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Interaction of heliquinomycin with single-stranded DNA inhibits MCM4/6/7 helicase

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Takafumi Sugiyama[1], Makoto Chino[2], Toshiki Tsurimoto[3], Naohito Nozaki[4] and Yukio Ishimi[1,*]

[1]College of Science, Ibaraki University, 2-1-1 Bunkyo, Mito, Ibaraki 310-8512; [2]Parmaceuticals Group, Nippon Kayaku Co. Ltd., 31-12 Shimo 3-Chome, Kita-ku, Tokyo 115; [3]Department of Biology, Faculty of Sciences, Kyushu University, 6-10-1 Hakozaki, Fukuoka 812-8581; and [4]Bio-Frontier Research Center, Tokyo Institute of Technology, 4259 Nagatsuda, Yokohama, Kanagawa, Japan

*Yukio Ishimi, Ibaraki University, 2-1-1 Bunkyo, Mito, Ibaraki 310-8512, Japan. Tel: +81-29-228-8439, Fax: +81-29-228-8439, email: ishimi@mx.ibaraki.ac.jp

The antibiotic heliquinomycin inhibited cellular DNA replication at IC_{50} of 2.5 μM without affecting level of chromatin-bound MCM4 and without activating the DNA replication stress checkpoint system, suggesting that heliquinomycin perturbs DNA replication mainly by inhibiting the activity of replicative DNA helicase that unwinds DNA duplex at replication forks. Among the DNA helicases involved in DNA replication, DNA helicase B was inhibited by heliquinomycin at IC_{50} of 4.3 μM and RECQL4 helicase at IC_{50} of 14 μM; these values are higher than that of MCM4/6/7 helicase (2.5 μM). These results suggest that heliquinomycin mainly targets actions of the replicative DNA helicases. Gel-retardation experiment indicates that heliquinomycin binds to single-stranded DNA. The single-stranded DNA-binding ability of MCM4/6/7 was affected in the presence of heliquinomycin. The data suggest that heliquinomycin inhibits the DNA helicase activity of MCM4/6/7 complex by stabilizing its interaction with single-stranded DNA.

Keywords: biochemistry/enzyme inhibitors/molecular biology/replication and recombination.

Abbreviations: MCM, minichromosome maintenance; HQ, heliquinomycin; BrdU, bromodeoxyuridine.

A hetero-hexameric MCM2-7 protein complex that has been identified as a component of the DNA replication licensing system that ensures a single round of DNA replication per cell cycle, probably functions as a replicative DNA helicase that drives unwinding of DNA duplex prior to semi-conservative DNA synthesis at replication forks (*1*–*4*). One problem related to the function of the MCM2-7 complex is that an isolated MCM2-7 complex does not exhibit definite DNA helicase activity *in vitro*, while one of the MCM subcomplex, MCM4/6/7 hexamer does. Recently, it has been reported that MCM2-7 complex exhibits DNA helicase activity under selected conditions (*5*). *In vivo*, it is probable that the DNA helicase activity of MCM2-7 complex is generated by the interaction with other proteins including CDC7, CDC45 and GINS (*6*, *7*), although structure of the activated form of MCM2-7 helicase remains to be determined. It is suggested that DNA helicases of RECQL4 (*8*, *9*) and DNA helicase B (*10*) are also involved in DNA replication. ATP-binding site of RECQL4 is required for initiation of DNA replication in *Xenopus* egg extract (*11*). A mouse temperature-sensitive mutant cell, which harbors a mutated DNA helicase B gene, is defective in DNA replication (*12*, *13*). Microinjection of a dominant-negative form of mouse DNA helicase B results in G1 arrest in mouse cells (*14*). Thus, these two DNA helicases play roles in DNA replication, and the roles may be distinct from those of MCM helicase.

Heliquinomycin, which is an antibiotic (*15*, *16*), is known to inhibit cellular DNA replication and RNA synthesis at IC_{50} of 1.4–4 and 3.8 μM, respectively. To elucidate its cellular targets for the inhibition of DNA replication, we examined the effects of heliquinomycin on activities of several DNA helicases including MCM4/6/7 complex and those of the DNA polymerase-α/primase complex (*17*). The results indicate that the MCM4/6/7 helicase is the most sensitive to heliquinomycin among the enzymes examined. It was also observed that heliquinomycin inhibited the ATPase activity of the MCM4/6/7 complex in the presence of single-stranded DNA but it hardly inhibited the activity in the absence of the DNA, raising a possibility that heliquinomycin inhibits the MCM4/6/7 helicase activity by interfering with its interaction with the single-stranded DNA.

Here, we examined the effect of heliquinomycin on checkpoint systems and on the activity of other DNA helicases that are involved in DNA replication. The results show that heliquinomycin does not significantly activate DNA replication stress checkpoint system or double-stranded DNA brakes. Human DNA helicase B exhibits similar sensitivity to heliquinomycin as the MCM4/6/7 helicase does. As to mechanism of the inhibition, it was found that heliquinomycin directly binds to single-stranded DNA. The interaction of heliquinomycin with single-stranded DNA affected binding of MCM4/6/7 helicase and DNA helicase B with single-stranded DNA. Thus, it is strongly suggested that heliquinomycin inhibits MCM4/6/7 helicase by interfering its interaction with single-stranded DNA.

Materials and Methods

BrdU labelling of HeLa cells

HeLa cells were cultured in DMEM supplemented with 8% newborn calf serum. Cells cultured on cover slips were incubated with dimethyl sulfoxide or increasing concentrations of heliquinomycin for

1 h and then pulse-labelled with 20 μM bromodeoxyuridine (BrdU) for 20 min (*17*). For extraction of chromatin-unbound proteins, HeLa cells were extracted by incubating with a buffer (10 mM PIPES, pH 7.0, 0.1 M NaCl, 0.3 M sucrose, 3 mM $MgCl_2$ and 0.5% Triton X-100) for 10 min at room temperature before fixation. After being washed with phosphate-buffered saline (PBS), the cells were fixed by incubation with 4% paraformaldehyde in PBS for 5 min at room temperature. The cells were washed with PBS, and then permeabilized and blocked by incubation with 0.1% Triton X-100, 0.02% SDS and 2% non-fat dried milk in PBS for 1 h at room temperature. Incubation of the cells with anti-MCM4 rabbit antibody (sc-22779, Santa Cruz Biotech., Santa Cruz, CA, USA) was performed for overnight at 4°C in the above blocking solution. The cells were washed with the same solution and then incubated with Cy3-conjugated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 1.5 h at 37°C in the blocking solution. Then, they were re-fixed, treated with 4 M HCl for 30 min at room temperature and incubated with rat anti-BrdU antibody (Clone BU1/75, Harlan Sera Laboratory, Belton, UK) followed by the incubation with FITC-conjugated anti-rat antibody (Cappel, Organon Teknika Corp., Durham, NC, USA). Positive immunoreactivities were detected with fluorescence microscopy (BX-9000, Keyence, Osaka).

