Inhibition of Chromosome Separation in Fertilized Starfish Eggs by Kalihinol F, a Topoisomerase I Inhibitor Obtained from a Marine Sponge

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Kalihinol F, a naturally occurring diterpene from a marine sponge, Acanthella sp., inhibited chromosome separation in fertilized starfish (Asterina pectinifera) eggs but allows the first cleavage to occur, thereby forming unseparated metaphase chromosomes which were elongated between the two daughter cells. The chromosomes were eventually torn off in the embryonic cells. Most of the cells gradually lost the chromosomes during the cell cycle progression. The embryonic development halted at the morula stage just before the onset of blastulation. The mitotic failure occurred when kalihinol F was applied to a fertilized egg during the second meiotic process, but not after the completion of the second meiotic division. Kalihinol F inhibited topoisomerase I activity in vitro, but had no effects on activities of DNA polymerases α, β, and γ, and of topoisomerase II. These results suggest that the topoisomerase I plays an essential role in meiosis II in this species.

Key words: fertilized egg; kalihinol F; meiosis II; starfish; topoisomerase I

Early development proceeds through successive events that are well ordered in space and time. Although the major morphogenetic events, such as oocyte maturation, fertilization, cleavage, and blastulation, have been shown to follow a precise temporal sequence, the timing of biochemical events corresponding to this sequence has not well defined. Oocytes in full-grown ovary are arrested at the germinal vesicle stage (first meiotic prophase), but they can be stimulated to resume meiotic maturation divisions by hormonal stimuli.1-3 The process of maturation is characterized by the germinal vesicle breakdown (GVBD), completion of meiosis I and II, which produce polar bodies I and II, respectively, and formation of the female pronucleus. The developmental period following fertilization is characterized by an abbreviated cell cycle consisting of rapid synchronous cell divisions and DNA synthesis in all cells within the embryo.1,2 Completion of the rapid cleavage period is followed by the immediate activation of a new developmental program, blastulation, in which randomly arranged embryonic cells become organized into an epithelium and further into a hollow blastula.3

Studies of the molecular mechanisms of these developmental changes would be facilitated by the availability of chemicals influencing development in various ways. In attempts to find such chemicals, we have used starfish eggs as a test material, since these eggs can be obtained in great numbers by treatment of the ovary with 1-methyladenine (1-MeAde).4 We searched for chemicals that prevent the normal chromosomal separation in the fertilized starfish...
(Asterina pectinifera) egg, leading to an eventual arrest of development at the stage just prior to blastulation. We obtained one such compound from an extract of a marine sponge and identified it as kalihinol F (Fig. 1), a diterpene with three isonitrile groups obtained from a marine sponge. This paper describes the effects of kalihinol F on cellular events in A. pectinifera embryos and its possible mode of action.

Materials and Methods

Culture of starfish embryos and bioassay for the blastulation-inhibitory activity. The starfish A. pectinifera were collected from the coastal waters off Japan during their breeding season and kept in natural sea water at 15°C in laboratory aquaria. Oocytes and sperm were removed from ovarian and testicular fragments, respectively. Experiments were carried out at 20°C and artificial sea water (ASW; Jamarin Laboratory, Osaka) was used throughout. Oocytes were induced to mature by the treatment with 1 μM 1-MeAde (Sigma, St. Louis, Missouri, U.S.A.). Maturing oocytes were fertilized by the addition of a diluted sperm suspension at 40 min after the start of the 1-MeAde treatment. Fertilized eggs were washed three times with ASW, and then allowed to develop in ASW until they reached the desired developmental stages.

A test sample was dissolved in MeOH to give the final concentration of 1 mg/ml. Then the solution was diluted with ASW to the desired concentrations containing MeOH, the concentration of which did not exceed 1% (vol/vol). MeOH at these concentrations did not affect embryonic development up to the blastula stage. The suspensions of embryos at various stages were placed into the diluted solutions. The embryos were periodically observed for any cytological changes.

