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Reviewer Comments

The manuscript reports the results of cell culture studies that were conducted to study the effect of zinc followed by cisplatin on select malignant pleural mesothelioma cell lines. While the execution of the cell culture experiments has been described, several details regarding the clarity of some methods (FFFPE-embedding of cell blocks) are poorly described in the experimental section and must be clarified. The authors seem to be unaware of the complex hydrolysis of the cisplatin molecule in aqueous solutions, which dramatically depends on the chloride concentration (Journal of Chromatography B 772, 2002, 273-281) and has direct implications in the context of intravenously infusing patients with this anticancer drug (Metallomics 8, 2016, 1170-1176). Blood plasma, for example, contains about 105 mM chloride, while the intracellular chloride concentration is about 25 mM, which drives the hydrolysis of cisplatin to a hydrolysis product which then 'binds' to the DNA and induces apoptosis. In light of this importance of chloride in the mechanism if anticancer activity (Metallomics 6, 2014, 2126-2133) the authors must provide the chloride concentration of their cell culture medium during the cisplatin treatment as this will determine if predominantly the intact cisplatin molecule was uptaken into the cells or one if its hydrolysis products. In addition, some statements of the conclusion are speculative and are not supported by the experiments that were conducted (one cannot infer that the results which were obtained with Zn can be directly extrapolated to Cd as Zn is an essential element and Cd adversely affects cells at rather low concentrations). If these major deficiencies and some minor changes (see detailed comments below) are addressed the manuscript can be reevaluated fur publication.

Author comments: We thank the reviewer for taking the time to review our manuscript and exceedingly appreciate his suggestions.

Detailed comments:

Line 62: It should read 'offers the best'. Author comments: Edited

Line 66: It should read 'by exposure to metal ions, such as zinc'. Author comments: Edited

Line 77: Please clarify what the abbreviation 'MT2A' refers to (metallothioneine II?). Author comments: Edited

Line 76+77: 'impedes' is a strong word. It should read 'heavy metal exposure during'. Author comments: 'Impedes' was edited to "inhibits". We have adopted the wording

‘‘heavy metal exposure during’.

Line 84: It should read ‘the median’. Author comments: Edited

Line 103: It should read ‘MTs are involved in zinc homeostasis’. Author comments: Edited

Line 104: This sentence is unclear and must be reformulated.

Author comments: We deleted this sentence, as the previous sentence already explains that MTs are involved in zinc homeostasis.

Line 105/106: It should read ‘reduced zinc intake’. As far as I know if zinc intake is reduced MT expression should be down and not upregulated. Please clarify and reformulate this sentence.

Author comments: Line 105 edited. Line 106: Thank you very much for finding this error. In fact, it is downregulation of MT expression, not upregulation.

Line 111: It should read ‘underlying factor of platinum resistance’. Edited

Line 141: It should read ‘ZnSO4’ (‘4’ superscript). Author comments: Edited

Line 145: The volume of ‘Staurosporin’ that was added should be included similar to digitonin. Author comments: Edited (25 µl)

Line 149: It is unclear why DMSO was used to dissolve cisplatin as many researchers use a cisplatin solution that is intravenously injected into patients which does not contain DMSO.

Author comments: DMSO was the preferred stock solution for us, as Cisplatin shows solubility of 10mg/ml in DMSO, while only 1 mg/ml in water or saline. Furthermore, cisplatin changes to the *trans* form in aqueous solutions and is more instable as in DMSO. However, during the preparation of the dilutions in the 96-wells, we used the respective growth medium of the tested cells, which is comparable to the aqueous solution. The highest concentration of DMSO per well was 0.008 µM. It should not have any effects to the cells, but we wanted to rule out any effects and thus run a control. We clarified in the text, that DMSO was only used for the preparation of stock solutions (line 149 ff.).

Line 152: Please briefly explain what the difference is between technical and biological triplicate.

Author comments: Explained as follows: A technical triplicate is performed during one measurement, as the condition is measured three times. The measurement was repeated twice, to generate a biological triplicate (experiment set up and measurement were performed three times in a row).

Line 155: It is unclear why cells were embedded in cell blocks (the purpose is inly stated later on, but should be presented at the beginning of the paragraph). In line 157 it is stated that cells were incubated for 48 or 77 h with zin, but in line 143 it is stated that cells were incubated for 6 hours. Please explain the difference between these experiments.

Author comments: We added an explanation, why cells were embedded in FFPE blocks in line 159 ff.

In cell state analysis, we treated the cells also with cisplatin. To let the cells, adhere to the bottom of the 96-well plate, we incubated them for 6 hours and after that, we added cisplatin.

No cisplatin treatment was performed on cell culture flasks (line 157), as we aimed to embed the cells in FFPE blocks for subsequent gene expression analysis of MTs. With this experimental setup we wanted to investigate the difference in gene expression with different zinc concentrations after 48 h and after 77h of incubation. As we did not aim to analyze apoptosis of cells in this setup, we did not treat cells with cisplatin. Furthermore, we would have not enough cells to embed after treatment with cisplatin. This is not clear in the text. Therefore, we added the information in line 188.

Line 156: Please replace 'Mio' by 'million'. Author comments: Edited

Line 182: 'in treated cell lines' is unclear. Does this refer to just Zn-treated and/or Zn- and cisplatin treated cells. Author comments: We noticed this when editing line 157, which is why we have already changed this sentence so far (now line 188)

Line 211: The 'false discovery rate' is unclear and should eb briefly explained what this means and why it was employed. Author comments: We explained the 'false discovery rate' in line 216 and 218.

