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# Identification of a Novel Small Molecule Inhibitor Against SARS Coronavirus Helicase

**Jin-Beom Cho[1†], Jin-Moo Lee[1†], Hee-Chul Ahn[2], and Yong-Joo Jeong[1*]**

[1]*Department of Bio and Nanochemistry, Kookmin University, Seoul 02707, Republic of Korea*
[2]*Department of Pharmacy, Dongguk University-Seoul, Goyang 10326, Republic of Korea*

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*Corresponding author
Phone: +82-2-910-5454;
Fax: +82-2-910-4415;
E-mail: jeongyj@kookmin.ac.kr

†These authors contributed equally to this work.

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A new chemical inhibitor against severe acute respiratory syndrome (SARS) coronavirus helicase, 7-ethyl-8-mercapto-3-methyl-3,7-dihydro-1H-purine-2,6-dione, was identified. We investigated the inhibitory effect of the compound by conducting colorimetry-based ATP hydrolysis assay and fluorescence resonance energy transfer-based double-stranded DNA unwinding assay. The compound suppressed both ATP hydrolysis and double-stranded DNA unwinding activities of helicase with $IC_{50}$ values of 8.66 ± 0.26 μM and 41.6 ± 2.3 μM, respectively. Moreover, we observed that the compound did not show cytotoxicity up to 80 μM concentration. Our results suggest that the compound might serve as a SARS coronavirus inhibitor.

**Keywords:** SARS, helicase, ATP hydrolysis, dsDNA unwinding, inhibitor

Severe acute respiratory syndrome (SARS) occurred in Guangdong, China in 2002 and was rapidly transmitted all over the world. Although the SARS pandemic was controlled in a year through global efforts, SARS has been regarded as a serious concern owing to the unavailability of effective medicine or vaccine. In fact, SARS patients were treated with conventional antiviral drugs, such as ribavirin, type-I interferon, and so on, during the initial outbreak. However, it was hard to determine which treatment was beneficial to SARS patients [16]. Some worsened the symptoms of patients. Therefore, it is necessary to develop effective inhibitors against SARS for possible outbreak in the future.

SARS was caused by a novel coronavirus, SARS coronavirus (SCV), which is a single-stranded (ss) RNA positive-strand virus with a genome of about 29.7 kb [12, 15]. From the SCV genome, pp1a and pp1ab polyproteins are synthesized and smaller functional proteins are produced by proteinases [7, 9]. This proteolysis generates several non-structural proteins (nsPs), such as RNA polymerase and NTPase/helicase, which are major components of the membrane-bound viral replicase complex [7, 17]. Generally, viral polymerase and helicase are regarded as good targets for the development of antiviral inhibitors [3, 5, 6]. Of the several SCV nsPs, it is noted that helicase is an attractive target because of its indispensability in viral genome replication.

Helicases are motor proteins that couple the released energy from nucleoside triphosphate hydrolysis with double-stranded (ds) nucleic acid unwinding into single-stranded nucleic acids [13, 14]. In fact, many attempts have been made to identify inhibitors of SCV helicase and test the inhibitory effect of the helicase as well as viral replication [1, 2, 10, 11, 18, 19]. Previously, we have published the isolation of ssRNA aptamers specific to SCV helicase [8]. We have also shown that aryl diketoacids and dihydroxychromone derivatives inhibit double-stranded DNA unwinding activity [10, 11]. In addition to synthetic compounds, natural compounds were screened to identify the inhibitors of SCV helicase, which resulted in the finding of myricetin and scutellarein as ATP hydrolysis inhibitors [20].

In the present study, we have found a new chemical compound, designated as EMMDPD hereafter, with a

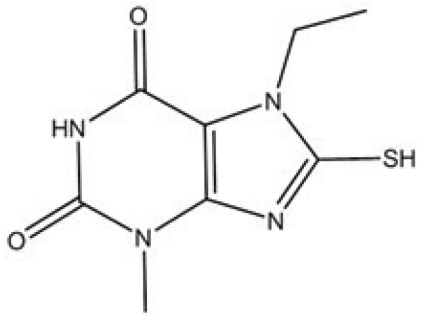


**Fig. 1.** Chemical structure of 7-ethyl-8-mercapto-3-methyl-3,7-dihydro-1H-purine-2,6-dione.

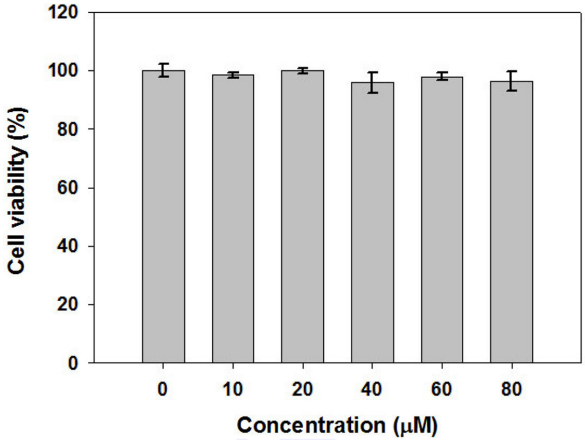


**Fig. 3.** Measurement of cell viability in the presence of 7-ethyl-8-mercapto-3-methyl-3,7-dihydro-1H-purine-2,6-dione.

novel structure, 7-ethyl-8-mercapto-3-methyl-3,7-dihydro-1H-purine-2,6-dione (Fig. 1), as part of our ongoing efforts to identify novel small compounds that can inhibit SCV helicase activity *in vitro*. EMMDPD does not show cytotoxicity, and its inhibitory effect against SCV helicase has never been reported.

