Long-term cultivation using ineffective MDM2 inhibitor concentrations alters the drug sensitivity profiles of PL21 leukaemia cells

Martin Michaelis¹, Florian Rothweiler², Constanze Schneider², Tamara Rothenburger², Marco Mernberger³, Andrea Nist⁴, Thorsten Stiewe³⁴ and Jindrich Cinatl Jr.²*¹

¹Industrial Biotechnology Centre and School of Biosciences, University of Kent, Canterbury CT2 7NJ, UK, ²Institut für Medizinische Virologie, Klinikum der Goethe-Universität, Paul Ehrlich-Str. 40, 60596 Frankfurt am Main, Germany, ³Institute of Molecular Oncology, Philipps-University, 35037 Marburg, Germany, and ⁴Genomics Core Facility, Philipps-University, 35037 Marburg, Germany
*Corresponding author. Email: cinatl@kent.ac.uk

(Received 30 August 2019; Accepted 15 November 2019)

Abstract
Acquired MDM2 inhibitor resistance is commonly caused by loss-of-function TP53 mutations. In addition to the selection of TP53-mutant cells by MDM2 inhibitors, MDM2 inhibitor-induced DNA damage may promote the formation of TP53 mutations. Here, we cultivated 12 sublines of the intrinsically MDM2 inhibitor-resistant TP53 wild-type acute myeloid leukaemia cell line PL21 for 52 passages in the presence of ineffective concentrations of the MDM2 inhibitor nutlin-3 but did not observe loss-of-function TP53 mutations. This suggests that MDM2 inhibitors select TP53-mutant cells after mutations have occurred, but do not directly promote TP53 mutations. Unexpectedly, many sublines displayed increased sensitivity to the anti-cancer drugs cytarabine, doxorubicin, or gemcitabine. Consequently, therapies can affect the outcome of next-line treatments, even in the absence of a therapy response. This finding is conceptually novel. A better understanding of such processes will inform the design of improved therapy protocols in the future.

Keywords: Cancer; Drug response; Leukemia; Resistance; Therapy

Introduction
MDM2 (Mouse Double Minute 2) inhibitors, which activate p53 by inhibiting MDM2-mediated p53 degradation, are under development for the treatment of TP53 wild-type cancer [1]. The MDM2 inhibitor idasanutlin is currently investigated in clinical phase II and III trials for acute myeloid leukaemia (AML; NCT02670044, NCT02545283).

Resistance formation of TP53 wild-type cancer cells to MDM2 inhibitors commonly results in the formation of TP53 mutations as resistance mechanism [2-8]. TP53 mutations may be the consequence of the selection of pre-existing TP53-mutant cell subpopulations or the induction of de novo TP53 mutations [3,5-7]. De novo TP53 mutations may be the consequence of the selection of cells in which TP53 mutations have occurred by chance and which would have disappeared in the absence of the selection pressure induced by an MDM2 inhibitor. However, MDM2 inhibitors may also actively promote the formation of TP53 mutations by inducing DNA damage [9-12].

© The Author(s) 2020. This is an Open Access article, distributed under the terms of the Creative Commons Attribution licence http://creativecommons.org/licenses/by/4.0/, which permits unrestricted re-use, distribution, and reproduction in any medium, provided the original work is properly cited.
Objective
We used the AML cell line PL21 to investigate whether MDM2 inhibitor-induced DNA damage may promote the formation of TP53 mutations in the absence of a selection pressure. PL21 AML cells are TP53 wild-type (Table 1) but intrinsically resistant to nutlin-3 (an MDM2 inhibitor closely related to idasanutlin [9]), as indicated by a nutlin-3 IC$_{50}$ of 20.49 μM (Figure 1, Table 1). Nutlin-3-sensitive cells display nutlin-3 IC$_{50}$ values in the very low micromolar range, while nutlin-3 concentrations above 20 μM are associated with non-specific, p53-independent effects [3,7]. Twelve PL21 sublines were cultivated for 52 passages in the presence of nutlin-3 10 μM. The emergence of TP53 mutations in response to nutlin-3 treatment would indicate mutagenic effects that promote the formation of TP53 mutations also in the absence of a selective pressure on p53.

