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Reversible paralysis of *Schistosoma mansoni* by forchlorfenuron, a phenylurea cytokinin that affects septins

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Abstract

Septins are guanosine-5′-triphosphate-binding proteins involved in wide-ranging cellular processes including cytokinesis, vesicle trafficking, membrane remodeling and scaffolds, and with diverse binding partners. Precise roles for these structural proteins in most processes often remain elusive. Identification of small molecules that inhibit septins could aid in elucidating the functions of septins and has become increasingly important, including the description of roles for septins in pathogenic phenomena such as tumorigenesis. The plant growth regulator forchlorfenuron (FCF), a synthetic cytokinin known to inhibit septin dynamics, likely represents an informative probe for septin function. This report deals with septins of the human blood fluke *Schistosoma mansoni* and their interactions with FCF. Recombinant forms of three schistosome septins, *Sm*SEPT5, *Sm*SEPT7.2 and *Sm*SEPT10, interacted with FCF, leading to rapid polymerization of filaments. Culturing developmental stages (miracidia, cercariae, adult males) of schistosomes in FCF at 50 – 500 μM rapidly led to paralysis, which was reversible upon removal of the cytokinin. The reversible paralysis was concentration-, time- and developmental stage-dependent. Effects of FCF on the cultured schistosomes were monitored by video and/or by an xCELLigence-based assay of

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motility, which quantified the effect of FCF on fluke motility. The findings implicated a mechanism targeting a molecular system controlling movement in these developmental stages: a direct effect on muscle contraction due to septin stabilization might be responsible for the reversible paralysis, since enrichment of septins has been described within the muscles of schistosomes. This study revealed the reversible effect of FCF on both schistosome motility and its striking impact in hastening polymerization of septins. These novel findings suggested routes to elucidate roles for septins in this pathogen, and exploitation of derivatives of FCF for anti-schistosomal drugs.

Keywords

Schistosome; Forchlorfenuron; Septin; Cytokinin; Miracidium; Cercariae; xCELLigence; Phenylurea

1. Introduction

The septins are cytoskeletal proteins and filaments involved in diverse cellular processes (Cao et al., 2009; Mostowy and Cossart, 2012). The assembly of septins into higher-order structures such as filaments, rings and gauzes (Rodal et al., 2004) is central to their functions, such as in establishing membrane diffusion barriers and scaffolds as well as in vesicle trafficking (Beites et al., 1999; Longtine and Bi, 2003; Dobbelaere and Barral, 2004; Kinoshita, 2006; Spiliotis et al., 2008) and cytokinesis, for which they were first described in yeast (Hartwell, 1971). The filaments formed by these guanosine-5′-triphosphate (GTP)-binding proteins are ordered assemblies of hetero-oligomeric complexes (Sirajuddin et al., 2007). The number of septin genes within the genomes of eukaryotes varies, ranging from one in some algae to 13 in humans (Nishihama et al., 2011; Russell and Hall, 2011), and splice variants occur (Russell and Hall, 2011). Accordingly, there can arise numerous combinations of septins in functional filaments in different species and tissues.

This has allowed for the recent description of four *Schistosoma mansoni* septins, which have received the names *Sm*SEPT5, *Sm*SEPT7.1, *Sm*SEPT7.2 and *Sm*SEPT10, based on their sequence similarity with human homologues (Zeraik et al., 2013). However, the assembly and specific roles of these proteins in schistosomes has yet to be investigated.

Forchlorfenuron, (FCF; *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea ($C_{12}H_{10}ClN_3O$)), a small molecule synthetic plant cytokinin, interferes with the assembly of septin filaments in both yeast and mammalian cells (Iwase et al., 2004; Hu et al., 2008; DeMay et al., 2010). FCF stabilizes septin structures in a specific and reversible way, while not affecting other cytoskeletal components such as actin filaments and microtubules. How FCF alters septin organization in metazoan cells is not understood (DeMay et al., 2010) but, nevertheless, it represents a tractable, promising tool for the investigation of septin function. In yeast, FCF induced the appearance of septin filaments outside of the bud neck, which vanished upon removal of FCF (Iwase et al., 2004). In the filamentous fungus *Ashbya gossypii*, similar ectopic septin structures were observed. These septin fibers induced by FCF were shown to be stable structures. Therefore FCF was considered a septin stabilizing drug, since septin fibers, highly dynamic structures, were no longer dynamic upon FCF treatment and thus do

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not recover from photo-bleaching (DeMay et al., 2010). These fibers, although stable, were readily dissolved upon FCF removal, indicating that FCF is necessary to induce ectopic septin polymerization and necessary for its maintenance (DeMay et al., 2010). The effect promoted by FCF in mammalian cells has shown to phenocopy the small interfering (si)RNA treatment (Hu et al., 2008), providing a simpler and reliable alternative to study septin function. It also has the compelling advantages that its effects are reversible once FCF is washed out of the culture, and it has a low level of cytotoxicity for cultured human tumor cells (Hu et al., 2008). Decreased cell migration was observed upon FCF treatment (Hu et al., 2008), and the motility of amoeboid T lymphocytes was seriously affected by septin depletion (Tooley et al., 2008). It has been postulated that septin filaments function as a molecular corset, providing rigidity at the cell cortex to support efficient motion of T cells and that septin might tune actomyosin forces during motility (Tooley et al., 2008).

Here, we report on the interaction of FCF with schistosome septins and its consequences for filament assembly. This phenylurea derivative enhanced the formation of higher-order structures by *S. mansoni* septins in vitro. Moreover, putative stabilization of septin polymers within the schistosome correlated with the motility of its developmental stages. The impact of FCF on the motor activity of schistosomes might therefore contribute to the elucidation of the function of septins in this pathogen and may be worthy of further investigation for potential anti-schistosomal activity.

2. Materials and methods

2.1. Ethics statement

Mice infected with *S. mansoni* were obtained from the Biomedical Research Institute (BRI), Rockville, MD, USA and housed at the Animal Research Facility of the George Washington University Medical School, USA which is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC no. 000347) and has an Animal Welfare Assurance on file with the National Institutes of Health (NIH), USA, Office of Laboratory Animal Welfare, OLAW assurance number A3205-01. All procedures employed were consistent with the Guide for the Care and Use of Laboratory Animals. Maintenance of the mice and recovery of schistosomes were approved by the Institutional Animal Care and Use Committee of the George Washington University.

2.2. Developmental stages of schistosomes

Biomphalaria glabrata snails and Swiss-Webster mice infected with the NMRI (Puerto Rican) strain of *S. mansoni* were supplied by Drs. Matt Tucker and Fred Lewis, BRI under NIH-NIAID contract HHSN272201000005I. Four developmental stages were investigated - adult worms, eggs, miracidia and cercariae. In brief, adult worms were recovered from mice by portal perfusion; schistosome eggs were isolated from livers of the mice (Dalton et al., 2007) and miracidia obtained by hatching the eggs. Miracidia were used immediately. Cercariae were obtained by shedding infected snails under bright light for 2 h at 23 °C. Adult worms were cultured under 5% CO_2 at 37 °C in DMEM, supplemented with 10% FBS and 1×penicillin/streptomycin as described (Dalton et al., 1997; Mann et al., 2009; Zeraik et al., 2013).

