

June 6th, 2016.

Dear Editor,

Re: Manuscript #7898 Tachyplesin 1 as a potential antimicrobial agent against *Burkholderia pseudomallei*: an *in vitro* and *in silico* approach

In the following pages, I have maintained the answers from the first round of comments in **blue** the second round of author comments in **purple**. My comments on these changes are in **brown**.

The revisions go further to answering the original comments made, but are not yet satisfactory for some of the points.

Comments of Reviewer 1 to the revised manuscript and rebuttal.

Editor's comments

The reviewers raise a number of important points which must be addressed in a revised manuscript.

1. Potential problems with your calculations and some experimental protocols
We thank the reviewer for pointing out the flaws. All calculations and protocols have been checked and any errors have been rectified.
Some still exist and need attention. See below.
2. Lack of justification for following TP1 as lead, especially given its toxicity to mammalian cells.
Justification has been included in the manuscript (Discussion, Lines 445-454)
OK, but needs to go further, see below.
3. Limited *in silico* analysis performed, and the lack of any experimental confirmation of the results. This gives me the impression that this part of the work is very preliminary.
Additional *in silico* analysis has been performed and discussed to further strengthen the conclusions derived. These have been included in the revised

manuscript (Lines 241-287; 374-437; 565-613).

Needs more justification of using the approach, give the reader a brief review of where this type of *in silico* approach has led to drug development outcomes, or outcomes that would support the potential of the approach in this area. One paper describing work in a similar area of this article is not enough.

We have edited and additional information have been added to the existing *in silico* work in the introduction, lines 90-102. We hope that the information will further support the approach taken in our study.

OK

4. This, and the toxicity of TP1 also leave me feeling somewhat misled by the title of your manuscript, which must be addressed if you choose to revise your manuscript.

The title has been modified from “Tachyplesin 1 as a Potential Antimicrobial Agent against *Burkholderia pseudomallei*: an *in vitro* and *in silico* approach” to “Antimicrobial activity of Tachyplesin 1 against *Burkholderia pseudomallei*: an *in vitro* and an *in silico* approach” to better portray our study.

Ok.

Reviewer 1

1. Basic reporting

- 1.1 English is in need of tightening in some places. Some sections could be more succinct. The abstract is a good example.

We have amended the language throughout the manuscript to improve flow and comprehension. The abstract has also been re-written to improve clarity (lines 28-47).

English is OK, but with room for improvement. No more comments here. There are mis-spellings and format errors to correct.

Spellings and formatting errors have been corrected throughout the manuscript.

OK.

- 1.2 The *in silico* work is not adequately introduced (for me, a non-expert in that

field).

Introduction to the *in silico* study has been included in the introduction for better understanding (lines 82-98).

One of the studies which incorporate both *in vivo* and *in silico* techniques was seen with Le et al. (2015) where molecular docking was carried out together with toxicity studies to predict possible binding targets of AMP DM3 where a strong affinity was observed towards autolysin and pneumococcal surface protein A (pspA). Moreover, the pneumolysin and pspA structure used in their study was modelled based on autolysin.

In this section I would like to see better explanation of the outcomes of the *in silico* approaches. Autolysin, PspA etc need clearer explanation and their relevance to this study. The added text goes some way to answering the question, but not far enough for me.

We have added additional explanation of the outcomes of the *in silico* approaches (Lines 287-288). Justification for the use of autolysin, pneumolysin and PspA (Lines 275-279;293-306) have also been added to improve clarity.

OK

- 1.3 Figure 1 is not needed; data is in the text. Legends need more information so the individual figures are stand alone.

Figure 1 has been removed as suggested and replaced with Figure 2. Precise and constructive information has been added to all the figure legends to improve clarity and allow it stand alone.

OK.

- 1.4 I think there are two studies, first the lab experiments to justify and underpin *in silico* studies (which I am not convinced that they do), and second the *in silico* experiments following a clearly justified strategy (which is not apparent in this draft).

Initially, *in vitro* experiments were carried out to identify potential AMPs with activity against *B. pseudomallei*. There, TP1 was selected for further investigation where the inhibitory concentrations, cytotoxicity, and microscopy studies were performed. Subsequently, molecular docking was carried out on *E. coli* lipopolysaccharide, *S. pneumoniae* and homology

modelled *B. pseudomallei* PDB structures in order to hypothesize the AMP interactions (Lines 241-287). The findings of the TP1 molecular docking on 26 *B. pseudomallei* PDB structures was also done and summarized in the results (Lines 423-437) and the data was made available as supplementary (Supplementary 6-8).

Ok, the link between *in vitro* and *in silico* sections is there but could be made stronger. In the introduction I'd like to see evidence from the literature that the approach taken works and clear review of literature in the wider area to justify the validity of the approach taken; this has not yet been provided as it needs to go far beyond reference to Le et al 2015.

We have further improved the link between the *in vitro* and *in silico* studies and additional references (Sarojini et al., 2010; Alves et al. (2013); Al-Sohaibani & Murugan (2012) covering a wider area have been included to support the validity of the approach taken (Lines 90-102).

