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The nongenotoxic carcinogens naphthalene and *para*-dichlorobenzene suppress apoptosis in *Caenorhabditis elegans*

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Naphthalene (1) and *para*-dichlorobenzene (PDCB, 2), which are widely used as moth repellents and air fresheners, cause cancer in rodents and are potential human carcinogens. However, their mechanisms of action remain unclear. Here we describe a novel method for delivering and screening hydrophobic chemicals in *C. elegans* and apply this technique to investigate the ways in which naphthalene and PDCB may promote tumorigenesis in mammals. We show that naphthalene and PDCB inhibit apoptosis in *C. elegans,* a result that suggests a cellular mechanism by which these chemicals may promote the survival and proliferation of latent tumor cells. In addition, we find that a naphthalene metabolite directly inactivates caspases by oxidizing the active site cysteine residue; this suggests a molecular mechanism by which these chemicals suppress apoptosis. Naphthalene and PDCB are the first small-molecule apoptosis inhibitors identified in *C. elegans.* The power of *C. elegans* molecular genetics, in combination with the possibility of carrying out large-scale chemical screens in this organism, makes *C. elegans* an attractive and economic animal model for both toxicological studies and drug screens.

Carcinogens cause the majority of human cancers[1,2]. Based on their modes of action, carcinogens can be classified into two groups: genotoxic carcinogens, which directly mutagenize or damage genomic DNA, and nongenotoxic carcinogens, which promote the survival and proliferation of latent tumor cells. Many studies have focused on investigating the mechanisms by which genotoxin-induced DNA damage contributes to the development of cancers[3,4]. By contrast, the mechanisms of nongenotoxic carcinogenesis are not well understood[5]. One way that nongenotoxic carcinogens can promote cancer is by disabling the apoptotic machinery that removes cells that are at risk of becoming cancerous[6]. As a result, agents that inhibit apoptosis often promote cancer development[7].

Naphthalene and PDCB are marketed to consumers as moth repellents and air fresheners. They are 'high production volume' chemicals (that is, they are consumed in excess of one million pounds per year) used as industrial plasticizers and are ubiquitous environmental pollutants. The National Toxicology Program (NTP) and the International Association for Research on Carcinogens (IARC) classify naphthalene and PDCB as rodent carcinogens that are "reasonably anticipated to be human carcinogens"[8–10]. These chemicals do not seem to cause DNA damage in most assays, but their nongenotoxic mechanisms of action are not known[8,9].

C. elegans is a good animal model for investigating the effects of nongenotoxic carcinogens on apoptosis. Of the 1,090 somatic cells born during the development of *C. elegans* hermaphrodites, 131 cells invariably undergo apoptosis[11,12]. Unlike mammals, *C. elegans* does not develop somatic tumors and seems to have no somatic apoptotic responses to genotoxic stresses[13]. As a result, it is easier to interpret phenotypes caused by suspected nongenotoxic carcinogens in *C. elegans* than in mammals. The regulation of apoptosis in *C. elegans* is less complex than in mammals, but key apoptotic genes are conserved between nematodes and humans[14]. These include *egl-1*, *ced-9*, *ced-4* and *ced-3*, which are necessary for nearly all apoptosis occurring in *C. elegans* somatic tissues. In addition, genes that are important for other aspects of apoptosis, including the specification of cell death (for example, *ces-1* and *ces-2*)[15,16], cell corpse engulfment (for example, *ced-1* and *ced-2*)[17,18], the kinetics of apoptosis (*ced-8*)[19] and the degradation of chromosomal DNA (*nuc-1* and *cps-6*)[20,21], are also conserved. The proteins EGL-1, CED-9, CED-4 and CED-3 have mammalian homologs (BH3-only proteins, Bcl-2 family proteins, Apaf-1 and caspases, respectively) that play very similar roles in regulating apoptosis in mammals[22]. In both mammals and *C. elegans*, activation of proapoptotic EGL-1/BH3-only proteins initiates apoptosis by binding to and inhibiting the activity of CED-9/Bcl-2 family proteins. This leads to the activation of CED-4/Apaf-1, which in turn activates CED-3/caspases, the key enzymatic executioners of apoptosis[22].

Caspases are a family of cysteine proteases that induce apoptosis by cleaving specific cellular protein substrates after aspartate residues[23]. The *C. elegans* caspase CED-3 has substrate specificities similar to those of the human caspase-3, one of the major cell-death executioners[24]. Therefore, molecules that affect apoptosis and CED-3

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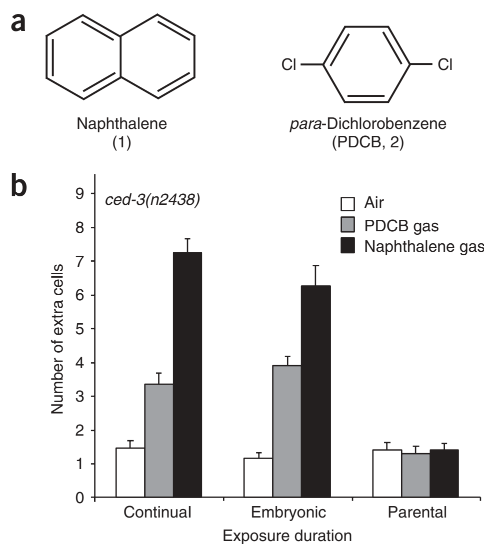

