Author’s response to reviews

Title: Tomatidine and analog FC04-100 possess bactericidal activities against Listeria, Bacillus and Staphylococcus spp

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The authors response letter has also been included as a supplementary file and would be easier to read in our opinion.

RESPONSES TO REVIEWERS

PHAT-D-17-00039

Old title: Tomatidine and analog FC04-100 possess bactericidal activities against the small colony variants of some Firmicutes including those of Listeria monocytogenes and are bactericidal in combination with aminoglycosides against their normal phenotype

New title: Tomatidine and analog FC04-100 possess bactericidal activities against Listeria, Bacillus and Staphylococcus spp.
REVIEWERS’ COMMENTS AND RESPONSES

Gian Maria Rossolini (Reviewer 1):

This submission reports on an extended characterization of the antibacterial activity of Tomatidine, a natural product from plants, against normal phenotypes and SCV of some Gram-positive species belonging to the Firmicutes, and on characterization of FC04-100, a modified tomatidine derivative, along the same line.

Activity against SCV of some species (e.g. staphylococci) can be an interesting feature given their role in some persistent infections. The relevance of activity against other species (e.g. Bacillus) remains less clear, but those results support a more general activity of these compounds and therefore could be worthy of reporting.

The paper is overall well written, but some issues should be considered for manuscript improvement, as detailed below.

1. Title: the reason for mentioning Listeria as outstanding species is unclear; it was one of the several species tested, but most additional experiments were carried out with staphylococci.

   ANSWER: the title was modified to be more inclusive/general

2. L. 37-50: this part of the introduction could be condensed by reducing the taxonomic details.

   ANSWER: The introduction now starts directly by a description of the Bacillales.

3. L. 54: here you might wish to mention the importance of MRSA also in veterinary settings (also considering the focus of the paper).

   ANSWER: The reference to LA-MRSA was added.
4. L. 149 and ref. 24: ref 24 does not seem appropriate; the correct CLSI reference for broth microdilution methodology is M07-A10 (2015).

ANSWER: The reference was corrected.

5. L. 222-224: the dosages and mode of administration of tomatidine and FC04-100 should be better described here.

ANSWER: The in vivo model of infection was removed from the manuscript as suggested by this Reviewer (comment #10 below).


ANSWER: The expression “low reversion rate” was removed and replaced by “were stable”. The sentence now reads “The SCV isolates selected for the rest of the study were stable and kept their phenotype without a GEN selection pressure”.

7. Results, text and Table 1: the lowest MIC values should be determined and reported (<0.03 is not precise).

8. Results, MIC values (Tables 1 and 2): presentation of the MIC data of tomatidine and FC against various strains, alone and in combination with GEN, could be condensed and streamlined; these data could probably be easier to follow if presented in a single Table and discussed in a single subsection of results.

ANSWER: The MIC tests were redone to determine the precise MICs. In this instance, the MIC was found to be 0.03 ug/mL. Also, Tables 1 and 2 were combined and additional MICs were obtained to unify the tables. The results are now discussed together in a single subsection of results.

9. Results, kill kinetics: as mentioned for MIC testing, even in this case an effort should be done at presenting together data for bactericidal activity of tomatidine and FC, rather than in separate sections, to facilitate comparative analysis and streamline the text.

ANSWER: The results are now discussed together in a single subsection of results.
10. Results: the significance of data from the pulmonary infection model appears to be quite preliminary; these data should probably deserve to be expanded (also in combination with antibiotics) and reported in a future publication.

ANSWER: We agree that the in vivo data does not fit well in this manuscript. This part of the manuscript was removed.

11. Authors contributions: what are the Xs?

ANSWER: Our manuscript version did not have the Xs. We will double check that section after transfer to the online submission platform.

12. Fig. 7 legend: per gland? Please clarify

ANSWER: The Figure of the in vivo data was removed.

Stefania Stefani (Reviewer 2):

The major problem of this paper is that it uses different assays with different samples of strains. The present data are not homogeneous, in this form, and difficult to follow.

My further advice is to shorten all sections (in particular Introduction, M&M and Discussion), and homogenize the data with a common aim.

ANSWER: These comments are similar to those of Reviewer #1 (see answers above). In general, the manuscript was shortened, Tables 1 and 2 were combined and the results are now discussed together in a single subsection of results to facilitate comparative analysis.

Background

Lines 37-54: too long general introduction. Reduce. ANSWER: The taxonomic details were removed.

Lines 84-85: this sentence is redundant. Delete. ANSWER: Done.
Line 64: ref. 9 refers to P. aeruginosa, and not to S. aureus. ANSWER: It is true that the mode of entry of aminoglycosides was first demonstrated in P. aeruginosa but we are taking here of the antibiotic mode of action not the bacteria. This is the original article.

Fig. 1: eliminate. ANSWER: We do not understand how showing the structure of the FC04-100 analogs is a problem for the manuscript.

Methods

Most of the methods used in this study are already published. Shorten this section and provide only the related ref. ANSWER: The in vivo experiments were removed from the manuscript. When similar methods were published, the references were provided to recognize previous authors and their work but the additional details that were provided describe important nuances or distinctions.