OK, but correct this, as only 2 not 3 targets are listed: Le et al. (2015) performed toxicity studies together with molecular docking to predict possible binding targets of AMP DM₃ using Autodock Vina where a strong affinity was demonstrated towards three targets; *Streptococcus pneumoniae* virulence factors; autolysin and pneumococcal surface protein A (PspA)

2. Experimental design

- 2.1. The strategy for screening against clinical isolates, testing anti-biofilm effects and cytotoxicity is OK. The choice of TP1 for further study is not well justified.

Currently, among the AMPs which were reported to demonstrate potential to inhibit *B. pseudomallei* include LL-37 (Kanthawong et al. 2012), PG1 (Sim et al. 2011), bovine lactoferrin (Puknun et al. 2013), phospholipase A2 (Samy et al. 2015), and SMAP-29 (Blower et al. 2015). There are more potential AMPs are yet to be tested against *B. pseudomallei*. TP1 was shown to exert broad-spectrum antimicrobial activity against a wide range of Gram-negative (i.e. *E. coli*, and *S. typhimurium*) and Gram-positive bacteria (i.e. *Staphylococcus aureus*) (Nakamura et al. 1988; Ohta et al. 1992). To date, the inhibition activity of TP1 on *B. pseudomallei* is yet to be reported. We aim to understand the mode of action of AMPs, specifically on TP1 and to identify potential interaction targets on *B. pseudomallei*. The justification for selection of TP1 has now been added to Discussion (Lines 448-456).

This argument needs to be made after line 303. experiments are performed on 3 AMPs and then narrow to what is the poorest performer – justification based on the evidence presented and with reference to other literature is needed at this point. The discussion is too late to address this issue, although it is an appropriate place to revisit.

Thank you for your suggestion. After careful consideration, we have included the justification for the selection of TP1 for further study after our results at Lines 325-332. Besides potential inhibition reports from the literature, we have also selected TP1 for further study based on the preliminary screening outcome before proceeding to determine the inhibition concentration. Therefore, we felt that by justifying our selection at this part will enable readers to understand our approach and enhance the overall flow of the manuscript.

OK

- 2.2. The *in silico* section does not have a strategy justifying the validity of the experimental design, and the *in silico* studies research question is not clearly stated and definitely not aligned to the stated aim of the manuscript in general: AMPs as

alternative therapies in the background of resistance to conventional antibiotics. Plus, other comments in the comments to authors.

The statement of the aim of the study has now been revised to improve clarity and include both the *in vitro* and *in silico* studies. The *in silico* study have also been modified to achieve our objectives (Kindly refer to point 1.4 and Lines 100-109).

OK, but I'd like to see a line or two before line 372 to highlight the problem and under study and the specific objective of this part of the study. It is not needed to be detailed, but at present there is no context to help the reader understand the direction or relevant of the section to come.

Thank you for highlighting. We have added a paragraph at (lines 407-413) just before the *in silico* study to improve the transition from the *in vitro* to the *in silico* study.

OK

3. **Validity of the findings**

- 3.1. I think there are some basic errors in the research (possibly at calculation or interpretation stage) that are concerning; e.g. 10e12+ CFU per ml in a microplate well culture, stating a reduction of 7.8 log CFU/ml to 4.2 log CFU/ml is a 2-fold reduction. Plus, other comments in the comments to authors.

We thank the reviewer for pointing out this error. All data has been checked and errors have been rectified. For justification on 10e12+ CFU per ml in a microplate well culture, please refer to point 5.5.4.2. We have corrected the CFU/mol reduction description in the results section in the “Time-kill kinetic assay” (Lines 309-318) and “Inhibition activity of AMPs against *B. pseudomallei* in biofilm state” (Lines 320-340).

5.5.4.2 is incorrect. There is either a mistake in the arithmetic, error in dilution technique or contamination in diluents. I do not believe you would get more than 10e10 per ml. This is a figure based on my lab experience and the values routinely obtained by students and researchers in my lab. For *Escherichia coli*, a similar sized bacterium, pelleted by centrifugation from a litre of culture (about 10e12-13 CFU) would have a volume around 1 ml- so any CFU per ml value that is higher is not

physically possible; 10e15 CFU would have a wet volume of 100ml by this calculation. CFU/ml values above 10e10 are possible in fermenters where growth media, wastes, temperature, oxygen etc are controlled to optimal levels; something impossible in the microplate.

We have plated out the diluent on fresh NA in each replicate in order to check for any contamination and the diluent used in each replicate did not show any signs of contamination.

As for the calculation, we did not detect any errors. For your information, we have used the formula: $CFU = (\#colony \times dilution\ factor) / volume\ plated\ in\ ml$. Moreover, we have repeated the experiment to the best we could and still obtain similar results. In our lab, the number of *B. pseudomallei* CFU/ml using a similar protocol (with a starting culture of ~10e5) generally range from 10e8 to 10e17. There were also instances where the bacteria still continue to grow even after 24-hour incubation.

On the side note, we also believe that when *B. pseudomallei* was grown in low nutrient conditions/exposed to antibiotics, it may become dormant or grow at a very slow rate. Once the bacteria are transferred onto a nutrient rich media/antibiotics removed from the media, it resumes its usual growth rate and multiplies rapidly. In our case, NA is definitely a much richer media than RPMI 1640. This is one of the hallmarks of *B. pseudomallei* which challenges the current antibiotic treatment. The dormancy of *B. pseudomallei* was also reported by Frangoulidis et al. (2008) where a German patient who travelled to Thailand has a melioidosis relapse after 10 years even after combined chemotherapy and surgical revision of the abscess. Anutrakunchai et al. (2015) also reported that *B. pseudomallei* was able to persist in a dormant and non-dividing state during nutrient depletion, and spontaneously switch to fast growth in a nutrient rich media.