Isolation of an inhibitory substance. Specimens of a marine sponge, Acanthella sp. (192 g, wet weight), were collected off the coast of Cape Sada, Ehime Prefecture, Japan, and frozen immediately after collection. The sponge was cut into small pieces and steeped in MeOH. The MeOH extract was concentrated in vacuo to afford an aqueous solution, which was acidified to pH 4 by the addition of 1 N HCl and extracted with ethyl acetate. The bioactive ethyl acetate soluble fraction (800 mg) was put through reversed-phase HPLC (ODS) using 0–100% CH3CN in H2O as the eluent to afford a bioactive substance (10.4 mg) as a white powder.

Physico-chemical analysis of the inhibitory substance. 1H and 13C NMR spectra were recorded on a JEOL GSX500 spectrometer (500 MHz for 1H, 125 MHz for 13C). 1H and 13C NMR chemical shifts were referred to solvent peaks: δH 3.30 (residual CHD3OD) and δC 49.8 for CD3OD. FABMS, HR-FABMS, and GC-EIMS were measured on a JEOL SX102A spectrometer. UV and IR spectra were recorded on a Shimadzu UV-160A and a JASCO FT/IR-5300 spectrometer, respectively.

Embryo fixation and nuclear staining. Embryos were fixed with 3.5% formaldehyde in ASW for 30 min at room temperature, permeated with phosphate buffered saline (PBS) containing 0.5% Nonidet P-40 for 1 h, and then rinsed with PBS three times. DNA was counterstained with 25 ng/ml 4′,6-diamidino-2-phenylindole (DAPI; Nacalai, Kyoto) dissolved in PBS, in the dark for 1 h. The embryos were mounted on glass slides with a drop of glycerol containing 10% 10 μM Tris-HCl adjusted to pH 8.0 and 2.3% 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma) as an antifluorescence bleaching agent, and covered with a coverslip. The specimens were examined using a fluorescence microscope (Orthoplan, Leitz, Wetzlar, Germany).

Microinjection of FITC-labeled dextran. Microinjection into an oocyte and measurement of injection volumes were done according to the methods of Hiramoto9 and Kishimoto.7 Oocytes were held between two coverslips separated by two pieces of double-stick tape during microinjection and observation.9 An oocyte was injected with an aqueous 64-kDa fluorescein-labeled dextran (Molecular Probes, Oregon, U.S.A.) solution at 1% of the total oocyte volume and to give a final concentration of 200 μg/ml fluorescein-labeled dextran in the oocyte cytoplasm. The oocyte was induced to mature by the treatment with 2 μM 1-MeAde, inseminated 40 min after 1-MeAde treatment, and incubated in ASW containing 5 μg/ml Hoechst 33342 (Molecular Probes) and a test sample (6 μg/ml). Nuclei were observed at the 4-cell stage in live embryos by using a differential interference contrast (DIC) and fluorescence microscope (Optiphot II, Nikon, Tokyo) equipped with an objective of UV-F 40× (NA = 1.30, oil immersion), epi-fluorescence (EFD-3, Nikon), and confocal fluorescence apparatus (Insightplus, Meridian Instruments Inc., Michigan, U.S.A.). Their images were taken with a CCD camera (MicroImager, Xillix Technologies Corp., Richmond, Canada).8,10

BrdU labeling. Embryos taken from a culture dish every 10 min were labeled with 5 mM bromodeoxyuridine (BrdU; Sigma) dissolved in ASW for 9 min. After the 9-min labeling, they were shaken for 20 s in 2% 2-mercaptoacetic acid in ASW (pH 9.4) to remove their fertilization envelope. The denuded BrdU-labeled embryos were pre-fixed with 3.5% for-
maldehyde in ASW for 15 min at room temperature. 
After they were washed with PBS twice, embryos 
were post-fixed with cold acetone on a dry ice-ethanol 
batch for 15 min and rinsed twice with PBS at room 
temperature. Embryos in each sample were processed 
for detection of the BrdU incorporated into nuclei by 
immunostaining.

