Arch. Pharm. Res. Vol.21, No.3, pp. 344-347, 1998

Isolation of a Multidrug Resistance Inhibitor from *Aconitum pseudo-laeve* var. *erectum*

Dae Keun Kim[+], Hyog Young Kwon, Kang Ro Lee*, Dong Kwon Rhee and Ok Pyo Zee

College of Pharmacy, Sung Kyun Kwan University, Suwon 440-746, Korea

(Received January 14, 1998)

To overcome multidrug resistance (MDR) in cancer chemotherapy, we prepared various plant extracts and searched for a component which is effective for inhibition of MDR. MDR inhibition activity was determined by measuring cytotoxicity to MDR cells using multidrug resistant human fibrocarcinoma KB V20C, which is resistant to 20 nM vincristine and expresses high level of *mdr1* gene. Of various plant extracts, the MeOH extract of the root of *Aconitum pseudo-laeve* var. *erectum* was found to have potent inhibitory activity on MDR. The bioassay-guided fractionation of the MeOH extract of the plant led to the isolation of an alkaloid, lycaconitine, as an active principle. And the IC_{50} of lycaconitne for KB V20C cells was 74 μg/ml.

Key words : Multidrug Resistance (MDR), *Aconitum pseudo-laeve* var. *erectum*, Lycaconitine

INTRODUCTION

Cancer chemotherapy has been successful in the cure of many malignancies, such as testicular cancer, Hodgkin's disease, and childhood leukemias, but also faced with the obstacles of tumors that respond poorly to chemotherapy, such as non-small cell lung cancer and gastrointestinal malignancies. In addition, there are many cancers, such as breast cancer and non-Hodgkin's lymphoma, that initially may respond to chemotherapy and then relapse either during or after therapy.

One of the major side effects to the treatment of human malignancies is the acquisition of broad based anticancer drug resistance by tumor cells. The exposure of malignant cells or tumor cell lines to a single hydrophobic cytotoxic agent of natural origin frequently results in the emergence of cell populations exhibiting resistance to other structurally unrelated natural products. This phenomenon has been termed multidrug resistance (MDR). Cell lines that display this MDR phenotype are usually resistant to the vinca alkaloids (vincristine and vinblastine), anthracyclines, epipodophyllotoxins (VP-16 and VM-26), actinomycin D, colchicine, and taxol and are due to overexpression of 170 kDa-phosphoglycoprotein (Beck, 1987; Fojo *et al.*, 1985; Gottesman & Pastan, 1988, 1993; Ueda *et al.*, 1987). Therefore, this type of MDR is called pgp-MDR.

Another type of MDR is caused by overexpression of multidrug resistance associated protein (MRP) which is encoded by *mrp* gene. The *mrp* gene is different from *mdr1* gene (Mirski *et al.*, 1987; Taylor *et al.*, 1991; Grant *et al.*, 1994) and is poorly reversed by modulators that are effective in cells overexpressing *mdr1* (Cole *et al.*, 1989, 1991; Cole, 1992). This type of MDR is called MRP-MDR.

Various plant extracts have been used to alleviate disease and maintain good health. Although great deal of investigations on the components and pharmacology of Korean plants has been performed, studies of the Korean plants on the MDR modulation was quite few. Since lack of useful MDR modulators in clinical use and high incidence of MDR in cancer chemotherapy, needs for potent MDR inhibitor are ever increasing. Therefore we examined the effects of various plants extracts on inhibition of multidrug resistance. Using bioassay-guided fractionation and repeated column chromatography, we have isolated one lycoctonine type alkaloid, which was characterized to lycaconitine (Fig. 1) by the physicochemical and several spectral data. This compound was isolated from this plant for the first time. In this paper, we report the isolation and structral elucidation of alkaloid and the inhibitory activity of lycaconitine on MDR cells.

MATERIALS AND METHODS

Instrument

Melting point was measured by Gallenkamp Melt-

Correspondence to: Kang Ro Lee, College of Pharmacy, Sung Kyun Kwan University, Suwon 440-746, Korea
[+]Present address: College of Pharmacy, Woosuk University, Cheonju 565-800, Korea

ing Pointing apparatus (uncorr.). ^{1}H-NMR spectra were run at 500 MHz and ^{13}C-NMR at 125 MHz and recorded by Brucker AC-500. The EI/MS (70 eV) was determined by a VG-VSEQ. The UV spectrum was recorded on Shimadzu UV 240 UV-Visible recording spectrophotometer.

Chemicals and drugs

Vincristine (VCR) and other chemicals were obtained from Sigma Chemical Co.

