Author’s response to reviews

Title: Sequential decitabine and carboplatin treatment increases the DNA repair protein XPC, increases apoptosis and decreases proliferation in melanoma.

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Reviewer reports:

David Melton (Reviewer 1):

Major changes required

1. Provide a more balanced consideration in the abstract, introduction and discussion of the relationship in melanoma and other cancers between levels of NER activity, levels of NER proteins and sensitivity/resistance to chemotherapy with platinating agents.

We thank reviewer 1 for this feedback. An additional paragraph has been included in the introduction describing previous studies that have reported high levels of members of the NER pathway in cancer, in particular ERCC1. We have previously published a review article on this topic: Bowden NA. (2014) Nucleotide excision repair: why is it not used to predict response to platinum-based chemotherapy? Cancer Letters 346(2). When writing this review article it became apparent that vast majority of previous studies investigating NER in cancer have focused on ERCC1. The focus of the manuscript under review is the global genome repair branch of NER, in particular XPC, a different component of the NER pathway with a different function to ERCC1. Therefore, we have explained this in more detail. The following has been added to page 3:

“Several previous studies have found an association between high NER protein and mRNA levels and platinum chemoresistance (reviewed in [6]). The NER component ERCC1 has been extensively studied as a predictive biomarker for response to chemotherapy. To date, high levels of ERCC1 before platinum chemotherapy have been associated with poor response in melanoma [7], non-small cell lung cancer [8, 9], head and neck [10], gastric [11, 12], bladder [13] and
oesophageal cancer [14]. Small molecule inhibitors of the ERCC1-XPF complex have been developed and shown to potentiate cisplatin efficacy in the A375 melanoma cell line [15] and H460 and H1299 lung cancer cell lines [16]. Further to this, in a melanoma mouse xenograft model loss of ERCC1 resulted in sensitivity to cisplatin [17]. To date, the study of NER in relation to platinum chemoresistance has largely focused on ERCC1.

In addition to the evidence supporting ERCC1 as a biomarker of platinum chemoresistance, our previous research has shown that the GGR damage detection branch of NER, does not function correctly in melanoma. We have found that the three GGR components XPC, DDB1 and DDB2 do not respond to UV treatment in melanoma cell lines, resulting in reduced repair of UV-induced DNA damage [17, 18]. We also identified that melanoma tumours with low XPC expression have significantly shorter survival [17].”

Reviewer 1 comment: “The high mutational load in melanoma (and non-melanoma skin cancers) can be explained by continuous skin exposure to the DNA-damaging UV component of sunlight rather than by an NER defect.”

In response to reviewer 1’s comment we have included the following sentence and references in the introduction, page 4:

“This was further supported by analysis of melanoma genomes, that concluded somatic mutations active gene promoters is caused by a decrease in the levels of nucleotide excision repair (NER) activity [21, 22].”

New references:


2. Many of the effects reported here are small and are not seen in all the melanoma lines studied. Significance values for some of the differences observed are not always provided (Figure 4). Some of the data (Fig 5A) are not displayed in a way that allows the link suggested by the authors between decitabine-induced increases in XPC expression and
chemosensitivity to be tested (pooled growth data for all 4 lines is shown, while only two lines show a significant increase in decitabine-induced XPC expression). The discussion of causal changes between demethylation, increased XPC expression and chemosensitivity should be moderated to properly reflect these limitations. The results part of the abstract should also be rewritten to reflect this.

We thank the reviewer for this feedback and the excellent summary of each figure that highlights the strengths and weaknesses of the data. Significance values were missing from Figure 4 in error and has been corrected. Reviewer 2 has also made the request for separate cell line data for Figures 5 and 6 and this had been added to the revised manuscript. The discussion and the abstract have been edited to reflect the limitations and highlighted with track changes in the uploaded revised version of the manuscript.

Minor changes required

3. Change the title so that the reader does not presume that a causal link between demethylation, increased XPC expression and chemosensitivity is established in this paper.

The title has been changed to “Sequential decitabine and carboplatin treatment increases the DNA repair protein XPC, increases apoptosis and decreases proliferation in melanoma.”

4. Comment on the high level of apoptosis observed in all untreated melanoma lines.

The level of apoptosis in the untreated cell lines in this study ranged from 6.5% to 11.3% which is consistent with previous papers using the same cell lines. We have commented on this and added the references to the results.

“Baseline levels of apoptosis ranged from 6.5% to 11.3% which is consistent with previous reports for Sk-Mel-28 [44, 45] MM200 [44, 45] Mel-RM [45, 46] and me4405 [45] cell lines.”

5. Line 310 delete "Remarkable"

This change has been made

6. Line 378 change "probable" to possible

This change has been made
Aaron G. Smith (Reviewer 2): Overall the data is solid, however a few minor points outlined below should be addressed by the authors.

1. **Figure 1.** I think it is important that these experiments are accompanied by XPC protein analysis by western blotting.

The authors agree with this comment and include XPC western blot data for the baseline and 0.26uM decitabine dose in Figure 3 to allow for comparison to the response to carboplatin alone and decitabine/carboplatin combination. We are very happy to include the data in Figure 1 if the reviewer or editor believes it is better placed in figure 1. We did not collect XPC Western blot data for the 10uM decitabine treatment as this dose was not used for any further experiments, it was used to ensure global demethylation occurs in the cell lines using decitabine and that a subsequent increase in XPC mRNA was occurring.

