Impact of drug solvents on *C. elegans* pharyngeal pumping

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**ABSTRACT**

*C. elegans* provides a multi-cellular model organism for toxicology and drug discovery. These studies usually require solvents such as dimethyl sulfoxide (DMSO), ethanol or acetone as a vehicle. This raises the need to carefully consider whether the chemical vehicles used in these screens are anodyne towards *C. elegans*. Here, we use pharyngeal pumping as a bioassay to assess this. Pharyngeal pumping is a visually scoreable behaviour that is controlled by environmental cues activating sensory and integrative neural signalling to coordinate pharyngeal activity. As such it serves as a rich bioassay to screen for chemical modulation. We found that while pumping was insensitive to high concentrations of the widely used drug solvents ethanol and acetone, it was perturbed by concentrations of DMSO above 0.5 % v/v encompassing concentrations used as drug vehicle. This was manifested as an inhibition of pharyngeal pump rate followed by a slow recovery in the continued presence of the solvent. The inhibition was not observed in a neuriligin mutant, *nlg-1*, consistent with DMSO acting at the level of sensory processing that modulates pumping. We found that *bus-17* mutants, which have enhanced cuticle penetration to drugs are more sensitive to DMSO. The effect of DMSO is accompanied by a progressive morphological disruption in which internal membrane-like structures of varying size accumulate. These internal structures are seen in all three genotypes investigated in this study and likely arise independent of the effects on pharyngeal pumping. Overall, these results highlight sensory signalling and strain dependent vehicle sensitivity. Although we define concentrations at which this can be mitigated, it highlights the need to consider time-dependent vehicle effects when evaluating control responses in *C. elegans* chemical biology.

**1. Introduction**

One approach to developing robust experimental systems to define drug mode of action and/or toxicology is through the use of simple model organisms [1–3]. This approach represents an intermediary between *in vitro* and *in vivo* platforms. *Caenorhabditis elegans* is an attractive model organism for medium and high-throughput screening. Among the advantages one can highlight its easy manipulation, a short life cycle, low-cost and accessibility to whole organism imaging. This experimental tractability is further supported by its genetic tractability, significant conservation of the proteome and genetic pathways with humans [4–6]. *C. elegans* is a particularly suitable model organism for approaches where genetic manipulations need to be combined with pharmacological studies. This flexibility has led to discovery platforms and mode of action investigations for nematicides, compounds with anti-aging properties as well as a range of therapeutic interventions [7–9].

The employment of model organisms in high-throughput screening is viewed as a promising strategy to identify genes or drugs potentially relevant to health and fitness as well as to target key determinants in disease. In particular, the ability to generate large numbers of animals allied to high throughput observational measurement lends itself to the development of whole organism bioassays [10–14]. In the context of *C. elegans*, motility and to some extent feeding behaviour, have been successfully used strategies [15–17].

In the case of *C. elegans* feeding behaviour the ability to rapidly present and remove bacteria allows the investigator to trigger and readily score increases in pharyngeal pumping. The response reflects a sensory detection and downstream neuronal signalling to execute an increase in the frequency of the contraction-relaxion cycle that can be visually observed or recorded in the intact worm [18,19]. This provides a quantitative readout that is underpinned by a range of transmitter pathways and embedded molecular targets providing an excellent model system *per se* for drug screening and has been used to identify receptor targets. More widely the pharyngeal system can be combined with the intrinsic genetic tractability to heterologously express drug targets from a range of different species including humans in which the bioassay...
embedded in pharyngeal pumping can be used to investigate the expressed drug target [20]. Finally, the pharynx is a sensitive biosensor that can provide a sub-lethal readout of drug toxicity [21,17]. This all evidences the value of the pharyngeal system as an important platform of drug investigation in the intact worm [22,23].

A confound of pharmacological approaches in the intact organism is control of drug access to the internal tissues and organs of the worm due to the cuticle. The presence of this surrounding exoskeleton can make drug access more difficult to predict [24–26]. However, mutations that increase cuticular permeability and/or judicious utilization of suitable drug vehicle can help overcome these limitations [2].

Chemical compound screens are regularly performed in popular amphipathic solvents like dimethyl sulfoxide (DMSO) or dimethyl formamide (DMF). However, studies identify differential interference of the biological assays by distinct classes of drug solvent. In the case of DMSO, which is the stock solvent in many industrial and commercial libraries per se effects on C. elegans physiology and homeostasis are documented. These solvent effects include effects on fertility, lifespan or feeding mechanisms [27–34].