Preparation of human DNA helicase B

cDNA of human DNA helicase B was synthesized from mRNA extracted from HeLa cells by the RT-PCR method (Invitrogen, Carlsbad, CA, USA), and they were cloned into the baculovirus vector, pAcHLT-A. DNA helicase B was cloned to be expressed as a $(his)_6$-DNA helicase B fusion protein. High-5 cells were infected with the recombinamt baculovirus for 2 days. The recombinant helicase B protein in the lysates of the infected cells was purified by performing nickel-nitrilotriacetic acid (Ni-NTA) (Qiagen, Hilden, Germany) affinity column chromatography as follows (*17*). The purification involved suspending the infected cells in a lysis buffer consisting of 10 mM Tris–HCl (pH 7.5), 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM sodium phosphate buffer, 10 mM $Na_4P_2O_7$, and protease inhibitors (Pharmingen, BD, San Jose, CA, USA). The mixture was incubated for 40 min on ice, and insoluble components were separated by centrifugation at 40,000 rpm (TLS55; Beckman, Fullerton, CA, USA) for 40 min at 4°C. To 1 volume of the clarified lysate, 1/10 the volume of Ni-NTA agarose was added, and the mixture was incubated for 1 h at 4°C on a rocking platform. The agarose beads were then collected by centrifugation and thoroughly washed with buffer A [50 mM sodium phosphate buffer (pH 6.0), 300 mM NaCl, and 10% glycerol] containing 20 mM imidazole. Next, the beads were washed once with buffer B [50 mM Na-phosphate buffer (pH 8.0), 300 mM NaCl and 10% glycerol] containing 20 mM imidazole, and the proteins bound to the beads were eluted by adding buffer B containing increasing concentrations of imidazole (50, 100, 200 and 300 mM) at a volume equivalent to 1 bed. This was followed by incubation for 5 min at 4°C on a rocking platform and separation of the beads by centrifugation. These eluates (100, 200 and 300 mM) were pooled and diluted to decrease the NaCl concentration to 50 mM, and the solution thus obtained was concentrated using Amicon Ultra4 (Millipore, Bedford, MA, USA). The concentrated proteins were loaded on a MonoQ column (GE Healthcare, Piscataway, NJ, USA), and the bound proteins were eluted using a linear NaCl gradient (0.1–0.6 M). The helicase B protein was eluted with approximately 0.4 M NaCl and they were concentrated using Microcon 30 (Millipore) after the salt concentration had decreased to 0.1 M. The sample was fractionated after glycerol gradient centrifugation, as reported (*18*).

Purification of human DNA polymerase ε

High-5 cells were co-infected with recombinant baculoviruses producing four subunits (His-p261, p59, p17 and p12) of human DNA polymerase ε. DNA polymerase ε complex was purified using Ni-NTA agarose beads, as described above. The eluates were fractionated by MonoQ column chromatography. Antibodies against p261 (sc-12728, Santa Cruz Biotech.) and p59 (sc-8805, Santa Cruz Biotech.) were used to detect the complex and a fraction containing these two subunits was used to examine the sensitivity to heliquinomycin. DNA polymerase activity was measured using a reaction mixture (20 μl) containing 20 mM Tris–HCl (pH 7.9), 3.3 mM 2-mercaptoethanol, 0.2 mg/ml bovine serum albumin, 5 mM $MgCl_2$, 0.25 mg/ml activated DNA, 100 μM each of dATP, dGTP and dTTP, 50 μM of dCTP, 3 μCi (α-^{32}P) dCTP, and DNA polymerase-ε complex in the presence of heliquinomycin at the indicated concentrations. The reaction was terminated by the addition of 30 μl of sodium pyrophosphate (0.17 M) and 50 μl of sperm DNA (1 mg/ml). Further, 1 ml of 5% trichloroacetic acid was added, and the acid-insoluble radioactivity trapped on a glass fibre filter was measured in a liquid scintillation cocktail.

DNA helicase activities

A human MCM4/6/7 complex was prepared (*19*) and its DNA helicase activity was measured, as reported previously (*18*), except for some minor modifications. The standard reaction mixture (20 μl) contained 50 mM Tris–HCl (pH 7.9), 20 mM 2-mercaptoethanol, 10 mM ATP, 10 mM magnesium acetate, 0.5 mg/ml bovine serum albumin, 1–2.5 fmol of a 17-mer oligonucleotide annealed to M13mp18 DNA and indicated amounts of MCM4/6/7 complex or DNA helicase B in the presence or absence of heliquinomycin at the indicated concentrations. This mixture was incubated at 37°C for 40 min, and the products were analysed by using 12% polyacrylamide gel electrophoresis. The radioactivity on the plate was detected by using a Bio-Image Analyzer (FLA3000; Fuji, Tokyo).

Gel-retardation assay

The standard reaction mixture (15 μl) contained 25 mM HEPES–KOH (pH 8), 1 mM dithiothreitol, 0.5 mM ATPγ-S, 10 mM magnesium acetate, 50 mM sodium acetate, 0.25 mg/ml bovine serum albumin, 20 fmol of a 5′-labelled oligonucleotides (17-, 37- and 56-mer) and ~130 ng sample of human MCM4/6/7 complex, 300 ng sample of DNA helicase B or 100 ng sample of SV40 T antigen in the presence or absence of heliquinomycin at the indicated concentrations. The mixture was incubated at 30°C for 40 min, and the products were electrophoresed in 5% polyacrylamide gel containing 5% glycerol and 6 mM magnesium acetate\ in 0.5 × TBE. For gel-retardation assay of double-stranded DNA, 366 base pairs of human RPA3 gene digested with BamH1 and Not1 was used (*20*). The DNA fragment eluted from agarose gel was labelled at 5′-ends, and 100 fmol of the fragments was added to the above reaction mixture. After incubation, the products were analysed by 3% polyacrylamide gel electrophoresis. The radioactivity on the plate was detected by using a Bio-Image Analyzer. Filter binding experiment was performed in the same reaction condition as the gel-retardation assay using the labelled 56-mer DNA. After incubation at 30°C for 40 min, the reaction mixture was filtrated through membrane filter (Millipore, MF, 0.45 μm pore size). The filter was washed six times with the reaction buffer and the radioactivity on the filter was measured with liquid scintillation counter.

Immunoblotting

Proteins were separated by SDS–polyacrylamide gel (10 or 12%) electrophoresis. After the proteins in the gel were transferred to Immobilon-P transfer membrane (Millipore), the membrane was incubated for 1 h at room temperature with a blocking buffer (EzBlock, ATTO, Tokyo, Japan) diluted by three-fold with TBS plus 0.1% Triton X-100 or 5% bovine serum albumin in TBS plus 0.1% Triton X-100 for detection of Chk1 phosphorylated at S345. The membrane was then incubated overnight at 4°C with 0.5–1 μg/ml of 1st antibody in the diluted blocking buffer. After washing the membrane with TBS containing Triton X-100, it was incubated for 2 h at 27°C with 2nd antibody conjugated with horseradish peroxidase (BioRad, Hercules, CA, USA). After washing, the membrane was incubated with SuperSignal West Pico Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) and a chemiluminescent signal was detected by Light-Capture (ATTO, Tokyo). Preparation of anti-MCM4 antibody was reported (*19*). A mouse monoclonal antibody against human RPA2 (SSB34) (*21*) was kindly provided by J. Hurwitz.

