



## Revealing the anticancer potential of candidate drugs *in vivo* using *Caenorhabditis elegans* mutant strains

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### ABSTRACT

Drug repurposing is used as a strategy for finding new drugs for cancer. The process is a faster and a more cost-effective way of providing new indications for drugs that can address emerging drug resistance and numerous side effects of chemotherapeutic drugs. In this study, the *in vivo* anticancer potential of itraconazole, disulfiram, etodolac, and ouabain were assessed using five different *C. elegans* mutant strains. Each strain contains mutations in genes involved in different signaling pathways such as Wnt (JK3476), Notch (JK1107 and BS3164), and Ras-ERK (SD939 and MT2124) that result to phenotypes of sterility, infertility, and multivulva formation. These same signaling pathways have been shown to be defective in several human cancer types. The four candidate drugs were tested on the *C. elegans* mutant strains to determine if they rescue the mutant phenotypes. Both ouabain and etodolac significantly reduced the sterile and infertile phenotypes of JK3476, JK1107, BS3164, and SD939 strains ( $p=0.0010$ ). Finally, itraconazole and etodolac significantly reduced multivulva formation ( $p=0.0021$ ). The degrees of significant phenotypic rescues of each mutant were significantly higher than vehicle only (1% DMSO). Therefore, this study demonstrated that the four candidate drugs have anticancer potential *in vivo*, and etodolac had the highest anticancer potential.

### Introduction

Drug repurposing is being used as a strategy for finding new drugs for cancer [1]. This approach is a faster and a more cost-effective way of finding new drugs for certain diseases [2]. Compared to the conventional way of drug discovery, concerns on toxicity are circumvented in drug repurposing because the selected drugs already have well-documented safety profiles. Thus, there would be a shorter amount of time needed from compound identification up to pre-clinical studies in drug development [3]. The main principle behind this approach is polypharmacy [4], which assumes that all drugs can influence different pathways. The numerous off-target effects become an opportunity to find new indications for the existing drugs.

Despite the developments in understanding the agents that target pathways of tumorigenesis, many types of cancer lack efficacious treatment. Thus, alternative approaches are being sought to find new cancer treatments that may be effective, available, and affordable. One of these approaches is the use of simple *in vivo* models, such as the free-living transparent roundworm *Caenorhabditis elegans*, in elucidating the mechanisms of repurposed drugs. Mutations in the signaling pathways, such as in Wnt, Notch, and Ras-ERK signaling cascades, may lead to cancer in humans. These mutations that lead to defective signaling pathways can

also be observed in *C. elegans*, however, instead of forming cancer, the *C. elegans* mutant strains become sterile, infertile, and form multivulva (Muv) [5]. Thus, drugs that can reverse these mutant phenotypes in *C. elegans* may also potentially reverse cancer in humans (Fig. 1).

The mutations in the Wnt, Notch, and Ras-ERK signaling pathways exemplified by *C. elegans* strains are already established to promote carcinogenesis in humans since these pathways control different aspects of cell proliferation, cell differentiation, cell cycle progression, cell fate and cell death [6–8]. The development of tissue stem cells necessitates activation of the Wnt pathway, which acts as a signaling cascade that mediates the proliferation of cells through growth factors [9]. Loss of function mutations of  $\beta$ -catenin, an essential component of this pathway, is associated with several types of cancer such as colorectal cancer, hepatocellular carcinoma, melanoma, pancreatic cancer, and adrenocortical carcinoma [10]. Similarly, the Ras-ERK pathway also acts as a mediator of growth factors. Point mutations of the Ras receptor and several downstream kinases (e.g. RAF, MEK, and ERK) lead to constitutive activation of this pathway. The aberrant cell proliferation from the prolonged activation of this pathway is observed in non-small cell lung cancer, hairy cell leukemia, melanoma, and papillary thyroid cancer [11]. Lastly, different cues from the cellular microenvironment are processed by the Notch pathway through the direct contact of cells. Pat-

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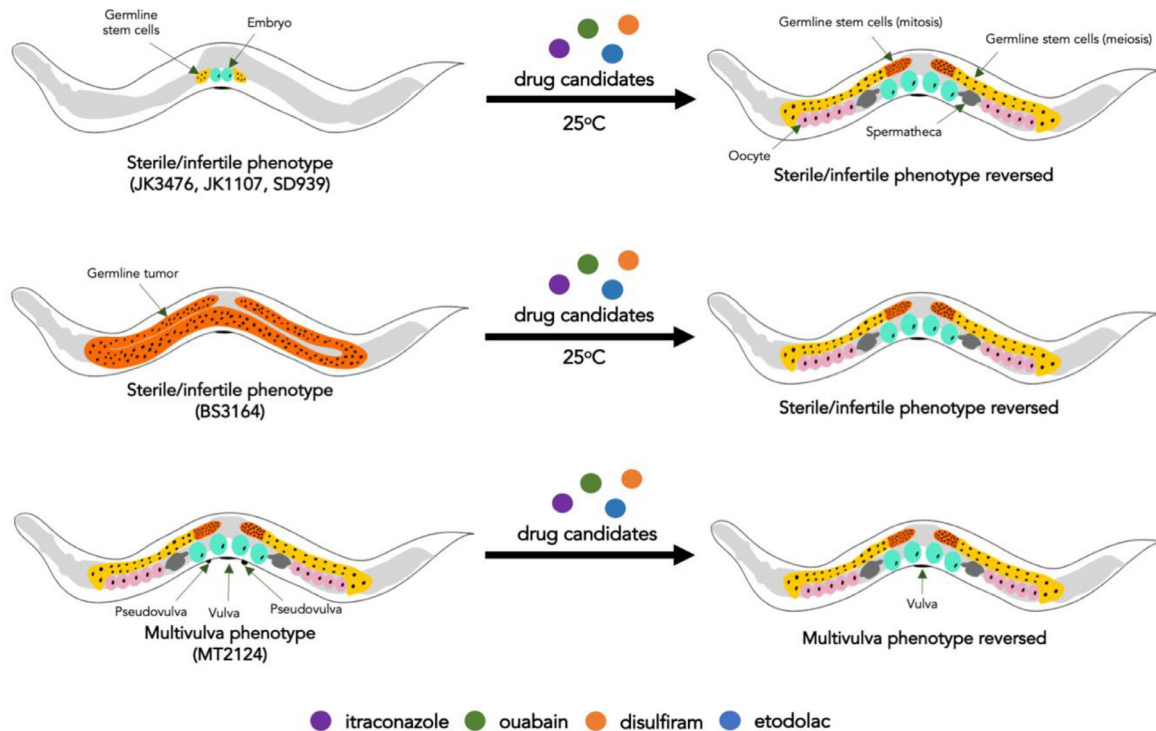


