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Progressive metabolic impairment underlies the novel nematicidal action of fluensulfone on the potato cyst nematode *Globodera pallida*

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ABSTRACT

Background: Fluensulfone is a new nematicide with an excellent profile of selective toxicity against plant parasitic nematodes. Here, its effects on the physiology and biochemistry of the potato cyst nematode *Globodera pallida* have been investigated and comparisons made with its effect on the life-span of the free-living nematode *Caenorhabditis elegans* to provide insight into its mode of action and its selective toxicity.
Results: Fluensulfone exerts acute effects (≤1 h; ≥100 μM) on stylet thrusting and motility of hatched second stage *G. pallida* juveniles (J2s). Chronic exposure to lower concentrations of fluensulfone (≥3 days; ≤30 μM), reveals a slowly developing metabolic insult in which *G. pallida* J2s sequentially exhibit a reduction in motility, loss of a metabolic marker for cell viability, high lipid content and tissue degeneration prior to death. These effects are absent in adults and dauers of the model genetic nematode *Caenorhabditis elegans*.
Conclusion: The nematicidal action of fluensulfone follows a time-course which progresses from an early impact on motility through to an accumulating metabolic impairment, an inability to access lipid stores and death.

1. Introduction

Plant parasitic nematodes (PPNs) present a major burden on agriculture, with every crop plant grown vulnerable to infection by at least one nematode species [1]. Annual yield losses directly attributable to PPNs are estimated at $125 billion [2], resulting from a 12.3% yield reduction in the major human food staples [3]. There are few efficacious methods for controlling PPNs, with many chemical nematicides being banned due to their unacceptable non-target toxicity and environmental impact [4]. New, environmentally safe methods for control of PPNs are required.

Fluensulfone ((5-chloro-2-(3,4,4-trifluorobut-3-enylsulfonyl)-1,3-thiazole); Nimitz®) is a new nematicide belonging to the heterocyclic fluoroalkenyl sulfones. It is a contact nematicide that is used for protecting a range of crops from PPNs. The toxicity profile of fluensulfone is superior to anticholinesterase and fumigant nematicides [5] yet the mechanism of action of fluensulfone is currently unknown. Fluensulfone has direct nematicidal activity against a number of PPNs, including *Meloidogyne* spp. and is effective at low micromolar concentrations in vitro [6–10]. Fluensulfone exposure results in irreversible paralysis that is characterised by a rod-shaped body posture. The rod-shaped posture in the presence of fluensulfone is distinctive from that seen in the presence of organophosphate and carbamate nematicides, which cause a shrunken, "wavy" posture [8]. These observations are consistent with a novel mode of action for fluensulfone relative to other nematicides.

The non-parasitic model nematode *Caenorhabditis elegans* has also been utilised to investigate the actions of fluensulfone. This served to substantiate that its mechanism of action is distinct from anticholinesterase nematicides such as aldicarb and macrocyclic lactone anthelmintics, such as ivermectin [11]. Furthermore, these observations support the selective toxicity of fluensulfone towards PPNs as the species that have been investigated are typically 50-fold more sensitive to fluensulfone than *C. elegans* [6,8,9,11]. This suggests that fluensulfone may act differently in PPNs and *C. elegans*.

The potato cyst nematodes (PCN; *Globodera pallida*, *G. rostochiensis*) that are economically important crop pathogens in an estimated 47 countries [12] are also susceptible to fluensulfone [13]. The susceptibility of *G. pallida* to fluensulfone was used to probe the mechanism underpinning its potent nematicidal action in PPNs by a systematic analysis of the time-dependent effects of fluensulfone on second-stage juvenile (J2) behaviour, metabolism and survival. We observed distinct low concentration effects with chronic exposure to fluensulfone making *G. pallida* selectively vulnerable compared to *C. elegans*. We show that this susceptibility is imparted by a progressive metabolic impairment and a novel mode of action of fluensulfone. The observation that the selective efficacy of fluensulfone against *G. pallida* is revealed by a critical interplay between concentration and time of exposure presents a

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paradigm shift in the analysis of nematicide action. It is clear that excellent selective toxicity and crop protection may be achieved by chemicals that have a longer time-course of nematicidal action than previously considered.

2. Materials and methods

2.1. C. elegans culture and assays

2.1.1. C. elegans culture

N2 Bristol strain *C. elegans* were cultured on 50 mm Petri dishes containing NGM (nematode growth medium) seeded with bacteria, *E. coli* OP50, according to standard protocols [14]. Behavioural assays in liquid were conducted in M9 solution (KH_2PO_4 3 g, Na_2HPO_4 6 g, NaCl 5 g, 1 M $MgSO_4$ 1 ml, in 1 l distilled water).

2.1.2. Fluensulfone exposure

Fluensulfone was stored at 5 °C in the presence of desiccant silica granules. It was included in agar plates by first dissolving it in 100% acetone and then adding it to the NGM agar so that the final acetone concentration was 0.5% of the total NGM volume.

2.1.3. C. elegans lifespan assay

The effects of prolonged exposure of *C. elegans* to fluensulfone were investigated by utilising a standard, solid media life span assay [15], in which worms were exposed to different concentrations of fluensulfone for >20 days. L4 + 1 day old adult *C. elegans* were picked onto OP50-seeded NGM plates modified with different concentrations of fluensulfone. 50 worms were used for each concentration tested and for the control. To prevent starvation, surviving worms were picked at intervals onto new seeded NGM plates with the same concentration of drug every 2 days. Worms were prodded and those that failed to move or show pharyngeal pumping were deemed dead and were removed from the experiment. Bagged worms (in which larvae hatch inside the gravid adult) were not scored as dead and were censored from the experiment. The number of bagged worms was recorded.

