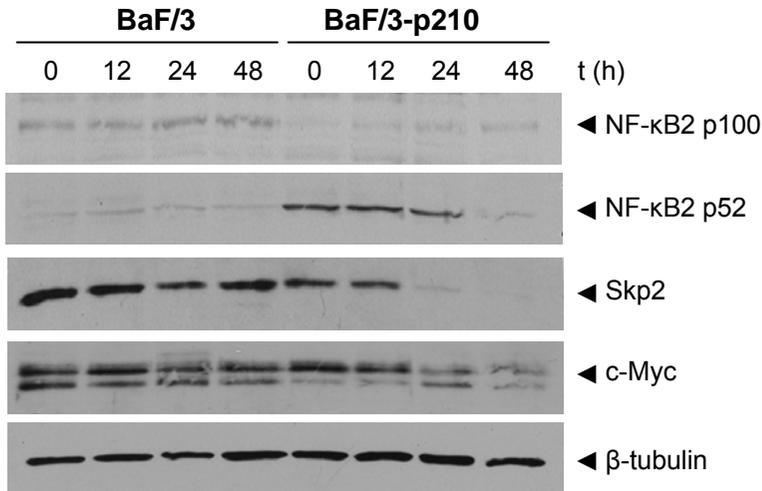


Figure 5: Bortezomib inhibits the activation of NF-κB2 in Bcr-Abl1 expressing cells.

6A

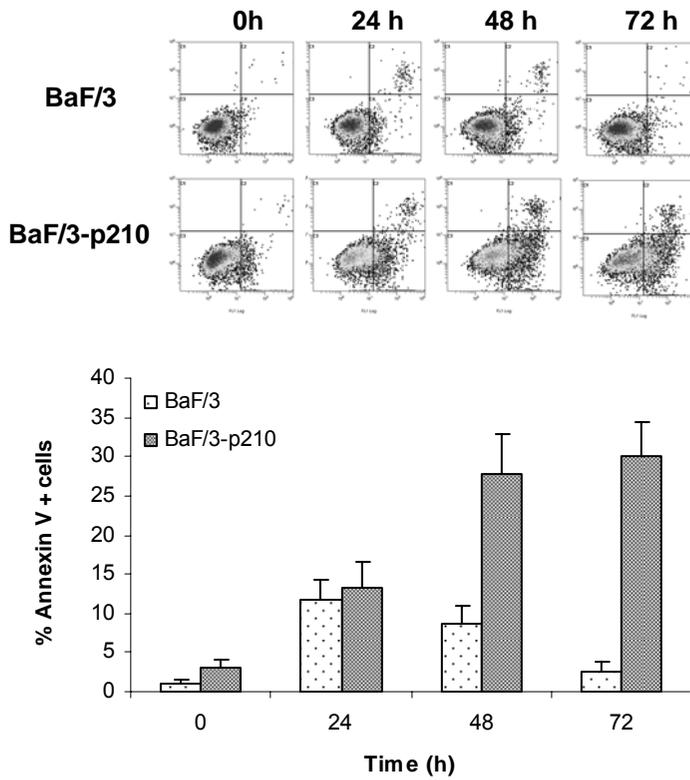
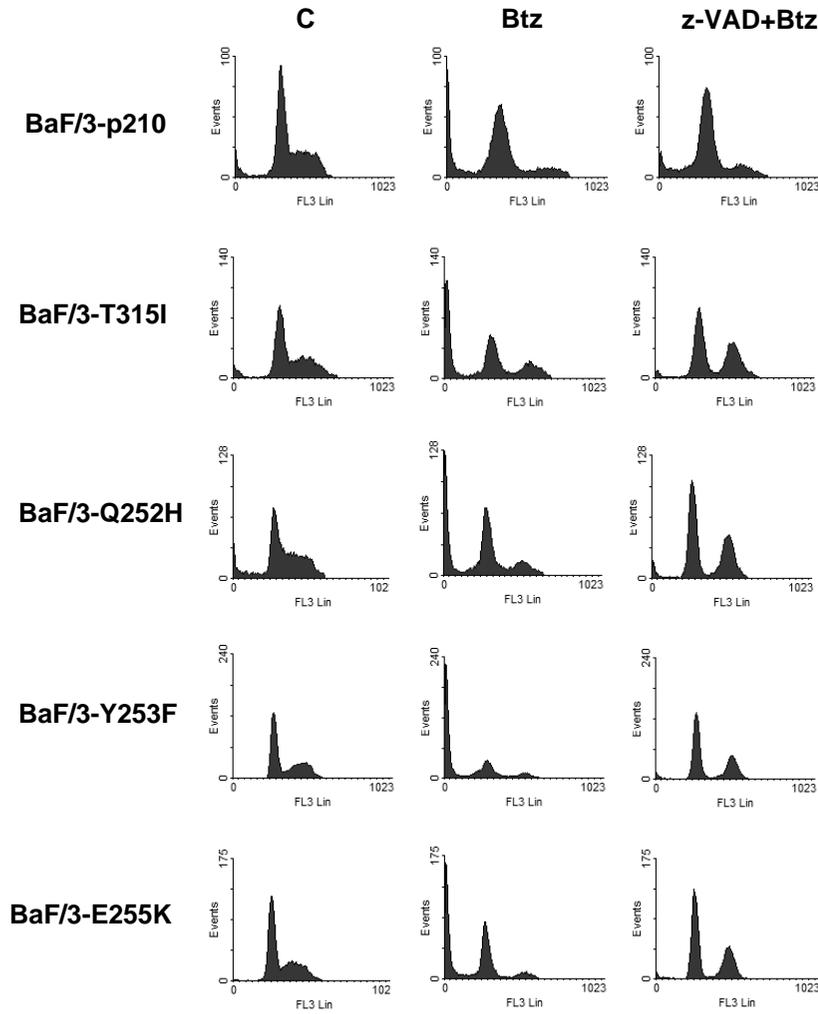


