Identification and Synthesis of DDI-6, a Quinolinol Analog Capable of Activating Both Caenorhabditis elegans and Mouse Spermatozoa

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Sperm activation is an essential process by which the male gametes become capable of fertilization. Because the process in Caenorhabditis elegans is readily reproducible in vitro, this organism serves as an excellent model to investigate it. C. elegans sperm activation in vivo occurs during spermiogenesis. Membranous organelles (MOs) contained within spermatids fuse with the plasma membrane, resulting in extracellular release of their contents and relocation of some proteins indispensable for fertilization from the MO membrane onto the sperm surface. Intriguingly, these cytological alternations are exhibited similarly in mouse spermatozoa during the acrosome reaction, which also represents a form of sperm activation, prompting us to hypothesize that C. elegans and mice share a common mechanism for sperm activation. To explore this, we first screened a chemical library to identify compounds that activate C. elegans spermatozoa. Because a quinolinol analog named DDI-6 seemed to be a candidate sperm activator, we synthesized it to use for further analyses. This involved direct dechlorination and hydrogenolysis of commercially available 5-chloro-8-quinolinol, both of which are key steps to yield 1,2,3,4-tetrahydro-8-quinolinol, and we subsequently introduced the sulfonamide group to the compound. When C. elegans spermatids were stimulated with solvent alone or the newly synthesized DDI-6, approx. 3% and approx. 28% of spermatids became MO-fused spermatozoa, respectively. Moreover, DDI-6 triggered the acrosome reaction in approx. 20% of mouse spermatozoa, while approx. 12% became acrosome-reacted after mock stimulation. Thus, DDI-6 serves as a moderately effective activator for both C. elegans and mouse spermatozoa.

Key words  quinolinol analog; sperm activation; Caenorhabditis elegans; mouse; membranous organelle fusion; acrosome reaction

Introduction

Spermatozoa from many animal species need to be activated before they become competent to fertilize oocytes. 1) During Caenorhabditis elegans spermiogenesis—the final phase of spermatogenesis—motile spermatozoa are formed by extension of pseudopods from round spermatids 2–4) (Fig. 1A). Sperm activation occurs during this process and allows membranous organelles (MOs), specialized secretory vesicles that are contained within spermatids, to fuse with the plasma membrane (PM) 2–4) (Fig. 1A). This results in the extracellular release of MO contents 2–4) and the relocation of some SPE-9 class proteins, which are indispensably required for fertilization, from the MO membrane onto the sperm PM. 4) In the mouse, the acrosome reaction is one event representing sperm activation. 1) Intriguingly, as shown in Fig. 1B, cytological alternations that occur during the acrosome reaction are similar to those exhibited during C. elegans MO fusion. 4) For instance, one involves the extracellular release of acrosomal contents, 1) and another is the relocation of IZUMO1, a sperm protein that is essential for gamete fusion, onto the surface of the spermatozoon's equatorial segment, where spermatozoa fuse with the oocyte's PM. 5)

Sperm activation is important for successful male reproduction, but its molecular basis is largely unknown in any animal species. Cytological similarities of sperm activation between C. elegans and the mouse imply that these two species might have a common molecular basis for this process. To explore this, we can utilize C. elegans for studies of sperm activation; C. elegans spermiogenesis, including sperm activation, is readily reproducible in a simple, chemically defined medium, 6) enabling us to seek for compounds that induce or block C. elegans spermiogenesis. If such compounds show similar effects on mouse sperm activation, those compounds would be useful to identify molecules that are commonly involved in the two species. Indeed, we have identified a compound named DDI-1 that blocks C. elegans spermiogenesis in vitro, and several DDI-1 analogs acted as probes to uncover a hitherto unknown pathway for C. elegans spermiogenesis. 6)

In this study, screening of a chemical library identified several compounds to induce spermiogenesis in vitro in C. elegans spermatids. Then, we synthesized a quinolinol analog named DDI-6—one of the positive compounds—and showed its capability to trigger both MO fusion in C. elegans spermatozoa and the acrosome reaction in mouse spermatozoa. These results suggest that DDI-6 could be used as a probe to identify a conserved mechanism for both C. elegans and mouse sperm activation.

