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A novel benzodiazepine derivative that suppresses microtubule dynamics and impairs mitotic progression

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers’ comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

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Reviewer 1

Advance summary and potential significance to field

The article submitted by Pirani et al describes the effects of a new benzodiazepine derivative (termed 1g), which alters the formation of the mitotic spindle and the elongation of microtubules in interphase cells, without a direct interference with tubulin assembly. 1g functions in a dose-dependent manner in cultured HeLa cells as well as in Drosophila neuroblasts. The authors propose that it could open the way to the identification of novel class(es) of antimitotic compounds. Overall, this study is interesting in describing quite convincingly the antimitotic effects of 1g and in showing that other ways that the direct targeting of tubulin could be effective to limit cell proliferation. I understand it could be too extensiveto better frame mechanism of action of the 1g compound, but the authors should consolidate a few experimental approaches and better quantify some of their experiments to fully support their conclusions.

Comments for the author

My first point is that although most of the data shown globally convince the reader that the conclusions are right, there are some points that would deserve better quantification. In this respect, unlike the data of Fig 1c, those shown in Fig 1a and 1b were not quantified at all. To me it's an important point because I feel a clear discrepancy between the images shown in Fig 1c and the hitograms of Fig 1d. Obviously some cells (blurred in the background of the fluorescence images) display an interphase cell morphology, which does not match with a 100% blockade upstream of the anaphase. What do these cells correspond to ? cells that did not enter mitosis or that underwent mitotic slipage ? Also this set of images (Fig 1c) does not look consistent with that of Figs 1a and b.

In Fig 2, the cells treated with 1g clearly display inefficient spindle assembly and some microtubule clusters that make cells look like if they underwent multipolar spindle formation. My first question is: what happens to the spindle of such cells if the observations last until 90 min or more ? Also, the persistence of microtubule asters in Fig 2a does not match the aspect of cells in Fig 1c. Could the authors quantify the proportion of cells that displayed 2 asters, with or without additional nucleation sites ? (this question could be of importance with regard to my remark about nucleation in the next paragraph). Also there is a point that could be have been discussed if not addressed by the authors: If the asters of 1g-treated cells contain shorter microtubules (according to the data of Fig 3), how is it that such shorter distance did not allow the formation of shorter spindles ? Have the authors considered the possibility that 1g inhibits not only microtubule growth but also the assembly of the spindle midzone ?

In Fig 3, the authors show that microtubule growth rates decreased in the presence of 1g. First, I do not see to which extent the decrease in the mean growth length brings a different information, i.e. if the microtubules grow more slowly for the same mean duration, then the mean length of growth will decrease in the same proportion. It seems to me also that the movie and the kymograph reveal that EB1 displays some blinking and thus that microtubule elongation is not continuous in the presence of 1g (the kymograph shows short tilted stretches with a global staircase look). This could reveal that elongation functions almost normally for short periods of time but that it is frequently interrupted.
I agree that Fig 1f suggests that 1g does not affect directly microtubule nucleation + elongation. However such curves only reveal the evolution of the total polymer length in the sample, but cannot discriminate between the presence of many short microtubules and that of less abundant but longer microtubules.
Also, if the assembly was performed using GTP (not GMPCPP but I did not find the information) microtubules may undergo dynamic instability, which could possibly mask an effect on elongation. The authors should thus take a look at the microtubules assembled with 1g to ensure that the curves only reveal elongation, without a compensatory effect on nucleation.

In Fig 4d, the authors measured the duration of mitosis, meaning that cells could actually exit mitosis.

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Again, is this phenotype contrasting with that of HeLa cells shown in Figs 1 and 2? Finally, do the cells that exit mitosis in the presence of 1g die downstream of the spindle assembly checkpoint activation ?

Finally, the question that has been completely eluded in this study is: could the authors provide some experimental evidence that 1g does not function at all through its effect on AMPA receptors ? Indeed, the literature is not completely devoid of relations between AMPA receptors and neuronal microtubule-associated proteins (MAP1B, MAP2), which could in turn control microtubule stabilisation and thus elongation.

Reviewer 2

Advance summary and potential significance to field

The sumitted manuscript shows that a new compound named 1g interferes with mitotic spindle assembly. It also shows that 1g does not interfere directly with tubulin. As expected from a compound with such an effect exposure to 1g triggers the SAC and leads to mitotic arrest.

These are interesting, but rather preliminary results regarding the effect of 1g. Some of the main conclusions listed in the abstract need to be confirmed and the effect on microtubule dynamics needs to be properly documented.

Comments for the author

The following points are critical.
1. Basic data on the effect of 1g on microtubule dynamics are incomplete. In the sentence starting with "The microtubule +tip protein EB1 has been commonly used" the authors claim that 1g treatment decreased "microtubule growth length" and "growth speed".
-Microtubule dynamic instability is defined by four parameters: growth and shrinkage rates, rescue and catastrophe frequencies. The authors must measure these and avoid unconventional terms like "microtubule growth length" (which are misleading). They should be able to derive these parameters from their data.