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2.3. Expression and purification of septin complexes

Aiming to express a protein complex comprising three *S. mansoni* septins, the gene encoding *SmSept10* was ligated in a plasmid pACYCDuet-1 vector (Novagen, EMD Millipore Billerica MA, USA) and those encoding *SmSept5* and *SmSept7.2* into pETDuet-1 (Novagen). *SmSept7.2* was expressed as a histidine (His)-Tag fusion at the N-terminus whereas fusion peptides were not introduced into the two other septin constructs. This strategy was used to facilitate the purification by affinity chromatography of the septin complex by eliminating forms of *SmSept5* and *SmSept10* not bound to *SmSept7.2*. The septins were co-expressed in *Escherichia coli* BL21 (strain DE3) with induction by isopropyl β-D-1-thiogalactopyranoside (IPTG) at 0.4 mM for 16 h at 18 °C in Luria Broth (LB). Cells were harvested by centrifugation at 4,000 *g* for 40 min at 4 °C and resuspended in 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 12% glycerol, 10 mM β-mercaptoethanol (binding buffer). After sonication, the lysates were centrifuged at 20,000 *g* for 30 min at 4 °C, after which supernatants were subjected to affinity chromatography on 1 ml of TALON cobalt-resin (Clontech, Mountain View, CA, USA). After several washes with binding buffer, complexes were eluted from the resin with 500 mM imidazole in the same buffer. A sequential purification was undertaken using a column of Superdex-200 (HR 10/30 GE Healthcare Life Sciences, Pittsburgh, PA, USA) fitted to an AKTA purifier liquid handling system (GE Healthcare Life Sciences). Eluates were analyzed by SDS-PAGE. High molecular mass fractions containing the heterocomplex were pooled and examined by MS, dynamic light scattering (DLS) and electron microscopy (see Sections 2.4 – 2.6).

2.4. MS of the purified complexes

Samples for MS were obtained from the polyacrylamide gel containing the recombinant septin complex purified from size exclusion chromatography. The gel was stained with Bio-safe Coomassie Blue G250 (Bio-Rad, Hercules, CA, USA) and destained with water. Gel strips corresponding to detected bands were further destained with 50% acetonitrile solution, dehydrated in 100% acetonitrile and rehydrated in 100 mM ammonium bicarbonate. The gel strips were diced and incubated with DTT (10 mM), followed by alkylation with iodoacetamide (55 mM). After washing steps with ammonium bicarbonate and 50% acetonitrile, the gel pieces were dehydrated in 100% acetonitrile, dried in a Savant SpeedVac concentrator and rehydrated in a solution of trypsin (Promega, Madison, WI, USA) for 16 h at 37 °C to fragment the proteins into peptides. Solutions containing peptides were purified using a C_{18} ZipTips (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions followed by direct infusion electrospray tandem MS (micrOTOF-QII, Bruker, Karlsruhe, Germany) to obtain fragmentation spectra for the liberated peptides. The spectra were interrogated using MASCOT (Matrix Science, Boston, MA, USA), utilizing a database of the proteins predicted encoded by the genome of *S. mansoni* to identify the peptides.

2.5. Transmission electron microscopy (TEM) of septin complexes

The recombinant *S. mansoni* septin complex at 2.2 mg/ml in binding buffer was dialyzed against 15 mM Tris-HCl pH 8.0 containing 40 mM NaCl, 2 mM $MgCl_2$, 0.5 mM GTP, 2 mM DTT in the presence of FCF (25 and 75 μM) or DMSO (control) for 30 min at 4 °C. To

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visualize polymerization of septins, aliquots (10 μl) were applied to glow-discharged carbon-coated grids and stained with 2% uranyl acetate. The grids were examined in a Tecnai-12 TEM (FEI, Hillsboro, OR, USA) under regular-dose conditions at an accelerating voltage of 80 keV.

2.6. DLS

The *S. mansoni* septin complex(es) at 0.2 mg/ml in binding buffer containing 5 mM GTP was dialyzed against 15 mM Tris-HCl pH 8.0, 40 mM NaCl, and 15 mM DTT in the presence of 75 μM of FCF or DMSO for 30 min at 4 °C. Samples were dispensed into 12 μl quartz cuvettes, inserted into a Zetasizer μV detector (Malvern Instruments, Worcestershire, UK) controlled with Zetasizer version 6.32 software (Malvern Instruments), and DLS from an 830 nm laser was measured at an angle of 90° and at 22°C. DLS was used to determine molecular size.

2.7. Effects of FCF on cultured schistosomes

FCF (Sigma-Aldrich, St. Louis, MO, USA) solution (250 mM) was prepared in DMSO (or ethanol). Aliquots of this stock were dispensed into culture medium, in increasing concentrations from 0 to 500 μM, for cercariae and adult stages of *S. mansoni*, and into water, from 0 to 200 μM, for miracidia. Control groups containing DMSO were included. To assess the reversibility of the FCF effect, the worms were transferred to fresh culture media without FCF. Phenotypic changes were documented with the assistance of a Zeiss Axio Observer A.1 inverted microscope coupled to a Zeiss AxioCam ICc3 camera. Bright field movies were recorded using the time series mode of the Carl Zeiss LSM 710 system equipped with a Zeiss EC Plan-Neofluar 5x/0.16NA objective. The images were captured consecutively, at 10 frames per second. In some experiments, samples of the schistosomes were removed from the culture and incubated in the fluorophores fluorescein diacetate (FDA), at 2.0 μg/ml, which stains live schistosomes, and propidium iodide (PI), at 0.5 μg/ml, which stains dead schistosomes, using previously reported methods (Peak et al., 2010; Rinaldi et al., 2012). In order to assess the effect of FCF on the F-actin structure, cercariae exposed to 500 μM FCF for 3 h were fixed in 4% paraformaldehyde for 1 h at 4 °C. Subsequently, the worms were stained with Alexa Fluor 568 phalloidin (Invitrogen, Life Technologies, Grand Island, NY, USA) at 165 nM in PBS containing 1 % BSA for 30 min at 25 °C. The slides were mounted with Fluoromount-G (EMS) after which the samples were air-dried and confocal micrographs collected with the Carl Zeiss LSM 710 system equipped with a Zeiss Plan-Apochromat 63x/1.40 oil DIC objective.

2.8. Real time assessment of motility of schistosomes and of egg hatching

The effect of FCF on fluke motility and egg hatching/miracidium motility was evaluated and quantified employing an xCELLigence DP system (ACEA Biosciences, San Diego, CA, USA) originally designed to monitor cellular events in real time by measuring electrical impedance across inter-digitated microelectrodes integrated on the bottom of tissue culture E-plates, see http://www.aceabio.com/main.aspx (Ke et al., 2011). This real time cell assay (RTCA) platform is useful for evaluating motility and egg hatching of other helminth parasites (Smout et al., 2010). The motility test with adult male schistosomes was performed

