nature portfolio

Peer Review File

PARP inhibitors enhance reovirus-mediated cell killing through the death-inducing signaling complex (DISC) with an associated NF-kB-regulated immune response.

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors describe a novel synergistic interaction between Reovirus RT3D and PARP inhibitor talazoparib that is independent of HR deficiency or DDR modulation, but due to talazoparib effects on dsRNA sensing and extrinsic apoptosis pathways. The finding that the activation of PARP1 by RT3D and its inhibition by talazoparib is phenocopied by dsRNA agonists and RIG-I signaling opens the door to the translation of dsRNA or RIG-I agonists in combination with talazoparib or other PARP inhibitors. The study examines in depth the multiple steps occurring in response to RT3D, or dsRNA agonists, and talazoparib, revealing interactions with the DISC complex and NFκB-mediated inflammatory signaling. These PARP interactions have not been previously characterized. Evaluation of combination efficacy in an immunocompetent mouse melanoma model demonstrated significant extension of mouse survival, protection from tumor rechallenge, and modulation of the immune TME.
The quality of the data is very sound and thorough, appropriate techniques were used, and the results strongly support the conclusions. This manuscript presents a new combination strategy (RT3D synergizing with talazoparib) to treat melanoma and likely other solid tumors. The comprehensive examination of the mechanism of action revealed the efficacy of dsRNA agonists in place of RT3D, providing a potentially more clinically relevant combination strategy. These results are very significant and will be of interest to those in the fields of cancer experimental therapeutics and immunotherapy, and oncolytic viruses.

Specific Comments.
1. Olaparib was in the screen; what was its Z-score (Fig 1A)? If the effect of talazoparib is phenocopied by PARP1-/- (Fig 2), would any PARP1 inhibitor work?

2. Very significant combination effect in vivo (Fig 1F, G). It is unfortunate that the experiment was terminated at day 90 so 'cures' couldn't be identified. Did any mice in the combination group (from F, it looks like slow tumor growth may be occurring) or the 2 remaining mice in the RT3D group have tumors at sacrifice?

3. ln154, either present data or don't include in Results.

4. Fig. S5C does not show a difference in μ1c expression with talazoparib (ln182); however, it looks like there is an effect on σ3.

5. If the modest activity of veliparib compared to talazoparib or oliparib (ie., Fig S9) is due to trapping (veliparib has low trapping), what about other PARP inhibitors with varied trapping activity? If PARP1 trapping is the dominant mechanism of inhibition (ln473), would veliparib + RIG-I siRNA phenocopy talazoparib + SC siRNA?

6. What was the tumor size for surviving mice at sacrifice at the end of the experiment in Fig 6C? Was RT3D significantly different from vehicle? Why was this experiment and in Fig 1 terminated on day 90? Fig 6F and G are not described in Results (not FACS analysis; ln390).

7. It would improve the manuscript to include some in vitro data with the mouse 4434 melanoma cells to demonstrate that the

RT3D - talazoparib combination is acting in a similar fashion as in the human cancer cells. For example, RT3D is much more effective in vivo in the mouse tumors (Fig 6B, C) than in human tumors (Fig 1F, G). Is this due to different apoptosis pahways, virus replication, etc?

8. Data on trapping (ln478) should be shown. This is important in supporting the hypothesis that inhibitor trapping is a key function of PARP inhibition in combination therapy.

9. Fig 7 is not very clear about the pathways affected by combination therapy, or as a consequence of PARP inhibition, for example, interactions between RIG-I and PARP1. Almost all the illustrated proteins are elevated by combination therapy (except for PARP1), thus indicating 'High' expression is not that informative, as opposed to illustrating the effects on signaling pathways.

Minor issues
Be consistent with naming, use talazoparib throughout and not BMN 673 in some places (ie., Fig 1 legend), and similarly for other names.

ln 118. insert (Fig. S1A)

Fig 1B. Should be 0.005, 0.05, 0.5 M.

Fig. 1E. How were PI+ cells quantified, counts in a field (like in D), or percentage from image cytometer?

ln 140. In Fig S2, C is not indicated.

ln153. Fig. 1 doesn't include data for other oncolytic viruses.

ln314. Shouldn't it read "...36 hours post-infection" (as in Fig) instead of "...prior to cell death"?

ln317. Synergy ("synergistic interaction") isn't measured.

ln322/Fig S9B. Should be Fig S9C and ln326 should be Fig S9B.

Fig S9. There is no mention of Fig S9D in the text.

Reviewer #2

(Remarks to the Author)
I congratulate the authors on this nice work. It has shown the novelty of the combination of PARPi with RT3D (dsRNA sensing) that led to the suppression of PARylation of DISC leading to cell death.

The authors showed very eloquently the mechanism of action of the combination both in vitro and in vivo , and ruled out the commonly thought synergism based on increased DNA damage or increased viral replication leading to cell death.

This concept clearly open many translational ideas to the field of melanoma treatment with viral and non-viral dsRNA sensing compounds.

Reviewer #3

(Remarks to the Author)
Currie and team have done a commendable amount of work. The use of PARP inhibitors in combination with oncolytic viral therapy is of great interest to the scientific community at the moment. The work detailed in the manuscript is comprehensive and provides a very thorough overview of the synergistic mechanism of action of talozoparib and RT3D in the context of cancer. However, given the expansive amount of work, the focus of the discovery tends to shift while reading the paper. I would highly suggest to re-group the main discoveries of this project and present the work in a more concise fashion. For example, pick the 3 most important pathways from the proteomics analysis that you have worked on (apoptotic proteins, RIG-1 pathway and NFkB pathway) and demonstrate that in one figure. Showing the proteomic analysis 3 times in 3 different figures, pointing to a different target (albeit that they are associated to each other) is driving the focus away from the main discovery of the project. Start off with why and how you chose to hypothesize that cell kill as a result of RT3D and talazoparib may be associated with RIG-1 and NFkB.

Here is the list of corrections:
1) Cite reference in line 82. Reference for unsuccessful attempts?
2) Supplementary table 1, explain what is + and -. What do the green and pink colors indicate? Move line 116-122 to methods and rephase the results section. Supplementary figure 1 A, “infected” is misspelled.
3) Supplementary table 2, what are the numbers in red. Add legend to show red indicates high cell kill. Supplementary figure 2, color the bars not just the standard deviation bars.
4) Keep the concentrations of drugs consistent in all the figures. Either pick micromolars or nanomaolars. Use the same decimal places in all graphs such as MOI of 0.1 or 0.1000

5) SRB in shorthand. Expand the first time in brackets you use shorthand.
6) Figure1A, instead of showing the numbers in red, indicate the drugs. In figure 1B, color the bars, change y axis to either log of cell survival or % of cell survival or fold change. Check consistency of drug concentrations. What is the vehicle, DMSO? Indicate DMSO or whatever the vehicle was. Indicate the concentration of talazoparib in 1C. In 1F, color the points in the same color that the line is colored (example orange line have orange points of standard deviation). 1E, y axis is % of dead cells.
7) Line 148: Remove entire section. It does not add much to the data and is not well explained. PARP western blots are not mentioned. Remove section all together.
8) Re-check the conclusion made on figure S4A (line 162-166). Is COMET assay explained somewhere in the methods?
9) Section "synthetic lethality is not mediated by enhanced viral replication" can be removed all together. Re-check the conclusions drawn from the western blots (line 181-183). Conclusions don't match the westerns. Integrate figure S5A,B into the previous section.
10) Figure 2D, missing significance bars. Change y axis to % survival or log or fold change. Rephrase what is shown in 2E,F (line 199-201). What is the vehicle in 2F? Explain why it is expected that the vehicle treated in G3 and G9 will have fewer cells that vehicle treated of PARP+/+. 2G seems to have the right format for plotting bar graphs. The bars are colored, and the standard deviation is in black. Use this format for all the bar graphs.
11) Figure 3A, change bars to colors, not enough variations of grey to distinguish the bars. Caspase3, cFLP and c-IAP1 is not needed. Limit it to caspase8 and PAR. Introduction to proteomic data for the first time in 3F. Include RIG-1 and NFkB data here as well. Explain the 3 pathways you chose to focus from the proteomic data. 3H bar graphs have 4 distinct color for each condition. Use the same colors for the same conditions throughout the manuscript. Use this graph to format all the other graphs. It does not make sense to show 24 and 48h time point in 3K. You do not show the time point in 3J. It raises unnecessary questions. Just show 36h time point assuming that the lysates used in J were also from 36h time point. Where is the loading control for J and K?
12) Remove section "RT3D plus talazoparib-induced cell death is mediated by TRAIL and TNF". It is great that you show the association of TRAIL and TNF but it is not necessary since you describe the DISC components in detail in the previous sections. Having this section does not add too much but shifts the focus of the paper.
13) The results from figure 4A and B may be an over interpretation. Cytokine levels are unaltered by siRNA of RIPK1, CASp8 and FADD. They are only affected when treated with talazoparib or a combination of RT3D+talazoparib to begin with. Re-interpret the results and rephrase the section. Change bar graphs in 4B to colored bars as in the previous figure. Keep the color scheme consistent across the manuscript. Combine 4C with proteomic data shown in the previous section. Supplementary figure 9B and 9C have been interchanged, they don't match the wording (line 320-326). Supplementary figure 9D is not accounted for in the text. You have to talk about all the figures. Modify color scheme in supplementary figure 10. Which cells were used for gene silencing? Mention in the results section. Which cells were co-treated in figure S10B? Was it the WT cells or those with silencing of caspase-8, RIPK1 and FADD.
14) Combine proteomics results from figure 5A in one figure. Consolidate all the proteomics data in one figure.
15) Change the standard of deviation points in figure 6B to the same color as the line joining the points. Text from line 390-396 does not match the figures. Wording for figure 6F and 6G is missing. There is no increase in foxp3 upon RT3D injection, recheck wording in line 392. Re-assess figure 6I.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors comprehensively responded to the Reviewer's comments and revised the manuscript.

