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RESEARCH ARTICLE

Using *Caenorhabditis elegans* as a model organism for evaluating extracellular signal-regulated kinase docking domain inhibitors

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Abstract We have recently identified several novel ATP-independent inhibitors that target the extracellular signal-regulated kinase-2 (ERK2) protein and inhibit substrate phosphorylation. To further characterize these compounds, we describe the use of *C. elegans* as a model organism. *C. elegans* is recognized as a versatile and cost effective model for use in drug development. These studies take advantage of the well characterized process of vulva development and egg laying, which requires MPK-1, the homolog to human ERK2. It is shown that treatment of *C. elegans* eggs or larvae prior to vulva formation with a previously identified lead compound (**76**) caused up to 50% reduction in the number of eggs produced from the adult worm. In contrast, compound **76** had no effect on egg laying in young adult or adult worms with fully formed vulva. The reduction in egg laying by the test compound was not due to effects on *C. elegans* life span, general toxicity, or non-specific stress. However, compound **76** did show selective inhibition of phosphorylation of LIN-1, a MPK-1 substrate essential for vulva precursor cell formation. Moreover, compound **76** inhibited cell fusion necessary for vulva maturation and reduced the multivulva phenotype in LET-60 (Ras) mutant worms that have constitutive activation of MPK-1. These findings support the use of *C. elegans* as a model organism to evaluate the selectivity and specificity of novel ERK targeted compounds.

Keywords Signal transduction · Extracellular signal-regulated kinase · Docking domain inhibitors · Model organism

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Introduction

The extracellular signal-regulated kinase-1 and 2 (ERK1/2) proteins belong to the mitogen-activated protein (MAP) kinase superfamily and are important regulators of cell growth, differentiation, inflammation and apoptosis (Pearson et al. 2001). Activation of the ERK1/2 proteins occurs in response to a variety of extracellular stimuli, which regulate plasma membrane receptors and induce the sequential activation of the well-defined pathway involving Ras G-proteins, Raf kinases, and the MAP or ERK kinase-1 and 2 (MEK1/2) proteins. MEK1/2, the only known ERK1/2 activators, regulate ERK proteins through dual-phosphorylation of threonine (T) and tyrosine (Y) residues within a TEY motif (Lewis et al. 1998). The activated ERK proteins may phosphorylate and regulate the activity and function of more than 70 different substrates, which include transcription factors, other kinases, signaling proteins, and structural proteins (Lewis et al. 1998; Pearson et al. 2001). ERK1/2 activities are tightly regulated through a balance between upstream MEK1/2 activation and inactivation by phosphatases (Shapiro 2002). However, a shift in the balance towards uncontrolled activation of the ERK1/2 signaling pathway is a hallmark of a variety of cancers (Cohen 1999). Therefore, targeted inhibition of ERK signaling is viewed as a potential approach for cancer chemotherapy (Bollag et al. 2003; English and Cobb 2002; Reuter et al. 2000; Sebolt-Leopold 2004; Thompson and Lyons 2005; Wallace et al. 2005).

Given the diversity of ERK substrate proteins and the importance of the ERK pathway in normal cell function, one approach to the development of more effective and less toxic chemotherapeutic agents involves the selective inhibition of ERK activity that contributes to the disease state while preserving ERK functions in the context of normal

cells. To this end, we have used computational, biophysical, and biological approaches to identify and characterize small molecular weight compounds that interact with ERK proteins and interfere with substrate docking interactions (Chen et al. 2006; Hancock et al. 2005). The initial set of compounds was designed *in silico* to interact with a groove that is situated between the common docking (CD) and ED domain (Tanoue et al. 2001). The CD/ED docking domain has been shown to regulate protein interactions between ERK1/2 and the transcription factor ELK-1 (Zhang et al. 2003), and kinases such as p90RSK-1 (Dimitri et al. 2005). Both ELK-1 and p90RSK-1 are important regulators of cell proliferation in response to growth stimuli (Gille et al. 1995; Janknecht et al. 1993). In addition, ELK-1 and p90RSK-1 contain two important docking sites termed the D-domain and the FXFP motif or F-site, which are involved in determining substrate interactions with ERK and other MAP kinases (Fantz et al. 2001; Jacobs et al. 1999).

Computational methods for identifying low-molecular weight ERK inhibitors take advantage of the 3-dimensional structure of ERK2, which was solved by X-ray crystallography in its unphosphorylated and phosphorylated forms (Canagarajah et al. 1997; Zhang et al. 1994). Based on these structures, work in our laboratories has identified biologically active lead compounds with the potential to target the CD/ED domain using *in silico* modeling of the unphosphorylated (Hancock et al. 2005) or phosphorylated ERK2 structure (Chen et al. 2006). Ongoing studies are aimed at characterizing and improving the efficacy of these lead compounds.

Caenorhabditis elegans (C. elegans) is recognized as a powerful model organism for screening potential drug compounds and validating drug efficiency prior to more costly and time consuming *in vivo* studies (Artal-Sanz et al. 2006). *C. elegans* offers a variety of advantages in the drug development process. For example, the genetics, biochemical pathways, and developmental processes of *C. elegans* have been well characterized and share many basic features with higher organisms, including humans. In addition, *C. elegans* studies are cost effective. The organisms are easy to maintain in the laboratory and can be grown on agar plates or liquid medium with *E. coli* as a food source. Moreover, the reproductive life cycle of *C. elegans* is quite short, taking 3.5 days from egg to fully mature adult. Thus, developmental processes can be studied in a relatively short period of time. Lastly, many *C. elegans* mutant strains are readily available and can be used to explore protein functions and mechanisms of drug action.

Many important signal transduction pathways found in humans are conserved in *C. elegans.* For example, the development of the vulva structure and subsequent egg laying involves the highly conserved LET-23/LET-60/LIN-45/MEK-2/MPK-1 signaling pathway, which is homologous to the mammalian epidermal growth factor receptor (EGFR)/Ras/Raf/MEK/ERK signaling pathway (Lackner and Kim 1998). MPK-1 (also called Sur-1) shares approximately 80% homology with human ERK2 in the amino acid sequence and is the only ERK ortholog in *C. elegans* (Wu and Han 1994). As expected with such a high degree of homology, the amino acid sequences that are important for substrate recognition by the CD/ED docking domain are identical in ERK2 and MPK-1. Activated MPK-1 phosphorylates multiple downstream proteins including the LIN-1 ETS domain transcription factor (Jacobs et al. 1998). LIN-1 plays a critical role in the regulation of vulval cell fate (Miley et al. 2004), which is consistent with the requirement for the MPK-1 pathway in vulva formation and egg laying (Lackner and Kim 1998). LIN-1 is a repressor of vulva cell fate through its interactions with other transcription factors (Tan et al. 1998). However, phosphorylation of LIN-1 by MPK-1 may regulate vulva formation by relieving transcription repression and promoting gene expression (Miley et al. 2004; Tiensuu et al. 2005). As with many ERK substrates, LIN-1 contains an F-motif and a D-domain that are important determinants for interaction with MPK-1 (Miley et al. 2004). Importantly, evidence supports the interactions of the D-domain of substrate proteins with the CD/ED domain on ERK2 (Abramczyk et al. 2007).

