Correspondence on "Synergy and antagonism between allosteric and active-site inhibitors of Abl tyrosine kinase"

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Abstract:

Soellner published on the interplay between allosteric and ATP-competitive inhibitors of ABL kinase, showing the latter preferably bind to different conformational states of ABL compared to allosteric agents that specifically target the ABL myristate pocket (STAMP) and deducing that asciminib cannot bind to ABL simultaneously with ATP-competitive drugs. These results are to some extent in-line with ours, although our analyses of dose-response matrices from combinations of asciminib with imatinib, nilotinib or dasatinib, show neither synergy nor antagonism, but suggest additive antiproliferative effects on BCR-ABL dependent KCL22 cells. Furthermore our X-ray crystallographic, solution NMR and isothermal titration calorimetry studies show that asciminib can bind ABL concomitantly with type-1 or -2 ATP-competitive inhibitors to form ternary complexes. Concomitant binding of asciminib with imatinib, nilotinib or dasatinib might translate to benefit some chronic myeloid leukaemia patients.

Structural biology studies indicate that the cytosolic Abelson ABL1b and ABL2b tyrosine kinases are auto-regulated by their myristoylated *N*-termini, which interact with a myristate-binding pocket in the catalytic SH1 domain, remote from the binding site of adenosine triphosphate (ATP), from which the phosphate group is transferred to substrates. This results in the adoption of closed, catalytically inactive conformations of the enzymes.[1] In haematopoietic stem cells, unfaithful repair of damaged DNA can result in a reciprocal chromosome translocation which leads to the expression of chimeric BCR-ABL1 oncoproteins, in which the *N*-terminal cap region of ABL1 and hence the myristoyl group is absent, resulting in the loss of the auto-regulatory mechanism which contributes to the constitutive activation of the ABL1 kinase domain. The dysregulated kinase activity of BCR-ABL1 causes chronic myeloid leukaemia (CML).

Whereas ATP-competitive ABL1 kinase inhibitors provide effective treatments for CML, such that many patients have an almost normal life-expectancy, some patients do not respond to therapy (primary resistance), lose response (secondary resistance), or experience intolerance.[2] Therefore, there is a medical need for alternative drugs which can be used either as monotherapy or in combination with ATP-competitive drugs, either to improve responses or to prevent the emergence of drug-resistant BCR-ABL1 mutations.

The myristate–binding pocket within the ABL SH1 kinase domain is also druggable as shown by the relatively weak, prototype compound GNF-2,[3,4] and by the potent and specific, FDA-approved drug asciminib (Scemblix®),[5-7] which are regarded as being allosteric inhibitors.[8]

Soellner and co-workers have published their findings from studies on combinations of ATP-competitive inhibitors and allosteric inhibitors that bind to the myristate pocket.[9] In experiments using mouse BaF3 cells engineered to be dependent upon the kinase activity of BCR-ABL1, they report weak antagonism of asciminib with ATP-competitive inhibitors, with combination indices (CI) ranging from 1.24 with nilotinib to 1.44 for dasatinib (the CI scale for antagonism is 1 - ∞, for synergy CI ranges between 1 and zero)[10]. The authors then rationalise the observed weak antagonism by the fact that ATP-site inhibitors favour an open global conformation, while allosteric inhibitors bind to a closed global conformation. For type-2 inhibitors, the same conclusion was previously drawn from NMR and SAXS experiments,[11,12] and Johnson *et al* corroborate this by applying a protease accessibility assay as a novel means to distinguish open

and closed global ABL conformations. Using the protease accessibility assay, Johnson *et al* could show that an ATP-site ABL inhibitor which binds to an αC helix-out conformation with moderate affinity allows the kinase to adopt a closed global conformation. Importantly, this inhibitor in combination with asciminib was synergistic, with a combination index of 0.71. These data indicate that ATP-site inhibitors and allosteric inhibitors of ABL can be synergistic if they bind to the same global conformation.

From their observation of weak antagonism between asciminib and ATP-site inhibitors in cellular experiments, Johnson *et al* conclude that "clinical ATP-competitive inhibitors cannot bind simultaneously to ABL with allosteric inhibitors"[9]. While we appreciate the quality of the cellular combination experiments, we disagree with the conclusion that weak antagonism with combination indices between 1.24 and 1.44 means that both inhibitors cannot bind to ABL kinase at the same time. In our opinion, this would simply suggest that the binding affinity of the second drug is slightly weaker in the presence of the first drug, but both drugs can still bind simultaneously.