Results and Discussion

DDI-6 Discovered by Screening of a Chemical Library

We screened a chemical library to obtain compounds capable of activating spermiogenesis in spermatids from C. elegans
him-8(tm611) males (for details, see “Experimental”). Eventually five compounds were found to trigger spermiogenesis in approx. 20–80% of total spermatids with no records about their activities on any biological phenomena. One of such compounds, named DDI-6, was a quinolinol analog and chosen for further analyses.

**The Quinolinol Analog DDI-6 Synthesized Using a Four-Step Procedure** We produced DDI-6 chemically to use it for *C. elegans* and mouse sperm activation assays. The synthesis started by dechlorination of 5-chloro-8-quinolinol (compound 1a) and its derivatives (compounds 1b–1d) with magnesium metal, but these reactions failed to yield 8-quinolinol derivatives. Therefore, we screened for reaction conditions using a palladium catalyst (Table 1). When compound 1a was treated with 10% palladium on carbon in various solvents, such as ethanol (EtOH) alone, tetrahydrofuran (THF) alone, and a mixture of THF, methanol (MeOH) and acetic acid (AcOH) (2:3:3), dechlorination did not occur (Table 1, entries 1–3). However, we found that a reaction performed in a mixture of THF and MeOH (1:2) could produce 1,2,3,4-tetrahydro-8-quinolinol (compound 3) without formation of 8-quinolinol derivatives such as compounds 2a–2d (Fig. 2 and Table 1, entry 4). The reaction was slightly improved in a mixture of THF and MeOH (1:1) to give compound 3 with a 72% yield (Table 1, entry 5). As shown in Fig. 2, N-sulfonylation of compound 3 was then conducted with pyridine and 4-acetamidobenzensulfonyl chloride to form compound 4 with a 56% yield. Deacetylation of the acetamido group in compound 4 under aqueous acidic conditions produced N-(4-amino benzensulfonyl)-1,2,3,4-tetrahydro-8-quinolinol (compound 5; Fig. 2), and subsequent treatment of this with methyl chlorofomate afforded the final product, DDI-6, with a 39% yield (Fig. 2).

**DDI-6 Triggers MO Fusion in *C. elegans* Spermatozoa**

We determined the ratios of cells completing spermiogenesis (spermatozoa formed with MO fusion) after stimulation of spermatids with several activators including the newly synthesized DDI-6 (Fig. 3). In the absence of activators (control), approx. 97% of total cells exhibited rounded morphology, and the PMs of the same cells were fluorescently stained with FM1-43 (Figs. 3A, B). Thus, neither pseudopod extension nor MO fusion were triggered substantially in spermatids in the absence of activators (Table 1).
absence of activators.

When spermatids were stimulated with 50 µM DDI-6, pseudopods were extended from approx. 28% of the cells. Moreover, punctate signals were observed by FM1-43-staining in nearly all of the pseudopod-bearing cells (Figs. 3A, B). We also tested the bacterial protease mixture Pronase (Pron) and the bacterial serine protease Proteinase K (ProK) as positive control activators. Indeed, 200 µg/mL Pron and ProK activated approx. 88 and approx. 99% of spermatids, respectively, to initiate spermiogenesis (Figs. 3A, B). Patterns of pseudopod extension and MO fusion with DDI-6 were indistinguishable from those induced by Pron and ProK (Fig. 3A), indicating that DDI-6 partly but significantly produced pseudopod-extended sperm with MO fusion.

Because Pron and ProK are water-soluble proteases, perhaps these two enzymes extracellularely degrade spermatid proteins, leading to initiation of spermiogenesis. Indeed, the previous genetic study implied that the C. elegans serine protease TRY-5 might be a physiological activator for spermiogenesis. Contrarily, DDI-6 indicated a low solubility in water, while it was highly soluble in organic solvents such as acetone, chloroform, dimethyl sulfoxide (DMSO), and EtOH (data not shown). This property presumably provides the PM-permeability to DDI-6, because of the PM’s hydrophobicity,
so that the compound might act within cells. So far several agents have been reported to induce *C. elegans* spermiogenesis in vitro, such as monensin (cationic ionophore),\(^{14}\) triethanolamine (TEA, weak base),\(^{15}\) trifluoperazine (TFP, calmodulin inhibitor),\(^{12}\) chlorpromazine (CPZ, calmodulin inhibitor),\(^{12}\) N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7, calmodulin inhibitor),\(^{12}\) 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS, chloride channel blocker),\(^{16}\) wortmannin (phosphatidylinositol-3 kinase inhibitor),\(^{17}\) labile zinc,\(^{18}\) and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, mitogen-activated protein kinase activator).\(^{19}\) In particular, predicted targets of TFP, CPZ, W7, wortmannin, and AEBSF are intracellular proteins, thereby DDI-6 possibly interacts with intracellular molecules to induce spermiogenesis in *C. elegans* spermatids.