Fig. 1. Screening potential anticancer drugs using *C. elegans*

A phenotypic screen using different *C. elegans* mutant strains can be developed for discovering drugs for cancer. Each strain corresponds to a signaling pathway mutation, which lead to the development of a mutant phenotype that is a surrogate outcome for cancer. The anticancer potential of drug candidates can be determined by the reversal of the mutant phenotypes.

terns of mutations in the different domain of the Notch receptor are associated with different types of cancers such as T cell acute lymphocytic leukemia, adenoid cystic carcinoma, triple negative breast cancer, chronic lymphocytic leukemia, mantle cell leukemia, B cell lymphoma, and squamous cell carcinoma of the skin, lung, head and neck [12].

Previously, itraconazole (anti-fungal drug), ouabain (cardiac glycoside), disulfiram (aldehyde dehydrogenase inhibitor), and etodolac (non-steroidal anti-inflammatory drug) were shown to have anticancer activities *in vitro*. Itraconazole is originally used as an anti-fungal drug by inhibiting fungal cytochrome P450, specifically 14- $\alpha$  demethylase, which is an enzyme involved in ergosterol synthesis [13]. The anticancer potential of itraconazole has been demonstrated in basal cell carcinoma [14,15], leukemia [16], and breast cancer cell lines. Ouabain is a member of a class of drugs called cardiac glycosides. These are drugs used for the treatment of heart failure by inhibiting the Na-K-ATPase pump found in cardiomyocytes [17]. Specific anticancer effects of ouabain were shown in *in vitro* studies using lung tumor cells and adrenocortical cells wherein these cancer cell types were suppressed by inhibiting focal adhesion kinase and apoptosis, respectively [18,19]. Disulfiram is used to treat chronic alcoholism by irreversibly inhibiting aldehyde dehydrogenase [20]. Combination of this drug with copper is an effective anticancer regimen because this complex induces aldehyde dehydrogenase activity, suppresses NF- $\kappa$ B signaling and creates an imbalance of ROS levels, which contribute to cytotoxicity in different cancer cell types [20]. *In vitro* data have shown the effectivity of the drug against temozolomide-resistant glioblastoma [21], inflammatory breast cancer [22], prostate cancer [23], and non-small cell lung cancer [24]. Lastly, etodolac is classified as a non-steroidal anti-inflammatory drug. This drug inhibits cyclooxygenase, which synthesized prostaglandins from arachidonic acid. This drug affects tumor formation by decreasing the expression of cyclin D and inhibits thymidylate synthase, which prevents progression of cell cycle and increases the sensitivity of the tumor to other chemotherapeutic drugs such as 5-fluorouracil [25]. These ac-

tivities were shown in experiments involving breast cancer [26] and head and neck squamous cells [27].

In spite of the prevalence of *in vitro* data, *in vivo* evidence on the anticancer potential of these four candidate drugs is lacking. This study determined the *in vivo* anticancer potential of itraconazole, disulfiram, etodolac, and ouabain by their ability to rescue the mutant phenotypes of the *C. elegans* mutant strains.

## Materials and methods

### Cultivation of *C. elegans* strains

A wild type (N2) and five mutant *C. elegans* strains were obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota. The mutant strains were JK3476 (*ceh-22(q632) sma-1(e30) V; nT1(qIs51) (IV;V)*), JK1107 (*glp-1(q224) III*), BS3164 (*unc-31(e189) glp-1(ar202) III*), SD939 (*mpk-1(ga111) unc-79(e1068) III*) and MT2124 (*let-60(n1046) IV*). These strains are described in Table 1. The *E. coli* OP50 strain, which is used to feed the worms, was also obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota.

All of the *C. elegans* strains were maintained using a modified protocol by T. Stiernagle from Wormbook [28]. Briefly, 6-10 nematodes were transferred into a single culture plate containing nematode growth medium (NGM) (NaCl, peptone, bacteriological agar, 1M CaCl<sub>2</sub>, 5mg/mL ethanol, 1M MgSO<sub>4</sub>, 1M KPO<sub>4</sub>) layered with a condensed *E. coli* OP50 solution. The culture plates were kept at 15 °C.

### Preparation of candidate drugs, positive controls, and negative control

The four candidate drugs and the anticancer drug controls were purchased from Abcam and AdooQ Bioscience. For each bioassay, an anticancer drug that targets the relevant signaling pathway was used as the positive control: PRI-724 (Wnt control), DAPT-GSI (Notch control) and

**Table 1**  
Characteristics of different *C. elegans* mutant strains and their associated human cancer types.

Strain	Characteristics	Examples of Associated Human Cancer
<b>JK3476*</b>	Signaling pathway: Wnt/ $\beta$ -catenin [29]	Papillary thyroid carcinoma [30]
	Type of mutation: Loss-of-function	Pediatric acute lymphoblastic leukemia [31]
<b>JK1107*</b>	Mutant phenotype: Sterile and infertile at 25 °C	Skin squamous cell carcinoma [32]
	Signaling pathway: Notch [33,34]	Cutaneous squamous cell carcinoma [35]
<b>BS3164*</b>	Type of mutation: Loss-of-function	Small cell lung carcinoma [36]
	Mutant phenotype: Sterile and infertile at 25 °C	Urothelial carcinoma [37]
<b>SD939*</b>	Signaling pathway: Notch [38]	Diffuse large B-cell lymphoma [39]
	Type of mutation: Gain-of-function	Acute lymphoblastic leukemia [40]
<b>MT2124</b>	Mutant phenotype: Sterile and infertile at 25 °C	Breast carcinoma [41]
	Signaling pathway: Ras-ERK [42,43]	Melanoma [44]
	Type of mutation: Loss-of-function	Non-small cell lung cancer [45]
	Mutant phenotype: Sterile and infertile at 25 °C	Colorectal cancer [46]
	Signaling pathway: Ras-ERK [47]	Cutaneous melanoma [48]
	Type of mutation: Gain-of-function	Acute myeloid leukemia [49]
	Phenotype: Multivulva formation	Juvenile myelomonocytic leukemia [50]

\* Wild type phenotype at 15 °C.