The goal of these studies was to use *C. elegans* as a model organism to further evaluate lead compounds that we have identified to be promising inhibitors of ERK substrate phosphorylation. Our studies indicate that small molecules targeting MPK-1, the homolog to human ERK2, can inhibit its interactions with LIN-1 substrates and disrupt the development of the vulva and inhibit subsequent egg laying capabilities. The findings suggest that *C. elegans* is a useful model to evaluate the specificity and toxicity of promising lead compounds that target ERK2 interactions with specific substrate proteins and relevant physiological processes in a whole organism.

Materials and methods

Reagents ERK docking domain inhibitors were identified using computer aided drug design as previously described (Hancock et al. 2005) and purchased from ChemBridge (San Diego, CA). Compounds were dissolved in 100% DMSO and stored at −20° in 25 mM stock solutions. The maximal concentration of DMSO did not exceed 2% in the working solutions used in experiments involving *C. elegans*. LIN-1 and phospho-threonine MAPK/CDK substrate antibodies were obtained from Santa Cruz Biotech (Santa Cruz, CA) and Cell Signaling (Danvers, MA), respectively. Antibodies against phosphorylated ERK MAP kinase and α-tubulin were purchased from Sigma (St. Louis, MO).

General maintenance and strains of C. elegans *C. elegans* used in these studies included the wild-type N2, the transgenic CL2070 (*dvIs70*), and BA17 (*fem-1 (hc17)*) strains. Additional strains included OH103 (*mgIs21*), SU93 (*jcIs1*) and MT2124 (*let-60(n1046)*) that were obtained from the *Caenorhabditis* Genetic Center (University of Minnesota, Minneapolis, MN). All strains were cultured in nematode growth medium (NGM, 3 g/L NaCl, 17 g/L agar, 2.5 g/L peptone, 5 mg/L cholesterol, 25 mM KPO_4 (pH 6), 1 mM $MgSO_4$ and 1 mM $CaCl_2$) with *Escherichia coli* (*E.coli*) OP50 strain as the food source (Brenner 1974; Stiernagle 2006). *C. elegans* strains were maintained at 20°C except the BA17 (*fem-1 (hc17)*) strain, which was incubated at 25°C (Wilson et al. 2006).

Worm synchronization Two methods were used to obtain age-synchronized eggs. In the first method, adult hermaphrodites were transferred onto fresh NGM plates and allowed to lay eggs for 2–4 h and then removed. All the eggs released in that time period have been shown to be well synchronized (Epstein and Shakes 1995). In the second approach, *C. elegans* were grown in liquid medium and eggs were collected using the alkaline hypochloride method (Sulston and Hodgkin 1988). Briefly, *C. elegans* were added to a flask containing 200 mL of S Basal medium (100 mM NaCl, 50 mM potassium phosphate (pH 6) and 5 mg/L cholesterol) and fed with concentrated *E. coli* OP50. After shaking for 4 or 5 days at 20°C, the worms were transferred to a 50 mL sterile conical centrifuge tube and centrifuged at 1,000 rpm for 2 min (Stiernagle 2006). The supernatant was removed and the worm pellet was washed twice with M9 buffer (3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 5 g/L NaCl and 1 mM $MgSO_4$ $7H_2O$) followed by digestion with ten volumes of fresh hypochlorite solution (1.0–1.3% sodium hypochlorite, 500 mM potassium hydroxide). The samples were mixed by inversion, centrifuged, and the supernatant was removed. The digestion procedure was repeated until the worms broke apart and the carcass material was no longer visible (Sulston and Hodgkin 1988). The collected eggs were washed once with water, three times with M9 buffer, and then transferred to 10 cm NGM plates and kept at 20°C. The L1, L2, L3, L4 larvae and young adult worms were then collected after 10, 24, 34, 46 and 58 h, respectively, based on previous studies (Epstein and Shakes 1995). The stages of *C. elegans* development were confirmed by microscopy.

Microscopy *C. elegans* were transferred into a 20–30 µL drop of M9 buffer in the center of glass slide using a worm pick and gently covered by a coverslip. Excess M9 buffer was removed with filter paper in order to make sure that the *C. elegans* did not move. The worms were observed using a Nikon E800 Epi-fluorescence microscope (Image Systems, Columbia, MD) and captured with a Hamamatsu CCD camera. Worms were also observed and images captured under light microscopy. Captured images were processed and analyzed using IPlab software (Scanalytics, Fairfax, VA).

Immunoblot analysis Various staged larvae were washed three times using cold phosphate buffered saline (PBS) and then lysed with 50 µL of tissue lysis buffer (TLB; 20 mM Tris-base, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 10% glycerol, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM benzamidine) followed by sonication for 15 one second pulses on ice. The lysates were clarified by centrifugation at 20,000×g for 1 h and added to 2X SDS-PAGE sample buffer at a 1:1 ratio. Approximately 60 µg of total protein was separated by SDS-PAGE, transferred to PVDF membrane (Perkin Elmer Life Sciences; Boston, MA) and blocked for 1 h with 5% nonfat dry milk in Tris-buffered saline (TBS-T; 50 mM Tris-base, pH 7.4, 0.15 M NaCl, and 0.1% Tween-20). Following incubation with primary antibodies in TBS-T containing 1% BSA solution for 1 h, membranes were washed several times in TBS-T solution and incubated with HRP conjugated secondary antibodies (0.1 µg/ml). Membranes were washed extensively with TBS-T and immunoreactivity was detected by enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, England).

Egg-laying assay *C. elegans* egg laying was evaluated using previously described methods (Dempsey et al. 2005; Trent et al. 1983). Briefly, synchronized worms at different stages were incubated with the indicated test compounds on NGM plates. Young adult worms were transferred onto fresh plates (4 plates of 2 worms for each treatment). The number of eggs released from each worm was counted on the 4th day of the worm life cycle or as indicated in the text.

Survival assay Synchronized eggs from the BA17 (*fem-1 (hc17)*) temperature-sensitive strain were cultured on NGM plates containing *E. coli* OP50 at 25°C. At this temperature, the resulting adult worms are sterile and therefore can be used to evaluate survival (Wilson et al. 2006). At L4 stage, ninety BA17 larvae for each group were fed OP50 with and without compound **76**. The worms were transferred onto fresh NGM plates containing food with and without **76** every other day and counted each day until all worms were dead. Worms were scored as dead if they had no movement response to touch stimulus.