**DDI-6 Also Triggers the Acrosome Reaction in Mouse Spermatzoa** Because DDI-6 seemed to be a moderate activator of *C. elegans* spermiogenesis (Fig. 3), we examined its capability to induce the acrosome reaction in mature spermatzoa from the cauda epididymis by Coomassie Brilliant Blue (CBB)-staining\(^{20}\) (Fig. 4). When mouse spermatzoa were mock stimulated, approx. 12% became acrosome-reacted (Figs. 4A, B). Therefore, it was likely that such levels of acrosome-reacted sperm would arise spontaneously in control treatments.

After being treated with 100 µM DDI-6, approx. 20% of all spermatzoa were acrosome-reacted. Because it is well known that the calcium ionophore A23187 can induce the acrosome reaction,\(^{21}\) this drug was also examined as a positive control activator. As expected, approx. 82% of sperm were acrosome-reacted in the presence of 10 µM A23187. Moreover, DDI-6-induced acrosome-reacted sperm were similar to A23187-induced acrosome-reacted sperm in cytology. These results demonstrate that DDI-6 is also a moderate activator for the acrosome reaction in mature mouse spermatzoa. It is worthwhile to note that A23187 does not affect *C. elegans* sperm activation.\(^{14,15}\)

**DDI-6 Can Be Utilized to Investigate a Mechanism for Sperm Activation Shared by *C. elegans* and Mice** There is an evolutionary distance of approximately one billion years between *C. elegans* and rodents,\(^{22}\) and genes controlling reproductive traits generally evolve faster than somatic genes.\(^{23-25}\) However, it is not totally unlikely that these two species have a common mechanism for sperm activation. We have previously reported that *C. elegans* SPE-45 acts during gamete fusion as a functional equivalent to mouse IZUMO1, despite only approx. 9% identity of the entire amino acid sequences between these two proteins.\(^{26,27}\) Our present findings also imply that *C. elegans* and the mouse might have a common target of DDI-6 in signaling pathways for MO fusion and the acrosome reaction. Because there are no known effects for DDI-6 in any biological phenomena, we are currently seeking molecules that might interact directly or indirectly with DDI-6 in *C. elegans* and mouse spermatzoa by a combination of biochemical and genetic approaches. Identification of such DDI-6-related factors would provide important information regarding a common mechanism for sperm activation that are shared by *C. elegans* and mice.

From a human clinical aspect, the discovery of DDI-6 suggests a possible strategy in screening for compounds that can activate *C. elegans* spermatzoa. To develop therapeutic approaches or testing drugs for treating human male infertility, seed compound screening using mammals might be replaced by *C. elegans*-based screening, at least in part. Given that it is preferable to minimize the use of mammals such as mice for drug development, the availability of *C. elegans* for such screening is worthy of consideration. Moreover, it is also important to identify an elemental structure(s) of DDI-6 that...
is required for sperm activation. Studies on the structure–activity relationship of DDI-6 would lead to elucidating how it acts on sperm activation and possibly to developing it as clinically useful drugs.

Conclusion

We hypothesized that *C. elegans* and the mouse might have a common molecular basis of sperm activation. To explore this, we sought compounds that could activate both *C. elegans* and mouse spermatozoa. Using chemical library screening, we identified a quinolinol analog named DDI-6 as a candidate sperm activator. To use DDI-6 for further sperm activation assays, the compound was synthesized through a four-step procedure with 5-chloro-8-quinolinol as the starting material. This newly synthesized DDI-6 was tested for its capacity to trigger MO fusion in *C. elegans* spermatozoa and the acrosome reaction in mouse spermatozoa, both of which are pivotal events for sperm activation. DDI-6 could partly but significantly activate spermatozoa from those two species. These data imply that signaling pathways for MO fusion and the acrosome reaction might share a target of DDI-6.

