Author’s response to reviews

Title: Evaluation of novel N-acetylalaninate prodrugs that selectively induce apoptosis in prostate cancer cells

Authors:

  Christopher A McGoldrick (cmcgoldr@uthsc.edu)
  Yu-Lin Jiang (jiayu@musc.edu)
  Marianne Brannon (leverone@etsu.edu)
  Koyamangalath Krishnan (krishnak@etsu.edu)
  William L Stone (stone@etsu.edu)

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Author’s response to reviews: see over
We very much appreciate the constructive points raised by the two reviewers and believe that revisions detailed below have improved the quality of this submission.

**Reviewer 1**

**Comment 1:** Despite the relevance of the assays, the authors must clarify and justify the differences obtained between cell lines and why the studies are not done in all.

**Response:** We now provide a brief rationale for the cell lines chosen for study in the Background section of the paper (lines 119-125). We now state:

In this study, the effectiveness of the S-NPAA, and three other similar prodrugs (Figure 3), was evaluated in tumorigenic (LNCaP, DU145, PC3) and non-tumorigenic (RWPE-1) prostate cell lines as well as COS-7 cells overexpressing human OPH (COS-7-OPH). We have previously characterized the expression of OPH in LNCaP, RWPE-1, COS-7 and COS-7-OPH cell lines [24]. Moreover, Kumar *et al.* [3] have characterized the degree of Akt activation in RWPE-1, LNCaP, DU145 and PC3 cells as well as the basal levels of oxidative stress.

Depending upon the purpose of the experiment is was not necessary to use all cell lines. For example, the main purpose of the data in Figure 5A was to demonstrate that the NPAA prodrug could cross the plasma membrane (of any mammalian cell). We demonstrated this in four different cell lines. A secondary purpose of the Figure 5A experiment was to demonstrate that increased OPH expression could result in increased GSH depletion. This was most clearly demonstrated by comparing COS-7 cells to COS-7-OPH cells where the only difference is the level of OPH expression. COS-7 cells are from the kidney of the African green monkey and not relevant to prostate cancer. For Figure 5C, the primary purpose was to compare NPAA induced GSH depletion among the various prostate cell lines (tumorigenic and non-tumorigenic): the COS-7 monkey kidney cells were, therefore, not relevant for Figure 5C.

Similarly, for the data in Figure 6A the primary goal was to determine if NPAA treatment increased cellular oxidative stress in cells overexpressing OPH. Four cell lines were tested, two with OPH overexpression (LNCaP and COS-7-OPH) and two with low OPH expression (COS-7 and RWPE-1). Both cell lines with OPH overexpression had increased levels of protein carbonyls (oxidative stress biomarker). Caspase 3 activation was measured in all cell lines (Figure 6B) since the primary purpose was to see how basal levels of oxidative stress AND the degree of OPH expression influences apoptosis induced by NPAA.

We have modified the text in the manuscript to be more transparent about the rationale for why particular cell line lines were used or not used (see lines 120-125, 342-347, 355-358, 389 and 408-411).
Comment 2: They should explain, besides the OPH levels, if other mechanisms could contribute to results observed (ex. Gene mutations, basal antioxidant levels...?)

Response: The Discussion section of the paper (lines 473-485) does mention the role of intrinsic oxidative stress as an additional mechanism. We now expand this section to address this second concern of reviewer 1.

Lines 487-492 now state:

It is also likely that the basal antioxidant levels in cancer cells could be an additional variable that could influence the effectiveness of pro-oxidant drugs like S-NPAA. Cancer cell often have a high level of GSH to cope with their high level of intrinsic oxidative stress [29-31]. In addition to mutations resulting in Akt activation, mutations in antioxidant enzymes or mutations resulting in increased ROS production (e.g., many mitochondrial mutations) would also be important determinants of pro-oxidant drug efficacy.

Other comments from reviewer 1:

Comment 3: The contribution of AKT activation is only a hypothesis, it is not proved in the article.

