A New Nonpeptidic Inhibitor of 14-3-3 Induces Apoptotic Cell Death in Chronic Myeloid Leukemia Sensitive or Resistant to Imatinib

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ABSTRACT

Resistance of chronic myeloid leukemia (CML) to tyrosine kinase inhibitor imatinib mesylate (IM) is most often due to point mutations in the Bcr-Abl fusion gene. T315I mutation (resulting in substitution of Ile for a Thr residue at the “gatekeeper” position 315) raises particular concern, because it also provides resistance to second-generation kinase inhibitors already approved for clinical use (nilotinib and dasatinib). Much effort is therefore focused on alternative molecular-based strategies. Previous studies proved that binding to 14-3-3 scaffolding proteins leads to cytoplasmic compartmentalization and suppression of proapoptotic and antiproliferative signals associated with Bcr-Abl protein kinase, hence contributing to leukemic clone expansion. Here we investigated the effect of 14-3-3 inhibition disruption on hematopoietic cells expressing the IM-sensitive wild type Bcr-Abl and the IM-resistant T315I mutation.

Using a virtual screening protocol and docking simulations, we identified a nonpeptidic inhibitor of 14-3-3, named BV02, that exhibits a remarkable cytotoxicity against both cell types. c-Abl release from 14-3-3α, promoting its relocation to nuclear compartment (where it triggers transcription of p73-dependent proapoptotic genes) and to mitochondrial membranes (where it induces the loss of mitochondrial transmembrane potential) combined with c-Abl enhanced association with caspase 9 (a critical step of sequential caspase activation further contributing to c-Abl pro-apoptotic function) has a prominent role in the effect of BV02 on Bcr-Abl-expressing cells. In conclusion, BV02 may be considered as a treatment option for CML and, in particular, for more advanced phases of the disease that developed IM resistance as a consequence of Bcr-Abl point mutations.

Introduction

The tyrosine kinase (TK) inhibitor imatinib mesylate (IM) has revolutionized the prognosis of chronic myeloid leukemia (CML), with best complete hematologic and cytogenetic response rates of 98 and 87%, respectively, after 5 years of follow-up (Druker et al., 2006). However, there is growing concern for the development of resistance, most often as a result of the emergence of Bcr-Abl point mutations. At the protein level, Bcr-Abl point mutations may either distort the configuration of Abl kinase, rendering it unable to adopt the inactive conformation to which IM binds, or change the identity of residues that directly contact IM (Weisberg et al., 2007). The greatest therapeutic challenge is posed by the substitution of highly conserved Thr residue at position 315, controlling the access to a hydrophobic pocket of the enzymatic active site, by Ile (T315I). In fact, T315I mediates resistance not only to IM but also to second-generation Abl kinase inhibitors highly effective in patients with CML who failed IM therapy (Druker, 2008). The need for new strategies to treat IM-resistant CML has stimulated considerable efforts to develop compounds targeting key functional motifs distant from the ATP-binding pocket of Bcr-Abl protein or, alternatively, kinase downstream effectors.