Tissue culture

Cell lines used in this study were same as the previous report (Park *et al.*, 1996). The human fibroblast carcinoma KB cells obtained from ATCC were grown in RPMI 1640 media with 5% fetal bovine serum, and 0.1 mg/ml kanamycin at 37°C in 5% CO_2. VCR-resistant KB V20C cells (generous gift of Dr. Cheng at Yale University, School of Medicine) were developed from the parental KB cells by stepwise selection for resistance with increasing concentration of VCR and cultured in the presence of 20 nM concentration of VCR. These cells have been shown to overexpress P-glycoprotein by Western blot (Chen *et al.*, 1993) and used for Pgp-MDR inhibition study. KB 7D cells (generous gift of Dr. Cheng at Yale University, School of Medicine), which overexpress the *mrp* gene and display MRP-MDR phenotype (Gaj *et al.*, 1995), were cultured in the presence of 7 μM concentration of etoposide.

Cytotoxicity assay

The KB V20C cells were maintained in drug free media 3 days before determination of the concentration capable of inhibiting 50% growth. Cytotoxicity assays were performed in triplicate by plating $1\times10_4$ cells in each well of a 96-well plate. The cells were incubated with 20 nM concentration of VCR in the absence or presence of plant extract (or component) at 5% CO_2 and 37°C for 72 hr. Subsequently, the SRB assay (Skehan *et al.*, 1990) was used to measure the cytotoxic effect.

Extraction and isolation

The roots of *A. pseudo-laeve* var. *erectum* (Ranunculaceae) were purchased from a commercial supplier in Seoul, Korea, in 1995. A voucher specimen is deposited in the herbarium of College of Pharmacy, Sung Kyun Kwan University. 500 g of the crude material was extracted three times with MeOH by reflux for 5 hours below 50°C. The resulting MeOH extract was subjected to evaporation and suspended in water, followed by the successive solvent partition with CH_2Cl_2, EtOAc and n-BuOH, and finally gave 18 g of CH_2Cl_2 soluble fraction, 6 g of EtOAc soluble fraction, 10 g of n-BuOH soluble fraction and 35 g of water soluble fraction, respectively. And each fraction was examined for the anti-MDR activity in vitro, and the n-BuOH soluble fraction was found to have anti-MDR activity against KB V20C cells. The n-BuOH soluble fraction was loaded on silica gel column and separated by gradient solvent system of $CHCl_3$:MeOH:H_2O (150:25:2 → 80:20:2) as eluents. Silica gel chromatography yielded four sub-fractions, and the active first fraction (Table I) was rechromatographed with Lobar-A column eluting with a mixture of chloroform: MeOH (20:1) to afford an active compound, 20 mg of compound **1**.

Compound **1** (lycaconitine). mp. 112~113°C; IR ν_{max}^{Nujol} 3455 (OH), 1720 (C=O); UV (EtOH) λ_{max}: 278, 230 nm; EI-MS (m/z): 668(M^+); ^{1}H-NMR ($CDCl_3$, 500 MHz, ppm): δ 8.08 (1H, d, *J*=7.0 Hz, Ar-H), 7.65 (1H, t, *J*=7.0 Hz, Ar-H), 7.54 (1H, t, *J*=7.0 Hz, Ar-H), 7.26 (1H, d, *J*=7.0 Hz, Ar-H), 3.40, 3.33, 3.32, 3.25 (each 3H, s, 4×OCH_3), 2.92 (1H, s, H-17) and 1.06 (3H, t, *J*=7.0 Hz, $NCH_2\underline{CH_3}$); ^{13}C-NMR ($CDCl_3$, 125 MHz, ppm): δ 176.5 (C-1", 4"), 164.2 (Ar-CO), 133.7 (C-4'), 132.9 (C-2'), 131.1 (C-5'), 130.1 (C-6'), 129.5 (C-3'), 126.9 (C-1'), 90.8 (C-6), 88.5 (C-7), 83.9 (C-1, 14), 82.5 (C-16), 77.5 (C-8), 69.6 (C-18), 64.5 (C-17), 58.1 (14-OCH_3), 57.8 (6-OCH_3), 56.3 (16-OCH_3), 55.8 (1-OCH_3), 52.3 (C-19), 50.9 ($N\underline{C}H_2CH_3$), 50.2 (C-9), 49.0 (C-11), 46.1 (C-13), 43.2 (C-5), 38.2 (C-10), 37.5 (C-4), 34.0 (C-2",

