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Short Communication

Lability in sulfur acidic cultivation medium explains unstable effects of CDK inhibitors on *Cyanidioschyzon merolae* cell proliferation

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We previously showed that nuclear DNA replication (NDR) is regulated by a checkpoint monitoring the occurrence of organelle DNA replication (ODR) in a unicellular red alga *Cyanidioschyzon merolae*. These analyses depended on the use of chemical CDK inhibitors such as CDK2 inhibitor II and roscovitine, but subsequent analyses yielded conflicting results depending on the experimental conditions. In the present study, we identified significantly short half-lives of the used chemicals in the sulfur acidic cultivation medium, which reconciles the discrepancy among these results.

Key Words: CDK; cell cycle; *Cyanidioschyzon merolae*; inhibitor; red algae

Cyanidioschyzon merolae is a unicellular red alga living in acidic hot springs, and was introduced as a novel model eukaryotic cell to elucidate various fundamental cell processes because it contains the simplest structure among eukaryotic cells. The complete genome sequences of the nucleus, mitochondria, and chloroplast were determined, and various tools for molecular genetics analyses have been developed (Fujiwara et al., 2013; Imamura et al., 2009; Kobayashi et al., 2010; Kuroiwa, 1998; Matsuzaki et al., 2004; Nozaki et al., 2007; Taki et al., 2015). *C. merolae* has been extensively used for studying organelle division processes, cell cycle regulation, and structural biology.

In a previous study, we investigated a mechanism underlying the coordination of organelle DNA replication (ODR) with nuclear DNA replication (NDR) in *C. merolae* (Kobayashi et al., 2009). Under periodic light-dark cultivation conditions, the algal cell cycle is arrested at the G1 phase in the dark, and initiated by illumination. Detailed analyses revealed that both mitochondria and chloroplast genomes replicate upon the onset of illumination, and then NDR occurs afterward. As the underlying mechanism, we revealed that an increase of intracellular Mg-ProtoIX, an intermediate of chlorophyll biosynthesis produced in the chloroplast, induced by the ODR occurrence was recognized as a retrograde signal from the chloroplast to the nucleus to activate CDKA, responsible for activating NDR.

During these experiments, specific inhibitors for cyclin-dependent kinases (CDK) were conveniently used to analyze the underlying mechanism. CDK2 inhibitor II is a commercially available inhibitor for animal CDK2 and plant CDKA (Merck, Darmstadt, Germany) (Davis et al., 2001). As expected, biochemical and physiological analyses revealed that this compound inhibited the relevant CDK (CDKA) activity and NDR in *C. merolae* (Kobayashi et al., 2009). Another CDK inhibitor used was roscovitine (Merck, Darmstadt, Germany), that is known as a general inhibitor for various CDK types, as revealed by its inhibition of cdc2/cyclin B, cdk2/cyclin A, cdk2/cyclin E, and cdk5/p53 in mammalian cells (Xie et al., 2016). Our results indicated that CDK inhibitor II inhibited only NDR, while roscovitine inhibited both ODR and NDR, and, thus, it was suggested that ODR is under the control of a CDK other than CDKA in *C. merolae*. Because both CDK inhibitors inhibit NDR at the cell cycle S phase, their addition to the medium was expected to result in the growth arrest. However, little inhibitory effect on OD_{750} increases was observed during 3 days after adding the drugs to the medium at the concentration used in the previous DNA replication analyses, 600 nM or 5 μM of CDK2 inhibitor