Experimental

**Materials** The Core Library used for screening of compounds was provided from the Drug Discover Initiative (DDI, at the University of Tokyo, Japan). Compounds contained in the library were stored as 10 mM solutions in DMSO at −80 °C until use. Pron and A23187 were purchased from Sigma-Aldrich (St. Louis, U.S.A.), ProK from Nacalai Tesque (Kyoto, Japan), and FM1-43 from Invitrogen (Carlsbad, U.S.A.). Other chemicals that we used were of the highest quality available.

**Animals** The *C. elegans him-8(tm611) IV* strain (CA257) was provided from the Caenorhabditis Genetic Center (CGC, at the University of Minnesota, U.S.A.). This strain was used in this study as wild-type worms. ICR mice (approx. 10-week-old males) were purchased from Japan SLC (Shizuoka, Japan). This study was approved by the Institutional Animal Care and Use Committee (permission number: K19-34) and carried out according to the Setsunan University Animal Experimentation Regulations.

**Microscopy** Most worm handling, such as picking and dissecting, were carried out under SZ61 or SZX10 dissecting microscopes (Olympus, Tokyo, Japan). To capture digital differential interference contrast (DIC) or fluorescent images, we used a BX53 microscope carrying a DP72 CCD camera with cellSens software (Olympus).

**Screening for Compounds Triggering *C. elegans* Spermiogenesis** The Core Library from the DDI contained 9600 entries that are structurally divergent to each other. To obtain *in vitro* activators for *C. elegans* spermiogenesis, we primarily screened 480 entries of the Core Library as described below (see “*C. elegans* Spermiogenesis Assay”). Since 1% DMSO (solvent control) triggered spermiogenesis in approx. 3% of total *C. elegans* spermatids, compounds capable of activating more than 20% of total cells were subjected to a secondary screening. The selected compounds were tested to induce spermiogenesis *in vitro* at concentrations of 50 and 100 µM. If those compounds exhibited significant activities in a concentration-dependent manner, we judged that they are positive as *C. elegans* spermiogenesis activators.

**Mouse Acrosome Reaction Assay** This assay was based on the previous report by Larson and Miller.26) Sperm were released from the cauda epididymis of ICR males into 200-µL drops of Human Tubal Fluid (HTF) medium (omitting bovine serum albumin (HTF-(BSA) medium) and allowed to stand at 37 °C for 30 min in 5% CO₂/95% air. Then, 2.0 × 10⁶/mL epididymal sperm were incubated with either of 1% DMSO (solvent control as a mock), 100 µM DDI-6 or 10 µM A23187 in 50 µL of HTF-(BSA) medium at 37 °C for 30 min in 5% CO₂/95% air. To quench the acrosome reaction by fixing sperm, 100 µL of 1 × phosphate-buffered saline (1 × PBS; 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl and 2.7 mM KCl) containing 4% paraformaldehyde was added to the sperm suspension. After 20-min incubation at room temperature, cells were washed three times by centrifugation at 2200 × g for 5 min at room temperature in 200 µL of 100 mM ammonium acetate, pH 9.0, and then re-suspended in 100 µL of the same buffer. A 10-µL aliquot from the sperm suspension was put onto a slide glass and air-dried at room temperature. To stain the acrosomes, 50 µL of Staining Solution (0.22% CBB G-250, 50% MeOH and 10% AcOH) was added to the air-dried cells. After 10-min incubation at room temperature, cells were washed three times with ultrapure water in a chamber to remove excess CBB G-250. The stained cells were mounted with 10 µL of 1 × PBS, and digital DIC images were acquired as described in “Microscopy.”

**Synthesis of DDI-6** Chemicals required to synthesize DDI-6 were used without any purification unless otherwise stated. Below are the procedures for synthesis of intermediates and the final products. Each product name is the same as those shown in Fig. 2.

**Equipments** Spectra of ¹H-NMR (400 MHz) and ¹³C-NMR (100 MHz) were measured on an ECZ 400S spectrometer (JEOL, Tokyo, Japan). Chemical shifts for ¹H-NMR were determined as δ values by comparing with that of tetramethylsilane as an internal standard, and coupling constants are shown in Hz. The following abbreviations are used to express spin multiplicity: s, singlet; d, doublet; t, triplet; m, multiplet; and br, broad. Chemical shifts for ¹³C-NMR were determined in ppm by comparing with that of the center line of a triplet for deute-
riochloroform, on the basis of the assumption that it appears at 77.16 ppm. Data on \(^1\)H-NMR and \(^{13}\)C-NMR of each compound are shown in “Supplementary Materials.” High-resolution mass spectroscopy (HR-MS) was carried out with electron ionization (EI) on a JEOL JMS-700T spectrometer. Analytical TLC was performed using Silica Gel 60F\(_{254}\), precoated analytical plates (Merck, Darmstadt, Germany). For flash chromatography, SilicaFlash P60 (Silicycle, Quebec, Canada) was used unless otherwise noted.

