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Cytotoxic compounds against cancer cells from *Bombyx mori* inoculated with *Cordyceps militaris*

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Abstract

Two compounds, 3'-deoxyinosine and cordycepin, were isolated from *Bombyx mori* inoculated with *Cordyceps militaris*. In the bioassay examining cytotoxicity against cancer cells, both compounds showed toxicity against A549, PANC-1 and MCF-7 cancer cells.

Key words: Cytotoxic compound; *Bombyx mori*; Structural identification; *Cordyceps militaris*

The genus *Cordyceps* belongs to Ascomycota phylum, Sordariomycetes class, Hypocreales order, Clavicipitaceae family. *Cordyceps* fungi have parasitic nature on insects' larvae and pupae, or even on adult insects.[1)] Many species of *Cordyceps* have been used as traditional Chinese medicines from ancient times. In the studies about *Cordyceps* sp., a lot of bioactivities have been reported, such as immunomodulatory activity, antioxidant activity, antitumour activity and cancer cell cytotoxicity.[2-17)]

Cordyceps militaris is a valuable specie of the genus *Cordyceps*. It parasitizes lepidopteran pupa and forms fruiting bodies. Natural *C. militaris* is very expensive because of its less production. However, recently, artificial cultivation of the fruiting bodies was succeeded and since then the fungus has become a widely-used functional food and a subject of research. Although several biological and chemical studies about the mycelia and the fruiting bodies have been reported, there are few studies about its host, lepidopteran larvae infested with the fungus.[4,7,8,10,12,13,15-20)] In the screening experiments, we found the cytotoxicity of the crude extracts of *Bombyx mori* inoculated with *C. militaris* and started this study.

Here, we describe the isolation and identification of two cytotoxic compounds against cancer cells from the infested *B. mori*.

Larvae bodies of the infested *B. mori* were extracted with EtOH and then with acetone. After the solutions were combined and dried up under reduced pressure, the dried materials were extracted with *n*-hexane, EtOAc and MeOH, respectively. The EtOAc soluble part was fractionated by repeated chromatography. As a result, two compounds (**1** and **2**) were purified (Fig. 1).

Compound **1** was identified to be (2*R*,3*R*,5*S*)-2-(6-aminopurin-9-yl)-5-(hydroxymethyl)oxolan-3-ol (cordycepin), which was isolated from the cultured mycelia of *C. militaris* in 1950.[21)] Cordycepin is famous for its various bioactivities, such as antioxidative activity and cancer cell cytotoxicity.[9,14,16,17,22,23)] All the data of **1** including the CD spectrum were identical with those of commercially available cordycepin.

Compound **2** was identified as 3'-deoxyinosine that has been isolated from okra (*Abelmoschus esculentus*) [24)] and it was previously reported that this compound showed antiprotozoal activities.[25,26)] However, there is no other report of isolation of **2** from natural sources.

Cytotoxicity of compounds **1** and **2** against cancer cells (A549, PANC-1 and MCF-7) was tested (Fig. 2). Compounds **1** and **2** showed dose-dependent cytotoxicity against PANC-1 and MCF-7 cancer cells. On the other hand, **1** also showed dose-dependent cytotoxicity against A549 cell, but the cytotoxicity of **2** against the cells was weak and there was no dose-dependency. Both the two compounds showed the strongest cytotoxicity against MCF-7 cells at 30 μM. In the previous studies, **1** has shown cytotoxicity against A549 and MCF-7 cancer cells.[16,17,22,23] The cytotoxicity of **1** against PANC-1 cancer cell is reported here for the first time. This is also the first time for reporting the cytotoxicity against cancer cells of **2.**

