Identification Of Genes Conferring Resistance To Furan In *Caenorhabditis Elegans*

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Abstract

Furan is a heterocyclic organic compound which is a liver carcinogen in mice and rats, associated with short-term liver toxicity and hepatic pathology; however, the mechanism of induction of these acute effects by furan is unknown. The aim of this study was to examine the toxicity of furan in *C. elegans* and identify the genes in mutagenised *C. elegans* responsible for resistance to furan, with the aim of identifying a mechanism of action of furan as a toxin in *C. elegans*. It would then be possible to test if the mechanism of acute toxicity of furan in *C. elegans* and mammals is conserved.

The growth of L1 larvae and brood size of N2 *C. elegans* were studied, and the effect of furan on these endpoints was measured by constructing full dose-response curves (n>3 for each point). Furan was toxic in both assays (EC_{50} ~2.7mM) and 30mM furan killed all offspring. N2worms (P_0) were mutagenised with Ethyl Methane Sulphonate (EMS), and F_1 generation worms at L4 stage were incubated with 30mM furan for three days. No offspring were obtained from non-mutagenised worms, but several lines of furan-resistant worms were obtained from mutagenised worms. Resistance to furan was shown to segregate as a recessive trait in crosses with N2 worms, and five independent resistant lines were shown to form three complementation groups. Both lines from one complementation group showed a high incidence of males (HIM) phenotype. The approximate location of this furan resistant gene was mapped on the left arm of chromosome V for resistant line 2. Future work will involve the fine mapping and identification of furan resistance genes.

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Abbreviation

BDA	cis-2-butene-1, 4-dial
C. elegans	*Caenorhabditis elegans*
C. I.	Confidence Interval
EMS	Ethane Methyl Sulfonate
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
GSH	Glutathione
HIM	High Incidence of Male
LB	Luria-Bertani
NGM	Nematode Growth Medium
NTP	National Toxicology Program
NRC	National Research Council
O. D.	Optimal Density
P450/CYP	Cytochrome P450
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
SNP	Single Nucleotide Polymorphism
TEMED	N,N,N,N- Tetramethylethylenediamine
UHP	Ultra High Purity

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Chapter 1 Introduction

1.1Furan

1.1.1 General properties and derivatives of furan

Furan (Figure 1.1) is an aromatic heterocyclic organic compound produced when wood, especially pine-wood, is distilled. It is also known as furane or furfuran. Furan is a colourless liquid which is toxic, flammable, highly volatile, and it also has a low boiling point close to room temperature (31.4°C). Furan is not very soluble in water; its water solubility is approximately 1% at room temperature, but it is quite soluble in alcohol or organic solvents. Ethanol and corn oil were the most common vehicles to study furan in biological models.

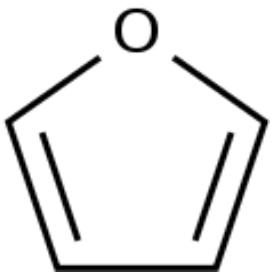


Figure 1.1 The chemical structure of furan (Modified from commons. wikimedia. org).

Furan and its derivatives are naturally occurring compounds, which can be found at low levels in many foods and drinks. In some food preparations, furan has been associated with the flavour of foods. Several derivatives of furan are very toxic (Kellert *et al*, 2008). Tetrahydrofuran is produced when furan undergoes a catalytic hydrogenation reaction with a palladium catalyst. Tetrahydrofuran is a commonly used organic laboratory solvent across a range of temperatures, but it is quite dangerous if inhaled or ingested.

1.1.2 Occurrence and usage of furan

Furan is a common chemical that used in industry to produce nylon, lacquers, insecticides, pharmaceuticals and other commercial compound (Moser *et al*, 2009). A recent survey (2004) carried out by the US Food and Drug Administration (FDA) showed that furan has been found in cigarette smoke, air pollution and is at high level in a variety of canned or jarred foods that undergo heat treatment, e.g. coffee and baby food (Moser *et al*, 2009, Peterson *et al*, 2005, FDA, 2005 and Durling *et al*, 2006). The European Food Safety Authority (EFSA) analysed furan concentrations for 273 different kinds of baby food, and the result showed that the highest furan concentration can be up to 112μg/kg. Assuming a standard jar of 234g baby food was eaten every day, there will be up to 26 μg furan exposures to human per day. Several studies have focused on the formation of furan in food, and thermal degradation of carbohydrates has been shown to be the mechanism whereby furan occurs in foods (Peterson *et al*, 2005 and Durling *et al*, 2006).

1.1.3 Furan toxicity and cancer

1.1.3.1 Furan toxicity and carcinogenicity

Since furan has been found in the food and environment, studies were carried out to characterise its toxicity. Previous experiment in rats (NTP, 1993) compared liver weight for both male and female rats after 90 days furan treatment (2, 4, 8mg/kg/day) to control, finding that there was a significant increase for liver weight in furan treated group, which indicates that furan has an adverse effect on liver weight. Histological examination of the livers showed that there was fibrosis in the bile ducts, necrosis and hyperplasia. Thus furan has marked toxic effects in the liver.

Studies in mice and rats have found that furan is carcinogenic after oral administration, inducing cholangiocarcinomas and hepatocellular carcinomas in liver. Gavage administration of furan in male and female F344/N rats for 2 years bioassay (NTP, 1993) showed that the incidence of hepatic cholangiocarcinomas, hepatocellular adenoma, hepatocellular carcinoma, and mononuclear cell leukaemia were increased in furan treated groups. A 2 year bioassay was also carried out in male and female B6C3F1 mice, and furan increased the incidence of hepatocellular adenoma, hepatocellular carcinoma and benign pheochromocytoma in a dose-dependent manner (NTP, 1993).

As a result of the carcinogenicity of furan in rodents, furan has been listed as a possible human carcinogen by the National Toxicology Program (NTP) in 2000.

1.1.3.2 Oxidation of furan

Burka *et al* (1993) compared the binding reactivity of furan with proteins between uninduced and induced F344 male rat liver microsomes, and showed that metabolism was needed to activate furan to a form which can react with protein. Subsequent investigation showed that the metabolism of furan was initiated by cytochrome P450 2E1-mediated oxidation (Kedderis *et al*, 1993; Peterson *et al*, 2005 and Kellert *et al*, 2008).

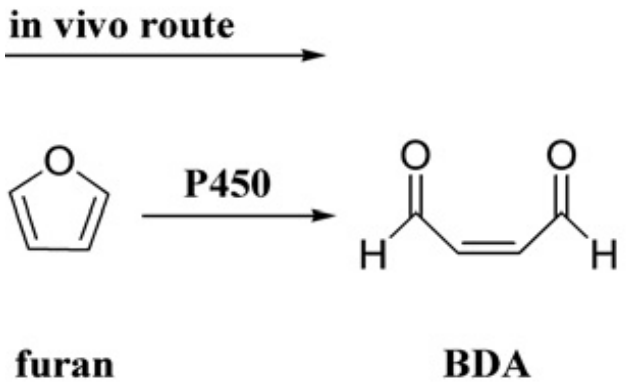


Figure 1.2 Metabolism of furan to *cis-2-butene-1*, 4-dial in vivo (Modified from Peterson *et al*, 2005). Furan oxidation was initiated by cytochrome P450 and metabolite BDA is produced.

Several studies have proved that *cis*-2-butene-1,4-dial (BDA) is an initial product during the oxidation of furan in vivo (Figure 1.2) (Kedderis *et al*, 1993; Peterson *et al*, 2005 and Kellert *et al*, 2008). BDA is a reactive metabolite that efficiently reacted with glutathione (GSH) to form mono- and bis-GSH reaction products (Figure 1.3). This metabolite is likely to react readily with protein, and this may have some role in furan-induced liver toxicity.

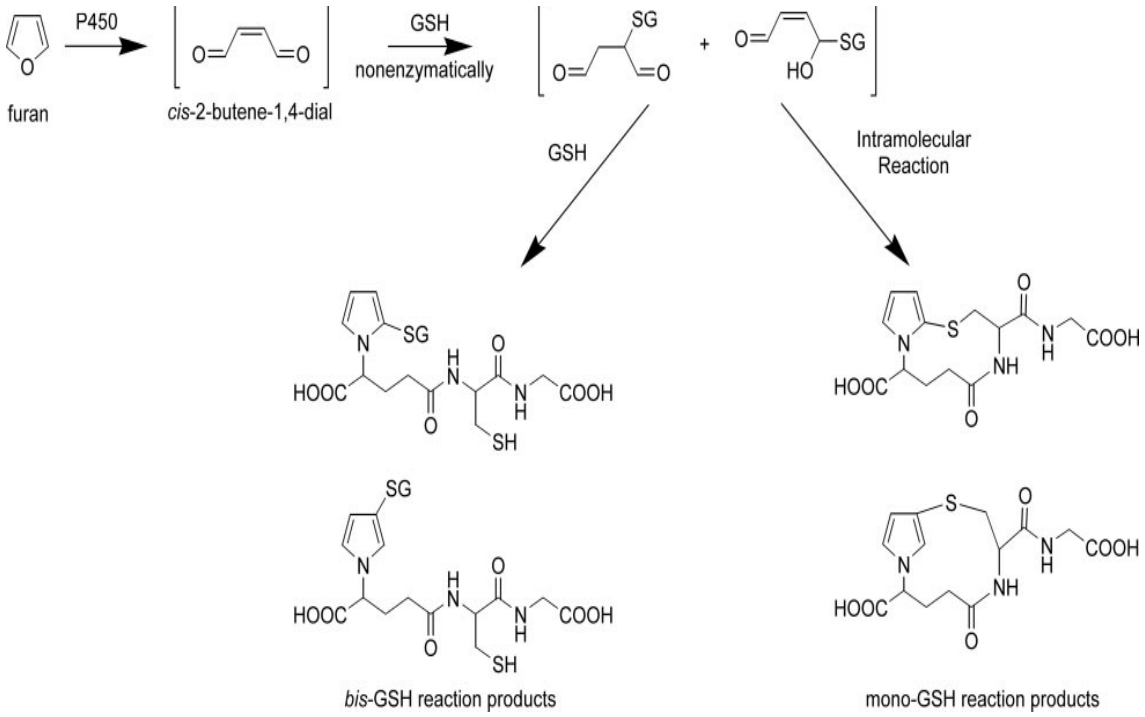


Figure 1.3 Reaction of *cis*-2-butene-1,4-dial with GSH to form mono- and bis-GSH reaction products (Modified from Peterson *et al*, 2005). *Cis-2-butene-1*,4-dial is the initial metabolite of P450-mediated furan oxidation. It can react with GSH to form different products.

1.1.3.3 Cytochrome P450 2E1

Cytochrome P450 (CYP) is a superfamily of haemoproteins that can be found throughout the biological kingdoms. The name was given due to its characteristic Soret peak formed at wavelength near 450nm. CYPs metabolise both endogenous and exogenous compounds and play an important roles in enzymatic metabolism of xenobiotics. In human, it is distributed

mainly in the inner membrane of the mitochondria or the endoplasmic reticulum, and it is a key element in human oxidative metabolism. P450 2E1 is a member of the CYP superfamily, which is involved in the metabolism of xenobiotics in the body. Although it is only involved in the oxidative metabolism for a range of small polar molecules, it is a mediator in lots of important drug interactions.

1.1.3.4 Mechanisms of furan toxicity/ carcinogenicity

The mechanisms behind the carcinogenic effect of furan are unclear. Although genotoxicity is a possible mechanism for the carcinogenicity of furan (Byrns *et al*, 2006), the evidence for genotoxicity is weak, since there are numerous assays which fail to show genotoxicity of furan. For example, the classical in vitro genotoxicity assay in bacteria, the Ames test, showed a negative result with five different strains of *Salmonella typhimurium* with and without S9 (S9 is a preparation of liver homogenate, specifically the supernatant after centrifugation at 9000g) from rat or Syrian hamster liver (NTP, 1993). Wilson *et al* (1992) carried out studies in both $B6C3F_1$ mice and F344/CrIBr rats and found that there was no induced unscheduled DNA synthesis when these rodents were administered furan at a frankly carcinogenic dose. This experiment showed that there was no induced DNA damage in hepatocytes for $B6C3F_1$ mice, or F344/CrIBr rats, and in view of the high incidence of tumours at these dose levels of furan, is strong evidence against a genotoxic mechanism of action. Burka *et al* (1991) showed that there was no detectable radioactivity in DNA from the livers of rats treated with [^{14}C] furan, which showed that furan and its metabolites did not covalently bind to DNA. As a contrast, Kellert *et al* (2008) showed that BDA (furan metabolite) is genotoxic in L5178Y tk+/- mouse lymphoma cells, while furan was inactive. Furan showed increased chromosomal aberrations in lymphocytes of B6C3F1 mice; DNA and chromosomal damage in CHO cells, but these latter endpoints show a tenuous connection

to the liver-specific carcinogenicity of furan. Together, these results suggest that the mechanism of furan toxicity does not follow a genotoxicity pathway.

Several studies characterised that furan carcinogenicity follows a non-genotoxic pathway. Fransson-Steen *et al* (1997) carried out a bioassay in B6C3F1 mice and Fisher 344 rats with varying doses of furan. The mice and rats were compare to control directly and after short-term gavage administration, showing that furan has effects on liver biology, inducing an increase in hepatic necrosis, apoptosis, increased DNA synthesis and bile duct hyperplasia at early time points. It is hypothesised that the acute hepatotoxic effects of furan are the reason for the subsequent development of tumours in liver, and that the acute toxicity is mediated through a receptor-mediated mechanism.

To sum up, there was only weak evidence to support the mechanism of acute furan toxicity followed a genotoxicity pathway and the preponderance of evidence indicated that the mechanism of furan is most likely to follow a non-genotoxic pathway.

1.2 Caenorhabditis elegans

1.2.1 *C. elegans* as a biological model organism

Caenorhabditis elegans (*C. elegans*) is a free living nematode, which inhabits soil and leaf-litter environment in many parts of the world (Hope, 1999). It has become as a very important model organism in various fields since it was first introduced by Dr Sydney Brenner for animal development and behaviour studies in 1965 (Riddle *et al* , 1997). Not only have 60-80% of human genes been identified in *C. elegans* homologues (Kaletta and Hengartner, 2006), but also 12 out of 17 known signal transduction pathways in human are conserved in C. *elegans* (NRC, 2000). The success of *C. elegans* as a model in study of neuroscience,

developmental biology, signal transduction, cell death, aging, and RNA interference has attracted increased attention in the fields of biomedical and environmental toxicology (Leung *et al*, 2008).

There are several features for *C. elegans* which make it a powerful tool in biological research. First of all, it is cheap and easy to maintain in laboratory conditions. *C. elegans* is normally kept on a nematode growth medium agar plate seeded with *E. coli* OP50 as a food source. Secondly, *C elegans* has a short hermaphroditic life cycle of around 3 days at 25°C, and also produces a large number of offspring (>300). It makes large-scale production of animals within a short period of time possible (Hope, 1999). Because *C. elegans* has a small body size, it can be easily transferred and manipulated in various assays, such as *in vivo* assays conducted in a 96-well plate. Furthermore, it is a perfect model for cell biology, developmental biology and histology due to its transparent body, which can give clear observation of all cells in the body (Leung *et al*, 2008). Finally, the development of genetic and molecular tools provides a variety of choices to manipulate and study *C. elegans* at the molecular level.