14-3-3 are a highly conserved family of 28- to 33-kDa acidic proteins consisting of seven members (β, γ, ε, σ, ζ, τ, and η).
the phosphorylated forms of $\beta$ and $\gamma$ were initially described as $\alpha$ and $\delta$, respectively (Aitken, 2006). They associate with more than 300 partners to regulate critical events such as cell cycle progression, DNA damage response, apoptosis, protein trafficking, signal transduction, cytoskeletal rearrangements, metabolism, and transcriptional regulation. Structural studies proved the dimeric nature of 14-3-3 proteins that can function as homo- or heterodimers. Each monomer consists of nine $\alpha$-helices arranged in an antiparallel array to surround an amphipathic groove, forming a highly conserved binding site for client proteins (Xiao et al., 1995). 14-3-3 binding is primarily dependent on ligand phosphorylation. Two main consensus motifs recognized by all 14-3-3 isotypes have been identified. They correspond to RSX-pS/T-XP (mode I) and RXXX-pS/T-XP (mode II) sequences, where pS/T denotes phospho-Ser or phospho-Thr and X any other amino acid except Cys. Crystal structures of phosphopeptide-bound 14-3-3 complexes demonstrated that pS/T binding occurs within amphipathic grooves at either edge of the central channel of 14-3-3 dimer. An additional mechanism of 14-3-3 binding is provided by the phosphorylation status of specific Ser/Thr residues. In particular, Ser58 phosphorylation by Akt regulates 14-3-3 dimerization and ligand binding site for client proteins (Xiao et al., 1995). 14-3-3 consists of nine $\alpha$-helices arranged in an antiparallel array to form a dimeric structure proved by NMR and crystallography (Dubois et al., 1997; Powell et al., 2002; Yoshida et al., 2005). Accordingly, 14-3-3$\beta$ phosphorylation at Thr233 phosphorylation by caspase kinase I negatively regulates c-Raf binding, and Ser184 phosphorylation by JNK promotes the release of forkhead box O 3a transcription factor and c-Abl TK (all residue numbers refer to 14-3-3$\alpha$). In particular, Ser58 phosphor-

**Materials and Methods**

**Structure-based-pharmacophore model generation.** Protein Data Bank entry 1ywt corresponds to crystallographic structure of 14-3-3$\alpha$ isoform in complex with a mode I-binding motif phosphopeptide (Wilker et al., 2005). This structure was imported in LigandScout 1.0 software (InteLigand Software-Entwicklungs und Consult GmbH, Vienna, Austria), and the peptide ligand was selected for pharmacophore generation using default parameters supplied by the software and in agreement with Catalyst 4.10 platform later employed for database searching (Catalyst 4.10; Accelrys Inc., San Diego, CA). Excluded volume spheres, corresponding to regions in which the presence of a ligand is not allowed, were automatically added to the pharmacophoric model. The final model consisted of a large number of features, illustrated in detail in the Supplemental material. A MD simulation has been applied to investigate the stability of interactions involving hydrogen bonds by means of GROMACS 3.3 software (http://www.gromacs.org; see Supplemental data for further details). Topology file for the phosphopeptide ligand was generated using PRODRG server (Schüttelkopf and van Aalten, 2004).

**Database Searching.** Once simplified by MD simulation, the pharmacophoric model was used as a search query for screening the Asinex Gold Collection (Asinex Ltd., Moscow, Russia). Catalyst 4.10 was used to perform the screening, whereas molecular weight and Lipinski’s properties were computed by means of Cerius2 (ver. 4.10L; Accelrys).

**Docking Studies.** To prepare input structures of selected compounds for docking calculations, a geometry optimization was performed using MacroModel 8.5 with OPLSA 2005 force field, the Polak-Ribiere conjugate gradient method, and a convergence of 0.001 kJ·mol$^{-1}$·Å$^{-1}$ (Mohamadi et al., 1999; Kaminski et al., 2001). Charges were computed by means of MOPAC2007 software (http://openmopac.net) using the AM1 Hamiltonian. All docking studies were performed by means of GOLD software, version 3.0.1, with ChemScore scoring function (Verdonk et al., 2003). For each ligand, 50 independent GA runs were performed, with a maximum number of 10$^5$ GA operations on a set of five islands with a population size of 200 individuals. Remaining GA parameters were kept to their default values. The binding site cavity was defined with a radius of 10 Å around the phosphopeptide. The X-Score program (Wang et al., 2002) was then used with default parameters to rerank results from GOLD (Verdonk et al., 2003) for each compound.

**MIFs Analysis.** MIFs were calculated by means of GRID version 22 (Molecular Discovery Ltd. Pinner, Middlesex, UK) to analyze how favorable interaction regions for a specific probe are located in the 14-3-3$\beta$ binding site. Minimum energy points of these MIFs were then computed and used as a tool to analyze the docking results of selected compounds. In details, probes DRY and C1 were selected to calculate MIFs referring to hydrophobic interactions, N1 and O for H bond donor and acceptor interactions, and PO4 for describing negative interaction regions. The determination of MIF minimum points was carried out by means of Minim and Filmap programs (both implemented in the GRID package) using a threshold value of 0 kcal/mol (see Supplemental Fig. S1).

