Author's response to reviews

Title: Resveratrol abrogates the Temozolomide-induced G2 arrest leading to mitotic catastrophe and reinforces the Temozolomide-induced senescence in glioma cells

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Author's response to reviews: see over
Dear Editor

Please find enclosed the revised manuscript entitled “Resveratrol abrogates the Temozolomide-induced G2 arrest leading to mitotic catastrophe and reinforces the Temozolomide-induced senescence in glioma cells”, by E. C. Filippi-Chiela et al., submitted for publication in BCM Cancer as a Research Article.

Below, we provide a detailed answer to all questions raised by the referees and we included in the manuscript the suggestions made and the answers to the queries raised.

Looking forward to hear from you,

Yours sincerely,

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REVIEWER#1
Title: Resveratrol abrogates the Temozolomide-induced G2 arrest leading to mitotic catastrophe and senescence in glioma cells
Version: 1 Date: 16 January 2013
Reviewer: Renata Cozzi
Reviewer's report:
The manuscript is certainly interesting and presents a large amount of data that deserve a reorganization to make them more readable. Many results present in the supplementary figures should be retrieved in the canonical figures since they are essential for the overall work.

We worked on the reorganization so that the sequence of presentation of the data in the result section follows the sequence of the figures. Attending the suggestion of putting more figures in the main body of the manuscript, we integrated the data presented in Supplementary Figure 5 regarding the data of the primary GBM culture with Supplementary Figure 1 (survival and autophagy) and Figure 5 (senescence induction).

MAJOR COMPULSORY REVISIONS
1. First of all, how were resveratrol doses chosen? How were treatment times chosen?
   Times and doses of treatments were defined by dose and time-response curves, as published previously by our group [1, 2]. In order to approximate in vitro to in vivo conditions, we opted for the lowest concentration with an effect. In this case we also focused more on long term effects than short term effects.

2. Results section
   • Page 8 line 3 and Fig1: which TMZ dose reduces cell number? Perhaps 1000 µM but p values are missing both for R and TMZ single treatments
     We added the statistics for the single treatments.

     • Supp Fig 5A and page 8 lines 4-6: TMZ treatment per sé does not show any toxicity in primary glioma cells
     We added this sentence to more accurately reflect the results of the primary GBM culture: “A primary GBM culture which was resistant to TMZ was sensitive to Rsv and to RT (Supp. Fig. S1).”

     • Fig 2 FigS3 FigS5 and page 8 lines 16: as far as autophagy induction, RT treatment effect seems more likely to be additive with respect to R and TMZ alone
     We changed the sentence to: “RT induced higher levels of autophagy in an additive manner with respect to Rsv and TMZ alone”

   Fig 3a FigS4 and page 8 lines 22-29: The authors state that “resveratrol did not alter cell cycle distribution …….. “, but in R treated samples DNA profiles are quite different from control ones!!!. On the other hand from literature data it is well known that in cancer cells R treatment is able to cause cell cycle delay both at G1/S transition and during S progression (eg. Ahmad et al, Clin Cancer Res 2001 ;7:1466–
Indeed, we also showed previously that Rsv induced S-G2/M cell cycle arrest after 24h of treatment, but cell cycle distribution appeared normalized after 48h, suggesting a recovery in U87 cells [1]. We altered the first sentence of the paragraph from lines 22 to 29, so that this text refers only to U87. We also altered the sentence related to U251 cells, in which Rsv induced a significant S-phase arrest and slightly reduced TMZ-induced G2/M arrest, although the effect is less clear due to the S phase-arrest induced by Rsv, as described in the text. Concerning U138 cells, we added the significance signs related to Rsv in the Supp Fig 4 and added a sentence to the text.

• Fig 3B how was “DNA damage index” on Y axis calculated since the scoring of damage was performed as “arbitrary value range from 0-4”?
In Material and Methods section we have this description – “One hundred nuclei were scored according to the amount of DNA present in the tail and the tail length. Each nuclei received an arbitrary value range from 0-4 (0, undamaged; 4, maximally damaged) [3], and at least 100 nuclei per slide were evaluated”. We correct the end of this paragraph removing the expression ‘at least’, since we always counted 100 nuclei (and not ‘at least’). Thus, the DNA damage index is generated from the addition of the damage level (0 to 4) of one hundred cells. Thus, the maximum DNA damage index is 400. The evaluation was done by a PhD student [4] specialized in this analysis and which was blind to the treatments she was analyzing. We added “blindly” to the text to reflect this fact.