Figure 6: Bortezomib induces caspase dependent apoptosis in Bcr-Abl1 expressing cells.

6B



6C

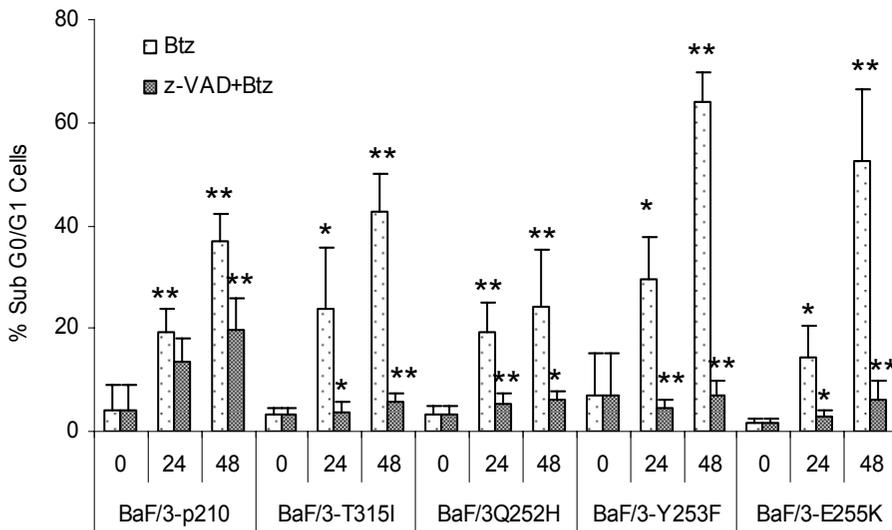


Figure 6: Bortezomib induces caspase dependent apoptosis in Bcr-Ab11 expressing cells.

7

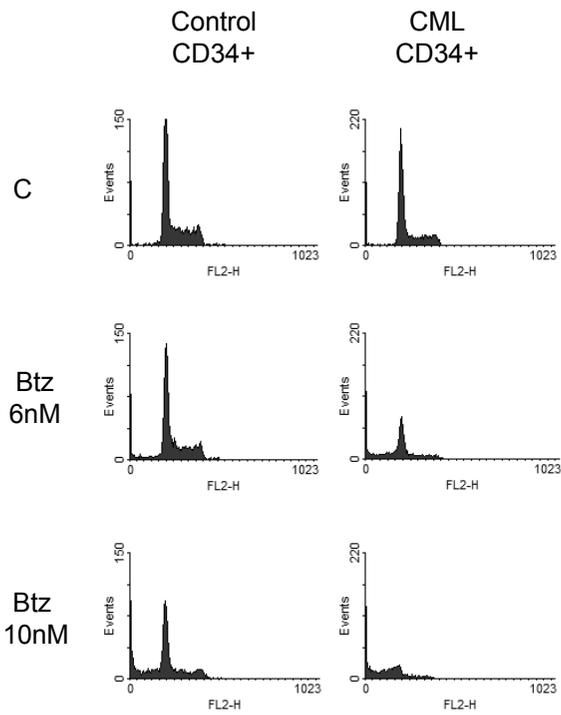


Figure 7: Bortezomib induces cell death in primary CD34+ cells from a CML patient.