Lethality assay L4 stage wild-type N2 larvae were transferred into a 24-well cell culture plate containing 1 mL of S-medium with up to 500 µM of compound **76** and lethality

was measured as previously described (Dengg and van Meel 2004). Compound **76** became insoluble at concentrations of 1,000 μM or greater. Briefly, worms were incubated with the test compound at 20°C and the number of dead worms was recorded after 24 h. Worms treated with 2% DMSO was used as a vehicle control. Each experiment had eight worms per treatment and was repeated in three independent studies.

Stress-response assay An evaluation of non-specific stress responses due to the test compounds was performed using the CL2070 strain. The CL2070 strain contains a GFP reporter which is controlled by the heat shock promoter *hsp-16-2*. The *hsp-16-2* promoter is activated following exposure to heat shock (35°C for 2 h) or oxidative stress (Strayer et al. 2003). Synchronized L4 larvae were incubated with OP50 containing 100–500 μM of compound **76** at 20°C for 24 h. The activation of the *hsp-16-2* promoter was measured by observing expression of GFP reporter using fluorescence microscopy. Heat shock treated worms that were allowed to recover at 20°C for 12 h were used as a positive control.

Expression, purification and phosphorylation of LIN-1 The plasmid pAT2 encoding the fusion protein glutathione-S-transferase (GST):LIN-1 (C-terminal amino acids 241–441) was kindly provided by Dr. Kerry Kornfeld (Washington University School of Medicine, St. Louis, MI). BL-21 *E. coli* transformants were induced with 400 μM isopropyl thiogalactoside (IPTG) for 4 h at 37°C and protein extracts were obtained using BugBuster protein extract reagent (Novagen) supplemented with protease inhibitors. The GST:LIN-1 fusion protein was isolated by incubating with Glutathione Sepharose 4B (GE Healthcare, Piscataway, NJ), washed with excess 50 mM Tris (pH 8.0), and eluted with 10 mM glutathione in 50 mM Tris-HCl (pH 8.0). The glutathione was removed by dialysis for 2 h at 4°C against 25 mM Tris-HCl (pH 7.5), 5% Glycerol, 0.2 M NaCl, and 0.1% 2-mercaptoethanol. Samples were concentrated using an Amicon Ultra-4 Centrifugal Filter Unit, 10 kDa cutoff (Sigma) and aliquots were stored at −80°C. Purified GST: LIN-1 was incubated in the absence or presence of 10 ng active p42 ERK2 MAP kinase (New England Biolabs, Beverly, MA) in MAP kinase reaction buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM DTT, 1 mM EGTA, 0.01% Brij 35, pH 7.5) supplemented with 200 μM ATP at 30°C for 30 min. In some cases, the active p42 MAP kinase was preincubated with the test compound at room temperature for 15~20 min prior to adding the GST:LIN-1 substrate with test compounds. Following the kinase reaction GST-LIN-1 was re-isolated with Glutathione Sepharose 4B and incubated in the absence or presence of 2.5 μg λ-phosphatase (New England Biolabs) in λ-phosphatase reaction buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM $MgCl_2$, 2 mM DTT, 0.1 mM EGTA, 0.01% Brij 35, pH 7.5). LIN-1 phosphorylation was also examined in the presence of active CDK1 (New England Biolabs). Histone H1 (Sigma) was used as a control substrate for CDK1 activity. The kinase or phosphatase reactions were stopped by addition of 2× SDS-PAGE sample buffer. Phosphorylation of LIN-1 or histone H1 was examined by immunoblotting with the phospho-threonine MAPK/CDK substrate antibody.

Vulva formation Following treatment in the presence or absence of the test compound, vulva structure and cells were observed by light and fluorescence microscopy in wild type N2, OH103, and SU93 strains. The OH103 strain expresses the *lin-11* promoter driving GFP expression in vulA, vulB1, vulB2, vulC and vulD cells and was used to show cell fate specification (Hobert et al. 1998). The SU93 strain, AJM-1:GFP, contains the apical junction-associated protein, AJM-1 (formerly JAM-1), fused to GFP to monitor fusion of the symmetric halves of the vulva in a single organ (Hurd and Kemphues 2003; Michaux et al. 2001; Mohler et al. 2002). Images of vulval cell fusion were recorded and the percentage of worms containing cell fusion was calculated in control and treatment conditions. Cell fusion defects in worms were counted if a gap between the tips of the cellular extensions from the anterior and posterior vulA-D cells was observed. At least 20 worms were examined under each condition.

Statistical analysis Comparisons between control and treatment groups were performed with two population *t*-test using Origin 6.0 software (Microcal Software, Northampton, MA). Statistical significance was indicated if p values were less than 0.05. The standard error of the mean is shown in the figures.

Results

MPK-1 activation during the C. elegans life cycle MPK-1 activity plays a key role in the development of the vulva structure in *C. elegans* (Lackner et al. 1994; Wu and Han 1994). MPK-1 activation was examined during the development of *C. elegans* by evaluating the levels of phosphorylated MPK-1 protein in whole organism protein extracts using a phospho-specific antibody. Phosphorylated MPK-1 was first observed in L2 stage and reached peak levels during L3 stage (Fig. 1). Subsequently, phosphorylated MPK-1 levels decreased in L4 stage and in young adult worms (Fig. 1). These results are in agreement with previous findings demonstrating that MPK-1 activation is required for vulva development (Lackner et al. 1994). Thus,

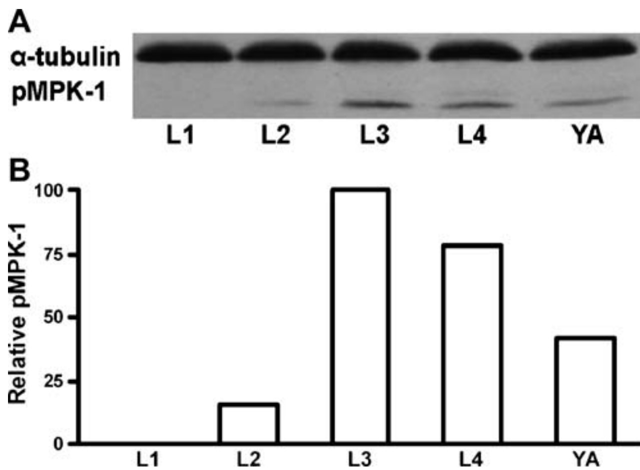

Fig. 1 Activation of MPK-1 during the *C. elegans* life cycle. Synchronized eggs were cultured as described in the methods and worms were collected at L1, L2, L3, L4 larvae and young adult stages. (**A**) Total protein was extracted and immunoblotted for active phosphorylated MPK-1 (pMPK-1) using a phospho-ERK MAP kinase antibody and α-tubulin as the protein loading control. (**B**) Quantification of the ratio of pMPK-1 to α-tubulin as measured by densitometry scanning

we sought to use the well-characterized process of vulva development and egg laying as a model for evaluating test compounds that are proposed to inhibit ERK interactions with substrate proteins.

