nature portfolio

Peer Review File

Complex mechanisms of topoisomerase II poisons revealed by whole-genome analysis

Corresponding Author: Dr Philipp Kapranov

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Here the authors have performed genome-wide mapping of a wide range of breaks induced by a wide range of topoisomerase II poisons in human cells. They show that different topoisomerase poisons behave differently and target different regions of the genome. On a qualitative level the findings of the manuscript are not unexpected – it not surprising that different poisons behave differently and have site specific effects. However, the extent to which the poisons differ is quite remarkable and this effort seems to be the most authoritative mapping of topoisomerase II induced breaks in human cells to date. I am highly enthusiastic about the manuscript and have only one major concern that should be addressed prior to publication:

Major concerns
1. My main concern about this manuscript is the question of dosing. One potential explanation for the differences observed between drugs is that dosing affects cell cycle progression, DNA damage, chromosome metabolism, etc, which in turn affects localization of breaks. Indeed, figure 5 shows this is the case for doxorubicin. I recognize that it is near impossible to perfectly match dosing because not all responses (e.g. breaks vs deaths) will scale in the same way between drugs. Doxorubicin is likely to be the exception and not the rule so it would be ideal to see another dose escalation experiment (as for Fig 5) for one of the other poisons that is not expected to have a dose dependent response (e.g. etoposide). I also think it would be reasonable for the authors to explain the challenges of dosage matching between the drugs and discussing this point about dosing the to best of their ability in the manuscript (i.e. to address the point without additional experimentation). Regardless, it needs to be addressed.

Minor concerns

2. For Figure 1A the authors should comment on whether the dispersed SSBs (that they removed) showed the same patterns as SSBs in the DMSO control.

3. There is no explicit mention of the enzymatic activity of topoisomerase II in the introduction. There should be a statement that it breaks one DNA duplex and passes another through the break to alter the number of linkages within or between DNA molecules.

4. PMID 28735753 should at the very least be cited and the results of this manuscript should be compared to the DSBs induced by END-Seq

5. The introduction should address the existence of the alpha and beta isoforms and explain that the data in this manuscript cannot distinguish between the two enzymes. I do not think this detracts from the story (the relative contributions of each isoform would be another paper) but this should be made clear.

6. Lines 197-223. Additional justification of the SICERII approach would be helpful. As currently written, it is not intuitive as to why differences between samples would be removed to analyze differences between conditions. (I think the approach makes sense, but it was difficult to read).

7. Figure 1B is very non-intuitive. Could this be depicted as a traditional Venn diagram instead, with the sphere for each condition overlapping in the center? Currently the diagram makes it seem as if there is no overlap between conditions.

8. Figures 1C-D are very non-intuitive ways to show the data. Would it be possible to show this data as a heat map of genomic loci instead, perhaps with some clustering between conditions?

9. Lines 262-265. Could the authors clarify why it is expected that SSBs should behave differently to DSBs (which are derived from two SSBs in proximity)? Is it because of processing of the DNA following DSB generation?

10. The differences to the Gittens et al study may merit further discussion. CC-seq involves phenol:chloroform extraction of topoisomerase-crosslinked DNA, which will result in loss of DNA molecules where a full length topoisomerase remains crosslinked (due to partitioning into the organic phase by full length proteins) i.e. CC-seq is biased towards TOP2:CCs that have been processed into peptides. CC-seq is therefore expected to have a high false negative rate. The greatly increased number of sites detected by Cao, Zhang and colleagues is entirely consistent with this limitation of CC-seq. The authors have been very diplomatic in how they refer to the Gittens study and I defer to their judgement on how best to relate their findings to the CC-seq data. However, I do think it would be very reasonable for them to point out the expected false negative rate of CC-seq as an explanation for the differences between their study and that of Gittens et al.

Reviewer #2

(Remarks to the Author)
This manuscript reports the location of DNA breaks after treating K562 cells with seven different drugs that target type II DNA topoisomerases.
Introduction line 44/45
"Topoisomerase II (TOP2) represents an essential, highly conserved and a well studied enzyme responsible for regulating DNA topology"
The introduction makes no mention of the fact there are two isoforms of TOP2, TOP2A and TOP2B which have different biological functions. This seems to be an oversight. In K562 both isoforms will be present in cycling cells. Both isoforms are briefly mentioned on page 23, line 648. But I think it needs to be stated that the method used here will detect breaks by both isoforms.

