Author's response to reviews

Title: Oral intake of Phenylbutyrate and vitamin D3 upregulates the Cathelicidin LL-37 in human macrophages: A dose finding study for treatment of tuberculosis

Authors:

Akhirunnesa Mily (mily@icddrb.org)
Rokeya Sultana Rekha (rokeya.sultana.rekha@ki.se)
SM Mostafa Kamal (ntrlnidch@yahoo.com)
Evana Akhtar (evana@icddrb.org)
Protim Sarker (protim@icddrb.org)
Zeaur Rahim (zeaur@icddrb.org)
Gudmundur H. Gudmundsson (ghrafn@hi.is)
Birgitta Agerbeth (birgitta.agerberth@ki.se)
Rubhana Raqib (rubhana@icddrb.org)

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Author's response to reviews: see over
We thank the reviewers for the very useful comments that have markedly improved the manuscript. Please find below point by point response to the reviewers’ comments

**Response to reviewer:**

**Reviewer 1:**

1. There are several important studies on the mechanisms of the vitamin D-cathelicidin-induced antimicrobial effects against mycobacteria, including Liu et al. (2006, 2007) through direct killing effects and Yuk et al. (Cell Host Microbe, 2009) and Campbell et al. (PLoS Pathogens, 2012) through antibacterial autophagy activation. Although the authors cited Liu paper, more discussion will nicely support the background of their clinical trial study.

**Response:** As suggested we have now added further description and more references in the discussion part (highlighted with yellow color) which increases the strength of the background for the clinical trial study.

2. Were there any side effects on the liver function in the volunteers participated in the study? For example, the AST/ALT ratio or other lab data should be shown in the treated subjects.

**Response:** None of the volunteers reported any notable side effects except for feeling excessive thirst and mild drowsiness by two volunteers. We have in addition, assessed liver and kidney functions of the volunteers during the study period by measuring SGPT and creatinine levels, respectively. None of the volunteers showed any increase in SGPT or creatinine levels after ingestion of phenylbutyrate (PB) alone or in combination with vitamin D₃ on day-4 or 8. One male volunteer in Group-I at day-0 had 66.49 IU/L of SGPT levels that was above the normal upper range of 56 IU/L. His SGPT levels declined on day-4 to normal levels (12.08 IU/L). A table has now been added as suggested by the Reviewer showing the SGPT and creatinine levels.

It is worth mentioning here that Phenylbutyrate is a FDA approved drug for urea cycle disorder and for the treatment of this disease much higher doses of Phenylbutyrate are used (100-200 mg PB/kg body weight/day).

3. The statistics and p-value should be clarified among groups in the figures.

**Response:** p-values are now added into the figures. The statistics are now clarified into the figure legend.

4. The reason why the group who intake PB 500 mg (neither high nor low dose) showed significance should be discussed.
Response: The 250 mg twice daily (b.d.) dose of PB with 5000 IU vitamin D3 once daily (o.d.) also induced LL-37 transcripts, but unlike the 500 mg b.d., did not result in upregulation of LL-37 expression. However, with the higher dose of PB (1g b.d.) a suppression of the transcriptional expression of LL-37 appeared, but increased 4 days after the PB supplementation (at day 8). The dose of PB 500 mg b.d. (1000 mg/day) of the adult volunteers was close to the concentration (600 mg for a 60 or 55 kg adult, twice daily i.e. 1200 mg/day; 10mg/kg/day) calculated by allometric scaling from the dose used in rabbits (25.54 mg/kg); this dose induced cathelicidin in rabbit mucosal epithelial cells. Butyrate is known to inhibit RNA and protein synthesis at high concentrations (de Haan 1986; Soliman M 2007; 2011). It is possible that in a similar fashion PB at high doses is inhibitory to the expression of LL-37 both at transcriptional and translational levels.

5. The authors need to be careful and clarify the terms ‘vitamin D3’, ‘25(OH)D3’, or ‘1,25(OH)D3’ throughout the paper.

Response: Thank you for pointing this out. We have checked all the terms throughout the paper and for more clarification we refer to vitamin D3 as the inactive form of vitamin D that is mainly produce by the body during sun exposure, in our study participants were supplemented with this form of vitamin D. 25-hydroxyvitamin D3 is the intermediate form, we have measured this form of vitamin D in plasma. The active form of vitamin D is 1,25-dihydroxyvitamin D3, that is the functional form for cellular function.

