Author's response to reviews

Title: Targeting of CD34+CD38- cells using Gemtuzumab Ozogamicin (Mylotarg) in combination with Tipifarnib (Zarnestra) in Acute Myeloid Leukaemia

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Author's response to reviews: see over
Dear Editor,

MS: 1399207949719418

Targeting of CD34+CD38- cells using Gemtuzumab Ozogamicin (Mylotarg) in combination with Tipifarnib (Zarnestra) in Acute Myeloid Leukaemia. Mays Jawad, Ning Yu, Claire H Seedhouse, Karuna Tandon, Nigel H Russell and Monica Pallis

We thank the reviewers for their time and for the insights they have both provided. We return a revised copy of the manuscript and detail below a copy of the reviewers’ comments in blue with our replies in red.

yours faithfully, Monica Pallis

Reviewer's report
Title: Targeting of CD34+CD38- cells using Gemtuzumab Ozogamicin (Mylotarg) in combination with Tipifarnib (Zarnestra) in Acute Myeloid Leukaemia
Version: 1 Date: 15 June 2012
Reviewer: Judith Karp

Reviewer's report:
The authors have conducted a provocative study that could form a basis for a novel combinatorial therapy for AML, perhaps with particular focus on older adults.
Major revisions:
1. The authors need to expand their explanations/discussion of their counter-intuitive and perhaps paradoxical findings. On the one hand, there appears to be strong CD33 expression in a presumptively primitive LSC population in 11/36 primary samples, raising the question of whether or not the true LSC population was being measured in those patients.
R1.1. We have added 2 references (41 and 42, page 18, lines 17-18) to work that has demonstrated CD33 expression in LSC but not HSC. There is also a potential explanation for this from an increasing body of evidence suggesting that the LSC is functionally more similar to the normal multipotent progenitor than to the true HSC (best shown in Goardon et al., Coexistence of LMPP-like and GMP-like leukemia stem cells in Acute Myeloid Leukemia, Cancer Cell 2011;19:138)
On the other hand, to the activity of GO in the LSC population in an apparently "off-target" CD33-independent fashion (previously reported by these investigators, but without any mention to potential mechanism -- is this simply related to free calicheamycin?). The last paragraph of the discussion states that the lack of relationship between GO and CD33 expression is unsurprising -- this off-handed statement requires succinct explanation and not just references.

R1.2
One experimentally derived potential explanation is antibody endocytosis and appears to be a dosage-related effect. We have included details of this and further discussion on page 20, lines 1-10.

2. The discussion surrounding chk2 activation and eventual cell death is highly speculative (pg 17), particularly since inhibition of DNA damage-induced chk2 activation has been demonstrated repeatedly to enhance drug cytotoxicity.

R1.2. In contrast to chk1 inhibition, the role of specific chk2 inhibitors in enhancing cancer cell toxicity remains controversial and we have included further discussion and references to elaborate this point (page 18, first paragraph)

3. Table 1 is critically important to the eventual clinical relevance of these preclinical data. As such, it might benefit from some clarification and perhaps breaking out some of the data into another table. The authors may want to consider reorganizing the table according to cytogenetic groupings (e.g., favorable, normal/intermediate, FLT3+, adverse, not done) -- alternatively, the table could be reorganized by level of CD34+/CD38- (e.g., from <1% to >50%). These groupings would provide some meaningful clinical context and perhaps illuminate potential clinically-relevant features that shed light on response to combinatorial therapy.

R1.3. We cannot group our data by FLT3, %CD34+CD38-, NPM1 or CD33 as this would undermine our findings (page 16, final paragraph) that none of these had a statistically significant impact on response. In the case of cytogenetics, we have argued (page 13, lines 1-4) that we did not include enough samples with abnormal cytogenetics for any conclusions to be drawn about their impact. However, we have taken on board the criticism and changed the Table: please see our response to Reviewer 2 (Replies R2.2 and R2.4, below) that offers our solution for sorting this mass of data.

Minor revisions:
1. Pg 10 (bottom): NPM1 is misspelled (NMP1)

R1.4
Thankyou. This has been altered (page 11, line 4).
It would be helpful for the reader if the two AML patient samples that did not show a defined leukaemic phenotype from the table making it easier to follow the discussion.

R2.1 We have done as requested. This has left the statistics for the CD34+CD38- subset unchanged, but has led to slight alterations in the data for bulk cells (now n=34 instead of n=36). These changes have not affected any levels of statistical significance except in the case of pgp which is now significantly associated with the effects of the combination (page 16, 2nd paragraph) and we have commented on this in the Discussion (page 20, lines 11-13).

The addition of two columns to Table 1 showing whether the sum of the individual toxicities was >100% or the effects of GO and Tip resulted in an additive or supra-additive (synergistic?) effect would make things easier for the reader to appreciate and evaluate the data.

R2.2 We found this comment really useful, and have added the requested columns, reorganised the whole Table in order of decreasing toxicity and separated bulk cells (Table 1A) from CD34+CD38- cells (Table 1B) as well as adding a Figure (Figure 1B) comparing the effects of the sum of the individual drugs versus the combination.