Other materials

Hybridoma cell producing monoclonal antibodies against DNA helicase B was prepared. Synthetic peptide (Sigma-Genosys) from the amino-terminal region of helicase B was coupled to keyhole limpet haemocyanin and used to immunize mice (*22*). Heliquinomycin was dissolved using dimethyl sulfoxide. Antibodies purchased are anti-BrdU (Harlan sera-lab), anti-phospho-Chk1 (Ser345) (Cell Signaling Tech., Beverly, MA, USA) and anti-γH2AX (ab2893-50,

Abcam., Cambridge, UK). Nucleotide sequences of 17-mer, 37-mer and 56-mer oligonucleotide are 5′-GTTTTCCCAGTCACGAC-3′, 5′-TCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATT-3′ and 5′-CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCATGTCTAGCATCACGG-3′.

Results

Effect of heliquinomycin on checkpoint systems

It has been shown that heliquinomycin inhibits DNA replication in various transformed cells at IC_{50} value of 1.4–4 μM where DNA synthesis was measured by incorporation of labelled thymidine (*16*) or BrdU (*17*) into DNA. Furthermore, cell cycle progression of HeLa cells during S phase was severely retarded in the presence of 3.5 μM of heliquinomycin (*16*). In the BrdU incorporation experiments, human HeLa cells were incubated with increasing concentrations of heliquinomycin for 1 h and then pulse-labelled for 20 min with BrdU in the presence of heliquinomycin, and the incorporated BrdU was detected with anti-BrdU antibody. To examine whether amounts of chromatin-bound MCM2-7 complex are affected by heliquinomycin, logarithmically growing HeLa cells were incubated in the absence or the presence of heliquinomycin for 1 h, the cells were extracted with CSK buffer containing Triton X-100 and the residual chromatin-bound MCM4 in these cells was detected with anti-MCM4 antibody (Fig. 1A). The amounts of MCM4 on chromatin were only slightly decreased in the presence of heliquinomycin (Fig. 1B). This result was supported by a biochemical experiment where chromatin-bound and -unbound MCM4 in total cellular MCM4 was examined using the same antibody (Fig. 2A). The quantitative data in Fig. 2A indicate that the amount of chromatin-bound MCM4 was not significantly decreased in the presence of heliquinomycin (Supplementary Fig. S1). Broad distribution in the gel of MCM4 in S and P fraction is probably due to the presence of different levels of phosphorylation of MCM4. It remains to be determined why mobility of MCM4 with the highest mobility is different between S and P fractions. Thus, the number of MCM2-7

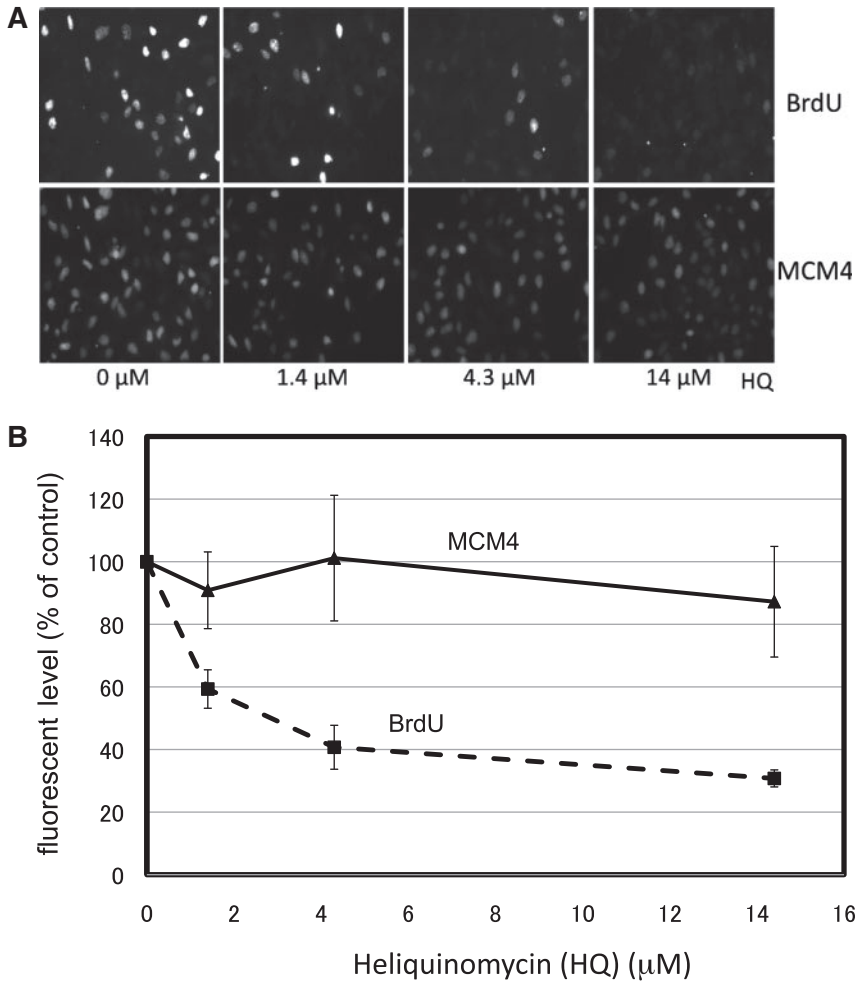

Fig. 1 Effect of heliquinomycin on chromatin-binding of MCM4 in HeLa cells. (A) The logarithmically growing HeLa cells were incubated with indicated concentrations of heliquinomycin for 1 h and then pulse-labelled with BrdU for 20 min. After removal of chromatin-unbound proteins, the incorporated BrdU in cells were detected by incubation with anti-BrdU antibody and then with FITC-labelled anti-rat antibody, and MCM4 on chromatin was detected with anti-MCM4 antibody (Santa Cruz Bio., sc-22779) and then with Cy3-labelled anti-rabbit antibody. (B) One hundred cells were selected, and the fluorescence intensity of FITC and Cy3 in those cells was quantified. The averaged level of the intensity in the cells cultured in the presence of heliquinomycin was expressed in comparison to that in the cells cultured in the absence of heliquinomycin. Two independent experiments were performed, and averages of the values were plotted with error bars.

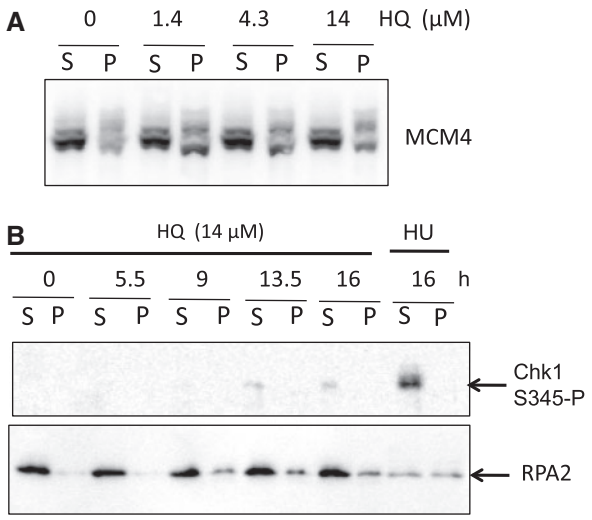

Fig. 2 Effect of heliquinomycin on checkpoint system.
(A) Logarithmically growing HeLa cells were incubated for 1 h with increasing concentration of heliquinomycin (0–14 μM). The cells were fractionated into Triton-soluble (S) and -insoluble (P) fractions (*19*). Proteins in these fractions were electrophoresed in polyacrylamide gel containing SDS. After transferred to filter, MCM4 protein on the filter were detected by using anti-MCM4 antibody (Santa Cruz Bio., sc-22779). (B) Logarithmically growing HeLa cells were incubated with heliquinomycin (14 μM) for 0, 5.5, 9, 13.5 and 16 h or they were incubated with 2 mM hydroxyurea (HU) for 16 h. After fractionation, phosphorylation of Chk1 at Ser345 and distribution of RPA2 were examined.

complex on chromatin may not be greatly affected by the incubation with heliquinomycin. Under the same conditions, incorporation of BrdU was inhibited by heliquinomycin, as reported (Fig. 1B).