In fact, there is overwhelming evidence that ATP-site and allosteric inhibitors can bind simultaneously to ABL1 to form ternary complexes: X-ray analysis of a crystal of $ABL1^{27-515}$ (throughout numbering is according to the sequence of the ABL1a splice variant, which has 19 less residues than ABL1b) in complex with imatinib that had been soaked with GNF-2 indicated that imatinib and this ligand could bind simultaneously to ABL1 (PDB entry 6HD6).[4] Furthermore, co-crystallisation of asciminib with a binary complex of human $ABL1^{27-515}$ (harbouring Thr315Ile and Asp363Asn point substitutions) also showed concomitant binding to form a ternary complex (PDB entry 5MO4; Figure 2, where TKI represents nilotinib).[5,6] In addition, earlier NMR work has shown that imatinib and GNF-5 (which is a close analogue of GNF-2, with similar weak activity)[4] can also bind simultaneously to ABL1 to form a ternary complex.[11]

In addition, we here present further evidence from solution-based studies for simultaneous binding of the allosteric inhibitor asciminib and ATP-site inhibitors to ABL1.

Firstly, we employed NMR spectroscopy to investigate the simultaneous binding of asciminib and dasatinib to ABL1 kinase. According to the cellular combination experiments of Johnson *et al*, dasatinib is the most antagonistic ATP-site binder in combination with asciminib.[9] However,

our NMR data unambiguously show that even dasatinib can bind to a binary complex of ABL1 and asciminib to form a ternary complex (Figure 1 and Supplementary Figures S1 and S2). Figure 1 shows a section of the NMR TROSY spectrum of apo $ABL1^{64-515}$ (black spectrum). The addition of asciminib (red) or dasatinib (blue) results in significant chemical shift changes, which clearly shows binding of both the compounds to the ABL kinase. The full spectra are shown in Supplementary Figure S1 and a quantitative analysis of the chemical shift changes is shown in Supplementary Figure S2. When dasatinib has been added to the $ABL1^{64-515}$-asciminib complex the TROSY spectrum (green) again shows significant chemical shift changes, indicating simultaneous binding of dasatinib and asciminib to ABL1 kinase. Additional experiments showed that the NMR TROSY spectra are identical, regardless of whether dasatinib was added to the ABL1-asciminib complex, or whether asciminib was added to the ABL1-dasatinib complex (Supplementary Figure S2). Thus, the order of addition of the inhibitors does not matter, supporting a binding scheme as depicted in Figure 2.

Secondly, we used isothermal titration calorimetry (ITC; Supplementary Figures S3 and S4) as an orthogonal biophysical method to quantify the binding affinities of asciminib to ABL1 and the ABL1-imatinib and ABL1-dasatinib complexes ($ABL1^{64-515}$ in all experiments). The affinity of asciminib to ABL1 complexes is generally in the sub-nanomolar range,[6] which is at the limit of quantification by ITC experiments and consequently the error bars are rather high. From an average of three independent titrations (representative titrations are shown in Supplementary Figure S3), the binding affinities of asciminib (mean dissociation constant, $K_D \pm$ SD n=3) were: 0.22 ± 0.14 nM for ABL1, 0.37 ± 0.17 nM for the ABL1-imatinib complex and 0.6 ± 0.5 nM for the ABL1-dasatinib complex (in a direct comparison the binary binding affinities of imatinib and dasatinib to $ABL1^{200-500}$ are reported to be 316 ± 25 nM and 2.0 ± 0.2 nM, respectively)[13]. Thus, the binding affinities of asciminib either to ABL1 with an empty ATP pocket, or to the ABL1-imatinib and ABL1-dasatinib complexes were identical within experimental error. Thus, the ITC experiments confirm that ternary complexes are formed between ABL1, ATP-site inhibitors and allosteric inhibitors, indicating that both classes of inhibitor can bind simultaneously.

Because the range of affinities was at the limit of quantification in the ITC experiments discussed above, we also conducted the reverse experiments, titrating imatinib (titrant) to either ABL1 or the ABL1-asciminib complex (Supplementary Figure S4; studies with nilotinib as

titrant were precluded because of its low solubility). The lower affinity of imatinib compared to asciminib was better quantifiable by ITC. Thus titration of imatinib to ABL1 gave a Kd of 44 nM (n=1) and titration of imatinib to the ABL-asciminib complex gave a Kd of 68 nM (n=1). Results from this reverse experiment fully corroborate our previous findings by ITC, NMR and X-ray crystallography that asciminib and ATP-site inhibitors can form ternary complexes.