Reviewer #3

(Remarks to the Author)
I am pleased to see a significant improvement in the manuscript. I applaud the team on addressing all the comments and making changes accordingly. I want to congratulate the authors on the excellent work that have done. I have some minor comments that are mostly editing errors.
1) Line 194: shown by western blot and PAR ELISA and was also manifest in vivo. Should be "manifested ".
2) I like that the color scheme is same in all the figures. Can you color the shapes according to the color on the bars of the graphs. Circles – purple, squares -blue, triangles-red. Just as you show in 5B
3) Is it possible that the label on figure 4E, that is PARP1 +/+ is interchanges with PARP1G9-/-? Should the left panel of blots be PARP1G9-/-?
4) In figure S9, either follow the labelling for x axis in A or B. I think label for A looks neater. Can you label B the same way? Can you assign p values between the bar graphs and denote them with a * on the graph? There is a lot of jargon to describe these figures. Such as "more profound effect", or " increased but not to the same extent". Please calculate the significance and describe in the text if the differences are significant or non-significant. Between D and E, call it uninfected or RT3D of 0. In S9E, Talazoparib and olaparib have similar expression of cleaved casp and PARP in D04 and MeWo. It is only in A375 and Mel64 that the expression of these proteins is higher with talazoparib treatment. It could be worthwhile to conclude that the PARP inhibitors have similar effect albeit there could be a dependency on the cell line (given that all cell lines are not exactly genetically uniform).
5) Please unify color scheme in figure 5 C, E and F.

6) In figure 6C, is RIG-I not supposed to show a band under all conditions of the SC siRNA? There is a band only when treated with RT3D and talazoparib.

Response to reviewers

We would like to thank all the reviewers for taking their time to read this manuscript and all their positive comments, ideas/suggestions as well as detailed feedback on our manuscript entitled: ***PARP inhibitors enhance reovirus-mediated cell killing through the death-inducing signaling complex (DISC) with an associated NF-κB-regulated immune response.*** *We have added additional figures in the supplementary data, and these additions are highlighted in the rebuttal. For ease of reference, we have also included the line numbers in the manuscript. Furthermore, we have moved all the proteomics data and collated it into one figure as sensibly requested by reviewer 3 and this has meant all the main figures in the manuscript have been reordered and renumbered. Again, this is explained in the rebuttal. We hope that this additional data we provide, and our detailed explanations will be satisfactory and that our manuscript can now be accepted for publication.*

Reviewer 1:

The authors describe a novel synergistic interaction between Reovirus RT3D and PARP inhibitor talazoparib that is independent of HR deficiency or DDR modulation, but due to talazoparib effects on dsRNA sensing and extrinsic apoptosis pathways. The finding that the activation of PARP1 by RT3D and its inhibition by talazoparib is phenocopied by dsRNA agonists and RIG-I signaling opens the door to the translation of dsRNA or RIG-I agonists in combination with talazoparib or other PARP inhibitors. The study examines in depth the multiple steps occurring in response to RT3D, or dsRNA agonists, and talazoparib, revealing interactions with the DISC complex and NFκB-mediated inflammatory signaling. These PARP interactions have not been previously characterized. Evaluation of combination efficacy in an immunocompetent mouse melanoma model demonstrated significant extension of mouse survival, protection from tumor rechallenge, and modulation of the immune TME.

The quality of the data is very sound and thorough, appropriate techniques were used, and the results strongly support the conclusions. This manuscript presents a new combination strategy (RT3D synergizing with talazoparib) to treat melanoma and likely other solid tumors. The comprehensive examination of the mechanism of action revealed the efficacy of dsRNA agonists in place of RT3D, providing a potentially more clinically relevant combination strategy. These results are very significant and will be of interest to those in the fields of cancer experimental therapeutics and immunotherapy, and oncolytic viruses.

Specific Comments.

1. Olaparib was in the screen; what was its Z-score (Fig 1A)? If the effect of talazoparib is phenocopied by PARP1-/- (Fig 2), would any PARP1 inhibitor work?

Response: The Z-scores for olaparib have been included in ***Supp Table 2B*** *(as shown below). The additional text in the manuscript now reads as follows:*

*Olaparib, another approved PARP-1/2 inhibitor was found to have a modest effect in terms of sensitisation to RT3D (Supplementary table 2B) compared to talazoparib. This may be due to talazoparib being a more effective PARP inhibitor (and may also reflects its greater PARP trapping capabilities) as will be discussed later in the manuscript. (****Results, line 123-127****).*

A.

	DE RT3D 0.01	DE RT3D 0.1	DE RT3D 0.5	DE RT3D 1.0	DE RT3D 5.0
Talazoparib (1 ! M)	-1.480456	**-4.651062**	**-7.104825**	**-10.243770**	**-9.015051**
Talazoparib (0.5 ! M)	-0.101188	**-4.033217**	**-5.041120**	**-7.273089**	**-8.148768**
Talazoparib (0.1 ! M)	0.089756	**-2.116922**	**-3.520491**	**-6.077790**	**-4.810190**
Talazoparib (0.05 ! M)	**-2.249301**	**-7.746680**	**-2.405891**	**-5.229588**	**-5.135364**
Talazoparib (0.01 ! M)	-0.604822	0.753134	**-2.710854**	-0.389711	**-2.074762**
Talazoparib (0.005 ! M)	-0.070781	1.025049	-0.361069	-0.417371	-1.128146
Talazioarub (0.001 ! M)	-0.659405	-1.027792	1.011949	0.092874	-0.552127
Talazoparib (0.0005 ! M)	0.121230	0.120058	-0.017374	0.144596	-1.319600

B.

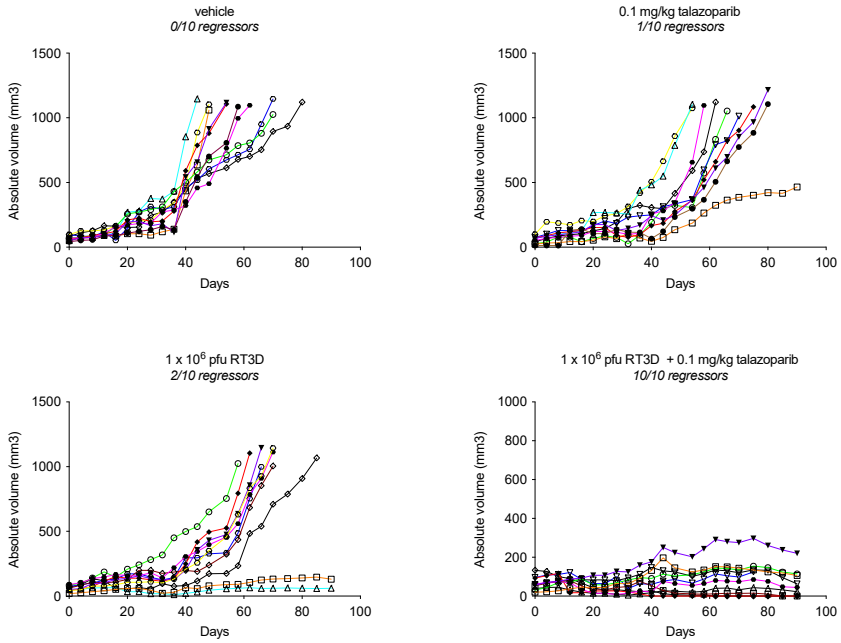
	DE RT3D 0.01	DE RT3D 0.1	DE RT3D 0.5	DE RT3D 1.0	DE RT3D 5.0
Olaparib (1 ! M)	0.402780	0.188810	-2.155600	-0.783270	0.580560
Olaparib (0.5 ! M)	-0.066660	-0.772010	-1.024420	-1.439050	-1.040350
Olaparib (0.1 ! M)	**-4.011290**	0.393870	**-2.278740**	-0.995440	-0.691920
Olaparib (0.05 ! M)	0.814240	1.110130	0.029930	0.194410	-0.271190
Olaparib (0.01 ! M)	-0.677620	-0.644460	**-3.651840**	-0.844930	0.999970
Olaparib (0.005 ! M)	0.382610	-0.225170	-1.562470	0.805680	-0.068510
Olaparib (0.001 ! M)	-0.448120	-0.430850	0.719560	0.262050	0.470130
Olaparib (0.0005 ! M)	0.361290	1.865040	-0.460030	0.787830	1.418870

Supplementary Table 2: Drug effect (DE) z score of RT3D and talazoparib (A) or RT3D and olaparib (B) with values <-2 being considered a profound sensitisation effect (highlighted in red).

*It is important to be very clear, however, that the nuclear PARP-trapping that we document in the manuscript is NOT the mechanistic basis of the co-operative interaction between RT3D and PARP inhibition that we have discovered. Our data show that RIG-I and PARP protein associate with one another, but this is not a nuclear event. Our data are strongly suggestive of the possibility that PARP inhibitors that are "PARP-trappers" mediate a stronger biological interaction between RIG-I and PARP protein, but we stop short of claiming that talazoparib is most effective because it locks PARP protein onto RIG-I. We plan to investigate this possibility in future experiments but, for now, we trust that the text that we have added (see below) will adequately cover this issue. We have included the following text in the manuscript: Next, we assessed if similar PARP-1 and RIG-1 interactions would occur following RT3D infection and co-treatment with other PARP-1 inhibitors (olaparib and veliparib) that are less potent than talazoparib. Interestingly, the interaction between PARP-1 and RIG-I appeared to be stronger and require a lower concentration of talazoparib (0.1-1μM) than was the case for olaparib, for which a concentration of 5-10 μM was required. Veliparib showed hardly any interaction between PARP-1 and RIG-I, even at 10 μM concentration (**Fig. S11E**). It is interesting to note that the relative levels of PARP inhibitor-mediated modulation of the interaction between PARP-1 and a nucleic acid-bound PRR precisely mirrors the respective potencies of talazoparib (most), olaparib (intermediate) and veliparib (least) in terms of their abilities to trap PARP on sites of DNA damage, as confirmed in **Fig. S11F**. These data align with previous reports [31, 32]. This observation raises the possibility that PARP inhibitors may have differential abilities to stabilise the PARP-1-RIG-I complex in a manner that may be analogous to PARP trapping on damaged DNA. (**Results, line 406-421**).*

2. Very significant combination effect in vivo (Fig 1F, G). It is unfortunate that the experiment was terminated at day 90 so 'cures' couldn't be identified. Did any mice in the combination group (from F, it looks like slow tumor growth may be occurring) or the 2 remaining mice in the RT3D group have tumors at sacrifice?