**BV02 Chemistry and Synthesis.** BV02 was purchased from Asinex (Moscow, Russia), which declares a purity higher than 95%. It was synthesized according to a previously published procedure (Bauer and Rademann, 2003). In brief, anhydrous reactions were run under a positive pressure of dry N$_2$. Thin-layer chromatography was carried out using thin-layer chromatography plates silica gel 60 F254 from Merck (Milan, Italy). $^1$H and $^{13}$C NMR spectra were recorded at 400 MHz on a DPX400 spectrometer from Bruker Avance (Milan, Italy). Chemical shifts relative to chloroform and tetramethylsilane are at $\delta$ 7.24 ppm at 6.00 ppm, respectively. Melting points were determined with a Gallenkamp melting point apparatus (Sanyo Gallenkamp, Loughborough, UK) and are uncorrected. 4-Aminoantipyrine (compound 1 in Fig. 1A: 158.7 mg, 0.78 mmol, 3.00 eq) was
dissolved in anhydrous dimethylformamide (1 ml). Next, trimellitic anhydride (compound 2 in Fig. 1A; 50.2 mg, 0.26 mmol, 1.00 eq) and N,N-dimethylamino pyrimidine (10.0 mg) were added and the resulting mixture was stirred at room temperature overnight (Fig. 1A). The suspension was diluted with H2O (5 ml) and then filtered under reduced pressure to obtain a white precipitate that was thoroughly washed with H2O, hexane and finally dried over P2O5. Compound purity was confirmed by high-performance liquid chromatography and sequencing were used to identify Bcr-Abl point mutations (Soverini et al., 2005). BV02 cytotoxicity was assayed in clonogenic assays, the best in vitro technique to quantify drug impact on cell reproductive integrity. In brief, we assessed the reduction of colony (aggregates containing >50 cells generated in 0.9% methylcellulose supplemented with 30% fetal calf serum) number in Bcr-Abl-expressing Ba/F3 cell lines and CD34+ in the presence of increasing doses of BV02 after 7- or 14-day incubation at 37°C in fully humidified atmosphere and 5% CO2. Linear and nonlinear regression analyses were used to calculate BV02 LD50 in Bcr-Abl-expressing Ba/F3 cell lines and CD34+ progenitors from patients with CML, respectively. Apoptosis induction and involved signals were assayed in Bcr-Abl-expressing Ba/F3 cell lines after 24-h exposure to 1 mM IM and 5 mM BV02 by means of cytofluorimetric analysis of Annexin V (F. Hoffmann-La Roche, Basel, Switzerland) and PI (Sigma) uptake, Western blot and IP/immunoblotting analyses, according to published methods (Mancini et al., 2009).

