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Identification of the first non-peptidic small molecule inhibitor of the c-Abl/14-3-3 protein–protein interactions able to drive sensitive and Imatinib-resistant leukemia cells to apoptosis

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ABSTRACT

An in silico structure-based ligand design approach resulted in the identification of the first non-peptidic small molecule able to inhibit protein–protein interactions between 14-3-3 and c-Abl. This compound shows an anti-proliferative effect on human leukemia cells either sensitive or resistant to Imatinib, in consequence of the T315I mutation. It also mediates c-Abl release from 14-3-3 in a way similar to that found in response to Imatinib treatment.

The constitutively activated tyrosine kinase (TK) activity of the Abl moiety of the p210 Bcr-Abl fusion protein is the causative event of chronic myeloid leukemia (CML).[1] Accordingly, TK inhibitors (Fig. 1) are currently used in CML, and, among them, Imatinib mesylate (**1**) represents the frontline therapy as it has revolutionized the treatment of this disease, improving the long-term survival of CML patients.[2]

However, despite the generally positive response to the clinical therapy, resistance to **1** emerged as a major problem in the treatment of CML, particularly in more advanced stages. Resistance to **1** is most often driven by Bcr-Abl point mutations (such as T315I, F317L, F359V) that can directly impair the binding of **1** or affect the conformation of the kinase domain required for the drug binding (Y253F, E255K in the ATP-binding loop; H396R in the activation loop; M351T, E355G, F359V in the catalytic domain).[3] The new generation of Bcr-Abl inhibitors, such as Dasatinib and Nilotinib (**2** and **3**, Fig. 1), overcomes the resistance associated with the great majority of Bcr-Abl mutations, with the only exception of T315I.[4] Moreover, compounds **5**–**9** (Fig. 1) are also known to inhibit T315I mutants resistant to **1**.[5] To explore the potentiality of alternative and/or complementary therapeutic strategies to overcome the drug resistance in CML, we focused on 14-3-3 proteins, which play a critical role in sub-cellular redistribution of pro-apoptotic proteins, such as Abl. In fact, binding of Abl to 14-3-3 is responsible for its prevalent cytoplasmic localization,[6] while disruption of the Abl/14-3-3 complex upon DNA damage leads Abl to nuclear translocation and activation of apoptosis.[6] Moreover, we have recently proved that p210 Bcr-Abl precludes post-translational modifications of 14-3-3 (namely, its phosphorylation at Ser186 for the σ isoform), preventing Abl nuclear translocation and reducing the apoptosis activation in CML cells.[7] All these results suggest that, in addition to the direct inhibition of the Bcr-Abl oncoprotein, targeting 14-3-3 with molecules able to affect its interactions with c-Abl (the residual c-Abl not rearranged with Bcr) may potentiate the current therapies for CML and be also effective against resistance to **1** due to the T315I mutation.[8] Accordingly, pharmaceutical research is now much more interested in discovery and developing small molecule inhibitors of protein–protein interactions that could provide novel opportunities for therapy. However, no small molecule non-peptidic inhibitors of 14-3-3 have been reported yet. The sole inhibitor of 14-3-3 known so far is a 20 amino acid polypeptide (thereafter referred to as R18) that binds the amphipatic groove of the protein through a WLDL sequence.[9] R18 is able to mask the region of the protein re-

