

Peer Review File

A naturally occurring polyacetylene isolated from carrots promotes health and delays signatures of aging



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Carolin Thomas et al: A novel naturally occurring polyacetylene isolated from carrots promotes health and delays signatures of aging

They identify isofalcarintriol (ISF), a carrot-derived phytochemical as a novel health promoting

Nrf2 activator and selective inhibitor of the mitochondrial ATP synthase. Evidence for Nrf2 activation and mitochondrial ATP synthase-mediated effects manifest in ROS signaling and AMPK activation in cells and *C. elegans*, resulting in stress adaptations such as increased oxidative stress resistance and somewhat enhanced mitochondrial biogenesis in

C. elegans. ISF-mediated stress signaling leads to improvement of the metabolic phenotype and endurance exercise performance of mice on high-fat diet, as well as mice and has the potential to counteract an age-related increase in frailty.

This is well written paper with extensive identification and experimentation on this compound using worms, cells and mice.

It is not a premise for healthy aging that energy should be targeted so their selection of this compound may not be the best approach in countering age associated defects.

Nevertheless, they do identify something of use and interest.

The effects they see on age associated parameters are significant but not dramatic, and it is not clear why this intervention is more useful than so many other natural substances that have been reported to slow aging. What is missing in the work is a discussion relating this to such other interventions and why this should be preferred. Many other natural substances increase the life span in mice, but here they see no effect.

Also, importantly, there is no safety results. Safety parameters, what was done? DNA damage, Senescence and other standard measures should be done in cells or mice.

There are a number of overinterpretations that should be either further experimentally explored, or the discussion toned down. This includes the mitochondrial parameters, Fig 4.

Some of the mitochondrial parameters are only minimally affected by the compound

The effect on ATP production. They identify this compound as an inhibitor of ATP but after

10 min ATP is minimally decreased and then increases at 48 hrs, Fig 3H

Fig S1. In E, Fig says 0.1 nM but legend says 1 nm ? And why no effect on lifespan at 10 nm ?

Fig 3H. ATP levels were slightly decreased after 10 nM but not after 1 nM in c.elegans

Fig 4. In Fig 4E the maximal respiration should be corrected for differences in baseline. The effects of ISF in fig 4B and C are rather minimal

1 Reply to Reviewers, NCOMMS-23-20515, Thomas, Erni *et al.*

2
3 (Authors' replies in red)

4 5 Reviewer 1:

6 They identify isofalcarintriol (ISF), a carrot-derived phytochemical as a novel health promoting Nrf2
7 activator and selective inhibitor of the mitochondrial ATP synthase. Evidence for Nrf2 activation and
8 mitochondrial ATP synthase-mediated effects manifest in ROS signaling and AMPK activation in cells
9 and *C. elegans*, resulting in stress adaptations such as increased oxidative stress resistance and
10 somewhat enhanced mitochondrial biogenesis in *C. elegans*. ISF-mediated stress signaling leads to
11 improvement of the metabolic phenotype and endurance exercise performance of mice on high-fat
12 diet, as well as mice and has the potential to counteract an age-related increase in frailty.

13 This is well written paper with extensive identification and experimentation on this compound using
14 worms, cells and mice.

15 We thank the reviewer for their positive and supportive comments.

16 It is not a premise for healthy aging that energy should be targeted so their selection of this compound
17 may not be the best approach in countering age associated defects. Nevertheless, they do identify
18 something of use and interest.

19 The most long-standing (and hence probably best studied) intervention to delay aging is calorie
20 restriction, first described by Clive McCay in 1937, as reviewed in reference 3 of the current
21 manuscript. Caloric restriction improves health by decreasing cardiac risk factors, improving insulin
22 sensitivity, and mitochondrial function thereby contributing to healthy aging (ref. 3). Besides that,
23 physical exercise promotes healthy aging by increasing physical endurance, cardiovascular health, and
24 metabolic function through an initial energy depletion during the exercise session. Consequently,
25 targeting energy metabolism by e.g. phytochemicals is in our opinion a very valid premise for healthy
26 aging, mimicking both calorie restriction as well as physical exercise.

27
28 The effects they see on age associated parameters are significant but not dramatic, and it is not clear
29 why this intervention is more useful than so many other natural substances that have been reported
30 to slow aging. What is missing in the work is a discussion relating this to such other interventions and
31 why this should be preferred.

32 We thank the reviewer for their valuable comment. While there are indeed other natural
33 compounds known to extend lifespan and promote health in model organisms, and possibly humans, it
34 should be noted that (i) IFT is similarly effective, (ii) IFT uses a different molecular mode of action, which
35 (iii) implies that IFT could be combined with other natural compounds to exert additive effects, (iv) is
36 readily available from carrots and particularly carrot peel as a by-product of carrot processing, and
37 most importantly (iv) IFT exerts biological activities at unusual low doses.

38 The Discussion section has been extended accordingly, now stating: *'Despite the existence of*
39 *known natural compounds exhibiting significant anti-aging effects in model organisms, isofalcarintriol*

40 *possesses several notable advantages over them, such as its versatile bioactivities even at remarkably*
41 *low doses. Published investigations on anti-aging structures such as resveratrol, curcumin, quercetin,*
42 *and epicatechin-3 gallate have demonstrated their impact on the lifespan of C. elegans at*
43 *concentrations ranging from 10 to 200 μ M (as sourced from <https://genomics.senescence.info/drugs/>).*
44 *In contrast, isofalcarintriol exhibits lifespan-extending properties at concentrations in the low*
45 *nanomolar range. Additionally, the bioactivity of resveratrol has been observed at concentrations of*
46 *300 mg/kg food which is 375-fold higher than the effective concentration of isofalcarintriol in mice (0.8*
47 *mg/kg water corresponding to 0.1 mg/kg body weight). Similarly, curcumin and green tea extract were*
48 *administered at concentrations of 2000 mg/kg food (PubMedID: 22451473), surpassing the dosage of*
49 *isofalcarintriol by a factor of 2,500 (PubMedID: 22451473).*

50 *Therefore, we anticipate that isofalcarintriol exhibits enhanced bioavailability and/or increased*
51 *potency as a natural anti-aging agent, different from previously documented structures. Furthermore,*
52 *the exceedingly low bioactive dosage facilitates its inclusion into a natural diet rich in carrots, which*
53 *has been widely and safely consumed by humans worldwide for centuries. Given the abundant*
54 *availability of carrots as source material, strategies can be envisaged to enrich our compound in the*
55 *form of a nutritional supplement that fosters the promotion of healthy aging.'*

56

57 Many other natural substances increase the life span in mice, but here they see no effect.

58 The National Institute on Aging Interventions Testing Program (ITP) has analyzed natural
59 compounds shown to extend lifespan in lower organisms, including resveratrol, green tea extract
60 (mainly including EGCG), curcumin, oxaloacetic acid, and lastly medium-chain triglycerides (PubMedID:
61 22451473). However, none of the tested compounds was lifespan extending in neither male nor
62 female mice. Consequently, and congruent with an increasing number of fellow researchers, we
63 believe that versatile effects on health and age-related frailty parameters are a more precise predictor
64 for healthy aging than pure lifespan analysis.