Heliquinomycin may inhibit progression of DNA replication forks by several mechanisms. It may inhibit replicative DNA helicases, DNA polymerases or topoisomerases, all of which are required for the progression of DNA replication forks. DNA polymerizing activity of polymerase-α was inhibited by heliquinomycin at IC_{50} of 6.5 μM (*17*) but DNA topoisomerases were insensitive to it (*16*). Thus, it is possible that DNA polymerases are mainly inhibited by heliquinomycin *in vivo*. If DNA polymerases are main targets of heliquinomycin, the DNA replication stress checkpoint system will be activated, since uncoupling of DNA unwinding and DNA synthesis occurs to generate single-stranded DNA region at replication forks. The question whether or not the DNA replication stress checkpoint system is activated in the presence of heliquinomycin was addressed (Fig. 2B). As a positive control, HeLa cells were incubated with hydroxyurea that inhibits DNA polymerase actions. Chk1 phosphorylated at Ser345 was detected in Triton-soluble fraction from HeLa cells treated with hydroxyurea (Fig. 2B, top). In the presence of heliquinomycin, the phosphorylated Chk1 was only slightly detected after incubation for 13.5 and 16 h, and the level was ~10-fold lower than that detected in hydroxyurea-treated HeLa cells. The change in distribution of RPA2 is detected from 2 h after incubation with hydroxyurea (*23*). Thus, to examine the distribution of RPA2 is probably more sensitive to detect uncoupling of the actions of replicative DNA helicase and DNA polymerases. After incubation with hydroxyurea for 16 h, RPA2 was evenly recovered in Triton-soluble and -insoluble chromatin-containing fraction (Fig. 2B, bottom). In the presence of heliquinomycin (14 μM), however, such the change in distribution of RPA2 was only slightly detected after incubation for 9 h. These results indicate that DNA replication stress checkpoint system is only slightly activated suggesting that heliquinomycin mainly inhibits the activities of replicative DNA helicases rather than the activities of DNA polymerases.

To examine whether heliquinomycin induces double-strand DNA break or not, HeLa cells treated with heliquinomycin were examined for binding of anti-γH2AX antibody, since phosphorylation of H2AX specifically occurs at double-stranded DNA brakes (Supplementary Fig. S2). The cell nucleus recognized with the antibody was not increased in the cells treated with heliquinomycin. In contrast, the cells with high level of fluorescence were clearly detected in the cells cultured in the presence of bleomycin. Quantitative data showing total fluorescent level also support these observations. These data suggest that heliquinomycin does not significantly induce double-stranded DNA brake.

Sensitivity of DNA helicase B to heliquinomycin

In vitro, heliquinomycin inhibits several enzyme activities with different efficiencies. Among the enzymes examined, MCM4/6/7 DNA helicase activity was the most sensitive to heliquinomycin ($IC_{50} = 2.5$ μM) (*17*). Two other DNA helicases of helicase B (*12*, *13*) and RECQL4 (*11*) are involved in cellular DNA replication. Recombinant human DNA helicase B of apparent molecular weight of 150 kDa was purified from baculovirus-infected insect cell lysate. It was purified to near homogeneity by successive purification of Ni-NTA agarose gel, MonoQ column chromatography and glycerol gradient centrifugation. DNA helicase B broadly sedimented at fraction no. 6–12 in the glycerol gradient centrifugation (*18*), suggesting that it mainly forms dimmer or trimmer (data not presented). The purified DNA helicase B exhibited DNA helicase activity that displaces 17-mer oligonucleotides annealed to M13 single-stranded DNA in an ATP-dependent manner. This enzyme can efficiently displace longer 37-mer oligonucleotides annealed M13 DNA; this is in contrast with the MCM4/6/7 helicase that cannot displace the 37-mer oligonucleotides (Supplementary Fig. S3). The human helicase B required the presence of hydrolysable ATP for the DNA helicase activity. Equal concentrations of ATP and Mg acetate of 0.3–10 mM were required for the reaction (data not presented). The sensitivity of helicase B to heliquinomycin was examined in the reactions containing 10 mM ATP and 10 mM Mg acetate where the sensitivities of other DNA helicases have been examined (*17*). The DNA helicase activity of helicase B was inhibited by heliquinomycin at IC_{50} of 4.3 μM (Fig. 3). Our group (*9*) and others (*8*) have reported that human RECQL4 exhibits DNA helicase

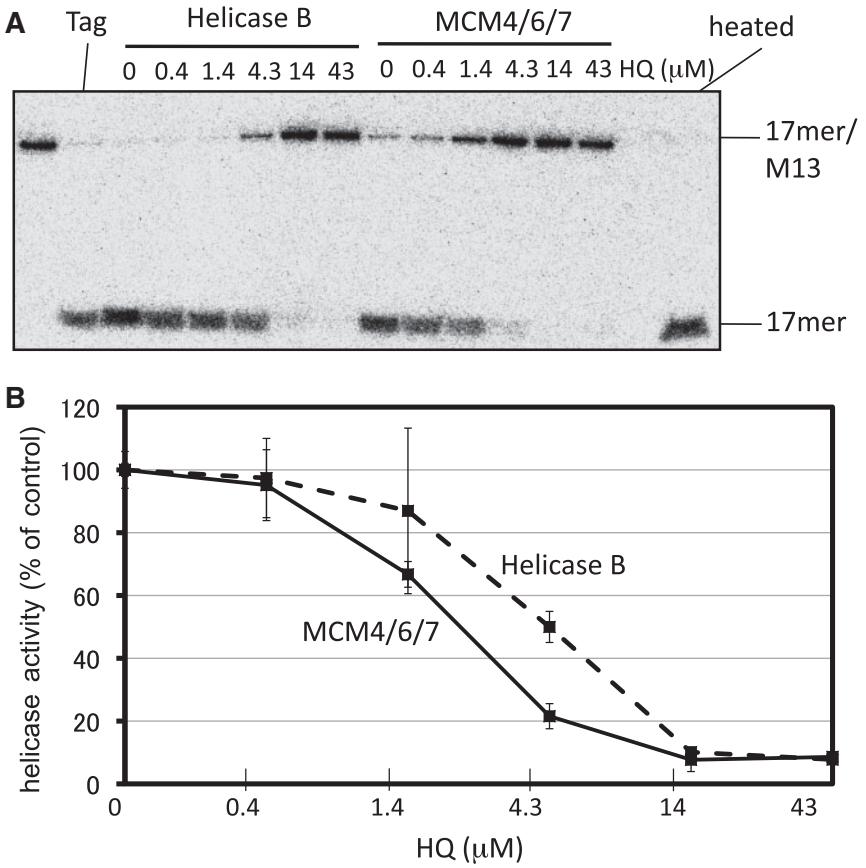

Fig. 3 Effect of heliquinomycin on the DNA helicase activities of the DNA helicase B and MCM4/6/7 complex. (A) The effects of increasing concentrations of heliquinomycin (HQ) on the DNA helicase activities of DNA helicase B (10 ng) and the MCM4/6/7 complex (100 ng) were examined. A 0.4 μl solution of dimethyl sulfoxide containing or lacking heliquinomycin was added to the reaction mixture. The final concentrations of heliquinomycin added to the reaction mixture are indicated at the top. The DNA helicase activity was measured as the activity that displaces 17-mer oligonucleotides annealed to the M13mp18 single-stranded DNA. (B) The proportion of the displaced 17-mer oligonucleotides in the total DNA was considered to be 100% in the control reaction mixture lacking heliquinomycin, and that recorded for the mixtures containing heliquinomycin was calculated in relation to this control reaction. The horizontal line is displayed at a logarithmical scale. Six independent experiments for DNA helicase B (Dashed line connecting filled boxes) and three experiments for MCM4/6/7 helicase (continuous line connecting filled boxes) were performed, and averages of the values were plotted with standard deviations.

activity. ATP-binding sites of this helicase is required for DNA replication using *Xenopus* egg extracts (*11*). The sensitivity of human RECQL4 to heliquinomycin was examined under the same reaction conditions. This helicase was inhibited by heliquinomycin at IC_{50} of 14 μM (Supplementary Fig. S4). Thus, among the three DNA helicases that are probably involved in DNA replication, MCM4/6/7 helicase is the most sensitive to heliquinomycin and DNA helicase B is also sensitive to it.

DNA polymerase-ε complex was purified from lysate of insect cells, which had been infected with the recombinant baculoviruses for expression of His-p261, p59, p17 and p12 subunits, by using Ni-NTA agarose and then by MonoQ column chromatograpy. The sensitivity of the DNA polymerase activity of the purified DNA polymerase-ε to heliquinomycin was examined by using activated DNA as a template-primer (Supplementary Fig. S5). DNA polymerase activity was inhibited at IC_{50} of 5.8 μM; the value is near to that of DNA polymease-α (17). These results indicate that heliquinomycin can inhibit several DNA helicases and DNA polymerases, suggesting that heliquinomycin does not interact specifically with MCM4/6/7 helicase to inhibit the activity (Supplementary Fig. S6). Since heliquinomycin does not inhibit DNA topoisomerase activity but it inhibits the activities of DNA helicases and DNA polymerases, heliquinomycin may interact with single-stranded DNA to inhibit these activities.

Binding of heliquinomycin to single-stranded DNA

To examine the interaction of heliquinomycin with single-stranded DNA, gel-retardation analysis was performed. After 5′-end labelled 56-mer single-stranded DNA (~17 kDa) was incubated with or without heliquinomycin, it was electrophoresed in native polyacrylamide gel (Fig. 4A). In the presence of 1.4 μM of heliquinomycin, the mobility of the single-stranded DNA was slightly retarded. From molecular weight of heliquinomycin (698 Da), the retarded DNA may bind several molecules of heliquinomycin. In the presence of 4.3–43 μM of heliquinomycin, the DNA migrated at the position of high molecular weight was detected. It is possible that the DNA bound to heliquinomycin was assembled into multimer.

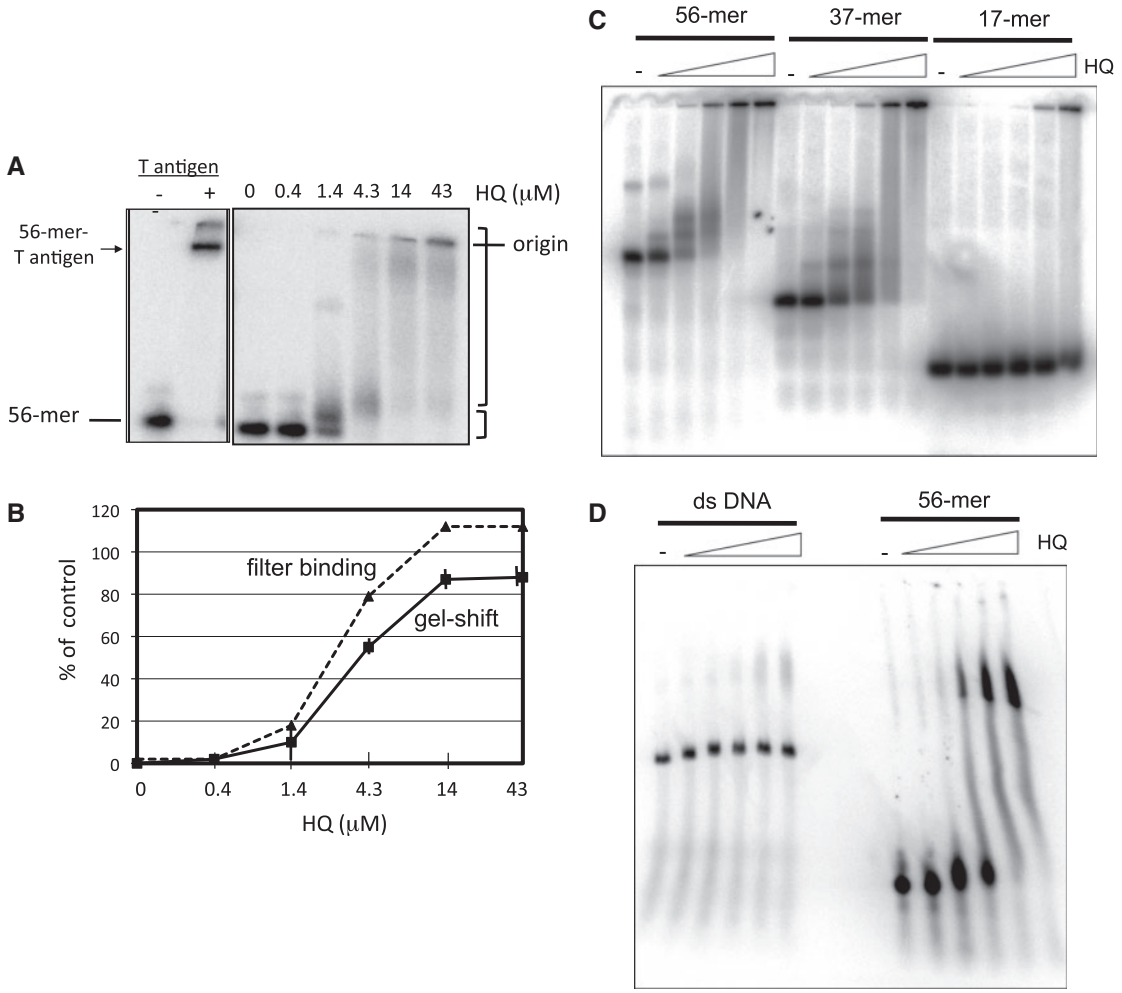

Fig. 4 Binding of heliquinomycin to single-stranded DNA. (A) 56-mer oligonucleotide labelled at 5′-end was incubated with increasing concentrations of heliquinomycin and electrophoresed on a native 5% polyacrylamide gel. After drying, radioactivity on the gel was detected. At the left, the DNA was incubated with or without SV40 T antigen DNA helicase in the absence of heliquinomycin. Positions of origin of electrophoresis and T antigen-56-mer complex are indicated. (B) As indicated on the right in (A), the radioactivity of DNA with retarded mobility and that of DNA without or low retarded mobility were measured. The proportion of the radioactivity with retarded mobility in the total DNA was determined (continuous line connecting filled boxes). Two independent experiments were performed, and averages of the values were plotted with error bars. Filter binding experiment of the labelled 56-mer and heliquinomycin was performed and the result was presented (dashed line connecting filled triangle). The proportion of the DNA on the filter in total DNA was determined. The horizontal line is displayed at a logarithmical scale. (C) Gel-retardation analysis using 17-, 37- and 56-mer oligonucleotides was carried out in the presence of increasing concentrations (0, 0.43, 1.4, 4.3, 14 and 43 μM) of heliquinomycin. (D) Gel-retardation analysis using 56-mer single-stranded DNA and 366 base pairs double-stranded DNA was carried out in the presence of increasing concentrations (0, 0.43, 1.4, 4.3, 14 and 43 μM) of heliquinomycin. A 100 fmol of the DNA was used. DNA was analysed by 3% polyacrylamide gel electrophoresis.