I am no pharmacologists but it seems that the term supra-additive is not well defined.

R2.3 The term supra-additive is indeed not standard – one usually hears that a positive interaction between two drugs is “additive”, or “synergistic” or that one drug “sensitises” to another. However, to perform a standard Chou and Talalay analysis for synergy, it must first be proved that Michaelis-Menten kinetics for individual drugs can be established (see T-C Chou. Drug combination studies and their synergy quantification using the Chou-Talalay method, Cancer Research 2010;70:440.). As the reviewer acknowledges, there is insufficient material on primary samples to do FACS analysis following a chequerboard set up of several doses of each drug individually plus the combination. For this reason we cannot fulfil the criteria that would allow us to use the term “synergy” with strict correctness. Neither can we use the term sensitisation, as neither drug appears to be conclusively either the sensitiser or the drug that is being sensitised. It is for this reason and after some consideration that the word “supra-additive” was chosen as a descriptive rather than a biometric term. However, the reorganisation of the Table and the new Figure 1B will help to summarise and clarify the drug interactions.

The fact that the authors have to exclude samples and which the sum of the individual treatment exceeds 100% shows that measuring % cell loss is probably a problematic way of investigating additive and synergistic effects. The reviewer is totally aware of the fact that using sorted primary AML CD34+/CD38- patient samples makes it impossible to establish IC 50 doses for each drug and investigate synergistic effects of the two drugs using this method.

Looking over the individual columns with the response of the bulk cells and the LSPC to the drugs alone or in combination and reading the material & methods I cannot really understand when an effect is called supra-additive and I do not know whether the Wilcoxon signed rank test is the appropriate test.

R2.4 With the Wilcoxon test we can compare the sum of the drugs used singly with the effect of the combination in paired groups, i.e. sample by sample, and determine whether the overall differences are statistically significant. An example of precedent for this way of organising the data and the use of the Wilcoxon test in a similar context is given in Morris et al. 2006 (reference 49 in the manuscript).
Figure 2:
The authors state: GO alone induced chk2 phosphorylation in primary cell culture in bulk cells and in the CD34+CD38−… (Figure 2b, c). Figure 2b includes only double treated cells according to the Figure legend. If space allows it would be helpful to include the single treatments on the different populations in Figure 2b.

R2.5 As flow cytometric histograms are on a logarithmic scale, the RFI changes we are measuring would not show up very clearly: the histograms are simply included as a demonstration that there is a chk2 activation response in each cell subset, but would not succeed in demonstrating differences between the effects of the drugs, which relies on quantitation.

Why is % phosphoCHK2 RFI vs untreated and % increase in phosphorchk2 used for the Y-axis in Figure 2a and Figure 2c respectively?
R2.6 Thankyou. There was no reason for the different titles. We have corrected the 2c legend to read the same as the 2a legend.

Figure 4:
Unfortunately, I do not understand what the experiments in Figure 4 really add to the message of the paper because the authors change (for technical reasons) the experimental set-up completely. Instead of using GO and Zarnestra they now use Daunorubicin treatment for two hours with or without pretreatment with a TOR inhibitor to enrich for dormancy.

R2.7. The experiment described in Figure 4 is especially aimed at those readers with a particular interest in DNA damage and repair, who might question the ability of DNA damaging agents to induce gamma H2A.X and chk2 (normally described in the context of the cell cycle homologous recombination response) in a subset of cells (i.e CD34+CD38− cells) that are assumed to be largely dormant. We reasoned that such a reader would require proof of a DDR involving γH2A.X induction in dormant cells. We have reworded the discussion paragraph (page 18, second paragraph, first 2 sentences), for clarity on this point.

It is also difficult to put these data in a clinical context because daunorubicin (in combination with cytarabin) has been used for the treatment of AML for several decades which has probably very little effect on leukemia initiating (stem) cells.

R2.8 On the one hand, patients do get cured on standard chemotherapy, albeit not enough patients! On the other hand, what the reviewer says makes good sense and doesn’t have a simple answer. We have shown induction of γH2A.X in dormant AML cells with daunorubicin, and this is a completely novel finding which may alter the way people think about damage in dormant cancer cells. Hitherto, attention tends to have been focused on membrane transporters and detoxification enzymes in cancer stem cells as a mediators of drug resistance, with little attention paid to what happens if the DNA does actually get damaged. In the current manuscript we have shown damage accumulation in dormant cells and suggested that this will make the cells susceptible to apoptosis, not that the apoptosis will definitely occur. In order to induce death the DDR has got to engage with the apoptotic machinery. Individual cell types, the type, extent and length of drug induced DDR induced by different doses of different drugs and the pro- versus anti-apoptosis molecular balance are all variables in this particular melting pot. We are planning further work on the roles of γH2A.X and chk2 in this context.

Minor essential revisions:
Figure 5:
The p-values should be included in Figure 5c to make it easier to understand.
R2.9. This has been done

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests:
I declare that I have no competing interests