Methods
PL21 cells (DSMZ, Braunschweig, Germany) were cultivated in the absence or presence of drug in Iscove’s modified Dulbecco’s medium supplemented with 10% foetal calf serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C. Cells were routinely tested for mycoplasma contamination and authenticated by short tandem repeat profiling.

The TP53 status was determined by next generation sequencing, and cell viability was measured using eight drug concentrations by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [3,7]. Based on the MTT data, concentrations that inhibit cell viability by 50% (IC$_{50}$) were determined using CalcuSyn (Biosoft, Cambridge, UK). Nutlin-3 was purchased from Selleck Chemicals via BIOZOL GmbH (Eching, Germany). Cytarabine was obtained from Tocris via Bio-Techne GmbH (Wiesbaden, Germany). Doxorubicin and gemcitabine were purchased from Teva GmbH (Ulm, Germany).

Results
All sublines had retained wild-type TP53 except for PL21rNutlin20XII and PL21rNutlin20XV, which displayed an M66L variant (Table 1). This variant was present in 386 (3.2%) out of 11,945 reads from the parental cell line and, hence not a de novo mutation induced by nutlin-3 treatment. If it had been of functional relevance, it would have been consistently selected by nutlin-3 treatment, as previously shown in other cell lines [5-7]. Thus, this observation does not suggest that nutlin-3 may directly induce TP53 mutations.

The 12 nutlin-3-treated PL21 sublines displayed an up to 3.1-fold variation in their nutlin-3 sensitivity (Figure 1, Table 1) and in their sensitivity to cytarabine (up to 6.7-fold), doxorubicin (up to 7.7-fold), and gemcitabine (up to 40.8-fold). Twelve PL21 sublines that had been cultivated for 52 weeks as control in parallel in the absence of nutlin-3 did not display any changes in their drug sensitivity profiles (Table 2).

Discussion
Since treatment of PL21 cells with ineffective nutlin-3 concentrations did not result in loss-of-function TP53 mutations, TP53 mutations in MDM2 inhibitor-adapted cells may be rather the consequence of selection processes than of drug-induced mutations. In agreement, a fraction of MDM2 inhibitor-adapted cell lines retains wild-type TP53 [3,7]. Unexpectedly, prolonged nutlin-3 treatment resulted in increased sensitivity of a fraction of sublines to cytarabine, doxorubicin, or gemcitabine. In this context, MDM2 inhibition has been shown to increase the cellular reactive oxygen species (ROS) levels [13,14], and higher ROS levels were associated with increased cytarabine sensitivity [15]. Cytarabine and anthracyclines are standard drugs for AML [16], and gemcitabine has recently been suggested as drug candidate for paediatric AML [17]. This may be of clinical relevance in AML patients in whom MDM2...
Table 1. Drug concentrations that reduce the viability of PL21 and its sublines cultivated for 52 weeks in the presence of nutlin-3 (20 μM) by 50% (IC$_{50}$) as indicated by MTT assay after 120 h of incubation.

