Author's response to reviews

Title: In vitro study on the schedule-dependency of the interaction between pemetrexed, gemcitabine and irradiation in non-small cell lung cancer and head and neck cancer cells.

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Author's response to reviews: see over
Dear Prof. Norton,

Please find enclosed the revised version of our manuscript. As far as possible, we have tried to modify our manuscript in line with the reviewers’ recommendations, in detail:

Comments of the first referee (Tina Li):

1. We agree with the reviewer that selecting two cell lines (one non-small cell lung cancer cell line and one head and neck cancer cell line) does not represent the heterogeneity and complexity seen in human cancers. As outlined in the last paragraph of the introduction, the main aim of the present study was to explore the interaction between pemetrexed and gemcitabine alone or combined with radiation using various treatment schedules. The two cell lines included in the study were selected based on previous literature indicating a potential interest for the pemetrexed-gemcitabine-radiation combination in these tumour types. The A549 NSCLC cell line was chosen given the three approved indications for pemetrexed in the treatment of these tumours. The CAL-27 SCCHN was included as radiotherapy in combination with gemcitabine is reported to be feasible and highly active in the treatment of locally advanced SCCHN.

We understand the reviewer’s concern that stating something about the “cell line dependency” of the interaction might be too preliminary when only two human tumour cell lines are included in the study. Therefore, we rephrased the title of the manuscript as "In vitro study on the schedule-dependency of the interaction between pemetrexed, gemcitabine and irradiation in non-small cell lung cancer and head and neck cancer cells”.

2. As suggested by the reviewer, there are indeed several limitations to in vitro studies that limit the direct translation of this work to the clinical arena. Nevertheless, we are convinced that laboratory studies may contribute to the optimisation of chemo-radiation protocols in the clinic in
at least two different ways. Firstly, in vitro studies can provide an important platform for selecting drugs that present a potential for radiosensitisation. Secondly, in vitro studies may help to improve the time schedule of chemo-radiotherapy regimens. In the present paper, we propose combined treatment assays, which measured the strength of the cytotoxic and radiosensitising effect of the triple combination of pemetrexed, gemcitabine and radiation. Results from our in vitro model suggest that the sequence 24h pemetrexed $\rightarrow$ 1h gemcitabine $\rightarrow$ radiation is the most rational design. Importantly, synergism was observed over the whole dose range (0-8 Gy), and was most obviously present at clinically relevant radiation doses (0-2 Gy). Though extrapolation of in vitro data to the clinic should be considered with caution, we believe that these experiments provide a strong experimental basis for future development of this triple combination in an in vivo and clinical setting.

3. Cell cycle data were re-evaluated and, as suggested by the third referee, FlowJo software was utilized. This software program provides a mathematical model to assess the fraction of cells in G$_1$, S and G$_2$/M. Previously, the cell cycle analysis indeed was subject to a more subjective interpretation, as with the WinMDI software, the boundaries of each cell cycle phase were positioned by the investigator himself. The objective re-evaluation of the cell cycle data using FlowJo software confirmed our previous results. Therefore, we judged it not absolutely required to implement a western blot analysis.

4. We strongly agree with the reviewer that evaluation of molecular and cellular mechanisms explaining the pemetrexed-gemcitabine-radiation interaction would be highly interesting to study more in depth. We realise that, by presenting just cell survival data, we only partially met the reviewer’s expectations of the manuscript.

However, the main goal of the study presented in this paper, was to focus on the schedule-dependency of pemetrexed and gemcitabine alone or combined with radiation. Given the reported radiosensitising potential of both gemcitabine and pemetrexed, this study, for the first time, describes a preclinical study evaluating the triple combination of pemetrexed, gemcitabine and radiotherapy. Obviously, with the schedule-dependency of in vitro interaction between pemetrexed, gemcitabine and radiation now being analysed, a more in depth study of the underlying molecular mechanisms is indeed our next research goal. Because there is a role of the cell cycle effect of gemcitabine for radiosensitisation, we started including some cell cycle data, and showed that the differences in radiosensitisation observed when pemetrexed was combined with radiation could not be explained by the pemetrexed-induced accumulation of cells in the S phase of the cell cycle.

At least in our opinion, further investigation of induction of apoptotic cell death and modulation of the expression of drug targets and signal transduction molecules would be beyond the scope of this paper. Therefore, we did not include additional experiments in this manuscript. However, as stated, we will surely elaborate on this highly relevant remark in a next study.

