Author's response to reviews

Title: gammaH2AX and Chk1 phosphorylation as predictive pharmacodynamic biomarkers of Chk1 inhibitor-chemotherapy combination treatments

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Author's response to reviews: see over
Response to reviewer’s comments

Reviewer: Alan Eastman

Major concerns
1. This manuscript sets out to determine which drugs combine well with a Chk1 inhibitor, and what biomarkers predict this response. A limitation of this study is its general lack of novelty because it has been preempted by many prior studies, albeit with a different Chk1 inhibitor. Many papers have assessed the activity of Chk1i in combination with other drugs; e.g., one recent paper directly compared multiple drugs (Xiao, Mol Cancer Therap, 2013). The use of phospho-Chk1 and gH2AX have also been well studied as response markers (e.g. Parsels, Clin Cancer Res 2011).

In our opinion, the major novelty of this study is that, in addition to using a novel Chk1 inhibitor, it is the first to examine a Chk1 inhibitor in combination with multiple drugs and evaluate response markers for these drugs in the same study. Previous studies have either focussed on Chk1i potentiation with multiple drugs but have not examined biomarkers of response (e.g. Xiao, Mol Cancer Therap 2013) or have looked at response markers of a Chk1 inhibitor in combination with a single chemotherapeutic drug (e.g. Parsels, Clin Cancer Res 2011). We acknowledge that some of this previously published work does pre-empt this study (and all relevant previously published work has been fully referenced) and this has been reflected in our choice of journal to submit this paper to. We feel this work is a useful addition to the scientific knowledge and therefore falls within the scope of BMC Cancer: “It is journal policy to publish work deemed by peer reviewers to be a coherent and sound addition to scientific knowledge and to put less emphasis on interest levels, provided that the research constitutes a useful contribution to the field.”

2. A major conclusion of this manuscript is that gH2AX would be a good marker to assess response in patients. Yet the results presented here show that cleavage of PARP, caspase 2 or 3 would be equally appropriate as markers. This raises the question of whether the appearance of gH2AX is a cause or consequence of the onset of apoptosis. I suspect it is a cause, but the authors would need to incubate cells with caspase inhibitors before they can make the conclusion that gH2AX is indeed causal (i.e., line 215).

We have not conducted the studies suggested by the reviewer (nor do we have the resource to conduct these studies). We have therefore changed line 215 to suggest that markers of apoptosis may also be useful biomarkers. Our main aim was to focus on gH2AX as this has some validation in the clinic as a biomarker of increased DNA damage.

3. The authors should consider the clinical schedule for administration of the drugs they use as continuous incubation for 72 h or 168 h has little relevance to bolus administration of most of the drugs to patients. Furthermore, while they use two different growth / survival assays, the end point in both is the same and neither can be said to assess survival. Both assays only measure viable cells and provide no data as to whether there are any dead cells in a culture. Hence, as used here they can only be said to assess growth inhibition. The comment on line 147 (“fraction of cells killed”) is therefore incorrect, and figure axis saying “survival” are also incorrect.

Modelling the in vivo tumour exposure using cells grown in culture is extremely difficult as plasma and tumour PK and metabolism have to be taken into account. For example, gemcitabine is given as a 30 minute IV infusion and has a plasma half-life of 42 to 94
The active metabolite (gemcitabine triphosphate) however has a terminal elimination half-life of 1.7 to 19.4 hours in PBMCs. We feel our approach is as valid as treating the cells for only one hour and then removing all drug.

Survival on the graph axis has been changed to Cell Viability and line 147 to “but also markedly reduced the viability of cells treated with gemcitabine”.

4. Many of the changes observed in Fig 2 could be attributed to differential cell cycle perturbation caused by the various drugs, yet it is unfortunate that no attempt was made to assess this important parameter.

The aim of this study was to identify usable biomarkers for potential clinical evaluation of V158411 in combination with various cytotoxic chemotherapy agents rather than understanding the mechanisms underlying potentiation. Such studies are always going to be based on the average of the cell population and a “snapshot in time” hence why we evaluated equitoxic doses of cytotoxic drug at the same time point. We have added a line to the text to suggest that some of the differences observed in protein biomarker changes may be a reflection of differential cell cycle perturbations induced by the various chemotherapeutic drugs.

