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ORIGINAL ARTICLE

The NF-κB pathway blockade by the IKK inhibitor PS1145 can overcome Imatinib resistance

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Imatinib represents at present the most attractive therapy for BCR-ABL positive leukemias, even though a percentage of CML patients develop resistance to this compound. For these resistant patients a therapeutic approach based on a combination of drugs is more likely to be effective. In the last years, constitutive NF-κB/Rel activity has been demonstrated in several hematological malignancies. As a result, NFkB/Rel-blocking approaches have been proposed as antineoplastic strategies. Furthermore, the identification of specific kinases within the NF-κB activation pathway offers a selective target to address tailored therapies. In the current study, we show that the IKK inhibitor PS1145 is able to inhibit the proliferation of CML cell lines and primary BM cells. Moreover, the addition of Imatinib increases the effects of PS1145 in resistant cell lines and BM cells from resistant patients, with a further increase of apoptosis and inhibition of proliferation and colony growth. Our data provide the rational for a new therapeutic approach, which combines Imatinib and the IKK inhibitor PS1145 in CML resistant patients.
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Introduction

Imatinib (Gleevec or Glivec, Novartis) represents the most attractive and revolutionary therapy for BCR-ABL-positive leukaemias. Reports of international phase 2 studies involving patients with chronic phase and advanced-phase CML have demonstrated that more than 90% of patients with interferon-resistant chronic-phase CML can achieve a complete hematological response and almost 50% a complete cytogenetic response.[1] In addition, Imatinib has been shown to be highly superior to interferon (IFN) plus low-dose cytarabine as first-line therapy in newly diagnosed chronic-phase CML in terms of hematological and cytogenetic responses and risk of progression to accelerated-phase or blast-crisis CML.[2] In fact, about 75% of patients with newly diagnosed chronic phase CML treated initially with imatinib achieve complete cytogenetic remission (CCyR).[3]

Although hematological and cytogenetic responses were less common in patients with accelerated or blast phases than in patients with chronic-phase CML, the clinical results are still favorable in comparison with those achieved with conventional therapy. However, most of the patients in blast crisis who initially respond to therapy with imatinib, after a variable period of remission, tend to relapse.[4] Taken together, these observations suggest that imatinib is more effective in early chronic phase CML than in more advanced phases of the disease.[5] Finally, imatinib therapy selects and favors the emergence of clones resistant to the drug.[6] Multiple mechanisms account for clinical resistance, some involving alterations in BCR-ABL itself. For example, reactivation of BCR-ABL signaling either through point mutation or gene amplification of BCR-ABL has been observed in Imatinib-resistant patients.[7,8] and in a number of Imatinib-resistant BCR-ABL-positive cell lines. In these cases, a combination therapy is more likely to be effective in eradicating BCR-ABL-positive leukemia and produce clinical benefits rather than the treatment with a single compound.

The activity of NF-κB/Rel transcription factors can down-modulate apoptosis in normal and neoplastic cells of the hematologic and of other origins, thereby contributing to maintaining survival of neoplastic clones survival and impairing response to therapy.[9,10]Alterations in NF-κB or IkB genes are documented in some hematologic neoplasias, while in others dysfunction in NF-κB/Rel-activating signaling pathways can be recognized.[11,12] Constitutive NF-κB/Rel activity has been demonstrated in several hematologic malignancies;[13–16] and it probably plays a central role in determining resistance to therapy.[17] Based on this evidence, NFkB/Rel-blocking approaches have been introduced in antineoplastic strategies.[11,18,19] Identification of specific kinases critical for NF-κB activation would offer selective targets necessary for tailored therapies and help to avoid any possible detrimental effects that could result with widespread inhibition of NF-κB.[20,21]

The IkB kinase IKK is a complex composed of three subunits, IKKα, IKKβ IKKγ. IKK phosphorilates two serines in the N-terminal regulatory domain of IKB proteins.[22] Phosphorilation of IkB by IKK tags IkB for polyubiquitination by a specific ubiquitin ligase. IkB proteins are rapidly degraded by the proteasome, thereby freeing NF-κB to enter the nucleus where it binds to DNA and activates transcription.[22] This complex may therefore represent an attractive target for molecular therapies.