^{1}H-NMR spectra (one-and two-dimensional) were recorded on a Jeol lambda-500 spectrometer (Jeol Ltd., Tokyo, Japan) at 500 MHz, while ^{13}C-NMR spectra were recorded by the same instrument at 125 MHz. HRESIMS data were measured by a JMS-T100LC mass spectrometer (Jeol Ltd., Tokyo, Japan). HPLC separation was performed with a Jasco Gulliver system (Jasco Co., Tokyo, Japan) using a reverse-phase HPLC column (Cosmosil PBr, ϕ20×250 mm, Nacalai tesque, Kyoto, Japan). C18 cartridges (Nihon Waters K.K., Tokyo, Japan) were used in the pro-processing of the samples. Silica gel plate (TLC Silica gel 60 F_{254}, Merck KGaA, Darmstadt, Germany) and silica gel 60N (Kanto Chemical Co.,Inc., Tokyo, Japan) were used for analytical TLC and for flash column chromatography, respectively.

Larvae of *B. mori*, which was inoculated with *C. militaris* by injection and then fruiting bodies of the fungus formed from the larvae. The larvae with the fruiting bodies were purchased from Nichihara Research & Development Laboratories, Inc. in 2014. Human adenocarcinoma A549, human pancreatic carcinoma PANC-1 and breast adenocarcinoma MCF-7 cells were obtained from ATCC cell line (VA, USA), and maintained in Dulbecco's modified eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin (all purchased from Invitrogen, CA, USA). All cultures were kept in a humidified atmosphere with 5% CO_2 at 37°C.

The larvae with the fruiting bodies (16.4 kg) was divided into the bodies of *B. mori* (14.3 kg) and the fruiting bodies (2.10 kg). The insect bodies were crushed and extracted with EtOH (40.0 L, 3 times) and then with acetone (20.0 L, 3 times). After the

solutions were combined and dried up under reduced pressure, the dried materials were extracted with *n*-hexane, EtOAc and MeOH for 2 times (2.0 L), respectively. The EtOAc-soluble part (45.0 g) was fractionated by silica gel flash column chromatography (99/1, 95/5, 80/20, 70/30, 50/50, 30/70 CH_2Cl_2/acetone; 99/1, 95/5, 80/20, 70/30, 50/50, 30/70 CH_2Cl_2/MeOH; 2.0 L each) to obtain 24 fractions (fractions 1 to 24). Fraction 21 (726 mg) was fractionated by C18 cartridges (50% MeOH, MeOH; 100 mL, 2 times, respectively) to obtain 3 fractions (fractions 21-1 to 21-3). Fraction 21-1 (520 mg) was further fractionated by reverse-phase HPLC (Cosmosil PBr, ϕ20×250 mm, UV 250 nm, 30% MeOH) to give 23 fractions (fractions 21-1-1 to 21-1-23), and fraction 21-1-17 was compound **1** (41.0 mg). Fraction 21-1-11 (5.3 mg) was separated by reverse-phase HPLC (Cosmosil PBr, ϕ20×250 mm, UV 250 nm, 20% MeOH) to give compound **2** (2.2 mg).

Compound **2**: white amorphous; ESIMS *m/z* 275 $[M+Na]^+$; ^{1}H-NMR (in CD_3OD) δ_H: 8.38 (s, H-2), 8.04 (s, H-8), 5.99 (d, *J*=2.1 Hz, H-1'), 4.65 (m, H-2'), 4.51 (m, H-4'), 3.90 (dd, *J*=12.2, 2.7 Hz, H-5'a), 3.67 (dd, *J*=12.2, 3.7 Hz, H-5'b), 2.33 (m, H-3'a), 2.02 (m, H-3'b), ^{13}C-NMR δ_C: 159.4 (C-6), 149.4 (C-4), 147.1 (C-2), 140.3 (C-8), 125.8 (C-5), 93.4 (C-1'), 82.8 (C-4'), 77.2 (C-2'), 63.9 (C-5'), 34.5 (C-3').