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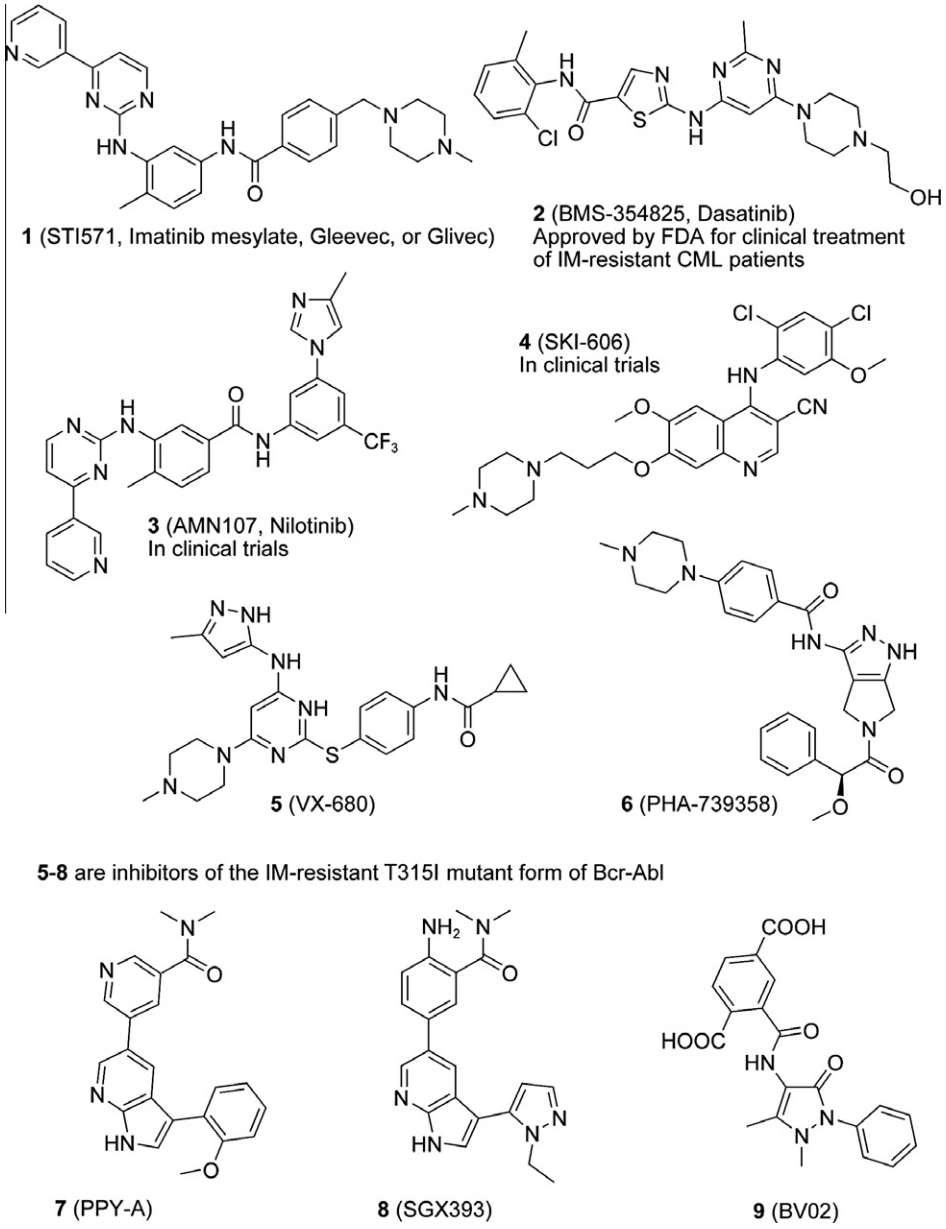

Figure 1. Schematic representation of current available inhibitors of Abl (**1**–**8**) and BV02 (**9**, discovered by a structure-based drug design approach). Compounds **5**–**8** are inhibitors of the T315I mutant form of Bcr-Abl resistant to **1**.

quired to bind client proteins, thus promoting Abl translocation to the nucleus.[6] In the attempt to identify compounds lacking in drawbacks of peptides, small non-peptidic molecules possibly able to disrupt the interactions between 14-3-3 and Abl have been searched by us by means of a computational protocol based on structure-based pharmacophore modeling, in silico virtual screening and molecular docking simulations. Structure-based pharmacophores are useful tools for virtual screening procedures because they codify important information derived from target–ligand interactions. Moreover, their combination with docking-based virtual screening procedure is known to significantly improve the probability of identifying putative hit candidates.[10]

The three-dimensional structure of the complex between 14-3-3σ isoform and the MARSH-phosphoS-YPA nonapeptide (protein data bank entry 1YWT)[11] was used to generate a map of the major interactions between the protein and its ligand by means of the software LIGANDSCOUT.[12]