Response: We agree with this comment and when making a connection between our results, Akt activation and oxidative stress we now always use the word “suggests.”

Comment 4: The authors must be more carefully when they said that the prodrugs tested “induced selectively apoptosis in prostate cancer cells”, because they use an in vitro study with cells lines and not primary cultures or animal models

Response: This point is well taken and we have changed the title of the paper to reflect this limitation. The title is now “In vitro evaluation of novel N-acetyllalaninate prodrugs that selectively induce apoptosis in prostate cancer cells.”

Comment 5: Figs 4, 5 B and C, 6 A and B and 8-B and Table 1 (Fig 10) should be increased n size to facilitate the visualization of results.

Response: We have increased the sizes as suggested.

Comment 6: In the figure legends the acronyms/abbreviations are not spelled which difficult the interpretation.
Response: We appreciate this concern and have examined all the figure legends and added definitions where appropriate (Figure 2, 4, 6). Figure 3 already had the needed abbreviations under the name for each organic structure.

Reviewer 2

Comment 1: They used LC-MS to show that HT29 colon cancer cells incubated with NPAA form the expected GSH-QM adduct: (a) In order to exclude the possibility of non-specific ester hydrolysis in phosphate buffer, a control experiment is expected by mixing GSH and S-NPAA in sodium phosphate buffer without rOPH and other conditions should be similar. (b) The mass spectra for analyzing the reaction mixture are expected to show either in the supporting information or for review at least.

Response:

(a) The work on HT29 colon cancer cells referred to by reviewer 2 was not ours but that of Hulsman et al. as referenced in the manuscript. Hulsman et al. have already demonstrated (by LC-MS) that the quinone methide resulting from the enzymatic hydrolysis of NO-ASA covalently reacts with GSH (to form a GS-QM adduct). The hydrolysis of our prodrug (NPAA) yields a quinone methide identical to that from the hydrolysis of NO-ASA. The results we briefly present in our manuscript are, therefore, confirmatory and not a unique finding.

We did in fact do the control experiment proposed by the reviewer 1 and found only an m/z for GSH and no m/z for GS-QM (see lines 222-223). Moreover, Figure 4 shows that in the absence of human OPH there is no GSH consumption in the presence of NPAA (and GSH). Similarly, when human OPH is inactivated with DFP (as shown in Figure 4) there is no GSH consumption in the presence of NPAA (and GSH). The GSH-QM adduct cannot form without some GSH consumption. For our experiment, we used rat liver OPH (rOPH) that was purified but still had some minor additional proteins present that were all identified by reverse phase nanospray LC-MS/MS and none were proteases or esterases. We have added some text (lines 331-336) to clarify and address the concern of reviewer 2.

(b) The Figure below shows the actual MS data requested by reviewer 2. We do not believe, however, that this figure needs to be included with the paper since the only relevant finding is the presence or absence of the m/z for GSH or the GSH-QM adduct. As noted above, our documentation for an m/z for GSH-QM is not unique and was already done by Hulsman et al. .
Comment 2: In figure 6, the author concluded that different activities of S-NPAA in different cell lines were caused by different level of OPH. It would be helpful to show the OPH level of different cell lines.

Response: Figure 6 shows data relevant to oxidative stress and apoptosis. Reviewer 2 is most likely referring to Figure 5C. The relative amounts of OPH in the various prostate cell lines have been extensively documented in our previously published paper [see ref 24]. We have also summarized this information in table 1. We have added text (lines 363-364) to clarify this issue.

Comment 3: It would also be interesting to compare the activity of S-NPAA with the known prodrug ANAA. It would be helpful for a reader if the author could show the structure of ANAA.

Response: While ANAA is a known substrate for OPH, it is not a prodrug and does yield a quinone methide in the presence of OPH. We have shown the structure of ANNA (both R- and S-stereoisomers) in our previously published paper [ref 24] and now provide a reference so the structure can be viewed (see line 315).