EMBO
Molecular Medicine

Inhibition of transcription by dactinomycin reveals a new characteristic of immunogenic cell stress

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 7th Nov 2019

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the two referees whom we asked to evaluate your manuscript.

You will see that their comments are overall supportive. Still, I would like to encourage you to carefully address all comments mentioned by referee 1. Indeed, we believe that a more thorough data analysis in vivo and the addition of clinical correlates would improve the translational and clinical aspect of the paper which is an important point for our scope.

We would therefore welcome the submission of a revised version within three months for further consideration and would like to encourage you to address all the criticisms raised as suggested to improve conclusiveness and clarity. Please note that EMBO Molecular Medicine strongly supports a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

The manuscript describes findings that could be potentially translated into the clinical setting. There is a increasing need for data that may help improve current immunotherapy treatments in cancer.

Referee #1 (Remarks for Author):

This is an original study that continues a series of manuscripts from the same principal investigators which have introduced the concept of immunogenic chemotherapy and given major insights into the functional relationship between dying cancer cells and their microenvironment. Here, by using

computational prediction analysis, they identify and select dactinomycin (DACT), a well-known anticancer agent, as an immunogenic cell death (ICD) inducer. They show that DACT treatment is accompanied by all known biochemical and phenotypic hallmarks of ICD (i.e., calreticulin exposure, type I interferon signalling, ATP and HMGB1 release) downstream of endoplasmic reticulum stress response and eIF2α phosphorylation. These data are validated and supported in in vivo and ex vivo mouse models. Indeed, cancer cells hit with DACT are well taken up by dendritic cells (DCs). Moreover, DACT, either alone or in combination with non ICD inducers (as cisplatin), is much more efficient in the context of an intact immune system. In particular, cognate immune effectors such as CD8 and CD4 T cells are required for an optimal therapeutic success, and the combination with anti-PD-1 blockade lead to long-term tumor growth control or even cure of disease. The notion that DACT is a well-known DNA intercalator and RNA transcription inhibitor, gives to the authors the rational to investigate whether and find out that inhibition of RNA synthesis is a common feature of immunogenic chemotherapeutics and thus represents yet another hallmark of cancer ICD. This is a very attractive conclusion and could benefit from further clarification and evaluation. The following points are suggested.
- Ex vivo analysis of CD8 T cell activation from MCA205 bearing mice following DACT treatment is not fully convincing. IFN-γ production in DACT group is not significantly higher than Ctr group, which can be at least in part related to CD8 T cell exhaustion and can explain why in this model DACT alone does not work. It would be useful to analyse CD8 T cell state and function either in other tumor models (e.g., WEHI 164) or in MCA205-derived tumors after co-treatment with PD-1 inhibitors.
- Adoptive cell therapy with CD8 T cells from DACT "responder mice" in nu/nu mice would be another compelling option.
- In vitro pulse of cancer cells with DACT significantly enhance calreticulin exposure, DC-mediated uptake of apoptotic bodies and likely tumor antigen cross-presentation. Perhaps the analysis of CD8 T cell activation following co-culture with DCs that have phagocytized cancer cell apoptotic bodies would give a global and more complete view of DACT-induced ICD.
Phagocytosis experimental Ctr at 4{degree sign}C should be shown to rule out the possibility of a mere juxtaposition of DCs with cancer cells instead of an effective taken-up.
- Authors only analyse preclinical settings. The retrospective analysis on publicly available databases of immune infiltrate in patients treated with DACT could help link the conclusion from experimental model back to the patients thereby strengthening the current study.
- Statistics is not uniform, for some experiment SD is reported, for others SEM. Please check.
- Please review few typos (ligand>ligands pag.4; Fig. EVA,K>Fig. EV3A,K pag.8; ionimycin>ionomycin pag.9; fibroscaroma>fibrosarcoma pag.9; crizotininb>crizotinib pag.15).

Referee #2 (Comments on Novelty/Model System for Author):

The in vitro and in vivo models used are appropriate and consistent with the aims of this work. Results are, therefore, consistent.

Referee #2 (Remarks for Author):

In this manuscript, Humeau and colleagues used artificial intelligence to identify anticancer agents that were predicted to induce ICD. They found DACT as a compound with high 'ICD score' and verified this result by both in vitro and in vivo assays. Finally, since DACT is a potent transcriptional/translational inhibitor, they verified whether this feature might represent an ICD requirement.
They have elegantly and convincingly demonstrated this hypothesis by significantly increasing our knowledge of the real impact of ICD in the treatment of neoplasms and provide us with new parameters to evaluate, in a predictive manner, the potential use of new and 'old' antineoplastic compounds.
Therefore, I recommend the manuscript for publication in EMBO Molecular Medicine.