PS1145 (Millennium, Cambridge, MA, USA) is a selective inhibitor of the IkB kinase IKK, the kinase inhibitor of NF-κB. Previously published studies clearly demonstrated that PS1145 is able to block the proliferation of multiple myeloma (MM) cells adherent to bone marrow stromal cells[23] and to block the protective effect of IL6 against dexamethasone-induced apoptosis.[24]

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Although at present very few data regarding the activity of PS1145 have been reported, this compound may represent a powerful therapeutic strategy in the leukemia setting.

Materials and methods

Compounds

PS1145, a gift from Millennium (Cambridge, MA, USA) is a selective inhibitor of IkB kinase IKK, the kinase inhibitor of NF-κB. PS1145 was received in lyophilized form, reconstituted in dimethyl sulfoxide (DMSO) and maintained at −20°C as a 10 mM stock. The optimal dose for *in vitro* experiments was established using ELISA and Western blot assays as described below in order to block IKB phosphorylation, and consequently NF-κB binding activity.

Imatinib, a gift from Novartis (Basel, Switzerland), was reconstituted in DMSO as 10mM stock and stored at −20°C.

Cell lines

K562 and KCL cell lines, both sensitive (s) and resistant (r) to imatinib, a kind gift from Carlo Gambacorti-Passerini (National Cancer Institute, Milan, Italy) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). For the K562 and KCL imatinib-resistant cell lines, media were supplemented with 0.5 and 0.2 μM Imatinib, respectively. The exact mechanism of resistance of these cell lines is unknown, nevertheless we excluded the presence of point mutations or gene amplification.

Patients

After informed consent, BM mononuclear cells were collected from 19 CML patients. In all, 15 out of 19 patients were in chronic phase (CP), three in accelerate phase (AP) and one in blastic phase (BP). The chronic and accelerated phases were defined according to published criteria.[1] Eight out of 15 CP patients were untreated at the time of collection. The remaining seven patients were under Imatinib therapy but they were characterized by the presence of upfront cytogenetic resistance (three out of seven) or they developed acquired resistance during Imatinib therapy. The cytogenetic resistance was defined as having more than 65% Ph positive methaphases after 1 year of imatinib therapy. In addition, BM samples were collected from seven healthy subjects as control. The patients in accelerated and blastic phases were under Imatinib therapy at the time of progression and of collection of samples.

Measurement of DNA binding activity of NF-κB

DNA binding activity was evaluated using an ELISA method (TransAM ELISA kit) following the manufacture's instructions. To prepare nuclear extract, 5×10^6 cells were washed with ice-cold phosphate-buffered saline (PBS) and incubated on ice on a shaker with 400 μl of cytosolic lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% NP-40, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride). After 15 min, nuclei wee separated by centrifugation at 5000 rpm for 10 min. The supernatant which contained the cytosolic proteins was then removed. The pellet, containing nuclei, was resuspended in 50 μl of a second lysis buffer provided by TransAM ELISA kit. After 30 min incubation, nuclei were clarified by high-speed centrifugation. Nuclear extract (20 μg) was assayed for the DNA binding activity of NF-κB according to the protocol of the TRANS AM Kit. Briefly the DNA binding motif of NF-κB (5′-GGGACTTTCC-3′) is coated to a 96-well plate. When nuclear extracts are added to the plate, transcriptionally active NF-κB binds to the DNA causing the exposure of an epitope which is recognized by a primary antibody directed against p65. A HRP-conjugated secondary antibody provides a sensitive colorimetric reaction which is quantified by spectrophotometry. Absorbances were read at 450 nm with a reference wavelength of 655 nM. All the experiments were performed in triplicate and the final numerical value is expressed as mean value ± standard deviation (s.d.).

