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Multidrug Resistance Reversal Activity of Methanol Extracts from Basidiomycete Mushrooms in Cancer Cells

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Abstract – Mushrooms have a long history of use in traditional medicine, and hundreds of novel constituents in mushrooms with miraculous biological properties have been identified recently. Although diverse effects for medicinal use of mushrooms such as anticancer activity are proven, their reversal activities of drug resistance in cancer cells was rarely reported so far. In the search for novel medicinal use of mushrooms, we tested the multidrug resistance (MDR) reversal activities of diverse mushrooms collected from Korea. Among, the mushroom extracts tested, *Cantharellus cibarius* (M02) and *Russula emetica* (M12) revealed MDR reversal activities of paclitaxel in the P-glycoprotein (Pgp)-positive HCT15 and MES-SA/dX5 cancer cells, but not in the Pgp-negative A549 and MES-SA cancer cells. In addition, these mushrooms also enhanced the cytotoxicity of doxorubicin, another well-kwown Pgp-associated anticancer drug against MES-SA/DX5 cells, but not against MES-SA cells. Meanwhile, the cytotoxicity of cisplatin, a well-known Pgp-non-associated anticancer drug, was not affected by the mushrooms all the cells tested. From these results, we suspected that some ingredients of M02 and M12 have Pgp-associated MDR reversal activities.
Keywords – Basidiomycete, *Cantharellus cibarius, Russula emetica,* cytotoxicity

Introduction

Mushrooms have been used as traditional foods, in worldwide, for a long time. They have also a long history of use in traditional medicine, but their medicinal activities are being supported by contemporary studies. There are various classes of primary and secondary metabolites in mushrooms and they exhibit significant antimicrobial, antiviral and antitumor activities (Shamtsyan *et al*., 2004; Patel and Goyal, 2012). The bioactive compounds of mushrooms include polysaccharides, proteins, fats, glycosides, alkaloids, volatile oils, tocopherols, phenolics, flavonoids, carotenoids, folates, ascorbic acid enzymes, and organic acids. Polysaccharides are the best known and most potent mushroom-derived substances with anti-tumor and immunomodulating properties (Xu and Bao, 2010).

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Drug resistance is one of the most significant impediments to successful chemotherapy of cancer. Intrinsic or acquired drug resistance refers to the simultaneous development of resistance in tumor cells to mechanistically or structurally unrelated agents, and this phenomenon has been termed multidrug resistance (MDR). A major form of MDR is overexpression of ATP-binding cassette (ABC) transporters, and P-glycoprotein (Pgp), the 170 KDa transmembrane glycoprotein product encoded by the mdr1 gene is a major form of ABC transporter overexpression in cancer cells. The mechanism of drug resistance in Pgp-expressing tumor cells is due to increased transport of various classes of anticancer agents out of cells, which results in decreased cellular accumulation and thus decreases the efficacy of the drug (s) (Lum *et al*., 1993). Anticancer drugs associated Pgp-mediated MDR include paclitaxel (TAX), doxorubicin (DOX), etoposide, actinomycin D, vinblastine, meanwhile, Pgp cannot affect the cytotoxicity of some anticancer drugs such as cisplatin (CDDP), carboplatin and 5-fluorouracil (Ueda *et al*., 1987).

So far, lots of compounds are reported their MDR

reversal activity, and some compounds isolated from natural products such as flavonoids, stilbenes also reported their MDR reversal activity in cancer cells (Min *et al*., 2007; Wesołowska, 2011). Meanwhile, there are little reports about MDR reversal activity of the extracts or single compounds from mushrooms.

In this study, we investigated the MDR modulating activity of extracts from diverse Basidiomycete mushrooms collected at Korea. To determine the reversal activity of each mushroom extract, we tested the cytotoxicity of TAX, a well-known Pgp-associated anticancer drug against Pgp-positive or –negative human cancer cells in the presence or absence of the mushroom extracts tested, in comparison with a well-known MDR reversal agent, verapamil (VER). Then, for the conformation of some mushroom's enhancing activity of TAX cytotoxicity against human tumor cells is associated with the inhibition of Pgp function, we also tested the effects of the mushrooms on the cytotoxicity of DOX, another well-known Pgp-associated anticancer drug, in couples with Pgp-non-associated anticancer drug, CDDP.

