Bacteria modulate tamoxifen-induced death via host fatty acid metabolism

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SUMMARY

Tamoxifen is a selective estrogen receptor (ER) modulator that is used to treat ER-positive breast cancer, but that at high doses kills both ER-positive and ER-negative breast cancer cells. We recapitulate this off-target effect in *Caenorhabditis elegans*, which does not have an ER ortholog. We find that different bacteria dramatically modulate tamoxifen toxicity in *C. elegans*, with a three-order of magnitude difference between animals fed *Escherichia coli*, *Comamonas aquatica*, and *Bacillus subtilis*. Remarkably, host fatty acid (FA) biosynthesis mitigates tamoxifen toxicity, and different bacteria provide the animal with different FAs, resulting in distinct FA profiles. Surprisingly these bacteria modulate tamoxifen toxicity by different death mechanisms, some of which are modulated by FA supplementation and others by antioxidants. Together, this work reveals a complex interplay between microbiota, FA metabolism and tamoxifen toxicity that may provide a blueprint for similar studies in more complex mammals.

INTRODUCTION

The bacteria that inhabit our body, known as our microbiota, influence many biological processes in both health and disease, and greatly contribute to our metabolic capacity (Koppel et al., 2017; Spanogiannopoulos et al., 2016). The bacteria from the gut microbiota are among the first cells to encounter orally ingested nutrients and xenobiotics. Therefore, it is perhaps not surprising that the microbiota can also impact our response to medications (Koppel et al., 2017). Several mechanisms have been identified by which microbes influence the host's drug response. First, the microbial composition can change in response to drugs. Known as dysbiosis, such altered composition can affect the host's response to therapeutic drugs (Diot et al., 2018; Maier et al., 2018). Second, bacteria can alter host drug availability, for instance by drug sequestration or modification (Koppel et al., 2017; Zhang et al., 2021). Third, the metabolic crosstalk between the microbiota and its host can modify host physiology, and consequently drug efficacy (Selwyn et al., 2015). All these processes can change drug action and it is therefore important to systematically identify which bacteria modulate the efficacies of which drugs, and to dissect the mechanisms involved.

Systematic identification of microbial effects on the host's drug response in humans or mammalian model systems is challenging because of their complex diet and microbiota, as well as relatively low scalability. Recently, we and others, have developed the nematode *Caenorhabditis elegans* and its bacterial diet as an interspecies model system to rapidly assess the animal's response to different drugs and how this response can be modulated by different bacteria (Garcia-Gonzalez et al., 2017)(Rosener et al., 2020; Scott et al., 2017). The application of high-throughput genetic screens not only in

the host, but also in the bacteria it consumes, make this a powerful model (Diot et al., 2018; Garcia-Gonzalez and Walhout, 2017; Watson et al., 2013; Watson et al., 2014). Recent studies illustrate the insights on host-bacteria-drug interactions that can be obtained using this model system. In a screen with 11 chemotherapeutic drugs and two bacterial diets, we found that three drugs, 5-fluoro-2'-deoxyuridine (FUDR), 5-fluoro-uracil (5-FU) and camptothecin (CPT) elicited a reproductive phenotype, two of which were modulated by the two bacteria, in opposite directions (Garcia-Gonzalez et al., 2017). Specifically, a diet of *Escherichia coli* rendered the animals more sensitive to FUDR, but less sensitive to CPT, relative to a diet of *Comamonas aquatica* (Garcia-Gonzalez et al., 2017). We found that bacterial pyrimidine metabolism, specifically the generation of 5-fluorouridine monophosphate (FUMP), was critical to FUDR toxicity in the animal (Garcia-Gonzalez et al., 2017; Scott et al., 2017).

Tamoxifen is a triphenylethylene belonging to the class of drugs known as selective estrogen receptor modulators (SERMs). SERMs bind to and either activate or repress the estrogen receptor (ER), a nuclear hormone receptor (NHR) transcription factor (Shelly et al., 2008). Tamoxifen is commonly used to treat ER-positive breast cancers, as well as other estrogen-dependent ailments such as ovarian or endometrial carcinoma (Jordan, 2003; Shaguffa and Ahmad, 2018). A significant proportion of ER-negative breast cancers also appear responsive to tamoxifen, but the underlying biology remains to be uncovered (Manna and Holz, 2016). There has also been increasing clinical interest in the potential of tamoxifen for treating various other cancer types (Clifford et al., 2020). The potential repurposing of tamoxifen for the treatment of non-ER-positive breast

malignancies requires an understanding of the potential alternative mechanisms of action of this drug, especially in ER-negative models.

The *C. elegans* genome encodes more than 250 NHRs (Reece-Hoyes et al., 2005). Most of these are paralogs of HNF4 α and there is no ER ortholog. We find that tamoxifen is toxic in *C. elegans* at higher doses and that three bacterial species, *E. coli*, *C. aquatica*, and *Bacillus subtilis*, confer dramatically different levels of sensitivity to tamoxifen toxicity, with an EC₅₀ ranging three orders of magnitude. We identify a role for host fatty acid (FA) metabolism in modulating tamoxifen toxicity. We further find that the FA composition of *C. elegans* is greatly modulated by bacterial diet. Most strikingly, we find that different bacteria modulate *C. elegans* tamoxifen toxicity through different death mechanisms. Taken together, we show that a complex interplay between bacteria, FA metabolism and different mechanisms of death modulate tamoxifen toxicity in *C. elegans*.

