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Point-by-point response to reviewers' comments

We again thank all reviewers for their excellent additional recommendations, great input and helpful suggestions. By including these points into our manuscript, it helped to improve the scientific power of our paper. We added our responses below the reviewer's suggestion (blue) and highlighted all novel changes in red in the manuscript.

Reviewer 1

1. Another method needs to be used for apoptosis detection.
2. Without any mechanistical study data and in vivo data, the authors at least need to show whether modulation (for example knock down of Top I or II) of TopI and Top II expression or the cells with different TopI and TopII expression response differently to the P8-D6.

Response:

1. We thank the reviewer for this remark and constructive feedback. A second method to prove apoptosis induction is indeed an important prerequisite to confirm the cellular effectiveness of P8-D6. Aware of this important point, we validated our caspase 3/7 method to quantify apoptosis by a second method using flow cytometry and Annexin V/7AAD staining as a complementary method in our previous study (doi: 10.1177/17588359211059896: Figure 2D, Supplements Material 3). This study proofed the effectiveness of P8-D6 on ovarian cancer. As these two methods delivered similar results, we decided to use the caspase 3/7 assay for the preceding studies. Moreover, this method has the advantage that it is performable in a plate-based formats and allows a direct and robust comparison between 2D and 3D set-ups. To extend the present publication with this important aspect, we added an additional explanatory section in the result part.
2. To classify P8-D6 as a topoisomerase poison similar to camptothecin or etoposide, P8-D6 was analysed by "in vivo complex of enzyme" bioassay (https://doi.org/10.3390/molecules25071524), which is the gold standard to detect topoisomerase poisons (Figure ICE – below, interesting part bordered in red). This method is suitable to investigate the potency of a drug stabilizing cleavable DNA/Topo complexes.
   Main advantage of the ICE Assay is that changes in the ability of cell divisions are only caused by the drug itself and not by changes in the expression of essential enzymes during the replication process. Changing the expression of Topo I or Topo II (i.e. by knockout/ knock down) would significantly interfere with the cell proliferation process itself. Therefore, we choose this method to better investigate the mechanism of action of P8-D6
   The investigation of the Topo I / Topo II expression level in different breast cancer and ovarian cancer cells was already done by using western blot (Figure 2). The results showed comparable levels of Topo I expression. The Topo II expression varied in the different cell lines. SkBr3 and MCF-7 cells, which have an increased Topo II expression, responded more sensitive to P8-D6 treatment (lower IC50 values) compared to UF-182 and MDA-MB231. Thus, overexpression of the topoisomerases seems to have a promoting effect.

We agree that mechanistic studies are very important and thank you again for this valuable comment. To address this point, we added a sentence / section in Line 227.

Figure ICE: (https://doi.org/10.3390/molecules25071524):

