Author's response to reviews

Title: Kinetics of mycolactone in human subcutaneous tissue during antibiotic therapy for Mycobacterium ulcerans disease.

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Author's response to reviews: see over
22/02/2014
The Editor,
BMC Infectious Diseases
Dear Editor,

We are most grateful for the attention given our manuscript and the Reviewers who all agreed that our manuscript would be a useful addition to scientific knowledge on Buruli ulcer disease.

Below is a point-by-point response to each of the comments raised by reviewers.

Thank you very much and we hope to learn more through this review process.

Sincerely,
Dr. Fred Stephen Sarfo

Reviewer Comments

Reviewer 1
This study evaluates the response to antibiotic therapy of Buruli ulcer patients in Ghana in terms of the concentration of the mycolactone toxin. Antibiotic therapy reduces the bacterial burden and there seems to be no difference between patients treated with rifampicin and streptomycin (RS) for 8 weeks and patients treated with RS for 2 weeks followed by 6 weeks of rifampin and clarithromycin (RC). Whether this is also true for mycolactone is unstated in the paper but appears to be likely.

1. The authors should determine whether slow healers were more often on one regimen or the other.

Response: There was no significant difference in median time to healing and rate of healing between the patients treated with rifampin and streptomycin (RS) for 8 weeks and patients treated with RS for 2 weeks followed by 6 weeks of rifampin and clarithromycin (RC). A sentence to this effect has been included in the results section and it is discussed. No significant differences were noted in mycolactone kinetics between the two groups (Figure shown to reviewer below but not included in revised manuscript).
2. In the Discussion, the authors might underline the practical value of knowing that mycolactone may be higher in the centre of pre-ulcerative lesions for future studies evaluating mycolactone as a diagnostic marker for infection as well as in monitoring the response to therapy.

Response: We agree with the reviewer and have inserted a statement to this effect in the discussion of the revised manuscript.

3. Legend for Figure 3: Were the values used those obtained by mass spec or cytotoxicity?

Response: They were mass spectrometry values. This has been clarified in the figure legend in the revised manuscript.

4. In either case, there appear to be missing baseline values in comparison to figure 1.

Response: Figures 3B-D show subset analysis of lesion forms and categories (Fig. 3B category I lesions; Fig. 3C- nodular lesions; fig 3D-category I ulcers). Figure 3A is for all lesions.

Figure 3 graphs: For each category, there is one extreme outlier. The graphs would be more meaningful if the y-axis were presented on two segments as done in figure 1. For each graph, it should also be possible to use solid symbols for patients receiving 8 weeks RS and open symbols for those receiving 2RS+6RC. The symbols should also be larger.

Response: Symbols have been made larger and y-axis has been segmented for figures 3A-C. Please find below a graph of mycolactone concentrations before, during and after antibiotic therapy for RS8 and RS2RC6 groups. We have not included this figure in the manuscript because we felt it did not add much to the information presented.
Minor comments

Abstract:
Background, line 6: delete concentration

Done.

Methods, line 2-3: mycolactone concentration was

Done.

Conclusion, Line 3: may be useful

Done.

Methods:

Monitoring, line 4: twice weekly

Patients were monitored every 2 weeks (modified in revised manuscript).

Quantitative cytotoxicity, line 2: measures
Corrected:

Mass spec: define UFLC, UPLC acronyms

_UFLC and UPLC have been defined among list of acronyms and abbreviations at the bottom of revised manuscript according to format of BMC journal._

Use either rifampin or rifampicin throughout

_Rifampin has been used throughout._

Reviewer 2

The paper is generally well written, and seems to cite most of the relevant papers in the field though there seems to be some selective referencing.

1. This study involves human subjects and – according to current Good Clinical Practice standards- should be registered as such. As there is no mention of a registration number, and this reviewer could not find the study protocol on the web, the authors should try explain where it was registered, or alternatively, why this registration requirement was not met and why perhaps registration was omitted; perhaps it would be best to explain also, when the study was designed and conducted, and when the Ethics Committee approved the study, as this current demand was not necessary before 2007, and some Ethics Committees have only recently started to insist on abiding with the GCP rule.