**Table 2**  
Concentrations used for each candidate drug ( $\mu\text{g/mL}$ ) and their corresponding conversion in molarity ( $\mu\text{M}$ ).

Itraconazole		Disulfiram		Etodolac		Ouabain		PRI-724		DAPT-GSI		U0126	
$\mu\text{g/mL}$	$\mu\text{M}$	$\mu\text{g/mL}$	$\mu\text{M}$	$\mu\text{g/mL}$	$\mu\text{M}$	$\mu\text{g/mL}$	$\mu\text{M}$	$\mu\text{g/mL}$	$\mu\text{M}$	$\mu\text{g/mL}$	$\mu\text{M}$	$\mu\text{g/mL}$	$\mu\text{M}$
0	0.1	0	0.3	0	0.3	0	0.2	0	0.2	0	0.2	0	0.3
1	1.4	1	3.4	1	3.5	1	1.7	1	1.5	1	2.3	1	2.6
10	14	10	34	10	35	10	17	10	15	10	23	10	26
100	142	100	337	100	348	100	171	100	152	100	231	100	263
200	283	200	674	200	696	200	342	125	190	125	289	145	381
400	567	370	1248	390	1357	400	684	200	304	200	462	290	762
600	850	400	1349	400	1392	600	1026	250	380	250	578	580	1524
800	1134	600	2023	600	2088	800	1368	400	607	400	925	1000	2628
1000	1417	800	2698	800	2784	1000	1710	470	714	500	1156		
		1000	3372	1000	3480			500	759	600	1387		
								600	911	800	1850		
								800	1215	1000	2312		
								1000	1518				

U0126 (Ras-ERK control). All the drugs were dissolved in 1% dimethyl sulfoxide (DMSO) to produce the desired concentrations for each bioassay (Table 2). The negative control used was 1% DMSO for all assays.

**Sublethal bioassay**

The sublethal concentration is the highest possible concentration that would produce a percent survival of more than 90% (or less than 10% death) at 72 h of incubation with the drug candidate. The bioassay is from a modified protocol by Jiang et al. [51]. Briefly, 15–20 age-synchronous nematodes in the L4 stage were added to culture plates containing NGM and OP50 *E. coli*. Different concentrations of each candidate drug were prepared in 1% DMSO (Table 2). A volume of 100  $\mu\text{L}$  of the test solution containing the drug were added to the culture plates. The plates were incubated at 15 °C for 24, 48, or 72 h. Then, the number of live, missing, and dead worms were recorded. Experimental set-ups for each time point and drug concentration were done in triplicates. The percentage survival for each set-up was calculated using the following formula:

$$\text{Percent survival (\%)} = \frac{\text{Total no. of worms} - \text{total no. of missing worms} - \text{total no. of dead worms}}{\text{Total no. of worms} - \text{total no. of missing worms}} \times 100$$

For each candidate drug, the percent survival was interpolated based on the line of best fit calculated using the values for each concentration. Additional concentrations were added as needed to give further resolution. Then, the percent survival for the interpolated concentration was experimentally determined.

**Determination of anticancer potential of the drug candidates**

**Egg-laying bioassay**

Sterility is the inability of the worm to produce and lay eggs. This bioassay is used to measure the decrease in sterility of the sterile strains JK3476, JK1107, BS3164, and SD939 when treated with the candidate drugs. The bioassay was based on a protocol by A. Hart in Wormbook [52] with some modifications. Twenty late L4 larvae were transferred to culture plates containing NGM and 100  $\mu\text{L}$  of condensed OP50 *E. coli* solution. The plates were incubated at 15 °C (permissive temperature) or 25 °C (restrictive temperature) with or without the corresponding 100  $\mu\text{L}$  of candidate drug using the sublethal concentration. The restrictive temperature is the temperature at which the sterile phenotype can be produced for each of the mutant strain, while the permissive temperature is the optimal temperature for egg production. Then, the number of eggs were counted for 120 h in 24-h intervals. Each experimental set-up for each temperature and each treatment group was done in triplicates.

**Fertility bioassay**

Infertility is the inability to produce eggs that hatch. This bioassay is used to measure the increase in fertility of the infertile strains JK3476, JK1107, BS3164, and SD939 when treated with the candidate drugs. The bioassay was based on a protocol by A. Killeen and C. Marin de Evsikova [53] with some modifications. Twenty L1 to L2 larvae were transferred to culture plates containing NGM and 100  $\mu\text{L}$  of condensed OP50 *E. coli* solution. The larvae were allowed to grow until the L4 stage with shifts in incubation temperature from 15 °C (permissive temperature) to 25 °C (restrictive temperature) with or without the corresponding 100  $\mu\text{L}$  of candidate drug using the sublethal concentration. The restrictive temperature is the temperature at which the infertile phenotype can be pro-

duced for each of the mutant strain, while the permissive temperature is the optimal temperature for egg hatching. Fertility was measured by counting the number of F1 progeny. The number of hatched and unhatched eggs were also counted for 120 h in 18-h intervals. Each experimental set-up for each temperature shift and each treatment group was done in triplicates. The percentage of hatched eggs in each of the experimental set-up was calculated using the following formula:

$$\text{Percent of hatched eggs (\%)} = \frac{\text{Total no. of eggs laid} - \text{Total no. of unhatched eggs}}{\text{Total no. of eggs laid}} \times 100$$

*Multivulva reduction bioassay*

The multivulva (Muv) phenotype is the presence of at least one pseudovulva, in addition to the normal vulva, along the ventral side of the worms when viewed with a stereoscope at 40X magnification. This bioassay is used to measure the reduction of the Muv phenotype of the MT2124 mutant strain when treated with the candidate drugs. The assay was based on a protocol by Hara and Han [54]. Twenty larvae of the MT2124 strain were transferred to culture plates containing NGM and 100 µl of condensed OP50 *E. coli* solution. The nematodes were incubated for 120 h at 15 °C with or without the corresponding 100 µl of candidate drug using five varying concentrations that differ be-