The method used to detect the breaks in the DNA will not distinguish between TOP2A and TOP2B.
Introduction line 53 "both strands of DNA are cleaved" it would be better to say "may be" rather than "are" as single strand breaks do occur as is mentioned somewhere else in this manuscript.

page 8, line 206 - 100uM doxorubicin is a very very high concentration for this drug.

page 14 line 378 - more references regarding doxorubicin effects would improve this manuscript.
e.g.
Chromatin as an old and new anticancer target.
Neefjes J, Gurova K, Sarthy J, Szabó G, Henikoff S.
Trends Cancer. 2024 Aug;10(8):696-707. doi: 10.1016/j.trecan.2024.05.005. Epub 2024 Jun 1.
PMID: 38825423 Free PMC article. Review.
Intercalating TOP2 Poisons Attenuate Topoisomerase Action at Higher Concentrations.
Atwal M, Swan RL, Rowe C, Lee KC, Lee DC, Armstrong L, Cowell IG, Austin CA.
Mol Pharmacol. 2019 Oct;96(4):475-484. doi: 10.1124/mol.119.117259. Epub 2019 Aug 9.
PMID: 31399497 Free PMC article.

page 16 & 17

The sequence consensus of the cleavage sites is analysed and compared to some previously published in vitro cleavage sites. UNFORTUNATELY for reasons that are not clear the authors use a different numbering system for their cleavage consensus sites which is very confusing. In this manuscript they use 0, when the method used by multiple groups is to assign the base immediately 5' to the break as -1, this is confusing and needs to be corrected both in the text and in figures 7 and S2. Also on page 21 & 22 lines 593to 606.

Despite the large number of sites there does not seem to be a consensus for cleavage sites without drugs.

It would be interesting to know how the sites obtained by whole genome methods such as this manuscript compare with the seminal work on cleavage sites by Spitzner and Muller and Sander and Hsieh
A consensus sequence for cleavage by vertebrate DNA topoisomerase II.
Spitzner JR, Muller MT.
Nucleic Acids Res. 1988 Jun 24;16(12):5533-56. doi: 10.1093/nar/16.12.5533.
PMID: 2838820 Free PMC article.
Drosophila topoisomerase II double-strand DNA cleavage: analysis of DNA sequence homology at the cleavage site.
Sander M, Hsieh TS.
Nucleic Acids Res. 1985 Feb 25;13(4):1057-72. doi: 10.1093/nar/13.4.1057.
PMID: 2987816 Free PMC article.

And how the amsacrine sites in cells compares to those in vitro with TOP2A and TOP2B
Amsacrine-promoted DNA cleavage site determinants for the two human DNA topoisomerase II isoforms alpha and beta.
Marsh KL, Willmore E, Tinelli S, Cornarotti M, Meczes EL, Capranico G, Fisher LM, Austin CA.
Biochem Pharmacol. 1996 Dec 13;52(11):1675-85. doi: 10.1016/s0006-2952(96)00516-3.
PMID: 8986129

And how the mitoxantrone sites in this study compare to the 8 base pair mitoxantrone cleavage site in patients treated with mitoxantrone.

DNA topoisomerase II in therapy-related acute promyelocytic leukemia.
Mistry AR, Felix CA, Whitmarsh RJ, Mason A, Reiter A, Cassinat B, Parry A, Walz C, Wiemels JL, Segal MR, Adès L, Blair IA, Osheroff N, Peniket AJ, Lafage-Pochitaloff M, Cross NC, Chomienne C, Solomon E, Fenaux P, Grimwade D.
N Engl J Med. 2005 Apr 14;352(15):1529-38. doi: 10.1056/NEJMoa042715.
PMID: 15829534 Free article.

