Fluensulfone is a nematicide with a mode of action distinct from anticholinesterases and macrocyclic lactones

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Abstract

Plant parasitic nematodes infest crops and present a threat to food security worldwide. Currently available chemical controls e.g. methyl bromide, organophosphates and carbamates have an unacceptable level of toxicity to non-target organisms and are being withdrawn from use. Fluensulfone is a new nematocide of the fluoroalkenyl thioether group that has significantly reduced environmental impact with low toxicity to non-target insects and mammals. Here, we show that the model genetic organism Caenorhabditis elegans is susceptible to the irreversible nematicidal effects of fluensulfone. Whilst the dose required is higher than that which has nematicidal activity against Meloidogyne spp. the profile of effects on motility, egg-hatching and survival is similar to that reported for plant parasitic nematodes. C. elegans thus provides a tractable experimental paradigm to analyse the effects of fluensulfone on nematode behaviour. We find that fluensulfone has pleiotropic actions and inhibits development, egg-laying, egg-hatching, feeding and locomotion. In the case of feeding and locomotion, an early excitation precedes the gross inhibition. The profile of these effects is notably distinct from other classes of anthelmintic and nematocide: the inhibition of motility caused by fluensulfone is not accompanied by the hypercontraction which is characteristic of organophosphates and carbamates and C. elegans mutants that are resistant to the carbamate aldicarb and the macrocyclic lactone ivermectin retain susceptibility to fluensulfone. These data indicate fluensulfone’s mode of action is distinct from currently available nematicides and it therefore presents a promising new chemical entity for crop protection.

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1. Introduction

Plant parasitic nematodes (PPNs) are responsible for yield losses in excess of 20% in some crop species [1] and an average yield reduction of 12.3% in the world’s 40 major food staples [2]. These yield losses amount to an estimated financial burden of at least $125 billion per annum [3]. PPNs have been controlled through extensive use of chemical nematicides, predominantly the fumigant nematicide methyl bromide. The use of methyl bromide is now restricted due to its ozone-depleting properties [4]. More recently carbamate- and organophosphate-based nematicides, such as aldicarb and fosthiazate [5], have served as replacements for methyl bromide. Both carbamates and organophosphates are anticholinesterases and many have now been banned or their use highly restricted due to concerns over their toxicity to non-target invertebrates, mammals and humans, and their impact on the environment [6–11].

Fluensulfone, or MCW-2, (5-chloro-2-(3,4,4-trifluorobut-3-enyl)sulfonyl)-1,3-thiazole) is a member of the fluoroalkenyl thioether group (Fig. 1). It has nematicidal actions [12–14] and lacks many of the drawbacks of other chemical controls, as exemplified by its relatively low toxicity to non-target organisms [14]. Fluensulfone is effective against a number of PPNs including species of Meloidogyne [14–18] which are responsible for a significant proportion of nematode crop yield loss. Recently fluensulfone has been shown to significantly reduce root infection and penetration by Meloidogyne javanica [19], Meloidogyne incognita [15] and Meloidogyne arenaria [18].

Fluensulfone affects the motility and body posture of PPNs [19]. In this respect, fluensulfone has an action similar to other compounds that have been widely used for crop protection; the organophosphates, the carbamates and the avermectins.

Here we investigated the mode of action of fluensulfone using the free-living model genetic nematode, Caenorhabditis elegans as an experimental platform. C. elegans has conserved physiology and pharmacology with parasitic nematodes and has been success-
A hypomorphic mutation in the vesicular acetylcholine transporter for effects of fluensulfone on action to that of aldicarb and ivermectin. We provide evidence experiment with 5 replicates mean ± s.e.mean; representative of two experiments conducted on separate occasions. Exposed to 1 mM fluensulfone showed an irreversible paralysis (Bonferroni’s multiple comparisons test). Over 24 h the control group (0.1% acetone) also became immobile, however following the wash, these worms recovered, whilst those exposed to 1 mM fluensulfone showed an irreversible paralysis (p < 0.001; one way Anova, Bonferroni’s multiple comparisons test). The data presented are from one experiment with 5 replicates mean ± s.e.mean; representative of two experiments conducted on separate occasions.

fully used to determine the mode of action of anthelmintic compounds with efficacy against nematodes that parasitise human, livestock and companion animals [20–24]. Indeed, for ivermectin and emodepside molecular genetic studies in C. elegans were key to this process [21,23]. Model hopping between C. elegans and PPNs is less well developed but is an experimental approach that might be exploited to provide insight into the mode of action of nematocides. Therefore, in order to provide further insight into the nematocidal action of fluensulfone we have conducted behavioural and electrophysiological investigations in C. elegans and compared its action to that of aldicarb and ivermectin. We provide evidence for effects of fluensulfone on C. elegans following acute and chronic exposure and by analysing the profile of its actions and comparing it with aldicarb and ivermectin resistant mutants we show that the mode of action of fluensulfone is distinct.

2. Materials and methods

2.1. Culturing C. elegans

C. elegans were cultured at 20 °C on nematode growth medium (NGM) plates [25] that were seeded with Escherichia coli (OP50 strain). These plates are referred to as OP50/NGM plates. N2 Bristol strain was used as the wild-type reference strain. Two further strains were used in this study: CB113 unc-17(e113) which carries a hypomorphic mutation in the vesicular acetylcholine transporter [26] and DA1316 avr-14(ad1302); avr-15(ad1051); glc-1(pk54) which carries functional null mutations in three genes encoding glutamate-gated chloride channels [27]. Strains were obtained from the C. elegans Genetics Center, CGC (University of Minnesota, USA). C. elegans were stage synchronised for some experiments by collecting populations of eggs and allowing them to develop over a defined time-frame to each larval stage and adult.