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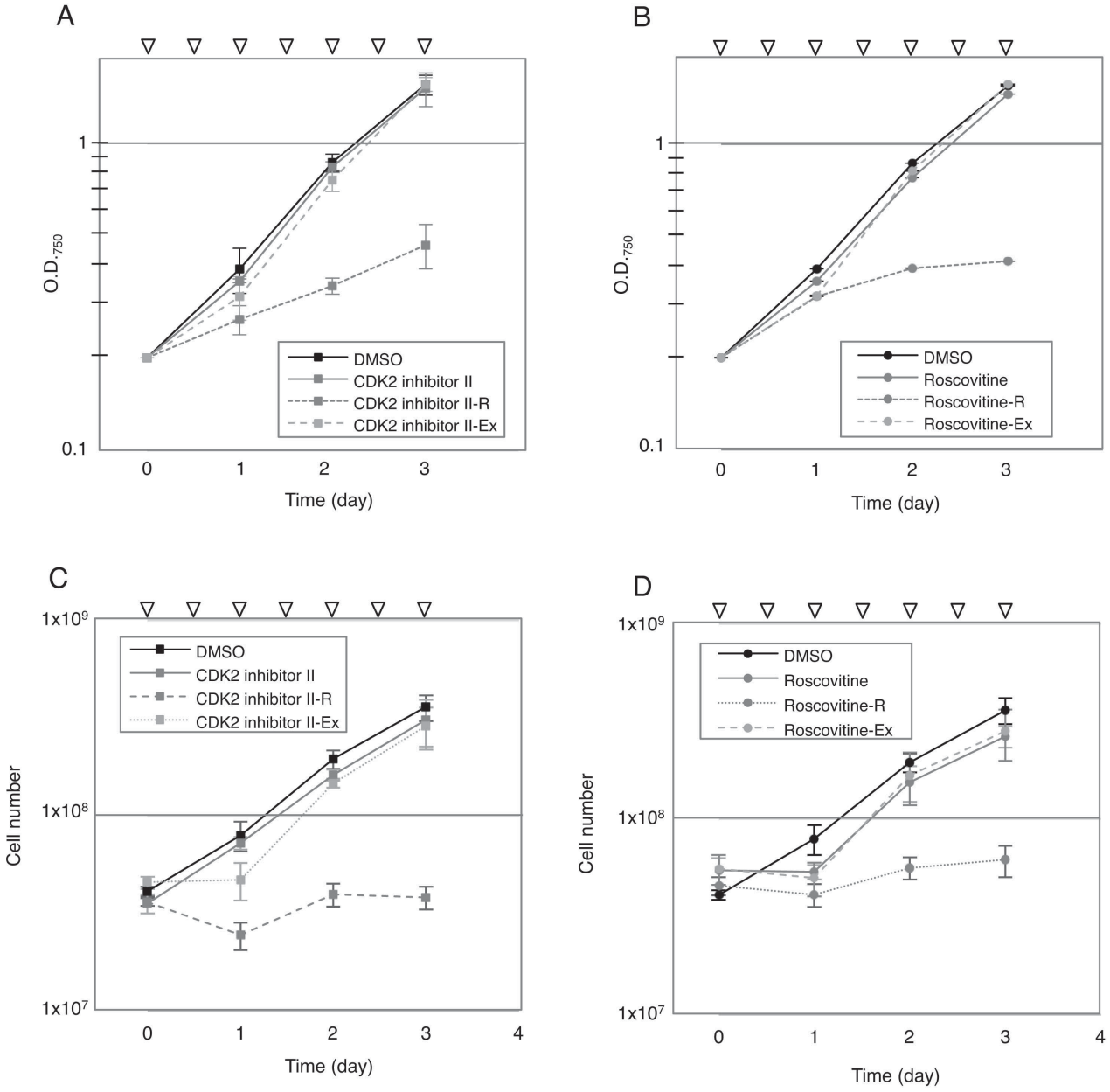

Fig. 1. Effect of CDK2 inhibitor II and roscovitine on cell growth.

When the OD_{750} of *C. merolae* cultures reached 10, cells were diluted to an OD_{750} of 0.2 and cultivated under continuous illumination (50 μmol photons m^{-2} s^{-1}) at 42°C, bubbled with air supplemented with 3% CO_2. The composition of MA2 medium was described previously (Kobayashi et al., 2010). Each inhibitor was added to the medium at time 0 (600 nM CDK2 inhibitor II; A and C, 5 μM Roscovitine; B and D) or repeatedly at 12 h intervals (R). Excess amounts of inhibitors (Ex) were also added at time 0 (4.8 μM CDK2 inhibitor II; A and C, 40 μM Roscovitine; B and D). Samples were collected at the indicated time points. A and B display changes in the OD_{750} C and D displays changes in cell numbers. Each data point represents the average of three independent experiments (n = 3 ± S.D.). White arrowheads indicate the points of repeated inhibitor additions.