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as described (Smout et al., 2010) with the addition of a motility baseline determined over 16 h with 100 µl of media alone before individual flukes were introduced into to each well in three 16 well E-plates. This motility baseline step is a modification to the procedure of Smout et al. (2010) and is desirable due to elevated inter-well baseline variability of the xCELLigence DP platform in comparison with the xCELLigence SP instrument (Smout et al., 2010). In brief, the motility of flukes was registered for ∼24 h before adding 500 µM FCF, or DMSO as vehicle control, in 200 µl media per well. Approximately 6 h later FCF was removed, after which the schistosomes were washed with 1x PBS before fresh medium free of FCF was added to the wells. Recovery of motility after removal of FCF was assessed for ∼16 h. Thereafter, the worms were removed from the E-plates and the final baseline for the plate ascertained for ∼16 h. Controls with individual male schistosomes cultured in media with DMSO (or ethanol) but not FCF and wells with no flukes ('background' wells) were included in the assays. To evaluate the motility of miracidia following hatching/release from eggs, 5,000 eggs in 200 µl of 0.1x PBS, 50 µM or 200 µM FCF per well in E-plates were induced to hatch by transfer from physiological medium into 0.1x PBS under bright light at 23 °C for ∼16 h. Controls included wells of eggs exposed to DMSO (or ethanol) in 0.1x PBS and non-viable eggs - eggs that had been killed immediately before the assay by incubation at 80 °C, 15 min. A motility baseline with 100 µl of 0.1X PBS was performed, dispensing schistosome eggs into E-plates. The motility for both adult flukes and miracidia hatching from eggs was monitored every 15 s and the motility index was calculated as described (Smout et al., 2010). The motility index for each E-plate well, i.e. individual flukes or 5,000 eggs, was calculated as the S.D. over 800 data points of the cell index (CI) difference from the rolling average over 20 data points (Smout et al., 2010). Statistical analyses were performed with Graphpad prism 5.0 (Smout et al., 2010). Triplicate biological replicates were undertaken. In parallel, eggs incubated and induced to hatch under these same conditions, as described for the xCELLigence assay, were maintained in 24-well plastic tissue culture plates (Corning Life Sciences, Corning, NY, USA) for microscopical observation (Zeiss Axio Observer A.1 inverted microscope fitted with a Zeiss AxioCam ICc3 camera) of hatching.

3. Results

3.1. Schistosome septins form heterocomplexes

The recently described septins from *S. mansoni* display traits commonly verified in septins, including a conserved GTPase domain containing the G1, G3 and G4 motifs, the septin unique element (SUE), prior to the C-terminal region and the prediction of a coiled-coil structure at the C-terminal domain (Zeraik et al., 2013). Accordingly, it was anticipated that they organize into filamentous heterocomplexes, as in yeast, *Drosophila* and human cells (Barral and Kinoshita, 2008). In order to investigate whether *S. mansoni* septins form such hetero-filaments, the *Sm*SEPT5, *Sm*SEPT10 and *Sm*SEPT7.2 were co-expressed in *E. coli;* we envisioned the formation of a heterocomplex concomitantly with their expression. After lysis, procedures were performed in a high concentration of NaCl, which dismantles septin filaments into oligomers (Sirajuddin et al., 2007). Only *Sm*SEPT7.2 had a fused His-tag and could be expected to bind directly to the cobalt affinity matrix column during chromatographic purification; the other two recombinant septins could only be co-purified

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as a consequence of molecular interaction with this septin. The eluate from the affinity column was submitted to gel filtration chromatography; and the resulting profile indicated several populations, suggesting heterogeneity in the composition of the purified complex of the three recombinant septins. Elution fractions corresponding to higher apparent molecular masses of ∼300-400 kDa contained the hetero-complex comprised of the three proteins (Fig. 1). Analysis by MS of that fraction confirmed that the septin hetero-complex comprised all three recombinant *S. mansoni* septins; peptides corresponding to each of them were detected (Supplementary Table S1). Analysis of the high molecular mass fraction by TEM revealed short rod-like structures (Fig. 2) of similar appearance to those of human septin hetero-oligomers in solution with a high salt concentration (Sirajuddin et al., 2007).

3.2. Polymerization of schistosome septin is hastened by FCF

The cytokinin FCF is a small organic compound that affects septin dynamics in mammalian cells, inducing the assembly of larger structures, among other attributes (Hu et al., 2008). Aiming to verify how schistosome septin assembly is affected by this synthetic phenylurea, septin filament formation was compared in the presence or absence of FCF at 25 μM (Hu et al., 2008). The septin complexes were dialyzed into low salt buffer to initiate polymerization into higher-order structures and the process monitored over time using TEM (Fig. 3). Polymerization of septins has a lag phase and even in the presence of FCF, long filaments were not observed within the first minute of dialysis (Fig. 3A, F, respectively). By 20 min, small quantities of long single filaments were evident in the control (Fig. 3B), as were numerous long filaments in the presence of FCF (Fig. 3G). Similar enhancement in polymerization of septin complexes was observed in FCF at 75 μM (not shown). After dialysis for 3 h, mature bundles were formed regardless of the presence of FCF or not (Fig. 3C - E and H - I). This suggested that whereas FCF hastened the polymerization of schistosome septins, the cytokinin did not influence how these septins interacted with each other within higher-ordered structures. TEM micrographs indicated that septins of *S. mansoni* form two types of bundles - curved and straight. Septin filaments can switch from one shape to the other within the same bundle, which suggests that the lateral interactions between the filaments within the bundle are polymorphic. To illustrate this phenomenon, we imaged characteristic shapes of bundles of filaments formed in the absence of FCF (Fig. 3C-E). Fig. 3C revealed a mostly straight bundle that terminates in a ring, Fig. 3D shows a long, straight bundle, and Fig. 3E presents an ensemble bundle of both rings and straight filaments. Similar structures were observed in bundles that assembled in FCF (Fig. 3H, I).

DLS was performed to assess the size of filament particles formed early during septin polymerization. Particle size distributions were obtained for the *S. mansoni* septin complexes before the dialysis and after dialysis for 30 min in the presence or absence of FCF. After dialysis, larger particles were present in the solution, compared with the sample maintained at high ionic strength, both in the presence or absence of FCF (Fig. 4). However, the solution with FCF showed larger particles in solution compared with the control sample. This indicated that schistosome septins undergo expedited polymerization induced by the decrease of ionic strength and that this process was hastened even further by FCF.

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3.3. FCF reversibly paralyzes schistosomes

In view of the influence of FCF on the dynamics of polymerization of schistosome septins, additional investigation was undertaken to examine potential phenotypic effects of this small molecule on developmental stages of schistosomes in vitro. The addition of FCF to culture media had a striking effect on adult stages of these schistosomes – it paralyzed both adult female and male flukes. The worms ceased to move, as observed by light microscopy, in a dose- and time-dependent manner. In 500 μM FCF, paralysis was apparent within 10 min, and by 3 h in 300 μM FCF, all movement by adult schistosomes ceased (Supplementary Movie S1). Yet, strikingly, the paralysis reversed quickly (almost immediately) when the flukes were transferred to culture medium free of FCF (Supplementary Movie S1).

FCF at 500 μM promoted paralysis of cercariae within a few minutes, clearly evident by loss of motor activity of the tail (Supplementary Movie S2). After 3 h in 500 μM FCF, motility could be restored after removal of FCF (Supplementary Movie S2). At 200 μM FCF, reduction of movement was also pronounced, but only after ∼ 3 h (Supplementary Movie S2). Miracidia exhibited exquisite sensitivity; by 30 min in 50 μM FCF, the otherwise highly motile larvae stopped moving and/or displayed only lethargic swimming (Supplementary Movie S3). FCF at 200 μM for 30 min caused miracidia to clump together, with minimal movement discernible in the clumps (Supplementary Movie S3). After 1 h in 200 μM FCF, miracidia had died, as determined by the inability to both exclude PI and uptake FDA, fluorophores that distinguish dead and live schistosomes, respectively (Peak et al., 2010) (Supplementary Fig. S1).