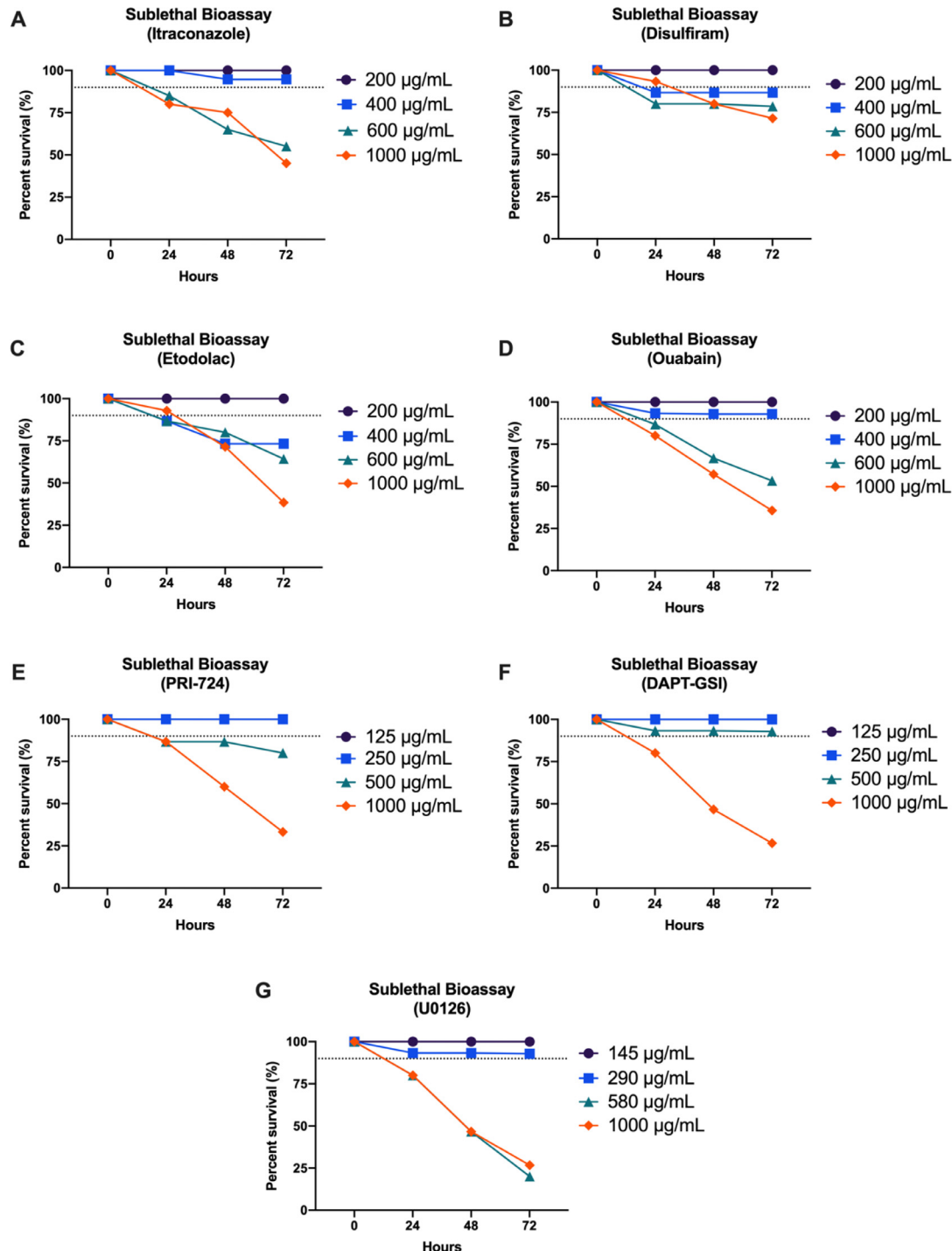


Fig. 2. Percent Survival of *C. elegans* N2 strain exposed to the different drug candidates (A)–(D) and positive controls for each of the signaling pathway (E)–(G) for 72 h ( $n = 15\text{--}20$  per concentration). The broken line represents 90% survival where concentrations above this value are considered as sublethal.

**Table 3**

Sublethal concentrations of candidate drugs and positive controls with the corresponding percent survival for each *C. elegans* strain at 72 h of incubation.

Drug	Sublethal concentration*	<i>C. elegans</i> strain	Percent survival (%)
Itraconazole	400 µg/mL	N2	94.74
		JK3476	93.33
		JK1107	92.86
		BS3164	92.86
		SD939	90.00
Disulfiram	370 µg/mL	MT2124	92.31
		N2	92.31
		JK3476	92.31
		JK1107	92.31
		BS3164	93.33
Etodolac	390 µg/mL	SD939	92.86
		MT2124	92.86
		N2	92.31
		JK3476	92.86
		JK1107	92.86
Ouabain	400 µg/mL	BS3164	92.86
		SD939	92.86
		MT2124	92.86
		N2	92.86
		JK3476	92.86
PRI-724	470 µg/mL	JK1107	92.86
		BS3164	92.86
		SD939	92.86
		MT2124	92.86
		N2	93.33
DAPT-GSI	500 µg/mL	JK3476	92.86
		N2	92.86
		JK1107	92.86
		BS3164	92.86
		N2	92.86
U0126	290 µg/mL	SD939	92.86
		MT2124	92.86
		N2	92.86
		SD939	92.86
		MT2124	92.86

\* See Table 2 for the molarity equivalents of each concentration.

tween drugs. Vulva induction was observed during the development of the worms from the L3 stage to adulthood. Each experimental set-up for each treatment group was done in triplicates. The percentage of worms with Muv phenotype was calculated using the following formula:

$$\text{Percent of Muv phenotype (\%)} = \frac{\text{Total no. of Muv phenotype worms}}{\text{Total no. of worms}} \times 100$$

### Statistical analyses

Data were processed using Graph pad (Prism 8) software. The data from treatment groups of each candidate drug were compared to the positive and negative control groups using one-way ANOVA with post-hoc Tukey HSD (Honestly Significance Difference) and Bonferroni and Holm test for multiple comparison.

## Results

### Sublethal concentrations of candidate drugs and positive controls

The sublethal concentrations for each candidate drug and the positive control anticancer drugs were initially determined in order to use as basis for dosing in the different bioassays.

The *C. elegans* N2 strain demonstrated a decrease in the survival when exposed to increasing concentrations of itraconazole, disulfiram, etodolac, and ouabain in a dose-dependent manner (Fig. 2). This same trend of reduced survival was also observed in the five *C. elegans* mutant strains when treated with the candidate drugs. The sublethal concentrations of each of the candidate drugs for every *C. elegans* strain were determined by interpolation (Table 3). These concentrations had consistent results in all of the strains.