Given the large number of sites using this genome wide approach it would have been nice to see a fuller analysis of the cleavage site consensus sequences. The numbering needs to be amended to match the previously published cleavage site consensus sequences to prevent confusion in the field.

Reviewer #3

(Remarks to the Author)
Topoisomerase type II (TOP2) plays essential roles in maintaining genomic integrity, functioning to alleviate supercoiling DNA during replication and transcription. TOP2 poisons have been used as frontline chemotherapeutic agents due to their ability inhibit TOP2 function. While double-strand breaks (DSBs) and single-strand breaks (SSBs) generated by TOP2 poison treatment can be carcinogenic and may cause severe side effects in patients. In this study, the authors used their previously developed SSiNGLe method and investigated the genome-wide DNA breaks induced by seven different TOP2 poisons in human cells.

Their study provided a comprehensive comparison of TOP2 cleavage regions identified from the treatment of seven different drugs. In addition, the authors validated these breaks by comparing them with alternative detection methods, and studied the cleavage patterns related to genome functions and sequence preferences. The research further provided genome-wide evidence on opposing effects on TOP2 activity of doxorubicin depending on drug concentration and genomic context. Their findings have scientific and clinical relevance in further understanding TOP2 cleavage profiling and the implications for the efficacy of chemotherapeutic treatments.

Suggestions and comments as below:
1) One dataset in Figure 3 warrants further examination. In Figure 3, the highest concentration of 100uM Doxorubicin was used and it showed the least effect among other drugs. Since the subsequent analysis in this paper showed the dose-dependent relationship of Doxorubicin to induce or inhibit DNA breaks, it would be beneficial to include the odds ratio with Doxorubicin treatment at 1uM as well.

2) Although the study showed the cleavage site profiling and sequence preferences were likely driven by the particular TOP2 poison used, other results in this paper also showed the difference of probability from different doses of Dox. Therefore, the low percentage of commonly shared regions in Figure 1B might be partially attributable to suboptimal concentrations of some poison agents. To strengthen the argument on the low overlapping among cleavage profiling, it would be advantageous to conduct titrations for each drug, to use the optimal concentrations to regenerate Figure 1B. This approach will ultimately enhance the characterization of TOP2 cleavage profiles and thorough guides for therapeutic considerations.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I am satisfied with the revisions the authors have made and think that the manuscript is now suitable for publication.

Reviewer #2

(Remarks to the Author)
My comments have now been addressed.

Additional comments

On line 101 the word "un-understood" is used this does seem to be a word meaning not understood, I think it would read/flow better if it said
"highly complex and not fully understood".
rather than
"highly complex and remain mostly un-understood".

Line 235-237
"Most (87%) of the total sequence represented by these regions was found in individual drug treatments and merely 187bp was shared across all 7 drugs (Figure 1B)."

What was the sequence of the share 187bp? and genomic location of this shared 187bp?

Reviewer #3

(Remarks to the Author)
The revision addressed my concerns from the initial review. The additional experiments the authors performed on amsacrine and teniposide titration to higher doses further supports their conclusion of drug-specific TOP2 cleavage pattern. The data generated in this paper serves a good reference for future TOP2 studies.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Here the authors have performed genome-wide mapping of a wide range of breaks induced by a wide range of topoisomerase II poisons in human cells. They show that different topoisomerase poisons behave differently and target different regions of the genome. On a qualitative level the findings of the manuscript are not unexpected – it not surprising that different poisons behave differently and have site specific effects. However, the extent to which the poisons differ is quite remarkable and this effort seems to be the most authoritative mapping of topoisomerase II induced breaks in human cells to date. I am highly enthusiastic about the manuscript and have only one major concern that should be addressed prior to publication:

Major concerns

1. My main concern about this manuscript is the question of dosing. One potential explanation for the differences observed between drugs is that dosing affects cell cycle progression, DNA damage, chromosome metabolism, etc, which in turn affects localization of breaks. Indeed, figure 5 shows this is the case for doxorubicin. I recognize that it is near impossible to perfectly match dosing because not all responses (e.g. breaks vs deaths) will scale in the same way between drugs. Doxorubicin is likely to be the exception and not the rule so it would be ideal to see another dose escalation experiment (as for Fig 5) for one of the other poisons that is not expected to have a dose dependent response (e.g. etoposide). I also think it would be reasonable for the authors to explain the challenges of dosage matching between the drugs and discussing this point about dosing the to best of their ability in the manuscript (i.e. to address the point without additional experimentation). Regardless, it needs to be addressed.