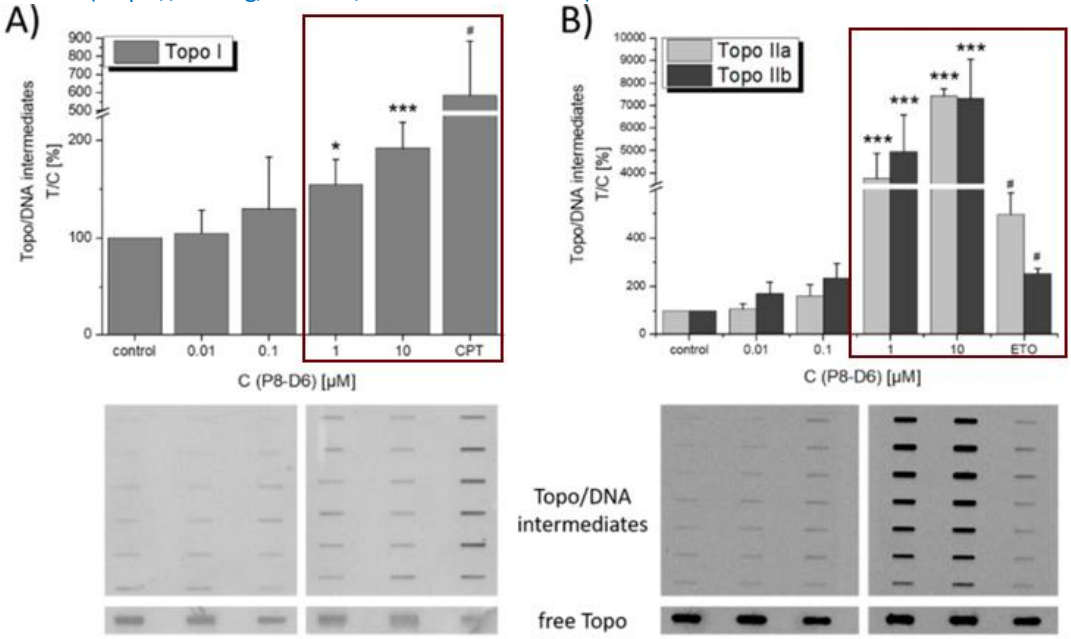


Figure 5. Stabilization of cleavable complexes in HT-29 cells after 1 h incubation with P8-D6, as measured with the ICE assay for (**A**) Topo I, compared to camptothecin (50 µM), and (**B**) Topo IIa and IIb, compared to etoposide (50 µM). Graphs show the results of at least five independent experiments, expressed as mean + SD and relative to the solvent control. Below the graphs, images of representative membranes are displayed. Significant differences were calculated by one-way ANOVA, followed by Fisher's LSD test. "*", "**", and "***" indicate a significant difference to the respective no-effect level with $p < 0.05$, 0.01, and 0.001, respectively. The significant difference between solvent and positive control was calculated using Student's *t*-test ($p < 0.05$) and is indicated with "#".

Reviewer 2

Thank you for addressing my previous comments. Minor revisions must be addressed prior to publication.

1. Please delete either lines 316-320 or 327-331. They are identical.
2. Delete line 321, it is identical to line 332 and cut's of making it an incomplete sentence.

Response: Thanks for these comments. We agree with the reviewer and have now changed/deleted the text section.

Reviewer 3

The manuscript is well revised from cancers-1405794. However, it is difficult to be understood that TOPO I was localized in the cytoplasm. Did the author detect colocalization of P8-D6/TOPO I in the nucleus? At least, it was not apparent from Figure 2A

Response: We thank you for your positive feedback and the additional question. Previous studies by Danks et al. 1996 (https://cancerres.aacrjournals.org/content/56/7/1664.long) analysed the human anaplastic astrocytoma cells for amount and localization (nucleus and cytoplasm) of topoisomerase I after treatment with topotecan. Their results showed that Topo I dissociated from nucleoli within 60 min after treatment. The cytoplasmic content of Topo I increased 50-100%.

Figure Topo I localisation: (https://cancerres.aacrjournals.org/content/56/7/1664.long – Figure 3):

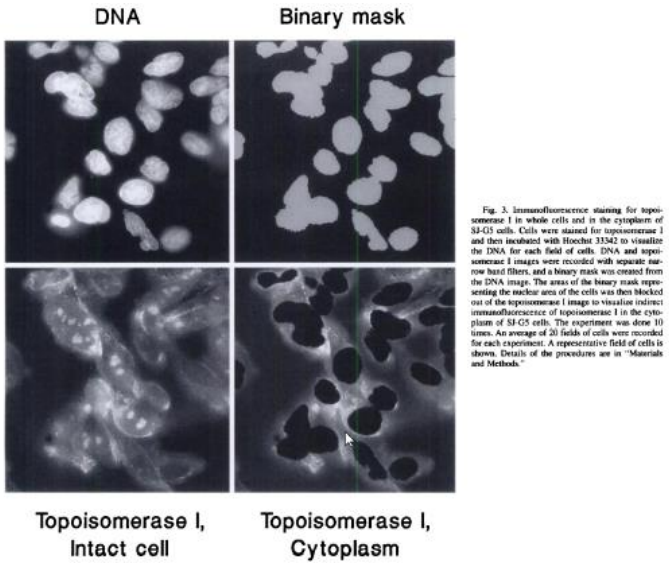


Fig. 3. Immunofluorescence staining for topoisomerase I in whole cells and in the cytoplasm of SJ-G5 cells. Cells were stained for topoisomerase I and then incubated with Hoechst 33342 to visualize the DNA for each field of cells. DNA and topoisomerase I images were recorded with separate narrow band filters, and a binary mask was created from the DNA image. The areas of the binary mask representing the nuclear area of the cells was then blocked out of the topoisomerase I image to visualize indirect immunofluorescence of topoisomerase I in the cytoplasm of SJ-G5 cells. The experiment was done 10 times. An average of 20 fields of cells were recorded for each experiment. A representative field of cells is shown. Details of the procedures are in "Materials and Methods."

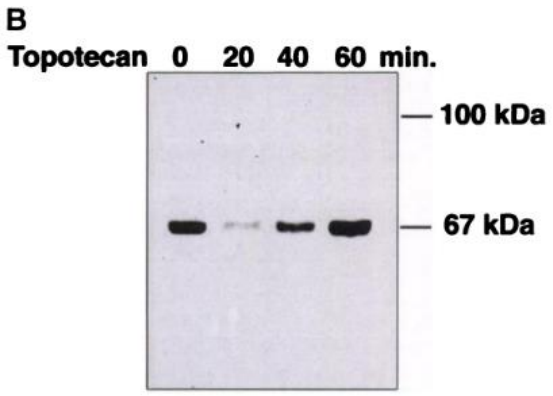
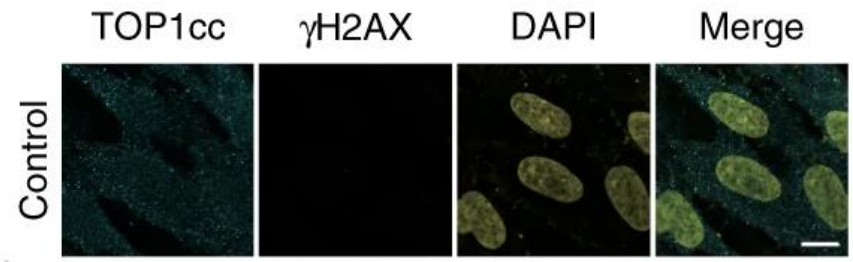