1.2.2 Anatomy

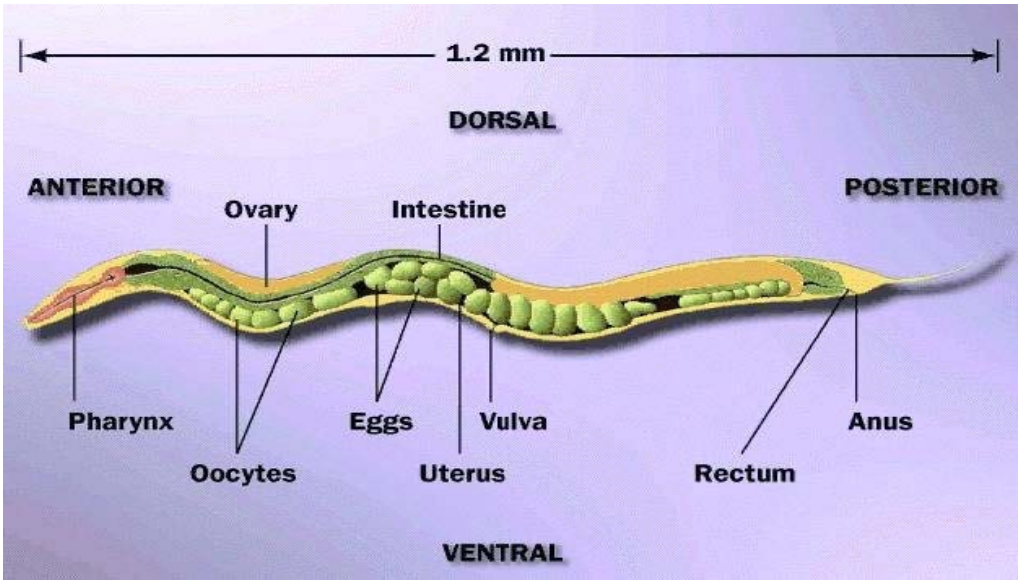


Figure 1.4 Structure of *C. elegans* (Taken from *C. elegans* neural network project). This is a cartoon of *C. elegans*. The scale represents the length of the worms and the name of each antomical feature is labelled as indicated.

C. elegans is a small worm with a maximum length and diameter of 1.2mm and 80μm, respectively. As shown in figure 1.4, *C. elegans* has an unsegmented, cylindrical body shape. It becomes tapered at both ends. The body consists of two tubes, known as outer tube (body wall) and inner tube (gut). The two tubes are separated by the pseudocoelomic space and the whole body shape is maintained by the internal hydrostatic pressure (Strange 2006).

C. elegans has two sexes, hermaphrodites and males (Albert *et al*, 2008). Hermaphrodites have 959 somatic cells, and are the dominant sex in the population, whereas, males comprise approximately 1/1000 of the total population, with 1031 somatic cells in the body. Figure 1.5 shows a comparison between male and hermaphrodite, with the male shorter and thinner than the hermaphrodite and with a fan-shaped tail for clasping during mating. The fan itself is only apparent in the adult. There are cellular differences at younger developmental stages that

cause a swelling in the tail that is visible under a typical dissecting microscope of L3 and L4 larvae.At the genetic level, hermaphrodites have five autosomes and two X chromosomes; males also have five autosomes but only one X chromosome due to non-disjunction in the germ line of hermaphrodite. The males are designated as XO, distinguishing them from the XX hermaphrodite. The hermaphrodites can self fertilise and produce up to 300 offspring, whereas the males have to mate with hermaphrodites to carry out reproduction. Once the male has mated with the hermaphrodite, there can be more than 1000 offspring produced. Mating rarely happens in normal conditions due to the low occurrence of males.

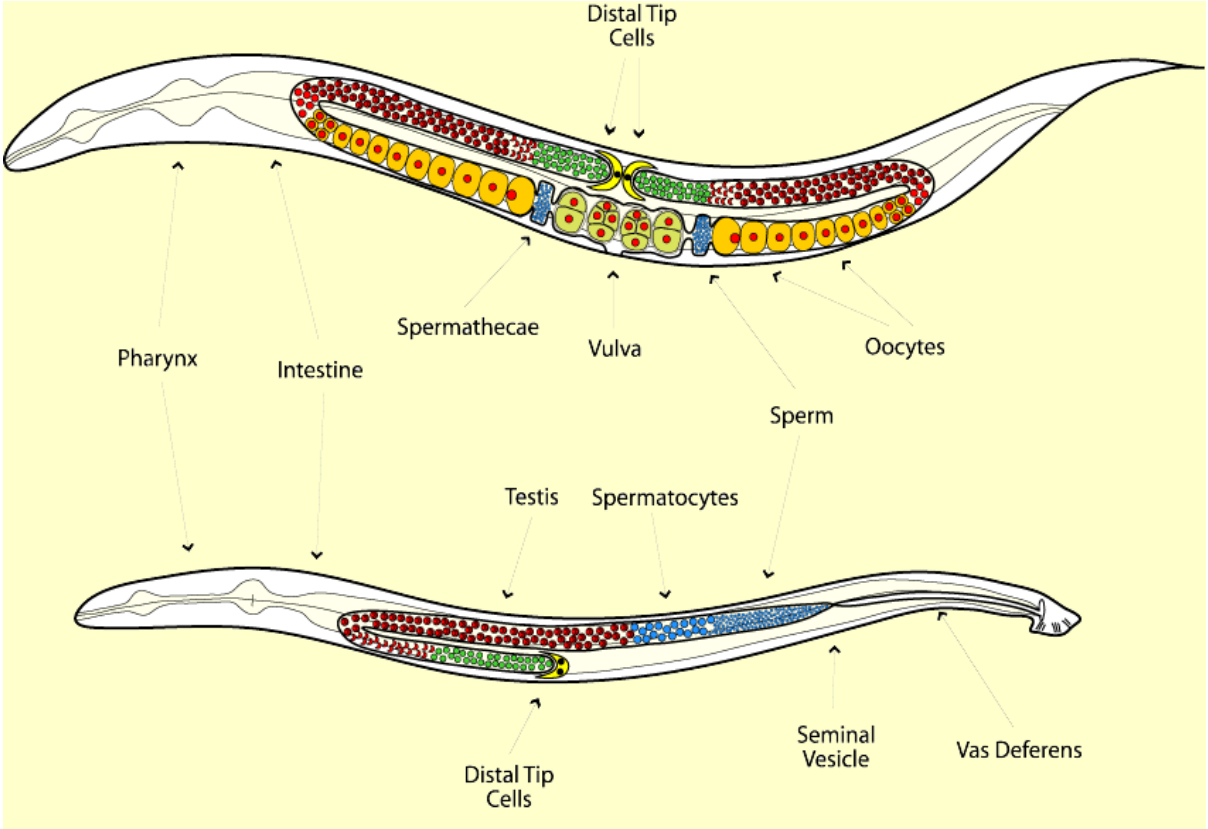


Figure 1.5 Comparison of hermaphrodites and male *C. elegans* (Taken from Hansen, D. and Pilgrim D., 1998). The hermaphrodite is shown above the male in this figure. The male is shorter and thinner than the hermaphrodite and with a fan-shaped tail for clasping during mating.

1.2.3 Life cycle

C. elegans has a short generation time. The length of the entire life cycle can vary between 3 days at 25°C to 6 days at15°C. The normal growth temperature is between 15°C and 25°C. Any temperature higher or lower than this range will inhibit the growth of *C. elegans* and the optimal growth temperature is 20°C. More than 300 progeny can be produced at this temperature over the course of 3-4 days. The whole life cycle can be divided into 2 main stages: embryonic and postembryonic (figure 1.6).

The embryonic stage is the first stage in the life cycle. As shown in figure 1.6, this stage can be sub-divided into two stages, known as *in utero* development and *ex utero* development. This stage lasts over 14 hours at 20°C.

The postembryonic stage includes four larval stages (L1-L4) and adulthood. Each larval stage is marked with a moult, and larval stage lasts over 43 hours at 20°C. L1 stage (~12 hours) is the first larval stage after hatching, the worm is about 250μm in length and it will arrest if there is no food supplied. There are two possible options at L2 stage dependent on the conditions. If the growth conditions are normal, the worm is about 360-380μm long and it will go to L3 stage after about 8 hours; but if the worms are crowded, starved or at high temperature, they will go to an alternative L3 stage known as dauer. At this stage, the worm is about 400μm in length, but it is much thinner than the normal L3 stage worms and can survive up to 4 months. The worm will go to L4 stage as soon as the growing conditions return to normal. L4 stage is the last stage before the adulthood, during which the gonad produces sperm. A further 10 hours will bring the worms into a young adult stage. Another 8 hours development is needed in order to have the capability to lay eggs.

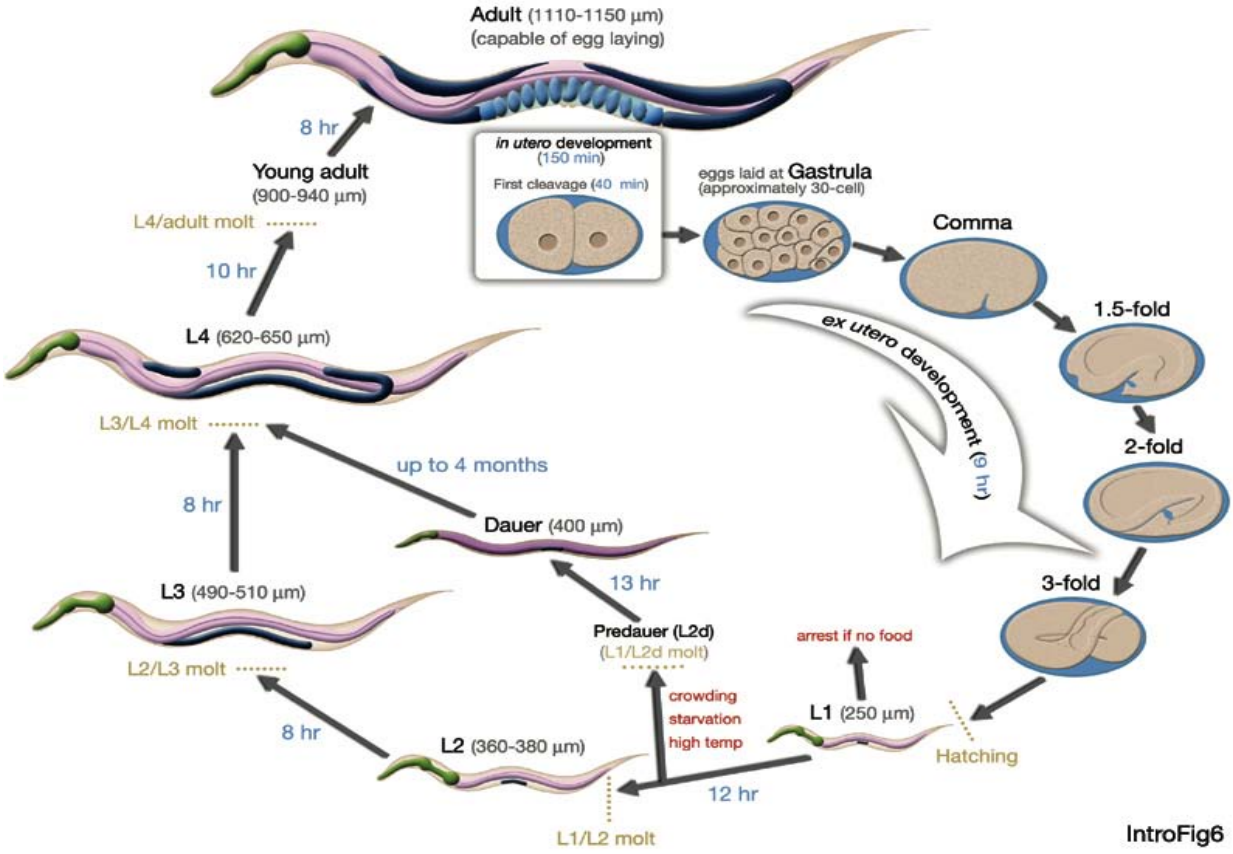


Figure 1.6 The life cycle of *C. elegans* (taken from wormatlas.org). The number in red colour showed different conditions; the number in blue colour showed the length of each stage and the length of worm at each stage was shown next to the stage name in micrometers.

1.3 Genetic Mapping

1.3.1 Single Nucleotide Polymorphism

Single Nucleotide Polymorphism (SNP) is a single nucleotide variation happens in genetic sequence with an appreciable frequency in the population. Such polymorphisms normally involve changes from a C to T or an A to a G; however changes such as an insertion or deletion of a nucleotide are common as well. SNP have become valuable genetic markers of human diseases (Kruglyak, 1999). There are also high density marker set for mapping genetic mutations in C. elegans (Wicks *et al*, 2001).

Single Nucleotide Polymorphism mapping has become an important tool in *C. elegans* studies since it was first introduced in 2001(Wicks *et al*). The purpose of SNP mapping is to generate high resolution maps of chromosomes in order to identify a mutated gene and investigate its functions. The advantage of using SNP mapping is that it is possible to narrow down the location of a mutation to a defined area. N2 wild-type *C. elegans* and the related Hawaiian CB4856 *C. elegans* are used as parental strains for SNP mapping. The Hawaiian CB4856 *C. elegans* is a strain isolated from Hawaii. It shows a high density of polymorphisms in comparison to the N2 wild-type. The difference of genomic sequence between Hawaiian CB4856 and N2 occur every 1000bp on average. The online SNP database can provide the information about the SNP exist between CB4856 and N2, and their genetic position on the chromosome (Wormbase and Wormbook).

There are normally two phases involved in SNP mapping. The first phase is known as chromosome mapping; it is similar to traditional two-factor mapping, and the purpose is to identify the general location of the gene of interest. The second phase is called interval mapping, which also known as three-factor is mapping. The aim of this phase is to place the gene of interest in an interval between two SNPs; subsequently, the gene of interest can be further investigated. SNP detection in these two phases is performed by using only SNPs that change a restriction site (snip-SNP). PCR amplification, restriction enzyme digestion and gel electrophoresis are required to detect the SNP.

1.3.2 Whole genome sequencing

C. elegans has long been used to identify genes involved in biological processes. Random mutagenesis and subsequent isolation of mutants defective in a given process is normally involved in the traditional genetic approach. The identification of the resistant gene in a

mutant strain is a painstaking process that involves mapping with genetic and/or single-nucleotide polymorphism (SNP) markers.

Whole genome sequencing is a process that determines the complete DNA sequence of an organism, and is now a viable proposition for sequencing *C. elegans* mutants with the advent of high-throughput sequencing technologies. It is a time saving approach to pinpoint the position of mutations induced by various chemicals in genetic model organisms. Whole genome sequencing has been used to identify model genes in EMS mutagenised *C. elegans*, but current approaches require mapping of the mutant to a small region of the genome, since mutagenesis typically induces large numbers of mutations throughout the genome (Sarin *et al*, 2008 and Shen *et al*, 2008).