Figure 1 Naphthalene and PDCB cause nonheritable apoptosis suppression in *C. elegans*. (**a**) Chemical structures of naphthalene and PDCB. (**b**) Naphthalene and PDCB inhibit apoptosis in *C. elegans*. *ced-3(n2438)* animals were exposed to normal air (white bars), PDCB-containing gas (gray bars) or naphthalene-containing gas (black bars) for different durations. 'Continual' indicates that animals were exposed continuously to the chemical from the L4 larval stage until their F1 progeny were scored as L4 larvae. 'Embryonic' indicates that embryos from unexposed parental animals were exposed to the chemical until they were scored 2 d later as L4 larvae. 'Parental' indicates that parental *ced-3(n2438)* animals were continuously exposed to the chemical, but their progeny were removed from the chemicals as embryos 1 h after being laid and scored 2 d later as L4 larvae. The *y* axis represents the average number of inappropriately surviving cells in the anterior pharynx of *ced-3(n2438)* animals. Error bars, s.e.m.; 20 animals were scored for each condition.

caspase activity in *C. elegans* may also affect apoptosis in mammals and have clinical relevance[25–27]. In this study, we show that naphthalene and PDCB suppress apoptosis in *C. elegans* by inhibiting CED-3 caspase activity. We propose that these chemicals promote cancer development in humans by suppressing apoptosis.

RESULTS

Naphthalene and PDCB inhibit apoptosis in *C. elegans*

To examine the mechanisms by which naphthalene and PDCB may act as carcinogens, we investigated the possibility that naphthalene and PDCB might inhibit apoptosis in *C. elegans* by counting extra surviving cells in the anterior pharynx of individual *C. elegans* animals. Normally, 16 cells in the anterior pharynx of wild-type (*N2*) *C. elegans* animals undergo apoptosis during embryonic development. Mutations in genes that regulate apoptosis can cause some or all of these cells to inappropriately survive. For example, in the *ced-3(n2438)* mutant, which carries a partial loss-of-function mutation in the *ced-3* gene, an average of 1.5 extra living cells are seen in the anterior pharynx[21]. When we grew *ced-3(n2438)* animals in an incubator with naphthalene mothballs (**Fig. 1a**), an average of four to nine extra living cells were seen in the anterior pharynx (**Fig. 1b** and **Supplementary Fig. 1** online). This result suggests that naphthalene substantially enhances the cell-death defect of *ced-3(n2438)* mutants. We found that another moth repellent, PDCB, results in similar but weaker inhibition of cell death (**Fig. 1**). Exposure of *C. elegans* animals to either of these chemicals during embryogenesis (the stage when most somatic cell deaths occur) was sufficient to suppress apoptosis (**Fig. 1b**), suggesting that these chemicals can penetrate the semi-impermeable eggshell to inhibit apoptosis in the embryo. In addition, untreated progeny from treated animals did not maintain the enhanced cell survival phenotype (**Fig. 1b**), indicating that the apoptotic defects caused by naphthalene and PDCB are not heritable. Therefore, these chemicals suppress apoptosis in *C. elegans* by a nongenotoxic mechanism.

Oil solvents allow hydrophobic drug delivery in *C. elegans*

To determine the relationship between naphthalene or PDCB concentrations and apoptosis inhibition, we counted the number of inappropriately surviving cells in the anterior pharynx of *ced-3(n2438)* mutants exposed to serial dilutions of naphthalene and PDCB dissolved in either air or water. Gaseous naphthalene consistently suppressed apoptosis in *C. elegans* at concentrations less than 4 μM (**Fig. 2a** and **Supplementary Fig. 1**), which are the gaseous concentrations that humans normally encounter when using naphthalene as a moth repellent or an air freshener. In comparison, for aqueous naphthalene, substantially higher concentrations (~100 μM) were needed for the compound to have an inhibitory effect on apoptosis (**Fig. 2a**). However, in both gaseous and aqueous naphthalene assays, the number of extra cells observed can vary widely from assay to assay because of the low aqueous solubility of these chemicals and the difficulties of working with gases (**Supplementary Fig. 1**). This variation precludes the use of either assay for quantitative analysis of apoptosis inhibition by these two chemicals. For this reason, we tested the possibility of using other solvents to dissolve naphthalene and PDCB. Soybean oil is not toxic to *C. elegans* but can dissolve molar amounts of naphthalene and PDCB. As worms cannot live in liquid oil culture, we exposed them to the chemicals by overlaying the entire surface of the nematode growth medium (NGM) culture plates with soybean oil solutions. Worms live and develop normally at the

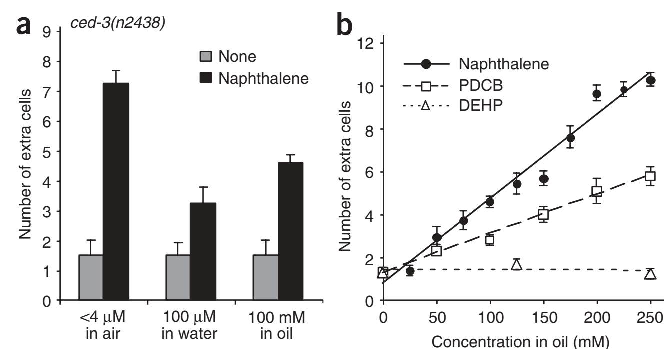

Figure 2 The inhibitory activity of naphthalene and PDCB toward apoptosis in different solvents. (**a**) The concentrations of naphthalene needed to suppress apoptosis in *C. elegans* are affected by the solvent conditions. *ced-3(n2438)* animals were exposed to different concentrations of naphthalene in air, water and oil as indicated, and the numbers of extra living cells were scored. Based on the vapor pressure, the maximum concentration of naphthalene in air is ~4 μM. (**b**) Naphthalene and PDCB inhibit apoptosis in a concentration-dependent manner in soybean oil. *ced-3(n2438)* animals were exposed to chemicals dissolved in soybean oil at the indicated concentrations, and the numbers of extra cells were scored. The *y* axis represents the average number of extra cells in the anterior pharynx of *ced-3(n2438)* mutants. Error bars, s.e.m.; 20 animals were scored at each solvent condition and at each concentration point in soybean oil.