Effect of MPK-1 inhibition on egg laying In the first set of studies to characterize the model, we established the time course for egg laying in controls and worms treated with a lead ERK inhibitor compound. Our studies focused on a putative ERK inhibitor, referred to as compound **76**, that we have previously reported to inhibit ERK substrate phosphorylation in cultured mammalian cells (Hancock et al. 2005). Synchronized eggs were isolated and grown in the absence or presence of **76** or the MEK inhibitor, U0126, added to the OP50 food source. When worms reached adulthood (day 3), the number of eggs produced were counted each day for the next 5 days (days 3 through 8). Untreated worms showed peak egg production at day 4 (Fig. 2A), which is consistent with previous studies (Byerly et al. 1976). Worms treated with **76** produced 50% fewer eggs at day 4 compared to untreated controls (Fig. 2A). As a control, treatment with U0126 also inhibited the number of eggs produced at day 4 and is consistent with the requirement for MPK-1 in this process.

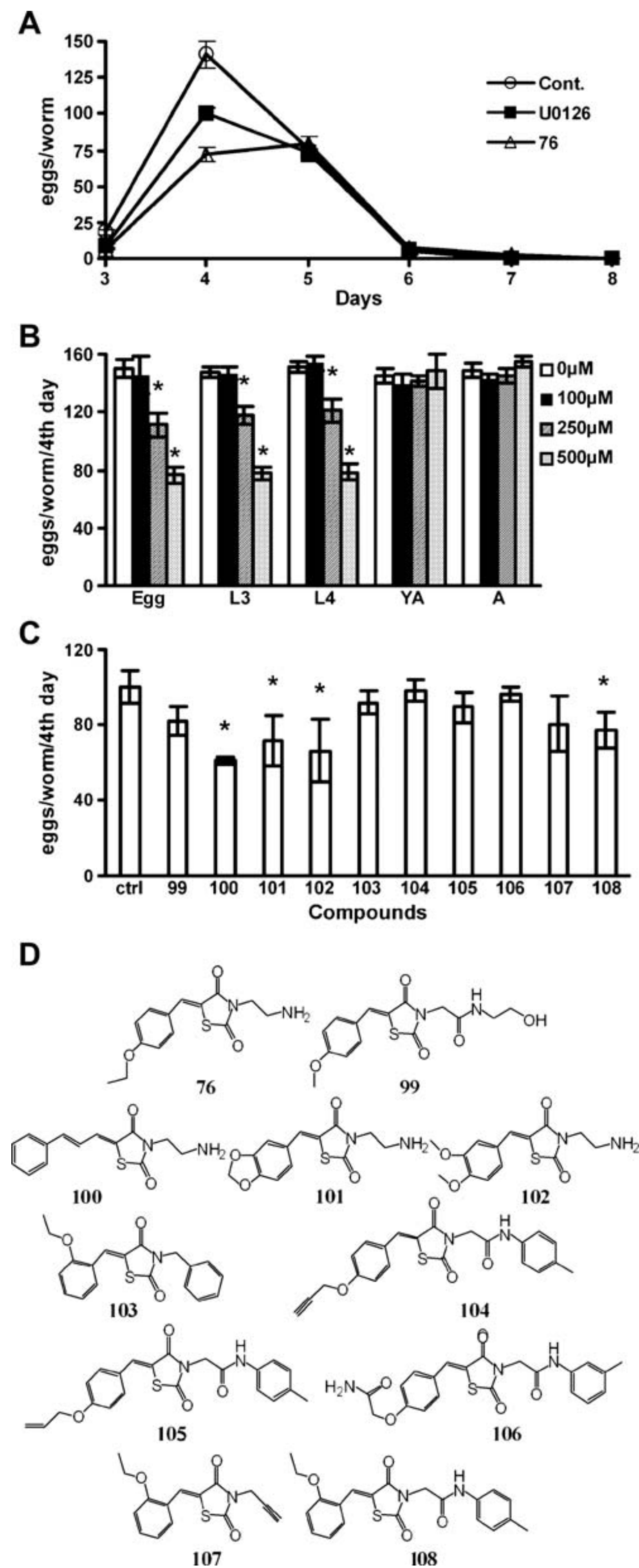

Fig. 2 Effects of ERK docking domain inhibitors on *C. elegans* egg laying. (**A**) Synchronized eggs were grown on NGM plates in the absence (Control) or presence of U0126 (50 μM) or compound **76** (500 μM). The number of eggs released was recorded from the 3rd day to the 8th day of the worm life cycle. Eggs from eight worms in each group were counted. (**B**) Synchronized eggs, L3, L4, young adult (YA) or adult worms (A) were incubated with 100, 250 or 500 μM of compound **76** and then the number of eggs laid was calculated during the 4th day of the worm life cycle. (**C**) Synchronized L4 larvae were treated with the indicated compounds (500 μM) whose structures were identified by *in silico* modeling to be similar to **76** and the number of eggs laid was calculated during the 4th day of the worm life cycle. (**D**) Chemical structures for **76** and structurally similar compounds **99**–**108**

The next set of experiments established the developmental stage in which **76** affected egg laying behavior. Synchronized eggs, L3, L4, young adult, or adult worms were incubated with OP50 supplemented with different concentrations of **76** and the number of eggs per worm was determined on day 4 of the *C. elegans* life cycle as described above. Egg laying was inhibited in a dose dependent manner when compounds were added to eggs, L3, or L4 larvae (Fig. 2B). In contrast, treatment of young adult or adult worms with compound **76** had no affect on the number of eggs laid by these worms. These data are consistent with the requirement for MPK-1 in vulva development and that inhibition of MPK-1 functions prior to vulva formation influences egg laying capacity.

To evaluate additional compounds with structural similarities to **76**, a computer aided drug design similarity search was performed and ten new compounds were obtained from commercial sources. The similarity search was based on MAC_BITS chemical fingerprints quantified using the Tanimoto Index as implemented in the program MOE (Chemical Computing Group) and as previously described (Macias et al. 2005). These compounds were evaluated for inhibition of egg laying with four compounds (**100**, **101**, **102**, and **108**) causing significant inhibition of egg laying similar to **76** (Fig. 2C). The structures of **76** and the similarity search compounds are shown in Fig. 2D. These studies identify a class of compounds that appear to inhibit *C. elegans* egg laying by targeting MPK-1 function. It is anticipated that members of this class of compounds will be amenable to lead optimization studies with the goal of maximizing the affinity and specificity of the compounds as inhibitors of ERK.