1.4 *C. elegans* as model organism in furan toxicity studies

The purpose of this study was not focused on the liver carcinogenicity of furan, but on the mechanism of acute furan toxicity. Previous work by C. Budd (2008) carried out a toxicity test for furan in *C. elegans* at different larval stages and found that furan had an inhibitory effect with a dose-dependent manner on brood size, L1 growth and lethality, which shows the *C. elegans* is a good model for furan toxicity studies. In the same study, the EC_{50} value for these toxic effects of furan was about 7mg/L. This is similar potency to the dose of 2mg/kg/day that is carcinogenic in rat (Kellert *et al*, 2008 and Moser *et al*, 2009). Our hypothesis is that the mechanism of acute toxicity of furan in *C. elegans* is conserved in mammals, and we sought to test this hypothesis by determining the mechanism of toxicity of furan in *C. elegans*.

1.5 Aim

The aim of this study was to examine the toxicity of furan in *C. elegans* and establish a screening method to examine whether it was possible to obtain furan-resistant worms, followed by the isolation of furan-resistant mutant worms. It also aimed to map the position of genes responsible for resistance to furan in mutant *C. elegans* in order to further identify the genes, with the aim of identifying a mechanism of action of furan as a toxin in *C. elegans*. It would then be possible to test if the mechanism of acute toxicity of furan in *C. elegans* and mammals is conserved.

Chapter 2 Materials and Methods

2.1. Experiment materials

LB (Luria-Bertani) broth, LB agar Agar and Tryptone were produced by Melford laboratory LTD. TEMED, Tween 20, Ethanol, Furan, Sodium Azide and Bleach solution were produced by Sigma. Primer sets and quick load 100bp ladder were provided by New England Biolabs. PCR Reddy mix was ordered from Thermo Scientific. DNeasy Blood & Tissue Kit was obtained from QIAGEN. The rest of chemical used were obtained from Fisher Scientific.

2.1.1 Recipes of reagents

LB Broth

Tryptone	10g
NaCl	10g
Yeast extract	5g
H_2O	1L

LB Agar

Tryptone	10g
NaCl	10g
Yeast extracts	5g
Micro Agar	10g

H_2O	1L

NGM (Nematode Growth Medium) Agar

NaCl	3g
Agar	17g
Tryptone	2.5g
Cholesterol (5mg/ml in ethanol)	1ml
H_2O	975ml

Additives after autoclaving;

$CaCl_2$	1M	1ml
$MgSO_4$	1M	1ml
Potassium Phosphate	1M pH=6	25ml

K Medium

KCl	2.38g
NaCl	3.09g

Freezing Down Solution

NaCl	5.85g
KH_2PO_4	6.8g
Glycerol	300g

NaOH 1M 5.6ml

Addictive after autoclaving;

$MgSO_4$ 0.1M 3ml

S Basal

NaCl 0.1M

Potassium Phosphate pH=6 0.05M

Cholesterol (5mg/ml in neat ethanol) 1ml/L

Trace Metals Solution

Disodium EDTA 1.86 g (5 mM)

FeSO4 7H2O 0.69 g (2.5 mM)

MnCl2 4H2O 0.20 g (1 mM)

ZnSO4 7H2O 0.29 g (1 mM)

CuSO4 5H2O 0.025g (0.1 mM)

Dissolve in 1L water; aliquot into 50 ml conicals and store in dark.

S Medium

S basal 1L

Potassium Citrate 1M pH=6 10 ml

Trace Metal solution	1M	10ml
$CaCl_2$	1M	3ml
$Mg\ SO_4$	1M	3ml

Egg Isolation Bleach Solution

NaOCl	1M	4ml
NaOH	10M	1ml
H_2O		5ml

M9 Buffer

KH_2PO_4		3g
Na_2HPO_4		6g
NaCl		5g
$MgSO_4$	1M	1ml
H_2O		1L

Lysis buffer

Tris-HCl (pH=8.0)		1ml
KCl		0.373g
$MgCl_2$		0.051g

IGEPAL (Nonidet P40)	450µl
Tween 20	450µl
Gelatin	10µg
Proteinase K (10mg/ml)	500µl

The lysis buffer was kept at -20°C.

TBE buffer (10×)

Tris	108g
Bomic Acid	55g
EDTA (pH=8.0) 0.5M	40ml

12% TBE-PAGE Gel

Polyacrylamide (30%)	40ml
H_2O	50ml
10× TBE buffer	10ml
TEMED	100µl

The solution was kept at 4°C. The gel was polymerised by adding 10µl per ml of Ammonium persulphate (APS. 0.1g/ml).

PCR Reddy Mix (1.1x working concentration)

Thermoprime plus DNA polymerase	1.25 units
Tris-HCL (pH=8.0)	75mM

$(NH4)2SO_4$	20mM
MgCl2	1.5mM
Tween 20	0.01% (v/v)
dATP, dCTP, dGTP and dTTP	each at 20mM

The master mix was stored at 4°C.

Loading buffer

Glycerol	5ml
Bromophenol blue	25µl
Xylene cyanol FF	25µl
10 X TBE	1ml

TEN buffer

Tris- HCL (pH=8.0)	1M	0.5ml
EDTA (pH=8.0)	0.5M	20µl
NaCl	5M	0.6ml
H_2O		8.9ml

2.2 General methods

2.2.1 Bacterial culture

The strain *E. coli* OP50 was used in this study as the food source for *C. elegans*. An LB agar plate was streaked with *E. coli* OP50 under sterile conditions and then incubated at 37°C overnight. A single colony of *E. coli* OP50 was picked and inoculated into a 20ml universal

tube containing 5ml of LB broth under sterile conditions. The tube was then incubated at 37°C on a shaker with shaking at 240rpm overnight. Both the plate and the liquid medium were stored at 4°C. The plates were re-streaked every 4 weeks.

2.2.2 Preparation of NGM agar plate

NGM agar was melted by microwaving. Once melted, the NGM agar was put in a 60°C water bath for 30 minutes. Subsequent steps were then carried out in a class II hood. For every 100ml of NGM agar, 100µl of 1M $CaCl_2$, 100µl of 1M $MgSO_4$ and 2.5ml of 1M Potassium phosphate buffer (pH=6) were added. The NGM agar was mixed and used to prepare 9cm plates and 3.5cm plates. For large plates, NGM agar was directly poured in and the amount used was just sufficient to cover the bottom of the plate; 4ml of NGM agar was added into each small plate. All the plates were left in the hood to cool down. *E. coli* OP50 was added after the agar had set. The amount added for large and small plates was 2ml and 150µl. The small plates were then left in the hood to air dry. The *E. coli* OP50 was spread by a sterilized glass spreader for the large plate. As soon as all the plates were dried, they were incubated at 37°C overnight. On the next day, the plates were taken out and ready to use.

2.2.3 Egg isolation

Egg isolation is also called “bleaching”. It is a method used to isolate eggs from a mixed population or to eliminate contamination.

Several plates of N_2 gravid hermaphrodite worms were chosen to bleach. The worms were washed off with 3-4ml of K-medium and transferred into a 15ml centrifuge tube. The tube was vortexed for 4 minutes as soon as an equal amount of bleach solution was added. Centrifugation was carried out at 500g for 1 minute. The supernatant was discarded and the

pellet was washed with 4ml of K-medium. The centrifugation and washing were repeated for 3 times. Finally, the pellet was re-suspended in 2ml of fresh K-medium and transferred into a 6-well plate. The plate was sealed with parafilm and incubated at 15°C overnight to allow L1 worms to hatch.

2.2.4 Freezing worms

An over populated plate with large numbers of L1 worms was washed with 3 ml of K-medium. The K-medium with worms was then transferred in to a 15ml tube. Equal amount of freezing down solution was added and mixed by vortexing. 1ml aliquots were dispensed into eppendorf tubes. The eppendorf tube was labelled clearly and stored at -80^{0}C.

2.2.5 Agarose pad preparation

A 2% (w/v) agarose solution was used to make agarose pads on microscope slides. First of all, an ordinary microscope slide was placed between two microscope slides held together by tape (Figure 2.1). The 2% (w/v) agarose solution was then melted by microwaving with 50% power for 1 minute. A drop of agarose was placed in the middle of the central slide and another slide was used to cover the agarose in a perpendicular orientation as shown in figure 2.2. Finally, the top slide was removed after 1 minute to allow the agarose to set and the slide was labelled clearly with a furan concentration. The steps were repeated to give sufficient agarose pads for the assay.

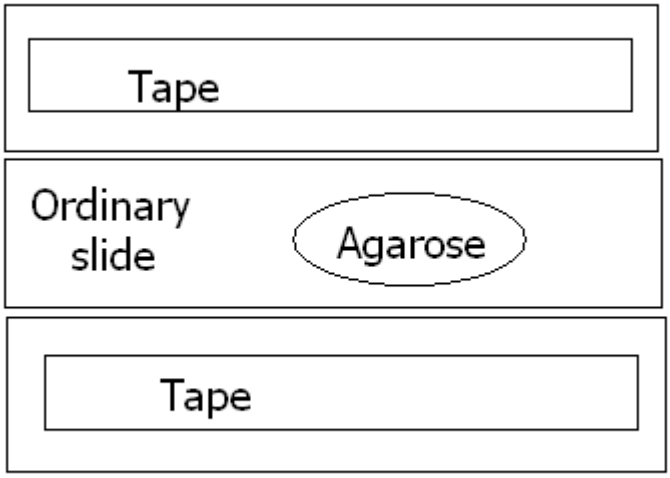


Figure 2.1 Preparation of agarose pads on microscope slides. An ordinary microscope slide was placed between two microscopy slides held together by tape**.** A drop of agarose was placed in the middle of the central slide. This figure was modified from C. Budd, MRes thesis (2008).

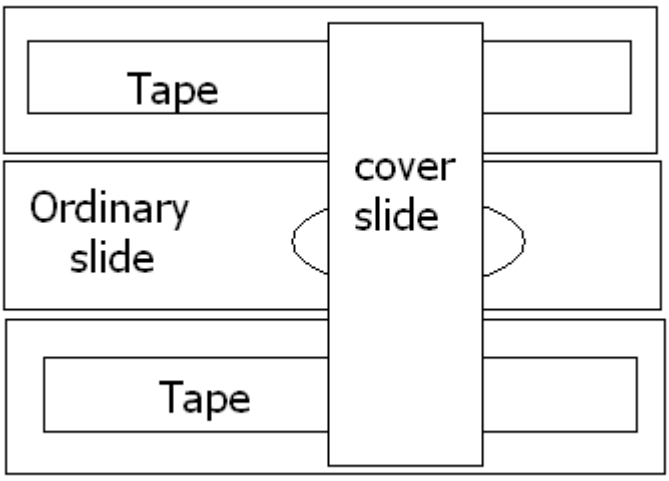


Figure 2.2 Preparation of cover slide on the top of agarose. Another slide was used to cover the agarose in a perpendicular orientation. It was then removed after 1 minute of agarose setting time and labelled clearly with a furan concentration. This figure was modified from C. Budd MRes thesis (2008).

10µl of each sample was pipetted individually on the agarose pads. A cover slip was then carefully placed on top. A Scion 1310M CCD camera and Visicapture (Scion corporation version CFW 1310) software were used to record photographs for the worms. The objective of the microscope was set at 10X in order to give all the images at the same scale. The images of 30 worms per each concentration were recorded. The images of 10 L1 worms were also recorded before the assay to determine the initial size of the worms.

The measurement of worm size in pixels was achieved by outlining the edge of the body with magnetic lasso tool in Adobe Photoshop CS4. A known area was labelled and the number of pixels was recorded first. The number of pixels was then divided by the known area to give $1mm^2$ = 1,709,750 pixels, so that the worm size in pixels can be converted to mm^2. The data was then analysed using GraphPad Prism 5; the model used in this experiment was log agonist vs. response and the equation was Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope)).

2.3 *C. elegans* toxicity assays

Methods used in these toxicity assays were modified from paper published by Bischof *et al* in 2006, and *C. elegans*: Methods and applications; Methods in Molecular Biology (Bischof *et al*, 2006 and *C.elegans* book).

All the toxicity assays were performed in liquid culture. The furan was dissolved in ethanol due to the poor solubility of furan in water. Different concentrations of ethanol vehicle (shown as percentages) were tested in both L1 growth assay and brood size assay for optimisation.

2.3.1 L1 growth assay

This assay was carried out with larval stage 1 (L1) worms. The aim of this assay was to test the effect of furan on growth and development of *C. elegans*.

A plate of gravid hermaphrodite worms was bleached and the eggs were kept in a 6-well plate at 15°C overnight to synchronise the *C. elegans* at L1 stage. 1ml of cultured E. coli OP50 was transferred into an Eppendorf tube and spun at 2000g for 5 minutes after overnight incubation. The supernatant was then discarded and the pellet was re-suspended in 1ml of S-medium. The procedure was repeated with L1 worms, but the centrifugation was at 500g for 1 minute. The absorbance of the bacterial culture was adjusted to 0.7 O.D. (Optical Density) at 600nm, with the spectrophotometer blanked with S-medium. The number of L1 worms in S-medium was adjusted to give 6-8 worms/µl.

Small scale liquid culture was set up in a 24-well plate, with final volume of 400µl of *E. coli* OP50, S-medium and furan at different concentrations. 5µl of L1 worm suspension was added in each well before the furan. Triplicates of each concentration were used to assess viability.

Once the liquid cultures were set up, the 24-well plate was sealed with parafilm to prevent evaporation. A damp paper was also used to wrap the plate to maintain humidity. The plate was then kept in an air tight plastic container and incubated at 15°C for 3 days.

The worms were immobilised by adding 4µl of 1M sodium azide to each well after 3 days incubation. The contents of each well was then transferred into eppendorf tubes and centrifuged at 500g for 1 minute. Most of the supernatant was discarded; only about 15µl was left to re-suspend the pellet.

2.3.2 Brood size assay

This assay was carried out with larval stage 4 (L4) worms.

A plate of gravid hermaphrodite worms was bleached and the eggs were cultured in a 6-well plate at 15°C overnight. The L1 worms were then transferred onto a 9cm NGM plate and kept in a 15°C incubator for 3 days to get L4 stage worms.

1ml of freshly cultured *E. coli* OP50 was transferred into an Eppendorf tube and spun at 2000g for 5 minutes after overnight incubation. The supernatant was then discarded and the pellet was re-suspended in 1ml of S-medium. The absorbance of the bacterial culture was adjusted to 0.7 O.D. at 600nm.

Small scale liquid culture was set up in a 48-well plate, with final volume of 400µl of *E. coli* OP50, S-medium and furan at different concentrations. A single L4 worm was added in each well; furan were then added. Triplicates of each concentration were also used to assess vaiability.

Once the liquid cultures were set up, the 48-well plate was sealed with parafilm to prevent evaporation. A damp paper was also used to wrap the plate to maintain humidity. The plate was then kept in an air tight plastic container and incubated at 15°C for 3 days.

The number of offspring in each well was counted under a microscope after 3 days incubation. The data was then analysed as described in chapter 2.3.1.

2.4 EMS (Ethyl Methane Sulphonate) Mutagenesis and screening

2.4.1 EMS Mutagenesis

A single N2 worm was picked and transferred onto a 9cm NGM agar plate. The plate was incubated at 20^0C for a week to generate a large population. The reason for doing this is that the DNAs of all the worms are from the same N2 worm. Before mutagenesis, the worms were synchronised at L4 stage and a frozen stock of this N2 sample was made and kept at -80^0C.