OK, I will put this simply. For whatever reason the authors have obtained a number of bacteria per ml (up to 6 orders of magnitude higher) that is far in excess of what is thought normal for bacteria, what is the normal upper limit in my experience and what is physically possible. The paper should not be published with this obvious error. The error casts doubt on all the viable count data, is there a problem with those values only above 10e10/ml or is it something affecting all readings? Who knows. I note that for each

manipulation a fresh sterile tip should be used as bacteria often stick to the tip and give a false high concentration if the same tip is used down the dilution series. How to fix this:

1. Perform dilution series from a turbid culture of known optical density and plate for CFU/ml values. Do in triplicate with three independent cultures. Do for *Escherichia coli* (or similar model bacterium) and *Burkholderia pseudomallei* cultures preferably at similar optical densities.
2. Enumerate the bacteria present by counting cells under the microscope using a haemocytometer.
3. Repeat at least one representative treatment from the figures for normalisation.
4. Analyse results.
5. Review results in figures, amending CFU/ml as necessary or submitting results and detailed method as a supplementary figure to show this abnormal result is true and peculiar to *Burkholderia*. Literature should be surveyed where other *Burkholderia* researchers have enumerated CFU/ml in cultures.

4. Comments for the author

- 4.1 The development of antimicrobial resistance is a serious public health concern. Some bacteria, e.g. *Burkholderia pseudomallei* also have high levels of intrinsic resistance to antibiotics. The development of further resistance in *B. pseudomallei* means there are some strains now resistant to many previously usable antibiotics, and increasingly for some people melioidosis does not respond to antibiotic treatment. Antimicrobial peptides (AMPs) are a good alternative candidate to conventional “antibiotics”.

The manuscript under review examines AMPs as a potential treatment for melioidosis by first screening the activity of a range of AMPs against a range of *B. pseudomallei* isolates. LL-37 and PG1 show best range and lowest MICs, but TP1 is chosen for further study without convincing justification.

Justification for the selection of TP1 has been provided base on the similar query raised by Reviewer #2 (Lines 448-456).

See 2.1 above.

Kindly refer to 2.1.

OK

- 4.2 No comparison of AMP activity profiles is made to any data that might be available for the isolates tested (e.g. API20 NE profile, clinical presentations, antibiotic sensitivity profile). Anti-biofilm activity is tested as an expansion of the data, this is a good strategy but there are serious problems with the presentation and interpretation of this data (see specific comments). SEM is used to begin to give some gross mechanism data, but the evidence presented does not really support the conclusions made. In vitro toxicity testing with a range of cell lines suggests that TP1 is more toxic to mammalian cells than *B. pseudomallei*, but no consideration is given to how this finding may suggest that TP1 is unsuitable for therapeutic use for melioidosis.

Kindly refer to point 5.5.2 in the specific comments, for the justifications on AMP activity profile comparison, point 5.5.5 on the data presentation of the anti-biofilm activity, point 5.5.6 on SEM, and point 5.5.7 for cytotoxicity.

Ok will comment in specific sections mentioned.

- 4.3 A substantial section is devoted to *in silico* analysis of TP1 and identification of proteins that TP1 may interact with. I do not consider this my area of expertise, but make some high-level comments in the specific details.

The *in silico* study was incorporated in this manuscript to predict the possible protein binding targets of TP1. We have revised the *in silico* study from docking TP1 directly on *B. pseudomallei* PDB structures to docking TP1 on *E. coli* LPS (previously reported by Kushibiki et al. (2014)) and *S. pneumoniae* structures (used for docking with DM3 by Le et al. (2015)), modelling *B. pseudomallei* protein structures based on those structures and then carrying out TP1 docking on the model. We believe that our current approach will provide a more concrete prediction of the possible protein binding targets of TP1 compared to our previous attempt. This has now been included in the revised manuscript (Lines 240-285; 372-435; 564-612).

ok

I do feel the manuscript is long enough without this information and this section

would be best left out and used as the basis of a separate manuscript.

We thank the reviewer for this suggestion, however, we believe that both the *in vitro* and *in silico* study performed in this study will allow us to achieve the aim to identify potential AMP (TP1) and elucidate the mechanism of action of this AMP on *B. pseudomallei*.

ok

- 4.5 Overall, the figures presented need legends that supply information to make the figure stand alone. For example, the concentrations of AMPs used must be stated clearly in the legend. I do not believe that supplementary information is appropriate for an online journal – it is either needed for the paper (include it) or not (leave it out).

Figure legends have been edited with additional information to make them stand alone. The supplementary data from the previous submission has been replaced with the additional data that will support our observation.

OK.

5. Specific comments

5.1 Abstract

Too long and rambling. It needs to be re-written to be more succinct and highlight findings.

The abstract has been shortened, focusing more on the findings (lines 28-47).

Ok

5.2 Introduction.

- 5.2.1 Line 64. What do the authors mean by “phenotypically mutate”? Is biofilm formation more correctly described as phenotypic adaptation, without necessarily requiring any mutation, which I would class as a genotypic change.