**Protein Analysis.** Western blot and IP/immunoblotting analyses were performed on proteins obtained from whole cells, nuclear fractions, and mitochondrial membranes according to published methods (Mancini et al., 2005, 2007). In brief, whole-cell lysates were obtained from 2 × 10⁷ cells in buffer A [10 mM Tris, pH 8.0, 150 mM NaCl, 10 mM iodosacetamide, 1% CHAPS, and 0.02% sodium azide supplemented with protease inhibitors: trypsin and pepsin inhibitors, leupeptin, antipain, Na3VO4, and phenylmethylsulfonyl fluoride (all from Sigma)]. Nuclear lysates were obtained from naked nuclei recovered from 2 × 10⁷ cells kept in buffer B [10 mM NaCl, 5 mM MgCl2, 10 mM phosphate buffer, and 0.1% Tergitol-type detergent NP40 (all from Sigma) supplemented with protease inhibitors] by means of three sonication rounds [10-s pulses using a Hight Intensity Ultrasonic Processor/Sonicator from Cole-Parmer Instruments (Vernon Hills, IL) equipped with 2-mm tips]. Mitochondrial membranes were separated from whole-cell lysates by 30-min centrifugation at 14,000 g in 2 ml of buffer C [20 mM HEPES, pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1% potassium metabisulphite, and protease inhibitors in 250 mM sucrose] at 4°C. Proteins or IP products obtained through overnight
incubation with primary antibodies in buffer D (250 mM NaCl, 15 mM MgCl₂, 40 mM HEPES, 60 mM glycerophosphate supplemented with protease inhibitors) were resolved in SDS-polyacrylamide gel electrophoresis. Gels were then transferred onto nitrocellulose membranes (Whatman Schleicher and Schuell, Dassel, Germany), labeled with primary and secondary antibodies in Tris-buffered saline with 5% bovine serum albumin (Sigma) and 0.1% Tween 20 (Sigma). The antibodies were purchased from Millipore (Billerica, MA), Cell Signaling Technology (Danvers, MA), and Santa Cruz Biotechnology (Santa Cruz, CA). Signals were visualized by the enhanced chemiluminescence detection system from Pierce (Rockford, IL). Signal intensities in single blots obtained from three individual experiments were quantified by mean of a dedicated software (VisionWorksLS; UVP, LLC, Upland, CA). Such software attributes a numerical value to signals of chemiluminescent substrates transferred on highly sensitive radiographic films (Pierce), thereby allowing a comparative analysis of protein levels in untreated and drug-exposed samples. The statistical significance of differences in signal intensities relative to cell treatments was assessed by mean of paired Student's t test; p < 0.05 was kept as limit for statistical significance.

Cytofluorimetric Analysis of Cell Cycle Distribution and Caspase 8 Activation. Cell cycle distribution was performed on 10⁶ cells fixed overnight in 70% ethanol and treated with 1 μg/μl PI and RNase (Sigma) at 37°C for 30 min. PI uptake was measured by mean of a FACScan flow cytometer set at >580 nm and dedicated software (both from BD Biosciences). Caspase-8 activity was detected with a commercial kit (Carboxyfluorescein FLICA Apoptosis Detection kits; Immunochemistry Technologies LLC, Bloomington, MN) according to the manufacturer's instructions. In brief, cells labeled with fluorochrome-bound inhibitor of caspase (FLICA: 300 μM/well at 37°C for 1 h in 5% CO₂, fully humidified atmosphere) were quantified by mean of cytofluorimetric analysis at an excitation range from 488 to 492 nm and an emission range from 515 to 535 nm. Apoptosis was quantified as the level of fluorescence emitted from FLICA probes bound to caspases. Nonapoptotic cells appeared unstained, whereas cells undergoing apoptosis were brightly fluorescent. Caspase-8 activity was quantified by mean of dedicated software (DIVA; BD Biosciences).

Results

Structure-Based Pharmacophore Model Generation. Nonpeptidic compounds able to affect interactions between 14-3-3 and their client proteins are not yet known. The ninemino acid, mode I-phosphopeptide MARSH-pS-YPA in complex with 14-3-3-σr (Protein Data Bank entry 1yw7) has been selected as starting point to generate a structure-based pharmacophoric model, in turn used as a search query for a virtual screening protocol aimed at identifying small molecules targeting the 14-3-3 binding site (Wilker et al., 2005). A preliminary model accounting for the large number of interactions found in the complex was then simplified as described in the Supplemental data, thus resulting in the final model consisting of two hydrogen bond acceptor features (HBA1 and HBA2), one hydrogen bond donor feature (HBD4), one negative ionizable feature (NEG.ION.), two hydrophobic features (HYD1 and HYD2), and eight excluded volumes (shown as gray spheres in Fig. 1B).

Database Searching. The final pharmacophoric model was then used as a three-dimensional query for screening a database of approximately 2 × 10¹⁰ commercially available compounds. The 99 compounds selected were further reduced to 87 entries by application of the Lipinski’s rule of five that allows the prediction of the drug likeness of a compound on the basis of several molecular properties, such as H bond acceptors (no more than 10), H bond donors (no more than five), molecular mass (lower than 500 Da), and octanol-water partition coefficient (lower than five) (Lipinski, 2000). Compounds with no more than one violation of the Lipinski’s rule were kept.

