CANCER

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Response to Comment on "PP2A inhibition sensitizes cancer stem cells to ABL tyrosine kinase inhibitors in BCR-ABL$^+$ human leukemia"

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LB100 sensitizes resistant chronic phase CML stem and progenitor cells to TKIs and spares healthy bone marrow cells.

Perrotti and colleagues make several comments that are backed by the many years of research his group has conducted into the role of protein phosphatase 2A (PP2A) as a tumor suppressor in chronic myeloid leukemia (CML) (*1*). In particular, they have demonstrated that PP2A is functionally inactivated in blast crisis (BC) CML, the late stage of CML, through the inhibitory activity of the BCR-ABL–regulated SET protein (*2*). They have explored SET-sequestering and PP2A-interacting activating drugs as potential therapeutics in CML (*3*). In our study, we have clearly acknowledged the importance of PP2A and its network in regulating multiple biological processes in cancer development and progression, including CML (*4*). We highlighted the complexity of PP2A's structure and its multiple substrates that can either negatively or positively regulate numerous signaling cascades involved in proliferation, cell cycle control, adhesion/migration, etc. Its activity can also be moderated by posttranslational modifications, multiple protein-protein interactions, and altered subcellular localization, which make its roles even more complicated. Thus, the complex role of PP2A and its ultimate effects on the growth of malignant human cells and their progression, as well as on cancer stem cells and their responses to drug treatment, remain poorly defined.

In the paper under discussion, we report the results of screening the Prestwick Chemical Library for effects on Abelson helper integration site–1 (AHI-1; a scaffold oncoprotein)–transduced cells, with the goal of identifying druggable targets involved in AHI-1–mediated pathways, as well as compounds that might act synergistically with a tyrosine kinase inhibitor (TKI) to kill TKI-insensitive cells. The results implicated a PP2A inhibitor (cantharidin) as active in this screen. We then explored this interesting finding in the context of the current understanding of how PP2A modulates various signaling pathways, specifically the suggested interaction of PP2A with AHI-1 and BCR-ABL in primitive CML cells from chronic phase (CP), but not BC, TKI nonresponder patients. Overall, our findings suggest an approach to targeting TKI nonresponder CML stem/progenitor cells by inhibiting PP2A in combination with a TKI. Specifically, our findings have the context of our experience with AHI-1–mediated signaling, as well as combining TKIs with other compounds, in this case PP2A inhibitors, to assess potential synergistic effects of the combination and the mechanisms behind these findings.

The independent findings of cantharidin-induced PP2A inhibition, along with the direct physical association of the PP2A PR55α subunit and β-catenin with AHI-1, led us to investigate whether and how an AHI-1–mediated PP2A–β-catenin–BCR-ABL–Janus kinase 2 complex might alter the response and resistance of CML stem/progenitor cells from CP patients to TKI treatment. Perrotti's comments and past work do not make much mention of this specific interaction and the as yet undefined mechanisms that control the growth and drug response or resistance of CP CML cells, which was a key point of interest for our study.

Perrotti *et al.* questioned why PP2A assays to determine the activity of PP2A in response to PP2A-inhibiting drugs (PIDs) were performed in CML cell lines but not in primary CML leukemia stem cells, stem/progenitor cells, and normal healthy cells. This concern oversimplifies the goal of our study, which was to first determine the specificity of PIDs, particularly the second generation PIDs LB100 and LB102. To undertake these specificity experiments using two different carefully selected PP2A activity assays, it seemed reasonable to use well-studied BCR-ABL$^+$ cell lines [namely, K562 cells, a BCR-ABL$^+$ myeloid BC cell line and an imatinib mesylate (IM)–resistant (IMR) derivative and BV173 cells, a BCR-ABL$^+$ B-ALL cell line]. The results demonstrated a consistent and lineage-independent selective inhibition of PP2A activity with both LB100 and LB102. We agree that it would be interesting to repeat these assays in TKI nonresponder stem/progenitor cells if that were feasible. Unfortunately, the numbers of patient cells required precluded this. However, Perrotti's group has already demonstrated that PP2A activity is much higher in CD34$^+$ CP-CML cells as compared to CD34$^+$ BC-CML cells (>10-fold) (*2*), which shows that PP2A is active in CML CP stem/progenitor cells and that this activity is reduced in CML BC patient cells. Together, this supports our suggestion that it could be of interest to target PP2A in patients with CP CML.

Perrotti *et al.* also asked why our results in K562 and K562-IMR cells did not show the TKI-mediated SET protein expression change reported by his group. We are fully aware of this discrepancy and, therefore, carefully evaluated it in our study. However, we were unable to confirm their finding, as discussed in our report. One difference in the two studies noted was that different cells were used, and in their studies, reduced expression of SET protein in K562 cells with TKI treatment was not convincing despite the obvious reduction in *SET* RNA. We appreciate the Gene Expression Omnibus (GEO) data brought up by Perrotti but feel that comments on these data

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should acknowledge all GEO profiles to avoid bias. Although the 15 GEO profiles selected from replicates of two cell lines support his point, other samples, including CML CD34$^+$CD38$^-$ cells, actually show no notable changes in *SET* RNA or contradict Perrotti's claim (for example, GDS4047/210231_x_at and GDS4047/213048_s_at). We acknowledge that a potential mechanism of forskolin-induced PP2A activation by induction of adenylate cyclase/cyclic adenosine monophosphate activity was not correctly described in our manuscript because Perrotti's study observed the activation of PP2A with the forskolin analog 1,9-dideoxy-forskolin, which is supposedly lacking adenylate cyclase–inducing activity. However, an alternative mechanism for activation of PP2A is still lacking.