Experimental

Mushroom material – The fresh fruiting bodies of Basidiomycete mushrooms were collected at Yuseong, Daejeon, Korea, in 2011, and the mushroom was identified by one of the authors (S.J.S.). A voucher specimen (43A001-021) was deposited at the herbarium of the College of Pharmacy, Chungnam National University, Korea.

Cancer cell culture – The human uterine sarcoma cell lines, MES-SA and its Pgp-associated MDR subline, MES-SA/DX5 were purchased from the American Type Culture Collection, and the colorectal adenocarcinoma cell line, HCT15 and human non-small cell lung cancer cells, A549 was provided by the National Cancer Institute (NCI). All cell cultures were maintained using RPMI1640 cell growth medium (Gibco, NY, USA) supplemented with 5% fetal bovine serum (FBS) (Gibco) and grown at 37 °C in a humidified atmosphere containing 5% CO_2 (Choi *et al*.,1996).

***In vitro* cytotoxicity assays** – All of the experimental procedures followed the NCI's protocols with some minor changes based on the sulforhodamine B (SRB) method as described previously. Stock solutions of each compound were prepared in DMSO and diluted in culture media by at least 200-fold for cell cytotoxicity assays. For each experiment, cell toxicity was calculated by comparing the number of cells surviving at time zero (Tz) with those

Table 1. Inhibition of tumor cell proliferation by methanol extracts of mushrooms

Mushrooms Extracts	IC_{50} (μg/ml) [a]		
	A549	MES-SA	HCT15
Tylopilus nigerrimus (M01)	> 100.0	58.71	> 100.0
Cantharellus cibarius (M02)	> 100.0	> 100.0	> 100.0
Pleurotus cornucopiae (M03)	> 100.0	> 100.0	> 100.0
Amanita virosa (M04)	67.19	42.35	> 100.0
Trametes dickinsii (M05)	> 100.0	> 100.0	> 100.0
Coprinus comatus (M06)	> 100.0	> 100.0	> 100.0
Russula cyanoxantha (M07)	> 100.0	> 100.0	> 100.0
Pleurotus salmoneo (M08)	> 100.0	> 100.0	> 100.0
Boletus fraternus (M09)	> 100.0	> 100.0	> 100.0
Armillariella tabescens (M10)	97.16	> 100.0	> 100.0
Daedaleopsis tricolor (M11)	> 100.0	> 100.0	> 100.0
Russula emetica (M12)	> 100.0	> 100.0	> 100.0
Formitella fraxinea (M13)	> 100.0	> 100.0	> 100.0
Russula densifolia (M14)	> 100.0	> 100.0	> 100.0
Grifola frondosa (M15)	68.07	72.36	> 100.0
Lactarius subvellereus (M16)	> 100.0	> 100.0	> 100.0
Russula pseudodelica (M17)	> 100.0	> 100.0	> 100.0
Russula japonica (M18)	>100.0	> 100.0	> 100.0

[a]IC_{50} value of methanol extracts of mushrooms against each cancer cell line, which was defined as a concentration (μg/mL) that caused 50 % inhibition of cell proliferation *in vitro*

surviving at the end of the 72 h experiment, referred to as DT for cells incubated with serial dilutions of the drug, or CC for control cells incubated without drug. If DT is greater than or equal to Tz, the net percent of cell growth inhibition was calculated as (DT Tz)/(CC Tz)/Tz 100. All data represent the average values of at least three wells per experiment. (Skehan *et al*., 1990; Ryu *et al*., 1992)

Drug resistance reversal assay – The cells were incubated with serial dilution of each anticancer drug in the presence or absence of the extract of each mushroom (50 μg/ml) or VER (10 μM) for 3 days. The procedure for calculations of survival fractions was identical to that of cytotoxicity assay. In this assay, the controls were wells that contained each mushroom extract or VER without anticancer drugs.

Results and Discussion

Cell cytotoxicities of mushrooms – We tested the tumor cell cytotoxicity of the mushrooms tested in this experiment for determination of the concentrations in use for the MDR reversal activity (Table 1). Among the mushrooms, the M01, M04 and M15 revealed minor cell cytotoxicities at high concentration (100 μg/ml). These mushrooms revealed cell growth inhibitory effect more than 30% against at least one kind of cancer cells tested. From these results, we selected mushrooms and determined the concentration tested for the MDR reversal activity. That is, we tested all the mushrooms except M01, M04 and M15 at the concentration of 50 μg/ml for MDR reversal activity.