In this study we use Bristol N2 (wild-type), bus-17 and nlg-1 C. elegans strains to test the effect of the widely used drug solvents DMSO, ethanol and acetone. bus-17 is one of a class of genes (bus-2, bus-4, bus-12, srf-3, bus-8) that encode proteins that impact on the composition of the worm cuticle which when mutated increase worm permeability to drugs [35]. We chose pharyngeal pumping as an integrated bioassay in C. elegans. We show that DMSO has an effect on the pharyngeal activity in the wild type N2 strain, leading to inhibition of the pharyngeal pumping in a short-term. This effect was recovered ~3 h after initial exposure in the continued presence of DMSO and was not seen in nlg-1 mutants that are known to control sensory processing of environmental cues [36]. In contrast this inhibition appeared more pronounced in bus-17 mutants, a genetic background used to sensitize bioassays to drug exposure. Finally, and independent of the modulation of the expression of sensory effects of DMSO and its modulation by cuticle integrity, we describe morphological changes of internal body composition following prolonged exposure to DMSO. These effects arise over a time course that would be encountered in some drug screening paradigms. Overall, this highlights the need to analyse an underling modulatory effect of organic solvents at concentrations that might be otherwise well tolerated in drug action. Thus, when using simple drug exposure strategies in whole organism bioassays of C. elegans these confounding vehicle effects should be considered and controlled for.

2. Methods

2.1. Culturing of C. elegans

Wild-type, Bristol N2, the mutant strains nlg-1 (ok259) X and bus-17 (e2800) X, were obtained from the Caenorhabditis elegans Genetic Centre (University of Minnesota, Minneapolis, MN, USA). nlg-1 and bus-17 strains were backcrossed 6 and 3 times respectively with Bristol N2 strain. All strains were grown and maintained at 20 °C under standard conditions [50]. Worm age was synchronized by picking L4 larval stage animals to new plates 18 h prior to performing the behavioral and/or imaging experiments.

2.2. Making assay plates

50 μl, 5 μl or 2.5 μl volumes of 100 % analytical grade DMSO, ethanol or acetone were soaked into 5 ml of NGM-medium of individual wells of a 6-well plate. This gave desired final concentrations of each solvent: 1 %, 0.75 %, 0.5 %, 0.25 %, 0.1 % and 0.05 % (V/V). This is equivalent to the following m M predicted concentrations of each solvent: DMSO ~ 141 mM, 105.75 mM, 70.5 mM, 35.25 mM, 14 mM, 7 mM; ethanol ~ 171 mM, 128.25 mM, 85.5 mM, 42.75 mM, 17 mM, 8.5 mM; acetone ~ 136 mM, 13.5 mM, 6.7 mM.

After adding the desired volumes of solvents, plates were immediately sealed and left for 12 h at RT to allow equilibration of the solvent into wells, as previously described [43]. Then 50 μl of E. coli OP50 (OD600 of 0.8 AU) were deposited on top of the vehicle laced agar and incubated for a further 12 h at RT (~20–23°C) (Fig. 1).

2.3. Measuring pharyngeal pumping activity

Pharyngeal activity was scored by measuring frequency of pharyngeal pumping, where a single pharyngeal pump is one contraction–relaxation cycle of the terminal bulb of the pharyngeal muscle. This was visually scored by counting the number of pharyngeal pumps for 1 min using a binocular dissecting microscope (Nikon SMZ2000; ×63). 1-day-old adult worms (L4 + 1) grown at 20 °C were picked onto the middle of the bacterial lawn (OD660 of 0.8 AU seeded the day before), where they were exposed to solvent for up to 24 h. After 10 min (time 0) the pharyngeal pumping was recorded using a hand counter. Five consecutive measurements (1 min each) at each time point were made and the mean of pharyngeal pumping rate was calculated. Pumping was monitored at the time-points: 0 min (10 min. post picking to food laced observation place with and without vehicles), 15 min, 30 min, 1 h, 3 h, 4 h, 5 h, 6 h and 24 h.