The worms were washed off from the plate into a 15ml centrifuge tube by M9 buffer, when most of the worms were in the L4 stage. The worms were then centrifuged at 200g for 30 seconds. The supernatant was discarded and the pellet was washed again with fresh M9 buffer. The centrifugation was repeated as before. The supernatant was discarded and the pellet was resuspended in 2ml of M9 buffer.

A 0.1M EMS solution was made up in the fume cupboard by adding 20µl of liquid EMS to 2ml of M9 buffer in another 15ml centrifuge tube. The solution was mixed. 2ml of the suspension of washed worms was added to the 2ml of EMS solution to give a final concentration of 0.05M.

The tube was sealed with parafilm and placed on a rocker at 20^0C for 4 hours. All the tubes and tips contaminated with EMS were soaked with an equal volume of inactivation solution (0.1M NaOH, 20% w/v $Na_2S_2O_3$) and kept in the fume cupboard for 24 hours prior to disposal.

The worms were washed twice with M9 buffer after mutagenesis and transferred onto a new NGM agar plate. The worms were then left on a plate for 2 hours recovery. 10 healthy

looking late L4 worms were pick up onto a small 3cm NGM agar plate after recovery and marked as P_0. 20 groups of P_0 worms were prepared and incubated at 20^0C for 20 hours. P_0 adults were washed off from the plates, the eggs were left on the plates to hatch and labelled as F_1. The F_1 was then incubated at 20^0C for 3 days to get L4 worms. The L4 stage F_1 was ready for F_2 screening assay.

2.4.2 F_2 screening assay

The F_2 screening assay was based on the results from the *C. elegans* toxicity assay, And was carried out in a large scale liquid culture. The concentration of furan used in this assay was 30mM, which was found to kill 100% of wild type worms.

The absorbance of the bacterial culture was adjusted to 0.7 O.D. at 600nm. Each group of L4 stage F_1 was washed into an individual 200ml flask with 35.9ml of S-medium. For each flask, 4ml of E. coli OP50 at 0.7 O.D. and 0.1ml of 12M furan in neat ethanol were then added to give a final concentration of furan at 30mM (ethanol concentration of 0.25 % ,v/v). All the flasks were sealed and incubated with a shaker at 15^0C for 3 days.

A group of wild type L4 worms with 30mM furan and a group of mutant L4 worms without furan were carried out at the same time as positive and negative control.

After 3 days incubation at 15^0C, the content of each flask was centrifuged at 500g for 5 minutes. The supernatant was discarded and the pellet was re-suspended into 2ml of M9 buffer. All the samples were transferred individually onto new 9cm NGM agar plates and stored at 20^0C for 3 days. For each group from the same flask, every single surviving worm

(F_2) was picked up and grown on a 3cm plate. The plates were labelled clearly with group number and self-fertilised at 25^0C for a week to produce a large population.

The brood size assay and L1 growth assay was carried out for every F_2 worm as described before; wild type worms were tested alongside as a control. The worms were kept if they were resistant to furan. Frozen stocks for every resistant F_2 group were made and stored at -80^0C. A complementation assay was carried out in the next step.

2.4.3 Complementation assay

The aim of this assay was to check if the different furan resistance lines were from the same gene. L4 hermaphrodites of each resistant group and N_2 control were heat shocked at 30^0C for 6 hours. The worms were then recovered at 20^0C to lay eggs. Approximately 1% of offspring were males after the heat shock treatment. 6 males from one group were picked up and transferred onto a new 3cm plate; meanwhile, 1 virgin L4 hermaphrodite from the other group was picked up and transferred onto the same plate. The mating was then carried out at 15^0C. The cross mating was repeated for the rest of the groups. The mating with L4 hermaphrodite in the same group was used as a positive control, whereas, a mating with N2 wild type L4 hermaphrodite was used as a negative control. The gravid hermaphrodite for each plate was picked up and placed in 30mM furan for selection after laying eggs for 24 hours and the F_1 generation was checked for viability. If the F_1 heterozygous offspring survived, it means the mutation is in the same gene. Otherwise, the mutations are in different genes. The eggs left on the plate were allowed to grow. The percentage of males was checked to make sure the mating was successful. Frozen stocks for different complementation groups were made and stored at -80^0C.

2.4.4 High Incidence of Males (HIM) test

High incidence of males was obtained in one of the complementation groups during the study. The furan-resistant gene in this group may also confer a high incidence of males genotype. The aim of this test was to determine if HIM was a significant difference compared to controls.

30 L4 hermaphrodites from this group were picked and self-fertilised in a 9cm NGM agar plate at 25^0C for 3 days (~1000 offspring). The number of males was then counted for every 1000 worms. The test was carried out in triplicate and N2 wild type with worms from other complementation groups were carried out alongside as controls. A t-test was taken to check if the high incidence of males in this complementation group was significant compared to N2 wild type and other groups.

2.4.5 Genetic mapping of furan-resistance genes

6 Hawaiian strain males were picked up and transferred onto a new 3cm plate; meanwhile, 1 virgin L4 hermaphrodite from one of the complementation groups was picked up and transferred onto the same plate. The mating was then carried out at 15^0C. The breeding was repeated for each of the groups. The mating with L4 Hawaiian hermaphrodite was used as a negative control. The gravid hermaphrodite and Hawaiian males for each plate were picked up and transferred onto new plates after laying eggs for 24 hours. This was repeated for 3 days. The eggs left on the plate were allowed to grow up to L4 stage. The percentage of males was checked to make sure the mating was successful.

The genotype for all the F_1 generation should be heterozygous. The F_1 generation from day 2 was kept in 50µl of lysis buffer and stored at -80^0C for genetic mapping. The F_1 generation

from day 3 was picked up for selection at the furan concentration of 30mM as soon as they were in L4 stage. The F_1 was taken out from the liquid culture after 2 days and lysed in 50µl of lysis buffer for genetic mapping. The surviving F_2 generation was allowed to self-fertilise individually and generated a large population for DNA preparation. The worms were then mixed to give a pool for DNA purification. The DNA samples were stored at -80^0C. Approx. 200 F_2 recessive homozygous worms were lysed in 200µl of lysis buffer and stored at -80^0C for genetic mapping.

2.5 Polymerase Chain Reaction (PCR) and Single Nucleotide Polymorphism (SNP) mapping

Methods used in PCRs were modified from paper published by Davis *et al* (2005) and Wicks *et al* (2001).

As described in chapter 1.2.2, *C. elegans* has 6 chromosomes. Three snip-primers were designed for each chromosome, two for each arm and one for the centre. Therefore, 18 PCR primer sets were used for SNP mapping. The details for these 18 primers are shown below in table 2.1.

	Clone	Allele	P1	P2	Enzyme	Length of PCR product	RE fragment size
I	Y18H1A	pkP1096	aagcgaaccaatcagcag	tcacgccaaaattaatggg	DraI	402	152 /251
I	B0041	pkP1097	atgcaggcattcgacgaatc	gtcgaaaaagcgcacaattc	DraI	448	110/103/235
I	C37A5	pkP1071	ctcatgcatgatttcgaggg	aaatccaacaggagcaggac	EcoRI	526	244/282
II	T01D1	pkP2101	aagaggtgttcttctgcagc	accatccacgcagttcattc	DraI	618	45/173/400
II	T13C2	pkP2107	tccacactatttccctcgtg	gagcaatcaagaaccggatc	DraI	494	300/70/125
II	F15D4	pkP2116	ttcccattttcctcccag	tcaaaaacccagacactgg	DraI	516	n/a
III	K02F3	pkP3045	ggcataccatagtatgcggtac	gactcttgggattcttgggaac	MseI	333	25/170/135/ 30
III	F31E3	pkP3076	cattaggaagtgatgcaagtgg	tggatttgagaggtgtccatag	AvaII	489	195/295
III	Y111B2A	pkP3077	ctaggcaccaatcattacaagc	tttgttgccctgatttacatcc	MseI	520	n/a
IV	K03H6	pkP4065	gtcattgtcagcaggtttccc	ttcaaagtcttgatggcttctg	HpaII	457	246/212
IV	E03H12	pkP4066	ccaaacaacctacagaaaatgc	aagatattcatgcgtcgtagtg	DraI	583	458/126
IV	Y105C5B	pkP4067	gaatttcaggtgttggaagg	tgctctgaaaaaattggctg	DraI	305	192/78/36
V	F36H9	pkP5076	cggaaaattgcgactgtc	attaggactgcttggcttcc	DraI	493	85/79/330
V	VC5	pkP5097	gtgctaattccagaaatgatcc	tagtgttcatagcatcccattg	DraI	511	82/430
V	Y51A2D	pkP5082	caggcatattacatgggatagg	caatctcacctccattctgtg	DraI	393	n/a
X	C52B11	pkP6010	aaagcagtaagtaggccctag	caccactatctacatatgc	MseI	343	240/104
X	F45E1	pkP6110	tttcttgacacctccggtag	ctcactctggtctttttccg	EcoRI	775	519/257
X	R03E1	pkP6139	aattttgaagcgccgtgg	aagctccacccaatcagag	MseI	250	n/a

Table 2.1 Details of 18 primer sets used in the experiment. Three snip-primers were designed for each chromosome, two for each arm and one for the centre. Therefore, 18 PCR primer sets were used for SNP mapping. The RE fragment sizes in this table were for N2 wild type.

2.5.1 PCR and Gel electrophoresis

Optimisation for PCR conditions was carried out in advance by varying the concentrations of primers, the concentrations of genomic DNA, annealing time, annealing temperature and the number of cycles.

50 worms at L4 stage were picked up and lysed in a 500 µl PCR tube containing 200µl of lysis buffer. The genomic DNA was isolated by the following step: first of all, the tube was

frozen at -80 ^{0}C for over an hour, and then heated for 1 hour at 65 ^{0}C, followed by 1 hour at 95 ^{0}C, finally the tube was cooled down to 4 ^{0}C.

The PCR reaction was optimised by adding 2µl of the DNA solution, 1µl of primers and 30µl of PCR Reddy mix. PCR reactions for sample without DNA solution were carried out alongside as a negative control. The optimal conditions were shown below:

95 ^{0}C for 40 seconds---------- Denaturing

58 ^{0}C for 40 seconds---------- Annealing

72 ^{0}C for 40 seconds ---------- Elongation

And 35 cycles were carried out for each stage.

The amplification of DNA sequence was then confirmed by running the PCR product on a 12% polyacrylamide gel electrophoresis. A 100 low base pair ladder was used as the marker. 9µl of PCR product with 1µl of running buffer was loaded into each slot and ran at 80V for 100 minutes. The gel was first staining in UHP water contains Ethidium Bromide for 15 minutes with shaking, and then washed in UHP water for 3 times. The duration for each washing was 5 minutes. Finally, the gel was exposed with UV light in the BIO-RAD Gel Doc 2000^{TM} Gel Documentation Systems. The image of gel was taken by the connected camera and recorded in the software called Quantity One®.

2.5.2 DNA quantification

The aim for doing this DNA quantification was to make sure that same amount of DNA product was used for restriction enzyme digestion, gel loading and enough DNA for sequencing.

DNA stock solution was first prepared at the concentration of 10mg/ml. The stock was then diluted with water to the concentrations of 3 mg/ml, 1 mg/ml, 0.3mg/ml and 0.1mg/ml. Hoechst dye at concentration of 10mg/ml was prepared as well. Hoechst dye was mixed with TEN buffer (pH=8) to get the final concentration of 5µg Hoechst/ml, i.e. 1:2000.

1µl of DNA sample was added into 1ml of TEN/Hoechst. The solution was mixed and stored in a dark place for 5 minutes in order to show the correct concentrations. The reading was then taken by the Varian Cary Eclipse Fluorescence Spectrophotometer. A standard curve was first set up at excitation 346nm and emission 460nm by using the known DNA solution (final DNA concentration was between 0mg and 10mg); TEN buffer only and Hoechst with water were used as controls. Quartz cuvette was used for the assay and the Fluorimeter was blanked with distilled water. The unknown samples were then tested and the concentrations were worked out by the standard curve.

2.5.3 Restriction Enzyme Fragment Length Polymorphism (RFLP)

The aim of this experiment was to identify the general location of the furan resistance gene in mutant worms.

PCR products for mutant worms were first prepared for all the primers as described in chapter 2.4.1. N_2 and Hawaiian strains were carried out alongside as control. The concentration for each PCR product was determined by DNA quantification assay. The equal amount of DNA for each primer was mixed with 1µl of 10 times enzyme buffer and 1µl of specified restriction enzyme (details in table 2.5.1.1). The samples were mixed and incubated at 37^0C for at least 4 hours. They were then loaded on the 12% polyacrylamide gel to check the polymorphisms.

Chapter 3 Results

3.1 Toxicity of ethanol vehicle

As described in chapter 1.1.1, furan is sparingly soluble in water, but soluble in ethanol. Ethanol is a toxic organic solvent; therefore, the effect of ethanol in the toxicity assays was tested. The maximum non-toxic concentration of ethanol used in the toxicity test had to be determined in order to avoid an effect caused by the ethanol vehicle.

3.1.1 Effect of ethanol on brood size in N2 *C. elegans*

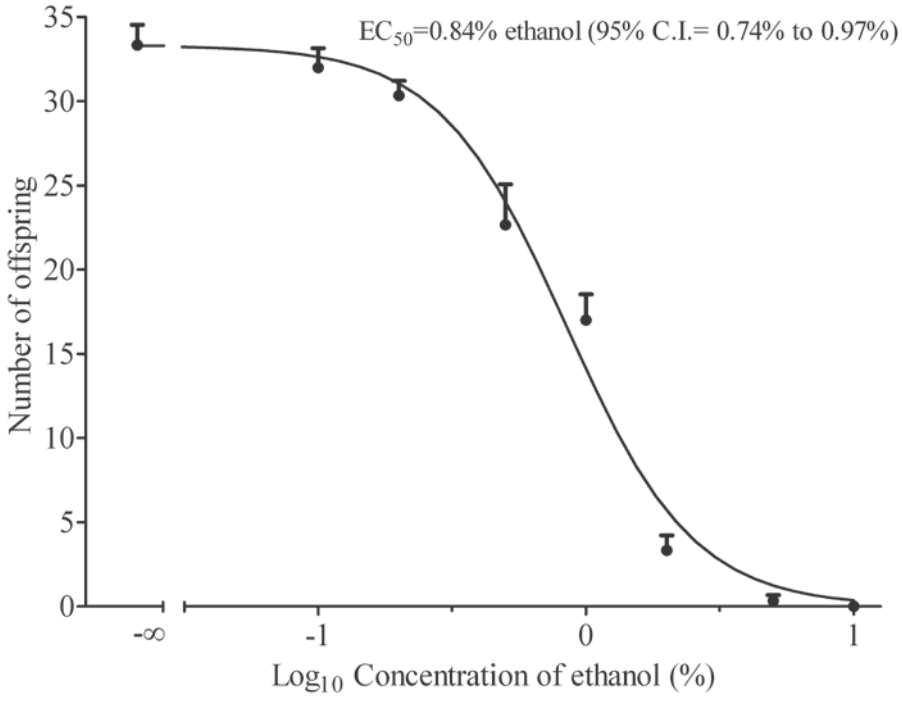


Figure 3.1 Effect of ethanol vehicle control on brood size in N2 *C. elegans*. The brood size assay was performed in a 48-well plate as described in chapter 2.3.2 This graph shows the results from a single experiment. Each concentration of ethanol was tested in triplicate, the means of brood size and the standard deviations (bars) are shown in the graph. The data was fitted into the model of log agonist vs. response (see details in chapter 2.3.2) and a non-linear regression dose response curve was produced by using GraphPad Prism 5. Similar results were obtained in five independent experiments.