*Response: The individual curves from each CD1 mouse implanted with human A375 cells (n of 10) for each treatment cohort group have been included to increase clarity for the reader **(Fig S3)**. These data show that combination treatment slowed the growth of all mice and led to tumour regression of 10/10 mice. RT3D alone had 2/10 mice with growth retardation, while talazoparib alone caused regression in 1/10 mice. We sacrificed mice on Day 90 because of a cost/benefit decision to curtail animal husbandry costs and because CD1 mice are immunodeficient and, therefore, cannot be used to test for immune memory in rechallenge experiments. These details are explained in the manuscript. The added text reads as follows: Individual tumour volumes (n of 10) per treatment cohort are summarised (**Fig. S3**). **Results, line 150-151).***



Supplementary Figure 3: CD1 nude mice carrying A375 tumour xenografts showing individual size of tumours for each treatment cohort (n of 10) consisting of vehicle (10% DMAc, 6% Solutol and 84% PBS), 0.1 mg/kg talazoparib, 1x10^6 pfu RT3D or 1x10^6 pfu RT3D + 0.1 mg/kg talazoparib.

3. In154, either present data or don't include in Results.

Response: *These data have now been removed. In the previous version of the manuscript, this read: "We are currently investigating the effect of talazoparib combination with the HSV-1 DNA oncolytic viruses, and this has shown a significant synergistic effect following the loss of HSV-1 induced PARylation in the presence of talazoparib (data not shown)."*

4. Fig. S5C does not show a difference in μ1c expression with talazoparib (ln182); however, it looks like there is an effect on σ3.

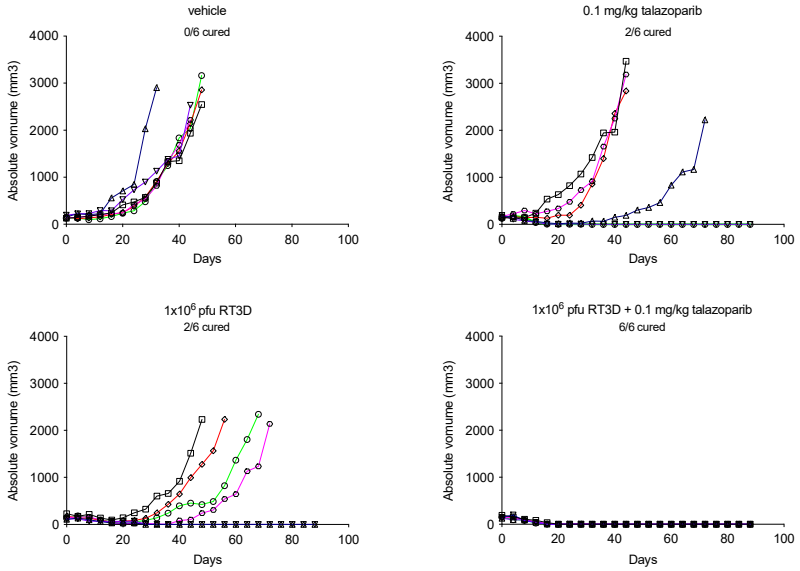
Response: *We thank the reviewer for taking highlighting this. The previous manuscript in the text (line 182-184) read: Talazoparib exposure increased μ1C production in A375 and MeWo, but not D04 cells. However, σ3 production was unaltered in all cell lines (Fig. S5C).*
*In the revised manuscript, we have changed the subtitle for this section to read "RT3D/talazoparib enhanced cell death is not mediated by enhanced viral replication" and we have removed mention of synthetic lethality in this place. In the text, we have rephrased the relevant sentences as follows: "Addition of talazoparib to RT3D infected cells did not result in any significant increase in production of intact, replication-competent RT3D in the one-step growth curve assays (Fig S5A, B). Interestingly, although μ1C protein production was unaltered in all cell lines (in keeping with the one-step growth curve data), there was a modest increase in σ3 production in A375 and MeWo, but not D04 cells (Fig S5C). This observation is likely to reflect variations in the ability of RT3D to redirect different cell lines to produce specific reoviral proteins. Nonetheless, taken together, these data clearly show that the combinatorial effect of talazoparib and RT3D cannot be explained in terms of increased viral replication (Fig. S5A, B)." (**Results, line 179-187**).*

5. If the modest activity of veliparib compared to talazoparib or olaparib (i.e. Fig S9) is due to trapping (veliparib has low trapping), what about other PARP inhibitors with varied trapping activity? If PARP1 trapping is the dominant mechanism of inhibition (ln473), would veliparib + RIG-I siRNA phenocopy talazoparib + SC siRNA?

Response: *We profiled 3 PARP inhibitors (talazoparib, olaparib and veliparib) in combination with RT3D and found similar results, albeit with varying levels of activity. We believe that the dominant reason for talazoparib being the most active agent in combination with RT3D relates to its inherently greater activity as a PARP inhibitor. Although we cannot exclude the fact that some of this greater efficacy may be due to PARP trapping, we do not have data to show that this is a dominant effect. The reviewer raises an interesting question in asking if veliparib + RIG-I siRNA would phenocopy talazoparib + SC siRNA, but we are uncertain how such experiments would directly answer the question of PARP trapping. In effect, we would be testing a combination versus a single-agent approach and, even if we showed equivalence, this might simply be due to a combinatorial efficacy. Because of these uncertainties, we have not tried to perform the requested experiments.*

6. What was the tumor size for surviving mice at sacrifice at the end of the experiment in Fig 6C? Was RT3D significantly different from vehicle? Why was this experiment and in Fig 1 terminated on day 90?

Response: *Please note that these data are now* ***Figure 7*** *due to reshuffling of figures due to collating all the proteomic data into Fig 3. The individual curves from each BL6 immunocompetent mouse implanted with mouse 4434 cells (n of 6) for each treatment cohort group have been included to increase clarity to the reader (****Fig S13****). Combination treatment slowed the growth of all mice and led to complete cure of 6/6 mice, RT3D alone cured 2/6 mice, talazoparib alone had 2/6 mice, while vehicle had no cures. We sacrificed mice on Day 90, except for the cured mice that were rechallenged, as summarised in Fig 7D. Naïve mice implanted with 4434 cells developed tumours and these continued to grow exponentially, while the cured mice from combination (6/6), RT3D alone (2/6) or talazoparib alone (2/6) groups demonstrated protection against tumour challenge. The new text in the manuscript now reads as follows: Individual tumour volumes for each mouse (n of 6) per treatment group are summarised where all mice in the combination (RT3D/talazoparib) group were cured (6/6) compared to 2/6 in the single agent counterparts (0.1 mg/kg talazoparib) or (1x10^6 pfu RT3D) as shown in the individual curves (Fig S13). Next, we assessed memory response in the cohort of mice cured by re-challenging with 4434 cells on the contralateral flank. No tumour growth occurred in this cohort of mice (whilst, in parallel, naïve mice developed large tumours over time) (Fig. 7D). (****Results, line 441-448****).*



Supplementary Figure 13: Black/6 female mice carrying 4434 tumours showing individual size of tumours for each treatment cohort (n of 6) consisting of vehicle (10% DMAc, 6% Solutol and 84% PBS), 0.1 mg/kg talazoparib, 1x10^6 pfu RT3D or 1x10^6 pfu RT3D + 0.1 mg/kg talazoparib.

Fig 6F and G are not described in Results (not FACS analysis; ln390).

*Response: We thank the reviewer for bringing this to our attention This was an oversight and has now been rectified. Please note that the figures are now Fig 7F and 7G due to collating all the proteomic data into Fig 3. The text in the manuscript now reads as follows: In support of these findings, FACS analysis of in vivo tumour samples was carried out. Tumour volumes of mice used to profile the immune infiltrate by FACS analysis were measured up to Day 12 (Fig. 7F) and their weights measured prior to profiling the immune infiltrate by FACS analysis at time of harvest (Fig. 7G). (**Results, line 457-460**).*

7. It would improve the manuscript to include some in vitro data with the mouse 4434 melanoma cells to demonstrate that the RT3D - talazoparib combination is acting in a similar fashion as in the human cancer cells. For example, RT3D is much more effective in vivo in the mouse tumors (Fig 6B, C) than in human tumors (Fig 1F, G). Is this due to different apoptosis pathways, virus replication, etc?

*Response: We appreciate this suggestion and, as a result, these data have now been included and are summarised in **Fig S12**. The text in the manuscript reads as follows: We firstly determined the effect of the RT3D plus talazoparib combination in vitro in 4434 BRAF-mutant mouse melanoma cells to assess whether it was similar to that seen for the human cell lines. Indeed, addition of talazoparib enhanced the effect of RT3D as shown by MTT cell viability assay (**Fig. S12A**). Western blotting confirmed an increase in caspase-8, caspase-3 and PARP cleavage in response to RT3D/talazoparib compared to either agent alone (**Fig. S12B**). Furthermore, RT3D plus talazoparib-induced IFN-β secretion (**Fig. S12D**) from supernatants collected from cells that were used to analyse SRB cell viability staining (**Fig. S12C**) and the increase in IFN-β correlated with an increase in dying cells. (**Results, line 426- 435**).*

To answer the reviewer's question as to whether RT3D is much more effective in vivo in the mouse tumors (Fig 6B, C, now Fig 7B C) than in human tumors (Fig 1F, G), we hypothesize that mouse tumours, which are grown in a BL6 immunocompetent mouse background, will have a more favourable tumor microenvironment enriched with immune cells that are lacking in the CD1 mice in which the A375 xenograft cell lines are grown. Indeed, there were an increase in tumour-infiltrating immune cells in the microenvironment following RT3D/talazoparib treatment (Fig 7E) as measured by increased expression of transcripts associated with immune subsets. Additionally, when we assessed memory response in the cohort of mice cured by re-challenging with 4434 cells on the contralateral flank, no tumour growth occurred in this cohort of mice (whilst, in parallel, naïve mice developed large tumours over time) (Fig. 7D).