Western blot for IkBα

To establish the ideal dose of PS1145 to be used in our experiments, K562 and CML BM cells were incubated with increasing doses of PS1145 for 24–48–72 h and then were analyzed using Western blotting to detect whether the block of IkBα phosphorilation was blocked. After incubation, 10^7 cells were washed twice in PBS and lysed in 500 μl of extraction buffer containing 50 mM TrisHCl pH8.0, 150 mM NaCl, 1% NP40 (wt/vol), 0.5% Sodium Deoxycolato, 0.1%SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin and 1 mM sodium orthovanadate. Cells lysates were incubated on ice for 30 min followed by centrifugation at 1200 *g* for 10 min at 4°C. Protein concentration was determined by Protein Assay Dye Reagent (Bio-Rad Laboratories, Hercules, CA. USA) and values were normalized accordingly. Protein (50 μg) was denatured in 3 × SDS sample buffer by boiling for 5 min, resolved on a SDS 10% polyacrylamide gel and transferred to nitrocellulose membranes. The membrane was blocked for 1 h at room temperature with 5% bovine serum albumin in 1 × Tris-buffered saline (TBS) and then probed overnight at 4°C with Phospho-IkBα (Ser32/36) monoclonal antibody (Cell Signaling Technology) at 1:2000 dilution in 1% BSA 1 × TBS-tween buffer. Detection was performed using anti-mouse IgG AP-linked secondary antibody for 1 h at 4°C and the product was detect by CDP-Star chemiluminescence reagent as specified by the manufacturer (PerkinElmer Life Sciences). Blots were then stripped with stripping buffer (62.5 mM Tris HCl pH 6.8 and 2% SDS) for 1 h at 42°C and reprobed with anti-β-actin monoclonal antibody (clone AC-74, Sigma-aldrich) at 1:1500 dilution in 1% BSA 1 × TBS Tween for 2 h at 4°C. Detection was repeated as described for Phospho-IkBα.

Immunofluorescence assay

To provide evidence of the effective blockage of NF-κB shuttle activity induced by PS1145, an immunofluorescence assay using an anti p65 monoclonal antibody was performed. Cytospins were prepared using K562 and KCL cells before and after treatment with 20 μM PS1145 for 6 h and TNF stimulated cells as control. Cytospins were fixed with 4% PFA and permeabilized with 0.1% Triton X-100 for 3 min. Cells were blocked with PBS containing 10% BSA for 45 min. Cells were then stained with a rabbit polyclonal antibody against NF-κB p65 (Santa Cruz Biotechnology) for 2 h at room temperature and washed three times with PBS for 3 min. The antibody–antigen complexes were detected by incubation for 30 min with a secondary goat anti rabbit Alexa Fluor 568 immunoglobulin G antibodies (1:1000, Molecular probes). Cells were washed in PBS for 5 min and treated with DAPI for 5 min to stain the nucleus. Coverslips were mounted with Mowiol and the cells were subsequently analyzed with Leica Gmbh fluorescence microscope using Qfluoro software (Leica Microsystem).

Proliferation assay

To assess the proliferation activity of BCR-ABL positive cells after PS1145 and Imatinib treatment, K562s, K562r, KCls, KClr were incubated with 1 μM Imatinib, 20 μM PS1145 or with the combination of the two drugs. Incubation with the vehicle alone (DMSO) was performed as control. Similar experiments were set up for BM primary cells collected from CML patients and healthy volunteers. Proliferation was evaluated at different time points (12, 24, 48 or 72 h) using MTT assay. 10^5 cells were plated in triplicate in 96-well plates in a volume of 100 ml/well of culture medium. After 24 and 48 h of incubation (37°C, 5% CO_2) 10 μl of WST-1/ECS solution (Electro Coupling Solution) were added to each well after 2 h of incubation in standard culture conditions. The plate was shaken thoroughly for 1 min on a shaker and the absorbance of the treated and untreated samples was measured using a microplate reader (BioRad) at 460 nm. The final numerical value is expressed as mean value $\pm$ standard deviation (s.d.).