Table I. Cell viability of P-glycoprotein-mediated MDR reversal activity and cytotoxicity of subfactions (**I-IV**) of n-BuOH extract of *Aconitum pseudo-laeve* var. *erectum* to cancer cells[1)]

sample \ con.	1 mg/ml			100 μg/ml			10 μg/ml		
	KB	KB V20C	KB7D	KB	KB V20C	KB7D	KB	KB V20C	KB7D
I	41.1	16.4	28.6	99.5	56.6	89.9	96.2	95.3	94.2
II	8.3	22.0	9.6	94.2	50.2	85.5	95.3	92	97.4
III	22.7	26.1	6.6	96.9	88.9	93.8	97.4	106.8	100.1
IV	42.5	27.3	6.1	98.5	105.6	95.1	97.9	100.3	98.5

[1)]Cell viability represents the concentration μM of a compound required for 50% inhibition of cell growth. Each fraction was examined at least at three concentrations in duplicate.

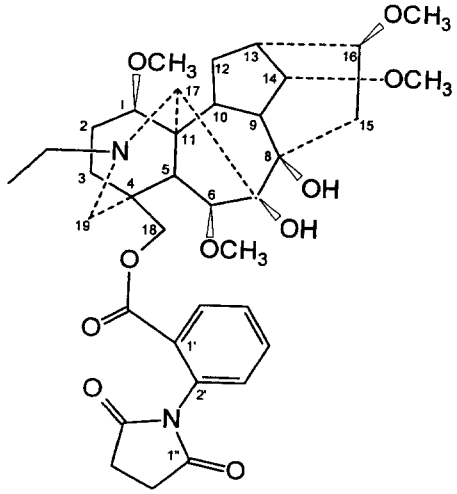

Fig. 1. The structure of lycaconitine.

3"), 33.6 (C-15), 32.1 (C-3), 28.7 (C-12), 26.1 (C-2), 14.0 ($NCH_2\underline{C}H_3$).

RESULTS AND DISCUSSION

To search for a MDR inhibitor from Korean medicinal plants, a variety of plants were extracted with MeOH and their cytotoxicity on MDR cells were measured. The MeOH extract of the roots of *Aconitum pseudo-laeve* var. *erectum* NAKAI (Ranunculaceae) showed significant modulating activity of Pgp-MDR against KB V20C but not MRP-MDR. Using a bioassay-guided fractionation and repeated column chromatography, we have isolated an active principle belonging to a family of lycoctonine type alkaloid.

Compound **1**, mp 112~113°C, was obtained as an amorphous powder and showed positive reaction to Dragendorffs reagent. The ^{1}H-NMR spectrum showed signals corresponding to an N-ethyl group, and four sharp singlets of methoxyl groups (δ 3.40, 3.33, 3.32, 3.25 ppm). The aromatic protons of compound **1** exhibited signals for two triplets protons and two doublets of a 1, 2-disubstituted aromatic system. 13C-NMR spectrum showed the three carbonyl groups [(δ 176.5 (C-1", 4"), 164.2 (Ar-CO)], four methoxyl groups (δ 58.1, 57.8, 56.3, 55.8 ppm) and six aromatic signals (δ 133.7, 132.9, 131.1, 130.1, 129.5, 126.9). The above results suggest that compound **1** is a lycoctonine type alkaloid. The chemical structure of the active compound was determined as lycaconitine on the basis of chemical and spectral evidences, together with comparison of its data with those reported in the literature (Yu De quan *et al.*, 1983, Shamma, M. *et al.*, 1979, Sakai, S. I. *et al.*, 1978).

Table II. Cell viability of Pgp (P-glycoprotein)-mediated MDR reversal activity and cytotoxicity of compound **1**[1)]

sample \ con.	0.5 mg/ml		0.1 mg/ml		0.01 mg/ml
	KB	KBV20C	KB	KBV20C	KB
compound **1**	85.3	19.4	93.4	43.6	96.0

[1)]Cell viability represents the concentration (μM) of a compound required for 50% inhibition of cell growth. Compound **1** was examined at least at three concentrations in duplicate.

Lycaconitine was found to have potent inhibitory activity on pgp-MDR but not on MRP-MDR. Its concentration capable of inhibiting 50% growth was 74 g/ml (Table II). Lycaconitine had such potent pgp-MDR inhibition that only 74 g/ml was required to reduce multidrug resistant population into half, but cytotoxicity to KB cells was unmeasurably large amount, demonstrating that it works preferably for Pgp-MDR inhibition rather than cytotoxicity to cancer cells. Considering the fact that no availability of commercial MDR inhibitors and ever increasing MDR patients made this compound highly feasible as a MDR inhibitor. Further studies on action mode and any other pharmacological effects should be performed.