SCV helicase was overexpressed in *E. coli* and purified as described previously [8]. To examine the ATP hydrolysis activity of SCV helicase, we measured the amount of inorganic phosphate ($P_i$) released from ATP. The released $P_i$ during ATP hydrolysis was monitored by the malachite green and ammonium molybdate (AM/MG reagent) method [4, 10]. Briefly, a 25 μl solution containing 400 nM SCV helicase pre-mixed with 0.5 μl of EMMDPD in 50 mM Tris/HCl (pH 6.6) was placed on 96-well plate. After 5 min incubation at 25°C, ATP hydrolysis was started by addition of another 25 μl solution containing 100 mM NaCl, 10 mM $MgCl_2$, 4 mM ATP, and 4 nM circular ssDNA M13 in 50 mM Tris/HCl (pH 6.6) to each well. Further incubation was performed for 10 min at 37°C and the reaction was stopped by adding 200 μl of AM/MG reagent (0.034% malachite green, 1.05% ammonium molybdate, and 0.04% Tween 20 in 1 M HCl). The developed color was monitored by measuring at 620 nm, and the released amount of $P_i$ was calculated using a standard curve. Fig. 2A displays the relative inhibitory effect (%) of ATP hydrolysis by EMMDPD. To determine the $IC_{50}$ value of EMMDPD, ATP hydrolysis reactions were performed in various concentrations of EMMDPD and the inhibitory effect was measured. Through this analysis, we obtained the $IC_{50}$ value of EMMDPD (8.66 ± 0.26 μM for ATP hydrolysis).

dsDNA unwinding experiments were also performed in the presence of EMMDPD. To measure the dsDNA unwinding activity, we measured dsDNA unwinding using fluorescence resonance energy transfer (FRET), as described previously [10, 11]. To determine the $IC_{50}$ value,

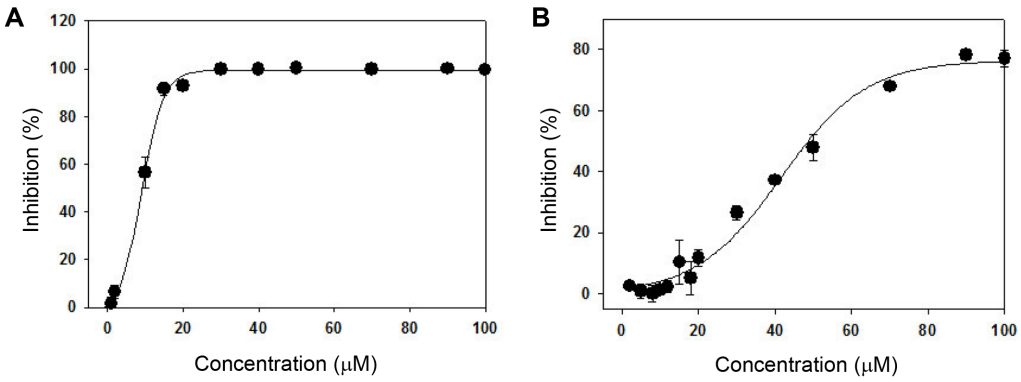


**Fig. 2.** Percentage inhibition of SCV helicase by 7-ethyl-8-mercapto-3-methyl-3,7-dihydro-1H-purine-2,6-dione.
(**A**) ATP hydrolysis activity. (**B**) dsDNA unwinding activity.

dsDNA unwinding reactions were performed in various concentrations of EMMDPD and inhibition was measured (Fig. 2B). Through this analysis, we obtained the $IC_{50}$ value of EMMDPD (41.6 ± 2.3 μM for dsDNA unwinding).

Cell viability in the presence of EMMDPD was examined by MTT assay using WI-38 cells that were maintained at 37°C in an incubator with atmosphere of 5% $CO_2$ and cultured in DMEM containing 10% heat-inactivated fetal calf serum, 1% non-essential amino acid, streptomycin (100 μg/ml), and penicillin (100 units/ml). The starting cell density was $6 \times 10^4$ cells/ml. After 24 h, EMMDPD was added to a final concentration of 10, 20, 40, 60, and 80 μM and the cells were incubated for 48 h. Then, MTT solution was added to each well and the supernatant was removed after 2 h incubation. The produced formazan crystal was dissolved in dimethyl sulfoxide and the absorbance at 540 nm was read using a scanning multiwell spectrophotometer. Fig. 3 shows that EMMDPD was not cytotoxic to WI-38 cells at any concentration tested. At up to 80 μM concentrations of EMMDPD, cell viability was more than 95% of that of control cells (0 μM EMMDPD).

In conclusion, we have demonstrated that EMMDPD is a novel chemical inhibitor of SCV helicase, which suppressed ATP hydrolysis as well as dsDNA unwinding activities without cytotoxicity. It is of particular interest that the inhibitory compounds reported to date used to exhibit selectivity towards only ATP hydrolysis or dsDNA unwinding. Whereas aryl diketoacids and dihydroxychromone derivatives selectively inhibit dsDNA unwinding activity, but not ATP hydrolysis [10, 11], we have found that a few natural compounds, such as myricetin, scutellarein, and baicalein, selectively inhibit only ATP hydrolysis [20]. However, it is unclear why EMMDPD inhibits both activities at present owing to lack of structural information. Because of its dual inhibitory effect, we expect EMMDPD to hold good potential for the development of antiviral material after further investigation.

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