EMBO Molecular Medicine

A *Klebsiella pneumoniae* antibiotic resistance mechanism that subdues host defences and promotes virulence

Timothy Kidd, Grant Mills, Joana Sá-Pessoa, Amy Dumigan, Christian Frank, José Insua, Rebecca Ingram, Laura Hobley, and José Bengeochea

Corresponding author: José Bengoechea, Queen's University Belfast

Review timeline:

Submission date:	14 November 2016
Editorial Decision:	15 December 2016
Revision received:	22 December 2016
Editorial Decision:	09 January 2017
Revision received:	11 January 2017
Accepted:	12 January 2017

Transaction Report:

(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.)

Editor: Céline Carret

1st Editorial Decision 15 December 2016

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. Although the referees find the study to be of potential interest, they also raise a few concerns that need to be addressed in the next final version of your article.

You will see from the comments below that both referees are enthusiastic about the work and only minor issues are raised. Notwithstanding, for our scope and interest, the manuscript does not provide any direct clinical implications at this stage. I would like to encourage you to thoroughly discuss the clinical consequences and implications of your findings to increase their medical relevance.

We would welcome the submission of a revised version for further consideration and depending on the nature of the revisions, this may be sent back to the referees for another round of review.

Please note that it is EMBO Molecular Medicine policy to allow only a single round of revision and that, as acceptance or rejection of the manuscript may depend on another round of review, your responses should be as complete as possible.

I look forward to receiving your revised manuscript.

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

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

the medical impact is definitely medium if not low; there is no immediate consequence. This is a basic research study.

Referee #1 (Remarks):

The multidrug resistance of Klebsiella is a great problem worldwide. In particular the authors aimed to elucidate what is the factor which develop colistin resistance in Klebsiella Previous studies demonstrated that this was due to a mutational inactivation of mgrB gene. Still teh authors wanted to clarify this at molecular level.
Through a series of studies, they reach the point that lipopolysaccharide lipid A is the chemical factor which confers resistance to the colistin by adding new chemical moieties to the pre-existing lipid A. It seems that in particular the presence of positively charged groups present in aminoarabinose and 2-amino ethanol work withdrawing the cation peptides, colistin included. This is a known mechanism; the presence of a further fatty acid, the 2-hydroxy-myristic was also shown. But for this no explanation/ speculation is given at molecular level. Additionally and intriguingly, the authors demonstrate that these chemical addition to the lipid A have the effect to render Klebsiella lipid A a weak stimulator of innate immunity. This was demonstrated in vitro and in vivo by a plethora of immunological experiments. So these mutations at chemical level switch the Klebsiella straint to be colistin resistant and also more virulent. This is certainly a novel finding, since it is thought that a higher resistance is paid by the bug with a low virulence.
I trust these are novel and interesting findings and the work is really good and experiments are well-conceived and carried out. The only part which is missing is at molecular level, there is no clear-cut explanation of the action/contribute (if any) of 2-hydroxy miristate to the resistance to polymixixns, only a vague statement; and there is no hypothesis of how this new lipid A bled can bind to TLR4/MD2 binary system in a different fashion, here maybe a deeper look at literature is worth. Maybe a couple of statements and a hypothesis could be advanced.

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

This study is focused on the mechanism of antimicrobial peptide resistance in K. pneumoniae. Specifically, mutants deficient in the function of mdrB gene are analyzed. The authors used a comprehensive approach to link modifications in lipid A to regulatory and functional genes in K. penumoniae and to the host responses. The technical quality is exceptional. Novelty is in the comprehensive approach, which takes previous speculations onto scientifically valid levels.

Referee #2 (Remarks):

An exceptional quality study that establishes functional and mechanistic links between mutational modifications of lipid A to specific genes and lipid A structures in K. pneumoniae and in the model hosts. The only minor suggestion is to rephrase the statement regarding the "conventional wisdom" or "dogma" that antibiotic resistance is linked to reduced bacterial fitness. This wisdom has been challenged in many publications in the last five years and by the simple fact of rapid spread in antibiotic resistance in clinics.

