Author’s response to reviews

Title: A new assay for measuring chromosome instability (CIN) and identification of drugs that elevate CIN in cancer cells

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Author’s response to reviews: see over
Dear Editor,

Below are point-by-point responses to the referees comments. All changes in the text are marked in red. In addition, we have carried out one experiment recommended by referee 3 and two experiments required by referee 4. They are presented as Additional file 1.

We hope that the revised version of the manuscript will be suitable for publication in BMC Cancer.

Sincerely yours,

Vladimir Larionov,
LMP, NCI, NIH

**Reviewer’s 1 report**

1) The authors state that the EGFP cassette loaded onto the HAC is protected from silencing by a chS4 insulator. Does this mean that all the CHO and HT1080 clones containing the HAC have identical levels of GFP expression, or is there variability? For example, on page 11, 2nd paragraph, the authors write that “Ten BS-resistant clones that expressed the GFP transgene were isolated…”. Were there any GFP negative BS-resistant clones? This point should be clarified, as the presence of variable GFP levels between clones could hint at epigenetic effect on GFP expression, possibly affecting the FACS readings. Quantitive RT-PCR data on GFP expression would be useful.

In this work, all blasticidin resistant clones obtained via MMCT expressed GFP. Based on our experience with MMCT transfer of other HACs, sometimes we have observed GFP negative BS-resistant clones but they are very rare. We also observed heterogeneity in the level of GFP expression between independently obtained Bsr-resistant clones.

However, for this work, we chose the HT1080 HAC-containing clone that exhibited stable GFP expression for almost a year. As we observed a strong correlation between FACS and FISH data, we conclude that there is no detectable effect of epigenetic GFP silencing on flow cytometry data.

2) The FACS analysis is conducted 2 weeks after the treatment with the aneugens compounds. If a cell gains growth advantages because of gain/loss of an essential chromosome, it would quickly take over the whole population during the 14 days period. The majority of the cells would either contain the HAC or not, depending on the HAC status of the founder cell. Thus, the aneugenic potential of the compound under test could be under/over estimated. Are three replicates sufficient to exclude this scenario? In figure 4, some of the treatments show much larger error bars than others. Could this be due to a founder effect? This point should be clarified and discussed.

This is very good question and indeed it is difficult to exclude the possibility that treatments by aneugens may affect the rate of cell division as a result of loss or gain of natural chromosomes. Therefore, we agree that the HAC status of the founder cell may affect the ratio of HAC-containing and HAC-less cells after 10-14 cell divisions. However, if this was so, such events should theoretically be stochastic and as a result
each independent measuring would produce different results. The raw FACS data of three independent populations for six drug treatments have standard deviations of less than 1%. SAHA and Docetaxel treatment had a std larger than 1%, (1.2% and 1.8% respective). Thus we do not believe that the aneugens significantly alter the rate of cell division.

However, to further address this point, we made nine independent measuring of HAC loss after treatment by taxol and peloruside A. The results were reproducible and the std small (Peloruside: SD±0.9%, Taxol: SD±1.1%), therefore we concluded that three replicates for each drug are sufficient. In a new version of the manuscript, we included these data in the Methods.

3) In the legend to Figure 5 the authors write “Our model assumes that when mis-segregation occurs during mitosis, one daughter cell will inherit a HAC while the other daughter cell does not”. What about non-disjunction errors, where one cells inherits two copies of the chromosome and the other none? How does the model take this phenomenon into account? This point should be discussed.

We agree with this comment and in the revised version of the manuscript we included a statement about non-disjunction event when describing a model (Figure 5 legend).

In theory, should a cell inherit two copies of the HAC by non-disjunction, the subsequent probability of complete HAC loss by that cell would be lower than a cell carrying one copy of the HAC. The model would therefore underestimate the rate of HAC loss. However, this assumes that each copy of the HAC segregates independently. Oddly we have found this not to be so. We have isolated and characterized HT1080 clones containing two copies of HAC in >70% of cells. The frequency of HAC loss in these clones was indistinguishable from those containing a single copy of the HAC. A similar phenomenon was previously described for yeast artificial chromosomes (YAC). While the mechanism is not yet clear, these observations suggest that two copies of HAC do not segregate independently from each other. Therefore, non-disjunction events should not significantly affect the mathematical model.

4) Figure 2 would be more informative with the addition of a map of the HAC vector showing the various markers, and a phase contrast image of the GFP expressing cells.

In accordance to the reviewer’s suggestion, we have included a map of the HAC vector in Figure 2B and a phase contrast image of the GFP expressing cells in Figure 2C.

Minor essential revisions

1) Nocodazole is misspelled in Table 1, 2 and 3, and Figure 4.

Thank you. Corrections were made.

2) Figure 2 legend should clarify which probe has been used for the FISH in Figure 2B, as some of the endogenous chromosomes appear to be labeled.