Perrotti *et al.* queried whether the effect of PIDs is a cytostatic one. We are not claiming that PIDs alone can act as an agent to induce apoptosis in CML cells. Our data indicate that PIDs have a marginal effect on apoptosis on their own but clearly enhance the apoptotic effects of TKIs, as demonstrated in apoptosis assays with increased caspase 8 and caspase 3 cleavage. We agree that elucidating whether cell cycle modulation (PID-induced mitotic catastrophe) may specifically enhance TKI-mediated apoptosis is worthy of further investigation. However, we chose to focus on studying downstream signaling effects of the combination treatment and how PP2A associates with AHI-1. Perrotti also neglects to acknowledge that our study demonstrates an obvious reduction in proliferation, as well as an increase in apoptosis. He points out that PIDs alone induced apoptosis in "only" 40% of cells, whereas the combination treatment increased apoptosis to 50% of cells. It should be noted that these data are specifically derived from TKI nonresponder cells, and the results achieved are statistically significant ($P < 0.04$). Importantly, the concentrations of PIDs we chose to use did not display any toxicity in CD34$^+$ healthy bone marrow cells in colony-forming cell (CFC) assays and, in combination with TKIs, did not increase the known toxicity of TKIs.

Perrotti *et al.* further suggest that the reduction in CFCs and long-term culture-initiating cells we detected when primary CML cells were exposed to PID and TKI was due to their cell cycle arrest and consequent impaired proliferation, rather than a sensitization of the cells to TKIs. This statement is likely based on our in vitro cell line data. We have not yet assessed the impact of combination treatment with PIDs and TKIs on cell cycle status in primary CML samples, so it would be speculative to argue for or against this possibility. However, if we are to similarly use our cell line data as a model of the effects observed in primary samples, then we should also acknowledge the marked reduction in proteins essential for BCR-ABL–mediated leukemogenesis. To demonstrate that the observed effects of combining PIDs and TKIs are due to synergism between the two drugs, rather than an individual cytostatic effect of PIDs compounded with TKI-mediated effects, we would like to emphasize the mechanistic evidence for a PID-mediated sensitization of CML cells via the β-catenin pathway. We observed increased phosphorylation of the T41 and S45 residues of β-catenin after treatment of the cells with either PID alone or IM and PID for 24 hours before the eventual total protein reduction at 48 hours. The temporal sequence of these phosphorylation changes followed by a reduction in total protein supports the concept of synergy in the combined delivery of two drugs, resulting in decreased stability and a consequent degradation of β-catenin along with the suppression of downstream transcriptional pathways. We also found, similar to other studies (*5*), that BCR-ABL inhibition with TKIs blocks phosphorylation of the Y86 residue. As indicated in our suggested model, this could be a mechanism contributing to the observed effects of a combined TKI and PID treatment strategy. In our discussion, we indicate experiments to further study this model, including targeted mutagenesis of these residues to mimic the drug-induced effects.

Perrotti *et al.* also question why total β-catenin was not reduced with PID treatment alone or in short hairpin RNA (shRNA)–PP2Ac–K562-IMR cells, etc. Our model does not anticipate that PID treatment alone causes these changes, but rather we expect to see them only when both BCR-ABL and PP2A are targeted, as seen with PID and TKI or shRNA-PP2Ac and TKI. It should also be noted that we observed a reduction in AKT protein in PID and TKI-treated K562-IMR cells but recognize that further investigation is required to determine whether there is a temporal relationship between the observed effects in β-catenin phosphorylation and AKT/glycogen synthase kinase-3β–mediated stabilization.

We would also like to emphasize that the activity of PP2A and its specific interacting proteins, pathways, and networks are differentially regulated during the progression of CML from CP to BC and/or in specific subpopulations of CP CML stem cells and progenitor cells from TKI responders versus nonresponders. We purposely used the K562-IMR cell line to investigate these signaling mechanisms because K562 cells are already very sensitive to TKI treatment. This rationale is similar to why we chose to use stem/progenitor cells from TKI nonresponders to examine the efficacy of the PID and TKI combination treatment. As pointed out by Perrotti, such a finding was recently demonstrated in another study, which suggested that PP2A interrupts glucose metabolism in BCR-ABL$^+$ B-ALL (*6*). Nevertheless, dual inhibition of PP2A (LB100) and glucose-6-phosphate dehydrogenase effectively eradicated BCR-ABL$^+$ malignant cells. Interestingly, a recent study demonstrated that pharmacologic inhibition of PP2A (LB100) achieves durable immune-mediated antitumor activity when combined with programmed cell death protein-1 blockade in colon cancer and melanoma models, highlighting the translational potential of PP2A inhibition in combination with checkpoint inhibition (*7*).

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