8. Data on trapping (ln478) should be shown. This is important in supporting the hypothesis that inhibitor trapping is a key function of PARP inhibition in combination therapy.

*Response: We apologise to the reviewer for having made this matter rather difficult to follow in the manuscript. In the amended version, we have tried to be very clear on the fact that the effect of RT3D and PARP inhibition is mediated through the interaction between RIG-I and PARP-1. The relative ability of the individual PARP inhibitors to promote the interaction between RIG-I and PARP-1 and to drive the resulting effects on apoptosis and inflammation precisely mirror the relative PARP-trapping potencies of the respective drugs. Thus, the effects are seen most strongly with talazoparib, then olaparib and, finally, more weakly with veliparib. We confirmed in our studies (**Fig S11F**) that the 3 PARP inhibitors are capable of trapping PARP enzyme on sites of damaged DNA in the same rank order (talazoparib, olaparib, veliparib) that we document for their effects on the RIG-I-PARP-1 interaction. We suggest, both in the Results section and the Discussion, that this phenomenon may be seen as analogous to PARP-trapping … but we wish to be completely clear that we are NOT claiming that PARP-trapping on damaged DNA explains the mechanistic basis for the biology we have discovered. We hope that the following text that is now in the manuscript makes these points very clearly.*

This may be due to talazoparib being a more effective PARP inhibitor (and may also reflect its greater PARP trapping capabilities) as will be discussed later in the manuscript. ***(Results, line 125-127****).*

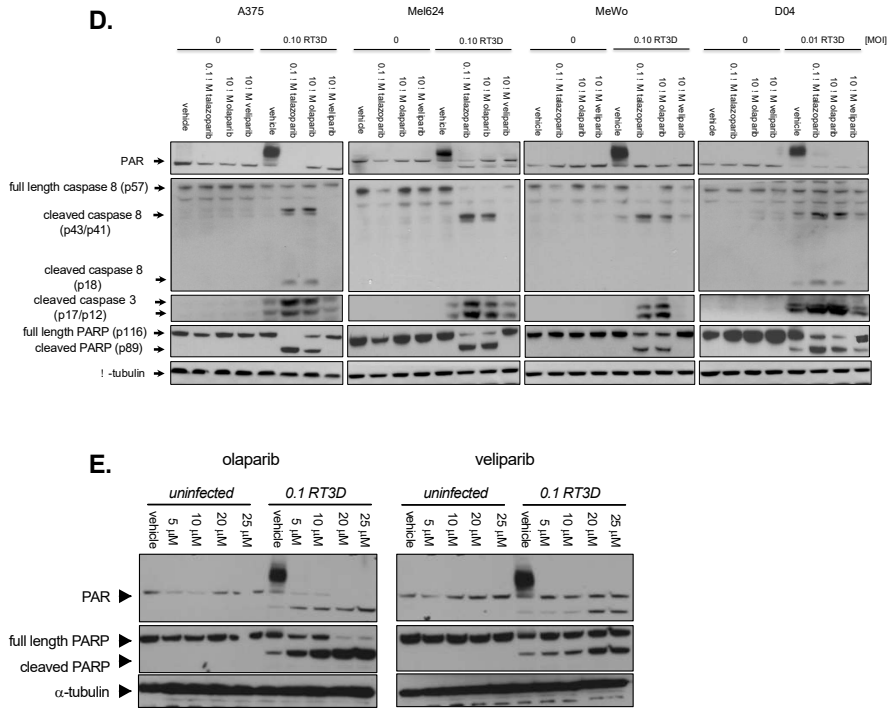
*Given our findings that RNA sensor agonists can trigger PARylation, and since PARP-1 has nucleic acid binding capabilities, we investigated whether PARP-1 interacts directly with RNA sensors, such as RIG-I, using a PARP-1-Trap agarose assay. Our data demonstrated an increase in PARP-1 and RIG-I interaction following RT3D infection and this was increased further by talazoparib treatment (****Fig. 6E****). Next, we assessed if similar PARP-1 and RIG-1 interactions would occur following RT3D infection and co-treatment with other PARP-1 inhibitors (olaparib and veliparib) that are less potent than talazoparib. Interestingly, the interaction between PARP-1 and RIG-I appeared to be stronger and require a lower concentration of talazoparib (0.1-1 μM) than was the case for olaparib, for which a concentration of 5-10 μM was required. Veliparib showed hardly any interaction between PARP-1 and RIG-I, even at 10 μM concentration (****Fig. S11E****). (****Results, line 402-413****).*

It is interesting to note that the relative levels of PARP inhibitor-mediated modulation of the interaction between PARP-1 and a nucleic acid-bound PRR precisely mirrors the respective potencies of talazoparib (most), olaparib (intermediate) and veliparib (least) in terms of their abilities to trap PARP on sites of DNA damage, as confirmed in ***Fig. S11F****. These data align with previous reports [31, 32]. This observation raises the possibility that PARP inhibitors may have differential abilities to stabilise the PARP-1-RIG-I complex in a manner that may be analogous to PARP trapping on damaged DNA. (****Results, line 414-421****).*

*The dsRNA sensor, RIG-I, has previously been shown to promote apoptosis and cell death in hepatocellular carcinoma (HCC) whilst activating NF-κB in macrophages[35]. Combination of RT3D plus talazoparib significantly increased RIG-I expression and massively upregulated RIG-I pathway components at the protein level, findings that correlated with the loss of PARylation. Our data point to a novel interaction between RIG-1 and PARP-1, which is amplified following RT3D plus talazoparib treatment compared to either agent alone. We posit that, following binding to dsRNA (or its mimics), the dsRNA sensor, RIG-I, can bind to and activate PARP-1 and, thus, simultaneously inhibit extrinsic apoptosis and serve as a platform for inflammatory NF-kB signalling. We further propose that combination therapy with PARP inhibition effectively removes this PARylation-driven block on apoptosis but also functionally "traps" PARP-1 on dsRNA-bound RIG-I, in an analogous fashion to that described for PARP-trapping on DNA[31, 32, 36, 37]. This leads to a phenotype of increased cancer cell death with enhanced cytokine production. Indeed, talazoparib has been shown to be a potent PARP-trapper on DNA, superior to either olaparib or veliparib (as confirmed in our own analyses). We confirmed talazoparib as a more effective and potent PARP-trapper on damaged DNA than either olaparib or veliparib, which correlated with greater efficacy of talazoparib in terms of triggering apoptosis, NF-κB signalling, cytokine production and RIG-I/PARP1 interaction. (****Discussion, lines 541-559****).*

*The data are presented below. We have now included a western blot (****Fig S9E****) to emphasise that both olaparib and veliparib enhance RT3D killing, as does talazoparib albeit at different concentrations.*

*The text in the manuscript now reads as follows: "Additionally, we found talazoparib (0.1 μM) led to enhanced apoptotic markers (caspase-8, caspase-3 and PARP cleavage) when compared to olaparib or veliparib, which were given at a higher concentration of 10 μM (Fig. S9D). Olaparib appeared to be more effective in enhancing RT3D-induced apoptosis compared to veliparib, as shown in the apoptotic markers by Western blot analysis. We found a higher dose of veliparib 20-25 μM was necessary to see a similar effect to that shown with olaparib at 5-10 μM (Fig. S9E). (****Results, line 352-359****).*



Supplementary Figure 9: D. *A375, Mel624, MeWo and D04 cells were treated with 0.1 µM talazoparib, 10 µM olaparib or 10 µM veliparib and thereafter infected with RT3D [MOI of 0.1] for 48 hours. Western analysis was carried out to assess PAR expression, caspase 8 and PARP cleavage. Equal loading of proteins was assessed by probing for α-tubulin.* ***E.*** *A375 cells were treated with a concentration range of olaparib or veliparib (5-25 µM) and thereafter infected with RT3D [MOI of 0.10] for 48 hours. Western analysis was carried out to assess PAR expression and PARP cleavage. Equal loading of proteins was assessed by probing for α-tubulin.*

*In addition to showing RIG-I and PARP-1 interaction following RT3D and talazoparib treatment (**Fig 6E**), we have now included supplementary data (**Fig S11E**) to show that RIG-I and PARP-1 interaction following RT3D infection is increased following olaparib and veliparib treatment (albeit at higher doses), as we previously described in the manuscript with talazoparib as shown below.*

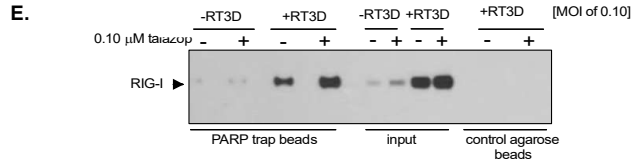
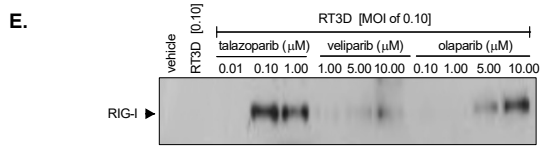
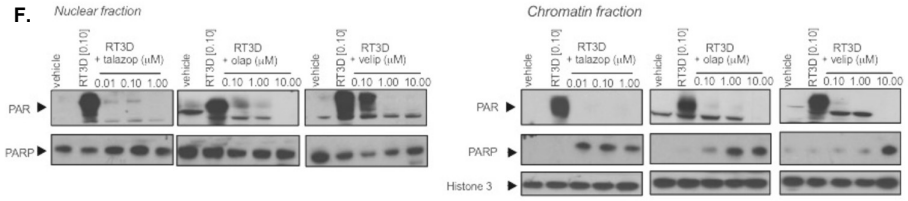


Fig 6E*: Talazoparib enhances RT3D-induced interferon signaling through RIG-I. A375 cells were treated with talazoparib at 0.1 µM and RT3D [MOI of 0.1]. PARP-trap agarose IP was performed on the lysates and Western analysis carried out to assess interaction between PARP-1 and RIG-I.*



Supplementary Figure 11E*: A375 cells were treated with RT3D [MOI of 0.1] plus talazoparib (0.01, 0.1 and 1 µM), veliparib (1, 5 and 10 µM) or olaparib (0.1, 1, 5 and 10 µM). A PARP trap agarose assay was carried out to assess PARP-1 and RIG-I interaction by IP. Western analysis was carried out to assess interaction between PARP-1 and RIG-I.*

We also found talazoparib to be a more effective and potent PARP-trapper on damaged DNA than either olaparib or veliparib as shown in Fig S11F and have included this as requested by the reviewer.



