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Computational techniques are valuable tools for the discovery of protein–protein interaction inhibitors: The 14-3-3σ case

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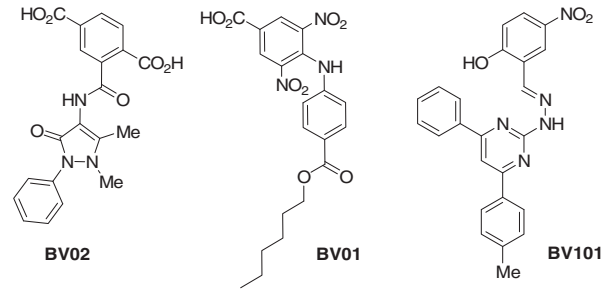
ABSTRACT

Targeting the binding site of 14-3-3 proteins lets the release of partner proteins involved in cell cycle progression, apoptosis, cytoskeletal rearrangement and transcriptional regulation and may therefore be regarded as an alternative strategy to integrate conventional therapeutic approaches against cancer. In the present work, we report the identification of two new small molecule inhibitors of 14-3-3σ/c-Abl protein–protein interaction (BV01 and BV101) discovered by means of computational methods. The most interesting compound (BV01) showed a lethal dose (LD_{50}) in the low micromolar range against Ba/F3 murine cell lines expressing the Imatinib (IM)-sensitive wild type Bcr-Abl construct and the IM-resistant Bcr-Abl mutation T315I. BV01 interaction with 14-3-3σ was demonstrated by NMR studies and elucidated by docking. It blocked the binding domain of 14-3-3σ, hence promoting the release of the partner protein c-Abl (the one not involved in Bcr rearrangement), and its translocation to both the nuclear compartment and mitochondrial membranes to induce a pro-apoptotic response. Our results advance BV01 as a confirmed hit compound capable of eliciting apoptotic death of Bcr-Abl-expressing cells by interfering with 14-3-3σ/c-Abl protein–protein interaction.

Inhibition of protein–protein interaction gained a significant interest in pharmaceutical research, providing novel opportunities for the therapy of several human diseases.[1–3] 14-3-3 is a family of highly conserved and ubiquitous proteins involved in multiple regulatory activities by interacting with more than 300 cellular partners.[4] Among the seven 14-3-3 isoforms currently characterized, 14-3-3σ is the only one that has been directly correlated to cancer so far.[5] In fact, 14-3-3σ interaction with Raf1, p53, c-Abl, Bad, HDAC or Cdc25 have been linked with cancer pathogenesis and progression.[6] Accordingly, 14-3-3σ may be considered as a suitable target for cancer therapy. However, due to the relatively recent understanding of the 14-3-3σ roles and to the difficulties connected to the development of protein–protein interaction inhibitors, effective anticancer agents targeting 14-3-3σ are not currently available. In 2010, two compounds were reported by Ottman[7] as able to stabilize the complexes formed by plant 14-3-3s and cellular partners. Very recently a peptide-mimetic inhibitor of human 14-3-3/ligand interaction has been discovered by means of small molecule microarray techniques.[8] The only small-molecule compound able to interfere with 14-3-3σ/c-Abl protein–protein interaction has been



LD_{50} = 1.04 μM (wt)
LD_{50} = 1.47 μM (T315I)

LD_{50} = 1.41 μM (wt)
LD_{50} = 1.70 μM (T315I)

LD_{50} = 0.65 μM (wt)

Fig. 1. Chemical structure of BV02 (reference hit compound), BV01 and BV101. LD_{50} calculated against Ba/F3 cells expressing the wt and the T315I mutated Bcr-Abl are reported, when available.

