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Reply to Correspondence on "Synergy and Antagonism between Allosteric and Active-Site Inhibitors of Abl Tyrosine Kinase"

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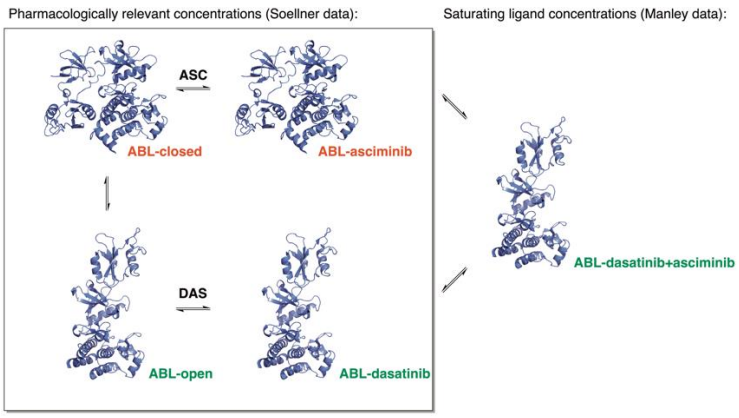
Abstract:

Manley and co-workers provide data demonstrating that at super-pharmacological concentrations (300 μM), a ternary complex between Abl, asciminib, and ATP-competitive inhibitors is possible. The work in our manuscript concerns the interplay of asciminib (and GNF-2) with ATP-competitive inhibitors at pharmacologically relevant concentrations (Cmax = 1.6–3.7 μM for asciminib). Manley and co-workers do not question any of the studies that we reported, nor do they provide explanations for how our work fits into their preferred model. Here, we consider the data presented by Manley and co-workers. In addition, we provide new data supporting the findings in our Communication. Asciminib and ATP-competitive inhibitors do not simultaneously bind Abl at pharmacologically relevant concentrations unless the conformation-selectivity for both ligands is matched.

TOC graphic. At pharmacologically relevant concentrations, asciminib and clinical ATP kinase inhibitors cannot simultaneously bind to Abl kinase. Manley and co-workers correspond that at saturating concentrations (concentrations that are not achievable in a human), asciminib and dasatinib can simultaneously bind to Abl kinase.



The correspondence by Manley and co-workers describes the combination index (CI) values we reported between asciminib and clinical ATP-competitive inhibitors as "weak antagonism". However, the CI values we observe in our cellular synergy models fall squarely into "antagonism" [1]. Moreover, with ATP-competitive inhibitors that stabilize the open kinase conformation, we observe increased antagonism with asciminib as the fraction of Abl inhibited increases (Table 1)

[2]. This is an expected trend for two compounds that cannot simultaneously bind the target (at the concentrations studied). In contrast, with DAS-CHO-II [3,4], an ATP-competitive inhibitor of Abl that binds the αC-helix-out, closed conformation, we observe increased synergy with asciminib as the fraction of Abl inhibited increases (Table 1). While our main text listed only CI_{75} values for the cellular synergy studies (which are solely considered in the Manley et al. Correspondence), the Supporting Information of our Communication lists all CI values we obtained [2].

Table 1. Combination indices for ATP-competitive inhibitors in combination with asciminib in BCR-Abl/BaF3 cells [2].

	CI_{50}	**CI_{75}**	**CI_{90}**	**CI_{95}**
dasatinib + asciminib	1.29	1.44	1.63	1.79
ponatinib + asciminib	1.13	1.44	1.84	2.18
DAS-CHO-II + asciminib	0.82	0.80	0.74	0.71

In their Correspondence, Manley and co-workers cite the existence of crystal structures with allosteric inhibitors (e.g., asciminib) co-bound with ATP-competitive inhibitors (e.g., nilotinib) as evidence that a ternary complex is possible (PDB: 5MO4). We agree that such a complex is possible, however, a crystal structure does not report on binding at pharmacological concentrations. A prominent example in the kinase field is imatinib and c-Src. There are crystal structures of this complex (PDB: 2OIQ) [5], however, imatinib does not bind c-Src at pharmacologically relevant concentrations (IC_{50} > 30 μM; imatinib mean steady-state Cmax = 5.3 μM) [5,6].

