Author's response to reviews

Title: The isothiocyanate class of bioactive nutrients covalently inhibit the MEKK1 protein kinase

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Author's response to reviews: see over
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Dear Editor, BMC Cancer

We were pleased with the thorough reviews that we received for our manuscript. We are grateful that the reviewers appreciated the significance of our study in its examination of a molecular mechanism of action for the dietary ITC chemopreventives.

While the overall tone of the reviews was positive, each reviewer raised some specific points. We have addressed each of these comments in turn in the response to reviewers on the following pages, and have modified the manuscript as indicated therein. The most prevalent concern, raised by all three reviewers, related to the concentrations of PEITC that we used in our experiments, so we have responded to that comment in the first few paragraphs below, without addressing it to a particular reviewer.

We hope that we have adequately addressed all of the reviewers concerns. We look forward to your decision regarding publication of our manuscript.

Thank you for your consideration.

Sincerely,

Janet V. Cross
All three reviewers questioned the doses of PEITC used in our studies. Therefore, we respond to this concern here, with other reviewer specific comment addressed below.

We agree that the concentrations of PEITC used in these studies are higher than reported physiologic conditions, although they are similar to those used in many studies in which new control mechanisms are sought. In our experiments examining endogenous proteins, we observe complete inhibition of endogenous MEKK1 in LnCAP cells at a 50µM concentration and fairly complete inhibition at 25µM (Figure 7); these concentrations are growth-inhibitory concentrations in the literature, as cited by Dr. Srivastava. To examine the molecular details of the reaction, we used in many experiments overexpressed MEKK1, and found that this overexpressed MEKK1 required higher concentrations of PEITC for complete inhibition. For example, the 50µM dose only very partially inhibited the overexpressed full length MEKK1 in Figure 1 and the 25µM dose in Figure 2A only partially inhibited the catalytic domain fragment. We suspect that since the modification and inhibition of MEKK1 is covalent and irreversible, the PEITC is consumed in the reaction, and are therefore not surprised that it requires more PEITC to inhibit the overexpressed than the less abundant endogenous protein. We would suggest that the observed partial inhibition of endogenous MEKK1 (Figure 7) at the concentrations listed by Dr. Srivastava seems likely to be sufficient to bring about a physiological effect (ie cell death). As noted in our discussion, the lower end of our dose response range (where we observe partial inhibition of MEKK1 activity) is consistent with the concentration ranges used in previous studies examining PEITC induced apoptosis, of which we reference nine.

While the plasma concentration of ITCs attained clinically have been determined to be far less than the 12.5-25µM doses needed to inhibit MEKK1 in this study, no one has yet successfully measured the intracellular concentrations of ITCs in target tissues. It has been demonstrated in cell culture systems that the ITCs accumulate in cells where they can reach levels much higher than that present in the extracellular media. Extrapolating these findings to an intact organism, one would predict that the intracellular levels of ITCs may be much greater than that measured in the circulating plasma. Therefore, when taken together, we would suggest that MEKK1 inhibitory concentration of PEITC may well be attainable clinically when considering the intracellular concentrations in target tissues.

As for toxicity, we do appreciate that these doses would likely be toxic to cells over longer-term incubation. However, all of our assays were performed at very short time points in order to both study very proximal events in the signaling pathways and to avoid any effects due to loss of cells or signaling effects secondary to apoptosis. As mentioned above and discussed in our manuscript, we would contend that inhibition of MEKK1 may actually contribute to the PEITC induced cell death observed at later time points, as MEKK1 normally provides a survival signal in the cell.

Response to Reviewer 1, Dr. Sanjay Srivastava

1. **Concentrations of ITCs are very high.** Please see discussion above.

2. **Finding that PEITC inhibits JNK activation by MEKK1 is in contrast with published reports that demonstrate that PEITC activates JNK.** We are aware that other groups have published studies that show that PEITC activates JNK, and that this activation is required for induction of
apoptosis. In experiments that we have not shown, we have likewise observed activation of JNK by PEITC treatment alone. The data that we present in Figures 7 and 8 demonstrates that PEITC inhibits activation of JNK by hyperosmotic shock (i.e., sorbitol), an MEKK1-dependent signaling pathway. These results are intended to demonstrate that inhibition of MEKK1 resulted in the inhibition of downstream signaling to SAPK, but not the other MAPKs, demonstrating that the inhibition is specific to the MEKK1 dependent pathways. We would contend that the activation of JNK by PEITC proceeds via a distinct (MEKK1-independent) pathway. However, our experiments demonstrate that, when MEKK1 is inhibited by PEITC prior to addition of an MEKK1 dependent activating stimulus such as hyperosmotic shock, the dominant effect is inhibition of the pathway, reflected both in the direct inhibition of MEKK1, as well as the inhibition of the downstream target, SAPK/JNK. The lack of inhibition of the other MAPKs indicates the specificity of the inhibition and confirms that it is not a non-specific or toxic effect.