2. In the abstract, the authors state that "1g promotes a ……..reduction in microtubule growth during interphase and mitosis". However, at the end of the manuscript, when referring to Drosophila neuroblasts they report that they "do not observe major alterations in the overall architecture of the interphase microtubules network in 1g treated cells, suggesting that this compound may not induce the secondary effects observed with other anti-microtubule agents." All this is very confusing in several regards.
-Firstly, they should clarify the statement in the abstract because it contradicts their conclusions.
-Secondly, they must explain what they mean by "secondary effects observed with other anti-microtubule agents". The disassembly of the interphase cytoskeleton caused by colchicine, for instance, is not a secondary, but the primary effect of colchicine: depolymerisation of microtubules.
-Lastly, and most importantly, the authors must provide evidence supporting the alleged lack of effect of 1g on " the overall architecture of the interphase microtubules network" in neuroblasts. All panels in figure 4 show cells in mitosis. I could not find any data on the architecture of the microtubules network in neuroblasts in interphase. This should be rather straightforward. It would only take extending the recording time for 15 more minutes after cytokinesis, just until the mother centriole loses the PCM, to show that 1g-treated neuroblasts that went through mitosis displaying aberrant spindles can arrange the full microtubule cytoskeleton that is very conspicuous in untreated Drosophila neuroblasts.

3. The authors refer to "..in the presence of DMSO (control)…." in different parts of the manuscript. However, they do not mention (I could not find) the actual DMSO concentration used as control. This is important. DMSO is not innocuous. Above a certain concentration it will bring about mitotic phenotypes. This is particularly worrying in the experiments shown in Figure 4.
-What is the concentration of DMSO in the "DMSO" and "1g" panels in Figure 4a?

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-What is the concentration of DMSO in the “DMS0”, “5uM”, “10uM”, and “20uM” columns in Figure 4 b, c, and d?
-Can we be sure that the effects shown in d are not caused by DMSO?

4. At the highest concentration (20µM) 1g delays anaphase onset in Drosophila neuroblasts for only 6 minutes. This is totally unexpected; 6 minutes is a very short delay indeed. Exposure to colchicine and analogs results in very long mitotic arrest in these cells. The same goes for taxol and analogs.
-Why would that be?
-20µM is a relatively high concentration. How sure can we be that the observed effect is not due to a contaminant?
-And, again, what is the concentration of DMSO in the 20µM 1g experiment?
-Please, run colcemide or taxol treatment experiments in parallel as positive control.

Other important issues.

5. I do not understand the meaning of the dotted circles in Figure 4a, panel 13:00. Do they mean that a tripolar spindle resulted in double cytokinesis (i.e one mother cell, three daughters)?
-Please explain.
-If so, what is the frequency of such an event?
-All three resulting products must be lethal (2n/3 for each). Please, discuss

6. In the abstract, the authors state that “1g does not interfere directly with tubulin, nor perturbs microtubules assembly in vitro.”
- This sentence is misleading. I guess (correct me if I am wrong) that if the in vitro assay was done in a cell free extract, xenopus egg extract, etc, 1g would work just as well as it does in vivo. The issue -still my guess- is not in vivo versus in vitro but a simple buffer versus a protein rich medium that contains MAPs, etc. Is that the case?

7. I am confused as to why they find it “Interesting” (abstract) that 1g also triggers SAC-dependent mitotic delay. Is that not the unavoidable consequence of disrupting spindle assembly?

8. Why do the authors think this 1g is more promising than other anti-mitotic drugs? They claim so, but fail to explain it.

9. Figure 1 c-e. The abnormal mitotic spindle caused by exposure to 1g is the main part of the manuscript and needs to be well documented. Cells shown in these panels are far too small to appreciate relevant details. Please provide enlarged higher resolution figures to document well this critical point.

Minor
—What the authors call “microtubules dynamicity” is normally referred to as “microtubule dynamics”
—“mitotic cells to be delayed by a SAC-dependent activation”. “”SAC-dependent mitotic delay” would be more appropriate.
—“suggesting that cells remained arrested “in the early phases of mitosis”. The actual phase of arrest by SAC activation is “prometaphase” (as the authors state later on in the manuscript). —
All Figures: show 1g and DMSO final concentration in each panel
—Figure 3f: captions are impossible to read.

First revision

Author response to reviewers' comments

Reviewer 1:
Point 1:
My first point is that although most of the data shown globally convince the reader that

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the conclusions are right, there are some points that would deserve better quantification. In this respect, unlike the data of Fig 1c, those shown in Fig 1a and 1b were not quantified at all.

We have now quantified the mitotic index after 16hrs treatment (now panel C) corresponding to figure 1A and B.

To me it's an important point because I feel a clear discrepancy between the images shown in Fig 1c and the hitograms of Fig 1d. Obviously some cells (blurred in the background of the fluorescence images) display an interphase cell morphology, which does not match with a 100% blockade upstream of the anaphase. What do these cells correspond to ? cells that did not enter mitosis or that underwent mitotic slipage ? Also this set of images (Fig 1c) does not look consistent with that of Figs 1a and b.