**Immunostaining.** Embryos were attached to a glass 
slide coated with poly-L-lysine (Sigma) and incubated 
in a moist chamber at 37°C in the following order: (i) 
4 N HCl for 20 min to denature the DNA; (ii) 10% 
skim milk in PBS for 10 min to block the non-specific 
reaction; (iii) mouse monoclonal antibody against 
BrdU (Dako, Glostrup, Denmark) diluted to 1:100 
with PBS containing 5% skim milk for 1 h; (iv) 
horseradish peroxidase (HRP)-conjugated goat 
monoclonal antibody against mouse immunoglobulin 
G (Bio-Rad, Hercules, California, U.S.A.) diluted 
to 1:200 with PBS containing 5% skim milk for 
1 h. Between each incubation step, embryos were 
rinsed with PBS three times at room temperature. 
Detection was done using 3,3'-diaminobenzidine 
(Dojin, Kumamoto) and H2O2.

**Topoisomerase I and II assay.** Mouse 
topoisomerase I was purified from Ehrlich ascites 
tumor cells with some modifications as described 
earlier.11-13 Activity was assayed by relaxation of 
supercoiled plasmid DNA as described below. 
The reaction mixture in 20 μl contained 50 mM Tris-
HCl, pH 8.0, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM 
dithiothreitol, 10% glycerol, 30 μg/ml bovine serum 
albumin, 1 μl of test compounds in dimethylsul-
foxide, one unit of topoisomerase I, and 0.2 μg each 
of the supercoiled pT2GN plasmid DNA. One unit 
of the enzyme was defined as the minimal amount of 
activity required to relax 0.2 μg of supercoiled 
pT2GN DNA under the conditions used. After incu-
bation at 37°C for 15 min, drugs in the reaction 
mixtures were extracted with an equal volume of 
chloroform and then with 1-butanol saturated with 
25 mM Tris-HCl, pH 8.0, and 10 mM EDTA. 
The aqueous phases were mixed with 4 μl of the dye/SDS 
stop solution, then analyzed by electrophoresis on 
0.8% agarose gels. 
Recombinant human topoisomerase IIα was 
purified and the decatenation activity assayed as 
described previously.14

**Results**

**Purification and identification of a substance in-
hibiting starfish embryogenesis**

During the course of our screening program for 
specific inhibitors of starfish oocyte maturation and 
embryogenesis from marine sponges, we found that a 
MeOH extract of the marine sponge, *Acanthella* sp., 
inhibited blastulation. Bioassay-guided purification 
as described under “Materials and Methods” result-
ed in the isolation of a bioactive substance which 
caused aberrant embryogenesis and halted develop-
ment at the morula stage. The FABMS spectrum of 
the active substance revealed the presence of [M + 
H]⁺ ion at m/z 384. The IR spectrum suggested the 
existence of hydroxyl and isonitrile groups. The ¹H 
and ¹³C NMR spectra of the active substance were 
identical to those of kalihinol F, a diterpene with 
three isonitrile groups reported by Patra et al.5) 
Therefore, the active substance was unambiguously 
deidentified as kalihinol F (Fig. 1).

**Inhibition of starfish embryogenesis by kalihinol F**

When fertilized eggs were treated with kalihinol F 
(3 μg/ml), the development of starfish embryos was 
arrested at the morula stage, prior to formation of 
blastulae, as shown in Fig. 2. The minimum effective 
concentration was 1.6 μg/ml (data not shown). 
Nuclear staining using DAPI indicated that the 
majority of the cells in a kalihinol-F-treated embryo 
did not contain nuclei.

When fertilized eggs were cultured in the presence
of kalihinol F (3 μg/ml), a 30-min delay of the first cell division was observed. Apparent normal cleavages progressed up to the sixth cell division on schedule, and then embryonic development was arrested prior to the seventh cell division (Fig. 3). It is suggested that kalihinol F affected the first chromosomal duplication and/or distribution before the first cleavage.