65 Independently, there are examples for compounds which do extend lifespan in specific
66 laboratories when applied to inbred mouse lines, while showing no effects in the ITP setting, employing
67 a genetically heterogenous background.

68

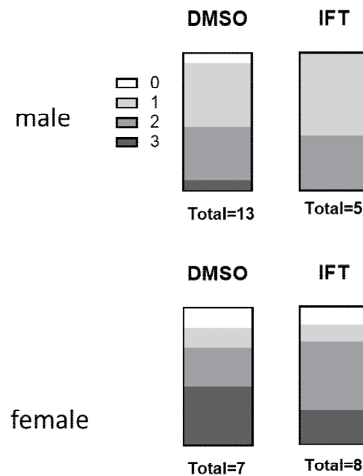
69 Also, importantly, there is no safety results. Safety parameters, what was done? DNA damage,
70 Senescence and other standard measures should be done in cells or mice.

71 We performed a short-term dose finding and toxicity study in young WT mice where IFT was
72 administered ad libitum in the drinking water over a period of 2 weeks. We included three
73 concentrations, 0.025, 0.25, and 2.5 mg/kg body weight. These concentrations were far below toxic
74 doses of other, previously studied polyacetylenes. For example, a single oral gavage of 300 mg/kg body
75 weight of Falcarinol, a structurally related polyacetylene, did previously not show any signs of toxicity
76 according to published data (PubMedID: 32905447).

77 We have added the above as an additional information in line 302-305 and line 310 of the main
78 manuscript, and have added Fig. S10A,B and S12E-F to the supplementary information: *'Most*
79 *importantly, a prior two-week toxicity study in animals on chow diet did not show any elevation of liver*
80 *toxicity parameter such as ALAT and ASAT levels in the blood when applying a maximum dose of 2.5*
81 *mg/kg isofalcarintriol (Fig. S10A, B), assuming that long-term treatment with the compound in lower*
82 *doses is save as well. The high-fat diet applied in this study serves to induce overweight and challenge*
83 *the metabolic phenotype, provoking a decrease in glucose sensitivity and promoting insulin resistance*

84 as typically seen in obese and diabetic individuals. During the 6 months course of this high-fat diet
85 study, no major differences in body mass (Fig. S12A), body composition (fat mass and lean mass) (Fig.
86 S12B), blood lipid levels (Fig. S12C, D), and liver toxicity parameters (Fig. S12E, F) were detected
87 between treated and control animals.'

88 In addition, we had analyzed the occurrence of tumors by necropsy of 2+ year old animals that
89 have been on IFT treatment (0.1 mg/kg) for at least 1 year. As a result, we did not observe any increase
90 in the number of formed tumors (pls see **Review Figure 1**). As depicted in Fig. S13, IFT-treated animals
91 had a similar life expectancy as control animals, indicating no major safety concerns of the compound.



92

93 **Review Figure 1: Number of tumors at endpoint in aged mice on IFT or DMSO**

94

95 There are a number of overinterpretations that should be either further experimentally explored, or
96 the discussion toned down. This includes the mitochondrial parameters, Fig 4. Some of the
97 mitochondrial parameters are only minimally affected by the compound.

98 We thank the reviewer for their comment, giving us the opportunity to further elaborate on
99 the primary concept of the initial screen (Fig. 1B). As can be seen in this figure, the initial screen was
100 intentionally performed for compounds that reduce cellular ATP content to a limited extent only. This
101 was based on the fact that a more prominent reduction in ATP would induce apoptosis and hence
102 induce toxic side effects in cells, nematodes and mice.

103 Those mildly ATP reducing compounds (Fig. 1B) were further analysed for NRF2 activation
104 (Figs. 1D and E). After having established IFT as an inhibitor of ATP synthase (Fig.3) previously
105 established ATP inhibitors (Bz-423, Piceatannol) were added to the experiments depicted in Figs. 1B,
106 D and E to test for comparable effects, also on lifespan (Figs. 3E and F).

107 Very consistently and as to be expected, the experiments in Fig. 4 indeed depict a limited (while
108 statistically highly significant) effect of the corresponding compounds, including IFT. This, however,
109 reflects the purpose of the screening design: only a limited impairment of mitochondrial activity will
110 promoted lifespan, whereas a pronounced inhibition of the electron transfer chain (ETC) will cause
111 reduced lifespan and/or lethality. This reflects a no-linear ('hormetic') dose-response mechanism,
112 where limited ETC inhibition promotes longevity, while strong inhibition of the ETC causes increased
113 mortality. One of the senior authors (M.R.) has shown this repeatedly for many interventions in the
114 past, and the mechanism is known as mitochondrial hormesis or mitohormesis.

115

116

117 The effect on ATP production. They identify this compound as an inhibitor of ATP but after 10 min ATP
118 is minimally decreased and then increases at 48 hrs, Fig 3H

119 This indeed is an essential part of the biochemical mechanism. As also for e.g. health-
120 promoting exercise, IFT causes a reduction of ATP for a limited period of time only. The initial reduction
121 is sensed by AMP-dependent protein kinase (AMPK/aak-2 in *C. elegans*; Figs.3L, M) to compensatorily
122 induce mitochondrial biogenesis (Figs. 6F-H) which leads to a secondary increase in ATP levels at later
123 time points, paralleled by increased exercise capacity (which is based on mitochondrial mass) (Figs. 6
124 and 7).

125

126 Fig S1. in E, Fig says 0.1 nM but legend says 1 nm ? And why no effect on lifespan at 10 nm?

127 We thank the reviewer for bringing this inconsistent labeling to our attention. Fig.S1E indeed
128 depicts a lifespan assay using IFT at the concentration of 0.1 nM (concentration at the panel has been
129 corrected; concentration in the legend remains).

130 Regarding the second question: Panel F has depicted (and still depicts) IFT at an concentration
131 fo 10 nM. What the reviewer possibly refers to, are panels C and D? These depict experiments using
132 Alnusone, where independent experiments showed inconsistent results. Such inconsistencies rarely
133 occur in lifespan assays, but have not been observed in any other independent repetition throughout
134 the manuscript.

135 Lastly, it should be noted that all lifespan data and statistics are listed in Table S6.

136

137 Fig 3H. ATP levels were slightly decreased after 10 nM but not after 1 nM in *c.elegans*

138 We do not necessarily expect significant changes at lower doses in whole-body lysates of
139 nematodes. Rather, reductions in ATP are to be expected in specific tissues of the worm, which cannot
140 be analyzed individually for technical reasons. For this reason and indeed, ATP levels were significantly
141 decreased at 10 nM only but not after 1 nM in *C. elegans* using three replicates containing pooled
142 whole worm samples; however, and notably, ATP levels were decreased in *C. elegans* in the
143 experiments shown in Fig. 4G. Taken together, we believe there is ample evidence for a transient
144 decrease in ATP levels, also when taking the epistatic dependency on AMPK/aak-2 into account (pls
145 see above).