Supplementary Figure 11F. *A375 cells were treated with RT3D [MOI of 0.1] plus talazoparib (0.01, 0.1 and 1 μM), olaparib or veliparib (0.10, 1 and 10 μM). Differential trapping of PARP1-DNA complexes was determined by Western blot analysis of nuclear and chromatin-bound fractions. Blots were probed for PAR and PARP for both nuclear and chromatin fractions. Histone H3 was used as a positive marker for chromatin bound fractions and as a loading control).*

9. Fig 7 (**now Fig 3E**) is not very clear about the pathways affected by combination therapy, or as a consequence of PARP inhibition, for example, interactions between RIG-I and PARP1. Almost all the illustrated proteins are elevated by combination therapy (except for PARP1), thus indicating 'High' expression is not that informative, as opposed to illustrating the effects on signaling pathways.

*<u>Response:</u> With respect, we disagree with this view. For us, the consistency of the changes across a host of proteins serving similar cellular functions really makes the point that the biological effects of RT3D/PARPi are extremely powerful and co-ordinated. We used STRING analysis to obtain the data **[now Fig 3E]**. Briefly, we focused on proteins upregulated by RT3D compared to vehicle. Thereafter, we looked at proteins that were even more upregulated by the addition of talazoparib to RT3D (combination) and built a network using STRING based on high confidence molecular interactions and clustered them by using the MCL algorithm. This methodology was one of the ways in which the top key pathways (RIG-I, NF-κB and apoptotic proteins) were highlighted to us. Subsequently, we hypothesised that upregulation of these pathways was a critical component in enhancing RT3D/talazoparib-induced cell death. The remarkable interaction between RIG-I and PARP1, for example, was evaluated in detail precisely due to the discovery of the RIG-I pathway being upregulated when RT3D/talazoparib were used in combination compared to either agent alone.*

Reviewer 2:

I congratulate the authors on this nice work. It has shown the novelty of the combination of PARPi with RT3D (dsRNA sensing) that led to the suppression of PARylation of DISC leading to cell death.

The authors showed very eloquently the mechanism of action of the combination both in vitro and in vivo , and ruled out the commonly thought synergism based on increased DNA damage or increased viral replication leading to cell death.

This concept clearly opens many translational ideas to the field of melanoma treatment with viral and non-viral dsRNA sensing compounds.

Response: *We would like to thank Reviewer 2 for his/her very encouraging and positive comments on this manuscript. We agree and are hopeful that these findings will lead to future translational research in the field of melanoma (and perhaps TNBC) in combining PARP inhibitors and dsRNA-sensing compounds.*

Reviewer 3:

Currie and team have done a commendable amount of work. The use of PARP inhibitors in combination with oncolytic viral therapy is of great interest to the scientific community at the moment. The work detailed in the manuscript is comprehensive and provides a very thorough overview of the synergistic mechanism of action of talazoparib and RT3D in the context of cancer. However, given the expansive amount of work, the focus of the discovery tends to shift while reading the paper. I would highly suggest to re-group the main discoveries of this project and present the work in a more concise fashion. For example, pick the 3 most important pathways from the proteomics analysis that you have worked on (apoptotic proteins, RIG-1 pathway and NF-κB pathway) and demonstrate that in one figure. Showing the proteomic analysis 3 times in 3 different figures, pointing to a different target (albeit that they are associated to each other) is driving the focus away from the main discovery of the project. Start off with why and how you chose to hypothesize that cell kill as a result of RT3D and talazoparib may be associated with RIG-1 and NF-κB. What is the biology?

Response: This observation has been well noted and acknowledged fully by the authors. We have now collated all the proteomic data into one figure (Fig 3), and this has been explained in the manuscript. The sub-heading for this section now reads: **PARP-1 regulates PARylation induced by RT3D infection and enhances death-inducing signalling complex (DISC)-mediated apoptosis.** (***Results, line 222-223).*** The *findings are explained in detail* ***(Results, line 223-249)*** and reads as follows:

*To further investigate the mechanistic interaction between RT3D and talazoparib, we analysed the proteome in a non-biased way by mass spectrometry. Briefly, we clustered the proteins into expression profiles across the four treatment groups (vehicle, 0.1 μM talazoparib, RT3D [MOI of 0.1] or combination and then carried out pathway enrichment analysis on each cluster. Proteins were clustered into expression profiles across our treatment cohort groups: vehicle, 0.1 μM talazoparib, RT3D [MOI of 0.1] or combination and then pathway enrichment analysis was carried out on each cluster. We identified the top three pathways that were enriched in clusters in which the highest expression was seen with combination treatment and the lowest expression in untreated cells, with the RT3D or talazoparib single-agent treatments showing intermediate expression, higher than untreated but lower than the combination treatment (**Fig 3A**). Proteomic analyses following RT3D plus talazoparib highlight profound increases in the RIG-I (**Fig 3B**), NF-κB (**Fig 3C**) and apoptotic pathways associated with cell-surface death domains, in particular the death-inducing signaling complex (DISC), which includes Caspase-8 and RIPK1 (**Fig 3D**).*

*In addition, we analyzed our data using STRING and a summary of significant proteins following RT3D/talazoparib treatment is highlighted as a graphical abstract (**Fig 3E**). Briefly, we focused on proteins upregulated by RT3D compared to vehicle. Thereafter, we looked at proteins that were even further upregulated by the addition of talazoparib to RT3D (combination). We then built a network using STRING based on high-confidence molecular interactions and clustered them by using the MCL algorithm.*

The remainder of this report focuses on these 3 key pathways and dissects the mechanisms through which they play a role in enhancing RT3D/talazoparib-induced cell death.

We trust that this represents an improvement in data presentation.

Main Comments:

1.Cite reference in line 82. Reference for unsuccessful attempts?

Response: We have added references for studies of oncolytic viruses combined with surgery, chemotherapy, radiotherapy and immunotherapy. We have also amended the text to indicate that these studies have not resulted in new registrations or change of practice, as follows: "As yet, attempts to augment their efficacy and clinical relevance through combination with standard-of-care therapies, including surgery, chemotherapy, radiotherapy and immune checkpoint blockers have been largely unsuccessful[6-13] and have not led to new registrations or change of practice." (***Introduction, line 80-84***).

2. Supplementary table 1, explain what is + and -. What do the green and pink colors indicate? Move line 116-122 to methods and rephase the results section. Supplementary figure 1A, “infected” is misspelled.

Response: Line 116-122 (older version of the manuscript) has now been moved to the methods section and incorporated into the Drug Screen and Cell Titer Glo (CTG) assay methods section (***Methods, line 602-611***) as requested. *The spelling of infected in Supplementary figure 1A has been corrected. To clarify, (-) sign indicates vehicle (DMSO) while (+) sign indicates a positive control staurosporine (0.5 μM) known to cause increased cell death in cancer cells. The green and pink colors were a color theme used from excel and had no significant meaning. The table has now been amended to avoid any confusion to readers.*

3. Supplementary table 2, what are the numbers in red. Add legend to show red indicates high cell kill. Supplementary figure 2, color the bars not just the standard deviation bars.

Response: The red numbers represent the Drug Effect (DE) z score of RT3D and talazoparib (Supplementary table 2A) or RT3D and olaparib (Supplementary table 2B) with values of less than -2 being considered a profound sensitisation effect. These numbers correlate with the red dots in the waterfall plot (Fig 1A) for RT3D and talazoparib. We have now included more waterfall plots showing different MOIs of RT3D used in the drug screen to emphasise how robust the Z-scores between RT3D and talazoparib are as displayed below. The previous manuscript displayed an example of this with only one MOI [0.1] used as an example and all the waterfall plots that are now in Figure 1A are summarised below:

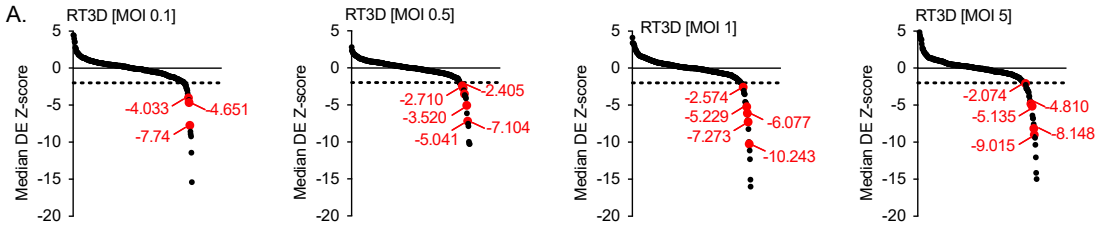


Fig 1: RT3D combination with talazoparib exerts a synergistic effect. A. *Results from a high-throughput screen experimental set up in the A375 melanoma cell line showing the RT3D multiplicity of infection [MOI] (ranging from 0.1-5) and a range of different drug inhibitors [ranging from 0.0005 μM -1 μM]. Cells were analysed 72 hours post-infection using the Cell Titer Glo (CTG) Assay. The z score (DE effect z score = virus effect z score) is plotted on the y axis and was obtained from the standardised value from (i) the median of triplicate samples normalised to virus only versus untreated (ii) the mean of virus only versus untreated and (iii) the median absolute deviation between (i) and (ii) - also known as Z-MAD. The z score plots are shown for talazoparib combined with RT3D at MOI of 0.1, 0.5, 1 and 5 (highlighted in red dots) as displayed in the waterfall plots*

4**.** Keep the concentrations of drugs consistent in all the figures. Either pick micromolars or nanomaolars. Use the same decimal places in all graphs such as MOI of 0.1 or 0.1000.