It is notable to us that the crystal structures (PDB: 5MO4 and 6HD6) are both in the αC-helix-in, open kinase conformation. Two of the authors of the Correspondence published NMR studies where they find that GNF-5 (an allosteric inhibitor of Abl) stabilizes the closed kinase conformation (αC-helix-out) and imatinib stabilizes the open kinase conformation (αC-helix-in) [7]. In our Communication, we also found that allosteric inhibitors of Abl (GNF-2 and asciminib) stabilize the αC-helix-out, closed conformation of Abl. The αC-helix-out, closed conformation of Abl is incompatible with the αC-helix-in, open kinase conformation [3,8]. In the αC-helix-out conformation, which is stabilized by asciminib, the αC-helix is rotated outward and the conserved Lys-290 and Glu-305 ion pair is disrupted. This disruption is the allosteric mechanism by which asciminib inhibits the catalytic function of Abl kinase. In contrast, all clinical ATP-competitive inhibitors of Abl stabilize the αC-helix-in, open kinase conformation by hydrogen bonding to Glu-305 on the αC-helix. This mismatch of conformation is the basis for the antagonism we observe between asciminib and clinical ATP-competitive inhibitors. Consistent with the crystal structures of the ternary complex, we reported in our Communication that saturating concentrations of DAS-DFGO-II (an ATP-competitive inhibitor that stabilizes the αC-helix-in, open kinase conformation [3,4]) + asciminib leads to an αC-helix-in, open kinase (Figure 2B in our Communication) [2].

Manley and co-workers next present NMR studies demonstrating that at very high concentrations (300 μM of each ligand) asciminib and dasatinib can simultaneously bind Abl. We do not dispute that it is possible for both ligands to be simultaneously bound at saturating concentrations of each ligand. Our Communication explored *pharmacologically relevant* concentrations (the steady-state

Cmax of asciminib ranges from 1.6–3.7 μM, depending on dose [9], and the steady-state Cmax of dasatinib ranges from 67–163 nM, depending on dose [10]). The concentrations used in the NMR studies are not pharmacologically relevant.

Previous NMR studies by these authors demonstrate divergent and structurally incompatible conformations for GNF-5 and imatinib, consistent with our published work [2,7]. We asked Manley and co-workers for their primary NMR data to determine whether asciminib and dasatinib individually bind to divergent kinase conformations, but this request was denied.

Next, Manley and co-workers present isothermal titration calorimetry (ITC) experiments of asciminib binding Abl vs Abl in complex with an ATP-competitive inhibitor (and vice versa). The authors note that they are at the limit of quantification with measuring the K_D of asciminib. With that caveat, we note that the mean K_D of asciminib for apo Abl (0.22 ± 0.14 nM) increases when Abl is saturated with asciminib (K_D = 0.6 ± 0.5 nM). Similarly, they measure an increase in the K_D of imatinib for Abl (44 nM, n=1) compared to the K_D of imatinib for the Abl-asciminib complex (68 nM, n=1). Despite increased K_D values and the high level of error in their measurements, Manley and co-workers conclude that both ligands are binding simultaneously. ITC can be used to evaluate cooperative vs anti-cooperative binding of multiple ligands to one protein (synergy vs antagonism), however, parameters other than the K_D are required to evaluate potential cooperativity [11,12]. We asked Manley and co-workers if they would share the primary ITC data and/or the stoichiometry values for each measurement, but this request was denied. In the absence of the primary data, we cannot interpret synergy or antagonism from their ITC data.

We note that all data that we have presented uses full-length protein in a cellular context (either full-length c-Abl or Bcr-Abl). Allosteric inhibitors of Abl kinase are well established to have construct-dependent binding [13]. Importantly, the NMR and ITC studies were performed by Manley and co-workers using a truncated form of Abl (residues 64–515) that is not post-translationally modified. It is possible that the use of artificial kinase constructs by Manley and co-workers is contributing to their interpretation of their data.

Finally, Manley and co-workers describe cancer cell line studies with asciminib in combination with imatinib, nilotinib, or dasatinib in KCL22 cells. Using a Zero Interaction Potency (ZIP) model, they observe additivity (rather than synergy or antagonism). Their work disagrees with studies we have recently performed with KCL22 cell lines (see below). We asked Manley and co-workers if they would share the primary data for their cellular studies, but this request was denied.

In 2018, Vasta et al. reported that allosteric Abl inhibitors were competitive with binding of a dasatinib-BODIPY tracer [15]. Using NanoBRET target engagement cellular assays (Promega, Madison, WI), we explored the interplay of dasatinib-BODIPY with asciminib. In our communication, we observed antagonism between asciminib and dasatinib at pharmacologically relevant concentrations in two cellular assays: BCR-Abl transformed BaF3 cells and an Abl cellular thermal shift assay [2]. NanoBRET thus represents a third, orthogonal assay using full-length Abl in a cellular environment (HEK293 cells). Using the NanoBRET assay, we find that asciminib is competitive with a dasatinib-BODIPY tracer and we measure an EC_{50} = 4 nM for asciminib (Figure 1A) at 300 nM dasatinib-BODIPY. When the concentration of the tracer is

increased, the EC_{50} value of asciminib dramatically increases (at 1 µM dasatinib-BODIPY, $EC_{50,\ asciminib}$ = 680 nM; at 3 µM dasatinib-BODIPY, $EC_{50,\ asciminib}$ > 1µM) (Figure 1A). We observe strong competition between dasatinib-BODIPY and asciminib, consistent with antagonistic binding.