5. As advised by the reviewer, two papers published by Adjei et al evaluating the effect of different sequences and schedules of gemcitabine and pemetrexed in solid tumours, including NSCLC, are now mentioned in the manuscript (paragraph 6 of the discussion).
"In the clinic, a phase I trial in patients with advanced solid tumours suggested that the sequence of gemcitabine administered on days 1 and 8 with pemetrexed administered on day 8, 90 minutes after gemcitabine was well tolerated and recommended for further study [46]. However, a few years later, the same research group conducted a phase II trial of three schedules of pemetrexed and gemcitabine as front-line therapy for advanced NSCLC. In this trial, the pemetrexed-gemcitabine schedule was less toxic compared with other sequences and, by obtaining a confirmed response rate of 31%, was the only schedule that met the protocol-defined efficacy criteria [47]. As such, both preclinical and clinical data support the sequential pemetrexed-gemcitabine schedule in NSCLC.”

**Comments of the second referee (Elisa Giovannetti):**

**General comments**

1. As suggested by the reviewer, additional data on the role of DNA repair with respect to the radiosensitising effect of gemcitabine were included (paragraph 4 of the background).

"Recently, Pauwels et al could grant a role for cell cycle perturbations and activation of the extrinsic apoptotic pathway in the radiosensitising effect of gemcitabine [10]. On the other hand, it has been suggested that radiosensitisation by gemcitabine may be primarily explained by the significant inhibition of DNA repair following combined radiation and gemcitabine treatment. DNA repair pathways using short DNA patches, such as non-homologous end joining and base excision repair, are thought not to play an important role in gemcitabine-mediated radiosensitisation [9,11]. Instead, homologous recombination, a long-patch DNA repair pathway, has been argued to be the target for gemcitabine to enhance cellular radiosensitivity [9]. Moreover, the role of the mismatch repair (MMR) system, an intermediate-patch DNA repair pathway, may be of relevance [12]. A dramatic increase of nucleotide misincorporations in gemcitabine-treated (MMR deficient) cells was demonstrated, presumably due to dNTP pool imbalances (particularly dATP depletion) [8,13]. Van Bree et al showed that MMR proficiency reduced radiosensitisation after 24h incubation with a low dose of gemcitabine, suggesting that the MMR status might affect the recovery from gemcitabine treatment [14]."

2. As advised, we now mention the fact that pemetrexed is one of the best-known substrates for the enzyme folylpolyglutamate synthase (paragraph 6 of the background).

"Pemetrexed enters the cell mainly by a reduced folate carrier system. Once inside the cell, pemetrexed is an excellent substrate for the enzyme folylpolyglutamate synthase (FPGS) [20], which rapidly converts pemetrexed to its active polyglutamate derivatives that have a substantially higher potency for inhibition of TS and GARFT [21]. It is believed that polyglutamation of pemetrexed plays a profound role in determining both the selectivity and the antitumour activity of this agent."

3. It has indeed been reported that DNA damaging agents can also produce morphological changes of cells, such as increased cell size and therefore protein content (Van Nguyen et al, J. Exp. Med. 2007). As indicated by the reviewer, this might result in an overestimated viability obtained by the SRB assay (RISTIC-FIRA et al, J. Exp. Clin. Cancer Res. 2009). We recognize that we cannot give evidence whether or not the A549 and CAL-27 cells increase in size when incubated with the antifolate pemetrexed. Microscopically, we could not observe any morphological changes of the cells. In the literature, no previous data on the influence
of antifolates on the cell size of CAL-27 cells are available. Regarding the A549 cell line, previous growth inhibition experiments using the SRB assay were performed to determine the cytotoxicity of pemetrexed, LY309887 and lometrexol (Smith et al, Br. J. Cancer 2000; Lu et al, Clin. Cancer Res. 2000; Smith et al, Clin. Cancer Res. 2001; Lu et al, Clin. Cancer Res. 2001). All of these studies indicated that the antifolate-induced growth inhibition was determined using the SRB assay as previously described (Skehan, J. Natl. Cancer Inst. 1990), without mentioning any correction for increased cell size. As such, we assume that incubation with antifolates does not affect the size of A549 cells.

4. In the 7th and 8th paragraph of the discussion, we now explore in more detail the possible tumour-related molecular abnormalities that might affect the responsiveness to gemcitabine-pemetrexed combinations. For the reviewer’s interest, tumour-related molecular characteristics of A549 and CAL-27 cells are presented in the table below.