• Fig3C how do the authors explain the slight induction of #H2AX, ATM and Chk2 after R treatment?
Rsv induced a slight DNA damage, which was not significant, as shown by comet assay (Fig 3C). Indeed, several other works described Rsv as a inducer of DNA damage in cancer cells, and the mechanism of DNA damage may involve the mobilization of copper (which is present in the fetal bovine serum used in cell culture) and oxidative damage. [5-8]. In agreement to our data, Tyagi et al showed that Rsv induced S-phase arrest accompanied by an increase of expression and activity of ATM, Chk2 and gammaH2AX in ovarian cancer cells [6]. We added the sentence above to the discussion (page 11). It is interesting to notice that in several parameters (DNA damage, cell cycle arrest, increase in nuclear size) Rsv induces an increase which is afterwards reversed, which is not the case of TMZ.

• Fig 4C what on X axis? And on Y axis? Page 9 lines 26-27: how do the authors explain the presence of micronuclei after R treatment?
We added the axis titles in Fig 4C. The presence of micronuclei after Rsv treatment may be caused by DNA damage, as discussed above. This observation is in agreement with data from Mitrut et al, which described a dose-dependent induction of micronuclei after Rsv treatment in primary gastric adenocarcinoma cell cultures [9].
Page 9 lines 32-34 and Fig 4C: R treatment induces a strong (significant?) increase in large nuclei considered by the authors a feature of senescence. Anyway from Fig 5B R treatment does not seem to induce senescence as measured by SA-beta-gal assay. How do the authors explain this discrepancy? In this regard, the title of the manuscript should be changed avoiding the statement that Resveratrol leads to senescence. Perhaps the large nuclei are polyploidy cells that together with micronuclei induction could explain the induction of DDR signalling. In other word R treatment induces DNA double strand breaks and chromosome malsegregation in U87 cells through an inhibitory effect on topoisomerase II (see Leone et al, Cancer Letters 295 (2010) 167–172)

We added the statistics to the Fig 4C and we changed the title of the manuscript as suggested. Previously we described that Rsv induced senescence in C6 rat glioma cells after 12 days with medium change every two days containing new Rsv [2]. Here, we treated cells during 48h, followed by drug removal and growth in drug-free medium for 7 days. Maybe the induction of senescence by Rsv requires the constant addition of new drug, or more than 7 days to stain positively for SA-beta-gal.

In our validation experiment of nuclear size as an indicator of senescence we found that 35 out of 39 large nuclei were also positive for SA-β-gal (Fig. 2 of [10]). In this case we used cells treated for 10 days. We hypothesize that nuclear increase occurs prior to SA-β-gal induction and that the cells which presented increased nuclear size at day 2 of treatment were eliminated from the population after 7 days. We added a discussion paragraph (third paragraph) to focus on the effects of resveratrol alone and we discuss this apparent discrepancy.

- MINOR ESSENTIAL REVISIONS
  o Correct paragraph numbers in Mat and Met section
    Done
  o DCFH assay is not described in Mat and Met
    Added
  o Page 8 line 3 change “cell number of gliomas” in “number of glioma cells”
    Done
  o Fig 3 legend: page 13 line 23: R is missing
    Added
  o Fig 3A and Supp Fig 4: the sum (subG1-G1-S-G2) should be 100
    Reviewed and corrected

DISCRETIONARY REVISIONS
I would suggest to reorganize the figures since it is hard to find data and results as they are currently organized
As mentioned above, we reorganized the figures. Thanks for the suggestion.
Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
'I declare that I have no competing interests'
Reviewer’s report
Title: Resveratrol abrogates the Temozolomide-induced G2 arrest leading to mitotic catastrophe and senescence in glioma cells
Version: 1 Date: 19 January 2013
Reviewer: Enrique Castellon
Reviewer’s report:
Manuscript BMC Cancer: Resveratrol abrogates the Temozolomide-induced G2 arrest leading to mitotic catastrophe and senescence in glioma cells. Eduardo C. Filippi-Chiela, et al.

