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Inhibitors **Hot Paper**

Synergy and Antagonism between Allosteric and Active-Site Inhibitors of Abl Tyrosine Kinase

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Abstract: *Allosteric inhibitors of Abl kinase are being explored in the clinic, often in combination with ATP-site inhibitors of Abl kinase. However, there are conflicting data on whether both ATP-competitive inhibitors and myristoyl-site allosteric inhibitors can simultaneously bind Abl kinase. Here, we determine whether there is synergy or antagonism between ATP-competitive inhibitors and allosteric inhibitors of Abl. We observe that clinical ATP-competitive inhibitors are not synergistic with allosteric ABL inhibitors, however, conformation-selective ATP-site inhibitors that modulate the global conformation of Abl can afford synergy. We demonstrate that kinase conformation is the key driver to simultaneously bind two compounds to Abl kinase. Finally, we explore the interaction of allosteric and conformation selective ATP-competitive inhibitors in a series of biochemical and cellular assays.*

The fusion protein BCR-Abl is created by the translocation of chromosomes 19 and 22 and is the causative factor in Chronic Myelogenous Leukemia (CML).[1] The ATP-competitive Abl inhibitor imatinib is very effective in increasing CML patient survival.[2] Unfortunately, over time, many patients develop resistance to imatinib and several second-generation Abl inhibitors.[3–5] An allosteric inhibitor of Abl, GNF-2, was discovered in an attempt to combat resistance.[6] Both NMR and crystallographic data demonstrate that GNF-2 binds to the myristate pocket of Abl.[7,8] The myristate pocket is located within the kinase domain of Abl and is employed biologically to regulate Abl kinase. Specifically, Abl is inactivated when its myristoylated N-terminal tail binds to the myristate pocket.[9] Myristate binding kinks the I-helix of the kinase domain, which then serves as a docking site for the SH2 domain of Abl. The docking of the SH2 domain to the kinase domain causes the linker between the kinase domain and the SH2 domain to coil. The SH3 domain then clamps down on this coiled linker, which reorients the N- and C-lobes of the kinase domain, rendering it inactive. Crystallographic data show that GNF-2 causes a similar kinking of the I-helix when bound in the myristate pocket.[8] Together, these data suggest that GNF-2 binding alters the global of conformation of Abl. Asciminib is a next-generation analog of GNF-2 that is currently being evaluated in the clinic, in combination with approved ATP-competitive ABL kinase inhibitors.

Since their discovery, there has been strong interest in combining myristoyl pocket allosteric inhibitors (e.g, GNF-2 and asciminib) with ATP-competitive inhibitors, with the belief that the combination would be synergistic.[8] In the clinic, asciminib is being combined with ATP-competitive inhibitors in an effort to reduce or eliminate resistance.[8] Many groups have reported synergy between GNF-2 and various ATP-competitive inhibitors.[7,8,10] There have been, however, contradictory reports suggesting that these combinations are not synergistic.[11] Furthermore, it has been shown that both imatinib and GNF-2 affect the global structure of Abl; however, they cause Abl to adopt different conformations.[12] With these data in mind, we decided to investigate the relationship between ATP-competitive and allosteric inhibitors of Abl using biochemical and cellular assays.

There are several conflicting claims in the literature regarding whether ATP-competitive inhibitors of Abl are synergistic with allosteric Abl inhibitors.[8,11] We decided to perform an analysis of combinations between GNF-2 and asciminib and four approved ATP-competitive inhibitors of Abl (dasatinib, imatinib, nilotinib, and ponatinib) in a BaF3 cell line transformed by BCR-Abl. BCR-Abl transformed BaF3 cells are growth-dependent on Abl kinase activity in the absence of IL-3. Using the Chou–Talalay method,[13,14] we determined the combination index (CI) for each of the four combinations in an assay of cellular proliferation.[15] To determine a CI value between two drugs, the effect of each drug upon cell growth at varying concentrations is measured. Then, the effect of the two together at varying concentrations of both is determined. The CI is determined using equation (1) in the Supporting Information.[15] A $CI < 1$ is synergistic, $CI = 1$ is additive, and $CI > 1$ is antagonistic. All analytical data for these measurements can be found in the Supporting Information. As shown in Table 1, we found that none of the inhibitor combinations tested led to synergy in BCR-Abl/BaF3 cells. Our data are in contrast to several previous reports that claim synergy using less rigorous analyses.[7,8,10] In

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Table 1: Combination Indices (CI_{75}) for various ATP-competitive inhibitors in combination with GNF-2 or asciminib in BCR-Abl/BaF3 cells.