Quantitative data of the gel-retardation experiment are shown in Fig. 4B. Filter binding experiment supports the conclusion in the gel-retardation experiment that heliquinomycin binds to the 56-mer DNA in a concentration-dependent manner (Fig. 4B). Next, 17- and 37-mer single-stranded DNA were used for gel-retardation experiment to examine the requirement of length of single-stranded DNA for binding to heliquinomycin (Fig. 4C). The mobility of 37-mer DNA was shifted in a heliquinomycin concentration-dependent manner but higher concentration was required for the shift in comparison of the 56-mer DNA. The mobility of 17-mer DNA was shifted to the highly retarded position only in the presence of 43 μM heliquinomycin, suggesting that interaction of heliquinomycin with single-stranded DNA requires length of the DNA. Binding of heliquinomycin with double-stranded DNA was examined (Fig. 4D). Shifted DNA band was only slightly detected in the presence of 14 and 43 μM heliquinomycin, and the shift was not evident in comparison to single-stranded 56-mer DNA. These results indicate that heliquinomycin specifically binds single-stranded DNA.

It has been shown that binding of MCM4/6/7 hexameric complex to single-stranded 37-mer and 53-mer DNA was detected under the condition where the binding of these DNA with MCM4/6/7 complex was fixed with glutaraldehyde (*24*). The complex of MCM4/6/7 and the 56-mer DNA was hardly detected under the present reaction condition without fixation (Fig. 5A). In the presence of 1.4–43 μM heliquinomycin, however, the band of the 56-mer DNA bound to the MCM4/6/7 hexamer was clearly detected. It is probable that this band is a single MCM4/6/7 hexamer

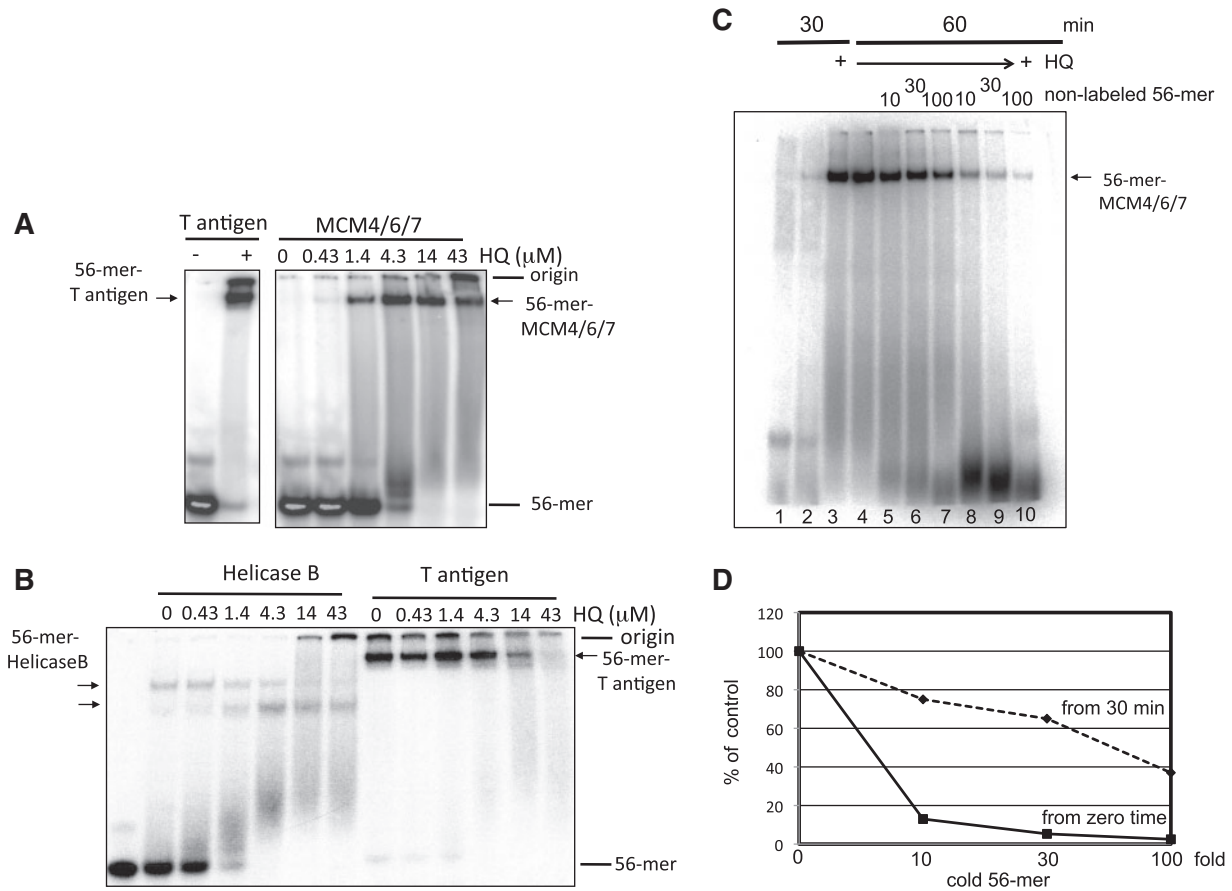

Fig. 5 Effect of heliquinomycin to single-stranded DNA binding of DNA helicases. (A) The labelled 56-mer oligonucleotide was incubated with a fixed amount MCM4/6/7 complex in the absence or the presence of increasing concentrations of heliquinomycin and electrophoresed. An arrow on the right indicates a complex of MCM4/6/7 hexamer and the 56-mer DNA. At the left, the DNA was incubated with or without SV40 T antigen in the absence of heliquinomycin. Positions of origin of electrophoresis and MCM4/6/7-56-mer complex are indicated. (B) The labelled 56-mer oligonucleotide was incubated with fixed amounts of helicase B or SV40 T antigen in the absence or the presence of increasing concentrations of heliquinomycin and electrophoresed. Arrows indicate complexes of T antigen and Helicase B with 56-mer DNA. (C) Labelled 56-mer oligonucleotide was electrophoresed after incubation for 30 min with (lane 2) or without MCM4/6/7 (lane 1). The 56-mer was incubated with MCM4/6/7 complex in the presence of 4.3 μM heliquinomycin for 30 min (lane 3) or 60 min (lane 4). After the labelled 56-mer was incubated with MCM4/6/7 complex in the presence of 4.3 μM heliquinomycin for 30 min, and then excess amounts (10-, 30- and 100-fold) of non-labelled 56-mer DNA to the labelled 56-mer were added to the reactions. These mixtures were further incubated for 30 min (lanes 5–7). The excess amounts of non-labelled 56-mer DNA were added to the reaction mixture at zero time of incubation, and the mixtures were incubated for 60 min (lanes 8–10). The products were electrophoresed on 5% polyacrylmide gel. (D) Radioactivities of the shifted bands (MCM4/6/7 and 56-mer complex) in (C) were quantified. The level of the radioactivity from the reaction mixtures containing 10-, 30- and 100-fold non-labelled 56-mer from zero time or after 30 min incubation was expressed in comparison to the radioactivity of the band derived from the mixture incubated for 60 min in the presence of heliquinomycin.