Response: We have now corrected this and chosen to go with μM throughout the manuscript and use the same decimal places in the graphs such as 0.1 for MOI.

5. SRB in shorthand. Expand the first time in brackets you use shorthand.

*Response: We have now included the full wording of SRB (Sulforhodamine B) when it’s first mentioned (**Results**, **line 137**) and is also explained in the materials and methods section (**Methods, line 647**).*

6. Figure1A, instead of showing the numbers in red, indicate the drugs. In figure 1B, color the bars, change y axis to either log of cell survival or % of cell survival or fold change.

Response: *All red dots in the waterfall plot represent the Drug Effect (Z score) between talazoparib and RT3D. The numbers have now been included beside the red dots and correlate with the supplementary table 2A for readers to understand better as explained in point 3 above.*

-Check consistency of drug concentrations.

Response: *This has now been done. For the melanoma cell line experiments, we initially started by using the concentrations used in the drug screen for talazoparib (Fig 1C and supplementary Fig 1A and 2A) at 0.005, 0.05 and 0.5 μM. Any mechanistic work carried out for talazoparib was done at 0.1 and 1 μM for consistency. In HeLa cells (both wild-type and PARP1-deficient), we used 0.01 and 0.1 μM talazoparib as we were keen to compare two concentrations in both the wild-type and PARP1-deficient cells to see if a lower concentration of talazoparib (0.01μM) had the same effect as a higher concentration (0.1 μM). This was, indeed, the case (Fig 2F and Fig 2G) in terms of cell survival. For any further experiments, we used 0.1 μM. For the work with the SUM149 TNBC cell line (Supp Fig 2C), this BRCA1 mutant cell line is known to be very sensitive to PARP1 inhibitors. This required us to reduce the concentration of talazoparib used (already established in publications with our collaborators). This is why we used much lower concentrations of talazoparib (0.0001, 0.001 and 0.01 μM) compared to the other TNBC cell lines (Cal51 and 4T1), where we used 0.01, 0.1 and 1 μM talazoparib.*

-What is the vehicle?

Response: *The vehicle is DMSO, and this has now been corrected in the figures.*

-Indicate the concentration of talazoparib in 1C.

Response: *For clarity, Fig 1B and 1C have now been reshuffled for all the figures to fit in due to addition of the waterfall plots in Fig 1A. We have now included the concentration of talazoparib (Figure 1B) which is 0.05 μM.*

-In 1F, color the points in the same color that the line is colored (example orange line have orange points of standard deviation). 1E, y axis is % of dead cells.

Response: *We agree with the reviewer and have made amendments to this. Fig 1E (y axis) is now % PI positive cells (Dead cells). We have also taken note of the colour points in Fig 1F, and the points are now in the same color that the line is colored.*

7. Line 148: Remove entire section (Talazoparib does not enhance cell kill in combination with other types of RNA oncolytic viruses). It does not add much to the data and is not well explained. PARP western blots are not mentioned. Remove section all together.

Response: *We have taken note of this comment and agree with the reviewer. This section has now been removed from the manuscript*

8. Re-check the conclusion made on figure S4A (line 162-166).

Response: *We have looked again at the data presented in the section "Synergistic activity of RT3D plus talazoparib and its effects on DNA damage repair." We stand by our interpretation of the data. Talazoparib as a single agent causes an increase in the G/M cell cycle population. As has previously been reported, RT3D increases the S phase fraction as a single agent. Together, talazoparib and RT3D increase the sub-G1 population in both A375 and MeWo cell lines. We have corrected sub-G0 to sub-G1. The text in the manuscript now reads as follows: In contrast, RT3D infection increased the fraction of cells in S phase. Combination of RT3D and talazoparib caused a marked increase in the sub-G1 phase, which is indicative of apoptosis and consistent with the enhanced cell killing observed following RT3D/talazoparib (Fig. S4A). (**Results, line 159-163**).*

-Is COMET assay explained somewhere in the methods?

*Response: We apologise for not including this in the manuscript. This has now been included (**Methods, line 697-708**).*

9. Section "synthetic lethality is not mediated by enhanced viral replication" can be removed all together. Re-check the conclusions drawn from the western blots (line 181-183). Conclusions don't match the westerns. Integrate figure S5A,B into the previous section.

*Response: We respectfully disagree that these data can be removed altogether, but we do accept that our rather definitive statements could benefit from being softened a little. We feel that it is important to retain this section as some researchers in the oncolytic virus field still hold the view that oncolytic viruses mediate their effects almost exclusively through cell lysis that results from viral replication. We stand by our conclusion that there is no significant increase in viral replication in the context of talazoparib/RT3D combination treatment. Most importantly, the one-step growth assays show no significant difference in production of intact, replication-competent virus from infections performed in the absence or presence of talazoparib. In line with this observation, the levels of viral proteins within the cell lines tested show no increase in μ1C production in the absence or presence of talazoparib. However, there is increased σ3 production in 2 of the 3 cell lines tested, likely reflecting variations in the ability of RT3D to redirect the protein synthetic pathways within different cells. Nonetheless, the gold-standard assay for viral replication (the one-step growth curve) is entirely consistent with our conclusion that production of intact, replication-competent RT3D is not increased by talazoparib and, therefore, the reason for the observed enhanced cell killing with the RT3D-talazoparib combination cannot be ascribed to viral replication. In the revised manuscript, we have changed the subtitle for this section to read "RT3D/talazoparib enhanced cell death is not mediated by enhanced viral replication" and we have removed mention of synthetic lethality in this place. In the text, we have rephrased the relevant sentences as follows: "Addition of talazoparib to RT3D infected cells did not result in any significant increase in production of intact, replication-competent RT3D in the one-step growth curve assays. (Fig S5A, B). Interestingly, although μ1C protein production was unaltered in all cell lines (in keeping with the one-step growth curve data), there was a modest increase in σ3 production in A375 and MeWo, but not D04 cells (Fig S5C). This observation is likely to reflect variations in the ability of RT3D to redirect different cell lines to produce specific reoviral proteins. Nonetheless, taken together, these data clearly show that the combinatorial effect of talazoparib and RT3D cannot be explained in terms of increased viral replication (Fig. S5A, B)." (**Results**, **line 179-187**).*

10. Figure 2D, missing significance bars. Change y axis to % survival or log or fold change.

Response: The significant bars have now been added and the graph replotted to make it easier to see the difference in effect between HeLa PARP1$^{+/+}$ and HeLa PARP1$^{-/-}$ (clone G3 and G9 clones). The y axis is now represented as % of cell survival

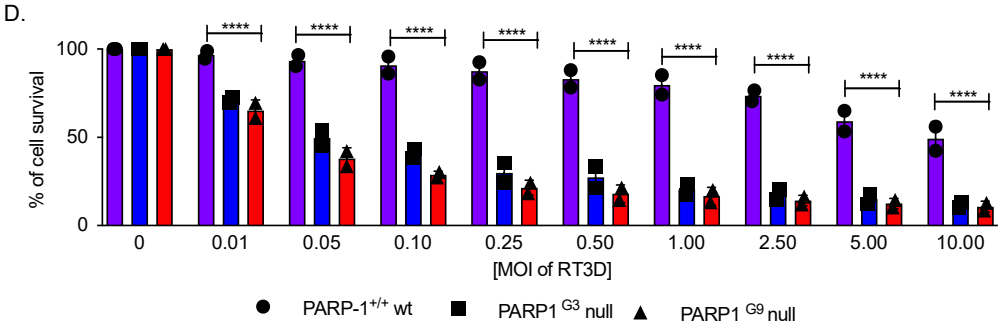


***Fig 2D.** RT3D sensitivity was assessed in HeLa PARP-1 paired cells: PARP-1+/+ (wild-type) and PARP-1-/- (clones G3 and G9), where cytotoxicity was measured by MTT assay 72 hours post-infection.*

-Rephrase what is shown in 2E,F (line 199-201).

Response: *We apologise for any ambiguity in the original text, which read as "Genetic loss of PARP-1 in G3 and G9 cells following RT3D infection mirrored PARP-1$^{+/+}$ cells following RT3D infection in the presence of talazoparib in terms of cell death, as shown by crystal violet and SRB assay (Fig 2E, F). Similar effects were also seen in HeLa PARP-mutant cell lines A7 and F7 mutants, which lack efficient PARylation activity, despite expressing PARP1 as previously described [18] when compared to wild-type PARP-1 (Fig 2G)."(line 199-203 of previous manuscript). For greater clarity, this text now reads as follows: "As anticipated, PARP-1$^{+/+}$ cells showed enhanced cell kill when RT3D was combined with talazoparib. In contrast, in matched cells with genetic PARP-1 loss (G3 and G9), a high degree of sensitivity to RT3D infection alone was seen and this was not enhanced by the addition of talazoparib. Interestingly, the sensitivity of PARP-1$^{-/-}$ cells to RT3D alone was equivalent to that seen in wild-type PARP-1$^{+/+}$ cells with the combination of RT3D and talazoparib (Fig 2E, F). Similarly, the A7 and F7 cells, which lack PARP-1 enzymatic function as previously described [16] showed greater levels of sensitivity to RT3D which were not further increased with talazoparib treatment. Again, the sensitivity of A7 and F7 cells to RT3D alone appeared equivalent to that for wild-type PARP-1$^{+/+}$ cells treated with combined RT3D-talazoparib (Fig 2G)".* ***(Results, line 201-210)***.

-What is the vehicle in 2F?

Response: *The vehicle is DMSO, and this has now been corrected in the figures.*

-Explain why it is expected that the vehicle treated in G3 and G9 will have fewer cells that vehicle treated of PARP+/+.