	Dasatinib	Imatinib	Nilotinib	Ponatinib
GNF-2	1.21	1.73	1.39	1.36
Asciminib	1.44	1.31	1.24	1.44

each of the 8 combinations, we observed antagonism (CI > 1) between allosteric and ATP-competitive inhibitors of Abl.

Because there are reports where synergy has been shown for select combinations, we wanted to confirm our BCR-Abl/BaF3 results using a cellular target engagement assay (CETSA_ for Abl (DiscoverX InCELL Pulse ABL1 Target Engagement Assay). This assay reports on the cellular binding, and thus stabilization, of Abl kinase. Using the InCELL Pulse cellular target engagement assay, we performed Chou–Talalay analyses for each of the eight allosteric + ATP-competitive inhibitor pairs (Table 2). Full analytical data for each combination can be found in the Supporting Information. Consistent with our findings using the BCR-Abl/BaF3 cell line, the combinations were all antagonistic (CI > 1.00) with one exception. The lone exception was that ponatinib is additive with GNF-2, while antagonistic in combination with asciminib.

Table 2: Combination Indices (CI_{95}) for ATP-competitive inhibitors in combination with GNF-2 or asciminib in the InCELL Pulse cellular target engagement assay.

	Dasatinib	Imatinib	Nilotinib	Ponatinib
GNF-2	1.13	2.16	1.44	0.96
Asciminib	1.27	1.71	1.52	1.46

Together, our cellular data demonstrate that clinical ATP-competitive inhibitors cannot bind simultaneously to Abl with allosteric inhibitors. Given the mechanism for biological regulation via the myristate pocket, we reasoned that allosteric inhibitors should induce a change in the global conformation of Abl.

Abl activity is biologically regulated using conformational dynamics. In its inactive state, Abl kinase adopts a compact "closed" conformation in which the SH2 and SH3 domains bind to the kinase domain and prevent catalysis.[9] When activated, the kinase adopts an "open" conformation where the structure is elongated and the SH2 domain moves to a "top-hat" location on the N-lobe of the kinase domain (Figure 1 A).[16,17] One of the key differences between the open vs. closed conformation is the accessibility of the linker between the kinase domain and SH2 domain. When activated, the global conformation of Abl is "open" and the SH2-KD linker is exposed.[16,17]

There are Abl constructs that stabilize both the "closed" and "open" conformations of Abl. SH3 engaged Abl (SH3eng, also reported as HAL9) is an Abl construct in which the global conformation is "closed" and the SH2-kinase linker is bound by the SH3 domain and inaccessible.[18] In contrast, A337N Abl has been shown to promote an "open" conformation, leading to an accessible SH2-kinase linker.[19]

Our lab has developed a protease accessibility assay to assess the global conformations of protein kinases that is based on the accessibility of the linker between the kinase and SH2 domains.[20] We previously demonstrated using this method that thermolysin, a bacterial protease, can cleave several kinases at a site on the linker (see Supporting Information for list of kinases known to be compatible with our methodology).[20] Furthermore, the rate of cleavage reports on kinase conformation ("open" versus "closed"). A construct that is more open will have a more exposed linker, be proteolyzed more quickly by thermolysin, and thus have a shorter half-life (Figure 1).