The aim of this experiment was to establish the effect of ethanol on brood size in N2 *C. elegans* and find the maximum non-toxic concentration of ethanol used in the furan test in order to avoid an effect caused by the ethanol vehicle.

A 100% inhibition of brood size was achieved by the highest two concentrations (10% and 5% ethanol). At these two concentrations, the parental hermaphrodites were killed, whereas the parental worms tested at other concentrations were still viable. A t-test was used to determine if there was a significant effect on brood size at different concentrations of ethanol vehicle. The mean brood size for control (no ethanol) was 33 ± 2.1 (mean $\pm$ S.D.). At 0.25% ethanol, the mean brood size was 30 ± 1.5 (mean $\pm$ S.D.). There was no significant effect on brood size compared to the control (P=0.11). At 0.5% ethanol, the mean brood size was 23 ± 4.2 (mean $\pm$ S.D.). There was a significant effect on brood size compared to the control (P=0.01). Thus, there was no effect of ethanol on brood size at 0.25% ethanol, but at 0.5% there was.

Figure 3.1 shows that ethanol had a significant inhibitory effect on the brood size and the EC_{50} =0.84% ethanol (95% C.I.=0.74% to 0.97%). Similar results were obtained in five independent experiments and the $EC_{50} = 0.97 \pm 0.37$% ethanol (mean $\pm$ S.D.). There was a considerable overlap of means and 95% confidence intervals; therefore, the EC_{50} value was relatively constant and indicated that the experiment was reproducible. A maximum non-toxic concentration of ethanol of 0.25 % was obtained; therefore, this was chosen as a solvent for furan toxicity assays on brood size.

3.1.2 Effect of ethanol on L1 growth in N2 *C. elegans*

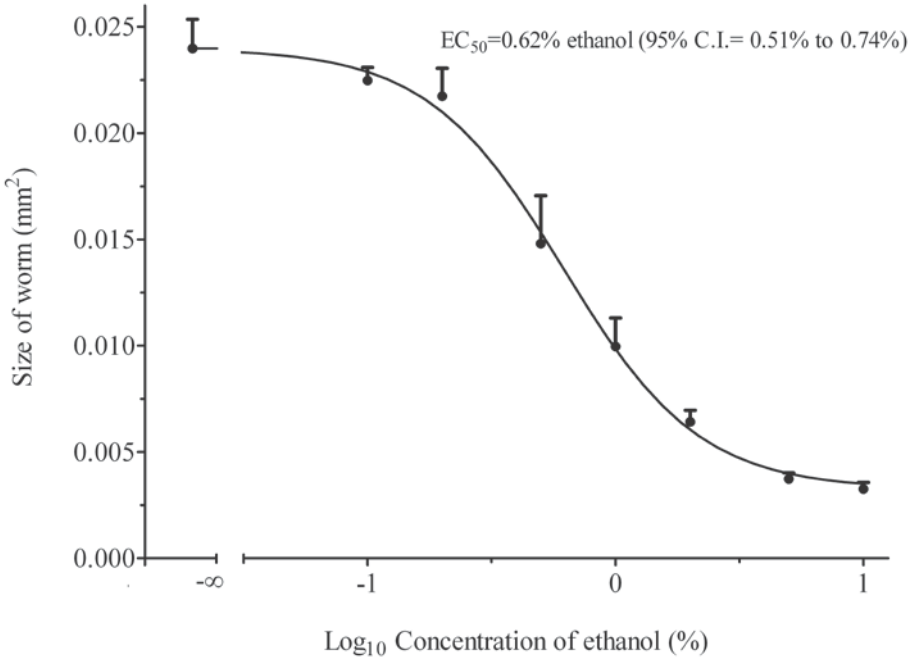


Figure 3.2 Effect of ethanol vehicle control test on L1 growth in N2 *C. elegans*. The L1 growth assay was performed in a 24-well plate as described in chapter 2.3.1. This graph shows the results from a single experiment. Each concentration of ethanol was tested in triplicate. The initial size of L1 worms in this experiment was 0.0026mm^2. The means worm size and standard deviations (bars) are shown in the graph. The data was fitted into the model of log agonist vs. response (see details in chapter 2.3.1) and a non-linear regression dose response curve was produced by using GraphPad Prism 5. Similar results were obtained in five independent experiments.

The aim of this experiment was to establish the effect of ethanol on L1 growth in N2 *C. elegans* and find the maximum non-toxic concentration of ethanol used in the furan test in order to avoid an effect caused by the ethanol vehicle.

The initial size of L1 worm in this experiment was $0.0026 \pm 0.0009\text{mm}^2$ (mean $\pm$ S.D.). A 100% inhibition on L1 growth was achieved by the highest two concentrations (10% and 5% ethanol). The inhibitory effect increased with increasing concentration of ethanol and the highest inhibition occurred at 10% ethanol. A t-test was used to determine if there was a

significant effect on L1 growth at different concentrations of ethanol vehicle. The mean size for control (no ethanol) was $0.0240 \pm 0.0031 mm^2$ (mean $\pm$ S.D.). At 0.25% ethanol, the mean size was $0.0218 \pm 0.0012 mm^2$ (mean $\pm$ S.D.). There was no significant effect on L1 growth compared to the control (P=0.27). At 0.5% of ethanol, the mean size was $0.0148 \pm 0.0051 mm^2$. There was a significant effect on L1 growth compared to the control (P=0.008). Thus, there was no effect of ethanol on brood size at 0.25% ethanol, but at 0.5% there was.

Figure 3.2 shows that ethanol had a significant inhibitory effect on the L1 growth and the EC_{50} =0.62% ethanol (95% C.I.=0.51% to 0.74%). Similar results were obtained in five independent experiments and the $EC_{50} = 0.87 \pm 0.24\%$ ethanol (mean $\pm$ S.D.). There was a considerable overlap of means and 95% confidence intervals; therefore, the EC_{50} value was relatively constant and indicated that the experiment was reproducible. A maximum non-toxic concentration of ethanol at 0.25 % was obtained; therefore, this was chosen as a solvent for furan toxicity assays on L1 growth.

3.2 Effect of furan in toxicity tests

The aim of this experiment was to establish the effect of furan in both N2 and Hawaiian *C. elegans*, and also determine if the Hawaiian strain is more sensitive to furan than N2 worms.

3.2.1 Effect of furan on brood size in N2 *C. elegans*

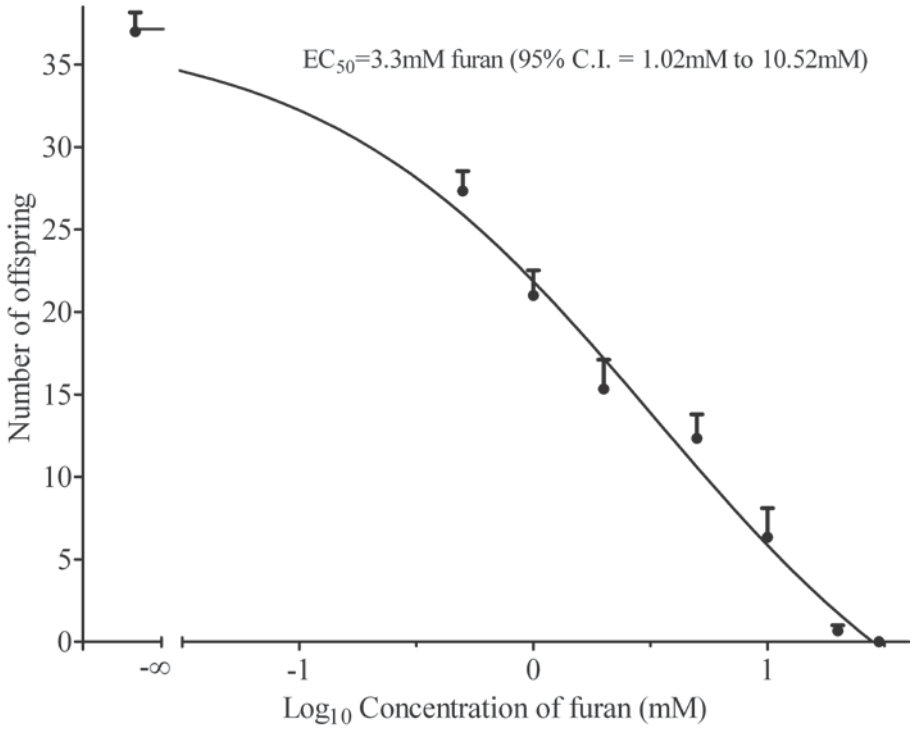


Figure 3.3 Effect of furan on brood size in N2 *C. elegans*. A brood size assay was performed as described in figure 3.1, to study the effect of different concentrations of furan.

The aim of this experiment was to establish the effect of furan on brood size in N2 *C. elegans*.

Figure 3.3 shows that the effect of furan on brood size in N2 followed a dose response curve and the EC_{50} =3.3mM furan (95% C.I.=1.02mM to 10.52mM). The inhibitory effect on brood size increased with increasing concentration of furan and the highest inhibition occurred at

30mM furan. At this concentration, 100% inhibition was achieved and the parental hermaphrodites were immobile, whereas the parental worms tested in other concentrations showed some movement. Similar results were obtained in five independent experiments and the EC_{50} =2.9 ± 0.92mM furan (mean ± S.D.). Therefore, the EC_{50} value was relatively constant and indicated that the experiment was reproducible. A 100% inhibitory concentration of furan at 30mM was obtained; therefore, this was chosen as a selection concentration for the screening assay based on inhibition of brood size.

3.2.2 Effect of furan on L1 growth in N2 *C. elegans*

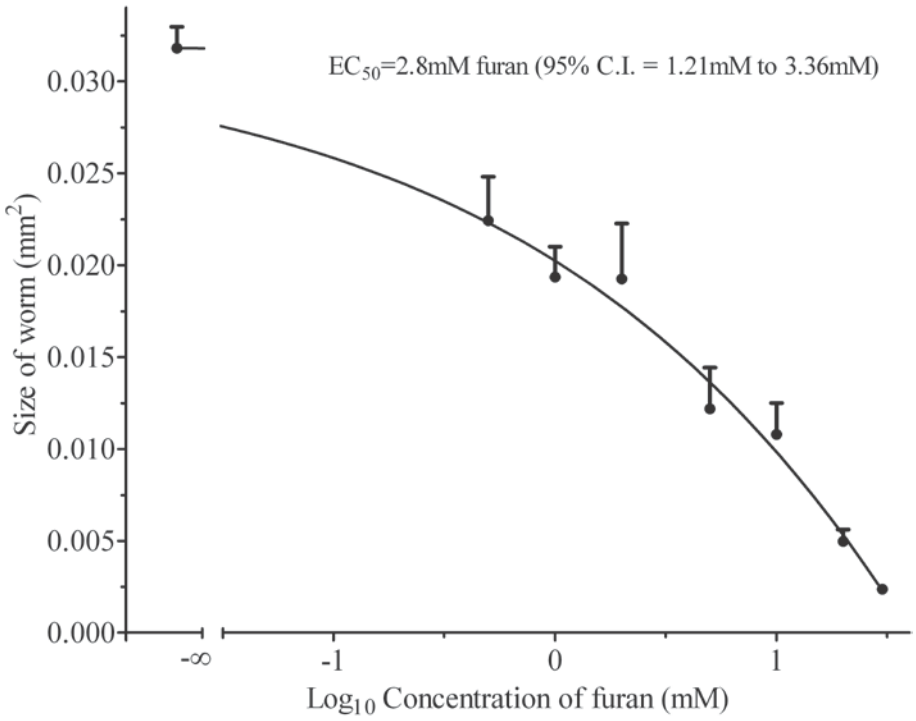


Figure 3.4 Effect of furan on L1 growth in N2 *C. elegans*. The initial size of L1 worm in this experiment was 0.0023mm ^{2} . An L1 growth assay was performed as described in figure 3.2, but with the indicated concentration of furan.

The aim of this experiment was to establish the effect of furan on L1 growth in N2 *C. elegans.*

Figure 3.4 shows that the effect of furan on L1 growth in N2 followed a dose response curve and the EC_{50} =2.8mM furan (95% C.I.=1.21mM to 3.36mM). The inhibitory effect on L1 growth increased with increasing concentration of furan and the highest inhibition occurred at 30mM furan. At this concentration, 100% inhibition was achieved and the parental hermaphrodites were killed, whereas the parental worms tested in other concentrations showed some movement. Similar results were obtained in five independent experiments and the EC_{50} =2.5 ± 0.79mM furan (mean ± S.D.). Therefore, the EC_{50} value was relatively constant and indicated that the experiment was reproducible. A 100% inhibitory concentration of furan at 30mM was obtained; therefore, this was chosen as a selection concentration for the screening assay based on inhibition of L1 growth.

3.2.3 Effect of furan on brood size in *C. elegans* CB4856 (Hawaiian)

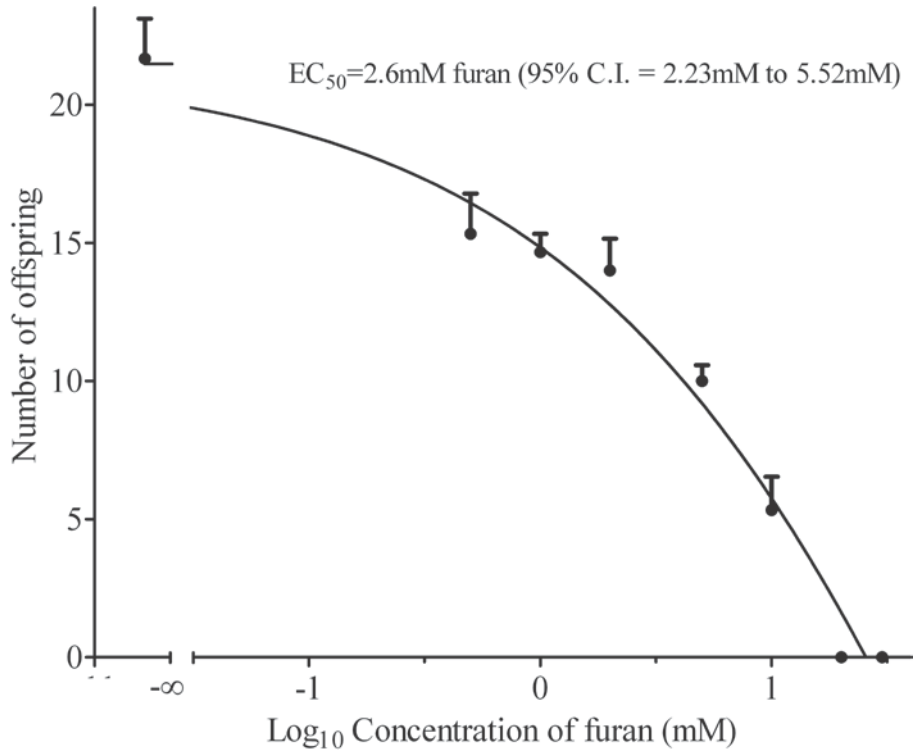


Figure 3.5 Effect of furan on brood size in Hawaiian *C. elegans*. A brood size assay was performed as described in figure 3.1, to study the effect of different concentrations of furan.