Reviewer 2:
Major Compulsory Revisions
1. The results of bactericidal activity in the model of MTB infection are clear however the authors did not include a control group of healthy subjects without any intervention (No PB no vitamin D) I think if the authors have this results it should be included in the manuscript and make the analysis against this group.

Response: We would like to point out here that the bactericidal activity results of day-0 (pre-dosing) serve as the control for all groups during the intervention, hence no separate control group was used.

2. Other recommendation is that probably the results could be more reliable if in the bactericidal assay the authors use lower rate of infection 5:1 instance to multiplicity of infection (MOI) of 25:1.

Response: Before starting the actual study, we optimized the bactericidal assay using various MOI- 10:1, 25:1, 50:1, 100:1 and 1000:1. We found that 25:1 or 50:1 gave better result than lower MOI which gave zero values. A Table is given below to show the differences.
MOI 10:1   MOI 25:1   MOI 50:1   MOI 100:1   MOI 1000:1
CFU       CFU       CFU       CFU       CFU
5         16        41        25        100
2         45        45        88        750
0         23        24        44        300
3         12        56        46        336
0         8         15        580       1000
2.0 ± 0.9 20.8 ± 6.5 36.2 ± 7.4 156.6 ± 105.2 497.2 ± 163.9

Other authors have also used higher MOI such as 50:1 e.g. Welin et al, 2011; Henao et al 2007; Cappelli et al 2001.

Minor Essential Revisions

3. Only 500 mgs of PB with vitamin D were effective and 500 mgs twice a day did not demonstrate this effect. It could be interesting to discuss for the authors why this administration did not work as well as just 500 mgs.

Response:

The active form of vitamin D₃ (1,25-dihydroxyvitamin D₃) is a potential inducer of antimicrobial peptide (Liu et al, 2006/2007). Our previous in vitro study has shown that PB alone can induce LL-37 expression in lung epithelial cell line and costimulation with 1,25-dihydroxyvitamin D₃ further boosts the expression to several folds in a synergistic fashion. In the present in vivo study we have found that oral intake of PB alone (500 mg b.d.) induces LL-37 mRNA (about 3 fold induction, but not statistically significant). However, intake of PB (500 mg b.d.) with vitamin D₃ (5000 IU o.d.) shows a synergistic effect in inducing LL-37 expression on both mRNA and peptide levels. We do not know the molecular mechanism behind this induction. PB is a histone deacetylase inhibitor, that induces hyperacetylation of chromatin leading to changes in gene expression. Our hypothesis is that vitamin D₃ may boost this effect in combination with PB.

4. It is interesting that they detected LL37 in lymphocytes besides to MDM, There very few information in the literature about that and deserve some comments in the manuscript.

Response: Various cell types including T cells are known to express LL-37 (Agerberth B 2000; Kin NW 2011). However, to our knowledge this is the first study to report enhanced in vivo induction of LL-37 in lymphocytes after oral supplementation with PB alone or in combination with vitamin D₃. Intracellular conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D₃ takes place in both macrophages and T cells (Fabri-Modlin 2011) that may control the intracellular level of 1,25-dihydroxyvitamin D₃ for immunomodulatory responses. Thus, the finding of the induction of LL-37 in lymphocytes with PB plus vitamin D3 underscores its significance in TB infection since T lymphocytes are very important cell of the cell-mediated host defense against tuberculosis.
Reviewer 1 & 2:

Statistical review: I do not feel adequately qualified to assess the statistics.

Response: Expression of LL-37 peptide or transcripts over time between different groups was analyzed by two-way (treatment and time) repeated measures ANOVA; post-hoc test (Holm-Sidak test) was conducted when the interactions between group-time were significant. Differences within group over time were analyzed by one-way ANOVA. ANOVA on ranks was applied when results were not normally distributed. P-values ≤ 0.05 were considered significant.

We have addressed all the comments raised by the Reviewers and have made all the modifications as suggested. We hope that the MS will now be accepted in BMC Pulmonary Medicine.

Thank you

Yours Sincerely,

Rubhana Raqib
Scientist
Nutritional Biochemistry Laboratory
Center for Vaccine Sciences
International Centre for Diarrhoeal Disease Research, Bangladesh (icddr, b)
Phone- 880-2-9840523-32, Ext-2404
Fax: +880-28823116 / +880-28812529
Email: rubhana@icddrb.org