A notable phenomenon evident in schistosomes that were exposed to FCF was that the paralysis was readily reversed when FCF was removed from the culture. In cercariae and adult males, after 24 h in contact with FCF, at 200 and 300 μM, respectively, motility was restored upon removal of FCF. Lower concentrations of 50 to 100 μM did not cause visible effects in cercariae and adults when evaluated for up to 24 h. On the other hand, FCF at concentrations ≥1 mM killed cercariae within minutes. FCF treatment did not appreciably impact the actin filament structure, as phalloidin staining was performed on cercariae after 3 h in the presence of 500 μM FCF, and the F-actin structure appeared unaffected (Supplementary Fig. S2).

The effects of incubation time and FCF concentration for these several, morphologically discrete, developmental stages are summarized in the Table 1. Control groups of these stages (adults, miracidia, cercariae) cultured with vehicle (DMSO) alone exhibited the expected movements for their developmental stages; none of the developmental stages examined displayed paralysis in the absence of FCF (Supplementary Movie S1).

3.4. Paralysis of schistosomes and inhibition of egg hatching quantified by xCELLigene RTCA

Motility of adult flukes was monitored by RTCA, before and during the incubation with 500 μM FCF for ∼ 6 h and after removal of the phenylurea from the media. ACEA's xCELLigence system uses E-plates, multi-well microtitre plates bearing gold electrodes across which changes in conductivity are monitored and output reported as a CI (Ke et al.,

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2011). While originally designed for monitoring cultured cells attached to the E-plate surface, we revealed in 2010 that this system is ideal for monitoring movements of parasitic helminths as they move or writhe in vitro (Smout et al., 2010). A motile worm will constantly change the degree of contact with the E-plate and hence the CI output varies with each twitch. The amplitude of this variation is used to monitor the degree of schistosome motility in response to external stimuli such as a drug or xenobiotic, or a pharmacological agent.

The flukes incubated in FCF exhibited near complete paralysis (∼3 - 7% motility) in comparison with flukes cultured in its absence (92-116% motility) (Fig. 5). After removal of FCF, the flukes recovered near complete motility (87% motility) within 10 h. It was noted that after the treatments were removed and fresh media applied, the vehicle control also showed reduced motility (67%) followed by complete recovery. The reason for this is unclear, but changing the medium may be disruptive in this assay such that movement of the schistosomes is perturbed temporarily until the culture conditions stabilize.

The egg hatch method developed by Smout et al. (2010) directly measures the numbers of hatched larval nematodes that migrate through mesh and fall onto the xCELLigence electrodes to be detected. This technique was employed with caution for schistosomes because the hatched egg releases a ciliated, motile miracidium that does not maintain contact with the electrodes on the base the well of the E-plate. Despite this limitation, we anticipated that the system could monitor egg hatching indirectly. Akin to the assay of motility for adult schistosomes (above), the motility of 5,000 eggs in 0.1 X PBS was measured and the output represented the combination of the number and amount of movement of the hatching eggs and miracidia. Initially we confirmed there were no significant differences in the FCF solvents on egg hatching by comparing ethanol and DMSO without dissolved FCF (Supplementary Fig. S3A). The reduced motility index with 50 μM or 200μM FCF was apparent (Fig. 6A shows FCF in DMSO and Supplementary Fig. S3B shows FCF in ethanol). Converting the motility index to % motility allowed comparisons between the solvents (DMSO versus ethanol) (Fig. 6B). This graph has been limited to 12 h duration; after this point the vehicle control motility index (100% motility) drops below twice the empty well motility index (0% motility) and artificially amplifies data variability % motility as a proportion of the vehicle control. Whereas all FCF treatments significantly reduced hatching/movement over time ($P < 0.001$), the FCF in DMSO was ∼25% more effective than FCF in ethanol (Fig. 5B). FCF at 200 μM in DMSO induced the most pronounced reduction (down to 37%) within 1 h after induction of egg hatching, and caused complete cessation of hatching and miracidial movement by 11 h. It was also noted that 50 μM FCF in ethanol or DMSO initially induced ∼120% motility (hyper-motility response?) that waned over the next ∼ 4 h. In parallel, these effects of FCF on hatching of eggs and motility of miracidia released from these eggs, as registered and quantified by RTCA, were directly observed and confirmed by microscopical examination (not shown).

4. Discussion

The septins are classified into four subgroups and it has been postulated that the septin heterocomplexes contain septin representatives from different subgroups in specific

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positions (Kinoshita, 2003). *Schistosoma mansoni* only displays proteins from three of these four subgroups, which are those with members present in the complex whose structures have been previously determined by X-ray diffraction (Sirajuddin et al., 2007). Successful observation of oligomers composed of recombinant septins from schistosomes from these three groups suggests that their assembly into higher-order structures is similar to that reported for orthologous septins (Kinoshita et al., 2002; Versele and Thorner, 2005; John et al., 2007).

Filaments of schistosome septins examined here organized themselves into curved and straight bundles, similar to those observed in other species (see Kinoshita, 2006), suggesting a conserved form of organization. Kinoshita et al. (2002) reported that upon actin disruption, septins tend to reorganize from long linear bundles to uniform rings, and considered that the rings could represent a default assembly or a septin storage form. Ring forms are abolished and septin recovers the linear structures after removal of cytochalasin, a fungal metabolite that depolymerizes actin filaments. It is possible that discrete forms of organization are also relevant in septins of *S. mansoni*. To address these kinds of questions, functional analyses in studies reported previously have employed antibody probes and RNA interference (Correnti et al., 2005; Morales et al., 2008) but the existence of multiple septin genes, splice isoforms, potential redundancy, plasticity and the wide diversity of septin functions can obfuscate the interpretation of results. It would be informative to determine the presence of these complexes in the parasite itself, perhaps with antisera to pull down such complexes from extracts of schistosomes. This would be challenging, however, because cytoskeletal proteins generally are difficult to solubilize and harsher treatments tend to destroy associations of septins.

The synthetic phenylurea FCF can stabilize septins, although its mechanism of action is unknown (Kim et al., 2010; Hagiwara et al., 2011; Wasik et al., 2012). The stabilized septin filaments are not functional and previous studies have shown that FCF treatments phenocopy the effects of septin depletion by RNAi, such as mitotic and cell migration defects (Hu et al., 2008). Nonetheless, here we used FCF to probe schistosome septins in vitro as well as cultured schistosomes in order to determine if the approach could identify whether the compound also interacts with septin/septin filaments of schistosomes and/or results in an informative phenotype. These assays revealed that FCF enhances the formation of septin fibers early in polymerization of *S. mansoni* septins, further confirming the specific effect of this cytokinin on a wide range of septins and species. The responses to exposure to FCF by the developmental stages of *S. mansoni* were striking. Motility of the flukes was severely compromised but, similarly strikingly, its effect was reversible - fully or partially - when FCF was removed from the culture medium. This swift reversal of the phenotype suggested a specific mechanism of action rather than a generalized toxicity. In like fashion, the effects of FCF on fungi and yeast are rapid and reversible (Iwase et al., 2004; De May et al., 2010). Since the action of FCF was relatively slow (10 min - 3 h) whereas the reversal of its effects was swift, the kinetics of binding of FCF to septins might be significantly slower than that of dissociation.