A BAC clone containing an amplified synthetic alphoid DNA dimer was used as the FISH probe. One monomer of this dimer is alphoid DNA consensus sequence carrying
the tetO sequence, while another monomer is alphoid DNA from chromosome 17. This probe gives a low signal with centromeric regions of several endogenous chromosomes. In the new version of the manuscript, we included this clarification into the Methods.

2) Figure 3 does not show the A and B.

   Thank you. Figure 3 has been modified.

Reviewer’s 2 report:

1. Two columns in table 3 are not impressive. You can do three and more times and show the error and variation. The readers would like to know the comparison of this test and conventional micronuclei formation test.

   We modified and included means of standard deviation (± SD) of two independent experiments into Table 3. In each experiment more than 400 cells were analyzed and the standard deviations are very low. Therefore, data of additional parallel experiments should not affect significantly the frequencies of MNi. Conventional micronuclei formation test gave similar results with tested drugs.

2. The in the table 1 should be compared to the MNi data

   We compared the MNi assay to a novel HAC-based assay described in this manuscript. This comparison is on page 14. Firstly, we concluded that “all of the analyzed drugs induced micronuclei formation in the HAC-containing HT1080 cells similar to that reported for other cell lines [27]” (page 14) (Table 3). Then, we compared data presented in Table 2 (HAC-based assay) and Table 3 (MNi assay) and made a conclusion that there is no detectable correlation between the frequencies of MNi formation (that varied between 37% and 63%) and the rates of HAC loss determined by FISH and FACS. MNi assay measures the total number of cells with chromosome abnormalities. This includes the cells undergoing to apoptosis (potentially dead cells), the cells with transient MNi (the MNi and chromosome within will be reabsorbed in the next cell cycle), and the cells that will loss MNi (chromosome) at the next cell division.

   In contrast, the HAC-based assay measures the frequency of HAC loss among viable cells recovered after drug treatment. Therefore, absence of correlation between MNi and HAC loss data is expected. In our work, we demonstrated that a HAC-based assay is much more sensitive and reproducible compared to MNi assay. Specifically, the raw FACS data of three independent populations for drug treatments have standard deviations of less than 1% (see page 13). Therefore, HAC-based assay allowed us to rank the compounds with the same or similar mechanism of action, based on their effect on the rate of chromosome loss. The identification of new compounds that increase chromosome mis-segregation rates is important to target the CIN phenotype in cancer cells. Such ranking is practically impossible when using MNi assay but doable by HAC-based assay.

3. Provide concentration of each drug in the Table 2.

Concentrations of each drug used in this study are presented in Table 1. The same concentrations of drugs were used for experiments described in Table 2.

3a. (7! Should be 71 in Reversine line)
Thank you for this comment. Correction is made.

4. **Fig2B, especially in the middle panels are very dark. Improve the quality.**

Thank you for this comment. The background should be dark and only the HAC visualized with the HAC specific probe should be seen as a red dot in this panel.

5. **What are 3 paired (up and bottom) panels of Fig3? Are they the results of triplicate experiments?**

Thank you for this comment. Yes, this is the result of triplicate experiments. Corrections were made in Figure 3 and Figure 3 legend.

6. **In Fig4. Docetaxel should be placed instead of Dxetaxel.**

Thank you. The correction was made.

7. **On page 12, they mentioned IC50, but not data is shown. Please provide the IC50 of the tested drugs.**

For each drug, IC50 value was determined. Concentrations of drugs when ~50% of cells remain viable are presented in Table 1. To clarify this point, we made a corresponding change on page 13.

8. **The author use “the references therin” in many places. I am not sure this is acceptable with the journal’s style.**

Thank you. We will recheck the journal’s style.

**Reviewer’s 3 report:**

Minor issues

1. **The authors state that higher doses of compounds do not appear to increase the rate of HAC loss, but It would be interesting to know whether lower doses (higher viability than IC50) exhibit a linear response at the HAC level. This is, is there a correlation between cell death and HACs rate? This could help discriminating when the drug effects are produced directly through chromosomal instability or other mechanisms may be also affected. This issue can be addressed at authors discretion in the discussion of the results.**

   Thank you for very good comment. We have performed the experiments with lower doses of drugs (higher viability than IC50), i.e. with concentrations $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$ and $\frac{1}{16}$ of the concentration corresponding to IC50. Indeed, for some drugs in our analysis the frequency of HAC loss were dose independent while for others displayed a linear dose-dependence.

   For example, HAC loss was decreased in an almost linear fashion after treatment by VP16 (which is inhibitor of TOP2) with lower doses. In contrast, our analysis revealed a non-linear dose-dependent decrease in HAC loss frequencies for nocodazole and taxol, which were previously reported as compounds that interact with the mitotic spindle
Thus, the Reviewer is right, suggesting that such experiments can indicate different mechanisms of action of a drug. In a new version of the manuscript, this issue was addressed in the Results section (page 14).

*Page 13, 3rd line, there’s a misspelling of “dose”*

Thank you, corrections are made.