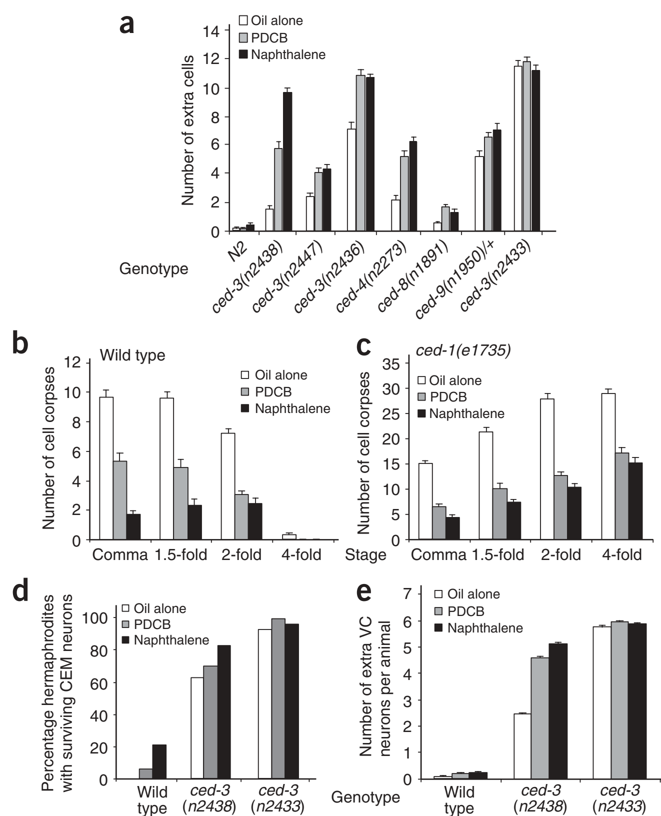

Figure 3 Naphthalene and PDCB suppress apoptosis in different genetic backgrounds and in multiple cell types in *C. elegans*. The indicated animals were exposed to soybean oil alone (white bars), 250 mM PDCB (gray bars) or 200 mM naphthalene (black bars). (**a**) Naphthalene and PDCB enhance cell survival in multiple *C. elegans* cell-death mutants. The numbers of extra cells in the anterior pharynges of L3 and L4 larvae were counted using Nomarski optics (*y* axis); 20 animals were scored in each experiment. (**b**,**c**) Naphthalene and PDCB inhibit embryonic cell death. Cell corpses were counted in (**b**) N2 and (**c**) *ced-1(e1735)* animals. Embryos were scored at the comma, 1.5-fold, two-fold and four-fold stages. The *y* axis represents the average number of cell corpses scored in the head region of embryos. Error bars, s.e.m.; 20 embryos were scored at each embryonic stage. (**d**) Naphthalene and PDCB inhibit sex-specific deaths of CEM neurons in hermaphrodite animals. The number of ectopically surviving CEM neurons was scored in wild-type, *ced-3(n2438)* and *ced-3(n2433)* hermaphrodite animals. The *y* axis represents the percentage of hermaphrodites with at least one inappropriately surviving CEM neuron. All strains also carry an integrated transgene (*smIs26*) that contains a $P_{pkd-2}gfp$ construct that specifically labels CEM neurons. Thirty animals were scored in each experiment. (**e**) Naphthalene and PDCB inhibit postembryonic cell death. The number of VC and VC-like neurons was counted in wild-type, *ced-3(n2438)* and *ced-3(n2433)* animals. The *y* axis represents the average number of extra VC-like neurons per animal (Pn.aap cell, $n = 1, 2, 9, 10, 11, 12$). VC and VC-like cells were scored in the presence of the *nIs96* transgene, which contains a $P_{lin-11}gfp$ construct that specifically labels VC and VC-like cells in the ventral nerve cord[30]. Fifty animals were scored in each experiment. Error bars, s.e.m.

interface of the oil and the NGM medium, which is seeded with OP50 (a nematode food source), and they are directly exposed to chemicals dissolved in the oil.

At concentrations between 25 mM and 250 mM in soybean oil, naphthalene and PDCB each generated a nearly linear increase in the number of extra surviving cells (**Fig. 2b**). Notably, in a weak *ced-3(n2438)* mutant that normally has only 1.5 extra cells, exposure to 200 mM naphthalene in oil (or less than 4 μM of naphthalene gas) generated almost as many extra surviving cells as did the strong loss-of-function *ced-3(n2433)* mutation (**Fig. 3a**), suggesting an almost complete inhibition of apoptosis. PDCB enhanced the average number of extra cells in the *ced-3(n2438)* mutant to a maximum of six (**Fig. 2b**); this suggests that PDCB is less effective than naphthalene as an inhibitor of apoptosis. In contrast, another aromatic chemical, di(2-ethylhexyl)phthalate (DEHP, **3**), did not suppress apoptosis in *C. elegans* at any of the concentrations tested (**Fig. 2b**). As expected from their relatively large log octanol/water partition coefficients (~3.3 for naphthalene and ~3.5 for PDCB), naphthalene and PDCB molecules probably enter cells less efficiently from oil than from water or air. Therefore, to cause a similar increase in the number of extra cells, a much higher concentration of naphthalene or PDCB was required in soybean oil than in water or air (**Fig. 2a**). However, this soybean oil–based chemical delivery method generated consistent and reproducible results in assaying apoptosis suppression and can be used as a general method for analyzing the effects of chemicals (especially hydrophobic ones) on *C. elegans*.