2.1.4. C. elegans dauer survival assay

To generate *C. elegans* dauers, NGM plates of mixed stage N2 worms that had recently exhausted their OP50 *E. coli* food source were placed at 28 °C for 7 days. Subsequently, around 20 dauers were picked into Petri dishes containing control and fluensulfone solutions made in M9 buffer with 0.01% bovine serum albumin (BSA). There were 5 dishes for each treatment. Mortality was scored up to 20 days. To assess mortality, dauers were prodded and those that failed to move were scored as dead and were removed from the dish.

2.2. G. pallida culture and assays

2.2.1. G. pallida culture and hatching of J2s

G. pallida were cultured on potato plants (*Solanum tuberosum* "Desiree") according to published protocols [16]. To induce J2 hatching, cysts were placed in a solution of 1 part potato root leachate (PRL) to 3 parts ddH_2O at ~20 °C in the dark. Potato root leachate was prepared by soaking the washed roots of 3 week old potato plants for 24 h in tap water (80 g/l) followed by sterilisation through a 0.45 um filter. Only J2s that had hatched within the previous 24 h were selected for experiments.

2.2.2. Stylet thrusting assays with G. pallida

Stylet thrusting assays were conducted in 20 mM HEPES buffer made up with ddH_2O (0.01% BSA), with pH adjusted to 7.4 with NaOH. J2s were pipetted into test solutions in a small volume chamber, typically a 30 mm Petri dish. The number of stylet thrusts per minute was counted at various time points in the presence of 5-HT (5-hydroxytryptamine or serotonin), fluensulfone or mixtures at the concentrations indicated. A single movement of the stylet knob forwards and then backwards to its original position was counted as one stylet thrust. Control assays were conducted in the presence of 20 mM HEPES with 0.5% acetone, as indicated in figure captions. All assays were conducted at room temperature (approximately 20–22 °C). All drug solutions were made on the day of use.

2.2.3. G. pallida coiled posture assays

A coiled worm was defined as any worm that was bent in such a way that either its head or tail was in contact with another part of its body, with this posture consistently maintained for the 10 s observation period. ~10 J2 *G. pallida* were soaked in either fluensulfone or vehicle (acetone) control and were scored for percentage coiling at the given time.

2.2.4. G. pallida motility and granular appearance assays

Assays were performed to examine the effects of prolonged exposure to fluensulfone on the motility and gross appearance of *G. pallida* and its internal tissue. J2s were washed with ddH_2O and transferred to 30 mm Petri dishes containing either fluensulfone or acetone (in 0.01% BSA). When scoring motility, J2s that were rod shaped and failed to move during a 10 s observation were defined as immotile.

2.2.5. Tissue integrity

It was noted that during prolonged exposure to fluensulfone the J2s became darker and looked "granular". This was accompanied by a progressive loss of structure in the internal tissue organization for the oesophageal-stylet system and gastrointestinal tract. The proportion of worms that appeared "granular" was scored.

2.2.6. Metabolic viability assay; MTT assay

MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide) is a tetrazolium salt that undergoes a reduction reaction in metabolically active cells and changes from a weak yellow colour to a dark purple colour as it is converted into an insoluble formazan product (see [17]for review). This compound has been used extensively to assay metabolic and proliferative activity in cell cultures [18–20] and has been adapted for use in *C. elegans* to measure worm death [21,22]. We have adapted this method to assay mortality and metabolic activity in *G. pallida*. J2 *G. pallida* were washed in ddH_2O and transferred to compound solutions made in ddH_2O (0.01% BSA), in which they were soaked for up to 14 days. Throughout this time, ~10 J2s were removed from the drug solutions, washed in ddH_2O and then placed in a 24 well plate in 5 mg/ml MTT solution made in ddH_2O. The well plates were gently rotated in the dark for 24 h. After 24 h in the MTT solution, each well was observed using a dissecting microscope at ×45 magnification (Brunel BMSZ stereomicroscope) and worms were scored for the presence or absence of staining. The J2s soaked in the control solution typically showed dark purple staining at their anterior end.

2.2.7. Lipid content of G. pallida; Nile red with isopropanol fixation

This protocol was modified from methods used to quantify lipid stores in *C. elegans* [23]. J2s were washed in ddH_2O and transferred to compound solutions. They were then soaked in the compound solutions for up to 10 days under slow constant rotation. At 5 and 10 days, J2s were removed and washed 3 times in M9 buffer. The worms were then pelleted by centrifugation and 200 μl M9 with 0.01% Triton X-100 was added. The worms were again pelleted by centrifugation and 200 μl of M9 with 40% isopropanol was added. The worms were incubated in 40% isopropanol for 3 min with constant agitation and then pelleted before the supernatant was removed. 200 μl Nile red solution (6 μl 0.5 mg/ml Nile red in acetone) per 1 ml M9 with 40% isopropanol was added to the pellet and the worms incubated in the dark for 2 h with constant rotation. The worms were washed in M9 with 0.01% Triton X-100 three times and left in 200 μl M9 with 0.01% Triton X-100 in the dark for 30 min. The worms were subsequently washed in M9 with 0.01% Triton X-100 and mounted onto 2% agarose pads for imaging.

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Images were taken using a Zeiss Axioplan 2 microscope under a FITC filter block. For each time point, images were taken using the same exposure time (500 ms) and with the same light intensity. ImageJ software was used to quantify fluorescence intensity in the whole worm. The image (8-bit) was processed by subtracting the background to correct for any unevenness in the background light intensity. The threshold level of each image was then adjusted to distinguish between the worm and the background. The threshold of each image was adjusted to the same level. The worm was then selected using the wand tool and the integrated density value was measured. The data were normalised as a percentage of the mean integrated density value of the control group where the control group were freshly hatched (<24 h) J2s processed and imaged in parallel.