Enhancing Activities of TAX cytotoxicity in cancer cells by mushrooms – Firstly, we tested the effects of mushrooms on the cytotoxicity of TAX against Pgp-positive HCT15 in comparison with Pgp-negative A549 human tumor cells. Among the mushrooms tested, M02 and M12 enhanced the cytotoxicity of TAX against HCT15 cells, remarkably. At 0.01 uM, TAX did not effect on the cell growth of HCT15 cells, meanwhile, the cell growth rate was reduced about 50% in the presence of M02 or M12 at the same concentration of TAX. At 0.1 μM, TAX inhibited the HCT15 cell growth about 50%, and in the presence of M02 and M12, TAX induced cell death about 35 and 20%, respectively. On the other hand, all the mushrooms tested did not effect on the cytotoxicity of TAX against A549 cells, remarkably (Fig. 1). For the conformation of MDR reversal activity of M02 and M12, we also tested the effects of the mushrooms on the cytotoxicity of TAX against Pgp-overexpressed MES-SA/DX5 human MDR cancer cells in comparition with its

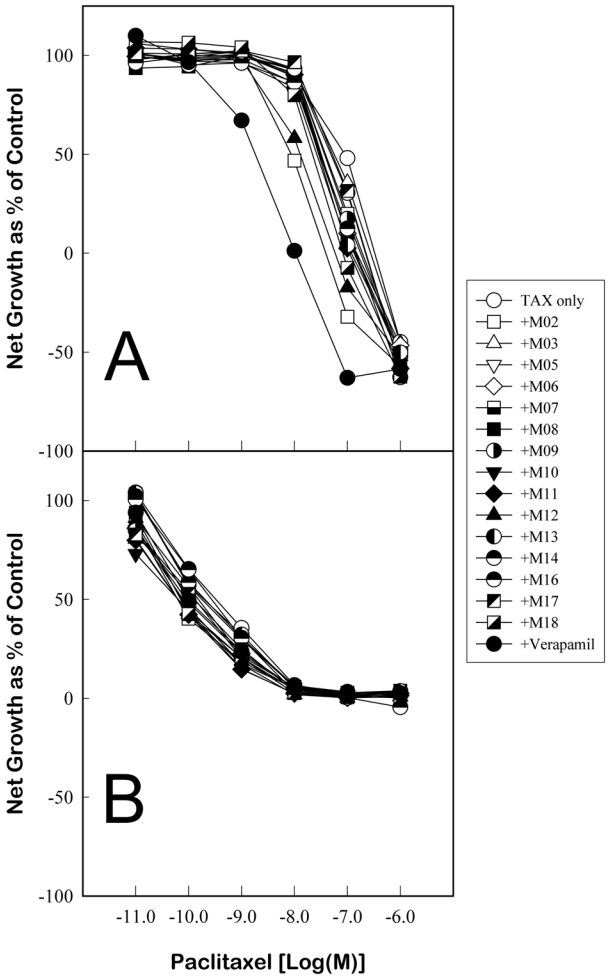

Fig. 1. Effects of mushroom extracts and VER on the cytotoxicity of TAX against HCT15 (A) human colorectal and A549 (B) human non-small cell lung cancer cells *in vitro*. The cells were cultured with serial dilution of TAX in the absence or presence of each mushroom extracts (50 μg/ml) and VER (10 μM). Cell survival fractions were assessed after continuous drug exposure for 3 days by SRB assay.

Pgp-negative parental MES-SA cancer cells. M02 enhanced the cytotoxicity of TAX at 0.01, 0.1 and 1.0 μM, and M12 also enhanced the cytotoxicity of TAX at 1.0 μM against MES-SA/DX5 cells, significantly. VER, a well-known MDR reversal agent, also enhanced the cytotoxicity of TAX against the MES-SA/DX5 cells at the same concentration range of M02, significantly. Meanwhile, M02, M12 and VER did not effect on the cytotoxicity of TAX against MES-SA cells at all the concentrations tested. From these results, we strongly supposed that M02 and M12 enhanced the cell cytotoxicity of TAX by inhibiting the Pgp-mediated drug efflux (Fig. 2).