1st Revision - authors' response 22 December 2016

We appreciate the referee’s efforts to assess our manuscript. We are glad that both of them share our enthusiasm about this work. Both referees raised two minor issues which we have met as follows (answer right after the referee’s comment which is blue marked):

Referee 1:

The multidrug resistance of Klebsiella is a great problem worldwide. In particular the authors aimed to elucidate what is the factor which develop colistin resistance in Klebsiella Previous studies demonstrated that this was due to a mutational inactivation of mgrB gene. Still the authors wanted to clarify this at molecular level. Through a series of studies, they reach the point that lipopolysaccharide lipid A is the chemical factor which confers resistance to the colistin by adding new chemical moieties to the pre-existing lipid A. It seems that in particular the presence of positively charged groups present in aminoarabinose and 2-amino ethanol work withdrawing the cation peptides, colistin included. This is a known mechanism; the presence of a further fatty acid, the 2-hydroxy-myristic was also shown. But for this no explanation/ speculation is given at molecular level. Additionally and intriguingly, the authors demonstrate that these chemical addition to the lipid A have the effect to render Klebsiella lipid A a weak stimulator of innate immunity. This was demonstrated in vitro and in vivo by a plethora of immunological experiments. So these mutations at chemical level switch the Klebsiella straint to be colistin resistant and also more virulent. This is certainly a novel finding, since it is thought that a higher resistance is paid by the bug with a low virulence.

I trust these are novel and interesting findings and the work is really good and experiments are well-conceived and carried out.

We thank the referee for her/his very positive assessment of our study. S/he has nicely summarized the main findings our work while putting them in the context of the state-of-the-art.

The only part which is missing is at molecular level, there is no clear-cut explanation of the action/contribute (if any) of 2-hydroxy miristate to the resistance to polymixixns, only a vague statement;

Our findings (this work and our recent publication Llobet et al Proc Natl Acad Sci U S A. 2015 112(46):E6369-78) sustain the important role of 2-hydroxy myristate modification on *Klebsiella* resistance to polymyxins. To generalize that the presence of a hydroxyl group on a lipid A secondary acyl chain is a bacterial mechanism to evade innate immune defences warrants further studies. However, and providing additional support to this notion, hydroxylation on the 3′-linked secondary acyl chain of *Vibrio cholerae* also promotes resistance to antimicrobial peptides (Hankins et al Mol Microbiol. 2011 81(5): 1313–1329). Interestingly, the fact that other Gram negative pathogens also synthesize lipid A species that possess a hydroxyl group on a secondary acyl chain ([*Salmonella*, Gibbons et al J Biol Chem. 2000;275:32940–32949], [*Pseudomonas*, Kulshin et al Eur J Biochem. 1991;198:697–704] [*Legionella*, Zharinger et al Prog Clin Biol Res. 1995;392:113–139], [*Acinetobacter*, Beceiro et al Antimicrob Agents Chemother. 2011 55(7):3370-9]) could indicate that this lipid A modification is an evolutionary conserved mechanism playing a role in host survival; reinforcing the notion that this lipid A modification may play a more important role in polymyxins/antimicrobial peptide resistant than previously anticipated. In this context, we do agree with the referee's view that additional investigations testing different pathogens are required to explain mechanistically its role in resistance, and most likely also, in outer membrane stabilization.
In the revised version of the discussion, we have included part of this discussion and expanded briefly what we hypothesize could be the role of this modification (lines 400-408).

and there is no hypothesis of how this new lipid A bled can bind to TLR4/MD2 binary system in a different fashion, here maybe a deeper look at literature is worth. Maybe a couple of statements and a hypothesis could be advanced.

Following the referee's advice, we have included in the discussion (lines 459-470) new text summarizing very briefly how *mgrB*-controlled lipid A structure may limit TLR4/MD2 activation. Our speculations are based on the seminal work by Park and coworkers showing the structural basis of LPS recognition by TLR4/MD2 complex (Park et al *Nature* 458, 1191-1195).

