nature portfolio

Peer Review File

A new class of natural anthelmintics targeting lipid metabolism

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

There is a lack of anthelmintics for human and veterinary use and an urgent need for new compounds with mechanisms of action that are distinct from existing classes. The authors performed a screen of 2,300 compounds in free-living models, identifying fatty acids from avocados as promising leads that cause both development defects and impair adult worm motility and viability. These compounds were then screened against various parasitic nematodes and found to kill worms in vitro, and there is in vivo evidence of their activity from data they reduce worm and egg burden of H. polygyrus infected mice. Genetic and biochemical experiments indicate that these compounds act to disrupt mitochondrial function and increase ROS through inhibition of fatty acid metabolism via POD-2 binding. I have two major comments and several minor comments below.

Major comments.

1. Please provide data on all compounds screened – not just the selected compounds in the supplemental table. This would be best provided as a table with SMILES IDs to convey structural identity of the compounds. The authors note that all compounds other than those screened in figure 1 were negative, but it is important to nevertheless provide the data as this information is valuable to the field to know what not to re-screen.

2. Unless limited by journal policy on figure number, I suggest POD-2 data be moved from supplemental fig ure13 to a main figure. This is key data to support the claim of the mechanism for this class of compounds and this would make it more accessible.

Minor Comments

1. Line 50 – when describing praziquantel, reword to remove ‘voltage gated’ and just state ‘calcium channels’ (understand older literature had implicated voltage-gated channels, more recent evidence indicates TRP channels, so it would be more accurate not to specify).

2. Paragraph lines 81 – 88 could be expanded to include more methodological detail. For example, the drug dose, treatment window, phenotypic endpoints measured.

3. In Figure 2 and 3, LD50 is provided in tables. It would be easier to compare these values between drug treatments if some measurement such as standard error or 95% confidence interval was added. Variation is expected, and I notice for example one panel reports N2 LD40 around 5 for avocadene acetate, then another 12.

Points 4-8 in the following comments refer to Figure 3.

4. Can the authors comment on why different compounds are used for different parasite experiments? E.g. Avo A on parasites, avocadene acetate on C. elegans.
5. It is noted in methods that numbers reflect three replicates, so perhaps it would be informative to put mean +/- st. error in the tables under percent lethality.
6. It is important to state male or female Brugia, since the different sexes have pronounced differences in drug response.
7. It is unclear on MF experiments – is this quantified motility, or qualitative scoring? For example, does 50% lethality at 4 micromolar mean 50% of worms were dead by eye (scoring 3000 different worms?), or that movement (or some other metric) was measured and reduced in the population by 50%?
8. In vivo mouse experiment – state the dose in mg/kg in legend under panel D.

9. In Figure 4 – are worms viable after paralysis in panel A and C (or not moving because dead?). If they are not viable, is that a reason why the OCR could be flat in D? Related to this point, different concentrations of drugs are used in different assays, and so if these could all be noted in the legend it be much easier to understand (e.g. concentration of drug used in panels E,F,G?).

10. Line 493 – state the concentration or percent of DMSO.

11. Supplemental figure 1 – in the legend, state how long was drug treatment.

12. Supplemental figure 8. Are there outcomes that are quantifiable from this data? I gather the Y axis is a composite of many phenotypes. But can this be broken up into quantifiable measurements of viability, movement, length/size, or fecundity?

Reviewer #2 (Remarks to the Author):

The Fahs et al manuscript describes a very thorough investigation of the potential utility of avocado fatty acids and alcohols against parasitic nematodes and its mechanism of action. I think it their discovery represents an important potential new tool in the battle against parasitic nematodes. I can easily imagine supplements to livestock food that might increase the health of the animals. The manuscript was truly a pleasure to read. I enthusiastically recommend its publication. I have no major issues with the paper and have only minor suggestions for improvement.

Lines 87-88: A little bit of an unusual cell line. Perhaps providing some rationale for the choice would be useful.

Paragraph beginning on line 89: I am curious to know whether there were structural analogs in the library that were not identified as hits? Were the two indicated in lines 117-118 part of the Spectrum library? Details about true negatives can help the reader better understand the important parts of the structure that confers activity, which comes up later in the manuscript with their docking experiments.

Figure 1a: the smaller pics below do not readily demonstrate the indicated phenotype. I tis not clear how a single shot can illustrate slow growth, sterility, uncoordination, and Emb (without showing dysmorphic embryos). Obviously, the details of some of these phenotypes are well laid out in Figure 2, which is really nice. However, since Fig 1is a nice opportunity to visually demonstrate the strength of the phenotype, perhaps illustrating the whole-well phenotype to compare to control would be more useful? Consider revising.

Lines 222-231: How do you control for death/dying impacting OCR?