3. Define which cell line is used in each experiment. The figure legends have been modified such that each occurrence of “cells” is now qualified with the cell type in which the experiment was performed.

4. Figures should be labeled with ITC concentrations. All figures examining dose responses have been modified to indicate the concentration of PEITC or Bio-ITC used in each lane. The legends have been simplified by removing this now duplicate information.

5. Use of term in vivo. We have replaced the single occurrence of “in vivo” with “treatment of intact cells”.

Reviewer 2, Ann M Winter-Vann

This reviewer has indicated only Discretionary Revisions. While stopping short of performing additional experiments, we have addressed the comments based on information that is already available to us.

1. What about other conditions that signal to ERK1/2? Concentration of sorbitol is quite high. We did not examine stimuli other than hyperosmotic shock, as the purpose of this experiment was simply to demonstrate that PEITC inhibits MEKK1 dependent signals to its downstream target SAPK. These results support the conclusion that the inhibition is specific (i.e., does not affect other MAPK pathways) and therefore is not due to some non-specific or toxic effect of the compound. As the hyperosmotic stress stimulus allowed us to examine all three MAPK pathways in parallel, we did not explore the effects of PEITC on signaling induced by other stimuli. The concentration of sorbitol used in this experiment is a fairly standard dose for activating all three MAPK pathways. Therefore, we have not examined other concentrations in this experiment.

2. Dietary levels of ITCs are lower than the amount required to see inhibition of MEKK1? What about exposure over longer periods of time? For question regarding ITC concentrations, please see discussion above. We chose to use short time points for these experiments so that we could examine very proximal events in the signaling responses to PEITCs. At later time points, PEITC would induce both changes in gene expression and apoptosis and these events would be expected
to lead to secondary effects on the pathways that we were examining. For example, SAPK is often activated (likely through an MEKK1 independent pathway) as a consequence of apoptosis. Using short time point allows us to avoid these confounding effects.

3. **Additional demonstrations of the stability of modification?** As indicated in our discussion, our data suggests that the modification of MEKK1 is irreversible. This is based primarily on the observation that the modification is stable to boiling in SDS lysis buffer containing 75mM DTT. Moreover, as the reviewer pointed out, the PEITC modification was not competed by incubation with excess Bio-ITC. We have not examined longer time points in these experiments.

4. **What is the fate of the modified MEKK1?** It is possible that ITC modification, in addition to quenching the catalytic activity of the protein may promote its eventual degradation. Similar amounts of overexpressed catalytic domain protein were always recovered from the PEITC treated and untreated samples (e.g. Figure 6 shown and Figure 2A, not shown) at the short time points that we examined. Longer time points or pulse chase experiments would allow us to address this question more thoroughly. However, as the focus of this study was on the covalent modification and inhibition of MEKK1 by the ITCs, we have not examined this possibility in detail. The idea that the PEITC modified MEKK1 could act as a dominant negative is provocative, and something that would be valuable to consider in future studies.

Reviewer 3, Ssang-goo cho

**Major Compulsory revisions:**

1. **What is the human health significance of the irreversible modification by dietary ITCs?** While the health benefits of the ITCs have been well studied and broadly accepted, very little is understood about their molecular mechanism of action. At the cellular level, the ITCs induce new gene expression and apoptosis and they clearly act as cancer chemopreventives in animal models. The chemical structure of the ITCs suggest that they would react with cysteine residues on proteins, suggesting that the molecular mechanism of action may involve covalent modification of critical cell signaling molecules that would lead to the biological responses that have been characterized. Our demonstration the MEKK1 is modified and inhibited by ITCs is a proof of concept that these chemicals can covalently modify intracellular signaling molecules in a functionally significant way. This work opens the door to future studies that should allow us to define the role of targets of covalent modification in the known biological effects of ITCs. These studies are ongoing, but we feel beyond the scope of the current manuscript. We would hypothesize (as discussed in the manuscript) that stable irreversible modification of MEKK1 could participate in the ITC mediated induction of apoptosis, as MEKK1 normally provides a cell survival signal. The induction of apoptosis by ITCs is postulated to contribute to their anti-cancer activities by promoting the death of incipient cancer cells.

