Reviewer's report

Title: Adenoviral Delivery of Pan-Caspase Inhibitor p35 Enhances P450 Gene-directed Enzyme Prodrug Therapy using Cyclophosphamide

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Reviewer: Peter F Searle

Reviewer's report:

This manuscript shows that co-expression of the baculoviral p35 caspase inhibitor reduces apoptosis caused by the viral vector alone or in response to cyclophosphamide activation, resulting in a modest increase in survival of the infected U251 cells.

The apparent benefit of this is to permit a greater amount of CPA to be activated, which has potential to increase the bystander effect — i.e. killing of non-infected cells by diffusion of the activated CPA metabolites.

In Figure 4A, the cell density in the right-hand panel appears almost twice that in the left panel. Note that increasing culture density (if the proportion of ‘factory’ cells remains the same) can increase the killing of bystander cells, due to more prodrug activation in the culture as a whole, and shorter diffusion distances. This might contribute to the apparent increase in apoptotic bystander cells. However, the reduced co-localisation of TUNEL and CYP2B6 staining with the Ad-2B6/p35 virus appears significant, and it is unlikely culture density could contribute to this effect. Panel B appears to confirm increased killing of bystander cells by the Ad-2B6/p35 virus, although considering the error bars, statistical analysis would be helpful.

The authors go on to show that expression of p35 does not reduce the ability of a co-infected oncolytic adenovirus Onyx017 to support the replication and release of the Adeno-2B6/p35 virus (Figure 5). They also show (Figure 6) that co-infection of Adeno-2B6/p35 with Onyx017 permits greater CPA-dependent bystander activity, relative to co-infection of Ad-2B6 with the oncolytic virus. Considering that the MOI used should have resulted in infecting all the U251 cells, and also the timescale of the experiment, the benefit of co-infection with Onyx017 is presumably due to increased copy number of the CYP1B6-expressing viral genomes, thus increasing the level of enzyme in the cells. Onward virus spread is unlikely to be contributing to the efficacy in this experiment.

Conversely, the experiment shown in Figure 5 does not address whether CPA activation inhibits viral replication and release (or the impact of p35 expression on this).

It should be noted that the use of a 60:40 ratio of ‘factory’ U251 cells to the target 9L/LacZ target cells is not a very stringent test for a high bystander activity — #10% factory cells would be a more realistic reflection of what might be
achievable clinically. Nevertheless, the experimental design suffices to make the point that prolonging the survival of the infected ‘factory’ cells increases the bystander effect.

- Discretionary Revisions
1) Since the benefit of p35 expression appears to be in respect of bystander cytotoxicity, I recommend including ‘bystander’ in the title.

2) page 14: Lines 13 and 15 of main paragraph suggests that increased spread of the viruses induced by ONYX-017 may contribute to the efficacy. Since the MOI of 7.5 or 15 for the E1-deleted Adeno-2B6 and Adeno-2B6/p35 viruses should be sufficient to infect essentially all the U251 cells, and also considering the short timescale, the major benefit is probably from amplification of the viral genomes within the initially infected cells, leading to greater expression of CYP2B6 in these cells, rather than there being much contribution from viral spread. I suggest rewording to clarify this.

- Minor Essential Revision
3) page 7: please indicate how the virus was quantitated, for definition of the MOI — is this virus particles per cell, infective focus units per cell, plaque forming units per cell...

4) page 8: presumably 4-OOH-CPA is a misprint for 4-OH-CPA?

5) page 8: indicate species and source of anti-2B6 monoclonal antibody.

6) Page 9: Top line — is anti-rabbit IgG the correct species?

7) Page 9 line 6 — “system” spelled “ystem”

8) Page 9: please describe 9L cells and their source.

9) Check that the two panels in Fig. 4A are from the same experiment, and consider possible reasons for the apparent differences in cell density in the two images.

10) Fig. 4B — a statistical test on these results would be useful.

11) Figure legends — please clarify whether means ±SD relate to multiple experiments, or multiple replicate wells in a single experiment — in which case, how reproducible were the results between experiments?

12) page 23: Figure 3 legend — “chemosensitive” is spelled incorrectly.

13) page 24: Figure 5 legend — “ONYX-107” should presumably read “ONYX-017”

14) page 24: Figure 6 legend — “elution” makes no sense here — was “evaluation” intended?

- Major Compulsory Revisions
None.

Level of interest: An article whose findings are important to those with closely
related research interests

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I declare that I have no competing interests