The reviewer should note that the experimental design is different between figure 1A,B,G and figure 1D. In figure 1 A,B,G, we have filmed an asynchronous population of cells. Since the doubling time for Hela cells is around 24hrs (ei. all the cells should enter mitosis within 24hrs) in case of mitotic arrest the number of cells in mitosis should increase as the time of treatment increases (in absolute, up to 100% at 24hrs).
Quantification of the mitotic index has been performed after 16hrs of treatment.
In figure 1D, in order to confirm that the accumulation of rounded cells we observed were cells arrested in metaphase, as the rounded phenotype suggests, we have arrested cells at the G2/M transition by treatment with the CDK1 inhibitor RO 3306, then released them in a synchronized fashion and followed their progression through mitosis. The blurred cells are cells in interphase and correspond to cells that have not entered mitosis for two reasons: first, since the doubling time for Hela cells is 24hrs and the RO treatment was of 16hrs, not all the cells would have reached the G2/M transition during the time of treatment. The second and main reason is that after long RO treatment not 100% of the G2/M arrested cells are able to reenter the cell cycle and some remain blocked. However, cells released from the block reenter the cell cycle in a synchronized fashion, allowing the comparison at fixed time of the mitotic progression under different conditions.
At 5µM, we do not believe that cell undergo mitotic slippage, since none has been observed when cells were filmed for 24hrs (movie S2). At longer time treatment, cells that arrest in mitosis have been documented to undergo apoptosis (Parenti et al 2016) (text line 124).

The apparent discrepancies between figure 1D and 1E come from the fact that in figure D we have only quantified cells that have entered mitosis. We have quantified their mitotic stage repartition thru time. We have now added this precision in the figure 1E (and figure legend) by changing % of cells for % of mitotic cells.

We have performed again the experiment 1C (now 1D) and thus the corresponding quantification (figure 1E) with the exact same drug concentration as in the other figures (2.5uM and not 2uM). We have acquired better resolution images on a SP5 confocal microscope (objective 63X), instead of the wide field DMRXA microscope (objective 40X) previously used. Furthermore, the materials and methods were previously included in the figure legends and thus very succinct. In the revised manuscript, we have now included a full materials and methods section describing in more details the experimental approaches.
We have also repeated experiment 1B and obtained equivalent results.
We have now described and discussed the results in more details in the text.

<u>Point 2:</u>

In Fig 2, the cells treated with 1g clearly display inefficient spindle assembly and some microtubule clusters that make cells look like if they underwent multipolar spindle formation. My first question is: what happens to the spindle of such cells if the observations last until 90 min or more ? Also, the persistence of microtubule asters in Fig 2a does not match the aspect of cells in Fig 1c. Could the authors quantify the proportion of cells that displayed 2 asters, with or without additional nucleation sites ?

To address the first question, we have repeated the experiment 2A and now filmed for 90min

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HeLa cells expressing both tubulin-GFP and H2BmCherry (to visualize DNA) in presence of DMSO or 1g. Selected time points are now presented in a new figure 2B We have now quantified bipolar spindle formation (panel C), chromosome congression (panel D) and asters numbers (panel E) and added the graphs to figure 2.
We have also described the phenotype in more details in the text. Lines 130-156

Also there is a point that could be have been discussed if not addressed by the authors: If the asters of 1g-treated cells contain shorter microtubules (according to the data of Fig 3), how is it that such shorter distance did not allow the formation of shorter spindles ? Have the authors considered the possibility that 1g inhibits not only microtubule growth but also the assembly of the spindle midzone?

We have now discussed line 266 the fact that when treated with 5µM 1*g*, mitotic Hela cells do not just form a shorter spindle, but are unable to form a bipolar spindle. “Moreover, additional microtubule asters are detected, that fails to coalesce into a bipolar structure. This inability to form a bipolar spindle suggests that not only the growth of MT is altered but also the structure of the metaphase spindle, such as the organization/bundling of the interpolar microtubules. ”

Point 3:
First, I do not see to which extent the decrease in the mean growth length brings a different information, i.e. if the microtubules grow more slowly for the same mean duration, then the mean length of growth will decrease in the same proportion

-As stated by the reviewer, the data on growth rate and growth length are redundant. We have thus removed the graph on growth length.

It seems to me also that the movie and the kymograph reveal that EB1 displays some blinking and thus that microtubule elongation is not continuous in the presence of 1g (the kymograph shows short tilted stretches with a global staircase look). This could reveal that elongation functions almost normally for short periods of time but that it is frequently interrupted.

-We have now derived additional information from our data collected in figure 3. These data are now presented in figure 3 E,F and added to the text line 178-190.
The inferred number of pause (figure 3F) is not statistically different in cells treated with 1*g* or DMSO, nor is the median Fgap speed (text lines 198). As a consequence, since the microtubule growth speed is decreased, the frequency time and length of pauses is significantly increased.