**Reviewer’s 4 report**

**Major Compulsory Revisions:**

1 - The proposed assay is based on the quantification of the EGFP intensity coming from the artificial chromosome. Although the high frequency for HAC missegregation (10%) confers sensitivity to the assay, it also over-estimates the rate of normal chromosome missegregation. This reviewer would like to see this issue addressed in the text, and if possible a correction factor should be introduced in the mathematical model in order to have a more realistic approximation of CIN rate for the endogenous chromosomes.

   Indeed, this assay is not to calculate the rate of natural chromosome loss caused by drugs. Instead, the usefulness of this assay is to rank drugs in their ability to cause chromosome instability (CIN). In principle, introduction of a correction factor to estimate the rate of endogenous chromosome loss is possible. (*However, the rate of natural chromosome loss would be dependent on chromosome, cell type, and mutations that a particular cancer cell line has acquired. A correction factor would thus be of limited value in a wider context.*) Nevertheless we do know that the mitotic stability of the HAC (without drug treatment) is approximately 10-fold less than stability of natural chromosomes (~1 x 10^-3) in HT1080. We propose that destabilization of natural chromosomes in response to drug treatment will be increased proportionally to that observed for HAC. Such assumption is reasonable because the HAC contains a functional kinetochore and its behavior during mitotic divisions does not differ from that of normal chromosomes. Also, if there is a defect of DNA replication, the HAC will be affected as well as normal chromosomes. In a new version of the manuscript, this issue was discussed in Discussion (page 16).

2 - In the methods is stated that FACS profiles were acquired 14 days after treatment to quantify HAC loss. The justification was that EGFP protein half-life is one day, and distinguishable differences between treated and untreated samples were only found 10 days after drug washout. It is not completely clear why this is happening. Is it an inherent limitation of the technique due to low EGFP loss, or is the assay measuring the cumulative effects of chromosome missegregation after drug treatment? The authors should provide the FACS profiles of cells before, after drug treatment and different time points after washout.

   Thank you for a very good comment. We have performed the experiment suggested by the Reviewer. FACS profiles of cells before, after drug treatment and at different time points after washout were measured for two drugs (taxol and ixabepilone) and used to calculate rates of HAC loss. As seen from a new Figure S1C (Additional file 1), indeed EGFP is not so stable in HT1080 cells and as a consequence the sampling time has a broad interval (from ~5 to 14 days) without a significant effect on the rate of
HAC loss. Thus the Reviewer is right. We made corresponding changes in a new version of the manuscript on page 13 and pages 16-17.

3 - HAC missegregation has no toxic effects, however loss of one or more chromosomes may increase CIN and compromise cell viability. The mathematical model used to calculate CIN rates assumes that the drug effect is limited to one cell cycle, and that spontaneous HAC loss after drug exposure does not change. If that was the case, distinguishable differences should be observed two days after treatment (considering EGFP half-life). The loss of HAC is proposed to measure CIN rates, but taking into account that cells are analysed 14 days after drug washout, it suggests that the assay is measuring viable aneuploid cells. In fact, micronuclei formation assays 20 hours after drug treatment don’t corroborate the calculated CIN rates. Especially for nocodazole and pelorusive A treatments the differences are quite significant, indicating that HAC loss is measuring viable aneuploid cells. The authors should carefully address this point, by showing total chromosome number before and after drug treatment within different time points. This quantification is essential to demonstrate that chromosome loss is limited to the first cell cycle, thereby validating the mathematical model.

To address this comment, the kinetics of MNs formation was analyzed after drug treatment at different time points after washout. The highest frequency of MNi was observed 24 hrs after drug washout – see a new Figure S1A, B (Additional file 1). After 24 hrs, a frequency of MNi did not differ from that observed for untreated cells. Based on these results, we can conclude that chromosome loss predominantly occurs during the first cell division. In a new version of the manuscript, these results are included into the description of the mathematical model (see Figure 5 Legend). We agree that the assay is measuring viable aneuploid cells. However this measuring strongly correlates with rates of HAC loss during the first cell division after drug washout. Therefore independently on the kinetics of HAC (chromosomes) loss in response to drug treatment, the proposed assay allows ranking the drugs on their effect on chromosome instability (CIN). It is important because CIN may be a target for cancer therapy as has been recently shown by R.H. Medema’s group (reviewed by Janssen et al: Targeting the mitotic checkpoint to kill tumor cells. *Hormones & Cancer* 2011, 2: 113-116).

**Minor Essential Revisions**

4 - Nocodazole is misspelled on tables 1, 2, 3, and on figure 4.

Thank you for the comment. The corrections were made.

5 - Docetaxel is misspelled on figure 4.

Thank you for the comment. The correction was made.

6 - Apoptosis is misspelled in the results section.

Thank you for the comment. The correction was made.

7 - On figure 5, in the equations related to F2 generation what is mentioned as P1 should be P2.

Thank you. The correction was made.