<table>
<thead>
<tr>
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<th>A549</th>
<th>CAL-27</th>
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<tbody>
<tr>
<td>p53</td>
<td>wt</td>
<td>mt</td>
</tr>
<tr>
<td>dCK activity</td>
<td>6.02 nM/h/mg protein</td>
<td>5.02 nM/h/mg protein</td>
</tr>
<tr>
<td>EGFR</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>K-ras</td>
<td>mt</td>
<td>wt</td>
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"In A549 cells, it has been demonstrated that pemetrexed, at its IC\textsubscript{50} and IC\textsubscript{75} levels, significantly upregulated the hENT1 carrier, potentially facilitating gemcitabine cytotoxicity [22]. Moreover, being an inhibitor of de novo purine biosynthesis (because of the blockade of the key enzyme GARFT), pemetrexed was shown to increase the expression of dCK as a compensatory mechanism [22]. The dCK activity of untreated A549 and CAL-27 cells was reported to be highly comparable (resp. 6.02 and 5.02 nmol/h/mg protein) and a weak positive correlation between dCK activity and the radiosensitising effect of gemcitabine has been reported [48], suggesting that enhancement of hENT1 and dCK expression by pemetrexed in the pemetrexed \rightarrow gemcitabine sequence strongly supports this combination.

In addition, several studies showed that TS expression is significantly correlated with pemetrexed sensitivity both in a preclinical and clinical setting [22,49]. Functional inactivity and mutations of p53 were shown to differentially affect the expression and activity of TS [50], potentially influencing the response of A549 (wt p53) and CAL-27 (mt p53) cells to pemetrexed-based treatment. Nevertheless, different conclusions regarding the relationship between functional p53 status and sensitivity to pemetrexed have been obtained, possibly depending on the phenotypic/genotypic background of the model system used [29,51-53]. Similarly, the role of p53 on the ability of gemcitabine to induce a cytotoxic and radiosensitising effect is not yet completely elucidated [6,54,55], making further mechanistic unravelling of the pemetrexed-gemcitabine-radiation combination highly warranted."

5. As advised by the reviewer, two clinical studies evaluating the effect of different sequences and schedules of gemcitabine and pemetrexed in solid tumours, including NSCLC, are now mentioned in the manuscript (paragraph 6 of the discussion; see also response to comment 5 of the first referee).

6. At present, the cellular or molecular mechanisms underlying the radiosensitising effect of pemetrexed have not yet been elucidated. It has only been demonstrated that induction of apoptotic cell death is not
responsible for the radiosensitisation. This is now mentioned in the 5th paragraph of the discussion.

"Moreover, tumour cell apoptosis was not found to be responsible for pemetrexed-induced radiosensitisation in human colon carcinoma cells [43]. Thus, the differential radiosensitisation induced by pemetrexed cannot be explained at present. A number of causes appear conceivable (such as differences in drug toxicity levels, growth characteristics of the cell lines investigated, levels of drug-inhibited enzymes, or intracellular pemetrexed polyglutamation), and further assessment of the molecular mechanisms underlying the radiosensitising potential of pemetrexed seems crucial.

7. We enlarged the DNA histograms presented in figure 2. As suggested by the third referee, the histograms showing the cell cycle phase percentages were deleted and replaced by a table (table 2).

Minor revisions

1. As suggested, we added “(and not toxic)” to the sentence describing the aim of the study.

2. The A549 cell line indeed originates from a lung adenocarcinoma and is not a squamous carcinoma cell line. This mistake was corrected.

3. In the paragraph on the cytotoxicity of pemetrexed alone, the statement “data not shown” was removed.

4. In the text on IC50 values, commas were substituted with points.

5. In the figure legends, MTA was always used for pemetrexed and dFdC for gemcitabine.

Comments of the third referee (Wainer Zoli):

1. As pointed out in the response to comment 1 of the first referee, we understand that selecting two cell lines (one non-small cell lung cancer cell line and one head and neck cancer cell line) does not represent the heterogeneity and complexity seen in human cancers. As outlined in the last paragraph of the introduction, the main aim of the present study was to explore the interaction between pemetrexed and gemcitabine alone or combined with radiation using various treatment schedules. The results report data on the cell survival of (1) pemetrexed combined with radiation; (2) pemetrexed combined with gemcitabine; (3) pemetrexed combined with gemcitabine and radiation, each using different treatment schedules. As previous studies on the interaction between either pemetrexed and gemcitabine or pemetrexed and radiation often included no more than two cell lines (see table below), it is, at least in our opinion, not fundamental to include a larger panel of cell lines.
2. As discussed in general comment 4 and 6 of the second reviewer, we now explore in more detail the possible tumour-related molecular mechanisms that might contribute to the interaction between pemetrexed and gemcitabine, as well as to the radiosensitising effect of pemetrexed.

3. The rationale for the specific exposure times to investigate the cytotoxicity of pemetrexed-gemcitabine combinations is now discussed in the Methods section ("Cytotoxicity experiments").