For initial analyses we quantified differences between freshly hatched worms and worms after an extended time post-hatching ('starved'). As J2s are a non-feeding stage, worms deplete their lipid stores after hatching [24,25]. Therefore, one group of J2s was washed in ddH_2O and left for 14 days to deplete lipid stores. The other group were freshly hatched within 24 h from the same cysts and used as a sample with lipid stores that were replete. The technique was used to map the stainable lipid content of *G. pallida* J2s treated with fluensulfone. J2s were soaked in ddH_2O in the presence of vehicle (0.5% acetone), 10 μM fluensulfone, 30 μM fluensulfone, or 30 μM aldicarb. Worms were removed at 5 and 10 days to stain for lipids. (Worms treated with 30 μM fluensulfone were not stained at 10 days due to the high mortality that occurs with this treatment). For each experimental group the lipid content is expressed as a percentage of control, where control is the group exposed to vehicle at the same time-point and processed and imaged in parallel with the experimental groups.

2.3. Statistical analysis

Data are shown as the mean ± S.E.M, except where stated. Student's *t*-test, one-way ANOVA and two-way ANOVA were used where appropriate and were followed by the stated post hoc tests (with significance level set at $P < 0.05$). All statistics were carried out using GraphPad Prism software. The number of individual worms and experimental repeats used to perform the statistical analysis for each experiment is stated in the figure legends, as is the specific statistical test employed.

2.4. Materials

Chemicals and drugs were obtained from standard suppliers unless stated otherwise. Fluensulfone (technical grade) was supplied by ADAMA Agricultural Solutions Ltd. Fluensulfone is shown in units of micromolar (μM) where 1 μM is equivalent to 0.29 ppm or 0.29 mg l^{-1}. 5-HT (serotonin creatinine sulphate), MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide), aldicarb and Nile red were obtained from Sigma Aldrich.

3. Results

3.1. High concentrations of fluensulfone have acute effects on G. pallida behaviours

We have previously investigated the effect of 300 μM fluensulfone and above (≥87.6 ppm) on *C. elegans* behaviour and have shown that it impairs locomotion and feeding [11]. The acute effects of fluensulfone on *G. pallida* behaviour were investigated to allow a comparison with *C. elegans*.

J2 *G. pallida* exposed to a vehicle control solution in liquid moved in an uncoordinated and irregular manner, rarely adopting any consistent posture. At any time up to 5 h, only around 10–15% of J2s were observed in a coiled posture. In contrast, within 30 min of exposure to 500 μM fluensulfone (146 ppm), 60–70% of J2s adopted a coiled posture and the proportion of worms exhibiting this was maintained for up to 5 h (Fig. 1) however no coiling was observed at 24 h (n = 5 wells of 10 worms per well). J2s adopting the coiled posture also moved slowly relative to J2s in the control solution. This effect was concentration-dependent as a lower proportion of J2s exhibited coiling in the presence of 10 and 100 μM fluensulfone (Fig. 1).

3.2. Fluensulfone impairs stylet activity of G. pallida

The majority of PPNs possess a specialized mouth spear known as the stylet, which is crucial in the parasitic life cycle, as it is required for egg hatching, host invasion and feeding. Given the importance of stylet activity in plant parasitism, the effects of fluensulfone on stylet behaviour were investigated.

J2s were exposed to fluensulfone and control solutions for up to 14 days. Stylet thrusting was counted in the presence of fluensulfone. Little or no stylet activity was observed in worms treated with vehicle alone throughout the course of the experiment. 30 μM fluensulfone did not stimulate stylet activity, whilst ≥200 μM fluensulfone showed a modest but consistent increase in activity to 7 stylet thrusts per minute at 5 h exposure (Fig. 2A). However, the stimulatory effect of concentrations of 200 and 500 μM fluensulfone was transient and no significant stimulation of stylet thrusting occurred at 24 h. Interestingly, the time-course of the increase in stylet activity paralleled that for the appearance of coiled posture suggesting the two behaviours may be linked to a common upstream response.

5-HT has been found to vigorously stimulate stylet behaviour in a number of other PPN species, including *Meloidogyne spp.* [26,27]. To assess the effects of fluensulfone on 5-HT activated stylet thrusting, the J2s were pre-incubated with either vehicle or fluensulfone and then moved to 10 mM 5-HT, in the continued presence of fluensulfone or vehicle, and stylet thrusting was scored after 10 min incubation in 5-HT. In other words, the J2s were exposed to fluensulfone for increasing times post-hatching and then their stylet response to a short 10 min application of 5-HT was scored.

Worms in the control group showed a robust increase in stylet thrusting after being removed from vehicle control and treated with 10 mM 5-HT for up to 14 days post-hatching (Fig. 2B) indicating that the age of the J2s in terms of time after hatching does not in of itself impair their ability to response to 5-HT for up to 14 days. In contrast, 500 μM fluensulfone significantly reduced 5-HT-induced stylet activity relative to the vehicle control after 1 and 5 hour exposure, with a 64%

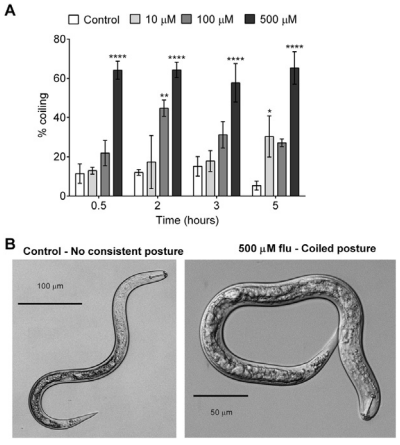

Fig. 1. Acute exposure to fluensulfone induces a coiled posture in *G. pallida*. A) 10 juveniles were placed in ddH_2O containing fluensulfone and vehicle and visually scored over 5 h for percentage coiling. A worm's posture was defined as coiled if either the anterior or posterior of the worm was looped back over its body and this posture was maintained for the 10 second observation period. (n = 5 plates per treatment, mean ± s.e mean, two-way ANOVA with Dunnett post hoc tests, $P < 0.0001$). B) Representative images of juveniles after 15 min in the presence of vehicle (left) and 500 μM fluensulfone (right).