Response: Ethical permission was given by the local ethics committee dated May 18th, 2009 for the present study. The Institutional Review Board did not require that we register the study and we were not aware of the emerging recommendation that all studies involving human subjects should be registered. Retrospective registration is not possible. The data presented in this study result from pursuing one of the study objectives of an ethically approved protocol as stated when the results were presented at the WHO Buruli ulcer meeting in 2011; this reference has been added to the manuscript.

2. As it was impossible to see the study protocol at the registration site, it would be helpful if reviewers were provided the text of this protocol in full.

Response: The protocol for the study is described in the methods section.

3. From the paper it is impossible to see how the sample size (number seems 80) of study participants was calculated; perhaps it was a convenience sample? Or was there a target number of adults in whom consecutive biopsies could be obtained? And if so, how many were deemed necessary to answer the primary study goal? On page 10, a median age (14 yrs) is mentioned but no breakdown of numbers of adults versus children is given; please provide these details or refer to the other paper in press for further details. From the actual number of observations however, with very few observations at week 6 and notably, week
12, it seems that this paper reflects proof-of-principle, or rather observational data alone, and not a study designed to really explore the question whether mycolactone measurement provides a biomarker to tailor treatment duration.

Response: As stated in the last sentence of the background section, this is a proof-of-principle study to evaluate the impact of antibiotic therapy on the kinetics of mycolactone within subcutaneous tissue in *M. ulcerans* infected lesions. There were no pre-existing data on which to base calculations of sample size. The small number of samples at weeks 6 and 12 were due to the ethical difficulty of taking punch biopsy samples from healing lesions as stated in the manuscript. The median age of patients is given as 14 with a range from 5-70 years; this in itself shows that the majority of patients were children.

4. As the primary goal of the study was to explore whether mycolactone could serve as a biomarker for response to therapy, how did the study team think about the validity of their model? In a recent paper by Converse et al (PLoS Negl Trop Dis 2014), infected mouse footpads were sacrificed, homogenised and assayed in whole-thereby securing that a well defined tissue concentration would truly correlate with total amount of mycolactone present in the lesion, with a well defined inoculum of *M. ulcerans*, and that sampling error could not be a confounding factor of tissue concentration found; in human lesions however, this problem cannot be solved- even with fairly large tissue volumes as obtained by 4mm (not 3mm; resulting in 1mL of tissue) punch biopsies. The authors should discuss this problem in the Discussion Section. It might be that e.g., the weak correlation between mycolactone concentration and time to healing (page 12 top) might partly be explained by this methodological problem.

Response: We agree with the reviewer and have included a statement in the discussion to reflect the fact that a biopsy taken from a lesion may not be reflective of mycolactone concentration within the entire lesion. However our findings will provide a useful guide to researchers interested in investigating this field in the future.

5. The authors mention that the mycolactone assay was described in detail in an ‘additional’ material file but this reviewer was unable to see this material. There have been rumours about difficulties with the extraction technique to reliably detect mycolactone in human tissue samples. In their recently published paper (Converse et al PNTD 2014) tissue samples were stored in 100% ethanol with no loss of signal during shipment; could the authors explain why their transport medium was either equally good, or should they perhaps admit that their protocol was potentially flawed in this respect? The entire discussion about discrepant mycolactone concentrations as measured by MSMS and cytotoxicity assays (page 13) might just be explained otherwise, e.g. by methodological flaws in assay, transport medium etc. Note that in the Converse paper, fluorescent thin layer chromatography had much lower detection level.

Response: The information in the additional file which reviewer could not access has now been incorporated into the main manuscript as requested by reviewer 3. The main methodological difference between our sampling technique and that by Converse et al is that whilst we immediately snap froze skin biopsies in liquid nitrogen in an amber coloured
tube to prevent photo-degradation of mycolactone, Converse et al harvested mouse footpads into 100% ethanol. The two approaches have not been compared so it is impossible to make a judgement as to which is better. Again we relied predominantly on mass spectrometry and cytotoxicity for mycolactone detection and quantification whereas Converse et al employed thin layer chromatography which is less sensitive as the reviewer correctly points out. Mass spectrometry can detect mycolactone in the order of 10 picogram Samples for cytotoxicity and for mass spectrometry were processed in the same way so this cannot account for the differences.