<table>
<thead>
<tr>
<th>TP53 status</th>
<th>Nutlin-3 IC$_{50}$ (μM)</th>
<th>Cytarabine IC$_{50}$ (ng/mL)</th>
<th>Doxorubicin IC$_{50}$ (ng/mL)</th>
<th>Gemcitabine IC$_{50}$ (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL21</td>
<td>P72R$^1$</td>
<td>20.49 ± 6.61</td>
<td>19.42 ± 6.28</td>
<td>56.12 ± 7.50</td>
</tr>
<tr>
<td>PL21'Nutlin$^{20}$I</td>
<td>P72R</td>
<td>19.84 ± 3.69 (–1.03)$^2$</td>
<td>7.12 ± 1.68 (–2.73)</td>
<td>24.37 ± 2.59 (–2.30)</td>
</tr>
<tr>
<td>PL21'Nutlin$^{20}$II</td>
<td>P72R</td>
<td>18.32 ± 3.05 (–1.12)</td>
<td>24.4 ± 8.58 (1.26)</td>
<td>64.85 ± 8.17 (1.16)</td>
</tr>
<tr>
<td>PL21'Nutlin$^{20}$III</td>
<td>P72R</td>
<td>17.81 ± 2.01 (–1.15)</td>
<td>11.62 ± 2.44 (–1.67)</td>
<td>23.16 ± 3.84 (–2.42)</td>
</tr>
<tr>
<td>PL21'Nutlin$^{20}$V</td>
<td>P72R</td>
<td>7.86 ± 3.11 (–2.61)</td>
<td>15.86 ± 0.67 (–1.22)</td>
<td>10.24 ± 8.16 (–5.48)</td>
</tr>
<tr>
<td>PL21'Nutlin$^{20}$VI</td>
<td>P72R</td>
<td>18.25 ± 2.83 (–1.12)</td>
<td>10.42 ± 0.54 (–1.86)</td>
<td>78.59 ± 1.01 (1.40)</td>
</tr>
<tr>
<td>PL21'Nutlin$^{20}$VII</td>
<td>P72R</td>
<td>20.00 ± 0.71 (–1.02)</td>
<td>31.57 ± 3.80 (1.63)</td>
<td>51.99 ± 22.53 (–1.08)</td>
</tr>
<tr>
<td>PL21'Nutlin$^{20}$VIII</td>
<td>P72R</td>
<td>21.18 ± 1.93 (1.03)</td>
<td>9.96 ± 1.12 (–1.95)</td>
<td>38.66 ± 4.55 (–1.45)</td>
</tr>
<tr>
<td>PL21'Nutlin$^{20}$IX</td>
<td>P72R</td>
<td>18.29 ± 1.44 (–1.12)</td>
<td>8.28 ± 2.11 (–2.35)</td>
<td>49.20 ± 19.50 (–1.14)</td>
</tr>
<tr>
<td>PL21'Nutlin$^{20}$X</td>
<td>P72R</td>
<td>22.94 ± 1.28 (1.12)</td>
<td>9.24 ± 4.37 (–2.10)</td>
<td>22.44 ± 2.99 (–2.50)</td>
</tr>
<tr>
<td>PL21'Nutlin$^{20}$XIV</td>
<td>P72R</td>
<td>20.25 ± 3.97 (–1.01)</td>
<td>4.71 ± 0.58 (–4.12)</td>
<td>26.08 ± 5.95 (–2.15)</td>
</tr>
<tr>
<td>PL21'Nutlin$^{20}$XV</td>
<td>P72R, M66L</td>
<td>24.29 ± 2.00 (1.19)</td>
<td>31.76 ± 1.78 (1.64)</td>
<td>30.25 ± 3.81 (–1.86)</td>
</tr>
</tbody>
</table>

$^1$Polymorphism that does not affect p53 function.

$^2$Fold change relative to PL21.
Figure 1. Drug sensitivity profiles of the AML cell line PL21 and its sublines cultivated in the presence of nutlin-3 (10 μM) for 52 weeks. Concentrations that inhibit cell viability by 50% (IC50, mean ± SD from three independent experiments) as determined by MTT assay after 120 h incubation and IC50 fold changes relative to PL21 were determined for nutlin-3 (A), cytarabine (B), doxorubicin (C), and gemcitabine (D). * P < 0.05 relative to PL21.
inhibitor treatment may modify the efficacy of next-line therapies, even if there is no response to MDM2 inhibitor therapy.