II or roscovitine, respectively (Figs. 1A and B). To explain this, we wondered if these inhibitors might be unstable and rapidly lose their activity in the sulfur acidic cultivation medium, MA2, since we previously found that acid lability in MA2 medium masks the physiological effects of exogenously added abscisic acid (ABA) (Kobayashi et al., 2016).

Because of the instability of the compounds in the acidic medium, periodic addition of the compounds to the medium may accomplish the growth inhibitory effect. To examine this, we repeatedly added the same dose of inhibitors to the medium at 12-hour intervals and observed cell growth. With the new protocol, we found that both CDK2 inhibitor II and roscovitine markedly inhibited cell growth (Lines-R in Figs. 1A and B). Accumulation of the inhibitor was not the reason for growth inhibition, because adding 8-fold higher doses of the inhibitors (4.8 μM and 40 μM, respectively) at the initial time point only slightly affected the growth (Lines-Ex in Figs. 1A and B). Increases of cell numbers during the cultivation were also measured during the time course, shown in Figs. 1C and D. While severe inhibition of cell number increase was observed by repeated addition of both drugs, it was also observed during the first 24 hours by the addition of CDK2

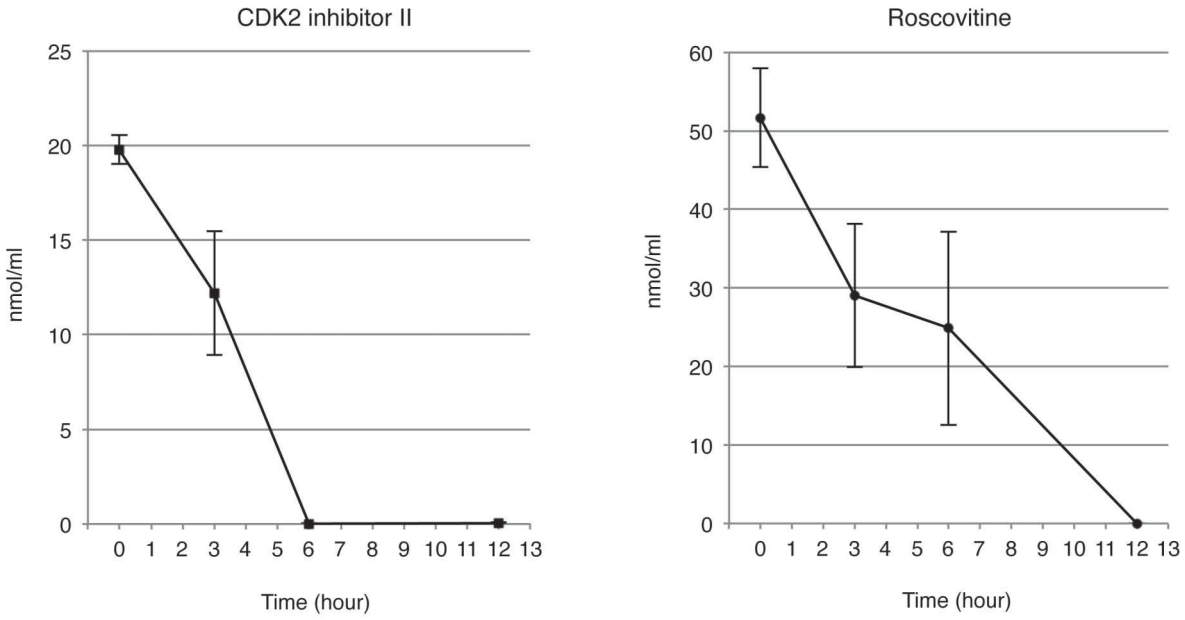

Fig. 2. Degradation of CDK2 inhibitor II and roscovitine in MA2 medium.