Response: we agree that this is a very important point since the differences among the drugs could potentially be explained by the effects of specific dose used in these experiments. However, as the reviewer mentions, it is very hard to match physiologically equivalent doses of each drug. Therefore, to address this issue, we have performed the dose escalation experiments with two additional drugs, amsacrine and teniposide. We found that just like in the case of doxorubicin, the strength of the enrichment of breaks induced by each of the drug had a strong positive or negative correlation with the concentration in various types of genomic elements.

However, each of the three drugs had a unique pattern of such correlations. For example, as shown in the Figure 5A, doxorubicin had negative correlations in all the regulatory regions (TSS-flanking regions, insulators or enhancers), while both amsacrine and teniposide showed strong positive correlations in enhancers and insulators (Figure 5A). However, each of the 3 drugs had a unique pattern of the dose-dependent correlations. For example, while the enrichment of teniposide-induced

breaks in the regions flanking TSSs (±200-5000bp) had a strong positive correlation with the concentration, those induced by amsacrine had no correlation (Figure 5A).

These results further support the conclusion that each drug has a specific effect that can not be explained by differences of the dosage. *However, they also show that each drug has concentration-dependent effect. We further confirm it by showing that the genomic profile of the breaks induced by each of the 3 drugs, not only doxorubicin, is unique (Figure 5B, C and Supplementary Figure 2B, C).*

However, we do not think that this totally unexpected. Even though doxorubicin is known to have a dual effect on TOP2—poison at lower concentrations and inhibitor at higher doses—it is also quite possible that even for drugs that always function as TOP2 poisons, the effect would be dependent on the concentration. For example, differences in local chromatin environment and/or heterogeneity of TOP2ccs might also result in differential sensitivities of TOP2ccs to various other TOP2 poisons besides doxorubicin.

We added discussion of these results and how their address the issue of dose matching in the Discussion. We revised ***Figures 1B–D, 2, 3, 4, 6, 7A, and 8*** *and* ***Supplementary Figure 1*** *using the highest concentrations of amsacrine and teniposide. Additionally, the panels for showing different concentrations of amsacrine and teniposide have been updated in* ***Figures 5A, 7C-D*** *and* ***Supplementary Figures 2B, 2C, and 3****. The corresponding* ***Supplementary Tables 1–8 and 10–11*** *(with the exception of Supplementary Table 9) have also been updated accordingly.*

Minor concerns

2. For Figure 1A the authors should comment on whether the dispersed SSBs (that they removed) showed the same patterns as SSBs in the DMSO control.

Response: we now include the comparison of the patterns of all SSBs for each drug treatment — the vast majority of which are represented by the breaks that are removed after constructing the TOP2 cleavage site — with those from the DMSO control. Indeed, the patterns of all SSBs from the drug treatments are very similar to those from DMSO treated cells.

3. There is no explicit mention of the enzymatic activity of topoisomerase II in the introduction. There should be a statement that it breaks one DNA duplex and passes another through the break to alter the number of linkages within or between DNA molecules.

Response: we have added this information.

4. PMID 28735753 should at the very least be cited and the results of this manuscript should be compared to the DSBs induced by END-Seq

Response: we have performed this comparison using the Nalm6 data from that study. We chose Nalm6 since it represents a leukemia cell line just like K562 used in our work. For each TOP2 cleavage region detected by SSiNGLe in each drug treatment, we calculated the difference between the normalized END-seq signal the etoposide treated and untreated Nalm6 cells. We found that the cleavage regions found in etoposide and teniposide, either shared or unique, and in amsacrine (shared) treatments did have significantly higher END-seq signal in the etoposide-treated Nalm6 cells. We now include these results in the revised manuscript text and Figure 2C.