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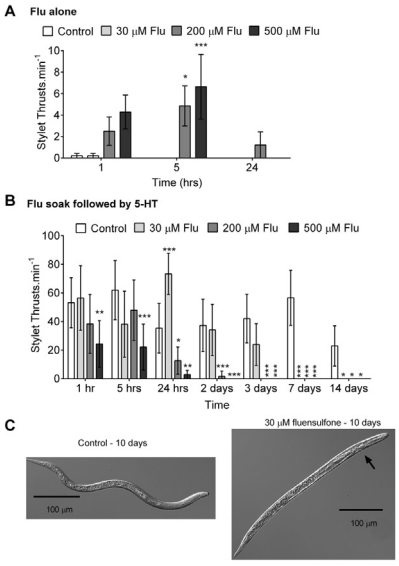

Fig. 2. The effects of fluensulfone on *G. pallida* stylet activity. A) J2 *G. pallida* were soaked for up to 14 days in fluensulfone (flu) or ddH_2O (control) and stylet activity was scored during this period (n = 14 worms, mean ± s.e mean, data pooled from two separate experiments, two-way ANOVA with Bonferroni post hoc tests, $P < 0.001$). B) At each time point, J2s were transferred to 10 mM 5-HT and stylet activity was scored after 10 min. After ≥3 days 5-HT-induced stylet thrusting was reduced at all concentrations, including 30 μM, most likely due to nematicidal effects. (n = 16, mean ± s.e mean shown, data pooled from two separate experiments, two-way ANOVA with Sidak post hoc tests, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). C) Representative images of worms soaked for 10 days in vehicle and 30 μM fluensulfone.

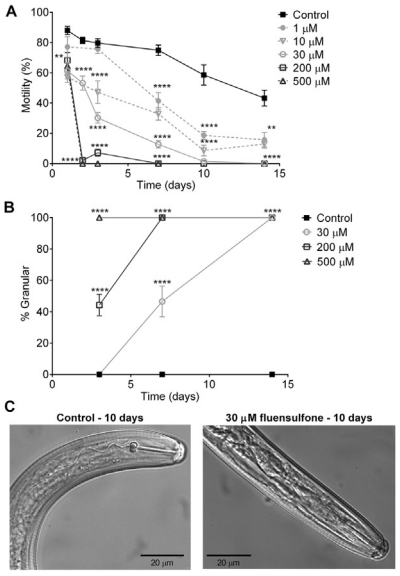

Fig. 3. Prolonged exposure of J2 *G. pallida* to fluensulfone induces a progressive increase in paralysis, which is followed by death. A) J2s were exposed to fluensulfone in ddH_2O in 2 separate experiments and scored for motility up to 14 days. J2s that were rod-shaped and failed to move were deemed immotile. Worms from different groups of cysts were soaked in 7 dishes with around 10 worms per dish (mean % motility ± s.e mean, two-way ANOVA with Bonferroni post hoc tests, $P < 0.0001$). B + C) Immotility in the presence of ≥30 μM fluensulfone was followed by a darkening of appearance and the apparent loss of integrity and structure of internal organs, with worms appearing "granular". B) No J2s in the control treatment group appeared granular after 14 days whereas there was a progressive increase in the number of J2s that were granular in appearance in the presence of ≥30 μM fluensulfone. Granular appearance was quantified by visual scoring in the first (A) experiment (mean ± s.e mean from 14 dishes each with ~10 worms, data pooled from two separate experiments, two-way ANOVA with Bonferroni post hoc tests $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). C) Representative images of J2s treated with a control solution (left) and 30 μM fluensulfone (right) for 10 days. Note the darkened appearance and distortion of internal structures.

reduction in the rate of stylet activity when compared to control-treated worms. Both 200 and 500 μM fluensulfone reduced stylet activity in the presence of 5-HT relative to the control at 24 hour exposure and at all other time points up to 14 days. No 5-HT-induced stylet activity was observed after 2 days in 500 μM fluensulfone and 3 days in 200 μM. It was of interest to note that 5-HT-induced stylet thrusting was enhanced by 108% relative to the vehicle control after 24 hour soaking in 30 μM fluensulfone (Fig. 2B) but this effect was not sustained and at later time-points 5-HT stimulated stylet thrusting was inhibited by fluensulfone (Fig. 2B). This inhibitory action of fluensulfone on 5-HT-induced stylet thrusting followed a similar time-course to the appearance of a rod-shaped posture in the J2s (Fig. 2C). Previous work has described that *M. javanica* exposed to fluensulfone for 24–48 h also adopt a rod-shaped posture when paralysed [8].

3.3. Fluensulfone progressively immobilises G. pallida J2s

Motility gradually decreased in worms exposed to vehicle alone, with 88% of worms motile after 1 day, falling to 43% motility at 14 days (Fig. 3A). Across a wide range of concentrations including the high doses that cause acute motility/coiling effects there was a significant time- and concentration-dependent reduction in motility. Exposure to 500 μM fluensulfone for 24 h elicited a 24% decrease in percentage motility relative to the control, whilst 24 hour exposure to 200 μM induced a 20% reduction in motility. All worms exposed to 500 μM fluensulfone were immotile after 2 days, whilst 200 μM fluensulfone induced complete immotility at 2–7 days. Exposure to 30 μM fluensulfone also resulted in a 27% decrease in the number of motile J2s relative to the vehicle control at 24 h. Whilst the onset of complete paralysis was relatively rapid in the presence of 200 and 500 μM fluensulfone, 30 μM elicited a slower progressive increase in percentage immotility, with immotility in all worms requiring 10–14 days exposure in both experiments. Significant reductions in motility of 40% and 50% occurred in the presence of 1 and 10 μM fluensulfone respectively at 10 days exposure.