However, because of the size of the peptide ligand, the pharmacophoric model was characterized by a high complexity in terms of the number of pharmacophoric features (Table 1S of Supplementary data). As a consequence, it was simplified by keeping hydrogen bond interactions with distances and angles stabilized to default values during molecular dynamics runs (Table 2S of Supplementary data). The final pharmacophoric model was used as a three-dimensional query to screen in silico a database of about 200,000 commercially available compounds, resulting in 99 entries that were further pruned to 87 by application of the Lipinski's rule of five. Selected compounds were then docked by means of GOLD software[13] into the 14-3-3 binding site, and ligand–target interactions were evaluated by a consensus scoring approach based on both ChemScore[13] and XScore scoring functions.[14] Moreover, the best docked conformation of each ligand was checked for its ability to map at least three pharmacophoric features of the model, as well as GRID minima corresponding to the regions of most favor-

able interaction between the protein and five different chemical probes, as found by GRID software.[15] As a result, 14 compounds that were able to satisfy such constraints were purchased and submitted to biological tests.

Preliminary cell proliferation assays conducted in Ba/F3 cells transduced with the wt Bcr-Abl construct led to the identification of BV02 (**9**, Fig. 1) as the most proficient compound in terms of cytotoxic potential (data not shown). BV02 cytotoxicity was assayed in clonogenic assays, an in vitro technique used to quantify the drug impact on cell reproductive integrity.[16] Further experiments showed that BV02 induced a significant dose-dependent reduction of reproductive integrity of Ba/F3 cells expressing both wt and T315I Bcr-Abl protein, with LD_{50} values of 1.04 ± 0.09 and 1.47 ± 0.12 μM, respectively (Fig. 2A).[17] The cytotoxicity of BV02 (5 μM for 24 h) in both cell types was, at least partly, driven by the induction of apoptosis, measured by cytofluorimetric analysis of annexin V and propidium iodide uptake (Fig. 2B).[17] BV02 had a similar anti-proliferative effect on early hematopoietic progenitors ($CD34^+$) isolated from CML patients who developed resistance to **1** in consequence of T315I Bcr-Abl mutation (Fig. 2C).[17] Taken together, these results showed that the inhibition of 14-3-3 upon BV02 in vitro treatment is effective against Bcr-Abl-expressing cells either sensitive or resistant to **1**.

To elucidate the mechanism driving apoptotic death induction in response to BV02, we investigated whether the compound owns the ability of releasing c-Abl from 14-3-3σ and promoting its relocation at sub-cellular compartments, that is, the nucleus and mitochondrial membranes. Western blot or immunoprecipitation (IP)/immunoblotting technique was used to perform protein analysis.[18] In Ba/F3 cell line transduced with the wt Bcr-Abl construct, BV02 (5 μM for 24 h) mediates c-Abl release from 14-3-3 in a way similar to that found in response to treatment with **1**, through events encompassing 14-3-3σ phosphorylation by c-Jun N-terminal kinase (Fig. 3A).[6] As a consequence, c-Abl translocated to the nuclear compartment, where it is expected to trigger p73- and p53-dependent pro-apoptotic signals. c-Abl nuclear import concerned the whole length protein (145 kDa) and the p120 kDa fragment generated by interaction with caspase 9 (Fig. 3B).[19] The significant increase of cyclin-dependent kinase inhibitor $p27^{Kip1}$ in response to BV02 suggests that a common mechanism for the nuclear import of proteins involved in cell proliferation and survival could be based on their release from 14-3-3 (Fig. 3B). Moreover, BV02-induced c-Abl translocation to mitochondrial membranes in the full-length and caspase-induced cleaved isoforms (mainly the 60 kDa fragment, Fig. 3C). At this level, c-Abl integration was associated with a significant increase of caspase 8 (18 kDa) and caspase 9 (35 kDa) cleaved fragments, a critical event for their dissipation in response to BV02 (Fig. 3C).[20] All these events were elicited by BV02 also in Ba/F3 cell line expressing the T315I Bcr-Abl mutation (Fig. 4A–C).