Any reason not to include Sup Fig 7 among the main display items? Seems like an important observation that is central to the main conclusions.

Paragraph starting at 286: The authors suggest that their mitochondrial results led them to hypothesize that lipid metabolism might be targeted by the AFAs. OK, but there is literature already showing this to be true (see PMID: 26077472, 34397122, 34095930) (which they later discuss). The authors should consider working this information into the rationale of the POD-2 results section and perhaps introducing that information earlier (i.e., another rationale for looking a the mitochondrial function and morphology).

I am curious- is there any data showing that communities that grow and consume avocadoes have lower nematode burdens than those who do not? Probably not, but worth a gander in the literature.

Technical:
-First results section, including figures: concentrations used and the number of technical and/or biological repeats should be indicated.
-Consider adding p values to text stating statistical differences where none were provided in the text (for example, Lines, 225, 231, 268, 272 etc.).

A few more complements:
-Fig 1c, Fig 2- really nice analyses.
-Paragraph of line 129: Impressive.
-Lines 213-215: Impressive.
-Lines 147-148: Remarkable.

Abu Dhabi, September 17, 2024

Dear Nature Communications reviewers,

Thank you for reviewing our manuscript. We appreciate the time you have spent and are grateful for all the remarks that you have raised for improving it.

Kindly find below point-by-point responses to your comments (indicated in blue font). All corresponding modifications to the text are highlighted in yellow in the revised manuscript file. During the revision process, we also made a number of minor editorial changes (that do not change any of the data or conclusions), which are highlighted in cyan in the main text.

Reviewer #1 (Remarks to the Author):

There is a lack of anthelmintics for human and veterinary use and an urgent need for new compounds with mechanisms of action that are distinct from existing classes. The authors performed a screen of 2,300 compounds in free-living models, identifying fatty acids from avocados as promising leads that cause both development defects and impair adult worm motility and viability. These compounds were then screened against various parasitic nematodes and found to kill worms in vitro, and there is in vivo evidence of their activity from data they reduce worm and egg burden of H. polygyrus infected mice. Genetic and biochemical experiments indicate that these compounds act to disrupt mitochondrial function and increase ROS through inhibition of fatty acid metabolism via POD-2 binding. I have two major comments and several minor comments below.

Major comments.

1. Please provide data on all compounds screened – not just the selected compounds in the supplemental table. This would be best provided as a table with SMILES IDs to convey structural identity of the compounds. The authors note that all compounds other than those screened in figure 1 were negative, but it is important to nevertheless provide the data as this information is valuable to the field to know what not to re-screen.

Thank you for pointing this out. We agree that negative screen data is valuable. We have added the whole Spectrum library table with compound names, catalog numbers and chemical formulas in **Supplementary Table 1**.

2. Unless limited by journal policy on figure number, I suggest POD-2 data be moved

from supplemental figure13 to a main figure. This is key data to support the claim of the mechanism for this class of compounds and this would make it more accessible.

Thank you for asking for this change to make the manuscript more accessible to the readers. We have now added the most relevant panels from Supplementary Figure 13 and rearranged **Figure 5** accordingly to allow the readers more immediate access to these data.

Minor Comments

1. Line 50 – when describing praziquantel, reword to remove 'voltage gated' and just state 'calcium channels' (understand older literature had implicated voltage-gated channels, more recent evidence indicates TRP channels, so it would be more accurate not to specify).

Thank you for this suggested improvement. We have made the requested change.

2. Paragraph lines 81 – 88 could be expanded to include more methodological detail. For example, the drug dose, treatment window, phenotypic endpoints measured.

We appreciate and agree with making the methodological detail more accessible and comprehensive and thank you for pointing this out. The primary screen is described in more detail in the Materials and Methods section. We have also added more information in the Results section and, to make the phenotypes clearer, we amended the figures in **Figure 1a**.

3. In Figure 2 and 3, LD50 is provided in tables. It would be easier to compare these values between drug treatments if some measurement such as standard error or 95% confidence interval was added. Variation is expected, and I notice for example one panel reports N2 LD40 around 5 for avocadene acetate, then another 12.

We agree that adding SEM to the tables for **Figure 2 and 3** is an important addition and have added these whenever possible. Singlet experiments, due to parasite availability, have been clearly marked.

For **Figure 3c**, we have changed our calculations of the variance compared to the original submission to make things more consistent with practice for SEM calculations, leading to minor differences between this version and that in the original submission. That is, we determined the LD50 for each individual biological repeat separately, and then calculated the average LD50 and standard error. Previously, all the technical and biological replicates were combined.