Naphthalene and PDCB are general inhibitors of apoptosis

To investigate whether the *ced-3(n2438)* mutation is specifically required for the apoptosis-inhibitory activity of naphthalene and PDCB, we counted the number of extra cells in different cell-death mutant strains exposed to these chemicals. The apoptosis-inhibitory activity of naphthalene and PDCB was not limited to *ced-3(2438)* mutants (**Fig. 3a**). These two chemicals increased the number of extra cells in *ced-3(n2447)*, *ced-3(n2436)*, *ced-4(n2273)* and *ced-8(n1891)* mutants, all of which were already partially defective in cell death, as they contain loss-of-function mutations that affect either different cell-death genes or different residues of the *ced-3* gene (the *n2438*, *n2447* and *n2436* mutations result in the amino acid substitutions G474R, S446L and L269F, respectively). Naphthalene and PDCB also enhanced the cell-death defect caused by a gain-of-function mutation (*n1950*) in the *ced-9* gene (**Fig. 3a**), which results in a G169E substitution that blocks the binding of EGL-1 to CED-9 and hence increases the death-inhibiting activity of CED-9 (refs. 28,29). These results suggest that naphthalene- and PDCB-mediated inhibition of apoptosis is dependent neither on the nature of mutations nor on mutations in a specific

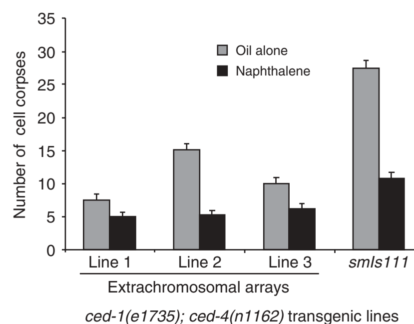

Figure 4 Naphthalene treatment suppresses activated CED-3 (acCED-3)-induced apoptosis in the absence of CED-4. The *y* axis represents the average number of cell corpses in four-fold stage *ced-1(e1735)*; *ced-4(n1162)* transgenic embryos containing either the P_{egl-1}acCED-3 extrachromosomal transgenes or the integrated transgene (*smIs111*) and grown with or without naphthalene (black and gray bars, respectively). At least 40 embryos from each of the three independent transgenic lines were scored; 15 embryos were scored from the integrated line. Error bars, s.e.m.

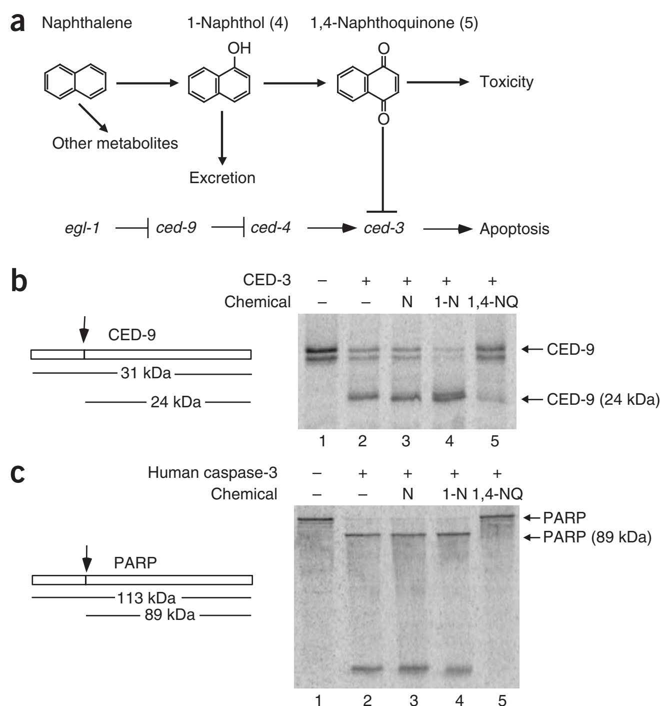

Figure 5 A naphthalene metabolite, 1,4-naphthoquinone, inhibits the activities of CED-3 and caspase-3 *in vitro*. (**a**) Naphthalene (N) metabolism. One of the major naphthalene metabolic products is 1-naphthol (1-N), which is either excreted or further metabolized into reactive 1,4-naphthoquinone (1,4-NQ). Naphthalene also generates other metabolic products that are not shown here. The *C. elegans* central cell-death pathway, which contains the cell-killing genes *egl-1*, *ced-4* and *ced-3* and the cell-death inhibitor *ced-9*, is also shown. (**b**,**c**) 1,4-naphthoquinone can inactivate CED-3 or human caspase-3 *in vitro*. 10 ng of purified CED-3 (**b**) and human caspase-3 (**c**) were incubated with different chemicals as indicated for 15 min at 30 °C: (–), 0.1% DMSO in CED-3 buffer; N, 100 μM naphthalene; 1-N, 100 μM 1-napthol; 1,4-NQ, 100 μM 1,4-naphthoquinone. [^{35}S]Methionine-labeled CED-9 or PARP proteins were then added into the reactions at 30 °C for 2 h and then the reaction mixtures were resolved on 12% SDS polyacrylamide gels.

cell-death gene. Notably, naphthalene and PDCB did not increase the number of extra cells seen in the strong loss-of-function mutant *ced-3(n2433)* (**Fig. 3a**), indicating that inhibition of apoptosis, rather than transformation of cell fates, causes the synergistic increase in extra cells.

Next we examined whether naphthalene and PDCB inhibit apoptosis in wild-type *C. elegans* animals by counting the number of cell corpses at different stages of embryogenesis, when most apoptosis occurs and when apoptotic cell corpses can be easily identified under Nomarski optics by their characteristic refractive appearance[12]. Naphthalene and PDCB substantially reduced the number of cell corpses observed in wild-type embryos (**Fig. 3b**) as well as the number of persistent cell corpses seen in engulfment-defective *ced-1(e1735)* mutant embryos (**Fig. 3c**). This result suggests that these chemicals generally suppress apoptosis during embryogenesis. We then followed the fates of four male-specific cephalic companion (CEM) neurons that are generated in both males and hermaphrodites during embryogenesis but undergo apoptosis specifically in hermaphrodite animals. PDCB and naphthalene inhibited the deaths of CEM neurons in wild-type hermaphrodite worms: 6% of PDCB-treated and 21% of naphthalene-treated hermaphrodites had at least one surviving CEM neuron, whereas oil-treated hermaphrodite controls had no surviving CEMs (**Fig. 3d**). These results suggest that naphthalene and PDCB are capable of inhibiting apoptosis in wild-type animals but have far greater effects on animals in which apoptosis is already compromised.