Compared with adult worms and cercaria, the miracidia showed the most marked sensitivity to FCF; FCF was lethal for miracidia after exposure for 1 h to 200 μM of the phenylurea.

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This might suggest that septins have a critical role in this developmental stage. This is consistent with the earlier findings that septins exhibit distinctive patterns of localization among developmental stages of the schistosome, indicating they perform development-related functions (Zeraik et al., 2013). Also, the motility of miracidia exiting hatching eggs appeared to be influenced by the solvent used as vehicle for FCF, with formulations with DMSO enhancing efficiency of FCF over those with ethanol. A possible explanation for this phenomenon may be related to the ability of DMSO to perturb lipid bilayers (Anchordoguy et al., 1992), and hence its presence might favor uptake of FCF by the schistosome cells.

Immunolocalization of septins in schistosomes revealed the prevalence of septin filaments in the muscles of this blood fluke – septin co-localized with F-actin fibers (Zeraik et al., 2013). Accordingly, we now hypothesize that the paralysis phenotype from exposure to FCF may result from interference of muscle contraction in which interaction between actin and septin filaments is expected to be essential for normal function of muscular fibers. Given that the phenotype was promptly reversed upon removal of FCF, there is likely to be a specific, defined mechanism involved rather than generalized disorganization of the filaments. This mechanism may be related to the perturbed polymerization of septin filaments observed in the presence of FCF.

In view of the striking reversible paralysis phenotype described here, and increased understanding of septins in the pathobiology of human diseases ranging from neurodegeneracy to oncogenesis (Kartmann and Roth, 2001), deeper investigation is warranted to elucidate the exact mechanism of action of this phenylurea derivative on schistosome septins. Considering the paralysis phenotype, it is possible to propose the therapeutic use of FCF for schistosomiasis. Indeed, an analogous paralysis is displayed when worms are cultured in praziquantel (Chavasse et al., 1979; You et al., 2013). A drawback is that high doses of FCF were required to achieve a visible phenotype in schistosomes, which would limit utility of the compound as an oral medication. Nonetheless, developing derivatives of FCF with higher affinity to schistosome septins may be warranted, thus facilitating a tractable pharmacological regimen. FCF has horticultural value; for example, the European Food Safety Authority approves its use for foliar outdoor treatment of commercial crops of grapes, kiwi fruits and other plants, and its toxicity has been assessed, http://www.efsa.europa.eu/en/efsajournal/doc/2862.pdf. In rats, FCF displays an acute oral toxic dose of 4,918 mg/kg, close to the threshold of 5,000 mg/kg at which a substance is considered non-toxic. Chronic oral exposure to FCF in animal models caused minimal side effects, limited to loss of body weight and kidney inflammation in long-term treatments (2 years with ∼100 mg/kg/day dosage) (United States Environmental Protection Agency, 2004). These findings indicate that FCF does not harm mammals.

Lastly, the development of an automated and quantitative schistosome egg hatch assay based on the xCELLigence system presented here is also noteworthy. This novel application expands the catalogue of species of helminth parasites and developmental stages amenable (e.g. Smout et al., 2010; Silbereisen et al., 2011; Tritten et al., 2013; You et al., 2013) to functional analysis in real time without the need for laborious, and more subjective, assessment by microscopy.

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Supplementary Material

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Highlights

- Schistosome septins assemble into complexes and higher-order structures, including filaments and rings
- Polymerization of schistosome septins hastened in forchlorfenuron (FCF), a phenylurea cytokinin
- FCF led to a striking paralysis phenotype in cercariae and adult schistosomes
- The paralysis was reversible, but FCF dose- and time-dependent

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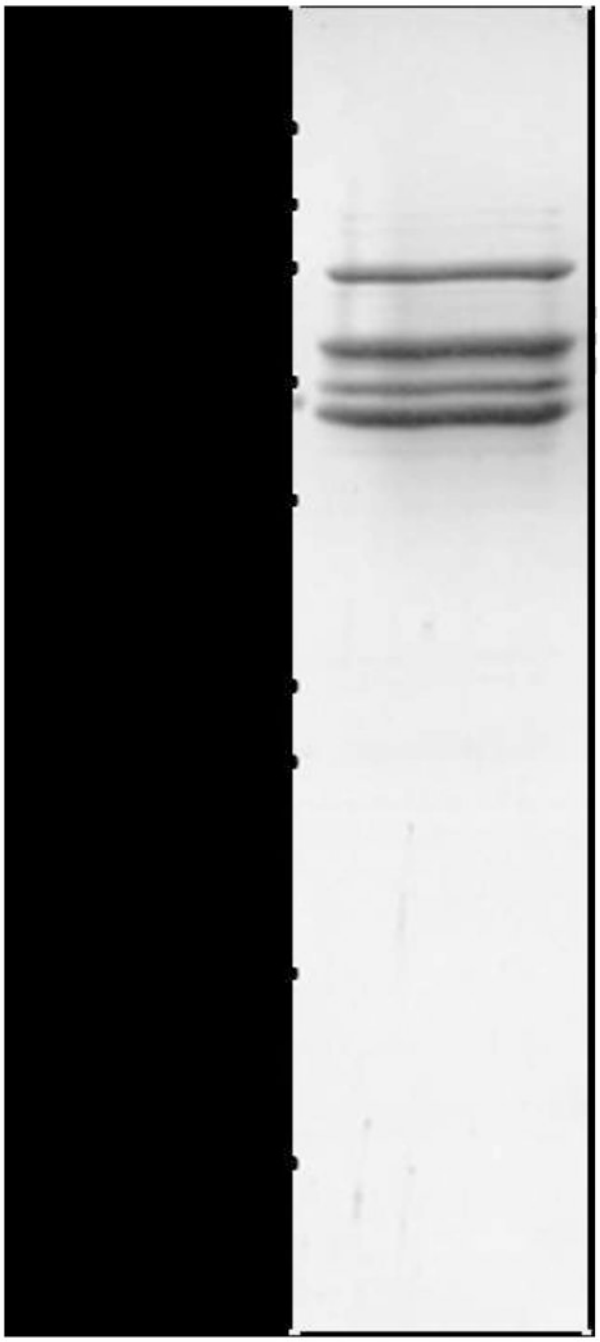

Fig. 1.

Schistosoma mansoni septins form hetero-oligomeric complexes. SDS-PAGE analysis of the purified hetero-complex. Note that three bands display sizes similar to those expected for *Sm*SEPT5 (51.3 kDa), *Sm*SEPT10 (48.1 kDa) and *Sm*SEPT7.2 (59.2 kDa). The band at ∼75 kDa is indicative of the presence of bacterial GroEL protein. Size standards in kilodaltons (kDa) are shown.