5. The introduction should address the existence of the alpha and beta isoforms and explain that the data in this manuscript cannot distinguish between the two enzymes. I do not think this detracts from the story (the relative contributions of each isoform would be another paper) but this should be made clear.

Response: we have added this information.

6. Lines 197-223. Additional justification of the SICERII approach would be helpful. As currently written, it is not intuitive as to why differences between samples would be removed to analyze differences between conditions. (I think the approach makes sense, but it was difficult to read).

Response: if we understood the comment correctly, it seems that this reviewer is concerned about removal of the SSBs that are found only in some but not all of the 3 replicates. However, this is not what we did. In fact, we included all the SSBs from each replicate in the analysis. We tried to modify the text to clarify this point.

7. Figure 1B is very non-intuitive. Could this be depicted as a traditional Venn diagram instead, with the sphere for each condition overlapping in the center? Currently the diagram makes it seem as if there is no overlap between conditions.

Response: we modified figure 1B by replacing the Venn diagram with stacked bars — one for each drug treatment. Considering there are 7 types of drugs, a traditional Venn diagram would make it difficult to distinguish the differences among the drugs.

8. Figures 1C-D are very non-intuitive ways to show the data. Would it be possible to show this data as a heat map of genomic loci instead, perhaps with some clustering between conditions?

Response: this comment is somewhat hard to address because the current plots represent the standard way to show the incremental increase in the discovery with the addition of each new drug. The plots start with teniposide, the drug that generated the largest lengths of the TOP2 cleavage regions (either total in the panel C or shared with other drugs in the panel D), and then continue with the 2nd, 3rd, … largest contributors. We suggest to keep the original here.

9. Lines 262-265. Could the authors clarify why it is expected that SSBs should behave differently to DSBs (which are derived from two SSBs in proximity)? Is it because of processing of the DNA following DSB generation?

Response: in this case, we found that breaks detected by SSiNGLe tend to overlap with the etoposide-induced breaks detected by CC-seq on the same strand. If all or most of the breaks were represented by DSBs, then there should be no strand preference since breaks would be equally distributed on the two strands. We tried to further clarify this point in the revised text.

10. The differences to the Gittens et al study may merit further discussion. CC-seq involves phenol:chloroform extraction of topoisomerase-crosslinked DNA, which will result in loss of DNA molecules where a full length topoisomerase remains crosslinked (due to partitioning into the organic phase by full length proteins) i.e. CC-seq is biased towards TOP2:CCs that have been processed into peptides. CC-seq is therefore expected to have a high false negative rate. The greatly increased number of sites detected by Cao, Zhang and colleagues is entirely consistent with this limitation of CC-seq. The authors have been very diplomatic in how they refer to the Gittens study and I defer to their judgement on how best to relate their findings to the CC-seq data. However, I do think it would be very reasonable for them to point out the expected false negative rate of CC-seq as an explanation for the differences between their study and that of Gittens et al.

Response: this is an interesting comment that prompted us to do more investigation. Actually, while the reviewer is correct about the potentially high false negative rate of CC-seq, the total genomic coverage of TOP2 cleavage regions detected by CC-seq was not less than that detected by our method. However, at least one reason for the low overlap between our technique and CC-seq appears to be a relatively high false positive rate of the latter. We compared the TOP2 regions from both studies with the CUT&TAG data. As it can be seen in the revised Figure 2B, the SSiNGLe-detected TOP2 cleavage regions had much higher TOP2A and TOP2B occupancy scores for each of the 7 drug treatments than the CC-seq regions—the median CUT&TAG scores for the SSiNGLe regions ranged from 2.79 to 4.52 while the CC-seq regions fell within the range of 2.2-2.26. While each of the 3 studies (CUT&TAG, CC-seq and SSiNGLe) were done in different human cell lines thus complicating direct comparisons of SSiNGLe and CC-seq, these results suggest that the small overlap between SSiNGLe and CC-seq might be caused by a relatively high false positive rate in the latter method. We added this information and the discussion in the Results and Discussion parts of the revised

manuscript.

