Author's response to reviews

Title: Characterization and drug sensitivity profiling of primary malignant mesothelioma cells from pleural effusions

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

Please find enclosed a revised version of our manuscript (MS: 9437319041276791) entitled “Characterization and drug sensitivity profiling of primary malignant mesothelioma cells from pleural effusions”.

The specific comments of the reviewers have been taken into account and the corresponding changes are included in the revised manuscript as follows.

I: Response to reviewer Leticia G G Leon

Major comments:
1. Not all samples contain sufficient material for testing a wide range of cytotoxic drugs, thus all drugs have not been tested on all primary cell isolates. We agree with the reviewer that this is a limitation of this study.

The method and the drug plates have been developed over time to better correspond to the clinical requirements and Pemetrexed, Carboplatin and Cisplatin were not included in the first drug plates. Nevertheless our data show an interesting individual cytotoxicity profile which warrants further attention.

The experiments were performed on primary cells established freshly after receiving them from the patients; thereby it is not possible to perform the experiments again in similar conditions for this unique patient cohort. Furthermore, we have previously shown that the effect of Pemetrexed is difficult to show in similar experimental setups (reference 16), therefore in our future experiments we plan to further limit the number of drugs tested and we aim to use modified multi-parameter arrays to measure tumour specific cytotoxicity. This is now better discussed on line 238-241 in the article.

2. We do not apply GI50 or IC50 for the calculation of the in vitro relevant concentrations. The method does not allow direct GI50 calculation for those drugs that are not effective in the highest concentration because it would require more than four dilution steps including even very high concentrations. The method is a drugs sensitivity assay (and not an extreme drug resistance assay) and it operates in the concentration ranges that are achievable in the human body. The present method was developed to be clinically useful and practical. To test drug sensitivity at concentrations that greatly exceed the in vivo toxic limits might have some theoretical interests (such as calculation of GI50, ID50 etc values) but has very limited practical relevance for the actual clinical decision making. Including very high concentration ranges would not only compromise the number of drugs that can be tested in parallel on limited amount of clinical material, but would also be incompatible with the industry standard plate printing protocols that work perfectly in the clinically relevant concentration range but have serious solubility limitations when operated at greatly aggravated concentrations.

3. The calculation of the drug efficiency has been published previously (reference 44). It is based on the different degrees of each sensitivity of each different concentrations, the
software calculated by weighted counting of the survival percentage and the drug concentration.

\[
\text{Efficiency} = \frac{((100-\text{Live}\%\ 125)\ln(125) + (100-\text{Live}\%\ 25)\ln(25) + (100-\text{Live}\%\ 5)\ln(5) + (100-\text{Live}\%\ 1)\ln(1))}{\ln(125) + \ln(25) + \ln(5) + 1}
\]

*Under the calculation:*

Where Live\% 125 is the percentage of surviving cells at maximum drug concentration in proportion to the control

Where Live\% 25 is the percentage of surviving cells at 5x diluted drug concentration in proportion to the control

Where Live\% 5 is the percentage of surviving cells at 25x diluted drug concentration in proportion to the control

Where Live\% 1 is the percentage of surviving cells at 125x diluted, minimal drug concentration in proportion to the control

The drug effect was measured at four different concentrations that are comparable with the dose range achieved in the human body. To create a single measure of the effect of the four independent measurements that represent drug response curves with variable steepness a single value measure, the killing efficiency was introduced where each measurement contribute to the final value, that is calculated as a weighted sum of the individual drug effects where the weighting factor is the natural logarithm of the drug dilution. The value is normalized on a scale of 0 to 100. This factor is more informative than the ID50 or GI50 because these later values denote only the inflection point of the drug titration curve but completely ignore the steepness of the curve. To correct this many scholars also require the measurement of ID90 or GI90 a value that is unattainable for highly drug resistant samples. Moreover characterization of drug response using two independent factors makes any comparison highly impractical. On the other hand using killing efficiency allows the incorporation of all measurements in a single figure and permits the linear ranking of all drugs based on their effect in the physiologically relevant concentration range.

The description of the calculation of the adjusted drug efficiency has been updated, see line 381-383. The software is not an open source. It is developed by the authors and it has not been tested in other laboratories.

4. We agree that an increased number of samples would definitely increase the robustness of the statistical analysis. However, malignant mesothelioma is a rear malignancy and the effusions that our hospital receives do not exceed 10/year. Many of these samples do not contain sufficient amount of malignant cells to establish primary cell cultures for subsequent cytotoxicity tests. Based on our experiences with the techniques used in this paper we now try to test effusions containing fewer cells and at the same time test tumor specific cell death caused by a lower number of selected drugs. Because of this it is not meaningful to perform additional experiments with the setup described in this paper.

**Minor comments:**
1. Specified that the indicated concentration is the highest used concentration on line 373.

The drug dilution was created as a universal four steps 5x dilution series. That means that the highest used concentration corresponds to 1x dilution followed by 5x, 25x and 125x dilutions. The highest concentration specifies the entire series, inclusion of additional values are redundant and superfluous.

2. For the selection of cytotoxic drug concentrations we used with the clinically relevant concentration as reference and the dilutions were selected to cover a broad spectrum of varying sensitivities for individual patients. We changed the way to express concentrations to 1:1, 1:5, 1:25 and 1:125.

II: Response to reviewer Elisa Paolicchi

Major comments:
1. Additional file 2 is now added containing information about patient age and gender distribution. It has to be noted that the study was not aimed at this stage as a clinical study but rather as a methodological one. No information is available about asbestos exposure.

2. An explanation for the great variability in chemo-sensitivity of primary cells has been added at line 238-241 in the article.

In most cases the effusions represent the first diagnostic material before initiation of chemotherapy treatment, thus 9 patients were chemo naive and only 3 had received previous chemotherapy. Patients with recurrent pleural effusions have received Pemetrexed and Carboplatin or Cisplatin after the first sample establishing the diagnosis. We do not have information concerning the stage of the disease for each patient. This information is now added in the manuscript; see line 319. When looking at the chemo-resistance profile patients receiving previous chemotherapy are not different from the other patients in their \textit{in vitro} resistance profile to the clinically relevant drug combinations.

3. Information is now added see line 342-345. All benign samples were derived from patients with non-malignant effusions. They included admixture of reactive mesothelial cells and inflammatory cells, without further information of their etiology and without any morphological sign of malignancy. Effusions containing benign mesothelial cells are not expected to be affected by chemotherapeutical agents in clinically relevant concentrations; however, we could observe such effect for some drugs, probably due to drug effect on dividing cells.

4. Explanation added, see line 284-288.

Minor comments:
5. Changed according to reviewer’s suggestion.
6. More recent articles are now added references 26-28; line 95.

7. See answer to Leticia G G Leon’s major comment 1.

Please also note that the corresponding author has been changed from Adam Szulkin to Katalin Dobra. We hope that this revised version will now be acceptable for publication in BMC Cancer and I am looking forward to your response in due course.

Sincerely,
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