Finally, we present the analysis of studies with asciminib combination studies using a human CML cell line. GNF-2 was not included as a comparator, since it is more than 100-fold less potent than asciminib and relatively unselective, inhibiting the proliferation of cells not expressing BCR-ABL1 by unknown mechanisms.[5]

Results of the effects of asciminib in combination with imatinib, nilotinib and dasatinib on the proliferation (72 hour incubation, with the level of cell growth determined relative to DMSO treated cells) of KCL-22 cells (derived from a blast crisis CML patient)[14,15] have been published, but the combination matrices were not analysed.[5] Here our analysis of the combination matrices using the SynergyFinder version 2.0 web-application are presented.[16] This analysis does not indicate any compelling evidence for either synergy or antagonism using Zero Interaction Potency (ZIP) synergy scoring, which aims to be superior to other models, such as BLISS.[17] Thus, the ZIP scores for the combinations of asciminib with nilotinib, imatinib and dasatinib, were -0.652, -1.535 and -0.699, respectively (Figure 3) and since a ZIP synergy score > 5 is predicted to lead to synergistic effects, a score < -5 is predicted to lead to antagonism and in cases where $-5 < ZIP < 5$ the interaction is likely to be additive, our analysis indicates merely additive effects with the three combinations. Similarly, Bliss combination scores (where values < -10 indicate antagonistic interactions, values between -10 and +10 indicate additive interactions, and when > 10 the interaction between two drugs is likely to be synergistic) for nilotinib, imatinib and dasatinib were -0.877, -2.107 and 1.016 respectively, also indicating additivity for each of the pairs.

Additivity between asciminib and ATP-competitive TKIs in inhibiting BCR-ABL1 driven KCL-22 cell proliferation may be rationalised based upon the somewhat simple scheme (Figure 2), whereby once the ternary complex has been formed, dissociation of either ligand will still leave the kinase in an inhibited state. Thus, such combinations could then be considered to be a ‘belt and braces’ approach to inhibit the proliferation of CML cells.

Our biophysical and *in vitro* data are supported by the published *in vivo* efficacy of asciminib in combination with nilotinib in a KCL-22 xenograft mouse of CML.[5] Thus whereas twice-daily oral administration of either asciminib (30 mg/kg b.i.d.) or nilotinib (75 mg/kg b.i.d.) initially lead to strong tumour regression, animals eventually relapsed with tumours harbouring either asciminib- or nilotinib-resistent BCR-ABL1 mutations (median time after start of treatment to relapse 38 and 52 days respectively; Sanger sequencing). However, when tumour-bearing animals were treated with a combination of asciminib (30 mg/kg b.i.d.) and nilotinib (75 mg/kg b.i.d.), complete tumour regression was achieved and maintained for at least 3 months beyond the discontinuation of the 10 week drug treatment period. Although formation of a ternary complex need not be invoked to explain these findings, the results must arise from asciminib preventing the emergence of nilotinib-resistant mutations and nilotinib preventing the emergence of asciminib-resistant mutations,[6] and are inconsistent with there being any strong antagonistic effect between the two drugs.

Drug synergy between combination partners is not necessarily required for clinical benefit, since additive effects are often sufficient, but synergy and antagonism (which is undesirable) scoring remains an important parameter to be evaluated when considering combination therapies.[18] However, there is a lack of consensus in the literature as to how to precisely characterise drug combination effects.[19] Also in this case, when asciminib is combined with ATP-competitive inhibitors we cannot irrefutably decide whether there is additivity (as our data suggest) or weak antagonism (as suggested by Soellner and coworkers) in the anti-proliferative effects on cells of the drugs. Nevertheless, given the ongoing clinical trials with asciminib and ATP-site ABL inhibitors,[20-22] it is important to stress that asciminib and ATP-site inhibitors such as imatinib, nilotinib, or dasatinib can indeed bind to ABL simultaneously, and each inhibit BCR-ABL1 kinase activity by their respective mechanisms.

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Legends for Figures

Figure 1. Representative sections of the two-dimensional ^{15}N-^{1}H TROSY NMR spectra of various ABL1$^{64-515}$-ligand complexes show shifts in the resonances and thus binding of either dasatinib (blue spectrum) or asciminib (red) to apo ABL1$^{64-515}$ (black) as well as formation of the ternary complex of ABL1$^{64-515}$-asciminib+dasatinib (green). For full spectra and measurement conditions see Supplementary Figure S1.

