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Direct Binding Assay for the Detection of Type IV Allosteric Inhibitors of Abl

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Supporting Information

ABSTRACT: Abelson (Abl) tyrosine kinase is an important cellular enzyme that is rendered constitutively active in the breakpoint cluster region (BCR)-Abl fusion protein, contributing to several forms of leukemia. Although inhibiting BCR-Abl activity with imatinib shows great clinical success, many patients acquire secondary mutations that result in resistance to imatinib. Second-generation inhibitors such as dasatinib and nilotinib can overcome the majority of these mutations but fail to treat patients with an especially prevalent T315I mutation at the gatekeeper position of the kinase domain. However, a combination of nilotinib with an allosteric type IV inhibitor was recently shown to overcome this clinically relevant point mutation. In this study, we present the development of a direct binding assay that enables the straightforward detection of allosteric inhibitors which bind within the myristate pocket of Abl. The assay is amenable to high-throughput screening and exclusively detects the binding of ligands to this unique allosteric site.

Abelson (Abl) tyrosine kinase is involved in many cellular pathways and is associated with several human diseases.[1−3] It is composed of a kinase domain, two Src homology domains (SH2, SH3), a C-terminal domain which defines its intracellular localization, and a myristoylated N-terminal cap region which is crucial for enzyme regulation.[4] In normal cells, Abl is predominantly found in an autoinhibited inactive state which is stabilized by the N-terminal myristoylation site.[5,6] In the oncogenic breakpoint cluster region (BCR)-Abl fusion protein, the N-terminus of Abl and its self-inhibiting mechanism are lost, resulting in a constitutively active form of the kinase which has been shown to be an important driver in chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL).[7]

All inhibitors approved by the FDA so far are ATP-competitive and bind completely (type I) or partially (type II) within the ATP binding site of the kinase domain (Figure 1a).[8] Innovative drug development efforts led to the discovery of imatinib as a successful type II BCR-Abl inhibitor for the treatment of CML.[9] However, after a positive initial response to treatment with imatinib, a significant number of patients relapse. This event is mainly attributed to the acquisition of specific point mutations in Abl, particularly the T315I (Abl1a numbering) gatekeeper mutation.[10] None of the FDA-approved drugs are able to overcome this mutation. Therefore, research efforts have intensified to find novel inhibitors for the treatment of imatinib-resistant patients carrying the T315I mutation.[11]

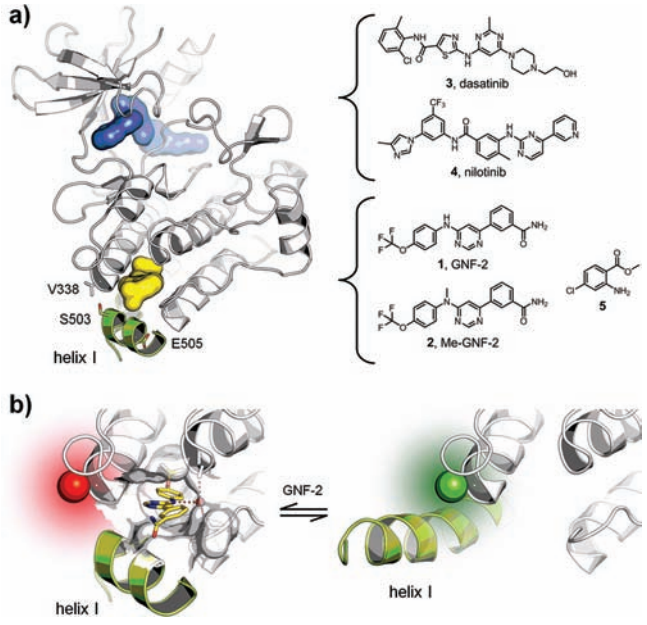

Figure 1. Schematic representation of Abl. (a) The kinase domain of Abl is shown with inhibitors addressing the active site (blue) and the myristate pocket (yellow). Labeling positions analyzed in this study are indicated (sticks). (b) Helix I is bent in the inactive conformation of Abl (left, PDB code 3k5v), while being straight in the active one (right, PDB code 2z60). This conformational change is reported by the attached fluorophore (spheres).