In *C. elegans* hermaphrodites, a small percentage of somatic cells (18 of 131 cell deaths) undergo apoptosis during postembryonic development[11]. Six of them are ventral cord cells that are posterior daughters of the anterior daughters of the anterior daughters of P1, P2 and P9–P12 neuroblast cells. In strong loss-of-function *ced-3* mutants, these cells survive and differentiate into class VC motor neuron–like cells that express the *lin-11* gene[30]. Naphthalene and PDCB substantially increased the number of extra VC-like cells in the ventral cord of *ced-3(n2438)* mutants, from an average of 2.5 extra VC-like cells in control (oil-treated) animals to around 5 extra cells in chemical-treated animals, suggesting that naphthalene and PDCB also suppress postembryonic apoptosis in *C. elegans* (**Fig. 3e**). Again, naphthalene and PDCB did not increase the number of extra VC-like cells in strong *ced-3(n2433)* mutants; this provides further support for the supposition that inhibition of apoptosis, but not transformation of cell fates, causes the increase in VC-like cells. Altogether, these results indicate that naphthalene and PDCB inhibit apoptosis in multiple developmental stages, in multiple cell types and in various genetic backgrounds. These chemicals are the first small-molecule apoptosis inhibitors identified in *C. elegans*.

In addition to inhibiting apoptosis, naphthalene and PDCB have some nonspecific toxic effects on *C. elegans*. The two chemicals slow down development, reduce brood size and cause some lethality at the same concentrations as those that affect apoptosis, although individuals that have been exposed to the chemicals appear anatomically and behaviorally normal (unpublished data).

Naphthalene inhibits activated CED-3–induced apoptosis

We next examined the possibility that naphthalene and PDCB inhibit apoptosis by affecting one of the core components of the cell-death pathway, such as *egl-1*, *ced-9*, *ced-4* or *ced-3*. To determine whether

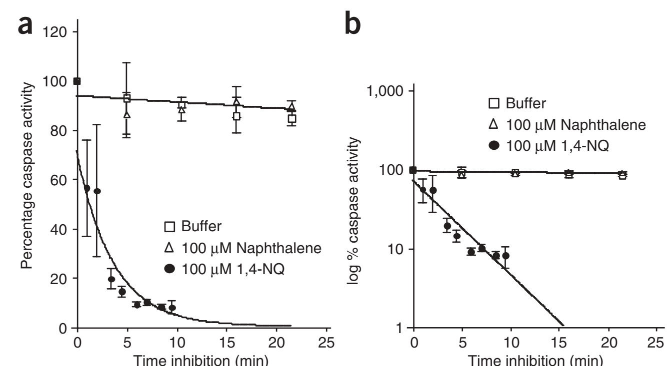

Figure 6 Kinetics of human caspase-3 inactivation by 1,4-naphthoquinone. (**a**) We monitored caspase-3 activity by observing the generation of fluorescent AMC from the caspase-3 substrate Ac-DEVD-AMC. We preincubated ∼10,000 units of purified recombinant caspase-3 in buffer alone (□), in 100 μM naphthalene (△) and in 100 μM 1,4-naphthoquinone (●) for the indicated amounts of time. Aliquots were then taken out and measured for caspase-3 activities as described in methods. Each data point is an average of three independent experiments. Error bars, s.d. (**b**) Plot of the log of % caspase activity based on data in **a**. The slope of this plot is $-K_{inact}$.