2. **ITC concentrations high and duration of ITC exposure too short.** Please see the discussion above regarding the concentrations of PEITC used. (Repeating from above) We chose to use short time points for these experiments so that we could examine very proximal events in the signaling responses to PEITCs. At later time points, PEITC induces changes in gene expression and apoptosis and these events would be expected to lead to secondary effects on the pathways...
that we were examining. For example, SAPK is often activated (likely through an MEKK1 independent pathway) as a consequence of apoptosis, though perhaps not as an initial event in cell signaling. Using short time point allows us to avoid these confounding effects.

Minor Revisions:

1 and 2. Controls to ensure equal amounts of protein. We include an equal loading control in Figure 6 that confirms that the exogenously-expressed kinase domain of MEKK1 is not altered by PEITC treatment, while activity is completely abolished. We have similar loading control blots for most of the experiments, but chose not include them in the aggregate figures as they would double the size of the figures. We would be happy to provide the data if the reviewer would like to see it.

Regarding endogenous MEKK1 protein, (e.g. Figure 7), our available reagents are insufficiently sensitive to allow us to observe endogenous MEKK1 expression, despite our best efforts. We suspect that the reviewer might be considering whether protein degradation may play a part in the loss of MEKK1 activity. As mentioned above, while we are certain from the results shown that kinase activity is blocked without loss of protein (Figure 6), and indeed is blocked in vitro (Figure 2B) we cannot conclude that endogenous MEKK1 is not lost following our short-term treatment or at later times through a secondary mechanism. We have previously published (Deak, et al, manuscript reference 7) that MEKK1 is targeted for degradation by caspases during apoptosis, which could happen after PEITC treatment, at later times. We have not yet examined the ultimate fate of the PEITC-modified protein in detail.

3. Effect of ITCs on activity of purified ASK1. Since ASK1 was insensitive to PEITC treatment in cells, we have not examined its sensitivity in vitro. If the reviewer is seeking evidence for the specificity of inhibition by PEITC, we point out that the insensitivity of the CV mutant of MEKK1 to PEITC is a preferable representation of specificity, as it is an identical protein except for one amino acid change.

4. Explanation of lane 2 on Figure 6. The legend for Figure 6 has been modified to indicate that this lane contained the molecular weight markers.

5. Explanation for decreased phospho-p38 at highest dose of PEITC. We agree that some inhibition of p38 activation appears evident at the highest dose of PEITC. However, the inhibition of SAPK occurs at far lower doses of PEITC treatment. Our discussion focused on the marked difference between the various MAPKs, with SAPK being fairly sensitive to PEITC pretreatment while ERK and p38 were mostly resistant. We have modified the results section to indicate that PEITC does slightly inhibit p38 activation at the highest dose (page 12, bottom), but suspect that the effect on p38 might be due to cell toxicity effects.

6. Change biotin to Bio-ITC. Change made as requested.

7. Change room temp to temperature and insert degree symbols. Changes made as requested.
8. **Define cell type used in each experiment.** The figure legends have been modified such that each occurrence of “cells” is now qualified with the cell type in which the experiment was performed.

**Discretionary Revisions:**

1. **Kinase assay data for MEKK1 treated with other chemopreventive ITCs.** Two of the experiments presented in the paper have been repeated with sulforaphane (the LnCAP experiments examining endogenous MEKK1 and the in vitro inhibition of MEKK1 wild type vs. CV). The results obtained with sulforaphane are referred to in the manuscript as data not shown. The dose ranges used in the sulforaphane experiment were not optimal (e.g. in the in vitro experiment, all of the doses chosen were completely inhibitory). Therefore, we feel that, while consistent in the conclusions, these experiments do not lend themselves to a compelling figure.

2. **Quality issues with Figure 8.** The figures as presented are as clean as we could generate using the reagents available to us at the time these experiments were completed. We feel that the data clearly supports the conclusions that we have made in the manuscript. We would be happy to share the primary blot data, if the reviewer would like to examine it.