2.4. Imaging worms to assess motility posture and integrity and appearance of internal compartments

After the indicated time of exposure to solvents in solid agar plates, the whole worm posture (loss of naïve body bends against straight or coiled forms) was observed and imaged. During these observations we noted the apparent disruption in the appearance of the worm’s internal structures. This included the accumulation of internal membrane-like structure. These structures were captured by imaging with a Nikon Eclipse X. After incubation on untreated or solvent containing agar the worms were transferred into 0.5 μM M9 buffer on a thin 2% agarose pad containing sodium azide (10 mM).

These mounted worms were then covered with a 24 × 24 mm cover slip and were observed with a 40–63X objective magnification for more than 10 min after being placed onto the sodium azide agarose pad. At least 5 independent worms per condition were analyzed and imaged. Images were captured through a Hamamatsu Photonics camera and acquired using IC Capture 2.2 software and composed with Abode Photoshop® (Adobe Systems) and ImageJ (NIH) softwares.

2.5. Statistical analysis

Statistical analyses were made using GraphPad Prism software. Data are expressed as means ± s.e.m. The statistical tests and post-hoc analyses are indicated individually in graphs.

3. Results

3.1. DMSO impacts on C. elegans feeding behaviour in short-term and in a reversible manner

To determine the effect of the solvents DMSO, ethanol and acetone on feeding behaviour we monitored the pharyngeal pumping over time in the wild type Bristol N2 strain. First, we measured the frequency of pumping in the presence of each solvent (1%) relative to untreated conditions (Fig. 1). We did not observe any effects on the bacterial lawns in control and solvent loaded plates. We observed a strong reduction of the pharyngeal pumping rate of N2 (wt) worms in presence of 1% (v/v) (141 mM) DMSO after 3 h of exposure to the solvent (Fig. 2). In contrast, we did not find any reduction in pharyngeal pumping when animals feed in the presence of ethanol (1% (v/v); 171 mM) and/or acetone (1% (v/v); 136 mM).

We also studied the effect of solvent on pumping rate at lower
concentrations to analyse the threshold of the effect on feeding behaviour. We found that N2 animals feeding on bacteria in the presence of low concentrations of DMSO (0.05 %–0.1 % (v/v); 7–14 mM) do not have a reduction in the pumping rate when compared to untreated animals (Fig. 3A). For the rest of the solvents, no significant differences were observed when comparing the rate of pumping at lower concentrations relative to the highest concentration tested (1 % (v/v)) (Fig. 3A).

Next, we monitored the pharyngeal pump rate at increasing times following exposure to solvent. We wanted to analyse what the longer-term exposure to these concentrations of solvents would be by monitoring pumping after 24 h of incubation at the distinct doses. Protracted exposure to 1 % (v/v) ethanol (171 mM) and acetone had no effect on the pump rate when compared to untreated worms. In keeping with the observations made when considering short exposure to DMSO we noted inhibition in the pump rate steadily increased up to a maximal inhibition at the previously investigated 3 h time-point (Fig. 3A). Surprisingly, by 4 h the pumping had recovered to pre-exposure levels and this recovered pumping was maintained for up to 24 h despite worms being continually exposed to 1% v/v DMSO throughout. This indicates a recovery from the inhibition in the face of persistent solvent exposure. For the other DMSO doses, no changes across the time course were observed compared to the untreated group (Fig. 3B).

We also monitored pharyngeal pumping in the presence of these solvents in the bus-17 mutants. When considering these solvents, acetone showed no impact on the pharyngeal pump in bus-17. Surprisingly, we observed an apparent sensitivity to ethanol not observed in N2 (wt) animals (Fig. 4). This is a surprise as our previous experiments suggested that solvents like ethanol readily equilibrate across the cuticle [43]. We also noted a shift in the sensitivity of pumping in the bus-17 worms. This is highlighted by the DMSO at a concentration of 0.1 % (v/v), which failed to inhibit pumping in the N2 (wt) animals, causing a significant inhibition of pharyngeal pumping (Fig. 4). Although the bus-17 mutants seemed to be sensitized to the DMSO induced inhibition, pumping recovered to pre-exposure levels after a peak inhibition in the bus-17 mutants. Thus, as for N2 (wt), pumping recovers in the bus-17 mutants despite the protracted incubation in the DMSO.

