Author's response to reviews

Title: Potent inhibition of rhabdoid tumor cells by combination of flavopiridol and 4OH-Tamoxifen

Authors:

Velasco Cimica (vcimica@notes.cc.sunysb.edu)
Melissa E Smith (msmith@aecom.yu.edu)
Zhaikai Zhnag (zzhang@aecom.yu.edu)
Deepti Mathur (dm322@cornell.edu)
Sridhar Mani (sridhar.mani@einstein.yu.edu)
Ganjam V Kalpana (ganjam.kalpana@einstein.yu.edu)

Version: 4 Date: 16 June 2010

Author's response to reviews: see over
The Editor, BMC Cancer

Dear Sir or Madam:

We thank the editors for arranging this set of reviews for our manuscript entitled, “Potent inhibition of rhabdoid tumor cells by combination of flavopiridol and 4OH-Tamoxifen.” We appreciate the efforts in critically reviewing the manuscript and are pleased to note that many of the previous concerns have been satisfied. We are addressing the remaining concerns below:

**Rebuttal to the reviewer (Magali Oliver):**

1. **Effect of TAM alone.**
   - We agree with the reviewer and have included the effect of 4OH-Tam alone in Figure 2 as panels C and G and have corrected the statement as follows. “Combination of flavopiridol and 4OH-Tam inhibits the growth of rhabdoid tumor cells” (page 2 and 9).
   - We also apologize for the lack of clarity in the statement “Increasing time of exposure…. We meant that 4OH-Tam did not significantly enhance cytotoxicity in a time-dependent manner as treatment of MON cells for days 1, 3, or 5 days gave almost the same survival curves. However, 4OH-Tam decreased cell survival in a concentration-dependent manner. To clarify this point within the text we have changed the sentence to, “We also tested the effect of 4OH-Tam alone and found that 4-OH-Tam decreased cell survival significantly (Figure 2C and G). However, increasing time of exposure, from one to three or five days, to 4-OH-Tam alone, did not result in further decreases of cell survival (Figure 2C and G). Thus, while flavopiridol and 4OH-Tam combination resulted in a time dependent decrease of survival, effect of 4OH-Tam alone was independent of time of exposure” (page 10).

2. **Quality and interpretation of figures 5 and 6B,C and role of cell cycle.**
   - It is clear enough in the paper that either total or nuclear p21 was measured in the different figures. However, I am still not convinced by the data on p21 presented in figure 5. …..
   - The method for quantifying the amount of nuclear p21 staining has now been added to the materials and methods section as follows: “The percentage of cells with nuclear expression of p21 was quantified by counting 250 to 300 individual cells, noting whether or not their nuclei showed positive staining above the background. The background staining was defined as an intensity of staining at or below the intensity of the negative control (i.e. any background staining that occurred in the absence of a p21-specific antibody)” (page 9).
   - It is clear from the data presented that flavopiridol induce a G2 arrest that is p53 dependent. However, the G2 arrest induced by flavopiridol+TAM shown in figure 4A is not reproduced in the new figure 6B added in this manuscript (control for p53 silencing experiment)…
To clarify the reviewers concern, we have now carried out statistical analyses of the cell cycle profiles in Figures 3A, 4A, and 6B. Our analyses show that there is a statistically significant (P<.05) G2 arrest in response to 100nM flavopiridol and in response to 100nM flavopiridol with 5µM 4OH-Tam in all these experiments. The P-values for all three panels, Figure 3A, 4A, and 6B have now been included within the figures and manuscript text (figures 3A, 4A, and 6B, manuscript pages 11 and 14). However, when p53 is silenced, the cell cycle profile is similar to that of untreated cell cycle profile and is not statistically significant (p=1.000). Therefore, it is clear that there is no G2 arrest when p53 is silenced.

Nevertheless, as the reviewer suggested, the data on apoptosis are very convincing and per the reviewer’s suggestion we have further emphasized the induction of apoptosis by flavopiridol and 4OH-Tam (versus emphasizing the cell cycle arrest) within the text describing these figures. For example, on page 11 we now state the following, “Very high levels of cell death are induced by these treatments and this likely plays the major role, compared to induction of cell cycle arrest, in inducing cytotoxicity in response to treatment with these drugs.”

3. Lack of statistics in figure 7.

The time point used for graphs 7J and 7K is 24 hrs (rather than the stated 12 hr). There was a typographical error which may have been the reason for discrepancies between the figures, which were correctly pointed out by the reviewer. We truly apologize for the confusion and have corrected this error within the manuscript text and figure legends.

Since the data and the interpretation of the data in Figure 7 have been confusing, we have added a much simplified figure to display the data with statistics in a new Figure 7 and have included the original as a supplemental figure. The data in the new figure represent caspase induction at the 24 hr time point and include error bars (±SEM) and statistical analysis (new Figure 7, panels A-D), which support our conclusions about caspase activation in response to treatment and p53-silencing. The statistical analyses (P-values) have been added to the text in order to support our conclusions.

4. p53 status in RT.

We thank the reviewer for pointing out the reference about the p53 status in RT cells. Since we do not know if p53 is indeed WT in MON cells we have modified our statement as follows in our second submission: “Most RTs express p53, however: a percentage of RTs do show mutations within the p53 gene [44-46]. Some RT cell lines express p53 at high levels or with increased nuclear distribution, however; the p53 pathway has been tested and considered to be functionally intact [42, 44]. The mutation status of p53 in MON RT cells has not been determined but the proper expression of p53 and responsiveness of p21WAF1 to p53 levels leads us to believe that the p53 pathway is intact in these cells” (page 18).

Sincerely,

Ganjam V. Kalpana
Professor