Docking Studies and MIFs. Compounds selected from virtual screening were docked to 14-3-3r binding site by means of GOLD software and then ranked on the basis of both ChemScore and XScore scoring functions (Verdonk et al., 2003). MIF computations were also performed when best docked conformations did not perfectly match the pharmacophoric model. In particular, points of minimum of MIFs were calculated, thus identifying the regions of most favorable interaction between the five probes and protein (see Supplemental Figs. S1 and S2). Finally, results of docking studies for selected compounds were compared with distribution of these minima into the groove. Fourteen compounds in which the docking pose fit at least three features of pharmacophoric model and that were in agreement with localization of the minima have been selected and evaluated for biological effects. BV02, purchased from Asinex, was the only compound among the 14 selected exhibiting a remarkable in vitro cytotoxicity on Bcr-Abl-expressing cells (Fig. 1A and data not shown).

BV02 Effects on Bcr-Abl-Expressing Cells. We first confirmed the differences in response to IM of pro-B murine cell line Ba/F3 expressing either the wt or the T315I-mutated Bcr-Abl construct. To this purpose, we evaluated drug impact on cell reproductive integrity [i.e., the ability of generating colonies (aggregates containing >50 cells) in semisolid culture media (0.9% methylcellulose)]. Such a method allowed the plotting of dose-response curves and of IM LD₅₀ (0.41 ± 0.01 μM for Ba/F3 cells expressing the wt Bcr-Abl and >10 μM for Ba/F3 cells expressing T315I mutation) (Fig. 2A, top and bottom left). Apoptosis induction in response to IM (1 μM for 24 h) was apparent in wt Bcr-Abl-expressing Ba/F3 cells, but not in Ba/F3 cells expressing T315I-mutation (Fig. 2B, first and third bars). BV02 induced a significant dose-dependent reduction of reproductive integrity of Ba/F3 cells expressing the wt and T315I-mutated Bcr-Abl with LD₅₀ of 1.04 ± 0.09 and 1.47 ± 0.12 μM, respectively (Fig. 2A, top and bottom right). Moreover, BV02 (5 μM for 24 h) recruited both cell types into the sub-G₁ phase of cell cycles and induced their apoptotic death (Fig. 2, B and C, second and fourth bars). Apoptosis induction in response to BV02 was further confirmed by caspase 8 activation, similar to that elicited by IM in wt Bcr-Abl-expressing Ba/F3 cells (Fig. 2D, first, second, and fourth bars). BV02 antiproliferative and proapoptotic effects were also seen in Ba/F3 cells expressing another Bcr-Abl point mutation associated with in vivo IM resistance [i.e., a Glu-to-Lys substitution at position 255 of Ab1 ATP-binding loop (E255K)] (Barthe et al., 2002). Results concerning BV02 effects on reproductive integrity, survival, and sub-G₁ recruitment of Ba/F3 cells expressing the E255K mutation are shown in Supplemental Fig. S3, A–C). BV02 effects were then investigated in CD34⁺ cells from patients with CML who were in blast crisis and developed in vivo IM resistance associated with the outcome of T315I Bcr-Abl mutation. BV02 elicited an antiproliferative response in CD34⁺ cells from all three patients, with LD₅₀ values of 0.5, 2.5, and 0.7 μM (Fig. 2E).
BV02 Effects on c-Abl Interaction with 14-3-3

Reactivation of proapoptotic or antiproliferative signals after their release from the 14-3-3 binding site is the most likely cause of BV02 cytotoxic effects on Bcr-Abl-expressing cells.