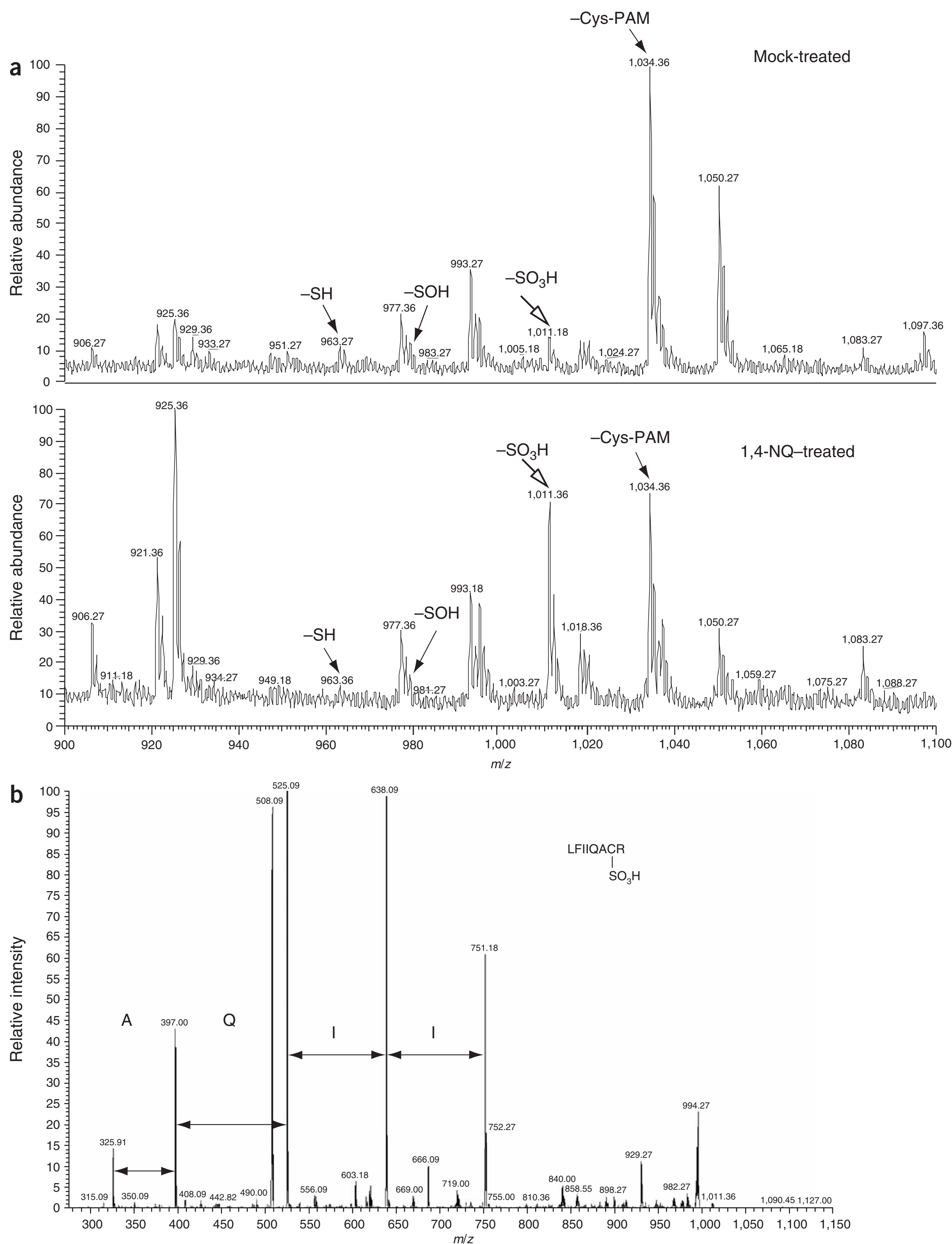

Figure 7 vMALDI-LTQ mass spectra of trypsin digests of caspase-3 treated with 1,4-naphthoquinone (1,4-NQ) and mock. (**a**) Caspase-3 was incubated with buffer alone (top) or with 100 μM 1,4-NQ (bottom), resolved on SDS-PAGE, digested in gel with trypsin and analyzed by vMALDI-LTQ. Four LFIIQACR peptide species were identified that contain the active site cysteine: the unmodified one (-SH, $M+H^+$ 963.5 Da), the one with sulfenic acid (-SOH, $M+H^+$ 979.5 Da), the one with cysteic acid indicated by empty arrows (-SO_3H, $M+H^+$ 1,011.5 Da) and the one with acrylamide adduct (PAM-Cys, $M+H^+$ 1,034.5 Da). (**b**) MS/MS spectrum that identifies the *m/z* 1,011.5 peak as the active site cysteine–containing peptide, in which the active site cysteine residue is modified as cysteic acid.

naphthalene and PDCB act upstream or downstream of the CED-3 caspase, we ectopically expressed an activated version of the CED-3 protease (acCED-3) in the *ced-1(e1735); ced-4(n1162)* mutant under the control of the *egl-1* promoter (P_{egl-1}acCED-3)[21], which directs gene expression in cells that normally undergo apoptosis[31]. CED-4 is a direct activator of the CED-3 zymogen[32], and the *ced-4(n1162)* mutation blocks virtually all apoptosis[33]. As a result, *ced-1(e1735); ced-4(n1162)* mutants have few cell corpses[33]. By contrast, *ced-1(e1735); ced-4(n1162)* mutants carrying the P_{egl-1}acCED-3 transgenes had an average of 8–15 unengulfed cell corpses at the four-fold embryonic stage in the three independent transgenic lines scored (**Fig. 4**). As the number of cell corpses varied among the three transgenic lines, possibly because of the unstable nature of extrachromosomal transgenic arrays, we also generated a stable, integrated P_{egl-1}acCED-3 transgenic line (*smIs111*) that indeed has more cell corpses (an average of 27 cell corpses) than the first three transgenic lines (**Fig. 4**). To determine whether naphthalene can inhibit cell killing that is induced by acCED-3, we counted the number of cell corpses in each of these transgenic lines with and without naphthalene treatment and found that naphthalene treatment substantially reduced the number of cell corpses in *ced-1(e1735); ced-4(n1162)* transgenic embryos (**Fig. 4**). Because naphthalene suppresses acCED-3–mediated cell killing in the *ced-4(n1162)* null mutant, naphthalene likely acts either on the CED-3 caspase itself or on a target or targets functioning downstream of or in parallel to *ced-3*.

1,4-Naphthoquinone inactivates caspases *in vitro*

Aspects of naphthalene metabolism are conserved from fungi to humans, including the catalysis of naphthalene to naphthols and naphthoquinones (**Fig. 5a**)[8,34]. Naphthols are relatively inert and can be excreted in urine. By contrast, naphthoquinones are electrophilic, reactive molecules that have been suggested to mediate the toxic and carcinogenic effects of naphthalene[8,35]. Given that caspases are cysteine proteases with thiols at their active sites, we investigated whether naphthalene or its major metabolites inhibit caspases *in vitro*.

Purified CED-3 protease cleaves [^{35}S]methionine-labeled CED-9, an endogenous CED-3 substrate, yielding a smaller (24 kDa) cleavage product (**Fig. 5b**)[36]. Naphthalene and 1-naphthol (**4**) do not affect CED-3 activity (**Fig. 5b**), whereas 1,4-naphthoquinone (1,4-NQ, **5**) strongly inhibits CED-9 cleavage (**Fig. 5b**), suggesting that 1,4-NQ inhibits the activity of CED-3. 1,4-NQ also inhibits the activity of human caspase-3, a key executor caspase for apoptosis in human cells (**Fig. 5c**). 1,4-NQ inhibits cleavage of [^{35}S]methionine-labeled poly (ADP-ribose) polymerase (PARP) (**Fig. 5c**), a human caspase-3 substrate, but naphthalene and 1-naphthol do not; this suggests that naphthoquinones may also inhibit apoptosis in human cells. Together, these results suggest that the inhibitory activity of naphthalene towards apoptosis in *C. elegans* may be attributable to its quinone metabolites.