The aim of this assay was to establish the effect of furan on brood size in Hawaiian *C. elegans* and determine if the Hawaiian strain is more sensitive to furan than N2 worms.

Figure 3.5 shows that effect of furan on brood size in Hawaiian followed a dose response curve and the EC_{50} =2.6mM furan (95% C.I.=2.23mM to 5.52mM). The inhibitory effect on brood size increased with increasing concentration of furan and the highest inhibition occurred at 20mM and 30mM furan. At these concentrations, 100% inhibition was achieved and the parental hermaphrodites were killed, whereas the parental worms tested at other concentrations showed some movement. Similar results were obtained in five independent experiments and the EC_{50} =2.9 ± 0.43mM furan (mean ± S.D.). Therefore, the EC_{50} value was relatively constant and indicated that the experiment was reproducible. A t-test showed that

the EC_{50} for Hawaiian worms was similar to that for N2 (P>0.99), which indicated that furan had a similar inhibitory effect on brood size in Hawaiian and N2 worms.

3.2.4 Effect of furan on L1 growth in *C. elegans* CB4856 (Hawaiian)

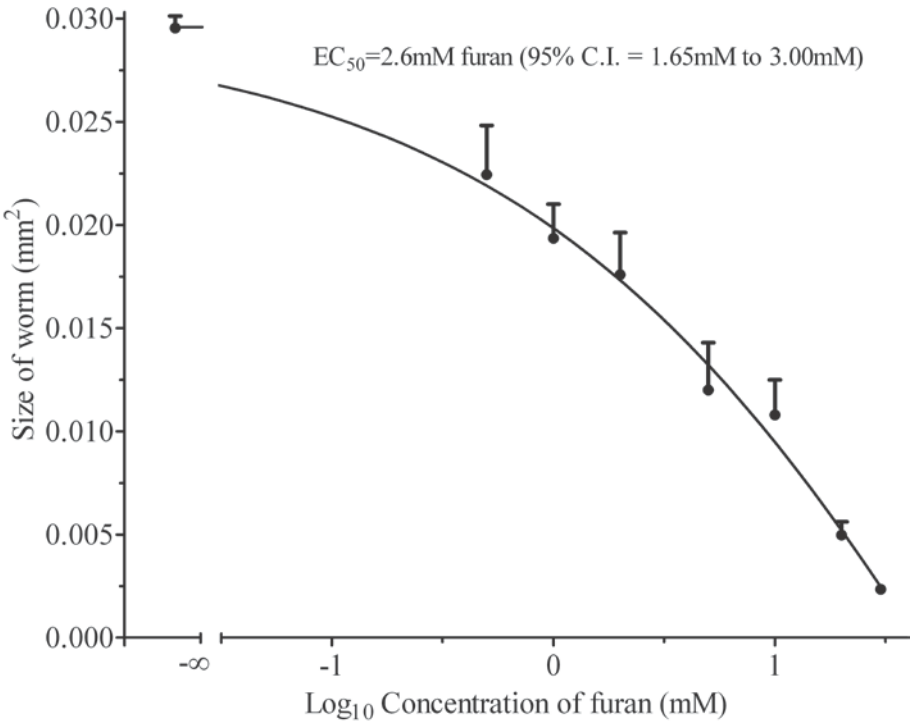


Figure 3.6 Effect of furan on L1 growth in Hawaiian *C. elegans*. The initial size of L1 worm in this experiment was $0.0023mm^2$. An L1 growth assay was performed as described in figure 3.2, but with the indicated concentration of furan.

The aim of this experiment was to establish the effect of furan on L1 growth in Hawaiian *C. elegans* and determine if Hawaiian strain worms are more sensitive to furan than N2 worms.

Figure 3.6 shows that effect of furan on L1 growth in Hawaiian worms followed a dose response curve and the EC_{50} =2.6mM furan (95% C.I.=1.65mM to 3.00mM). The inhibition effect on L1 growth increased with increasing concentration of furan and the highest inhibition occurred at 30mM furan. At this concentration, a 100% inhibition was achieved and the parental hermaphrodites were killed, whereas the parental worms tested in other

concentrations showed some movement. Similar results were obtained in five independent experiments and the EC_{50} =2.6 ± 0.61mM furan (mean ± S.D.). Therefore, the EC_{50} value was relatively constant and indicated that the experiment was reproducible. A t-test result showed that the EC_{50} for Hawaiian strain worm was similar to that for N2 (P=0.87), which indicated that furan had a similar inhibitory effect on L1 growth in Hawaiian and N2 worms.

3.3 Mutagenesis of N2 worms and isolation of furan-resistance worms

3.3.1 Isolation of resistant lines

The aim of this experiment was to create furan resistant lines for characterisation and mutant gene identification.

18 groups of 10 N2 worms (P_0) were mutagenised with Ethyl Methane Sulphonate (EMS), and self-fertilised to give an F_1 generation of ~100 worms (per group). The F_1 generation worms grew to L4 stage and were incubated with 30mM furan for three days (3600 genomes were screened). A group of 10s test non-mutagenised N2 worms with furan were carried out alongside as a negative control and a group of 10s test mutagenised N2 worms without furan as a positive control (see details in chaper 2.4).

Thirteen furan-resistant worms were obtained from mutagenised worms grown in the presence of furan, but only five of them could be propagated further and were characterised in the furan toxicity assays. The mutagenesis protocol itself was not responsible for poor growth of F_2 worms, as worms cultured without furan gave offspring and a normal brood size was obtained. The presence of surviving worms at F_2 required mutagenesis of the P_0 worms,

as there were no offspring in non-mutagenised worms treated with furan, but in mutagenised worms there were. The mutagenesis protocol is essential to create the furan resistant lines for characterisation and mutant gene identification.

3.3.2 Characterisation of resistant lines

The aim of this experiment was to establish the effect of furan in resistant mutants and determine if mutant worms are more resistant to furan than N2 wild types.

3.3.2.1 Comparison of N2 wild type and resistant mutants on brood size

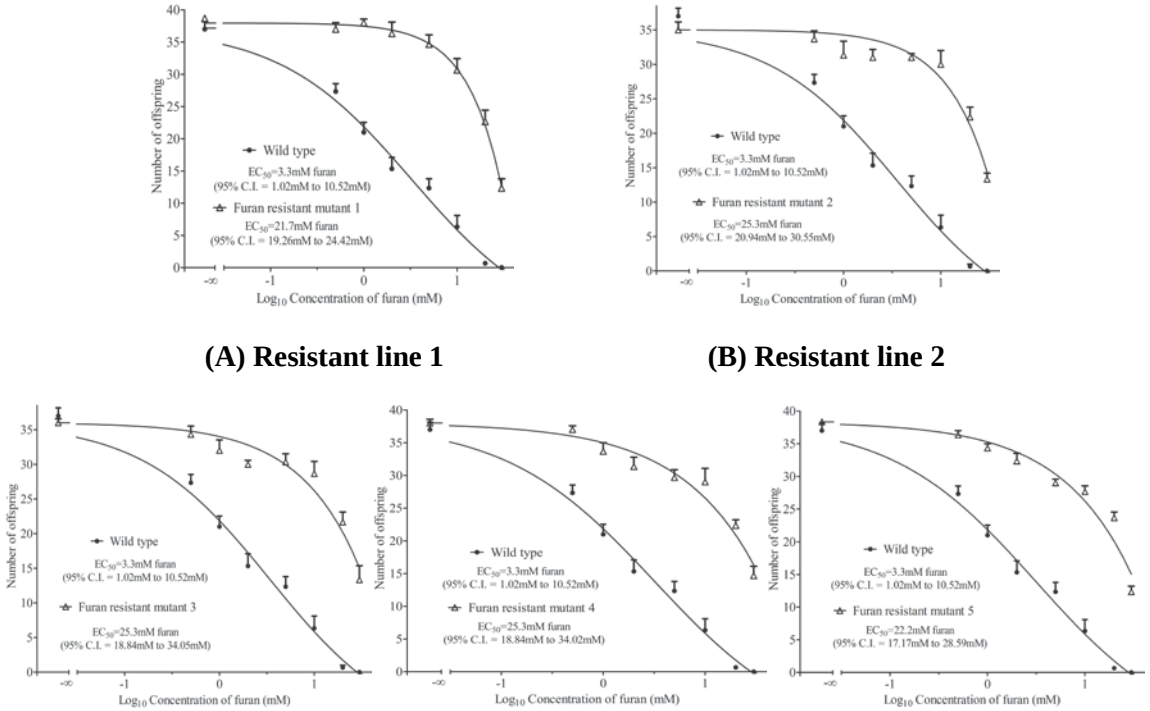


Figure 3.7 Comparison of N2 wild type and resistant mutants on brood size. Five resistant lines were isolated, propagated and the resistance to furan characterised. N2 wild type was characterised in parallel as a control. The brood size assay was performed in a 48-well plate as described in chapter 2.3.2. This graph shows the results from a single experiment. Each concentration of furan was tested in triplicate, the mean brood size was represented as dots for N2 and triangles for mutants and the standard deviations were also shown as bars in the graph. A non-linear regression dose response curve was obtained from all five independent resistant lines (A-E). Similar results were obtained in three independent experiments.

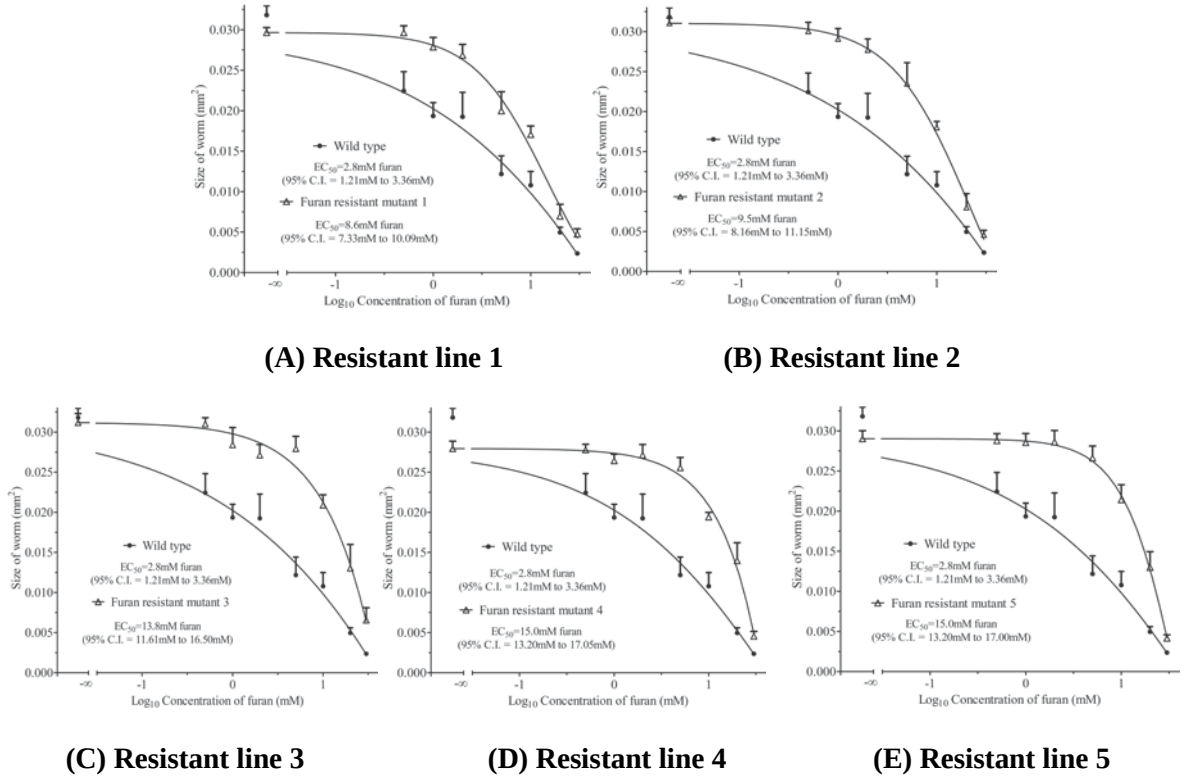
The aim of this experiment was to establish the effect of furan on brood size in resistant mutants and determine if mutant worms are more resistant to furan than N2 wild types.

Figure 3.7A shows that effect of furan on brood size in both N2 wild type and resistant line 1 followed a dose response curve, but with different EC_{50}s. A 100% inhibitory effect of furan was achieved at the concentration of 30mM (no growth) in N2 wild type, but there was no 100% inhibitory effect of furan obtained in resistant worms. The maximum concentration of furan that can be dissolved in 0.25% ethanol is 30mM; therefore, there was only part of a dose responsive curve for resistant worms. The brood size in resistant worms was constrained to 0 at the maximum effect of furan. An EC_{50} of 3.3mM furan (95% C.I.=1.02mM to 10.52mM) for N2 wild type and an EC_{50} of 21.7mM furan (95% C.I.=19.26mM to 24.42mM) for resistant line 1 was estimated. Similar results were obtained in three independent experiments, and the average from all 3 experiment shows an $EC_{50}=2.9\pm0.92$mM furan (mean $\pm$ S.D.) for N2 wild type and the $EC_{50}=26.4\pm4.7$mM furan (mean $\pm$ S.D.) for resistant line 1. There was no overlapping of the means and 95 % confidence intervals and the EC_{50} for resistant line 1 is much higher than for N2 wild type. These results suggested that there was a significant different between N2 wild type and resistant line 1 on brood size i.e. mutant worms are more resistant to furan than N2 wild type. Similar results were also obtained in another four resistant lines (figure 3.7 B-E). The data for control and all five resistant lines are shown below in table 3.1).

Name of line tested	**EC_{50} and 95% C.I. (mM) From data shown in fig. 3.7**	**EC_{50} ± S.D. (mM) Mean from 3 independent experiments**
N2 wild type	3.3; 1.02 to 10.52	2.9±0.92
Resistant line 1	21.7; 19.26 to 24.42	25.9±4.2
Resistant line 2	25.3; 20.94 to 30.55	22.4±5.1
Resistant line 3	25.3; 18.84 to 34.05	24.3±4.3
Resistant line 4	25.3; 18.84 to 34.02	26.1±5.8
Resistant line 5	22.2; 17.17 to 28.59	23.8±6.1

Table 3.1 The EC_{50} for N2 wild type and all five resistant lines. The maximum concentration of furan can be dissolved in 0.25% ethanol is 30mM; therefore, there was only a part of dose response curve for resistant worms. The brood size in resistant worms was constrained to 0 at the maximum effect of furan. The EC_{50} and 95% C.I. (mM) in figure 3.7 are given in column 2. Similar results were obtained in three independent experiments and the EC_{50} value is shown as mean $\pm$ S.D. in column 3.