Here we investigated BV02 effects on subcellular distribution of c-Abl, the proapoptotic function of which is precluded by persistent 14-3-3 ligand associated with p210 Bcr-Abl TK (Mancini et al., 2009). In wt Bcr-Abl-expressing Ba/F3 cells,
BV02 (5 μM for 24 h) promoted c-Abl release from 14-3-3σ, abolished c-Abl expression in the cytoplasm (as IM did), but had no influence on 14-3-3σ levels (Fig. 3A). It is noteworthy that the p145 c-Abl form was the only one present in the cytoplasm, whereas the p120-kDa fragment generated by caspase-dependent cleavage was completely absent either in control or treated cells (Fig. 3A) (Barilà et al., 2003). As expected, BV02 had no influence on phosphorylation of c-Abl and p210 Bcr-Abl at Tyr245 (the residue located at linker region and required for both protein enzymatic activity) (Fig. 3A). c-Abl release from 14-3-3σ in response to BV02 induced its translocation into the nuclear compartment similar to that elicited by IM (Fig. 3B). In the nuclear compartment c-Abl was seen as 145 kDa full-length protein and 120 kDa fragment (Fig. 3B). The concomitant increment of cyclin-dependent kinase inhibitor p27<sup>Kip1</sup> in response to BV02 supports the idea that the release from 14-3-3 is a common mechanism for nuclear import of proteins whose 14-3-3 binding causes cytoplasmic compartmentalization and “loss of function” associated with p210 Bcr-Abl TK (Fig. 3B) (Fujita et al., 2002).

Moreover, BV02 promoted c-Abl targeting to mitochondrial membranes in the full-length (145-kDa) form and cleaved fragments, in particular the 60-kDa form resulting from caspase-induced cleavage (Fig. 3C). A previous study proved that c-Abl association with caspase 9 promotes the caspase 9 phosphorylation-dependent auto-cleavage, a critical step of DNA damage-induced auto-processing of caspase 9 leading to the activation of caspase 3 and apoptosis in response to genotoxic stress (Raina et al., 2005). Here we saw that c-Abl release from 14-3-3σ in response to BV02 and IM is associated with c-Abl enhanced interaction with caspase 9 and a remarkable increase of caspase 9 p35 cleaved subunit both in cytoplasmic compartment and at mitochondrial membranes (Fig. 3, A and C).

Caspase 9 is the apical member of intrinsic apoptotic pathway: it triggers sequential activation of caspases 3, 7, and 6, leading in turn to the activation of caspase 8, the apical member of extrinsic pathway (Inoue et al., 2009). Accordingly, caspase 9 activation in response to BV02 and IM was followed by caspase 8 cleavage into the p18 active fragment both in the cytoplasmic compartment and at mitochondrial membranes (Fig. 3, A and C). It is noteworthy that caspase 8 activation in response to either IM or BV02 evoked the cleavage and mitochondrial targeting of p15 fragment of the BH3-interacting death agonist Bid (Fig. 3C) (Billen et al., 2008). Such an event may further contribute to the outer mitochondrial membrane permeabilization. BV02-induced 1) c-Abl release from 14-3-3σ, 2) nuclear import, 3) mitochondrial membrane targeting associated with the activating cleavage of caspases 9 and 8, 4) nuclear translocation of p27<sup>Kip1</sup>, 5) cleavage, and 6) mitochondrial accumulation of Bid were also seen in IM-resistant Ba/F3 cells expressing T315I and E255K Bcr-Abl mutations (Fig. 4, A–C, and Supplemental Fig. S4, A–C). As in wt Bcr-Abl-expressing Ba/F3 cells, the exposure to BV02 reduced p145-kDa c-Abl but did not change 14-3-3σ levels (Fig. 4A). Therefore, BV02 had no influence on 14-3-3σ phosphorylation at Tyr245 (Fig. 4A and Supplemental Fig. S3C). As in the IM-sensitive cell context, c-Abl dissociation from 14-3-3σ was not contingent upon Tyr245 dephosphorylation (Fig. 4A and Supplemental Fig.
On the contrary, a slight increase (p < 0.05) of Tyr245 phosphorylation of normal and Bcr-rearranged c-Abl was apparent in Ba/F3 cells expressing the T315I mutation after exposure to BV02 (Fig. 4A). This increase may be ascribed to the release of unknown signal(s) that integrate phospho-Tyr signaling and/or protein stability. Further studies are required to elucidate the matter.

