Author's response to reviews

Title: Protection of p53 wild type cells from taxol by nutlin-3 in the combined lung cancer treatment

Authors:

Sergey V Tokalov (Sergey.Tokalov@oncoray.de)
Nasreddin D Abolmaali (Nasreddin.Abolmaali@oncoray.de)

Version: 2 Date: 23 January 2010

Author's response to reviews: see over
Dear Miss Judith Gorton,

Thank you very much for your letter with the suggestions and queries of the reviewers. We found their comments very helpful and we have responded to the all criticisms and changed the manuscript accordingly.

For your convenience, I will go through all points raised by the reviewers (comments are indicated by bold), explain our response (blue) and include changes in the respective parts of the text (indicated in red).

Yours sincerely,

Dr Sergey V Tokalov

Miss Judith Gorton
The BioMed Central Editorial Team

Tel: +44 (0) 20 3192 2013
e-mail: editorial@biomedcentral.com
Web: http://www.biomedcentral.com/

Dresden, 23 January, 2010
Reviewer's report
Title: Protection of p53 wild type cells from taxol by nutlin-3 in the combined lung cancer treatment
Version: 1 Date: 21 October 2009
Reviewer: Marzia Pennati
Reviewer's report:
This manuscript describes the effect of different compounds (5-fluorouracil, camptothecin, roscovitine, and Nutlin-3) in combination with taxol in cell lines differing in the TP53 gene status. The basic finding is that exposure to Nutlin-3 protects wild-type p53 cells (A549) from taxol, whereas the same combination treatment induces mitotic arrest and apoptosis in p53-deficient cells (FaDu and H1299). The manuscript is badly written and the results and discussion are very poor. In my view, the paper adds little to the literature and do not provide significant evidence concerning the effects of Nutlin-3 in combination with taxol. Although the idea of the research is interesting, the paper does not warrant publication in its present form. My recommendation would be to explore further the mechanism responsible for the effects mentioned above before re-submitting the manuscript for consideration.

First of all we wish to express our appreciation for the efforts of Prof. Marzia Pennati to improve our manuscript, practical advices and especially positive response to the total idea the research.

According to Reviewer recommendation additional information concerning the mechanism of the described effects was added in the chapter Background:

Cancer is a complex family of diseases, characterized by the deregulation of normal control pathways for cellular growth. Lung cancer (LC) is the leading cause of death among human malignancies and is among the most threatening of them due to its disappointing response to therapy [1]. Development of LC, which can be separated roughly into small cell lung cancer (SCLC) or non-small cell lung cancer (NSCLC), it usually involves multiple genetic abnormalities, including mutations in the tumor suppressor gene p53 with a higher mutation frequencies at advanced diseases. One of the most common changes on this way is mutation in the tumour suppressor TP53 gene with a mutations frequency of 50% and 70% in NSCLC and SCLC, respectively [2-4]. Such genetic abnormality is shown to be associated with a poorer survival prognosis and increased cellular resistance to therapy [5-7]. Thus, there is an urgent need for development of target-driven novel class of anti-cancer drug against this deadly disease.

The discovery of new cancer-related therapeutic targets is mainly based on the identification of genes involved in pathways selectively exploited in cancer cells [8,9]. For example, the lack of wt p53 (the product of TP53 gene) in cancer cells can be utilized for therapeutic advantage by selective killing of p53 deficient (p53") cancer cells and by protecting p53 wild type cells (p53wt) at normal proliferation rates using antagonistic drugs [10,11]. It was demonstrated that certain anticancer drugs could selectively arrest p53wt cells in G1 or G2 phases of the cell cycle by activation of the p53 pathway and thereby protects them from antimitotic agent. E.g. taxol, which simultaneously kills and/or blocks p53" cancer cells during mitosis [12].
However, genotoxic drugs are known also to express p53-independent features and could affect the sensitivity of tumor cells to antimitotic agents. They can trigger multiple molecular events including activation of p53-independent checkpoints and thus may partially protect the cancer cells during chemotherapy [3]. This can be avoided by using agents targeted specifically at the p53 pathway. In proliferating cells that are not subjected to stress, p53 level is tightly controlled by its negative regulator MDM2, which binds p53 and modulates its transcriptional activity and stability [3,13-15]. MDM2 is an E3 ubiquitin ligase that binds the tumor suppressor and facilitates its ubiquitin-dependent degradation [16]. The MDM2 binding domain overlaps with the transcriptional activation domain of p53, and therefore MDM2 binding also inhibits the transcriptional activity of p53, thus effectively impairing its function [17]. Disruption of the p53-MDM2 interaction, therefore, provides an attractive strategy for activating p53. It was shown that nutlin-3 could selectively disrupt the interaction between p53 and MDM2 [18] inducing cell cycle arrest in normal murine and human cells [3,13-15,19] cells without initiation of apoptosis. This presents unique opportunities for p53-dependent modulation of the cell cycle of the proliferating p53wt cells of the intact surrounding tissues to protect them from the taxol during chemotherapy of p53−/− tumors [20]. Studies with these compounds have strengthened the concept that selective, non-genotoxic p53 activation is a viable alternative to current cytotoxic chemotherapy, but clinical validation is still pending. Further investigations are necessary to elucidate the effects of these compounds.

