Author's response to reviews

Title: TUCAN/CARDINAL/CARD8 and apoptosis resistance in non-small cell lung cancer cells

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Version: 2 Date: 27 April 2006

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Authors’ response to reviewers comments

Title: TUCAN/CARDINAL/CARD8 and apoptosis resistance in non-small cell lung cancer cells. Manuscript ID: 1584855777941573

Reviewer: Dr. R. Ramesh
We thank the reviewer for the valuable comments that allowed us to improve the manuscript.

Reviewer’s concern:
1. “Figure 1-The authors in the results section (pg#9) and discussion (pg#13) state that procaspase-9 activation was not seen in H460 and GLC4 cells after CDDP treatment but could see only after addition of cytochrome c and dATP. Although the statement can be assumed to be somewhat true, it is not clear whether the activation observed in cells treated with CDDP alone was significant compared to untreated cells. Per Figure 1C, CDDP treatment alone induced 1.5 times cleavage compared to untreated control (0.5 times increase). If it is significant then the wording has to be rephrased. Additionally, in Figure 1B semiquantitative analysis of the change in caspase-9 levels would be of help in examining the results of Figure 1C. For example, if change/increase in caspase-9 levels in Figure 1B at 48h (time point common to both Figure 1B and 1C) is 1-2 fold in cells treated with cyt.c/dATP compared to those that were not, then the caspase-9 activity in Figure 1C would correlate as there is an approximate 2 fold increase in activity. The authors should do this comparison and rephrase the sentence in the results and discussion section. This may also change the interpretation for this figure though not the final conclusion.”

Authors’ response:

Statistical analysis revealed that the suggested 0.5 fold increase in the activation of caspase-9 observed in H460 cells treated with cisplatin for 48 h towards the untreated control is statistically not significant (p=0,06). The p-values have now been included
and this point has been further addressed in the material and methods, results and discussion sections (page 9, lines 1-4; page 10, lines 4-6; page 13, lines 13-14).

Furthermore, the cleavage of caspase is not a quantitative assay and does not directly correlate with caspase activity also due to factors like IAPs that can modulate caspase activity downstream of cleavage. Therefore, in our opinion it is not possible to compare the values obtained by semiquantitative analyses of levels of caspase-9 cleavage in Figure 1B to levels of caspase-9 activity in Figure 1C.

2. “Figure 4- the authors in the results section for figure 4A (pg. 12) indicate that mRNA for TUCAN in cells stably expressing TUCAN SiRNA was inhibited by 70%. However, per Figure 4A it appears that inhibition of mRNA is not greater than 60%. This needs to reexamined and corrected. Additionally, it is recommended that the authors include a figure showing downregulation of TUCAN protein expression in addition to mRNA inhibition”.

Authors’ response:
We reexamined the mRNA level of TUCAN in H460 cells stably transfected with TUCAN siRNA and found in fact a 65% inhibition of mRNA TUCAN expression level. This has been corrected in the Results section and in Figure 4A and (page 12, lines 8-10).

Following the reviewer’s suggestion, we included the data showing the downregulation of TUCAN at the protein level on Western blot in an additional Figure 4B and changes in the Material and Methods and Results sections were made accordingly (page 7, lines 16-18; page12, line 9-10) and in the Legend for figures (page 20, line 9). Former Figure 4B has been renamed into Figure 4C (page12, line 15).

Minor Concerns:

1. “At what time point the CDDP was added to the cells and how long the cells were treated prior to analyses for SubG1 population (Fig. 4B)?

Authors’ response:
The cells were seeded 24h before treatment and after that the cisplatin was applied for 48h prior to SubG1 analyses. This information has been inserted in the Material and Methods section of the manuscript (page 5, lines 8-12).

2. “It would be better if the authors provide a rational in the methods or results section for using IC80 rather than IC20 or IC50 in the study”.

Authors’ response:
We used the IC80 concentration of cisplatin (7µM) that inhibits 80% of tumor growth in MTT assays. The use of this concentrations in the experiments is somewhat arbitrary, but is routinely used in our laboratory because it elicits clearly detectable levels of apoptosis in the cell lines used and at the same time represents a clinically relevant concentration of cisplatin, which in plasma samples from patients’ is around 10µM after 1h of drug administration (Korst et al, Clin Cancer Res, 1998). To address this point, we have now included a sentence on the issue in Material and Methods section (page 5, lines 10-12).

Discretionary Revisions:
“Have the authors tested treating the cells stably expression TUCAN siRNA at IC20 or IC50 rather than IC80 as the differences in restoration of CDDP sensitivity following downregulation of TUCAN might compulsory at IC80”.

Authors’ response:
For reasons mentioned above, we selected IC80 concentrations. If TUCAN would have any relevance in modulating apoptosis this would have been revealed in the experiments.
Reviewer: Dr. P. Daniel

We thank the reviewer for the valuable comments that allowed us to improve the manuscript.

1. "In the figure 4, efficient shRNA knockdown of CARD8 protein expression should be documented by Western blot analysis”.

Author’s response:
Following the reviewer’s suggestion, we included the data showing the downregulation of TUCAN at the protein level as assessed by Western blotting. Results are depicted in an additional Figure 4B and changes in the Material and Methods and Results sections were made accordingly (page 7, lines 16-18; page12, line 9-10) and in the Legend for figures (page 20, line 9). Former Figure 4B has been renamed into Figure 4C (page12, line 15).

2. “Figure 4: In the same vein: processing of caspase-9 and caspase-9 activity should be shown in the transfectants”.

Authors’ response:
We have now included results obtained in caspase-9 activity assays in an additional figure 4D (page 20, line 16-18), which did not show alterations in cisplatin-induced caspasase-9 activation between the parental and transfected cells. This is in agreement with our conclusion that TUCAN is not involved in modulating caspase-9 in these cells. Accordingly changes have been made in the Material and Methods and Results sections (page 9, lines: 1-4; page 12, line 15-18).

3. “Figure 4: In the same system, the authors should provide evidence that the inhibition of caspase-9 has an impact on drug sensitivity to cisplatinum induced apoptosis. This could be done either by LEHD-fmk peptide inhibitor or cysteinyl mutant of casp9 that would interfere with apoptosome function. Such a positive control for caspase-9 function in this system is mandatory. APAF-1 knockdown should be considered as well.”
Authors’ response:
In this system caspase-9 activity is already suppressed and considering its apoptotic function can be assumed to have an impact on drug sensitivity/response (Ferreira et al. Cancer Res., 2001). Indeed, one could debate about redundancy in this system and that other caspases or caspase-independent mechanisms may compensate for this. This, however, does not alter the fact that apoptosome-dependent caspase-9 activation is suppressed, and it is our primary interest and aim to elucidate the molecular cause of this inhibition. A further suppression of caspase-9 with a peptide inhibitor (LEHD), which by nature have limited specificity, in cells that already demonstrate a blockade in caspase-9 would not be helpful in this context. Moreover, in the LEHD-AFC assays no activity was detected. A number of reports have elaborated on, and shown, the involvement of caspase-9 and the apoptosome in drug-induced apoptosis (see for example Liu JR et al., Cancer Res, 2002; Soengas MS et al., Nature, 2001). Studies to further investigate the role of the apoptosome, Apaf-1 and caspase-9, in our system in relation to drug sensitivity in a broader context may be of interest in light of what has already been reported but is outside the scope of the current study.

4. “Figure 4: the negative data reported here would be become far more reliable if the authors could show that CARD8 knockdown does not interfere with other stimuli that trigger the mitochondrial pathway apart from cisplatinum.”

Authors’ response:
The DNA-damaging agent cisplatin is well known for its potent activation of the mitochondrial apoptotic route. Following the reviewer’s suggestion, we also demonstrated that the downregulation of TUCAN did not interfere with another stimulus that targets the intrinsic apoptotic pathway, the topoisomerase inhibitor etoposide (VP16). The H460-derived stable transfectants were treated with 2,5 µM etoposide for 48h and the amount of cell death was assessed by PI staining assay. As shown in the added Figure 4C, the level of apoptosis in transfectants was similar to the level in H460 wild type cells and ranged between 20-25%. Accordingly changes have been made in the Material and Methods (page 9, line 1-4) and Results (page 12, line 10-15) and in the Legend for figures (page 20, lines 14) sections.
5. “The authors should consider corroborating their data in a second, independent cell line system.”

Authors’ response:
In line with this suggestion, we have studied the interaction between TUCAN and procaspase-9 in SW1573 cells that express high level of TUCAN (See Figure 2). As we described in manuscript (page 11, line 18-21), we could not detect an interaction between these proteins in this cell line, in line with our observations in H460 cells.

Minor Concerns:
1. Na3Vo4: Capitalize the „O”
   This has been corrected (page 8, line 7)

2. Primers and probe for real time PCR should be indicated.
   This has been indicated (page 6, lines 18-21)