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.

4. This, and the toxicity of TP1 also leave me feeling somewhat mislead 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.

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 DM_3 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.

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.

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.

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.

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.

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.

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 permeablise 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 µg/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.

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.

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%?

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).

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.

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 10e12 or 10e13 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 to CFU/ml of final culture in the paper cited.

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.

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.

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 1x10e4 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 x 10e4 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 it's *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?

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.

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.

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.

ii) Interactions are described which what I believe are secreted proteins (e.g. pneumolysin), the cellular location should be considered when interpreting the data.

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.