3.4. Fluensulfone compromises the internal integrity of G. pallida

After 2 days exposure to 200 and 500 μM fluensulfone we noted that some immotile worms began to take on a granular appearance of their internal structures and tissues. This coincided with the internal organs, the oesophagus and stylet, becoming indistinct and the overall internal structure becoming darkened. Granular worms did not move, were rod-shaped and did not respond when prodded. Worms exhibiting this deteriorated appearance increased with time, and exhibited progression to a more complete disintegration of internal organs (Fig. 2C). We conclude that this granular appearance provides a visual indication of tissue necrosis and worm mortality.

J2s were scored for indistinguishable internal organs with a granular appearance, an indicator of tissue degeneration and a direct read-out of death (Fig. 3B, C). The disintegration of internal structures was most apparent at the anterior of the worm, where the structures of the pharynx and the stylet disappeared and tissues became darker (Fig. 3C). All J2s were granular at 3 days in the 500 μM treatment group. At 3 days, around 40% of worms treated with 200 μM fluensulfone were granular in appearance and 100% were granular at 7 days exposure. No worms appeared granular at 3 days exposure to 30 μM though this increased with time, with around 40% of worms granular at 7 days and 100% at 14 days. Control-treated worms did not exhibit any deterioration of internal structures, even after 21 days (data not shown).

The results from these experiments suggest that the effects of fluensulfone on motility and morphology are concentration-dependent and that the time course of these effects varies with concentration. Whilst ≥200 μM fluensulfone has acute behavioural effects on J2 *G. pallida*, culminating in paralysis and death at 2–3 days on the other hand, concentrations of 30 μM induce a slower

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progressive increase in paralysis, reaching full paralysis and death at 10–14 days exposure.

3.5. Fluensulfone does not affect life-span of C. elegans

The systematic analysis of fluensulfone across dose and time highlighted the progressive nature of its nematicidal activity. Previous observation regarding the potential selective toxicity for PPNs over the model nematode *C. elegans* had restricted investigation to shorter times of exposure due to the short life-cycle of *C. elegans* (egg to adult in 3 days). Therefore, we tested the effect of fluensulfone on *C. elegans* in a paradigm that allows exposure for a longer period of time using a life-span assay [15]. In the presence of vehicle alone, significant death began after 11 days and all worms were dead after 20 days consistent with previous studies [15] (Supplementary Fig. 1A). The highest concentration of fluensulfone tested, 500 μM, shortened life-span with worms dying after 3 days consistent with the acute toxicity we observed for *G. pallida*. In contrast, 10 and 100 μM fluensulfone, doses that accumulate a clear nematicidal effect in PPNs, did not affect lifespan of *C. elegans*.

The infective J2 stage of sedentary endoparasitic PPNs has been compared to the non-feeding *C. elegans* dauer stage [28] and therefore we tested whether or not this life-stage of *C. elegans* might be more similar to PPNs in terms of fluensulfone susceptibility. Therefore, dauers were soaked in fluensulfone and lifespan and mortality were scored (Supplementary Fig. 1B). In the presence of vehicle alone, very few dauers died throughout the course of the experiment, with 85% still alive after 20 days. The survival curves of dauers in the presence of 10, 30 and 100 μM fluensulfone did not differ from the vehicle control, with few worms dying over 20 days. Only 500 μM caused a reduction in survival, with a large number of deaths occurring at 10 days and 100% mortality at 14 days. These observations show that *C. elegans* is less sensitive to fluensulfone than *G. pallida* and other PPNs, indicating selective nematicidal activity against PPNs.

3.6. Fluensulfone alters G. pallida metabolism prior to its nematicidal effects

To better understand the process leading to death, the tetrazolium dye MTT was utilised [21]. MTT detects cellular levels of NAD(P)H and can be used to determine cell metabolic viability. Typically, the majority of untreated J2s soaked in MTT for 24 h were strongly stained purple in the head region, with fewer staining at the posterior (Fig. 4A). Worms were soaked in a concentration range from 1 to 500 μM fluensulfone, with the carbamate aldicarb as a comparison (Fig. 4B). Aldicarb was used to allow comparison with a previously used chemical control for PPNs that acts as a nematostatic and is not nematicidal [4]. Throughout the experiment, nearly 100% of control treated worms exhibited clear MTT staining, with >95% of worms generating robust staining even after 14 days post-hatching. In contrast, exposure to the highest concentration of fluensulfone tested, 500 μM for 24 h, resulted in a substantial reduction in the percentage of worms staining, with only 35% of worms showing any staining as compared to the 99% that stained in the control treatment group (Fig. 4B). This suggests that metabolic impairment occurs in tandem with the decrease in motility. After 3 days exposure to 500 μM fluensulfone, none of the J2s stained, indicating full metabolic impairment and death, again consistent with the distinct acute effect on worm viability that is non-selectively seen in *C. elegans* and PPN. 200 μM fluensulfone had a similar effect on MTT staining to 500 μM, with some reduction in the number of worms staining after 1 day exposure in one experiment and almost no observable staining after 3 days.