... and in the chapter Discussion:

... Our study in agreement with previous findings [30] showed that nutlin-3 in low concentrations (1-10µM) induced depletion of the S-phase fraction, causing arrest at G1/S and/or G2/M phases in p53wt A549 cells without apparent apoptosis. Last may be explained by the known fact that the induction of apoptotic genes alone sometimes is not sufficient to provoke apoptosis, as the high levels of cell cycle inhibitor, such as p21, dominantly lead to the cell cycle arrest [23]. This was supported using MI-43 (another compound that disrupted Mdm2-p53 binding) which at low concentrations induced remarkable induction of p53 leading to 90% cells arrested of A549 cells at the G1 phase without apparent apoptosis, although Puma and Noxa were also induced. However, at higher drug concentration cells underwent apoptosis even with a moderate further increase of Puma and Noxa. [24]. Previous studies show that elevated concentrations of nutlin-3 induced p53- and p21-dependent cell cycle arrest and p53-dependent cell death in different p53wt tumor cell lines including A549 [14,15,17,18,20,24,26,28,30]. The p53 activation by nutlin-3 has been shown to lead cell cycle arrests in normal human [3,14] fibroblasts, endothelial [15] and epithelial [19] cells without initiation of apoptosis. Therefore, NSCLC cell line A549 seems to be more sensitive to induction of apoptosis through activation of p53 pathway and our results might be translated to the control cell lines mentioned above.

Lung cancer is the leading cause of death among human malignancies [31] and is among the most threatening of them due to its disappointing response to therapy [1,32]. Combining molecular-targeted agents with irradiation is a highly promising avenue for cancer research and patient care [2,33,34]. Development of lung cancer involves multiple genetic abnormalities including mutation in p53 gene [6], with a
mutations frequency of 50% in advanced NSCLC [4]. This was shown to be associated with a poorer survival prognosis and increased resistance to ionizing radiation and chemotherapy [7]. Our data suggest that nutlin-3 might be helpful in NSCLC treatment when combined with mitotic poisons.

Taxol and other mitotic chemotherapeutics are frequently used together with genotoxic drugs activating the p53 pathway in wild-type p53 cells via genotoxic stress [10]. However, the usefulness of DNA damaging agent is limited by their ability to activate p53-independent checkpoint mechanism in cancer cells with mutant p53 [11]. The nutlin-3 works solely through stabilization and activation of p53 gene. Therefore, protection by nutlin-3 is strictly dependent on the p53 status of the cells [20]. Using MDM2 antagonist nutlin-3 as selective activator of p53 pathway, we have shown that induction of cell cycle arrest can protect p53wild NSCLC (A549) cells from the cytotoxicity of taxol selectively killing of p53null PSCC cells (FaDu) and p53null NSCLC (H1299) cells. Our results confirmed previously reported synergistic interaction of the nutlin-3 / taxol treatment of the p53null cancer cells and protection of normal proliferating fibroblasts or p53wild colon cancer cells [3]. Although the experiments described in this report use taxol as a mitotic inhibitor, nutlin-3 halted cell cycle progression at the G1/S and G2/M phases and can thus attenuate the activity of S-phase- and M-phase-specific drugs. For example, treatment of normal proliferating fibroblasts or keratinocytes with nutlin-3 protects these cells against gemcitabine and Ara-C killing proliferating p53null cancer cells in S-phase [28]...