Evaluation of non-specific effects and general toxicity of compound 76 The effective concentration of **76** used in the *C. elegans* egg laying studies shown in Fig. 2 was relatively high (250–500 μM) and approximately 4–6 times higher than effective concentrations used in previous studies with cultured mammalian cells (Hancock et al. 2005). In order to exclude the possibility that inhibition of egg laying behavior is caused by non-specific toxicity due to high concentrations of test compound, we evaluated the effect of **76** on the life span of *C. elegans.* As shown in Fig. 3, the presence of 500 μM compound **76** had no significant effect on the average life span as compared to untreated worms.

The potential for toxicity of **76** was further assessed using a lethality assay where *C. elegans* were exposed to increasing concentration of test compound. Compound **76** at concentrations of up to 500 μM had no effect on lethality after 1 day exposure (data not shown). Increasing the concentrations of **76** to 1,000, 1,500, 2,000 μM were lethal to approximately 0%, 10%, 25% of the worms, respectively (data not shown). However, **76** became insoluble at these doses so the effective lethal dose can not be accurately determined. Nonetheless, these data suggest that concentrations of **76** that inhibit egg laying are not due to general lethality of the compound or effects on *C. elegans* life span.

Additional evidence for **76** effects on non-specific stress responses was evaluated using the transgenic strain CL2070 containing the HSP-16-2 promoter driving GFP expression. The HSP-16-2 promoter responds to general stress stimuli, which can be evaluated by the degree of GFP expression. As shown in Fig. 4, the dose of **76** that inhibited egg laying had no effect on HSP-16-2 dependent GFP expression. These data further support the findings that the effects of **76** on egg laying were not due to a non-specific stress response or toxicity to the organism.

Compound 76 inhibition of egg laying involves targeting MPK-1 mediated LIN-1 phosphorylation MPK-1 regulation of vulva formation involves phosphorylation of the transcription factor LIN-1, which is homologous to human Elk-1 (Jacobs et al. 1998; Miley et al. 2004). LIN-1, which contains a D-domain that may interact with the CD/ED domain on ERK2, is an important cell fate regulator of vulva cells (Miley et al. 2004). To test whether **76** targets MPK-1, the C-terminus of LIN-1, which contains the D-domain and phosphorylation sites was purified as a GST fusion protein and incubated with activated ERK2 in the presence or absence of **76**. Active ERK2 enhanced the phosphorylation of LIN-1 as detected with a phospho-threonine MAP kinase substrate specific antibody (Fig. 5A). The ERK-mediated LIN-1 phosphorylation, (pT)LIN-1, was inhibited in the presence of **76** or following phosphatase treatment (Fig. 5A). Treatment with compound **76** had no effect on the level of phosphorylated ERK,

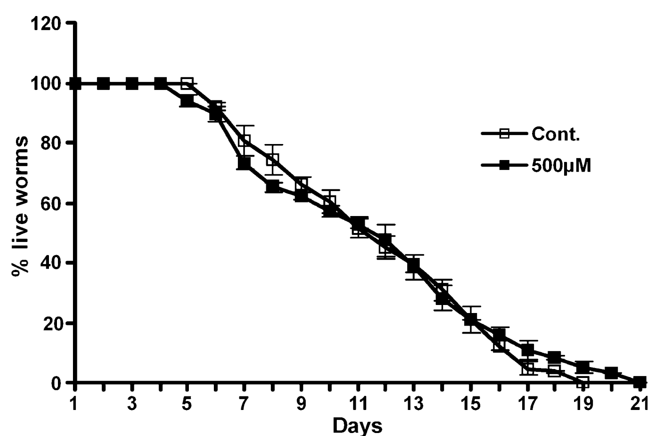

Fig. 3 Compound **76** does not affect *C. elegans* life span. At L4 stage, the BA17 *(fem-1 (hc17)) C. elegans* strain was exposed to **76** (500 μM) or DMSO as a control. Three plates containing 30 worms each were used for each condition. Worms were transferred onto new plates every other day in the presence or absence of compound **76**. The number of live animals was recorded each day for 20 days

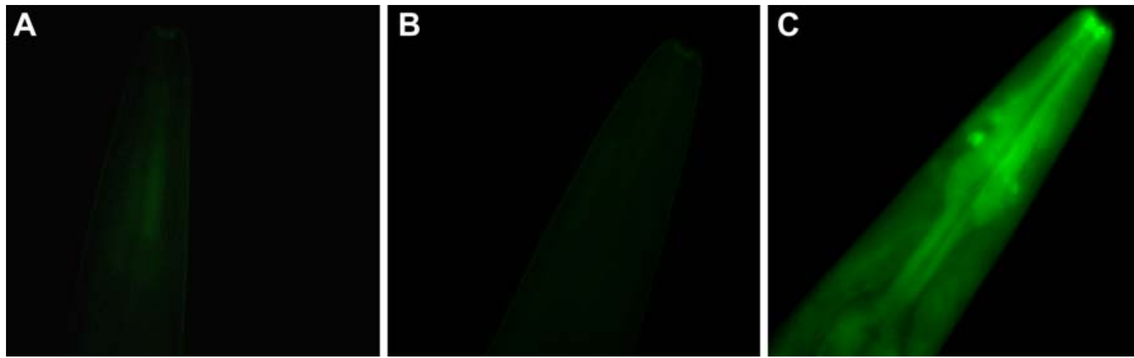

Fig. 4 Compound **76** does not induce Hsp-16 stress response. Fluorescent images of L4 larvae from the CL2070 strain containing a GFP reporter, which is controlled by heat shock promoter *hsp-16-2,* following 24 h treatment with 2% DMSO as a control (**A**) or 500 µM of compound **76** (**B**). As a positive control worms were heat shocked at 35°C for 2 h followed by a 12 h recovery at 20°C (**C**). Fluorescent images were taken using the same exposure time and are representative of at least three independent experiments

which supported the concept that the effects of **76** on LIN-1 phosphorylation were due to disruption of protein-protein interactions. Activated ERK2 caused a dramatic retardation of LIN-1 gel mobility (Fig. 5A), which is due to phosphorylation as observed previously (Tan et al. 1998). This was confirmed by demonstrating that phosphatase treatment could restore the faster migrating form of LIN-1 (Fig. 5A). Compound **76** restored some of the faster migrating forms of LIN-1 and could be due to partial inhibition of all ERK-mediated LIN-1 phosphorylation sites or selective inhibition of some sites (Fig. 5A). To further confirm the specificity of the ERK-mediated LIN-1 phosphorylation, LIN-1 was incubated with activated cyclin dependent kinase 1 (CDK1 or Cdc2), another proline directed serine/threonine kinase. Again, LIN-1 phosphorylation was evaluated by the phospho-threonine (pT) specific antibody that recognizes proline directed phosphorylations mediated by CDK or MAP kinase proteins. No phosphorylation of LIN-1 by CDK1 was observed (Fig. 5B). Importantly, CDK1-mediated threonine phosphorylation of a histone substrate, (pT) histone, was unaffected by **76** (Fig. 5B). These findings help support the specificity for **76** in the inhibition of MPK-1 mediated phosphorylation of LIN-1 but not general inhibition of proline directed phosphorylation events.

