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Response

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In reply: Dr Amin and his colleagues touch on a series of aspects of Stat3 activation and NPM/ALK signaling. Their key comments, stemming mostly from their previously published work,[1] relate to: (1) potential involvement of Jak3 in NPM/ALK activation, (2) possible role of Jak3 in Stat3 activation in the NPM/ALK-expressing cells, and (3) specificity of the WHI-131 and WHI-154 compounds originally described as Jak3 inhibitors. The most controversial is the statement that Jak3 activates NPM/ALK. This conclusion is based on the observation that the WHI compounds inhibit tyrosine phosphorylation of NPM/ALK.[1] Given that NPM/ALK tyrosine kinase is a fusion protein that is constitutively dimerized, its activation is also constitutive and results from cross-phosphorylation of the catalytic domains of the ALK portion. This fact alone calls into question the specificity of the WHI inhibitors and suggests that they act on NPM/ALK directly. We have, indeed, demonstrated inhibition of NPM/ALK enzymatic activity by the WHI compounds[2] using two different methods. In retrospect, it is not particularly surprising that WHI compounds inhibit more than one kinase. First, all known tyrosine kinase inhibitors show substantial degree of cross-reactivity, often with kinases that are structurally overall quite diverse but seem to share the tri-dimensional shape of their ATP-binding pocket. Second, the WHI compounds need to be used in μM doses (5–14 μM for WHI-154 and 10–75 μM for WHI-131). These relatively high concentrations enhance the possibility of cross-reactivity with other tyrosine kinases. The recent finding[3] that WHI compounds inhibit MAPK and PI3K/Akt pathways in mast cells in the Jak3-independent manner is consistent with their reactivity with non-Jak3 kinases.

We did consider early on the possibility that Jak3 might be functionally downstream of NPM/ALK in activation of STAT3. However, a number of diverse experiments to address this very issue[2] convinced us that Jak3 participation is not critical for Stat3 activation by NPM/ALK. Accordingly, using the NPM/ALK-positive as well as ALK-negative T-cell lymphoma cells in which Stat3 is activated by the cytokine common gamma chain-associated Jak1/Jak3 complex,[4] we observed that the NPM/ALK-expressing cells are significantly much more affected by the WHI compounds in regard to their growth, proliferation, cell cycle progression, viability and Stat3 phosphorylation.[2] The opposite was true for the potent pan-Jak inhibitor (Jak I, Calbiochem) that is effective in low nM doses against all members of the Jak/Tyk family including Jak3. These observations not only suggested that WHI inhibitors act on NPM/ALK directly but also indicated that the compounds, in particular WHI-131, are rather poor inhibitors of Jak3. The lack of involvement of Jak3 in Stat3 phosphorylation in the NPM/ALK-positive cells was further documented by NPM/ALK cell transfection and siRNA-mediated depletion studies performed in the ALK-negative BaF3 cells and NPM/ALK-positive Karpas 299 cells, respectively[2] and the usage of a novel, highly effective and specific Jak3 inhibitor (IC 50 of 1 nM to inhibit the *in vitro* Jak3 kinase activity.[5]

In regard to features of the experimental models discussed by Dr Amin *et al*, it is possible that BaF3 cells express small (or various, possibly, subclone dependent) amount of endogeneous Jak3. Even if present, this small amount of Jak3 would likely be not effective enough to cause the easily detectable Stat3 phosphorylation. Perhaps more importantly, treatment of the NPM/ALK-expressing BaF3 cells with as much as 1 μM of the pan-Jak inhibitor (IC50 of 5 nM for Jak3) had no effect on Stat3 phosphorylation in such cells.[2] In regard to the efficiency of siRNA-mediated depletion, it varies greatly in our experience depending on the given siRNA, cell type, and protocol used. As documented in our publications,[2,6] the effect may exceed 90% of depletion as determined by comparative Western blot studies.

The Figure 1 presented in Dr Amin's letter, raised some questions in our minds. First, even if Jak3 is expressed in BaF3 cells (seemingly at low concentration, as discussed above), it is not clear why Jak3 would be phosphorylated in the parental, NPM/ALK nontransfected, presumably cytokine-starved BaF3 cells. BaF3 cells are strictly dependent on IL-3, the cytokine that signals in the Jak3-independent fashion. Second, Stat3 is not phosphorylated in the parental BaF3 cells.[2,7] This observation may be interpreted as additional evidence that Jak3 is not involved in activation of Stat3 in these cells. Third, the drug dose used was rather high and still not very effective (40 μM of the more potent WHI-154 yielded only 50% of inhibition of Jak3 phosphorylation). By comparison, we see almost total inhibition of Stat3 phosphorylation in the NPM/ALK-transfected BaF3 cells with 10 μM of the compound.[2] Fourth, it is surprising that the Jak3 phosphorylation was examined after 12 h from exposure to the drug, whereas tyrosine inhibitors including this one[2] inhibit cell signaling within 1 h from application. The delay raises a concern for secondary events impacting on the result. Finally, Jak kinases typically cross-phosphorylate each other rather than autophosphorylate.

Therefore, the observed effect of WHI-154 compound on Jak3 phosphorylation might reflect inhibition of a non-Jak3 kinase.

Noteworthy, studies performed by Inghirami and his colleagues[8] also indicate that NPM/ALK activates Stat3 independently of Jak3. Accordingly, treatment of NPM/ALK-expressing cells by one of the early developed kinase Jak2/Jak3 inhibitors, AG490, had no effect on Stat3 phosphorylation in such cells. Perhaps more convincingly, NIH 3T3 cells transfected with NPM/ALK deletional mutant (residues 118–138) were unable to bind Jak3 but retained the ability to induce Stat3 phosphorylation. Finally, it is worth mentioning that several tyrosinase kinases including certain growth factor receptors, src, and bcr/abl, are capable of activating Stat3 seemingly directly and independently of Jaks.[9] Activation of Stat3 by NPM/ALK independently of Jak3 and, apparently, of other members of the Jak/Tyk family, is in keeping with such alternative mechanism of Stat3 activation.

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