The effect of lower concentrations of fluensulfone on MTT staining followed a slower time-course. Exposure to 30 μM fluensulfone had no effect on the percentage of worms with observable MTT staining after 1 or 2 days exposure in both experiments (Fig. 4B). At 3 days, 27% of juveniles treated with 30 μM had no visible staining and with longer exposure there was a progressive decrease in the percentage of worms with

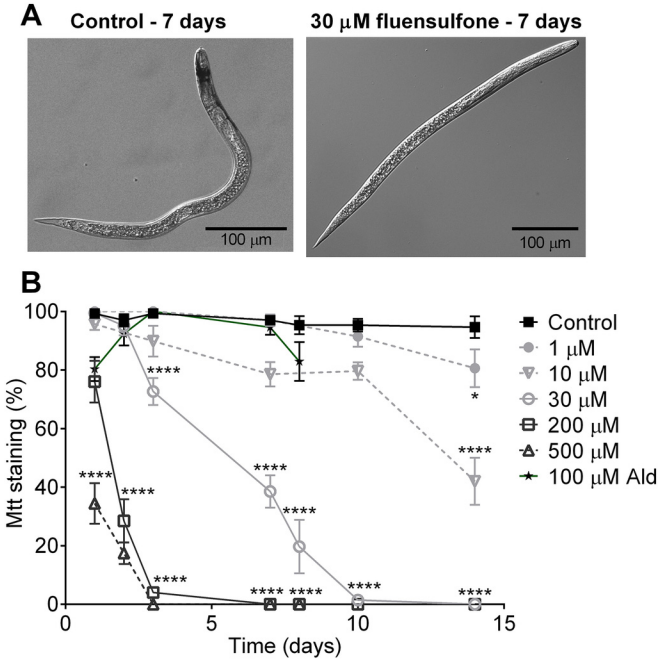

Fig. 4. Prolonged exposure to fluensulfone causes a progressive reduction in the number of *G. pallida* that stain in the presence of MTT. A + B) J2 *G. pallida* were soaked in fluensulfone or aldicarb in ddH_2O for up to 14 days and were subsequently moved, washed and transferred to wells containing MTT solution. After staining, worms were visually scored for staining. A) Representative images of J2s treated with control (left) and 30 μM fluensulfone solutions for 7 days stained with MTT. B) The control treated worms persistently stained, predominantly around the anterior region up to 14 days, whereas there was a progressive decrease in the number of worms that stained in the presence of fluensulfone (Worms from different batches of cysts were soaked in 14 dishes and around 10 worms were removed for staining at each time point, data pooled from two separate experiments, mean ± s.e mean, two-way ANOVA with Bonferroni post hoc test, ****$P < 0.0001$). Ald, aldicarb.

MTT staining (Fig. 4A, B). After 10 days exposure to 30 μM, staining was almost completely absent and no staining was observed at 14 days. This reduction in the percentage of total worms showing MTT staining closely maps onto the decrease in motility that occurs in the presence of fluensulfone (Fig. 3A).

A smaller reduction in the percentage of worms showing clear MTT staining was also observed in worms treated with 10 μM fluensulfone, at 7 and 10 days, with a 19% and 15% reduction in staining relative to the control, respectively. There was a sharp drop in percentage staining between 10 and 14 days, with only 42% of worms treated with 10 μM staining at 14 days compared to 95% of control-treated worms. This suggests that longer exposure to 10 μM fluensulfone converges on a similar metabolic endpoint to 30 μM, with complete immotility and death. A significant, 14% reduction in percentage staining also occurred after 14 days exposure to 1 μM fluensulfone, relative to the vehicle control.

J2s were also exposed to 100 μM aldicarb as a comparison (Fig. 4B). Overall, no reduction in MTT staining occurred as a result of exposure to aldicarb and percentage staining did not differ from the control at 8 days. This confirms that the carbamate aldicarb is nematostatic rather than nematicidal and reinforces a novel, irreversible nematicidal effect of fluensulfone on PPNs relative to other carbamate treatments [8].

3.7. Fluensulfone treatment induces a spatial shift in the pattern of MTT staining in G. pallida J2s

In addition to the MTT reporting on viability we noted that fluensulfone treatment also caused a shift in the distribution of staining as the drug executed the pathways that led to death (Fig. 5). To quantify this unexpected qualitative observation we scored the pattern of staining in individual worms. Batches of worms were processed for MTT

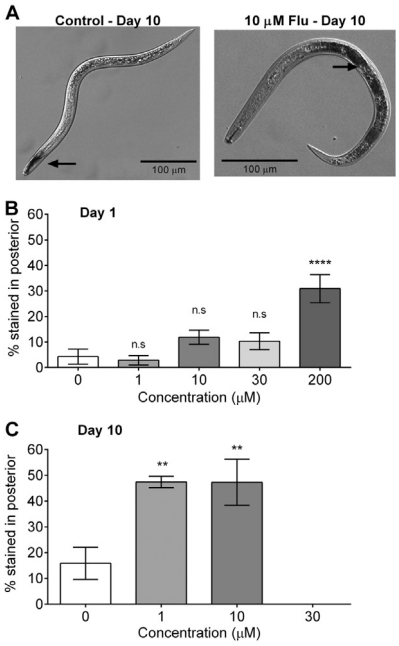

Fig. 5. Fluensulfone induces a shift in the distribution of MTT staining from the anterior to the posterior. A) Representative images of MTT stained worms after 10 days treatment. Note the head staining in the control-treated J2 (left) and the posterior staining in the 10 μM fluensulfone-treated J2 (right) (as indicated by arrows). B + C) After 1 day exposure, J2s in the control, 1, 10 and 30 μM treatment groups stain little in the posterior. A higher proportion of J2s treated with 200 μM fluensulfone stain in the posterior/tail region relative to the control. After 10 days exposure, control-treated J2s continue to stain mainly in the head region, whilst a greater proportion of J2s treated with 1 and 10 μM fluensulfone stain in the posterior/tail ($n = 7$ groups of worms from 1 experiment, mean ± s.e mean, one-way ANOVA with Dunnett post hoc tests, $^{**}P < 0.01$; $^{****}P < 0.0001$).