3.2. The sensory processing deficient-mutant nlg-1 is refractory to DMSO induced pumping inhibition

We tested the impact of DMSO on the feeding behaviour of the neuroligin-deficient mutant nlg-1 (ok259). These mutants present sensory processing deficits against a wide range of mechanical, chemical and gustatory cues [36,37]. First, we measured the frequency of pharyngeal pumping in nlg-1 mutants in untreated conditions and we found that nlg-1 animals have a low frequency of pumping on food compared to wild-type (Fig. 5) [38]. Secondly, we tested the pharyngeal behaviour of nlg-1 animals incubated with the highest dose (1% (v/v)) of DMSO tested in the previous experiments with N2 (wt) animals. nlg-1 mutants did not show a decrease of the feeding rate in presence of this
3.3. Chronic exposure to DMSO induces a disruption of normal body posture during locomotion and further morphological changes in the internal structures

During observation of the effects of solvent on pharyngeal function we noticed disruption of the worm’s gross motility and morphology particular to exposures longer than 3 h. To gain further insight we investigated the consequence of exposing worms to this solvent on the anatomical integrity of the treated worms.

We incubated the worms for 3 h, where the maximum inhibition of pharyngeal pumping had been observed, at concentrations between 0.05–1% of DMSO. Under control conditions the C. elegans posture was described by changes to the sinusoidal body’s bends as the worm bends its body during crawling in “S” shapes (Fig. 6A). In contrast this was disrupted in the worms exposed to 1% (v/v) DMSO (Fig. 6A). This was reflected in a flattening of the typical S posture expressed by the worms when moving on food. This flattening during movement on food was more obvious in bus-17 animals and in some cases the worms lost all undulatory appearance and adopted a straight posture, such postural changes were modest in N2 (wt) or nlg-1 animals (Fig. 6A). This postural effect was reversed after 24 h when the affected worms were removed to a DMSO free bacterial agar plate, returning to a normal pattern of locomotion. With respect to these observer-based descriptions of motility posture no changes were noted at concentrations lower than 1% DMSO (from 0.1–0.75%).

When observing the shift in worm’s motility we recognized a change in the appearance of the internal organization of the worm under light microscope. At concentration of 1% DMSO, which are used in some drug treatments, we noted that the worms started to accumulate internal membrane-like inclusions. These structures, which are not observed in age matched untreated worms, accumulated along the entire worm’s pseudocoelom surrounding the pericellular (Fig. 6B). We also observed a progressive accumulation of these internal membrane-like inclusions which ranged from between 3 and 10 μm after 1% DMSO dosing for 24 h in the N2 (wt), nlg-1 and bus-17 mutants (Fig. 6C). They were not observed at the DMSO doses described below 1%. The appearance of these internal membrane-like inclusions was sustained over time with no clearance after 24 h of chronic exposure (Supplement to Fig. 6). The formation of internal membrane-like inclusions appeared selective to DMSO solvent exposure and was not observed in animals after chronic exposure to ethanol or acetone.

4. Discussion

Our study highlights the importance of choosing a suitable solvent
during experimental approaches based on drug treatments of *C. elegans*. Our analysis was motivated by our attempts to improve throughput for whole organism approaches focussed on using pharyngeal pumping as a bioassay for drug action. We and others have usefully developed this using two-way ANOVA (Bonferroni multiple comparisons test).

**Fig. 4.** Ethanol induces a modest and reversible inhibition of pharyngeal pumping in *bus-17* mutants. *bus-17* deficient-mutants feeding in presence of DMSO (0.05–1 %) (A), as well as ethanol (1%) (B), present a significant reduction over time (showing with a light grey shadow block on the graph) in pumping rate compared to animals in untreated conditions (**** p ≤ 0.0001; n = 5). Acetone does not affect the pharyngeal pumping over time (as, P > 0.05. n=6) (C). Data represent the mean ± s.e.m of pharyngeal pumping rate. The means were calculated from data collected from repeats of the same experiment conducted on different days. Statistical analysis was performed using two-way ANOVA (Bonferroni multiple comparisons test).