General comment:
This work deals with the potential mechanism by which Resveratrol (Rsv) may potentiate the toxicity of Temozolomide (TMZ) in glioma cells. As authors state, TMZ induces formation of O6-methyl-guanine which impairs DNA replication. On the other hand, O6-methyl-guanine-DNA methyltransferase (MGMT) repairs most of the DNA damage produced by TMZ. Therefore, it is well known that status of expression, activity or promoter methylation of MGMT strongly correlate with TMZ resistance in glioblastoma. In the last years, several drugs and natural compounds (including Rsv) has been studied and proposed as combined therapy in order to potentiate TMZ effects (for updated review see Nakada et al, Front Oncol. 2012;2:98. The strategy for enhancing temozolomide against malignant glioma). It is not clear what is really novel in the present paper. The authors claim that Rsv does not reduce the DNA damage response (DDR), instead, it forces cell to go through the cell cycle inducing mitosis catastrophe. It would be interesting to check the status of MGMT in glioma cells used and evaluate whether Rsv have any effect on it.

We agree that much has been published on the topic, but the focus is mainly on ROS and autophagy. We focused on the DDR signaling and the cell cycle, which are centrally involved in the mechanism of TMZ toxicity. Furthermore, the molecular mechanisms that control mitotic catastrophe and the chronic effect of acute treatments with cytotoxic drugs in cancer cells are poorly known.

In order to more clearly answer the two initial questions, we prepared an integrated figure (below) with the data organized according to the effect of Rsv on TMZ-treated cells.

We agree that MGMT plays a fundamental role in TMZ resistance, but if the effects of Rsv on the cell cycle were mediated by inhibiting MGMT, we would expect DNA damage to be increased in Rsv+TMZ, which was not the case (upper box). Most importantly, if the effect was exclusively on MGMT, a more damaged DNA should increase the arrest at G2, not abrogate it. Therefore we focused our efforts on trying to identify the signaling that links DNA damage to the cell cycle machinery. A more detailed analysis is given in the next answer.
Even when the author's hypothesis is interesting, no direct evidence about the actual mechanism of Rsv action is provided. How does Rsv inhibit TMZ-induced cell cycle G2-arrest? This main mechanistic question remains unsolved. Actually, it is not even addressed. Additionally, many flaws should be properly clarified.

This figure organizes the results according to the effect that Rsv has on TMZ-treated cells. As already mentioned, no effect was found on the level of DNA damage index (upper box), arguing against an involvement of MGMT or other direct regulators of DNA repair on the effect of Rsv. The initial DDR signaling components had their
phosphorylation increased by Rsv over TMZ (middle box), suggesting a target positively regulated at the initial recognition and signaling of DNA damage.

Phosphorylation of Wee1 and Histone H3, two targets (the former direct, the latter indirect) of Chk1/2, were decreased by Rsv in TMZ treated cells (lower box), suggesting that Rsv inhibits Chk1/2 directly or indirectly, which could be responsible for the lack of cell cycle arrest. This could also explain the dual effect on Cdk1 Y15 phosphorylation, which was increased by Rsv at 24h (perhaps through Myt1), but which was reduced by Rsv 48h later, at which time the inhibitory effect on Chk1/2 might have taken over. However, we feel that this time-specific mechanism is too speculative to be added to the manuscript.

Following you suggestion, we added a sentence to try to summarize all molecular data in a hypothetical paragraph in the end of discussion section: “In summary, considering the effect of Rsv on TMZ-treated cells we noted that there was no effect on DNA damage, but the early steps of DDR signaling, \( \gamma \text{H2AX}, \text{pATM} \) and pChk2, Rsv increased the levels induced by TMZ. On the other hand, effectors of DDR signaling that regulate the cell cycle were decreased by Rsv in TMZ treated cells, mainly pWee1, pCdk1 and Histone H3 phosphorylation, which are targets of Chks. This indicates that Rsv somehow potentiates the early steps of DDR signaling while blocking the late stages, probably at the level of Chk1/2, which may be responsible for the lack of TMZ-induced arrest”.

Specific comments (Compulsory revision):

- In Material and Methods section it is stated that 3 glioma cell lines and a glioma primary culture were used in the study. However, only results from one cell line are showed and discussed. Why those cell lines were chosen? Do they represent different stages of glioblastoma? From which type of glioblastoma was obtained the primary culture?

All cell lines used here are human glioblastoma cell lines, while primary culture was obtained from a grade IV glioma (i.e. glioblastoma). We used these cell lines because they present different expression profiles of tumor suppressors (such as p53, PTEN and INK4a/Arf proteins), oncogenes (such as Ras) and MGMT protein (table below). We added the most relevant information concerning these proteins to the text (methods section).