Specific comments:
1. The authors base their conclusions only on cancer cell lines. It would be important to confirm their hypothesis in normal cell lines.

According to Reviewer 1 require the next information was added in the chapter Discussion:

Previous studies show that elevated concentrations of nutlin-3 induced p53- and p21-dependent cell cycle arrest and p53-dependent cell death in different p53wild tumor cell lines including A549 [14,15,17,18,20,24,26,28,30]. The p53 activation by nutlin-3 has been shown to lead cell cycle arrests in normal human [3,14] fibroblasts, endothelial [15] and epithelial [19] cells without initiation of apoptosis. Therefore, NSCLC cell line A549 seems to be more sensitive to the induction of apoptosis through the activation of p53 pathway and our results might be translated to the control cell lines mentioned above.

2. How was defined the treatment schedule to be used for the drug combination? Authors should clarify this point.

According to Reviewer 1 require the next information about treatment schedule to be used for the drugs alone and in combinations was added in Chapter: Flow cytometry
To have a possibility for comparison of our results with previous findings [3,12-15,18,19,21-27] and taking into account that in solid tumor-derived cell lines in which p53-dependent apoptosis is usually delayed for 24 h [18] the cells were placed in culture medium (2×10^5 cells/mL) 1 day before exposure to 5-fluorouracil (1, 3, 10 and 30 µM), camptothecin (10, 30, 100 and 300 nM), nutlin-3 (1, 3, 10 and 30 µM), roscovitine (1, 3, 10 and 30 µM) and taxol (1, 3, 10, 30 and 100 nM). All compounds were pursued from Sigma (Germany). To test an advantage of the nutlin-3 in the selective protect p53<sup>wt</sup> A549 cells from taxol, all cell cultures according to [3,28] were incubated for a 24 hours period in parallel with 5-fluorouracil (3µM), camptothecin (10nM), roscovitine (10µM) and nutlin-3 (3µM) respectively. Then, taxol (10nM) was added and cell cultures were incubated for another 24 hours period.

3. The effects of the treatment with the different compounds (alone or in combination) on cell proliferation should be reported.

In presented work we would like to show that proposed therapeutic strategy using nutlin-3 allowed protect p53<sup>wt</sup> but not p53<sup>−/−</sup> cells from taxol.

It was shown in the chapter Results that pretreatment with nutlin-3 selectively protected p53<sup>wt</sup> A549 cells from taxol, dramatically reducing the proportion of apoptotic cells from 35 ± 6% (taxol) to 2 ± 1% (nutlin-3 plus taxol, p<0.001). At the same time no significant changes in the proportion of apoptotic cells were registered in FaDu and H1299 cell after administration of the taxol alone or in combination with nutlin-3.

Taking into account that our "data showing the perturbation of the cell cycle after treatment with the different compounds (alone or in combination) are convincing" (see below), we did not investigate cell proliferation in more details.

4. The data showing the perturbation of the cell cycle after treatment with the different compounds (alone or in combination) are convincing, but it would be interesting to know the effects of the treatment on the expression/activity of the proteins involved in cell cycle regulation.

Thank you for this interesting question. We are glad to see that the data showing the perturbation of the cell cycle after treatment with the different compounds (alone or in combination) were convincing for the Reviewer 1. We are going to investigate the effects of the treatment on the expression/activity of the proteins involved in cell cycle regulation in the nearest future to get more detailed schedule for the combined treatment.