Response: *We apologise if this was not clear in the original text. We trust that the amended text above(from* ***Results, line 201-210*** *provides greater clarity. However, to explain further, the same number of cells (PARP1$^{+/+}$ and PARP1$^{-/-}$ (G3 and G9 clones)) were plated in each well for all experiments (see crystal violet staining in Fig 2E). All conditions with RT3D MOI 0 and DMSO control (i.e. 0 μM talazoparib) are normalised at 100% cell survival (see Fig 2F). For the PARP1$^{+/+}$ cells, there is reduced % survival at RT3D MOI 0 following treatment with 0.01 μM and 0.1 μM talazoparib alone (blue and red lines, respectively). This is due to the sensitivity of PARP$^{+/+}$ wild-type cells to PARP inhibition (talazoparib). However, as anticipated, there was no effect of talazoparib alone in the G3 and G9 clones as these clones are deficient in PARP-1. Therefore, at RT3D MOI 0, cell survival remains at 100% with 0.01 μM and 0.1 μM talazoparib alone (blue and red lines, respectively).*

-Fig 2G seems to have the right format for plotting bar graphs. The bars are colored, and the standard deviation is in black. Use this format for all the bar graphs.

Response: *This has been acknowledged and most of the graphs are now represented like this (as requested by reviewer). A small number of graphs needed to have other colors as they had a range of different caspase inhibitors (Fig 4A) or a range of talazoparib, olaparib and veliparib doses (Fig S9A, B). We, therefore, had to use a range of other colors to accommodate this. We appreciate the reviewer's comment and believe the data is easier to visualise for the readers.*

11. Figure 3A, change bars to colors, not enough variations of grey to distinguish the bars.

Response: *This has been amended (now presented how Fig 2G is as requested by reviewer).*

-Caspase3, cFLIP and c-IAP1 is not needed. Limit it to caspase8 and PAR.

Response: *We have now removed the cFLIP and c-IAP1 blots as suggested and kept caspase 8, caspase 3 and PAR (Fig 4B).*

-Introduction to proteomic data for the first time in 3F. Include RIG-1 and NF-κB data here as well. Explain the 3 pathways you chose to focus from the proteomic data**.**

Response: As requested, the proteomics data have now been collated in ***Fig 3*** *and all the pathways dissected in this manuscript are summarised under the sub-heading:* ***PARP-1 regulates PARylation induced by RT3D infection and enhances death-inducing signalling complex (DISC)-mediated apoptosis*** *(****Results, line 222-223****).* The *findings are explained in detail (****Results, line 223-249****). We trust that this represents an improvement in data presentation.*

-3H bar graphs have 4 distinct colors for each condition. Use the same colors for the same conditions throughout the manuscript. Use this graph to format all the other graphs.

Response: We have used the same color for the same conditions in most of the graphs in the manuscript (now presented how Fig 2G is presented as requested by reviewer). As previously mentioned, some graphs needed to have other colors as they had a range of different caspase inhibitors (Fig 4A) or a range of talazoparib, olaparib and veliparib doses (Fig S9A, B). We, therefore, had to use a range of other colors to accommodate this.

-It does not make sense to show 24 and 48h time point in 3K. You do not show the time point in 3J. It raises unnecessary questions. Just show 36h time point assuming that the lysates used in J were also from 36h time point**.**

Response: Please note that Fig 3K and 3J are now ***Fig 4I and 4J*** *due to moving and collating the proteomics data as requested. We apologise if these figures were not described clearly.*

*To clarify, they are two separate experiments. Fig 4I is an immunoprecipitation assay as described in the methods section (****line 745-755****) where lysates were incubated with PAR antibody and Protein G Sepharose beads overnight and then probed for PAR, Caspase-8, FADD, TRADD and RIPK1 at 36 hours (Fig. 4I). Input protein analysis also confirmed that RT3D-induced PARylation was inhibited by talazoparib in the lysates (as shown in* ***Fig. S7A****). On the other hand, figure 4J is a caspase 8 immunoprecipitation assay as described in the methods section (****line 735-745****) where lysates treated with RT3D and/or talazoparib at 0, 24 and 36 hours post infection were probed for RIPK1, FADD, PAR and caspase 8. Input protein analysis also confirmed that RT3D-induced PARylation was inhibited by talazoparib at 24 and 36 hours as well as confirmation of increase of caspase 8 cleavage (as an apoptotic marker) at 36 hours following RT3D/talazoparib compared to RT3D alone (Fig. S7B). In order to make this issue clearer for the reader, we have modified the text to read as follows: Having shown that combined RT3D-talazoparib enhanced apoptosis mediated by the DISC, we explored the possibility of an interaction between DISC components and poly(ADP)-ribose (PAR) chains following RT3D infection. Firstly, we carried out an immunoprecipitation assay to pull down PAR, where PAR antibody was added to lysates in the presence of protein G-agarose beads and cleared lysates run by Western analysis. Our data revealed PAR interaction with Caspase-8 (as well as FADD, TRADD and RIPK1) at 36 hours. These interactions were reduced by talazoparib (****Fig. 4I****). Input protein analysis confirmed that RT3D-induced PARylation was inhibited by talazoparib (****Fig. S7A****).*

*Conversely, we carried out a second experiment where we pulled-down caspase-8 using the Complex II immunoprecipitation assay as detailed in the methods section. Our results revealed an interaction between Caspase 8 and PAR (by 36 hours) that, again, was attenuated in the presence of talazoparib (****Fig. 4J****). The interaction of DISC components, RIPK1 and FADD, with caspase-8 was also inhibited by talazoparib and this correlated with an increase in cleaved caspase-8 as shown in the input data (****Fig. S7B****). Taken together, these data suggest that RT3D induces PARylation of DISC components to inhibit apoptosis, and that this effect is reversed by talazoparib, leading to enhanced cell death. (****Results, line 282-298****).*

We trust that this amended text improves the clarity of data presentation.

-Where is the loading control for J and K?

Response: The loading control and input for the immunoprecipitation pull downs were in the supplementary figures (Fig S7).

12. Remove section "RT3D plus talazoparib-induced cell death is mediated by TRAIL and TNF". It is great that you show the association of TRAIL and TNF, but it is not necessary since you describe the DISC components in detail in the previous sections. Having this section does not add too much but shifts the focus of the paper (Fig. supp 8).

*Response: We respectfully request that these data are retained in the manuscript. We believe it is important to demonstrate the importance of RT3D-induced TRAIL and TNF-α secretion (**Supp Fig 8**) which are known to play a role in activating the DR4/DR5 and TNF-α receptors. These data point to an autocrine feedback loop that plays an important role in activating the DISC components (**Supp Fig 10A**). Our data also implicates this feedback loop in the NF-κB pathway (**Supp Fig 10B**). These findings may have therapeutic consequences, including opportunities for TRAIL/TNFα receptor agonism and immune activation through NF-κB pathway activation. We highlighted the importance of this axis in the Discussion in the original submission, but we have now added another sentence to stress this point more clearly. This text is as follows: "RT3D-induced cancer cell death involves TRAIL-mediated apoptosis and is associated with activation of Caspase-8-mediated extrinsic apoptosis[18, 19]. While we observed an increase in RT3D-induced TRAIL and TNF-α plus their receptors, DR4/DR5 or TNFR1/TNFR2, respectively, this was not increased further by combination with talazoparib. Nonetheless, it is important to recognise that modulation of TRAIL/TNFα signalling may represent a means of enhancing the therapeutic efficacy of RT3D/PARP inhibitor combinations." **(Discussion, line 509-515)**.*

13. The results from figure 4A and B (**now fig 5A and B**) may be an over interpretation. Cytokine levels are unaltered by siRNA of RIPK1, CASp8 and FADD. They are only affected when treated with talazoparib or a combination of RT3D+talazoparib to begin with. Re-interpret the results and rephrase the section.

Response: We respectfully disagree with this comment and stand by our original interpretation. Following the clear evidence from the cytokine array assay in Fig 5A, we sought to evaluate the roles of RIPK1, Casp8 and FADD (all implicated in the combinatorial therapeutic effect) in cytokine release. We agree that there is hardly any cytokine release when RIPK1, Casp 8 and FADD siRNA alone are transfected into cells (as shown in the purple bars in Fig 5B) and these levels are comparable to scrambled (SC) alone. Clearly, input siRNA does not drive a cytokine response and neither does silencing RIPK1, Casp8 or FADD. This is also true in the presence of talazoparib (yellow bar) where SC, RIPK1, Casp8 and FADD siRNA are not associated with a cytokine response. In contrast, with both RT3D alone and with RT3D and talazoparib, there is a significant increase in CCL5, CXCL8, CXCL1 and CXCL10. These data correlate exactly with the cytokine assay shown in Fig 5A. The important role of the DISC components (RIPK1, Casp8 and FADD) in mediating cytokine release following RT3D or RT3D/talazoparib treatment is confirmed since the release of each of these cytokines is significantly attenuated when we use specific siRNA against RIPK1, Casp8 and FADD.

-Change bar graphs in 4B to colored bars as in the previous figure. Keep the color scheme consistent across the manuscript.

*Response: We have now amended the bar graphs as requested in keeping with the color scheme. Please note that Fig 4B is now **Fig 5B** due to collating all the proteomic data as one (Fig 3).*

-Combine 4C with proteomic data shown in the previous section.

*Response: The proteomics data have now been collated in **Fig 3** and all the pathways dissected in this manuscript are summarised under the sub-heading: **PARP-1 regulates PARylation***

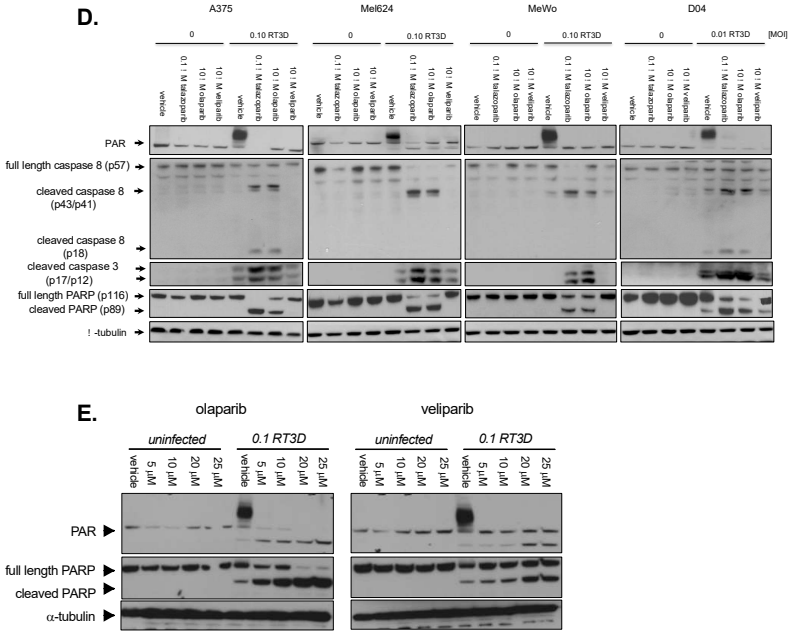
induced by RT3D infection and enhances death-inducing signalling complex (DISC)-mediated apoptosis *(**Results, line 220-223**).* The *findings are explained in detail (**Results, line 223-246**).*
We trust that this represents an improvement in data presentation

-Supplementary figure 9B and 9C have been interchanged, they don't match the wording (line 320-326).