Reviewer #2 (Remarks to the Author):

1. This manuscript reports the location of DNA breaks after treating K562 cells with seven different drugs that target type II DNA topoisomerases.
Introduction line 44/45
"Topoisomerase II (TOP2) represents an essential, highly conserved and a well studied enzyme responsible for regulating DNA topology"
The introduction makes no mention of the fact there are two isoforms of TOP2, TOP2A and TOP2B which have different biological functions. This seems to be an oversight. In K562 both isoforms will be present in cycling cells. Both isoforms are briefly mentioned on page 23, line 648. But I think it needs to be stated that the method used here will detect breaks by both isoforms.
The method used to detect the breaks in the DNA will not distinguish between TOP2A and TOP2B.

Response: we now state this clearly in the Introduction.

2. Introduction line 53 "both strands of DNA are cleaved" it would be better to say "may be" rather than "are" as single strand breaks do occur as is mentioned somewhere else in this manuscript.

Response: good point, we fixed this in the Introduction.

3. page 8, line 206 - 100uM doxorubicin is a very very high concentration for this drug.

Response: as mentioned by the reviewer 1, matching physiologically-equivalent concentrations for multiple drugs in any given biological system is very complex. Therefore, in the original version of the paper, we have chosen to use maximum published concentrations of each drug to ensure that we would detect an effect of each drug since the treatment time was relatively short (1 hour). Furthermore, reducing the time of treatment to 30 minutes did not cause major changes. It is somewhat hard to imagine secondary effects at such short times even if the concentration of some drugs was too high.

We also performed dose titration experiments for doxorubicin in the original version and two additional drugs (teniposide and amsacrine) in the revised version. While each concentration of each drug appears to have some unique genomic profiles, for each drug we have also observed strong correlations between the dose and enrichment of DNA breaks in particular genomic elements (Figure 5A). In addition, we have observed a correlation between the enrichment of specific nucleotides and the concentration (Figure 7). Therefore, we did not observe any obvious signs of artifactual effects at

higher concentrations for any of the 3 tested drugs.

4. page 14 line 378 - more references regarding doxorubicin effects would improve this manuscript.
e.g.
Chromatin as an old and new anticancer target.
Neefjes J, Gurova K, Sarthy J, Szabó G, Henikoff S.
Trends Cancer. 2024 Aug;10(8):696-707. doi: 10.1016/j.trecan.2024.05.005. Epub 2024 Jun 1.
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Intercalating TOP2 Poisons Attenuate Topoisomerase Action at Higher Concentrations.
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Mol Pharmacol. 2019 Oct;96(4):475-484. doi: 10.1124/mol.119.117259. Epub 2019 Aug 9.
PMID: 31399497 Free PMC article.
Response: done.

5. page 16 & 17

The sequence consensus of the cleavage sites is analysed and compared to some previously published in vitro cleavage sites. UNFORTUNATELY for reasons that are not clear the authors use a different numbering system for their cleavage consensus sites which is very confusing. In this manuscript they use 0, when the method used by multiple groups is to assign the base immediately 5' to the break as -1, this is confusing and needs to be corrected both in the text and in figures 7 and S2. Also on page 21 & 22 lines 593to 606.
Response: we have replaced "0" with "−1" in the Figures 7, S3, S4 (previous S2), Tables S8, S9 and throughout text.

6. Despite the large number of sites there does not seem to be a consensus for cleavage sites without drugs.

It would be interesting to know how the sites obtained by whole genome methods such as this manuscript compare with the seminal work on cleavage sites by Spitzner and Muller and Sander and Hsieh
A consensus sequence for cleavage by vertebrate DNA topoisomerase II.
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Sander M, Hsieh TS.

Nucleic Acids Res. 1985 Feb 25;13(4):1057-72. doi: 10.1093/nar/13.4.1057. PMID: 2987816 Free PMC article.

Response: indeed, we could not define a strong consensus TOP2 cleavage site due to the major differences among the drugs. Nonetheless, we detected the preference for C at the −1 position which was consistently found in multiple other studies. This observation is also partially consistent with the findings of the two studies above which found enrichment of pyrimidines (C or T) at this position. We have included the discussion of these findings in the revised Discussion.