Measurement of cell viability and apoptosis

Cell viability and apoptosis were evaluated in cell lines and primary BM cells at established time points (12, 24, 48 or 72 h) using Trypan blue dye exclusion and flow cytometry for the detection of annexin V positive cells. Cell lines and BM cells incubated with PS1145, Imatinib or the combination of both or the control samples were labeled with annexin V conjugated with fluorescein isothiocyanate and 7-aminoactinomycin D (7-AAD). Briefly, cells were washed once in PBS and once in 1 $\times$ binding buffer, then 5 μl each of annexin V-FITC and 7-AAD was added to the cells. Cells were incubated at room temperature for 15 min, after which 300 μl 1 $\times$ binding buffer was added and cells were analyzed by flow cytometry. Apoptotic cells were defined as annexin V positive and 7-AAD negative.

Colony growth assay

BM mononuclear cells from healthy subjects and from CML patients were plated in methylcellulose containing human growth factors (G-CSF, GM-CSF, IL3 and EPO) to test their ability to give origin to colony growth in semisolid culture after treatment with PS1145, Imatinib and their combination. Appropriate control samples were plated for each experiment. The assay for multilineage colony-forming units (CFU-Mix), erythroid bursts (BFU-E), and granulocyte-macrophage colony-forming units (CFU-GM) was carried out as described elsewhere.[25] Progenitor cell growth was evaluated according to previously published criteria.[25]

Results

DNA binding activity in CML cell lines and primary cells

The DNA binding activity of NF-κB was measured in K562 and KCL cells sensitive and resistant to Imatinib using an ELISA method. As shown in Figure 1 panel a, PS1145 was able to reduce the NF-κB binding activity in K562 and KCL by 92 and 87%, respectively, when compared to controls. Similar results were obtained in resistant cells K562r and KCLr showing an inhibition of NF-κB activity of 93 and 82%, respectively. Imatinib incubation followed by TNF stimulation resulted in a modest effect on NF-κB activity with a 20 and 16% inhibition in K562 and KCL cells, respectively. Similar results were obtained in BM cells from CML patients (Figure 1 panel b). PS1145 reduced NF-κB activity by 67 $\pm$ 11% (mean value $\pm$ s.d.; range 52–85%) in BM cells from sensitive (patients 1–8 of Figure 1) and resistant CML patients (patients from 9 to 15 of Figure 1).

Immunofluorescence assay

In order to assess the effect of PS1145 on NF-κB nuclear localization, immunofluorescence was performed on treated