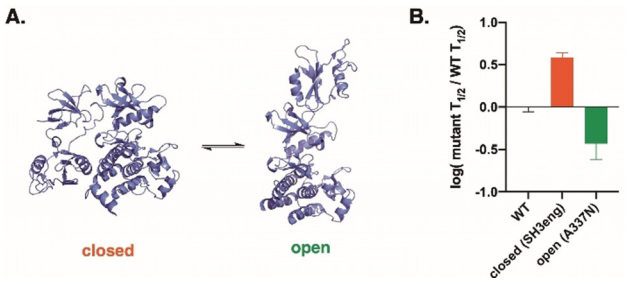

Figure 1. Closed and open global conformations of Abl. A) The closed and open forms of Abl exist in equilibrium. PDB codes: 2FO0 (closed) and 1OPL (open). B) Protease accessibility assay for Abl with controls of closed and open Abl kinase mutants (SH3eng and A337N, respectively). To visualize the mutational impact of open vs. closed conformation, we represent the data as log(mutant Abl $T_{1/2}$/ WT Abl $T_{1/2}$). $T_{1/2}$ is the half-life (in min) for Abl in the presence of thermolysin. Data represent the average and standard deviation for $n = 3$ replicates (with 3 technical replicates each).

We confirmed that Abl is selectivey cut within the SH2-kinase linker by thermolysin (see Supporting Information for details on the cleavage site). As controls to evaluate our limited proteolysis assay with Abl, we used constructs of Abl previously reported to be open and closed (A337N and SH3eng, respectively) to validate that the thermolysin half-life corresponds to known conformations of the protein (Figure 1).

After verifying our limited proteolysis assay with known Abl mutant proteins, we used our assay to determine the conformation of Abl when bound to a panel of allosteric and ATP-competitive inhibitor (Figure 2). We found that each of the four ATP-competitive clinical inhibitors (dasatinib, imatinib, nilotinib, ponatinib) stabilize the open conformation of

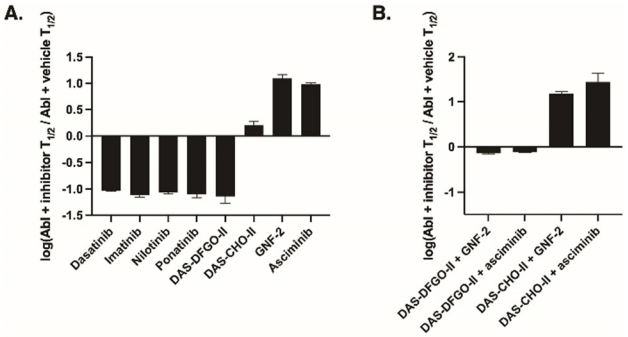

Figure 2. Effect of ATP-competitive and allosteric inhibitors on Abl conformation. To visualize the compounds that stabilized the open vs. closed conformation, we represent the data as log(Abl + inhibitor $T_{1/2}$/ Abl + vehicle Abl $T_{1/2}$). A) Dasatinib, imatinib, nilotinib, ponatinib and DAS-DFGO-II stabilize the open conformation of Abl. GNF-2, asciminib, and DAS-CHO-II stabilize the closed conformation of Abl. B) Inhibitors which stabilize the closed conformation (e.g., DAS-CHO-II) when combined with allosteric inhibitors (e.g., GNF-2, asciminib) both bind the closed conformation of Abl.

Abl. We found that GNF-2 and asciminib stabilize the closed conformation of Abl. Thus, we thus hypothesize that the antagonism observed between allosteric and ATP-competitive inhibitors is due to their binding divergent global conformations.

In previous studies with c-Src kinase, we found that inhibitors that bind the αC-helix out conformation stabilize the closed global conformation of c-Src.[20] Thus, we reasoned that αC-helix-out (CHO) inhibitors of Abl could bind simultaneously with allosteric inhibitors, potentially leading to synergistic combinations. We previously characterized DAS-CHO-II as the first crystal-structure confirmed αC-helix-out inhibitor of Abl.[21] We have also previously reported DAS-DFGO-II, an inhibitor that stabilizes the open conformation (DFG-out, αC-helix-in) of Abl. Using our limited proteolysis assay, we confirmed that the global conformation of Abl when bound by DAS-CHO-II was "closed" (Figure 2A). We next showed that that the global conformation of Abl was "closed" when bound by saturating concentrations of DAS-CHO-II and either allosteric inhibitor: GNF-2 and asciminib (Figure 2B). In contrast, DAS-DFGO-II combined with either allosteric inhibitor led to conformationally-neutral Abl, suggesting again that both compounds cannot simultaneously bind Abl. Together, these data explain the antagonism observed between the clinical ATP-competitive inhibitors with allosteric inhibitors of Abl.