Conclusion

Our data do not provide evidence that MDM2 inhibitors may exert mutagenic effects that would promote the formation of loss-of-function TP53 mutations. MDM2 inhibitors rather seem to select TP53-mutant cells after mutations have occurred. Surprisingly, we found that cultivation of PL21 cells in the presence of ineffective nutlin-3 concentrations resulted in increased drug sensitivity in a substantial fraction of sublines. This is conceptually important, because our findings show that non-effective therapies can affect the outcome of next-line therapies. A better understanding of such processes may inform therapy protocols in the future. Our study also illustrates how cancer cell lines as permanent preclinical model systems can be used to produce findings that cannot be made in the clinics, because different treatment schedules cannot be compared in the same patient.

Author Contributions. J.C. and M. Michaelis designed and conducted the study. C.S., F.R., T.R., M. Mernberger, and A.N. performed experiments. All authors analysed data. M. Michaelis and J.C. wrote the initial manuscript draft. All authors read and approved the final version.

Funding Information. The work was supported by the Hilfe für krebskranke Kinder Frankfurt e.V. (J.C.), the Frankfurter Stiftung für krebskranke Kinder (J.C.), the Deutsche José Carreras Leukämie-Stiftung (J.C., T.S.), and the Kent Cancer Trust (M. Michaelis).

Publishing Ethics. The authors confirm that

1. the manuscript has been submitted only to the journal – it is not under consideration, accepted for publication or in press elsewhere. Manuscripts may be deposited on pre-print servers;

Table 2. Drug concentrations that reduce the viability of PL21 sublines cultivated separately for 52 weeks by 50% (IC50) as indicated by MTT assay after 120 h of incubation. Values are represented as means ± S.D. of at least three independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>Nutlin-3 IC50 (μM)</th>
<th>Cytarabine IC50 (ng/mL)</th>
<th>Doxorubicin IC50 (ng/mL)</th>
<th>Gemcitabine IC50 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL21</td>
<td>20.49 ± 6.61</td>
<td>19.42 ± 6.28</td>
<td>56.12 ± 7.50</td>
<td>24.56 ± 1.34</td>
</tr>
<tr>
<td>PL21I</td>
<td>18.82 ± 1.60</td>
<td>19.54 ± 1.81 (−0.01)</td>
<td>52.38 ± 10.77 (−1.07)</td>
<td>26.46 ± 4.40 (1.08)</td>
</tr>
<tr>
<td>PL21II</td>
<td>18.51 ± 2.21</td>
<td>16.97 ± 3.53 (−1.14)</td>
<td>56.36 ± 11.67 (1.00)</td>
<td>25.35 ± 4.48 (1.03)</td>
</tr>
<tr>
<td>PL21III</td>
<td>22.20 ± 1.43 (1.08)</td>
<td>19.83 ± 6.34 (−1.02)</td>
<td>62.51 ± 9.17 (1.11)</td>
<td>24.73 ± 2.49 (1.01)</td>
</tr>
<tr>
<td>PL21IV</td>
<td>20.07 ± 4.68</td>
<td>18.05 ± 0.63 (−1.08)</td>
<td>59.78 ± 1.14 (1.07)</td>
<td>21.32 ± 6.00 (−1.15)</td>
</tr>
<tr>
<td>PL21V</td>
<td>21.86 ± 2.29 (1.07)</td>
<td>22.36 ± 6.06 (1.15)</td>
<td>53.26 ± 14.64 (1.05)</td>
<td>23.47 ± 2.30 (−1.05)</td>
</tr>
<tr>
<td>PL21VI</td>
<td>24.11 ± 7.44 (1.18)</td>
<td>22.74 ± 5.42 (1.17)</td>
<td>53.94 ± 11.53 (−1.04)</td>
<td>21.53 ± 3.60 (−1.14)</td>
</tr>
<tr>
<td>PL21VII</td>
<td>17.63 ± 1.88 (−1.16)</td>
<td>24.02 ± 7.83 (1.24)</td>
<td>54.24 ± 1.74 (−1.03)</td>
<td>26.06 ± 2.70 (1.06)</td>
</tr>
<tr>
<td>PL21VIII</td>
<td>21.84 ± 3.70 (1.07)</td>
<td>18.30 ± 2.95 (−1.06)</td>
<td>63.33 ± 1.41 (1.13)</td>
<td>26.15 ± 3.41 (1.06)</td>
</tr>
<tr>
<td>PL21IX</td>
<td>20.41 ± 5.44 (−1.00)</td>
<td>18.86 ± 4.44 (−1.03)</td>
<td>51.51 ± 5.14 (−1.09)</td>
<td>23.44 ± 2.73 (−1.05)</td>
</tr>
<tr>
<td>PL21X</td>
<td>19.99 ± 7.26 (−1.03)</td>
<td>22.52 ± 4.92 (1.16)</td>
<td>57.70 ± 13.11 (1.03)</td>
<td>23.57 ± 1.04 (−1.04)</td>
</tr>
<tr>
<td>PL21XI</td>
<td>21.66 ± 1.49 (1.05)</td>
<td>18.53 ± 2.25 (−1.05)</td>
<td>59.06 ± 8.41 (1.05)</td>
<td>21.10 ± 7.11 (−1.16)</td>
</tr>
<tr>
<td>PL21XII</td>
<td>21.58 ± 3.42 (1.05)</td>
<td>20.41 ± 2.42 (1.05)</td>
<td>52.86 ± 4.53 (−1.06)</td>
<td>24.87 ± 2.67 (1.01)</td>
</tr>
</tbody>
</table>