RESULTS

Tamoxifen Kills both ER-Positive and ER-Negative Cancer Cells

We first confirmed that tamoxifen can kill ER-negative breast cancer cells (Bekele et al., 2016). Specifically, we compared the effects of tamoxifen on ER-positive T-47D cells, which rely on ER for proliferation, to the effects of tamoxifen on ER-negative MDA-MB-231 cells (Lacroix and Leclercq, 2004). Drug toxicity can be assessed through two parameters: growth rate and fractional viability. The growth rate (GR) index is calculated by the relative number of live cells over time in presence or absence of drug and integrates both cytostatic and lethal effects of a drug (Hafner et al., 2016). In contrast,

fractional viability only considers the lethal effect of a drug (Schwartz et al., 2020). We first calculated the GR index and, as expected, observed a decrease in the growth rate of T-47D cells incubated with tamoxifen in a dose-dependent manner (**Figure 1A**). While lower doses of tamoxifen did not affect the growth rate of MDA-MB-231 cells, we found that concentrations greater than 10 μ M resulted in a reduction in the population growth rate, indicating either a reduced cell proliferation rate or increased cell death rate (**Figure 1A**). When we computed fractional viability, we found that a dose of tamoxifen greater than 10 μ M, killed both T-47D and MDA-MB-231 cells effectively (**Figure 1B**). This indicates that tamoxifen toxicity relies on two distinct mechanisms depending on the dose: at low doses, tamoxifen slows the growth of ER-positive, but not ER-negative cells, whereas at high doses, tamoxifen kills independently of whether the cells express ER. Since this cell death does not depend on ER, it can be considered an off-target effect.

Bacteria Differentially Modulate Tamoxifen Toxicity in *C. elegans*

C. elegans does not have an obvious ER ortholog (Reece-Hoyes et al., 2005; Taubert et al., 2011). Therefore, we wondered whether we could use *C. elegans* to study off-target tamoxifen toxicity in an intact animal. Since we previously found that the bacterial diet consumed by *C. elegans* can greatly affect the response to chemotherapeutic drugs (Garcia-Gonzalez et al., 2017), we supplemented increasing doses of tamoxifen to animals fed either of three different bacterial diets: *E. coli*, *C. aquatica* or *B. subtilis*. Visual inspection showed dramatic differences in tamoxifen toxicity, depending on bacterial diet: there was little effect on animal development on *E. coli*, while animals fed *C. aquatica* displayed developmental arrest or delay at high drug concentrations, and animals fed

B. subtilis were exquisitely sensitive to tamoxifen, even at low micromolar concentrations (Figure 1C). We observed many arrested first larval stage (L1) animals in the presence of high concentrations of tamoxifen, and therefore quantified tamoxifen toxicity by counting the proportion of animals that failed to develop at different doses of the drug. The resulting dose response curves (DRCs) confirmed that tamoxifen is more than three orders of magnitude more toxic to animals fed *B. subtilis* than to animals fed *E. coli* (Figure 1D). Together, these results show that tamoxifen is toxic to *C. elegans* at high doses and that bacteria greatly modulate this toxicity.

***C. elegans* Fatty Acid Synthesis Protects Against Tamoxifen Toxicity**

When fed *E. coli*, tamoxifen is only toxic to *C. elegans* at high micromolar doses (Figure 1D). We hypothesized that *C. elegans* metabolism may modulate tamoxifen toxicity. Therefore, we screened an RNAi library of 1,495 *C. elegans* metabolic genes (in *E. coli*) for increased or decreased drug toxicity. This library includes genes with known metabolic functions that make up the genome-scale iCEL1314 metabolic network model, as well as other genes that are predicted to encode metabolic enzymes (Yilmaz et al., 2020; Yilmaz and Walhout, 2016). We did not find any genes that, upon RNAi knockdown, rendered the animals less sensitive to tamoxifen. We did, however, identify three genes for which RNAi knockdown led to increased tamoxifen toxicity (Figure 2A). These genes include *dhs-19*, which encodes a short-chain dehydrogenase of unknown function that localizes to lipid droplets (Liu et al., 2018) and is predicted to function in retinol metabolism (Yilmaz et al., 2020). The other two genes, *elo-3* and *elo-6*, both encode elongases predicted to be involved in long chain FA biosynthesis (Walker et al., 2021; Watts, 2009). We

generated a *dhs-19* deletion mutant by CRISPR/Cas9 genome editing and confirmed that this strain is more sensitive to tamoxifen (**Figure 2B, Figure S1**).

While mutant strains are available for *e/o-3* and *e/o-6*, we could not use these animals because their development is strongly impaired even in absence of drug exposure (data not shown). Both *e/o-3* and *e/o-6* function in FA biosynthesis (**Figure 2C**). We wondered if these two genes specifically affect tamoxifen toxicity, or if FA metabolism is more broadly involved even though no other genes were captured in the RNAi screen. To test this, we performed full tamoxifen DRC experiments for the knockdown of 33 genes encoding enzymes predicted to be involved in FA elongation and desaturation (Walker et al., 2021)(**Figure 2C**). In addition, we included eight genes encoding acyl-CoA synthetases that catalyze the addition of co-enzyme A (CoA) to FAs and eight genes encoding enzymes that remove CoA. We calculated the difference between the area under the curve (Δ AUC) for each RNAi condition + tamoxifen relative to *E. coli* (**Figure 2D**). Statistical significance was evaluated on the original DRCs (**Figure S2, Tables S1 and S2**). Remarkably, knockdown of most FA biosynthesis genes tested significantly increased tamoxifen toxicity (**Figure 2E, Figure S2, Table S1**). These results show that *C. elegans* FA synthesis is broadly involved in mitigating tamoxifen toxicity. This points to a systems-level involvement of FA biosynthesis rather than a single FA species.

Next, we asked whether genes involved in mitochondrial and peroxisomal FA degradation also modulate tamoxifen toxicity and found that, of 33 genes tested, only *ech-8* appeared to increase tamoxifen toxicity, but this effect was not statistically significant (**Figure 2F, Figure S3**). Knockdown of 13 of the 33 FA degradation genes tested led to a decrease in tamoxifen toxicity, with only *daf-22* RNAi being statistically

significant (**Table S2**). This result shows that the biosynthesis, but not degradation, of long chain FAs is critical to mitigate tamoxifen toxicity in *C. elegans*.

Bacterial Diets Modulate *C. elegans* Fatty Acid Profiles

Previously, it has been shown that different strains of *E. coli* diets affect the FA composition of *C. elegans* (Brooks et al., 2009). Since *C. elegans* FA synthesis modulates tamoxifen toxicity, we hypothesized that the three bacterial species used herein may elicit different FA profiles in the animal. To test this, we measured both bacterial and *C. elegans* FA profiles by gas chromatography-mass spectrometry (GC-MS) (**Table S3** and **S4**). The bacterial FA profiles showed dramatic differences: *E. coli* mainly contained the saturated FAs (SFAs) palmitic acid and stearic acid, cyclopropane FAs (CPFAs), and the mono-unsaturated FAs (MUFAs) oleic acid, palmitoleic acid and cis-vaccenic acid; *C. aquatica* mainly contained the MUFAs oleic acid and palmitoleic acid; and *B. subtilis* almost exclusively contains monomethyl branched chain FAs (mmBCFAs), which agrees with previous findings (Kaneda, 1977) (**Figure 3A**).

The FA profiles of *C. elegans* fed the three bacteria reflected the three bacterial diets in some but not all respects. One striking observation was that the proportion of each of the different poly-unsaturated FAs (PUFAs) is diet-independent (**Figure 3B**). This indicates that the animal can effectively convert different dietary FAs into PUFAs by elongation and/or desaturation. Oleic acid is the primary substrate for PUFA synthesis and is present in *E. coli* and *C. aquatica* but absent in *B. subtilis* (**Figure 3A**). This could indicate that PUFAs are generated by a combination of FA metabolic steps that yield this

even-chained MUFA from odd-chained mmBCFAs in animals fed *B. subtilis* or synthesizes them de novo.

Another striking observation is that most other types of FAs are quite different in proportion in *C. elegans* fed different bacterial diets. Most notable are the near absence of MUFAs, and presence of C15 and C17-ante-iso mmBCFAs in animals fed *B. subtilis* (**Figure 3B**). The differences between animals fed the other two bacteria are more subtle, with a greater proportion of the MUFA cis-vaccenic acid in *C. aquatica*-fed animals, relative to those fed *E. coli* (**Figure 3B**). Altogether, the different bacterial diets result in a different proportion of the different types of FAs: high overall levels of MUFAs in animals fed *E. coli* or *C. aquatica* and high overall levels of mmBCFAs in animals fed *B. subtilis* (**Figure 3B**, bottom). Taken together, these results show that *C. elegans* maintains its PUFA content in a narrow and well-defined regime, while it can tolerate different proportions of the other types of FAs.

Dietary Fatty Acids Modulate Tamoxifen Toxicity

Our findings so far lead us to consider two models: either tamoxifen treatment could alter the animal's FA profile, leading to diet-dependent toxicity, or differences in *C. elegans* FA composition elicited by bacterial diet may modulate tamoxifen toxicity. To discriminate between these two models, we first measured bacterial and *C. elegans* FAs in the presence or absence of tamoxifen and found that the drug did not modulate the animal's FA profiles (**Table S5**). Thus, we did not find evidence to support the first model.

To evaluate the second model, we first asked whether supplementation of a FA cocktail, containing equimolar amounts of the PUFAs arachidonic and linoleic acid, the

SFAs lauric and myristic acid, and the MUFA oleic acid (Sharma et al., 2013), would modulate tamoxifen toxicity in animals fed either of the three bacterial diets. We found that FA cocktail supplementation suppressed tamoxifen toxicity but only in animals fed *C. aquatica*; it had no effect on tamoxifen toxicity in animals fed *E. coli* or *B. subtilis* (**Figure 4A-C**). Next, we tested the effects of the supplementation of individual FAs and found that MUFAs such as cis-vaccenic acid increased tamoxifen toxicity on animals fed *E. coli*, but not *C. aquatica* (**Figure 4D and 4E, Figure S5**). Since *C. aquatica*-fed animals contain more cis-vaccenic acid than *E. coli*-fed animals (33% vs 20%, **Figure 3B**), this suggests that this difference may contribute to increased tamoxifen toxicity on the *C. aquatica* diet.

Supplementation of individual PUFAs such as α -linoleic acid decreased tamoxifen toxicity on animals fed *C. aquatica*, (**Figure 4F and 4G, Figure S5**). On an *E. coli* diet PUFAs had no effect. However, animals are generally tolerant to tamoxifen, which limits the ability to observe decreased tamoxifen toxicity. Since the proportion of PUFAs is similar in animals fed each of the three bacterial diets (**Figure 3B**), this indicates that the ratio of PUFAs to other FAs is more relevant than absolute levels. Surprisingly, supplementation of either MUFAs or PUFAs abolished the growth of *B. subtilis*, and therefore these FA could not be tested for tamoxifen toxicity modulation in *C. elegans* fed these bacteria (**Figure 4H and 4I**). Together with the observation that knockdown of genes involved in FA synthesis increases tamoxifen toxicity, these results point to a complex interplay between dietary FA, FA synthesis and tamoxifen toxicity.

The FA cocktail is stabilized by vitamin E, a potent antioxidant that inhibits FA oxidation (Traber and Atkinson, 2007). Therefore, we performed a control experiment by supplementing only vitamin E. We found that vitamin E had no effect on tamoxifen toxicity

in animals fed either *E. coli* or *C. aquatica*, indicating that it is the FAs in the FA cocktail that mitigate toxicity in animals fed *C. aquatica*. To our surprise, we found that vitamin E supplementation did suppress tamoxifen toxicity in animals fed *B. subtilis* (**Figure 4J-L**). However, N-acetylcysteine, another antioxidant that can rescue the growth of *C. elegans* on iron-deficient *E. coli* mutants (Zhang et al., 2019) did not affect tamoxifen toxicity (**Figure 4M**). These results indicate that FA oxidation, but not the general accumulation of reactive oxygen species (ROS), affects tamoxifen toxicity in *C. elegans* when fed *B. subtilis*. Since FAs rapidly turnover in the animal's membranes (Dancy et al., 2015), the observation that FA biosynthesis mitigates tamoxifen toxicity (**Figure 2E**) could be explained because it replenishes oxidized FAs.

Taken together, these results indicate that FA or vitamin E supplementation can mitigate tamoxifen toxicity differently depending on which bacteria the animals are fed, which suggests that different, diet-dependent toxicity mechanisms are involved.

Bacteria Elicit Different Tamoxifen-Induced Death Mechanisms

Lipid metabolism has been linked to different mechanisms of cell death, especially apoptosis and ferroptosis (Galluzzi et al., 2018; Garbarino and Sturley, 2009). In fact, it has been shown that cell survival is dependent on lipid homeostasis, and both excessive MUFAs or SFAs have been shown to be pro-apoptotic, depending on the cell lines (Garbarino and Sturley, 2009). Further, several MUFAs have been reported to protect against ferroptosis (Magtanong et al., 2019), which is characterized by lipid peroxidation, while others, specifically cis-vaccenic acid have been reported to induce cell death (Nakamura et al., 2018). PUFAs, most notably di-homo-gamma-linoleic acid, have been

shown to induce ferroptosis in both human cells and in *C. elegans* (Perez et al., 2020). Given these connections, we asked whether apoptosis contributes to tamoxifen toxicity in *C. elegans*. To test this, we used *ced-3(n717)* mutant animals that cannot execute apoptosis, because they harbor a substitution in the acceptor splice site of exon 7 encoding for the caspase p10 domain of the CED-3 protein (Yuan et al., 1993).

Remarkably, loss of *ced-3* greatly increased sensitivity to tamoxifen in animals fed either *E. coli* or *C. aquatica* but had no effect on animals fed *B. subtilis* (**Figure 5A-C**). This result suggests that, when fed *E. coli* or *C. aquatica*, tamoxifen toxicity employs apoptosis, and that *ced-3* mutant animals switch to another, more potent mode of death that is suppressed when apoptosis is functional. We additionally observed that a high dose of vitamin E could mitigate tamoxifen toxicity in *ced-3* mutant animals fed *C. aquatica* but not *E. coli* (**Figure 5A and 5B**). This indicates that, in the absence of *ced-3*, tamoxifen toxicity in animals fed *C. aquatica* involves FA oxidation, similar to what we observed with wild type animals fed *B. subtilis*.

Finally, we used expression profiling by RNA-seq to ask whether tamoxifen supplementation results in the modulating the expression of genes involved in FA metabolism or oxidative stress in *C. elegans*. As discussed above, tamoxifen does not affect the animal's FA profiles and accordingly, the expression of FA biosynthesis or degradation genes was not systematically altered upon tamoxifen exposure, with the exception of *fat-5* which showed a three-fold increase, and *ech-6* which was reduced by ~two-fold (**Figure 5D**). We did, however identify increased expression of R05D8.9 (**Figure 5D**), which encodes a predicted 17 β -hydroxysteroid dehydrogenase 14 (17 β HSD14) homolog. In humans, 17 β HSD14 enzymes are associated with the response

to tamoxifen in breast cancer (Sivik et al., 2012). These enzymes are further known to interconvert different steroids, suggesting that R05D8.9 may be directly involved in the detoxification of tamoxifen in *C. elegans*. In addition, the expression of several CYP genes, which encode cytochrome P450 enzymes, is induced by tamoxifen supplementation (**Figure 5D**). Cytochrome P450 enzymes have been reported to modify tamoxifen in humans (Singh et al., 2011), and are a source of oxidative stress as their enzymatic cycle involves significant leakage of electrons (Hrycay and Bandiera, 2015). Taken together, tamoxifen does not transcriptionally rewire FA metabolism. In contrast, it induces the expression of ROS-generating detoxifying enzymes, that may contribute to FA oxidation.

DISCUSSION

Tamoxifen Off-Target Mechanisms: Fat Metabolism, Oxidative Stress and Microbiota

In this study, we present *C. elegans* as a model to study off-target, ER-independent, mechanisms of tamoxifen toxicity using concentrations in the range of drug-levels accumulating in patients (Kisanga et al., 2004; Robinson et al., 1991). We found that FA biosynthesis and composition of the animals are major drivers of tamoxifen toxicity, and that this toxicity relies on the potential of the drug to induce oxidative stress and lipid peroxidation. Moreover, we found that both the mechanism and the degree of tamoxifen toxicity are greatly modulated by bacteria, where *B. subtilis* induces a non-apoptotic

mechanism that is three orders of magnitude more severe than apoptotic mechanism elicited by tamoxifen in the presence of either *E. coli* or *C. aquatica* (**Figure 6**).

In the *C. elegans* model, bacteria form a source of nutrition, can be pathogenic, and can inhabit the gut as the animal ages (Felix and Braendle, 2010). Both in the laboratory and in the wild, the animals are constantly exposed to bacterial metabolism which provides nutrients and contributes to animal physiology (Yilmaz and Walhout, 2014). Our findings illuminate the possible effects the microbiota may have on tamoxifen off-target effect toxicity and the treatment of ER-independent malignancies (Clifford et al., 2020). It is tempting to speculate that tamoxifen activity could be modulated by perturbations of the microbiota using antibiotics or probiotics. Notably, *B. subtilis*, which is present in a large proportion of commercially available probiotics (Joerger and Ganguly, 2017) may affect patients treated with tamoxifen. Our findings of the role of FA metabolism and oxidative stress further suggest that studies of nutritional intake of fats and vitamin E in humans may shed light on whether the off-target mechanisms discovered herein are conserved.

FA Modulation of Tamoxifen Toxicity

We uncovered complex relationships between bacteria, host FA metabolism, apoptosis, and tamoxifen toxicity. The literature about the relationship between FAs and cell survival integrates two major components: distinct FA-species display different oxidation potentials (Gardner, 1989), and different potentials to induce alternative cell-death mechanisms (Garbarino and Sturley, 2009; Magtanong et al., 2019). Our data using lipophilic ROS-scavenging vitamin E and apoptosis-deficient animals suggest that

tamoxifen-toxicity relies on these two entangled components. MUFAs are generally described as ‘healthy’ FAs, as they buffer the oxidative stress of the ROS-sensitive PUFAs (Magtanong et al., 2019). To our knowledge, there is only one report on the effects of the MUFA cis-vaccenic acid, which found that cis-VA accumulation inhibits cell survival, which is opposite to other MUFAs tested in parallel (Nakamura et al., 2018). Overall, the literature is consistent with the observation of extreme tamoxifen toxicity in *B. subtilis*-fed animals that do not provide the animal with MUFAs, and in *C. aquatica*-fed animals that accumulate cis-vaccenic acid. Further studies are required to systematically determine the effects of different FAs on cell survival and tamoxifen toxicity in human cells.

***dhs-19*: a Potential Integrator of FA Metabolism and Oxidative Stress**

Knockdown of or mutations in *dhs-19* enhance tamoxifen toxicity in animals fed *E. coli*. The precise molecular or metabolic function of DHS-19 is unknown. It encodes a dehydrogenase and is a marker of lipid droplets (Zhang et al., 2012), which are essential for FA homeostasis in all eukaryotes (Olzmann and Carvalho, 2019). Based on enzyme homology, DHS-19 is predicted to act in retinoid metabolism, through the interconversion of retinol and retinal (Walker et al., 2021). This reaction precedes the synthesis of retinyl palmitate which consumes palmitoyl-CoA, which is a precursor for the synthesis of palmitoleic and vaccenic acid, both of which potentiate tamoxifen toxicity in *C. elegans* when animals are fed *E. coli*. Retinoids are also potent ROS scavengers and have been shown to act synergically with vitamin E to reduce lipid oxidative stress (Tesoriere et al., 1993). Together, this suggests that the increase in tamoxifen toxicity by *dhs-19*

perturbation could result from a complex integration of metabolic cues involving oxidative stress and retinoid metabolism.

You are What you Eat: *C. elegans* FA Composition is Determined by its Diet

C. elegans is a remarkable animal with a fixed lineage in which wild type animals develop deterministically to adults with precisely 959 somatic nuclei (Sulston and Horvitz, 1977; Sulston et al., 1983). Therefore, one would assume that all adult animals are essentially the same. Here we show that wild type animals can exhibit marked differences in their FA composition, depending on which bacterial diet they are fed. To our knowledge, *C. elegans* FA composition has only been studied in animals fed *E. coli*, and the profiles observed in our experiments are consistent with the only available previous study (Brooks et al., 2009). Here, we observe much more drastic changes in FA composition in animals fed *C. aquatica* or *B. subtilis*, relative to those fed *E. coli*. First, the MUFA, mmBCFA or CPFAs content of the animals strongly reflects the dietary input; a diet of *C. aquatica* is rich in MUFAs, and a diet of *B. subtilis* is rich in mmBCFAs, and these FAs accumulate in the animals. This result demonstrates that the animal's membranes are permissive to varying levels of these types of FAs. In contrast, the PUFA composition of *C. elegans* is largely independent of its bacterial diet. While PUFAs are essential dietary FAs in humans, *C. elegans* synthesize PUFAs de novo (Watts and Browse, 2002). This result indicates that *C. elegans* PUFA levels are sensed, and that metabolic fluxes in FA biosynthesis pathways are adjusted to maintain relative levels in a tight regime. It is therefore perhaps not surprising that PUFA deficiencies have been associated with developmental and neuronal defects in *C. elegans* (Connor and Watts, 2019). Future

studies are required to reveal to what the extent wild type *C. elegans* exhibit other fitness changes in response to differences in FA composition.

Cell Death and Organismal Death

How does cell-death connect to organism death? Fifty years after Horvitz and Sulston first described apoptosis in *C. elegans* (Sulston and Horvitz, 1977), it remains unclear how cell death mechanisms communicate and how they relate to organismal phenotypes including death. Other open questions that remain to be answered are: What is the exact nature of the triggers directing decisions in the induction of alternative cell-death mechanisms? And how do bacteria affect cell-death potentials? This study provides a foundation for future studies to address fundamental questions related to cell-death and organismal fate.

ACKNOWLEDGEMENTS

We thank members of the Walhout lab and Job Dekker for discussion and critical reading of the manuscript. This work was supported by grants from the National Institutes of Health DK068429 and GM082971 to A.J.M.W., GM127559 to M.J.L., AG058950 to C.O.P. and GM122393 to A.G.G. Several bacterial and nematode strains used in this work were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440).

AUTHOR CONTRIBUTIONS

C.D., A.G.G. and A.J.M.W. conceived the study. C.D. and A.G.G. performed most experiments with technical help from M.W., H.D., Y.R. and A.F.V. performed the fatty acid measurements under supervision of C.P.O. H.N. engineered the $\Delta dhs-19$ mutant strain. M.H. and C.D. performed the mammalian cell experiments under the supervision of M.L. H.Z. and C.D. performed and analyzed the RNA-seq experiments. The manuscript was written by C.D., and A.J.M.W. with editing by all other authors.

DECLARATION OF INTERESTS

All authors have no competing interests.

FIGURE LEGENDS

Figure 1. Tamoxifen Kills ER-Negative Breast Cancer Cells and Animals

(A, B) The growth rate index (A) and fractional viability (B) of ER-positive (T-47D) and ER-negative (MDA-MB-231) breast cancer cells plotted as a function of increasing concentrations of tamoxifen.

(C) Bright-field images showing *C. elegans* supplemented with increasing doses of tamoxifen, fed *E. coli*, *C. aquatica*, and *B. subtilis*. Images were taken at 2x magnification after 48h exposure to tamoxifen.

(D) Dose response curves of *C. elegans* supplemented with increasing doses of tamoxifen, fed *E. coli*, *C. aquatica*, and *B. subtilis*. Data are represented as mean $\pm$ SEM of three biological replicates.

Figure 2. Host Long Chain Fatty Acid Synthesis Modulates Tamoxifen Toxicity

(A) Screening a metabolic gene RNAi library identified three genes (*dhs-19*, *elo-3* and *elo-5*) that, when knocked down, increase TAM toxicity. Animals were fed *E. coli* HT115 expressing double stranded RNA as indicated. Control indicates *E. coli* containing vector control plasmid (pL4440). Bright-field images were taken at 2x magnification after a 48h exposure to tamoxifen.

(B) Tamoxifen dose response curves comparing $\Delta dhs-19$ mutant animals to wild type animals. See also **Figure S1**.

(C) *C. elegans* fatty acid biosynthesis pathway. Gene names are colored by enzymatic function: FA elongation in blue, FA desaturation in pink, and acyl-CoA synthesis in purple.

(D) Cartoon illustrating calculation of differential area under curve (ΔAUC) used in panels E and F. AUCs were calculated using average DRCs and statistical significance was assessed by performing 2way anovas using Graphpad Prism (v9) on original DRCs and are reported in **Table S1** and **S2**. See also **Figures S2** and **S3**.

(E, F) Bar graph showing ΔAUC values obtained from average full DRCs for animals exposed to RNAi of indicated genes.

Figure 3. Long Chain Fatty Acid Profiles of Bacteria and *C. elegans* Fed Different Bacteria

(A, B) Bubble plots showing long chain fatty acid proportions measured by GC-MS in the indicated bacteria (A), and animals fed the indicated bacteria (B). Bubble sizes are indicative of FA ratios within the sample of interest and are not indicative of absolute quantities. Ratios are available in **Table S3** and **S4**. Data are represented as mean of

three to six biological replicates. Statistical pairwise comparisons are available in **Table S5**.

Figure 4. Long Chain Fatty Acid Supplementation Modulates Tamoxifen Toxicity

(A-M) Unless otherwise indicated, fatty acids and vitamin E were supplemented to bacterial culture medium, as described previously (Devkota et al., 2021).

(A-C) DRCs of tamoxifen toxicity with and without FA cocktail supplementation on animals fed *E. coli* (A), *C. aquatica* (B) or *B. subtilis* (C).

(D,E) DRCs of tamoxifen toxicity with and without PUFA (ALA) supplementation on animals fed *E. coli* (D) or *C. aquatica* (E).

(F) Growth curves of *B. subtilis* supplemented with PUFAs ALA or GLA. For clarity, no error bars are reported in the graph, n=3.

(G,H) DRCs of tamoxifen toxicity with and without MUFA (VA) supplementation on animals fed *E. coli* (G) or *C. aquatica* (H).

(I) Growth curves of *B. subtilis* supplemented with MUFAs VA, PA, or OA. No error bars are reported in the graph, n=3.

(J-L) DRCs of tamoxifen toxicity with and without vitamin E supplementation on animals fed *E. coli* (J), *C. aquatica* (K) or *B. subtilis* (L).

(M) DRCs of tamoxifen toxicity with and without the antioxidants vitamin E and N-acetyl-cysteine (NAC). Culture: NAC was supplemented in the bacterial culture medium as for vitamin E. Medium: NAC was supplemented to the NGM.

DRC: dose response curve, FA: fatty acid, MUFA: mono-unsaturated FA, PUFA: poly-unsaturated FA, ALA: alpha-linoleic acid, GLA: gamma-linoleic acid,

DGLA: di-homo-gamma-linoleic acid, VA: cis-vaccenic acid, PA: palmitoleic acid, OA: oleic acid. Statistical analysis of DRCs was conducted by performing 2-way Anova using GraphPad Prism (v9) and are reported in **Table S6**. Larger panel of fatty acid supplements was tested in **Figure S5**.

Figure 5. Bacteria Affect the Modulation of Tamoxifen Toxicity by Apoptosis

(A) Bright-field images (left) and bar graph (right) of wild type and $\Delta ced-3$ animals fed *E. coli*.

(B) Bright-field images (left) and bar graph (right) of wild type and $\Delta ced-3$ animals fed *C. aquatica*.

(C) Bright-field images (left) and bar graph (right) of wild type and $\Delta ced-3$ animals fed *B. subtilis*.

(D) Volcano plot distribution of genes which expression changed significantly in animals fed *E. coli* and exposed to 400 μ M tamoxifen (FDR<0.1, >2-fold change). Pink indicates genes encoding enzymes participating in FA metabolism, and red indicates genes encoding for enzymes that may be involved in the detoxification of tamoxifen.

Figure 6. Model of Interplay Between Bacteria, Fatty Acid Metabolism, and Death Pathways Affecting Tamoxifen Toxicity

STAR * METHODS

RESOURCE AVAILABILITY

Lead contact

Further information and requests for reagents may be directed to, and will be fulfilled by the corresponding author A.J.M. Walhout (marian.walhout@umassmed.edu).

Materials availability

All requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact. All reagents will be made available on request after completion of a Materials Transfer Agreement.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

C. elegans

C. elegans N2 (Bristol) was used as the wild-type strain. Prior to all experiments, animals were maintained on Nematode Growth Media (NGM) and fed a diet of *E. coli* OP50. The *C. elegans ced-3(n717)* mutant strain was kindly provided by the Francis lab (UMass Medical School) and was first described in (8598288). The *C. elegans dhs-19(VL1313)* mutant strain was generated using CRISPR-Cas9 genome editing (Kim et al., 2014).

Bacterial Strains

Escherichia coli BW25113, *E. coli* HT115, *Comamonas aquatica* DA1877, and *Bacillus subtilis* sp168 were grown in LB, overnight at 37°C to stationary phase under agitation (200 rpm). The dietary effects on tamoxifen toxicity described herein were independent of the *E. coli* strain tested. Experiments were conducted using *E. coli* BW25113, unless stated otherwise.

508

509 **METHOD DETAILS**

510 **Chemotherapeutic Drugs containing NGM preparation**

511 Tamoxifen citrate (Selleckchem, S1972) was dissolved to 150 mM in sterile dimethyl
512 sulfoxide (DMSO), aliquoted and stored at -20°C. Stock solutions were diluted in series
513 to 200X the final concentration and added at a final volume of 0.5% to NGM agar heated
514 to 55°C. Drug containing NGM plates were dried overnight at room temperature. NGM
515 was supplemented with antibiotics for experiments in 96-well plate format, and 2 mM
516 IPTG for RNAi experiments. 96-well and 48-well plates were seeded with 15 µL and 30
517 µL of bacterial cultures, respectively. Plates were then dried for 6h at room temperature
518 and kept overnight at room temperature before being used. *E. coli* cultures were
519 concentrated 1-2X, and *C. aquatica* DA1877 and *B. subtilis* sp168 strains were
520 concentrated 2-4X prior to seeding onto NGM agar plates, to normalize for post-seeding
521 bacterial growth.

522

523 **RNAi screen**

524 We tested a collection of 1,645 individual *E. coli* HT115 strains expressing dsRNA against
525 a unique *C. elegans* metabolic gene, and originating from the ORFeome (Rual et al.,
526 2004) or the Ahringer (Kamath et al., 2003) libraries. The screens were performed using
527 96-well plates containing NGM supplemented with tamoxifen, 50 µg/mL ampicillin, and 2
528 mM IPTG. We used a low dose of 300 µM tamoxifen to identify knockdowns that
529 aggravate tamoxifen toxicity, and a high dose of 600 µM tamoxifen identify knockdowns
530 that mitigate tamoxifen toxicity. Approximately 20 L1-arrested animals were added to

each well. Two days later, hits were scored visually using a dissecting microscope as knockdowns leading to a difference in animal drug response relative to animals fed an *E. coli* strain containing the empty dsRNA vector backbone (pL4440).

***C. elegans* Dose-Response Curves**

Dose-response curves (DRCs) were performed in 48-well plates with six to twelve technical replicates. Fifty to one hundred L1-arrested animals were plated onto each well and incubated at 20°C for 48 hours. Animals that developed beyond the L1 stage were counted under a dissecting microscope. The percentage of animals that did not developed was calculated relative to the total number of animals developed in the control condition. For Δ AUCs in Figures 3D-F, we used the average AUCs from the DRCs of the *E. coli* BW25113 and *E. coli* HT115 pL4440 control conditions.

Bacterial Growth Curves

Bacteria were first grown overnight in LB at 37°C and shaking at 200 rpm. Next, bacterial cultures were diluted 1:200 and grown in LB supplemented with 1% of FA supplements (or ethanol vehicle as a control). Cultures were then grown for 20h at 37°C and shaking at 200 rpm, in an Eon™ Microplate Spectrophotometer (BioTek) and absorbance at 600 nm was measured every 900 s.

Fatty Acid and Vitamin E supplementations

Undiluted fatty acid cocktail and PUFAs solutions were supplemented at 1% v.v. in LB for overnight growth. MUFAs were first diluted 1:10 in ethanol and supplemented at 1% v.v.

in LB for overnight growth. Undiluted vitamin E was supplemented from 0.1% to 10% in LB for overnight growth.

***C. elegans* FA Measurements**

Fatty acid measurements in bacteria and animals were performed as described before (Perez and Van Gilst, 2008). Briefly, lipids were extracted from whole animals using a 2:1 chloroform:methanol mixture and dried in liquid nitrogen. A calibrated phospholipid or triacylglycerol standard was added to the extracted lipids before separation by solid-phase exchange chromatography. Purified lipids were then converted into fatty acid methyl esters by incubation with methanol/2.5% H₂SO₄ before GC/MS analysis. All data are presented as percentage of the total fraction of fatty acids measured in the sample.

Mammalian Cell Culture

MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (Corning, 10-017-CV). T-47D cells were grown in RPMI-1640 media (Gibco, 11875119). All cell culture media were supplemented with 10% FBS (Hyclone, SH30910.03, lot AYG161519), 2 mM glutamine (Corning, 25-005-CI), and penicillin/streptomycin (Corning, 30-002-CI). Cells were cultured in a humidified incubator at 37°C with 5% CO₂. In addition, cell lines were maintained at low passage numbers and regularly tested for mycoplasma.

Mammalian Drug Sensitivity Assays

Measurement of lethal fraction (LF), fractional viability (FV), and normalized growth rate inhibition (GR) were performed using the FLICK assay as previously described (Richards et al., 2021). Briefly, cells were seeded in 96-well optical-bottom black-walled plates (Greiner Bio-One, 655090) in 90 μ L of media and allowed to adhere overnight. Density was optimized for each cell line depending on the growth rate of the cells and the duration of the assay. Dilutions of tamoxifen (Selleckchem, S1972) were prepared at 10x final concentration in media containing 50 μ M SYTOX green (Thermo Fisher Scientific, S7020). 10 μ L of drug combinations were supplemented to the cell culture media and fluorescence signals (excitation = 504 nm, emission = 523 nm) were recorded at the indicated time points on a Tecan Spark multimode plate reader. Total cell fluorescence was evaluated by lysing untreated cells at the start and experimental conditions at the end of the assay, using a supplement of 0.1% Triton X-100 (Fisher Scientific, BP151-500) diluted in PBS.

Gene expression profiling by RNA-seq

Total RNA was extracted using TRIzol (ThermoFisher), followed by DNase I (NEB) treatment and purified using the Direct-zol RNA mini-prep kit (Zymo research). RNA quality was verified by agarose gel electrophoresis. Briefly, multiplexed libraries were prepared using Cel-seq2 (Hashimshony et al., 2016). Two to three biological replicates were sequenced with a NextSeq 500/550 High Output Kit v2.5 (75 Cycles) on a Nextseq500 sequencer. Paired end sequencing was performed; 13 cycles for read 1, six cycles for the illumina index and 73 cycles for read 2. Analysis were performed as

previously detailed (Giese et al., 2020), using a homemade DolphinNext pipeline (Yukselen et al., 2020).

DATA AND CODE AVAILABILITY

RNA-seq data have been deposited at GEO.

This study does not report original code.

SUPPLEMENTAL INFORMATION

Supplemental information includes five Supplemental Figures and six Supplemental Tables.

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Diot, et al. Figure 1

Diot *et al.* Figure 6