staining with and without fluensulfone treatment. J2s in the vehicle control solution had staining restricted to the head region with little staining in the posterior or tail throughout the time-course. For the purpose of this analysis posterior is defined as the region 50% from the midpoint of the worm to the tip of the tail. 91% of worms showing strong anterior staining after 1 day in the control solution and 80% staining in the head after 10 days (Fig. 5A). Treatment with 1, 10 and 30 μM fluensulfone for 1 day had no effect on the distribution of staining, with the majority of worms staining in the head region rather than the posterior/tail, that is similar to worms treated with vehicle alone (Fig. 5B). Exposure to 200 μM fluensulfone for 1 day reduced the overall percentage of worms that stained to 55%: Of the remaining worms that did show staining 56% were exclusively stained in the posterior. Treatment with 1 and 10 μM fluensulfone for 10 days also resulted in a shift in staining from the head to the posterior/tail (Fig. 5C). Of the worms that did stain after treatment with 1 and 10 μM fluensulfone, 52% and 60% stained in the posterior/tail, respectively, as compared to 17% in the control treatment group. These results indicate a general trend of MTT staining shifting from the anterior of the worm to the posterior after treatment with fluensulfone. Furthermore this redistribution precedes the overall loss of staining that is a consequence of prolonged fluensulfone treatment and associated with death.

3.8. Nile red staining indicates that fluensulfone decreases G. pallida J2 lipid consumption

The reduction of MTT staining suggests that fluensulfone affects *G. pallida* metabolism and the shift in the distribution of staining may reflect shifts in regional metabolic activity. To investigate the metabolic status of J2s, a modified Nile red lipid staining technique was utilised as a means of determining lipid content and how this may be affected by fluensulfone treatment. This was rationalized on the basis that lipid reserves are the major metabolic resource available to the free living J2 stage. The ability of the method to detect predicted changes in lipid content was validated by conducting the analysis on freshly hatched J2 *G. pallida* compared to 'starved' worms that were 14 days post-hatch (Supplementary Fig. 2). The intensity of Nile red fluorescence was reduced by >60% in starved worms relative to freshly hatched worms (Supplementary Fig. 2A, B) consistent with the ability to detect changes in the internal lipid content of *G. pallida*.

Worms treated with vehicle, 30 μM fluensulfone and 100 μM aldicarb were subjected to Nile red staining after 5 days. Worms treated with vehicle, 10 μM fluensulfone and 100 μM aldicarb were also stained at 10 days. (Worms treated with 30 μM fluensulfone were not stained at 10 days due to the high mortality that occurs with this treatment). Cholinesterase inhibitors, such as oxamyl, have been found to reduce lipid consumption in PPNs by reducing movement and thus conserving energy and so aldicarb provided a useful comparison for fluensulfone [29].

Treatment with 30 μM fluensulfone for 5 days had no effect on *G. pallida* lipid content relative to the vehicle control (Fig. 6A). Treatment with 10 μM fluensulfone for 10 days however, resulted in 50% higher fluorescence relative to the control (Fig. 6B), suggesting that lipid consumption is reduced in the presence of fluensulfone. Aldicarb had no effect on *G. pallida* lipid content relative to the control, contrary to reports for other cholinesterase inhibitors [29].

4. Discussion

This study confirms in vitro evidence for efficacy of fluensulfone against the PCN *G. pallida* and the investigations across different concentrations and times of exposure of freshly hatched J2s to fluensulfone reveal high and low concentration effects on *G. pallida* behaviour and physiology that have not been previously reported.

4.1. Fluensulfone has rapid high concentration effects on G. pallida J2 behaviour, similar to effects on C. elegans

The high concentrations of fluensulfone (≥200 μM) that exerted a rapid acute effect on *G. pallida* encompassing coiling and stylet behaviour, are in a similar range to the concentrations that elicited effects on *C. elegans* behaviour [11,30]. These effects may result from interference with neural signalling pathways, given their rapidity and the strong neural regulation of the locomotion and feeding behaviours that are affected. A possibility is that these high concentration effects represent a form of stress response. For example, *C. elegans* stress responses are known to involve increased 5-HT signalling [31] which could account for the transient stimulation of pharyngeal pumping and stylet thrusting elicited by fluensulfone.

4.2. Fluensulfone has low concentration effects on G. pallida J2 behaviour, not seen in C. elegans

Chronic exposure of freshly hatched J2s to fluensulfone at low concentrations (≤30 μM) impacted on their viability by a mechanism which progressed from a reduction in motility and metabolic activity (as indicated by MTT staining) followed by the appearance of tissue necrosis and death. During this time-course lipid levels were preserved relative to controls.

These observations indicate that low concentrations of fluensulfone progressively impair *G. pallida* metabolic capability. To our knowledge, this mechanism of action is unique amongst nematicides and anthelmintics. The organophosphates and carbamates both act via disruption of neural signalling pathways, as do the majority of anthelmintics, such as the macrocyclic lactones [32]. These compounds acutely affect motility by impairment of neural pathways that allow movement. Fumigant nematicides are thought to act via the rapid block of integral metabolic activity, resulting in nematode death [33]. Fluopyram [34] acts through a similar mechanism by inhibition of succinate

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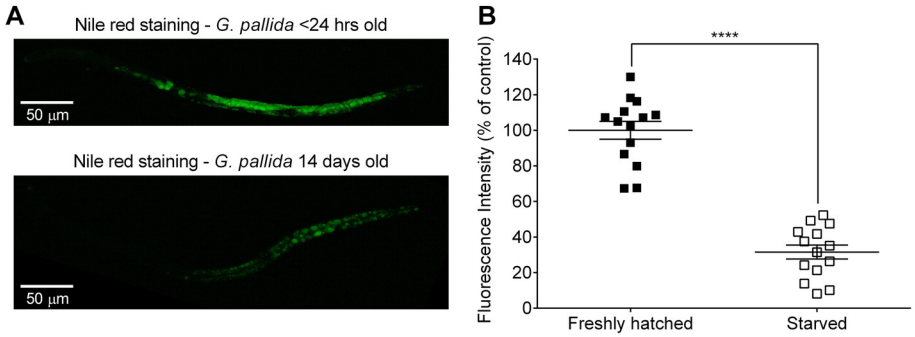