We thank the reviewer for pointing for clarifying the term. This The term “phenotypically mutate” has been deleted from the sentence and the sentence was reconstructed to “Over the years, *B. pseudomallei* has been reported to resist the commonly used antibiotics (increased usage of ceftazidime and

amoxicillin/clavulanic acid in treatment), and also to the ability to form biofilm *in vitro* and *in vivo*.” to improve clarity (Lines 61-64).

ok

- 5.2.2 Line 71. What is meant by “inoculation” – can this mode of dissemination be described.

The word “inoculation” was replaced with “through an open wound” to better relay the intended meaning. (Line 55)

ok

- 5.2.3 Line 86. I think “synthetic” rather than “synthesized” better describes the authors’ intent here.

The word “synthesized” was replaced with “synthetic” as suggested (Line 70).

Ok

- 5.2.4 Line 88. The sentence starting “They are also highly potential ...” doesn’t make sense to me. Perhaps highly potent?

The phrase “also highly potential” has been removed from the sentence in order to improve clarity. The sentence now reads “Furthermore, they act on slow-growing or even non-growing bacteria due to the ability to permeabilise and form pores within the cytoplasmic membrane (Batoni *et al.* 2011).” (Line 73-75)

Ok

- 5.2.5 Line 90. Begin the sentence with Groups of ...

The sentence was amended as suggested to “Groups of AMP’s i.e., defensins, cathelicidins, and dermicins have previously been reported to show potential against various pathogens (Wiesner & Vilcinskas 2010).” (Line 74-76)

ok

- 5.3 The overall strategy of the *in silico* approach to drug design here needs to be clearer; i.e. identify something that works and use an *in silico* approach to generate hypothesis for mechanism of action and new designs for better acting drugs. Specific examples where this approach has been successful should be briefly described.

The manuscript has been revised to include a different approach of the *in silico* analysis and additional references that supports the approach has been added (Le et al. (2015). Please refer to point 1.4 and the revised manuscript (Line 93-98).

This is not deep enough, and needs a wider review citing the seminal works/reviews in the area.

5.4 Methods.

5.4.1 Peptide storage and Preliminary screening.

Please indicate the concentrations of stocks and tests for the preliminary screening. It would also be helpful to include molar concentrations and g/l concentrations for each.

The AMP stock concentrations have been added to the “Peptide storage and handling” section (Line 123-139) while the concentrations used in the preliminary screening have been added in Line 144. Besides that, the “molar concentrations in μM and $\mu\text{g/ml}$ ” have also been added into the supplementary file 3.

ok

5.4.2 MIC and MBC of planktonic cells.

5.4.2.1 Why was RPMI used as the medium? The methods do not look like internationally accepted methods for measuring MIC or MBC that would be used for “antibiotics” so should be given a brief justification. Dilution of the cells in

RPMI will give a final concentration of 0.8x RPMI, which should be commented on. The same comments apply to the testing of anti-biofilm activity.

Thank you for highlighting this point. Additional information has been added to the “peptide storage and handling” section in methods where serum free RPMI was used to dilute the AMPs prior to the experiment (Lines 137-138).

ok

Justification for the use of RPMI in our experiments has also been included in the discussion (Lines 531-540).

Needs to be a brief explanation in the methods section, as well as this in the discussion.

We have added a brief explanation at lines 140-143 in Methods: Peptide storage and handling, “The AMPs were diluted in 0.01% acetic acid containing 0.2% bovine serum albumin (BSA) for the preliminary screening whereas the dilution of AMPs with serum free Roswell Park Memorial Institute 1640 media (RPMI 1640; Life Technologies) was done to minimize the dilution of the RPMI used in subsequent experiments.”

OK, the authors have mis-understood my request. Please simply justify the use of RPMI over other media commonly used for antimicrobial assays, e.g. Muller Hinton. Is it important to use RPMI or can any medium be used? If important can you explain why?

- 5.4.2.2 Please indicate more than the range of concentrations, i.e. doubling dilutions for 200microM? Or state the actual concentrations tested. The same comments apply to the testing of anti-biofilm activity.

Range of concentrations have now been clearly indicated as “two-fold increase” in the “MIC and MBC” section (Line 158), and “Inhibition activity of AMPs against *B. pseudomallei* in biofilm state” (previously anti-biofilm activity) section (Line 188-193) in the methods. The methods for “Inhibition activity of AMPs against *B. pseudomallei* in biofilm state” (Line 185-203) have been revised to improve clarity.

ok

- 5.4.2.3 Assays were performed in a U-shaped microplate, followed by measurement of absorbance at 570nm. I think flat bottomed microplates would be needed for Absorbance readings.

In the U-shaped plates, we are aware that the depth will vary with different position of reading. Therefore, our reader was programmed as such that the

readings were done at exactly the same point for each well. In this case the reader reads from the bottom of the microplate.

Ok. The methods should end the relevant sentence ... using a programme for U shaped microplates.

Thank you for your kind suggestion. We have included the above words at line 172 in the sentence, “Following incubation at 37°C for 24 hours, the plates were subjected to optical density (OD) at 570 nm readings with a microplate absorbance reader (Tecan Sunrise™, Switzerland) using the settings for U-bottom microtiter plates.”