5. The results in Fig 3 seem inconsistent at times but the authors fail to address or explain this. In particular, in HT29 cells, 62.5 nM V158411 inhibits Chk1 as assessed by p296Chk1, yet no gH2AX is observed in combination with gemcitabine until 1 μM V158411. In contrast, gH2AX is observed at 62.5 nM in the equivalent Colo25 experiment. Is there some reason why these results are so different between cell lines? Equally confusing is the observation that the addition of V158411 has no additional impact in gemcitabine treatment in both cell lines despite the fact it is supposed to be collapsing replication forks (Fig 3B).

Some of these differences are a reflection of the time component of the response to Chk1 inhibitor plus chemotherapeutic agent treatment (assessed in more detail in Fig 4). In HT29 cells, 62.5nM V158411 does induce gH2AX in combination with gemcitabine, it is much weaker than that induced by camptothecin. We have added the following two sentences to highlight this: “In HT29 cells, the induction of γH2AX by V158411 was more pronounced in combination with camptothecin than with gemcitabine whilst in Colo205 cells, treatment with V158411 induced γH2AX by roughly equal amounts in combination with either gemcitabine or camptothecin.”

The perceived lack of additional impact on camptothecin and gemcitabine treatment in Fig 3B is most likely due to the time at which the biomarker changes were observed. This is assessed in greater detail in Fig 4B. We have added the sentences “Chk1 inhibition in combination with chemotherapy induced DNA damage was predicted to have dramatic effects on replication fork stability and cell cycle arrest. However, at the time point studied, no difference in pChk1 (S317) or pChk1 (S345) levels could be perceived between cells treated with DNA damaging agent alone or in combination with V158411.” to reflect this.

6. The experiments to demonstrate lack of response in p53 wildtype cells are inadequate because the cell lines used are not isogenic. As a minimum this limitation should be noted. However, there is also a problem in understanding why p53 might protect cells. Consider the text on lines 310 – 320. Gemcitabine stalls cells in S phase, and when Chk1 is inhibited, the replication forks collapse. It is not correct that these cells progress through S phase and enter a lethal mitosis, which p53 might protect from, as the absence of dNTPs in cells on
gemcitabine means that the cells remain in S phase. There is no rationale to suppose that p53 would impact the collapse of replication forks.

We have added the following from line 248 “Whilst the HT29 and HCT116 cell lines are both derived from the same tissue type, they are non-isogenic and further studies are warranted in matched isogenic cell line pairs.” We do not have access to the HCT116 p53 isogenic cell lines.

The reviewer’s statement “It is not correct that these cells progress through S phase and enter a lethal mitosis, which p53 might protect from, as the absence of dNTPs in cells on gemcitabine means that the cells remain in S phase.” is not supported by the literature. The recent study by Del Nagro et al (Cell Cycle 2014) clearly demonstrates that p53 deficient cells treated with gemcitabine and a Chk1 inhibitor exit S-phase and undergo a premature mitosis and that this requires p53 to be inactive. We have added the Del Nagro ref to the text to support this.

Minor revisions requested:
8. Lines 49/50 should clarify that the Mre11 complex signals to ATM while 9-1-1 signals to ATR. On line 47 it should be noted that ATR signals in response to single-strand regions of DNA.

We have added “respectively” to the end of the sentence on line 50 to clarify this. We have changed the sentence to “The serine-threonine checkpoint kinases Chk1 and Chk2 are often described as the “central transducers” of the DDR and are activated by the ATM kinase in response to DNA breaks and ATR kinase by single-stranded regions of DNA and form the key link between the sensing kinases ATM / ATR and the cell cycle machinery.”

9. Line 64 is misleading as all the agents listed inhibit replication fork progression. Etoposide inhibitors topoisomerase II.

We have changed “replication fork progression” to “DNA unwinding”.

10. The antibody to pCdc2-Y15 can not discriminate phospho-Cdc2 from phospho-CDK1 (despite what suppliers frequently say). Furthermore, changes in this phosphorylation can not be said to be due to mitotic entry.