Fig. 6. Prolonged treatment with fluensulfone results in elevated lipid levels. J2 *G. pallida* were soaked in either ddH_2O (control), 10 µM fluensulfone (flu), 30 µM fluensulfone or 30 µM aldicarb (aldi) for up to 10 days. At 5 days (A) and 10 days (B), worms were removed from the drug solutions, washed, fixed and then stained with Nile red to assess lipid content. At 10 days, worms soaked in 10 µM fluensulfone had elevated lipid levels relative to the control group and worms treated with aldicarb ($n = 12–18$ worms, data were normalised as a % of the control, individual data points and mean ± s.e mean shown, one-way ANOVA with Dunnett post hoc tests, $P < 0.0001$). C) Representative images of control (left) and 10 µM fluensulfone-treated J2s (right) at 10 days stained with Nile red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dehydrogenase [35] a key component of the tricarboxylic acid cycle (TCA) and oxidative phosphorylation metabolic pathways [36]. These compounds elicit a maximal effect relatively rapidly, whereas the effect of fluensulfone accumulates over time to achieve efficacious PPN death.

Intriguingly the low concentration time-dependent effect of fluensulfone, equivalent to 0.3 to 10 ppm, is highly selective compared to *C. elegans*. Chronic exposure to the same concentrations of fluensulfone that inhibit *G. pallida* motility and are progressively nematicidal was found to have no effect on *C. elegans* adults or dauers. In contrast, nematicides and anthelmintics that act via neural disruption are typically toxic towards both PPNs and *C. elegans* [32] and the metabolic poison fluopyram is also rapidly toxic towards *C. elegans* [30].

4.3. Fluensulfone imparts an accumulating metabolic insult to G. pallida

The exact mechanism of fluensulfone metabolic toxicity in *G. pallida* is not clear but interesting observations here provide some indications. The chronic nature of fluensulfone effects on *G. pallida* motility and MTT staining suggests that it is unlikely to directly inhibit oxidative phosphorylation. Oxidative phosphorylation is the metabolic pathway that is required for synthesis of ATP, the key molecule for cellular energy transfer [36]. As such, inhibition of oxidative phosphorylation would rapidly inhibit metabolism, behaviour and result in rapid death, as is the case with fluopyram and sodium azide [30]. The large difference in sensitivity of *G. pallida* and *C. elegans* suggests that fluensulfone acts on aspects of metabolism that are essential to PPN survival but not the non-parasitic *C. elegans*.

The progressive nature of fluensulfone action in *G. pallida* suggests a slowly building insult that would be predicted to impair infectivity and viability across a time-course that culminates in death within 10 days of hatching. The reduced consumption of *G. pallida* lipid stores in the presence of fluensulfone relative to both control and aldicarb treated worms suggests that fluensulfone may affect the ability of PPNs to access their lipid reserves. This could be due to interference with β-oxidation, the process by which fatty acids are broken down to generate acetyl-CoA and NADH, which are then used in the TCA cycle and oxidative phosphorylation, respectively [36]. Fluensulfone could also impair lipolysis, which is required for the generation of fatty acids from triglyceride lipid stores [37]. The observation that fluensulfone induces a shift in MTT staining from the head to the posterior of *G. pallida* J2s may indicate a shift in metabolic activity to the posterior/tail, or alterations to metabolism occurring in the posterior/tail. This could be consistent with altered lipid metabolism, as PPN lipid stores are found predominantly in the posterior/tail, as verified by Nile red staining.

Finally, it is worth noting that the efficacy of fluensulfone varies between nematode species with the most susceptible being *Meloidogyne* spp. [8]and the least susceptible nematode tested to date being *Caenorhabditis elegans* [11]. Migratory plant parasitic nematodes *Bursaphelenchus xylophilus*, *Aphelenchoides besseyi*, *Aphelenchoides fragariae*, *Ditylenchus dipsaci*, *Pratylenchus penetrans*, *Pratylenchus thornei* and *Xiphinema index* show an intermediate susceptibility [6]. In this study we provide evidence that the susceptibility of the potato cyst nematode *G. pallida* would appear to be greater than the migratory nematodes but less than the root knot nematodes *Meloidogyne* spp. This difference in sensitivity to the nematicidal actions of fluensulfone between nematode species may reflect differences in pharmacokinetic parameters, for example in the bioaccumulation of fluensulfone within the nematode. It could also result from different sensitivities of the molecular target for fluensulfone. These two explanations are not exclusive and both may play a role.

5. Conclusion

Fluensulfone has a unique and highly selective mechanism of action compared to other chemicals used to control PPNs with its nematicidal activity resulting from progressive impairment of PPN metabolic capability (Graphical abstract). This impairment of metabolism occurs selectively in *G. pallida* and not *C. elegans*. This work highlights the power of using a systematic investigation of the effect of different concentrations and times of exposure that better mimic the way these complex and highly specialized organisms encounter chemical treatments in the soil. The significance of this approach is that it opens a route to understanding enhanced selectivity and suggests potential parasitic specializations in metabolism that make for efficacious targeting of PPNs. The approach that targets key metabolic activities that might be specializations of parasitic nematodes has the exciting potential for improved pest management through selective but efficacious crop protectants.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.pestbp.2017.01.009.

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