A class of pyrimidines have shown antiproliferative activity against BCR-Abl-expressing cells[12] and received great attention from oncologists worldwide, as they are also capable of inhibiting BCR-Abl (T315I) when applied in combination with ATP-competitive inhibitors such as imatinib and nilotinib.[13] These pyrimidines, which include GNF-2 (**1**), were discovered

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to act via a unique, truly allosteric (type IV) mechanism and bind to a remote site outside of the ATP binding cleft, thus providing an alternative mechanism of inhibition (Figure 1). The binding of the myristoylated N-terminus of Abl within the myristate binding pocket (MP) stabilizes the autoinhibited inactive state of Abl.[5] Similarly, the binding of ligands such as GNF-2 can mimic this interaction by promoting a ~90° bend in helix I of Abl (Figure 1b), thereby allowing the adjacent SH2 domain of Abl to interact with the kinase domain in an autoinhibitory manner.[14,15] In contrast, helix I extends away from the kinase domain in active Abl and disrupts the interactions between the SH2 domain and the kinase domain.

Here we report the development of a direct binding assay which is based on ligand-induced conformational changes of helix I. We show that this system is a strong and reliable tool for the identification of ligands which bind to the MP and can be readily applied to high-throughput screening formats. This assay technology (FLiK, "fluorescent labels in kinases") was originally developed for high-throughput screening of type II and III kinase inhibitors by labeling either the highly flexible activation loop[16,17] or the glycine-rich loop[18] of the target kinase with acrylodan, a thiol-reactive fluorophore.[19] Changes in protein conformation lead to differences in the microenvironment of acrylodan and alter its characteristic dual emission spectrum.

Specific labeling of the protein with acrylodan is accomplished by using site-directed mutagenesis to introduce a cysteine at a site on the protein that will be perturbed by ligand-induced conformation changes. Since two recent studies provided evidence that bending of helix I does not require the presence of SH2 and SH3 domains,[13,20] we generated recombinant Abl constructs consisting of only the catalytic domain (as described elsewhere[15]). A comparison of published crystal structures with either a straight or bent conformation of helix I suggested three amino acids (V338, S503, or E505) as potential labeling sites for the fluorophore, since a significant change in the microenvironment of acrylodan would be expected at these positions during the transition from the active to the inactive state (Figure 1). Initial experiments suggested that covalent labeling of V338C with acrylodan was the most sensitive to conformation changes and therefore the most suitable for the FLiK assay. This residue is close but not part of the MP in either Abl conformation, suggesting that labeling of V338C with the fluorophore should not disturb ligand binding to the MP.

The crystal structures also revealed that three native cysteines (C305, C330, and C464) are exposed on the surface of the protein (Figure S1). As these cysteines may be susceptible to nonspecific labeling by acrylodan, they were to be substituted by site-directed mutagenesis. While C330 was conservatively mutated to serine, C305 was found to be conserved as a valine among several Src family members, including cSrc, HCK, Lck, Lyn, BLK, YES, FIN, FGR, and Abl (Figure S2), and was therefore substituted by a valine. C464 is located on a loop C-terminal to helix H inside the MP (Figure S1)[15] and is conserved in sequence (Figure S2) and structure (Figure S3) among several Src family members, suggesting an important but unknown function of this residue within the MP. To avoid any unwanted alterations of the native binding site, we decided to leave this cysteine in the recombinant construct. Mass spectrometric and FLiK-based analyses suggested that this cysteine is not prone to labeling (Figure S4). In MS/MS measurements of tryptic digests of acrylodan-labeled Abl (C305V/C330S/V338C), only the desired C338 was found to be modified by acrylodan (Figure S5).