2.2. Drugs and chemicals

Fluensulfone (batch number: 2010-291-12-7; 97% purity) was provided by Makhteshim Agan UK (Berkshire, UK). Fluensulfone was prepared in 100% acetone and then mixed into the final solution at a concentration of 0.5% acetone of the total solution. Fluensulfone was prepared as fresh stock in acetone each day of use. Spectrophotometric analysis of centrifuged compound samples indicated that this protocol was sufficient to deliver a non-particulate solution. NGM agar plates containing fluensulfone were prepared by making a stock solution of 200 mM fluensulfone in acetone. Dilutions of fluensulfone in acetone were made from the stock solution. 750 µl of fluensulfone were added to 150 ml of NGM (nematode growth medium agar; after the NGM had been autoclaved and as it was cooling) to give the desired final concentration. For vehicle control plates exactly the same procedure was followed with the omission of fluensulfone. The final maximum concentration of acetone in the plates was 0.5%. Control (vehicle) plates were made with 750 µl acetone in 150 ml NGM.

The NGM plates were poured 3 days before use and were seeded with 50 µl OP50 1 day before use. For electrophysiological experiments all fluensulfone-containing solutions were made using Dent’s saline (in mM; 140 NaCl, 10 HEPES, 10 D-glucose, 6 KH2PO4, 6g Na2HPO4, 5 g NaCl, 1 ml 1 M MgSO4, H2O to 1 l).

Some experiments were conducted in M9 phosphate buffer (3 g KH2PO4, 6 g Na2HPO4, 5 g NaCl, 1 ml 1 M MgSO4, H2O to 1 l).

2.3. Lethal effects of fluensulfone at different stages of C. elegans development

C. elegans progresses through 4 larval stages, from egg to adulthood in 3.5 days (at 20 °C) [28]. To assess the impact of fluensulfone on survival, worms of each developmental stage were soaked in fluensulfone for 24 h. The worms were then washed several times and placed back on OP50/NGM plates to test for survival. For each assay 50–100 stage-synchronised worms at L1, L2 and L3 (these stages are hard to distinguish and were assayed together; L2/3, L4 or one day old adult were soaked in fluensulfone in phosphate buffer) for 24 h (100 µM, 300 µM, 1 mM) or in vehicle (0.1% acetone). The worms were then washed several times and placed
on OP50/NGM plates and lethality was scored as a % of the total number of worms in each sample. Worms that were completely immotile and unresponsive to prodding and that had a granular appearance suggesting disintegration of internal structures were defined as dead. The experiment was conducted on two separate occasions with five replicates for each developmental stage. The data presented are from one experiment which is representative of both.

2.4. Assaying paralysis induced by fluensulfone at different stages of C. elegans development

For these assays worms of different developmental stages were incubated in liquid with and without fluensulfone for up to 24 h, and paralysis was scored. 400 μl of M9 phosphate buffer with either fluensulfone (100 μM, 200 μM or 1 mM) or vehicle (0.5% acetone) was put into each well of a 24 well plate (5 replicates for each fluensulfone concentration). 5 μl suspension of age synchronised C. elegans (L1, L2/3, L4 or one day old adult) was added to each well. Each well contained approximately 50–100 worms. The number of worms not moving at 1, 2, 3, 4, 5, 6 and 24 h was determined. The experiment was conducted on two separate occasions with five replicates. The data presented are from one experiment which is representative of both.

In a further experiment, to investigate reversibility of paralysis, C. elegans L2/3 larvae were soaked in either 1 mM fluensulfone or 0.5% acetone for 6 h and then washed several times in M9 phosphate buffer before placing on NGM plates and scoring for paralysis.

2.5. Effects of fluensulfone on C. elegans growth and developmental timing

For these experiments the development of C. elegans from egg to adult in the presence of vehicle or fluensulfone (100 μM, 300 μM and 1 mM) was observed. 10 one day old adult worms were placed on OP50-seeded NGM plates containing either vehicle, 100 μM, 300 μM or 1 mM fluensulfone and were allowed to lay eggs for 1 h at which point the adult worms were removed and discarded. The plates were incubated for up to 96 h at 20 °C and the state of development of the eggs and hatching larvae was scored at 24, 43, 66 and 96 h. Developmental stage was scored as egg, <L4 (encompassing L1 and L2/3 larval stages), L4 or adult. This was performed with 5 plates for each experimental condition and was repeated on 3 separate occasions.

2.6. Effects of fluensulfone on egg-laying and egg hatching

One day old adult worms were individually transferred to OP50/NGM plates that contained either vehicle or 1 mM fluensulfone. After 1 h the adult worms were removed and discarded and the number of eggs laid was counted. The plates were re-inspected 24, 48 and 72 h later. For each experiment 5 control plates and 10 plates with fluensulfone were analysed. At 24 h the number of eggs that had hatched was scored. Subsequently, at 48 and 72 h hatched larvae were observed for development. The experiment was conducted on three separate occasions. The data presented are from one experiment which is representative of three.

2.7. Effects of fluensulfone on embryo viability

These assays tested the effect of in utero exposure to fluensulfone on embryo viability. Gravid adult worms were placed on OP50/NGM plates containing either vehicle or 1 mM fluensulfone for 1 h at which point the adult worms were removed to OP50/NGM plates without fluensulfone or vehicle. Each subsequent hour, for up to 5 h, the adult worm was moved to a new plate. The eggs that were laid on each plate were inspected and scored at 24 h and 48 h for hatching. The experiment was conducted three times with between 5 to 10 plates for each treatment group. The data presented are from one experiment which is representative of three. A subset of unhatched eggs was further subjected to DIC microscopy analysis to determine whether or not unhatched eggs showed evidence of fertilisation (i.e. contained a worm embryo).