Thank you also for pointing out that our submitted manuscript could be interpreted as having some slight inconsistency in the way we described the strength of the effects. While the LD50 values indeed do show some variability between experiments, they show a more pronounced difference at different larval stages. Specifically, in *C. elegans* N2 strain, avocadene acetate LD50 is 5µM (+-0.3) in L1, whereas it is 12.5µM (+-0.61)

in L4. We have revised the text to ensure we mention the larval stage used in each case to clarify.

Diverse AFA commercial sources as well as batches from the same company can also add to the variability which we dealt with by analyzing each batch purity by NMR and determining the LD50 value of each AFA batch upon purchase on controls. These practices are now described more fully in the Material and Methods section.

Points 4-8 in the following comments refer to Figure 3.

4. Can the authors comment on why different compounds are used for different parasite experiments? E.g. Avo A on parasites, avocadene acetate on C. elegans.

The commercial availability of pure AFA compounds has been a challenge during the study, leading us to develop a way to mitigate these challenges. First, we tested each compound separately in control experiments and then we developed a way to check by NMR the compounds we received as well as checking activity using controls. We now describe these steps in the Material and Methods section.

We tested each of the four individual compounds and their mixtures (Avo A and Avo B) in *C. elegans* (Figure 2) and *H. contortus* (Supp Table 2). The results from these experiments (along with the primary screen results) confirmed that the acetate forms are strongest. Therefore, we subsequently performed all experiments using only the acetate forms either as Avocatin A (a mixture of avocadene acetate and avocadyne acetate) or as a pure avocadene acetate (available from Sigma).

We have clarified this in the text (Lines 177-179).

5. It is noted in methods that numbers reflect three replicates, so perhaps it would be informative to put mean +/- st. error in the tables under percent lethality.

Thank you for this observation. We have added the SEM, except for the data that are derived from a single biological replicate due to the limited availability of parasitic nematodes for experimental assays.

6. It is important to state male or female Brugia, since the different sexes have pronounced differences in drug response.

The initial experiments in Figure 3b were done using mixed sexes. We have repeated these tests on separate sexes and added the results to **Figure 3b**. Indeed, the males are more sensitive than the females and we now report that.

7. It is unclear on MF experiments – is this quantified motility, or qualitative scoring? For example, does 50% lethality at 4 micromolar mean 50% of worms were dead by eye (scoring 3000 different worms?), or that movement (or some other metric) was measured and reduced in the population by 50%?

Thank you for pointing out this gap in our original description. We have now changed the Figure to reflect our recent experiment with accurate (manual counting) Mf counts and lethality scores. In the previous Mf experiment, we could not count accurately by eye since we were scoring large populations of Mfs (~3000). The immotile worms never recovered.

8. In vivo mouse experiment – state the dose in mg/kg in legend under panel D.

Thank you for pointing this out. We have made this important change.

9. In Figure 4 – are worms viable after paralysis in panel A and C (or not moving because dead?). If they are not viable, is that a reason why the OCR could be flat in D? Related to this point, different concentrations of drugs are used in different assays, and so if these could all be noted in the legend it be much easier to understand (e.g. concentration of drug used in panels E,F,G?).

Thank you for pointing out a possible confusion in our descriptions of the results . We have amended the text to reflect the fact that, at the lower concentration (10 μM) some of the worms do indeed recover, but not at higher concentration (25 μM); and that all OCR experiments were done at doses that induced neither developmental arrest nor lethality in the treated L1 stage, as the measurements were done at L4.

In addition to amending Figure 4 to reflect these changes, we have modified Supplementary Figure 4 to show the recovery data over time.

For Figure 4, we have updated the drug concentrations in the legend. The experiments represented in Figure 4g and h were conducted at the earlier stages of the study on L1s using the AFA aliquots from the Spectrum collection library, which was less potent (10μM treatments were sub-lethal to L1 in that library batch). In panel h, the treatment was on L4s, hence the sub-lethal concentration is higher.

10. Line 493 – state the concentration or percent of DMSO.

We have added 1% DMSO to the text.

11. Supplemental figure 1 – in the legend, state how long was drug treatment.

We have added the duration to the legend.

12. Supplemental figure 8. Are there outcomes that are quantifiable from this data? I gather the Y axis is a composite of many phenotypes. But can this be broken up into quantifiable measurements of viability, movement, length/size, or fecundity?

In the RNAi experiments, (**Supplementary Figure 7** in the revised document), we quantified L1 development into fertile adults. We have modified the Y axis to reflect the L1 development score.

Reviewer #2 (Remarks to the Author):

The Fahs et al manuscript describes a very thorough investigation of the potential utility of avocado fatty acids and alcohols against parasitic nematodes and its mechanism of action. I think it their discovery represents an important potential new tool in the battle against parasitic nematodes. I can easily imagine supplements to livestock food that might increase the health of the animals. The manuscript was truly a pleasure to read. I enthusiastically recommend its publication. I have no major issues with the paper and have only minor suggestions for improvement.

