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BIOMEDICAL SCIENCES
NOVEL-RESULT

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

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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].

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 PL21^{r}Nutlin^{20}XII and PL21^{r}Nutlin^{20}XV, 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

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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.

	TP53 status	Nutlin-3 IC_{50} (μM)	Cytarabine IC_{50} (ng/mL)	Doxorubicin IC_{50} (ng/mL)	Gemcitabine IC_{50} (ng/mL)
PL21	P72R[1]	20.49 ± 6.61	19.42 ± 6.28	56.12 ± 7.50	24.56 ± 1.34
PL21^{r}Nutlin^{20}I	P72R	19.84 ± 3.69 (−1.03)[2]	7.12 ± 1.68 (−2.73)	24.37 ± 2.59 (−2.30)	20.38 ± 4.43 (−1.21)
PL21^{r}Nutlin^{20}II	P72R	18.32 ± 3.05 (−1.12)	24.4 ± 8.58 (1.26)	64.85 ± 8.17 (1.16)	32.11 ± 5.12 (1.31)
PL21^{r}Nutlin^{20}III	P72R	17.81 ± 2.01 (−1.15)	11.62 ± 2.44 (−1.67)	23.16 ± 3.84 (−2.42)	10.54 ± 2.46 (−2.33)
PL21^{r}Nutlin^{20}V	P72R	7.86 ± 3.11 (−2.61)	15.86 ± 0.67 (−1.22)	10.24 ± 8.16 (−5.48)	0.84 ± 0.31 (−29)
PL21^{r}Nutlin^{20}VI	P72R	18.25 ± 2.83 (−1.12)	10.42 ± 0.54 (−1.86)	78.59 ± 1.01 (1.40)	14.37 ± 0.40 (−1.71)
PL21^{r}Nutlin^{20}VII	P72R	20.00 ± 0.71 (−1.02)	31.57 ± 3.80 (1.63)	51.99 ± 22.53 (−1.08)	10.74 ± 4.11 (−2.29)
PL21^{r}Nutlin^{20}VIII	P72R	21.18 ± 1.93 (1.03)	9.96 ± 1.12 (−1.95)	38.66 ± 4.55 (−1.45)	17.50 ± 2.37 (−1.40)
PL21^{r}Nutlin^{20}IX	P72R	18.29 ± 1.44 (−1.12)	8.28 ± 2.11 (−2.35)	49.20 ± 19.50 (−1.14)	9.55 ± 1.03 (−2.57)
PL21^{r}Nutlin^{20}X	P72R	22.94 ± 1.28 (1.12)	9.24 ± 4.37 (−2.10)	22.44 ± 2.99 (−2.50)	24.13 ± 1.60 (−1.02)
PL21^{r}Nutlin^{20}XII	P72R, M66L	16.51 ± 4.32 (−1.24)	13.24 ± 1.50 (−1.47)	29.54 ± 17.59 (−1.90)	23.53 ± 13.48 (−1.04)
PL21^{r}Nutlin^{20}XIV	P72R	20.25 ± 3.97 (−1.01)	4.71 ± 0.58 (−4.12)	26.08 ± 5.95 (−2.15)	10.43 ± 2.94 (−2.35)
PL21^{r}Nutlin^{20}XV	P72R, M66L	24.29 ± 2.00 (1.19)	31.76 ± 1.78 (1.64)	30.25 ± 3.81 (−1.86)	34.27 ± 13.55 (1.40)

[1]Polymorphism that does not affect p53 function.
[2]Fold change relative to PL21.