<table>
<thead>
<tr>
<th></th>
<th>U87</th>
<th>U251</th>
<th>U138</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN</td>
<td>Mutated</td>
<td>Null</td>
<td>Mutated</td>
<td>[11, 12]</td>
</tr>
<tr>
<td>P53</td>
<td>Wild type</td>
<td>Mutated</td>
<td>Mutated</td>
<td>[13]</td>
</tr>
<tr>
<td>p14(^{ARF}/p16)</td>
<td>Deleted</td>
<td>Deleted</td>
<td>Deleted</td>
<td>[14]</td>
</tr>
<tr>
<td>Ras expression</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
<td>[15-17]</td>
</tr>
<tr>
<td>MGMT levels</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>[18]</td>
</tr>
</tbody>
</table>

We performed the experiments related to autophagy induction and cell cycle for all cell lines. Since U251 and U138 did not present a clear reversion in TMZ-induced cell cycle arrest by Rsv, we did not perform molecular analysis of mitotic catastrophe with these cells. We added a paragraph discussing these differences between cell lines on discussion section (second paragraph).
- In supplementary material, results of other cell lines are provided. This results show differences between cells. This should be properly discussed, indicating other differences between those cells that may explain the distinct responses.

As described above, we added the following paragraph to the discussion section: The effect of Rsv in increasing TMZ-induced toxicity and autophagy occurred in all glioma cells tested, demonstrating that it does not involve p53 pathway, since U251 and U138 cells are p53 mutant. The lack of correlation between autophagy and cytotoxicity in three GBM cell lines and the absence of reversion of the cytotoxicity by 3MA, despite a significant reduction in autophagy, suggest that this is not a central mechanism in the reduction of cell number. Indeed, autophagy induced by co-treatment seems to be protective rather than cytotoxic. In U138 cells, which have high levels of MGMT protein when compared to U251 and U87 cells, TMZ did not trigger G2-cell cycle arrest, while U87 and U251 cells arrested at G2 with TMZ, also suggesting a p53-independent arrest. Furthermore, in agreement with our data in U87 cells, Mhaidat et al showed that sensitivity of melanoma cells to TMZ was associated with MGMT status, G2 arrest and senescence entry, while no apoptosis was induced.

- Supplementary figure 5 is the only one showing primary culture results. Interestingly, those cells are not responding to TMZ alone (graph A). This should be discussed.

We transfer the results of primary cells (figure S5) to figure 5 (senescence) and figure S1 (autophagy and cell number) to integrate them into text and facilitate the understanding and interpretation of data. We added the following sentence to the results section – “A primary GBM culture which was resistant to TMZ was sensitive to Rsv and to RT” – and the following sentence to the discussion section – “primary glioma tumor tested was resistant to TMZ, while Rsv was cytotoxic alone and potentiated the effect of TMZ in these cells”.

- Figure 1 is difficult to understand. Apparently, there is no statistical difference (at least it is not indicated) between different concentrations of TMZ alone (comparing only white bars). Does it mean that TMZ between 100 and 1000 uM (alone) have no effect on cell number? The same occurs when comparing R30 regarding TMZ different concentrations (comparing all grey bars) and with R100 (comparing all black bars). Multi-group tests should be used. We performed this and added the statistical analysis. There is no difference between black bars in cotreatments, while letter ‘c’ corresponds to the difference (p<0.05) between T1000+R30 in relation to T100+R30 and T300+R30.

- A similar situation occurs when analyzing figure 2 B. We added all the statistics to the graph.

- References should be updated and carefully revised. For example reference 28 has nothing to do with resveratrol effects on prostate cancer. Ref. 29 has no
relation with Rsv and melanoma.
We accidentally opened an old endnote file which altered some references. We now manually revised all references.

- Discussion, including the above questions, should be extensively improved.
We added two new paragraphs in the discussion section, discussing the differences between cell lines, the effect of Rsv in these cells and the results of nuclear morphometric analysis and senescence induction.

**Level of interest:** An article of limited interest

**Quality of written English:** Not suitable for publication unless extensively edited

**Statistical review:** Yes, and I have assessed the statistics in my report.

**Declaration of competing interests:** I have no competing interests

**References**


