Author's response to reviews

Title: Identification of a Novel CK2 Inhibitor - Hematein (3, 4, 10, 6a-tetrahydroxy-7, 6a-dihydroindeno [2, 1-c] chroman-9-one) from a Natural Product Library

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Author's response to reviews: see over
Dear Editor-in-Chief,

Thank you for your review of our manuscript entitled “Identification of a Novel CK2 Inhibitor - Hematein (3, 4, 10, 6a-tetrahydroxy-7, 6a-dihydroindeno [2, 1-c] chroman-9-one) from a Natural Product Library”.

Please find our revised manuscript and our response to comments from both reviewers. We thank both reviewers for their experts, because their comments make this manuscript a better manuscript. We have done extensive experiments to address their comments. We have revised our manuscript according to their suggestions and answered point by point to each comment.

Sincerely,

David M. Jablons, M.D.
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Reviewer 1

General Comments

The work presented adds a new member (hematein) to the growing list of compounds that are inhibitors of protein kinase CK2 and display cytotoxic effect on tumour cell lines. No attempt is done to demonstrate a cause-effect relationship between the two effects.

Major compulsory revision

1. The authors adopt kinase assays different from those commonly applied in the literature, apparently relying only on the manufacturer’s protocols, without any bibliographic support. It looks especially strange that one kit exploits p53 serine-46 phosphorylation to quantify CK2 activity, whereas the CK2 site in p53 is not serine-46. The authors shouldn’t blindly trust the manufacturer’s claims and instead they should support the reliability of their methods with appropriate references. In any case experimental conditions (notably source and concentration of the protein kinase) should be better defined.

   Answer: We have further validated our previous results with the classical radioisotope CK2 kinase assay for determination of hematein IC$_{50}$ values, kinetic study, and kinase activity in cancer cells after treated with hematein. Changes made are reported in material and methods, results, Figure 2 and 3.

2. The crucial experiment showing the decrease of Akt S129 phosphorylation (Figure 6A) is biased by an ambiguous DMSO control (irregular intensity of the band). This should be replaced by a better experiment with statistically significant quantification of the outcome.

   Answer: We have performed additional experiments and replaced the AktS129 phosphorylation western blot figure with a better quality figure, and quantization is also performed which shows statistically significant. Changes of AktS129 phosphorylation are shown in Figure 4C.

3. Higher efficacy of hematein toward cancer cells as opposed to “normal” cells is an interesting outcome of this work. It rises however the issue of selectivity which has not been touched by the authors, while the selectivity of most other CK2 inhibitors (included TBB here used for sake of comparison) has been thoroughly examined on panels of numerous kinases. This gap should be filled at least by testing hematein on a few protein kinases (notably those belonging to the DYRK, PIM, HIPK, PKD and CDK families) which have been reported to be particularly
susceptible to CK2 inhibitors.

**Answer:** We have tested hematein on a panel of 48 kinases, including DYRK, PIM, HIPK, PKD and CDK families using KinaseProfiler™ service (Millipore, UK). Hematein does not have notable inhibition effects to DYRK, PIM, HIPK, PKD and CDK families compared to CK2 at 10 µM concentration. The results have been reported in Table 1.

4. The 100 uM concentration of effective compounds used to perform the in vitro experiment shown in Fig. 2 is exceedingly high, especially considering that the concentration of compounds used to perform the in cell experiment of Fig. 1 is lower (6-27 uM). This experiment should be repeated at 10 uM concentration.

**Answer:** We have repeated the kinase assay with 10 µM concentration and the results are shown in Figure 2A.

**Minor essential revisions.**

1. In Fig. 1 only 11 compounds out of the 400 compounds of the library are considered, only 3 of which display significant inhibition of cell proliferation. I presume that all the other compounds were devoid of any effect. If so, this should be clearly stated and the negative data mentioned as “not shown”.

**Answer:** We have mentioned that the negative data as “not shown” in Figure 1.

2. Fig. 3 (hematein structure) should be incorporated into Fig. 2, together with the structures of the other two library compounds which display significant CK2 inhibitory activity (4A6 and 4A9).

**Answer:** We have incorporated the structure of 4A6 and 4A9 together with the structure of hematein into Figure 2.
Reviewer 2

The authors should provide the following information:

1. What is the specificity of Hematein towards CK2? Does this inhibitor have activity towards other protein kinases (data indicating the effect of this inhibitor on a battery of other kinases is essential to prove that hematein is a specific inhibitor of CK2).

   Answer: We have tested hematein on a panel of 48 kinases using KinaseProfiler™ service (Millipore, UK). Hematein has strongest inhibition effect toward CK2 among all enzymes tested. The results have been reported in Table 1.

2. What is the nature of Hematein inhibition of CK2. Is it reversible or irreversible? Is the inhibition through ATP binding site? What is the nature of this inhibition with respect to ATP levels (competitive or non-competitive).

   Answer: We have done reversibility and kinetics experiments to further elucidate these questions. From the results of reversibility experiments, hematein exerts reversible effects towards CK2. Increasing the concentration of ATP in CK2 kinase assay does not show corresponding increase in IC$_{50}$ value of hematein. The results of kinetic experiments also support that hematein inhibits CK2 in an ATP non-competitive manner. The results are shown in Figure 3.

3. To indicate that Hematein is not effective in normal cells, it is important to show that it is able to enter these cells. Also, more than one cell line needs to be evaluated. What is the inhibition level in normal cells? Are these cells surviving despite reduction in CK2 or is there no inhibition of CK2 in these cells which could indicate that the inhibitor is not entering these cells.

   Answer: Hematein can also inhibit normal cells (CCL-211 and WI-38) in our study with higher IC$_{50}$ values than other cancer cells (A549, A427, HCT-116, and Hela). We also take advantage of the staining property of hematein and show that hematein can enter both cancer and normal cells. The results are shown in Figure 7.