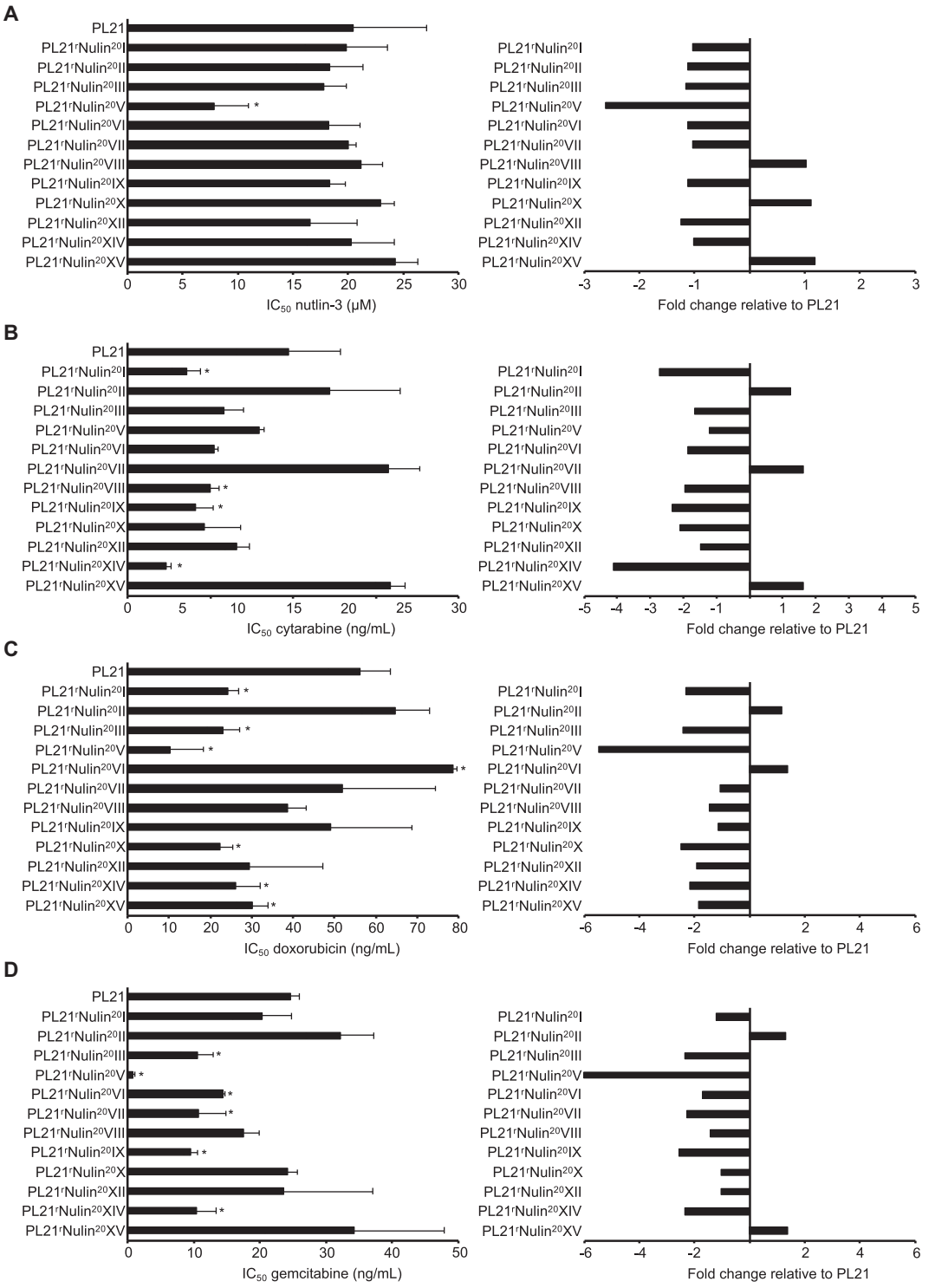

Figure 1. Drug sensitivity profiles of the AML cell line PL21 and its sublines cultivated in the presence of nutlin-3 (10 μM) for 52 weeks. Concentrations that inhibit cell viability by 50% (IC_{50}, mean ± SD from three independent experiments) as determined by MTT assay after 120 h incubation and IC_{50} fold changes relative to PL21 were determined for nutlin-3 (A), cytarabine (B), doxorubicin (C), and gemcitabine (D). * $P < 0.05$ relative to PL21.

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

	Nutlin-3 IC_{50} (μM)	Cytarabine IC_{50} (ng/mL)	Doxorubicin IC_{50} (ng/mL)	Gemcitabine IC_{50} (ng/mL)
PL21	20.49 ± 6.61	19.42 ± 6.28	56.12 ± 7.50	24.56 ± 1.34
PL21I	18.82 ± 1.60 (−1.09)[1]	19.54 ± 1.81 (−1.01)	52.38 ± 10.77 (−1.07)	26.46 ± 4.40 (1.08)
PL21II	18.51 ± 2.21 (−1.11)	16.97 ± 3.53 (−1.14)	56.36 ± 11.67 (1.00)	25.35 ± 4.48 (1.03)
PL21III	22.20 ± 1.43 (1.08)	19.83 ± 6.34 (−1.02)	62.51 ± 9.17 (1.11)	24.73 ± 2.49 (1.01)
PL2IV	20.07 ± 4.68 (−1.02)	18.05 ± 0.63 (−1.08)	59.78 ± 1.14 (1.07)	21.32 ± 6.00 (−1.15)
PL21V	21.86 ± 2.29 (1.07)	22.36 ± 6.06 (1.15)	53.26 ± 14.64 (1.05)	23.47 ± 2.30 (−1.05)
PL21VI	24.11 ± 7.44 (1.18)	22.74 ± 5.42 (1.17)	53.94 ± 11.53 (−1.04)	21.53 ± 3.60 (−1.14)
PL21VII	17.63 ± 1.88 (−1.16)	24.02 ± 7.83 (1.24)	54.24 ± 1.74 (−1.03)	26.06 ± 2.70 (1.06)
PL21VIII	21.84 ± 3.70 (1.07)	18.30 ± 2.95 (−1.06)	63.33 ± 1.41 (1.13)	26.15 ± 3.41 (1.06)
PL21IX	20.41 ± 5.44 (−1.00)	18.86 ± 4.44 (−1.03)	51.51 ± 5.14 (−1.09)	23.44 ± 2.73 (−1.05)
PL21X	19.99 ± 7.26 (−1.03)	22.52 ± 4.92 (1.16)	57.70 ± 13.11 (1.03)	23.57 ± 1.04 (−1.04)
PL21XI	21.66 ± 1.49 (1.05)	18.53 ± 2.25 (−1.05)	59.06 ± 8.41 (1.05)	21.10 ± 7.11 (−1.16)
PL21XII	21.58 ± 3.42 (1.05)	20.41 ± 2.42 (1.05)	52.86 ± 4.53 (−1.06)	24.87 ± 2.67 (1.01)

[1]Fold change 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.

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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;

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.

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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

3.7 /5

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

4.8 /5

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

2.8 /5

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

doi:10.1017/exp.2019.1.pr2

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

Is the article written in clear and proper English? (30%)	5/5
Is the data presented in the most useful manner? (40%)	5/5
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%)	5/5
Does the introduction give appropriate context? (25%)	5/5
Is the objective of the experiment clearly defined? (25%)	5/5

Analysis

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

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doi:10.1017/exp.2019.1.pr3

Review 3: Long-term cultivation using ineffective MDM2 inhibitor concentrations alters leukaemia cell drug sensitivity profiles

Reviewer: Georg Hempel[1]

[1]Westfälische Wilhelms-Universität MünsterInstitut 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

4.3 /5

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

4.0 /5

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

3.8 /5

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