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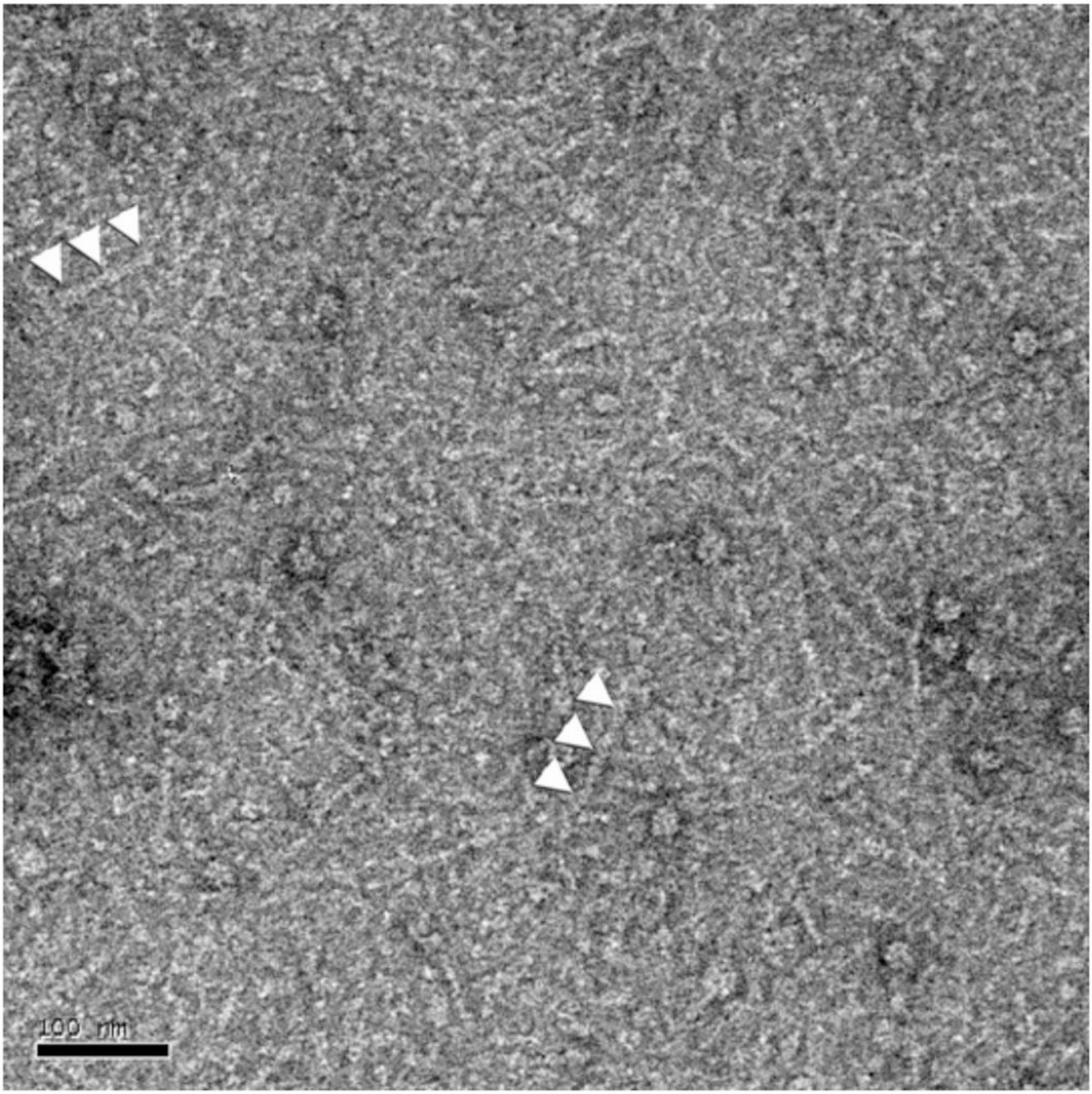

Fig. 2.

Transmission electron micrographs of negatively stained *Schistosoma mansoni* septins showing heterogeneous complexes that had assembled (indicated by white arrowheads). Scale bar, 100 nm.

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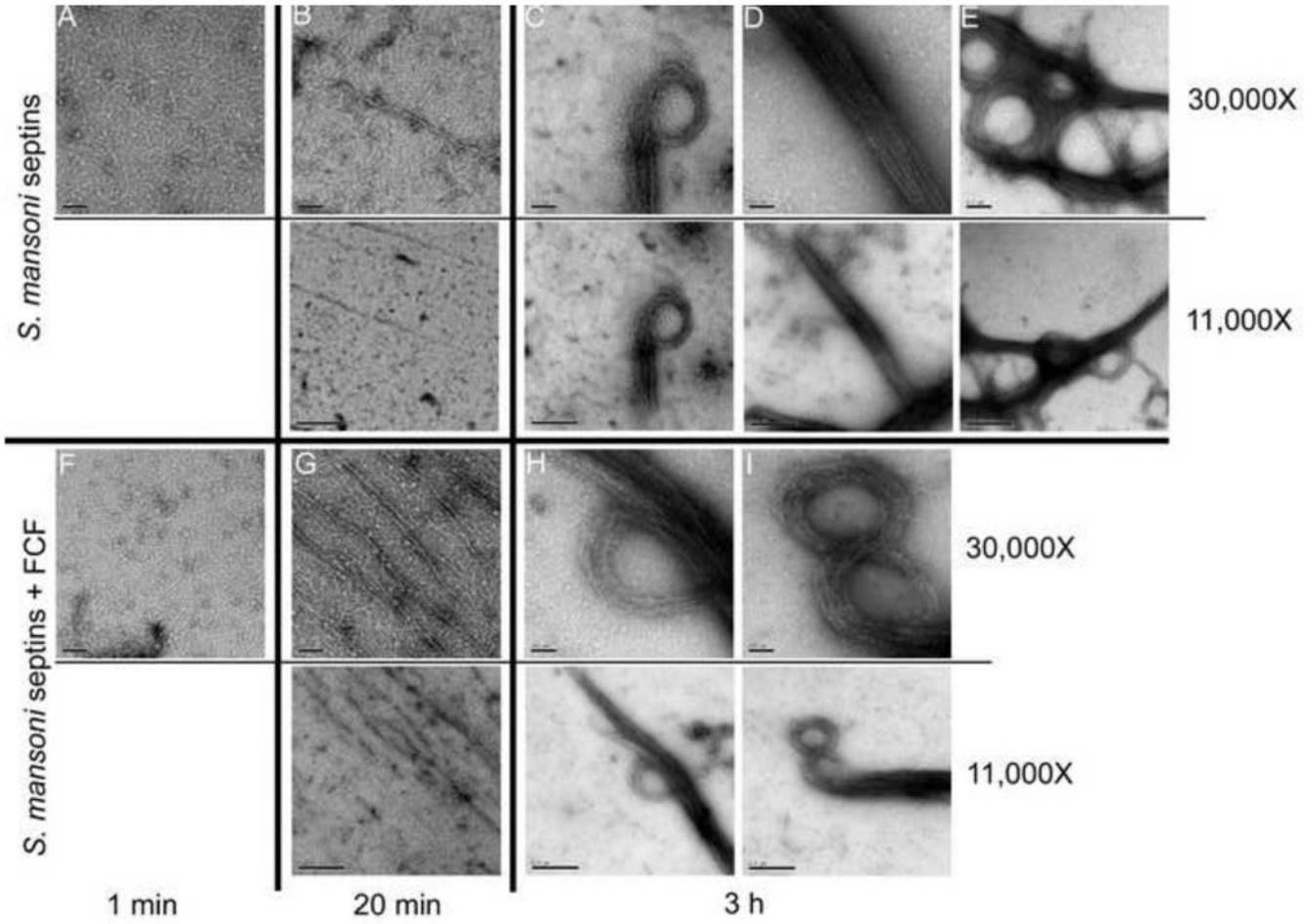

Fig. 3.