To investigate the kinetics of caspase inactivation by 1,4-naphthoquinone, we used the fluorogenic caspase-3 substrate Ac-DEVD-AMC (where AMC is 7-amino-4-methylcoumarin) to monitor caspase-3 activity in the presence or absence of naphthalene or 1,4-naphthoquinone. Incubation of 1,4-naphthoquinone with caspase-3 caused time-dependent inactivation of the caspase (**Fig. 6a**), suggesting that 1,4-naphthoquinone may be an irreversible inhibitor of caspase-3 (ref. 37). As shown by the slope of the semilogarithmic plot of caspase activity over time (**Fig. 6b**), the first-order rate of caspase inactivation (K_{inact}) is 0.11 min^{-1}, indicating that 11% of the caspase is inactivated per minute by 100 μM 1,4-NQ.

1,4-NQ oxidizes the catalytic cysteine residue of caspase-3

To investigate the nature of caspase-3 inactivation by 1,4-NQ, we carried out mass spectrometry analysis of caspase-3 incubated with or without 1,4-NQ. We found that in samples treated with 1,4-NQ, the tryptic caspase-3 peptides containing the active site cysteine residue Cys163 (LFIIQACR) existed in four species differing from one another by modifications of Cys163 (**Fig. 7**). One of the species corresponds to the unmodified form of the peptide (-SH) at 963.5 *m*/*z* and was seen in both 1,4-NQ–treated and untreated samples. The relative mass of the second species (979.5 *m*/*z*) is shifted up 16 Da, indicating the addition of an oxygen atom and the generation of a sulfenic acid (-SOH) group in the peptide. Oxidation of the -SH group to the sulfenic acid, which is reversible under reducing conditions, was observed in both 1,4-NQ and mock-treated samples (albeit as a minor species), suggesting that the active site Cys163 can undergo low-level spontaneous oxidation. At 1,011.5 *m*/*z*, the third and most abundant species, which is shifted by the mass of three oxygen atoms (48 Da), corresponds to the peptide with a cysteic acid (-SO_3H) group at the Cys163 residue, probably a product of consecutive oxidation reactions facilitated by 1,4-NQ. This species is barely visible in the mock-treated sample. The fourth LFIIQACR peak, at 1,034.5 *m*/*z*, represents acrylamide-modified Cys163 (Cys-PAM). Cys-PAM is routinely generated during SDS-PAGE (because of free cysteines labeled by residual acrylamide) and was observed in both 1,4-NQ–treated and mock-treated samples. However, the relative abundance of this species was substantially reduced in the 1,4-NQ–treated sample because of the corresponding increase of the cysteic acid–containing species. Notably, all the other cysteine residues in caspase-3 were detected only in unmodified forms or in the Cys-PAM forms. This suggests that 1,4-NQ specifically catalyzes the oxidation of the Cys163 residue at the caspase-3 active site. Taken together, these data suggest that 1,4-NQ specifically inhibits caspase-3 by oxidizing its active site Cys163 residue.

DISCUSSION

Cancer development occurs through a multistep process in which cells accumulate mutations that collectively promote inappropriate cell survival and proliferation[38]. Because inhibition of apoptosis is a requisite step during carcinogenesis[39,40], environmental factors that inhibit apoptosis can facilitate cancer development. Carcinogens are thought to promote cancer in two qualitatively different ways[41]. Genotoxic carcinogens cause cancer by mutating tumor-suppressor genes and proto-oncogenes. By contrast, nongenotoxic carcinogens are thought to act through various mechanisms to promote the survival and proliferation of latent tumor cells. Understanding the ways that nongenotoxic carcinogens cause cancer is therefore a critical issue in adopting appropriate measures and regulations to reduce both the exposure to carcinogens and the incidence of cancer in humans.

Naphthalene and PDCB are unlikely to be genotoxic carcinogens[8]. However, the mechanisms by which they cause cancer are unknown. In this study, we showed that naphthalene and PDCB inhibit apoptosis in *C. elegans* by a nongenotoxic mechanism, a result that suggests a mechanistic basis for their carcinogenic activities in mammals. This finding, along with the observations that other nongenotoxic carcinogens such as 2-acetylaminofluorene (**6**)[42], nafenopin (**7**)[43] and tetrachlorodibenzo-*p*-dioxin (TCDD, **8**)[44] also suppress apoptosis (both in cell culture and in rodents), suggests that a subclass of nongenotoxic carcinogens may promote tumorigenesis by suppressing apoptosis.

We also demonstrate that naphthalene seems to suppress apoptosis by inactivating caspases. Specifically, the naphthalene metabolite

1,4-naphthoquinone inactivates caspases *in vitro* by oxidizing the active site cysteine residue, suggesting a molecular mechanism by which naphthalene suppresses apoptosis. PDCB shares physical and chemical properties with naphthalene and may suppress apoptosis by a similar mechanism. Indeed, some classes of quinone molecules have been proposed to be caspase inhibitors[45]. Thus naphthalene, PDCB and their quinone metabolites may serve as valuable pharmacological leads for the development of therapeutic drugs that inhibit caspases.