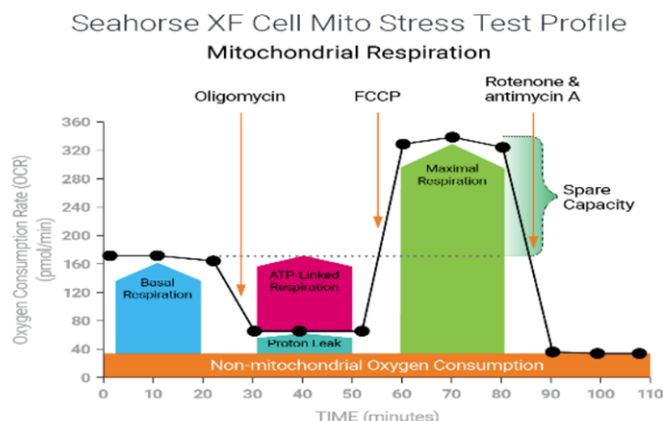
146

147 Fig 4. In Fig 4E the maximal respiration should be corrected for differences in baseline. The effects of
148 ISF in Figs. 4B and C are rather minimal

149 As already stated above, the limited effects of IFT are reassuring since mechanistically
150 essential, rather than being a reason to be concerned.

151 To our knowledge, and also according to the Agilent Seahorse Cell Mito Stress user manual
152 ([https://www.agilent.com/cs/library/usermanuals/public/XF_Cell_Mito_Stress_Test_Kit_User_Guide](https://www.agilent.com/cs/library/usermanuals/public/XF_Cell_Mito_Stress_Test_Kit_User_Guide.pdf)
153 .pdf), the maximal respiration is calculated by subtraction of the non-mitochondrial oxygen
154 consumption rate after injection of rotenone and antimycin A (see Figure below). Maximal respiration

155 rates represented in Fig 4 were calculated as suggested there. Corrections for differences in baseline
156 would result in the calculation of the spare capacity which was not the aim here.



157
158 Moreover, the effects of IFT in Fig. 4B and C are in a similar magnitude – or even stronger –
159 than the established ATP synthase inhibitor piceatannol (PubMedID: 10425214) and Bz-423
160 (PubMedID: 30022951). Consequently, the effect of IFT is rather profound in our opinion.

161
162 Taken together, we believe that we have addressed the reviewer’s questions, and/or have
163 implemented their valuable suggestions, and think that the revised version of the manuscript may now
164 be acceptable for publication. Sincerely, we hope that the reviewer will share our view.

165
166
167

168 Reviewer 2:

169 The 1,200 single compounds were purchased from a company for this study in the first step of
170 bioactivity screening on ATP levels in HepG2 cells and Nrf2 activation in HEK293. Why used a cancer
171 cell line HepG2, not a normal cell line such as HEK293 to measure the compound effect on ATP levels,
172 as the ROS levels or/and mitochondria bioenergetic status are believed to be different to normal cell
173 lines?

174 We thank the reviewer for this very relevant question.

175 Firstly, we wish to point out that the cell line used primarily served to perform the majority of
176 experiments, HepG2, is very widely used for metabolism-related cell culture studies. This is reflected
177 by the fact that the search term "HepG2" generates 812,000 entries in GoogleScholar. Importantly, the
178 well-established metabolic flexibility of HepG2 cells make them the ideal tool to study changes in
179 mitochondrial capacity, as performed in the current study (Fig. 1B and others).

180 By contrast, number of GoogleScholar entries for the search term ("HEK293" AND "HEK-293") is
181 64,700 combined, i.e. less than 8% of the corresponding HepG2 entries. This may be due to the fact
182 that HEK cells are embryonic, and (more importantly) carry an unstable chromosome set, as described
183 repeatedly as ‘hypotriploid’, with increasing loss of chromosome during passaging. Hence, the main
184 advantage of HEK293 cells is their transfectability, especially with reporter constructs as used in the
185 current manuscript (Figs. 1C and D).

186 Thirdly and in addition to their impaired metabolic flexibility, HEK293 cells cannot be used in
187 Seahorse-based experiments, due to their almost non-adhesive nature of growth in cultured media.

188 Lastly and most importantly, all findings in HepG2 and HEK293 cells have been fully replicated and
189 validated in nematodes, and (where possible) in mice. This indicates that the cell-based findings
190 presented are not cell-line specific, but rather can be translated into living organisms.

191

192 If it was a structurally unknown compound, how it was known as a diacetylene as described in line
193 114? On the other hand, isofalcarintriol is indicated/labeled as 1, 1a, or IFT in Figure 1 and Figure S1
194 that is somewhat confusing. Whether the data in both figures were obtained using commercial
195 compound 1 or asymmetry-synthesized 1a that should be clearly described, as the compound purity
196 was not the same in 1 and 1a (Figure S3 and Table S3).

197 The compound was contained in a commercially obtained library. The compounds contained
198 were isolated from plant extracts. Regarding the compound of interest in this manuscript, the supplier
199 of the library (AnalytiCon Discovery) did not provide a name or C.A.S. number, however did provide a
200 structure, based on their in-house analyses.

201 We apologize for the inconsistencies in the usage of the commercially obtained compound '(1)'
202 and the asymmetry-synthesized compound '(1a)', and have corrected this accordingly.

203 In brief, only the initial screening experiments depicted in Figs. 1B, D and E were performed
204 using IFT (1), while all subsequent experiments were performed with the synthesized IFT (1a).

205

206 Figure S1: Typo in panel C or D as which data is for 1 nM alnusone treatment? Why 10- gingerol did
207 not test with 10 nM, and why isofalcarintriol was tested with 0.1 nM, not 1 nM in this experiment?

208 The labeling was corrected (pls see comment to Reviewer #1), and panels were adjusted so
209 that they all show 10 nM concentrations. Alnusone and Gingerol were both tested in three
210 concentrations (1nM, 10nM, 100 nM). It should be noted that all lifespan data and statistics are listed
211 in Table S6.

212 Panels C and D **both** depict experiments using Alnusone at 10 nM, where independent
213 experiments showed inconsistent results. Such inconsistencies rarely occur in lifespan assays, but have
214 not been observed in any other independent repetition throughout the manuscript. We included both
215 data sets to make them accessible to the reviewers and future readers.

216 Lastly, the requested lifespan using 1 nM isofalcarintriol (1a) was (and still is) depicted in the
217 main Fig. 1.

218

219 Figure 1: The name of HEK293 cell line should be indicated in the legend to panel G. It is also suggested
220 to switch panel G data to panel E following panel D in Figure 1 that would be easier to compare and
221 understand both data sets.