Lines 110-111: provide reference for USA 100 and USA 300 MRSA strains used in this study. ANSWER: The source (ATCC) is now provided.

Page 9 - Invasion assay: provide the MOI (multiplicity of infection) used to infect Calu-3 cell line. ANSWER: The MOI is now indicated in the M&M section.

Lines 204-206: I do not understand the meaning of trypsinization before cell lysis, if the aim is to recover intracellular bacteria. It is unnecessary. ANSWER: Perhaps, but to be sure these polarized cells are lysed and that no small clumps remained we preferred to do it. It seems more reproducible this way in our hands.

Results

Lines 237-252: these results are expected. Delete this paragraph and fig. 2. ANSWER: Although well known for S. aureus, to our knowledge, only sporadic reports have described SCVs from
various species of Bacillus and from Listeria. It was critical here to show that the mutants and colonies selected on gentamicin were typical SCVs. This add credibility to the manuscript.

Lines 267-270: expected results. Eliminate. ANSWER: Yes expected, if true respiratory deficient SCVs but this had to be demonstrated and validated.

Line 270: In the result section, not report reference, if not necessary. ANSWER: The reference was removed.

Lines 272-274: Delete. Not in the aim of this study. ANSWER: Sure ok, this section was removed.

Lines 288-89: Delete. In the result section, not report reference, if not necessary. ANSWER: The sentence and reference were removed.

Lines 293-296 and figure 3: Why did not the authors show the time kill curve of SCVs in comparison? ANSWER: Figure 3D shows very explicitly the killing of SCVs. The detailed kill curves (Figures 3A, B, C) were necessary to better show the bactericidal synergy.

Lines 303-337. The paragraph "Bactericidal activity of the TO analog, FC04-100, against L. monocytogenes SCVs and synergy with aminoglycosides against prototypic strains and MRSA" joins two different aspects of this article and needs to be separate and totally re-worked. ANSWER: This section and the previous one were re-worked as suggested by Reviewer #1.

Lines 305-308: why did the authors analyze the bactericidal activity of FC04-100 only against L. monocytogenes ATCC 13932? ANSWER: We are not sure of the meaning of the question; the reason for the species or for the specific strain? We tested Listeria because Tomatidine showed no bactericidal activity and because Listeria is notoriously difficult to kill and we tested that strain because it is a reference strain.
Lines 328-337 and table 3: why did the authors analyze the activity of FC04-100 and its synergistic effect with aminoglycosides against prototypic strains of MRSA? ANSWER: MRSA strains are the most clinically important species of the Bacillales. They are often multi-resistant and deserved special attention. Combining an aminoglycoside and a β-lactam to FC04-100 offers a new alternative to combat these strains.

Lines 352-374: Also in this paragraph, the authors join experiments related to two different aspects, with different aims and performed on different strains. It is very hard to follow! ANSWER: The paragraph was divided. As specifically indicated in the M&M section strain SH1000 was used in the biofilm assay because it produces important amounts of biofilm, whereas infection of pulmonary cells was done with a cystic fibrosis relevant SCV (CF07-S).

Moreover, the authors did not detail the data obtained.

Lines 354-357 and 377-382: data were not well described (i.e FC04-100 can kill S. aureus; the activity of GEN was significantly improved; increasing doses of FC04-100 can significantly decrease the bactericidal counts in the lung). ANSWER: The results related to Figure 5 (formerly lines 354-357) and Figure 6 are now more detailed. The section related to the in vivo activity was removed (formerly lines 377-382).

Line 360: SH100…correct with SH1000. ANSWER: Done.

Lines 366-367: not in this study. Delete. ANSWER: Yes, in this study as well. Figure 6 shows the intracellular killing of S. aureus by both Tomatidine and FC04-100 done in parallel experiments. The sentence was clarified.

Fig. 6: the authors did not show the MOI used to infect Calu-3 cell line and they did not report the % of bacteria/host cells. ANSWER: The MOI is clearly indicated in the M&M section.

Lines 384-388: This sentence should be moved in the materials and methods section. Delete. ANSWER: Figure 7 (in vivo data) was removed from the suggestion of Reviewer #1.
Discussion

This section needs a revision.


This part is too long, with many repetitions and descriptions of already published studies, without any deduction and conclusion related to the present study. ANSWER: This part on the structure-activity relationship of Tomatidine (and analogs) was considerably shortened and only information relevant to this manuscript was provided with also a conclusion.

Lines 436-437: No conclusions reached. ANSWER: see answer to previous comment above.

Lines 458-461: Delete. Out of context and not related to the experiment of this study. The author did not analyze intracellular activity of L. monocytogenes in this study. Which is the correlation with S. aureus intracellular activity? ANSWER: This part was removed.

Lines 468-471: this aspect of the study was not described in detail (nor in the results and in the discussion section). Even the authors stated (lines 473-74) that this part needs further improvements. ANSWER: More details are now provided in the results section as mentioned in one of the responses above. Noteworthy, we did not state “that this part needs further improvements”. On the contrary, we meant that these very interesting intracellular and intra-biofilm activities should support future studies on this class of antibiotics! The sentence was modified for clarity.