3.3.2.2 Comparison of N2 wild type and resistant mutants in terms of L1 growth



(A) Resistant line 1 **(B) Resistant line 2**

(C) Resistant line 3 **(D) Resistant line 4** **(E) Resistant line 5**

Figure 3.8 Comparison of N2 wild type and resistant mutants on L1 growth. Five resistant lines were isolated, propagated and the resistance to furan characterised. N2 wild type was carried in parallel as a control. The initial size of L1 worm in this experiment was 0.0023mm^2. The L1 growth assay was performed in a 24-well plate as described in chapter 2.3.1. This graph shows the results from a single experiment. Each concentration of furan was tested in triplicate, the mean worm size was represented as dots for N2 and triangles for mutants, and standard deviations are shown as bars in the graph. A non-linear regression dose response curve was obtained from all five independent resistant lines (A-E). Similar results were obtained in three independent experiments.

The aim of this assay was to establish the effect of furan on L1 growth in resistant mutants and determine if mutant worms are more resistant to furan than N2 wild types.

Figure 3.8A shows that effect of furan on L1 growth in both N2 wild type and resistant line 1 followed a dose response curve, but with different EC_{50}s. A 100% inhibitory effect of furan was achieved at a concentration of 30mM (no offspring) in N2 wild type, but there was no 100% inhibitory effect of furan obtained in resistant worms. The maximum concentration of furan that can be dissolved in 0.25% ethanol is 30mM; therefore, there was only part of a

dose responsive curve for resistant worms. The worm size in resistant worms was constraint to $0.0023mm^2$ at the maximum effect of furan. The EC_{50} =2.8mM furan (95% C.I.=1.21mM to 3.36mM) for N2 wild type and the EC_{50} =8.6mM furan (95% C.I.=7.33mM to 10.09mM) for resistant line 1 were obtained. Similar results were obtained in three independent experiments. The EC_{50} $=2.5 \pm 0.79$mM furan (mean $\pm$ S.D.) for N2 wild type and the EC_{50} $=8.6 \pm 2.2$mM furan (mean $\pm$ S.D.) for resistant line 1. There was no overlapping of the means and 95 % confidence intervals and the EC_{50} for resistant line 1 is much higher than for N2 wild type. These results suggested that there was a significant different between N2 wild type and resistant line 1 in the term of L1 growth i.e. mutant worms are more resistant to furan than N2 wild type. Similar results were also obtained in another four resistant lines (figure 3.8 B-E). The data for control and all five resistant lines are shown below in table 3.2).

Name of line tested	EC_{50} and 95% C.I. (mM) From data shown in fig. 3.8	EC_{50} ± S.D. (mM) Mean from 3 independent experiments
N2 wild type	2.8; 1.21 to 3.36	2.5±0.79
Resistant line 1	8.6; 7.33 to 10.09	8.6±2.2
Resistant line 2	9.5; 8.16 to 11.15	10.4±3.1
Resistant line 3	13.8; 11.61 to 16.50	14.3±4.3
Resistant line 4	15.0; 13.20 to 17.05	16.1±5.8
Resistant line 5	15.0; 13.20 to 17.00	13.8±6.1

Table 3.2 The EC_{50} for N2 wild type and all five resistant lines. The maximum concentration of furan can be dissolved in 0.25% ethanol is 30mM; therefore, there was only a part of dose response curve for resistant worms. The worm size in resistant worms was constrained to the initial size of 0.0023 at the maximum effect of furan. The EC_{50} and 95% C.I. (mM) in figure 3.8 are given in column 2. Similar results were obtained in three independent experiments and the EC_{50} value is shown as mean ± S.D. in column 3.

3.3.3 Incidence of male offspring

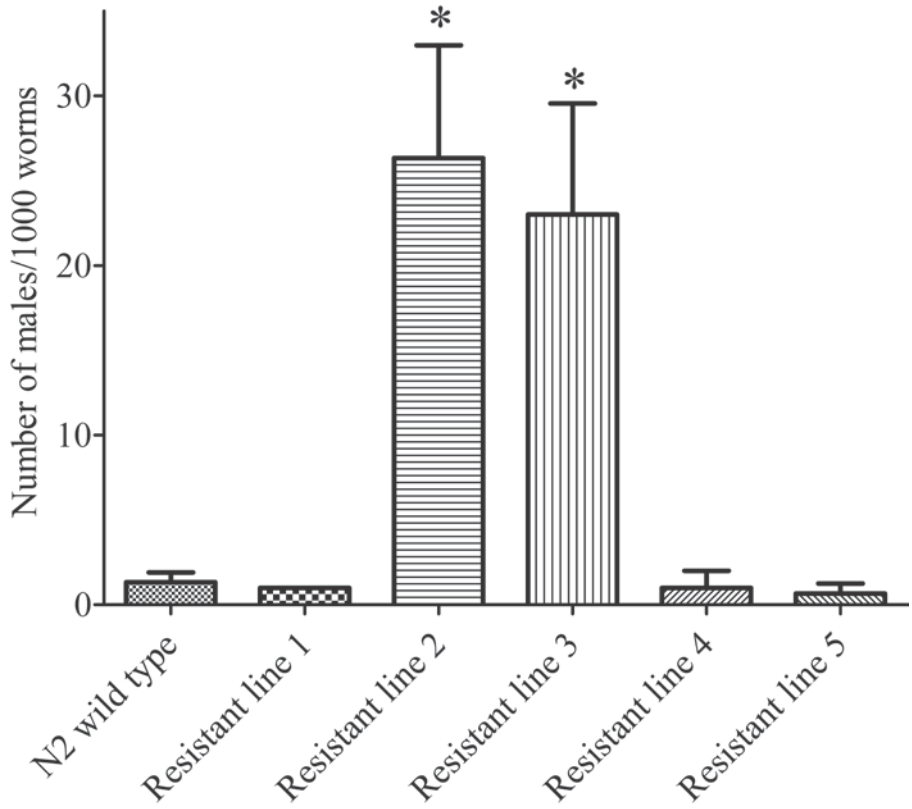


Figure 3.9 Comparison of N2 wild type and different resistant lines in term of high incidence of males. 30 L4 hermaphrodites from one of the resistant lines were picked and self-fertilised in a 9cm NGM agar plate at 25°C for 3 days (~1000 offspring). The number of males was then counted for every 1000 worms. The test was carried out in triplicate and N2 wild type was alongside as a control. The procedure was repeated with all the resistant lines. ANOVA& Dunnett tests were then used to check the significance of high incidence of males in all the resistant lines compared to N2 wild type. The mean number of males for N2 wild type and mutant lines are shown by columns, and the standard deviation s are shown as bars.

It was noticed that there were numerous males present in resistant line 2 and 3 worms. This experiment set out to determine if there is a significant increase in the number of male offspring in the furan-resistant lines compared to control.

The result shown in figure 3.9 demonstrated that there was a significant increase in the incidence of males in both resistant line 2 and resistant line 3; therefore, the HIM phenotype

was found in both independently-derived lines. *C. elegans* has about 20,000 genes, the chance for a single mutation occurring is therefore 1/20,000. If these two mutant genes have no linkage with each other, the chance for both mutations occuring at the same time will be 1/400,000,000. Since Him is present in both resistant lines, this most likely means that the Him mutation is linked to furan resistance.

3.3.4 Complementation groups

The aim of this assay was to check if the different furan resistance lines involved the same gene. 6 males from one group were picked up and transferred onto a new 3cm plate; meanwhile, 1 virgin L4 hermaphrodite from the other group was picked up and transferred onto the same plate. Mating was then carried out at 15°C for 24 hours. The cross mating was repeated for the rest of the groups. A mating with L4 hermaphrodites in the same group was used as a positive control, whereas a mating with an N2 wild type L4 hermaphrodite was used as a negative control. After 24 hours with males, the gravid hermaphrodite from each plate was picked up and placed in 30mM furan for selection, and the viability of the F_1 generation in the presence of furan was determined using the conventional brood size assay.

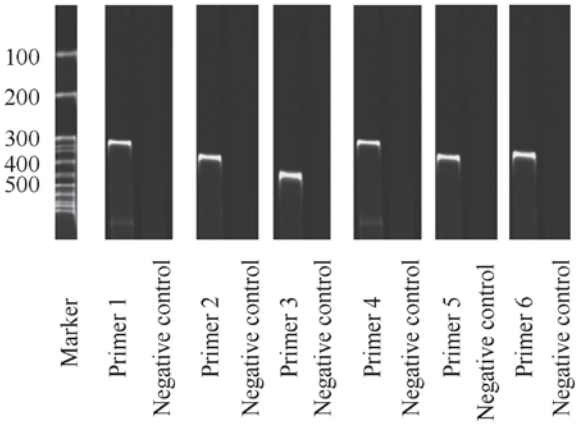
There was no F_1 survival for offspring produced by mating with N2 wild type (n=5), whereas F_1 worms did survive when mating with the same resistant line (table 3.3), hence, the furan resistant alleles are recessive mutations. If the number of offspring is less than 8 worms, it will not be defined as same complementation group. The results of crosses between groups showed that three complementation groups were present among the five independent resistant lines. Resistant line 1 was in one complementation group, resistant lines 2 and 3 were in another complementation group, and resistant lines 4 and 5 were in the third complementation group.

Hermaphrodite / Male	N2 wild type	Resistant line 1	Resistant line 2	Resistant line 3	Resistant line 4	Resistant line 5
N2 wild type	0	N.D.	N.D.	N.D.	N.D.	N.D.
Resistant line 1	0	28.6±1.8	N.D.	N.D	N.D.	N.D.
Resistant line 2	0	2.6±1.5	32±2.2	N.D.	N.D.	N.D.
Resistant line 3	0	5.6±2.8	30±2.4	35±2.8	N.D.	N.D
Resistant line 4	0	4.6±2.2	0	1.6±0.8	29±2.4	N.D.
Resistant line 5	0	2.6±1.8	7.6±3.8	0	33±1.9	28±1.5

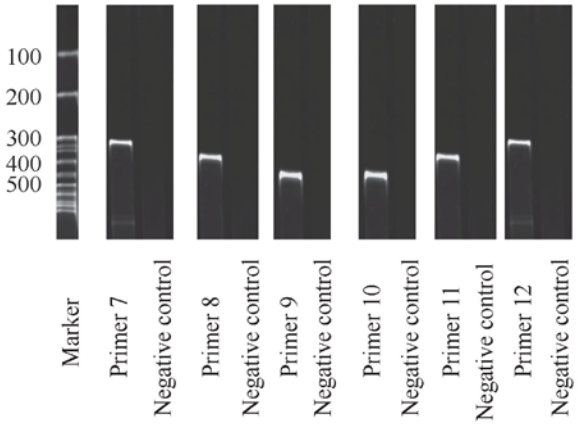
Table 3.3 Complementation test for furan-resistant worms. 6 males from one group were placed on the same plate as 1 virgin L4 hermaphrodite from the indicated group, and mating was then carried out at 15°C for 24 hours. The gravid hermaphrodite from each plate was placed in 30mM furan for selection and the F_1 generation was counted after three days. The number of offspring survived in the F_1 generation is shown as mean ± S.D, from n=5 breeds. N.D. = not done.

3.4 Detection of Single Nucleotide Polymorphism (SNP)

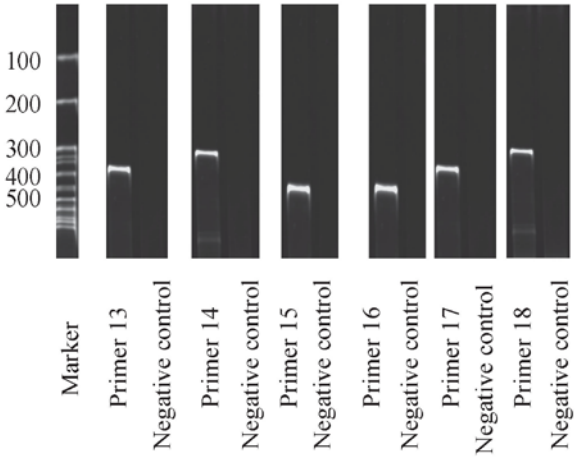
3.4.1 Specificity of PCR primers



(A) Primer 1-6 with negative control



(B) Primer 7-12 with negative control



(C) Primer 13-18 with negative control

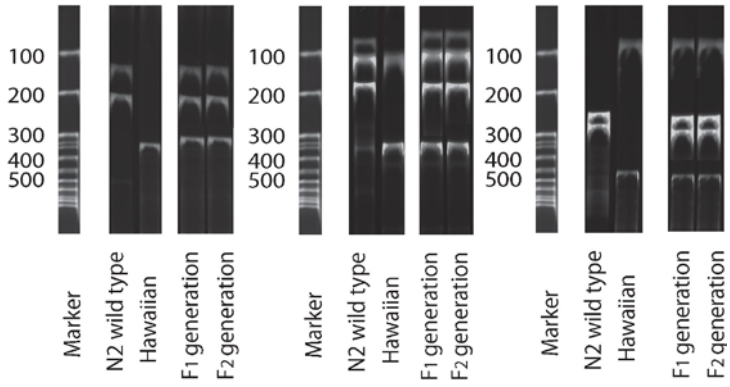
Figure 3.10 PCR primer optimisation. 100bp DNA ladder was used as a marker and PCR reactions for sample without DNA solution were carried out alongside as a negative control (see details in chapter 2.5.1). (A) Primer 1-6 with negative control, all the primers and marker were from the same gel. (B) Primer 7-12 with negative control, all the primers and marker were from the same gel. (C) Primer 13-18 with negative control. All the primers and marker were from the same gel.

Three snp-primers were designed for each chromosome, two for each arm and one for the centre. Therefore, 18 PCR primer sets were used for SNP mapping. The aim of this experiment was to optimise the PCR conditions for all the primer sets.

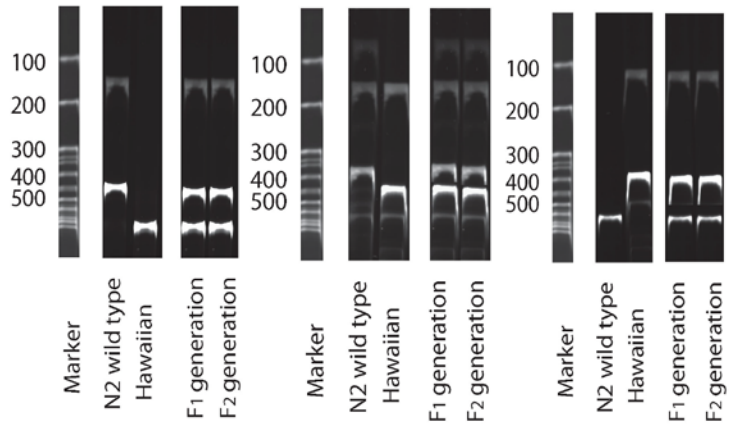
Optimisation for PCR conditions was carried out by varying the concentrations of primers, the concentrations of genomic DNA, annealing time, annealing temperature and the number of cycles.

According to table 2.1, the bands of PCR product shown in figure 3.10 were the right length for all the primer sets, and strong signals were obtained as well; therefore, the above method was chosen for all the PCR reactions.