Finally, Figure 5A and 5B describes the theoretical binding mode of BV02 found with our docking calculations. In particular, the carboxylic group at the C5 of BV02 terephthalic moiety corresponds to the phosphate group of the phosphopeptide used to generate the pharmacophoric model. Its binding pocket is lined by K49, R56, R129 and Y130. Hydrogen bond interactions also occur between K122 and N175 with the carbonylic group at the C3 of the pyrazole ring and the NH group of the amide moiety, respectively. Moreover, the hydrophobic interactions involving the aromatic ring of the Tyr residue of the phosphopeptide are mimicked by the two methyl groups of the pyrazole ring of BV02.

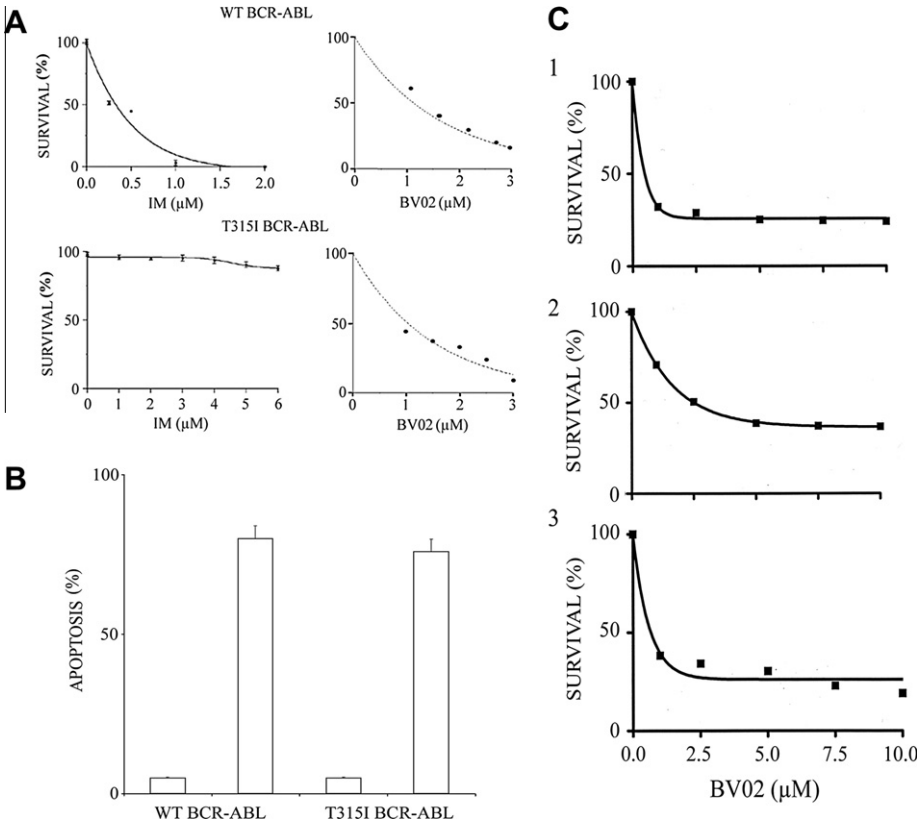

Figure 2. Effects of BV02 on Bcr-Abl-expressing cells. (A) Activity of BV02 toward reproductive integrity of Ba/F3 cells expressing the wt Bcr-Abl sensitive to **1** (upper panels) or the T315I mutation resistant to **1** (lower panels). (C) Activity of BV02 toward $CD34^+$ cells isolated from bone marrow samples of 3 (CML) patients in blast crisis who developed in vivo resistance to **1**. (B) Cytofluorimetric analysis of annexin V and propidium iodode uptake was used to measure apoptosis induction in response to BV02 (5 μM for 24 h) in Ba/F3 cells expressing the wt or the T315-mutated Bcr-Abl protein. Results shown in sections A and B represent the mean values ± SD of three individual experiments.