5. The authors should verify the mechanism by which Nutlin-3 protects cells from taxol.

According to Reviewer 1 require and to explain the mechanism by which nutlin-3 protect p53<sup>wt</sup> cells from taxol next information was added in the chapter Background:
For example, the lack of wt p53 (the product of TP53 gene) in cancer cells can be utilized for therapeutic advantage by selective killing of p53 deficient (p53⁻/⁻) cancer cells and by protecting p53 wild type cells (p53wt) at normal proliferation rates using antagonistic drugs [10,11]. It was demonstrated that certain anticancer drugs could selectively arrest p53wt cells in G₁ or G₂ phases of the cell cycle by activation of the p53 pathway and thereby protects them from antimitotic agent. E.g. taxol, which simultaneously kills and/or blocks p53⁻/⁻ cancer cells during mitosis [12]. However, genotoxic drugs are known also to express p53-independent features and could affect the sensitivity of tumor cells to antimitotic agents can trigger multiple molecular events including activation of p53-independent checkpoints and thus may partially protect the cancer cells during chemotherapy [3]. This can be avoided by using agents targeted specifically at the p53 pathway. In proliferating cells that are not subjected to stress, p53 level is tightly controlled by its negative regulator MDM2, which binds p53 and modulates its transcriptional activity and stability [3,13-15]. MDM2 is an E3 ubiquitin ligase that binds the tumor suppressor and facilitates its ubiquitin-dependent degradation [16]. The MDM2 binding domain overlaps with the transcriptional activation domain of p53, and therefore MDM2 binding also inhibits the transcriptional activity of p53, thus effectively impairing its function [17]. Disruption of the p53-MDM2 interaction, therefore, provides an attractive strategy for activating p53. It was shown that nutlin-3 could selectively disrupt the interaction between p53 and MDM2 [18] inducing cell cycle arrest in normal murine and human cells [3,13-15,19] cells without initiation of apoptosis. This presents unique opportunities for p53-dependent modulation of the cell cycle of the proliferating p53wt cells of the intact surrounding tissues to protect them from the taxol during chemotherapy of p53⁻/⁻ tumors [20].

6. The apoptotic rate should be better determined using more specific assays for apoptosis (i.e., TUNEL assay, caspase-9 and caspase-3 catalytic activity).

We are full agreeing with this comment. There are several assays which are very specific for the different stages of apoptosis. However, we would like to show the protective effect of nutlin-3 for p53wt cells only. We believe that PI is good enough for this purpose. PI has been widely used for the analysis of the apoptotic cells proportion in the recent investigations (See References from this manuscript: Carvajal et al., 2005, Kranz et al., 2006, Merten et al., 2006, Tokalov et al., 2007, Sun et al., 2008, Vassilev et al., 2009).

7. On the flow cytometry on DNA context for the different treatments, how many independent replications were performed before to draw the conclusions?

According to Reviewer 1 require a number of independent replications was added in the chapter Statistics:

Statistics
The experimental results are expressed as the mean ± standard deviation (mean ± s.d.) of 6 several independent experiments.
8. The compounds reported in “Methods” section (“Flow cytometry”, pag 4) are different from drugs used in the paper.

We are sorry for this mistake. Corresponding changes were performed in the chapter Flow Cytometry:

…the cells were placed in culture medium (2x10^5 cells/mL) 1 day before exposure to 5-fluorouracil (1, 3, 10 and 30 µM), camptothecin (10, 30, 100 and 300 nM), nutlin-3 (1, 3, 10 and 30 µM), roscovitine (1, 3, 10 and 30 µM) and taxol (1, 3, 10, 30 and 100 nM).

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

Manuscript has been edited by one of our naturally English speaking colleagues and all of the found errors were excluded.

Reviewer’s report
Title: Protection of p53 wild type cells from taxol by nutlin-3 in the combined lung cancer treatment
Version: 1 Date: 31 December 2009
Reviewer: Giorgio Zauli
Reviewer’s report:
The study of Tokalov and Abolmaali is interesting as it shows a potential therapeutic role of Nutlin-3, a non-genotoxic activator of the p53 pathway in association with taxol in the combined lung cancer treatment. Therefore, the Authors propose a therapeutic strategy protecting normal cells from taxol while increasing apoptosis selectively in p53-deficient cells using nutlin-3.

May we take this opportunity of thanking Prof. Giorgio Zauli for this comment and positive response to the article?

Minor Essential Revisions:
1) there are some spelling errors throughout the text that need to be amended;

We are very sorry for these mistakes. The text of manuscript was checked again and corresponding errors were excluded.

2) there are important References missing, such as the effect of Nutlin-3 on primary cells relevant for cancer progression, such as endothelial cells, osteoclasts and fibroblasts.

Please add the following references and a statement discussion your current findings with those of other Authors:
MDM2 antagonist Nutlin-3 suppresses the proliferation and differentiation of human pre-osteoclasts through a p53-dependent pathway.
Zauli G, Rimondi E, Corallini F, Fadda R, Capitani S, Secchio P.
Antiangiogenic activity of the MDM2 antagonist nutlin-3.

Many thanks for this comment. According to Reviewer 2 suggestion all the mentioned above references were added in the manuscript.

Quality of written English: Needs some language corrections before being published

Manuscript has been edited by one of our naturally English speaking colleagues.