2. **Figure 1.** Have the authors analysed XPC levels and associated methylation of the CpG island using primary melanocytes, and moreover the response to decitabine. This is particularly important if the central tenet of the paper is that methylation at the CpG island or additional site is responsible for low levels of XPC and hence impaired GG-NER in melanoma.

At the suggestion of Reviewer 2, the CpG island and shores have been assessed in melanocytes and are methylated in a similar pattern to melanoma cells. The effect of decitabine on melanocyte methylation is difficult to assess as the mechanism of action of DNA methyltransferase inhibitors is passive blocking of methylation during replication. As melanocytes have low replication rates in vitro and do not replicate in vivo the blocking of methylation does not occur to the same extent as in melanoma cells, therefore the effect of decitabine differs significantly. We have added a supplementary figure 1 showing the methylation patterns in the XPC CpG island and shores in melanocytes and the 4 melanoma cell lines used in the study and added the following to the discussion.

“The same pattern of methylation was also seen in a melanocyte cell line (Supplementary Figure 2) suggesting it is a lineage-specific epigenetic pattern.

Methylation within the CpG island shores was present in melanocytes and the 4 melanoma cell lines in this study. The methylated regions were partially demethylated by decitabine in the melanoma cell lines. As melanocytes have low replication rates in vitro and do not replicate in vivo the blocking of methylation does not occur to the same extent as in melanoma cells, therefore the effect of decitabine is not seen in melanocytes.”
3. Figure 4. I assume these data are lacking statistical analysis as at least Me4405 and Mel-RM would appear to be significant. The poor/lack of response of the Sk-Mel-28 line would suggest it is a good candidate for exogenous over-expression studies to further support a role for XPC in he observed sensitisation to Carboplatin (see comment below).

Statistical analysis was performed, the significance levels were left off the figure during submission in error. These have now been added to Figure 4.

4. Figure 5. I would suggest that all lines should be independently shown in panel A rather than pooled.

This is an excellent suggestion and we have edited Figure 5 to show all cell lines independently in Panel A.

5. Figure 5 (B). Why has only the dual treatment been shown in this figure. The vehicle and single agent controls also need to be reported here and quantification of senescence performed.

We have added supplementary figure 1 showing the baseline, single agent and double agent senescence results for all 4 melanoma cell lines. Presence/absence of senescence has been reported as quantification was not accurate due to low staining levels in small numbers of cells. In the future, we will aim to use a more quantitative assay for senescence.

6. Figure 6B. As for Fig 5B it is important to show all controls (vehicle and single agent).

Figure 6 has been edited to include all controls and single agent treatments.

7. Figure 6. Given the effect of XPC knockdown on apoptosis and cell viability is modest, despite robust attenuation of XPC induction, the effect of exogenous expression of XPC expression would be useful to clarify if the effects that are being observed are indeed related to XPC expression. Given the Sk-mel-28 line was the only line in which decitabine did not induce XPC and was refractory to increased apoptosis in response to decitabine/carboplatin treatment, it would seem like a good line to chose for such an experiment.

This is a good suggestion and we have addressed this shortcoming of the study in the discussion with the addition of the reviewer’s comment:
The importance of XPC in the response to combined treatment was investigated. When XPC was knocked down with siRNA during decitabine treatment minor changes were seen in apoptosis and proliferation. Given the effect of XPC knockdown on apoptosis was minor, despite robust attenuation of XPC, the effect of exogenous overexpression of XPC expression is required to further clarify if the effects that are being observed are indeed related to XPC expression.

General comment: As GG-NER is also pivotal to the repair of UV induced DNA lesions have the authors tested the effect of decitabine treatment of the response and resolution of UV induced CPD's and/or 6-4-photoproducts? The inclusion of such data would strengthen the argument that the increased sensitivity of the cells to DNA damage was directly related to restored NER capacity.

This is an excellent suggestion and we will endeavor to assess repair of UV induced lesions in a future study. It was not performed for this study as the focus was on investigating decitabine to reinstate sensitivity to carboplatin as a potential new treatment combination. We have previously published 2 papers describing reduced repair of UVA and UVB induced CPDs and 6-4PPs in relation to NER deficiency in melanoma (Murray, 2016 and Budden, 2016), the suggested study would be an excellent confirmation study of both the current paper under review and our previous work.

General comment: MITF is central to melanoma proliferation and viability and even the DNA damage response. Was the expression of MITF examined to preclude it from being responsible for the carboplatin sensitisation observed in figure 4, given that XPC siRNA knockdown in Figure 6 only had a minor effect on the apoptotic response?

This is a very good comment, although MITF was not assessed in this study we have added mention of the potential role it may play in the results of this study.

“The melanocyte-lineage specific master regulator, MITF may additionally contribute to the apoptotic response to decitabine and carboplatin. Although this requires further investigation, MITF is involved in melanoma proliferation and survival (reviewed in [53]) and has been associated with DNA repair in melanoma [54]. Regardless of the role of the XPC response and its importance, here we have identified a potential combination treatment in melanoma.”

We have a follow-up manuscript in preparation where we assessed demethylation across the genome of 16 melanoma cell lines after decitabine treatment. The reviewer raises a very good point and we will investigate MITF expression in the larger cell line study.