1 Fold change relative to PL21.
2. all listed authors know of and agree to the manuscript being submitted to the journal; and
3. the manuscript contains nothing that is abusive, defamatory, fraudulent, illegal, libellous, or obscene.

**Conflict of Interest.** The authors declare none.

**Data Availability.** All data are included in the manuscript.

**References**


---

Peer Reviews

Reviewing editor: Dr. Michael Nevels
University of St Andrews, Biomolecular Sciences Building, Fife, United Kingdom of Great Britain and Northern Ireland, KY16 9ST

This article has been accepted because it is deemed to be scientifically sound, has the correct controls, has appropriate methodology and is statistically valid, and met required revisions.

doi:10.1017/exp.2019.1.pr1

Review 1: Long-term cultivation using ineffective MDM2 inhibitor concentrations alters leukaemia cell drug sensitivity profiles

Reviewer: Lukasz Skalniak Dr.

Date of review: 11 September 2019
Published online:

Conflict of interest statement. Reviewer declares none.

Comments to the Author: Michaelis and co-workers aim at the verification of the hypothesis of the generation of TP53 mutations upon the treatment of p53wt PL21 cells with MDM2 antagonist, nutlin-3. Also, increased susceptibility of nutlin-treated cells to three anti-cancer drugs is reported. While this study is important, the manuscript suffers from several critical conceptual mistakes which largely decrease its impact.

Score Card

Presentation

Is the article written in clear and proper English? (30%) 4/5
Is the data presented in the most useful manner? (40%) 4/5
Does the paper cite relevant and related articles appropriately? (30%) 3/5

Context

Does the title suitably represent the article? (25%) 5/5
Does the abstract correctly embody the content of the article? (25%) 5/5
Does the introduction give appropriate context? (25%) 4/5
Is the objective of the experiment clearly defined? (25%) 5/5

Analysis

Does the discussion adequately interpret the results presented? (40%) 3/5
Is the conclusion consistent with the results and discussion? (40%) 3/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%) 2/5
Review 2: Long-term cultivation using ineffective MDM2 inhibitor concentrations alters leukaemia cell drug sensitivity profiles

Reviewer: Oliver Krämer

Date of review: 29 September 2019
Published online:

Conflict of interest statement. Reviewer declares none.

Comments to the Author: The work describes that resistance against nutlin can sensitize leukemic cells to standard chemotherapy. The manuscript is well written and informative. The data are presented clearly and I have only minor criticism.