I agree that Fig 1f suggests that 1g does not affect directly microtubule nucleation + elongation. However, such curves only reveal the evolution of the total polymer length in the sample, but cannot discriminate between the presence of many short microtubules and that of less abundant but longer microtubules.
Also, if the assembly was performed using GTP (not GMPCPP but I did not find the information) microtubules may undergo dynamic instability, which could possibly mask an effect on elongation. The authors should thus take a look at the microtubules assembled with 1g to ensure that the curves only reveal elongation, without a compensatory effect on nucleation.

-Question on figure 3F-turbidity assay:
Turbidity measurements are classically used to analyze the effect of drugs or MAPs on microtubule assembly, including microtubule nucleation and elongation. If 1g had a particular effect on nucleation, this would have been seen in the lag-phases at different drug concentrations (i.e., the time between incubation at 35 °C and the beginning of turbidimetry increase). A compound that would enhance nucleation would shorten this lag-phase (e.g. GMPCPP), while a compound that decreases nucleation would have had the inverse effect. In the present case, we do not observe any effect of the drug on nucleation (same lag-phases at different concentrations), on elongation (sigmoid) phase, nor on the plateau of polymerization (total mass of microtubules assembled).

We have now included these points in the result section line 192-203. We have also included the method used to perform the turbidimetry assay in the materials and methods section of the manuscript.

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Point 4:
In Fig 4d, the authors measured the duration of mitosis, meaning that cells could actually exit mitosis. Again, is this phenotype contrasting with that of HeLa cells shown in Figs 1 and 2? Finally, do the cells that exit mitosis in the presence of 1g die downstream of the spindle assembly checkpoint activation ?

We observe similar spindle assembly defects in both Hela cells and Drosophila neuroblasts (Fig2B and Fig4F-new panels). However, in Drosophila, after a delay neuroblasts are able proceed through a defective mitosis, giving rise to three cells most likely aneuploid. This difference can be explained by several factors. First of all, in contrast to the treatment of Hela cells in culture, in Drosophila experiments whole brains were incubated in the 1*g* solution. The drug thus needs to penetrate the tissue and the dividing neuroblasts may thus be exposed to a lower effective drug concentration. Secondly, the difference between the effect of 1*g* on Drosophila and human cells may be caused by their different in genome size. Indeed, Drosophila neuroblasts contain only four chromosomes which is a huge difference compared to human cells that exhibit 46 chromosomes. Consequently, proper attachment of kinetochores is an easier task for Drosophila cells compared to Hela cells. In agreement with that, the group of Karess has demonstrated that in *Drosophila* cells under unstressed conditions, a functional SAC is not required for spindle assembly and chromosome segregation. However, when fly cells are subjected to mitotic poisons, like Taxol or nocodazole, the SAC becomes unsatisfied leading to a persistent mitotic arrest as a direct consequence of SAC unsatisfaction (Figure S1). Our live imaging studies in the presence of 1g (Figure 4) suggests that the SAC is transiently activated accounting for the mitotic delay. The formation of a spindle albeit abnormal, still allows a slower progressive attachment of the kinetochores to the microtubules, ending up satisfying the SAC. This situation of SAC satisfaction delay is similar to the one observed in Hela cells treated with the lower concentration of 1*g* (1□M). However, for Hela cells the delay for proper chromosome attachment is longer.

We have now discussed this point in the text line 109 and in discussion line 255-263.

The fate of the daughter cells subjected to 1*g* treatments has not been investigated in deep details. However, two recent reports using fly mutants (and only correlative analyses) have suggested that aneuploid neuroblasts are arrested in the following interphase and start a differentiation program (Caous et al., Nature communication, 2015; Godendeau et al., Nature communications, 2015). We haven't performed long live imaging experiments to assess if the resulting aneuploid Nbs and aneuploid presumptive GMCs in presence of 1*g* are able to complete another cell cycle (and if they eventually become arrested in interphase). These experiments will require extensive analyses and to set up new protocols to quantify the levels of chromosome segregation defects in live dividing Nbs and GMCs (that are very small cells) and to analyze their fate over long periods. This is interesting, but will be technically difficult. Moreover, I presume that these analyses would need to be confirmed in mutant backgrounds to draw general rules from it. These questions are really exciting and deserve to be addressed, but we feel they are probably beyond the scope of this initial story on the 1*g* compound, especially given the report format of JCS, which is very short.

We have now better characterized the phenotype observed in presence of 1*g* by quantifying the frequency of the formation of tripolar spindle (added in the text line 224and new Fig 4E).

Point 5:
Finally, the question that has been completely eluded in this study is: could the authors provide some experimental evidence that 1g does not function at all through its effect on AMPA receptors ? Indeed, the literature is not completely devoid of relations between AMPA receptors and neuronal microtubule- associated proteins (MAP1B, MAP2), which could in turn control microtubule stabilisation and thus elongation.

Although it is true, that some authors have reported a link between AMPA receptors and microtubules, our molecule binds the receptor with a Kd>100 μM, as reported in the paper by Micale et al. (Bioorganic & Medicinal Chemistry 16 (2008) 2200–2211), which means a no-pharmacological interaction. Since our experiments have been performed at much lower concentrations than Kd, we can exclude the interaction with AMPA receptor and therefore the

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involvement of AMPA in the microtubule modulation described in this study.
We have now added this point in the discussion line 236.

