Author's response to reviews

Title: RITA (Reactivating p53 and Inducing Tumor Apoptosis) is efficient against TP53abnormal myeloma cells independently of the p53 pathway. A comparative study with nutlin3a.

Authors:

Sylvanie Surget (sylvanie.surget@orange.fr)
Géraldine Descamps (geraldine.descamps@univ-nantes.fr)
Carole Brosseau (carole.brosseau@inserm.fr)
Vincent Normant (vincent.normant@etu.univ-nantes.fr)
Sophie Maiga (sophie.maiga@inserm.fr)
Patricia Gomez-Bougie (Patricia.Gomez@univ-nantes.fr)
Nadège Gouy-Colin (nadege.gouy@chu-nantes.fr)
Catherine Godon (catherine.godon@chu-nantes.fr)
Marie C Béné (mariecbe@gmail.com)
Philippe Moreau (philippe.moreau@chu-nantes.fr)
Steven Le Gouill (steven.legouill@chu-nantes.fr)
Martine Amiot (martine.amiot@inserm.fr)
Catherine Pellat-Deceunynck (Catherine.pellat-Deceunynck@univ-nantes.fr)

Version: 2
Date: 14 February 2014

Author's response to reviews: see over
Dear Editor

We submit our revised article entitled “RITA (Reactiving p53 and Inducing Tumor Apoptosis) is very efficient against TP53abnormal myeloma cells independently of the p53 pathway, a comparative study with nutlin3a” by Surget et al for consideration for publication in BMC Cancer. In this revised version, we provide the answers to all concerns raised by the reviewers, as detailed below. We hope that our revision article is now suitable for publication.

I certify that all authors reviewed the manuscript and have no conflict of interest to declare and that this work is not submitted in another journal and has not been previously published elsewhere.

Yours sincerely

Catherine Pellat-Deceunynck
Responses to reviewers

Reviewer 1
Reviewer: Hareth Nahi
Reviewer’s report
Surget et al described the mechanisms of cell death using 2 novel drugs Nutlins and RITA in Myeloma cell lines and primary malignant plasma cells from newly diagnosed and relapsing multiple myeloma (MM) patients. The effect of RITA in hematological malignancies without 17p deletion was already described 2008 (PMID: 18341636); this work was not referred to.

Response: the reference has been added

The percentage of the p53-deleted clone in Chronic Lymphocytic Leukemia is considered to be of clinical importance if it is above 20%, otherwise patients below 20% are not considered to be p53 deleted.

Response: In the paper the reviewer mentioned (Nahi et al, Br J Haematol 2008), authors assessed efficiency of RITA in AML and CLL. They reported that AML cell death induced by 1 µM RITA was not different within samples with or without del(17p) (p=0.15 Figure 1A), while samples del(17p)+ were less sensitive to lower doses than samples del(17p)-. In MM, we did not assess efficiency of RITA with doses under 1 µM because the total number of primary purified cells was too low to perform dose-response assays. It is possible that del17p impacts the response to lower doses of RITA. We can’t answer this question. In the paper of Nahi et al, CLL samples were much more resistant to RITA, IC_{50} values ranged from 10 µM to 25 µM, and no significant differences were found between samples with or without del(17p), whatever the dose used, (Figure 1C). Thus, in at least 3 different hematological malignancies (CLL, AML, MM), it appears 1) that cells are highly differently sensitive to RITA (nM to µM), 2) that correlation between del(17p) and sensitivity to RITA is dose-dependent in CLL, and 3) that there is no correlation in MM and AML.

In Myeloma, as described by Avet-Loiseau H et al, the clone should be at least 60% to be considered of importance or even to be called a deleted 17p.

Response: We perfectly agree with the reviewer that the clinical cut-off value of del(17p) is 60%, we did not claim any other cut-off value. The samples harbored a del(17p) in less than 20% or in more than 60% of cells. Thus in this cohort a cut-off of 20% or 60% provides the same results.