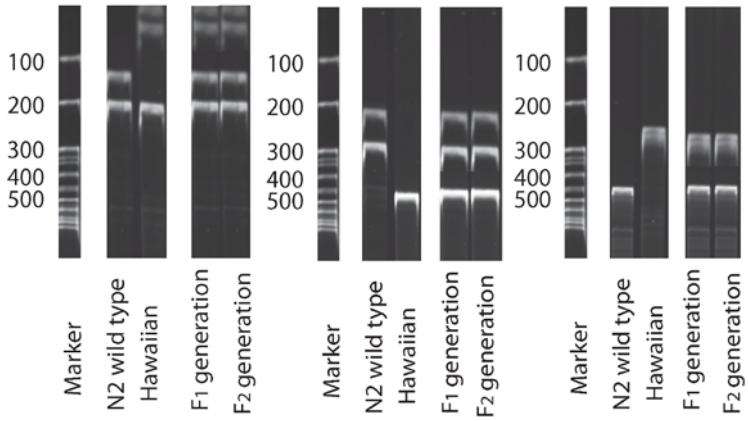
3.4.2 Characterisation of Restriction Fragment Length Polymorphism (RFLP) between N2 and Hawaiian worm DNA



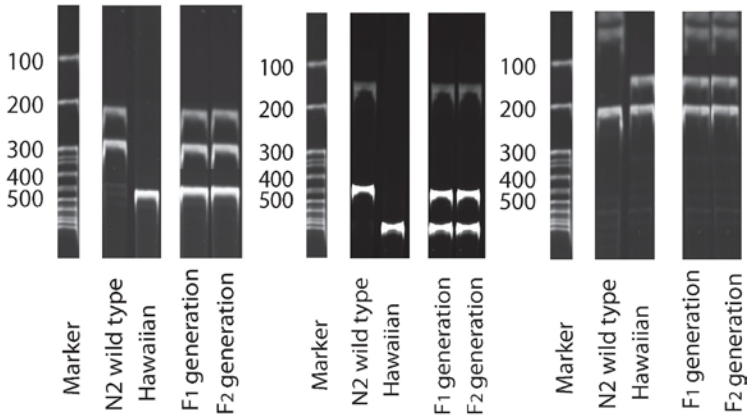
(A) Restriction enzyme digestion for primer 1-3 on chromosome I



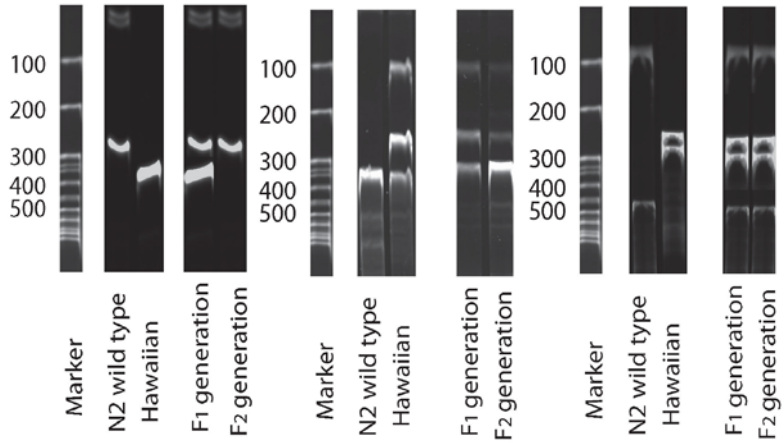
(B) Restriction enzyme digestion for primer 4-6 on chromosome II



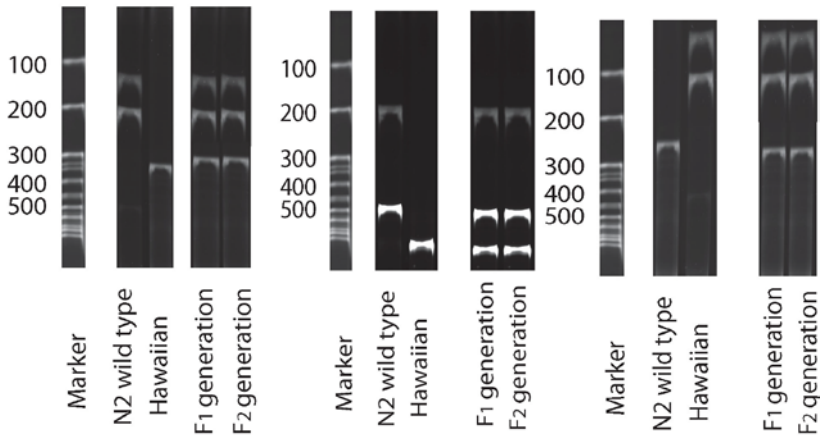
(C) Restriction enzyme digestion for primer 7-9 on chromosome III



(D) Restriction enzyme digestion for primer 10-12 on chromosome IV



(E) Restriction enzyme digestion for primer 13-15 on chromosome V



(F) Restriction enzyme digestion for primer 16-18 on chromosome X

Figure 3.11 Restriction Fragment Length Polymorphism for all six chromosomes in *C. elegans*. This was the result from complementation group 2; the restriction enzyme digestion for each primer was run on the same gel along with a 100bp DNA ladder as a marker. RFLP for F_1 generation of mutant worms was carried out in advance to confirm the success of breeding. (A) Restriction enzyme digestion for primer 1-3 on chromosome I; (B) Restriction enzyme digestion for primer 4-6 on chromosome II; (C) Restriction enzyme digestion for primer 7-9 on chromosome III; (D) Restriction enzyme digestion for primer 10-12 on chromosome IV; (E) Restriction enzyme digestion for primer 13-15 on chromosome V; (F) Restriction enzyme digestion for primer 16-18 on chromosome X.

The aim of this experiment was to identify the approximate location of the furan resistant gene in mutant worms.

Figure 3.11 shows the SNP detection for complementation group 2; the restriction enzyme digestion for each primer was run on the same gel along with a 100bp DNA ladder as a marker. RFLPs are shown in both N2 and Hawaiian strains for all the primers. The length of restriction fragment for N2 was same as shown in table 2.1.

An heterozygous genotype was shown in all of the F_1 generations of mutant worms, but this varied in the F_2 generations. Figure 3.11A-D showed the restriction enzyme digestion for primer 1-12 on chromosome I-IV, and all twelve primers showed a heterozygous genotype; therefore, the furan resistant gene was not on these chromosomes. Figure 3.11F showed the restriction for primer 16-18 on sex chromosome X. All three primers showed a heterozygous genotype, which indicated that the furan resistant gene also had no linkage with the sex chromosome. Figure 3.11E represents the restriction enzyme digestion for primer 13-15 on chromosome V. Primer 15 showed a heterozygous genotype, whereas, primer 13 showed an entirely N2 homozygous genotype and primer 14 showed a mainly N2 genotype. This finding suggested that the furan resistance gene is linked to this region of the arm of chromosome V.

As described in chapter 3.3.4, high incidence of males was found in this complementation group. Both *him*-5 and *him*-7 were found in this region of chromosome V; therefore, *him*-5 and *him*-7 may be linked to furan resistance in this complementation group. Further SNP detection in this region is needed to confirm a linkage of this mutation more precisely.

Figure 3.11 represents the Restriction Fragment Length Polymorphism for all six chromosomes in C. elegans and the approximate location of furan resistant mutation was identified on the arm of chromosome V for resistant line 2 in complementation group 2. The SNP detection was not carried out in the other two complementation groups due to time limitation.

Chapter 4 Discussion and Conclusions

4.1 Toxicity studies of furan in *C. elegans*

The first aim of this study was to examine the short term (acute) toxicity of furan in *C. elegans*. In the present study, furan showed an dose-dependent inhibitory effect on both brood size and L1 growth. This finding supports a previous study by C. Budd (2008). EC_{50} values were obtained at 2.9 ± 0.92mM furan (mean $\pm$ S.D.) for brood size assay and 2.5 ± 0.79mM furan (mean $\pm$ S.D.) for L1 growth assay. Comparison of data showed that there is no significant difference in furan toxicity between L1 growth and brood size. The finding matches the previous work by C. Budd (2008), but the EC_{50} was a bit higher (~200mg/kg furan) in this study. This could possibly be caused by the evaporation of furan. As described in chapter 1.2.1, furan is a highly volatile toxin; it is therefore very sensitive to the temperature. The experiment was carried under normal laboratory conditions: -the furan was left on ice all the time, the well plate was sealed with parafilm and wrapped with clean film to prevent evaporation, and similar results were obtained in three independent experiment as well, but the evaporation was not tested during the study. Evaporation of furan may occur, thereby reducing the concentration of furan in liquid culture and increasing the EC_{50}. This could be improved by carrying out the experiment in an air-conditioned environment at 15°C all the time and testing the evaporation of furan during the experiment.

4.2 Mutagenesis and screening

4.2.1 EMS mutagenesis

There are approximately 20,000 genes in the nematode *C. elegans* and around 14,000 genomes were screened in this study (approximately 3600 genomes were screened each time and the experiment was repeated for four times). On average, 2,000 genomes need to be screened to find a mutation in a gene of interest when using 50mM EMS, therefore around 7 mutations are expected to be obtained. In this study, 5 mutations were obtained which is approximately the number expected, which indicates that the EMS mutagenesis protocol was successful. Previous studies by Bruinsma *et al* (2008) mutagenised *C. elegans* with 50mM EMS and isolated worms that were resistant to high dietary zinc. The mutagenesis protocol itself was not responsible for poor growth of F_2 worms, as worms cultured without furan gave offspring and a normal brood size was obtained. The presence of surviving worms at F_2 required mutagenesis of the P_0 worms, as there were no offspring in non-mutagenised worms treated with furan, but in mutagenised worms there were.

4.2.2 Isolation and characterisation of furan resistant worms

As described in chapter 3.3, five resistant lines were isolated and characterised after screening. The EC_{50} values were about 10-fold higher for mutant lines compared to N2 wild type on brood size and around 4-fold higher on L1 growth. Comparisons of brood size and L1 growth for each resistant line showed that L1 growth was about 2-fold higher than brood size, but there was no significant difference between L1 growth and brood size; therefore, the sensitivity of furan on L1 growth was similar to brood size. Thus maybe the L1 metabolic pathway is functional, but not fully developed, in the embryonic stage of resistant worms.

Another possibility is that there might be a metabolic enzyme, membrane transporter or receptor that only works in the L1 stage, but not in the embryonic stage of resistant worms.

A 100% inhibitory effect of furan was achieved for both brood size assay and L1 growth assay at the concentration of 30mM in N2 wild type, but there was no 100% inhibitory effect of furan in resistant worms; therefore, it was only possible to characterise a part of the dose response curve for furan in resistant worms. The maximum furan concentration that can be obtained using the permissible concentration of ethanol vehicle (0.25%) was 30mM. Consequently, there is some uncertainty in the dose-response analysis, as the full response is not known, and this leads to comparatively high estimates of variability. As shown in figure 3.7 and 3.8, the slope of the furan dose responsive curve at the furan concentration of 20-30mM for resistant worms was much steeper than N2 wild type. It may be that there is an entirely different mechanism operating at the 20-30 mM concentration range, for example, it may be associated with the toxicity of furan as a solvent.

4.2.3 Incidence of male offspring

The result shown in chapter 3.3.3 demonstrated that there was a significant increase in the incidence of males in both resistant line 2 and resistant line 3; therefore, the Him phenotype was found in both independently-derived lines. Further tests on resistant line 2 in this study showed that the furan-resistance gene is linked to chromosome V, at the left arm; therefore, *him*-5 and *him*-7 (located on chromosome V) are candidates for the furan resistance gene in resistant line 2. There are about 20,000 genes in the genome of *C. elegans*; the chance of a single gene having a mutation is 1/2000. If the two Him phenotypes arise from unlinked mutations in genes that have no linkage with furan resistance or each other, this would occur about once every 4,000,000 mutants; since there are >10 *him* genes, this is still comparatively

unlikely. Since Him phenotypes are present in both resistant lines, the most likely explanation is that the Him mutation is linked to furan resistance. A reduced brood size was also observed in these 2 resistant lines, which is the same phenotype found in *him*-5 and *him*-7 worms (Wormbase). However, the genetic mapping of line 2 shows that this gene is likely to be distant from *him*-5 and *him*-7 on chromosome V, and further tests are needed to investigate the linkage of *him*-5 and *him*-7 with the furan resistance gene. An alternative test is to investigate the sensitivity of these two Him mutants to furan.

4.3 Detection of Single Nucleotide Polymorphisms (SNP)

The second aim of this study was to identify the genes in mutant *C. elegans* responsible for resistance to furan, with the aim of identifying a mechanism of action of furan as a toxin in *C. elegans*.

Initially, the RFLP analysis was carried out for F_2 generation worms without checking the genotype of the F_1 generation, and there was a strong N2 band present in all the PCR products; therefore, the SNP mapping had failed. The reason may be that the offspring were produced before mating with Hawaiian worms and went through the selection. This was improved by checking the genotype of F_1 generation in advance of using the F2 worms.

In the present study, SNP mapping for furan-resistant line 2 was done and the result showed that the approximate location of the furan-resistant mutation was on the left arm of chromosome V; the resistance gene could be on the left of F36H9 or between primer F36H9 and VC5 (details in table 2.1) for resistant line 2 in complementation group 2. The interpolated genetic position of F36H9 is V: -17.62 and VC5 is V: 0.56. Similar SNP detection was not carried out in the other two complementation groups due to time

limitations. Future work will involve fine mapping around these 2 primers for resistant line 2, SNP mapping for the rest of the resistant lines, and whole genome sequencing for N2 wild-type and all resistant lines.

4.4 Mechanism of action of furan as a toxin in *C. elegans*

Current work has showed that furan has an inhibitory effect on brood size and L1 growth in N2 wild type *C. Elegans*. Furan resistant worms were characterised and an approximate location of one furan-resistant gene was found on the arm of chromosome V for resistant line 2. There are several possible mechanisms to change the toxicity of furan in mutant worms.

One such mechanism could involve altering the metabolism of furan by the worm. The mutant worm may carry a gene with a novel function that detoxifies furan, so that furan is metabolised into a less toxic form by a metabolic enzyme, and is more rapidly destroyed or excreted. As described in chapter 1.1.3.2, oxidation of furan might be needed to activate its toxicity (Burka *et al*, 1991). The metabolism of furan is initiated by cytochrome P450 2E1-mediated oxidation in rodents(Kedderis *et al*, 1993; Peterson *et al*, 2005 and Kellert *et al*, 2008), and it is possible that there may be an orthologous cytochrome P450 in *C. elegans* that catalyses the oxidation of furan to a toxic moiety. However, resistance to furan is unlikely to be caused by mutation in one specific P450, because there are more than 80 CYP genes in *C. elegans* and they have overlapping substrate specificity. However, there is a large cytochrome P450 cluster of nine genes at V: -7.5, and an EMS-induced deletion could result in the loss of all these genes, about 10% of the total CYP genes in *C. elegans*. A deletion of this magnitude might well affect furan toxicity in worms. It is impossible to knock out all of the *cyp* genes, because the *C. elegans* would no longer be viable. Another possibility is that a gene mutation

on chromosome V affected a gene that regulates a signalling pathway that is essential for furan toxicity; however the nature of this signalling pathway remains a matter for speculation.

4.5 Conclusions and future work

The current study examined the toxicity of furan in *C. elegans* and showed a dose-dependent effect on brood size and L1 growth. After EMS mutagenesis, furan resistant worms were isolated and characterized, and the rough location of furan resistant gene for resistant line 2 was mapped on the left arm of chromosome V. Future work will involve the fine mapping and identification of furan resistance genes. Once the resistance genes are identified, work will be focused on the identification of the mechanisms that cause furan toxicity in *C. elegans* and test if the mechanism of toxicity of furan is conserved between *C. elegans* and mammals.

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