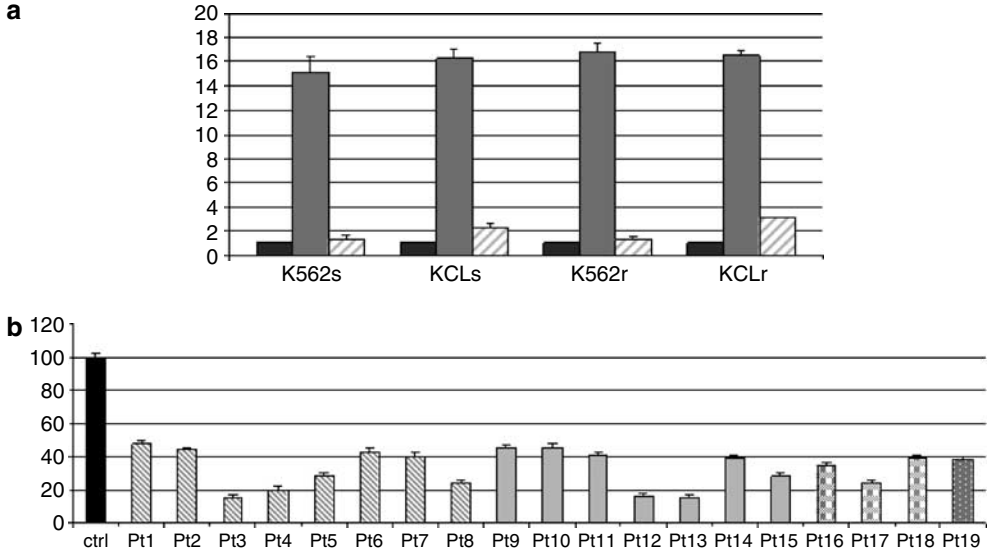

Figure 1 (**a**) DNA binding activity of NF-κB evaluated by ELISA in K562 and KCL cells sensitive (s) and resistant (r) to Imatinib therapy. The value of 1 has been attributed to the basal level of NF-κB DNA binding activity (black columns). The NF-κB activity after TNF stimulus (gray columns) and after PS1145 treatment (ruled columns) is expressed as folds of activity respect to the control. (**b**) Percentage of inhibition of NF-κB binding activity after PS1145 incubation in CML patients when compared to controls which represent the 100% of activity. Pts 1–8 are in CP and sensitive to Imatinib, Pts 9–15 are in CP and they are resistant to Imatinib. Pts 16–17–18 are in accelerated phase and patient 19 in blastic phase. All the experiments have been performed in triplicate and expressed as mean value.

and untreated K562 cells. As shown in Figure 2, the *in vitro* incubation with PS1145 resulted in an unbalanced distribution of NF-κB with a predominance of cytoplasmatic localization (Figure 2b). In contrast, untreated control cells showed both, cytoplasmatic and nuclear localization of NF-κB (Figure 2a).

Western blot

Western blot assay carried out in K562s and K562r, demonstrated that the phosphorilation of Ikbα is strongly reduced after incubation with PS1145 at the concentration of 10 and 20 μM (Figure 3)

Effect on proliferation, viability and apoptosis

In sensitive cell lines K562s and KCLs incubated with 1 μM Imatinib, the MTT assay detected a 48 and 37% decrease in cell proliferation, respectively. By contrast no effects on proliferation were observed in resistant cell lines K562r and KCLr incubated in the same conditions. Interestingly, incubation with 20 μM PS1145 for 48 h, reduced the proliferation rate of both sensitive and resistant cells (Figure 4a). In sensitive K562s and KCLs the proliferation was inhibited by 47 and 40%, respectively, and in resistant cells K562r and KCLr a 33 and 40% inhibition was observed. Moreover, treating these resistant cells with both 1 μM Imatinib and 20 μM PS1145 for 48 h strongly increased the inhibition of cellular proliferation with a reduction of cell growth by 90 and 82%, respectively.

Similar results were obtained by incubating BM cells with PS1145 (Figure 4b). After 24 h of treatment, proliferation in these cells was decreased by 31%±12.8 (mean value±s.d., range 11–55%) while a more significant reduction of 47%±15.1 was seen after 48 h (range 29–79%). As observed in cell lines, resistant patients also (patients from 9 to 15 in Figure 4b, c) showed a reduction in cell proliferation after PS1145 incubation. Imatinib treatment of BM cells from Imatinib sensitive patients resulted in a significant inhibition of proliferation (mean reduction of 41±11%; range 29–55%) while, as expected, no significant effects on cell proliferation was observed in CML patients resistant to Imatinib therapy. The combination of both compounds, Imatinib and PS1145, increased the inhibition of cell proliferation compared to PS1145 alone: 47±12% after 24 h and 71.2±8.7% after 48 h.

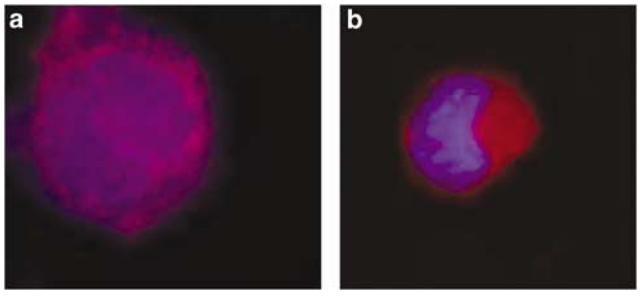

Figure 2 Immunofluorescence assay using the antibody against the subunit p65 of NF-κB in K562 cell line. (**a**) Control sample. NF-κB is localized in both cytoplasm and nucleous. (**b**) K562 after treatment with 20 μM PS 1145. NF-κB is mainly localized within the cytoplasm.