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recently discovered by our research group using computer-aided methods.[9]

This compound (BV02; Fig. 1) inhibits the interaction between 14-3-3σ and c-Abl in vitro with a lethal dose 50 (LD_{50}) in the low micromolar range (LD_{50} = 1.04 μM). BV02 promoted the nuclear localization of c-Abl and stimulated the pro-apoptotic signals in Ba/F3 cells expressing the wt and the T315I Bcr-Abl construct.[10,11] Due to its peculiar mechanism of action, BV02 was considered as a candidate for the treatment of Chronic Myeloid Leukemia (CML), and particularly to overcome the drug resistance associated with the disease progression. Unfortunately, later NMR studies revealed that BV02 underwent to a spontaneous chemical cyclization at room temperature, being the 'open' 2-carbamoyl benzoic moiety in equilibrium with its ftalimidic 'closed' form (see Supplementary data). This equilibrium is pH dependent and was observed either by solubilising the open derivative purchased from Asinex vendor (purity >95%) or the one obtained by chemical synthesis.[11] From a medicinal chemistry point of view, the simultaneous existence of two closely related equilibrium structures of the same hit compound could represents an obstacle for undertaking further 'hit to lead' optimization steps, since the identity of the chemical moiety responsible for the observed biological activity could not be easily determined. Accordingly, BV02 was not further investigated, but the identification of closely related analogues that may not undergo to such rearrangement is currently under study.

In the present work we wish to report how the application of molecular modeling coupled with biophysical and biochemical techniques could efficiently lead to the identification of protein–protein interaction inhibitors. Considering the chemical stability problem experienced with BV02, computational results have been carefully revised in order to discard those compounds that may suffer from possible spontaneous chemical rearrangements at room temperature.

The Virtual Screening (VS) protocol based on the sub-sequential application of pharmacophore modeling and ligand docking, which has been previously described,[9] has been used in this work to screen the whole Asinex database in search for potential 14-3-3σ/c-Abl protein–protein interaction inhibitors. In addition, to overcome the steric restrictions intrinsic in structure-based pharmacophoric modeling, high throughput docking (HTD) has been also applied alongside the VS. In the previously described approach the virtual screening was performed against the Asinex Gold collection, which consists of about 200,000 compounds. Here, we used the whole Asinex library, which contains more than 600,000 small molecules. Therefore, it should not be surprising if different results were obtained with respect to those previously discussed.[9] In addition, only compounds endowed with better scoring values than BV02 were selected from the database. As a result, a small set of the best ranking compounds identified by both the VS and the HTD protocols were purchased for a preliminary biological evaluation. Among them, compounds BV01 and BV101 (Fig. 1) showed considerable cytotoxicity against Bcr-Abl-expressing Ba/F3 cells and were further investigated.

BV01 is the 4-(4-hexyloxycarbonyl-phenylamino)-3,5-dinitrobenzoic acid and presents a different scaffold than the reference compound BV02. The only similarity is represented by the negative charge distributed on the 'head' of the inhibitor, which, according to docking studies, should interact in the 14-3-3σ amphipathic groove in close proximity of the cluster of basic residues consisting of K49, R56, R60 and R129 (Fig. 2). This is also in agreement with the orientation of the phosphate group of the bound phospho-peptide in the crystal structure (PDB code: 1YWT).[12]

The 'tail' of BV01 is represented by an aliphatic and very flexible lipophilic chain, whereas BV02 shows a conjugated aromatic system. The Tanimoto coefficient, which describes the similarity between two compounds based on the presence or absence of molecular fragments,[13] was herein used to compare BV01 and BV101 against the reference BV02. The calculation of the Tanimoto coefficient was out carried with Discovery Studio 3.0[14] by using both the FCFP_6 and ECFP_6 sets of fingerprints, which gave for BV01 a value of 0.1489 and 0.1351, respectively. This means that that this compound is considerably different from BV02. BV101 is the 4-nitro-2-[(4-phenyl-6-p-tolyl-pyrimidin-2-yl)-hydrazonomethyl]-phenol and, similarly to BV02, shows very few degrees of freedom. However, the chemical diversity with respect to BV02 is high as in the case of BV01. The Tanimoto similarity index calculated by using both the FCFP_6 and ECFP_6 sets of fingerprints is 0.094 and 0.1065, with respect to BV02. Due to the lack of the carboxylic group, BV101 shows a less pronounced negative charge on the scaffold 'head' with respect to the other compounds. Its