Despite its prominent application as a model organism for studying basic biological processes such as programmed cell death and aging, *C. elegans* has not been used as an animal model for therapeutic drug screens or for pharmacological or toxicological studies. One major reason is that there is no reliable and established method for delivering compounds into *C. elegans*, especially hydrophobic molecules, which often have interesting and potent pharmacological activities. Here we have developed a simple and reliable method to expose *C. elegans* to water-insoluble chemicals using a nontoxic oil solvent that is spread onto standard worm culture plates. Using this method, we have shown that two hydrophobic chemicals, naphthalene and PDCB, inhibit *C. elegans* apoptosis in a concentration-dependent manner, and we have thus established the validity of this method as a drug delivery protocol for *C. elegans*. This method will allow screening of all sorts of compounds in *C. elegans* and make *C. elegans* an ideal and inexpensive animal model for disease study and for compound screens. Regulatory agencies such as the NTP and IARC have primarily relied on rodent models to evaluate the hazardous risks of environmental, workplace and household chemicals. However, because of the high cost and slow timescale of rodent bioassays, only 900 out of thousands of commercial chemicals have been evaluated by IARC. *C. elegans* may thus become an inexpensive and expeditious alternative animal model for toxicology studies and for evaluation of chemicals for nongenotoxic carcinogenicity.

METHODS

Strains. We maintained *C. elegans* strains using standard procedures[46]. The wild-type strain was *N2*. We used the following alleles[30]: linkage group (LG)I: *ced-1(e1735)* and *smIs13*; LGIII, *ced-4(n2273)*, *ced-4(n1162)* and *ced-9(n1950)*; LGIV: *ced-3(n2438)*, *ced-3(n2447)*, *ced-3(n2436)* and *ced-3(n2433)*; LGV: *him-5(e1490)* and *unc-76(e911)*; LGX: *ced-8(n1891)* and *nIs96*. We generated *ced-9(n1950)*/+ heterozygous animals by crossing *ced-9(n1950); him-5(e1490)* males into *unc-76(e911)* hermaphrodites, and we examined the resulting non-Unc cross-progeny.

Chemical treatment of *C. elegans* and phenotypic analyses of treated animals. We scored extra surviving cells and cell corpses as previously described[19]. We dissolved naphthalene (Sigma, CAS # 91-20-3), PDCB (Acros, CAS# 106-46-7) and DHEP (Acros, CAS# 117-81-7) in 100% soybean oil (Crisco). We placed L4 larval stage hermaphrodite animals onto standard NGM plates seeded with OP50. Oil solutions containing the chemicals were spread (2–3 ml) onto each plate so that the NGM surface was completely covered by oil. Worms live at the interface of the NGM medium and the oil. We scored F1 progeny for various defects, including apoptosis inhibition. In all experiments, the naphthalene and PDCB concentrations were 200 mM and 250 mM, respectively, unless otherwise noted. To expose worms to naphthalene or PDCB gas, we grew the animals at room temperature (22 °C) on standard NGM plates placed in a 0.5-liter non-airtight plastic storage box containing solid naphthalene or PDCB. To expose worms to aqueous naphthalene and PDCB, we incubated adult *C. elegans* animals overnight in M9 buffer with these chemicals diluted from DMSO stocks ($<1\%$ DMSO final concentration). We collected L1 larvae after 12 h, transferred them to NGM plates seeded with OP50 and grew them under normal conditions until analysis.

Germline transformation. We performed microinjections as described previously[47]. $P_{egl\text{-}1}$acCED-3 constructs (50 μg ml^{-1}) were injected into *ced-1(e1735); ced-4(n1162)* animals along with 50 μg ml^{-1} of the pTG96 plasmid (which directs GFP expression in most cells throughout development[48]) as an injection marker.

***In vitro* caspase assays.** We purified CED-3 and caspase-3 as described previously[24]. [^{35}S]Methionine-labeled CED-9 and PARP proteins were synthesized using the TNT-coupled rabbit reticulocyte lysate system (Promega). For *in vitro* caspase assays, purified CED-3 or caspase-3 was preincubated with the chemicals for 15 min at 30 °C before the [^{35}S]methionine-labeled substrate was added and digestion allowed to proceed for 2 h. Reactions were terminated by adding 2× SDS loading buffer and the reaction mixtures resolved on 12% SDS-PAGE gels, which were then dried and visualized by autoradiography.

Caspase activity assays using fluorogenic substrates. We determined caspase-3 activity by monitoring the cleavage of the fluorogenic substrate Ac-DEVD-AMC. We preincubated 4 μl (approximately 40,000 units) of purified caspase-3 (ref. 24) on ice with 16 μl of the chemical dissolved in caspase buffer (20 mM PIPES, 75 mM NaCl, 2.5 mM EDTA, 0.1% CHAPS and 7.5% sucrose). At specific time points, 5 μl of the reaction was taken out and diluted 100 times into 500 μl of the assay buffer (caspase buffer with 10 mM DTT and 10 μM fluorogenic substrate). Substrate cleavage was monitored at a constant temperature of 25 °C using a 360-nm excitation wavelength and a 480-nm emission wavelength and recorded once per second for 120 s. Under these conditions, fluorescence readings increased linearly for greater than 20 min.

Mass spectrometry. We performed the caspase-3 inhibition reaction by incubating 100 ng of caspase-3 with 100 μM 1,4-NQ (final concentration) in 20 μl CED-3 buffer (50 mM Tris at pH 8.0, 0.5 mM EDTA and 0.5 mM sucrose) for 2 h at room temperature (22 °C). The mock reaction was carried out under the same conditions without adding 1,4-NQ. Both samples were resolved on SDS-PAGE. Two caspase subunits were excised from the gel and then digested in gel with trypsin. The tryptic peptides were analyzed with a vMALDI-LTQ (Thermal Electron) mass spectrometer.

Note: Supplementary information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

D.K. performed most of the experiments. D.K. and D.X. designed and interpreted most of the experiments. Y.H.L. and J.Q. performed the mass spectrometry analysis and related data analysis. D.K. and D.X. wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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