*Response: Thank you for highlighting this. We have now rectified the interchange in the manuscript which was an oversight. The text now reads as follows: Cell viability analysis by crystal violet staining showed that, like talazoparib, olaparib increased RT3D-induced cell death, while veliparib had modest effects on cell viability (Fig. S9C). These results confirm that PARP inhibition generally enhances the effects of RT3D on NF-κB signalling, where talazoparib caused a more profound effect compared to other PARP inhibitors. In addition, cytokine induction was increased with olaparib and veliparib, but not to the same extent as with talazoparib (Fig. S9B). (**Results, line 346-352**).*

-Supplementary figure 9D is not accounted for in the text. You have to talk about all the figures.

*Response: These data are now discussed in the manuscript. In addition, we have included a western blot (**Fig S9E**) to emphasise that both olaparib and veliparib enhance RT3D killing, as does talazoparib albeit at different concentrations.*
*The text in the manuscript now reads as follows: "Additionally, we found talazoparib (0.1 μM) led to enhanced apoptotic markers (caspase-8, caspase-3 and PARP cleavage) when compared to olaparib or veliparib, which were given at a higher concentration of 10 μM (Fig. S9D). Olaparib appeared to be more effective in enhancing RT3D-induced apoptosis compared to veliparib, as shown in the apoptotic markers by Western blot analysis. We found a higher dose of veliparib 20-25 μM was necessary to see a similar effect to that shown with olaparib at 5-10 μM (Fig. S9E). (**Results, line 352-359**).*



Supplementary Figure 9: D. *A375, Mel624, MeWo and D04 cells were treated with 0.1 μM talazoparib, 10 μM olaparib or 10 μM veliparib and thereafter infected with RT3D [MOI of 0.1] for 48 hours. Western analysis was carried out to assess PAR expression, caspase 8 and PARP cleavage. Equal loading of proteins was assessed by probing for α-tubulin.* ***E.*** *A375 cells were treated with a concentration range of olaparib or veliparib (5-25 μM) and thereafter infected with RT3D [MOI of 0.10] for 48 hours. Western analysis was carried out to assess PAR expression and PARP cleavage. Equal loading of proteins was assessed by probing for α-tubulin.*

-Modify color scheme in supplementary figure 10.

Response: We have now amended the bar graphs as requested in keeping with the color scheme.

-Which cells were used for gene silencing? Mention in the results section.

*Response: Thank you for bringing this to our attention. A375 cells were used in the gene silencing studies and the cell line has now been made clear in the figure legends (**Fig S10**) as well as in the main body of the manuscript (**Results, line 367**) to clarify this point.*

Which cells were co-treated in figure S10B? Was it the WT cells or those with silencing of caspase-8, RIPK1 and FADD.

*Response: The cells treated in Fig S10B are A375 cells without any siRNA treatment as the main aim of the experiment was to investigate if use of the neutralising anti-TRAIL [2E5] or anti-TNF-α [D1B4] antibodies would reduce RT3D/talazoparib-induced NF-κB signalling. We have now included mention of A375 cells (**Results, line 370**) and apologise as this was not made clear in the manuscript.*

14. Combine proteomics results from figure 5A in one figure. Consolidate all the proteomics data in one figure.

*Response: The proteomics data have now been collated in **Fig 3** and all the pathways dissected in this manuscript are summarised under the sub-heading: **PARP-1 regulates PARylation induced by RT3D infection and enhances death-inducing signalling complex (DISC)-mediated apoptosis** (**Results, line 222-249**).*

15. Change the standard of deviation points in figure 6B to the same color as the line joining the points.

Response: This has now been amended as requested by the reviewer

-Text from line 390-396 does not match the figures.

*Response: We thank the reviewer for bringing this to our attention. This was an oversight and has now been rectified. This has now been corrected and made to match the figures. Please note that **Fig 6 is now Fig 7** in the manuscript. We have corrected the text to match the figures and reads as follows: In support of these findings, FACS analysis of in vivo tumour samples was carried out. Tumour volumes of mice used to profile the immune infiltrate by FACS analysis were measured up to Day 12 (**Fig. 7F**) and their weights measured prior to profiling the immune infiltrate by FACS analysis at time of harvest (**Fig. 7G**). Our data revealed an increase of CD3+, CD8+ and CD4+ cells with RT3D plus talazoparib (**Fig. 7H**). (**Results, line 457-461**).*

Wording for figure 6F and 6G is missing.

*Response: We thank the reviewer for bringing this to our attention. This was an oversight and has now been rectified. Please note that the figures are now Fig 7F and 7G due to collating all the proteomic data into Fig 3. The text in the manuscript now reads as follows: In support of these findings, FACS analysis of in vivo tumour samples was carried out. Tumour volumes of mice used to profile the immune infiltrate by FACS analysis were measured up to Day 12 (**Fig. 7F**) and their weights measured prior to profiling the immune infiltrate by FACS analysis at time of harvest (**Fig. 7G**). (**Results, line 457-460**).*

-There is no increase in foxp3 upon RT3D injection, recheck wording in line 392. Re-assess figure 6I.

Response: The summary on foxp3 data has now been reworded to correlate with the findings and reads as follows: There was no increase in foxp3+ cells following RT3D injection, when compared

to vehicle or talazoparib alone. These levels were increased following RT3D/talazoparib treatment, but this was non-significant (Fig. 7I). (***Results*, *line 461-464***).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors comprehensively responded to the Reviewer's comments and revised the manuscript.

Reviewer #3 (Remarks to the Author):

I am pleased to see a significant improvement in the manuscript. I applaud the team on addressing all the comments and making changes accordingly. I want to congratulate the authors on the excellent work that have done. I have some minor comments that are mostly editing errors.
1) Line 194: shown by western blot and PAR ELISA and was also manifest in vivo. Should be "manifested ".

This has now been changed to "and was also manifested *in vivo* (Fig 2C)."

2) I like that the color scheme is same in all the figures. Can you color the shapes according to the color on the bars of the graphs. Circles – purple, squares -blue, triangles-red. Just as you show in 5B

This has been done except for figure 5E and F. The author who conducted this work has the R script files to analyse these graphs and is currently working on it. We hope to get this done by the next round of edits.

3) Is it possible that the label on figure 4E, that is PARP1 +/+ is interchanges with PARP1G9-/-? Should the left panel of blots be PARP1G9-/-?

Apologies for this oversight, this has now been corrected.

4) In figure S9, either follow the labelling for x axis in A or B. I think label for A looks neater. Can you label B the same way? Can you assign p values between the bar graphs and denote them with a * on the graph? There is a lot of jargon to describe these figures. Such as “more profound effect”, or “ increased but not to the same extent”. Please calculate the significance and describe in the text if the differences are significant or non-significant. Between D and E, call it uninfected or RT3D of 0.

In figure S9, the axis in A has now been used for B as well as requested, and the text has been replaced to better clarify what has been observed:

“In addition, the cytokines CCL5, CXCL1 and CXCL10 induced by RT3D was increased with talazoparib at 1 μM, but not with olaparib and veliparib at 1 μM (Fig. S9B).”

Between D and E, the labeling has been made consistent, instead of vehicle it is ‘0’ uM drug, and instead of 0 RT3D, it is now labelled ‘uninfected’.

In S9E, Talazoparib and olaparib have similar expression of cleaved casp and PARP in D04 and MeWo. It is only in A375 and Mel64 that the expression of these proteins is higher with talazoparib treatment. It could be worthwhile to conclude that the PARP inhibitors have similar effect albeit there could be a dependency on the cell line (given that all cell lines are not exactly genetically uniform).

In figure S9E, data is only for the A375 cell line. Perhaps you are referring to figure S9D? If so, cleaved caspase and cleaved PARP are increased with the combination of either RT3D-talazoparib or RT3D-olaparib vs. RT3D alone across all cell lines, but not for RT3D-veliparib vs. RT3D. The text has been changed for clarity:

“Additionally, we found either talazoparib or olaparib in combination with RT3D led to a greater increase in apoptotic markers (caspase-8, caspase-3 and PARP cleavage) when compared to RT3D alone, which was not seen with veliparib (Fig. S9D). However, olaparib was used at a far higher dose (10 μM) than talazoparib (0.1 μM). We found a higher dose of veliparib 20-25 μM was necessary to see an effect similar effect as that seen with olaparib at lower doses of 5-10 μM (Fig. S9E).”

5) Please unify color scheme in figure 5 C, E and F.

This has been done except for figure 5E and F. The author who analysed this data is currently working on it and we hope to get this done in the next round of edits.

6) In figure 6C, is RIG-I not supposed to show a band under all conditions of the SC siRNA? There is a band only when treated with RT3D and talazoparib.

RIG-I is IFN induced, and increased IFN signaling is a consequence of combination therapy. This is demonstrated in figure 6A. Here, RIG-I is expressed with combination therapy along with other IFN stimulated proteins such as pSTAT1 and pIRF3. Furthermore, proteomic data in figure 3 also shows that RIG-I has low expression basally (in A375), and this is increased with combination therapy. Figure 6C correlates with these previous data, and by using siRNA of RIG-I, the expression seen in the combination therapy is lost.