Author's response to reviews

Title: Pharmacological targeting of valosin containing protein (VCP) selectively kills canine lymphoma cells by inducing DNA damage

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Author's response to reviews: see over
We thank the reviewers for their time in reviewing our manuscript and for their positive comments. Please find below our responses to the issues raised by each reviewer.

Reviewer 1.

Major point 1. This is an interesting question, but not one that we are able to answer using our experimental design. Increases in apoptosis and cell cycle arrest occur almost concomitantly around 12-24h post-Eey1 treatment, and we have no clear evidence that G1 arrest occurs first. All cells die after 48h (presumably by apoptosis) at the dose of Eey1 that we use in these experiments. Whether or not this is preceded by G1 arrest in all cells remains to be determined.

Major point 2. We have performed this experiment, unfortunately the result was not conclusive. PBMCs are not a cell line, but a primary culture of mononuclear cells (mostly lymphocytes) that are non-proliferative and do not survive for long periods in culture. These are therefore freshly isolated at the beginning of each experiment, and the number of cells that we recover at the end of the experiment is quite a bit lower than the number that we plate initially (even in absence of any treatment). The loss of cells is probably due to a combination of damage incurred during the purification process and attrition in culture. Because of this, there is an intrinsic high level of γH2AX expression in our PBMCs, and we do not observe a clear increase in presence of Eey1, quite possibly due to this high background.

We were therefore unable to determine if normal lymphocytes also die by a DNA damage-associated mechanism. We do however wish to point out that our use of PBMCs was only meant to illustrate a differential sensitivity to Eey1 between normal and cancerous cells (Fig 2). As we discuss in the text, we feel this provides proof of principle that there is a therapeutic window that could be clinically exploitable. Whether normal lymphocytes are killed by Eey1 via a similar mechanism as cancerous lymphocytes is an interesting question, but one that is unrelated to the goal of our study.

Major point 3. If we understand correctly, the reviewer is requesting these experiments as proof that the observed effects of Eey1 are due to inhibition of VCP, and not to off-target effects (i.e., if similar effects of Eey1 are observed in VCP-negative cells, this would indicate that Eey1 is acting independently of VCP). Unfortunately, VCP is constitutively expressed in all cells and is absolutely critical for life. Knockdown of VCP rapidly induces apoptosis through ER stress and other mechanisms. We acknowledge that we cannot exclude the possibility that Eey1 acts, at least in part, by acting on targets other than VCP. We can however mention that we are conducting a follow-up study using a VCP inhibitory compound that is structurally unrelated to Eey1, and that it induces apoptosis in CLBL-1 cells via a DNA-damage related mechanism (and not ER stress or inhibition of autophagy), as we observed for Eey1. It would therefore seem very unlikely that two unrelated VCP inhibitors should both induce apoptosis via the same mechanism, and that both would be via off-target effects.

Minor point 1. This information was added to the legend for Figure 1.

Minor point 2. We found little of interest for VCP in the oncomine database. We cite all of the published studies of VCP expression in human cancers in the discussion.
Minor point 3. If the reviewer is referring to Fig 7B, it was Cdkn1a (a.k.a. p21WAF1/CIP1) that was measured and not Cdkn2a.

Reviewer 2.

Main point 1. We feel that our finding that the P53/ATM DNA damage response pathway is activated by Eey1 provides good evidence that DNA damage is inducing apoptosis in our cell model. Nonetheless, we agree with reviewer that this is not rigorous proof. We have therefore modified the text (including the title) to tone down this conclusion as suggested.

Main point 2. Regarding Fig. 3A, please note that there are far fewer total cells present in the 3 micromolar Eey-treated sample (the DAPI stain may not be obvious if the reviewer viewed the image in black and white), so that the proportion of cells that are TUNEL-positive is higher. We refer the reviewer to the quantitative analysis of the experiment (Fig. 3B), which is perhaps more compelling. Regarding Figure 4C/D, we agree that the changes in the height of the G0/G1 peak in response to Eey1 is not obvious, but that is not very surprising given the modest (c. 5%) increase in the proportion of quiescent cells (see Fig 4A). What Fig 4C/D best illustrates is the loss of cells in the S phase; note the reduced height of the plateau between the G0/G1 and G2 peaks in Fig 4D compared to Fig 4C.

Main point 3. We actually had performed these types of positive controls (both for ER stress and autophagy), but did not include them in our original manuscript. We agree with the reviewer that they should be included, and have added a new supplemental figure and modified the text accordingly.

Main point 4. Reviewer 1 raised a similar issue, please see our response to his major point 2. We speculate that not all cell types respond to Eey1 by accumulating DNA damage (at least not to the same extent); this may be something specific to lymphoma cells. It is also possible that Eey1 causes DNA damage in many cell types, and that the extent to which this causes apoptosis relates to the rate of proliferation of specific cell types. Indeed, non-proliferative cells (such as PBMCs) are typically very resistant to DNA damage-inducing chemotherapeutic agents. Further experiments will be required to test these hypotheses, but this goes well beyond the intended scope of our study.

Minor point 1. We chose the abbreviation Eey1 following the lead of other authors whose also use it (ex: PMID 24685158, PMID 23393593, PMID 23481258). We would not object to using a different abbreviation if the reviewer specifies which should be used instead.

Minor point 2. We believe that we have cited the major relevant studies in the introduction and discussion (i.e., refs 14, 15, 21-23, 39-41). If the reviewer knows of an additional study to cite, we would be happy to do so.