Figure 2. Scheme showing potential equilibria for the engagement of asciminib (a type-4 inhibitor)[8] binding to the myristate pocket of ABL and an ATP-competitive tyrosine kinase inhibitor (TKI; type-1 or -2 inhibitors) to the ABL kinase, which itself has catalytically active (DFG-in) and inactive (DFG-out) conformations,[8] indicated by ABL^{act} and ABL^{inact}.

Figure 3. Combinations of asciminib with ATP-competitive BCR-ABL1 inhibitors do not predict to synergistic interactions. Human blast crisis CML derived KCL-22 cells, which express BCR-ABL1, were incubated for 72 hours with asciminib combined with either nilotinib, imatinib or dasatinib. The combination matrices were analysed using the SynergyFinder 2.0 web-application, resulting in Zero Interaction Potency (ZIP) scores of were -0.652, -1.535 and -0.699, for the combinations of asciminib with nilotinib, imatinib and dasatinib respectively. The corresponding Bliss scores for nilotinib, imatinib and dasatinib were -0.877, -2.107 and 1.016 respectively.

Figure 1.

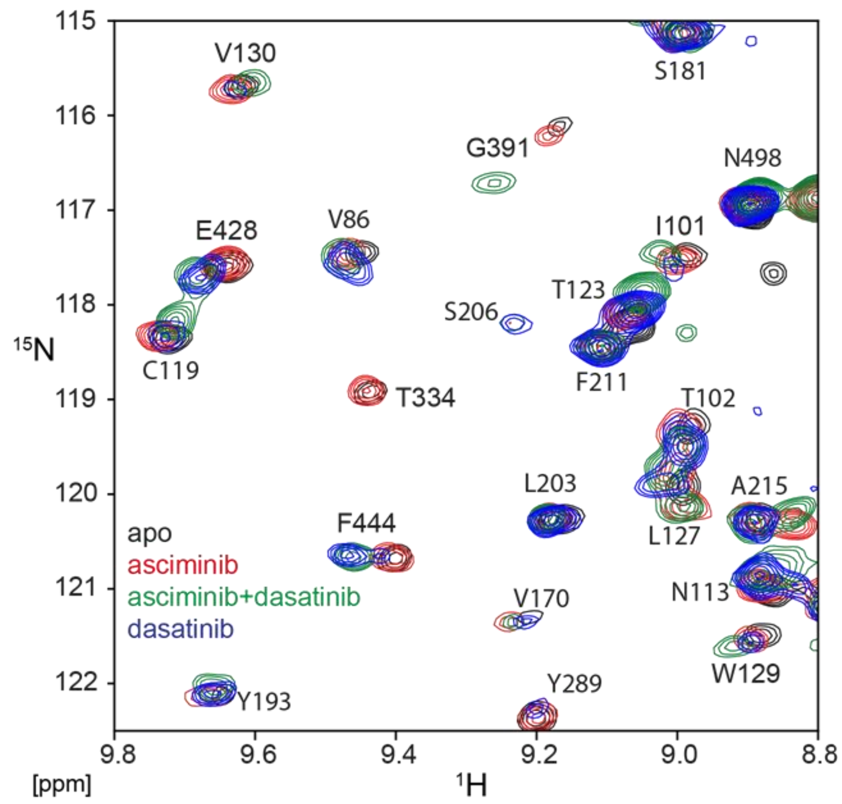

Figure 2.

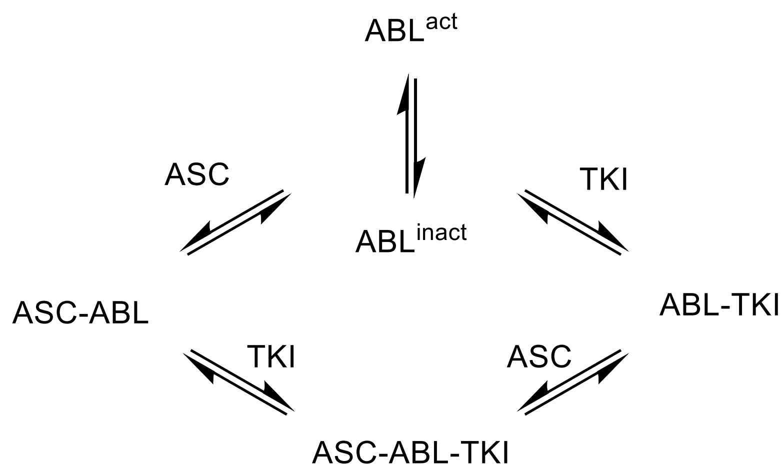

Figure 3.