222 We fully agree. The sequence of the figure panels as well as the adaptation of the figure legend
223 has been changed accordingly.

224

225 5. There are some flaws in the compound structure characterization and elucidation in this study. The
226 authors described significant impurities contained in compound 1 (Table S3) and were not able to
227 assign some spectra data for the compound. Then, in Figure 2A, how the correlation data assigned in
228 the compound could be given only based on the HMBC data without the support of NOESY and HBQC
229 analysis/data. In Figure S3, some impurities were observed in the eSFC chromatograms for 1 (A), ent-
230 1b (B), ent-1a (C), 1b (E); in addition, only selected retention times (10 to 18 min) are shown in Figure
231 S3. It is not clear whether there are other impurities/peaks present in any chromatogram out of the
232 time range. The bioactivity data can not be directly compared within impure compounds.

233 Please accept our apologies for essential parts of the **Supplemental Information (SI)** missing
234 in the primary submission. It appears that, due to data volume / size issues this (originally separate
235 file) has not been processed during upload of the original manuscript. We have now resized the file,
236 and included it into the revised version of the Supplemental Information (SI), specifically **pages 36 to**
237 **210 (sic)**.

238 Accordingly, the initially missing spectra for isofalcarintriol (**1, NP017896**) can now be found in
239 the SI (Spectra to Configurational Assignment of Isofalcarintriol) including ¹H, ¹³C, HSQC, HMBC, COSY
240 NMR spectra. Full length eSFC chromatograms can be found in the SI (SFC Data to Configurational
241 Assignment of Isofalcarintriol). The initial screening was conducted with isofalcarintriol (**1, NP017896**)
242 as provided by AnalytiCon Discovery GmbH and follow-up biological evaluation was conducted with
243 synthetic isofalcarintriol (**1a**).

244

245 6. All the NMR spectral profiles of major synthesized compounds in this study should be provided along
246 with the spectral data in the supplementary information. All the anti-1,2- diol structure drawing is not
247 accurate that should be corrected.

248 Again (pls. see above), please accept our apologies for essential parts of the **Supplemental**
249 **Information (SI)** missing.

250 The full synthetic descriptions as well as spectral data have been amended to the SI file in total
251 190 pages have been added. Regarding the anti-1,2- diol structures: During the synthesis the
252 compounds are cyclic 4,5 disubstituted 2,2-dimethyl-1,3-dioxolanes (e.g. *anti*-alkyne 4a). After
253 deprotection the compounds are converted to the corresponding linear compounds which are *syn*-
254 configured. The linear compounds are not drawn in their fully extended zig-zag configuration to keep
255 them in line with their precursor structures.

256

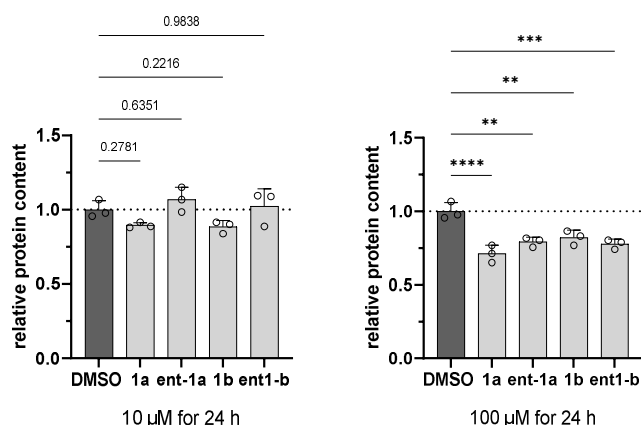
257 Please uniform all the compound numbers with bold-faced throughout the text.

258 As stated above and also applicable here, we made the adaptations in the text accordingly.

259

260 Figure 2 and Figure S2: Any explanation for why 100 μM 1a, 1b, 1c and 1d compound treatments
261 showed much less fold activation of Nrf2 compared to their 10 μM treatments?

262 As shown both functionally and by mass spec in this manuscript, IFT is an ATP synthase inhibitor
263 which, as all such inhibitors, eventually will impair growth and induced apoptosis. Accordingly, the
264 reduced activation might be explained by the toxicity of the compound at high concentrations leading
265 to a decrease in cell number (see **Review Figure 2**, below), and most likely also to significant changes
266 in cellular signaling pathways, thus resulting in attenuated Nrf2 signaling.



267

268 **Review Fig. 2: Cell protein content upon treatment with compound variants at 10 vs. 100 μM**

269

270 Figures S4 and S5: In these two figures, the importance of the C10 to C17 moiety and the hydroxy
 271 group at C3 in the compound structure were demonstrated. However, the biotin-2 isofalcarintriol (15)
 272 was inactive in HEK293 cells, conversely, it retained some activity in HepG2 cancer cells. What made
 273 this bioactivity discrepancy in both cell lines? In addition, what is the rationale to use an inactive
 274 compound probe to do protein pulldown experiment for their specific targeting/binding protein(s) in
 275 HEK293 cells?

276 As outlined in lines 171-174, as well as in the Supplementary Information, some cell lines
 277 (including HepG2) express specific biotin transporters in their plasma membrane which enables them
 278 to take up biotin as well as biotin-labelled compounds. However, and by contrast, HEK293 cells lack
 279 such transporters, and thus are unable to take up biotin-labelled compounds (pls see for details
 280 PubMedID 26021457).

281 This difference explains why biotin-isofalcarintriol appears to be inactive in intact HEK293, i.e.
 282 solely due to the lack of cellular uptake. Given that information, the biotin pulldown protocols were
 283 adapted accordingly: While biotin-IFT treatment was performed in intact HepG2 cells, where its uptake
 284 is possible, the treatment of HEK293 was performed in lysed cells, allowing the compound to access
 285 proteins directly (pls see Fig. S6).

286

287 Figure 3: In panel K, it needs to provide the cell image treated with azide488 probe alone to compare
 288 with the cells treated with IFT-azide488 conjugate.

289 Indeed, and we wish to apologize for suboptimal labeling: Both the “DMSO” solvent control
 290 and “IFT-alkyne” condition in Fig. 3K were all co-treated with azide488. The figure legend was adapted
 291 accordingly, to avoid further misunderstanding.

292

293 The concentrations used for both cell lines and C. elegans assays have 1000-fold differences (μM vs.
 294 nM), please explain the reason. Please delete one repeated word “including” in line 832.

295 This is a well-known issue, which the authors’ biological lab (M.R.) as well as many other labs
 296 have experienced for a significant number of different compounds.

297 E.g. in a previous publication on arsenite we observed similarly high differences in effective
298 compound concentrations between cells and *C. elegans* (PubMedID 23534459). Arsenite was used in
299 concentrations up to 100 μ M in HepG2 cells. By contrast, Arsenite was lifespan-extending in a 1000-
300 fold lower concentration in *C. elegans* (100 nM).