2.8. Effects of fluensulfone on motility

In liquid C. elegans makes a stereotypical flexing movement around the midpoint of its body called ‘thrashing.’ Thrashing assays were performed in M9 buffer supplemented with 1 mg ml–1 bovine serum albumin (BSA). Individual stage-synchronised worms e.g. L2/3, were placed in M9 buffer with fluensulfone (100 μM–1 mM) and after the specified length of time of incubation (5 min up to 3 h) the number of thrashes performed in 30 s scored by visual observation with a binocular microscope. Vehicle control groups were paired i.e. performed at the same time as the fluensulfone treatment groups. ‘n’ is the number of worms tested (10 in the first experiment and 5 in the repeat). The experiment was conducted on two separate occasions and the data presented are from one experiment which is representative of both.

2.9. Effects of fluensulfone on C. elegans feeding behaviour

C. elegans feeding behaviour is supported by the pharyngeal system [29]. The pharynx is a muscular tube leading from the mouth to the intestines and it has an associated pharyngeal nervous system. Feeding may be scored by visual observation of the contraction–relaxation cycle, pump, of the terminal bulb of the pharynx in the freely moving worm. One day old adult or L4 C. elegans were transferred to either fluensulfone or vehicle containing OP50/NGM plates. Feeding was measured by counting pharyngeal pumping while the worm was on the OP50 bacterial lawn at intervals for up to 1 h. This experiment was conducted on two separate occasions for n = 15 worms and n = 6 worms, respectively. The data are presented from the first experiment which is representative of both.

Pharyngeal activity was also measured in a cut head preparation. For this, the head of the worm was cut from the body at the level of the pharyngeal-intestinal valve, just anterior to the terminal bulb. This was achieved using a small razor blade fragment in a blade holder as previously described [30]. The cut head was placed in Dent’s saline with either fluensulfone or vehicle. In these experiments 300 mM 5-HT was included in the Dent’s saline to stimulate pharyngeal pumping.

2.10. Effect of fluensulfone on food-dependent behaviour

The behaviour of C. elegans is highly regulated by the presence of its food source, bacteria. [31]. In the presence of E. coli OP50 the sinusoidal movement of the worm occurs at about half the frequency of movement off bacteria. We assessed the effect of fluensulfone on the rate of body bends on and off food. For the experiments off food, one day old adult C. elegans were picked to an NGM plate without OP50 and allowed to move for 1 min to remove any adhering bacteria. They were then moved to another NGM, ‘test’, plate without OP50 and after 2 min recovery the frequency of body bends was monitored by visual observation at intervals for the next 2 h. The test plates contained either fluensulfone or vehicle. To test the effect of fluensulfone on movement on food, one day old adult worms were transferred to the bacterial lawn of an OP50/NGM plate and after 1 h and 5 h the number of body bends in 30 s was counted. Experiments were performed on
more than one occasion, with a parallel control on each day. \( n \) is the number of worms observed (7–30).

On a high quality food source wild-type \( C. \) elegans typically dwell on the bacterial lawn and exhibit a very low frequency of leaving the food patch, a behaviour termed ‘food leaving.’ However, if the density of the bacteria decreases [32] or is detected as noxious [33] the frequency of food leaving is increased. We tested whether or not fluensulfone caused food leaving. For this NGM plates were prepared with either vehicle or with fluensulfone at concentrations of 100 \( \mu \)M or 500 \( \mu \)M or vehicle. Plates were seeded with 50 \( \mu \)l OP50 (optical density 0.8) 1 day before use. 7 wild-type one day old worms were transferred to a single plate, on a spot away from the bacterial lawn and left for 5 min. The plates were then observed for 2 h and the number of leaving events was noted. A food leaving event was defined as an incident in which the whole body of the worm left the bacterial lawn and the worm did not immediately reverse back onto the bacteria. For each plate of worms a leaving rate was calculated as leaving events/worm/minute. The experiment was conducted on separate occasions over several days and on each day a control plate was assayed in parallel with the fluensulfone treatment group. \( n \) is the number of plates that were scored.

2.12. Electrophysiological analysis of the effects of fluensulfone

Neural and muscular activity of \( C. \) elegans can be captured using extracellular electrophysiological recordings made via a suction electrode placed over the mouth of the worm [34,35]. The signal, called the electropharyngeogram (EPG), provides a read-out of the activity of the pharyngeal system which underpins feeding behaviour but is more detailed and precise than whole organism observations of pharyngeal pumping. EPG recordings were made from head preparations of one day old adult \( C. \) elegans as previously described [36]. Signals were analysed using AutoEPG software [37].

Once suction was applied onto the preparation 5 min were allowed to ensure that the seal between the head and the pipette was stable. Dissected pharynxes were exposed to a perfusion at a constant rate of 4.5 ml min\(^{-1}\). For the first 5 min, a perfusion of Dent’s saline was applied prior to any drug application to gain a pre-drug control for that preparation. Experiments were conducted on several separate occasions.

2.12. Comparing the effects of fluensulfone with aldicarb and ivermectin

2.12.1. Measuring the effect on worm length

Anticholinesterase compounds such as aldicarb elicit a marked contraction of \( C. \) elegans body wall muscle, manifest as a decrease in overall worm length [38]. To test whether or not fluensulfone has a similar effect, assays for contraction were conducted for both fluensulfone and aldicarb on L4 \( C. \) elegans. Assays were conducted as previously described [38]. Individual L4 larvae were imaged on food plates in the absence of compound and then placed on agar plates containing either 1 mM fluensulfone or 500 \( \mu \)M aldicarb. Further images were captured at a one hour time-point after placing them on the compound. Software ImageJ which provides a skeleton image of the worm was used to measure its length. Measurements were normalised to the initial length for each individual worm. The experiment was conducted on a second occasion for L4 plus one day old adult worms. \( n \) is the number of worms that were measured.

2.12.2. Comparing the effects of aldicarb and fluensulfone on egg-laying

These experiments deployed a strain which is defective in cholinergic signalling and thus resistant to aldicarb [26]. For these experiments L4 plus one day old hermaphrodites, either wild-type or \( unc-17(e113) \), were placed on OP50/NGM plates containing either vehicle, 1 mM fluensulfone or 500 \( \mu \)M aldicarb. 3 plates were set-up for each experimental condition. After 1 h the number of eggs on each plate was counted.