Critique:
1. I would like to have more information on the PL21 cell line. What is known about the driving oncogene(s)? Where are they from? Which type of leukemia? AML, CML, others…?
2. Line 110
The authors write
TP53 mutations MDM2…
I think that this sentence is incomplete. Was its second part accidentally deleted?
3. Line 115
The authors write
MDM2 inhibition has been shown to increase the cellular reactive oxygen species (ROS) levels…
Do the PL21 sublines have increased ROS levels?
4. Figure 1: Which values reach statistical significance in ANOVA analysis?
5. Title: Maybe
…alters leukaemia cell drug sensitivity profiles
is maybe better written as
…alters the drug sensitivity profiles of PL21 leukaemia cells
This would be more precise and not 5 nouns in a row (leukaemia cell drug sensitivity profiles), which is not so easy to read and immediately understand.
6. If the authors wish, they may additionally discuss some recent HDM2 inhibitors that are under investigation, see for example, Conradt, L. … Schneider, G., Int. J. Cancer 2003, May 15.
7. I suggest that the authors add some speculation/discussion why some of the sublines behave different than the others.
### Score Card

**Presentation**

<table>
<thead>
<tr>
<th>Question</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Is the article written in clear and proper English? (30%)</td>
<td>5/5</td>
</tr>
<tr>
<td>Is the data presented in the most useful manner? (40%)</td>
<td>5/5</td>
</tr>
<tr>
<td>Does the paper cite relevant and related articles appropriately? (30%)</td>
<td>4/5</td>
</tr>
</tbody>
</table>

**Context**

<table>
<thead>
<tr>
<th>Question</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Does the title suitably represent the article? (25%)</td>
<td>4/5</td>
</tr>
<tr>
<td>Does the abstract correctly embody the content of the article? (25%)</td>
<td>5/5</td>
</tr>
<tr>
<td>Does the introduction give appropriate context? (25%)</td>
<td>5/5</td>
</tr>
<tr>
<td>Is the objective of the experiment clearly defined? (25%)</td>
<td>5/5</td>
</tr>
</tbody>
</table>

**Analysis**

<table>
<thead>
<tr>
<th>Question</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Does the discussion adequately interpret the results presented? (40%)</td>
<td>5/5</td>
</tr>
<tr>
<td>Is the conclusion consistent with the results and discussion? (40%)</td>
<td>5/5</td>
</tr>
<tr>
<td>Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)</td>
<td>5/5</td>
</tr>
</tbody>
</table>

https://doi.org/10.1017/exp.2019.1 Published online by Cambridge University Press
Review 3: Long-term cultivation using ineffective MDM2 inhibitor concentrations alters leukaemia cell drug sensitivity profiles

Reviewer: Georg Hempel

1Westfälische Wilhelms-Universität Münster
Institut für Pharmazeutische und Medizinische Chemie- Klinische Pharmazie,
Münster, Germany

Date of review: 15 November 2019
Published online:

Conflict of interest statement. Reviewer declares none.

Comments to the Author: The abbreviation MDM-2 should be spelled out when first used.

How many different concentrations were used to determine the IC50-values? This information should be given in the Methods section. The source of the drugs used in the experiments should also be added.

Tables 1 and 2: Drug concentrations given in µM would be better. Please add information if means or median are given, standard deviations or ranges and the number of experiments per value given result.

Score Card

Presentation

Is the article written in clear and proper English? (30%) 5/5
Is the data presented in the most useful manner? (40%) 4/5
Does the paper cite relevant and related articles appropriately? (30%) 4/5

Context

Does the title suitably represent the article? (25%) 4/5
Does the abstract correctly embody the content of the article? (25%) 4/5
Does the introduction give appropriate context? (25%) 4/5
Is the objective of the experiment clearly defined? (25%) 4/5

Analysis

Does the discussion adequately interpret the results presented? (40%) 4/5
Is the conclusion consistent with the results and discussion? (40%) 4/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%) 3/5