6. Initially two biopsies were taken- one to establish the diagnosis, and the other for mycolactone detection- see page 7, top. Further on the same page 7 the authors state that diagnostic confirmation was done by FNA-while initially no mention was made of FNA for the diagnosis in the first place. Perhaps the authors should explain in more detail WHICH procedures exactly were done in their study participants, and for which purpose; as PCR already confirms the diagnosis with a less invasive procedure than with 4mm biopsies, the protocol should have been crystal-clear about the necessity to perform these additional biopsies for diagnostic reasons; again reviewing the study protocol might be helpful for the reviewers and editors to elucidate these points. On page 11, ‘Mycolactone concentration during and after antibiotic therapy: further biopsies were taken at 6 and/or 12 weeks from patients whose lesions were healing slowly (Figures 2A and 2B)- here seems as if indeed, up to 6 (six) consecutive biopsies were taken in some study participants.

Response: An FNA was used to confirm the diagnosis using PCR for IS2404. One of the two punch biopsies collected was used to establish the diagnosis by microscopy for acid-alcohol fast bacilli and for quantitative culture whilst the other biopsy was used for measuring mycolactone concentration at baseline. These points have been clarified under the methods section.

7. It seems-from reference 18-that perhaps the study was primarily designed to address yet another question- i.e., whether streptomycin as used in the WHO standard treatment guideline could be reduced to 2 weeks- as 4 weeks had already been shown to be non-inferior to 8 weeks of streptomycin use (ref 21). The authors should provide the reference 18 for reviewers to be able to appreciate in full the work that they refer to.

Response: The emphasis of the present manuscript does not relate to the issue of using streptomycin for 2 weeks compared with 8 weeks. The authors do not wish to distract readers towards a separate clinical issue. Reference 18 has been provided to the reviewer. Please note that this reference is supplied as a confidential document to the editor because we could not find a way to attach this document.

8. In their discussion, data are really over-stretched and over-interpreted in that too many assumptions are piled. IF mycolactone kinetics are such that antimicrobial treatment stops production, then wash-out may take several weeks-6?12? and stating that mycolactone PRESENCE helps tailoring treatment is obviously incorrect. At best it might help understand antimicrobial treatment if treatment stops production-not presence-of mycolactone. IF their
assay would show arrest of mycolactone production—e.g. expression array of mycolactone synthase activity—their statement would make sense; now it does not.

Response: It is not our intention to over-interpret the data in our discussion and we have made some changes in the light of these comments. Although it is possible that mycolactone may persist in tissue after the organisms have been killed, there is no evidence that this is so at present, and our finding that there were viable organisms in some lesions after antibiotic treatment is against the idea. Our findings are the first to raise this issue and they are a reason for designing further experiments to investigate whether mycolactone continues to have significant biological effects after antibiotic treatment has been successful. This is why we consider our findings a useful contribution to literature.

Minor comments:

1. Background – line 3: why do authors mention mycolactone to be a novel polyketide molecule: are there older ones that this reviewer does not know about? Mycolactone was described 15 years ago so why should it be novel in the first place? If no reason can be given please omit the word: novel.

Response: The word ‘novel’ has been deleted.

2. Page 8 – diagnosis was based on….PCR by FNA? Or tissues biopsy quantitative culture? Or both?

Response: Diagnosis was primarily confirmed by PCR from samples obtained using FNA. Quantitative culture and microscopy were ancillary.

The authors use the words skin and tissue as if this is entirely exchangeable— but my guess is that tissue might be preferred as 1mL of tissue in a biopsy, undoubtedly, subcutaneous tissue will have been sampled which is probably actually to be preferred as typically most of the micro-organisms, as well as mycolactone may be present in subcutaneous fat rather than in skin.

Response: All punch biopsies contained skin as well as subcutaneous tissue both of which can be correctly referred to as tissue. However we agree with the reviewer that most of the mycolactone measured were in the subcutaneous tissue. We have modified text accordingly.

3. Page 9- top: …. Which measure.. consider rephrasing; that measures

Response: which measure has been changed to ‘that measures’

4. Page 11, top: There was a wide variation of concentration in all types of lesion with median (range) of 437ng/ml (136-2589; n=18) in nodules, 311ng/ml (148-834; n=14) in plaques, 443ng/ml (114-3020; n=45) in ulcers and 895ng/ml (457-2689; n=4) in oedematous lesions using the cytotoxicity assay (Figure 1A). If I add these numbers it is 81 (45 ulcers, 36-
non-ulcerated (plaque, nodule, edema) not 80; in table 1, not 45 but rather 44 ulcers are listed- please correct the text (or the table)

Response: the ulcers totalled 44 and not 45 as stated in the text. This has been corrected.