Cdc2 and CDK1 are the same protein, we assume the reviewer is referring to CDK2? It is true that the 12aa centred around Y15 are identical between CDK1 and CDK2 and therefore discrimination between the two is not possible. We have changed the sentence “Treatment with camptothecin followed by V158411 induced an increase in mitotic entry (determined by a decrease in pCdc2 (Y15) and an increase in phH3 (S10)) compared to treatment with camptothecin alone.” to “Treatment with camptothecin followed by V158411 induced an abrogation of DNA damage induced arrest and an increase of cells into mitosis (determined by a decrease in pCdc2 (Y15) and an increase in phH3 (S10)) compared to treatment with camptothecin alone.”

Loss of phosphorylation of CDK1 on Y15 is necessary for exit from G2 and entry into mitosis and this, coupled with an increase in phH3 (S10), is highly suggestive of exit from G2 and entry into mitosis.
11. Justification should be given as to why anchorage independent cultures were investigated. Similarly, what was the intent of performing experiments in low oxygen or low serum? A clear reason for each experiment needs to be provided.

Both anchorage independent culture and hypoxia are more reflective of in vivo tumour growth than 2D growth on plastic. We have added this comment to the text.

12. Line 293. There are already a number of papers that have published the lack of sensitization to 5FU, but the authors might find it interesting to note that several of those same papers have shown sensitization with fluorodeoxyuridine.

We are not certain which exact references the reviewer is referring to here. There are indeed several reports showing no sensitisation of 5-fluorouracil by a Chk1 inhibitor (including their own work, Montano et al Mol Cancer Therap 2012) but also studies showing potentiation (e.g. Xiao, Mol Cancer Therap 2013; Ganzinelli et al, Clin Cancer Res 2008). The only studies we are aware of comparing sensitisation to 5-fluorouracil and 5-fluorouridine focussed on ATM or ATR depletion by siRNA and PARP inhibitors (Heuhls et al, Cancer Res 2011; Geng et al, PLoS One 2011).

13. Line 306. It is correct that the greatest sensitization has been seen with antimetabolites, but the greatest sensitization in this class has been reported with cytarabine and hydroxyurea.

The Xiao study, referenced in line 306 and probably the most extensive study to date, demonstrated the greatest potentiation with gemcitabine and cladribine. We have changed especially to including.

14. Line 328, gemcitabine is administered to mice at doses below the MTD as it is in humans. The difference in schedule of 3 days vs. weekly is a minimal difference given the difference in species.

When gemcitabine is administered to tumour bearing mice at the MTD using the same schedule as in humans (q7d) there is little efficacy observed compared to administration on a q3d schedule. This would suggest that there are clear species differences and that different schedules are necessary to see optimal efficacy.

Reviewer: Hatim Allawi

1- The authors submit a well planned experimental evaluation of the effect of combination therapy of the Chk1 inhibitor V158411 with a number of cytotoxic chemotherapeutic agent. They use p53 mutant colon cancer cell line for in vitro testing of the effect of V158411 combo therapy.

2- Data is clear and results are well documented and the manuscript is easy to follow and is well written.

3- More background on the choice of colon cell lines as a model would be helpful.

We selected HT29 cells as these have been used extensively by both ourselves and others to assess potentiation of cytotoxic chemotherapy drugs by Chk1 inhibitors. We used Colo205 cells as a second model as previous in house studies also demonstrated potentiation of gemcitabine cytotoxicity by V158411. We therefore felt this was a suitable second cell line to study.
We have added the following to the results section (line 144): “The p53 mutant HT29 colon carcinoma cell line has been extensively used to evaluate the potentiation of cytotoxic chemotherapy by Chk1 inhibitors [19-23] and was therefore used as the main test system for this study.”

4- Some explanation of the chemical properties of V158411 (i.e, source, structural features, purity level, etc.) that would not compromise any confidential information would be beneficial to the reader.

We have referenced the patent that includes V158411 within it (ref 18) which describes the general chemotype etc of the molecule. We are currently preparing another manuscript that will describe the discovery and structure of V158411. The compound was synthesised in house and is >98% pure by LC-MS and NMR analysis.