bound to the 56-mer DNA from the position on the gel (*24*). The findings that both the binding of heliquinomycin to single-stranded DNA and the stimulation of binding of MCM4/6/7 hexamer to single-stranded DNA are detected at similar concentration (1.4 μM) of heliquinomycin, suggest that the binding of heliquinomycin with single-stranded DNA stabilizes the interaction of the DNA with MCM4/6/7 hexamer. The fact that IC_{50} (2.5 μM) of MCM4/6/7 helicase activity is near to the concentration of 1.4 μM where the stable complex of the MCM4/6/7 hexamer and the single-stranded DNA is formed, suggests that the effect of heliquinomycin on single-stranded DNA-binding activity of MCM4/6/7 hexamer is closely related to the inhibition of DNA helicase activity of the complex.

DNA helicase B binds to the 56-mer DNA to form two bands at high molecular weight positions (Fig. 5B). It is possible that these two bands are different in number of helicase B molecules bound to the DNA. At 4.3 μM of heliquinomycin, proportion of these two bands was changed. Correlation between the modulation of single-stranded DNA-binding activity by heliquinomycin and the inhibition of DNA helicase activity suggests a possibility that heliquinomycin affects the interaction of helicase B with single-stranded DNA to inhibit its DNA helicase activity. Incubation of SV40 T antigen with 56-mer DNA generated a distinct band with retarded mobility. It is probable that this band is a complex of a single T antigen hexamer and the 56-mer DNA (*24*). The binding of T antigen to the DNA was not largely affected with 14 μM of heliquinomycin but it was completely inhibited with 43 μM. These data indicate that there is correlation between the sensitivity to heliquinomycin of DNA helicase activities and of single-stranded

DNA-binding activities among the DNA helicases of MCM4/6/7, DNA helicase B and T antigen.

Stability of the complex of MCM4/6/7 hexamer and 56-mer single-stranded DNA formed in the presence of heliquinomycin (4.3 μM) was examined (Fig. 5C). After incubation of the MCM4/6/7 hexamer with the labelled 56-mer DNA in the presence of heliquinomycin for 30 min, excess amounts (10-, 30- or 100-fold) of non-labelled 56-mer DNA in comparison to the labelled 56-mer DNA was added to the reaction, and the mixture was then incubated for 30 min. The level of the complex of MCM4/6/7 and labelled 56-mer DNA was gradually decreased in proportion to the amounts of non-labelled DNA (lanes 5–7). As a control, when the non-labelled DNA was added to the reaction from zero time of incubation, the level of the complex of MCM4/6/7 and labelled 56-mer was severely decreased (lanes 8–10). These results indicate that the complex of MCM4/6/7 hexamer and labelled 56-mer single-stranded DNA formed in the presence of heliquinomycin is stable at a certain level.

Discussion

The data presented suggest that heliquinomycin mainly inhibits DNA unwinding prior to DNA synthesis at the DNA replication forks, since heliquinomycin does not significantly activate the DNA replication stress checkpoint system. Among the three DNA helicases that are probably involved in DNA unwinding at the replication forks, an MCM4/6/7 helicase that is a core enzyme of MCM2-7 helicase is the most sensitive to heliquinomycin, and DNA helicase B is also relatively sensitive. It has been shown that heliquinomycin binds to single-stranded DNA by gel-retardation analysis and filter binding experiment. This binding of heliquinomycin to single-stranded DNA may inhibit translocation of these helicases on single-stranded DNA. Heliquinomycin inhibited not only the helicase activity of the MCM4/6/7 complex but also the single-stranded DNA-dependent ATPase activity of the complex that was measured in the presence of single-stranded DNA (*17*). However, it did not inhibit the ATPase activity of the complex in the absence of single-stranded DNA. Thus, heliquinomycin may inhibit the single-stranded DNA-dependent ATPase activity and DNA helicase activity of the MCM4/6/7 complex by affecting its ability to interact with single-stranded DNA.

Heliquinomycin, which has been isolated as an antibiotic, inhibits human cell DNA replication at IC_{50} of 1.4–4 μM and also inhibits RNA synthesis at IC_{50} of 3.8 μM (*16*). These notions are not inconsistent with the conclusion that heliquinomycin interacts with single-stranded DNA to inhibit DNA replication. Gel-retardation analysis shows that heliquinomycin interacts with single-stranded DNA at lower concentration (1.4 μM). This concentration is almost comparable to that by which DNA helicase activity of MCM4/6/7 complex is inhibited, suggesting that interaction of heliquinomycin with single-stranded DNA inhibits the DNA helicase activity. In the presence of 1.4 μM heliquinomycin, the interaction of MCM4/6/7 helicase and single-stranded DNA was greatly stabilized. It is probable that such the change leads to the inhibition of the DNA helicase activity. So far, six DNA helicases were examined for the sensitivity to heliquinomycin (17 and data presented here). SV40 T antigen that is hardly inhibited with heliquinomycin stably bound to single-stranded DNA in gel-retardation analysis (Fig. 5). Distinct complexes of RECQL4 with single-stranded DNA were also detected in this analysis, suggesting that RECQL4 tightly binds to single-stranded DNA (data not presented). Such the distinct complex formation with single-stranded DNA was not detected for heliquinomycin-sensitive DNA helicases, MCM4/6/7 complex and DNA helicase B, suggesting that these two DNA helicases do not tightly bind to single-stranded DNA. Thus, the weak interaction with single-stranded DNA may render these two DNA helicases sensitive to heliquinomycin. As to the mechanism of the binding of heliquinomycin to single-stranded DNA, we speculate that heliquinomycin may structurally bind to single-stranded DNA. Such the interaction may lead to assembly of multiple single-stranded DNA that migrates at highly retarded position in gel-shift analysis. In relation to this point, it is possible that heliquinomycin may inhibit several DNA helicases by its ability to stimulate annealing of separated two DNA. However, the data suggest that heliquinomycin does not stimulate annealing of complementary DNA in the DNA helicase reaction (Supplementary Fig. S7). Further analysis is required to understand the mechanism of the binding of heliquinomycin to single-stranded DNA and the formation of the complex with high molecular weight.

Structural analysis of archaeal MCM complex indicates that MCM hexamer forms toroidal structure with a central hole (*25*). Two models for mechanism of DNA unwinding are postulated. As a snapshot of the one model, one of displaced single-stranded DNA is located in the hole and another single-stranded DNA is located outside of the hexamer complex. During the reactions of ATP hydrolysis, local structural changes in the domains, which interact with single-stranded DNA inside the hole of the MCM hexamer, may occur to translocate the helicase on single-stranded DNA. If heliquinomycin interacts with the single-stranded DNA inside the hole, it will stabilize the interaction of MCM complex and the single-stranded DNA to perturb translocation of MCM complex on single-stranded DNA.