2.12.3. Comparing the effects of fluensulfone and the macrocyclic lactone ivermectin on motility

Ivermectin inhibits \( C. \) elegans behaviour through a family of glutamate-gated chloride channels [23] and a strain carrying mutations in three of these channels, DA1316, is highly resistant to the effects of ivermectin [39]. Here we deployed this strain to determine whether or not fluensulfone exerts its effects on \( C. \) elegans behaviour through glutamate-gated chloride channels. The thrashing rate of wild type or DA1316 L2/L3 larvae exposed to vehicle or fluensulfone for 1, 2 and 3 h was scored. The experiment was conducted on two separate occasions for 10 worms for each treatment group. The data presented are from one experiment which is representative of both.

2.13. Statistical analysis

Data are shown as the mean ± S.E.M. Student’s \( t \) tests, One-way ANOVA and two-way ANOVA were used as indicated and followed by Bonferroni post-tests. For all data statistical significance was set at \( p < 0.05 \). All statistical analyses were carried out using GraphPad Prism software (GraphPad version 5). The number of individual worms used to perform the statistical analysis for each experiment is stated in the figure legends, as is the specific statistical test employed. For all statistical testing of the EPG data repeated measures tests were used. The data shown are pooled from replicates or multiple experiments as indicated.

3. Results

3.1. Fluensulfone has nematicidal action against \( C. \) elegans

24 h soaking of \( C. \) elegans in 1 mM fluensulfone resulted in high levels of mortality at all developmental stages (Table 1) whilst worms that were soaked in the vehicle control (0.5% acetone) exhibited >90% survival. Fluensulfone is therefore clearly effective against \( C. \) elegans. The threshold for the effect on survival occurred at around 100–300 \( \mu \)M (Table 1). In a further analysis the motility of L2/3 larvae was scored at intervals during the 24 h fluensulfone exposure period and subsequently 6 h and 24 h after washing and recovery on OP50/NGM plates (Fig. 1). Incubation in liquid for 24 h impaired motility in all the experimental groups, including the vehicle control however the effect of fluensulfone on inhibiting motility is clearly apparent at the early time-points up to 6 h, when the majority of the control group were still motile (Fig. 1). In order to determine whether or not there was a difference in susceptibility of the different developmental stages to the inhibitory effects of fluensulfone on motility larval and adult stages were soaked in fluensulfone for 3 h. After this treatment the vehicle control groups and fluensulfone treated L1 and adult worms were still motile. However, incubation with fluensulfone induced paralysis in larval stages L2/3 and L4 (Fig. 2A). The threshold for the effect of fluensulfone on L4 was 100 \( \mu \)M whilst, for L2/3 it was 100 \( \mu \)M and at 1 \( \mu \)M about half the population of worms were paralysed after 3 h (Fig. 2A). The inhibition of motility by 1 mM fluensulfone on L2/3 larvae was irreversible after 24 h exposure (Fig. 1) or after a shorter 6 h exposure (Fig. 2B). These data indicate that fluensulfone has nematicidal action against \( C. \) elegans, similar to the effect previously reported against plant parasitic nematodes [14]. Interestingly, whilst the nematicidal actions of fluensulfone occur at
all developmental stages (Table 1), the larval stages, L2/3 and L4, appear most susceptible to the paralytic action (Figs. 1A and 1B).

3.2. Fluensulfone causes L1 larval arrest

Worms were grown from eggs to adults in the constant presence of vehicle (control) or fluensulfone to assay the concentration-dependence of effects of fluensulfone on development. 1 mM fluensulfone prevented any development past L1 (Fig. 2C, data not shown). Lower concentrations of fluensulfone delayed development (Fig. 2D): 100 μM fluensulfone caused a slight delay as at 66 h fewer worms had reached the adult stage whilst at 300 μM no worms reached the adult stage at 66 h and some failed to reach L4 (Fig. 2D). Thus fluensulfone delays development in a concentration-dependent manner.

3.3. Fluensulfone inhibits egg-laying, egg hatching and reduces egg viability

Adult hermaphrodites laid fewer eggs in the presence of 1 mM fluensulfone (Fig. 3A). Indeed, visual observation of C. elegans gravid adult hermaphrodites left on 1 mM fluensulfone for 24 h showed that eggs were retained rather than laid, and that the worms exhibited a “bagging behaviour” (sometimes known as matricalid hatchig) in which the retained embryos hatch inside the adult. (See Suppl. Video 1 as an example of a worm with a swollen appearance due to ‘bagging.’)

About one fifth of the eggs that were laid on 1 mM fluensulfone failed to hatch (Fig. 3B). Of the remaining eggs that did hatch, none progressed beyond L1 larvae (data not shown).

Fluensulfone was also found to reduce the viability of eggs in utero (Fig. 3C). In these experiments gravid adult worms were exposed to 1 mM fluensulfone for just one hour and then removed from the compound and placed on a new agar plate to lay eggs. The eggs that were laid were monitored for hatching. Intriguingly, viability was unaffected in those eggs that were laid 1 h after removal from fluensulfone however eggs that were laid 2–5 h later showed significantly reduced hatching. Indeed, even after 48 h a significant number of these eggs had not hatched. We hypothesised that exposure of the gravid adult worm to fluensulfone had affected some time-critical event underpinning embryo viability in utero and one possibility we considered is that fluensulfone inhibited the process of self-fertilization which occurs prior to egg laying. However, DIC microscopy analysis of a subset of the un-hatched eggs showed that they harboured worm embryos (Fig. 2D) indicating that the effect of fluensulfone is not on the self-fertilization that occurs within the adult hermaphrodite, but rather after this process: Most likely the compound is nematicidal to the developing embryo.