ACKNOWLEDGEMENT

This work was supported by the research grant from Kyungki Pharmaceutical Research Center (KPRC).

REFERENCES CITED

Beck, W. T., The cell biology of multidrug resistance. *Biochem. Pharmacol.*, 36, 2879-2887 (1987).

Chen, H.-X., Bamberger, U., Heckel, A., Guo, X. and Cheng, Y.-C., BIBW22, a dipyridamole analog, acts as a bifunctional modulator on tumor cells by influncing both p-glycoprotein. *Cancer Res.*, 53, 1974-1977 (1993).

Cole, S. P. C., The 1991 Merck Frost Award. Multidrug resistance in small cell lung cancer. *Can. J. Physiol. Pharmacol.*, 70, 313-329 (1992).

Cole, S. P. C., Downers, H. F. and Slovak, M. L., Effect of calcium antagonists on the chemosensitivity of two drug resistant human tumor cell lines which do not overexpress P-glycoprotein. *Br. J. Cancer,* 59, 42-46 (1989).

Cole, S. P. C., Chanda, E. R., Dicke, F. P., Gerlach, J. H. and Mirski, S. E. L., Non-P-glycoprotein-mediated multidrug resistance in a small cell lung cancer cell line: evidence for decreased susceptibility to drug-induced DNA damage and reduced levels of topoisomerase II. *Cancer Res.*, 51, 3345-3352 (1991).

Fojo, A., Akiyama, S. I., Gottesman, M. M. and Pastan, I., Reduced drug accumulation in multiply drug resistant human KB carcinoma cell lines. *Cancer Res.*, 45, 3002-3007 (1985).

Gaj, C. L., Anyanwutaku, I. O., Coli, S. C., Chang, Y.,

and Cheng, Y.C., Reversal of multidrug resistance associated protein mediated multidrug resistance in a human carcinoma cell line by the V-enanciomer of verapamil. Proceedings of the American Association for Cancer Research 36, 346 (1995).

Gottesman, M. M. and Pastan, I., The multidrug transporter, a double-edged sword. *J. Biol. Chem.*, 263, 12163-12166 (1988).

Gottesman, M. M. and Pastan, I., Biochemistry of multidrug resistance mediated by the multidrug transporter. *Ann. Rev. Biochem.*, 62, 385-427 (1993).

Grant, C. E., Valdimarsson, G., Hipfner, D. R., Almquist, K. C., Cole, S. P. and Deeley, R. G., Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. *Cancer Res.*, 54, 357-366 (1994).

Mirski, S. E. I., Gerlach, J. H. and Cole, S. P. C., Multidrug resistance in a human small cell lung cancer cell line selected in Adriamycin. *Cancer Res.*, 47, 2594-2598 (1987).

Park, J. D., Kim, D. S., Kwon, H. Y., Son, S. K., Lee, Y. H., Baek, N. I., Kim, S. I., and Rhee, D. K., Effect of Ginseng saponin on modulation of multidrug resistance. *Arch. Pharm. Res.* 19, 213-218 (1996).

Sakai, S. I., Shimna, N., Hasegawa, S. and Okamoto, T., On the alkaloids of *Aconitum gigas* and the structure of a new base, gigactonine. *Yakugaku Zasshi*, 98, 1376-1384 (1978).

Shamma, M., Chinnasmy, P., Miana, G. A., Khan, A., Bashir, M., Salazar, M., Patil, P. and Beal, J. L., The alkaloids of *Delphinium cashmirianum. J. Nat. Prod.* 42, 615-623 (1979).

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Jonathan, T. W., Bokesch, H., Kenney, S., and Boyd, M. R. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* 82, 1107-1112 (1990).

Taylor, C. W., Dalton, W. S., Parrish, P. R., Gleason, M. C., Bellany, W. T., Thompson, F. H., Roe, D. J., and Trent, J. M. Different mechanisms of decreased drug accumulation in doxorubicin and mitoxantrone resistant variants of the MCF7 human breast cancer cell line. *Br. J. Cancer,* 63, 923-929 (1991).

Ueda, K., Cardarelli, C., Gottesman, M. M. and Pastan, I., Expression of a full-length cDNA for the human *mdr1* gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc. Natl. Acad Sci. USA.*, 84, 3004-3008 (1987).

Yu De-quan and Bhupesh, C.D., Alkaloids of *Aconitum barbatum. Planta Med.*, 49, 85-89 (1983).