Looking at the samples that the authors worked with (table 2) only samples 15-19 are to be considered to have del17p. None of these did responded neither to RITA or Nutlin3a, beside sample 18 on RITA. The authors concluded that RITA was effective independently of the 17p deletion and might be of interest to p53 mutated patients resistant to therapy.
We also agree that the number of samples with del(17p) was low. We obtained samples during
the reviewing process and fortunately 3 were del(17p)+. Thus, we have re-analyzed response of 22
patients including now 8 with del(17p) involving at least 68% of cells. Among patients with
del(17p), 2 were highly sensitive (98-100% of cell death), 3 were sensitive (32-38%) and 3 were
resistant (0-9%): the median cell death induced by RITA was 35% in samples with del(17p) and
18% in samples without del(17p), p=0.80. Thus, samples with del(17p) were as sensitive to RITA as
samples without.

Concerning nutlin3a, all samples with del(17p) were resistant to nutlin3a while samples lacking
del(17p) were sensitive (p=0.034). As underlined in the discussion, we proposed that RITA could be
helpful for some (not all) patients with del(17p), although individual sensitivity can’t be predicted.
In MM cell lines, sensitivity to RITA was highly heterogeneous and not related to TP53 status in
contrast to nutlin3a.

Sorry, but I cannot see any effect of RITA on the few patients/cell lines with p53 deletion and
the conclusion is totally wrong. Other wise the methodology of the work was satisfactory.

Response: We disagree with the reviewers concerning the conclusion: our results are not wrong.
We show that sensitivity toward RITA can’t be predicted, that only 29% of samples (n=22) and 25%
of cell lines (n=32) were sensitive (defined by at least 50% of cell death induced by 1 µM) and that
this sensitivity was independent from del(17p) in primary samples, and from TP53 status and p53
expression in myeloma cell lines.

Reviewer 2
Reviewer: Manujendra Saha
Comments:
In this study, Surget et al, demonstrated p53-independent anti-myeloma activity of RITA and
compared such activities with nutlin, an MDM2 antagonist. RITA which was originally described
as a wild type p53 activating small molecular later showed its anti-tumor activities in cancer cells
with mutant p53 as well. Similarly, anti-tumor activities of nutlin have not been limited to wild
type p53 alone. Because of the complexities of the p53 signaling pathway, small molecules can
indeed target several apoptotic pathways to exert their anti-tumor activities. To identify a
binding target for RITA is still remaining a challenge. Nevertheless, anti-myeloma activities of
RITA in a broad spectrum of multiple myeloma (MM) cell lines or patient samples irrespective of
p53 mutation/deletion status would be clinically significant. However, the major limitation of
this study relies on the fact that the authors did not explain how RITA is inducing apoptosis in
wild type or mutant p53 harboring MM cells without requiring activation of p53 or any of the
pro-apoptotic proteins (e.g., Noxa and Bax). In addition, there are some issues in the manuscript
as mentioned below which must be addressed in order to get it publish.
Major problems:
In this study the authors examined the apoptotic effect of RITA in a panel of MM cell lines (Fig. 1 and Table 1) and primary MM samples. This study goes beyond the results of the previous studies of RITA in MM cells which described its wild type p53-dependent activities.

However, the rationale for choosing different cell lines of either wild type or mutant p53 status in different experiments but for similar purpose is not clear. For example, the authors used KMS12PE as a representative of mutant p53 cell line to examine the effect of expression of p53 and its downstream targets upon stimulation by RITA or nutlin (Fig. 2). Whereas XG5 cell line was used for p53 knockdown experiments to examine the effect of p53 knockdown on apoptosis induction by these two agents. Using XG5 cells in both of the experiments would be an ideal representative. Similarly, to examine and compare whether RITA or nutlin whether they can activate p53 signaling pathway as described in Fig. 2 the authors should consider using H929 or MM.1S cells as a representative of wild type p53 cell line model. Since previous reports have shown the modulation of p53 and its targets upon RITA treatment of these cells. It would be important see if the results are consistent with the previous reports. Moreover, according to the data presented here, XG5 cell line which harbors mutant p53 is the most sensitive cell line to RITA (LD50 0.007 μ M) (Table 1). Therefore, using XG5 cell lines as a mutant p53 model would be the best choice.