Effects of M02 and M12 on the cytotoxicity of DOX and CDDP against human tumor cells – To conform the Pgp-mediated MDR reversal activity of M02 and M12

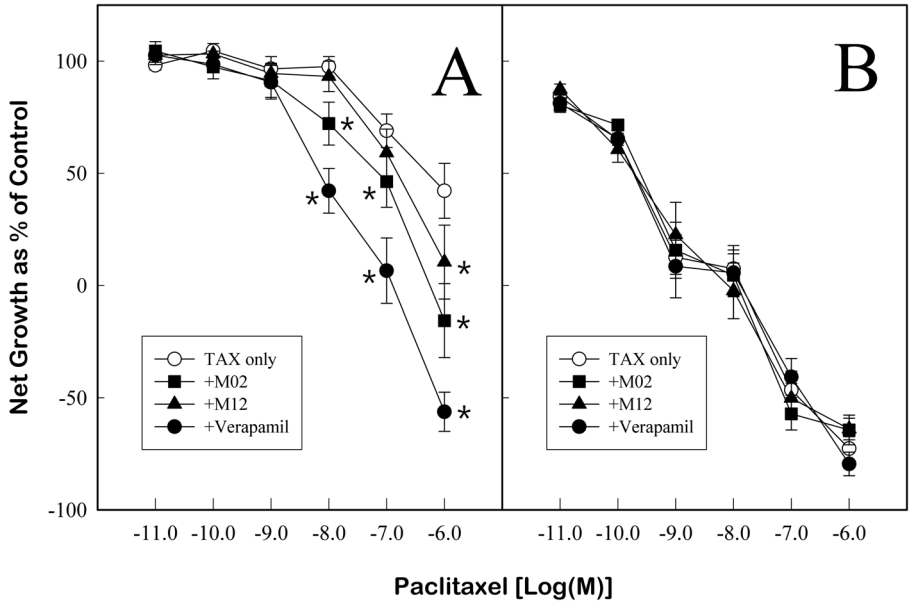

Fig. 2. Effects of mushroom extracts and VER on the cytotoxicity of TAX against MES-SA-DX5 (A) and MES-SA (B) human uterine cancer cells *in vitro*. The cells were cultured with serial dilution of TAX in the absence or presence of each mushroom extracts (50 μg/ml) and VER (10 μM). Cell survival fractions were assessed after continuous drug exposure for 3 days by SRB assay.
*Significantly different from the data of TAX only at the same concentration ($p < 0.05$)

Table 2. Effects of M02, M12 and VER on the cytotoxicity of DOX and CDDP against human tumor cells

Reversal agent	DOX (IC50, μM)[a]		CDDP (IC50, μM)	
	MES-SA	MES-SA/DX5	MES-SA	MES-SA/DX5
none	0.003 ± 0.0004[b]	2.43 ± 0.41	5.90 ± 0.32	7.36 ± 0.52
M02 (50 μg/ml)	0.004 ± 0.0006	0.25 ± 0.04*	4.71 ± 0.37	8.04 ± 0.44
M12 (50 μg/ml)	0.003 ± 0.0003	0.82 ± 006*	5.64 ± 0.30	8.25 ± 0.47
VER (10 M)	0.004 ± 0.0003	0.08 ± 0.004*	6.12 ± 0.27	7.69 ± 0.39

[a]IC50 : the concentration that cause 50% cell growth inhibition
[b]Data are presented as mean ± SEM of at least three distinct experiments
*Significaltly different from control ($p < 0.05$)

in cancer cells, we also tested the effects of the M02 and M12 on the cytotoxicity of DOX, a well-known Pgp-substrate anticancer drug, and CDDP, a well-known Pgp-non-associated anticancer drug, against MES-SA and MES-SA/DX5 cells. The IC_{50} value of DOX against MES-SA and MES-SA/DX5 cells are 0.003 ± 0.004 and 2.43 ± 0.41 μM, respectively. In the presence of M02, M12 and VER, the IC_{50} of DOX against MES-SA/DX5 cells are significantly reduced to 0.25 ± 0.04, 0.82 ± 0.06 and 0.08 ± 0.004, respectively. Meanwhile, the cytotoxicity of DOX against MES-SA in the presence of all the MDR reversal agent tested are not significantly changed in comparison with control. In the cytotoxicity test of CDDP, the M02, M12 and VER did not effect on the cytotoxicity of CDDP against MES-SA and MES-SA/DX5 cells, significantly (Table 2). These results strongly support that the cytotoxicity enhancing activity of M02 and M12 are mediated by inhibition of Pgp.

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