Reviewer 2:

Point 1:
Basic data on the effect of 1g on microtubule dynamics are incomplete. In the sentence starting with "The microtubule +tip protein EB1 has been commonly used" the authors claim that 1g treatment decreased "microtubule growth length" and "growth speed".
-Microtubule dynamic instability is defined by four parameters: growth and shrinkage rates, rescue and catastrophe frequencies. The authors must measure these and avoid unconventional terms like "microtubule growth length" (which are misleading). They should be able to derive these parameters from their data.

As suggested, we have removed growth length. We now generated new graphs providing information on number of nucleation events (Fig3E), growth rate (Fig3D), pause numbers and duration (Fig3F).
In presence of 1*g*, no shrinkage events followed by regrowth were detected. The algorithm therefore cannot infer shrinking rates from the data. This information has been added in the text line 180-188.

Point 2:
2. In the abstract, the authors state that "1g promotes a ……..reduction in microtubule growth during interphase and mitosis". However, at the end of the manuscript, when referring to Drosophila neuroblasts they report that they "do not observe major alterations in the overall architecture of the interphase microtubules network in 1g treated cells, suggesting that this compound may not induce the secondary effects observed with other anti-microtubule agents." All this is very confusing in several regards.
-Firstly, they should clarify the statement in the abstract because it contradicts their conclusions.

- We have made the suggested changes in the abstract in order to address this confusion; The reduction in microtubule growth was measured in interphase cells. In non-motile interphase cells, the slowing down in growth speed results in a modest alteration in the overall microtubule network. However, the remodeling of microtubules into a mitotic spindle and the dynamic attachment of kinetochores, requires a 10-fold increase in the turnover rate of microtubules, thereby amplifying the effect of the 1*g* compound. This is discussed in the discussion of the manuscript line 2242-254. We also discuss the fact that 1*g* may also alter the organization of the mitotic spindle in addition to microtubules growth (lines 266-269).

-Secondly, they must explain what they mean by "secondary effects observed with other anti-microtubule agents". The disassembly of the interphase cytoskeleton caused by colchicine, for instance, is not a secondary, but the primary effect of colchicine: depolymerization of microtubules.

- In agreement with the reviewer we have now removed "secondary "and rewrote the discussion

-Lastly, and most importantly, the authors must provide evidence supporting the alleged lack of effect of 1g on " the overall architecture of the interphase microtubules network" in neuroblasts. All panels in figure 4 show cells in mitosis. I could not find any data on the architecture of the microtubules network in neuroblasts in interphase. This should be rather straightforward. It would only take extending the recording time for 15 more minutes after cytokinesis, just until the mother centriole loses the PCM, to show that 1g-treated neuroblasts that went through mitosis displaying aberrant spindles can arrange the full microtubule cytoskeleton that is very conspicuous in untreated Drosophila neuroblasts.

- We have now repeated the experiment presented in figure 4 and performed longer time-lapse. We are now providing in a new panel- figure 4G a longer time sequence of 1*g* treated neuroblasts,

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where we can see the formation of the interphase aster following a defective division.

Point 3:
The authors refer to "..in the presence of DMSO (control)…." in different parts of the manuscript. However, they do not mention (I could not find) the actual DMSO concentration used as control. This is important. DMSO is not innocuous. Above a certain concentration it will bring about mitotic phenotypes. This is particularly worrying in the experiments shown in Figure 4.
-What is the concentration of DMSO in the "DMSO" and "1g" panels in Figure 4a?
-What is the concentration of DMSO in the "DMS0", "5uM", "10uM", and "20uM" columns in Figure 4 b, c, and d?
-Can we be sure that the effects shown in d are not caused by DMSO?

In the revised manuscript, we are now providing a Materials and Methods section. In the Chemical compounds paragraph, we now describe the DMSO concentration used in the experiments. "1*g* was dissolved in DMSO at a stock concentration of 50mM. Further dilutions were performed in tissue culture medium. DMSO control treatment is equivalent to the amount of DMSO present in the highest 1*g* treatment concentration used in the experiment. The final DMSO concentrations in the assays were therefore inferior or equal to 0.01% in Hela cell experiments and 0.04% for Drosophila tissue experiments".

Point 4:
4. At the highest concentration (20µM) 1g delays anaphase onset in Drosophila neuroblasts for only 6 minutes. This is totally unexpected; 6 minutes is a very short delay indeed. Exposure to colchicine and analogs results in very long mitotic arrest in these cells. The same goes for taxol and analogs.
-Why would that be?