301 This aligns with the observed variations in concentration as outlined in this manuscript (μ M in
302 cells vs nM in *C. elegans*). This discrepancy might result from differences in uptake, distribution, and
303 metabolism of the compound (cellular vs organismal).

304

305 11. Table S4: The experiment to analyze the spatial distribution of 1a in *D. carota* should be described
306 with details, such as the extraction conditions (volume ratio, time, temperature, etc). Why bother to
307 create isotope labeled 1a for this experiment? The calibration curve of concentration for 1a can be
308 established and used to quantify the compound content in the extracts. The numbers in Table S4
309 should be more clearly explained. Actually, this experiment and experimental data did not add
310 significance to this study.

311 As stated above, the experimental data/description had been erroneously not been part of the
312 initial submission; apologies again! Full description can be now found in the revised SI (section
313 'Extraction and Quantification of Isofalcarintriol from *D. carota*').

314 The quantification curve was indeed established via the standard addition method, but for
315 normalization an internal standard (isotope labeled isofalcarintriol (**12**)) was used. The crude extracts
316 contain a plethora of different compounds through a variety of retention times therefore the isotope
317 labeled isofalcarintriol (**12**) was chosen as internal standard (exactly matching in retention time with
318 **1a**) for the extracts to compensate for any matrix effects on ionization.

319 To our knowledge, isofalcarintriol (**1a**) has not been described in the literature in turn the
320 natural source/abundance has not been characterized. The confirmation of natural occurrence and the
321 quantification in *D. carota* are first reported in this study.

322

323 Scheme S1 content does not match with the description in lines 161-162 as "an extraction procedure
324 and liquid chromatography–mass spectrometry (LC-MS) separation method" Scheme S2 is missing.
325 Actually, the current scheme S1 content belongs to the missing scheme S2.

326 Apologies for the mistake. Scheme S2 has been renamed to Scheme S1.

327

328 Table S5: Several identified proteins (more than 10) showed much higher fold changes than both ATP
329 synthase subunits in mitochondria; however, those were not discussed or carried out further validation
330 in this study. It is believed that the interesting observation for compound 1a on promoting health or
331 delaying signatures of aging would not just relate to inhibition of ATP levels, ATP synthase activity,
332 mitochondria biogenesis, and other particularly mentioned activities in this study. To strengthen the
333 novelty and the pharmacological effects and applications of compound 1a, more extensive discussion
334 and/or in-depth molecular/biological activity studies should be considered.

335 We thank the reviewer for this important comment. The current research project and
336 importantly the initial part, i.e. the initial screen, aimed to identify compounds that exert a short-term
337 depletion of ATP. Hence and after evaluating the mass spec interaction data thoroughly, our priority

338 became clearly to further investigate on the two subunits of the ATP synthase that were, in
339 independent experimental approaches, identified as interaction partners of IFT.

340 While not included into the initial version of the manuscript, we also had performed a gene
341 annotation enrichment analysis showing overrepresented functional pathways of biotin-pulldown
342 protein hits. This analysis is now included as Supplemental Table 6 in the Supplemental Information.
343 As the reviewer may now appreciate, the category 'Formation of ATP by chemiosmotic coupling' was
344 most highly overrepresented (53fold) compared to other significantly enriched cellular pathways,
345 which include cellular transport processes and signaling by GTPases (Table S6). Hence, and based on
346 both independent experimental as well as bioinformatics evidence, we believe, that the choice of the
347 most relevant target is scientifically justified.

348 Accordingly, the following paragraph was added to the main text (line 179-184): *Strikingly, a*
349 *functional annotation analysis revealed that the category "formation of ATP by chemiosmotic*
350 *coupling", including both subunits of the ATP synthase, was the highest overrepresented (53x)*
351 *biological function compared to other significantly enriched cellular pathways such as cellular transport*
352 *processes and signaling by GTPases (Tab. S6). Together with the data on partial ATP depletion the*
353 *evidence points towards mitochondrial energy metabolism being the main pathway targeted by IFT.*

354

355 Figure S7: it is difficult to see the shape differences between the round and tubular-shaped
356 mitochondria in isofalcarintriol-treated vs. DMSO-treated mitochondria, respectively. This data can be
357 improved by using transmission electron microscopy (TEM) to examine the treated mitochondria
358 shape/structure.

359 We agree with the reviewer that more advanced approaches would be ideal to further
360 substantiate this observation. Since this potential change in morphology is not relevant for the
361 mechanism presented, we have now rephrased the corresponding sentences to keep the statement
362 more general (line 215-222), as follows: *'During confocal microscopy, we observed potential indications*
363 *of differences in shape between round isofalcarintriol-treated mitochondria and DMSO-treated*
364 *tubular-shaped mitochondria (Fig. S7F). [...] However, to further quantify differences in mitochondrial*
365 *dynamics, advanced microscopy analyses would need to be conducted in future studies.'*

366

367 Figure 5: Which cells were used in panel A? In panel C: the bar of Ctl Oex meant for IFT treated group
368 (the bar color is not correct)? The Figure S10 data can be merged into Figure 5 (panel I to K), as the
369 effects on HepG2 and HT-29 cells were much significant than that on MCF-7 cells. The method of soft
370 agar colony formation assay should be described in more detail.

371 HepG2 cells were used in Panel A; the figure legend was expanded accordingly. Both bars in
372 Panel C reflect samples treated with IFT, thus the color scheme is slightly different than in other panels.
373 Following to the reviewer's suggestion, parts of Figure S10 have now been incorporated in Figure 5.
374 The methods description of the soft agar colony formation assay was extended as well (line 540-553):
375 *In short, 5% (w/v) agar stock solution was prepared in PBS, autoclaved and equilibrated at 50°C. A 0.5%*
376 *agar solution was prepared and added as bottom layer to 12 well plates. The cells were detached off*
377 *the cell culture plate by trypsin and 500-2,000 cells per ml were mixed with compound and liquid agar*
378 *(final concentration of 0.3%). The mixture was added on top of the bottom layer of agar and allowed*
379 *to solidify for 30 min at RT. Cell culture media was added on top of each well. The plates were incubated*
380 *2-3 weeks and the media was changed every 3-4 days. After that, the colonies were stained with 0.01%*
381 *crystal violet (Sigma Aldrich; #V5265-500ML) and analyzed with a Leica M165FC microscope with Leica*

382 camera DFC 3000G. The number and size of colonies was calculated with ImageJ and the Plugin
383 ColonyCounter.

384

385 Figure 6: what cells were used in panel F? Typo in line 905, two panel F indicated.

386 Thank you for bringing these issues to our attention. As above, HepG2 cells were used in Panel
387 F; the figure legend was changed accordingly. Moreover, the typo in line 905 was corrected.