OK

5.4.2.4 How was the MIC decided upon, i.e. how much inhibition was needed?

Additional sentences have been incorporated in the MIC and MBC experiment section for better clarification. “The MIC was obtained at the lowest concentration of AMPs which showed reduction in the absorbance compared to the untreated after 24- hour incubation, whereas the MBC was determined based on observation of bacterial growth on NA after 24-hour incubation.” (Lines 166-169).

What is needed is a definition of what measure of Absorbance is scored as a reduction i.e. it could be 10%, 50%, 90%?

Thank you for the additional clarification. We have added “10%” at line 174 clarify the absorbance. The sentence now reads “The MIC was observed at the lowest concentration of AMPs which showed a 10% reduction in the absorbance compared to the untreated after 24- hour incubation.”

A 10% reduction is not sufficient in my opinion to indicate inhibition. I’m happy to reconsider if literature evidence can be shown to demonstrate this is a valid measure. I advise re-analysis of the data and use of either 50% reduction or better 90% reduction as the measure of inhibition. The paper should not be accepted with MIC as a 10% reduction.

5.4.2.5 Please explain exactly how the time to kill curve was performed, first I don’t see how A570 (as stated in the manuscript) can be used for this, unless there is a high inoculum and reduction in absorbance due to cell lysis is measured. Plating should be used, and the Sieuwerts method stated used plating- but how much medium was

used? I think the experiment states taking 24 x hourly readings from 0.1ml total volume.

An initial inoculum of approximately 10e5 CFU/ml was used in each well (three wells per concentration of antimicrobial agents per time point) and the growth was monitored throughout the experiment. In order to determine viability of the bacteria at each time point, samples were plated onto nutrient agar concurrent with the microplate readings. Moreover, every time a well was used for plating, it will be discarded as the reduced volume will affect the readings and bacterial count at subsequent time points. The “Time-kill assay” section has been revised to improve clarity (Lines 171- 183).

Ok. This is clearer, but further clarity is needed re what is meant by triplicate. Three technical replicates at the same time (not replicated) or three biological replicates (performed on different occasions).

Thank you for your suggestion. We have edited “triplicates” to “Three technical replicates were performed on three different occasions” to relay the work done. (Lines 176-177, 189-190)

OK

5.5 Results.

5.5.1 Preliminary screening.

Figure 1 is not needed; the information is in the text. For TP-1 83/100 strains were

susceptible, but this was calculated at 83.33% (it is 83%). The use of 100.00% also is 2 too many decimal places. Please also indicate here or in the methods the criteria for classification as sensitive.

Thank you for pointing out the mistake made. Figure 1 has been deleted as suggested and the numbering of figures have been changed. The decimal places have also been amended. Criteria for classification of sensitive has been included in the methods as “Isolates were categorized as “sensitive” when no growth was observed after 24-hour incubation on NA.” (Lines 149-150).

Ok, please indicate the definition of the growth/no growth boundary.

We have amended lines 155-157 to include the definition of the growth/no growth boundary,” Isolates were categorized as “sensitive” when no growth

was observed and categorized as “resistant” if there one or more colonies grown after 24-hour incubation on NA.”

OK.

- 5.5.2 No comparison of AMP activity profiles is made to any data that might be available for the isolates tested (e.g. API20 NE profile, clinical presentations, antibiotic sensitivity profile). This should be done, even if there is no correlation observed, and this may only be possible for TP1 where there are sensitive and insensitive isolates.

We did not observe any correlation between the AMP activity profiles and the antibiotic susceptibility data. For your reference, we have included the AMP profile and antibiotic susceptibility of the tested strains were included in the supplementary file 1 and 2. Furthermore, we have briefly stated in the discussion (Lines 467-471) that “In general, the activity of the AMPs was not affected by the antimicrobial susceptibility of the *B. pseudomallei* isolates. A similar observation was reported by Mataraci & Dosler (2012) where their tested strain, methicillin-resistant *S. aureus* (MRSA) ATCC 43300 were susceptible to AMPs indolicidin and cecropin (1-7)–melittin A (2-9) amide (CAMA).”(Lines 467-471)

ok

- 5.5.3 Please justify selection of TP1 as the AMP chosen for further study, LL37 and PG1 hit all the isolates and are more potent (lower MIC/MBC).

Kindly refer to point 2.1.

ok

- 5.5.4 Time-kill assay

- 5.5.4.1 The section repeats the results description, please rewrite to be more concise.

Time-kill assay results have been revised for better clarity. (Lines 310-318)

ok

5.5.4.2 Figure 2 is of concern, the untreated reaches a concentration of 10^{12} or 10^{13} cfu/ml, and what the methods suggest is in a microplate well. My experience is that this number of cfu may be obtained from a litre of bacteria grown overnight in a rich medium, not per ml in 0.8x RPMI in a microplate. I suggest checking calculations and if they are correct then the method used has some deficiency or artefact. I am happy with the data being used for time to kill (with limits of detection) based on those platings that do not grow bacteria, but not with the enumeration of cfu, which is clearly incorrect.