Line 215: Since 'be observed after 48 h' is not backed by any evidence whatsoever, it is suggested to add 'data not shown' after this statement. It is suggested to state 'expression by Zn could already'. Author comments: Edited

Line 216: 'triangleCP' is unclear and it must be explained what this refers to.

Author comments: We thank the reviewer for this observation. This was an error by us, as we aimed to write $2^{-\Delta Cp}$ instead of ΔCP. However, we added an explanation of the $2^{-\Delta Cp}$ in line 223.

Line 218: A statement about the results for cell line H2452 must be added. It is unclear why no results for cell line MRC5 (data for this cell line are shown in Figure 2) are reported?

Line 235: It should read 'NCI-H2052 require 50-70 µM of Zn to achieve a notable reduction in the apoptosis of cisplatin.'. Author comments: Edited (now line 243f.)

Line 237: It should read 'correlated with the increasing concentration of Zn'. Author comments: Edited (now line 245)

Line 249: After 'be detected in the serum' a relevant reference must be provided. The abbreviation 'TNM' is unclear and must eb explained.

Author comments: The reference provided at the end of the sentence also includes the statement that MTs could be detected in serum. In addition, we added another reference, providing this statement and placed it after 'be detected in the serum' (now line 257).

TNM is a classification of malignant tumors describing the size of the tumor and if it

has already spread. T describes the tumor size, N the spread of cancer nerbay lymph nodes and M describes metastases (spread of cancer into other organs of the body). As it is not crucial to describe TNM classification in detail in our study, we explained it shortly as 'classification system of malignant tumors' in line 258.

Line 251: 'enriched source' must be reformulated for which specific type of disease MTs may represent biomarkers.

Author comments: We edited the sentence as follows:'… MTs act as an enriched source of biomarkers in malignant tumors.'

Line 257: 'MTs have been shown to regulate inhibition of apoptosis' is cryptic/unclear. More detail must be provided so it is clear to the reader how MTs are linked to apoptosis at a molecular level.

Author comments: This sentence builds on the previous one about oxidative stress reduction. We have reformulated the sentence and hope that the context is more clearly now.

Line 269'270: It should read 'concentration was also observed by'. Author comments: Edited (now line 279).

Line 273: It should read 'overexpression thus generating'. Author comments: Edited (now line 282).

Line 275/276: Thee statement 'this cell line seems to show no change in apoptotic effect' does not accurately describe Figure 2B and must be reformulated.

Author comments: We reformulated the statement and hope it is more understandable now. (Now line 284 ff.)

Line 286: It should read 'strongly influenced by zinc'. Author comments: Edited (now line 297).

Line 289: 'Without, it would lose' must be reformulated to make sense.

Author comments: We reformulated the statement as follows: 'Without this complex domain, it would lose its DNA binding ability and could not induce cell cycle arrest or mediate signals for apoptosis'. (Now line 300).

Line 291: It should read 'Zinc homeostasis is a complex'. Edited (now line 302).

Line 292: Please remove 'until now'.

Author comments: We removed 'until now' but added 'processes being incompletely understood'. (Now line 302).

Line 299: Please reformulate 'others than zinc'.

Author comments: We reformulated as follows:'… to all heavy metals, others additional metals beside than zinc may be considered…' (now line 310 f.).

Line 316-318: This sentence is speculative as all experiments were conducted with Zn, but not Cd.

Author comments: We are unsure if the sentence is meant: 'The observed effect by zinc supplementation in this study could be speculated to be an important issue for MPM

patients smoking cigarettes, which contain heavy metals like cadmium.' In fact, this is speculative, and therefore we stated that we 'speculate' it, based on the observations during zinc supplementation in this study. Since MTs show a high binding affinity to all heavy metals (incl. cadmium), we speculate, that smoking may have severe effects during cisplatin therapy. However, this has to be considered in further studies.
To clearly delineate that this is speculation, we have changed the paragraph slightly as follows:' homeostasis processes paving the way for clinical application.
Based on the observed effect by zinc supplementation in this study, it could be speculated to be an important issue for MPM patients smoking cigarettes, which contain heavy metals like cadmium. Smoking during a platinum-based chemotherapy thus may lead to an early therapy failure. However, this has to be validated in further studies.

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Figure 1: The authors must explain why no standard deviation bars are provided for the bars which correspond to the expression of MT2A in the various cell types as a function of the Zn concentration in the cell culture medium. The connotation of the y-axis is unclear and must be explained in the figure caption. It is unclear why no expression of MT2A is provided for the H2452 cells since data for all other cell types were measured. The fact that the data were measured 77 h after zinc supplementation should be mentioned early on in the caption and not toward the end.

Author comments: We thank the reviewer for these important suggestions. We added the error bars in Figure 1. We also explained the y -axis. The expression of MT2A is provided for the NCI-H2452 cells. However, we let out the MRC-5 cells, as these were irrelevant and only served as a healthy control. We focused on MPM cell lines in this Figure. We also mentioned the fact that the data were measured after 77h after zinc supplementation earlier in the caption (line 448 ff.).

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Figure 2: The cisplatin concentration that was used to induce apoptosis must be provided in the figure caption. The figure caption needs to clearly explain how many h the cells were exposed to Zn and then how long they were exposed to cisplatin.

Author comments: We thank the reviewer for raising these points. We added the information, that measurements of the apoptotic response were performed 48 h after treatment with cisplatin, and also added the used concentration of cisplatin (10 µM). We explained that cisplatin was added to cells after 6 h of incubation with varying zinc concentrations (line 456 ff.).