### Candidate drugs reduces the sterility of *C. elegans* mutant strains

To determine the effect of itraconazole, disulfiram, etodolac, and ouabain on the Wnt, Notch, and Ras-ERK signaling pathways, the sterility of the JK3476, JK1107, BS3146, and SD939 mutant strains was observed by counting the number of eggs produced by each strain when exposed to each drug candidate for 120 h at 15 °C (permissive temperature) or 25 °C (restrictive temperature). The highest average number of eggs laid by all *C. elegans* strains was at 48 h of exposure with each drug candidate and anticancer drug control at 15 °C and 25 °C (Figure 3). Thus, the specific reference period used to compare the effects of the drugs across the wild type and mutant strains was selected at 48 h.

The effects of treatment with the different candidate drugs for 48 h on the average number of eggs laid was compared using the *C. elegans* strains at 25 °C (Fig. 4, Supplementary Table 1). None of the drugs including the positive control significantly affected the egg laying capacity of the N2 strain. Relative to the vehicle only, all of the candidate drugs significantly increased egg production of all the mutant strains. Etodolac treatment produced the significantly highest egg production in JK1107 and BS3164 ( $p=0.0010$ ), while ouabain treatment produced the significantly highest egg production in JK3476 and SD939 ( $p=0.0010$ ).

### Candidate drugs increases fertility of *C. elegans* mutant strains

To further investigate the effect of the candidate drugs on the Wnt, Notch, and Ras-ERK signaling pathways, the fertility of the JK3476, JK1107, BS3146, and SD939 mutant strains was observed by counting the number of hatched and unhatched eggs produced by each strain when exposed to each drug candidate for 120 h at 15 °C (permissive temperature) or 25 °C (restrictive temperature). Then, the percent of hatched eggs were calculated.



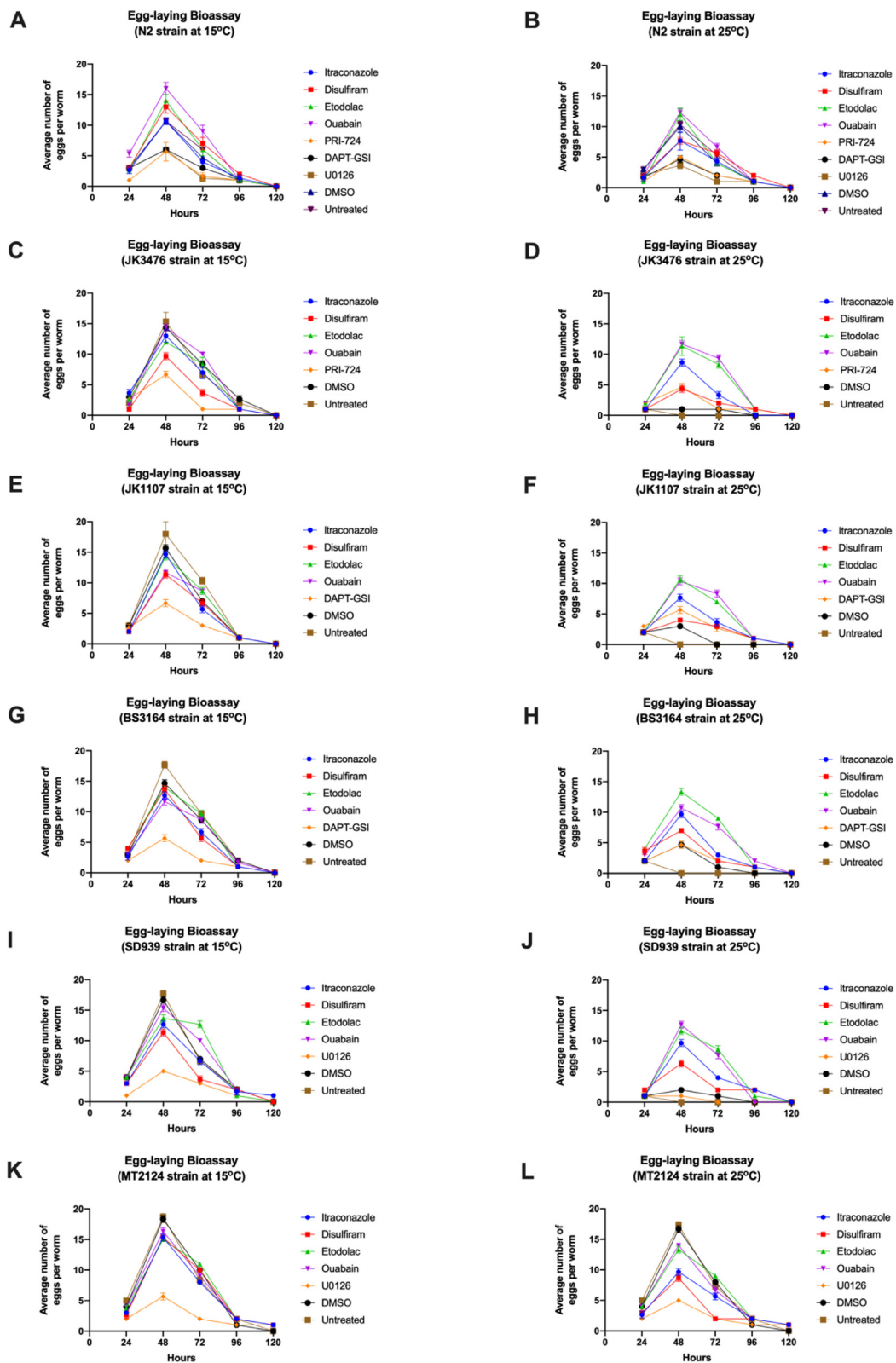


Fig. 3. Average number of eggs produced by the wild type (A) and (B) and mutant *C. elegans* strains (C)–(L) incubated with the corresponding candidate drugs and controls for 120 h at 15 °C and 25 °C. (n=20 per strain)