*Effect of compound **76** on vulva formation* Having established that **76** may affect egg laying by inhibiting MPK-1 mediated phosphorylation of the LIN-1 substrate involved in vulva formation, the next series of experiments examined structural changes in the vulva following treatment with **76**. The overall vulva structure as viewed by light microscopy did not appear to change in L4 larvae treated with or without **76** (Fig. 6A and B). The first set of experiments took advantage of transgenic strains, OH103 (*lin-11::GFP*) and SU93 (*ajm-1::GFP*), expressing GFP driven by a promoter that is activated in vulva or uterine cells (see Methods for description of strains). In these studies, eggs from the OH103 strain were treated with and without **76** and GFP patterns in L3 or L4 larvae were examined. As shown in Fig. 6C and D, GFP expression could be observed in vulva precursor cells. However, no distinct changes in vulva cell patterns were observed in controls or **76** treated worms and control and treated cells contained a similar number of GFP positive cells, respectively (Fig. 6G). Next, eggs from the transgenic strain SU93, which express the apical junction-associated AJM-1-GFP chimeric protein and can evaluate vulva cell fusion during L4 stage (Gupta et al. 2003; Hurd and Kemphues 2003; Inoue et al. 2002), were treated with and without **76**. As compared to controls, treatment with **76** caused an apparent defect in vulva cell fusion as shown by the 2 fold inhibition of the joining of the two symmetric halves of homologous cells that will form the functional vulva structure (Fig. 6E, F and H). These findings indicate that while **76** does not significantly influence the generation of vulva precursor cell types, it does inhibit the ability for these cells to form a functional vulva.

*Effects of **76** on the LET-60 multivulval phenotype* Constitutively active LET-60 is the *C. elegans* ortholog to the human RAS protein that is mutated in a variety of human cancers. Active LET-60 causes constitutive activation of the MPK-1 pathway and results in the generation of a multivulval (Muv) phenotype (Beitel et al. 1990). The Muv phenotype of this mutant strain includes one functional vulva and one or more pseudovulvae, which present themselves as round or other asymmetric invaginations (Fig. 7A). Inhibition of MPK-1 pathway with the MEK inhibitor U0126 has been shown to reduce the Muv phenotype in LET-60 expressing worms (Reiner et al. 2008). Therefore, this transgenic strain is a useful tool for characterizing the efficiency of putative ERK pathway inhibitors on vulva formation. The Muv phenotype was found in 92.3% of mutant worms and the average number of vulvae was 2.75 in the DMSO vehicle-treated controls

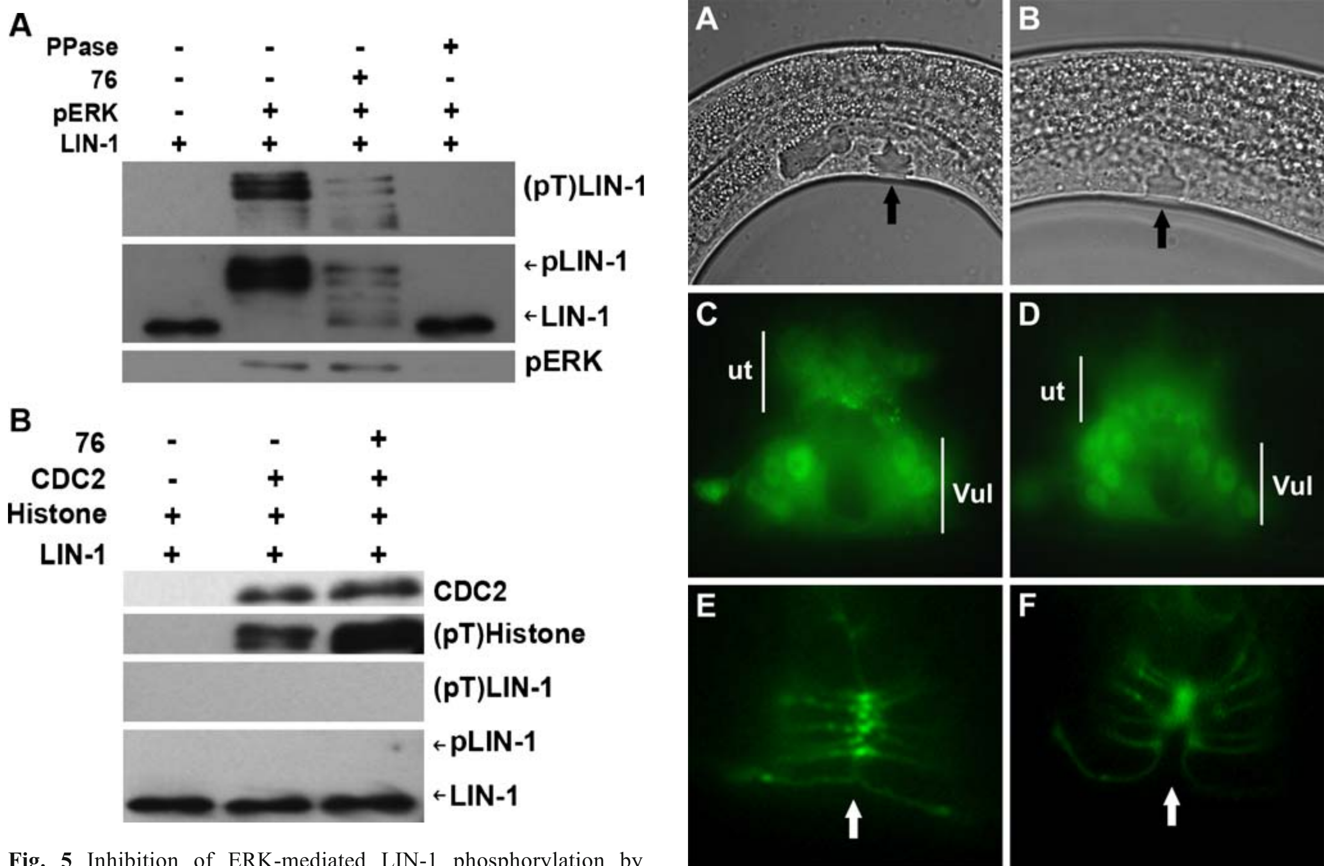

Fig. 5 Inhibition of ERK-mediated LIN-1 phosphorylation by compound **76**. (**A**) GST tagged LIN-1 protein was incubated with active ERK2 MAP kinase in the absence or presence of **76** (100 μM) or λ-phosphatase (PPase). The proteins were immunoblotted with antibodies against LIN-1, phospho-threonine MAPK/CDK substrates (pT), total LIN-1, and phosphorylated MAP kinase (pERK) in the top, middle, and lower panels, respectively. (**B**) Effect of compound **76** on LIN-1 and histone phosphorylation by CDK1. Histone and GST-LIN-1 proteins were incubated with CDK1 (CDC2) kinase in the absence or presence of 100 μM compound **76**. The proteins were detected with antibodies against CDC2, phospho-threonine MAPK/CDK substrates (Histone and LIN-1), and total LIN-1 in the top, middle, and bottom panels, respectively

(Fig. 7A, D and E). In contrast, only 23% of the worms incubated with **76** showed the Muv phenotype and the average vulva number per worm was reduced to 1.32 (Fig. 7B, D, and E). As a control and in agreement with previous reports (Reiner et al. 2008), the MEK inhibitor, U0126, also inhibited the Muv phenotype in LET-60 mutant worms (Fig. 7C, D, and E).