388

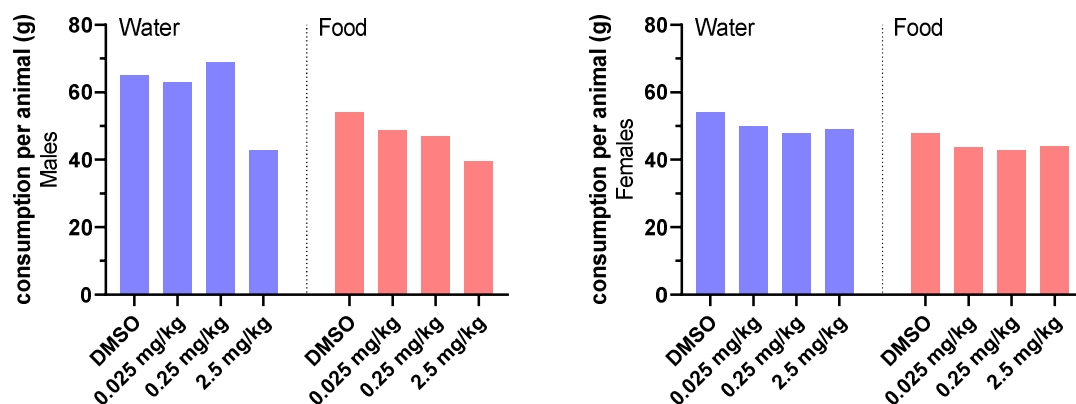
389 Figure 7: the compound dose was used 0.1 mg/kg/day in mouse experiments. How this dose was
390 determined, and no dose-dependent effect of the compound was addressed in 3 this in vivo mouse
391 study.

392 Before initiating the long-term mouse study, we had performed a short dose-finding / toxicity
393 study in young WT mice by administration of IFT ad libitum in the drinking water over two weeks. We
394 included three concentrations, 0.025, 0.25, and 2.5 mg/kg body weight. In our experience, an
395 appropriate conversion factor to predict effective concentrations in mice based on previously
396 established doses used in *C. elegans* is between 10-100. Consequently, the lowest mouse dose of 0.025
397 mg/kg body weight corresponds to $100 \times 1 \text{ nM} = 100 \text{ nM}$. The calculation was performed assuming an
398 average body mass of 35 g and 4 g of water consumption per day per mouse.

399 We observed decreased water and food consumption of the mouse cage with the highest
400 concentration of IFT (2.5 mg/kg) tested in a preliminary dose finding experiment (pls see **Review Figure**
401 **3**). A possible explanation might be the avoidance of the typical bitter taste of polyacetylenes in
402 elevated concentrations, as it has been described previously (PubMedID: 12797757). To guarantee
403 identical water and food consumption between groups, we decided to continue with a rather mild
404 concentration of IFT of 0.1 mg/ kg were no such differences were observed in the preliminary dose-
405 finding experiment. Later analysis showed no difference of water and food consumption upon 0.1
406 mg/kg long-term IFT application (**Review Figs. 4 and 5**).

407

408

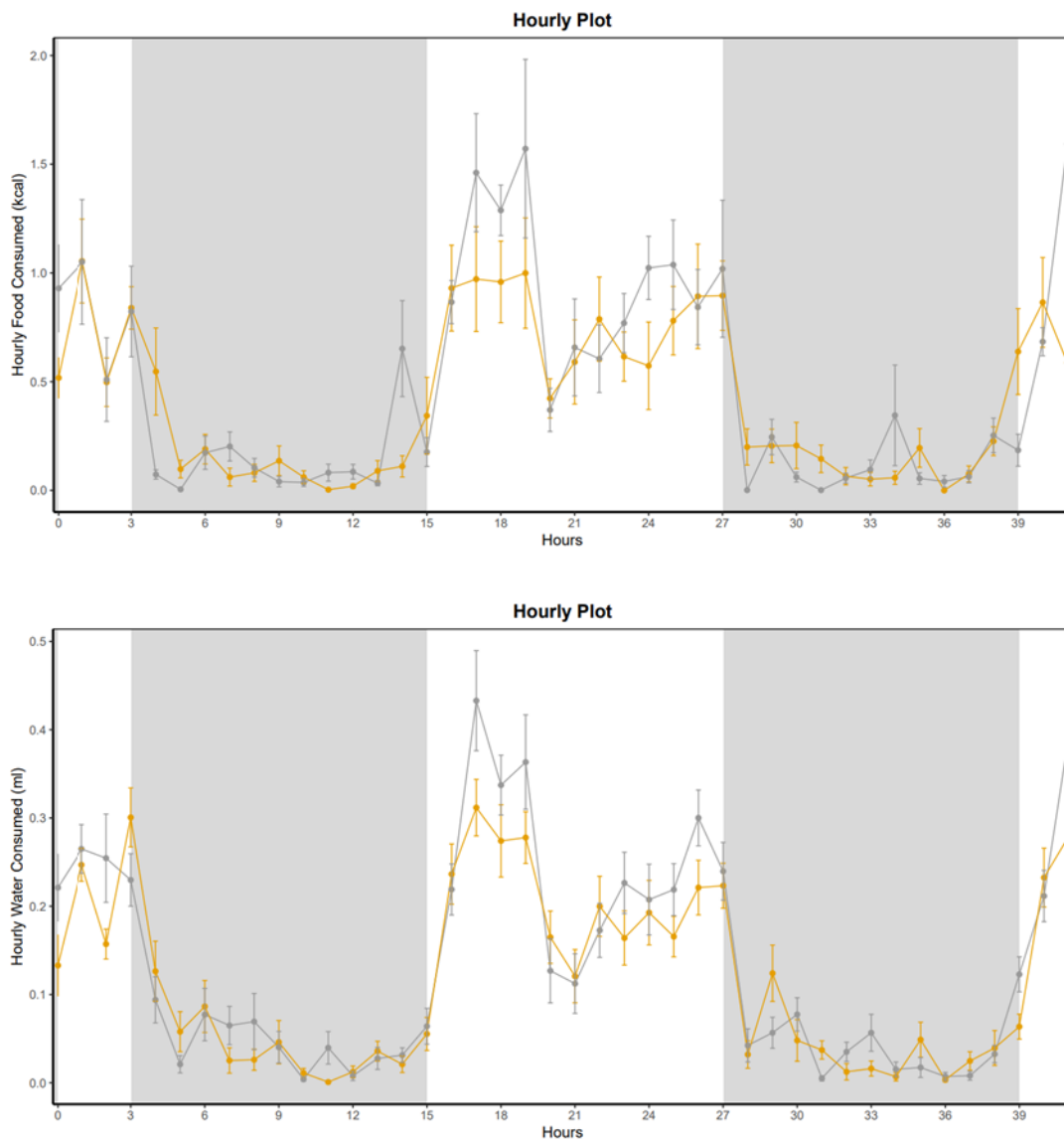


409

410 **Review Fig. 3:** Accumulative water and food consumption of male and female mice on chow diet after
411 two weeks of IFT treatment; consumption per cage was assessed, and divided by the number of