Despite the critical role of 14-3-3 ligand in the balance between life and death, no 14-3-3 antagonists with pharmacological applications have been developed so far. The only known compound capable of blocking 14-3-3 interaction with most or all of its client proteins is R18 (otherwise named difopein), a high-affinity peptide ligand binding with the amphipathic groove of all 14-3-3 isoforms (Wang et al., 1999). R18 was widely used to probe 14-3-3 participation in various signaling pathways. Here we used R18 to confirm that c-Abl release from 14-3-3 after the inhibition of the 14-3-3 binding site triggers the chain of events leading to apoptotic death of Bcr-Abl-expressing cells. In Ba/F3 cells expressing the wt Bcr-Abl, R18 (25 μM for 24 h) promoted c-Abl release from 14-3-3-3r (Fig. 5). Moreover, it induced c-Abl nuclear import and translocation to mitochondrial membranes and was associated with the increase of caspase 8 cleaved fragments (Fig. 5). It is noteworthy that c-Abl nuclear translocation was significantly enhanced by R18 association with IM, suggesting that the two compounds may have additive effects on 14-3-3 interaction(s) with client proteins (Fig. 5).

Discussion

TK of the Abl moiety of the p210 Bcr-Abl fusion protein constitutively activated by the coiled-coil domain at the N terminus of BCR is the causative event of CML (McWhirter et al., 1993). Accordingly, IM, a 2-phenylaminopyrimidine derivative having a specific inhibitory activity against c-Abl and Bcr-Abl TK, has revolutionized the disease prognosis (Druker, 2008). However, secondary resistance (defined as the loss of IM response during treatment) emerged as a major problem with IM therapy in CML, particularly in more advanced phases of the disease. IM resistance is most often driven by Bcr-Abl point mutations that directly impair drug binding to the Abl TK domain (T315I, F317L, and F359V) or preclude the fusion protein conformational changes required for drug binding either in the P-loop (M244V, G250E, Q252H/R, and Y253F/H) or near the activation loop (E255K) (Weisberg et al., 2007). The development of other therapeutic
options is therefore crucial to overcome drug resistance in CML.

Previous studies demonstrated that p210 Bcr-Abl TK induces an overexpression of 14-3-3, which therefore binds substrates with proapoptotic and antiproliferative effects. This event causes their “loss of function” through cytoplasmic compartmentalization, thereby contributing to the illegitimate expansion of CML hematopoiesis. Accordingly, the dephosphorylation of 14-3-3 binding motifs and the phosphorylation of 14-3-3 at critical binding residues restore the functions of Bad, p27kip1, and forkhead O3a, and of Bax and c-Abl, respectively, through events encompassing their release from 14-3-3 (Tsuruta et al., 2004; Yoshida et al., 2005; Dong et al., 2008; Mancini et al., 2009). Targeting 14-3-3 binding sites may be therefore considered a useful strategy to induce apoptotic death and growth arrest of leukemic progenitors. We applied a virtual screening protocol to a database of approximately 200,000 commercially available non-peptidic compounds. We found that among 14 compounds selected in agreement with the filters applied during the virtual screening and docking studies, the one named BV02 exhibits a remarkable cytotoxicity against Bcr-Abl-expressing cells either sensitive or resistant to IM (Figs. 1 and 2, A and C, and Supplemental Fig. S3A). c-Abl repartitioning among subcellular compartments is one component of BV02 cytotoxicity. First, the inhibition of 14-3-3 binding motifs and the phosphorylation of 14-3-3 at critical binding residues restore the functions of Bad, p27kip1, and forkhead O3a, and of Bax and c-Abl, respectively, through events encompassing their release from 14-3-3 (Tsuruta et al., 2004; Yoshida et al., 2005; Dong et al., 2008; Mancini et al., 2009). Targeting 14-3-3 binding sites may be therefore considered a useful strategy to induce apoptotic death and growth arrest of leukemic progenitors. We applied a virtual screening protocol to a database of approximately 200,000 commercially available non-peptidic compounds. We found that among 14 compounds selected in agreement with the filters applied during the virtual screening and docking studies, the one named BV02 exhibits a remarkable cytotoxicity against Bcr-Abl-expressing cells either sensitive or resistant to IM (Figs. 1 and 2, A and C, and Supplemental Fig. S3A). c-Abl repartitioning among subcellular compartments is one component of BV02 cytotoxicity. First, the inhibition of 14-3-3 binding site in response to BV02 promotes c-Abl release in the Tyr245 phosphorylated isoform, confirming that the two protein interactions are independent from c-Abl activating phosphorylation (Yoshida et al., 2005; Mancini et al., 2009). Once released from 14-3-3, c-Abl relocates to the nuclear compartment and mitochondrial membranes, where it evokes apoptotic death signals (Figs. 3 and 4, Supplemental Fig. S4). Previous studies proved that apoptosis after c-Abl nuclear import is driven by its ability to directly phosphorylate p73 and promote p73 acetylation to increase the transcription rate of proapoptotic genes (Yuan et al., 1999; Costanzo et al., 2002; Tsai and Yuan, 2003). Moreover, c-Abl release from 14-3-3 is followed by its enhanced association with caspase 9 (Figs. 3A and 4A). Such an event promotes caspase 9 phosphorylation and auto-cleavage required for its own full activation and sequential activation of caspase 8, the apical member of extrinsic apoptotic pathway through caspases 3, 6, and 7 (Figs. 3 and 4, Supplemental Fig. S4) (Raina et al., 2005; Inoue et al., 2009). Furthermore, caspase 9 activation is involved in c-Abl cleavage and, in particular, in the generation of the 120- and 60-kDa fragments, the former preferentially imported into the nuclear compartment and the latter preferentially targeted to mitochondrial membranes (Barilà et al., 2003). The loss of mitochondrial membrane integrity may be further potentiated by endoplasmic reticulum stress through events involving the activation of protein kinase Cδ (Ito et al., 2001; Qi and Mochly-Rosen, 2008).