We truly appreciate this comment and share in the enthusiasm that these compounds could provide new avenues for research and treatments.

Lines 87-88: A little bit of an unusual cell line. Perhaps providing some rationale for the choice would be useful.

We appreciate this observation and we have added a brief explanation in the text. We use U2-OS cells in our assays routinely to perform toxicity studies because of their flattened morphology to help imaging-based toxicity analysis [1-2]. Since we selected compounds that showed no toxicity for this particular study, this aspect was not highlighted in the original submission.

1.Bray MA, Singh S, Han H, Davis CT, Borgeson B, Hartland C, Kost-Alimova M, Gustafsdottir SM, Gibson CC, Carpenter AE. Cell Painting, a high-content image-based assay for morphological profiling using multiplexed fluorescent dyes. Nat Protoc. 2016 Sep;11(9):1757-74.

2.Pearson YE, Kremb S, Butterfoss GL, Xie X, Fahs H, Gunsalus KC. A statistical framework for high-content phenotypic profiling using cellular feature distributions. Commun Biol. 2022 Dec 22;5(1):1409.

Paragraph beginning on line 89: I am curious to know whether there were structural analogs in the library that were not identified as hits? Were the two indicated in lines 117-118 part of the Spectrum library? Details about true negatives can help the reader better understand the important parts of the structure that confers activity, which comes up later in the manuscript with their docking experiments.

We agree that negative results are important and have added the compound names and chemical formulas in **Supplementary Table 1** for all the compounds in the library we tested. We report the furans (avocadenofuran and avocadynofuran) in Figure 1 that are the closest matches and are indeed negative in our assays. In addition to the Spectrum library, as described, these results also led us to extract other similar compounds from avocado seeds detailed in Figure 1b (persin, persenone A) that were found to be toxic. We report on these in the manuscript.

Figure 1a: the smaller pics below do not readily demonstrate the indicated phenotype. It is not clear how a single shot can illustrate slow growth, sterility, uncoordination, and Emb (without showing dysmorphic embryos). Obviously, the details of some of these

phenotypes are well laid out in Figure 2, which is really nice. However, since Fig 1 is a nice opportunity to visually demonstrate the strength of the phenotype, perhaps illustrating the whole-well phenotype to compare to control would be more useful? Consider revising.

Thank you for this comment. We illustrated the whole well in **Figure 1a** showing a representation of the phenotypes observed for AFA.

Lines 222-231: How do you control for death/dying impacting OCR?

We are grateful for pointing this out as it is indeed confusing, allowing us to clarify this point in the text. For OCR experiments, L1s were treated with sub-lethal concentrations of Avo Ac, the treatment did not induce any developmental arrest or lethality and the OCR measurements were performed 44 hours later, on the L4 stage animals. The animal counts were performed before the experiments and the animals were viable.

Any reason not to include Sup Fig 7 among the main display items? Seems like an important observation that is central to the main conclusions.

Thank you for this comment. We have added the mitochondrial phenotype to **Figure 4**.

Paragraph starting at 286: The authors suggest that their mitochondrial results led them to hypothesize that lipid metabolism might be targeted by the AFAs. OK, but there is literature already showing this to be true (see PMID: 26077472, 34397122, 34095930) (which they later discuss). The authors should consider working this information into the rationale of the POD-2 results section and perhaps introducing that information earlier (i.e., another rationale for looking a the mitochondrial function and morphology).

Thank you for pointing this out. We have added this point to the text (line 356). It is correct that the mentioned papers were part of the rationale that led us to look at the mitochondrial phenotypes. However, we didn't reproduce their results in worms and we didn't find a link between the AFA effect and *C. elegans* VLCAD. One explanation could be that these studies were based on data from cancer cell lines.

I am curious- is there any data showing that communities that grow and consume avocados have lower nematode burdens than those who do not? Probably not, but worth a gander in the literature.

We were also curious about this but we didn't find scientifically proven data supporting this possibility, probably because these compounds are more concentrated in the non-edible part (seeds).

Technical:
-First results section, including figures: concentrations used and the number of technical and/or biological repeats should be indicated.

We have added this to the Results and the Materials and Methods sections.

-Consider adding p values to text stating statistical differences where none were provided in the text (for example, Lines, 225, 231, 268, 272 etc.).

Thank you for pointing this out. We added the p-values in the text.

A few more complements:
-Fig 1c, Fig 2- really nice analyses.

-Paragraph of line 129: Impressive.

-Lines 213-215: Impressive.

-Lines 147-148: Remarkable.

Thank you Peter!

Peter Roy

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have been responsive and addressed all of the comments from the initial review.

Reviewer #2 (Remarks to the Author):

The authors have satisfactorily addressed all of my comments.