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:** Elisa Giovannetti

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The present manuscript describes the triple combination of pemetrexed (MTA), gemcitabine (dFdC) and irradiation using various treatment schedules, and demonstrates, for the first time, a strong synergistic interaction produced by the schedule of 24h MTA followed by 1h dFdC, which combined with irradiation produced a clear radiosensitising effect. The results apply to two different cell lines, originating from non-small cell lung cancer (NSCLC) and head and neck cancer.

Overall, the manuscript deals with an issue of topical interest, which has not yet been the focus of many studies. Furthermore, radiotherapy, gemcitabine and pemetrexed are commonly used in the treatment of these tumours, and the fundings of this preclinical study, if further developed, might lead to clinically relevant applications.

The experiments are well conducted, and the results summarized appropriately. However there are some revisions that might clarify several points to the reader:

1. Introduction: The authors mentioned the mode of action of gemcitabine of producing a "chain termination" after its incorporation into DNA. Therefore they should be encouraged to speculate whether DNA repair might play an important role with respect to gemcitabine radiosensitizing effect on the tumour cells.

2. The paragraph on pemetrexed presents most of the basic findings related to the topic, with the exception of the fact that pemetrexed is one of the best known substrates for the enzyme folylpolyglutamate synthase (Km=9.8 microM, Habeck et al., Mol Pharmacol 1995) and it is believed that polyglutamation of pemetrexed plays a profound role in determining both the selectivity and the antitumour activity of this agent.

3. Methods and Results: the authors used the SRB assay to assess growth inhibition by antifolates. However, antifolates may increase cell size, and hence protein content of the cells. This may affect the results of the SRB assay, which is based on protein measurement. Since this does not affect all types of cells, the authors should give evidence whether their cells increase in size, which may affect the SRB results.

4. Several recent studies suggested that a main challenge of chemotherapy/radiotherapy relies on the identification of molecular markers predictive of response that may help in the selection of drugs best suited to the individual
patient. Were the cell lines used in this study characterized for tumour-related molecular abnormalities which can affect responsiveness to drugs (i.e. deoxycytidine kinase or thymidylate synthase expression levels; p53, RAS or EGFR mutations)?

5. Discussion: As correctly pointed out by the Authors “the [gemcitabine-MTA] combination has been examined in vitro with different human tumour cell lines, resulting in controversial schedule-dependent interactions.” However, they should cite the clinical study evaluating 3 different dosing schedules for MTA and gemcitabine as front-line therapy for stage IIIB or IV NSCLC. This study showed that the optimal regimen for greater efficacy and fewer severe toxicities was: Day 1: MTA (500 mg/m2) followed by gemcitabine (1250 mg/m2) and Day 8: gemcitabine (1250 mg/m2), repeated every 21 days (Adjei et al. 40th ASCO; June 5-8, 2004; New Orleans. Abstract 7070, and Ma CX, Nair S, Thomas S, et al. Randomized phase II trial of three schedules of pemetrexed and gemcitabine as front-line therapy for advanced non-small cell lung cancer. J Clin Oncol (2005) 23(25):5929–5937.). In this trial the schedule MTA gemcitabine was less toxic compared with the other sequences and was the only schedule that met the protocol-defined efficacy criteria, obtaining a confirmed response rate of 31%. Therefore, both preclinical and clinical data support the schedule MTA-gemcitabine in NSCLC.

6. In agreement with previous data, the Authors suggested that “the S phase enrichment is not the primary mechanism for radiosensitisation by pemetrexed”. Is there precedent literature suggesting other mechanisms underlying these results?

7. In order to assist the reader, the plots in Figure 2, as well as the labels should be enlarged for easy identification of the content. Furthermore, several results of the Figures are already summarized in the text and in the Tables.

Minor Revisions

Introduction: “The aim of the present study is the exploration of the cytotoxic (and not toxic) effects of combinations of pemetrexed and gemcitabine alone or combined with irradiation using various treatment schedules in two human carcinoma cell lines”.

Methods: A549 are adenocarcinoma and not “squamous lung carcinoma” cells

Results:
- The statement on data not shown (“as for example in the PANC-1 pancreatic cell line, no dose-response relationship was observed and concentrations up to 1500 µM induced only 30% cell kill (data not shown)” should be removed.
- The text on IC50 values should be corrected by substituting commas with points

Figure legends: use always MTA for pemetrexed and dFdC for gemcitabine

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.

**Declaration of competing interests:**

I declare that I have no competing interests