Prior to using this mutated construct for the characterization of type IV Abl inhibitors, we first assessed the impact of these substitutions on enzyme activity of wild-type and mutated Abl (labeled and unlabeled) using standard activity-based assays. We found that all constructs displayed similar ATP K_m values (as compared to the corresponding wild-type construct) and all proteins were inhibited by dasatinib (**3**) and nilotinib (**4**) with similar IC_{50} values, suggesting that neither the introduced mutations nor the subsequent acrylodan labeling had a major effect on enzyme activity or its affinity for known inhibitor molecules (Figure S6).

Having biochemically characterized the acrylodan-labeled Abl, we then analyzed its behavior in the FLiK assay. In the apo form (no ligand bound), the labeled protein exhibited an emission spectrum with a major maximum at 474 nm and a secondary maximum (shoulder) at 510 nm (Figure 2a).

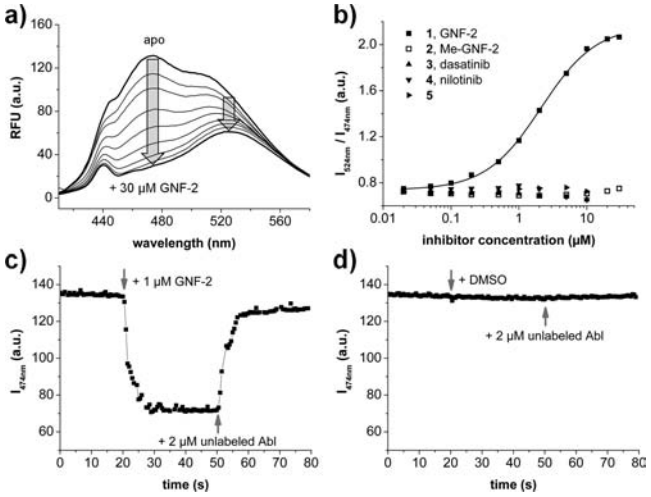

Figure 2. FLiK assay. (a) Stepwise addition of GNF-2 to acrylodan-labeled Abl (C305V/C330S/V338C) results in a significant and dose-dependent change in the fluorescence. (b) The K_d can be determined from plotting the logarithmic concentration of GNF-2 against the ratio of the two acrylodan emission peaks. No significant change in the ratio is observed for Me-GNF-2 and other control inhibitors. Due to intrinsic compound fluorescence, titration of **3**−**5** did not exceed 10 μM. (c) The binding of GNF-2 is fast and reversible. (d) Addition of vehicle alone had no effect.

Stepwise increase of the GNF-2 concentration resulted in a significant change in the fluorescence spectrum of acrylodan such that the first emission peak (474 nm) decreased dramatically over the course of the titration, while the second peak was less affected, shifted from 510 to 524 nm, and became the dominant peak in the GNF-2-saturated form of Abl.

With this assay, it is possible to directly determine the dissociation constant of inhibitors as described previously.[16,18] This is done by monitoring the ratio of both acrylodan emission peaks ($R = I_{524\ nm}/I_{474\ nm}$) in the presence of increasing GNF-2 concentrations. Plotting these ratiometric values against the inhibitor concentration on a logarithmic scale produced a sigmoidal binding curve for GNF-2, with an inflection point corresponding to $K_d = 2.1 \pm 0.4\ \mu$M (Figure 2b). The affinity of GNF-2 for the MP of Abl was consistent ($n = 7$) across two independent preparations (i.e., expression, purification, and labeling) of the labeled Abl (C305V/C330S/

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V338C) mutant and is in a similar range as compared to an NMR-based study in which a ligand-induced change in the chemical environment of V506 was measured by ^{15}N-HSQC.[20] Addition of the N-methylated version of GNF-2 (**2**, Me-GNF-2) as a known negative control[12] gave no response in our FLiK assay, confirming that the change in fluorescence of acrylodan is due to binding of GNF-2 to the MP and not due to an unspecific interaction between compound and fluorophore.