3.4. Fluensulfone elicits acute inhibition of motility in liquid

In order to further characterise the effect of fluensulfone on C. elegans motility we conducted experiments to quantify its inhibitory action on thrashing behaviour. We focused on L2/3 larval stages given the greater susceptibility of this stage to fluensulfone and in view of its potential equivalence to the PPN infective larval stage, J2. After 3 h incubation with 100 μM to 1 mM fluensulfone the thrashing rate was significantly inhibited, maximal inhibition occurring with 1 mM (Fig. 4A). A time-course analysis for the inhibitory effect of fluensulfone indicated that the effect gradually increased over the course of 1 h (Fig. 4B).

3.5. Fluensulfone elicits acute inhibitory effects on feeding behaviour

The effect of fluensulfone on the rate of pharyngeal pumping was investigated in worms grazing on an OP50 E. coli bacterial lawn on OP50/NGM plates with fluensulfone compared to vehicle control. After 1 h both 300 μM and 1 mM fluensulfone caused a significant and reversible inhibition of pharyngeal pumping relative to the vehicle control (Fig. 5A). Experiments were also conducted on cut-head preparations in which the pharynx is directly exposed to the fluensulfone containing solution. In this preparation pharyngeal pumping was stimulated by the application of 300 nM 5-HT. Fluensulfone caused as similar, although more variable, level of inhibition after 1 h exposure in the cut head preparation compared to the intact worm (Fig. 5B) suggesting that access of fluensulfone to the internal organs of the worm is not a limiting factor in terms of its mechanism of action. The variability between the data sets for the intact worms assays and cut head experiments may be due to the fact that in the latter 5-HT was used to stimulate pumping.

The susceptibility of the larval stage L4 to fluensulfone was also tested and we found that after 1 h exposure of the intact larva to 300 μM fluensulfone pharyngeal pumping was completely inhibited (Fig. 5C) providing further evidence that the larval stage of C. elegans is more susceptible to fluensulfone than adult. This analysis was not performed on L2/3 larvae as their small size makes accurate visual scoring of pharyngeal pumping technically difficult.

3.6. Fluensulfone elicits complex effects on C. elegans food dependent locomotion

C. elegans moves at about twice the rate off a bacterial lawn compared to on bacteria. Fluensulfone (500 μM) inhibited the frequency of body bends in one day old adult hermaphrodites off food after 2 h exposure (Fig. 6A). Surprisingly the opposite effect was observed on food i.e. 500 μM and 1 mM fluensulfone transiently stimulated the frequency of movement (Fig. 6B). This stimulation was observed at 1 h exposure but not at a later time-point after 5 h exposure (Fig. 6C). These data suggest that the acute effects of fluensulfone on the locomotion rate of adult C. elegans...
are context-dependent. That is, when worms are exposed to fluen- sulfone on a bacterial lawn their movement is transiently stimu- lated, but when they are exposed to fluen sulfone in the absence of food only an inhibition of movement is observed. This is an intriguing observation suggesting an interaction between fluen- sulfone and the behavioural state of the worm.

In a further series of experiments we tested whether or not fluen sulfone affected the preference of C. elegans to dwell on food and found that 500 µM fluen sulfone more than doubled the frequency of food leaving events (Fig. 6D). Therefore the increased locomotion observed in the presence of fluen sulfone on a bacterial lawn is accompanied by an increased tendency to move off the food. The food-leaving induced by fluen sulfone led us to question whether or not fluen sulfone causes aversion. In a standard assay for avoidance, in which a drop of the test chemical is placed in front of a freely moving worm [40], we repeatedly observed that worms would swim through a drop of 1 mM fluen sulfone: 8 worms were tested and only one worm reversed when it contacted the drop of fluen sulfone. Thus it would appear that C. elegans do not show an aversive response to fluen sulfone.

3.7. Electrophysiological recordings indicate rapid and reversible effects of fluen sulfone on neuromuscular signalling in the pharyngeal system

Electrophysiological recordings of the activity of the pharyngeal system of adult hermaphrodite C. elegans were made via an extracellular suction electrode placed on the mouth of the worm (Fig. 7A). These experiments were paired i.e. the control was performed on the same day as fluen sulfone test as there is some day to day variability in pump rate. In the first series of experiments 30 and 300 µM fluen sulfone were compared to control and both elicited a significant increase in pump frequency (control 0.0722 ± 0.02 Hz, n = 3; 30 µM 0.183 ± 0.03, n = 4, p < 0.05; 300 µM 0.4333 ± 0.07, n = 3, p < 0.001; one way Anova, Bonferroni’s post-hoc test). In a further series we found that the spontaneous activity of the pharynx was increased by 10 min application of 50 and 100 µM fluen sulfone but inhibited by 500 µM (Fig. 7B). An analysis of concentrations spanning 10–500 µM indicated a bell-shaped concentration response curve (Fig. 7C). Notably, both the excitation and inhibition elicited by fluen sulfone were rapidly reversible (Fig. 7B). Furthermore, an analysis of the time-course of the effect of 500 µM fluen sulfone showed that the inhibition observed after 10 min application was preceded by a transient excitation (Fig. 7D).

3.8. A comparison of the effects of fluen sulfone and the carbamate aldicarb

Both fluen sulfone and aldicarb inhibit C. elegans motility (Fig. 4B). Aldicarb also has an inhibitory effect on feeding, though not as marked as the inhibition observed for fluen sulfone (Fig. 5C). Taken together these observations suggest that fluen sulfone and aldicarb might have a similar mechanism of action. In the first instance, we investigated this further by observing the
behaviour of one day old adult worms or L2/3 that had been placed on OP50/NGM plates containing either 1 mM fluensulfone or 1 mM aldicarb and left for 24 h. Worms on vehicle control plates were actively moving at this time (data not shown). Both the fluensulfone and aldicarb treated worms were severely affected by the exposure to compound and were immobile and distended due to the retention of eggs. However, the fluensulfone treated worms showed no evidence of pharyngeal pumping (Suppl. Video 1), compared to the aldicarb treated worms (Suppl. Video 2) which were still feeding, albeit at a greatly reduced rate compared to controls.