"The experimental conditions adopted in this study, including time of exposure to gemcitabine and pemetrexed, are similar to those selected in previous studies [10,22,29,30]. In simultaneous experiments, treatment exposure time was 24h. To reduce the variation in drug efficacy associated with time of drug addition relative to plating time, the sequential schemes examined the effect of both a 1-h and a 24-h gemcitabine exposure on the cytotoxicity of pemetrexed. Since several clinical studies have indicated that administration of gemcitabine and pemetrexed immediately after each other is well tolerated and clinically active [31-33], no wash out with drug-free medium was included in the sequential treatment schedules."
4. The specific exposure times to examine pemetrexed-radiation combinations and cell cycle perturbations are now explained in the Methods section.

Chemoradiation experiments:

"The same time intervals as in a previous study investigating the schedule-dependency of gemcitabine treatment combined with radiation were included [34]."

Cell cycle experiments:

"Hence, cells were treated with pemetrexed using the same treatment schedules as adopted in the pemetrexed-radiotherapy experiments, but without subsequent radiation. As such, cell cycle perturbations at the time of irradiation were measured."

5. Cell cycle data were re-evaluated and, as suggested, FlowJo software was utilized. This software program provides a mathematical model to assess the fraction of cells in G₁, S and G₂/M. Previously, the cell cycle analysis indeed was subject to a more subjective interpretation, as with the WinMDI software, the boundaries of each cell cycle phase were positioned by the investigator himself. The objective re-evaluation of the cell cycle data using FlowJo software confirmed our previous results. Moreover, the histograms showing the cell cycle phase percentages were deleted from figure 2 and replaced by a table (table 2).

6. We agree with the reviewer that the colorimetric SRB assay does not distinguish between viable and dead cells. With the schedule-dependency of the in vitro interaction between pemetrexed, gemcitabine and radiation now being analysed, a more in depth study of the underlying mechanisms of the interaction is obviously our next research goal. At least in our opinion, further investigation of induction of apoptotic cell death would be beyond the scope of this paper. Therefore, we did not include additional apoptosis experiments in this manuscript. However, as stated, we will surely elaborate on this highly relevant remark in a next study.

7. The cell doubling times of A549 and CAL-27 cells (28h and 33h, resp.) are now reported in the Methods section ("Cell lines"). It has previously been reported that, when sufficient time is allowed for delayed radiation-induced cell death and when exponential cell growth is guaranteed during the assay, the results of the colorimetric SRB assay are highly comparable to those obtained with the clonogenic assay in moderately radiosensitive cell lines (Pauwels et al, Cancer Chemother. Pharmacol. 2003). Therefore, in our experiments, cells were allowed to undergo at least six doubling times after irradiation. A549 cells were incubated for 7 days and CAL-27 cells for 8 days after radiation before survival assessments by the SRB assay were performed. Plating densities assured exponential growth during the experiments, so that control cells did not show confluence at the time of cell fixation and staining.

8. In the text, the term "feasible" was only used to describe the combination of radiotherapy with gemcitabine in the treatment of locally advanced SCCHN (Specenier et al, Ann. Oncol. 2007), and was not used to describe the presented results on the pemetrexed-gemcitabine-radiation combination. We strongly agree with the reviewer that more in depth studies of the molecular mechanisms underlying the interaction are highly
warranted. As stated, a follow-up study will focus on this issue. In the last paragraph of the discussion, we literally say that “the 24h MTA → 1h dFdC → RT turned out to be the preferred schedule for combined administration with radiotherapy in our preclinical model system”. Moreover, as we already stressed in our conclusion, extrapolation of \textit{in vitro} data to the clinic indeed should be considered with caution. In order to emphasise this even more, we discuss this comment now in more detail in the manuscript.

Abstract:

“Results from our \textit{in vitro} model suggest that the sequence 24h MTA → 1h dFdC → RT is the most rational design and would, after confirmation in an \textit{in vivo} setting, possibly provide the greatest benefit in the clinic.”

Conclusion:

“This study characterises, for the first time, the interactions between gemcitabine, pemetrexed and radiotherapy. Results from our \textit{in vitro} model suggest that the sequence 24h MTA → 1h dFdC → RT is the most rational design. Further mechanistic unravelling of the pemetrexed-gemcitabine-radiation combination is certainly needed. As extrapolation of \textit{in vitro} data to the clinic should be considered with caution, the experiments provide a strong experimental basis for future development of this triple combination in an \textit{in vivo} setting.”

Taken together, the reviewers’ profound evaluation of our manuscript has helped us to change it in such a way that the evaluation of the pemetrexed-gemcitabine-radiation combination has become more concise. Our manuscript in its revised form has benefited from it and we hope to have convinced you and the reviewers to reconsider our manuscript for publication in \textit{BMC Cancer}.

Looking forward to a positive response,

Yours sincerely,

An Wouters