CDK2 inhibitor II or Roscovitine were added to MA2 medium at time 0 (CDK2 inhibitor II 20 μM, roscovitine 50 μM) and kept under illumination (150 μmol photons m^{-2} s^{-1}) at 42°C, bubbled with air supplemented with 3% CO_2. Samples were collected at the indicated time points. Stability of CDK2 inhibitor II and Roscovitine were measured using HPLC as described previously (Davis et al., 2001; Xie et al., 2016), with minor modifications. Samples of CDK2 inhibitor II were separated on a reversed-phase column, Symmetry C8 (150 × 4.6 mm; Waters) using a Nexera X2 HPLC system (Shimadzu). The following gradient (solution A = acetonitrile, solution B = 5 mM ammonium acetate) with a flow rate of 1 mL/min was applied: 0.5 min = 10% B, 0.5–20 min = 10–70% B, 20–25 min = 70–100% B, 25–28 min = 100% B, and 28–33 min = 100–10% B. CDK2 inhibitor II was detected using a photodiode array detector at 395 nm, and produced a peak at 11.6 min. Standard curves were made with authenticated standards. Samples of Roscovitine were separated on a reversed-phase column, Symmetry C18 (250 × 4.6 mm; Senshu) using a Nexera X2 HPLC system. Isocratic separations were performed using a hexane: 2-propanol (80:20 v/v) mobile phase with a flow rate of 1 mL/min. Roscovitine was detected using a UV detector at 260 nm, and produced a peak at 2.5 min. Standard curves were made with authenticated standards. Each data point represents the average of three independent experiments (n = 3 ± S.D.).

Table 1. Drug stability under high acidity or high temperature conditions.

Incubation condition	0 h	pH 7.4, 25°C, 24 h	pH 7.4, 42°C, 24 h	pH 2, 25°C, 24 h
CDK2 inhibitor II	19.79 ± 0.77 μM	15.93 ± 2.43 μM	3.25 ± 0.92 μM	9.05 ± 4.83 μM
Roscovitine	51.7 ± 6.27 μM	52.71 ± 13.9 μM	41.56 ± 7.67 μM	0 ± 0 μM

inhibitor II (Ex 4.8 μM, Fig. 1C) or roscovitine (5 μM or Ex 40 μM, Fig. 1D) at the initial time point. Thus, increase in cell number, which is relevant to the function of CDK inhibitors, is more sensitive to these drugs than the increase of the cell mass.

The half-lives of the two compounds in the *C. merolae* cultivation medium were analyzed by HPLC analysis. Doses of the compounds were added to the MA2 medium and incubated under usual *C. merolae* cultivation conditions (50 μmol photons m^{-2} s^{-1}, bubbled with air supplemented with 3% CO_2). The medium was sampled periodically, and the drug concentrations were quantitated. As shown in Fig. 2, the concentrations of these compounds reduced over time, and half-lives of CDK2 inhibitor II and roscovitine in the medium were calculated to be around 3 hours and 6 hours, respectively. These analyses showed that both inhibitors had mostly disappeared within 12 hours under the cultivation conditions, which is consistent with the increase of cell numbers (Figs. 1C and D). To identify determinants of the drug lability, CDK2 inhibitor II and roscovitine were incubated in MA2 medium under standard cultivation conditions, except pH and temperature were controlled as indicated in Table 1. The remaining drugs were quantitated after 24 hours. CDK2 inhibitor II was rapidly degraded under both high temperature (pH 7.4, 42°C) and low pH (pH 2, 25°C) conditions. Roscovitine was stable at high temperature (pH 7.4, 42°C) but unstable under low pH (pH 2, 25°C) condition.

In this study, we found that CDK2 inhibitor II and roscovitine are rather unstable under *C. merolae* cultivation conditions. From a structural point of view, CDK2 inhibitor II contains an acid-labile hydrazone structure, whereas roscovitine is highly basic and protonated under acidic conditions. As mentioned above, ABA also possesses acid-labile hydroxy groups (Supplementary Fig. S1). These molecular characteristics are well-consistent with our experimental observation. Thus, we should be especially cautious when using inhibitory drugs to analyze cellular mechanism of organisms cultivated in extremely acidic conditions.

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Supplementary Materials

Supplementary figure is available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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