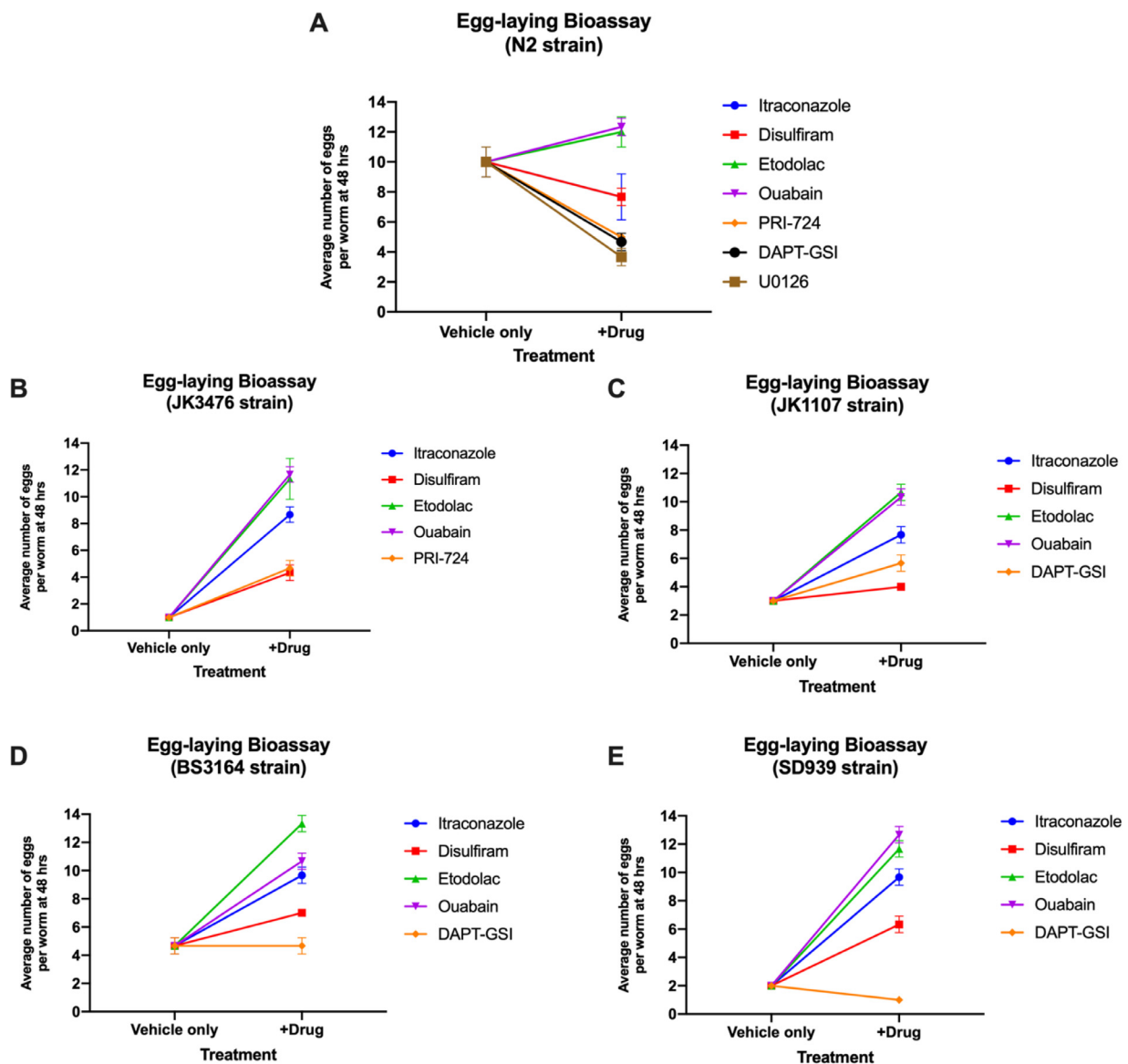


Fig. 4. Average number of eggs produced by the wild type (A) and mutant (B)–(E) *C. elegans* strains incubated with the corresponding candidate drugs and controls for 120 h at 15 °C and 25 °C. ( $n=20$  per strain).

The candidate drugs and anticancer drug controls significantly decreased the percentage of hatched eggs of the N2 strain (Fig. 5, Supplementary Table 2). Relative to the vehicle only, all of the candidate drugs significantly increased the percentage of hatched eggs of all the mutant strains. Etodolac treatment produced the significantly highest percentage of hatched eggs in JK3476, JK1107, and SD939 ( $p=0.0010$ ), while ouabain treatment produced the significantly highest egg production in BS3164 ( $p=0.0010$ ).

#### Candidate drugs prevent the formation of the Muv phenotype in the MT2124 mutant strain

To augment the sterile and infertile phenotypes, another phenotypic endpoint, multivulva formation, was used to assess the effect of the candidate drugs on the development of the Muv phenotype. The effect of the candidate drugs on the development of the Muv phenotype was observed by counting the number of worms that produced the multivulva phenotype for 120 h at 15 °C (Figure 6, Supplementary Table 3). All of the candidate drugs produced significantly decreased percentage of Muv formation in the MT2124 strain ( $p=0.0021$ ) compared to vehicle only in a dose-

dependent manner. Etodolac and itraconazole treatment of the MT2124 strain produced the significantly lowest percentage of Muv formation ( $p=0.0021$ ).

#### Ranking of each candidate drug based on rescuing of the mutant phenotype of each *C. elegans* mutant strain

Etodolac treatment consistently ranked the highest among all the candidate drugs in rescuing the mutant phenotypes of the different mutant strains (Table 4). Additionally, ouabain consistently ranked second in rescuing 3 out of 5 mutant strains.

#### Discussion

This study aimed to evaluate the anticancer potential of itraconazole, disulfiram, etodolac and ouabain using the mutant strains of *C. elegans*. The results of the assays show that all of the candidate drugs were able to significantly rescue the mutant phenotypes in each strain. Among these, etodolac was able to revert all of the mutant phenotypes with the consistently highest level. This drug may affect tumor formation by de-