7. And how the amsacrine sites in cells compares to those in vitro with TOP2A and TOP2B Amsacrine-promoted DNA cleavage site determinants for the two human DNA topoisomerase II isoforms alpha and beta.
Marsh KL, Willmore E, Tinelli S, Cornarotti M, Meczes EL, Capranico G, Fisher LM, Austin CA.
Biochem Pharmacol. 1996 Dec 13;52(11):1675-85. doi: 10.1016/s0006-2952(96)00516-3. PMID: 8986129

Response: this is a good question. In our revised work, we have performed dose escalation studies with 2 new drugs, including amsacrine. Using these data, we have updated the amsacrine consensus site which now has some key similarities with previously published consensus from the Marsh et al study. Specifically, we found the aforementioned preference of C at −1, a strong preference for A at +1 and also preference for G at +2 which was also consistent with that study. However, we detected enrichment of A at +4 which was not found by Marsh et al. We now include these results and the discussion of possible reasons for the differences in the revised Results section.

8. And how the mitoxantrone sites in this study compare to the 8 base pair mitoxantrone cleavage site in patients treated with mitoxantrone.

DNA topoisomerase II in therapy-related acute promyelocytic leukemia.
Mistry AR, Felix CA, Whitmarsh RJ, Mason A, Reiter A, Cassinat B, Parry A, Walz C, Wiemels JL, Segal MR, Adès L, Blair IA, Osheroff N, Peniket AJ, Lafage-Pochitaloff M, Cross NC, Chomienne C, Solomon E, Fenaux P, Grimwade D.
N Engl J Med. 2005 Apr 14;352(15):1529-38. doi: 10.1056/NEJMoa042715.
PMID: 15829534 Free article.
Response: this is also a very good question. In fact, yes, one of the TOP2 cleavage regions detected in this work in the mitoxantrone treatment overlapped the 8 bp TOP2 hotspot in PML gene previously found in cancer patients treated by this drug. The probability of this event occurring by random change was <2.16E-16 (Proportion Test test). We added this information to the Results section of the revised manuscript.

9. Given the large number of sites using this genome wide approach it would have been nice to see a fuller analysis of the cleavage site consensus sequences. The numbering

needs to be amended to match the previously published cleavage site consensus sequences to prevent confusion in the field.

Response: we could not find a consensus motif in this work with the exception of the preference for C at the −1 position. This feature is quite consistent across multiple studies, including this one and several previous ones that we cite in the Discussion of the revised version, that used different drugs in different biological systems.

However, defining true TOP2 cleavage consensus would be tricky in any in vivo study which uses drugs because any consensus cleavage site would be heavily influenced by the sequence preference of the drugs, and also, conceivably, by the biological system itself. The latter might explain some of the differences with the previous studies observed in this work. We think that the best way to establish the pure enzyme cleavage site consensus without the confounding effects of the drugs and biological systems is to perform cleavage in vitro using a purified enzyme— potentially derived from the self-trapping mutants (doi:10.1101/2024.06.17.599352) — corresponding to specific TOP2 isoform and purified genomic DNA. SSiNGLe would be ideally suited for this topic, however, this would be beyond the scope of this work and our team does not have access to the purified TOP2 enzymes.

Reviewer #3 (Remarks to the Author):

Topoisomerase type II (TOP2) plays essential roles in maintaining genomic integrity, functioning to alleviate supercoiling DNA during replication and transcription. TOP2 poisons have been used as frontline chemotherapeutic agents due to their ability inhibit TOP2 function. While double-strand breaks (DSBs) and single-strand breaks (SSBs) generated by TOP2 poison treatment can be carcinogenic and may cause severe side effects in patients. In this study, the authors used their previously developed SSiNGLe method and investigated the genome-wide DNA breaks induced by seven different TOP2 poisons in human cells.

Their study provided a comprehensive comparison of TOP2 cleavage regions identified from the treatment of seven different drugs. In addition, the authors validated these breaks by comparing them with alternative detection methods, and studied the cleavage patterns related to genome functions and sequence preferences. The research further provided genome-wide evidence on opposing effects on TOP2 activity of doxorubicin depending on drug concentration and genomic context. Their findings have scientific and clinical relevance in further understanding TOP2 cleavage profiling and the implications for the efficacy of chemotherapeutic treatments.