Inhibition of chromosomal separation in fertilized eggs by kalihinol F

The immature oocyte contains a germinal vesicle with a 4N chromosome set. Meiotic maturation is initiated by the addition of 1-MeAde, which causes GVBD 30 min later and subsequent oocyte maturation renders the oocytes fertilizable. Maturing oocytes, i.e. eggs, were inseminated at 40 min after the start of the 1-MeAde treatment. In order to determine the time when chromosomes of a fertilized starfish egg separate into two daughter cells, chromosomes were stained with DAPI and observed by epifluorescence microscopy. An inseminated egg completes meiosis I, releasing the first polar body containing a 2N chromosome set, at 60 min after the start of the 1-MeAde treatment (Fig. 4j) and meiosis II, releasing the second polar body containing an N chromosome set, at 90 min, followed by the formation of the female pronucleus (Fig. 4l). The female pronucleus fuses with the male pronucleus at 110 min (Fig. 4m), and then the fused pronucleus starts the first mitotic process. The sister DNA molecules are disentangled and then pulled to opposite poles of the cells. At 120 min, chromosomes separate (Fig. 4n) and at 130 min the first cleavage takes place (Fig. 4o).

When chromosomes of an egg which was treated with kalihinol F (3 μg/ml) from fertilization were stained with DAPI and observed in a similar manner, first and second polar bodies were expelled, pronuclei formed, and they fused at the same time as the control egg (Fig. 4a, b, c, d). However, kalihinol F caused the delay of chromosome separation at 120 min after the start of 1-MeAde treatment (Fig. 4e) and the arrest at the metaphase-like stage continued for 30 min (Fig. 4f, g, h). The chromosomes were eventually torn off 10 min later (Fig. 4i). Magnifications of the micrographs shown in Fig. 4 h, i exhibited the unseparated chromosomes remaining at the center of the cell (Fig. 5a, a') and the chromosomes elongated between the two daughter cells (Fig. 5b, b'). Because the metaphase-anaphase transition requires completion of DNA replication and segregation, it is considered that the delay of chromosome separation was caused by the failure of DNA synthesis, recombination, or chromatin condensation/decondensation.

Kalihinol-F-sensitive period during meiotic and mitotic cell-cycle progression

We next examined the effective period during which kalihinol F arrested the first cleavage. When fertilized eggs were treated with kalihinol F by 80 min after the start of 1-MeAde treatment, the delay of cell division and of chromosome separation were observed, as shown in Fig. 6. The embryos treated with kalihinol F later than 90 min after the start of 1-MeAde treatment developed normally to proceed to the blastula stage. These results indicate that kalihinol F specifically affects the meiotic process before the formation of the second polar body and female pronucleus. Because this stage precedes chromosome DNA synthesis and pronuclear fusion, kalihinol F could not affect this DNA replication and subsequent DNA recombination.

DNA synthesis in the kalihinol-F-treated embryo

We attempted a BrdU pulse labeling experiment to examine whether DNA synthesis takes place in kalihinol-F-treated embryos from the first to fifth mitotic cell division cycles. As described in the "Materials and Methods", fertilized eggs or embryos were pulse-labeled with BrdU for 10 min and the replicated DNA was detected with an anti-BrdU antibody. Due to the high background, DNA syntheses during the 1- to 2-cell stages were undetectable. However, DNA synthesis occurred at the 4- to 32-cell stages as revealed by the presence of BrdU-positive cells (Figs. 7 and 8). The reciprocal DNA syntheses and cleavages took place through the cell cycle progression, as shown in Fig. 8. These results suggest that kalihinol F does not affect DNA synthe-
Fig. 4. Inhibition of Chromosome Separation Caused by Kalihinol F during M-Phase of the Cell Cycle.
Fertilized eggs were cultured in the presence (a, b, c, d, e, f, g, h, i, a', b', c', d', e', f', g', h', i') or absence of kalihinol F (3 μg/ml) (j, k, l, m, n, o, p, q, r, j', k', l', m', n', o', p', q', r') fixed at the indicated time (min) after 1-MeAde treatment, stained with DAPI, and observed under a fluorescence microscope (a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r) or a differential interference contrast microscope (a', b', c', d', e', f', g', h', i', j', k', l', m', n', o', p', q', r'). Small letters in micrographs, f, m, and n, indicate female pronuclei, male pronuclei, and zygotic nuclei, respectively. Arrows indicate polar bodies. Bar indicates 50 μm.