Fig. 6. *A*, immunoblot for topoisomerase I of preparations of nuclear proteins of SJ-G5 cells treated with 5 μM TPT for 0, 20, 40, or 60 min. Cells were incubated for the indicated times with TPT, after which nuclear and cytoplasmic proteins were separated by rapid fractionation of the cells, followed by SDS-PAGE. Proteins were transferred to nitrocellulose paper, and immunoblots were done to detect topoisomerase I by standard methods (see "Materials and Methods"). The experiment was done eight times; each set of samples was run on at least two SDS gels. A representative blot is shown. *B*, immunoblot for topoisomerase I of preparations of cytoplasmic proteins of SJ-G5 cells treated with 5 μM TPT for 0, 20, 40, or 60 min. See the legend to Fig. 6A for details of the methods.

Zhao et al. published 2020 in nature communications a fluorescence staining of TOP1cc on IMR90 (human diploid fibroblasts). In these results TOP1 is also located in the cytoplasm and nucleus. (https://doi.org/10.1038/s41467-020-14652-y).

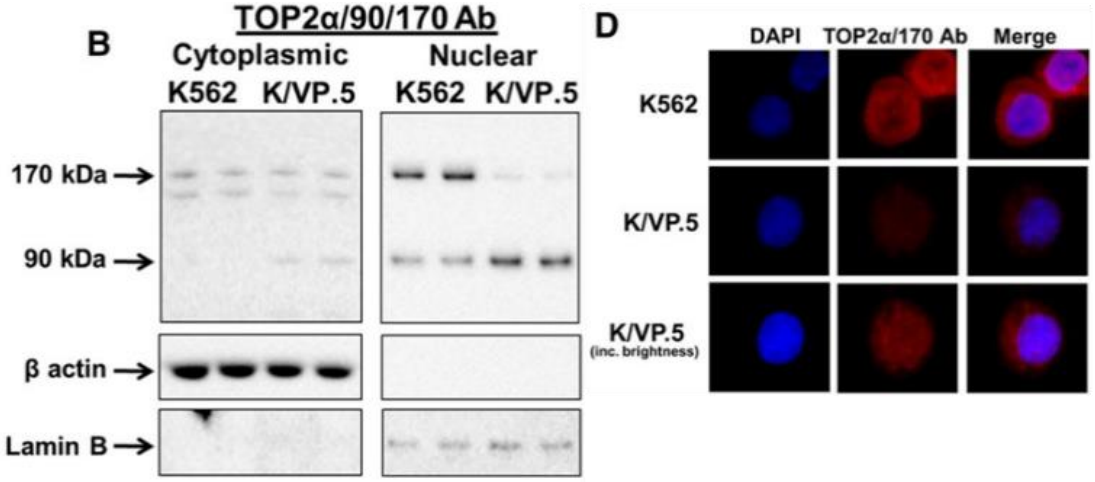
Figure Top1cc localisation: (https://doi.org/10.1038/s41467-020-14652-y – Figure 2):

Top1cc is stained in the nucleus and the cytoplasm.



Kanagasabai et al. showed 2018 similar results of localisation for Top2a (https://doi.org/10.1124/mol.117.111567)

Figure Top2α localisation: (https://doi.org/10.1124/mol.117.111567 – Figure 2):
Top2α is detected cytoplasmic and nuclear by western blot and fluorescence microscopy.



Thus, topoisomerases are predominantly located in the nucleus, but are also detectable in the cytoplasm. This effect might be caused by the drug treatment itself (Danks et al. 1996). For visualization of P8-D6 in BC cells we used two different methods. On the one hand, we incubated the cells with P8-D6 for 24 h and stained the cells with Hoechst (white) and CellTracker (red) using live cell imaging (Live Cell Image Solution) (Figure 2A/B i; supplement material 1B-C). With these images (supplement material 1B-C) we quantified the fluorescence intensity of P8-D6 which is located in the nucleus area (Figure 2C) using ZEN (black edition, Zeiss). These results confirm our hypothesis “Fractions of P8-D6 reach the nucleus”. On the other hand, we treated BC cells with P8-D6 for 24 h, measured the autofluorescence of P8-D6, fixed the cells and stained Topo I. By merging these images, a colocalization of P8-D6/Topo I in the nucleus is detectable (Figure 2A ii; supplement material 1A). For better transparency and more detail view we added a supplement figure (supplement material 1). The colocalization of P8-D6/Topo I and the images for quantification are shown in detail.

Sincerely,

Dirk Bauerschlag

Inken Flörkemeier