Suggestions and comments as below:
1) One dataset in Figure 3 warrants further examination. In Figure 3, the highest concentration of 100uM Doxorubicin was used and it showed the least effect among other drugs. Since the subsequent analysis in this paper showed the dose-dependent

relationship of Doxorubicin to induce or inhibit DNA breaks, it would be beneficial to include the odds ratio with Doxorubicin treatment at 1uM as well.

Response: we included the odds ratios of different concentrations in the revised Supplementary Table 4.

2) Although the study showed the cleavage site profiling and sequence preferences were likely driven by the particular TOP2 poison used, other results in this paper also showed the difference of probability from different doses of Dox. Therefore, the low percentage of commonly shared regions in Figure 1B might be partially attributable to suboptimal concentrations of some poison agents. To strengthen the argument on the low overlapping among cleavage profiling, it would be advantageous to conduct titrations for each drug, to use the optimal concentrations to regenerate Figure 1B. This approach will ultimately enhance the characterization of TOP2 cleavage profiles and thorough guides for therapeutic considerations.

Response: we agree that this is a very important point since the differences among the drugs could potentially be explained by the effects of specific dose used in these experiments, which is also raised by Reviewer 1. However, as the reviewer 1 mentions, it is very hard to match physiologically equivalent doses of each drug. Therefore, to address this issue, we have performed the dose escalation experiments with two additional drugs, amsacrine and teniposide. We found that just like in the case of doxorubicin, the strength of the enrichment of breaks induced by each of the drug had a strong positive or negative correlation with the concentration in various types of genomic elements. ***However, each of the three drugs had a unique pattern of such correlations. These results further support the conclusion that each drug has a specific effect that can not be explained by differences of the dosage.*** *Please see the response to the comment 1 by the Reviewer 1.*

We revised ***Figures 1B–D, 2, 3, 4, 6, 7A, and 8*** *and* ***Supplementary Figure 1*** *using the highest concentrations of amsacrine and teniposide. Additionally, the panels for showing different concentrations of amsacrine and teniposide have been updated in* ***Figures 5A, 7C-D*** *and* ***Supplementary Figures 2B, 2C, and 3****. The corresponding* ***Supplementary Tables 1–8 and 10–11*** *(with the exception of Supplementary Table 9) have also been updated accordingly.*

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

I am satisfied with the revisions the authors have made and think that the manuscript is now suitable for publication.

Response: We really appreciate the constructive comments and suggestions that significantly improved the manuscript.

Reviewer #2 (Remarks to the Author):

My comments have now been addressed.

Response: We really appreciate the constructive comments and suggestions that significantly improved the manuscript.

Additional comments

On line 101 the word "un-understood" is used this does seem to be a word meaning not understood, I think it would read/flow better if it said "highly complex and not fully understood". rather than "highly complex and remain mostly un-understood".

Response: Done.

Line 235-237 "Most (87%) of the total sequence represented by these regions was found in individual drug treatments and merely 187bp was shared across all 7 drugs (Figure 1B)." What was the sequence of the share 187bp? and genomic location of this shared 187bp?

Response: The 187 bp shared region is located at chr4:153944749-153944936 (HG19) and the coordinates and the sequence information have now been included in the Supplementary Data 1. Interestingly, this shared sequence maps to a genomic region that likely has regulatory properties as evidenced by the strong ChIPseq signal for the H3K4me1 chromatin mark associated with enhancers and a transcription start of a long non-coding RNA. We have included this information to the text and added Supplementary Figure 1 showing the genomic context of the region.

Reviewer #3 (Remarks to the Author):

The revision addressed my concerns from the initial review. The additional experiments the authors performed on amsacrine and teniposide titration to higher doses further supports their conclusion of drug-specific TOP2 cleavage pattern. The data generated in this paper serves a good reference for future TOP2 studies.

Response: We really appreciate the constructive comments and suggestions that significantly improved the manuscript.