Discussion

The ERK1/2 signaling pathway represents a key target for the development of clinically effective anticancer agents. To date, several inhibitors of upstream ERK regulators including tyrosine kinase receptors, Ras, Raf, and MEK proteins have been developed with limited clinical success

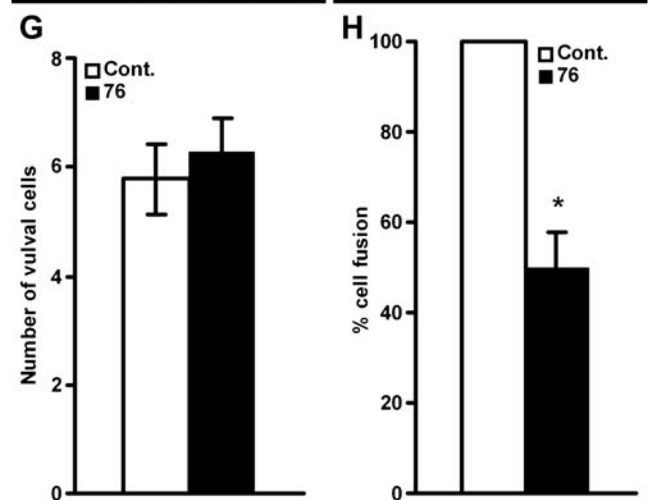

Fig. 6 Effect of compound **76** on vulva formation. Gross structural morphology of vulva in wild-type N2 strain treated in the absence (**A**) or presence (**B**) of 500 μM compound **76**. Organization and fusion of vulva cells was observed using the OH103 strain, which expresses GFP driven by the *lin-11* promoter in uterine (ut) and vulva (vul) cells, and the SU93 strain, which expresses the AMJ-1-GFP chimeric protein during vulva cell fusion. GFP expression in untreated (**C** and **E**) and **76** treated (**D** and **F**) worms was examined by fluorescence microscopy in the OH103 (**C** and **D**) or SU93 (**E** and **f**) strains. **g** Quantification of the number of GFP positive vulval (Vul) precursor cells in the absence or presence of **76** in the OH103 strain. (**H**) Quantification of percentage of vulva in SU93 worms showing fusion of the two symmetric halves in the absence or presence of compound **76**. 20 worms were analyzed under each condition

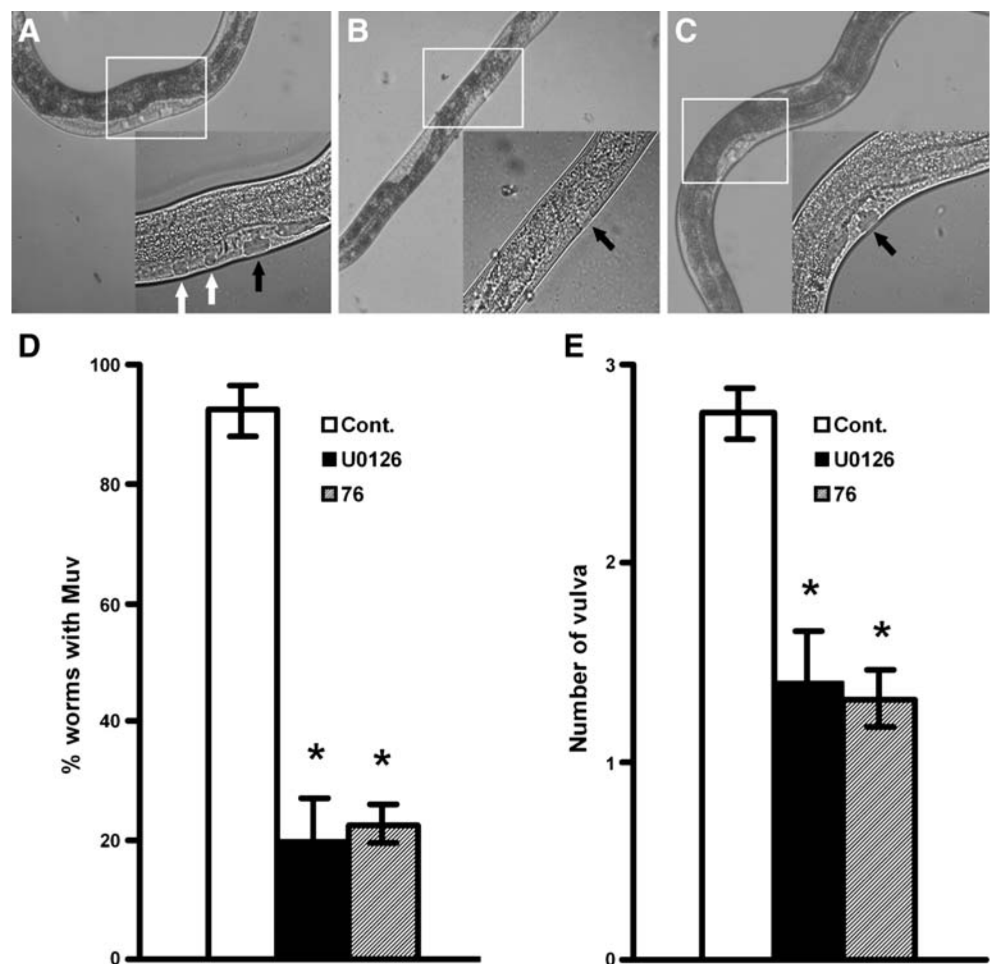

Fig. 7 Compound **76** reduces Ras induced multivulval (Muv) phenotype. Eggs from the *Let-60* (n1046gf) strain containing the ortholog to human Ras mutations were treated in the absence or presence of 500 μM of compound **76** or 50 μM of the MEK inhibitor U0126. Images show control (**A**), **76** treated (**B**), or U0126 treated (**C**) L4 stage worms. The boxed areas in the insets of each panel show the vulva and pseudovulvae marked by the black and white arrows, respectively. The graphs show the percentage of worms containing the Muv phenotype (**D**) and the average number of vulvae per worm (**E**) under each condition. The microscopic images in panels **A**, **B** and **C** were taken at 100× and the insets were magnified 600×. Fifteen to twenty worms were analyzed under each condition