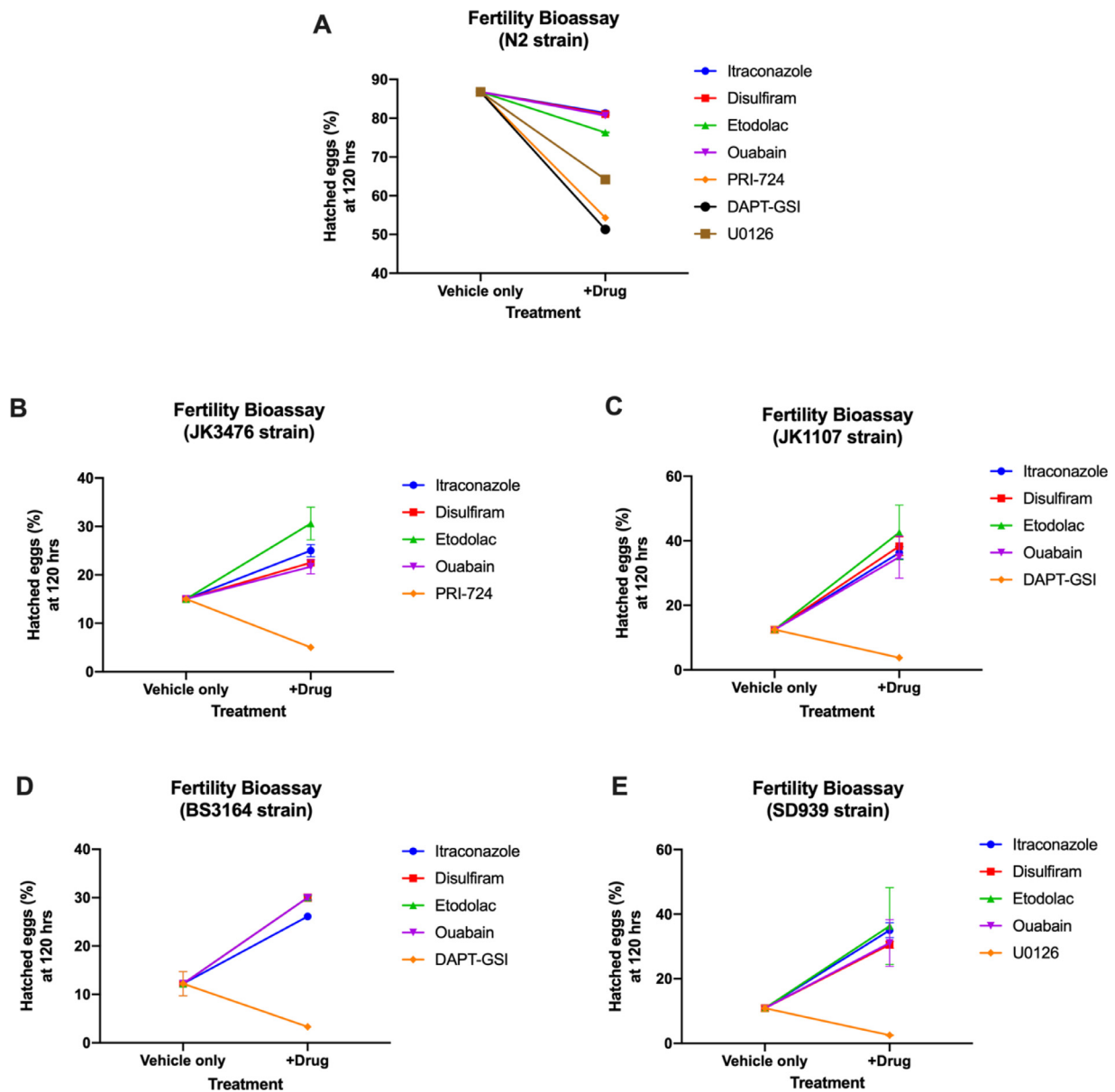


Fig. 5. Percent hatched eggs based on the number of hatched and unhatched eggs of wild type (A) and mutant (B-E) *C. elegans* strains incubated with the corresponding candidate drugs and controls for 120 h at 15 °C and 25 °C. (n=20 per strain).

**Table 4**  
Ranking of drug candidates based on the degree of phenotypic rescue observed in each mutant strain using the different bioassays. (1 – highest, 4 – lowest).

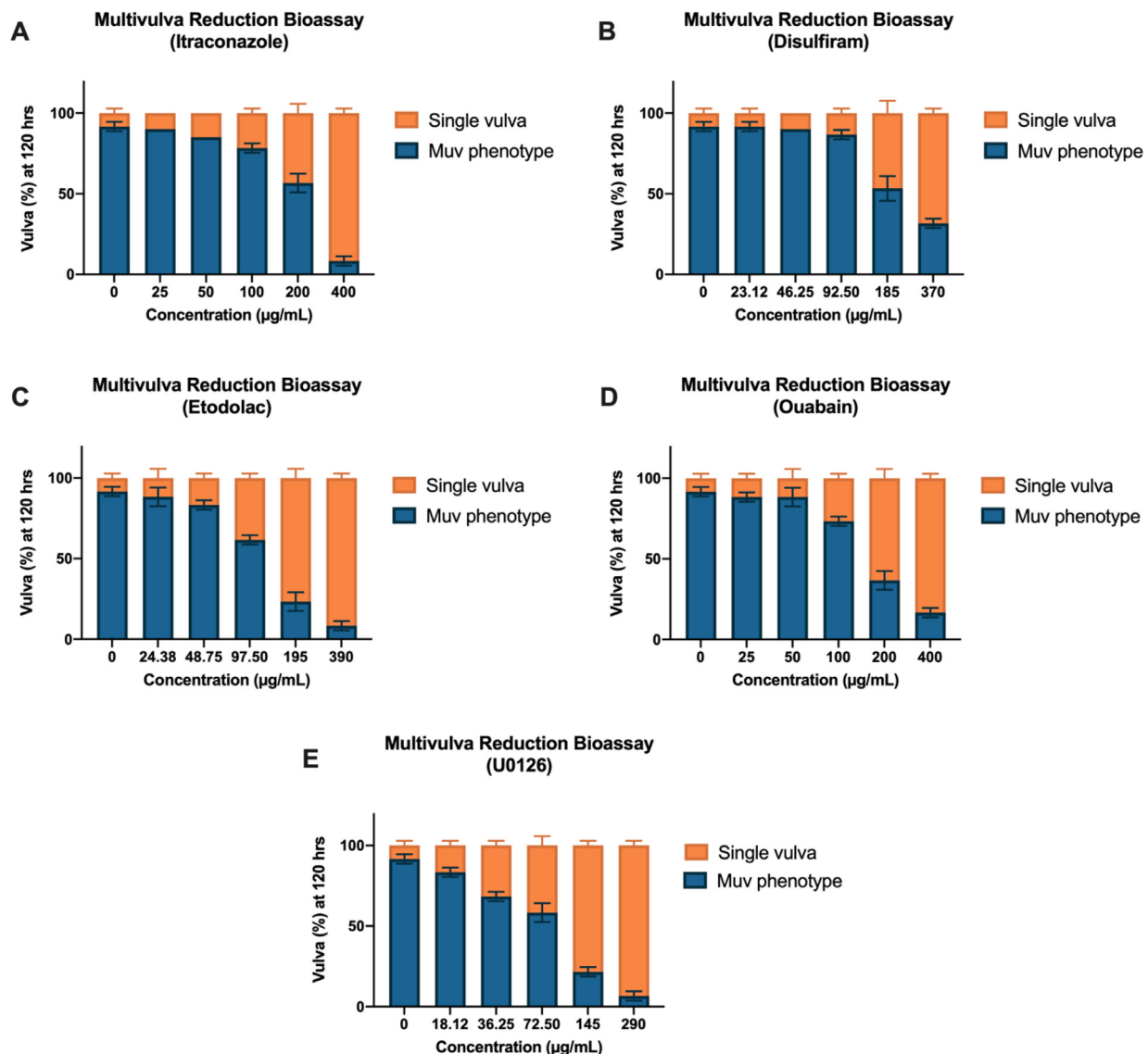
Rank	<i>C. elegans</i> mutant strains				
	<i>JK3476</i>	<i>JK1107</i>	<i>BS3164</i>	<i>SD939</i>	<i>MT2124</i>
1	Etodolac	Etodolac	Etodolac	Etodolac	Etodolac
2	Ouabain	Disulfiram	Ouabain	Ouabain	Itraconazole
3	Itraconazole	Itraconazole	Disulfiram	Itraconazole	Ouabain
4	Disulfiram	Ouabain	Itraconazole	Disulfiram	Disulfiram