The CFU/ml of bacteria that we have obtained from our study is possible to achieve and the results we obtained are reproducible, based on the replicates that have been made. When supplied with rich media (i.e. LB) *B. pseudomallei* K96243 was able to continue growing even in 96-microplate wells after 24-hours. Naturally, the number of cfu will exceed 10^{13} . In order to address the concern, the experiment was repeated with the same strain (no AMP exposure) in LB instead of RPMI. The growth actually reached to approximately 10^{15} CFU/ml. We have included the data (in a separate file) of the absorbance and CFU of K96423 culture in LB for reference. Moreover, using a 96 well plate, the growth of *B. pseudomallei* K96243 in a nutrient rich media was also reported higher than that of modified Vogel and Bonner medium (Anutrakunchai et al. 2015).

I do not believe this. I couldn't find any reference to CFU/ml of final culture in the paper cited.

Kindly refer to point 3.1.

Sorry, don't believe the result. See 3.1. For context a lab rat might weigh 100g, by analogy, you are telling us that you easily get 100kg rats (that is bigger than me) and in routine culture you get 10tonne rats. My judgement is that you are making an error somewhere that needs to be demonstrably fixed- i.e. with evidence, not just changing the numbers- before publication is considered.

5.5.4.3 The comparison with CAZ, a drug in clinical use against *B. pseudomallei* is a good control. Are the amounts CAZ used clinically relevant?

The amount of CAZ used was the same concentration as the commercially available CAZ discs available (30 µg/ml) (Kindly visit the webpage http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CT0412&c=UK&lang=EN for more information).

Ok

5.5.5 Anti-Biofilm activity

5.5.5.1 The presentation and interpretation of results needs to be thought about and redone. The first line states, the number of ... biofilm forming cells (7.84 log CFU/ml) was reduced by two fold (4.2 log CFU/ml) This is almost a 3 log reduction in CFU! (1000-fold reduction). I would also use CFU per biofilm in the assay well, not the concentration in the suspension released from the biofilm.

The error in calculation for this experiment has been amended. CFU/biofilm was used (amended in the figures) instead of CFU/ml as we were enumerating the number of CFUs from the biofilm in each well. Thank you for your suggestion.

ok

5.5.5.2 In figure 3 DJK5 is *'d with a significant effect, but the graph does not support this. If there is a reduction it is very small.

The error in figure 3 has been amended. There was no significant reduction observed.

ok

5.5.5.3 An important set of findings here concern LL37 and PG1, and CAZ and MRP. LL37 is not active against the biofilm, while PG1 appears more active than TP1, and should be discussed regarding the choice of AMP to study further.

Overall, TP1 was selected for further study based on the observation from the preliminary screening, before the other experiments were conducted (i.e. MIC, MBC, Time-kill, etc.). Both TP1 and PG1 were able to inhibit *B. pseudomallei*

in both planktonic and biofilm state. However, LL-37 exerted its antimicrobial activity only on planktonic *B. pseudomallei* cells but not on *B. pseudomallei* cells in the biofilm state. Although PG1 appears more active than TP1, Sim et al (2011) has already reported the susceptibility of *B. pseudomallei* to PG1. As per our knowledge, activity of TP1 on *B. pseudomallei* is yet to be reported. Therefore, we further investigated TP1 in order to ascertain if the data will contribute to the development of anti- *B. pseudomallei* agents. These points have been added to improve clarity (lines 445- 456; 473-477).

Ok. But as noted above the succinct justification should be given in the results at the moment of choice. Line 303.

Kindly refer to point 2.1.

OK.

- 5.5.5.4 CAZ and MRP, the classical antibiotics appear most active, this should be discussed and the experiment should be repeated with isolates resistant to these antibiotics to demonstrate benefit of the AMPs.

The manuscript has been revised appropriately. The activity of CAZ and MRP have been stated in the revised manuscript (Lines 522-524). We have observed that the activity of the AMPs were not affected by the antimicrobial susceptibility of the *B. pseudomallei* isolates, also suggested by Mataraci & Dosler (2012). (Lines 467-471).

ok

- 5.5.5.5 Fig 4 should be discussed with correlation to planktonic MIC/MBCs, MIC planktonic is 221 microM, maximum anti-biofilm effect is seen at 442 microM.

Thank you for pointing that out. Figure 3 (previously figure 4) has been discussed accordingly (Lines 501-504), “This observation was similar to

Anutrakunchai et al. (2015) where the drug susceptibilities of *B. pseudomallei* biofilm were much higher than those of planktonic cells.” They have also stated that *B. pseudomallei* in biofilm state have reduced growth and metabolic activity where most antibiotics exhibit their effect on actively dividing cells.

Ok. But the * and statistical differences between treatments is unclear. Which treatments are different? There seems to be two sets of comparisons but no explanation as to what is being compared.

We have added the sentence “There was a significant decrease in the number of cells when exposed to all the TP1 concentrations as compared to the untreated cells (0 μ M)” at lines 369-371 to state the significance of the treatments.

OK, noting test used is also stated.

5.5.5.6 Also looking at the anti-biofilm effect here the limit of detection (LoD) of the test should be indicated, I wonder why the graph plateaus 1×10^4 CFU per ml? How many colonies on the plate does this correspond too? And are we at the LoD?

The number of colonies on the plate in each replicate was about 10 and slightly lesser as the TP1 concentration increase. Thank you for pointing out such an important point. Yes, we are at the limits of detection for the bacterial growth on NA (which explains why the graph plateaus at 1×10^4 CFU/biofilm). We have added the indication of LoD of the anti-biofilm and the time-kill experiments in the discussion (lines 524-529).

ok

5.5.5.7 Fig 5 looks at the key treatments, but values differ from Fig 3. The legends really need to state the concentrations of each antibiotic and AMP used. The figure legends for all the figures have been updated with additional information (i.e., concentration) to make them stand alone.