We also tested highly potent inhibitors that bind in the ATP binding site (DFG-in and DFG-out conformations) of Abl to assess whether conformational changes in Abl distinct from the MP would be detected by our new FLiK assay. The type I inhibitor dasatinib (**3**) stabilizes the active "DFG-in" form of the kinase, while the type II inhibitor nilotinib (**4**) stabilizes the inactive "DFG-out" form.[21,22] Both Abl-inhibitor complexes differ by a very significant conformational change in the activation loop of the kinase. Addition of both inhibitors to acrylodan-labeled Abl did not induce a response in the FLiK assay (Figure 2b), highlighting that this assay is insensitive to other common conformational changes within the kinase domain aside from those undergone by helix I, which occur specifically upon binding of type IV allosteric inhibitors.

The small molecule **5** was reported in a NMR-based study to bind to the MP with moderate affinity (K_d = 6 μM) but was unable to induce bending of helix I due to a steric clash with I502.[20] Although known to bind to this site, titration of **5** up to 10 μM in the FLiK assay did not result in a significant change in the ratio of the two emission peaks of acrylodan (Figure 2b), thereby confirming that detected compounds must in fact induce the required bend in helix I and that this event is required to alter the acrylodan emission spectrum.

In addition to using the assay to obtain valuable data about affinities of allosteric Abl inhibitors, it is also possible to measure the kinetic parameters for the association and dissocation of MP ligands. Due to the greater change in acrylodan emission at 474 nm with ligand binding (Figure 2a), this wavelength was used to monitor the binding kinetics of GNF-2 in real-time. As shown in Figure 2c, the assay can reliably detect fast and reversible binding of GNF-2. Addition of vehicle alone (Figure 2d) or the negative control Me-GNF-2 (Figure S7) did not induce the same type of response.

Finally, to facilitate the use of our assay in formats compatible with high-throughput screening, we placed acrylodan-labeled Abl in 384-well microtiter plates, measured the emission spectra in the presence and absence of GNF-2 (Figure 3a), and found a striking similarity to the spectral behavior observed in the cuvette format (Figure 2a). We then assessed the quality of the assay by measuring the ratiometric fluorescence in response to negative (DMSO) and positive (20 μM GNF-2) controls (Figure 3b) and determined Z' = 0.84 ± 0.05 (n = 5), suggesting a high robustness of the assay in HTS formats. Traditionally, Z' > 0.5 is considered suitable for a quality high-throughput screening assay.[23]

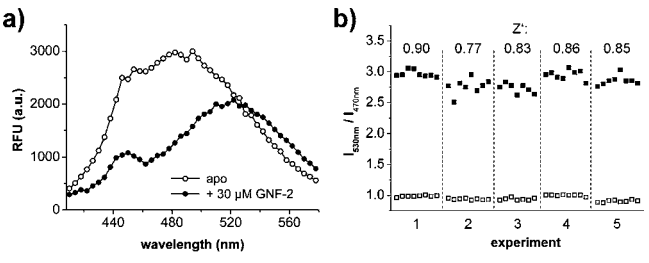

Figure 3. FLiK assay in the microtiter plate format. (a) Spectra of ligand-free and GNF-2-bound acrylodan-labeled Abl resemble the spectra measured in cuvettes (see Figure 2a). (b) Ratio of the two acrylodan emission peaks in the apo form (open squares) and with 20 μM GNF-2 (filled squares), used to calculate Z' for the assay.

In summary, we have established a novel direct binding assay for the detection of type IV allosteric inhibitors addressing the myristate pocket of Abl. We show that this assay is robust and compatible with high-throughput screening in microtiter plates and will serve as a powerful tool for exclusively detecting conformational changes which occur in the vicinity of the MP. These changes are associated directly with conformational changes in helix I of Abl, which occur when type IV allosteric inhibitors bind within the MP of Abl.

■ ASSOCIATED CONTENT

Supporting Information

Details on compound synthesis, protein production (construct design, expression and purification), the sequence and structural alignment, mass spectroscopic analyses, and representative plots of the activity-based assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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