creasing the expression of cyclin D and inhibits thymidylate synthase, which prevents progression of cell cycle and increases the sensitivity of the tumor to other chemotherapeutic drugs such as 5-fluorouracil [55]. Several mechanisms have been hypothesized to explain the antitumor effects of etodolac in terms of the Wnt, Notch, and Ras-ERK pathways. An analogue of etodolac introduced to hepatoma [56] and multiple myeloma cells [57] showed inhibition of the Wnt pathway by preventing the nuclear translocation of  $\beta$ -catenin. Meanwhile, similar

COX-2 inhibitors have been shown to inhibit the Notch [58] and Ras-ERK [59] signaling by inhibition of NICD and MAPK phosphorylation activity, respectively. Additionally, the effectivity of ouabain may be due to its inhibitory effect on the Na<sup>+</sup>/K<sup>+</sup> ATPase pump. This is exemplified by the hypothesized interaction of the pump to activators of the Ras-ERK signaling pathway such as Src [60].

There are still certain aspects of the anticancer properties of candidate drugs that are difficult to harness in the *C. elegans* model such as





**Fig. 6.** Percent Muv formation of MT2124 mutant strain incubated with different concentrations of the corresponding candidate drugs (A)–(D) and positive control (E) for 120 h at 15 °C. ( $n=20$  per strain). The Muv phenotype is the presence of at least one pseudovulva, in addition to the normal vulva, along the ventral side of the worms when viewed with a stereoscope at 40X magnification. The difference between single vulva % and Muv % per concentration is the value of strain that reverted to wild-type vulva.

safety, dose range, and delivery [61,62]. Thus, further evaluation is warranted using other *in vivo* models such as rodents. Given the significant effects of etodolac and ouabain using the *C. elegans* model, these can be recommended for further evaluation using other *in vivo* models.

Aside from determining anticancer potential of the candidate drugs, this study was also able to demonstrate the role of *C. elegans* as a model for drug discovery. The transparent body and short life cycle of *C. elegans* enable faster generation of an observable response, simplicity in detecting outcomes (e.g. fluorescence imaging), and ease in generating large amounts of worms [6–8;63–65]. As observed in the phenotypic assays, results can be produced within 168 h. (7 days) wherein worm cultivation can be done in 48 hours and the subsequent observation period for 120 h.. With these known features, the nematode can be used as a model that allows large scale screening of drugs, which may reduce the number of drugs for further testing in more complicated *in vivo* models. This may significantly reduce time and resources. In terms of drug repurposing, this may also further reduce the cost of re-evaluating the new indication for the drug and the time spent for evaluation. Furthermore, several studies have suggested improvements

on the *C. elegans* model to make it more efficient for drug screening. More fluorescent markers can be added not only as a food additive, but also as a transgene, which may make counting of progeny more effective especially when used in an automated setting [63;66–68].

## Conclusions

The anticancer potential of itraconazole, disulfiram, etodolac, and, ouabain was demonstrated in this study using phenotypic assays of *C. elegans* mutant strains. Specifically, the specific ability of these drugs to affect the Wnt, Notch and Ras-ERK signaling pathways were shown since the drugs were able to significantly rescue sterility, infertility, and Muv formation compared to vehicle only. Several *in vitro* studies have already confirmed much of the anticancer effects of the four candidate drugs by promoting apoptosis, activating autophagy [69], or producing reactive oxygen species [70]. Some evidences also demonstrated that these drugs can target specific signaling pathways such as Wnt and NF- $\kappa$ B [71]. The findings of this study augments these previous studies by

showing the robustness of these drugs in targeting the pathways, which lead to carcinogenesis in humans.

The conventional process of drug discovery entails the use of many resources, which would take years before application to human studies [3]. The *in vivo* system using *C. elegans* can effectively bridge *in vitro* and mammalian *in vivo* studies because the model can complement the simplicity of the system in cell cultures, while providing a faster and more perceptible development of phenotypic endpoints, which are more complex and harder to attain in rodents and non-human primates [72]. These aspects are clearly depicted in the phenotypic assays used in this study. Differences in egg-laying, fertility, and Muv formation were already observable as early as 48 hours post exposure to the drug at a certain restrictive temperature with the use of a stereoscope. Furthermore, the advantages of using *C. elegans* is elaborated by the role of the model as part of the drug screening process. Assessment of potential drugs may reduce the pool of candidate anticancer drugs to be tested, so that the drugs chosen to be tested in more expensive and longer validation studies in mammals may be more likely to provide a beneficial effect.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### CRediT authorship contribution statement

**Paul Mark Medina:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Supervision. **Jozelle Marie Ponce:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Christian Alfredo Cruz:** Formal analysis, Writing - review & editing.

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### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2020.100940.

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