Compounds **1** and **2** were tested the cell viability against A549, PANC-1 and MCF-7 cancer cells through MTT assay.[27] Cells were seeded at 1×10^4 cells/cm^2 in 96-well plates and cultured for 24 h. Cells were incubated with compounds **1** and **2** (1, 10, 30 μM, respectively), sulforaphane (SFN, 30 μM) as indicated concentrations for 48 h. Sulforaphane (SFN; 30 μM) was used as positive control. After the incubation, the growth medium was removed and the cells were given 100 μL of 0.05% MTT solution, then incubated for 4 h. After the cells were incubated with 100 μl of lysis buffer [20% SDS, 50% *N*,*N*-dimethyl formamide (DMF), pH 4.7], absorbance was measured by a microplate reader (Bio-Rad Laboratories, CA, USA) at 595 nm. All incubations were carried out at 37°C in 5% CO_2. All data are shown as means ± SD. Differences among the all groups were evaluated using a 1-way analysis of variance (ANOVA) followed by the Dunnet. A *P* value less than 0.05 and 0.01 were considered statistically significant.

Author contribution

Hirokazu Kawagishi and Hiroshi Nishida designed the experiments. Weitao Qiu performed the experiments. Jing Wu, Jea-Hoon Choi, Hirofumi Hirai, Hiroshi Nishida and Hirokazu Kawagishi contributed to discussions. Weitao Qiu, Hiroshi Nishida and Hirokazu Kawagishi wrote the manuscript.

Disclosure statements

No potential conflict of interest was reported by authors.

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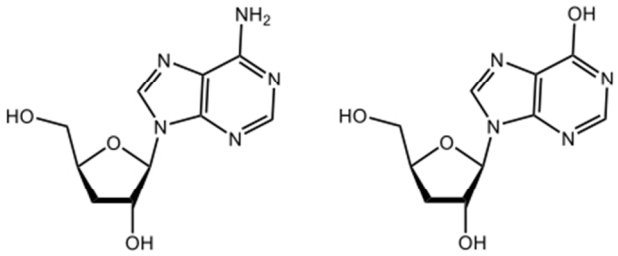
Legend to figure

Fig. 1. Structures of compounds **1** and **2**.

Fig. 2. Cytotoxicity of compounds **1** and **2** against A549, PANC-1 and MCF-7 cancer cells tested by MTT assay.

Notes: The concentrations of compounds **1** and **2** were adjusted to be 1 μM, 10 μM and 30 μM, respectively. Sulforaphane (SFN; 30 μM) was used as positive control. All data are shown as means ± SD (n=5-6). Differences among the all groups were evaluated using a 1-way analysis of variance (ANOVA) followed by the Dunnet test.

* indicates significant difference compared with CT ($P < 0.05$); ** indicates significant difference compared with CT groups ($P < 0.01$).



1 **2**

Fig. 1 Qiu et al

Fig.1

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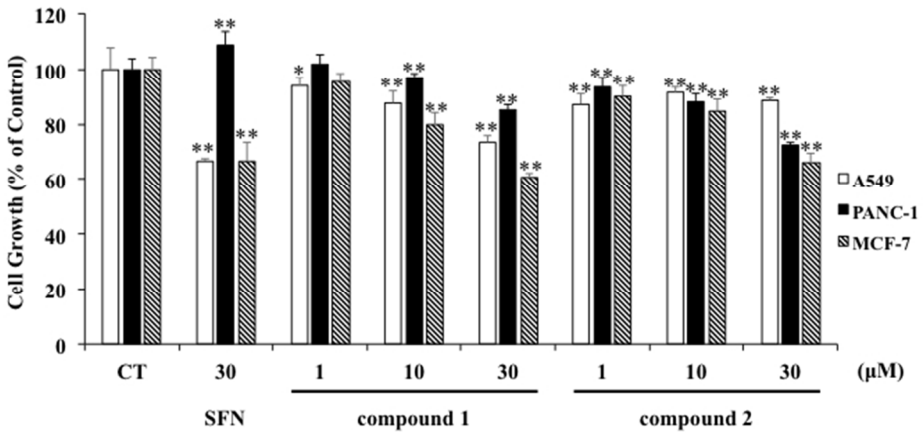

Fig. 2 Qiu et al

Fig.2

254x190mm (72 x 72 DPI)