To investigate this further we characterised the paralysis induced by both compounds in more detail. Aldicarb is an anticholinesterase that has been extensively studied for its paralytic effects in C. elegans [41]. At a concentration of 500 µM to 1 mM it produces a characteristic hyper-contraction of body wall muscle and shortening of body length. Visual observation of adult C. elegans shortly after being treated with fluensulfone indicated that its mode of paralysis is distinct from that of aldicarb as hyper-contraction was not observed. To quantify this, measurements were made from images captured of L4 larvae before and after exposure to either 500 µM aldicarb or 1 mM fluensulfone. These clearly indicate that whilst aldicarb shortens body length, fluensulfone does not (Fig. 8A). Thus, whilst both fluensulfone and aldicarb inhibit larval motility (Fig. 4B) the differences in the effects of these compounds on body contractile state and length indicate the mode of action is different. In a further experiment on one day old adults we also found that aldicarb elicited shortening in contrast to fluensulfone for which there was no overall change in body length (data not shown).

Analysis of the susceptibility of an aldicarb resistant mutant unc-17 to fluensulfone provided further evidence that this compound does not exert its inhibitory effects via an anticholinesterase mode of action. unc-17 encodes the acetylcholine vesicle transporter [26] and the hypomorphic mutation in the strain used in this study confers reduced release of acetylcholine at the body wall neuromuscular junction and thereby a marked resistance to the actions of cholinesterase inhibitors. To test whether or not fluensulfone can break C. elegans unc-17 aldicarb resistance we exposed 1 day old adult worms (unc-17 or wild-type) to either 1 mM fluensulfone or 500 µM aldicarb for 24 h on OP50/NGM plates and the number of paralysed worms was counted. unc-17 were highly resistant to aldicarb but as susceptible to fluensulfone as wild-type worms (Fig. 8B). Egg-laying is also a C. elegans behaviour which is susceptible to the effects of aldicarb [42] and therefore we tested whether or not unc-17 mutants were susceptible to the effects of aldicarb in the egg-laying assay. Whilst egg-laying was similar in unc-17 vehicle control and aldicarb (500 µM) treated worms

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**Fig. 3.** Effect of acute exposure to fluensulfone on C. elegans reproduction and development. (A) 10 gravid worms were put on each plate seeded with OP50, and allowed to lay eggs for 1 h in the presence of either vehicle (control) or 1 mM fluensulfone (F). After 1 h the adult worms were removed from the plates and the number of eggs laid counted. Control n = 5; F, n = 10; where ‘n’ is the number of plates. Data are expressed as the mean ± s.e.mean eggs per plate, p = 0.001 unpaired Student’s t-test. (B) Eggs that were laid on fluensulfone (1 mM) were scored for hatching 24 h later. Control, n = 5; Fluensulfone (F), n = 10; where ‘n’ is the number of plates; mean ± s.e. mean, p < 0.001 unpaired Student’s t-test. Eggs that hatched on fluensulfone did not develop past L1 (see Fig. 2C). (C) In utero effects of fluensulfone. 10 gravid hermaphrodite worms were put on each of 10 OP50/NGM plates, and exposed to either vehicle control or 1 mM fluensulfone (F) for 1 h. They were removed to new plates (without fluensulfone or vehicle) every hour up to 5 h after removal from fluensulfone. The eggs laid on the plates were scored for hatching 24 h later. mean ± s.e. mean, ***p < 0.001 compared to vehicle control, one way ANOVA with Bonferroni’s post hoc test. Eggs that hatched from fluensulfone treated animals started feeding at the time of hatching (data not shown). (D) A subset of un-hatched eggs from this experiment were examined by DIC microscopy. The image shows the typical appearance of an egg which failed to hatch indicating that it contains an embryo and therefore was fertilised. The percentage of fertilised embryos was the same for the 1 mM fluensulfone and vehicle control treatment groups (>90% of eggs).

**Fig. 4.** Acute exposure to fluensulfone inhibits the rate of C. elegans movement in liquid. (A) L2/L3 larvae were placed individually in M9 buffer containing either vehicle or fluensulfone (F). After 3 h the number of thrashes in 30 s was counted for each worm (n = 10 worms, mean ± s.e. mean; ***p < 0.001, ***p < 0.001 unpaired Student’s t-test compared to vehicle control). (B) The thrashing rate of L2/L3 larvae in saline was measured immediately prior to placing them either in saline containing either vehicle (control), 1 mM fluensulfone (F) or 500 µM aldicarb (ald). Time zero is when the worms were transferred. The number of thrashes made by each worm in 30 s was visually scored at intervals for the next hour. n = 5 worms, mean ± s.e.mean, ***p < 0.001 compared to control for F and ald, unpaired Student’s t test.
the *unc-17* mutant, was very susceptible to the inhibitory effects of fluensulfone on egg-laying (Fig. 8C).

3.9. A comparison of the effects of fluensulfone and the macrocyclic lactone ivermectin

Ivermectin elicits an inhibitory paralysis of *C. elegans*, slows development [27], and inhibits pharyngeal pumping [30] and in this respect shows some similarities to the profile of effects of fluensulfone. Ivermectin elicits its actions through a family of glutamate-gated chloride channels [23]. In order to test whether or not these channels are involved in mediating the actions of fluensulfone we tested an ivermectin resistant strain for susceptibility to fluensulfone using the thrashing assay. The thrashing rate of L2/L3 larvae of the ivermectin resistant strain was not impaired following 3 h exposure to ivermectin (Fig. 9A) but it was still susceptible to the actions of fluensulfone (Fig. 9B). However, there was a small, but significant reduction in the magnitude of the fluensulfone inhibition in the ivermectin resistant strain (one way Anova, Bonferroni’s multiple comparisons test). To further determine whether or not this ivermectin resistant strain showed any cross-resistance with fluensulfone we compared the effect of fluensulfone (1 mM for 1 h) on pharyngeal pumping in adult wild-type and the ivermectin resistant strain. Mutations in *avr-15*, present in this strain, confer a high resistance to the inhibitory effects of ivermectin on pharyngeal pumping [30]. In this functional assay fluensulfone caused the same level of inhibition in both wild-type and ivermectin resistant worms (Fig. 9C). To reconcile this observation with the small but significant shift in the susceptibility of the ivermectin resistant strain to fluensulfone in the thrashing assay we suggest that the mutations in the ivermectin strain, which are in inhibitory glutamate-gated chloride