(Bollag et al. 2003; English and Cobb 2002; Kohno and Pouyssegur 2003; Kohno and Pouyssegur 2006; Sebolt-Leopold 2004; Wallace et al. 2005). Thus, new approaches for inhibiting ERK signaling events and cell proliferation are needed. The ubiquitous nature of ERK1/2 expression in all cell types presents a challenge for the development of ERK pathway inhibitors that are selective for ERK and its functions in promoting cancer cell growth and survival while preserving ERK actions in normal cells. We have recently described an approach using computational and biological methods to identify ERK inhibitors that have the potential to be substrate selective such that ERK activation is intact but the ability for ERK to interact with and regulate substrates involved in cell proliferation is inhibited (Chen et al. 2006; Hancock et al. 2005). To our knowledge, these studies are the only ones to report the identification of ERK inhibitors that act independent of ATP binding and have the potential to be substrate selective. Other studies have recently described the identification of pyrazolylpyrrole-based compounds that inhibit ERK proteins by competing with ATP binding (Aronov et al. 2007; Ohori et al. 2005).

Our findings support the use of *C. elegans* as a model organism for identifying, screening, and characterizing potential lead pharmacological agents that target specific signaling proteins. We have applied this model to evaluate the specificity and toxicity of a promising lead compound (**76**) that is predicted to target protein interactions with an ERK docking domain, referred to as the common docking (CD) domain. The *C. elegans* model is recognized to offer many advantages in the drug discovery and development process. In the context of the current studies, the ERK signaling process in *C. elegans* and its involvement with vulva formation and egg laying is well-characterized. Others have also used *C. elegans* models to evaluate inhibitors of Ras proteins, which are commonly mutated in human cancers (Reiner et al. 2008). This report took advantage of the Ras-induced multivulval phenotype for analysis of inhibitory compounds similar to the current studies (Fig. 7). While the possibility exists that lead compound **76** is reducing egg laying by affecting other targets in the Ras-Raf-MEK-ERK pathway, our previous reports demonstrate that the inhibitory effects of **76** are at the level of ERK interactions with downstream substrates, such as p90RSK-1 or Elk-1, and does not interfere with ERK interactions with upstream regulatory kinases, such as MEK1/2 (Hancock et al. 2005). This is consistent with

other reports that demonstrate that the CD domain is more important for ERK interactions with substrates such as p90RSK-1 but not for interactions with MEK proteins (Robinson et al. 2002).

One of the concerns of using the *C. elegans* model for drug discovery is the determination of the effective dose for a lead compound. The test compounds used in these studies were provided to the worms through the *E. coli* food source. Thus, the effective concentration absorbed through the intestinal lumen is not known. *C. elegans* also has an outer exoskeleton or cuticle that is synthesized and shed during each larvae stage and may provide protection from environmental chemicals (Page and Johnstone 2007). The presence of the exoskeleton could likely interfere with the absorbance of test compounds not ingested through the food source. Regardless, the concentrations of **76** used in these studies did not appear to induce general toxicity or reduce the worm life span.

In addition to affecting vulva formation, inhibition of MPK-1 may affect other processes, such as germline development during meiosis, that impact egg laying behavior (Lee et al. 2007). In *C. elegans* hermaphrodites, spermatogenesis occurs transiently during L3 and L4 larval stages whereas oogenesis occurs from L4 until adulthood (Lee et al. 2007). In male worms, spermatogenesis occurs from L3 until adulthood (Lee et al. 2007). Thus, it is possible that inhibition of MPK-1 with **76** in larval stages up to and including L4 could affect germline maturation, subsequent fertilization, and ultimately egg laying. However, treatment of young adult worms with **76** did not affect egg laying behavior indicating that oocyte maturation at this time of the life cycle and subsequent fertilization by mature sperm was not likely affected (Fig. 2).

An important finding of these studies demonstrated that **76** inhibited ERK-mediated LIN-1 phosphorylation (Fig. 5). As LIN-1 is an important regulator of precursor vulva cell fate (Tiensuu et al. 2005). Reduced LIN-1 phosphorylation and activity could explain the observed defects in vulva formation (Fig. 6). LIN-1 contains a D-domain (also called DEJL site or *d*ocking site for *E*RK or *J*NK, *L*XL residues) and an F-site (also called a DEF motif or *d*ocking site for *E*RK, *F*XFP residues) (Fantz et al. 2001). These residues make contacts with distinct docking domains on ERK proteins to confer substrate specificity and it is thought that these domains play a role in determining what residues will be phosphorylated (Fantz et al. 2001). The CD and ED domains, which correspond to residues D316/D319 and T157/T158, respectively, on ERK2 are the best characterized regions involved in substrate interactions (Tanoue et al. 2000). The CD/ED docking domain residues have been shown to coordinate protein interactions between ERK and substrates containing the D-domain, which include the dual specificity phosphatase MKP-3 (Zhang et al. 2003), ELK-1 (Abramczyk et al. 2007; Fantz et al. 2001), RSK-1 (Dimitri et al. 2005), caspase-9 (Martin et al. 2008). Another ERK2 docking pocket consisting of residues L198, L232, L235, and Y261 may mediate ERK2 interactions with the F-site on substrates (Dimitri et al. 2005; Sheridan et al. 2008). The fact that LIN-1 contains both a D-domain and an F-site may account for the partial effects of **76** on inhibiting ERK-mediated phosphorylation (Fig. 5). While the findings suggest that **76** may disrupt ERK interactions with LIN-1 by targeting the region around the CD/ED domain, the exact nature of the binding interactions between **76** and ERK have yet to be determined.

Our findings indicate that inhibition of egg laying may be a result of the improper formation or assembly of vulva cells (Fig. 6). These findings suggest that the fusion of homologous cells from each half of the vulva is inhibited in the presence of **76**. One explanation for this observation is that inhibition of MPK-1 mediated LIN-1 phosphorylation by **76** prevents expression of LIN-39, a transcription factor that regulates the process of vulva cell fusion (Wagmaister et al. 2006). Alternatively, **76** may directly inhibit phosphorylation of LIN-39, which has been demonstrated to be a substrate of MPK-1 *in vitro* (Wagmaister et al. 2006). Another consideration is that **76** may affect other signaling pathways, such as the Wnt pathway, which regulates vulva development potentially through regulation of LIN-39. Nonetheless, future examination of these potential interactions along with other signaling events in the *C. elegans* model will help further define the mechanisms of action for novel kinase targeted compounds.

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