Fig. 5. Abnormal Cleavage Caused by Kalihinol F.
Eggs were inseminated at 40 min after 1-MeAde addition, and fertilized eggs were cultured in the presence of kalihinol F (3 μg/ml) from fertilization, fixed 150 min (a, a') or 160 min after 1-MeAde addition (b, b'), and stained with DAPI. (a) and (b) show fluorescence images, and (a') and (b') DIC images. Bar indicates 50 μm.

Fig. 6. Periods of Kalihinol-F-treatment Which Results in a Cleavage-arrest in the Fertilized Eggs.
Eggs were inseminated at 40 min after the start of 1-MeAde treatment. The fertilization envelope elevates soon after the penetration of sperm (a circular head with a tail). Arrows indicate the periods during which kalihinol F was included in the culture.
Kalihinol F (3 μg/ml)  

FIG. 7. BrdU-Incorporation into Chromosome of Kalihinol-F-treated Embryos.

Fertilized eggs which were cultured in the presence (a) or absence of kalihinol F (3 μg/ml) (b) were pulse-labeled with BrdU for 10 min, fixed 10 min later, and stained with mouse anti-BrdU monoclonal antibody and HRP-conjugated goat anti-mouse immunoglobulin antibody. Embryos fixed 180 min (a) and 190 min after 1-MeAde treatment (b) are passing the 4- and 8-cell stages, respectively. Bar indicates 50 μm.

Fig. 8. Cell Cycle Progression and BrdU-Incorporation in Kalihinol-F-treated Embryos.

Fertilized eggs which were cultured in the presence (closed circle) or absence of kalihinol F (3 μg/ml) (open circle) were pulse-labeled with BrdU for 10 min, fixed 10 min later, and stained with mouse anti-BrdU monoclonal antibody and HRP-conjugated goat anti-mouse immunoglobulin antibody. The percentage of cells containing an immunoreacted chromosome was counted for 200 embryonic cells in each pulse-labeled culture. S3, S4, and S5 indicate the third, fourth, and fifth S phase of the cell cycle in normal embryos, respectively.

treated embryos, we injected immature oocytes with fluorescein-labeled dextran with the molecular mass of 64 kDa. As demonstrated by Hiramoto and Kaneda,9 fluorescein-labeled dextran with the molecular mass of 39 kDa or more is impermeable across the nuclear envelope in starfish cells. The dextran-injected oocytes were induced to mature, fertilized, cultured in the presence of kalihinol F, and observed with an epifluorescence and confocal fluorescence microscope after they reached the 4-cell stage. It was found that the fluorescein-labeled dextran was excluded from the nuclei at the center of cell in the kalihinol F-treated embryo (Fig. 9a) in spite of the fiber-like DNA elongated between daughter cells (Fig. 9b), indicating the presence of a nuclear envelope impermeable to 64-kDa dextran.9,10 These results show that a normal nuclear envelope can form around unseparated chromosomes in the kalihinol-F-treated embryo, thereby supporting the occurrence of the DNA synthesis during the interphase of a cell cycle.

Inhibition of DNA topoisomerase I activity in vitro by kalihinol F

Since kalihinol F caused aberration in chromosome separation in the first mitosis following pronuclear fusion after meiosis II, where topoisomerases I and/or II are involved in meiotic recombination, we examined the effects of kalihinol F on the activities of DNA topoisomerases I and II. Nearly complete inhibition of topoisomerase I was demonstrated at the concentration of 115 μg/ml, as shown in Fig. 10, whereas no inhibition of topoisomerase II was observed (data not shown), suggesting kalihinol F to be a specific inhibitor of topoisomerase I. Topoisomerase I inhibitors include a natural compound, camptothecin, and its synthetic analogues, topotecin and irinotecan. Included in the

 sis. In accordance with these results, the activities of DNA polymerases α, β, and γ were unaffected by kalihinol F when they were examined in vitro (data not shown).

Formation of the nuclear envelope surrounding unseparated chromosomes in the kalihinol-F-treated embryo

To demonstrate the presence of the nuclear envelope that surrounds chromosomes in kalihinol-F-
category are minor groove binders such as bisbenzimidazols, Hoechst 33258 and 33342, and indolocarbazols derived from rebeccamycin. All these compounds have a planar structure with aromatic rings. Kalihinol F obtained from a marine sponge is the first aliphatic compound specifically inhibiting topoisomerase I activity.