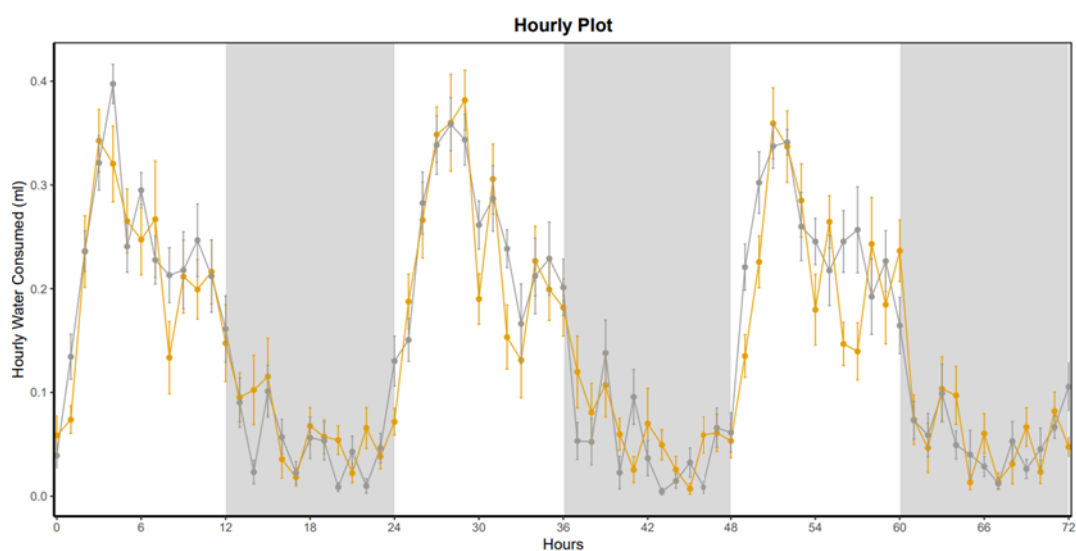
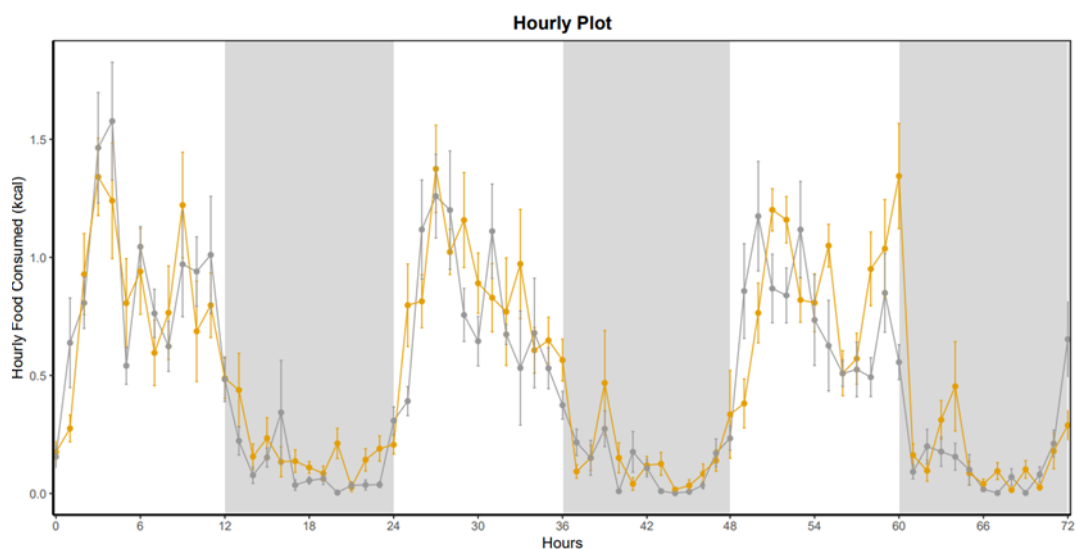
412 animals per cage. Animals of same sex and dose were housed in the same cage, hence no statistical
413 analyses were performed.

414



415

416 **Review Fig. 4:** Hourly water and food consumption in male mice on chow diet showing no significant
417 differences between DMSO (gray) and IFT (orange) animals (0.1 mg/kg body weight) at months 5 of
418 treatment. Food consumed: $p = 0.94$; Water consumed: $p = 0.41$; Two-way ANOVA. Data are
419 represented as average \pm SEM.



420

421 **Review Fig. 5:** Hourly water and food consumption in female mice on chow diet showing no significant
 422 differences between DMSO (gray) and IFT (orange) animals (0.1 mg/kg body weight) at month 5 of
 423 treatment. Food consumed: $p = 0.22$; Water consumed: $p = 0.99$; Two-way ANOVA. Data are
 424 represented as average \pm SEM.

425

426 In panel R, lower lymphocytes numbers could not be referred directly to decrease age-related
 427 inflammation in female mice, as a mixture of immune cells was contained in lymphocytes. To refer
 428 compound effect on age-associated inflammation, proinflammatory cytokines could be measured in
 429 mouse plasma or serum.

430 We thank the reviewer for suggesting this (as it turned out later, very valuable) experiment.
 431 We have now quantified cytokine levels in female blood plasma samples, and have implemented these
 432 data in the revised version of the manuscript (Fig. 7R, SI Tab. S9).

433 Correspondingly, the following text was added: *Line 354-357: Additionally, levels of anti-*
 434 *inflammatory cytokines IL-4 and IL-10 were increased in female plasma samples (Fig. 7R) while pro-*
 435 *inflammatory cytokines were unchanged (Tab. S9), indicating decreased age-related inflammation*
 436 *promoted by isofalcarintriol (1a).*

437 *Line 660-662: Cytokines in blood plasma samples were processed with the V-PLEX Proinflammatory*
438 *Panel 1 Mouse Kit (MSD, Rockville, MD, USA) and analyzed with a Sector Imager Microplate Reader*
439 *(MSD, Rockville, MD, USA) according to manufacturer's instructions.*

440

441 Taken together, we believe that we have addressed the reviewer's questions, and/or have
442 implemented their valuable suggestions, and think that the revised version of the manuscript may now
443 be acceptable for publication. Sincerely, we hope that the reviewer will share our view.