- As expected, the new experiments we have now performed with Taxol and nocodazole treatments of neuroblasts result in very long delays (now presented in a new figure S1D,E). In addition, treated neuroblasts do not proceed through cytokinesis, but rather undergo a mitotic slippage-like behavior. The phenotype is clearly different from the one observed with 1*g*, where treated neuroblasts manage to assemble a spindle (although tripolar in many cases) and segregate their chromosomes. Our *in vitro* experiment with purified microtubules (Fig. 3G) indicates that unlike Taxol or colchicine, 1*g* does not directly target microtubules directly. We therefore propose that it rather targets a key regulators of MT polymerization. This difference in the mode of action can account for the strong difference in the phenotype observed.

Since we hypothesize that 1*g* targets a MAP (manuscript discussion), for sake of comparison, we have now added in a new supplementary Fig. S1 the analysis of Msps/TOG/XMAP215 RNAi. Msps is a MAP whose down regulation has been described to severely disrupt the integrity of the mitotic spindle (Cullen et al., JCB 1999). Whereas the mean duration of mitosis for control cells (DMSO treated and wild type neuroblasts) is 5.8±0.1 min (n=43), it is of 15.5±0.8 min n=44 for the msps-depleted neuroblasts and of 10.6±0.5 (n=42) for 1*g* treated neuroblasts. These results reveal a comparable range of mitotic delay.

We have now included those results in the text line 218.

-20µM is a relatively high concentration. How sure can we be that the observed effect is not due to a contaminant?

-Reviewer 2 raised the issue of the possible presence of contaminants. At the time of synthesis, the purity of the 1*g* compound was analyzed by 1H NMR and 13 C NMR. Furthermore, prior to use, HLPS-MS-MS was performed on the compound in PBS and the only relevant peak was that one of 1g. These elements are now indicated in the materials and methods section under the "Small molecules inhibitor" section.

And, again, what is the concentration of DMSO in the 20µM 1g experiment?

- Concentration of DMSO (see answer point 3): For all the panels of figure 4, DMSO is present at

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0.04%, which correspond to the DMSO concentration present in the 20uM 1*g* treatment.

-Please, run colcemide or taxol treatment experiments in parallel as positive control.

-We have now run Taxol and Nocodazole treatment experiments as positive control and have included them in a new supplementary Figure S1

Point 5:

I do not understand the meaning of the dotted circles in Figure 4a, panel 13:00. Do they mean that a tripolar spindle resulted in double cytokinesis (i.e. one mother cell, three daughters)?
-Please explain.

-In neuroblasts exposed to 1*g*, we had observed the formation of tripolar spindle resulting in the initiation of a double cytokinesis which can lead to the abnormal formation of three daughter cells (Fig.4A, 1*g*-dotted lines 13min). However, in the new films with longer time lapse that we have performed for the revised manuscript, we could observe in some instance the regression of the cytokinesis furrow. We have now discussed the variability of the phenotype in the text line 226-230.

-If so, what is the frequency of such an event?

-As requested by the reviewer, we have quantified the frequency of the formation of tripolar spindles (79% of the 20¤M treated neuroblasts n=42). All chromosome segregation defects quantified in Fig. 4C are associated with tripolar spindle formation and the formation of two central spindles, inducing furrowing. We have now added a new graph (Fig 4E) quantifying this phenotype. The graph also includes the quantification of the additional movies we have performed. However, as mentioned above the prolonged time lapses have revealed that the outcome is multiple. Some divisions proceed through double cytokinesis leading to the abnormal formation of three daughter cells (Fig.4A, 1*g*-dotted lines 13min). We could also observe in some cases the fusion of two of the three daughter cells. We film neuroblasts in a whole tissue and very frequently in the late time points after cytokinesis, the progeny neuroblast remains in the plane of focus but the GMC like progeny moves out of the plan of focus, making it very difficult to illustrate and quantify precisely the outcome.

All three resulting products must be lethal (2n/3 for each). Please, discuss

-In view of the chromosome segregation observed (Fig. 4A, DNA, time 9 min in the presence of 1g), it is likely that the cytokinesis results in the formation of aneuploid neuroblast and aneuploid ganglion mother cells (GMC).
The fate of the daughter cells subjected to 1*g* treatments has not been investigated in deep details. However, two recent reports using fly mutants (and only correlative analyses) have suggested that aneuploid neuroblasts are arrested in the following interphase and start a differentiation program (Caous et al., Nature communication, 2015; Godendeau et al., Nature communications, 2015). We haven't performed long live imaging experiments to assess if the resulting aneuploid Nbs and aneuploid presumptive GMCs in presence of 1*g* are able to complete another cell cycle (and if they eventually become arrested in interphase). These experiments will require extensive analyses and to set up new protocols to quantify the levels of chromosome segregation defects in live dividing Nbs and GMCs (that are very small cells) and to analyze their fate over long periods. This is interesting, but will be technically difficult. Moreover, I presume that these analyses would need to be confirmed in mutant backgrounds to draw general rules from it. These questions are really exciting and deserve to be addressed, but we feel they are probably beyond the scope of this initial story on the 1*g* compound, especially given the report format of JCS, which is very short.

Point 6:
In the abstract, the authors state that "1g does not interfere directly with tubulin, nor perturbs microtubules assembly in vitro."
- This sentence is misleading. I guess (correct me if I am wrong) that if the in vitro assay was done in a cell free extract, xenopus egg extract, etc, 1g would work just as well as it does in vivo. The

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issue -still my guess- is not in vivo versus in vitro, but a simple buffer versus a protein rich medium that contains MAPs, etc. Is that the case?

The *in vitro* microtubule polymerization assay was not performed in a cell free extract, but in a minimal buffer: purified tubulin in polymerization buffer (10 % glycerol, 1 mM GTP, 0.02 % DMSO in BRB80 buffer (80 mM K-Pipes, 1 mM EGTA, 1mM $MgCl_2$, pH 6.8 with KOH)). No others cellular proteins (MAPS) were present in the assay. We have now a new section named **Turbidimetry assay** describing in details this assay in the Materials and Methods section.

Point 7:
I am confused as to why they find it “Interesting” (abstract) that 1g also triggers SAC-dependent mitotic delay. Is that not the unavoidable consequence of disrupting spindle assembly?
Indeed, we agree “interesting” is not appropriate transition word and we have removed it from the abstract line 36.

Point 8:
Why do the authors think this 1g is more promising than other anti-mitotic drugs? They claim so, but fail to explain it.
This is a good suggestion and we have now discussed in the text why we believe that 1*g* is a promising anti-mitotic drug (lines 276-283).

Point 9:
Figure 1 c-e. The abnormal mitotic spindle caused by exposure to 1g is the main part of the manuscript and needs to be well documented. Cells shown in these panels are far too small to appreciate relevant details. Please provide enlarged higher resolution figures to document well this critical point.

In order to obtain better resolution images, we have now repeated experiment 1C, acquired Z stack images on a SP5 confocal microscope (objective 63x) instead of the previous wide field DMRXA (40x objective). New panels are now included in figure 1D and the quantification analysis has been repeated (Fig. 1E).

Minor points:
—What the authors call “microtubules dynamicity” is normally referred to as “microtubule dynamics”
—“mitotic cells to be delayed by a SAC-dependent activation”. “”SAC-dependent mitotic delay” would be more appropriate.
—“suggesting that cells remained arrested “in the early phases of mitosis”. The actual phase of arrest by SAC activation is “prometaphase” (as the authors state later on in the manuscript).

—All Figures: show 1g and DMSO final concentration in each panel
—Figure 3f: captions are impossible to read.

- We have replaced microtubule dynamicity by microtubule dynamics
- We have replaced “mitotic cells to be delayed by a SAC-dependent activation” by “SAC-dependent mitotic delay”
- We have replaced “early phases of mitosis” by prometaphase
- We have indicated the treatment concentration on each panel
- We have replaced the low-resolution figure 3F by a new panel.

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Second decision letter

MS ID#: JOCES/2019/239244

MS TITLE: A novel benzodiazepine derivative that suppresses microtubules dynamics and impairs mitotic progression

AUTHORS: Vittoria Pirani, Mathieu Metivier, Emmanuel Gallaud, Alexandre Thomas, Siou Ku, Denis Chretien, Roberta Ettari, Regis Giet, Lorenzo Corsi, and Christelle Benaud
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The paper by Pirani et al. identifies and characterizes the effects of a new compound called 1g, which functions to prevent normal mitotic spindle assembly and mitotic progression without interacting with tubulin.
This work opens the way to the identification of alternative molecular targets which could be valuable to function as new antimitotics to kill cancer cells. This is important due to the increasing problems caused by intrinsic or acquired resistance to tubulin-interacting drugs.

Comments for the author

The revised version of the article by Pirani et al addressed all my previous comments. In my view, the authors provided satisfactory explanations and additional elements that comforted their results and made some aspects of their work clearer and easier to understand. They appropriately added the corresponding arguments to the main results and to the discussion sections. Thus, I may now recommend in favor of publication in the JCS.

Reviewer 2

Advance summary and potential significance to field

The authors have made substantial changes that help to clarify results and claims. However, some fundamental issues remain unclear to me.

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Comments for the author

For instance, I do not understand why the authors do not stick to the standard nomenclature and provide (whatever the values) the four parameters that define “microtubule dynamic instability” (i.e. growth and shrinkage rates, rescue and catastrophe frequencies). “Nucleation events”, “pause numbers”, etc are very confusing. I am at a loss with the statement that in presence of 1g, no shrinkage events take place: are Mts frozen?

Are the authors convinced that the interphase aster (new panel figure 4G) in 1g treated neuroblasts is identical to the aster in the DMSO treated neuroblasts? To me, the control aster looks more prominent and with longer microtubules. This is one of the main claims of the manuscript. It should be thoroughly documented.

I also find it hard to understand how the disarranged mitotic figures assembled following 1g treatment do not trigger the SAC.

Having said this, I see and acknowledge the improvement in this revised version. The observations and conclusions reported here will be of interest to cell biologists.