To date, a number of antibiotics have been isolated and some of them are used as anti-cancer drugs. They interact with double-stranded DNA to break the DNA or to crosslink the complementary DNA. The results presented here suggest that heliquinomycin binds to single-stranded DNA to inhibit DNA replication. It may be the first case that the antibiotic exhibits its effect by interacting mainly with single-stranded DNA. Heliquinomycin may be useful to examine cellular responses when progression of DNA replication forks is specifically perturbed.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

References

1. Tye, B.K. (1999) MCM proteins in DNA replication. *Annu. Rev. Biochem.* **68**, 649–686
2. Bell, S.P. and Dutta, A. (2002) DNA replication in eukaryotic cells. *Annu. Rev. Biochem.* **71**, 333–374
3. Forsburg, S.L. (2004) Eukaryotic MCM proteins: beyond replication initiation. *Microbiol. Mol. Biol. Rev.* **68**, 109–131
4. Masai, H., Matsumoto, S., You, Z., Yoshizawa-Sugata, N., and Oda, H. (2010) Eukaryotic chromosome DNA replication: where, when, and how? *Annu. Rev. Biochem.* **79**, 89–130
5. Bochman, M.L. and Schwacha, A. (2008) The Mcm2-7 complex has *in vitro* helicase activity. *Mol. Cell* **31**, 287–293
6. Moyer, S.E., Lewis, P.W., and Botchan, M.R. (2006) Isolation of the Cdc45-MCM2-7-GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase. *Proc. Natl Acad. Sci. USA* **103**, 10236–10241
7. Gambus, A., Jones, R.C., Sanchez-Diaz, A., Kanemaki, M., van Deursen, F., Edmondson, R.D., and Labib, K. (2006) GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. *Nat. Cell Biol.* **8**, 358–366
8. Xu, X. and Liu, Y. (2009) Dual DNA unwinding activities of the Rothmund-Thomson syndrome protein, RECQL4. *EMBO J.* **28**, 568–577
9. Suzuki, T., Kohno, T., and Ishimi, Y. (2009) DNA helicase activity in purified human RECQL4 protein. *J. Biochem.* **146**, 327–335
10. Seki, M., Enomoto, T., Hanaoka, F., and Yamada, M. (1987) DNA-dependent adenosinetriphosphatase B from mouse FM3A cells has DNA helicase activity. *Biochemistry* **26**, 2924–2928
11. Sangrithi, M.N., Bernal, J.A., Madine, M., Philpott, A., Lee, J., Dunphy, W.G., and Venkitaraman, A.R. (2005) Initiation of DNA replication requires the RECQL4 protein mutated in Rothmund-Thomson syndrome. *Cell* **121**, 887–898
12. Seki, M., Kohda, T., Yano, T., Tada, S., Yanagisawa, J., Eki, T., Ui, M., and Enomoto, T. (1995) Characterization of DNA synthesis and DNA-dependent ATPase activity at a restrictive temperature in temperature-sensitive tsFT848 cells with thermolabile DNA helicase B. *Mol. Cell. Biol.* **15**, 165–172
13. Tada, S., Kobayashi, T., Omori, A., Kusa, Y., Okumura, N., Kodaira, H., Ishimi, Y., Seki, M., and Enomoto, T. (2001) Molecular cloning of a cDNA encoding mouse DNA helicase B, which has homology to *Escherichia coli* RecD protein, and identification of a mutation in the DNA helicase B from tsFT848 temperature-sensitive DNA replication mutant cell. *Nucleic Acids Res.* **29**, 3835–3840
14. Taneja, P., Gu, J., Peng, R., Carrick, R., Uchiumi, F., Otto, R.D., Gustafson, E., Podust, V.N., and Fanning, E. (2002) A dominant-negative mutant of human DNA helicase B blocks the onset of chromosomal DNA replication. *J. Biol. Chem.* **277**, 40853–40861
15. Chino, M., Nishikawa, K., Umekita, M., Hayashi, C., Yamazaki, T., Tsuchida, T., Sawa, T., Hamada, M., and Takeuchi, T. (1996) Heliquinomycin, a new inhibitor of DNA helicase, produced by *Streptomyces sp.* MJ929-SF2. I. Taxonomy, production, isolation, physico-chemical properties and biological activities. *J. Antibiotics* **49**, 752–757
16. Chino, M., Nishikawa, K., Yamada, A., Ohsono, M., Sawa, T., Hanaoka, F., Ishizuka, M., and Takeuchi, T. (1998) Effect of a novel antibiotic, heliquinomycin, on DNA helicase and cell growth. *J. Antibiotics* **51**, 480–486
17. Ishimi, Y., Sugiyama, T., Nakaya, R., Kanamori, M., Kohno, T., Enomoto, T., and Chino, M. (2009) Effect of heliquinomycin on the activity of human minichromosome maintenance 4/6/7 helicase. *FEBS J.* **276**, 3382–3391
18. Ishimi, Y. (1997) A DNA helicase activity is associated with an MCM4, -6, and -7 protein complex. *J. Biol. Chem.* **272**, 24508–24513
19. Ishimi, Y., Komamura-Kohno, Y., Kwon, H.-J., Yamada, K., and Nakanishi, M. (2003) Identification of MCM4 as a target of the DNA replication block checkpoint system. *J. Biol. Chem.* **278**, 24644–24650
20. Nakaya, R., Takaya, J., Onuki, T., Moritani, M., Nozaki, N., and Ishimi, Y. (2009) Identification of proteins that may directly interact with human RPA. *J. Biochem.* **148**, 539–547
21. Kenny, M.K., Schlegel, U., Furneaux, H., and Hurwitz, J. (1990) The role of human single-stranded DNA binding protein and its individual subunits in simian virus 40 DNA replication. *J. Biol. Chem.* **265**, 7693–7700
22. Kimura, K., Nozaki, N., Saijo, M., Kikuchi, A., Ui, M., and Enomoto, T. (1994) Identification of the nature of modification that causes the shift of DNA topoisomerase II beta to apparent higher molecular weight forms in the M phase. *J. Biol. Chem.* **269**, 24523–24526
23. Ishimi, Y., Komamura-Kohno, Y., Karasawa-Shimizu, K., and Yamada, K. (2004) Levels of MCM4 phosphorylation and DNA synthesis in DNA replication block checkpoint control. *J. Struc. Biol.* **146**, 234–241
24. Sato, M., Gotow, T., You, Z., Komamura-Kohno, Y., Uchiyama, Y., Yabuta, N., Nojima, H., and Ishimi, Y. (2000) Electron microscopic observation and single-stranded DNA binding activity of the Mcm4,6,7 complex. *J. Mol. Biol.* **300**, 421–431
25. Brewstera, A.S., Wanga, G., Yua, X., Greenleafa, W.B., Carazob, J.M., Tjajadia, M., Kleina, M.G., and Chen, X.S. (2008) Crystal structure of a near-full-length archaeal MCM: Functional insights for an AAA^{+} hexameric helicase. *Proc. Natl Acad. Sci. USA* **105**, 20191–20196