Next, we determined the K_D value of dasatinib-BODIPY to be 54 nM for Abl (Figure 1B). In the presence of 1 µM asciminib, we obtained a K_D value of 550 nM for dasatinib-BODIPY (Figure 1B). Thus, we observe antagonism between dasatinib-BODIPY and asciminib binding in both directions.

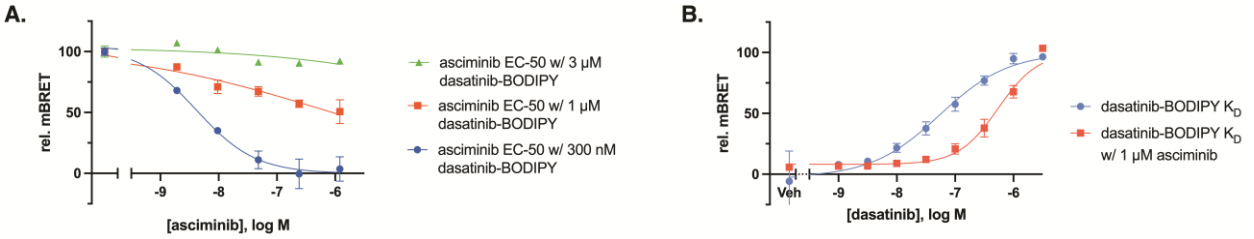


Figure 1. Competition is observed between dasatinib-BODIPY and asciminib in nanoBRET target engagement assays. A. The EC_{50} value of asciminib is measured in the presence of different concentrations of dasatinib-BODIPY. At 300 nM dasatinib-BODIPY the $EC_{50,\ asciminib}$ = 4 nM, at 1,000 nM dasatinib-BODIPY the $EC_{50,\ asciminib}$ = 680 nM, and at 3,000 nM dasatinib-BODIPY the $EC_{50,\ asciminib}$ > 1,000 nM. B. We measured the K_D of dasatinib-BODIPY in the presence of varied concentrations of asciminib. Without asciminib, the $K_{D,\ dasatinib\text{-}BODIPY}$ = 54 nM and at 1,000 nM asciminib the $K_{D,\ dasatinib\text{-}BODIPY}$ = 550 nM. These data demonstrate strong competition between the binding of dasatinib-BODIPY and asciminib in cells at pharmacologically relevant concentrations.

Our Communication explored synergy in three patient-derived CML cell lines: AR230, K562, and LAMA84 [2]. Here, we present Chou-Talalay synergy data in the cell line examined by Manley and co-workers: KCL22. In KCL22 cells, we find that the combination of dasatinib + asciminib is antagonist (CI_{50} = 1.40), and DAS-CHO + asciminib is synergistic (CI_{50} = 0.75) using Chou-Talalay synergy analysis (see Supporting Information for details) [1]. As a control, we evaluated the combination of dasatinib + nilotinib (two ATP-site inhibitors) and found this combination to be additive (CI_{50} = 0.96) as expected for two compounds that cannot occupy the kinase simultaneously [1]. One possible explanation for the discrepancy between our results and those of Manley and co-workers is their inclusion of higher concentrations of each drug in their synergy analysis. Another literature report using KCL22 cells, found that dasatinib + asciminib was antagonistic at lower concentrations (<ED_{80}) and synergy at the highest concentrations tested [16].

In the KCL22 data from Manley and co-workers there are distinct areas of both antagonism and synergy in their ZIP analysis. As single agents, we measured the GI_{50} values for asciminib and dasatinib in KCL22 cells to be 1 and 0.02 nM, respectively. The concentrations we selected for our synergy studies for asciminib and dasatinib were centered around the individual GI_{50} values (0.06–4 nM for asciminib and 0.001–0.08 nM for dasatinib). In contrast, Manley and coworkers

surveyed concentrations far beyond the GI_{50} values we measured (0.024–100 nM for asciminib and 0.002–10 nM for dasatinib). As previously noted, our request to analyze their primary data was denied.

We agree with Manley and co-workers that synergy is not required for clinical benefit. Pre-clinical studies have clearly shown that combinations of asciminib + ATP-competitive inhibitors lead to a reduction of resistance [16,17], an ongoing issue with single-agent Abl inhibitors [18]. We are impressed with the pre-clinical and clinical data for asciminib [19].

While Manley and co-workers mention no concern with our data, we present additional information in this Reply that supports the findings published in our Communication. The data at pharmacologically relevant concentrations presented by Manley and co-workers support our position. We believe that our data and conclusions are robust, clear, and definitive: at pharmacologically relevant concentrations, asciminib and clinical ATP kinase inhibitors cannot simultaneously bind to Abl kinase.

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