Participation of c-Abl in death signals elicited by IM, including caspase activation and Bid accumulation, conflicts with a previous study showing that IM inhibits c-Abl proapoptotic function (Kumar et al., 2003). However, it must be noted that in the cited study, c-Abl inhibition was achieved by higher IM doses (10 μM) than those used in our study (1 μM).

Previous studies showed that Bcr proteins encoded by the nonrearranged gene or by the c-Abl-rearranged gene interact with 14-3-3 (otherwise referred to as Bap-1) and 14-3-3β (Reuther et al., 1994). Such interactions occur at the N-terminal Ser/Thr kinase domain encoded by the first exon of Bcr (Braselmann and McCormick, 1995). Nevertheless, in our experience, p210 Bcr-Abl was neither bound to 14-3-3 nor released from 14-3-3 and allowed to translocate into the nuclear compartment in response to IM (Mancini et al., 2009). Indeed, in the Bcr-Abl fusion protein, Bcr interaction with 14-3-3 is abrogated by Tyr autophosphorylation at the Bcr first exon (Liu et al., 1993; Peters and Smithgall, 1999). Moreover, Bcr phosphorylates 14-3-3 at Thr233, but not at Ser184, the JNK substrate involved in c-Abl interaction (Clokie et al., 2005). However, further investigation is required to elucidate whether 14-3-3 phosphorylation at Thr233 has a role in 14-3-3 binding with Bcr-Abl proteins. To conclude, our study supports the idea that the inhibition of 14-3-3 binding site may be considered for the treatment of CML and, in particular, to overcome the outcome of drug resistance associated with the disease progression (Dong et al., 2008). To our knowledge, BV02 represents the first nonpeptidic inhibitor of 14-3-3 proteins potentially useful for clinical purposes. Further work for the improvement of BV02 for clinical purpose is currently in progress.

Authorship Contributions

Participated in research design: Mancini, Corrada, Petta, Barbieri, Manetti, Bottà, and Santucci.

Conducted experiments: Mancini, Petta, Barbieri, and Santucci.

Performed data analysis: Corrada, Manetti, and Bottà.

Wrote or contributed to the writing of the manuscript: Mancini, Corrada, Petta, Barbieri, Manetti, Bottà, and Santucci.

References


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