Response: Our initial rationale was precisely to show that our results were not dependent on the cell line but we understand that it can be confusing. Thus, experiments in NCI-H929 cells have been added (WB in Figures 2 and 3). Experiments in XG5 cell line is cells have been added (WB in Figures 2 and 3)

Specific points:
1. Inconsistent results have been described in Fig. 2A and B for p53 expression by RITA treatment in MDN cells. For example, p53 is downregulated in MDN cells treated with RITA (Fig. 2A), whereas it is clearly up-regulated in all the subcellular fractions of RITA-treated MDN cells (Fig. 2B). Such discrepancies should be discussed.

Response: New experiments with MDN were performed. Discrepancies between Fig2A and 2B were due to different intensity of cell death and thus different level of protein cleavage and degradation. At t=3h, RITA did not induce any cell death.

2. While describing the results of co-localization of p53 in MM cells treated with nutlin or RITA the authors described the different time course (3 hr and 24 hr) of the studies (2nd paragraph of page 7), however as shown in the Fig. 2A, there is no such time course.

Response: We apologize for results of co-localization that were missing. They have been added.
3. Did the authors examine the effect of RITA in normal hematopoietic cells particularly at high concentration of RITA (>10 μM)?

Response: We examined effects of RITA and nutlin3a in normal PBMCs. Results have been added (Figure 1).

4. Figure labelling (Fig. 3) is incorrect and confusing. It is not clear what concentration ranges of RITA or nutlin were used for each experiments. LD50 of RITA in XG5 has been shown as 0.007 μM (Table 1), however, surprisingly, a much higher LD50 of RITA (~0.05) in the same cell line is shown in Fig. 3A (right panel).

Response: Figure labeling has been improved in Figure 3. We agree with the reviewer that LD50 values were higher in Shcont cells when compared to parental cells (values were already included within the legend). These weak differences are frequent in sh cells and might be related to selection. This is the precise reason why shp53 cells have to be compared to shCont cells (performed at the same time) and not to parental cells.

5. The level of p53 knockdown for the representative blots should be shown.

Response: We evaluated p53 silencing in NCI and XG5: the level of extinction in NCI-H929 was calculated in untreated and nutlin3a-treated cells: constitutive and nutlin3a-induced p53 expression was decreased by 63% and 68%, respectively (now indicated in the legend). In XG5, silencing level could not be calculated: indeed, p53 was not detectable in shp53 cells unless overexposure, which then induced saturation of the constitutive signal.

Minor points:
1. The cleavage bands for caspase-3 and -9 instead of showing just inactive forms should be shown.

Response: Cleavage forms of caspases have been added.

2. The reduced β-actin band in the shRNA mediated p53 knocked down XG5 cells treated with RITA indicates an unequal loading.

Response: We have performed a new WB with equal loading. Because the reviewer underlined that different cell lines were used and that the rationale for using one or other cell line was unclear, we removed XG6 and included NCI-H929. Of note, the same results were found showing that they were not dependent on the cell line.
3. The authors should show the level of expression of DR5 in cells treated with RITA or nutlin by Western blot at least in cell lines.
Response: DR5 expression was assessed by flow cytometry and not WB because WB could not be performed in primary samples. Figure 3 shows that RITA induced Noxa expression in both sh Ct and shp53 cells. In contrast, nutlin3a-induced Noxa expression was impaired in shp53 wt cells.

4. Noxa is clearly up-regulated by RITA in MDN cells (Fig. 2A). This should be discussed.
Response: It is discussed.

5. Inclusion of the data for either NAN6 or NAN8 cell line as a null p53 model as described in the text (Page 11) would further strengthen the p53-independent activity of RITA in MM cells.
Response: Experiments with NAN8 cells have been added in Figure 2

6. Same wording (“arguing against an involvement of p53”) has been used multiple times. Please consider different wording.
Response: Several sentences “arguing against an involvement of p53” have been removed or modified.

Reviewer 3
Jean-Christophe Bourdon
Reviewer’s report:
This is a very interesting and important work. It deserves publication in BMC.

Quality of written English: Needs some language corrections before being published
Response: Manuscript has been (re)edited by American Journal Expert.