(46.0 ± 5.2 and 43.6 ± 13.2, respectively; *n* = 3 plates of 10 worms) exposure to ivermectin (Fig. 9A) but it was still susceptible to the actions of fluensulfone (Fig. 9B). However, there was a small, but significant reduction in the magnitude of the fluensulfone inhibition in the ivermectin resistant strain (one way ANOVA, Bonferroni’s multiple comparisons test). To further determine whether or not this ivermectin resistant strain showed any cross-resistance with fluensulfone we compared the effect of fluensulfone (1 mM for 1 h) on pharyngeal pumping in adult wild-type and the ivermectin resistant strain. Mutations in *avr-15*, present in this strain, confer a high resistance to the inhibitory effects of ivermectin on pharyngeal pumping [30]. In this functional assay fluensulfone caused the same level of inhibition in both wild-type and ivermectin resistant worms (Fig. 9C). To reconcile this observation with the small but significant shift in the susceptibility of the ivermectin resistant strain to fluensulfone in the thrashing assay we suggest that the mutations in the ivermectin strain, which are in inhibitory glutamate-gated chloride...
channels, reduce the intrinsic inhibitory tone in the motornervous system. Thus, in the ivermectin resistant strain, fluensulfone exerts its inhibitory action against a background of heightened excitability in the motor circuits making it slightly less effective at inducing an inhibition. It is well recognised that mutations in signalling pathways other than the molecular target can alter susceptibility to a nematicidal compound (see e.g. [43]).

Taken together these data indicate that the mode of action of fluensulfone is distinct from that of ivermectin.

4. Discussion

4.1. C. elegans as a model to study the mode of action of fluensulfone

Here we have shown that fluensulfone elicits a nematicidal action on C. elegans similar to its effects on PPNs [14]. The threshold effect on development is observed at 100 µM from exposure from egg onwards (equivalent to 29.2 ppm or 29.2 mg l⁻¹). The electrophysiological assays resolved discrete effects of fluensulfone at lower concentrations of compound i.e. 30 µM (8.76 ppm or 8.76 mg l⁻¹). The irreversible inhibitory effect of fluensulfone on C. elegans motility following exposure in liquid, and the observation that it persists after prolonged washing, is similar to the effect reported in M. javanica although in this species the threshold concentration for the inhibitory effect was as low as 0.5 mg l⁻¹ [14]. A higher dose of fluensulfone, 8 mg l⁻¹ reversibly inhibited M. javanica hatching [14] and fluensulfone also inhibits egg-hatching in C. elegans although higher doses are required. It will be interesting to test the susceptibility of other species of nematode to fluensulfone to investigate the extent to which the susceptibility to the effect of fluensulfone varies across the phylum. The decreased susceptibility of C. elegans compared to M. incognita could reflect altered pharmacokinetics, e.g. metabolism, or different sensitivities of the molecular target(s) to the compound. Interestingly,
C. elegans exposed to fluensulfone on agar were less susceptible than those exposed in liquid. In the latter the worms are deprived of food and it is possible this may contribute to an increased susceptibility to the lethal effects of the compound.

The qualitative similarity of the effects of fluensulfone on *M. incognita* and *C. elegans* prompted us to use *C. elegans* as a model to allow for the quantification and study of its effects on a number of nematode behaviours that are not easily assayable in sedentary endoparasitic PPNs, including feeding and reproduction and furthermore as a route to a genetic analysis of its mode of action. Using this approach we have shown that fluensulfone inhibits *C. elegans* egg-laying, larval development and feeding in addition to its effects on motility and egg hatching. One might predict therefore, that fluensulfone would have a similar range of actions against the PPNs.

4.2. Embryos and larval stages are most susceptible to fluensulfone

Whilst all developmental stages succumbed to prolonged 24 h exposure to fluensulfone (Table 1) an analysis at earlier time points has revealed that the mobility of L2/3 and L4 larvae is most readily impaired by fluensulfone. Notably, for L2/3, after just 6 h exposure, this paralytic effect is irreversible. Whilst L1 larvae were less susceptible to the paralytic effects of fluensulfone nonetheless L1 that had been hatched from egg on 1 mM fluensulfone failed to progress past this developmental stage. These assays reveal profound effects of fluensulfone on *C. elegans* development in which it induces complete L1 larval arrest in developing populations and exerts rapid paralysis, within 1 h, against L2/3 and L4. Furthermore, the effect of fluensulfone on pharyngeal pumping in L4 is also more marked than in adult. Preferential sensitivity of different nematode developmental stages has also been observed for other anthelmintics e.g. emodepside [44] and ivermectin [45]. These differences in susceptibility may be due to differences in access of the compound e.g. different cuticle permeability. Alternatively, it may be due to different levels of expression of the receptor or receptors through which the compound exerts its actions. The observations of the relatively rapid time-course of the effects of fluensulfone in adult *C. elegans*, albeit at higher concentrations than for L2/3 larvae,
suggests that the difference in susceptibility of the developmental stages of *C. elegans* is more likely to be due to differences in expression of receptors rather than in pharmacokinetic properties.