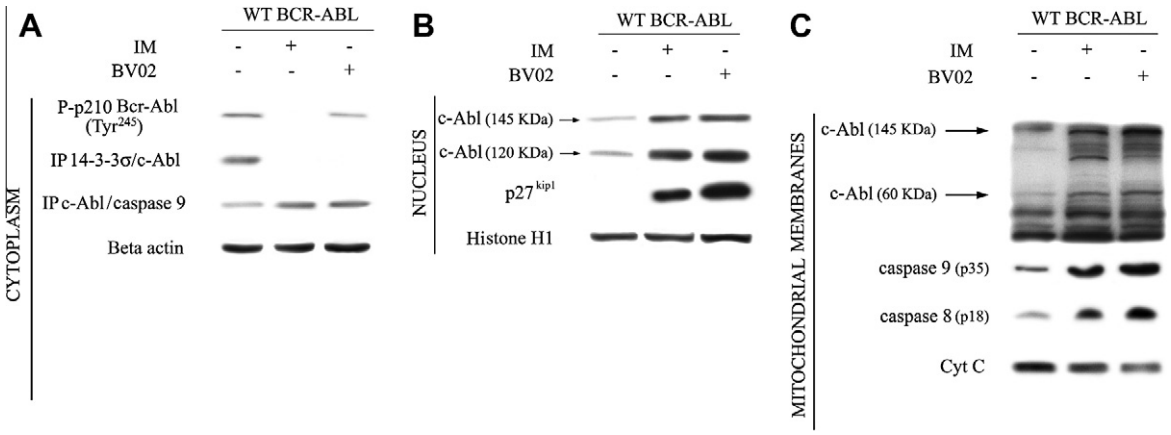

Figure 3. Effects of BV02 on c-Abl sub-cellular relocation in wt Bcr-Abl-expressing Ba/F3 cells. Protein expression and interactions in cytoplasmic (A) and nuclear (B) compartments and in mitochondrial membranes (C) were analyzed by means of Western blot or IP/immunoblotting of either untreated or after 24 h exposure to 1 μM **1** or 5 μM BV02. β-Actin, histone H1 and cytochrome c (Cyt c) levels served as controls for loading of cytoplasmic, nuclear and mitochondrial membrane proteins, respectively. Identical results were obtained in two additional experiments. Signal intensities in single blots were measured by a dedicated software to assess the statistical significance of differences elicited by either **1** and BV02. See Ref. 18 for further details.

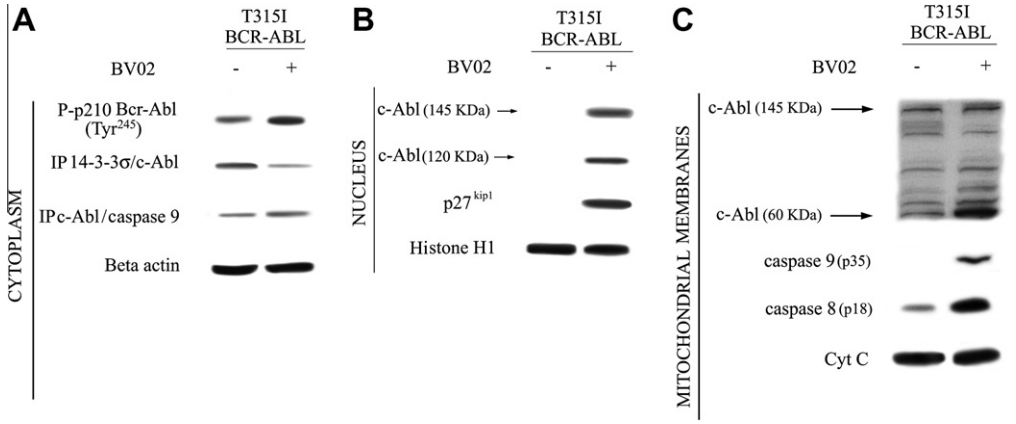

Figure 4. Effects of BV02 on c-Abl sub-cellular relocation in Ba/F3 cells expressing T315I Bcr-Abl mutation. See legend to Figure 3 and Ref. 18 for details.