1,2,3,4-Tetrahydro-8-quinolinol (Compound 3)

Compound 1a (5-chloro-8-quinolinol: 1.26 g, 7.00 mmol) was dissolved in 14 mL of a mixture containing THF and MeOH (1 : 1), added to 10% Pd/C (744.9 mg, 0.70 mmol), and hydrogenated at 1 atm in H\(_2\) gas at room temperature. After being stirred for 24.5 h, the reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. The crude product was purified by recrystallization with CHCl\(_3\) to afford compound 3 (724.0 mg, 72%) as a pale orange crystal.

Analytical data on compound 3 was as follows: \(^1\)H-NMR (CD\(_3\)OD, 400 MHz) \(\delta\): 2.05–2.15 (m, 2H), 2.89 (t, \(J = 6.1\) Hz, 2H), 3.41–3.49 (m, 2H), 6.78 (d, \(J = 7.5\) Hz, 1H), 6.82 (d, \(J = 8.1\) Hz, 1H), 7.20 (d, \(J = 8.1\), 7.5 Hz, 1H); \(^{13}\)C-NMR (CDCl\(_3\), 100 MHz) \(\delta\): 22.9, 24.7, 25.1, 46.7, 116.5, 119.0, 121.5, 125.0, 128.4, 128.6, 131.7, 137.4, 143.5, 152.6, 169.4; HR-MS (EI) Calcd for C\(_{15}\)H\(_{16}\)N\(_2\)O\(_3\)S ([M]+) \(m/z\): 346.0987. Found: 346.0986.

N-(4-Acetamidobenzensulfonyl)-1,2,3,4-tetrahydro-8-quinolinol (Compound 4)

Compound 3 (300 mg, 2.01 mmol) was dissolved in 4 mL of CH\(_3\)CN, and acetamidobenzensulfonyl chloride (516.4 mg, 2.21 mmol) and pyridine (240 µL, 3.02 mmol) were added to the solution at 0°C. After stirring the reaction mixture for 2 h at room temperature, the reaction was quenched by addition of saturated, aqueous NH\(_4\)Cl at 0°C. The mixture was extracted with CHCl\(_3\) and the organic phase was washed with brine and then dried over anhydrous Na\(_2\)SO\(_4\). After filtration to remove solid Na\(_2\)SO\(_4\), the resulting filtrate was concentrated under reduced pressure. The crude product was subjected to flash chromatography and then eluted in a mixture of hexane and AcOEt with a linear gradient from 3 : 1 to 7 : 3 ratios to afford DDI-6 (70.3 mg, 39%) as a yellow solid. Analytical data on the final product, DDI-6, was as follows: \(^1\)H-NMR (CDCl\(_3\), 400 MHz) \(\delta\): 1.76 (t, \(J = 6.5\), 6.5 Hz, 2H), 1.87 (t, \(J = 6.5\), 2H), 3.74 (t, \(J = 6.5\), 2H), 3.80 (s, 3H), 6.54 (d, \(J = 7.4\) Hz, 1H), 6.85 (br s, 1H), 6.95 (d, \(J = 8.1\), 1H), 7.08 (dd, \(J = 8.1\), 7.7, 1H), 7.44 (d, \(J = 8.5\), 2H), 7.52 (d, \(J = 8.5\), 2H), 7.71 (brs, 1H); \(^{13}\)C-NMR (CDCl\(_3\), 100 MHz) \(\delta\): 23.1, 25.2, 46.9, 53.0, 116.8, 117.9, 120.6, 125.0, 128.4, 131.7, 137.4, 143.5, 152.6, 169.4; HR-MS (EI) Calcd for C\(_{25}\)H\(_{22}\)N\(_2\)O\(_3\)S ([M]+) \(m/z\): 362.0936. Found: 362.0935.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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