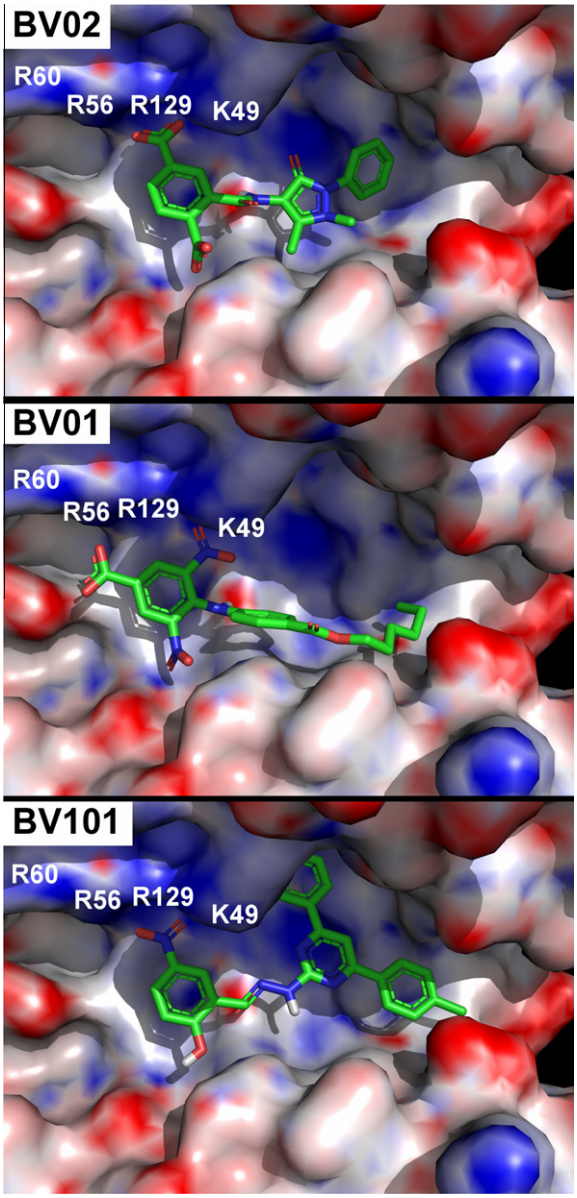


Fig. 2. Docking-based binding mode of BV02 (the reference hit compound), BV01 and BV101 within the amphipathic groove of 14-3-3σ (coordinates of the protein were taken from the crystallographic structure coded by PDB 1YWT). The surface of 14-3-3σ is coloured according to its electrostatic potential. Blue = positive charge. Red = negative charge. Colour intensity is proportional to the charge density. The negatively charged 'heads' of BV02 and BV01 interact in a positively charged region of the protein (basic residues are labelled).

interaction with the amphipathic groove of 14-3-3σ is mediated by hydrophobic interactions of the 'tail' and a few hydrogen bonds performed by the polar groups of the 'head'. Docking simulations of BV01 and BV101 were performed with the GOLD program (ChemScore function)[15–17] toward the crystallographic structure of 14-3-3σ (PDB code: 1ywt).[12] Both BV01 and BV101 display key interactions with the amphipathic groove of 14-3-3σ, in accordance with those previously observed for BV02 and for the R18 peptide (Fig. 2).[9,18]

In the case of BV01, the presumed interaction with 14-3-3σ was further supported by transferred NOEs experiments (tr-NOEs). The 2D homonuclear experiment shows the tr-NOEs between BV01 and the protein 14-3-3σ (Fig. 3). The highlighted region shows the difference between the spectra recorded with and without 14-3-3σ. In the presence of 14-3-3σ, the strong, negative NOE cross-peaks with the same sign as the diagonal resonances and involving all BV01 signals are visible. The reference sample of BV01 alone, on the other hand, shows no or very weak NOEs, as expected for a molecule of this size. The change of intensity of the NOE peaks of the ligand in the presence of 14-3-3σ indicates a weak interaction of BV01 with 14-3-3, with the K_D of the complex 14-3-3/BV01 being >1 μM.