**Fig. 5.** Neuroligin mutants are refractory to the inhibitory effect of DMSO in pharyngeal pumping.

*nlg-1* mutants feeding in presence of DMSO sustained their pharyngeal pumping, without inhibition over time. There were no significant differences between different concentrations of DMSO at any of the individual time points (ns, P > 0.05; n=7–10). Data represent the mean ± s.e.m of pharyngeal pumping rate. The means were calculated from data collected from repeats of the same experiment conducted on different days. Statistical analysis was performed using two-way ANOVA (Bonferroni multiple comparisons test).
We had previously shown that the synaptic protein neuroligin (NLG-1) was an important extrapharyngeal determinant of pharyngeal pumping. *nlg-1* mutants present a reduced pumping that suggests that neuroligin organizes extrapharyngeal circuits that regulate the pharynx, highlighting the role of neuroligin in discretely impacting functional circuits underpinning complex behaviours [38]. Interestingly, *nlg-1* mutants lost their sensitivity to DMSO. This does not rule out important contribution of direct perturbation effects of key cells involved in co-ordinating pharyngeal pumping but does highlight that DMSO at 1% and above concentrations can provide an additional selective modulation of sensory modalities. This could imply interference with sensory detection of food in the neural circuits that regulate pharyngeal activity. This is consistent with sensory processing defects in *nlg-1* previously described [38], as the lack of an aversive response by the normally aversive chemical 1-octanol, or the defects in the processing of two conflicting chemosensory inputs [37].

Interestingly, we observed a response to 1% (v/v) (141 mM) DMSO at the whole worm level disrupting the *C. elegans* posture typical of untreated worms. These observations showed that DMSO exposed animals, do not likely induce lethal changes in metabolism. However, we did note changes in worm posture from which they recovered and consistent with effects of the solvent on the body wall muscle system that controls motility [49]. In addition, we observe exposure to DMSO...
resulted in the formation of a disrupted morphology. This coincided with the appearance of internal membrane-like structures. These changes seem to be particular to DMSO relative to the solvents tested in this study. These structures appear membranous in structure however at the light microscopic level we cannot resolve if these are double membrane vesicles or single lipid boundaries defined by visible lipid droplets that are present and dynamically change in nematodes [45,46]. Both remain a possibility and the accumulation of DMSO and solvents within the pseudocoelom, following external exposure, would be well placed to trigger signaling and metabolic changes that disrupt cellular and wider sub-compartment integrity. The size and features of the internal membrane-like structures would be compatible with DMSO triggering the accumulation of corpuscular apoptotic cells [47], disrupted and enlarged lipid droplet trafficking [45] or the activation of the recently recognized cellular vacuolization that arises through the processes of methosis [48]. One speculation is these structures are a stress response and/or metabolic disruption, potentially compromising the intra and/or extra cellular integrity, and that is subject to regulation by the scavenging systems of the worm encompassed in coelomocytes. We observe these changes in the extreme of prolonged DMSO exposure at the upper range at which it is used for drug vehicle. However, the selective effect of DMSO relative to other solvents tested, alerts to potential confounds associated with chronic dosing experiments in which DMSO is the background vehicle. Indeed, their appearance within the worms pseudocoelom and persistence following DMSO removal would be consistent with this idea (Fig. 5B).

In conclusion, here we provide direct evidence for a differential effect of molar equivalents of a common drug solvent DMSO used in the investigation of drug administration in C. elegans. These data highlight that the function of the pharynx, the neuromuscular organ that controls feeding is not affected by DMSO up to concentrations of 0.1 %. However, higher concentrations including those that are used for vehicle controls impact on pharyngeal function. Although, we have limited the investigation of these solvents in solid phase in this study, it is worth considering that the effective concentrations of solvents impacting on physiological parameters such as development, fitness or complex behaviour could vary in different media i.e. axenic liquid culture. In the current paper we directly monitor pharyngeal pumping as this is feasible in the agar plate format. Higher throughput assays are achieved in liquid culture in which animals are supported by bacteria in solution rather than those plated in solid phase on agar [10,11]. The confounds in which DMSO disrupts feeding as mapped out in this paper is likely to pertain to other experimental formats that utilize DMSO dissolved drugs as a central factor to their approach. DMSO while acting as an excellent drug vehicle screening tool has shown to have a large number of non-target effects on the body. Further studies are needed to understand the long-term effects of DMSO exposure and the potential for off-target effects.

Conflict of Interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

**Fernando Calahorro:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Funding acquisition, Writing - original draft. **Lindy Holden-Dye:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing - review & editing. **Vincent O’Connor:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2021.06.007.

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