Schistosoma mansoni septin complexes assemble into filaments in vitro. Transmission electron micrographs of negatively stained septin complexes after dialysis against low salt solution with DMSO (A - E) or Forchlorfenuron (FCF) (F - I) for: 1 min (A, F), 20 min (B, G) or 3 h (C - E, H, I). The diversity of higher-order structures formed by *S. mansoni* septins is especially evident after 3 h of dialysis. Nominal magnification for each panel is shown on the right.

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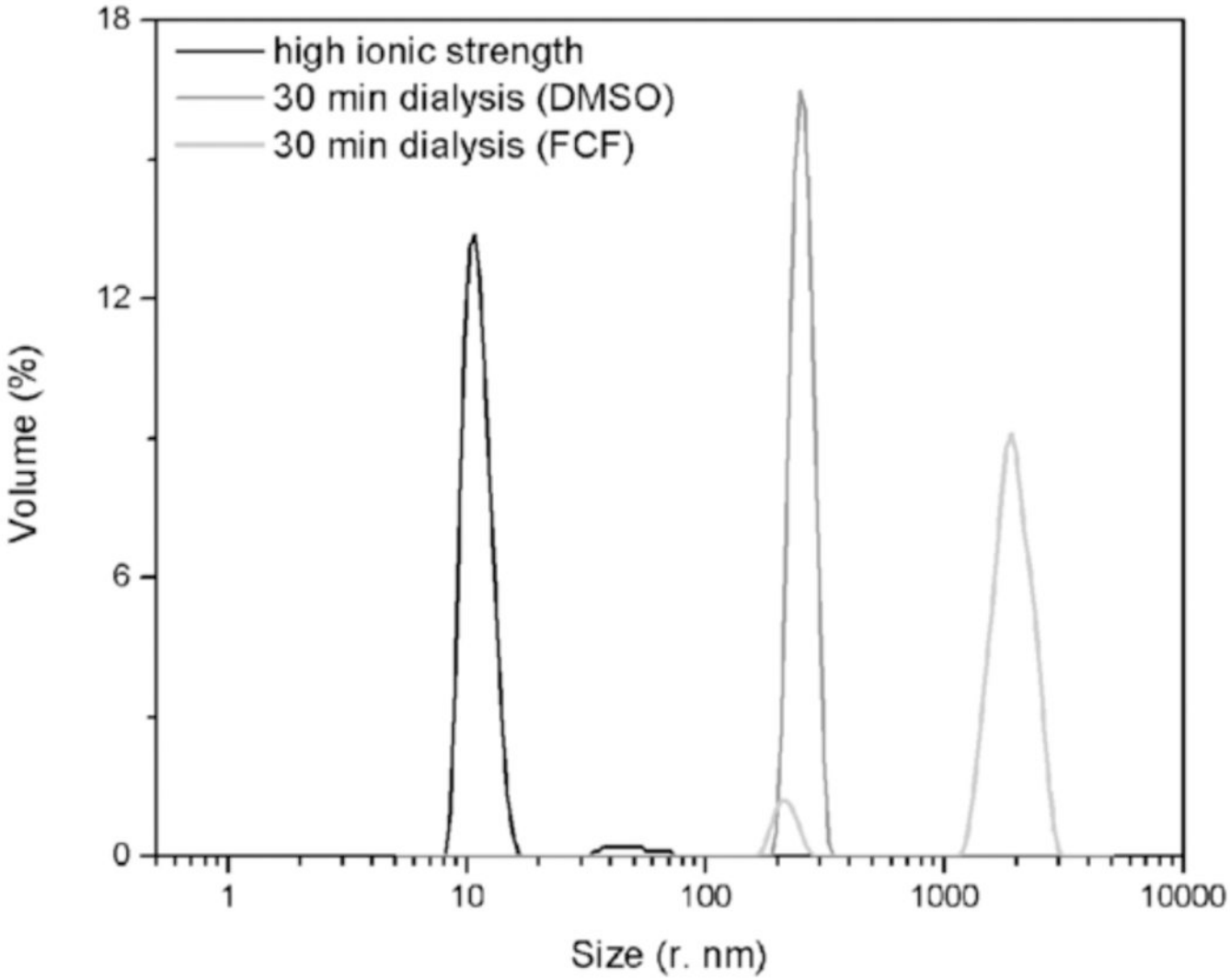

Fig. 4.

Forchlorfenuron (FCF) hastens polymerization of complexes of schistosome septins. A volume weighted differential size distribution is shown for the purified *Schistosoma mansoni* septin complexes maintained in high ionic strength (black) and after 30 min dialysis against low salt buffer containing DMSO (dark gray) or 75 μM of FCF) (light gray).

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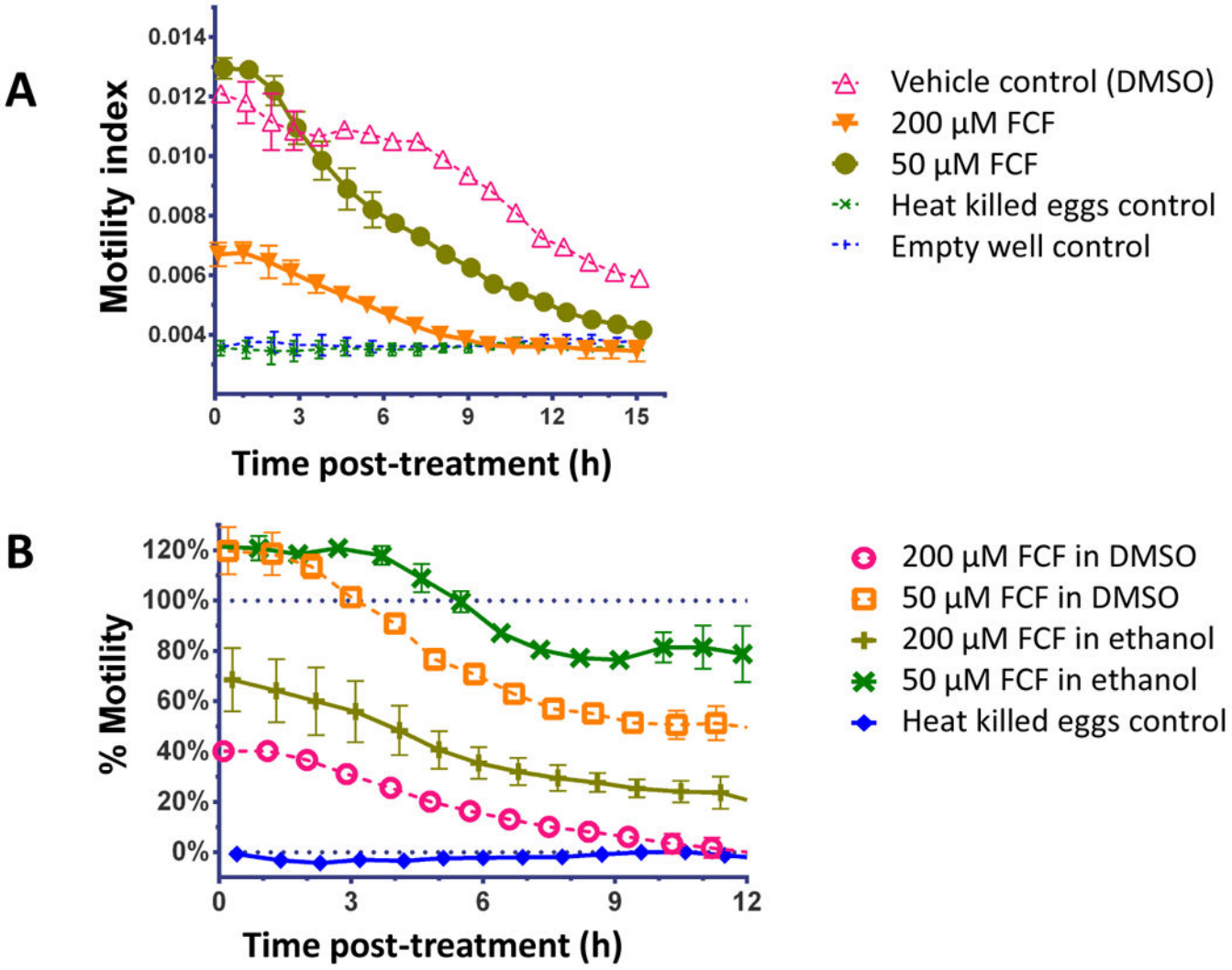