Results of our previous studies underscored the critical role of 14-3-3σ ligand in the cytoplasmic compartmentalization and 'loss of function' of pro-apoptotic c-Abl protein coded by the Abl allele not involved in the rearrangement with Bcr.[10] To assess whether the inhibition of 14-3-3σ binding domain by BV01 and BV101 elicits a cytotoxic effect, we used Ba/F3 cell lines expressing either the wt IM-sensitive Bcr-Abl construct or IM-resistant mutated construct coding for T315I. It is worth noting that both compounds, unlike IM, did not directly inhibit Bcr-Abl (wt) even at high concentrations, further indicating that the cellular activity of BV01 and BV101 could be due to a different mechanism of action, namely the inhibition of the 14-3-3/c-Abl protein–protein interaction (data not shown).

BV01 exhibited a considerable cytotoxicity against Ba/F3 cells expressing the wt and the IM-resistant T315I mutated Bcr-Abl construct with a LD_{50} of 1.41 and 1.70 μM, respectively (see Fig. S2, Supplementary data).[19] As previously reported for BV02,[9] BV01 cytotoxicity on Bcr-Abl-expressing cells was due to the release of residual normal c-Abl from 14-3-3σ, followed by its nuclear import and translocation to mitochondrial membranes where it induces a pro-apoptotic response. Immunoprecipitation (IP)/immunoblotting technique was used to analyze the protein subcellular distribution after treatment with BV01.[9] In Ba/F3 cell line transduced with the wt Bcr-Abl construct, BV01 (5 μM, 24 h) promoted the release of the full-length c-Abl protein (145 kDa) from the cytoplasmic complex with 14-3-3σ and its nuclear import although to a lesser extent compared to IM (1 μM, 24 h) (Fig. 4A and B). Once in the nucleus, c-Abl is expected to trigger p73- and p53-dependent pro-apoptotic signals. Moreover, BV01 induced the relocation of both the full length (145 kDa) and the cleaved (60 kDa) c-Abl at mitochondrial membranes (Fig. 4C). Such relocation drives the mitochondrial membrane dissipation that precedes apoptotic death. Our previous report proved that c-Abl dissociation from 14-3-3σ, promoting its relocation at the nuclear