Intriguingly, exposure of gravid adult hermaphrodites to 1 mM fluensulfone had nematicidal effects on their embryos *in utero*. The exposure time to fluensulfone was just 1 h duration and a high proportion of the eggs that were subsequently laid failed to hatch. Inspection of the un-hatched eggs revealed that they had been fertilised, therefore the nematicidal effect *in utero* occurs post-fertilisation. This is a remarkable effect and is consistent with fluensulfone gaining rapid access to the internal structures of the worm which then elicit prolonged detrimental effects not only on the adult worms but also on their progeny. Such an effect in plant parasitic nematodes could conceivably contribute to the reported nematicidal effects of fluensulfone following systemic application [15]. Other systemic nematicides have been reported to have inhibitory effects on egg hatching for various species of PPN [46–48].

### 4.3. Fluensulfone gains rapid access to the worm

Further evidence that fluensulfone can rapidly access the internal organs of the worm is provided by comparing the data for the inhibition of feeding behaviour elicited by fluensulfone either in the intact organism or in cut head preparations. Similar inhibition was observed in both assays. Moreover, these effects were fully reversible. The time-course of the inhibitory effect of fluensulfone on pharyngeal pumping in L4 larvae, which was rapid and with complete inhibition after 1 h exposure, also suggests that the diffusional barriers for fluensulfone to access the internal tissues of the worm are minimal.

### 4.4. Fluensulfone may induce L1 larval arrest by inhibiting feeding

The observation that fluensulfone inhibits feeding may be related to the fact that it induces L1 larval arrest: There is a known link between feeding and arrested development. Thus, L1 can enter a diapause state when embryos hatch where food is scarce, during which they do not moult to the next larval stage until food becomes more abundant [49]. Whilst L1 diapause can continue for several days, mortality increases as the length of starvation increases. It is possible that larvae arrest as they cannot feed, even in the presence of abundant food. Fluensulfone could also inhibit development through interfering with the larval moulting process. Whilst other chemicals have been shown to induce larval and embryonic arrest, the mechanisms by which this arrest is induced...
are not known [49]. The inhibition of mitochondrial respiration by sodium azide, which inhibits cytochrome C in the electron transport chain, results in the arrest of the cell cycle and hence embryonic arrest in *C. elegans* [50]. Inhibition of mitochondrial function is one mechanism through which fluensulfone could inhibit egg hatching.

### 4.5. Transient excitatory effects of fluensulfone precede behavioural inhibition

In the whole organism assays measuring the effect of fluensulfone on feeding we noted that the inhibition of feeding that occurred within 1 h exposure was accompanied by a transient excitation; an increase in the frequency of body bends and an increased tendency of the worm to leave the food lawn, called ‘food leaving.’ A parsimonious explanation of this is that the inhibition of feeding prompts the worm to adopt the behavioural pattern it exhibits in the absence of food i.e. a faster frequency of movement called ‘roaming.’ This is based on a number of studies showing that *C. elegans* mutants with impaired feeding exhibit increased roaming behaviour [51].

The electrophysiological recordings of the effect of fluensulfone on the pharynx also revealed a transient excitatory effect which preceded the inhibition suggesting that perhaps this excitation is intrinsically linked to the longer latency inhibition. By comparison, the pyrethroid pesticides also cause an excitation [52]. However, unlike the pyrethroids the excitatory effect of fluensulfone is not sustained nor is it detected in the whole animal studies measuring feeding behaviour.

### 4.6. Fluensulfone does not act like organophosphates, carbamates or ivermectin

The profile of actions of fluensulfone in *C. elegans* is distinct from compounds that work through the cholinergic system, e.g. both levamisole and aldicarb. These agents contract body wall muscle and cause shortening and spastic paralysis [20]. Fluensulfone on the other hand does not cause hypercontraction. Further evidence that fluensulfone does not share a mode of action with cholinesterase inhibitors is provided by the observation that *unc-17*, an aldicarb resistant mutant [17], was sensitive to fluensulfone. Intriguingly, it appeared hypersensitive. This suggests that in this mutant decreased excitatory transmission has rendered the worm more sensitive to a compound which has an inhibitory action on the neuromuscular system.

The effects of fluensulfone share some similarities with ivermectin [30,45] however the data presented here indicate a distinct mode of action. Thus whilst ivermectin and fluensulfone are similar in that they both elicit paralysis after prolonged exposure, ivermectin does not elicit transient excitatory actions on either locomotion or feeding [30,53]. We have shown that a strain that is highly resistant to ivermectin (because it carries mutations in three of the genes that encode ivermectin receptors [39]) is still sensitive to fluensulfone. Together these results indicate that the glutamate-gated chloride channels which mediate the effect of ivermectin are not a site of action for fluensulfone.

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**Fig. 9.** A comparison of the effect of fluensulfone and ivermectin on *C. elegans*. The thrashing rate of strain DA1316 which is ivermectin resistant (r) was compared to wild-type (wt) in the presence of either vehicle (control), (A) ivermectin (ivm; 100 nM for 1 h) or (B) fluensulfone (F; 1 mM for 3 h). n = 10 worms, ‘***’ *p* < 0.001; one way ANOVA with Bonferroni post hoc test. ‘ns’, not significant. (C) The effect of fluensulfone on pharyngeal pumping in wild-type or ivermectin resistant worms after 1 h exposure, n = 10 worms; ‘***’ *p* < 0.001; one way ANOVA with Bonferroni post hoc test.
5. Conclusions

These studies have shown that fluensulfone has a broad range of effects that impact on nematode reproduction, development, feeding and motility and ultimately has nematicidal action. Whilst *Caenorhabditis elegans* is less susceptible to fluensulfone than *C. elegans* feeding and motility and ultimately has nematicidal action. Whilst *C. elegans* model hopping approach between concentrations. Precisely how fluensulfone exerts its effects, and importantly how it achieves its selective toxicity, remains to be determined, however on the basis of this study we suggest that a model hopping approach between *C. elegans* and PPNs may be informative in this regard.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pestbp.2014.01.004.

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