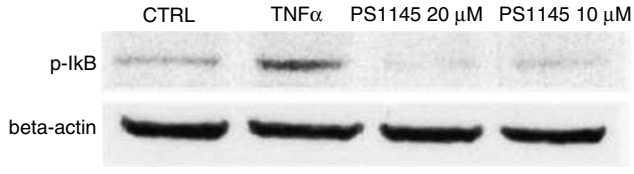

Figure 3 Western blot showing the phosphorilation status of IKBa in control K562 cells (line 1), after TNFa stimulus (line 2), and after the incubation with PS1145 20 μM (line 3) and 10 μM (line 4).

Apoptosis assay

The reduced proliferation observed in CML cells after 20 μM PS1145 treatment is largely due to the induction of apoptosis. As shown in Figure 5, in K562s and KCls the percentage of apoptotic cells was 38 and 35%, respectively after 48 h of incubation. Similarly, it was 35% in K562r and 37% in KClr in resistant cells incubated at the same conditions. The same sensitive lines incubated only with 1 μM Imatinib for 48 h showed a percentage of apoptotic cells equalling 32 and 41%. In resistant cell lines number of apoptotic cells did not significantly increase after Imatinib incubation when compared to the control samples (7 *vs* 9% in K562r and 9 *vs* 9.5% in KCLr).

By contrast, the combination of 20 μM PS1145 and 1 μM Imatinib induced a significant increase of apoptosis, in K562r from 7 to 69% and in KCLr from 9 to 71%.

Similar results were obtained in BM cells from CML sensitive and resistant patients where a significant amount of apoptotic cells were induced by the combination of Imatinib and PS1145. In the seven resistant patients studied, the addition of Imatinib induced a mean percentage of apoptotic cells of 47±8%. The same experiments carried out in BM cells from healthy volunteers did not result in any significant changes with respect to apoptosis.

Colony growth assay

Cellular resistance to Imatinib was confirmed by robust hematopoietic colony formation in the presence of 1 μM of Imatinib. As shown in Figure 6, the *in vitro* incubations of BM samples from sensitive patients with 1 μM of Imatinib resulted in a marked inhibition of CFU-GM compared to the control. Mean value of CFU-GM: 7±4 *vs* 50±5. In contrast, no significant inhibition of colony growth was detected after incubation of BM cells from resistant patients when compared to the untreated control cells. The mean value of CFU-GM 48±6 *vs* 47±6. PS1145 treatment resulted in a significant reduction of colony growth in both sensitive and resistant patients: in sensitive patients CFU-GM were 19±2 and in resistant patients 14±4. The addition of PS1145 and Imatinib did not significantly increase the effect of Imatinib in sensitive BM cells: 6±5. However, an additional effect was noted in resistant patients (mean value of CFU-GM of 5±6) where the combination of the two drugs induced a block of colony growth similar to that induced by Imatinib in sensitive patients. As shown in Figure 6, in normal BM samples neither the incubation with PS1145 alone nor the combination of Imatinib and PS1145 was able to induce colony growth inhibition.

Discussion

NF-κB is an inducible transcription factor that plays an important role in the expression of a variety of genes involved in cell survival.[21,26,27] The biological activity of NF-κB is tightly controlled by its inhibitor protein IkBα, which binds to and sequesters the transcription factor in the cytoplasm.[28] IkBα, the