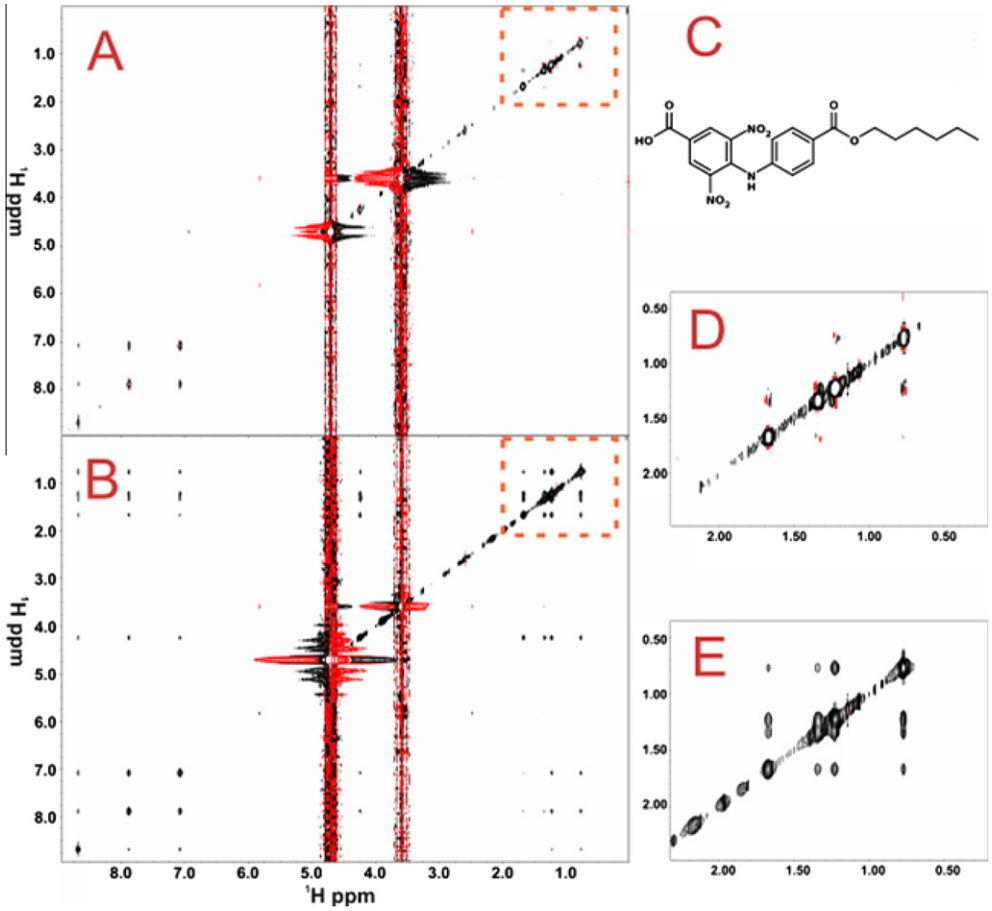


Fig. 3. 14-3-3σ interactions with BV01 studied by tr-NOEs. NOESY spectra of free BV01 (A) and in presence of 14-3-3σ in ratio 10:1 (B) recorded at 320 ms mixing time. In presence of 14-3-3σ, strong, negative NOE cross-peaks with the same sign as the diagonal resonances and involving all BV01 signals are visible. (D) and (E) represent the enlargement of highlighted regions in (A) and (B), respectively.

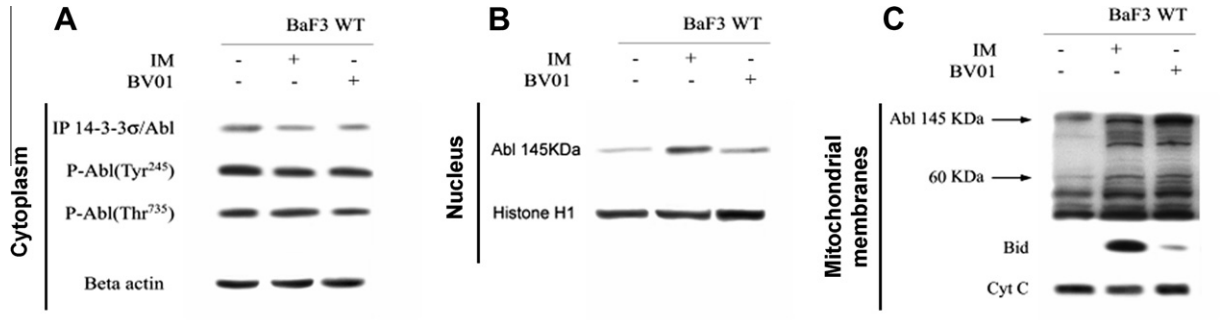


Fig. 4. Effects of BV01 on the relocation of c-Abl in Ba/F3 cells expressing the wt Bcr-Abl. Beta actin, Histone H1 and cytochrome c (Cyt c) levels served as controls for loading of cytoplasmic (A), nuclear (B) and mitochondrial membranes proteins (C), respectively. Identical results were obtained in two additional experiments.