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General comment by Reviewer #1: The manuscript describes findings that could be potentially translated into the clinical setting. There is a increasing need for data that may help improve current immunotherapy treatments in cancer.
This is an original study that continues a series of manuscripts from the same principal investigators which have introduced the concept of immunogenic chemotherapy and given major insights into the functional relationship between dying cancer cells and their microenvironment. Here, by using computational prediction analysis, they identify and select dactinomycin (DACT), a well-known anticancer agent, as an immunogenic cell death (ICD) inducer. They show that DACT treatment is accompanied by all known biochemical and phenotypic hallmarks of ICD (i.e., calreticulin exposure, type I interferon signalling, ATP and HMGB1 release) downstream of endoplasmic reticulum stress response and eIF2α phosphorylation. These data are validated and supported in in vivo and ex vivo mouse models. Indeed, cancer cells hit with DACT are well taken up by dendritic cells (DCs). Moreover, DACT, either alone or in combination with non ICD inducers (as cisplatin), is much more efficient in the context of an intact immune system. In particular, cognate immune effectors such as CD8 and CD4 T cells are required for an optimal therapeutic success, and the combination with anti-PD-1 blockade lead to long-term tumor growth control or even cure of disease. The notion that DACT is a well-known DNA intercalator and RNA transcription inhibitor, gives to the authors the rational to investigate whether and find out that inhibition of RNA synthesis is a common feature of immunogenic chemotherapeutics and thus represents yet another hallmark of cancer ICD. This is a very attractive conclusion and could benefit from further clarification and evaluation.

Our response: We appreciate the encouraging comments by reviewer #1.

Point 1 raised by Reviewer #1: Ex vivo analysis of CD8 T cell activation from MCA205 bearing mice following DACT treatment is not fully convincing. IFN-γ production in DACT group is not significantly higher than Ctr group, which can be at least in part related to CD8 T cell exhaustion and can explain why in this model DACT alone does not work. It would be useful to analyse CD8 T cell state and function either in other tumor models (e.g., WEHI 164) or in MCA205-derived tumors after co-treatment with PD-1 inhibitors. Adoptive cell therapy with CD8 T cells from DACT "responder mice" in nu/nu mice would be another compelling option.

Our response: We thank the reviewer for this comment. Since the tumors are relatively small after DACT treatment in the WEHI 164 model, the number of tumor-infiltrating lymphocytes is low, rendering it difficult to perform adoptive transfer experiments. However, we have addressed the question about interferon-gamma production by mean of quantitative RT-PCR (new Fig. 6). The tumors from DACT-treated mice contained more mRNA coding for interferon-gamma than control tumors from untreated mice. These results were obtained 9 days after DACT treatment, when due to the small tumor size, cytofluorometric analyses of the tumor-infiltrating lymphocytes is problematic. However, to obtain information on the contribution of the immune system, we depleted T lymphocytes (with CD4 and CD8-specific antibodies), showing that this maneuver abolished the therapeutic effects of DACT. We performed additional experiments in which we injected a neutralizing interferon-gamma specific antibody, showing that this also impaired tumor growth reduction by DACT (new Fig. 6).

Finally, we rechallenged mice that had been cured from established WEHI164 sarcoma by DACT-based chemotherapy with the same cells (WEHI164 cells injected into the opposite flank), finding that the mice had developed a protective immune response that

precluded the growth of the sarcoma cells. As a control, such cells rapidly formed tumors in naïve mice, as determined in the same experiment (new Fig. 5I-K).

Point 2 raised by Reviewer #1: In vitro pulse of cancer cells with DACT significantly enhance calreticulin exposure, DC-mediated uptake of apoptotic bodies and likely tumor antigen cross-presentation. Perhaps the analysis of CD8 T cell activation following co-culture with DCs that have phagocytized cancer cell apoptotic bodies would give a global and more complete view of DACT-induced ICD.

Our response: To assess if DACT-treated tumors induce activation and maturation of DCs, we co-cultured DACT-treated MCA205 cells with BMDCs during 24 h and measured the percentage of co-stimulatory molecule CD86 and of MHC class II positive CD11c cells. DACT was indeed able to increase these markers of DC activation (Fig. 4A-D, Fig. S3).

Point 3 raised by Reviewer #1: Phagocytosis experimental Ctr at 4{degree sign}C should be shown to rule out the possibility of a mere juxtaposition of DCs with cancer cells instead of an effective taken-up.

Our response: As requested, the phagocytosis experiment has been repeated including additional controls. As expected, DACT-treated tumor cells were significantly less phagocytosed at 4°C as compared to 37°C standard environmental conditions. This new data is included in the revised version of the manuscript (Fig. 4B, Fig. S2).

Point 4 raised by Reviewer #1: Authors only analyse preclinical settings. The retrospective analysis on publicly available databases of immune infiltrate in patients treated with DACT could help link the conclusion from experimental model back to the patients thereby strengthening the current study.

Our response: We have done our best to locate databases describing the molecular properties of the immune infiltrate in clinical tumor specimens from patients under DACT treatment. Commensurate with the fact that DACT is only rarely used in modern oncology (mostly in the context of rare pediatric tumors), we were unable to find this information.

Minor point 5 raised by Reviewer #1: Statistics is not uniform, for some experiment SD is reported, for others SEM. Please check.

Our response: We have reformulated the statistic evaluation part in the Material and Methods section and pointed out the statistical methods and the basis of their usage: SD is used to show variation of experimental replicates when data come from one representative experiment. SEM is used to show variation of *ex vivo* data or when the mean of at least three independent experiment is shown.

