Author's response to reviews

Title: Anthracyclin, proteasome activity and mult-drug-resistance

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Author's response to reviews:

Dear Sir,

we have taken all comments from the reviewers into account, would like to thank both reviewers for their valuable input, and hope that the manuscript is now acceptable for publication.

We would like to answer to the specific comments as follows:

Reviewer 1

Minor Essential Revisions
1. The discussion section is highly focused. To further improve the discussion, additional possible mechanisms like interfering with the topoisomerase II activity to restore chemosensitivity of tumor cells by inhibition of the proteasome by anthracyclines should be discussed in brief (e.g. Ogiso Y, Tomida A, Lei S, et al. Proteasome inhibition circumvents solid tumor resistance to topoisomerase II-directed drugs. Cancer Res 2000; 60: 2429-34.)

Added.

Editorial changes
1. Page 6 line 9; change to "German collection of microorganism and cell cultures"

Changed.

2. Page 6 line 18; change to "were added"

Changed.

3. Page 7 last paragraph; Drug accumulation assay. Although cited as described elsewhere, a brief description of the method for determining drug concentration in cytoplasm and nucleus as displayed in figure 3 should be included.

Added.

4. Page 8 line 11; delete the sentence "ECV304 cells were maintained......." and insert in the following sentence "Twelve hours.... ECV 304 cells...."

Done.

5. Page 9 line 4-6; better so say "Experimental data are presented as mean +/- standard error of the mean from at least three independent experiments."

Changed.

6. Page 11 line 19; "accumulation of daunorubicin".....

Added.

7. Page 13 line 16; please complete the sentence "...can both be activated ???"

Completed.

8. Page 13 line 19; delete "possible"

Deleted.
9. Figure 1 and figure 3A. If possible, significances and p-values should be included in the figures and legends

Reviewer 2
1) What is the rationale for a possible activity of anthracyclines as proteasome inhibitors? The background provided in the Introduction section of the manuscript describes the ability of P-glycoprotein inhibitors such as cyclosporin and ritonavir to act also as proteasome inhibitors. However anthracyclines are substrates for P-glycoprotein. The hypothesis for the experimental approach is stated in the Results section but it might be more appropriate to introduce it earlier in the text and to provide references to support such an approach.

This part of the manuscript was moved to the Introduction Section.

2) What is the level of accumulation of daunorubicin in KB8.5 cells incubated with verapamil and daunorubicin? This result would allow a better comparison between the inhibitory effects of the different compounds used to inhibit both the proteasome and P-glycoprotein.

Data from the literature was added to the Discussion to address this point.

3) The results of the incubations with anthracyclines, verapamil and MG-132 do not support the statement presented in the Abstract and the Discussion sections in which the authors suggest that P-glycoprotein and proteasome have overlapping substrate specificities. Could the inhibition of the proteasome be affecting P-glycoprotein expression at the molecular level?

The Abstract and Discussion sections were changed.

The proteasome appears to be involved in the post-translational regulation of P-gp. However, since P-gp has a long half-life (14-24h) the impairment of P-gp function at 45 minutes is more likely a direct effect. This aspect is now addressed in the Discussion section.

4) What is the effect on P-glycoprotein activity provoked by MG-132 alone?
In general, most inhibitors can be considered as high-affinity substrates, which compete with low-affinity substrates for binding. Answering this question seems difficult since P-gp function is usually assessed by its function as a pump-protein. Therefore, this question could only be answered in an ATPase-assay using a highly-purified P-gp fraction if MG-132 interferes with the ATP-binding site of P-gp.

5) What is the cytotoxicity of MG-132 in ECV304 and KB8.5 cells? The levels of apoptosis are evaluated after 24 hours, but the accumulation of daunorubicin is analyzed after 45 minutes of treatment with MG-132.

At 45 minutes, no toxicity can be observed. This was added to the Results Section.

6) Do all these inhibitors have some structural properties in common that could suggest a general mechanism of action on both proteasome and P-glycoprotein?

No. Ritonavir, cyclosporin A, anthracyclines, Verapamil and MG-132 are structurally unrelated. However, eliminating a large number of structurally unrelated drugs from the cells, it is a known feature of P-gp.

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Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

1) The authors state in the Introduction section that vinblastine is able to inhibit P-glycoprotein, this affirmation is not supported by the literature cited in the manuscript.
Corrected.

2) What is the reason to use either doxorubicin or daunorubicin to evaluate the accumulation of the drug in the presence of a proteasome inhibitor?
Both drugs were used because they give a fluorescent signal.

3) The manuscript needs English revision
The manuscript was carefully revised for orthographical and grammatical errors.

Sincerely

F. Pajonk