We apologize for the errors in data presentation and have amended accordingly. The figure legends have been included with additional information to make it clear and concise (refer to figure legends).

Ok,

5.5.5.8 In Fig 5 LL37 is *'d, but this is an increase in CFUs and should be highlighted as such. I would recommend highlighting (and testing for) the treatments that reduce bacterial numbers.

We apologize for the error. We have re-interpreted the results and found that there was no significant inhibition observed when the cells were exposed to LL-37.

ok

5.5.6 SEM

5.5.6.1 A small number of cells in one image per treatment is given. Only 2 cells are in

6A. If any conclusion is to be made on cell dimensions this needs to be on a substantial number of cells, e.g. 6 fields of view containing >50 cells and from three independent experiments. The data should then be graphed, and analysed with appropriate statistics.

Thank you for pointing that out. Our intention of carrying out SEM was to observe if the physical changes in the bacterial membrane after TP1 exposure. This observation will give us a clue to the mode of action of TP1 on *B. pseudomallei*. Therefore, we did not repeat the experiments.

Ok. SEM section improved.

5.5.6.2 Line 290. I'm not certain on the statement that cells are 5 micro m in length. In Fig 6B there look like pairs of cells that have divided, but are still attached, and here the pair is not 5 micro m in length. It may be that complimentary techniques, e.g. using a membrane stain and fluorescence microscopy, is needed to confirm this.

We have amended the section as best as we could to support our findings.

Ok. SEM section improved.

5.5.6.3 Line 290. Where there are references made to specific features in an SEM image these should be indicated with e.g. an arrow.

We have added yellow arrows in the images to indicate the specific features as requested. Therefore, the description of Fig 5 (Previously Fig 6) was expanded.

ok

5.5.6.4 Figs 6B and D. Is the “debris”, debris of killed cells and are they blisters and

bubbles on the cells? Or maybe it is aggregating protein? Perhaps an image set of an AMP and a resistant isolate would help as a comparison? Overall, the interpretations from the SEM are not supported by the data presented.

“Debris” seen in the SEM images are mixtures of dead cells, and aggregated proteins. Blisters are bubbles can only be seen on intact cells or cells which were undergoing membrane blebbing due to TP1 exposure. At the positions indicated by red arrows, there were no signs of intact cells or cells which were undergoing membrane blebbing due to TP1 exposure. By amending as much as we could, we hope the interpretations will support the data presented. In our opinion, we believe that an isolate resistant to TP1 will only have a few blisters and dimples on the bacterial membrane but not enough to disrupt the membrane integrity. Some cells may not have any changes in the membrane structure and will look similar to the bacteria cells before the exposure of TP1.

ok

5.5.7 Cytotoxicity.

5.5.7.1 *In vitro* toxicity testing with a range of cell lines suggests that TP1 is more toxic to mammalian cells than *B. pseudomallei*. What does this mean for the therapeutic use of TP1, as alternative therapies are the proposed aim of the study?

At the moment, TP1 is not suitable for therapy due to its *in vitro* cytotoxicity unless certain modification was done (i.e. to reduce non-specific binding). As more data is needed to modify the AMP, we believe that our findings will contribute to the existing literature in order for it to specifically target either bacteria cells or cancer cells.

Ok, should be in the discussion.

5.5.7.2 Perhaps this should also be compared to LL37 and PG1, where there is data in the literature already published showing at least for LL37, and it is more toxic to bacterial cells than mammalian cells. The discussion (e.g. section beginning Line 465) considers action as an anti-cancer agent, which is an aside that does not address the key findings that suggest TP1 will be toxic to human cells at doses that

are anti-bacterial and anti-biofilm. Armed with these toxicity results, would an animal or human ethics committee approve animal experiments or human trials? I think the answer would be no.

The justification for TP1 cytotoxicity was added to the discussion (Line 553-557) where modification is needed to specify its binding target. Regarding the usage and cytotoxicity effect of LL-37 and PG1, kindly refer to the discussion, lines 557-563.

Ok, but isn't this also one of the reasons to perform the *in silico* experiments and should be mentioned as such?

At the moment, PDB structures of A549, AGS, and HEPG2 are yet to be reported. In order to carry out molecular docking, we need the PDB structures of the target protein, peptide molecule structures and a docking program. Therefore, we did not carry out *in silico* study to further justify our cytotoxicity observation.

OK, can't say I am convinced of the why, but not my area of expertise.

5.5.8 *In silico* molecular docking.

5.5.8.1 This is not my area of expertise, and I am not qualified to comment on the specific methodology. The actual proteins interacting with TP1 are not clearly identified. I would be more overt in reference to Figure 3. I feel this table would also benefit from predicted or known cellular location of the target protein (i.e. are they surface exposed).