Minor point 6 raised by Reviewer #1: Please review few typos (ligand>ligands pag.4; Fig. EVA,K>Fig. EV3A,K pag.8; ionimycin>ionomycin pag.9; fibroscaroma>fibrosarcoma pag.9; crizotininb>crizotinib pag.15).

Our response: We apologize for these oversights. The manuscript has been re-edited and orthographic flaws have been addressed.

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General comment by Reviewer #2: The in vitro and in vivo models used are appropriate and consistent with the aims of this work. Results are, therefore, consistent.
In this manuscript, Humeau and colleagues used artificial intelligence to identify anticancer agents that were predicted to induce ICD. They found DACT as a compound with high 'ICD score' and verified this result by both in vitro and in vivo assays. Finally, since DACT is a potent transcriptional/translational inhibitor, they verified whether this feature might represent an ICD requirement.
They have elegantly and convincingly demonstrated this hypothesis by significantly increasing our knowledge of the real impact of ICD in the treatment of neoplasms and provide us with new parameters to evaluate, in a predictive manner, the potential use of new and 'old' antineoplastic compounds.
Therefore, I recommend the manuscript for publication in EMBO Molecular Medicine.

Our response: We are very grateful to reviewer #2 for his encouraging support of our work.

2nd Editorial Decision 5th Mar 2020

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now supportive of publication and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments.

***** Reviewer's comments *****

Referee #1 (Remarks for Author):

The authors have addressed all my suggestions.

Referee #2 (Remarks for Author):

The authors produced new data to answer the reviewer's criticisms and included them in the revised manuscript. In my opinion, they cleared any point raised by the reviewer, and the new data also contribute to increasing the overall quality of the manuscript. I, therefore, recommend the manuscript for publication in the present form.

2nd Revision - authors' response 25th Mar 2020

The authors performed the requested editorial changes.

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Corresponding Author Name: Oliver Kepp, Guido Kroemer

Journal Submitted to: EMBO Mol Med
Manuscript Number: EMM-2019-11622-V2

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For in vitro (imaging, cytometry, luminescence assay or ELISA), data from more than 1000 cells per condition were analyzed, with experimental triplicates or quadruplicates. In addition, at least three independent experiments were performed. For ex vivo data, sample size was chosen based on available results from similar experiments to reach statistical significant results. When possible, results were confirmed with different methodology to avoid technical bias.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We used "BiostaTGV" software to calculate the number of animals needed to reach statistical significance, based on expected results.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Imaging data was assessed in quadruplicates; the singlet that deviated most from the mean was exlcuded and data analysis was conducted using the resulting triplicate dataset from which further dead or dying cells were excluded. In animal experimentation, animals that had to be sacrificed during the experiment due to an ethical endpoint other than tumor size were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Randomization procedure was applied. Please see below for description.
For animal studies, include a statement about randomization even if no randomization was used.	When tumors became palpable, their size were measured and the mice where distributed into groups, so that the day of the first treatment, the mean tumor size of each group was identical.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Randomization was performed and following tumor size was measured without any further changes. Mice that reached ethical endpoints were sacrificed.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done.
5. For every figure, are statistical tests justified as appropriate?	One-sided unpaired Student's t-tests were performed to compare each condition to its respective control. A pairwise multiple comparisons test with a Benjamin-Hochberg correction was used to compare different conditions within one dataset. For tumor growth analysis, a type II ANOVA test was used and for survival analysis, a LogRank test was applied (both using the freely available TumGrowth software package).
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Plotting the density of the distributions of imaging data with the functions "plot" from the "graphics" R package and the function "density" from the "stats" R package showed that they usually followed a normal distribution.

Is there an estimate of variation within each group of data?	Yes, data are presented as means ± SD of three experimental replicates if one representative among at least three independent experiment is depicted or as means ± SEM of three independent experiment.
Is the variance similar between the groups that are being statistically compared?	For animal experimentation, variance was analyzed with ANOVA and individual tumor growth curves are depicted in the manuscript.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Reference and/or clone numbers are indicated in the material and methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cells have been purchased from the ATCC or Sigma Aldrich, provided by collaborators or were genetically altered as described in the manuscript. All cell lines were regularly tested for the absence of mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	C57Bl/6 wild-type, Balb/c wild-type and nu/nu female mice came from Envigo. They were 7 to 10 weeks when starting the experiment. Animals were housed in the animal facility at the Gustave Roussy Cancer Center in a pathogen–free, temperature-controlled environment with 12 h day and night cycles and received water and food ad libitum.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal experiments were conducted in compliance with the EU Directive 63/2010 and with protocols 2018071210276451_n2018_051_16095, 201903131451670_n2019_017_19749 or 2019072311495586_n2019_050_21586 and were approved by the ethical committee of the Gustave Roussy Cancer Center (CEEA IRCIV/IGR no. 26, registered at the French Ministry of Research).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We complied to the guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	gRNAs, PCR probes/primers and information on sequences used for genetic modification are available as supplemental material.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	A dataset from the National Cancer Institute was used and the link is indicated in the material and methods section.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	A computational model was used. It is available on Github with the link indicated in the material and methods section.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA