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Citation: Genes & Genetic Systems (2016), 91(4): 201-207

Issue Date: 2016

URL: http://hdl.handle.net/2433/230413

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Type: Journal Article

Textversion: publisher

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FUdR extends the lifespan of the short-lived AP endonuclease mutant in *Caenorhabditis elegans* in a fertility-dependent manner

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(Received 14 September 2015, accepted 24 May 2016; J-STAGE Advance published date: 31 August 2016)

The anticancer drug 5-fluorouracil (5-FU) and its metabolite 5-fluoro-2′-deoxyuridine (FUdR) inhibit thymidylate synthase and induce uracil bases in DNA. FUdR is commonly used for inhibiting fertility when measuring the lifespan of the nematode *Caenorhabditis elegans*. However, it is not known whether DNA damage induced by FUdR affects lifespan. EXO-3 is an apurinic/apyrimidinic endonuclease in *C. elegans*, and we reported previously that deletion of the *exo-3* gene causes reproductive abnormalities and decreased lifespan. In this study, we found that FUdR extended the lifespan of *exo-3* mutants. We measured the lifespan of multiple germline mutants to examine whether this lifespan extension effect was dependent on fertility. In the presence of a *fem-1* mutation, which causes a deficiency in sperm production, FUdR did not extend the lifespan of the *exo-3* mutant. In *glp-1* mutants, which do not develop gonads, the *exo-3* mutant was not short-lived, and FUdR did not extend its lifespan. These results suggest that the lifespan extension effect of FUdR depends on fertility and the presence of gonads. *fem-3* mutants, which do not produce oocytes, had increased lifespan in the presence of FUdR, independent of the *exo-3* mutation. It is possible that the *fem-3* mutant was susceptible to the lifespan extension effect of FUdR. From these results, we suggest that FUdR affects the lifespan of *C. elegans* in two ways: by interfering with fertility, which extends lifespan, and by inducing DNA base damage, which reduces lifespan.

**Key words:** 5-fluoro-2′-deoxyuridine (FUdR), *Caenorhabditis elegans*, *exo-3*, fertility, lifespan

**INTRODUCTION**

5-fluorouracil (5-FU) is an antimetabolite drug used in cancer treatment (Longley et al., 2003; Kim et al., 2009). It induces base damage by being incorporated into DNA and RNA (McNeill et al., 2009). 5-fluoro-2′-deoxyuridine (FUdR) is a metabolite of 5-FU. It binds to thymidylate synthase and strongly inhibits its activity. Thymidylate synthase is an enzyme that converts dUMP to dTMP. This is the sole synthetic pathway for thymine in the cell. Therefore, DNA replication and cell growth are strongly inhibited in the presence of 5-FU and FUdR. In addition, FUdR and 5-FU treatment increase uracil misincorporation into DNA, because inhibition of thymidylate synthase causes accumulation of dUMP.

DNA base damage such as 5-FU and uracil is mainly processed by base excision repair (BER). In this pathway, DNA glycosylases recognize damaged bases in DNA, and excise them by cleaving the N-glycosidic bond to produce an apurinic/apyrimidinic (AP) site. AP endonuclease then incises DNA at the AP site, and the resulting single-strand break (SSB) is resynthesized by DNA polymerase and DNA ligase (Dalhus et al., 2009). Because misincorporated 5-FU and uracil are converted to AP sites by DNA glycosylases, the number of AP sites is also increased by treatment with 5-FU (McNeill et al., 2009). In the nematode *Caenorhabditis elegans*, AP endonucleases and mismatch repair process 5-FU to induce checkpoint-mediated autophagy (SenGupta et al., 2013).

FUdR is widely used when measuring the lifespan of *C. elegans* to suppress worm reproduction and the growth of progeny (Mitchell et al., 1979). It is believed that FUdR does not affect lifespan or cause severe side effects in adult worms because somatic cell division does not occur.
in the adult stage (Sulston and Horvitz, 1977). FUdR significantly reduces the time and effort required to measure lifespan. However, recent studies have revealed that FUdR extends the lifespan of some mutant worms, including tub-1 and gas-1 mutants, but the mechanism has not been elucidated (Aitlhadj and Stürzenbaum, 2010; Van Raamsdonk and Hekimi, 2011). In addition, it was reported that inhibition of DNA synthesis by FUdR improves protein homeostasis and increases stress resistance, extending healthspan via a fertility pathway that regulates sexual development (Angeli et al., 2013). Therefore, studies on the side effects of FUdR on worm lifespan are needed.

Here, we aimed to verify the effect of FUdR on lifespan from the viewpoint of DNA damage. Because 5-FU induces DNA base damage (McNeill et al., 2009), we first hypothesized that FUdR adversely affects the lifespan of BER gene mutants. To date, FUdR has not been shown to induce DNA damage in C. elegans. We previously reported that deletion of the exo-3 gene, but not the apn-1 gene, results in decreased lifespan in the absence of FUdR in a ung-1-dependent manner (Kato et al., 2015). EXO-3 and APN-1 are AP endonucleases (Shatilla et al., 2005; Schlotterer et al., 2010; Zakaria et al., 2010; Yang et al., 2012), and UNG-1 is a uracil DNA glycosylase in C. elegans (Nakamura et al., 2008). In this study, we measured the lifespan of the exo-3 mutant in the presence of FUdR, and found that FUdR extended the lifespan of the mutant. To investigate the mechanism of lifespan extension by FUdR, we conducted lifespan analyses using germline mutants. We focused on fem-1, glp-1 and fem-3 as genes involved in fertility (Nelson et al., 1978; Barton et al., 1987; Priess et al., 1987). In fem-1 mutant worms, which do not produce sperm, lifespan extension by FUdR was not observed. In glp-1 mutant worms, which do not develop gonads, the exo-3 mutant was not short-lived, and FUdR treatment did not result in lifespan extension. FUdR extended the lifespan of fem-3 mutant worms (which do not produce oocytes) independent of the exo-3 mutation. We suggest that there are both positive and negative effects of FUdR on worm lifespan.

![Lifespan of the exo-3 mutant in the presence of FUdR](image-url)
MATERIALS AND METHODS

Strains and culture conditions The *C. elegans* strains used in this study were as follows: Bristol N2, BA17 [fem-1 (hc17) IV] (Nelson et al., 1978), JK816 [fem-3 (q20) IV] (Barton et al., 1987) and CF1903 [glp-1 (e2141) III] (Priess et al., 1987) were obtained from the Caenorhabditis Genetics Center, University of Minnesota. TM4374 [exo-3 (tm4374) I] (Kato et al., 2015) was kindly provided by Shohei Mitani (Tokyo Women’s Medical College) of the National Bioresource Project for the Nematode. N2 and exo-3 mutant worms were grown and maintained at 20 °C, while fem-1, glp-1 and fem-3 mutant worms were grown and maintained at 16 °C. Worms were cultured on standard NGM agar plates and fed *Escherichia coli* strain OP50 (Morinaga et al., 2009).

Lifespan measurement To synchronize the worms, embryos were prepared by the standard alkaline bleach method, and eggs were hatched overnight in M9 buffer without food. L1 worms were grown at 25 °C for 2.5 days. Adult worms were transferred to an NGM plate containing 50 μM FUdR, and their lifespan was measured by transferring worms daily to fresh plates using a platinum pick. As it was assumed that the worms were damaged by picking, we conducted all measurements at 25 °C to finish the experiments in a short period. Worms that did not respond to touch were counted as dead (Kato et al., 2015).

Mean lifespans were calculated from the results of three independent experiments, and are presented as means ± SD in figures. For statistical analysis, Student’s t-test was carried out, and data were considered significantly different when the *P* value was < 0.05.

RESULTS

FUdR extends the lifespan of the *exo-3* mutant worm To examine whether FUdR affected the lifespan of *C. elegans* via DNA damage induction, we measured the lifespan of *exo-3* mutant worms in the presence and absence of FUdR. We found that the lifespan of short-lived *exo-3* mutants was restored, and indeed extended,
FUdR increased the mean lifespan of exo-3 mutants by 50% (in the absence and presence of FUdR, the mean lifespan of exo-3 mutants was 9.73 days and 14.52 days, respectively, and the P value from a t-test was < 0.05) (Fig. 1C). Furthermore, exo-3 mutants had a significantly longer lifespan than N2 worms on FUdR-containing plates (mean lifespan was 14.52 days and 10.74 days, respectively, and the P value from the t-test was < 0.05). FUdR extends the lifespan of the exo-3 mutant in a sperm- and gonad-dependent manner. Worms homozygous for mutations in exo-3 show abnormalities in reproduction (Kato et al., 2015), and we assumed that FUdR extended the lifespan of exo-3 mutant worms by interfering with fertility. To examine this hypothesis, we measured the lifespan of multiple germline mutants. fem-1 (hc17) mutant worms do not produce sperm at 25 °C (Nelson et al., 1978). In the absence of FUdR, fem-1;exo-3 double mutants had a decreased lifespan compared with fem-1 mutants (Fig. 2A). The mean lifespan of the fem-1 and fem-1;exo-3 mutants was 10.94 and 9.82 days, respectively (Fig. 2C). However, in the presence of FUdR, fem-1;exo-3 mutants had a similar lifespan to fem-1 mutants (Fig. 2B). In the presence of FUdR, the mean lifespans of fem-1 and fem-1;exo-3 mutants were 10.09 and 10.16 days, respectively, and the P value from the t-test was > 0.05 (Fig. 2C). Worms homozygous for the glp-1 (e2141) allele do not develop gonads at 25 °C (Priess et al., 1987). glp-1;exo-3 double mutant worms had a normal lifespan similar to glp-1 mutants in the absence of FUdR (Fig. 3A). The mean lifespans of glp-1 and glp-1;exo-3 worms were 12.52 and 12.15 days, respectively, and the P value from the t-test was > 0.05 (Fig. 3C). In addition, FUdR did not extend the lifespan of glp-1;exo-3 mutants (Fig. 3B). In the presence of FUdR, the mean lifespan of glp-1 and glp-1;exo-3 mutants was 12.70 and 13.57 days, respectively, and the P value from the t-test was > 0.05 (Fig. 3C).

Inhibition of oocyte production results in longevity in the presence of FUdR. To further investigate the
FUdR extends lifespan of mutant worms

Fig. 4. Lifespan of fem-3 mutants in the presence of FUdR. (A and B) Survival plots of fem-3 (q20) mutants in the absence (A) and presence (B) of 50 μM FUdR at 25 °C. The fem-3 mutant does not produce oocytes when grown at 25 °C. To measure lifespan, worms were synchronized at the L1 stage and grown at 25 °C. Adult worms were placed on normal NGM plates, or NGM plates containing 50 μM FUdR, and transferred to a fresh plate every day. (C) Mean lifespan of the fem-3 mutants. Data shown are the mean ± SD from three independent experiments. * denotes a significant difference (P < 0.05) as determined by t-test; n.s., not significant.

relationship between worm fertility and lifespan extension by FUdR, we measured the lifespan of fem-3 (q20) mutant worms, which do not produce oocytes at 25 °C (Barton et al., 1987). In the absence of FUdR, fem-3;exo-3 mutants had a normal lifespan similar to fem-3 mutants (Fig. 4A). Mean lifespans of the fem-3 and fem-3;exo-3 mutants were 8.68 and 9.62 days, respectively, and the P value from the t-test was > 0.05 (Fig. 4C). Also, in the presence of FUdR, fem-3;exo-3 mutants had a similar lifespan to fem-3 mutants (Fig. 4B). On FUdR-containing plates, the mean lifespans of fem-3 and fem-3;exo-3 mutants were 12.72 and 12.05 days, respectively, and the P value from the t-test was > 0.05 (Fig. 4C). Importantly, FUdR extended the lifespan of fem-3 mutants independently of mutations in exo-3 (P values from t-tests of fem-3 vs. fem-3 with FUdR and of fem-3;exo-3 vs. fem-3;exo-3 with FUdR were both < 0.05).

DISCUSSION

FUdR as a DNA damage inducer in C. elegans

Whether FUdR induces DNA base damage in C. elegans is not known, and further investigation is needed to reveal if FUdR affects worm lifespan via induction of DNA base damage. Contrary to our initial expectation, FUdR extended the lifespan of the exo-3 mutant (Fig. 1B). This result suggests that FUdR affects worm lifespan via the BER pathway. Our result conflicts with a previous report that 5-FU induces base damage (McNeill et al., 2009). We measured the level of AP sites in C. elegans by an aldehyde reactive probe assay, but found no significant increase in AP sites caused by FUdR treatment (data not shown). Inhibition of DNA synthesis and induction of base damage are conflicting effects of FUdR. It is possible that in the presence of a higher concentration of FUdR (for example, 50 μM in this study), DNA synthesis is strongly inhibited so that misincorporation of 5-FU and uracil rarely occurs. Lower concentrations of FUdR might induce base damage in C. elegans. It has been reported that lower concentrations of 5-FU (< 5 μM) induce AP endonuclease- and mismatch repair-mediated autophagy in C. elegans (SenGupta et al., 2013). Another possible reason why we did not detect any difference in the number of AP sites following FUdR
Effect of reproductive inhibition on lifespan  In *C. elegans*, it is recognized that lifespan is related to reproduction. For example, killing the germline precursor cells Z2 and Z3 in larvae increases longevity (Arantes-Oliveira et al., 2002). The *glp-1* mutant, in which the formation of gonads is prevented, also lives longer (Berman and Kenyon, 2006). We previously reported that deletion of the *exo-3* gene causes abnormality in reproduction, including decreased self-brood size (Kato et al., 2015). In this study, we found that the *exo-3* mutant has a short lifespan dependent on production of oocytes and gonads. When worms lacked oocytes (*fem-3;exo-3*) or gonads (*glp-1;exo-3*), *exo-3* mutants were not short-lived (Figs. 3A and 4A). These results suggest that deletion of the *exo-3* gene caused decreased lifespan via reproduction (especially production of oocytes). Lifespan extension by FUdR was not observed when worms were deficient in sperm or gonads (Figs. 2B and 3B). This suggests that lifespan extension by FUdR was related to worm reproduction and fertility. Because FUdR extended the lifespan of oocyte-less *fem-3* mutants independently of mutations in *exo-3* (Fig. 4B), it is possible that *fem-3* mutants were susceptible to the lifespan extension effect of FUdR. FUdR is very useful when measuring worm lifespan. However, it may not be suitable for measuring the lifespan of mutants that show abnormal reproduction.

**FUdR affects lifespan in two ways** In this study, we suggest that deletion of the *exo-3* gene caused a short lifespan via the fertility pathway, and that lifespan is restored by inhibiting oocyte production or gonad development. However, in the presence of FUdR, the lifespan of *exo-3* mutants was increased, not just restored, compared with N2 worms (Fig. 1B). This phenomenon cannot be explained by the hypothesis that FUdR extends the lifespan of *exo-3* mutants by interfering with fertility. Taking DNA base damage induction by FUdR into account might explain this phenomenon. We hypothesize that FUdR affects lifespan in two opposing ways (Fig. 5). By interfering with fertility in a specific manner, FUdR extends worm lifespan. However, FUdR also reduces worm lifespan by inducing DNA base damage, resulting in AP sites and SSBs. In N2 worms, it seems that FUdR does not affect lifespan because these two effects are almost equal and counteract each other. In *exo-3* mutant worms, AP sites are not efficiently converted into SSBs. SSBs are more harmful than DNA base damage and AP sites in the short term, and therefore deletion of the *exo-3* gene is advantageous for survival in the presence of FUdR. FUdR extends the lifespan of *exo-3* mutants because *exo-3* mutants were barely affected by the negative aspects of FUdR treatment compared with the N2 worms. FUdR may only influence specific aspects of nematode fertility. In worms lacking *fem-1* function, these aspects may still be present, which explains the slight decrease in lifespan observed in *fem-1* mutant worms treated with FUdR (Fig. 2C). The *glp-1* mutant tested does not possess gonads; therefore, lifespans were affected by neither the *exo-3* mutation nor FUdR (Fig. 3C). It is possible that the *fem-3* mutant was susceptible to the lifespan extension effect of FUdR because the mutant was long-lived independent of the *exo-3* mutation (Fig. 4C).

We thank the National Bioresource Project for the Experimental Animal Nematode *C. elegans* of Japan (Mitani laboratory). Some strains were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). This work was financially supported in part by Grants for Excellent Graduate Schools and by Grants-in-Aid for Scientific Research (#24510071 and 16H00545) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We are also grateful to the Shiseido Female Research Science Grant for supporting Q.-M. Zhang-Akiyama.
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