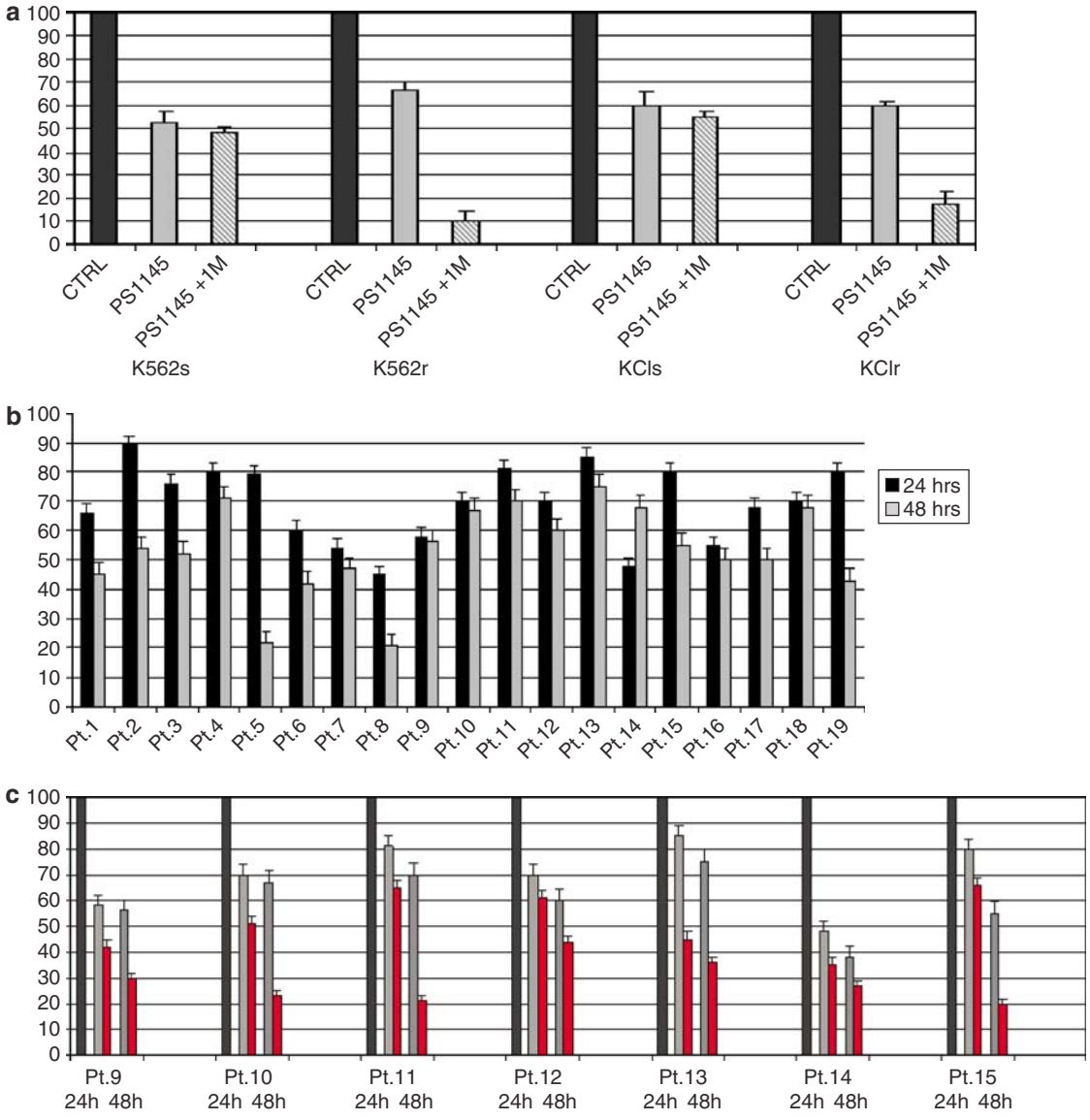

Figure 4 (**a**) Percentage of inhibition of proliferation evaluated by MTT assay in cell lines sensitive and resistant to Imatinib after PS1145 and PS1145 plus Imatinib incubation compared to the control. (**b**) Percentage of inhibition of cell proliferation induced by PS1145 in BM cells obtained from sensitive patients (pts 1–7), resistant patients (pts 8–15), AP patients (Pts 16–18) and BC patient (pt 19). (**c**) Percentage of inhibition of cell growth when compared to controls (black columns) in BM cells from resistant patients (pt 8–15) after PS1145 incubation (gray columns) and Imatinib and PS1145 incubation (red columns) after 24 h (first column) and 48 h (second column).