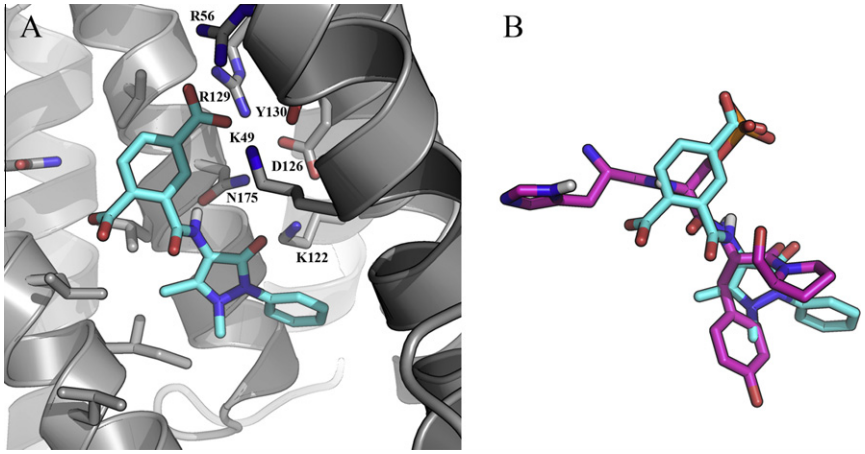

Figure 5. (A) Binding mode of BV02 (cyan sticks) on the structure of 14-3-3σ. (B) Superimposition of BV02 and the phosphopeptide (magenta sticks) used to generate the pharmacophoric model. Some of the residues of the peptide are not shown for clarity.

In summary, BV02 represents the first non-peptidic inhibitor of 14-3-3 proteins, discovered by application of a virtual screening approach. In addition to lack the drawbacks of peptidic drugs, BV02 belongs to the first generation of 14-3-3 inhibitors that could be used in Bcr-Abl-associated diseases to overcome the resistance to the traditional drugs.

Finally, our results further support the idea that in silico virtual screening is a valuable tool for the identification of new hit/lead

compounds and it should be routinely added to the protocols for drug discovery. In fact, application of sequential filters (pharmacophores, molecular docking and dynamics) to databases of commercially available compounds allows the researcher to avoid the time and money consuming step of synthesis and/or to apply testing procedures less expensive than a high throughput screening.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.08.019.

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16. In vitro clonogenic assay technique: this technique allows to calculate the lethal dose 50 (LD_{50}) through the reduction of number of colonies (defined as aggregates containing more than 50 cells generated in 0.9% methylcellulose, addition with 30% fetal calf serum) in response to scalar doses of drug.
17. We confirmed BV02 anti-proliferative effect on early hematopoietic progenitors ($CD34^+$) isolated from CML patients who developed resistance to **1** in consequence of T315I Bcr-Abl mutation. Using indirect immuno-magnetic technique, $CD34^+$ cells were isolated from mononuclear cell fractions of bone marrow samples purchased at Miltenyi Biotec (Miltenyi Biotec, Bergisch Gladbach, Germany). Their content, measured by cytofluorimetric analysis of CD34 expression, was greater than 85% in all three cases. Moreover, high-performance liquid chromatography (D-HPLC) and sequencing let identify Bcr-Abl point mutation associated with in vivo resistance to **1**.
18. General method for Western blot or immunoprecipitation (IP)/immunoblotting technique: lysates through sonication from 2×10^7 whole cells, naked nuclei (isolated in low salt/detergent buffer: 10 mM NaCl, 5 mM $MgCl_2$, 10 mM phosphate buffer and 0.1% NP40) or mitochondrial membranes (isolated by 30′ centrifugation at 14,000 rpm in 2 mL lysis buffer: 20 mM Hepes pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 0.1% PMS) were resolved in SDS–PAGE, transferred in nitrocellulose membranes, and labeled with primary and secondary antibodies. β-Actin, histone H1 and cytochrome c were used as internal controls for cytoplasmic, nuclear and mitochondrial membrane protein loading. Signal intensities from three repeated experiments were quantified by a GS-700 Imaging densitometer from BioRad, equipped with a dedicated software (Launch VisionWorks LS, Upland, CA).
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