Fig. 5.

Motility (%) of adult male schistosomes before, during and after incubation in 500 µM Forchlorfenuron (FCF). The percentage of motility is calculated after subtraction of the background signal detected in the wells before worms were added and relative to worm motility prior to treatment. Treatments were added at time 0 h and removed at 7 h (indicated by the red arrow). Data points are the average of ≥5 replicates ± S.E.M. For clarity, data points are shown at hourly intervals (symbol points have been nudged 0.2 h left or right to reduce overlaps).

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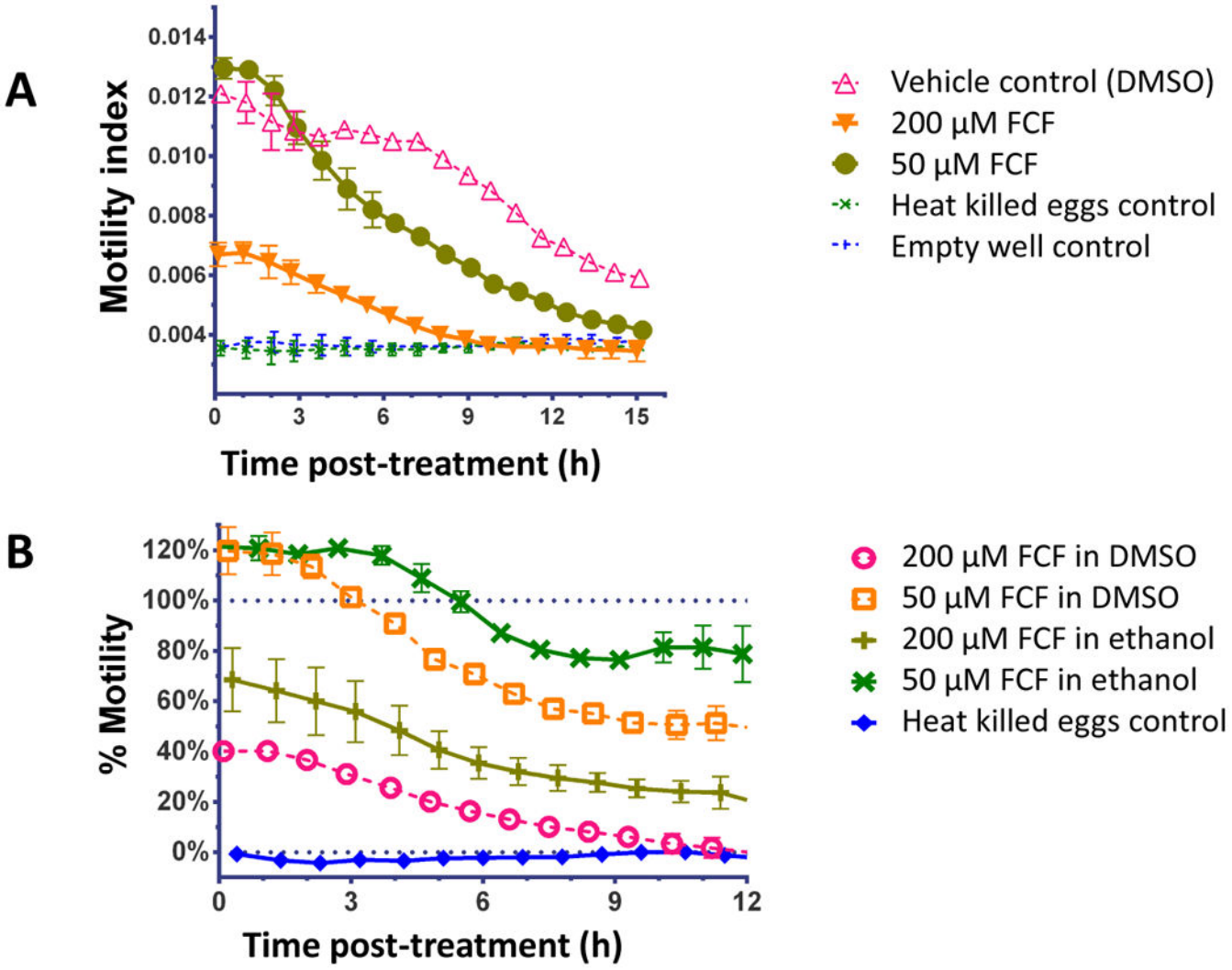

Fig. 6.

Motility of schistosome miracidia during the egg hatching quantified by the xCELLigence Real Time Cell Analyzer DP platform. (A) Motility Index of miracidia hatching from the eggs exposed to light in 0.1X PBS with Forchlorfenuron (FCF) at 50 µM or 200 µM FCF prepared in DMSO as solvent. (B) Motility (%) of miracidia hatching from eggs with FCF prepared in ethanol or DMSO. These curves range from 0% empty well background to 100% vehicle control.

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Table 1

Effects of forchlorfenuron (FCF) on developmental stages of *Schistosoma mansoni*

Stage	FCF	10 min	30 min	1 h	3 h	24 h	2-3 h removed[a]	24 h removed[a]
Adults	<100 μM	ne	ne	ne	ne	ne		
	300 μM	ne	ne	ne	paralysis	paralysis		recovery
	500 μM	paralysis	paralysis	paralysis	paralysis	death	recovery	
Cercariae	<100 μM	ne	ne	ne	ne	ne		
	200 μM	ne	ne	ab	paralysis	paralysis		recovery
	500 μM	paralysis	paralysis	paralysis	paralysis	death	recovery	
Miracidia	50 μM	ab	ab					
	200 μM	ab	paralysis, clumping	death				

P, paralysis; ne, no effect apparent; ab, abnormal behavior (including slowed movement, lethargy).

[a]Phenotype observed after incubation with FCF for the indicated period followed by removal of FCF and replacement with FCF-free medium.