Given our biochemical data demonstrating that DAS-CHO-II can bind to Abl simultaneously with allosteric inhibitors, we wanted to evaluate this combination in the cell-based assays. We first evaluated synergy using the Chou-Talalay method in BCR-Abl Ba/F3 cells. Consistent with our conformation-dependent hypothesis, the combination of DAS-CHO-II with asciminib is synergistic with a $CI_{75} = 0.71$. This contrasts the antagonism ($CI > 1$) observed between clinical ATP-competitive inhibitors and asciminib in this same assay (data for clinical inhibitors is found in Table 1). We next used the Abl CETSA assay to assess the combination of DAS-CHO-II and asciminib. Consistent with the BCR-Abl/BaF3 results, we found DAS-CHO-II + asciminib was synergistic ($CI_{95} = 0.76$). This contrasts the antagonism observed between the clinical ATP-competitive inhibitors and asciminib in this assay (data for clinical inhibitors is found in Table 2). In contrast to the synergy observed with DAS-CHO-II, DAS-DFGO-II was antagonistic in both assays (BCR-Abl/BaF3 $CI_{75} = 1.11$; Abl CETSA $CI_{95} = 2.18$). Together, these data demonstrate that matched kinase conformation is required for dual binding of allosteric and ATP-competitive inhibitors of Abl.

Given our results in cellular engagement and engineered cellular models, we wanted to determine whether DAS-CHO-II and asciminib would be synergistic in cancer cell lines. Toward this goal, we performed Bliss synergy analyses with three CML cell lines growth-dependent on BCR-Abl activity: AR230-S, K562-S, and LAMA84-S. Cell proliferation was used as a readout. In each cell line, we observe synergy between DAS-CHO-II and asciminib (Figure 3).

In conclusion, we have described a better understanding of the interplay between allosteric and ATP-competitive inhibitors of Abl. Since the discovery of imatinib to treat

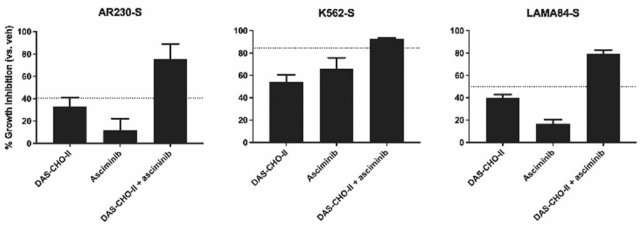

Figure 3. Bliss synergy analysis of DAS-CHO-II + asciminib in three CML cell line models. The dotted line represents the predicted additivity between a combination of asciminib + DAS-CHO-II. The combination of DAS-CHO-II + asciminib is synergistic in all three cell lines.

CML, there has been intense interest in the development of Abl inhibitors. Moreover, there are a wealth of ATP-competitive Abl inhibitors in the clinic, along with allosteric Abl inhibitors GNF-2 and asciminib. Using a protease accessibility assay, we determined the global conformation of Abl when bound to each inhibitor. GNF-2 and asciminib bind to the closed conformation of Abl. In contrast, the clinical ATP-competitive inhibitors bind to an open conformation. We then turned to conformation selective inhibitors of Abl we previously reported. DAS-CHO-II binds Abl in an αC-helix-out conformation, which we demonstrated stabilized the closed kinase conformation. We found that synergy between ATP-competitive and allosteric inhibitors of Abl requires a "match" of kinase conformation. In biochemical and cellular assays, GNF-2 and asciminib were synergistic only with inhibitors that stabilize the closed conformation of Abl. These findings highlight the need to consider protein kinase conformation when undertaking medicinal chemistry and pharmacological efforts to drug protein kinases. Specifically, as more allosteric kinase inhibitors are discovered, to achieve the full benefit of drug-drug combinations on a single target, kinase conformation must be taken into account.

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Conflict of Interest

The authors declare no conflict of interest.

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