Second revision

Author response to reviewers' comments

Reviewer 2

For instance, I do not understand why the authors do not stick to the standard nomenclature and provide (whatever the values) the four parameters that define “microtubule dynamic instability” (i.e. growth and shrinkage rates, rescue and catastrophe frequencies). “Nucleation events”, “pause numbers”, etc are very confusing. I am at a loss with the statement that in presence of 1g, no shrinkage events take place: are Mts frozen?

Assessing microtubule dynamics in a whole cell can be challenging. Labelling tubulin results in an abundant signal which allows single microtubules tracking only on the cell edge.
However, the presence of spatial difference in microtubule dynamic within a cell has been demonstrated (Applegate *et al*. J Struct Biol., 2011). Therefore, in a whole cell context, the EB1-tracking methods provides a more global information, since it allows the tracking of all the growing microtubule within the cell.
For this reason, in the present study, we have analyzed EB1-GFP comets dynamics using plusTipTraker software tracking algorithm and not whole microtubules. The clustering algorithm has been proven to be robust and been used in many publications since it has been developed. However, there is a caveat to the method because EB1-GFP labels only growing microtubules. The data linked to non-growing microtubules such as shrinking or phase transition have to be interpolated. Altogether the measure inferred from the analysis are not directly comparable to the classical parameters of microtubules dynamic (growth and shrinkage rate, rescue and catastrophe frequencies) due to the different in the method of analysis (Matov *et al*., Nat methods, 2010; Applegate *et al*. J Struct Biol., 2011). The method based on a “trajectory reconstruction by subtract linking reveals only those shrinkage events followed by a rescue within a highly constrained spatial and temporal window”. The developers also “estimate that only 20-30% of the shortening events are capture by the method.” In order to infer shrinking rate, shrinkage in one microtubule has to be followed by subsequent phase of regrowth generating detectable EB1 comets on the same trajectory. Therefore, the algorithm misses unrescued shrinkage events. As a consequence, only catastrophes followed by a rescue are identified and we cannot deduce the frequency of each separately.
In control cells, we could infer a shrinkage rate of 32.88±2.16 $\mu m.min^{-1}$ (which was not statistically different in DMSO treated cells 34.5±1.7 $\mu m.min^{-1}$). In presence of 1*g*, no shrinkage

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events were detected by the algorithm in the analyzed cells. This could be explained by the fact the main events occurring were terminal shortenings and shrinkage followed by short or slow growth phases that do not produce detectable EB1-comets within the temporal window analyzed. We are now providing these results and discuss those in the text line 187. We also amended the text and changed “dynamic instability” into microtubule growth (abstract, text line 80, 158, 190).

Are the authors convinced that the interphase aster (new panel figure 4G) in 1g treated neuroblasts is identical to the aster in the DMSO treated neuroblasts? To me, the control aster looks more prominent and with longer microtubules. This is one of the main claims of the manuscript. It should be thoroughly documented.

Our result indicated that the formation of the interphase aster is not significantly impeded by the 1*g* treatment, which was not obvious in the previous version submitted. We have therefore performed additional time-lapse experiments using a different brighter microtubule marker (mcherry alpha-tubulin) coupled slightly different imaging approaches to better visualize the interphase aster (now described in material and methods lines 349, 329). These new experiments are now illustrated in a new additional panel, Figure 4 panel H. We have also added some precision in the discussion (line 245), since we do not wish to state that 1*g* does not affect at all the interphase network but rather has milder effect than microtubules binding agents.

I also find it hard to understand how the disarranged mitotic figures assembled following 1g treatment do not trigger the SAC.

In Hela cells the SAC is activated (results line 105-107 and figure 1F), resulting in mitotic arrest. We also observe SAC activation in drosophila neuroblasts. This activation is illustrated by a delay in NEB to anaphase progression and consist of a transient SAC activation and not a mitotic arrest (result line 215-218, Figure 4, discussion line 243).
Our data indicate that the effect of 1*g* on microtubules is milder than that of Nocodazole or Taxol. Upon 1*g* treatment microtubules retain some ability to grow. This defective microtubule growth seems nevertheless sufficient to attach all neuroblast kinetochores (albeit the abnormal mitotic spindle) and with significant time delay to satisfy the SAC. The same phenotype has been described for several MAPs (such as minispindle) whose alteration perturbs microtubule growth and mitotic spindle assembly (Cullen *et al*. 1999, Gallaud *et al*. 2014, Ramdas *et al*. 2016, Cesario *et al*. 2006) (msps control presented in the manuscript: result line 224 fig S1D). One could imagine that in view of the relatively low number of chromosomes that need to be attached (8 chromosomes), if enough time is given, a disarranged mitotic spindle can still attach the 16 kinetochores and satisfy the SAC. For sake of clarity we have reformulated the discussion line 261-273.

Third decision letter

MS ID#: JOCES/2019/239244

MS TITLE: A novel benzodiazepine derivative that suppresses microtubules dynamics and impairs mitotic progression

AUTHORS: Vittoria Pirani, Mathieu Metivier, Emmanuel Gallaud, Alexandre Thomas, Siou Ku, Denis Chretien, Roberta Ettari, Regis Giet, Lorenzo Corsi, and Christelle Benaud
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

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