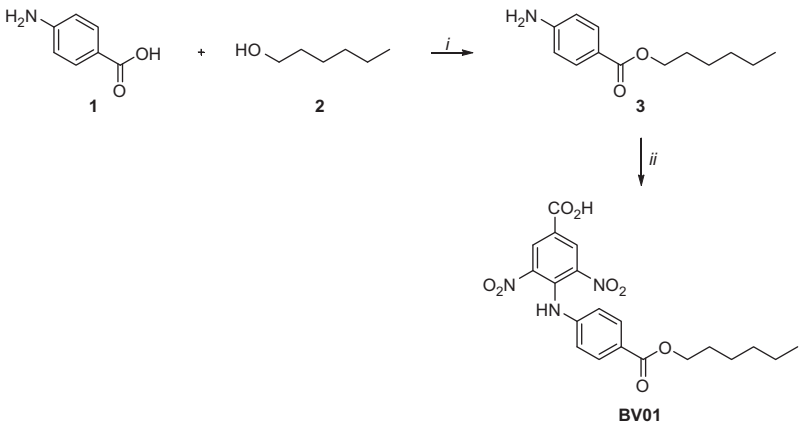
compartment and mitochondrial membranes upon Bcr-Abl inhibition by IM, is induced by the phosphorylation of 14-3-3σ at a critical residue (Ser184) for c-Abl ligand.[10] Results presented here confirmed that c-Abl release restoring its pro-apoptotic function may be achieved by the inhibition of 14-3-3σ protein–protein interactions by BV01.

For BV101, similar methods as described above were used to probe its biological activity in Ba/F3 and 32D murine hematopoietic progenitor cells expressing the wt Bcr-Abl construct. The calculated LD_{50} values for BV101 were 2.84 and 0.65 μM, respectively, and were thus comparable with those obtained for IM and BV01 (Fig. S3, Supplementary data). However, considering that BV101 analogues were also reported to be active against different targets (e.g. DDX3 helicase),[20] further studies are ongoing to asses whether this compound could be considered as a valuable hit compound. Nevertheless, these biological results supported the high potency of the computational methods here applied in identifying valuable small molecular inhibitors of the 14-3-3σ/c-Abl protein–protein interaction.

Considering the promising results showed by BV01 in vitro, we have also investigated its synthetic feasibility. As described in Scheme 1, the 'tail' of this compound, hexyl 4-aminobenzoate (**3**), was easily synthesized starting from *p*-aminobenzoic acid (**1**) and 1-hexanol (**2**) by a classical DCC coupling approach using DMAP as base. The intermediate **3** was then reacted with the commercial 4-chloro-3,5-dinitrobenzoic acid in the presence of DIPEA to obtain the desired compound in good yield (see Supplementary data for details). This synthetic approach can be exploited to generate a wide collection of highly functionalized analogues for SAR studies, by simply using different building blocks in each synthetic step.

In conclusion, BV01 and BV101 have been discovered by means of computational methods as inhibitors of the 14-3-3σ/c-Abl protein–protein interaction. Their anti-proliferative activity against cell lines expressing the wt Bcr-Abl was found to be similar to that of IM which is the first line drug for treatment of CML. More importantly, BV01 was shown to inhibit the proliferation of IM-resistant cells expressing the T315I Bcr-Abl mutation. Altogether these results suggest that BV01 acts with an alternative mechanism of action than IM, namely the inhibition of the 14-3-3σ/c-Abl protein–protein interaction.

Up to date, only one peptidomimetic inhibitor[8] and two small molecule stabilizers[7] of 14-3-3 protein–protein interactions have been identified by means of experimental procedures (microarray-assisted screening and X-ray crystallography), while the only non-peptidic inhibitor of 14-3-3σ (BV02)[9] has been discovered using computational techniques. It should be pointed out that, although all these experimental and theoretical methodologies need further biological investigations, experimental methods are in general more time and money consuming than computer-aided approaches. Without obfuscating the essential role of the experimental methods, we just wish to underline that, in the case of 14-3-3σ, hit compounds showing micromolar affinity could be rapidly discovered by means of molecular modeling. Therefore, in depth biological investigations could be concentrated on a reduced number of molecules, selected on the basis of theoretical results. Accelerating the drug discovery process would produce large benefits, especially for 'hot topic' projects such as those involving



Scheme 1. Reagents and conditions: (i) DCC, DMAP, DCM, rt, 18 h; (ii) 4-chloro-3,5-dinitrobenzoic acid, DIPEA, THF, rt, 24 h.

the discovery of protein–protein interaction inhibitors or the modulation of the 14-3-3σ activity.

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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.2011.09.011.

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