Referee 2:

An exceptional quality study that establishes functional and mechanistic links between mutational modifications of lipid A to specific genes and lipid A structures in K. pneumoniae and in the model hosts. The only minor suggestion is to rephrase the statement regarding the "conventional wisdom"

or "dogma" that antibiotic resistance is linked to reduced bacterial fitness. This wisdom has been challenged in many publications in the last five years and by the simple fact of rapid spread in antibiotic resistance in clinics.

We thank the referee's comments on our work.

As suggested we have rephrased the statement concerning the "dogma" that antibiotic resistance is linked to reduce bacterial fitness. Additionally, and as indicated by the Editor, we have expanded the clinical implications of our work highlighting the notion that antibiotic resistance is not inexorably linked to reduce fitness but may result even in increase virulence (lines 480-496), as this manuscript demonstrates.

2nd Editorial Decision 09 January 2017

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

Ethics:
please indicate the age of the mice used and each instances, the exact n

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

2nd Revision - authors' response 11 January 2017

Authors made requested editorial changes.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Professor Jose Bengoechea
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2016-07336

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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 $\chi 2$ 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We have performed extensive studies of this type in the past. The group size is the minimum number that is sufficient to reliably define a specific functional parameter taking into account the biological variability.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Animals were randomized for interventions but researches processing the samples and analysing the data were aware which intervention group corresponded to which cohort of animals (page 22).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals 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.	Experimental intervention was randomized (page 22)
For animal studies, include a statement about randomization even if no randomization was used.	Animals were randomized to the treatment groups (page 22)
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.	Not applicable
4.b. For animal studies, include a statement about blinding even if no blinding was done	blinding was not performed (page 22)
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	All analyses were performed using GraphPad Prism for Windows (version 5.03) software (page 34). Normality was tested with the Kolmogorov–Smirnov test.
Is there an estimate of variation within each group of data?	All analyses were performed using GraphPad Prism for Windows (version 5.03) software. Variation within each group of data was determined by Brown-Forsythe test (page 34)
Is the variance similar between the groups that are being statistically compared?	Not always (page 34). Statistical analyses were performed using the two-tailed t test, or when the requirements were not met, by the Mann-Whitney U test. P-values of <0.05 were considered statistically significant. Survival analyses were undertaken using the Log-rank (Mantel-Cox) test with Bonferroni correction for multiple comparisons (α=0.008).

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jij.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	α-tubulin (1:3000; Sigma-Aldrich T6074);anti-IκBα (1:1000; Santa Cruz Biotechnology sc-847), anti-phospho-p38 (1:1000; Cell Signaling #4511), anti-phospho-SAPK/JNK (1:1000; Cell Signaling #9251) and anti-phospho-ERK (1:1000; Cell Signaling #9101). Immunoreactive bands were visualised by incubation with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (1:5000; Bio-Rad 170-6515) or goat anti-mouse immunoglobulins (1:1000; Bio-Rad 170-6516).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Immortalised BMDM (iBMDM) cells (BEI Resources, NIAID, NIH: Macrophage Cell Line Derived from Wild Type Mice, NR-9456). Cells were tested fro Mycoplasma contamination (page 33)

* 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.	Wild-type immunocompetent C57BL/6 (females, 8-9 weeks of age) from a breeding colony kept at Queen's University Belfast (page 22). Animals were provided food and water ad libitum.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Home licence PPL2700 (page 22)
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.	The animal experiment were performed under ASPA 1986 (PPL2700), and adhered to ARRIVE and NC3Rs guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable
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.	Not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable
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.	Not applicable
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.	Not applicable

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	Not applicable
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).	Not applicable
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).	Not applicable
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Not applicable
22. 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.	Not applicable

G- Dual use research of concern

23. 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.	No to the best of my knowledge.