5. It seems that the Discussion Section overstretches the importance of the findings.

Response: This has already been alluded to under major comments by Reviewer.

6. It seems more logical to simply conclude that mycolactone detection seems to correlate with healing over time, and that perhaps future studies address whether the assay-provided, the threshold of detection is improved, such as one reported by Spangenberg, Kishi and Converse- could possibly help to monitor treatment and perhaps one day, tailor treatment duration?

Response: The threshold of detection in the Spangenberg reference is not lower than in our methods and the value of this methods and the value of this method, if it can be made to work consistently which is not currently the case, is that it ma be possible to use it in endemic countries. However we agree that our conclusion could be modified and this has been done.

Reviewer 3

Sarfo et al tackle an important question here, looking at the relationship between mycolactone levels, bacterial burden and disease state in different presentations of *M. ulcerans* infection.

I have a few issues that the authors should consider.

Major:

I would have liked to have seen substantially more detail on the establishment of mycolactone detection sensitivity for the cytotox assay and MS assays. These data should be included in the main body of the manuscript, rather than an additional file. In this vein, rather than just showing fitted curves for these experiments (Supp figure 1) it would be more informative to see the original data plotted too, including biological replicates or means of replicates with error spread indicators for these spiking experiments. Spiking different tissue samples would be particularly important to understand method variability. There was simply not enough information in the methods or results to assess how the spiking experiments were performed. At the moment it reads that only a single dose of mycolactone was spiked, but Supp Fig 1 shows dose response curves. Much more detail is needed in this section to give the reader an understanding of the variation in the extraction method used and the inherent variability of the assays used. Establishing the limit-of-detection for these methods developed together with an understanding of the variability of the assay is critical to enable sensible interpretation of the data from real clinical specimens. Once a more sophisticated assessment of the methods has been performed then the data from the real clinical specimens may need to be reinterpreted.
Response: We have incorporated details of methods and results of mycolactone quantification by cytotoxicity and mass spectrometry experiments. We have now shown both the fitted plots and raw plots of replicates with SEM of dose-response between mycolactone and cytotoxicity as supplementary figures 1A and B. As expected, there was variation in dose-response at each spiking concentration of mycolactone, hence we ran all samples in triplicate and the average of those triplicates was taken as the concentration of mycolactone in that sample.

We spiked 4mm skin biopsies with 1ug of mycolactone followed by extraction and quantification by both cytotoxicity assay and mass spectrometry to determine recovery following extraction. Three (3) independent experiments were done and the average recovery of mycolactone using cytotoxicity was between 64-100% and mass spectrometry was between 11-18%. This information has now been included in the main text.

Minor:

1. Abstract: We sought to measure the concentration of mycolactone concentration within lesion”… to many “concentrations”
   
   Response: corrected.

2. Why use both Pearson and Spearman correlation analysis? Spearman rho correlation coefficients were not cited in the ms. Were the data normally distributed? Perhaps use Spearmann instead of Pearson as the former is a non-parametric test, based on rank and does not assume normality.

   Response: We have used Pearson correlation analysis because the data distribution of mycolactone measured using mass spectrometry followed a normal distribution. We have deleted Spearman rho from the manuscript as suggested.

3. Background: “Variations in the side chains can give rise to small differences among the family of mycolactones”. Meaning not clear here.

   Response: We mean to say that variation in the structure of the polyketide side chain is responsible for differences in potency/virulence of mycolactone congeners. This has been rectified.

4. Monitoring the clinical response to antibiotic treatment: “The maximum diameter and the diameter at right angles were measured and the average diameter was used”. I assume it's the lesion but need to make clear.

   Response: we have inserted the phrase ‘the maximum diameter of the lesion’.... to make the sentence more intelligible.

5. Eighty patients with clinically confirmed Mu were all PCR positive. One hundred percent clinically confirmed correct cases is unusually high. Would it be more accurate to say 80 patients with laboratory-confirmed (IS2404 PCR) were selected for the study?
Response: The sentence in Subjects and Samples has been modified to reflect the fact that only PCR confirmed cases were selected for this study.