Discussion

Our experiments show that the treatment of fertilized starfish eggs with kalihinol F caused the abnormality of chromosome separation at the first cleavage. Similarly, incomplete chromosome separation at the first cleavage has been seen in aphidicolin-treated zygotes of the starfish. Aphidicolin inhibits the DNA synthesis that occurs in the period from pronuclear formation to fusion of fully developed pronuclei. Although kalihinol F had a similar effect on starfish eggs to that of aphidicolin, kalihinol F did not inhibit chromosomal DNA synthesis. Assays for enzymes involved in DNA replication and recombination in vivo revealed that kalihinol F inhibited the activity of DNA topoisomerase I without affecting the activities of DNA topoisomerase II and of DNA polymerases α, β, and γ. Therefore, kalihinol F caused incomplete chromosomal separation through inhibition of the activity of topoisomerase I without affecting DNA syntheses. Topoisomerase I modifies DNA topology in connection with a number of nuclear processes, such as chromatin condensation/decondensation and recombination by producing single-stranded breaks, thereby allowing rotation of the broken strand around the intact strand. On the other hand, topoisomerase II transports a double-helical portion of DNA through a cut involving both strands of another double-helical region. In our preliminary experiments, the treatment of fertilized starfish eggs with camptothecin (10 ng/ml or greater), a specific topoisomerase I inhibitor, caused a failure of sister chromosome separation in the first cleavage as kalihinol F did, whereas similar treatment with etoposide (50 μg/ml), a specific topoisomerase II inhibitor, did not affect the chromosome separation (data not shown). These results suggest that topoisomerase I is an essential enzyme for chromosome separation at the first mitosis of the embryo in this species. The effective concentrations of kalihinol F and camptothecin to inhibit chromosome separation in starfish embryonic cells are considerably lower than those to inhibit the topoisomerase I activity in vitro. The difference of the response indicates the unusually high sensitivity of the fertilized starfish egg at the stages prior to the first mitotic process. The kalihinol-F-sensitive period is restricted to meiosis II before pronuclear formation (Fig. 6). Development of the embryo at the 2-cell stage was unaffected by kalihinol F at the concentration of as high as 50 μg/ml (data not shown).

In contrast to starfish ovary, full-grown sea urchin ovary egg is filled with eggs which have completed meiosis I and II, and which contain a pronucleus with a fully decondensed haploid chromosome set. Kalihinol F (50 μg/ml) did not affect chromosome separation of fertilized sea urchin eggs, which cleaved normally (data not shown). Therefore, it is possible that kalihinol F inhibits chromatin decondensation during meiosis II through prevention of topoisomerase I activity.

Normally, cell division takes place in concert with mitotic division, which depends on chromosome and centrosome cycles. On the other hand, in the fertilized kalihinol-F-treated starfish egg, the chromosome cycle is restrained while the centrosome cycle advances (Figs. 4 and 5). The delay of the first mitotic division caused by kalihinol F can be explained as follows. In the first mitotic division, chromosome pairs are arranged at the equator of the spindle in a typical bipolar mitotic apparatus. However, sister chromatids cannot separate to form daughter chromosomes because the sister chromatids might be aberrant due to the failure of chromosome decondensation before pronuclear formation. On the other hand, the centrosome continues movement toward the formation of four poles from the original two, while the chromosomes persist in a metaphase-like configuration. The four poles join a tetrapolar spindle which remains connected to sister chromatids. In the subsequent step, the poles pulled individual sister chromatids apart toward them along the newly-formed spindle axis, dividing the chromatids into two groups, thereby producing apparent daughter
chromosomes as elongated sister chromatids. It is noteworthy that chromosomal DNA replication takes place in most of the cells (Fig. 8). This implies that partial decondensation of chromosomes takes place in the daughter cells.

Further experiments are obviously necessary to investigate the role of topoisomerase I in situ in producing a normal haploid chromosome set in starfish eggs. The results of this study will be the basis for using kalihinol F as a tool to investigate the mechanism of chromatid condensation/decondensation during meiosis II.

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