Reviewer’s report

Title: The interaction between pemetrexed, gemcitabine and irradiation: in vitro study to the cell line and schedule dependency.

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Reviewer: Wainer Zoli

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The manuscript by An Wouters at al. aims to evaluate the in vitro activity of the triple combination (MTA, dFdC and irradiation) in two cell lines from non-small cell lung carcinoma and from squamous cell carcinoma of the head and neck, using various treatment schedules.

Whilst the study is undoubtedly interesting, an in depth methodological revision is needed of the experiments carried out.

Major points

1. The authors use two cell lines derived from two different tumor histotypes. A larger panel of cell lines is needed (if possible, at least two different cell lines for each histotype).

2. In the Introduction, the authors provide a reasonably good explanation of the mechanisms of action of pemetrexed and gemcitabine. However, in the Discussion they are not totally clear on how the drugs’ mechanisms of action are connected to their reported activity, especially at molecular level. This aspect should be explained better in the text.

3. Although the authors select specific exposure times to examine the cytotoxicity of the investigated drugs, it is difficult to find, or at least to deduce, a clear rationale for these choices. For example, why did the authors utilize pemetrexed for 24 hours and gemcitabine for 1hour OR 24 hours? This should be explained in the Methods section.

4. The authors select specific exposure times to investigate cell cycle perturbations, but they do not specify why they chose such exposure times. This should be elucidated. Moreover, it would be interesting to observe what happens to cell cycle after irradiation and after the investigated sequences. This would possibly make it easier to understanding the potential molecular interaction between drugs and irradiation.

5. The cell cycle analysis on DNA fluorescence histograms is not
supported by WinMDI software. “No model or algorithm available” is clearly written in a WinMDI tutorial easily downloadable from Purdue University internet site. Cell cycle data should therefore be re-evaluated utilizing Modfit or other software that provides the correct mathematical model or algorithm. Moreover, why did the authors show two different types of images/graphs/histograms to represent cell cycle data? The same illustration type should be used (i.e., Excel histograms OR histogram plots) and a table should be added to clarify the cell cycle phase percentages.

6. It is fundamental to perform an apoptosis assay (e.g. Tunel or Ann-V assay) to confirm the observed cytotoxicity. In fact, without a specific cell death assay, it is difficult to be completely sure that the detected reduction in cell survival/proliferation is really dependent on cell death rather than on a generalized block of cell cycle/proliferation.

7. The authors state that survival rates after chemoradiation experiments were determined 7-8 days after the end of treatment, but they do not report what the cell line doubling times were. It is clear that a cell line with a very low/fast doubling time should not be utilized for an “extended” SRB assay because control cells could show confluence or senescence. This aspect should be reported and commented on.

8. No molecular/ baseline cell line profiles are shown. Thus, given the small number of cell lines analyzed, how can the authors’ generalize their conclusions or hypothesize them in a clinical setting? Moreover, although it is fair to say that the regimen is active, it is incorrect to affirm that it would be “feasible” without an in vivo approach or at least a cytotoxic assay on in vitro normal cells. These experiments should be carried out or this issue should be clarified in the text and the term “feasible” removed.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

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