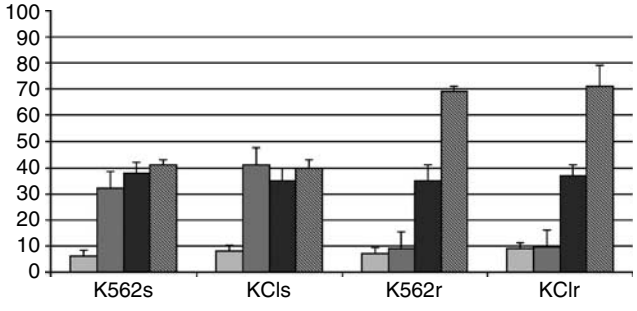

Figure 5 Percentage of apoptotic cells evaluated by FACS for the detection of annexin V positive cells in K562 and KCL sensitive and resistant to Imatinib in control cells (light gray columns), after the incubation with Imatinib (dark gray columns), PS1145 (black columns), and Imatinib and PS1145 combination (ruled columns).

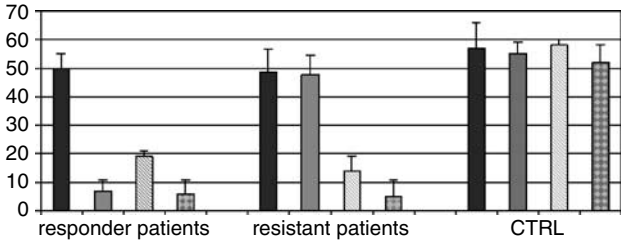

Figure 6 Mean value of CFU-GM grown from Imatinib sensitive and resistant cells and normal BM cells (CTRL), in control cells (black colums), after incubation with Imatinib (gray columns), PS1145 (ruled columns) and with imatinib and PS1145 combination (squared columns).

best characterized member of the IkB family is a protein mainly regulated by phosphorilation. IKK-mediated phosphorilation of IkBα 32/36 serine residues triggers its ubiquitination and subsequent degradation via the proteasome dependent proteolysis, thereby releasing NF-κB to the nucleus to function as a transcription activator.[29,30] It has been demonstrated that c-ABL inhibits IkBα degradation by inducing its accumulation in the nucleous.[31,32] c-ABL is a protein that actively shuttles between the cytoplasm and the nucleus.[33] Interestingly IkBα also actively undergoes nucleus-cytoplasm shuttling. IkBα enters the nucleus to complex with NF-κB and to disable its transcriptional activity by promoting nuclear export.[31] The functional inhibition of NF-κB therefore serves as one of the mechanisms for the proapoptotic function of c-ABL.[31] The finding that the inhibition of NF-κB by c-Abl requires its nuclear localization is of great significance because the oncogenic Bcr-Abl, which is mainly cytoplasmatically localized, is unable to share the same activity. On the contrary Bcr-Abl can potently activate NF-κB which has been demonstrated to be essential for Bcr-Abl to induce transformation.[34] This completely opposite effect on the NF-κB mediated cell survival pathway seems to represent a key point that differentiates the oncogenic Bcr-Abl from the growth suppressive function of normal c-Abl. Moreover this provides the rationale for targeting IkBα phosphorilation in BCR-ABL positive leukemias.

Our study clearly demonstrated that cells obtained from CML patients undergo growth arrest and apoptosis *in vitro* after NF-κB inhibition by PS1145, a pharmacologic blocker of the IKK kinase. Importantly, a significant apoptotic response could also be obtained in CML patients resistant to imatinib and this response is more profound in Imatinib resistant cells that are treated with the combination of Imatinib and PS1145. Although at present the mechanisms underlying the induction of apoptosis in CML imatinib resistant cells treated with the Imatinib and PS1145 combination is at present a pure object of speculation, these data clearly suggest an intriguing approach to induce apoptosis in imatinib resistant cells probably based on the presence of imatinib resistance itself. The combination of Imatinib and the IkB inhibitor could therefore represent a valid approach to be tested *in vivo* for the treatment of CML patients resistant to Imatinib therapy.

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