Thank you for highlighting this issue. We have moved the list for the potential *B. pseudomallei* protein structure and possible interaction from molecular docking in supplementary 6 and 7. We have revised the *in silico* strategy to include the potential binding site the common peptide or inhibitor binding targets for lipopolysaccharide (LPS) of *Escherichia coli*, and *Streptococcus pneumoniae*. First, we carried out docking based on Kushibiki et. al (2012) where TP1 binds to the lipopolysaccharide (LPS) of *E. coli*. Secondly, we carried out docking on autolysin, pneumolysin, and pspA which were the virulence factors for *S. pneumoniae* according to previous paper by Le et al, 2015. Next, the *E. coli* and *S. pneumoniae* molecule sequences were subjected to BLAST against *B. pseudomallei* sequence database. After that, homology modelling was carried out on the *B. pseudomallei* sequence obtained from

BLAST and then another round of docking was performed to visualize the interaction of TP1 and the model protein.

Ok, but in the article please write PspA (as you are referring to the protein) and not pspA.

Thank you for pointing that out. We have amended pspA to PspA throughout the manuscript.

OK

- 5.5.8.2 I did struggle to see the strategy justifying the *in silico* experiments, especially as this could easily be tailored to the design of an anti-melioidosis agent that is potent against *B. pseudomallei* while not affecting human cells at therapeutic concentrations. In addition, the relevance of AMP binding to proteins is only addressed at the end of the discussion, as possibly a route to aiding the clearance of bacterial lysis products. So, again I wonder if the strategy is viable and would like to see this clearly explained.

Please refer to the above response (5.5.8.1). We have revised the manuscript and included common binding targets in the revised section on “Possible TP1 interactions with protein targets from *in silico* molecular docking study (Lines 240-285; 372-435; 564-612).

Ok, this will also be supported by greater detail in the justification of using *in silico* approach, eg 5.3. But I am not truly convinced.

The results section dealing with the *in silico* experiments is essentially a data dump, and impenetrable to this non-expert. To be relevant to this article a hypothesis-driven, logically argued presentation of results, highlighting the specific problem under investigation (i.e. understanding the interaction of TP1 with target cells to shortcut chemical modifications to improve potency and reduce toxicity to human cells?), the hypothesis under test using the *in silico* experiments, the aim of these experiments, then the results themselves, then the analysis- and particularly with some interpretation of what the ranges of interaction energies indicative of a functional interaction between TP1 and bacterial surface component would be.

We have amended the *in silico* study to the best of our abilities, adding additional information and justification to help the readers to comprehend

our study. Kindly refer to the manuscript (Lines 293-306) for the revised version.

OK

Two points need justification:

- i) The paper is concerned with *Burkholderia pseudomallei*, but in silico work involves both *Escherichia coli* and *Streptococcus pneumoniae*, and so the reasoning here needs to be clearly explained and limitations discussed. For *E. coli* interactions with LPS are described, further clarity is needed as O-antigen portions (the PS) between *E. coli* and *B. pseudomallei* are likely to be different. *S. pneumoniae* is Gram-positive.

We have chosen *E. coli* for molecular docking study based on 2 reasons:

1. The interaction of TP1 and *E. coli* has been reported by Kushibiki et al, 2014 where the interaction will be a reference for the docking methods used in the *in silico* study.
2. We have used LPS model from *E. coli* to predict the interaction with *B. pseudomallei* as they are both Gram negative bacteria. We are aware of the limitations of using *E. coli* and have included the points in lines 614-623 of the discussion. Better LPS model of *B. pseudomallei* may be further explored.

We have selected *Streptococcus pneumoniae* for molecular docking for a few reasons: -

1. TP1 was reported to bind to both Gram negative and Gram positive bacteria (Imura et al. 2007; Ohta et al. 1992).
2. TP1 binds to autolysin, pneumolysin, and PspA (at the binding sites; referenced from Le et al, 2015) with negative interaction energy, indicated a strong interaction with the molecules.
3. When BLAST was carried out on all the 3 structures against *B. pseudomallei* protein sequence database, only the PspA sequence resulted in YD repeat-containing protein (accession: CFU00865).
4. There are similarities in the proteins between the Gram positive and Gram negative bacteria. In a study by Alloing et al. (1990), *S. pneumoniae* Ami proteins exhibit homology with components of the oligopeptide permeases

of *Salmonella typhimurium* and *E. coli*. In addition, autolysin exists in the peptidoglycan bacterial cell walls, which applies to both Gram positive and Gram negative bacteria (Beveridge 1999).

Taking into consideration of the above points, we have carried out homology modelling of the *B. pseudomallei* protein using PspA as a template. The validity of our model has been verified and shown satisfactory results based on Verify 3D, ERRAT and PROCHECK. We have also added additional justification at lines 293-306.

- ii) Interactions are described which what I believe are secreted proteins (e.g. pneumolysin), the cellular location should be considered when interpreting the data. We have added the following statement in the discussion, “At the moment, the exact role of the YDP of *B. pseudomallei* has yet to be reported. However, from the verification of the homology modelled *B. pseudomallei* protein based on PspA, and the membrane blebbing observed in the SEM analysis., we hypothesize that the YDP may contribute to a surface protein on *B. pseudomallei* where it interacted with TP1 molecules.” (Lines 617-621).

As a final point, I found the discussion an overlong rehash of results with added discussion that often tries to answer original comments, but which needed to come earlier. The discussion is not the place to justify an approach, but is the place to discuss the impact of a particular approach. I think the discussion needs more thought and less words.

We have amended the discussion to the best of our abilities and with your suggestions.

OK