Reviewer #2 (Remarks to the Author):

Review on the manuscript entitled “A novel naturally occurring polyacetylene isolated from carrots promotes health and delays signatures of aging”

Comments:

1. The 1,200 single compounds were purchased from a company for this study in the first step of bioactivity screening on ATP levels in HepG2 cells and Nrf2 activation in HEK293. Why used a cancer cell line HepG2, not a normal cell line such as HEK293 to measure the compound effect on ATP levels, as the ROS levels or/and mitochondria bioenergetic status are believed to be different to normal cell lines?
2. If it was a structurally unknown compound, how it was known as a diacetylene as described in line 114? On the other hand, isofalcarintriol is indicated/labeled as **1**, **1a**, or IFT in Figure 1 and Figure S1 that is somewhat confusing. Whether the data in both figures were obtained using commercial compound **1** or asymmetry-synthesized **1a** that should be clearly described, as the compound purity was not the same in **1** and **1a** (Figure S3 and Table S3).
3. Figure S1: Typo in panel C or D as which data is for 1 nM alnusone treatment? Why 10-gingerol did not test with 10 nM, and why isofalcarintriol was tested with 0.1 nM, not 1 nM in this experiment?
4. Figure 1: The name of HEK293 cell line should be indicated in the legend to panel G. It is also suggested to switch panel G data to panel E following panel D in Figure 1 that would be easier to compare and understand both data sets.
5. There are some flaws in the compound structure characterization and elucidation in this study. The authors described significant impurities contained in compound **1** (Table S3) and were not able to assign some spectra data for the compound. Then, in Figure 2A, how the correlation data assigned in the compound could be given only based on the HMBC data without the support of NOESY and HBQC analysis/data. In Figure S3, some impurities were observed in the eSFC chromatograms for **1** (A), **ent-1b** (B), **ent-1a** (C), **1b** (E); in addition, only selected retention times (10 to 18 min) are shown in Figure S3. It is not clear whether there are other impurities/peaks present in any chromatogram out of the time range. The bioactivity data can not be directly compared within impure compounds.
6. All the NMR spectral profiles of major synthesized compounds in this study should be provided along with the spectral data in the supplementary information. All the anti-1,2-diol structure drawing is not accurate that should be corrected.
7. Please uniform all the compound numbers with bold-faced throughout the text.
8. Figure 2 and Figure S2: Any explanation for why 100 μ M **1a**, **1b**, **1c** and **1d** compound treatments showed much less fold activation of Nrf2 compared to their 10 μ M treatments?
9. Figures S4 and S5: In these two figures, the importance of the C10 to C17 moiety and the hydroxy group at C3 in the compound structure were demonstrated. However, the biotin-

isofalcarintriol (15) was inactive in HEK293 cells, conversely, it retained some activity in HepG2 cancer cells. What made this bioactivity discrepancy in both cell lines? In addition, what is the rationale to use an inactive compound probe to do protein pulldown experiment for their specific targeting/binding protein(s) in HEK293 cells?

10. Figure 3: In panel K, it needs to provide the cell image treated with azide488 probe alone to compare with the cells treated with IFT-azide488 conjugate. The concentrations used for both cell lines and *C. elegans* assays have 1000-fold differences (μM vs. nM), please explain the reason. Please delete one repeated word “including” in line 832.
11. Table S4: The experiment to analyze the spatial distribution of **1a** in *D. carota* should be described with details, such as the extraction conditions (volume ratio, time, temperature, etc). Why bother to create isotope labeled **1a** for this experiment? The calibration curve of concentration for **1a** can be established and used to quantify the compound content in the extracts. The numbers in Table S4 should be more clearly explained. Actually, this experiment and experimental data did not add significance to this study.
12. Scheme S1 content does not match with the description in lines 161-162 as “an extraction procedure and liquid chromatography–mass spectrometry (LC-MS) separation method”.... Scheme S2 is missing. Actually, the current scheme S1 content belongs to the missing scheme S2.
13. Table S5: Several identified proteins (more than 10) showed much higher fold changes than both ATP synthase subunits in mitochondria; however, those were not discussed or carried out further validation in this study. It is believed that the interesting observation for compound **1a** on promoting health or delaying signatures of aging would not just relate to inhibition of ATP levels, ATP synthase activity, mitochondria biogenesis, and other particularly mentioned activities in this study. To strengthen the novelty and the pharmacological effects and applications of compound **1a**, more extensive discussion and/or in-depth molecular/biological activity studies should be considered.
14. Figure S7: it is difficult to see the shape differences between the round and tubular-shaped mitochondria in isofalcarintriol-treated vs. DMSO-treated mitochondria, respectively. This data can be improved by using transmission electron microscopy (TEM) to examine the treated mitochondria shape/structure.
15. Figure 5: Which cells were used in panel A? In panel C: the bar of Ctl Oex meant for IFT treated group (the bar color is not correct)? The Figure S10 data can be merged into Figure 5 (panel I to K), as the effects on HepG2 and HT-29 cells were much significant than that on MCF-7 cells. The method of soft agar colony formation assay should be described in more detail.
16. Figure 6: what cells were used in panel F? Typo in line 905, two panel F indicated.
17. Figure 7: the compound dose was used 0.1 mg/kg/day in mouse experiments. How this dose was determined, and no dose-dependent effect of the compound was addressed in

this *in vivo* mouse study. In panel R, lower lymphocytes numbers could not be referred directly to decrease age-related inflammation in female mice, as a mixture of immune cells was contained in lymphocytes. To refer compound effect on age-associated inflammation, proinflammatory cytokines could be measured in mouse plasma or serum.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

The authors have responded to most of my questions, made necessary amendments to the revised manuscript, and provided abundant supplementary information as requested; however, one last question to be discussed below:

Ethyl acetate and ethanol are two different polarity solvents, so the chemical ingredients to be extracted by them could be quite different. Why these two solvents were used to do crude extract preparation from carrots, and what were their initial concentration (%) used? What were the total yields of the EA and ethanol-extracted crude extracts? Typo in the paragraph of "10 Extraction and Quantification of Isofalcarintriol from *D. carota*" on page 61 of the Supplementary Information: The solid "mater"

Reply to Reviewers

Reviewer #1: no remarks to authors

Reviewer #2 (reviewer's comments in red)

The authors have responded to most of my questions, made necessary amendments to the revised manuscript, and provided abundant supplementary information as requested; however, one last question to be discussed below:

We thank the reviewer for their positive comments.

Ethyl acetate and ethanol are two different polarity solvents, so the chemical ingredients to be extracted by them could be quite different. Why these two solvents were used to do crude extract preparation from carrots, and what were their initial concentration (%) used? What were the total yields of the EA and ethanol-extracted crude extracts?

The chemical properties of the solvents used for extractions are indeed very different and have been selected to represent a wide range of extraction conditions used for extraction of previously reported falcarinol-type polyacetylenes from *D. carota* (pls see Supplementary Reference 4, Figure 2 within, exemplifying the differences between pentane and EA extraction methods).

Hence and initially, we have used three solvents for the extractions, namely pentane, EA and ethanol (pls see Supplemental Table 4 for details); only by using the latter two, 1a was obtained to a relevant extent, and EA was exclusively used for follow-up experiments (pls see Supplemental Table 4 for details).

Referring to the initial concentration of solvents used: For the extraction, 2 ml/g carrots of the respective solvent was used. This previously missing information has been added to the Supplemental Methods, page 3, first paragraph.

The respective yields for each solvent are also now provided in Supplemental Table 4.

Typo in the paragraph of "10 Extraction and Quantification of Isofalcarintriol from *D. carota*" on page 61 of the Supplementary Information: The solid "mater"

Thank you for bringing this to our attention; the corresponding typo has been corrected into "matter".